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PCR-based Calibration Curves for Studies of Quantitative Gene Expression in Human Monocytes: Development and Evaluation

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Background: **Quantitative reverse transcription-PCR (RT-PCR) used to detect small changes in specific mRNA concentrations is often associated with poor reproducibility. Thus, there is a need for stringent quality control in each step of the protocol.**

Methods: **Real-time PCR-based calibration curves for a target gene, tissue factor (TF), and a reference gene, β-actin, generated from PCR amplicons were evaluated by running cDNA controls. In addition, the reverse transcription step was evaluated by running mRNA controls. Amplification efficiencies of calibrators and targets were determined. Variances within and between runs were estimated, and power statistics were applied to determine the concentration differences that could reliably be detected.**

Results: **Within- and between-run variations (CVs) of cDNA controls (TF and β-actin), extrapolated from reproducible calibration curves (CVs of slopes, 4.3% and 2.7%, respectively) were 4–10% (within) and 15–38% (between) using both daily and "grand mean" calibration curves. CVs for the β-actin mRNA controls were 12% (within) and 19–28% (between). Estimates of each step's contribution to the total variation were as follows: $CV_{RT\text{-}PCR}$, 28%; CV_{PCR}, 15%; CV_{RT}, 23% (difference between $CV_{RT\text{-}PCR}$ and CV_{PCR}). PCR efficiencies were as follows: β-actin calibrator/target, 1.96/1.95; TF calibrator/target, 1.95/1.93. Duplicate measurements could detect a twofold concentration difference (power, 0.8).**

Conclusions: **Daily PCR calibration curves generated from PCR amplicons were reproducible, allowing the use of a grand mean calibration curve. The reverse transcription step contributes the most to the total variation. By determining a system's total variance, power analysis may be used to disclose differences that can be reliably detected at a specified power.**

Changes in patterns of gene expression may be observed during various disease states *(1–3)*. Quantitative estimates of specific mRNAs may provide new insights into health and disease issues and may be used in a wide range of clinical applications, such as adjusting the effect of treatment during minimal residual disease, monitoring the response to chemotherapy, or detecting bacterial and viral pathogens *(4–7)*. To achieve reliable quantification of mRNAs in biological samples, the quality and reproducibility of a system are of major importance. Quantitative reverse transcription-PCR (RT-PCR)[1] is a method of choice to detect small changes in mRNAs from cell lysates *(8–11)*. However, this approach is often characterized by lack of reproducibility. Clearly, there is an urgent need for stringent quality control in every step. RT-PCR requires the synthesis of high-quality cDNA libraries generated from highly heterogeneous mRNA pools collectively encoding virtually all of the polypeptides synthesized in a cell. Furthermore, quantitative PCR requires quantification of the amount of synthesized PCR amplicons in the log-linear phase of the PCR. Systems in which amplicons can be monitored in the reaction vessels by the use of fluorescence as a reaction progresses in real time have substantially improved over the last 5 years and aided in the fulfillment of this requirement *(4, 10, 12–14)*. Accurate quantification of mRNA molecules also depends on the amplification efficiency during the reaction. Quantitative RT-PCR assays assume that the PCR efficiencies are equal for all samples in a run *(15, 16)*.

Quantification of mRNAs in a cell lysate requires that the lysate is accompanied by appropriate calibrators and controls (both mRNA and cDNA), which should all be included in the same run *(12, 17)*. Homologous calibrators

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[1] Nonstandard abbreviations: RT-PCR, reverse transcription-PCR; TF, tissue factor; IL, interleukin; LPS, lipopolysaccharide; and Cp, crossing point.

may be generated either by use of linearized vectors containing the cDNA sequence of interest or obtained by cloning *(18, 19)* or by use of specific PCR amplicons. Because cloning is more labor-intensive, we have developed calibrators based on purified, specific PCR amplicons.

The aim of this study was to develop and evaluate a protocol to quantify any mRNA in monocyte lysates based on reverse transcription and real-time PCR. For these purposes, we produced specific PCR amplicons to generate calibration curves suitable for quantification of amplified specific cDNAs. Individual PCR efficiencies were determined for targets and calibrators. Reproducibility was evaluated by running individual calibration curves over a period of 1 year and by quantifying specific transcripts in long-term mRNA as well as cDNA controls. The minimum difference that could be reliably detected was determined by estimating the variances *(20)* and by applying power statistics to the system *(21)*. Variations attributable to mRNA isolation and RT-PCR efficiencies were adjusted by simultaneous quantification of the endogenous housekeeping gene (β-actin or β_2-microglobulin) in the same lysate. To date, we have successfully produced calibration curves for the quantification of cDNA for tissue factor (TF), tumor necrosis factor-α, urokinase plasminogen activator receptor, interleukin-10 (IL-10), MCP-1, β_2-microglobulin, and β-actin. The results for β-actin and TF quantification are presented here.

Materials and Methods

HUMAN MONOCYTES

Isolation of monocytes. Monocyte were isolated on a large scale from EDTA whole blood (450 mL) from blood donors by density gradient centrifugation, purified by elutriation to a purity of >90%, and cryopreserved in RPMI 1640 (Gibco) containing 250 mL/L acid-treated fetal calf serum (Flow Laboratories) or 100 mL/L dimethyl sulfoxide (article no. 2931; Merck) at −150 °C as described previously *(22)*.

Monocyte stimulation. Human monocytes respond to lipopolysaccharide (LPS) stimulation by the synthesis and release of immunoregulatory and inflammatory mediators such as IL-1, IL-6, IL-8, and tumor necrosis factor-α *(23, 24)*. This approach was used to produce specific cDNA calibrators for specific mRNA quantification from genes that are not constitutively expressed in human monocytes. Monocytes were thawed, resuspended in 50 mL/L acid-treated fetal calf serum in RPMI containing 20 mL/L of a penicillin/streptomycin solution (cat. no. P0906, containing 5000 units/mL penicillin and 5 mg/mL streptomycin; Sigma) and seeded at a density of 5×10^4 cells in microtiter plates (Costar 3595; Corning). Cells were cultured in the absence or presence of 300 pg of *Escherichia coli* LPS (0.55:B5; BioWhittaker) per well in a total volume of 200 μL for 3 h at 37 °C. Cells were harvested for mRNA isolation by centrifugation (500*g* for 7 min at 20 °C), the medium was aspirated, and the cells were lysed in 150 μL of lysis/binding buffer according to the instructions accompanying the Dynabeads mRNA Purification Kit® (product no 610.12, Dynal Biotech ASA).

RT-PCR

mRNA isolation. mRNA was isolated using the Dynabeads mRNA Purification Kit *(25, 26)*.

Reverse transcription reaction. The enzymatic conversion of a heterogeneous mRNA population to a high-quality cDNA library was performed by first testing various commercially available reverse transcriptases: Omniscript RT Kit (cat. no. 205111; Qiagen); First Strand cDNA Synthesis Kit for RT-PCR (AMV; cat. no. 1 483 188; Roche Diagnostics GmbH); Superscript (Life Technologies); and Ready · To · Go T-primed First Strand Kit (cat. no. 27-9263-01; Amersham Pharmacia Biotech). Their efficiency, sensitivity, parallellism, linearity, and convenience of use were validated. β-Actin mRNA isolated from diluted solutions of human monocytes and commercially available mRNA from human liver cells (PolyA$^+$ RNA, Adult Liver, Human; cat. no 775013; Stratagene) were quantified using reverse transcription and PCR β-actin calibration curves. Each reverse transcription enzyme was handled according to the manufacturer's instructions. In our systems, Omniscript reverse transcriptase was the best according to the criteria (see above) set out in our work, and reverse transcription was performed as follows: 8 μL of mRNA solution was added to 12 μL of reverse transcription mixture [2.5 mM oligo(dT) primer; 1 unit of RNase inhibitor (PE Applied Biosystems); 10× reverse transcription buffer; 0.5 mM each of dATP, dCTP, dGTP, and dTTP; 4 U of Omniscript Reverse Transcriptase; and RNase-free water (Omniscript RT Kit; cat. no. 205111)]. The reverse transcription step was performed in a gradient cycler (Gene Amp PCR System 2400; PE Applied Biosystems) at 37 °C for 60 min and 95 °C for 5 min. The cDNA libraries were stored at 4 °C.

PCR. We added 2-μL of a cDNA mixture to 18 μL of PCR Mastermixture (3 mM $MgCl_2$, 0.25 mM primers, 2 μL of LightCycler™ Fast Start Master SYBR Green), and amplification was performed in a LightCycler (cat. no. 2239264; Roche Diagnostics). PCR primer design and optimizations were carried out with software downloaded from the Internet Center for Genome Research (http://www-genome.wi.mit.edu/genome_software/other/primer3.html). GenBank accession no. J02846 was used for designing TF primers, and D28354 was used for β-actin. Primers were designed to distinguish cDNA and genomic DNA/pseudogenes *(27)*. The following primers were used: 5′-ggcatcctcaccctgaagta-3′ and 5′-ggggtgttgaaggtctcaaa-3′ for β-actin and 5′-gaccgtagaagatgaacggact-3′ and 5′-ggagggaatcactgcttgaa-3′ for TF. The amplicons were sequenced (CEQ200), and homology with other sequences was examined using the NCBI gene BLAST™ website (www.ncbi.nlm.nih.gov/blast).

LightCycler protocol. A four-segment LightCycler PCR amplification and melting curve protocol was used for online detection of β-actin and TF cDNA, using SYBR Green I. The protocol included a 10-min denaturation step at 95 °C to activate the DNA polymerase, followed by 35 amplification cycles at 95 °C for 1 s, 60 °C for 10 s, and 72 °C for 10 s. Melting curve analyses were performed at 95 °C for 0 s, 65 °C for 15 s, and 95 °C for 0 s. Temperature transition rates were set to 20 °C/s in all segments except the final insert in the melting curve analysis, for which the rate was 0.1 °C/s. The protocol ended with a cooling segment (40 °C). LightCycler software version 3.01 was used to estimate the threshold cycle numbers (crossing points; Cp). The reproducibilities of the calibration curves (slopes and intercepts) and controls were estimated by use of the "second derivative maximum method", in which the software based on default values determines the log-linear area. The limiting dilutions were calculated by the "fit points" method, in which the user may decide the log-linear part and thereby increase the sensitivity.

PREPARATION OF CALIBRATORS

mRNA was isolated from human monocytes incubated with LPS as described above. A cDNA library was synthesized (see above) and used to produce the different calibrators. Specific PCR primers for each calibrator were used in large-scale amplification to obtain quantities of the specific PCR amplicons measurable by ultraviolet detection. Each tube was examined by melting point analysis to assure that only one specific product was present before it was included in the PCR amplicon pool.

Purification of PCR calibrators. The pools of PCR amplicons were purified conventionally by phenol–chloroform extraction and ethanol precipitation and dissolved in 100 μL of H_2O.

Quantification of PCR calibrators by ultraviolet spectrophotometry and limiting dilutions. We measured the absorbance of 70 μL of the purified PCR calibrators at 260/280 nm and calculated the PCR amplicon concentrations and purity. We checked the amplicon numbers, calculated from the DNA concentration by taking into account the size of each specific PCR calibrator (β-actin, 203 bp; TF, 211 bp), the mean molecular weight of the nucleotide bases (340.5 g/mol), Avogadros number (6.022×10^{23}), and the volume, by analyzing several dilutions containing amplicon numbers down to 1–0.1, using the fit point method and the melting curve analysis to search for amplification products *(28)*.

Testing diluents: effects of various matrixes. Known amounts of TF amplicons were added to different solutions [water and MS2 RNA (10 μg/mL; article no. 165948; Roche Diagnostics)] and a nonexpressing TF cDNA library (synthesized from human monocytes and examined for the presence of TF) to examine the matrix effect. PCR was performed, and the Cp values were recorded.

Dilution of PCR calibrators to generate calibration curves. After ultraviolet quantification, the PCR calibrators were diluted severalfold with carrier nucleic acid solution [MS2 RNA (10 μg/mL)] in siliconized tubes (Corning Costar) until they reached the concentration that became the highest calibrator (10^{11} to $\sim 10^5$ amplicons). We then prepared 10-fold dilutions from the highest calibrator until no theoretical amplicon could be obtained. Generally five calibrators were used for each calibration curve. The calibrators and LightCycler Fast Start Master SYBR Green mixture were prepared in separate rooms. The reproducibilities of the calibration curves were examined by calculating mean and range values for each dilution (Fig. 1). The Cp values for the calibrators obtained from each run were examined for outliers by use of Grubbs test (GraphPad).

Handling and storage of cDNA for calibration curves. The stock calibrators [10^{11} copies/μL diluted in MS2 RNA solution (10 μg/mL)] and stored at −70 °C. After thawing, the working calibrator solutions (10^5) were stored at 4 °C for 1 month. From these working solutions dilution series were made for each run.

CONTROLS

Lysate-based cDNA libraries. The cDNA controls were made by synthesizing cDNA libraries from mRNAs isolated from different numbers ($2.5–10 \times 10^4$) of LPS-stimulated human monocytes. The controls were stored at 4 °C. Target values as well as within- and between-run

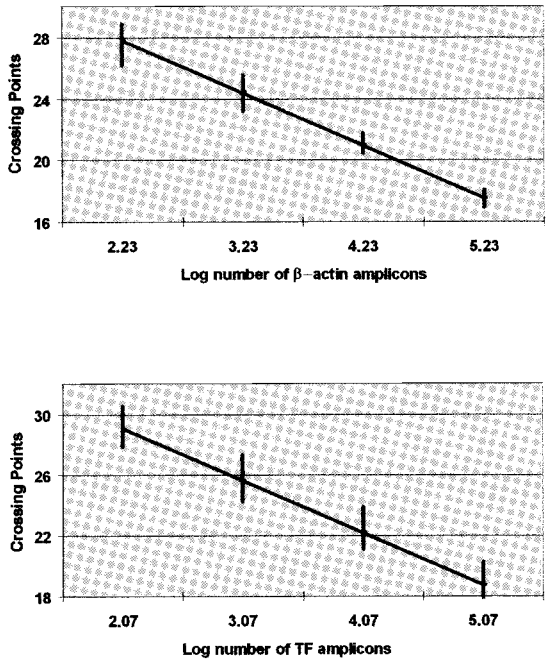

Fig. 1. Reproducibility of the calibration curves for β-actin (*top*: n = 57) and TF (*bottom*: n = 14).

Calibration curves were constructed over a period of 1 year. Between-day CVs calculated on the *y*-intercepts (mean Cp, 36.00 and 37.05) and slopes (mean, −3.46 and −3.43), were 2–4% and 4–8% for β-actin and TF, respectively.

variation were calculated based on both daily calibration curves and a "grand mean" calibration curve. (Because the present LightCycler Software lacks the ability to import a grand mean calibration curve, a representative calibration curve was chosen as the grand mean calibration curve.)

mRNA controls. PolyA$^+$ RNA from adult human liver cells (cat. no. 775013; Stratagene) was diluted in RNase-free water to a concentration of 1 ng/μL, aliquoted, and stored at −70 °C. An aliquot was thawed and diluted further to concentrations of 200 and 20 pg/μL, and all blends were included each time a reverse transcription reaction was performed.

PCR amplification efficiencies

The PCR efficiencies were determined in calibrators and targets (cDNA libraries obtained from LPS-stimulated human monocytes, containing both the target and housekeeping genes TF and β-actin, respectively). Serial dilutions of targets and calibrators (in 10 μg/mL MS2 RNA solution) were PCR-amplified, and the Cp values of each dilution were recorded. Cp values were plotted against the log of the initial template concentrations. Slopes were calculated using Statgraphics® Plus (Manugistics). PCR efficiencies were calculated as $E = 10^{-1/\text{slope}}$ *(29, 30)*.

Correction for differences in the PCR amplification efficiencies. The calculation of efficiency-corrected results is based on the equation: $T = T_0 \times E^{\text{Cp}}$, where T is the number of amplicons synthesized when the Cp is at the detection threshold, E is the efficiency of the calibrator or the target, and T_0 is the initial number of molecules *(4, 29)*. Assuming that the number of amplicons synthesized at the Cp should be the same in the calibrator and the target reaction, the following equation may be used to correct for the systematic error caused by differences in PCR efficiencies in the calibrators and targets:

$$T_{0\ \text{eff.corr.}} = \mathrm{T}_0 \times E_{\text{std}}^{\text{Cp}} / E_{\text{target}}^{\text{Cp}}$$

T_0 and Cp are obtained from the LightCycler run. E_{std} and E_{target} must be determined for each new calibrator and target. The efficiency correction (eff.corr.) is performed by downloading data files from the LightCycler to Microsoft Excel.

Experimental design for estimation of variances and power statistics in the present RT-PCR assay

Six replicates of three dilutions (giving 8, 2.7, and 0.9 ng of mRNA in the reverse transcription step) of PolyA$^+$ RNA (from adult human liver cells; cat. no. 775013; Stratagene), aliquoted (1 ng/μL) and stored at −70 °C, were analyzed for β-actin mRNA by RT-PCR on 6 separate days.

Variance components (within- and between-day) and total variance were estimated by nested ANOVA *(20)*. The *F*-test was used to compare variances in the different sets of concentrations *(20)*. The sample size needed to detect differences with a certain reliability was estimated by computer software (Sample Power Release 2.0 in SPSS 11.0; SPSS Inc.).

Results

Quality control of cDNA calibrators

The β-actin cDNA used to generate calibration curves consisted of a 203-bp amplicon with specific melting point of 89 °C, calculated by melting curve analysis in the LightCycler. The sequenced amplicon had the expected nucleotide composition. The TF primers produced a 211-bp amplicon with a specific melting point of 80 °C and the expected sequence. Results of ultraviolet spectrophotometry indicated that the phenol–chloroform-purified β-actin and TF cDNA calibrator stock solutions contained 79.8 μg/mL (3.4×10^{11} amplicons) and 88.5 μg/mL (3.7×10^{11} amplicons), respectively. These amplicon numbers were examined by use of limiting dilutions. Generally no PCR products were detected in dilutions with amplicon numbers <1.

Choice of diluent solutions

The amplification efficiency, which is the fraction of templates replicated during each reaction cycle, is a crucial factor for the reliability of quantitative PCR *(15–17, 31)*. Because the efficiency of the PCR reaction may be influenced by several factors, such as diluent composition *(15)*, different solutions were examined by amplifying known amounts of TF amplicons in different matrix solutions. It appeared that the Cp values differed depending on diluent composition (data not shown). Highly consistent results, however, were obtained when we diluted the targets in either MS2 RNA solution or a solution similar to our cDNA libraries (nonexpressing TF cDNA library). The usefulness of MS2 RNA (10 μg/mL) as a stabilizer was further evaluated by estimating the β-actin cDNA numbers in various mixtures of MS2 RNA and cDNA library solution. It appeared that the MS2 RNA solution over a wide range gave similar β-actin cDNA results on PCR amplification (data not shown). In addition, these results also indicated that there was no inhibition of the PCR.

Reproducibility of the calibration curves

Calibration curve performance was evaluated with use of data collected from 57 β-actin and 14 TF calibration curves over a period of 1 year. Between-day variations, calculated from the *y*-intercepts (mean Cp values, 36.00 and 37.05) and slopes (mean, −3.46 and −3.43; given by the LightCycler Software after each new run) were 2–4% and 4–8% for β-actin and TF, respectively. Mean and range values for each calibrator's Cp values were calculated to show the reproducibility (Fig. 1). As shown in Fig. 1, the calibration curves were linear over four orders of magnitude, which indicates constant PCR efficiency over the concentration range studied. We improved reproducibility by increasing the concentration of calibrators in the

PCR. A search for outliers in each series of calibrators, using Grubbs test, showed no significant outliers ($P > 0.05$). The lower limits of the method using the "standard deviation method" were 172 copies for β-actin and 118 for TF (see *Materials and Methods*).

PCR amplification efficiency

The Cp values of the targets were converted to concentrations with use of our calibration curves. The amplification efficiencies of the calibrators and targets must be the same to make these readings. Similar slopes in plots of dilutions of calibrators and targets indicated equal efficiencies. A linear plot indicated constant efficiency over the concentration range studied. However, because the efficiencies of the calibrators and the targets may differ, we corrected the target results to observe the consequences (Table 1). The PCR efficiencies were calculated to 1.96 (range, 1.84–2.07; n = 57) and 1.95 (range, 1.84–2.03; n = 7) for the β-actin calibrators and targets and 1.95 (range, 1.84–2.03; n = 15) and 1.93 (range, 1.92–1.95; n = 7) for the TF calibrator and target, respectively. We found no significant differences between the PCR efficiencies of β-actin and TF targets ($P = 0.72$).

Table 1. Within- (CV_w) and between-day (CV_b) variation for long-term lysate-based cDNA controls and long-term mRNA controls.

	Daily calibration curve			Grand mean calibration curve	
	$T_{0\ mean}$	CV_w, %	CV_b, %	$T_{0\ mean}$	CV_b, %
Long-term lysate-based cDNA controls[a]					
β-Actin DNA (n = 21)	1.9×10^4	5			
	1.2×10^2	9			
	6.8×10^3		18	6.5×10^3	15
	1.1×10^3		31	1.1×10^3	26
TF cDNA (n = 15)	1.3×10^3	4			
	0.8×10^2	10			
	5.1×10^3		26	5.0×10^3	29
	0.9×10^2		34	0.9×10^3	38
Long-term mRNA controls[b]					
β-Actin in PolyA$^+$ RNA (n = 17)	1.8×10^4	12			
	3.6×10^4		19	3.8×10^4	23
	5.8×10^3		23	6.2×10^3	28
	6.7×10^2		25	7.3×10^2	26

[a] Pooled cDNA libraries obtained from mRNA isolated from LPS-stimulated human monocytes ($2.5–10 \times 10^4$) were examined every time PCR calibration curves for β-actin and TF were performed. The numbers of targets (T_0) were estimated from both a daily and a grand mean calibration curve, and target mean ($T_{0\ mean}$) and CV were calculated. Within- and between-day variations were calculated on different cDNA libraries. No runs were discarded.

[b] β-Actin concentrations in known amounts of PolyA$^+$ RNA (Adult Liver) were quantified using reverse transcription (Omniscript) and β-actin PCR calibration curves. The number of β-actin mRNAs (T_0) was estimated from both a daily and a grand mean calibration curve, and β-actin mRNA mean ($T_{0\ mean}$) and CV were calculated. No runs were discarded.

Evaluation of the PCR calibration curves

The reproducibility of measured β-actin (n = 57) and TF (n = 15) concentrations in lysate-based cDNA controls at different concentrations were estimated either by use of daily run calibration curves or a grand mean calibration curve (see *Materials and Methods*). Within-run variation (CV) was 4–10%, whereas the between-run CV was 18–38% for β-actin and TF. Similar results were obtained when we used a grand mean calibration curve, indicating that the system is sufficiently stable for the same calibration curve to be used over time (Table 1).

Evaluation of RT-PCR

To evaluate the variation in both the reverse transcription and the PCR step, we quantified β-actin mRNA in dilutions of mRNA (isolated from commercial human liver cells), using both the individual calibration curves and a combined calibration curve (Table 1). The within-run CV was 12%, and the between-run CV was 19–28%, depending on input target concentrations. β-Actin concentrations obtained from dilutions of mRNA (20 pg/μL, 200 pg/μL, and 1 ng/μL) showed a linear relationship, indicating that the RT-PCR are similar over the concentration range studied.

Reliable detection of concentration differences

The minimum difference that can be detected in our system depends on the variances and the power (reliability) chosen. Variance component analysis was performed on data collected from experiments on 6 separate days (see *Materials and Methods* and Table 2). There were no statistical differences in the concentration range studied. Using within-run data at a power of 0.8, we needed duplicate measurements to detect differences of 100%. A sample size of four would be needed within a run to detect differences of 40%. The total variation in the system indicated that a sample size of three would be needed to detect differences at 100%; however, to detect a difference as small as 40%, a sample size of seven would be required (Table 3).

Discussion

Measurements of specific mRNAs through quantitative RT-PCR poses numerous problems; in fact, it requires the isolation of intact mRNA species, quantitative reverse transcription, and quantitative PCR amplification *(4, 32, 33)*. In the present study, the first step focused on the development and evaluation of PCR amplicon-based calibrators to generate calibration curves that could be used to quantify specific target mRNAs. In addition, various cDNA lysate-based controls were generated to evaluate within- and between-run variations in the PCR step. The PCR efficiencies were determined for calibrators and targets to allow for use of the calibration curves. The second step involved using the developed calibration curves to quantify β-actin concentrations in mRNA controls to evaluate the overall reproducibility of the reverse

transcription and PCR steps. The final stage of the study involved an experiment to determine the number of replicates needed to detect a certain concentration difference between samples in the assay.

Our homologous PCR calibrators consist of purified, specific PCR amplicons, compared with the calibrators used by other authors, which were vectors containing the sequence of interest *(18, 32, 34, 35)*. The choice of purification method is of great importance because of the reliability of the A_{260} measurements. If a cDNA library is used as target, cDNA content should be as small as possible if phenol–chloroform extraction and ethanol precipitation are used. Another method could be purification of the PCR amplicons from a gel.

To evaluate the between-run reproducibility of the calibration curves, we collected data over a period of 1 year. The between-run variation (CV) of the slopes of the calibration curves was 4% for β-actin and 8% for TF. The between-run variation (CV) of the *y*-intercept was 2% and 4%, respectively, and in line with results reported by Kruse et al. *(19)*. This indicates that highly reproducible calibration curves over at least four orders of magnitude may be generated based on the synthesis of PCR amplicons.

To evaluate the LightCycler PCR performances, we ran long-term lysate-based cDNA controls at different concentrations for two cDNAs (β-actin and TF). The controls were lysate based to closely mimic the matrix of the samples to be quantified. As expected, the concentrations affected the reproducibility. In line with the observations of Rasmussen *(29)*, we found within-run variations (CVs) between 4% and 11%, whereas the between-run variations ranged from 18% to 38% (Table 1). To the best of our knowledge, very little information has been reported concerning between-run variation when these types of controls and concentrations are used *(9, 19)*.

According to the basic PCR equation, the generated copy number (T) at a certain cycle is a function of the initial copy number (T_0), the PCR efficiency (E), and the cycle number (Cp) *(17, 29)*. The efficiency of the PCR describes the kinetics during the reaction, and maximum efficiency ($E = 2$) is seldom achieved because of various inhibiting factors, such as dilution composition and GC content *(17)*. Separate PCR efficiencies should determined for targets and calibrators to allow for conversion of the Cp values to concentrations based on calibration curves. The PCR efficiencies for targets and calibrators in our systems (1.96/1.95 for β-actin calibrator/target and 1.95/1.93 for TF calibrator/target) showed small differences that may be attributable to variations in dilution composition and the more complex nature of the cDNA library as a starting material. Slack et al. *(32)* determined that PCR efficiencies for their calibrators and targets were 98% and 94%, respectively ($E = 1.96$ and 1.88, using our terms), and "suggested this as little to no decrease in PCR efficiencies". The company Roche *(30)* advises their customers working with relative quantification and external calibrators to use a common calibration curve for any target as long as the amplification efficiency does not differ more than 0.05. The effect of the differences in PCR efficiencies is exponentially dependent on the cycle number and increases with each cycle. We found no significant difference in PCR efficiencies between the targets presented in this report. However, one should be aware that quantification of other targets may give significant differences in PCR efficiencies compared with the housekeeping gene. The need for an efficiency-correcting calculation will then depend on the accuracy required for a given application. This can be done by multiplying the results by a given factor (*Materials and Methods*).

The reverse transcription is another critical step in quantitative analyses *(4)*. This step converts a complex mRNA population into a cDNA library that can be analyzed further by either microarrays or PCR. To evaluate this step, we used RT-PCR to quantify the β-actin mRNA concentrations in solutions containing known amounts of mRNA. Commercially available reverse transcriptases do not always convert the mRNAs to cDNA libraries in a satisfactory way (data not shown). In line with results reported by others *(33)*, we found that Omniscript was the optimal enzyme, based on our criteria (see *Materials and Methods*). This was used to further evaluate variations in the reverse transcription step. The variations, which include variations both from the reverse transcription and PCR steps, were 12% (within-run CV)

Table 2. Variance components of present RT-PCR system.[a]

	Within day (SD)	Between day (SD)	Total variance (SD)	No. of observations
Undiluted	0.19	0.23	0.30	36
Diluted 1:3	0.23	0.17	0.28	36
Diluted 1:9	0.24	0.22	0.33	36
All samples pooled	0.21	0.22	0.30	108

[a] PolyA$^+$ RNA (1 ng/μL) was thawed and further diluted (1:3 and 1:9) on 6 separate days, and RT-PCR was quantified (six samples from each dilution) for β-actin (see *Materials and Methods*).

Table 3. Sample size necessary to detect mRNA concentration differences at power 0.8 and 0.9.[a]

Target concentration difference to be detected	Corresponding cycle number difference (ΔCp)	Sample size at power 0.8	Sample size at power 0.9
Within-day variance SD, 0.21			
40%	0.5	4	5
100%	1.03	2	3
200%	2.06	1	2
Total variance SD, 0.30			
40%	0.5	7	9
100%	1.03	3	4
200%	2.06	1	2

[a] Based on calculations of variance components (Table 2) and using Sample Power 2.0 (SPSS Inc.).

and 19% and 25% (between-run CV; Table 1). Kleiber et al. *(6)* presented data on the reproducibility in their one-step TaqMan HCV RT-PCR test (within-run CV, 17–26%; between-run CV, 21–30%; n = 60) which were slightly higher than in our system. Slack et al. *(32)*, who used a two-step system, also reported between-run variations slightly higher than ours (31–43%; n = 4). Both the reverse transcription and the PCR steps will contribute to the total variation of the described RT-PCR method *(36)*. On the basis of our CVs, exemplified by the cDNA control with a $T_{0\,mean}$ concentration of 6.5×10^3 amplicons and a between-day CV [$CV_{b\,(PCR)}$] of 15% and the mRNA control with a $T_{0\,mean}$ concentration of 6.2×10^3 amplicons and a $CV_{b\,(RT\text{-}PCR)}$ of 28% (Table 1), and with use of ANOVA, the reverse transcription reaction appears to contribute the most to the overall variation (CV_{RT} = 23%).

Our CVs are calculated from concentrations of a given transcript in the samples. If CVs instead are calculated from the Cp values (equivalent to a logarithm of the concentration), the CVs necessarily are substantially lower. Thus, our data, using the Cp values for calculation, indicate a CV_{PCR} of 0.7%, CV_{RT} of 1.8%, and $CV_{RT\text{-}PCR}$ of 2.0% compared with a CV_{PCR} of 15%, CV_{RT} of 23%, and $CV_{RT\text{-}PCR}$ of 28% observed when the calculated concentration of transcripts derived from the calibration curves is used. Because CVs are calculated to predict the stability of the analysis when comparing analyte concentrations, we believe that the CV should be reported accordingly.

The probability of detecting a true difference of a given magnitude can be determined using test power analysis *(21)*. Such analyses give the sample size needed to obtain a specified difference with a certain reliability or power. Determination of the minimum difference in mRNA molecules that could be differentiated in our RT-PCR system was performed by quantifying the β-actin concentrations in diluted mRNA solutions in an experiment designed to determine the within- and between-run variability (see *Materials and Methods* and Table 3). These results indicate that to reliably detect, at a power of 0.8, a twofold difference (100%) between samples, duplicate measurements have to be used. However, to detect a difference as small as 40%, a sample size of four is needed. Gentle et al. *(37)* performed a similar experiment, testing the PCR step by use of purified PCR product as target molecule, and concluded that a difference of 52% in target molecules can be detected between runs with a sample size of four. Quantitative RT-PCR has been used extensively over the last few years to evaluate hepatitis C virus RNA and HIV-2 RNA load and expression of minimal residual disease *(38–40)*. The vast majority of reports conclude that despite variations, RT-PCR enables evaluation of the diseases in question.

Finally, we examined the PCR amplification efficiencies of the housekeeping gene β-actin and TF to allow for normalization of variations in mRNA isolation and RT-PCR efficiencies. The mean slope and PCR efficiency were −3.50 and 1.95 for β-actin cDNA and −3.43 and 1.93 for TF (difference not significant; Fig. 2). In our system, β-actin may be used to normalize for such variations in the range studied without correcting for differences in efficiency. As pointed out, corrections for amplification efficiencies may be made. It should be noted, however, that for every new target gene to be quantified, the amplification efficiencies have to be estimated to qualify for such adjustments.

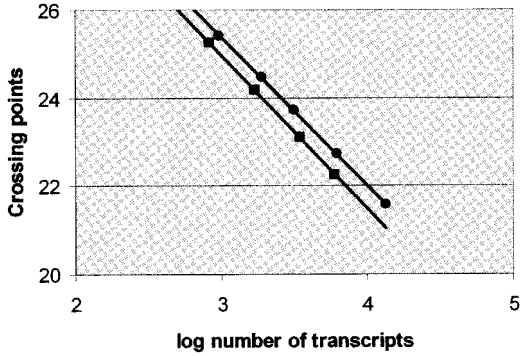

Fig. 2. PCR amplification efficiencies of β-actin (●) and TF (■).

Serial dilutions of cDNA libraries containing both β-actin and TF cDNA were quantified, and the Cp values for each dilution were recorded. PCR amplification efficiency (*E*) was 1.96 for β-actin cDNA and 1.93 for TF cDNA. Seven different cDNA libraries were examined. Representative data are shown.

In conclusion, we have developed reproducible PCR-based calibration curves for TF and β-actin mRNA quantification. The reproducibility of the PCR amplicon-based calibration curves indicates no need to insert the PCR product into a vector to generate calibration curves. If suitable software is available, the reproducibility of the calibration curves indicates that they may be stored between runs, provided that a reference sample is at hand to assure the quality. Differences in PCR efficiencies between targets and calibrators can be compensated with use of suitable software. The evaluation of the present RT-PCR system showed reproducible results that can detect twofold concentration differences. Finally, the present system may be used to produce calibration curves suitable for quantification of any mRNA.

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