METHODS **25**, 386–401 (2001)
doi:10.1006/meth.2001.1261, available online at http://www.idealibrary.com on IDEAL®

An Overview of Real-Time Quantitative PCR: Applications to Quantify Cytokine Gene Expression

Annapaula Giulietti[1], Lut Overbergh[1], Dirk Valckx, Brigitte Decallonne, Roger Bouillon, and Chantal Mathieu[2]

Laboratory for Experimental Medicine and Endocrinology (LEGENDO), Catholic University of Leuven, U.Z. Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium

The analysis of cytokine profiles helps to clarify functional properties of immune cells, both for research and for clinical diagnosis. The real-time reverse transcription polymerase chain reaction (RT-PCR) is becoming widely used to quantify cytokines from cells, body fluids, tissues, or tissue biopsies. Being a very powerful and sensitive method it can be used to quantify mRNA expression levels of cytokines, which are often very low in the tissues under investigation. The method allows for the direct detection of PCR product during the exponential phase of the reaction, combining amplification and detection in one single step. In this review we discuss the principle of real-time RT-PCR, the different methodologies and chemistries available, the assets, and some of the pitfalls. With the TaqMan chemistry and the 7700 Sequence Detection System (Applied Biosystems), validation for a large panel of murine and human cytokines and other factors playing a role in the immune system is discussed in detail. In summary, the real-time RT-PCR technique is very accurate and sensitive, allows a high throughput, and can be performed on very small samples; therefore it is the method of choice for quantification of cytokine profiles in immune cells or inflamed tissues. © 2001 Elsevier Science (USA)

Key Words: cytokines; quantification; real-time reverse transcription polymerase chain reaction; TaqMan.

Cytokines are regulatory proteins, which play a central role in the immune system by modulating immune responses, including lymphocyte activation, proliferation, differentiation, survival, and apoptosis. They are low-molecular-weight proteins secreted by many different cell types, most prominently by lymphocytes, antigen-presenting cells, monocytes, endothelial cells, and fibroblasts. Although their classification is rather confusing, i.e., their names do not always indicate their most important function, cytokines can be classified into different groups, such as interleukins (IL-1 to IL-23), interferons (e.g., IFN-α, IFN-γ), colony-stimulating factors, tumor necrosis factors (TNFs), tumor growth factors (e.g., TGF-β), and chemokines (e.g., MCP-1, MIP-1)(1–5).

To elucidate the immune and pathological pathways involved in many inflammatory reactions, autoimmune diseases, and transplant rejections, it is important to quantify the cytokines involved. Indeed, knowing the local cytokine profiles is essential to gaining insight into the immune processes involved (6, 7). Because the tissue samples available to be analyzed are often too small to allow quantification of cytokines at the protein level, the detection of mRNA is widely used to investigate the cytokine profiles at sites of immune infiltration or inflammation. Moreover, cytokine protein detection, by techniques such as ELISA, allows only a limited number of cytokines to be analyzed from a single sample.

At present, a variety of methods are used to quantify mRNA expression, such as Northern blotting, *in situ* hybridization, RNase protection assays, cDNA arrays, and reverse transcription polymerase chain reaction (RT-PCR). Quantitative RT-PCR is the method of choice used to quantify the mRNA expression of cytokines, which are often expressed at very low levels. It is the most sensitive and accurate of the quantification methods (8). Since the discovery of PCR (9) numerous applications have been described to quantify the results, such as semiquantitative and quantitative competitive RT-PCR and its latest innovation quantitative "real-time" RT-PCR. In the semiquantitative method PCR

[1] Both authors contributed equally to this review.
[2] To whom correspondence should be addressed. Fax: 00 32 16 345934. E-mail: chantal.mathieu@med.kuleuven.ac.be

1046-2023/01 $35.00
© 2001 Elsevier Science (USA)
All rights reserved.

product accumulation is measured during the exponential phase of the reaction by interruption of the PCR after an experimentally determined number of cycles (10–12). In this method it is extremely important that PCR product is measured during the exponential phase of the reaction. Drawbacks of this system are the relatively small linear range, as well as the fact that the results obtained will at a maximum be "semi" quantitative.

Alternatively, quantitative competitive RT-PCR has been widely used for cytokine mRNA analysis (13–16). This method requires coamplification of a "competitor" with the unknown sample in the same tube. This internal control consists of target DNA or RNA that has been slightly modified. For cytokine quantification, mosaic RNAs containing sequences of several cytokines or RNAs consisting of slightly modified sequences of individual cytokines have been used (14). Thus, one primer set is used to amplify the target and the competitor simultaneously, although they can be distinguished from each other, for instance, by difference in length or restriction sites. The amount of mRNA is quantified by titration of an unknown amount of target template against a dilution series of known amounts of internal competitor. Although this method provides a strategy for accurate quantification, the design of internal standards is technically complicated and validation of the technique is labor intensive. Moreover, this may still not result in absolute quantification because possible differences in efficiency between control and target will remain undetected (17).

Irrespective of the PCR amplification method used, several techniques are used for the detection of PCR products, such as agarose gel electrophoresis and ethidium bromide staining (11), fluorescence labeling and analysis using polyacrylamide gels (14), and radioactive labeling and Southern blotting or detection by phosphorimaging (18). All these techniques require intensive and laborious post-PCR manipulation, make use of hazardous chemicals, and carry a potential risk for laboratory contamination.

The introduction of the new procedure based on fluorescence–kinetic RT-PCR enables quantification of the PCR product in "real-time." This sensitive and accurate technique measures PCR product accumulation during the exponential phase of the reaction. The technique is much faster than the previous endpoint RT-PCR as it is designed to provide information as rapidly as the amplification process itself, thus requiring no post-PCR manipulations.

PRINCIPLE OF REAL-TIME PCR

Two important findings led to the discovery of real-time PCR: first, the finding that the *Taq* polymerase possesses $5' \rightarrow 3'$-exonuclease activity (19); second, the construction of dual-labeled oligonucleotide probes, which emit a fluorescence signal only on cleavage, based on the fluorescence resonance energy transfer (FRET) principle (20, 21). In the TaqMan assay these two important findings are combined (22). In this real-time PCR method the *Taq* polymerase enzyme cleaves an internal labeled nonextendable probe, the so-called "TaqMan" probe, during the extension phase of the PCR. The probe is dual-labeled, with a reporter dye, e.g., FAM (6-carboxyfluorescein), at one end of the probe and a quencher dye, e.g. TAMRA (6-carboxytetramethylrhodamine), at the other extremity. As long as the probe is intact (in its free form), fluorescence energy transfer occurs through which the fluorescence emission of the reporter dye is absorbed by the quenching dye. On nuclease degradation of the probe during the PCR, the reporter and quencher dyes are separated, and the reporter dye emission is no longer transferred to the quenching dye (no more FRET), resulting in an increase of reporter fluorescence emission (e.g., for FAM at 518 nm). This process occurs in every cycle and does not interfere with the exponential accumulation of PCR product.

More recently, other sophisticated systems have been developed, such as molecular beacons, scorpions, and hybridization probes. These systems all rely on the FRET principle, although without the need for hydrolysis by the nuclease activity of the *Taq* polymerase. Finally, the use of dsDNA-binding dyes, such as SYBR Green, was found to be very useful in detecting PCR product formation. Using this system, the need for an expensive, although specific probe can be avoided.

Using any of the developed chemistries, the increase in fluorescence emission can be read by a sequence detector in "real time," during the course of the reaction, and is a direct consequence of target amplification during PCR. A computer software program calculates a ΔRn using the equation $\Delta Rn = Rn^{+} - Rn^{-}$, where Rn^{+} is the fluorescence emission of the product at each time point and Rn^{-} is the fluorescence emission of the baseline (22, 23). Thus, this value expresses the probe degradation during the PCR process. The computer software constructs amplification plots (Fig. 1A) using the fluorescence emission data that are collected during the PCR amplification. The ΔRn values are plotted versus the cycle number. During the early cycles of the PCR amplification, the ΔRn values do not exceed the baseline. An arbitrary threshold is chosen, based on the

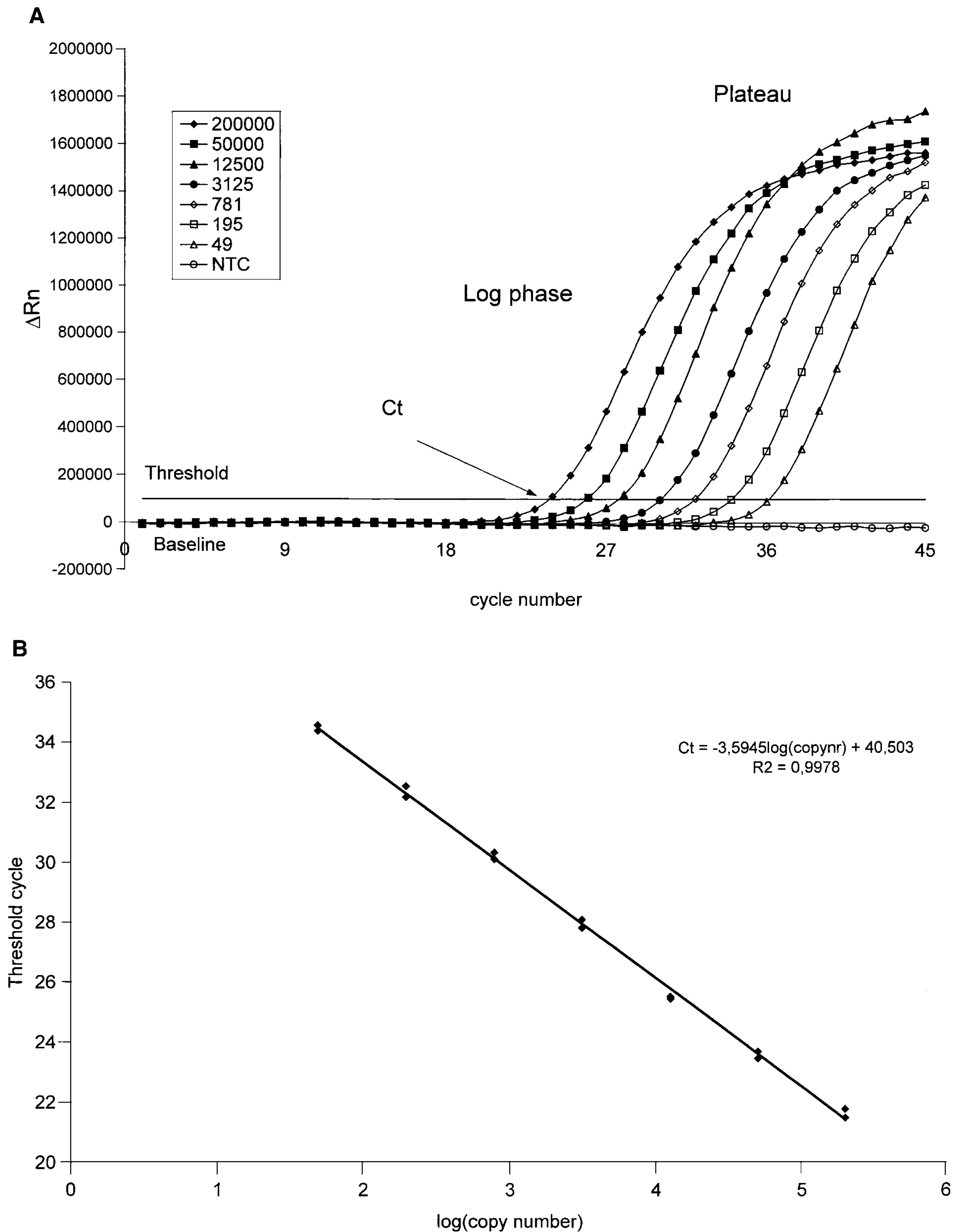

FIG. 1. (A) Amplification plots of IL-4 plasmid cDNA. Fivefold serial dilutions of IL-4 plasmid cDNA were amplified by real-time RT-PCR using the ABI Prism 7700 SDS. The software constructs amplification plots, where ΔRn is plotted against cycle number. (B) Standard curve for IL-4. C_T values are plotted against input cDNA copy number.

variability of the baseline, usually determined as 10 times the standard deviation of the baseline, set from cycles 3 to 15. This can be manually changed for each individual experiment if necessary. Threshold cycle (C_t) values are then calculated by determining the point at which the fluorescence exceeds this chosen threshold limit. C_t is reported as the cycle number at this point. Therefore, C_t values decrease linearly with increasing input target quantity (Fig. 1B). This can be used as a quantitative measurement of the input target.

The high specificity of this method is due to a complementarity between the set of primers, the internal probe, and the target. Indeed, a fluorescence signal will be generated only if the probe is annealed to the target sequence during PCR amplification.

INSTRUMENTATION AND DETECTION CHEMISTRIES AVAILABLE

Real-time PCR is a very powerful technique. Therefore, it is a technology in wide expansion, offering an increasing range of possibilities in instrumentation and chemistries.

A. Instrumentation

At present, it is possible to choose between a variety of competing instruments, the most important characteristics of which are summarized in Table 1. All the instruments available run the reaction as a closed-tube system, diminishing the chances of contamination.

The ABI Prism 7700 Sequence Detection System (SDS) from Applied Biosystems (Foster City, CA) was the first commercially available thermocycler for real-time PCR. Laser light induces the fluorescence. Continuous fluorescence wavelength detection from 500 to 660 nm allows multiplex PCR by the use of multiple fluorophores in a single reaction. The instrument can be used for assays based on hydrolysis probes, molecular beacons, and dsDNA-binding dyes. One run has a capacity of 96-well positions and takes about 2 h to complete. The result, however, can be viewed and analyzed only when the amplification run is completed. Numerous publications describe use of the ABI Prism 7700 SDS to quantify cytokine mRNA expression, convincingly illustrating that it is capable of providing reliable results using TaqMan probes, for instance, in studies on cytokine quantification in murine tissues (24), β-cell islet grafts (25, 26), pancreatic tissue (27–30), lymphoid organs such as spleen and lymph nodes (31–33), thymus (34), spinal cord (35), intestine (36, 37), and peritoneal macrophages (38). Examples from studies on cytokine quantification in humans include, for instance, studies on UV-irradiated skin (39), myocardium (40), peripheral blood mononuclear cells (41), and synovial tissues (42).

Another system from Applied Biosystems is the Gene Amp 5700 SDS. This system, like the 7700 SDS, can carry out real-time RT-PCR using the TaqMan assay, molecular beacons, or the DNA-binding dye SYBR Green I. The 5700 SDS differs from the 7700 SDS in use of a halogen lamp instead of a laser. It is a less

TABLE 1

Real-Time PCR Instruments

Company	PCR system	Sample format	Max sample number
Applied Biosystems	ABI Prism 7700 SDS	Microplate	96
www.appliedbiosystems.com		Tubes	
	GeneAmp 5700 SDS	Microplate	96
	ABI Prism 7900 HT SDS	Tubes	96, 384
		Microplate	
Bio-Rad	iCycler iQ (thermal cycler plus optical module)	Microplate	96
www.bio-rad.com		Tubes	96
Cepheid[a]	Smart Cycler	Tubes	16
www.cepheid.com			
Corbett Research	Rotor-Gene	Tubes	32
www.corbettresearch.com		Strip tubes	72
Roche Molecular Biochemicals	LightCycler	Capillaries	32
biochem.roche.com			
Stratagene	Mx4000 Multiplex	Microplate	96
www.stratagene.com	Quantitative PCR		

[a] Designed by Cepheid, but distributed by Fisher Scientific (United States and Japan) or by Eurogentec (Europe).

expensive alternative, but allows only single-wavelength detection. In the literature the use of the Gene Amp 5700 in combination with SYBR Green I technology to quantify cytokine mRNA has been exemplified in lung infections by *Legionella pneumophila* (43).

Very recently, Applied Biosystems launched the ABI Prism 7900HT, which has the same specifications as the 7700 SDS, but is designed especially for very high throughput applications. The process is completely automated, it is able to prepare the plates that will load the sequence detector and it has the ability to handle microtiter plates ranging in size from 96 to 384 wells. The system also offers an optional automation accessory capable of loading up to eighty-four 384-well plates into the instrument for a 24-h, hands-free operation.

The LightCycler from Roche Molecular Biochemicals (Mannheim, Germany) performs real-time RT-PCR in borosilicate glass capillaries which can hold up to 20-μl of sample. Fluorescence excitation is made by a blue light-emitting diode and is read by three silicon photodiodes with different-wavelength filters, allowing detection of spectrally distinct fluorophores. Therefore, multiplex PCR can be performed. Moreover, the fluorescence data can be visualized during PCR amplification. The combination of using air for rapid thermal cycling and the high surface-to-volume ratio of the capillaries allows a complete PCR run of 30–40 cycles to be performed in 20–30 min (44). For cytokine analysis, the LightCycler is most often used in combination with the dsDNA binding dye SYBR Green I. This method has been validated in several studies, e.g., in murine retinas (45) and human synovial membranes, and in dendritic cells and skin punction biopsies (46). The system can also be used in combination with TaqMan probes, for example, in dendritic cells (47).

Advantages of the LightCycler, compared with the 7700 SDS are its lower price, the ability to view the data while PCR amplification is still in progress, and the high throughput, since one run can be completed in 20–30 min. Furthermore, analyzing the specificity of the results by performing melting curves adds to the advantages, as it makes the use of dsDNA-binding dyes such as SYBR Green I more reliable (see next section). Disadvantages are, however, the use of capillaries as opposed to tubes, their use being less practical for the investigator. Moreover, the small sample format, allowing only 32 wells to be analyzed simultaneously, is a disadvantage. This is especially important when performing quantification studies, where most often many samples have to be compared within a single experiment. Taking into account that some of the wells are used for a standard curve, and all unknown samples are performed in duplicate wells, only a limited number of samples can be analyzed simultaneously.

The iCycler iQ from Bio-Rad Instruments (Hercules, CA) is another option for the real-time PCR. It has an optical module that can be connected in the thermal cycler, measuring fluorescence emission during the PCR. The fluorescence emission can be viewed during the course of PCR amplification. According to the manufacturer's specifications, the iCycler is able to multiplex four different fluorophores per sample tube. Moreover, the 96 samples are tracked simultaneously, thereby providing a very fast assay.

A new option is the Mx4000 Multiplex from Stratagene (La Jolla, CA). This sequence detector instrument is able to detect multiple-fluorescence PCR chemistries, including TaqMan probes, hybridization probes, and molecular beacons. The design of the sample block accommodates samples in a variety of formats (96-well plates, 8-strip tubes, or individual tubes). The light source for the Mx4000 system is a quartz tungsten halogen lamp that generates a broad excitation range of 350 to 750 nm, again allowing the performance of multiplex PCR. The system generates real-time amplification plots that can be viewed as the PCR run progresses.

The Smart Cycler System has recently become available from Cepheid (Sunnyvale, CA). The system can be operated with molecular beacons, scorpions, hybridization probes, TaqMan probes, or SyberGreen I. An advantage of this system is its high flexibility, as it contains 16 different modules. Each module can be individually programmed and has its own optical subsystem, being able to detect four different fluorophores in one reaction. Different operators can define the parameters for each reaction and different runs can be carried out at the same time for individual experiments. With the software it is also possible to view the data as soon as they are collected. A disadvantage of this system, especially accounting for quantification analysis, is the small sample number.

The Rotor-Gene, designed by Corbett Research (Mortlake, Sydney, Australia), is a "centrifugal" thermal cycler comparable to the LightCycler. It uses two light sources, a blue one that emits fluorescence at 470 nm and a green one at 530 nm. Either 32 or 72 samples can be loaded in one run, depending on the tube format. A wide variety of fluorophores can be detected.

The last four instruments described have become commercially available only very recently. Therefore, at present, publications validating these instruments are not yet available.

B. Detection chemistries

Currently a range of different possibilities in detection chemistries are available for real-time PCR. All of

them can be used in the different instruments described above.

1. *Hydrolysis or TaqMan Probes*

Three oligonucleotides are used: a forward primer, a reverse primer, and a probe (Fig 2). All of them are specific for the target and are able to bind to it. The TaqMan assay uses a probe technology that exploits the 5′ → 3′-nuclease activity of an enzyme, the most commonly used being *Taq* polymerase (19). The assay efficiency is largely dependent on this 5′ → 3′ nuclease activity. In this regard one should be careful in choosing a suitable polymerase. Indeed, some polymerases available on the market appear not to be suitable for real-time RT-PCR (48), even though the manufacturers claim they possess 5′-exonuclease activity. The probe is an oligonucleotide with a reporter dye at the 5′ end and a quencher dye at the 3′ end. The fluorescent reporter dye is attached covalently to the 5′ end and can be FAM (6-carboxyfluorescein), TET (tetrachloro-6-carboxyfluorescein), JOE (2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein), HEX (hexacholoro-6-carboxyfluorescein), or VIC. The reporter is quenched by TAMRA (6-carboxytetramethylrhodamine), bound to the 3′ end by a linker arm. DABCYL [4-(4′-dimethylaminophenylazo)benzoic acid] can also be used as a quencher dye (49), but its use is much more prevalent in the molecular beacon probes. An advantage of using DABCYL in the TaqMan probes is its reduced autofluorescence compared with TAMRA. When the probe is intact the quencher dye absorbs the fluorescence of the reporter dye due to the proximity between both. The proximity between quencher and fluorophore permits FRET, and fluorescence emission does not occur. By the 5′-exonuclease activity of the *Taq* polymerase the probe is hydrolyzed and the reporter dye is separated from the quencher, resulting in an increase in fluorescence emission. During PCR amplification, if the target of interest is present, the probe specifically anneals to the target. The *Taq* polymerase cleaves the probe, allowing an increase in fluorescence emission. This increase in fluorescence is measured cycle by cycle and is a direct consequence of the amplification process (22, 23). TaqMan probes, or hydrolysis probes, have been widely used for real-time RT-PCR for research purposes or diagnosis, such as gene detection (50), virus quantitation (51, 52), molecular quantification of micrometastases in cervical cancer (53), and cytokine quantification (24), just to quote some examples. In an exponentially increasing number of publications use of the TaqMan probes for cytokine quantification is described, conclusively showing that the results are very specific and sensitive, which is important when analyzing target genes with very low expression levels.

2. *Molecular Beacons*

These are probes that form a stem-and-loop structure from a single-stranded DNA molecule (Fig 3). A fluorophore is linked to one end of the molecule and a quencher is linked to the other end (54). Fluorescence is quenched when the probe is in a hairpin-like structure (stem-and-loop structure) due to the proximity between quencher and fluorophore allowing FRET (20, 21).

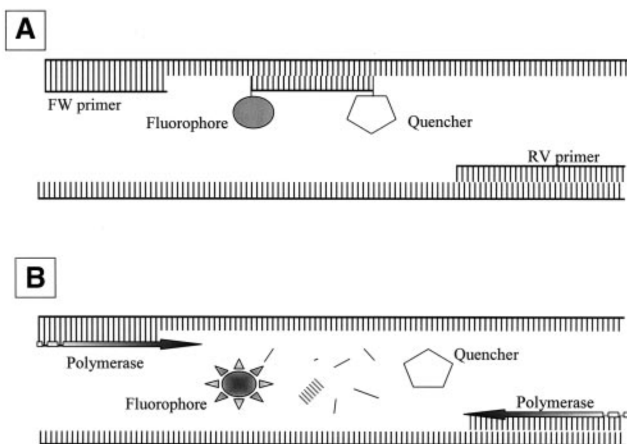

FIG. 2. TaqMan system. (A) After denaturation, primers and probe anneal to the target. Fluorescence does not occur because of the proximity between fluorophore and quencher. (B) During the extension phase, the probe is cleaved by the 5′ → 3′ enzymatic activity of *Taq* polymerase. Thereby quencher and fluorophore are separated, allowing fluorescence emission from the reporter dye. FW, forward; RV, reverse.

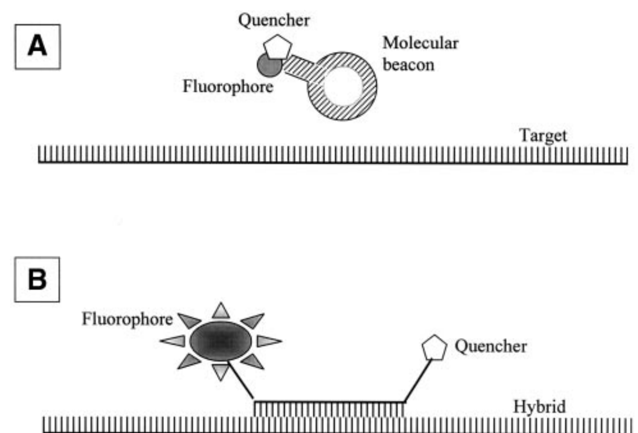

FIG. 3. Molecular beacons. (A) The probe has a stem-and-loop hairpin shape, so that quencher and fluorophore are in very close proximity, avoiding fluorescence emission. (B) As soon as probe hybridizes to the target, the probe is conformationally changed to a linear structure, separating quencher and fluorophore. This results in an increase in fluorescence emission.

When the probe sequence in the loop anneals to a complementary nucleic acid target sequence, a conformational change allows the formation of a linear structure whereby FRET no longer occurs, increasing the fluorescence emission. Molecular beacons are especially suitable for identifying point mutations. They can distinguish targets that differ by only a single nucleotide and they are significantly more specific than conventional oligonucleotide probes of equivalent length (55, 56). A description of the methodology to use molecular beacons to detect single nucleotide polymorphisms is published in this issue (57). Molecular beacons have been used mainly for mutation detection (58, 59), quantification of pathogens (60, 61), virus replication (62), and gender detection in embryos (63). Multiplex PCR for detection of different retroviruses has also been documented (64).

3. Scorpions

The reaction is carried out with two oligonucleotides: a primer and a fluorescent molecule that combines the primer and probe function (65). The primer is linked to a specific probe sequence that is held in a hairpin–loop form. This configuration brings the fluorophore in close proximity with the quencher and avoids fluorescence. As soon as annealing between the primer–probe and the target occurs, the hairpin is opened and the fluorophore and quencher are separated, resulting in an increase in fluorescence emission. Compared with molecular beacons and TaqMan probes, scorpions are faster and are able to produce a much stronger fluorescence signal (66). Scorpions allow a very specific PCR amplification, because fluorescence emission occurs only when the specific target is present in the reaction, enabling hybridization between the probe–primer and the target. Scorpions represent a relatively new chemistry, validated for mutation detection, but most likely it will be adapted to other assays (66).

4. Hybridization Probes

In this system four oligonucleotides are used: two primers and two probes (Fig. 4). Hybridization probes have a single label, one with a donor fluorophore and one with an acceptor fluorophore. The sequences of the two probes are selected so that they can hybridize to the target sequences in a head-to-tail arrangement, bringing the two dyes very close to each other, allowing FRET. The acceptor dye in one of the probes transfers energy, allowing the other one to dissipate fluorescence at a different wavelength. The amount of fluorescence is directly proportional to the amount of target DNA generated during the PCR process (67). This method has been convincingly validated in studies using the LightCycler instrument. Examples of its use are detection of minimal disease after therapy (68), mutation detection (69–71), pathogen detection (72), and viral load quantification (73).

5. SYBR Green I

SYBR Green I is a DNA-binding dye that incorporates into dsDNA (Fig 5). It has an undetectable fluorescence

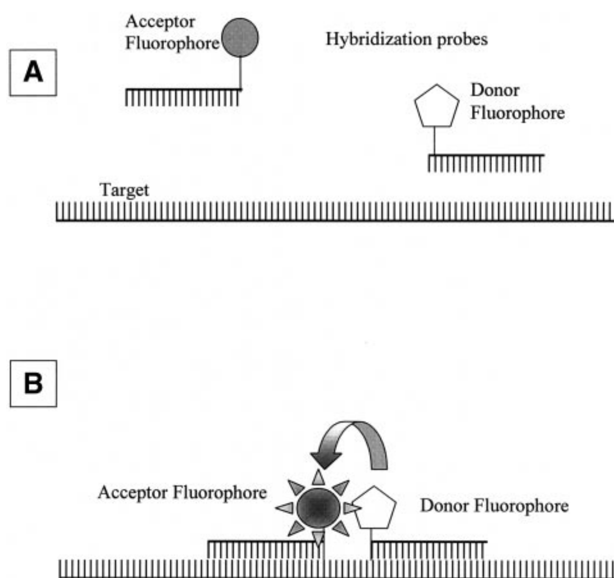

FIG. 4. Hybridization probes. (A) Two probes each carrying one fluorophore, a donor and an acceptor. (B) The probes anneal to the target in a head-to-tail conformation bringing acceptor and donor fluorophores in close proximity, allowing fluorescence emission.

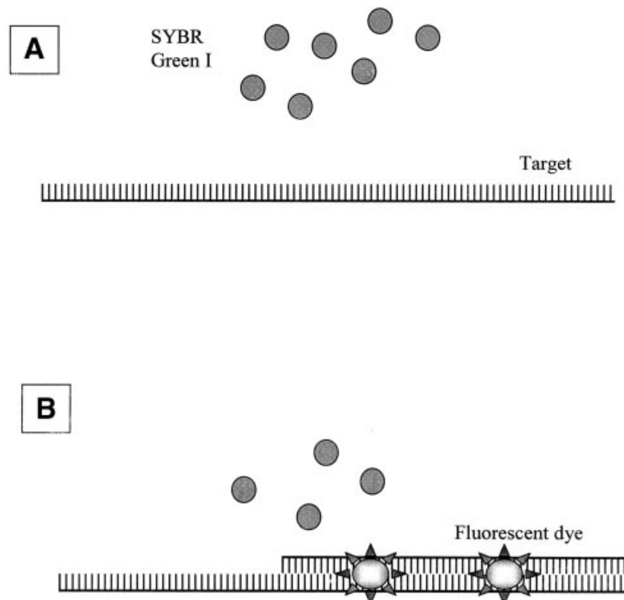

FIG. 5. DNA-binding dyes (SYBR Green I). (A) The dyes free in the solution do not emit fluorescence light. (B) As soon as the SYBR Green binds to the dsDNA, target fluorescence occurs.

when it is in its free form, but once bound to the dsDNA it starts to emit fluorescence. Its greatest advantage is that it can be used with any pair of primers for any target, making its use less expensive than that of a probe. However, specificity is diminished due to the risk of amplifying nonspecific PCR products (45). Indeed, SYBR Green I binds to any dsDNA, detecting not only the specific target, but also nonspecific PCR products and primer dimers. This is a major disadvantage. There are a few ways of handling this problem, comparing melting curves being one of them (74). The LightCycler is capable of analyzing melting curves of the reaction, similar to the RotorGene, the Smart Cycler, the iCycler, and the Mx4000. In this way, the fraction of fluorescence originating from the target can be distinguished from that originating from primer dimers or specific amplification products. Once the melting curves are established, it is possible to set the software to acquire fluorescence above the primer dimers' melting temperature but below that of the product. Second, a careful design of the primers and optimization of the reaction conditions can reduce the formation of primer dimers to a level that is important only for very low copy number detection. Furthermore, techniques such as Hot Start PCR can reduce primer dimer formation (75). The SYBR Green I method has been successfully used for many applications using the LightCycler, e.g., for cytokine quantification (45, 46, 76) and viral load detection (77).

QUANTIFICATION

To quantify the results obtained by real-time RT-PCR, two different methods are commonly used: the standard curve method and the comparative threshold method.

In the standard curve method a sample of known concentration is used to construct a standard curve. This standard can be purified plasmid dsDNA, *in vitro*-transcribed RNA, *in vitro*-synthesized ssDNA (78), or any cDNA sample expressing the target gene. The concentration of these DNA or RNA samples can be measured spectrophotometrically at 260 nm and converted to the number of copies using the molecular weight of the DNA or RNA. For absolute quantification of mRNA expression absolute standards have to be used, for instance, *in vitro*-transcribed RNA. However, this implicates a labor-intensive construction of cDNA plasmids that need to be reverse transcribed *in vitro*. Furthermore, the stability of such standards has to be taken into account. Because of these difficulties, this method is used only for applications where absolute quantification is essential to obtain reliable results, e.g., in quantification of viral load (51, 79).

More often used for quantification are cDNA plasmid standards. For quantification of mRNA expression, however, these will result only in a relative quantification, because variations in efficiency of the reverse transcription step are not controlled. An 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 variations. Furthermore, cDNA plasmids can be stored for long periods, aliquoted at −20°C, without significant degradation. Correction for inefficiencies in RNA input or reverse transcriptase is performed by normalization to a housekeeping gene (see next section). To our experience, a great advantage of using this method is that the standard curve that is included in each PCR run provides an extra control and correction on the PCR efficiency of each individual run, making interassay comparisons more reliable.

An alternative method used for relative quantification is the comparative C_T method. A formal description of the comparative C_T method is included in this issue (80). In this method arithmetic formulas are used to calculate relative expression levels, compared with a calibrator, which can be for instance a control (non-treated) sample. The amount of target, normalized to an endogenous housekeeping gene and relative to the calibrator, is then given by $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T(\text{sample}) - \Delta C_T(\text{calibrator})$, and ΔC_T is the C_T of the target gene subtracted from the C_T of the housekeeping gene. The equation thus represents the normalized expression of the target gene in the unknown sample, relative to the normalized expression of the calibrator sample. Importantly, for the $\Delta\Delta C_T$ method to be applicable, the efficiency of PCR amplification for the target gene must be approximately equal to that for the housekeeping gene. For every individual target gene this has to be tested, by determining how the ΔC_T(sample) and ΔC_T(calibrator) vary with template dilution. If the efficiencies of the two are not the same, and in our hands this is quite often the case, another method for quantification has to be used, for example, the standard curve method. Advantages of using this system are that no standards have to be constructed and that the 96 wells can be fully applied for unknown samples, saving time and money. A disadvantage however, is that the efficiencies of amplification of housekeeping and target gene have to be similar to obtain reliable results.

NORMALIZATION

A reliable quantitative RT-PCR method requires correction for experimental variations in individual reverse transcription and PCR efficiencies. Indeed, differences in efficiency of the reverse transcription will result in an amount of cDNA that does not correspond to the starting amount of RNA. Furthermore, because of the exponential nature of the PCR, minor differences in PCR amplification efficiency will result in major differences in PCR product (81). However, this problem is minor using the real-time PCR technique, as opposed to endpoint PCR quantification techniques. This is because quantification is based on C_T values, which are determined very early in the exponential phase of the reaction (Fig. 1A). Indeed, differences in efficiency become apparent only during the exponential phase or in the plateau phase, while essentially no changes in C_T values are measured. An example of this is shown in Fig. 1A, where endpoint analysis would provide results completely out of range for the third dilution of the standard, while the C_T value is correct.

The normalization to a housekeeping gene is currently the most acceptable method to correct for minor variations due to differences in input RNA amount or in efficiencies of reverse transcription. An ideal "housekeeping" gene should be expressed at a constant level among different tissues of an organism, at all stages of development, and should not be affected by the experimental treatment itself. Finding a gene with these characteristics is not always straightforward. The most common genes used as housekeeping gene are β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), rRNA (ribosomal RNA), and hypoxanthine–guanine phosphoribosyltransferase;, however other genes also are used, such as cyclophilin (82), mitochondrial ATP synthase 6 (83), and porphobilinogen deaminase (84). Choosing the ideal housekeeping gene for each experiment is very important to ensure the credibility of the results.

β-Actin mRNA encodes a cytoskeleton protein and is expressed among almost all cell types. It is widely used to normalize results in real-time RT-PCR, although there is some evidence that its expression can be changed under various types of treatment and diseases, e.g., porcine immune cells and tissues (85). Our experience with β-actin has shown that its use is valuable for most of the experiments carried out in our laboratory. Indeed, β-actin copy numbers of different samples under different types of treatments are in most cases constant, not interfering with the final result.

GAPDH is another gene widely used as a "housekeeping" gene. GAPDH is an abundant glycolytic enzyme, present in most cell types. This enzyme can participate in different cellular processes. Despite its prevalent use as a housekeeping gene, there is plenty of evidence that this gene is not a suitable endogenous control for quantification assays. Indeed, in several recent articles the use of GADPH as an endogenous control has been severely criticized because of numerous situations where its expression is influenced by the experimental treatment or condition (78, 81, 86, 87).

It should be noted that the optimal choice of housekeeping gene is dependent on the specific experimental treatment and tissue. Prior to each set of experiments a suitable housekeeping gene should be chosen that gives the most reproducible results. Sometimes, however, finding a relevant housekeeping gene can be a nightmare to the investigator. Indeed, some experimental treatments can change the size or composition of an organ, e.g., by inducing splenomegaly, rendering the tissue unsuitable for comparison to a normal control tissue. Other treatments, such as irradiation, can change the expression level of several housekeeping genes, again making normalization very difficult (33). In these cases, a correction factor can be used to compare the treated samples with the controls. Starting from the hypothesis that a housekeeping gene should differ only because of minor differences in input RNA levels or differences in reverse transcription efficiency, the mean value for a housekeeping gene in a control group should be similar to the mean value of the same housekeeping gene in the treated group. If this is not the case, the housekeeping gene is influenced by the treatment itself, and normalization will result in wrong conclusions. The use of a correction factor, where the mean of the housekeeping gene in the control group is divided by the mean of the housekeeping gene in the treated group, can solve this problem. This correction factor is used to correct for the variation in housekeeping gene due to the treatment itself, in each individual sample (33).

Another situation where the use of a housekeeping gene is not possible is in experiments where different kinds of cells are cultured together in different cell:cell number combinations, but the target expression by only one of the cell populations is of interest. In this case normalization to a cell-specific gene can be used, e.g., CD11b for macrophages (88), although again one has to determine if in this specific experimental setup this gene really acts as a "housekeeping" gene. Another possibility in this case is to normalize to the input cell number (Stoffels *et al.*, unpublished results).

TABLE 2

Primer and Probe Sequences for Murine Cytokines and Other Immune-Related Factors

		Sequence (5′–3′)	Length (bp)[a]	Accession No.[b]	gDNA[c]
IL-1β	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[d]	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			
IL-15	FW	CATCCATCTCGTGCTACTTGTGTT	126	U14332	–
	RV	CATCTATCCAGTTGGCCTCTGT		AB006745	
	TP[d]	AGGGAGACCTACACTGACACAGCCCAAAA			
IL-17	FW	GCTCCAGAAGGCCCTCAGA	142	NM010552	–
	RV	AGCTTTCCCTCCGCATTGA		U35108	
	TP	CTCTCCACCGCAATGAAGACCCTGA			
IL-18	FW	CAGGCCTGACATCTTCTGCAA	105	NM008360	–
	RV	TCTGACATGGCAGCCATTGT		AJ002364	
	TP	CTCCAGCATCAGGACAAAGAAAGCCG			
IFN-γ	FW	TCAAGTGGCATAGATGTGGAAGAA	92	K0083	–
	RV	TGGCTCTGCAGGATTTTCATG		M74466	
	TP[d]	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	
CD40	FW	GTCATCTGTGGTTTAAAGTCCCG	91	M83312	+
	RV	AGAGAAACACCCCGAAAATGG		M94129	
	TP	AGCCCTGCTGGTCATTCCTGTCGTG			
CD40	FW	CTCAAACTCTGAACAGTGCGCT	88	X65453	+
Ligand	RV	GGCAGGTCCTAACTGACTTGCT			
	TP[d]	AGGGAAGACTGCCAGCATCAGCCCT			
iNOS	FW	CAGCTGGGCTGTACAAACCTT	95	U43428	–
	RV	CATTGGAAGTGAAGCGTTTCG		L23806	
	TP	CGGGCAGCCTGTGAGACCTTTGA			
MCP-1	FW	CTTCTGGGCCTGCTGTTCA	126	L13763	–
	RV	CCAGCCTACTCATTGGGATCA		U12470	
	TP	CTCAGCCAGATGCAGTTAACGCCCC			
IL-1Ra	FW	CCTCGGGATGGAAATCTGCT	133	M74294	+
	RV	CCAGATTCTGAAGGCTTGCAT		L32838	
	TP	TTTCATTCAGAGGCAGCCTGCCG			

TABLE 2—*Continued*

		Sequence (5′–3′)	Length (bp)[a]	Accession No.[b]	gDNA[c]
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	
TNF-Rp55	FW	GCTGACCCTCTGCTCTACGAA	132	X57796	+
	RV	GCCATCCACCACAGCATACA		M88067	
	TP	CTGTTCAGAAATGGGAAGACTCCGCCC		M76655	
GAPDH	FW	TCACCACCATGGAGAAGGC	168	M32599	+
	RV	GCTAAGCAGTTGGTGGTGCA		U09964	
	TP	ATGCCCCCATGTTTGTGATGGGTGT			
β-Actin	FW	AGAGGGAAATCGTGCGTGAC	138	X03672	+
	RV	CAATAGTGATGACCTGGCCGT		V01217	
	TP	CACTGCCGCATCCTCTTCCTCCC		J00691	

Note. FW, forward primer; RV, reverse primer; TP, TaqMan probe, dual-labeled with 5′FAM and 3′TAMRA; IL, interleukin, IFN-γ, interferon-γ; TNF-α, tumor necrosis factor α; TGF-β, transforming growth factor β; iNOS, inducible nitric oxide synthetase; MCP-1, monocyte chemoattractant protein 1; IL-1Ra, interleukin 1 receptor antagonist; MIP3α, macrophage inflammatory protein 3α; IP10, interferon γ-inducible protein; TNF-R, TNF receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

[a] Amplicon length in base pairs.

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

[c] PCR amplification on genomic DNA.

[d] Antisense probe.

VALIDATION OF CYTOKINE REAL-TIME RT-PCR

Here we describe the validation of the real-time RT-PCR technique to quantify murine and human cytokines as well as other factors playing a role in the immune system. We use the ABI Prism 7700 SDS in combination with the TaqMan chemistry. A specific set of primers and internal fluorogenic probe were designed and optimized for each of the target genes of interest. The probe, a hydrolysis probe, was dually labeled with a reporter dye FAM (6-carboxyfluorescein) covalently attached to the 5′ end of the oligonucleotide and a quencher dye TAMRA (6-carboxytetramethylrhodamine) at the 3′ end. With the objective of acquiring a relative quantification of transcription, cDNA plasmid standards were constructed for each individual target. Finally, to correct for variations in input RNA amounts and efficiency of reverse transcription, an endogenous "housekeeping" gene (in this case β-actin) was also quantified and used to normalize the results.

A. RNA Extraction

Depending on the tissue to be extracted, different methods are used for RNA extraction. Isolated tissues are either used immediately or frozen in liquid nitrogen and stored at −80°C until use. TRIzol reagent (Life Technologies, Gaithersburg, MD) is used to extract total RNA from most tissues of interest, such as spleen, lymph nodes, heart, brain, spinal cord, intestine, thymus, and lung, as described by the manufacturer. However, this method does not give reliable results for pancreatic tissue, probably due to the high concentration of endogenous RNase. Therefore, total RNA from pancreatic tissue is extracted using the SV Total RNA Isolation System from Promega (Madison, WI). This method results in highly pure, DNA-free RNA, since the protocol includes a DNase treatment. Total RNA from small amounts (up to 1×10^6 cells) of cultured cell lines, peritoneal murine macrophages, human peripheral blood mononuclear cells, and pancreatic β cells is extracted using the High Pure RNA isolation kit (Roche Diagnostics Corp. Indianapolis, IN), which again combines RNA extraction and DNase treatment in a spin-column system. If total RNA of a larger amount of cells has to be extracted, ranging from 1×10^6 to 1×10^7 cells, either the TRIzol Liquid Suspension reagent (Life Technologies) or the RNeasy mini kit (Qiagen Inc, Valencia, CA) is used. Both result in pure RNA, although no DNase treatment is included. The concentration of the purified total RNA is determined spectrophotometrically at 260 nm.

B. cDNA synthesis

Total RNA is reverse transcribed in two steps. First, 5 μM oligo(dT)$_{16}$ (Applied Biosystems) is added to 0.5–1 μg of total RNA, heated to 70°C for 10 min, and subsequently cooled on ice. Second, 100 U of Superscript II reverse transcriptase (Life Technologies) is added in the presence of 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.5 mM dNTPs and kept at 42°C for 80 min. For every reaction set, one RNA sample is performed without Superscript II RT (RT-minus reaction) to provide a negative control in the subsequent PCR. To minimize variation in the reverse transcription reaction, all RNA samples from a single experimental setup are reverse transcribed simultaneously. For subsequent PCR amplification maximum 0.5 μl of each cDNA sample is used per 25 μl PCR -mixture. Use of larger amounts of cDNA in the PCR is avoided, since this may inhibit efficient PCR amplification.

C. Designing Primers and Probes

Primers and probes for all target sequences were designed using the computer program Primer Express, a software program specially provided with the 7700 SDS (Applied Biosystems) (Tables 2 and 3). The default parameters of this program are set very narrow; most important are the T_m of the primers and probe and the amplicon length. The melting temperature (T_m) of the primers is 58–60°C, while the T_m of the probe should be at least 10°C higher, approximately 68–70°C, to anneal to the target sequence during the extension step of the PCR (PCR extension is performed at 60°C). An advantage of these narrow temperature ranges is that the temperature cycle conditions for PCR amplification are identical for all targets. When designing the probe, the presence of guanidine at the 5′ end has to be avoided, because this base slightly quenches the reporter signal, even after probe cleavage. Furthermore, the probe should contain more C's than G's; if this is not the case, the antisense probe is used (e.g., for IL-6, IL-15, IFN-γ, CD40 ligand in Table 2). Amplicon lengths should be chosen as short as possible: between 50 and 150 bp, although, in our experience, amplicons up to 300 bp amplify efficiently. On the other hand, amplicons shorter than 90 bp are avoided, because they are more difficult to visualize on agarose gel and to purify on DNA purification columns (see construction of cDNA plasmids below).

When designing the primers, special care is taken to prevent coamplification of genomic DNA. For all the targets the primers are located in two different exons. Moreover, in the case of small introns, either one of the primers or the probe is located on an intron–exon junction. Even with these precautions, it is still not excluded that the primer set will not coamplify genomic DNA. This is indeed the case for CD40, IL-1Ra, MIP3α, and TNF-R (Table 2), where either the forward or reverse primer or the probe is located on an intron/exon boundary. In these cases, designing a second primer also located on a boundary or in the next exon might provide a solution to this problem. Finally, if the genomic DNA sequence of the target cDNA has not been published (e.g., CD40 ligand, fractalkine in Table 2), primers cannot be designed on different exons and the coamplification of genomic DNA cannot be avoided. In this case treatment of the RNA with RNase-free DNase is necessary. In our hands treatment of the RNA with the total RNA purification kit from Roche efficiently removes contaminating DNA without significant degradation of the RNA sample.

For each target gene different concentrations of $MgCl_2$ (3–9 mM) and primers (100–900 nM) are tested to optimize the PCR. The exact $MgCl_2$ and primer concentrations are not mentioned here, because, in our experience, optimal conditions may differ considerably depending on the company where the primers are synthesized. Therefore we suggest titrating always for optimal primer and $MgCl_2$ concentrations. However, identical thermal cycling conditions are used for all targets: 15 s at 94°C and 1 min at 60°C, with a total of 35 (β-actin) to 45 cycles.

When optimizing the PCR for each individual target gene, different controls are performed, as described in detail previously (24). Essentially, controls are performed to confirm the specificity and size of the PCR product, as well as to exclude coamplification of genomic DNA. For all murine target genes listed in Table 2 the possible coamplification of genomic DNA is indicated in the last column. For all human cytokines optimized (Table 3) all primer–probe sets are specifically amplifying the cDNA, not coamplifying genomic DNA.

Murine μ-actin as well as GAPDH coamplifies genomic DNA, due to the known presence of corresponding pseudogenes (89, 90). In this case the following is used to subtract the percentage of genomic DNA contamination:

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

However, due to the very high mRNA expression of both "housekeeping genes," contaminating genomic DNA has, in all experiments we have performed, always been less than 1% of the cDNA sample, thus being negligible.

D. PCR Amplification

PCRs are performed in an ABI Prism 7700 SDS. A Power Macintosh 7200 linked to the sequence detector

is programmed with the reaction conditions. PCR amplifications are performed in a total volume of 25 μl, containing 0.5 μl cDNA sample, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 10 mM EDTA, 60 nM Passive Reference 1, 200 μM dNTPs, 3–9 mM $MgCl_2$, 100–900 nM of each primer, 100 nM TaqMan probe, and 0.625 U AmpliTaqGold (Applied Biosystems).

PCR amplifications are always performed in duplicate or triplicate wells, using the following conditions: 10 min at 94°C, followed by a total of 35 to 45 two-temperature cycles (15 s at 94°C and 1 min at 60°C). Intra- and interassay variability appears to be very low, as described previously (24).

E. Quantification Using the Standard Curve Method

To quantify the results we constructed cDNA plasmid standards, consisting of purified plasmid DNA, specific for each individual target. By including a serial dilution of such a standard in each PCR run, with known amounts of input copy number, the target gene can be quantified in the unknown samples. For each dilution, ΔRn was measured and plotted against cycle number. By plotting Ct values against the known input copy number, a standard curve is generated, with linear range covering 7–8 log units (Fig. 1).

To construct the cDNA standards, total RNA was extracted from a tissue with known expression of the target, most often the spleen, and cytokine cDNA fragments were generated by RT-PCR using the same primers as described above. Each of these amplicons was purified on silica columns (QIAquick PCR purification; Qiagen, Chatsworth, CA) and cloned into pGEM-Teasy (Promega Corp.). Ligated fragments were transformed into DH5α-competent cells (Life Technologies) and plasmid DNA was prepared using silica cartridges (Nucleobond AX plasmid purification; Macherey-Nagel, Düren, Germany). The exact sequence of cloned amplicons was analyzed by cycle sequencing (Thermosequence fluorescence labeled primer sequencing kit),

TABLE 3

Primer and Probe Sequences for Human Cytokines

		Sequence (5′ → 3′)	Length (bp)[a]	Accession No.[b]
IL-1α	FW	CGCCAATGACTCAGAGGAAGA	120	X02531
	RV	AGGGCGTCATTCAGGATGAA		X03833
	TP	AGCACCTTTTAGCTTCCTGAGCAATGTGAAA		
IL-2	FW	AACTCACCAGGATGCTCACATTTA	148	NM000586
	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	NM000585
	RV	AACACAAGTAGCACTGGATGGAAA		X91233
	TP	CTGCTGGAAACCCCTTGCCATAGCC		
IFN-γ	FW	TCAGCTCTGCATCGTTTTGG	120	X01992
	RV	GTTCCATTATCCGCTACATCTGAA		J00219
	TP	TTGGCTGTTACTGCCAGGACCCATATGT		
TGF-β	FW	CAGCAACAATTCCTGGCGATA	136	NM000660
	RV	AAGGCGAAAGCCCTCAATTT		Y00112
	TP	CTGCTGGCACCCAGCGACTCG		
TNF-α	FW	TCTTCTCGAACCCCGAGTGA	151	M10988
	RV	CCTCTGATGGCACCACCAG		X02910
	TP	TAGCCCATGTTGTAGCAAACCCTCAAGCT		X02159

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

[a] Amplicon length in base pairs.

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

with M13 universal primers, using the ALFexpress instrumentation (Amersham Pharmacia, Uppsala, Sweden). cDNA plasmid concentrations were measured by optical density spectrophotometry (Pharmacia, Uppsala, Sweden) and the corresponding copy number was calculated using the equation

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

Serial 10- or 5-fold dilutions of the resulting plasmid clones, e.g., ranging from 10^7 down to 10^2 input cDNA copies, are used as a standard curve in each PCR run. Dilutions of the plasmid clones are stored aliquoted at −20°C. To minimize interassay variability, all samples analyzed from a single experiment are performed with the same dilution series of a standard.

POTENTIAL AND PITFALLS

The analysis of cytokines plays a central role in the understanding of inflammatory reactions, autoimmune diseases, and transplant rejections, resulting in better insight into the different immunological pathways involved. Because the samples obtained to analyze (e.g., tissue biopsies) are often too small to allow quantification of cytokines at the protein level, quantification at the mRNA level is increasingly used. Indeed, detection methods such as ELISA are not sensitive enough for the analysis of cytokines expressed at very low levels.

Real-time RT-PCR as described in this review is a revolutionary technique in this field, and is the method of choice to quantify cytokine mRNA levels from organs, body fluids, or cell cultures. The technique is very fast, accurate, and sensitive, compared with previously described endpoint RT-PCR methods, and has a decreased potential for PCR contamination as it is performed in a "closed tube" chamber. PCR amplification is combined with product detection, allowing continuous monitoring of PCR product during the reaction. The method is very sensitive, allowing the analysis of very small amounts of RNA. Moreover, in contrast to ELISA, many different target genes can be quantified from a single RNA sample.

Although the "real-time" RT-PCR is a very powerful technique, it needs extensive and accurate optimization to be reliable. Different points have to be taken into account, such as avoiding coamplification of genomic DNA, interassay variability, and normalization to a relevant housekeeping gene.

In our experience the best way to avoid coamplification of genomic DNA is designing specific primer sets that do not react on genomic DNA. In this way an additional DNase treatment on the RNA sample can be avoided. This is preferable because of the often low expression levels of cytokines in the samples to be analyzed, necessitating an RNA extraction with very good efficiency. If not, some of the cytokines of interest might be expressed below the detection limit.

As mentioned, the system allows a very high throughput, as one run, containing a maximum of 42 samples in duplicate wells and a standard curve, is performed within 2 h. When performing experiments with large numbers of samples (e.g., from different treatment groups), the results from different runs will have to be compared. Therefore, it is very important to minimize interassay variability. This can be handled by preparing one universal PCR mixture and a single dilution series of the standard, and subsequently running all the samples from a single experimental setup using this same master mixture. Minor differences in PCR efficiency between the different runs will be corrected for by using the same dilution series of the standard curve.

Finally, normalization to a relevant "housekeeping gene" is not always straightforward. This problem is not only important in the real-time RT-PCR technique, but is inherent to all quantitative RNA expression techniques. However, finding a good "housekeeping gene" that is not influenced by the experimental treatment itself is very important, and it is often necessary to test different housekeeping genes to find the best suitable for each individual experiment.

Once all parameters are optimized and all factors are carefully controlled, the method allows a very high throughput of accurate results. We have been working with this method to quantify cytokine mRNA expression levels in our laboratory for more than 5 years and the applicability of the process has been confirmed in different studies approaching diabetes prevention and treatment in the nonobese diabetic mouse (NOD mouse), as well as in transplantation studies (24–38). In our experience, it is obvious that the use of this technique, in the many studies performed, has definitely resulted in better insight into the important immunological mechanisms playing a role in autoimmune diseases, such as Type 1 diabetes, and transplantation-related immunology.

CONCLUSION

Overall, real-time RT-PCR makes the quantification of mRNA expression easier, quicker, and more sensitive. With careful optimization it can be very accurate and reliable, making it ideal as the standard method for

cytokine mRNA quantification. Using the TaqMan technology, very specific and reliable results are obtained. Furthermore, designing new primers and probes for an expanding range of new target genes of interest, for instance, the chemokines or the human cytokines, is easily performed.

This growing list of cytokines, which can be analyzed using real-time RT-PCR, will definitely lead to an exponential increase in publications in the cytokine field, in both research and clinical applications.

REFERENCES

1. Thomson, A. (1998) The Cytokine Handbook, 3rd ed., Academic Press, San Diego.
2. Corwin, E. J. (2000) *Biol. Res. Nurs. Pt. I* **2,** 30–40.
3. Rengarajan, J., Szabo, S. J., and Glimcher, L.H. (2000) *Immunol. Today* **21,** 479–483.
4. Glimcher L. H., and Murphy, K.M. (2000) *Genes Dev.* **14,** 1693–1711.
5. Baggiolini, M., and Loetscher, P. (2000) *Immunol. Today* **21,** 418–420.
6. Rabinovitch, A. (1998) *Diabetes Metab. Rev.* **14,** 129–151.
7. Dai, Z., and Lakkis, F.G. (1999) *Curr. Opin. Immunol.* **11,** 504–508.
8. Wang, T., and Brown, M.J. (1999) *Anal. Biochem.* **269,** 198–201.
9. Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., and Erlich, H. (1986) *Cold Spring Harb. Symp. Quant. Biol.* **51,** 263–273.
10. Montgomery, R.A., and Dallman, M.J. (1997) *Cytokine* **9,** 717–726.
11. Kuschnaroff, L.M., Valckx, D., Goebels, J., Rutgeerts, O., Heremans, H., Froyen, G., and Waer, M. (1997) *Scand. J. Immunol.* **46,** 459–467.
12. Fox, C.J., and Danska, J.S. (1997) *J. Immunol.* **158,** 2414–2424.
13. Zhou N.M., Matthys, P., Polacek, C., Fiten, P., Sato, A., Billiau, A., and Froyen, G. (1997) *Cytokine* **9,** 212–218.
14. O'Garra, A., and Vieira, P. (1992) *Curr. Opin. Immunol.* **4,** 211–215.
15. Gilliland, G., Perrin, S., Blanchard, K., and Bunn, H.F. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 2275–2729.
16. Barth, S., Kleinhappl, B., Gutschi, A., Jelovcan, S., and Marth, E. (2000) *Inflamm. Res.* **49,** 266–274.
17. Raeymarkers, L. (1993) *Anal. Biochem.* **214,** 582–585.
18. Hanson, M.S., Cetkovic-Cvrlje, M., Ramiya, V.K., Atkinson, M.A., Maclaren, N.K., Singh, B., Elliott, J.F., Serreze, D.V., and Leiter, E.H. (1996) *J. Immunol.* **157,** 1279–1287.
19. Holland, P.M., Abramson, R.D., Watson, R., and Gelfand, D.H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7276–7280.
20. Stryer, L. (1978) *Annu. Rev. Biochem.* **47,** 819–846.
21. Cardullo, R.A., Agrawal, S., Flores, C., Zamecnick, P.C., and Wolf, D.E. (1998) *Proc. Natl. Acad. Sci. USA* **85,** 8790–8794.
22. Heid, C.A., Stevens, J., Livak, J.K., and Williams, P.M. (1996) *Genome Res.* **6,** 986–994.
23. Gibson, U.E.M., Heid, C.A., and Williams, P.M. (1996) *Genome Res.* **6,** 995–1001.
24. Overbergh, L., Valckx, D., Waer, M., and Mathieu, C. (1999) *Cytokine* **11,** 305–312, doi:10.1006/cyto.1998.0426.
25. Casteels, K.M., Waer, M., Laureys, J., Valckx, D., Depovere, J., Bouillon, R., and Mathieu, C. (1998) *Transplantation* **65,** 1225–1232.
26. Gysemans, C.A., Waer, M., Valckx, D., Laureys, J.M., Mihkalsky, D., Bouillon, R., and Mathieu, C. (2000) *Diabetes* **49,** 1992–1997.
27. Casteels, K.M., Mathieu, C., Waer, M., Valckx, D., Overbergh, L., Laureys, J.M., and Bouillon, R. (1998) *Endocrinology* **139,** 95–102.
28. Casteels, K.M, Waer, M., Bouillon, R., Depovere, J., Valckx, D., Laureys, J.M., and Mathieu, C. (1998) *Clin. Exp. Immunol.* **112,** 181–187.
29. Gysemans, C.A., Pavlovic, D., Bouillon, R., Eizirik, D.L., and Mathieu, C. (2001) *Diabetologia* **44,** 567–574.
30. Chen, M.-C., Proost, P., Gysemans, C., Mathieu C., and Eizirik, D.L. (2001) *Diabetologia* **44,** 325–332.
31. Sefrioui, H., Billiau, A.D., Overbergh, L., Rutgeerts, O., and Waer, M. (1999) *Transplantation* **68,** 1560–1567.
32. Overbergh, L., Decallonne, B., Waer, M., Rutgeerts, O., Valckx, D., Casteels, KM., Laureys, J., Bouillon, R., and Mathieu, C.(2000) *Diabetes* **49,** 1301–1307.
33. Billiau, A.D., Sefrioui, H., Overbergh, L., Rutgeerts, O., Goebels, J., Mathieu, C., and Waer, M. (2001) *Transplantation* **71,** 292–299.
34. Decallonne, B., Overbergh, L., Casteels, K.M., Gysemans, C., Bouillon, R., and Mathieu, C. (2000) *Diabetologia* **43,** 1302–1308.
35. Kuschnaroff, L.M., Overbergh, L., Sefrioui, H., Sobis H., Vandeputte, M., and Waer, M. (1999) *J. Neuroimmunol.* **99,** 157–168.
36. Liu, Z., Geboes, K., Colpaert, S., Overbergh, L., Mathieu, C., Heremans, H., de Boer, M., Boon, L., D'Haens, G., Rutgeerts, P., and Ceuppens, J.L. (2000) *J. Immunol.* **164,** 6005–6014.
37. Liu, Z., Geboes, K., Heremans, H., Overbergh, L., Mathieu, C., Rutgeerts, P., and Ceuppens, J. (2001) *Eur. J. Immunol.* **31,** 1550–1560.
38. Overbergh, L., Decallonne, B., Valckx, D., Verstuyf, A., Depovere, J., Laureys, J., Rutgeerts, O., Saint-Arnaud, R., Bouillon, R., and Mathieu, C. (2000) *Clin. Exp. Immunol.* **120,** 139–146.
39. Brink, N., Szamel, M., Young, A.R., Wittern, K.P., and Bergemann, J. (2000). *Inflamm. Res.* **49,** 290–296.
40. Birks, E.J., Burton, P.B.J., Owen, V., Mullen, A.J., Hunt, D., Banner, N.R., Barton, P.J.R., and Yacoub, M.H. (2000) *Circulation* **102,** III352–III358.
41. Härtel, C., Bein, G., Müller-Steinhardt, M., and Klüter, H. (2001) *J. Immunol. Methods* **249,** 63–71.
42. Takahashi, M., Funato, T., Ishii, K.K., Kaku, M., and Sasaki, T. (2001) *J. Lab. Clin. Med.* **137,** 101–106, doi: 10.1067/mlc.2001.112508.
43. Brieland, J.K., Jackson, C., Hurst, S., Loebenbergh, D., Muchamuel, T., Debets, R., Kastelein, R., Churakova, T., Abrams, J., Hare, R., and O'Garra, A. (2000) *Infect. Immun.* **68,** 6567–6573.
44. Wittwer, C.T., Ririe, K.M., Andrew, R.V., David, D.A., Gundry, R.A., and Balis, U.J. (1997) *Biotechniques* **22,** 176–181.
45. Simpson, D.A.C., Feeney, S., Boyle, C., and Stitt, A.W. (2000) *Mol. Vis.* **6,** 178–183.
46. Blaschke, V., Reich, K., Blaschke, S., Zipprich, S., and Neumann, C. (2000) *J. Immunol. Methods* **246,** 79–90.
47. Goriely, S., Vincart, B., Stordeur, P., Vekemans, J., Willems, F., Goldman, M., and De Vit, D. (2001) *J. Immunol.* **166,** 2141–2146.
48. Kreuzer, K.A., Bohn, A., Lass, U., Peters, U.R., and Schmidt, C.A. (2000) *Mol. Cel. Probes* **14,** 57–60, doi: 10.100/mcpr.1999.0284.
49. Kreuzer, K.A., Bohn, A., Lupberger, J., Solassol, J., le Coutre, P., and Schmidt, C.A. (2001) *Clin. Chem.* **47,** 486–490.

50. Killgore, G.E., Holloway, B., and Tenover, F. (2000) *J. Clin. Microbiol.* **38,** 2516–2519.
51. Martell, M., Gomez, J., Esteban, J.I., Sauleda, S., Quer, J., Cabot, B., Esteban, R. and Guardia, J. (1999) *J. Clin. Microbiol.* **37,** 327–332.
52. Oleksiewicz, M.B., Donaldson, A.I., and Alexandersen, S. (2001) *J. Virol. Methods* **92,** 23–35.
53. Van Trappen, P.O., Gyselman, V.G., Lowe, D.G., Ryan, A., Oram, D.H., Bosze, P., Weekes, A.R., Shepherd, J.H., Dorudi, S., Bustin, S.A., and Jacobs, I.J. (2001) *Lancet* **357,** 15–20.
54. Tyagi, S., and Kramer, F.R. (1996) *Nat. Biotechnol.* **14,** 303–308.
55. Bonnet, G., Tyagi, S., Libchaber, A., and Kramer, F.R. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 6171–6176.
56. Kaboev, O.K., Luckkina, L.A., Tret'iakov, A.N., and Bahrmand, A.R. (2000) *Nucl. Acids Res.* **28,** e94.
57. Mhlanga, M.M., and Malmberg, L. (2001) *Methods,* **25,** in press.
58. Smit, M.L., Giesenford, B.A.J., Vet, J.A.M., Trijbels, F.J.M., and Blom, H.J. (2001) *Clin. Chem.* **47,** 739–744.
59. Durand, R., Eslahpazire, J., Jafari, S., Delabre, J.F., Mamorat-Khuong, A., di Piazza, J.P., and Le Bras, J. (2000) *Antimicrob. Agents Chemother.* **44,** 3461–3464.
60. Fortin, N.Y., Mulchandani, A., and Chen, W. (2001) *Anal. Biochem.* **289,** 281–288, doi: 10.1006:abio.2000.4935.
61. Chen, W., Martinez, G., and Mulchadani, A. (2000) *Anal. Biochem.* **280** 160–172, doi: 10.1006/abio.2000.4518.
62. Lewin, S.R., Vesanen, M., Kostrikis, L., Hurley, A., Duran, M., Zhang, L., Ho, D.D., and Markowitz, M. (1999) *J. Virol.* **73,** 6099–6103.
63. Pierce, K.E., Rice, J.E., Sanchez, J.A., Brenner, C., and Wangh, L.J. (2000) *Mol. Hum. Reprod.* **6,** 1155–1164.
64. Vet, J.A.M., Majithia, A.R., Marras, S.A.E., Tyagi, S., Dube, S., Poiesz, B.J., and Kramer, F.R. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 6394–6399.
65. Whitcombe, D., Theaker, J., Guy, S.P., Brown, T., and Little, S. (1999) *Nature Biotechnol.* **17,** 804–807.
66. Thelwell, N., Millington, S., Solinas, A., Booth, J., and Brown, T. (2000) *Nucl. Acids Res.* **28,** 3752–3761.
67. Bernard, P.S., and Wittwer, C.T. (2000) *Clin. Chem.* **46,** 147–148.
68. Emig, M., Saussele, S., Wittor, H., Weisser, A., Reiter, A., Willer, A., Berger, U., Hehlmann, R., Cross, N.C., and Hochhaus, A. (1999) *Leukemia* **13,** 1825–1832.
69. Bernard, P.S., Ajioka, R.S., Kushner, J.P., and Wittwer, C.T. (1998) *Am. J. Pathol.* **153,** 1055–1061.
70. Neoh, S.-H., Brisco, M.J., Firgaira, F.A., Trainor, K.J., Turner, D.R., and Morley, A.A. (1999) *J. Clin. Pathol.* **52,** 766–769.
71. Cane, P.A., Cook, P., Ratcliffe, D., Mutimer, D., and Pillay, D. (1999) *Antimicrob. Agents Chemother.* **43,** 1600–1608.
72. Bellin, T., Pulz, M., Matussek, A., Hempen, H.-G., and Gunzer, F. (2001) *J. Clin. Microb.* **39,** 370–374, doi: 10.1128/JCM.39.1.370-374.2001.
73. Schalasta, G., Eggers, M., Schmid, M., and Enders, G. (2000) *J. Clin. Virol.* **19,** 175–185.
74. Ririe, K.M., Rasmussen, R.P., and Wittwer, C.T. (1997) *Anal. Biochem.* **245,** 154–160, doi: 10.1006/abio.1996.9916.
75. Kellogg, D.E., Rybalkin, I., Chen, S., Mukhamedova, N., Vlasik, T., Siebert, P.D., and Chenchick, A. (1994) *Biotechniques* **16,** 1134–1137.
76. Blaschke, S., Schulz, H., Schwarz, G., Blaschke, V., Müller, G.A., and Reuss-Bosrt, M. (2001) *J. Rheumatol.* **28,** 12–21.
77. Schröter, M., Zöllner, B., Schäfer, P., Laufs, R., and Feucht, H.H. (2001) *J. Clin. Microb.* **39,** 765–768, doi: 10.1128/JCM.39.2.765-768.2001.
78. Bustin, S.A. (2000) *J. Mol. Endocrinol.* **25,** 169–193.
79. Moody, A., Sellers, S., and Bumstead, N. (2000) *J. Virol. Methods* **85,** 55–64.
80. Livak, K.J., and Schmittgen, T.D. (2001) *Methods* **25,** 402–408.
81. Freeman, W.M., Walker, S.J., and Vrana, K.E. (1999) *Biotechniques* **26,** 112–125.
82. Zhong, H., and Simons, J.W. (1999) *Biochem. Biophys. Res. Commun.* **259,** 523–526.
83. Gerard, C.J., Andrejka, L.M., and Macina, R.A. (2000) *Mol. Diagn.* **5,** 39–46.
84. Cassinat, B., Zassadowski, F., Balitrand, N., Barbey, C., Rain, J.D., Fenaux, P., Degos, L., Vidaud, M., and Chomienne, C. (2000) *Leukemia* **14,** 324–328.
85. Foss, D.L., Baarsch, M.J., and Murtaugh, M.P. (1998) *Anim. Biotechnol.* **9,** 67–78.
86. Barroso, I., Benito, B., Garcia-Jimenez, C., Hernández, A., Obregón, M.J., and Santisteban, P. (1999) *Eur. J. Endocrinol.* **141,** 169–179.
87. Suzuki, T., Higgins, P.J., and Crawford, D.R. (2000) *Biotechniques* **29,** 332–337.
88. Javorschi, S., Labrouche, S., and Freyburger, G. (2000) *Biotechniques* **28,** 1116–1124.
89. Ng, S.Y., Gunning, R.E., Ponte, P., Leavitt, J., Shows, T., and Kedes, L.(1985) *Mol. Cell. Biol.* **5,** 2720–2732.
90. Hanauer, A., and Mandel, J.L. (1984) *EMBO J.* **3,** 2627–2633.