General Introduction to Data Analysis

The aim of this Technical Note is to explain the principle of absolute quantification analysis and to guide you through the experimental set up and analysis of data. It begins with a general introduction to data analysis.

Results Editor

The starting point for data analysis is the Results Editor. Before an analysis method has been set, the Results Editor home screen will display the plate layout and thermal cycling program. Each stage where readings have been made will have its own tab showing a graph of raw data.
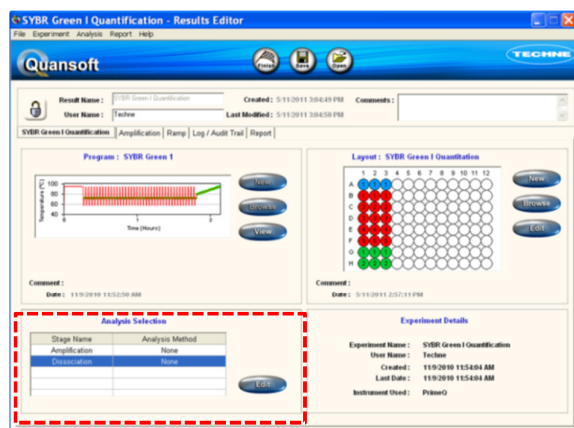

Figure 1: The Results Editor home screen.

Each stage where readings have been made will have a separate results tab enabling analysis of the data for that particular stage.

*The Analysis Selection box displays the stage name as assigned in the program setup. Only those stages that have been assigned with reads are displayed since stages without reads have no data to analyse. Double-click on a stage name, or highlight a stage name then click on **Edit** to launch the Analysis Wizard Selection screen.*

Selecting the Analysis Method

The Analysis Selection box on the Results Editor home screen allows the user to define the method of analysis to be applied to the readings gathered during the PCR run. Highlighting a stage name and pressing the Edit button will launch the Analysis Wizard Selection screen and allow an analysis method to be assigned for that stage.
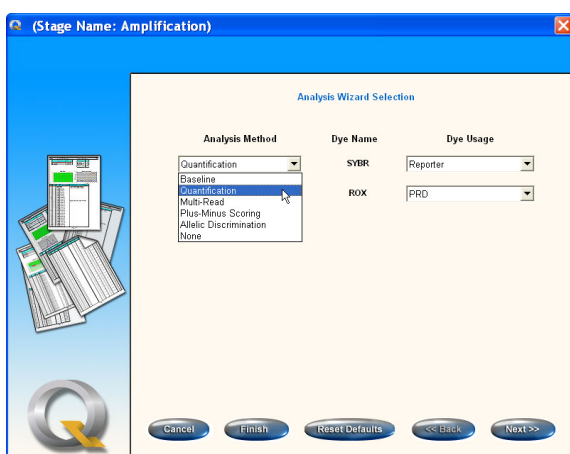

Figure 2: The Analysis Wizard Selection screen.

Analysis Method: *The drop-down menu lists analysis types appropriate to the selected stage. These will be dependent on the number of reads and the number of cycles programmed into the run.*
Dye name: *The name of the dyes selected in the program setup will be displayed.*
Dye Usage: *Assign a Dye Usage from the list in the drop-down menu. There will be one dye usage box for each read present in the stage.*
Cancel: *Aborts the procedure and takes the user back to the Results Editor.*
Finish: *Accepts all the default analysis settings for the analysis method chosen and closes the Wizard.*
Reset defaults: *Returns all analysis parameter settings to the defaults.*
Back/Next: *Allows the user to move between screens in the Analysis Wizards.*

The Analysis Wizards

Once an analysis method has been defined, a series of default settings will automatically analyse the data. The defaults can be viewed in the Analysis Wizards and edited if required. The intuitive Analysis Wizards explain in detail the mathematics behind the analysis settings and will lead you through each stage of the analysis setup. With experience, analysis can be quickly performed using the Analysis Properties displayed in the Parameters (PAR) box feature found on each data graph.

Analysis Method Options

The following analysis options are available in Quansoft:

Baseline

This simple analysis method allows for correction of differences in background fluorescence. It is also incorporated into many of the other analysis methods.

Quantification

Quantification analysis is used to determine the absolute or relative quantity of a target DNA template in a given test sample by measuring the cycle-to-cycle change in the fluorescent signal. The fluorescent signal increases proportionately to the amount of amplified DNA and quantification is performed either by comparison of the fluorescence of a PCR product of unknown concentration with that of several dilutions of an external standard, or by comparing the fluorescence of one product relative to another. To be able to make this comparison, the fluorophore is measured at a point in the amplification where the reaction efficiency can be considered optimal. This is generally around the cycle at which an increase in fluorescence is first detected.

Dissociation curve

Dissociation curve analysis can add to the information obtained from the PCR. Also known as melting curve analysis, it measures the temperature at which the DNA strands separate into single strands. This provides a measurement of the melting temperature or Tm, taken as the point at which 50% of the double stranded DNA (dsDNA) molecules are dissociated. Using the easy-to-program 'ramp' function, PrimeQ will perform a thermal ramping program that can be used to determine the Tm of the PCR product. This analysis provides the user with extra confidence in experiments using intercalating dye chemistry for identifying amplification of non-specific products or contamination.

Plus/minus scoring

This analysis exploits PrimeQ's fluorescence technology to determine with ease and accuracy the presence or absence of a PCR product in any given sample. Input data can either be kinetic (where readings are taken throughout the amplification stage) or end-point (readings taken at the end of the run). The software scores the samples as positive or negative according to user-defined thresholds.

Allelic discrimination

Users of PrimeQ have the option of this powerful technique capable of detecting single nucleotide differences (SNPs). It can be used to discriminate between genotypes, mutations and polymorphisms within or between samples simply by comparing the fluorescence signal obtained using allele-specific, dye-labeled probes.

Multi-read

This is a simple end point analysis method which will report the average fluorescence of a selected number of readings. It is useful for assays other than PCR, for example fluorescence-based DNA assays, where just the fluorescence of a sample needs to be measured; in this way PrimeQ can be used as a simple fluorescence plate reader or fluorimeter.

Analysis method: Absolute Quantification

This analysis method determines the "absolute" concentration of an unknown sample by comparison to standards of known concentration using a standard curve. An alternative method of relative quantification whereby a relative value determined by the comparison between a reference and unknowns, is also available in Quansoft and is discussed in more detail in the Technical Note T008-002A: Relative Quantification Analysis.

Both approaches use quantification cycle (Cq) calculation as the basis of the quantification. The Cq is the cycle number at which the concentration of the amplicon (measured by fluorescence) reaches a set threshold. The Cq is inversely proportional to the initial template concentration. Quansoft can use one of two methods to calculate Cq values: fit points or first derivative maximum; further details are given below.

Absolute quantification	Uses known standards to generate a standard curve and calculates the "absolute" value of the unknowns e.g. copies/well, µg/ml etc.
Relative quantification	Uses known standards to generate a standard curve for each reporter and compares the absolute concentrations of the two reporters in the same well (ratio of one to the other).
Relative Cq	Subtracts the Cq value of a reference from an unknown amplified from the same sample and compares to a control sample ($2^{-\Delta\Delta Cp}$ method).

Table 1: *Quantification analysis options. Absolute quantification is described in this Technical Note.*

Setting up and running the experiment

Quantification analysis is a kinetic analysis method and requires fluorescence readings to be taken at each cycle of the amplification stage. Therefore a reading for each reporter and a passive reference dye (PRD), if used must be included at the end of the extension step of each cycle. If intercalating dye chemistry is used, it is also advisable to include a dissociation curve stage at the end of the amplification to check for non-specific amplification or contamination (see Technical Note T008-006A: Dissociation Curve Analysis for further details).

Standards of known concentration or copy number amplified by the same primers as the target must also be prepared. This is to ensure that the reaction efficiency is the same between the standards and unknowns. We recommend you use at least three standards diluted serially at 10-fold dilutions. For increased precision, all reactions should be performed in duplicate or triplicate. The standards need to be defined in the plate layout and the concentrations added to the Well Information table in the Plate Layout Editor.

During the run, the real-time collection of data can be monitored on the Run Screen. The plate layout shows the fluorescence curve on a per-well basis and the temperature profile plot indicates how far the run has progressed. When the run has completed, results can be viewed in the Results Editor with data from each stage of the run located under its own tab.

Quantification Analysis Wizard setup

Once the PCR has completed, open the results file and from the Results Editor home screen set up the Quantification analysis as described below.

- In the Analysis Selection box, highlight the stage name for analysis to be applied and click **Edit**. The Analysis Wizard will launch.

- Select **Quantification** from the drop-down menu in the Analysis Method selection box.

- Assign a dye usage for each of the reads (e.g. Reporter, PRD etc.) and click **Next**. The Quantification Analysis Wizard will launch.

- If a PRD was assigned in the dye usage menu, the next screen will offer the option of PRD correction. Click **Next**.
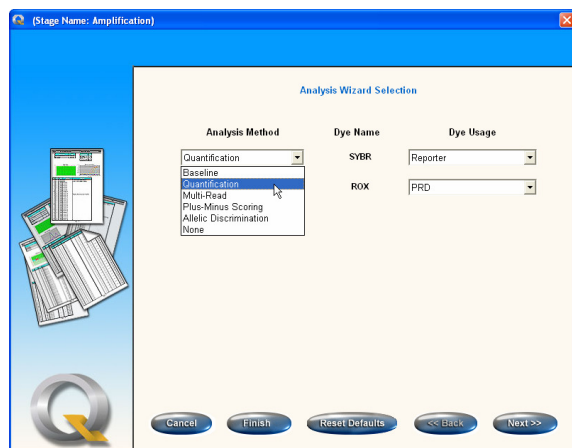

Figure 3: *Analysis Wizard Selection.*

Select the Quantification analysis method and assign dye usages.

Baseline Correction

Follow the directions in the Operator's Manual in section 4.5 to select an appropriate baseline correction method. Note that the default parameters may not always be suited to the fluorescent data, especially if an increase in fluorescence is first seen to occur prior to 10 cycles of amplification. It is recommended to check the baseline correction parameters whilst viewing the fluorescent data graphs and adjusting as necessary using the Analysis Properties displayed in the PAR box (see below). Click **Next** to progress to the next screen.

Cq Calculation Method

Quansoft offers two methods to calculate Cq values: fit points or first derivative maximum. The latter is an automatic method dependent entirely on mathematical calculations and requires no user input.
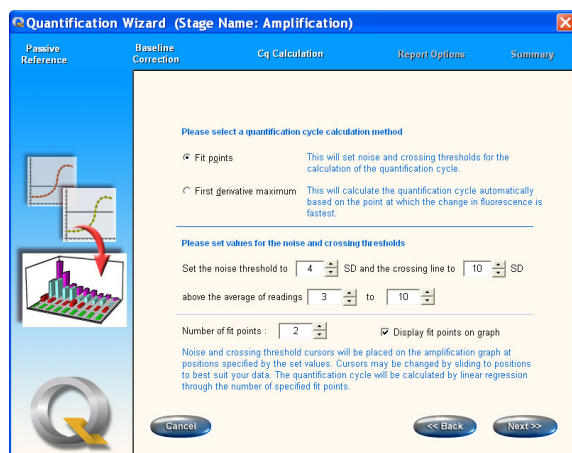
Figure 4: *Select the appropriate Cq calculation method:*

Fit points: (Default): Involves setting noise and crossing thresholds. This is the most flexible option and is useful for quantifying low copy number targets.

First derivative maximum: Calculates Cq values automatically based on the point at which the rate of change is fastest. As this method uses the shape of the curve to determine Cq, there is no user-input in the analysis of the data. This method can be useful for analysing high copy number templates (over 1000 copies).

The fit points method is more flexible and involves the setting of thresholds. The crossing line is a threshold line ideally positioned where the reaction efficiency is at its highest and most constant for each reaction curve. The Cq is the cycle at which an amplification curve intercepts the crossing line and measuring fluorescence at the Cq for each well will allow the most reliable well-to-well comparison.
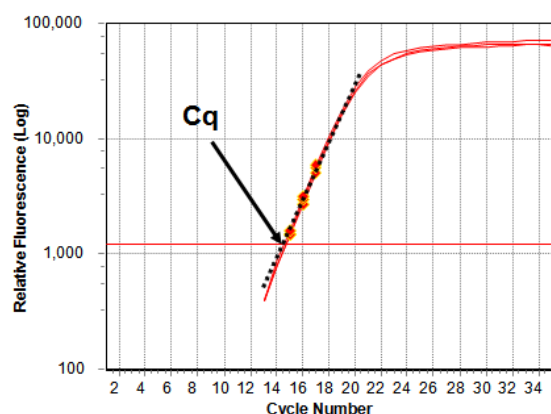
Figure 5: *Calculation of Cq using fit points.*

The fit points are the next few fluorescence readings above the crossing line (shown here as a horizontal red line. The Cq is determined by calculating a linear regression through the fit points. The Cq is the point where the regression line intercepts the crossing line.

Once you have set the Cq calculation method, click **Next** to progress to the next screen.

Report Options and Summary

The Report Options screen allows you to select data to appear in a printable report. The selection can be changed from the Report tab in the Results Editor if necessary. The Summary screen provides a quick checklist of the parameters set up in the Analysis Wizard.

• Click **Back** to change any settings or **Cancel** to abort the procedure.

• Click **Finish** to complete the set up.

Viewing the analysed results

Click on the results tab for the stage which has been set up for Quantification analysis. The analysed data will be displayed as a series of graphs similar to those shown in **Figure 6**. Individual graphs can be closed or enlarged for easier viewing.
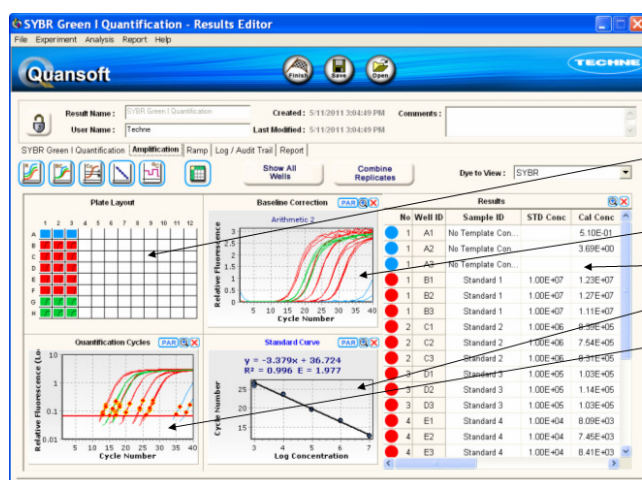
Figure 6: *The layout of data in the Results Editor screen for Quantification analysis with a standard curve.*

Plate layout with individual well view of fluorescent data.

Baseline correction graph.

Results table.

Standard curve.

Quantification cycle graph (fit points method only).

Clicking an individual well or a selection of wells in the plate layout will highlight just the selected well(s) on the graphs. Clicking the **Show All Wells** button will re-select all wells.

The Quantification Cycle Graph (Fit Points Analysis Only)

The results (with or without baseline correction) are plotted on a scale of log fluorescence vs. cycle number. The graph displays two horizontal threshold lines at positions defined in the Analysis Wizard. The blue line represents the noise threshold and the red line the crossing line. Fit points are shown as orange points above the crossing line.

Quansoft performs a linear regression through the fit points of each individual amplification curve to determine the intersection with the crossing line. This is then recorded as the Cq value. The Cq will be a fractional cycle number calculated for each well.
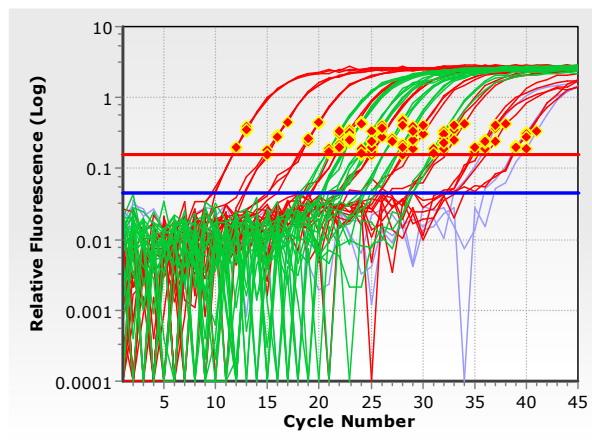

Figure 7*: The Quantification Cycle graph.*

The two threshold cursors are placed at the following default positions:

Noise threshold cursor: 4 SD above the average of all readings x to y
Crossing line cursor: 10 SD above average of all readings x to y

Where: x = 3 (or 1 if the number of readings is less than 10)
y = 10 (or 2 if the number of readings less than 10)

Note: x and y can be changed as appropriate.

You can manually adjust the position of the threshold lines by dragging with the mouse to positions better suiting the data. The position can also be changed from within the Analysis Properties accessed by clicking on the PAR button next to the Cq graph. The noisy data below the noise line can also be removed for clarity. Changing the position of the cursors will update the standard curve graph. This can be useful for finding the best fit such that the R^2 value (correlation coefficient) is as close to 1 as possible (see The Standard Curve Graph below).

The Standard Curve Graph

The standard curve graph is a plot of the Cq for each standard vs. log concentration and is displayed for both fit points and first derivative maximum analysis. It will only be displayed if standards have been defined with their concentration in the plate layout. The concentration of each standard should be added to the Well Information table in the Plate Layout Editor.
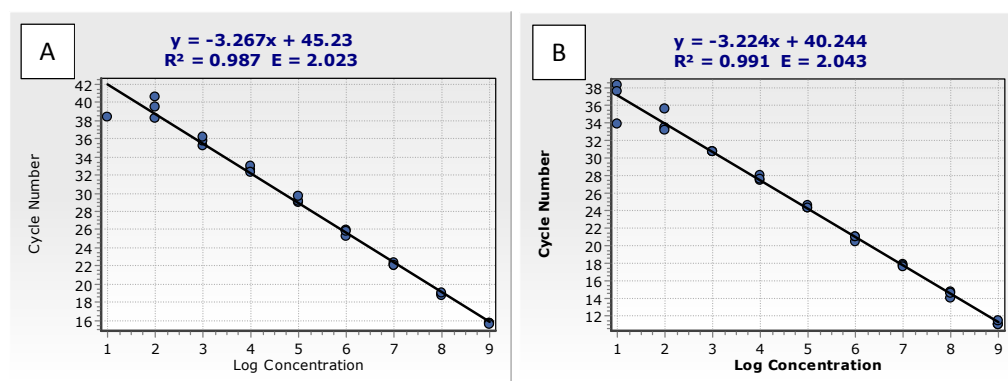

Figure 8*: Standard curves calculated using fit points (A) or first derivative maximum (B). Note that changing the calculation method will lead to differences in Cq so it is important to be consistent in analysis methods when comparing experiments.*

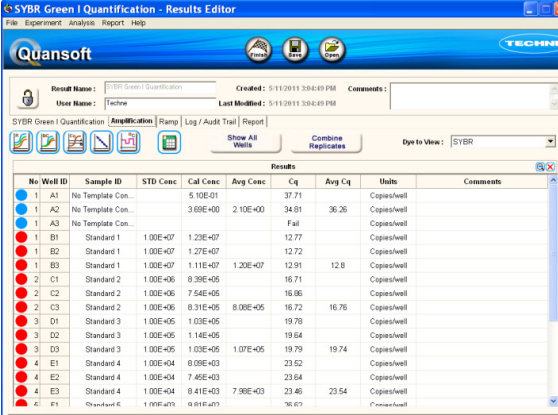Linear regression is used to generate a straight line of best fit through the points, y = mx + c whereby:

- y = the slope; ideally this should be approximately -3.32.

- c = the value at which the line intercepts the y axis.

- R^2 = Correlation coefficient (mean squared error of the determination).A perfect correlation has a value of 1.

- E = efficiency of the reaction whereby E = $10^{-1/slope}$. The ideal value is 2 for 100% efficiency (i.e. a doubling of target every cycle). A value less than 2 suggests that the PCR is sub-optimal while above 2 suggests the amplification of non-specific products. When optimizing individual reactions for multiplexing, it is important for both PCRs to have a near equal efficiency (typically in the range of 1.95 to 2.05).

Changing the position of the red crossing line cursor on the quantification cycle graph will update the standard curve graph. This can be used to optimise the fit of the standard curve for the data. The standard curve is then used to calculate the starting concentration of unknowns by reading their Cq values from the graph. In Quansoft this is performed automatically and the calculations are displayed in the results table.

The Results Table

Concentrations are calculated for the unknowns based on a comparison of Cq values with standards of known concentration. The results are displayed in the results table.

Figure 9: *The Results Table.*

No: *Well number.*
Well ID: *Location of well.*
Sample ID: *User-supplied or default name of well.*
STD Conc: *Concentration of standard.*
Cal Conc: *Calculated concentration of sample.*
Avg Conc: *Mean concentration obtained for all replicates of a sample.*
Cq: *Quantification cycle for sample.*
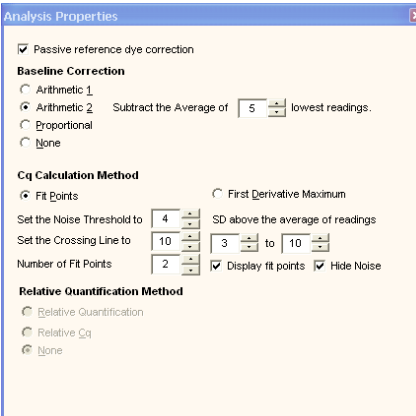**Avg Cq:** *Mean Cq obtained for all replicates of a sample.*
Units: *Unit of concentration.*
Comments: *User inputted text.*

Note: *Data can be easily exported to Excel.*

Viewing and Changing the Parameters

The analysis parameters as set up in the Quantification Wizard can be changed at any time and can be easily accessed via the PAR box on any of the graphs. You can change any of the parameters, and in doing so, the changes will be reflected immediately in the adjacent graphs.

Figure 10: *Analysis Properties.*

Click the **PAR** *button next to any of the graphs to bring up the Analysis Properties for Quantification. If any settings are changed, the data will be recalculated and the graphs and results table updated accordingly.*

PRD and baseline correction parameters can be adjusted as required.

Changing the Cq Calculation Method will automatically update your graphs and in turn this will result in different Cq determinations.

Changing the position of the cursors manually will automatically update the values in the Analysis Properties.

PrimeQ Report

If a printed report of the analysed data is required, open the Report tab of the Results Editor to view the Quantification analysis report. To change any of the report contents, click on the **Report Options** icon to open up the Report Options box. Tabs will display the report options relevant for each stage. Change as appropriate and click **Done** to finish. The report can be printed or saved as a PDF file for future reference.

Saving

Saving will overwrite any previous analysis, therefore to preserve a particular analysis set up, click on **File** followed by **Save As…** to save as a different file name.

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