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The Pitfalls of Cut-off Values in Real-time, Quantitative PCR: Should Target Density or Amplification Cycle Threshold be used for Interpreting qPCR Results?

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Abstract

Quantitative real-time PCR (qPCR) methods are now routinely used and have advanced to allow for high-throughput amplification of multiple targets in a single qPCR run. Where algorithms and comparisons are used to interpret the results of different assay-sets (primers and probe for one target), or multiple assay-sets in a run (a reaction-set), between labs, or with different reagents, the recommendations for analysis should be updated to account for heterogeneity in assay-set performance.

Introduction

Cycle of quantification (Cq) cut-off values are routinely applied to qPCR to ensure results reproducibility [1] and to control for amplification and fluorescence artifacts [2]. Normally, amplification for a specific target where the Cq is lower than the cut-off value will be interpreted as positive, while amplification with Cq above the cut-off value will be interpreted as equivocal or negative. Concerns have been raised that the use of Cq values that are either too high or low can result in the reporting of false positive or false negative results, respectively [3]. Further, an arbitrary Cq value does not account for variations in the efficiency of different assay-sets. Assays can be optimised for sensitivity, specificity, precision, accuracy, and limit of detection (LOD); however, differences in the calibration or standard curves remain, resulting in variation in the calculated concentration when applying a single Cq cut-off across multiple assay-sets.

In a hypothetical reaction-set, containing assay-sets A and B, we presume both assay-sets are well optimised, with efficiency of 90-110%, the correlation coefficient >0.98, the LOD equivalent to ≤10-100 copies, inter- and intra-assay variations <0.1 standard deviation (SD), and an accuracy ratio within ± 0.1. Assay-A has a calibration curve equation of y = -3.5x + 36.356 and is more efficient at detecting its respective target compared to Assay-B with the calibration curve y = -3.1x + 39.345. If a Cq cut-off value of 35 is applied to both assays, then positive samples with a $\log_{10}$ concentration of <1 unit and <2 units will be excluded from the analysis for Assay-A and Assay-B, respectively. A sample is thereby 100 times more likely to be reported as positive when amplified with Assay-A compared to Assay-B just because Assay-A is more efficient at amplifying its target. These differences have been observed in a study, where up to a 1000-fold difference was reported between different assays detecting SARS-CoV2 [4]. Further, where an algorithm is applied to interpret results within a typing scheme (Table 1), the use of an arbitrary Cq cut-off may incorrectly type a sample as Type 1, due to the higher efficiency of Assay-A where the copy number of the target is close to the limit of detection.

Uniform Cq cut-off values applied across multiple assay-sets hinder our ability to interpret findings from molecular qPCR reaction-sets holistically, and biases toward more efficient assay-sets. While this may be inconsequential when comparing a single target, for example, seasonal Influenza within the same setting over time, it compromises our ability to compare qPCR results across different settings where different assay-sets or reagents have been used to amplify the same target. Further, this complicates the comparison of findings for several targets amplified in the same cohort. For example, molecular qPCR methods are currently being optimised to detect over 100 serotypes of *Streptococcus pneumoniae* to monitor the impact of routine pneumococcal conjugate vaccine (PCV) immunization on serotype distribution [5,6]. Using a single Cq value as a cut-off to interpret these results will not account for variations in the efficiency of the multiple assay-sets targeting these serotypes and would introduce some bias when comparing the prevalence of one serotype to another in the same cohort.

Alternatively, the use of concentration density calculated from the calibration curve would correctly account for variation in the efficiency of assay-sets, as the performance of each assay-set would be normalised to allow for interpretation and comparison of results from different assay-sets. For instance, we have previously published a detection and typing method that included an algorithm applied to three assay-sets to distinguish *S. pneumoniae* individual serotypes 6A, 6B, 6C, and 6D within serogroup [7]. In our method, the algorithm mistyped or failed to detect a small proportion of serotype 6A (3%) and 6B (6%) that were previously detected using standard culture-based methods, as the arbitrary Cq cut-off value did not adjust for assay-set efficiencies. If we re-analyse the same dataset using the concentration density of 1 $\log_{10}$ colony-forming units/ml as a cut-off and apply the algorithms, the method is now able to correctly type 33% (3/9) of 6A previously mistyped as 6B. Further, an additional 50% (2/4) of the samples typed as Serotype 6A and 33% (1/3) as Serotype 6B that were missed when using a Cq cut-off, were correctly typed by using a density-based cut off. This equates to a 7.1% increase in sensitivity for serotype 6A (87.2 and 94.3% for Cq and Density cut-offs, respectively), a <1% increase for 6B (93.9 and 94.7%, respectively), and no change in specificity of our assay when compared with the referent culture-based Quellung serotyping method.

There are, however, some limitations to using a density-based cut-off:

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Table 1. Hypothetical typing algorithm based on assay-sets A and B.

Assay-sets	Type 1	Type 2
Assay-A	+	-
Assay-B	+	+

- The cut-off density should not be lower than the LOD for the assay.
- The guidelines for Minimal Information for Publication on Quantitative Real-Time PCR Experiments (MIQE) should be applied together with the linear equations used to calculate density.
- Where possible standard calibration curves should be included in each qPCR experiment, or alternatively a Levy Jennings Plot should be constructed to ensure calibrators included on different plates remain in the same range (i.e., they do not vary by more than 2 SD of the average).

Conclusion

In conclusion, Cq values are specific to a particular assay-set and cannot be compared directly between different settings, assay-sets or within a reaction-set due to variations in efficiency. To circumvent this limitation, density should be calculated from the calibration curves and used as cut-off to allow for comparison, as the performance of each qPCR reaction will be normalised.

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