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Analytical Biochemistry 345 (2005) 102–109
ANALYTICAL BIOCHEMISTRY
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mRNA and 18S–RNA coapplication–reverse transcription for quantitative gene expression analysis

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Received 20 April 2005
Available online 10 August 2005

Abstract

Fluorescence-based reverse transcription real-time quantitative polymerase chain reaction (RT–QPCR) is a highly sensitive method for the detection and quantitation of mRNA. To control and correct for sample variability, some common housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, and ubiquitin are often used as endogenous standards. Other internal calibrators such as 18S–ribosomal RNA (18S–RNA) have also been used, but further methodological concerns arise given that ribosomal RNA lacks the 3′ poly-A tail typically associated with messenger RNA. To take advantage of the constant expression levels of 18S–RNA and the precision of oligo-(dT) primed first-strand synthesis, we have developed a method that combines oligo-(dT) with an 18S–RNA-specific primer in the initial reverse transcription (RT) reaction. This strategy, termed coapplication reverse transcription (Co–RT), allows for the analysis of multiple target genes with the advantages of 18S–RNA normalization from a single RT reaction. In this article, we describe Co–RT and present tissue distribution and expression level analysis of several target genes using this method. Co–RT provides increased sensitivity and higher accuracy than do the standard random primed RT methods.

Keywords: Housekeeping genes; 18S–ribosomal RNA; GAPDH; β-actin; Ubiquitin; Reverse transcription; Real-time quantitative polymerase chain reaction

In gene expression analysis, both quality and quantity of messenger RNA (mRNA)[1] attained may vary from sample to sample. To control for such variations, endogenous standards or internal controls have been employed for normalization of gene expression levels when evaluated by Northern blot, reverse transcription polymerase chain reaction (RT–PCR), and reverse transcription real-time quantitative polymerase chain reaction (RT–QPCR) methods. The use of endogenous genes as internal standards requires that the gene be expressed at a constant level among different tissues at all stages of development and be unaffected by experimental treatment [1]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, ubiquitin, and 18S–ribosomal RNA (18S–RNA) are several of the most common endogenous standards currently in widespread use. Despite reports that the expression levels of GAPDH and β-actin can vary widely in some tissues or cell types in response to experimental manipulation [1–4], studies using these genes as endogenous standards can still be found in current publications [5–7].

In our effort to study gene expression by RT–QPCR, we found that expression levels of GAPDH, β-actin, and ubiquitin have significant variability across multiple

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[1] *Abbreviations used*: mRNA, messenger RNA; RT–PCR, reverse transcription polymerase chain reaction; RT–QPCR, reverse transcription real-time quantitative polymerase chain reaction; GADPH, glyceraldehyde-3-phosphate dehydrogenase; 18S–RNA, 18S–ribosomal RNA; RT, reverse transcription; Co–RT, coapplication reverse transcription; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAP-I, pancreatitis-associated protein I; Rab-7, Ras-related GTP-binding protein family member 7.

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doi:10.1016/j.ab.2005.07.028

tissues. Based on results from this study, we conclude that the use of these endogenous genes for normalization is unsuitable as standards. With increasing regularity, ribosomal RNA is becoming a favored standard when used in conjunction with RT–PCR and RT–QPCR methods. Several recent studies have also concluded that the use of ribosomal RNA as an endogenous standard was consistently the best choice when compared with other methods in a variety of cellular systems [2,8,9]. Even so, several methodological concerns regarding the use of 18S–RNA remain. The most critical is that 18S–RNA is ribosomal and does not contain a poly-A tail. This suggests that 18S–RNA might not be truly representative of the overall cellular mRNA population. More important is the practical limitation of using 18S–RNA as an internal standard. To use 18S–RNA, random oligomers are needed to prime the initial reverse transcription (RT) reaction rather than the oligo-(dT) primer that is often preferred when evaluating mRNA expression. Random primers in the RT reaction might affect the accuracy and reproducibility of the RNA quantitative analysis [10,11].

Theoretically, methods using oligo-(dT) rather than random oligomers in the RT priming strategy preferably produce a single initiation event rather than multiple initiations per individual mRNA [12,13]. This in turn eliminates the unpredictability of the cDNA synthesis step resulting from the multiprime reaction, thereby making the analysis more quantifiable. To achieve the aim of using 18S–RNA for normalization in an oligo-(dT) primed RT, we designed a coapplication reverse transcription (Co–RT) method. This improvement to the methodology uses an 18S–RNA sequence-specific primer combined with oligo-(dT) in the initial RT reaction, an approach that has been employed successfully in our recent gene expression tissue profiling and regulation studies using two-step QPCR [14,15]. In the current article, we describe the developed Co–RT method and compare it with the standard random primed RT in gene expression studies.

Materials and methods

Animal tissue collection and total RNA isolation

Animals were housed and cared for according to National Institutes of Health guidelines for humane treatment of laboratory animals and the Animal Welfare Act in a program accredited by the American Association for Accreditation of Laboratory Animal Care under a protocol approved by the Schering–Plough Research Institute's Animal Care and Use Committee. Male and female Sprague–Dawley rats (~250–300 g, Charles River Laboratories, Wilmington, MA, US) were kept under controlled conditions of humidity, light, and temperature. The animals were euthanized, and isolated tissues were snap frozen in liquid nitrogen. Tissues were stored at −80 °C until use. Total RNA was isolated from 16 rat tissue samples with TRI-Reagent (cat. no. TR118, Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's protocol. RNA purity was verified by agarose gel electrophoresis followed by ethidium bromide staining and determination of $OD_{260/280}$ with an absorption spectrometer.

Reverse transcription

Prior to RT, contaminating genomic DNA was removed by treating with DNase-I (cat no. 18068-015, Invitrogen, Carlsbad, CA) at room temperature for 15 min followed by 37 °C for 30 min. RNA samples from each tissue were adjusted to 100 ng/μl, and the RT reaction was carried out with the SuperScript First-Strand Synthesis System for RT–PCR (cat. no. 11904-018, Invitrogen). Co–RT reaction conditions were as follows. First, 3 μg total RNA, 2.5 μg oligo-$(dT)_{12–18}$ primers, and 2.5 μM of 18S–RNA-specific primer (5′-GAGCTGGA ATTACCGCGGCT-3′) were combined with 10 μl of 10 × reaction buffer, 5 μl of 10 mM dNTP, 20 μl of 25 mM $MgCl_2$, 10 μl of 0.1 M dithiothreitol (DTT), and 5 μl of RNase inhibitor to a final reaction volume of 100 μl. For the Co–RT reaction, the mixture with oligo-(dT) and 18S–RNA primers was preincubated for 2 min at 42 °C, and then 250 units of SuperScript II reverse transcriptase was added and incubated at 42 °C for 100 min. In the random hexamer primed RT reaction, 500 ng random primers (conditions compatible with TaqMan Ribosomal RNA Control Reagents Kit, cat. no. 4308329, Applied Biosystems) were substituted for the oligo-(dT) and 18S–RNA-specific primers. The mixture was preincubated at 25 °C for 2 min. Following the addition of reverse transcriptase, the reaction was maintained at 25 °C for 10 min and then transferred to 42 °C for 100 min. For the comparative studies, the Co–RT and random primer RT reactions were carried out simultaneously. The RT reactions were stopped by heating the mixture at 70 °C for 15 min, chilling to 4 °C, and then diluting 1:25 with H_2O in a 96-well plate and storing at −30 °C.

Real-time quantitative PCR

Primers and probes for QPCR (Table 1) were designed with the assistance of Primer Express 2.0 software. Particular care was made to design primer/probe amplicons within 1 kb of the poly-A tail for efficient and complete first-strand cDNA synthesis. Specificity of each primer pair was analyzed by agarose gel electrophoresis and confirmed to give a single amplified band of the appropriate size. The QPCR was performed with Platinum Quantitative PCR SuperMix-UDG (cat. no. 11730-017, Invitrogen)

Table 1
Primers and probes for RT and QPCR

Target gene	GenBank number	Primer/Probe sequence	Orientation	Location	Amplicon (bp)	Proximity to poly-A (bp)
18S-RNA	V01270	GGGAGGTAGTGACGAAAAATAACAAT	QPCR-F	507–532		
		TTGCCCTCCAATGGATCCT	QPCR-R	589–607	101	33
		(VIC)-CGAGGCCCTGTAATTGGAATGAG TCCACT-(TAMRA)	QPCR-P	545–573		
		TACCACATCCAAGGAAGGCAGCA	PCR-F	456–478		
		TGGAATTACCGCGGCTGCTGGCA	PCR-R	614–636	180	4
		GAGCTGGAATTACCGCGGCT	RT-Primer	621–640		
GAPDH	M17701	CCTGCCAAGTATGATGACATCAA	QPCR-F	778–800		
		AGCCCAGGATGCCCTTTAGT	QPCR-R	831–850	73	383
		(VIC)-TGGTGAAGCAGGCGGCCGAG-(TAMRA)	QPCR-P	806–825		
		CATGCCGCCTGGAGAAACCTGCCA	PCR-F	761–784		
		TGGGCTGGGTGGTCCAGGGGTTTC	PCR-R	1033–1056	296	177
β-Actin	NM 031144	ATCGCTGACAGGATGCAGAAG	QPCR-F	925–945		
		TCAGGAGGAGCAATGATCTTGA	QPCR-R	980–1001	76	127
		(VIC)-AGATTACTGCCCTGGCTCCTAGCACC AT-(TAMRA)	QPCR-P	947–974		
		GAGGCTCTCTTCCAGCCTTCCTTCCT	PCR-F	775–800		
		CCTGCTTGCTGATCCACATCTGCTGG	PCR-R	1056–1081	306	47
Ubiquitin	BC 060312	CCGGCGGGCACTGAT	QPCR-F	1038–1052		
		CATTTTTAACAGAGGTTCAGCTATTACTG	QPCR-R	1110–1138	101	61
		(6FAM)-CATTACTCTGCACTCTAGCCATTTGC CCC-(TAMRA)	QPCR-P	1055–1083		
		ATGCAGATCTTCGTGAAGACCCTGA	PCR-F	787–811		
		TGTTGCTTACCATGCAACAAAACCT	PCR-R	1146–1170	383	29
PAP-1	M98049	TTCTTGGCATCCATGGTCAA	QPCR-F	268–287		
		CATCCACCTCCATTGGGTTCT	QPCR-R	348–368	101	361
		(6FAM)-TTGGACTCCATGACCCCACTCTTG GT-(TAMRA)	QPCR-P	320–345		
		TCTTATCACAGGTGCAAGGAGAAGACT	PCR-F	68–94		
		TGACAGGATGTGCTTCAGGACAAACTA	PCR-R	561–587	520	142
Villin	XM 237288	AGCACCTGTCCACTGAAGATTTC	QPCR-F	2412–2434		
		TGGACGCTGAGCTTCAGTTCT	QPCR-R	2524–2544	132	649
		(6FAM)-CTTCTCTGCGCTGCCTCGATGG AA-(TAMRA)	QPCR-P	2464–2487		
		CCGAGTTGGGAAACTCTGGGGACTGGAGCC	PCR-F	2229–2258		
		GGGGGGTGGGGAGGAGGCTTGAAGGCAGGG	PCR-R	2555–2584	355	209
Rab-7	BC072470	CCTGCATCATGGCTTGCTT	QPCR-F	1970–1988		
		TATTGGCATCACGCTCATCTCT	QPCR-R	2049–2070	101	37
		(6FAM)-AGCACTTCCCCTCCAGAAGTCTACA TTCTAGGG-(TAMRA)	QPCR-P	1994–2026		
		ATTACAGAGTGTTAGAGACTCAAATTT	PCR-F	1463–1489		
		TGTTCTTGTTAAATTTTATTGGCATCA	PCR-R	2060–2086	623	21

Note. Amplicon primer sets are designated as QPCR-F (5′ forward), QPCR-R (3′ reverse), or QPCR-P (probe). Amplicon probes are labeled as indicated with either 5′-VIC/3′-TAMRA (carboxytetramethylrhodamine) or 5′-6FAM (6-carboxyfluorescein)/3′-TAMRA.

using the manufacturer's protocol following full optimization of the primer/probe PCR conditions. QPCR was carried out in a 96-well plate in 25 μl reaction volume containing Platinum SuperMix (12.5 μl), ROX reference dye (0.5 μl), 50 mM magnesium chloride (1–2 μl), and a 1:25 diluted cDNA template (5 μl). When reactions were multiplexed with the target gene and 18S–RNA standard in the same well, forward and reverse primers (200 nM each) and FAM-labeled probe (100 nM) were used for the target gene and primers (100 nM each), with the VIC-labeled probe (50 nM) for 18S–RNA supplemented with an additional 1 unit (0.2 μl) Platinum Taq DNA polymerase (cat. no. 10966-026, Invitrogen). All QPCR reactions were analyzed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with a standard two-step cycling program of 40 cycles at 95 °C for 15 s and at 60 °C for 1 min.

Generation of external calibration curves

External calibration curves for target genes and 18S–RNA were prepared with double-stranded cDNA frag-

ments. For 18S–RNA, the DNA fragment (185 bp) was generated by PCR using the forward primer 5′-TACCA CATCCAAGGAAGGCAGCA-3′ and the reverse primer 5′-TGGAATTACCGCGGCTTGCTGGCA-3′. cDNA fragments for each target gene were approximately 300 to 500 bp in length and encompassed the region of the gene-specific primer/probe amplicons shown in Table 1. Double-stranded DNA fragments were gel purified and quantified by OD_{260} at several concentrations. Calibration curves were prepared as fourfold serial dilutions, with 10 concentrations starting from 1×10^{-5} pmol. Calibration curves for both 18S–RNA and target genes were run in parallel with RT reactions from 16 tissues. All sample concentration points were determined in triplicate. Target gene levels were determined from at least two independent RT reactions, which were then subjected to two independent QPCR reactions. Levels of target gene RNA are reported as the numbers of molecules per nanogram total RNA.

Results

Expression of 18S–RNA, β-actin, ubiquitin, and GAPDH in rat tissue

Expression levels of typically used control genes, 18S–RNA (GenBank No. V01270), β-actin (GenBank No. NM031144), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank No. M17701), and ubiquitin (GenBank No.BC060312), are shown for 16 common rat tissues (Fig. 1). Overall levels of 18S–RNA were the highest when compared with either β-actin, GAPDH, or ubiquitin in all tissues evaluated (Fig. 1; cf. panel A with panels B, C, and D). The 18S–RNA showed consistent expression levels across all 16 rat tissues, with the average at $1.3 \times 10^7 \pm 3.6 \times 10^5$ molecules/ng RNA. The ratio of the highest to lowest tissue levels was 1.47. Conversely, all other endogenous control genes showed various differential expression levels among the tissues, as shown inFigs. 1B–D. β-Actin expression averaged $9.6 \times 10^4 \pm 1.7 \times 10^4$ molecules/ng RNA, with a ratio of the highest to lowest tissue levels of 16.9. GAPDH expression averaged $1.0 \times 10^4 \pm 3.2 \times 10^3$ molecules/ng RNA, with a ratio of the highest to lowest tissue levels of 32.9. The mRNA for ubiquitin showed the least tissue variability among these three genes, with expression levels averaging $7.16 \times 10^2 \pm 1.87 \times 10^2$ molecules/ng RNA, with a ratio of the highest to lowest tissue levels of 12.5. The ratio of ubiquitin expression was 4.2 even when testis was not included in the analysis.

Determination of suitable primer concentration for rat 18S–RNA RT reaction

Four concentrations of 18S–RNA-specific primers (0.2, 1.0, 5.0, and 25 μM) were combined with the standard

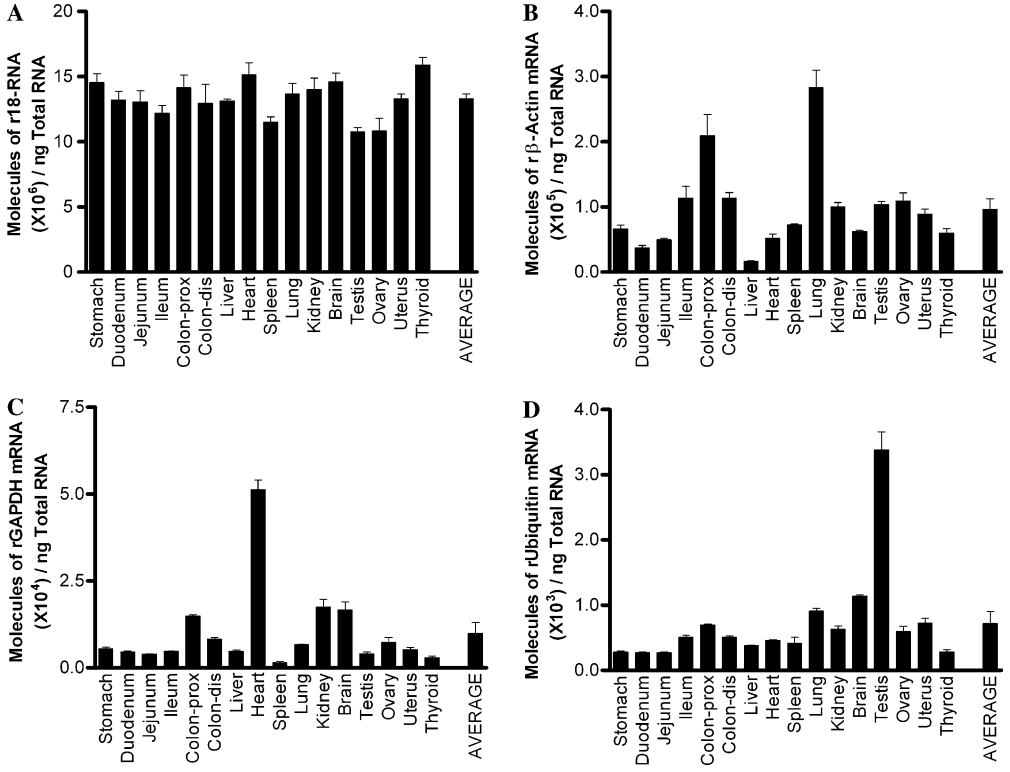

Fig. 1. Expression of 18S-RNA, β-actin, and GAPDH in rat tissues. Total RNA was isolated from 16 rat tissues, and equal amounts of RNA were used in the RT with random hexamers. Expressions of 18S RNA (A), β-actin (B), GAPDH (C), and ubiquitin (D) were analyzed by QPCR. All samples were assayed in triplicate, and each real-time PCR experiment was repeated at least twice on two different occasions. Values are means and SEM.

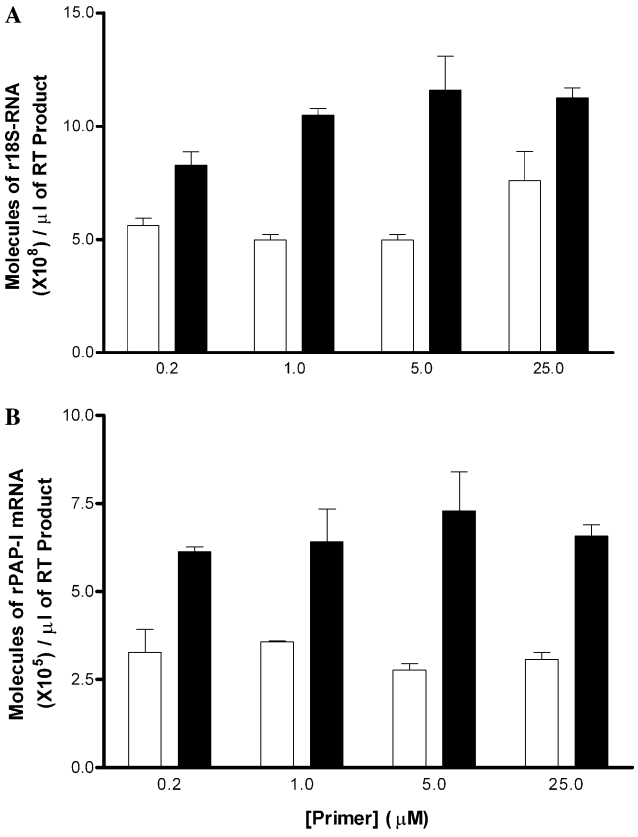

Fig. 2. Primer concentrations for 18S–RNA in Co–RT. Total RNA from rat small intestine was isolated. Four concentrations (0.2, 1.0, 5.0, and 25.0 µM) of 18S–RNA-specific primer and 0.5 µg oligo-$(dT)_{12-18}$ primers were combined into 20 µl Co–RT reaction with either 15 ng/µl (□) or 30 ng/µl (■) total RNA. The expressions of 18S–RNA and rPAP-1 genes were analyzed by QPCR. Results are reported as the numbers of 18S–RNA molecules (A) and of PAP-I mRNA molecules (B) detected from each microliter of the RT products.

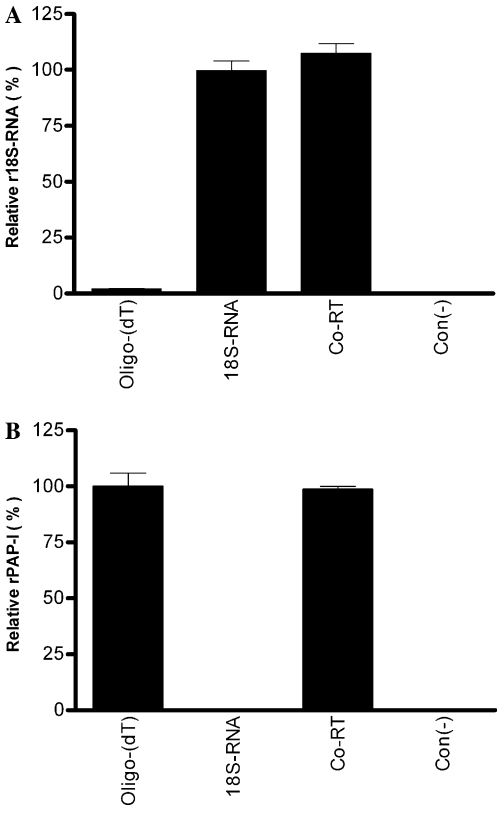

Fig. 3. RT efficiencies for 18S–RNA and PAP-I mRNA with different RT primers. RT was performed with 600 ng total RNA isolated from rat small intestine in each 20-µl reaction volume. The RT primers were 0.5 µg of oligo-(dT) (Oligo-(dT)), 2.5 µM of 18S–RNA-specific primer (18S–RNA), 0.5 µg of oligo-(dT) and 2.5 µM of 18S–RNA-specific primer (Co–RT), and the mixture of oligo-(dT) and 18S–RNA primers but without reverse transcriptase (Con(–)). The transcription efficiency for the 18S–RNA amplicon was set to 100% for the 18S–RNA-specific primer alone. The transcriptional efficiency for the rPAP-I amplicon was set to 100% for the reaction containing the oligo-(dT) primer alone.

0.5 µg of oligo-$(dT)_{12-18}$ primer and two total RNA concentrations of 15 and 30 ng/µl from the small intestine in 20 µl RT reaction. QPCR was performed to evaluate the expression of 18S–RNA and the target gene pancreatitis-associated protein I (PAP-I). Fig. 2A shows similar 18S–RNA levels ($\sim 5 \times 10^8$ molecules) across all four RT primer concentrations when 15 ng RNA was used. When 30 ng RNA was used, overall 18S–RNA levels increased, but only at primer concentrations of 1.0 and 5.0 µM did 18S–RNA increase by a factor of 2. At the lowest primer concentration of 0.2 µM, only 80% of the 1.0- and 5.0-µM signals were achieved. Furthermore, for the target gene (PAP-I), all four concentrations of 18S–RNA-specific primer showed similar levels of PAP-I mRNA when using 15 ng of total RNA/µl and approximately twofold more when the starting concentration of total RNA was doubled (Fig. 2B).

Comparison of the RT efficiencies for 18S–RNA and the target gene with different RT primer combinations

RT efficiency of four different primers with rat small intestine RNA was evaluated by QPCR (Fig. 3). First, 18S–RNA priming was measured using oligo-(dT), 18S–RNA-specific primer, 18S–RNA-specific primer combined with oligo-(dT) primer, and the negative control (combined RT reaction without reverse transcriptase) (Fig. 3A). Reactions indicated that 18S–RNA-specific primer alone or in combination with oligo-(dT) primer resulted in the same high measurable levels of 18S–RNA. The oligo-(dT) primer alone had a priming efficiency of approximately 2% and was similar to the no reverse transcriptase negative control. In contrast for the target gene, PAP-I (Fig. 3B), only RT reactions primed with oligo-(dT) alone or combined with 18S–RNA-specific primer resulted in efficient priming of PAP-1 mRNA. Priming efficiency levels of 18S–RNA specific for 18S–RNA or oligo-(dT) for mRNA species were unaffected when used in combination.

Tissue distributional gene expression: comparative analysis of Co–RT and RT with random primers

The expression levels of the target genes, PAP-I, villin, and Ras-related GTP-binding protein family member 7

(Rab-7), were evaluated by RT–QPCR in 16 rat tissue samples using either the Co–RT methodology or the standard protocol (manufacturer's recommendation) with random primers for the initial RT reaction as described. Fig. 4 illustrates both tissue distribution and message expression level variability for these selected target genes. Gene expression profiles determined by the Co–RT and standard random prime RT methods were essentially the same, with target gene expression either positive or negative for tissues examined. In each instance, mRNA expression levels were slightly but consistently higher when the Co–RT method was employed. Both PAP-I and villin gene expressions were biased to the gastrointestinal tract, and the relative tissue levels were unchanged regardless of the method used. In contrast, Rab-7 expression was observed in all tissues. However, the levels of Rab-7 expression when measured by both methods were highly discordant. In particular, in high-expression tissues such as lung, heart, colon, and ilium, the random prime RT levels were only 8 to 20% of the levels measured using the Co–RT method.

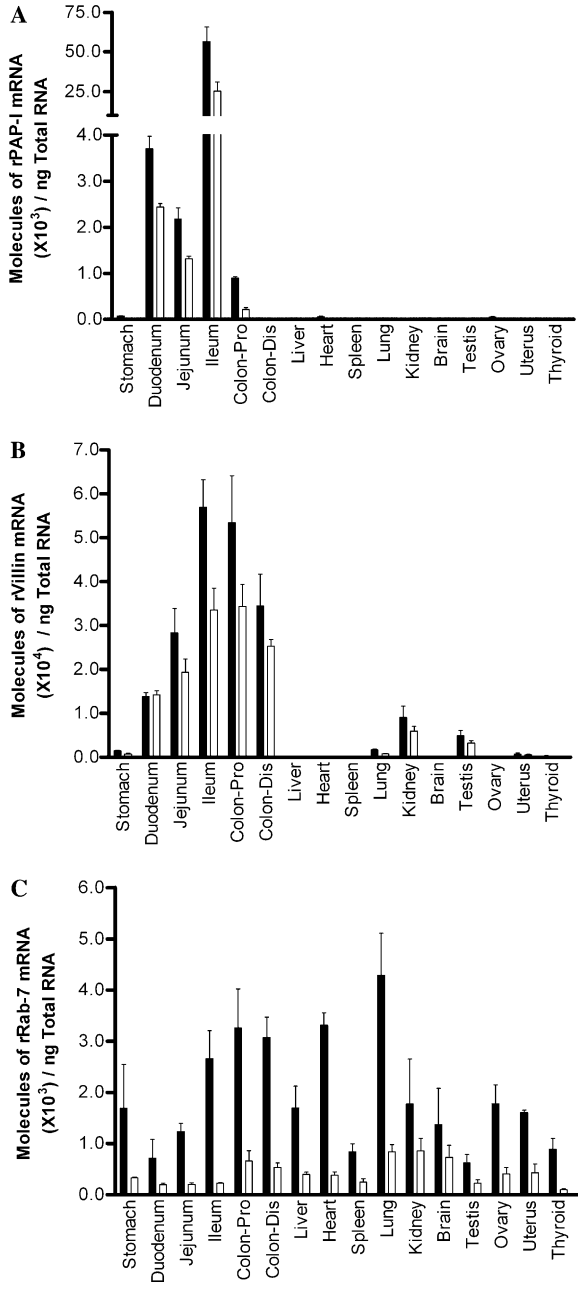

Fig. 4. Gene expression analysis comparing Co–RT and RT using random primers. Equal amounts of total RNA from 16 rat tissues were used in either Co–RT or RT with random hexamers. Expression levels of PAP-I (A), villin (B), and Rab-7 (C) were determined by QPCR. Evaluations of each gene using Co–RT (■) and random primed RT (□) were performed simultaneously on a single 96-well plate, and QPCR reactions were designed to be multiplexed with the target genes and 18S–RNA control in the same well. Quantitative analysis was performed by counting the amount of the mRNA molecules of the target genes detected from each nanogram of total RNA used in the RT followed by normalization with the level of 18S–RNA from the same well.

Discussion

Results of this study indicated that the expression levels of β-actin, GAPDH, and ubiquitin vary significantly among different tissues, with the ratios of the highest to lowest tissue expression levels at 16.9, 32.9, and 12.5, respectively (Fig. 1). β-Actin, GAPDH, and ubiquitin are wildly expressed in most cell types and have roles in specialized cellular processes such as cell proliferation and differentiation [16,17], membrane transport and fusion [18], protein degradation, and mRNA synthesis [19,20]. Therefore, it is not surprising that the tissue expression levels of these genes show variations as a consequence of differences in the cellular organization and functional activities of those tissues. Other examples of fluctuating expression levels of β-actin and GAPDH in tissue and cell cultures following different experimental conditions or during the period of treatment have been documented [1,11,21,22]. The current study indicates that the expression of β-actin, GAPDH, and ubiquitin varies among tissues in animals. This finding is consistent with previous reports regarding tissue RNA analysis with β-actin as the internal control [5,23,24] and Northern blot analysis examining GAPDH mRNA levels from six different rat tissues [25]. We conclude that none of these genes serves as an acceptable endogenous standard for tissue comparison analysis.

Fig. 1 indicates that 18S–RNA levels are more consistent across the 16 rat tissues as compared with β-actin, GAPDH, and ubiquitin. The ratio of highest to lowest tissue expression levels was only 1.5. Other reports have also concluded that 18S–RNA behaves reliably as a useful endogenous standard for mRNA expression studies under different experimental conditions or treatments [2,4,8,26]. The results presented in the current analysis further demonstrate its usefulness as a standard in the comparative study of mRNA expression among different tissues or organs.

Although 18S–RNA is superior to β-actin, GAPDH, and ubiquitin as a standard, several technical limitations on its use in RT–QPCR remain. Being a ribosomal RNA, 18S–RNA lacks the typical 3′ poly-A tail found on most mRNA species and, as a consequence, is not efficiently reverse transcribed using oligo-(dT) as a primer. Instead, efficient RT of 18S–RNA must be performed using random primers, an approach reported to be unsatisfactory for the quantitative analysis of mRNA [10,11]. Random priming in itself can be highly variable because the resulting cDNA products are both difficult to predict and cumbersome to analyze. Conversely, RT using oligo-(dT) is direct; given a single annealing event, each mRNA results in a single cDNA [13].

To integrate the advantages of 18S–RNA as an endogenous standard with the predictable efficiency of oligo-(dT) primed reverse transcription, we have developed a modified procedure termed coapplication reverse transcription. In the Co–RT method, two individual primers, oligo-(dT) and an 18S–RNA sequence-specific primer, are mixed in the initial RT reaction. In theory, each mRNA and 18S–RNA will be represented by a single cDNA and each individual reaction retains its own internal control. By reverse transcribing both internal control and target gene in the same tube, identical conditions for each are ensured. Variability in sample preparation and in both enzymatic steps, RT and subsequent PCR amplification, is internally controlled for the target gene and 18S–RNA endogenous standard. This multiplexing approach provides better reproducibility and uses time and reagents more efficiently than do two independent reactions on the same sample.

Accordingly, the 18S–RNA-specific primed and oligo-(dT) primed reactions should not interfere with each other. The experiment using four concentrations of 18S–RNA primer and two concentrations of total RNA showed that detected 18S–RNA levels were consistent with the changes of input RNA when the primer concentrations were between 1 and 5 μM (Fig. 2A). Thus, 2.5 μM was selected as the standard concentration of the 18S–RNA-specific primer in the Co–RT reaction. For the example target mRNA, represented by PAP-I in Fig. 2B, its detected levels were dependent only on the amount of input RNA and were unaffected by 18S–RNA primer concentration. These results indicated that the 18S–RNA-specific primer reaction does not interfere with the oligo-(dT) primed reaction in this Co–RT method. Fig. 3 compares the relative levels of these genes from the RT with those of the 18S–RNA primer and oligo-(dT) primer alone or paired together. When 18S–RNA levels are assessed using the 18S–RNA-specific primer, no differences are observed when compared with the Co–RT reaction. Similarly, PAP-I mRNA levels measured the same in both the oligo-(dT) alone and Co–RT reactions. This comparison further demonstrates that there is no cross-interference between 18S–RNA-specific primer and oligo-(dT) primed reactions using the Co–RT approach.

QPCR analysis of target gene expression has employed both oligo-(dT) and random primer strategies. Common perception suggests that either scheme is applicable for the study of poly-A mRNA, although the manufacturer's instructions (see Materials and methods) indicate that random hexamer primers are preferred when ribosomal RNA is used as the endogenous standard for normalization. Fig. 4 compares Co–RT with random prime-based RT for three genes: PAP-I, villin, and Rab-7. Both methods showed PAP-I (Fig. 4A) and villin (Fig. 4B) to be expressed with primarily high levels in the gastrointestinal tract. Results were consistent with previous reports on PAP-I and villin gene expression studies [27,28]. However, for both genes the Co–RT method generated slightly higher mRNA levels in all tissues, suggesting that Co–RT may provide greater sensitivity than the random prime RT reaction. Fig. 4C showed that Rab-7 has broad tissue expression, consistent with its role as a regulator of vesicle trafficking [29], but the levels of Rab-7 were highly discordant between the two methods.

The apparent increased Rab-7 expression levels using the Co–RT method was anticipated. RT priming with oligo-(dT) always initiates 3′ of the primer/probe amplicon, whereas random primers will anneal along the entire length of the mRNA. Some fraction of the random primers will initiate 3′ of the amplicon, depending on the length of the mRNA and the distance of the amplicon from the poly-A tail. Our strategy to fix the amplicon within 1 kb of the poly-A tail for each target gene (Table 1) was intended to improve productive first-strand synthesis by limiting mRNA length and secondary structure, two characteristics that may affect the efficiency of the RT reaction [13,30]. This Co–RT approach appears to be more accurate than the random primer method. Continued implementation of the Co–RT method with greater numbers of target genes will offer better understanding of this notion.

An improvement in RT–QPCR methodology that provides better precision and throughput when evaluating gene expression levels in a variety of tissues has been presented. In instances where tissue distribution is an important criteria, the Co–RT method is an effective means of increasing efficiency through the use of oligo-(dT) primed RT reaction and 18S–RNA normalization. A single Co–RT reaction can be employed for the analysis of many independent target genes, an important consideration when the RNA sample is of limited availability. The use of the Co–RT method takes advantage of 18S–RNA for normalization while improving throughput and sensitivity.

Acknowledgments

Thanks go to Michael Graziano for a critical review of the manuscript and to Chaogang He for technical help.

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