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# Evaluation of real-time PCR data

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**ABSTRACT: If real-time PCR is to be of much worth to its user, some idea regarding the reliability of its data is essential. We discuss here some of the problems associated with interpreting numerical real-time PCR data that lend themselves to analytical evaluation. We translate into the language of molecular biology some of the criteria which are used to evaluate the performance of any new method (linearity, precision, specificity, limit of detection and quantification). (J Biol Regul Homeost Agents 2004; 18: 212-4)**

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## INTRODUCTION

The real-time polymerase chain reaction (PCR) is one of the most widely used techniques in modern molecular biology. It allows the reliable detection and quantification of nucleic acid sequences. Any PCR reaction can be divided into two segments: an exponential growth phase and a plateau. Theoretically, during the exponential growth phase, there is a quantitative relationship between amount of starting target sequence and amount of PCR product at any given cycle:

$$X_n = X_o (1+E)^n$$

Where $X_n$ is the amount of target sequence at cycle n, $X_o$ is the initial amount of target, and E is the amplification efficiency. The E values are included between 0 (no amplification) and 1 (every amplicon is replicated every cycle).

Real-time PCR instruments detect amplicons as they accumulate, by measuring an increase in fluorescence resulting from DNA synthesis (1). This fluorescence is generated when adding either double-stranded DNA binding dyes or fluorogenic probes in the amplification mix. Amplification curves are constructed by plotting the increase in fluorescence versus cycle number. They have three segments : an early background phase (the signal from the PCR is weaker than the background signal of the system), an exponential phase (sufficient product has accumulated to be detected and quantitated above background) and a plateau. With real-time PCR, the quantitative information that is present during the exponential phase is extracted from the amplification curve.

This technology is evolving rapidly with the introduction of new chemistries and instrumentation.

If real-time PCR is to be of much worth to its user, some idea regarding the reliability of its data is essential. For instance, the ability of real-time PCR to produce a change in signal for a defined change of the analyte quantity (analytical sensitivity) is very low. Consequently, a twofold DNA concentration change is approximately the smallest significant difference which can be observed between two samples.

We will describe and discuss here from a critical point of view some of the problems associated with interpreting real-time PCR results that are numerical and lend themselves to analytical evaluation.

### *Threshold cycle number*

How does real-time quantification work? Most quantitative approaches assume that E is constant (during the exponential phase) for any given PCR. Several dilutions of an external standard are amplified together with unknown samples. After PCR, each amplification curve is defined by its threshold cycle number (Ct). Ct values are fractional cycle numbers where amplification fluorescence levels reach a fixed threshold. Fluorescence threshold levels are set at the same position (over the background and below the plateau values) for all reactions that are compared. Standard curves are then constructed by plotting horizontally the log of the initial standard copy numbers and vertically the corresponding Ct values. A linear regression is carried out. Ct values of unknown samples are converted to concentration using the calibration curve Equation [2].

### *PCR efficiency*

The efficiency (E) of a PCR reaction can be deduced from the slope (S) of standard curves:

$$E = 10^{-1/S} - 1$$

Repeated runs of the same standard curve give variations around 1% in slope. This affects the

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variation in E (around 2%). This approach assumes that the efficiency of any given PCR is constant over a range of initial template concentrations. The effect of efficiency is exponentially dependent on cycle number. If E = 1, amplicon quantity is duplicated every cycle. If E = 0.8, amplicon quantity is only duplicated every 1.2 cycle.

*Quantification*

Final quantitative results are always reported relative to something (DNA or cDNA copies per milliliter of blood, per cell…). If relative changes are important (relative quantification), the denominator may be an endogeneous control (such as a house-keeping gene). If absolute changes in copy number are important, then the denominator must be invariant (absolute quantification). Careful use of controls is critical, in order to demonstrate that the choice of denominator is adapted to the analytical problem.

In the standard curve method, the input amount for unknown samples is calculated from the standard curve of a specific gene (and normalized to the input amount of a reference gene if the quantification is relative). This approach requires the construction of one standard curve for each individual PCR.

With the comparative Ct method (3-5), normalized amount of target gene is calculated from the difference of Ct (ΔCt) between target and control genes. For the ΔCt calculation to be valid, the efficiencies of each target and control amplifications must be approximately equal.

Other quantification methods (of restricted use) are based on the calculation of E for each individual reaction (6), without the need for calibration curves or even validation experiments.

*Specificity*

PCR specificity is strongly dependent on primer design. Mis-priming events are detected by post-PCR manipulations. With real-time PCR, amplification and detection of target are performed simultaneously (without post-PCR manipulations). In case of non-specific amplification (not a desirable situation), amplicon detection must be specific.

The disadvantage of using a double stranded-DNA dye for real time PCR is that the dye detects any double-stranded DNA generated during PCR (7). Specific amplicons and primer dimers, for instance, are detected equally as well. It is often said (but not always true) that the use of fluorogenic probes eliminates this problem (8).

*Precision*

Real-time PCR studies must include estimates of “within-run” and “total” standard deviations. Each should be determined at various target concentrations that are ideally in an appropriate biological matrix. Within-run precision associated with Ct values are of about 1% of variation coefficient for copy numbers ranging from 100 up to 100,000 copies of target DNA. For lower copy numbers, the precision on Ct values is poor (coefficient of variation >1%).

*Accuracy*

For absolute quantification, the accuracy of external standard quantification depends entirely on the accuracy of the standards. Care is thus needed for the design, synthesis, purification and calibration of DNA standards. Absorbance at 260 nm, fluorescence measurement with DNA dyes and limiting dilution assays are ways to calibrate standards. If your quantification method is relative, it is not important to know the actual copy number of the standards.

*Detection limit*

The detection limit is often defined as the concentration corresponding to a signal 3 standard deviations above the mean for a calibrator that is free of analyte. This definition is not operational for real time PCR. There is no noise in real-time PCR. Theoretically, even a single copy of a target DNA is amplified by PCR and thus generates an amplification curve above the fluorescence noise. However, at very low analyte concentrations, the solution composition is not homogeneous. The sampling error (Poisson error) determines the lowest concentration of target DNA that can be detected. In practice, 10 copies of target DNA per PCR vessel is the lowest concentration which is amplified each time a PCR assay is performed.

*Qualification limit*

The quantification limit is often defined as the analyte concentration corresponding to a signal 10 standard deviations above the mean for a calibrator that is free of analyte. Again, this definition is not operational for real-time PCR. PCR quantification limit may be set around 100 copies of target DNA per PCR vessel (9). Indeed, above this value, coefficients of variation on Ct values are more or less constant. Under this value of 100 copies, coefficients of variation are comparatively larger.

*Linearity*

The dynamic range of real-time PCR is of at least six order of magnitude. Within this range, the relationship between log of target DNA and Ct values is linear.

However, deviations from linearity may be observed. On one hand, for high analyte copy numbers, partial or total inhibition of PCR amplification is sometimes observed. On the other hand, the sampling error explains the underestimate

observed for the quantification of target copy numbers lower than 100.

## Conclusions

Real-time PCR is a powerful technique that gives quantitative answers difficult to obtain with end point PCR. The challenge of today is to design experimental protocols that are rigorously validated. Each step of any real-time PCR based assay must be controlled, from sampling to PCR, including manipulations like extraction and reverse transcription. The evaluation of analytical parameters such as linearity, precision, accuracy, specificity, recovery, limit of detection, robustness, reference interval (and so on) helps us to achieve this goal. Statistical tools such as control charts allow us to study, in the real-time PCR process, the impact of various factors that change over time.

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