ACADEMIC PRESS

Biochemical and Biophysical Research Communications 294 (2002) 347–353

BBRC

www.academicpress.com

Validation of a quantitative method for real time PCR kinetics

Weihong Liu and David A. Saint*

Department of Physiology, University of Adelaide, Adelaide, SA 5005, Australia

Received 30 April 2002

Abstract

Real time RT-PCR is the most sensitive method for quantitation of gene expression levels. The accuracy can be dependent on the mathematical model on which the quantitative methods are based. The generally accepted mathematical model assumes that amplification efficiencies are equal at the exponential phase of the reactions for the same amplicon. However, no methods are available to test the assumptions regarding amplification efficiency before one starts the real time PCR quantitation. Here we further develop and test the validity of a new mathematical model which dynamically fits real time PCR data with good correlation ($R^2 = 0.9995 \pm 0.002$, $n = 50$). The method is capable of measuring cycle-by-cycle PCR amplification efficiencies and demonstrates that these change dynamically. Validation of the method revealed the intrinsic relationship between the initial amount of gene transcript and kinetic parameters. A new quantitative method is proposed which represents a simple but accurate quantitative method.

Keywords: Real time PCR; Kinetics; Mathematical model; Quantitation of gene expression

The reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive method for the detection of specific mRNAs, in particular for low abundance genes [1–7]. 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 [8–11]. Kinetic quantitative assays have largely overcome the limitation of classical 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. This has made real time PCR a routine technique in molecular biology [12].

Two major approaches of real time PCR quantitation, the absolute or relative quantitative method, are currently in use [6,9,13–19]. The standard curve method for either absolute or relative quantitation is based on the construction of standard curve of cycle number at a threshold (C_T) vs initial input amount of total RNA or copy number. This method assumes an approximately equal amplification efficiency of PCR among the diluted samples of the same gene, thus producing a linear relationship of C_T vs initial input amount of total RNA or copy number. The comparative C_T method, a currently broadly adopted relative method, uses a non-convergence and very straight forward mathematical model which fits only the initial phase of the reaction to model the kinetics of PCR. This method relies on several assumptions including (1) approximately equal amplification efficiencies between target and reference genes and (2) a constant amplification efficiency close to 1 in the exponential phase of PCR [20]. C_T is used in both absolute and relative methods since it is generally assumed that in the exponential phase of PCR, where C_T is determined, the reaction components are not limiting, and thus amplification efficiency should be identical. However, there are no methods yet available for evaluating whether the overall amplification efficiency in the exponential phase is indeed constant and equal between reactions.

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 [21]. Similarly, differences in amplification efficiency have been found in DNA samples for real time PCR, and an efficiency compensation protocol

* Corresponding author. Fax: +61-8-8303-3356.
E-mail address: david.saint@adelaide.edu.au (D.A. Saint).

0006-291X/02/$ - see front matter
PII: S0006-291X(02)00478-3

has been developed recently for human DNA samples [22]. This indicates that the kinetic parameters of real time PCR vary for individual reactions, even for the same gene. Therefore, more accurate quantitation could be achieved by consideration of the kinetics of each individual reaction. For example, amplification efficiency in the early exponential phase of each reaction could be calculated from individual reaction kinetics, instead of assuming a constant ($E = 1$) and identical amplification efficiency for all reactions. Based on the kinetics in the early exponential phase of individual real time PCR, we have developed an amplification efficiency-based quantitative method [21]. In the present study, we demonstrated that amplification efficiency varies over the early exponential phase of the reaction and we applied a sigmoidal mathematical model that well fits the whole kinetic process of real time PCR. We then evaluated the validity of various assumptions employed in current real time PCR by simulation of real time PCR kinetics using this new mathematical model. The studies revealed that the amplification efficiency of real time PCR is changed dynamically during the course of the reaction. The association of changes in kinetic parameters and the initial amount of gene level are clarified, and a new quantitative method based on the mathematical model is proposed and experimentally validated.

Materials and methods

Total RNA extraction and reverse transcription reaction. Heart tissue was used for total RNA extraction in this study. Rats were killed by exsanguination under CO_2 anaesthesia. Hearts were removed and snap frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Total RNA was isolated using TRIzol Reagent (Life Technologies, Frederick, MD, USA), 2 μg of total RNA was treated with DNase (Life Technologies, Frederick, MD, USA) and reverse-transcribed using random hexamer primer and SYBR Green RT-PCR reagents (PE Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

Synthesized cDNA corresponding to 100 ng total RNA was used for real time PCR. *GAPDH* and a two-pore potassium ion channel gene *TREK-1* were chosen for PCR amplification. Primers were designed using Primer Express Software (PE Applied Biosystems, Foster City, CA) according to the software guidelines.

The primer sequence is:
GAPDH
Forward: 5′-ATGTTCCAGTATGACTCCACTCACG-3′,
Reverse: 5′-GAAGACACCAGTAGACTCCACGACA-3′,
TREK-1
Forward: 5′-CGGCCGAGTTCAAGGAAA-3′,
Reverse: 5′-CACGCTGGAACTTGTCGTAGAT-3′.

All primers were purchased from GenSet (GenSet Pacific, Australia). SYBR Green PCR assays were performed on cDNA samples in 96-well optical plates on an ABI Prism 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). For each 25 μl SYBR Green PCR reaction, 2.5 μl cDNA, 1.5 μl sense primer (5 μM), 1.5 μl anti-sense primer (5 μM), 12.5 μl SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA), and 7 μl PCR-grade water were mixed together. The parameters for a two-step PCR were 95 °C for 10 min, 1 cycle, then 60 °C for 1 min, 95 °C for 15 s, 40 cycles. The specificity of the amplified products was examined in 3% agarose gel.

Simulation method. R_n vs Cycles were fitted with the following equation using SigmaPlot (SigmaPlot, version 5.0, SPSS, Richmond, CA, USA):

$$R - R_b = \frac{R_{max}}{1 + \exp(-((n - n_{1/2})/k))} \quad (1)$$

$$R_n = R - R_b, \quad (2)$$

where R is fluorescence dye strength in real time PCR, R_n is background-removed fluorescence dye strength in real time PCR at cycle n, R_b is the background fluorescence dye strength, R_{max} the maximal fluorescence dye strength, $n_{1/2}$ the cycle number when fluorescence dye strength is half of the R_{max}, k the slope factor of increase in fluorescence dye strength.

Obviously, amplification efficiency is not constant over the whole PCR process, since the PCR kinetic curve eventually saturates (implying that efficiency must drop to zero). Thus, we define amplification efficiency on a cycle to cycle basis according to the mathematical model currently applied in the real time PCR quantitation [20].

Amplification efficiency can be written as:

$$E_n = \frac{R_n - R_{n-1}}{R_{n-1}} \quad (3)$$

or

$$R_n = R_{n-1} + R_{n-1} * E_n, \quad (4)$$

where E_n is the amplification efficiency at cycle n, and where R_n and R_{n-1} are the fluorescence dye strength at cycle n and $n - 1$.

For simplicity in simulation, we assume $R_b = 0$, thus, a combination of Eqs. (1)–(3) gives:

$$E_n = \left\{1 + \exp\left(-\frac{n - 1 - n_{1/2}}{k}\right)\right\} \Big/ \left\{1 + \exp\left(-\frac{n - n_{1/2}}{k}\right) - 1\right\}. \quad (5)$$

The association of real time PCR kinetics with R_{max}, $n_{1/2}$, and k was investigated by simulation using Eq. (1), and the relationship between amplification efficiency and k and $n_{1/2}$ was simulated using Eq. (5).

The initial amount of gene transcripts, as represented by R_0 in the simulation, is defined by setting $n = 0$ in Eqs. (1) and (2), or written as:

$$R_0 = \frac{R_{max}}{1 + \exp(n_{1/2}/k)}. \quad (6)$$

How R_0 is reflected by real time kinetics was investigated using Eq. (6).

Quantitation of GAPDH with known concentration using the new mathematical model. To test the validity of this new method, a 171 bp GAPDH RT-PCR fragment was purified from the agarose electrophoresis gel using UltraClean 15 DNA Purification Kit (MO BIO Laboratories, CA, USA) and used for real time PCR assay. A series of dilutions (2, 4, 6, 8, and 10 times) were made from a 1× sample with GAPDH concentration at 1.6×10^{-3} ng/μl. The kinetic data from real time PCR is fitted using the sigmoid mathematical model and initial amount of GAPDH as represented by R_0 is calculated using Eq. (6).

Results

Amplification quality by SYBR Green two-step PCR protocol

An exclusive two-step PCR protocol has been broadly applied in SYBR Green PCR system [23]. In combination with Primer Express Software for real time PCR primer design and ABI Prism 5700 Sequence Detection System, SYBR Green two-step PCR confers a high

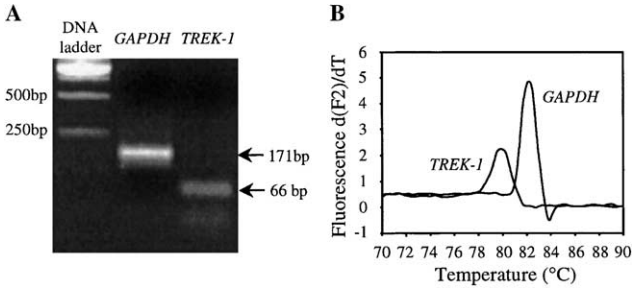

Fig. 1. Amplification specificity by real time PCR. (A) Real time PCR products visualized in 3% Agarose gel, a single band for both *GAPDH* and *TREK-1* is observed with the size as predicted. (B) PCR product dissociation curves (melting temperature analysis) for both *GAPDH* and *TREK-1*.

success rate for specific amplification. To validate the application of mathematical model, the specificity of the real time PCR product was examined by both running the product in agarose gel and dissociation curve analysis; both demonstrated that the PCR protocol employed produced the specific gene product without non-specific amplification or primer–dimer formation. Fig. 1A shows single *GAPDH* and *TREK-1* fragment with the predicted size of 171 and 66 bp, respectively. Dissociation curve plots (melting temperature analysis) showed only one peak for each product of *TREK-1* and *GAPDH*, with melting temperatures (T_m) values of 80 and 83 °C, respectively (Fig. 1B), thus indicating that no primer–dimer formation had occurred during the PCR [23].

The sigmoidal mathematical model fits the whole kinetic process of real time PCR

The generally accepted mathematical model in which a constant PCR amplification efficiency is assumed [20], is a non-convergence function. It cannot reflect the whole kinetic process of real time PCR, since product growth will eventually be saturated due to either limiting of reaction components or declining enzyme activity, or both. We applied a sigmoidal mathematical model to fit real time PCR data, and found this model fitted well the kinetics of the whole real time PCR process. In 50 sets of real time PCR kinetic data from a reference gene, *GAPDH*, goodness of fit coefficients of 0.9995 ± 0.002 ($n = 50$) were achieved (for example see Fig. 2). The technique has also been successfully applied to several other genes (*TWIK-1*, *TWIK-2*, *TASK-1*, *TASK-2*, *TASK-3*, *TREK-1*, *TREK-2*, *TRAAK*, and *KCNK6,* as well as subunits for L-type and T-type Calcium channels). Other kinetic parameters from the data fitting are R_{max}: 1.6–2.5 (1.57 ± 0.14, $n = 50$); k: 2.1–2.9 (2.20 ± 0.10, $n = 50$); and $n_{1/2}$: 21.24–36.37 (33.37 ± 0.67, $n = 50$). The input parameters used in the validation simulations were then chosen from within these ranges to closely approximate the real situation.

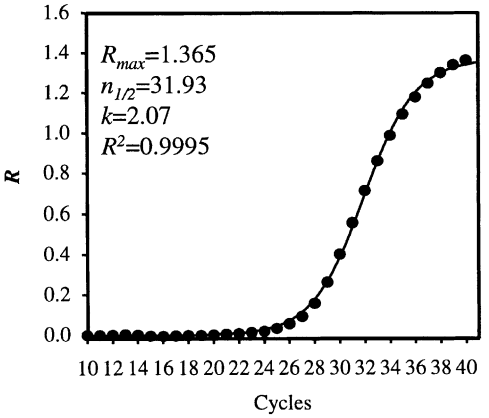

Fig. 2. A representative real time PCR. R_n vs Cycles plot fitted using Eq. (1). Filled circles are experimental data from real time PCR, the line shows the best fit of the equation, which gives the parameters shown.

The mathematical model revealed that the amplification efficiency is changed dynamically

Simulation using Eq. (5) demonstrated that the amplification efficiency can change from cycle to cycle. The amplification efficiencies are relatively stable in the early stage of the reaction, then decay to approach zero at cycle 40 (Figs. 3B and D). Such a dynamic change in amplification efficiency presumably reflects the cycle-dependent depletion of PCR reaction components and/or a decline in polymerase activity due to polymerase binding to its own amplification products (a classical end product inhibition) [24].

Figs. 3A and C show the kinetic process of real time PCR with constant $n_{1/2}$ and k, respectively. The corresponding amplification efficiency kinetics in Figs. 3B and D demonstrated that a smaller k is associated with a higher initial amplification efficiency. Change in $n_{1/2}$ is not associated with significant change of initial amplification efficiency, but a smaller $n_{1/2}$ indicates an earlier decay of the amplification efficiency. Table 1 summarizes the relationship between the amplification efficiencies in the early stages of real time PCR and $n_{1/2}$ and k. Filled circles in Fig. 3E are kinetic data from an experimental real time PCR, open cycles are calculated amplification efficiencies. Because the equation used to calculate efficiency has a small denominator in the early stages of the PCR reaction, small random fluctuations in efficiency are greatly magnified, and hence the apparent large fluctuations in calculated efficiency are not reflective of true changes. After about the 20th cycle, calculated amplification efficiency for the reaction followed a similar exponential decay as seen in simulations.

A higher end-point R does not necessarily mean a higher initial level of gene transcript

In semi-quantitative RT-PCR, the end PCR product after certain number of cycles are compared to deter-

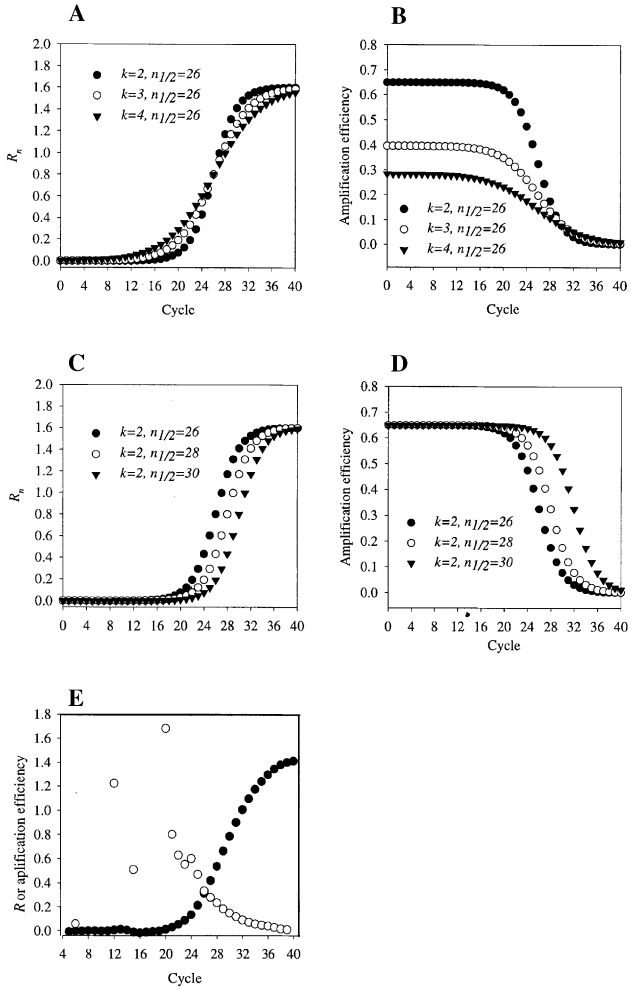

Fig. 3. Amplification efficiencies are changed dynamically. (A and C) Simulated real time PCR R_n vs Cycles plots with R_{max} 1.6 for each reaction but different other kinetic parameters as indicated in the figures. (B and D) The amplification efficiency vs Cycles plots for (A) and (C), respectively. Both show that the amplification efficiencies decay exponentially from mid phase of the reaction. (B) shows that a smaller k is associated with a higher amplification efficiency, (D) reveals that the difference of $n_{1/2}$ indicated is mainly associated with mid and late phase amplification efficiencies of the reactions. (E) shows the kinetic data and calculated amplification efficiencies from an experimental real time PCR.

Table 1
Amplification efficiencies of real time PCR at amplification cycle 8 of simulated real time PCR

$n_{1/2}$	$k = 1$	$k = 2$	$k = 3$	$k = 4$
20	1.72	0.65	0.39	0.27
24	1.72	0.65	0.39	0.27
28	1.72	0.65	0.40	0.28
32	1.72	0.65	0.40	0.28

mine the gene expression level; the higher the product is, the higher expression level is believed [25,26]. This assumption has been questioned, especially when the quantitation is used as clinical diagnostic assay or disease screening [27]. To clarify this, we simulated how the

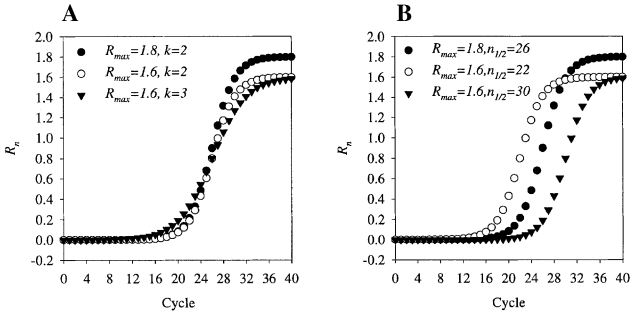

Fig. 4. A higher end-point R does not mean a higher initial gene transcripts. In (A) all reactions have the same $n_{1/2} = 26$, in (B) all reactions have the same $k = 2$, other kinetic parameters are indicated in the figures. See details in the text.

end-point R and other kinetic parameters in real time PCR are associated with the initial level of gene transcripts, as represented by R_0. In Fig. 4A, the ratio of R_0 (○)/R_0 (●) is 0.89, and R_0 (▼)/R_0 (●) is 67.2, indicating that the sample with a smaller R but a higher k can have a higher initial amount of gene transcripts than the sample with a higher R but a smaller k. In Fig. 4B, the ratio of R_0 (○)/R_0 (●) is 6.57, and R_0 (▼)/R_0 (●) is 0.12, indicating that the sample with a smaller R and $n_{1/2}$ can have a higher initial amount of gene transcripts than the sample with a higher R and $n_{1/2}$.

A lower C_T does not necessarily mean a higher initial level of gene transcript

The standard curve method is based on the construction of C_T vs initial input amount of total RNA (or copy number). It would seem reasonable to assume that a lower C_T represents a higher initial amount of gene transcripts [28]. However, this is true only when the two reactions being compared have identical amplification efficiencies. We demonstrate this using the simulation as shown in Fig. 5. In Fig. 5, C_T for three individual reactions was 14.5 (●), 20.5 (○), and 18 (▼); note that, although reaction ▼ has a higher C_T, in fact it had a 3.79-fold greater initial amount of gene transcript than that of reaction ●.

Reactions having equal starting amounts of total RNA but varying amounts of polymerase activity and other components have distinct kinetics

To test the validity of the proposed quantitative method, we applied the method to real time PCR reactions that amplified the same gene (*GAPDH*) having the same initial amount of total RNA but different amounts of SYBR Green PCR Master Mix. Fig. 6 shows that the kinetic processes of the reactions were different. Ten microliter SYBR Green PCR Master Mix resulted in a larger R_{max} than the other reactions; a

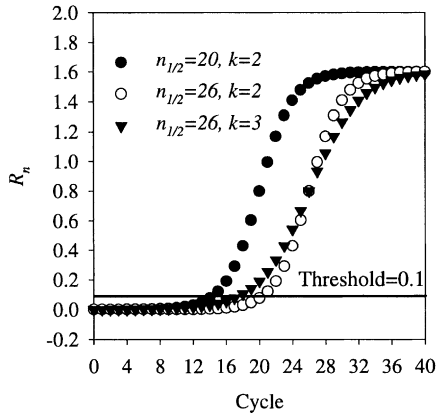

Fig. 5. A lower C_T does not mean a higher R_0. All reactions have the same R_{max} 1.6 but different other kinetic parameters as indicated in the figure. C_T in reactions, •, ○, and ▼ are 14.5, 20.5, and 18, respectively. See details in the text.

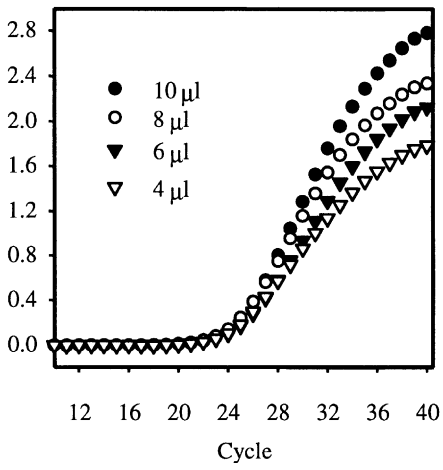

Fig. 6. Real time PCR data with same input amount of total RNA but varying SYBR Green PCR Master Mix (see details in the text).

decrease in SYBR Green PCR Master Mix led to a reduction of amplification efficiency. However, the initial amount of gene transcripts calculated from Eq. (6) is almost same for the samples, indicating that the difference in kinetics does not necessarily reflect a difference in initial amount of gene. Table 2 summarizes the results of the samples.

R_0 represents a quantitative approach for real time PCR assay

The fitting of PCR kinetic data using Eq. (1) in this study took into account the random fluorescence dye background, R_0, and derives the initial gene transcripts based on the whole kinetic process. To further test the validity of this quantitative method, real time PCR was performed on the samples with known initial concentrations of *GAPDH* gene fragment. The derived R_0 among the diluted samples showed a similar fraction relationship as expected from the dilution factor. Table 3 summarizes the results of the experiment.

Discussion

An appropriate mathematical model is important for accurate quantitation of gene transcripts in real time PCR. The currently applied model, or $R_n = R_0*(1+E)^n$, is a non-convergence function by which the growth of real time PCR product is viewed as an unlimited process, which, although patently unrealistic, is perhaps a reasonable approximation for the early stages of the reaction. The model also assumes an amplification efficiency which is constant [15,20], an assumption which is obviously not true for the whole course of the reaction, but, again, may be a reasonable approximation for the early stages. Indeed, our simulation demonstrates that amplification efficiencies during the early exponential phase for an individual reaction are relatively constant. However, although this may be true in the initial stages of the reaction, it cannot be true throughout the whole process. Amplification of template by PCR is a process involving multiple components, including amount of templates, primers, ions, nucleotides, enzyme activity, and reaction temperature. Except for the reaction temperature, which is well controlled, all of these components are likely to be dynamically changed as the reaction progresses and to subsequently affect amplification efficiency. Since the reaction eventually saturates, amplification efficiency must eventually fall to zero.

As well as the assumption of constant amplification efficiency, current quantitation methods also rely on the assumption of equal amplification efficiencies among

Table 2
Summary of kinetic data from samples with different polymerase for real time PCR

Polymerase[a] (µl)	R^2	R_{max}	k	$n_{1/2}$	E^b	C_T	R_0
10	0.9986	2.783	2.605	30.60	0.47	15.95	2.3×10^{-5}
8	0.9988	2.340	2.635	30.23	0.46	15.90	2.4×10^{-5}
6	0.9989	2.155	2.684	30.93	0.45	16.55	2.1×10^{-5}
4	0.9982	1.791	2.749	30.443	0.44	16.20	2.7×10^{-5}

[a] SYBR Green PCR Master Mix was used.
[b] Amplification efficiency at cycle 8 was calculated using Eq. (2).

Table 3
Real time PCR assay on samples with known concentration of *GAPDH* ($n = 5$)

Dilution times	R_0 (mean ± SE)	(R_0 of dilution 1)/R_0
1	$0.001542 \pm 7.3 \times 10^{-5}$	1
2	$0.000817 \pm 8.3 \times 10^{-5}$	1.9
4	$0.000409 \pm 3.4 \times 10^{-5}$	3.8
6	$0.000261 \pm 1.5 \times 10^{-5}$	5.9
8	$0.000197 \pm 1.3 \times 10^{-5}$	7.8
10	$0.000156 \pm 8.2 \times 10^{-6}$	9.9

standard samples and between standard and measured samples. In the comparative C_T method, another assumption is that amplification efficiency is 1 in the early exponential phase [20]. However, in practice it has been observed that trivial variations in reaction components, thermal cycling conditions, and mispriming events during the early stage of PCR can greatly affect the end yield of the amplified product [29]. It therefore seems highly unlikely that amplification efficiency would be exactly 1 and equal between different reaction mixes. Indeed, we have shown that early stage amplification efficiencies obtained from experimental real time PCR data are often considerably less than 1 and often not equal between individual samples [21]. Thus, in practical real time PCR, if the above assumptions are not actually satisfied, one could not expect reliability of quantitation.

The simulation study demonstrated that a higher endpoint R or a lower C_T does not necessarily reflect a higher initial level of gene transcript. Even for the same gene from the same tissue sample, the kinetics can be different due to variation of sample loading, primer concentration, enzyme, and nucleotide amount. Therefore, to maximize accuracy, quantitation of gene transcripts from each individual real time PCR should be determined from a combined consideration of R_{max}, $n_{1/2}$ and k as indicated from Eq. (6). Since R_{max}, $n_{1/2}$, and k can be obtained by fitting the real time PCR data using Eq. (1), the initial amount of gene level can easily be derived from R_0. If there is neither non-specific amplification nor primer-dimer formation in the real time PCR, the derived R_0 will reflect the initial amount of gene transcripts. Either normalization or relative comparison between genes can be derived from the calculated initial amount of gene level as represented by R_0.

Acknowledgment

This work was supported by grants from the Australian NHMRC.

References

[1] D.A. Rappolee, A. Wang, D. Mark, Z. Werb, Novel method for studying mRNA phenotypes in single or small numbers of cells, J. Cell Biochem. 39 (1989) 1–11.

[2] T. Wang, M.J. Brown, mRNA quantification by real time TaqMan polymerase chain reaction: validation and comparison with RNase protection, Anal. Biochem. 269 (1999) 198–201.

[3] A. Al-Taher, A. Bashein, T. Nolan, M. Hollingsworth, G. Brady, Global cDNA amplification combined with real-time RT-PCR: accurate quantification of multiple human potassium channel genes at the single cell level, Yeast 17 (2000) 201–210.

[4] G. Brady, M. Barbara, N.N. Iscove, Representative in vitro cDNA amplification from individual hemopoietic cells and colonies, Methods Mol. Cell. Biol. 2 (1990) 17–25.

[5] G. Brady, N.N. Iscove, Construction of cDNA libraries from single cells, Methods Enzymol. 225 (1993) 611–623.

[6] N. Steuerwald, J. Cohen, R.J. Herrera, C.A. Brenner, Analysis of gene expression in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT-PCR, Mol. Hum. Reprod. 5 (1999) 1034–1039.

[7] T.B. Morrison, J.J. Weis, C.T. Wittwer, Quantification of lowcopy transcripts by continuous SYBR Green I monitoring during amplification, Biotechniques 24 (1998) 954–958, see also pp. 960, 962.

[8] L.G. Lee, C.R. Connell, W. Bloch, Allelic discrimination by nick-translation PCR with fluorogenic probes, Nucleic Acids Res. 21 (1993) 3761–3766.

[9] K.J. Livak, S.J. Flood, J. Marmaro, W. Giusti, K. Deetz, Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization, PCR Methods Appl. 4 (1995) 357–362.

[10] C. Orlando, P. Pinzani, M. Pazzagli, Developments in quantitative PCR, Clin. Chem. Lab. Med. 36 (1998) 255–269.

[11] P.M. Holland, R.D. Abramson, R. Watson, D.H. Gelfand, Detection of specific polymerase chain reaction product by utilizing the 5′–3′ exonuclease activity of *Thermus aquaticus*, Proc. Natl. Acad. Sci. USA 88 (1991) 7276–7280.

[12] S.A. Bustin, Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays, J. Mol. Endocrinol. 25 (2000) 169–193.

[13] C. Aberham, C. Pendl, P. Gross, G. Zerlauth, M. Gessner, A quantitative, internally controlled real-time PCR assay for the detection of parvovirus B19 DNA, J. Virol. Methods 92 (2001) 183–191.

[14] M.L. Collins, C. Zayati, J.J. Detmer, B. Daly, J.A. Kolberg, T.A. Cha, B.D. Irvine, J. Tucker, M.S. Urdea, Preparation and characterization of RNA standards for use in quantitative branched DNA hybridization assays, Anal. Biochem. 226 (1995) 120–129.

[15] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (2001) 2002–2007.

[16] M.S. Rajeevan, S.D. Vernon, N. Taysavang, E.R. Unger, Validation of array-based gene expression profiles by real-time (kinetic) RT-PCR, J. Mol. Diagn. 3 (2001) 26–31.

[17] M.W. Pfaffl, M. Hageleit, Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR, Biotechnol. Lett. 23 (2001) 275–282.

[18] T.D. Schmittgen, B.A. Zakrajsek, A.G. Mills, V. Gorn, M.J. Singer, M.W. Reed, Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods, Anal. Biochem. 285 (2000) 194–204.

[19] D.A. Simpson, S. Feeney, C. Boyle, A.W. Stitt, Retinal VEGF mRNA measured by SYBR green I fluorescence: a versatile approach to quantitative PCR, Mol. Vis. 6 (2000) 178–183.

[20] K.J. Livak, ABI Prism 7700 Sequence Detection System. User Bulletin 2. PE Applied Biosystems, 1997.

[21] W. Liu, D.A. Saint, A new quantitative method of real time RT-PCR assay based on simulation of PCR kinetics, Anal. Biochem. Mar. 1 302 (1) (2002) 52–59.
[22] J. Meijerink, C. Mandigers, L. van De Locht, E. Tonnissen, F. Goodsaid, J. Raemaekers, A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR, J. Mol. Diagn. 3 (2001) 55–61.
[23] PE Biosystems, SYBR Green PCR Master Mix and RT-PCR Protocol, 1999.
[24] P. Kainz, The PCR plateau phase—towards an understanding of its limitations, Biochim. Biophys. Acta 1494 (2000) 23–27.
[25] F. Ferre, Quantitative or semi-quantitative PCR: reality versus myth, PCR Methods Appl. 2 (1992) 1–9.
[26] R.A. Montgomery, M.J. Dallman, Semi-quantitative polymerase chain reaction analysis of cytokine and cytokine receptor gene expression during thymic ontogeny, Cytokine 9 (1997) 717–726.
[27] M.H. Sokoloff, C.L. Tso, R. Kaboo, S. Nelson, J. Ko, F. Dorey, R.A. Figlin, S. Pang, J. deKernion, A. Belldegrun, Quantitative polymerase chain reaction does not improve preoperative prostate cancer staging: a clinicopathological molecular analysis of 121 patients, J. Urol. 156 (1996) 1560–1566.
[28] U.E. Gibson, C.A. Heid, P.M. Williams, A novel method for real time quantitative RT-PCR, Genome Res. 6 (1996) 995–1001.
[29] D.Y. Wu, L. Ugozzoli, B.K. Pal, J. Qian, R.B. Wallace, The effect of temperature and oligonucleotide primer length on the specificity and efficiency of amplification by the polymerase chain reaction, DNA Cell Biol. 10 (1991) 233–238.