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Real-time PCR for gene quantitation

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Abstract
Real-time PCR has become one of the most widely used methods of gene quantitation because it has a large dynamic range, can be highly sequence-specific, has little to no post-amplification processing, and is amenable to increasing sample throughput. Its simplicity, specificity and sensitivity, together with its potential for high throughput and the ongoing introduction of new chemistries, more reliable instrumentation and improved protocols, has made real-time RT-PCR the benchmark technology for the detection and/or comparison of RNA levels. Typical uses of real-time PCR include pathogen detection, gene expression analysis, single nucleotide polymorphism (SNP) analysis, analysis of chromosome aberrations, and most recently also protein detection by real-time immuno PCR. Starting with the theory behind real-time PCR, this review discusses the key components of a real-time PCR experiment, including one-step or two-step PCR, absolute versus relative quantitation.

Keywords: Real time PCR, quantitation, absolute, relative

1. Introduction
Many cellular decisions concerning survival, growth and differentiation are reflected in altered patterns of gene expression and the ability to quantitate transcription levels of specific genes has always been central to any research into gene function (Zamorano *et al*., 1996) [29]. More recently, the emergence of molecular medicine has resulted in the increased use of techniques able to quantitate levels of RNA in clinical diagnostics. Four methods are in common use for the quantification of transcription: northern blotting and in situ hybridisation (Parker and Barnes, 1999) [17], RNAse protection assays (Hod, 1992; Saccomanno *et al*., 1992) [10, 20] and the reverse transcription polymerase chain reaction (RT-PCR) (Weis *et al*., 1992) [28]. A fifth method, cDNA arrays, is still limited in its use by cost considerations (Bucher, 1999) [2]. Northern analysis is the only method providing information about mRNA size, alternative splicing and the integrity of RNA samples. The RNAse protection assay is most useful for mapping transcript initiation and termination sites and intron/exon boundaries, and for discriminating between related mRNAs of similar size, which would migrate at similar positions on a northern blot. In situ hybridisation is the most complex method of all, but is the only one that allows localisation of transcripts to specific cells within a tissue. The main limitation of these three techniques is their comparatively low sensitivity (Melton *et al*., 1984) [16]. Real-time PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step. This is achieved using a variety of different fluorescent chemistries that correlate PCR product concentration to fluorescence intensity. Reactions are characterized by the point in time (or PCR cycle) where the target amplification is first detected. This value is usually referred to as cycle threshold (Ct), the time at which fluorescence intensity is greater than background fluorescence. Consequently, the greater the quantity of target DNA in the starting material, the faster a significant increase in fluorescent signal will appear, yielding a lower Ct. It is the most sensitive and the most flexible of the quantification methods (Wang and Brown, 1999) [27] and can be used to compare the levels of mRNAs in different sample populations, to characterise patterns of mRNA expression, to discriminate between closely related mRNAs, and to analyse RNA structure. RT-PCR can also circumvent time-consuming and technically demanding cloning steps and generate reagents, such as full-length complementary DNA (cDNA) inserts for cloning, or arbitrarily primed enhanced sequence tag cDNA libraries.

2. Theory of Real time PCR
PCR can be broken into four major phases the linear ground phase, early exponential phase, loglinear (also known as exponential) phase, and plateau phase (Tichopad *et al*., 2003) [23]. During the linear ground phase (usually the first 10–15 cycles), PCR is just beginning, and fluorescence emission at each cycle has not yet risen above background. Baseline fluorescence is calculated at this time. At the early exponential phase, the amount of fluorescence has reached a threshold where it is significantly higher (usually 10 times the standard deviation of the baseline) than background levels. PCR reaches its optimal amplification period with the PCR product doubling after every cycle in ideal reaction conditions. Finally, the plateau stage is reached when reaction components become limited and the fluorescence intensity is no longer useful for data calculation (Bustin, 2000) [3]. When quantifying mRNA, realtime PCR can be performed as either a one-step reaction, where the entire reaction from cDNA synthesis to PCR amplification is performed in a single tube, or as a two-step reaction, where reverse transcription and PCR amplification occur in separate tubes.

2.1 One-step real-time PCR
One-step real-time PCR is thought to minimize experimental variation because both enzymatic reactions occur in a single tube. However, this method uses an RNA starting template,

which is prone to rapid degradation if not handled properly. Therefore, a one-step reaction may not be suitable in situations where the same sample is assayed on several occasions over a period of time. One step protocols are also reportedly less sensitive than two-step protocols (Battaglia *et al*., 1998) [1].

2.2 Two-step real-time PCR

Two-step real-time PCR separates the reverse transcription reaction from the real-time PCR assay, allowing several different real-time PCR assays on dilutions of a single cDNA. Because the process of reverse transcription is notorious for its highly variable reaction efficiency (Mannhalter *et al*., 2000) [15], using dilutions from the same cDNA template ensures that reactions from subsequent assays have the same amount of template as those assayed earlier. Data from two-step real-time PCR is quite reproducible with Pearson correlation coefficients ranging from 0.974 to 0.988 (Vandesompele *et al*., 2002a) [24]. A two-step protocol may be preferred when using a DNA binding dye (such as SYBR Green I) because it is easier to eliminate primer-dimers through the manipulation of melting temperatures. However, two-step protocols allow for increased opportunities of DNA contamination in real-time PCR.

3. Types of real time quantification

3.1 Absolute Quantitation

Absolute quantitation uses serially diluted standards of known concentrations to generate a standard curve. The standard curve produces a linear relationship between Ct and initial amounts of total RNA or cDNA, allowing the determination of the concentration of unknowns based on their Ct values (Heid *et al*., 1996) [6, 3]. This method assumes all standards and samples have approximately equal amplification efficiencies (Souaze *et al*., 1996). In addition,the concentration of serial dilutions should encompass the levels in the experimental samples and stay within the range of accurately quantifiable and detectable levels specific for both the real-time PCR machine and assay. The PCR standard is a fragment of double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), or cRNA bearing the target sequence. While a DNA standard for two-step real-time PCR can be synthesized by cloning the target sequence into a plasmid (Gerard *et al*., 1998), purifying a conventional PCR product (Liss, 2002) [12], or directly synthesizing the target nucleic acid. The standard used must be a pure species. DNA standards have been shown to have a larger quantification range and greater sensitivity, reproducibility, and stability than RNA standards (Pfaffl *et al*., 2004) [18]. However, a DNA standard cannot be used for a one-step real-time RT-PCR due to the absence of a control for the reverse transcription efficiency (Giulietti, *et al*., 2001) [8].

3.2 Relative quantitation

Relative quantitation is also known as the comparative threshold method (2^{-Cq} method). This method eliminates the need for standard curves and mathematical equations are used to calculate the relative expression levels of a target relative to a reference control or calibrator such as a nontreated sample or RNA from normal tissue. The amount of target, normalized to an endogenous housekeeping gene and relative to the calibrator, is then given by $2^{-\Delta\Delta Cq}$, where $\Delta\Delta Cq = \Delta Cq$ (sample) - ΔCq (calibrator), and ΔCq is the Cq of the target gene subtracted from the Cq of the housekeeping gene (Livak and Schmittgen, 2001) [14].

In brief,

ΔCq	= Cq (GOI) – Cq
$\Delta\Delta Cq$	= ΔCq (sample) – ΔCq (calibrator)
Fold difference	= $2^{-\Delta\Delta Cq}$

4. Amplification efficiency

Amplification efficiency of the reaction is an important consideration when performing relative quantitation. Past methods of calculating gene expression have assumed the amplification efficiency of the reaction is ideal, or 1, meaning the PCR product concentration doubles during every cycle within the exponential phase of the reaction (Gibson *et al*., 1996) [6]. However, many PCRs do not have ideal amplification efficiencies, and calculations without an appropriate correction factor may overestimate starting concentration (Liu and Saint, 2002). Current mathematical models make assumptions of reaction kinetics and usually require its accurate measurement. Traditionally, amplification efficiency of a reaction is calculated using data collected from a standard curve (Rasmussen, 2001) [19]

5. Housekeeping genes and normalization

In real-time quantitative PCR experiments, specific errors will be introduced due to minor differences in the starting amount of RNA, quality of RNA or differences in the efficiency of cDNA synthesis and PCR amplification. In order to minimize these errors and correct for sample-to-sample variation, a cellular RNA is simultaneously amplified with the target, which serves as an internal reference against which other RNA values can be normalized. The most common housekeeping genes used for normalization are β-actin, a cytoskeletal protein, and glceraldehyde-3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme (Gilliland *et al*., 1990) [7] and ribosomal RNA (rRNA). These genes should theoretically be expressed at a constant level among different tissues of an organism, at all stages of development, and their expression levels should also remain relatively constant in different experimental conditions (Thellin *et al*., 1999) [22]. It is necessary to normalize the data of RT-qPCR experiments with an internal control gene to draw valid conclusions (Bustin, 2000) [3] Importantly, selection of the housekeeping gene for each specific experiment should be made very carefully as the reliability of the results depends on the choice of the most relevant housekeeping gene according to the cells of interest and specific experimental treatments. Standard housekeeping genes should be validated for each experiment to call it as a reference gene in RT-qPCR experiment (Livak and Schmittgen, 2001) [14]. Vandesompele *et al*. (2002b) [25] stated that most steadily expressed reference gene should have least deviation from the mean cycle threshold value (CT) across different samples used in the study. Minimum Information for publication of quantitative real-time PCR Experiments (MIQE) guidelines suggested usage of multiple reference genes for normalisation (Bustin *et al*., 2009) [4]. Wan *et al*. (2010) [26] recommended usage of reference gene with similar expression level as that of test gene, with cycle threshold value ranging from 15 to 30. Stability of expression of reference gene was assessed by the

variation (standard deviation and coefficient of variation) in expression across different samples examined (Kozera and Rapacz, 2013) [11]. They also supported the use of reference genes showing a value of standard deviation of Cq value below one.

6. Conclusion

Real-time technology has significantly extended the use and scope of RT-PCR assays, with the potential for quantification of mRNA targets a particular advantage. With the use of appropriate standard curves, absolute copy numbers of mRNA can easily be calculated. In addition, kinetic RT-PCR assays are readily standardised, making the comparison of results from different laboratories easier and more reliable. Therefore, real-time RT-PCR must be the method of choice for any experiments requiring sensitive, specific and reproducible quantification of gene expression.

7. References

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