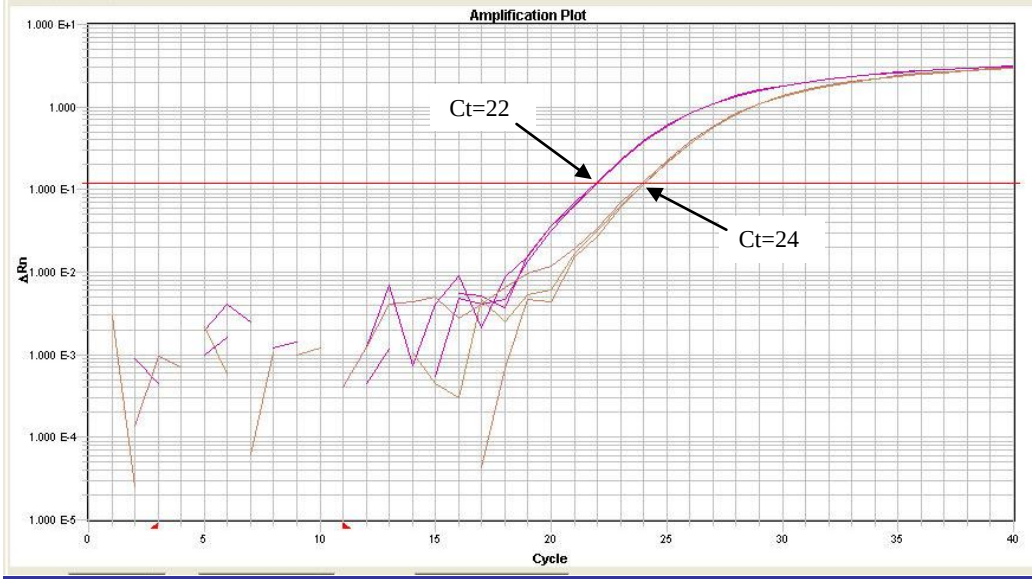
Information on qPCR results



What does qPCR measure?

If you are measuring gene expression, qPCR will tell you how much of a specific mRNA there is in your samples. You amplify a small region of this mRNA with oligos and a fluorescent probe. The qPCR machine measures the intensity of fluorescence emitted by the probe at each cycle. During the first cycles, there is not enough fluorescence to be detected, but the reaction rapidly produces more and more amplicons and the fluorescence builds up. A qPCR curve has typically an exponential phase followed by a plateau phase. The Ct measure needs to be taken in the exponential phase, where the curve is linear.

Most of the time, a qPCR experiment will give a "relative expression", which is a variation of the expression of a gene between two samples.

Definitions of the terms found in the analysis

Endogenous control

The control is the gene that does not vary between all of the samples tested.
For example: GAPDH, ACTB, TBP, HPRT, PPIA, YWHAZ

Calibrator

The calibrator is the sample that all others are compared to. It's the "untreated", or "time zero". The RQ of the calibrator is 1 because it does not vary compared to itself.

Ct = PCR cycle

A typical qPCR run has around 40 cycles. The Ct is the value where the PCR curve crosses the threshold, in the linear part of the curve. It's the value that will be used for the analysis. The higher the Ct (30-35), the less the mRNA detected is present, because you need more cycles

of amplification to detect the fluorescence. If the Ct has a small value (10-15), the gene is highly expressed. Endogenous controls typically have a smaller Ct then regular genes.

Delta Ct = $Ct_{\text{gene test}} - Ct_{\text{endogenous control}}$

Delta Delta Ct = $\Delta Ct_{\text{sample1}} - \Delta Ct_{\text{calibrator}}$

Delta Ct SD = Standard Deviation
The standard deviation is calculated by the software with the delta Ct value of the technical triplicates. The triplicates are valid when the SD is smaller than 0.25. If the SD is over 0.25, the RQ value is considered unreliable.

RQ = Relative quantification = $2^{-\Delta\Delta Ct}$

The RQ is your fold change compared to the calibrator (untreated sample, time zero, etc.). The calibrator has a RQ value of 1. All samples are compared to the calibrator.

A RQ of 10 means that this gene is 10 times more expressed in sample x then in the calibrator sample. A RQ of 0,1 means that the gene is 10 times less expressed.

We consider a RQ significant when there is a minimum of two-fold change: RQ of more than 2 or less then 0,5. This is within the variations of the technique. You might get a RQ of 0.8 one day and easily get one of 1.2 the next day. If you are looking for a small fold (1.5-fold), you need perfect samples very very clean, do technical quadruplicates, consider using 2 assays for this gene, and use positive samples with a known fold-change.

RQmin and RQmax = range of possible RQ values defined by the standard error of the delta Ct. Confidence interval set à 95%.

If you need a real biological standard error of the RQ value, you need to have done biological triplicates and calculate the standard error on the average delta Ct values or the RQ values of the biological triplicates. If you are using the 7900HT, the software DataAssist can do that for you (www.applied biosystems.com).

General data quality

When analysing your results, look at your PCR curves, they need to be nice and parallel. Technical replicates have to be within 0.5 cycles. Cts should be <35. If you suspect that a curve is not normal, take it out of the analysis. If you have curves coming out after 35, do not use them, or be very careful and make sure that your technical replicates are good.

If you don't have access to the PCR amplification curves and you want to verify if the qPCR experiment went well, you can use the Ct SD or the DeltaCt SD. This will tell you if the technical triplicates were within range (0.5 cycles). A DeltaCt SD of less than 0.25 is good. This error is not a good error to validate the biological significance of your results; it only validates the method's measurements. You need to do biological replicates to get accurate results.

If all your genes are coming out later than expected (especially the endogenous controls), and your results look bad in general: check your RNA. It's the most common source of bad results. Make sure your RNA is not degraded (on gel or on a bioanalyzer) and that your RNA is not contaminated (verify the ratios 260/280 and 260/230).

Analysis with multiple endogenous controls

Usually, only one endogenous control is used, but using two or three controls will improve the accuracy of your results. Test a few controls and choose the ones with less variation possible between samples (you can use software such as Genorm to select the best endogenous controls). If you are using the Lightcycler 480, it's possible to do an analysis directly with multiple controls. If you are using the 7900HT, download the software DataAssist at www.appliedbiosystems.com to do the analysis with multiple controls. This software can also do an analysis with your biological replicates and make a T test between groups.

Interesting article on qPCR methods for publication

The MIQE guidelines

The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. Clin Chem. 2009 Apr;55(4):611-22. Epub 2009 Feb 26.