Molecular and Cellular Probes (2002) **16**, 99–110
doi:10.1006/mcpr.2002.0405, available online at http://www.idealibrary.com on IDEAL®

A method for the rapid construction of cRNA standard curves in quantitative real-time reverse transcription polymerase chain reaction

S. Fronhoffs[1], G. Totzke[2], S. Stier[1], N. Wernert[3], M. Rothe[3], T. Brüning[1], B. Koch[1], A. Sachinidis[1], H. Vetter[1] and Y. Ko[1]*

[1]*Medizinische Universitäts-Poliklinik Bonn, Wilhelmstr. 35–37, 53111 Bonn, Germany,* [2]*Abteilung für Immunologie und Zellbiologie, Institut für experimentelle Dermatologie, Röntgenstr. 21, 48149 Münster, Germany,* [3]*Institut für Pathologie der Universität Bonn, Sigmund-Freud-Str. 25, 53105 Bonn, Germany*

(Received 11 June 2001; accepted for publication 3 January 2002)

Quantification of nucleic acids, especially of mRNA, is increasingly important in biomedical research. The recently developed quantitative real-time polymerase chain reaction (PCR) – a highly sensitive technology for the rapid, accurate and reproducible quantification of gene expression – offers major advantages over conventional quantitative PCR. Transcript quantification is performed in the exponential phase of the PCR reaction through extrapolation of fluorescence signals from a standard calibration curve which represents the initial copy number for a given fluorescence signal. We have developed a method for gene transcript quantification which is based on a LightCycler™ – assisted real-time PCR in combination with a simple and rapid approach for the construction of external cRNA standards with identical gene sequences as the target gene. Synthesis of cRNAs was performed by *in vitro* transcription with T7 RNA polymerase followed by reverse transcription and real-time PCR. We applied this approach for transcript quantification of eukaryotic initiation factor 3 p110 (*EIF3S8*) mRNA in normal testicular tissue. We also present a rapid and simple strategy for the construction of cRNA standards for use in real-time PCR.

KEYWORDS: quantitative real-time PCR, mRNA quantification, cRNA standards.

INTRODUCTION

Due to the progress made in sequencing the human genome and in identifying clinically-relevant genes, measurement of gene expression has become an increasingly important tool in basic research and for clinical applications in such areas as genetics, infectious diseases and oncology. Because of this, fast, reliable and accurate methods for the quantification of transcription are required.

A number of methods for mRNA quantification have been developed over the last two decades. Originally, Northern blotting and RNase protection assays were developed,[1,2] but were less sensitive than polymerase chain reaction (PCR)-based methods. With the introduction of PCR technology[3] and its combination with reverse transcription (RT), there have been attempts to measure mRNA levels using relatively invariant mRNAs, such as β-actin, as an internal standard.[4,5] In 1989, Wang *et al.*[6] wrote that, ideally, target mRNA could be most accurately quantified by first using an internal standard possessing the same sequence and primer recognition sites as the target itself, and then, by co-amplification of both

* Author to whom correspondence should be addressed: E-mail: yonko@uni-bonn.de

0890–8508/02/020099 + 12 $35.00/0

in the same tube, to produce identical amplification efficiencies. Unfortunately, using this method, PCR products from one standard and one target sequence could not be differentiated. Therefore, internal standards were developed which either used allelic deletion variants of the target gene, or incorporated restriction enzyme sites to facilitate the discrimination of standard and target mRNAs.[7–14] In the last decade, the competitive reverse transcription polymerase chain reaction (RT-PCR) became the gold standard in quantification of especially low-abundance level mRNAs.[15–19] Problems with this method include the need for significant laboratory experience, a relatively long protocol, especially in cases featuring large sample numbers, and the need for post-PCR sample processing.

With the recent introduction of real-time fluorescence PCR technology, a rapid and simple method for the detection of nucleic acids became available.[20–23] High throughput, a wide dynamic range of quantification, and the lack of post-PCR sample processing are the key advantages of this method. Quantification of mRNA using fluorescence PCR technology has been shown to be both precise and reproducible.[24–27]

We describe a method of mRNA quantification which combines the advantages of fluorescence PCR technology (based on the LightCycler™ system) with a modified approach for the simple and rapid construction of external cRNA standards. [14] This modification uses identical sequences as the target gene itself and co-amplification of standard and target mRNA, in parallel. This approach should be applicable for measuring transcription of any gene of interest. As an example, we describe the quantification of eukaryotic initiation factor 3 p110 (*EIF3S8*) mRNA in normal germ cells of testicular tubules. The TATA binding protein (*TBP*) gene transcript amount was used as an endogenous reference against which *EIF3S8* mRNA was normalized.

MATERIALS AND METHODS

Tissue samples

We examined 10 human testicular biopsies which were collected during infertility diagnoses. Patients were 19 to 49 years old. All patients gave their informed consent, and the study was approved by the Ethics Committee of the Rheinische Friedrich-Wilhelms University Bonn. In order to preserve histomorphology, parts of each sample were frozen in −150°C isopentane (precooled in liquid nitrogen) for RNA extraction. Histological evaluation was done on frozen or paraffin sections stained with hematoxylin and eosin (H&E). In all cases, biopsies were evaluated as normal and free of preneoplastic germ cell alterations.

Cryo-microdissection and RNA extraction

We microscopically selected areas from normal testicular parenchyma on frozen sections and trimmed the tissue blocks to the size of these areas at −20°C using a sterile scalpel. We then cut 30 20-µm-thick sections from these blocks using a cryostat and immediately placed them into liquid nitrogen. Histology was again verified during this process at regular intervals on H&E-stained sections.

Total cellular RNA was isolated from micro-dissected tissues using Trizol (Gibco-BRL, Karlsruhe, Germany) after evaporation of the liquid nitrogen. RNA extraction was performed according to the manufacturer's instructions, using 750 µl of Trizol for 30 slides. Genomic DNA potentially present in RNA samples was removed by incubating the RNA with RNase free DNase I (Roche, Mannheim, Germany). RNA quantification was performed by spectrophotometry at 260 nm, and integrity of the RNA was verified by electrophoresis of 4 µg of total RNA on a 2% agarose gel stained with ethidium bromide.

Construction of cRNA standards for the *EIF3S8* target gene (GeneBank™ Accession No. U46025) and the endogenous reference gene *TBP* (GeneBank™ Accession No. M55654)

Synthesis of cDNA

Four µg total cellular RNA from normal testicular parenchyma were reverse-transcribed into first strand cDNA. Samples were incubated with Moloney murine leukemia virus (MMLV) reverse transcriptase (200 U/µl) for 5 min at 25°C, 5 min at 30°C, 90 min at 37°C and 5 min at 95°C in a total reaction volume of 40 µl containing 1 × RT-buffer (50 mM Tris-HCl – pH 8·3 – 75 mM KCl, 3 mM $MgCl_2$), 10 mM dithiothreitol, 0·5 mm of each dNTP, 50 U of RNA-guard (Pharmacia, Freiburg, Germany) and 100 pmol of random hexamer primers.

Primer design for optimal PCR

Following reverse transcription, we performed specific *EIF3S8* and *TBP* cDNA amplification with the primers shown in Table 1.[28] Primers were designed

Table 1. Primers used for conventional and real-time PCR amplification of *EIF3S8* and *TBP* cDNA. Optimal primers are shaded.

TBP			
5′-1	5′-GCTCTTCCACTCACAGACTC-3′	3′-1	5′-GCCAGTCTGGACTGTTCTTC-3′
5′-2	5′-CACCACTCCACTGTATCCCTC-3′	3′-2	5′-CCAACTTCTGTACAACTCTAG-3′
5′-3	5′-CTGGGATTGTACCGCAGCTG-3′	3′-3	5′-GAAGTCCAAGAACTTAGCTGG-3′
EIF3S8			
5′-1	5′-AAAAGGCACCCACCACAGAT-3′	3′-1	5′-CAGGTTGGGGTTGTAGTCATAGA-3′
5′-2	5′-GCTAAGAAGAAGCACGACAGGAAAT-3′	3′-2	5′-CCAGGTTGGGGTTGTAGTCATAG-3′
5′-3	5′-CTAAGAAGAAGCACGACAGGAATC-3′	3′-3	5′-CCAGGTTGGGGTTGTAGTCATA-3′

Table 2. Primers used for the in *vitro* transcription of *EIF3S8* and *TBP* cRNA standards and fluorescence hybridization probes for LightCycler™-assisted real-time PCR of *EIF3S8* and *TBP* mRNA.

Primer	*EIF3S8*	*TBP*
5′-cRNA-construction-primer	5′-GGATCCTAATACGACTCACTATAGGGAGGGC-TAAGAAGAAGCACGACAGGAAAT-3′	5′-GGATCCTAATACGACTCACTATAGGGAGGGC-TCTTCCACTCACAGACTC-3′
3′-cRNA-construction-primer	5′-TTTTTTTTTTTTTTTCCAGGTTGGGGTTGTAGT-CATAG-3′	5′-TTTTTTTTTTTTTTTGCCAGTCTGGACTGT-TCTTC-3′
FL-hybridization-probe	5′-GACCCTTTCCCACTCCCCGCCTT-FL-3′	5′-CCGTGGTTCGTGGCTCTCTTATCCTC-FL-3′
LCR-hybridization-probe	5′-LCR-TGTCCTCCTCCTCCTCATCCAGGC-3′	5′-LCR-TGATTACCGCAGCAAACCGCTTG-3′

FL: fluorescein; LCR: LightCycler™-Red 640.

to have a size of about 20 bp and an approximately 50% G/C content. The fragment sizes were 200–400 bp. PCR was performed in a total volume of 50 µl consisting of 5 µl 10 × reaction buffer [10 mM Tris-HCl, (pH 8·3), 50 mM KCl, 1·5 mM $MgCl_2$, 0·001% gelatin], 34·8 µl sterile water, 4 µl dNTPs (0·2 mM dATP, dTTP, dCTP, dGTP), 1 µl of each primer (20 pmol), 0·5 µl Taq-polymerase (5 U/µl, Amersham Life Science, Cleveland, USA) and 4 µl cDNA. PCR was performed on a thermal block cycler (Perkin-Elmer, model TC-1, Weiterstadt, Germany) after an initial DNA-denaturation (5 min at 95°C) step as follows: 35 cycles of denaturation for 60 s at 94°C, annealing for 60 s at 60°C, extension for 90 s at 72°C, final extension for 20 min at 72°C. Twenty µl of each sample were loaded on a 1·5% conventional agarose gel, and were visualized by ethidium bromide after electrophoresis.

Construction of modified primers

The optimal primer pairs were modified as described by Totzke *et al.*[14] with a T7-promoter sequence at the 5′-end of the 5′-primer and with an oligo-dT at the 5′-end of the 3′-primer (Table 2). Incorporation of T7 promoter sequence onto the 5′-primer is essential to perform *in vitro* transcription with the T7 RNA polymerase. Oligo-dT at the end of the 3′-primer generates a cRNA with a poly(dA) tail at the 3′-end. These modifications can be uniformly performed for all transcripts of interest. Amplification of *EIF3S8* and *TBP* cDNA was performed as described above using the optimized primer pairs and the modified optimized primer pairs. After' PCR amplification, 20 µl of each sample was separated by electrophoresis on a conventional 1·5% agarose gel and visualized by ethidium bromide staining.

In vitro transcription

PCR products amplified with the modified primer pairs were quantified spectrophotometrically at 260 nm. One µg of PCR product was used for *in vitro* transcription (MEGAscript T7 kit, Ambion, Austin, TX, USA). Two µl of reaction buffer, 2 µl of each NTP (75 mM ATP, CTP, GTP, UTP), 2 µl enzyme mix and 1 µg of PCR product in a reaction volume of 20 µl were incubated at 37°C overnight. cDNAs were removed by digesting with 1 µl (2 U) of RNase-free DNase I for 15 min at 37°C. cRNAs were precipitated by adding 30 µl nuclease free water and 25 µl 7·5 M LiCl and subsequent incubation at −20°C for at least

30 min. After 15 min of centrifugation at 14,000 rpm, supernatant was removed and 50 µl of 70% EtOH were added. After another 15 min centrifugation at 14,000 rpm, supernatant was removed and the pellet was dissolved in 20 µl diethyl pyrocarbonate-dH_2O (DEPC-H_2O) and stored at −80°C.

Quantification and dilution of cRNA standard

Quantification of cRNAs was performed spectrophotometrically at 260 nm after incubation at 60°C for 5 min. The measurements of cRNA concentration were performed in duplicate and then converted to the molecule number.

$$N\ (\text{molecules per } \mu l) = \frac{C\ (\text{cRNA}\} \mu g/\mu l)}{K\ (\text{fragment size /bp})} 182{\cdot}5 \times 10^{13}$$

The formula shown above gives the molecules per µl (N), if the concentration of the cRNA (C) is known in relation to the fragment size (K) multiplied by a factor derived from the molecular mass and the Avogadro constant. The dilution series of the cRNA (10^4 to 10^{11} molecules) was performed in the presence of tRNA (Roche, Mannheim, Germany). A tRNA concentration of 1 : 100 in DEPC-H_2O was found to provide the best condition (data not shown). Aliquots of the dilution series were frozen at −80°C until used for experiments.

Real-time RT-PCR

Reverse transcription of samples and cRNA standards

Samples with 2 µg of total RNA from normal testicular tissues and the *EIF3S8* or *TBP* cRNA standard dilution series in the range from 10^4 to 10^{11} molecules were reverse-transcribed, as previously described, in a parallel procedure.

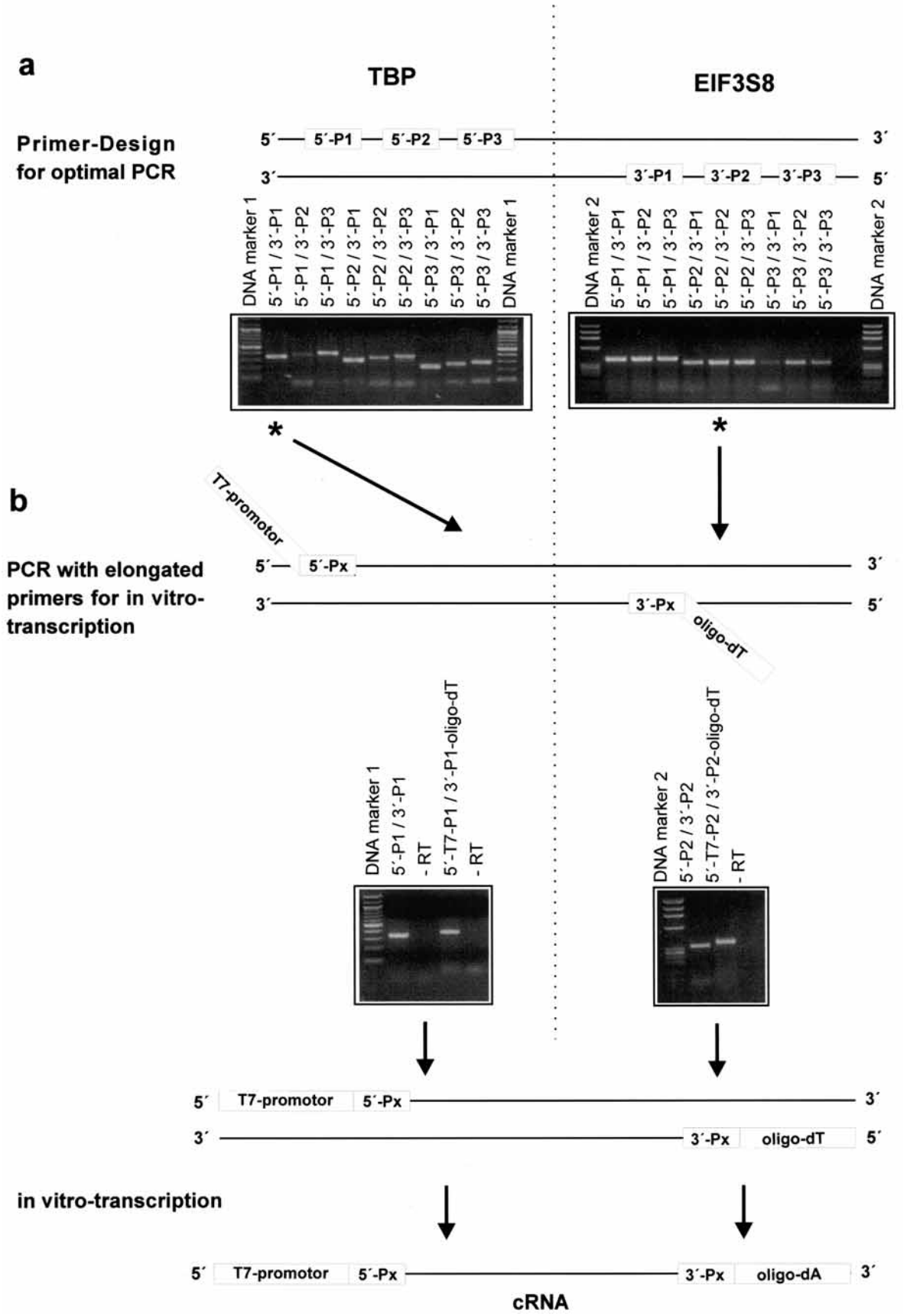
Optimizing real-time PCR using SYBR Green

In order to optimize the real-time PCR conditions for quantitation the optimal $MgCl_2$ concentration was determined. Two µl of DNA Master SYBR Green I (Roche, Mannheim, Germany), 1 µl of each primer (20 pmol) (Table 1) and 1–5 mm $MgCl_2$ in a final volume of 18 µl were mixed in a pre-cooled capillary and 2 µl of cDNA were added. After 2 min of denaturation (at 95°C), 40 PCR cycles were performed with 0 s denaturation at 95°C, 5 s annealing at 60°C and 10 s extension at 72°C. Melting curves were obtained following a denaturation period of 0 s at 95°C, at a start temperature of 65°C and an end temperature of 95°C, with a temperature increase of 0·2°C/s. PCR and melting procedures were detected in real time with the LightCycler™ Instrument (Roche, Mannheim, Germany).

Real-time PCR and quantification of samples

Reverse-transcribed cRNA standards and samples were amplified in parallel by PCR on a LightCycler™ using additional sequence-specific hybridization probes in combination with the LightCycler™ DNA Master Hybridization Probes Kit (Roche, Mannheim, Germany). Primers used for amplification are shown in Table 1 and hybridization probes (TIB Molbiol, Berlin, Germany) of *EIF3S8* and *TBP* cDNAs are presented in Table 2. In principle, hybridization is performed in addition to primer annealing during LightCycler™ PCR with two different short oligonucleotides which hybridize to two adjacent internal sequences of the PCR fragment during the annealing phases. One probe is 5′ labelled with LightCycler™-Red fluorophore and the other with fluorescein. Probes get into close proximity after specific hybridization resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores when the donor fluorophore fluorescein is stimulated by the light source of the LightCycler™. Parts of the absorbed energy are transferred to the acceptor fluorophore, LightCycler™-Red, the emitted fluorescence of which is finally measured. The PCR reaction mixture contained 2 mM $MgCl_2$, 20 pmol of both PCR primers, 1 pmol of each hybridization probe, 2 µl of LightCycler™ DNA Master Hybridization Mix (Roche, Mannheim, Germany) and 2 µl of reverse-transcribed

Fig. 1. Synthesis of *in vitro* transcribed *EIF3S8* and *TBP* cRNA standards. (a) Amplification of *TBP* and *EIF3S8* cDNA using different combinations of 5′-primer (5′-Px) and 3′-primer (3′-Px). PCR results are viewed on ethidium bromide stained agarose gels. Optimal primer pairs (*) were selected and used in all further experiments. (b) Optimal primer pairs were elongated at the 5′-end of the 5′-primer with T7-promoter (5′-T7-Px), and at the 5′-end of the 3′-primer with oligo-dT (3′-Px-oligo-dT). For control, *TBP* and *EIF3S8* cDNA were amplified using optimal and modified primer pairs. The use of modified primer pairs results in larger fragments, as demonstrated by ethidium bromide stained agarose gel. Synthesis of cRNA was performed by *in vitro* transcription with T7 RNA polymerase. DNA marker 1, 100 bp DNA ladder; DNA marker 2, DNA molecular weight marker Φ × 174 DNA-Hae III digest (Hae III); -RT, control without MMLV reverse transcriptase.


a
TBP
EIF3S8
Primer-Design
for optimal PCR
5´
5´-P1
5´-P2
5´-P3
3´
3´-P1
3´-P2
3´-P3
5´
DNA marker 1
5´-P1 / 3´-P1
5´-P1 / 3´-P2
5´-P1 / 3´-P3
5´-P2 / 3´-P1
5´-P2 / 3´-P2
5´-P2 / 3´-P3
5´-P3 / 3´-P1
5´-P3 / 3´-P2
5´-P3 / 3´-P3
DNA marker 1
DNA marker 2
5´-P1 / 3´-P1
5´-P1 / 3´-P2
5´-P1 / 3´-P3
5´-P2 / 3´-P1
5´-P2 / 3´-P2
5´-P2 / 3´-P3
5´-P3 / 3´-P1
5´-P3 / 3´-P2
5´-P3 / 3´-P3
DNA marker 2
*
*
b
T7-promotor
PCR with elongated
primers for in vitro-
transcription
5´
5´-Px
3´
3´
3´-Px
oligo-dT
5´
DNA marker 1
5´-P1 / 3´-P1
- RT
5´-T7-P1 / 3´-P1-oligo-dT
- RT
DNA marker 2
5´-P2 / 3´-P2
5´-T7-P2 / 3´-P2-oligo-dT
- RT
5´
T7-promotor
5´-Px
3´
3´
3´-Px
oligo-dT
5´
in vitro-transcription
5´
T7-promotor
5´-Px
3´-Px
oligo-dA
3´
cRNA

samples in a final volume of 20 µl. Following 3 min of denaturation (at 94°C), we performed 45 PCR cycles with 3 s of denaturation at 94°C, 20/15 s (*EIF3S8/TBP*) of annealing at 63/60°C (*EIF3S8/TBP*) and 20 s of extension at 72°C. All experiments were performed in duplicate.

The sample copy numbers were calculated using the LightCycler™ analysis software (Roche, Mannheim, Germany). The larger the starting quantity of the target molecule, the earlier a significant increase in fluorescence caused by FRET was detected during monitoring of the PCR by the LightCycler™. The LightCycler™ software system analyzed the spectral data collected at the end of the annealing phase of each cycle and plotted fluorescence intensity versus cycle number. The threshold cycle (Ct), defined as the fractional cycle at which the fluorescence signal becomes significantly different from the baseline signal, was manually determined by the fit-point method provided by the LightCycler™ software. The cRNA standard curve was generated by the LightCycler™ software system by plotting the Cts against the logarithm of the calculated initial copy numbers. The unknown initial sample copy numbers were then automatically calculated from their Cts, as compared to the cRNA standard curve.

RESULTS

Primer design for optimal PCR

The best amplification resulted with the primer pairs 5′-P1/3′-P1 for *TBP* and 5′-P2/3′-P2 for *EIF3S8*. A strong product without nonspecific fragments and without abundant primer dimers was observed (Fig. 1a).

Generation of EIF3S8 and TBP cRNA standards

The optimal primer pairs were modified at the 5′-end of the 5′-primer with a T7-promoter sequence and at the 5′-end of the 3′-primer with an oligo-dT sequence. As a control *EIF3S8* and *TBP* cDNAs were then amplified with the optimal and the modified primer pairs and the results were visualized (Fig. 1b). The PCR performed with the modified primer pairs produced expectedly larger products. The *in vitro* transcription gave a cRNA yield of C = 0·232 µg/µl (K = 378; N = $0{\cdot}11 \times 10^{13}$ molecules/µl) for *EIF3S8* and C = 3·133 µg/µl (K = 398; N = $1{\cdot}43 \times 10^{13}$ molecules/µl) for *TBP*. Based on these calculations, dilution series were performed for both cRNAs in the range from 10^4 to 10^{11} molecules.

Optimizing real-time PCR

SYBR Green assisted real-time PCR with different concentrations of $MgCl_2$ (1–5 mM) showed an exponential amplification of *EIF3S8* starting at 10 cycles of PCR. The best amplification efficiency was achieved at a $MgCl_2$ concentration of 2 mM (Fig. 2a). Melting curve analysis showed a single melting peak without demonstrable amounts of primer dimers or nonspecific fragments (Fig. 2b). All further experiments were then performed with 2 mM $MgCl_2$.

cRNA standard curves

For real-time RT-PCR analysis of *EIF3S8* and *TBP* mRNA expression in human testicular tissue we established standard curves for *EIF3S8* and *TBP* using the serially diluted cRNA obtained by *in vitro* transcription. Fig. 3a shows the standard curve (10^5 to 10^{10} transcripts) for the *TBP* gene and Fig. 3b shows the standard curve (10^4 to 10^{10} transcripts) for the *EIF3S8* gene. The dynamic range was wide (at least six orders of magnitude). A strong linear relationship with a correlation coefficient of $r^2 > 0{\cdot}99$ between the fractional cycle number and the log of the starting copy number was demonstrated for both cRNA standard curves in all experiments. The efficiencies of the amplifications (E) of *TBP* and *EIF3S8* cRNAs were calculated according to the equation: $E = 10^{1/m} - 1$, where m is the slope of the standard curve as given by the LightCycler™ software, and was found to range in all experiments between 88·7% and 100% and between 87·4% and 100%, respectively.

Quantification

In order to determine the total amount of *TBP* transcripts in 2 µg of total tissue RNA we reverse-transcribed and amplified tissue samples during real-time RT-PCR in parallel with the cRNA standards (Fig. 4a). The total amount of *TBP* transcripts was determined using the standard curve as described. The analysis gave a range from 98,560 to 30,630,000 transcripts in 10 different normal human testicular tissues. We applied the same approach for the expression analysis of *EIF3S8* gene in the 10 normal human testicular tissues (Fig. 4b) and between 685,200 and 24,050,000 transcripts were found in the various samples.

To minimize errors derived from the isolation of total RNA, *TBP* transcripts were used as an endogenous RNA control.[29] In order to obtain a normalized

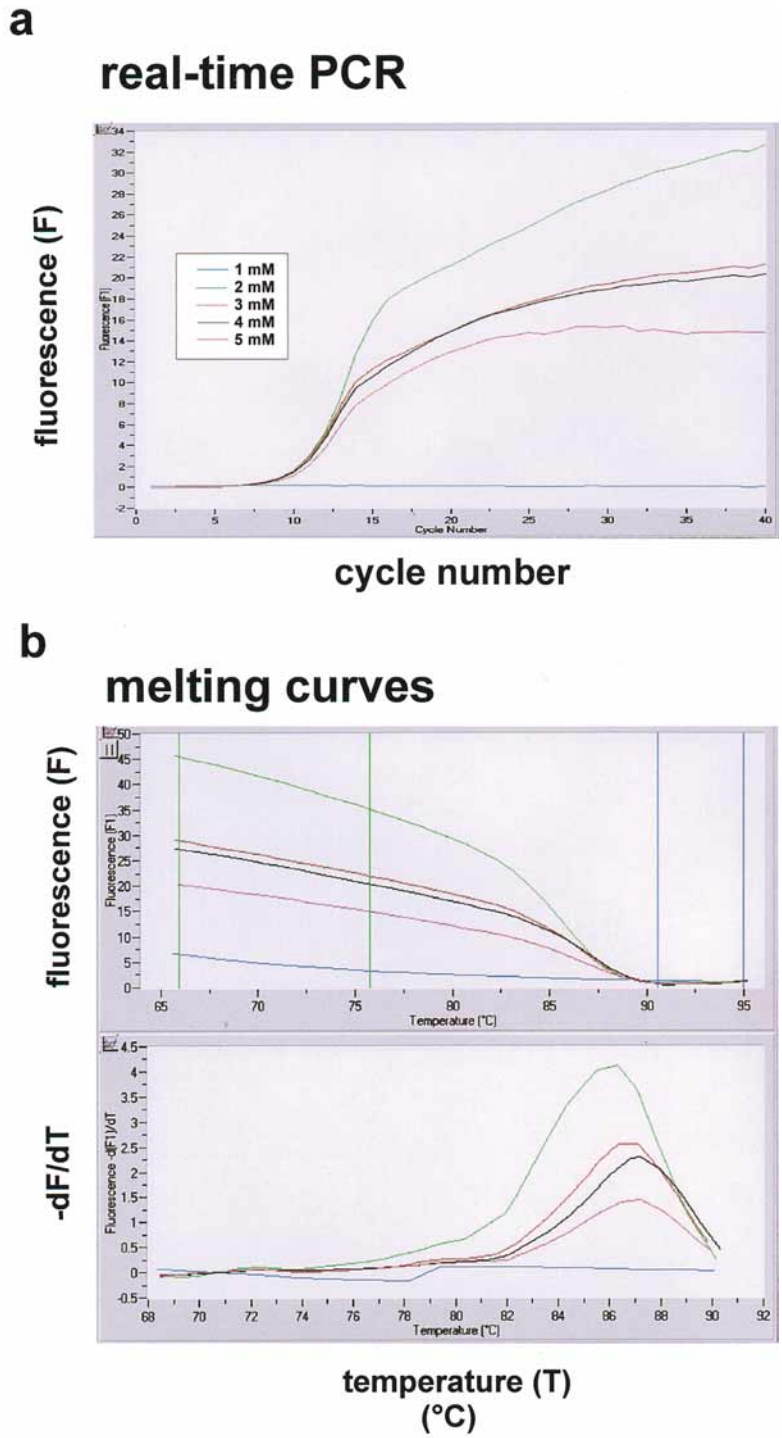


Fig. 2. SYBR Green assisted LightCycler™ real-time PCR of *EIFS38* cDNA using different $MgCl_2$ (1–5 mM) concentrations (a) Five *EIF3S8* cDNAs were amplified using the selected primer pairs indicated in Table 1 with $MgCl_2$ concentrations ranging from 1 to 5 mM. Plotting fluorescence data against cycle number, the highest amplification was obtained with 2 mM $MgCl_2$. (b) Melting curve analysis for each sample was performed by plotting the first negative derivative of the fluorescence (F) with respect to temperature (T) against temperature [(-dF/dT) vs T] and showed a single melting maximum for each sample indicating specific amplification without primer dimer.

value for *EIF3S8* mRNA for each tissue sample, the *EIF3S8* amount was divided by the corresponding *TBP* amount. The relative amount of *EIF3S8* transcripts ranged from 0·785 to 6·952 with a mean value of $3{\cdot}661 \pm 1{\cdot}395$ (*EIF3S8/TBP*, SD $\pm$ 5% confidence interval).

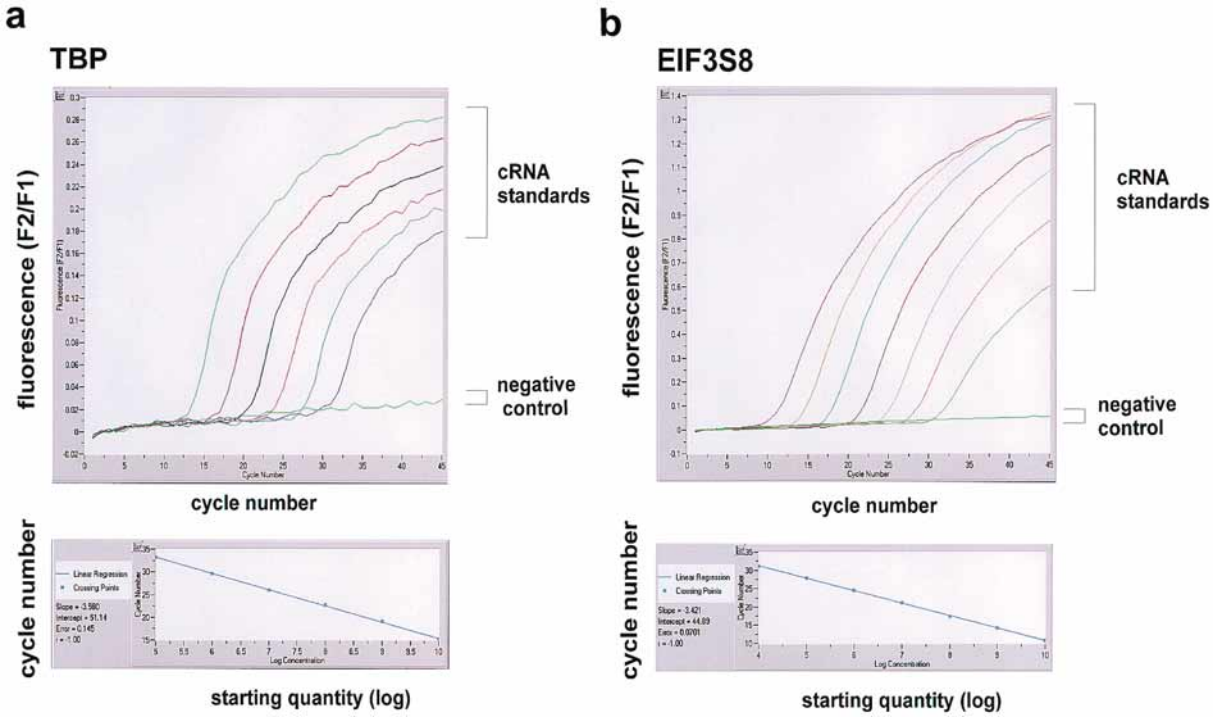


Fig. 3. Construction of *TBP* and *EIF3S8* cRNA standard curves. LightCycler™-assisted real-time PCR of *in vitro* transcribed (a) serially diluted *TBP* cRNAs ranging from 10^5 to 10^{10} transcripts and (b) serially diluted *EIF3S8* cRNAs ranging from 10^4 to 10^{10} transcripts using the primer pair and sequence specific hybridization probes given in Tables 1 and 2. Plotting fluorescence against cycle number, the fractional cycles (threshold cycle) at which the fluorescence signal becomes significantly different from the baseline signal were manually determined by the fit-point method provided by the LightCycler™ software. The fractional cycles were plotted against the logarithm of the initial transcript numbers indicating a precise linear relationship with a correlation coefficient of r^2>0·99. A negative amplification control was performed with a sample containing no cRNA.

DISCUSSION AND CONCLUSIONS

With increasing demand for the quantitation of gene expression, simple, rapid, accurate and reproducible methods are required for use in clinical and routine laboratory applications. To fulfill these criteria we have developed a method for gene transcript quantitation which is based on a LightCycler™-assisted quantitative real-time RT-PCR in combination with a simple and rapid approach for the construction of external and simultaneously-amplified cRNA standards with gene sequences identical to the target gene.

Recently, quantitative fluorescence-based real-time RT-PCR has been established for both commercially-available systems (the ABI PRISM™ 7700 Sequence Detection System and the LightCycler™).[23–26,30] In direct comparison, both real-time PCR systems appeared to quantify gene transcription with equal sensitivity and reliability.[31,32] An advantage of LightCycler™ technology is that it permits the use of two sequence-specific fluorescence hybridization probes as briefly described in 'Materials and Methods'. The LightCycler™ also allows faster PCRs and the ability to discriminate DNAs through melting curve analysis.[23,30] A disadvantage of this method is that it requires more complicated design of hybridization probes which have to be constructed uniquely for each target gene. Probes must also fulfill certain criteria, such as the ability to simultaneously anneal and must perform at higher melting temperatures compared to the primer pair to ensure efficient hybridization during the annealing phase. Alternatively, LightCycler™-assisted quantitative real-time RT-PCR can be performed using the double-stranded DNA (dsDNA) dye SYBR green.[26,33] However, this approach lacks the specificity of labelled hybridization probes and requires post-PCR melting curve analysis to detect primer-dimer and nonspecific PCR products or treatment with anti-Taq antibodies.[26] Therefore, we chose specifically labeled hybridization probes, to perform quantitative real-time PCR and used SYBR green only to optimize PCR conditions as previously described.

Quantitative real-time RT-PCR has already been used for the measurement of gene expression in a variety of fields, such as oncology,[29,31,34–46] hematology,[47,48] and microbiology.[32,33,49–53] This method has been shown to possess several advantages over conventional quantitative RT-PCR. First, it is simple,

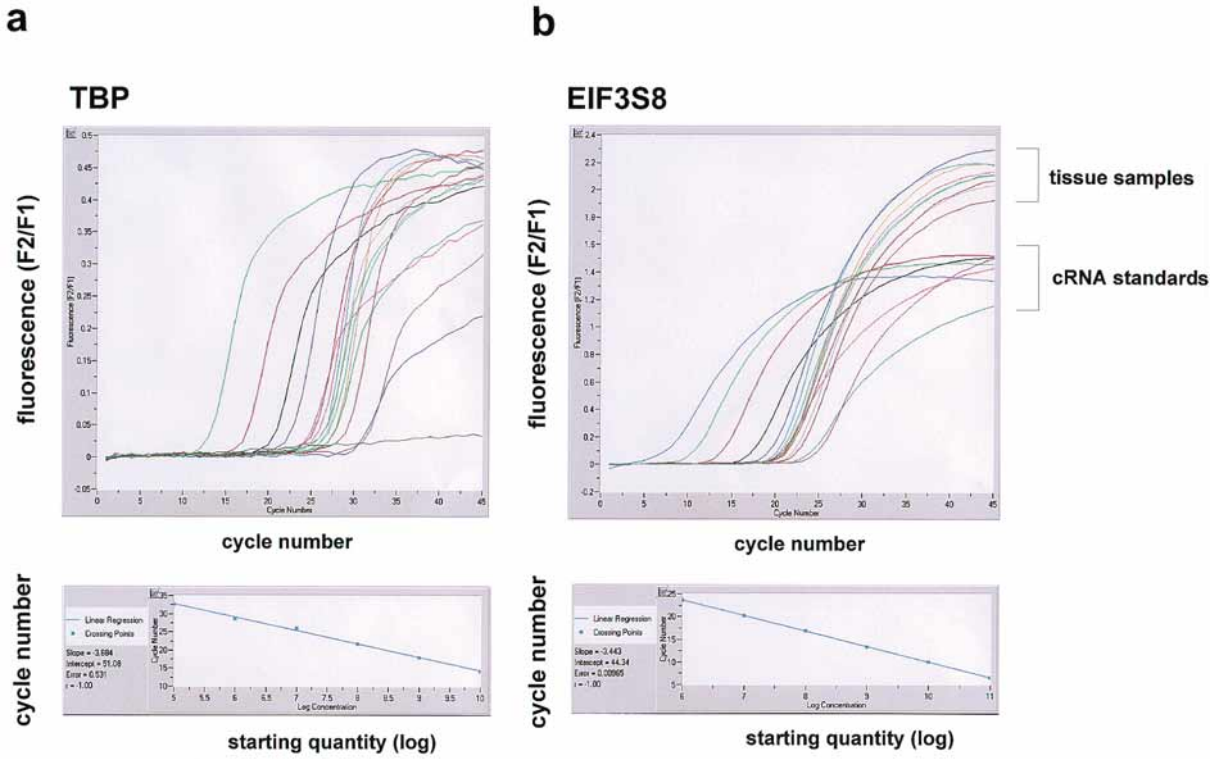


Fig. 4. Quantitation of *TBP* and *EIF3S8* mRNA in 2 µg of total human testicular tissue RNA. (a) Parallel LightCycler™-assisted real-time PCR of serially diluted *TBP* cRNAs ranging from 10^5 to 10^{10} transcripts and of 10 samples using the primer pair and sequence specific hybridization probes given in Tables 1 and 2. (b) Parallel LightCycler™-assisted real-time PCR of serially diluted *EIF3S8* cRNA ranging from 10^6 to 10^{11} transcripts and of 10 samples using the primer pair and sequence specific hybridization probes given in Tables 1 and 2. Quantitation of sample mRNA is achieved by extrapolation of fluorescence signals at the threshold cycle against the standard curves which represent the initial transcript numbers for a defined fluorescence signal.

rapid, possesses a wide dynamic range of quantitation and allows higher sample throughput in comparison to alternative methods. Second, it is performed in a closed-tube system and does not require post-PCR sample processing, thereby reducing the risk of sample contamination and other errors due to sample manipulation. Third, in contrast to the endpoint analysis of conventional PCR, real-time PCR allows for the calculation of RNA amounts during the exponential phase when none of the reaction components are rate-limiting. Since the fluorescence, which is proportional to the amount of amplified DNA, is measured at the end of the annealing phase of each PCR-cycle the log-linear phase of the amplification reaction can easily be detected. Quantification of mRNA is achieved by extrapolation of fluorescence signals from test samples against standard curves which represent the initial copy numbers for a defined fluorescence signal. Several reports have demonstrated that quantitative real-time PCR is an accurate and reproducible method with a high specificity and sensitivity and a high intra- and inter-assay precision. It is also able to detect as few as 10 initial template copies.[24–27,32,33,42,48]

Accurate transcript quantitation using real-time RT-PCR depends on the construction of standard curves. To date, different approaches for the development of external standard curves used in combination with LightCycler™-based real-time PCR include the use of constitutively-expressed genes, such as β-actin and 16S ribosomal RNA[26,41,50,54] and plasmid clones containing the cDNA of interest as a template.[32,33]

In the approach presented here, external *in vitro* synthesized cRNA standards[13,14] are used to obtain a calibration curve, thereby offering some important advantages. The method for construction of cRNA standards is simple, rapid to perform and should be applicable for measuring transcripts from any gene of interest. Samples and cRNA standards can be reverse-transcribed and amplified in a parallel procedure using identical primer pairs and hybridization probes. In addition, this approach allows the parallel amplification of cRNA standards with a sequence identical to the target gene itself.

A precise linear relationship ($r^2 = >0{\cdot}99$) between the threshold cycles and the log of the initial copy number was demonstrated for both the *EIF3S8* and the *TBP* cRNA standard curves in all experiments.

The amplification efficiency of *EIF3S8* and *TBP* cRNA standards, based on the slope of the exponential phase during PCR as calculated by the LightCycler™ software, ranged from 87·4% to 100% and 88·7% to 100%, respectively, in different runs. As indicated in Figures 4a and 4b, all samples were found to show equal amplification efficiencies as compared to the *TBP* cRNA standard curves but slightly higher efficiencies as compared to the *EIF3S8* cRNA standard curves. Since the external use of cRNA standards requires equal amplification efficiencies for samples and standards to determine the absolute transcript amount we used this approach for the relative measurement of *EIF3S8* mRNA in performing tissue comparisons. However, given equal amplification efficiencies of samples and corresponding cRNA standard curves, absolute quantitation can be performed. To circumvent the problem of amplification efficiency one might use internal cRNA standards and hybridization probes labeled with different LightCycler™-Red (LCR) fluorophores. However, this approach is more laborious and decreases sample throughput.

As with to other quantitative PCR techniques, LightCycler™ real-time PCR is affected by the quality of the RNA and by the efficiency of reverse transcription both of which are known to account for most of the variability in quantitative RT-PCR. To minimize these errors and to correct for sample-to-sample variation, mRNA amounts were normalized against an endogenous reference. TBP, which is a subunit of the transcription factor IID (TFIID), was chosen because no *TBP* pseudogenes are known.[29] To normalize sample mRNA amounts, a *TBP* cRNA standard curve was established to quantify the amount of *TBP* transcripts in each sample. *EIF3S8* mRNA level was normalized with respect to the corresponding *TBP* amount and gene expression levels were subsequently determined by comparison of the normalized sample target amounts.

In conclusion, we present a method for the rapid and simple construction of external cRNA standards with sequences identical to the target itself and which can be used to obtain calibration curves for the quantitation of gene expression in LightCycler™-assisted quantitative real-time RT-PCR. This approach should be applicable for the measurements of transcripts for any gene of interest and might become a new standard for mRNA quantitation.

ACKNOWLEDGEMENTS

We thank Dr. Olfert Landt (TIB MOBIOL, Tempelhofer Weg 11–12, 10829 Berlin, Germany, Fax: +49 30 78799499) for the construction of the LightCycler™ hybridization probes.

REFERENCES

1. Berk, A. J. & Sharp, P. A. (1977). Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. *Cell* **12**, 721–32.
2. Thomas, P. S. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proceedings of the National Academy of Science of the United States of America* **77**, 5201–5.
3. Mullis, K. B. & Faloona, F. A. (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in Enzymology* **155**, 335–50.
4. Raff, T., van der Giet, M., Endemann, D., *et al.* (1997). Design and testing of beta-actin primers for RT-PCR that do not co-amplify processed pseudogenes. *Biotechniques* **23**, 456–60.
5. Kreuzer, K. A., Lass, U., Landt, O., *et al.* (1999). Highly sensitive and specific fluorescence reverse transcription-PCR assay for the pseudogene-free detection of beta-actin transcripts as quantitative reference. *Clinical Chemistry* **45**, 297–300.
6. Wang, A. M., Doyle, M. V. & Mark, D. F. (1989). Quantitation of mRNA by the polymerase chain reaction [published erratum appears in Proc Natl Acad Sci U S A 1990 Apr;87(7):2865]. *Proceedings of the National Academy of Science of the United States of America* **86**, 9717–21.
7. Kanangat, S., Solomon, A. & Rouse, B. T. (1992). Use of quantitative polymerase chain reaction to quantitate cytokine messenger RNA molecules. *Molecular Immunology* **29**, 1229–36.
8. Martino, T. A., Sole, M. J., Penn, L. Z., *et al.* (1993). Quantitation of enteroviral RNA by competitive polymerase chain reaction. *Journal of Clinical Microbiology* **31**, 2634–40.
9. Scadden, D. T., Wang, Z. & Groopman, J. E. (1992). Quantitation of plasma human immunodeficiency virus type 1 RNA by competitive polymerase chain reaction [published erratum appears in *J Infect Dis* 1993 Apr;167(4):995]. *Journal of Infectious Diseases* **165**, 1119–23.
10. Taniguchi, A., Kohsaka, H. & Carson, D. A. (1994). Competitive RT-PCR ELISA: a rapid, sensitive and non-radioactive method to quantitate cytokine mRNA. *Journal of Immunological Methods* **169**, 101–9.
11. Riedy, M. C., Timm, E. A., Jr. & Stewart, C. C. (1995). Quantitative RT-PCR for measuring gene expression. *Biotechniques* **18**, 70–4, 76.
12. Ruster, B., Zeuzem, S. & Roth, W. K. (1995). Quantification of hepatitis C virus RNA by competitive reverse transcription and polymerase chain reaction using a modified hepatitis C virus RNA transcript. *Analytical Biochemistry* **224**, 597–600.
13. Vanden Heuvel, J. P., Tyson, F. L. & Bell, D. A. (1993). Construction of recombinant RNA templates for use as internal standards in quantitative RT-PCR. *Biotechniques* **14**, 395–8.
14. Totzke, G., Sachinidis, A., Vetter, H., *et al.* (1996). Competitive reverse transcription/polymerase chain reaction for the quantification of p53 and mdm2 mRNA expression. *Molecular and Cellular Probes* **10**, 427–33.

15. Souaze, F., Ntodou-Thome, A., Tran, C. Y., *et al.* (1996). Quantitative RT-PCR: limits and accuracy. *Biotechniques* **21**, 280–5.
16. Zimmermann, K. & Mannhalter, J. W. (1996). Technical aspects of quantitative competitive PCR. *Biotechniques* **21**, 268–72, 274–9.
17. Hayward, A. L., Oefner, P. J., Sabatini, S., *et al.* (1998). Modeling and analysis of competitive RT-PCR. *Nucleic Acids Research* **26**, 2511–8.
18. Freeman, W. M., Walker, S. J. & Vrana, K. E. (1999). Quantitative RT-PCR: pitfalls and potential. *Biotechniques* **26**, 112–22, 124–5.
19. Vu, H. L., Troubetzkoy, S., Nguyen, H. H., *et al.* (2000). A method for quantification of absolute amounts of nucleic acids by (RT)-PCR and a new mathematical model for data analysis. *Nucleic Acids Research* **28**, E18.
20. Holland, P. M., Abramson, R. D., Watson, R., *et al.* (1991). Detection of specific polymerase chain reaction product by utilizing the 5′-----3′ exonuclease activity of Thermus aquaticus DNA polymerase. *Proceedings of the National Academy of Science of the United States of America* **88**, 7276–80.
21. Higuchi, R., Fockler, C., Dollinger, G., *et al.* (1993). Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (N Y)* **11**, 1026–30.
22. Livak, K. J., Flood, S. J., Marmaro, J., *et al.* (1995). Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods and Applications* **4**, 357–62.
23. Wittwer, C. T., Herrmann, M. G., Moss, A. A., *et al.* (1997). Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* **22**, 130–1, 134–8.
24. Heid, C. A., Stevens, J., Livak, K. J., *et al.* (1996). Real time quantitative PCR. *Genome Research* **6**, 986–94.
25. Gibson, U. E., Heid, C. A. & Williams, P. M. (1996). A novel method for real time quantitative RT-PCR. *Genome Research* **6**, 995–1001.
26. Morrison, T. B., Weis, J. J. & Wittwer, C. T. (1998). Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques* **24**, 954–8, 960, 962.
27. Bustin, S. A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays [In Process Citation]. *Journal of Molecular Endocrinology* **25**, 169–93.
28. Ko, Y., Totzke, G., Schiermeyer, B., *et al.* (1995). Reverse transcriptase-polymerase chain reaction (RT-PCR): a sensitive method to examine basic fibroblast growth factor-induced expression of the early growth response gene-1 (egr-1) in human umbilical arterial endothelial cells. *Molecular and Cellular Probes* **9**, 215–22.
29. Bieche, I., Laurendeau, I., Tozlu, S., *et al.* (1999). Quantitation of MYC gene expression in sporadic breast tumors with a real-time reverse transcription-PCR assay. *Cancer Research* **59**, 2759–65.
30. Wittwer, C. T., Ririe, K. M., Andrew, R. V., *et al.* (1997). The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. *Biotechniques* **22**, 176–81.
31. Kreuzer, K. A., Lass, U., Bohn, A., *et al.* (1999). LightCycler technology for the quantitation of bcr/abl fusion transcripts. *Cancer Research* **59**, 3171–4.
32. Nitsche, A., Steuer, N., Schmidt, C. A., *et al.* (1999). Different real-time PCR formats compared for the quantitative detection of human cytomegalovirus DNA. *Clinical Chemistry* **45**, 1932–7.
33. Morrison, T. B., Ma, Y., Weis, J. H., *et al.* (1999). Rapid and sensitive quantification of Borrelia burgdorferi-infected mouse tissues by continuous fluorescent monitoring of PCR. *Journal of Clinical Microbiology* **37**, 987–92.
34. Mensink, E., van de Locht, A., Schattenberg, A., *et al.* (1998). Quantitation of minimal residual disease in Philadelphia chromosome positive chronic myeloid leukaemia patients using real-time quantitative RT-PCR. *British Journal of Haematology* **102**, 768–74.
35. Bieche, I., Olivi, M., Champeme, M. H., *et al.* (1998). Novel approach to quantitative polymerase chain reaction using real-time detection: application to the detection of gene amplification in breast cancer. *International Journal of Cancer* **78**, 661–6.
36. De Kok, J. B., Schalken, J. A., Aalders, T. W., *et al.* (2000). Quantitative measurement of telomerase reverse transcriptase (hTERT) mRNA in urothelial cell carcinomas. *International Journal of Cancer* **87**, 217–220.
37. de Kok, J. B., Ruers, T. J., van Muijen, G. N., *et al.* (2000). Real-time quantification of human telomerase reverse transcriptase mRNA in tumors and healthy tissues. *Clinical Chemistry* **46**, 313–8.
38. Pfitzner, T., Engert, A., Wittor, H., *et al.* (2000). A real-time PCR assay for the quantification of residual malignant cells in B cell chronic lymphatic leukemia. *Leukemia* **14**, 754–66.
39. Nakao, M., Janssen, J. W., Flohr, T., *et al.* (2000). Rapid and reliable quantification of minimal residual disease in acute lymphoblastic leukemia using rearranged immunoglobulin and T-cell receptor loci by LightCycler technology. *Cancer Research* **60**, 3281–9.
40. Eckert, C., Landt, O., Taube, T., *et al.* (2000). Potential of LightCycler technology for quantification of minimal residual disease in childhood acute lymphoblastic leukemia. *Leukemia* **14**, 316–23.
41. Emig, M., Saussele, S., Wittor, H., *et al.* (1999). Accurate and rapid analysis of residual disease in patients with CML using specific fluorescent hybridization probes for real time quantitative RT-PCR. *Leukemia* **13**, 1825–32.
42. Donovan, J. W., Ladetto, M., Zou, G., *et al.* (2000). Immunoglobulin heavy-chain consensus probes for real-time PCR quantification of residual disease in acute lymphoblastic leukemia. *Blood* **95**, 2651–8.
43. Saussele, S., Weisser, A., Muller, M. C., *et al.* (2000). Frequent polymorphism in BCR exon b2 identified in BCR-ABL positive and negative individuals using fluorescent hybridization probes [In Process Citation]. *Leukemia* **14**, 2006–10.
44. Nakanishi, H., Kodera, Y., Yamamura, Y., *et al.* (2000). Rapid quantitative detection of carcinoembryonic antigen-expressing free tumor cells in the peritoneal cavity of gastric-cancer patients with real-time RT-PCR on the lightcycler [In Process Citation]. *International Journal of Cancer* **89**, 411–7.
45. Kamihira, S., Dateki, N., Sugahara, K., *et al.* (2000). Real-time polymerase chain reaction for quantification of HTLV-1 proviral load: application for analyzing

aberrant integration of the proviral DNA in adult T-cell leukemia. *International Journal of Hematology* **72**, 79–84.
46. Saffroy, R., Lemoine, A., Brezillon, P., *et al.* (2000). Real-time quantitation of bcr-abl transcripts in haematological malignancies [In Process Citation]. *European Journal of Haematology* **65**, 258–66.
47. Smith, R. D., Li, J., Noguchi, C. T., *et al.* (2000). Quantitative PCR analysis of HbF inducers in primary human adult erythroid cells. *Blood* **95**, 863–9.
48. Blaschke, V., Reich, K., Blaschke, S., *et al.* (2000). Rapid quantitation of proinflammatory and chemoattractant cytokine expression in small tissue samples and monocyte-derived dendritic cells: validation of a new real-time RT-PCR technology [In Process Citation]. *Journal of Immunological Methods* **246**, 79–90.
49. Pahl, A., Kuhlbrandt, U., Brune, K., *et al.* (1999). Quantitative detection of Borrelia burgdorferi by real-time PCR. *Journal of Clinical Microbiology* **37**, 1958–63.
50. Mathews, S. A., Volp, K. M. & Timms, P. (1999). Development of a quantitative gene expression assay for Chlamydia trachomatis identified temporal expression of sigma factors. *FEBS Letters* **458**, 354–8.
51. Loeffler, J., Henke, N., Hebart, H., *et al.* (2000). Quantification of fungal DNA by using fluorescence resonance energy transfer and the light cycler system. *Journal of Clinical Microbiology* **38**, 586–90.
52. Schalasta, G., Eggers, M., Schmid, M., *et al.* (2000). Analysis of human cytomegalovirus DNA in urines of newborns and infants by means of a new ultrarapid real-time PCR-system [In Process Citation]. *Journal of Clinical Virology* **19**, 175–85.
53. Schaade, L., Kockelkorn, P., Ritter, K., *et al.* (2000). Detection of cytomegalovirus DNA in human specimens by LightCycler PCR [In Process Citation]. *Journal of Clinical Microbiology* **38**, 4006–9.
54. Schmittgen, T. D. & Zakrajsek, B. A. (2000). Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR [In Process Citation]. *Journal of Biochemical and Biophysical Methods* **46**, 69–81.