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Statistical methods for efficiency adjusted real-time PCR quantification

Joshua S. Yuan[1, 2], Donglin Wang[3] and C. Neal Stewart Jr.[2]*

[1]UTIA Genomics Hub, The University of Tennessee, Knoxville, TN, USA
[2]Department of Plant Sciences, The University of Tennessee, Knoxville, TN, USA
[3]Department of Statistics, The University of Tennessee, Knoxville, TN, USA

The statistical treatment for hypothesis testing using real-time PCR data is a challenge for quantification of gene expression. One has to consider two key factors in precise statistical analysis of real-time PCR data: a well-defined statistical model and the integration of amplification efficiency (AE) into the model. Previous publications in real-time PCR data analysis often fall short in integrating the AE into the model. Novel, user-friendly, and universal AE-integrated statistical methods were developed for real-time PCR data analysis with four goals. First, we addressed the definition of AE, introduced the concept of efficiency-adjusted ΔΔCt, and developed a general mathematical method for its calculation. Second, we developed several linear combination approaches for the estimation of efficiency adjusted ΔΔCt and statistical significance for hypothesis testing based on different mathematical formulae and experimental designs. Statistical methods were also adopted to estimate the AE and its equivalence among the samples. A weighted ΔΔCt method was introduced to analyze the data with multiple internal controls. Third, we implemented the linear models with SAS programs and analyzed a set of data for each model. In order to allow other researchers to use and compare different approaches, SAS programs are included in the Supporting Information. Fourth, the results from analysis of different statistical models were compared and discussed. Our results underline the differences between the efficiency adjusted ΔΔCt methods and previously published methods, thereby better identifying and controlling the source of errors introduced by real-time PCR data analysis.

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1 Introduction

Real-time PCR is a powerful, economical, rapid, and high-throughput technique for assaying gene expression with broad applications in clinical studies, diagnostics, forensics, food technology, pathogen detection, and functional genomics [1–4]. The technique has been adopted quite widely because of the apparent advantages over other gene expression methodologies such as Northern blot analysis: a much larger dynamic range, higher sensitivity, smaller sample amount requirements, and less labor. Even though real-time PCR has become one of the most important enabling technologies in the genomic era, the real or perceived unreliability of real-time PCR data has engendered serious concerns [1, 4]. Both proper experimental design and reliable statistical analysis are the bases for the accuracy and reproducibility of results. Furthermore, without these, data interpretation and conclusions could be faulty. Proper experimental design in-

Correspondence: Professor C. Neal Stewart, Jr., Department of Plant Sciences, The University of Tennessee, Knoxville, TN 37996, USA
E-mail: nealstewart@utk.edu
Fax: +1 865 846 1989

Abbreviations: AE, amplification efficiency; **PAE,** percentile amplification efficiency

*E-mail: syuan@utk.edu

volves the inclusion of suitable internal and external controls, sufficient replication, and appropriate and efficient cDNA synthesis methods [4, 5]. The optimal statistical treatment for real-time RT-PCR requires precise data modeling, integration of amplification efficiency (AE), and rational outlier exclusions. While real-time PCR experiments can be performed for both absolute and relative quantification, the focus of this paper will be on the relative quantification, which is the more prevalent application in biology [4].

Previous publications laid down the foundations for mathematical methods for real-time PCR data analysis [6, 7]. The standard curve method and ΔΔCt method are described in these articles. Both methods involve calculation based on Ct, the cycle number at which logarithm-transformed fluorescence crosses a threshold. The standard curve method calculates the AE and expression ratio based on Ct numbers from a serial dilution of templates [6]. The ΔΔCt method assumes the percentile amplification efficiency (PAE) is 100% (doubling of PCR products *per* cycle) and calculates the ratio based on $2^{-\Delta\Delta Ct}$ [7]. Even though these papers have been significant contributions for analysis of real-time PCR data, robust statistical treatments are largely lacking, especially in terms of confidence interval estimation and *P* value [8]. Statistical treatments are crucial to obtain meaningful and accurate interpretation of real-time PCR data, since large variation may be observed with different mathematical methods without appropriate statistical analysis [9]. A recent influx of publications for statistical modeling of real-time PCR data is reflective of bioinformaticians' desire to address these issues. However, the problem is still acute for "messy" data sets.

Statistical analysis of real-time PCR data involves data modeling for ΔΔCt calculation, AE calculation, quality control, and outlier exclusion. Most statistical modeling methods include data quality control functions based on linear regression models [8]. KOD and Grubbs' test have been proposed to be outlier exclusion methods and can be integrated for data analysis [9]. Statistical methods have been developed to derive ΔΔCt based on pair-wise tests, ANOVA, and GEE models [10]. In 2006, Yuan *et al*. proposed fitting real-time PCR data into ANCOVA and multiple regression models for ΔΔCt calculation, data quality control, and an equal AE test. Here, the regression models are especially powerful tools for real-time PCR data analysis. However, the current models often include inappropriate assumptions in relation to AE, which limits the models application to appropriate data sets. Some models assume that all PCR reactions should have PAE of 100%, and others assume the equal PCR amplification efficiencies among samples or genes [7, 8]. Either assumption is sometimes violated in actual experiments, since amplification efficiencies in real-time PCR experiments can typically vary between 70 and 100%. When data from lower AE or unequal amplification efficiencies are analyzed with the current available models, the inferred result could be much different from actual gene expression, which is a major source of unreliability of real-time PCR technology.

AE is probably the most important concept in real-time PCR quantification, since the development of the technique is based on the assumption that PCR products double each cycle, but it can be a confusing issue to practitioners. When the percentile PCR AE is not 100%, the quantification must be adjusted by the AE. PCR AE has been presented as variably from 1 to 2, or 0 to 1 (0–100%) [11, 12]. In order to avoid the confusion, we use AE to represent the multiplication of PCR product increase during each cycle, which is between 1 and 2, and PAE to represent the percentage of full AE capacity, which is between 0 and 1 or 0 and 100%. The mathematical conversion between AE and PAE has been controversial as well. Some researchers define AE as 1 added to PAE as shown in Eq. (1) [11], while others define AE as 2 powered by PAE as shown in Eq. (2) [4]. In this article, we adopted the second definition to have AE equal to 2 powered by PAE as shown by Eq. (2), because of the advantages in statistical modeling and estimation. AE and PAE are normally calculated through standard curve for Ct or fluorescent signal strength during the amplification [8, 11]. The concept and calculation of AE and PAE will be discussed in detail in the article since they are important in calculating AE adjusted ΔΔCt and relative gene expression abundance. Even though several papers have been published in calculating AE, few have thoroughly discussed a precise universal statistical model with AE integrated in ΔΔCt and ratio calculation. Proper AE-integrated statistical models for ratio calculation and significance estimation are therefore the key issue for accurate real-time PCR quantification nowadays.

$$AE = 1+PAE \quad (1)$$

$$AE = 2^{PAE} \quad (2)$$

where AE is the amplification efficiency (1–2) and PAE is the percentile amplification efficiency (0–1).

Besides the efficiency adjustment, recent advances of real-time PCR quality control involving

multiple controls require a proper statistical model for ΔΔCt calculation integrating more than one control [13, 14]. A weighted ΔΔCt method is also presented, and integrating efficiency adjusted ΔΔCt should provide optimal accuracy.

Overall, in this article, we present efficiency adjusted and weighted ΔΔCt methods for gene expression analysis with real-time PCR as well as the statistical models implementing the method. Several linear model based methods and SAS programs will provide new approaches for efficiency-integrated real-time PCR data analysis. The concept of $\Delta\Delta Ct_{adjusted}$ and an efficiency-adjusted quantification method are first introduced. The definition of AE and its calculation based on simple linear regression models are discussed. Statistical models are then developed for efficiency adjusted ΔΔCt calculation for single concentration design, standard curve design, and a mixed design. The weighted ΔΔCt models are included for experiments with more than one internal control. Two-way ANOVA and multiple regression combined with an unbalanced linear combination are the main statistical approaches employed. SAS programs are developed for each model and a test sample set is analyzed with different statistical methods. Comparison of efficiency adjusted quantification approach with previous models has indicated that analysis of low-quality real-time PCR data may miscalculate the target gene expression ratio if AE effects are not explicitly considered. Moreover, it highlights the importance of proper experimental design and condition optimization for real-time PCR. The pros and cons for each proposed models and designs are also discussed. The new methodology also provides a more precise and high-throughput alternative for statistical analysis of low quality real-time PCR data.

2 Materials and methods

2.1 Real-time PCR experiments

Arabidopsis thaliana (Col1) plant growth, RNA extraction, and real-time PCR experiments were carried out as described [15]. The real-time PCR experiments were conducted using a standard protocol recommended by the manufacturer. Basically, approximately one microgram of total RNA was synthesized into cDNA using iScript cDNA synthesis kit (BioRad Laboratories). The cDNA was then diluted into one to four and one to sixteen serial dilutions. Real-time PCR experiments were carried out with duplication for each concentration with an ABI 7000 Sequence Detection System (Applied Biosystems). After the experiment, the Ct number was extracted for both reference gene and target gene with auto baseline and manual threshold of 0.4613. In addition to the Ct number, the fluorescence measurements were also downloaded. Besides the dataset with one internal control, we included a dataset with two internal controls (ubiquitin and tubulin) to illustrate weighted ΔΔCt estimation.

2.2 $\Delta\Delta Ct_{adjusted}$ and efficiency adjusted quantification method

As discussed in Section 1, we adopted the PAE definition in Eq. (2). According to the equation, PCR product amount during the reaction can be defined by Eq. (3), where PCR product equals AE raised to the power of cycle number n and then multiplied by original template amount. Since Ct is also a cycle number by definition, it can replace the n in Eq. (3) with Ct. From Eq. (3), we can derive Eq. (4). The goal of real-time PCR quantification is to calculate the absolute or relative abundance of original template amount (P_0) as shown in Eq. (4).

$$P = P_0(\mathrm{AE})^n = P_0(2^{\mathrm{PAE}})^n = P_0 \times 2^{n\times \mathrm{PAE}} \tag{3}$$

where P is the PCR product amount for a given PCR cycle, P_0 the original template amount, n is the number of PCR cycles.

$$P_0 = \frac{P}{2^{\mathrm{Ct}\times \mathrm{PAE}}} \tag{4}$$

The purpose of relative quantification is to derive the ratio of target gene expression between a treatment sample and a control sample after normalized by the internal reference gene. Equation (5) provides the universal solution for relative quantification, where the P_0 for each sample will be first normalized against the internal reference gene and then compared between the samples. Since Ct is derived from the cycle number for a given threshold, the PCR product amount (P) is equal among all the samples and genes and therefore can be cancelled out. The relative abundance of original template for the target gene between samples can thus be presented as follows in Eq. (5).

$$\begin{aligned}\mathrm{Ratio} &= \frac{P_{0\_Tgt\_Trt}/P_{0\_Ref\_Trt}}{P_{0\_Tgt\_Con}/P_{0\_Ref\_Con}} \\ &= \frac{2^{Ct\_Ref\_Trt\times PAE}/2^{Ct\_Tgt\_Trt\times PAE}}{2^{Ct\_Ref\_Con\times PAE}/2^{Ct\_Tgt\_Con\times PAE}}\end{aligned} \tag{5}$$

where Con is the abbreviation for control sample, Tgt the abbreviation for target gene, Trt the abbre-

viation for treatment sample, and Ref is the abbreviation for reference gene.

In the traditional $2^{-\Delta\Delta Ct}$ method without efficiency adjustment, the ratio can be calculated directly from the Ct numbers as shown in the Eq. (6).

$$\text{Ratio} = 2^{-\Delta\Delta Ct} = \frac{2^{Ct\_Ref\_Trt} / 2^{Ct\_Tgt\_Trt}}{2^{Ct\_Ref\_Con} / 2^{Ct\_Tgt\_Con}} \quad (6)$$

The differences between Eqs. (5) and (6) are that the Cts in Eq. (5) have been adjusted by PAE. The generalized efficiency adjusted ΔΔCt ($\Delta\Delta Ct_{adjusted}$) can therefore be defined as the differences for the Ct numbers that are adjusted by PAE. From Eq. (5), the $\Delta\Delta Ct_{adjusted}$ can be calculated and defined as in Eq. (7). Equation (7) is the basis of statistical models in Sections 2.4 and 2.5 of the article

$$\begin{aligned}\Delta\Delta Ct_{adjusted} = {} & (Ct_{Tgt\_Trt} \times PAE_{Tgt\_Trt} \\ & - Ct_{Ref\_Trt} \times PAE_{Ref\_Trt}) \\ & - (Ct_{Tgt\_Con} \times PAE_{Tgt\_Con} \\ & - Ct_{Ref\_Con} \times PAE_{Ref\_Con})\end{aligned} \quad (7)$$

2.3 The estimation of PAE

A key component for the $\Delta\Delta Ct_{adjusted}$ calculation is the PAE of PCR. We hereby present the linear regression models for PAE estimation in both standard curve design and single concentration design. The PAE can be estimated for a group of reactions or a single reaction by simple linear regression model. In 2006, Yuan *et al*. proposed a simple linear regression model to calculate and perform quality control on the PAE based on the standard curve design as shown in Eq. (8). The PAE can be defined as $-\beta_{lcon}$ [8, 12, 16].

$$Ct = \beta_0 + \beta_{lcon} X_{lcon} + \varepsilon \quad (8)$$

where X_{lcon} is the logarithm 2 based transformation of serial diluted concentration.

Even though the Eq. (8) was developed for standard curve design, an alternative simple linear regression model can be used for single concentration design by estimating PAE from fluorescence measurements for each amplification cycle [11, 12]. Linear models for AE estimation has been discussed before, however, previous research either models data with Eq. (1) or uses logarithm 10-based transformation, both of which lead to no direct estimation and test of PAE in the model. A better way to calculate PAE in fluorescence can be derived from logarithm 2-based transformation of Eq. (3) as shown in Eq. (9).

$$P_{log} = P_{0log} + n \times PAE \quad (9)$$

where P_{log} is the logarithm 2 transformed PCR product amount (or fluorescence), P_{0log} the logarithm 2 transformed original template amount, and n is the number of amplification cycle.

According to Eq. (9), a simple linear regression model can be developed as shown in Eq. (10).

$$P_{log} = \beta_0 + \beta_x X_c + \varepsilon \quad (10)$$

where P_{log} is the logarithm 2 transformed PCR product amount (or fluorescence reading) as shown in fluorescence data, and X_c is the cycle number n.

This model will be the basis for PAE calculation for fluorescence data in the later part of the article. The PAE can be directly estimated thorough testing the β_x. Based on this model, we can also perform the data quality control for the PCR to test if PAE is equal to 100%. When β_x equals to 1, the PAE equals to 100%.

The above simple linear regression models can be readily implemented with SAS 9.1 (SAS Institute, Cary, NC) as shown in Supporting Information File 1 and 2 for fluorescence data and standard curve data, respectively. Supporting Information File 3 is the input file for the single concentration design fluorescence data, and Supporting Information File 4 is the input file for Ct value from standard curve design. Both data formats are as shown in Tables 1 and 2. For the fluorescence data analysis, multiple filtering methods can be used to determine the data point representing the exponential phase of PCR, which is not the focus of this paper. In order to generate the dataset for downstream analysis, we filtered the input data by a range based on the observation of amplification curve. Typical real-time PCR data can be presented as a plot of logarithm transformed fluorescence against cycle number as shown in Fig. 1. A linear range can be observed in the plot, in which the amplification curves for different samples are parallel to one another. The linear range represents the logarithm phase of PCR and is used for AE calculation. After the data filter, the fluorescence intensity representing the PCR product amount is then subjected to logarithm 2-based transformation. The logarithm transformed fluoresce signal (PLOG) and cycle number (CY) are then fitted for a simple linear regression in Eq. (10). The slope represents the PAE, and the test of slope = 1 will render the test of PAE's equivalence to 100%. In the same way, we can analyze the standard curve data with Eq. (8) except that the logarithm-transformed concentration should be in reverse proportion to Ct.

Besides the model above, several other ways can be used for point estimation and equivalence test of

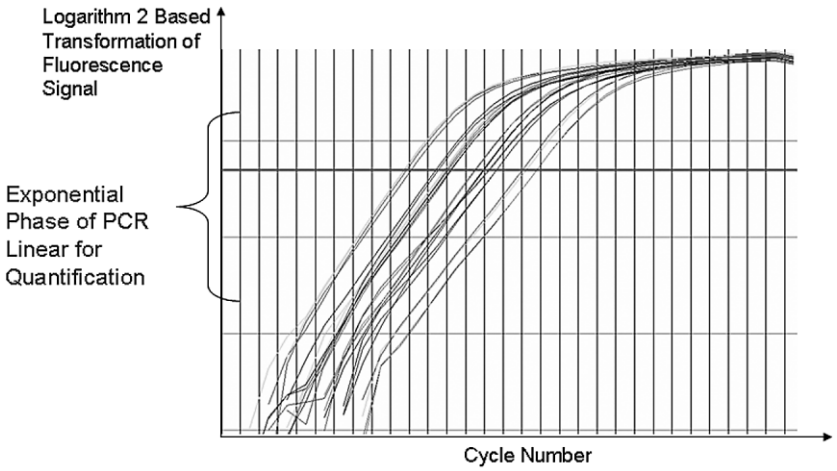

Figure 1. Linear region for PCR quantification. The exponential phase of PCR reaction can be used to analyze PAE, and it is a linear region in the plot of logarithm-based fluorescence against cycle number.

AE. Yuan *et al*. [8] proposed to use Type III sums of squares of multiple regression model to test the equivalence of AE. More generalized linear models as shown in Figs. 2 and 3 can be used for estimation of equivalence of PAE for different treatment and samples with different design.

2.4 Statistical modeling of $\Delta\Delta Ct_{adjusted}$ for fluorescence data

The efficiency adjusted ΔΔCt in Eq. (7) can be analyzed with linear models. Previous research has fitted real-time PCR data into ANCOVA, multiple regression and ANOVA models [8–10]. Fitting real-time PCR data into linear models is a significant step toward the precise real-time PCR data analysis with appropriate quality controls. However, most of the current models are designed to derive ΔΔCt directly with the assumption of 100% PAE, and therefore cannot be used in the analysis of low-quality data. Yuan *et al*. [8] proposed an efficiency adjusted ANCOVA model for low quality data analysis, but the model still requires equal AE for each gene between different samples, an assumption which might be commonly violated. In reality, real-time PCR data could have PCR amplification efficiencies deviating from 1 for different cDNA samples and different genes. This is probably the rule rather than the exception. The assumptions over AE therefore compromise the application of these statistical models in practice. Novel models with universal applications based on Eq. (7) are necessary for AE integrated statistical analysis. Moreover, previous linear models by Yuan *et al*. [8] were developed for analyzing Ct values from standard curve design only, statistical models need to be developed for efficiency adjusted analysis of fluorescence data. Here we present unbalanced linear combination of group effect for a two-way ANOVA model and intercept estimation of multiple regression model as two options for the $\Delta\Delta Ct_{adjusted}$ calculation for fluorescence data analysis with single concentration design. The same models can also be used for standard curve design data. The pros and cons of each statistical model will be discussed later.

Table 1. Input fluorescence data of single concentration design

Cycle	Fluorescence	Sample	Group
1	0.003895	1	1
…			
16	0.251703	1	1
17	0.488688	1	1
18	0.925395	1	1
…			
42	7.278038	2	4

The data include PCR cycle number and fluorescence measurements (in arbitrary units) for each PCR cycle. We have included two samples for each group, and four groups for comparison. The four groups are the reference and gene in the control sample, as well as the reference and target gene in the treatment sample.

2.4.1 *Two-way ANOVA model and unbalanced linear combination*

The fluorescence measurements do not yield an explicit Ct number, however, we can consider Ct as

Table 2. Input data for standard curve design

Treatment	Gene	Concentration	Ct	Group
Water (2 h)	UBQ	16	16.8847	1
Water (2 h)	UBQ	16	17.205	1
Water (2 h)	UBQ	4	18.972	1
Water (2 h)	UBQ	4	19.0513	1
Water (2 h)	UBQ	1	21.2514	1
				…
Ala (2 h)	MT7	1	25.367	4

The data has the relative concentration and Ct numbers as well as different treatments and genes.

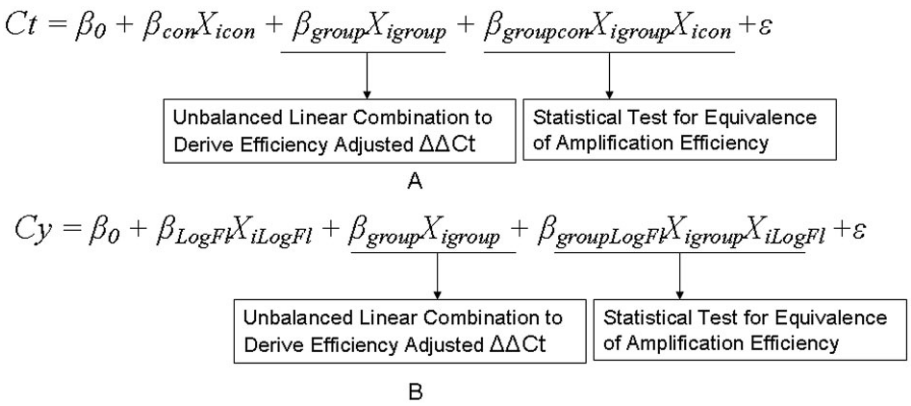

Figure 2. Statistical estimation of real-time PCR data using ANOVA model with cycle or Ct number as dependent variable. (A) Presents the efficiency adjusted ΔΔCt estimation from unbalanced linear combination of two-way ANOVA model for data from standard curve design. (B) Presents efficiency adjusted ΔΔCt estimation for fluorescence data from the single concentration design.

the cycle number for a given PCR product amount. When the sample effect is randomized, the sample dataset in Supporting Information File 3 can be fit into the models in Fig. 2B. The traditional ΔΔCt can be calculated by the ANOVA model by contrasting and estimating the linear combinations of means for cycle number of different groups as shown in Eq. (11). The $\Delta\Delta Ct_{adjusted}$ can be tested and estimated in the same way except that the combination of means needs to be adjusted by PAE as shown in Eq. (12). The adjustment in combination often results in an unbalanced linear combination.

$$\Delta\Delta Ct = (\mu_1 - \mu_2) - (\mu_3 - \mu_4) = \mu_1 - \mu_2 - \mu_3 + \mu_4 = 0 \quad (11)$$

$$\Delta Ct_{adjusted} = \mu_1 \times PAE_1 - \mu_2 \times PAE_2 - \mu_3 \times PAE_3 + \mu_4 \times PAE_4 = 0 \quad (12)$$

where μ_1–μ_4 is the mean of cycle number for groups 1–4, respectively, PAE_1–PAE_4 are the percentile amplification efficiency for groups 1–4, respectively.

Both equations fit into the general linear combination model as shown in Eq. (13). For ΔΔCt estimation in Eq. (11), the linear combination is balanced with c_i equals to 1 or –1, representing 100% PAE. For $\Delta\Delta Ct_{adjusted}$ estimation in Eq. (12), the linear combination is unbalanced with c_i equals to PAE or –PAE for each group.

$$\Delta\Delta Ct = \sum_{i=1}^{r} c_i \mu_i \quad (13)$$

Equation 12 can be realized by unbalanced linear combination of group effects as shown in Fig. 2B for fluorescent data of single concentration design. The null hypothesis for the test in Eq. (12) is that the $\Delta\Delta Ct_{adjusted}$ equals to 0 when offset by combined mean of PAE, which indicates no changes in target gene expression between the treatment and control sample. The alternative hypothesis is that the target gene expression changes significantly between the samples. A low *P* value will favor the alternative hypothesis. The model is implemented in SAS as shown in Supporting Information File 1. The model statement establishes the statistical model for the two-way ANOVA, where CY (cycle number) depends on the logarithm 2-based transformation of PCR product amount (fluorescence reading), different groups, and their combinatorial effects. The $\Delta\Delta Ct_{adjusted}$ are then derived from contrasting and estimating the unbalanced linear com-

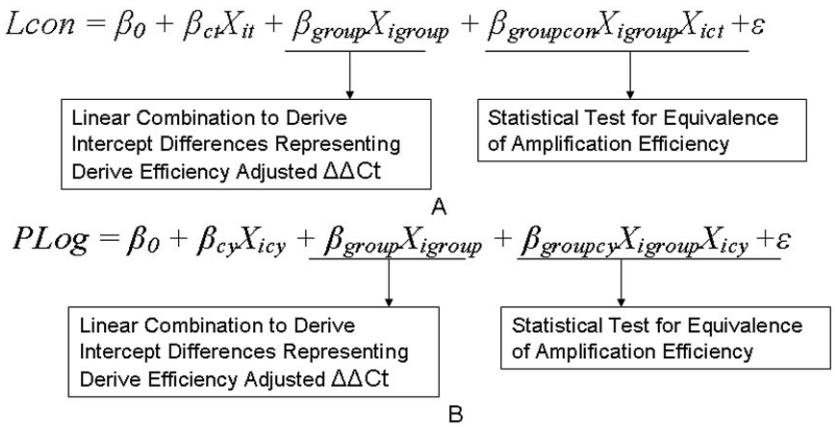

Figure 3. Statistical estimation of real-time PCR data and balanced linear combination of intercept to derive efficiency adjusted ΔΔCt. (A) Presents the multiple regression model for standard curve design and the estimation efficiency adjusted ΔΔCt. (B) Presents the same model for analyzing fluorescence data with single concentration design.

bination of PAE adjusted means. The SAS output provides both point and confidence level estimation for $\Delta\Delta Ct_{adjusted}$ as shown in Table 4. Moreover, the SAS output also gives a *P* value to estimate the statistical significance of gene expression differences.

2.4.2 *Multiple regression model and linear combination of intercepts*

The ANOVA model provides a conceptually simple approach to estimate the $\Delta\Delta Ct_{adjusted}$. However, the method needs to calculate the grouped PAE for each gene and treatment combination before the $\Delta\Delta Ct_{adjusted}$ calculation. Moreover, since the PAE is estimated for a group and the variation of PAE within the group is not integrated in the model, the model might potentially underestimate the variation of $\Delta\Delta Ct_{adjusted}$. A more conceptually complicated, but more direct approach for $\Delta\Delta Ct_{adjusted}$ estimation is through linear combination of intercepts in multiple regression model. Considering the simple linear regression model in Eq. (10), the $\beta_c X_c$ represents the cycle number multiplied by PAE. If we take into account the simple linear regression for the four groups respectively as shown in Eq. (14), a linear combination of these four groups by adding groups 1 and 4 and subtracting groups 2 3 will give the Eq. (14), since the $P_{\log}$ for each group will be equal at a given Ct number and terms can be cancelled out.

$$
\begin{aligned}
&P_{\log G1} = \beta_{0G1} + \beta_{xG1} X_{cG1} + \varepsilon \qquad (14)\\
&P_{\log G2} = \beta_{0G2} + \beta_{xG2} X_{cG2} + \varepsilon\\
&P_{\log G3} = \beta_{0G3} + \beta_{xG3} X_{cG3} + \varepsilon\\
&P_{\log G4} = \beta_{0G4} + \beta_{xG4} X_{cG4} + \varepsilon\\
&\Rightarrow -(\beta_{0G1} - \beta_{0G2} - \beta_{0G3} + \beta_{0G4})\\
&= \beta_{xG1} X_{cG1} - \beta_{xG2} X_{cG2} - \beta_{xG3} X_{cG3} + \beta_{xG4} X_{cG4}
\end{aligned}
$$

In Eq. (14), the right portion of the equation is the $\Delta\Delta Ct_{adjusted}$ as shown in Eqs. (12 and 13), and the left portion of the equation is the linear combination of the intercepts, which will be equal to the linear combination of the intercept of the multiple regression model as shown in Fig. 3B if no interaction among groups existed. Basically, for multiple linear regression model, $\Delta\Delta Ct_{adjusted}$ can be estimated from linear combination the intercept of different groups. Conceptually, for either model in Fig. 3, if cycle or Ct number equals to 0, the product or template concentration represents the initial amount. Therefore, the contrast and combination of intercepts will derive $\Delta\Delta Ct_{adjusted}$ as also visualized in Figs. 4A and B.

The model can be easily implemented in SAS as shown in Supporting Information Files 1 and 2. The model statement in the procedure helps to establish multiple regression for logarithm transformed PCR product amount (PLOG) with group and cycle number (CY). The output of linear combination includes the mean and confidence interval estimation of $\Delta\Delta Ct_{adjusted}$ as shown in Table 3. It should be noted that the variation for the $\Delta\Delta Ct_{adjusted}$ is much larger in this analysis as compared to the two-way ANOVA model, and the resulting test is not significant.

2.5 Statistical modeling of $\Delta\Delta Ct_{adjusted}$ for standard curve

Even though the $\Delta\Delta Ct_{adjusted}$ can be calculated in the real-time PCR experiments with fluorescence data from single concentration design, the design may lead to more variation than the standard curve design, which allows a large dynamic range of the quantification [5]. Moreover, the standard curve allows to observe amplification inhibition at earlier stage of PCR, while fluorescence data only reflect the efficiency above the baseline level. The previously published linear models can be modified to estimate $\Delta\Delta Ct_{adjusted}$ for standard curve design [8].

The principle and procedure for fluorescence data analysis can be applied to analyze $\Delta\Delta Ct_{adjusted}$ for the standard curve design as shown in Figs. 2 and 3. The same unbalanced linear combination of cycle number can be used for $\Delta\Delta Ct_{adjusted}$ estimation in the statistical model with Ct as the dependent variable. The linear combination of intercepts in the statistical model with logarithm transformed concentration as the dependent variable can also be used for $\Delta\Delta Ct_{adjusted}$ estimation. The only difference between the modeling of fluorescence data and standard curve data is that logarithm transformed fluorescence data are in linear proportion to the cycle, while the logarithm transformed concentration is in reverse linear proportion to the Ct number. Therefore, the linear combination equation is reversed in modeling the two types of data.

Table 3. PAE estimation

Group	Design	PAE	STD	*P* value
1	Standard curve	0.9216	0.0349	0.089
2	Standard curve	0.8774	0.0348	0.024*
3	Standard curve	0.9067	0.0223	0.014*
4	Standard curve	0.8983	0.0305	0.029*
1	Single concentration	0.9571	0.0352	0.254
2	Single concentration	0.9469	0.0284	0.095
3	Single concentration	0.8782	0.0105	<0.0001**
4	Single concentration	0.8902	0.0271	0.023*

The PAE estimation for each group is listed for both designs. Ct values were used for standard curve design and single concentration stands for fluorescence data from single concentration design. * indicates a p value smaller than 0.05 and ** indicates a p value smaller than 0.01.

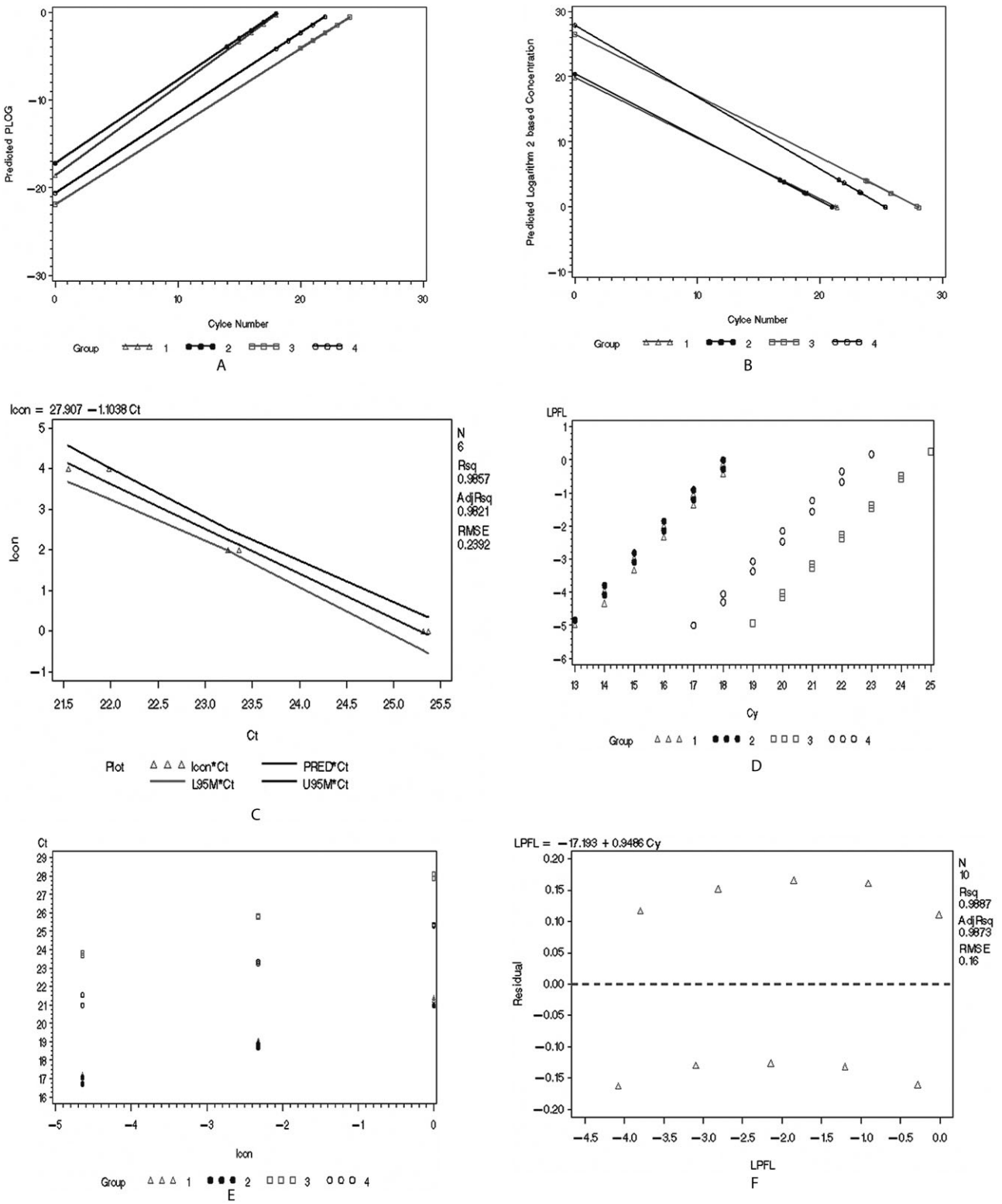

Figure 4. The visualization of data. The intercepts for the multiple regression models in single concentration design and standard curve design are shown in (A) and (B), respectively. (C) Presents the 95% confidence interval (D) and (E) are the regression data point for fluorescence data and Ct value of standard curve, which shows clear differences among four groups. (F) Is the residual plot for regression model. PLOG is the abbreviation for logarithm transformed PCR product, Icon is the abbreviation for logarithm transformed concentration, and LPFL ist the abbreviation for logarithm transformed fluorescence signal.

Table 4. Comparison of different methods

Type	Design	Test	Mean	Error	95% CI	*P*	Ratio	Reference
ΔΔCt	Single concentration	ANOVA	–1.90	0.17	(–2.24, –1.55)	<0.01	3.73	[9]
	Standard curve	*T*-test	–2.34	0.27	(–2.53, –2.15)	<0.01	5.06	[8]
		Wilcoxon	–2.11	N/A	(–2.56, –2.36)	<0.01	4.32	[8]
		ANCOVA	–2.26	0.19	(–2.65, –1.86)	<0.01	4.78	[8]
$\Delta\Delta Ct_{adjusted}$	Single concentration	ANOVA (PAE adjusted, UBLC)	–1.25	0.16	(–1.57, –0.93)	<0.01	2.38	This paper
		Multiple regression (LCI)	–1.17	0.95	(–3.1, 0.76)	0.23	2.25	This paper
	Standard curve	ANCOVA (PAE adjusted, UBLC)	–1.47	0.17	(–1.82, –1.11)	<0.01	2.77	This paper
		Multiple regression (LCI)	–3.41	1.68	(–6.79, 0.15)	0.06	7.42	This paper
	Mixed design	Multiple regression (LCI)	–1.19	0.63	(–2.43, 0.05)	0.06	2.28	This paper
$\Delta\Delta Ct_{weighted}$	Standard curve	ANOVA weighted	–2.84	0.39	(–3.64, –2.67)	<0.01	7.16	This paper
	Standard curve	ANOVA Weighted PAE adjusted	–2.23	0.36	(–2.98, –1.49)	<0.01	4.69	This paper

Statistical analysis results are presented for different models for ΔΔCt and $\Delta\Delta Ct_{adjusted}$. The mean estimation, standard error, 95% confidence interval, *P* value, predicted ratio, and references are presented in the table. UBLC stands for unbalanced linear combination. LCI stands for linear combination of intercepts.

The statistical models are implemented in SAS as shown in Supporting Information file 2, and the data output is summarized in Table 4.

2.6 Statistical modeling of $\Delta\Delta Ct_{adjusted}$ for mixed design

The fluorescence data from a standard curve design can also be analyzed: a mixed design. A three-way ANOVA model can be used and the $\Delta\Delta Ct_{adjusted}$ can be estimated through the linear combination of intercepts. The main advantage is that the design enlarges the sample size for $\Delta\Delta Ct_{adjusted}$ estimation and allows estimation of AE using two approaches. The three-way ANOVA model is implemented in the Supporting Information File 5, which is based on dataset in Supporting Information File 6. The analysis results yield a smaller *P* value as compared to the linear combination of intercepts from either fluorescence data of single concentration design or Ct value from standard curve design because the increase in the effective sample size.

2.7 Statistical modeling of $\Delta\Delta Ct_{weighted}$ for data with multiple controls

Besides the AE, another important issue of real-time PCR analysis is having the proper internal control. Multiple genes as internal controls have been proposed to reduce systemic error and improve the accuracy of analysis [14]. Most of the current models are developed for experimental designs including only one control. A modified linear combination of group effects in the ANOVA model allows the analysis of real-time PCR data with multiple controls as shown in Eq. (15), where *W* is the weight equaling to 1 divided by number of control genes. In the case inclusion of two internal genes, the weight should be 0.5. Supporting Information File 8 provides a data set with multiple controls, and Supporting Information File 7 is the SAS implementation of the Eq. (15).

$$\Delta\Delta Ct_{adjusted} = \mu_1 \times PAE_1 - \mu_2 \times PAE_2 - \mu_3 \times PAE_3 \times W + \mu_4 \times PAE_4 \times W - \mu_5 \times PAE_5 \times W + \mu_6 \times PAE_6 \times W \quad (15)$$

where 3, 4, 5, and 6 represent the data from two reference genes for both control and treatment samples. When multiple reference genes are included, *W* can be equal to 1 divided by total number of reference genes.

3 Results and discussion

The main advantage of the statistical models presented is the ability to perform analysis without AE restrictions, which is an important improvement from our previous statistical treatment of real-time PCR data [8]. The concepts and modeling of $\Delta\Delta Ct_{adjusted}$ and $\Delta\Delta Ct_{weighted}$ are novel.

3.1 The estimation of PAE

The estimation of AE is presented in Table 3, where the statistical test of slope's equivalence to 1 renders the *P* value. A small *P* value indicates PAE is significantly different from 1 or 100%. Even though the interaction effects between group and concentration are not significant under the ANOVA model for standard curve design, the data in Table 3 indicate real differences among sample AE. Moreover, the PAEs are significantly different from 100% in several cases. The efficiency adjusted data analysis is therefore highly recommended for such data. It would, therefore, not be appropriate to apply our previously reported models [8] in this case. Hence, we analyzed the data with different statistical models for efficiency adjusted ΔΔCt presented above.

3.2 The comparison of statistical models for $\Delta\Delta Ct_{adjusted}$ and ΔΔCt

The comparison of the ΔΔCt and $\Delta\Delta Ct_{adjusted}$ as shown in Table 4 highlights the importance for efficiency adjustment in the analysis of this data set. The ΔΔCt estimation is different from the $\Delta\Delta Ct_{adjusted}$. The ΔΔCt estimation gives a lower *P* value and larger differences among gene expression values as compared to the $\Delta\Delta Ct_{adjusted}$ with PAE adjustment by unbalanced linear combination. The efficiency adjustment for this particular dataset is important for appropriate data interpretation. As shown by Table 1, most of the PCRs for this dataset have PAE less than 1 and amplification efficiencies also differ from one another. In the case of low AE, the unadjusted ΔΔCt might overestimate or underestimate the target gene expression differences depending on the PAE for each group of PCR. Since the error for PCR increases exponentially during the reaction, a 10% PAE difference might result in huge data distortion if the Ct differences are large enough.

If we examine Figs. 4A and B, the intercepts represent the initial template amount and are used to calculate $\Delta\Delta Ct_{adjusted}$ in linear combination of intercepts for multiple regression. The differences of intercepts could be quite different from those in our observed range, which illustrates the effects of amplified error. For ΔΔCt analysis, we are analyzing data based on the observed regions. If calculations are not adjusted by PAE, the resulting interpretation could be very misleading. The efficiency problem shown in this dataset is quite common from our experience, and it underlines the fact that it is impossible to make assumptions of equal PCR amplification efficiencies or 100% PAE unless proper tests are performed. Thus, the appropriateness of using the quantification method is highly dependent on data quality. In the case where high quality data are available, ΔΔCt and $\Delta\Delta Ct_{adjusted}$ would yield similar results [8], and the traditional ΔΔCt method could be used since the analysis would not overestimate the errors. However, for messy data, the $\Delta\Delta Ct_{adjusted}$ method is a much better choice, because otherwise assuming a 100% PAE will miscalculate the ratio estimation significantly.

3.3 The errors for real-time PCR

The differences between $\Delta\Delta Ct_{adjusted}$ and ΔΔCt also stress the importance of experimental condition optimization and experimental design. In this particular dataset, we used a 2 × 3 design where duplicate biological samples and three concentrations were tested. We found that increasing sample size significantly decreases the error and makes the test more robust. For this dataset, there should be differential target gene expression among different treatments, however, it was not significant for several test models using our small sample size. Moreover, it is also important to optimize the cDNA synthesis and the primer design. If the PCRs for all groups have PAE approximately 1, the ΔΔCt method can be directly applied and the test will be more robust.

3.4 The comparison of different statistical methods for $\Delta\Delta Ct_{adjusted}$

Different methods can be used for $\Delta\Delta Ct_{adjusted}$ calculation. As discussed before, it becomes apparent that integrating PAE in the ANOVA model with unbalanced linear combination will give a much smaller error than the linear combination of intercepts from the multiple regression model. The discrepancy could result from two effects, the underestimation of error for caused by the pooling of PAE in ANOVA model, and the inflation of the error by linear combination of intercepts. The results shown in Table 3 confirmed our concerns. All the standard errors in PAE integrated ANOVA models were smaller than that from the multiple regression model. When sample sizes are small, PAE-adjusted ANOVA may be a better choice of analysis because

error values will be less. However, it is a less conservative approach.

How could error be inflated in the linear combination of intercepts? In Fig. 4C, we can see the 95% confidence interval for the predicted value will form a curve with the smallest interval at the mean observation. This imposes a particular problem for the linear combination of intercepts in the multiple regression model, since the errors at the intercept may be much inflated because of their large distance from the mean of observed values. The linear combinations of intercepts by multiple regression model may overestimate the error for $\Delta\Delta Ct_{adjusted.}$ As we can see from Figs. 4D and E, there is a clear difference in the target gene expression between the samples, however, the error is enlarged by the linear combination of intercepts resulting in a statistically nonsignificance result in the analysis. Unless we have a larger sample size and smaller standard error for the predicted variables, the multiple regression models should be used only with great caution. However, multiple regression is the most conservative model for the $\Delta\Delta Ct_{adjusted}$ calculation since errors for all the effects are analyzed. In order to increase the sample size and dynamic range, a mixed design of analysis of fluorescence at different concentrations might be a valid alternative for linear combination of intercepts with multiple regression model.

Considering the limitations for both types of $\Delta\Delta Ct_{adjusted}$, efficiency adjusted quantification should not be the default choice for real-time PCR data analysis. If experiments are carefully controlled, and equal amplification efficiencies are assured that are required by the traditional $\Delta\Delta Ct$ method, the $\Delta\Delta Ct$ method may be a better choice for data analysis because it is effective within the observed data range and would not introduce additional errors. The test provided both in Methods Part III and our previous publication [8] will help to test whether PAE is 100% and equal among all groups.

3.5 The weighted $\Delta\Delta Ct$

Besides the efficiency adjustment, more internal control genes in the experiment may also result in more robust data [14]. The $\Delta\Delta Ct_{weighted}$ can be used to perform linear combination of multiple controls to derive point estimation and test for the gene expression ratio. As shown in Table 4, adding another internal control gene coding for tubulin resulted in a different ratio estimation. The assumption that the internal control gene is always expressed consistently can be as easily violated, which would lead to interpretive errors. Therefore, including multiple internal control is always a recommended practice, especially for clinical studies requiring accuracy for diagnoses, treatments, *etc*. Regardless of the number of control genes, proper efficiency adjustment can often improve the data quality (Table 4).

3.6 The future of real-time PCR data analysis

Since the metric of statistical analysis of real-time PCR data is $\Delta\Delta Ct_{adjusted}$, not the ratio of gene expression, the confidence interval and standard error should therefore be presented for $\Delta\Delta Ct_{adjusted}$ instead of the ratio. However, the confidence level for the ratio can be derived from the confidence interval of $\Delta\Delta Ct_{adjusted}$.

Our research herein highlights the importance of integrating AE in the statistical analysis of real-time PCR data. In addition, it helps to explain a common phenomenon in functional genomics, in which the real-time PCR-derived gene expression ratio (treated *vs.* control) is often higher than that of an equivalent microarray experiment [17, 18]. One explanation is that real-time PCR tends to overestimate the gene expression ratio if AE is not integrated into the data analysis, and thus, the typical interpretation of gene up-regulation appears to be greater than it is in reality. An alternative explanation was that the ratio expansion for real-time PCR could be due to lower AE for the gene at a lower expression level in the two groups being compared. In either scenario, the problem becomes more severe when the gene expression ratios are high, since the larger Ct differences can result in amplified error. Using the methods outlined here should yield real-time RT-PCR estimates that are more congruent with microarray data. With some modification, the method can also be used to estimate the AE at different cycle number as previously described, or to analyze the multiplex data with more accuracy [19, 20]. The research also underscores the importance for the scientific community to establish standard procedures for real-time PCR data analysis, since an improper treatment may lead to amplified error in case of real-time PCR data analysis. In turn, this could lead to faulty biological interpretations and conclusions.

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4 References

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