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THEORETICAL CONSIDERATIONS FOR THE APPLICATION OF COMPETITIVE POLYMERASE CHAIN REACTION TO THE QUANTITATION OF A LOW ABUNDANCE mRNA: ESTROGEN RECEPTOR

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Abstract—The necessary theoretical considerations for the development of a correct quantitative analysis of a low abundance messanger RNA (mRNA), estrogen receptor mRNA, by competitive polymerase chain reaction (PCR) are presented together with a series of experimental data. When compared to other methodologies currently utilized for RNA quantitation, this PCR application proved to be a very reliable, rapid and sensitive method. Furthermore, the PCR-based quantitative method described is of particular interest since it does not require the use of radiolabled compounds.

Recent reports have indicated that the polymerase chain reaction (PCR‡) can be usefully applied not only for qualitative, but also for quantitative analysis of selected (mRNAs). Several systems based on co-amplification of target and standard DNA (stDNA) have been described. Most of them require time-consuming manual operations [1], and careful titrations of PCR efficiencies [2, 3]. Furthermore, most of these quantitative analyses are critically dependent on the number of cycles performed [4]. A further development of quantitative PCR methods is the competitive PCR [5] where the cDNA of interest is co-amplified with a series of dilutions of a standard cDNA. The concentration of the mRNA is then calculated by direct comparison with the standard cDNA. In the present paper, the competitive PCR methodology was applied to the quantitative analysis of a rare mRNA like the estrogen receptor (ER) mRNA, which exhibits an abundance in target tissues that varies from 0.02% (uterus) to 0.001% (brain) of total mRNA. As an internal standard, a deletion mutant of ER mRNA was inserted into an expression plasmid, allowing the synthesis of the standard, sense, RNA to be added to the uterine tissue homogenate. Theoretical considerations, supported by the experiments reported here, indicate that the use of such an internal standard allows quantitative analysis of cDNAs which underwent a high number of amplification cycles rendering the technique particularly sensitive.

MATERIALS AND METHODS

Preparation of the internal standard. The pGEM3z plasmid (Promega, Madison, WI, U.S.A.) containing the *Eco*RI fragment of the rat ERcDNA [6], corresponding to the 1800 bp of the coding region, 210 nucleotides of the 5′-untranslated region and 74 nucleotides of the 3′-untranslated region, was digested with *Not*I (Boehringer Mannheim, Mannheim, Germany) in order to delete 66 bp corresponding to nt 212–278 of the receptor coding region. Following purification via agarose gel electrophoresis, the digested plasmid (pGEM3Z-ERmut) was ligated on itself. The pGEM3Z-ERmut was used as a template for transcription by the T7 polymerase according to the Promega transcription protocol. Synthesis of the RNA (standard RNA) was performed in the presence of 30 μCi of [^{3}H]-UTP. The efficiency of the reaction was calculated on aliquots by scintillation counting on TCA precipitable material. The template was then digested with RNAse-free DNAse. After phenol-chloroform extraction the newly synthesized ^{3}H standard RNA was ethanol precipitated, resuspended in water and stored in aliquots at −70°.

RNA preparation. Total RNA (utRNA) was isolated from rat uterine tissue by the method of guanidium thiocianate extraction [7]. The utRNA, resuspended in water, was quantitated by absorbance reading (at 260 nm) and stored in aliquots at −70° until used.

cDNA synthesis. The reverse transcription reaction was performed using, as primers, a mixture of four ER-specific oligonucleotides, each at a concentration of 0.05 μg/μL. The 3b, 4b, 5b and 6b primers used encompassed the 516–1140 bp of the coding region (3b: 5′-GTCGGAGAGTCTCTCTCGGCC-3′; 4b: 5′-GTTCTTATCGATGGTGCATTG-3′; 5b: 5′-CTTAGTGTGCTTGATCACAAG-3′; 6b: 5′-TTGATCGTGGAGATTCAAGTC-3′) and were chosen according to previous analysis of ER amplification products [8]. Primer mixture (2 μL)

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‡ Abbreviations: PCR, polymerase chain reaction; ER, estrogen receptor, utRNA, uterus RNA; stDNA, standard DNA; TBE, Tris borate EDTA.

was added to 3 and 5 μL of utRNA and standard RNA, respectively. utRNA and standard RNA were used at the concentration shown for each experiment in the Results. The 10-μL mixture containing oligonucleotides, standard RNA and utRNA was heated at 90° for 3 min, then cooled at room temperature for 30 min. Transcription buffer (14 μL) (50 mM Tris–HCl pH 8.2, 40 mM KCl, 6 mM $MgCl_2$, 20 mM DTT, 1.2 mM dNTP) and 1 μL (40 units) of AMV reverse transcriptase (Stehelin, Basel, Switzerland) were added. Incubation was carried out for 1 hr at 42°.

Amplification method. One tenth of the cDNA reaction mixture was combined with 10 μL of 10 × PCR buffer (Promega), 5 μL of 4 mM dNTP, 2 μL of 0.2 μg/μL primers 2a and 2b (2a: 5′-GCTCTGGGCGAGGTGTACGTG-3′; 2b: 5′-AGCCCCCAGACTATTGG ACC-3′) and 2.5 units of Taq polymerase (Promega) to a final volume of 100 μL. The reaction mixture was covered with a layer of mineral oil (Perkin Elmer, Norwalk, CT, U.S.A.). The thermal profile of the amplification reaction involved an initial 10 min denaturing step at 94° followed by the indicated number of cycles of: (a) primer annealing at 48° for 1 min 15 sec, (b) extension at 72° for 2 min 30 sec, (c) denaturation at 94° for 1 min 15 sec.

Quantitative analysis. Each PCR reaction mixture (20 μL) was subjected to electrophoresis in 3% (w/v) agarose gel in TBE buffer (90 mM Tris, 90 mM borate, 2 mM EDTA). Gels were stained with ethidium bromide and photographed with Polaroid type 667 film [9]. The photographic negatives were scanned by the AppleScanner (Apple Computer, Cupertino, CA, U.S.A.) laser densitometer and the data analysed by AppleScan, ImageFolder and CricketGraph programs on a Macintosh computer (Apple Computer). The linearity range of the curve linking the absorbance reading to the concentration of DNA stained with the ethidium bromide stain was experimentally determined.

Northern analysis. The northern and slot blot analyses were done as described previously [9]. Briefly, 25, 10, 5, 1 and 0.1 μg of utRNA, together with 50 pg of standard RNA, were denatured in 50% (v/v) formamide and 6.7% (v/v) formaldehyde then run on a 0.75% (w/v) agarose denaturing gel. The blotting and hybridizations were done under the conditions previously described [10] using the pGEM3Z plasmid containing the *Eco*RI fragment of the rat ERcDNA as a probe [6]. The plasmid was labeled by the "Multiprime DNA labeling system" (Amersham, Little Chalfont, U.K.) with [α-^{32}P]-dCTP to a specific activity of 2 × 10^8 dpm/μg cDNA. Hybridization was carried out for 36 hr at 42° in the presence of 5 × 10^6 dpm/mL of ^{32}P labeled plasmid. The filters were washed four times for 30 min with 1 × SSC, 0.1% SDS at 50° and exposed to an X-ray film (Hyper-film HP; Amersham) in the presence of intensifying screens at −70°.

Slot blot analysis. Aliquots of 25, 10, 5 and 1 μg of utRNA were spotted onto a nitrocellulose filter (Schleicher and Schuell, Dassel, Germany) using a slot blot apparatus (Minifold II; Schleicher and Schuell) [10]. To determine the range of linearity of the autoradiographic signal, known dilutions (50, 10, 1 and 0.5 μg) of the standard RNA were processed in parallel with utRNA.

The nitrocellulose filter was hybridized with a ^{32}P labeled probe under the same conditions described in the previous paragraph.

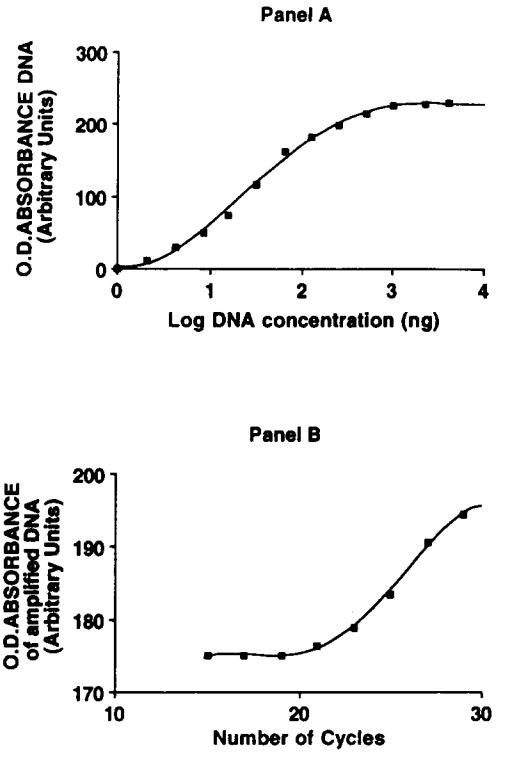

Fig. 1. Panel A: determination of the range of linearity for O.D. readings of various concentrations of cDNA. Panel B: accumulation of amplified DNA at various cycles of PCR.

RESULTS

Setting the conditions for cDNA amplification

With preliminary experiments it was determined that concentrations of DNA between 5 and 600 ng produced a linear increase in optic density readings (Fig. 1, panel A). The subsequent studies were therefore performed by analysing concentrations of amplified DNA of about 10–100 ng.

To establish the number of amplification cycles needed to produce the correct amount of DNA in a reverse transcription-PCR experiment, 10 μg of utRNA were processed as described in Materials and Methods and the amplification was done for a variable number of cycles (15, 17, 19, 21, 23, 25, 27 and 29). As shown in Fig. 1 panel B the required O.D. reading was obtained with 21–29 cycles of amplification. It has to be underlined that at 29 cycles the amplification process had not reached a plateau. As demonstrated in the next paragraph, however, the use of an internal standard allowed performance of the analysis during the exponential phase of the amplification process.

Accumulation of PCR co-amplified products: theoretical considerations

The amplification of DNA via PCR amplification is a repetitive process of DNA synthesis, which does not necessarily have the same efficiency (ε) at each cycle. Description of the trend in accumulation of reaction products is based on the following equation:

$$A_n = A_{n-1}(1 + \varepsilon_n) \quad (1)$$

where A_n and ε_n are, respectively, the concentration of DNA accumulated and the efficiency of the reaction at the cycle n. Therefore at the first cycle:

$$A_1 = A_0(1 + \varepsilon_1) \quad (2)$$

where A_0 is the initial concentration.

At the second cycle:

$$A_2 = A_1(1 + \varepsilon_2) \text{ or else: } A_2 = A_0(1 + \varepsilon_1)(1 + \varepsilon_2). \quad (3)$$

Indicating ε_i the value of the efficiency of the i cycle; at cycle n the reaction can be described as follows:

$$A_n = \pi_{i=1}^{n}(1 + \varepsilon_i)A_0 \quad (4)$$

where

$$\pi_{i=1}^{n} = (1 + \varepsilon_1)(1 + \varepsilon_2)(1 + \varepsilon_3) \ldots (1 + \varepsilon_n).$$

When a second DNA fragment is co-amplified, a formula of the same type can be applied:

$$B_n = \pi_{i=1}^{n}(1 + \varepsilon_i)B_0. \quad (5)$$

Provided that the amplification process uses (a) the same primers, (b) similar nucleotide concentration and (c) molecules of similar sizes, ε_i can be considered to be the same for molecules A and B. On the bases of this hypothesis, dividing (4) by (5) it follows that:

$$A_n/B_n = \pi_{i=1}^{n}(1 + \varepsilon_i)A_0/\pi_{i=1}^{n}(1 + \varepsilon_i)B_0$$

or

$$A_n/B_n = A_0/B_0. \quad (6)$$

This formula states that, independent of changes in the reaction efficiency within the different cycles, the ratio between the concentrations of the two amplified DNA species is constant during the whole amplification process. As a consequence of the hypothesis that ε_i is identical for the two species of DNA, the two functions $A_n = f(n)$ and $B_n = f(n)$ describing the trend of accumulation as a function of the number of cycles are the same. For the initial cycles the efficiency ε_i is constant and an exponential behaviour is expected, that is:

$$A_n = (1 + \varepsilon)^n A_0 \quad (7)$$

$$B_n = (1 + \varepsilon)^n B_0$$

Typically, a 100 μL PCR mixture is composed of: 2.5 Taq polymerase (7.5×10^{10} enzyme molecules); 5 μL 4 mM dXTP (4.8×10^{16} molecules); 0.8 μg primers (7.0×10^{13} molecules); 10 pg DNA (internal standard) (1.5×10^{8} molecules); 10 pg DNA (to be quantitated) (0.9×10^{8} molecules).

Maximum efficiency of the reaction can be achieved for the initial cycles, as experimentally demonstrated by the exponential progression of the reaction. However, assuming that $\varepsilon = 1$, by cycle 18 the number of molecules of DNA (stDNA + DNA) will be about 2×10^{14}; the number of nucleotides remaining will be 4×10^{16}; the number of primers remaining will be 1×10^{13}, while the enzyme (2.5 U of the enzyme incorporates 3×10^{20} molecules of dXTP per cycle) will still be in excess. Due to the shortage of primers, the reaction will not progress exponentially, but ε_i will decrease with the rate of amplification. It was experimentally demonstrated that at later stages both A_n and B_n exhibited a linear trend with n:

$$A_n = p + qn$$

$$B_n = r + sn.$$

We described this linear trend by the following equations:

$$A_n = (a + bn)A_0 = aA_0 + bA_0n \quad (8)$$

$$B_n = (a + bn)B_0 = aB_0 + bB_0n.$$

The formulas (7) and (8) are special cases of the general formula (6) differing as a result of the different trend of ε_i at the various stages of amplification. Thus they must exhibit the general characteristics of formula (6): the trend with n is the same for the two species and A_n and B_n are proportional to A_0 and B_0, respectively. In order to take these two features into account, in the equations (8) adopted during the linear range, the proportion relative to the initial concentrations was considered and the same values of a and b were assumed. It is evident that not only the absolute values of A_n B_n, but also the angular coefficients bA_0 and bB_0, should be proportional to the initial concentration A_0 and B_0.

Accumulation of PCR co-amplified products by a different number of cycles

utRNA (10 μg) was reverse transcribed with 100 pg, 30 pg and 10 pg of the internal standard (standard RNA). Aliquots containing one-tenth of the cDNA mixtures were subjected to 15, 17, 19, 21, 23, 25, 27 and 29 cycles of amplification using the primers reported in the Materials and Methods. The absorbance values of the DNA bands stained with ethidium bromide were plotted versus the number of cycles. Due to the limited sensitivity of the ethidium bromide staining method used to visualize the amplification products, the reaction could not be followed through all of the exponential phase. Generally, the DNA accumulated could be effectively quantitated only after cycle 20. Starting from cycle 20, the progression of the reaction was linear, as demonstrated by the linear regression coefficient (Fig. 2, panels A, B and C). In order to check the theoretical model presented above, which implies a direct correlation between the initial concentrations of cDNA and the progression of the reaction, several amplification reactions were performed in parallel on samples containing a fixed amount of utRNA and 1:3 serial dilution of the standard RNA. In accordance with that expected from the theory, the ratio between the absolute values A_n and B_n as well as the ratio between the slopes or their angular coefficients should be

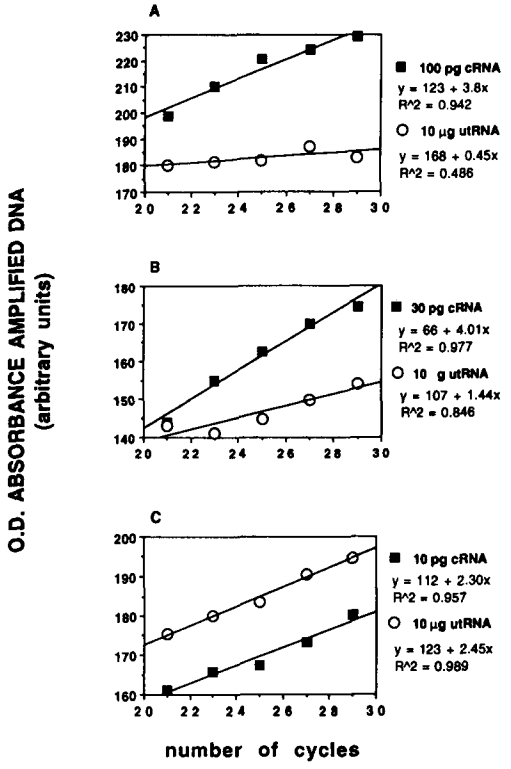

Fig. 2. Accumulation of PCR co-amplified products following different numbers of amplification cycles. utRNA (10 µg) and 100, 30 and 10 pg of standard RNA (cRNA) were reversed transcribed. One tenth of the cDNA reaction mixture was subjected to 15, 17, 19, 21, 23, 25, 27 and 29 cycles of amplification; the reaction products were separated by gel electrophoresis and visualized by ethidium bromide staining. The quantitation of the bands was done by densitometric scanning of a photograph of the gel. For the data concerning cycles 21–29 the linear regression was calculated and the best fit curve plotted (panels A, B and C).

Table 1.

	Amounts of internal standard (µg)		
	100	30	10
Internal standard slope value	3.8	4.0	2.3
ERmRNA slope value	0.4	1.4	2.4
Internal standard/ERmRNA	8.4	2.8	0.9

The data are calculated on the bases of the regression lines shown in Fig. 2.

proportional to the ratio A_0/A_0. In the experiment performed it was expected, therefore, that the ratio between the slopes describing the progress of the reaction for the standard RNA and the utRNA should differ by a factor of 3 in the different reactions. As shown in Table 1, the ratios between the slopes of the two reactions calculated on the basis of the data presented in Fig. 2 are 8.4, 2.8 and 0.94. As expected, these values differ by a factor of 3. When the reaction was performed with 10 µg of utRNA and 10 pg of standard RNA, the ratio between the two slopes was close to 1, indicating that the initial concentrations of the two species of RNA were almost identical. On the basis of the present results, the concentration of ERmRNA in the uterus was about 1 pg/µg utRNA.

Accumulation of PCR co-amplified products at a fixed number of cycles

utRNA (1 µg) was reverse transcribed with a series of dilutions of standard RNA. Aliquots containing one-tenth of the cDNA mixtures were amplified for a fixed number of cycles (25). An initial experiment was performed utilizing a broad range of standard RNA dilutions (1000, 100, 10, 1, 0.1 and 0.01 pg). As shown in Fig. 3 panel A the ratio between uterine and standard amplified DNAs approaches 1 at a concentration of standard RNA between 10 and 1 pg. In order to obtain a more precise quantitative determination of the concentration of ERmRNA in the sample analysed, the experiment was repeated using another series of dilutions within a narrower range of standard RNA concentrations (0.1–10 pg). At this point, the exact concentration of ERmRNA could be determined and proved to be equal to 1 pg/µg utRNA, a value very close to the amount determined by the quantitative analysis of the accumulation of PCR co-amplification products with a variable number of amplification cycles (Fig. 3, panel B).

Quantitation of ERmRNA using different amounts of total RNA

In order to establish the sensitivity and the existence of a range of linearity of the above-examined quantitative methods, ERmRNA was quantitated starting with different amounts of utRNA. When the absorbance of the bands obtained following the amplification of the different concentrations of utRNA was plotted versus the concentration of utRNA, a linear curve was obtained, indicating that the quantitation of the ERmRNA was linear in a wide range of concentrations (Fig. 4). The same value of ERmRNA concentration (1 pg/µg of total RNA) was obtained when the determination was performed starting with 25, 10, 5, 1 and 0.1 µg of total utRNA (data not shown). When the determination was performed with 0.01 µg or less of total utRNA, no band of amplified DNA was observed on agarose gel after 25–30 cycles of amplification. When the DNA was amplified for over 40 cycles no specific product of amplification was observed and a smear at high molecular weight was present on the agarose gel (data not shown). A loss of specific PCR products and their conversion to random-length higher molecular weight fragments after excessive cycling has already been reported in the literature [11].

Quantitative analysis of ERmRNA content by northern and slot blot analysis

The quantitation of ERmRNA was also performed

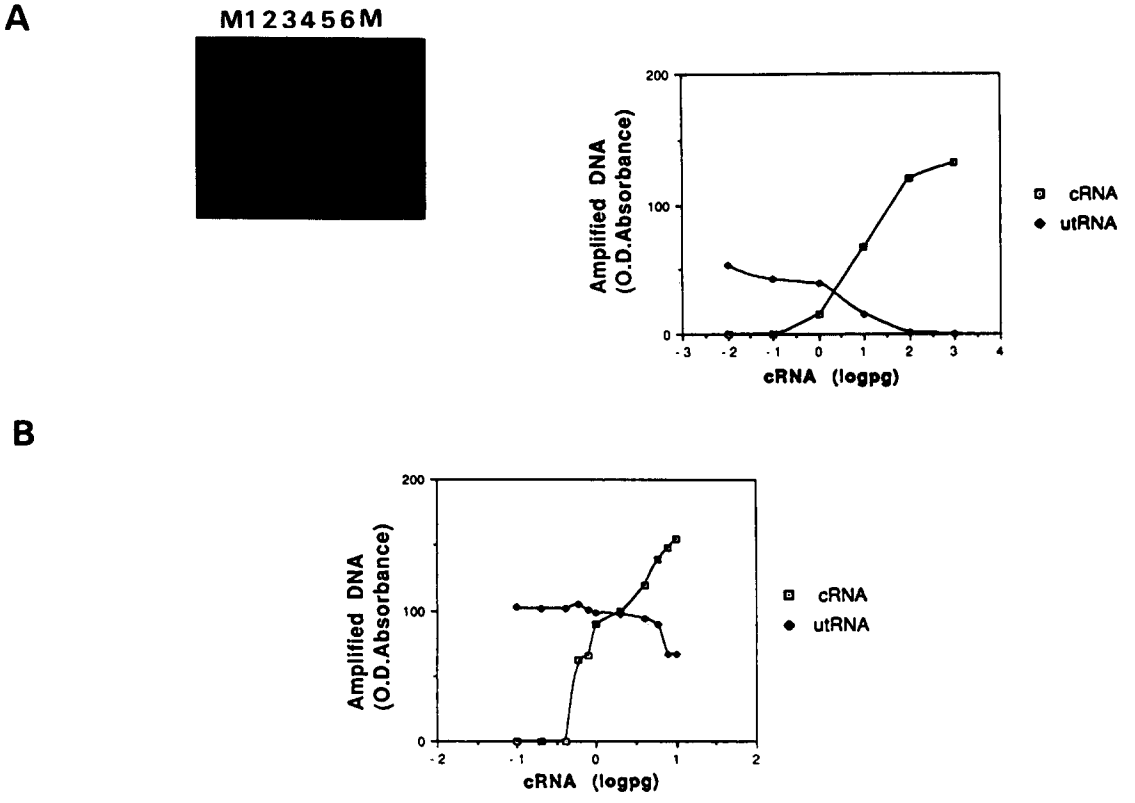

Fig. 3. Standard dilution test. Panel A left: ethidium bromide staining of PCR products (after 25 cycles) separated on 3% agarose gel. Lane M: molecular weight markers. Lanes 1–6: 1 μg total RNA from rat uterus and various amounts of standard RNA (cRNA) dilutions (0.01, 0.1, 1, 10, 100 and 1000 pg), respectively. Panel A right: plot of the densitometric scan of the stained gel. Panel B: plot of the densitometric scan of a second series of PCR cycles done with different standard RNA (cRNA) dilutions (0.15, 0.22, 0.34, 0.52, 0.79, 0.83, 1.06, 2.75, 2.16, 6.3 and 9.5 pg).

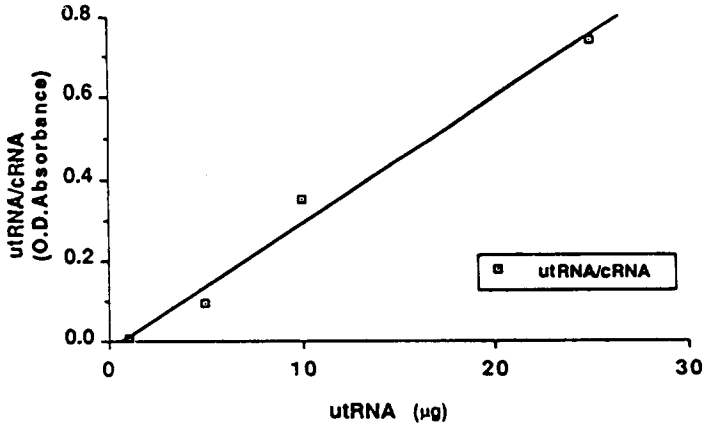

Fig. 4. Linearity curve of the PCR reaction. utRNA (25, 10, 5, 1, 0.1 μg) was subjected to 30 cycles of PCR amplification in the presence of a fixed amount of internal standard. The concentration of the amplified ERmRNA was linear in a wide range of utRNA concentrations utilized for the analysis.

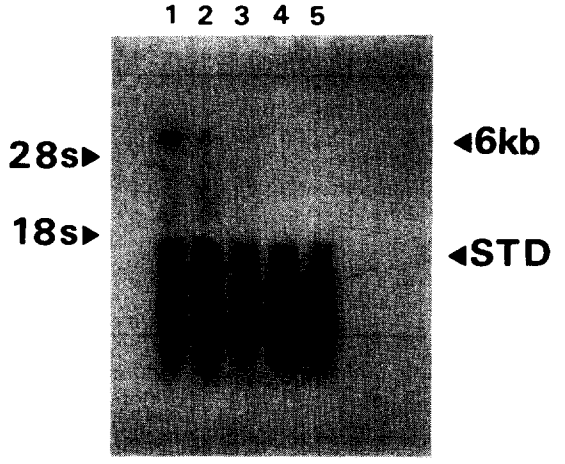

Fig. 5. Northern blot analysis. utRNA (25, 10, 5, 1 and 0.1 μg) (lanes 1–5) and 50 pg of standard RNA were subjected to northern analysis as described in Materials and Methods. The quantitation of the ERmRNA was done by scanning the X-ray film and by direct comparison with the absorbance values obtained for the standard RNA.

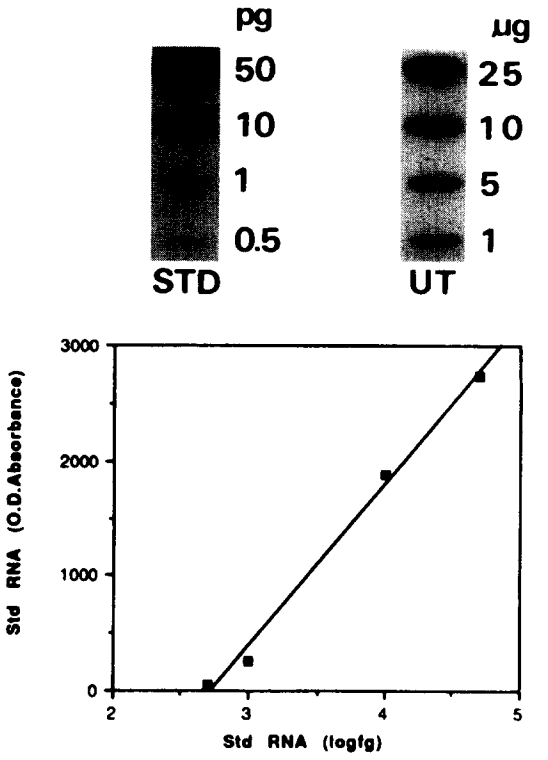

Fig. 6. Slot blot analysis. Various concentrations of standard RNA (STD) were filtered in parallel to different amounts of utRNA (UT). Following hybridization with a ^{32}P-labeled probe, the filters were exposed to X-ray films. The densitometric scanning of the bands of the standard RNA permitted description of the linearity curve shown in the lower panel and therefore quantitation of the ERmRNA.

at different concentrations of total utRNA by northern and slot blot analysis. For the northern analysis, 50 pg of standard RNA processed in parallel with several concentrations of total utRNA were run on agarose denaturing gel, blotted and hybridized as described in Materials and Methods. Within 15 days of exposure time, the ERmRNA could be detected in samples of a minimum of 5 μg of total utRNA. The quantitation of the ERmRNA was obtained by O.D. scanning of the autoradiograph and was calculated to be 1.5 pg/μg utRNA (Fig. 5).

The same concentrations of total utRNA also underwent slot blot analysis. As shown in Fig. 6, this further quantitation also established that the concentration of ERmRNA was of about 0.9 pg/μg total utRNA.

DISCUSSION

The aim of the present study was to demonstrate that the PCR can be utilized for a rapid, sensitive and non-isotopic determination of ERmRNA. The present study substantiates the observations by Nakayama *et al.* [12] and, in addition, presents the theoretical basis for correct application of the methodology.

The criticisms of the use of PCR in the quantitation of small amounts of specific mRNAs can be summarized as follows: (1) prior to amplification, the mRNA needs to be reverse transcribed into cDNA. Due to the high variability of this reaction, the subsequent quantitative analysis cannot provide an absolute value of RNA concentration and lacks the necessary reproducibility. (2) The number of amplification cycles performed may influence the efficiency of the amplification. (3) The synthesis of non-specific amplification products may perturb the progression of the reaction. (4) The resolution of the quantitative analysis is limited. (5) The ability to recognise heterogeneity in mRNAs is limited (due to transcripts of duplicated genes).

The data presented here demonstrate that the intrinsic variability of the reverse transcription can be of no influence on the overall quantitation, provided that the appropriate standard is utilized. Furthermore, the correct choice of the internal standard allows amplification of the standard and of the cDNA of unknown concentration to be carried out with the same set of primers: in this way the synthesis of undesired products of amplification can be limited (especially if the sequence of the primer is such as to permit a particularly high annealing temperature). With regard to the number of cycles performed, the theoretical considerations presented, substantiated by the data reported, indicate that the PCR can be successfully applied once an appropriate choice of internal standard is made. Finally, the limits of resolution of the present method are not any different from those described for other methodologies and the restrictions in the possible recognition of mRNA heterogeneity can often be overcome by the use of selected primers for both reverse transcription and amplification.

It is therefore concluded that, when appropriately

utilized, the competitive PCR represents a rapid and very sensitive method for the quantitation of low abundance mRNAs. Of particular interest is the fact that this methodology does not require the use of radiolabeled compounds.

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