iCycler iQ™ Multi-Color Real Time PCR Detection System

Operating Instructions

Catalog Number
170-8740

Safety Information

Important: **Read this information carefully before using the** iCycler iQ Real Time PCR Detection System.

Grounding

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

Servicing

The only user-serviceable parts of the iCycler are the lamp and filters. *There are no other user-serviceable parts for this instrument*. When replacing the lamp or filters, remove *ONLY* the outer casing of the iCycler Optical Module for lamp and filter replacement. Call your local Bio-Rad office for service for all other service.

Power Switch

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

Temperature

For normal operation the maximum ambient temperature should not exceed 30 °C (see Appendix A for specifications).

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

Do not operate the iCycler Optical Module in extreme humidity (>90%) or where condensation can short internal electrical circuits or fog optical elements.

Notice

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

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

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

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

The iCycler is intended for laboratory research applications only.

Table of Contents

Section 1
Introduction

The Polymerase Chain Reaction (PCR)* has been one of the most important developments in Molecular Biology. PCR has greatly accelerated the rate of genetic discovery, making critical techniques relatively easy and reproducible.

The availability of technology for kinetic, real time measurements of a PCR in process greatly expands the benefits of the PCR reaction. Real-Time analysis of PCR enables truly quantitative analysis of template concentration. Real-Time, on-line PCR monitoring also reduces contamination opportunities and speeds time to results because traditional post PCR steps are no longer necessary. A wide range of fluorescent chemistries may be employed to monitor the PCR in progress.

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

The iCycler iQ Real Time PCR Detection System builds on the strengths of the iCycler thermal cycling system. The iCycler iQ system features a broad spectrum light source that offers maximum flexibility in selecting fluorescent chemistries. The filter based optical design allows selection of the optimal wavelengths of light for excitation and emission, resulting in excellent sensitivity and discrimination between multiple fluorophores. The 350,000 pixel array on the CCD detector allows for simultaneous imaging of all 96 wells every second. This results in a comprehensive data set illustrating the behavior of the data during each cycle. Simultaneous image collection insures that well-to-well data may reliably be compared. The iCycler iQ system reports data on the PCR in progress in Real Time, allowing immediate feedback on reaction success. All of these features of the iCycler iQ system hardware were built to promote reliability and flexibility.

The iCycler iQ Real Time Detection System Software includes the features that make software easy and useful. The software is designed for convenience - offering speedy setup and analytical results. The functions are presented graphically to minimize hunting through menus. Tips on usage are available as your mouse glides over the buttons - and the tips can be turned off when you no longer need to see them. The iCycler software automatically analyzes the collected data at the touch of a button yet leaves room for significant optimization of results based on your analysis preferences.

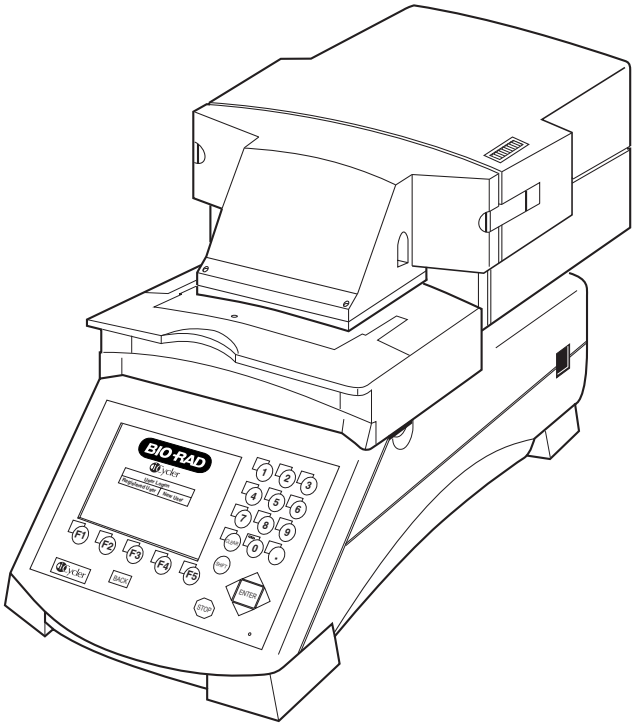


Fig. 1.1. Optical Module Upgrade to iCycler Thermal Cycler.

1.1 iCycler iQ System Description

The optical module houses the excitation system and the detection system. The Excitation system consists of a fan-cooled, 50-watt tungsten halogen lamp, a heat filter (infrared absorbing glass), a 6-position filter wheel fitted with optical filters and opaque filter "blanks", and a dual mirror arrangement that allows simultaneous illumination of the entire sample plate. The excitation system is physically located on the right front corner of the optical module, with the lamp shining from right to left, perpendicular to the instrument axis. Light originates at the lamp, passes through the heat filter and a selected color filter, and is then reflected onto the 96 well plate in the thermal cycler by a set of mirrors. This light source excites the fluorescent molecules in the wells.

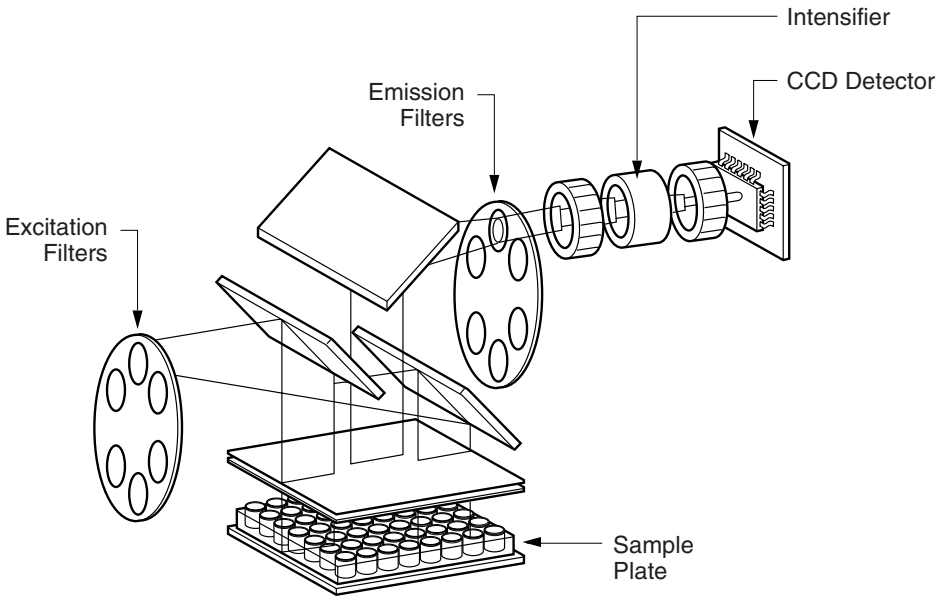


Fig. 1.2. Representation of Optical Detection System layout.

The detection system occupies the rear two-thirds of the optical module housing. The primary detection components include a 6-position emission filter wheel, an image intensifier, and a CCD detector. This filter wheel is identical to the wheel in the excitation system and is fitted with colored emission filters and opaque filter "blanks". The intensifier increases the light intensity of the fluorescence without adding any electrical noise. The 350,000 pixel CCD allows very discrete quantitation of the fluorescence in the wells. Fluorescent light from the wells passes through the emission filter and intensifier and is then detected by the CCD.

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

At the right side of the optical module are two connectors (see Figure 1.3):

- Round 9-pin power connector: This provides power to the optical module via the optical system power supply. Note: Always turn power switch on the power supply to the OFF position before connecting this connector.
- Parallel-port connector: This uses a cable that is 25 pin male-to-male and connects to the computer. The computer requires an IEEE 1284 compatible, 8-bit bi-directional, or EPP type, parallel port. Data are transferred to the computer via this cable.

At the right rear corner of the reaction module is a single connector.

- Miniature phone plug connector: This senses when the handle is lifted. When the handle is lifted, the emission filter wheel shifts to the home position, blocking light to the intensifier and the CCD detector.

At the left rear corner of the iCycler thermal cycler is a single connector.

- Serial connector: The iCycler program directs the operation of the iCycler via this cable.

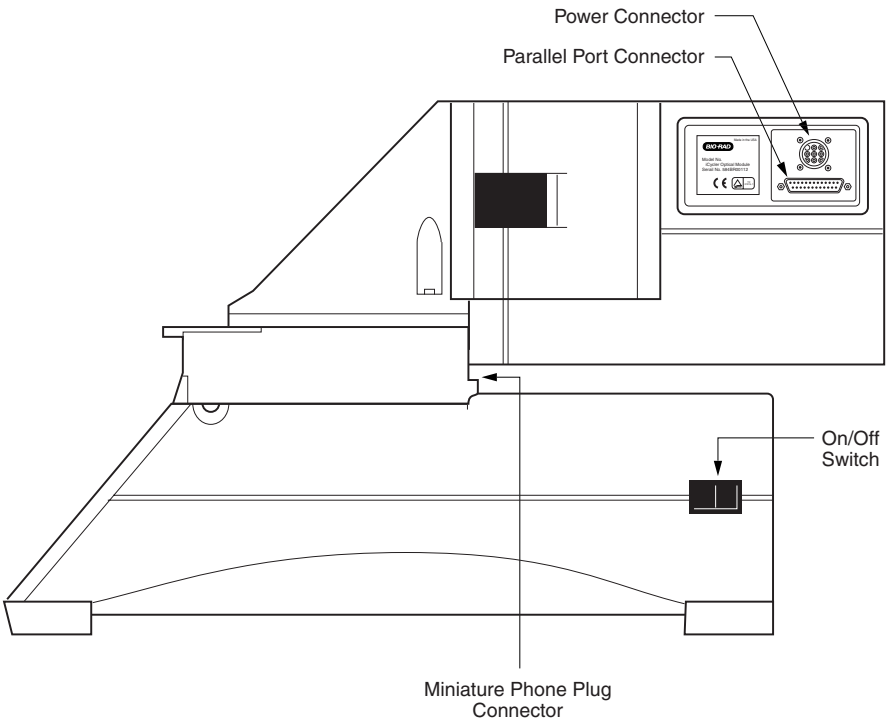


Fig. 1.3. Side View of iCycler iQ Real-Time Detection System showing cable connections.

1.2 iCycler iQ Filter Description and Instructions

Before running the iCycler program, be sure the correct filters have been installed. In addition, if the system has been moved prior to use, it is necessary to check the alignment of the mask. This procedure is discussed in Section 6.2.2. All filters are mounted in holders (see Figure 1.4). The filter holders are held in filter wheels and may be changed. Each filter wheel holds six filters. Every position in a filter wheel must have a filter or an opaque filter blank to avoid damage to the CCD detector. The first position in each filter wheel is designated as the "home" position and must always contain an opaque filter blank.

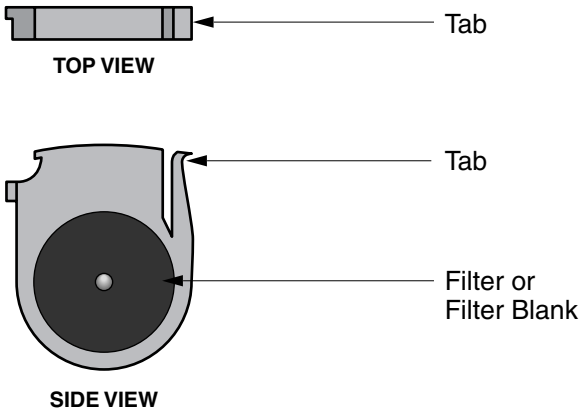


Fig. 1.4. Filter in filter holder.

To change filters, proceed as follows:

1. Turn off the power to the Optical Module.
2. Release the two black latches on each side of the Optical Module. Slide the housing backwards 2–3" (5–8 cm), exposing a black case, the filter wheel housing. It is not necessary to remove the housing or cables.
3. Remove the two rubber plugs on the top of the filter wheel housing by pulling them straight upward. These plugs shield the filter wheels. The excitation filters are located in the slot on the right side of the instrument; the emission filters are located in the slot in the center of the instrument (see Figure 1.5). Changing both types of filters is similar.
4. Turn the filter wheels to the desired positions using the ball end hex driver. As long as the power to the Optical Module is off, the filter wheels may be turned freely in either direction.
5. To remove a filter, grasp it on both sides with the filter removal pliers and squeeze the tab in; gently pull the filter up and out.
6. To insert a filter, grasp the filter with the pliers and insert it into a vacant slot. For the excitation filters, the tab on the filter is toward the front of the instrument. For the emission filters, the tab on the filter is toward the right of the instrument. Be sure that every position in the filter wheel has either an excitation or emission filter or a filter blank. **Record the position of filters to compare later with the plate setup**. (See Section 5.2)
7. After the filters or filter blanks have been inserted, replace the rubber plugs over the slots of the filter wheels.
8. Move the camera housing forward and re-attach the latches.

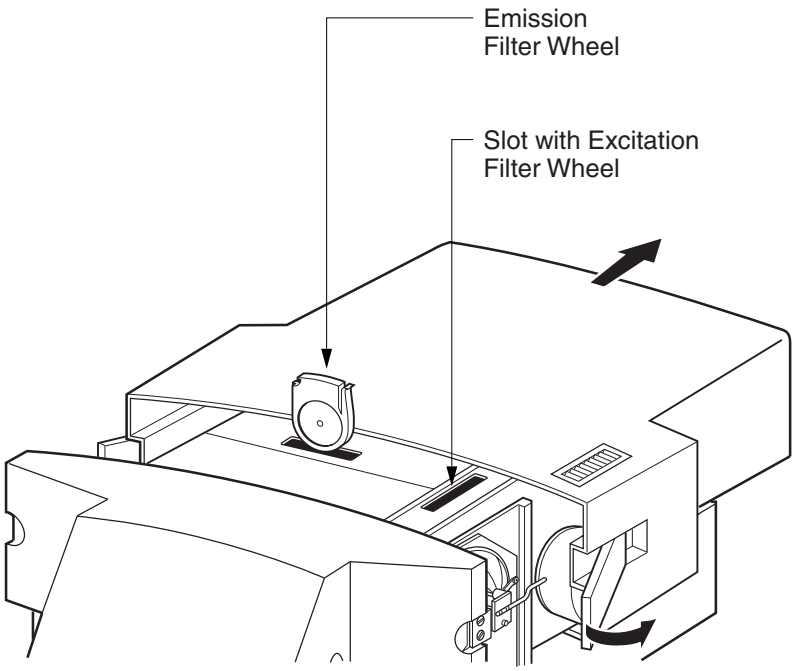


Fig. 1.5. Installing the filters.

Section 2
The iCycler iQ Real Time PCR Detection System for Single and Multi-Color Experimentation

2.1 Introduction

The iCycler iQ Detector can simultaneously collect light from as many as four fluorophores in 96 wells and separate the signals into those of the individual fluorophores. This allows monitoring of two or more amplifications simultaneously on the same plate or in each well.

At each data collection step in real time, fluorescent light from each monitored well is measured through each filter pair. Since there is one filter pair for each fluorophore on the plate, each data collection step may require as many as four readings. For example, if there are probes labeled with FAM, HEX, Texas Red® and Cy™5 in a well, then at each data collection step, light from the well must be measured using the FAM filter pair, the HEX filter pair, the Texas Red filter pair and the Cy5 filter pair. The software then splits the signals into the contributions of each individual fluorophore. Using these data, separate amplification plots are displayed and at the end of the experiment, separate standard curves are automatically calculated for each fluorophore and unknown concentrations are determined on an individual fluorophore basis.

Every experiment on the iCycler iQ system requires well factor and pure dye calibration data in order to separate the signals of the individual fluorophores from the combined measured light. These two concepts are presented in detail in this chapter; understanding them will make it possible to rapidly optimize experimental protocol development and to collect the best possible optical data.

2.2 Quick Guide to Single or Multi-Color Experimentation

1. Allow the camera to warm up for 30 minutes. Power up the iCycler and log onto the instrument. Load the iCycler software. If the iCycler or the iQ detector has been moved since the last experiment, enter Imaging Services in the Run Time Central module and check the alignment of the masks. See Section 6.2.
2. If necessary, conduct a Pure Dye Calibration protocol to collect the data required to separate the signals from overlapping fluorophores. Calibration data are required for each fluorophore/filter pair combination on the experimental plate. See Section 2.4.
3. Prepare the experimental PCR reactions in a 96-well Thin Wall plate (catalog number 223-9441). Place a sheet of Optical Quality sealing tape (catalog number 223-9444) on the top of the 96-well plate. Use the tape applicator (flat plastic wedge) to smooth the tape surface. Avoid touching the surface of the sealing tape with gloved fingers. Tear off the white strips that remain on the sides of the tape. If individual sample tubes or strips of tubes are to be used, you must seal the tubes with the appropriate caps. Note that a minimum of 8 sample tubes is required to prevent tube crushing when using the green anticondensation ring. If the ring is not present, a minimum of 14 sample tubes must be present.
4. Create and save the thermal protocol in the Protocol Workshop. The thermal protocol specifies the dwell times and set point temperatures, the number of cycles, steps and repeats, and the step(s) at which data collection are to occur. See Section 5.1.

5. Create and save the Plate Setup in the Protocol Workshop. The process of creating the Plate Setup includes choosing the appropriate Filter Wheel Setup file. Choose a Filter Wheel Setup that includes all the fluorophores that you want to monitor. See Section 5.2.5. Finally, in the Plate Setup window, indicate what fluorophores are to be monitored in which wells and define the sample type, and for Standards, enter the quantity and units of measure. Check these entries in the 'View Plate Setup' tab before proceeding. See Section 5.2.
6. Ensure that the positions of the filters in the excitation (lamp) and emission (camera) filter wheels are in the exact same position as defined by the filter wheel setup chosen in Step 5. See Section 5.2.5.
7. If you will be using an external well factor plate, (see Section 2.3) place the well factor plate in the iCycler; otherwise, place the experimental plate in the iCycler. Click the View Protocols tab in the Protocol Library and select the desired Thermal Protocol; click the View Plate Setup tab in the Protocol Library and select the desired plate setup and then click **Run**.
8. In the Run Prep tab, confirm that the desired protocol and plate setup files are selected. Enter the reaction volume. Indicate the type of protocol (PCR Quantification/Melt Curve or Pure Dye Calibration) and the Well Factor Source, then click **Begin Run**. (Figure 2.1)
9. Enter a name for the data file. Data are saved during the running protocol. The run will not begin without a data filename.

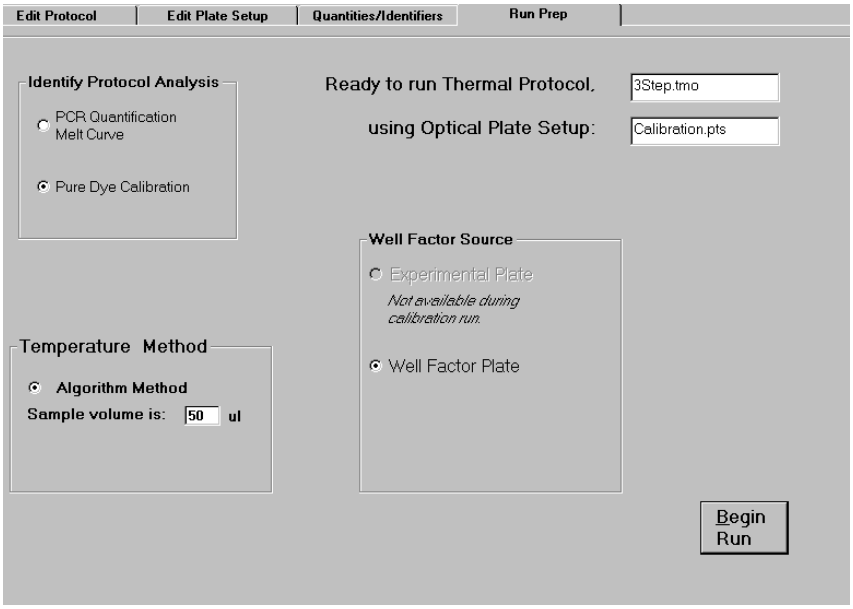


Fig. 2.1.

10. Well factors from the Experimental Plate will be collected automatically after you click Begin Run and the protocol will execute immediately afterwards without any user intervention. If you are using a Well Factor Plate, the protocol will begin execution and after about five minutes, the external well factors will be collected and the iCycler will go into Pause mode. During the Pause, remove the well factor plate and replace it with the experimental plate and then click **Continue Running Protocol**. (See Figure 2.6)
11. After data collection on the PCR reaction plate begins, the PCR Amp Cycle plot will be displayed and the software will open the Data Analysis module. It is not possible to make adjustments to the PCR Amp Cycle plot while data are being collected. You can change the monitored fluorophore or adjust the size of the plot during steps at which data are not collected.

2.3 Well Factors

Well factors are used to compensate for any system or pipetting non-uniformity in order to optimize fluorescent data quality and analysis. Well factors must be collected at the beginning of each experiment. Well factors are calculated after cycling the filter wheels through all monitored positions while collecting light from a uniform plate. Well factors may be collected directly from an experimental plate or indirectly from an external source plate.

The better and easier source of well factors is the actual experimental plate. Well factors collected from the experimental plate are called dynamic well factors. The only requirement for using dynamic well factors is that *each monitored well must contain the same composition of fluorophores*. Within each dye layer the fluorophore must be present at the same concentration, however, all dye layers need not have the same concentration. If all the wells on a plate have, for example, 50 nM fluorescein, 100 nM HEX, 125 nM Texas Red and 200 nM Cy5, you can use dynamic well factors because the fluorophore composition is the same in every well. If some of the wells have 100 nM fluorescein and others have 200 nM fluorescein, then you cannot use dynamic well factors and you must use external well factors. Collection of dynamic well factors is a completely automated process initiated by clicking the Experimental Plate radio button in the Well Factor Source box of the Run Prep screen (see Figure 2.1).

In most experiments using DNA-binding dyes like SYBR® Green I or ethidium bromide, dynamic well factors cannot be used. When the template DNA is denatured, the fluorescence of the intercalators is not sufficiently high to calculate statistically valid well factors. There are two solutions to this problem: (1) use external well factor plate or (2) for experiments with SYBR Green I, spike the master mix with a small volume of dilute fluorescein solution (see Section 2.3.2). This dilute fluorescein results in sufficient fluorescence at 95 °C so that good dynamic well factors can be calculated and it will not interfere with the PCR.

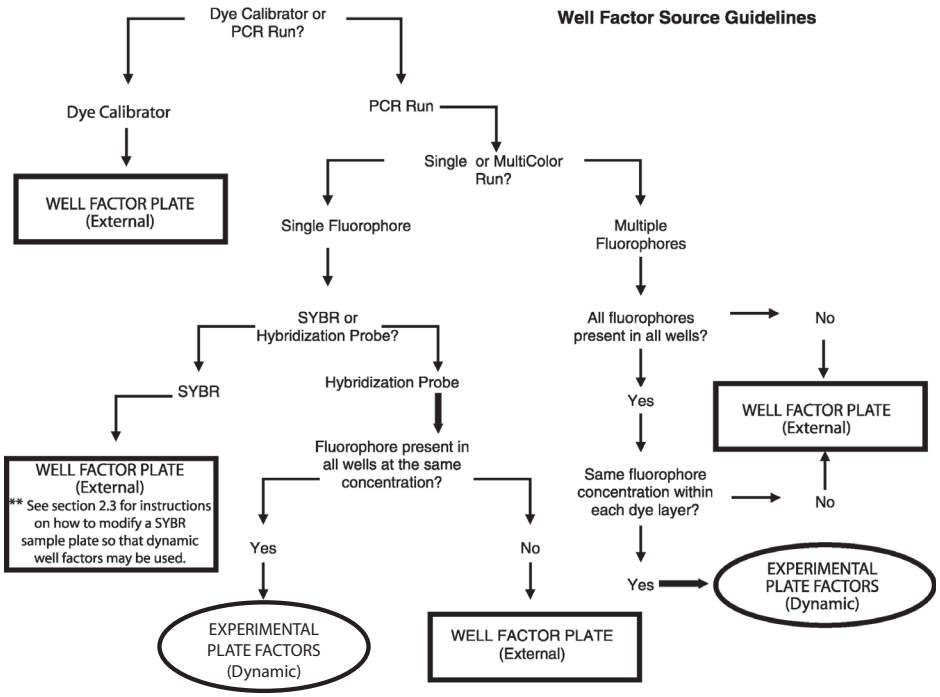


Fig. 2.2.

2.3.1 Well Factor Source: Experimental Plate

Dynamic well factors are collected the first time that the experimental plate is heated above 90 °C. This is particularly important if the mode of detection employs a probe with any secondary structure (the secondary structure must be relaxed for proper calibration). Because dynamic well factors are not collected until after the plate is heated to 90 °C at least once, optical data collection cannot be specified in the thermal protocol until after a step in which the temperature is programmed to exceed 90 °C.

2.3.2 Using the Experimental Plate for Well Factors

When you select the experimental plate as the source of well factors, the software automatically inserts a short protocol in front of the first step at which the temperature exceeds 90 °C. This protocol, Dynamicwf.tmo, includes 90 seconds at 95 °C. You may want to take that into consideration when creating your thermal protocol. For example, if you normally heat your reaction mixture to 95 °C for 10 minutes prior to amplification, you can accomplish the same thing by programming an initial cycle of 8 minutes and 30 seconds at 95 °C when using the experimental plate for well factors.

During this inserted cycle, each filter pair to be used during the experiment is briefly moved into position and optical data are collected from the plate and the well factors are calculated. While the well factor data are being collected a message is displayed in the Run Time Central screen. (Figure 2.3)

In order to use dynamic well factors on a plate monitored with SYBR Green I, bring the master mix to a final concentration of 10 nM fluorescein. First make a 1 µM solution by a 1:1000 dilution of the 1 mM stock Fluorescein Calibration Dye (catalog number 170-8780) in PCR buffer (10 mM Tris, pH 8.0, 50 mM KCl, 3 mM MgCl2). Then add 1 part of the 1 µM dilution to each 99 parts of master mix. For example, mix 10 µl of 1 µM fluorescein with 990 µl of master mix to yield a final concentration of 10 nM fluorescein.

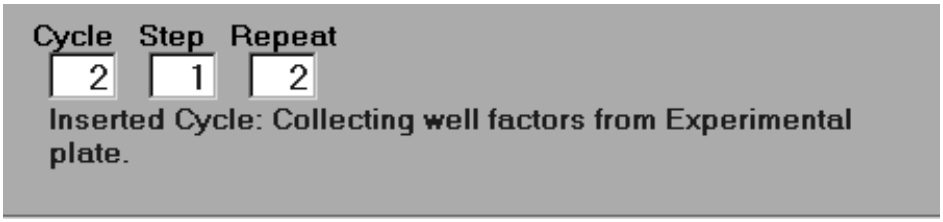


Fig. 2.3.

Once well factors are collected from the experimental plate, they are written to the opd file, and the software continues to execute the programmed protocol.

2.3.3 Well Factor Source:Well Factor Plate

The external well factor approach must be employed if there are varying concentrations or types of fluorophores in the individual wells of a plate. For example, you must use an external well factor plate if some wells on a plate have 100 nM fluorescein while others have 200 nM fluorescein, or you must use them if some wells contain fluorescein and others contain Texas Red (as in a Pure Dye Calibration protocol).

External well factors are not collected on the experimental plate, but rather on a separate calibration plate containing the same *volume* as the experimental plate in each individual well; *i.e.*, if the experimental plate will contain 50 µl of sample in each well, then the calibration plate must contain 50 µl of fluid in each well. External well factors must be collected in a type of plate and with a sealing mechanism identical to that of the experimental plate. If a single fluorophore is being monitored in the experiment, then you can make a well factor plate using only that fluorophore, but you may have to determine the optimum concentration of fluorophore. If you are monitoring more than one fluorophore, you must use the External well factor plate solution (catalog number 170-8794) provided by Bio-Rad. You can also use this solution to collect well factors for single-fluorophore experiments.

2.3.4 Preparing the External Well Factor Plate

Use the Bio-Rad External Well Factor Solution (catalog number 170-8794) supplied as a 10x concentrate.

Dilute the 10x solution 1 part to 9 with ddH_2O. The volumes used in each well of both the well factor and the experimental plate must be identical. You need only fill the wells on the well factor plate that correspond to wells that will be monitored on the experimental plate. For example, if your experimental plate is loaded with 50 µl in each well of columns 5 and 6, then you must put 50 µl of diluted external well factor solution in each well of column 5 and column 6 of the well factor plate.

- Pipet 50 µl of 1x well factor solution into each well of the well factor plate. Cover the plate with a piece of optically clear sealing film and briefly spin the plate to bring all the reagents to the bottom of the wells.
- You can use Imaging Services (Section 6.2) to confirm that the 1x well factor solution gives a strong, but not saturated image somewhere in the exposure range of 80–640 ms for each filter pair.

2.3.5 Using the External Well Factor Plate

The external well factor plate is used in experiments in which the concentration of fluorophore varies across the PCR reaction plate, including Pure Dye Calibration experiments.

- Place the external well factor plate into the iCycler and close the lid.
- From the Protocol Library select the thermal protocol and plate setup files and click **Run**.
- In the RunPrep screen, click the External Plate radio button in the Well Factor Source box. Choose the other conditions (reaction volume and type of protocol) based on the experimental plate, and click **Begin Run**.

  After you click Begin Run, the iCycler automatically inserts a 3-minute protocol, Externalwf.tmo, in front of your thermal protocol.

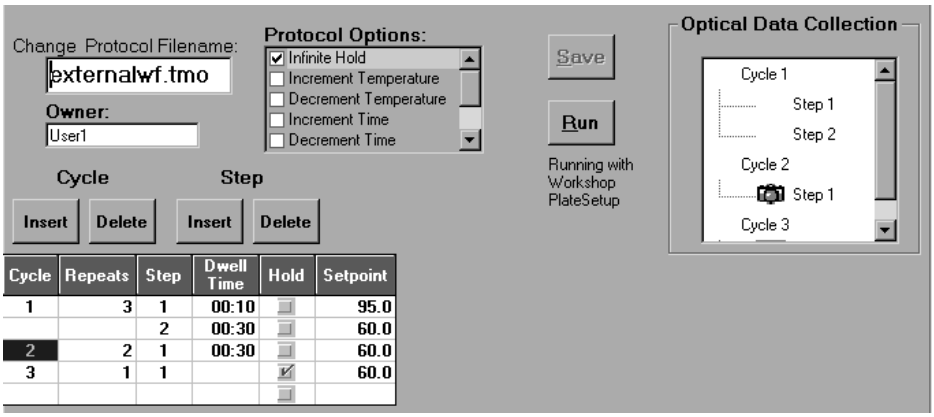


Fig. 2.4.

This protocol (Figure 2.4) will cycle the well factor plate three times between 95 °C and 60 °C. Then it will hold at 60 °C for one minute while each filter pair to be used during the PCR is briefly moved into position and optical data are collected and the well factors are calculated.

While the well factors are being collected a message is displayed in Run Time Central (Figure 2.5).

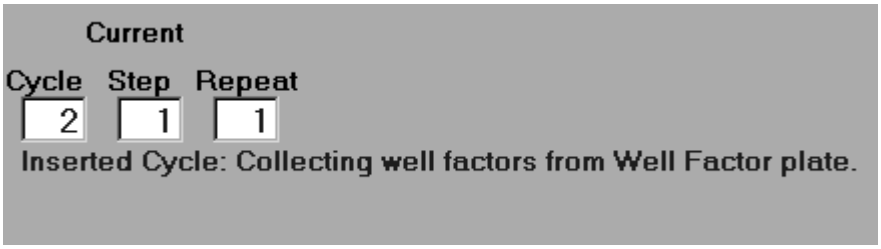


Fig. 2.5.

- After well factors are calculated the iCycler pauses. Remove the well factor plate and insert the PCR reaction plate and click **Continue Running Protocol**. (Figure 2.6)

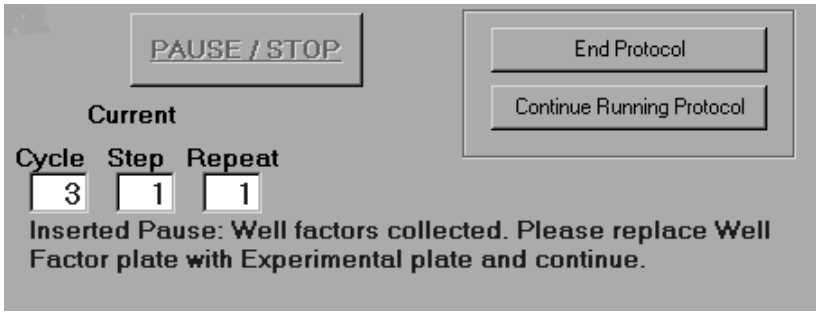


Fig. 2.6.

2.4 Pure Dye Calibration and RME Files

Pure dye calibration data are required for every experiment, even those that monitor only one fluorophore. In contrast to well factors which must be collected at the beginning of each

experiment, pure dye calibration data persist from experiment to experiment. When a new combination of fluorophore and filter pair are added to the analysis a new pure dye calibration must be done.

The pure dye calibration data are used to separate the total light signal into the contributions of the individual fluorophores. The data are collected by executing a Pure Dye Calibration protocol on a plate containing replicate wells filled with a single pure dye calibrator solution; there is a different calibrator solution for each fluorophore. These data are written to the file *RME.ini* found at C:\Program Files\Bio-Rad\iCycler\Ini.

Pure dye calibration data are collected on as many as 16 combinations of fluorophore and filter pairs per protocol (up to four fluorophores and up to four filter pairs). At the end of the calibration protocol, the data are automatically written to the RME file: one entry for each fluorophore/filter pair combination. Once pure dye calibration data are written to the RME file they are valid on all subsequent experiments using the same fluorophore/filter pair combinations. As more pure dye calibration protocols are executed, the RME file is updated with data for the new fluorophore/filter pair combination. If a fluorophore/filter pair combination for which an RME entry exists is repeated in a subsequent pure dye calibration protocol, the new results are written to the RME file and the old results are overwritten.

If a plate setup is specified that includes a fluorophore/filter pair combination for which pure dye calibration data do not exist, an alert message will be presented when the experiment is initiated. The RME file must be updated with the necessary pure dye calibration data before conducting the experiment. It is a good practice to archive RME files before running new pure dye calibration protocols. Archive the RME files by renaming them or moving them from the C:\Program Files\Bio-Rad\iCycler\Ini folder.

2.4.1 An example Pure Dye Calibration and RME file

In the RME file, the pure dye calibration data are organized first by fluorophore. Within each fluorophore group, there is an entry for each filter pair that was used to monitor the fluorophore calibrator solution. For example, under the fluorophore, Texas Red, may be entries for the 490/530, the 530/575, the 548/595; the 575/620 and the 635/680 filter pairs. Each subsequent fluorophore listed in the RME file will have entries corresponding to one or more of these same filter pairs.

Consider an example in which a pure dye calibration protocol is run on FAM-490 and Cy5-635. The resulting RME.ini file would have the structure (example values)

```
[FAM-490]
490/20X_!_530/30M=5.384598e+003
635/30X_!_680/30M=1.565864e+001

[CY5-635]
490/20X_!_530/30M=2.951601e+001
635/30X_!_680/30M=6.064747e+003
```

Now assume that another pure dye calibration is conducted with FAM-490 and Texas Red 575. The RME file will be updated to show

[FAM-490]
490/20X_!_530/30M=5.332098e+003
635/30X_!_680/30M=1.565864e+001
575/30X_!_620/30M=3.864038+001

[Texas Red-575]
490/20X_!_530/30M=1.40998e+001
575/30X_!_620/30M=4.737024e+004

[CY5-635]
490/20X_!_530/30M=2.951601e+001
635/30X_!_680/30M=6.064747e+003

Notice that the value for FAM-490 through the 490/530 filter pair has been slightly changed; it was updated when that fluorophore/filter pair combination was evaluated again in the second pure dye calibration. The values for Cy5-635 and for FAM-490 with the 635/680 filter pair are unchanged since these combinations were not tested in the second pure dye calibration. New entries appear for Texas Red-575 with two filter pairs, and a new entry appears in the FAM-490 section for the 575/620 filter pair.

The second RME file would be appropriate for an experiment with FAM-490 and Texas Red-575 together or FAM-490 and Cy5-635 together, or any of the three fluorophores alone, but it would not be appropriate for an experiment with both Texas Red-575 and Cy5-635 because there are no entries in the RME file for the combination of Texas Red and the 635/680 filter pair (the pair used to monitor Cy5) and the combination of Cy5 and the 575/620 filter pair (the pair used to monitor Texas Red). A pure dye calibration with Texas Red and Cy5 must be conducted before they can be used together on a plate. After this third pure dye calibration, the RME file would have the structure

[FAM-490]
490/20X_!_530/30M=5.632098e+003 – unchanged
635/30X_!_680/30M=1.565864e+001 – unchanged
575/30X_!_620/30M=3.864038+001 – unchanged

[Texas Red-575]
490/20X_!_530/30M=1.40998e+001 – unchanged
575/30X_!_620/30M=4.81645e+004 – re-evaluated
635/30X_!_680/30M=2.64573e+000 – new value

[CY5-635]
490/20X_!_530/30M=2.951601e +001 – unchanged
635/30X_!_680/30M=6.123537e+003 – re-evaluated
575/30X_!_620/30M=4.746395e+001 – new value

After the third pure dye calibration, any combination of the three fluorophores could be used on the same plate. Conversely, if the three fluorophores are run in a pure dye calibration simultaneously, the resulting RME would allow any combination as well.

2.4.2 Preparing a Pure Dye Calibration Plate

The supplied Bio-Rad Pure Dye Calibration solutions (catalog number 170-8792) must be used to collect the pure dye calibration data necessary for separating the signals of overlapping fluorophores. The solutions are prepared from singly-labeled oligonucleotides and are supplied at the working concentration. It is important that all pure dye calibrations are conducted using the solutions at the supplied concentration without any dilution. As many as four different calibration solutions may be evaluated on a single plate.

- Pipet 50 µl of the first calibration solution into ten wells.
- Repeat for up to three more calibration solutions **all in separate wells**.
- Cover the plate with a piece of optically clear sealing tape and briefly spin the plate to ensure that all reagents are at the bottom of the wells.
- Because the plate from which you are collecting the pure dye calibration data does not contain the same volume and concentration of each fluorophore in each monitored well, you must prepare an External Well Factor plate to collect well factors. Prepare an External Well Factor plate as described (Section 2.3.4) and place it in the iCycler.

For intercalator detection you can prepare of calibration solution of 500 pg/µl DNA and a 1:100,000 dilution of SYBR Green or 1 µg/ml ethidium bromide.

2.4.3 Running a Pure Dye Calibration Protocol

- Create a thermal protocol that contains two cycles. The first cycle should be 1 minute at 55 °C followed by 10 repeats of a second cycle, also at 55 °C for 1 minute. Choose to collect data for real-time analysis (yellow camera icon) at the second cycle. (Figure 2.7)

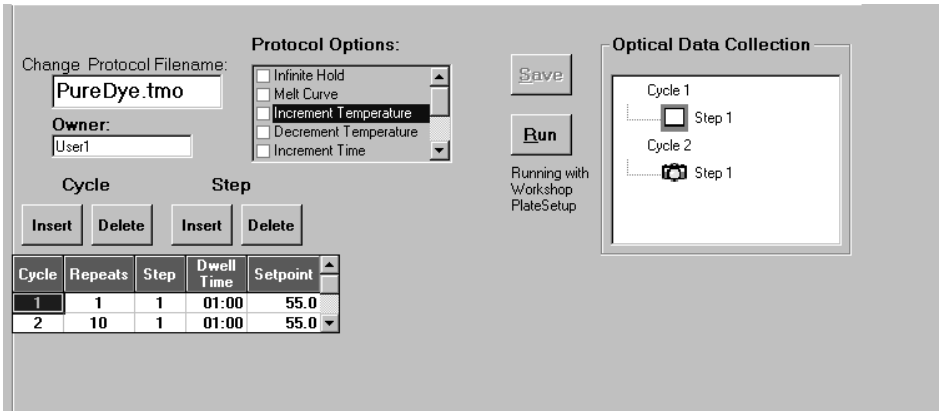


Fig. 2.7.

- Create a plate setup file that shows the location of the ten filled wells for each fluorophore being calibrated. The plate setup below indicates that the fluorescein pure dye calibration solution is in wells B2-B11, the Texas Red pure dye calibration solution is in wells D2–D11, the HEX pure dye calibration solution is in wells F2–11, and the Cy5 pure dye calibration solution is in well H2–11. (Figure 2.8).

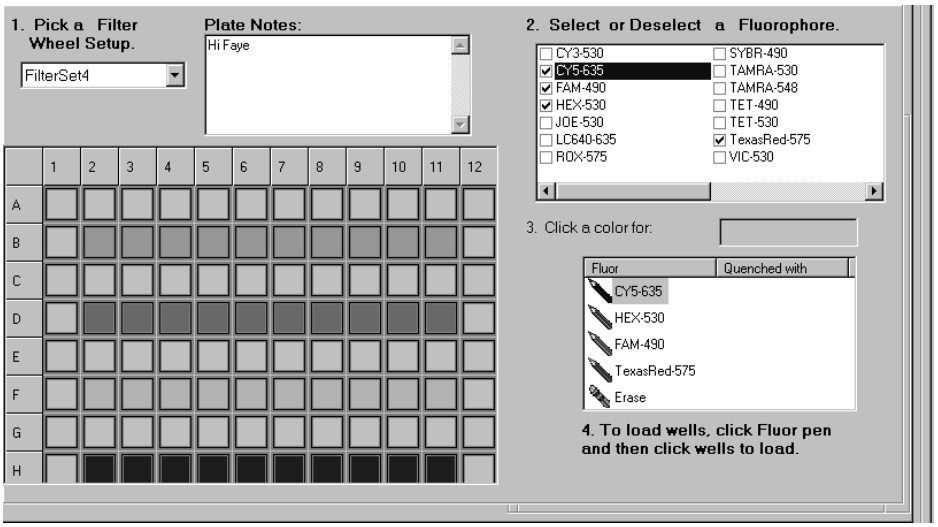


Fig. 2.8.

- With the External Well Factor plate inside the iCycler, go to the Protocol Library and select the correct thermal protocol file and then select the correct plate setup file, and click **Run**.
- In the RunPrep screen, enter the volume of reagent on the pure dye calibration plate, and choose Pure Dye Calibration as the type of protocol. **Note**: when Pure Dye Calibration is chosen Experimental Plate is grayed out for the source of Well Factors (Figure 2.9).
- Click **Begin Run**.
- Enter a name for the optical data file.

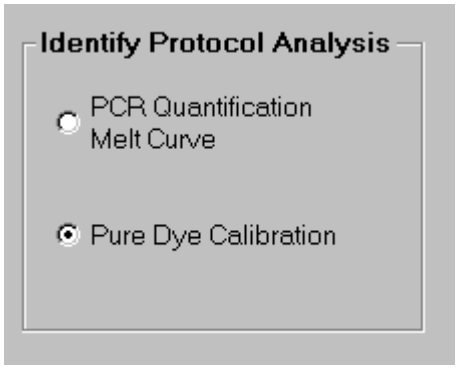


Fig. 2.9.

- After you name the data file, the iCycler will briefly cycle the well factor plate, collect optical data and calculate well factors. After the factors are calculated, the iCycler enters pause mode. (See Figure 2.6.)
- Remove the external well factor plate and replace it with your pure dye calibration plate and then click **Continue Running Protocol**.

- The iCycler will then execute the pure dye calibration protocol and as soon as sufficient data are collected, the protocol will automatically terminate and you will be alerted. (Figure 2.10) Typically it only takes about four cycles to collect the necessary calibration data. The calibration results will be automatically written to the RME.ini file. You may discard the opd file.

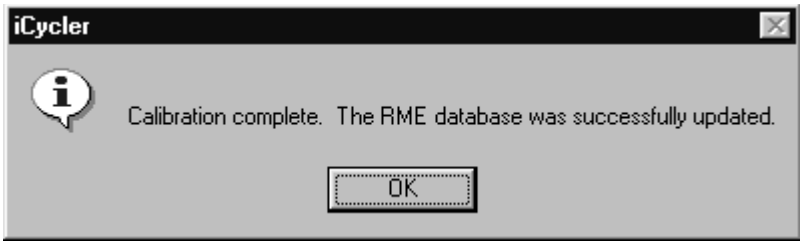


Fig. 2.10.

2.5 Converting from version 1.440 (1.0)

2.5.1 Data Files

Real-time PCR files collected with version 1.440 of the software may be opened in multi-color versions of the software as long as the RME.ini file is present at C:\Program Files\Bio-Rad\iCycler\Ini and that file has an entry for FAM/SYBR. The correct form of the entry is

[FAM/SYBR]
485DF22_!_535RDF45=4.021013e+003

At installation of the software, an RME file with an appropriate entry is copied into the proper location.

In all versions after 2.1.880, the RME data are automatically saved with the optical data in the OPD file, so that you may take your data to other computers for analysis. If you open data files collected in version 1.440 and then save them again in version 2.1.880, then the RME data will be saved along with the original data.

Note: If the contents of the RME.ini file are deleted, data sets collected with version 1.440 will not be available until the missing FAM/SYBR entry shown above is replaced. This does not apply to version 1.440 files that were opened and saved again under version 2.1.880 because the RME values are already embedded in those files.

2.5.2 Thermal protocols

Thermal protocols created in version 1.440 will run in all later versions.

2.5.3 Plate Setups

Plate setups created in version 1.440 cannot be used in later versions of the software; they must be recreated and saved in the new version of software. Plate setup files from v1.440 may be viewed in the protocol libraries, but not run or edited in subsequent versions. Viewing or Printing from the protocol library facilitates easy recreation of these plate setups in the new software version. When the cursor highlights a plate setup created in version 1.440 a message is displayed indicating that this file must be updated. (See Figure 2.11.)

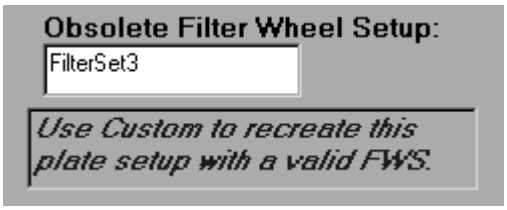


Fig. 2.11.

2.6 Converting from Version 1.880 (2.1)

There is no conversion needed for files created in version 1.880 (2.1).

Section 3
Introduction to the iCycler Program

3.1 Organization of the Program

The iCycler program allows you to create and run thermal cycling programs on the iCycler and to collect and analyze fluorescent data captured by the Optical Module. The operation of each instrument is controlled by separate parts of the iCycler Program. Operation of the iCycler thermal cycler is controlled by a segment of the program called 'Protocols'. Protocol files are thermal cycling programs that direct the operation of the iCycler. The Protocol files also specify when data will be collected during the thermal cycling run. Protocol files are stored with a '.tmo' extension. The details of setting up protocol files are described in Section 5.1.

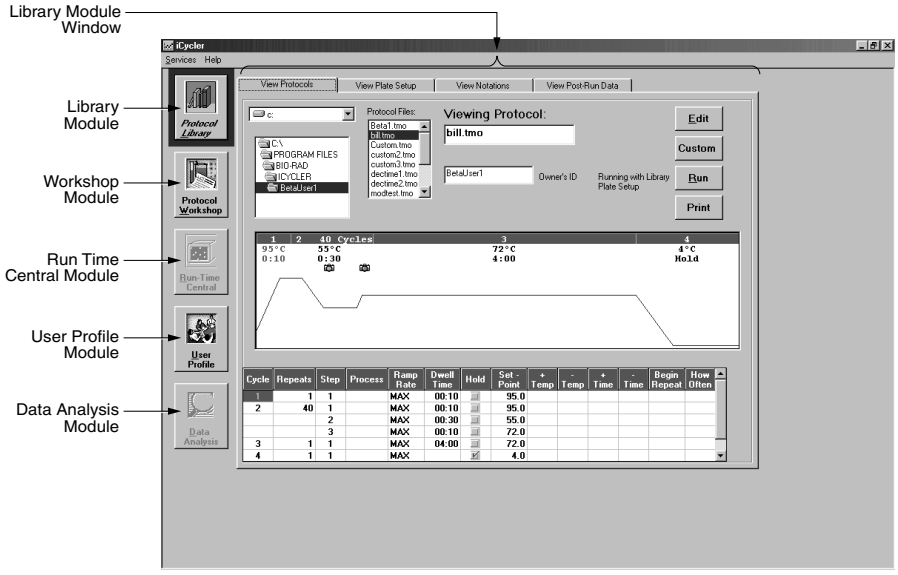


Fig. 3.1 Layout of a screen.

Operation of the Optical Module is controlled by the part of the program called 'Plate Setup'. This portion of the program allows you to specify from which sample wells data will be collected, the type of sample in each well (*e.g.*, standard, unknown, control, etc.) and the fluorophores to be monitored. Plate setup files are stored with a '.pts' extension. The details of

setting up Plate Setup files are described in Section 5.2. In order to run a thermal cycling program and collect fluorescent data both a Protocol file and a Plate Setup file must be specified.

The iCycler Program is organized into five sections, called 'modules'. These are the Library Module, the Workshop Module, the Run Time Central Module, the Data Analysis/Real Time Analysis Module, and the User Profile Module. Icons representing each of the modules are always shown on the left side of the screen. The module that is opened is displayed with a highlighted border while the names of the other modules have plain borders. Figure 3.1 shows the first screen you see when you open the iCycler Program: the names of all of the modules are listed on the left side of the screen with the Library Module icon highlighted, and the Library Module window is displayed on the entire right side of the screen. Each module has a different function, described on the next page.

3.1.1 The Library Module

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

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

3.1.2 The Workshop Module

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

- Edit existing Protocol files
- Edit existing Plate Setup files
- Write new Protocol files
- Write new Plate Setup files
- Save new and edited Protocol files
- Save new and edited Plate Setup files
- Initiate a run using new or edited Protocol and Plate Setup files.

Since Protocol and Plate Setup files are written and saved separately, you may "mix and match" a different file from each category in new experiments.

3.1.3 The Run Time Central Module

Once settings are confirmed in the Run Prep section of the Workshop Module, the iCycler Program transfers you directly into the Run Time Central Module where you may monitor the progress of the reaction, including the start time and completion time for the experiment, the current cycle, step, and repeat number, the thermal activity of the iCycler thermal cycler.

3.1.4 The Data Analysis/Real Time Analysis Module

This module may be accessed in either of two ways.

- It opens automatically from the Run Time Central Module when the iCycler iQ Real Time PCR Detection System begins collecting fluorescence data which are being analyzed in real time. This allows you to monitor a reaction as it occurs.
- You may open a stored data set from the Library Module and the data will automatically be presented in the Data Analysis Module.

This module allows you to:

- View experimental data
- Optimize data
- Assign threshold cycles for all standards and unknowns
- Construct standard curves
- Determine starting concentrations of unknowns
- Conduct statistical analyses.

3.1.5 The User Profile Module

The User Profile Module is independent of the other four modules. This module allows you to define default settings for the iCycler thermal cycler and to communicate to the iCycler Program where the filters are located in the filter wheel of the iCycler iQ Real Time PCR Detection System.

Note: In this release, the User Profile module is inactive, see section 5.2.5 for more information regarding Filter Wheel Setup.

3.2 Definitions and Conventions

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

- A “window” refers to the view of the iCycler Program found on the computer screen.
- Active buttons across the top of a window are referred to as ‘tabs’.
- A text box refers to a field in the window that you can type in.
- A field box refers to a region in the window that you cannot type in but provides information about the program.
- A dialog box refers to a region in the window that allows you to make a selection.
- All active buttons and tabs are printed in bold type in the text descriptions and figure legends. For example, the **Edit** button is always printed in bold since selecting this will result in some action by the iCycler Program.

3.3 Thermal Cycling Parameters

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

3.3.1 Temperature and Dwell Time Ranges

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

- Zero Dwell Times. When the dwell time is set to 00:00, the iCycler will heat or cool until it attains the setpoint temperature and then immediately begin heating or cooling to the next setpoint temperature.
- Infinite Dwell Times. When a cycle is not repeated, the dwell time at any step in that cycle may be specified as infinite by using the Infinite Hold option. This means that the instrument will maintain the specified temperature until you interrupt execution. When an infinite dwell time is programmed within a protocol at some step other than the last step, the instrument will go into Pause mode when it reaches that step and will hold that setpoint temperature until you select the **Continue Running Protocol** button in the Run Time Central Module (see Chapter 6).

3.3.2 Advanced Programming Options

The following are advanced programming options. Discussion of the use of these functions can be found in Section 5.1.4.

- Ramp Rate and Ramp Time: The ramp rate is the speed with which the iCycler changes temperatures between the steps of a cycle, or between cycles. The ramp time is the time interval over which a temperature increase or decrease is attained. The default condition is for the iCycler to adjust temperatures at the maximum ramp rate with the minimum ramp time. The iCycler allows you to change temperatures at a fixed rate less than the maximum (Ramp Rate), or you may choose to change the temperature over a fixed time interval (Ramp Time). Ramp rates are adjustable to 0.1 °C /sec, and ramp times are adjustable to 0.1 sec. Ramp rates must fall within the range of 0.1 to 3.3 °C per second for heating and 0.1 to 2.0 °C per second for cooling. Ramp times cannot result in ramp rates outside those specified above, and if they do, the ramp time will be adjusted so that it results in an allowable ramp rate.
- Automatic Increment/Decrement of Temperature or Dwell Time Setpoints: You may program an automatic periodic increase or decrease in the step temperature (Increment Temp or Decrement Temp) and/or dwell time (Increment Time or Decrement Time) in a repeated cycle.

  Temperature increments or decrements may be as little as 0.1 °C per cycle. You may make the increase or decrease as frequently as every cycle, and the increase or decrease can begin following any cycle. The temperature increment or decrement may be as large as desired, as long it does not result in temperatures which are outside the temperature limits described above.

  Time increments or decrements may be as low as 0.1 second per cycle. You may make the increase or decrease as frequently as every cycle, and the increase or decrease can begin following any cycle. The time increment or decrement may be as large as desired, as long as it does not result in a dwell time in any cycle that is outside the limits described above.

Section 4
The Protocol Library Module

From the Protocol Library module you may examine saved Protocols, Plate Setups, and Notations prior to selection of files for editing. The Protocol Library facilitates review of and opening Post-run Data files.

At the top of the Protocol Library window are four tabs. Select any of these tabs to open the associated window.

- **View Protocols**: Provides information on the thermal parameters for the protocol specified and indicates when data will be collected and analyzed (see Figure 4.1);
- **View Plate Setup**: Provides information about the location of sample wells and the fluorophores that will be analyzed in each (see Figures 4.2, 4.3, 4.4);
- **View Quantities and Identifiers**: Displays information entered by the user when the protocol was created (see Figure 4.5);
- **View Post-Run Data**: Opens stored data files (see Figure 4.6).

From the Protocol Library module you may also **Edit**, **Run**, or **Print** the existing Protocol or Plate Setup, or create (**Custom**) a new file. Selecting **Edit**, **Run**, or **Custom** will transfer you to a window in the Protocol Workshop module (see Section 5 for details).

4.1 View Protocols

The **View Protocols** window of the Protocol Library module is the default window that appears when the iCycler program is opened (see Figure 4.1.) You may also enter this window by selecting the **View Protocols** tab at the top of the Protocol Library module.

The lower two-thirds of the window displays the file identified in the Protocol Filename text box both graphically and in spreadsheet format (see Figure 4.1). The graphical display shows the reaction temperature (on the y-axis) as a function of time (on the x-axis). In the graphical display:

- The bar across the top of the graphical display shows the cycle number;
- The numbers below the bar indicate the setpoint temperature for each step in the cycle (*i.e.*, the y-axis on the graph) and the dwell time specified for that step (*i.e.*, the x-axis on the graph).
- The presence of a camera icon on a particular cycle of the graphical display indicates that optical data will be collected at that step. A gray camera icon indicates that data will be collected for post-run analysis. A yellow camera icon indicates that quantitative data will be collected and analyzed in real time. A green camera icon indicates collection of melt curve data.

Details of specialized options, such as automatic increment and decrement of temperature or dwell time are provided in the spreadsheet but not in the graphical display. These functions are described in Section 5.1.4.

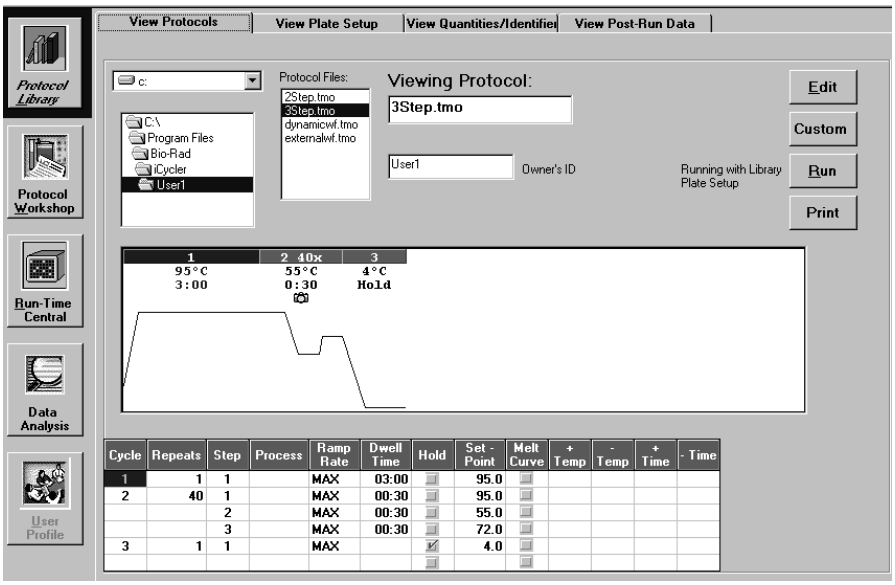


Fig. 4.1. Protocol Library / View Protocols Window. This is the default window that appears upon opening the iCycler Program.

The upper third of the window displays the following protocol file information.

- The Drive Location: The Protocol files shown here are stored on the C drive.
- The Directory Tree: The Protocol files shown here are stored in the User1 folder.
- The Protocol Filename menu: A list box of all protocol file names in the directory identified in the Directory Tree. All protocol filenames have a .tmo extension.
- The Protocol filename text box: The file name of the protocol displayed in the window.
- Owner's ID box: The log on name of the creator of the current protocol.

The right side of the window has the following active buttons. Selecting each of these buttons results in the following:

- **Edit**: Transfers the selected Protocol file to the Protocol Workshop/Edit Protocol window; this allows you to edit the protocol displayed on the screen (see Section 5.1).
- **Custom**: Transfers to the Protocol Workshop/Edit Protocol window; this allows you to create a new protocol (see Section 5.1). Selecting the Custom button rather than the Edit button allows you to write a new protocol beginning with a simple one-cycle, three-step thermal cycling program.
- **Run**: Transfers the selected Protocol file to the Protocol Workshop/Run Prep window; this allows you to run the currently displayed protocol (see Section 5.4).
- **Print**: Prints the spreadsheet section of the Protocol displayed on the screen.

4.2 View Plate Setup

Select the View Plate Setup tab near the top of the Protocol Library window to review stored Plate Setup files. You may choose to review these files looking only at the sample and standards assignments, the fluorophores assigned to each well, or by the quantities assigned to the standards defined. The default setting will show the Samples view (see Figure 4.2).

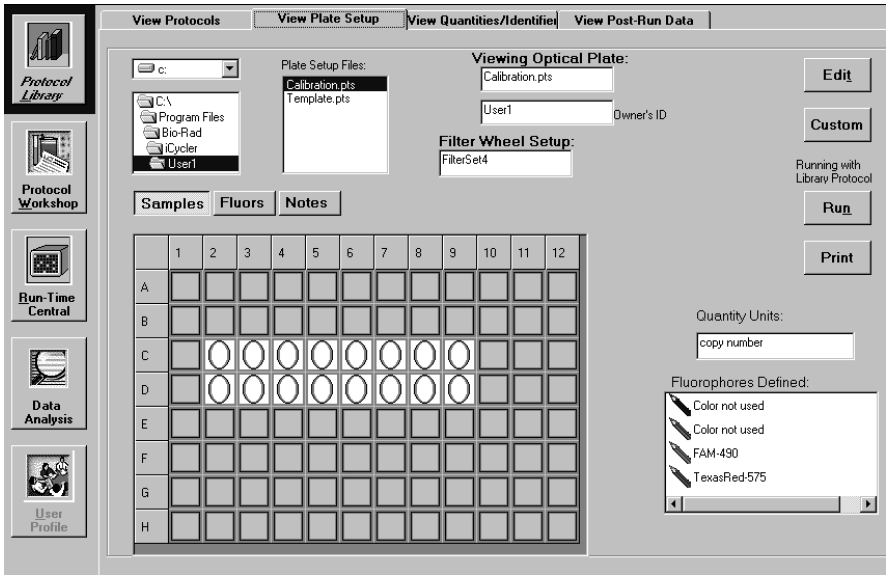


Fig. 4.2. Protocol Library / View Plate Setup Window, Samples View.

Several parts of this window are similar to the **View Protocols** window described above (compare Figures 4.1 and 4.2.)

The Plate Setup window displays the following plate setup file information:

- The Drive Location: Plate setup files are stored on the C drive.
- The Directory Tree: The directory location of the current plate setup; typically, plate setup files are stored in the User1 folder.
- The Plate Setup Filename menu: A list box of all plate setup file names in the directory identified in the Directory Tree; all plate setup filenames have a .pts extension.
- The Plate Setup Filename text box: The filename of the plate setup displayed in the window.
- Owner information box: The name of the creator of the current plate setup.

The View Plate Setup window also has the following active buttons:

- **Edit**: Transfers the selected Plate Setup file to the Protocol Workshop/Edit Plate Setup window; this allows you to edit the plate setup displayed on the screen (see Section 5.2).
- **Custom**: Transfers to the Protocol Workshop/Edit Plate Setup window; this allows you to create a new plate setup (see Section 5.2). Selecting the Custom button rather than Edit button allows you to design a new plate setup beginning with a blank plate layout.
- **Run**: Transfers to the Protocol Workshop/Run Prep window; this allows you to run the currently displayed protocol (see Section 5.4).

- **Print**: Prints the Plate Layout section of the Plate Setup file displayed on the screen.

The following information is displayed in the View Plate Setup window but not in the View Protocols window.

- The Filter Wheel Setup field box in the center of the window identifies the filter wheel setup used for collecting the data (see Section 1.2 for a discussion of how to insert filters into the filter wheel).
- The Units field box on the right side of the window indicates the units of measure that was used in setting up the standards. Units may be either copy number, micromole, nanomole, picomole, femtomole, attomole, milligram, microgram, nanogram, picogram, femtogram, or attogram.
- The Fluorophores Defined field box identifies each fluorophore with a corresponding color icon.
- The Plate Layout, an 8 by 12 grid comprising the lower portion of the View Plate Setup window, identifies the type of samples and their location on the plate. Selecting one of the three active buttons, **Samples**, **Fluors**, and **Notes**, immediately above the Plate Layout will display different information about the samples. The Protocol Library/View Plate Setup window, Samples view is the default window which appears when the View Plate Setup window is opened (Figure 4.2); in this case the **Samples** button is highlighted. Selecting the Fluors or Notes buttons toggles the grid to display the following information:
- **Samples** button: Displays the location of wells in the plate that will be assayed, and identifies those wells by icons specified as standards, unknowns, blanks, controls, pure dyes, or customized (see Figure 4.2).
- **Fluors** button: Displays the fluorophore to be analyzed in each well (see Figure 4.3). Wells that will be analyzed are shown in a color. The fluorophore associated with the color is identified in the Fluorophores Defined field box. Wells that will not be analyzed displayed in gray.
- **Notes** button: Displays any notes written about the plate setup.

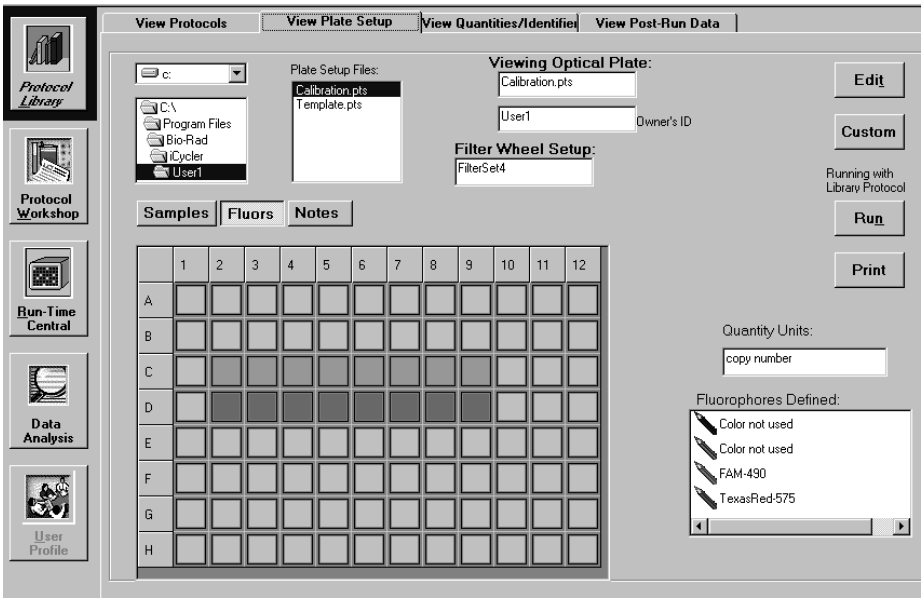


Fig. 4.3. Protocol Library / View Plate Setup window, Fluors view.

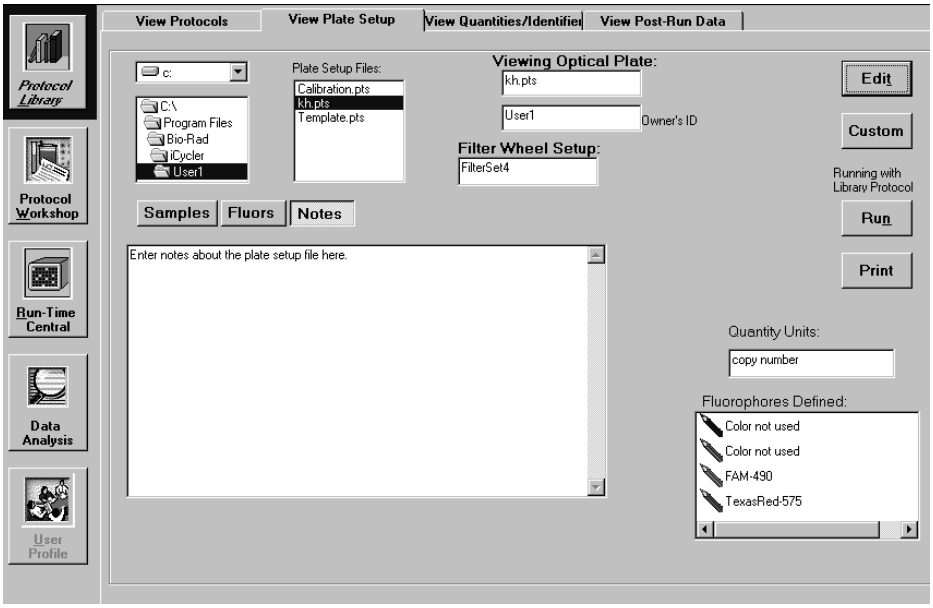


Fig. 4.4. Protocol Library / View Plate Setup window, Notes view.

4.3 View Quantities and Identifiers

Displays information about each individual well on the plate, one dye layer at a time.

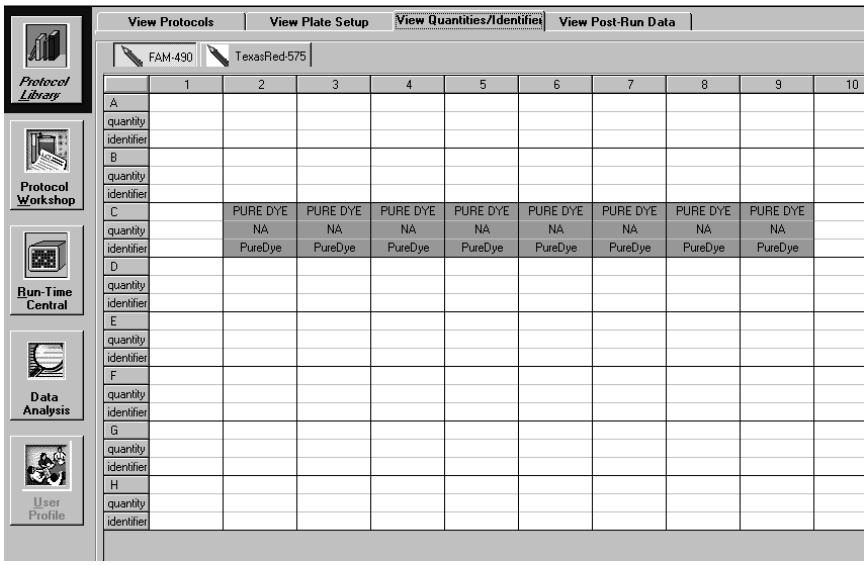


Fig. 4.5. Protocol Library / View Notations window.

4.4 View Post-Run Data

The View Post-Run Data window (Figure 4.6) may be used to open results of saved data files. To enter the View Post-Run Data window from another window in the Protocol Library, select the **View Post-Run Data** tab.

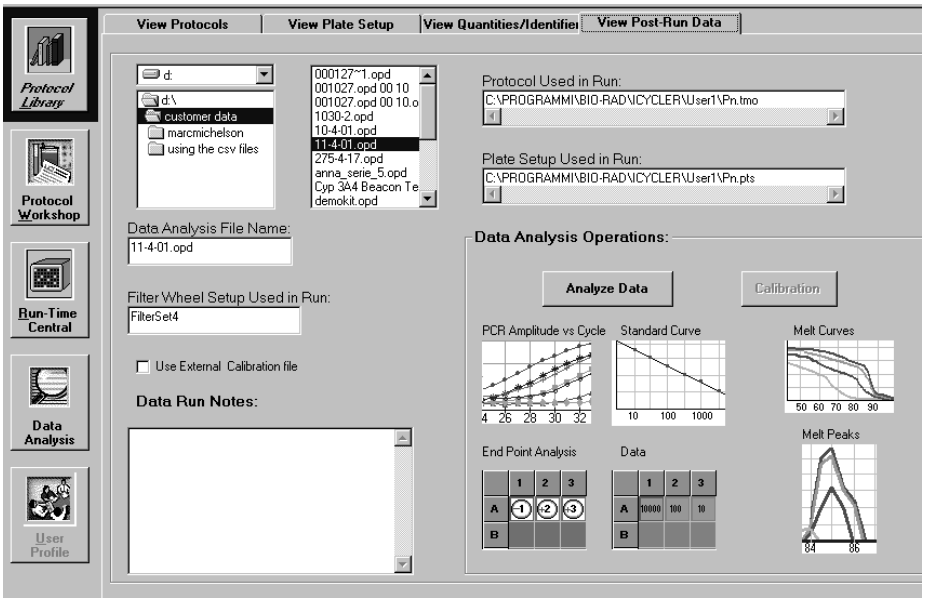


Fig. 4.6. Protocol Library / View Post-Run Data Window.

This window contains the following information:

- Drive location: To select the drive for the stored data file.
- Directory Tree: To select the folder containing the stored data file.
- Data Analysis Filename: Post-Run Data filenames have an .opd extension ("optical data").
- The Filter Wheel Setup Used in Run:
- The Protocol Used in Run: This is the filename and path for the Protocol file.
- The Plate Setup Used in Run: This is the filename and path for the Plate Setup file.
- Data Run Notes: These are notes entered by the user at the time of the run.

Choose **Analyze Data** for quantitative or melt curve experiments. Choose **Calibration** to reapply pure dye calibration data.

Caution: When you click **Calibration**, the current RME file will be updated with the information contained in the stored pure dye calibration run. If you want to prevent modification to the RME file, copy it to another folder before opening the saved pure dye calibration.

Section 5
The Protocol Workshop Module

The Protocol Workshop module allows you to create and make changes to Protocols and Plate Setups. Section 5.1 describes the layout of the Edit Protocol window and a discussion on writing and editing protocol files. Section 5.2 describes the organization of the Edit Plate Setup window and explains how to write and edit plate setup files. For Quick Guides relating specifically to Melt Curve, please see Section 8.2.

There are four tabs across the top of the Protocol Workshop window (Figure 5.1) which permit editing various aspects of protocols and plate setups:

- **Edit Protocol**: in this window you may specify the thermal parameters for the protocol and indicate when the data will be collected and analyzed (see Section 5.1, Edit Protocol.)
- **Edit Plate Setup**: in this window you may specify the location of the samples, standards, and the kinds of fluors that will be analyzed in each well (see Section 5.2, Edit Plate Setup.)
- **Quantities/Identifiers**: This window displays information entered in the Edit Plate setup window on an individual dye-layer basis.
- **Run Prep**: in this window you confirm the protocol file, plate setup file, and conditions for the run; this is the last step before running a protocol (see Section 5.4, Run Prep.)

Select each of these tabs to open the associated window.

5.1 Edit Protocol

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

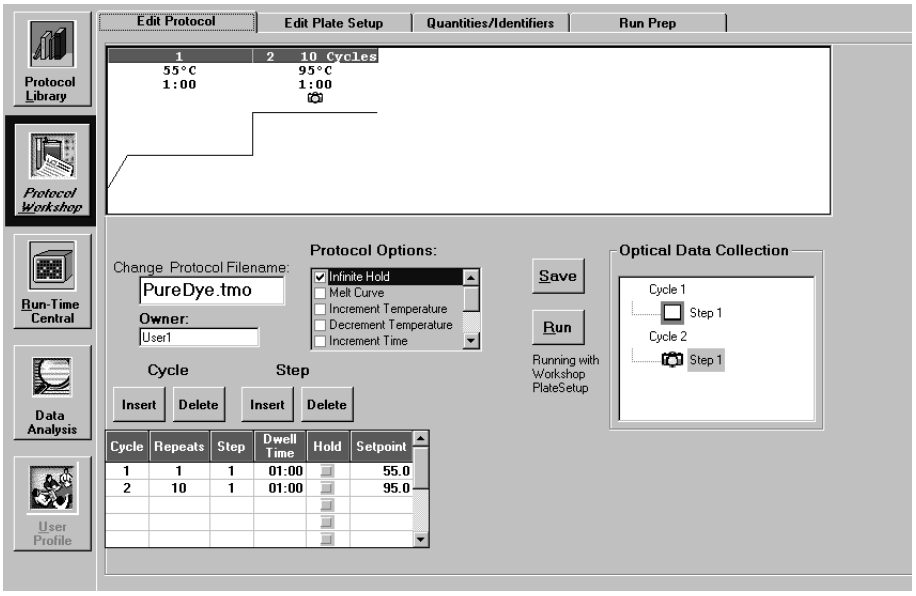


Fig. 5.1. Protocol Workshop / Edit Protocol Window. This shows the minimum programming spreadsheet.

A graphical display of the currently loaded protocol, showing reaction temperatures (on the y-axis) and dwell times (on the x-axis), is shown in the upper one-third of the window. The actual thermal protocol is displayed in an adjustable spreadsheet at the bottom of the window.

The center of the window contains:

- The **Protocol Filename** text box contains the protocol filename; the **Owner** field box contains the name of the person who saved the protocol;
- The **Protocol Options** box contains advanced options that can be applied to the protocol (see Section 5.1.2);
- The **Save** and **Run** buttons are used to save changes to the protocol and to run the protocol, respectively (see Section 5.1.5);
- The **Optical Data Collection** box may be used to specify the step at which the data are collected. This is described in Section 5.1.4.

The procedures for creating new protocols and editing existing ones are summarized in Sections 5.1.1 and 5.1.2. The graphical display and the thermal programming options are described in Sections 5.1.3 and 5.1.4, respectively. Programming in the spreadsheet and specifying optical data collection are described in detail in Sections 5.1.5 and 5.1.6, respectively, and the saving of protocol files is detailed in Section 5.1.7.

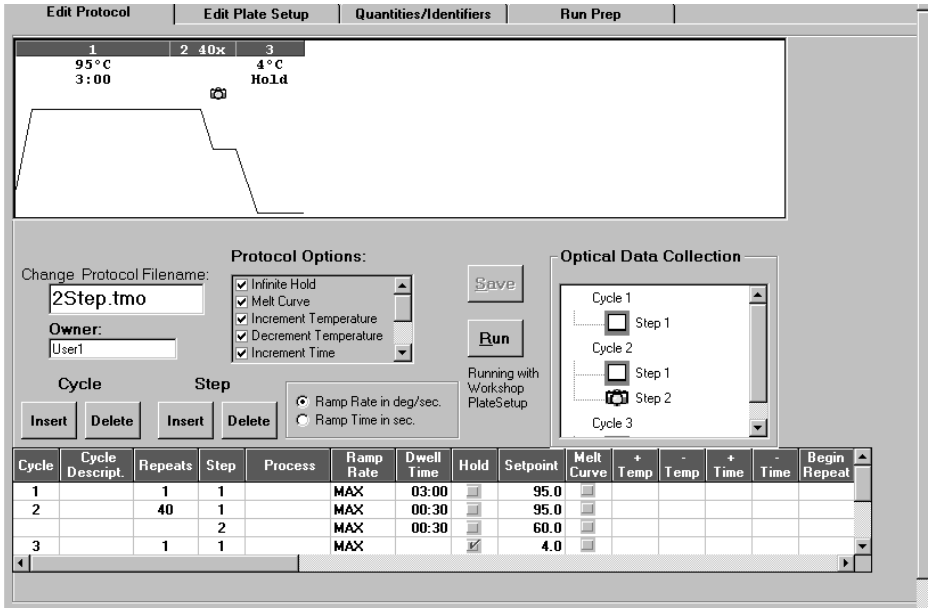


Fig. 5.2. Protocol Workshop / Edit Protocol Window, as it appears with all of the Protocol Options selected.

5.1.1 Quick Guide to Creating a New Protocol

1. From the Protocol Library, select the *View Protocols* tab.
2. Click **Custom**. The *Edit Protocol* window of the Protocol Workshop will open.
3. Start in the spreadsheet at the bottom left of the window and fill in the thermal cycling protocol. As you make changes in the spreadsheet, they are reflected in the graphical representation at the top of the window. The cycle being edited is shown in blue on the bar across the top of the graph and is highlighted in blue in the spreadsheet.
   - Double click in a time or temperature field to change the default settings.
   - Insert a new cycle in front of the current cycle by clicking **Insert Cycle** and then clicking anywhere on the current cycle in the spreadsheet. Insert a cycle after the last cycle by clicking **Insert Cycle** and then clicking anywhere on the first blank line at the bottom of the spreadsheet. If you right mouse click on the Insert Cycle button, you can choose to insert a One-, Two- or Three-step cycle. Click the Insert Cycle button again to deselect it.
   - Delete cycles by clicking **Delete Cycle** and then clicking anywhere on that cycle in the spreadsheet. Click the Delete Cycle button again to deselect it.
   - Insert a step in front of the current step by clicking **Insert Step** and then clicking on the current step. If you right mouse click on the Insert Step button, you can choose to insert the new step before or after the current step. Click the Insert Step button again to deselect it.
   - Delete steps by clicking **Delete Step** and then clicking anywhere on the line containing the step to be deleted. Click the Delete Step button again to deselect it.
4. If you want to add any protocol options, choose them by clicking in the check box next to its description in the Protocol Options box.

- If you select Infinite Hold, a new column will appear in the spreadsheet. To specify that a temperature be held indefinitely, click in the Hold column of the spreadsheet next to the temperature to be maintained. A red check mark will appear in the Hold column.
- If you select Melt Curve, three new columns appear. The first is a check box which you click to indicate the cycle for the melt curve data collection. Two additional columns, + Temp and - Temp, will also appear. Enter the increment (or decrement) to be accomplished at each cycle, and the total number of repeats of the cycle. The melt curve will begin at the temperature listed in the setpoint field and increase or decrease by the amount specified in the + Temp or - Temp field, respectively, with each repeat of the cycle. A green camera icon will appear in the optical Data Collection Box.
- If you select Increment or Decrement of Temperature or Time, three new columns will appear. Specify the amount of change in temperature or time, the first cycle at which the change is to occur and the frequency with which the change is to occur. For example, to increase time by 5 seconds beginning with the third cycle and to further increase the time by 5 seconds every other cycle after that you would enter 00:05 in the + Time column, 3 in the Begin Repeat column and 2 in the How Often column.
- If you select Ramping, another box will appear in which you indicate if you wish to specify the Ramp Rate or Ramp Time. Double click in the Ramp Rate column and enter a value or choose Min or Max from the pull down menu.
- You can enter a description of the cycle or the step by first checking Cycle Name or Step Process, respectively, from the Protocol Options Box. Click inside the Cycle Description or Process box and enter a name or choose one from the pull down menu.

5. In the Optical Data Collection box, specify the cycles at which fluorescent data are to be collected for quantitative experiments.
   - Click once in a square to indicate that data are to be collected for post-run analysis. A camera with a gray lens will appear in the box and on the graphical display.
   - Click a second time to indicate that fluorescent data are to be collected and analyzed in real time. The lens on the camera icon will turn yellow.
   - Click a third time to deselect data collection for that cycle. The camera icon will disappear.
6. Double click in the *Change Protocol Name* field and enter a new name for the protocol.
7. Click **Save**. A Save dialog box will appear, click **Save** again.

Note: You may save plate setup and protocol files to the iCycler folder or any subfolder of the iCycler folder.

5.1.2 Quick Guide to Editing a Stored Protocol

1. From the Protocol Library, select the *View Protocols* tab.
2. In the top left corner of the window, select the drive where the stored plate setup file resides.
3. Use the directory tree to locate the folder containing the stored protocol file.
4. Select the desired file from the *Protocol Files* box.
5. Click **Edit**. The *Edit Protocol* window of the Protocol Workshop will open.

6. Start in the spreadsheet at the bottom left of the window and fill in the thermal cycling protocol. As you make changes in the spreadsheet, they are reflected in the graphical representation at the top of the window. The current cycle begin edited is shown in blue on the bar across the top of the graph and is highlighted in blue in the spreadsheet.

    - Double click in a time or temperature field to change the settings.
    - Insert a new cycle in front of the current cycle by clicking **Insert Cycle** and then clicking anywhere on the current cycle in the spreadsheet. Insert a cycle after the last cycle by clicking **Insert Cycle** and then clicking anywhere on the first blank line at the bottom of the spreadsheet. If you right mouse click on the Insert Cycle button, you can choose to insert a One-, Two- or three-step cycle. Click the Insert Cycle button again to deselect it.
    - Delete cycles by clicking **Delete Cycle** and then clicking anywhere on that cycle in the spreadsheet. Click the Delete Cycle button again to deselect it.
    - Insert a step in front of the current step by clicking **Insert Step** and then clicking on the current step. If you right mouse click on the Insert Step button, you can choose to insert the new step after the current step. Click the Insert Step button again to deselect it.
    - Delete steps by clicking **Delete Step** and then clicking anywhere on the line containing the step to be deleted. Click the Delete Step button again to deselect it.

7. If you want to add any protocol options, choose them by clicking in the check box next to its description in the *Protocol Options* box.

    - If you select Infinite Hold, a new column will appear in the spreadsheet. To specify that a temperature be held indefinitely, click in the Hold column of the spreadsheet next to the temperature to be maintained. A red check mark will appear in the Hold column.
    - If you select Melt Curve, three new columns appear. The first is a check box which you click to indicate the cycle for the melt curve data collection. Two additional columns, + Temp and - Temp, will also appear. Enter the increment (or decrement) to be accomplished at each cycle, and the total number of repeats of the cycle. The melt curve will begin at the temperature listed in the setpoint field and increase or decrease by the amount specified in the + Temp or - Temp field, respectively, with each repeat of the cycle. A green camera icon will appear in the optical Data Collection Box.
    - If you select Increment or Decrement of Temperature or Time, three new columns will appear. Specify the amount of change in temperature or time, the first cycle at which the change is to occur and the frequency with which the change is to occur. For example, to increase time by 5 seconds beginning with the third cycle and to further increase the time by 5 seconds every other cycle after that you would enter 00:05 in the + Time column, 3 in the Begin Repeat column and 2 in the How Often column.
    - If you select Ramping, another box will appear in which you indicate if you wish to specify the Ramp Rate or Ramp Time. Double click in the Ramp Rate column and enter a value or choose Min or Max from the pull down menu.
    - You can enter a description of the cycle or the step by first checking Cycle Name or Step Process, respectively, from the Protocol Options Box. Click inside the Cycle Description or Process box and enter a name or choose one from the pull down menu.

8. In the Optical Data Collection box, specify the cycles at which fluorescent data are to be collected for quantitative experiments.
    - Click once in a square to indicate that data are to be collected for post-run analysis. A camera with a gray lens will appear in the box and on the graphical display.
    - Click a second time to indicate that fluorescent data are to be collected and analyzed in real time. The lens on the camera icon will turn yellow.
    - Click a third time to deselect data collection for that cycle. The camera icon will disappear.
9. If you want to write over your old protocol, click **Save**.
10. If you want to rename your protocol, double click in the *Change Protocol* Name field and enter a new name for the protocol and click **Save**. A Save dialog box will appear, click **Save** again.

Note: You may save the plate setup and protocol files to the iCycler folder or any subfolder of the iCycler folder.

5.1.3 Graphical Display

The graph at the top of the Protocol Workshop / Edit Protocol window shows a pictorial display of the temperature cycling program (Figure 5.2.) The bar across the top of the graphical display indicates the cycle number for each section of the protocol; the active cycle (*i.e.*, the one being edited) is highlighted. The setpoint temperature and the dwell time for each step are displayed below the bar. Note that occasionally space limitations do not permit display of all temperature and time settings.

You can expand the time axis in the graphical display by holding down the **Ctrl** key on the keyboard while dragging the cursor over a section of the graph (the cursor will turn to a two-headed arrow). When the left mouse button is released, the time axis will expand. Use the scroll bar at the bottom of the graph to move across the time axis of the graph. To zoom out, place the cursor anywhere in the area of the graph and click the left mouse button.

When optical data collection is specified (see Figure 5.2 and Section 5.1.6), a camera icon is shown below the temperature at the step(s) the data will be collected. A camera icon with a gray lens indicates that optical data will be collected for post-run analysis. A camera icon with a yellow lens indicates that optical data will be analyzed in real time.

Placing the cursor anywhere over the graphical display and pressing the right mouse button displays the following menu:

- **Copy Graph**: allows you to copy the graph to the clipboard so that it can be pasted to another application such as a text or a spreadsheet program.
- **Print Graph**: allows you to print a copy of the graph.

5.1.4 Spreadsheet and Protocol Options

The bottom third of the Protocol Workshop / Edit Protocol window displays a spreadsheet that shows each cycle and step in the protocol. There are five columns which are always present and which must be specified for any protocol (see Figure 5.1.) These are:

- Cycle: a group of up to 9 steps (numbered 1–9) that are repeated; there may be up to 9 cycles (numbered 1 to 9) in a protocol;
- Repeats: the number of times a cycle is repeated; cycles may be repeated up to 600 times; the repeat number is displayed only for the first step of a multi-step cycle.

- Step: an individual temperature or dwell time event; each cycle may have up to 9 steps;
- Dwell time: the time the step is maintained at the specified temperature; this may vary from 1 sec (0:01) to 99 min, 59 sec (99:59);
- Setpoint: the specified temperature that the reaction step will achieve; this may be within the range of 4.0 to 100.0 °C.

In addition, a number of options may be specified for each step. All of these options are first selected from the Protocol Options check box. Selecting an option from this check box will result in one or more additional columns being displayed in the spreadsheet (see Figure 5.2.) Choose any of these additional options as follows:

1. In the Protocol Options check box, select a check box corresponding to the desired new protocol; a check will appear in the box and one to three new columns will appear in the spreadsheet.
2. Use the scroll bar on the right side of the Protocol Options check box to scroll through the options.
3. Enter the necessary information in the new column(s) in the spreadsheet as described below.

If all of the options are selected, the spreadsheet displays the columns shown in Figure 5.2.

The following list describes the function of the Protocol Options, indicates the Heading in the spreadsheet associated with the protocol, and indicates how to activate these.

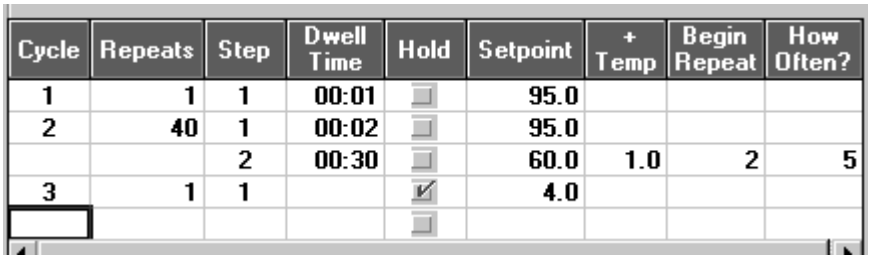
Protocol Option	Spreadsheet column heading	Description of function Specifying the function
Infinite hold	Hold	This function allows you to specify a step that has an infinite dwell time. From the Protocols Options box, select **Infinite Hold**; a new column, “Hold” will appear in the spreadsheet. In the “Hold” column of the spreadsheet, Click to select an indefinite pause for that step. Click the **check box** a second time to deselect the pause; the checkmark will disappear from the box.
Melt Curve	Check Box	Click this box to generate a melt curve. The melt cycle will begin at the Setpoint temperature and then change by the increment or decrement set in one of the next two columns. The final temperature of the melt cycle is determined by the number of repeats and the amount of increment or decrement.
	+ Temp	Indicate the incremental increase in temperature desired with each repeat in the melt cycle.
	- Temp	Indicate the incremental decrease in temperature desired with each repeat in the melt cycle.
Increment Temperature	+ Temp	This function allows you to specify a step in which the temperature is increased at successive cycles. When this function is chosen, you must specify the temperature increase (the “+ Temp” column in the spreadsheet), the cycle (repeat) at which the temperature increase is to be initiated (the “Begin Repeat” column in the spreadsheet), and how often the temperature is to be increased (the “How Often?” column in the spreadsheet). 1. From the Protocol Options box, select **Increment Temperature**. Three columns will appear in the spreadsheet and must be specified: (see Figure 5.3). 2. In the “+ Temp” column of the spreadsheet, select the cell corresponding to the step at which you want the temperature to increase; the selected cell will be active and appear with a heavy border. 3. Enter the desired temperature increase (in °C); the adjacent cell in the “Begin Repeat” column will appear with a heavy border. 4. Enter the repeat at which the increase is initiated; the adjacent cell in the “How Often?” column will appear with a heavy border. 5. Enter how often (in repeat number) the temperature is to be increased. For example, assume the temperature is to be increased by 1 °C beginning on the second repeat, then increased by an additional 1 °C every fifth repeat. In the column “+ Temp” enter 1.0, in the column “Begin Repeat” enter 2, and in the column “How often?” enter 5 (see Figure 5.3.)

Protocol Option	Spreadsheet column heading	Description of function Specifying the function
Decrement Temperature	- Temp	This function allows you to specify a step in which the temperature is decreased at successive cycles. 1. From the Protocol Options box, select **Decrement Temperature**; three columns will appear in the spreadsheet and must be specified: (see Figure 5.2). 2. In the "- Temp" column of the spreadsheet, select the cell corresponding to the step at which you want the temperature to decrease; the selected cell will be active and appear with a heavy border. 3. Enter the desired temperature decrease (in °C); Select **Enter**; the adjacent cell in the "Begin Repeat" column will appear with a heavy border. 4. Enter the repeat at which the decrease is initiated; the adjacent cell in the "How Often?" column will appear with a heavy border. 5. Enter how often (in repeat number) the temperature is to be decreased. For example, assume the temperature is to be decreased by 1 °C beginning on the second repeat, then decreased by an additional 1 °C every fifth repeat. In the column "- Temp" enter 1.0, in the column "Begin Repeat" enter 2, and in the column "How often?" enter 5.
Increment Time	+ Time	This function allows you to specify a step in which the time is increased at successive cycles. 1. From the Protocol Options check box, select **Increment Time**; three columns will appear in the spreadsheet and must be specified: (Figure 5.2). 2. In the "+ Time" column of the spreadsheet, select the cell corresponding to the step at which you want the time to increase; the selected cell will be active and appear with a heavy border. 3. Enter the desired time increase (in min and sec); the adjacent cell in the "Begin Repeat" column will appear with a heavy border. 4. Enter the repeat at which the increase is initiated; the adjacent cell in the "How Often?" column will appear with a heavy border. 5. Enter how often (in repeat number) the time is to be increased. For example, assume the time is to be increased by 5 sec beginning on the second repeat, then increased by an additional 5 sec every third repeat. In the column "+ Time" enter 00:05, in the column "Begin Repeat" enter 2, and in the column "How often?" enter 3.

Protocol Option	Spreadsheet column heading	Description of function Specifying the function
Decrement Time	- Time	This function allows you to specify a step in which the time is decreased at successive cycles. 1. From the Protocol Options check box, select **Decrement Time**; three columns will appear in the spreadsheet and must be specified (see Figure 5.2): 2. In the "- Time" column of the spreadsheet, select the cell corresponding to the step at which you want the time to decrease; the selected cell will be active and appear with a heavy border. 3. Enter the desired time decrease (in min and sec); the adjacent cell in the "Begin Repeat" column will appear with a heavy border. 4. Enter the repeat at which the decrease is initiated; the adjacent cell in the "How Often?" column will appear with a heavy border. 5. Enter how often (in repeat number) the time is to be decreased. For example, assume the time is to be decreased by 2 sec beginning on the tenth repeat, then decreased by an additional 2 sec every fifth repeat. In the column "- Time" enter 00:02, in the column "Begin Repeat" enter 10, and in the column "How often?" enter 5.
Ramping	Ramp Rate	This function allows you to adjust the rate of temperature change between successive steps in a cycle. You may specify a rate of change (ramp rate) or you may specify the time (ramp time) required to achieve the setpoint temperature. The default setting is the maximum ramp rate, which is indicated by "MAX" in the Ramp Rate field in the spreadsheet. For heating, the maximum ramp rate is 3.3 °C/sec. For cooling, the maximum ramp rate is 2.0 °C/sec. For both heating and cooling the minimum ramp rate ("MIN") is 0.1 °C/sec. The ramp time may be as short as 1 sec or as long as 99:59 sec, as long as it does not result in a ramp rate greater than the maximum or less than the minimum. From the Protocol Options check box, select **Ramping**; a Ramp Rate dialog box will appear above the spreadsheet and a new column, "Ramp Rate" will appear in the spreadsheet (see Figure 5.2.) In the Ramp Rate dialog box, select the radio button to indicate whether you are specifying the ramp rate or the ramp time.

Protocol Option	Spreadsheet column heading	Description of function Specifying the function
		In the “Ramp Rate” column, select the cell corresponding to the step in which you want to specify the ramp rate; the selected cell will be active, appear with a heavy border, and an arrowhead will appear on the right side of the cell.
		Select the **Down Arrow**; the cell will become active and MAX will become highlighted.
		To choose the minimum ramp time, select MIN; “MIN” will replace “MAX” in the cell.
		To specify a ramp time or ramp rate, type a number in the active cell. Note that if you enter a value for either the ramp time or the ramp rate which exceeds the maximum ramp rate, the value is changed to “MAX”; if you enter a value that results in a ramp rate slower than the minimum ramp rate, the value is changed to “MIN”.
Cycle Name	Cycle Description	This function allows you to add cycle descriptions to a protocol.
		From the Protocol Options check box, select **Cycle Name**; a new column, “Cycle Descript.” will appear in the spreadsheet.
		In the “Cycle Descript.” column of the spreadsheet, select the cell corresponding to the step at which you want to specify the description; the selected cell will be active and appear with a heavy border and a scroll arrow on the right side of the cell.
		Select the **Down Arrow**; a predefined list of descriptive names will be displayed.
		Select one of the descriptors to select that name. Alternatively, enter other designations by typing in the active cell, then pressing **Enter**.
Step Process	Process	This function allows you to add descriptions for each step within a cycle. This option may be activated in the same manner as Cycle Name above.

Note that if an error is made in programming, at least one of the fields in the spreadsheet is highlighted in yellow. For example, if a step with 30 cycle repeats and a dwell time of 40 sec is programmed for a time decrement of 10 seconds beginning on the fifth cycle and decreasing by 10 seconds every other cycle, the field in the "- Time" column containing 00:10 will be highlighted in yellow since by the 13th cycle the dwell time of that step would be less than zero. This problem may be corrected by reducing the decrement time to 3 seconds or less, by changing the cycle to begin the decrement to 24 or higher, or by changing the time of the occurrence to every sixth cycle. Note that the iCycler program will allow you to save a protocol in which a highlighted cell is found, although it will not allow you to run the protocol.



Cycle	Repeats	Step	Dwell Time	Hold	Setpoint	+ Temp	Begin Repeat	How Often?
1	1	1	00:01	☐	95.0			
2	40	1	00:02	☐	95.0			
		2	00:30	☐	60.0	1.0	2	5
3	1	1		☑	4.0			
				☐				

Fig. 5.3. Protocol Workshop / Edit Protocol Window, showing entries in the spreadsheet for Increment Temperature.

5.1.5 Editing Cycles and Steps

Cycles and Steps in the thermocycling program may be inserted and deleted using the **Insert** and **Delete** buttons above the spreadsheet. A new cycle may consist of one, two, or three steps.

- To insert a cycle:
  a. Right mouse click the **Insert** button under "Cycle"; an active box will appear showing **1-step**, **2-step**, and **3-step**. (Note, a one-step cycle is the default setting.)
  b. Select the appropriate number of steps; the active box will disappear and the **Insert** button will be highlighted.
  c. In the spreadsheet, select a cell within a cycle that will follow the inserted cycle; a new cycle will be inserted into the protocol.
- To delete a cycle:
  a. Click the **Delete** button under "Cycle"; the **Delete** button will be highlighted.
  b. In the spreadsheet, select a cell within the cycle to be deleted; all of the steps in the cycle will be deleted.
- To insert a step:
  a. Click the **Insert** button under "Step"; the **Insert** button will be highlighted.
  b. In the spreadsheet, select a cell in the step that will follow the inserted step; a new step will be inserted into the cycle.
  c. (Note, by placing the cursor over the **Insert** button then clicking the right mouse button, an active box appears which allows you the option of inserting the new step **Before** or **After** the step in the spreadsheet; the default is **Before**.)
- To delete a step:
  a. Click the **Delete** button under Step; the **Delete** button will be highlighted.
  b. In the spreadsheet, select a cell in the step to be deleted; the selected step in the cycle will be deleted.

If you inadvertently select any of the Insert or Delete buttons, you may deselect it by clicking it again.

5.1.6 Optical Data Collection box

The Optical Data Collection box at the right side of the Edit Protocol window allows you to specify the step(s) in which data will be collected for real time analysis or for post-run analysis (see Figure 5.2). Identify the step(s) at which data will be analyzed as follows:

- Click once on a camera box at a step in the cycle at which data are to be collected; a gray camera icon will appear in the box to indicate that collected data are to be saved for possible post-run analysis.

- Click on the camera box a second time; the gray camera icon will change to a yellow camera icon indicating that data are to be analyzed and displayed in real time.
- Clicking on the camera box a third time will clear the icons.
- If you program a melt curve cycle, a green camera icon will appear in the Optical Data Collection box. It appears and disappears as you click the melt curve option in the thermal protocol options box.

In addition to a camera icon appearing in the appropriate step in the Optical Data Collection box, a camera icon will also appear at the appropriate step in the graphical display at the top of the Protocol Workshop / Edit Protocol window.

Data may be collected during one or more steps in any one cycle. However, data may not be collected in more than one cycle.

5.1.7 Saving the Protocol

After you have finished editing the protocol, save the file.

- Edited Protocols may be saved with the existing name by clicking **Save**. The old protocol will be overwritten with the new protocol.
- Edited and new Protocols may be saved with new names as follows:
  1. To rename an existing protocol or to name a new protocol, doubleclick with the left mouse button in the Protocol Filename text box; the current filename will be highlighted.
  2. Type a new name in the text field.
  3. Click **Save**; a Save Protocol As dialog box will appear on the screen with '.tmo' added to the filename.
  4. Click **Save** to save the protocol with the given name or select **Cancel** to return to the Protocol Workshop.

5.2 Edit Plate Setup

The plate setup file contains information about the samples and fluorophores in the sample plate.

In the Protocol Workshop/Edit Plate Setup window, existing plate setups may be modified and new plate setups may be created. Sections 5.2.1 and 5.2.2 summarize the procedures for writing new plate setup files and for editing existing plate setup files, respectively. A plate setup includes the well positions of samples and standards, the identification of replicates, the concentrations of standards, and information about which fluorophores are included in the assay.

There are two views in the Edit Plate Setup window that display slightly different information depending on whether the **Samples** (Figure 5.4) or **Fluorophores** (Figure 5.5) tab is selected. Sections 5.2.3 and 5.2.4 provide detailed explanations on identifying the proper samples and fluorophores, respectively, in the plate layout. Section 5.2.5 describes the filter wheel setups and fluorophore selection. Section 5.2.6 describes saving plate setup files.

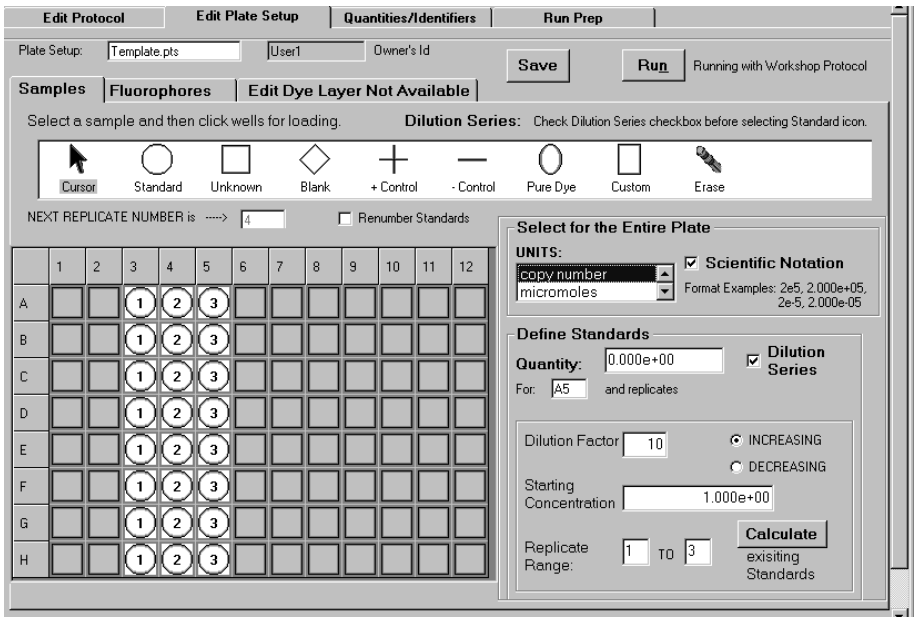


Fig. 5.4. Protocol Workshop/Edit Plate Setup Window, Samples view.

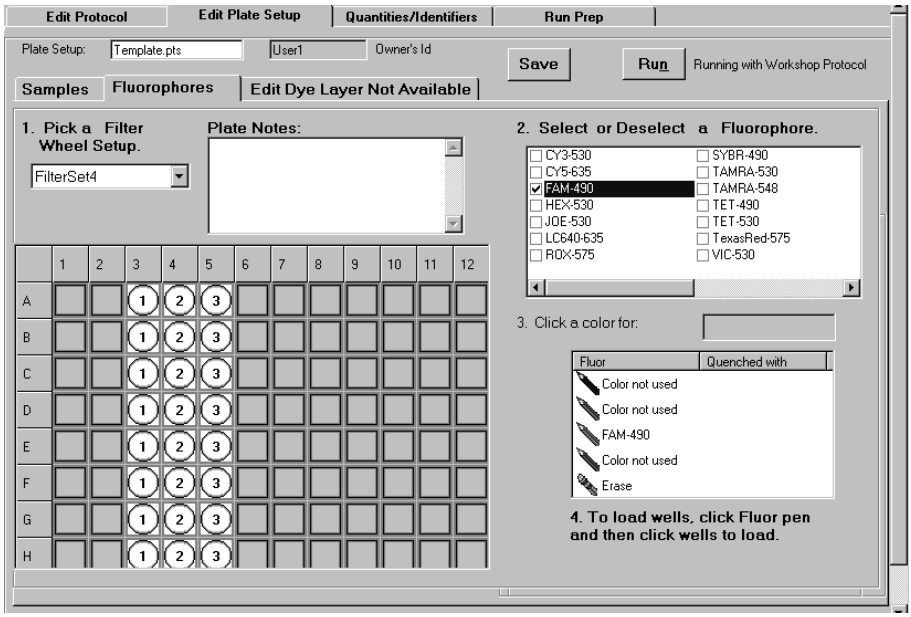


Fig. 5.5. Protocol Workshop / Edit Plate Setup Window, Fluorophores view. From this window you may specify the well locations for the selected fluorophores.

The following information is common to all windows. The lower two-thirds of the window shows the Plate Layout. If you are creating a new plate setup, the Plate Layout will be blank. If you are editing an existing plate setup, those well positions which have been previously defined will be indicated on the layout. Near the top of the window are the following:

- The **Plate Setup Filename** text box contains the file name.
- The **Owner** text box contains the name of the user who created the file name.
- The **Save** and **Run** buttons may be used to save changes to the protocol and to run the protocol, respectively.
- The **Samples** button opens a window containing information about the location of the standards and samples, and the quantity of each standard. This is discussed in detail in Section 5.2.3.
- The **Fluorophores** button opens a window containing information about the filter wheel setup and the fluorophores used in each well. This is discussed in detail in Section 5.2.4.

5.2.1 Quick Guide to Creating a New Plate Setup

1. From the Protocol Library, select the View Plate Setup tab.
2. Click **Custom**. The Edit Plate Setup window of the Protocol Workshop will open.
3. Specify the type of sample in each well. Choose the appropriate icon from the box and then click in a well to define it.
    - As you specify each Standard, you must also enter the quantity in the Define Standards box. If your standards are a dilution series, you can define the quantity of the first standard and the software will calculate the concentrations of all the other standards.
    - Click the top left corner to select all 96 wells at once.
    - Click on a number or letter to select an entire column or row, respectively.
    - Drag across wells to specify replicate standards or unknowns.
    - If you make a mistake, use the Erase icon. You can erase the settings in all wells at once by clicking in the top left corner of the plate.
    - You can renumber the standards or unknowns by entering a number in the box labeled Next Replicate Number.
4. Click **Fluorophores**. A set of instructions for defining the fluorophores will be displayed.
5. Choose one of the filter wheel setups available from the pull down menu, a set of fluorophores will be displayed.
6. Click the box next to the desired fluorophore.
7. Click on a crayon icon to associate the fluorophore with a color.
8. Repeat Steps 6 and 7 to specify additional fluorophores.
9. Click a crayon icon for the first fluorophore and then click on each well to be monitored for that fluorophore.
    - Click the top corner to select all 96 wells at once.
    - Click on a number or letter to select an entire column or row, respectively.

- If you make a mistake, use the Erase icon. You can erase the settings in all wells at once by clicking in the top left corner of the plate.

10. Repeat Step 9 for each additional fluorophore.
11. Double click in the Plate Setup text box and enter a new name for the plate setup file.
12. Click **Save**.

5.2.2 Quick Guide to Editing a Stored Plate Setup

1. From the Protocol Library, select the View Plate Setup tab.
2. In the top left corner of the window, select the drive where the stored plate setup file resides.
3. Use the directory tree to locate the folder containing the stored plate setup file.
4. Select the desired file from the Plate Setup Files box.
5. Click **Edit**. The Edit Plate Setup window of the Protocol Workshop will open.
6. Specify the type of sample in each well. Choose the appropriate icon from the box and then click in a well to define it.
   - Numbering of standards and unknowns will begin where it last left off.
   - You can change the definition of a well by clicking over it with the new sample type or you can clear the definition by using the Erase icon.
   - As you specify each Standard, you must also enter the quantity in the Define Standards box. If your standards are a dilution series, you can define the quantity of the first standard and the software will calculate the concentrations of all the other standards.
   - Click the top corner to select all 96 wells at once.
   - Click on a number or letter to select an entire column or row, respectively.
   - Drag across wells to specify replicate standards or unknowns.
   - If you make a mistake, use the Erase icon. You can erase the settings in all wells at once by clicking in the top left corner of the plate.
   - You can renumber the standards or unknowns by entering a number in the box labeled Next Replicate Number.
7. Click **Fluorophores** and look in the Fluorophore Palette box. If you do not need to add additional fluorophores to this list, skip to step 12 below.
8. Choose one of the filter wheel setups available from the pull down menu, a set of fluorophores will be displayed in the Fluorophore Selection box.
9. Click the box next to the desired fluorophore in the Fluorophore Selection box.
10. Click on a crayon icon in the Fluorophore Palette box to associate the fluorophore with a color.
11. Repeat Steps 9 and 10 to specify additional fluorophores.

12. Click a crayon icon for the first desired fluorophore and then click on each well to be monitored for that fluorophore.
    - Click the top corner to select all loaded wells at once.
    - Click on a number or letter to select an entire column or row, respectively.
    - If you make a mistake, use the Erase icon.
13. Repeat Step 12 for each additional fluorophore.
14. If you want to save the file under a new name, double click in the Plate Setup text box and enter a new name for the plate setup file.
15. Click **Save**.

5.2.3 Edit Plate Setup/Samples

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

5.2.3.1 Icon functions

The toolbar menu contains the following icons that may be used to identify the types of samples in each well of the Plate Layout (see Figure 5.4).

Icon	Description
Cursor	Used to select a specific well in the Plate Layout.
Standard	Used to identify wells that contain known amounts of the template being assayed. Wells in the Plate Layout identified as standards are given sequential numbers when they are selected. Replicate wells containing the same concentrations of standards are labeled with the same number (see Section 5.2.3.4.) The Define Standards box on the right side of the Edit Plate Setup window is active when the Standard icon is active and a well containing the standard is selected; the concentration of the template in each of the Standards must be specified in the Quantity text box and the units of measure are specified from the Units list box (see Section 5.2.3.3).
Unknown	Used to identify wells that contain samples with an unknown quantity of the template being assayed. Wells in the Plate Layout identified as unknowns are given sequential numbers when they are selected; replicate wells containing the same amount of template are indicated by the same number (see Section 5.2.3.4.)
Blank	Used to identify wells without any reaction mixture.
+Control	This icon allows you to identify wells with a sample containing the compound of interest; in Endpoint Assays, this is useful to define the range of the results.
-Control	Used to identify wells that contain samples missing one or more of the parts of the reaction mixture; in Endpoint Assays, this is useful to define the range of the results.
Pure Dye	Used to identify wells containing pure fluorophore for pure dye calibration protocols.
Custom	Used to identify wells with unique descriptors.
Erase	Used to clear identification from previously specified wells.

5.2.3.2 Activating and Using Icons from the Toolbar Menu to Specify Well Locations (see Figure 5.4)

Select an **Icon** from the Toolbar menu to activate it; the icon will appear highlighted.
In the Plate Layout, specify a sample location by selecting the desired well(s); the icon will appear in the designated well(s).

Multiple wells may be selected in several ways:

1. To specify an entire column, select one of the numeric column headings;
2. To specify an entire row, select one of the alphabetic row designations;
3. To select a group of wells, place the cursor over one well, hold down the left mouse button, then drag the cursor over the desired wells.

In these cases, icons will appear in the designated column, row, or group. It is important to use one of these methods for specifying wells containing replicates of the same standards or samples. Icons appear in the highlighted wells after the mouse button is released. Note that previously specified wells are overwritten with the new icon when they are highlighted a second time.

5.2.3.3 Defining Standards

1. Click **Dilution Series** to bring up box for specifying the dilutions. This checkbox is available when the "cursor" icon is selected.
   - Enter value in **Dilution Factor**. The default is 10 for a 10-fold dilution series.
   - Enter **Starting Concentration**. To use scientific notation, for example, change the 1.000e+00 default setting to the correct starting concentration. All exponential values are considered positive by default, so it is not necessary to enter a plus sign.
   - Click whether it is **increasing** or **decreasing**.
   - Enter the **Replicate Range** to be included in the standards value calculation. For example, standard replicates numbered 1 to 5.
   - Click **Calculate** to apply specifications.
2. Select the **"Standard"** icon and highlight wells for Standard 1. Continue selecting for each Standard and its replicates as specified under dilution series.
3. Use **Renumber Standards** box or **Next Replicate Number** to renumber if a mistake is made in numbering.
   - For example, if your standard replicates are numbered 1, 2, 3, and 5, you can check **Renumber Standards** box to renumber them as 1, 2, 3, and 4.
4. To check that standards have been properly calculated, use the cursor to click on the wells containing standards and view the quantity in the **Define Standards Quantity** box.
5. Quantities of the standards may be changed by repeating step 1.
6. Proceed with defining the rest of plate setup, defining fluorophores, and save.
7. Plate setup with samples, fluorophores, and quantities can be viewed using the **Quantities/Identifiers** tab of the Protocol Workshop.

Note: If you specify your standards to range from replicate group 1 through 5, the calculation assumes that the fold dilution is constant over the entire range. This means if you specify a 10-fold dilution factor, beginning with 1e10 for replicate 1, the software will assign values of 10^9 for replicate 2, 10^8 for replicate 3, 10^7 for replicate 4, and 10^6 for replicate 5. If you only define standards 1, 2, 3, and 5 (no standard 4), the software will still assign a value of 10^6 for replicate 5.

5.2.3.4 Renumbering Standards and Unknowns

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

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

If renumbering or editing standards results in a gap in the numbering sequence, *e.g.* there are standards 1, 2, 3, and 5, click the box Renumber Standards to assign them 1, 2, 3, and 4.

5.2.4 Edit Plate Setup/Fluorophores

The Edit Plate Setup window, Fluorophores view, allows you to specify which fluorophores are being used and in which wells they are to be assayed. You can get to the Edit Plate Setup window by choosing **Edit** or **Custom** from the View Plate Setup window of the Protocol Library. The choice of Edit or Custom affects the presentation of the Fluorophores view of this window.

- If you chose Custom, then you must choose a filter wheel setup and specify fluorophores before making well assignments (Step 1 below).
- If you chose Edit, then first inspect the list of fluorophores presented in the Fluorophore Palette box. If the desired fluorophores are present, then proceed with editing (Step 6 below). If the desired fluorophores are not present in the box, then choose a new filter wheel setup (Step 1 below), if necessary, before specifying the fluorophores.

Procedure:

1. Choose one of the Filter Wheel Setups from the pull down list box (see Figure 5.5). As you make your selection, a list of fluorophores will appear in the Fluorophore Selection box.
2. In the Fluorophore Selection box, choose a fluorophore by clicking in the check box next to its name. The remainder of the fluorophores will be grayed out after you make a selection. To deselect a fluorophore, click the check box again.
3. Now assign a color to the fluorophore by clicking on one of the crayon icons in the Fluorophore Palette box. You must choose a crayon that is not yet assigned to a fluorophore (one labeled *Color Not Used*).
4. You may repeat steps 2 and 3 until as many as four fluorophores have been selected and assigned colors.
5. Now you are ready to describe which wells are to be monitored for which fluorophores. Click on the crayon icon of the desired fluorophore in the Fluorophore Palette box. The name of the fluorophore will be highlighted.
6. Click on each well to be monitored for this fluorophore. As you select a well, it will be filled in with the color of the crayon. You can click the top left corner of the plate layout to select all loaded wells. You can click on a number or letter to select an entire column or row, respectively. You can also drag the mouse across contiguous regions of the plate layout.

7. Repeat Steps 5 and 6 for each additional fluorophore.
8. If you want to change the fluorophores to be monitored in a well, you must first use the Erase icon to clear the well of the existing fluorophores. Use this tool in the same way as one of the crayon tools. Then repeat Steps 6 and 7 for the new fluorophores.
9. When you have defined the fluorophores and the wells to be monitored on the plate, double click in the Plate Setup field at the top left of the window and enter a name for the plate setup file and click **Save**. A standard Save dialog box will open and you click **Save** again.

   Note: You may save plate setup and protocol files to the iCycler folder or any subfolder of the iCycler folder.

5.2.5 Filter Wheel Setup and Fluorophore Selection

The information in the Filter Wheel Setup file tells the software where to position the excitation and emission filter wheels in order to collect optical data on each individual fluorophore. The available filter wheel setups are FilterSet 4 and FilterSet 5. (Plate setup files created with different versions of the filter wheel setups must be updated before they can be used with the multi-color releases. See Section 2.5.3.) **It is critically important that the excitation and emission filters are in the correct positions in the filter wheels, please confirm that the filters are in the proper location regularly.**

In the tables below for each current filter wheel setup are the positions for the filters, their optical characteristics, and the recommended fluorophores.

FilterSet4

Position	Excitation	Emission	Recommended Fluorophores
2	490/20X	530/30M	Fluorescein (FAM),SYBR Green
3	530/30X	575/20M	HEX, TET, VIC, JOE, Cy3
4	545/30X	585/20M	TAMRA/Cy3
5	575/30X	620/30M	Texas Red, ROX
6	635/30X	680/30M	Cy5, LC640

FilterSet5

Position	Excitation	Emission	Recommended Fluorophores
2	490/20X	530/30M	Fluorescein (FAM),SYBR Green
3	530/30X	575/20M	HEX, TET, VIC, JOE, Cy3
4	53030X	620/30M	Ethidium Bromide
5	610/30X	660/20M	LC640
6	660/30X	710/20M	Cy5.5, LC705

Note: The filter designation 490/20X indicates that this filter will allow light from 480/500 nm to pass through. The first number, 490, indicates the center of the wavelength of light. The second number, 20 indicates the total breadth of wavelengths of light that can pass through it. X indicates excitation only and M indicates emission only types of filters. Excitation and emission filters are not interchangeable.

Fluorophore Selection for Multiplex PCR: Any fluorophore for which there is a filter pair and a pure dye calibration solution may be used successfully in a single-color experiment, but careful consideration must be given to the choice of fluorophores in multicolor experiments. Successful multiplex PCR analysis depends on optimized assay design. A critical element in the design of multiplexed real time PCR assays is the selection of fluorophores; the more spectrally discrete the fluorophores, the better the data quality.

Guidelines for fluorophore selection.

1. For every fluorophore on the plate, the detector must collect data with a unique corresponding filter pair. If you are using three fluorophores on a plate, then the software will expect data from three different filter pairs.

2. Fluorophores are selected from the Fluorophores view of the Edit Plate Setup screen in the Protocol Workshop. The fluorophore names are each appended with a number representing the wavelength of the excitation filter that will be used to measure it. For example, FAM-490 means that the software will position the filter wheels so that the 490/530 filter pair is used to take fluorescein (FAM) measurements; Texas Red-575 indicates that the 575/620 filter pair will be used to take measurements. It is this information (the appended number) along with the filter wheel setup that tells the software (1) what filter pair to use and (2) where that filter pair is located.

3. You may not choose two fluorophores that are both appended with the same number because that would mean that two different fluorophores would be measured with only one filter pair.

4. Some fluorophores appear more than once in the fluorophore list, but are appended with different numbers. This means that there is more than one possible filter pair that can be used to measure that fluorophore. Note that RME data (pure dye calibration data) are specific to fluorophore/filter pair combinations, so if you have RME entries for TET-490, they are not applicable to the use of TET-530.

5. Some combinations of fluorophores create overlapping spectra that are difficult to resolve in multiplex experiments and should be avoided. For example, if the signal of fluorophore A is stronger through the filter pair for fluorophore B, than the signal of fluorophore B is through the filter pair for fluorophore B, you should not use A and B in multiplex experiments. This is true, for example, of HEX and TAMRA. The high quantum efficiency of HEX (relative to TAMRA) makes the HEX signal through the TAMRA filter pair stronger than the TAMRA signal through the TAMRA filter pair.

6. We have not exhaustively tested all possible combinations of fluorophores, but our best recommendation is to use the set or some subset of FAM, HEX, Texas Red and Cy5 when combining multiple fluorophores in the same well.

Fluorophore Selection Table for Multiplexing

	FAM	HEX	TET	JOE	VIC*	Cy3	TAMRA	Texas Red	ROX	Cy5
FAM		+++	+++	++	+++	+++	+++	+++	+++	+++
HEX	+++							+++	+++	+++
TET	+++						+ +	+++	+++	+++
JOE	++						+	+++	+++	+++
VIC*	+++						+	+++	+++	+++
Cy3	+++							+	+	+++
TAMRA	+++		+ +	+	+			++	++	+++
Texas Red	+++	+++	+++	+++	+++		++			+++
ROX	+++	+++	+++	+++	+++	+	++			+++
Cy5	+++	+++	+++	+++	+++	+++	+++	+++	+++	

+++ Indicates the best fluorophore combination for multiplex experiments.

++ Indicates a fluorophore combination that should work for multiplex, but is not optimal.

+ Indicates the least optimal fluorophore combination for multiplex experiments.

* To calibrate the iCycler iQ for VIC please contact Technical Support at 1-800-4BIORAD.

5.2.5.1 Fluorophore Selection Box

When a Filter Wheel Setup file is selected, the fluorophores defined in that setup are listed in the Fluorophore Selection box. Next to the name of each fluorophore is a check box. As you click in the check box next to a fluorophore, its name is highlighted and you must assign a color to it in the Fluorophore Palette box. You may select as many as four fluorophores from the Fluorophore Selection box and assign a color to each of them in the Fluorophore Palette box. You should only select fluorophores for which you have filters, and those filters must be in the positions specified by the Filter Wheel Setup file chosen above.

5.2.5.2 Fluorophore Palette Box

Each time you choose a fluorophore from the Fluorophore Selection box you must assign a color to it in the Fluorophore Palette box. Click on a crayon associated with the fluorophore and use it to select wells to be monitored for that fluorophore. The same rules apply as when using the sample selection tools:

- Click the top corner to select all loaded wells at once.
- Click on a number or letter to select an entire column or row, respectively.
- If you make a mistake, use the Erase icon.

5.2.5.3 Plate Notes

Enter notations to be saved with the plate setup file.

5.2.6 Saving a new Plate Setup

After you have completed the Plate Setup in the **Samples** and **Fluorophores** views in the Edit Plate Setup window, save the file as follows:

- Edited Plate Setups may be saved with the existing name by clicking **Save** ; the old plate setup will be overwritten with the one.
- Edited and new Plate Setups may be saved with a new name by doubleclicking in the Plate Setup Filename text box; the old filename will be highlighted.
  a. Type a new name in the text field.
  b. Click **Save**; a Save Plate Setup As dialog box will appear on the screen with '.pts' added to the text name.
  c. Click **Save** to save the plate setup with the given name or select **Cancel** to return to the Protocol Workshop.

It is possible to run a plate setup without first saving it by selecting the **Run** button; the Run Prep window will appear (see Section 5.3). In this case, '(M)' is placed before the plate setup name indicating that the saved plate setup has been modified.

5.3 Run Prep

Selecting the Run button from either the Protocol Library or the Protocol Workshop opens the Run Prep window (Figure 5.6). This window allows you to confirm the following before starting the thermal cycling procedure:

- The Protocol file selected for the run.
- The Plate Setup file selected for the run.

You must indicate

- The reaction volume
- The type of protocol. Choose PCR Quantification/Melt Curve for all runs except for Pure Dye Calibrations.
- The source of Well Factors.

Click Begin Run to start the run. A dialog box will open automatically so that you can name the data file. Data will be written to the file at the end of each cycle, if the non-data collection steps are sufficiently long that writing to file will not interfere with data collection. All data are automatically saved at the conclusion of the experiment.

5.3.1 Autosave

Data are now automatically saved during a run to a file name designated by the user before the run occurs. The software will estimate the time required to save data from each cycle during protocol execution. If there is sufficient time during the non data collection steps, the data will be saved at the end of each cycle. Otherwise date are saved automatically at completion of the protocol.

1. Click **Run** after selecting a **Protocol** and **Plate Setup** from the **Protocol Library** Module.
2. The **Run Prep** window will open, displaying the **Thermal Protocol** and **Optical Plate Setup** files selected.
3. Click **Begin Run** and a dialog box "**Select Optical Data File Name**" will open (Figure 5.7). The software creates a default name for you from the date and time setting on your computer. (*e.g.* **Data 19-Apr-01 1103**)
4. Rename the .opd file and select where the file is to be saved. Click **Save** and the run will proceed.
5. If you do not wish to rename your file at that time, the software saves the file under the default name (Data with the current date). You may rename the file after the run is over.

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

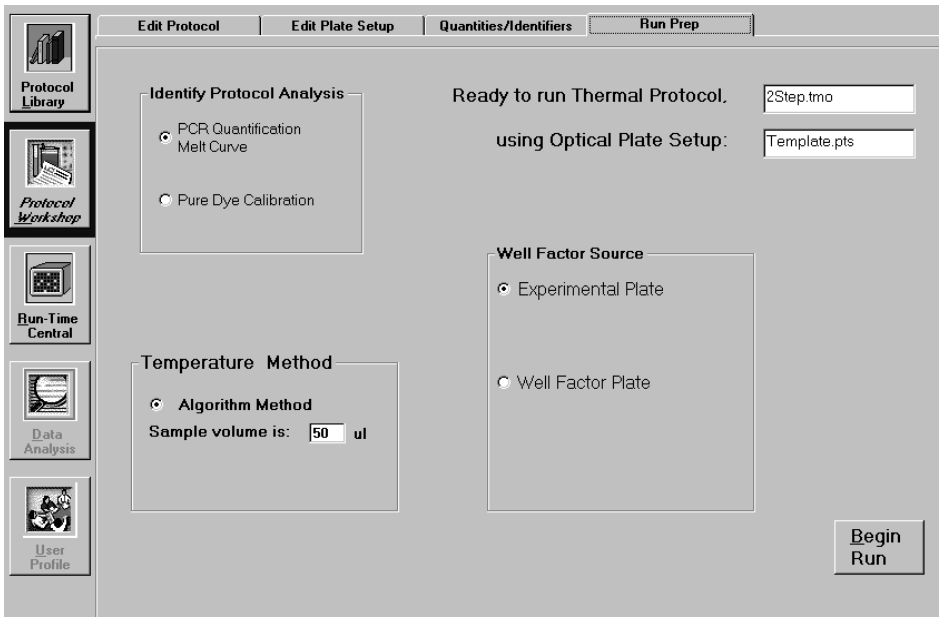


Fig. 5.6. Protocol Workshop / Run Prep Window.

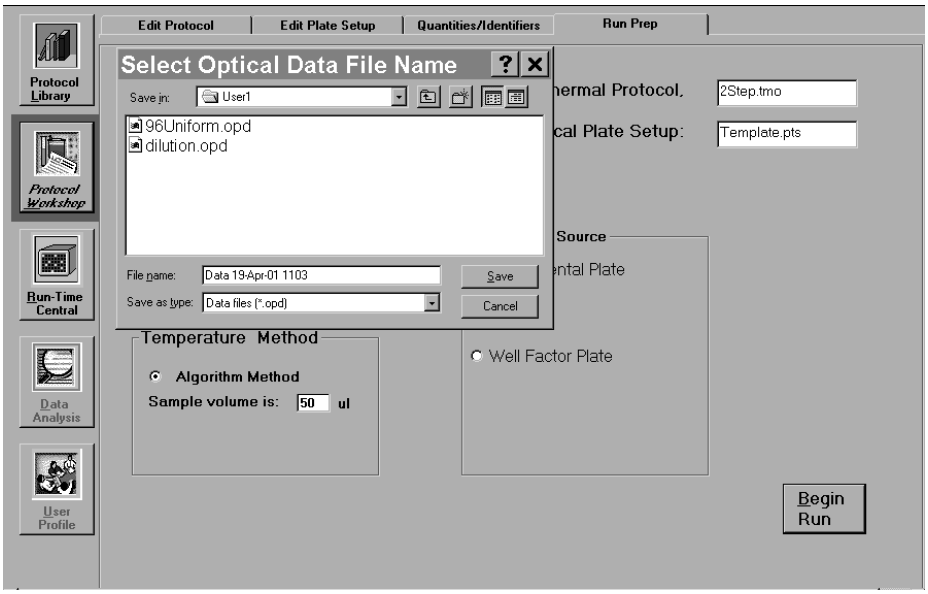


Fig. 5.7. Select Optical Data File Name window before run begins.

Section 6
The Run-Time Central Module

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

6.1 Thermal Cycler

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

- **Pause/Stop**: Used to stop a running protocol.
- **Running Protocol** and **Running Plate Setup**: These buttons allow you to toggle the display on the bottom half of the screen between one describing the thermal behavior of the iCycler and one monitoring the fluorescence of the individual wells as the protocol is carried out. These buttons are discussed in greater detail in the following pages.

Field boxes in the upper part of the window display the following information:

- **Start Time**: The time the protocol was initiated.
- **Stop Time (est)**: The approximate time the protocol will finish.
- **Dwell Time**: This is the programmed Dwell time of the current step or of the next step if the instrument is heating or cooling between steps.
- **Dwell Time Remaining**: This box displays the time remaining in the current step.
- **Current Cycle, Step, and Repeat**: These boxes show the cycle, step and repeat in progress.

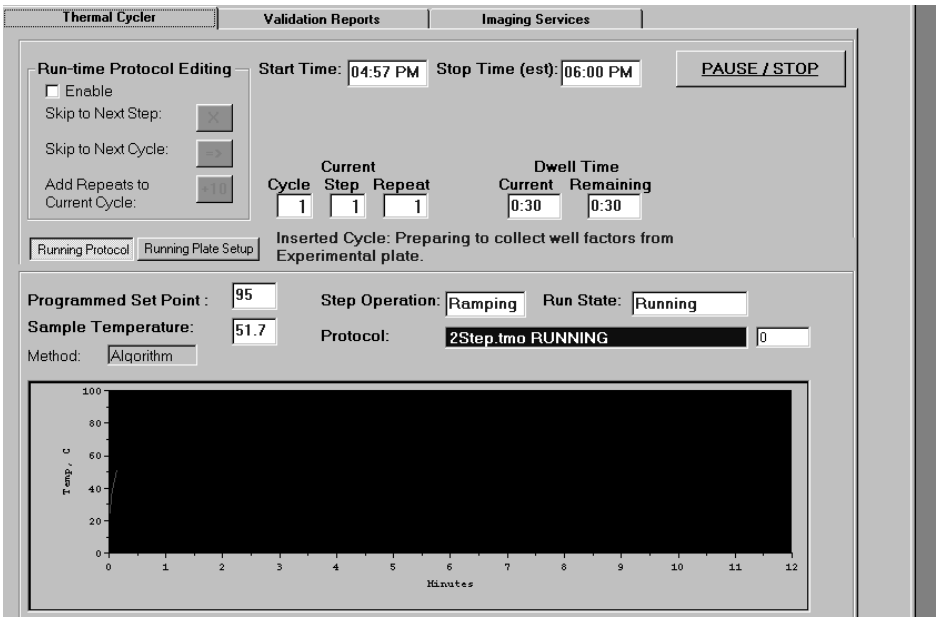


Fig. 6.1. Run Time Central window, Running Protocols view.

6.1.1 Run-Time Protocol Editing

There are three choices available for modifying a protocol during a run: **Skip to Next Step**, **Skip to Next Cycle** and **Add 10 Repeats**. During a run, these options are available by accessing the **Running Protocol** tab in the **Run Time Central** module. The options are listed in the **Run Time Protocol Editing** box and are accessed by clicking the **Enable** box (Figure 6.2).

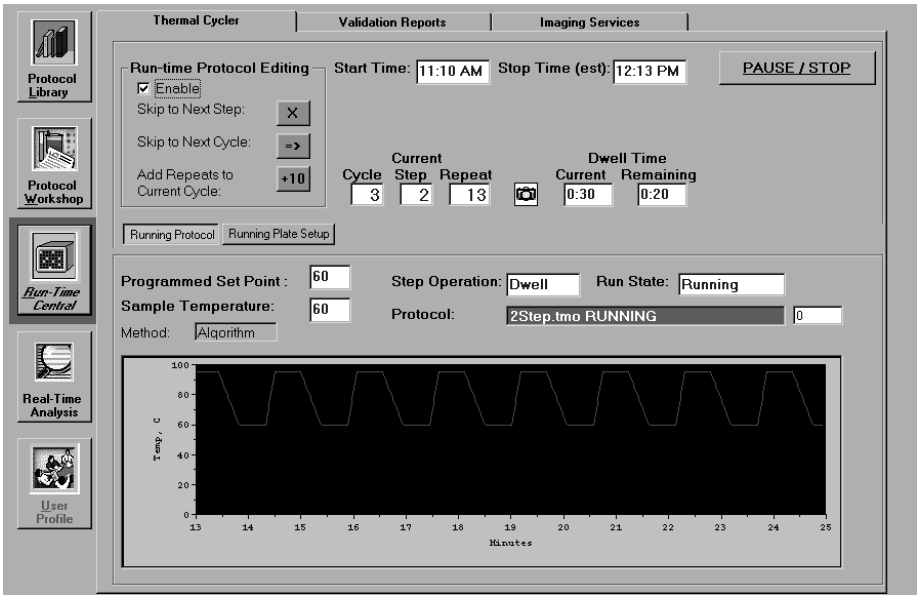


Fig. 6.2. Run-Time Protocol Editing Options to modify run as it is occurring.

6.1.1.1 Skip to Next Step

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

Note: The error message "**Action Denied**" will appear if you click this option when it is unavailable. This will NOT interrupt or terminate the run.

6.1.1.2 Skip to Next Cycle

- Click **Skip to Next Cycle** to complete the current repeat of the present cycle before skipping to skip to the next cycle. For example, you can use this feature when your samples have clearly crossed the threshold, and you want to skip to the melt cycle of your protocol.
- This feature is available when the protocol is running, when the base is ramping, and when the run is in pause mode.

6.1.1.3 Add 10 Repeats

- Click **Add 10 Repeats** for adding additional repeats to the current cycle. This button may be clicked multiple times, but is limited to 600 repeats total.
    - For example, it may be necessary to add more repeats to a run in an experiment amplifying low copies of DNA to allow all samples to cross threshold. Click **Add 10 Repeats** to an amplification cycle of 30 repeats to make it 40 repeats.
- **Add 10 Repeats** may be combined with **Skip to Next Cycle** to reduce the number of repeats in a cycle. Click **Skip to Next Cycle** during the dwell time of the current repeat to cancel the remaining repeats of the current cycle, then immediately click Add **10 Repeats**.
    - For example, to reduce the number of repeats from 50 repeats to 25 repeats, click **Skip to Next Cycle** during the dwell of repeat 5. Immediately click **Add 10 Repeats** twice. This will add 20 repeats to the 5 repeats that have already occurred, resulting in 25 repeats total for the cycle.
- This feature is available when the protocol is running, when the base is ramping, and when the run is in pause mode.

Note: Modifications to the protocol are updated on the protocol displayed on the base module or within the thermal information displayed on the **Thermal Cycler** tab of the **Run-Time Central** module.

6.1.2 Running Protocol

When the **Running Protocol** button is selected, the bottom half of the screen displays thermal information about the currently running protocol. The programmed setpoint temperature and the current temperature of the sample are displayed at the top left.

A graph of the sample temperature is shown in the graphical display at the bottom of the screen.

To the right of the Temperature boxes are the following:

- **Step Operation**: This field box displays *Ramping* or *Dwell* to show the current operation of the instrument.
- **Protocol**: This field box shows the name of the currently running protocol.
- **Run State**: This field box displays the status of the run, either Running or Paused.

6.1.3 Running Plate Setup

Click the **Running Plate Setup** button to view the fluorophores selected in the plate setup. In Run Time Central window, **Running Plate Setup** view, the bottom half of the screen shows the plate layout of the current plate setup on the left side of the window and the filename of the plate setup and the Filter Wheel Setup file on the right side of the window. Each well that is defined to contain the monitored fluorophore is shown in its associated color. (For example, in Figure 6.2 all defined wells contain fluorescein.) Select any other defined color icon in the Fluorophore Palette box on the right to show wells monitored for that fluorophore. Select the bottom four-colored square to show those wells containing multiple fluorophores.

Note: The Graph function in Run Time Central window, **Running Protocol** view is not enabled in this version of the software.

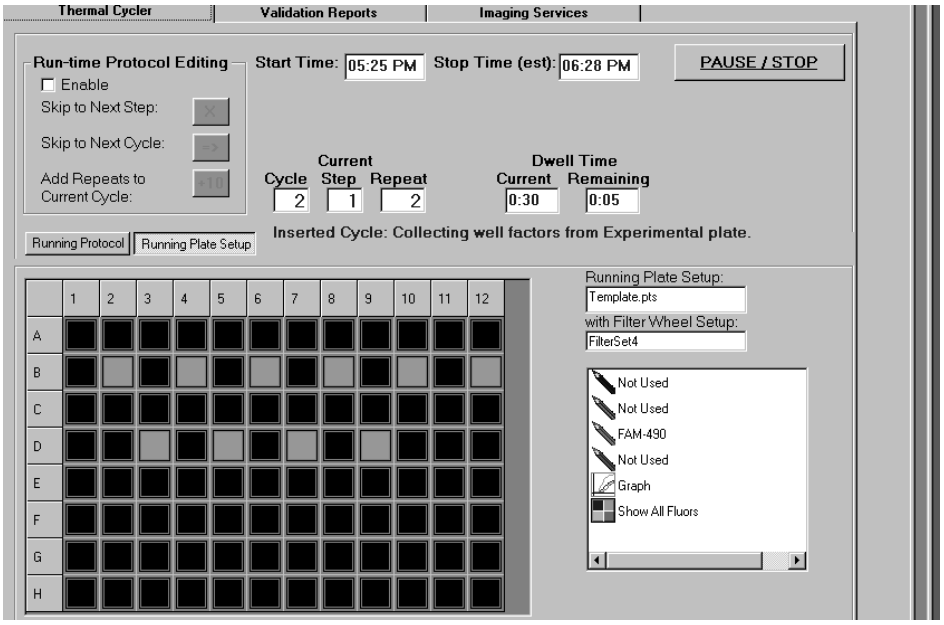


Fig. 6.2. Run Time Central window, Running Plate Setup view.

6.1.4 Pause / Stop

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

Clicking **Pause / Stop** brings up a new dialog box on the screen. Click **Continue Running Protocol** to resume the thermal cycling protocol. Click **End Protocol** to terminate the experiment.

When you pause the thermal cycler beyond the specified dwell time, the pause appears on the graphical display as a lengthening of that step along the time axis. After the run is complete, see the validation report from iCycler Thermal Cycler to confirm any times that the iCycler was paused (see iCycler Instruction Manual, Section 4.3). Validation Report Tab in this screen is not active.

6.2 Imaging Services

The main use of Imaging services is to adjust the masks, but there are several others uses. You may also use it to capture an image of an experimental plate to check the response of a probe or to assess the completion of a reaction. With a plate image you can obtain fluorescence readings for each of the 96 individual wells. The Imaging Services screen is shown in Figure 6.3.

Note: This window is automatically closed when you select another TAB in the software.

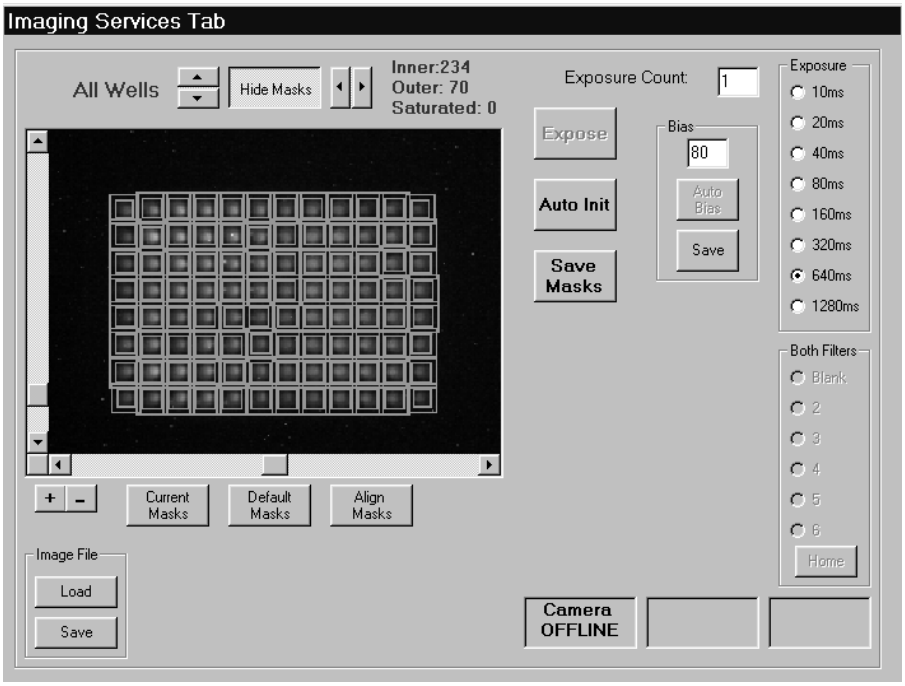


Fig. 6.3.

6.2.1 Description

- At the top of the screen are the average inner and outer readings for the 96 wells on the plate and the number of saturated pixels in the image. When you select a single well the surrounding mask becomes blue and the inner and outer readings and saturation of that particular well are displayed.
- The arrow keys at the top are used to manually position the masks.
- There are radio buttons to position the filter wheels and to control exposure length.
- There are boxes indicating whether an iQ optical module is connected (camera is online/offline) if intensifier is ON/OFF and another one that reports the lid (door) status.
- The AutoBias button initiates automatic adjustment of system bias and that value can be saved.
- There is a button to initiate Exposure and a box (Exposure count) to control the number of exposures that are collected and averaged before display.
- The AutoInit button begins the automated process of selecting the best fitting masks and aligning them.
- There are a series of buttons below the image used to help in manual adjustments.
- Image files may be saved from or loaded into Imaging Services.

6.2.2 Adjusting the Masks

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

Each individual mask consists of a pair of concentric squares. The inner square should be centered on the well so that all fluorescent signals from the well fall within it. The outer square surrounds the inner one, and all signal collected within the region defined by the outer square, except for the signal from the region defined by the inner square, is considered background. A data point for a well is calculated by first taking the difference between the inner and outer readings (well signal minus background) at a particular moment in time.

Auto Init automatically positions each individual mask in an optimal position. There are also manual methods for adjusting the position of masks.

The process of adjusting the masks consists of

- autobiasing the detector
- capturing an image
- loading the mask file
- adjusting the masks
- saving the mask file

Procedure: You must capture an image of a 96-well plate with some fluorophore in each well. It does not matter which fluorophore is used as long as it is compatible with one of the filter pairs in your detector. You may use the External Well Factor solution in combination with any filter pair. The same volume and concentration of solution should be present in every well.

1. Dilute 600 µl of 10X external well factor solution with 5.4 ml of ddH2O and pipet 50 µl of 1X solution into each well of a 96-well plate. Cover the plate with a piece of optically clear sealing tape, spin it briefly to bring all solution to the bottom of the wells and place the plate in the iCycler.
2. Open the iCycler software and open Imaging Services.
3. Click **AutoBias** to initiate the process of detector optimization. When the correct bias is reached, the setting in the Bias box will be shown in bold and the Save button will become active. **Save** the bias setting so that the next time bias optimization is initiated, it will begin with this saved setting and make the process of optimization faster.
4. **Home** the excitation and emission filter wheels (they move together).
5. Move the filter wheels to the appropriate position, depending on the fluorophore, by clicking the associated radio button.
6. Click **Expose**. An image of the plate will be displayed. Examine the image for saturated pixels which are shown in magenta. If you detect saturated pixels, reduce the exposure time and collect another exposure. Continue reducing exposure time until no saturation is detected. If your first image does not show any saturation, increase the exposure time until saturated pixels are detected, then reduce exposure time to the longest time that does not result in saturated pixels.
7. Open up the mask file, by placing the mouse over the image of well A1 (the top left well), and click while holding down the Shift key. Alternatively click **Show Masks,** then use the arrow keys to move the masks so that the top left mask is approximately centered over well A1.
8. Click **Auto Init** and the software will search for the best fitting masks and optimize their positions. An alert is displayed when mask alignment is completed or if it fails. If automatic mask alignment fails, repeat steps 8 and 9; if it fails again, try the manual adjustment as described below.

9. When the masks are properly set, click **Save Masks**. The new positions will be automatically written to the mask96.ini file.

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

Manual Adjustment to Mask Alignment: If the Auto Init feature fails to align the masks, the plate image may be either too small or too large for the masks. The software will bring up the a mask file that best fits the current image.

1. Visually inspect the mask and use the + or - button to enlarge or shrink the masks, respectively.
2. Make gross adjustments to the masks by clicking the arrow buttons above the displayed image. Move the masks so that most of the wells are centered inside the inner squares.
3. When it is approximately correct, click **Align Masks**. Concentrate on aligning the center columns with the arrow keys before clicking Align Masks. **The mask can be improved by clicking Align Masks a second time since the final quality of the masks is a function of the beginning quality**.
4. Any changes made to the masks may be discarded by clicking **Default Masks**.
5. Visually confirm the mask alignment and then click **Save Masks**.

6.2.3 Checking Mask Alignment

It is a good practice to periodically inspect the alignment of the masks. Click Current Masks to see them. If it looks good, then exit the screen. Otherwise click **Align Masks** to fine tune your alignment and then click **Save Masks**.

6.2.4 Image File

You can save plate images to a file by clicking **Save** and recall stored ones by clicking **Load**. With either choice, a standard Windows Save or Open dialog box opens up to choose the destination or source files, respectively. (Saved as .isi files.)

Section 7
Data Analysis Module

Introduction

The Data Analysis Module is where data are presented and analyzed. When the iCycler software opens, the Data Analysis icon is gray and the module is not active. You can enter the Data Analysis module automatically from Run-Time Central during the execution of a protocol, or you can enter it by opening a stored data file from the Protocol Library.

A run always begins in Run-Time Central. At the beginning of the second repeat of the real-time data collection step, the Data Analysis icon will become active. At the same time, the PCR Quantification window of the Data Analysis module will open, displaying the PCR Amp Cycle plot with data from the first repeat of the data collection step. The PCR Amp Cycle plot will be updated at the beginning of each repeat of the data collection step and will lag by one cycle. For example, at the beginning of the fourth repeat of the data collection step, data from cycle 3 are added to the plot.

As data are collected and the PCR plot is updated, they are displayed on the PCR Amp Cycle Plot in Background Subtracted mode. Do not change the analysis mode to PCR Base Line Subtracted during data collection steps. All other analysis options are only available after the protocol is completed and all data have been collected.

7.1 Quick Guide to Collecting and Analyzing Data

1. Create and save the protocol and plate setup files in the Protocol Workshop, and start the run. Select a name for the data file.
2. The PCR Amp Cycle plot will automatically display data after the second repeat of the data collection step. **Do not change the Analysis mode during data collection**.
3. When the protocol is complete, choose PCR Baseline Subtraction from Analysis Mode.
4. From the PCR Quantification window select a fluorophore to analyze.
5. Make adjustments to the data analysis parameters. You may adjust:
   A. Baseline cycles - to calculate the background fluorescence
   B. Threshold -to establish Threshold Crossing
   C. Data Analysis Window - to establish the data points to be evaluated for threshold crossing.
   D. Type of digital filtering.
   E. Selection of Wells included in the Analysis.
   F. If data were collected for post-run analysis (not for real-time analysis (gray camera), see Section 5.1.6) you may combine these data with the real-time data.
6. Once all adjustments are made, print the standard curve, PCR amplification graph or export the data to a spreadsheet program.

7.2 A Guide to Data Analysis

Data analysis may begin as soon as thermal cycling ends, or once a saved data set is opened. No more than one data file may be analyzed at a time.

7.2.1 The Data

7.2.1.1 Opening Stored Data

Begin by opening a stored data set, or as soon as the PCR protocol is completed.

- To open a stored data set, click on the View Post-Run Data tab in the Protocol Workshop module. Navigate the directory until you find the data set and then click **Analyze Data** in the Data Analysis Operations box.

7.2.1.2 Applying a New RME to Stored Data

When a real-time PCR (optical) data file is saved (in the proprietary OPD format) the RME values are written to the file. Any time that the optical data file is subsequently opened with the iCycler program, the data are analyzed using the values that were in the RME.ini file when the optical data were collected. It is possible to overwrite the RME values stored within the OPD file with the ones in the current RME.ini file, thus changing the analysis of the experimental data. This feature protects you from losing valuable experimental data if a pure dye calibration was conducted incorrectly.

In order to apply the current RME values to a stored data set, click the box labeled 'Use External Calibration File' in the View Post-Run Data tab of the Protocol Library (Figure 7.1). When the optical data file is opened the new RME values will be applied to the data. The existing RME values will be overwritten if you save the file again. If the software is exited without explicitly saving the data set again the original RME values will be restored. Optical data may be analyzed repeatedly with different RME files simply by exchanging the location of stored (inactive) RME files with the active RME file. **The only active RME file is the one stored at C:\Program Files\Bio-Rad\iCycler\Ini.**

Note: It is recommended that the optical data file is saved under a different name before saving the file with new RME values. This ensures that the original file is always maintained for reference.

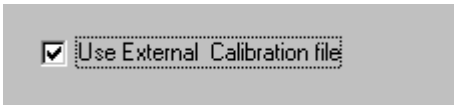


Fig.7.1. To select an external calibration file.

7.2.2 Background Subtracted

When a stored data set is opened, or when a protocol reaches the completion, the PCR Amp Cycle plot is displayed in the Background Subtracted view.

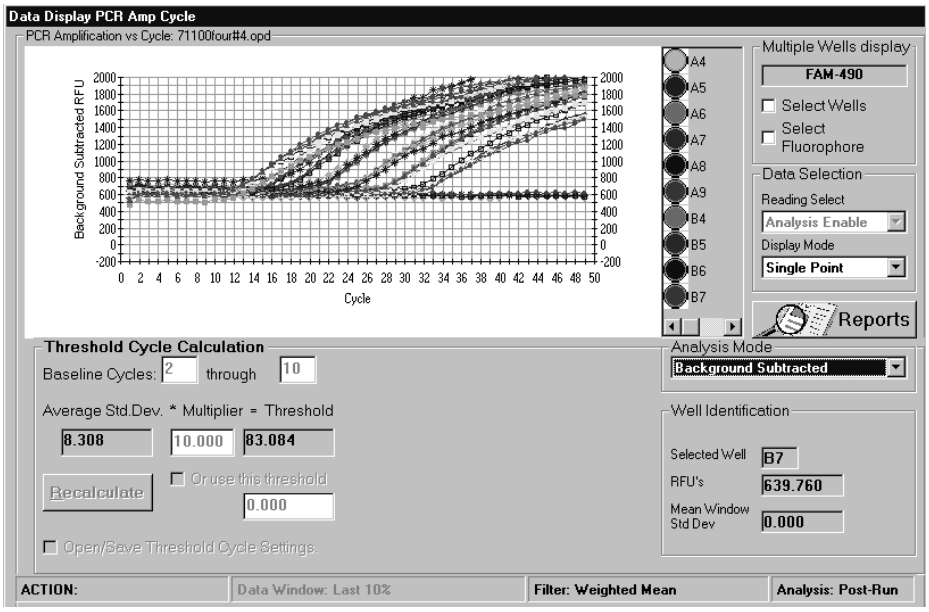


Fig. 7.2. Background Subtracted Plot.

Display Options

- Data are displayed one fluorophore at a time. Choose the active fluorophore by first clicking Select Fluorophore in the top right corner of the PCR Quantification window (Multiple Wells display, Figure 7.2) and then selecting a fluorophore from the list box which appears in the lower right-hand corner (Figure 7.3).

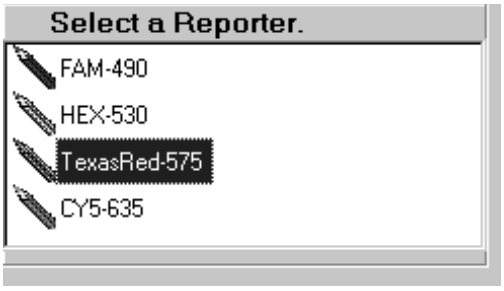


Fig. 7.3. Fluorophore Selection.

- You can **select** data for viewing from all wells or from any subset of the wells. To display data from a single well, click its colored button in the list of wells to the right of the plot. Hold down the shift key to select contiguous wells, or use Control to select non-contiguous wells. Press S to **show all** wells again, or right mouse click on the plot and select Show All Traces from the active box.

- You can **zoom** on any section of the plot by pressing Control while you drag the mouse across the part of the plot in which you wish to zoom. Press R to **restore** the plot to its original size, or right mouse click on the plot and choose Restore Graph from the active box.

Note: You must click the mouse somewhere on the plot before any of the well selection or zoom/restore features are active.

- Clicking the mouse on a trace in the plot, shows information about the data and the well in the **Well Identification** box at the bottom right of the screen (Figure 7.6). In addition to identifying the well, the mean fluorescence reading for that cycle and the standard deviation of all readings collected at that cycle are also shown.
- To **rescale the axes**, place the cursor in the plot area, click the right mouse button and select Adjust Graph from the active box. In the Adjust PCR Amp Cycle Chart box (see Figure 7.4), select the values for the new maximum and minimum RFU or use the arrow buttons to select the number of cycles and then click OK. You can choose a **logarithmic display** for the RFU by checking the Log RFU Axis box.

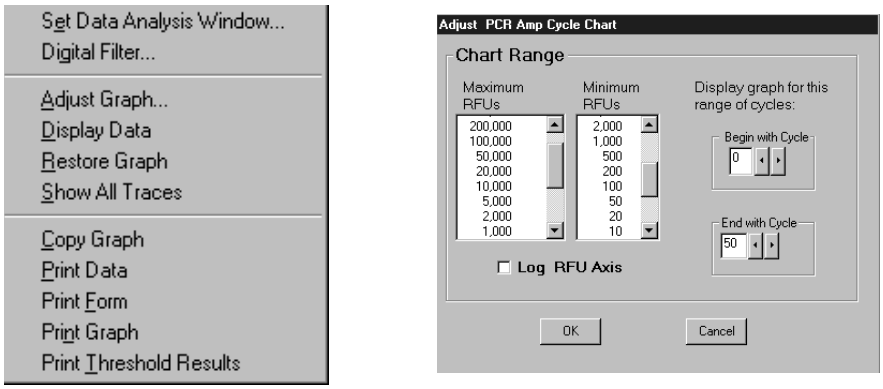
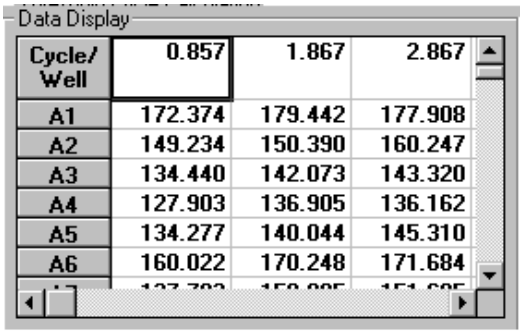


Fig. 7.4. Adjust Graph Dialog Box

- To display the individual readings for each well, click the right mouse button over the plot and choose Display Data. A small spreadsheet will appear in the bottom left of the window. At the top is the cycle number and down the side is the well number. Use the scroll bars to navigate the data. (Figure 7.5) **Note**: These data may be directly exported to text and spreadsheet programs.



Cycle/ Well	0.857	1.867	2.867
A1	172.374	179.442	177.908
A2	149.234	150.390	160.247
A3	134.440	142.073	143.320
A4	127.903	136.905	136.162
A5	134.277	140.044	145.310
A6	160.022	170.248	171.684

Fig. 7.5. Display Data Spreadsheet

Print Options

- You can print the graph, the displayed data, the threshold cycle assignments or the entire window. Click the right mouse button inside the plot and choose **Print Data**, **Print Graph**, **Print Threshold Results** or **Print Form**.

Copy Options

- You can copy the graph to the clipboard for import into another program. Click the right mouse button inside the plot and choose **Copy Graph**, then paste into the destination programs.

Analysis Options. (See discussion below.) Set parameters for data analysis in the Data Selection box.

- **Reading Select**: Choose *Analysis Enable* to display and analyze the data collected for real time analysis. Choose *Collect Enable* to analyze all data collected during the experiment, including data not intended for real time analysis. See Section 7.3.7.
- **Display Mode**. Choose *Single Point* to represent the data collected during each cycle as one single point. By default, the mean of the readings over the last 10% of each cycle is used for single point display. Choose *All Candidates* to see all individual readings displayed for every well at each cycle.

  For easiest viewing and analysis, choose Analysis Enable and Single Point and the appropriate dye layer.

7.2.3 PCR Base Line Subtracted Analysis Mode

You must choose PCR Base Line Subtracted before threshold cycles are assigned and before standard curves are constructed.

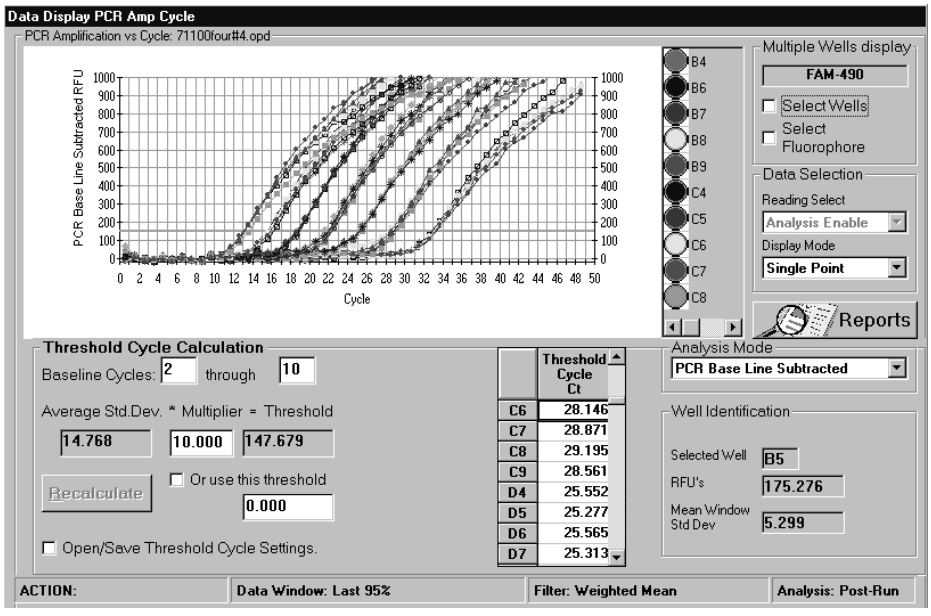


Fig. 7.6. PCR Base Line Subtracted Plot.

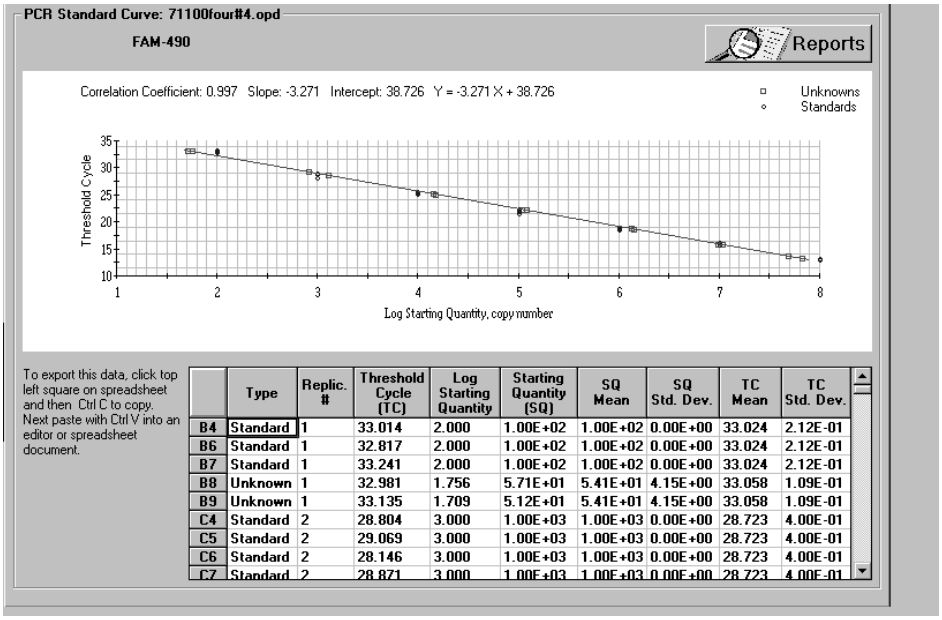
Once you choose PCR Base Line Subtracted, the software will calculate the threshold for the experiment and display it as a red line. Then the threshold cycle for every well included in the analysis is calculated. When the calculations are complete, another spreadsheet will open listing the threshold cycle assignment for each well.

Printing and Copying the Threshold Cycle Assignments. You can print the threshold cycle assignments by clicking the right mouse button over the PCR Amp Cycle plot and choosing **Print Threshold Results** from the active box.

Click in the top left corner of the spreadsheet to select all the data and then press "Control C" to copy the data to the clipboard. These data can be copied from the clipboard directly into text or spreadsheet programs.

7.2.4 Standard Curve Calculation and Determination of Unknowns

Once PCR Base Line Subtraction has been carried out and the threshold cycles have been assigned for all standards, a standard curve is constructed. Click the third tab labeled PCR Standard Curve to display the graph. At the top of the plot is the correlation coefficient and , (see Figure 7.7) the slope and intercept of the standard curve.



	Type	Replic. #	Threshold Cycle (TC)	Log Starting Quantity	Starting Quantity (SQ)	SQ Mean	SQ Std. Dev.	TC Mean	TC Std. Dev.
B4	Standard	1	33.014	2.000	1.00E+02	1.00E+02	0.00E+00	33.024	2.12E-01
B6	Standard	1	32.817	2.000	1.00E+02	1.00E+02	0.00E+00	33.024	2.12E-01
B7	Standard	1	33.241	2.000	1.00E+02	1.00E+02	0.00E+00	33.024	2.12E-01
B8	Unknown	1	32.981	1.756	5.71E+01	5.41E+01	4.15E+00	33.058	1.09E-01
B9	Unknown	1	33.135	1.709	5.12E+01	5.41E+01	4.15E+00	33.058	1.09E-01
C4	Standard	2	28.804	3.000	1.00E+03	1.00E+03	0.00E+00	28.723	4.00E-01
C5	Standard	2	29.069	3.000	1.00E+03	1.00E+03	0.00E+00	28.723	4.00E-01
C6	Standard	2	28.146	3.000	1.00E+03	1.00E+03	0.00E+00	28.723	4.00E-01
C7	Standard	2	28.871	3.000	1.00E+03	1.00E+03	0.00E+00	28.723	4.00E-01

Fig. 7.7. Standard Curve.

Below the plot of the standard curve is a spreadsheet that lists the type of sample, sample number, threshold cycle, calculated log of starting amount (copy number in this example), starting amount, and, if the sample is part of a replicate group, the standard deviations of threshold cycle assignments and calculated starting amounts for each well.

Printing Standard Curves and Data. Click the right mouse button over the graph to display an active box in which you may choose to **Print Graph** or **Print Data** from this box. You may also copy the graph to the clipboard by choosing **Copy Graph** from the active box and then paste directly into a text or spreadsheet program.

7.3 Adjust the Threshold Cycle Parameters

There are several data analysis parameters that may be modified after PCR Base Line Subtraction has been completed. You may change the:

- Range of baseline cycles
- Threshold value
- Number of data points used from each cycle in the analysis

- Digital filtering
- Wells included in the analysis

Changes to any of these results in changes to the threshold cycle assignments and the standard curve.

Consider the following example of 96 replicates in the file 96uniform.opd. PCR Base Line Subtraction has been carried out and threshold cycles assignments have been made with the default values for baseline cycles, threshold, number of data points and it includes all wells. Under the default conditions, the mean threshold cycle for the 96 repeats was 24.3 with a standard deviation of 0.557 cycles. (See Figure 7.8.)

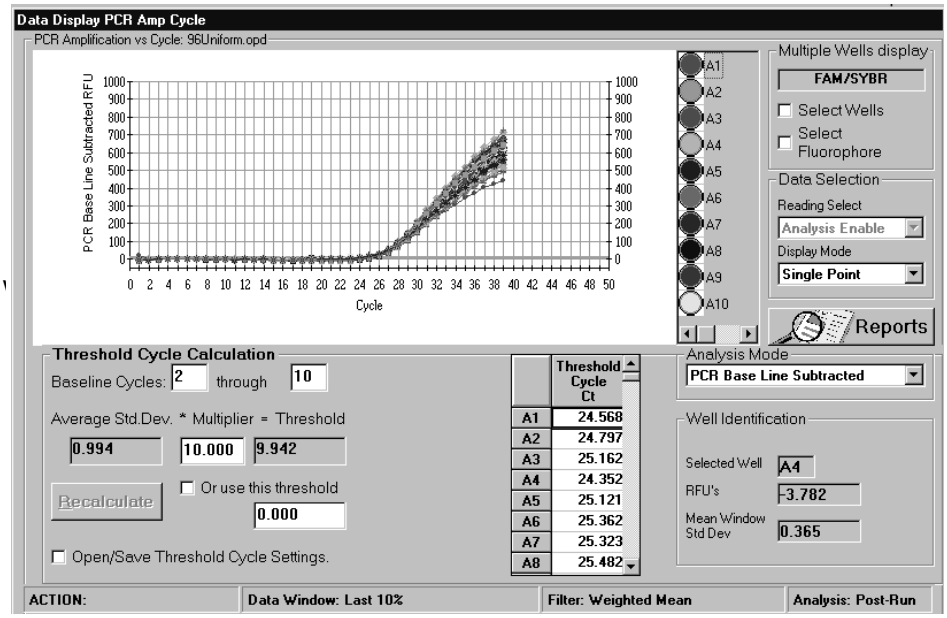


Fig. 7.8. PCR Base Line Subtraction of 96-well uniformity data.

7.3.1 Baseline Cycles

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

Click in either box to highlight the current value and then type a new value. You may not include cycle 1 in this calculation. The range must be at least 3 cycles. Click **Recalculate** once you have chosen the cycles. After extending the baseline from cycle 2 to cycle 23, the mean threshold cycle becomes 24.8 with a standard deviation of 0.164 cycles. (Figure 7.9)

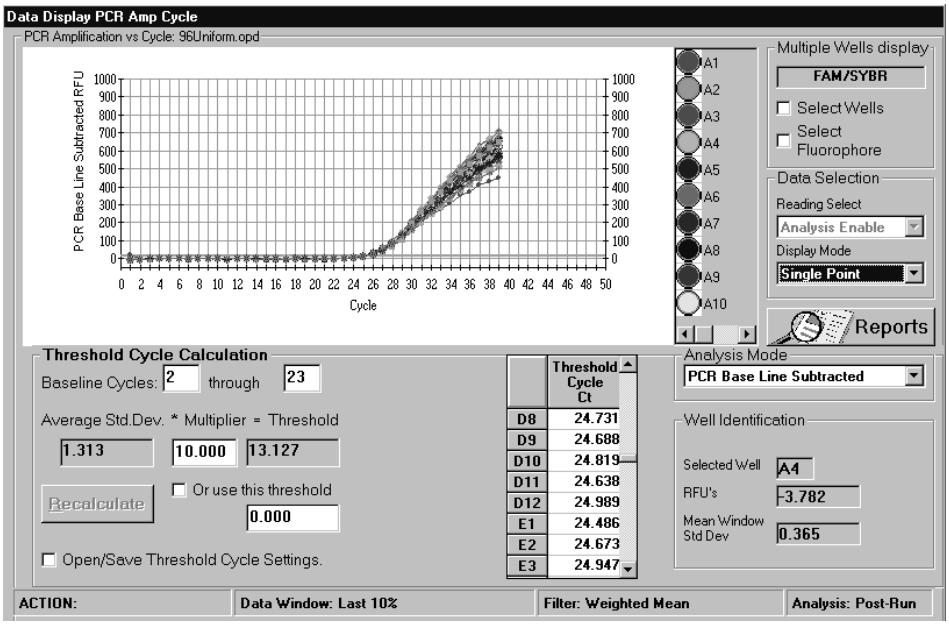


Fig. 7.9. Adjusting Baseline Cycles.

7.3.2 Threshold Value

By default the threshold value is 10 times the mean standard deviation of fluorescence in all wells over the baseline cycles. This multiplier may be changed by highlighting the value in the **Multiplier** box and entering the desired value. Press **Recalculate** to accept the new value.

Alternatively, you can directly enter a value for the threshold. Click in the box labeled **Or Use this Threshold** and then enter a value in the box below it. Click **Recalculate** to accept the change.

Observing the data with logarithmic y-axis (Figure 7.10) can be very useful in trying to choose a baseline; it can also make it easier to identify anomalous wells or outliers. Click the right mouse button on the graph and choose **Adjust Graph** from the active box. Click **Log Axis** and then **OK** in the active box.

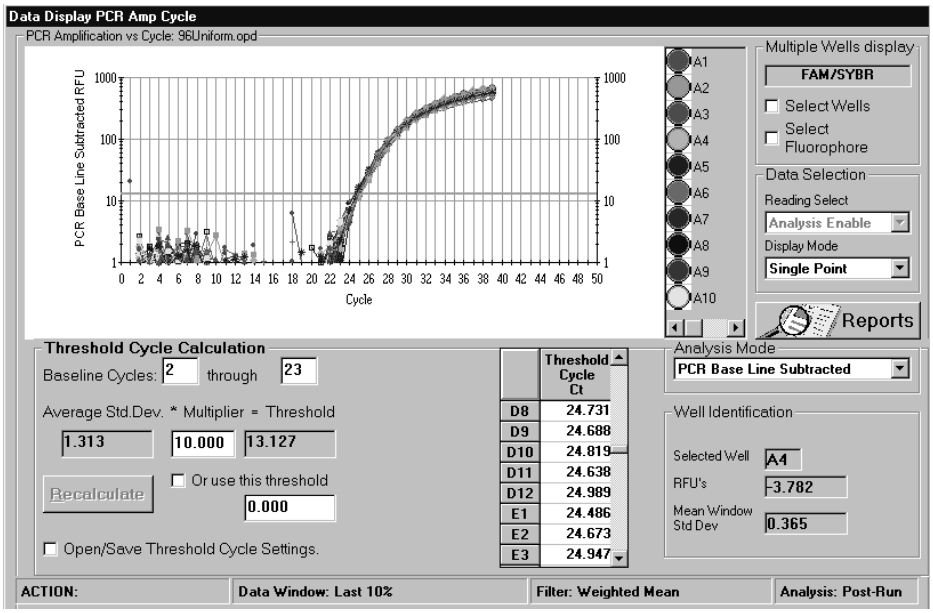


Fig. 7.10. Log Display.

In this example, the threshold may be set as high as 80 or 90 counts without significantly affecting the uniformity of the threshold cycle assignments. As the traces rise above 100 counts, the slope begins to change, indicating the end of exponential amplification, and you should not consider setting threshold above this point.

7.3.3 Data Analysis Window

The PCR Base Line subtracted plots are constructed using the mean of the readings taken over the last 10% of each cycle. The number of data points collected depends on the exposure time and the dwell time at the cycle. You can change the percentage of data points used (the window) and you can choose to use data from the beginning of the cycle, the end of the cycle or anywhere in between.

1. Click the right mouse button on the PCR Amp Cycle plot and choose Set Data Analysis Window from the active box.

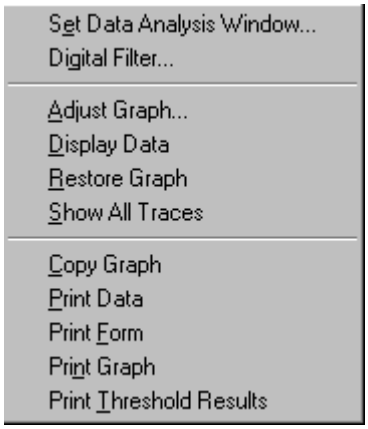


Fig. 7.11. The Active Window in the PCR Amp Cycle Plot

2. If you want data from the **Beginning of the Cycle** or the **End of the Cycle**, click the appropriate box and use the up and down arrows to select the percentage of data points. Then click **OK** to reanalyze the experiment.

3. To center the analysis around data collected somewhere else in the cycle, first click the middle button, **Pick Window From Plot** (Figure 7.12). The instructions will be displayed in the window.

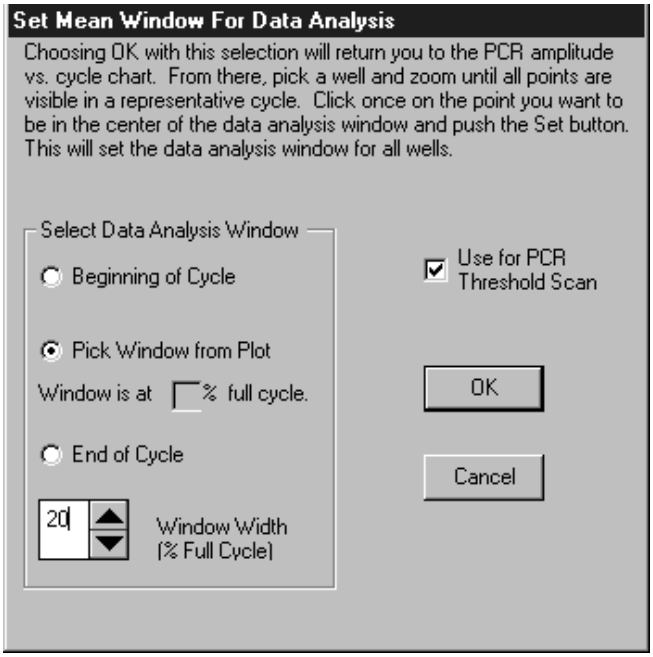


Fig. 7.12. Set Data Analysis Window.

4. Use the up and down arrow keys to set the width of the data window. If you choose 20, for example, then the analysis will include 10% of the data points on either side of the center point. Click **OK** to return to the PCR Amp Cycle Plot.

5. The PCR Amp Cycle plot will be displayed in the All Candidates mode, showing every single data point for each well. (Figure 7.13)

Note: The use for PCR Threshold Scan box must be checked. If this box is not checked the display will be updated but threshold calculations will not be updated.

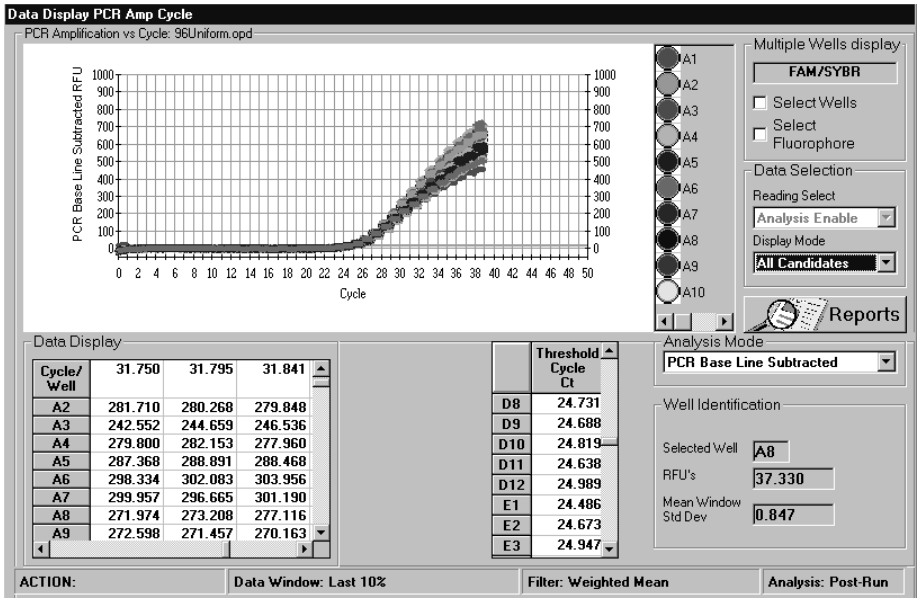


Fig.7.13. All Candidates Display Mode.

6. Choose any single well by clicking on its button to the right of the plot, and then while holding down the Control key, drag the mouse across a section of the plot to zoom in. You may zoom more than once. (Figure 7.14)

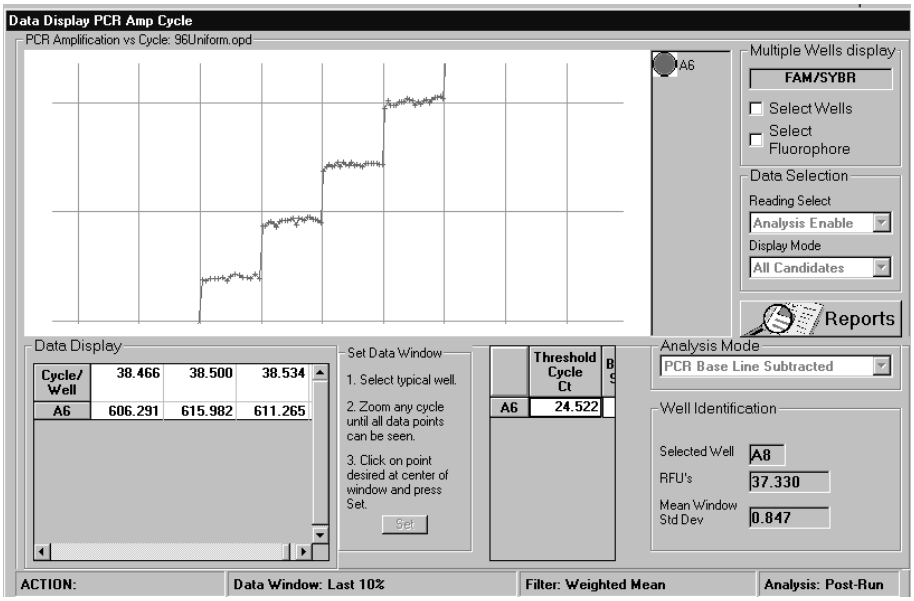


Fig. 7.14. Zoomed in on a Single Well.

7. Choose the center point of the next analysis. As you place the pointer on a data point, its reading and cycle number will be highlighted in the Data Display box.

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

7.3.4 Digital Filtering

There are two intra-cycle data filtering options available: a weighted mean and a rolling boxcar. You can access them by a right mouse click on the PCR Amp cycle plot. Choose Digital Filter from the pull down list. The default filter is the weighted mean and it is the only one available during data acquisition.

Fig. 7.15. Set Digital Filters.

The weighted mean is determined by the equation

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

where:

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

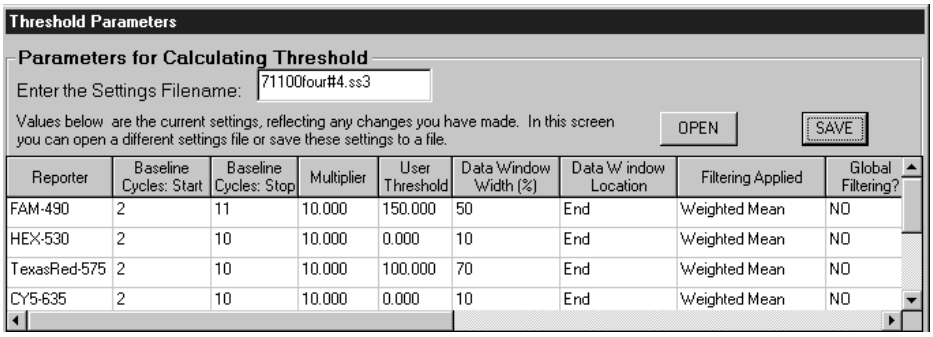
The rolling box car filter is the arithmetic mean of data readings i - (w-1) to i where w is the filter width. For example if you want to calculate the 20th data point (i=20) and the width is 4 (w=4), you take the mean of data points 17 through 20. Then data point 21 is the mean of data points 18–21,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

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

7.3.5 Saving Optimized Data Analysis Parameters

Optimized data analysis parameters may be saved and recalled. After adjusting the baseline cycles, the threshold, the data analysis window and the filtering options for a particular fluorophore, click in the box at the bottom left of the window labeled Open/Save Threshold Cycle Settings (the box does not appear until after you perform PCR Baseline Subtraction) and then click **Save**. To open saved settings, first select the fluorophore, then click the Save/Open Settings box and then click **Open**.

Note: The check box acts as a toggle bringing up/closing the Threshold Parameters dialog box (Figure 7.16).



Reporter	Baseline Cycles: Start	Baseline Cycles: Stop	Multiplier	User Threshold	Data Window Width (%)	Data W indow Location	Filtering Applied	Global Filtering?
FAM-490	2	11	10.000	150.000	50	End	Weighted Mean	NO
HEX-530	2	10	10.000	0.000	10	End	Weighted Mean	NO
TexasRed-575	2	10	10.000	100.000	70	End	Weighted Mean	NO
CY5-635	2	10	10.000	0.000	10	End	Weighted Mean	NO

Fig. 7.16. Open/Save Threshold Cycle Settings.

7.3.6 Select Wells

This feature allows you to eliminate one or more wells from the data analysis, effectively permitting you to conduct more than one analysis on a plate. You can eliminate entire columns or rows or you may eliminate individual wells.

1. In the PCR Quantification window in the upper right hand corner (see Figure 7.13), click Select Wells and a dialog box will open up. (Figure 7.17) The check box acts as a toggle, bringing up or closing the Select Wells dialog box.

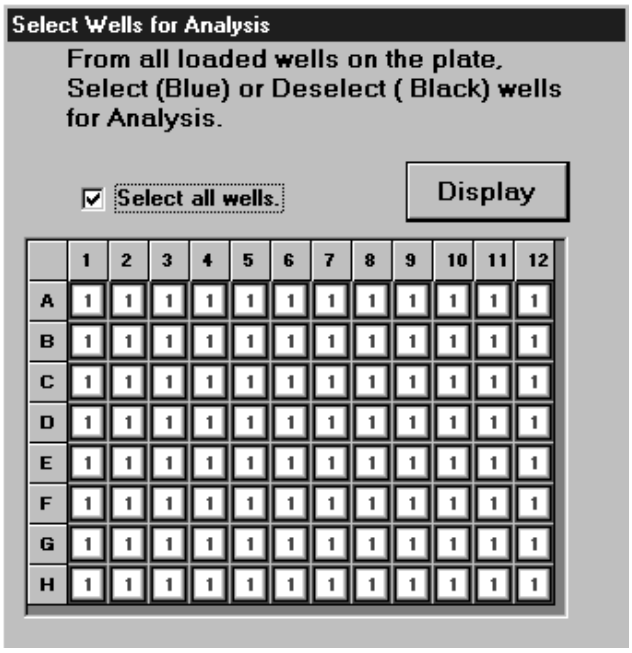


Fig. 7.17. Select Wells Window.

2. To eliminate a row or column, click the appropriate letter or number, respectively. To eliminate an individual well, click inside the well. As you click on wells, the blue border surrounding the well changes to black.

3. After choosing all wells to be eliminated, click **Display**. It will take a few moments for the data to be removed.

4. To add data back to the analysis, reverse this procedure. You can add back wells one at a time, or an entire row or column at once. Alternatively, you may click the **Select all wells** box to restore all data to the analysis.

Note: Data are not permanently removed by this procedure. They are eliminated only from the present analysis. You do not have to restore the data before saving the file.

7.3.7 Collect Enable

Data may be collected for reference but not viewed in real-time by indicating a "grey camera" (see Section 5.1.6) in the thermal protocol. These data may be added to the existing data by choosing Collect Enable from the Reading select field. These data may be analyzed separately by using "Set Data Analaysis Window" Feature.

1. From the Data Selection box of the PCR Amp Cycle Plot, choose Collect Enable in the Reading Select field. The data collected with the gray camera will be combined with the existing data. (Figure 7.18)
2. Carry out analyses as described previously. If you want to exclude the data collected with the yellow-camera, use the Set Data Analysis Window.
3. Selecting Analysis Enable from the Reading Select field will remove the Collect Enable data from the display and analysis.

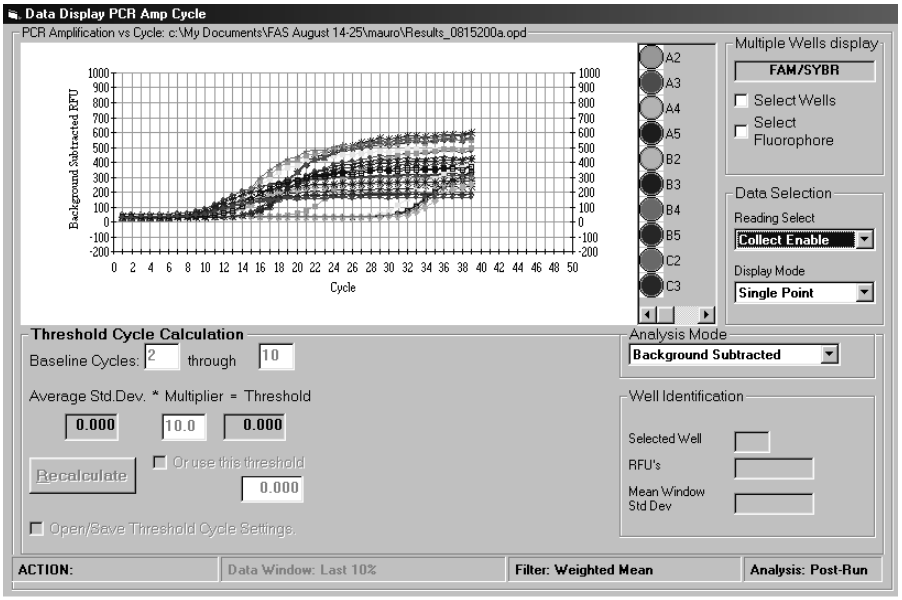


Fig. 7.18. Selecting Collect Enable.

7.4 PCR View/Save Data Window

Enter this window by selecting the first tab in the Data Analysis module. In this window, you can save a data set and you can also view the data after analysis is complete.

7.4.1 View Data

There are three radio buttons in this window that control the data that are displayed on the plate layout in this window. (Figure 7.19)

- Threshold Cycle. Superimposes the calculated threshold cycle for each well over the plate layout.
- Calculated Concentration. Shows starting concentrations calculated for unknowns from the standard curve.
- Standard Quantities. Shows the defined quantities for each standard on the plate layout. The units of measure defined in the plate setup are shown in an adjacent text box.

You can tell which wells, if any, are eliminated from the present analysis by referring to the PCR View/Save Data window. Wells that have been eliminated are shown in black rather than in the color of the fluorophore.

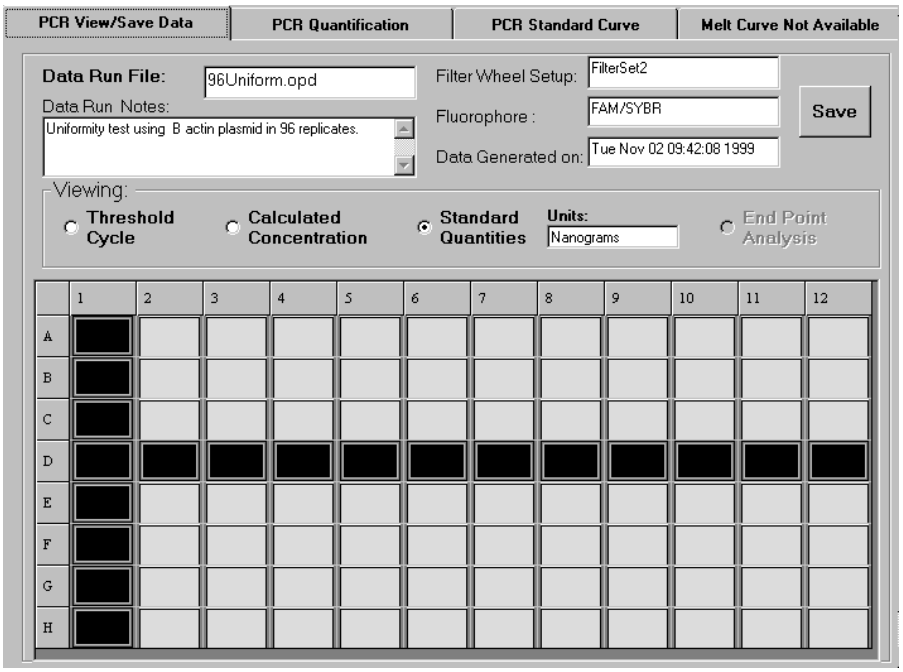


Fig 7.19. The PCR View/Save Data Window.

7.4.2 Save Data

Data may be saved to file for future reference and analysis. You must save the file in the proprietary .OPD format for future analysis. From the PCR View/Save window:

- Enter a name for the file in the box labeled Data Run File.
- Enter any notes about the experiment in the Data Run Notes box.
- Click **Save**. A Save dialog will open, and .OPD will be appended to the file name. Click **Save** in the dialog box.

Section 8
Melt Curve Functionality and New Features in v.2.3

8.1 Introduction

This section of the manual guides you through the use of the melt curve functionality of the software and provides information on the new features included in this version. To begin using your new version 2.3 iCycler iQ software you must also upgrade the Firmware on the base iCycler thermal cycler to version 2.032 or later. For complete details on upgrading the base Firmware see Appendix H. Sample data files of melt curve runs are included on the install CD-ROM for v. 2.3. These files will not be automatically installed. If you would like to use them, copy them to your computer to the following folder: C:\Programs Files\Bio-Rad\iCycler.

What Is Melt Curve?.

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

When Should I Use Melt Curve?

Three major applications for melt curve are: peak identification (number of amplified products), characterization of molecular beacons, and allelic discrimination. The first two applications are typically used as a guide for improving real-time PCR assay development. The third, allelic discrimination, is any assay used to distinguish or detect mutations between sequences of DNA. Single Nucleotide Polymorphism (SNP) Identification is one type of allelic discrimination assay. One way in which SNP detection can be done is to use the melt curve functionality in this software with fluorescent resonance energy transfer (FRET) assays. These applications are discussed in detail in this section.

8.1.1 Peak Identification

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

DNA-binding dyes, such as SYBR Green I, are typically used for non-specific visualization of amplified product. SYBR Green I binds to double-stranded DNA and fluoresces 50-100 times brighter than when not bound to DNA. Since these dyes bind to any double-stranded DNA molecule, they cannot distinguish between multiple products amplified in the same reaction. SYBR Green I can be used to monitor real-time amplification with a particular primer pair. The number of amplified products can then be identified by melt curve analysis. At temperatures below the T_m of the amplified product, SYBR Green I will bind to the double-stranded DNA and fluoresce brightly. As the T_m is reached, the DNA denatures and

releases SYBR Green I, causing a sharp decline in fluorescence. This decrease in fluorescence is plotted as Fluorescence vs. Temperature. Plotting the negative first derivative of this data versus the temperature change (–dF/dT vs. Temperature) results in a melting peak and T_m for each amplified product. The T_m for each peak is dependent on the length of the amplified DNA as well as the G/C content of the sequence. Primer-dimers, which are typically shorter in length, usually melt at a much lower T_m than the intended product and are therefore easy to distinguish. Secondary or non-specific products can be of varying lengths and sequences and therefore have a large range of possible melting temperatures.

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

8.1.2 Characterization of Molecular Beacons

Fluorescently labeled molecular beacon probes are used for specific quantitation of a particular product in real-time PCR assays. Melt curve analysis can assist in characterizing a molecular beacon for real-time PCR amplification by distinguishing the best temperature for data collection.

Molecular beacons are labeled with a fluorophore at the 5' end and a quencher molecule at the 3' end. They consist of a probe sequence, complementary to the target DNA and 2 arm sequences of 5 or more nucleotides flanking both sides of the probe. The arms are not complementary to the target, but they do complement one another. When the arms anneal, the molecular beacon assumes a hairpin structure, bringing the 5' and 3' ends of the molecule together. This brings the fluorophores and quenchers in close proximity, allowing the fluorescence to be quenched. When a beacon is bound to its DNA target, the 5' and 3' ends are maximally separated, allowing fluorescence detection of DNA.

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

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

temperature of the complementary arms is reached, there is a sharp increase in fluorescence as all of the molecular beacons open and the fluorophores at the 5' end are no longer quenched.

In reactions with molecular beacons and the single-stranded complements, fluorescence is usually highest at low temperatures. This is the point at which the probes are bound to the targets and the fluorophores are maximally separated from the quenchers. As the temperature increases and the T_m of the probe:target hybrids are reached, there is a marked change in fluorescence as the molecular beacons are released from the target DNA.

Melt curve can assist in designing a molecular beacon real-time PCR assay by determining the best temperature at which fluorescent data should be collected. Both the T_m of the arms and the T_m of the probe:target hybrid are best revealed in the negative first derivative plot (-dF/dT vs. Temperature). Fluorescent data generated during an amplification protocol should be collected at a temperature at which the unbound molecular beacons are closed (below the T_m of the arms) and the probes bound to the DNA targets are maximal. This will ensure the maximum signal-to-noise ratio in the experiment. See Section 8.5.2 for further discussion.

8.1.3 Single Nucleotide Polymorphism (SNP) Detection

The T_m of a double-stranded DNA molecule is usually based on both strands of DNA being exact complements of each other. If one strand of the DNA contains a single nucleotide polymorphism (SNP), this double-stranded complex will have a lower T_m than a complex with an exact match. Melt curve takes advantage of this difference in T_m to verify a SNP with fluorescently labeled probes. This makes it possible to distinguish mutant and wild-type homozygotes from heterzygotes in allelic discrimination studies. Therefore, melt curve analysis can be used as an alternative to other SNP detection methods that utilize molecular beacon probes or minor-groove binder hybridization probes.

Two gene sequences with a difference of only one base pair can be distinguished from one another by melt curve using a single fluorescent probe. For allelic discrimination, a probe sequence is designed to exactly match one allele (wild-type) yielding a T_m that is higher when bound to the wild-type allele than when it is bound to the mutant allele. The probe will not bind as tightly to the mutant allele containing the SNP, resulting in the lower T_m.

Fluorescent resonance energy transfer (FRET) is one technique chosen for SNP detection by melt curve[1,2]. A primer set is designed to amplify both alleles with an internal label (*e.g.* Texas Red or Cy5) near the 3'end of the reverse primer. The probe is designed to be an exact complement to the amplified wild-type allele, and is labeled at the 3'end with FAM. This same probe is positioned to bind the amplified mutant allele with the mismatch centered in the middle of the probe. This aids in making the DNA hybrid less stable so that the difference in T_m between wild-type and mutant can be resolved by melt curve.

Melt curve analysis can distinguish between wild-type and mutant homozygotes or identify heterozygotes. Below the T_m, the probe will anneal to both amplified alleles. The Cy5 signal will be high due to FRET from the FAM probe. As the mutant allele T_m is reached, the probe dissociates from the allele, causing a significant decrease in fluorescence with a large change in slope (as seen in the Fluorescence vs. Temperature plot). A second noticeable decrease in fluorescence will occur as the wild-type allele T_m is reached. The changes in slope seen in the Fluorescence vs. Temperature plot will yield distinct melt curve peaks in the negative first derivative plot (-dF/dT vs. temperature). Therefore, the melt curve peaks will provide distinct T_m values for the wild-type and mutant alleles.

[1] Neoh, S-H, Brisco, M J, Firgaira, F A, Trainor, K J, Turner, D R, Morley, A A. Rapid detection of the factor V Leiden (1691 G>A) and haemochromatosis (845G>A) mutation by fluorescence resonance energy transfer (FRET and real time PCR. J Clin Pathol 1999; 52:766-769.

[2] Lyon, E, Millson, A, Phan, T, Wittwer, C T. Detection and identification of base alterations within the region of Factor V Leiden by fluorescent melting curves. Mol. Diag.;3:203-210.

8.1.4 Description of New Software Features

8.1.4.1 Skip to Next Step, Skip to Next Cycle, Add 10 Repeats

These features have been added to the iCycler iQ software to enhance the versatility of a running protocol. You now have the ability to change characteristics of the protocol while the run is in progress. Skip to the Next Step allows you to cancel the remaining dwell time of the current step of a cycle. The protocol will then proceed to the next step of the cycle. This feature is used for cycles with more than one step but no repeats. Skip to the Next Cycle cancels all remaining repeats in the current cycle and proceeds to the next cycle. This type of feature is useful when you have collected enough data for a particular cycle and do not want to wait for the remaining programmed repeats to finish. Finally, Add 10 Repeats permits you to add an additional 10 repeats to a cycle which is currently in progress. This modification can be used multiple times for as many as 600 total repeats for any cycle. Application of these features is described in detail in Section 6.1.1.

8.1.4.2 Software Compatibility

The iCycler iQ software can now be installed and operated on the following Windows platforms:

Windows 95	Windows ME
Windows 98	Windows NT4
Windows 98SE	Windows 2000

8.1.4.3 Plate Setup Modifications

Several new features have been added to the Edit Plate Setup window in the Protocol Library. When defining standards in a plate setup (under the Samples tab), quantities can now be entered in scientific notation (1e5 or 2.374e-04). The units (*e.g.* copy number, nanograms) can also be defined for each replicate set of standards. If standards are to be defined as a dilution series, the software automatically calculates and labels the concentrations for each standard set after the starting quantity and dilution factor are defined. When selecting fluorophores for these samples, you may enter plate setup notes in the Plate Notes text box. Finally, an additional tab as been added into Protocol Library next to the Plate Setup tab labeled Quantities/Identifiers. This tab provides an overall view of a selected plate setup by illustrating the type of sample in each well and the quantities of any defined standards in each dye layer.

8.1.4.4 Reports

A Reports button is now available for every data analysis window of the iCycler iQ software. These reports provide customizable, detailed documentation of the completed experiment (Figure 8.1). Information such as general data (*e.g.* user and file names), run and data parameters, and the data itself, in graphical and numerical format can be printed or saved to a specified file name. You may select various types of reports, which differ in the amount of information displayed.

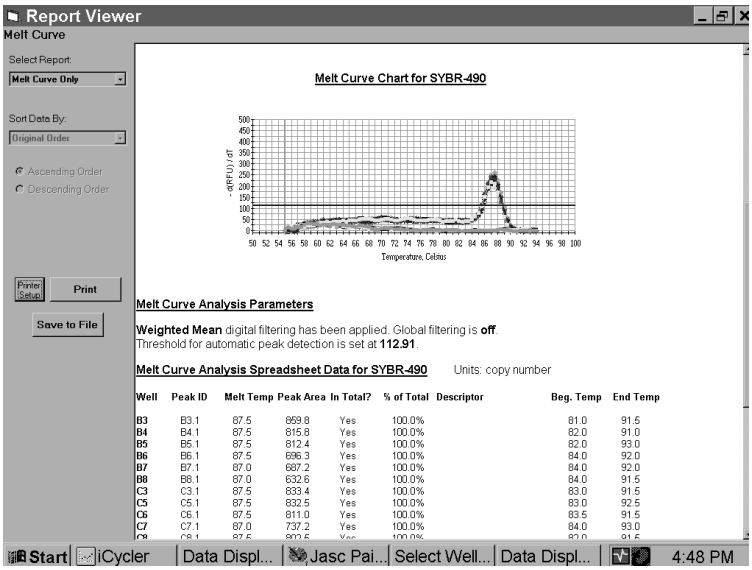


Fig. 8.1. Example of information stored in a particular report.

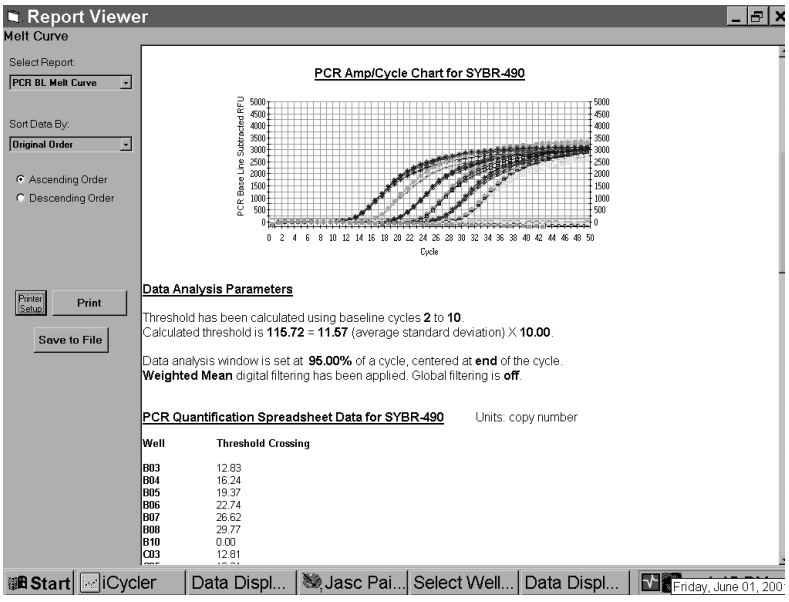


Fig. 8.2. Example of a report file that includes amplification data.

8.1.4.5 Autosave

All data are now saved automatically during a protocol run and on completion of the run. Autosave is activated at the start of a protocol run after the user names the .opd file with its location. The autosave feature prevents data loss in situations such as a power failure or system crash. This feature is described in detail in Section 6.1.1.

8.2 Quick Guides to Creating Melt Curve Protocols and Plate Setups

8.2.1 Quick Guide to Creating a Melt Curve Protocol

1. From the **Protocol Library**, select the **View Protocols** tab.
2. To create a new melt curve protocol, click **Custom** to open the **Edit Protocol** window. To add a melt curve protocol to an existing amplification protocol, select the appropriate amplification protocol file from the **Protocol Library**. Click **Edit** to open the **Edit Protocol** window.
3. If you are creating a new amplification and melt curve protocol, follow the instructions for creating an amplification protocol on pages 29–30 of this manual. To create a melt curve protocol only or to add a melt curve protocol to an existing amplification protocol:
   - Insert a new cycle by selecting **Insert Cycle** and clicking on the first available blank line in the spreadsheet (the melt curve cycles should be placed after any existing amplification cycle). If you are creating a custom protocol, you can delete any default cycler step by selecting **Delete Cycle** or **Delete Step** and clicking on the existing default setting.
   - Under **Setpoint**, enter 95 °C, followed by entering 1:00 minute for the dwell time. This will be the initial denaturation step.
   - If the beginning temperature of the melt curve is any temperature other than 95 °C, insert a second cycle by selecting **Insert Cycle** and clicking on the first available blank line. Enter the temperature at which you wish to begin your melt curve and enter 1:00 minute for the dwell time. This step serves to adjust all DNA products to the appropriate temperature at the start of the melt curve.
   - Insert another new cycle in the next available blank line. This cycle will be used to generate the melt curve data.
   - In the **Protocol Options** window, click on the box to the left of the **Melt Curve Option**. This will add 3 additional columns to the spreadsheet entitled **Melt Curve**, **+ Temp**, and **– Temp**.

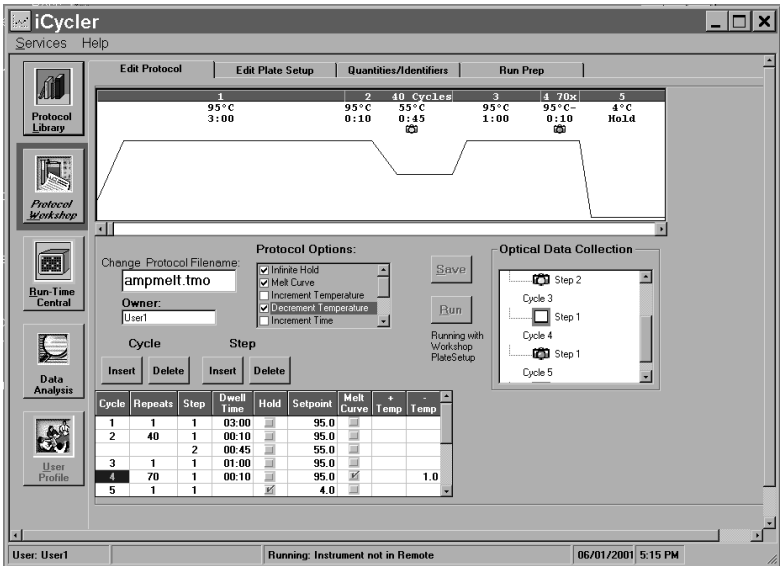


Fig. 8.3. Melt curve protocol designed for decrement temperatures of 1.0 °C with a starting temperature of 95 °C and ending at 25 °C. Data is collected at cycle 4 as indicated by the lighted green camera in the Optical Data Collection Box.

- Check the box under the Melt Curve column only at the cycle where you intend to collect melt curve data. This will place a green camera under this cycle number in the **Optical Data Collection Box**.
- Enter the temperature at which you wish to begin the melt curve under **Setpoint** (4 °C–100 °C).
- Enter the increment (**+ Temp**) or decrement (**- Temp**) values in which the temperatures will change during the melt curve between the starting and ending temperatures. This number can be as low as 0.1 °C increments (Note: typical increment values are 0.3–0.5 °C for SYBR Green I and FRET melt curves and 1.0 °C decrements for molecular beacon melt curves).
- Enter an appropriate number of repeats under the **Repeats** column of this cycle (2–600 repeats), based on the desired beginning and end temperatures and the temperature increments/decrements previously chosen
    - For example, a desired starting temperature of 95 °C and ending temperature of 25 °C at 1.0 °C increments would require 70 repeats (Figure 8.3).
    - If an inappropriate number of repeats is entered based on the starting temperature and increment/decrement values, the + Temp or – Temp box will be highlighted in yellow. This is followed by an error message when attempting to save the protocol (Figure 8.4). In this figure 100 repeats of 0.5 °C would increase the temperature beyond the maximum of 100 °C. You must change the number of repeats, the Setpoint temperature or the increment/decrement value before the protocol can be properly saved.

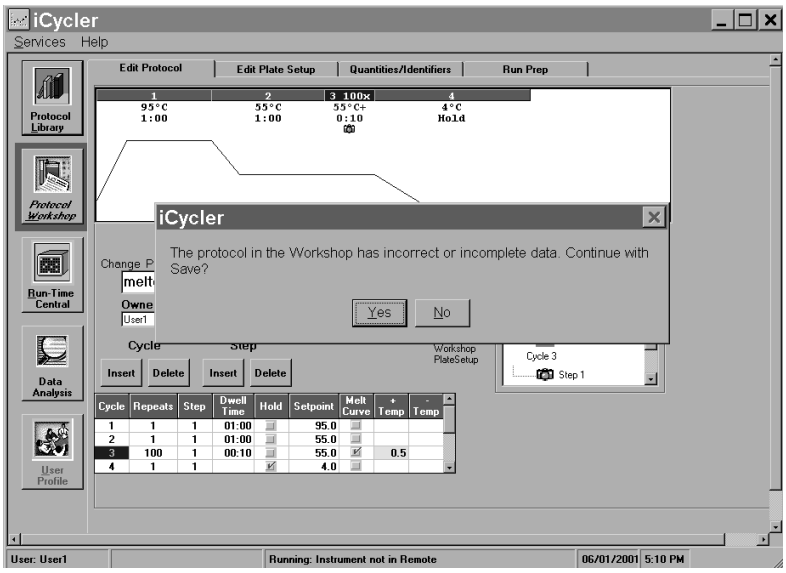


Fig. 8.4. When an inappropriate value is placed in the repeats column of a melt curve protocol, the + Temp or –Temp column will be highlighted. When trying to save this protocol, an error message will appear, alerting you that there is an error in the protocol.

- Finally, enter an appropriate dwell time for data collection under the Dwell Time column. Dwell times for melt curve will vary based on the number of fluorophores you using for an individual experiment.
    - The minimum dwell times necessary for data collection are:

      1 fluorophore = 7 sec

      2 fluorophores = 14 sec

      3 fluorophores = 22 sec

      4 fluorophores = 33 sec
    - We recommend using a slightly higher dwell time than the minimum values so that more data points are collected at each repeat (*e.g.* 1 fluorophore = 10 sec).

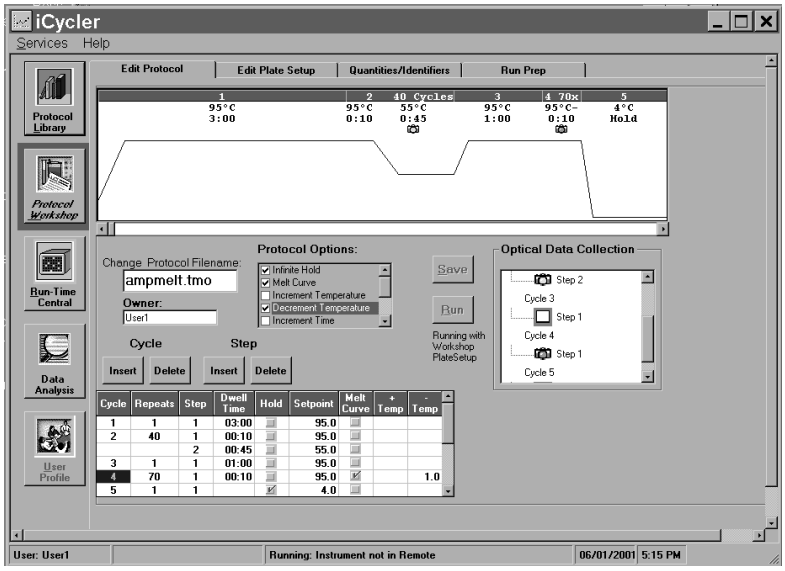


Fig. 8.5. A protocol was designed with a melt curve immediately following amplification. Amplification data is collecting at the annealing temperature (Cycle 2, Step 2) as indicated by the lighted yellow camera in the Optical Data Collection Box. Melt curve data is collected in cycle 4 as indicated by the lighted green camera in the Optical Data Collection Box.

- If desired, insert a final hold cycle. Click **Insert Cycle**, click on the next blank line, enter the desired hold temperature under the **Setpoint** column, and finally, check the box under the Hold column (**Note**: a dwell time is not necessary for a hold cycle).

4. Save the protocol by entering a file name in the box labeled **Change Protocol Filename** and click **Save**. Your new protocol will be saved under this file name in the iCycler folder. You can bring up this protocol at any time by clicking the **View Protocols** tab in the **Protocol Library**.

8.2.2 Quick Guide to Defining Standards in Plate Setups

1. Click **Dilution Series** to bring up box for specifying the dilutions. This checkbox is available when the "cursor" icon is selected.
   - Enter value in **Dilution Factor**. The default is 10 for a 10-fold dilution series.
   - Enter **Starting Concentration**. To use scientific notation, for example, change the 1.000e+00 default setting to the correct starting concentration. All exponential values are considered positive by default, so it is not necessary to enter a plus sign.
   - Click whether it is **increasing** or **decreasing**.
   - Enter the **Replicate Range** to be included in the standards value calculation. For example, standard replicates numbered 1 to 5.
   - Click **Calculate** to apply specifications.
2. Select the **"Standard"** icon and highlight wells for Standard 1. Continue selecting for each Standard and its replicates as specified under dilution series.
3. Use **Renumber Standards** box or **Next Replicate Number** to renumber if a mistake is made in numbering.
   - For example, if your standard replicates are numbered 1, 2, 3, and 5, you can check **Renumber Standards** box to renumber them as 1, 2, 3, and 4.

4. To check that standards have been properly calculated, use the cursor to click on the wells containing standards and view the quantity in the **Define Standards Quantity** box.
5. Quantities of the standards may be changed by repeating step 1.
6. Proceed with defining the rest of plate setup, defining fluorophores, and save.
7. Plate setup with samples, fluorophores, and quantities can be viewed using the **Quantities/Identifiers** tab of the Protocol Workshop.

Note: If you specify your standards to range from replicate group 1 through 5, the calculation assumes that the fold dilution is constant over the entire range. This means if you specify a 10-fold dilution factor, beginning with 1e10 for replicate 1, the software will assign values of 10^9 for replicate 2, 10^8 for replicate 3, 10^7 for replicate 4, and 10^6 for replicate 5. If you only define standards 1, 2, 3, and 5 (no standard 4), the software will still assign a value of 10^6 for replicate 5.

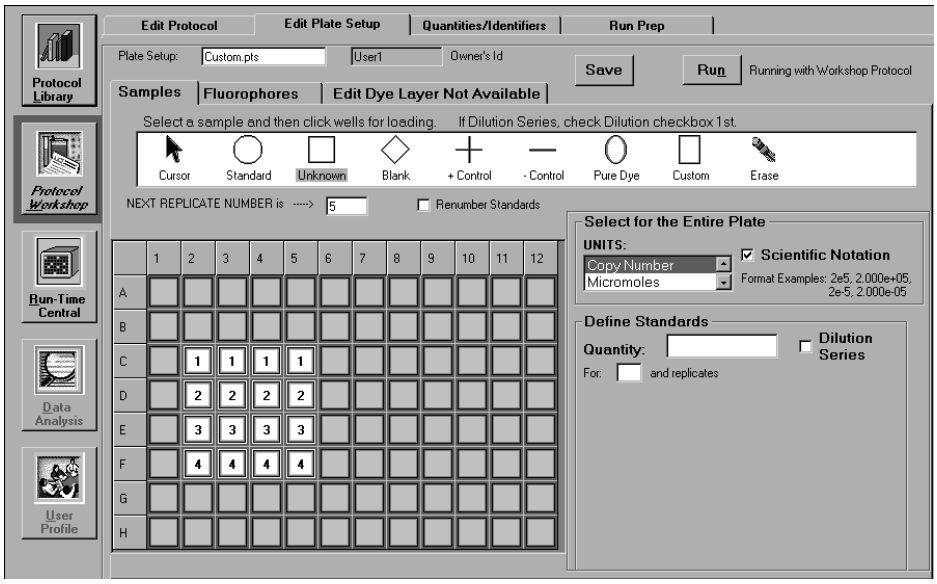


Fig. 8.6. Protocol Workshop/Edit Plate Setup Window before defining Standards.

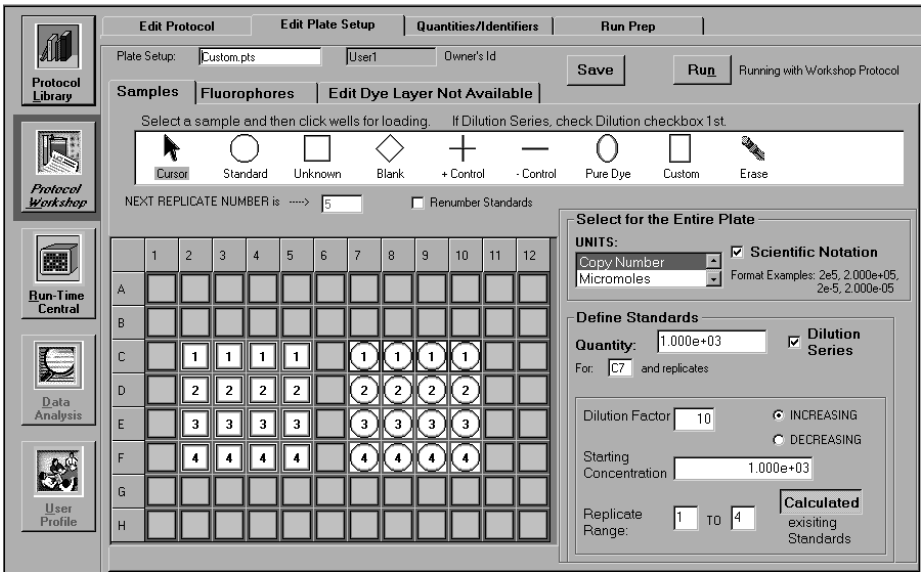


Fig. 8.7. Protocol Workshop/Edit Plate Setup Window defining standards using the Dilution Series and Scientific Notation features.

Fig. 8.8. Quantities/Identifiers tab of Protocol Workshop.

8.3 Collecting and Analyzing Melt Curve Data

8.3.1 Data Display

Melt Curve data are accessed in the Data Analysis Module under the **Melt Curve** tab. The data are displayed as **Fluorescence vs. Temperature** and **–dF/dT vs. Temperature**.

1. Fluorescence vs. Temperature plots fluorescent data in real-time as temperature increases or decreases (Figure 8.9).
2. -dF/dT vs. Temperature displays the negative first derivative of the Fluorescence vs. Temperature plot over temperature. The software identifies peaks and assigns melting temperatures from this plot (Figure 8.10).

Note: It may be necessary to adjust the height of the graphs so that the data is fully displayed and peaks can be clearly seen. The height of the plots can be adjusted by right clicking on the graph and adjusting the graph X and Y minimum & maximum values as described in Display Options in Section 7.2.

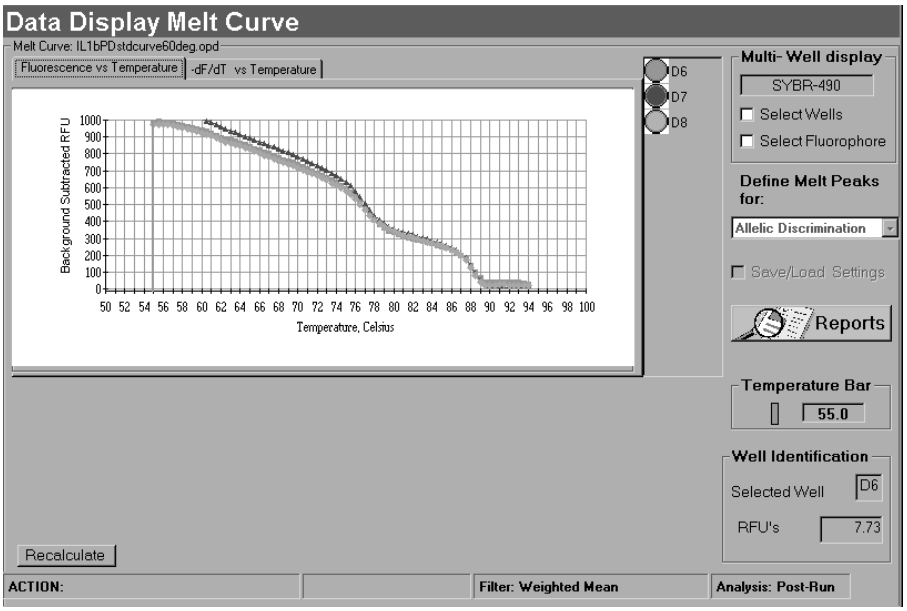


Fig. 8.9. Fluorescence vs. Temperature plot of melt curve data.

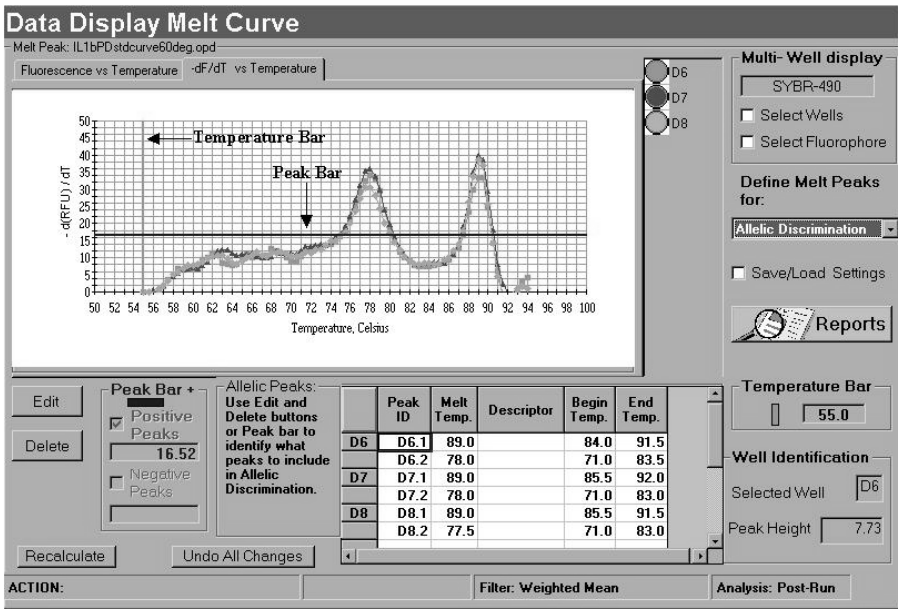


Fig. 8.10. –dF/dT vs. Temperature plot displaying melting peaks of amplified PCR product. Use Temperature Bar and Edit feature to edit begin and end temperature for peak identification. Use Peak Bar and Delete feature to select peaks for peak identification and melt temperature.

8.3.2 Identifying Peaks

1. Peaks are displayed in a spreadsheet under the **–dF/dT vs. Temperature** plot.
   - Each peak is given a **Peak ID** number. For example, Peak ID D6.1 means well D6, peak 1. Peak ID D6.3 means well D6, peak 3.
   - The order in which peaks are displayed are from highest to lowest melting temperature.
2. Additional information for each peak is given based on the type of analysis chosen. The information provided for each peak includes **Melt Temp.**, **Area**, peak **Begin Temperatur**e and peak **End Temperature**.
   - **Melt Temp**. The melting temperature (T_m) assigned for each peak. This is typically the temperature at the highest point of the peak.
   - **Descriptor**. Available for typing in any desired descriptive information for the individual peak or well.
   - **Area**. Area information is displayed in 3 different columns:

     Peak's Area – total area under the peak.

     Fraction of Total Area – displays the percentage of the total area under the trace for the specified well that is attributed to the peaks selected in the **Include in Total Area Column**.
   - **Begin Temp**. Defines the temperature at which each peak begins.
   - **End Temp**. Defines the temperature at which each peak ends.
3. The number of peaks displayed in the spreadsheet is dependent on the location of the **Peak Bar**.

8.3.2.1 Adjusting Identified Peaks

1. **Peak Bar**- The blue horizontal line displayed across the graph can be manually adjusted with the cursor to change which peaks are to be included in the spreadsheet
   - To adjust, click on bar and move with cursor.
   - Click the **Recalculate** button to update the spreadsheet when changes are made.
   - Only the peaks above the peak bar are identified and displayed in the data spreadsheet.
   - Peaks are renumbered in the data spreadsheet to reflect any changes.
   - The placement of the peak bar (Y-axis value) is also displayed to the left of the spreadsheet.
2. **Temperature Bar**- The orange vertical line across the graph can be moved with the cursor to identify a temperature anywhere on the graph.
   - The temperature where the bar is placed is displayed in the box to the right of the graph.
   - The temperature bar is typically used in the editing feature to identify the beginning or end temperature of a peak.

8.3.2.2 Editing Peaks

The begin and end temperature defined for a peak may be adjusted with the edit feature. This is only necessary when you want to adjust the temperatures the software identifies automatically. This also results in the recalculation of the area.

1. Click the **Edit** button. This button changes to **Editing** when in edit mode.
2. Click either the **Begin Temp.** or **End Temp.** box for that peak.
3. Move the **Temperature Bar** to the temperature preferred for the peak and release the mouse. The new temperature will be displayed in the selected **Begin Temp.** or **End Temp.** box and in the Temperature Bar box to the right of the spreadsheet.
4. To add the same edited temperature to another sample, click the **Begin Temp.** or **End Temp.** for that sample and click on the **Temperature Bar** again. The same edited temperature will be assigned to that sample.
5. Click the **Editing** or **Recalculate** button when finished to leave the edit mode.
6. To retrieve the original temperatures at any time, click **Undo All Changes**.

8.3.2.3 Deleting Peaks

The delete feature is an alternative to the peak bar adjustment for identifying peaks. You may delete any peak identified by the software that is not significant for data analysis.

1. Click the **Delete** button. This button changes to **Deleting** when in delete mode.
2. Select peak to be removed by clicking on the appropriate **Peak ID** box. Multiple peaks can be deleted from an individual well or from different wells at one time.
3. Click the **Deleting** or **Recalculate** button when finished to exit delete mode.
4. To retrieve the deleted peaks, click **Undo All Changes**.

Note: At least one peak must be identified in the spreadsheet for every well included in the data analysis. Attempting to delete all peaks for a given well results in an error message displayed at the bottom of the display: Action Denied! Deleting only peak for this well is not allowed.

8.3.3 Define Peaks Display

The data columns within the spreadsheet present various types of information depending on the analysis mode selected in the **Define Peaks Display**. The types of analysis include **Allelic Discrimination**, **PCR Product**, **Qualifying Probes**, or **Other Analysis**. The order of the data presentation and type of data displayed are changed for each type of analysis; however, the data are not affected by which analysis is chosen.

8.3.4 Save/Load Settings for Melt Curve Analysis

Modifications to the melt curve data using the **Edit** and **Delete** features may be saved and viewed at a later time. Melt curve parameters can be saved in the same method as saving PCR quantification parameters with the **Save/Load Settings** box. Multiple sets of parameters can be made for the same data file.

1. Click **Save/Load Settings** box after making changes.
2. The threshold Parameters box comes up with changes displayed in a spreadsheet (see Figure 8.13).
3. A default name is automatically assigned to the **Modified Melt Peaks .ss3 file**, beginning with "MP" followed by the file name previously assigned to the opd file. This .ss3 file will be saved in the iCycler folder.
4. Click **Save**.
5. To bring up saved settings at a later time, click Save/Load Settings box, then click **Open** in **Threshold Parameters** box to apply settings to melt curve data.

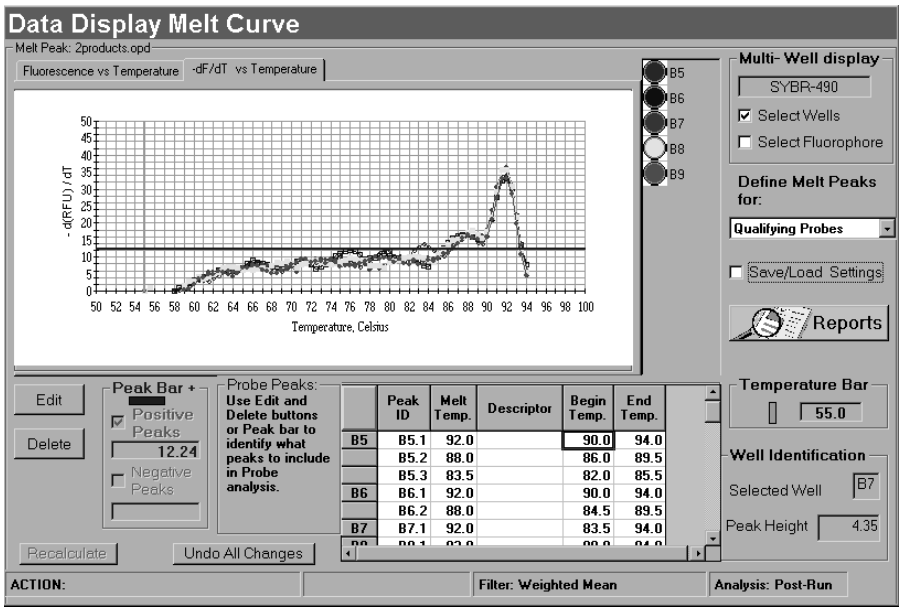


Fig. 8.11. Melt Curve data display of peaks and melt temperature before modifications are made.

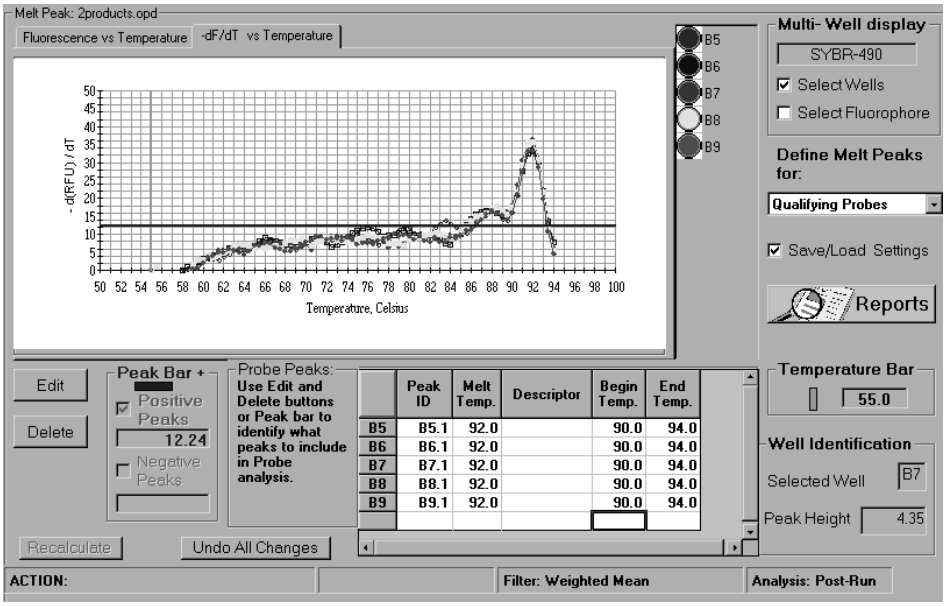


Fig. 8.12. Melt Curve data display after deleting peaks to isolate only the 92 °C product peaks for all wells. The Begin Temp. for well B7 was edited to change from 92 °C to 90 °C. Changes made are recorded in Figure 8.13.

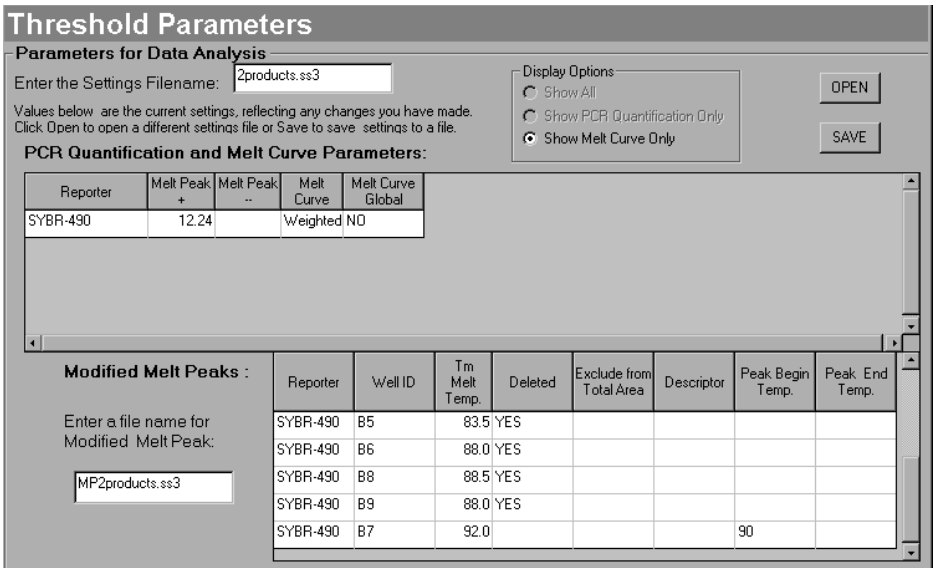


Fig. 8.13. Clicking Save/Load Settings brings up the Threshold Parameters window to open or save settings. Deleted peaks and edited temperatures can be identified by the Well ID and Melt Temperatures.

8.3.5 Quick Guide to Collecting and Analyzing Melt Curve Data

1. Assign a name to the data file before the run begins. Melt curve protocols can be run alone or immediately after an amplification protocol.
2. When the protocol is complete, click tab labeled **PCR View/Save Data**. Enter any notes about the run in **Data Run Note**s box and click **Save**. The .opd file name will be the same name assigned before run.

3. From **Melt Curve** tab, select **Fluorescence vs. Temperature** plot or the **–dF/dT vs. Temperature** plot.
4. Adjust the graph as needed to see data clearly. You may access graph adjusting feature by right clicking in the graph.
5. Make further adjustments on the **–dF/dT vs. Temperature** plot:
   - Adjust **Peak Bar** with cursor to include desired peaks for analysis.
   - If necessary use **Delete** feature to eliminate any undesired peaks.
   - Click **Recalculate** to obtain melting temperatures for the desired peaks.
6. Click **Reports** for Melt Curve Data Report. If amplification is coupled to melt curve, the report will contain information from both runs.
7. Click **Save/View Settings** to save analysis parameters.

8.4 Interpretation of Data

8.4.1 Peak Identification

Amplification with DNA-binding dyes (*e.g.* SYBR Green I) provides non-specific quantitation of DNA. Therefore, the presence of non-specific products during the amplification could affect the overall dynamic range of the standard curve and possibly decrease the efficiency of the reaction. IL-1b plasmid was amplified with SYBR Green I in a 5-fold dilution series from $1x10^4$ copies/well to 16 copies/well with primers containing a GGG/CCC overlap at their 3' ends. These primers were designed to produce significant primer-dimers that interfere with the amplification of the specific product. As seen in the amplification plot in Figure 8.16, the higher concentrations of plasmid ($1x10^4$, $2x10^3$) can be resolved from one another (~2.5 cycles apart). However, the lower dilutions cannot be resolved. Furthermore, it appears that amplification also occurred in the zero template control. Overall, this standard curve produced a correlation coefficient of 0.95 and a slope = -2.427 (~158% efficiency). Since efficiency is >100%, this suggests that more than one product was amplified and being monitored in real-time.

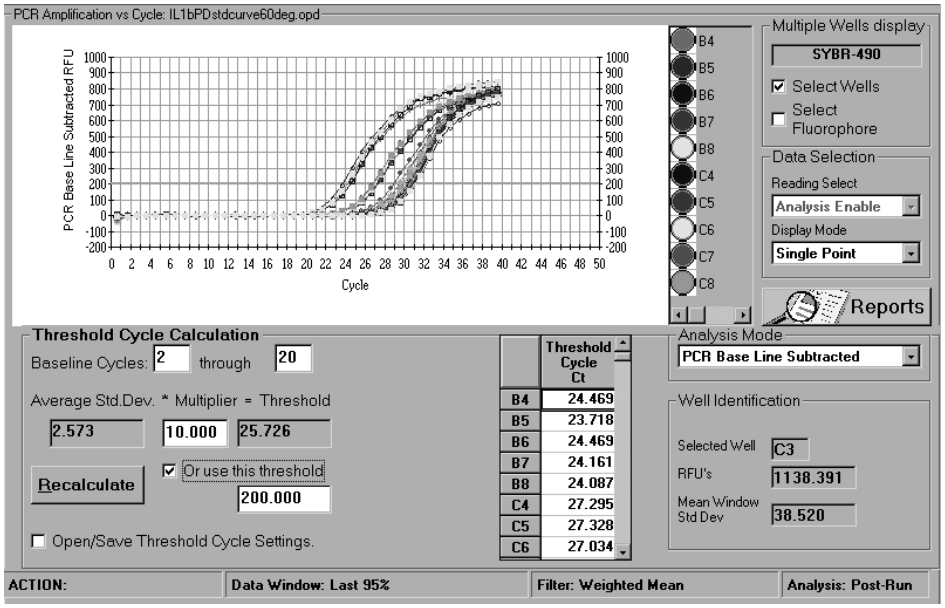


Fig. 8.14. Amplification of IL-1b plasmid (5-fold dilution series) with SYBR Green I using primers designed to produce primer-dimers.

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

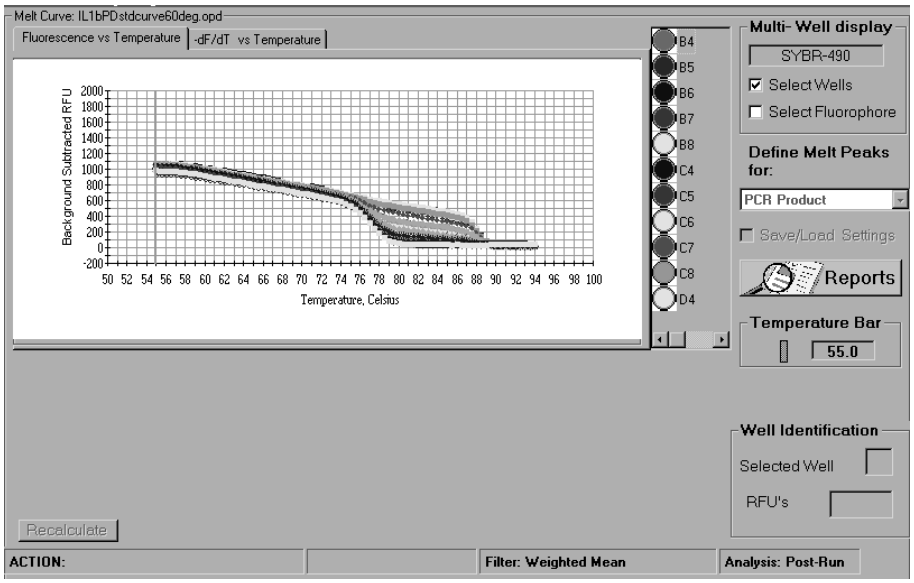


Fig. 8.15. Fluorescence vs. temperature plot of data collected during the melt curve run protocol between 55 °C – 95 °C (80 repeats, 0.5 °C increments) following amplification of IL-1b with SYBR Green I.

A negative first derivative plot is then generated from this data and presented as the rate of change in fluorescence over temperature or **–dF/dT vs. Temperature** (Figure 8.16). This graph represents the amplified DNA products as distinct melting peaks with specified melting temperatures (T_m):

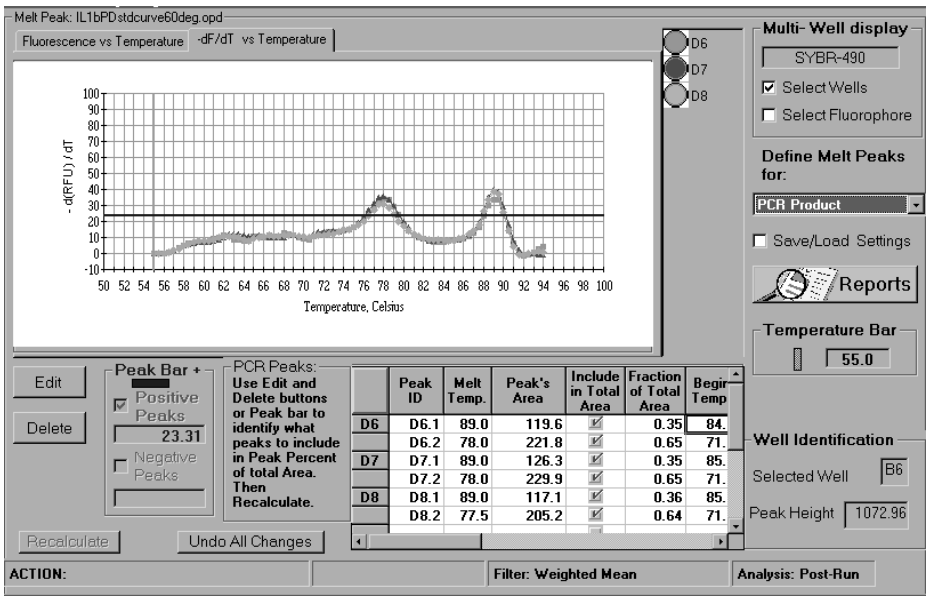


Fig. 8.16. Negative first derivative plot of melt curve data collected after amplification of IL-1b plasmid (4x10^2 copies/well) with SYBR Green I using primers designed to produce primer-dimers.

Figure 8.16 reveals melt curve data generated after amplification of wells containing $4x10^2$ copies/well of IL1b plasmid with SYBR Green I (3 replicates). Two peaks are identified in the melt curve with melting temperatures of 89 °C and 78 °C. Multiple peaks confirm the presence of multiple amplification products including the specific product of interest, plus additional non-specific products. In this example, the additional non-specific peak is due to the presence of primer-dimers since the amplification was performed with primers containing a GGG/CCC overlap at the 3'ends. Primer-dimers typically melt at a lower T_m than the specific amplified product due to their shorter length. Other secondary products will melt at a large range of melting temperatures. This is because T_m of each product is dependent on the length of the product as well as the percentage of G/C in the DNA sequence.

Based on the information obtained from the melt curve, it is apparent that a significant amount of primer-dimers (78 °C peak) were present during amplification. The melt curve results from the zero-template control wells (Figure 8.17) show that all amplification in these wells were due to primer-dimer (single melt curve peak at 78 °C). Overall, the presence of primer-dimers resulted in competition for reaction reagents and in a reduction of the dynamic range of the standard curve.

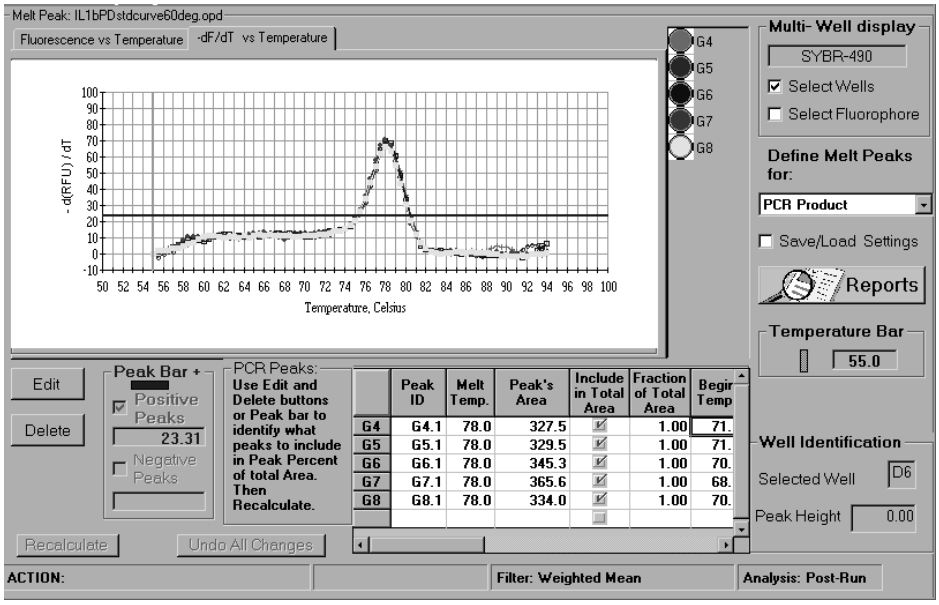


Fig. 8.17. Melt curve data collected in the zero template control wells for amplification of IL-1b. These wells contained primers only, yet produced a product peak at 78 °C, indicating that the amplified product is completely primer-dimer.

Melt curve analysis was used in this example to identify the source of the assay problems. In this particular example, primer-dimer formation was the major contributor to the decrease in dynamic range and efficiency of the reaction. Upon redesigning the primers, a second standard curve was generated as a 10-fold dilution series ($1x10^7$ – $1x10^2$ copies/well with a zero template control) using SYBR Green I (Figure 8.18).

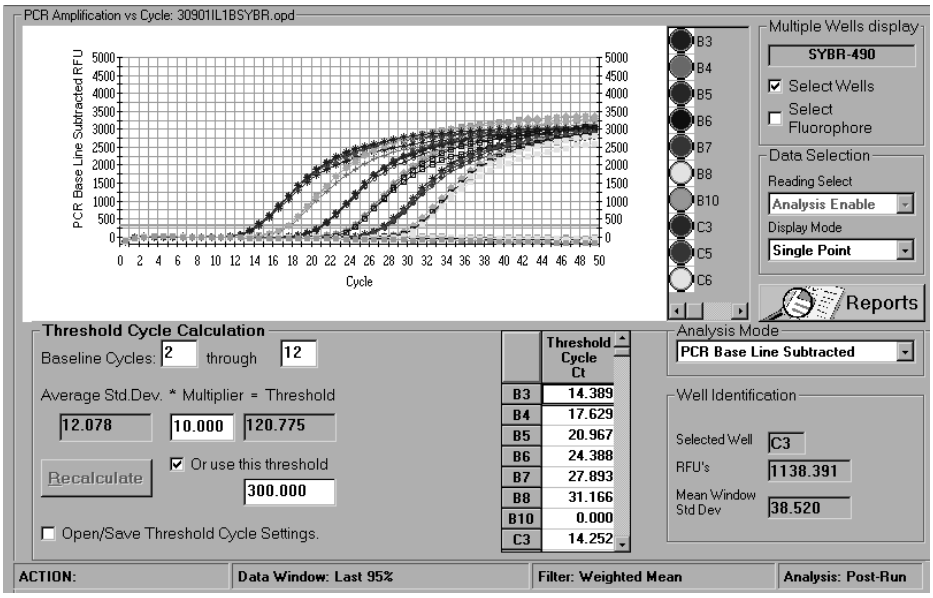


Fig. 8.18. 10-fold dilution series of IL-1b plasmid using redesigned primers with SYBR Green I. Concentrations ranged from $1x10^7$ – $1x10^2$ copies/well with a set of replicates as a zero template control.

This standard curve produced a correlation coefficient of 1.00 and a slope = -3.358 (~100 % efficiency). Furthermore, no amplification occurred in the zero template control wells. Melt curve analysis of the reactions revealed that all wells (with the exception of the zero template controls) produced a single amplified product at a T_m of 87–87.5 °C, the T_m of the intended product (Figure 8.19).

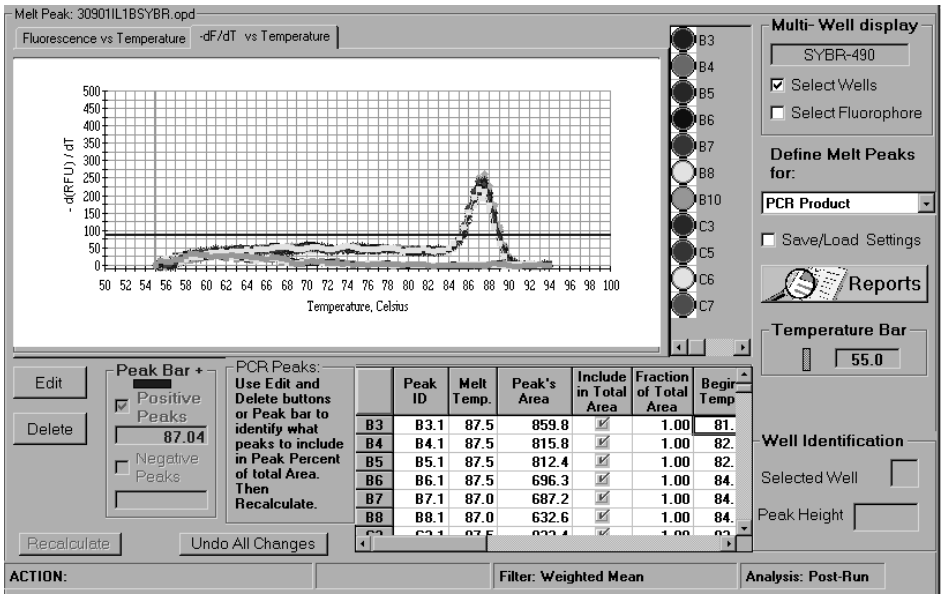


Fig. 8.19. Melt curve data collected after IL-1b amplification with redesigned primers. All wells in the standard curve produced a single amplification product. The zero template controls produced no amplified product.

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

8.4.2 Characterization of Molecular Beacons

Melt curve analysis can also aid in developing molecular beacon assays for real-time PCR. A melt curve can be used to assess the design of a molecular beacon. It can also be used to approximate an annealing temperature for the amplification protocol. In this example, a melt curve was performed on a molecular beacon labeled with FAM and quenched with DABCYL. The beacon was melted alone or in the presence of a single stranded complement that binds to the probe portion of the beacon. The conditions used for the protocol consisted of complete denaturation at 95 °C for 1 minute followed by melting at 1 °C decrements for 20 seconds from 95 °C to 50 °C. The fluorescence vs. temperature was plotted in real time during the melt curve run (Figure 8.20).

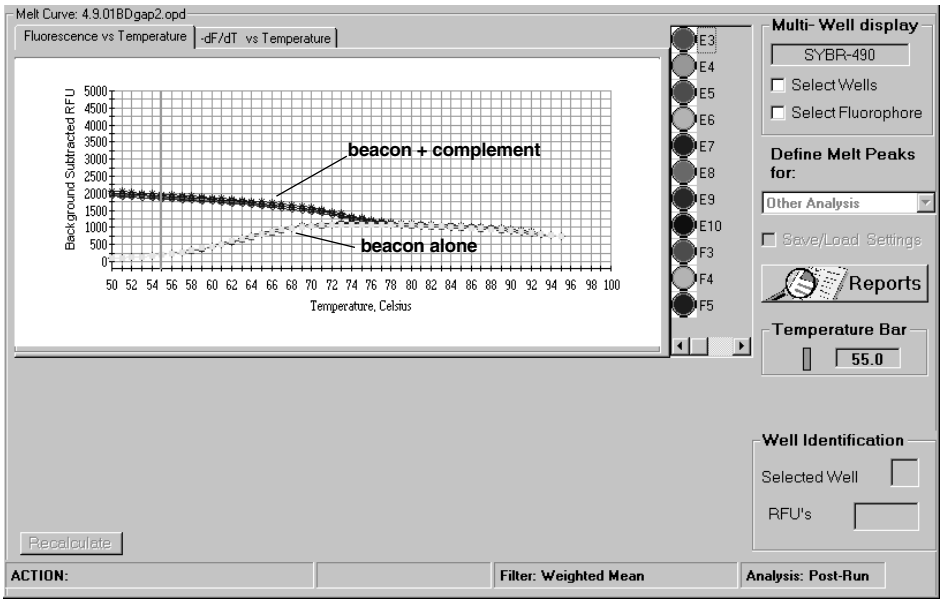


Fig. 8.20. Fluorescence vs. Temperature plot of a melt curve protocol for a FAM beacon alone or in the presence of a single stranded complement template.

The negative first derivative plot of fluorescence vs. temperature (**- dF/dT vs. Temperature**) provides information as to whether the beacon was designed correctly. Figure 8.23 shows distinct melting peaks for beacon alone and beacon mixed with complement template. The graph indicates that the beacon began to bind to its template around 80 °C (an increase in fluorescence or positive peak). By 65 °C, all of the available template is bound to beacon probes. The beacon alone trace shows that the highest rate of beacon arms closing beginning at 75 °C (a decrease in fluorescence or negative peak). By 53 °C, all of the beacon arms have closed. This information indicates that this beacon was designed correctly. The beacon will bind to its template around 80 °C before the arms of the unbound beacons close at 75 °C. Once the beacons are closed, they are unavailable to bind to template. Therefore, the temperature at which the arms close must be lower than the temperature at which the beacon binds to template.

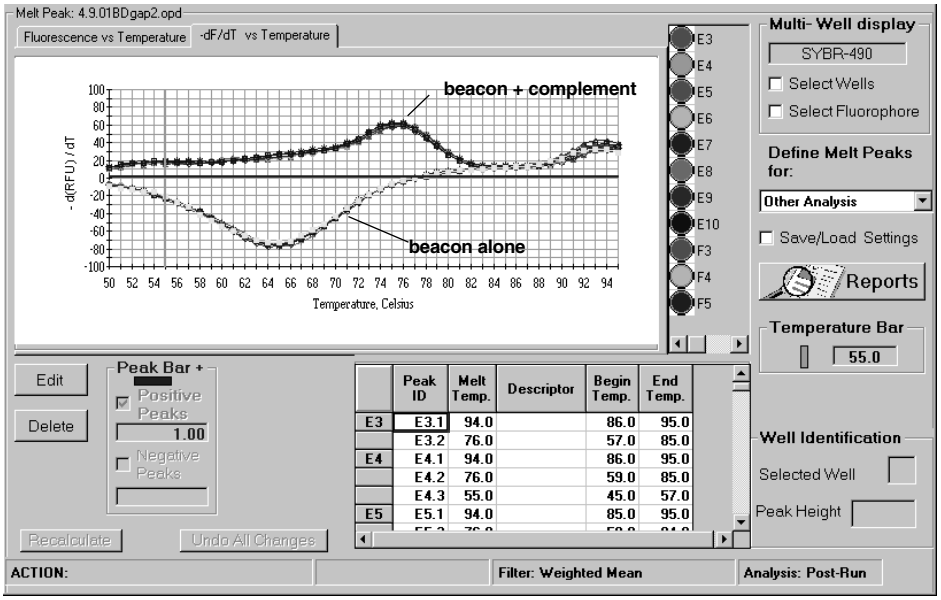


Fig. 8.21. Negative first derivative (-dF/dT vs. Temperature) plot of fluorescence vs. temperature of a FAM beacon alone or in the presence of a single stranded complement template. The graph demonstrates the rate of change in fluorescence, with the peaks representing the maximun rate of change.

This plot also can assist the user in choosing the appropriate annealing temperature for amplification. When a molecular beacon probe anneals to its target, the total fluorescence is recorded as amplification data. As amplification products increase, the fluorescence also continually increases. Since the total fluorescence is detected, it is important to choose an annealing temperature where fluorescence of molecular beacon to target is high, and background fluorescence from unbound beacon is low. Based on the data presented in Figure 8.21, the best annealing temperature would be between 50–53 °C. All available template is bound to probe by 65 °C. However, the background fluorescence of molecular beacon alone is still extremely high at this temperature. The arms of the remaining beacons not bound to template will not close completely until 53 °C. By choosing an annealing temperature between 50–53 °C, the total fluorescence collected for data analysis is primarily from molecular beacon probe binding to template with minimal background fluorescence. Therefore, melt curve analysis is a simple, quick assay for examining molecular beacon design as well as determining the appropriate annealing temperature for amplification.

8.4.3 Single Nucleotide Polymorphism (SNP) Detection

Melt curve is also a convenient way of distinguishing between two genes differing by only a single nucleotide polymorphism (SNP). Several papers have described using fluorescence resonance energy transfer (FRET) for SNP detection with melt curve[1,2]. It is a common technique used for allelic discrimination. In the example below (Figures 8.22–8.24), vectors containing the wild-type p53 tumor suppressor gene (Figure 8.22) or the dominant-negative mutant (Figure 8.23) (differing only by a G/A transition at nucleotide 1017) were amplified alone or in a 1:1 heterozygote mixture (Figure 8.24). The same primer set was used to amplify the wild-type and the mutant genes. The reverse primer was labeled internally with Cy5 near the 3' end. A FAM probe labeled at the 3' end, with position 1017 in the middle of the probe, was used to detect the products previously amplified with the Cy5-labeled primer. When the FAM probe hybridizes to the Cy5-labeled product, FRET occurs from FAM to Cy5 upon excitation of the FAM fluorophore. This Cy5 signal is collected during the melt curve run. As the T_m of the probe binding to either wild-type or mutant product is reached, the probe is released from its target and the Cy5 signal diminishes. As seen in Figures 8.22 and 8.23, there was a 6 °C shift in the T_m of the probe binding to wild-type target (T_m = 67.2 °C) as opposed to the probe binding to mutant target (T_m = 61.2 °C). The melting temperatures for both peaks could be resolved from one another in a 1:1 heterozygote mixture (Figure 8.24). These data demonstrate that melt curve can be used for allelic discrimination between wild-type and mutant alleles differing by a single base pair mutation.

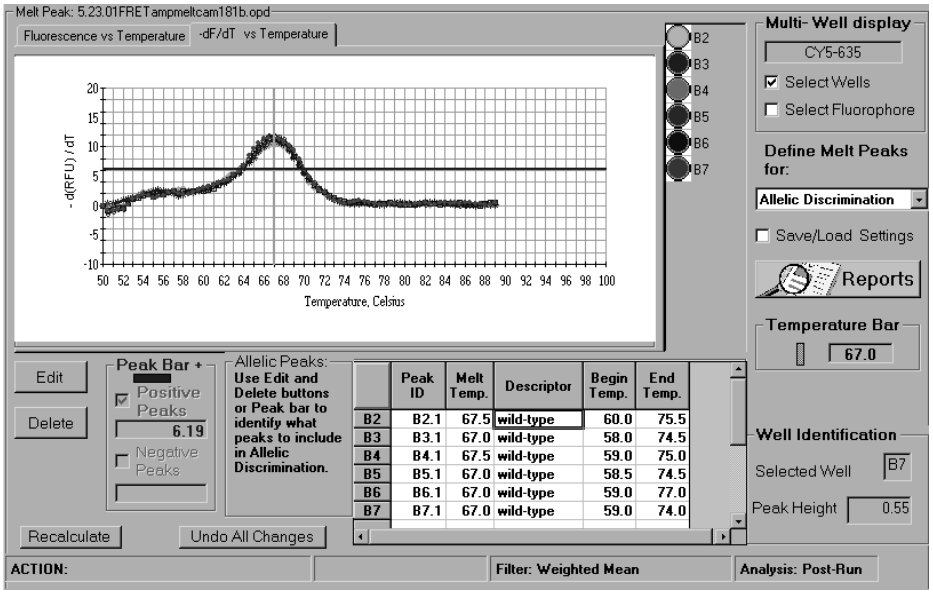


Fig. 8.22. Melt curve plot of the amplified p53 wild-type.

[1] *Ibid*

[2] *Ibid*

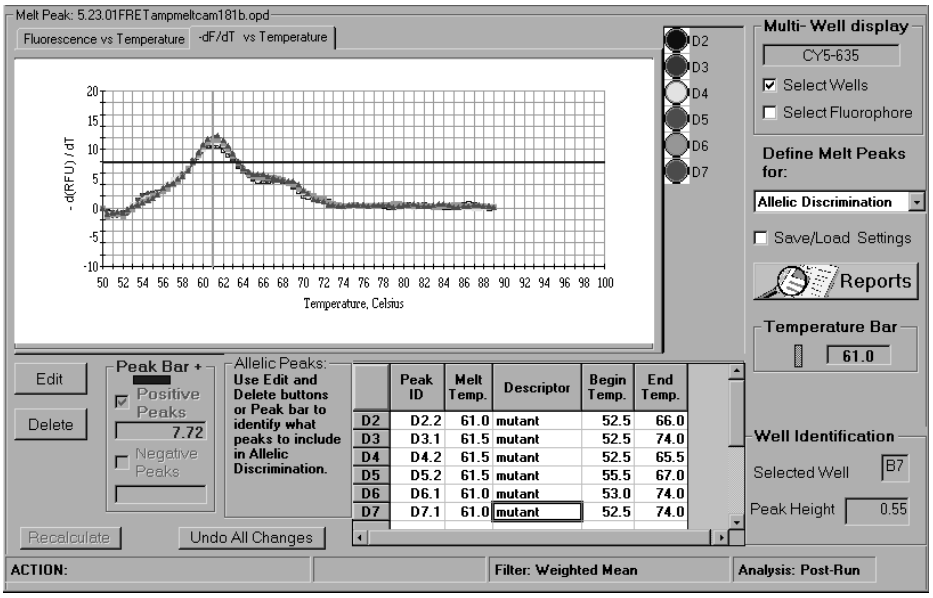


Fig. 8.23. Melt curve plot of the amplified dominant-negative p53 mutant allele.

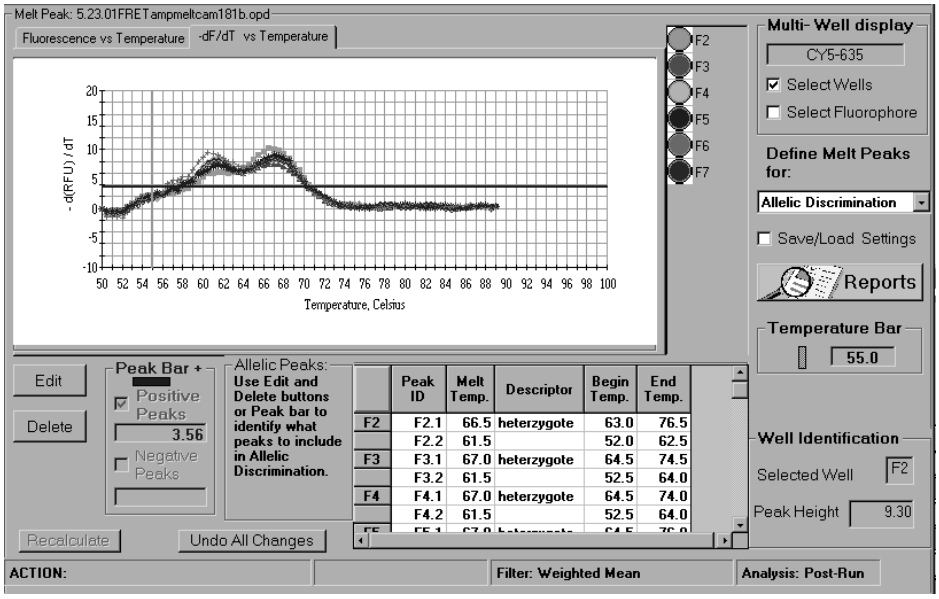


Fig. 8.24. Melt curve plot of the amplified 1:1 mixture of both wild-type and mutant p53 alleles (heterozygotes).

Section 9
User Preferences

9.1 User Preferences Module Not Enabled in this version of Software

The User Preferences Module includes customization features so that each user's preferred protocol, plate setup, and analysis parameters are automatically accessed as that user signs into the software. These features are not enabled in this version. An update of this section will be sent with each upgrade detailing the features and use of this module.

Section 10
Care and Maintenance

10.1 Cleaning the unit

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

10.2 Replacing the lamp

Before replacing the lamp turn the instrument off and to allow it to cool down for at least 15 minutes.

The lamp is located on the right side of the Optical Module immediately behind the right latch (Figure 10.1). To replace the lamp:

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

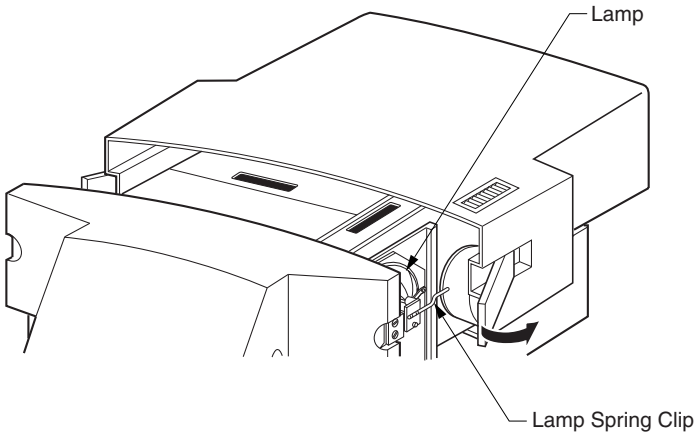


Fig. 10.1. Replacing the lamp.

Appendix A
Specifications

Input Power	100–250 VAC ± 10% 50–60 Hz
	250 VA max., 95W typical
Environmental	
Operating temperature	15–30 °C
Storage temperature	-20–30 °C
Humidity	0–90%, noncondensing
Descriptive, Optical Module	
Size	width = 12.2” (31. cm)
	height = 7” (17.8 cm)
	depth = 17” (43.2 cm)
Weight	15 lbs (6.8 kg) net max
Power Supply Size	width = 3.75” (9.5 cm)
	height = 2.56” (6.5 cm)
	depth = 7.4” (18.8 cm)
Power Supply Weight	2 lbs (0.9 kg) net max
Data communications	bidirectional PC parallel port
Regulatory Compliance	TUV
	CE

Appendix B
Minimum Computer Specifications

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

- Microsoft Internet Explorer (v5.0 or higher)
- Any IBM-compatible computer with a 400 Mhz or faster Pentium processor
- Operating system with Windows 95, Windows 98, Windows 2000, and Windows NT US version
- 128 Mb RAM minimum
- 6 GB Harddrive minimum
- CD ROM drive
- 1.44 MB capacity 3.5” floppy disk drive
- Mouse
- 57.6 kB Serial port
- Bi-directional Parallel port (EPP)
- 1024 x 768 x 256 colors minimum Screen resolution
- IEEE 1284 Parallel Port Cable (m-m)

The following are also recommended:

- USB port for printer
- A USB compatible color printer
- A modem with an Internet connection (suggested for downloading new releases)
- An uninterrupted power supply (500 VA or more) for the iCycler and the computer
- Use of shielded (rather than unshielded) cables

Appendix C
Warranty

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

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

The warranty does not apply to fuses.

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

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

This instrument is an Authorized Thermal Cycler. Its purchase price includes the up-front fee component of a license under the patents on the Polymerase Chain Reaction (PCR) process, which are owned by Hoffmann-LaRoche Ltd, to practice the PCR process for internal research and development using this instrument. The running royalty component of that license may be purchased from Perkin-Elmer or obtained by purchasing Authorized Reagents. This instrument is also an Authorized Thermal Cycler for use with applications licenses available from Perkin-Elmer. Its use with Authorized Reagents also provides a limited PCR license in accordance with the label rights accompanying such reagents. Purchase of this product does not itself convey to the purchaser a complete license or right to perform the PCR process. Further information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at The Perkin-Elmer Corporation, 850 Lincoln Centre Drive, Foster City, California, 94404.

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

Appendix D
Product Information

Catalog Number	Product Description
170-8720	**iCycler with 96 x 0.2 ml Reaction Module**, includes iCycler base with the 96 x 0.2 ml iCycler Reaction Module, In-Sample temperature Probe (0.2 ml tube size), Comprehensive Instruction Manual, Quick Reference Card, PCR tubes, and power cord.
170-8703	**iCycler 96 x 0.2 ml Reaction Module**, includes 96 x 0.2 ml iCycler Reaction Module, In-Sample temperature Probe (0.2 ml tube size)
170-8740	**iCycler Optical System**, includes iCycler Optical Upgrade for 96-well reaction module, optical power supply, basic filter set, iCycler iQ validation solution, software interface CD-ROM, optical quality 96-well PCR plates, communication cables, power cord, instructions.
170-8714	**iCycler 0.2 ml Immersion Probe/Sample Cap**
223-9441	**96-well 200 µl Thin Wall PCR Plates**, 25 per box
223-9442	**96-well 200 µl PCR Plate Caps**, for 223-9441, 300 per box
223-9444	**Optical Quality Sealing Tapes**, optimized for use with 223-9441, 60 sheets
170-8777	**iCycler iQ Filter Set,** FAM/SYBR
170-8778	**iCycler iQ Filter Set,** VIC/HEX/TET/Cy3
170-8779	**iCycler iQ Filter Set,** Cy3/TAMRA
170-8781	**iCycler iQ Filter Set,** ROX/TexasRed
170-8782	**iCycler iQ Filter Set,** Cy5
170-8783	**iCycler iQ Filter Set,** Ethidium Bromide
170-8792	**iCycler iQ Calibrator Dye Solution Set**
170-8794	**iCycler iQ External Well Factor Solution**
170-8793	**iCycler iQ FAM Calibrator Solution,** 5 per package
170-8795	**iCycler iQ HEX Calibrator Solution,** 5 per package
170-8797	**iCycler iQ Cy5 Calibrator Solution,** 5 per package
170-8799	**iCycler iQ Texas Red Calibrator Solution,** 5 per package

Appendix E
Error Messages and Alerts

E.1 Software Startup

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

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

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

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

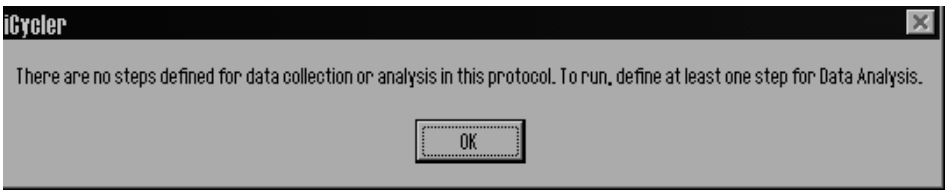
E.2 Protocol Workshop and Protocol LIbrary

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

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

Cause: You have created a thermal protocol without real time data analysis. A gray camera icon has been selected for at least one step (collect and save data), but there is no step with a yellow camera icon (analyze data in real time).

Solution: Add a data analysis step, one with a yellow camera icon, to the thermal protocol, or click the existing gray camera icon to change it to a yellow one.



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

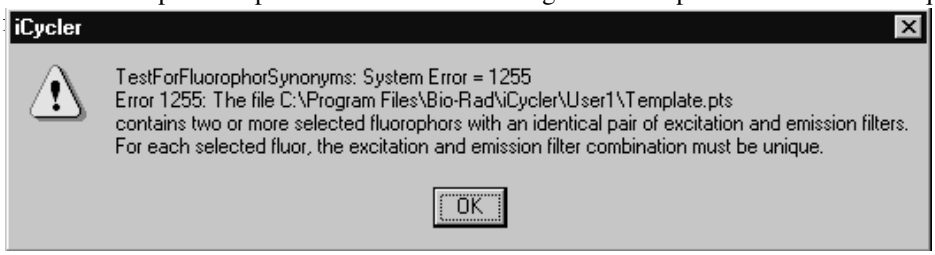
Solution: Specify data collection for real time analysis (define a step with a yellow camera icon) at one step of the protocol.

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

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

Cause: You are trying to view a plate setup file created in a previous version of software that contains two or more fluors defined for the same filter pair. This file cannot be opened. For example, FAM490 and TET490 were both defined in the same plate setup. Each fluorophore on a plate must be associated with a unique filter pair. In this case, there are two fluors to be measured by the same filter pair (490/520).

Solution: The plate setup must be recreated matching each fluorophore chosen with a unique



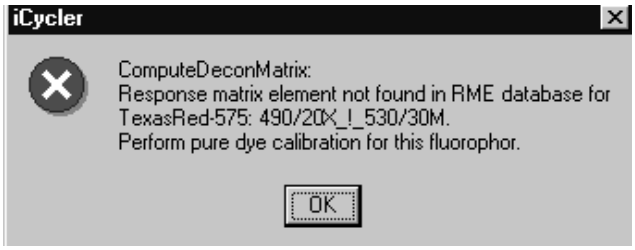
Cause: You are trying to save or run a plate setup in which two or more fluorophores are associated with the same filter pair. In every experiment, there must be a unique filter pair combined with each fluorophore. A plate setup that calls for both FAM490 and TET 490 on the same plate is not valid and cannot be saved or executed.

Solution: Edit the plate setup file in the Protocol Workshop. In the Fluorophore view of the Edit Plate Setup window, click Clear Setup and begin be redefining the fluorophores on the plate. You may choose only those combinations of fluorophores and filter pairs so that no filter pair is used to read more than one fluorophore.

Cause: This happens if you use a version of software after 1.800 to try to open a stored OPD file collected with version 1.440 of the software.

Solution: The pure dye calibration data contained in the RME file must have an entry for FAM/SYBR fluorophores in combination with the 485/535 (replaced with the 490/530 filter pair). The RME file that is installed with all versions after 1.880 includes an entry for this combination, but if that RME file is deleted, it is not possible to recreate the correct value through a pure dye calibration protocol. You must edit the RME file in a word processing application and add the following lines.

[FAM/SYBR]
485DF22_!_535RDF45=4.021013e+003



Cause: This alert is similar to the previous one, except that this one will list one or more fluorophore/filter pair combinations other than FAM/SYBR with the 485/535 filter pair. In this case, a plate setup file has called for the use of a fluorophore/filter pair combination (in this example, Texas Red 575 with the 490/530 filter pair) for which pure dye calibration data do not exist.

Solution: You must first conduct a pure dye calibration in which the desired fluorophore/filter pair combination is evaluated.

Cause: You clicked the radio button for Pure Dye Calibration in the Run Setup screen, but the plate setup file listed in the Run Setup screen has one or more wells defined to have more than one fluorophore. In a pure dye calibration protocol, no well may have more than one fluorophore.

Solution: If the plate setup file is in error, correct it. If any wells have more than one fluorophore, deselect them from the pure dye calibration plate setup file, or make a new pure dye calibration plate in which no more than one fluorophore is in any well.

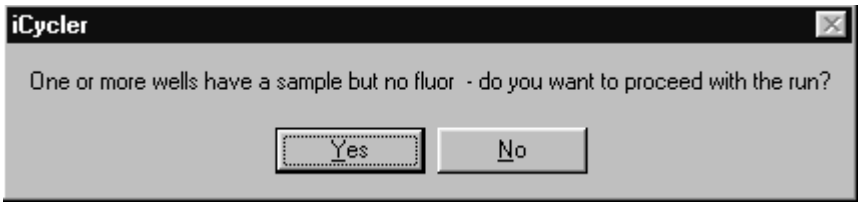
Cause: No fluorophore has been defined in the plate setup file.

Solution: Define at least one fluorophore on the experimental plate in the Plate Setup file.

E.3 Run Time Central

Cause: You have not chosen to use each fluorophore defined for your plate setup.

Solution: This is just a reminder to check your plate setup. It is not necessary to use all defined fluorophores in any experiment.



Cause: You are trying to use a plate setup file in which some wells defined to contain sample are not defined for a fluorophore. No fluorescent data will be collected on that well.

Solution: If you want fluorescent data collected on those wells that are not presently defined for fluorophore, assign a fluorophore to them in the Edit Plate Setup window of the Protocol Workshop.

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

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

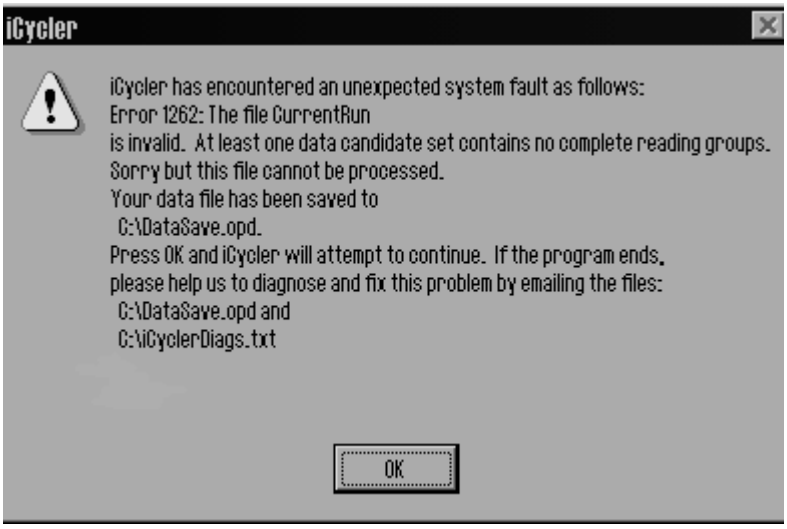
Cause: The lid has been opened while an experiment is in progress, or the lid is not closed completely.

Solution: Close the lid securely. If the lid is opened during an experiment, the software will pause the protocol until the camera lid is closed and you click **Continue Running Protocol**.

Cause: Light of too much intensity has triggered the protective circuitry of the iQ optical module. If this occurs during execution of a protocol, the software will put the instrument is Pause until power is restored to the intensifier.

Solution. Correct the cause of the intensifier shutdown before toggling the switch to the iQ detector power supply off and then back on. Click Continue Running Protocol.

The most likely causes of intensifier shutdown are associated with the filter wheels and filters. Shutdown can occur if unfiltered light reaches the intensifier, so be sure that each position of the filter wheel has either a filter or a blank. This can also occur if filters are not in their correct positions as defined in the filter wheel setups; make sure that the filters are in the correct positions in both the excitation and emission filter wheels. Finally, extremely concentrated solutions of fluorophores can trigger the intensifier's protective circuits.

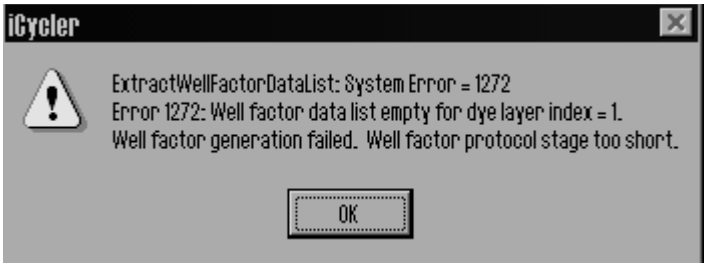


Cause: The data collection step of the protocol was not sufficiently long to collect at least one complete reading group. A complete reading group consists of a separate set of exposures taken with each filter pair. Since there is one filter pair for each fluorophore on the plate, a complete reading group can consist of as many as four sets of exposures.

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

If this occurs while the software is trying to collect dynamic well factors at the beginning of the experiment, you can edit the Dynamicwf.tmo file to extend the dwell times of the single step in the second cycle (the cycle with the gray camera icon). If you do edit the Dynamicwf.tmo file, we suggest that you first save the original under a different name and restore it afterwards. Dynamicwf.tmo is found in the User1 folder.

If this happens while the software is trying to collect external well factors, it probably means that the Well Factor solution was not properly diluted and it is saturating the detector, even at the shortest exposure time of 10 ms.

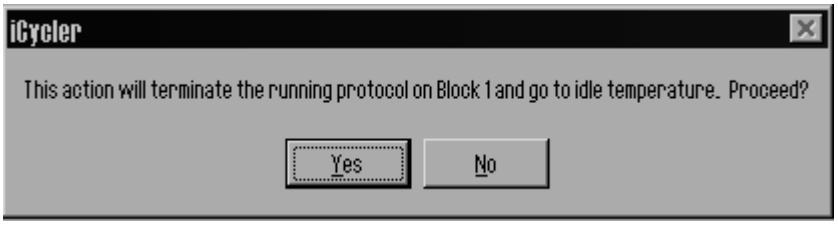


Cause: The data collection step of the cycle inserted for external well factor calculation was not sufficiently long.

Solution: Edit the thermal protocol file Externalwf.tmo and extend the dwell time of the data collection step (the single step in the second cycle).

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

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



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

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

E.4 Data Analysis

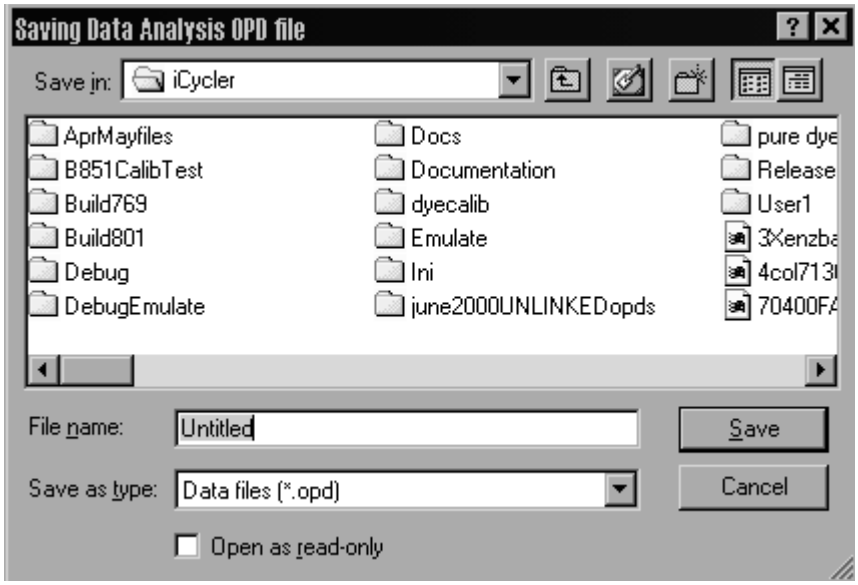
Cause: You have tried to perform some other data analysis before PCR Base Line subtraction. Until PCR Base Line subtraction is completed, no other analysis options may be carried out.

Solution. Choose PCR Base Line Subtraction.

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

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

E.5 Exiting Software



Cause: The Save data dialog box opens automatically if you try to exit the software without saving the last data set collected, or if you have modified the data set in some way (for example, you have applied a new RME file to these data by using the 'Use External Calibration File' box.).

Solution: Save data files before exiting the software.

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

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

Appendix F
Hardware Error Messages

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

Message	Possible Cause(s)	Possible Solution(s)
`Can't allocate image buffer (low memory)`	When the dll starts, an image buffer is allocated in memory. This message appears if not enough memory is available.	1. Close other applications. 2. Reboot the computer. Note: Sometimes applications may cause "memory leaks", slowly depleting available RAM. Rebooting resets the memory.
`No bi-directional port at nnnh.`	The port address selected does not point to a valid parallel port. If the system was working previously, the port settings have been changed in the BIOS or under Settings.	1. Reset BIOS values. 2. Check under Settings to see if the port address was changed for LPT1.

`Failed 1st Readback`
 	
and
 	
`Failed 2nd Readback`
	The camera has failed to respond to a test command. This usually occurs if the camera is not powered up, if the cable is unplugged, or if there is a bad cable.	1. Check the camera to be sure the power is on.
		2. Check the cable connection.
		3. Replace the cable

`Timeout waiting for IMGRDY bit`	The camera failed to respond after an exposure command was sent successfully.	Turn the camera off, then on to reset it.

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

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

Appendix G
Description of iCycler iQ Data Processing

1. Fluorescent data may be collected for real-time analysis or for post-run analysis. Data are simultaneously collected from all 96 wells during the entire dwell time of the steps defined for analysis; the number of individual readings on each well depends on the exposure time and the dwell time. With a 30-second dwell time and a 160-ms exposure, as many as 25 data points may be collected at each cycle for every well.

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

3. Data collection begins at an exposure time of 640 ms by default. The pixels in the camera are monitored for saturation at each cycle and if a significant number of pixels are saturated, then the iQ Detector will automatically reduce exposure time in half. The next time saturated pixels are detected, the exposure time will be reduced by half again and this will continue each time the detector measures significant saturation. Eventually all data are normalized to the 640-ms exposure time; that is, data collected at 320 ms are doubled and data collected at 160 ms are quadrupled.

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

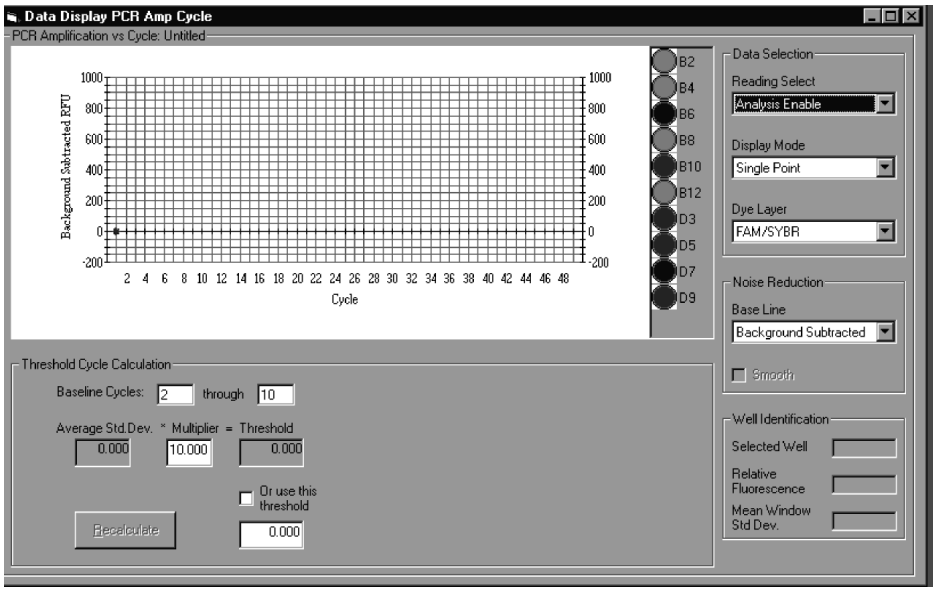


Fig G.1. The PCR Amp Cycle Plot.

There are two views of the data available: Background Subtracted and PCR Base Line Subtracted.

- By default, the Background Subtracted data displayed in the PCR Amp Cycle plot are the mean of the last three net readings in the cycle. The Background Subtracted data are also normalized to a constant exposure time.
- By default, the PCR Base Line Subtracted data displayed in the PCR Amp Cycle plot are calculated by first fitting a straight line through Background Subtracted data of cycles 2 through 10. This line is extended beyond the tenth cycle for each subsequent cycle. Next the actual background subtracted reading (inner-outer) at each cycle is subtracted from the value calculated for each cycle from the straight line fit made above, and then plotted against cycle number.

5. From the PCR Base Line Subtracted plot, the software will calculate the **threshold** fluorescence for the experiment and then determine the **threshold cycle** for each well. The threshold is the level of fluorescence that is considered to be significantly above the background level of fluorescence measured in the early cycles of the amplification. By default, this is 10 times the mean of the standard deviation of the fluorescence reading of each well over the beginning 10 cycles, excluding Cycle 1.

6. A graph is constructed by plotting the log of the beginning concentration of template in each standard well against that well's threshold cycle. A least squares linear regression is fit to these data to produce a standard curve. Next the beginning concentration of each well defined as an unknown is calculated by mapping the threshold cycle of that well to the standard curve. If the unknown was amplified in replicate samples, then a standard deviation is also calculated for the beginning concentration.

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

		A1		H1	
Cycle #	**Exposure Time**	**Inner**	**Outer**	**Inner**	**Outer**
1	640	350	118	400	150
2	640	360	129	405	154
5	640	355	122	400	147
10	640	360	125	405	150
15	640	365	125	408	154
20	640	412	135	460	165
25	320	315	69	350	88

   A. The Background Subtracted graph is constructed by plotting the net (inner-outer) reading for each well at each cycle. The net difference at cycle 25 was doubled to normalize the exposure time to 640 ms.

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

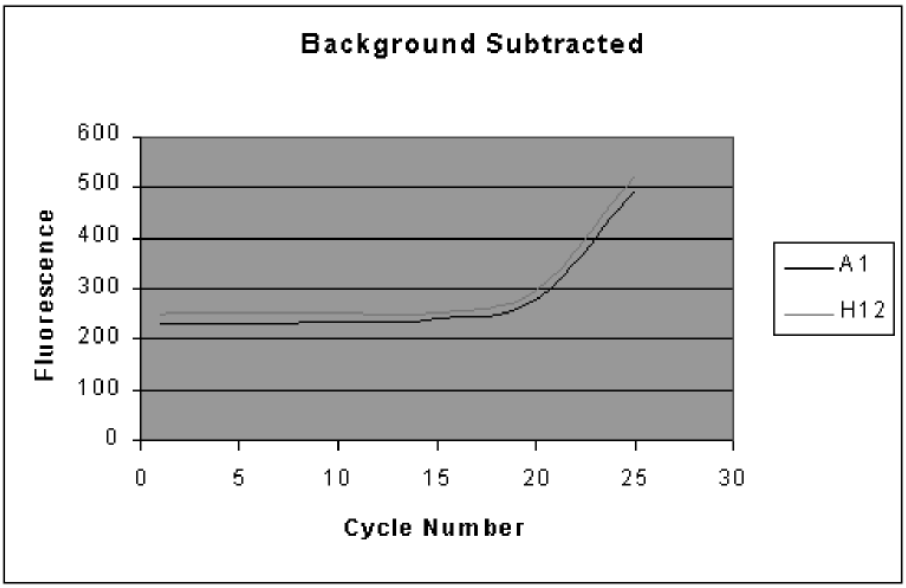


Fig.G.2. Background Subtracted Plot.

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

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

Fluorescence = Cycle * (0.490) + 230.2

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

Fluorescence = Cycle * (0.092) + 251.5

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

	Background subtracted		**Predicted**		**Final**	
Cycle #	**A1**	**H12**	**A1**	**H12**	**A1**	**H12**
1	232	250	230.7	251.6	1.3 -	1.6
2	231	251	231.2	251.7	-0.2	-0.7
5	233	253	232.7	251.9	0.3	1.1
10	235	252	235.1	252.4	-0.1	-0.4
15	240	254	237.6	252.9	2.4	1.1
20	277	295	240.0	253.3	37.04	1.7
25	493	525	242.5	253.8	250.5	271.2

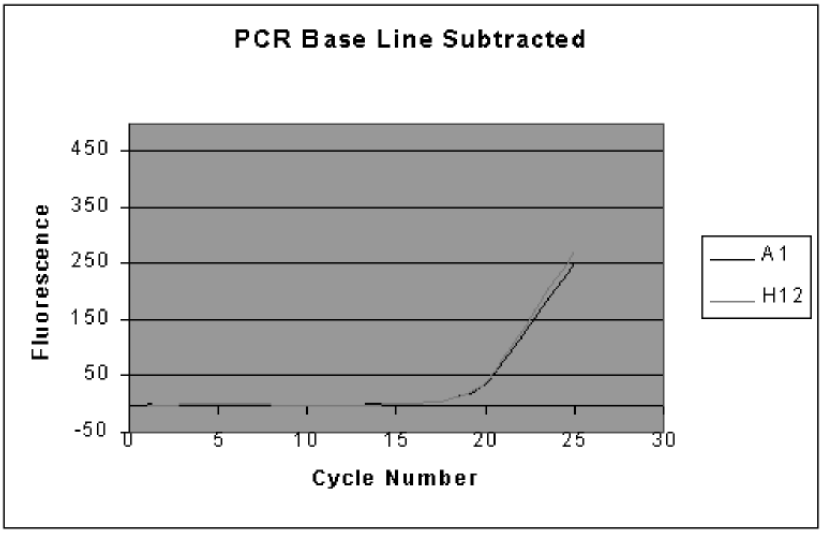


Fig. G.3. PCR Base Line Subtracted Plot.

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

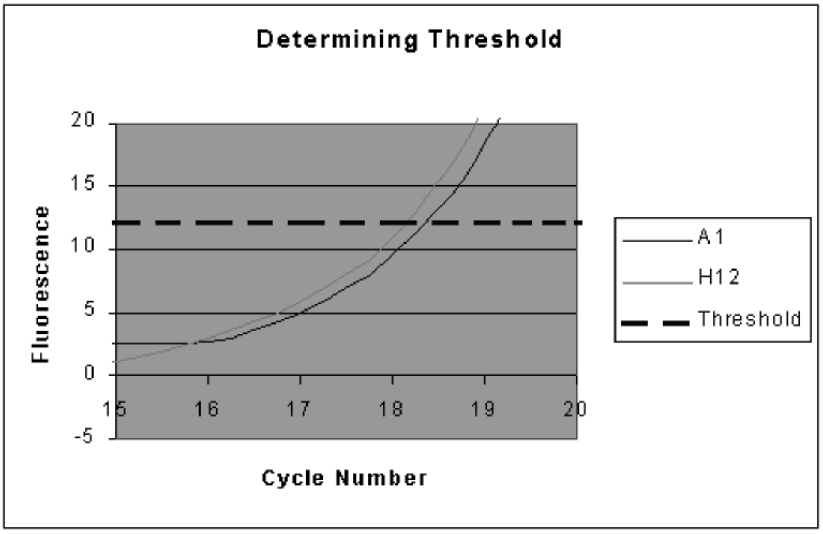


Fig. G.4. Determining Threshold Level.

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

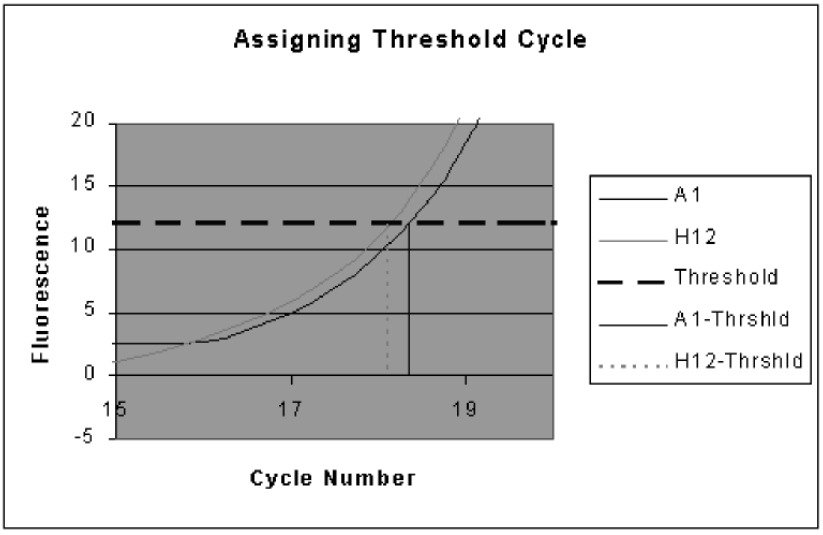


Fig. G.5. Assigning a Threshold Cycle.

7. **Understanding the standard curve**

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

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

Correlation coefficient:

The r value is the correlation coefficient.

> The correlation coefficient is a measure of how well the predicted values, *e.g.* the standard amounts entered for a sample "fit" with the experimental data. That is, how well the data from the experiment fit to the values assigned to the standards.
>
> The correlation coefficient is a number between 0 and 1. If there is no relationship between the predicted values and the actual values then the correlation coefficient is 0 or very low (the predicted values are no better than random numbers).As the strength of the relationship between the predicted values and actual values increases so does the correlation coefficient. A perfect fit gives a coefficient of 1.0. Thus the higher the correlation coefficient the more accurately your experimental data fit to the expected values.

Efficiency:

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

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

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

At 100% efficiency the template doubles after each cycle during exponential amplification. Several design factors influence efficiency such as the length of the amplicon, the G/C content of the amplicon and secondary structure. The dynamics of the reaction itself can also influence efficiency. Variations in the dynamics can result from such sources as the enzymes used in the reaction and non-optimal reagent concentrations. When performing multiplex reactions or when comparing relative curves, as in gene expression experiments, the goal is to have the efficiencies of all reactions to be equal to make valid comparisons throughout.

Appendix H
Uploading New Versions of Firmware

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

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

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

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

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Germany *Ph. 089 318 84-177, Fx. 089 318 84-123* ***Hong Kong*** *Ph. 852-2789-3300, Fx. 852-2789-1257* ***India*** *Ph. (91-124)-6398112/113/114, Fx. (91-124)-6398115*
Israel *Ph. 03 951 4124, Fx. 03 951 4129* ***Italy*** *Ph. 34 91 590 5200 , Fx. 34 91 590 5211* ***Japan*** *Ph. 03-5811-6270, Fx. 03-5811-6272*
Korea *Ph. 82-2-3473-4460, Fx. 82-2-3472-7003* ***Latin America*** *Ph. 305-894-5950, Fx. 305-894-5960* ***Mexico*** *Ph. 52 5 534 2552 to 54, Fx. 52 5 524 5971*
The Netherlands *Ph. 0318-540666, Fx. 0318-542216* ***New Zealand*** *Ph. 64-9-4152280, Fx. 64-9-443 3097* ***Norway*** *Ph. 47-23-38-41-30, Fx. 47-23-38-41-39*
Russia *Ph. 7 095 979 98 00, Fx. 7 095 979 98 56* ***Singapore*** *Ph. 65-2729877, Fx. 65-2734835* ***Spain*** *Ph. 34-91-590-5200, Fx. 34-91-590-5211*
Sweden *Ph. 46 (0)8-55 51 27 00, Fx. 46 (0)8-55 51 27 80* ***Switzerland*** *Ph. 061-717-9555, Fx. 061-717-9550* ***United Kingdom*** *Ph. 0800-181134, Fx. 01442-259118*

Sig 0101

4006200 Rev B