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Comparing real-time quantitative polymerase chain reaction analysis methods for precision, linearity, and accuracy of estimating amplification efficiency

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ABSTRACT

New methods are used to compare seven qPCR analysis methods for their performance in estimating the quantification cycle (C_q) and amplification efficiency (E) for a large test data set (94 samples for each of 4 dilutions) from a recent study. Precision and linearity are assessed using chi-square (χ^2), which is the minimized quantity in least-squares (LS) fitting, equivalent to the variance in unweighted LS, and commonly used to define statistical efficiency. All methods yield C_qs that vary strongly in precision with the starting concentration N_0, requiring weighted LS for proper calibration fitting of C_q vs $\log(N_0)$. Then χ^2 for cubic calibration fits compares the inherent precision of the C_qs, while increases in χ^2 for quadratic and linear fits show the significance of nonlinearity. Nonlinearity is further manifested in unphysical estimates of E from the same C_q data, results which also challenge a tenet of all qPCR analysis methods — that E is constant throughout the baseline region. Constant-threshold (C_t) methods underperform the other methods when the data vary considerably in scale, as these data do.

The development of real-time quantitative polymerase chain reaction (qPCR)[1] methods has greatly facilitated the quantification of small amounts of genetic material [1]. The target substance is amplified through a cyclical heating/cooling process, during which the amount of the target roughly doubles in early cycles. Unfortunately this early amplification is not directly observable in most procedures, because the optical fluorescence that is commonly used to monitor the reaction progress is dominated by background contributions. Eventually the product fluorescence rises above the background, in the growth phase; but within a few cycles thereafter the process begins to saturate in the approach to the final plateau stage. Typical data are illustrated in Fig. 1, which includes profiles for four initial concentrations of the target gene, giving curves similar in shape but shifted along the cycle axis. Data for different known starting amounts of the target can be used to determine the amount of an unknown, through calibration procedures based on the exponential growth equation,

$$y = y_0 E^x, \tag{1}$$

where E is the amplification efficiency, ranging from $E = 1$ (no amplification) to $E = 2$ (perfect doubling), x is the cycle number, and y represents the fluorescence signal from the target gene, which is assumed to be proportional to the number of target molecules N. Calibration can be accomplished by associating certain cycle location indices with fixed amounts of the amplified target material. For these location benchmarks, which are labeled collectively as C_q (quantification cycle, see below), Eq. (1) implies:

$$y_q = y_0 E^{C_q}, \tag{2}$$

and a plot of C_q vs $\log(N_0)$ provides the desired calibration relation, where N_0 is the number of target molecules before amplification.

In the two decades that real-time fluorescence monitoring of the PCR reaction has been in use [2], many procedures have been described for analyzing the resulting data [3–53]. Some of these are directed toward a better determination of C_q. Others attempt to estimate the starting amount y_0 through Eq. (2) or variants thereof. This requires estimation of E, since initial hopes that E could be taken as 2 for cycles in the baseline region [11] have

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[1] *Abbreviations and symbols used:* qPCR, quantitative polymerase chain reaction; y and y_0, fluorescence signal above baseline at cycle x and at cycle 0; E, amplification efficiency; C_q, quantification cycle; y_q, signal at $x = C_q$; N_0, initial amount of target nucleic acid in sample; C_t, threshold cycle, where $y = y_q$; FDM and SDM, cycles where y reaches its maximal first and second derivatives, respectively; C_{y0}, intersection of a straight line tangent to the curve at the FDM with the baseline-corrected x-axis; LS, least squares; χ^2, chi-square; w_i, statistical weight for ith data point; σ^2 and σ, variance and standard deviation; S, sum of weighted, squared residuals (="Chisq" in KaleidaGraph fit results, = χ^2 when $w_i = 1/\sigma_i^2$); ν, statistical degrees of freedom, = # of data points − # of adjustable parameters; SE, parameter standard error; FPLM, DART, Miner, Cy0, 5PSM, LinReg, and FPK, published qPCR analysis algorithms.

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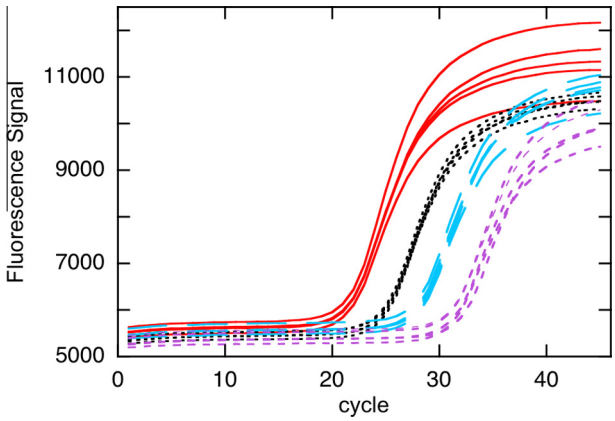

Fig.1. Representative qPCR fluorescence curves, from the 94 × 4 Reps technical dataset in Ref. [1]. Shown are 5 of the 94 replicates at each of the 4 dilutions, spanning a starting concentration range of 1000.

not been borne out [23,38]. The ultimate goal is reliable estimation of y_0 from the data for a single experiment. This goal has proved elusive and will likely remain so, because there is no direct experimental information about y from the early cycles, where the signal is buried in the background. Thus any attempt to extrapolate back to cycle 0 requires assumptions about E in this region. While it may be possible to bolster such assumptions by accumulated experience for specific genetic targets, estimating y_0 purely from single-run data will likely have to retain the assumption of constant E in this region until methods can be devised to permit its direct estimation here. Even if E can be determined, most y_0 methods also require reliable estimates of C_q. Accordingly, in this paper we emphasize the estimation of C_q and its subsequent use in calibration when data are available for multiple dilutions.

Fig. 2 shows several commonly used C_q benchmarks for idealized qPCR trajectories. The first- and second-derivative maxima (FDM and SDM) are the (noninteger) cycle values where those derivatives reach their maxima. C_t is the cycle where the fluorescence reaches a specified threshold level y_q above the baseline. C_{y0} is the intersection of a line tangent to the curve at the FDM with the baseline-subtracted signal level [37]. If the curves are all of the same shape, shifted along the cycle axis, then all of these markers are equally valid for calibration [38]; and the optimal choice is that

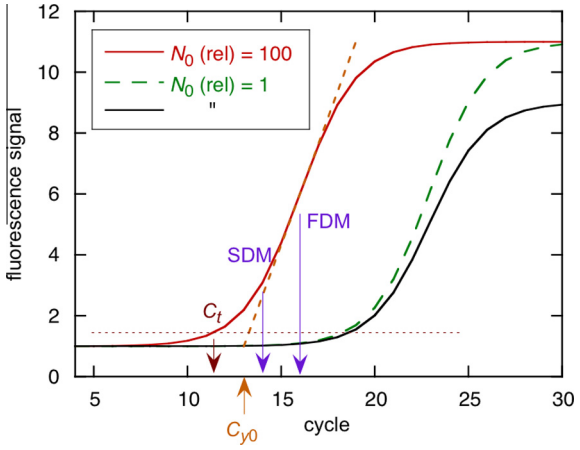

Fig.2. Synthetic qPCR curves, showing for the first (highest-N_0) curve 4 common location indices used as C_q: C_t = 11.4 (for y_q = 0.44, horizontal dashed line), C_{y0} = 13.0, SDM = 14.0, FDM = 16.0. Curves were generated with the logistic equation, $y = b + a/[1 + \exp(c(x_0 - x))]$, with baseline b = 1 and amplitude a = 10 for the first two, with a reduced to 8 for the third. The second and third curves (N_0 = 1) share a common x_0 (=FDM) of 22.89, consistent with E = 1.95.

which can be determined most precisely. However, for the purpose of estimating y_0 with Eq. (2), C_q must be taken within the range where the amplification is thought to follow Eq. (1); this exponential growth phase is commonly taken to end at the SDM or earlier. Note that the FDM, SDM, and C_{y0} are all insensitive to scale changes of the sort shown for N_0 = 1 in Fig. 2. However, C_t is sensitive to scale, as it is based on an absolute threshold level. For this reason the data are often scaled to a common plateau level ("normalized") by the instrumental software prior to analysis. With data like those in Fig. 1, where most curves do not achieve a clear plateau level, such scaling is difficult to implement without fitting to a functional form containing a plateau parameter, like the logistic function used to produce Fig. 2.

A major deficiency in the development of new qPCR analysis methods has been the lack of suitable data sets on which new methods can be compared objectively with existing methods. Recently Ruijter et al. [1] have taken a big step toward remedying that deficiency, in a comprehensive examination of some of the more popular qPCR analysis methods in medical diagnosis applications and in more purely mathematical tests. Their study employed large data sets, analyzed by 9 methods to obtain estimates of C_q and E, and they have made all these data and results available for further such work. Here we illustrate how the χ^2 statistic can be used to assess precision and linearity in the C_q estimates when data are available for multiple dilutions of the target gene. Although some of our results are specific to the data set used for this illustration, the methods will be straightforwardly applicable to results for other test data sets when they become available.

χ^2 is the minimization target in weighted and unweighted regression, equivalent to the estimated variance for the latter. Accordingly, it has a simple physical significance, leading to its use in defining the *statistical efficiency*: Increases in χ^2 are equivalent to proportionate increases in the experimental effort (number of data values) needed to maintain a stated precision. In calibration fitting, its dependence on the choice of calibration function (cubic, quadratic, linear) is thus a simple quantitative measure of the importance of nonlinearity. By contrast, the widely used R (R^2) has no such simple interpretation, though it is mathematically related to χ^2 (see below).

In the following sections, we show that the C_q values for the 94 × 4 Reps technical data set from Ref. [1] (Fig. 1) require weighted least squares for proper calibration analysis — a result that is likely to be generally applicable to C_q calibration fitting but appears not to have been noted before. We find that the methods examined in Ref. [1] vary by more than a factor of 3 in statistical efficiency but typically show efficiency losses <20% from nonlinearity. We also address the estimation of PCR amplification efficiency, and we argue that estimates of E from C_q for multiple N_0 *do* pertain to the early cycles of amplification. The resulting E estimates challenge a basic assumption of most y_0-estimation methods: that E is constant over the baseline region. Finally, we identify the source of the poorer performance by some of the qPCR analysis methods as an experimental flaw that produces variability in the scale of the data profiles, leading to the aforementioned systematic errors in C_t when it is taken as C_q.

Materials and methods

Weighted regression

In ordinary least squares (LS), estimates of the parameters in the fit model function are obtained through procedures that minimize the sum of squared residuals [54–56],

$$S = \sum \delta_i^2 = \sum [f(a, b, c, \ldots; x_i) - y_i]^2, \quad (3)$$