The mitochondrial housekeeping gene 16S is inappropriate as an internal standard in comparative studies of rare mitochondrial transcripts using S1-nuclease protection assays

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Abstract

The analysis of rare mitochondrial transcripts derived from the L-strand of the mitochondrial genome requires a sensitive method such as the S1-nuclease protection assay. We examined whether the ribosomal mitochondrial transcript 16S is suitable as an internal standard in a multiplex S1-nuclease protection assay for the measurement of different mitochondrial transcripts. For reliable quantification of rare mitochondrial transcripts with the RNase protection assay, a minimum of 2 μg of total RNA is necessary. Standard curves of 16S RNA produced with total RNA from human kidney, liver, brain, and a human neuroblastoma cell line (SH-SY5Y) revealed dose-response relationships that were saturated already at less than 0.5 μg of total RNA. Therefore, 16S is inappropriate as an internal standard for analyzing mitochondrial transcripts with RNase protection assays when more than 0.5 μg of total RNA have to be analyzed.

Introduction

Analysis of steady-state RNA levels using techniques like real-time polymerase chain reaction (PCR), Northern blotting or ribonuclease (RNase) protection assay usually includes the measurement of an internal standard to control for errors between samples.[1,2] Transcripts of housekeeping genes serve as internal standards, and equal transcript levels of housekeeping genes in the measured samples prove the analysis of equal amounts of RNA. An important criterion for a suitable internal standard is an unaltered transcript level during the cell cycle and under the experimental conditions used. For the analysis of transcript levels of nuclear genes, steady-state RNA levels of different housekeeping genes, such as actin, GAPDH, or ribosomal RNAs, are commonly used as internal standards. For quantitative comparison of mammalian mitochondrial (mt) RNAs, two potential internal controls are the structural transcripts from the 16S and 12S ribosomal genes.[3-6]

As transcript levels of housekeeping genes and rarely transcribed genes can differ by several orders of magnitude, caution is necessary when steady-state levels of rare transcripts are examined together with highly expressed control transcripts. The amount of RNA examined and the corresponding signal intensity of all transcripts must have a linear relationship within the same range of total RNA analyzed. Appropriate validation of housekeeping genes in any new experimental system is crucial.

The RNase protection assay is a highly sensitive and specific method that offers the possibility of simultaneous detection and quantification of multiple mRNA targets in a single RNA sample.[7,8] Its specificity is higher than that of Northern blotting or RT-PCR.[9,10] Therefore, RNase protection assays are widely used.[11,12] The major advantage of the RNAse protection assay in comparison to the real-time reverse transcription PCR (real-time RT-PCR) is the direct measurement of RNA levels without error-prone amplification steps. Errors because of contamination with genomic DNA-fragments are precluded in the RNase protection assay. This is especially important for the measurement of transcripts from genes without intron/exon boundaries like mt RNAs. A differentiation between mt cDNA and small fragments of mt genomic DNA, which even can be present after DNase digestion, is not possible

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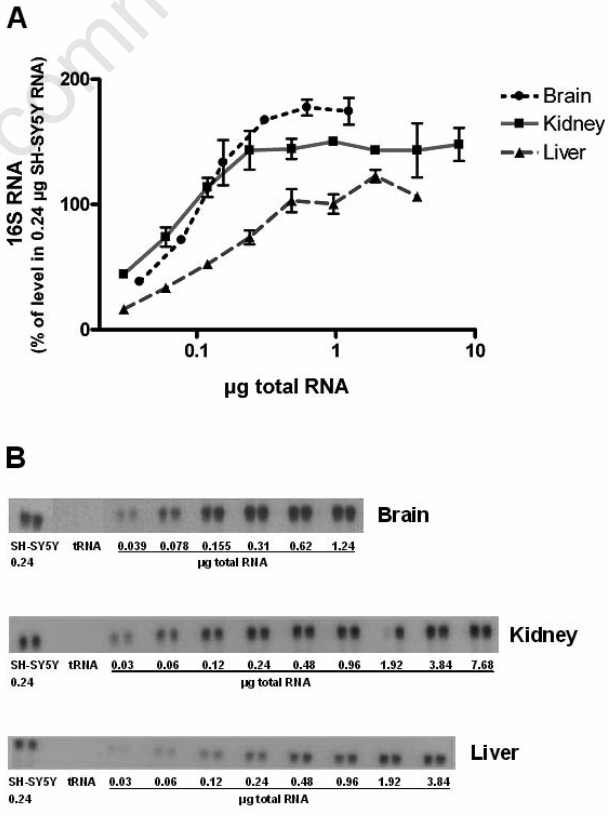


Figure 1. Standard curves of mitochondrial 16S steady-state RNA levels measured by solution hybridization/ S1-nuclease digestion in total RNA from human kidney (■), liver (▲), and brain (●), expressed as % of the16S RNA level in 0.24 μg total RNA of SH-SY5Y cells ± SD (A) and the corresponding hybridization signals on the X-ray films (B). All RNA samples were hybridized with 10^5 cpm 16S antisense cRNA per sample in duplicate (for 1.92 μg of total RNA from kidney, one pellet was lost during the experiment); SD ranged between 1.36 and 15.11% of the mean. No specific signals above background were seen with yeast tRNA as the negative control. Note the logarithmic X-axis of the graph in (A).

with RT-PCR. We previously described rare non-coding transcripts derived from the L-strand of the ATPase 6/8 region of the porcine mt genome.[13] In order to analyze these transcripts in more detail and in a different species, we developed a multiplex S1-nuclease protection assay for human mt transcripts. With this assay, transcripts of eight different mt genes of the L- and H-strand can be measured in a dose-response relationship within a concentration range of 1-20 μg of total RNA. Northern blotting was performed to prove the specific length and the integrity of the transcripts and to demonstrate the superior sensitivity of the RNase protection assay. In order to check whether 16S is appropriate as an internal standard for the RNase protecion assay, we performed standard curves of mitochondrial 16S steady-state RNA levels with total RNA from different human tissues and from cultured cells of the human neuroblastoma cell line SH-SY5Y.

Materials and Methods

RNA

RNA from tissue was prepared according to standard methods with guanidine isothiocyanate and a CsCl-gradient.[14] RNA from cultured SH-SY5Y cells was prepared as described previously,[15] according to a method of Gough.[16] RNA integrity was checked by analyzing an aliquot on an ethidium bromide-stained, non-denaturing agarose gel. RNA concentration was determined by measuring the optical density at 260 nm.

S1-nuclease protection assay

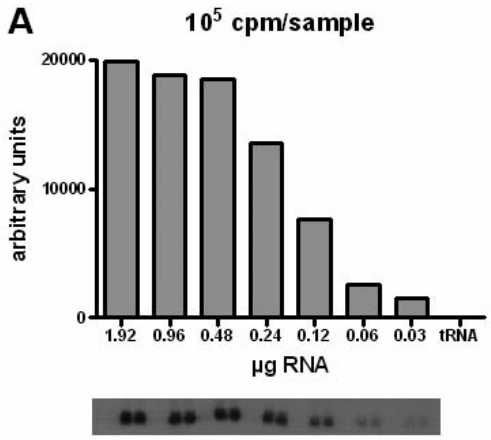
S1-nuclease protection assay was performed as described previously.[17] RNA samples were hybridized with 10^5 cpm or 10^6 cpm ^{32}P-labelled 16S antisense cRNA per sample at 80°C overnight. Yeast tRNA was used as the negative control.

Northern blotting

Northern blotting was done with 1% de-naturing agarose formaldehyde gels according to standard methods[14] using the AlkPhos Direct labeling and detection kit (Amersham Pharmacia Biotech, Freiburg, Germany).

Data analysis and presentation

Hybridization signals on X-ray films (shown underneath the respective bar graphs and in Figure 1B) were scanned with an Epson 9000 scanner and the Epscan-ScanPack 2 software analysis program. Quantification of the signals was achieved with the NIH Image 1.62 program. GraphPad Prism 4.0 Software (GraphPad, San Diego, California, USA) was used for graphical presentation.



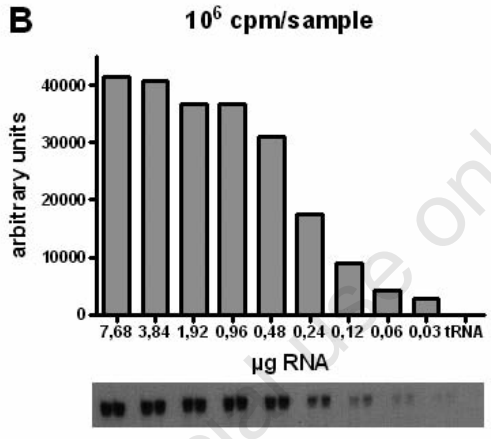


Figure 2. Histograms of mitochondrial 16S steady-state RNA levels in total RNA from human SH-SY5Y neuroblastoma cells as measured by the S1-nuclease protection assay performed with 10^5 cpm 16S antisense cRNA per sample (A) and 10^6 cpm 16S antisense cRNA per sample (B). Hybridization signals on the X-ray films are shown underneath the respective column (duplicates of each RNA sample). No specific signals above background were seen with yeast tRNA as the negative control.

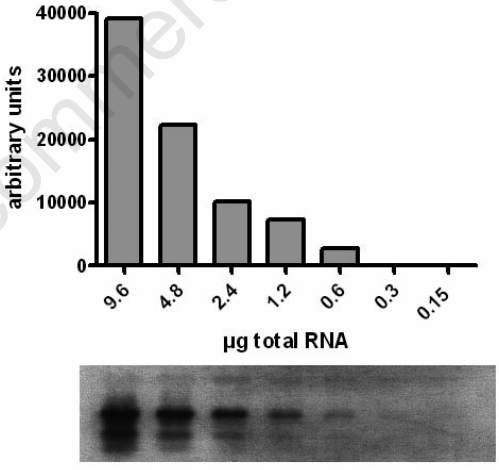


Figure 3. Histogram of mitochondrial 16S steady-state RNA levels in total RNA from human SH-SY5Y neuroblastoma cells as measured by Northern blotting. Hybridization signals on the X-ray film are shown underneath the respective column.

Results and Discussion

Measurements of mitochondrial 16S steady-state RNA levels by S1-nuclease protection assay in total RNA from human kidney, liver, brain, and a human neuroblastoma cell line (SH-SY5Y) revealed dose-response relationships that were saturated already at less than 0.5 μg of total RNA (Figures 1 and 2A). Even if samples were hybridized with a 10-fold higher concentration of labeled 16S probe, the saturation level of the assay was shifted only from approximately 0.5-1 μg of total RNA (Figure 2). When total RNA from SH-SY5Y cells was analyzed by Northern blotting, standard curves with a linear range of 0.6-9.6 μg were obtained (Figure 3).

16S is an appropriate standard for analyzing mt transcripts with Northern blotting[5] (see our data), but not for RNase protection assays in which more than 0.5 μg of total RNA needs to be analyzed. Northern blotting is at least 10-fold less sensitive than S1-nuclease protection

assays and, therefore, only of limited value for the analysis of rare transcripts[8,9] (see our data). A reliable quantification of rare mt transcripts with the RNase protection assay, however, needs at least 2 μg of total RNA; an amount far beyond the linear range of the 16S standard curve. Thus, in our assay, 16S RNA levels could be measured separately in additional samples of diluted total RNA, but must not be quantified in the same sample as levels of rare mt transcripts. Alternatively, low-level nuclear-encoded transcripts might be applicable as an internal standard in the multiplex RNase protection assay.[18-20]

Our results show that steady-state levels of 16S RNA differ between different tissues and between tissue and cancer cells (Figure 1). Furthermore, steady-state levels of mt rRNAs can vary under certain conditions, for example in knock-out models,[21] aging,[18] and disease.[20] Therefore, the use of mt rRNAs as housekeeping genes, as well as the use of housekeeping genes in general,[19] is limited to comparisons within a specific type of tissue/cell and has to be validated carefully for any experimental condition. The limitations mentioned above apply to all RNA quantification methods, including gene expression microarrays[20] and deep sequencing.[22]

Steady-state levels of mt protein-coding transcripts are low[23] and approximately 50- to 100-fold less abundant than transcripts from ribosomal genes.[24] The ratio of the transcript levels of 16S RNA and protein coding mt genes is similar to the ratio of the expression levels of structural nuclear genes and rare nuclear transcripts. Thus, the applicability of transcripts from abundant genes as an internal standard in multiplex assays is limited. Therefore, as shown in this paper, the simultaneous measurement of rare transcripts and highly expressed housekeeping genes in multiplex S1-nuclease or RNAse protection assays needs careful analysis of the concentration range in which the internal standard shows a linear dose-response relationship.

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