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The Use of Real-Time Reverse Transcriptase PCR for the Quantification of Cytokine Gene Expression

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Real-time reverse transcriptase polymerase chain reaction (RT-PCR) is becoming a widely used method to quantify cytokines from cells, tissues, or tissue biopsies. The method allows for the direct detection of PCR product during the exponential phase of the reaction, combining amplification and detection in a single step. Using TaqMan chemistry (Applied Biosystems, Foster City, CA) and the ABI Prism 7700 Sequence Detection System (Applied Biosystems), we validated a large panel of murine and human cytokines, as well as other factors playing a role in the immune system, such as chemokines and apoptotic markers. Although the method allows fast, sensitive, and accurate quantification, different control assays are necessary for the method to be reliable. By construction of complementary DNA (cDNA) plasmid clones, standard curves are generated that allow direct quantification of every unknown sample. Furthermore, the choice of a reliable housekeeping gene is very important. Finally, coamplification of contaminating genomic DNA is avoided by designing sets of primers located in different exons or on intron–exon junctions. In conclusion, the real-time RT-PCR technique is very accurate and sensitive, allows high throughput, and can be performed on very small samples. The development of real-time RT-PCR has resulted in an exponential increase in its use over the last couple of years, and the method has undoubtedly become the standard for quantifying cytokine patterns, clarifying many functional properties of immune cells and their associated diseases. (J Biomol Tech 2003;14:33–43)

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Many cellular functions are regulated by changes in gene expression. Thus, quantification of transcription levels of genes plays a central role in the understanding of gene function and of abnormal alterations in regulation that may result in a disease state. The innovation of the real-time polymerase chain reaction (PCR) technique played a crucial role in molecular medicine and clinical diagnostics. Examples are the quantitation of relative gene expression (as described herein), detection of minimal residual disease,[1] cancer diagnostics,[2] pathogen detection,[3] and quantitation of viral load.[4] Other applications include detection of genetically modified organisms in food samples,[5] measurements of DNA or transgene copy number, and allelic discrimination.[6]

In the field of immunology, many studies have shown that chronic inflammatory disorders, autoimmune diseases, and transplant rejections are closely associated with specific changes in the balance between pro- and anti-inflammatory cytokines in the affected cells or tissues. Quantification of the cytokines involved is essential for gaining more insight into the immune processes involved. Because tissue samples available for analysis are often too small to allow quantification of cytokines at the protein level, analysis of messenger RNA (mRNA) is widely used to investigate the cytokine levels at sites of immune infiltration or inflammation. In interpreting the results, however, one should always take into account that a discrepancy may exist between mRNA and protein levels. Although post-transcriptional or post-translational modifications may play a role in the target genes of interest, different publications have described a good correlation between cytokine mRNA levels quantified by real-time PCR and protein levels quantified by enzyme-linked immunosorbent assay.[7,8]

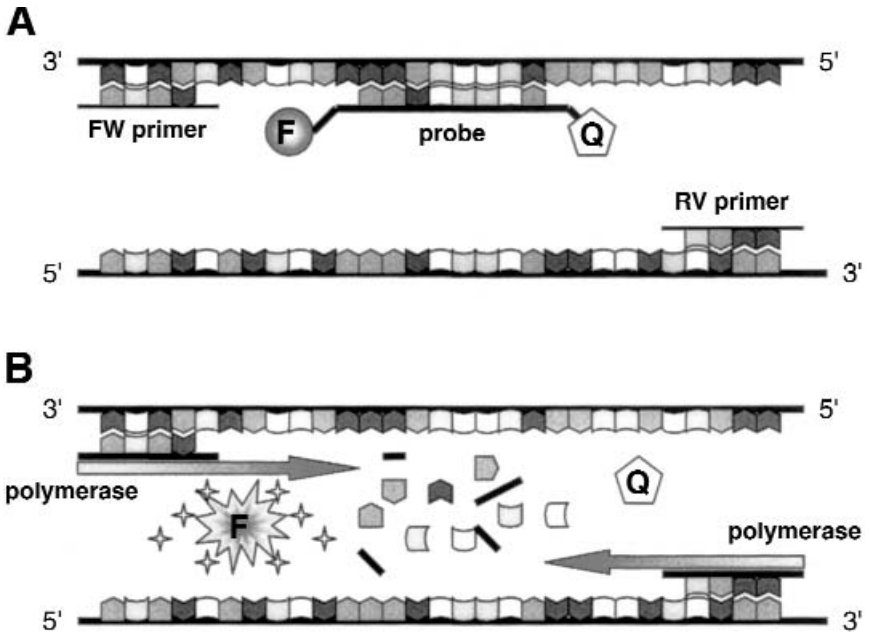

FIGURE 1

Schematic representation of the TaqMan principle. **A:** Primers and probe anneal to the target gene. Fluorescence emission does not occur because the probe is still intact. **B:** During the extension phase of the PCR reaction, the probe is cleaved by the 5′–3′ exonuclease activity of the Taq polymerase, allowing fluorescence emission. FW, forward; RV, reverse; F, fluorophore; Q, quencher dye.

Real-time quantitative reverse transcriptase PCR (RT-PCR), which is the latest innovation in the field of PCR technology, provides a sensitive, reproducible, and accurate method for determining mRNA cytokine levels in tissues or cells. The method is based on the detection of a fluorescent signal produced and monitored during the amplification process, without the need for post-PCR processing.[9] The method has been available for more than 5 years and has seen an exponential increase in use over the last 2 years.

PRINCIPLE OF REAL-TIME RT-PCR

The discovery of the real-time PCR technique as it is used today was made possible by two important findings. First, the Taq polymerase has, apart from its polymerase activity, a 5′–3′ exonuclease activity.[10] Second, dual-labeled fluorogenic oligonucleotide probes have been created which emit a fluorescent signal only upon cleavage, based on the principle of fluorescence resonance energy transfer.[11] In the TaqMan assay (Applied Biosystems, Foster City, CA), which was the first real-time PCR assay developed, these two principles are combined. In this system a probe, the so-called TaqMan probe, is designed to anneal to the target sequence between the classical forward and reverse primers (Fig. 1).The probe is dually labeled, with a reporter fluorochrome (e.g., 6-carboxyfluorescein, or FAM) at one end and a quencher dye (e.g., 6-carboxy-tetramethyl-rhodamine, or TAMRA) at the 3′ end. Importantly, in its intact form, the fluorescence emission of the reporter dye will be absorbed by the quencher dye. The probe has a melting temperature (T_m) approximately 10°C higher than the T_m of the primers, in order to anneal to the amplicon during the extension phase of the PCR process (which is performed at 60°C). Consequently, the probe will be degraded during the extension phase by the 5′–3′ exonuclease activity of the Taq polymerase. This will result in an increase in reporter fluorescence emission because reporter and quencher are separated. The amount of fluorescence released is directly proportional to the amount of product generated in each PCR cycle and thus can be applied as a quantitative measure of PCR product formation.

As the technology has become more commonly used, other sophisticated chemistries have been developed to directly measure PCR product accumulation by fluorescence emission. Examples include molecular beacons, Scorpions, hybridization probes, and minor groove binder (MGB) probes (e.g., Eclipse, TaqMan MGB). Other new technologies include ResonSense probes, light-up probes, and Hy-Beacon probes.[12,13] Finally, the use of double-stranded DNA minor groove binding dyes, such as SYBR Green I, is a cheaper, widely used alternative, where the need for an expensive probe can be avoided.

One can choose among a diversity of competing instrumentations that have recently been launched on the market.[12,13] All of them run the PCR reaction as a closed tube and measure product accumulation in real time during the course of PCR amplification. Differences between the instrumentations are the sample format (tubes, microplates, strip tubes, capillaries, etc.), the maximum sample number (ranging from 16 to

384), the length of a run (ranging from 30 min to 2 h), the light source (halogen or laser), the fluorescence wavelength detection, the possibility of performing single or multiplex (i.e., measuring different fluorescence emissions simultaneously) PCR reactions, the availability of melting curve analysis, and finally the price. Some of the instruments are designed primarily for high-throughput applications, whereas others allow more flexibility. Therefore, the choice for an instrument will depend on the specific applications to be performed.

With any of the developed chemistries on any of the developed instrumentations, a software package is provided that measures the increase in fluorescence emission in real time, during the course of the reaction. This increase in fluorescence emission is directly related to the increase in target amplification. In the ABI Prism 7700 Sequence Detection System (SDS) (Applied Biosystems), for example, the software calculates a ΔRn using the equation $\Delta Rn = Rn^{+} - Rn^{-}$, with Rn^{+} being the fluorescence emission of the product at each time point and Rn^{-} being the fluorescence emission of the baseline.[9] The ΔRn values are plotted against cycle number, resulting in amplification plots for each sample (Fig. 2). Threshold values (Ct) are then determined as the cycle number at which the fluorescence emission (ΔRn) exceeds a chosen threshold, which is usually 10 times the standard deviation of the baseline (this threshold level can, however, be changed manually if desired). Software will plot Ct values of unknown samples on the standard curve to determine the starting copy numbers of the unknown samples. Alternatively, the Ct values can be used as a direct quantitative measurement.

VALIDATION OF THE REAL-TIME PCR SYSTEM FOR CYTOKINE ANALYSIS

Because the research in our laboratory mainly focuses on the pathophysiology of autoimmune diseases such as type 1 diabetes,[14–16] we were very interested in obtaining a high-throughput and reliable system for quantifying cytokine levels in immune cells, particularly in immune and invaded tissues. Therefore, we began optimizing a real-time RT-PCR system for a panel of murine cytokines about 5 years ago. In that time, the list of murine cytokines and other factors playing a role in the immune system that we validated using the real-time RT PCR system has grown continuously, we have recently expanded our studies to the human system.

We use the ABI Prism 7700 SDS in combination with TaqMan chemistry, using a specific set of primers and an internal fluorogenic probe for each target of interest. In designing these primer sets, special care was taken to avoid co-amplification of contaminating genomic DNA. With the objective of requiring an absolute quantification of transcription, we constructed cDNA plasmid standards for each target gene of interest.

RNA EXTRACTION AND cDNA SYNTHESIS

Total RNA has been extracted from many different tissues or cells. Isolated tissues are either used immediately or snap frozen in liquid nitrogen and stored at −80°C until use. Two different methods for total RNA extraction from tissues (such as spleen, lymph node, heart, brain, spinal cord, intestine, thymus, lung, pancreas) have been tested and used: TRIzol reagent (Life Technologies, Gaithersburg, MD) and, more recently, the SV Total RNA Isolation System from Promega (Madison, WI). Although extracting total RNA using the SV Total RNA Extraction Kit is somewhat more complicated and takes longer, it results in highly pure, DNA-free RNA, because the protocol includes a DNAse treatment. Moreover, the components of the kit are less toxic to the investigator. For pancreatic tissue, the TRIzol method does not give reliable results,

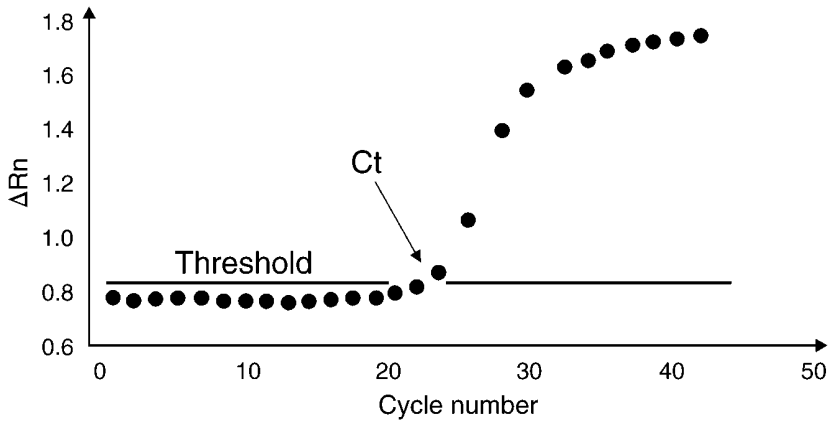

FIGURE 2

PCR amplification plot. Fluorescence emission is measured continuously during the PCR reaction and ΔRn (increase in fluorescence emission, from which the background fluorescence signal is subtracted) is plotted against cycle number. The threshold cycle (Ct) is the cycle at which the fluorescence exceeds a chosen threshold.

probably because of the high endogenous RNAse concentrations in this tissue.

For extraction of total RNA from small amounts (up to 10^6 cells) of cultured cells, peritoneal murine macrophages, human peripheral blood mononuclear cells, and pancreatic β-cells the High Pure RNA Isolation Kit from Roche Diagnostics (Indianapolis, IN) is used. This kit combines RNA extraction and DNAse treatment in a spin column system. For extraction of total RNA from a larger amount of cells, both the RNeasy Mini Kit (Qiagen, Chatsworth, CA) (up to 10^7 cells) and the TRIzol Liquid Suspension reagent (Life Technologies) are used. Both methods result in pure RNA, although no DNAse treatment is included. The concentration of purified total RNA is determined spectrophotometrically at 260 nm.

Target RNA is reverse transcribed using the Superscript II reverse transcriptase enzyme (Life Technologies). In a first step, 5 μM Oligo$(dT)_{16}$ is added to 0.5–1 μg of total RNA and annealed at 70°C for 10 min. Then, 100 U Superscript II reverse transcriptase is added in the presence of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 5 mM unlabeled deoxynucleotides (dNTPs) and incubated at 42°C for 80 min. For each experiment, RT-minus controls (i.e., RNA samples that are treated similarly but without addition of Superscript II RT enzyme) are included to provide a negative control for subsequent PCR reactions. For PCR amplification, a maximum of 1 μL of cDNA is used per 50 μL PCR. Larger amounts are avoided because they may significantly inhibit PCR amplification. To minimize variations in reverse transcriptase efficiency, all samples from a single experiment are reverse transcribed simultaneously.

PRIMER AND PROBE DESIGN

With the ABI Prism 7700 SDS instrumentation, Primer Express software is provided to design the primers

TABLE 1

Primer and Probe Sequences for Murine Cytokines[a]

		Sequence (5′–3′)	Length (bp)[b]	Accession[c]	gDNA[d]
IL-1-β	FW	CAACCAACAAGTGATATTCTCCATG	152	M15131	–
	RV	GATCCACACTCTCCAGCTGCA		X04964	
	TP	CTGTGTAATGAAAGACGGCACACCCACC			
IL-2	FW	CCTGAGCAGGATGGAGAATTACA	141	X01772	–
	RV	TCCAGAACATGCCGCAGAG		M16760	
	TP	CCCAAGCAGGCCACAGAATTGAAAG		AF195956	
IL-4	FW	ACAGGAGAAGGGACGCCAT	95	M25892	–
	RV	GAAGCCCTACAGACGAGCTCA		X05253	
	TP	TCCTCACAGCAACGAAGAACACCACA			
IL-5	FW	AGCACAGTGGTGAAAGAGACCTT	117	X06270	–
	RV	TCCAATGCATAGCTGGTGATTT		X06271	
	TP	CTGTTGACAAGCAATGAGACGATGAGG			
IL-6	FW	GAGGATACCACTCCCAACAGACC	141	X54542	–
	RV	AAGTGCATCATCGTTGTTCATACA		M20572	
	TP	CAGAATTGCCATTGCACAACTCTTTTCTCA			
IL-7	FW	ATTATGGGTGGTGAGAGCCG	257	X07962	–
	RV	GTTCATTATTCGGGCAATTACTATCA		M29054	
	TP	CCTCCCGCAGACCATGTTCCATGT			
IL-10	FW	GGTTGCCAAGCCTTATCGGA	191	M37897	–
	RV	ACCTGCTCCACTGCCTTGCT		M84340	
	TP	TGAGGCGCTGTCATCGATTTCTCCC			
IL-12 p40	FW	GGAAGCACGGCAGCAGAATA	180	M86671	–
	RV	AACTTGAGGGAGAAGTAGGAATGG		S82420-6	
	TP	CATCATCAAACCAGACCCGCCCAA			
IL-13	FW	GGAGCTGAGCAACATCACACA	142	M23504	–
	RV	GGTCCTGTAGATGGCATTGCA		L13028	
	TP	CGGGTTCTGTGTAGCCCTGGATTCC			

(continued)

and fluorogenic probes. The primary object of this program is to design sets of primers and internal probes that can be run under universal thermal cycling conditions (15 s at 94°C and 1 min at 60°C). Thus, the default parameters of the software are set to be very narrow. Most important are the melting temperature (T_m) of the primers and probe, and the amplicon length. The T_m of the primers should be 58–60°C, whereas the T_m of the probe must be at least 10°C higher (thus approximately 68–70°C), in order to anneal to the target during the extension phase of the PCR reaction. For amplicon lengths the rule is "the shorter the more efficient"; default parameters for amplicon lengths are set between 50 and 150 bp.

To avoid co-amplification of contaminating genomic DNA, primers are (where possible) designed on different exons or intron–exon boundaries. With the Primer Express software, these primer sets must be searched manually.

For specific applications where no primer sets can be found using these stringent parameters, the parameters can, of course, be changed manually. In this case, the first parameter we change is amplicon length; in our experience, amplicon lengths up to 250 bp have been found to amplify efficiently.

At present, large panels of murine cytokines (Table 1), murine chemokines and other immune related factors (Table 2), human cytokines (Table 3), and apoptotic markers (Table 4) have been validated. In each

TABLE 1 (continued)

Primer and Probe Sequences for Murine Cytokines[a]

		Sequence (5′–3′)	Length (bp)[b]	Accession[c]	gDNA[d]
IL-15	FW	CATCCATCTCGTGCTACTTGTGTT	126	U14332	–
	RV	CATCTATCCAGTTGGCCTCTGT		AB006745	
	TP	AGGGAGACCTACACTGACACAGCCCAAAA			
IL-17	FW	GCTCCAGAAGGCCCTCAGA	142	NM_010552	–
	RV	AGCTTTCCCTCCGCATTGA		U35108	
	TP	CTCTCCACCGCAATGAAGACCCTGA			
IL-18	FW	CAGGCCTGACATCTTCTGCAA	105	NM_008360	–
	RV	TCTGACATGGCAGCCATTGT		AJ002364	
	TP	CTCCAGCATCAGGACAAAGAAAGCCG			
IFN-γ	FW	TCAAGTGGCATAGATGTGGAAGAA	92	K00083	–
	RV	TGGCTCTGCAGGATTTTCATG		M74466	
	TP	TCACCATCCTTTTGCCAGTTCCTCCAG		M28381	
TNF-α	FW	CATCTTCTCAAAATTCGAGTGACAA	175	M13049	–
	RV	TGGGAGTAGACAAGGTACAACCC		Y00467	
	TP	CACGTCGTAGCAAACCACCAAGTGGA			
TGF-β1	FW	TGACGTCACTGGAGTTGTACGG	170	M13177	–
	RV	GGTTCATGTCATGGATGGTGC		L42460	
	TP	TTCAGCGCTCACTGCTCTTGTGACAG		L42459	
MIC-1	FW	CCAACCAGAGCCGAGAGGA	113	NM_011819	–
	RV	GTTGACGCGGAGTAGCAGCT		AJ011967-8	
	TP	CCGGATACTCAGTCCAGAGGTGAGATTGG			
GM-CSF	FW	GCCATCAAAGAAGCCCTGAA	114	X02333	–
	RV	GCGGGTCTGCACACATGTTA		X03020	
	TP	ACATGCCTGTCACATTGAATGAAGAGGTAGAAG			

FW, forward primer; RV, reverse primer; TP, TaqMan probe dual-labeled with 5′FAM and 3′TAMRA; IL, interleukin; IFN-γ, interferon-gamma; TNFα, tumor necrosis factor alpha; TGF-β, transforming growth factor-beta; iNOS, inducible nitric oxide synthetase; MIC-1, macrophage inhibiting cytokine-1; GM-CSF, granulocyte–macrophage colony-stimulating factor.

[a]Adapted from Giulietti et al.[12] with permission from the publisher (Elsevier Science USA, New York, NY).

[b]Amplicon length in base pairs.

[c]Genbank accession number of cDNA and corresponding gene available online at http://www.ncbi.nlm.nih.gov/

[d]PCR amplification on genomic DNA.

TABLE 2

Primer and Probe Sequences for Murine Chemokines and Other Immune-Related Factors[a]

		Sequence (5′–3′)	Length (bp)[b]	Accession[c]	gDNA[d]
MCP-1	FW	CTTCTGGGCCTGCTGTTCA	126	L13763	–
	RV	CCAGCCTACTCATTGGGATCA		U12470	
	TP	CTCAGCCAGATGCAGTTAACGCCCC			
Fractalkine	FW	GGGTGGCCATGTTTGCTTAC	140	U92565	+
	RV	CAGGCAAGCAGCTCACACTG			
	TP	TCCCCCGTAGCTGTGGCAGTAACTCAT			
MIP3α	FW	CCAGGCAGAAGCAAGCAACT	96	AJ222694	+
	RV	TCGGCCATCTGTCTTGTGAA		AB015137	
	TP	TGTTGCCTCTCGTACATACAGACGCCA			
IP10	FW	GCCGTCATTTTCTGCCTCAT	127	AF227743	–
	RV	GCTTCCCTATGGCCCTCATT		M33266	
	TP	TCTCGCAAGGACGGTCCGCTG		L07417	
CD40	FW	GTCATCTGTGGTTTAAAGTCCCG	91	M83312	+
	RV	AGAGAAACACCCCGAAAATGG		M94129	
	TP	AGCCCTGCTGGTCATTCCTGTCGTG			
CD40 ligand	FW	CTCAAACTCTGAACAGTGCGCT	88	X65453	+
	RV	GGCAGGTCCTAACTGACTTGCT			
	TP[d]	AGGGAAGACTGCCAGCATCAGCCCT			
iNOS	FW	CAGCTGGGCTGTACAAACCTT	95	U43428	–
	RV	CATTGGAAGTGAAGCGTTTCG		L23806	
	TP	CGGGCAGCCTGTGAGACCTTTGA			
PAF-AH	FW	CCTGCAAGCTGGAATTCTCC	123	U34277	–
	RV	CCCATTAGATGCCAAGCCAA			
	TP	TTCTCATGGTCTCGGAGCCTTCAGGAC			
PAF-R	FW	CAACGAGGGCGACTGGATT	97	D50872	+
	RV	GACACCCAAAAAGGCCACACT			
	TP	TCCTGTGCAACGTGGCTGGCTG			
IL1Ra	FW	CTGGGAAAAGACCCTGCAAG	91	NM_031167	–
	RV	CCAGCAATGAGCTGGTTGTTT		L32833	
	TP	TGCAAGCCTTCAGAATCTGGGATACTAACCA			
PGS2	FW	TGGTGCCTGGTCTGATGATG	159	M64291	–
	RV	GTGGTAACCGCTCAGGTGTTG		D28235	
	TP	CCACCATCTGGCTTCGGGAGCA			
ICAM 1	FW	CCGCAGGTCCAATTCACACT	143	X52264	–
	RV	TCCAGCCGAGGACCATACAG		X15372	
	TP	CAGCTCGGAGGATCACAAACGAAGCT		M90546	
TNF-Rp55	FW	GCTGACCCTCTGCTCTACGAA	132	X57796	+
	RV	GCCATCCACCACAGCATACA		M88067	
	TP	CTGTTCAGAAATGGGAAGACTCCGCCC		M76655	

FW, forward primer; RV, reverse primer; TP, TaqMan probe dual-labeled with 5′FAM and 3′TAMRA; MCP-1, monocyte chemoattractant protein 1; MIP3α, macrophage inflammatory protein 3 alpha; IP10, interferon gamma inducible protein; iNOS, inducible nitric oxide synthetase; PAF-AH, platelet activating factor acetyl hydrolase; PAF-R, platelet activating factor receptor; IL1-Ra, interleukin 1 receptor antagonist; PGS2, prostaglandin synthetase 2; ICAM 1, intercellular adhesion molecule 1; TNF-Rp55, TNF-receptor.

[a]Adapted from Giulietti et al.[12] with permission from the publisher (Elsevier Science USA, New York, NY).

[b]Amplicon length in base pairs.

[c]Genbank accession number of cDNA and corresponding gene available online at http://www.ncbi.nlm.nih.gov/

[d]PCR amplification on genomic DNA.

TABLE 3

Primer and Probe Sequences for Human Cytokines[a]

		Sequence (5′–3′)	Length (bp)[b]	Accession[c]	gDNA[d]
IL-1α	FW	CGCCAATGACTCAGAGGAAGA	120	X02531	–
	RV	AGGGCGTCATTCAGGATGAA		X03833	
	TP	AGCACCTTTTAGCTTCCTGAGCAATGTGAAA			
IL-2	FW	AACTCACCAGGATGCTCACATTTA	148	NM_000586	–
	RV	TCCCTGGGTCTTAAGTGAAAGTTT		J00264	
	TP	TTTTACATGCCCAAGAAGGCCACAGAACT			
IL-4	FW	CCACGGACACAAGTGCGATA	149	M13982	–
	RV	CCCTGCAGAAGGTTTCCTTCT		M23442	
	TP	TCTGTGCACCGAGTTGACCGTAACAGAC			
IL-10	FW	GTGATGCCCCAAGCTGAGA	138	AF043333	–
	RV	CACGGCCTTGCTCTTGTTTT		U16720	
	TP	CCAAGACCCAGACATCAAGGCGCA			
IL-12p40	FW	TGGAGTGCCAGGAGGACAGT	147	AF180563	–
	RV	TCTTGGGTGGGTCAGGTTTG		AY008847	
	TP	ATGGTGGATGCCGTTCACAAGCTCAA			
IL-15	FW	GGAGGCATCGTGGATGGAT	143	NM_000585	–
	RV	AACACAAGTAGCACTGGATGGAAA		X91233	
	TP	CTGCTGGAAACCCCTTGCCATAGCC			
IFN-γ	FW	TCAGCTCTGCATCGTTTTGG	120	X01992	–
	RV	GTTCCATTATCCGCTACATCTGAA		J00219	
	TP	TTGGCTGTTACTGCCAGGACCCATATGT			
TGF-β	FW	CAGCAACAATTCCTGGCGATA	136	NM_000660	–
	RV	AAGGCGAAAGCCCTCAATTT		Y00112	
	TP	CTGCTGGCACCCAGCGACTCG			
TNF-α	FW	TCTTCTCGAACCCCGAGTGA	151	M10988	–
	RV	CCTCTGATGGCACCACCAG		X02910	
	TP	TAGCCCATGTTGTAGCAAACCCTCAAGCT		X02159	

FW, forward primer; RV, reverse primer; TP, TaqMan probe dual-labeled with 5′FAM and 3′TAMRA; IL, interleukin; IFN-γ, interferon-gamma; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha.

[a]Adapted from Giulietti et al.[12] with permission from the publisher (Elsevier Science USA, New York, NY).

[b]Amplicon length in base pairs.

[c]Genbank accession number of cDNA and corresponding gene available online at http://www.ncbi.nlm.nih.gov/

[d]PCR amplification on genomic DNA.

table the possible co-amplification of genomic DNA is indicated (last column).

PCR AMPLIFICATION

PCR amplifications are performed on the ABI Prism 7700 SDS, using 96-well microtiter plates. They are performed in a total volume of 25 μL, containing 0.5 μL cDNA sample, 1× buffer A (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 10 mM EDTA, 60 nM Passive Reference 1), 200 μM dNTPs, 3–9 mM $MgCl_2$, 100–900 nM of each primer, 0.625 U AmpliTaqGold (Applied Biosystems), and 100 nM TaqMan probe (Eurogentec, Liege, Belgium). For each target gene, different $MgCl_2$ (3–9 mM) and primer (100, 300, and 900 nM) concentrations are tested to optimize the PCR amplification, and all other components are maintained constant. PCR amplifications are always performed in duplicate or triplicate wells, using the universal temperature cycles: 10 min at 94°C, followed by 35–45 two-temperature cycles (15 s at 94°C and 1 min at 60°C).

TABLE 4

Primer and Probe Sequences for Murine Apoptosis Related Factors

		Sequence (5′–3′)	Length (bp)[a]	Accession[b]	gDNA[c]
Fas	FW	CTGCGATGAAGAGCATGGTTT	208	M83649	–
	RV	CCATAGGCGATTTCTGGGAC			
	TP	TGCGATTCTCCTGGCTGTGAACACTG			
Fas-ligand	FW	AAGAAGGACCACAACACAAATCTG	234	U58995	–
	RV	CCCTGTTAAATGGGCCACACT			
	TP	TGCAGAAGGAACTGGCAGAACTCCG			
Bcl-2β	FW	CTTAGAAAATACAGCATTGCGGAG	194	M16506	+
	RV	GGATGTGCTTTGCATTCTTGG			
	TP	TTCCTGCATCTCATGCCAACGGG			
Bcl-xL	FW	CACTGTGCGTGGAAAGCGTA	127	U51278	+
	RV	AAAGTGTCCCAGCCGCC		U78030-1	
	TP	CAAGGAGATGCAGGTATTGGTGAGTCGG			
Bax-α	FW	GTTTCATCCAGGATCGAGCAG	238	L22472	+
	RV	CCCCAGTTGAAGTTGCCATC			
	TP	AGCTGAGCGAGTGTCTCCGGCG			
Bcl-6	FW	ATGTACAGCCATCTCCCGCT	134	U41465	+
	RV	TTAGGGACTTGCCTGGCACT			
	TP	AATGCCTGTGGCCAACCCTTTTCC			
Bid	FW	TGGCAGTGCTTGGAGCTACA	140	BC002031	–
	RV	CCTCCAGCTCTTGGCGAGTA		AC006404	
	TP	TGAGGTCAGCAACGGTTCCGGC			
c-FLIP	FW	GCAACCCAGACACTGCACAA	148	U97076	+
	RV	CGTCTCCTGCCTTGCTTCAG			
	TP	AGAAGCCCTCCAGCTCATCCTCTGTGT			
c-jun	FW	CCTGTCCCCTATCGACATGG	93	J04115	+
	RV	CTTTTCCGGCACTTGGAGG		X12740	
	TP	TCCTCATGCGCTTCCTCTCTGCCT			
c-myc	FW	TGAGCCCCTAGTGCTGCAT	137	AF076523	–
	RV	AGCCCGACTCCGACCTCTT		L00038-9	
	TP	CTTCTTGCTCTTCTTCAGAGTCGCTGCTG			
c-fos	FW	CTCCTTCTCCAGCATGGGC	81		–
	RV	GGGATAAAGTTGGCACTAGAGACG		J00370	
	TP	TCAACACACAGGACTTTTGCGCAGATCT			

FW, forward primer; RV, reverse primer; TP, TaqMan probe dual-labeled with 5′FAM and 3′TAMRA; FLIP, Flice inhibitory protein.

[a]Amplicon length in base pairs.

[b]Genbank accession number of cDNA and corresponding gene available online at http://www.ncbi.nlm.nih.gov/

[c]PCR amplification on genomic DNA.

The PCR reactions performed on the ABI Prism 7700 SDS have an absolute requirement for a reference dye (ROX, which is included in buffer A). Initially, a buffer containing this reference dye could only be purchased from Applied Biosystems as part of a complete kit, resulting in high expenses. Recently, however, the necessary buffer has become available in different formulations or can be purchased from other companies. Furthermore, competing companies are also providing dual-labeled fluorogenic probes. Wider availability of reagents should result in lower costs for performing real-time PCR amplifications.

QUANTIFICATION

To quantify the results obtained by real-time RT-PCR, we use the standard curve method. With this method,

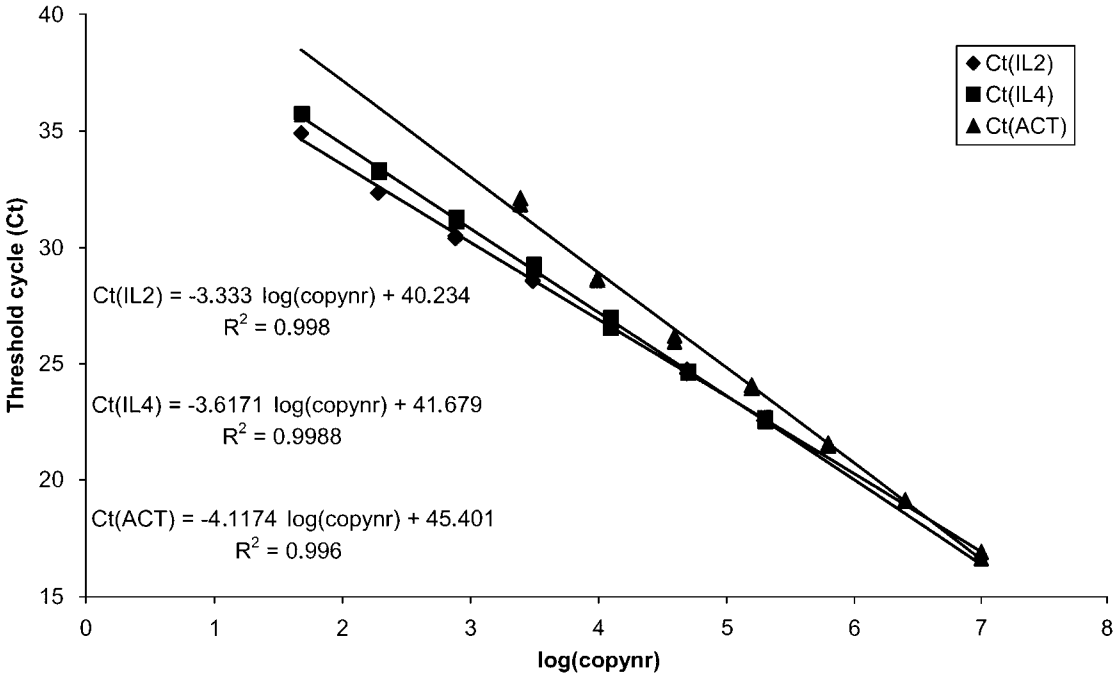

FIGURE 3

Standard curves for IL2, IL4, and β-actin (ACT). Threshold cycle values are plotted against input cDNA copy number. The differences in slope and *y*-intercept for the cytokine standard curves compared with the actin standard curve indicate a difference in amplification efficiency and sensitivity, respectively. If the amplification efficiencies were identical, the three standard curves should have the same slope and should be superimposed.

quantification results in absolute copy numbers per cell, total RNA concentration, or total tissue. For constructing a standard curve, either RNA or DNA can be used. In our laboratory, we routinely use cDNA plasmid standards. The advantage of using cDNA plasmids is that, once they are constructed, by cloning the target PCR fragment into a suitable plasmid vector, they can be easily prepared in very large amounts. Therefore, numerous experiments can be performed using the same dilutions of one standard, minimizing interassay variation.

To construct cDNA plasmid standards, total RNA is extracted from a tissue that abundantly expresses the target gene. The target gene of interest is amplified by classical qualitative RT-PCR, using the same primers as for real-time RT-PCR. The amplified target is purified on silica columns (e.g., the QIAquick PCR purification kit; Qiagen), ligated into the pGEM-Teasy plasmid (Promega), and transformed into DH5α-competent cells (Life Technologies). Finally, plasmid DNA is extracted using silica cartridges (Nucleobond AX plasmid purification; Macherey-Nagel, Düren, Germany), and cDNA plasmid concentrations are measured spectrophotometrically (Pharmacia, Uppsala, Sweden). The corresponding copy number is calculated using the equation 1 μg of 1000-bp DNA = 9.1×10^{11} molecules.

As an alternative to the standard curve method, the comparative Ct method is often used.[17,18] An advantage using this method is that no standards need to be constructed, and all 96 wells can be applied for unknown samples (except for the calibrator samples). A disadvantage, however, is that the efficiencies of target and housekeeping gene must be identical. If not, the results will not be reliable. As shown in Figure 3, this is not always the case. The differences in slope and *y*-intercept for the cytokine standard curves compared with the actin standard curve indicate a difference in amplification efficiency and sensitivity, respectively.

NORMALIZATION

In each quantitative RT-PCR method, specific errors will be introduced due to minor differences in starting amount of RNA or differences in efficiency of cDNA synthesis and PCR amplification. Therefore, a reliable quantitative RT-PCR method requires correction for these experimental variations. At present this

is most often performed by normalization to a housekeeping gene. Finding a suitable housekeeping gene (i.e., a gene that is constant during the experimental conditions) is, however, not always straightforward. Therefore, different housekeeping genes should be tested in each experimental setup to find the most suitable one (not influenced by the experimental treatment). In the search for the "ideal" reference gene, a kit (Applied Biosystems) can be purchased with a range of housekeeping genes, to test which one is the most suitable in a particular setup.

Many recent articles have discussed the problem of housekeeping genes, and it turns out that the housekeeping genes most commonly used are subject to variation in numerous experimental conditions, bringing into question the reliability of the results obtained. In particular, the use of GAPDH and of β-actin has been severely criticized.[19–21] Ribosomal RNA (rRNA) may be a more valuable alternative, because its expression is less likely to vary under conditions that change the levels of mRNA expression. Drawbacks of using rRNA, however, are that it cannot be used for quantification of samples when starting from mRNA, or when oligo $d(T)_{16}$ is used for cDNA synthesis. Another drawback is its very abundant expression relative to mRNA expression levels of target genes.

Other alternative genes have been proposed for normalization in specified conditions. For instance, for expression profiling of T helper cell differentiation,[22] the search for a suitable housekeeping gene was performed using a microarray approach. Although this method may find a reliable reference gene, it is a rather sophisticated approach and cannot be performed in every laboratory. Moreover, the search must be repeated for each individual experimental setup.

An alternative to normalization by a housekeeping gene could be normalization to an irrelevant exogenously added reference gene.[23] In this case, an unrelated purified RNA is added to the tissue or cells before RNA extraction. Another method that has been proposed is to normalize to the input cell number or total RNA concentration (when working with tissues).[19] Because none of the methods used today is completely satisfactory, the search for a more universal method in normalizing the results continues.

CONCLUDING REMARKS

Real-time PCR, as described in this review, is a revolutionary technique and is becoming the standard method for quantifying cytokine mRNA levels from organs, cells, or cell cultures. Compared with previously used endpoint PCR assays, the technique is very fast, accurate, and sensitive, and it has a decreased potential for PCR contamination. Evidently, the assay described herein for quantifying cytokine gene expression can be easily extrapolated to other classes of mRNA. Overall, the technique has enabled scientists to gain a better insight into many immunological mechanisms and diseases in a fast and relatively automated way.

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