

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, co-amplification 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

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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)

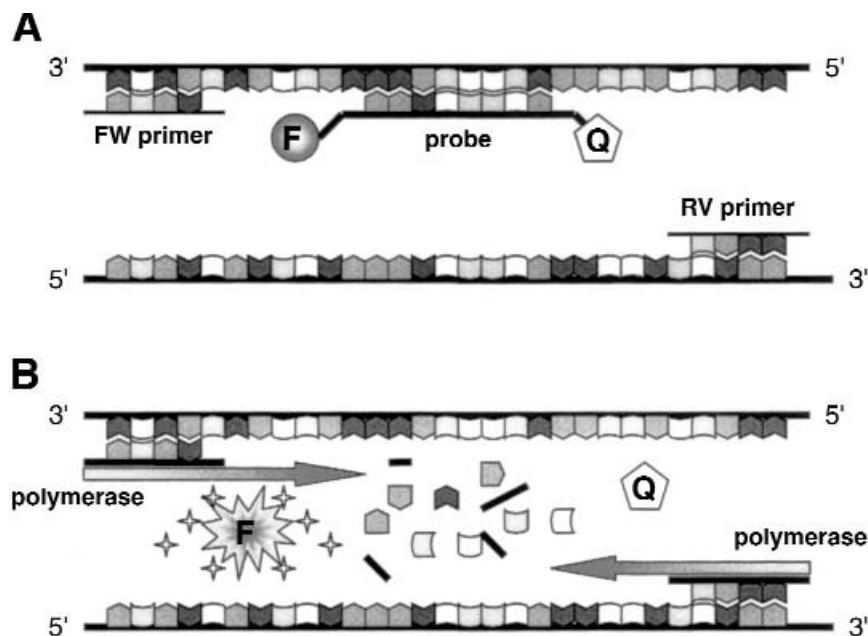
KEY WORDS: real-time RT-PCR, TaqMan, cytokine.

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 (C_t) 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 C_t values of unknown samples on the standard curve to determine the starting copy numbers of the unknown samples. Alternatively, the C_t 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,

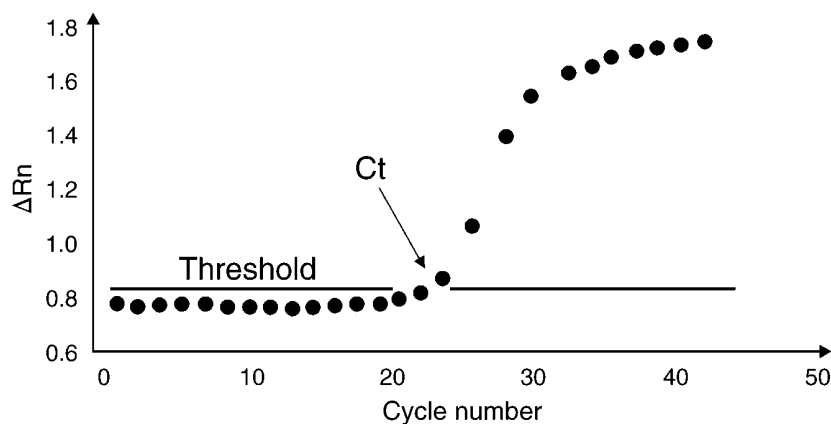


FIGURE 2

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 (C_t) 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 μ M Oligo(dT)₁₆ is added to 0.5–1 μ g 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 MgCl₂, 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 μ L of cDNA is used per 50 μ L 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 I

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 X04964 | – |
| | RV | GATCCACACTCTCCAGCTGCA | | | |
| | TP | CTGTGTAATGAAAAGACGGCACACCCACC | | | |
| IL-2 | FW | CCTGAGCAGGATGGAGAATTACA | 141 | X01772 M16760 AF195956 | – |
| | RV | TCCAGAACATGCCGACAGAG | | | |
| | TP | CCCAAGCAGGCCACAGAATTGAAAG | | | |
| IL-4 | FW | ACAGGAGAAGGGACGCCAT | 95 | M25892 X05253 | – |
| | RV | GAAGCCCTACAGACGAGCTCA | | | |
| | TP | TCCTCACAGCAACGAAGAACACCACA | | | |
| IL-5 | FW | AGCACAGTGGTAAAAGAGACCTT | 117 | X06270 X06271 | – |
| | RV | TCCAATGCATAGCTGGTGATTT | | | |
| | TP | CTGTTGACAAGCAATGAGACGATGAGG | | | |
| IL-6 | FW | GAGGATACCACTCCCAACAGACC | 141 | X54542 M20572 | – |
| | RV | AAGTGCATCATCGTTGTTTCATACA | | | |
| | TP | CAGAAATGCCATTGCACAACCTCTTTTCTCA | | | |
| IL-7 | FW | ATTATGGGTGGTGAGAGCCG | 257 | X07962 M29054 | – |
| | RV | GTTCAATTATTCGGCAATTACTATCA | | | |
| | TP | CCTCCCGCAGACCATGTTCCATGT | | | |
| IL-10 | FW | GGTTGCCAAGCCTTATCGGA | 191 | M37897 M84340 | – |
| | RV | ACCTGCTCCACTGCCTTGCT | | | |
| | TP | TGAGGCGCTGTCATCGATTTCTCCC | | | |
| IL-12 p40 | FW | GGAAGCACGGCAGCAGAATA | 180 | M86671 S82420-6 | – |
| | RV | AACTTGAGGGAGAAGTAGGAATGG | | | |
| | TP | CATCATCAAACCAGACCCGCCCAA | | | |
| IL-13 | FW | GGAGCTGAGCAACATCACACA | 142 | M23504 L13028 | – |
| | RV | GGTCCTGTAGATGGCATTGCA | | | |
| | 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 AB006745 | – |
| | RV | CATCTATCCAGTTGGCCTCTGT | | | |
| | TP | AGGGAGACCTACACTGACACAGCCCAAAA | | | |
| IL-17 | FW | GCTCCAGAAGGCCCTCAGA | 142 | NM_010552 U35108 | – |
| | RV | AGCTTTCCCTCCGCATTGA | | | |
| | TP | CTCTCCACCGCAATGAAGACCCTGA | | | |
| IL-18 | FW | CAGGCCTGACATCTTCTGCAA | 105 | NM_008360 AJ002364 | – |
| | RV | TCTGACATGGCAGCCATTGT | | | |
| | TP | CTCCAGCATCAGGCAAAGAAAGCCG | | | |
| IFN- γ | FW | TCAAGTGGCATAGATGTGGAAGAA | 92 | K00083 M74466 M28381 | – |
| | RV | TGGCTCTGCAGGATTTTCATG | | | |
| | TP | TCACCATCCTTTTGGCAGTTCCTCCAG | | | |
| TNF- α | FW | CATCTTCTCAAAAATTCGAGTGACAA | 175 | M13049 Y00467 | – |
| | RV | TGGGAGTAGACAAGGTACAACCC | | | |
| | TP | CACGTCGTAGCAAACCACCAAGTGG | | | |
| TGF- β 1 | FW | TGACGTCACTGGAGTTGTACGG | 170 | M13177 L42460 L42459 | – |
| | RV | GGTTCATGTCATGGATGGTGC | | | |
| | TP | TTCAGCGCTCACTGCTTGTGACAG | | | |
| MIC-1 | FW | CCAACCAGAGCCGAGAGGA | 113 | NM_011819 AJ011967-8 | – |
| | RV | GTTGACGCGGAGTAGCAGCT | | | |
| | TP | CCGGATACTCAGTCCAGAGGTGAGATTGG | | | |
| GM-CSF | FW | GCCATCAAAGAAGCCCTGAA | 114 | X02333 X03020 | – |
| | RV | GCGGGTCTGCACACATGTTA | | | |
| | TP | ACATGCCTGTACATTGAATGAAGAGGTAGAAG | | | |

FW, forward primer; RV, reverse primer; TP, TaqMan probe dual-labeled with 5' FAM and 3' TAMRA; IL, interleukin; IFN- γ , interferon-gamma; TNF α , 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.

^aAdapted from Giulietti et al.¹² with permission from the publisher (Elsevier Science USA, New York, NY).

^bAmplicon length in base pairs.

^cGenbank accession number of cDNA and corresponding gene available online at <http://www.ncbi.nlm.nih.gov/>

^dPCR amplification on genomic DNA.

TABLE 2Primer 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 | CCAGCCTACTCATTTGGGATCA | | U12470 | |
| | TP | CTCAGCCAGATGCAGTTAACGCCCC | | | |
| Fractalkine | FW | GGGTGGCCATGTTTGCTTAC | 140 | U92565 | + |
| | RV | CAGGCAAGCAGCTCACACTG | | | |
| | TP | TCCCCCGTAGCTGTGGCAGTAACTCAT | | | |
| MIP3 α | 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 | AGAGAAAACACCCCGAAAATGG | | M94129 | |
| | TP | AGCCCTGCTGGTCAATTCCTGTCTGTG | | | |
| CD40 ligand | FW | CTCAAACCTCTGAACAGTGCCT | 88 | X65453 | + |
| | RV | GGCAGGTCCCTAACTGACTTGCT | | | |
| | TP ^d | AGGGAAGACTGCCAGCATCAGCCCT | | | |
| iNOS | FW | CAGCTGGGCTGTACAAAACCTT | 95 | U43428 | - |
| | RV | CATTGGAAGTGAAGCGTTTCG | | L23806 | |
| | TP | CGGGCAGCCTGTGAGACCTTTGA | | | |
| PAF-AH | FW | CCTGCAAGCTGGAAATTCCTC | 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 | TGCAAGCCTTCAGAAATCTGGGATACTAACCA | | | |
| 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 | CTGTTTCAGAAATGGGAAGACTCCGCC | | M76655 | |

FW, forward primer; RV, reverse primer; TP, TaqMan probe dual-labeled with 5' FAM and 3' TAMRA; MCP-1, monocyte chemoattractant protein 1; MIP3 α , 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.

^aAdapted from Giulietti et al.¹² with permission from the publisher (Elsevier Science USA, New York, NY).

^bAmplicon length in base pairs.

^cGenbank accession number of cDNA and corresponding gene available online at <http://www.ncbi.nlm.nih.gov/>

^dPCR amplification on genomic DNA.

TABLE 3Primer and Probe Sequences for Human Cytokines^a

| | | Sequence (5'-3') | Length (bp) ^b | Accession ^c | gDNA ^d |
|---------------|----|---------------------------------|--------------------------|------------------------|-------------------|
| IL-1 α | FW | CGCCAATGACTCAGAGGAAGA | 120 | X02531 | - |
| | RV | AGGGCGTCATTCAGGATGAA | | X03833 | |
| | TP | AGCACCTTTTAGCTTCCTGAGCAATGTGAAA | | | |
| IL-2 | FW | AACTCACCAGGATGCTCACATTTA | 148 | NM_000586 | - |
| | RV | TCCCTGGGTCTTAAGTGAAAAGTTT | | J00264 | |
| | TP | TTTTACATGCCCAAGAAGGCCACAGAACT | | | |
| IL-4 | FW | CCACGGACACAAGTGCGATA | 149 | M13982 | - |
| | RV | CCCTGCAGAAGGTTTCCTTCT | | M23442 | |
| | TP | TCTGTGCACCCGAGTTGACCGTAACAGAC | | | |
| IL-10 | FW | GTGATGCCCAAGCTGAGA | 138 | AF043333 | - |
| | RV | CACGGCCTTGCTCTTGTTTT | | U16720 | |
| | TP | CCAAGACCCAGACATCAAGGCGCA | | | |
| IL-12p40 | FW | TGGAGTGCCAGGAGGACAGT | 147 | AF180563 | - |
| | RV | TCTTGGGTGGGTCAAGTTTG | | AY008847 | |
| | TP | ATGGTGGATGCCGTTTACAAGCTCAA | | | |
| IL-15 | FW | GGAGGCATCGTGATGGAT | 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 | TAGCCCATGTTGTAGCAAACCCCTCAAGCT | | 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.

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^bAmplicon length in base pairs.

^cGenbank accession number of cDNA and corresponding gene available online at <http://www.ncbi.nlm.nih.gov/>

^dPCR 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 μ L, containing 0.5 μ L cDNA sample, 1 \times buffer A (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 10 mM EDTA, 60 nM Passive Refer-

ence 1), 200 μ M dNTPs, 3–9 mM MgCl₂, 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 MgCl₂ (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-2 β | FW | CTTAGAAAATACAGCATTGCGGAG | 194 | M16506 | + |
| | RV | GGATGTGCTTTGCATTCTTGG | | | |
| | TP | TTCTGCATCTCATGCCAACGGG | | | |
| Bcl-xL | FW | CACTGTGCGTGGAAGCGTA | 127 | U51278 U78030-1 | + |
| | RV | AAAGTGTCCCAGCCGCC | | | |
| | TP | CAAGGAGATGCAGGTATTGGTGAGTCGG | | | |
| Bax- α | FW | GTTTCATCCAGGATCGAGCAG | 238 | L22472 | + |
| | RV | CCCCAGTTGAAGTTGCCATC | | | |
| | TP | AGCTGAGCGAGTGTCTCCGGCG | | | |
| Bcl-6 | FW | ATGTACAGCCATCTCCCCT | 134 | U41465 | + |
| | RV | TTAGGGACTTGCCCTGGCACT | | | |
| | TP | AATGCCTGTGGCCAACCCTTTTCC | | | |
| Bid | FW | TGGCAGTGCTTGGAGCTACA | 140 | BC002031 AC006404 | – |
| | RV | CCTCCAGCTCTTGGCGAGTA | | | |
| | TP | TGAGGTCAGCAACGGTTCCGGC | | | |
| c-FLIP | FW | GCAACCCAGACACTGCACAA | 148 | U97076 | + |
| | RV | CGTCTCCTGCCTTGCTTCAG | | | |
| | TP | AGAAGCCCTCCAGCTCATCCTCTGTGT | | | |
| c-jun | FW | CCTGTCCCCTATCGACATGG | 93 | J04115 X12740 | + |
| | RV | CTTTTCCGGCACTTGGAGG | | | |
| | TP | TCCTCATGCGCTTCTCTCTGCCT | | | |
| c-myc | FW | TGAGCCCCTAGTGCTGCAT | 137 | AF076523 L00038-9 | – |
| | RV | AGCCCGACTCCGACCTCTT | | | |
| | TP | CTTCTTGCTCTTCTTCAGAGTCGCTGCTG | | | |
| c-fos | FW | CTCCTTCTCCAGCATGGGC | 81 | J00370 | – |
| | RV | GGGATAAAGTTGGCACTAGAGACG | | | |
| | TP | TCAACACACAGGACTTTTGCAGATCT | | | |

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

^aAmplicon length in base pairs.^bGenbank accession number of cDNA and corresponding gene available online at <http://www.ncbi.nlm.nih.gov/>^cPCR 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,

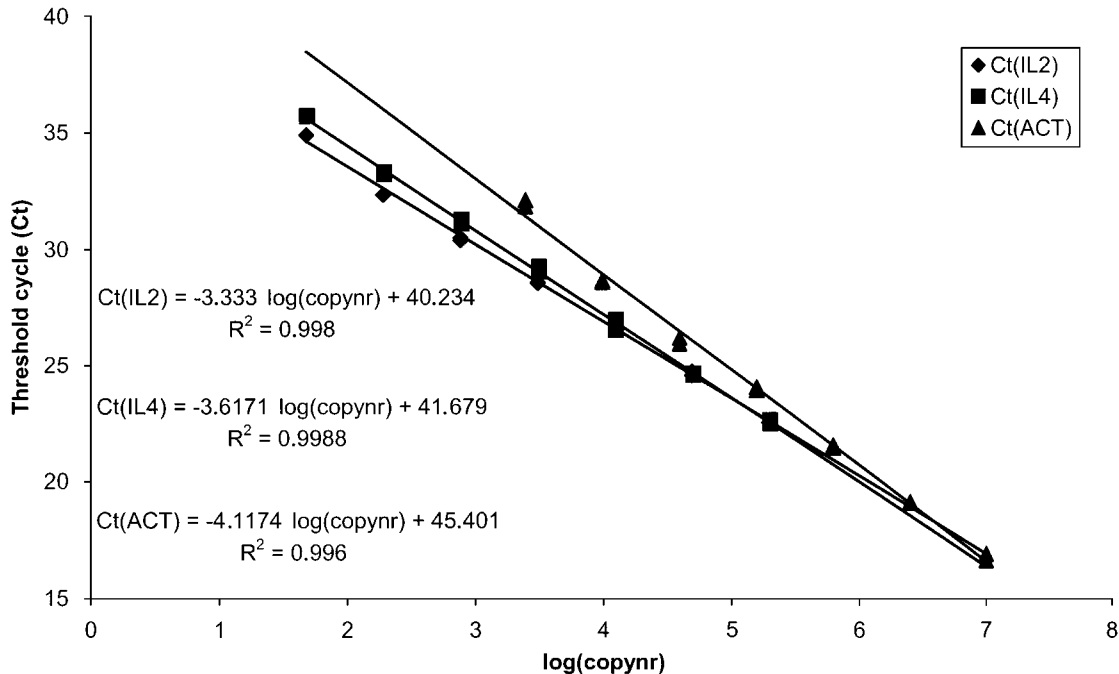


FIGURE 3

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 DH5 α -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 calcu-

lated using the equation $1 \mu\text{g}$ 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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