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A New Quantitative Method of Real Time Reverse Transcription Polymerase Chain Reaction Assay Based on Simulation of Polymerase Chain Reaction Kinetics

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Real-time reverse transcription (RT) PCR is currently the most sensitive method for the detection of low-abundance mRNAs. Two relative quantitative methods have been adopted: the standard curve method and the comparative C_T method. The latter is used when the amplification efficiency of a reference gene is equal to that of the target gene; otherwise the standard curve method is applied. Based on the simulation of kinetic process of real-time PCR, we have developed a new method for quantitation and normalization of gene transcripts. In our method, the amplification efficiency for each individual reaction is calculated from the kinetic curve, and the initial amount of gene transcript is derived and normalized. Simulation demonstrated that our method is more accurate than the comparative C_T method and would save more time than the relative standard curve method. We have used the new method to quantify gene expression levels of nine two-pore potassium channels. The relative levels of gene expression revealed by our quantitative method were broadly consistent with those estimated by routine RT-PCR, but the results also showed that amplification efficiencies varied from gene to gene and from sample to sample. Our method provides a simple and accurate approach to quantifying gene expression level with the advantages that neither construction of standard curve nor validation experiments are needed.

The reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive method for the detection of specific mRNAs (1, 2). In contrast with classic techniques, such as Northern blot analysis and RNase protection assays, which require large amounts of total RNA, RT-PCR assay is capable of quantifying mRNA levels from samples as small as individual cells (3–6). The recent introduction of fluorescence techniques to PCR, together with instrumentation able to amplify, detect, and quantify mRNA levels, has formed the basis of kinetic or real-time RT-PCR assays (7–10). Kinetic quantitative assays have largely overcome the limitation of classic RT-PCR quantitation strategies such as RT-PCR with dot-blot analysis and competitive RT-PCR, and have significantly simplified the process of producing reproducible quantitation of low-abundance mRNAs (11).

Quantitation of mRNA transcription by real-time RT-PCR can be either absolute or relative (9, 12–14). Unlike endpoint RT-PCR, real-time quantification is defined by C_T (threshold cycle number) at a fixed threshold where PCR amplification is still in the exponential phase and the reaction components are not limiting gene amplification. Absolute quantification requires the construction of an absolute standard curve for each individual amplicon to calculate the precise copy numbers of mRNA transcripts per cell or unit mass of tissue (8). Two relative quantitation methods, the standard curve method and comparative C_T method, have been developed (15). In the standard curve method, the input amount for unknown samples is calculated from the standard curve of a specific gene, and normalized to the input amount of a reference gene which is also calculated from its standard curve. The comparative C_T method, as previously described by Livak (15), detects the relative gene expression with the formula $2^{-\Delta\Delta C_T}$. This formula is based on the assumptions that the amplification efficiencies of the "reference" and "target" gene are approximately equal and that the amplification efficiency is close to 1 (15).

The SYBR Green real-time RT-PCR assay with the same instrumentation and software as TaqMan real-time RT-PCR has also been broadly employed and similar quantitative methods based on TaqMan real-time

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TABLE 1

Primers for Two-Pore Potassium Channel Genes and *GAPDH*

Gene and GeneBank Accession No.	Forward primer (5′–3′)	Reverse primer (5′–3′)	Size (bp)
TWIK-1 (AF033017)	AAGCCTTCTGCATCATCTACTCTGT	ACCAGGGCCGTCAGGAA	67
TWIK-2 (AF281305)	GCGTGCCTATCACCATGCTA	TGGGTGAGCAGCAGTGACA	64
TASK-1 (AF065162)	CTACTACGAGCGCTGGACTTTCT	TAGTCGCCGAAGCCGATG	78
TASK-2 (NM021542)	ACAAGATCCTACAGGTGGTGTCTG	GAAAGTCTGGTTCCCGGTGAT	68
TASK-3 (AF192366)	GACGCCCTCGAGTCGGA	AGGCGGACCTCTTCTGCTTT	65
TREK-1 (NM010607)	CGGCCGAGTTCAAGGAAA	CACGCTGGAACTTGTCGTAGAT	66
TREK-2 (AF196965)	ATCCGGGTCATCTCAACTATTCTT	GACAGCAGGGATCGTCACAA	69
TRAAK (AF056492)	GTCCAGTGGTTGCCAAATGC	TGGTGGGCTTTGGAAATGAT	73
KCNK6 (AF110521)	TCAGAGACAAGCAACTGGGATCT	ATAACCGGTGGTGGTGAGGAT	72
GAPDH (M32599)	ATGTTCCAGTATGACTCCACTCACG	GAAGACACCAGTAGACTCCACGACA	171

RT-PCR have been applied (6, 16–18). In our application of SYBR Green real-time RT-PCR, we have found that the amplification efficiency of PCR is often not close to 1, and amplification efficiencies of the reference and target genes are not always approximately equal. Similarly, amplification efficiency differences have been found in DNA samples for real-time PCR, and an efficiency compensation protocol has recently been developed for human DNA samples (19). In the present study, we simulate the kinetics of real-time PCR using experimentally determined parameters. We demonstrate that the simulation can well describe the early exponential phase of the PCR, and we develop a new method for normalization of target gene expression level to a reference gene, based on the dynamics of the reaction. In the new method, the amplification efficiency of an individual reaction is calculated from the kinetics of the reaction and used for quantitation and normalization. We also simulate the relative standard curve method and comparative C_T method, and compare these with our new quantitation method. The accuracy by our method is comparable to that of the standard curve method and greater than that of the C_T method in the simulation. Using the new method, we quantified the expression levels of nine two-pore potassium channel genes in rat cardiac left ventricle. The new method obviates the need for the construction of the relative standard curve and the need for series of validation experiments which are the prerequisite for the comparative C_T method (15), hence providing a more accurate, faster, and more efficient quantitation method.

MATERIALS AND METHODS

Preparation of Total RNA and Real-Time RT-PCR. Rats were killed by exsanguination under CO_2 anaesthesia. Hearts were removed, snap-frozen in liquid nitrogen, and stored at −80°C until RNA extraction. Total RNA was isolated using TRIzol Reagent (Life Technologies, Inc., Frederick, MD), 2 μg of total RNA was treated with DNase (Life Technologies, Inc.) and reverse-transcribed using random hexamer primer and SYBR Green RT-PCR reagents (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Synthesized cDNA corresponding to 100–200 ng total RNA was used for real-time PCR.

Nine rat or mouse genes of two-pore potassium channels and a rat housekeeping gene, *GAPDH*, were selected for real-time RT-PCR analysis. Gene-specific primers (detailed in Table 1) were designed using Primer Express Software (PE Applied Biosystems) according to the software guidelines. All primers were purchased from GeneSet (GenSet Pacific Pty. Ltd., Australia). SYBR Green PCR assays for all specific genes were performed on cDNA samples in 96-well optical plates on an ABI Prism 5700 Sequence Detection System (PE Applied Biosystems). *GAPDH* assays were run parallel to each different sample. For each 25-μl SYBR Green PCR, 2.5 μl cDNA, 1.5 μl sense primer (5 μM), 1.5 μl antisense primer (5 μM), 12.5 μl SYBR Green PCR Master Mix (PE Applied Biosystems), and 7 μl PCR-grade water were mixed together. The PCR parameters were 95°C for 10 min, 1 cycle, then 60°C for 1 min, 95°C for 15 s, 40 cycles.

Simulation of PCR kinetics. The initial growth of PCR product can be dynamically expressed as (15)

$$X_n = X_0 * (1 + E)^n, \quad [1]$$

where X_n is number of amplified molecules at cycle n, X_0 initial number of template molecules, E amplification efficiency, and n number of cycles.

In fluorescence dye real-time PCR, X_n is proportional to reporter fluorescence R, so Eq. [1] can be written as

$$R_n = R_0 * (1 + E)^n, \quad [2]$$

where R_n is reporter fluorescence at cycle n, and R_0 initial reporter fluorescence. Following the original description of amplification efficiency in real-time PCR and its adoption by others (15, 20), we still adopt E in

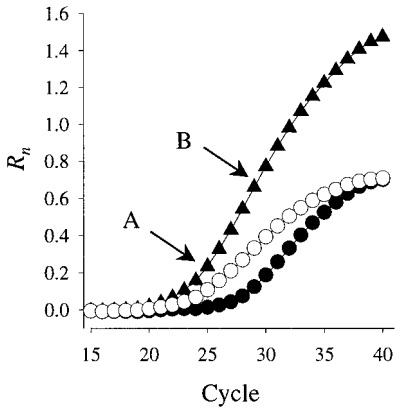

FIG. 1. Calculation of amplification efficiency from the kinetic PCR curve. Three curves were chosen representing *GAPDH* (▲) and two different target genes (○ and ●). R_n is measured at two thresholds along the exponential phase as shown at A and B. Amplification efficiency is calculated using Eq. [3].

Eq. [2] as amplification efficiency though it reflects only a fraction of extra yield from a preceding cycle (i.e., at maximum theoretical efficiency, when the product is doubling with each cycle, $E = 1$; a reaction that gave no product with each cycle would give $E = 0$).

Since the reaction is performed in a closed tube containing a small amount of reaction mixture (25–50 μl), the reaction kinetics can be affected by all components in the reaction mixture, including reporter dye, nucleotide concentration, primer concentration, and initial copy number (or concentration) of template. Because the reporter dye, nucleotide, primer concentration, and enzymatic activity can become limiting to the rate of synthesis of amplicon, the rate of synthesis of amplicon will slow and eventually cease. Thus, Eq. [1] or [2] can only mathematically describe the initial exponential phase of the reaction. We therefore focused on the exponential phase, and simulated the initial PCR kinetics based on Eq. [2].

Quantitative methods. Amplification efficiencies of reference and target genes are not always approximately equal, as revealed by the different slopes of the exponential phase and different maxima for R in real-time PCR (Fig. 1). Thus, we modified the quantitative method by calculating the amplification efficiency for each PCR kinetic curve.

According to Eq. [2], the amplification efficiency can be determined by

$$E = \left(\frac{R_{n,\mathrm{A}}}{R_{n,\mathrm{B}}}\right)^{-(C_{\mathrm{T,A}} - C_{\mathrm{T,B}})} - 1, \qquad [3]$$

where $R_{n,\mathrm{A}}$ and $R_{n,\mathrm{B}}$ are R_n at arbitrary thresholds A and B in an individual curve, respectively, and $C_{\mathrm{T,A}}$ and $C_{\mathrm{T,B}}$ are the threshold cycles at these arbitrary thresholds (Fig. 1).

R_n of a reference gene and R_n of a target gene at the same threshold (i.e., R_n are equal) can be expressed as

$$R_{n,\mathrm{R}} = R_{0,\mathrm{R}} * (1 + E_{\mathrm{R}})^{C_{\mathrm{T,R}}}, \qquad [4]$$

$$R_{n,\mathrm{T}} = R_{0,\mathrm{T}} * (1 + E_{\mathrm{T}})^{C_{\mathrm{T,T}}}, \qquad [5]$$

where R_n, E, and C_{T} have their usual meanings and the added subscripts R and T denote reference and target genes, respectively.

If $E_{\mathrm{R}} \neq E_{\mathrm{T}}$, two approaches can be applied for normalization:

1. From Eqs. [4] and [5], normalization or $R_{0,\mathrm{T}}/R_{0,\mathrm{R}}$ can be expressed as

$$R_{0,\mathrm{T}}/R_{0,\mathrm{R}} = \frac{(1 + E_{\mathrm{R}})^{C_{\mathrm{T,R}}}}{(1 + E_{\mathrm{T}})^{C_{\mathrm{T,T}}}}. \qquad [6]$$

2. At any cycle, the reporter dye strength can be also written as

$$R_{n,\mathrm{R}} = R_{0,\mathrm{R}} * (1 + E_{\mathrm{R}})^{n}, \qquad [7]$$

$$R_{n,\mathrm{T}} = R_{0,\mathrm{T}} * (1 + E_{\mathrm{T}})^{n}. \qquad [8]$$

Thus, normalization can be also expressed as

$$R_{0,\mathrm{T}}/R_{0,\mathrm{R}} = \frac{R_{\mathrm{n,T}} * (1 + E_{\mathrm{R}})^{n}}{R_{n,\mathrm{R}} * (1 + E_{\mathrm{T}})^{n}}. \qquad [9]$$

If $E_{\mathrm{R}} = E_{\mathrm{T}} = E$, similarly, two approaches can be used for normalization: $R_{0,\mathrm{T}}/R_{0,\mathrm{R}}$ can be simplified from Eqs. [6] and [9]:

$$R_{0,\mathrm{T}}/R_{0,\mathrm{R}} = (1 + E)^{\Delta C_{\mathrm{T}}}, \qquad [10]$$

$$R_{0,\mathrm{T}}/R_{0,\mathrm{R}} = R_{n,\mathrm{T}}/R_{n,\mathrm{R}}, \qquad [11]$$

where $\Delta C_{\mathrm{T}} = C_{\mathrm{T,R}} - C_{\mathrm{T,T}}$.

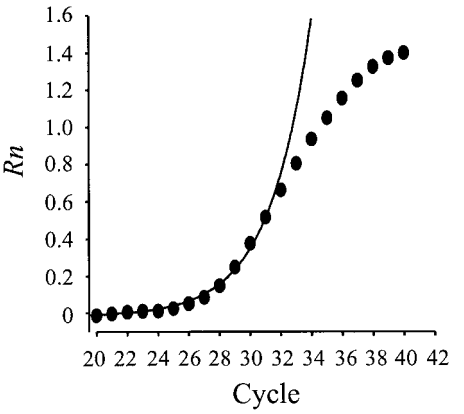

FIG. 2. Simulation of exponential phase of real-time PCR. ●, Experimental data; —, simulated curve. The simulated curve fits well the exponential phase of the experimental data.

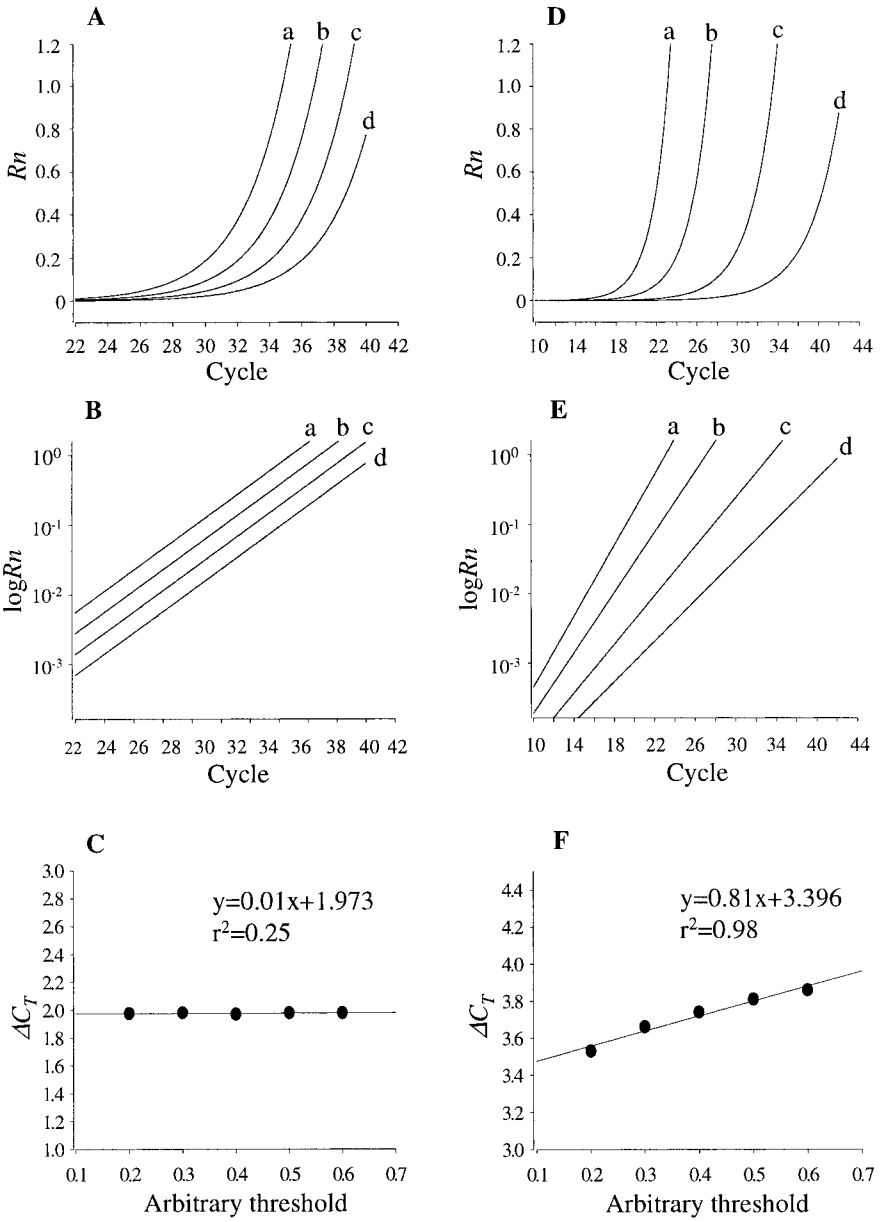

FIG. 3. Simulation of real-time PCR at different initial amounts of template and amplification efficiencies. (A) Constant amplification efficiency ($E = 0.42$) and four different initial amounts of template represented by R_0: 5.0×10^{-6}, 2.5×10^{-6}, 1.25×10^{-6}, and 0.625×10^{-6} (a, b, c, and d, respectively). (B) The logarithm scale of R_n of (A). (C) ΔC_T is plotted against different thresholds. (D) Constant initial amount of template (2.5×10^{-6}) and four different amplification efficiencies: 0.8, 0.65, 0.5, and 0.4 (a, b, c, and d, respectively). (E) and (F) are similar to (B) and (C).

When Eq. [10] is used to calculate the gene expression level relative to the 1× sample from real-time PCR, it becomes

$$R_{N,b}/R_{N,a} = (1 + E)^{-\Delta\Delta C_T}, \qquad [12]$$

where $R_{N,a}$ and $R_{N,b}$ are normalized R_0 by Eq. [10] for samples a and b, respectively. $\Delta\Delta C_T$ is the difference of ΔC_T for samples a and b.

RESULTS

Simulation Studies

R_0 and amplification efficiencies were set up in the range that was derived from real-time RT-PCR experiments by fitting Eq. [2] to the early exponential phase of the PCR kinetic data. By using Eq. [2], we simulated the exponential phase of a real-time PCR kinetic curve with the parameters (R_0 and amplification efficiencies) derived from the reaction curve; the reconstructed

TABLE 2

Relative Gene Expression Levels from Simulated Real-Time PCR Calculated by $2^{-\Delta\Delta C_T}$ and the New Methods

Curve	R_0	Relative to 1× sample[a]	$2^{-\Delta\Delta C_T}$	$(1 + E)^{-\Delta\Delta C_T}$	$R_{n,T}/R_{n,R}$[b]
a	5.0×10^{-6}	—	—	—	—
b	2.5×10^{-6}	1	1	1	1
c	1.25×10^{-6}	0.5000	0.2553	0.5021	0.5000
d	0.625×10^{-6}	0.2500	0.0647	0.2503	0.2500

[a] Gene expression level relative to 1× sample is calculated directly from the assumed R_0 in Fig. 3A.
[b] R_n was measured at cycle 32.

curve fits well for most of the exponential phase of the experimental curve as shown in Fig. 2.

Simulation revealed that both the initial amount of template and the amplification efficiency affect C_T. In Fig. 3A, a, b, c, and d simulate the exponential phase of real-time PCR with the same amplification efficiency and different R_0. C_T measured at a fixed threshold of 0.1 for a, b, c, and d are 28.1, 30.08, 32.05, and 34.03, respectively, demonstrating that the larger initial amount of template needs fewer cycles to reach a threshold for detection. A logarithmic scale for R_n revealed that the exponential phases of a, b, c, and d are parallel to each other (Fig. 3B), reflecting the equal amplification efficiency. Similar to the validation experiments for detection of equal amplification efficiency (15), C_T difference (ΔC_T) at a series of arbitrary thresholds for two reactions was plotted against the thresholds; a linear regression line for this relationship with a slope not significantly different from zero indicates equal amplification efficiencies (Fig. 3C). Figure 3D shows the results of the simulated kinetic processes a, b, c, and d with the same initial template concentration but different amplification efficiencies. At a fixed threshold of 0.1, C_T for a, b, c, and d are 19.13, 22.45, 27.22, and 33.41, respectively, demonstrating that a higher amplification efficiency results in fewer cycles being required for detection at a fixed threshold. In contrast to the simulations with equal amplification efficiency in Fig. 3A, plotting R_n on a logarithmic scale yields a nonparallel relationship between the exponential phases of the reactions (Fig. 3E), and the ΔC_T plot reveals a slope significantly different from zero (Fig. 3F). Therefore, the logarithmic plot of R_n or the ΔC_T plot from the early exponential phase of real-time PCR can be used for detection of whether amplification efficiencies of two reactions are equal.

We also used the comparative C_T method to calculate relative gene expression levels simulated in Fig. 3A to compare the results obtained by our method. Curve a was used as reference, gene expression levels in b, c, and d were normalized to a, and curve b was used as calibrator (1× sample). Gene expression levels relative to the 1× sample were calculated directly from the assumed R_0, also calculated by $2^{-\Delta\Delta C_T}$ and Eq. [12] with an amplification efficiency of 0.42 for all curves. In comparison to the results directly calculated from the initial R_0, the results obtained by our method using Eq. [12] were more accurate than those from the $2^{-\Delta\Delta C_T}$ method, as shown in Table 2. The $2^{-\Delta\Delta C_T}$ method led to 1.96 and 3.87 times smaller estimates for curves c and d, respectively. Our simulation also demonstrated that the smaller the initial amount of template, the more significant the underestimation resulting from the $2^{-\Delta\Delta C_T}$ method.

To test whether the quantitation method using the calculated amplification efficiency and Eq. [6] or [9] is comparable to the relative standard curve method, we simulated the relative standard curve method for normalization and calculation of relative gene expression levels. Figures 4A and 4C show simulations of the exponential kinetic curves of a reference gene and a target gene, respectively, with amplification efficiencies of 0.4 for the reference gene and 0.6 for the target gene. Five series of input amounts were set for the construction of standard curves. In reality, input amounts for the construction of standard curves can be either total RNA or dilutions of the sample (12). Here, we represented the input amount as the multiple of an elementary R: 4, 1, 0.25, 0.0625, and 0.015625 times R were chosen; $R \times 10^{-6}$ was used for the reference gene and $R \times 10^{-7}$ for the target gene. A threshold of 0.02 was used for the measurement of C_T from the simulated curves. The standard curves and the regression equations are shown in Figs. 4B and 4C. Four simulated samples with assumed input amounts of 2, 1, 0.5, and 0.25 for the target gene and the same input amount of 1 for the reference gene were quantified, normalized, and calculated relative to the 1× sample, and compared with the results obtained by the new method. The amount of genes input was calculated from the simulated reactions using the regression equation obtained from the standard curves. Table 3 shows the results obtained by both the standard curve method and our method. Though the normalization values from the two methods differ, the relative gene expression levels are approximately the same.

Relative Quantitation Using Amplification Efficiencies

Figure 5 shows the amplification efficiencies of *GAPDH* and nine two-pore channel genes calculated

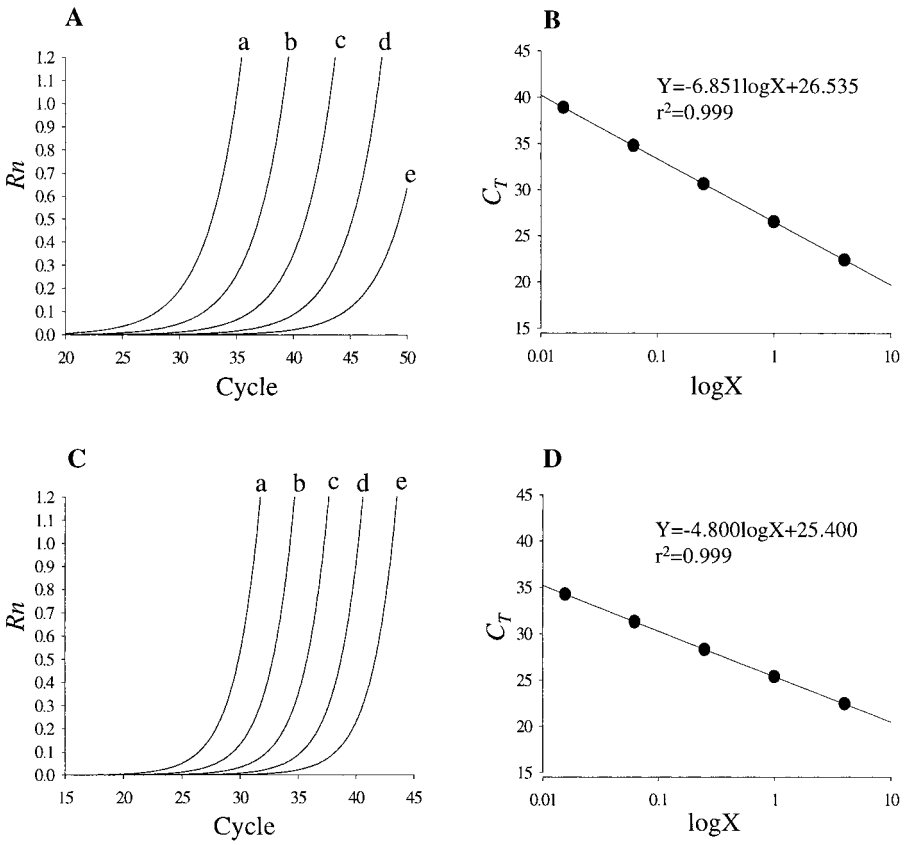

FIG. 4. Simulation of relative standard curve method for quantitation and normalization. Simulated curves (a–e) from five different input amounts (see in text) of a reference gene (A) with amplification efficiency 0.4 and a target (C) with amplification efficiency 0.6 were used for construction of the standard curves (B and C). Y represents C_T, and X, the input amount.

using Eq. [3]. This result demonstrated that amplification efficiencies were not equal each to other for most of the amplified genes, and no reaction reached an amplification efficiency of 1. Because the amplification efficiency of most target genes is not equal to that of the reference gene, the method using Eq. [6] or [9] is the preferred approach, since these equations can be applied directly without considering whether the amplification efficiencies of both target and reference genes are equal. To study whether the nucleotide G and C content of the amplicons affected amplification efficiencies we plotted amplification efficiencies against GC content of amplicons (Fig. 5B). It was found that the GC content of amplicons has no significant effect on amplification efficiency ($P = 0.37$). Normalization of nine selected two-pore potassium genes from rat left ventricles to *GAPDH* is illustrated in Fig. 5C. This result is consistent with, but more accurate than, the relative expression levels of two-pore potassium genes reported from studies in which routine RT-PCR was employed (21–24).

TABLE 3

Normalization and Relative Gene Expression Levels by the Standard Curve Method and the New Method

Target gene				Reference gene				Standard curve method		New method	
Assumed input	C_T	Calculated input	Calculated R_0	Assumed input	C_T	Calculated input	Calculated R_0	Normalization	Relative level	Normalization	Relative level
2	23.9	2.0503	2×10^{-7}	1	26.55	0.9950	10^{-6}	2.0639[a]	1	0.2000*	1
1	25.4	1	1×10^{-7}	1	26.55	0.9950	10^{-6}	1.0051	0.4870	0.1000	0.5000
0.5	26.85	0.4988	0.5×10^{-7}	1	26.55	0.9950	10^{-6}	0.5013	0.2429	0.0500	0.2500
0.25	28.35	0.2429	0.25×10^{-7}	1	26.55	0.9950	10^{-6}	0.2441	0.1183	0.0250	0.1250

[a] The value is used as 1× sample for relative gene expression level.

DISCUSSION

In this study we describe a rapid and accurate method for quantitation and normalization of gene expression level, on the basis of the simulation of real-time PCR kinetics. The method is simple and independent of standard curve construction or validation experiments as required in the relative standard curve or comparative C_T methods (15). The precision of quantitation is central for comparison of low-abundance genes, but the precision of quantitation in real-time PCR can be affected by small variations between samples. For example, the accuracy of sample dilution for construction of the standard curve is very important for accurate quantitation (15). Since our method does not need additional series of samples for construction of the standard curve, the error due to dilution inaccuracy is greatly reduced, or obviated entirely if real-time PCR for the reference and target genes is performed in one tube. In our method, the amplification efficiency is calculated from the kinetic curves and the initial amount of gene transcripts derived therefrom, thereby eliminating the need for validation of equality of amplification efficiency of the reference and target genes. Though the relative standard method is proposed to be used for quantitation when amplification efficiencies of the reference and target gene are not equal (15), it assumes that the amplification efficiencies of different samples for the same gene are equal. This assumption is often not valid, as confirmed by direct calculation of amplification efficiencies in our experiments and other reports (19). If this equality does not hold, optimization experiments are required until approximate equality is set up.

In comparison with the standard curve method and comparative C_T methods, our method has been demonstrated to be the most accurate as shown in Tables 2 and 3. In the standard curve method, normalization is based on the calculation of input amount of total RNA or the dilutions of the samples (15). This normalization does not represent the fraction of the target gene transcripts to the reference gene transcripts; thus, gene expression level relative to a 1× sample has to be used. The comparative C_T method, based on the ratio of derived initial target gene transcripts to initial reference gene transcripts (15), is the least accurate method if amplification efficiencies of target and reference genes are not equal to 1. In reality, the comparative C_T method could result in significant inaccuracy because of: (1) an amplification efficiency less than 1, (2) unequal amplification efficiencies of the target and reference genes, and (3) a very low abundance of the target gene which can exacerbate the inaccuracy.

Since normalization by our method is based on the derived R_0 of both reference and target genes, which represent the fraction of target gene transcripts to reference gene transcripts, this normalization can be used directly for comparison among different samples, and the expression level relative to a 1× sample can be calculated from the normalization results. The method

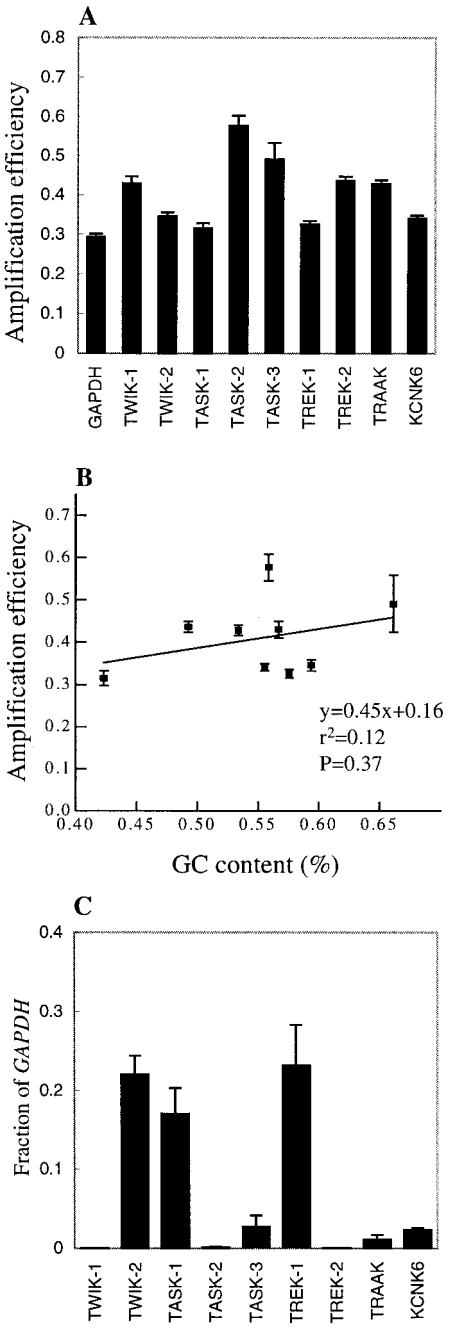

FIG. 5. Quantitation and normalization of two-pore potassium channel genes with the new methods. (A) Amplification efficiencies of *GAPDH* and two-pore potassium channel genes. The amplification efficiencies are: *GAPDH*, 0.29 ± 0.01 (31); *TWIK-1*, 0.43 ± 0.02 (13); *TWIK-2*, 0.35 ± 0.01 (16); *TASK-1*, 0.32 ± 0.01 (13); *TASK-2*, 0.58 ± 0.03 (13); *TASK-3*, 0.49 ± 0.04 (6); *TREK-1*, 0.33 ± 0.01 (28); *TREK-2*, 0.44 ± 0.01 (13); *TRAAK*, 0.43 ± 0.01 (12); *KCNK6*, 0.34 ± 0.01 (13). Value in parentheses is duplicate number. (B) Amplification efficiencies are plotted against the nucleotide GC contents in the amplicons. (C) Normalization of the expression levels of two-pore potassium channel genes to *GAPDH*.

can also be used for absolute quantitation of gene transcripts if the relationship between R_0 and gene copies is determined by parallel real-time RT-PCR with known copies of the target and reference genes.

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