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Flow Cytometric Quantification of Competitive Reverse Transcription-PCR Products

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Background: **Competitive PCR of reverse transcribed mRNA sequences is used to quantify transcripts, but the usual approaches are labor-intensive and time-consuming. We describe the non-gel-based quantification of competitive reverse transcription (RT)-PCR products with use of microparticles and flow cytometry.**
Methods: **PCR products of a target sequence and an internal control sequence (competitor) were labeled during PCR using digoxigenin (DIG)- and dinitrophenol (DNP)-labeled primer, respectively, allowing specific binding to microparticles coated with the corresponding antibody. Both amplification products were biotinylated to enable fluorescence labeling with streptavidin-R-phycoerythrin. The mean fluorescence intensity of each microparticle population, corresponding to the amount of bound PCR product, was measured in a flow cytometer. We constructed microparticles coated with antibodies against DIG and DNP to specifically capture PCR products derived from target and competitor sequences, respectively.**
Results: **As required for a reliable competitive PCR assay, nearly identical kinetics were found for the amplification of target and competitor sequences when using only one competitive primer. The method was applied to examine interleukin-8 expression in human lymphocytes after x-irradiation. One hour after irradiation, the concentration of transcripts decreased by half.**
Conclusions: **The flow cytometric assay for the quantification of competitive RT-PCR products avoids additional hybridization steps and antibody labeling. The use of paramagnetic microparticles would also enable the complete automation of this method.**

A change in gene expression is often associated with the onset or progression of pathologic alterations. To date, identification of differentially expressed sequences and studies of their physiologic role in pathogenesis have revealed hundreds of novel marker genes for more precise clinical diagnosis *(1)*. Traditional techniques for analyzing gene expression changes, such as Northern blot hybridization, RNA in situ hybridization, and the RNase protection assay, are not useful for routine diagnostics. Gene expression microarray technology is useful for discovering new diagnostic and prognostic indicators rather than for analyzing a large number of clinical probes with respect to one or few disease-relevant factors.

In terms of speed and specificity, reverse transcription-PCR (RT-PCR)[1] is the method of choice for the clinical routine diagnosis of gene expression, in particular when the amount of sample material is limited. However, the high tube-to-tube variability requires the analysis of PCR products exclusively from the exponential phase of the amplification and/or the use of a coamplified competitor standard probe *(2)*. Comparative RT-PCR, which detects relative changes in the transcript amount related to a housekeeping gene, is commonly used but often suffers from the assumption that the reference point is stable *(3)*. In contrast, competitive quantitative RT-PCR has been shown to enable absolute quantification of transcript concentrations *(4, 5)*. In principle, the target sequence is coamplified with an internal standard (competitor), which is usually a homologous sequence comprising an insertion or deletion detectable, for example, by size (gel electrophoresis) or sequence (heterologous probe). The internal standard competes with the target sequence for primers and enzyme during the PCR, thus reducing the amplification signal of the target sequence when the internal standard is in excess. In contrast, as the amount of the internal standard decreases, the amplification signal of the target increases. However, one essential require-

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[1] Nonstandard abbreviations: RT-PCR, reverse transcription-PCR; DIG, digoxigenin; DNP, dinitrophenol; IL-8, interleukin 8; PBS, phosphate-buffered saline; and BrdU, 5-bromo-2-deoxyuridine.

ment for the availability of the internal control sequence is that its reverse transcription and amplification rates are nearly identical to those of the target. If these criteria are fulfilled, the unknown copy number of a target sequence can be reliably determined in a titration-like experiment using known amounts of a competitor.

Competitive RT-PCR has been used frequently for a broad range of applications, including the analysis of viral load *(6–9)*, gene expression changes *(4, 5, 10, 11)*, genomic alterations *(12)*, and leukemia *(13)*.

The most important requirement for a future molecular diagnostic assay will be automation. In particular, this means turning from gel-based procedures to automatable online systems. Such systems are the ELISA-based DIANA assay *(14)* or real-time PCR *(15)*. A powerful alternative is the flow cytometric assay for quantifying PCR products with use of paramagnetic microparticles *(16)*. In this assay, PCR products are labeled with both digoxigenin (DIG) and biotin during amplification by means of labeled oligonucleotides. This double labeling of amplification products enables the simultaneous binding to microparticles coated with anti-DIG antibodies and fluorescence labeling using the biotin-specific dye streptavidin-R-phycoerythrin. Finally, the mean fluorescence intensity per microparticle is determined by highly sensitive flow cytometric analysis. This assay allows the detection of very small amounts of PCR product (down to 0.4 fmol) in a linear range useful for reliable semiquantification of expressed gene sequences.

In this report, we describe a modified assay that enables the quantification of competitive PCR products. For this purpose, we used one competitive primer specific for both the target and competitor sequences. Leygue et al. *(17)* previously showed that the use of just one competing primer is sufficient for reliable quantification. To quantify target and competitor PCR products separately, amplification products are labeled during PCR with DIG and dinitrophenol (DNP), respectively. This enables the selective capture of PCR products by microparticles coated with anti-DIG or anti-DNP antibodies and their separate quantification after fluorescence labeling in the flow cytometer in parallel.

To test the feasibility of this assay we examined the expression of the interleukin-8 (IL-8) gene in x-irradiated human lymphocytes. IL-8 is a chemoattractant cytokine that induces rapid mobilization of progenitor cells and pluripotent stem cells in response to irradiation *(18)*.

Materials and Methods

x-irradiation

We irradiated 2 mL of human whole blood (EDTA) in a 5-cm Petri dish at room temperature with 100 kV of x-rays through a 1-mm beryllium window (Müller; 970 μm Al filter; 10 mA; E_{max}, 30–50 keV; for 6 Gy, 28.5 cm and 1.52 Gy/min). The absorbed dose was monitored using a calibrated dosimeter (Unidos).

RNA preparation

Irradiated and nonirradiated blood samples were incubated for 1 h at 37 °C in an atmosphere of 5% CO_2. Samples were then mixed with 2 mL of phosphate-buffered saline [PBS; 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L phosphate buffer (pH 7.4)] and carefully transferred to a 15-mL centrifugation tube containing 3 mL of lymphocyte separation medium (ICN). After centrifugation at 400*g* for 20 min, the buffy coat was removed and transferred into a fresh tube. The lymphocytes were washed twice by centrifugation with 15 mL of PBS at 400*g* for 10 min. The pellet was then ready for RNA preparation (RNeasy Mini Kit; QIAGEN) as recommended by the supplier. Putative DNA contaminants were removed by DNase I treatment (Ambion). The RNA content was determined by photometry, and the quality of the RNA was verified by standard gel electrophoresis.

construction of the plasmids pIC-1-ΔIL-8 and pBLUE-IL-8

To obtain a truncated IL-8 RNA standard (competitor), the vector pIC-1 was constructed. For this purpose, the adaptor for a poly(A) tail was generated by boiling 1 μg of each oligonucleotide (5′-CGC TTT TTT TTT TTT TTT TTT TTG CA-3′ and 5′-GGC CTG CAA AAA AAA AAA AAA AAA AAA GCG AGC T-3′) for 5 min and cooling the sample to room temperature. The vector pBLUE-polyA was constructed by cloning the adaptor into pBluescript SK+ (Stratagene) digested with *Not*I and *Sac*I. The adaptor for the universal primer binding site was generated as described above, using the oligonucleotides 5′-TCG AGC TTA CAA CGG TTG CTA TGT CGC GGG CCG CA-3′ and 5′-AGC TTG CGG CCC GCG ACA TAG CAA CCG TTG TAA GC-3′, and was cloned into pBLUE-polyA double digested with *Xho*I and *Hin*dIII. To confirm the cloning steps leading to the novel vector named pIC-1, cycle sequencing was performed using the M13 forward primer and the Sequi Therm EXEL™ Long-Term™ DNA Sequencing Kit (Biozym). The sequencing reaction was analyzed on a LiCor 4000 automated DNA sequencer (MWG Biotech). The vector pIC-1 enables the fusion of an A_{20} tail to a sequence of interest, its in vitro transcription, and RT-PCR of the artificial transcript using the DNP-labeled universal primer.

To generate the plasmid pIC-1-ΔIL-8, human lymphocyte total RNA was used as template for RT-PCR using the primers 5′-AAC TGC AGT GCA GAG GGT TGT GGA GAA GT-3′ and 5′-GCT CTA GAC AAC CCT ACA ACA GAC CCA CA-3′. The 153-bp amplification product and the vector pIC-1 were digested with *Xba*I and *Pst*I and subsequently gel-purified to remove small digestion products. Ligation and transformation procedures were carried out according to standard protocols.

pBLUE-IL-8 was constructed by cloning the 193-bp IL-8 RT-PCR product using the primers 5′-AAA ACT GCA GTG ATG GAA GAG AGC TCT GTC T-3′ and

5′-GCT CTA GAC AAC CCT ACA ACA GAC CCA CA-3′ into pBluescript SK+ digested with *Pst*I and *Xba*I.

IN VITRO TRANSCRIPTION

A linear fragment containing the T3 promoter, the universal primer binding site, the truncated IL-8 sequence, and the poly(A) tail was obtained by amplification of the plasmid pIC-1-ΔIL-8 using the primers M13 Forward and M13 Reverse. After gel purification (QIAquick gel extraction reagent set; QIAGEN), amplification product was added to the in vitro transcription mixture (Promega) as recommended by the supplier. The in vitro transcript was digested with DNase I as described above and quantified by photometry.

REVERSE TRANSCRIPTION AND PCR

Reverse transcription was performed using 100 ng of human lymphocyte RNA, 1 amol/L to 10 fmol/L of the in vitro transcript probe (competitor), both preheated at 65 °C for 5 min and placed on ice immediately, 50 pmol of oligo(dT)$_{18}$ primer, 200 U of OMNISCRIPT reverse transcriptase (QIAGEN), 40 units of rRNasin RNase inhibitor (Promega), and the deoxynucleotide triphosphates (500 μmol/L final concentration) in a total volume of 20 μL. Samples were incubated at 37 °C for 1 h and finally heated at 95 °C for 3 min. For PCR, 1 μL of the reverse transcription sample was used.

PCR was carried out in a 25-μL volume containing *Taq* DNA polymerase (1 U per 25-μL PCR; QIAGEN) mixed with 1 μL of Taq Start antibody (CLONTECH) as recommended by the supplier, 10 pmol of primer IL8s (5′-TGA TGG AAG AGA GCT CTG TCT-3′-DIG), 10 pmol of IL8as (5′-CAA CCC TAC AAC AGA CCC ACA-3′-biotin), 10 pmol of universal primer (5′-GCT TAC AAC GGT TGC TAT GTC-3′-DNP), and 0.4 μL of reverse transcription sample. After heat denaturation at 94 °C for 4 min, 35 cycles at 92 °C for 0.5 min, 60 °C for 0.5 min, and 72 °C for 1 min were carried out. Final polymerization was for 5 min at 72 °C. Amplifications were performed in a Mastercycler (Eppendorf).

In parallel to the flow cytometric analyses, PCR products were separated by gel electrophoresis on a 4% NuSieve gel (Biozym).

DOT-BLOT ASSAY

To determine the binding efficacy of different antibodies to PCR products labeled with the corresponding antigen, we performed a dot-blot assay. β-Actin cDNA was amplified as described previously *(16)*, using the sense primer labeled with DIG, DNP, or 5-bromo-2-deoxyuridine (BrdU; Eurogentec). After PCR purification (QIAGEN), 50 ng, 5 ng, 500 pg, 50 pg, and 5 pg of the PCR products were spotted onto a positively charged nylon membrane (Roche Diagnostics). Blots were incubated with mouse anti-DIG (Roche Diagnostics), rat anti-DNP (ZYMED Laboratories), and mouse anti-BrdU monoclonal antibody (Dako), respectively, as recommended by the suppliers. After a wash step, blots were treated with goat anti-mouse antibody (Sigma) and rabbit anti-rat antibody (ICN), respectively, both conjugated with alkaline phosphatase. Signals were obtained using colorimetric substrates (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; Roche Diagnostics) according to the instructions of the supplier.

COUPLING OF ANTIBODIES TO MICROPARTICLES

Rabbit anti-mouse IgG antibody (200 μg; Sigma) and rabbit anti-rat IgG antibody (200 μg; ICN), respectively, were covalently coupled to 50 μL of carboxylated paramagnetic 3.15-μm microparticles (Spherotech) with use of the Carbodiimide Kit (Polysciences). For binding the mouse anti-DIG and the rat anti-DNP antibodies, respectively, microparticles were mixed with the antibody in a 1:2 ratio and were incubated for 1 h at 4 °C. Antibody-coated microparticles were stored at 4 °C.

FLOW CYTOMETRIC ASSAY

Before capture of the PCR products, antibody-coated magnetic particles were washed twice in 500 μL of 3× binding buffer [15 mmol/L Tris (pH 7.5), 1.5 mmol/L EDTA, 150 mmol/L NaCl] in a magnetic holder (Promega). After washing, 1 μL of beads was incubated with 1 μL of a purified PCR product [purification as described previously *(16)*] and 1 μL of 90 g/L streptavidin-R-phycoerythrin (Sigma) by rotating at room temperature for 15 min. Finally, the beads were washed with 500 μL of PBS containing 0.4 mL/L Tween 20 and resuspended in 1.5 mL of Tris-EDTA.

Flow cytometric analysis of 10 000 microparticles/sample was performed using a PAS III flow cytometer (Partec). The excitation light was provided by a 25 mW argon laser operated at 488 nm. The orange emission was measured with a 575–605 nm bandpass filter (EM 590). The data were analyzed using FloMax instrument software. In particular, only the fluorescence intensity of monosized microparticles was quantified after gating of the corresponding scatter signals.

Results

In principle, the feasibility and speed of flow cytometric quantification of single PCR products has been shown previously *(16)*. The analysis of competitive PCR products, however, requires the separate quantification of two different amplification products derived from target and competitor sequences. In contrast to gel-based procedures, flow cytometric detection is unable to differentiate between sizes of PCR products, but it can simultaneously quantify the amounts of several PCR products after their fluorescent labeling and physical separation, e.g., by microparticles. Therefore, each PCR product has to contain a certain chemical label that allows specific capturing of amplicons using microparticles coated with antibodies against this label. In addition to DIG, we tested two other chemical labels that can be covalently bound to PCR

primer: DNP and BrdU. Different amounts of β-actin PCR products labeled with DIG, DNP, and BrdU, respectively, were dotted onto a nylon membrane. The blots were incubated independently with antibodies against one of the three labels. After incubation with a secondary antibody labeled with alkaline phosphatase, each blot was subjected to a colorimetric assay. High specificity with no cross-reaction was observed for each antibody (data not shown). This demonstrated that all three labels are appropriate in principle for flow cytometric analysis of labeled PCR products.

To determine the binding efficiency of the antibodies after coupling to microparticles, defined amounts of biotinylated PCR products additionally labeled with DIG, DNP, or BrdU were captured using microparticles coated with the antibody against the corresponding label. After fluorescent labeling of the immobilized PCR products with streptavidin-R-phycoerythrin, the fluorescence intensity of the microparticles was determined by flow cytometry. Microparticles coated with antibodies against DIG and DNP showed comparable capacities to bind labeled PCR products, whereas anti-BrdU microparticles had lower efficiency (Fig. 1).

Rather than generating internal deletion or insertion variants of the target sequence, which are usually used as competitors for standard competitive PCR assays, we cloned a 43-bp shortened fragment of the IL-8 cDNA sequence into a novel vector (pIC-1) designed to generate competitor sequences. In contrast to standard procedures, this competitor shared only one primer binding site with the target sequence (downstream), whereas the second target-specific primer sequence (upstream) was replaced by a universal primer site. Thus, only the primer binding both template sequences served as the competitive element during the PCR reaction (Fig. 2A). After a PCR of that kind, we obtained DIG- and biotin-labeled amplification products from the target sequence as well as DNP-

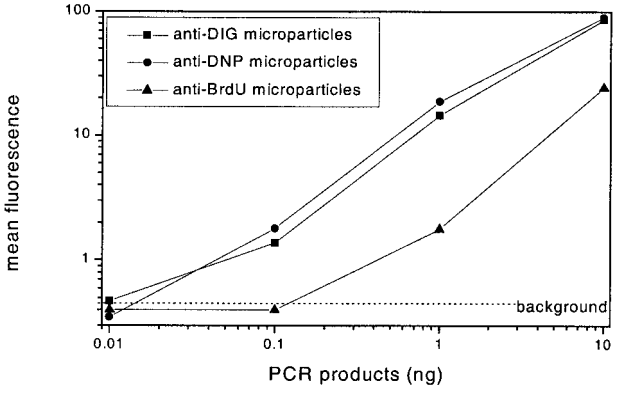

Fig. 1. Efficiencies of antibody-coated microparticles in capturing PCR products.

Fixed amounts of PCR products labeled with DIG (■), DNP (●), and BrdU (▲) were incubated with microparticles coupled with an antibody against one of these labels. After fluorescent labeling of the captured products with streptavidin-R-phycoerythrin, the mean fluorescence of 10 000 microparticles was determined by flow cytometry.

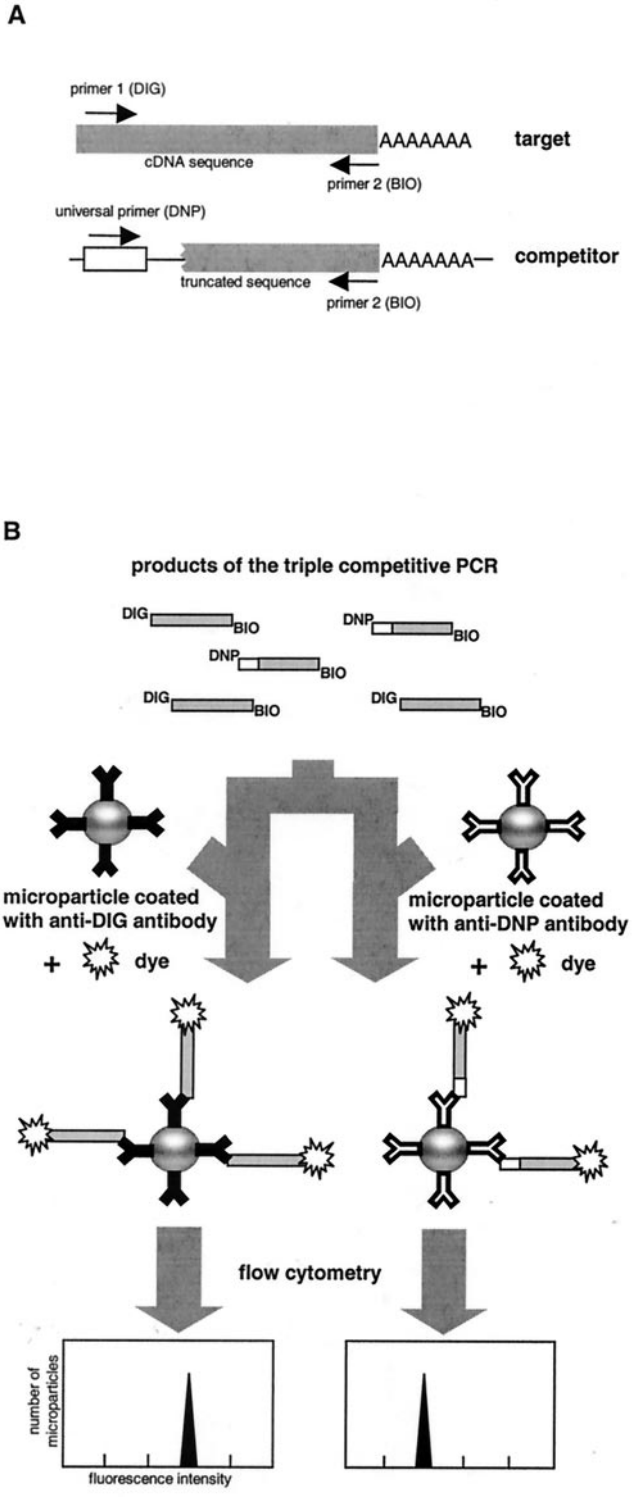

Fig. 2. Schematics showing the target and competitor sequences used for the flow cytometric assay (*A*) and the assay itself (*B*).

(*A*), target and competitor sequences share a downstream region including the binding site for biotinylated (*BIO*) primer 2. In the upstream region of the sequences, the binding site of the target-specific primer 1 labeled with DIG was replaced by a binding site for a DNP-labeled universal primer in the competitor sequence. (*B*), PCR products were immobilized using microparticles coated with anti-DIG and anti-DNP antibodies, respectively. Simultaneously, the bound PCR products were both fluorescently labeled with streptavidin-R-phycoerythrin. Flow cytometry was used to quantify the fluorescent signals per microparticle.

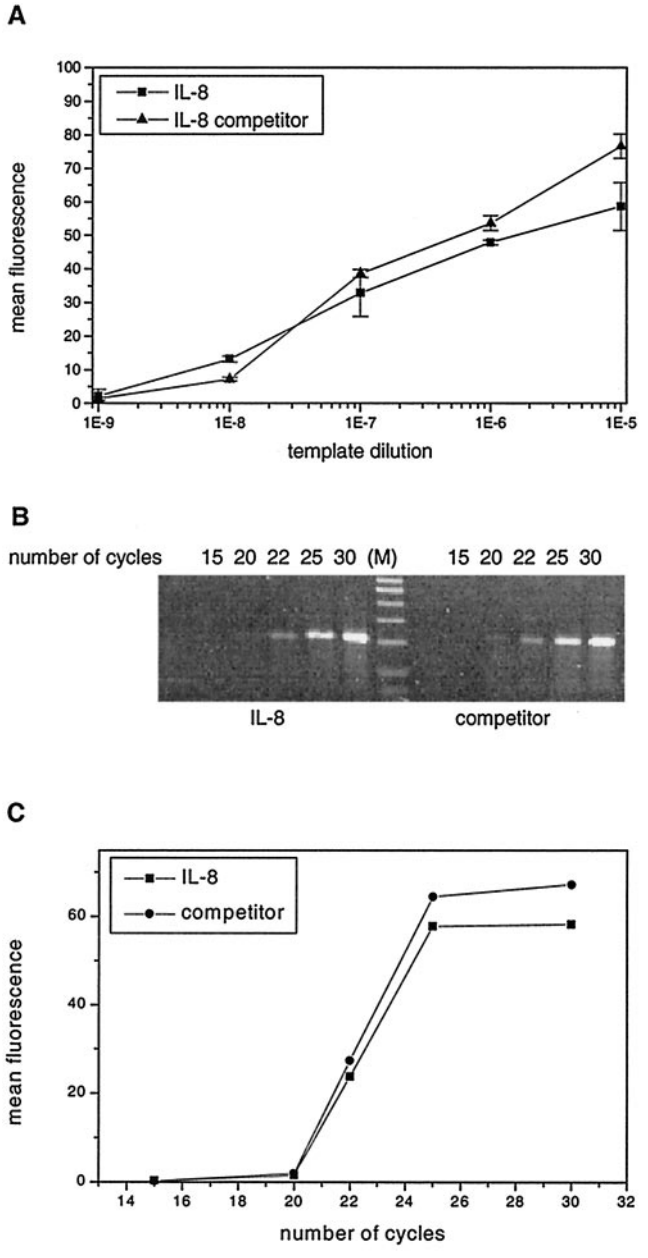

Fig. 3. Kinetics of target and competitor amplification.

(*A*), different dilutions of plasmids containing IL-8 and IL-8 competitor cDNA, respectively, were used for PCR. Amplification products were immobilized on microparticles coated with the corresponding antibody. After fluorescent labeling, samples were measured in a flow cytometer. *Error bars*, SD. PCR products isolated after 15, 20, 22, 25, and 30 cycles were also separated by gel electrophoresis and stained with ethidium bromide (*B*). The same amplification products were captured by microparticles coated with anti-DIG and anti-DNP antibodies, respectively. Immobilized products were fluorescently labeled and quantified in the flow cytometer (*C*). *M* indicates the molecular size marker.

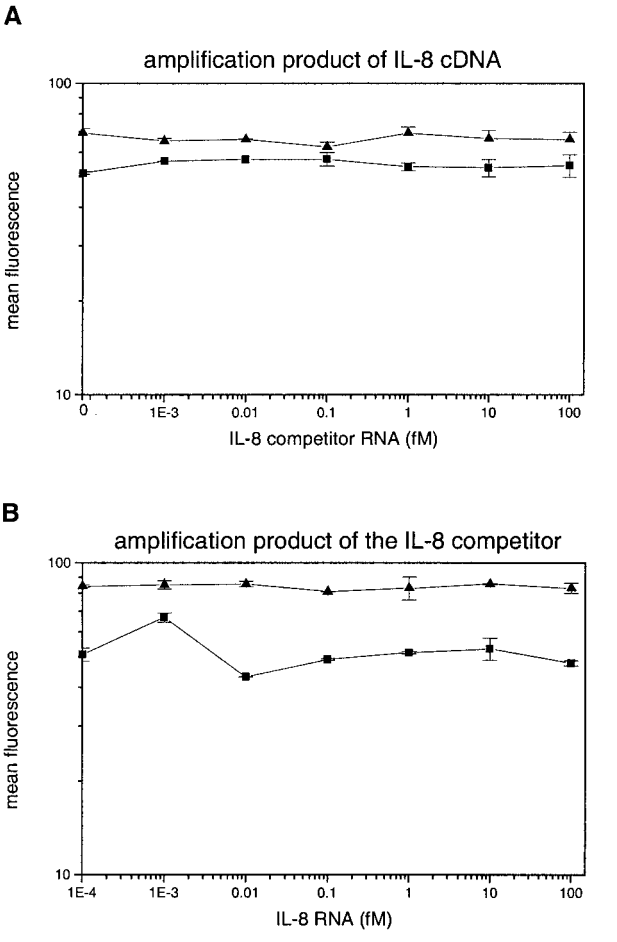

Fig. 4. Analysis of competitive template effects.

To assess the competition between the two templates during PCR, a constant amount of IL-8 cDNA (target) was coamplified in duplicate with increasing amounts of an IL-8 competitor-containing plasmid and captured by anti-DIG microparticles (*A*). Products after 31 (■) and 35 (▲) amplification cycles were used. In parallel, constant amounts of IL-8 competitor were coamplified in duplicate with increasing amounts of IL-8 cDNA and captured by anti-DNP microparticles (*B*). After labeling, microparticles were analyzed in duplicate in the flow cytometer for the fluorescence intensity. No competition was observed in either case over a seven-log dynamic range. *Error bars*, SD.

and biotin-labeled amplification products from the competitor sequence (Fig. 2B). To quantify the amount of each amplification product, the PCR sample was analyzed in two separate steps. Microparticles coated with anti-DIG antibodies were used to examine the target-specific amplification product, and microparticles coated with anti-DNP antibody were used to examine the competitor-specific amplification product. Simultaneously with immobilization on the microparticles, the amplification products were fluorescently labeled with streptavidin-R-phycoerythrin and were subsequently quantified by flow cytometry.

To ensure comparable amplification kinetics of the target and the competitor, we selected primers with an identical base content and products with similar lengths (competitor, 207 bp; target, 193 bp). As shown in Fig. 3, the amplification kinetics of the target (pBLUE-IL-8) and competitor sequences (pIC-1-ΔIL-8) were comparable. Almost identical plots in the exponential phase of the amplification were obtained with different dilutions of the template over four-log ranges (Fig. 3A) as well as after different PCR cycle numbers using a fixed amount of template (Fig. 3, B and C). Moreover, the presence of the competing sequence in the PCR reaction did not influence

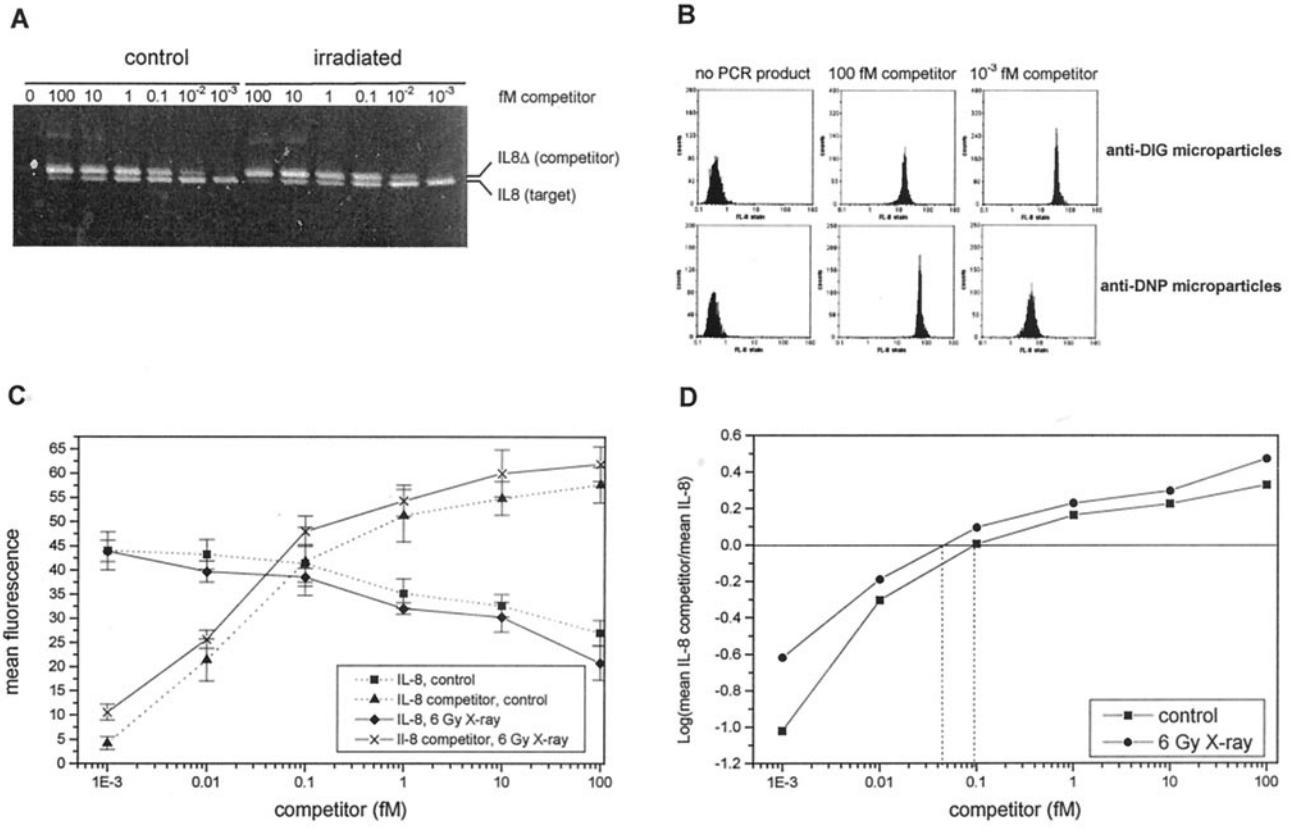

Fig. 5. Competitive RT-PCR quantification of IL-8 expression in human lymphocytes before and after x-irradiation.

Various amounts of IL-8 competitor RNA were mixed with a constant amount of total RNA isolated from unirradiated and irradiated (6 Gy) human lymphocytes, respectively. After reverse transcription, PCR was performed in duplicate using two IL-8- and one competitor-specific primer. PCR products were separated on a 4% NuSieve agarose gel and stained with ethidium bromide (*A*). In parallel, each PCR product was immobilized for the flow cytometric assay by capturing with anti-DIG and anti-DNP microparticles, respectively. After labeling, the fluorescence intensity per microparticle was determined by flow cytometry (*B*). The mean fluorescence of PCR products derived from IL-8 and IL-8 competitor was plotted against the amount of competitor RNA added (*C*). To compare the quantification of IL-8 expression in unirradiated and irradiated cells, the log ratios of the mean fluorescence derived from IL-8 competitor and IL-8 target product were plotted against the initial amount of IL-8 RNA competitor added to reverse transcription mixture (*D*). The equivalence point of each probe is defined as the value where the curve intersects the *zero line* (represented by the *vertical lines*). The *dashed lines below* indicate the resulting amounts of IL-8 transcript. *Error bars*, SD. fM, fmol/L.

the amplification rate of the target and competitor, respectively, as shown for template amounts varying over a six-log dynamic range at two different cycle numbers (Fig. 4).

The described flow cytometric assay was used to examine the influence of ionizing radiation (6 Gy; 100 kV x-ray) on the expression of the cytokine IL-8 in human lymphocytes. For this purpose, fresh whole blood was irradiated in vitro and cultured for 1 h. To analyze expression of the gene in irradiated and unirradiated blood cells, total RNA was isolated from each sample. Because the reverse transcription step is known to be the source of most of the variability in quantitative RT-PCR, we used a RNA competitor containing the truncated IL-8 sequence in fusion with a poly$(A)_{20}$ tail generated by in vitro transcription. Before reverse transcription, cellular and competitor RNA was treated with DNase I to remove putative genomic and plasmid DNA contamination. DNase I-treated competitor RNA was additionally examined for remaining DNA content by PCR using plasmid-specific primers. No amplification product was observed in the dilutions subsequently used for competitive RT-PCR (data not shown). A fixed amount (100 ng) of total RNA was added to a set of different amounts of competitor RNA (1 amol/L to 10 fmol/L) and was reverse transcribed using an oligo$(dT)_{18}$ primer. For each mixture, two PCR reactions were performed using the IL-8-specific DIG- and biotin-labeled (competitive) IL-8 primers as well as the competitor-specific universal primer (DNP labeled). The quality of the products was evaluated by conventional gel electrophoresis (Fig. 5A). In parallel, amplification products were each incubated with anti-DIG and anti-DNP microparticles, respectively, fluorescently labeled, and analyzed in duplicate by flow cytometry (Fig. 5B). Because the fluorescence intensity of amplification setups containing no template DNA (negative control) was insignificantly higher than that of the pure beads, primer-dimer formation can be excluded. The fluorescence intensity of each microparticle population was graphed against the amount of added competitor RNA (Fig. 5C). The obtained SD was 3–20% for single probes (10% on average). The curves calculated from the

two titration experiments have the expected course: saturation at high template concentration and linearity in the range where the curves cross (equivalence points).

To analyze the relationship of the two amplification products in each sample, calibration curves were constructed by plotting the ratio of the mean fluorescence intensities of captured amplification products derived from competitor and captured amplification products derived from the target against the amount of the competitor RNA present in the sample (Fig. 5D). The IL-8 mRNA content thus determined in unirradiated lymphocytes was twice as high (90 amol/L per 100 ng of total RNA) as in irradiated cells (45 amol/L per 100 ng of total RNA). The competitive assay was linear in the range of 10 amol/L to 1 fmol/L, including the equivalence point (r^2 = 0.969 for irradiated cells and 0.982 for unirradiated cells).

Discussion

Over the past 10 years, the development and application of molecular techniques have initiated a revolution in clinical diagnostics. The availability of user-friendly diagnostic reagent sets, especially for gene amplification techniques, and robotic automation has enabled the rapid, sensitive, and economic routine clinical analysis of even small amounts of disease-related factors. However, despite its frequently demonstrated reliability, the competitive PCR assay has not been inserted into the clinical routine. Classic techniques described for the quantification of competitive PCR products, such as gel electrophoresis combined with densitometry *(5)*, are barely automatable. However, the great potential of this technique obviously lies in the automatic analysis of separated PCR products *(19)*. For this purpose, various assays based on microtiter plates have been considered, including immobilization of PCR products followed by hybridization using labeled target- and competitor-specific probes, e.g., ELISA *(20)* or scintillation proximity assay *(8)*; automated separation and quantification by HPLC *(21)*; capillary zone electrophoresis *(22)*; or separate quantification of competitive amplification products by real-time PCR *(15, 23)*. The major advantage of real-time PCR over other techniques is the online quantification of PCR products without the need for post-PCR treatment. This, however, requires identical amplification kinetics for target and competitor but different hybridization kinetics or labels for the fluorescent probes needed to distinguish between the two sequences.

The flow cytometric quantification of competitive PCR products described here fulfills the requirements of future diagnostic technology. In contrast to conventional competitive RT-PCR methods, we used only one competing primer. Because three primers consequently had to be used instead of two to amplify target and competitor, it was necessary to examine more precisely the amplification kinetics of both templates. Neither differences in the PCR kinetics analyzed at the exponential and plateau phases nor inhibitory effects of one of the templates were identified. Although the competition was weaker than in conventional methods, leading to a lower slope for the calibration curve, small differences in expression were reliably detected, as demonstrated. An additional criterion for the usefulness of this method was the binding efficiency of the microparticles. Although the binding capacities of the anti-DIG and anti-DNP microparticles were comparable and therefore suitable for multiple quantification, the efficiency of the anti-DNP microparticles was slightly higher (~5%) in all experiments. To overcome this limitation, the amounts of antibodies coupled to the microparticles could be adjusted or the values obtained for the anti-DIG microparticles could be multiplied by a correction factor. However, essential differences from our results for IL-8 expression are not likely.

These results are in total agreement with data from a semiquantitative RT-PCR study of IL-8 expression in x-irradiated human lymphocytes (E. Pascher, E. Severin, and W. Göhde, unpublished results); in that study, in contrast to the increased expression shown by other probands, IL-8 expression was decreased in the proband examined. Moreover, this proband showed an unusually high antioxidative capacity. As postulated by Remick and Villarete *(24)*, the expression of IL-8 correlates directly with the intercellular balance of antioxidants and reactive oxygen intermediates arising after ionizing radiation. Furthermore, high concentrations of antioxidative proteins, such as superoxide dismutases, before treatment mediate a significant decrease in induction of cytokine mRNA by radiation *(25)*.

In the present study, we aimed to develop an automatable assay for the quantification of competitive RT-PCR products appropriate for analysis on a large scale. For this purpose, we combined two automatable technologies already successfully applied in clinical routine diagnostics: paramagnetic microparticles and flow cytometry. Microparticles containing a ferruginous core or surface can be handled on a large scale by robotic devices using magnetic separation. Examples include high-throughput DNA isolation and purification *(26, 27)*. Because we used paramagnetic microparticles in our assay, fully automated quantification of competitive PCR products, as described here, could be performed using appropriate robotic devices. In addition to classic applications, such as isolation and purification, the diagnostic possibilities of paramagnetic microparticles have recently been reported *(28)*. Various molecular applications of clinical relevance, e.g., detection of viral sequences *(29)*, minisequencing of single-nucleotide polymorphisms *(30)*, and real-time measurement of enzyme activity *(31)*, have been adapted for microparticles. All of these applications used flow cytometry as the final detection system.

Flow cytometry is a highly sensitive detection technology for analyzing the size, shape, and fluorescent signals of cells or particles at different wavelengths in parallel. Recently it was shown that flow cytometers coupled with automated sample-handling devices can process 9–10

samples/min from 96-well microplates *(32)*. To optimize the present method for high-throughput applications, both anti-DIG and anti-DNP microparticles should be measured in one step. For this purpose, microparticles with an individual fluorescent label different from the detection signal could be used. In this way, microparticle populations can be reliably distinguished by flow cytometry, as described by Fulton et al. *(33)*, who showed that up to 64 different microparticle populations are measurable independently by use of this technique.

The quantification of multiplex PCR products described here is not limited to the competitive PCR assay. Other PCR assays requiring coamplification of an internal control, such as the detection of infective microorganisms, can also be taken into consideration.

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