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Journal of Immunological Methods 259 (2002) 55–64

JIM
Journal of Immunological Methods

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Cytokine mRNA quantification by real-time PCR

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Received 12 March 2001; received in revised form 18 May 2001; accepted 2 August 2001

Abstract

Real-time PCR represents a new methodology that accurately quantifies nucleic acids. This has been made possible by the use of fluorogenic probes, which are presented in two forms, namely hydrolysis probes (also called TaqMan probes) and hybridisation probes. We decided to apply this methodology to cytokine mRNA quantification and this led us to the development of a protocol that provides an easy way to develop and perform rapidly real-time PCR on a Lightcycler instrument. It was made possible by the use of freely available software that permits a choice of both the hydrolysis probe and the primers. We firstly demonstrated that the reproducibility of the method using hydrolysis probes compares favourably with that obtained with hybridisation probes. We then applied this technique to determine the kinetics of IL-1ra, IL-1β, IL-5, IL-13, TNF-α and IFN-γ induction upon stimulation of human peripheral blood mononuclear cells (PBMC) by phytohaemagglutinin (PHA). Finally, the method was also used successfully to demonstrate that IFN-α induces IL-10 mRNA accumulation in human monocytes. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Lightcycler; TaqMan; Real-time PCR; Cytokine; IL-10; IFN-α

1. Introduction

RT-PCR has become a popular technique with which to obtain insight into the complexity of the immune response. The easy detection of cytokine mRNA trancripts in a limited number of cells where the corresponding protein could barely be measured is probably one of the major advantages of the technique. Nevertheless, RT-PCR suffers from the drawback that it is difficult to quantify accurately the amount of these transcripts. To circumvent this, several (semi)-quantitative RT-PCR techniques have been developed during the last decade, including real-time (or kinetic) PCR, which appears nowadays to be the most accurate.

Real-time PCR is so called because the amplicon accumulation can be directly monitored during the PCR process, using fluorogenic probes. Two kinds of such probes are currently used, namely the hydrolysis probes (TaqMan probes) that take advantage of the $5' \rightarrow 3'$ exonuclease activity of *Thermus aquati-*

Abbreviations: PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; FCS, foetal calf serum; LPS, lipopolysaccharide; CN, copy number.

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PII: S0022-1759(01)00489-6

cus DNA polymerase (Holland et al., 1991; Heid et al., 1996; Livak et al., 1995), and the hybridisation probes that use the fluorescence resonance energy transfer (FRET) phenomenon (Wittwer et al., 1997b; Lay and Wittwer, 1997). Usually, TaqMan probes are used on a GeneAmp 5700 or an ABI PRISM 7700 apparatus (Applied Biosystems), and FRET probes on a Lightcycler apparatus (Roche Diagnostics, Brussels, Belgium) (Wittwer et al., 1997a). Here, we describe a simplified strategy to develop and perform a real-time PCR for cytokine mRNAs using TaqMan probes on a Lightcycler. First, we present results obtained on both instruments for reproducibility testing of mouse IL-9 DNA quantification. Thereafter, we applied this protocol to develop real-time PCR for human IL-1ra, IL-1β, IL-5, IL-13, TNF-α, IFN-γ and β-actin mRNAs, permitting us to monitor the induction of these cytokines upon polyclonal activation of human peripheral blood mononuclear cells (PBMC). Finally, real-time PCR for human IL-10 mRNA allowed us to evaluate further the induction of this cytokine by IFNα in monocytes.

2. Materials and methods

2.1. Cells

PBMC were prepared from healthy donors by centrifugation of heparinized venous blood on Lymphoprep (Nycomed, Oslo, Norway). Human monocytes were isolated from PBMC by a two-cycle clumping method as described in a previous study (Stordeur et al., 1995). Cells were cultured in RPMI 1640 medium + 10% foetal calf serum (FCS), in a 5% CO_2 atmosphere incubator, at 2 million cells/ml. Phytohaemagglutinin (PHA) and lipopolysaccharide (LPS) were purchased at Sigma-Aldrich (Bornem, Belgium), and IFN-α (IFN-α2b, Intron A®) came from Schering-Plough (Brinny) (Innishannon, Ireland).

2.2. Total RNA isolation and reverse transcription

Total RNA was isolated using a commercially available reagent (Tripure™, Roche Diagnostics) following the manufacturer's instructions. In some cases, total RNA was treated with 10 units of RQ1 RNase-free DNase (Promega, Madison, WI) for 30 min, in order to avoid amplification of contaminating genomic DNA (see footnote "c" of Table 1). After the addition of 500 μl of Tripure™ to inactivate DNase, total RNA was extracted once again. Reverse transcription of mRNA was carried out as follows: 8 μl of water containing 500 ng of total RNA were added to 2 μl of oligo dT primer (0.5 μg/μl), and incubated at 65 °C for 10 min. Samples were chilled on ice, and 10 μl of RT mix containing the following components were added: (1) 4 μl 5 × RT buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$); (2) 2 μl deoxynucleotide triphosphate mix (10 mM each); (3) 0.2 μl bovine serum albumin (1 mg/ml); (4) 0.6 μl (25 U) human placental ribonuclease inhibitor (RNAguard®, Pharmacia Biotech, Sweden); (5) 1 μl (200 U) M-MLV reverse transcriptase (Gibco Life Technologies, Scotland, UK); (6) 0.2 μl H_2O; (7) 2 μl dithiothreitol (100 nM). The samples were then incubated at 37 °C for 60 min.

2.3. Primers and probes

Except for the mouse IL-9 TaqMan probe that was kindly provided by Applied Biosystems (Foster City, CA), all primers and probes used in this study (sequences listed in Table 1) were synthesized at Biosource Europe (Nivelles, Belgium) and designed with the Primer 3 software (Rozen and Skaletsky, 2000; http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The default parameters of the program were applied, except for the following: product size 70–90 bp, primer size 18–27 bp, primer T_m 58–62 °C with a max T_m difference of 2.0 °C, product T_m 0–85 °C, max self and 3′ self complementarity for primers = 6.00, max poly-X = 3, primer and Hyb Oligo penalty (penalty weights for primer pairs) = 2.0, Hyb Oligo T_m 68–72 °C, max self and 3′ self complementarity for Hyb Oligo = 6.00, max poly-X = 3. Within the proposed oligonucleotides, primers and probe were selected following these criteria (in order of importance): (1) intron spanning if possible, (2) no G in 5′ for the probe, (3) no more than two G or C within the five last nucleotides in 3′ for the primers, (4) more C than G in the probe.

Table 1
Oligonucleotides for real-time PCR

mRNA targets	Oligonucleotides (5′ → 3′)[a]	Product size (bp)	Final concentration (nM)[b]
IL-1ra	F264: GAAGATGTGCCTGTCCTGTGT	80	F 900; R 900
	R343: CGCTCAGGTCAGTGATGTTAA		
	P291: 6Fam-TGGTGATGAGACCAGACTCCAGCTG-Tamra-p		
IL-1β	F176: ACAGATGAAGTGCTCCTTCCA	73	F 600; R 900
	R248: GTCGGAGATTCGTAGCTGGAT		
	P207: 6Fam-CTCTGCCCTCTGGATGGCGG-Tamra-p		
IL-5	F83: AGCTGCCTACGTGTATGCCA	71	F 300; R 900
	R153: GCAGTGCCAAGGTCTCTTTCA		
	P104: 6Fam-CCCCACAGAAATTCCCACAAGTGCATT-Tamra-p		
IL-10	F409: CATCGATTTCTTCCCTGTGAA	74	F 600; R 900
	R482: TCTTGGAGCTTATTAAAGGCATTC		
	P431: 6Fam-ACAAGAGCAAGGCCGTGGAGCA-Tamra-p		
IL-13	F155: TGAGGAGCTGGTCAACATCA	76	F 900; R 900
	R230: CAGGTTGATGCTCCATACCAT		
	P187: 6Fam-AGGCTCCGCTCTGCAATGGC-Tamra-p		
TNF-α	F275: CCCAGGGACCTCTCTCTAATC	84	F 900; R 900
	R358: ATGGGCTACAGGCTTGTCACT		
	P303: 6Fam-TGGCCCCAGGCAGTCAGATCATC-Tamra-p		
IFN-γ	F464: CTAATTATTCGGTAACTGACTTGA	75	F 600; R 900
	R538: ACAGTTCAGCCATCACTTGGA		
	P491: 6Fam-TCCAACGCAAAGCAATACATGAAC-Tamra-p		
β-actin	F976: GGATGCAGAAGGAGATCACTG	90[c]	F 300; R 300
	R1065: CGATCCACACGGAGTACTTG		
	P997: 6Fam-CCCTGGCACCCAGCACAATG-Tamra-p		
Mouse IL-9 (TaqMan probe)	F91: GGCARCAGAGACACCAATTACCT	143	F 300; R 300
	R233: TGGCATTGGTCAGCTGTAACA		
	P184: 6Fam-CTCTCCGTCCCAACTGATGATTGTACCAC-Tamra-p		
Mouse IL-9 (hybridisation probes)	F91: GGCATCAGAGACACCAATTACCT	143	F 300; R 900
	R233: TGGCATTGGTCAGCTGTAACA		
	P163: AACGTGACCAGCTGCTTGTGT-flourescein		
	P185: LCred 640-TCTCCGTCCCAACTGATGATT-p		

[a] F, R and P indicate forward and reverse primers and probes, respectively; numbers indicate the sequence position.
[b] Final concentration of forward (F) and reverse (R) primers.
[c] Except for IL-5, all primers were chosen to span intronic sequences so that gennomic DNA amplification is not possible, excepted for β-actin for which a 112-bp longer band is obtained. If contaminating genomic DNA is detected using this size difference on agarose gel, a DNase digestion of all of the RNA samples coming from the same experiment is performed.

2.4. PCR on the GeneAmp 5700 for mouse IL-9 DNA

The PCR reaction was carried out in a 50-μl final volume containing: (1) H_2O up to 50 μl; (2) 25 μl Universal PCR Master Mix (Applied Biosystems); (3) 1 μl of 6 pmol/μl for each forward and reverse primer (final concentration 300 nM); (4) 1μl of 4 pmol/μl TaqMan probe (final concentration 200 nM); (5) 5 μl of plasmid dilution. After an initial denaturation step at 94 °C for 10 min, temperature cycling was initiated. Each cycle consisted of 94 °C for 15 s and 60 °C for 60 s, the fluorescence being read at the end of this second step. In total, 45 cycles were performed.

2.5. PCR on the Lightcycler with hybridisation probes (mouse IL-9 DNA)

The PCR reaction was carried out in a 20-μl final volume containing: (1) H_2O up to 20 μl; (2) 2 μl DNA Master Hybridisation Probes 10× (DNA Master Hybridisation Probes Kit; Roche Diagnostics); (3)

5 μl 25 mM $MgCl_2$; (4) 1 μl of 6 pmol/μl forward and 3 μl of 6 pmol/μl reverse primers (final concentration 300 and 900 nM, respectively); (5) 1 μl of 4 pmol/μl of each of both hybridisation probes (final concentration 200 nM); (6) 0.3 μl anti-Taq DNA polymerase antibody (Platinum® *Taq* antibody, Gibco Life Technologies); (7) 1 μl of plasmid dilution. After an initial denaturation step at 95 °C for 30 s, temperature cycling was initiated. Each cycle consisted of denaturation at 95 °C for 0 (zero) second, hybridisation at 59 °C for 10 s, and elongation at 72 °C for 10 s. The fluorescent signal was acquired at the end of the hybridisation step (F2/F1 channels, fluorimeter gains regulated on 1 for F1, 15 for F2 and 30 for F3, with color compensation). A total of 45 cycles were performed.

2.6. PCR on the Lightcycler with TaqMan probes

The PCR reaction was carried out in a 20-μl final volume containing: (1) H_2O up to 20 μl; (2) 2 μl DNA Master Hybridisation Probes 10 × (DNA Master Hybridisation Probes Kit; Roche Diagnostics); (3) 5 μl 25 mM MgCl2; (4) 1, 2 or 3 μl of 6 pmol/μl forward and reverse primers (final concentration 300, 600 or 900 nM, see Table 1); (5) 1 μl of 4 pmol/μl TaqMan probe (final concentration 200 nM); (6) 0.3 μl anti-Taq DNA polymerase antibody (Platinum® *Taq* antibody, Gibco Life Technologies); (7) 1 μl cDNA or standard dilution. After an initial denaturation step at 95 °C for 30 s, temperature cycling was initiated. Each cycle consisted of 95 °C for 0 (zero) second and 60 °C for 20 s, the fluorescence being read at the end of this second step (F1/F2 channels, fluorimeter gains regulated on 8 for F1, 2 for F2 and 4 for F3, without color compensation). A total of 45 cycles were performed.

2.7. Standard curves and results expression

mRNA levels were expressed either in absolute copy numbers or in relative copy numbers normalised against β-actin mRNA. This was achieved by constructing, for each PCR run, a standard curve from serial dilutions of a purified DNA. This latter consisted of a PCR product that included the quantified amplicon, and that was prepared by "classical" PCR from cDNA positive for the concerned target mRNA. These PCR products used as standards were purified from agarose gel following standard procedures, at the end of which the copy number was calculated as described (Overbergh et al., 1999). Detailed information concerning these standards is given in Table 2. For human IL-5 and mouse IL-9,

Table 2
Oligonucleotides for standard preparation

mRNA targets	Oligonucleotides (5′ → 3′)[a]	Product size (bp)	Conditions for "classical" PCR[b]
IL-1ra	F43: CTCCTCTTCCTGTTCCSTTC R493: CTTCGTCAGGCATATTGGT	451	A = 56, Mg = 1.5
IL-1β	F59: CTTCATTGCTCAAGTGTCTGAA R553: ACTTGTTGCTCCARARCCTGTC	495	A = 58, Mg = 1.5
IL-10	F296: TTTACCTGGAGGAGGTGATG R771: TTGGGCTTCTTTCTAAATCGT	476	A = 56, Mg = 1.5
IL-13	F23: GCTCCTCAATCCTCTCCTGT R507: GCAACTTCAATAGTCAGGTCCT	485	A = 56, Mg = 1.0
TNF-α	F83: ACCATGAGCACTGAAAGCAT R488: AGATGAGGTACAGGCCCTCT	406	A = 58, Mg = 1.5
IFN-γ	F154: TTGGGTTCTCTTGGCTGTTA R632: AAATATTGCAGGCAGGACAA	479	A = 58, Mg = 1.5
β-actin	F745: CCCTGGAGAAGAGCTACGA R1253: TAAAGCCATGCCAATCTCAT	509	A = 58, Mg = 1.5

[a]F and R indicate forward and reverse primers, respectively; numbers indicate the sequence position.
[b]Conditions, for all targets, were as follows: denaturation at 95 °C for 20 s, annealing (temperature as stated (A)) for 20 s and elongation at 72 °C for 45 s, for a total of 35 cycles. $MgCl_2$ concentration (Mg, mM) was as stated. For the complete procedure, see (Stordeur et al., 1995, PCR for IFN-γ).

the serial dilutions were made from a purified plasmid (mouse IL-9 plasmid was kindly provided by Dr. Jean-Christophe Renauld from the Ludwig Institute, Brussels, Belgium, and human IL-5 plasmid was purchased from the American Type Culture Collection, Manassas, VA).

The mRNA copy numbers were calculated for each sample from the standard curve by the instru-

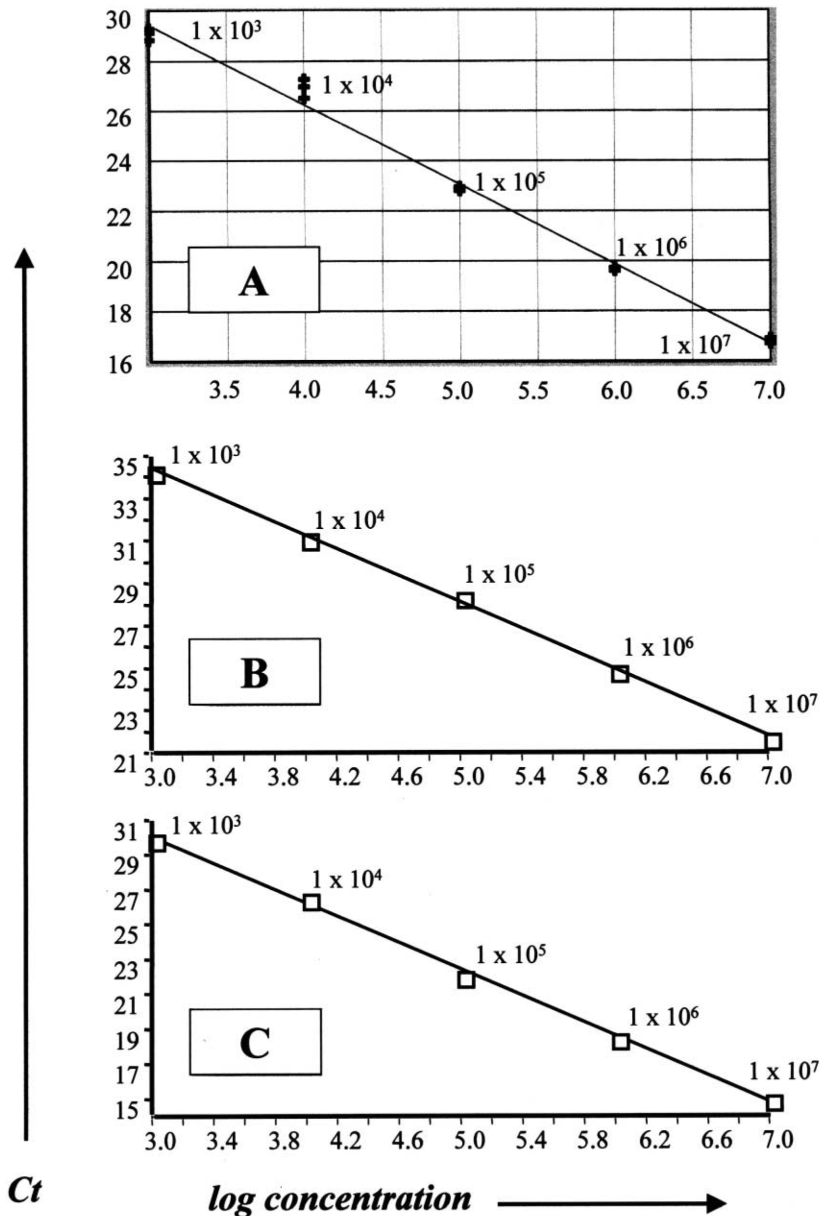

Fig. 1. ***Standard curves for mouse IL-9 reproducibility***. Standard curves were constructed with plasmid dilutions ranging from 10^3 to 10^7 copies (for details, see text). A typical curve is shown for the TaqMan probe on GeneAmp 5700 (A), for hybridisation probes (B) and the TaqMan probe (C) on Lightcycler.

ment software, using the C_t value ("Arithmetic Fit Point analysis" for the Lightcycler). Results were expressed in absolute copy numbers, or in copy numbers calculated relative to unstimulated cells, after normalisation against β-actin mRNA, as follows.

For each sample, a corrected cytokine mRNA copy number (CN) was first calculated:

$$\text{Corrected cytokine mRNA CN} = (\text{cytokine mRNA CN}/\beta\text{-actin mRNA CN}) \times \beta\text{-actin mRNA CN of unstimulated cells}$$

Then the relative copy number was obtained from the formula:

$$\text{Relative CN}(\%) = (\text{corrected cytokine mRNA CN}/\text{corrected cytokine mRNA CN of unstimulated cells}) \times 100$$

This normalisation against the housekeeping gene is possible only if both PCR (cytokine + housekeeping genes) present the same efficiency. This latter has been calculated for each PCR run from the slope of the standard curve:

$$\text{Efficiency}\,(E) = [10^{(1/\text{slope})}] - 1$$

and found to be nearly similar for all PCR reactions presented in this study. If that had not been the case, we could have expressed our results in $\Delta\Delta C_t$ instead

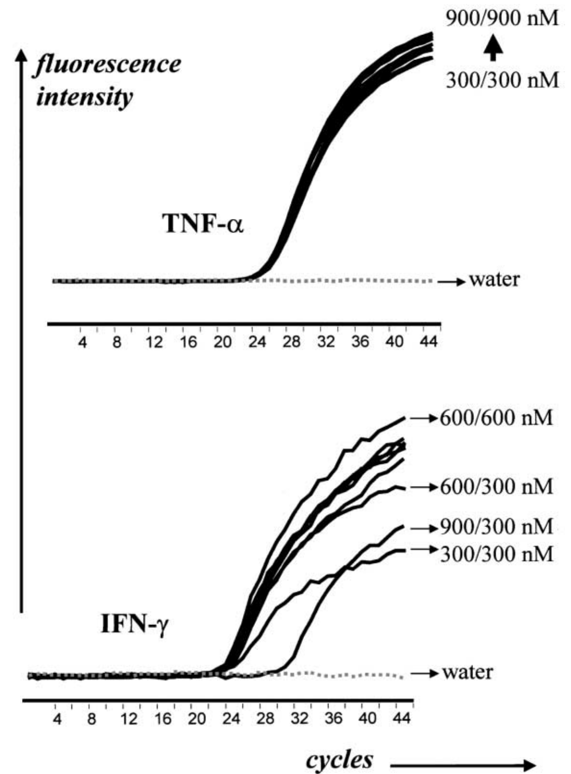

Fig. 2. ***Typical results for primer titration***. Three different concentrations of each primer may be used: 300, 600 and 900 nM. Each concentration of forward primer is tested in combination with each concentration of reverse primer in a real-time PCR reaction starting with 10^5 copies of purified standard. The curves obtained with the different combinations of primer concentration were nearly similar for TNF-α (top panel), but quite different for IFN-γ (bottom panel). In this latter case, we choose the primer concentrations that give the curve with the higher slope, and the lower C_t value, i.e. here 600/600 nM.

Table 3A
Coefficient of variation (CV) (%)

CV for mouse IL-9 ($n = 20$)		Plasmid dilution (numbers of copies)		
		1×10^7	1×10^5	1×10^3
Intra-run assays				
Lightcycler	Hybridisation probes	8.81	7.41	8.78
	TaqMan probe	8.12	7.05	6.48
Gene Amp 5700	TaqMan probe	6.95	8.83	11.49
Inter-run assays				
Lightcycler	Hybridisation probes	21.95	12.91	30.61
	TaqMan probe	16.84	14.64	17.28
GeneAmp 5700	TaqMan probe	31.16	20.32	40.71

of relative copy numbers. In this case, for each sample, two "ΔC_t" can be calculated:

$$\Delta C_t\ \text{cytokine} = C_t\ \text{cytokine of the sample} - C_t\ \text{cytokine of unstimulated cells}$$

$$\Delta C_t\ \beta\text{-actin} = C_t\ \beta\text{-actin of the sample} - C_t\beta\text{-actin of unstimulated cells}$$

and used for the determination of the $\Delta\Delta C_t$: (for each sample)

$$\Delta\Delta C_t = \left[1 + \left(E_{cy}/E_{act}\right)\right]^{-(\Delta C_t\,\text{cytokine} - \Delta C_t\,\beta\text{-actin})} \times 100$$

where E_{cy} = efficiency of cytokine PCR and E_{act} = efficiency of β-actin PCR.

3. Results

3.1. Use of TaqMan chemistry on the Lightcycler: reproducibility of mouse IL-9 DNA quantification

We transposed to the Lightcycler the protocol recommended by Applied Biosystems for TaqMan probe use on a GeneAmp 5700. To confirm that this protocol gave the same results on both types of apparatus, we compared the reproducibility of mouse IL-9 DNA quantification on the two instruments, for the two kinds of probes (except the hybridisation probes that could not be used on GeneAmp 5700). This was carried out with three dilutions of a plasmid that contained the coding sequence of the cytokine. The PCR were performed for each plasmid dilution 20 times in one experiment (intra-run coefficient of variation (CV)), and once in 20 different experiments (inter-run CV). The copy numbers were calculated for each dilution from a standard curve constructed with serial dilutions of the plasmid (Fig. 1). The CV of the copy numbers was calculated for each dilution (Table 3A). We found (1) that the

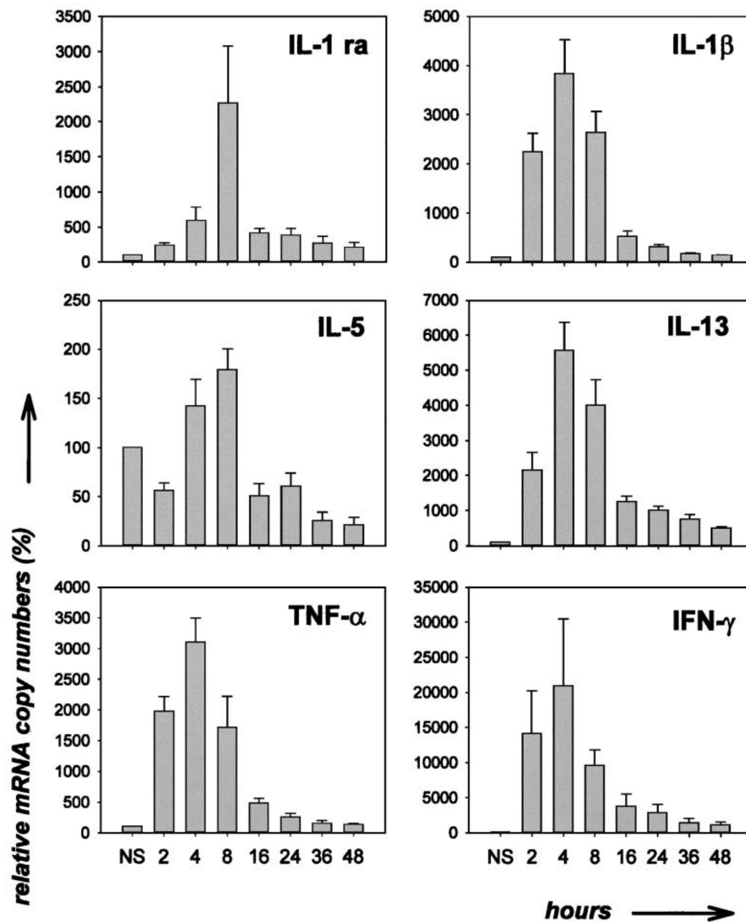

Fig. 3. ***Kinetic study for cytokine mRNAs***. PBMC were cultured in medium alone (NS columns) or stimulated with 1 μg/ml PHA, for the indicated time periods. Real-time PCR was performed for the different cytokine mRNAs, in four independent experiments. The results were expressed in mRNA copy numbers calculated relative to non-stimulated (NS) cells, after normalisation against β-actin. The mean + S.E.M. (error bars) of the four experiments is represented.

TaqMan probe on the Lightcycler offered a better reproducibility than hybridisation probes, especially for low copy numbers in inter-run assays, and (2) that the intra-run reproducibility was similar and satisfactory for the two instruments, while the Lightcycler globally presented a better but unsatisfactory inter-run reproducibility. On this basis, we decided to express our results in copy numbers calculated relative to unstimulated cells, after normalisation against β-actin.

3.2. Quantification of human IL-1ra, IL-1β, IL-5, IL-13, TNF-α and IFN-γ mRNAs

The strategy proposed here to develop real-time PCR is simple and includes the following steps: (1) primers and probe choice with the primer 3 software; (2) preparation of the standard by "classical" PCR; (3) primer titration. We successfully applied it for different cytokine mRNAs and efficiently found that the only necessary adaptation was the primer titration (Fig. 2). This technique was then used in a kinetic study of human PBMC stimulated with PHA. We quantified the mRNA levels of IL-1ra, IL-1β, IL-5, IL-13, TNF-α, IFN-γ and β-actin at different times of culture. Fig. 3 illustrates the capacity of this system to quantify cytokine mRNAs. A similar pattern, i.e. a rapid but transient induction, peaking around 4–8 h after PHA addition, was seen for the different cytokines.

3.3. Human IL-10 transcript quantification: monocytic IL-10 mRNA induction by IFN-α

We developed IL-10 mRNA quantification in order to study the capacity of IFN-α to enhance IL-10 mRNA levels, which was demonstrated in previous studies (Schandené et al., 1996; Aman et al., 1996). First, we evaluated the reproducibility of the method

Table 3B
Coefficient of variation (CV) (%)

Intra-run CV for human IL 10 ($n = 20$)	
LPS-stimulated PBMC[a]	6.94

[a]PBMC were stimulated for 6 h with 1 μg/ml LPS, and total RNA was extracted. Real-time PCR for IL-10 was performed 20 times in one run, and the CV was calculated from absolute copy numbers.

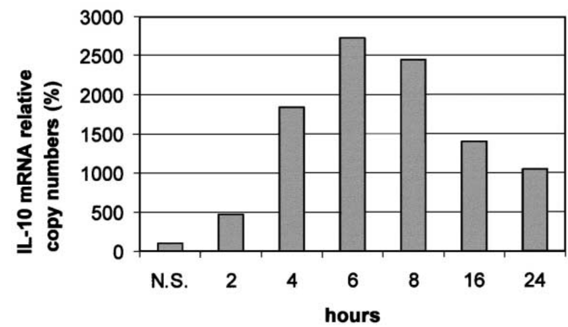

Fig. 4. ***Induction of IL-10 mRNA in human monocytes by IFN-α***. Human monocytes were cultured in medium alone (NS column) or stimulated with 5000 IU/ml IFN-α for the indicated time periods. Cells were lysed and total RNA was extracted for IL-10 mRNA quantification by real-time PCR. Results are expressed in mRNA copy numbers calculated relative to non-stimulated (NS) cells after normalisation against β-actin and represent the mean of three different experiments.

for IL-10 mRNA, using a cDNA from LPS-stimulated PBMC. The coefficient of variation obtained was satisfactory as shown in Table 3B. Data presented in Fig. 4 confirmed that IFN-α induces a clear and transient induction of IL-10 mRNA in purified monocytes.

4. Discussion

The development of real-time PCR to quantify a (c)DNA copy number represents a major step forward in PCR technology. It is now routinely applied in cancer for the evaluation of minimal residual disease (Nakao et al., 2000; Verhagen et al., 2000), as well as for the detection of bacterial and viral infections (Lyons et al., 2000; Yun et al., 2000; Josefsson et al., 2000). Apart from these applications, real-time PCR has been used little to date for cytokine mRNA quantification (Brink et al., 2000; Overbergh et al., 1999; Blaschke et al., 2000). However, as demonstrated by Wang and Brown (1999), real-time PCR gives similar results than the RNase protection assay, a technique widely used to quantify cytokine mRNAs (these authors demonstrated a strong correlation between the two techniques, with a higher sensitivity for the kinetic PCR). In the same vein, a recent study showed that real-time PCR for IL-16 mRNA quantification gave the same results as competitive conventional RT-PCR (Blaschke et al.,

2000). These observations led us to develop real-time PCR for cytokine mRNA quantification using TaqMan chemistry on a Lightcycler. The use of FRET technology requires the simultaneous choice of four different oligonucleotides (two primers and two probes) and no available software existed for such a design, whereas the selection of TaqMan probes and primers was possible through freeware on the Net. Moreover, the successful use of hydrolysis probes on a Lightcycler has already been described (Kreuzer et al., 1999).

The protocol for kinetic PCR with TaqMan probes on the GeneAmp 5700 was readily transposed. In order to optimise the technique, we investigated different conditions and found that a unique protocol can be used for different target mRNAs. This was exactly as suggested by Applied Biosystems for the GeneAmp 5700, the only adjustment required for a new mRNA target design being the titration of primer concentrations (300, 600 or 900 nM of each primer).

We evaluated the reproducibility of the protocol and found satisfactory intra-run coefficients of variation for both apparatus, and a better reproducibility for TaqMan probes compared to hybridisation probes, especially for inter-run assays. These results validated the use of TaqMan probes on a Lightcycler. Nevertheless, because of the inter-run coefficients of variation, we expressed our results in copy numbers calculated relative to a reference sample, after normalisation against a housekeeping gene. As far as the intra-run coefficients of variation are concerned, similar results have been obtained in previous studies (Overbergh et al., 1999, Bolufer et al., 2000, Gerard et al., 1998).

The methodology described here was easily and successfully applied to the quantification of several cytokine genes. It was first used to determine the magnitude and the kinetics of induction of cytokine mRNAs upon polyclonal activation of PBMC. Then, we took advantage of the technique to specify the effects of IFN-α on IL 10 mRNA accumulation in human monocytes. Indeed, it has been previously suggested that IL-10 might mediate some of the anti-inflammatory properties of type I interferons (Schandené et al., 1996; Aman et al., 1996). However, the influence of IFN-α on the production of IL-10 by monocytes is controversial and seems to depend on the activation system considered (Pawelec et al., 1999; Hermann et al., 1998). The high sensitivity of the real-time PCR method permitted us to demonstrate unambiguously that IFN-α triggers IL-10 mRNA induction in monocytes in the absence of any other stimulus. Apart from the data presented in this paper, we also successfully used the same approach to demonstrate an impaired synthesis of IL-12 (p35) mRNA by neonatal dendritic cells (Goriely et al., 2001), providing thereby a molecular basis for the deficient Th1-type immune responses in the newborn.

In conclusion, we provide here a simple strategy to perform and develop quantitative real-time PCR for cytokine mRNA quantification. A unique protocol is used for different target mRNAs, the only adjustment being the primer titration, so that real-time PCR for a new target mRNA is rapidly developed. This is, in part, due to the use of the primer 3 software that permits the simultaneous choice of the probe and the primers, leading to successful oligonucleotide design. In this way, it is possible to avoid the use of a fluorescent dye (e.g., SYBR Green or ethidium bromide), which is less sensitive and less specific than the probe. We suggest that this technique has many advantages for researchers wishing to quantify cytokine mRNAs, and could provide powerful insights into the complexities of the cytokine network.

Acknowledgements

We are grateful to Dr. Jean-Christophe Renauld for the mouse IL-9 plasmid. We thank Wivine Burny, Xavier Duhant and Bérengère Nowak for their help in standards preparation. This work was supported by a grant from Roche Diagnostics Belgium. L. Poulin is a research fellow of the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture. A. de Lavareille (Télévie Programme) and S. Goriely are supported by the Fonds National de la Recherche Scientifique.

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