C_{rt}, a relative threshold method for qPCR data analysis on the QuantStudio 12K Flex system with OpenArray technology

Introduction

Data analysis associated with quantitative real-time PCR (qPCR) depends upon the concept of threshold cycle (C_t): the cycle at which the level of fluorescence from accumulating amplicons crosses a defined threshold. The most common method of quantitation, based on this measurement, can be referred to as the C_t method, or "baseline threshold" method [1]. An alternative method called the C_{rt} method, or "relative threshold" method, has proven to be more robust for analyzing data generated on the Applied Biosystems™ QuantStudio™ 12K Flex Real-Time PCR System using Applied Biosystems™ OpenArray™ plates. OpenArray plates are unique to the QuantStudio 12K Flex system—instead of wells, the OpenArray plates contain 3,072 through-holes that enable very low-volume (33 nL) reactions. The C_{rt} method accounts for these low reaction volumes and associated differences in fluorescence levels by analyzing the amplification curve from each through-hole individually.

Here we describe the application of the C_{rt} method on the OpenArray platform and compare its performance to the traditional C_t method. We show that on the OpenArray platform, the C_{rt} values correlated very well to C_t values generated by the baseline threshold method. The relative threshold method gave reproducible C_{rt} values for replicate assays run on the same plate, and the variation between sample replicates was consistently lower than with the C_t method. In addition, fold-change measurements between normal and test samples were comparable between the two methods.

Following MIQE recommendations [1], we will use C_q when referring generically to fractional cycle values. When distinguishing between the traditional baseline threshold method and the relative threshold method, we will use C_t and C_{rt}, respectively.

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OpenArray data analysis

The OpenArray plate consists of 3,072 through-holes that each hold a reaction volume of 33 nL (Figure 1). Hydrophilic and hydrophobic coatings enable the reagents to stay in the bottomless through-holes via surface tension. The low reaction volume and the very nature of the array through-holes can result in differences in the absolute height of the amplification curve or variability in the baseline fluorescence in early cycles. Thus, there is potential for greater variation across the plate than with other platforms, when using traditional analysis methods such as the C_t method.

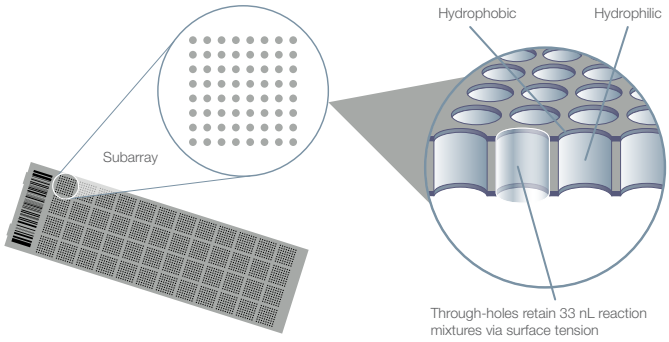

Figure 1. OpenArray sample plate showing through-holes.

Unlike the C_t method, which considers all the curves for a specific target to determine the threshold, the C_{rt} method sets a threshold for each curve individually that is based on the shape of the amplification curve, regardless of the height or variability of the curve in its early baseline fluorescence. The method first estimates a curve that models the reaction efficiency from the amplification curve. It then uses this curve to determine the relative threshold cycle (C_{rt}) from the amplification curve. The C_{rt} algorithm computes a C_q that is roughly in the middle of the exponential growth region. Thus, the difference between the C_t method and the C_{rt} method lies in the steps used to determine the C_q (see Appendix, Figure 5).

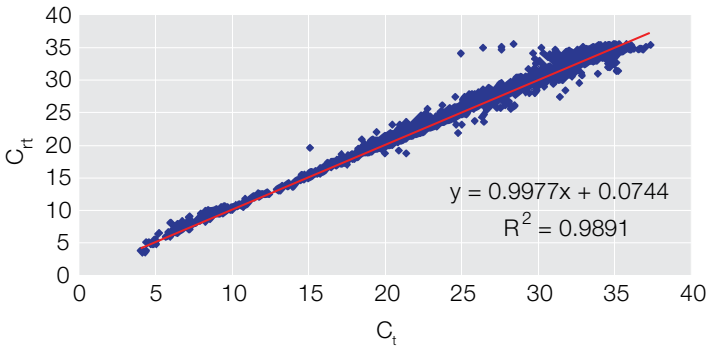

Figure 2. Strong correlation between C_{rt} and C_t. A total of 7,710 individual TaqMan Assays with a wide range of C_q values (~4 to ~37) were run on the OpenArray platform. C_{rt} and C_t values were generated for each through-hole, and strong correlation (R^2 = 0.9891) was observed for the two methodologies.

To test the robustness of the method, thousands of individual Applied Biosystems™ TaqMan™ Assays were run on the OpenArray platform, and the C_{rt} and C_t values generated for each through-hole were compared (Figure 2). C_q values spanned ~10 log units (~4 to ~37) and included hundreds of targets. Strong correlation (R^2 = 0.9891) was seen for the two sets of C_q values, indicating that the two methodologies give comparable C_q values across a large dynamic range. Although correlation in the high C_q range is very good, for gene expression analysis we recommend removing C_{rt} values >28 (C_{rt} = 28 is ~1 copy) to avoid results that may be stochastic. Table 1 provides a summary and comparison of the two methods.

Table 1. Comparison of baseline threshold (C_t) and relative threshold (C_{rt}) methods.

	C_t method	C_{rt} method
Baselining	Amplification curve–specific	No baselining
Threshold	Target-specific	Curve-specific
C_q	Target-level C_t values	Curve-level
Curves	Amplification curves	Reaction efficiency curves

Results derived from C_{rt} and C_t are comparable

To confirm reproducibility of the algorithm across a plate on the OpenArray system, we ran total RNA converted to cDNA from normal liver and liver carcinoma tissue samples in duplicate on the Applied Biosystems™ TaqMan™ OpenArray™ Human Cancer Panel (Cat. No. 4475391). This panel consists of TaqMan Assays targeting 624 genes that have been implicated in cancer, and 24 endogenous control genes. We found that the C_{rt} values were reproducible for replicate assays across the same OpenArray plate. Previous studies using a larger number of technical replicates showed greater repeatability with C_{rt} compared to C_t. Of the 2,589 technical replicate sets (n = 2 or 4) tested in this study, the average standard deviation (SD) of the C_{rt} was lower more than 60% of the time when compared to the C_t (data not shown).

When we compared the SD of the C_t (n = 2) to the SD of the C_{rt} with the OpenArray Cancer Panel data set, we saw very good reproducibility with both algorithms (Figure 3). However, the C_{rt} replicates had greater repeatability as measured by the average SD of the C_q (average SD of C_{rt} = 0.111; average SD of C_t = 0.168). These results were consistent with the earlier study in which more than 60% of the time the SD of the C_{rt} was lower.

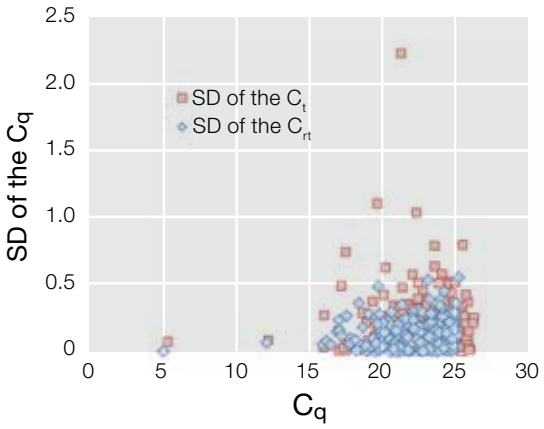

Figure 3. C_{rt} gives smaller standard deviations than the C_t method. Duplicate cDNA samples from normal and carcinoma tissue were run on the TaqMan OpenArray Human Cancer Panel on the QuantStudio 12K Flex system. The data were analyzed using either the C_{rt} or C_t method. Only genes with C_{rt} <25 and good amplification quality (Amp Score > 1.1, C_q confidence >0.8, see reference 2) were included. The C_{rt} replicates had greater repeatability, as measured by the average SD of the C_q (average SD of C_{rt}: 0.111; average SD of C_t: 0.168). Blue diamonds = C_{rt}; red squares = C_t.

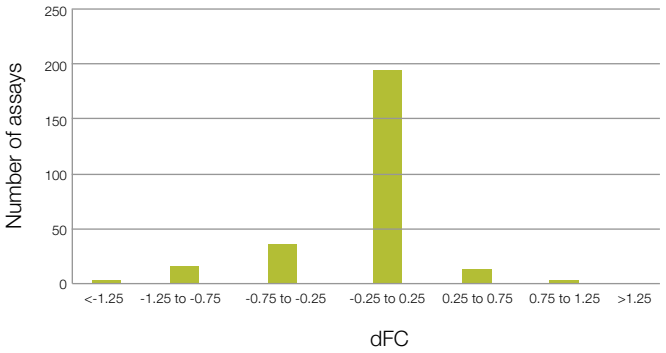

Figure 4. Fold-change analysis: C_{rt} is comparable to C_t. Fold changes (FC) between cancer and normal cells were determined using the $2^{-\Delta\Delta C_q}$ method. The range and distribution of difference between FCs from the C_{rt} method and from the C_t method (dFC) is shown. The FC differences are binned in 0.5 increments.

Fold change results

When we compared fold change (FC) values using C_{rt} and C_t, we found that they were very similar. Figure 4 shows the differences between FCs (for normal vs. carcinoma liver samples) obtained using C_{rt} vs. C_t. FC was calculated using the $2^{-\Delta\Delta C_q}$ method [2]. We found that the majority of targets had FC values within ±1 (equivalent to $\Delta\Delta C_q$ = ~0.5), and more than 70% of the data had $\Delta\Delta C_q$ of <0.25. Assays that had the largest FC between normal and cancer tissue (FC >10) also had large FC differences between the methods. In this range, a small difference in the $\Delta\Delta C_q$ can result in a larger FC difference.

Conclusion

To account for the small sample volume (33 nL) and inherent differences in the nature of the OpenArray plates (through-holes vs. wells), we developed a new quantitation method for the OpenArray platform on the QuantStudio 12K Flex system. Unlike the C_t method, the C_{rt} method computes the threshold using the individual curve. We found that for the OpenArray system, the C_{rt} method of analyzing qPCR data is clearly superior to the traditional C_t method. The C_{rt} method produces lower variation across replicate samples while maintaining the same dynamic range. Relative gene expression results are comparable. In addition, the new method enables easier handling and faster analysis of large experimental data sets since you do not have to reanalyze the entire set when additional experiments are completed.

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Appendix

The relative threshold (C_{rt}) method

The relative threshold method calculates C_q values for each individual amplification curve, and no information is needed from the other curves. The amplification curve is first set to a relative scale by setting the minimum relative fluorescence value to 0 and the maximum value to 1. A curve that models the reaction efficiency is calculated on a 0–1 scale such that the early cycles are around 1 and the later cycles are close to 0. Using an empirically predetermined reference fluorescence value and a proprietary algorithm, a common point on the reaction efficiency curve is identified and used to map back to the original amplification curve. This fractional cycle value is ultimately reported as the C_q. A step-by-step description is shown in Figure 5.

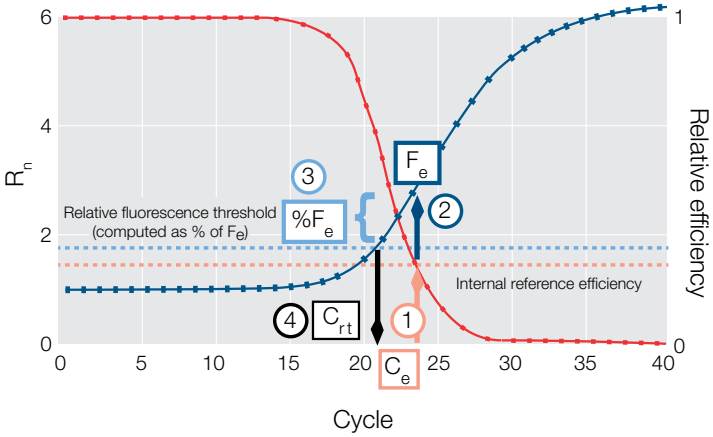

Figure 5. The relative threshold (C_{rt}) method. The figure and steps below describe how the C_{rt} is calculated. The amplification curve is in blue; the model of the reaction efficiency curve is in red; the y-axis on the left goes with the blue amplification curve, and the y-axis on the right goes with the red curve. C_{rt} is determined in four steps:

1. A predetermined internal reference efficiency level (pink dotted line) is used to identify the fractional cycle (C_e) where the reaction efficiency curve (model) reaches a specific value.
2. The fluorescence level (F_e) corresponding to the fractional cycle C_e on the amplification curve is determined.
3. The relative fluorescence threshold (light blue dotted line) is a curve-specific threshold computed as a specific percentage of F_e (%F_e).
4. The C_{rt} is computed as the fractional cycle where the amplification curve crosses the Relative Fluorescence Threshold.

References

1. Bustin SA, Benes V, Garson JA et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55:611–622.
2. Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System—multi-well plates and array card experiments. PN 4470050, Rev A.

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