

Protocol Sheet

Eppendorf® Mastercycler® ep *realplex* instrument setup instructions for qBiomarker Copy Number PCR Arrays

Important points before starting

- Please read the handbook supplied with the qBiomarker Copy Number PCR Array, paying careful attention to the “Safety Information” and “Important Notes” sections, before beginning this procedure.
- Make sure the correct thermal block cycler (silver or aluminum) is in place.
- Turn on the Mastercycler instrument; the main switch is located at the back of the instrument. Turn on the computer linked to the instrument and log on to Windows® XP.

Procedure

1. Start *realplex* software version 2.2.
2. Enter “Username” and “Password” when the “Login” dialog box appears and click “OK” to proceed with the login. The “Plate Layout” window (Figure 1) will appear.

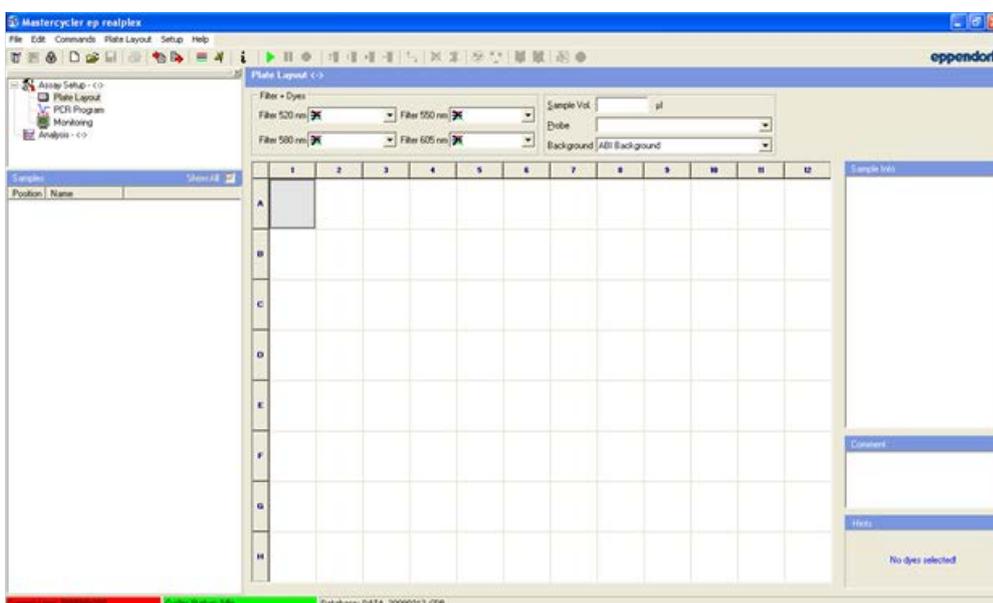


Figure 1. “Plate Layout” window.



Background calibration

1. Before setting up an experiment, it is necessary to perform background calibration using an empty, capped A plate. Click on "Setup," then "Background Calibration." A list of previously performed calibrations will appear. Click "New..." to open the following dialog box (Figure 2):

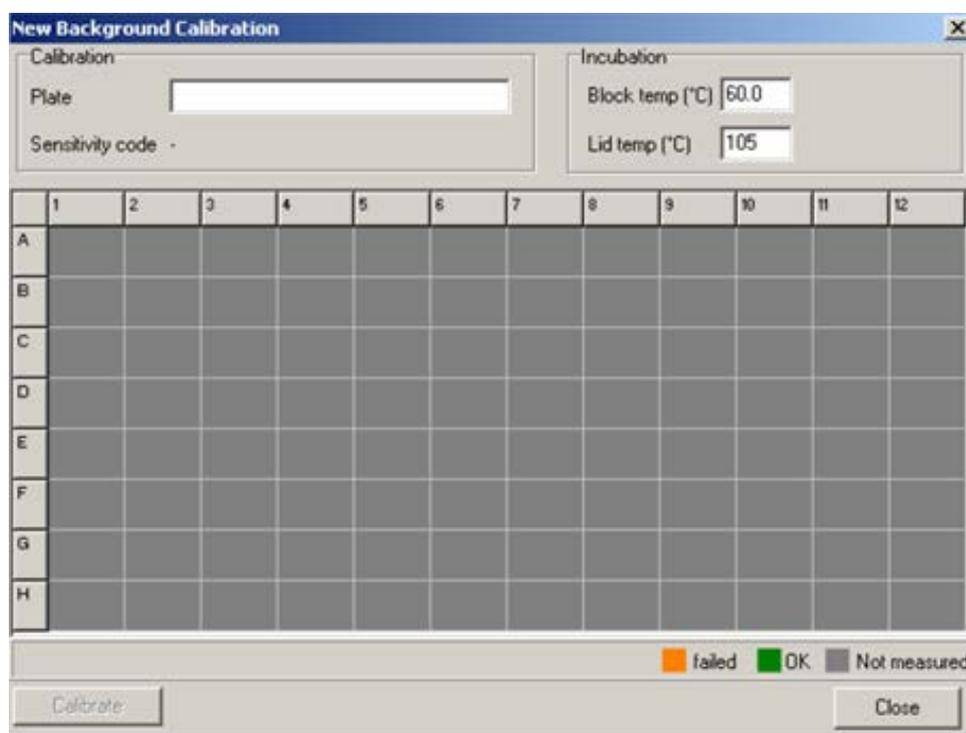


Figure 2. "New Background Calibration" dialog box.

2. Enter "QIAGEN A Plate" as the name of the background plate. The default block and lid temperatures (60.0°C and 105°C, respectively) do not need to be changed. Insert the background plate into the Mastercycler with well A1 at the upper left corner; slide the heated lid closed, pull the sealing clamp into the closed position and click "Calibrate." Click "OK" to begin calibration.
3. After the first reading, the software will prompt you to turn the plate. Open the *realplex* instrument and turn the background plate so that well A1 is in the lower right corner. Then close the instrument and click "OK" to continue the calibration.
4. If background calibration was successful, the plate layout will be highlighted in green and a dialog box will appear with the message "Calibration successfully finished." Click "OK" to continue with the experiment setup.

Note: If background calibration fails in some positions, those wells will be highlighted in orange. Failure may be a result of fluorescence contamination on the plate or in some wells of the thermoblock. Retry calibration with a new A Plate; if this also fails, clean the thermoblock as instructed in the instrument manual.

qBiomarker Copy Number PCR Array template setup

1. In the "Plate Layout" window, specify the following setup parameters:
 - Filter 520 nm: SYBR®
 - Sample Vol: 25 µl
 - Probe: SYBR Green
 - Background: QIAGEN A plate
2. Select all wells, then right-click in the blue area and select "Unknown" to complete plate layout (Figure 3).

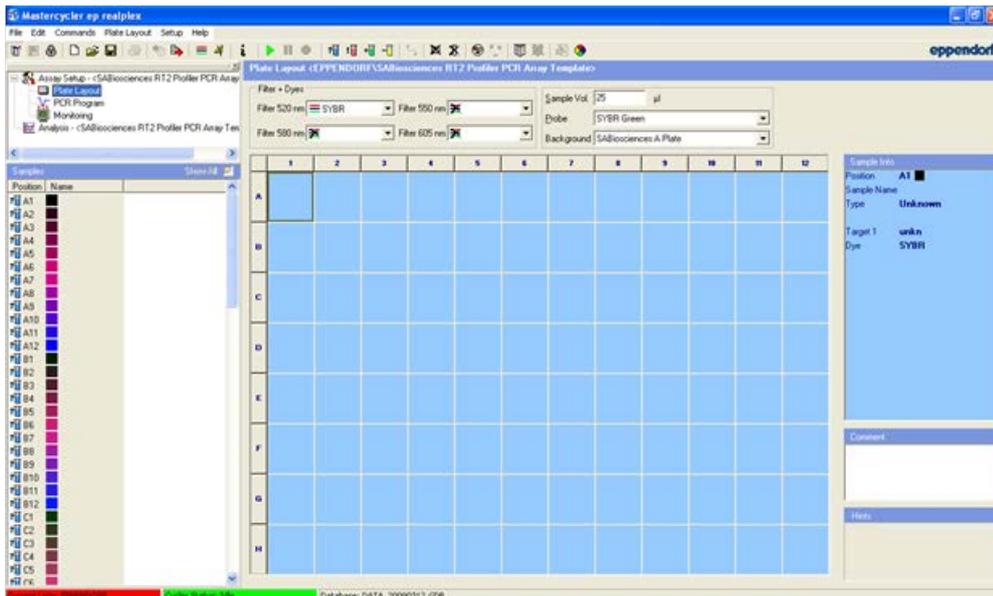


Figure 3. Complete "Plate Layout" window.

3. Select "PCR Program" in the navigator window at the upper left corner of the screen. The default PCR program will appear (Figure 4).

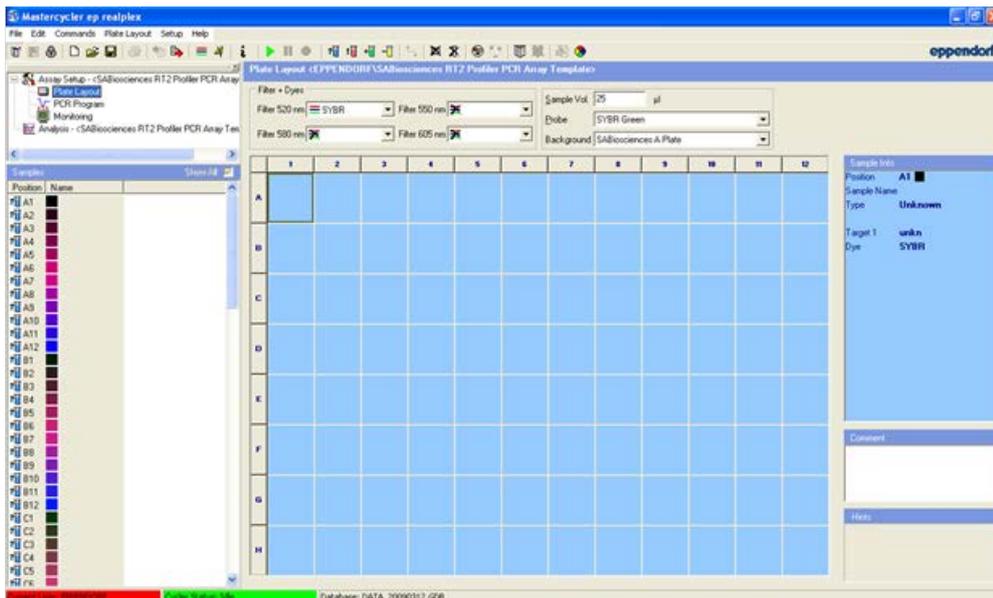


Figure 4. "PCR Program" window.

4. Edit the default program to create a qBiomarker Copy Number PCR Array template. Right-click in segment 2, 3, or 4, then click "Delete." All three segments will be deleted.
5. Right-click on "END." Select "Add Step", then select "2 Step Cycle" and click "OK" (Figure 5).

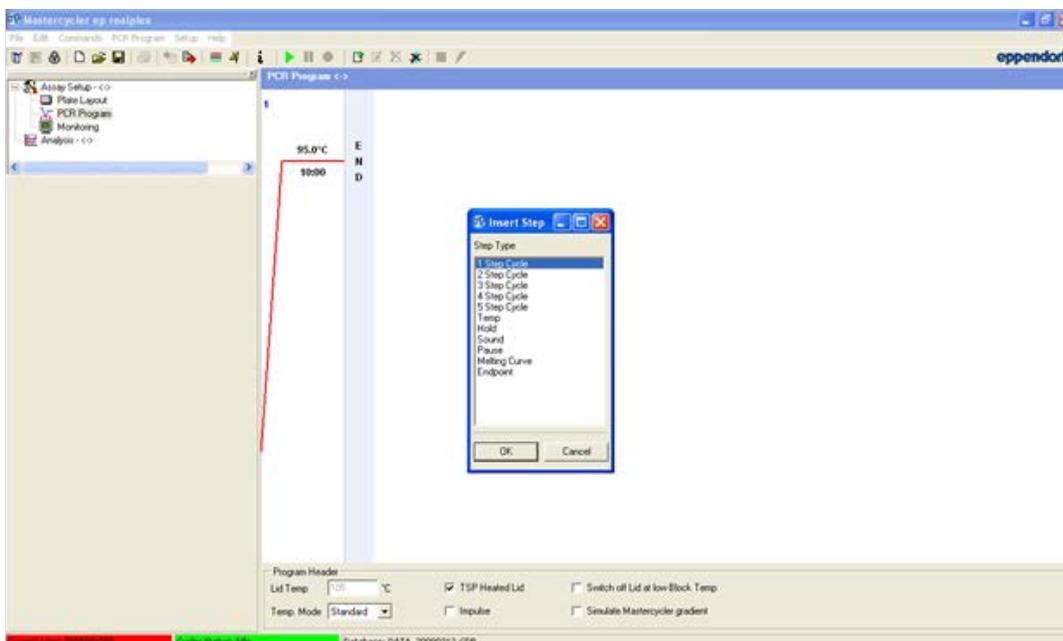


Figure 5. Adding 2-step cycle.

- Right-click on "END." Select "Add Step", then select "Melting Curve" and click "OK" (Figure 6).

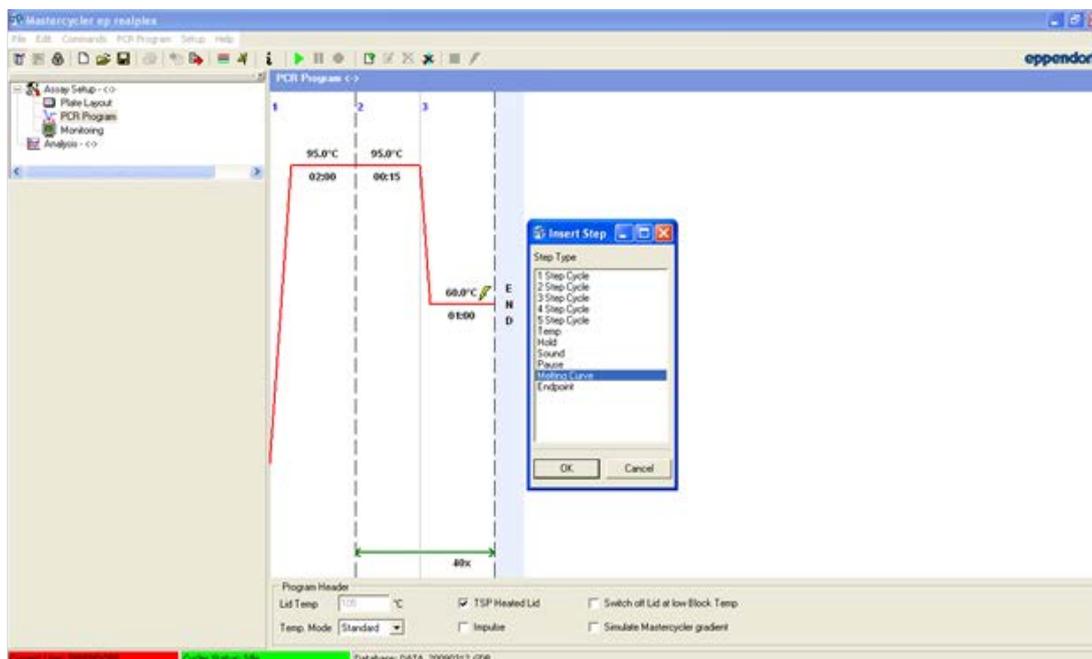


Figure 6. Adding the melting curve.

- Change the length of the heat activation step to 10 minutes. To do so, click on 02:00 in segment 1 and enter 10:00. The final parameters for the PCR program should be as shown below:

Program	Segment	Temperature (°C)	Hold (mm:ss)	Cycles
Heat activation	1	95.0	10:00	1
PCR cycle	2	95.0	00:15	40
	3	60.0	01:00	
Melt curve	4	95.0	00:15	1
	5	60.0	00:15	
	6		20:00	
	7	95.0	00:15	

Temperature step parameters

- Double-click on the  icon in segment 3. The "Temp Step" window will appear. Uncheck "Gradient" and change the "Ramp [%]" to 26**. The final "Temp Step" parameters should appear as in Figure 7. Click "OK."

Note: 26% is the recommended ramp rate for the silver block. The recommended ramp rate for the aluminum block is 35%.

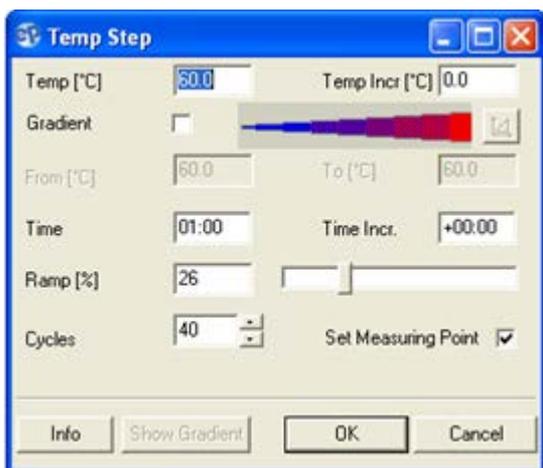


Figure 7. "Temp Step" parameters.

2. When the "PCR Program" setup is complete, the window should appear as in Figure 8.

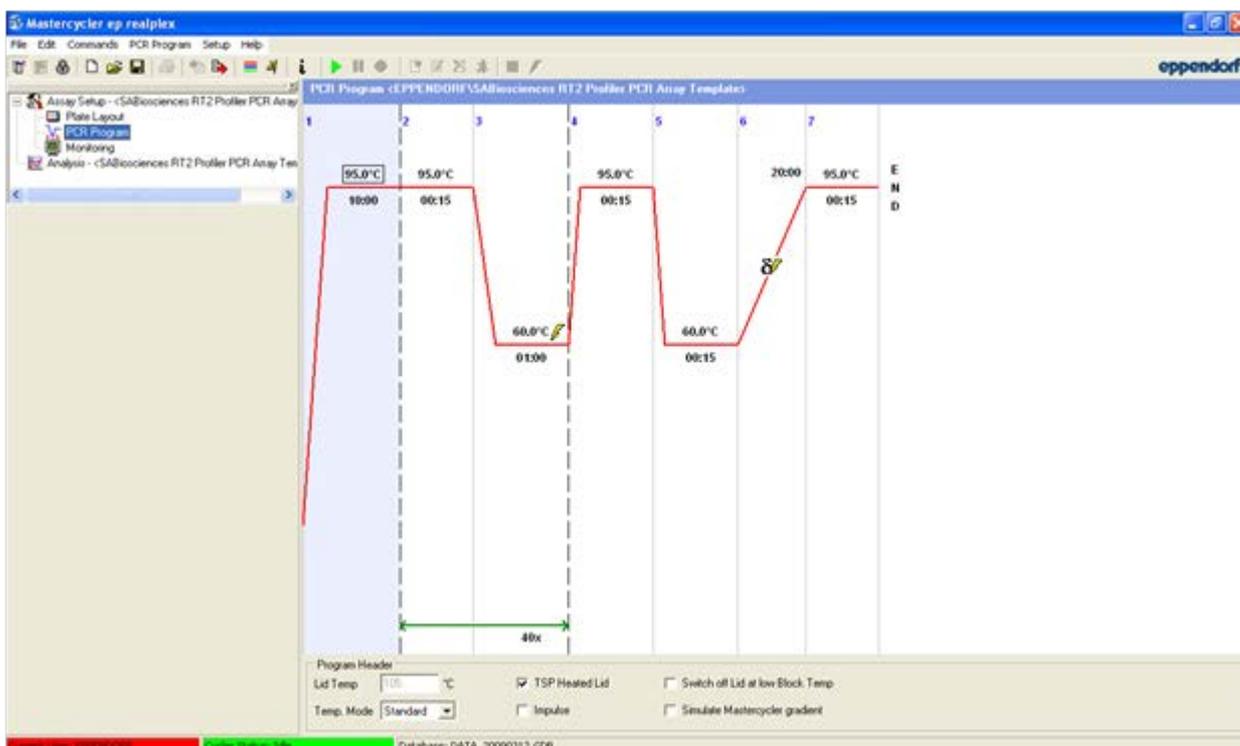


Figure 8: Final "PCR Program" screen.

3. Click "File" and select "Save As." In the "Assay Name" field enter the filename "qBiomarkerCNPCRRArraySilver" or "qBiomarkerCNPCRRArrayAluminum" depending on which sample block is being used. From the dropdown list for "Assay Type," select "Template." Click "OK."

Performing real-time PCR detection

1. When the PCR array is prepared and centrifuged, bring the plate to the realplex instrument to load.
2. Make sure the correct thermal block cycler (silver or aluminum) is in place.
3. If the *realplex* instrument is not on, turn it on using the main switch at the back of the instrument. Turn on the computer linked to the instrument and log on to Windows XP.
4. Start *realplex* software version 2.2.
5. Enter "Username" and "Password" when the "Login" dialog box appears and click "OK" to proceed with the login. The "Plate Layout" window will appear.
6. Wait for the LED on the top of the instrument to turn green. Make sure the instrument has been warmed up for at least 15 minutes before starting an assay.
7. Push the sealing clamp back into the open position, then slide the heated lid back. Place the PCR array plate on the thermoblock with well A1 in the upper left corner.
8. Slide the heated lid forward and pull the clamp down.
9. In the "Plate Layout" window, click "File" then "Open." Select the previously saved "qBiomarkerCNPCRRArraySilver" or "qBiomarkerCNPCRRArrayPCRRArrayAluminum" run template as appropriate, then click "OK."
10. Click "File," then "Save as." Save the file under a new filename as an "Assay."
11. Click the  button to start the PCR program.
12. The dialog box "Start PCR" will appear. Ensure that "QIAGEN A Plate" is selected as the background plate, then click "OK." Real-time data acquisition can be viewed in the "Monitoring" window.

Data analysis

1. Click "File," then "Open Assay" (Figure 9) and find the run file to be analyzed. Alternatively, upon completion of a PCR experiment, a dialog box will appear prompting you to start analysis. Click "Yes."

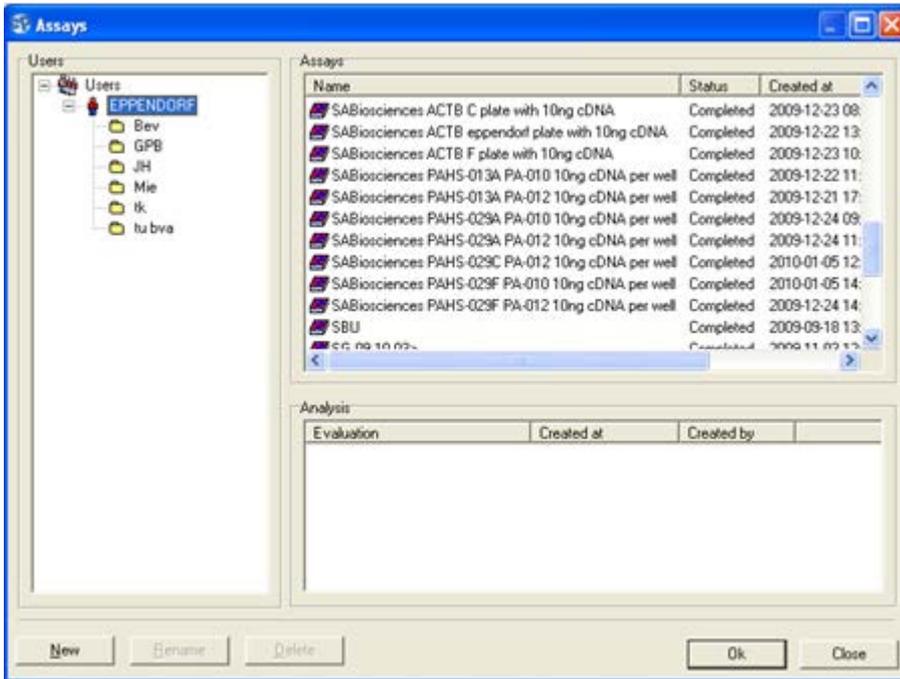


Figure 9. Opening the run file.

Note: It is possible to open a data file for analysis during an ongoing assay. A message will appear alerting you that the file will be opened for data analysis only. Click "OK" to view the assay in the analysis window. You will not be able to view plate layout or PCR program information for the completed assay in this mode.



Figure 10. Analysis only.

- In the navigator window at the upper left corner, click on "Analysis." The top panel (Figure 11) features a "Type of Application" combo box. Click on the ▾ button to select an analysis module.



Figure 11. "Analysis" panel.

3. Select "Quantification" (Figure 12) as the type of application to obtain C_T values. Numerical values are listed in the "Sample/Analysis" window to the left of the screen while amplification curves are shown in the main work area as a function of fluorescence and cycle number. As a default, the curves are shown in linear scale; to view the curves in log scale, check the "Log Scale" box in the top panel. Click on the  button to save the file with results.
4. To view a selection of samples, you can highlight the desired wells in the "Sample/Analysis" window or in the "MiniPlate Layout" at the lower left part of the screen.

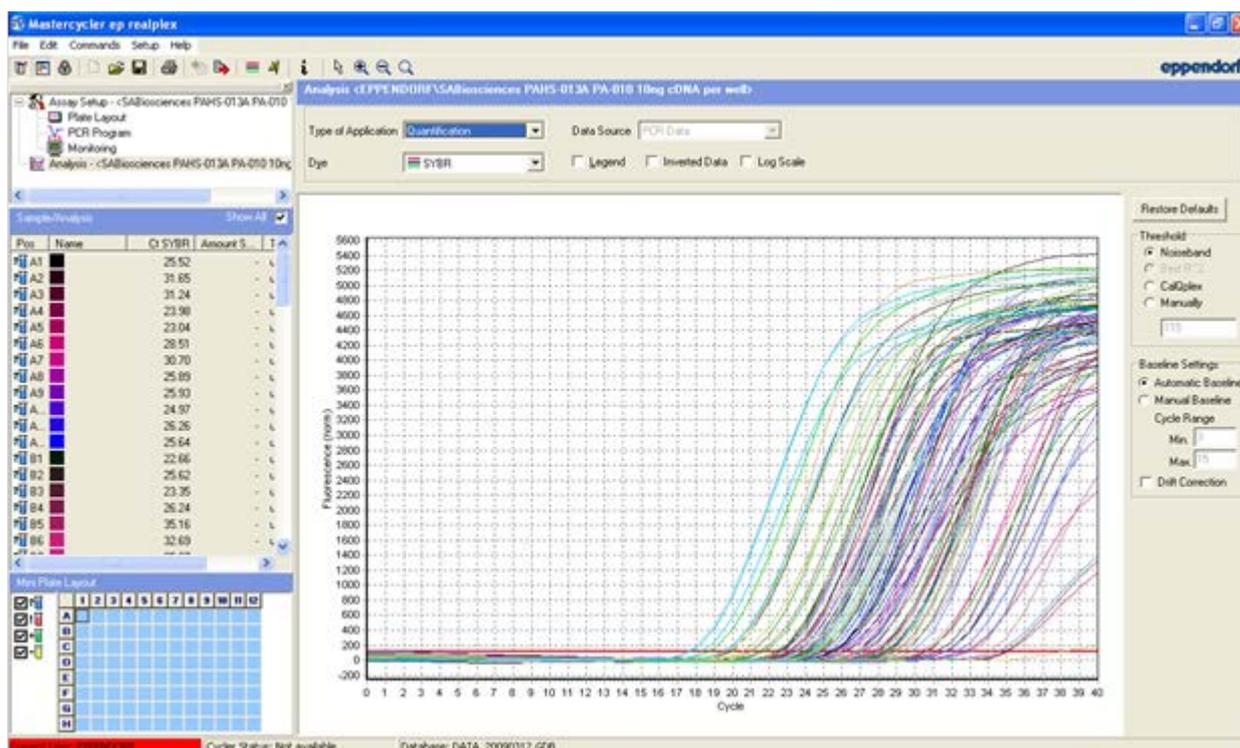


Figure 12. "Quantification Analysis" window.

5. The "Threshold" should be set at the same level for all of the PCR arrays in your experiment. The threshold should be set using the log view of your amplification data and set somewhere in the lower 1/3 of your amplification curves. A good indication that the threshold is set in the proper location is that the values of the PPC assays on catalogued arrays should be between 18 C_T and 22 C_T .
6. To set the "Baseline," use the "Automatic Baseline" setting.

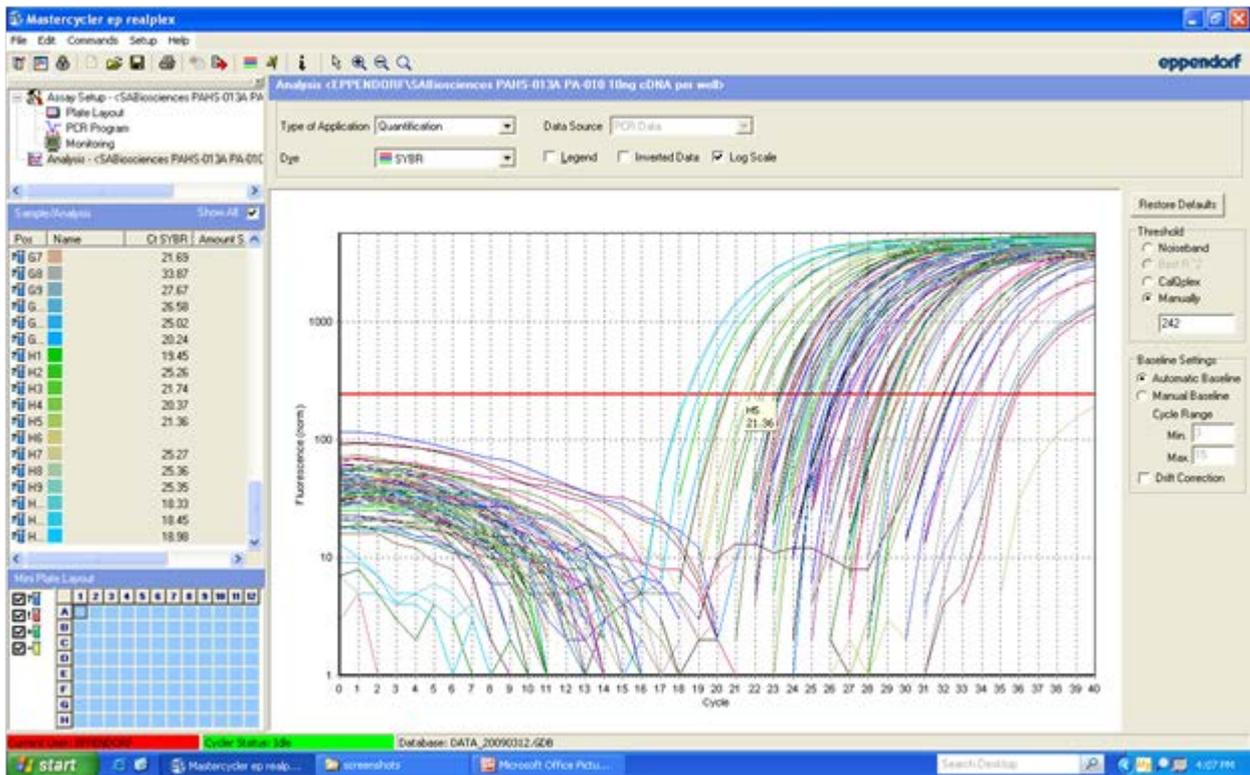


Figure 13. Log view of raw data.

7. To export C_T values, right-click in the “Sample/Analysis” window and select “Save Results As.” Export results in text format to view results as a spreadsheet. Click “OK.” When prompted, choose the file destination, filename, and type. Click “Save” to export.
8. To export results from a selection of wells, highlight the desired wells in the “Sample/Analysis” window before right-clicking to save results. Under “Include,” click the radio button for “Selected Samples” before proceeding with the export.

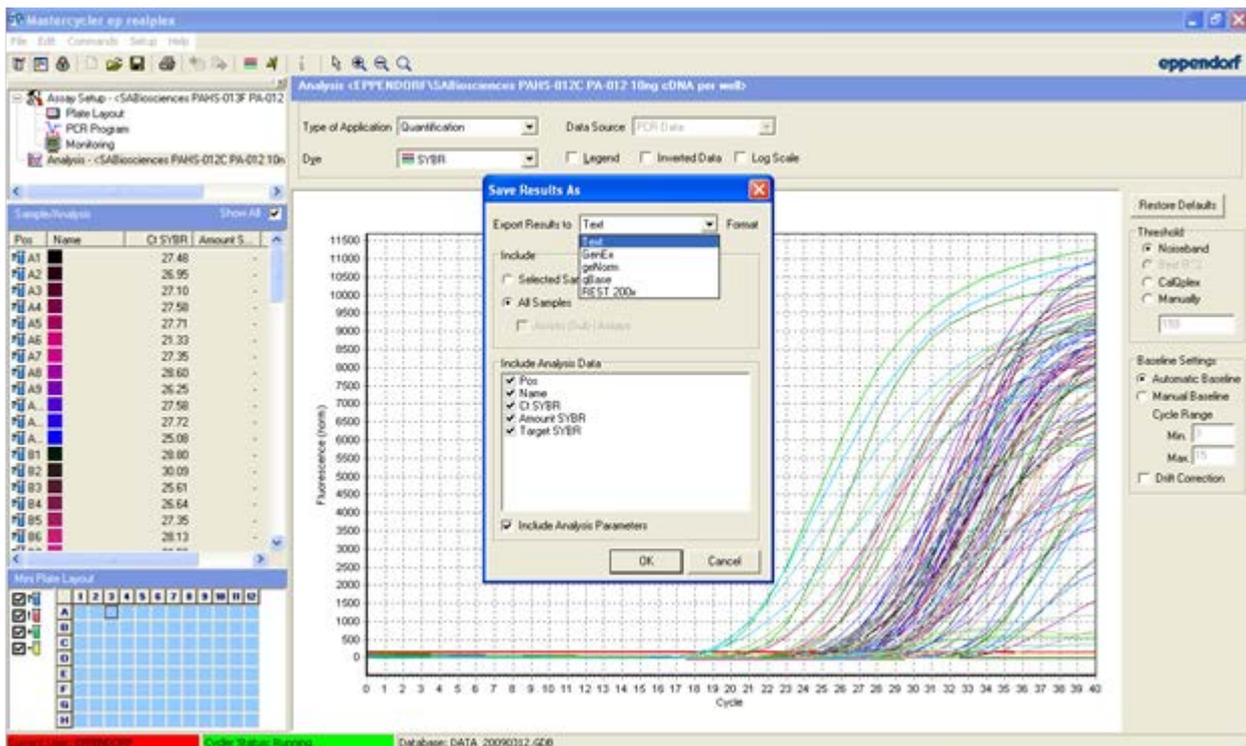


Figure 14. Exporting the C_T results.

Note: You can also export the entire run file containing all setup information, raw data, and analyses as a *.asy file. This will allow you to open the file using the *realplex* software on another computer. To do so, go to "File" and "Export." The dialog box "Export assay to file" will appear. Select the destination for the file and enter a filename. The file type should be realplex-Exchange (*.asy). Click "Save" to finish.

9. To import a file, open the *realplex* software and create a new document by clicking on the  button (or go to "File" and "New Assay"). You will not be able to import a file if an assay is currently in progress. Go to "File," then "Import." In the "Import Assay from File" dialog box, find the file you wish to import. Click "Open."
10. To proceed with the melt curve analysis, select "Melting Curve" as the type of application in the top panel. The "Sample/Analysis" window will show the wells and corresponding melting point values. Peak curves are shown in the main working window and below that is a plot of fluorescence vs. temperature. Again, a selection of curves may be viewed by selecting the desired wells in either the "Sample/Analysis" or "MiniPlate Layout" windows.
11. Melting points will be analyzed only for those peaks that are above the threshold. The default threshold is 33%, but this can be changed by editing the value at the right of the screen. Alternatively, the threshold can be manually adjusted by moving the horizontal red line. Below the default value, the temperature that is displayed represents the temperature at the vertical green line. This line can be moved to any peak maximum to determine the melting point.

- To export Tm values, right-click in the "Sample/Analysis" window and select "Save Results As." Export results in text format to view results as a spreadsheet. Click "OK." When prompted, choose the file destination, filename, and type. Click "Save" to export.

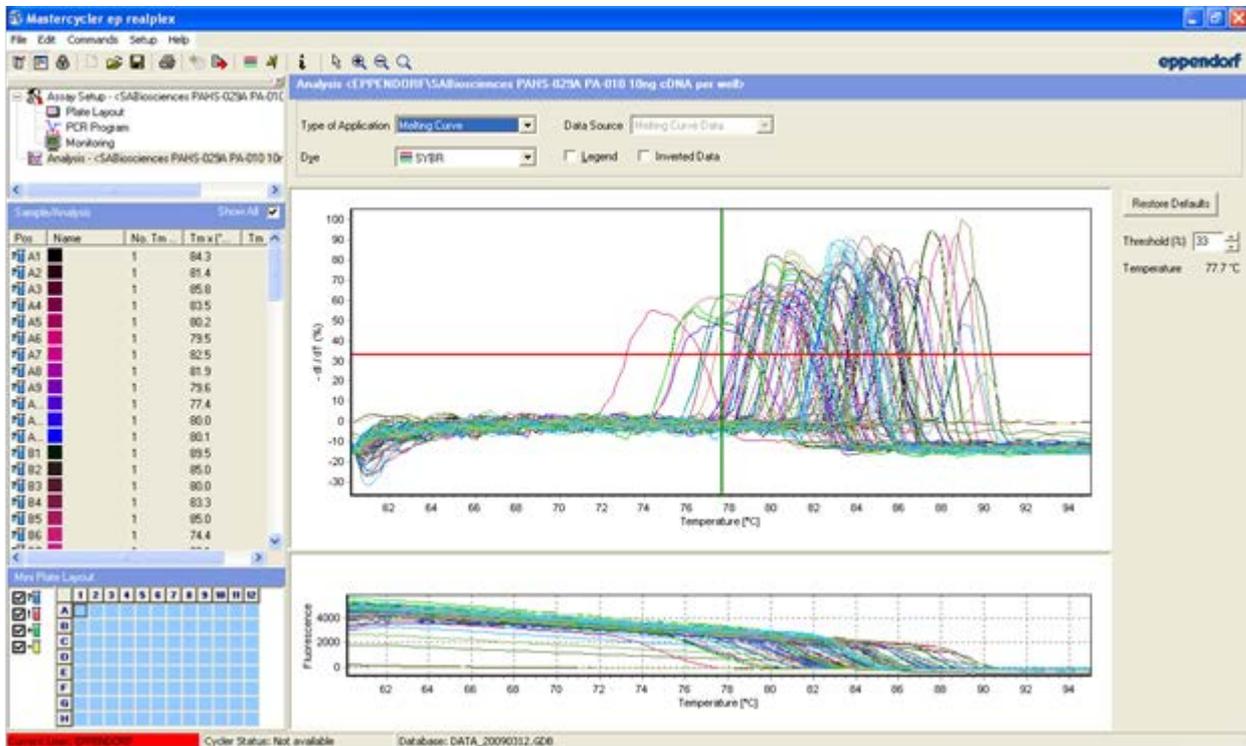


Figure 15. Melting curve analysis.

The qBiomarker Copy Number PCR Arrays are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

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