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# Quantification of Cytokine mRNA Expression by RT-PCR and Electrochemiluminescence

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**Variable gene expression constitutes a major mechanism for controlling cell development and cell function. To investigate these changed mRNA levels, a sensitive and quantitative assay is required. We describe a quick and easy method to quantify specific mRNAs by a combination of PCR and an electrochemiluminescent (ECL) detection of the amplified products. Total cellular RNA is reverse-transcribed and amplified with a biotinylated forward primer and a Tris (2,2'-bipyridine) ruthenium (II) (TBR)-labeled reverse primer. The amplification product is captured on streptavidin-coated paramagnetic beads and quantified by ECL detection using the QPCR system 5000. The results can be converted to quantitative values with an external standard curve. This method permits accurate and reliable quantitation of cytokine mRNA expression.**

**T**he quantitative measurement of specific mRNA species is of major importance to approach many fundamental questions in biology. Therefore, various strategies have been developed for quantitation of cDNA by PCR-based methods,<sup>(1-4)</sup> most of them utilizing the principle of competitive PCR (C-PCR) in which a synthetic cDNA fragment is co-amplified with the target cDNA segment.

We were interested in comparing the expression of cytokine genes in numerous samples of antigen-specific T-cell clones. C-PCR is not suitable for this purpose because quantitative analysis of C-PCR reactions is tedious, with a large number of tubes per sample and because of separation of the two products by electrophoretic techniques or high performance liquid chromatography (HPLC), time-consuming Southern blot analysis, the use of radioactive isotopes, and tedious digitalization of the results. Therefore, we looked for a quantitative PCR method that is simple and fast and one in which the results could be digitalized easily—to provide numeric data—so that many samples can be processed at the same time.

Recently, DiCesare and co-workers<sup>(5)</sup> described an electrochemiluminescent (ECL)-based detection method for the quantitation of PCR-amplified DNA. This method involves the labeling of the amplified product by an oligonucleotide primer or probe coupled to Tris (2,2'-bipyridine) ruthenium (II) (TBR) chelate. TBR emits light at 620 nm in an electrochemical cell under appropriate redox

conditions, allowing for direct and specific quantitation of DNA sequences.

Here, we describe a protocol for directly quantitating PCR product formation by electrochemiluminescence and how these data can be used to determine the starting template concentration. In the present test, a single amplification is sufficient; therefore, several samples can be analyzed at the same time. No separation is required to quantify the amplified products. Although we used this method to quantify cytokine mRNA expression in long-term antigen-specific T-cell clones, it can be applied to any mRNA of interest.

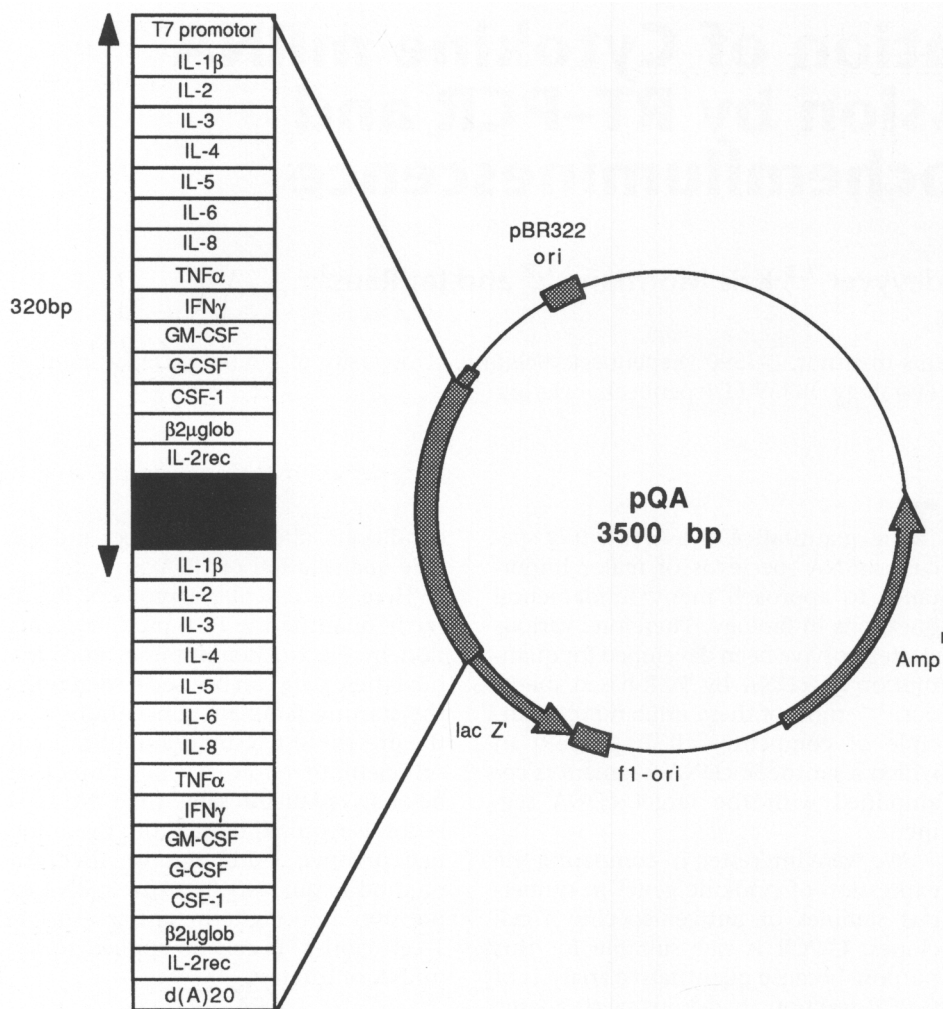
## MATERIALS AND METHODS

### Preparation of mRNA Cytokine Standards

The PCR standard RNAs (st-RNAs) were obtained from two nonhomologous, cloned synthetic DNA constructs, pQA and pQB, comprising the cytokine primer pairs (kindly provided by SANOFI Elf Bio Recherches, Labège, France)<sup>(6)</sup> (Fig. 1).

The structures pQA and pQB were linearized with *EcoRI*, and 2  $\mu$ g was transcribed into RNA using the T7 polymerase transcription kit (Boehringer Mannheim) followed by an RNase-free DNase treatment to remove the DNA template. The obtained standard RNAs (st-RNA<sub>A</sub> and st-RNA<sub>B</sub>, respectively) were extracted with water-saturated phenol-chloroform (24:1; vol/vol) and precipitated with ethanol. Poly(A) RNA

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**FIGURE 1** mRNA cytokine standards. The structures pQA and pQB (not shown, similar to pQA but containing IL-10-specific primers) were linearized with *EcoRI*, and 2  $\mu$ g was reverse-transcribed into RNA using the T7 polymerase transcription kit (Boehringer Mannheim) followed by an RNase-free DNase treatment to remove the DNA template. st-RNA<sub>A</sub> and st-RNA<sub>B</sub>, respectively, were extracted with water-saturated phenol–chloroform (24:1; vol/vol) and precipitated with ethanol. Poly(A) RNA was purified using the mRNA separator kit (Clontech, Palo Alto, CA) and quantitated by absorbance at 260 nm. The mRNA standards were used as an internal standard in the competitive PCR assay and as an external standard in the QPCR system 5000.

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### RNA Extraction and Reverse Transcription

T cells grown in suspension<sup>(7)</sup> were sedimented by centrifugation (1200 rpm, 10 min, 4°C) and washed twice with ice-cold PBS prior to RNA extraction. Total RNA was extracted using the RNeasy B method (Qiagen, Crawley, UK) method (Biotecx Laboratories, Inc., Houston, TX).<sup>(8)</sup> Total RNA (9.8  $\mu$ l, 1  $\mu$ g)

or st-RNA<sub>A</sub> or st-RNA<sub>B</sub> (100 ng) was reverse-transcribed into first-strand cDNA using the Reverse Transcription System of Promega (Leiden, The Netherlands). cDNA priming was performed in the presence of oligo(dT). The cDNA was extracted and precipitated, and the pellet was dissolved in 50  $\mu$ l of TE buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA) and used as such in the amplification reactions.

### RT-PCR

One microliter of cDNA was added to the following amplification mixture: 5  $\mu$ l of 10 $\times$  *Taq* polymerase buffer (100 mM Tris-HCl at pH 8.4, 100 mM KCl, 15 mM MgCl<sub>2</sub>, 0.1% gelatin), 2.5  $\mu$ l of 10 mM

dNTP mix, 0.25  $\mu$ l of *Taq* polymerase (5 U/ $\mu$ l) (Ampli $Taq$  DNA Polymerase, Perkin-Elmer), 5 pmoles of sense primer (5'), and 5 pmoles of antisense primer (3') specific for one of the cytokines,<sup>(6)</sup> in a total volume of 50  $\mu$ l. To use the primers in the QPCR system 5000 (Perkin-Elmer, Zaventem, Belgium), we labeled the PCR antisense primers at the 5' end with TBR and the PCR sense primers with biotin using modified phosphoramidites (Applied Biosystems) during oligosynthesis. Primers specific for  $\beta$ 2-microglobulin<sup>(6)</sup> served as a positive control of the PCR and the integrity of the cDNA. The amplification profile used was 20 sec at 95°C, for denaturation; 20 sec at 55°C, for annealing; 40 sec at 72°C, for extension in 28–35 cycles (GeneAmp 9600, Perkin-Elmer). Thereafter, samples were incubated for an additional 7 min at 72°C, to ensure complete extension of all PCR products.

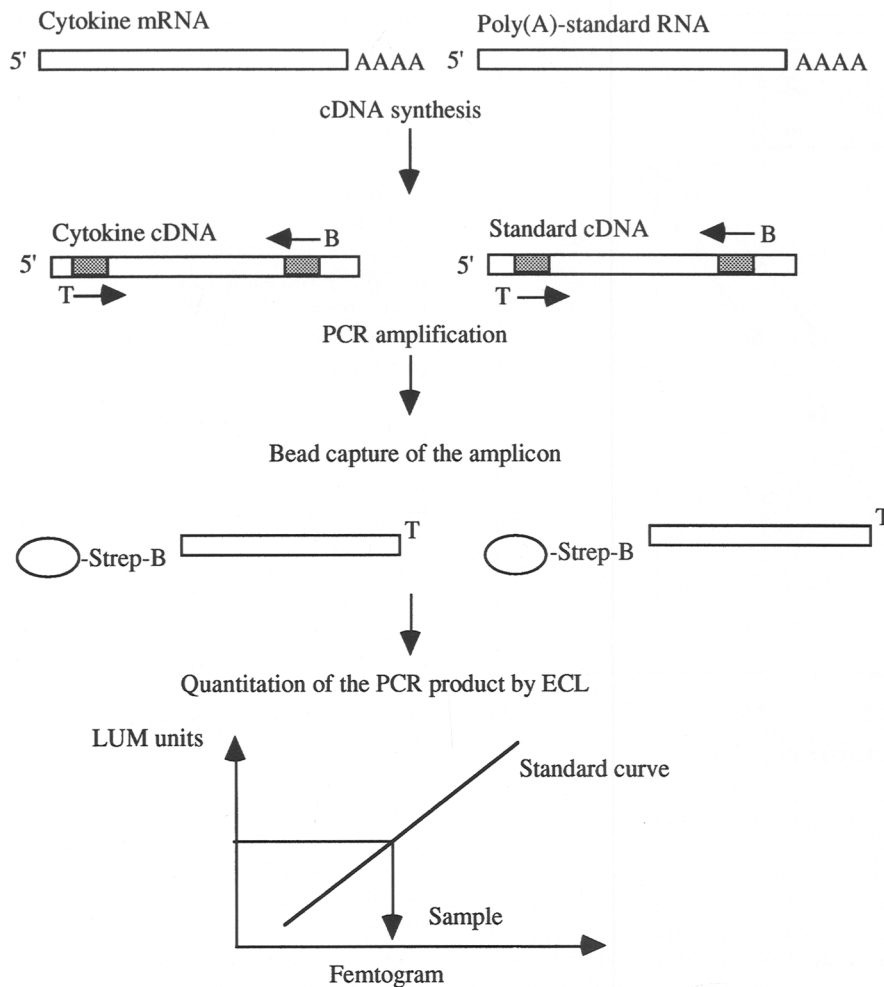
### Quantitation of Amplification Products

To quantitate PCR products using the QPCR system 5000 (Perkin-Elmer), the following procedure is used: The amplified products (5  $\mu$ l) are resuspended in 35  $\mu$ l of 1 $\times$  PCR buffer (10 mM Tris-HCl at pH 8.4, 10 mM KCl, 4 mM MgCl<sub>2</sub>, 0.1% gelatin) in the specific QPCR Sample Tubes. QPCR Streptavidin Dynabeads (2 mg/ml) (Perkin-Elmer) are resuspended and vortexed at low speed to minimize foam formation. Fifteen microliters (30  $\mu$ g/assay) of a homogenous mixture of Dynabeads is added to the QPCR Sample Tube containing the PCR-amplified product to be captured and analyzed. The mixture is incubated at room temperature for 15 min. Three hundred and forty-five microliters (total assay volume 400  $\mu$ l) of QPCR Assay Buffer, containing the coreactant tripropylamine (TPA), is added, the tube is placed in the carousel of the QPCR system 5000, and the electrochemiluminescent (LUM) units are measured. An appropriate blank sample (QPCR Assay Buffer) is used to measure the background level of the system, and parallel to each sample measurement, an external standard curve is measured, making quantification of the samples possible.

### RESULTS

#### Outline of the Assay

The principle of the assay is illustrated



**FIGURE 2** Principle of the quantitative RT-PCR. A synthetic RNA containing the specific cytokine primers and an internal nonsense sequence was synthesized.<sup>(6)</sup> Total sample RNA and known concentrations of synthetic RNA are converted into cDNA and amplified using the same primers. The PCR antisense primers were labeled at the 5' end with TBR, as a reporter group for ECL detection, and the PCR sense primers with biotin for capturing the amplified templates with streptavidin-coated magnetic beads. The specific amplicon is captured by streptavidin-coated magnetic beads and quantified by measurement of ECL using the QPCR system 5000.

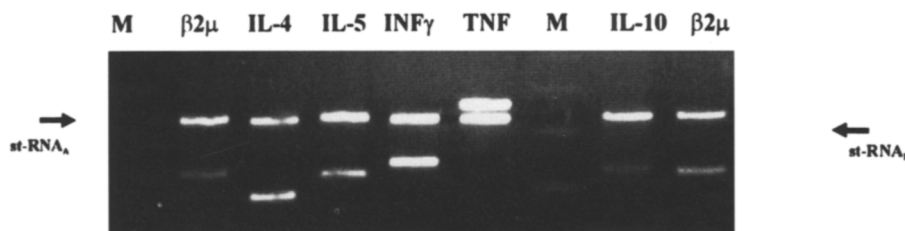
in Figure 2. One microliter of sample cDNA is subjected to PCR amplification using labeled primers. The PCR antisense primers are labeled at the 5' end with TBR as a reporter group for ECL detection, and the PCR sense primers with biotin for capturing the amplified templates with streptavidin-coated mag-

netic beads. Simultaneously—using the same PCR reaction mix—a standard curve is generated in separate tubes, with a heterologous cDNA template, which differ from the target except for the flanking primer-template regions, which are identical. The amplified cytokine and standard cDNA, both biotinylated at one 5' end, are captured by streptavidin-coated magnetic beads. The bond templates are quantified using the QPCR system 5000. The amount of cytokine mRNA initially present is evaluated from the standard curve.

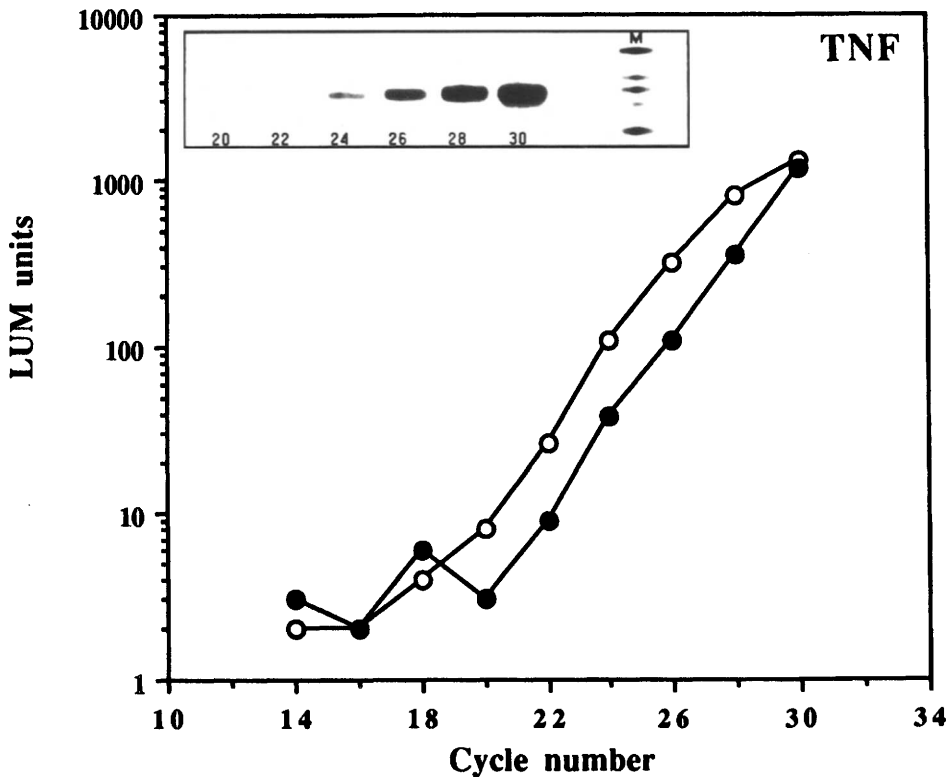
### Generation of the Standard Curve

The PCR amplification efficiency and specificity for several cytokines and housekeeping genes were evaluated. All PCR primer pairs and PCR conditions gave rise to only one discrete band of expected size for standard and target (Fig. 3). The identity of the PCR product was confirmed further by hybridization analysis of the amplification product with digoxigenin-labeled internal probes (data not shown).

Although the cytokine target and standard share the same primer template, the intervening DNA sequences differ, which could give rise to different efficiencies of the amplification of targets and standards because of differences in denaturation characteristics or other factors. It was therefore necessary to determine empirically whether the target and the synthetic standard sequence are amplified with similar efficiencies. One can plot, therefore, the log of the LUM units against the cycle number for the experimental and standard target. One microgram of total RNA from phytohemagglutinin (PHA)-activated lymphocytes and 0.05 ng of standard mRNA were reverse-transcribed and amplified using PCR. Five microliters of PCR amplification product was measured in the QPCR system 5000. The amplification kinetics obtained for tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) are shown in Figure 4. The similarity of the slopes of the linear portion of the resulting two curves is indicative of the similarity of the amplification efficiency between target and standard cDNA. The amplification kinetics of target and standard pairs were compared for all cytokines studied and for  $\beta$ 2-microglobulin. In all cases, the target and standard pairs amplified with similar kinetics.



**FIGURE 3** PCR analysis of cytokine mRNAs. st-RNA<sub>A</sub> or st-RNA<sub>B</sub> was coreverse-transcribed with total RNA of PHA-stimulated lymphocytes. All PCR reactions were carried out for 35 cycles. PCR products were separated on a 1% agarose gel, and bands were revealed with ethidium bromide staining.

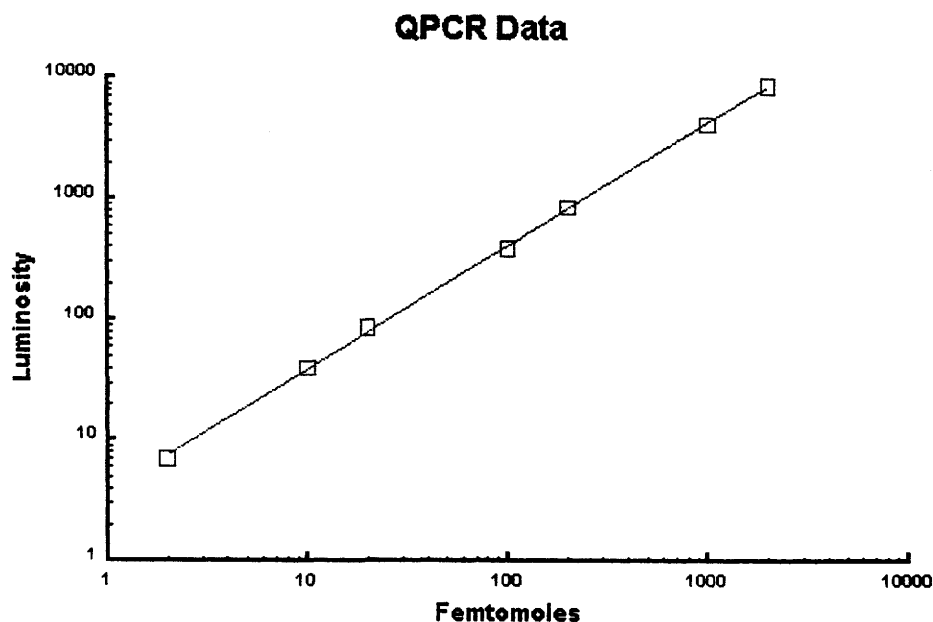


**FIGURE 4** Kinetics of amplification of the cytokine target and standard (st-RNA<sub>B</sub>) cDNA. One microgram of total RNA from PHA-activated lymphocytes and 0.05 ng of mRNA standard (st-RNA<sub>B</sub>) were reverse-transcribed and amplified as described for 14–30 cycles. Ten microliters of the reactions were resolved on a 1% agarose gel, TNF amplification products were visualized using a digoxigenin-labeled TNF probe (*inset*) ( $M=653-517-453-394-298$ -bp; DNA molecular weight marker VI, digoxigenin-labeled), and 5  $\mu$ l was used in a QPCR assay. The data were plotted as a function of the LUM units vs. cycle number. (●) TNF standard; (○) TNF target.

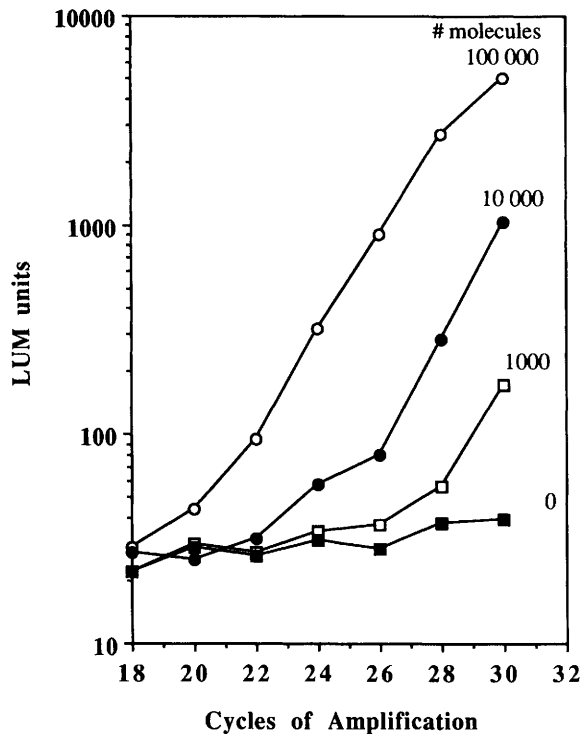
### Validation of the Quantitative RT-PCR

To examine the sensitivity and linearity of the ECL-based detection system, a 370-bp amplicon from the interleukin-4 (IL-4) st-RNA<sub>A</sub> was generated using IL-4-specific primers.<sup>(6)</sup> The product was purified and quantified spectrophotometrically. Serial dilutions of the product were prepared (2000:1 fmoles), and each dilution was analyzed by the QPCR system 5000. The lowest detectable amount was ~2 fmoles, and the highest was ~2 pmoles (Fig. 5). This level of sensitivity is important not only to ensure the detection of weak positives but also to allow the reduction of the number of cycles. The assay cutoff value, as defined by the mean of blank readings (three measurements of the PCR reaction mixture without cDNA, mean=2 LUM units) plus three times the standard deviation of the background signal, was 7 LUM units. The QPCR system 5000 provided a linear response over a range of more than three orders of magnitude of PCR product concentrations.

The influence of the starting template concentration and the number of amplification cycles on the amplification efficiency ( $E$ ) was examined by amplification of serial dilutions of a known amount of cytokine st-RNA<sub>A</sub> (IL-4). Samples were evaluated from the reaction mixtures from cycles 18–30. The signal intensity (LUM units) of each product was plotted as a function of the number of cycles (Fig. 6). A comparison of the slopes of the linear parts of the curves (0.241, 0.245, and 0.245 for  $10^5$ ,  $10^4$ , and  $10^3$  starting molecules, respectively) indicates that parallel curves are obtained according to the initial amount of cDNA used, until a plateau is reached. From the above curves, we could estimate the amplification efficiency obtained with the IL-4-specific primers as follows: It is known that the PCR reactions started with  $10^5$ ,  $10^4$ , etc., cDNA molecules and produced the same amount of amplified fragments after 23.7, 27.7, etc., cycles, respectively, when the cycle numbers are measured by interpolation on the horizontal line corresponding to 250 LUM units in Figure 6. This means that the 10-fold difference in starting cDNA concentrations can be compensated by adapting the number of cycles (e.g., the difference of four cycles for the curves obtained with



**FIGURE 5** Sensitivity and linearity of the quantitative RT-PCR assay. A 370-bp amplicon from the IL-4 st-RNA<sub>A</sub> was generated using the IL-4-specific primers. The product was purified and quantified by absorbance at 260 nm. Serial dilutions of the product were prepared (2000:1 fmoles), and each dilution was analyzed by the QPCR system 5000.

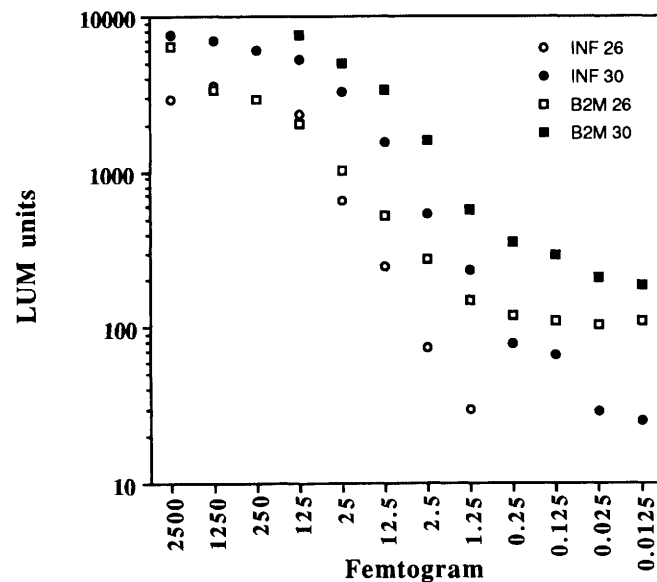


**FIGURE 6** Curves relating final signal intensity to cycle number starting with  $10^3$ ,  $10^4$ , and  $10^5$  molecules of cytokine st-RNA<sub>A</sub> (IL-4). The slopes and linear regression coefficients are 0.241 and 0.999, respectively, for  $10^5$  starting molecules, 0.245 and 0.992 for  $10^4$  starting molecules, and 0.245 and 1.0 for  $10^3$  starting molecules.

$10^5$  and  $10^4$  starting cDNA molecules). Therefore, the amplification efficiency  $E$ , calculated from the first two curves according to the formula  $Y=X \times E^n$  (with  $X$  corresponding to the amount of starting cDNA and  $n$  to the number of PCR cycles) equals  $10^{1/4}$  that is,  $E=1.8$ . By performing the same calculation for the curve interval between  $10^4$  and  $10^3$  starting cDNA molecules, we obtained 2.15 as  $E$  value, indicating a small decrease of the amplification efficiencies when the starting template concentration is high.

The accuracy of quantitation necessitates that the PCR reaction is stopped when the production of DNA is within the exponential growth phase. As the quantity of initial cDNA can influence the number of cycles before reaching the plateau, we determine the dynamic range for the cytokine-specific PCR products. Therefore, cDNA reverse-transcribed from different mRNA quantities was amplified and the amount of accumulated specific DNA was determined by ECL. The dynamic range of the QPCR system 5000 was great. As shown in Figure 7, the 100- to 1-fg range could be quantified at 26 cycles. The 0.1-fg to

1-ag range needed 30 cycles or more, according to the amplification efficiency. The same was true for all cytokines studied.



**FIGURE 7** Dynamic range of the quantitative RT-PCR assay. Serial dilutions (2.5 pg–2.5 ag) of st-RNA<sub>A</sub> (INF- $\gamma$ ,  $\beta$ 2- $\mu$ Glob) were amplified for 26 ( $\circ$ ,  $\square$ ) or 30 ( $\bullet$ ,  $\blacksquare$ ) cycles. The amplified product was then quantified with the QPCR system 5000. All data were corrected for the assay background (negative control, 0 pg).

### Reproducibility of the RT-PCR and ECL Detection

We evaluated the tube-to-tube and day-to-day variations in the quantification assay as follows: Total RNA mixes of PHA-stimulated T lymphocytes were aliquoted into three tubes, and the RT-PCR reaction (TNF,  $\beta$ 2- $\mu$ Glob) was carried out as described. The same procedure was repeated on a different day to determine the day-to-day variations. Tube variations were not significantly different at the 5% levels, as shown by Student's  $t$ -test ( $P=0.235$ ). The mean values of TNF,  $\beta$ 2- $\mu$ Glob and the ratio (TNF/ $\beta$ 2- $\mu$ Glob $\times$ 1000) of amplification day 1 and amplification day 2 were not significantly different ( $P>0.05$ ).

To determine the reproducibility of the integral method (the reverse transcription, PCR amplification, and ECL-based quantification), five identical experiments were performed, each starting with the reverse transcriptase reaction. Five samples of purified cytokine standard mRNA<sub>A</sub> (0.1 pg) were reverse-transcribed, and 1  $\mu$ l ( $1/20$ ) of the five cDNA samples were amplified with the TNF $\alpha$ -specific primers. The reproducibility of the reverse transcription and amplification step was good [coefficient of variation (CV): 12.8% at 24 cycles and 14.3% at 30 cycles]. We further evaluated the variability in RNA extraction and degra-

dation and in reverse transcriptase yield, by adding known amounts of standard mRNA to the cells before digestion and RNA extraction. Jurkat cells, negative for IL-10 mRNA, were spiked with known concentrations of st-RNA<sub>B</sub> before and after RNA extraction. Then RNA was reverse-transcribed to cDNA, which was amplified with IL-10-specific primers. The amplification products were evaluated by the QPCR system 5000. The recovery of spiked st-RNA<sub>B</sub> was 75% for concentrations >1 pg and ~50% for concentrations <1 fg.

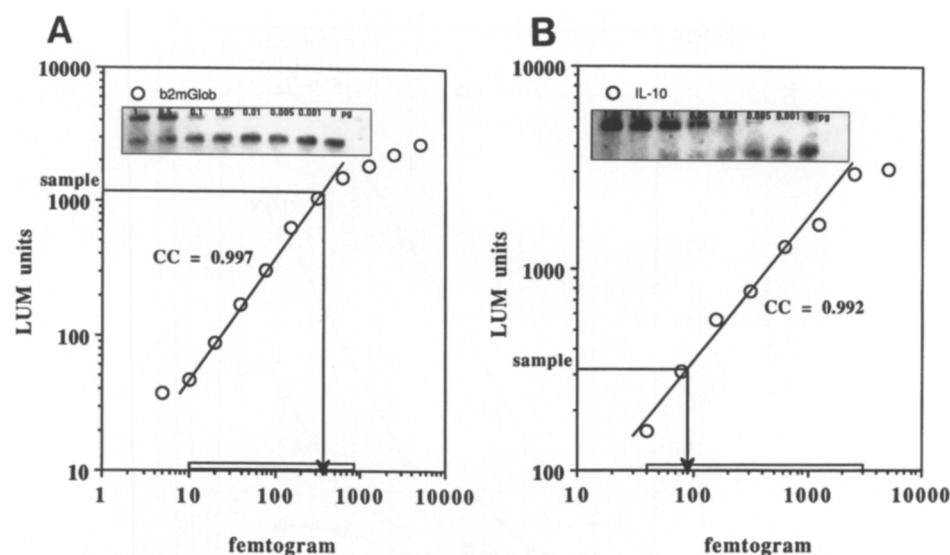
### RT-PCR to Determine the Levels of Cytokine mRNA in T-cell Clones

The assay was used to determine cytokine mRNA levels in small samples of T-cell clones. Because the amounts of products are only proportional to the amounts of starting target mRNA during the exponential phase of the amplification, the PCR reaction was stopped at 26 cycles. Samples that did not fall within the linear range of the standard curves were reevaluated after one-half or one-tenth dilution of the sample cDNA. To avoid false-negative results, negative samples were reevaluated after more amplification cycles.

To detect significant differences in cytokine mRNA expression between T-cell clones, normalization of the data is required so that the expression among samples can be compared with a common denominator. We chose the expression of  $\beta$ 2-microglobulin mRNA as an approximate reference for the relative amounts of cDNA obtained from different numbers of cells per culture. The mean advantage is that the reference mRNA ( $\beta$ 2- $\mu$ Glob) and the target mRNA (cytokine) are treated in the same way from the beginning of the procedure, thus providing an internal control for the amount of intact RNA successfully isolated and converted into cDNA.

An example of the procedure is shown in Figure 8. The results obtained were compared with the classical competitive PCR, using the same mRNA standard as competitor. Both tests showed good agreement.

The cytokine profile of heat shock protein-specific T-cell clones, isolated from three healthy individuals, is shown in Figure 9. Comparing the cytokine profile obtained for the T-cell clones with that of PHA-stimulated T-cell blasts



**FIGURE 8** Analysis of cytokine mRNA levels by competitive PCR vs. the QPCR system 5000. (A) Competitive RT-PCR: One microliter of sample cDNA was mixed with known amounts of cytokine standard cDNA. The mixture was subjected to amplification in the presence of digoxigenin-dUTP. The amplified products were electrophoresed on a 2% agarose gel and visualized with the digoxigenin luminescent detection kit (*inset*). Densitometric analysis was performed with the QuantiGel Software (Stratagene) and revealed 46 fg of IL-10 mRNA and 414 fg of  $\beta$ 2- $\mu$ Glob mRNA. (B) ECL detection. Two standard curves (IL-10 and  $\beta$ 2- $\mu$ Glob) were generated. Horizontal bars show the concentration range of the standard curve from which specific mRNA amounts can be accurately calculated. Simultaneously, 1  $\mu$ l of sample cDNA was amplified and the resulting amplified products (5  $\mu$ l) were then quantified with the QPCR system 5000. A total of 88 fg of IL-10 and 327 fg of  $\beta$ 2- $\mu$ Glob mRNA was detected.

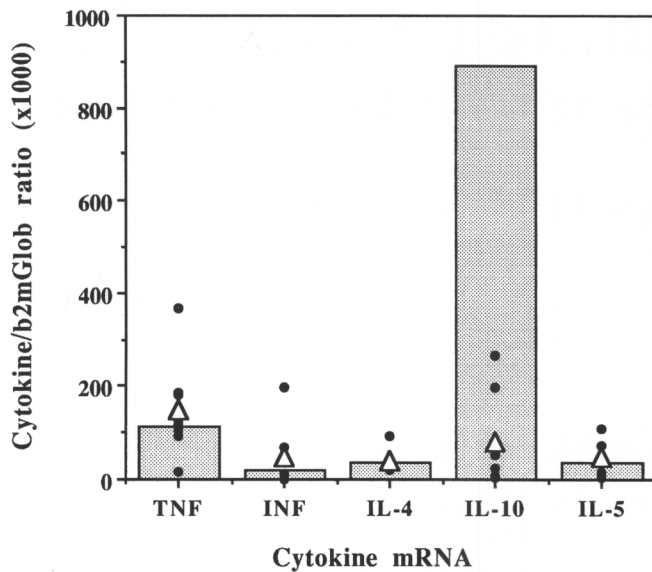
shows that TNF and  $\gamma$  interferon (INF- $\gamma$ ) mRNA levels of the clones were substantial, whereas relative low IL-10 mRNA levels were observed. Therefore, we can conclude that these clones display a T<sub>H</sub>1-like cytokine profile.

### DISCUSSION

The quantitative analysis of competitive PCR reactions is labor intensive involving the separation of the two PCR products by electrophoretic techniques or HPLC. In most cases, quantification has been performed with an isotopic label. Furthermore, competitive amplification is tedious because multiple PCR reactions have to be performed to titrate one sample with the internal standard. In this paper we describe a quantitative PCR method that uses only commercially available reagents (5' biotinylated oligonucleotide, 5' TBR-labeled oligonucleotide, streptavidin-coated paramagnetic beads, QPCR assay buffer) and the QPCR system 5000 equipment. Furthermore, in the present assay, a single amplification is sufficient, so that several samples can be analyzed at once and

there is no need to separate PCR products by gel electrophoresis. In agreement with other QPCR studies, we were able to measure the relative amounts of cytokine mRNA with reasonable accuracy. The QPCR system 5000 allows direct and quick quantification of PCR amplification products at different moments of amplification, without the need of potential carcinogens such as ethidium bromide and radioactivity. Combined use of the GeneAmp 9600 thermocycler and the QPCR system 5000 allows quantification of PCR products within 3 hr. The post-PCR steps are carried out automatically, with a low coefficient of variation and an enlarged dynamic range ( $10^{-18}$  to  $10^{-15}$  M) of the ECL-based detection method.

The method, however, has inherent limitations. Careful optimization of the PCR reaction is necessary to avoid false-positive detection, caused by nonspecific amplifications. To compare mRNA amounts in various samples, accurate values can be obtained using an external or internal standard (i.e., DNA that can be amplified with the same primer but differs in internal sequence or length). The



**FIGURE 9** Cytokine profile of antigen-specific long-term T-cell clones. Data are given as a ratio of the cytokine mRNA content and the  $\beta$ 2-microglobulin, reporter gene content of the sample. (Bars) The cytokine profile of PHA-stimulated T-cell blasts. (●) Individual measurements for each T-cell clone studied. (Δ) The mean cytokine content of the T-cell clones studied.

cytokine standard mRNA used in this study, has a nonhomologous sequence with the target cytokine cDNA; therefore, the standard was used as an external standard, in parallel reaction tubes, and in the exponential phase of the PCR amplification. However, the use of an internal standard is possible, when the PCR reaction is performed with only a biotinylated primer, and, subsequently, a liquid hybridization of the PCR products is performed with either a standard or a cytokine-specific TBR-labeled probe, after duplicating the QPCR Sample Tubes.<sup>(9)</sup> However, we believe that an internal RNA standard is only required for viral RNA targets, because the RNA extraction and the reverse transcription yield can be controlled in eukaryotic cells by studying the expression of housekeeping genes. The sources of variability when measuring mRNA expression by QPCR are (1) the cell number, (2) the RNA extraction yield, (3) the degradation of mRNA during the extraction, (4) the reverse transcription efficiency, and (5) the amplification efficiency and rate. Therefore, to normalize these variables inherent in RNA preparation, cDNA synthesis and PCR reaction, and the variable cell numbers, we utilized the differential PCR technique using as reporter gene  $\beta$ 2-microglobulin. This means that the relative rather than ab-

solute levels of gene expression are measured by determining a ratio between PCR products generated by amplifications of the desired target cDNA and the endogenous reporter gene in separate reactions followed by comparing with the same ratio in another sample.

In conclusion, with each step optimized and validated, the test offers a convenient new possibility of accurately monitoring cytokine mRNA levels in T cells, in a broad dynamic range. The method can be adapted to any gene and is therefore well suited for research use and routine clinical diagnostics. The simplicity of this method for quantification of PCR amplification products should broaden the use and applications of QPCR, particularly for a high throughput for routine analysis.

#### ACKNOWLEDGMENTS

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