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Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta C_T}$ Method

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The two most commonly used methods to analyze data from real-time, quantitative PCR experiments are absolute quantification and relative quantification. Absolute quantification determines the input copy number, usually by relating the PCR signal to a standard curve. Relative quantification relates the PCR signal of the target transcript in a treatment group to that of another sample such as an untreated control. The $2^{-\Delta\Delta C_T}$ method is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments. The purpose of this report is to present the derivation, assumptions, and applications of the $2^{-\Delta\Delta C_T}$ method. In addition, we present the derivation and applications of two variations of the $2^{-\Delta\Delta C_T}$ method that may be useful in the analysis of real-time, quantitative PCR data. © 2001 Elsevier Science (USA)

Key Words: reverse transcription polymerase chain reaction; quantitative polymerase chain reaction; relative quantification; real-time polymerase chain reaction; Taq Man.

Reserve transcription combined with the polymerase chain reaction (RT-PCR) has proven to be a powerful method to quantify gene expression (1–3). Real-time PCR technology has been adapted to perform quantitative RT-PCR (4, 5). Two different methods of analyzing data from real-time, quantitative PCR experiments exist: absolute quantification and relative quantification. Absolute quantification determines the input copy number of the transcript of interest, usually by relating the PCR signal to a standard curve. Relative quantification describes the change in expression of the target gene relative to some reference group such as an untreated control or a sample at time zero in a time-course study.

Absolute quantification should be performed in situations where it is necessary to determine the absolute transcript copy number. Absolute quantification has been combined with real-time PCR and numerous reports have appeared in the literature (6–9) including two articles in this issue (10, 11). In some situations, it may be unnecessary to determine the absolute transcript copy number and reporting the relative change in gene expression will suffice. For example, stating that a given treatment increased the expression of gene *x* by 2.5-fold may be more relevant than stating that the treatment increased the expression of gene *x* from 1000 copies to 2500 copies per cell.

Quantifying the relative changes in gene expression using real-time PCR requires certain equations, assumptions, and the testing of these assumptions to properly analyze the data. The $2^{-\Delta\Delta C_T}$ method may be used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments. Derivation of the $2^{-\Delta\Delta C_T}$ equation, including assumptions, experimental design, and validation tests, have been described in Applied Biosystems User Bulletin No. 2 (P/N 4303859). Analyses of gene expression data using the $2^{-\Delta\Delta C_T}$ method have appeared in the literature (5, 6). The purpose of this report is to present the derivation of the $2^{-\Delta\Delta C_T}$ method, assumptions involved in using the method, and applications of this method for the general literature. In addition, we present the derivation and application of two variations of the $2^{-\Delta\Delta C_T}$ method that may be useful in the analysis of real-time quantitative PCR data.

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1. THE $2^{-\Delta\Delta C_T}$ METHOD

1.1. Derivation of the $2^{-\Delta\Delta C_T}$ Method

The equation that describes the exponential amplification of PCR is

$$X_n = X_0 \times (1 + E_X)^n, \quad [1]$$

where X_n is the number of target molecules at cycle n of the reaction, X_0 is the initial number of target molecules. E_X is the efficiency of target amplification, and n is the number of cycles. The threshold cycle (C_T) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. Thus,

$$X_T = X_0 \times (1 + E_X)^{C_{T,X}} = K_X \quad [2]$$

where X_T is the threshold number of target molecules, $C_{T,X}$ is the threshold cycle for target amplification, and K_X is a constant. A similar equation for the endogenous reference (internal control gene) reaction is

$$R_T = R_0 \times (1 + E_R)^{C_{T,R}} = K_R, \quad [3]$$

where R_T is the threshold number of reference molecules, R_0 is the initial number of reference molecules, E_R is the efficiency of reference amplification, $C_{T,R}$ is the threshold cycle for reference amplification, and K_R is a constant.

Dividing X_T by R_T gives the expression

$$\frac{X_T}{R_T} = \frac{X_0 \times (1 + E_X)^{C_{T,X}}}{R_0 \times (1 + E_R)^{C_{T,R}}} = \frac{K_X}{K_R} = K. \quad [4]$$

For real-time amplification using TaqMan probes, the exact values of X_T and R_T depend on a number of factors including the reporter dye used in the probe, the sequence context effects on the fluorescence properties of the probe, the efficiency of probe cleavage, purity of the probe, and setting of the fluorescence threshold. Therefore, the constant K does not have to be equal to one. Assuming efficiencies of the target and the reference are the same,

$$E_X = E_R = E,$$

$$\frac{X_0}{R_0} \times (1 + E)^{C_{T,X} - C_{T,R}} = K, \quad [5]$$

or

$$X_N \times (1 + E)^{\Delta C_T} = K, \quad [6]$$

where X_N is equal to the normalized amount of target (X_0/R_0) and ΔC_T is equal to the difference in threshold cycles for target and reference ($C_{T,X} - C_{T,R}$).

Rearranging gives the expression

$$X_N = K \times (1 + E)^{-\Delta C_T}. \quad [7]$$

The final step is to divide the X_N for any sample q by the X_N for the calibrator (cb):

$$\frac{X_{N,q}}{X_{N,cb}} = \frac{K \times (1 + E)^{-\Delta C_{T,q}}}{K \times (1 + E)^{-\Delta C_{T,cb}}} = (1 + E)^{-\Delta\Delta C_T}. \quad [8]$$

Here $-\Delta\Delta C_T = -(\Delta C_{T,q} - \Delta C_{T,cb})$.

For amplicons designed to be less than 150 bp and for which the primer and Mg^{2+} concentrations have been properly optimized, the efficiency is close to one. Therefore, the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by

$$\text{amount of target} = 2^{-\Delta\Delta C_T}. \quad [9]$$

1.2. Assumptions and Applications of the $2^{-\Delta\Delta C_T}$ Method

For the $\Delta\Delta C_T$ calculation to be valid, the amplification efficiencies of the target and reference must be approximately equal. A sensitive method for assessing if two amplicons have the same efficiency is to look at how ΔC_T varies with template dilution. Figure 1 shows the

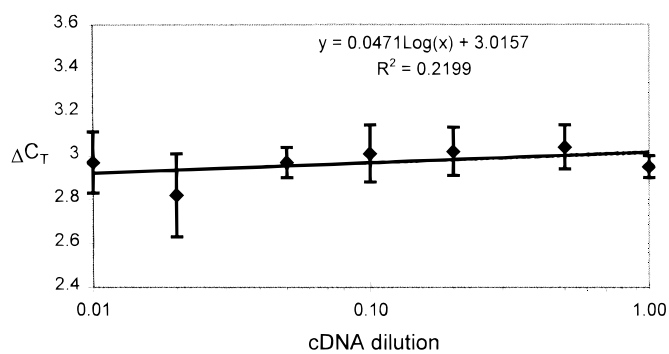

FIG. 1. Validation of the $2^{-\Delta\Delta C_T}$ method: Amplification of cDNA synthesized from different amounts of RNA. The efficiency of amplification of the target gene (c-*myc*) and internal control (GAPDH) was examined using real-time PCR and TaqMan detection. Using reverse transcriptase, cDNA was synthesized from 1 μg total RNA isolated from human Raji cells. Serial dilutions of cDNA were amplified by real-time PCR using gene-specific primers. The most concentrated sample contained cDNA derived from 1 ng of total RNA. The ΔC_T ($C_{T,c-myc} - C_{T,GAPDH}$) was calculated for each cDNA dilution. The data were fit using least-squares linear regression analysis ($N = 3$).

results of an experiment where a cDNA preparation was diluted over a 100-fold range. For each dilution sample, amplifications were performed using primers and fluorogenic probes for c-*myc* and GAPDH. The average C_T was calculated for both c-*myc* and GAPDH and the ΔC_T ($C_{T,myc} - C_{T,GAPDH}$) was determined. A plot of the log cDNA dilution versus ΔC_T was made (Fig. 1). If the absolute value of the slope is close to zero, the efficiencies of the target and reference genes are similar, and the $\Delta\Delta C_T$ calculation for the relative quantification of target may be used. As shown in Fig. 1, the slope of the line is 0.0471; therefore, the assumption holds and the $\Delta\Delta C_T$ method may be used to analyze the data. If the efficiencies of the two amplicons are not equal, then the analysis may need to be performed via the absolute quantification method using standard curves. Alternatively, new primers can be designed and/or optimized to achieve a similar efficiency for the target and reference amplicons.

1.3. Selection of Internal Control and Calibrator for the $2^{-\Delta\Delta C_T}$ Method

The purpose of the internal control gene is to normalize the PCRs for the amount of RNA added to the reverse transcription reactions. We have found that standard housekeeping genes usually suffice as internal control genes. Suitable internal controls for real-time quantitative PCR include GAPDH, β-actin, β_2-microglobulin, and rRNA. Other housekeeping genes will undoubtedly work as well. It is highly recommended that the internal control gene be properly validated for each experiment to determine that gene expression is unaffected by the experimental treatment. A method to validate the effect of experimental treatment on the expression of the internal control gene is described in Section 2.2.

The choice of calibrator for the $2^{-\Delta\Delta C_T}$ method depends on the type of gene expression experiment that one has planned. The simplest design is to use the untreated control as the calibrator. Using the $2^{-\Delta\Delta C_T}$ method, the data are presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the untreated control. For the untreated control sample, $\Delta\Delta C_T$ equals zero and 2^0 equals one, so that the fold change in gene expression relative to the untreated control equals one, by definition. For the treated samples, evaluation of $2^{-\Delta\Delta C_T}$ indicates the fold change in gene expression relative to the untreated control. Similar analysis could be applied to study the time course of gene expression where the calibrator sample represents the amount of transcript that is expressed at time zero.

Situations exist where one may not compare the change in gene expression relative to an untreated control, for example, if one wanted to determine the expression of a particular mRNA in an organ. In these cases, the calibrator may be the expression of the same mRNA in another organ. Table 1 presents mean C_T values determined for c-*myc* and GAPDH transcripts in total RNA samples from brain and kidney. The brain was arbitrarily chosen as the calibrator in this example. The amount of c-*myc*, normalized to GAPDH and relative to brain, is reported. Although the relative quantitative method can be used to make this type of tissue comparison, biological interpretation of the results is complex. The single relative quantity reported actually reflects variation in both target and reference transcripts across a variety of cell types that might be present in any particular tissue.

1.4. Data Analysis Using the $2^{-\Delta\Delta C_T}$ Method

The C_T values provided from real-time PCR instrumentation are easily imported into a spreadsheet program such as Microsoft Excel. To demonstrate the analysis, data are reported from a quantitative gene expression experiment and a sample spreadsheet is described (Fig. 2). The change in expression of the *fos–glo–myc* target gene normalized to β-actin was monitored over 8 h. Triplicate samples of cells were collected at each time point. Real-time PCR was performed on the corresponding cDNA synthesized from each sample. The data were analyzed using Eq. [9], where $\Delta\Delta C_T = (C_{T,Target} - C_{T,Actin})_{Time\ x} - (C_{T,Target} - C_{T,Actin})_{Time\ 0}$. Time x is any time point and Time 0 represents the 1× expression of the target gene normalized to β-actin. The mean C_T values for both the target and internal control genes were determined at time zero (Fig. 2, column 8) and were used in Eq. [9]. The fold change in the target gene, normalized to β-actin and relative to the expression at time zero, was calculated for each sample using Eq. [9] (Fig. 2, column 9). The mean, SD, and CV are then determined from the triplicate samples at each time point. Using this analysis, the value of the mean fold change at time zero should be very close to one (i.e., since $2^0 = 1$). We have found the verification of the mean fold change at time zero to be a convenient method to check for errors and variation among the triplicate samples. A value that is very different from one suggests a calculation error in the spreadsheet or a very high degree of experimental variation.

In the preceding example, three separate RNA preparations were made for each time point and carried through the analysis. Therefore, it made sense to treat each sample separately and average the results after the $2^{-\Delta\Delta C_T}$ calculation. When replicate PCRs are run on the same sample, it is more appropriate to average

C_T data before performing the $2^{-\Delta\Delta C_T}$ calculation. Exactly how the averaging is performed depends on if the target and reference are amplified in separate wells or in the same well. Table 1 presents data from an experiment where the target (c-*myc*) and reference (GAPDH) were amplified in separate wells. There is no reason to pair any particular c-*myc* well with any particular GAPDH well. Therefore, it makes sense to average the c-*myc* and GAPDH C_T values separately before performing the ΔC_T calculation. The variance

TABLE 1

Treatment of Replicate Data Where Target and Reference Are Amplified in Separate Wells[a]

Tissue	c-*myc* C_T	GAPDH C_T	ΔC_T (Avg. c-*myc* C_T − Avg. GAPDH C_T	$\Delta\Delta C_T$ (Avg. ΔC_T − Avg. $\Delta C_{T,Brain}$)	Normalized c-*myc* amount relative to brain $2^{-\Delta\Delta C_T}$
Brain	30.72	23.70			
	30.34	23.56			
	30.58	23.47			
	30.34	23.65			
	30.50	23.69			
	30.43	23.68			
Average	30.49 ± 0.15	23.63 ± 0.09	6.86 ± 0.17	0.00 ± 0.17	1.0 (0.9–1.1)
Kidney	27.06	22.76			
	27.03	22.61			
	27.03	22.62			
	27.10	22.60			
	26.99	22.61			
	26.94	22.76			
Average	27.03 ± 0.06	22.66 ± 0.08	4.37 ± 0.10	−2.50 ± 0.10	5.6 (5.3–6.0)

[a] Total RNA from human brain and kidney were purchased from Clontech. Using reverse transcriptase, cDNA was synthesized from 1 μg total RNA. Aliquots of cDNA were used as template for real-time PCR reactions containing either primers and probe for c-*myc* or primers and probe for GAPDH. Each reaction contained cDNA derived from 10 ng total RNA. Six replicates of each reaction were performed.

Sample #	Well	Type	Gene	Time (h)	Primer/Probe	C_T Time X	Mean C_T Time 0	$2^{\Delta\Delta CT}$	Mean Fold Change in gene expression	S.D.	C.V.
1	A1	UNKN	fos-glo-myc	0	PR1	22.3	21.9	1.023			
2	B1	UNKN	fos-glo-myc	0	PR1	22.0	21.9	0.786	1.02	0.228	22.4
3	C1	UNKN	fos-glo-myc	0	PR1	21.5	21.9	1.243			
4	D1	UNKN	fos-glo-myc	0.5	PR1	19.8	21.9	1.845			
5	E1	UNKN	fos-glo-myc	0.5	PR1	20.2	21.9	2.019	2.00	0.152	7.61
6	F1	UNKN	fos-glo-myc	0.5	PR1	20.0	21.9	2.149	2^-((20.0-21.7)-(21.9-22.5))		
7	G1	UNKN	fos-glo-myc	1	PR1	19.5	21.9	2.271			
8	H1	UNKN	fos-glo-myc	1	PR1	18.9	21.9	3.466	2.86	0.598	20.9
9	A2	UNKN	fos-glo-myc	1	PR1	19.2	21.9	2.855			
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1	A5	UNKN	Beta Actin	0	PR2	22.9	22.5				
2	B5	UNKN	Beta Actin	0	PR2	22.3	22.5				
3	C5	UNKN	Beta Actin	0	PR2	22.4	22.5				
4	D5	UNKN	Beta Actin	0.5	PR2	21.2	22.5				
5	E5	UNKN	Beta Actin	0.5	PR2	21.7	22.5				
6	F5	UNKN	Beta Actin	0.5	PR2	21.7	22.5				
7	G5	UNKN	Beta Actin	1	PR2	21.2	22.5				
8	H5	UNKN	Beta Actin	1	PR2	21.2	22.5				
9	A6	UNKN	Beta Actin	1	PR2	21.3	22.5				
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FIG. 2. Sample spreadsheet of data analysis using the $2^{-\Delta\Delta C_T}$ method. The fold change in expression of the target gene (*fos–glo–myc*) relative to the internal control gene (*β*-actin) at various time points was studied. The samples were analyzed using real-time quantitative PCR and the C_t data were imported into Microsoft Excel. The mean fold change in expression of the target gene at each time point was calculated using Eq. [9], where $\Delta\Delta C_T = (C_{T,Target} - C_{,Actin})_{Time\ x} - (C_{T,Target} - C_{,Actin})_{Time\ 0}$. The mean C_T at time zero are shown (colored boxes) as is a sample calculation for the fold change using $2^{-\Delta\Delta C_T}$ (black box).

estimated from the replicate C_T values is carried through to the final calculation of relative quantities using standard propagation of error methods. One difficulty is that C_T is exponentially related to copy number (see Section 4 below). Thus, in the final calculation, the error is estimated by evaluating the $2^{-\Delta\Delta C_T}$ term using $\Delta\Delta C_T$ plus the standard deviation and $\Delta\Delta C_T$ minus the standard deviation. This leads to a range of values that is asymmetrically distributed relative to the average value. The asymmetric distribution is a consequence of converting the results of an exponential process into a linear comparison of amounts.

By using probes labeled with distinguishable reporter dyes, it is possible to run the target and reference amplifications in the same well. Table 2 presents data from an experiment where the target (c-*myc*) and reference (GAPDH) were amplified in the same well. In any particular well, we know that the c-*myc* reaction and the GAPDH reaction had exactly the same cDNA input. Therefore, it makes sense to calculate ΔC_T separately for each well. These ΔC_T values can then be averaged before proceeding with the $2^{-\Delta\Delta C_T}$ calculation. Again, the estimated error is given as an asymmetric range of values, reflecting conversion of an exponential variable to a linear comparison.

In Tables 1 and 2, the estimated error has not been increased in proceeding from the ΔC_T column to the $\Delta\Delta C_T$ column. This is because we have decided to display the data with error shown both in the calibrator and in the test sample. Subtraction of the average $\Delta C_{T,cb}$ to determine the $\Delta\Delta C_T$ value is treated as subtraction of an arbitrary constant. This gives results equivalent to those reported in Fig. 2 where C_T values for nonreplicated samples were carried through the entire $2^{-\Delta\Delta C_T}$ calculation before averaging. Alternatively, it is possible to report results with the calibrator quantity defined as 1× without any error. In this case, the error estimated for the average $\Delta C_{T,cb}$ value must be propagated into each of the $\Delta\Delta C_T$ values for the test samples. In Table 1, the $\Delta\Delta C_T$ value for the kidney sample would become -2.50 ± 0.20 and the normalized c-*myc* amount would be 5.6× with a range of 4.9 to 6.5. Results for brain would be reported as 1× without any error.

2. THE $2^{-\Delta C'_T}$ METHOD

2.1. Derivation of the $2^{-\Delta C'_T}$ Method

Normalizing to an endogenous reference provides a method for correcting results for differing amounts of input RNA. One hallmark of the $2^{-\Delta\Delta C_T}$ method is that it uses data generated as part of the real-time PCR experiment to perform this normalization function. This is particularly attractive when it is not practical to measure the amount of input RNA by other methods. Such situations include when only limited amounts of RNA are available or when high-throughput processing of many samples is desired. It is possible, though, to normalize to some measurement external to the PCR experiment. The most common method for normalization is to use UV absorbance to determine the amount

TABLE 2

Treatment of Replicate Data Where Target and Reference are Amplified in the Same Well[a]

Tissue	c-*myc* C_T	GAPDH C_T	ΔC_T (Avg. c-*myc* C_T − Avg. GAPDH C_T)	$\Delta\Delta C_T$ (Avg. ΔC_T − Avg. $\Delta C_{T,Brain}$)	Normalized c-*myc* amount relative to brain $2^{-\Delta\Delta C_T}$
Brain	32.38	25.07	7.31		
	32.08	25.29	6.79		
	32.35	25.32	7.03		
	32.08	25.24	6.84		
	32.34	25.17	7.17		
	32.13	25.29	6.84		
Average			6.93 ± 0.16	0.00 ± 0.16	1.0 (0.9–1.1)
Kidney	28.73	24.30	4.43		
	28.84	24.32	4.52		
	28.51	24.31	4.20		
	28.86	24.25	4.61		
	28.86	24.34	4.52		
	28.70	24.18	4.52		
Average			4.47 ± 0.14	−2.47 ± 0.14	5.5 (5.0–6.1)

[a] An experiment like that described in Table 1 was performed except the reactions contained primers and probes for both c-*myc* and GAPDH. The probe for c-*myc* was labeled with the reporter dye FAM and the probe for GAPDH was labeled with the reporter dye JOE. Because of the different reporter dyes, the real-time PCR signals for c-*myc* and GAPDH can be distinguished even though both amplifications are occurring in the same well.

of RNA added to a cDNA reaction. PCRs are then set up using cDNA derived from the same amount of input RNA. One example of using this external normalization is to study the effect of experimental treatment on the expression of an endogenous reference to determine if the internal control is affected by treatment. Thus, the target gene and the endogenous reference are one in the same. In this case, Eq. [2] is not divided by Eq. [3] and Eq. [5] becomes

$$X_0 \times (1 + E_X)^{C_{T,X}} = K_X. \quad [10]$$

Rearranging gives the expression

$$X_0 = K_X \times (1 + E_X)^{-C_{T,X}}. \quad [11]$$

Now, dividing X_0 for any sample q by the X_0 for the calibrator (cb) gives

$$\frac{X_{0,q}}{X_{0,cb}} = \frac{K_X \times (1 + E_X)^{-C_{T,q}}}{K_X \times (1 + E_X)^{-C_{T,cb}}} = (1 + E_X)^{-\Delta C'_T}, \quad [12]$$

where $\Delta C'_T$ is equal to $C_{T,q} - C_{T,cb}$. The prime is used to distinguish this expression from the previous ΔC_T calculation (see Eq. [6]) that involved subtraction of C_T values for target and reference.

As stated in Section 1.1, if properly optimized, the efficiency is close to one. The amount of endogenous reference relative to a calibrator then becomes

$$2^{-\Delta C'_T}. \quad [13]$$

2.2. Application of the $2^{-\Delta C'_T}$ Method

An appropriate application of the $2^{-\Delta C'_T}$ method is to determine the effect of the experimental treatment on the expression of a candidate internal control gene. To demonstrate this analysis, serum starvation and induction experiments were performed (7). Serum starvation/induction is a commonly used model to study the decay of certain mRNAs (8). However, serum may alter the expression of numerous genes including standard housekeeping genes (9).

Gene expression was induced in NIH 3T3 cells by adding 15% serum following a 24-h period of serum starvation. Poly(A)$^+$ RNA was extracted from the cells and equivalent amounts were converted to cDNA. The amounts of β_2-microglobulin and GAPDH cDNA were determined by real-time quantitative PCR with SYBR Green detection (7). The relative amounts of β_2-microglobulin and GAPDH are presented using the $2^{-\Delta C'_T}$ equation where $\Delta C'_T = C_{T,\text{Time } x} - C_{T,\text{Time } 0}$ (Fig. 3). A statistically significant relationship exists between the treatment and expression of GAPDH but not for β_2-microglobulin (Fig. 3). Therefore, β_2-microglobulin makes a suitable internal control in quantitative serum stimulation studies while GAPDH does not. This example demonstrates how the $2^{-\Delta C'_T}$ method may be used to analyze relative gene expression data when only one gene is being studied.

3. STATISTICAL ANALYSIS OF REAL-TIME PCR DATA

The endpoint of real-time PCR analysis is the threshold cycle or C_T. The C_T is determined from a log–linear plot of the PCR signal versus the cycle number. Thus, C_T is an exponential and not a linear term. For this reason, any statistical presentation using the raw C_T

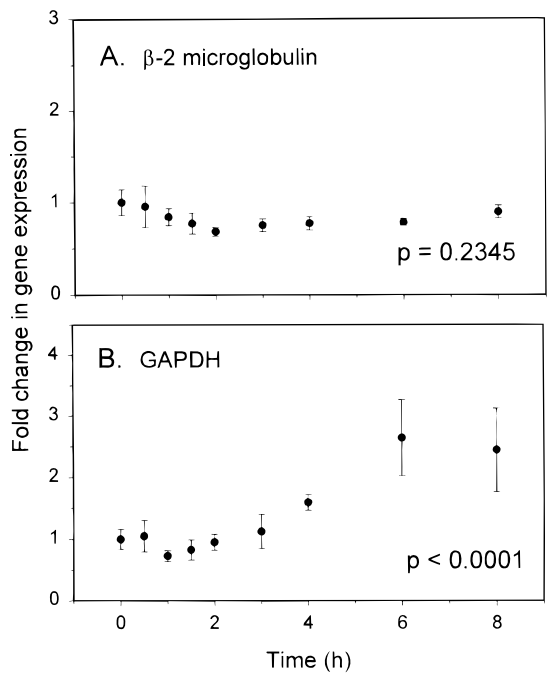

FIG. 3. Application of the $2^{-\Delta C'_T}$ method. The following experiment was conducted to validate the effect of treatment on the expression of candidate internal control genes. NIH 3T3 fibroblasts were serum starved for 24 h and then induced with 15% serum over an 8-h period. Samples were collected at various times following serum stimulation; mRNA was extracted and converted to cDNA. The cDNA was subjected to real-time quantitative PCR using gene-specific primers for β_2-microglobulin and GAPDH. The fold change in gene expression was calculated using Eq. [13], where $\Delta C_T = (C_{T,\text{Time } x} - C_{T,\text{Time } 0})$ and is presented for both β_2-microglobulin (A) and GAPDH (B). Reprinted from T. D. Schmittgen and B. A. Zakrajsek (2000) Effect of experimental treatment on housekeeping gene expression: Validation by real-time quantitative RT-PCR, *J. Biochem. Biophys. Methods* **46,** 69–81, with permission of Elsevier Science.

values should be avoided. As described within the previous sections of this article, presentation of relative PCR data is most often calculated along with an internal control and/or calibrator sample and is rarely presented as the C_T. An exception is when one is interested in examining the sample-to-sample variation among replicate reactions.

To demonstrate this, 96 replicate reactions of the identical cDNA were performed using real-time PCR and SYBR Green detection. A master mixture containing all of the ingredients was pipetted into individual tubes of a 96-well reaction plate. The samples were subjected to real-time PCR and the individual C_T values were determined. To examine the intrasample variation, the mean ± SD was determined from the 96 samples. If calculated from the raw C_T the mean ± SD was 20.0 ± 0.194 with a CV of 0.971%. However, when the individual C_T values were converted to the linear form using the term 2^{-C_T}, the mean ± SD was $9.08 \times 10^{-7} \pm 1.33 \times 10^{-7}$ with a CV of 13.5%. As demonstrated by this simple example, reporting the data obtained from the raw C_T values falsely represents the variation and should be avoided. Converting the individual data to a linear form using 2^{-C_T} more accurately depicts the individual variation among replicate reactions.

4. CONCLUDING REMARKS

Experimental design and data analysis from real-time, quantitative PCR experiments may be achieved using either relative or absolute quantification. When designing quantitative gene expression studies using real-time PCR, the first question that an investigator should ask is how should the data be presented. If absolute copy number is required, then the absolute method should be used. Otherwise, presentation of the relative gene expression should suffice. Relative quantification may be easier to perform than the absolute method because the use of standard curves is not required.

The equations provided herein should be sufficient for an investigator to analyze quantitative gene expression data using relative quantification. To summarize the important steps in the design and evaluation of the experiment: (*i*) select an internal control gene, (*ii*) validate the internal control to determine that it is not affected by experimental treatment, and (*iii*) PCR on perform dilutions of RNA or cDNA for both the target and internal control genes to ensure that the efficiencies are similar. Finally, statistical data should be converted to the linear form by the 2^{-C_T} calculation and should not be presented by the raw C_T values.

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