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Gene-specific monitoring of T7-based RNA amplification by real-time quantitative PCR

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T7-based RNA amplification is applied when there is insufficient RNA. The overall extent of amplification can be measured spectrophotometrically (i.e., quantifying RNA yields), but this measurement does not provide information about the RNA amplification of individual genes. Here we describe a method applying real-time quantitative PCR, which enables the monitoring of RNA amplification of individual genes. The amount of RNA before and after T7-based RNA amplification was determined by real-time quantitative PCR for three housekeeping genes: β-2-microglobulin, porphobilinogen deaminase, and serine dehydratase, which are, respectively, a high, intermediate/low, and low copy transcript. Real-time quantitative PCR appeared to be suitable to determine the extent of RNA amplification, as was reflected by the low intra- and inter-run coefficients of variation of cycle threshold of 1.1%–2.1%. The application of real-time quantitative PCR showed that T7-based RNA amplification is reproducible but might introduce a sequence-specific bias. Real-time quantitative PCR is a novel approach to monitor RNA amplification and is particularly suited to study RNA amplification of individual genes.

INTRODUCTION

With the completion of the human genome project, unraveling the function of genes and their relation to a certain phenotype is, at present, the principal challenge in genetic research. Microarray techniques have recently proven useful in the discovery of genes involved in physiological processes (1–3). One of the rate-limiting steps in microarray experiments is the availability of relatively large amounts of total RNA (10–100 µg) needed for use in standard microarray platforms. When only a limited amount of RNA is available (e.g., small tissue samples or laser-microdissected material), one must use methods either to amplify the RNA or to increase the signal intensity to overcome the RNA shortage (4–6). Several amplification protocols have been developed, of which T7-based RNA amplification is the most frequently used approach (7,8).

The extent of RNA amplification is in general quantified by the use of a spectrophotometer or fluorometer. Roughly, the polyadenylation [poly(A)$^+$] RNA content of a cell is estimated to be 3.3% of the total RNA content (7), so a spectrophotometric measurement of the antisense RNA can be used to calculate a rough estimate of the overall extent of amplification (9). However, microarray techniques and real-time quantitative PCR could be used to determine the extent of RNA amplification of single genes (6,9). More importantly, they could also give additional information about the linearity of T7-based RNA amplification (i.e., whether the relative abundance of different genes is preserved after RNA amplification).

Here we describe a detailed method to study T7-based RNA amplification of single genes by real-time quantitative PCR using molecular beacons (10). Three housekeeping genes were used for this purpose, β-2-microglobulin (*B2M*), porphobilinogen deaminase (*PBGD*), and serine dehydratase (*SDH*), which are, respectively, a high, intermediate/low, and low copy transcript. This is the first study in which a detailed method utilizing real-time quantitative PCR to monitor the characteristics of T7-based RNA amplification is described.

MATERIALS AND METHODS

Characteristics of Real-Time Quantitative PCR

Oligonucleotide and molecular beacon design. Oligonucleotides used for the real-time quantitative PCR experiments were designed according to the GenBank® sequences (rat PBGD mRNA X06827; rat B2M mRNA NM_012512; rat SDH mRNA J03863; and rat SDH gene X13119) using Oligo design software version 3.4 (Table 1). Molecular beacon probes were designed using the online folding program mfold (http://www.bioinfo.rpi.edu/applications/mfold/old/dna) (Table 1). The PBGD (I) molecular beacon was designed at 435 bp, PBGD (II) at 1344 bp, B2M at 501 bp, and SDH at 477 bp upstream from the poly(A) signal. Biolegio BV (Malden, The Netherlands) synthesized all oligonucleotides and molecular beacons.

Real-time quantitative PCR. Real-time quantitative PCR studies were carried out in a total volume of 50 µL containing 2 µL cDNA, 100 ng molecular beacon, 200 nM forward and reverse primers, 200 µM dNTPs, 10× AmpliTaq Gold® amplification buffer (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands), 4 mM $MgCl_2$, and 1.5 U AmpliTaq Gold DNA polymerase. PCR conditions were as follows: cycling was preceded by 10 min activation of AmpliTaq Gold at 95°C, followed by 46 cycles of 95°C for 30 s, 58°C for 45 s, and 72°C for 30 s. Real-time quantitative PCR was performed on the iCycler™ iQ (Bio-Rad Laboratories, Veenendaal, The Netherlands), and fluorescence was measured during each annealing step. The baseline was set at 10 times the standard deviation of the noise of 10 amplification cycles. Genomic rat DNA was included as a template in a separate reaction to assess the amplification of potential pseudogenes.

PCR efficiency of molecular beacon assays. The linearity of each assay was derived from the correlation coefficient of the standard curve (R^2). The efficiency of each real-time quantitative PCR was analyzed by determining the cycle threshold (C_T) values of five different dilutions of cDNA (corresponding with 1 µg/µL to 320 pg/µL total RNA) by plotting the C_T values against the log value of each dilution. A slope of -3.32 represents an efficiency of 100% (i.e., the amount of PCR products is doubled during each cycle: 2^n, where n is the number of cycles). Each real-time quantitative PCR was optimized so that for each gene the 95% confidence interval (CI) of the slope (95% CI) included -3.32.

Reproducibility of real-time quantitative PCR. Reproducibility of real-time quantitative PCR, as determined by whether or not serial reactions result in the same C_T value, was assessed by performing 20 times the same cDNA standard in two dilutions (5 and 250 times) within one run (intra-run variation) and between 20 separate runs (inter-run variation) using the PBGD (I) molecular beacon.

Linearity of First-Strand cDNA Synthesis

The linearity of first-strand cDNA synthesis was tested over a broad range of RNA concentrations by diluting total RNA from 1 µg/L to 100 pg/µL. First-strand cDNA transcription was performed, and C_T values were obtained by real-time quantitative PCR using the B2M molecular beacon. The C_T values were plotted against the log value of each RNA dilution for this experiment, and a slope of -3.32 would indicate equal first-strand cDNA synthesis efficiency for each dilution.

Table 1. Rat Sequences of Oligonucleotides and Molecular Beacons

Target	Sequence
B2M	
Sense oligonucleotide	5′-CGTGCTTGCCATTCAGAAAAC-3′
Antisense oligonucleotide	5′-TCTGAGGTGGGTGGAACTGAG-3′
Molecular beacon[a]	5′-TxR-CGCACGTTCAAGTGTACTCTCGC-CATCCACCCGTGCG-dabcyl-3′
PBGD (I)	
Sense oligonucleotide	5′-CCAGGTCCCTGTTCAGCAAG-3′
Antisense oligonucleotide	5′-CCCAGGTTCTCAGCAGCTAGC-3′
Molecular beacon[a]	5′-TxR-CCGACGGTTCCGGGCAGTGATTC-CAACCAACGTCGG-dabcyl-3′
PBGD (II)	
Sense oligonucleotide	5′-CGGTAACGGCGGCGCGGC-3′
Antisense oligonucleotide	5′-CGGTCTGTATGCGAGCCAGCTGAC-3′
Molecular beacon[a]	5′-TxR-CGGACCGAAGAAAACGGCTCAATGAT-GAGGGGGTCCG-dabcyl-3′
SDH	
Sense oligonucleotide	5′-CTGCCCAAGATCACCAGTG-3′
Antisense oligonucleotide	5′-CTCGATAGCAGTCACAGCCTCC-3′
Molecular beacon[a]	5′-Cy5-CGCACGCCCTGAAGCTGTTTTACGAA-CACCCCACGTGCG-BHQ_2-3′

[a] Molecular beacon sequences with corresponding fluorophore (5′), quencher (3′), and stem sequences (underlined); BHQ_2, black hole 2 quencher; TxR, Texas Red®.

Monitoring T7-Based RNA Amplification with Real-Time Quantitative PCR

One round of RNA amplification was performed on total rat kidney RNA (n = 4) (BD Biosciences Clontech, Erembodegem, Belgium) according to Baugh et al. (9) using their 10 µL scale protocol. One-tenth of the antisense RNA (n = 4) and of the unamplified RNA control (n = 1) was reverse-transcribed to cDNA using 0.5 µg random hexamers (Promega, Leiden, The Netherlands) and 200 U SUPERSCRIPT™ II reverse transcriptase (Invitrogen BV, Breda, The Netherlands) in a final reaction volume of 20 µL according to standard procedures. cDNA was diluted 10 times, and 2 µL were subsequently used in the real-time quantitative PCR for the housekeeping genes *PBGD*, *B2M*, or *SDH*.

Quantification of the Extent of RNA Amplification

The extent of amplification was quantified by measuring the A_{260} of the obtained antisense RNAs (n = 4) on a spectrophotometer (Shimadzu Benelux, Hertogenbosch, The Netherlands), with the assumption that total RNA consists of 3.3% of poly(A)$^+$ RNA.

In comparison, real-time quantitative PCR was used to determine the extent of the amplification of three housekeeping genes by comparative quantitation (11,12) using the following equation:

$$\frac{R_a}{R_0} = 2^{\Delta C_T} \qquad \text{[Eq. 1]}$$

In Equation 1, R_a/R_0 is the amount of antisense RNA (R_a) relative to the unamplified RNA (R_0). ΔC_T is the difference in C_T of unamplified RNA and antisense RNA ($C_{T,R0}$-$C_{T,Ra}$) after first-strand cDNA synthesis (Figure 1). The unamplified control is an RNA sample that underwent the same RNA amplification procedure, except for the in vitro transcription step, during which the sample was stored at -80°C. During each PCR cycle, the amount of target is doubled (2^{CT}).

Statistical Analysis

The difference in the extent of amplification monitored by PBGD (I) and (II) quantitative PCR assays was determined by a Student's t test, and differences between the three housekeeping genes were estimated by one-way analysis of variance (ANOVA). Differences were accepted as significant when $P <$ 0.05. All P values were two-tailed. Linear regression was performed using the Microsoft® Excel® Regression Analysis ToolPak (Microsoft Office® 2000).

RESULTS AND DISCUSSION

Characteristics of Real-Time Quantitative PCR

PCR efficiency of molecular beacon assays. All real-time quantitative PCR assays for B2M, PBGD (I) and (II), and SDH were optimized to obtain linear standard curves. Regression equations for the different molecular beacon assays were: PBGD (I): $y = -3.57x + 29.6$, $R^2 = 0.995$; PBGD (II): $y = -3.18x + 31.9$, $R^2 = 0.999$; B2M: $y = -3.27x + 24.1$, $R^2 = 0.999$; SDH: $-3.17x + 32.9$, $R^2 = 0.990$. Real-time quantitative PCR efficiencies were calculated from the slopes of the standard curves [$10^{(-1/\text{slope})}$], which resulted in efficiencies of 102% (E = 2.02) for B2M, 91% (E = 1.91) for PBGD (I), 106% (E = 2.06) for PBGD (II), and 107% (E = 2.07) for SDH. Each real-time quantitative PCR efficiency did not differ significantly from 100% (i.e., the 95% CI of each slope included -3.32 [B2M 95% CI (-3.11 to -3.43), PBGD (I) 95% CI (-3.91 to -3.23), PBGD (II) 95% CI (-3.35 to -3.01), and SDH 95% CI (-3.61 to -2.74)]).

Reproducibility of real-time quantitative PCR. The reproducibility of the real-time quantitative PCR assay (i.e., whether serial reactions result in the same C_T) was determined by real-time quantitative PCR using the PBGD (I) molecular beacon. Intra-run variation resulted in a mean C_T of 28.4 ± 0.3 (CV_{CT} = 1.1%) and 33.9 ± 0.7 (CV_{CT} = 2.1%) for, respectively, a 5 and 250 times diluted cDNA standard ($n = 20$). The inter-run mean C_T values for both dilutions were, respectively, 28.2 ± 0.4 (CV_{CT} = 1.4%, $n = 20$) and 34.1 ± 0.7 (CV_{CT} = 2.1%, $n = 20$). These CV_{CT} values are in line with a previously published study (13). Therefore, real-time quantitative PCR is reproducible and can be suitably applied in assays in which a precise estimate of target abundance is desirable (e.g., in the determination of the extent of RNA amplification of separate genes). Although real-time quantitative PCR leads to a small variation in C_T values of only 1%–2%, the variation in the extent of amplification, however, will automatically be much larger (10%–30%) because of the exponential character of this calculation ($2^{\Delta CT}$).

Spectrophotometric Measurement of the Extent of Amplification

The extent of amplification of four separate RNA amplification reactions versus one unamplified control reaction was determined spectrophotometrically. This resulted in 3.3 ± 0.9 µg antisense RNA, which corresponded with a mean amplification of 100.4 ± 28.4 times, with the assumption that total RNA consists of 3.3% of poly(A)$^+$ RNA, which is in line with the results of Baugh et al. (9).

Monitoring T7-Based RNA Amplification with Real-Time Quantitative PCR

Gene-specific extent of amplification. Real-time quantitative PCR analysis was applied to the antisense RNA and the unamplified control to calculate the extent of gene-specific RNA amplification of three housekeeping genes and resulted in a mean ΔC_T of 5.6 ± 0.2 for B2M, 6.6 ± 0.2 for PBGD (I), and 4.0 ± 0.4 for SDH. The mean extent of amplification was calculated from the ΔC_T using Equation 1, and this resulted in 47.7 ± 7.1 times for B2M, 99.1 ± 11.9 times for PBGD (I), and 16.1 ± 5.1 times for SDH. Comparing the results of PBGD (I), B2M, and SDH showed that a significant bias of amplification had occurred (ANOVA, $P < 0.001$). Particularly, the low copy transcript SDH showed a significantly lower extent of amplification, which raises the question whether the extent of amplification is related to the copy number. Similar studies using microar-

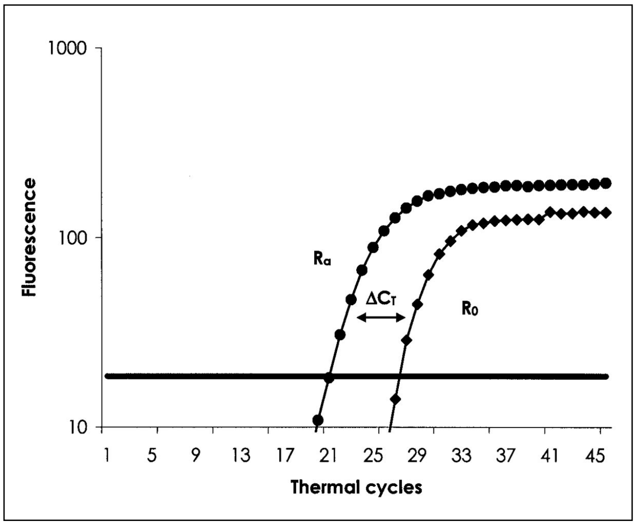

Figure 1. Example of calculation of the extent of T7-based RNA amplification by real-time quantitative PCR. Real-time quantitative PCR results of β-2-microglobulin (B2M) showing the plot of amplified RNA (R_a) and the unamplified control (R_0). The extent of amplification can be calculated by $2^{\Delta CT}$.

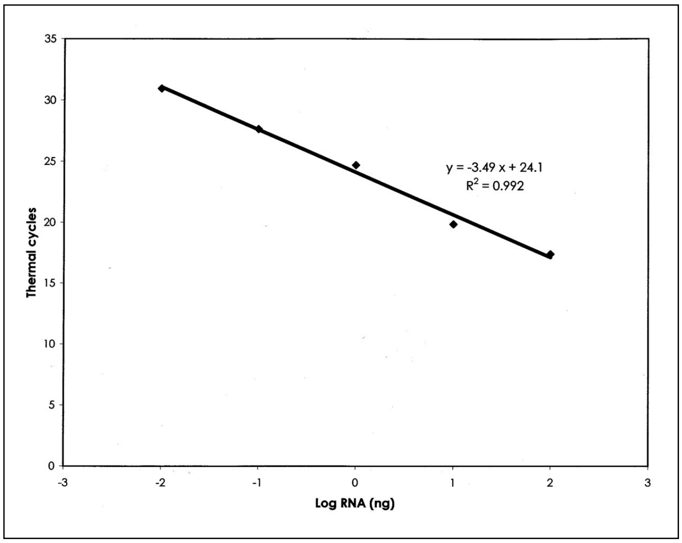

Figure 2. Efficiency of first-strand cDNA synthesis for different amounts of total RNA. Standard curve of real-time quantitative PCR using β-2-microglobulin (B2M) for RNA dilution series of 1 µg/µL to 100 pg/µL with the corresponding regression equation is shown.

rays to study target-dependent amplification were recently published (6,9), which showed that RNA amplification is not dependent on the initial copy number but rather on differences in the sequence itself. Therefore, we speculate that the observed bias of amplification might already be introduced during first-strand cDNA synthesis preceding the amplification step. Because of the GC-richness of some targets, secondary structures can easily be formed, which could perturb the efficiency of reverse transcription. Obviously, when mRNA copies of different targets are not equally reversed-transcribed to cDNA, this will result in a bias after amplification. In addition, it is not known whether first-strand cDNA synthesis is linear for different amounts of input RNA. To test the linearity of first-strand cDNA synthesis, we performed a real-time quantitative PCR experiment using B2M on a range of RNA dilutions. This resulted in a linear standard curve ($R^2 = 0.992$; Figure 2) and in a similar PCR efficiency (E = 93%, 95% CI -4.08 to -2.90) as obtained for the cDNA dilution series (E = 102%, 95% CI -3.11 to -3.43). For a given target gene, these results demonstrated that first-strand cDNA synthesis is linear for different amounts of total RNA (1 µg/µL to 100 pg/µL). However, the results of this experiment do not give information about the extent of reverse transcription efficiency for each separate target. Therefore, when gene expression levels of limited RNA samples are compared using T7-based RNA amplification, only relative quantification is valid, as this compares the intra-target differences (i.e., gene expression of a particular gene in sample vs. control).

Reproducibility of T7-based RNA amplification. In our study, real-time quantitative PCR was applied to monitor the reproducibility of RNA amplification (i.e., whether several separate RNA amplification reactions would result in the same extent of amplification). The results from four separate reactions showed that T7-based RNA amplification was reproducible [mean ΔC_T of 5.6 ± 0.2 for B2M, 6.6 ± 0.2 for PBGD (I), and 4.0 ± 0.4 for SDH], as small standard deviations were obtained for each of the three genes tested (SD < 0.5). Other studies, in which microarray techniques were used, have also shown that T7-based RNA amplification is a reproducible technique (6,9,14).

Distance of molecular beacon towards poly(A) signal. During in vitro transcription, antisense RNA is transcribed from the 3′ end, resulting in products with lengths of 500–1500 bp. Therefore, the position of the molecular beacon towards the poly(A) signal could influence the calculation of the extent of amplification. For this purpose, two molecular beacons were developed for one gene, located at a distance of 435 bp [PBGD (I)], and 1344 bp upstream from the poly(A) signal [PBGD (II)]. Real-time quantitative PCR experiments using both molecular beacons resulted in a significantly lower mean extent for PBGD (II) compared with PBGD (I) (16.1 ± 5.1 times vs. 99.1 ± 11.9 times, respectively, $P < 0.001$). Therefore, we fixed the position of other molecular beacons (B2M and SDH) at a distance of approximately 500 bp upstream from the poly(A) signal. Importantly, these results also show that when antisense RNA is used in real-time quantitative PCR or microarray experiments, the oligonucleotides or cDNA clones spotted on an array should represent the 3′ end of the transcripts.

In the present study, we give a detailed description of real-time quantitative PCR using molecular beacons to monitor T7-based RNA amplification, and we applied this method to monitor several characteristics of T7-based RNA amplification. Currently, microarray techniques are also being used to study differences after T7-based RNA amplification (6,9,14), but this requires more expertise and expensive facilities. At the moment, real-time quantitative PCR is a frequently used technique (15) that is relatively simple and inexpensive; therefore, it is accessible to most genetic laboratories. Results of the present study show that real-time quantitative PCR can be easily applied to monitor the extent of RNA amplification of individual genes. Application of real-time quantitative PCR demonstrated that T7-based RNA amplification is reproducible even though it might introduce a sequence-specific bias for the three housekeeping genes studied here. Ideally, the extent of amplification of all targets should be preserved during RNA amplification. Nevertheless, gene expression profiling is usually executed to compare profiles between two samples, for the identification of up- or down-regulated genes. For this purpose, a reproducible bias in amplification is acceptable between different targets, as long as two samples that are compared have both been amplified using the same amplification technique. In combination with very promising techniques like laser capture microdissection, T7-based RNA amplification will widen the frontier with which to perform gene expression profiling of even isolated single cells.

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REFERENCES

1. **Schena, M., D. Shalon, R.W. Davis, and P.O. Brown.** 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science *270*: 467-470.
2. **Chee, M., R. Yang, E. Hubbell, A. Berno, X.C. Huang, D. Stern, J. Winkler, D.J. Lockhart, et al.** 1996. Accessing genetic information with high-density DNA arrays. Science *274*:610-614.
3. **Duggan, D.J., M. Bittner, Y. Chen, P. Meltzer, and J.M. Trent.** 1999. Expression profiling using cDNA microarrays. Nat. Genet. *21*: 10-14.
4. **Ohyama, H., X. Zhang, Y. Kohno, I. Alevizos, M. Posner, D.T. Wong, and R. Todd.** 2000. Laser capture microdissection-generated target sample for high-density oligonucleotide array hybridization. BioTechniques *29*:530-536.
5. **Luo, L., R.C. Salunga, H. Guo, A. Bittner, K.C. Joy, J.E. Galindo, H. Xiao, K.E. Rogers, et al.** 1999. Gene expression profiles of laser-captured adjacent neuronal subtypes. Nat. Med. *5*:117-122.
6. **Scheidl, S.J., S. Nilsson, M. Kalen, M. Hellstrom, M. Takemoto, J. Hakansson, and P. Lindahl.** 2002. mRNA expression profiling of laser microbeam microdissected cells from slender embryonic structures. Am. J. Pathol. *160*:801-813.
7. **Brady, G.** 2000. Expression profiling of single mammalian cells—small is beautiful. Yeast *17*:211-217.
8. **Van Gelder, R.N., M.E. Von Zastrow, A. Yool, W.C. Dement, J.D. Barchas, and J.H. Eberwine.** 1990. Amplified RNA synthesized from limited quantities of heterogenous cDNA. Proc. Natl. Acad. Sci. USA *87*:1663-1667.
9. **Baugh, L.R., A.A. Hill, E.L. Brown, and C.P. Hunter.** 2001. Quantitative analysis of mRNA amplification by in vitro transcription. Nucleic Acids Res. *29*:e29.
10. **Tyagi, S. and F.R. Kramer.** 1996. Molecular beacons: probes that fluoresce upon hybridization. Nat. Biotechnol. *14*:303-308.
11. **Freeman, W.M., S.J. Walker, and K.E. Vrana.** 1999. Quantitative RT-PCR: pitfalls and potential. BioTechniques *26*:112-115.
12. **Bustin, S.A.** 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. *25*:169-193.
13. **Yang, J.H., J.P. Lai, S.D. Douglas, D. Metzger, X.H. Zhu, and W.Z. Ho.** 2002. Real-time RT-PCR for quantitation of hepatitis C virus RNA. J. Virol. Methods *102*: 119-128.
14. **Puskas, L.G., A. Zvara, L.J. Hackler, and P. Van Hummelen.** 2002. RNA amplification results in reproducible microarray data with slight ratio bias. BioTechniques *32*: 1330-1340.
15. **Walker, N.J.** 2002. Tech.Sight. A technique whose time has come. Science *296*:557-559.

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