Quantitative RT-PCR: limits and accuracy

Frédérique Souazé, A Ntodou-Thomé, C y Tran, W Rostène, P Forgez

Quantitative RT-PCR: Limits and Accuracy

BioTechniques 21:280-285 (August 1996)

F. Souazé, A. Ntodou-Thomé, C.Y. Tran, W. Rostène and P. Forgez
INSERM Unit 339, Hôpital Saint-Antoine, Paris, France

ABSTRACT

In this paper we determine the limits and accuracy of quantitative reverse transcription (RT)-PCR using a modification of the original protocol. The quantification of mRNA with this procedure requires a preliminary estimation of the target molecule (TM) concentration, established from experiments with an internal control molecule (ICM). A definitive quantification is then attained from serial dilutions of the reverse transcription reaction. The success of this latter step is dependent on maintaining an equivalent number of TM and ICM in the reaction. The purpose of our study was to evaluate the influence of the deviation between the TM and the ICM on the result. We show here that we can control the accuracy of the assay by fixing the limit of the TM/ICM ratio. Indeed, when the TM/ICM ratio is between 0.66 and 1.5 (i.e., the difference between TM and ICM is 1.5-fold), the final result has an error of approximately 10%. Exceeding this limit produces errors approaching 60%, as in the case of TM/ICM = 2. When the above conditions are respected, a difference as small as 20% between two samples can be determined with an accuracy of 95%.

INTRODUCTION

The reverse transcription polymerase chain reaction (RT-PCR) is the technique of choice for analyzing extremely low abundance mRNA derived from cells or tissues. Recently, a number of reports have described different quantitative procedures for analyzing mRNA steady-state levels. PCR is now a well-established method whose sensitivity is a principal advantage over other "similar" techniques, such as Northern blots, which only provide semi-quantitative results. The earliest PCR studies proceeded by comparing the amplified products from the target molecule (TM) with the amplified products from the cDNA of an abundant protein, such as actin or β-globulin. Quantification of specific mRNA molecules with RT-PCR was dependent on extrapolating the results from a control molecule. However, variations in the kinetics of the reverse transcription reaction could produce discrepancies between the calculated amount of molecules from the sample and the actual amount of molecules. For this reason, the best control molecules are internal control molecules (ICM). To ensure that the efficiency of the PCR is similar for both molecules, the ICM is created from a synthetic cRNA. This cRNA is identical to the TM except it possesses a small deletion in the amplified portion of the molecule. The resulting amplified products have different molecular weights and are distinguished easily on polyacrylamide gel electrophoresis.

Quantitative RT-PCR is based on the competitive status between both amplicons. The addition of an ICM in the RT-PCR creates competition between the TM and the ICM for the factors controlling the amplification process, such as nucleotides and primers (for review see Reference 2). The approach developed by Wang et al. (9) was based on the fact that during the exponential phase of PCR, the amount of TM could be quantified by extrapolating against the results obtained from the ICM, provided that the reaction efficiencies were identical. In order to properly set up a Wang assay, it is necessary to know the amount of the target RNA before the experiment commences and, for this reason, we adopted the modifications proposed by Nagano and Kelly (4). Consequently, a titration assay is used to estimate the quantity of target molecule. The quantitative assay is then performed using serial dilutions from an RT reaction containing equivalent amounts of TM and ICM.

The aim of our study was to evaluate how the ratio of TM to ICM affects the competition between the two amplicons and therefore the accuracy of quantitative RT-PCR. For these reasons, we devised a quantitative RT-PCR using a known number of two different cRNA molecules (one molecule was designed as the ICM and the other as the TM in nonspecific RNA). By manipulating one of the two molecules, we were able to assess the limits and the precision of quantitative RT-PCR.

MATERIALS AND METHODS

Internal Controls

Neurotensin receptor (NTR) cDNA was kindly supplied by Dr. Nakanishi. The coding region of NTR cDNA (-7 to 1301) (8) was inserted into the *Sma*I-*Bam*HI site of pT7/T3α18. An oligonucleotide containing a poly$(dA)_{45}$ was

Table 1. Summary of the Results Obtained from Titration (A) and Quantitative (B) Assays, and a Comparison of Two Samples in Which Quantity Differs by 20% (C)

	A Titration Assay	B Quantitative Assay					C Sensitivity	
Molecules IN cRNA Δ34	1 000 000	1 000 000	750 000	1 500 000	2 000 000	500 000	1 000 000	800 000
Molecules OUT (mean)	1 179 825	1 091 817	831 764	1 654 190	3 083 262	306 359	1 091 817	755 681
S.D.	345 505	130 920	62 059	132 370	534 665	65 239	130 920	136 790
S.D. as % Molecules OUT	29.3	12	7.4	8	17	21.3	12	18
% IN/OUT	15.25	8.4	9.8	9.3	35.1	63.2	8.4	5.9
Student's *t* Test							*P*<0.05	

A varying number of cRNA Δ34 molecules is assayed with 10^6 molecules of cRNA Δ96.

then inserted between the *Sal*I-*Bam*HI sites. Deletions of 34 and 96 nucleotides were made by deleting fragments *Nco*I-*Nhe*I and *Hin*cII-*Nco*I, giving the plasmids named pΔ34 and pΔ96, respectively. All enzymes, unless otherwise noted, were purchased from Life Technologies (Gaithersburg, MD, USA) and the reaction conditions were those as suggested by the manufacturers. The cRNA Δ34 and the cRNA Δ96 were obtained by in vitro transcription of 8 µg each of plasmid linearized at the *Sal*I site. The transcription reaction contained 40 mM Tris-HCl pH 7.2, 10 mM dithiothreitol (DTT), 6 mM $MgCl_2$, 4 mM spermidine, 80 U RNasin® Ribonuclease Inhibitor (Promega, Madison, WI, USA), 0.5 mM dNTP and 60 U of T7 RNA polymerase. Polymerase chain reactions were carried out in a final volume of 50 µL at 37°C for 1 h. The reaction mixture was treated with 10 U of RNase-free DNase I for 15 min at 37°C. The cRNAs were purified on oligo(dT) columns (Sigma Aldrich Techware, Milwaukee, WI, USA), following the procedure described by Aviv and Leder (1). After elution, the cRNAs were precipitated with ethanol and then diluted in diethylpyrocarbonate (DEPC)-treated water containing 1 U/µL of RNasin. cRNA quality was checked by electrophoresis in formaldehyde RNA gel (6), and cRNA concentration was estimated by spectrophotometric absorbance at 260 nm. The cRNA solution was diluted to 1×10^7 molecules/µL in DEPC-treated water, put into 10-µL aliquots and stored at -80°C.

Primer Labeling

Fifty picomoles of antisense PCR primer were 5′ ^{32}P end-labeled with 20 U of polynucleotide T4 kinase in 50 µL of 10 mM $MgCl_2$, 5 mM DTT, 70 mM Tris-HCl pH 7.6 and 100 pmol [γ-^{32}P]-ATP (3000 Ci/mM) (Amersham International, Little Chalfont, Bucks, England, UK) at 37°C for 30 min. The end-labeled oligonucleotide was subsequently purified on a microcolumn. The oligonucleotide concentration was estimated by counting 1 µL of eluent on a GF/C filter (Whatman, Maidstone, Kent, England, UK) in 5 mL of dry extract scintillation fluid (Optiphase; Wallac/Pharmacia, Brussels, Belgium).

Reverse Transcription Reaction

The primer RT-NTR (5′-GCT-GACGTAGAAGAG-3′) located at position 1069–1083 was used for reverse transcription of both cRNA molecules. Varying amounts of cRNA Δ34 and cRNA Δ96 were mixed with 1 µg RNA of total mRNA from Chinese hamster ovary (CHO) cells devoid of NTR mRNA, 50 pmol of RT-NTR primer in 20 mM Tris-HCl pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP, 1 U/µL RNasin and 200 U Moloney murine leukemia virus reverse transcriptase in a final volume of 30 µL for 1 h at 37°C.

Quantitative RT-PCR Conditions

Primers used in this procedure were S-NTR (5′-CCTTCAAGGCCAAGA-CCCTC-3′) and AS-NTR (5′-CAG-CCAGCAGACCACAAAGG-3′) at positions 521–540 and 947–966, respectively, giving a PCR product of 411 nucleotides for cRNA Δ34 and 349 nucleotides for cRNA Δ96. Primers were synthesized and purified using polyacrylamide gel electrophoresis. The PCR amplification was performed on 1:5 (vol:vol) of the RT reaction in a mixture containing 16 mM Tris-HCl pH 8.3, 40 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM concentration of each dNTP, 25 pmol of each primer (S-NTR and

AS-NTR), 10^6 cpm of a 5′ end-labeled [γ-^{32}P]ATP AS-NTR and 1 U of *Taq* DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) in a final volume of 50 µL. The amplification profile was divided into denaturation at 94°C for 30 s, annealing at 55°C for 1 min and extension at 72°C for 1 min 30 s. The 26 cycles were preceded by a denaturation at 95°C for 5 min and immediately followed by a final extension at 72°C for 10 min. The amplification was performed in a 500-µL GeneAmp® tube, containing 50 µL of Nujol mineral oil, in a DNA Thermal Cycler 480 (Perkin-Elmer).

The assay system consisted of two steps. Titration assays were performed with 10^6 molecules of cRNA Δ96 with increasing quantities (10^4 to 10^8 molecules) of cRNA Δ34. For precise RNA quantitative studies, a known number of cRNA Δ34 and cRNA Δ96 molecules were subjected to reverse transcription, followed by a series of 1:3 (vol:vol) dilutions and amplification by PCR. For the negative controls, the same procedure was applied to a RT reaction containing no RNA or cRNA. This control was made for each group of samples. Gel pieces from the negative control were excised, counted and used for background estimates. The experiment was rejected if the negative control contained visible bands.

Electrophoresis

Twenty microliters of each PCR was immediately loaded on 5% polyacrylamide gels in 90 mM Tris-borate and 2 mM EDTA. Electrophoresis was performed at 250 V for 3 h at room temperature in an ADJ2 apparatus (Owl Scientific, Woburn, MA, USA). After migration, the bands were stained in ethidium bromide and cut out before counting in 3 mL of scintillation fluid in a β-scintillation counter (Model LS 6000 SC; Beckman Instruments, Fullerton, CA, USA).

Data Analysis

After counting, the log of the cpm was plotted against the log of the ICM or the log of the RNA. A linear regression for each curve was calculated. The data generated from these curves were used to extrapolate the number of molecules in each sample. The experiments in Table 1 were performed five times and the results expressed as the average. The statistical significance of the results were analyzed using the Student's *t* test.

RESULTS AND DISCUSSION

The quantitative RT-PCR assay developed by Wang et al. (9) and modified by Nagano and Kelly (4) is the most sensitive technique available for the measurement of RNA. Proper implementation of this technique is dependent on completing two rounds of RT-PCR, consisting of titration and quantification assays. We performed here a series of tests to illuminate the critical steps and parameters controlling the accuracy of quantitative RT-PCR

In a typical RT-PCR, there is an unknown concentration of target molecule (TM) and a known concentration of an internal control molecule (ICM). The concentration of the TM is determined by extrapolating the data based on the ICM. For our experiments, the TM was known in advance, which permitted us to determine if the expected concentration resulting from the RT-PCR was equal to its known concentration. In our system, cRNA Δ34 was considered the target molecule (TM), contained in 1 µg of CHO RNA, and cRNA Δ96 was the ICM. The known number of starting molecules is referred to as "Molecules IN" and the number of molecules calculated as a result of the RT-PCR is referred to as "Molecules OUT".

The titration assay is a preliminary step, consisting of a competitive RT-PCR that determines the departure point for the final quantification of the mRNA. An increasing amount of ICM is added to the RNA sample, followed by a RT reaction and PCR. If the initial

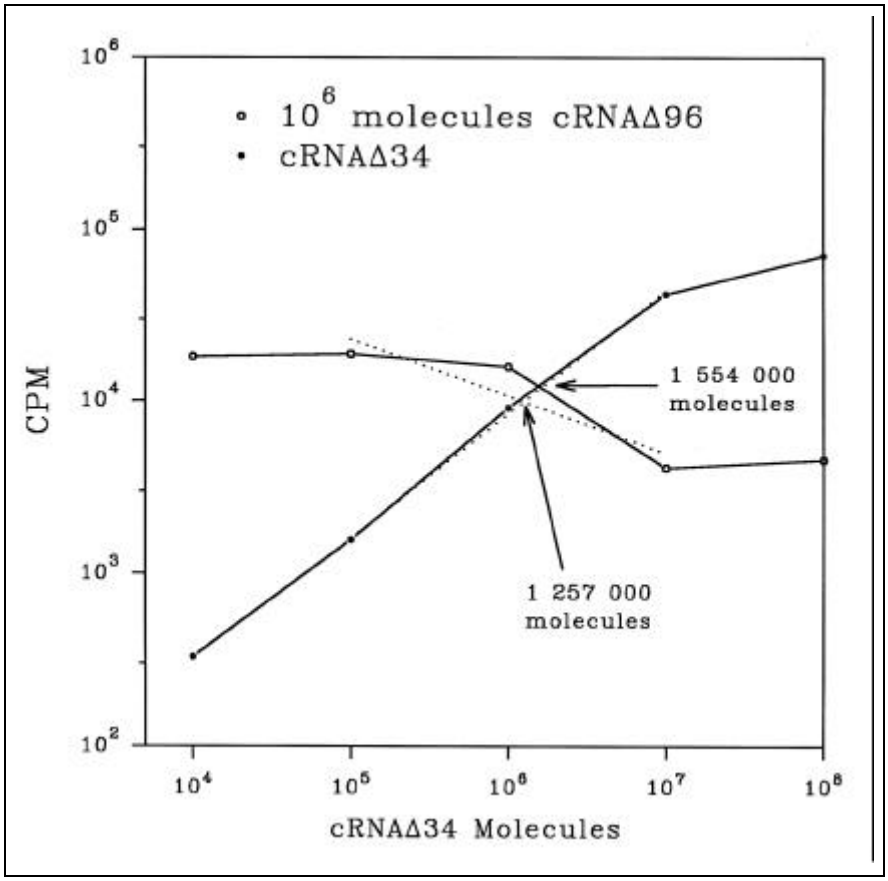

Figure 1. Titration assay. Each point containing 10^6 molecules of cRNA Δ96 and various numbers of molecules of cRNA Δ34 were reverse-transcribed and amplified as described in Materials and Methods. The amplification products were separated using polyacrylamide gel electrophoresis and detected by ethidium bromide staining. The cpm from the bands were counted after they were excised from the gel.

TM and ICM concentrations were identical, the resulting PCR products should be produced in equal amounts (5,6).

We evaluated the accuracy of the titration assay by determining the effect of increasing the number of cRNA Δ34 molecules on a constant quantity of ICM, i.e., 10^6 molecules of cRNA Δ96. The results were plotted using the formula Log (cpm) = f(Log ICM), and the estimated quantity of TM was calculated at the point where the two curves intersected. As shown in Figure 1, the results obtained from this procedure were inexact. The total number of molecules differed by approximately 25% (1.554×10^6 vs. 1.257×10^6), depending upon whether the calculation was made at the point were the curves crossed (solid lines) or at the point where the regressions crossed (dotted lines). In addition, while the average quantity of molecules obtained from the five experiments corresponded to what was expected, the value for the standard deviation was 30% of the final result (Table 1A). Finally, we also noticed that among these five experiments, the extreme values of the results varied up to 50% (data not shown). The titration assay, based on competitive PCR, was first described by Gilliland et al. (3). While this method is very accurate for DNA measurements, the authors noted that several pitfalls should be considered when applying this technique to the quantification of low abundance mRNAs. To eliminate the effect caused by differences in RT efficiency, which can vary up to 50% (2), a homologous cRNA internal control must be added to the RT reaction. In the case of the titration assay, where calculations are based on individual RT reactions, it is likely that these inconsistent results are caused by efficiency differences between the various RT reactions. Nevertheless, in many cases this procedure can be used when the experiment requires an accuracy of only 50%.

As can be seen from the results shown above, a second step (quantitative assay) is required to attain precise data with RT-PCR. By using the results obtained from the initial titration assay, a known quantity of RNA sample (TM) is mixed with the internal control so that the quantities of both molecules are equal. This mixture is then reverse-transcribed and a PCR is performed on samples that have been (1:3) serially diluted.

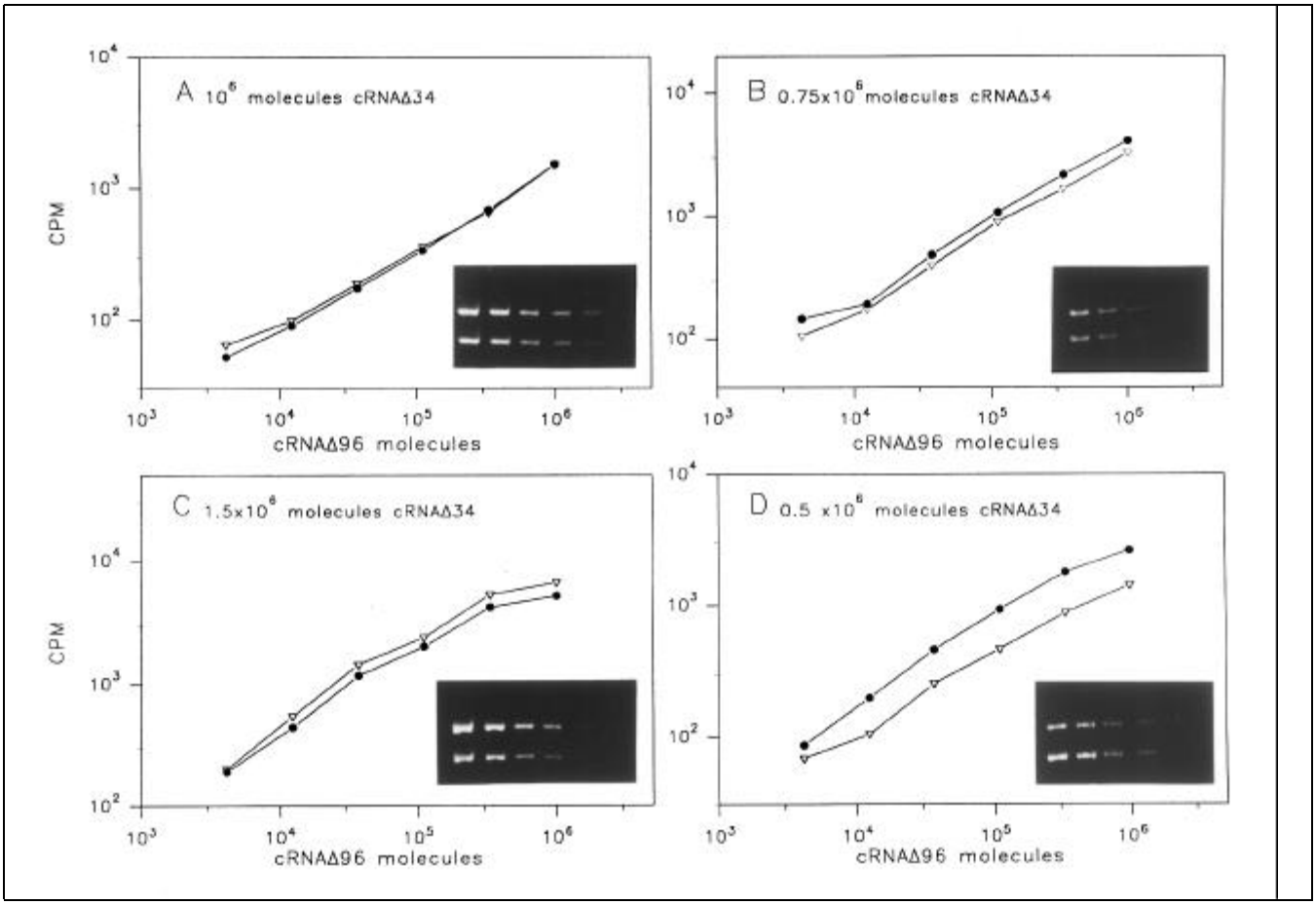

Figure 2. Visualization of the discrepancy between the amounts of TM and ICM. Varying quantities of cRNA Δ34 molecules (∇), as indicated in Panels A, B, C and D, and 10^6 molecules of cRNA Δ96 (●) were reverse-transcribed, followed by serial dilutions and amplification as described in Materials and Methods. The amplification products were separated using polyacrylamide gel electrophoresis and detected by ethidium bromide staining (see insert in each panel). The bands were excised from the gel and counted. The cpm from cRNA Δ34 and cRNA Δ96 were plotted against the quantity of cRNA Δ96 molecule contained in each dilution.

In order for RT-PCR to be quantitative, it is necessary to have nonbiased competition between the TM and the ICM during the exponential phase of the PCR. Unfortunately, nonbiased competition can be assumed only when the ratio of TM to ICM is 1:1. We designed experiments that purposely distorted the ratio of TM to ICM to determine how variations in this ratio influence the final quantification.

One way to visualize the effect of varying the TM to ICM ratio was to plot the cpm from ICM and from TM against the number of internal control molecules. The distances between the two curves illustrate the divergence between the amounts of TM and ICM. In Figure 2A, 10^6 molecules of cRNA Δ96 (ICM) was assayed with 10^6 molecules of cRNA Δ34 (TM). As expected, the curves were perfectly superimposed because TM and ICM were in nonbiased competition. However, variations in their respective concentrations induced biased competition effects. For example, when the amount of cRNA Δ34 was altered by 25% or 50% (i.e., by adding 0.75×10^6 or 1.5×10^6 molecules), the curves became slightly separated (Figure 2, B and C). The curves separated completely when the TM and ICM had a 2-fold concentration difference, as shown in Figure 2D.

The results from these same experiments were also quantified, as shown in Table 1B. A difference up to 1.5-fold between the TM and the ICM resulted in a deviation of approximately 10%, as determined from the known amount of "Molecules IN" and the calculated amount of "Molecules OUT". However, a divergence of 2-fold between the TM and the ICM caused the amount of Molecules OUT to be altered by 35%–60%, when compared to the number of Molecules IN (Table 1B).

To access to the accuracy of the assay, we represented the variation of the ratio of the Molecules IN/Molecules OUT as a function of the Molecules IN. The closer the Molecules IN/Molecules OUT ratio is to 1, the more precise is the assay. In Figure 3 we visualized the discrepancy caused by distorting the ICM to TM ratio. The practical limits of quantitative RT-PCR is depicted by a box that defines the situation where the ratio of Molecules IN/OUT remains linear with a variation of 10%. These conditions are satisfied when the TM/ICM ratio is between 0.66 and 1.5 (or the difference between TM and ICM is 1.5-fold). Outside these limits, the IN/OUT ratio diverged abruptly and the fidelity of the results subsequently diminished.

To test the sensitivity of quantitative RT-PCR, we analyzed the results from two different experiments where the ratio of TM and ICM was within the above-determined limits. In this analysis, we wanted to determine if minor differences between samples could be accurately quantified. As shown in Table 1C, two experiments were performed where the quantity of starting cRNA Δ34 differed by only 20%. A Student's *t* test was applied to the results of this quantification and were shown to be accurate at the level of 95% confidence.

From these experiments we determined some of the fundamental aspects controlling the accuracy and sensitivity of quantitative RT-PCR. The titration assay was a preliminary step employed to set up the quantitative assay. The quantitative assay was, however, necessary for very accurate quantification. By manipulating the ratio of TM and ICM, we were able to define the practical limits of quantitative RT-PCR. As a general rule, results are accurate to 90% if the difference between TM and ICM remains equal or under 1.5-fold. Lastly, we showed that this technique

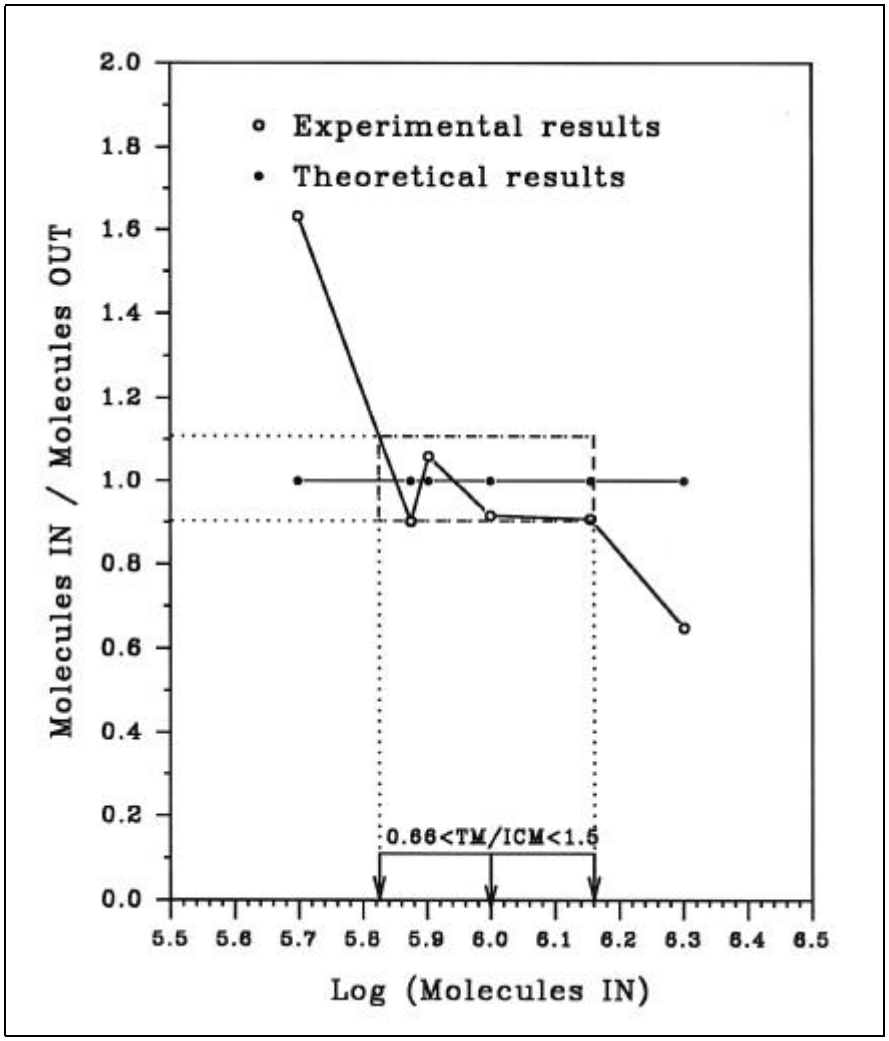

Figure 3. Accuracy of quantitative RT-PCR. A comparison between the experimental results and the theoretical results. This graph shows the variation between the number of molecules added at the beginning of the experiment and the number of calculated molecules (Molecules IN/Molecules OUT), against the number of molecules added at the beginning of the experiment (Molecules IN).

was sufficiently sensitive for measuring concentration differences as small as 20%. We demonstrated that under defined limits, quantitative RT-PCR is a very useful method to precisely quantify low abundance mRNA.

ACKNOWLEDGMENT

The authors wish to express their many thanks to Dr. Neil Insdorf for his invaluable help in the writing of the manuscript and for helpful discussions. The authors also thank Drs. Kelly and Nagano for introducing them to this method. This work was supported by a fellowship to F. Souazé from the Ministère de l'enseignement supérieur et de la recherche.

REFERENCES

1. **Aviv, H. and P. Leder.** 1972. Purification of biological active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA. *69*:1408-1412.
2. **Ferre, F., A. Marchese, P. Pezzoli, S. Griffin, E. Buxton and V. Boyer.** 1994. Quantitative PCR—an overview, p. 67-88. Polymerase Chain Reaction. Birkhauser Boston, Cambridge, MA.
3. **Gilliland, G., S. Perrin, K. Blanchard and H.F. Bunn.** 1990. Analysis of cytokine mRNA and DNA: detection and quantification by competitive polymerase chain reaction. Proc. Natl. Acad. Sci. USA *87*:2725-2729.
4. **Nagano, M. and P.A. Kelly.** 1994. Tissue distribution and regulation of rat prolactin receptor gene expression. Quantitative analysis by polymerase chain reaction. J. Biol. Chem. *269*:13337-13345.
5. **Piatak Jr., M., L. Ka-Cheung, B. Williams and J.D. Lifson.** 1993. Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species. BioTechniques *14:*70-81.
6. **Sambrook, J., E.F. Fritsch and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
7. **Stieger, M., C. Démollière, K. Ahlborn-laake and J. Mous.** 1991. Competitive polymerase chain reaction assay for quantification of HIV-1 DNA and RNA. J. Virol. Methods *34*:149-160.
8. **Tanaka, K., M. Masu and S. Nakanishi.** 1990. Structure and functional expression of the cloned rat neurotensin receptor. Neuron *4*:847-854.
9. **Wang, A.M., M.V. Doyle and D.F. Mark.** 1989. Quantitation of mRNA by the polymerase chain reaction. Proc. Natl. Acad. Sci. USA *86*:9717-9721.

Received 30 October 1995; accepted 31 January 1996.

Address correspondence to:

Patricia Forgez
INSERM Unit 339
Pavillon Raoul Kourilsky
Hôpital Saint-Antoine
184 rue du Faubourg Saint-Antoine
75012 Paris, France
Internet: forgez@idf.ext.jussieu.fr