Gene expression of *HIF-1α* and *XRCC4* measured in human samples by real-time RT-PCR using the sigmoidal curve-fitting method

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*Quantitative reverse transcription PCR (RT-PCR) has become an important tool for studying functional gene expression. However, the most often used cycle threshold (C_T)-based method, primarily related to the required amplification efficiency determination via serial dilution, can call into question the level of quantitative reliability and accuracy that can be achieved, in addition to the impracticalities inherent to C_T-based methodologies. In this study, an alternative method, named the sigmoidal curve-fitting (SCF) method, was compared with the classic C_T method for two target genes (*XRCC4 *and* HIF-1α*) and a reference gene (*HPRT*). The PCR conditions were optimized for each gene on a LightCycler® apparatus. Fluorescence data were fitted to a four-parametric sigmoidal function, and the initial messenger RNA (mRNA) copy number was determined by a theoretical fluorescence (F_0) value calculated from each fitting curve. The relative expression of the target gene versus that of the reference gene was calculated using an equation based upon these F_0 values. The results show that the F_0 value had a good linearity with the initial number of target genes between 10^7 and 10^1 copies. The reproducibility tests showed that the variations of initial target quantity were well reflected by F_0 values. Relative expression of target gene calculated by the SCF method and by the C_T method showed similar results. In our hands, the SCF method gave reliable results and a more precise error description of quantitative RT-PCR.*

INTRODUCTION

Quantitative reverse transcription PCR (RT-PCR), which detects fluorescence intensity during the amplification process, has greatly evolved and overcome the limitations of classical RT-PCR assays, giving an extraordinarily easy and accurate method to quantify nucleic acids (1,2). Thus quantitative RT-PCR, with a very large dynamic range (seven to eight orders of magnitude) and a high sensitivity (as few as 10 molecules), has been widely utilized in many aspects of biologic research including oncology (3–6). Nevertheless, the best method to quantify and calculate the absolute or relative expression of target genes by real-time PCR is still under debate. The standard curve method and comparative cycle threshold (C_T) method and their variants have been developed and used for relative expression quantification in different laboratories (7). 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 (8). The comparative C_T method detects and calculates relative gene expression using the formula $2^{-\Delta\Delta C_T}$ (9). This formula is based on the assumptions that amplification efficiencies of the reference and target genes are approximately equal and that the amplification efficiency is close to 2. Nevertheless, these two groups of methods use a common parameter: the value of C_T. C_T value is the fractional number of cycles required to reach a particular threshold fluorescence signal level. It can be decided manually or automatically using different methods and algorithms (10,11). In some apparatuses, such as the LightCycler®, the term crossing point or Cp is used for the same concept. Each amplification has its characteristic C_T value, which is determined by the initial concentration of target gene. Although widely used, these methods are always criticized for their inherent complexity and imprecision. The problem is due to the absence of information concerning exact amplification efficiency. As mentioned by many researchers, the variation of amplification efficiency is a non-negligible source in bias of PCR results (9,12–15). Another problem for C_T-based methods is that the exact variation of initial numbers of target gene, which is believed to vary between 6%–20% (8,16), cannot be directly quantitated by these methods.

Recently, a new quantification method has been proposed by Liu and Saint (17) and then revised by Rutledge (18). This method, called sigmoidal curve-fitting (SCF), appears to be

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an attractive one because it gives a simulation of the whole PCR process. The new method obviates the need for construction of relative standard curves and the need for a series of validation experiments that are the prerequisite for the comparative C_T method, providing a more accurate, faster, and more efficient quantification method.

To test this SCF method, we developed a quantitative RT-PCR assay to measure the expression of two genes (*XRCC4* and *HIF1*α) in human tumor samples both with the classic method (standard curve method) and with SCF. These genes were chosen because, in our laboratory, we believed they might be useful for predicting radiosensitivity. The gene product of *XRCC4* participates in the repair system of double-strand DNA breaks (19,20), while *HIF1*α is a subunit of the transcription factor, hypoxia-induced factor 1 (HIF-1), which is the most reactive gene in response to hypoxia, another source of radioresistance during radiotherapy (21–26). In this study, we compared the results obtained with both methods, we proposed an equation for relative quantification based upon the SCF method, and we tested its sensitivity and reproducibility by measuring *XRCC4*, *HIF1*α, and the housekeeping gene, *HPRT*, messenger RNA (mRNA) levels in different human cell lines and tissue samples.

Table 1. Oligonucleotide PCR Primers for Human *XRCC4*, *HIF-1*α, and *HPRT*

cDNA Species (GenBank® accession no.)	Primers	Exon Position	PCR Product (bp)
Human XRCC4 (NM_022550)	(f) 5′-AAGATGTCTCATTCAGACTTG-3′ (r) 5′-CCGCTTATAAAGATCAGTCTC-3′	Exon 3–4 Exon 5	233
Human HIF-1α (NM_001530)	(f) 5′-TTCACCTGAGCCTAATAGTCC-3′ (r) 5′-CAAGTCTAAATCTGTGTCCTG-3′	Exon 10–11 Exon 11–12	151
Human HPRT (NM_000194)	(f) 5′-GAAGAGCTATTGTAATGACC-3′ (r) 5′-GCGACCTTGACCATCTTTG-3′	Exon 3–4 Exon 6	177

f, forward; r, reverse.

MATERIALS AND METHODS

Biological and Patient Samples

The biological samples used in this study included cell lines and patient tumor samples. Two cancer cell lines were used to check the reproducibility of the experiment: HT29 cL.19A (human colorectal adenocarcinoma, kindly supplied by M. Laboise, INSERM U239, Paris, France) and HCT116 (human colorectal adenocarcinoma, supplied by J. Bourhis, Villejuif, France). Cells were grown in their recommended medium and controlled for mycoplasma contamination by DNA fluorochrome staining every month during the experiment. For clinical samples, 21 primary non-small cell lung cancer samples were collected (from June 2001 to November 2004) immediately after surgery. Tumor samples were examined by a pathologist and stored at -190°C according to culture collection guidelines. The total RNA of each sample was extracted, and the expression of the target genes and reference genes were measured.

Total RNA Extraction

Total RNA was extracted using the RNeasy® kit (Qiagen, Courtaboeuf, France) from either 10^7 cells or 30 mg tumor sample. Before RNA extraction, tumor fragments were crushed with stainless steel beads and homogenized at 30 Hz for 4 min with cold agitation in a MM300 Mixer Mill (Qiagen). Cells or homogenized tissue were lyzed, and RNA purification was performed according to the manufacturer's protocol. A DNase I digestion step was added to each extraction to further decrease genomic DNA contamination. RNA integrity was examined by electrophoresis on a 3% agarose gel, and total RNA concentration was measured by spectrophotometry at 260 nm.

Reverse Transcription

RNA (0.5 or 1 μg) was added during the reverse transcription process in order to examine the reproducibility of the experiment. After this step, only 0.5 μg RNA was used to further establish the standard amplification curves and to estimate the range of expression levels in tumor samples. Briefly, RNA was incubated with 20 U avian myeloblastosis virus (AMV) reverse transcriptase (Roche, Meylan, France),

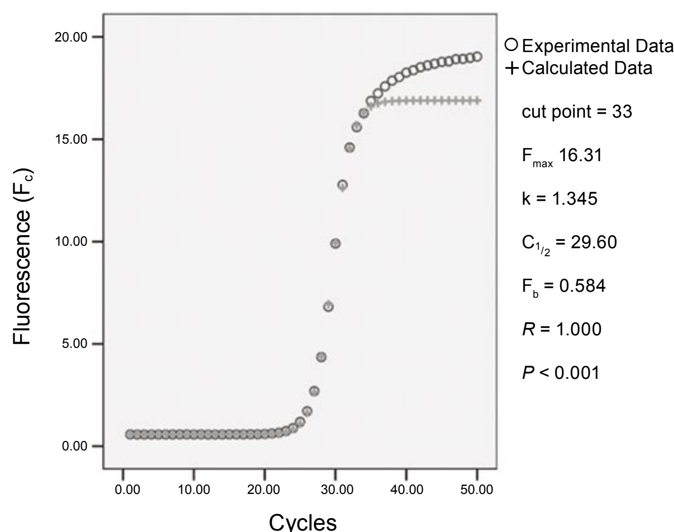

Figure 1. An example of simulation of PCR amplification by the sigmoidal curve-fitting (SCF) method (Equation 1). Experimentally derived fluorescence data set (circles) were plotted against a curve-fitting data set generated using Equation 1 (crosses). The cycles beyond the cut-off were excluded from the curve-fitting process (see Materials and Methods for additional details).

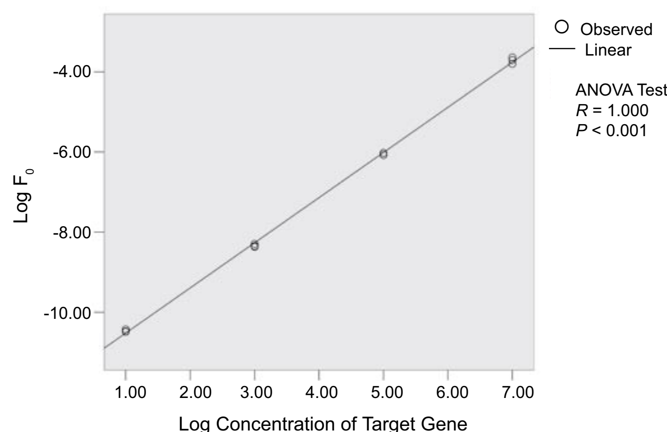

Figure 2. The target DNA concentration presented by F_0 values. The linear regression analysis generated by plotting the log of target DNA concentration versus log of F_0. The nearly perfect linear relation ($R = 1.000$, $P < 0.001$) indicated that F_0 directly reflected target DNA concentration. F_0, theoretical fluorescence; ANOVA, analysis of variance.

1 mM dNTP, 1.6 µg oligo$(dT)_{15}$ primer, and 40 U RNase inhibitor in a final volume of 20 µL. Reverse transcription was performed as follows: 25°C for 10 min, 55°C for 1 h (cDNA synthesis and destruction of the RNA portion of the RNA:cDNA hybrids by RNase H activity of recombinant AMV reverse transcriptase), and 5 min at 94°C (enzyme inactivation). Two types of controls were included: (*i*) the reverse transcription control for each extracted RNA contained all reagents except the AMV reverse transcriptase and (*ii*) the RNA control was reverse-transcribed without any RNA matrix.

Product DNA of each target gene and reference gene were amplified and purified to establish the standard curve and the calibrator. The DNA precipitation step was started by adding 1/10 volume 2 M sodium acetate to the cDNA reactions. Product DNA was precipitated with 2.5 volumes 100% ethanol (Sigma, St. Louis, MO, USA) at -80°C for 1 h. After centrifugation at 13,000× *g* for 45 min at 4°C, the supernatant was discarded, 500 µL 75% ethanol were added and after a second centrifugation at 13,000× *g* at 4°C for 30 min, the supernatant was removed. The product DNA pellet was dried and dissolved in 50 µL Tris-HCl.

Quantitative PCR of *XRCC4*, *HIF-1*α, and *HPRT* Genes

The primers used for the amplification reaction of each gene are listed in Table 1. They were designed and verified by dedicated software (Amplify 1.2), and primer sequences were analyzed by BLASTn for their specificity. To exclude contamination by residual genomic DNA, the primers were always chosen spanning two different exons. Real-time PCR amplification was performed on a LightCycler (Version 3.5; Roche). Each reverse transcription product and the purified cDNA were used for PCRs. The reverse transcription products from tumor samples were diluted to a concentration such that the C_T value of the diluted solution were well within the range of detection capacity of the real-time PCR apparatus that was determined by a standard curve. For the reaction, 2 µL pure or diluted reverse-transcribed products or the purified cDNA products were incubated with 4 mM $MgCl_2$, 2 µL ready-to-use SYBR® Green I Master mix (LightCycler FastStart DNA Master SYBR Green I; Roche), and 0.5 µM forward and reverse primers in a final volume of 20 µL. The conditions for PCR and melting curves performed after amplification were tested and optimized. The amplification product was then submitted to specific restriction endonuclease digestion (*Pvu*II for *XRCC4*, *Eco*RI for *HIF-1*α, and *Mbo*I for *HPRT*) and polyacrylamide gel electrophoresis (PAGE).

Gene Expression Quantification

The real-time PCR machine (LightCycler) measured the fluorescence of each sample in every cycle at the end of the elongation step. After amplification, all fluorescence data sets were then exported by the LightCycler software in the form of text files for SCF analysis. SigmaPlot® (Version 9; Systat Software, Richmond, CA, USA) was used to fit fluorescence readings with a nonlinear regression function. Real-time PCR can be precisely simulated using the four-parametric sigmoidal function:

$$F_C = F_b + \frac{F_{max}}{1 + e^{(C_{1/2} - C)/k}}$$

[Eq. 1] (18)

in which C is the cycle number, F_c is the reaction fluorescence at cycle C, F_{max} is the maximal fluorescence during the reaction, $C_{1/2}$ is the cycle at which fluorescence reaches half of F_{max}, k is related to the slope of the sigmoid curve (at $C = C_{1/2}$, the slope = $F_{max}/4k$), and F_b is the background reaction fluorescence. Because fluorescence intensity increase is proportional to the product concentration increase, the F_c calculated from Equation 1 directly reflects the number of products during amplification. Thus, in Equation 1, if C = 0, then the theoretical fluorescence (F_0) is given by the following formula:

$$F_0 = \frac{F_{max}}{1 + e^{(C_{1/2}/k)}}$$

[Eq. 2](18)

in which the deduced F_0 value is the direct reflection of the initial target quantity expressed in fluorescence units. Because the terminal phase of PCR has more complex kinetics that cannot be well simulated by the SCF method, a certain number of cycles with this portion were excluded. Selection of the cut-off cycle for all amplification curves was based upon the subset that produced the minimum-calculated F_0 value (18). A macro was programmed for data treatment (source code available upon request). After obtaining the F_0 value for each sample, relative expression of the gene was calculated by reference to the housekeeping gene

using Equation 4. In order to compare the results between SCF method and classic threshold method, the C_T value of each sample generated by the machine was recorded. It was determined by the second-derivation maximum method, which is calculated automatically by the LightCycler software (10).

Statistics

All statistics were performed using SPSS® 11 (SPSS Inc., Chicago, IL, USA). In the SCF method, variability of gene expression was expressed as the coefficient of variation (CV) of F_0 values [CV = (SD/Average) × 100%; SD, standard deviation] obtained in the same run or between runs performed on different days (interassay variability). In the standard curve method, the variation of gene expression cannot be evaluated by the CV of C_T values due to its exponential characteristics. An alternative method is used to estimate the theoretic variation of the initial number of target gene (8). This method uses the equation

$$\pm\%\text{Molecule}=[(E+1)^{SD}-1]\times 100\%$$

to estimate the variation of initial numbers of the target gene in percent molecules, in which SD is the standard deviation of the C_T value and E is the efficiency of each reaction. In our study, the efficiency of each reaction was approximately decided by introducing two standard calibrators with different concentrations of target gene in each run.

RESULTS

Specificity of PCR Amplification

To avoid nonspecific amplification, especially from residual genomic DNA, forward and reverse primers were always designed on two different exons of the targeted genes. Different melting temperatures (T_m) and $MgCl_2$ concentrations were tested in order to determine optimal amplification. The specificity of amplification was verified by melting curve analysis using the LightCycler software and gel electrophoresis.

Sigmoidal Curve-Fitting

As indicated in the methods section, real-time PCR can be precisely simulated using the four-parametric sigmoid function (Equation 1). After carefully configuring the cut-off cycle of each regression process, the resulting curve was highly correlated with the experimental data ($R > 0.9999$). Figure 1 shows an example of the simulation process. The F_0 value calculated from Equation 2, which is the direct counterpart of the initial target concentration, was then collected for further analysis.

Reproducibility of Gene Expression Measured by Both Methods

XRCC4 and *HPRT* cDNA amplification were used to test the reproducibility of the RT-PCR method. The intra- and interassay variability with targets from the same RNA preparations were examined. Four different RNA preparations were tested by RT-PCR. Reverse transcription was performed with 0.5 and 1 µg RNA, extracted from HT29 and HCT114 cells, and the resulting cDNAs were used in PCRs for *XRCC4* and *HPRT* genes. The value of C_T and F_0 of each reaction was recorded and compared. Intra-assay comparison consisted of triplicate PCRs using cDNA obtained from the four different RNA samples. The results are shown in Tables 2 and 3. The CV of F_0 was between 2.28% and 17.32% throughout the intra-assay comparison. In interassay experiments, the CV of F_0 calculated from PCRs performed on two different days did not exceed 26.6%. Interestingly, the theoretic variation of the initial numbers of target gene, calculated from the standard deviation of C_T using the method proposed by Rutledge (8), was comparable to the F_0 variation (Table 3).

F_0 Represents a Quantitative Approach for Real-Time PCR Assay

In order to further investigate the quantification sensitivity of the SCF

Table 2. Reproducibility of *XRCC4* and *HPRT* cDNA Amplification Calculated by SCF Method

	XRCC4 Expression Measured by SCF Method						*HPRT* Expression Measured by SCF Method					
Cell	Intra-Assay Variability			Interassay Variability			Intra-Assay Variability			Interassay Variability		
Samples	Mean F_0	SD	CV	Mean F_0	SD	CV	Mean F_0	SD	CV	Mean F_0	SD	CV
HT29 (0.5 µg)												
1:10	4.239E-09	3.632E-10	**8.57%**	4.807E-09	7.196E-10	**14.97%**	1.172E-08	5.718E-10	**4.88%**	1.397E-08	3.212E-09	**22.99%**
1:100	2.992E-10	2.091E-11	**6.99%**	2.909E-10	5.99E-11	**20.60%**	8.732E-10	4.485E-11	**5.14%**	1.056E-09	2.133E-10	**20.20%**
HT29 (1 µg)												
1:10	9.792E-09	2.237E-10	**2.28%**	8.703E-09	1.500E-09	**17.24%**	2.437E-08	1.288E-09	**5.28%**	3.134E-08	7.912E-09	**25.24%**
1:100	7.152E-10	9.578E-11	**13.39%**	7.151E-10	1.106E-10	**15.46%**	1.834E-09	3.176E-10	**17.32%**	2.353E-09	6.268E-10	**26.64%**
HCT116 (0.5 µg)												
1:10	1.771E-08	1.098E-09	**6.20%**	1.794E-08	1.661E-09	**9.26%**	2.972E-08	3.626E-09	**12.20%**	3.366E-08	4.977E-09	**14.79%**
1:100	1.428E-09	2.269E-10	**15.89%**	1.375E-09	1.554E-10	**11.30%**	2.104E-09	2.873E-10	**13.65%**	2.294E-09	3.011E-10	**13.12%**
HCT116 (1 µg)												
1:10	2.854E-08	1.233E-09	**4.32%**	2.873E-08	2.667E-09	**9.28%**	4.651E-08	1.530E-09	**3.29%**	5.487E-08	9.671E-09	**17.62%**
1:100	2.999E-09	4.374E-10	**14.58%**	2.747E-09	4.002E-10	**14.57%**	4.823E-09	5.691E-10	**11.80%**	4.301E-09	6.777E-10	**15.76%**

Samples were reverse-transcribed cDNA from 0.5 and 1 µg total RNA of HT29 and HCT116 cells. Diluted at 1:10 and 1:100 and amplified with *XRCC4* and *HPRT* primers. Intra-assay variability is for results from triplicate PCRs of cDNA obtained from RNA samples. Interassay variability was estimated from results obtained from PCRs performed on two different days. SCF, sigmoidal curve-fitting; F_0, theoretical fluorescence; SD, standard deviation; CV, coefficient of variation.

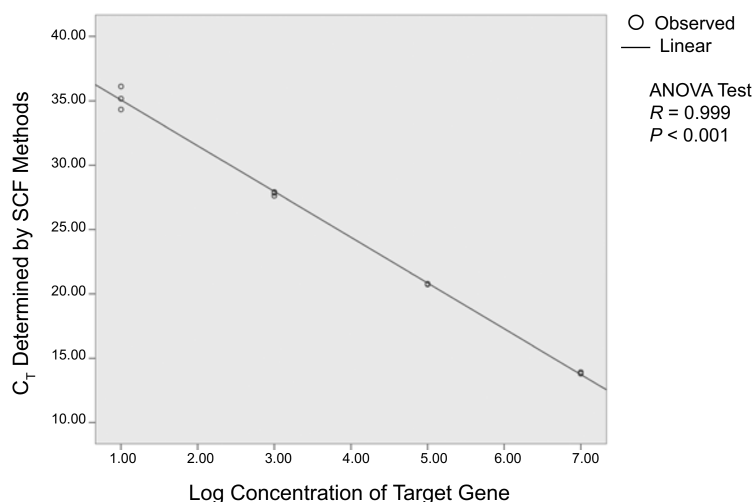

Figure 3. Standard curve of cycle threshold (C_T) values generated by the sigmoidal curve-fitting (SCF) method. A real-time PCR was performed using a series of dilutions of *HIF-1*α cDNA. The C_T values were generated by the equation $C_T = C_{1/2} - \ln(2+\sqrt{3}) \times k$ in which $C_{1/2}$ and k are parameters of Equation 1. These C_T values were plotted against the log target concentration. ANOVA, analysis of variance.

method, we detected the F_0 value in a PCR test using a serially diluted standard cDNA. In brief, the cDNA of the target gene was amplified, purified, quantified by spectrophotometry, and then diluted in four series covering six orders of magnitudes (10^7–10^1). Real-time PCR was done in triplicate. The F_0 values were plotted against the concentration of each dilution (Figure 2). The relationship between F_0 and the initial sample concentration was precisely linear with $R = 1$ and $P < 0.0001$ using the analysis of variance (ANOVA) test. This gave a strong indication that F_0 can serve as an independent parameter of the initial numbers of target gene.

C_T Value Calculated by the SCF Method

One of the automated methods to generate C_T value is to identify the fractioned cycle number at which the second deviation of fluorescence value reaches its maximum. This point can be found using Equation 1.

The first derivative of Equation 1 is:

$$\frac{\partial F}{\partial C} = \frac{F_{max} e^{(C_{1/2}-C)/k}}{\left(1+e^{(C_{1/2}-C)/k}\right)^2 k}$$

The second derivative of Equation 1 is:

$$\frac{\partial^2 F}{\partial C^2} = \frac{2 * F_{max} * (e^{(C-C_{1/2})/k})^2}{(1+e^{(C-C_{1/2})/k})^3 * k^2} - \frac{F_{max} * e^{(C-C_{1/2})/k}}{(1+e^{(C-C_{1/2})/k})^2 * k^2}$$

The extreme value of the second deviation of Equation 1 can be deduced by setting the third derivative to 0. Interestingly, the C_T value calculated using this deduction is quite simple, leading to Equation 3, in which the C_T value is decided by the $C_{1/2}$ value and the k value.

$$C_T = C_{1/2} - \ln(2+\sqrt{3}) \times k$$

[Eq. 3]

An example of the standard curve of C_T values calculated using this method is shown in Figure 3, in which a very good linear relationship between the C_T value and Log N_0 is presented, and inter-run variation is no more than 2.5%.

Relative Quantification of mRNA Expression in Human Tissues Using F_0

After verifying the method, 21 samples of non-small cell lung cancer were analyzed. For all samples, adequately diluted cDNA was amplified. Values were obtained from duplicated amplifications performed on two different days. A new mathematical model is presented to determine the relative quantification of a target gene in comparison to a reference gene (Equation 4). The relative arbitrary unit (RAU) of a target gene is calculated based on the F_0 value of an unknown sample versus a calibrator and expressed in comparison to a reference gene. Target gene mRNA levels were expressed as normalized ratios (*XRCC4/HPRT* and *HIF1*α*/HPRT*) in RAU using the following equation:

$$\text{R.A.U.} = \frac{F_{0\ \text{TargetGeneSample}} / F_{0\ \text{TargetGeneCalibrator}}}{F_{0\ \text{ReferenceGeneSample}} / F_{0\ \text{ReferenceGeneCalibrator}}}$$

[Eq. 4]

The results calculated with the SCF method (Equation 4) were compared with those obtained using the standard curve method (Equation 5, see the Discussion section). These two results were very highly correlated ($R = 0.953$) (Figure 4). Afterwards, all expression levels were calculated based upon the SCF method. Finally in all tumor samples, *XRCC4/HPRT* mRNA expression ranged from 0.10 to 7.42 RAU, with a median value of 0.38, and *HIF1*α*/HPRT* mRNA expression ranged from 0.40 to 29.02 RAU, with a mean value of 2.84.

DISCUSSION

In the present study we developed a quantitative RT-PCR method for two genes (*XRCC4* and *HIF1*α) relative to the *HPRT* housekeeping gene. We quantified the mRNAs using two different methods: a classical C_T method and a more recent SCF method. Both gave reliable and reproducible results. However, the SCF method seems to be easier to use and is based on fewer assumptions. For historical reasons, all of the quantification methods used for real-time PCR are based on the C_T.

Although the C_T value-based methods are the most popular method for either absolute or relative quantification, many uncertainties associated with these methods could make data interpretation difficult. In order to use these methods, we have to assume that the reaction efficiency is equal or nearly the same for all samples and calibrators throughout the intra- and interassay runs. Such an assumption has been reported to be patently invalid

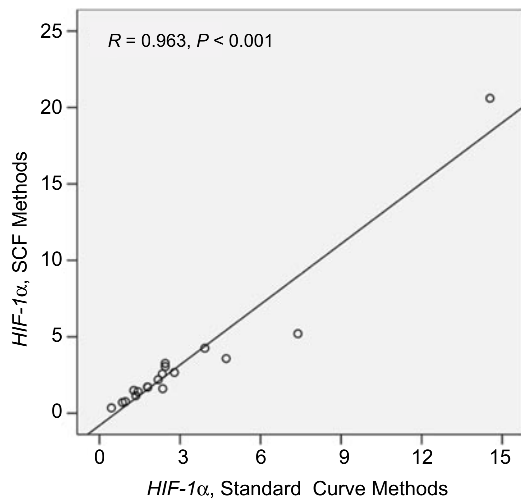

Figure 4. Relative expression calculated using the sigmoidal curve-fitting (SCF) method. The linear regression analysis between the relative arbitrary unit (RAU) values generated by the SCF method (Equation 4) and RAU values generated by the standard curve method (LightCycler software) showed a close relationship between both methods ($R = 0.953$ and $P < 0.001$).

for many cases in which amplification efficiency of samples have been determined (13). Many factors can influence amplification efficiency, including the presence of hemoglobin, fat, glycogen, cell constituents, Ca^{2+}, DNA or RNA concentration, and DNA binding proteins (14,15). Additionally, unknown tissue-specific factors and sequence-specific factors can influence amplification kinetics, which in turn can flaw results (27). Small differences in amplification efficiency cannot only produce large quantitative errors, but the frequency and the magnitude of these errors are virtually impossible to ascertain using a threshold approach. As the data of the reproducibility test shows in our study, the variation of initial input template, which is believed to vary between 6% and 21% (8), can be reflected directly by the variation of F_0 values. In contrast, the variation of C_T values, although giving a much smaller variation, cannot reflect the real variation of target quantity.

However, in order to circumvent such problems, many suggestions have been put forth. Pfaffl et al. (28) introduced the use of amplification efficiency of target and internal control genes measured and calculated from standard curves, leading to the following formula:

$$\text{Relative Expression} = \frac{(E_{\text{target}}+1)^{C_T\,\text{target (Calibrator-Sample)}}}{(E_{\text{ref}}+1)^{C_T\,\text{ref (Calibrator -Sample)}}} \quad \text{[Eq. 5]}$$

In this equation, E is the amplification efficiency, and ref refers to the reference or housekeeping gene. Equation 5 is used when the efficiency of target gene amplification and reference gene amplification are not equal to 1.

These calculations, however, are always based upon an external standard curve that does not have the same conditions as the test samples. Some have tried to calculate the amplification efficiency of each individual reaction and then use it for quantification and normalization (29–32). The concept of determining efficiency for each reaction is logical, but the methods to determine efficiency are somewhat operator-dependent, and the efficiency values derived from these methods are quite different from that obtained with a standard curve method, so it is difficult to compare these results. The study described here verified the work of Liu and Saint (17) and Rutledge (18), demonstrating that SCF fitted experimental data well. Because the SCF method does not need to use the external generated efficiency parameter, the uncertainty of calculation is decreased. In the reproducibility assay, the SCF method is able to directly detect variation in initial target gene quantity, which cannot be done using the standard curve method. When using series of diluted cDNA fragments of target gene over six orders of magnitude, SCF results show very linear correlations between F_0 values and initial concentrations. While the traditional standard curve

Table 3. Reproducibility of *XRCC4* and *HPRT* cDNA Amplification by C_T Method

Cell Samples	XRCC4 Expression Measured by C_T Method								HPRT Expression Measured by C_T Method							
	Intra-Assay Variability				Interassay Variability				Intra-Assay Variability				Interassay Variability			
	Mean C_T	SD	±%Mol	E	Mean C_T	SD	±%Mol	E	Mean C_T	SD	±%Mol	E	Mean C_T	SD	±%Mol	E
HT29 (0.5 µg)																
1:10	28.38	0.11	7.13%		27.79	0.65	42.26%		25.28	0.25	17.56%		25.47	0.27	19.59%	
1:100	31.77	0.10	6.46%	0.87	32.25	0.35	20.90%	0.72	28.83	0.06	3.96%	0.91	28.96	0.16	11.19%	0.94
HT29 (1 µg)																
1:10	27.34	0.08	5.44%		27.5	0.23	16.46%		24.19	0.04	2.69%		24.39	0.23	16.88%	
1:100	30.81	0.09	6.15%	0.94	30.99	0.21	14.93%	0.94	27.66	0.11	7.56%	0.94	27.79	0.17	12.22%	0.97
HCT116 (0.5 µg)																
1:10	26.73	0.07	4.82%		26.72	0.09	5.95%		24.25	0.03	1.98%		24.24	0.04	2.66%	
1:100	30.16	0.14	9.88%	0.96	30.31	0.19	12.97%	0.90	27.77	0.03	1.98%	0.92	27.75	0.08	5.40%	0.93
HCT116 (1 µg)																
1:10	25.89	0.04	2.71%		25.97	0.13	9.00%		23.53	0.04	2.77%		23.59	0.08	5.66%	
1:100	29.34	0.09	6.19%	0.95	29.44	0.12	8.28%	0.94	26.91	0.07	4.90%	0.98	26.93	0.06	4.22%	0.99

SD, standard deviation in cycle threshold (C_T) generated from replicate amplifications; E, efficiency of amplification; ±%Mol, the variation of initial molecule numbers of each dilution calculated with the formula $[(E+1)^{SD}-1] \times 100\%$ (8).

method gives a log-linear relationship between C_T values and initial target quantity, the SCF method gives a direct linear-linear relationship, which gives a clear indication of target quantity variation. Although we did not study the parameter indicating amplification efficiency here, the four parameters of each reaction can certainly give enough information to judge and monitor this problem (17,18). As we showed in this article, the C_T value of each amplification can be easily deduced from Equation 3, which indicates the interrelationship between the SCF method and the threshold method. Because the calculated F_0 value is more directly proportional to the initial target concentration than the C_T value, it is logical to use it for further relative gene expression instead of Equation 5. We propose to quantify normalized relative expression by Equation 4, which is deduced using the F_0 value. This equation is much more simple and understandable than the classic one, and no more information of amplification efficiency is required, thus introducing less error. When comparing the relative expression results of two methods (Figure 4), we observed a good correlation between two values from the same sample.

The problem of the reference gene is an important one, as many researchers believe the ideal reference gene does not exist. In order to give a more reliable indication of relative expression, the use of more than one reference gene has been proposed by certain researchers (33). If the classic $2^{-\Delta\Delta C_T}$ quantification method is used, which does not correct the amplification efficiency, the error will increase rapidly as more reference genes are added. If the revised quantification equation (Equation 5), which does provide a correction, is used, the calculation will have to be done manually. In contrast, the F_0-based calculation gives a more reliable tool to determine a baseline with different reference genes using their geographic mean of F_0 value.

For the reasons mentioned above, we believe that the SCF method has great potential and may help scientists and biologists in determining gene expression levels in human samples.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES

1. **Higuchi, R., C. Fockler, G. Dollinger, and R. Watson.** 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology (NY) *11*:1026-1030.
2. **Morrison, T.B., J.J. Weis, and C.T. Wittwer.** 1998. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. BioTechniques *24*:954-962.
3. **Schuler, F. and G. Dolken.** 2006. Detection and monitoring of minimal residual disease by quantitative real-time PCR. Clin. Chim. Acta *363*:147-156.
4. **Stahlberg, A., N. Zoric, P. Aman, and M. Kubista.** 2005. Quantitative real-time PCR for cancer detection: the lymphoma case. Expert Rev. Mol. Diagn. *5*:221-230.
5. **Schuster, R., N. Max, B. Mann, K. Heufelder, F. Thilo, J. Grone, F. Rokos, H.J. Buhr, et al.** 2004. Quantitative real-time RT-PCR for detection of disseminated tumor cells in peripheral blood of patients with colorectal cancer using different mRNA markers. Int. J. Cancer *108*:219-227.
6. **Mocellin, S., C.R. Rossi, and F.M. Marincola.** 2003. Quantitative real-time PCR in cancer research. Arch. Immunol. Ther. Exp. (Warsz.) *51*:301-313.
7. **Wong, M.L. and J.F. Medrano.** 2005. Real-time PCR for mRNA quantitation. BioTechniques *39*:75-85.
8. **Rutledge, R.G. and C. Cote.** 2003. Mathematics of quantitative kinetic PCR and the application of standard curves. Nucleic Acids Res. *31*:e93.
9. **Livak, K.J. and T.D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Methods *25*:402-408.
10. **Roche Diagnostic GmbH.** LightCycler-Software 4.05 Manual. Roche Diagnostic GmbH, Mannheim, Germany.
11. **Luu-The, V., N. Paquet, E. Calvo, and J. Cumps.** 2005. Improved real-time RT-PCR method for high-throughput measurements using second derivative calculation and double correction. BioTechniques *38*:287-293.
12. **Muller, P.Y., H. Janovjak, A.R. Miserez, and Z. Dobbie.** 2002. Processing of gene expression data generated by quantitative real-time RT-PCR. BioTechniques *32*:1372-1379.
13. **Dheda, K., J.F. Huggett, J.S. Chang, L.U. Kim, S.A. Bustin, M.A. Johnson, G.A. Rook, and A. Zumla.** 2005. The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. Anal. Biochem. *344*:141-143.
14. **Wilson, I.G.** 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. *63*:3741-3751.
15. **Arezi, B., W. Xing, J.A. Sorge, and H.H. Hogrefe.** 2003. Amplification efficiency of thermostable DNA polymerases. Anal. Biochem. *321*:226-235.
16. **Pfaffl, M.W., T.M. Georgieva, I.P. Georgiev, E. Ontsouka, M. Hageleit, and J.W. Blum.** 2002. Real-time RT-PCR quantification of insulin-like growth factor (IGF)-1, IGF-1 receptor, IGF-2, IGF-2 receptor, insulin receptor, growth hormone receptor, IGF-binding proteins 1, 2 and 3 in the bovine species. Domest. Anim. Endocrinol. *22*:91-102.
17. **Liu, W. and D.A. Saint.** 2002. Validation of a quantitative method for real time PCR kinetics. Biochem. Biophys. Res. Commun. *294*:347-353.
18. **Rutledge, R.G.** 2004. Sigmoidal curve-fitting redefines quantitative real-time PCR with the prospective of developing automated high-throughput applications. Nucleic Acids Res. *32*:e178.
19. **Lieber, M.R., Y. Ma, U. Pannicke, and K. Schwarz.** 2003. Mechanism and regulation of human non-homologous DNA end-joining. Nat. Rev. Mol. Cell Biol. *4*:712-720.
20. **Jackson, S.P.** 2002. Sensing and repairing DNA double-strand breaks. Carcinogenesis *23*:687-696.
21. **Clottes, E.** 2005. Hypoxia-inducible factor 1: regulation, involvement in carcinogenesis and target for anticancer therapy. Bull. Cancer *92*:119-127.
22. **Swinson, D.E., J.L. Jones, G. Cox, D. Richardson, A.L. Harris, and K.J. O'Byrne.** 2004. Hypoxia-inducible factor-1 alpha in non small cell lung cancer: relation to growth factor, protease and apoptosis pathways. Int. J. Cancer *111*:43-50.
23. **Williams, K.J., B.A. Telfer, D. Xenaki, M.R. Sheridan, I. Desbaillets, H.J. Peters, D. Honess, A.L. Harris, et al.** 2005. Enhanced response to radiotherapy in tumours deficient in the function of hypoxia-inducible factor-1. Radiother. Oncol. *75*:89-98.
24. **Zhong, H., A.M. De Marzo, E. Laughner, M. Lim, D.A. Hilton, D. Zagzag, P. Buechler, W.B. Isaacs, et al.** 1999. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res. *59*:5830-5835.
25. **Kim, S.J., Z.N. Rabbani, M.W. Dewhirst, Z. Vujaskovic, R.T. Vollmer, E.G. Schreiber, E. Oosterwijk, and M.J. Kelley.** 2005. Expression of HIF-1alpha, CA IX, VEGF, and MMP-9 in surgically resected non-small cell lung cancer. Lung Cancer *49*:325-335.
26. **Volm, M. and R. Koomagi.** 2000. Hypoxia-inducible factor (HIF-1) and its relationship

to apoptosis and proliferation in lung cancer. Anticancer Res. *20*:1527-1533.

27. **Tichopad, A., A. Didier, and M.W. Pfaffl.** 2004. Inhibition of real-time RT-PCR quantification due to tissue-specific contaminants. Mol. Cell. Probes *18*:45-50.
28. **Pfaffl, M.W.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. *29*:e45.
29. **Ramakers, C., J.M. Ruijter, R.H. Deprez, and A.F. Moorman.** 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. *339*:62-66.
30. **Tichopad, A., M. Dilger, G. Schwarz, and M.W. Pfaffl.** 2003. Standardized determination of real-time PCR efficiency from a single reaction set-up. Nucleic Acids Res. *31*:e122.
31. **Peirson, S.N., J.N. Butler, and R.G. Foster.** 2003. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. Nucleic Acids Res. *31*:e73.
32. **Liu, W. and D.A. Saint.** 2002. A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. Anal. Biochem. *302*:52-59.
33. **Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman.** 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol *3*:RESEARCH0034.

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