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Original Article

How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments

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ABSTRACT

We have examined the imprecision in the estimation of PCR efficiency by means of standard curves based on strategic experimental design with large number of technical replicates. In particular, how robust this estimation is in terms of a commonly varying factors: the instrument used, the number of technical replicates performed and the effect of the volume transferred throughout the dilution series. We used six different qPCR instruments, we performed 1–16 qPCR replicates per concentration and we tested 2–10 μl volume of analyte transferred, respectively. We find that the estimated PCR efficiency varies significantly across different instruments. Using a Monte Carlo approach, we find the uncertainty in the PCR efficiency estimation may be as large as 42.5% (95% CI) if standard curve with only one qPCR replicate is used in 16 different plates. Based on our investigation we propose recommendations for the precise estimation of PCR efficiency: (1) one robust standard curve with at least 3–4 qPCR replicates at each concentration shall be generated, (2) the efficiency is instrument dependent, but reproducibly stable on one platform, and (3) using a larger volume when constructing serial dilution series reduces sampling error and enables calibration across a wider dynamic range.

1. Introduction

Literature search (e.g. Pubmed) for scientific publications using the keyword "quantitative PCR" (qPCR) retrieves hundreds of thousands of hits, manifesting that qPCR has become mainstream life sciences technology [1–3]. It is widely acknowledged as the most sensitive method to quantify minute amounts of nucleic acids and its applications split into two main types referred to as: relative [4,5] and absolute [6–8] quantification. In relative quantification the analyte, often reverse-transcribed mRNA or microRNA, is quantified relative to an endogenous reference [4,5]. In absolute quantification the targeted nucleic acid (the analyte) is measured relative to a set of standards used to construct a standard curve [6–8]. The established name "absolute quantification", is rather confusing, since absolute value are never determined; a more appropriate name would be "calibration", as the concentration of the field sample in fact is measured "relative" to the concentrations of the standard samples.

The standard curve is also used to assess the performance of qPCR assay by estimating its efficiency [9] and optionally also determining the assay dynamic range, limit of detection and limit of quantification. For the estimation of PCR efficiency the standard used to construct the standard curve does not have to be calibrated. The efficiency (E) of PCR is defined as the fraction of target molecules that are copied in one PCR cycle [10,11]. A properly designed assay shall, in the absence of interfering substances in the sample matrix, amplify target DNA with at least 90% efficiency [12,13]. However, the experimental determination of PCR efficiency has been subject of many discussions, resulting even in some very inappropriate recommendations, such as to perform

Abbreviations: RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ANCOVA, analysis of covariance; E, PCR efficiency; Cq, cycle of quantification; GMO, genetically modified organism; ISO, International Organization for Standardization; IEC, International Electrotechnical Commission; RIN, RNA Integrity Number; NTC, no template control; FDA, food and Drug Administration; EPA, Environmental protection agency; CLSI, Clinical and Laboratory Standards Institute; MIQE, minimum information for publication of quantitative real-time PCR experiments.

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separate standard curves with few data points in every qPCR run to account for inter run variation. There is also a qPCR community that focuses on alternative procedures estimate PCR efficiency based on the analysis of individual amplification curves [14,15]. In spite of these heroic efforts, the standard curve remains the most reliable and robust approach to estimate PCR assay efficiency that is broadly accepted by the community [16], while some of the alternative approaches have found use as quality control tools in high-throughput setups [17–19].

The estimation of PCR efficiency by means of a standard curve involves generating a series of samples with controlled relative amounts of targeted template. These samples are usually constructed by serial dilution of a concentrated stock solution, most frequently using 10-fold dilution steps. The so prepared standard samples are analyzed by qPCR measuring the quantification cycle (Cq) using standard procedures. A plot of the Cq's versus the logarithm of the target concentrations is constructed and is expected to be linear with a negative slope. For a 10-fold dilution series the slope is −3.33 when $E = 100\%$. This follows from the assumption of a perfect doubling of the number of DNA template molecules in each step of the PCR (Eq. (1)).

$$N_x = N_0 2^x \tag{1}$$

Where N_x is the number of target molecules after x cycles and N_0 is the initial number of double stranded target molecules. If the initial template is single stranded, such as cDNA, the first PCR cycle produces its complement rather than doubling it (Eq. (2)).

$$N_x = N_0 2^{(x-1)} \tag{2}$$

In practice, perfect doubling of the number of molecules in every cycle is highly uncommon; rather a fraction only is copied, which is the PCR efficiency (E). Hence, E is a number expected to be between 0 and 1 and is frequently expressed as percentage (Eq. (3)).

$$N_x = N_0(1+E)^{(x-1)} \tag{3}$$

For example, let say a test tube contains 100 target molecules and after one amplification cycle it contains 180 molecules, $E = 80\%$, since 80% of the target molecules present were amplified. In practice we do not measure the number of amplicons; rather we measure the fluorescence (I) from dyes or probes present in the reaction mix that bind to the amplicons formed (Eq. (4)).

$$I = k'_{(\text{assay\_1})} \times N_x \tag{4}$$

The fluorescence (I) depends on the amount of amplicon formed (N_x) and k' is a proportionality constant. It reflects the amount of fluorescence produced per amplicon formed, and may change during the course of the reaction as the reporter/DNA ratio changes. The thermodynamics behind is complex, although some brave attempts to model it have been made [20]. Modelling the thermodynamics is, however, not needed in order to compare samples at a fluorescence threshold ($I_{\text{threshold}}$), which is the normal practice to analyze qPCR data, since at threshold all reactions based on the same assay contain the same number of amplicons (Eq. (5)):

$$I_{\text{threshold}} = k' \times N_0(1+E)^{(\text{Cq}-1)} \tag{5}$$

and the effect of k' cancels. Rearranging Eq. (5) produces the relation between PCR efficiency and the slope of the standard curve found in textbooks (Eq. (6)) [2,21].

$$E = 10^{-(1/\text{slope})} - 1 \tag{6}$$

The PCR efficiency depends on many factors including: (1) the assay performance, which depends on the primers' and template sequences and structures. Secondary structure and opportunity for undesired intra-molecular interactions reduce PCR efficiency; (2) the sample matrix, which may contain inhibitors and other interfering substances from the sample or carry overs agents from upstream processing steps; (3) reagents used and their concentrations. Essentially, any of the PCR reagents can be rate and performance limiting [22] including PCR protocol; and (4) competing reactions.

The samples shall be tested for inhibition, which is easy done using RNA or DNA spikes [23,24]. It can also be observed by performing a serial dilution [25]. In fact inhibition is often the cause of unrealistic PCR efficiency estimates ($E > 100\%$) as it is pronounced in the most concentrated samples leading to deviation from linearity. If ignored and mistakenly included in the linear fit, those samples reduce the slope leading to too high PCR efficiency estimates. In some cases inhibition is pronounced only in the upstream reactions such as the reverse transcription and not noticed in qPCR.

A template must be chosen for the assessment as well as the matrix. Choosing a matrix characteristic of the field sample the estimated efficiency will reflect the performance of the PCR assay in the actual samples that will be analyzed. This, however, requires pure matrix is available. Usually a new assay is first validated in a pure matrix devoid of interfering agents. Assays that show high PCR efficiency are robust and will be less prone to inhibition in complex matrices. Purified PCR product is often used as template for PCR efficiency estimates, because it is easy to produce. However, it often leads to side reactions because of its short length, and it does not reflect the effect of flanking sequences that may interfere with PCR by wrapping onto the template [26]. Such interference can be significant in the initial cycles of the PCR, when the original template is abundant, and influence the measured Cq. For validation of assays for gene expression profiling a cDNA library is a suitable source of long template molecules with representative secondary structures. Genomic DNA or plasmids containing the gene of interest can be used as standard for validation of assays for DNA analysis, preferably after excising a fragment containing the target sequence to remove interfering supercoiling [26]. Still another option is to use synthetic templates (e.g. gBlocks – IDT, GeneArt – LT).

The performance of new assays needs to be tested by means of specificity, efficiency and sensitivity (sometimes also for limits of detection and quantification). While properties of a good qPCR assay are well described by means of specificity in MIQE guidelines [16], where tests and optimal criteria are recommended, e.g. in silico BLAST (single unique complementarity), electrophoresis (single band of correct size), melt curve (single peak in target amplification, no peaks in NTC while Cq of NTC ≤40 can be ignored if ΔCq of NTC and target is ≤5), "no RT" control (ΔCq of no_RT and RT ≤5). Detailed piece of information about what parameters to use for optimal efficiency estimate using standard curves was missing and our work offers detailed evidence. The PCR efficiency is one of the most important indicator of the performance of a qPCR assay and is also required parameter for quantitative analysis when fold changes are calculated. Proper usage of PCR efficiency in qPCR analysis requires it is estimated with high precision. Inaccurate estimations of E_x can lead to substantial under- or overestimation of the calculated fold change, particularly when large differences in expression are measured (Fig. 1).

The aim of this study is to test the impact of three experimental factors on the precision of the estimated PCR efficiency: (1) the effect if qPCR instrument changes; (2) the effect of how many technical replicates are included; and (3) the effect of the volume transferred across dilutions.

2. Results

We tested three experimental factors in terms of precision of the estimated PCR efficiency: (1) the effect of the qPCR instrument; (2) the impact of the number of technical replicates; and (3) the effect