Session 9

Introduction to qPCR Analysis Software on the ABI 7000 (& 7500)

Exemplar: the CAL DOJ Nuclear/Mito Duplex

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Overview of a qPCR Protocol:

1. PCR Set-Up 2. Run qPCR 3. Analyze

Combine qPCR master mix with primer/probe mix to make a master mix cocktail.

Aliquot 16 uL of cocktail into each sample well (or tube).

Add 4 uL of standard or unknown to each well (or tube); include a DNA standard dilution series (e.g., 25 ng to 50 pg) and one negative control per run.

Cover plate (or tubes) with optically transparent seal or caps.

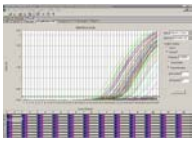
Set up the qPCR instrument (10min 95C; 45 cycles of 2-step qPCR (15s, 95C denature, then 60s, 60C anneal/extend)).

Load the samples into the real-time qPCR instrument, click "run," then come back in ~2 hours to **analyze data** (which takes a few minutes).

Goals of this Session

to become familiar with the basic steps in post-collection qPCR data analysis using the ABI Prism® 7000 Sequence Detection System (SDS)

to begin to determine the effects of various analysis parameters on a qPCR assay (e.g., the effects of changing the baseline settings, the effects of changing the cycle threshold, the use of manual vs. automated parameter settings)



The 7000 SDS Collection Software

used for both data collection and data analysis on the 7000 ("ABI Prism® 7000 SDS Software") three versions of the software:

- v. 1.0 - no smoothing of fluorescence data
  - Ct and baseline settings must be manually set
  - 'bug' in report printing (skips lines in printout, but Excel export works fine)
- v. 1.0.1 - same as v. 1.0, except report printing 'bug' is fixed
  - still downloadable(?) from ABI website
- v. 1.1 - non-optional smoothing algorithm of fluorescence data
  - Ct and baseline settings can be manually set or can be automatically set by the software

ABI supports v. 1.0 for the Quantifiler qPCR kits because it is what was used for ABI validations (we use v. 1.1, using manual Ct and baseline settings).

Now ABI offers v.1.2.3 for ABI 7000 and 7500 for forensic work (However CALDOJ currently uses v.1.3 not formally validated by ABI with Quantifiler kit)

The 7000 SDS Collection Software

You'll be using ver. 1.3 of the ABI 7500 Collection and Analysis Software – it is functionally very similar to ver. 1.1 of the ABI 7000 Software – but will analyze data from the 7000, 7300, and 7500 instruments.

For 7000 data, results are identical to ver. 1.1. (v.1.1 can analyze 7000 data, but not 7500)

Overview of a qPCR Protocol:

Basic Steps for Data Analysis (Manual)

Start ABI 7000 / 7500 SDS Collection software.

Open appropriate '.sds' data file (created from your qPCR run).

Select 'Setup/Plate' tabs to identify the DNA standard samples.

- Highlight DNA standard wells, then use 'Well Inspector' to label these samples by using the 'Task' tab.
- Use the 'Quantity' tab of 'Well Inspector' to enter appropriate template quantity.

Select 'Results/Amplification Plot' tabs to view data.

Set 'Manual Baseline' values ('Start (cycle)' and 'End (cycle)') to correct for assay-dependent variations in the background signal.

Set 'Manual Ct' (above baseline noise, but in exponential phase).

Press in menu bar to analyze all data using these settings and to generate quantifications for the unknown samples.

(Minimum steps required for analysis)

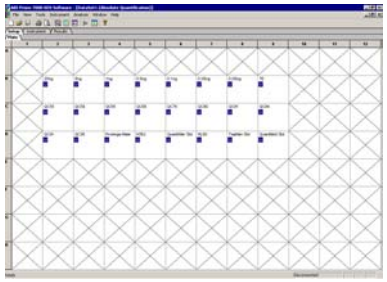
Example: Analysis of DataSet1

data obtained using nuTH01 nuclear qPCR assay (in duplex), from K. Swango's reproducibility/accuracy validation studies of nuTH01-mtND1 duplex qPCR assay (from file 030404a)

3.A. Open the qPCR (.sds) data file from the ABI 7000 software

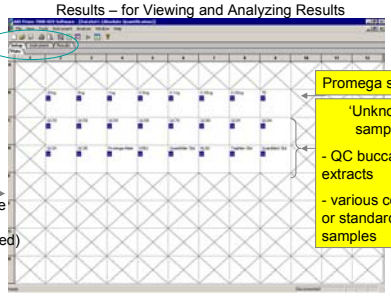
Example: Analysis of DataSet1

Open the ABI 7000 SDS (v. 1.1) software (use desktop icon)
Under 'File' menu, 'Open' the "DataSet1" .sds data file.



Example: Analysis of DataSet1

Tabs: Setup – for Sample Setup
Instrument – for Instrument Run Setup
Results – for Viewing and Analyzing Results



Promega std DNA

'Unknown' samples

- QC buccal swab extracts
- various control or standard DNA samples

omitted wells were "x'ed out" (undetected) prior to the run

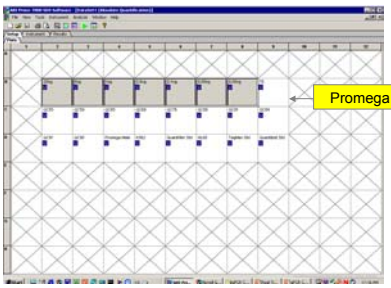
Example: Analysis of DataSet1

3.B. 'Task' the Calibration Standard DNA Samples for the Run, then Enter the Initial Template Quantities for the Standards

(NOTE: This step is often performed **prior** to the run. If, from run-to-run, the DNA standard samples are consistently run in the same wells, then this step can be further simplified by using a template plate document (.sdt) that contains default 'tasks' for the standards.)

Example: Analysis of DataSet1

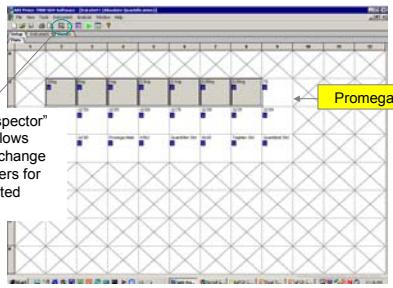
Highlight (e.g., click and drag) the DNA standard samples.
selecting sample wells is similar to selecting cells in MS ExcelTM



Promega std DNA

Example: Analysis of DataSet1

Use the "Well Inspector" icon to change the "Task" of the standard samples from Unknown (U) to Standard (S).

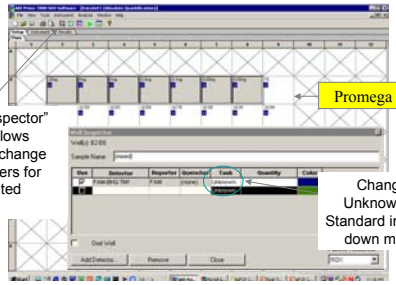


Promega std DNA

"Well Inspector" icon – allows user to change parameters for high-lighted samples

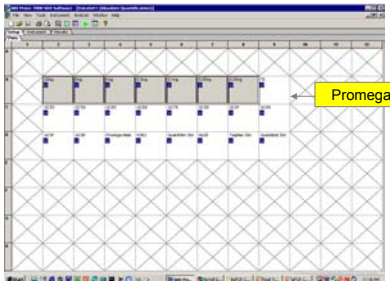
Example: Analysis of DataSet1

Use the "Well Inspector" icon to change the "Task" of the standard samples from Unknown (U) to Standard (S).



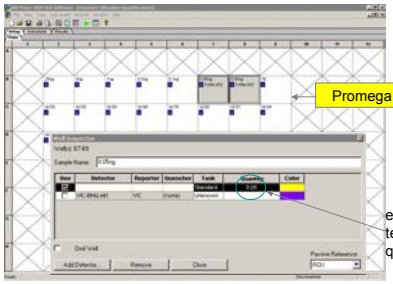
Example: Analysis of DataSet1

- Standards are now identified, and the next step is to specify the amount of template DNA in each sample.



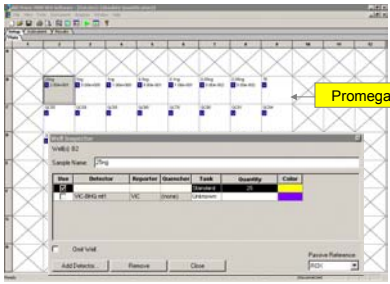
Example: Analysis of DataSet1

- Highlight standard samples and use the Well Inspector to enter the Quantity of template DNA in each standard sample.



Example: Analysis of DataSet1

- Repeat steps to use the Well Inspector to enter the amount of template DNA for each standard sample.

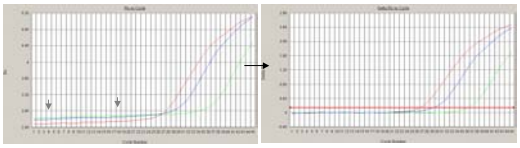


Example: Analysis of DataSet1

3.C. View the Amplification Plots and set the Threshold and the Manual Baseline Start and End values for analyzing the run.

Goals of step 3.C.:

- view data to identify any samples that might not have amplified properly (mainly to look at slopes of amplification plots)
- set the Baseline Start and End so that the 7000 software properly subtracts background fluorescence in the sample

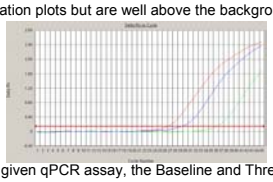


no Baseline correction (Rn) with Baseline correction (delta Rn)

Example: Analysis of DataSet1

Goals of step 3.C. (continued)

- set the Threshold so that the sample Ct's are in the exponential phase of the amplification plots but are well above the background noise

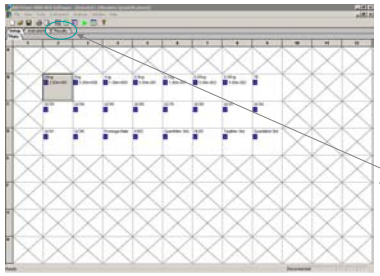


NOTE 1: For a given qPCR assay, the Baseline and Threshold parameters are usually quite stable, run-to-run, and are commonly set at suitable default values in the template plate document so that few (or no) manual changes are required at this step.

NOTE 2: Version 1.1 of the 7000 SDS software allows the program to automatically set the Threshold and Baseline values, although neither the ABI Quantifiler nor local validations use these features.

Example: Analysis of DataSet1

3.C. Specific Steps: Select the Results Tab to Examine the Amplification Plots and to set the Manual Baseline Start and End values and the Threshold value



Example: Analysis of DataSet1

- Select the sub-tabs to view the various Results sections .
- Select the 'Amplification Plot' sub-tab to set the analysis parameters.

Plate – results for the run ("Undet." because data is not analyzed yet)

Spectra – "raw" fluorescence per sample per detection filters A, B, C, D

Component – "raw" fluorescence plots (ROX ~ 700-1500 RFU)

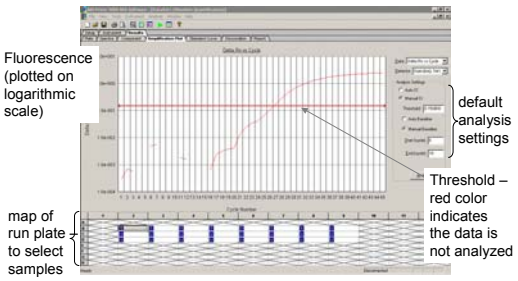
Amplification Plot – principal screen for setting analysis parameters

Standard Curve – calibration curve for the run

Report – displays selected results

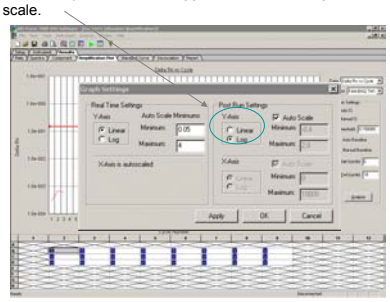
Example: Analysis of DataSet1

- Default view is logarithmic scale for y-axis.



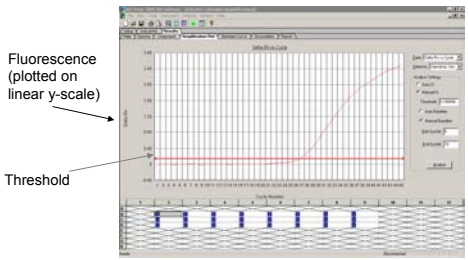
Example: Analysis of DataSet1

- Double-click on y-axis scale to toggle 'Post Run Settings' from log to linear scale.



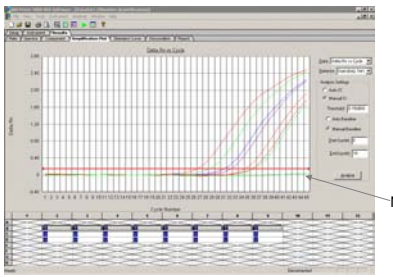
Example: Analysis of DataSet1

- View Amplification Plots for the samples. Look for amplification curves with unusual shapes, unusual slopes, or unexpected null amplifications. View the NTC for negative amp.`



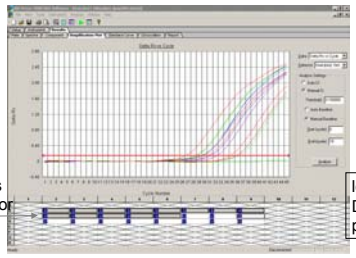
Example: Analysis of DataSet1

- View DNA standards – slopes of amp curves should be similar.
- View the NTC (TE-4) – expect null amp curve or very low copy.



Example: Analysis of DataSet1

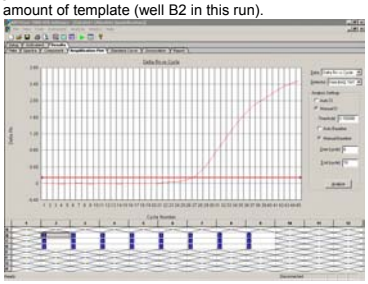
- View DNA standards. "CTRL-click" the unknown samples to visually compare the slopes of the amp curves for the unknowns to the slopes for the standard samples. Shallow slopes ➔ _____ ?



Example: Analysis of DataSet1

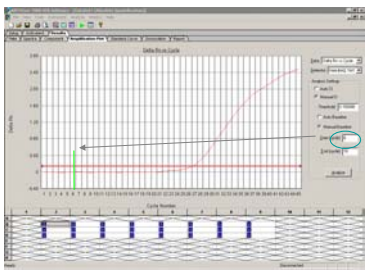
- Set the Baseline Start and End values.

First, highlight the well that represents the standard DNA sample with the highest amount of template (well B2 in this run).



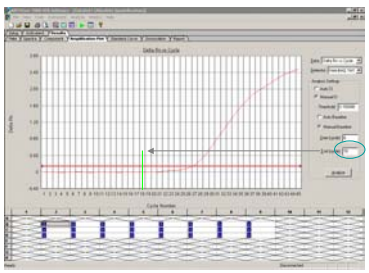
Example: Analysis of DataSet1

- Then set the Baseline Start at ~3-6 cycle numbers (avoid the first several cycles due to occasional noisy data).



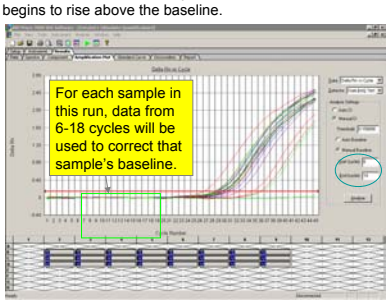
Example: Analysis of DataSet1

- Then set the Baseline Start at ~3-6 cycle numbers (avoid the first several cycles due to occasional noisy data).



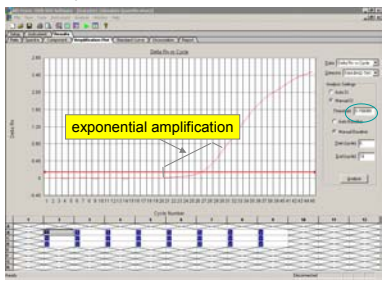
Example: Analysis of DataSet1

- Set the Baseline End value 2-3 cycle numbers before the amplification curve begins to rise above the baseline.



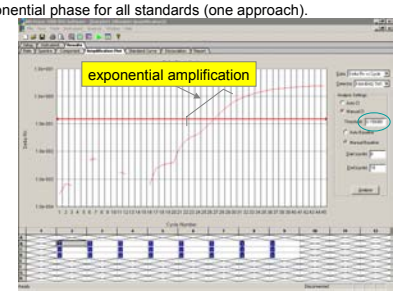
Example: Analysis of DataSet1

- Set the Threshold value for the qPCR run. The goal is for all amplification curves to cross the threshold in the exponential phase of amplification. Why in the exponential phase? _____



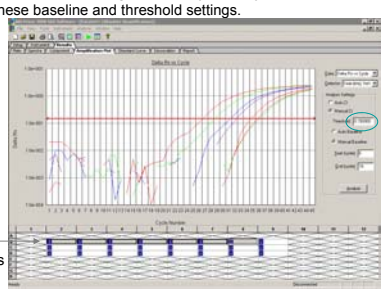
Example: Analysis of DataSet1

- Toggle the y-axis back to the logarithmic scale. The exponential phase is LINEAR when plotted on this scale. Set threshold in the middle of the exponential phase for all standards (one approach).



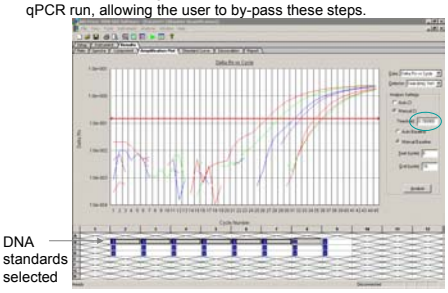
Example: Analysis of DataSet1

- Set threshold in the middle of the exponential phase for all standards (one approach) – looks good. Ready to analyze all of the data in the run with these baseline and threshold settings.



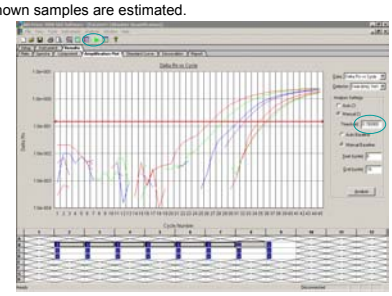
Example: Analysis of DataSet1

- Worth Repeating: Standard DNA sample values, as well as baseline and threshold settings, are often included in the template file for the qPCR run, allowing the user to by-pass these steps.



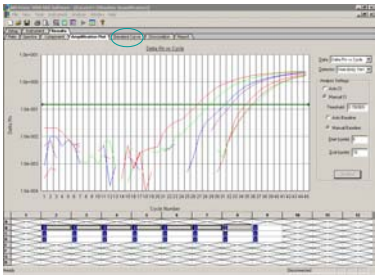
Example: Analysis of DataSet1

- 3.D. Analyze the data by clicking the green arrow or the Analyze button. This action calculates a standard curve from which DNA quantities for the unknown samples are estimated.



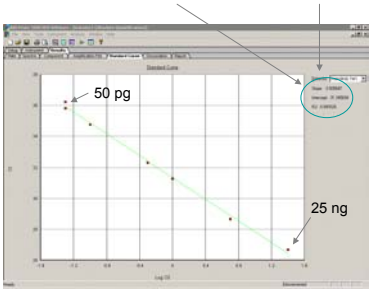
Example: Analysis of DataSet1

- Select 'Standard Curve' sub-tab under 'Results' tab to view and evaluate the standard (calibration) curve.



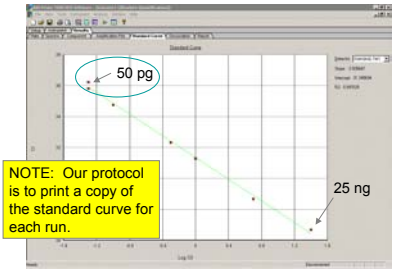
Example: Analysis of DataSet1

- Acceptable Standard Curve:
  Slope between –3 and –4 (or –4.5) R2 > 0.98



Example: Analysis of DataSet1

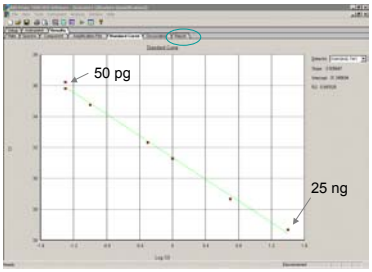
- If the standard curve is unacceptable due to an (obvious) outlier in the 50 pg samples, then the data can be re-analyzed by re-tasking that sample as an "Unknown."



Example: Analysis of DataSet1

- There are several ways to view the quantification results:

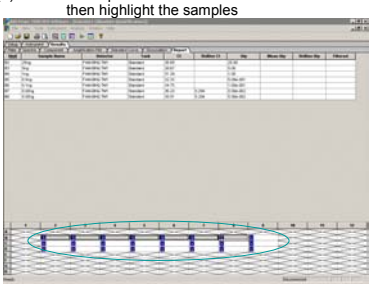
  (1) select the 'Report' sub-tab under the 'Results' tab -



Example: Analysis of DataSet1

- There are several ways to view the quantification results:

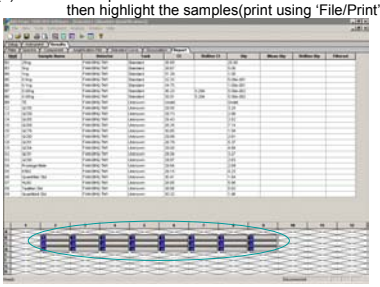
  (1) select the 'Report' sub-tab under the 'Results' tab - then highlight the samples



Example: Analysis of DataSet1

- There are several ways to view the quantification results:

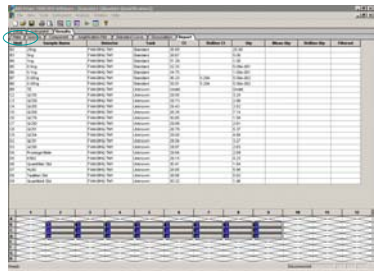
  (1) select the 'Report' sub-tab under the 'Results' tab - then highlight the samples(print using 'File/Print')



Example: Analysis of DataSet1

- There are several ways to view the quantification results:

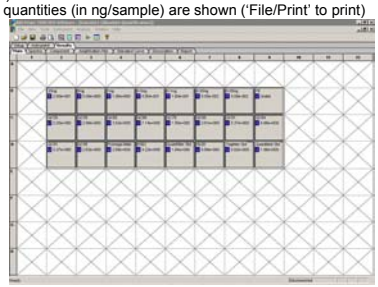
  (2) select the 'Plate' sub-tab under the 'Results' tab -



Example: Analysis of DataSet1

- There are several ways to view the quantification results:

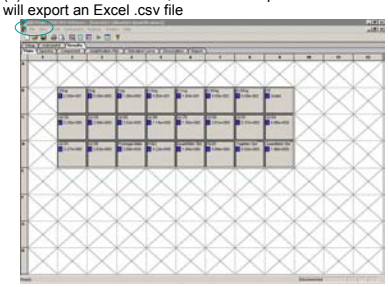
  (2) select the 'Plate' sub-tab under the 'Results' tab - quantities (in ng/sample) are shown ('File/Print' to print)



Example: Analysis of DataSet1

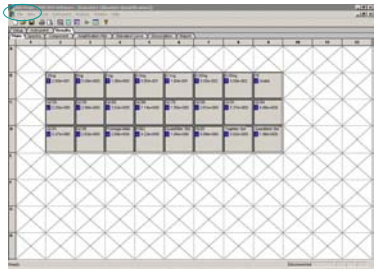
- There are several ways to view the quantification results:

  (3) Under the Main menu, select 'File/Export/Results' – this action will export an Excel .csv file



Example: Analysis of DataSet1

- Use 'File/Save As' to save the analyzed qPCR data, e.g., as "DataSet1Analyzed"



Example: Analysis of DataSet1 - Review

Launch the SDS collection/analysis software.

Open the appropriate .sds file for analysis.

If necessary, use 'Setup/Plate' tabs to identify standard DNA samples and to enter standard DNA quantities ('well-inspector').

Use 'Results/Amplification Plot' tabs to examine the data.

If necessary, set the Baseline Start and End values and the Threshold.

Analyze the data, evaluate the standard curve, print/view the quantification results. Use 'Save As' to save the analyzed file.