The iCycler iQ™ Detection System for Evaluating Reference Gene Expression in Normal Human Tissue

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Introduction

Real-time reverse transcription-polymerase chain reaction (RT-PCR) is a recently developed technique for quantitative assessment of specific RNA levels. In the TaqMan assay, *Taq* DNA polymerase hydrolyzes a specific probe during the elongation phase of the PCR. This results in the release and accumulation of a reporter fluorophore. The point during amplification when the accumulation of free fluorophore results in a measurable change in fluorescence is defined as the threshold cycle. There is a linear relationship between the log of the starting amount of template and the corresponding threshold cycle.

The quantity of specific RNA is usually expressed in relation to cell numbers, the amount of total RNA, or an internal reference gene. The use of an internal reference gene has the advantage of providing an internal standard that can compensate for variation in the quantity and quality of the RNA sample. When choosing a reference gene, however, it is important to choose a gene that is expressed at relatively constant levels in different tissues and under different experimental conditions. We have analyzed the expression of the three most commonly used reference genes, GAPDH, β-actin, and 18S rRNA, in ten normal human tissues, and we found that only 18S rRNA was expressed at relatively constant levels in these tissues.

Methods

Total RNA

Total RNA from liver, spleen, testis, stomach, and small intestine was purchased from Ambion, Inc. RNA from heart, mammary gland, prostate, skeletal muscle, and trachea was from Clontech Laboratories, Inc.

Standard Curve Construction

GAPDH and 18S rRNA plasmid cDNA and human genomic DNA was used for the standard curves. The concentration and purity of the DNA was assessed by A_{260}/A_{280} measurement and the corresponding copy numbers were calculated according to the molecular weight of the respective DNA. The following real-time PCR reaction mix was prepared:

12.5 µl Platinum SuperMix-UDG (Life Technologies)
2.5 µl 3 µM forward primer (see Table)
2.5 µl 3 µM reverse primer (see Table)
2.5 µl 2 µM probe
5.0 µl DNA

PCR conditions were 5 min at 95°C followed by 45 cycles of 15 sec at 95°C and 30 sec at 60°C. Primers and probes for 18S rRNA and GAPDH (Table) were from Scandinavian Gene Synthesis AB (Köping, Sweden). Primers and probe for β-actin were purchased from PE Biosystems. Indicated reactions were run in triplicate.

Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR was performed using the Bio-Rad iCycler iQ system essentially as described by Nilsson et al. (2001). The following reaction mix was prepared:

12.5 µl ThermoScript reaction mix (Life Technologies)
2.5 µl 3 µM forward primer
2.5 µl 3 µM reverse primer
2.5 µl 2 µM probe
0.5 µl ThermoScript Plus RT/Platinum *Taq* mix (Life Technologies)
0.5 µl RNase *OUT* RNase remover (40 U/µl)
4.0 µl RNA (200 ng)

Table. Oligonucleotide primer and probe sequences for quantitative real-time RT-PCR.

Gene	Oligonucleotides	Sequence*	PCR Product Size (bp)
GAPDH	Forward primer	5'-CACCAGGGCTGCTTTTAACTCTGGTA-3'	131
	Reverse primer	5'-CCTTGACGGTGCCATGGAATTTGC-3'	
	Probe	5'-fCCATCAAQGACCCCTTCATTGACCTCAACTACATGGTTTACATG-3'	
18S rRNA	Forward primer	5'-CGGCGACGACCCATTCGAAC-3'	99
	Reverse primer	5'-GAATCGAACCCTGATTCCCCGTC-3'	
	Probe	5'-fCCTATCAACQTTCGATGGTAGTCGCCGTGCC-3'	

* f denotes a fluorescein conjugated to the 5'-nucleotide; Q denotes a T with a conjugated dark quencher.

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For each reaction, 200 ng of DNase-treated tissue total RNA was used. The real-time RT-PCR conditions were 5 min at 60°C and 5 min at 95°C followed by 45 cycles of 15 sec at 95°C and 30 sec at 60°C. Data were collected during the annealing/extension step. All reactions were run in triplicate.

Results

Uniformity and Linearity of Standard Curve

Standard curves were generated by the iCycler iQ software by plotting the log of the DNA copy number against respective threshold cycles (C_T) (Figure 1). The standard curves, covering 4–7 orders of magnitude of template concentration, showed good linearity, with a correlation coefficient of 0.998 for each reference gene. The coefficient of variation at each concentration was <2% (0.24–1.94%), showing high uniformity of the real-time detection system.

Expression of GAPDH, β-Actin, and 18S rRNA Genes in Ten Different Human Tissues

Total RNA from ten normal human tissues was analyzed for transcript levels of GAPDH, β-actin, and 18S rRNA. The apparent transcript copy numbers were calculated by using the respective standard curves and were normalized to the respective amounts of total RNA (Figure 2). We found that GAPDH and β-actin gene expression varied up to 26- and 38-fold among the different tissues. ANOVA analysis showed statistically significant differences in GAPDH and β-actin levels ($p < 0.001$), while 18S rRNA gene expression level was relatively constant among the analyzed tissues ($p = 0.2457$).

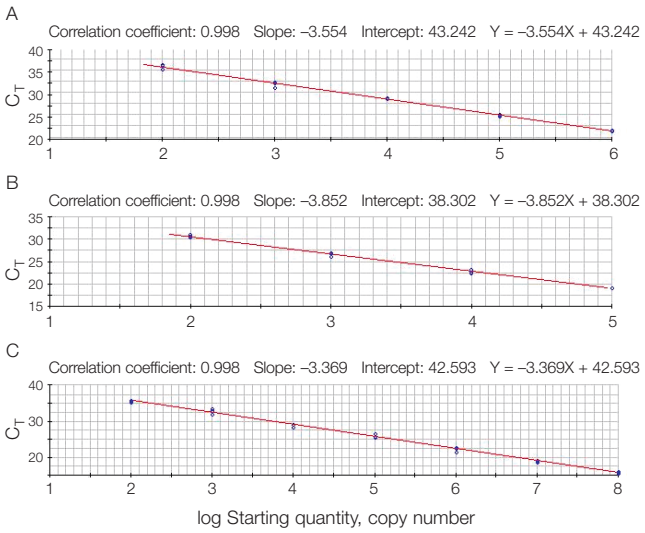

Fig. 1. Standard curves generated by running plasmid cDNA for GAPDH and 18S rRNA and genomic DNA for β-actin. A, GAPDH; B, β-actin; C, 18S rRNA.

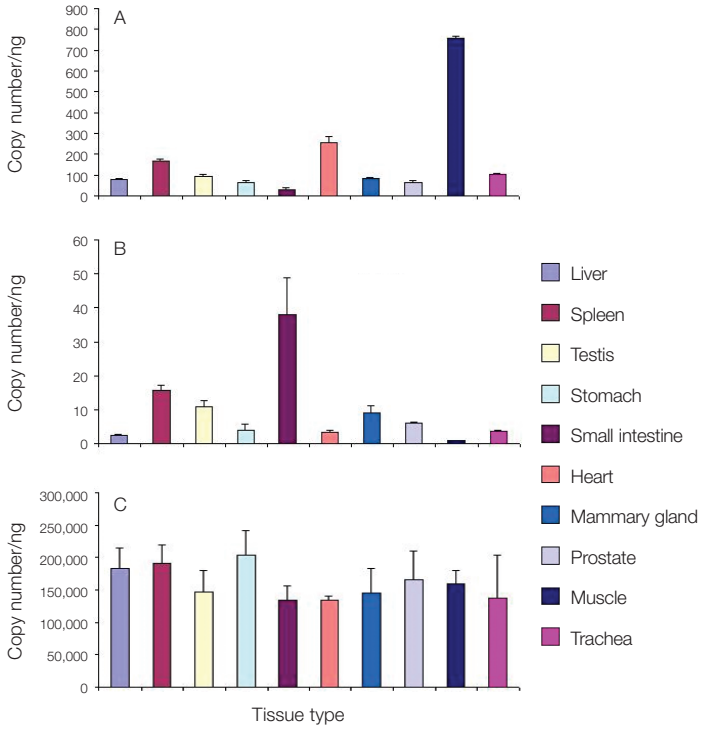

Fig. 2. Expression levels of GAPDH (A), β-actin (B), and 18S rRNA (C) in human tissues. Apparent copy numbers per nanogram of total RNA were calculated using the standard curves shown in Figure 1. Shown are mean values for triplicate samples with standard deviations indicated with error bars.

Discussion

We have evaluated the RNA expression levels of the three most commonly used internal reference genes in ten normal human tissues using the Bio-Rad iCycler iQ detection system. Proper selection of internal control genes is important when performing quantitative gene expression studies. We found that the expression of GAPDH and β-actin varied widely among the different organs, while the 18S rRNA levels were relatively constant. In our opinion, 18S rRNA appeared to be the best choice for a reference gene for RNA expression studies in the ten human tissues analyzed.

Reference

Nilsson J et al., Cloning, characterization, and expression of human LIG1, Biochem Biophys Res Commun 284, 1155–1161 (2001)

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