Research Report

Rapid Competitive PCR Using Melting Curve Analysis for DNA Quantification

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ABSTRACT

A rapid competitive PCR method was developed to quantify DNA on the LightCycler®. It rests on the quantitative information contained in the melting curves obtained after amplification in the presence of SYBR® Green I. Specific hybridization probes are not required. Heterologous internal standards sharing the same primer binding sites and having different melting temperatures to the natural PCR products were used as competitors. After a co-amplification of known amounts of the competitor with a DNA-containing sample, the target DNA can be quantified from the ratio of the melting peak areas of competitor and target products. The method was developed using 16S rDNA fragments from Streptococcus mutans *and* E. coli *and tested against existing PCR-based DNA quantification procedures.*

While kinetic analysis of real-time PCR is well established for the quantification of pure nucleic acids, competitive PCR on the LightCycler based on an internal standardization was found to represent a rapid and sensitive alternative DNA quantification method for analysis of complex biological samples that may contain PCR inhibitors.

INTRODUCTION

Real-time PCR instruments allow a detection of the produced dsDNA by measuring the fluorescence signal produced when intercalating dyes such as SYBR® Green I (Molecular Probes, Eugene, OR, USA) or sequence-specific labeled probes (17). For a quantification of initial template DNA, an externally standardized kinetic analysis based on the fluorescence threshold cycle number is established (8). However, as any other PCR method using external calibration, it rests essentially on the assumption of equal amplification efficiencies of sample and standards. Hence, its accuracy and even applicability depends critically on the purity of the template because the presence of PCR inhibitors that occurs frequently in biological samples may compromise results or even lead to wrong negatives. This problem is solved by internal standardization using housekeeping genes (6) or competitive PCR techniques that are characterized by a co-amplification of the target DNA and homologous (1–4) or heterologous internal standards (13,15) using only one pair of primers (9,16). A kinetic analysis of competitive real-time PCR would require the design and optimization of two different pairs of hybridization probes, one pair specific for the wild-type product and the other for the competitor, which are labeled by different fluorescent dyes, allowing a separate analysis of one reaction at two different wavelengths. The parallel application of more than one pair of hybridization probes requires a time-consuming optimization of PCR conditions, and several probes often have to be tested before reliable results are obtained. Such methods have not yet been published.

To take advantage of the rapid amplification process together with the fast and contamination-reducing, post-PCR analysis on the LightCycler® (Roche Molecular Biochemicals, Mannheim, Germany), in comparison to analysis on agarose (2), temperature gradient electrophoresis gels (5), or HPLC (3), we developed a competitive PCR method that uses the information contained in the melting curves obtained routinely after amplification by following the fluorescence while slowly heating up to 95°C. Although the basic concept of this approach has been mentioned briefly already by Ririe et al. (10), it has not yet been proved experimentally. In this paper, we show the applicability and limitations of a quantification of DNA with the LightCycler using competitive PCR.

MATERIALS AND METHODS

Preparation of Competitors for LightCycler PCR

A 224-bp DNA fragment of human muscle fructose-1,6-bisphosphatase cDNA (14) cloned in pGEM-T® (Promega, Madison, WI, USA) was used as starting material for the heterologous LightCycler competitors. For *Streptococcus mutans*, the binding sites of the forward primer Strmufo (5′-GGT-

Table 1. Application of Competitive PCR on the LightCycler on Experimental and Clinical samples in Comparison to Kinetically Analyzed Real-Time PCR and Conventional Competitive PCR on a Block Cycler

A. Quantification of *S. mutans* cells as an example for a species-specific competitive PCR on the LightCycler

Sample	*S. mutans* Cells/mL: Conventional Competitive PCR	Real-Time PCR (Kinetic Analysis)	LightCycler Competitive PCR
liquid culture 1	$1.0 \times 10^9 \pm 1.1 \times 10^8$	$1.4 \times 10^9 \pm 2.0 \times 10^8$	$8.0 \times 10^8 \pm 2.0 \times 10^8$
liquid culture 2	$1.0 \times 10^8 \pm 1.3 \times 10^7$	$9.0 \times 10^7 \pm 2.0 \times 10^7$	$1.3 \times 10^8 \pm 4.0 \times 10^7$
saliva sample 1	$1.2 \times 10^6 \pm 1.4 \times 10^5$	$4.2 \times 10^5 \pm 4.0 \times 10^5$	$1.0 \times 10^6 \pm 3.5 \times 10^5$
saliva sample 2	$2.3 \times 10^6 \pm 3.0 \times 10^5$	$2.8 \times 10^6 \pm 1.6 \times 10^6$	$1.7 \times 10^6 \pm 7.0 \times 10^5$
saliva sample 3	$2.2 \times 10^6 \pm 1.7 \times 10^5$	$2.4 \times 10^6 \pm 2.0 \times 10^6$	$1.4 \times 10^6 \pm 5.0 \times 10^5$

B. Quantification of total bacterial cells with competitive PCR on the LightCycler

Sample	*E. coli* Cells/mL; Stool Samples Cells/g: Conventional Competitive PCR	Real-Time PCR (Kinetic Analysis)	LightCycler Competitive PCR
culture TG 1	$6.0 \times 10^8 \pm 1.4 \times 10^8$	$6.1 \times 10^8 \pm 1.0 \times 10^8$	$9.2 \times 10^8 \pm 1.1 \times 10^8$
culture TG 2	$7.0 \times 10^8 \pm 2.2 \times 10^7$	$7.1 \times 10^8 \pm 3.0 \times 10^7$	$8.2 \times 10^8 \pm 1.8 \times 10^8$
culture of ×L-I	$5.8 \times 10^8 \pm 7.5 \times 10^7$	$2.3 \times 10^8 \pm 1.4 \times 10^8$	$2.4 \times 10^8 \pm 1.5 \times 10^8$
stool sample 1	$1.7 \times 10^{10} \pm 2{,}6 \times 10^9$	$1.75 \times 10^{10} \pm 9.0 \times 10^9$	$1.2 \times 10^{10} \pm 3.3 \times 10^9$
stool sample 2	$5.0 \times 10^9 \pm 1.4 \times 10^9$	$5.1 \times 10^9 \pm 2.0 \times 10^9$	$3.5 \times 10^9 \pm 2.0 \times 10^9$

The results represent the means and the SDs of five independent experiments.

CAGGAAAGTCTGGAGTAA-3′) and the reverse primer Strmure (5′-GCGTTAGCTCCGGCACTAAGCC-3′) were introduced on both sides of the 224-bp cDNA fragment. For the construction of the internal standard for the quantification of *E. coli* and total eubacteria, the binding sites of the foreword primer Eubactfo (5′-ACTACGTGCCAGCAGCC-3′) and the reverse primer Eubactre (5′-GGACTACCAGGGTATCTAATCC-3′) were introduced.

Cloning of 16S rDNA Fragments and Preparation of DNA

For the construction of plasmids containing bacterial 16S rDNA fragments a 282-bp product was obtained from cultured *S. mutans* using the primers Strmufo and Strmure and a 296-bp PCR product from *E. coli* with the primers Eubactfo and Eubactre. Amplification was performed in 50 µL with 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, preceded by denaturation for 7 min at 94°C, and followed by extension for 3 min at 72°C using 1.25 U AmpliTaq® DNA polymerase (Applied Biosystems, Weiterstadt, Germany). PCR products were ligated into the pGEM-T vector, and the resulting plasmids (pStrmu and pEubact) were controlled by DNA sequencing. Using the primers M13fo (5′-GTAAAACGACGGCCAGT-3′) and M13re (5′-AACAGCTATGACCATGA-3′) and the described plasmids as templates, respectively, competitor and target DNAs were amplified. The obtained DNAs were video-densitometrically quantified on an ethidium bromide-stained agarose gel using the phi 174 *Hae*III Marker (Stratagene, La Jolla, CA, USA), which contains a known amount of DNA.

The 16S rDNA fragments from the bacteria species *Actinobacillus actinomycetemcomitans, Eikenella corrodens, Porphyromonas gingivalis, Prevotella intermedia, S. mutans, Streptococcus sobrinus,* and *E. coli* were amplified using the primers Eubactfo and Eubactre and quantified on an agarose gel as described above. DNA from stool samples was prepared using the QIAamp® DNA Stool Mini Kit from (Qiagen, Valencia, CA, USA).

LightCycler PCR

PCR was performed on a LightCycler with 20 µL reaction mixture containing 2 µL LightCycler-DNA Master SYBR Green I (Roche Molecular Biochemicals), 6 mM $MgCl_2$, 2–4 µL DNA template, and 0.5 µM of both forward and reverse primers. For the quantification of *S. mutans*, Strmufo and Strmure were used while Eubactfo and Eubactre was utilized for quantification of eubacteria. For amplification, 45 cycles of 94°C for 0 s, 55°C for 10 s, and 72°C for 20 s (temperature transition of 20°C/s), preceded by denaturation at 94°C for 3 min, were used, and the fluorescence reading was taken at 72°C. The melting curve analysis was per-

formed with continuous fluorescence reading between 65°C and 95°C with a transition rate of 0.1°C/s. For the kinetic analysis, the second derivative maximum method was used. LightCycler competitive PCR was performed in the presence of target and competitor DNA. The polynomial calculation method included in the LightCycler software was used to calculate the melting peak areas from the raw fluorescence data obtained from melting of the PCR product.

Conventional Competitive PCR

Target and competitor DNA were co-amplified on a GeneAmp® PCR System 9600 (Applied Biosystems) in 50 µL reaction mixture with 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, preceded by denaturation for 7 min for 94°C, and followed by extension for 3 min at 72°C using 1.25 U AmpliTaq DNA polymerase.

RESULTS AND DISCUSSION

Melting curves are routinely obtained after PCR with the LightCycler to characterize the products by their melting temperature. The quantitative information about the PCR product that could be obtained by an integration of the melting curve peak is not used because it differs inevitably from capillary to capillary even if the same reaction conditions are applied. However, within one capillary, the proportion of the peak areas of separable peaks is expected to reflect the relative amounts of the respective products. To take advantage of this for competitive PCR, it is necessary that the melting temperatures of the products of the target and the competitor are sufficiently different to allow a reliable determination of the areas of the melting curve peaks formed by the two products.

This was experimentally tested for two bacteria-specific 16S rDNA-based reactions. One of them used primers specific for a defined bacteria species (*S. mutans*), and the other used a universal primer pair (Eubactfo and Eubactre) binding to highly conserved regions of bacterial 16S rDNA and was used to quantify cultured *E. coli* and total bacteria in biological samples.

The reliability of the quantification of competitive PCR based on melting curve analysis was controlled by a comparison of the results with those obtained by conventional competitive PCR methods developed earlier (11,12). The amplification and melting curve analysis of the PCR products from *S. mutans* and *E. coli* with the LightCycler showed a melting temperature of 89°C and 88°C, respectively. To obtain individually integrable melting curve peaks, the melting temperatures of target and competitor should differ by at least 3°C. In the plasmids used for the synthesis of the *S. mutans*- and eubacteria-specific competitors, this sequence is flanked by the respective primer binding sites. The amplification of competitor DNAs using these plasmids as template and the primer pairs Strmufo and Strmure or Eubactfo and Eubactre, respectively, with the LightCycler showed that the two competitors exhibited a melting temperature of approximately 85°C, which is 3°C–4°C lower than the melting temperature of the respective bacterial PCR products (Figure 1).

For studying competitive PCR on the LightCycler, calibration curves have been determined by titration and co-amplification of fixed amounts of target DNA with serial dilutions of competitor DNA on the LightCycler using SYBR Green I in the reaction mixture. The quantification of nucleic acids rests on the ratios of the peak areas obtained from the melting curves of the PCR products of target and competitor. The co-amplification of a defined amount of *S. mutans* 16S rDNA with serial dilutions of the *S. mutans*-specific competitor DNA in the LightCycler showed a linear relationship between the ratios of the peak areas of competitor and bacterial products and the initial amounts of the competitor template. As expected, if equimolar amounts of competitor and *S. mutans* 16S rDNA were co-amplified, the peaks of the products of the competitor and the bacterial DNA had nearly identical areas (Figure 1).

The co-amplifications of a known amount of 16S rDNA of *E. coli* and serial dilutions of eubacteria-specific competitor DNA in the presence of the universal primers showed a linear relationship between the ratios of the peak areas of competitor and bacterial amplificates and the initial amounts of competitor template (Figure 2A). However, if equimolar amounts of competi-

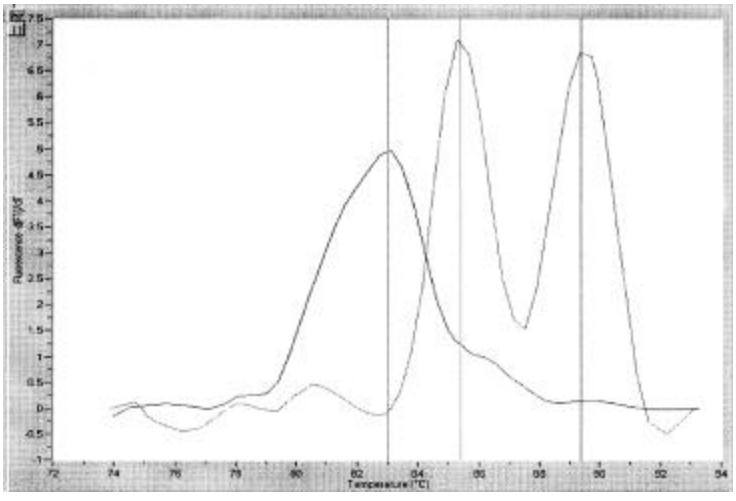

Figure 1. Melting peaks obtained after co-amplification of equal amounts of target and competitor. 10^4 molecules of *S. mutans*-specific competitor and bacterial DNA molecules, respectively, were co-amplified in the LightCycler. Melting peaks of target (A) and competitor (B) product. Primer dimers obtained in the negative control (C).

tor and *E. coli* 16S rDNA were co-amplified, then the peak of the bacterial product was much larger than that of the competitor product. To distinguish whether this was caused by different amplification efficiencies of bacterial DNA and the heterologous competitor or by the different fluorescence intensities of SYBR Green I integrated into bacterial and competitor DNA, the PCR products were analyzed on an ethidium bromide-stained agarose gel. The videodensitometric analysis of the visualized DNA fragments showed that the co-amplification of equivalent molar amounts of *E. coli* DNA and competitor template lead to equal amounts of amplification products (Figure 2B). This proved that the amplification efficiencies of the wild-type and the heterologous competitor are not significantly different and led to the conclusion that the differences of the areas of the melting curve peaks are related to differences of the specific fluorescence of the amplified DNAs in the presence of SYBR Green I. This result is in accordance to the finding of Ririe et al. (10) that the size and shape of melting curves obtained in the presence of SYBR Green I are a function of GC content, length, and sequence of PCR products.

To determine unknown target DNA concentrations with the competitive PCR on the LightCycler, it is necessary to characterize the relationship between the peak area ratio and the ratio of the initial amounts of competitor and target

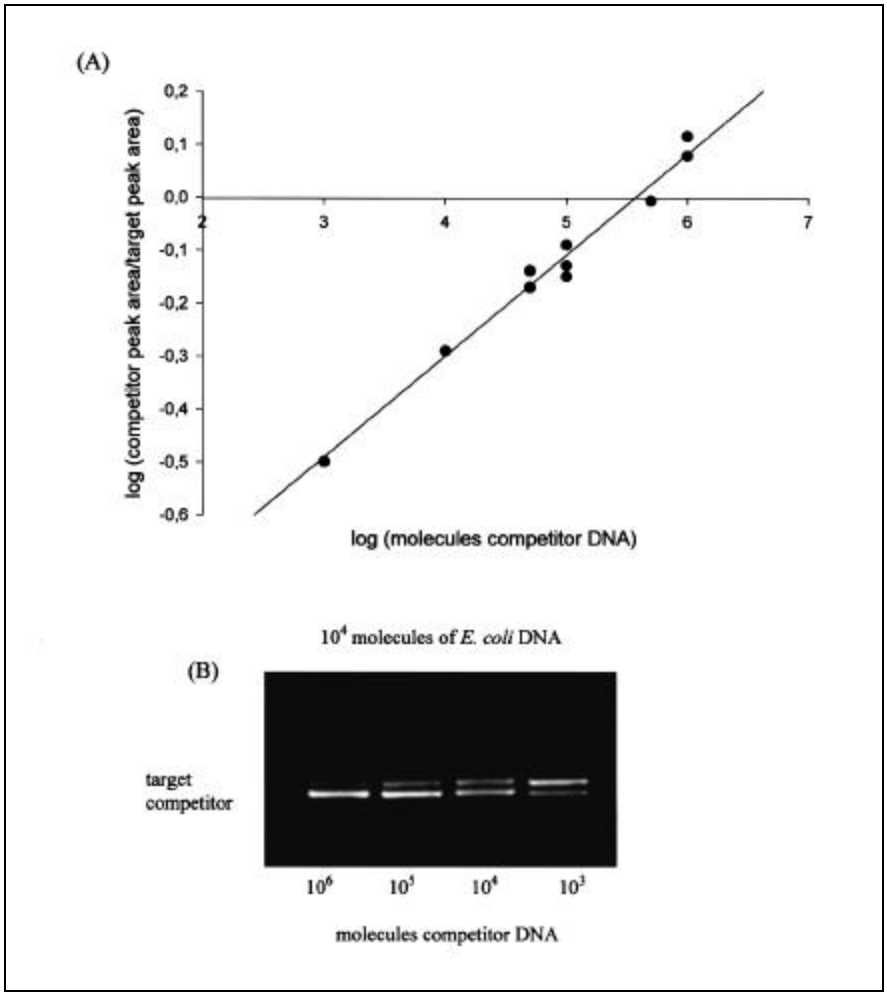

Figure 2. Competitive PCR on the LightCycler using *E. coli* 16S rDNA as target. 10^4 molecules of *E. coli* 16S rDNA were titrated with serial dilutions of competitor DNA and amplified with the LightCycler. (A) Plot of the experimental data obtained from the competitive PCR representing the logarithm of the peak area ratios of competitor and target product against the logarithm of the initial competitor DNA molecule number. (B) Analysis of the LightCycler competitive PCR products on a 1.5% agarose gel.

DNA over a wide range of target DNA concentrations. Corresponding results obtained for *S. mutans* and *E. coli* DNA are shown in Figure 3, A and B, respectively, for target DNA concentrations between 10^3 and 10^7 molecules/reaction. From the experimental results, calibration curves were obtained by a linear regression to the following equation:

molecules target DNA = 10^x, where x = log (molecules competitor DNA) - k - n log (competitor peak area/target peak area) [Eq. 1].

The developed method of LightCycler competitive PCR was applied to different biological samples, and the results were compared with those obtained by kinetically analyzed real-time PCR and conventional competitive PCR (12). The number of *S. mutans* cells was determined in two liquid cultures of this bacterial species and in three human saliva samples. While the two competitive PCR methods yielded similar results (Table 1), the kinetic analysis of the real-time PCR of the human saliva samples turned out to be compromised because the curves of fluorescence intensity versus cycle number were flat, not well S-shaped, and the calculated cell counts varied extremely from experiment to experiment. This was probably caused by the presence of PCR inhibitors in the saliva samples.

Also, *E. coli* cells were quantified in three liquid cultures containing the strains TG1, TG2, or XL-I using the universal primers (Table 1). Primers binding to conserved regions of the 16S rDNA have been proved as suitable tools for the quantification of total eubacteria by conventional competitive (11) and standard real-time PCR (7). The applicability of LightCycler competitive PCR using universal primers for the determination of total eubacteria amounts in mixtures of different bacterial strains was tested. For the characterization of the relationship between the peak area ratio and the ratio of the initial amounts of competitor and target DNA, defined amounts of 16S rDNA fragments from the bacterial strains *A. actinomycetemcomitans*, *E. corrodens*, *P. gingivalis*, *P. intermedia*, *S. mutans*, and *S. sobrinus* were used as target DNA. The obtained calibration curves were nearly identical to that obtained for *E. coli* (data not shown). Hence, the calibration curve shown in Figure 3B can be used for the quantification of eubacterial DNA. To prove this, a mixture of defined amounts of the 16S rDNA fragments of the bacteria mentioned above has been prepared containing a total amount of 1×10^5 molecules of DNA. By LightCycler competitive PCR, 1.2×10^5 molecules of DNA were obtained.

LightCycler competitive PCR for the quantification of eubacteria has also been successfully applied to DNA prepared from human stool samples. As shown in Table 1, the results obtained by conventional competitive PCR, real-time PCR, and LightCycler competitive PCR are comparable, which indicates that the purified DNA does not contain significant amounts of PCR inhibitors.

The results of this work prove LightCycler competitive PCR using melting curve analysis to be a rapid and reliable method for the quantification of DNA. It rests on information obtained from SYBR Green I fluorescence of the amplification product and does not require hybridization probes. Most important-

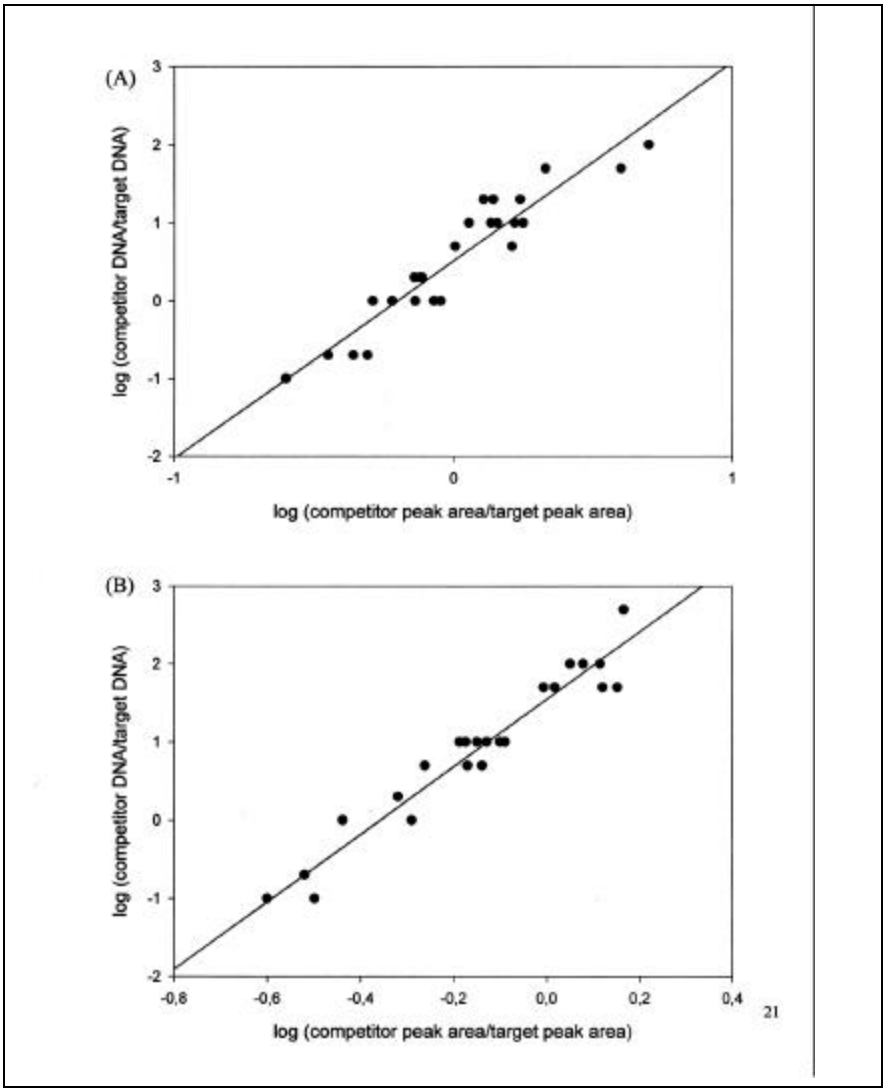

Figure 3. Calibration curves for competitive PCR on the LightCycler. Different known amounts of target DNA (between 10^3 and 10^7 molecules) were co-amplified with serial dilutions of competitor DNA in the LightCycler. The plot represents the logarithm of the ratio of the initial molecules of competitor and target DNA against the logarithm of the peak area ratios of competitor and target. (A) Calibration curve for *S. mutans*. By linear regression analysis, the parameters of Equation 1 were determined to be n = 2.6 and k = 0.55. (B) Calibration curve for *E. coli*. By linear regression analysis, the parameters of Equation 1 were determined to be n = 4.1 and k = 1.54.

ly, the developed method allows a reliable quantification of DNA in laboratory experiments and clinical studies in which biological samples often contain PCR inhibitors that critically interfere with the routine kinetic analysis on the LightCycler without internal standardization. This method takes advantage of the rapidity of the LightCycler and the accuracy of the competitive PCR using internal standards. Mean inter- and intra-serial coefficients of variation were 15%, 23%, and 34% for the determination of purified DNA, cells, and biological samples, respectively. This small scattering of the results proves the reproducibility of the competitive PCR on the LightCycler.

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NOTE ADDED IN PROOF

After the submission of this manuscript, a competitive PCR method using sequence-specific probes and melting curve analysis for the quantification of a human gene in breast cancer cells was published (Lyon, E., A. Millson, M.C. Lowery, R. Woods, and C.T. Wittwer. 2001. Quantification of HER2/neu gene amplification by competitive PCR using fluorescent melting curve analysis. Clin. Chem. *47*:844-851).

REFERENCES

1. **Becker-André, M. and K. Hahlbrock.** 1989. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). Nucleic Acids Res. *17*:9437-9446.
2. **Boer, P.H. and J. Ramamoorthy.** 1997. How to correct for errors in mRNA quantitation by competitive PCR due to heteroduplex formation of amplification products. Cell. Mol. Biol. *43*:841-850.
3. **de Kant, E., C.F. Rochlitz, and R. Herrmann.** 1994. Gene expression analysis by a competitive and differential PCR with antisense competitors. BioTechniques *17*:934-942.
4. **Gilliland, G., S. Perrin, K. Blanchard, and H.F. Bunn.** 1990. Analysis of cytokine mRNA and DNA: Detection and quantitation by competitive polymerase chain reaction. Proc. Natl. Acad. Sci. USA *87*:2725-2729.
5. **Henco, K. and M. Heibey.** 1990. Quantitative PCR: the determination of template copy numbers by temperature gradient gel electrophoresis (TGGE). Nucleic Acids Res. *18*:6733-6734.
6. **Kinoshita, T., J. Imamura, H. Nagai, and K. Shimotohno.** 1992. Quantification of gene expression over a wide range by the polymerase chain reaction. Anal. Biochem. *206*:231-235.
7. **Lyons, S.R., A.L. Griffen, and E.J. Leys.** 2000. Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. J. Clin. Microbiol. *38*:2362-2365.
8. **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.
9. **Nicoletti, A. and C. Sassy-Prigent.** 1996. An alternative quantitative polymerase chain reaction method. Anal. Biochem. *236*:229-241.
10. **Ririe, K.M., R.P. Rasmussen, and T. Wittwer.** 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal. Biochem. *245*:154-160.
11. **Rupf, S., K. Merte, and K. Eschrich.** 1999. Quantification of bacteria in oral samples by competitive polymerase chain reaction. J. Dent. Res. *78*:850-856.
12. **Rupf, S., K. Merte, and K. Eschrich.** 1999. Quantitative determination of *Streptococcus mutans* by using competitive polymerase chain reaction. Eur. J. Oral Sci. *107*:75-81.
13. **Siebert, P.D. and J.W. Larrick.** 1993. PCR MIMICS: competitive DNA fragments for use as internal standards in quantitative PCR. BioTechniques *14*:244-249.
14. **Tillmann, H. and K. Eschrich.** 1998. Isolation and characterization of an allelic cDNA for human muscle fructose-1,6-bisphosphatase. Gene *212*:295-304.
15. **Überla, K., C. Platzer, T. Diamantstein, and T. Blankenstein.** 1991. Generation of competitor DNA fragment for quantitative PCR. PCR Methods Appl. *1*:136-139.
16. **Wang, A., M.V. Doyle, and D.F. Mark.** 1989. Quantitation of mRNA by the polymerase chain reaction. Proc. Natl. Acad. Sci. USA *86*:9717-9721.
17. **Wittwer, C.T., M.G. Herrmann, A.A. Moss, and R.P. Rasmussen.** 1997. Continuous fluorescence monitoring of rapid-cycle DNA amplification. BioTechniques *22*:130-138.

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