Quantitative determination of gene expression in human lymphocytes assessed by reverse transcription–polymerase chain reaction coupled to high-performance liquid chromatography

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Abstract

Gene expression in human lymphocytes was assessed using reverse transcription and polymerase chain reaction amplification followed by ion-pair reversed-phase chromatography analysis. Competitive PCR was used to quantitate the desired cDNAs with a polivalent competitor adaptable to multiple novel mRNAs estimations with minor changes. Accuracy was $11.27 \pm 11.87\%$ ($n=7$), as determined using standards. The coefficients of variation of the assessment of human OK12b were 7% ($n=6$), 7.68 attmol/μg of total RNA, and 21% ($n=6$), 0.93 attmol/μg of total RNA. Sample-to-sample variation in the reverse transcription and in the quantity and quality of RNA was attenuated by normalising results to beta-actin mRNA expression. The correlation between the OK12b/β-actin ratio and competitive assessments of OK12b was 0.984, $n=6$. The correlation between HPLC results and an independent method based on radionuclide uptake by the product, detected by electrophoretic separation, was 0.848, $n=10$. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Quantitative analysis of mRNAs is becoming widely used in many experimental studies and clinical conditions. Friedhoff et al. (1995, unpublished observations) reported differential expression of novel gene fragments derived from lymphocytes of a pair of twins discordant for schizophrenia. These novel genes were expressed in lymphocytes of the

healthy twin but not in the lymphocytes of the schizophrenic twin and one of the genes has been detected in rat brain [1].

The objective of the present study was to develop a quantitative method for the study of these novel genes in lymphocytes in normal controls and patients with schizophrenia. A traditional approach, RNAse protection assay (RPA) was used by Friedhoff et al. (1995, unpublished observations) to confirm differential expression of the novel genes in a second sample of lymphocytes from the same pair of discordant twins 2 years later. As the genes appear to be detectable only in the non-schizophrenic twin,

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assessment of their expression in lymphocytes derived from other sample populations with schizophrenia requires a sensitive and quantitative method superior to that of conventional methods of mRNA measurement, such as Northern blots and hybridisation protection assays. Although ribonuclease-protected assays based on RNA hybridization are more sensitive than Northern assay, these assays still have some limitations. Highly specific activity probes labeled with ^{32}P must be used; a large amount of RNA is needed (25–50 μg) compared with RT-PCR (1.35 μg); the technique is labor-intensive, time-consuming, and requires skilled staff; and RNA degrades easily during the process. When we developed this technique in our laboratory for the analysis of OK12b RNA, the level of detection was 0.1 fmol. RPA lacks the sensitivity required to detect mRNA expressed at low levels. This is of particular importance for clinical studies in which gene expression is expected to be absent or at very low levels.

For this reason, the extremely sensitive RT-PCR has become the methodology more widely used for the examination of gene expression. This measurement has universal applicability: in clinical practice, quantitative or semi-quantitative RT-PCR is becoming the method of choice for monitoring certain diseases at different stages [2,3]; or for predicting treatment response during acute and chronic infection [4]. Moreover, the RT-PCR approach is ideal for certain clinical studies, where peripheral blood specimens may be the only practical tissue source and where the availability of tissue samples is limited. For our study we desired a method that could be conducted on a large number of samples and would allow sampling of different genes from the same lymphocyte RNA sample therefore we have established a two-step RT-PCR method. Although this method is an easy-to-use qualitative assay, quantitative determination of mRNA is associated with many difficulties due to the variability of the reverse transcription step, difficulties that increase exponentially during PCR. Amplification efficiency varies. Consequently, the method has gained a reputation for poor reproducibility, leading to concern over the validity of much of the data generated. We addressed this problem of poor reproducibility using combined relative PCR with competitive PCR. Relative PCR includes co-amplification with two sets of primers of

an endogenous beta-actin sequence and the target gene to be analysed. This permits corrections for variability in RNA quality, RNA quantification, and differences in the efficiency of the RT reaction, thus normalising cDNA input.

To assess the cDNA of interest, we used competitive PCR. Competitive PCR, as originally described [5,6], involves the addition of known amounts of a serially diluted exogenous competitor template to a fixed endogenous target cDNA, both with identical primer-binding sites. This method controls differences in PCR-reaction efficiencies and circumvents the high number of variables that affect the yield of PCR products. Both relative PCR and competitive PCR require a good and reliable method for the analysis and quantification of products.

We compared two different methods for the analysis of the PCR products of OK12b, one of the novel genes whose differential expression in lymphocytes of the twin pair discordant for schizophrenia was inconclusive by a second RPA analysis (Friedhoff, unpublished data). One method involved DNA amplification in the presence of a radiolabeled nucleotide and subsequent gel electrophoresis. Reaction products were quantified by excising the bands and counting the radioactivity [7]. As an alternative, we used ion-pair reversed-phase chromatography (IP-RP-HPLC) [8]. PCR products were separated and quantified by direct injection into the chromatographic system.

Although HPLC is a very important method for separation of PCR products and data acquisition, other alternatives are available for quantification. Recent developments in fluorescence energy-transfer systems allow quantitative real-time detection of the kinetics of product accumulation. Using this technique, the target sequence can be quantified during the extension phase of PCR amplification, in contrast to end-point quantitative methods.

Even though automation of real-time PCR techniques has some technical advantages and its use is extending widely, HPLC analysis of PCR products is still a solid and versatile tool and the necessary instruments are often available in analytical laboratories, so costly technology can be avoided. Furthermore it is a valuable tool for establishing conditions for the RT-PCR process. Another advantage is that it allows simultaneous isolation and

purification of PCR products for subsequent sequence analysis [9]

2. Experimental

2.1. RNA preparation and cDNA synthesis

Blood lymphocytes were separated on FicollPaque gradient (Amersham Pharmacia Biotech, Uppsala, Sweden). Total RNA was immediately isolated following the method of Chomczynsk and Sacchi [10]. RNAs were incubated with RNAse-free DNAse (EC 3.1.21.1, Promega, Madison, WI, USA) for 30 min at 37°C to eliminate DNA contamination, extracted with acid-equilibrated phenol/chloroform, then precipitated with 0.5 M NH_4Ac and EtOH. The RNA pellets were resuspended in nuclease-free water. The amount of RNA was determined spectrophotometrically and its integrity was confirmed in a denaturing agarose-gel electrophoresis run on MOPS buffer. Samples were stored at -80°C in DEPC-treated water until use.

For cDNA synthesis, 1 μg of total RNA was mixed with 0.5 μg of oligo-dT(15), incubated in a volume of 15 μl at 72°C for 5 min and rapidly cooled to 5°C. This nonspecific primer protocol permits storage of more stable cDNA samples for a number of different PCR reactions and reduces to some degree the differences in efficiencies of the RT reaction resulting from separate RT reactions conducted with gene-specific primers. Reverse transcription was performed at 37°C for 1 h, in a total volume of 20 μl containing: 50 U of Mu-MLV reverse transcriptase (EC 2.7.7.49, Promega, Madison MA, USA), 10 U of placental Rnase inhibitor, 4×0.5 mM dNTPs, 4 μl of 5× transcription buffer and the 15 μl of primed RNA. The RT reaction was followed by 5 min at 95°C and rapidly cooled to 5°C. Single-stranded cDNA was synthesized from 2–5 μg of total lymphocyte RNA. Aliquots of the RT reactions were stored at -20°C.

2.2. Primer sequences

The OK12b gene fragment, 201 bp in length, was cloned in a pcDNAII construct (In Vitrogen, Groningen, The Netherlands). The Primer Premier pro-

gram (PREMIER Biosoft International, Palo Alto, CA, USA) was used to design primers for PCR amplification. For OK12b the sense primer sequence was a 22-mer: 5′-GCTTTATCAGGCCAGGCACA-GT, the antisense primer was a 22-mer: 5′-CCACC-ACCAATGGCTAAGTTTT. These primers yielded a 160-bp PCR product. PCR amplification of the beta-actin cDNA with sense primer [5′-AAGAGAG-GCATCCTCACCCT] and antisense primer [5′-TACATGGCTGGGGTGTTGAA] yielded a 218-bp PCR product.

A pair of composite primers was designed to generate a nonhomologous competitor DNA fragment. These primers contained the primers for the target sequence OK12b pasted to the β-actin primers. The sense primer was: 5′-GCTTTATCAGGCCAG-GCACAGTAAGAGAGGCATCCTCACCCT, and the antisense primer was: 5′-CCACCACCAATG-GCT AAGTTTTT ACA TGG CTGG GGTGT TGAA. A 262-bp PCR product was obtained. All primers were synthesized by Amersham Pharmacia Biotech, Uppsala, Sweden.

2.3. Production of the competitor standard

The competitor standard was generated by two successive PCR amplifications. In the first PCR reaction, a DNA fragment was prepared by amplifying human genomic cDNA with low annealing stringency using the pair of composite primers for OK12b. This PCR product was diluted and used to perform a second PCR amplification with primers for the OK12b and [^{32}P]dATP. The radiolabeled 264-bp fragment generated was purified in a low melting agarose gel, extracted with phenol/chloroform, and precipitated. The amount of product was quantified by counting the radioactivity incorporated. The competitor was stored at -20°C in aliquots ng/μl.

2.4. PCR conditions

The PCR reaction was performed in a 50-μl volume containing 1×PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl (pH 8.8 at 25°C, 0.01% Tween-20), 1.5 mM $MgCl_2$, 100 μM each dNTPs, 1.25 U BioTaq DNA polymerase (source Termus aquaticus YT-1, Bioline, Kenilworth, NJ, USA), 15 pmol of each OK12b primer, and 3.75

pmol of each beta-actin primer. This solution was placed in 0.5-ml thin-wall tubes, which were introduced into a DNA Thermal Cycler (PE Biosystems, Foster City, CA, USA).

The PCR conditions involved initial denaturation at 95°C for 3 min followed by 25 cycles of denaturation at 95°C for 1 min, annealing at 62°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 7 min.

The reaction mixtures for reverse transcription and PCR were prepared as master mixtures to enhance accuracy.

The absence of contaminants was routinely checked by RT-PCR assays of negative control samples in which RNA samples were replaced with sterile water.

2.5. Analysis of PCR products

2.5.1. HPLC

The HPLC system was composed of a 600E multisolvent delivery system, a 717 plus Autosampler, a 2487 Dual lambda absorbance detector, and Millenium system-controller software, all obtained from Waters (Milford, MA, USA). A non-porous polymeric column ZORBAX Eclipse dsDNA 4.6 mm×75 mm, 3.5 μm particle size and an analytical Guard column from Agilent Technologies (Palo Alto, CA, USA) were used.

2.5.1.1. Mobile phase, stock solution. A 1 M solution of triethylammonium acetate (TEAA) was prepared from triethylamine (Romil, Cambridge, UK) and titrated to pH 7.0 with glacial acetic acid (Merck, Darmstadt, Germany); EDTA 1 mM was added to reduce the free concentration of polyvalent metal cations in solution. High purity water was used for buffer preparation.

Eluent A was prepared by diluting the stock solution to 0.1 M TEAA, 0.1 mM EDTA, pH 7.0.

Eluent B was 25% acetonitrile in 0.1 M TEAA, 0.1 mM EDTA, pH 7.0.

The gradient profile used for elution started with 40% eluent B which was increased linearly to 80% over 30 min followed by 100% A for 10 min of column conditioning. The flow-rate was 1 ml/min.

All samples were injected without further processing and run at room temperature.

2.5.2. Gel electrophoresis

Some of the samples were also analyzed by gel electrophoresis. In these samples PCR was performed in the presence of 170 kBq of [^{32}P]dATP. Twenty microliters of each PCR was electrophoresed on 1.5% agarose gel in 0.5×TBE buffer. Gels were stained with ethidium bromide, the appropriate bands were cut out of the gel, and radioactivity was measured in a scintillation counter.

2.5.3. Relative PCR

Various amounts of cDNA corresponding to RNA inputs of 5, 10, 20, 40 and 60 ng were amplified for 25 cycles using different primers for β-actin and OK12b. The concentration of β-actin primers was reduced by four. In these conditions, PCR was carried out in the exponential phase, yielding similar efficiencies for the OK12b and the β-actin products.

2.5.4. Competitive PCR

Different amounts of competitor DNA, ranging from 0.03 to 3 attomol, $n=5$, were added to a constant amount of cDNA (300 ng); in these tubes, OK12b and the competitor were amplified with the same set of primers. Products were analysed by HPLC and gel electrophoresis in some cases.

2.5.5. Quantification of PCR products

The peak areas of template and competitor were reported by the Millenium (Waters, Milford, MA, USA) software. The ratio of template area to competitor area was plotted against the amount of competitor added and expressed in attomol on a logarithmic scale. The point where this ratio is equal to $\log_{10}$(160 bp/262 bp) indicates an equal number of target and competitor molecules. Results were expressed as attomol per μg of cDNA.

From each relative experiment, the regression line representing $\log_{10}$ of cDNA input versus $\log_{10}$ of the area generated by beta-actin was used to derive a correction factor expressed as β-actin area/μg cDNA. This factor was used to correct the absolute OK12b concentration obtained from competitive experiments; after this correction results were expressed in attomol OK12b per β-actin area.

OK12b was coamplified with β-actin and relative values of OK12b versus β-actin were obtained by calculating the ratios of both areas for the different

cDNA inputs ($n=6$). The average ratio was used as an index of the relative concentration of both products.

Experimental values were multiplied by two because the number of single-stranded cDNA was measured using a double-stranded DNA competitor.

3. Results

Total RNAs prepared from blood lymphocytes were quantified using a spectrophotometer and had a A_{260}/A_{280} ratio of 1.8–2.0; 10-15 µg of total RNA was obtained from 10 ml of peripheral blood.

3.1. HPLC

PCR products were analysed without further treatment. Fig. 1 illustrates the application of HPLC to the analysis of RT-PCR products in relative and competitive experiments. Our HPLC system could separate DNA fragments in the 15–750 bp range in a size-dependent manner in 35 min, as assessed using a PCR molecular-mass marker. The minimum size difference required for full separation of two peaks in the 50–300 bp range was 5–20 bp. The primers differed in two bases and gave two peaks in our HPLC conditions. The performance of the PCR and HPLC method could not be evaluated independently because the original DNA in the sample had to be amplified for detection. There were no interfering peaks in the competitive PCR chromatograms or relative RT-PCR chromatograms.

3.2. Competitive PCR

3.2.1. Experiments with standards
Increasing amounts of target standard and competitor were amplified, in separate tubes to avoid competition, in amounts ranging from 5 to 500 fg ($n=11$). The logarithm of the areas generated by the amplified products had a linear relationship with the logarithm of the initial amount of DNA. The decline in efficiency was equal for both target DNA and competitor (Fig. 2).

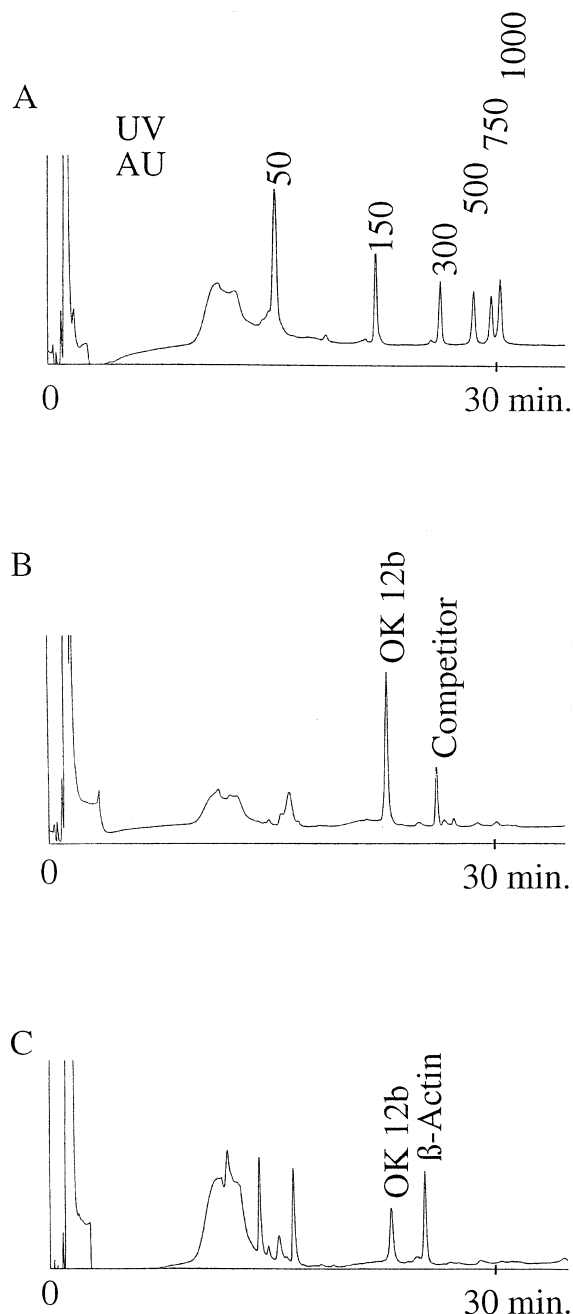The mean efficiencies in this titration experiment were 61.7% for OK12b and 67.3% for the competitor.

Fig. 1. HPLC analysis of RT-PCR products using an ion-pair reversed-phase column and separation by gradient elution; mobile phase: (A) 0.1 M TEAA, 0.1 mM EDTA, pH 7.0, (B) 0.1 M TEAA, 0.1 mM EDTA, 25% acetonitrile, pH 7.0; linear gradient 40–80% B in 30 min; flow-rate 1 ml/min at room temperature; UV detection at 254 nm. (a) PCR molecular mass marker, (b) competitive PCR products derived from 300 ng of total RNA and 0.03 attmol of competitor, (c) relative PCR products derived from 40 ng of total RNA.
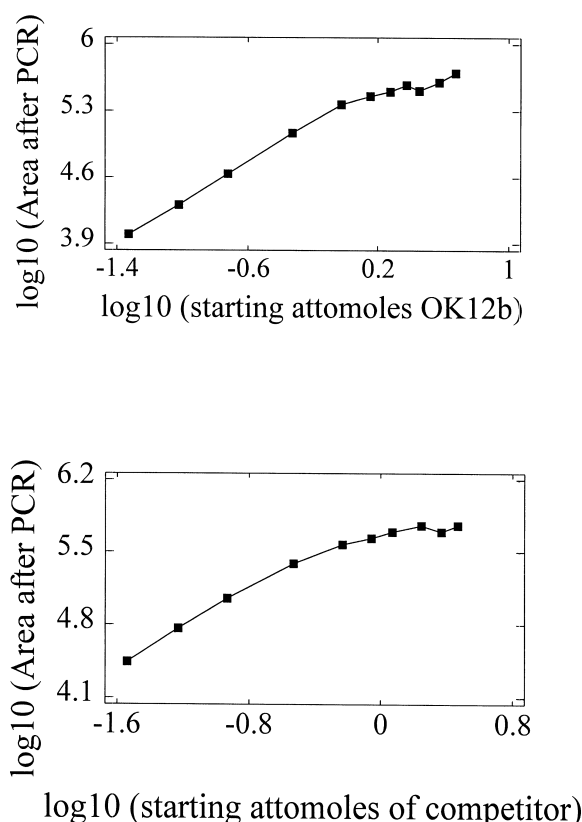
log10 (starting attomoles of competitor)

Fig. 2. Relationship between the logarithm of the amount of PCR product and the logarithm of the initial amount of sample DNA. Increasing amounts of target (OK 12b) and competitor were amplified and the products were analyzed by HPLC as described in the Experimental section. The logarithm of the areas (μV s) generated by the amplified products was plotted against the logarithm of the initial amounts of target and competitor.

As a result of these differences in efficiency, the slope of the regression line obtained from the competitive experiments was not the ideal value 1 but 0.84±0.11 (*n*=25).

3.2.2. Validation of competitive PCR

The accuracy of the competitive DNA assessment was evaluated by means of experiments performed with standards. Using seven different known amounts of target standard DNA, from 0.13 to 2.53 attomol, we performed a competitive experiment. Values obtained from these competitive assessments differed from predicted values by 11.27±11.87% (mean±SD) (Table 1).

Table 1
Relationship between known DNA input amounts and DNA calculated amounts

OK12b added (attomol)	OK12b observed (attomol)	Difference (%)
0.13	0.09	−30.8
0.32	0.24	−25.0
0.63	0.60	−4.8
0.95	0.94	−1.0
1.26	1.29	2.4
1.89	1.97	4.1
2.53	2.8	10.7

Competitive reactions performed using seven known starting amounts of target OK12b standard DNA. Each concentration was calculated from the regression lines obtained from each amount of input DNA standard. After HPLC analysis, log values of the ratio (OK12b area/competitor area) were plotted against log values of the competitor (attomol).

An inverse competitive experiment was carried out with variable amounts of target DNA versus a fixed amount of competitor (0.286 attomol). The observed concentration of competitor was 0.281 attomol, *n*=6 (CV: 1.8%) (observed, 0.281; predicted, 0.286 attomol).

3.3. Experiments with cDNA

Different starting amounts of the same cDNA (100, 200, 300 and 400 ng) were assessed for OK12b using four competitive PCR experiments on two independent RNA-derived cDNA samples. The resulting regression lines were parallel and the variability in attomol of OK12b measured was 20 and 18%, respectively (Table 2).

Reproducibility was assessed in two different cDNAs with different OK12b concentrations on six different days. Variability was highest when measuring a concentration of 0.93 attomol OK12b per μg of total RNA, coefficient of variation 21%, *n*=6. When assessing samples with a higher OK12b concentration, 7.68 attomol OK12b per μg of total RNA, the coefficient of variation was 7%, *n*=6. Slopes of all regression analyses were similar from day to day and on the same day between experiments: 0.84±0.11 (*n*=25).

Input RNA-derived cDNA can range from 100 to 400 ng improving sensitivity. In our conditions 0.05 attmol of OK12b cDNA can be assessed.

Table 2
Estimation of OK12b transcript using different cDNA starting amounts

	OK12b (attomol) observed in each experiment	OK12b (attomol) per µg cDNA
Ng cDNA RT101		
100	0.183	1.83
200	0.292	1.46
300	0.353	1.18
400	0.507	1.26
Ng cDNA RT156		
100	0.094	0.94
200	0.146	0.73
300	0.291	0.97
400	0.461	1.15

OK12b was analyzed by competitive PCR against 0.03, 0.12, 0.29, 0.58, 1.15 and 2.89 attomol of competitor using different amounts of cDNA input in two different RNAs, as described in Material and methods.

3.4. Relative RT-PCR

Results of kinetic studies (not shown) indicated that 25 cycles with an input RNA of 5 to 60 ng were
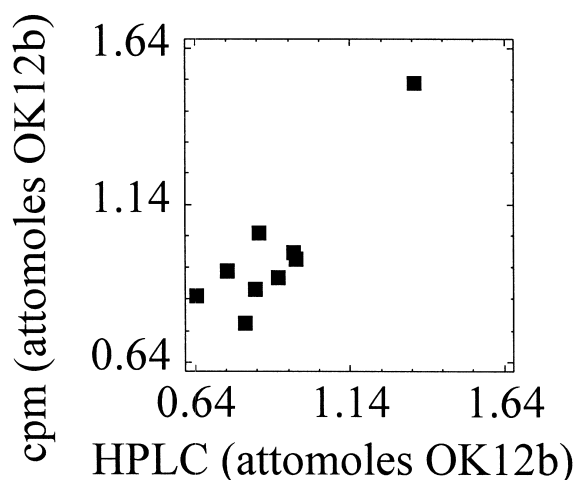

Fig. 3. Comparison of quantitative results obtained by two different methods. Ten different RNAs were assessed by RT-competitive PCR; concentrations were calculated from the regression lines generated by two different methods of analyzing PCR products: ion-pair reversed-phase chromatography and counting the radioactivity contained in PCR products separated by gel electrophoresis. Each point represents the result of an RT-competitive PCR experiment. A Pearson's correlation coefficient of 0.848 was obtained.

sufficient to observe a quantifiable signal of OK amplimer within the linear range of amplification.

There was a correlation between the absolute concentrations of OK12b obtained in competitive experiments and the ratio of the areas generated in relative experiments, $r = 0.952$ ($n = 6$).

The correlation between the OK12b/β-actin ratio and absolute OK12b concentrations, expressed as attomol OK12b/area β-actin, was stronger $r = 0.984$ ($n = 6$).

Comparison of quantitative results obtained by HPLC and by radioactivity methods yielded a correlation coefficient between systems of 0.848, $n = 10$ (data shown in Fig. 3).

4. Discussion

We describe a protocol for the quantification of mRNAs in human lymphocytes based on end-point product analysis. The critical element in the design of this system is the choice of the method to separate and quantify RT-PCR products. HPLC analysis offers a number of advantages over other available methods, including one-step separation and quantification.

The possibility of analysing PCR reaction products directly with no further effort than to load PCR reactions into the refrigerated auto-sampler reduces the risk of contamination since post-PCR sample handling is minimal. Furthermore computerised data acquisition and analysis make HPLC powerful, efficient, and productive. In addition, artefacts such as non-specific products are easily recognised and separated by their well-defined retention times that depend on their molecular masses. Moreover, it has the advantage over other systems that PCR products are not chemically modified by the process and can be recovered for other analysis. Some authors [9] propose its use as an alternative to PAGE for the purification of selected PCR products prior to sequencing. All these advantages make HPLC a critical tool not only for quantification after the system has been designed but also for establishing the conditions for the relative and competitive experiments as well. The composition of the PCR reaction mixture, cycle number, optimal amount of cDNA input, and adjust-

ment of the exponential phase of amplification can be determined with HPLC.

Different HPLC methods can be used, but the methods that use nonporous resin-based anion-exchange column packing [11] have the disadvantage of sequence-dependent retention of DNA fragments. Our system uses ion-pair reversed-phase HPLC for high-resolution separation of double-stranded DNA fragments [12] and its applicability for the separation and analysis of PCR products has been demonstrated [13–15]. In this system, the separation of double-stranded DNA (dsDNA) is determined by the chain-length of fragments, with longer dsDNAs eluting later than shorter-chain dsDNAs.

We have quantified using on-line UV absorbance detector, although the level of detection of the UV absorbance detector is moderate, its dynamic range is broad. In our system, the areas of the peaks generated by the PCR products range from 5000 to 600 000 μV s. Sensitivity of the detection could be improved using an on-line fluorescent detector: Arakawa et al. [16] reported a method using an on-column derivatization system, even though resolution was not affected by intercalation of the dye, the intensity of fluorescence was not completely proportional to DNA size. Another possibility is analysing the incorporation of a fluorescent labeled primer by the PCR product [17]. Furthermore, we used our conventional analytical HPLC system at room temperature, which we have used routinely to assess CNS neurotransmitter metabolites [18,19].

Quantitative PCR has evolved with recent developments in fluorescent systems that allow quantitative "real-time" detection of the kinetics of product accumulation. These methods require initial experiments with pure products to establish conditions for absolute quantification. Inasmuch as they do not require the separation of PCR products, they do not discriminate between the true product and PCR artefacts. Therefore, when using them, PCR conditions must be well-established to avoid non-specific binding that could lead to erroneous quantification. Multiplex analysis of levels of several cDNAs derived from different gene transcripts is also possible through the use of different dye combinations. However, the method is sophisticated and requires an expensive scanner for the detection of the different fluorophores. In contrast, HPLC discriminates PCR products before quantification. Different

PCR products can be analysed in the same chromatogram and product identity can be further assessed by collecting the peak and sequencing. Finally, the method is straightforward, requiring only evaporation of the mobile phase and dissolution in water.

In contrast, although RT-PCR technology was introduced years ago, it is still being optimised. RT and PCR are entirely different types of enzymatic reactions. Fluctuation in RT efficiency and inhibition of the PCR by the RT are common problems to all quantitative RT-PCR technologies [20].

To take into account these variations we performed relative measurements using the housekeeping gene, beta-actin as an internal standard. The relative PCR experiment provided a quick look at possible variations in expression and gave information on RNA quality and quantity. For these experiments, we prepared five different reactions containing different amounts of cDNA, from 5 to 60 ng ($n=5$). Although the number of reactions is high, the total amount of sample needed was only 135 ng of cDNA.

When preparing these PCR experiments, the relative concentration of each set of primers was adjusted to obtain similar amplification of β-actin and OK12b in the same PCR system [21]. The ratio of beta-actin primers relative to OK primers was 1:4. Other authors [22] reduced the β-actin product by delaying addition of the primers, but this requires additional handling and may be a source of contamination.

In analysing gene expression, the choice of the most appropriate reference for expression of the data is critical for potential comparisons between subjects and experimental conditions. The aim of absolute quantification is to determine the exact number of target mRNA molecules present in a given sample, but a valid question is whether it is meaningful to express results per μg of total RNA when this measure is dependent on many factors. Variability in the isolation of RNA from the blood sample, partial or total RNA degradation even under ideal conditions in which these variations could be controlled, and the UV measure of total starting RNA concentration are sources of irreproducibility [23]. The use of beta-actin as a normalisation factor can solve those problems to a great extent. Even in samples of varying integrity, it is possible to quantify mRNA expression by normalising to a housekeeping gene

[24]. Therefore, beta-actin regression lines were used to derive a factor for the relative normalisation of differences between cDNA samples.

Samples were routinely treated with DNase. In this case, controlling for the absence of genomic DNA is very important because the PCR product could be an intronless fragment, indistinguishable from genomic DNA. If any intron were present, this could be another source of irreproducibility.

To assess the cDNA of interest we used competitive PCR. One of the variables that make competitive PCR problematic is difference in the efficiency of the amplification rates of target and competitor. Previous studies [25] have shown that the sequence of the primers has the greatest effect on amplification efficiency, so we used a DNA competitor sharing identical primer binding sites with the target cDNA, which minimized differences in efficiency due to the primer-annealing step. We established five different reactions, each one with 300 ng of cDNA and amounts of competitor ranging from 0.03 to 3 attomol. When needed, sensitivity can be improved by increasing input cDNA to 400 ng.

In our case, the shortest DNA, OK12b, did not amplify more efficiently than the longest one (competitor), as theoretically expected [26]. A possible reason for this difference could be that they have different sequences between their respective primer binding sites. As a compensation, we did not detect hard-to-quantify heteroduplex molecules as additional reaction products in the HPLC chromatograms. In spite of these differences in size and sequence, the profile of the curves generated by both amplimers in the titration experiments were similar (Fig. 2). As a consequence, slope values in the competitive experiments were less than 1 (0.84). Aside from these differences, the accuracy of the determination was acceptable (Table 1). Part of the variability arises from the slightly different amplification rate observed when the initial number of molecules to be amplified is very low. In addition, the extreme points of the regression lines weighed most in regression analysis.

Our DNA competitor was carefully quantified by radioactive methods. We were aware that errors in this step could lead to aberrant results so we did not use spectrophotometric measures, which are less reliable. We confirmed competitor concentration by preparing an inverse competitive experiment and assessing the competitor against a plasmid that contained the OK sequence. In competitive experiments, the initial amounts of competitor and template were the same when the number of molecules became equal after the PCR. In these competitive assays, some authors prefer to express the competitor in femtograms instead of attomoles, although this unit of measurement is valid only for substances of identical size. We prefer to use attomoles, among other reasons, because the results of PCR products analysis can be proportional to the size of the products, as occurs in HPLC determinations or PCR performed in the presence of a labeled nucleotide. Furthermore, other methods of estimation using radiolabeled primers are affected by the number of single chains generated. Femtograms are not useful for studies comparing the expression of different genes or different-sized PCR fragments generated from the same gene; therefore, mRNAs of unknown genes must be expressed as molar concentrations, regardless of the method used for assessment.

The advantage of using DNA competitor [27] rather than an RNA standard is that DNA molecules are more stable and easier to handle than RNA molecules and partial degradation of RNA competitive standard is difficult to detect and a frequent source of error. Some authors [28] have reported that a change in the ratio of standard-to-target template occurring during reverse transcription causes poor reproducibility and decreases the accuracy of calculations of target mRNA.

In spite of the high number of reactions that have to be quantified, only 1.35 µg of total RNA sample was used. In terms of amount of sample needed for the analysis, RT-PCR cannot be compared to Northern or radioprotected hybridization assays, which require large amounts of RNA. RPA was initially established in our laboratory for the quantification of OK12b, but our level of detection was 0.1 fmol using 25–50 µg of total RNA.

Other methods, such as real-time methods, require pure products to prepare the calibration curve, as well as a large number of reactions. In any case, analysis time is reduced dramatically in these systems because they do not entail two consecutive processes since separation techniques are not needed.

Although a new generation of instruments with rapid air thermocycler and integrated fluorimeters has emerged, they are not always available to

research and clinical laboratories. HPLC systems are more widely distributed in these laboratories and can be used for the quantification of PCR products using the technique described in this article.

Accordingly, we have developed this system and demonstrated that ion-pair reversed-phase HPLC analysis of relative and competitive PCR products is an efficient method for quantifying, which produces results that correlate closely with those obtained by quantification of radiolabeled PCR products. Another important feature of this method is that it can be adapted to the assessment of other cDNAs with only minor changes to the method.

Acknowledgements

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