Comparing Algorithms for Calculating Amplification Efficiencies of Real-Time PCR

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Background

❖ Quantitative real-time RT-PCR is the most sensitive and widely used method for the measurement of gene expression. In real-time PCR, a fluorescent dye is used to monitor the amplification of target genes by a thermostable DNA polymerase.

❖ The three principles of real-time PCR quantification[1-3] are:
(1) The accumulation of fluorescence is proportional to accumulation of PCR product.
(2) The amplification efficiencies of all samples must be comparable.
(3) The amplification threshold used for analysis must be set within the exponential phase of the PCR to ensure that the amount of amplicons generated at the threshold cycle (C_t) truly reflects the initial template amount.

❖ The analysis of real-time PCR data has become the focus of mathematical modeling to increase quantification precision and accuracy. The standard curve method and the comparative C_t method (also known as the $\Delta\Delta C_t$ method) are two main approaches currently employed to analyze real-time PCR data.

Why is it important to know the amplification efficiency of the PCR for each gene target?

❖ The two methods above may potentially introduce biases in quantification, because they both are based on the assumption of equal amplification efficiencies among different samples. The latter comparative C_t method also assumes a constant efficiency of 100% for all PCR assays (Figure 1).

Figure 1. Principles of Real-Time PCR Quantification

$$R_n = R_0 \times (1+E)^n$$

R_n = fluorescence intensity at cycle n
R_0 = initial fluorescence intensity
E = Amplification Efficiency
C_t = threshold cycle

The $2^{-\Delta\Delta Ct}$ formula assumes E=1

$$\frac{R_{0\ sample1}}{R_{0\ sample2}} = \frac{(1+E_{sample2})^{Ct_{sample2}}}{(1+E_{sample1})^{Ct_{sample1}}}$$

❖ A difference of 5% in amplification efficiency between two initially equal samples can result in one sample having twice as much product after 26 cycles of PCR. (Figure 2)

Hence, corrections for differences in amplification efficiencies during PCR data analysis has been suggested to improve quantification accuracy.

Figure 2. Effects of Variations in Amplification Efficiency on the Threshold Cycle (C_t) Number

	C_t A	C_t B 95%	90%	85%	80%
A = 100% efficient PCR	1	1.0	1.1	1.1	1.2
	2	2.1	2.2	2.3	2.4
	3	3.1	3.2	3.4	3.5
B = PCR with indicated efficiency	4	4.2	4.3	4.5	4.7
	5	5.2	5.4	5.6	5.9
	6	6.2	6.5	6.8	7.1
	7	7.3	7.6	7.9	8.3
	8	8.3	8.6	9.0	9.4
	9	9.3	9.7	10.1	10.6
	10	10.4	10.8	11.3	11.8
	11	11.4	11.9	12.4	13.0
	12	12.5	13.0	13.5	14.2
	13	13.5	14.0	14.6	15.3
	14	14.5	15.1	15.8	16.5
	15	15.6	16.2	16.9	17.7
	16	16.6	17.3	18.0	18.9
	17	17.6	18.4	19.2	20.0
	18	18.7	19.4	20.3	21.2
	19	19.7	20.5	21.4	22.4
	20	20.8	21.6	22.5	23.6
	21	21.8	22.7	23.7	24.8
	22	22.8	23.8	24.8	25.9
	23	23.9	24.8	25.9	27.1
	24	24.9	25.9	27.0	28.3
	25	25.9	27.0	28.2	29.5
	26	27.0	28.1	29.3	30.7
	27	28.0	29.2	30.4	31.8
	28	29.1	30.2	31.5	33.0
	29	30.1	31.3	32.7	34.2
	30	31.1	32.4	33.8	35.4
	31	32.2	33.5	34.9	36.6
	32	33.2	34.6	36.1	37.7
	33	34.3	35.6	37.2	38.9
	34	35.3	36.7	38.3	40.1
	35	36.3	37.8	39.4	41.3

C_t A: Threshold cycle number for reaction A with 100% amplification efficiency
C_t B: Projected threshold cycle number for reaction B with the indicated efficiency

Methods for Estimation of PCR Amplification Efficiencies

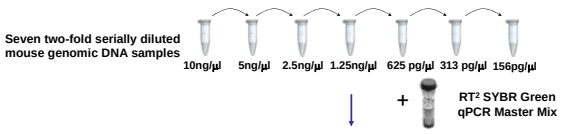
Various strategies have been proposed and practiced to estimate the amplification efficiency of PCR. These include:
(1) The slope-derived efficiency calculation from the standard curve method using threshold cycle (C_t) or crossing point (CP) values determined either from
❖ (a) The fit-point method OR
❖ (b) The second derivative maximum (SDM) of the four-parameter logistic model
(2) The single amplification plot methods to compute the efficiency values from individual PCR kinetic curves using comprehensive algorithms such as
❖ (a) The mid-value point regression (Data Analysis for Real-Time PCR or DART-PCR)[1] OR
❖ (b) The window-of-linearity algorithm (LinReg PCR)[2] OR
❖ (c) The noise-resistant iterative nonlinear regression (Real-Time PCR Miner)[3].

Study Aim

To directly compare various standard curve and amplification plot methods to obtain the amplification efficiency values for a panel of PCR assays performed on different real-time PCR instruments.

Experimental Design

Mouse Inflammatory Cytokines & Receptors RT² Profiler™ PCR Arrays (Cat # PAMM-011, SuperArray Bioscience, Frederick MD) each containing 89 gene-specific assays with "same exon" designs in a 96-well plate format were performed using seven standards, generated from two-fold serial dilutions of mouse genomic DNA (Cat # G3091, Promega, Madison, WI) from 10 ng/μl to 156 pg/μl, on three models of real-time PCR instruments: Bio-Rad iCycler iQ™ (Hercules, CA), Stratagene Mx3005P™ (Cedar Creek, Tx) and ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Each standard was mixed with RT² SYBR Green®/Fluorescein qPCR Master Mix (Cat # PA-011, SuperArray) for the iCycler iQ or with RT² SYBR Green®/Rox qPCR Master Mix (Cat # PA-012, SuperArray) for the Mx3005P and ABI 7500. Each mixture was aliquoted across separate PCR Arrays with each well containing 1 μl of the standard. PCR Arrays were run in triplicates for each standard. The amplification efficiency for each assay on the PCR Array was computed with the five methods mentioned above.



Methods to obtain the amplification efficiency

1. **Standard Curve Methods:** The slope of the standard curve (Log template amount vs C_t) can be used to determine the efficiency of the PCR reaction by the following equation:

$$\text{Efficiency} = [10^{(-1/\text{slope})}] - 1$$

Threshold Cycle (C_t) Determination:
• **Fit-Point Method:** Auto-baseline correction; manually set the threshold to lie within the exponential phase of all amplification curves from all plates
• **Second Derivative Maximum (SDM):** The Crossing Point (CP) at the SDM determined by Real-Time PCR Miner[3] with Four-Parameter Logistic Model (FPLM) fitting

2. **Amplification Plot Methods:** The slope of an individual amplification plot can be used to determine the efficiency of the PCR reaction using either one of the following algorithms:
   - **Data Analysis for Real-Time PCR (DART-PCR)[1]:** An Excel-based algorithm which uses baseline-corrected fluorescence data (delta R_n) to define the cycles of exponential amplification by determining the midpoint (M) of a log-plot of an amplification curve using maximum (R_{max}) and background (R_{noise}) fluorescence values, and to determine the linear slope of that region
   - **Window-of-Linearity Algorithm (LinReg PCR)[2]:** An interactive software which applies linear regression analysis on the log values of baseline-corrected fluorescence data (delta R_n) to define a "window" with the best linear fit, and to determine the slope of that region
   - **Real-Time PCR Miner[3]:** An algorithm which first identifies the exponential phase of the amplification curve by applying whole kinetic curve fitting based on FPLM, and then uses an iterative nonlinear regression and weighted average analysis to compute a final efficiency value

Results

Table 1. Comparisons Between Different Methods for the Estimation of Amplification Efficiencies of 89 SYBR Green Real-Time PCR Assays

	Average Efficiency			Avg. 95% Confidence Intervals					
	iCycler iQ	Mx3005P	ABI7500	iCycler iQ	Mx3005P	ABI7500	iCycler iQ	Mx3005P	ABI7500
Fit-Point Std Curve	95.2%	88.7%	87.7%	85.6% 107.2%	83.3% 94.2%	82.3% 93.0%			
SDM Std Curve	91.0%	90.1%	86.0%	85.7% 96.8%	85.0% 95.9%	80.9% 91.1%			
	Average Efficiency			Average CV in Triplicates			Average Difference Versus Std Curve Method**		
DART-PCR	117.1%	103.0%	96.7%	6.4%	4.9%	3.2%	+21.9%	+14.3%	+9.0
LinReg PCR	118.8%	96.9%	93.8%	10.5%	5.5%	4.3%	+23.6%	+8.1%	+6.1%
Real-Time PCR Miner	102.6%	103.9%	99.1%	4.4%	3.2%	1.8%	+11.5%	+13.8%	+13.1%

**Note: Results from the DART-PCR and LinReg PCR methods are compared with the fit-point method, as these methods use auto-baseline corrected fluorescence data. Likewise, Real-Time PCR Miner values are compared with those from the SDM method, as both of them use fluorescence data with no baseline correction.

❖ The Real-time PCR Miner-derived efficiencies for each assay were usually comparable across all three PCR instruments. All other methods showed platform-dependent efficiency estimations, where the highest average efficiency values were observed from the iCycler iQ, and the ABI 7500 gave the lowest values.
❖ The fit-point and SDM standard curve methods generated similar efficiency values for both the Mx3005P and ABI 7500. The values generated from the iCycler iQ showed a greater difference between the two methods, with a larger 95% CI from the fit-point method.
❖ In general, the standard curve methods gave lower efficiency values than the amplification plot methods.
❖ For all three single amplification plot methods, the smallest variation in efficiency values for replicate reactions was seen with ABI 7500 (CV of 1.8% to 4.3%), while the replicate efficiency values obtained from the iCycler iQ were found to be the least reproducible (CV of 4.4%-10.5%).
❖ When comparing the three algorithms, the efficiencies calculated from LinReg PCR showed the greatest variations among replicates (CV of 4.3% to 10.5%). In contrast, the Real-Time PCR Miner produced the smallest coefficients of variance in replicate efficiency values (1.8%-4.4%) for all three PCR instruments.

Table 2. Characteristics of the Different Methods for Amplification Efficiency Estimation

Methods	Description	Advantages	Drawbacks
Standard Curve Method	- Uses the slope of the standard curve based on serially diluted standards	- Most widely acceptable method - Can be adapted for absolute quantification	- Laborious - Consumes reagents - Assumes similar amplification kinetics between standards and samples and between different reactions
Amplification Plot Methods: Determine amplification efficiency from the actual slope of the amplification plot			
Data Analysis for Real-Time PCR (DART-PCR) Peirson SN et al NAR 2003	- An Excel-based algorithm which utilizes the linear portion of the log plot (i.e. exponential phase) - Based on the mid-value point regression	- No need to construct a standard curve - Allows the PCR efficiencies of individual reactions to be monitored	- Affected by baseline correction (i.e. noise) → Instrument-Dependent - Users have to know how to export raw fluorescence data from their PCR instruments
LinReg PCR Ramakers et al Neurosci Lett 2003	- An interactive software to identify the window-of-linearity	- No need to construct a standard curve - Allows the PCR efficiencies of individual reactions to be monitored	- Affected by baseline correction (i.e. noise) → Instrument-Dependent - Large variations across replicates - Users have to know how to export raw fluorescence data from their PCR instruments
Real-Time PCR Miner Zhao et al J Comput Biol 2005	- Applies whole kinetic curve fitting to identify the exponential phase - Uses iterative nonlinear regression and weighted average analysis to compute a final efficiency value	- Objective → No baseline correction or threshold setting required - Noise-resistant - Small variations between replicates - Instrument-Independent	- Users have to know how to export raw fluorescence data from their PCR instruments

Conclusions

This study demonstrates that the Real-time PCR Miner provides the best precision in efficiency estimation independent of the PCR instrument, while the precisions for other methods are platform-dependent. Hence, the Real-time PCR Miner, a completely objective and noise-resistant algorithm, is the ideal tool for estimating PCR amplification efficiencies.

References:
1.) Peirson SN, Butler JN, and Foster RG (2003). Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic Acids Research* **Vol 31** (14).
2.) Ramakers C, Ruijter JM, Deprez RHL, and Moorman AFM (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience Letters* **339**: 62–66.
3.) Zhao S and Fernald RD (2005). Comprehensive Algorithm for Quantitative Real-Time Polymerase Chain Reaction. *Journal of Computational Biology* **12(8)**: 1047–1064.