Short Technical Reports

Calibration and Storage of DNA Competitors Used for Contamination-Protected Competitive PCR

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ABSTRACT

DNA fragments used as standards in competitive PCR were precisely calibrated using HPLC and commercially available DNA molecular mass markers. The accuracy of calibration was reflected by data that differed by only 2% from the mean when two independently purified and calibrated competitor preparations were compared. Highly dilute competitor solutions were stable at -20°C for up to 1 year in the presence of carrier Hind*III-digested l DNA, but progressive loss of competitor DNA with increasing storage time was observed when carrier DNA was omitted from the solution. Applying 0.2 U uracil-DNA glycosylase (UDG) per assay of remaining temperature-stable activity did not effect the ratios of synthesized products. This study describes quality management in PCR quantitation that is useful for the measurement of multidrug resistance-associated protein (MRP) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene transcripts.*

INTRODUCTION

Competitive polymerase chain reaction (PCR) is a quantitative adaption of the PCR method in which a known number of copies of a synthetic RNA (2,5,11) or DNA (4,6,9) is co-amplified with the target sample in the same reaction tube. Perhaps the most critical step in quantitative PCR analysis is the introduction of accurately calibrated and consistent amounts of exogenous standards to the PCR tubes. However, highly diluted competitor preparations, which are required widely, include the possibility of distinct nonspecific interactions between standard nucleic acids and the storage tube wall and possible elevated degradation. Both of these factors may strongly influence the quality of results. Therefore, most of the manufacturers and suppliers advise using only freshly prepared competitor solutions, although pipetting inaccuracies are more frequent.

Therefore, the aim of this work was to select optimal techniques for standard calibration and reliable conditions for storage of highly diluted competitors, thus preventing quantitation artifacts by unstable preparations. To adopt the method for clinical routine analysis, an optimized protocol is proposed to determine relative levels of mRNA coding for multidrug resistance-associated protein (MRP), which is associated in multidrug resistance (3) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene transcripts, which are frequently used for normalization of quantitative PCR results (9).

MATERIALS AND METHODS

Cell Lines

The high-level, multidrug-resistant human T-lymphoblastoid cell line CCRF ADR5000, drug-selected by adriamycin as described earlier (7), and human skin fibroblasts were used.

RNA Isolation and cDNA Synthesis

Total RNA was isolated using RNAzol® "B" (Tel-Test, Friendswood, TX, USA). cDNA was synthesized from 1-µg aliquots of total RNA in a 20-µL standard reaction mixture containing avian myeloblastosis virus (AMV) reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 50 mM $MgCl_2$, 50 mM dithiothreitol, 2.5 mM spermidine), 5 U AMV reverse transcriptase, 0.5 mM of each dNTP (Promega, Madison, WI, USA), 10 U recombinant RNase inhibitor (AGS, Heidelberg, Germany), and 200 ng oligo(dT) (Pharmacia Biotech, Uppsala, Sweden).

Primers and DNA Competitors

Primers were designed using the automated Oligo™ 5.0 Primer Analysis Software (National Biosciences, Plymouth, MN, USA) and available sequence information. A homologous competitor fragment developed for MRP quantitation was generated using a modified site-directed mutagenesis protocol described by Förster (6). Briefly, the competitor fragment for the chosen PCR product was generated by PCR amplification of cDNA from the MRP-expressing CCRF ADR5000 cells with the appropriate 5′ primer and a 3′ linker primer (LP) carrying the original 3′ primer sequence on its 5′ end. Primers used in this procedure were MRP3 (5′-GCTCGTCTTGTC-CTGTTTCT-3′, 5′ primer), MRP4 (5′-CTCCACCTCCTCATTCGCAT-3′, 3′ primer) and MRP-LP (5′-MRP4-CCTTCTTCCA GTTCTTTACC-3′). A purified 355-bp fragment of the pMS1 plasmid carrying a heterologous (multifunctional) competitor DNA sequence (9) was used for GAPDH quantitation using primers hGAPDH1 (5′-CGTCTTCACC ACCATGGAGA-3′) and hGAPDHrc (5′-CGGCCATCAC GCCACAGTTT-3′).

Standard Competitive PCR Assay

Aliquots of reverse-transcribed RNA were amplified in 50-µL standard PCR mixtures containing 2 µL of each 3′ and 5′ primer (10 pmol/µL), 5 µL 10× *Taq* DNA polymerase buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, 0.01% [wt/vol] gelatin, pH 8.3), 1.5 U AmpliTaq® DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) and 8 µL dNTPs (0.2 mM each, [Promega]) using dUTP (Boehringer Mannheim, Mannheim, Germany) instead of dTTP. Amplifications were started with an initial 15-min incubation at 37°C with 0.2 U uracil-DNA glycosylase (UDG; Boehringer Mannheim) added to each reaction tube to prevent carryover contamination. A 10-min denaturation at 94°C was performed followed by manual addition of AmpliTaq DNA Polymerase at 72°C. After an initial PCR cycle to allow second-strand cDNA synthesis, aliquots of a serial dilution of the competitor fragment (appropriate 2–5 µL vol) were added. MRP amplification was performed with 30 cycles (94°C for 30 s, 53°C for 30 s and 72°C for 1 min) and GAPDH was amplified for 20–40 cycles (94°C for 30 s, 58°C for 30 s and 72°C for 45 s) using a GeneAmp® 9600 Thermal Cycler (Perkin-Elmer).

Purification and Storage of Competitor DNA Fragments

PCR-amplified standard fragments were purified by electrophoresis through a 2% (wt/vol) agarose gel. Ethidium bromide-stained bands were cut out, and DNA was isolated by using a Sephaglas™ Band Prep Kit (Pharmacia Biotech). The purified fragments were diluted to a final concentration of about 10 ng/μL with sterile water, and competitor working dilutions were prepared with water or an aqueous solution of *Hin*dIII-digested λDNA (1–10 ng/μL; AGS, Heidelberg, Germany) used as a carrier for competitor stabilization. All solutions were stored at -20°C in 1.7-mL Multi Twist Top Vials (Sorenson Bio Science, Salt Lake City, UT, USA).

Calibration and Quantitation of DNA Fragments by HPLC

Aliquots of competitor stock solution and Low DNA Mass Ladder™ (Life Technologies, Gaithersburg, MD, USA) composed of a mixture of six blunt-ended DNA fragments ranging from 100–2000 bp, containing 5–100 ng DNA, respectively, were chromatographed over a TSK DEAE-NPR column protected by a DEAE-NPR guard column (TosoHaas GmbH, Stuttgart, Germany). An HPLC system (Jasco Labor und Datentechnik GmbH, Gross-Umstadt, Germany) was used that consisted of a pump, low-pressure gradient former, degasser, UV/VIS detector and an autosampler (capacity: 84-well plate, suitable for 0.2-mL MicroAmp® Reaction Tubes [Perkin-Elmer]). Twenty-microliter aliquots of each competitive PCR sample were applied to the column. For HPLC, a mobile phase of binary composition consisting of Buffer A (25 mM Tris-HCl, 1 M NaCl, pH 9.0) and Buffer B (25 mM Tris-HCl, pH 9.0) was used, and a discontinuous gradient program was used as described earlier (8). Data aquisition and integration were performed with NINA Chromato-Graphic-System Software (Nuclear Interface GmbH, Münster, Germany). Differences in UV absorbance resulting from the different lengths of standard and target DNA fragments were adjusted using a correction factor, determined as follows: a 10-point titration analysis was performed in duplicate with both original primers and a primer set, which was replaced for the unlabeled by a Cy5™-labeled 5′ primer. Non-fluorescent samples were analyzed by HPLC and Cy5-labeled DNA by electrophoresis through a ReadyMix gel using an ALFexpress™ Personal DNA Sequencer (Pharmacia Biotech). Data were processed with the AlleliX V2.00.25 Fragment Analysis Software (Pharmacia Biotech). The correction factors that match the ratio of titrated cDNA amounts obtained by ALF/HPLC analysis were 0.665 for MRP and 0.4 for GAPDH.

RESULTS AND DISCUSSION

Calibration of Competitor DNA Fragments

When competitor DNA fragments used for quantitation of several cDNAs were synthesized and purified as described, recovery was 1–4 μg DNA from 6 amplification reactions. In a representative experiment (Figure 1), a purified heterologous 355-bp multifunctional competitor DNA fragment used for GAPDH cDNA quantitation was subjected to HPLC analysis and calibrated by a reference curve generated from a parallel run of a DNA mass ladder aliquot.

To assess the reproducibility of the calibration method, two separate preparations of the competitor fragment were independently calibrated. Standard dilutions yielding final competitor concentrations of 0.4, 2 and 4 amol per assay were prepared, and GAPDH transcripts were quantified in duplicate in a uniformly used human skin fibroblast cDNA sample. Initial cDNA amounts were calculated from the absorbance ratio of target-to-standard-derived products (A_t/A_s) following correction for UV absorbance differences. The high reproducibility of the calibration procedure was reflected by the measured cDNA concentrations (11.0 ± 0.2 and 10.5 ± 0.9 amol GAPDH cDNA/μg reverse-transcribed RNA), which differed by only 2% from the mean value. Considering the many

steps required for standard fragment preparation, these differences were small. Therefore, we recommend the procedure over traditional UV-absorbance measurement (6,11) or calibration by densitometric means (4).

Effect of Carrier DNA on Competitor Preservation and Enzymatic Amplification

For these experiments, the homologous MRP standard DNA fragment was used, producing a fragment 88 bp shorter than the target. As shown earlier, the competitor allowed reliable quantitation during both exponential and plateau phases of amplification (9). Carrier *Hin*dIII-digested λDNA was added to the standard solutions to stabilize the DNA and to block nonspecific binding of DNA to the storage tube. As shown in Figure 2A, the ratio of template-to-competitor-derived products increased due to loss of competitor with increasing storage time when carrier DNA was omitted from the solution. A standard deviation (SD) of about 49% was obtained when competitor solutions were subjected to three freeze/thaw cycles and when they were stored for more than 3 weeks. Conversely, substantially improved preservation of highly dilute competitors was achieved when carrier DNA was added before freezing. This effect was more obvious when 10 ng instead of 1 ng carrier DNA per µL competitor solution (SD ± 34% vs. ± 18%) were added (Figure 2, B and C). Greater amounts of carrier DNA showed no further effect. SD values of about 20% are in the range of inter-assay variations observed for PCR quantitation of MRP transcripts (9). Different slopes of the individual curves are purported to be the result of competition artifacts, which may be observed when the concentration of either target or competitor species is in great excess (5).

To assess the integrity of highly diluted competitors following long-term storage in the presence of 10 ng/µL carrier DNA, MRP cDNA was quantified in a unique CCRF ADR5000 cDNA sample using freshly prepared competitor solution and a competitor dilution series that had been stored for 1 year at -20°C. cDNA concentrations were 16.8 and 16.3 zmol (i.e., 10^{-21} mol) per µg reverse-transcribed RNA (SD ± 1.5%), respectively, indicating competitor stabilization under storage conditions. The enhanced stability is rather the result of reduction in loss of DNA due to nonspecific binding to storage tubes than of degradation, e.g., by ambient UDG from bacterial infections. Therefore, the long-term storage of larger batches

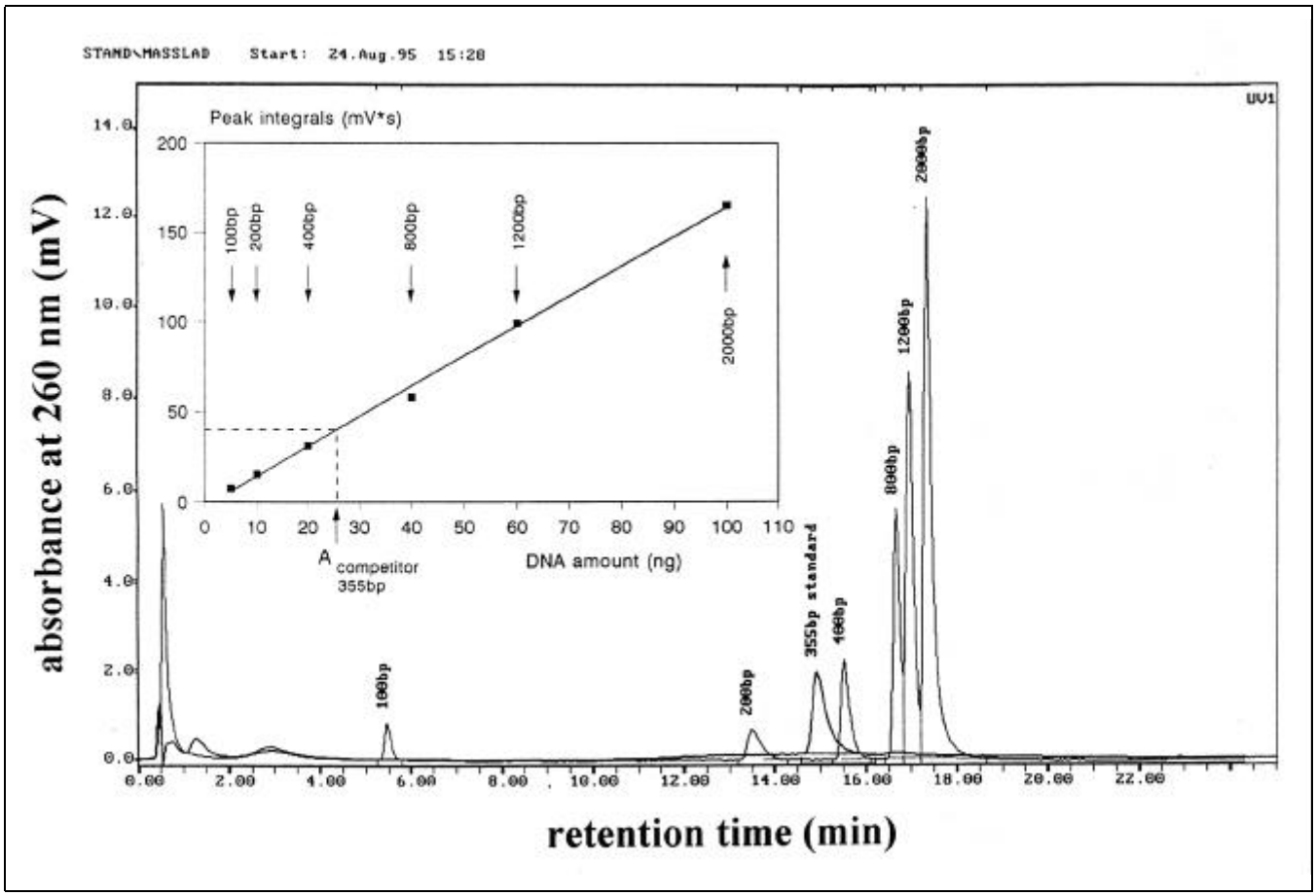

Figure 1. Competitor calibration by anion-exchange HPLC. Separation of a purified 355-bp multifunctional competitor DNA fragment and DNA mass ladder. Insert: calibration graph obtained from the peak integrals of the individual mass ladder components. The purified fragment was run in parallel and compared with the calibration curve to calculate the accurate competitor amount ($A_{competitor}$).

of highly diluted competitor DNA is possible. Consequently, the precision of transcript quantitation stretched over a long time period may be improved.

Experiments performed in such a way with stabilized competitors revealed another beneficial DNA side effect, which cannot be explained: the variability in amplification was diminished using heterologous standards, whose application is often limited by efficiency problems (9). As shown in Figure 3, for competitive amplification of GAPDH cDNA, the addition of 50 ng λDNA per assay with the standard formulation may be beneficial. A non-uniform accumulation of GAPDH products may be easily converted into a uniform one, resulting in a shift from a non-horizontal to a horizontal A_t/A_s curve.

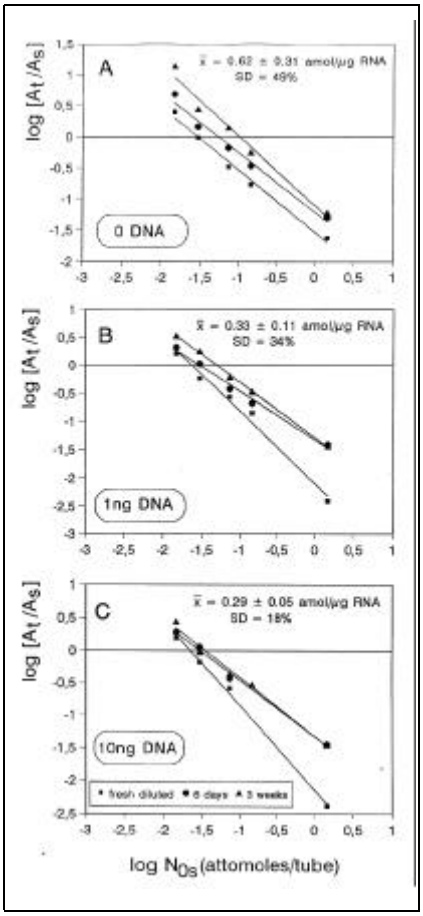

Figure 2. Effect of carrier DNA on stability of highly diluted competitor solutions. 100 ng of reverse-transcribed RNA from CCRF ADR5000 were co-amplified for 30 cycles with 4.1, 0.4, 0.2, 8.3×10^{-2} and 4.1×10^{-2} amol MRP competitor fragment, which was stored at -20°C up to 3 weeks. Target (340 bp) and competitor-derived (256 bp) amplification products were separated using HPLC. (A) Standard fragment without carrier DNA; competitor solutions containing 1 ng/µL (B) or 10 ng/µL λDNA (C). Values represent the mean ± SD of calculated initial MRP cDNA from the three respective curves.

Contamination-Free Standard Competitive PCR Assay

An optimized amplification procedure was adopted for quantitative PCR using calibrated and stabilized double-stranded DNA competitors. If such competitors are used to assay cDNA reverse-transcribed from mRNA in a sample, the amount of template will be theoretically underestimated by a factor of 2. This discrepancy can be overcome by applying single-stranded DNA as proposed by de Kant and coworkers (4) or, alternatively, by extension of thermal cycling by one cycle before adding the standards.

Conversely, strict avoidance of contamination is an essential feature for reproducible use of competitive PCR. Cross-contamination may be efficiently prevented by replacing the great majority of dT molecules with dU and by treating the PCR mixture with 0.2 U UDG before thermocycling. UDG has been shown to retain partial activity even after extensive incubation at 95°C and applying as little as 0.1 U of enzyme per assay (10). However, we observed negligible product degradation (<5%) following incubation of the sample at room temperature for as long as 12 h (i.e., throughout the period of automated HPLC analysis; data not shown). Therefore, effects of residual UDG activity on product ratios can be ruled out. But note that the precautions taken in this study require that dU-replaced competitors are added subsequent to UDG heat-inactivation.

In summary, we have described an optimized competitive PCR protocol. Although the approach described here is valuable in quantifying cDNA, the method does not address variability occuring during reverse transcription. Therefore, data normalization according to reference gene transcripts measured by the same procedure and expressed in the same sample, e.g., GAPDH cDNA, is required (9). To measure absolute cDNA amounts, the

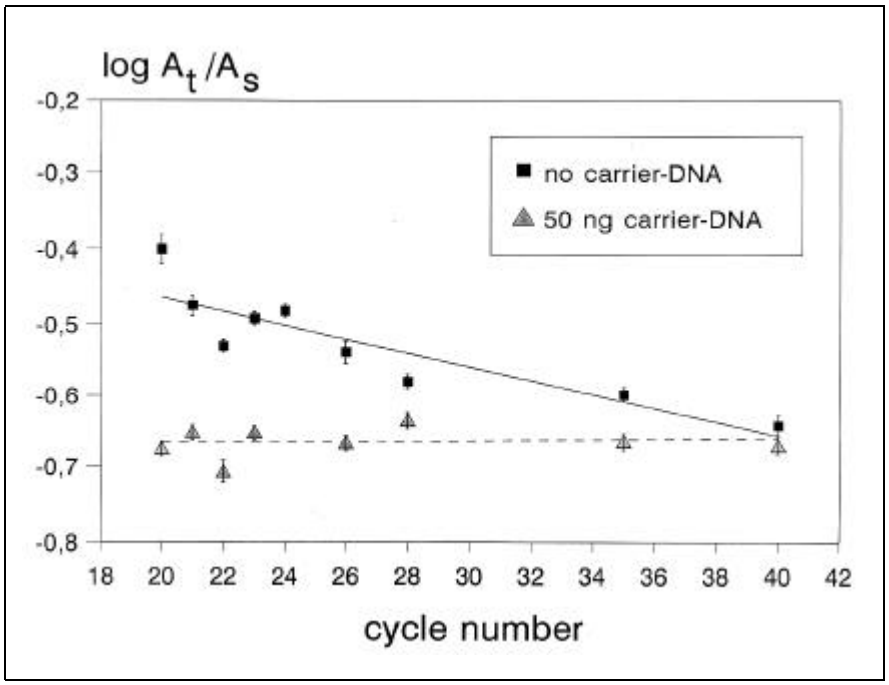

Figure 3. Effect of carrier DNA on amplification kinetics using a heterologous competitor DNA fragment. 50 ng reverse-transcribed RNA were co-amplified with 2.1 amol pMS1-derived competitor DNA fragment for the indicated number of cycles. Target (300 bp) and competitor (139 bp) products were analyzed by HPLC, data are expressed in terms of corrected A_t/A_s ratios. Continous line: no carrier DNA added. Dashed line: amplification in the presence of 50 ng λDNA DNA per tube. The vertical bars represent the SD of two separate experiments.

method is further limited by the requirement of a factor necessary to correct for UV absorbance differences with regard to different lengths of PCR products. This type of correction is unnecessary when using PCR enzyme-linked immunosorbent assay (ELISA) technology (1,9). The development of assays for simultaneous discrimination between homologous PCR products differing by at least one nucleotide is now in progress.

REFERENCES

1. **Alard, P., O. Lantz, M. Sabagh, C.F. Calvo, D.E. Weill, G. Chavanel, A. Senik and B. Charpentier.** 1993. A versatile ELISA-PCR assay for mRNA quantitation from a few cells. BioTechniques *15*:730-737.
2. **Becker-Andre, 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.
3. **Cole, S.P.C., G. Bhardway, J.H. Gerlach, J.E. Mackie, C.E. Grant and K.C. Almquist.** 1992. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science *258*:1650-1653.
4. **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.
5. **Duchmann, R., W. Strober and S.P James.** 1993. Quantitative measurement of human T-cell receptor Vβ subfamilies by reverse transcription-polymerase chain reaction using synthetic internal mRNA standards. DNA Cell Biol. *12*:217-225.
6. **Förster, E.** 1994. An improved general method to generate internal standards for competitive PCR. BioTechniques *16*:18-20.
7. **Gekeler, V., S. Weger and H. Probst.** 1990. MDR1/P-glycoprotein gene segments analyzed from various human leukemic cell lines exhibiting different multidrug resistance profiles. Biochem. Biophys. Res. Commun. *169*:796-802.
8. **Katz, E.D., W. Bloch and J. Wages.** 1992. HPLC quantitation and identification of DNA amplified by the polymerase chain reaction. Amplifications *8*:10-13.
9. **Köhler, T., D. Laßner, A.-K. Rost, B. Thamm, B. Pustowoit and H. Remke.** 1995. Quantitation of mRNA by Polymerase Chain Reaction - Nonradioactive PCR Methods. Springer-Verlag, Heidelberg.
10. **Mateo, R., E. Sico, L.F. Wang and J. Fung.** 1994. Uracil DNA glycosylase: minimizing residual enzyme activity after nested PCR. Focus *16*:92-94.
11. **Wang, A.M., M.V. Doyle and D.F. Mark.** 1989. Quantitation of mRNA by the polymerase chain reaction. Proc. Natl. Acad. Sci. USA *86*:9717-9721.

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