

Theory of gene expression quantification using real-time PCR

For the quantification of RNA a reverse transcription (RT) step precedes the actual real-time PCR. Depending on the amount of sample available, the characteristics of the RNA target (secondary structure) and the throughput required, there are two different approaches:

1. **Two-Step RT-PCR:** Reverse transcription of mRNA will lead to a cDNA archive, allowing for multiple analyses using real-time PCR.
2. **One-Step RT-PCR:** In a single-tube with single-enzyme system mRNA is reverse transcribed to cDNA and amplified using real-time PCR.

A species-specific endogenous reference gene is quantified. Examples for endogenous reference genes are the GAPDH (Glyceraldehyde-3-P-Dehydrogenase) gene, the β -actin gene or the 18S rRNA gene. The choice and validation of the optimal reference gene is crucial for the performance of the gene expression quantification assay. In parallel, the gene(s) of interest is (are) quantified. The endogenous reference gene is used for normalization between the individual samples. Comparison of the normalized fluorescence signals leads to quantification of the different expression levels. For instance, treated and untreated sample groups of an experiment can be compared.

In the figure below, so-called real-time PCR amplification plots are shown. Amplification plots are graphs plotting the normalized fluorescence signals of a real-time PCR reaction against the PCR cycle number. The ct value - or threshold cycle - is the PCR cycle at which a statistically significant increase of the fluorescence signal is first detected. Ct values form the basis for quantitative comparison of individual PCR reactions. The smaller the ct value is, the bigger the quantity of target cDNA in a given TaqMan[®] PCR reaction. In an ideal PCR reaction the number of target molecules doubles in each PCR cycle. Therefore, a difference in the ct value of 1 corresponds to a concentration difference of 2.

As an example, quantification of the expression levels of a gene of interest in treated and untreated cells of a cell culture is shown.

Panel A (untreated cells)

An untreated cell culture is used as a calibrator. The ct values of the endogenous reference gene (blue) and the gene of interest (green) are compared: Δct (untreated cells).

Panel B (treated cells 1)

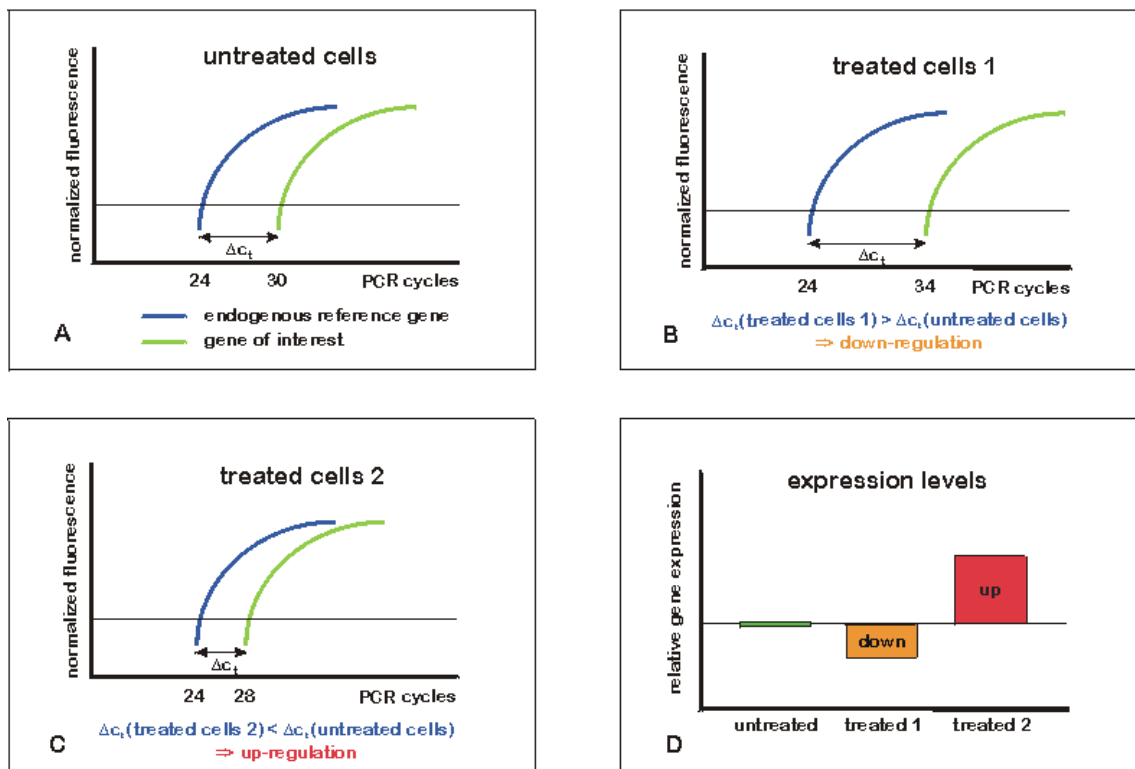
The ct values of the endogenous reference gene (blue) and the gene of interest (green) are compared: Δct (treated cells 1). As Δct of the treated cells 1 is 4 PCR cycles higher than Δct of the untreated cells, the gene of interest is down-regulated. The degree of down-regulation can be exactly quantified.

Panel C (treated cells 2)

The ct values of the endogenous reference gene (blue) and the gene of interest (green) are compared: Δct (treated cells 2). As Δct of the treated cells 2 is 2 PCR cycles lower than Δct of the untreated cells, the gene of interest is up-regulated. The degree of up-regulation can be exactly quantified.

Panel D (expression levels)

The relative gene expression levels of the gene of interest are shown for the different treatments of the cells. The expression level of the gene of interest in untreated cells is set arbitrarily to 1 (green). Down-regulation in the treated cells 1 (orange) and up-regulation in the treated cells 2 (red) of the gene of interest is shown.



Gene expression quantification is playing a key role in understanding the impact of therapeutic intervention in patients. At the same time, gene expression quantification studies are speeding up drug development significantly. In basic research it provides a powerful tool to address complex biological questions.