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Quantification of mRNA expression by competitive PCR using non-homologous competitors containing a shifted restriction site

Franz Watzinger, Elfriede Hörth and Thomas Lion*

Children's Cancer Research Institute, St Anna Kinderspital, A-1090 Vienna, Austria

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ABSTRACT

Despite the recent introduction of real-time PCR methods, competitive PCR techniques continue to play an important role in nucleic acid quantification because of the significantly lower cost of equipment and consumables. Here we describe a shifted restriction-site competitive PCR (SRS-cPCR) assay based on a modified type of competitor. The competitor fragments are designed to contain a recognition site for a restriction endonuclease that is also present in the target sequence to be quantified, but in a different position. Upon completion of the PCR, the amplicons are digested in the same tube with a single restriction enzyme, without the need to purify PCR products. The generated competitor- and target-specific restriction fragments display different sizes, and can be readily separated by electrophoresis and quantified by image analysis. Suboptimal digestion affects competitor- and target-derived amplicons to the same extent, thus eliminating the problem of incorrect quantification as a result of incomplete digestion of PCR products. We have established optimized conditions for a panel of 20 common restriction endonucleases permitting efficient digestion in PCR buffer. It is possible, therefore, to find a suitable restriction site for competitive PCR in virtually any sequence of interest. The assay presented is inexpensive, widely applicable, and permits reliable and accurate quantification of nucleic acid targets.

INTRODUCTION

Competitive PCR is a powerful tool for accurate quantification of DNA or RNA. The procedure relies on the co-amplification of the sequence of interest with a serially diluted synthetic DNA fragment of known concentration (competitor) using a single set of primers (1,2). The initial quantity of target molecules in the sample can be calculated from the ratio of competitor- and target-derived amplicons generated during PCR, provided that the target and competitor sequences are amplified with equivalent efficiency (3). The quantity of target DNA (cDNA) can be most conveniently assessed at the so-called equivalence point (EQP), at which the target- and competitor-derived amplification products display the same signal intensity, indicating identical amounts of target and competitor at the beginning of the PCR reaction. To approach the EQP as closely as possible, several PCR reactions covering a range of competitor concentrations must be set up for quantification of individual targets, thus rendering the procedure rather laborious. In principle, it is feasible to use a small number of reactions with competitor concentrations covering the range of interest, and to quantify the target outside of the EQP. For quantification beyond the EQP it is essential that the initial target/competitor ratio is not affected by the amplification process.

As equal amplification efficiency of competitor and target sequences is a necessary prerequisite for quantitative PCR assays, competitors are usually designed to resemble the target sequence as closely as possible. Many investigators have used highly homologous competitors differing only by the presence or absence of a unique restriction enzyme site. The strategies commonly used to separate and quantitate the PCR products include cutting either target or competitor with a single restriction endonuclease (4–7) or digesting both target and competitor with two different enzymes (8,9). The latter strategy may result in false quantification if the restriction enzymes do not cut with equivalent efficiency. In the former approach, in which a restriction site is present in either the competitor or the target fragment, incomplete digestion leads to false quantification because undigested products co-migrate with the fragments lacking the restriction site. In both approaches, digestion-resistant heteroduplexes are generated during PCR (4,10–12) that co-migrate upon electrophoretic separation with the non-digested homoduplex products. This may lead to a change in the ratio between target and competitor fragments, resulting in wrong or inaccurate quantification. To prevent errors in quantification by competitive PCR assays, different measures were taken to minimize or eliminate the formation of heteroduplexes. Some investigators resolved heteroduplexes by adding fresh PCR components prior to the last amplification cycle (11,13), others introduced an additional denaturation–renaturation step after PCR, resulting in a binomial distribution of homo and heteroduplexes (14). Mathematical models have

*To whom correspondence should be addressed. Tel: +43 1 40470 489; Fax: +43 1 40470 430; Email: lion@ccri.univie.ac.at
Present address:
Elfriede Hörth, Clinic for Radiotherapy and Radiobiology, University of Vienna, A-1090 Vienna, Austria

been described to permit quantification outside the EQP despite the generation of heteroduplexes (12,15). However, particularly in situations in which target and competitor differ by a single nucleotide, theoretical predictions based on mathematical models may be problematic (16).

In order to avoid the pitfalls of heteroduplex formation, we have designed non-homologous competitors that display equal size and identical primer binding regions, but contain an internal nucleotide sequence, which is different from the target sequence. It was demonstrated that non-homologous competitor molecules with a size identical to the target fragment are amplified with the same efficiency and prevent the formation of heteroduplexes (11,17,18).

In this report, we present a novel modification of non-homologous competitor molecules permitting precise quantification outside the EQP, which require a single restriction endonuclease digest for distinction between target and competitor. The technique is presented using mRNA quantification of the human multidrug resistance gene *MDR1* as a model.

MATERIALS AND METHODS

Generation of DNA and RNA competitors

The non-homologous competitor was designed to contain *MDR1*-specific primer binding sequences of ~20 bp at the ends encompassing a bacterial sequence lacking homology to human DNA. It was generated by amplification of the bacterial cloning vector pBluescript (pBS) II KS$^+$ with hybrid primers. The 5′ ends of these primers contained an *MDR1*-specific sequence, and the 3′ ends were homologous to the pBS sequence: MDR*hyb* 22a/pBS S 5′-AGT TTG CAG GTA CCA TAC AGT TGC CTA ATG AGT GAG CTA AC-3′, MDR*hyb* 24a/pBS AS 5′-CTG TAG CTG TCA ATC AAA GGA TGT TCT TTC CTG CGT TAT C-3′ (pBS sequence location 899–918 and 1137–1155, respectively, according to GenBank accession no. X52331). The plasmid sequence was selected to contain a natural recognition site for the same restriction endonuclease as the target, but the position within the competitor molecule was shifted. PCR was performed in 10 mM Tris–HCl, 50 mM KCl, 2 mM $MgCl_2$, 200 µM of each dNTP, 0.1 µM of forward and reverse primers, 5 ng pBS template and 1.25 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) in a total volume of 50 µl. The amplification profile consisted of 35 cycles of 40 s at 95°C, 30 s at 54°C and 40 s at 72°C. The cycles were preceded by an initial denaturation step at 95°C for 3 min, and followed by a final extension step at 72°C for 7 min. The competitor fragment generated by PCR was cloned into the *Eco*RV site within the polylinker sequence of Bluescript phagemid according to the manufacturer's recommendations (USB, Editorial Comments Vol.19. No1. pp 4–6). Identity and orientation of the cloned insert were determined by DNA sequencing using T3/T7 primers.

For the synthesis of DNA competitors, the plasmid was cut with *Bam*HI and *Hin*dIII (Roche, Basel, Switzerland), the insert-containing fragments were purified, and the competitor concentration measured by spectrophotometry and by comparison with a DNA Mass Ladder (Life Technologies, Paisley, UK) upon electrophoresis in an agarose gel. Aliquots of the competitor fragments were stored as a stock solution at a final concentration of 10^9 molecules per 5 µl at –20°C.

For the synthesis of RNA competitors, the plasmid was linearized and transcribed *in vitro* using the MAXIscript kit (Ambion, Austin, TX). The full length transcripts were purified from prematurely terminated transcription products by separation on polyacrylamide gels (5% acrylamide, 8 M urea), eluted overnight at 45°C in DEPC-treated water, extracted with acid-equilibrated saturated phenol–chloroform (pH 4.5) (Amresco, Solon, OH), and precipitated with 2.5 vol of 100% ethanol. The concentration of the synthesized RNA was measured by spectrophotometry and stored as a stock solution at a final concentration of 10^9 molecules per 5 µl at –70°C.

cDNA synthesis and PCR template preparation

When using RNA competitors, a semi-logarithmic serial dilution of *MDR1* RNA competitor molecules was added covering a range of 3–4 logs, depending on the range of expression of the target transcript. Total cellular RNA (1 µg) was mixed with 1 mM of each of the dNTPs, 25 µM pd$(N)_6$, 4 µl of nuclease-free water and incubated at 72°C for 5 min. The mixture was placed on ice for 1 min before the addition of 4 µl reaction buffer [50 mM Tris–HCl (pH 8.3), 75 mM KCl, 5 mM $MgCl_2$], 10 mM DTT, 1 U/µl RNasin (Promega, Mannheim, Germany), and 5 U/µl Moloney murine leukemia virus reverse transcriptase (Life Technologies). The reaction was incubated at 37°C for 45 min and, finally, the enzymes were inactivated by heating at 98°C for 3 min.

When using DNA competitors, 1 µg of total RNA extracted from the cells of interest was converted to cDNA as described above. Two microliters of the reverse transcription reaction corresponding to 100 ng total RNA were added to a semi-logarithmic serial dilution of the DNA competitor stock solution.

Competitive PCR

The following primers were used for specific amplification of competitor and target: MDR 22a S 5′-AGT TTG CAG GTA CCA TAC AG-3′ (location 100–119, according to GenBank accession no. M29441) and MDR 24a AS 5′-CTG TAG CTG TCA ATC AAA GG-3′ (location 140–159, according to GenBank accession no. M29443). The PCR mixture and the amplification profile were as described in generation of DNA and RNA competitors. An example of *MDR1* mRNA expression analysis by competitive PCR is shown in Figure 1A.

To assess the kinetics of amplification, target and competitor cDNA fragments were mixed at different ratios and co-amplified in the presence of ^{32}P-end-labeled primer (10^6 c.p.m. per reaction) over 12–40 cycles. Aliquots of each sample were separated on a 2% agarose gel, the bands were excised and the radioactivity determined by liquid scintillation counting (Fig. 2).

Restriction endonuclease digestion

All products were designed to show small, yet clearly detectable differences in length following digestion. After the restriction digest, only products <300 bp with a difference in length between 15 and 30 bp were obtained.

The competitor- and target-derived *MDR1* amplicons were digested in the PCR buffer using the restriction enzyme *Pvu*II (Roche). To test the applicability of this approach to a broad spectrum of target sequences, we have investigated a number of restriction endonucleases for their ability to cut efficiently in

Table 1. Optimum conditions for digestion of unpurified PCR fragments in PCR reaction buffer

Enzyme	Recognition sequence	Units/time	Application	Manufacturer
*Acc*II	CG/CG	10 U/1 h	1× PCR buffer	Amersham Pharmacia Biotech (Uppsala, Sweden)
*Cfo*I	GCG/C	5 U/1 h	1× PCR buffer	Roche
*Csp*6I	G/TAC	5 U/1 h	1× PCR buffer	MBI Fermentas (Buffalo, NY)
*Hpa*II	C/CGG	5 U/1 h	1× PCR buffer	Roche
*Mae*I	C/TAG	10 U/1 h	0.5× PCR buffer	Roche
			0.5× Enzyme buffer	
*Msp*I	C/CGG	5 U/1 h	1× PCR buffer	Roche
*Mvn*I	CG/CG	5 U/1 h	0.5× PCR buffer	Roche
			0.5× Enzyme buffer	
*Nde*II	/GATC	10 U/2 h	1× PCR buffer	Roche
*Rsa*I	GT/AC	5 U/1 h	1× PCR buffer	Roche
*Sau*3A	/GATC	5 U/1 h	1× PCR buffer	Roche
*Apa*I	GGGCC/C	10 U/3 h	1× PCR buffer	Roche
*Bam*HI	G/GATCC	10 U/3 h	1× PCR buffer	Roche
*Bgl*II	A/GATCT	10 U/1 h	1× PCR buffer	Roche
*Eco*RI	G/AATTC	5 U/1 h	1× PCR buffer	Roche
*Hin*dIII	A/AGCTT	5 U/1 h	1× PCR buffer	Roche
*Kpn*I	GGTAC/C	5 U/1 h	1× PCR buffer	Roche
*Pst*I	CTGCA/G	10 U/1 h	1× PCR buffer	Roche
*Pvu*II	CAG/CTG	10 U/1 h	1× PCR buffer	Roche
*Sma*I	CCC/GGG	5 U/1 h	1× PCR buffer	Roche
*Xho*I	C/TCGAG	10 U/1 h	1× PCR buffer	Roche

The panel of endonucleases displayed includes 10 enzymes with hexanucleotide and 10 with tetranucleotide recognition sequences. The conditions indicated permit complete digestion of 1 µg PCR product at the appropriate temperature (as recommended for each restriction endonuclease by the respective manufacturer).

PCR buffer. PCR products of different target genes including the human multidrug resistance gene *MDR1*, the multidrug resistance associated protein (*MRP*), the gene for the pi form of glutathione *S*-transferase (*GST-pi*) and the thymidylate synthase gene (*TS*) were tested by a panel of restriction endonucleases. We have identified 20 different enzymes providing adequate results under the conditions indicated in Table 1. Most of the restriction endonucleases revealed excellent enzymatic activity in PCR buffer. Only two of the enzymes (*Mae*I and *Mvn*I; Roche) required the addition of 50% (v/v) specific enzyme buffer (supplied by the manufacturer) to the PCR reaction to permit efficient digestion of the amplicons.

Quantitative analysis

Twenty microliters of each PCR mixture were electrophoresed in 2% TBE agarose gels containing ethidium bromide. Gels were photographed and the EQP was determined using a Kodak Digital Science™ DC120 Zoom Digital Camera and the 1D Image Analysis Software (Kodak, Rochester, NY). To account for the fact that ethidium bromide signal intensity obtained under UV light is dependent on the size of the respective DNA fragment, it may be necessary to consider the length of the products when determining the EQP (19,20). Our system, however, did not require the application of a correction factor to compensate for the influence of size on signal intensity, because the effect on the results was negligible. As a result of the small differences in size between target and competitor, application of the correction factor, for example 1.27 for the 100 bp signal for comparison with the 127 bp band, and 1.16 for the 172 bp fragment for comparison with the 199 bp band (19,21), would only result in minor differences not exceeding the intrinsic variability of the methodology used (0.5 log).

Figure 1B shows the quantitation of MDR1 transcripts in a model test system containing a known amount of cloned target (1×10^6 per reaction), and a serial dilution of competitor molecules ranging from 10^4 to 3×10^7. The result of competitive analysis showed the EQP at the expected position, in the reaction containing 10^6 competitor molecules (Fig. 1, lane 5). In a parallel competitive PCR experiment (not shown), a control gene transcript of *b2-microglobulin* (β2-*MG*) was quantified using the identical amount of template cDNA from the same preparation. In accordance with other publications, the result of quantification was expressed as the ratio between the target and the control gene (22). For the purpose of *MDR1* monitoring, the data were expressed as the number of transcripts per 10^6 transcript molecules of the control gene.

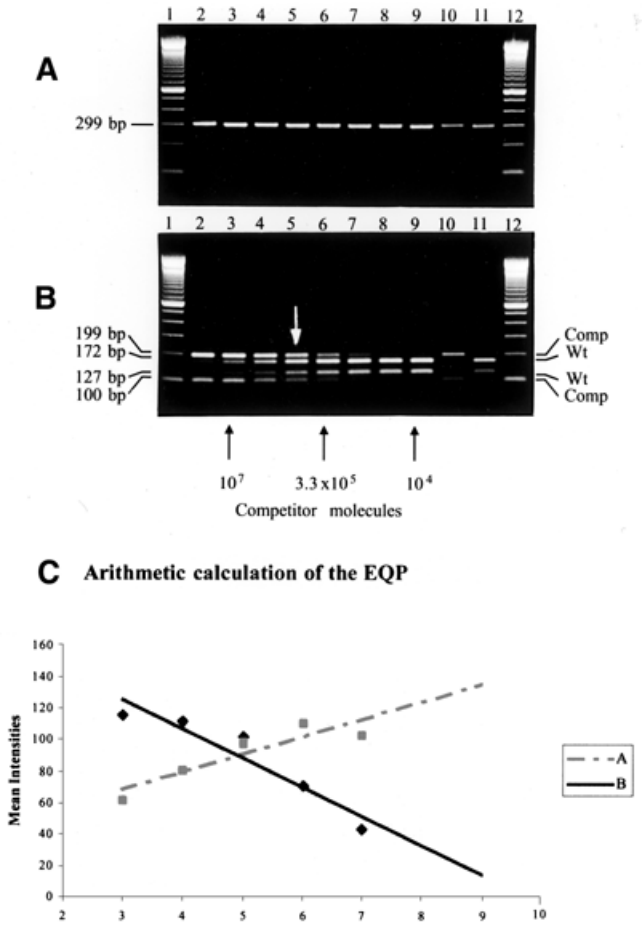

Figure 1. *MDR1* quantification using SRS-cPCR. Competitive PCR and restriction digest were carried out as described in Materials and Methods. Lanes 1 and 12 in each panel contain size marker (100 bp ladder). Lanes 10 and 11 represent amplification products of only competitor and target mRNA, respectively. (**A**) Twenty microliters PCR product was loaded on a 2% agarose gel. The target and the competitor products were identical in size (299 bp). Lanes 2–9 contain 10^6 molecules of an *in vitro* transcribed cloned human wild-type *MDR1* fragment and a semi-logarithmic dilution of the competitor RNA ranging from 10^4 to 3×10^7 molecules. (**B**) After digestion with the restriction endonuclease *Pvu*II, the wild-type PCR products were cleaved into 127 and 172 bp fragments, and the competitor PCR products into 100 and 199 bp fragments. The EQP is indicated by an arrow (lane 5). It was determined by comparison of signal intensities of the 100 and 127 bp fragments, or of the 172 and 199 bp fragments, respectively. The competitive amplification of an external control gene, β2-*MG*, permitting correction of target quantification (29) is not displayed. In competitive PCR reactions, the subdominant template yields visible products if it represents at least 1% of the total. Three competitor dilutions covering a range of 3 logs (lanes 3, 6 and 9, indicated by arrows) usually permit the visualization of target- and competitor-derived products in at least one of the reactions (lanes 3 and 6), thus permitting calculation of the target molecules. (**C**) The band intensities of competitor- and target-derived fragments were digitalized and measured by using the 1D Image Analysis Software. In the graph, the mean intensities from the larger competitor (line B) and the target (line A) fragments were used for the performance of a regression analysis. The EQP is indicated by the intersection of the two lines. The numbers on the abscissa correspond to the lane numbers on the agarose gel. Elimination of any data points between the extreme values has no effect on the EQP. This demonstrates that it is possible to reduce the number of competitor dilutions needed for quantitative analysis of target transcripts, thus rendering the assay less laborious.

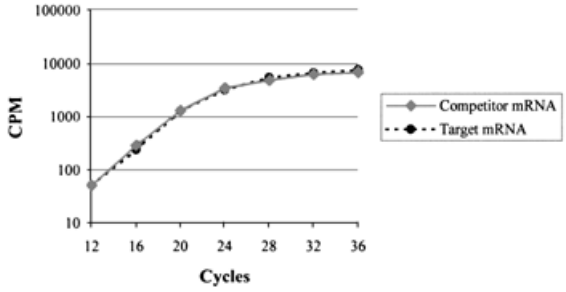

Figure 2. Amplification kinetics of endogenous human *MDR1* mRNA and competitor RNA in presence of [α-^{32}P]dCTP. Competitor (solid line) and target (broken line) mRNA were mixed at different ratios, and amplified for 12, 16, 20, 24, 28, 32, 36 and 40 cycles. The data are plotted as a function of c.p.m. versus cycle number. The amplification kinetics of mixtures were identical regardless of the target/competitor ratio. In the example displayed, the ratio was 1:1.

Amplification of β2-*MG* transcripts is a common approach to controlling factors such as RNA degradation and reverse transcription (RT) efficiency, permitting quantitative comparison between samples (23,24). When using constant starting amounts of RNA, highly consistent β2-*MG* signal intensities were observed indicating stable RNA quality and low variability of the RT step in our experimental setting. An additional indication of the low RT variability was provided by the analysis of multiple replicates of RNA samples, which revealed high reproducibility of the results.

RESULTS AND DISCUSSION

The competitive PCR approach presented is based on the use of a heterologous competitor, sharing only the primer binding sites with the target sequence. As a result of the low homology between the competing amplicons, this method has the advantage of avoiding the formation of heteroduplexes. This prevents incorrect quantification caused by co-migration of the heteroduplex product with the undigested fragments. Moreover, elimination of heteroduplex formation in competitive PCR assays permits the calculation of target copy numbers without the need to visualize the EQP, thus greatly reducing the number of PCR reactions per assay (Fig. 1C). The linear range of this competitive PCR approach is around 3 logs, for example, 1.5 logs above and below the EQP.

A key to successful and reliable competitive PCR is the identical amplification efficiency of competitor and target fragments. Under ideal conditions, the competitor/target ratio should remain constant throughout the amplification process. The kinetic analysis shown in Figure 2 revealed two identical curves within the exponential phase of PCR, indicating equivalent amplification efficiencies for both fragments co-amplified in the same reaction. The PCR kinetics was not affected by the lack of homology between the nucleotide sequences of the competing fragments. These observations were also made in a number of other quantitative PCR assays using the type of competitor described (not shown).

The use of either RNA or DNA competitors showed that both types permit measurement of changes in the relative amount of a specific target RNA with reasonable accuracy. DNA competitors are more convenient in terms of handling,

and are not prone to degradation during storage. A problem associated with the use of DNA competitors is underestimation of the concentration of mRNA target molecules. The error is most likely attributable to incomplete conversion of target RNA molecules to cDNA. This problem can be largely eliminated by using RNA competitors that are reverse transcribed in the same reaction as the target RNA. In our hands, however, the differences in quantitative results depending on the use of RNA or DNA competitors were not significant for a variety of target and competitor combinations including *MDR1*, *MRP* and *BCR/ABL* (data not shown). These observations indicate a low variability of the RT step under well-standardized experimental conditions. If the aim of the quantitative RT–PCR assay is calculation of the absolute number of target molecules, it is advantageous to use RNA competitors. If the assay is performed for detection of relative differences in the number of RNA molecules, for example, changes in RNA expression over time assessed by serial follow-up samples, the use of DNA competitors will be adequate, provided that the RT step has a low variability.

The only requirements for construction of the competitor type described herein are two hybrid primers. They permit generation of a competitor displaying the following features: (i) identical length to the target sequence, (ii) lack of homology to the target sequence, except of the primer binding sites and (iii) a recognition site for a restriction endonuclease which is present at a different position within the target sequence. The application of restriction endonucleases for discrimination between target- and competitor-derived PCR products is greatly facilitated by the finding that many common enzymes cut efficiently in PCR buffer (Table 1). This renders the entire procedure less time- and labor-intensive and permits selection of a suitable restriction endonuclease in virtually any sequence of interest, thus enabling quantitative analysis by the shifted restriction-site competitive PCR (SRS-cPCR) assay in any experimental settings.

The approach described has the advantage of being independent from the efficiency of the restriction enzymes used. Both target and competitor are digested concurrently in the same tube by the same enzyme, but at different positions. If incomplete digestion occurs, it affects both target and competitor to the same extent, and has no impact on the target/competitor ratio. Hence, the origin of molecules within the undigested band is not relevant for quantitative analysis.

In comparison with recently introduced real-time Q-PCR approaches (25–28), competitive PCR analysis is more laborious, but permits equally sensitive detection and precise target quantification at substantially lower cost. Competitive PCR approaches may, therefore, continue to play an important role in PCR-based quantification of DNA and RNA targets as long as the cost of RQ-PCR prevents many researchers from adopting this technique. The use of competitor molecules containing a shifted restriction site contributes to increased reliability and precision of competitive PCR assays.

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