qPCR Hand Calculations

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).

NOTE: These calculations apply when only one reference gene is being used. If you are using more than one reference gene (which is recommended) please use the document titled “qPCR Hand Calculations when using Multiple Reference Targets”

I. Calculating Normalized Relative Quantity (NRQ)
   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
         ![Image of Analysis Mode Change](image)
      b. In the Quantification tab, under the Amplification graph, uncheck all but one target
         ![Image of Amplification Graph](image)
      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.

<table>
<thead>
<tr>
<th></th>
<th>Standard</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHYF</td>
<td>E=113.4%</td>
<td>$R^2=0.983$ Slope=-3.038 y.int=26.116</td>
</tr>
</tbody>
</table>

   **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 Template Excel spreadsheet.

   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. Record the % Efficiency from your standard curves with the appropriate Cq threshold from CFX Manager in your Hand Calculation Template Excel Spreadsheet.

3. Convert the % Efficiency to the E value in your Hand Calculation Template Excel Spreadsheet:

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

4. Export all data sheets to Excel. **NOTE:** The only one you will need to work with is “Quantification Ct Results”.

5. Start filling in your Normalized Expression Table in the Hand Calculation Template Excel Spreadsheet with the Target name and whether or not it’s a Reference (REF) gene or Gene of Interest (GOI).

6. Using the Exported “Quantification Ct Results” Excel Spreadsheet copy and paste the information for Sample Name, Mean Cq, and SD Cq (Cq Std. Dev) into the Normalized Expression Table in the template. It will also be helpful at this point to record the number of wells (should be three unless you had to omit a technical replicate) in the appropriate column in the Error Bar Table in the template.
7. Calculate the Relative Quantity (RQ) for your REF and GOI using the Hand Calculation Template Excel Spreadsheet. This equation factors in the efficiency of your primers and calibrates the RQ values to the control sample that you have chosen.

\[ \text{Relative Quantity}_{Target} = E^{(C_{q(\text{Control})}-C_{q(\text{Treatment})})} \]

Where:
- \( E \) = Efficiency of primer set.
- \( C_{q(\text{Control})} \) = Average Cq for the control or untreated sample.
- \( C_{q(\text{Treatment})} \) = Average Cq for treated sample.
- Target = The gene of interest or reference gene

8. Calculate the normalized expression using the RQ’s you determined in step 7.
Normalized expression is the relative quantity of your target gene normalized to the quantities of the reference target gene:

\[ \text{Normalized Expression}_{\text{GOI}} = \frac{RQ_{sample\ (\text{GOI})}}{RQ_{sample\ (\text{REF})}} \]

Where:
- \( RQ \) = Relative quantity of a sample
- \( \text{GOI} \) = Gene of interest
- \( \text{REF} \) = Reference target which can include one or more targets for each sample
- \( n \) = Number of reference targets

II. Calculating Error

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

\[ \text{SD } RQ = \text{SD } C_{q\ (sample)} \times RQ_{sample} \times (\text{Ln } E_{\text{GOI}}) \]

Where:
- \( \text{SD } C_{q\ (sample)} \) = Standard Deviation of the Cq for the sample
- \( RQ_{sample} \) = Relative Quantity of the sample
- \( \text{Ln} \) = Natural Log
- \( E \) = Efficiency of primer set for that target
- \( \text{GOI} \) = Gene of interest (target)

10. Calculate the Normalization Factor (NF). The denominator of the normalized expression equation (Step 8) is referred to as the normalization factor. The normalization factor is the geometric mean of the relative quantities of all the reference targets for a given sample:
Where:
GOI = Gene of interest (target)
RQ = Relative quantity of the sample
n = Number of reference targets

NOTE 1: If you only have 1 reference gene, this means that the RQ of the reference gene for that sample is the normalization factor for the corresponding gene of interest.

NOTE 2: So why is the normalization factor important to calculate if you only have 1 reference gene? Inspection of normalization factors allows you to inspect possible experimental problems. Using approximately equal amounts of equal quality input material and stably expressed reference genes, the normalization factor values should be similar for all samples. According to qBase+ and the MIQE guidelines, a variation of 2- to 3-fold is generally acceptable (this is the experimental variation that you want to remove in the normalization process). Any higher variation should be treated with care.

11. Calculate the standard deviation for the normalization factor (SD NF):

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

Where:
NF = Normalization factor
RQ = Relative quantity of a sample
SD = Standard deviation
Ref = Reference target
n = Number of reference targets

NOTE: If you only have one reference target then the SD RQ = SD NF:

12. Calculate the standard deviation of the normalized relative quantity (SD NRQ)

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

Where:
NRQ = Normalized Relative Quantity
RQ = Relative Quantity
SD = Standard deviation
GOI = Gene of interest
13. 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.)

**NOTE:** If you are using only one reference gene skip to step 15.

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

\[ SE NF_n = NF_n \times \sqrt{\left( \frac{SE \ RQ_{sample \ (Ref \ 1)}}{n \times SE \ RQ_{sample \ (Ref \ 1)}} \right)^2 + \ldots + \left( \frac{SE \ RQ_{sample \ (Ref \ n)}}{n \times SE \ RQ_{sample \ (Ref \ n)}} \right)^2} \]

Where:
N = Number of reference targets
SE = Standard Error
NF = Normalization Factor
RQ = Relative Quantity
Ref = Reference gene (target)

**NOTE:** If you are using only one reference gene this should equal the value of the normalization factor.

15. Calculate the Standard Error for the normalized relative quantity of the gene of interest (SE NRQ):

\[ SE \ NRQ_n = NRQ_n \times \sqrt{\left( \frac{SE \ NF_n}{NF_n} \right)^2 + \left( \frac{SE \ RQ}{RQ} \right)^2} \]

Where:
SE = Standard Error
NRQ = Normalized Relative Quantity
NF = Normalization Factor
RQ = Relative Quantity

**NOTE:** If you are using only one reference gene then you can ignore the part of the equation that has the NF in it as it should equal 1.
YOU’RE DONE WITH HAND CALCULATIONS!

Now you can graph your results!