QUANTITATION OF mRNA BY COMPETITIVE RT-PCR AND SILVER STAINING OF POLYACRYLAMIDE GELS

SALMAN A. H. ALROKAYAN*

SUMMARY: We present here the development of a rapid, simple and reliable nonradioactive method for the quantification of mRNA by competitive reverse transcription polymerase chain reaction (cRT-PCR) and silver staining after the polyacrylamide gel electrophoresis (PAGE). This technique does not require any labeling or blotting procedures. In this method, the RNA is reverse transcribed in the presence of an internal cellular RNA standard (cRNA) and amplified by the PCR using specific set of primers. The silver staining and scanning densitometry of the polyacrylamide gel allows the detection and quantitation of the target sequence. The quantitation of the target mRNA is performed by comparison with the cRNA and expressed per µg of total cellular RNA (TC-RNA). This technique was used to study the platelet derived growth factor-A (PDGF-A) mRNA levels in the peripheral blood mononuclear cells (PBMN) from adult healthy human subjects (n = 10) as a measure of gene expression. We demostrate that use of cRT-PCR and silver staining provides sensitive and reproducible quantitation linear with the amount of PDGF-A mRNA in the sample. This simple method can be adapted to study the expression of any cellular or viral gene of known sequence and no radioactive substance or blotting method is required to quantify PCR products.

Key Words: PDGF-A mRNA, gene expression, silver staining, mRNA quantitation, cRT-PCR, PAGE.

INTRODUCTION

The responses of biological systems to various stimuli which influence mRNA levels can be monitored by reverse transcription of mRNA into cDNA and amplification by polymerase chain reaction (RT-PCR), several researchers have used this approach to study the gene expression in various studies (1-4) and to monitor the levels of platelet derived growth factor (PDGF) mRNA (5). Recently, real-time RT-PCR has also been used for the quantitation of specific mRNA species (6-7). Amplification and detection of rare mRNA transcripts by RT-PCR is routinely carried out in several moderately equipped molecular biology laboratories. Since PCR amplification is an exponential process, minor differences in any of the variables that control the reaction rate could lead to large differences in the yield of PCR product and that is why quantitation of mRNA present in the starting material is still complicated, labor intensive and costly. One way to diminish the influence of variable is to co-amplify an internal standard in the same reaction tube together with target template known as competitive RT-PCR (8). The ratio of target to competitor product is unaffected during

* From Department of Biochemistry, College of Science, King Saud University, Kingdom of Saudi Arabia.

amplification in the cRT-PCR, therefore, it is possible to determine precisely the starting target template concentration in the sample.

The most widely used techniques for the quantitation of mRNA include ethidium bromide stained agarose gel analysis, Southern transfer with subsequent hybridization of specific radioactive or non-radioactive probes and detection by densitometry, competitive PCR using non-homologous competitors containing a shifted restriction site (9-14) and real-time RT-PCR (6-7). All these methods require special and expensive equipment and chemicals that are not always available in most of the laboratories. Although the analysis of PCR products by agarose gel electrophoresis followed by staining with ethidium bromide is quick and the DNA products are readily recovered for subsequent analysis, however, this method is not ideal for quantitation of gene expression. In particular, separation on agarose gel lacks resolution, detection of very small quantities of DNA is neither possible nor the end product is permanent. In this report, we describe the development of a simple, rapid, non-radioactive and inexpensive method for the quantitation of PDGF-A mRNA as an example from peripheral blood mononuclear cells (PBMN) by cRT-PCR, silver staining and scanning densitometry of the polyacrylamide gels. This method can be applied to any newly discovered cellular or viral gene in a matter of days.

MATERIALS AND METHODS

Isolation of total cellular RNA

The peripheral blood mononuclear cells (PBMN) from 10 healthy adult human subjects were isolated by adding 5 ml of EDTA blood on the Ficoll-Paque (Pharmacia Biotech), and centrifugation at 1000 rpm for 20 min at 4°C. The RNA was isolated from these PBMN cells by the method of Chomezynski and Sacchi (15). The pellet was homogenized with 1 ml of solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate pH 5.2, 0.5% *N*-lauryl sarcosine, 0.1 M DTT and 0.1 M 2-mercaptoethanol). Sequentially, 0.3 ml of 2 M sodium acetate (pH 4.0), 3 ml water saturated phenol (pH 4.0) and 0.6 ml of choloroform-isoamyl alcohol (49:1) were added to the homogenized pellet. The aqueous phase was separated by centrifugation at 4.000 g for 20 min at 4°C, precipitated with 0.6 volume of isopropanol and washed in 75% ethanol. Finally, RNA was dissolved in 20 μl DEPC (diethyl-pyrocarbonate) treated sterile deionized (DI) H_2O and stored at -70°C until analyzed. RNA concentration was measured spectrophotometrically at 260 nm by diluting 1 μl of RNA in 249 μl of sterile DI H_2O using GeneQuant-II (Pharmacia Biotech). All RNA preparations had an A_{260} / A_{280} ratio of >1.8 and showed clear undegraded bands of RNA on 1.5% agarose gel electrophoresis in the presence of formaldehyde.

cDNA synthesis

Reverse transcription was carried out by using GeneAmp ®RNA PCR kit (Perkin Elmer). Briefly, 4 μl of 25 mM $MgCl_2$, 2 μl of 10 X PCR buffer II (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 2 μl of each 10 mM dNTP (dGTP, dATP, dTTP and dCTP), 1 μl of 20 U/μl RNAse inhibitor, 1 μl of 50 U/μl MuLV Reverse Transcriptase, 1 μl of 50 μM random hexamer, 1 μl (5-25 ng) of TC-RNA, 1 μl (10^6 copies) of pAW109 RNA were added in a 0.2 ml PCR tube, volume was made up to 20 μl with DEPC treated DI H_2O and incubated at room temperature for 10 min for the extension of the hexameric primers. Tubes were then incubated for reverse transcription reaction using a thermal cycler (Techne GENIUS, UK) at 42°C for 15 min, denatured at 99°C for 5 min and cooled at 5°C for 5 min.

Polymerase chain reaction (PCR)

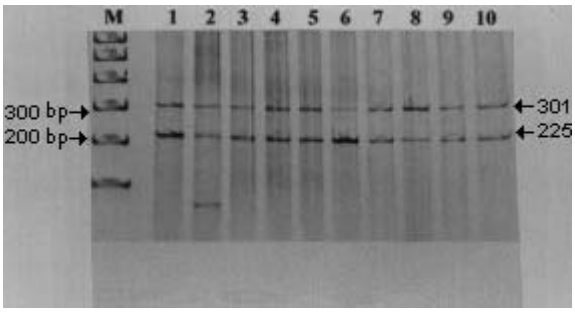
For PCR amplification, 10 μl of cDNA synthesized by reverse transcription was used with 2 μl of 25 mM $MgCl_2$, 4 μl of 10 X PCR buffer II, 0.5 μl of 15 μM PDGF-A forward primer (AW116, 5`-CTGCCATTCGGAGGAAGAG-3`), 0.5 μl of 15 μM reverse primer (AW117, 5`-TTGGCCACCTTGACGCTGCG-3`), and 0.25 μl of 5 U/μl AmpliTaq ®DNA polymerase. The PCR reaction was carried out in 200 μl thin walled tubes in a final volume of 50 μl. The reaction was started with incubation at 95 °C for 5 min. The amplification profile involve 25 rounds of denaturation at 94°C for 30 s, primer annealing at 60°C for 45 s, and extension at 72°C for 90 s in a thermal cycler. An additional extension step at 72°C for 7 min was also included at the end of the thermal profile and the reaction tubes were held at 4°C.

Polyacrylamide gel electrophoresis (PAGE), silver staining and quantitative analysis

For analysis of amplicons, the PCR products were electrophoresed in a Genephor apparatus (Pharmacia Biotech) using ready made 10% polyacrylamide gels (Pharmacia Biotech) at 150 V, 25 mA and 15 W for 2 hours in 0.5 X Tris-phosphate buffer to allow adequate resolution of the target and synthetic internal control amplification product. The gel was silver stained by DNA silver staining kit using Hoefer automated gel stainer (Pharmacia Biotech) or according to a previously described manual procedure (15).

Each pair of signals on silver stained PAGE corresponding to the target template mRNA (225 bp) and the cRNA (301 bp) was analyzed by scanning densitometry (Hewlett Packard) and quantitated by IQ software (MD ImageQuant Software version 3.22). Quantitation of the target mRNA was performed by comparison with the cRNA internal standard and

Figure 1: Representative silver stained 10% polyacrylamide gel electrophoresis (PAGE) to quantitate PDGF-A mRNA levels in PBMN derived from adult healthy human subjects (n=10). The isolated RNA was reverse transcribed into cDNA in the presence of 10^6 copies of AW109 cNA and subjected to 25 cycles of PCR amplification using PDGF-A specific primers. PCR products were resolved by PAGE and silver stainig as described in the methods and quantitated by scanning using IQ software. The upper band (301 bp) and lower band (225 bp) in lanes 1-10 correspond to standard AW109 cRNA and PDGF-A specific mRNA in 10 different samples from healthy subjects respectively (M=100 bp ladder).



expressed per mg of total cellular RNA which leads to linear relationship between RNA concentration and mRNA copy number. Integration of peak areas was calculated using the IQ software. The concentration of PDGF-A mRNA was calculated from the relative sample and control peak areas and the known number of molecules of cRNA added to the PCR reaction as described by Powell and Kroon (8):

$$\text{mRNA molecules/}\mu\text{g RNA} = \frac{\text{R x (copies of cRNA added)}}{(\mu\text{g of human RNA added})}$$

RESULTS AND DISCUSSION

To quantify the expression of PDGF-A mRNA by cRT-PCR, a small synthetic RNA (AW109) was used as an internal standard (cRNA). The pAW109 cRNA contains synthetic PCR primer sites for PDGF-A and a number of other genes thus serves as quantitative internal standard. The PCR primers used are RNA specific i.e. span exons only and therefore do not amplify the genomic DNA (8). The cRT-PCR amplified products i.e. internal control and template mRNA differ in size (301bp and 225 bp respectively) and allow resolution on agarose or polyacrylamide gel electrophoresis. Competitive RT-PCR products were detected by PAGE and silver staining. This represents an improvement over a previously published approach where a small synthetic RNA was used as an internal standard and detection system was based on radioactively labeled nucleotides that were incorporated during the amplification step (8). In the present study, blotting and radiation based detection system was replaced with silver staining, scanning densitometry and quantitation using computer software. Figure 1 shows the analysis of cRT-PCR products on PAGE after silver staining. The technique was used for the quantification of the PGDF-A mRNA recovered from PBMN of 10 healthy adult human subjects. The upper band (301 bp) represents the standard AW109 internal control product and lower band (225 bp) corresponds to the target PDGF-A mRNA template in the sample. The equivalence point, which is characterized by equal intensities of bands for internal standard, cellular RNA and marker defines the amount of specific RNA in the starting material, can be reliably detected by the eye. In addition, densitometric scanning of PAGE was used to accurately quantify smaller differences in the expression of specific mRNA. The average PDGF-mRNA copies or number of molecules/mg of cellular RNA are $29.29 \pm 4.83 \times 10^4$ (values are mean±SD) and the results are in line with the previous studies (8-10) where gene expression was measured using blotting procedures and radioactive or non-radioactive labeling.

The method described here, provides several advantages over other conventional methods which require much larger amounts of total cellular RNA, consume more time and do not give precise quantitation of mRNA species (11).

The method presented here allows direct and quick results that reflect the amount of PCR products at different stages of amplification, thus the amount of products obtained could be quantified during the exponential phase and quantitative PCR performed with much ease and accuracy. For instance the Northern blot technique requires at least 10 mg of RNA per lane, which is very difficult to obtain. By contrast, the present assay is both sensitive and quantitative enough to study the gene expression of mRNA species like PDGF-A present in small quantity recovered from small samples.

In conclusion, the method presented for the detection and quantitation of PDGF-A mRNA by cRT-PCR and silver staining of polyacrylamide gel is quick, inexpensive, involves less labor, uses only commercially available reagents and does not require any mutagenic/carcinogenic substance like ethidium bromide or radioactive compound. This method can be adapted to quantify any cellular or viral mRNA with known sequence within few days and automated without much difficulty.

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Correspondence:
Salman A. H. Alrokayan
Department of Biochemistry,
College of Science,
King Saud University,
P.O. Box 2455, Riyadh 11451,
KINGDOM OF SAUDI ARABIA.
e-mails: alrokyan@kacst.edu.sa
srokyan@ksu.edu.sa