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QUANTIFICATION OF MURINE CYTOKINE mRNAs USING REAL TIME QUANTITATIVE REVERSE TRANSCRIPTASE PCR

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Recently, a novel technique for "real time" quantitative Reverse Transcriptase-PCR which measures PCR-product accumulation during the exponential phase of the PCR reaction using a dual-labelled fluorogenic probe, has been developed. This method allows direct detection of PCR-product formation by measuring the increase in fluorescent emission continuously during the PCR reaction. Here we present data validating this PCR-method for the quantification of murine cytokines and other factors playing a role in immune regulation (IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p40, IL-13, IL-15, IFN-γ, TNF-α, TGF-β and iNOS). For each substance of interest, a set of primers and internal probe was designed, which specifically amplify the target cDNA, not co-amplifying contaminating genomic DNA. Furthermore, a corresponding reference plasmid cDNA clone was constructed, allowing direct quantification. Additionally, normalization to the housekeeping genes β-actin or GAPDH was performed. The assay is very sensitive and accurate. It is a "closed-tube" PCR reaction, avoiding time-consuming and hazardous post-PCR manipulations and decreasing the potential risk of PCR contamination.

Cytokines are important mediators of the immune system. Quantification of these products locally in inflammation sites, such as transplanted grafts or organs under autoimmune attack, is essential in gaining insight in the immune processes.[1–3]

Reverse Transcriptase PCR (RT-PCR) is a powerful method used to quantify the mRNA expression of cytokines, which are often expressed at very low levels. Different methods of (semi-) quantitative PCR and RT-PCR have been described for measuring cytokine mRNA levels.[4–6] One method measures PCR product accumulation during the exponential phase of the reaction.[7,8] To obtain semi-quantitative results it is extremely important that the PCR product is measured during the log phase of the reaction, thus before saturation is reached. This is the major difficulty using this method, because it necessitates interruption of the PCR reaction after an experimentally determined number of cycles. A drawback is the relatively small linear range, during which samples within a single experimental setup can be analysed.

Alternatively, quantitative competitive PCR can be performed.[5,6,9] This method requires co-amplification of an internal cDNA or RNA control (competitor) with the unknown sample in the same tube. The amount of mRNA is quantified by titration of an unknown amount of target template against a dilution series of known amounts of the standard. The internal control consists of target DNA or RNA that has been slightly modified. Thus, the same primers are used to co-amplify the target and the competitor, in such a way that they amplify with the same efficiency, although they can be distinguished from each other (by difference in length or restriction sites). Although this method provides a strategy for accurate quantification, the construction of internal standards is technically sophisticated and labour intensive.

For the detection of PCR products using either of these methods, several detection techniques can be used, all requiring excessive post-PCR manipulations. The most common used are agarose gel electrophoresis and EtBr-staining,[7] fluorescent labelling and analysis using polyacrylamide gels,[9] radioactive labelling and Southern blotting[8] or detection by phosphorimaging.[10] Major drawbacks using these classical detection systems are the use of hazardous chemicals and the

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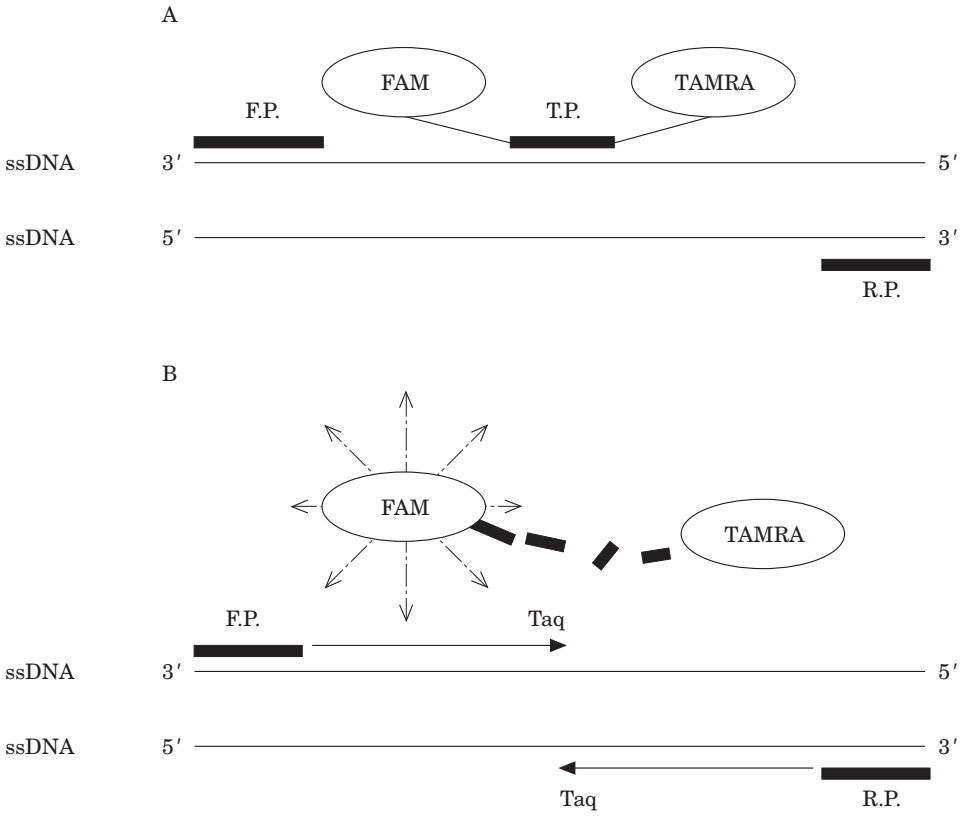

Figure 1. Schematic representation of the "TaqMan" principle.

During PCR annealing, primers and fluorogenic probe anneal to the denatured DNA template. As long as the probe is intact the 3′ located quencher dye (TAMRA) absorbs the fluorescent emission of the 5′ located reporter dye (FAM). During the extension phase, the probe is cleaved by the 5′nuclease activity of the Taq Polymerase, separating the reporter from the quencher signal, which results in an increase in fluorescence emission of the reporter dye. F.P., forward primer; R.P., reverse primer; T.P., fluorogenic probe; Taq, Taq Polymerase.

potential risk for laboratory contamination. Moreover, all these post-PCR manipulations are very time consuming.

In this article we describe a recently developed, quick and accurate technique for measuring PCR-product accumulation during the exponential phase of the reaction: real-time quantitative RT-PCR.[11,12] The system uses the ABI prism 7700 sequence detector (TaqMan; Perkin Elmer/ Applied Biosystems, Foster City, CA).

Real-time PCR uses a non-extendible fluorogenic probe to monitor PCR product formation continuously during the PCR reaction. This probe is an oligonucleotide, dually labelled with a reporter dye (FAM, 6-carboxyfluorescein) covalently attached at the 5′ end and a quencher dye (TAMRA, 6-carboxytetramethylrhodamine) covalently attached at the 3′ end (Fig. 1). The proximally located quencher dye absorbs the emission of the reporter dye as long as the probe is intact. During the extention phase of the PCR reaction, the hybridized probe is hydrolysed by the 5′-nuclease activity of the Taq-polymerase, separating the quencher from the reporter. This results in an increase in fluorescence emission of the reporter dye, which is quantitative for the initial amount of template. The ABI-prism 7700 sequence detector allows measurement of the fluorescent spectra of all 96 wells of the thermal cycler in real-time. Using the fluorescent emission data, collected during the PCR amplification, the software constructs amplification plots. The measurements from cycle 3 until 15 are considered as the baseline and its standard deviation is calculated. Ct (Threshold cycle) values are calculated by determining the point at which the fluorescence exceeds a threshold limit: 10 times the standard deviation of the baseline. On-line software determines Ct values and plots these to an included standardcurve with known concentrations to determine starting copy numbers of the unknown samples.

As this method combines PCR amplification and product detection in one single step, the technique is very fast and easy to perform, compared to classical RT-PCR techniques. Ct-values also provide accurate measurements over a wide range of starting quantities. Furthermore, the use of an internal probe, guarantees the specificity of the PCR product to be measured.

In the present paper we describe the validation of this technique for several murine cytokines and other

factors playing a role in the immune system. A specific set of primers and internal fluorogenic probe was designed and optimized for each of the target genes. To obtain standardized quantitative results, external controls were constructed, consisting of cDNA plasmid standards. To compensate for variations in input RNA amounts, and efficiency of reverse transcription, an endogenous "housekeeping" gene (β-actin or GAPDH) was also quantified, and results were normalized to these values.

RESULTS

Primer and probe design

Primers and probes for the cytokines and other substances of interest, as well as for the housekeeping genes β-actin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were designed using the computer program Primer Express (Perkin Elmer/Applied Biosystems, Foster City, CA) (Table 1). Except for β-actin, primers were always located in two different exons. Moreover, in case of small introns, either one of the primers or the probe were located on an intron-exon junction to prevent co-amplification of genomic DNA. The melting temperature (T_m) was 57–60°C. The probe T_m was at least 10°C higher than the primer T_m, thus approximately 70°C. The presence of a guanidine at the 5′ end was avoided, as this base slightly quenches the reporter signal, even after probe cleavage. The amplicon lengths were kept very short. Generally, amplicons up to 300 bp amplified efficiently.

For each target gene different $MgCl_2$ (3 to 9mM) and primer concentrations (100 to 200 nM) were tested to optimize the PCR amplification. However, for all cytokines, identical thermal cycling conditions were used: 15 s at 94°C and 1 min at 60°C, with a total of 40 to 45 cycles.

Use of cDNA standards

Specific external controls were constructed for all target genes of interest by cloning a partial cytokine cDNA fragment (obtained by PCR amplification) into a pGEM-Teasy plasmid vector (as described in Materials and Methods). The exact identity of the cloned amplicons was confirmed by sequence analysis (ALFexpress, Pharmacia, Uppsala, Sweden). Overall, in eight of the clones 100% sequence identity was found, while in the other six clones a maximum of two point mutations were present in the complete cloned sequence. Importantly, mismatches were not located in the primer or internal probe sequences, therefore PCR efficiency will not be altered. These plasmid clones allow the cytokine cDNA levels of the target samples to be quantified directly. Indeed, in each PCR run, serial dilutions of the corresponding plasmid clone were included, with known amounts of input copy number. In Figure 2A amplification plots of 10-fold serial dilutions of the IL12 plasmid clone are shown, with concentrations ranging from 1×10^{11} down to 1×10^{3} copies of input cDNA. For each dilution, ΔRn (increase in fluorescence emission due to template amplification, subtracted by the background fluorescence signal) was measured and plotted against cycle number. By plotting Ct values against the known input copy number, a standard curve is generated, with a linear range covering 8 log units (Fig 2B).

Normalization to a housekeeping gene

The PCR method chosen quantifies PCR product during the log phase of the reaction. This method requires equal input amounts of RNA for all samples. A housekeeping gene, which is present at constant amounts in all samples, can be used to correct for these minor variations.

In each experimental setup an aliquot of each target sample was analysed by quantitative PCR for β-actin or GAPDH mRNA expression, in order to normalize for inefficiencies in cDNA synthesis and RNA input amounts. Copy numbers obtained for β-actin (or GAPDH) for the target samples were divided by the highest β-actin (or GAPDH) value obtained in the experiment, resulting in a correction factor. Following PCR amplification and quantification of the cytokines, this factor was then used for normalization.

Control assays

Quality assurance

To exclude PCR amplification of contaminating genomic DNA different controls were performed. First, RT^- controls (samples containing RNA which was not reverse transcribed) were included in each PCR reaction. Second, a negative control containing genomic DNA was included. Finally, gel electrophoresis was performed to confirm the correct size of the amplicons and the absence of aspecific bands.

If RT^- controls were positive, as was the case for β-actin and GAPDH, two genes known to have corresponding pseudogenes,[14,15] the following equation was used to substract the percentage of genomic DNA contamination:

$$2^{[Ct(RT^+) - Ct(RT^-)]}$$

However, due to the very high mRNA expression of both these genes, contaminating genomic DNA was always less than 1% of the cDNA sample, thus being neglectable.

TABLE 1. Primer and probe sequences for the cytokines and other factors important in immune responses, as well as for β-actin and GAPDH

	Name	Sequence (5′→3′)	Amplicon length (bp)
IL-1	IL1-TP	CTG TGT AAT GAA AGA CGG CAC ACC CACC	152
	IL1-RV	GAT CCA CAC TCT CCA GCT GCA	
	IL1-FW	CAA CCA ACA AGT GAT ATT CTC CAT G	
IL-2	IL2-TP	CCC AAG CAG GCC ACA GAA TTG AAA G	141
	IL2-RV	TCC AGA ACA TGC CGC AGA G	
	IL2-FW	CCT GAG CAG GAT GGA GAA TTA CA	
IL-4	IL4-TP	TCC TCA CAG CAA CGA AGA ACA CCA CA	95
	IL4-RV	GAA GCC CTA CAG ACG AGC TCA	
	IL4-FW	ACA GGA GAA GGG ACG CCA T	
IL-5	IL5-TP	CTG TTG ACA AGC AAT GAG ACG ATG AGG	116
	IL5-RV	TCC AAT GCA TAG CTG GTG ATT T	
	IL5-FW	AGC ACA GTG GTG AAA GAG ACC TT	
IL-6	IL6-TP	GAG GAT ACC ACT CCC AAC AGA CC	141
	IL6-RV	AAG TGC ATC ATC GTT GTT CAT ACA	
	IL6-FW	CAG AAT TGC CAT CGT ACA ACT CTT TTC TCA	
IL-7	IL7-TP	CCT CCC GCA GAC CAT GTT CCA TGT	231
	IL7-RV	GTT CCT GTC ATT TTG TCC AAT TCA	
	IL7-FW	ATT ATG GGT GGT GAG AGC CG	
IL-10	IL10-TP	TGA GGC GCT GTC GTC ATC GAT TTC TCC C	190
	IL10-RV	ACC TGC TCC ACT GCC TTG CT	
	IL10-FW	GGT TGC CAA GCC TTA TCG GA	
IL-12p40	IL12p40-TP	CAT CAT CAA ACC AGA CCC GCC CAA	180
	IL12p40-RV	AAC TTG AGG GAG AAG TAG GAA TGG	
	IL12p40-FW	GGA AGC ACG GCA GCA GAA TA	
IL-13	IL13-TP	CGG GTT CTG TGT AGC CCT GGA TTC C	123
	IL13-RV	TGG GTC CTG TAG ATG GCA TTG	
	IL13-FW	AGA CCA GAC TCC CCT GTG CA	
IL-15	IL15-TP	AGG GAG ACC TAC ACT GAC ACA GCC CAA AA	126
	IL15-RV	CAT CTA TCC AGT TGG CCT CTG TTT	
	IL15-FW	CAT CCA TCT CGT GCT ACT TGT GTT	
iNOS	iNOS-TP	CGG GCA GCC TGT GAG ACC TTT GA	95
	iNOS-RV	CAT TGG AAG TGA AGC GTT TCG	
	iNOS-FW	CAG CTG GGC TGT ACA AAC CTT	
IFN-γ	IFN-γ-TP	TCA CCA TCC TTT TGC CAG TTC CTC CAG	92
	IFN-γ-RV	TGG CTC TGC AGG ATT TTC ATG	
	IFN-γ-FW	TCA AGT GGC ATA GAT GTG GAA GAA	
TGF-β	TGF-β-TP	TTC AGC GCT CAC TGC TCT TGT GAC AG	140
	TGF-β-RV	GGT TCA TGT CAT GGA TGG TGC	
	TGF-β-FW	TGA CGT CAC TGG AGT TGT ACG G	
TNF-α	TNF-α-TP	CAC GTC GTA GCA AAC CAC CAA GTG GA	175
	TNF-α-RV	TGG GAG TAG ACA AGG TAC AAC CC	
	TNF-α-FW	CAT CTT CTC AAA ATT CGA GTG ACA A	
β-Actin	β-actin-TP	CAC TGC CGC ATC CTC TTC CTC CC	148
	β-actin-RV	CAA TAG TGA TGA CCT GGC CGT	
	β-actin-FW	AGA GGG AAA TCG TGC GTG AC	
GAPDH	gapdh-TP	TGC ATC CTG CAC CAC CAA CTG CTT AG	236
	gapdh-RV	GGC ATG GAC TGT GGT CAT GA	
	gapdh-FW	TTC ACC ACC ATG GAG AAG GC	

Except for β-actin, forward (F.P) and reverse (R.P.) primer are always located in different exons. Fluorogenic probes (T.P) are FAM-labelled at the 5′end and TAMRA-labelled at the 3′end. All cDNA sequences were obtained from the Genbank database. Il, interleukin; IFN-γ, interferon-gamma; TGF-β, transforming growth factor β; iNOS, inducible Nitric Oxide Synthetase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

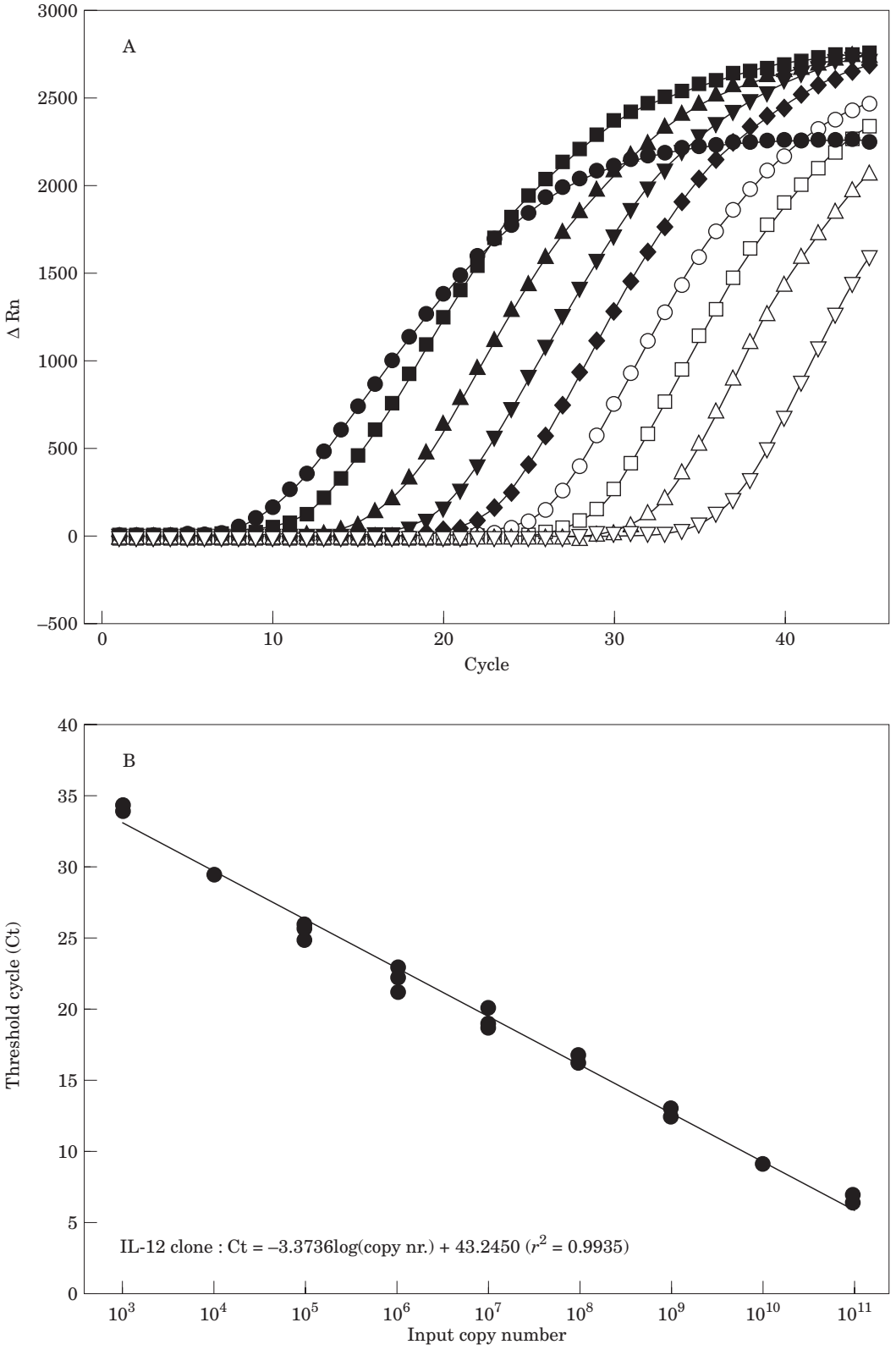

Figure 2. A: Amplification plots of IL12 plasmid cDNA. Tenfold serial dilutions of IL12 cDNA were PCR amplified using the TaqMan method. Fluorescence emmision is measured continuously during the PCR and ΔRn (increase in fluorescence emission, substracted by the background fluorescence signal) is plotted against cycle number. (●), 10^{11}; (■), 10^{10}; (▲), 10^9; (▼), 10^8; (◆), 10^7; (○), 10^6; (□), 10^5; (△), 10^4; (▽), 10^3. B: Standard curve for IL12, generated by plotting Ct values (obtained from the amplification plots in A) against input cDNA copy number.

TABLE 2. Reproducibility of the assay

Assay	Sample 1		Sample 2	
	Ct	Copynr	Ct	Copynr
1A	27.11	53 184	25.76	103 489
1B	27.33	47 717	26.22	82 372
1C	27.37	46 690	26.27	80 320
1D	26.81	61 622	25/73	104 975
Mean ± SE (1)		52 303 ± 3417		92 789 ± 6627
2A	27.00	50 595	25.64	103 864
2B	26.94	52 015	25.79	95 847
2C	27.03	49 696	25.69	101 156
Mean ± SE (2)		50 769 ± 675		100 289 ± 2354
3A	25.56	40 066	24 33	91 137
3B	25.37	45 372	24 14	103 877
Mean (3)		42 719		97 507
Mean ± SE (total)		49 622 ± 1989		96 337 ± 3209

Quantification for IFN-γ by PCR of two different cDNA target samples. PCR amplification was performed in 3 different runs (1, 2, 3) in quadruplicate (1A–D), triplicate (2A–C) or duplicate (3A,B) wells.

Efficiency tests

Reproducibility of the PCR method was examined by intra- and inter-assay amplification. PCR consumables were always prepared as a mixture, while cDNA aliquots were added individually to each well. The results from triplicate wells of 10 different target samples amplified for IFN-γ showed a minimal variation, with standard errors ranging from 2.4 to 12.3% (example in Table 2). Furthermore, inter-assay variation in PCR efficiency was examined by amplification of five different target samples during three different PCR runs. Although Ct values between different assays showed some variation, input copy numbers were very reproducible, due to a similar shift in Ct values in the included standard curve. Again, standard errors on the obtained relative input copy numbers were very low, ranging from 4.0 to 7.3% (Table 2). These tests conclusively demonstrate a high reproducibility of PCR amplification using the ABI Prism 7700 instrumentation.

Finally, efficiency of PCR-amplification of target cDNA versus external control plasmid DNA was examined, to measure colinearity of dilution. Serial 5-fold dilutions of both target cDNA and control plasmid cDNA were PCR amplified, as shown for β-actin (Fig 3). Each dilution was performed in duplicate wells. A standard curve was generated by plotting Ct values against relative input copy numbers. The linear range of dilution for the plasmid control as well as for the target covered more than 4 log units. Both curves showed a similar slope, demonstrating an identical amplification efficiency for target samples and control plasmid, over the wide range tested.

DISCUSSION

We validate a recently developed RT-PCR technique for the detection of murine cytokines and other factors playing an important role in immune regulation. The method combines PCR amplification and product detection in a single step, by continuously measuring PCR product accumulation throughout the PCR reaction.[11,12] By construction of cDNA plasmid clones for each target of interest, standard curves can be generated which allow direct quantification of every

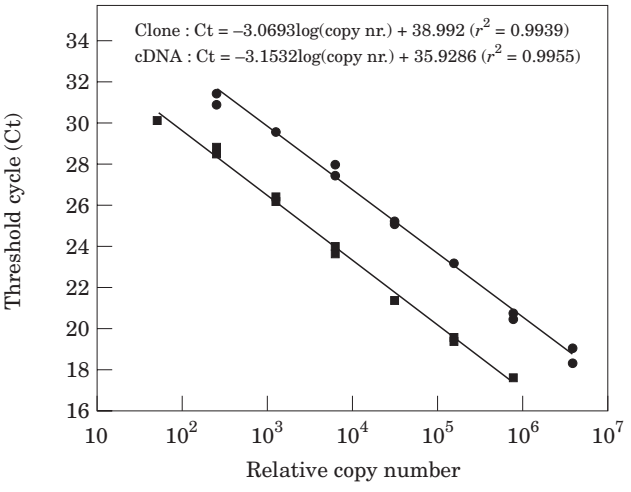

Figure 3. Efficiency of PCR amplification of target and plasmid control cDNA.

Fivefold serial dilutions of target and external control cDNA, prepared in duplicate wells were amplified for β-actin using the TaqMan system. The obtained Ct values are plotted against relative copy number. (●), clone; (■), cDNA.

unknown sample. Furthermore, minor differences in input RNA quantity and efficiency of cDNA synthesis are corrected for by normalization to β-actin or GAPDH.

We have conclusively demonstrated that amplification using this PCR technique is linear over a wide range of input copies (more than 8 log units), with high precision and reproducibility of amplification for input copy numbers ranging from 10^{11} down to 10^3 copies. Indeed, intra-assay as well as inter-assay variation was minimal, with standard errors of maximal 12.3 and 7.3%, respectively. Moreover, input cDNA levels as low as 100 copies could be detected, although with a somewhat higher intra assay variation (results not shown). This makes the method very attractive for mRNA quantification of different cytokines, often present at very low concentrations in transplanted grafts or organs under immune attack.

Amplification of contaminating genomic DNA was avoided by designing sets of primers located in different exons, or on intron/exon junctions. Using this approach a DNase treatment of the RNA samples prior to reverse transcription is unnecessary. Such DNase treatment can be very inconvenient, especially if small tissue samples or small amounts of cells are to be analysed, where loss of RNA sample may be critical.

The applicability of the method was illustrated in studies performed in our lab in non-obese diabetic (NOD) mice.[16,17] The NOD mouse is an animal model for human type-1 diabetes or insulin-dependent diabetes mellitus, an organ specific autoimmune disease characterized by destruction of β-cells in the pancreatic islets by cells of the immune system.[18] Both studies address the protection against type 1 diabetes using a combination treatment of analogues of 1,25-dihydroxyvitamin D3 and cyclosporin A. Using semi-quantitative RT-PCR, as described here, the protective effect of this combination therapy could be explained by effects on the type of immune cells infiltrating the pancreases[16] or transplanted islet grafts[17], as reflected by local cytokine production. In these studies, a significant increase of IL-4 was observed locally in the pancreases or in the transplanted grafts, whereas IL-12, IL-2 and especially IFN-γ levels were decreased. These findings are in agreement with other studies regarding the role of cytokines in the pathogenesis of autoimmune diabetes. The Th1 cytokines, IL-2 and IFN-γ, seem to be involved in the β-cell destructive infiltrate and are associated with destruction of syngeneic islet grafts and diabetes recurrence in NOD mice.[19] On the other hand, Th2 cytokines, more specifically IL4, seem to protect against type 1 diabetes and recurrence of the disease after islet transplantation.[20]

In conclusion, we have validated a recently developed technique for real-time PCR detection of several cytokines and other factors playing an important role in immune responses. The technique is fast, accurate and sensitive, compared to previously described RT-PCR methods, and has a decreased potential for PCR contamination as it is performed in a "closed tube" reaction. The method helps to gain more insight in the functional role of immune cells and the cytokines they produce in the destruction of transplanted tissues or organs under immune attack.

MATERIALS AND METHODS

RNA extraction and cDNA synthesis

Total RNA was extracted from different mouse tissues. Isolated tissues were used immediately or were frozen in liquid nitrogen and stored at −80°C until use. RNA extractions from spleen and lymph nodes were performed using TRIzol reagent (Life Technologies, Gaithersburg, MD), as described, while the classical guanidinium isothiocyanate method,[13] was used to isolate RNA from pancreases.

Target RNA (1–5 μg) was reverse transcribed using 100U SuperscriptII RT (Life Technologies, Gaithersburg, MD) at 42°C for 80 min in the presence of 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT, 0.5 mM dNTPs, 8 U RNasin (Promega Corp., Madison, WI) and 5 μM $Oligo(dT)_{16}$ (Perkin-Elmer/Applied Biosystems, Foster City, CA). For every reaction set, one RNA sample was performed without SuperscriptII RT (RT^- reaction) to provide a negative control in subsequent PCR reactions.

Primers and probes

PCR primers and fluorogenic probes for all target genes were designed using the computer program Primer Express and were purchased from Perkin-Elmer. The fluorogenic probes contained a reporter dye (FAM) covalently attached at the 5′end and a quencher dye (TAMRA) covalently attached at the 3′end. Extension from the 3′end was blocked by attachment of a 3′phophate group. Fluorogenic probes were HPLC purified.

cDNA standards

External controls were constructed consisting of plasmid standards for each target of interest, as well as for β-actin and GAPDH. Total RNA was extracted from spleen, and cytokine cDNA fragments were generated by RT-PCR using the same primers as described above. Each of these amplicons was purified on silica columns (QIAquick PCR purification; Qiagen, Chatsworth, CA) and cloned into pGEM-Teasy (Promega Corp, Madison, WI). Ligated fragments were transformed into DH5a competent cells (Life Technologies, Gaithersburg, MD) and plasmid DNA was prepared using silica cartridges (Nucleobond AX plasmid purification; Macherey-Nagel, Düren, Germany). The exact sequence of the cloned amplicons was analysed by cycle sequencing (Thermosequence fluorescent labelled primer sequencing kit; Amersham Pharmacia; Uppsala, Sweden) with M13 universal primers, using the ALFexpress instrumentation. cDNA plasmid concentrations were measured by optical density spectrophotometry (Pharmacia, Uppsala,

Sweden) and the corresponding copy number was calculated using the following equation:

$$1\text{–}\mu g \text{ of } 1000 \text{ bp DNA} = 9.1 \times 10^{11} \text{ molecules}$$

Serial dilutions from the resulting clones were used as standard curves, each containing a known amount of input copy number.

PCR amplification

PCR reactions were performed in the ABI-prism 7700 sequence detector, which contains a Gene-Amp PCR system 9600 (Perkin Elmer/Applied Biosystems, Foster City, CA). Reaction conditions were programmed on a Power Macintosh 7200, linked directly to the Sequence Detector.

PCR amplifications were performed in a total volume of 25 µl, containing 0.5 µl cDNA sample, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 10 mM EDTA, 60 nM Passive Reference 1, 200 µM dATP, dCTP, dGTP and 400 µM dUTP, 3 to 9 mM MgC_{12}, 100 to 200 nM of each primer, 0.625 U AmpliTaqGold and 0.25 U AmpErase Uracil N-Glycosylase (Perkin Elmer/Applied Biosystems, Foster City, CA). Each reaction also contained 100 nM of the corresponding detection probe (Table 1).

Each PCR amplification was performed in triplicate wells, using the following conditions: 2 min at 50°C and 10 min at 94°C, followed by a total of 40 or 45 two-temperature cycles (15 s at 94°C and 1–min at 60°C).

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