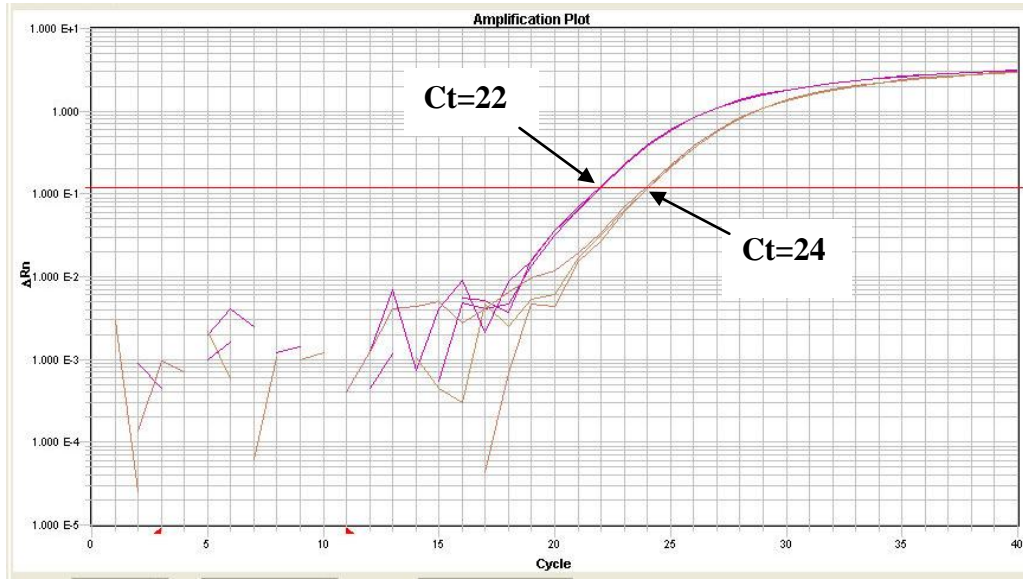


Understanding qPCR results



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 (if working with Taqman). 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 is a determined PCR cycle and represents the basic result of a qPCR experience. It's taken in the exponential phase, where the curve is linear. The **threshold** (red line) is placed in the linear phase, and the Ct is measured where the PCR curve **crosses** the threshold. The threshold is different for every qPCR assay (every gene tested), and is the same for all samples tested with this gene.

The principle of the qPCR is based on the fact that at each PCR cycle, the number of PCR products doubles. If there is a difference of 2 cycles between two reactions (see figure), we can say that there is 4 times more copies in the pink reaction than in the orange reaction.

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. It's also possible to determine an absolute quantification (copy number) of a gene, but this technique requires a standard : typically the cloning of the cDNA of the gene into a vector.

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 than regular genes.

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

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

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.

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

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 than 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 than 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 biological replicates, 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 error of the RQ value, you need to have done biological triplicates. Then, you can do an average of your RQs and do a Student T test between the different groups of biological triplicata. 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. 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 statistically accurate results.

If all of your genes are coming out later than expected (endogenous controls also coming out late), 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

Using two or more endogenous controls is strongly recommended, and 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 Via7, download the free software Expression Suite at www.appliedbiosystems.com and import the eds file. This software can also do an analysis with your biological replicates. If you are using the Lightcycler 480, it's possible to do an analysis directly with multiple controls.