outbreak, we are also adapting this protocol to a high-throughput format. It is expected that the current rapid method of prompt identification of this pathogen will allow prompt identification of this virus and thus facilitate control of the disease and provision of prompt and appropriate treatment to patients.

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References

Automated RNA Extraction by MagNA Pure Followed by Rapid Quantification of Cytokine and Chemokine Gene Expression with Use of Fluorescence Resonance Energy Transfer, Juergen Loeffler, Phillipp Swatoch, Diana Akhawi-Araghi, Holger Hebart, and Hermann Einsele (Eberhard-Karls-Universität Tuebingen, Medizinische Klinik, 72076 Tuebingen, Germany; * address correspondence to this author at: Medizinische Klinik, Abt. II, Labor Prof. Dr. med. H. Einsele, Ottfried-Mueller-Strasse 10, 72076 Tuebingen, Germany; fax 49-7071-294687, e-mail juergen.loeffler@med.uni-tuebingen.de)

Cytokines and chemokines, a superfamily of small cytokine-like molecules, are key mediators of immunity and inflammation. Detection of cytokines and chemokines in disease states provides useful diagnostic tools for investigating host responses to invading organisms, tumors, and trauma.

Different reverse transcription-PCR (RT-PCR) protocols have been described in the literature, including semiquantitative, quantitative, and competitive PCR techniques, but most of them were labor-intensive and time-consuming (1,2). Recently, real-time PCR assays have become a major tool for quantifying the number of DNA or cDNA copies in different settings and clinical materials. These assays offer a standardized, rapid, accurate, and reproducible method that combines rapid in vitro amplification with real-time quantification of the DNA or cDNA load. However, to date, only limited data are available for quantification of cytokine gene expression (3,4). In addition, there is a need for rapid, sensitive, reliable, and standardized methods for RNA extraction.

Here we present an automated standardized protocol for the extraction of RNA from human peripheral blood mononuclear cells (PBMCs), using the MagNA Pure LC system (Roche Diagnostics), combined with a rapid and easy-to-perform quantitative PCR assay, using the LightCycler (Roche Diagnostics) amplification and detection system. We applied this protocol to quantification of mRNA expression of the following immunorelevant genes: interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-18, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), macrophage-inducing protein-3α (MIP-3α), SCYB-11 (IFN-inducible T-cell-α chemoattractant; H174; CXCL11), intracellular adhesion molecule-1 (ICAM-1; CD54), and nuclear factor-κB (NFκB), the transcription factor and central mediator of immune response.

PBMCs were prepared from 50 mL of heparinized fresh venous blood obtained from healthy donors (n = 30). PBMCs were separated by centrifugation over Ficoll and resuspended in 1 mL of phosphate-buffered saline. The PBMC concentration was adjusted to 1 × 10⁶/mL in RPMI 1640 containing 100 mL/L fetal calf serum (Life Technologies). PBMCs were incubated at 37 °C in a 5% CO₂ atmosphere for 8 h with 10 mL/L phytohemagglutinin (PHA) or 1 mg/L lipopolysaccharide (LPS; Sigma) followed by centrifugation for 5 min at 2200 g. Lysis buffer (200 μL) was then added to the pellet, and the resuspended cells were pipetted into the MagNA Pure LC sample cartridge for subsequent RNA extraction.

For RNA extraction, we used the MagNA Pure LC RNA Isolation Kit I (Roche Diagnostics). In this automated process, the samples were lysed in a buffer containing a chaotropic salt and a RNase inactivator. Nucleic acids were bound to the surface of glass magnetic particles. After a DNase digestion step, unbound substances were removed by several washing steps, and purified RNA was eluted (elution volume, 100 μL) with a low-salt buffer followed by spectrophotometric quantification of RNA.

cDNA was synthesized with use of the First Strand cDNA Synthesis Kit (Roche Diagnostics). Briefly, 8 μL of RNA substrate, 2.2 μL of oligo-p(dT)₁₅ primer, and 0.8 μL of AMV reverse transcriptase were used for cDNA synthesis. After each reverse transcription, cDNA was quantified spectrophotometrically, based on the absorbance at
260 and 280 nm, and diluted to a concentration of 20 ng/μL.

cDNA was amplified and quantified with use of a standard LightCycler protocol and external standardization. This procedure is based on fluorescence resonance energy transfer with specific oligonucleotides (5). We used a standardized reaction mixture [Fast Start DNA Master Hybridization Probes Kit (Roche Diagnostics), containing 3 mM MgCl₂, 0.15 μM probes (TIB Molbiol), and 0.125 μM primers] and an identical PCR protocol (45 cycles of 3 s at 95 °C, 15 s at 54 °C, and 25 s at 72 °C) for all assays. After the LightCycler run, reaction products were separated by agarose gel electrophoresis in a 2% Tris acetate-EDTA agarose gel, and the DNA was stained with ethidium bromide for independent validation of the presence of an amplicon.

External standards were prepared by serial titration of purified target cDNA containing a known number of input copies. This cDNA was obtained by “classic” PCR assays that specifically amplified the concerned targets (4). The corresponding copy number was calculated after absorbance spectrophotometry, using an equation described previously by Overbergh et al. (3): 1 μg of 1000-bp DNA = 9.1 × 10¹³ molecules. Target DNA concentrations were calculated by comparing the cycle numbers of the log-linear phase of the samples with the cycle numbers of the external standards. Results for stimulated PBMCs were expressed by comparison with the copy number of unstimulated PBMCs.

In this study, we used a single, rapid, and easy-to-perform LightCycler RT-PCR assay for the quantification of the expression of 15 different mRNAs in PBMCs (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-18, TNF-α, IFN-γ, MIP-3α, SCYB-11, ICAM-1, and NFκB). For all targets, specific primers, probes, and external standards were established and evaluated (the sequences of the primers and probes are listed in Table 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol49/issue6/). Titration of target cDNA showed that the lower detection limit of the assay ranged between 10¹ copies (e.g., for TNFα or IFNγ; Fig. 1B) and 10³ copies (MIP-3α and SCYB-11). We achieved a lower detection limit of 10¹ copies for 13 of the 15 targets. The assay was linear over a wide range of input copies (10¹⁰–10³ copies for 13 of 15 targets). We also evaluated the reproducibility of the assay (see Fig. 1A for the results for six different targets). PCR was performed for each dilution in 15 independent experiments for interassay variation and three times in one experiment for intraassay variation. The results of the experiments for interassay variation revealed a mean value of 1.2 × 10¹ copies and a CV of 29% for an input number of 10⁶ copies, a mean value of 9.3 × 10³ copies (CV = 33%) for an input number of 10⁴ copies, and a mean value of 8.5 × 10¹ copies (CV = 50%) for an input of 10⁵ copies. We obtained CVs of 16% for PBMCs stimulated with LPS and 34% for PBMCs stimulated with PHA. The intraassay variation was lower: we achieved CVs between 4.1% (for 10⁶ copies) and 24% (for 10⁷ copies).

RNA extraction was based on a standardized, fully automated protocol by MagNA Pure that allows extraction of 32 RNA samples in parallel within 1.5 h, compared with 3 h for silica spin columns (RNeasy; Qiagen). The amount (3–6 μg of RNA from 10⁶ PBMCs) and purity (A₂₆₀ nm/A₂₈₀ nm, 1.8–1.9) of RNA were comparable to or higher than results achieved with manual extraction. After the reverse transcription step (2 h), the LightCycler-based PCR assay was finished within one additional hour.

To evaluate the capacity of the assay to quantify cytokine mRNA expression in human blood cells, we stimulated PBMCs from 30 different donors with PHA or LPS and compared the expression of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-18, TNF-α, IFN-γ, MIP-3α, SCYB-11, ICAM-1, and NFκB with the mRNA expression in unstimulated PBMCs. We were able to quantify, with a single protocol, the expression of all target genes in PBMCs obtained from 30 different donors. After PHA stimulation, we obtained markedly higher concentrations of IL-1α, IL-1β, IL-2, IL-4, IL-10, IL-12p40, TNF-α, IFN-γ, and MIP-3α mRNA, whereas stimulation with LPS led to increased expression of IL-1β, IL-12p40, TNF-α, IFN-γ, and MIP-3α (compared with unstimulated PBMCs). We observed no substantial changes in the expression of IL-6, IL-8, IL-18, SCYB-11, ICAM-1, and NFκB. Cytokines and chemokines are regulatory peptides that can be produced by almost every nucleated cell having pleiotropic regulatory effects on hematopoietic and many other cell types. In this report, we provide a simple assay for rapid, standardized analysis of the expression of 15 different immunorelevant genes. We used one protocol for all targets, which allowed us to adapt the method to new target RNA very rapidly and easily.

All primers were located in exons to prevent coamplification of genomic DNA. Amplicon lengths were 178–518 bp, which corresponds to the ideal length of amplicons for rapid cycling protocols. The melting temperature of the probes was, on average, 15 °C higher than the melting temperature of the primers. We titrated the MgCl₂ (1.5–5 mM) and primer concentrations (0.1–0.6 μM) to obtain an optimized PCR mixture and modified thermal cycling conditions to obtain an ideal annealing temperature (51–63 °C) and annealing time (5–20 s).

The Fast Start Master Hybridization Probes Kit is based on the “hot-start” PCR technology, which minimizes the generation of nonspecific PCR products. The protocol is based on oligonucleotide probes that hybridize to specific targets, providing a 10- to 100-fold higher sensitivity than conventional gel electrophoresis. In addition, the assay is run in closed glass capillaries. Postamplification analysis can be performed without opening the capillaries, minimizing the risk of carryover contamination. Moreover, this RT-PCR method also allows quantification of mRNA in cases where the amount of sample material is small, such as in biopsies (6) and in selected cell subpopulations. An initial number of only 10⁶ PBMCs allows quantification of 10 different cytokines (input of cDNA per Light-
Fig. 1. Intraassay reproducibility of IL-4 (A), and interassay reproducibility and relative expression of five cytokines (B).

(A), results are for three replicates of a series of dilutions (10^6-10^1 copies) of IL-4 external standard. (B), results are for serial dilutions (10^6-10^1 copies) of five cytokine external standards assayed 15 times. Also shown is the relative mRNA expression of these targets in stimulated (by LPS or PHA) and unstimulated (NS) PBMCs. Results for external standards are in absolute copy numbers (left y axis); results for PBMCs are in relative copy numbers (right y axis).
Cycler reaction is 2 μL. If probes are labeled with two different dyes (LCRed640 and LCRed705), two independent genes can be quantified in a single reaction, doubling the number of targets. Other methods, such as intracellular cytokine staining, require at least 10^6 cells for the quantification of only one cytokine. Thus the proposed method enables determination of different subpopulations of cells, e.g., precursor frequencies of T-cell subpopulations, or maturation states of defined cell populations. Finally, the method also provides an efficient tool to confirm selected data from gene array experiments.

Because of poor interassay reproducibility, cytokine concentrations were expressed in relative copy numbers. We thus achieved appropriate results when cDNA concentrations were measured spectrophotometrically before reverse transcription and PCR and when strong stimulators such as PHA and LPS were used (Fig. 1B). However, for weak stimuli, an internal control is needed to compensate for minor variations in the RNA concentration. This can be fulfilled by normalization against a housekeeping gene or ribosomal RNA.

Recently, several reports have been published that describe quantification of cytokine mRNA by real-time RT-PCR (4, 7–9). All of these protocols rely on manual RNA extraction by spin column technology [QIAamp Blood Kit (7)] or High Pure RNA isolation (8). However, to achieve a high sensitivity for the RT-PCR assay, standardized RNA extraction protocols with high-quality nucleic acid purification are mandatory. Our proposed protocol uses a fully automated assay with commercially available reagents, which reduces the risk of RNA degradation and allows several tests to be performed within 1 day. Furthermore, reports describing other RT-PCR assays have been limited to the detection of simian cytokine RNA (9) or describe the detection of only a very limited number of target genes (4, 7). Autschbach et al. (8) described a LightCycler-based assay for the detection of 35 mRNAs encoding cytokines and chemokines to define mediator profiles in inflammatory bowel disease. However, this protocol relies on the SYBR Green I detection format, which is less sequence specific than the hybridization probes format and requires additional melting curve analysis to exclude primer-dimers.

In conclusion, the proposed method for analysis of cytokine and chemokine gene expression is accurate, reliable, fast, and easy to perform and may help to prevent graft failure and reduce patient morbidity and mortality.

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Stability of NT-proBNP in Serum Specimens Collected in Becton Dickinson Vacutainer (SST) Tubes, Amitava Dasgupta,* Loretta Chou, Gertie Tso, and Lupe Nazarena (1 Department of Pathology and Laboratory Medicine, University of Texas-Houston Medical School, 6431 Fannin, MSB 2.292, Houston, TX 77030; 2 Memorial-Hermann Laboratory Services, Houston, TX 77030; * author for correspondence: fax 713-500-0730, e-mail Amitava.Dasgupta @uth.tmc.edu)

Atrial natriuretic peptides, B-type natriuretic peptide (BNP), and C-type peptides play important roles in the cardiovascular system (1–3). ProBNP is a 108-amino acid peptide that is secreted by the ventricle and is cleaved to physiologically active BNP (amino acids 77–108) and N-terminal fragment NT-proBNP (amino acids 1–76) (3). NT-proBNP is a useful marker for heart failure, including congestive heart failure (4–6). Recently, Roche Diagnostics marketed an automated NT-proBNP assay on the Elecsys analyzer. The assay uses polyclonal antibodies against NT-proBNP. The concentration of NT-proBNP in healthy individuals depends on both age and gender, with females and elderly patients having higher concentrations. The clinical sensitivity and specificity of NT-proBNP at a cutoff of 125 ng/L for male patients younger than 75 years are 81.6–91.7% (sensitivity) and 86.7–95.7% (specificity), depending on age. The corresponding sensitivity and specificity for female patients are 86.7–94.3% (sensitivity) and 57.8–84.9% (specificity), respectively. The negative predictive value of this test is almost 100% at a clinical threshold of 125 ng/L, according to the package insert (NT-proBNP; Roche Diagnostics).

A common practice in clinical laboratories is to draw blood in serum-separator tubes because of the advantage of the barrier gel, which facilitates rapid separation of serum from cellular constituents of blood and prevents hemolysis during prolonged storage. However, measured