What is Relative Quantitation?

Methods for relative quantitation of gene expression allow you to quantify differences in the expression level of a specific target (gene) between different samples. The data output is expressed as a fold-change or a fold-difference of expression levels. For example you might want to look at the change in expression of a particular gene over a given time period in a treated vs. untreated samples.

For this hypothetical study, you can choose a calibrator (reference) sample (i.e. untreated at day 0) and an endogenous control gene to normalize input amounts. For all samples, levels of both target and endogenous control genes would be assessed by realtime PCR. The results (target levels normalized to endogenous control levels) would then be expressed in a format such as "At day 30, sample A had a 10-fold greater expression level of the target gene than at day 0".

Terms you need to know their scientific meanings when you talk about relative quantitation

Active reference

An active signal used to normalize experimental results. Endogenous controls are an example of an active reference. Active reference means the signal is generated as a result of PCR amplification. The active reference has its own set of primers and probe.

Amplicon

A PCR product generated from a DNA or cDNA template.

The base line

The background fluorescence signal emitted during the early cycles of the PCR reaction before the real-time PCR instrument detects the amplification of the PCR product.

The calibrator

A sample used as the basis for comparative expression results. See also reference sample.

Ct

is the cycle number at which the fluorescence generated within a reaction crosses the threshold line. CT values are logarithmic and are used either directly (comparative CT method) or indirectly (interpolation to standard curves to create linear values) for quantitative analyses.

Endogenous control

A gene sequence contained in a sample that is used to normalize target quantities. In addition to the target sequence, an endogenous control is quantified as a means of correcting results that can be skewed by input nucleic acid loading differences. Endogenous controls are an example of an active reference.

Experimental replicates

An amplification that uses the same PCR reagents as another amplification and that uses template preparations from similar but not identical samples. Experimental replicates provide information about the overall precision of the experiment. For example, if you want to examine the effect of drug treatment on the level of a mouse mRNA, you would treat multiple mice identically with the drug to determine the variation of response in the mouse population. A group of ten mice would represent ten experimental replicates

Identical replicate (Technical Replicate)

An amplification performed in multiple wells using the same template preparation and the same PCR reagents. Identical replicates provide:

• Data preservation: If amplification fails in one well, replicates in other wells can potentially provide data. • Monitoring: Replicates can be used to monitor the precision of the PCR amplification and detection steps

Passive reference

A dye that provides an internal fluorescence reference to which the reporter dye signal can be normalized during data analysis. The reference dye does not participate in the PCR reaction. This normalization corrects for fluorescence fluctuations that are caused by changes in reaction concentration or volume. Failure to use a passive reference dye can compromise accurate

target quantitation. Applied Biosystems incorporates the internal passive reference dye ROX all of its real-time PCR chemistries.

Precision and Statistical Tests

Amplification and Detection Step: The degree to which identical replicates give similar values (degree of agreement). This type of precision can be used to monitor the accuracy of template and reagent pipetting, homogeneity of template, and instrument performance.

Experimental: The degree to which experimental replicates give similar values. Note: For relative quantitation, better precision (identical and experimental) enables smaller fold differences in nucleic acid copy number to be distinguished with greater statistical confidence.

Reference gene

An active fluorescence signal used to normalize experimental results. Endogenous and exogenous controls are examples of active references. An active reference means the signal is generated as the result of PCR amplification using its own set of primers/probe

Reference sample

A sample used as the basis for comparative expression results. See also calibrator.

Standards

A sample of known concentration used to construct a standard curve.

Target

An RNA or DNA sequence, or gene of interest.

Test sampel

A sample compared against a calibrator as a means of testing a parameter change (for ex., the expression level of a gene) after an intervention such as a drug treatment, tumor transformation, growth factor treatment and so on.

Threshold

A level of normalized reporter signal that is used for determination in real-time assays. The level is set to be above the baseline but sufficiently low to be within the exponential growth region of an amplification curve. The cycle number at which the fluorescence signal associated with a particular amplicon accumulation crosses the threshold is referred to as the CT.

4. Relative Quantitation of Gene Expression Requires Quantitation of Two Different Genes (Target and Endogenous Control)

To obtain accurate relative quantitation of a mRNA target, it is recommended to also evaluate the expression level of an endogenous control. By using an endogenous control as an active reference, you can normalize quantitation of targets for differences in the amount of total nucleic acid added to each reaction. For example, if you determine that a calibrator sample has a two-fold greater amount of endogenous control than a test sample you would expect that the calibrator sample was loaded with two-fold more cDNA than the test sample. Therefore, you would have to normalize the test sample target by two-fold to accurately quantify the fold-differences in target level between calibrator and test samples. Some factors that can cause total cDNA or RNA sample loading differences are: • Imprecise RNA measurement after extraction • RNA integrity • Inaccurate pipetting

Factors Affecting Accurate Real-Time PCR Results

A variety of factors must be considered when setting-up real-time PCR reactions. During the initial set up it is important to include identical replicates for each input amount. The use of these replicates can help in identifying precision issues. After performing a realtime PCR run, you can gauge the accuracy of the results. If identical replicate samples have a CT standard deviation >0.3 and/or a standard curve has a correlation coefficient (R2 value) <0.99, the accuracy of the data is questionable. Some experiments may only tolerate low variation among identical replicates, for example, if you are looking for lowfold changes in target expression. It is important to appreciate that due to statistical

distribution there is always a high level of CT variation when target quantities approach single copy (CT values of 34 - 40). Therefore, sample masses that yield CT values in this range will unavoidably give rise to poorer precision and consequently less power to detect low-fold changes. The following practices help to achieve accurate real-time PCR results.