Data Analysis on the ABI PRISM® 7700 Sequence Detection System: Setting Baselines and Thresholds

Overview

In order for accuracy and precision to be optimal, the assay must be properly evaluated and a few adjustments need to be made.

There are three important parameters to be assessed:

- **Baseline**

- **Threshold**

- **Ct value**

Data Analysis Tutorial

To accurately reflect the quantity of a particular target within a reaction, i.e. the amount of PCR product, it is critical that the point of measurement be accurately determined. Real-time analysis on the ABI PRISM® 7700 Sequence Detection System involves three principle determinants for more accurate, reproducible data.

Baseline Value

During PCR, changing reaction conditions and environment can influence fluorescence. In general, the level of fluorescence in any one well corresponds to the amount of target present. Fluorescence levels may fluctuate due to changes in the reaction medium creating a background signal. The background signal is most evident during the initial cycles of PCR prior to significant accumulation of the target amplicon. During these early PCR cycles, the background signal in all wells is used to determine the "baseline fluorescence" across the entire reaction plate. The goal of data analysis is to determine when target amplification is sufficiently above the background signal, facilitating more accurate measurement of fluorescence.

Threshold

The threshold is the numerical value assigned for each run, which reflects a statistically significant point above the calculated baseline.

Ct Value

The Threshold Cycle (Ct) reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold. The Ct value assigned to a particular well thus reflects the point during the reaction at which a sufficient number of amplicons have accumulated, in that well, to be at a statistically significant point above the baseline.

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Baseline Setting

The first step of data analysis is to accurately set the baseline. By default, the software sets the baseline from cycles 3-15.
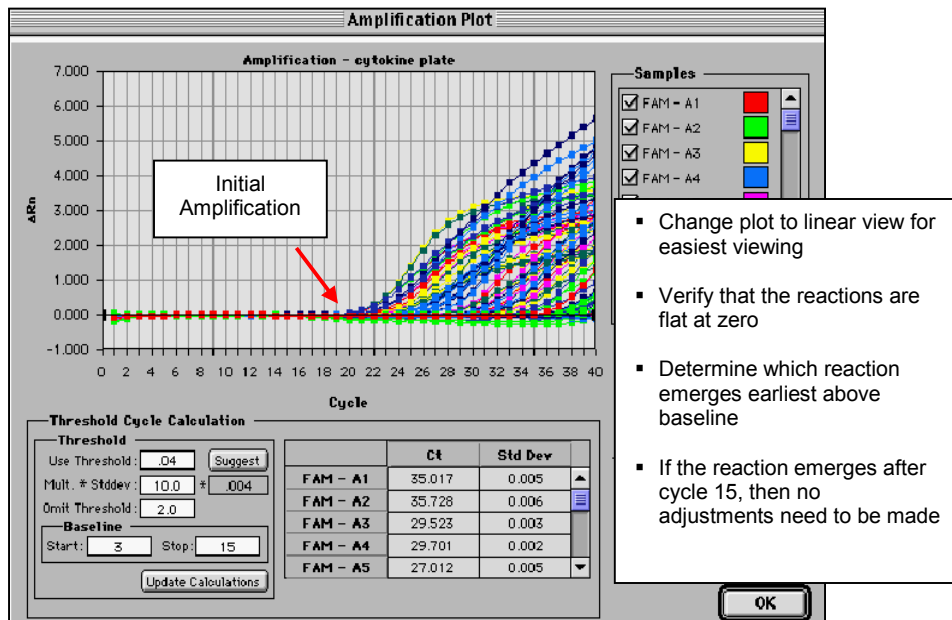
An example of this initial analysis is shown here:

Figure 1: Default Baseline
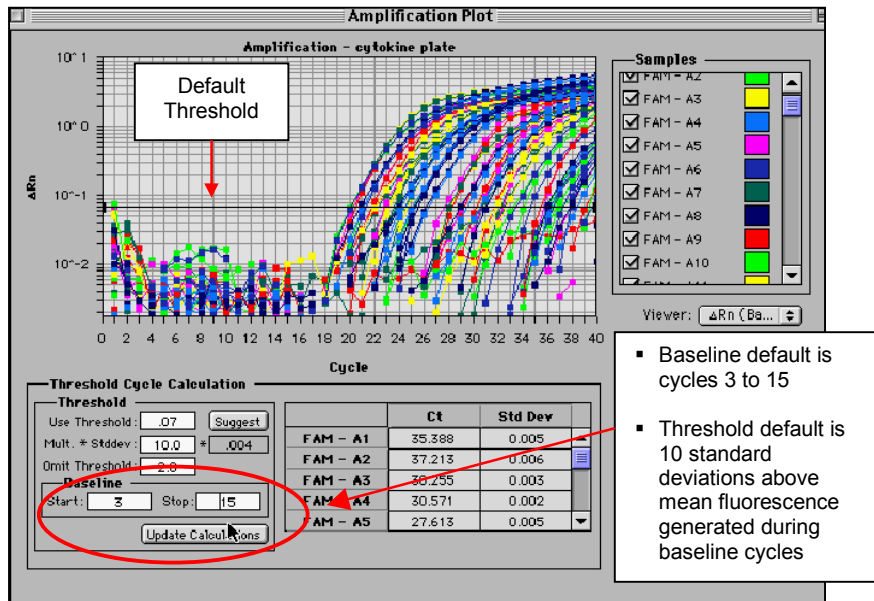
To establish if the baseline needs to be adjusted, it is important to determine which reaction in the assay emerges earliest above the baseline. In order to determine this, double-click on the y-axis and change the plot to Linear View.

Figure 2: Baseline Adjustment

If the first reaction emerges after cycle 15, then no adjustments to the baseline are necessary.
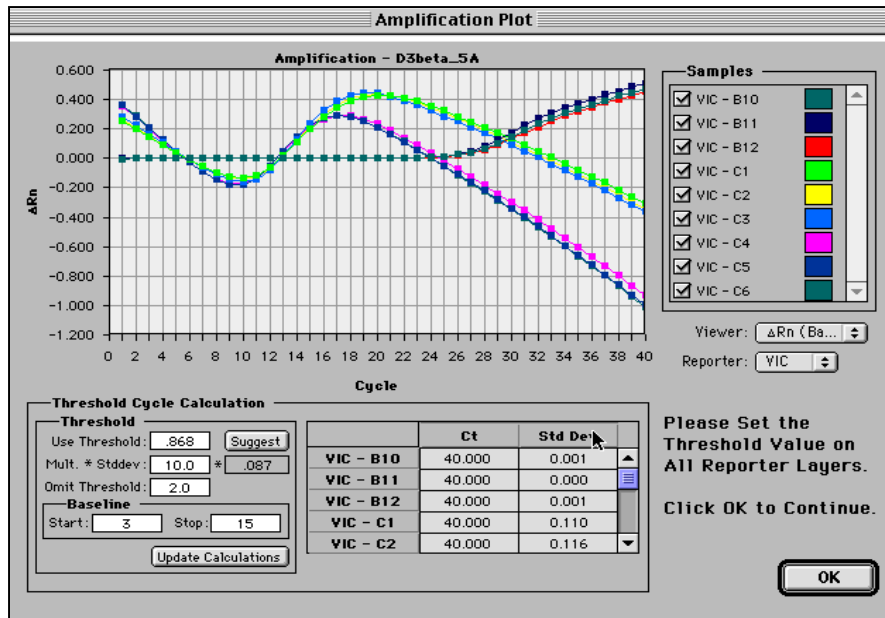
Adjusting the Baseline

A highly abundant target (i.e., 18s rRNA) will often amplify very early in the PCR cycles. In this type of assay, it is necessary to adjust the stop value of the baseline setting. As shown in the example below, the amplification plot displays the characteristics of a highly abundant target, and reflects the need to adjust the baseline.

Figure 3: Log View of Early Amplification
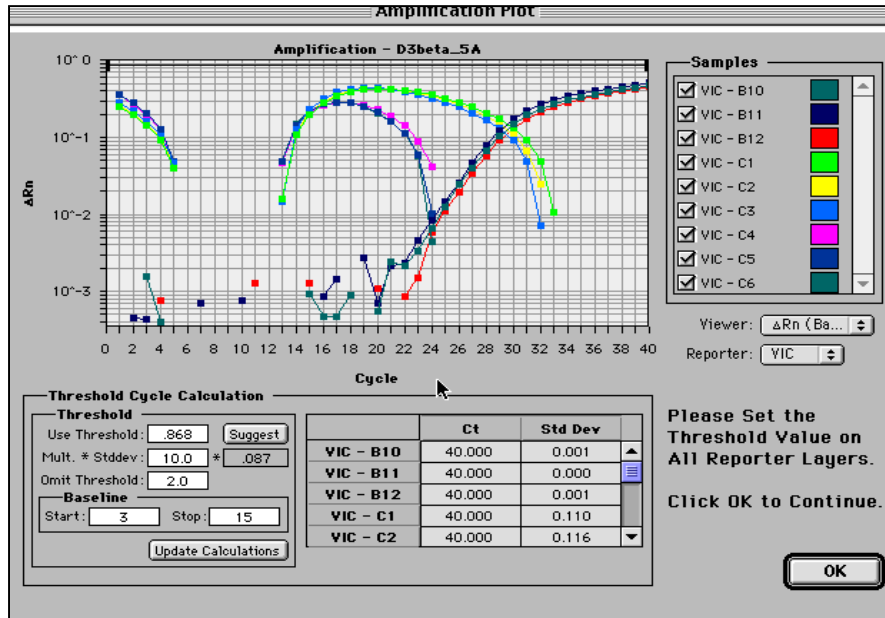
To determine the appropriate stop value, double-click on the y-axis and change the view to linear.

Figure 4: Linear View

For a quick check, adjust the Baseline stop value to six and hit the **Update** button. Next, determine the first cycle at which amplification is detected.

Adjust the stop value one to two cycles before the earliest amplification. In the example below the best stop value was determined to be at cycle 8.

Figure 5: Good Baseline Setting
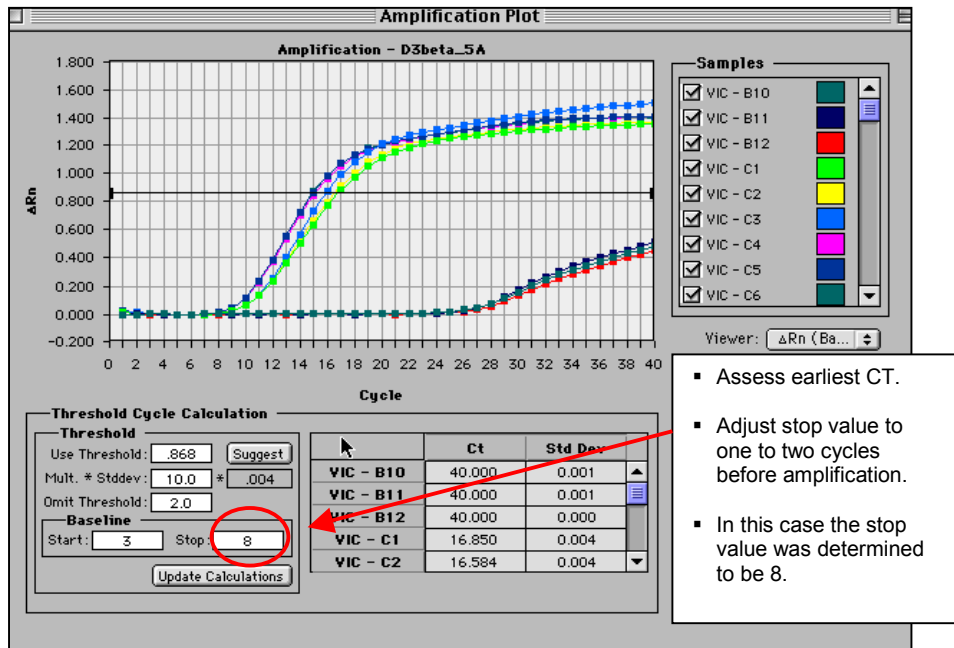
Double-click on the y-axis to return to log view.

Incorrect Baseline Settings
The following examples depict what an amplification plot will look like if the baseline stop value is set too high or too low.

Baseline Stop Value is Too Low

The settings in the example below were changed to cycles 3-8. Note that the plot begins to dip below zero around cycle 14. To fix a plot like this, extend the baseline stop back out to 15 and click on update calculations.

Figure 6: Baseline Setting Too Low
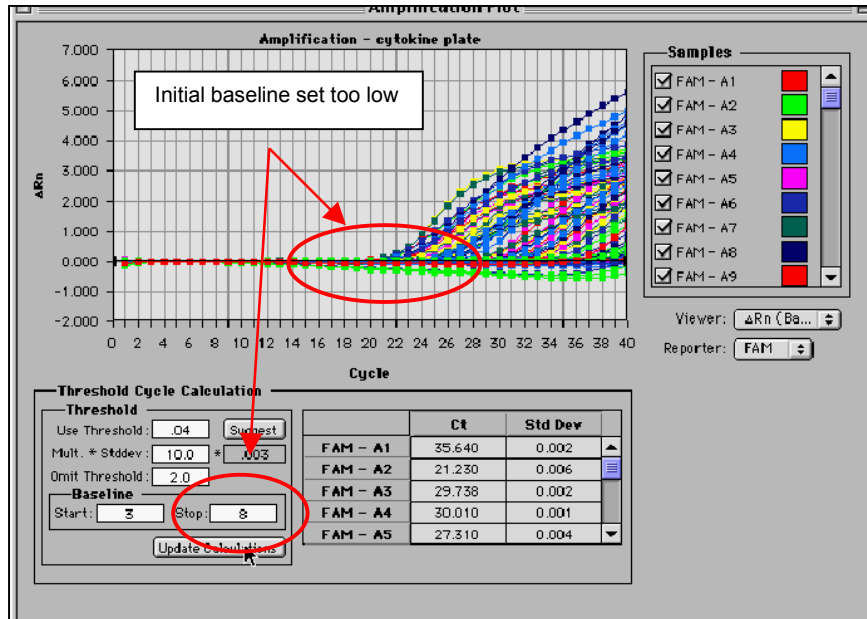
Baseline Stop Value is Too High

The settings in this plot were changed from 3-28. The fluorescence is not flat at zero; therefore the baseline stop is set to high.   To correct it, lower the baseline stop point to a value of 15. View the plot in linear view to assist in determining the best stop point.

Figure 7: Baseline Setting Too High

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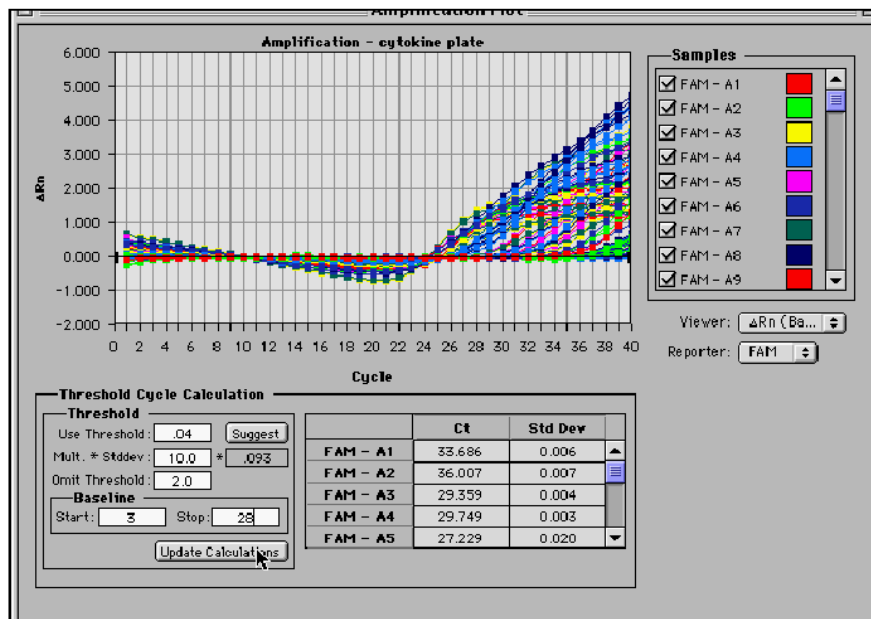
Threshold Value

Once the baseline has been set correctly, the software will automatically set the threshold at 10 standard deviations above the mean baseline fluorescence. At this point, the threshold can be adjusted manually by placing the cursor on the threshold line and dragging the line to the point on the amplification plot that follows these parameters:

1. Linear phase of exponential amplification.

The threshold should be placed in the region of exponential amplification across all of the amplification plots. This region is depicted in the log view of the amplification plots as the portion of the plot, which is linear. The threshold line should neither be placed in the plateau phase nor in the initial linear phase of amplification, which may be too low, and into the background fluorescence.

2. Precision maximization.

The threshold should be set at a point that maximizes the precision of replicates. It is common to find that the precision amongst the replicates increases as the amplification progresses further into the exponential phase of the reaction. Therefore, to assure maximal precision, the threshold value must be set within the exponential range above any background noise within the assay.

3. Sensitivity maximization.

The threshold should also be set to maximize the sensitivity of the assay. It is important that the threshold be placed at the point which best reflects all orders of magnitude in the assay(s) across the plate.

The example below depicts the ideal location for setting the threshold.

Note: There will usually be a "window" or range of values within which a threshold setting will fit the aforementioned criteria and yield optimum results.

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Figure 8: Good Threshold Setting
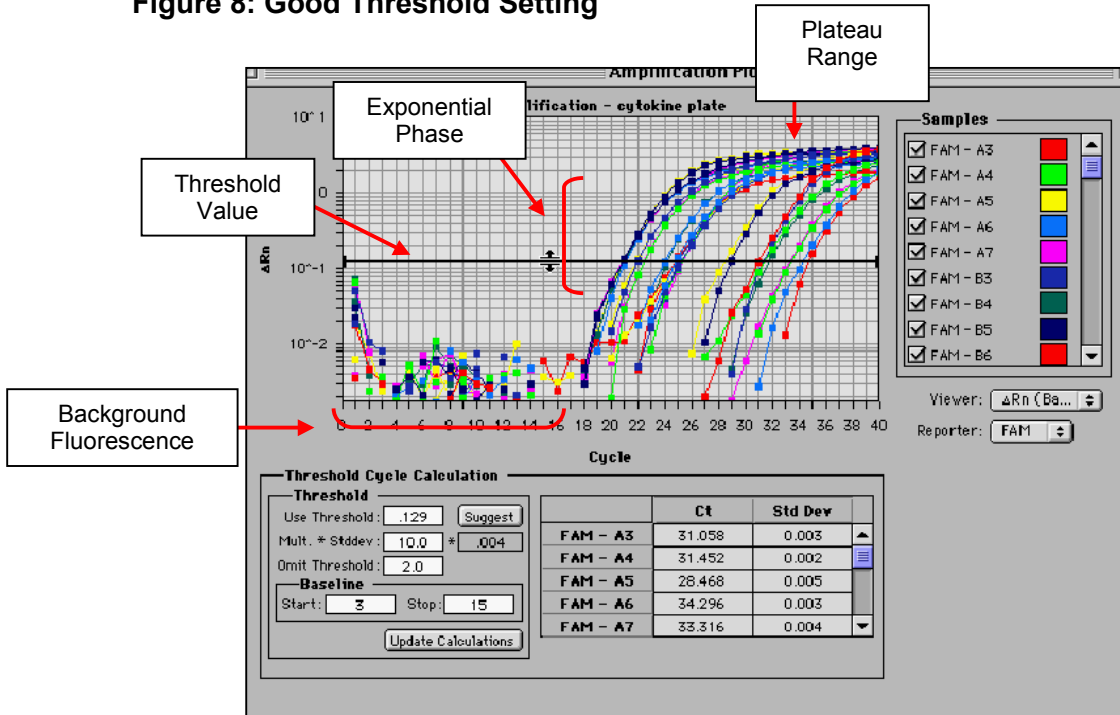
The Exponential phase of amplification is the most accurate place to take a measurement of the amplicon. The Exponential phase of amplification appears as the middle third of the reaction in the logarithmic view. The default numerical value assigned for the threshold depicts a value equal to a certain multiplier (default of 10) above the standard deviation of the mean baseline fluorescence. The default Threshold value may not be the best setting for a particular assay. There are two ways of determining the appropriate threshold setting: the standard curve method and the replicate method.

Standard Curve Method

The figures below depict the default setting for the threshold; it is too low and close to the background noise. The default threshold is also in a place where the replicates are not as tight. This may result in impaired precision for quantitative values
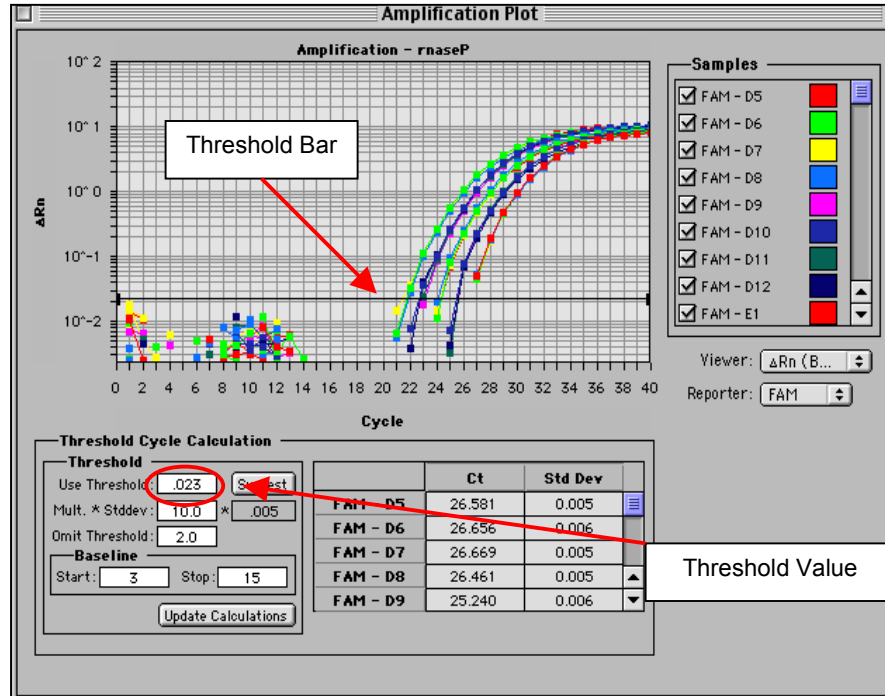
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Figure 9: Default Threshold Setting

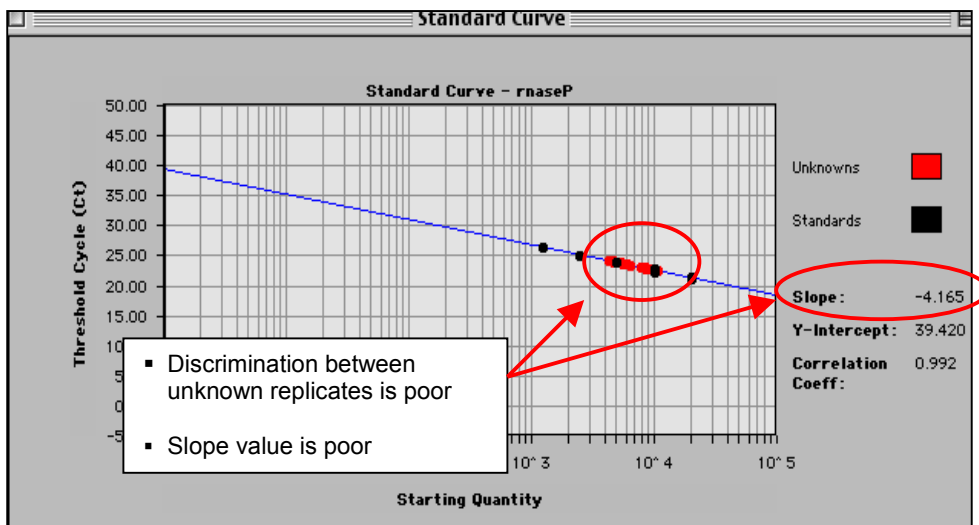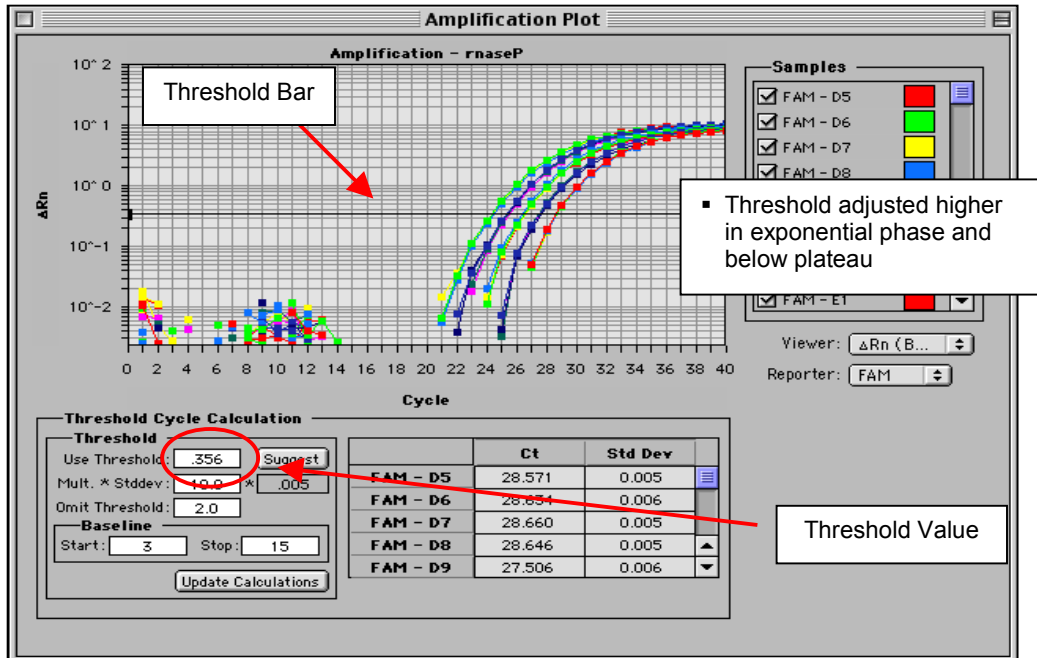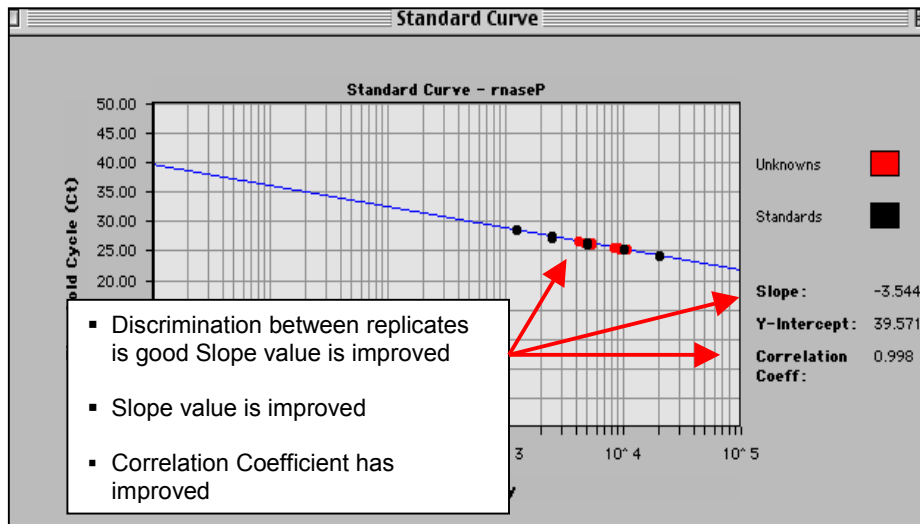**Figure 10: Corresponding Standard Curve Values**

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Figure 11: Adjusted Threshold

Moving the threshold higher in the exponential phase of amplification, as shown above, gave rise to more precise results.
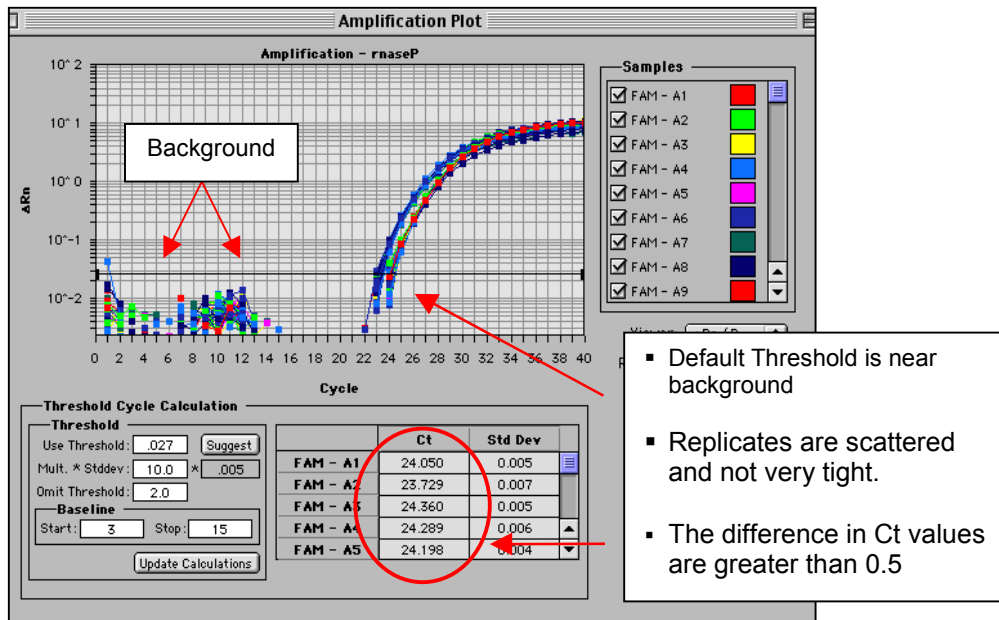
Figure 12: Adjusted Threshold

By adjusting the threshold, the Slope and Correlation Coefficient values have improved.

For Reference Only

Replicate Method:
When a standard curve has not been run, the replicate method can be used to properly set the threshold. In the figure below replicates of sample sets are being compared. Once again the default threshold is too low; The Ct's of the replicates are scattered a bit and the background is coming above the threshold.

Figure 13: Thresholds for Replicates

When the X-axis is expanded, it is easier to see just how spread apart the replicates are. The threshold can be easily set when viewed this way.

Figure 14: Expanded X-axis

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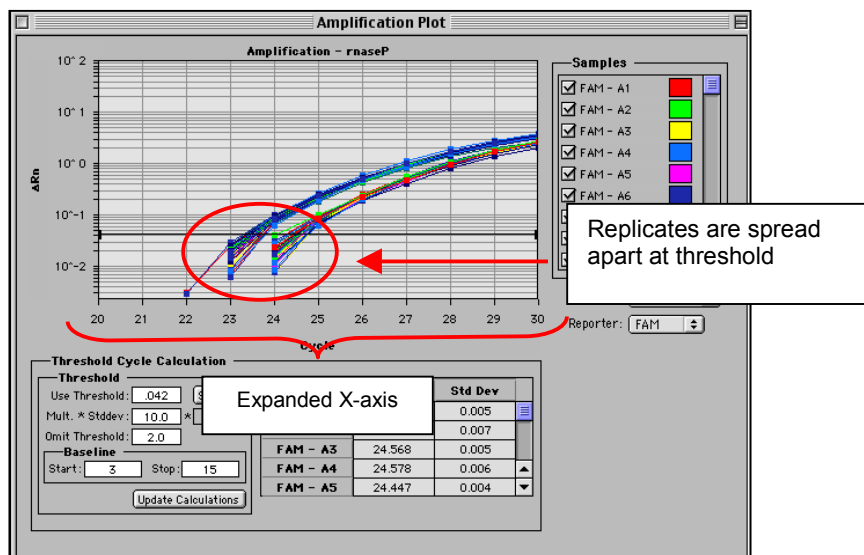
The threshold can be adjusted to improve the precision of the replicates. After adjustment, the Ct values of the replicates are much tighter.

Figure 15: Adjusted Threshold
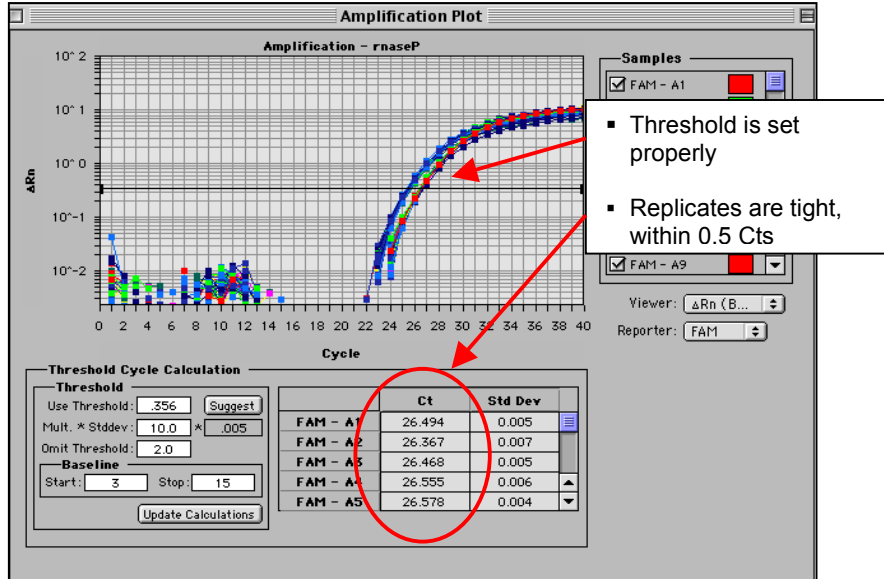
Data Analysis Summary
Step 1: Determine the correct Baseline Setting

Step 2: Set the proper Threshold

In most cases for a given assay (i.e. same primer and probe set) use the same threshold and baseline settings. Using the same settings from assay to assay will improve the precision and make the data more comparable from plate to plate and instrument to instrument.

▪ Setting appropriate Baselines and Thresholds will help to assess the quality of an assay

▪ If threshold and baseline settings are not helping with precision and accuracy, the assay may not have been properly optimized

Note: The threshold and baseline settings will not be saved. It is best to record the baseline and the numerical threshold and manually set them the next time the run is opened or another assay is performed.

Data Capture
After a run is complete, the data can be manipulated for presentation purposes.

After the appropriate settings have been determined, the Experimental Report, Amplification Plot, Standard Curve and other views can be printed out and filed.

The data can also be exported to Microsoft® Excel or an other spreadsheet program. When the Sequence Detection file is opened and all settings have been adjusted, go to File > Export > Experimental Report or Results. This will create a text file, which can be opened in programs like Microsoft® Excel and manipulated.

There are several tutorials on-line to help with some manipulations of data in an Excel like program.

Summary:
- The SDS software and instrumentation collects fluorescent data as amplicon accumulates.

- The data collected is displayed in an Amplification Plot that can be viewed in two ways: Linear and Logarithmic.

- The Threshold Cycle (Ct) is determined when the level of fluorescence from accumulating amplicon crosses the Threshold.

- Ct vs. Well position is a good way of determining outliers in your reaction.

- Standard curves can be used in the reaction to determine unknown quantities.

- The software displays the standard curve as well as the unknowns in the Standard Curve view.

- The quantities of the unknowns are determined automatically and displayed in the Experimental Report.

- Evaluating the Raw Data and Multicomponent View are ways to monitor the fluorescence in the reaction as the reaction progresses.

- Setting Baselines and Thresholds properly will increase the precision of a given assay.

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