

qPCR Hand Calculations

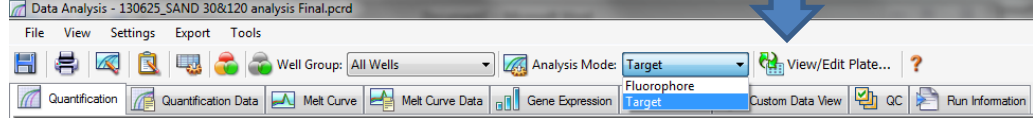
- Multiple Reference Genes

→ This word document will take you through the hand calculations needed to determine the Normalized Gene Expression, using the Pfaffl method for efficiency correction, when comparing expression to a Control sample (i.e. comparing all expression to WT, untreated, or a specific time point).

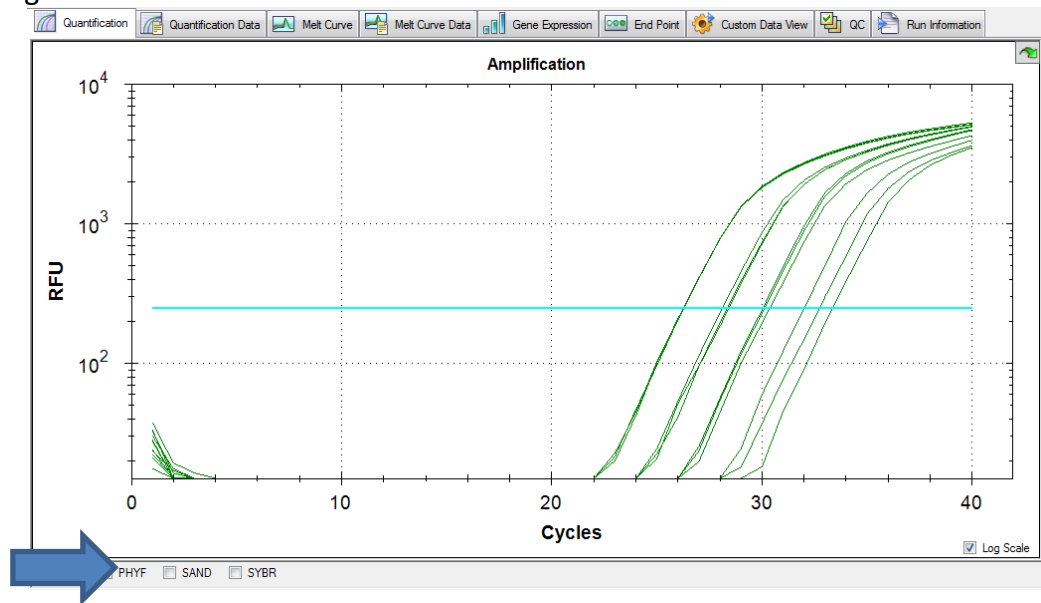
I. Using Bio-Rad CFX Manager to Export Data to Microsoft Excel

1. Set the Cq Threshold value the same for all targets and runs, the exact threshold value should be determined using your standard curve.

a. Change Analysis Mode from Fluorophore to Target



b. In the Quantification tab, under the Amplification graph, uncheck all but one target



c. Using the mouse cursor, click and drag on the horizontal line to set the threshold so that it follows these parameters:

- i. 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 (i.e. all amplification lines are parallel to one another. 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.

ii. Precision maximization:

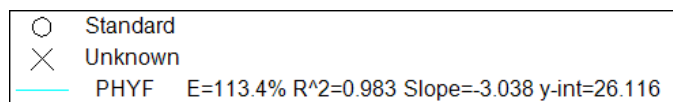
The threshold should be set at a point that maximizes the precision of replicates. It is common to find that the precision amongst the replicates increases as the amplification progresses further into the exponential phase of the reaction. Therefore, to assure maximal precision, the threshold value must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

iii. 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.

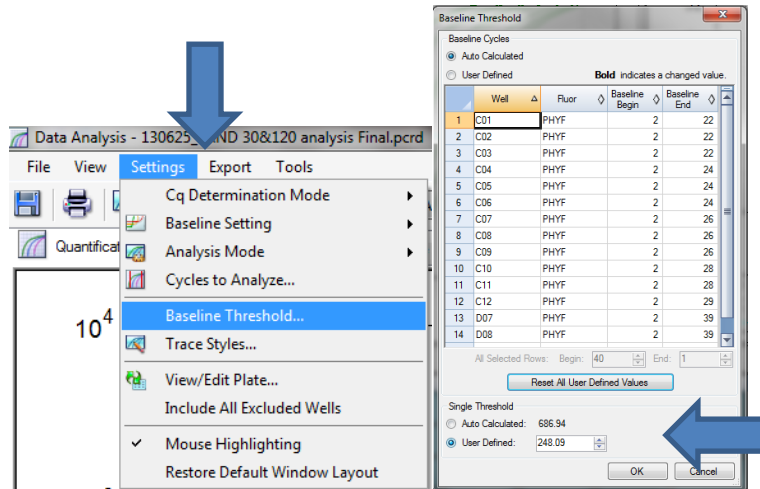
NOTE 1: There will usually be a “window” or range of values within which a threshold setting will fit the aforementioned criteria and yield optimum results. Using the standard curve method you should aim to get a % Efficiency close to 100% and R^2 close to 1.

NOTE 2: Each standard curve for your gene of interest (GOI) can have a different Cq threshold value, BUT the value you determine from the standard curve needs to be set and verified in your runs.



- d. To obtain what this value click “Settings” and select “Baseline Threshold”. Go down to the Single Threshold portion of the new window. Make sure the User Defined option is selected and record this value in the appropriate Hand Calculation Excel Spreadsheet Template.

Repeat this process for each of your standard curves and to set the appropriate Cq threshold values for each of the targets in each of your runs. Make sure you save this CFX Manager Run File!

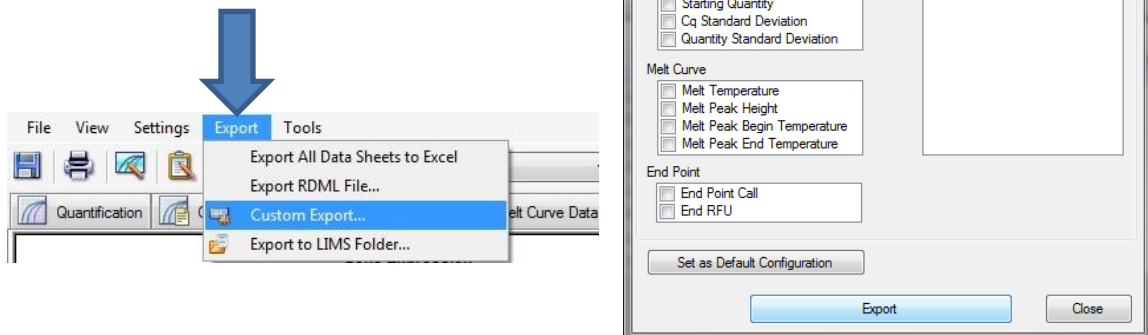


2. Open the Hand Calculation Excel Spreadsheet Template.
3. Record the % Efficiency from your standard curves with the appropriate Cq threshold from CFX Manager in your Hand Calculation Excel Spreadsheet Template.
4. Convert the % Efficiency to the E value in your Hand Calculation Excel Spreadsheet Template:

$$\% \text{ Efficiency} = (E - 1) * 100$$

$$E = (\% \text{ Efficiency} \times 0.01) + 1$$

5. Export your data to Excel by Clicking “Export” and choose “Custom Export...”
 - a. Choose .xls or .xlsx for the Export Format
 - b. The only columns you need exported (have a check mark by) are “Well”, “Target Name”, “Sample Name”, and “Cq”
 - c. Click Export
 - d. Save file to appropriate location
 - e. Close Export window



- Using the Exported Excel Spreadsheet fill in the information for Target and Sample Names. Copy and paste the Cq values into the appropriate column in the template.

II. Equations used in Hand Calculation Excel Spreadsheet Template

- Calculate the Relative Quantity (RQ) for your REF and GOI using the Hand Calculation Excel Spreadsheet Template. This equation factors in the efficiency of your primers and calibrates the RQ values to the control sample that you have chosen.

$$Relative\ Quantity_{Target} = E_{Target}^{(Cq(Control) - Cq(Treatment))}$$

Where:

E = Efficiency of primer set.

Cq (Control) = Average Cq for the control or untreated sample.

Cq (Treatment) = Average Cq for treated sample.

Target = The gene of interest or reference gene

- Calculate the Standard Deviation for the Relative Quantity (SD RQ):

$$SD\ RQ = SD\ Cq_{(sample)} \times RQ_{sample} \times (Ln\ E_{(Target)})$$

Where:

SD Cq_{sample} = Standard Deviation of the Cq for the sample

RQ_{sample} = Relative Quantity of the sample

Ln = Natural Log

E = Efficiency of primer set for that target

GOI = Gene of interest (target)

- Calculate the Standard Error for the Relative Quantity (SE RQ):

$$SE\ RQ = \frac{SD\ RQ_{sample}}{\sqrt{n_{(sample)}}}$$

Where:

SD RQ = Standard Deviation of the relative quantity for that sample

n = number of wells for that sample (i.e. how many technical replicates. You'll need to double check to see if you had to omit any.)

10. Calculate the Normalization Factor (NF). The normalization factor is the geometric mean of the relative quantities of all the reference targets for a given sample:

$$NF_{sample} = (RQ_{sample (Ref 1)} \times RQ_{sample (Ref 2)} \dots RQ_{sample (Ref n)})^{\frac{1}{n}}$$

Where:

GOI = Gene of interest (target)

RQ = Relative quantity of the sample

n = Number of reference targets

11. Calculate the Standard Deviation for the Normalization Factor (SD NF):

$$SD NF_{sample} = NF_{sample} \times \sqrt{\left(\frac{SD RQ_{sample (Ref 1)}}{n \times RQ_{sample (Ref 1)}}\right)^2 + \left(\frac{SD RQ_{sample (Ref n)}}{n \times RQ_{sample (Ref n)}}\right)^2}$$

Where:

NF = Normalization factor

RQ = Relative quantity of a sample

SD = Standard deviation

Ref = Reference target

n = Number of reference targets

12. Calculate the Standard Error for the Normalization Factor (SE NF):

$$SE NF_{sample} = NF_{sample} \times \sqrt{\left(\frac{SE RQ_{sample (Ref 1)}}{n \times RQ_{sample (Ref 1)}}\right)^2 + \dots + \left(\frac{SE RQ_{sample (Ref n)}}{n \times RQ_{sample (Ref n)}}\right)^2}$$

Where:

N = Number of reference targets

SE = Standard Error

NF = Normalization Factor

RQ = Relative Quantity

Ref = Reference gene (target)

13. Calculate the Normalized Relative Quantity (NRQ) using the RQ's you determined in step 7 and the NF you determined in step 10. This is also known as the normalized expression and is the relative quantity of your target gene normalized to the quantities of the reference target genes:

$$\text{Normalized Relative Quantity}_{GOI} = \frac{RQ_{\text{sample } (GOI)}}{NF_{\text{sample}}}$$

Where:

RQ = Relative quantity of the indicated sample

GOI = Gene of interest

REF = Reference target

NF = Normalization Factor for that particular sample

14. Calculate the Standard Deviation of the Normalized Relative Quantity (SD NRQ)

$$SD\ NRQ_{\text{sample } (GOI)} = NRQ_{\text{sample } (GOI)} \times \sqrt{\left(\frac{SD\ NF_{\text{sample}}}{NF_{\text{sample}}}\right)^2 + \left(\frac{SD\ RQ_{\text{sample } (GOI)}}{RQ_{\text{sample } (GOI)}}\right)^2}$$

Where:

NRQ = Normalized Relative Quantity

RQ = Relative Quantity

SD = Standard deviation

GOI = Gene of interest

15. Calculate the Standard Error for the Normalized Relative Quantity (SE NRQ):

$$SE\ NRQ_{\text{sample } (GOI)} = NRQ_{\text{sample } (GOI)} \times \sqrt{\left(\frac{SE\ NF_{\text{sample}}}{NF_{\text{sample}}}\right)^2 + \left(\frac{SE\ RQ_{\text{sample } (GOI)}}{RQ_{\text{sample } (GOI)}}\right)^2}$$

Where:

SE = Standard Error

NRQ = Normalized Relative Quantity

NF = Normalization Factor

RQ = Relative Quantity

GOI = Gene of Interest

YOU'RE DONE WITH HAND CALCULATIONS!

Now you can graph your results!