

A novel quantitative multiplex NASBA method: application to measuring tissue factor and CD14 mRNA levels in human monocytes

Peter B. H. van Deursen*, Adrie W. Gunther, Caroline C. Spaargaren-van Riel[1], Mark M. E. D. van den Eijnden[1], Hans L. Vos[1], Bob van Gemen, Dianne A. M. W. van Strijp, Nicole M. M. Tacken and Rogier M. Bertina[1]

Organon Teknika B.V., Boseind 15, PO Box 84, 5280 AB Boxtel, The Netherlands and [1]Leiden University Medical Center, Department of Hematology, PO Box 9600, 2300 RC Leiden, The Netherlands

Received July 12, 1999; Revised and Accepted July 15, 1999

ABSTRACT

A new method to quantify two individual mRNAs in a single NASBA reaction is described. In this study, tissue factor and CD14 mRNAs were used as a model system. RNA ratios of –4 to +4 log units were determined with good precision (within 0.3 log) and accuracy (within 0.2 log). By measuring both mRNAs in human monocytes that were stimulated with LPS, the multiplex Q-NASBA proved to be a successful tool to monitor the expression levels of two individual mRNAs in a single-tube amplification system. The method has potential in all fields in which quantitative information is needed on two individual RNAs.

INTRODUCTION

Over the past few years the quantification of nucleic acids with target sequence amplification methods has been accomplished in many ways. Theoretically the coamplification of internal standard nucleic acid sequences, either DNA or RNA, is superior to other quantification methods, provided that the amplification efficiencies are more or less equal (1,2). The major advantage of this method over methods that use external calibration curves is that both the target and the internal standard nucleic acid sequence are amplified in one and the same tube, meaning that the conditions for both nucleic acids are identical in each single quantification reaction. Whereas all other quantification methods are based on the assumption that the amplification efficiencies are identical between two individual reactions, the internal standard approach is not dependent on this. The method has been applied successfully for PCR (3), RT–PCR (4,5) and NASBA (6,7).

NASBA is an isothermal nucleic acid amplification method that amplifies RNA in a manner analogous to the replication of retroviruses (8,9). NASBA has been applied for the detection of viral genomes (6,7,10), viroids (11), rRNAs (12) and mRNAs (13–17). The NASBA reaction mixture contains oligonucleotide primers and three enzymes: AMV-RT, RNase H and T7 RNA polymerase for target specific amplification. In NASBA, nucleic acids are only a template for the amplification reaction if they are single-stranded in the primer binding regions. Since the NASBA reaction is isothermal, specific amplification of single-stranded RNA in the presence of double-stranded DNA is possible as long as denaturation of double-stranded DNA is prevented in the sample preparation procedure. This makes the method useful for specific mRNA detection in a background of genomic DNA even for intronless genes (13). NASBA proved to be a successful tool in the detection of mRNAs (13–17) and in the quantification of viral RNAs in combination with the internal standard approach (6,7). Combining these two applications makes NASBA a potentially very suitable approach for the quantification of mRNAs in DNA backgrounds. Whereas viral RNA loads are normally expressed as genome copies per sample volume, mRNAs levels are normalized to the amount of cells or to the total amount of RNA, which is also indicative for cell numbers. This has led to the idea to coamplify a second mRNA together with the target mRNA in a multiplex reaction. The second mRNA should be unique to the cells expressing the target RNA. In this way, the expression level of the target mRNA may be normalized. Quantification of both targets in one and the same amplification reaction simplifies the quantitative NASBA procedure considerably and makes it more suitable for future routine diagnostics. We selected tissue factor (TF) and CD14 mRNA expression in monocytes as a model system to develop the first multiplex quantitative NASBA assay.

The transmembrane glycoprotein TF is the major trigger of the blood coagulation cascade, since it is an essential cofactor for Factor VIIa in the activation of the Factors IX and X (18,19). TF is normally not present on cells of the blood or the vasculature. However, monocytes can express TF on their cell surface after stimulation by a variety of triggers, such as bacterial lipopolysaccharide (LPS), inflammatory cytokines (e.g. interleukin-1), tumor necrosis factor-α, immune complexes and infectious agents. TF level in monocytes therefore might be a key marker of a (pro-)thrombotic state in whole blood in various pathological situations. To study this hypothesis, a robust and sensitive method measuring TF mRNA levels would be extremely useful, since direct FACS measurement of the level of a transmembrane protein in a specific subset of blood-borne

*To whom correspondence should be addressed. Tel: +31 411 654 591; Fax: +31 411 654 311; Email: p.deursen@teknika.btl.akzonobel.nl

Table 1. Sequences of primers and probes used in detection of TF and CD14 mRNA

Oligonucleotide	Sequence	Positions[a]
TF primer 1 (TF-1)	5'-AAT TCT AAT ACG ACT CAC TAT AGG GAG AGG GCT GTC TGT ACT CTT CCG GTT A-3'	788–765
TF primer 2 (TF-2)	5'-GAA GGA ACA ACA CTT TCC TA-3'	575–594
TF capture probe (TF-C)	5'-B GCC TCC GGG ATG TTT TTG GCA AGG A-3'	596–620
TF wt detection probe	5'-E GTT CAG GAA AGA AAA CAG CCA-3'	656–676
TF Q detection probe	5'-E AAG TAA AGT CGA CAA GCA CAG-3'	–
CD14 primer 1 (CD14-1)	5'-AAT TCT AAT ACG ACT CAC TAT AGG GGG AGA AGT TGC AGA CGC AGC GGA-3'	192–170
CD14 primer 2 (CD14-2)	5'-GAA GCT AAA GCA CTT CCA-3'	17–34
CD14 capture probe (CD14-C)	5'-B CCT GTC CGG AGC TCA GAG GTT-3'	38–58
CD14 wt detection probe	5'-E CCA TGG AGC GCG CGT CCT-3'	74–91
CD14 Q detection probe	5'-E GCT ACG GGA TCC TCC CGG-3'	–

B, biotine; E, ECL label. The T7 promoter part in primer 1 sequences is underlined.
[a]TF mRNA sequence, GenBank accession no. M16553; CD14 mRNA sequence, GenBank accession no. M86511.

cell types is not within reach of most diagnostic laboratories. In addition to a method of RNA quantification, an accurate quantification of cell numbers might be critical in order to estimate the number of TF mRNA copies per monocyte. The amount of monocytes can be estimated by quantification of a mRNA that is rather unique for monocytes and is present in these cells at a relatively constant level. We selected CD14 mRNA as a possible candidate marker for the number of monocytes and developed a quantitative multiplex NASBA assay for the simultaneous measurement of the TF and CD14 mRNAs. In the present study we describe the development of this assay and its performance in measuring TF and CD14 mRNA levels during LPS stimulation of isolated human monocytes.

MATERIALS AND METHODS

Plasmids and RNA synthesis

The starting point for the TF constructs was an 823 bp long cDNA fragment that had been subcloned in the *Eco*RI site of pUC13 (20). The entire insert was subcloned in the plasmid pcDNA II (Invitrogen) that contains promoters for the T7 and SP6 RNA polymerases. This clone, which encompasses exons 1–5 of the TF gene, was named pTFwt. A CD14 cDNA fragment that had been cloned in the plasmid πH_3M was digested with *Xho*I, and a fragment of ~460 bp containing the 5' part of the cDNA was inserted in the *Xho*I site of pcDNA II to yield pCD14wt. The mut-constructs, which contained an ~20 bp long scrambled part in an otherwise identical construct, were generated from both wild-type (wt) plasmids using PCR techniques. The mut-constructs were used as the source for the internal control (Q-)RNAs and the scrambled sequences made specific detection of these RNAs possible (Table 1).

Using T7 RNA polymerase (Pharmacia), *in vitro* RNA was generated from pTFwt (TF WT RNA) and pTFmut (TF Q-RNA) after digestion with *Sac*I and from pCD14wt (CD14 WT RNA) and pCD14mut (CD14 Q-RNA) after digestion with *Bam*HI. The lengths of the *in vitro* RNAs were 933 nt for TF WT RNA and TF Q-RNA, including part of the flanking polylinker sequence, and 971 nt for CD14 WT RNA and CD14 Q-RNA. The RNA was treated with DNAse I (Pharmacia) to remove plasmid DNA. Next, the RNA was purified on an anionic exchange column (RNeasy spin column; Qiagen). RNA was quantified on the basis of its extinction using 1 OD_{260} = 40 µg/ml and diluted to the desired concentration with water. The RNA concentration was expressed as molecules/ml. All RNA stock solutions were stored at –70°C.

Monocyte isolation from whole blood and culture conditions

Monocytes were isolated from five human donors and were cultured on Teflon membranes as described in van den Eijnden *et al*. (21). We used a series of RNA preparations sampled at various time points during a 13 day culture of human monocytes in suspension, during which period the cells differentiate into macrophages. In addition, the monocytes were treated with 100 ng/ml LPS (Sigma) at the first day of *in vitro* culture for various periods of time (20 h at most).

RNA isolation from isolated monocytes and northern blotting

Total RNA from monocytes was purified as described by van den Eijnden *et al*. (21). RNA aliquots (~5 µg) were stored under ethanol at –70°C. The RNA was recovered by centrifugation and the nucleic acid pellet was resuspended in 100 µl water. Next the nucleic acid suspensions were diluted 100-fold in water, and 5 µl of these dilutions were used per NASBA reaction. Northern blot analysis was essentially as described in van den Eijnden *et al*. (21).

NASBA

The NASBA reactions were performed as described by Kievits *et al*. (8) with some modifications. A 5 µl sample of RNA was added to 10 µl NASBA pre-mixture [final concentration in 20 µl reaction mixture: 40 mM Tris–HCl, pH 8.5, 12 mM $MgCl_2$, 70 mM KCl, 15% v/v DMSO, 1 mM each dNTP, 2 mM each NTP, 0.2 µM of each primer (TF P1, TF P2, CD14 P1, CD14 P2; Table 1), 2×10^4 copies of TF Q-RNA, 1×10^5 copies of CD14 Q-RNA]. Reactions were incubated for 5 min at 65°C to

destabilize the secondary structure of the RNA and subsequently cooled down to 41°C to allow primer annealing. The amplification reaction was started by adding 5 µl enzyme mixture [0.43 g/l BSA (Boehringer), 0.016 U/µl RNase H (Pharmacia), 6.40 U/µl T7 RNA polymerase (Pharmacia), 1.28 U/µl AMV-RT (Seikagagu)]. Reactions were incubated for 90 min at 41°C. NASBA reactions were stored at –20°C.

Detection of NASBA products by ECL

NASBA reaction products were detected using ECL as described previously (22). Briefly, 5 µl of a 1 in 10 diluted NASBA reaction mixture was added to 20 µl hybridization mixture. The hybridization buffer (5× SSC) contained 2×10^{12} copies of an ECL [Tris(2,2-bipyryridine)ruthenium(II) complex]-labeled detection probe (Table 1) and 20 µg streptavidin-coated magnetic beads which were pre-coated with 2×10^{12} copies of a biotinylated capture probe (Table 1). Each NASBA reaction was analyzed with all four specific probe combinations: (i) TF capture probe and TF wt detection probe; (ii) TF capture probe and TF Q detection probe; (iii) CD14 capture probe and CD14 wt detection probe; and (iv) CD14 capture probe and CD14 Q detection probe. Hybridization was performed in a total reaction volume of 25 µl and the reactions were incubated for 10 min at 60°C. Subsequently, 300 µl assay buffer (100 mM tripropylamine, pH 7.5) was added and the tubes were placed in an Origen 1.5 ECL detection instrument (Organon Teknika).

Quantification of RNA levels

RNA quantification based on NASBA amplification and ECL detection has been reported previously for HIV-1 RNA (22) and HCV RNA (7). However, those studies describe the quantification of one WT RNA, using three internal standard RNAs. Here, we describe the quantification of two individual WT RNAs (TF and CD14) using two individual internal standard RNAs that are amplified with different primer pairs in a single reaction. This means that each WT RNA is quantified with only one internal standard RNA and, therefore, an altered quantification algorithm was applied. Nevertheless, the quantification of RNA levels is still based on the ECL signals and the amount of Q-RNA spiked per reaction. Basically, wt ECL and Q ECL-signals are measured over a range of wt RNA input at a fixed Q-RNA concentration. From these dose–effect curves, two correction factors (a and b) are derived that can be used to calculate wt mRNA concentrations from the ECL signals (wt-ECL and Q-ECL) and the Q-RNA concentration according to the following formula:

$$\log WT_{conc} = 1/a \times (\log WT_{ECL} - \log Q_{ECL} + \log Q_{conc}) - b/a$$

The factors a and b correct for differences in amplification rate between wt and Q-RNA, for differences in hybridization efficiencies of the various oligonucleotides and/or for possible differences in the specific activity of both detection probes. The constants a and b are specific for a particular set of reagents used in amplification, hybridization and detection and for the Q-RNA concentration used. For this study we have used for TF mRNA quantification a = 0.85, b = 1.00, TF-Q = 2×10^4 molecules per NASBA reaction and for CD14 mRNA quantification a = 0.81, b = 0.79 and CD14-Q = 1×10^5 molecules per NASBA reaction.

RESULTS AND DISCUSSION

The multiplex TF/CD14 Q-NASBA

Here we describe for the first time a multiplex quantitative NASBA assay, a method that is highly suitable for the simultaneous quantification of two mRNAs. Compared to other RNA amplification techniques such as RT–PCR, in NASBA RNA amplification is achieved in a homogeneous and isothermic manner. RNA itself is the template molecule for the NASBA reaction, whereas in RT–PCR one additional step is needed to generate the cDNA template for the amplification reaction. Moreover, in NASBA dsDNA is not amplified, which makes NASBA the preferred method for the specific detection of mRNAs in a background of genomic DNA. Removal of genomic DNA is not needed, not even for the detection of mRNAs from intronless genes (13). The multiplex Q-NASBA is based on the co-amplification of specific internal standard RNAs. In the case of TF/CD14 Q-NASBA, two internal standards (Q-RNAs) were generated, that were derived from the corresponding wt mRNAs. Each Q-RNA carries a unique sequence of ~20 nt which is a scrambled version of the wild-type sequence at that position. In this way, specific probes can be designed for WT and Q-RNA amplificates that are identical in length and in GC-content. This results in equal hybridization behavior of each probe on the specific RNA template. WT and Q-RNA are amplified with the same NASBA primers ensuring equal amplification efficiency for both template molecules. Finally, by directing the WT probes to a region that was split by an intron in the genomic DNA, these probes will only detect RNA molecules that have been generated from completely spliced mRNA.

For the multiplex TF/CD14 Q-NASBA, Q-RNAs were prepared for both TF and CD14 quantification by *in vitro* transcription of plasmid templates. Known amounts of both Q-RNAs were added to the NASBA reaction and were co-amplified with the WT mRNAs. From the ratio of the ECL signals obtained with the specific probes for WT and Q-amplificates, the WT RNA concentration in the original sample can be calculated using two correction factors, a and b, that have been determined in an experiment in which known amounts of RNA were added.

The dynamic range of the NASBA using *in vitro* generated RNAs

The multiplex TF/CD14 Q-NASBA was used to quantify different amounts of *in vitro* generated TF WT RNA and CD14 WT RNA (Table 2). Therefore, both WT RNA types were added at a concentration of 10^7, 10^6, 10^5, 10^4, 10^3 or no WT RNA copies per NASBA reaction. All possible input combinations between TF RNA and CD14 RNA were analyzed in a multiplex TF/CD14 Q-NASBA. The WT RNAs were co-amplified with the TF and CD14 Q-RNAs that were present at constant concentrations of 2×10^4 and 1×10^5 copies per NASBA reaction respectively.

In each single amplification reaction TF and CD14 RNA were detected if present in the NASBA reaction. TF and CD14 probes did not result in positive ECL signals in the reactions in which these RNAs were not added to the amplification reaction. The ECL results were used to quantify TF and CD14 RNAs (log copies) and were correlated to the actual input of these two RNAs. The outcome of TF quantification was independent of the amount of CD14 RNA that was co-amplified

Table 2. NASBA quantitation of TF and CD14 RNA

Input TF RNA (log copies)	Input CD14 RNA (log copies)						
	7.00 TF/CD14	6.00 TF/CD14	5.00 TF/CD14	4.00 TF/CD14	3.00 TF/CD14	no template TF/CD14	**TF result (mean ± SD)**
7.00	7.07/7.07[a]	7.26/6.26[a]	6.85/5.03[a]	6.95/3.94[a]	6.90/2.48[a]	7.33/neg	**7.06 ± 0.20**
6.00	5.93/6.81[a]	5.88/6.16	5.99/5.45	5.97/4.02	6.03/2.67	6.18/neg	**6.00 ± 0.10**
5.00	4.86/6.92[a]	4.96/6.12	4.96/5.45	4.87/4.22	4.93/2.72	4.97/neg	**4.92 ± 0.05**
4.00	3.92/6.78[a]	3.99/6.01	4.05/5.15	4.18/4.13	4.23/2.92	4.03/neg	**4.07 ± 0.12**
3.00	2.99/6.50[a]	2.96/5.86	3.15/5.14	3.02/3.37	3.01/3.32	2.78/neg	**2.98 ± 0.12**
no template	neg/6.90	neg/6.06	neg/5.13	neg/4.21	neg/3.16	neg/neg	**neg**
CD14 result (mean ± SD)	**6.83 ± 0.19**	**6.08 ± 0.14**	**5.23 ± 0.18**	**3.98 ± 0.32**	**2.88 ± 0.32**	**neg**	

neg, negative result.
[a]Marked data points are also presented in Figure 1 to determine the linearity of the assay.

Table 3. Reproducibility of multiplex NASBA (n = 3)

Input concentration (log RNA copies/reaction)		NASBA quantitative result (log RNA copies/reaction)	
TF RNA	CD14 RNA	TF RNA	CD14 RNA
7.00	3.00	7.20 ± 0.29	2.87 ± 0.24
5.00	5.00	5.07 ± 0.07	5.13 ± 0.16
3.00	7.00	2.95 ± 0.09	7.04 ± 0.26

in the same reaction tube and the same result was obtained regarding the CD14 quantification (Table 2). On the same data set the precision and accuracy of the assay was determined. Here precision is defined as fluctuations in quantitative results obtained on the same amount of TF RNA input (i.e., the standard deviation). The accuracy is defined as the difference between the outcome of the quantification and the actual input of TF RNA. The precision measured in the six individual reactions that were spiked with the same amount of TF RNA but with different amounts of CD14 RNA was ≤0.20 log units in all cases, whereas the accuracy varied between –0.08 and +0.08 log units. For CD14 RNA quantification the precision was ≤0.32 log units and accuracy was measured between –0.17 and +0.23 log units. Input ratios of 1:10 000 to 10 000:1 for TF and CD14 RNA were reliably detected by TF/CD14 Q-NASBA (Fig. 1). This means that RNA ratios can be measured over an eight log dynamic range: –4 to +4 log units of TF versus CD14 RNA with a good precision and accuracy.

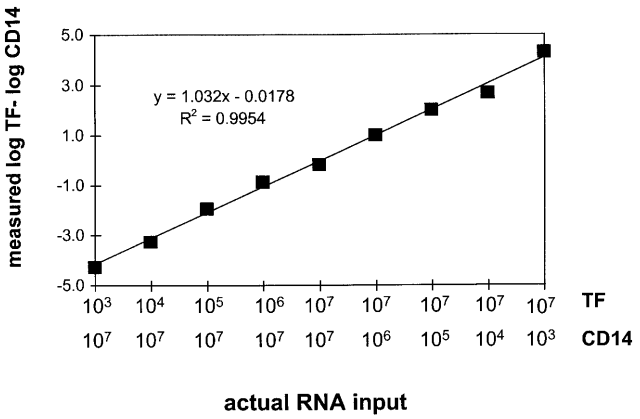

Figure 1. Dynamic range of the one-tube multiplex Q-NASBA.

Reproducibility of the results

The reproducibility of the TF/CD14 Q-NASBA assay was measured by analysis of three different RNA mixtures in three independent NASBA experiments. The three NASBA runs and the ECL-based detection were performed on three different days. The three RNA mixtures that were used for determination of the reproducibility consisted of the input ratios 10 000:1, 1:1 and 1:10 000 for TF to CD14 RNA. Results are presented in Table 3. For these RNA samples the precision was ≤0.29 log unit and the accuracy ranged from –0.13 to +0.20 log unit. These results prove that the TF/CD14 Q-NASBA assay can be used to quantify both mRNAs reproducibly in a dynamic range of 10^3–10^7 copies per NASBA reaction.

TF and CD14 expression in LPS-stimulated monocytes

Next, the multiplex Q-NASBA assay was evaluated in a second model system using total RNA isolated from monocytes. Here, TF and CD14 mRNA levels were measured upon LPS stimulation of isolated monocytes (Table 4; Fig 2A). Monocytes were incubated with LPS for 0, 2, 4, 8, 12 or 20 h. The cells were lysed and their purified total RNA was used directly as input in a multiplex TF/CD14 Q-NASBA. Each time-point was analyzed in triplicate in three individual experiments. The maximal standard deviation measured on the three individual NASBA quantifications was 0.15 log unit. Two hours after LPS stimulation TF mRNA levels had increased by 1.5 log unit. After that, TF mRNA levels returned to their pre-LPS levels over a period of

Table 4. Measuring TF mRNA and CD14 mRNA in monocytes after LPS incubation

Time (h)	TF mRNA (log copies/µg total RNA)	CD14 mRNA (log copies/µg total RNA)
0	6.19 ± 0.06	7.51 ± 0.08
2	7.69 ± 0.03	7.01 ± 0.16
4	7.19 ± 0.13	6.40 ± 0.15
8	6.12 ± 0.03	7.18 ± 0.11
12	5.59 ± 0.12	7.37 ± 0.11
20	6.01 ± 0.02	8.07 ± 0.03

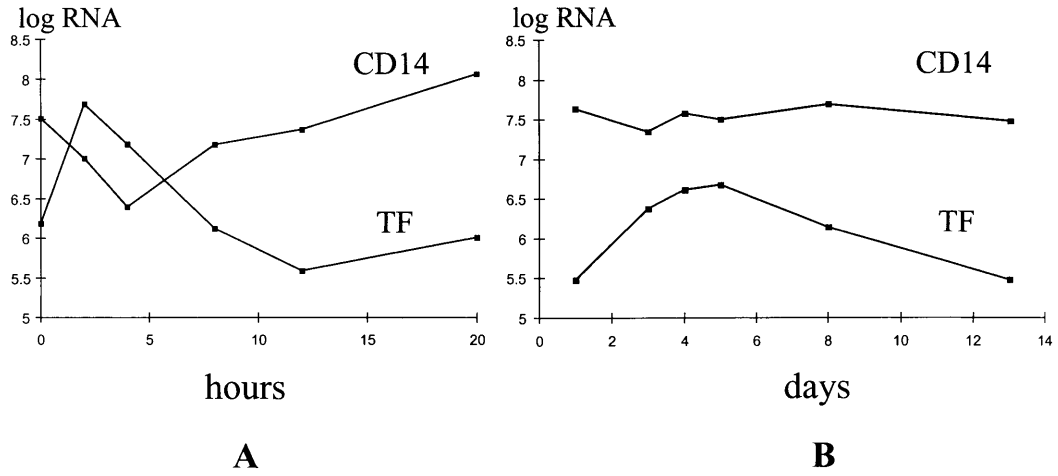

Figure 2. TF and CD14 mRNA levels in monocytes. (**A**) Isolated monocytes were treated with 100 ng/ml LPS at the first day of *in vitro* culture for various periods of time (20 h at most). (**B**) Human monocytes were cultured for various periods of time (13 days at most). Isolated total RNA was used as input for the multiplex Q-NASBA.

8 h. Surprisingly, CD14 mRNA levels dropped during the first hour of LPS incubation by 1 log unit, to return afterwards to levels slightly higher than in the pre-LPS cells. The same samples were also analyzed on a northern blot. NASBA TF quantification results correlate well with this semi-quantitative method (Fig 3).

TF and CD14 expression during a long-term culture of human monocytes

We have also determined TF and CD14 RNA levels in RNA that was purified from a similar monocyte culture during its differentiation into macrophages (Table 5; Fig 2B). During this process, the cells temporarily express a relatively low level of TF. Expression peaks at day 4 of culture (21). The results in Table 5 also shows that these low levels are detected with ease using the NASBA procedure that shows a 1 log increase of expression. After a 13 day culture period, the TF RNA levels had returned to their baseline value.

CD14 mRNA as marker for monocyte numbers

CD14 is frequently used as a marker for monocytic cells and we have used the CD14 signal as a marker for the number of monocytic cells in the blood. However, CD14, which acts as the LPS receptor on the cell surface, is in itself also subject to regulation. The presently observed changes of CD14 mRNA levels by the addition of LPS is an indication that CD14 mRNA expression cannot be used as an absolute measure of the number of monocytic cells. However, it remains at present the most useful marker for our purpose.

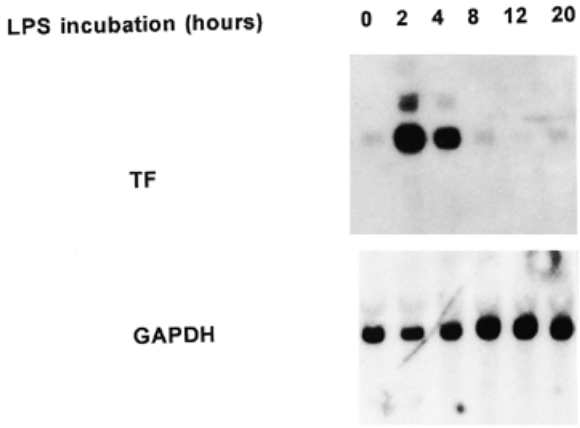

Figure 3. Northern blot analysis of total cellular RNA from LPS-stimulated human monocytes using a TF or a GAPDH specific probe.

Table 5. TF mRNA and CD14 mRNA levels during a 13 day culture of human monocytes

Time (days)	TF mRNA (log copies/µg total RNA)	CD14 mRNA (log copies/µg total RNA)
1	5.48 ± 0.02	7.64 ± 0.01
3	6.38 ± 0.09	7.35 ± 0.09
4	6.62 ± 0.20	7.58 ± 0.02
5	6.68 ± 0.13	7.50 ± 0.03
8	6.14 ± 0.04	7.69 ± 0.02
13	5.48 ± 0.04	7.48 ± 0.06

Applications of one-tube quantitative multiplex assay

An obvious future application of the multiplex TF-CD14 Q-NASBA is the measurement of TF mRNA in whole blood. Preliminary experiments using whole blood of healthy volunteers demonstrated the feasibility of a test in which lysed blood samples are spiked with both Q-RNAs. Obviously, different assay parameters (a,b) are needed, which will allow for correction of, among others, effects of RNA isolation. The determination of these parameters is complicated by the fact that human blood definitely contains copies of TF and CD14 mRNAs. For appropriate calibration, blood without these mRNAs is required (diluted human blood, blood of other species). Also more information on the effects of blood collection materials and procedures on TF mRNA levels is needed and optimal conditions for blood processing (storage time and temperature) need to be determined. Only then can the multiplex TF-CD14 Q-NASBA be used in clinical studies.

In conclusion, in this study it was shown for the first time that the multiplex Q-NASBA is a powerful tool for measuring two RNAs in a broad dynamic range with good precision and accuracy. The quantification method of two individual RNAs in a single tube assay might be useful for other NASBA applications. Both the assay performance and the simplicity of the assay make it very suitable for routine screening of RNA levels in clinical diagnostics.

ACKNOWLEDGEMENTS

We would like to thank Dr Pieter H. Reitsma for his valuable input during the initial phase of these studies. M.M.E.D. van den Eijnden was supported by a grant from the Netherlands Heart Foundation (91.066).

REFERENCES

1. Nedelman,J., Heagerty,P. and Lawrence,C. (1992) *Bull. Math. Biol.*, **54**, 477–502.
2. Nedelman,J., Heagerty,P.and Lawrence,C. (1992) *Comput. Appl. Biosci.*, **8**, 65–70.
3. Juriaans,S., Dekker,J.T. and de Ronde,A. (1992) *AIDS*, **6**, 635–641.
4. Scadden,D.T., Wang,Z.Y. and Groopman,J.E. (1992) *J. Infect. Dis.*, **165**, 1119–1123.
5. Piatak,M.J., Saag,M.S., Yang,L.C., Clark,S.J., Luk,K.C., Hahn,B.H., Shaw,G.M. and Lifson,J.D (1993) *Science*, **259**, 1749–1754.
6. van Gemen,B., van de Wiel,P., van Beuningen,R., Sillekens,P., Jurriaans,S., Dries,C., Schoones,R. and Kievits,T. (1995) *PCR Methods Appl.*, **4**, S177–S184.
7. Hollingsworth,R.C., Sillekens,P., van Deursen,P., Neal,K.R. and Irving,W.L. (1996) *J. Hepatol.*, **25**, 301–306.
8. Kievits,T., van Gemen,B., van Strijp,D., Schukkink,R., Dircks,M., Adriaanse,H., Malek,L., Sooknanan,R. and Lens,P. (1991) *J. Virol. Methods*, **35**, 273–286.
9. Compton,J. (1991) *Nature*, **350**, 91–92.
10. Leone,G., van Schijndel,H., van Gemen,B., Kramer,F.R. and Schoen,C.D. (1998) *Nucleic Acids Res.*, **26**, 2150–2155.
11. Nakahara,K., Hataya,T. and Uyeda. (1998) *Nucleic Acids Res.*, **26**, 1854–1885.
12. Morré,S.A., Sillekens,P., Jacobs,M.V., van Aarle,P., de Blok,S., van Gemen,B., Walboomers,J.M.M., Meijer,C.J.L.M. and van den Brule,A.J.C. (1996) *J. Clin. Microbiol.*, **34**, 3108–3114.
13. Heim,A., Grumbach,I.M., Zeuke,S. and Top,B. (1998) *Nucleic Acids Res.*, **26**, 2250–2251.
14. Darke,B.M., Jackson,S.K., Hanna,S.M. and Fox,J.D. (1998) *J. Immunol. Methods*, **212**, 19–28.
15. Blok,M.J., Goossens,V.J., Vanherle,S.J.V., Top,B., Tacken,N., Middeldorp,J.M., Christiaans,H.L., van Hooff,J.P. and Bruggeman,A. (1998) *J. Clin. Microbiol.*, **36**, 1341–1346.
16. Reitsma,P.H., van der Velden,P.A., Vogels,E., van Strijp,D., Tacken,N., Adriaansen,H. and van Gemen,B. (1996) *Blood Coagulat. Fibrinol.*, **7**, 659–663.
17. Sooknanan,R.L., Malek,L., Wang,W.I., Siebert,T. and Keating,A. (1994) *Exp. Hematol.*, **21**, 1719–1724.
18. Carson,S.D. and Brozna,J.P. (1993) *Blood Coagulat Fibrinol.*, **4**, 281–292.
19. Gailani,D.and Broze,G.J. (1991) *Science*, **253**, 909–912.
20. van der Logt,C.P.E., Reitsma,P.H. and Bertina,R.M. (1992) *Thromb. Haemost.*, **67**, 272–276.
21. van den Eijnden,M.M.E.D., Steenhauer,S.I., Reitsma,P.H. and Bertina,R.M. (1997) *Thromb. Haemost.*, **77**, 1129–1136.
22. van Gemen,B., van Beuningen,R., Nabbe,A., van Strijp,D., Jurriaans,S., Lens,P. and Kievits,T. (1994) *J. Virol. Methods*, **49**, 157–168.