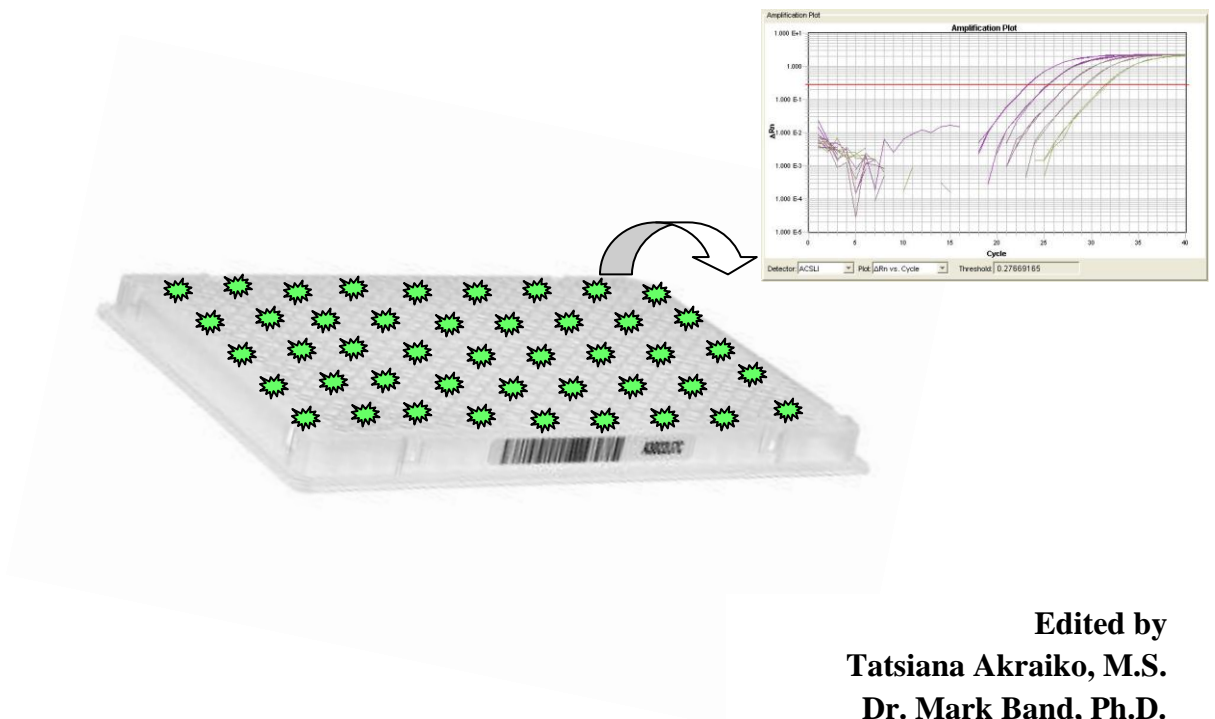




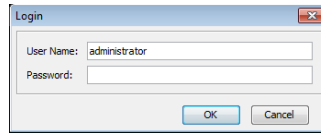
W.M. Keck Center 7900 HT Real-time Quantitative PCR Practical Operating Guide




W. M. Keck Center for Comparative and Functional Genomics
at the University of Illinois at Urbana-Champaign
Functional Genomics Unit

REAL-TIME PCR USER INSTRUCTIONS:

1. Seal your plate well. Make sure that the plate does not have any adhesive, or film protruding beyond the perforation. The robot cannot position sticky plates correctly inside of the instrument.
2. Spin your plate down in the centrifuge. Use Program #2, which was created specifically for spinning all real-time plates. In order to change centrifugation programs, you need to click RCL, press number 2, Enter and Start.
3. After centrifugation, start the SDS software by clicking on the SDS 2.4 icon on the desktop.



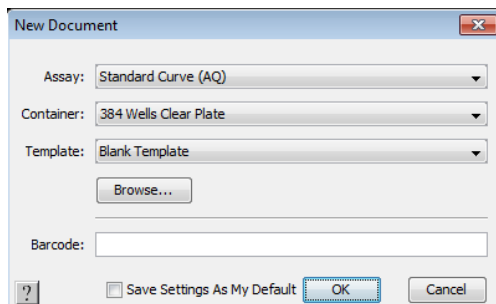
4. When the dialog box appears, just click . No password!

5. Create a new Plate Document within the SDS software by following the next steps:
 - a) Click  (or select **File > New**).
 - b) From the **Assay** drop-down list select the appropriate type of assay for your run. The majority of users prefer the **Standard Curve (AQ) Assay**, which allows adding the Dissociation Stage right after 40 cycles of PCR. In addition to that, when you create a Plate File for **Standard Curve (AQ)**, you do not need to enter information such as Sample Name, Calibrator Sample, etc. If you choose to run the **$\Delta\Delta C_t$ (RQ) Assay**, you will need to enter additional information before performing a run.

AQ – Absolute Quantification


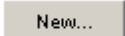
RQ – Relative Quantification

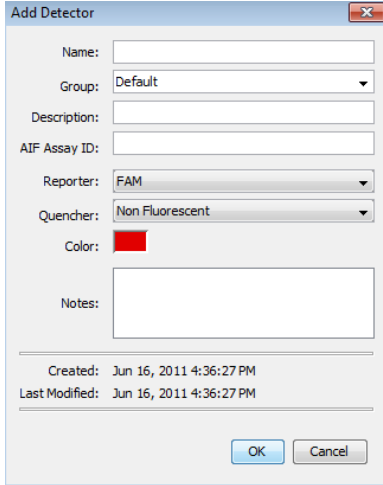
- c) **Container:** DO NOT CHANGE. Leave **384 Wells Clear Plate** selected.
- d) **Template:** Always select **Blank Template**.
- e) **Barcode:** Always scan the plate barcode for the overnight robotic run or if using the **Queue**. You may leave this field blank for a manual daytime run, when only a single plate is run. **PLEASE NOTE**, you **MUST** click in the barcode field first, and then scan the plate barcode. If you do not click the barcode field the assay type may change, for example, to Allelic Discrimination instead of Standard Curve (AQ). Check to make sure the barcode has been entered before proceeding.



- f) Click  after you configured the settings in the New Document dialog box.


6. Apply Detectors to a Plate Document

- a) Click  (or select **Tools > Detector Manager**).
- b) If you need to create a new detector, in the Detector Manager dialog box click . Enter a unique name for the detector, enter group name (PI Name), and select the appropriate reporter and quencher dyes for your assay. For a SYBR Green I assay, set the Reporter Dye to SYBR, and the Quencher Dye to Non Fluorescent.

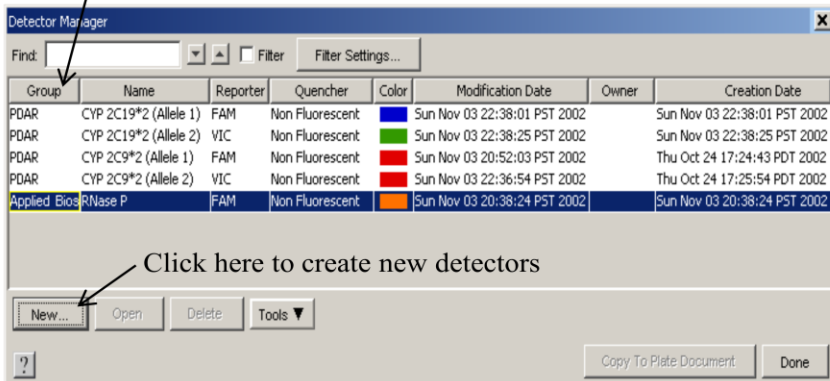


The 'Add Detector' dialog box contains the following fields and controls:

- Name:
- Group:
- Description:
- AIF Assay ID:
- Reporter:
- Quencher:
- Color:
- Notes:
- Created: Jun 16, 2011 4:36:27 PM
- Last Modified: Jun 16, 2011 4:36:27 PM
- Buttons: OK, Cancel


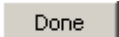
- c) Click . The software will save the new detector and display it in the detector list.
- d) In the Detector Manager dialog box, click on **Group**. This will allow sorting all detectors by Group Name. Select the detectors you want to apply to your plate document. You can select multiple detectors by pressing and holding the **Ctrl** key.

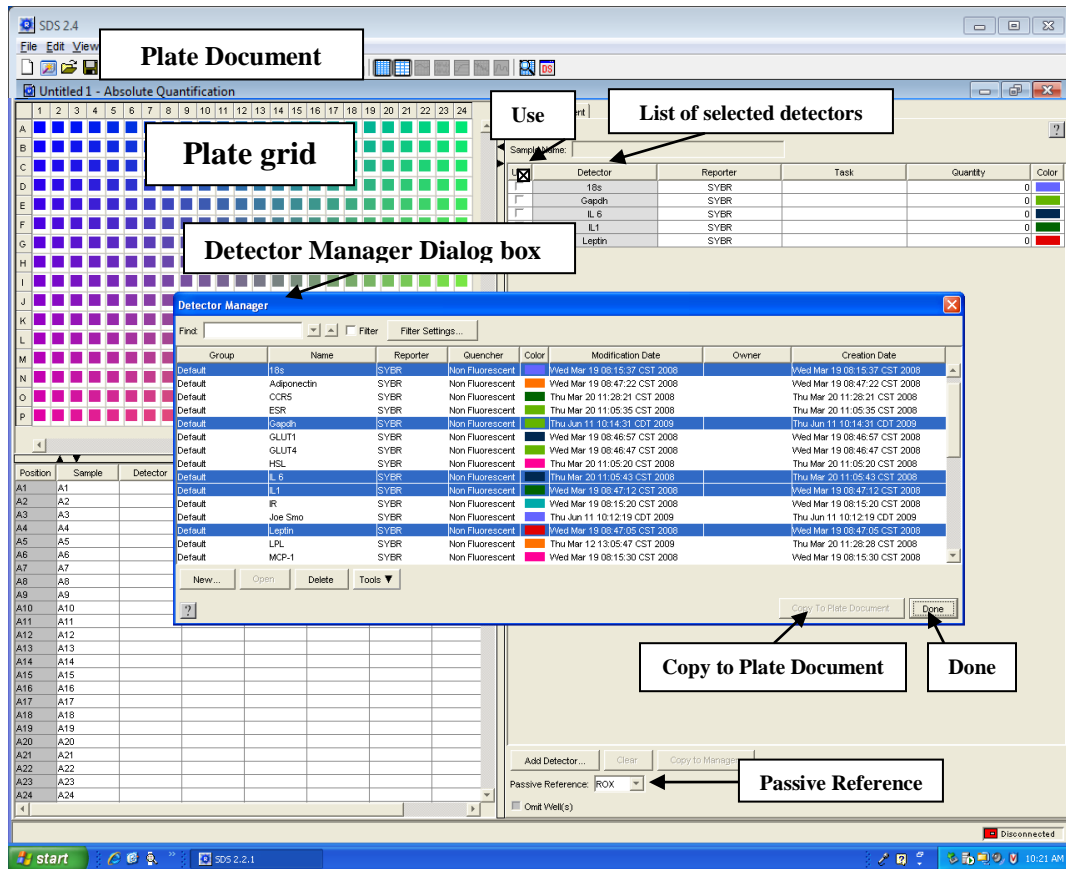
Click here to sort Detectors in alphabetical order





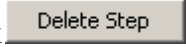

The 'Detector Manager' dialog box displays a table of detectors with the following columns: Group, Name, Reporter, Quencher, Color, Modification Date, Owner, and Creation Date. The 'Group' column header is highlighted, and an arrow points to it with the text 'Click here to sort Detectors in alphabetical order'. Below the table, the 'New...' button is highlighted with an arrow and the text 'Click here to create new detectors'. At the bottom right, the 'Copy To Plate Document' and 'Done' buttons are visible.

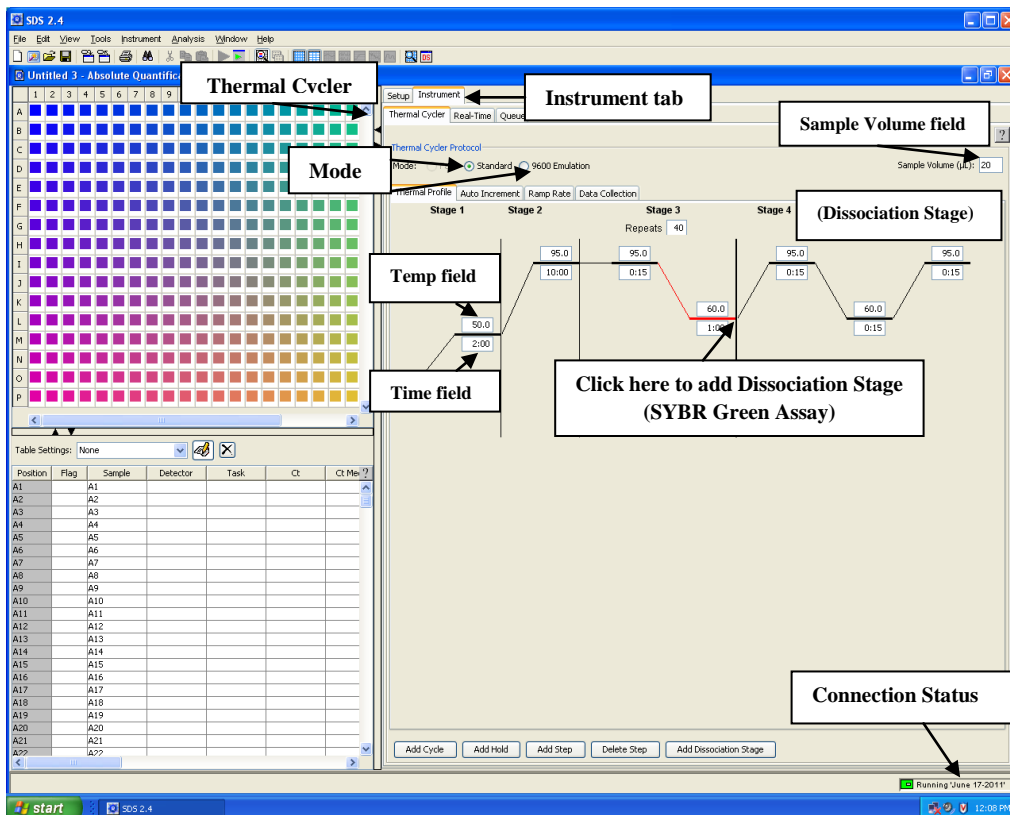
Group	Name	Reporter	Quencher	Color	Modification Date	Owner	Creation Date
PDAR	CYP 2C19*2 (Allele 1)	FAM	Non Fluorescent	Blue	Sun Nov 03 22:38:01 PST 2002		Sun Nov 03 22:38:01 PST 2002
PDAR	CYP 2C19*2 (Allele 2)	VIC	Non Fluorescent	Green	Sun Nov 03 22:38:25 PST 2002		Sun Nov 03 22:38:25 PST 2002
PDAR	CYP 2C9*2 (Allele 1)	FAM	Non Fluorescent	Red	Sun Nov 03 20:52:03 PST 2002		Thu Oct 24 17:24:43 PDT 2002
PDAR	CYP 2C9*2 (Allele 2)	VIC	Non Fluorescent	Red	Sun Nov 03 22:36:54 PST 2002		Thu Oct 24 17:25:54 PDT 2002
Applied Bios	RNase P	FAM	Non Fluorescent	Orange	Sun Nov 03 20:38:24 PST 2002		Sun Nov 03 20:38:24 PST 2002

- e) Click . The selected detectors will be added to your Plate Document. Click  to close the Detector Manager.




- f) In the plate grid, select the wells for the first detector. Use the **Ctrl** and **Shift** keys to select wells either individually or in groups. After that, click the check box for the corresponding detector in the **Use** column. Repeat this step to apply remaining detectors to all used wells on your plate.
- g) If you need to enter the plate bar code at this point (see step 5e), click **Tools > Document Information > Barcode**. Scan the plate bar code, and click .
- h) Select the appropriate **Passive Reference** from the drop-down list. If you are using an *Applied Biosystems* chemistry (SYBR Green or TaqMan), use the default reference dye, which is ROX. If you are using Master Mixes manufactured by any other company, make sure to select the correct reference dye.

7. Customize Thermal Cycler Conditions and Volume Settings for your experiment
 - a) Select the **Instrument tab** of the plate document and click on **Thermal Cycler**.
 - b) Select **Mode: Standard** (default) or **9600 Emulation**. **9600 Emulation Mode** matches that of the older ABI Prism 7700 instrument. We recommend that all new users select the default **Standard Mode**. Once you start your study using either mode, it is not recommended to switch from one to the other.
 - c) If you want to adjust any PCR parameter, you need to select it and enter a new value.
 - d) Click the step after which you want to modify Thermal Cycler Conditions. Click  , or  if you want to add a new stage or a new step respectively.
 - e) If you want to remove the step, highlight it and click  .
 - f) For a **SYBR Green Assay** performing a **dissociation curve analysis** is necessary. Click the step to the left of the stage you want to place the Dissociation Stage (after Stage 3, see picture below), and click  . The Dissociation Stage will be added to the PCR program.
 - g) Click the **Sample Volume (µL)** field and enter the correct reaction volume. All wells of your plate must have the same reaction volume.
 - h) You can customize the default temperature, ramp and data collection points according to your assay. The default settings are optimized for assays designed with the Primer Express program, or assays purchased from ABI.

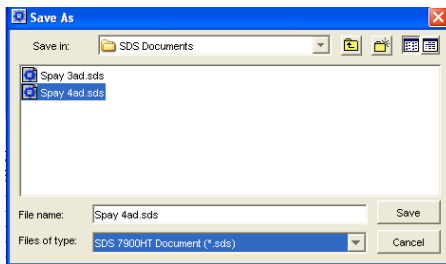


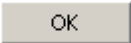
8. Saving the Plate Document

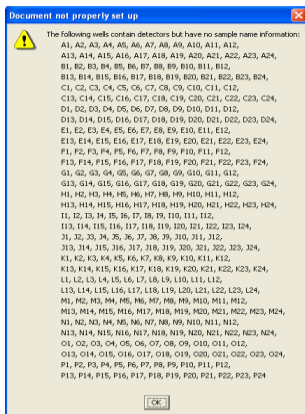
- a) Select **File > Save As**
- b) Save the file in your folder on the D drive. D:/AppliedBiosystems/SDS Documents/Your Folder. Please do not save your files in any other directory on the computer. Save the file as an SDS 7900HT Document (*.sds).


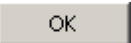
- c) In the File Name field enter a file name for the plate and click .

NOTE: File names should not be long, or have any characters such as: @, %, \$, *, !, /, +, &, (), etc.



- d) When you get this message below, click . It is a warning that sample names have not been entered, however this can be done after the run.

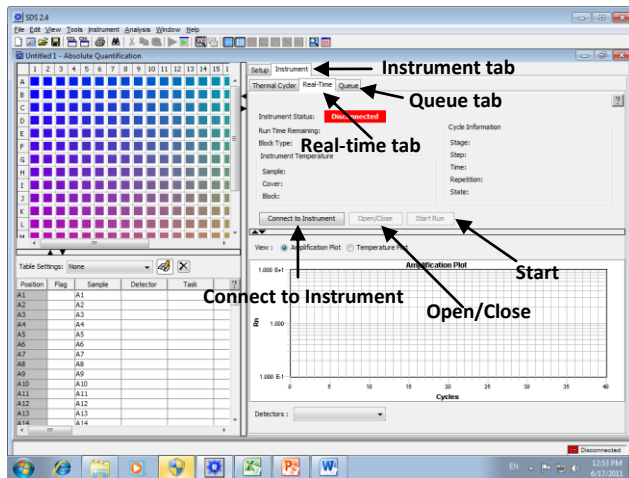
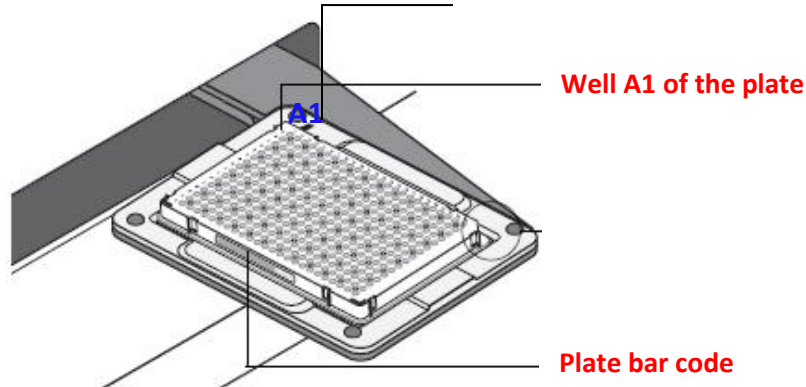


- e) Please do not create many subfolders within your folder. Excessive folders or very long filenames may corrupt the run.
- f) Please copy and delete your old files. Files that are more than one year old will be deleted without notification in order to save disk space.
- g) If you want to save the plate document as a template, and use it for a series of plates with identical **assay configurations**, you need to select **File > Save As**. In the **Files of type** field select SDS 7900HT Template (*.sdt). Click . For running the plate using the template file, you need to open a template file (*.sdt) with SDS 2.4 software and **Save it As** an SDS 7900HT Document (*.sds). Note, configure the new plate document, and enter the plate bar code by clicking **Tools > Document Information > Barcode** field. Click .

9. Running the Plate on the 7900HT Instrument

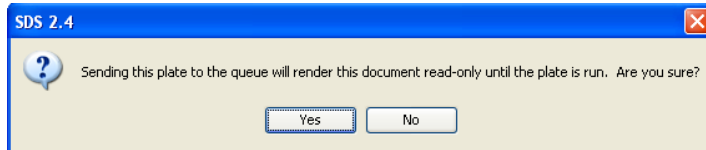
- a) To run the plate **manually** (during the daytime) create and save the file. Select the **Instrument tab** of the plate document. In the **Real-time tab**, click **Connect to Instrument**. The connection status box in the lower right hand corner should turn green. Click **Open/Close**. When the instrument tray rotates to the OUT position, place your plate as shown below.

- b) Click **Start**. **The A1 position on the instrument tray**

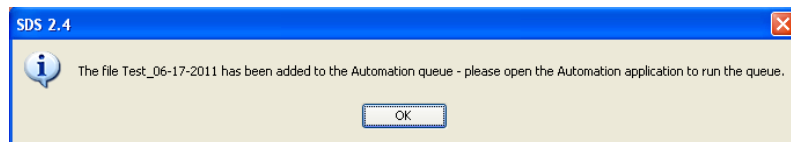


- c) To run the plate using the loading robot as a part of a group (**overnight run**), in the **Instrument tab** select the **Queue tab**. Click **Send To Queue**.

- d) Click **Yes** when you see the following message:



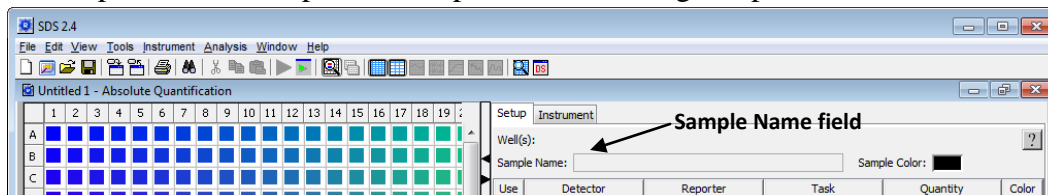
- e) Click **OK**.



After the run

10. Applying Sample Information

Open the SDS file. If you want to name your samples, in the plate grid select the replicate wells containing the first sample, and click the **Sample Name** field. Enter the sample name, and press **Enter**. Repeat this step for all remaining samples.

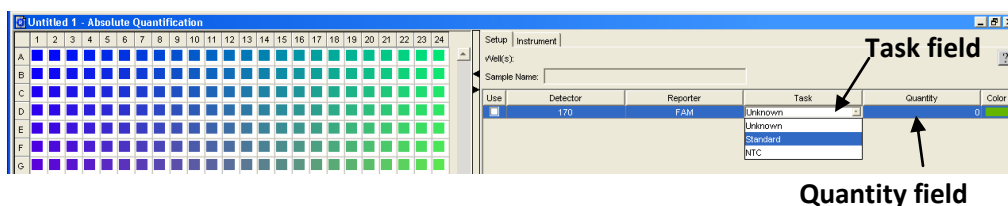


11. Assigning Tasks (Unknown, Standard, NTC) to the detectors

- Select the wells containing samples for a particular task, and click in the **Task** field. From the drop-down list select the appropriate task for the selected wells. Tasks are described in the table provided below.

Table 3-1 Detector Tasks of the SDS Software *Applied Biosystems User Guide (P/N 4351684, Rev A)*

Experiment	Task	Apply to...
Allelic Discrimination	Unknown	U all detectors of wells that contain PCR reagents and test samples.
	NTC	N all detectors of negative control wells that contain reagents for the PCR, but lack template.
Absolute Quantification	Unknown	U all detectors of wells containing PCR reagents and test samples for quantification.
	Standard	S the appropriate detectors of wells that contain PCR reagents and samples of known quantities. (See page 3-17 for instructions.)
	NTC	N all detectors of negative control wells that contain PCR reagents, but lack template.
Relative Quantification	Target	T all detectors of wells that contain PCR reagents for the amplification of target sequences.
	Endogenous Control	E all detectors of wells that contain reagents for the amplification of the endogenous control sequence.



- To create a standard curve for quantification of unknown samples, you need to select all wells containing the first standard, and then select **Standard** from the drop-down list in the **Task** column. After that, you need to assign quantities to the standard wells, by entering values in the **Quantity** field. Press **Enter**. Repeat this step for all standards on your plate (endogenous control, target transcript 1, target transcript 2, and so on).

NOTE: By using the same stock RNA or DNA to prepare standard curves for multiple plates, the determined quantities can be compared across all plates.

12. Analyzing the Real-Time Data


- a) Open your SDS file.
- b) Configure the analysis settings.
 - I) Select **Analysis**, and click **Analysis Settings**.
 - II) In the **Analysis Settings** dialog box, in the **Detector** drop-down list select either individual detector or **All Detectors**.
 - III) Select the method for data analysis: **Automatic Ct**, or **Manual Ct**.

If you choose to use the **Automatic Ct** method (default), the SDS software will calculate baseline and the threshold values automatically for either all detectors, or for each individual detector depending on what option was selected in step II.

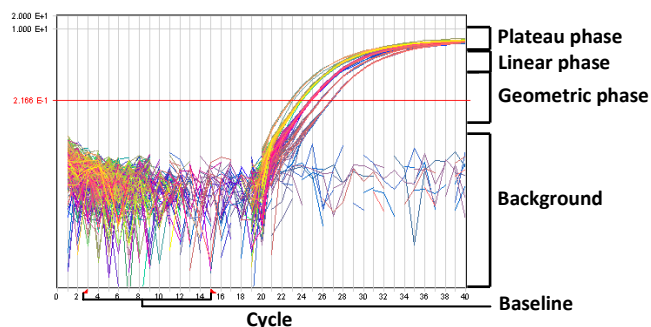
NOTE: Verify that the baseline and threshold were generated correctly. For this, check the amplification curves for each detector and make sure that the threshold is set within the geometric phase of PCR, and above the background (see the figure below).

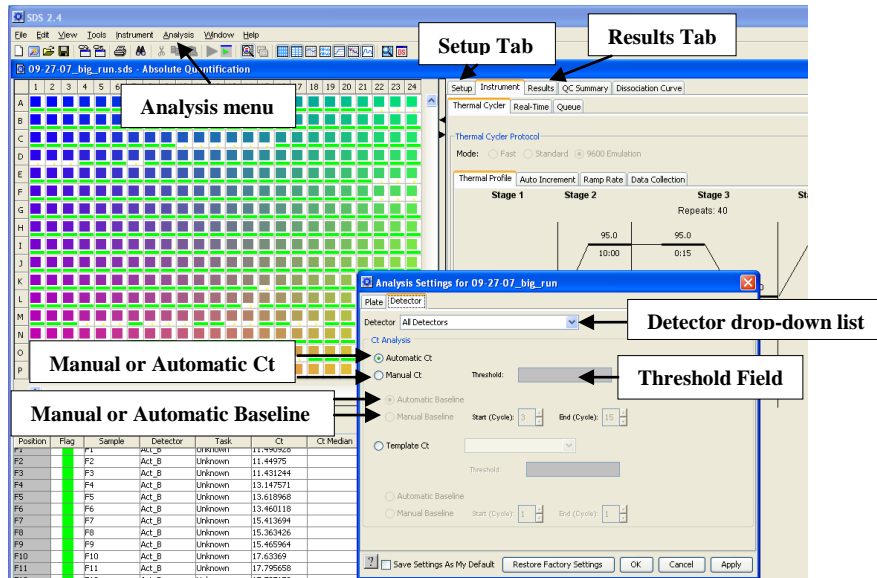
If you select the **Manual Ct** option, you can manually set the threshold for your detectors by entering a threshold value (start from 0.2) in the Threshold Field. After that, make adjustment by dragging and moving the threshold line (green) along the amplification plot. If you select **Manual Baseline** option, enter **Start** and **Stop** cycle values. Set the baseline for either all detectors or for each individual detector so that the amplification curve growth begins at a cycle number greater than the **Stop** baseline cycle. This is especially critical if your gene of interest is highly expressed (for example, Ct value is 8). If the baseline is set spanning cycle 3 to cycle 15, the SDS software will split the amplification curve into 2 parts, and you may get a Ct value of 15 instead of 8, the correct value. Make sure the baseline is set to end before the amplification curve starts to rise.

In general the automated function works very well and is the simplest to use. You can analyze your data without configuring the analysis settings. In this case, the software will use the default settings: **Automatic Ct** and **Automatic Baseline**.


Click  to accept the selected analysis settings and exit.

Typical Amplification Curve ([Applied Biosystems User Guide \(P/N 4351684, Rev A\)](#))






IV) Analyze the run data.

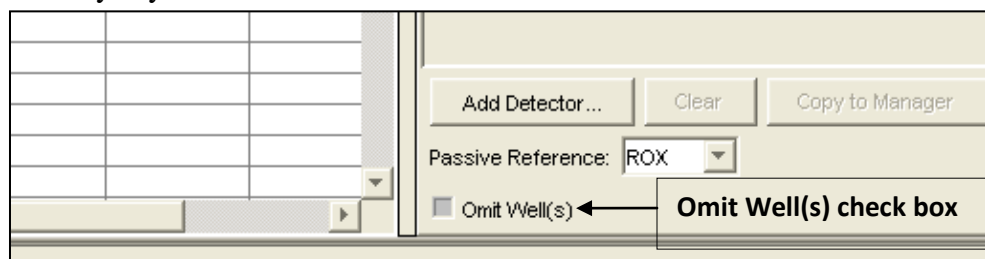
Click , or select **Analysis> Analyze**. The SDS software analyzes your data and displays the results in the Results tab. Check the results and choose either the **Automatic Ct** or **Manual Ct** method (as described in step III), click



V) Remove Outliers.

You can omit wells from use before or after the run. If you omit wells before running a plate, data from these wells will not be collected during the run. Therefore, it is recommended to exclude either empty wells, or wells with outliers from the analysis after you run a plate.

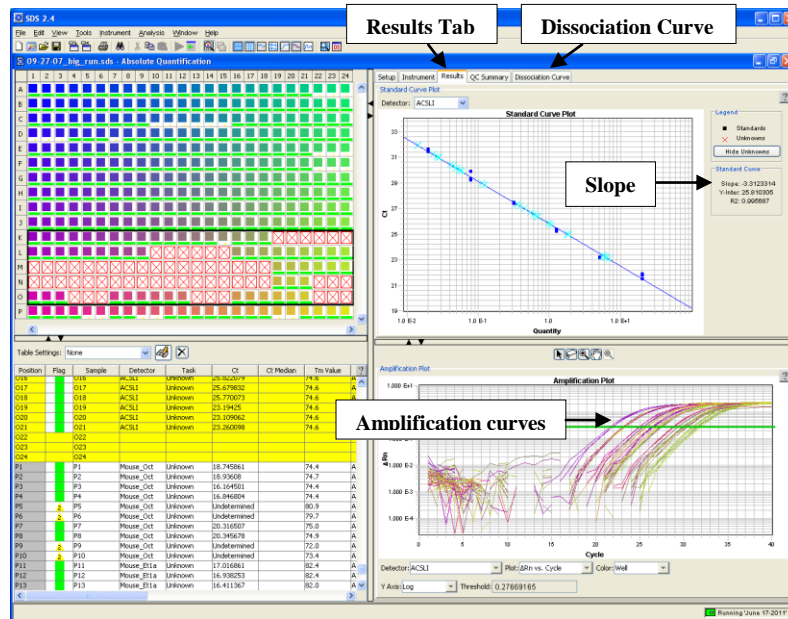
- Select the **Results Tab**
- Visually inspect technical replicates of the same sample by selecting them in the plate grid. These wells should produce similar Ct values. If Ct value in one of the wells significantly differs from the rest of the replicate wells, this well should be removed from the data analysis. To delete wells, in the plate grid select the well(s) that you want to omit, and select the **Setup Tab**.
- Click the Omit Well(s) check box near the bottom of the document. Omitted wells are marked .
- Reanalyze your data.



VI) Viewing the Standard Curve

If you ran an Absolute Quantification Assay and assigned quantities to the standard wells as explained in step the **11b**, the Results Table in addition to Ct values will have Quantity values. The software calculates a standard curve from Ct values of the diluted standards and extrapolates quantities for unknown samples based on their Ct values.

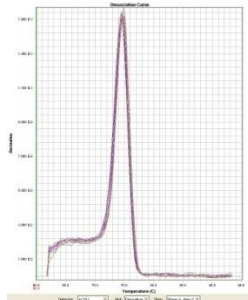
Click the **Results** tab, and view **Standard Curve** and **Amplification Plots**.



NOTE: The **Slope** of the standard curve provides information about the efficiency of the assay. If your assay is 100% efficient, the slope should be **-3.33**.

VII) Dissociation Curve Analysis (Only for SYBR Green Assay)

In order to see dissociation curve data, you need to click the **Dissociation Curve** tab. The tab displays the data from the selected wells of the plate grid. The data appears as a graph of the negative of the derivative ($-R_n$) versus temperature (T), and it visualizes the change in fluorescence at each temperature interval during the ramp. It is used to screen for non-specific amplification products.



This figure displays a single peak with a T_m of 74° C. This is an example of a specific amplification in the cDNA sample. If you see more than one peak, it indicates either primer-dimer formation, or non-specific amplification (more than one amplicon is detected).

13. Saving the Run Data

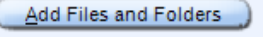

After the run, you **MUST** save the results to your CD or DVD. Files that are more than one year old will be deleted without notification.

a) Insert your disk.

b) Click on  icon.

c) Select “Data Disk”



d) Click on , find your file (D:/AppliedBiosystems/ SDS Documents/Your Folder), and after that click on .



NOTE: When the instrument is running, please minimize work on the computer (DO NOT CLOSE THE SDS FILE THAT IS IN USE).

14. The SDS 2.4 software can be downloaded from the ABI website

www.appliedbiosystems.com. **Support>Software Downloads>Applied Biosystems 7900 HT Fast Real-time PCR System>SDS Software v2.4 for 7900HT Fast.**

Please analyze the data in your lab.

15. You can export most plots (Dissociation Curve, Amplification, and Standard Curve) as

JPEG files. Right click the plot that you want to export, and select **Save Plot to Image File** from the menu. In the **Save As** dialog box, select your folder as the designated

directory, and enter the file name. Click .

a) To export analyzed run data as a tab-delimited text file, which is compatible with most spreadsheet applications, you need to click **File>Export**. Select the directory to which you would like to save the exported file, and select the type of data you would like to export. Enter a name for your file in the **File Name** text box, and click **Export**. You can open the exported file on your PC with Excel and continue data analysis.

b) If you have any question please contact the Functional Genomics Staff.

REFERENCES:

1. Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide. P/N 4351684 Rev. A.
2. Real-time PCR Systems Chemistry Guide. P/N 4348358 Rev. B.
3. www.appliedbiosystems.com