



Quantitative RT-PCR: Rapid Construction of Templates for Competitive Amplification

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Reverse transcription coupled with PCR (RT-PCR) is a valuable technique for detecting rare RNA species present in cells or tissues. Methodologies that extend RT-PCR to provide quantitative information have been described, thus coupling the extreme sensitivity of qualitative amplification with the added ability to quantitatively measure the level of RNA expression under different experimental conditions. In this report, we demonstrate the design and construction of a control template that can be used for competitive RT-PCR.

INTRODUCTION

Reverse transcription PCR^(a) (RT-PCR) methodologies are some of the most sensitive ways to obtain quantitative information regarding rare RNA species. Although several quantitative RT-PCR techniques have been demonstrated, one of the most useful and accurate amplification techniques for analyzing RNA levels is competitive RT-PCR (1-5). This assay is based on the competitive coamplification of known amounts of a control template within the experimental reactions. Competition for amplification reactants between the target template and the control template allows the determination of the equivalence point at which the control template and target template concentrations are equal. Careful construction of the control template is required to ensure precise quantitation of the target template.

In this report we demonstrate a simple and quick method for generating homologous competitive templates for RT-PCR and discuss their use. As illustrated in [Figure 1](#), four general steps are required for this purpose: 1) production of intact and truncated RNA templates, 2) determination of linear range of amplification, 3) determination of relative amplification efficiencies and 4) experimental determination of endogenous RNA levels.

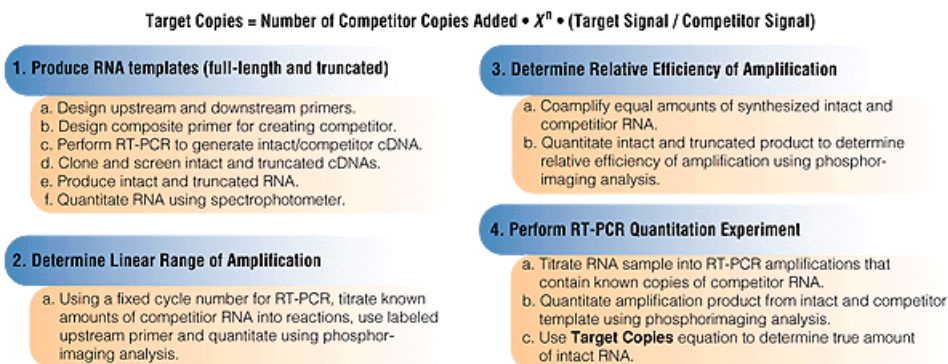


Figure 1. The formula for quantitation of target RNA in a sample. Formula parameters are generated in four steps. An outline of how each step is accomplished is listed, and specific details for each step are given in the text.

TEMPLATE DESIGN AND GENERATION

We chose human beta-actin RNA to demonstrate the construction of a template appropriate for quantitative RT-PCR. A precise comparison between a target RNA species and the added control template requires that the amplification efficiency be as similar as possible between the two RNA species. To minimize differences in the kinetics of primer annealing, which can lead to errors in quantitation, amplification of the internal control template must use the same primer set that is used to amplify the target template. Kinetic differences that may occur during the reverse transcription and amplification steps are also minimized by using a homologous control template sequence that is similar to the target RNA sequence (6,7). However, the control must be designed to allow discrimination between amplification products derived from both templates; the easiest way to distinguish between target and control products is by size difference. Although several strategies can be used to generate size-altered competitive templates, the amplification technique used here is simple, efficient and rapid.

Three primers were required in these experiments. Two primers, designated US and DS, were designed to generate an intact 511 bp product during RT-PCR amplification of the beta-actin RNA ([Figure 2](#)). An additional upstream primer (USc) was required solely for

construction of the competitive template by RT-PCR amplification. The USc primer was a composite of the US primer sequence and a sequence region located 132 nucleotides downstream in the RNA. When the USc and DS primers are used to amplify total RNA, sequences between the composite oligonucleotides are effectively eliminated from the amplified product to generate the delta beta-actin cDNA (Figures 2 and 3). The truncated template contains binding sites for the US and DS primers but is truncated relative to the native sequence. The difference in size between the full-length and truncated RT-PCR products allows discrimination between product generated from endogenous RNA and competitive RNA (cRNA) templates using the US and DS primers in RT-PCR.

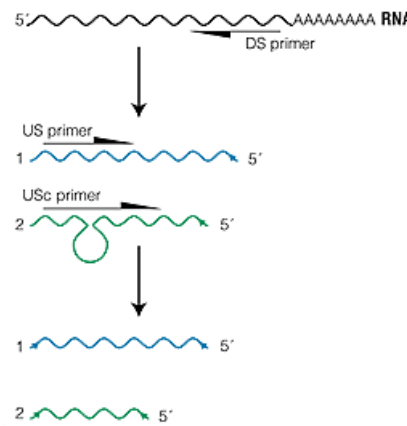


Figure 2. Primers required for competitive RT-PCR. The upstream sequence (US; 5'-TGTGATGGTGGGAATGGGTCAG-3') and downstream sequence (DS; 5'-TTTGATGTCACGCACGATTTCC-3') primers are complementary to the human beta-actin sequence and are designed to amplify a 511 base region of the mRNA. The upstream composite sequence (USc; 5'-TGTGATGGTGGGAATGGGTCAGGCCAACC GCGAGAAGATGACCCAG-3') primer is used to produce a truncated cDNA template of 318bp. The cDNA is used to generate a competitive cRNA transcript that is amplified with the US and DS primers.

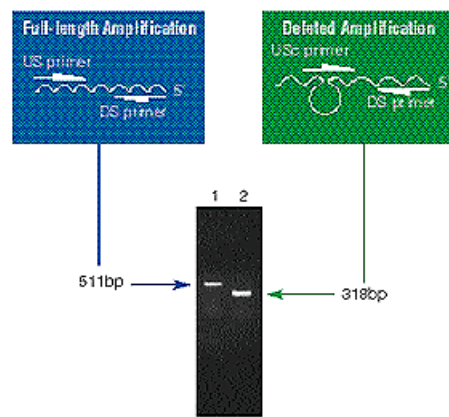


Figure 3. Generation of a truncated cDNA template compared with the native beta-actin product. Total RNA was isolated from human blood using Promega's SV Total RNA Isolation System (Cat.# Z3100). Five microliters (approximately 75ng) of the RNA preparation were amplified using Promega's Access RT-PCR System (Cat.# A1250) and primers US and DS (lane 1) or USc and DS (lane 2). The RNA was reverse transcribed (48°C for 45 minutes) and the reverse transcriptase inactivated (94°C for 2 minutes). This was followed by 40 amplification cycles of denaturation (94°C for 30 seconds), annealing (60°C for 1 minute) and extension (68°C for 1 minute) with a final extension step (68°C for 7 minutes). The amplification products were analyzed by 1.5% agarose gel electrophoresis to ensure that the intact and truncated cDNAs were produced (compare lanes 1 and 2). The RT-PCR products were purified, cloned and analyzed as described in the text.

The full-length and truncated amplification products were purified directly using Promega's Wizard[®] PCR Preps DNA Purification System^(b) (Cat.# A7170), and an aliquot was ligated directly into the pGEM[®]-T Easy Vector^(c,d) (Cat.# A1380), according to the *pGEM[®]-T and pGEM[®]-T Easy Vector Systems Technical Manual #TM042* (10,11). JM109 competent cells were transformed with the resulting ligation reaction, plated and screened by blue/white color selection. Potential positives were rapidly screened by PCR using the US and DS primers to check for insertion of the correct template sequence, and with the DS primer and a T7 primer to confirm the orientation of the inserted DNA. Plasmid DNAs isolated from one native clone and one competitive clone were used for preparation of RNA templates using Promega's RiboMAX[™] Large Scale RNA Production System^(e) (Cat.# P1300). The RNA was purified and the concentration of RNA was quantitated by spectrophotometric analysis (using one A₂₆₀ unit of single-stranded RNA = 40µg/ml [12]).

Careful quantitation of the RNA templates is critical, as all subsequent analyses will be based upon the determined RNA concentration. The RNA was further analyzed by agarose gel electrophoresis and aliquots were tested for residual plasmid DNA contamination by PCR amplification using the US and DS primers to confirm the quality and purity of the RNA templates (data not shown).

DETERMINATION OF LINEAR AMPLIFICATION RANGE

Possible differences in amplification efficiencies between target and internal control templates can be minimized by ensuring that the amplification reaction remains in the linear range (6,8). The linear range can be defined as the concentrations of starting template for which the reaction remains in the exponential phase of amplification for a given number of cycles. This range was determined for a 35-cycle amplification by titrating a calculated number of synthetic delta beta-actin RNA transcripts into standard reactions using Promega's Access RT-PCR System^(a), and quantitating the amount of ³²P-labeled US primer incorporated into the amplified product after gel electrophoresis and analysis (Figure 4). The accumulation of amplified product increases linearly up to 10⁸ copies of delta beta-actin RNA input. We chose to use 10⁴ copies of internal control RNA in subsequent experiments, as this was well within the linear range of amplification and provided sufficient product for detection.

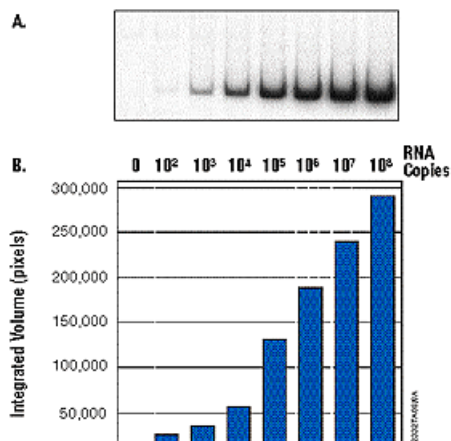


Figure 4. Determination of the linear amplification range. RNA was synthesized *in vitro* using Promega's RiboMAXTM RNA Production System. After production, the synthesized RNA was purified using Promega's SV Total RNA Isolation System and aliquots were quantitated by spectrophotometric analysis at 260nm. The number of RNA copies per microliter was determined by using the calculated molecular mass of each RNA to convert the concentration to moles/ml and multiplying by Avagadro's number (6.022×10^{23}). The linear range of amplification was determined for a 35-cycle amplification by titrating a known number of delta beta-actin cRNA transcripts into standard reactions that contained 50pmol US and DS primers and trace amounts of ³²P-labeled US primer (approximately 0.01-0.02pmol per reaction). Conditions for reverse transcription and PCR were as described in the legend to Figure 3, except 35 cycles were performed. **Panel A:** After amplification, equal amounts of each reaction were resolved by nondenaturing polyacrylamide gel electrophoresis and visualized by autoradiography. **Panel B:** The radioactive gels were scanned and analyzed by phosphorimaging analysis. The amount of radioactivity was determined by pixel integration of the region of each lane represented by the delta beta-actin RT-PCR product.

DETERMINATION OF RELATIVE AMPLIFICATION EFFICIENCY

A variety of sequence- and size-dependent factors may affect the efficiency of amplification of intact and truncated control templates. To directly determine the efficiency of amplification, equivalent copy numbers of synthesized intact beta-actin and delta beta-actin RNA transcripts were coamplified in the presence of trace amounts of ³²P-labeled US primer, and the resulting products were analyzed and quantitated by phosphorimaging analysis. Smaller templates are often amplified more efficiently than longer templates, and as can be seen in Figure 5, the delta beta-actin RNA template was amplified much more efficiently than the intact template. Phosphorimaging analysis was used to quantitate the amount of radioactivity present in the amplified product derived from the intact and truncated RNA templates, and the relative efficiency was calculated. The amplification efficiency per cycle may be calculated as: $X = (\text{delta beta-actin value}/\text{beta-actin value})^{1/n}$, where n is the number of amplification cycles. In these experiments, the delta beta-actin cRNA template was amplified an average of 1.058 times more efficiently than the full-length template per cycle ($X = 1.058$). This efficiency factor is then incorporated into the calculation of the number of endogenous target copies present in an unknown RNA sample.

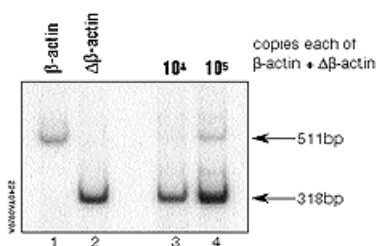


Figure 5. Determination of amplification efficiency of intact and truncated RNA. Equal numbers of copies of *in vitro* synthesized RNA (10⁴ copies, lane 3, or 10⁵ copies, lane 4) corresponding to intact beta-actin target or the delta beta-actin RNA product were coamplified using the US and

DS primer pairs. Conditions for reverse transcription and PCR were as in [Figure 4](#). Radioactivity in the band representing either the intact (upper band) or truncated (lower band) RT-PCR product was quantitated by phosphorimaging analysis, and these data were used to determine the amplification efficiency of the delta beta-actin target versus the beta-actin target as explained in the text. For comparison, lanes 1 and 2 contain amplified intact beta-actin RNA and delta beta-actin target, respectively.

QUANTITATION OF ENDOGENOUS BETA-ACTIN TRANSCRIPTS

Whole human blood was used as starting material for the isolation of total RNA using Promega's SV Total RNA Isolation System^(f). Quantitative RT-PCR amplifications were assembled by making a master mix that contained all of the components required for amplification plus 1×10^4 copies of the delta beta-actin RNA template per reaction. Trace quantities of ³²P-labeled US primer were included in the reactions to enable precise phosphorimaging quantitation of amplified material. Total RNA to be quantitated was titrated, 0.355ng to 22.2ng, into reactions and amplification performed as recommended in the Access RT-PCR System (9). Equivalent aliquots of each reaction were resolved by polyacrylamide gel electrophoresis and quantitated by radioactive detection ([Figure 6](#)). The reaction in lane 2 of [Figure 6](#) was used for quantitation, as this sample best represented the signal obtained when equivalent numbers of target and competitor molecules were present in a reaction (compare with [Figure 5](#), lane 3). After quantitation of the bands representing beta-actin and delta beta-actin, the number of endogenous delta beta-actin transcripts present in the total RNA samples can be calculated by:

$$\text{Target copies} = (\text{delta beta-actin copies added}) \cdot X^n \cdot (\text{beta-actin value}/\text{delta beta-actin value})$$

Where delta beta-actin copies in reaction = 10^4 (step 2), $X = 1.058$ (step 3), and $n = 35$ (number of amplification cycles). This equation generates a value of 2.03×10^4 endogenous beta-actin copies per 0.89 μ g of total RNA.

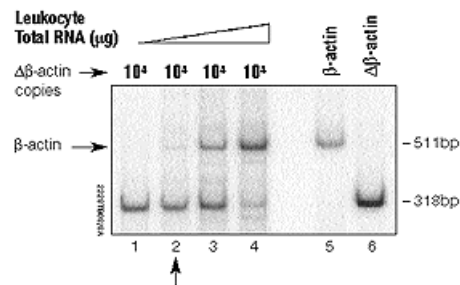


Figure 6. Quantitation of endogenous beta-actin RNA levels in leukocytes. RT-PCR amplifications were assembled by titrating increasing amount of human leukocyte total RNA into reactions that contained the US and DS primer in conjunction with trace amounts of ³²P-labeled US primer (lanes 1-4). Competitive delta beta-actin RNA (10^4 copies) was included in each amplification reaction. Conditions for reverse transcription and PCR were as in [Figure 4](#). Equal volumes of each reaction were resolved by nondenaturing polyacrylamide gel electrophoresis and analyzed by phosphorimaging analysis. Data from lane 2 was used to quantitate endogenous beta-actin RNA levels as described in the text. Lanes 5 and 6 contain amplified intact beta-actin RNA and delta beta-actin target, respectively.

CONCLUSIONS

This article introduces many of the techniques involved in one method of quantitative RT-PCR. The procedures are not a rigorous experimental determination of the levels of endogenous RNA species, but rather are designed and conducted for illustrative purposes. Statistically robust determinations require sample averaging over several experiments, perhaps with independently derived RNA samples. Extreme care should be exercised during quantitation of the amount of competitive RNA introduced into the amplifications, as this forms the basis of all subsequent experimental determinations. Measurements should be made within the linear range of amplification, and this value should be determined for each target amplified. The amplification efficiency between full-length and competitive RNAs can vary significantly, and this deviation should be incorporated into calculations when determining the copies of endogenous template. If carefully applied, competitive amplification techniques can provide the most sensitive quantitative analysis of RNA species present in a complex nucleic acid sample.

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Ordering Information

Product	Size	Cat.#
Access RT-PCR System	100 reactions	A1250
pGEM [®] -T Easy Vector System I	20 reactions	A1360
pGEM [®] -T Easy Vector System II	20 reactions	A1380
RiboMAX [™] Large Scale RNA Production System - T7		P1300
SV Total RNA Isolation System	50 preps	Z3100
Wizard [®] PCR Preps DNA Purification System	50 preps	A7170

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^(d)U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different bacteriophage RNA polymerase promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.

^(e)U.S. Pat. No. 5,552,302, European Pat. No. 0 422 217 and Australian Pat. No. 646803 have been issued to Promega Corporation for the methods and compositions for production of human recombinant placental ribonuclease inhibitor (PRI). Inhibitors of Angiogenin, which comprises a segment of human PRI, is the subject of U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687 assigned to the President and Fellows of Harvard College and exclusively licensed to Promega Corporation.

^(f)Patent Pending.

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