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6.1 Overview of Experimental Setup and Programming

Before running a protocol on the MiniOpticon system, you must tell the system what each well of the microplate contains, the sequence and duration of temperature incubations to use in the run, and when during the run the detector should collect fluorescence data. These instructions are given in plate and protocol files. The plate file specifies the contents of the wells, any descriptive well labels that the user assigns, the calibration files for the plate type and dyes used, and the quantities of any standards to be used in generating a standard curve. The protocol file specifies the order and parameters of temperature incubations, plate reads, temperature gradients, cycling (goto) steps, and melting procedures to be used in the experimental run. The protocol file also specifies the reaction volume, temperature control method, and heated lid settings.

Plate files and protocol files are created independently of each other. This design allows you to run different saved protocols with the same plate setup, or the same protocol with different saved plate setups, without having to recreate the plate and protocol files for each new combination. The master file links a specific plate file and a specific protocol file for use in the current run.

This chapter describes:

- The parts of the Master File, Plate Setup, and Protocol Setup windows
- How to create new plate and protocol files and how to assign them to a master file
- How to assign existing plate and protocol files, with or without modifications, to a master file
- How to reuse and edit existing master files

6.2 The Master File Window

Upon launching Opticon Monitor software (see Chapter 5), the Opticon Monitor display window will show either a new master file template (first launch), or the last master file used (subsequent launches). All setup and programming operations can be accessed from the Master File window. If another window is showing, click the *Master* button on the toolbar to view the Master File window.

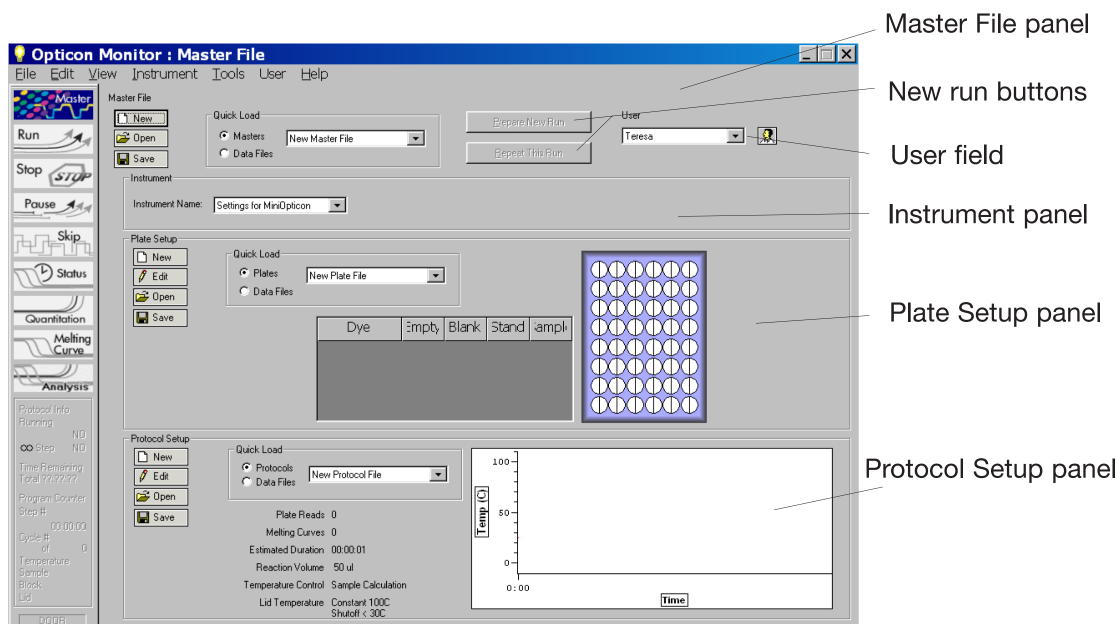


Figure 6-1 The Master File window

If the Master File window is displaying a master file from a previously run experiment, click on the *Prepare New Run* button in the top panel of the Master File window to clear the master-, plate-, and protocol-file templates. If you want to use the same, or a slightly modified plate setup and/or protocol for another run, follow the instructions in the section “Reusing Master Files” at the end of this chapter.

6.2.1 Parts of the Master File Window

The Master File window contains the following panels:

The Master File panel is used to create *New* master files, *Open* previously created files, and *Save* files — by clicking on the appropriate button on the left side of the panel. You can also open a saved master file by selecting it from the pull-down menu in the Quick Load field (see section 6.2.4, “Using the Quick-Load Menus”). The Master File panel also includes buttons for repeating a run or preparing a new run, described above, and a field for managing users, described in the next section.

The Instrument panel is used to select which instrument will be used to run the experiment if more than one instrument is connected to the computer. See section 6.2.3, ”Selecting an Instrument” for more information.

The Plate Setup panel is used to assign plate files to the master file. You can create a *New* file, *Open* a previously created file, *Edit* the file, and *Save* the file by clicking on the appropriate button on the left side of the panel. You can also open a file by selecting it from the pull-down menu in the Quick Load field (see section 6.2.4, “Using the Quick Load Menus”). Once a plate file is created or opened, a summary of the contents is displayed in the table and the plate diagram in the Plate Setup panel. See section 6.3.15, “Exiting the Plate Setup” for more information.

The Protocol Setup panel is used to assign protocol files to the master file. You can create a *New* file, *Open* a previously created file, *Edit* the file, and *Save* the file by clicking on the appropriate button on the left side of the panel. You can also open a file by selecting it from the pull-down menu in the Quick Load field (see section 6.2.4, “Using the Quick-Load Menus”). Once a protocol file is created or opened, a summary of the protocol and a graphical representation will appear in the Protocol Setup panel. See section 6.4.8, “Exiting the Protocol Setup” for more information.

6.2.2 Managing Users

The User field in the Master File panel specifies default storage locations for master, plate, protocol, and data files. Adding a user creates a folder with the user’s name and creates folders within that folder for saving the four types of files. If no specific user is specified, new files are saved in the Shared folder.

6.2.2.1 Adding New Users

To create a new user folder, click the user icon in the Master File window.

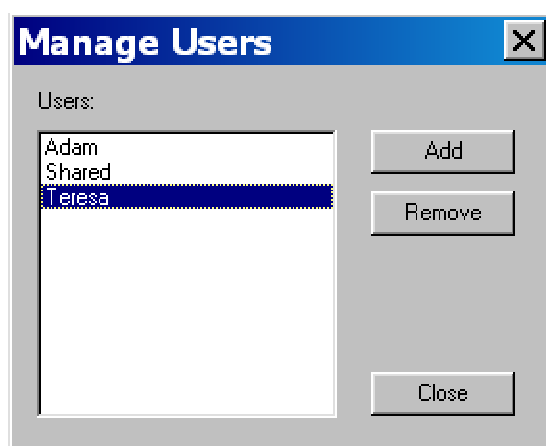


Figure 6-2. managing users

In the Manage Users window that appears, select *Add*. Enter the new user’s name in the New User window that appears and then click *OK*. The new user’s name will appear in the Users list.

6.2.2.2 Specifying a User

To select a user, choose a name from the drop-down list in the User field. Alternatively, click on the user icon, highlight a user name in the Mange Users window that appears, and then click *OK*. The user's folder will become the default save location for newly saved files.

6.2.2.3 Removing Users

To remove a user from the Opticon Monitor software, select the user's name in the Manage Users window and then click *Remove*. You will be asked to confirm deletion of the user, as **all data associated with the user will also be deleted**.

6.2.3 Selecting an Instrument

If more than one instrument is connected to the computer, use the drop-down list in the Instrument panel to select the instrument you wish to run. If the instrument you want to use does not appear on the drop-down list, make sure it is attached to the computer and then select *Scan For Instruments* from the *Instrument* pull-down menu. The instrument will then be listed on the drop-down list.

If you want to setup an experiment on a computer that is not currently attached to an instrument, choose *Settings for [an instrument]* from the drop-down list.

To re-initialize an instrument, for example if it were not properly connected to the computer upon launching Opticon Monitor software, select *Reinitialize Instrument* from the Instrument pull-down menu.

If you want the currently selected instrument to be automatically selected upon launching Opticon Monitor software, select *Set Default Instrument* from the Instrument pull-down menu.

The setup and analysis options available on other screens depend on what instrument is selected in the Instrument panel of the Master File window. This manual will document and illustrate the options available for the MiniOpticon system. **When *MiniOpticon* is selected in the Instruments panel, no more than two dyes may be assigned to a well.**

6.2.4 Using the Quick-Load Menus

The quick-load menus provide rapid access to any existing plate, protocol, master, or data files.

If the *Masters*, *Plates*, or *Protocols* option is selected in the respective panels, all of the available plate, protocol, or master files (but not data files) that have been saved to the Shared folder or individual user folders are displayed in the drop-down list. The files are listed along with their associated user as shown below.

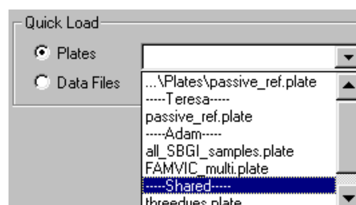


Figure 6-3. Quick-load drop-down menu

Scroll to locate the desired file in the drop-down menu, and select the file.

If the *Data Files* option is selected, all of the data files are listed in the drop-down menu. Selecting a data file will apply the plate, protocol, or master file that was used to generate that data file to the current master file.

6.3 Creating a Plate File

6.3.1 What Is a Plate File?

A plate file describes the experimental microplate. Opticon Monitor software uses the information in this file to interpret the raw data collected by the fluorescence detectors. The plate file specifies: (1) the type of microplate and dyes used in the experiment, so that the software can apply the appropriate calibration files to the data; (2) the “contents” classification (see section 6.3.6, “Specifying Well Contents”) of individual wells, which tells the software what wells to use in different types of calculations, such as background subtraction and standard-curve generation; (3) the quantities and units of any standards; and (4) well descriptions to help the user identify the specific reaction components.

6.3.2 Accessing the Plate Setup Window

To create a new plate file, click the *New* button in the Plate Setup panel of the Master File window. Alternatively, you may alter the currently loaded plate file by clicking the *Edit* button. In either case, the screen shown below will appear.

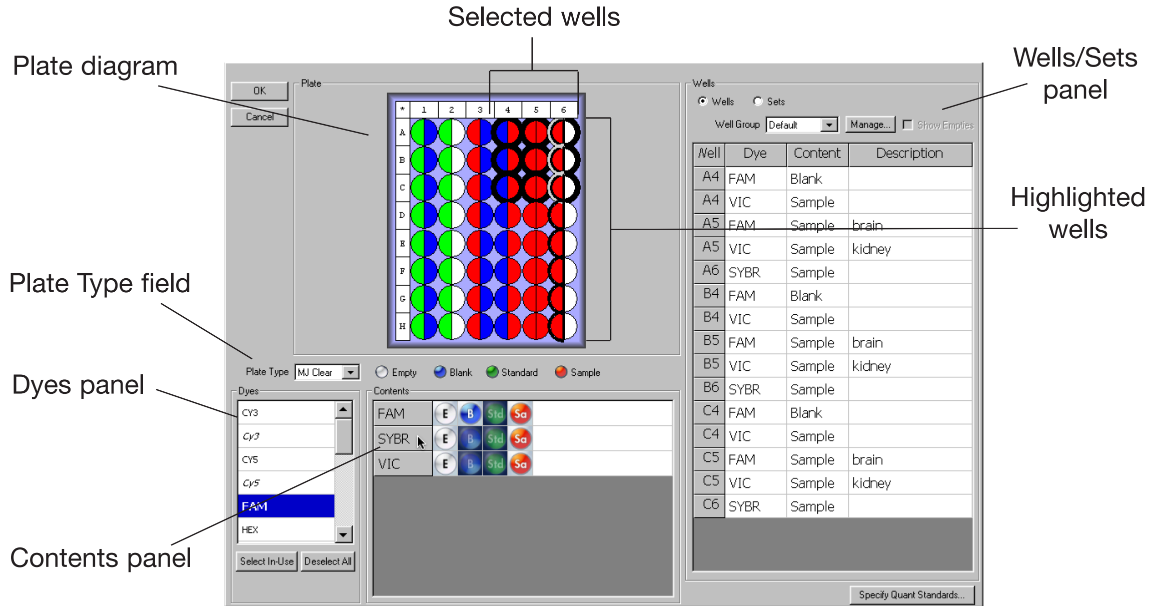


Figure 6-4 Plate Setup window

6.3.3 Parts of the Plate Setup Window

The Plate Setup window includes the following panels; these will be described in more detail in subsequent sections of this chapter:

The Plate diagram is a representation of the 48 wells that may be used in the experiment. The wells in the Plate diagram are color-coded according to the contents specified for each dye: white for empty, blue for blank, green for standards, red for sample, and orange for passive reference (see section 6.3.6, “Specifying Well Contents” and section 6.3.7, “Viewing Well Contents in the Plate Diagram”). A legend indicating which color corresponds to each type of reaction appears below the plate diagram. Note that the legend for passive reference only appears if this option is selected from the *Plate* pull-down menu.

The Plate Type field is used to indicate the type and well color (white or clear) of the vessels that will be used in the experiment.

The Dyes panel, in the lower left corner of the window, is used to select dye(s) to display in the Wells panel. All calibrated dyes are listed in this panel.

The Contents panel is used to assign contents to wells selected in the Plate diagram and to enter the descriptions that appear in the Wells panel. A dye must be listed in the Contents panel for the user to identify which wells contain that dye and what type of reaction contents have been specified for it.

The right-hand display panel displays either the Wells panel or the Sets panel, depending on which radio button is selected at the top of the panel. When *Wells* is selected, the panel displays a table listing the contents and descriptions of selected wells/dyes. Wells can also be assigned to Well groups in the Wells panel. When *Sets* is selected, buttons for creating and editing well sets appear, and the panel displays a table listing all sets that have been defined. Wells or sets can be sorted by clicking on the column headers in the table.

6.3.4 Beginning Plate Setup — Specifying the Plate Type and Dyes

The MiniOpticon system must be calibrated for both the type of reaction vessel and the dyes that will be used in an experiment. The system is precalibrated with settings for plates (or tubes) and many popular dye chemistries. Plate calibration files exist for white or clear wells. Dye calibration files exist for FAM, SYBR Green I, HEX, TET, and VIC dyes.

To begin a new plate setup, first select the type of vessel used from the drop-down list in the Plate Type field.

Next, select the dye(s) that will be used in the experiment, using the Dyes panel. Selecting a dye lists the dye in the Contents panel, allowing you to specify well contents for the dye. The content classifications and descriptions assigned to selected dyes in selected wells are listed in the Wells panel.

If you no longer want a dye to appear in the Contents and Wells panels, click on the name of the dye in the Dyes panel. If that dye has already been assigned to some wells, its name will be emboldened in the Dyes panel. To remove all dyes from the Contents and Wells panels, click on the *Deselect All* button at the bottom of the Dyes panel. Click the *Select In Use* button to list in the Contents and Wells panels all dyes that have been assigned to wells.

Note that only two dyes can be used in a given well. **If you assign more than two dyes to a single well, you will not be able to exit the Plate Setup window unless you correct the error.** On the other hand, many dyes can be used in a single plate, for example by using FAM and VIC in column 1 and SYBR Green and HEX in column 2.

Also be aware that although the software allows you to combine any two dyes in a well, it is difficult to discriminate signals from dyes with largely overlapping emission spectra. You should consider the spectra of dyes when designing your experiment.

6.3.4.1 Using Uncalibrated Dyes

Selecting a dye from the Dyes panel tells the software which dye-calibration files to apply to the fluorescence measurements. If the desired calibration file is not present for the dye/plate combination being used, it may be possible to use the setting for one of the precalibrated dyes, if one has an emission spectrum similar to that of the dye you want to use. Of course, any dye used must be excited at wavelengths between 470–500 nm. Please contact Bio-Rad if you would like calibration recommendations when using non-precalibrated dyes.

If it is not possible to use one of the precalibrated settings, it will be necessary to perform a calibration by selecting *Dye Calibration* from the *Tools* pull-down menu. Follow the directions in the Dye Calibration Wizard to create a calibration file(s) for the dye(s) being used (see Chapter 10).

6.3.5 Selecting and Deselecting Wells

To select wells, either for specifying contents for an experiment or, later, to view the data for a specific well, use the Plate diagram.

Move the cursor over an individual well, row letter, or column number to highlight the well or wells with a thin outline and darken the corresponding well coordinates (see wells in column 6 in Figure 6-4). Clicking on a highlighted well will select it. Selected wells appear heavily outlined (see wells A4 through C6 in Figure 6-4).

Select an individual well by clicking on the well.

Select a different well by clicking on that well. Previously selected wells will be deselected.

Select multiple wells by holding down the left mouse button and dragging the cursor over the wells to be selected, or hold down the control key and click on the individual wells you wish to select.

Select all wells in a column by clicking on the numbered column header. Select multiple columns by holding down the control key while clicking on the numbered column headers.

Select all wells in a row by clicking on the lettered box at the start of the row. Select multiple rows by holding down the control key while clicking on the lettered boxes.

Select all wells in the plate by clicking on the asterisk in the upper-left corner of the plate diagram.

Deselect a single one of several selected wells by holding down the control key while clicking on the well.

Deselect all wells by clicking on any selected well.

6.3.6 Specifying Well Contents

Well “contents” tell the software what type of reaction each dye will indicate in each well. The software uses this information when performing calculations, to identify which wells provide the fluorescence data needed for the calculations. The different content classifications and their uses are:

- Empty: Nothing has been added to the well. Fluorescence measurements are not used. Undefined wells are considered empty.
- Blank: The well contains an incomplete set of reactants (e.g., buffer only). Fluorescence intensity measurements from blank wells can later be subtracted from measurements taken from other wells.
- Quantitation Standard: The well contains a user-specified standard of known quantity (see section 6.3.13, “Specifying Quantitation Standards”). Fluorescence intensity readings from all quantitation standards are automatically used to plot a standard curve of log quantity vs. cycle number.
- Sample: The well contains an experimental sample. If quantitation standards are also used, the quantities of samples are automatically calculated by interpolating from the standards.
- Passive Reference: The dye is present in the well as a reference, to compensate for variability in concentration or volume of the samples. The fluorescence measured for the other dye in the well will automatically be divided by the fluorescence measured for the passive-reference dye. This designation is only available if *Passive Reference* has been selected from the *Plate* pull-down menu (see section 6.3.8, “Using Passive References”).

The defined contents for a well are dye-specific, so the contents of a well can be defined as a sample reaction using one dye and a blank reaction for another dye.

To specify contents:

1. Select the dye(s) for which you want to specify contents using the Dyes panel. The selected dye(s) will appear in the Contents panel adjacent to the Dyes panel. You may find it easiest to specify contents for all wells containing one dye, and then deselect that dye before selecting and specifying contents for another dye.
2. Select the well or group of wells for which contents are to be specified, using one of the methods described in the section “Selecting and Deselecting Wells”.
3. Indicate the type of reaction each dye is used for in the selected wells by clicking on one of the buttons to the right of the dye’s name in the Contents panel: *E* for empty, *B* for blank, *St* for quantitation standard, *Sa* for sample, or *PR* for passive reference (if this option has been selected in the *Plate* pull-down menu, see section 6.3.8, “Using Passive References”).

If you decide you no longer want the fluorescence data from a particular well to be used in the calculations for which it is specified, you can return to the Plate Setup window, select the well, and redefine its contents. This can be done even after the experiment has been run.

6.3.7 Viewing Well Contents in the Plate Diagram

Once the dyes in a set of wells has been designated for use as blanks, standards, samples, or reference, the corresponding wells in the Plate diagram will be color coded according to the contents: white for empty, blue for blank, green for standards, red for sample, and orange for passive reference. A legend appears below the Plate diagram.

Each well in the diagram is partitioned to show the contents assigned to different dyes in that well (see Figure 6-5). A dye must be selected in the Dyes panel before its contents will be displayed in the Plate diagram. The number of partitions is determined by either the number of dyes selected (in the default view) or the number of dyes the well can hold (if *Fixed Well Sections* is selected from the *Plate* pull-down menu). Note that if you change the display mode in the Plate Setup window, the plate diagram in the Plate Setup panel of the Master File window will change accordingly.

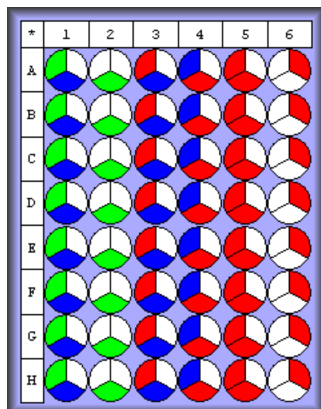
6.3.7.1 The Default Plate-Diagram Display

In the default Plate view, each well in the Plate diagram is partitioned into the same number of sections as there are dyes selected in the Dyes panel. For example, if a single dye is selected from the Dyes panel, the wells in the Plate diagram will not be partitioned and will show only contents assigned to the selected dye; if three dyes are selected in the Dyes panel, each well in the Plate diagram will be divided into thirds, as shown in Figure 6-5A. When multiple dyes are selected, the content classification for each dye is represented in the same section of each well, e.g., FAM in the top left section, SYBR in the top right, and VIC in the lower section. The dyes are displayed alphabetically, moving clockwise around the circle. Note that since a maximum of two dyes may be assigned to a given well in the MiniOpticon system, if three dyes are selected, one dye in each well must be designated as “Empty”.

6.3.7.2 Fixed Well Sections

Because the default display option partitions wells into the number of selected dyes, the display can become complicated if many dyes are viewed at once. In these cases, it may be easier to use the Fixed-Well-Sections display option.

A. Default Plate diagram



B. Fixed Well Sections

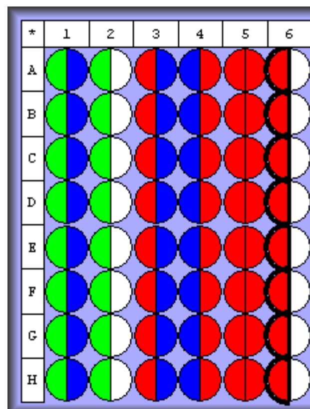


Figure 6-5. The two display modes for the Plate diagram. For both plates, FAM and VIC dyes were assigned to columns 1–5 and SYBR Green was assigned to column 6. The contents for SYBR Green are highlighted in the Fixed Well Sections display. Remember that the color represents the content classification, not the dye.

When *Fixed Well Sections* is selected from the *Plate* pull-down menu, the wells of the plate diagram are partitioned into sections corresponding to the maximum number of dyes that can be added to the well — two for the MiniOpticon and Opticon™ 2 systems, four for the Chromo4™ system.

If only one dye is assigned to a well in the MiniOpticon system, its content classification is displayed in the left half of the circle when *Fixed Well Sections* is selected. If two dyes are assigned to a well, they are ordered alphabetically, e.g., FAM in the left half, VIC in the right half. Therefore, if a dye is used in singleplex reactions in some wells and multiplex reactions in other wells, its contents may be represented in different halves of different wells. For example, in the plate diagram in Figure 6-5B, the contents for VIC are displayed in the right half of wells in column 1 and in the left half of the wells in column 2 (compare to the plate diagram on the left, in which the contents for VIC are always displayed in the bottom section).

6.3.7.3 Highlighting Contents for a Dye

To view the content classifications for a single dye, first click *Deselect All* at the bottom of the Dyes panel, and then select the dye whose contents you wish to view. Click on the dye again to deselect it.

You can also highlight the wells containing a specific dye when multiple dyes are selected. To do so, position the cursor over the name of the dye in the Contents panel: the well sections corresponding to that dye will be highlighted with a dark line in the Plate diagram (see column 6 in Figure 6-5B).

6.3.8 Using Passive References

If you wish to use a passive reference to standardize fluorescence across wells, select *Passive References* from the Plate pull-down menu. The orange circle for passive reference will then appear in the plate-diagram legend, and *PR* will be added to the buttons in the Contents panel. Any dye can be used as a passive reference, but only one dye can serve as a passive reference in a given well.

When a dye is designated as a passive reference in a well, the fluorescence measured for the other dye in that well is automatically divided by the fluorescence measured for the passive-reference dye. If, after you run the experiment, you decide you don't want this calculation to be made, return to the Plate Setup window and re-assign the well contents for that dye. Make sure you select all wells that contain the passive reference and change the contents to *E* (Empty—see section 6.3.6, "Specifying Well Contents").

6.3.9 Assigning Well Descriptions

To aid in sample identification, you can enter additional descriptions for individual wells or groups of wells, e.g., the target or the template source. This may be done most easily while you are specifying contents, but it can be done at any time.

Begin by selecting well(s) for which you want to enter descriptions, using the Plate diagram. Next, select the dyes for which you want to enter descriptions, using the Dyes panel. Finally, click on the white rectangular field in the line bearing the dye's name in the Contents panel, and type a description. The well label will be applied to the selected well or wells and appear in the Description column in the table in the Wells panel (see next section).

Alternatively, you can enter a description by clicking on an individual cell in the Description column of the table in the Wells panel and typing directly into the cell. You can also copy and paste a well description from one Description field in the table to another by using control+c to copy and control+v to paste. Finally, you can copy descriptions from an Excel file. When doing this, be careful to copy the labels into the correct column of the Wells information table.

6.3.10 Viewing Well Information in the Wells Panel

The panel on the right side of the Plate Setup window displays either the Wells panel or the Sets panel, depending on which radio button has been selected at the top of the panel. To view the information entered for selected dyes in selected wells, select *Wells*. The well-information table will then be displayed (see Figure 6-4).

To list well information in the table, you must first select well(s) in the Plate diagram and also select dye(s) in the Dyes panel. The columns in the well-information table list the Well coordinates, which of the selected Dye(s) have been assigned to each well, the Contents assigned to each selected dye, and any Description that has been entered. To list dyes with contents classified as "Empty", select *Show Empties* above the Wells table. This option is not available if *Fixed Well Sections* is selected: dyes assigned the "Empty" classification are not listed when *Fixed Well Sections* is selected.

The listing of wells in the plate information table can be sorted by *Well*, *Dye*, *Content*, or *Description* by clicking on the appropriate column heading.

The well-information table can also be used to enter additional descriptions of a well's contents. See the previous section for more information.

6.3.11 Defining Well Groups

The Well Groups function adds an extra level of organization to a plate, to simplify viewing of experiments that include many reaction types and to allow independent analysis of multiple experiments in the same plate.

Defining well groups is especially useful if two experiments with different quantitation standards are run simultaneously. By default, Opticon Monitor software uses all wells classified as standards to generate a standard curve. If the Manage Well Groups function is used to define groups of wells, then when one of those groups is selected, the Standards graph will include only the standards present in that particular group.

You can create, manage, and select well groups from the Plate Setup, Quantitation, Melting Curve, and/or Analysis windows. To define well groups from the Plate Setup window, select *Wells* at the top of the right-hand panel, and then click on the *Manage* button at the top of the Wells panel. The Well Groups Manager window, displaying a list of any defined groups and a plate diagram, will open.

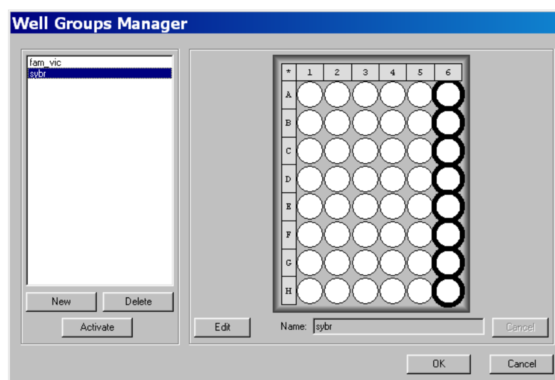


Figure 6-6. The Well Group Manager window with one group selected

To create a new group, click the *New* button at the bottom-left corner of the window. Select wells to include in the group using the plate diagram, enter a name in the Name field, and then click *Apply*. The newly defined group will appear in the list on the left side of the window.

You may define additional groups by clicking *Add New*. The same well may be added to multiple groups.

To edit an existing group, highlight its name in the list in the Well Groups Manager window, click *Edit*, and proceed as for new groups. To delete a group, highlight its name and click *Delete*.

To close the Well Groups Manager window, click OK or highlight one well group and click Activate. If you choose Activate, all the wells that are not in the activated group are not selectable — they are gray with an X through them — in the Plate diagram in the Opticon Monitor window. If you choose OK, the Opticon Monitor window will display the entire microplate (or whatever group was displayed when the Manager window was opened).

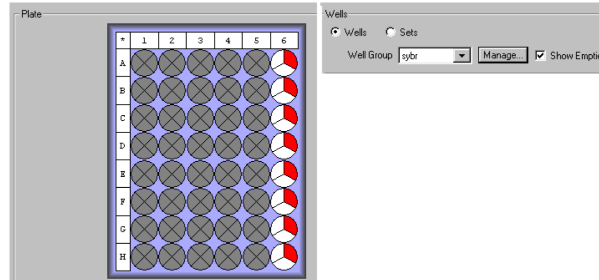


Figure 6-7. Plate diagram with one group activated.

To activate a different group, you may either re-select Manage and proceed as above, or simply select a defined group from the Well-group drop-down menu in the Wells panel. To display the entire microplate, choose default from the drop-down menu.

6.3.12 Creating Well Sets

If sets of replicate wells are defined in the Plate Setup window, Opticon Monitor software will calculate the mean and standard deviation of the fluorescence measured at each cycle in each of the wells in the set. You can then plot the mean and standard deviation in the Graphs panel of the Quantitation window, and you can view calculations based on the mean, maximum, and minimum C_T values (see Chapter 8). In addition, defining well sets, specifically a calibrator set, is required for relative expression analyses (discussed in the next section and in Chapter 8).

To create a well set, first select the *Sets* radio button at the top of the right-hand panel. This displays the Sets panel.

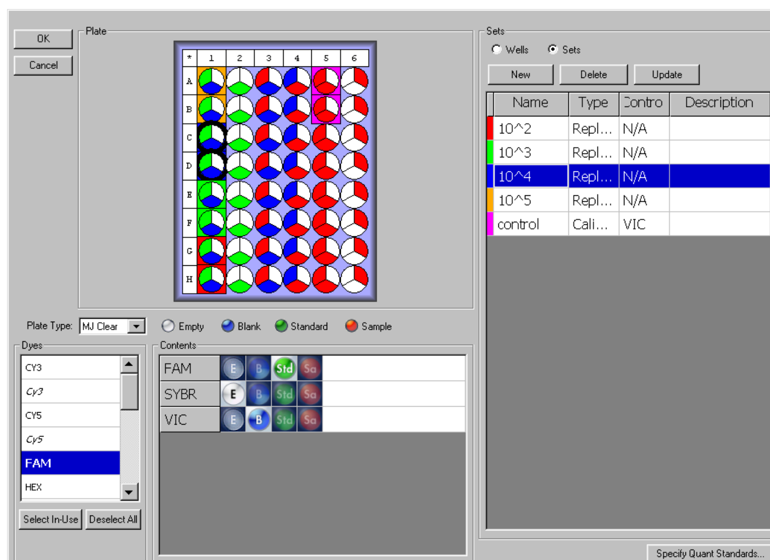


Figure 6-8. Plate Setup window displaying Sets panel.

To add a new set, click *New*. You may select wells to include in the set either before or after clicking *New,* using the plate diagram. When you click *New,* a new row with the name New Set will be added to the Sets table in the Sets panel. The bar to the left of the New Set row in the Sets table will be given a new color, and a box of the same color will be drawn around that set's wells in the Plate diagram (if wells were selected before clicking the *New* button). Please note the following:

- You may create well sets before or after assigning contents to the wells, but the same content must be assigned to every well in the set.
- If you select wells after creating the set, you must click *Update* to assign the new wells to the set.
- If you change the content assignment of wells after a set has been created, you must click *Update* to accept the changes.
- Well sets are not dye-specific. You do not have to create sets independently for each dye.

To name a set, click in the Name field that says *New Set*, highlight the words, and type in a new name. If you don't change the name, the next new set will be named "New Set 2". You can further define the set by clicking in the Description field and typing a description of the set.

By default, a new set is assigned the type "Replicate". You can change the type to Calibrator by clicking in the Type field and choosing *Calibrator* from the drop-down menu. Calibrators are used in expression analyses using the ΔC_T and $\Delta\Delta C_T$ methods (see Chapter 8). When you specify a set as a calibrator, you can select which dye is used for indicating the internal control by selecting the dye from the drop-down menu in the Control column. The internal-control dye is used only in $\Delta\Delta C_T$ calculations.

To edit a well set, highlight the row containing the set's name in the Sets table, and then select wells to include in the set using the Plate diagram. Remember to hold down the control key while selecting new wells if you want to keep the previously selected wells in the set. Click *Update* to accept the changes to the set.

To delete a well set, highlight the set's row in the Sets table, then click *Remove*.

6.3.13 Specifying Quantitation Standards

If you are using quantitation standards, after you have designated which wells contain the standards, click the *Specify Quant Standards* button in the bottom-right corner of the Plate Setup window. A pop-up window will appear listing all of the wells to which quantitation standards have been assigned, and indicating which dye(s) have reactions using standards in that well. You can sort the items by *Well*, *Dye*, or *Amount* (once amounts have been entered) by clicking on the appropriate column heading.

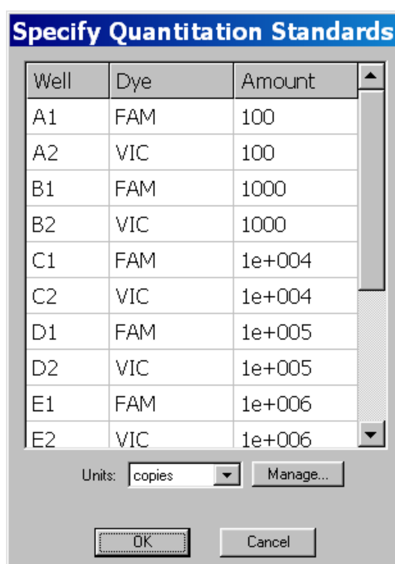


Figure 6-9. The Specify Quantitation Standards window

Enter the amount of each standard in the Amount fields. Alternatively, you can cut and paste multiple cells from an Excel worksheet. Numbers may be entered as integers or using scientific notation (e.g., 1e+004). Note that numbers greater than 1,000 and numbers entered in the form 1E4 will be converted to the form 1e+004.

After entering quantities, specify the Units of the standards by choosing ng, pg, moles, molecules, ge (genome equivalents), or copies from the drop-down menu. You can define additional units by selecting the *Manage* button.

Select *OK* to apply the Standard quantities to the plate file, or click *Cancel* to undo any changes to the Standard quantities. A standard curve will automatically be generated during analysis of the data using the values supplied.

To define additional units, first click the *Manage* button in the Specify Quantitation Standards pop-up window. Next, select *Add* in the Manage Standards window that appears, and then type the desired unit name in the Add Quantitation Standard Name window. To remove unit designations, highlight the designation in the Standards list in the Manage Standards window and click the *Remove* button.

6.3.14 Pasting Plate Information From Microsoft Excel

If on a previous run you exported a plate file to Microsoft Excel (see section 8.6.1, ”Exporting Data”), you may paste the plate information from the Excel file into the Opticon Monitor plate file. First, open the Excel file and select the type of plate information you want to paste (Well Name, Dye, Contents, and/or Description). Include the column headings to have these automatically applied in the Opticon Monitor software. Copy the selected cells to the clipboard (Control-c). Next, return to the Opticon Monitor screen and, in the Plate Setup window, select *Paste from Excel* from the *Edit* menu. The following window will appear:

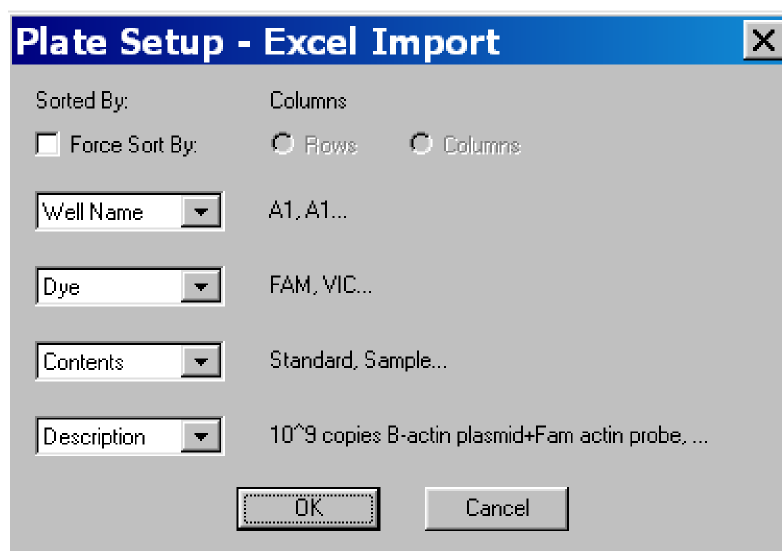


Figure 6-10. Pasting plate data from Excel.

If you copied the column headings specifying the type of plate information, these headings will automatically appear in the drop-down menus in the Plate Setup - Excel Import dialog box. If the headings were not selected, or if you want to reclassify the data, select the type of plate information from the drop-down menu. Note that the data in each column must match the format for the different plate information types (Well Name, Dye, Contents, or Description). Click *OK* to enter the plate information into the plate information table. Note that other plate parameters, such as plate and dye type, and values of quantitation standards, are not specified by the *Paste from Excel* command. You can, however, copy and paste quantitation standard data from Excel into the Specify Quantitation Standards window, in a separate step (see previous section).

6.3.15 Exiting the Plate Setup

Once you have finished entering plate file parameters, click the *OK* button in the upper-left corner of the Plate Setup window to return to the Master File window. Alternatively, if you wish to discard the plate file information and return to the Master File window, click *Cancel*.

When you click *OK*, a summary diagram and table of the newly assigned plate contents will appear in the Plate Setup panel of the Master File window. The plate diagram will use the same display (default or fixed-well-sections) as was last used in the Plate Setup window. The table will list all the dyes that have been assigned to wells and the number of wells assigned each content type (empty, blank, standard, sample). To highlight in the plate diagram the wells that contain a particular dye, position the cursor over the name of the dye in the table. To highlight only the wells that are assigned a particular content for a particular dye, place the cursor over the appropriate cell in the table.

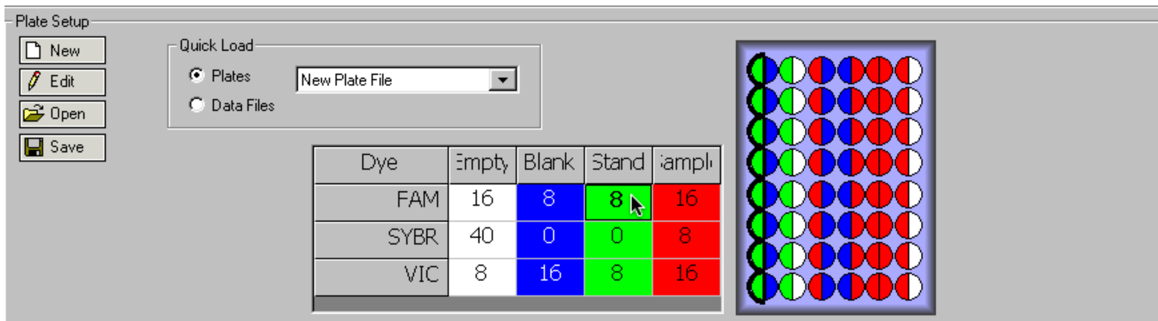


Figure 6-11. Plate Setup summary in master File window. FAM standards are highlighted.

6.3.16 Saving a Plate File

To save the newly created plate file, click the *Save* button from the Plate Setup panel in the Master File window. Enter an appropriate name in the File name field of the save window. Click the *Save* button to save the .plate file.

6.4 Creating a Protocol File

The protocol file contains the thermal-cycling parameters for an experimental run, and specifies when during the run the MiniOpticon detector will measure fluorescence. Protocol steps are entered and edited in the Protocol Setup window. A listing and graphical representation of the protocol are displayed for easy review.

Click the *New* button in the Protocol Setup panel of the Master File window to create a new protocol file.

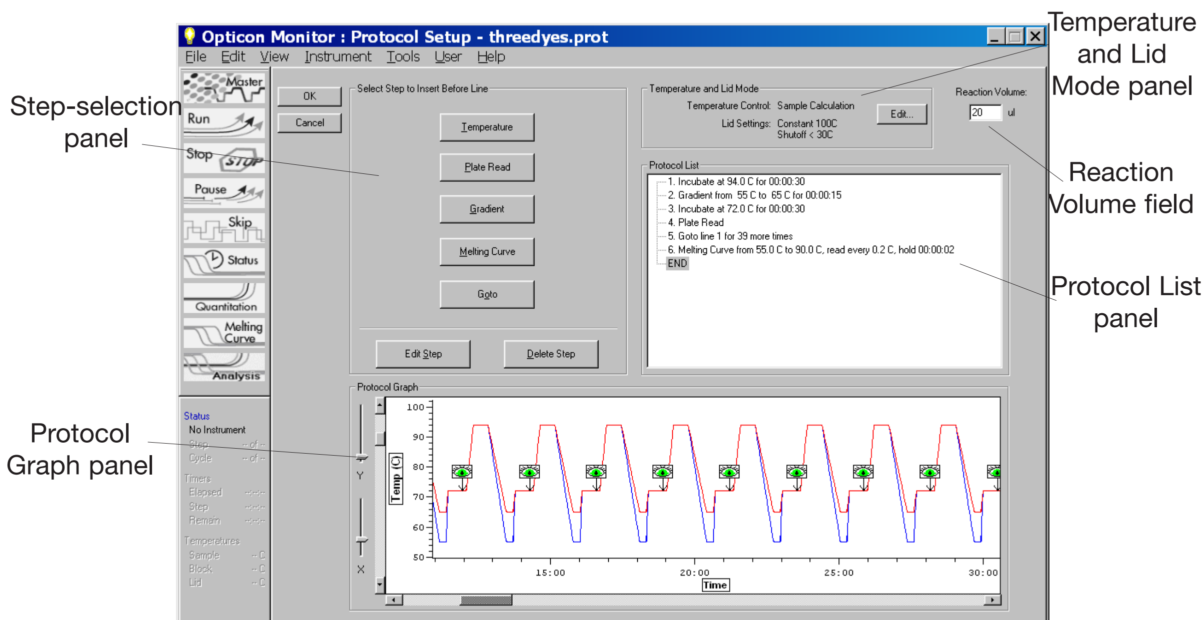


Figure 6-12. The Protocol Setup window

6.4.1 Parts of the Protocol Setup Window

The Protocol Setup window is made up of the following panels, which will be described in more detail in subsequent sections of this chapter:

The step-selection (“Select Step to Insert Before Line”) panel is used to add or edit different kinds of steps to the protocol. The long name is intended to remind you that new steps are inserted before the highlighted step in the protocol list.

The Temperature and Lid Mode panel is used to select options for controlling the temperature of the sample holder and for heating the lid.

The Reaction Volume field is for entering the volume of the wells, for use in temperature-control calculations.

The Protocol List panel displays the protocol steps in numbered order.

The Protocol Graph panel displays a graphical illustration of the protocol, showing temperature increases and decreases as well as plate reads. Each cycle of the protocol is represented.

6.4.2 Reaction Volume

For the Opticon Monitor software to accurately calculate and control the sample temperature, the user must enter the volume of reactions (in µl, including the volume of any oil or wax overlay) in the Reaction Volume field in the upper right-hand corner of the Protocol Setup window. See section 4.6, “Reaction Volume Recommendations” for additional information.

6.4.3 Choosing Temperature and Lid Control Modes

Click the *Edit* button in the Temperature and Lid Mode panel to display the Protocol Options window, and to specify the methods of temperature and lid control to be used in the run.

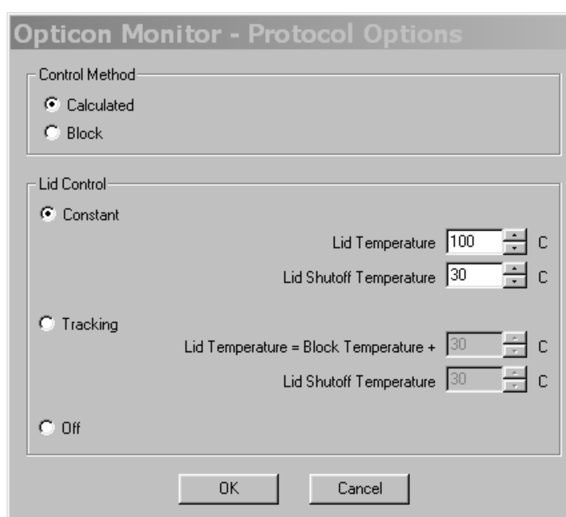


Figure 6-13. The Protocol Options window for choosing temperature and lid control modes.

6.4.3.1 Temperature Control Modes

The MiniOpticon system can control block temperature in two different ways. Each method has implications for the speed and accuracy of sample heating.

1. *Calculated* control is the default method of temperature control. It is the preferred method for most protocols, yielding consistent, reliable, and fast programs.

   With calculated control, the user-entered incubation periods specify how long the samples, rather than the block, should remain at the target temperature. The system estimates sample temperatures based on the block’s thermal profile, the rate of heat transfer through the sample tube, and the sample volume. Brief, precise block-temperature overshoots are used to bring samples to the correct temperature rapidly. Since this estimate is based on known quantities and the laws of thermodynamics, sample temperatures are controlled much more accurately than with block temperature control.

Because the samples are held at the target temperature for the user-specified duration when using calculated control, these durations can be shortened significantly compared to when using block control. Typically, optimal hold times for denaturation steps range from 5–30 seconds when using the calculated control method. Annealing and extension steps can also be shortened, but the periods for these will be reaction-specific. In addition to offering the simple convenience of spending less time running reactions, shorter protocols minimize false priming and help to preserve enzyme activity.

Calculated control also makes transferring protocols easier, because the instrument automatically compensates for vessel type and reaction volume.

2. When *Block* control is selected, the user-entered incubation periods specify how long the block should remain at the target temperature, without regard to sample temperature. Under block control, the temperature of samples always lags behind the temperature of the block, and that lag varies with sample volume and vessel type (typical lag time is 10–30 seconds). Therefore, the sample temperature is less precisely controlled in block-control mode than in calculated-control mode. Block control is used chiefly to run protocols developed on thermal cyclers that do not have the calculated-control option.

6.4.3.2 Lid Control Modes

When a sample is heated, condensation can form on the tube cap or the plate sealer. This changes the volume of the sample, the concentration of components, and thus the kinetics of the enzymatic reaction. Use of a heated lid minimizes condensation by keeping the upper surface of the reaction vessel at a temperature slightly greater than that of the sample itself.

The MiniOpticon system provides three options for controlling lid temperature: *Constant*, *Tracking*, or *Off*.

- The *Constant* method keeps the inner lid at a specified temperature (°C). This is the default method of control. To use constant lid-temperature control, select *Constant* and enter a Lid Temperature between 30°C and 110°C or use the arrows to scroll to the desired temperature. For low-profile tubes, a constant temperature of up to 5°C above the highest temperature in the protocol is recommended. You can also specify a sample-block temperature below which the heated lid will turn off. Enter a Lid Shutoff Temperature between 1°C and 50°C or use the arrows to scroll to the desired temperature.
- The *Tracking* method offsets the temperature of the heated inner lid a specified number of degrees relative to that of the sample block. Tracking is useful for protocols that have long incubations in the range of 30–70°C, where maintaining a high lid temperature may be counterproductive. An offset of 5°C above block temperature is adequate for most protocols. To use tracking lid-temperature control, select *Tracking* and enter the number of degrees, from 1°C to 45°C, that you wish the lid temperature to be maintained above the block temperature, using the format Lid Temperature = Block Temperature + __°C. You can also use the arrows to scroll to the desired temperature. To specify a sample-block temperature below which the heated lid will turn off, enter a Lid Shutoff Temperature between 1°C and 50°C or use the arrows to scroll to the desired temperature.

Note: Because there is no active cooling of the lid, a decrease in the lid temperature may not be observed during rapid cycling. Note also that the lid heats more slowly than the sample block due to its additional thermal mass.

- When *Off* is selected in the lid control panel, no power is applied to the heated lid. In this mode, condensation will occur at a rate consistent with the incubation temperature and the type of seal being used. This option is not recommended unless you are using a wax overlay.

Click the *OK* button to apply the temperature and lid control settings to the protocol, or choose *Cancel* to close the window without changing the settings. The Temperature Control and Lid Settings will appear in the appropriate fields of the Temperature and Lid Mode panel in the Protocol Setup window.

6.4.4 Entering a New Protocol

To create a protocol, simply select the steps you want to perform using the buttons in the Select Step to Insert Before Line panel. Clicking on one of the buttons opens a window that prompts you to enter specific parameters.

As you insert steps into a protocol, descriptions of the steps will appear in the Protocol List panel, and a graphical representation of each step's temperature and time period (in minutes) will appear in the Protocol Graph panel. To view the graphical display more clearly, use the sliders to the left of the display panel to zoom in and out on the X- and Y-axes. Use the scroll bars on the axes to position the graph appropriately.

Before beginning to enter a new protocol, note that the END step is highlighted in the Protocol List panel. Opticon Monitor software adds new steps before the step that is highlighted.

The following sections will describe the types of protocol steps you may enter, using the protocol below as an example.

6.4.4.1 Example protocol

1. Incubate at 94°C for 30 seconds to denature dsDNA.
2. Optimize annealing temperature by incubating at a range of 55°C to 65°C across the eight rows of the sample block, for 15 seconds.
3. Incubate at 72°C for 30 seconds to allow extension.
4. Read the fluorescence intensity of the Blank, Quantitation Standard, and Sample wells.
5. Sequentially repeat steps 1-4, 39 more times, then proceed to step 6.
6. Identify and determine the purity of reaction products by melting profile — raise the temperature from 55°C to 90°C, and read the fluorescence 2 seconds after every 0.2°C increase in temperature.

6.4.4.2 Temperature Step

A temperature step specifies incubation temperature and duration. The MiniOpticon system ramps the sample to this temperature at its maximum rate unless ramp-modifying instructions are added to the program (see the description of “Manual Ramp Rate” later in this section).

Click the *Temperature* button to enter a temperature incubation step (e.g., step 1 or step 3 from our example) into a protocol.

Figure 6-14. Temperature step window

Enter the desired temperature, from 0°C to 99.9°C, into the Set temperature to field or use the arrows to scroll to the desired temperature — 94°C in step 1 of our example.

Enter the desired incubation time, to a maximum of 18 hours, in the Maintain for field. Click in the hour: minute: or second field and either enter a time period or use the arrows to scroll to the desired time — 00:00:30 in step 1 of our example. Alternatively, you can select *Forever* to maintain the desired temperature indefinitely. A forever incubation step at the end of a protocol can be useful for holding reaction products at a sub-ambient temperature (we recommend 10°C) until they can be processed. In the graphical representation of the protocol, a forever incubation is indicated with an infinity sign (∞).

Click the *Insert* button to add the temperature step to the protocol without further modifications. The temperature step should appear as step 1 in the Protocol List panel, and a graphical representation of the temperature and duration should appear in the Protocol Graph panel. Note that the END step is again highlighted indicating that the next step will be added above the END step and therefore after step 1.

You can also choose to modify a temperature step before inserting it into the protocol. Available options include:

Manual ramp rate: Set a slower-than-maximum rate of heating or cooling. A slower-than-maximum ramp rate ranging from 0.1°C to 2.5°C per second can be specified.

Change temperature: Modifies a temperature step to allow a per-cycle increase or decrease of temperature (0.1°C to 10.0°C per cycle) each time the step is executed. This feature is useful when annealing stringency is a consideration, such as in a touchdown program.

In a touchdown program, the temperature selected for the annealing step begins higher than the calculated annealing temperature and incrementally decreases with each cycle, first reaching, and eventually falling below, the calculated annealing temperature. Thus the reaction begins at a temperature favoring high stringency in hybridization and increments to a lower stringency. The higher initial stringency favors the desired product by creating a high signal-to-noise ratio in the early amplification cycles.

Change time: Modifies a temperature step to allow a per-cycle lengthening or shortening of the hold time of the step (by 1–60 sec/cycle) each time the step is executed. This capability is useful for slowly increasing (typically by 2 to 5 seconds per cycle) the hold time during an extension step. The number of bases that the polymerase must incorporate during the extension step increases in later cycles because there are more template molecules and/or fewer active polymerase molecules. Allotting extra time for extension can increase the likelihood that synthesis will be completed.

Beep when completed: Modifies a temperature step so the instrument will beep when the target temperature is reached.

6.4.4.3 Gradient Step

The temperature gradient feature allows you to optimize denaturing or annealing conditions by incubating at several different temperatures simultaneously. The range of temperatures that can be achieved from front to back across the 48-well sample block can be as small as 1°C or as great as 16°C. The maximum programmable temperature is 99°C; the minimum programmable temperature is 35°C.

Click the *Gradient* button to insert a gradient step into a protocol.

Figure 6-14. Gradient step window

The minimum gradient temperature is assigned to the front row (row H) of the sample block and can range from 35°C to 98°C. In our example, the Front Temperature (Lower) field is set to 55. The maximum gradient temperature is assigned to the back row (row A) and can range from 31°C to 99°C. In our example, the Back Temperature (Higher) field is set to 65. The minimum temperature difference between the far left and far right columns is 1°C and the maximum difference is 16°C. A warning will appear if you attempt to enter a difference exceeding 16°C.

After entering the range of temperatures for the gradient, enter the desired incubation time in the Maintain Temperature for field by clicking in the hour: minute: or second: field and either entering a time period or using the arrows to scroll to the desired time — 00:00:15 in step 2 of our example. Alternatively, you can select *Forever* to maintain the desired temperature gradient indefinitely.

Click *Insert* to add the gradient step to the protocol without further modifications. The gradient step should appear as step 2 in the Protocol List panel. Note that the END step is again highlighted indicating that the next step will be added above the END step and therefore after step 2.

You can also choose to modify the gradient step before inserting it into the protocol by choosing the *Change Time* option (see section 6.4.4.2, “Temperature Step” for an explanation of this option).

6.4.4.4 Gradient Calculator

To accurately predict the temperature of each of the eight rows across the block during a gradient incubation, select *Gradient Calculator* from the *Tools* menu.

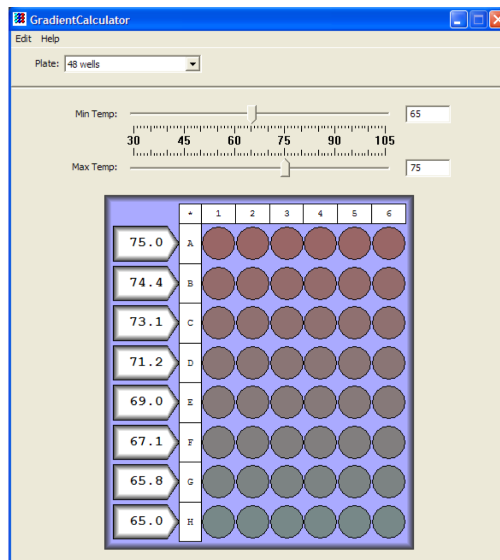


Figure 6-15. Gradient calculator

Set the minimum and maximum temperatures of the gradient step, using the sliders above the plate diagram. The arrow above each column will then display the predicted temperature of the column, and the wells in the diagram will be colored to reflect the gradient.

Note that the gradient temperature distribution is not linear. This is a natural consequence of the geometry of the Peltier-Joule heaters that underlie the block. Although not evenly distributed, the temperatures displayed are within ±0.4°C of the actual temperature attained by each well in a given column.

The predicted temperature for the row that yields the best results can be accurately transferred to a temperature step in a non-gradient protocol. Select *Copy* from the *Edit* menu to copy the temperatures to the clipboard for pasting into another program.

6.4.4.5 Plate Read Step

Insertion of a plate read step directs the MiniOpticon system to measure the fluorescence of the wells. The plate read begins immediately after the programmed end of the previous incubation step, step 3 in our example. The MiniOpticon system performs the plate read at the current incubation temperature, and then initiates the next step, step 5 in our example.

To insert a plate read step, click the *Plate Read* button. In our example, the plate read step should appear as step 4 in the Protocol List panel (step 3 is a temperature step, like step 1). A plate read appears as an eye icon in the graphical protocol display.

6.4.4.6 Adding Multiple Temperature, Gradient, or Plate Read Steps

To add additional temperature steps, gradient steps, or plate reads to your protocol, click the appropriate button and follow the directions for the specific step as outlined above.

6.4.4 7 Goto Step

The goto step abbreviates a protocol that has many repeating steps. When a goto step is executed, the protocol program returns to the step specified by the goto step, and repeats that step and all subsequent steps back to the goto step. After it has looped back to the goto step a specified number of times, the protocol program moves on to the step that follows the goto step.

Note: You cannot nest goto steps. In other words, a protocol loop cannot contain another goto step within this loop. You cannot include melting curve steps (see next section) within a goto loop.

Step 5 of our example protocol indicates that the protocol should return to step 1, repeat steps 1-4 39 additional times for a total of 40 cycles, and then proceed to step 6. To add the goto step, click the *Goto* button in the protocol creation panel.

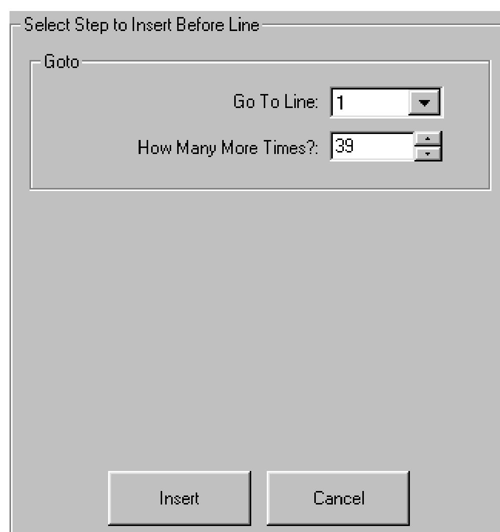


Figure 6-16. Goto step window

Enter the line number of the step to which the protocol program should return. You may also select the number of the step using the drop-down menu. In our example, enter 1 in the Go to line field, and 39 in the How many more times? field. Click the Insert button to add the goto step to the protocol.

6.4.4.8 Melting Curve Step

A melting curve can be generated to identify specific fragments and/or assess the homogeneity of a sample. Melting profiles are influenced by several factors, including the number and concentration of discrete fragments produced, the length and G+C content of each fragment, and buffer conditions.

Melting curves are often useful for verifying the identity of amplification products, as well as distinguishing positive internal controls from amplified products. In these cases, simply specify a melting curve after a cycling run, and the instrument will perform both procedures automatically. The MiniOpticon system can also be programmed to run a melting curve independently of a cycling protocol.

To add a melting curve step, click the *Melting Curve* button in the protocol creation panel.

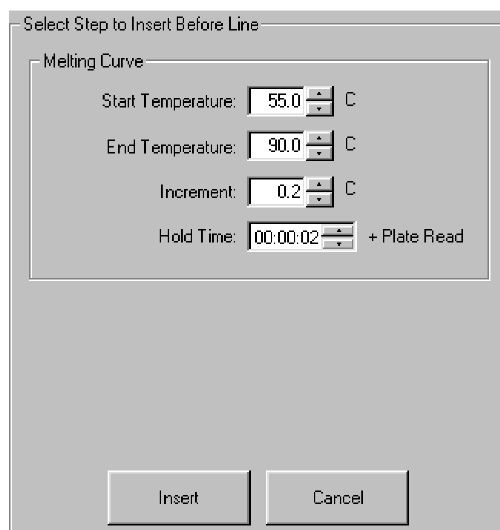


Figure 6-17. Melting curve step window

Enter a Start temperature (0.0°C to 99.0°C), and an End temperature (1.0°C to 100.0°C). In our example, the melting curve starts at 55°C and ends at 90°C.

Next, specify when during the melting curve step the MiniOpticon detector will measure fluorescence. Designate a temperature Increment between reads of 0.1°C to 10°C and a Hold Time (1 sec to 1 hr) for which the temperature increment should be maintained before the fluorescence is read. A temperature increment of 0.2°C and a hold time of 1–2 sec is often recommended.

Click the *Insert* button to add a melting curve step to the protocol. Only one melting curve step can be performed per protocol.

6.4.5 Editing a Protocol Step

To edit a protocol step, first click on the step in the Protocol List panel to highlight it, then click the *Edit Step* button in the protocol-editing panel. The existing parameters for the step will appear. After making the desired changes, click the *Replace* button to enter the edited step into the protocol, or click *Cancel* to leave the step unmodified.

6.4.6 Deleting a Protocol Step

To delete a protocol step, click on the step in the Protocol List panel to highlight it, then click the *Delete Step* button in the protocol-editing panel to remove the step from the protocol. The remaining protocol steps will automatically renumber.

6.4.7 Inserting a Protocol Step Between Existing Steps

To insert a protocol step between existing steps, highlight the step in the Protocol List panel that will follow the newly inserted step. All protocol steps are added immediately before the step that is highlighted in the Protocol List panel. Next, click the button corresponding to the type of step you would like to add.

6.4.8 Exiting the Protocol Setup

Once you have finished entering protocol file parameters, click the *OK* button in the upper-left corner of the Protocol Setup window to return to the Master File window. A graphical representation of the protocol and a summary of the total number of plate reads, melting curves, and the estimated run duration will appear in the Protocol Setup panel of the Master File window.

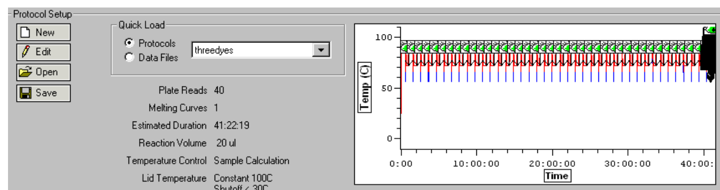


Figure 6-18. Protocol Setup panel of the Master File window

If you wish to discard the protocol file information and return to the Master File window, click *Cancel*.

6.4.9 Saving a Protocol File

To save the newly created protocol file, click the *Save* button in the Protocol Setup panel in the Master File window. Enter an appropriate name in the File name field of the Save window. Click the *Save* button to save the .prot file.

6.5 Saving a Master File

To save a master file, click the *Save* button in the Master File panel of the Master File window. Enter an appropriate name in the File name field of the save window. Click the *Save* button to save the .mast file.

You can also choose not to save this collection of component files and proceed directly to the run (see Chapter 7 for information on initiating a run).

6.6 Assigning Existing Plate and Protocol Files to a Master File

To assign existing plate and protocol files to a new or existing master file, either click the *Open* button in the panel of the Master File window corresponding to the type of file you wish to assign, or use the Quick Load menus to assign existing plate/protocol files to a master file (see section 6.2.4,"Using the Quick-Load Menus").

Clicking *Open* will display all the plate/protocol files in either the Shared folder or, if a user has been selected, in that user's folder. Select the desired file or, if the desired file has been saved to an alternate location, use the Windows browse screen to locate the file, and then click *Open*. The plate/protocol file will be applied to the master file, and a corresponding summary will appear in the Master File window.

To view the newly assigned plate or protocol file and/or make any necessary modifications, click the *Edit* button in the appropriate panel of the Master File window. The Plate Setup or Protocol Setup window will open, allowing you to modify the file parameters. Select *OK* to retain any modifications and return to the Master File window, or select *Cancel* to return to the Master File window without modifying the plate/protocol file. Select *Save* to save any changes to the plate/protocol file under the same or a newly assigned file name.

Click the *Save* button in the Master File panel to save any changes to the master file.

Important Note: If a component plate or protocol file is edited and saved under the same name, the edited file will replace the original file in all master files to which that file has been assigned.

6.7 Reusing Master Files

A new master file need not be created for every run. Existing master files may be reused without modifying the plate or protocol files, or the master file may be edited to accommodate changes such as a different arrangement of samples in the plate.

If a run has just completed, or if a data file for a previously completed run has just been displayed, and you wish to use the same master file, click on the *Repeat This Run* button. To clear the master file template, click on the *Prepare New Run* button. To display an existing master file, click the *Open* button in the Master File panel or use the Quick Load menu to open an existing master file. To access a recently used master file, select the desired master file from the list of *Recent Master Files* under the *File* menu.

Selecting *Open* will display all the master files in the Shared folder or, if a user has been selected, in that user's folder. Select the desired file or use the Windows browse screen to locate the file if it has been saved to a different location, and then click *Open*. The master file will be applied to the master file template and the corresponding plate and protocol file summaries will appear in the Master File window.

To use the master file without any changes, proceed to Chapter 7, "Run Initiation and Status".

You can also modify the master file before initiating a run, by editing the assigned plate or protocol files (*Edit* button), by substituting files (*Open* button or Quick Load), or by creating new component files (*New* button).

Click the *Save* button if you want to save any changes to the master file.

7 Run Initiation and Status

7.1 Running a Protocol **7-2**
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7.1 Running a Protocol

Before initiating a run, check that the appropriate master file is displayed. Click the *Master* button on the toolbar to access the Master File window if it is not currently displayed. If a run has just completed or a data file for a previously completed run has just been displayed, select the *Prepare New Run* button to clear the master file template, or select the *Repeat This Run* button to use the same master file (see Chapter 6 for instructions on how to create and edit master files).

Check the Status section in the run-status box in the bottom-left corner of the window to verify that the instrument is ready to begin a run (Figure 7-1, left). If the Status section indicates that No Instrument is attached (Figure 7-1, center) or that the Door is Open (Figure 7-1, right), correct the problem before continuing.

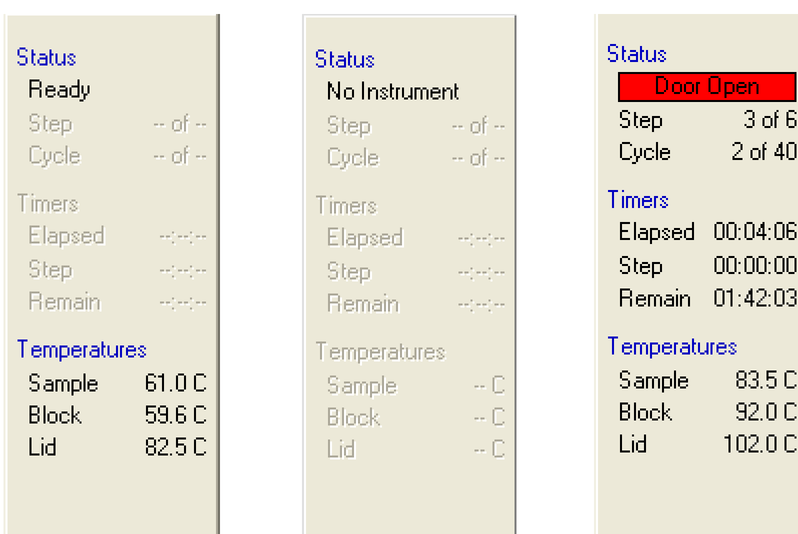

Figure 7-1 The run-status box indicates whether the instrument is ready.

Initiate the run by clicking the *Run* button on the toolbar. A Windows browse screen will appear asking you to name the file to which the data will be saved. Click *Save* to accept the default filename, or enter an appropriate file name and then click the *Save* button. The data file will be saved as a .tad (acquired data) file. The file will be saved in the data folder of the selected user (see “Specifying a User”, section 6.2.2.2).

The default data filename can be set using the *Filename Preferences* command under the *User* menu. The fields that can be included in the default filename are: username, year (two format choices), month (two format choices), day (two format choices), time (six format choices), and count, which adds an incremental count to the filename. In addition to these variable fields, the default file name can include constant characters, for example an underscore between fields. The fields and constant characters must be typed into the Data Filename Format field in the Filename Format window as in the following example:

<year1>_<month2><day1>_<time4>

This setting will result in a default filename formatted as follows:

2004_March15_102013PM

7.2 Interrupting a Run

Click the *Stop* button on the toolbar to halt the run at any time. The *Skip* button can be used to skip to the next step in the protocol file. **Note that if you click *Skip* while a Goto loop is being executed, the program will skip out of the loop and implement the next protocol step. Pressing *Skip* does not skip to the next step within the loop.** For example, if step 8 of a protocol says to goto step 2 for 39 times, and you press *Skip* during the 20th execution of step 5, the program will skip to step 9 of the protocol, not step 6.

7.3 Monitoring Run Status

7.3.1 Protocol Information on the Toolbar

A summary of run information is displayed on the bottom half of the toolbar.

Status		Status		Status	
Running		Reading Plate		Infinite Step	
Step	4 of 6	Step	5 of 6	Step	1 of 6
Cycle	1 of 1	Cycle	1 of 1	Cycle	1 of 1
Timers		Timers		Timers	
Elapsed	00:02:48	Elapsed	00:03:32	Elapsed	00:00:34
Step	00:00:17	Step	00:00:00	Step	00:00:00
Remain	01:43:16	Remain	01:42:23	Remain	01:47:03
Temperatures		Temperatures		Temperatures	
Sample	60.0 C	Sample	60.0 C	Sample	23.7 C
Block	60.0 C	Block	60.0 C	Block	23.8 C
Lid	92.0 C	Lid	99.0 C	Lid	50.0 C

Figure 7-2 The run-status box during a run

The run-status box includes three sections:

Status: If a run is in progress, this section indicates the current step and cycle number. This section also indicates when a plate read or an infinite incubation step is in progress, as well as if the instrument is open or disconnected (see Figures 7-1 and 7-2).

Time: This section displays an estimate of the how long the experiment has been running, how much longer the current step will last, and how long before the entire run is finished

Temperature: displays the current sample, block, and lid temperatures.

7.3.2 The Status Window

The Status window allows you either to monitor the system's progression through the protocol, by viewing the Thermal Cycler Status screen, or to monitor real-time data collection, by viewing the Optical Read Status screen. The Thermal Cycler Status screen is automatically displayed in the Status window when the run is initiated. To access the Status window after another screen has been displayed, click the *Status* button on the toolbar. The tabs in the lower-left corner of the Status window toggle the display between the *Thermal Cycler Status* and *Optical Read Status* screens.

7.3.2.1 Thermal Cycler Status Screen

Thermal profiles are the instrument are displayed in the Temperature Graphs panel at

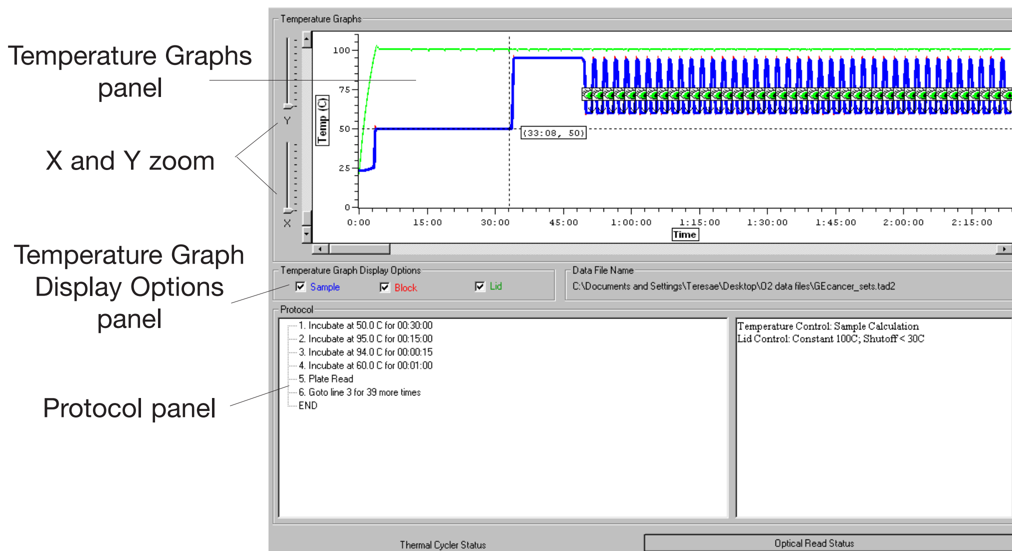

Figure 7-1. Thermal Cycler Status screen

the top of the Thermal Cycler Status screen. Select the appropriate boxes from the Temperature Graph Display Options panel to display a graph of the *Sample*, *Block*, and/or *Lid* temperatures over time. To clearly view the graph, use the X and Y zoom sliders to the left of the graph, or right click and drag a box around the area of the graph that you wish to magnify. The protocol is listed in the bottom panel of the window, with the current step highlighted. The END step is highlighted if the run has finished.

7.3.2.2 Optical Read Status Screen

Click the *Optical Read Status* tab to monitor real-time data collection in real time.

A graph of fluorescence versus cycle number can be displayed for selected dyes in

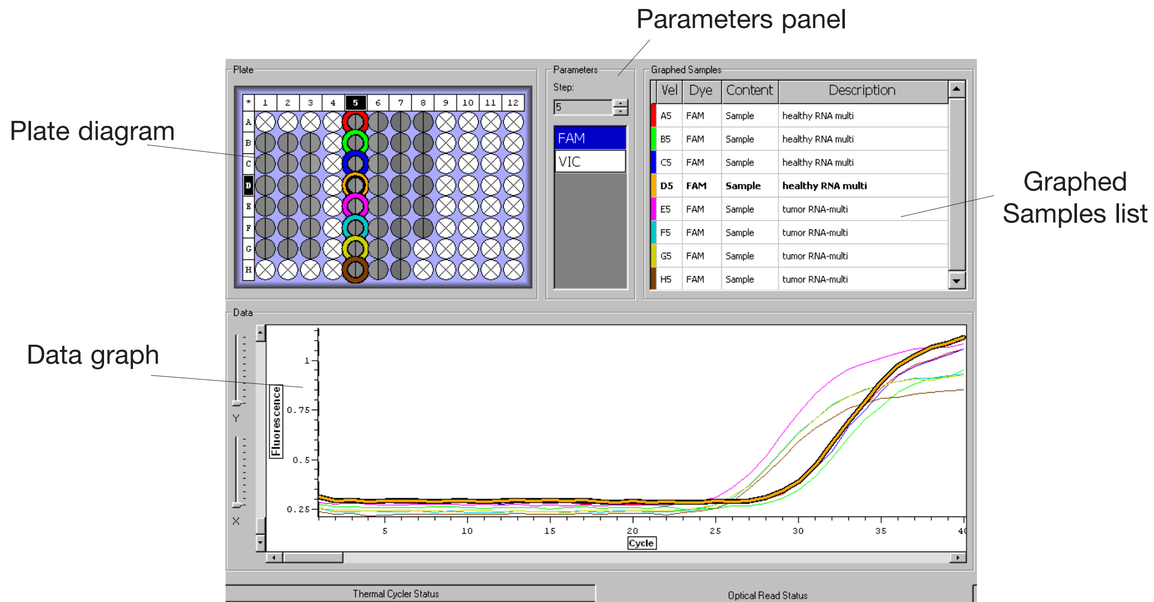

Figure 7-2. Optical Read Status screen

selected wells. Use the Plate diagram to select the wells you want to be displayed in the graph (see section 6.3.5, “Selecting and Deselecting Wells”, for additional information), and use the Parameters panel to the right of the Plate diagram to select the dye(s) you want displayed. Note that no data will appear in the Data graph until you select a well and a dye.

Use the Step field in the Parameters panel to display the signal intensity data for a particular step, if a plate read is included in more than one step of the protocol. This can be particularly useful if, for example, you wish to monitor a plate read in step 3 and later a melting curve in step 6.

Selected wells will appear outlined in color. The color outlining the well corresponds to the color of the well coordinates/descriptions in the Graphed Samples list and to the color of the fluorescence intensity trace in the Data graph.

Deselect all wells by clicking on any selected well. To deselect a subset of wells, hold

down the control key, and click on the well(s) you wish to deselect. The well(s) will no longer appear outlined in color, and the corresponding fluorescence intensity trace will be removed from the Data graph.

Deselect a previously selected dye by clicking on its name in the Parameters panel. Data for that dye will no longer appear in the Data graph for any of the selected wells.

To highlight the results for a particular sample in the plate diagram, Data graph, and the Graphed Samples list, do one of the following:

Move the cursor over a well on the Plate diagram. The column number and row letter coordinates of the well will be highlighted in the Plate diagram. The well will also be highlighted in the Graphed Samples list, and its trace will be thickened on the Data and Standards graph.

Move the cursor over a particular trace on the graph to thicken the trace and display the x and y coordinates corresponding to the position of the cursor on the trace. The corresponding well in the Plate diagram and the well coordinates in the Graphed Samples list will also be highlighted

Select a well from the Graphed Samples list to highlight the well coordinates in the Plate diagram and thicken its trace on the Data graph.

7.4 Performing Data Analysis During a Run

Opticon Monitor software allows users to analyze data while a protocol is running. Quantitation, Analysis, and Melting Curve windows can be used to view the data collected so far in the current run (see Chapter 8 for more details about these windows). It is also possible to analyze data collected during a previous run while a current run is in progress. To analyze previously gathered data during a run, select the *File->Open->Data File* path you would normally use to open a data file. The Status, Quantitation, Melting Curve and Analysis windows will display the data from the previous run and data analysis can be performed as usual in these windows. The Protocol Info in the run-status box (in the lower-left corner of the window) will continue to display the status of the current run. To close the data set and return to the current protocol running, select the *Reattach to Run* option located under the *File* menu.

It is also possible to open a second Opticon Monitor session while a run is in progress — simply follow the same steps used to open the initial session.

8 Data Analysis

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8.1 Analysis Features in Opticon Monitor Software

The analysis options available in Opticon Monitor software include absolute quantitation using standard curves; relative quantitation using ΔC_T and $\Delta\Delta C_T$; genotyping by clustering of samples on a scatter plot of C_T vs C_T; and melting curve analysis and calculation of melting temperature (T_m). This chapter describes how to perform these analyses.

8.2 Basic Principles of Real-Time Quantitation

Real-time fluorescence detection of PCR products allows one to calculate the initial quantity of template present in different samples. This is done by counting the number of PCR cycles required to reach a defined level of fluorescence (the threshold fluorescence). Because fluorescence intensity reflects the amount of product, samples that initially have more template will require fewer PCR cycles to reach the threshold than samples with less template. This strong relationship can be defined mathematically: the number of cycles required to reach threshold (i.e., the threshold-cycle or C_T value) is inversely proportional to the log of the initial template number. Therefore, C_T values can be used to calculate initial template quantities.

To calculate the initial quantity of template in a sample, one must first define the fluorescence threshold at which different samples and references will be compared. The simplest way to do this is by first plotting fluorescence vs. cycle number, and then positioning a threshold line on the graph at a point where the signals surpass background levels and begin to increase exponentially. The C_T for an individual sample is then defined as the cycle at which the sample's fluorescence trace crosses the threshold line.

The C_T values obtained in different samples are compared to the C_T of references to calculate either absolute or relative quantities of the template. If quantitation standards with a range of known amounts of template are used as references, one can generate a linear standard curve of the log of template quantity vs. C_T. The absolute quantity of initial template in unknown samples can then be interpolated from the standards, using the samples' C_T values. If quantitation standards are not available, the quantity of template in one sample can be calculated relative to another based on the difference in the C_T values, ΔC_T.

Opticon Monitor software automatically generates standard curves for absolute quantitation when standards are specified in the plate setup. The software can also estimate reaction efficiency and calculate ΔC_T values for relative expression analysis. The following sections will describe these functions in more detail.

8.3 Viewing Data and Performing Absolute Quantitation

The Quantitation window displays graphs of fluorescence intensity data and an automatically generated standard curve (if quantitation standards were specified during plate setup). In this window, you may adjust the data analysis options, position the threshold line, adjust the standard curve, and calculate the quantity of sample initially present in a reaction. If a run is in progress or has just completed, click the *Quantitation* button on the toolbar to view the quantitation data.

To access a previously generated data file, select *Open* and then *Data File* from the *File* menu. Select the desired file from the standard browsing window. To view a recently created or viewed data file, select the desired file from the *Recent Data Files* list accessible from the *File* menu. When the data file loads, the Quantitation window is displayed.

The Quantitation window has two screens: the Graphs screen and the Quantity Calculations screen. Toggle between these screens by clicking on the tabs at the bottom of the window. The features of these two screens are described in the following sections.

8.3.1 The Graphs Screen

The Graphs screen is the default Quantitation screen. The main purpose of this screen is to set parameters, including the fluorescence threshold, for accurately calculating template quantities. This screen can also be used to view graphs of the fluorescence data measured at each cycle of the protocol and to depict the relative intensities of fluorescence measured in all the wells in the plate during a particular cycle.

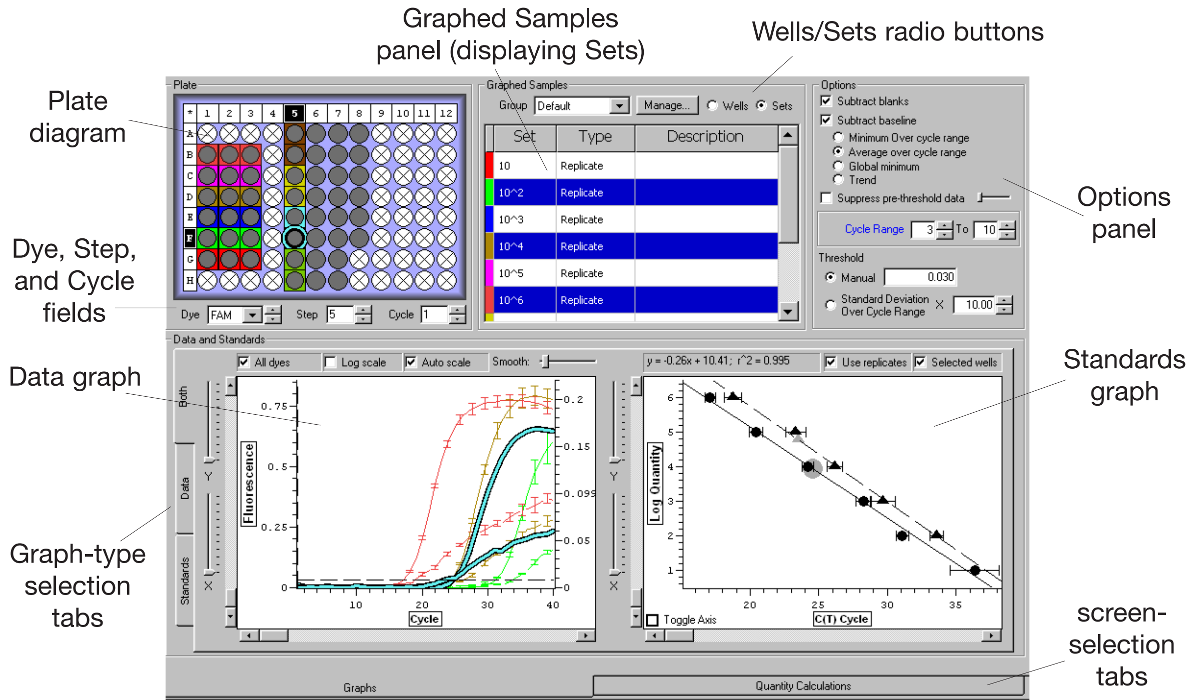

Figure 8-1. The Graphs screen of the Quantitation window

8.3.1.1 Parts of the Graphs Screen

The Graphs screen includes the following panels and fields. These will be discussed in more detail in subsequent sections of this chapter:

The Plate diagram is a representation of the wells in the microplate. It is used to select the wells for which data will be displayed in the Data graph. It can also depict the fluorescence intensity measured in each well and show well sets.

The Dye, Step, and Cycle fields below the Plate diagram are used to select the primary dye for which fluorescence data will be displayed, the protocol step for which data will be displayed (if the protocol included more than one plate-read step), and the cycle, if any, for which you wish to highlight data.

The Graphed Samples panel lists selected wells, when the *Wells* options is selected, and lists all well sets when *Sets* is selected. When *Sets* is selected, this panel can be used to select sets for which summary data will be displayed in the Data graph, or to select all wells in a given set. You can also manage well groups in this panel.

The Options panel is used to specify whether blanks should be subtracted from samples and standards, to select a baseline subtraction method, and to select a method for positioning the threshold line.

The bottom panel displays a *Data* graph of fluorescence (or log fluorescence) versus cycle number, a *Standards* graph of log quantity versus C_T cycle, or *Both* depending on which tab is clicked on the left side of the panel.

8.3.2 Using the Plate Diagram to View Fluorescence Intensity

The Plate diagram can display the approximate fluorescence intensity that was measured in each well during each amplification cycle. The shading of a well in the Plate diagram correlates with the signal intensity measured in that well for the specific Dye, Step, and Cycle specified in the fields below the diagram.

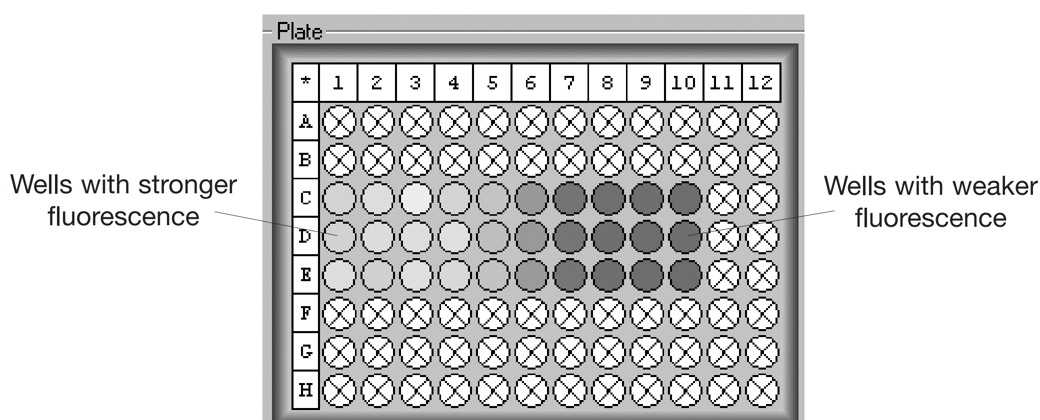

Figure 8-2 Well Intensities displayed in Plate diagram

Dark grey wells indicate weak or no signal, whereas white or light grey wells indicate strong signal. Normally, if you use the arrows adjacent to the Cycle field to scroll through the cycles, the wells will get lighter as the cycle number increases, indicating that the fluorescence intensity is increasing. By scrolling to the last cycle, you can get an indication of the endpoint fluorescence intensity in each well.

Use the Dye field to view the fluorescence intensity for a specific dye in both the Plate diagram and the Data graph (see the next section). To display the fluorescence data for a particular step, if a plate read was performed at more than one step of the protocol, enter the step number in the Step field or use the arrows to scroll to the desired step. Use the Cycle field to view (in the Plate diagram) the signal intensity measured during a specific cycle, as described above. You can also use the Cycle field to mark a particular cycle in the Data graph with a vertical dotted line.

8.3.3 The Data Graph

The Data graph plots the fluorescence measured at each cycle in selected wells. Additionally, the Data graph can plot the average of the fluorescence measured at each cycle in all the wells of selected sets. The standard deviation of the fluorescence in well sets is plotted as error bars on the average fluorescence trace.

Click the *Data* tab on the left side of the graph panel to display a large graph of fluorescence vs. cycle number. Note that no graph will be displayed until at least one well is selected.

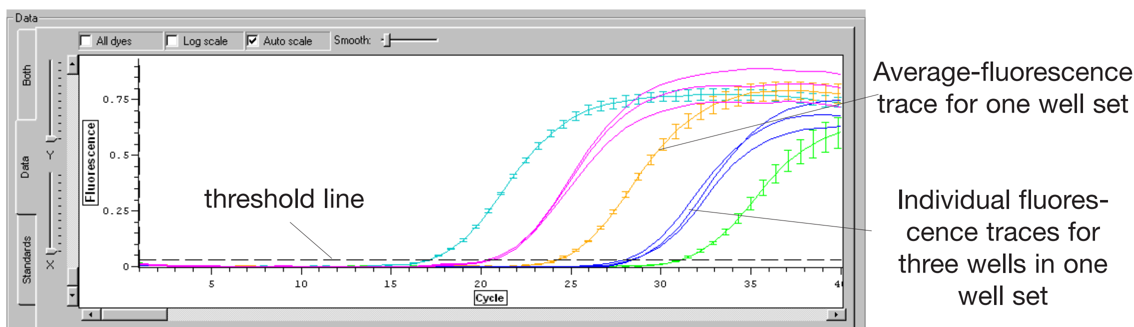

Figure 8-3. Fluorescence data graph.

To plot data for individual wells, use the Plate diagram to select the wells (see section 6.3.5, “Selecting and Deselecting Wells”). To plot the mean and standard deviation of fluorescence for a set of wells, choose Sets in the Graphed Samples panel and then click on the name of the set. If you no longer wish to plot the summary data, click on the well set again. To plot unaveraged data for every well in a particular set, click on the colored tab (in the Graphed Samples panel) to the left of the set you wish to display.

Selected wells will appear outlined in color in the Plate diagram. The color that outlines a well is the same as that used to color that well's fluorescence-intensity trace in the Data graph. When *Wells* is selected in the Graphed Samples panel, each well for which data are plotted is colored differently, and the color appears on the tab to the left of the well's coordinates in the Graphed Samples list. When *Sets* is selected in the Graphed Samples panel, the data for all wells in a given set are plotted with the same color, and that color appears on the tab to the left of the set's name in the Graphed Samples panel.

The fluorescence intensity data for the dye that is selected in the Dye field below the Plate diagram will be displayed in the Data graph. If the *All dyes* option is selected in the field at the top of the Data graph panel, the intensity data for the dye selected in the Dye field will be drawn with a solid line and the data for the other dye will be drawn as a dotted line of the same color.

Select the *Auto scale* option to adjust the scale of the y-axis to accommodate the fluorescence data displayed.

To display the log of fluorescence vs. cycle number, which may be useful when setting the threshold line (see section 8.3.5, "Positioning the Threshold Line"), select the *Log scale* option at the top of the Data graph panel.

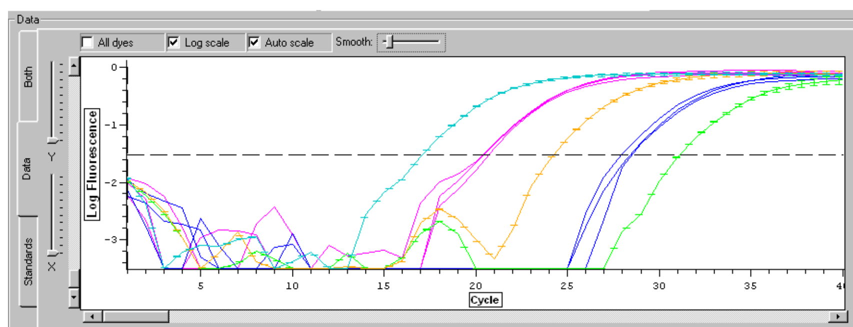

Figure 8-4. Log of fluorescence data

To expand regions of the graph, right click and drag a box around the area of the graph that you wish to magnify. Alternatively, use the X and Y sliders to the left of the graph to zoom along the x- and y-axes. The scroll bars along the axes can be used to position the region of interest in the display panel. Double-right-click on the graph panel to zoom out to full view.

Moving the cursor over a data trace will thicken the trace and display the C_T value along with the x and y coordinates corresponding to the current position of the cursor over the trace. In addition, the coordinates of the corresponding well will be highlighted in the Plate diagram, and the well information will be emboldened in the Graphed Samples list, if *Wells i*s selected. Moving the cursor over a well in the Plate diagram or a description in the Graphed Samples list will thicken the corresponding trace in the Data graph, highlight the well coordinates in the Plate diagram, and embolden the name in the Graphed Samples list (the C_T will not be shown on the graph, however).

The *Smooth* slider bar, located at the top of the graph panel, allows the user to smooth the data display by creating a running average over a variable number of points. For example, 3-point smoothing takes each particular data point, along with the point before and after it, and takes an average of the three. This average is then plotted on the graph in place of the original data point. The default Smooth setting is 3. The user can turn off all smoothing by setting the slider all the way to the left. Smoothing of the data for each dye is done independently, after selecting the dye in the Dye field under the Plate diagram. **Note that smoothing data can change the C_T value and therefore affect quantity calculations.**

8.3.4 Adjusting the Data Graph

You can choose from the following items in the Options panel of the Quantitation window, to adjust the data graph:

- *Subtract blanks*: If this option is selected, the fluorescence measured in all wells designated as blanks will be averaged and subtracted from the fluorescence measured in all wells designated as samples or quantitation standards, on a cycle-by-cycle basis. Note that this generally is not necessary for accurate quantitation.
- *Subtract baseline*: If this option is selected, a baseline fluorescence value (or set of values in the case of the *Trend* option), will be subtracted from the fluorescence data collected in each well. The value is calculated from the signals measured in each well and thus will vary from well to well. A baselining option must be selected before a standard curve will be plotted.

  There are four options for defining the baseline signal value for a well:

  - *Minimum over cycle range*: The baseline signal value for each well is defined as the minimum fluorescence value measured in that well over the range of cycles specified in the Cycle range boxes.
  - *Average over cycle range*: The baseline signal value for each well is defined as the average of the measured fluorescence in that well, calculated from the range of cycles specified in the Cycle range boxes.
  - *Global minimum*: The baseline signal is defined as the lowest fluorescence signal measured in the well, over all cycles.
  - *Trend*: Opticon Monitor software looks for an initial trend of increasing or decreasing fluorescence and calculates a baseline to subtract from the signals that will eliminate this trend. When this option is selected, *Global minimum* is used to set the baseline for the data from cycles beyond the trend.
- *Suppress pre-threshold data*: This option can be used to specify the earliest cycle for which data is displayed on the Data graph. If the option is selected and the slider is positioned all the way to the right, the fluorescence data for selected wells will be plotted only for the cycle at which the threshold fluorescence is surpassed (the threshold cycle) and for subsequent cycles. Moving the slider to the left increases the number of cycles preceding the threshold cycle that are displayed. When the slider is positioned all the way to the left, the earliest cycle for which fluorescence data are plotted will be 10 cycles before the threshold cycle.

- Threshold: These two options are for positioning the threshold line, which determines each fluorescence curve's threshold cycle (C_T) for use in quantitation of starting copy number (see next section,"Positioning the Threshold Line").

8.3.5 Positioning the Threshold Line

The threshold line, sometimes called the C_T line, appears as a dashed horizontal line on the Data graph. Normally, this line should be positioned such that, on a graph of baseline-subtracted data, it intersects the fluorescence traces at a point where the signal intensities surpass background levels and begin to increase exponentially. The threshold line should be positioned independently for each dye.

To adjust the position of the threshold line, select one of the Threshold options in the Options panel, or left-click and drag the threshold line to the desired position on the Data graph. If no threshold line appears on the graph, select *Manual* from the Threshold options and enter a value that is less than the maximum fluorescence value displayed on the y-axis of the graph. Next, drag the threshold line to the desired position.

Options for setting the threshold line include:

- *Manual*: The threshold line can be set manually by entering a threshold value for fluorescence intensity or by dragging the threshold line to the desired position on the graph.
- *Standard deviation over cycle range*: The threshold line can be set to a user-defined multiple of standard deviation above the mean fluorescence across the Cycle range specified by the user (in the panel above the Threshold options). A maximum of 10 standard deviations is allowed.

It is often useful to consider both the Data and the Standards graphs (if standards were used) when establishing the position of the threshold line. The best option for setting the threshold line can often be determined by observing the effects of each option on the fit of the quantitation standards to a linear standard curve (see the next section for more information). The effect the position of the threshold line has on the standard curve can easily be visualized by dragging the line up and down.

Viewing the log of fluorescence vs. cycle number, by selecting the *Log scale* option above the Data graph, may allow you to more accurately set the threshold line. In the log scale view, the threshold line should be placed in the linear portion of the curve, not low on the curve as it is in the fluorescence vs. cycle number view.

8.3.6 The Standards Graph

To display a large Standards graph, click the *Standards* tab on the left-hand side of the Graphs panel.

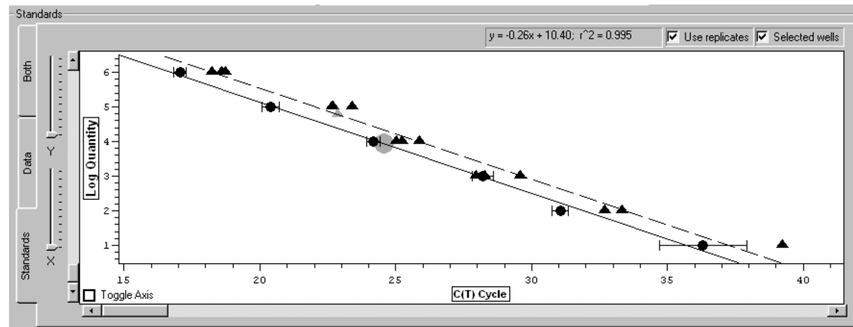

Figure 8-5. The Standards graph

Standard curves are automatically generated using the information provided in the Specify Quantitation Standards window during creation of the Plate file. The Standards graph(s) plots the base-10 logarithm of initial quantity (ng, copies, etc.) versus the C_T value for wells specified as standards for the dye selected in the Dye field (below the Plate diagram). A line is automatically fitted to these data, and the equation for this line is displayed above the Standards graph. If the *All dyes* option is selected above the Data graph, the standards data for the dye selected in the Dye field will be fitted with a solid line, while the data for the second dye will be fitted with a dashed line. The line equation for the dye selected in the Dye field will be the only equation displayed. Note that *All dyes* can only be selected when the Data graph is showing.

If well sets were created in the Plate Setup window, the mean C_T value of the replicate standards can be plotted, with error bars representing the standard deviation, by selecting *Use replicates* above the Standards graph. You must select and deselect *Use replicates* for each dye individually; selecting this option only affects the dye selected in the Dye field. To plot summary data for another dye, choose that dye in the Dye field and then click *Use replicates*.

Written above the Standards graph is the equation describing the vest-fit linear standard curve, and the r-square value of that line, for the dye selected in the Dye field. These items can help guide the positioning of the threshold. To view the equation and r-square value for another dye, change which dye is selected in the Dye field.

The line equation is of the form y = mx + b, where m is the slope of the line and b is the y-intercept. Theoretically, if perfect doubling of the template was achieved during each cycle, the slope of the line would be –0.30. When the threshold line is properly positioned, the slope should be close to this value.

The r^2 (r^2) value is the square of the correlation coefficient, often called the coefficient of determination. This number indicates what proportion of the variation in one variable is explained by variation in the other variable. In other words, the r^2 value tells how close to the regression line the data points lie. The value of r^2 can vary between 0 and 1, with values closer to 1 signifying a good fit. An r^2 value of 0.999 indicates that the linear standard curve explains 99.9% of the variation in the data. Ideally, when the threshold line is properly positioned, the r^2 value will be greater than 0.990.

Choose the *Selected wells* option to plot quantitation data from wells selected in the Plate diagram or Graphed Samples list. Sample data points are plotted with gray symbols. If a well set is selected in the Graphed Samples list, and if *Use replicates* is selected above the Standards graph, the mean fluorescence of the sample set will be plotted with error bars representing the standard deviation.

The *Toggle Axis* box in the bottom left corner of the Standards graph allows you to switch the x and y axes of the graph, if you have a preference for how the data are viewed. The default graph shows C_T on the x-axis and log quantity on the y-axis. Checking the *Toggle Axis* box switches the axes so that C_T is on the y-axis. Note that the r^2 value and the slope in the equation describing the standard curve also change. When log quantity is plotted along the x-axis, a slope of –3.32 indicates perfect doubling, and the y-intercept (b) indicates the sensitivity.

If a Standards graph is not automatically displayed when the Quantitation window is opened, check that the threshold line has been appropriately set on the Data graph, and that the quantitation standards have been defined in the Specify Quantitation Standards window (see section 8.3.7, “Changing the Values of Quantitation Standards”).

8.3.6.1 Adjusting the Standard Curve

If desired, you can adjust the standard curve by deselecting outlying points. Moving the cursor over a data point will increase the size of the point and highlight the corresponding well in the plate diagram, Graphed Samples list, and Data graph. To exclude a point from the standard curve, click on the point and it will turn red, indicating that it is no longer being used in the calculation of the line. The standard curve will automatically be recalculated. Click the point again to include the point in the calculation of the curve; the point will again appear black.

Note: If a deselected point is part of a well set, it will still be included in the average when Use Replicates is selected. To exclude the well from the average, you must remove it from the set, in the Plate Setup window. (See section 6.3.12 “Creating Well Sets”.)

8.3.7 Changing the Values of Quantitation Standards

If, during creation of the plate file, a mistake was made in entering the values of quantitation standards, or quantitation standards were not specified, you can change or add quantitation standards during the data-analysis phase.

To change the values of the standards, first click the *Master* button on the toolbar. Click the *Edit* button in the Plate Setup panel and make the desired changes to the quantitation standards, as described in Chapter 6. Click *OK* to return to the Master File window, and then click on the *Quantitation* button on the toolbar to continue analyzing data with the modified standards.

8.3.8 Managing Well Groups

By default, Opticon Monitor software uses all wells classified as standards to generate a standard curve. In some cases, such as when two experiments with different quantitation standards are run simultaneously, you may only want to use a subset of the standards, to generate a standard curve for quantitation of a subset of the samples. In these cases, the Manage Well Groups function can be used to define subsets of wells. When this is done, the Standards graph will include only the standards present in the selected group.

To define well groups, click the *Manage* button in the Graphed Samples panel. A window listing any defined groups will appear (groups may also be defined in the Plate Setup, Melting Curve, and Analysis windows). Follow the instructions in the section “Managing Well Groups” in Chapter 6 to create a new group, or to delete or edit an existing group.

To select a previously defined group, use the Group drop-down menu in the Graphed Samples panel. When a group is selected, all wells that are not members of that group will appear as empty (i.e., gray with an X through them) in the Plate diagram. The Standards graph will only plot data from wells in the selected group that are designated as standards. If no wells are designated as standards in the selected group, no standard curve will be drawn, and no quantity calculations will be performed on any wells.

To view data from all wells that have been assigned contents (i.e., all non-empty wells), choose *Default* from the Group drop-down menu in the Graphed Samples panel.

8.3.9 The Quantity Calculations Screen

The second screen of the Quantitation window, the Quantity Calculations screen, displays tables of data, calculations, and summary statistics for well sets and individual wells. Click the *Quantity Calculations* tab to display this screen.

Show All Wells and Sets   Show Selected Only   Efficiency as %   Copy to Clipboard

Sets

Efficiency	C(t)	copies	Avg C(t)	Max C(t)	Min C(t)	C(t) SD	Avg copies	Max copies	Min copies
73.9024%	22.3704	N/A	22.3938	22.9334	22.1056	0.381895	100000	100000	100000
86.3306%	32.6177	N/A	32.5566	32.6855	32.3355	0.157079	100	100	100
92.3221%	35.3488	N/A	35.8337	37.8399	34.8251	1.41862	10	10	10

Wells

Well	Dye	Content	Description	Efficiency	C(t)	copies
A5	VIC	Sample	healthy RNA multi	47.9968%	24.0292	23411.7
A5	FAM	Sample	healthy RNA multi	106.954%	27.2296	946.499
B5	FAM	Sample	healthy RNA multi	118.064%	27.3124	900.119
B5	VIC	Sample	healthy RNA multi	45.1865%	23.4567	33692.9
C5	FAM	Sample	healthy RNA multi	98.2233%	27.2426	939.084
C5	VIC	Sample	healthy RNA multi	104.042%	22.9583	46257.5
D5	FAM	Sample	healthy RNA multi	150.833%	27.3092	901.871
D5	VIC	Sample	healthy RNA multi	108.879%	24.0919	22497.7
E5	FAM	Sample	tumor RNA-multi	109.307%	23.4203	9543.4
E5	VIC	Sample	tumor RNA-multi	92.6098%	23.2842	37599.2
F5	FAM	Sample	tumor RNA-multi	160.984%	23.5831	8646.09
F5	VIC	Sample	tumor RNA-multi	36.4068%	22.5734	59083.8

Graphs   Quantity Calculations

Figure 8-6. The Quantity Calculations screen

Use the radio buttons at the top of the screen to *Show all wells and sets* or to *Show selected only. F*or the latter option, wells and sets are selected in the Graphs screen. Wells can be sorted as desired, by clicking on any of the column headers.

The Wells table lists for each dye in each (selected) well:

- the Well coordinates
- the Dye
- the Content classification (standard, sample, blank)
- any Description entered during plate setup
- the approximate reaction Efficiency, which is determined as follows: A line is fitted to three points—the point where the fluorescence trace crosses the threshold line and the points corresponding to the fluorescence intensity at the next two cycles. The slope of this line is the approximated reaction efficiency. The efficiency can also be listed as a percentage (100% efficiency means perfect doubling occurred and the slope = 2), by selecting *Efficiency as %* at the top of the screen.
- the C_T value, the cycle number at which the fluorescence intensity trace for the well intersects the threshold line on the Data graph
- the initial quantity of template, either user-specified (for standards) or calculated by interpolating from the standards (for samples), given in the units specified during plate setup

The Sets table lists, for each dye in each (selected) set (Use the slider at the bottom of the table to see hidden columns):

- the name of the well Set
- the Dye used in each set
- the Type of set (replicate or calibrator)
- the Content classification (standard, sample, blank) of wells in the set
- any Description given to the set
- the reaction Efficiency, calculated as described above, of the average-fluorescence trace for the set, that is, the trace drawn through the mean of the fluorescence measurements at each cycle
- the C_T value of the average fluorescence trace
- the template quantity calculated for the C_T value of the average fluorescence trace (in units specified during plate setup)
- the average (Avg) of the individual C_T values of wells in the set
- the maximum (Max) of the individual C_T values of wells in the set
- the minimum (Min) of the individual C_T values of wells in the set
- the standard deviation (SD) of the individual C_T values of wells in the set
- the template quantity calculated for the mean, maximum, and minimum individual C_T values of wells in the set
- the standard deviation of the template quantities calculated from the individual C_T values of wells in the set

Click the *Copy to Clipboard* button to copy the quantity calculations to the clipboard for pasting into word processing or spread sheet programs.

8.4 Melting Curve

Because the melting temperature of nucleic acids is affected by length, G+C content, and the presence of base mismatches, among other factors, products can often be distinguished by their melting temperature (T_m). A melting curve analysis can be used to verify that only the intended target was amplified, often eliminating the need for electrophoresis.

To generate a melting profile, you must use an indicator chemistry whose fluorescence depends on the presence of double-stranded DNA (e.g., SYBR Green I). To generate a melting curve, sample fluorescence is measured repeatedly as the temperature is gradually increased. A decrease in fluorescence is observed as melting progresses, with a sharp decrease occurring at the melting temperature. This sharp decrease appears as a peak in a plot of the negative first derivative of the fluorescence intensity over time.

If a run is in progress or has just completed, click the *Melting Curve* button on the toolbar to analyze the melting curve data. To access a previously generated data file, select *Open* and then *Data File* from the *File* menu. Select the desired file from the standard browse window. To view a recently created data file, select the desired file from the *Recent Data Files* list accessible from the *File* menu. Once the data file loads, click the *Melting Curve* button on the toolbar to analyze melting curve data.

8.4.1 Display

The Display screen is the default screen in the Melting Curve analysis window. In this window, you can display a graph of Fluorescence versus Temperature, –dI/dT versus Temperature, or both, for wells selected in the Plate diagram or for sets selected in the Graphed Samples panel (when *Sets* is selected).

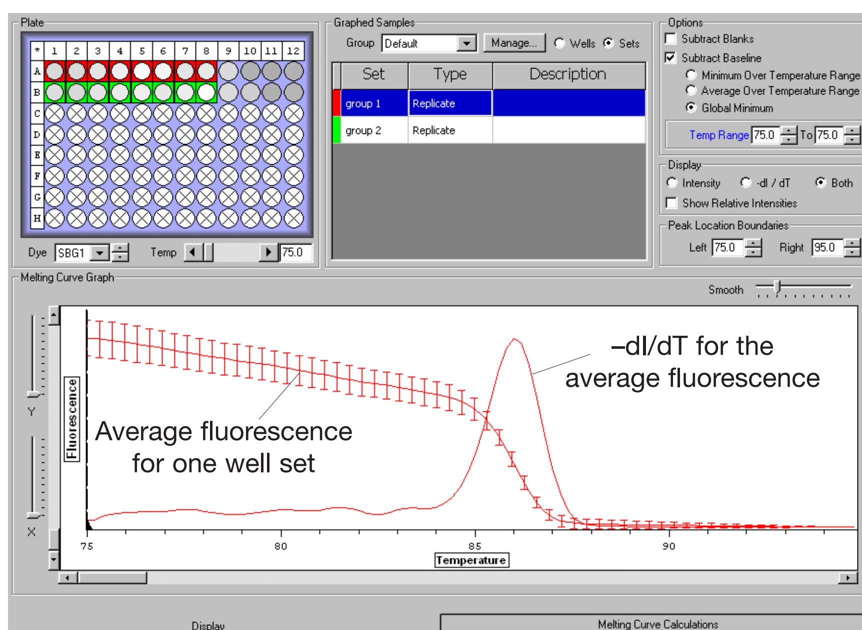

Figure 8-7. Melting curve analysis

8.4.1.1 Selecting Dyes, Wells, and/or Sets

Use the Dye field below the Plate diagram to choose the dye for which you wish to display melting curve data.

To display a list of all sets that have been defined, select *Sets* at the top of the Graphed Samples panel. Select a set by clicking on its row in the Graphed Samples list, and a mean melting curve will be constructed by averaging the fluorescence measurements at each temperature step for all wells in the set. Error bars representing the standard deviation at each point will appear on the graph (see Figure 8-7).

To plot the individual melting curves for all wells in a set, click on the colored tab to the left of the set's row in the Graphed Samples list. Use the Plate diagram to select individual wells to be included in the graph. See section 6.3.5, "Selecting and Deselecting Wells" for additional information.

Selected wells appear outlined in color. The color outlining the well corresponds to the colored tab in the Graphed Samples list and to the color of the trace in the melting curve graph. If *Wells* is selected in the Graphed Samples panel, each selected well will be represented by a different color; if *Sets* is selected, all wells in a set will be represented by the same color.

Deselect all wells by clicking on any selected well. To deselect a subset of wells, hold down the control key, and click on the well(s) you wish to deselect. The well(s) will no longer appear outlined in color, and the corresponding trace(s) will be removed from the graph. Deselect a set by clicking on its row in the Graphed Samples list. To deselect all individual wells in a set, click the colored tab to the left of the set's row in the Graphed Samples list.

8.4.1.2 Choosing Background-Subtraction Options

You can adjust the following data analysis options in the Options panel of the Melting Curve window:

- *Subtract Blanks*: If this option is selected, the fluorescence measured in all wells designated as blanks (blue) will be averaged and subtracted, as background, from the fluorescence measured in all wells designated as samples (red) or quantitation standards (green).
- *Subtract Baseline*: If this option is selected, the baseline signal, an absolute fluorescence value, will be subtracted from the fluorescence data collected in each well. This value is based on the signals measured in each well and therefore will vary from well to well.

  There are three ways to define the baseline signal value for a well:

  - *Minimum over temperature range*: The baseline signal value is defined as the minimum fluorescence value measured in the range of cycles specified in the Temp. Range field.
  - *Average over temperature range*: The baseline signal value is defined as the average of the measured fluorescence calculated from the range of cycles specified in the Temp. Range field.
  - *Global minimum*: The baseline signal is defined as the weakest fluorescence signal measured in the well. This value will be set to zero.

8.4.1.3 Display Options

Use the Display panel to choose how the melting curve data are graphed.

- Select *Intensity* to graph fluorescence intensity versus temperature.
- Select *–dI/dT* to graph the negative first derivative of the fluorescence intensity versus temperature. When sets are graphed, the –dI/dT curve is the negative first derivative of the mean fluorescence intensity trace of the set vs. temperature.

  The dotted vertical line, drawn in the same color as the corresponding trace, marks the maximum –dI/dT value, the temperature at which the rate of change in fluorescence is the greatest. This is defined as the melting temperature (T_m) of the product.

  The dotted horizontal line indicates the sharpness of the –dI/dT curve as the number of degrees Celsius over which the curve spans (i.e., the curve width) at half of the maximum -dI/dT value calculated for the well (i.e., the FWHM, or full width half max).
- Select *Both* to simultaneously display the Intensity and the -dI/dT graphs.
- Select *Show Relative Intensities* to display the relative intensities of the signals for the selected wells. If this option is not selected, the melting curves of all wells will be auto-scaled, so that the peaks are the same height.

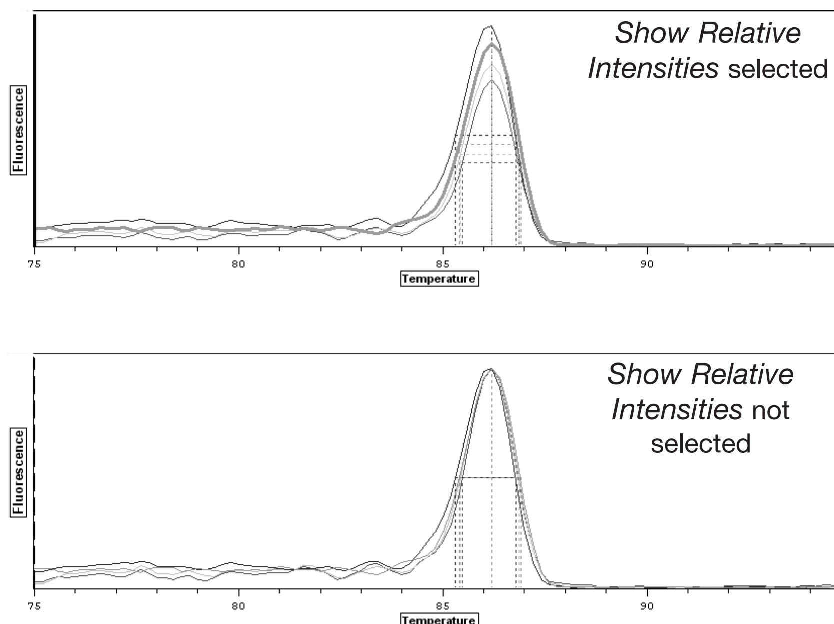

Figure 8-8. Melting curve with and without *Show Relative Intensities* selected

The Peak Location Boundaries field allows you to limit the range of temperatures over which Opticon Monitor software looks for peaks in the –dI/dT trace, thereby limiting the range of possible calculated melting temperatures (see Figure 8-9 below). This can be particularly useful for excluding unwanted peaks, or for determining the melting temperature for a second, smaller peak (e.g., when genotyping heterozygotes). You can set *Left* and *Right* peak location boundaries by entering the temperature or by using the arrows to scroll to the desired temperature. Alternatively, drag the peak location boundary guides to the desired location on the graph.

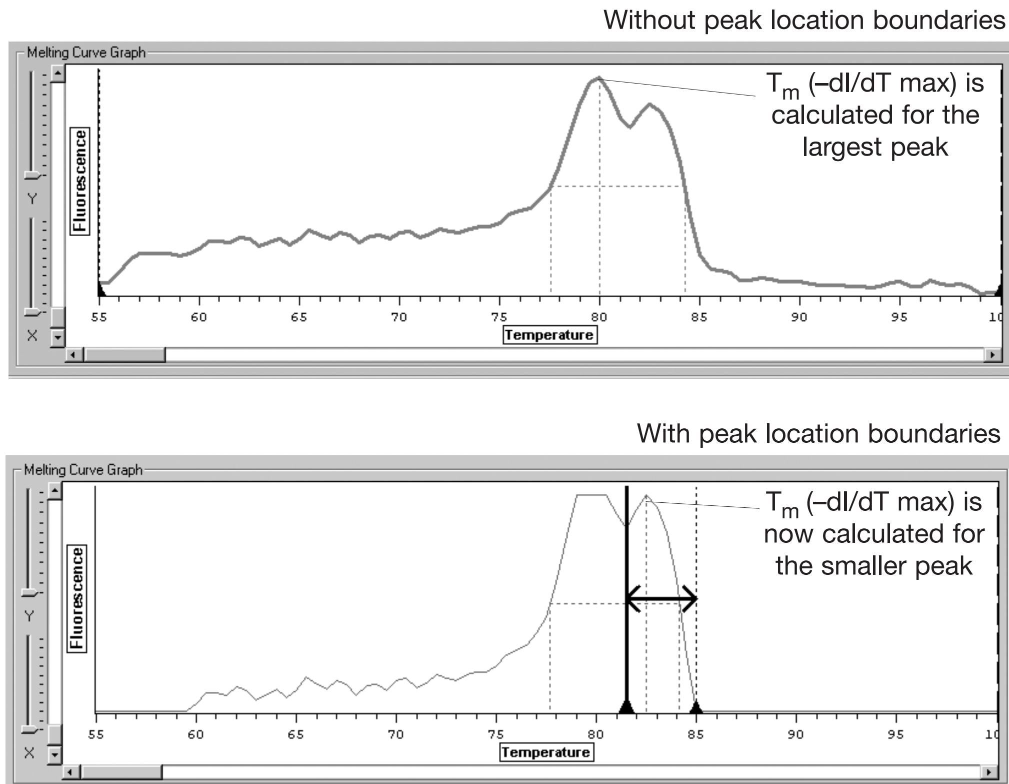

Figure 8-9. Using peak boundary markers to define peak regions

The Temp. field, located below the Plate diagram, can be used to highlight the results associated with a particular temperature, by drawing a dotted vertical line on the graph at the designated temperature. Either use the scroll bar to scroll to the desired temperature or enter the desired temperature in the box located to the right of the scroll bar. This field can also be used to display fluorescence intensity information in the Plate diagram (see section 8.3.2, “Using the Plate Diagram to View Fluorescence Intensity”).

The *Smooth* slider allows you to change the number of points that are used in calculating a running average to smooth the melting-curve graph. This can be particularly useful for resolving peaks when many reads have been collected over small increments in temperature, resulting in a choppy graph. The default setting for well-resolved data is 3.

8.4.2 Melting Curve Calculations

Click on the *Calculations* tab at the bottom of the Melting Curve window to display the melting curve calculations screen. This screen displays data tables for individual wells and well sets.

Show All Wells and Sets
Show Selected Only
Copy to Clipboard

Sets

Content	Description	FWHM	dI/dT ma	Tm	Avg Tm	Max Tm	Min Tm	SD Tm
Sample		6.845	0.07847	86	86	86.2	86	0.09682
Sample		7.063	0.07548	86.2	86	86.2	86	0.09682

Wells

Well	Dye	Content	Description	FWHM	-dI/dT max	Tm
B12	SBG1	Sample		48.16	0.0009487	83.8
A12	SBG1	Sample		3.39	0.0008159	85.8
A1	SBG1	Sample		5.128	0.07364	86
A4	SBG1	Sample		7.038	0.08454	86
A5	SBG1	Sample		7.119	0.09298	86
A6	SBG1	Sample		7.053	0.08682	86
A7	SBG1	Sample		7.034	0.07491	86
A2	SBG1	Sample		6.623	0.07321	86.2
A3	SBG1	Sample		6.966	0.07876	86.2
A8	SBG1	Sample		7.045	0.07309	86.2
B1	SBG1	Sample		5.653	0.06706	86.2
B5	SBG1	Sample		7.199	0.08161	86.2
B6	SBG1	Sample		7.283	0.07621	86.2

Graphs | Calculations

Figure 8-10. Melting curve calculations screen

Use the radio buttons at the top of the screen to either *Show all wells and sets* or *Show selected only*.

For each (selected) well, the Wells table lists:

- the Well coordinates
- the Dye used
- the Content classification (blank, standard, etc.)
- a Description of the well contents, if one was entered during creation of the plate file
- FWHM (Full Width Half Maximum): The width, in °C, of the –dI/dT peak, at half of the maximum –dI/dT value calculated for the well. This describes the sharpness of the peak
- the –dI/dT max, the peak value of the negative-first-derivative trace (between peak location boundaries, if specified)
- the T_m (–dI/dT Max): the temperature at which the peak –dI/dT occurs; this is the melting temperature of the main amplification product

For each (selected) well set, the Sets table lists:

- the name of the Set
- the Dye used
- the Type of set (replicate or calibrator)
- the Content classification (blank, standard, etc.)
- a Description of the well contents, if one was entered during creation of the plate file
- FWHM (Full Width Half Maximum): the width, in °C, of the peak of the –dI/dT of the average fluorescence, measured at half of the maximum –dI/dT value calculated for the set.
- the –dI/dT max, the peak value of the negative-first-derivative trace for the set
- the T_m (–dI/dT Max): the temperature at which the peak –dI/dT occurs. This is the melting temperature of the main amplification product
- the Avg T_m (–dI/dT Max): the mean of the melting temperatures calculated for the individual wells in the set
- the Max T_m (–dI/dT Max): the highest of the melting temperatures calculated for the individual wells in the set
- the Min T_m (–dI/dT Max): the lowest of the melting temperatures calculated for the individual wells in the set
- the SD T_m (–dI/dT Max): the standard deviation of the melting temperatures calculated for the individual wells in the set

Click the *Copy to Clipboard* button to copy the melting curve calculations to the clipboard for pasting into word-processing or spreadsheet programs.

8.5 Additional Analysis Options

Opticon Monitor software has two types of data-analysis options in addition to absolute quantitation and melting curve analysis: expression analyses, using delta-C_T or delta-delta-C_T calculations, and genotyping, using scatter plots. Expression and genotyping analyses are performed in the Analysis window, which is accessed by clicking the *Analysis* button on the side toolbar. Each analysis will be described in more detail in this section.

8.5.1 Relative-Expression Analysis

8.5.1.1 Relative Expression Using Delta-C_T and Delta-Delta-C_T

Because a sample's C_T value is dependent on both the efficiency of amplification and the amount of target initially present in the sample, the C_T and efficiency of two reactions can be used to calculate the relative initial quantity of target. If the efficiency of both reactions is 100%, that is, there is perfect doubling of product with each amplification cycle, the expression ratio between samples is equal to $2^{-\Delta C_T}$, where ΔC_T is the difference between the C_T values of the two reactions.

If a multiplex experiment is performed, the amount of target in different samples can be standardized to an endogenous reference before the relative expression of the target is calculated. In this case, assuming the efficiencies of the two reactions is equal and perfect doubling occurs, the relative expression ratio is equal to $2^{-\Delta\Delta C_T}$. The $\Delta\Delta C_T$ is calculated by first comparing the C_T of the target gene in each sample to that of the endogenous reference, yielding the ΔC_T of the sample, and then calculating the difference in ΔC_T between two samples.

Opticon Monitor software can calculate relative expression ratios using either ΔC_T or $\Delta\Delta C_T$. In both cases, equal efficiency and perfect doubling are assumed. When performing these analyses, it is good practice to examine the reaction efficiency of each target to verify that they are roughly equal. Reaction efficiencies are listed in the Calculations table accessed by clicking on the *Calculations t*ab at the bottom of the Analysis window.

8.5.1.2 Expression-Analysis Options in Opticon Monitor Software

The Graphs screen is the default screen in the Analysis window. In this screen, you select the type of analysis you want to perform, select the wells and/or sets to be analyzed, and view a graph of the analysis results.

To perform relative-expression analyses, select *Expression* from the drop-down menu in the Analysis Type field in the upper-right corner of the Analysis window. The following window will be displayed:

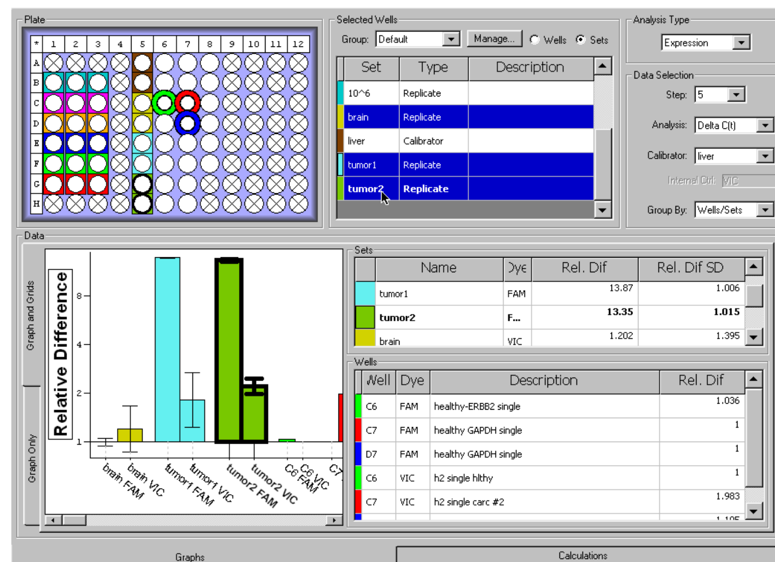

Figure 8-11. Expression analysis using the ΔC_T method. Results from one well set are highlighted.

Select the type of analysis you want to perform — *Delta* C_T or *Delta Delta* C_T from the drop-down list in the Analysis field in the Data Selection panel of the Analysis window.

To perform a relative-expression analysis, you must specify which sample or well set you will compare other samples to. This sample/set is called the calibrator. The calibrator is specified in the Plate Setup window, in the Type column of the Sets table. If you did not specify a calibrator during initial plate setup, you may do so at any time by returning to the Plate Setup windrow. The default Type is *Replicate*. To make a set a calibrator, choose *Calibrator* from the drop-down list in the Type column. If the calibrator includes more than one sample well, the average C_T will be used in the relative-expression calculation.

When performing $\Delta\Delta C_T$ analysis, in addition to specifying a calibrator sample or well set, you must specify which indicator dye is used for the internal control. This is done in the Control column of the Sets table in the Plate Setup window. Once a set is designated as a calibrator in the Type column, the internal-control indicator dye can be selected from the drop-down list in the Control column.

To continue expression analysis:

If more than one calibrator was specified in the Plate Setup window, select the Calibrator you wish to use for the current analysis from the drop-down list in the Data Selection panel of the Analysis window. If you are performing $\Delta\Delta C_T$ analysis, the indicator dye chosen for the internal control will automatically be listed below the Calibrator field.

To calculate the expression level of a target in samples or well sets relative to the calibrator, select wells from the Plate diagram or well sets from the table in the Selected Wells panel (when *Sets i*s selected). The relative expression ($2^{-\Delta C_T}$ or $2^{-\Delta\Delta C_T}$) calculated for selected wells or sets is plotted in the Data panel in the lower left corner of the window. If sets are plotted, error bars representing the standard deviation are shown. You can choose to group the bars in the graph by *Wells/Sets* or by *Dye* by selecting the appropriate item from the drop-down list in the Data Selection panel.

The relative expression for each selected well or set is listed in the Wells and Sets tables, respectively. The Sets table also lists the standard deviation of the relative difference. This information is also listed in the tables on the Calculations screen, accessed by clicking the *Calculations* tab at the bottom of the Analysis window. The relative difference listed in these tables is calculated as either $2^{-\Delta C_T}$ or $2^{-\Delta\Delta C_T}$, depending on whether *Delta* C_T or *Delta Delta* C_T is selected from the drop-down list in the Analysis field in the Data Selection panel. Note that the relative expression of the calibrator is, by definition, equal to 1.

You can hide the Wells and Sets panels and view a full-width graph by clicking on the *Graph Only* tab on the left side of the Data panel. To view the Wells and Sets panels again, click on the *Graph and Grid* tab.

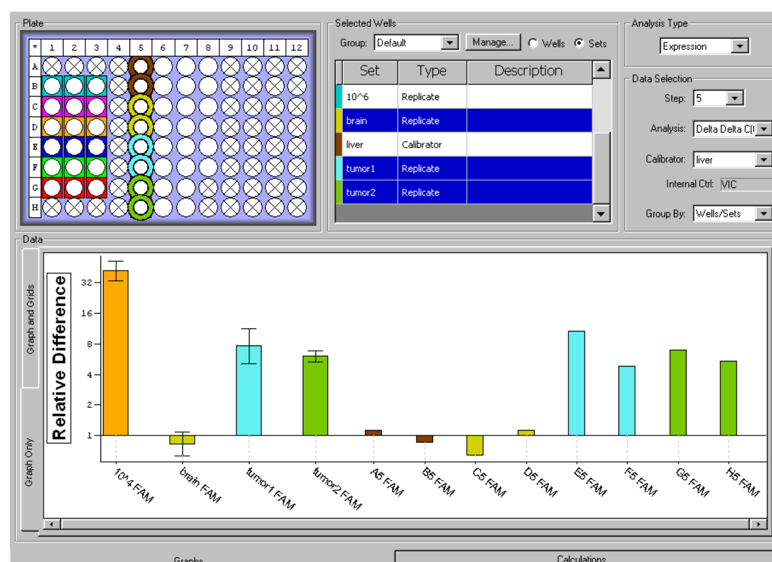

Figure 8-12, Expression analysis using the $\Delta\Delta C_T$ method; *Graph Only* tab selected.

8.5.1.3 Relative Expression Calculations Screen

Clicking the *Calculations* tab at the bottom of the Analysis window displays the Calculations screen. The tables on this screen list analysis calculations for the Analysis Type selected in the Analysis Graphs screen, for well sets and individual wells.

Show All Wells and Sets | Show Selected Only | Efficiency as % | Copy to Clipboard

Sets

Content	Description	copies	Efficiency	Rel. Dif	vg Rel. Di	lax Rel. Di	lin Rel. Di	Rel. Dif
Sample		N/A	2.12247	1	1.02091	1.28123	0.813485	1.254
Standard		N/A	2.18189	67.8532	65.099	67.665	61.4345	1.042
Standard		N/A	2.36804	35.0492	28.2084	41.4796	22.6104	1.314

Wells

Well	Dye	Content	Description	copies	Efficiency	Rel. Dif
A5	FAM	Sample	healthy RNA multi	946.499	2.06954	1.28123
B5	FAM	Sample	healthy RNA multi	900.119	2.18064	0.813485
C5	FAM	Sample	healthy RNA multi	939.084	1.98223	0.60442
D5	FAM	Sample	healthy RNA multi	901.871	2.50833	1.26623
E5	FAM	Sample	tumor RNA-multi	9543.4	2.09307	10.7169
F5	FAM	Sample	tumor RNA-multi	8646.09	2.60984	5.84926
G5	FAM	Sample	tumor RNA-multi	7926.44	2.14148	8.33566
H5	FAM	Sample	tumor RNA-multi	8364.8	1.95938	8.16863
B1	FAM	Standard	10^6	1e+006	2.49355	47.9259
B2	FAM	Standard	10^6	1e+006	2.14118	32.7229
B3	FAM	Standard	10^6	1e+006	2.394	40.2967
C1	FAM	Standard	10^5	100000	2.52542	115.847

Display | Calculations

Figure 8-13. Calculations screen showing relative expression analysis

When *Relative Expression* is selected on the Graphs screen, the Calculations tables list the reaction efficiency and relative difference calculated for each well and well set. For well sets, the efficiency and relative difference are calculated using the C_T of the average-fluorescence trace, that is, the trace drawn through the average of the fluorescence measured at each cycle for the wells in the set. The Sets table also lists the average of the relative differences calculated for each of the individual wells in the set, as well as the standard deviation and the relative difference for the maximum and minimum C_Ts in the set.

Click the *Copy to Clipboard* button to copy the quantity calculations to the clipboard for pasting into word processing or spread sheet programs.

8.5.2 Genotyping With Scatter Plots

In addition to their use in quantitation, C_T values can be used for genotyping of samples. Genotyping analysis can be performed on data from multiplex experiments in which different alleles were amplified using distinct primer sets and indicator dyes. To genotype, the samples are sorted into groups defined by which dyes emitted above-background signals following amplification: dye 1, dye 2, both dyes, or neither dye. Opticon Monitor software can sort the data by creating scatter plots of $C_{T(dye1)}$ vs. $C_{T\{dye2\}}$ or endpoint-fluorescence$_{dye1}$ vs. endpoint-fluorescence$_{dye2}$.

To create a scatter plot, select *Genotyping* from the drop-down menu in the Analysis Type field in the upper-right corner of the Analysis window. The window will display the following screen:

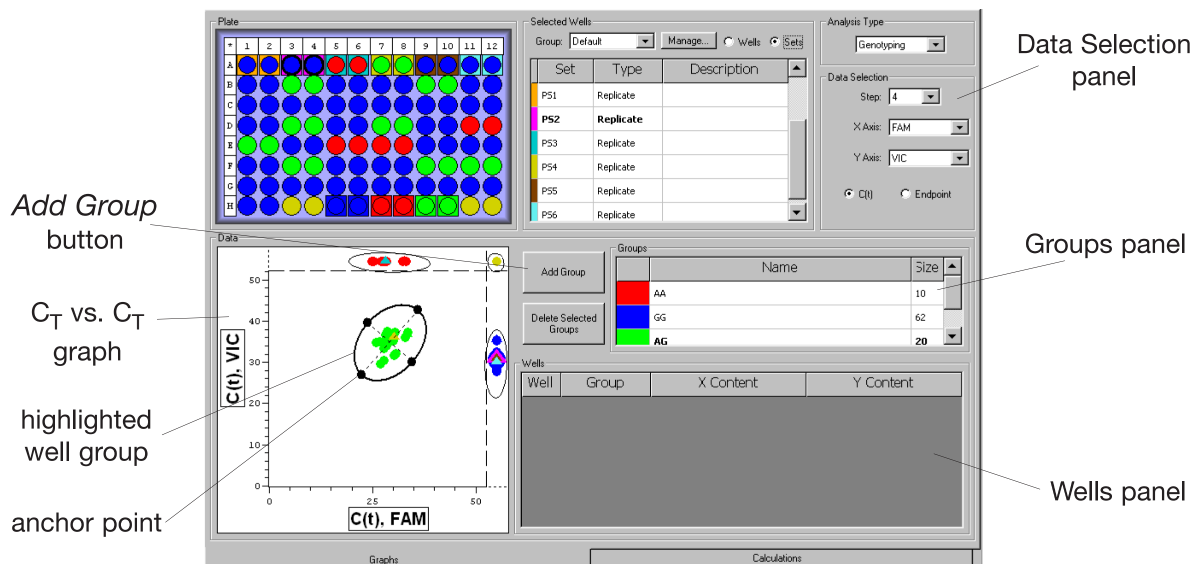

Figure 8-14. Genotyping analysis

In the Data Selection panel, select which protocol step you want to analyze, which dye you want displayed on each axis, and whether you want to compare the fluorescence data based on C_T or Endpoint. The graph in the lower left-hand corner plots data from all wells in the plate and from all defined sets. As shown in Figure 8-14, both endpoint and C_T plots can cluster data into groups based on which dye(s) had above-background signals.

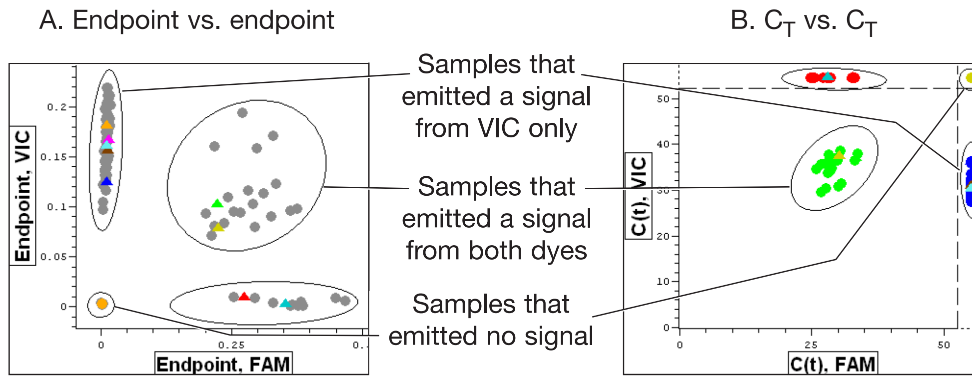

Figure 8-15. Genotyping scatter-plot options

The graph in Figure 8-15A plots the endpoint fluorescence data from each well in a two-color experiment, with the fluorescence from the FAM channel plotted along the x-axis and the fluorescence from the VIC channel plotted along the y-axis. The software has plotted the relative fluorescence intensities for each dye, as measured at the end of the run.

The graph in Figure 8-15B plots the C_T values obtained in the same experiment as in the graph in Figure 8-15A. The C_T for FAM is plotted along the x-axis and the C_T for VIC is plotted along the y-axis. The dashed lines at the top and right edges of the graph are separators that mark the last cycle of the experimental protocol. The fluorescence of those samples plotted above or to the right of these lines did not rise above threshold for at least one of the dyes during this experiment.

8.5.2.1 Identifying Individual Wells and Sets in the Scatter Plot

When the scatter plot is first constructed, the data from wells are plotted as black circles and, if *Sets* is selected in the Selected Wells panel, the data from sets is plotted as triangles colored as indicated by the tabs next to the set names. Positioning the mouse cursor over a symbol on the scatter plot highlights the corresponding well in the Plate diagram or the corresponding set in the Graphed Samples panel. Positioning the cursor over a well in the Plate diagram or an item in the Graphed Samples panel enlarges the corresponding symbol in the scatter plot. Selecting a well in the Plate diagram colors the symbols representing other wells gray in the scatter plot, while the selected well's symbol remains black.

8.5.2.2 Defining Subsets of Data in the Scatter Plot

Using the scatter plot, you can identify subsets of data based on the clustering pattern of the data points (Figure 8-15). For example, in genotyping, samples with the same genotype will tend to cluster in a similar C_T range. Users can select samples that cluster together and assign particular group names, e.g., the genotype, to the samples located in these clusters.

To group a cluster of data points, click on the *Add Group* button in the center of the Analysis window. This converts the mouse pointer to a set of cross hairs. Move the cross hairs to the periphery of a clustered data set in the scatter plot. Left-click the mouse to set an anchor point on one side of the data set, drag the cross hairs to the opposite edge of the data set, and left-click the mouse to mark the end of the set. Dragging the cross hairs perpendicular to the ellipse that was just created causes the ellipse to radiate outward. Expand and contract the radius to include the data points that you wish to analyze. When you are satisfied with the area that you have defined, left-click a final time to set the group.

If no wells have been selected in the Plate diagram, data points in the scatter plot will turn from black to a particular color (red, green, blue, etc.) when they are included in a set. If wells have been selected, then only the symbols corresponding to selected wells will change color when they are added to a set — the others will remain gray until they are selected or all wells are deselected in the Plate diagram.

When a group has been created, it will be listed in the Groups panel as a “New Group”, along with the number of data points that are in the group. To rename the group, highlight the group name, type the new name and then press the *Enter* key on the keyboard. To delete a group, highlight the group name and then click the *Delete Selected Groups* button.

Defined groups are color coded — the graphed data points included in the set and. the sample wells that correspond to these data points (in the Plate diagram) will be assigned the same color. This color will appear in the left-hand column in the Groups panel. You can change the color of a group by right-clicking on the color and selecting a different color from the palette that appears.

When a group is selected by clicking on a group name from the Groups panel, its member wells will be listed in the Wells panel along with their descriptions. Wells can be sorted by *Well*, *Group*, *X content*, or *Y Content* by clicking on the appropriate column header. Reverse the sort order by clicking the header again. Clicking the *Wells* header multiple times allows you to sort by column or row, in forward or reverse order.

8.5.2.3 Editing a Group

You can alter the size, shape, and position of an ellipse after it has been drawn. To change the dimensions of the ellipse, click on one of the four anchor points that lie on the ellipse. (These will appear when you position the mouse pointer on the ellipse, as in Figure 8-14). Position the pointer at the intersection of the axes with the ellipse.) Dragging the anchor points can alter both the shape and size of the ellipse. To move the ellipse without changing its dimensions, click on the ellipse (between anchor points) and drag it to a new location.

8.5.2.4 Calculations Table

Clicking the *Calculations* tab at the bottom of the Analysis window displays a table listing the group assignment, as well as the C_Ts and reaction efficiencies associated with each dye, for individual wells and well sets. The maximum, minimum, and standard deviation of the individual C_Ts of wells in each set is also listed. Clicking the *Copy to Clipboard b*utton allows you to paste the information in the table into spreadsheet programs.

Show All Wells and Sets   Show Selected Only   Efficiency as %   Copy to Clipboard

Sets

Name	Group	Description	C(t) X	C(t) Y	Efficiency X	Efficiency Y
AA control	AA		32.7868	0	1.50455	N/A
AG control	AG		33.2753	37.0046	1.42204	1.16163
GG control	GG		0	36.0531	N/A	1.22318

Wells

Well	Group	Description X	Description Y	C(t) X	C(t) Y	Efficiency X	Efficiency Y
A1	GG	PS#1	PS#1	None	30.4244	N/A	1.29507
A2	GG	PS#1	PS#1	None	31.0455	N/A	1.25473
A3	GG	PS#2	PS#2	None	31.1791	N/A	1.21554
A4	GG	PS#2	PS#2	None	30.4571	N/A	1.27457
A5	AA	PS#3	PS#3	27.8199	None	1.51729	N/A
A6	AA	PS#3	PS#3	28.3628	None	1.40779	N/A
A7	AG	PS#4	PS#4	30.445	38.6736	1.30362	1.08819
A8	AG	PS#4	PS#4	29.7616	36.4989	1.33031	1.11137
A9	GG	PS#5	PS#5	None	32.1065	N/A	1.19032
A10	GG	PS#5	PS#5	None	29.9608	N/A	1.30888
A11	GG	PS#6	PS#6	None	29.9419	N/A	1.31615
A12	GG	PS#6	PS#6	None	30.6667	N/A	1.24532

Display   Calculations

Figure 8-16. Calculations table showing genotyping analysis

8.6 Exporting and Printing Data

8.6.1 Exporting Data

For customized data analysis, Opticon Monitor software provides the option to write all the fluorescence data collected during a run to a comma-separated-values (CSV) file, readable by Microsoft Excel. The Export command creates a CSV file listing all fluorescence measurements (for selected wells) that were collected during all plate read and melting curve steps in an experimental run.

To export data for selected wells, choose *Export* (under the *Quantitation*, *Melting Curve*, or *Analysis* menu, depending on which window is being viewed — the exported data will be the same). Wells can be selected in the window that appears.

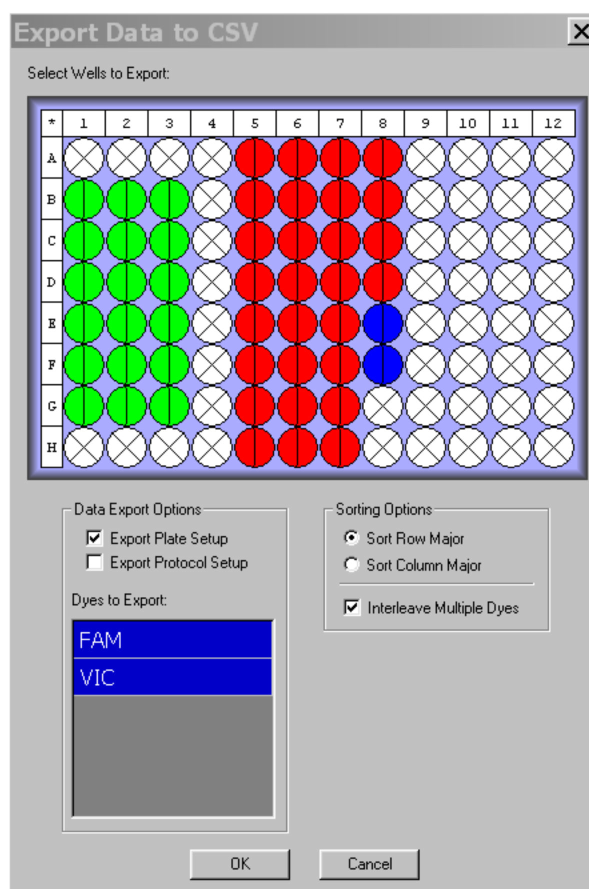

Figure 8-17. Exporting data

You can also select the dye(s) for which data will be exported by selecting from the Dyes to Export list (selected dyes are highlighted in blue). You can choose to sort the wells by row or by column; and can choose whether to *Interleave Multiple Dyes* (i.e., to list data for all dyes in each well together), or to first list the data for each well for one dye and then list the data for each well for the second dye.

In addition to exporting fluorescence data, you can choose to export the plate and/or protocol setup information by checking *Export Plate Setup* and/or *Export Protocol Setup*. The plate information that is exported includes the well number, dye used, well contents, and well descriptions. If the plate setup is exported, the information can later be pasted into a different master file to easily reconstruct the plate file (see section 6.3.14, "Pasting Plate Information from Microsoft Excel").

By default, the exported CSV file also lists analysis options (i.e., Subtract Blanks, Subtract Baseline, Baseline Start cycle, Baseline End cycle, and Suppress Pre-Threshold) that were chosen on the analysis screen for each protocol step at which data were collected.

8.6.2 Copying Results to Clipboard

The *Copy to Clipboard* command (under the *Quantitation*, *Melting Curve*, or *Analysis* menu, depending on which window is being viewed) allows the user to paste run information and graphical data into a variety of applications for presentation or storage. This command can also be implemented by clicking on the *Copy to Clipboard* button on the Calculations screen. The following items can be copied using *Copy to Clipboard* from the specified windows:

8.6.2.1 From the Quantitation Window

The *Data Graph* (a plot of fluorescence — or log fluorescence — vs. C_T cycle), *Standards Graph*, *Microtitre plate* (the Plate diagram), or *Quantity calculations* can be copied to the clipboard and pasted into several applications. Selecting *Quantity Calculations* from the *Copy to Clipboard* menu generates CSV tables to be pasted subsequently into an Excel spreadsheet. The tables have the same content as the Quantity Calculations screen, including information for both well sets and individual wells. See section 8.3.9, "The Quantity Calculations Screen", for more details on these tables. The tables can also be copied to the clipboard by clicking on the *Copy to Clipboard* button on the Quantity Calculations screen.

8.6.2.2 From the Melting Curve Window

The *Data Graph* (the plot of fluorescence intensity or –dI/dT or both), *Microtitre plate* (the Plate diagram) or *Melting Temp Calculations* can be copied to the clipboard. Selecting *Melting Temp Calculations* generates a CSV file with information identical to that shown on the Melting Curve Calculations screen, including both well sets and individual wells. See section 8.4.2, "Melting Curve Calculations", for more details of this table. This table can also be copied to the clipboard by clicking on the *Copy to Clipboard* button on the Melting Curve calculations screen.

8.6.2.3 From the Analysis Window

The *Data Graph* (the plot of C_T or Endpoint fluorescence or the plot of relative expression levels), *Microtitre Plate*, *Calculations* or *Analysis List* can be copied to the clipboard. Selecting *Calculations* generates a CSV file with information identical to that shown on the Calculations screen, including both well sets and individual wells. Selecting *Analysis List* generates a CSV file listing the Group Name, and the Dye 1/Dye 2 contents (standard, sample, etc.) for each sample belonging to a Group in the scatter plot (as defined by the user — see section 8.5.2.2, “Defining Subsets of Data in the Scatter Plot”). Selecting *Analysis List* does not copy the relative expression calculations.

8.6.3 Creating and Printing Reports

Opticon Monitor software can assemble a report of the real-time run and send it directly to a networked printer or save it as an HTML file. This can be done by selecting *Print HTML Report* from the *Quantitation*, *Analysis*, or *Melting Curve* pull-down menu in the appropriate window.

If *Print HTML Report* is selected from the *Quantitation* menu, the software creates a report that contains: the user’s name; the name and location of the data file on the Opticon computer’s hard drive; an indication of the active dye; the position of the C_T line; and an indication of whether the C_T level was set manually or “via signal-to-noise”. The report will also include the Plate diagram with selected wells highlighted; the Data graph of fluorescence (or log fluorescence) versus C_T cycle; and the Standards graph along with the equation detailing the fit of the data. The Plate diagram and the graphs will appear exactly as they do when *Print HTML Report* is selected, i.e., with or without error bars on the Standards graph, showing *All Dyes* or only the selected dye, etc.

The report also includes tables similar to those on the Calculations screen (see section 8.3.9). The tables will include all wells and sets or only selected wells and sets, depending on what radio button has been selected on the Calculations screen. When P*rint HTML Report* is selected, a window opens, allowing you to select which columns from each table you want to include in the report (see Figure 8-18).

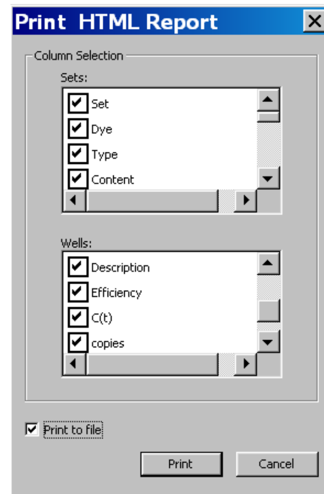

Figure 8-18. Printing from the Quantitation window

If *Print HTML Report* is selected from the Analysis window, the software creates a file that contains: the user's name; the name and location of the data file on the Opticon computer's hard drive; the dye graphed on each axis of the genotyping graph, and the plate type chosen during plate setup. The report also includes the Plate diagram as it appears when *Print HTML Report* is selected, as well as the Data graph that is showing (genotyping or relative expression). Note that only the small version of the Data graph will be included in the report, even if the *Graph Only* tab has been selected in the Analysis window.

The report printed from the Analysis window will also include tables similar to those appearing in the Calculations screen. You can select what columns to include when the Print HTML Report window opens.

If *Print HTML Report* is selected from the Melting Curve menu, the software creates a report that contains: the user's name; the name and location of the data file on the Opticon computer's hard drive; and an indication of Display Type, i.e., whether intensity, -dI/dT, or both are plotted in the graph. The report will also include the Plate diagram with selected wells highlighted; and the Data graph of fluorescence intensity and/or -dI/dT vs. temperature. Finally, the report will include a tables similar to those that appear on the Calculations screen. You can select what columns to include when the Print HTML Report window opens.

To save a report to the computer's hard drive, select mark the *Print to File* box near the bottom of the Print HTML Report window. When you then click *Print*, a Save window will open. Specify a name and location for the HTML file. The Plate diagram and graphs will be saved as separate .bmp files in the same location. Click *Save* to save the file.

To print the report directly to a printer, make sure the *Print to File* box is unmarked before you click *Print*.