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ISSN 1330-9862
(FTB-1464)

preliminary communication

Comparison of Three RT-PCR Based Methods for Relative Quantification of mRNA

Davorka Breljak[1], Andreja Ambriović-Ristov[2], Sanja Kapitanović[1], Tamara Čačev[1] and Jelka Gabrilovac[1]*

[1]Ruđer Bošković Institute, Division of Molecular Medicine, Bijenička c. 54, HR–10000 Zagreb, Croatia
[2]Ruđer Bošković Institute, Division of Molecular Biology, Bijenička c. 54, HR–10000 Zagreb, Croatia

Received: December 23, 2004
Revised version: April 27, 2005
Accepted: May 25, 2005

Summary

Comparison of three RT-PCR based methods: semi-quantitative, competitive and real-time RT-PCR for relative quantification of mRNA is presented. Aminopeptidase N expressed on human promyeloid HL-60 cell line, at basal and activated state, served as a model for comparison. HL-60 cells were stimulated with IFN-γ (6 ng/mL) for 72 h at 37 °C, total cellular RNA was isolated, reverse transcribed to cDNA and semi-quantitative, competitive and real-time RT-PCR were performed to obtain the relative levels of mRNA for aminopeptidase N. The data obtained showed that all three RT-PCR based methods gave reliable and comparable results, *i.e.* approximately twofold increase of aminopeptidase N mRNA on IFN-γ stimulated HL-60 cells. Thus, in spite of rapid advances made in the area of real-time RT-PCR, end-point RT-PCR such as competitive and semi-quantitative RT-PCR, although laborious and time consuming, may still remain useful techniques for relative mRNA quantification when small number of samples are to be analyzed.

Key words: aminopeptidase N, APN, HL-60, RT-PCR, real-time PCR, competitive PCR

Introduction

Quantitative measurement of specific gene expression is a critically important tool in understanding basic cellular mechanism and effects of various agents on cell function. The combined use of reverse transcriptase followed by polymerase chain reaction (RT-PCR) is a powerful technique for quantification of the mRNA levels (*1,2*). At least two serious problems exist in quantifying gene expression by RT-PCR. One problem arises from the fact that PCR is an exponential process where the amount of product increases exponentially. At a certain point, referred to as plateau phase, the rate of production slows and PCR product increases at an unknown rate. After this point has been reached, initial concentration of target DNA cannot be determined by extrapolation (*3–5*). In other words, even very small differences at the start of amplification process will have a large effect on the amount of product accumulated after limited number of cycles (*4,5*). The second problem with PCR-based measurement of gene expression is that it is difficult to ensure equal amount of RNA in each sample. To compensate for variation of the amount of RNA input, both target gene and a housekeeping gene that is not expected to vary in response to stimuli must be quantified (*6–8*).

*Corresponding author; E-mail: breljak@irb.hr

Various approaches have been developed to circumvent these problems of PCR-based gene quantification. One solution is the measurement of PCR products by semi-quantitative PCR, where the amounts of PCR products are measured during the exponential (*i.e.* log) phase of the PCR reaction, which occurs before saturation is reached (*4,5,9*). This is performed for both, the housekeeping and the target genes. Another method for PCR-based quantification is competitive PCR, where the competitor and target templates compete within the same PCR tube for enzyme, nucleotides and primer molecules (*5,9,10*). The amount of target is quantified by titration of constant, but unknown, amounts of target template against a serial dilution of known amounts of the competitor. Since competitor molecule differs in size from the target one, the two PCR products can be separated by agarose gel electrophoresis. Their relative intensities are used for evaluation of starting amount of the target template (*4,10*). Finally, the most recent studies have focused on the use of real-time PCR. Real-time PCR allows detection of PCR product during the exponential phase of the reaction, combining amplification and detection in one step, eliminating the need for post-PCR processing such as electrophoresis (*2,11–13*). Real-time monitoring of DNA amplification within the PCR reaction tubes is achieved by detecting the fluorescence. The amount of fluorescence released is directly proportional to the amount of product generated in each PCR cycle and thus can be applied as a quantitative measure of PCR reaction (*1,2,12*).

We have recently shown the effect of T cell-derived cytokine IFN-γ on the level of aminopeptidase N expression on human promyeloid HL-60 cell line (*14*). Aminopeptidase N (APN; CD13; E.C. 3.4.11.2) is involved in the regulation of peptide-mediated cellular response, regulating cell proliferation, adhesion, activation, differentiation and transformation (*15,16*). Therefore, reliable quantification of APN gene expression is essential in gaining insight in the role of APN in these processes.

Here we present the comparison of three RT-PCR--based methods for relative quantification of APN mRNA level: semi-quantitative, competitive and real time RT--PCR on the model of human promyeloid HL-60 cell line treated with IFN-γ. Advantages and limitations of each of these methods will be commented. We have shown that all three methods can give reliable and comparable results.

Material and Methods

Experimental design

HL-60 cell line (ATCC) was maintained in RPMI--1640 medium containing L-glutamine 3 mM, HEPES 20 mM, penicillin 0.1 g/L, streptomycin 0.1 g/L and 10 % fetal calf serum (FCS). Cells were maintained in a humidified atmosphere with 5 % CO_2 at 37 °C. HL-60 cells were seeded in 24-well culture plates ($2.5 \cdot 10^5$ cells/mL) in the presence of IFN-γ (6 ng/mL). Control samples were cultured in the medium without IFN-γ. At the end of incubation time, the cells were collected, washed in phosphate buffered saline (PBS) and total cellular RNA was extracted using High Pure RNA Isolation Kit (Roche) following the manufacturer's instruction. RNA concentration and its purity were estimated by measuring the absorbance at 260 and 280 nm. The quality and integrity of RNA were verified by agarose gel electrophoresis. Isolated RNAs were stored at –80 °C and subsequently used for semi-quantitative, competitive as well as real-time RT-PCR.

Semi-quantitative RT-PCR

Total cellular RNA (1 μg) was heated at 65 °C for 5 min and used as a template for the first strand cDNA synthesis. The reaction mixture contained 3 μg of random hexadeoxynucleotide primers (Roche), 1 mM of dNTP mix (Roche), 40 units of RNase Inhibitor (Roche), 10 mM of dithiothreitol (Sigma), 1× reverse transcription buffer (Roche) and 40 units of Moloney murine leukemia virus (M-MuLV) reverse transcriptase (RT) (Roche) in a final volume of 20 μL. The reaction mixture was incubated at 37 °C for 1 h, heated to 65 °C for 10 min and finally diluted 5× in DNase/RNase free water (Gibco). Since APN forward and reverse primers are within the 1st exon, different controls were performed to exclude PCR amplification of possible contaminating genomic DNA. First, RNase free DNase I digestion of the RNA samples prior to reverse transcription was done. Second, absence of genomic DNA was additionally confirmed by using intron-overspanning primers for the housekeeping gene ABL. The predicted size of PCR amplified ABL cDNA is 193 bp, while the same reaction gives a 793 bp fragment from genomic DNA (Table 1) (*17*). Third, non-reverse transcribed RNAs were also included in each PCR reaction to confirm the absence of genomic DNA. No PCR products were detected in cDNAs and RNAs indicating lack of genomic DNA in the samples (data not shown). Housekeeping gene ABL was also used as a control for variations in the input of RNA. Ribosomal protein large subunit P0 (RPLP0) was used as a second housekeeping gene to standardize the results. The sequences of the specific primers used for RT-PCR reactions are defined in Table 1. PCR was performed using 2 μL of 5× diluted first strand cDNA on a Perkin Elmer 9600 thermocycler in total volume of 20 μL with 1 unit of Taq polymerase (Roche), 0.2 mM of dNTP mix (Roche), 1× PCR buffer (Roche) and 0.20 μM of each primer. Reaction conditions used for semi-quantitative RT--PCR are shown in Table 2. PCR products were taken from successive PCR cycles and resolved by electrophoresis in 1.5 % agarose gel, stained with ethidium bromide (EtBr) and visualized under ultraviolet light by video image system (Pharmacia). The samples which were analyzed and compared to each other (control and IFN-γ treated samples) were loaded on the same gel following the same settings of the image analyses. Densitometric analysis of EtBr-stained gel bands was performed using Image Master VDS software 1.0 (Pharmacia).

Construction of the competitor

RNA was isolated from HL-60 cells, reverse transcribed to cDNA as described above and used as a template for the PCR amplification using specific primers for APN gene (Table 1). Pwo DNA polymerase (Roche) was used in order to obtain a blunt-ended RT-PCR product of 450 bp, which was then cloned into the *Eco*

Table 1. Primer sequences used for semi-quantitative RT-PCR

Gene	Forward (F) and reverse (R) primers (5′-3′)	Location	Gene Bank Accession No.	RT-PCR product size/bp
APN	**F** gccgtgtgcacaatcatcgcact **R** caccagggagcccttgaggtg	63–86 492–513	M 22324	450
ABL	**F** agcatctgactttgagcc **R** cccattgtgattatagcctaagac	462–480 631–655	M 14752	193
RPLP0	**F** ggcaccattgaaatcctgagtgatgtg **R** ttgcggacaccctccaggaagc	524–551 717–739	NM_001002	215

Table 2. Reaction conditions used for semi-quantitative RT-PCR

Gene	Denaturation	Annealing	Extension
APN	30 s at 95 °C	30 s at 61 °C	45 s at 72 °C
ABL	30 s at 95 °C	30 s at 55 °C	45 s at 72 °C
RPLP0	30 s at 95 °C	30 s at 57 °C	45 s at 72 °C

RV restriction site of pBlueSript II. To construct a plasmid to be used as a competitor, small partial internal deletion was introduced into the APN insert based on the data of published APN cDNA sequence (*18,19*). Briefly, plasmid containing 450 bp fragment of APN was digested with *StyI* and *AatII* (Roche), both ends were filled in with T4 DNA polymerase and religation resulted in the plasmid lacking 165 bp of the APN fragment. This plasmid, named APN competitor, was used in competitive RT-PCR. Concentration of the purified APN competitor was measured by the absorbance at 260 nm and corresponding copy number was calculated using the following equation: 1 μg of plasmid DNA contains $2.98 \cdot 10^{11}$ molecules. Serial dilutions of APN competitor, each containing a known input number, were stored at –20 °C until use.

Competitive RT-PCR

A volume of 2 μL of 5× diluted first strand cDNA, synthesized as described for semi-quantitative RT-PCR, was used as a template for competitive RT-PCR. Two-fold serial dilutions of APN competitor were coamplified with a constant amount of cDNA synthesized from HL-60. Competitive PCR was performed in total volume of 30 μL for 33 cycles under conditions shown in Table 2. Products of competitive PCR reactions were resolved by electrophoresis in 1.5 % agarose gel, stained with EtBr, and visualised under ultraviolet light by video image system (Pharmacia). Densitometric analysis of EtBr-stained gel bands was performed using Image Master VDS software 1.0 (Pharmacia). Amplified PCR products APN cDNA and APN competitor differ in size, therefore band intensities were corrected before the calculation of their ratio. The data were plotted as a function of the log of the ratio (APN cDNA/APN competitor) against the log of the copy number (APN competitor). The lines were drawn from the linear regression analysis of the data points and the calculation of X-intercepts was done. At this intercept, the amounts of target APN cDNA molecules and of APN competitors are identical. Since the amount of APN competitors was known, the amount of target APN cDNA molecules was then calculated.

Real-time RT-PCR

First strand cDNA synthesis was performed using TaqMan RT Reagents (Applied Biosystems), which are optimized for use in the real-time PCR and 5' nuclease detection methods according to manufacturer's protocol. Primers and probes for real-time PCR were designed by Applied Biosystems and supplied as Assay-on-Demand Gene Expression Assay Mix containing a 20× mix of unlabeled PCR forward and reverse primers as well as TaqMan MGB probe. Assay IDs were Hs00174265_m1 and Hs99999902_m1 for APN and RPLP0, respectively (http://www.appliedbiosystems.com). Real-time PCR was performed in a 50-μL volume using 5 μL of the first-strand cDNA template, 2.5 μL of 20× Assay-on-Demand Gene Expression Assay mix (Applied Biosystems), 25 μL of the 2× TaqMan Universal PCR Master Mix (Applied Bioystems) and 17.5 μL of DNase/RNase--free water (Gibco). Amplification and detection were performed using the ABI PRISM 7000 Sequence Detection System (SDS) (Applied Biosystems). Thermal cycling conditions included initial 2 min at 50 °C and 10 min at 95 °C followed by 40 two-step cycles including denaturation of 15 s at 95 °C and annealing/extension of 1 min at 60 °C. No template control (NTC) reactions, where the cDNA is substituted with DNase/RNase-free water, were included in each real-time PCR run to screen for possible contamination. To compensate for variations in the input of RNA amounts, and an efficiency of reverse transcription, housekeeping gene RPLP0 was quantified and results were normalized to these values. Analyses of real-time RT-PCR products were performed using the ABI PRISM 7000 SDS software (Applied Biosystems). Relative quantification of APN mRNA amount was accomplished by comparative Ct, *i.e.* $2^{-\Delta\Delta Ct}$ method (*20*). Control (untreated) samples were chosen as calibrators, *i.e.* as the basis for comparative results. Relative amounts of APN mRNA were normalized to housekeeping gene RPLP0 and expressed relative to the calibrator using the arithmetic formula:

$$\text{N-fold difference} = 2^{-[\Delta Ct\ (\text{IFN-}\gamma\ \text{treated}) - \Delta Ct\ (\text{control})]}$$

where $\Delta Ct = Ct\ (APN) - Ct\ (RPLP0)$. N-fold difference represents the fold change in APN mRNA expression between the control (untreated) and IFN-γ treated samples. The statistic analysis was performed according to manufacturer's instruction.

Results and Discussion

Reliable methods for quantification of mRNA levels are necessary for investigation of gene expression. Recently, by using semi-quantitative RT-PCR, we have shown that T-lymphocyte derived cytokine, IFN-γ, modulates the expression of APN on human promyeloid cell line HL-60 (*14*).

Here we present the comparison of three RT-PCR based methods for quantification of relative level of APN mRNA: semi-quantitative, competitive and real-time RT-PCR, using IFN-γ treated HL-60 cells as a model.

Semi-quantitative RT-PCR

The prerequisite for a PCR to be used for quantification is that PCR product is measured within the exponential phase of the PCR reaction, where the amount of amplified target is directly proportional to the input amount of target. Therefore, quantification of gene expression by semi-quantitative RT-PCR must be carried out during the exponential (log) phase of the PCR reaction and plateau phase must be avoided. Exponential phase of the PCR reaction can be determined empirically by amplifying equivalent amounts of cDNA over different number of PCR cycles or by amplifying dilutions of cDNA over the same number of PCR cycles (*4,5*). In this work we performed kinetic analysis by amplifying PCR products over different number of PCR cycles and showed that PCR reaction was exponential between cycles 25 and 30 (Figs. 1A and C). Subsequently, »fine tuned« kinetic analysis was performed within the given range of cycles (Figs. 1B and D). Similar kinetic analysis was also done for APN and another housekeeping gene RPLP0 (data not shown). Results of semi-quantitative RT-PCR using comparative kinetic analysis are shown in Fig. 2. Two samples to be compared, obtained from control and IFN-γ treated cells, were amplified for consecutive cycles within the exponential phase of PCR reaction. The optimal numbers of PCR cycles determined were 25–28, 25–28 and 20–23 for ABL, APN and RPLP0, respectively (Fig. 2A). The number of cDNA molecules for ABL, RPLP0 and APN in control and IFN-γ treated samples was derived from the intercept of the regression line with the Y-axis. At this intercept, the difference between the log of cDNA level is directly proportional to the difference of starting level of cDNA in control and IFN-γ treated samples (Fig. 2B). Finally, Fig. 2C summarizes the results obtained by semi-quantitative RT-PCR. Treatment of HL-60 cells with IFN-γ (6 ng/mL) for 72 h upregulated expression of APN mRNA for approximately twofold, while the expression of both housekeeping genes, ABL and RPLP0, was unchanged.

Although semi-quantitative RT-PCR allows quantification of mRNA level and requires only common laboratory equipment, one of the major drawbacks of this method is the poor dynamic range of the quantification. In addition, the need for determination of exponential phase of the PCR reaction for each gene as well as each sample to be quantified is time consuming and labor intensive.

Competitive RT-PCR

Perhaps the greatest advantage of competitive PCR is that the initial ratio of target and competitor remains constant through the PCR reaction. Therefore, there is no need for PCR to be performed exclusively during the exponential phase (*5,10*). In competitive RT-PCR, a competitor, containing the same primer sequences as the target fragment, competes within the same tube with a target for nucleotides, enzyme and primers. The competitor bears the same primer binding region, but the sequence between them is modified in such a way that it allows separation of the two PCR products during gel electrophoresis and the comparison of their relative amounts

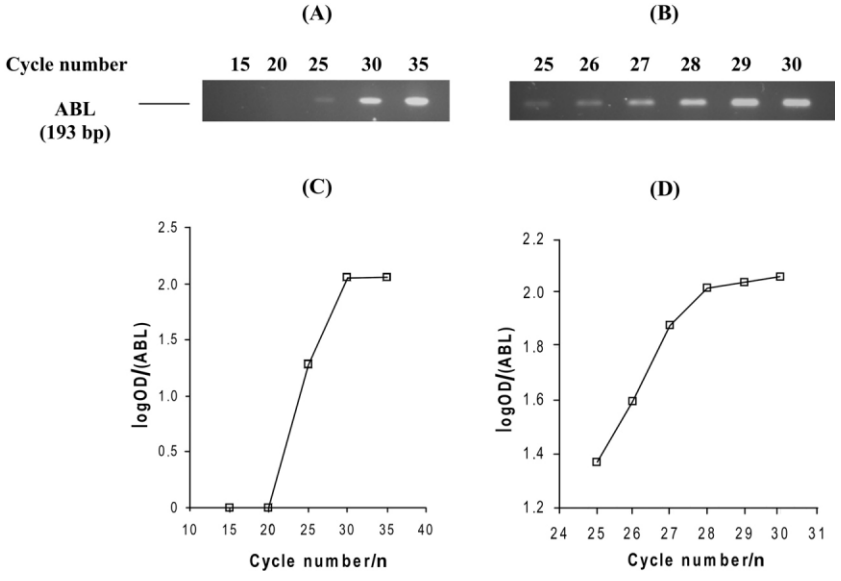

Fig. 1. Kinetic analysis of semi-quantitative RT-PCR. RNA was isolated from HL-60 cells, reverse transcribed to cDNA and semi-quantitative RT-PCR was performed using specific primers for ABL (Tables 1 and 2). **(A)** and **(B)** Ethidium bromide staining of RT-PCR products. **(C)** and **(D)** Graphic presentation of the data shown in **(A)** and **(B)** obtained by the Image Master VDS. The data were plotted as a function of the log OD of amplified RT-PCR product against the number of PCR cycles

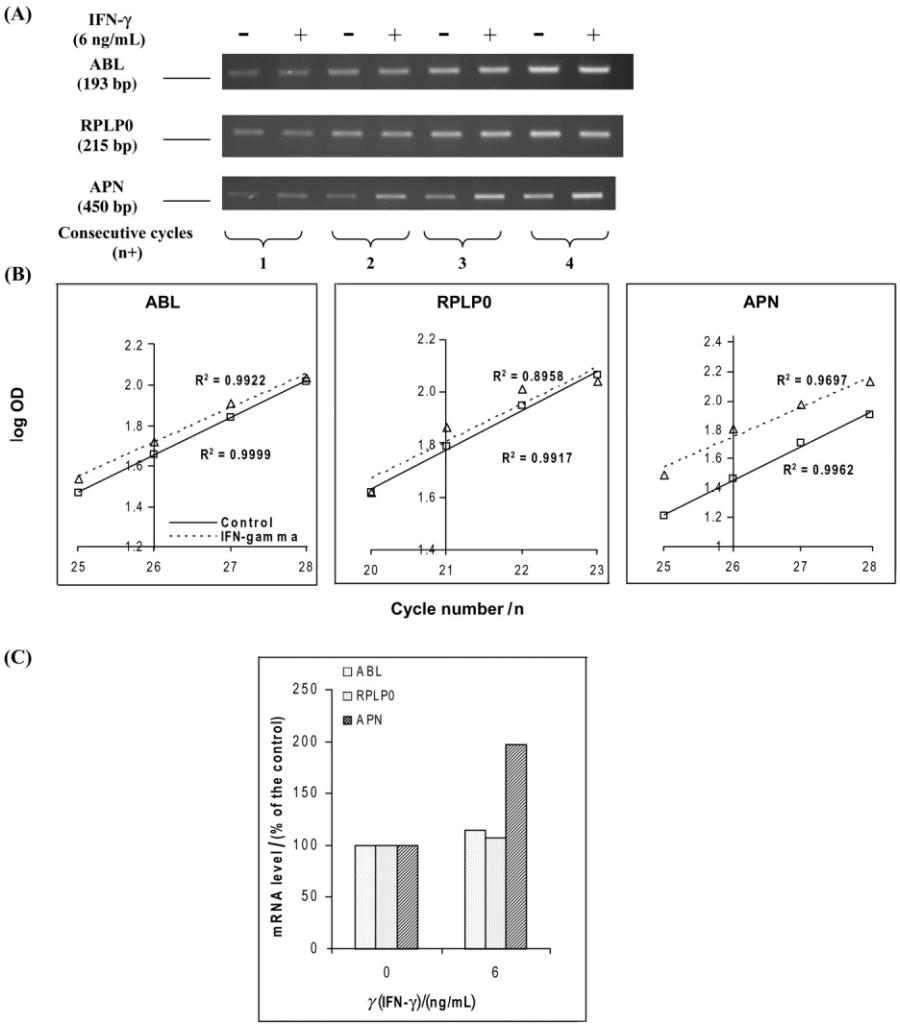

Fig. 2. Relative quantification of APN mRNA of IFN-γ treated HL-60 by semi-quantitative RT-PCR. HL-60 cells were cultured for 72 h with IFN-γ (6 ng/mL). Control (untreated) cells were incubated in the medium alone (without IFN-γ). At the end of incubation the cells were washed, total cellular RNA was isolated and semi-quantitative RT-PCR using kinetic analysis was performed. **(A)** Ethidium bromide staining of RT-PCR products. The samples which were analyzed and compared to each other (control and IFN-γ treated samples) were loaded on the same gel following the same settings of the image analyses. **(B)** Graphic presentation of the data shown in **(A)** obtained by the Image Master VDS. Log OD of amplified PCR products for APN and both housekeeping genes, ABL and RPLP0, was plotted against the number of PCR cycles. The lines were drawn from linear regression analysis of the data points. Relative number of cDNA copies was derived from the intercept of the regression line with the Y-axis. **(C)** Graphic presentation of the data shown in **(A)** and **(B)** obtained after 26 cycles (ABL and APN) and 21 cycles (RPLP0). Data are expressed as percentages of the control (cells cultured in medium, designated as 100 %). Each experiment was performed twice. Data of one of two experiments with similar results are presented

(*5,10*). Reviewing literature, there are no general rules for the construction of competitor molecules (*4,5,9*). Requirements for a good competitor are: (*i*) competitor must be amplified with the same efficiency as the target gene, (*ii*) the competitor should have the same primer binding sites as the target gene and (*iii*) the two amplified products of the target and competitor should be easily distinguished. Thus, the competitor can be engineered to be slightly larger or smaller than the target or a unique restriction site can be added or removed from the target requiring an additional step, *i.e.* digestion with restriction enzyme before gel electrophoresis (*4,5,10*).

For that purpose we have constructed the APN competitor that contains the same primer binding sequences as the target gene, *i.e.* fragment of APN cDNA, but differs from it in size. An example of competitive PCR experiment using a cDNA synthesized from HL-60 and cloned APN competitor is shown in Fig. 3. Two-fold serial dilutions of the cloned APN competitor were co-amplified with a constant amount of cDNA synthesized from HL-60 cells. The products were resolved by agarose gel electrophoresis and stained with EtBr. The expected sizes of the amplified APN cDNA fragment and APN competitor products were 450 and 285 bp, respectively. As expected, the amount of APN competitor product decreased as the competitor number decreased, whereas the amount of APN cDNA fragment increased (Fig. 3).

Although the competitor shares the same primer binding sites as the target APN cDNA, the intervening

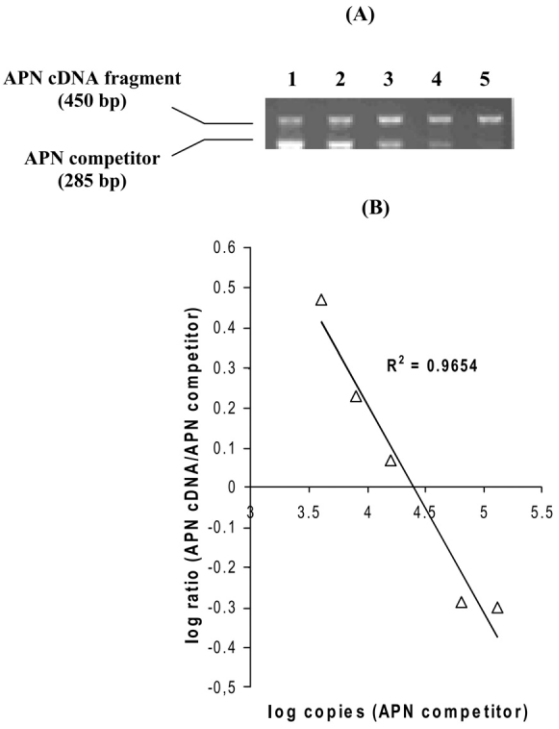

Fig. 3. Competitive RT-PCR analysis. 1 μg of total RNA isolated from HL-60 cells was used as a template for cDNA synthesis. Constant amount of cDNA was then amplified in competitive PCR in the presence of five two-fold serial dilutions of APN competitor. After 33 cycles PCR products were analyzed by 1.5 % gel electrophoresis following densitometric analysis of band intensity. **(A)** Ethidium bromide staining of RT-PCR products. Lanes 1-5: $128 \cdot 10^3$, $64 \cdot 10^3$, $32 \cdot 10^3$, $16 \cdot 10^3$ and $4 \cdot 10^3$ APN competitor, respectively. **(B)** Graphic presentation of the data shown in **(A)** obtained by the Image Master VDS. Due to the difference in size of amplified APN cDNA fragment and APN competitor, band intensities were corrected before the calculation of the ratio between them. Log of the ratio (APN cDNA/APN competitor) was plotted against the log of the copies (APN competitor). The lines were drawn from linear regression analysis of the data points. Number of APN cDNA copies was derived from the intercept of the regression line with the X-axis

DNA sequence differs, making it possible to amplify APN competitor and APN cDNA at different efficiencies. It is basic assumption of the competitive RT-PCR that the efficiency of amplification should be the same for the target of interest and competitor (*5,10*). In order to test whether amplification efficiency of APN cDNA fragment and APN competitor was identical, competitive PCR was performed using an APN cDNA and APN competitor at approximately equivalent concentration. The results of this experiment were shown in Fig. 4. Similar slopes of the lines drawn by linear regression analysis indicate that APN cDNA fragment and APN competitor have very similar amplification efficiencies. The ratios between amplified APN cDNA fragment and APN competitor remained constant in the range from 30–36 cycles (Fig. 4). Therefore, the APN competitor that we have constructed can be effectively used for measurement of relative amount of APN mRNA on IFN-γ treated HL-60 cells using competitive RT-PCR.

Results of competitive RT-PCR are shown in Fig. 5. The number of APN cDNA molecules in control and IFN-γ treated samples was derived from the intercept of the regression line with the X-axis. At this intercept the copy number of APN cDNA and APN competitor molecules are identical. The values obtained from plots for control and IFN-γ treated samples were $4.15 \cdot 10^3$ and $7.42 \cdot 10^3$ molecules, respectively, giving a change of approximately 1.8-fold (Fig. 5). This result is in agreement with that obtained by semi-quantitative RT-PCR, where 2-fold upregulation was obtained (Fig. 2).

One possible disadvantage of the use of DNA competitor in RT-PCR experiment is that there is no control of variations in the input of RNA amounts and the efficiency of the RT-step. One way to circumvent this problem is a construction of RNA competitor by *in vitro* transcription. Several reports have been published where RNA competitor was used for measurement of APN mRNA level in different cell types (*21–26)*. In our experiment, the correction of the inefficiencies in RNA input is performed by normalization to a housekeeping gene ABL using semi-quantitative RT-PCR, which shows that relative amount of ABL mRNA was very similar in control as well as in the IFN-γ treated samples (data not shown).

The principal advantage of using DNA competitor is that once it is constructed, by cloning the target PCR fragment into a suitable plasmid vector, it can be prepared in very large amount and stored for long periods at –20 °C without a risk of degradation. Therefore, numerous experiments can be prepared using the same dilutions of the competitor, minimizing inter-assay variations. In addition, any inaccuracies in the initial competitor yield determination will be consistent in all the experiments and will not affect quantification of relative changes in mRNA levels.

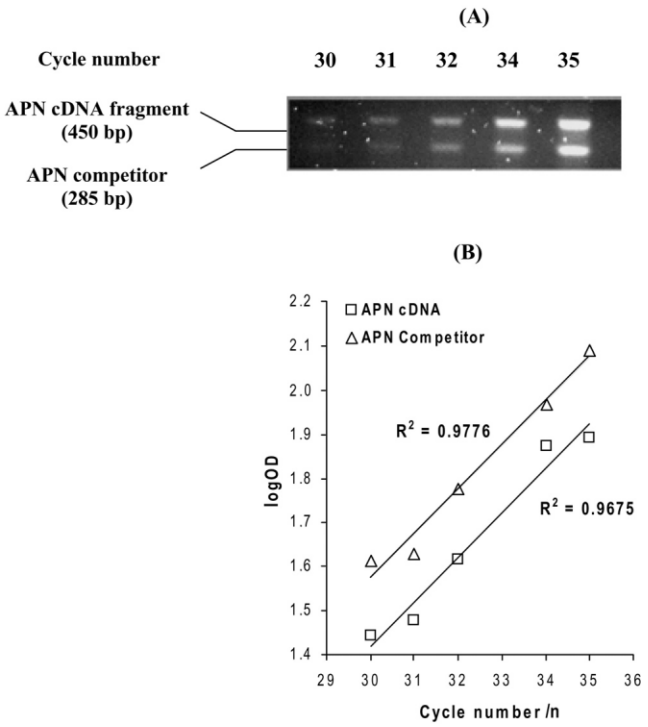

Fig. 4. Kinetics of amplification of the APN cDNA fragment and APN competitor. Competitive PCR was performed using cDNA from HL-60 cells and APN competitor synthesized as described in Material and Methods. Approximately equal molar quantity of APN cDNA fragment and APN competitor were added to a single PCR tube and competitive PCR was performed. After 30 to 35 cycles, amplified products were analyzed by 1.5 % gel-electrophoresis following densitometric analysis of band intensities. **(A)** Ethidium bromide staining of competitive PCR products. **(B)** Graphic presentation of the data shown in **(A)** obtained by the Image Master VDS. Due to the difference in size of amplified APN cDNA fragment and APN competitor, band intensities were corrected. Log of the OD of amplified PCR products (APN cDNA fragment and APN competitor) was plotted against the number of PCR cycles. The lines were drawn from linear regression analysis of the data points

By using DNA competitor, we were able to measure only a relative level of APN mRNA, because variation in efficiency at the RT-step was not controlled. Although competitive RT-PCR described here allows accurate relative quantification, the design and construction of competitor for each gene to be quantified is technically complex, labor intensive and time consuming. In addition, validation of amplification efficiency of target and competitor is necessary for the method to be reliable.

Real-time RT-PCR

Real-time RT-PCR has the ability to monitor the progress of PCR reaction as it occurs *(i.e.* in real time) and data are collected through the PCR process, rather than at the end of the PCR. Thus, real-time PCR allows detection of the accumulation of PCR products during the exponential phase of the PCR reaction (*1*,*2*,*11*,*13*).

Several chemistries are available for real-time monitoring of DNA amplification by fluorescence emission such as TaqMan probes, Molecular Beacons, Scorpions and SYBR Green I DNA binding dye (*2*,*11*,*27*). The primary advantage of TaqMan chemistry used in our study is that specific hybridization between probe and target resulted in generation of a specific fluorescence signal. The increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, nonspecific amplification is avoided (*2*,*12*). In this work we have used Assay-on-Demand Gene Expression Assay containing a mix of unlabeled PCR forward and reverse primers as well as TaqMan MGB probe. These assays are highly specific, designed to amplify target cDNA without amplifying the contaminating genomic DNA and can be run under identical conditions, saving researchers time needed to design and optimize PCR conditions (http://www.appliedbiosystems.com*).*

To quantify the results obtained by real-time RT-PCR, we have used the comparative Ct method, also known as $2^{-\Delta\Delta Ct}$, which is a convenient way to analyze the relative changes in gene expression (*20*,*27*,*28*). Comparative Ct method assumes that the amplification efficiency of the target gene, *i.e.* APN, and endogenous control, *i.e.* RPLP0, must be the same (*28*). One of the very important advantages of TaqMan Gene Expression Assays, which is used in our investigation, is that they have equal amplification efficiency (average 100 % efficiency; ±10 %) and it is not necessary to measure and compare their efficiency of amplification (http://www.appliedbiosystems.com).

Finally, we applied real-time RT-PCR for measuring the effect of IFN-γ on APN mRNA level on HL-60 cells. According to comparative Ct method, the Ct values of both the control and IFN-γ treated samples were normalized to housekeeping gene RPLP0 and relative differences in APN mRNA level were determined using the arithmetic formula $2^{-\Delta\Delta Ct}$. Results of comparative Ct methods of real-time RT-PCR showed that treatment with IFN-γ (6 ng/mL) for 72 h increased APN mRNA

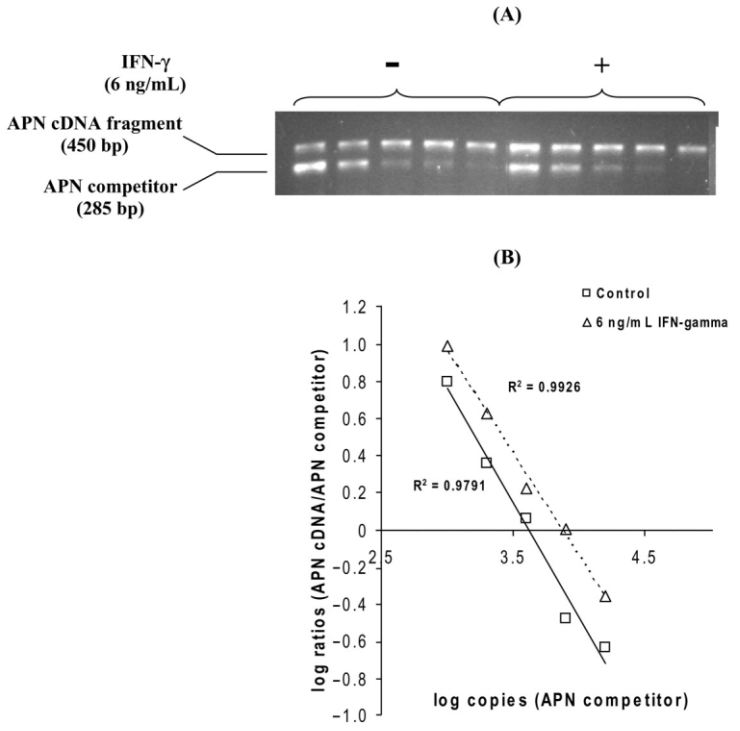

Fig. 5. Relative quantification of APN mRNA of IFN-γ treated HL-60 cells by competitive RT-PCR. HL-60 cells were cultured for 72 h with IFN-γ (6 ng/mL). Control (untreated) cells were incubated in the medium alone (without IFN-γ). At the end of incubation the cells were washed, total cellular RNA was isolated and competitive RT-PCR was performed. **(A)** Ethidium bromide staining of RT-PCR products. **(B)** Graphic presentation of the data shown in **(A)** obtained by the Image Master VDS. Due to the difference in size of amplified APN cDNA fragment and APN competitor, band intensities were corrected before the calculation of ratio between them. Log of the ratio (APN cDNA/ APN competitor) was plotted against log of the copies (APN competitor). The lines were drawn from linear regression analysis of the data points. Number of APN cDNA copies was derived from the intercept of the regression line with the X-axis. Each experiment was performed twice. Data of one of two experiments with similar results were presented

level on HL-60 cells approximately twofold (Table 3). Similar results were obtained with semi-quantitative and competitive RT-PCR as well (Figs. 2 and 5).

Since real-time RT-PCR method combines PCR amplification and product detection in one single step, it does not require laborious post-PCR analysis. Moreover, this method incorporates specialized software to simplify data analysis, which further reduces labor and time.

In each quantitative RT-PCR based methods, specific errors will be introduced due to minor differences in the starting amount of RNA or differences in efficiency of cDNA synthesis and PCR reaction. This problem is inherent to all quantitative RT-PCR-based methods including semi-quantitative, competitive and real-time RT-PCR, respectively. Therefore, a reliable quantitative RT-PCR method requires normalization for these experimental variations by using an endogenous control (*1,2, 7,8,29*). Housekeeping genes, which are expected to be expressed at a constant level and should not be affected by experimental conditions, are most often used as control for variation of input RNA and an efficiency of reverse transcription. Unfortunately, until now, an »ideal« housekeeping gene has not been found. Therefore, normalization to different housekeeping genes has been proposed (*1,2,6–8,30*). For determination of most stable housekeeping gene, several mathematical algorithms have been developed as software applications such as BestKeeper©, REST© and GeNorm (*31–32*). Two housekeeping genes, RPLP0 and ABL, were chosen in our study because they had been used in previous gene expression studies (*17,33*).

Table 3. Relative quantification of APN mRNA of IFN-γ treated HL-60 cells by real-time RT-PCR

γ(IFN-γ) ng/mL	APN Average Ct±SD	RPLP0 Average Ct±SD	ΔCt±SD	ΔΔCt	APN Fold change Relative to control
0	21.47±0.722	17.21±0.280	4.257±0.774	0	1.00 (0.58–1.71)
6	20.84±0.327	17.68±0.272	3.163±0.425	–1.094	2.13 (1.59–2.87)

HL-60 cells were cultured for 72 h with IFN-γ (6 ng/mL). Control (untreated) cells were incubated in the medium alone (without IFN-γ). At the end of incubation the cells were washed, total cellular RNA was isolated and real-time RT-PCR was performed. Relative amount of APN mRNA was calculated using the comparative Ct method

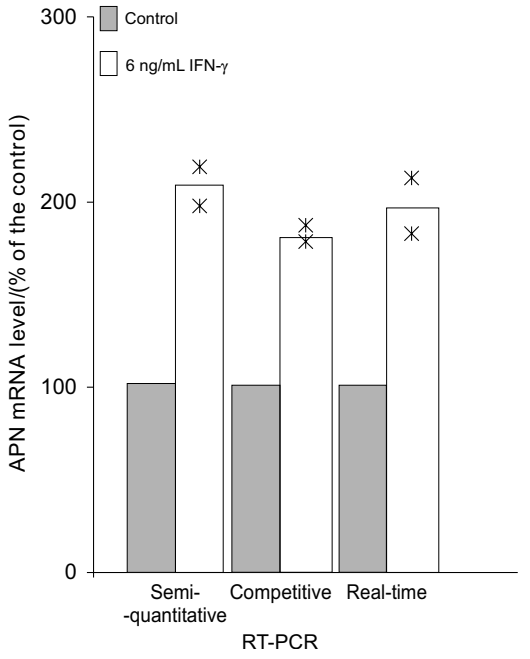

Fig. 6. Comparison of three RT-PCR-based methods for relative quantification of APN mRNA on IFN-γ treated HL-60 cells. HL-60 cells were cultured for 72 h with IFN-γ (6 ng/mL). Control (untreated) cells were incubated in the medium alone (without IFN-γ). At the end of incubation the cells were washed, total cellular RNA was isolated and semi-quantitative, competitive, and real-time RT-PCR were performed. Two independent experiments were done for each of three RT-PCR based methods. Individual values for each experiment were shown. The data are expressed as percentages of the control (cells cultured in medium, designated as 100 %)

The goal of quantitative RT-PCR is to determine the amount of RT-PCR product. For the measurement of RT-PCR products by either semi-quantitative or competitive RT-PCR, we used agarose gel electrophoresis and subsequent EtBr-staining. The major drawback of this detection system is the use of hazardous chemical EtBr. In addition, results should be transformed into numbers by image analysis, which is time consuming, labor intensive and depends on the software quality. Compared to semi-quantitative and competitive RT-PCR, referred to as end-point RT-PCR, real-time RT-PCR does not require post-PCR methods and thus is much faster and easier to perform.

Fig. 6 summarizes results obtained by three different RT-PCR based methods used to determine the relative amounts of APN mRNA on HL-60 cells treated with IFN-γ (6 ng/mL) for 72 h. Comparable results were obtained with semi-quantitative, competitive and real-time RT-PCR, *i.e.* approximately twofold increase of APN mRNA was found applying IFN-γ treatment. Thus, by using two additional RT-PCR based methods, such as competitive and real-time RT-PCR, we confirmed the twofold increase of APN mRNA on IFN-γ treated HL-60 cells for 3 days, which was determined by semi-quantitative RT-PCR (*14*).

In summary, the data presented in this study have shown that all three RT-PCR-based methods, semi-quantitative, competitive and real-time RT-PCR, can be used for relative quantification of mRNA for APN on promyeloid HL-60 cell line upon stimulation with IFN-γ. Thus, in spite of rapid advances made in the area of real-time RT-PCR, end-point RT-PCR such as competitive and semi-quantitative RT-PCR, although laborious and time consuming, may still remain useful techniques for relative mRNA quantification when small number of samples are to be analyzed.

Acknowledgments

This work was funded by grant No. 0098094 from the Croatian Ministry of Science, Education and Sport.

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Usporedba triju RT-PCR metoda za relativnu kvantifikaciju mRNA

Sažetak

U ovom su radu prikazane i uspoređene tri RT-PCR metode za relativnu kvantifikaciju mRNA: semi-kvantitativni, kompetitivni i »real-time« RT-PCR. Kao pokusni model upotrijebljena je promijeloidna stanična linija HL-60 koja eksprimira aminopeptidazu N. Stanice HL-60 linije inkubirane su s IFN-γ (6 ng/mL) tijekom 72 h pri 37 °C; izolirana je ukupna stanična RNA, reverznom transkripcijom sintetizirana je cDNA, a relativna razina mRNA za aminopeptidazu N mjerena je semi-kvantitativnim, kompetitivnim i »real-time« RT-PCR. Rezultati istraživanja pokazali su da sve tri RT-PCR metode daju pouzdane i usporedive rezultate, tj. uočena je dva puta veća količina mRNA za aminopeptidazu N u HL-60 stanicama stimuliranim s IFN-γ. Prema tome, unatoč brzom razvoju tehnologije »real-time« RT-PCR, klasični RT-PCR, kao što su kompetitivni i semi-kvantitativni RT-PCR, iako zahtjevni i dugotrajni, ipak mogu poslužiti za određivanje relativne količine mRNA na malom broju uzoraka.