

Research Article

Real-time reverse transcriptase–polymerase chain reaction (RT–PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I

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Summary Real-time quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) is the method of choice for rapid and reproducible measurements of cytokine or growth factor expression in small samples. Fluorescence detection methods for monitoring real-time PCR include fluorogenic probes labelled with reporter and quencher dyes, such as Taqman probes or Molecular Beacons and the dsDNA-binding dye SYBR Green I. Fluorogenic (Taqman) probes for a range of human and rat cytokines and growth factors were tested for sensitivity and compared with an assay for SYBR Green I quantification using real-time fluorescence monitoring (PE Applied Biosystems Model 7700 sequence detector). SYBR Green I detection involved analysis of the melting temperature of the PCR product and measurement of fluorescence at the optimum temperature. Fluorogenic probes provided sensitive and reproducible detection of targets that ranged from low (<10 copies/reaction) to high (>10⁷ copies/reaction) expression. SYBR Green I gave reproducible quantification when the target gene was expressed at moderate to high levels (≥1000 copies/reaction), but did not give consistently reproducible quantification when the target gene was expressed at low levels. Although optimization of melting temperature improved the specificity of SYBR Green I detection, in our hands it did not equal the reproducible sensitivity and specificity of fluorogenic probes. The latter method is the first choice for measurement of low-level gene expression, although SYBR Green I is a simple and reproducible means to quantify genes that are expressed at moderate to high levels.

Key words: fluorescent resonance energy transfer (FRET) probe, quantitative real-time PCR, SYBR Green I, Taqman.

Introduction

A number of methods are available for measurement of cytokine and growth factor mRNA expression including Northern Blot, RNase protection assays, *in situ* hybridization and quantitative reverse transcription–polymerase chain reaction (RT–PCR). Of these, quantitative RT–PCR is the only method that provides sufficient sensitivity and specificity to measure low level mRNA expression in small amounts of tissue, such as biopsy samples.¹ This is especially the case for some cytokines, such as IL-2 and IL-4, that are expressed at very low levels.¹ In spite of its advantages, previous methods for quantitative RT–PCR have been laborious and difficult to make consistently reproducible.² Conventional methods for quantifying PCR include measurement of end-stage product by Southern blotting or incorporation of radioactive label;³

use of known numbers of competitor DNA fragments during PCR,² end-point titration of cDNA and removal of aliquots of PCR reaction at successive cycle numbers to determine qualitative⁴ or quantitative⁵ differences in cytokine mRNA expression.

Recent parallel developments of equipment for real-time monitoring of fluorescence within PCR tubes and in the chemistry of fluorescent detection of PCR product have led to a major improvement in the ease and reproducibility of quantitative RT–PCR. Equipment for monitoring of PCR includes the Model 7700 and Model 5700 sequence detectors (PE Applied Biosystems, Foster City, CA, USA), and the LightCycler (Roche, Basel, Switzerland), Sentinel (Stratagene, La Jolla, CA, USA) and Rotorgene (Corbett Research, Mortlake, Australia) real-time PCR machines. Methods for fluorescence monitoring of PCR product employ fluorogenic probes based on fluorescent resonance energy transfer (FRET) systems such as Taqman and Molecular Beacons or the dsDNA-binding dye SYBR Green I.^{6–8} Real-time monitoring of the PCR reaction using these technologies allows faster and more accurate measurement of gene expression than conventional PCR quantification. In-tube fluorescent monitoring of the appearance of PCR product eliminates

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post-PCR analysis of product, thus removing the errors and time associated with these steps. It also enables quantification to be based on the early, exponential amplification phase of the reaction where substrates are not limiting and differences in efficiency of amplification have less effect on the results. There have been few reports to date of the use of real-time quantitative PCR for analysis of human or rat cytokines.^{7,9,10} Also the conditions used for quantitative PCR and the monitoring methods for fluorescent detection of PCR product have not been well characterized. Here we examine the optimum conditions for use of fluorogenic Taqman probes and SYBR Green I and compare the sensitivity and specificity of the two systems for measurement of cytokine and growth factor genes in human and rat cDNA.

Materials and Methods

RNA extraction and cDNA synthesis

Human liver core biopsies (1 mm diameter and 10 mm length) and rat kidney tissue samples (3 mm³) were immediately frozen in liquid nitrogen. Total RNA was prepared by the acid phenol-guanidine method¹¹ and reverse transcribed by methods we have previously described.¹² A yield of 5–15 µg of RNA was obtained from approximately 10 mg of tissue. All samples were reverse transcribed at the same time, diluted 1:10 in diethylpyrocarbonate-treated autoclaved H₂O (Sigma, St Louis, Mo, USA) and stored in aliquots at –70°C.

PCR standards

The PCR standards for each cytokine or growth factor consisted of known numbers of molecules of purified PCR product, prepared as previously described.⁵ PCR product was purified by gel electrophoresis followed by excision of the band of the correct molecular weight and separation of DNA (Wizard Minipreps, Promega, Madison, WI, USA). The concentration of the purified PCR product DNA was estimated by OD₂₆₀ and the number of copies/mL of standard were calculated according to the following formula:

$$\text{copies/mL} = \frac{6.023 \times 10^{23} \times C \times \text{OD}_{260}}{\text{MWt}}$$

where C = 5 × 10⁻⁵ g/mL for DNA and MWt = molecular weight of cytokine PCR product (base pairs × 6.58 × 10² g).

Standards were made to a concentration of 10⁸ copies/µL. Sodium azide preservative was added to a final concentration of 0.025% w/v and the solution sterile filtered through a 0.22-µm filter (Millipore, Bedford, MA, USA) and stored in aliquots at 4°C.

Primers and probes

Primers for amplification using SYBR Green I were based on published sequences for IFN-γ¹³ and glyceraldehyde-3-phosphate dehydrogenase (G3PDH).¹⁴ For growth arrest-specific gene 6 (Gas6) the sequences used were: forward primer, 5'-GAGGT-GTTCGAGAACGACCC-3'; reverse primer, 5'-CTGCATTCGTT-GACATCTTTGTC-3'. Sizes of PCR products amplified with primers for SYBR Green I analysis were: IFN-γ, 285 base pairs (bp); G3PDH, 456 bp; Gas6, 254 bp. Primers and fluorogenic probes were designed using Primer Express version 1.0 (PE Applied Biosystems) and are listed in Table 1. All probes were synthesized by PE Applied Biosystems. All primers and probes were designed either to cross large expanses of intronic sequence or to span an intron–exon boundary except for Gas6, for which the genomic

sequence has not been described. Fluorogenic primers and probes were limited to amplify products of 150 bases or less and could not be designed to cross large introns. In this case, one or more of the primers and probe were designed to cross an exon–intron boundary (Table 1). Non-reactivity of the primers and probe with contaminating genomic DNA was tested by the inclusion of controls that omitted the reverse transcriptase enzyme from the cDNA synthesis reaction (no RT controls).

Real-time PCR quantification using fluorogenic probes

Reaction conditions for fluorogenic PCR mixtures are shown in Table 2 (columns 2 and 3). The optimum concentration of probe, primers and MgCl₂ was determined in preliminary experiments. The thermal cycling profile consisted of: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; stage 3, 95°C for 15 s followed by 60°C for 1 min. Stage 3 was repeated for 40 cycles. In experiments using fluorogenic probe and primer sets either Buffer A or Universal Master Mix was used (Table 2).

Real-time PCR quantification using SYBR Green I

SYBR Green I dye (Sigma S9430) was supplied at 10 000× concentration in DMSO. It was stored in 10 µL aliquots at –80°C, and a working dilution of 50× solution in water was stored as multiple aliquots at –20°C. Each aliquot of working dilution was discarded after a single use. The reaction conditions and buffers used are shown in Table 2. For some experiments 6-carboxy-X-rhodamine (ROX) (Molecular Probes, Eugene, OR, USA) was used as a positive internal reference. Fifteen milligrams of ROX was dissolved in 0.5 mL DMSO (Sigma) and diluted to 600 nmol/L concentration with H₂O and stored at –20°C. All SYBR Green I or ROX solutions were protected from light. In experiments using SYBR Green I dye either Buffer A or Buffer II + ROX was used (Table 2).

As SYBR Green I is not specific for the PCR product and binds to primer dimers formed non-specifically during all PCR reactions, it was necessary to obtain an optimum temperature for analysis of specific product as previously described.⁷ This was performed on the PCR product obtained after amplification by raising the temperature successively through 1°C steps and comparing the melting temperature of specific cytokine or growth factor product with non-specific product obtained from a no-template control (NTC). The melting temperature profile on the Model 7700 Sequence Detector consisted of: stage 1, 95°C for 1 min then 55°C for 2 min; stage 2, 77°C for 1 min then a 1°C increase in temperature every 1 min up to a final temperature of 92°C at stage 17. Each cytokine or growth factor analysed by SYBR Green I was examined by this melting temperature profile. The optimum temperature was that which gave the maximum reading for specific product when the non-specific product could no longer be detected.

The optimum temperature determined from melting point analysis was then used for quantitative PCR using the following thermal cycling programme: stage 1, 95°C for 10 min. Stage 2 consisted of three steps: 95°C for 30 s with a 40-s ramp time to 55°C for 30 s. After a 20-s ramp time to 72°C for 1 min there was a 17-s ramp time before data acquisition at the optimum melting temperature for 30 s. Stage 2 was repeated for 40 cycles.

PCR buffers

The buffers used for these experiments were obtained commercially. They were PCR Buffer II, PCR Buffer A and Universal Master Mix (PE Applied Biosystems). PCR Buffer II when diluted to the final concentration in the PCR reaction mixture (1×) contained

Table 1 Fluorogenic probe and primer sets

Species	Target gene	Primer/probe	Sequence	Length (bp)
Human	IL-2	Forward	CCAGGATGCTCACATTTAAGTTTAC	87
		Reverse	GAGGTTTGAGTTCTTCTCTAGACTGA	
	IL-4	Probe	6FAM-TGCCCAAGAAGGCCACAGAACTGAA-TAMRA	101
		Forward	AACAGCCTCACAGAGCAGAAGAC	
		Reverse	GCCCTGCAGAAGGTTTCCTT	
	IL-10	Probe	6FAM-TGCTGCCTCAAGACACAACCTGA-TAMRA	82
		Forward	ACGGCGCTGTCATCGATT	
		Reverse	TTGGAGCTTATTAAGGCATTCTTC	
	TNF- α	Probe	6FAM-CTTCCCTGTGAAAACAAGAGCAAGGCC-TAMRA	87
		Forward	TCTGAACCCCGAGTGACA	
Reverse		GGCCCGCGGTTCA		
IFN- γ	Probe	6FAM-TAGCCCATGTTGTAGCAAACCCTCAAGC-TAMRA	103	
	Forward	AGCGGATAATGAACTCTTTTCTTAG		
	Reverse	AAGTTTGAAGTAAAAGGAGACAATTTGG		
Human/Rat	G3PDH	Probe	6FAM-TCTGTCACTCTCCTCTTCCAATTCTCAAAATG-TAMRA	87
		Forward	TGCACCACCAACTGCTTAGC	
		Reverse	GGCATGGACTGTGGTCATGAG	
	Gas6	Probe	VIC-CCTGGCCAAGGTCATCCATGACAACCT-TAMRA	113
		Forward	GACCTCGTCCAGCCGATTA	
		Reverse	TGCATTTTGTATTGGCCTTGA	
Rat	IL-2	Probe	6FAM-TCAGCCAGTTCAGCTCCTCATGCA-TAMRA	76
		Forward	CCCCATGATGCTCACGTTTA	
		Reverse	ATTTTCCAGGCACTGAAGATGTTT	
	IFN- γ	Probe	6FAM-TTGCCCAAGCAGGCCACAGAATTG-TAMRA	117
		Forward	AGTCTGAAGAACTATTTTAACTCAAGTAGCAT	
		Reverse	CTGGCTCTCAAGTATTTTCGTGTTAC	
	TGF- β	Probe	6FAM-CCTTTTGCCAGTTCCTCCAGATATCCAAGA	74
		Forward	CGTGGAAATCAATGGGATCAG	
		Reverse	GGAAGGGTCGGTTCATGTCA	
	Rat	TGF- β	Probe	6FAM-CGAGGTGACCTGGGCACCATCC-TAMRA

Exon/intron boundaries are shown as underlined bases. All primer/probe sets were designed to span intronic sequence, except Gas6 for which the genomic structure is unknown. bp, base pairs; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; Gas6, growth arrest-specific gene 6.

Table 2 Polymerase chain reaction mixtures for fluorogenic probes or SYBR Green I

	Buffer II + ROX (SYBR Green I)		Buffer A (SYBR Green I or Probe)		Universal Master Mix (Probe)	
Buffer II (10 \times)	2.5*	[1 \times] [†]	–	–	–	–
Buffer A (10 \times)	–	–	2.5	[1 \times]	–	–
Universal Master Mix (2 \times)	–	–	–	–	12.5	[1 \times]
dNTP (10 mmol/L)	0.5	[200 μ mol/L]	0.5	[200 μ mol]	–	[200 μ mol/L]
MgCl ₂ (25 mmol/L)	3.0	[3 mmol/L]	3.0	[3 mmol/L]	–	–
Taq polymerase (5 U/ μ L)	0.125	[0.625 U]	–	–	–	–
Gold Taq polymerase (5 U/ μ L)	–	–	0.125	[0.625 U]	–	–
SYBR Green I (50 \times) [‡]	0.25	[0.5 \times]	0.25 [§]	[0.5 \times]	–	–
Probe (20 μ mol/L)	–	–	0.25 [§]	[200 nmol/L]	0.25	[200 nmol/L]
ROX (600 nmol/L)	2.5	[60 nmol/L]	–	–	–	–
Forward primer (30 μ mol/L)	0.25	[300 nmol/L]	0.25	[300 nmol/L]	0.25	[300 nmol/L]
Reverse primer (30 μ mol/L)	0.25	[300 nmol/L]	0.25	[300 nmol/L]	0.25	[300 nmol/L]
cDNA (1:10 dilution)	5	–	5	–	5	–
H ₂ O	10.625	–	12.875	–	6.75	–
Total volume	25	–	25	–	25	–

*Figures denote μ l/reaction. [†]Figures in [] denote the final concentration in the reaction. [‡]SYBR Green I was diluted from the supplied 10 000 \times concentrate as described in the methods section. [§]Either SYBR Green I or fluorogenic probe was used. dNTP, deoxyribonucleoside triphosphate; ROX, 6-carboxy-X-rhodamine.

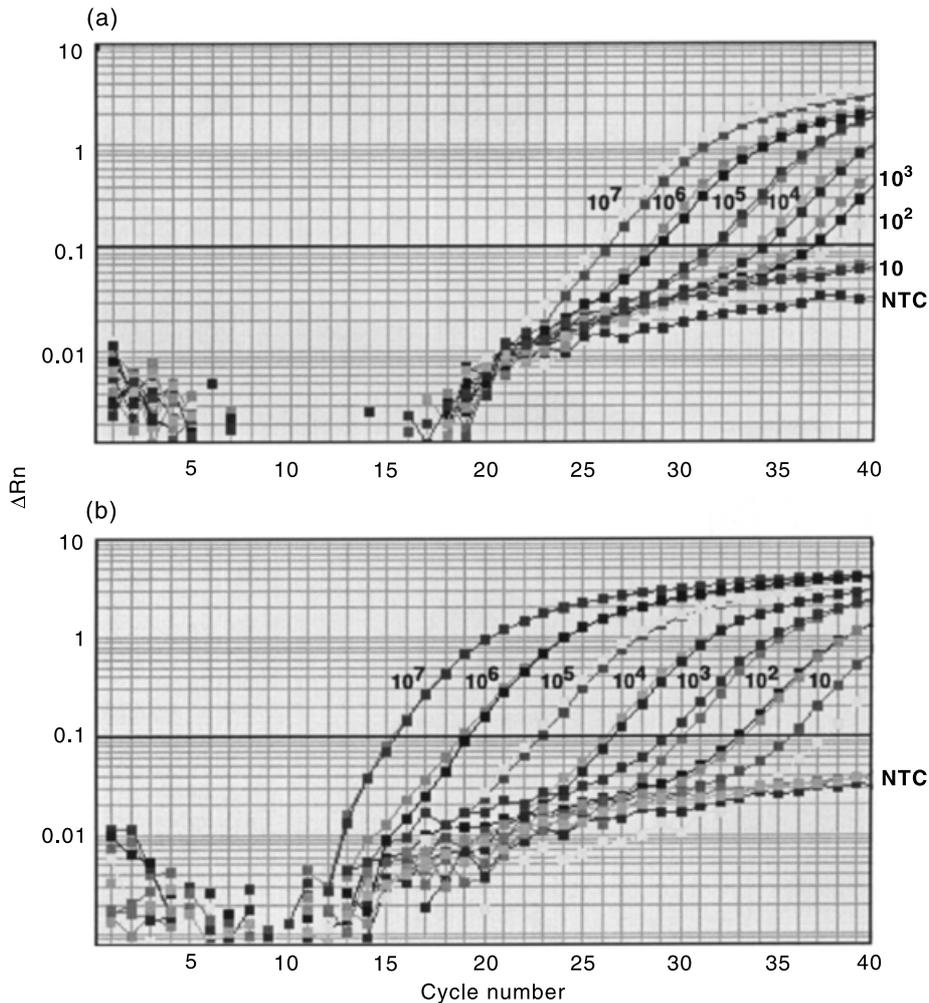


Figure 1 Efficiency of fluorogenic probe amplification in two different buffers. Amplification plots of IL-4 standards in (a) Buffer A and (b) Universal Master Mix. Amplification plots of standards ranged from 10^7 copies/reaction to 10 copies/reaction. Duplicate reactions are shown and often appear superimposed. No-template controls (NTC) were also included and did not amplify in either buffer system. The horizontal line shows the setting of the baseline. The point at which the amplification plot crosses the baseline is the C_T value.

50 mmol/L KCl and 10 mmol/L Tris HCl (pH 8.3). PCR Buffer A (1 \times) contained 50 mmol/L KCl, 10 mmol/L Tris HCl (pH 8.3), 10 mmol/L EDTA and 60 nmol/L ROX. Universal Master Mix (1 \times) contained 6% Glycerol, 50 mmol/L KCl, 10 mmol/L Tris HCl (pH 8.3), 7.5 mmol/L $MgCl_2$, 200 μ mol/L dATP, 200 μ mol/L dCTP, 200 μ mol/L dGTP, 400 μ mol/L dUTP, 0.05 U/ μ L AmpliTaq Gold, 0.01 U/ μ L Amperase UNG and 60 nmol/L ROX.

Data expression and analysis

For each assay a standard curve was prepared by serial dilution of a known number of cytokine or growth factor PCR product molecules. All samples to be compared were run in the same assay. After completion of the PCR amplification, data was analysed with the Sequence Detector version 1.7 software (PE Applied Biosystems). To maintain consistency, the baseline was set automatically by the software using data collected from cycle 3 to cycle 15 in most experiments. In some experiments it was necessary to manually override this setting to optimize analysis. The increase in intensity of fluorescence of the reporter dye (ΔRn)⁶ was plotted against the cycle number. The threshold cycle (C_T) was calculated by the sequence detection software as the cycle number at which the ΔRn crossed the baseline. Quantification of the samples by the software was calculated from the C_T by interpolation from the standard curve to yield a copy number of cytokine or growth factor cDNA.

Statistical analysis

Statview (Abacus Concepts Inc, Berkeley, CA, USA) was used for statistical analysis. The results are shown as mean \pm SD for three different PCR reactions. Reproducibility between assays was determined by linear regression analysis.

Results

Fluorogenic probe optimization and buffer reaction efficiency

Initial experiments examined the effect of concentration of primers, probe, Mg^{2+} and enzyme on efficiency of amplification in PCR. The optimum concentration of these is shown in Table 2. In addition, the effect of different PCR buffers was examined. This showed that Universal Master Mix was superior to other buffers tested for amplification of most fluorogenic probe and primer sets. Figure 1 shows an example of an amplification plot of human IL-4 standards, derived from IL-4 PCR product, in Universal Master Mix compared to Buffer A. Buffer A gave poor amplification of this target as shown by the high C_T values for the standards and the shallow slope of the amplification plot (Fig. 1a). Universal Master Mix gave much more efficient amplification as shown by the

Table 3 Sensitivity of fluorogenic probe and primer sets for cytokine detection

Fluorogenic probe	Sensitivity (copies/reaction)	NTC (C_T)	10 copies (C_T)	10^3 copies (C_T)	10^5 copies (C_T)
G3PDH	≤ 10	$> 40^*$	33.9 ± 0.6	27.8 ± 0.7	18.9 ± 2.8
IL-2	≤ 10	> 40	37.2 ± 0.6	30.5 ± 0.9	23.5 ± 1.1
IL-4	≤ 10	> 40	36.2 ± 0.7	29.1 ± 0.1	22.4 ± 0.2
IL-10	≤ 10	> 40	36.3 ± 0.3	29.0 ± 0.8	22.1 ± 0.4
IFN- γ	≤ 10	> 40	36.1 ± 1.0	28.4 ± 1.6	23.3 ± 0.5
TNF- α	≤ 10	> 40	34.9 ± 1.0	28.8 ± 1.2	22.4 ± 1.2
Gas6	≤ 10	39.0 ± 1.6	31.7 ± 1.1	26.8 ± 0.5	20.2 ± 0.1
TGF- β	≤ 10	> 40	29.1 ± 0.2	22.9 ± 0.2	17.4 ± 0.1

*The no-template control (NTC) was not amplified in the 40 cycle PCR reaction and a value of > 40 was allotted. All primers and probes were specific for human sequence and the results show amplification of human standards, except for the growth factors Gas6 and TGF- β , which were specific for rat sequence and used rat standards. C_T , threshold cycle; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; Gas6, growth arrest-specific gene 6.

lower C_T values for all standards and a steep amplification plot (Fig. 1b). Similar results were obtained when Universal Master Mix was compared to Buffer II and amplification in Universal Master Mix equalled or exceeded the efficiency compared to the other buffers for all primers and probes tested.

No amplification for cytokine or growth factor cDNA was observed in the no RT controls, showing that the primer and probe combinations did not amplify potential contaminating genomic DNA in samples. It also showed the absence of PCR contamination, a situation that was brought about by a combination of factors. The real-time system allowed analysis without requirement to open the PCR tube and the PCR set-up area was in a separate laboratory to the sequence detector. Also there were contamination control reagents in the Universal Master Mix that incorporated uracil-n-glycosylase and dUTP to prevent PCR product carry-over.

There was consistent amplification of G3PDH in the no RT control, although at a level of expression of $< 1\%$ of that observed when reverse transcriptase enzyme was included. These results for G3PDH were not due to contamination of the reactions with PCR product as the NTC were consistently negative. G3PDH appeared to be present in the samples at low levels, possibly due to small amounts of processed pseudogenes in genomic DNA contamination in the purified RNA. Although the G3PDH probe was labelled with VIC fluorophore, which allows multiplexing of the PCR reaction, multiplex reactions were not used for these experiments.

Sensitivity of fluorogenic probe detection

Sensitivity of detection of cytokine and growth factor standards with each of the primer/probe combinations is shown in Table 3. These results were obtained using the optimum concentration of probe and primer in the Universal Master Mix. The sensitivity of the method was indicated by the minimum number of copies of standard that could be consistently amplified and was < 10 copies for each cytokine or growth factor. A further indication of the sensitivity of the method was the C_T at low copy numbers. This is shown in Table 3 and for amplification of 10 copies/reaction the C_T ranged from 29.1 ± 0.2 to 37.2 ± 0.6 . The NTC was not amplified for all but one template, which meant that the C_T

was allotted the arbitrary value of > 40 in these 40 cycle reactions. The sensitivity of this technique is shown by the difference between the C_T for the NTC and for the 10 copy standard, which ranged from > 2.8 for IL-2 to > 10.9 for TGF- β (Table 3). This sensitivity reflects the high efficiency of amplification in these reactions, shown by the slopes of the standard curves, which were close to the theoretical optimum of -3.32 and correspond to 100% efficiency of amplification. This is shown in Table 3 where the difference in C_T between 100-fold differences in copy numbers is approximately 6.6. The primers and probes used for analysis of the human cytokines were thus able to reproducibly detect as few as 10 copies of standard per reaction. The results for rat G3PDH, IL-2, IL-4, IL-10 and IFN- γ were similar and each was able to amplify ≥ 10 copies of standards (data not shown).

This absolute quantification was obtained using a DNA-based standard curve derived from PCR product. It is also possible to obtain relative gene expression by serial dilution of a cDNA sample with high-level expression. We prepared standard curves of diluted cDNA and found that cDNA standard curves were linear with high correlations ($R^2 = 0.986 \pm 0.013$) similar to the linear standard curves of DNA standards. The mean slope of the cDNA standard curves (-4.0 ± 0.7) was different from that of the DNA standards (-3.25 ± 0.42) ($P = 0.01$) showing that cDNA standards amplify less efficiently than DNA standards.

Reproducibility of fluorogenic probe detection

The reproducibility of the fluorogenic probe detection system was tested in 25 kidney biopsy samples for a highly expressed target (G3PDH), for intermediate levels of expression (Gas6) and for low-level expression (IFN- γ). Two separate PCR reactions run on different days from stored aliquots of the same cDNA were compared by linear regression analysis (Fig. 2). There was highly reproducible measurement at all levels of gene expression when the C_T from each run were compared. The correlation coefficients (R^2) for C_T ranged from 0.74 ($P < 0.0001$) for G3PDH to 0.98 ($P < 0.0001$) for IFN- γ , showing that even at low levels of expression there was good reproducibility of amplification. The lower correlation coefficient for G3PDH compared to

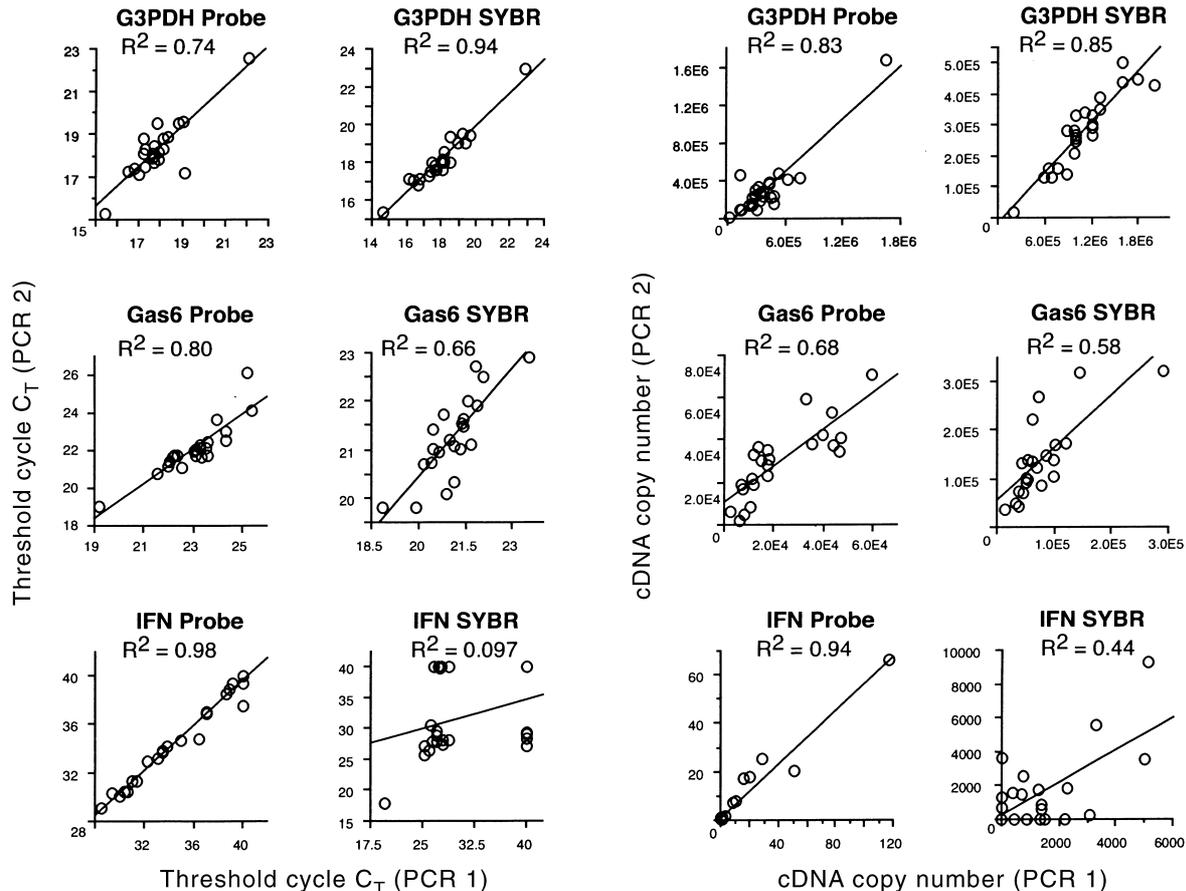


Figure 2 Reproducibility of amplification of samples using fluorogenic probes compared to SYBR Green I. Results are shown for glyceraldehyde-3-phosphate dehydrogenase (G3PDH), growth arrest-specific gene 6 (Gas6), and IFN- γ . Linear regression analysis of two separate PCR reactions amplified from aliquots of the same cDNA synthesis is shown. The horizontal axis shows the results of PCR1 and the vertical axis of PCR2. Comparison of reproducibility of threshold cycle (C_T) values for fluorogenic probe (column 1) and for SYBR Green I (column 2) and of the corresponding copy numbers calculated using external standards for fluorogenic probe (column 3) and SYBR Green I (column 4) are shown. The regression coefficient (R^2) is shown for each analysis. All results showed high reproducibility ($P < 0.0001$), except for SYBR Green I analysis of IFN- γ (P value = 0.076 for C_T and 0.004 for copy numbers).

IFN- γ was due in part to the smaller range of expression of G3PDH target in the samples, with most samples having C_T between 17 and 19, while for IFN- γ the high correlation reflects the wider range of C_T (from 29 to 40).

There was also very good reproducibility based on the number of copies of target, calculated from the external standards and shown in Fig. 2. There was a good correlation between PCR runs with R^2 ranging from 0.68 for Gas6 to 0.94 for IFN- γ . The correlation for estimated copy numbers for each of these three targets was in all cases lower than the correlation for the corresponding C_T , which was a pattern that was observed for all the cytokines and growth factors tested. This probably reflects the combined variations in amplification of both the sample and the standards required for the derivation of the number of copies.

Optimization of SYBR Green I quantification

Initial experiments examined the optimum concentration of primers, Taq polymerase enzyme and SYBR Green I dye.

These reaction conditions were found to be relatively constant for different primer/template combinations and are shown in Table 2. To obtain optimum specificity of detection of dsDNA with SYBR Green I, the temperature at which non-specific product had disappeared was determined by melting temperature analysis. Results of a typical analysis for no-template controls compared to specific targets are shown in Fig. 3. The multicomponent plot of the NTC (Fig. 3a) showed that at a temperature of 86°C the fluorescence signal from SYBR Green I was reduced to background levels. In contrast, there was still a high level of SYBR Green I fluorescence at a temperature of 86°C in the sample that contained cDNA template and the fluorescent signal did not decrease to background until a temperature of greater than 89°C (Fig. 3b). A melting temperature profile was performed for all cytokines and growth factors analysed by SYBR Green I and showed an almost identical pattern of melting. Consequently, a temperature of 86°C was selected as the optimum for IFN- γ , Gas6 and G3PDH, which had sizes ranging from 254 to 456 base pairs.

Sensitivity of SYBR Green I detection

The sensitivity of detection was tested in Buffer A and Buffer II + ROX and gave high efficiency in both buffer systems. The C_T for human/rat G3PDH and Gas6 in the two different buffer systems are shown in Table 4. There was little or no difference between the two buffers for efficiency of amplification as shown by the similar C_T values over the range of copy numbers tested, or of reproducibility, as shown by the low variability of the analysis. The main difference was that

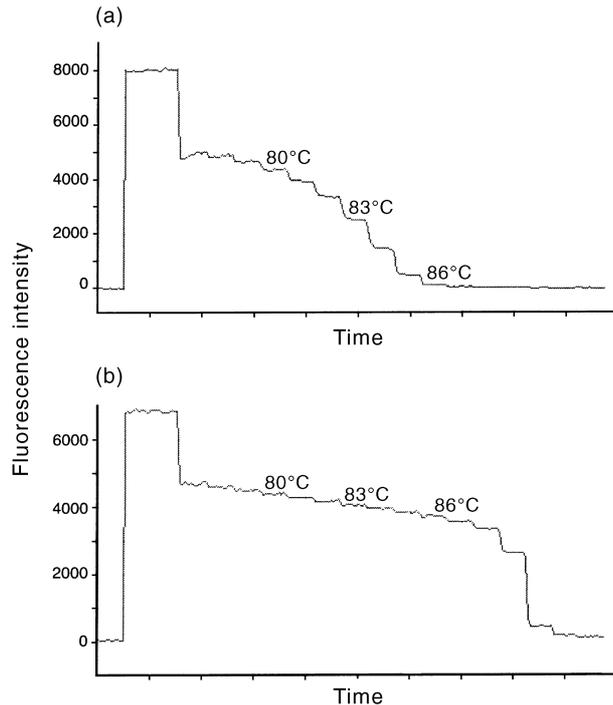


Figure 3 Optimization of fluorescence measurement for SYBR Green I by melting temperature analysis. The fluorescence signal generated by SYBR Green I binding to dsDNA amplified with primers for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) is shown in the no-template control (NTC) (a), compared to the positive control containing cytokine template (b). The melting temperature profile of the reaction consisted of an initial denaturation followed by re-annealing then a stepwise increase in temperature from 77°C to 92°C as described in the methods. At 86 °C there was no signal from the PCR artifact including primer-dimers present in the NTC while there was still significant signal in the positive control reaction.

Buffer A gave a lower background level for the NTC, shown by the C_T value of >40 cycles for both primer combinations tested.

Reproducibility of SYBR Green I detection

The reproducibility of the SYBR Green I detection system, tested in 25 kidney biopsy samples for a highly expressed target (G3PDH) for intermediate expression (Gas6) and for low-level expression (IFN- γ) is shown in Fig. 2. The aliquots of cDNA used for these experiments were from the same cDNA as used for fluorogenic probe quantification. Two separate PCR reactions run on different days from stored aliquots of the same cDNA were compared by linear regression analysis. There was highly reproducible amplification, based on comparison of C_T , for the highly expressed target G3PDH ($R^2 = 0.94$), where most samples expressed between 1×10^5 and 1.8×10^6 copies/reaction. There was also acceptable reproducibility for Gas6 ($R^2 = 0.68$; $P < 0.0001$), where most samples were expressed at intermediate levels of approximately 1×10^4 – 3×10^5 copies/reaction. Reproducibility of IFN- γ measurement using SYBR Green I was poor compared to the reproducibility of fluorogenic probes. SYBR Green I gave a poor correlation between two different PCR reactions ($R^2 = 0.10$) for C_T analysis compared to an R^2 of 0.98 for fluorogenic probe analysis of the same samples (Fig. 2). Similarly there was poor reproducibility of the SYBR Green I analysis for IL-2, which was also expressed at low levels (data not shown).

A further difference between SYBR Green I and fluorogenic probes was the different estimates of copy numbers obtained, particularly at low-level gene expression. This was most obvious for the number of copies/reaction of IFN- γ obtained with SYBR Green I (range 0– 9.3×10^3 , median 675) compared to fluorogenic probe (0–117, median 0.5). It was less apparent at higher level expression as shown by Gas6 (SYBR Green I range 1.2×10^4 – 3.2×10^5 , median 8.4×10^4 compared to fluorogenic probe range 2.2×10^3 – 7.1×10^4 , median 2.4×10^4). It was much less marked at the high level of expression of G3PDH (SYBR Green I range 2.1×10^4 – 2.0×10^6 , median 6.4×10^5 compared to fluorogenic probe range 1.1×10^4 – 1.6×10^6 , median 2.8×10^5).

Discussion

Two methods for fluorescence detection in real-time quantitative RT-PCR analysis of cytokine and growth factor expression were optimized and compared. Fluorogenic probes were

Table 4 Sensitivity of SYBR Green I detection in Buffer A and Buffer II + 6-carboxy-X-rhodamine (ROX)

	Buffer	Sensitivity (copies/reaction)	NTC	10 copies (C_T)	10^3 copies (C_T)	10^5 copies (C_T)
G3PDH	II + ROX	≤ 1000	33.7 ± 7.4	31.3 ± 3.5	22.4 ± 2.4	14.5 ± 0.9
	A	≤ 1000	> 40.0*	29.2 ± 1.6	22.6 ± 1.9	14.6 ± 1.1
Gas6	II + ROX	≤ 1000	35.2 ± 5.6	25.8 ± 0.5	20.8 ± 1.5	14.0 ± 0.1
	A	≤ 1000	> 40.0	27.7 ± 0.7	22.3 ± 1.6	14.9 ± 0.9

*The no-template control (NTC) was not amplified in the 40 cycle PCR reaction and a value of > 40 was allotted. G3PDH, glyceraldehyde-3-phosphate dehydrogenase; Gas6, growth arrest-specific gene 6; ROX, 6-carboxy-X-rhodamine.

extremely sensitive, detecting fewer than 10 copies of target per reaction and providing excellent reproducibility of analysis at this level of detection. These probes were specific for the processed form of the genes, present in mRNA and not the genomic form, as no RT controls were consistently negative for all cytokines and growth factors. No RT controls for G3PDH were always positive, due to the presence of processed pseudogenes in contaminating DNA in the RNA preparation.¹² In spite of this, the level of false positive expression of G3PDH in the no RT controls was always markedly less than the level of mRNA expression in the samples and did not affect accurate quantification. SYBR Green I analysis of genes expressed at moderate to high levels gave excellent reproducibility; however, the reproducibility was poor for IFN- γ , which was expressed at lower levels.

The fluorogenic probe system proved to be more difficult to optimize than SYBR Green I and was more sensitive to the buffer conditions used. The sensitivity of this system to the reaction conditions might have been due to the fluorogenic probe interfering with amplification by the forward and reverse primers or to dependence of probe cleavage on the reaction conditions. The original description, which used exonuclease cleavage of labelled probe for quantitative PCR, found that the presence of probe did not affect the efficiency of amplification.¹⁵ Subsequent studies have found that the efficiency of probe cleavage was dependent on a range of conditions, including the type and concentration of divalent cation¹⁶ or the type of DNA polymerase used.¹⁷ In spite of the dependence of the fluorogenic probe system on the reaction conditions, under optimum conditions, the reaction progressed with high efficiency (approximately 100%) leading to high sensitivity of detection. SYBR Green I amplified at optimal efficiency in a wider range of buffers than the fluorogenic probe system, possibly because it was not dependent on a labelled oligonucleotide that could potentially reduce the efficiency of amplification or that had a requirement for efficient nucleolytic cleavage.

The experiments shown used external standards for the quantification of cytokines or growth factors. These consisted of purified PCR products that spanned the target regions for forward and reverse primers used for fluorogenic probe or SYBR Green I detection. These standards were useful to estimate the sensitivity of the reaction and could also be used to derive an estimate of the number of copies of cytokine mRNA present in the unknown samples as we have previously described.¹² It is unlikely that this estimate is an accurate reflection of the actual number of cytokine mRNA molecules present in the sample for several reasons. The estimate does not take account of the efficiency of purification of RNA from the original sample or of cDNA synthesis during reverse transcription. Neither does it account for differences in efficiency of amplification between cDNA of samples and PCR product of standards. The results here show that DNA standards made from PCR product amplified more efficiently than cDNA standards in the fluorogenic probe assay. This latter difference might have been the basis of discrepancies between fluorogenic probes and SYBR Green I in the estimation of gene copy numbers. Measurement of IFN- γ expression by the fluorogenic probe method gave an estimate of copy numbers approximately 100-fold less than

the estimate using SYBR Green I. This difference was less marked for Gas6, which was expressed at higher levels and was barely detected for G3PDH, the gene expressed at the highest level. Less efficient amplification of samples than standards in the fluorogenic probe system would provide an explanation for this difference.

In spite of this, the use of the same external standards allows for the comparison of results obtained in separate PCR runs on different machines or in separate laboratories. They also provide a numerical estimate of the differences in cytokine expression levels between samples. Ribonucleic acid standards are likely to overcome some of the limitations of standards prepared from PCR product described, but are much more time consuming to prepare and are less stable than DNA standards.

There have been few reports examining the reproducibility and sensitivity of fluorogenic probes to measure low-level human cytokine mRNA expression especially with primers and probes for short amplicons.^{9,10} The efficiency of PCR amplification is higher for short amplicons, which should lead to improved sensitivity of detection.¹⁸ Quantification of murine,¹⁹ equine²⁰ and feline²¹ mRNA expression has also been reported using fluorogenic probes and the sensitivity of this system for murine IL-12 and β -actin has been examined.¹⁹ In this latter case the method was able to detect ≤ 1000 copies of IL-12 plasmid DNA and ≤ 100 copies of β -actin plasmid. Sensitivity of feline G3PDH primers and probe was reported to be ≤ 10 copies/reaction.²¹ The efficiency of all our fluorogenic primer/probe combinations, which were designed to amplify short amplicons, was tested and found to be highly sensitive, with detection of ≤ 10 copies/reaction.

The sensitivity of detection of cytokines or growth factors using SYBR Green I dye for the target genes that we examined was ≤ 1000 copies/reaction. SYBR Green I has previously been used for quantification of rat cytokines IL-1 β , IL-6, TNF- α , GM-CSF, granulocyte colony-stimulating factor (G-CSF) and MCP-1.⁷ Although this method was initially unable to measure < 1000 copies of template per reaction, its sensitivity was improved to ≤ 10 copies per reaction by increasing the temperature of fluorescence reading to 86°C and by using a heat-denaturable antibody to Taq polymerase enzyme to prevent PCR artifact formation.²² Our experiments using SYBR Green I dye also used high-temperature measurement of fluorescence at 86°C and a hot-start enzyme that was inactive until commencement of thermal cycling. This method reproducibly amplified G3PDH and Gas6, which were expressed at $\geq 10\,000$ copies per reaction. We were, however, unable to obtain reproducible measurement of IFN- γ , which was expressed at lower levels, ranging from 0 to 9300 (median 900) copies per reaction.

Our results demonstrate that the ability of SYBR Green I dye to reproducibly measure gene expression was thus, in our hands, limited to genes that were expressed at ≥ 1000 copies/reaction. The ability of SYBR Green I to reproducibly measure cytokines or growth factors that are expressed at lower levels might be improved by the use of alternative primers that are better able to specifically amplify the target. Further optimization of the reaction to favour amplification of the specific PCR product and minimization of PCR artifact could also improve sensitivity. Also, dedicated software

that can more accurately measure and control the temperature of PCR artifact melting, such as that available with some recently developed real-time PCR machines, might improve the sensitivity of detection.

In conclusion, we report the rapid and reproducible quantification of cytokine and growth factor mRNA in small samples using real-time RT-PCR. Two methods were used for fluorescent detection of PCR product: fluorogenic probes and SYBR Green I dye. Both methods gave rapid and reproducible amplification of gene expression at expression levels of >1000 copies/reaction. SYBR Green I was simpler and cheaper than fluorogenic probes as it could be used with any set of amplification primers. In contrast, fluorogenic probes were more difficult to design; more demanding in terms of the reaction conditions required for optimum amplification and were expensive to synthesize. The advantage of fluorogenic probes was that they were exquisitely sensitive and detected ≤ 10 copies of cytokine or growth factor per reaction for all primer and probe sets tested. This resulted in reproducible measurement of cytokines at low-level expression. Additionally, the internal probe increases the specificity of detection. In our experience, fluorogenic probes were thus superior to SYBR Green I for measurement of genes expressed at low levels of <1000 copies per reaction.

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