Evaluating RNA status for RT-PCR in extracts of postmortem human brain tissue

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Total RNA was extracted from 105 individual postmortem human brain samples representing a range of postmortem conditions. To improve upon parameters currently used to screen for RNA quality, electropherogram patterns generated by the Agilent Bioanalyzer 2100 were compared to the average score in random hexamer-primed reverse transcription real-time PCR for four housekeeping genes in each RNA sample. The ribosomal ratio (28S to 18S) was found to be unrelated to the housekeeping gene score ($r = -0.06$*;* $P = 0.50$*), and there was no threshold value in the ratio that could be applied to effectively categorize the RNA degradation. Although the housekeeping gene score correlated significantly with the percentage of area in the electropherogram corresponding to moderate to high molecular weight intact mRNA (*$r = 0.41$*;* $P = 0.0001$*), the best discriminator was determined to be the ratio of the 18S peak height to the highest peak in the tRNA to 18S rRNA baseline. Applying a lower boundary of 2.12 for the ratio allowed for the screening out of samples with the lowest housekeeping gene scores without excluding better-quality samples. This measure represents a marked improvement over the 28S to 18S ratio, which proved to be a misleading indicator of the state of the mRNA for use in random hexamer-primed reverse transcription PCR.*

INTRODUCTION

The ability to quickly assess mRNA quality using small amounts of total RNA has become increasingly important as the subsequent measures of mRNA transcripts have become more comprehensive and more expensive. Although a moderate degree of RNA degradation does not preclude meaningful results for reverse transcription PCR (RT-PCR) or microarray analysis (1), more extensive degradation necessitates the exclusion of those samples from further study. Screening RNA to exclude samples prior to cDNA synthesis or probe generation is clearly an advantage from the perspective of generating a sample set of adequate size that can then be processed as one group.

In the past, the evaluation of mRNA quality in total human RNA preparations relied heavily on estimating the ratio of the 28S to 18S ribosomal components seen when the RNA was subjected to gel electrophoresis. RNA from tissue samples are typically screened based on the observation that the nucleic acid content represented by 28S rRNA should be approximately twice the quantity of that of the 18S in total RNA samples for the mRNA quality to be acceptable (2). However, we were unable to locate the original data for this commonly accepted premise. Based on structural differences alone, it might be expected that the in situ stability of mRNA differs from rRNA. Certainly, RNases will eventually result in the loss of both components, although there are other factors under which in situ rRNA will be completely degraded but mRNA remains intact (3). Skrypina et al. (4) have demonstrated that under in vitro conditions, the decrease in the 28S to 18S ratio stimulated by magnesium correlates with the decrease in subsequent oligo(dT) RT-PCR signal. The relevance of this finding to mRNA changes in situ is unclear.

For the ribosomal ratio to be reflective of mRNA degradation requires that the mRNA integrity correspond more closely to the 28S than to the 18S integrity. With increased length, there is a greater statistical chance of cleavage (5). Thus, one would expect the 18S integrity to correlate better than 28S with the mRNA, as the length of 18S is more closely aligned with that of the average mRNA. An additional complication is that "hidden" breaks can be introduced into the larger ribosomal subunit of some eukaryotic organisms in vivo, leading to two molecules of equivalent size that cannot be distinguished from the smaller ribosomal subunit (6). It is not known if this process occurs in human tissue, although there are data that show a decrease in the ribosomal ratio with age in both humans (7) and mice (8). Other artifacts include the altered migration of the 18S rRNA due to variable electrophoretic behavior of different 18S conformational states (9).

It is now possible, using the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA), to visualize more components of the total RNA than was previously possible with standard gel electrophoresis. This has allowed us to examine the relationship between the total RNA components and mRNA quality. In particular, we were interested in the quality of the mRNA for real-time PCR. To evaluate the amplifiable pool of mRNA for RT-PCR, we measured the transcripts for four housekeeping genes [(*GAPDH*) (10,11), β-2-microglobulin (*β2M*) (12), neurofilament light polypeptide (*NEFL*) (13), and kynurenine formamidase (*KYNF*) (14–16)] that represent a range of genes from those with abundant expression such as *GAPDH* to the weakly-expressed constitutive enzyme, *KYNF*. Random hexamer priming was used for cDNA generation because previous work had shown it to be the most sensitive method for the RNA in our collection (17).

MATERIALS AND METHODS

Characteristics of the Samples

Tissue samples were obtained (17,19) from the dorsolateral prefrontal cortex, Brodmann area 46, of 105 individuals (Table 1). Three deaths were attributable to chronic disease (chronic obstructive pulmonary disease, lung cancer, and pancreatic/bladder cancer), and other factors causing death were automobile accidents, suicide, cardiac failure, and pulmonary embolism. The

Table 1. Demographic and Postmortem Characteristics of the Sample Set

Parameter	$\bar{x}$	SD	Range
Age (years)	43.9	9.0	19–64
Brain weight (g)	1428.2	133.3	1120–1900
Brain pH	6.5	0.3	5.8–7.0
Refrigeration interval[a] (h)	6.7	7.2	0–54
Postmortem interval[b] (h)	32.8	16.0	9–84

n = 105. $\bar{x}$, mean.
[a]Refrigeration interval: postmortem time at 4°C, prior to tissue acquisition and freezing at -80°C.
[b]The postmortem interval temperature was not known for the individual samples, complicating the interpretation of this time interval.

brain pH determinations were made on 0.4–0.8 g tissue homogenized in 10× volume-deionized, distilled water (pH 6.0–7.0). For the extraction of RNA, the tissue samples were homogenized in TRIzol® solvent (Invitrogen, Carlsbad, CA, USA), and the total RNA was isolated per the product protocol.

Generation of cDNA

DNase treatment of RNA. The samples were treated with RQ1 DNase (specificity not based on the double-stranded structure of nucleic acid; Promega, Madison, WI, USA) because the sample set was designated for subsequent studies of high copy number, intronless genes. RNA (20 µg) was digested with 2 µL RQ1 DNase in 100 µL containing 40 mM Tris-HCL, pH 8.0, 10 mM NaCl, 6 mM $MgCl_2$ incubated at 37°C for 15 min, then spiked with 4 µL 0.5 M EDTA, pH 8.0, and purified with an RNeasy® Mini Kit (Qiagen, Valencia, CA, USA).

Random Hexamer Reverse Transcription

cDNA was generated from 4 µg RNA using SuperScript™ II enzyme (Invitrogen). RNA concentration was measured by A_{260} in 1/8× Tris EDTA (TE) buffer, pH 8.0. The QIAquick® PCR cleanup kit (Qiagen) was used to remove the reverse transcription enzyme. cDNA was eluted with 50 µL of kit buffer, and then diluted 1:1 in 1/4× TE.

Real-Time PCR

The Brilliant® SYBR® Green QPCR Core Reagent kit (Stratagene, La Jolla, CA, USA), the TaqMan® Assays-on-Demand™ kit (Applied Biosystems, Foster City, CA, USA), and the Mx4000® (96-well format; Stratagene, La Jolla, CA, USA) were used for PCR (Table 2) in the following protocol: 1 cycle of 10 min at 95°C and then 40 cycles of 5 s at 95°C and 1 min at 60°C. The standard curves represented serial dilutions of cDNA prepared as above from human brain total RNA (BD Biosciences Clontech, Palo Alto, CA, USA). Due to sample number, the sample set was processed in two separate runs for each gene.

Calculation of RT-PCR Quality Score

The threshold cycle value for each housekeeping cDNA was determined, and the relative concentration was calculated from the standard curve. Tube-to-tube variations in the calculated concentration had been previously determined using similar samples (18% SEM for triplicates); replicate samples were not processed in this study. To correct for run-to-run variation, the concentration of each sample was ratioed to the mean value to generate a relative fold value. The fold value for each housekeeping gene was then summed with all four housekeeping genes and divided by four to create an average score.

Electropherograms of Total RNA

The RNA used to generate the electropherogram (via fluorescence detection of RNA eluting from a molecular sieve) on the Agilent Bioanalyzer 2100 was obtained after the TRIzol extraction step (no further processing). Approximately 700 ng RNA were applied to a RNA 6000 Nano LabChip® (note that the RNA was not heated prior to loading; Agilent Technologies), and subsequent steps of the protocol adhered to the brochure protocol.

Calculation of Elution Profile Parameters

The electropherogram was divided into the following seven windows:

Table 2. Primers, Probes, and Size of Product in Real-Time RT-PCR

Gene	Fluor Method	Size Product	Sense/Antisense Primers	Probe Sequence
GAPDH	SYBR Green	131	5′-GTCAAGGCTGAGAACGGGAAG-3′/ 5′-CCTTCGCCGTCTCCATAG-3′	N.A.
KYNF	SYBR Green	102	5′-GGAAGCCTTGAGGACCTAC-3′/ 5′-CCTTCGCCGTCTCCATAG-3′	N.A.
β2M	TaqMan[a]	N.A.	N.A.	5′-GATCGAGACATGTAAGC-3′
NEFL	TaqMan[a]	N.A.	N.A.	5′-CAGGACACGATCAACAAA-3′

RT-PCR, reverse transcription PCR; N.A., not applicable.
[a]TaqMan chemistry provided by Applied Biosystems' Assays-on-Demand kit. The exact product size and primer sequences are held proprietary by Applied Biosystems.

tRNA, tRNA to 18S baseline, 18S rRNA, the area underlying the 18S, the 18S to 28S baseline, 28S rRNA, and the area underlying the 28S. For each time segment, the area and range were determined.

Statistical Analyses

Linear regression analyses were carried out singly for the housekeeping gene score versus seven different regions (the interrelated components peak height and area) of the electropherogram and for the housekeeping gene score and five different premortem or postmortem variables.

RESULTS AND DISCUSSION

The expression levels of the housekeeping genes were significantly correlated overall ($r = 0.50$; $P = 0.0001$). When the housekeeping gene expression scores were normalized to a mean of 1.0, *GAPDH* exhibited the least sample to sample standard error (0.062), followed by *NEFL* (0.084), *KYNF* (0.095), and *β2M* (0.1098).

Premortem and postmortem factors (Table 1) were evaluated for their effect on mRNA quality. Only brain pH showed a significant correlation with the housekeeping gene expression score ($r = 0.233$; $P = 0.017$), which is in keeping with past experience in this laboratory (19) and others (20). Postmortem interval (PMI) did not correlate significantly with the housekeeping gene score ($r = -0.083$; $P = 0.40$) as documented by other laboratories working with postmortem human brain (20,21). The limitations of our results include the relative lack of samples with short PMI and the unknown body temperature before refrigeration. A study of continuously refrigerated dissected tissue has demonstrated a correlation between the length of the PMI and RNA integrity (22). However, there may be processes activated by removal of the brain from the cranium that are not apparent in the PMI period in situ (21). Animal studies will be necessary to rigorously determine the importance of such postmortem factors to this analysis.

There was no correlation between the ratio of 28S to 18S rRNA areas and total housekeeping gene score ($r = -0.06$; $P = 0.50$) (Figure 1). In addition, there was no positive correlation between any single housekeeping gene score and the 28S to 18S ratio. For this sample set, applying a cutoff value of 2.0 for the 28S to 18S would have resulted in the unnecessary exclusion of over one-third of the samples. Because the size of the fragments amplified were all less than 300 bp, and the reverse transcription priming utilized hexamers, it is certainly possible that many of the samples were substantially degraded and still allowed the amplification of the mRNA. However, that possibility cannot account for the finding of high ribosomal ratios corresponding to relatively poor-quality mRNA (Figure 1). In those samples, degradation may have occurred postmortem that affected the 18S and mRNA components more than the 28S components. Results similar to our data are apparent in the oligo(dT) RT-PCR of Malik et al. (23) for products up to 817 bases in length. In that study, retinal RNA maintained in situ for various lengths of time yielded good RT-PCR signal that did not appear to correlate with increasingly poor 28S to 18S ratios.

Other factors that may have obscured a correlation with 28S to 18S are the possible error introduced by variations in the percentage of mRNA content intrinsic to each sample preparation and variations

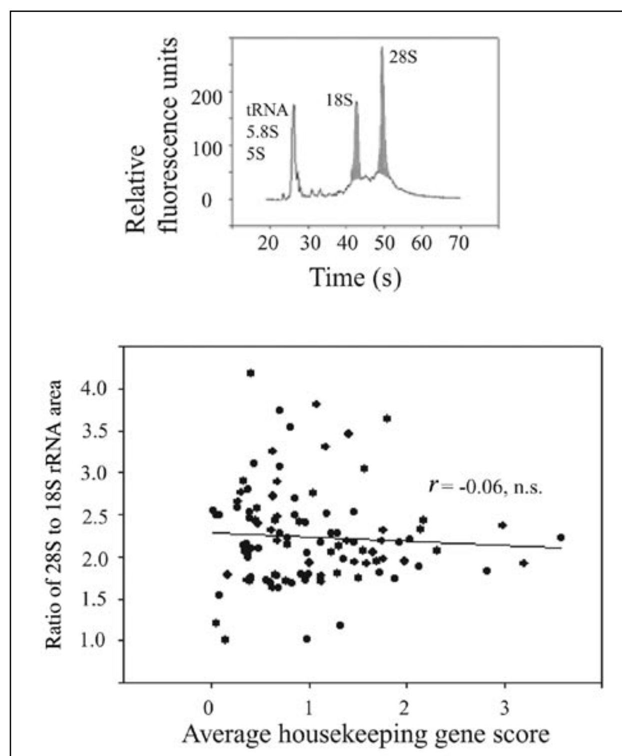

Figure 1. The lack of correlation between ribosomal ratio and mRNA quality, as measured by the average real-time RT-PCR score for the mRNA of four housekeeping genes (fold values, *GAPDH, KYNF, NEFL, β2M*). Upper panel: representative electropherogram, illustrating the areas (shaded) used for calculating the ribosomal ratio. Lower panel: graph and linear regression line, illustrating that the criterion of ribosomal ratio could not be used to screen for mRNA quality. RT-PCR, reverse transcription PCR; n.s., nonsignificant.

attributable to the DNase step. Because of the large sample size in this study, variations in the percentage of mRNA content not attributable to degradation should have been well distributed over samples with different electropherogram characteristics, and thus should not have significantly biased the regression results. Magnesium-stimulated endogenous RNase-like activity (including RNA autocatalysis) during the RQ1 DNase incubation is a concern (4,17). However, previous work in our laboratory and others have shown that the effect of such an incubation (<4 h) is greatest for oligo(dT)-primed reverse transcription, but is minimal (i.e., within the error of an RT-PCR) for random hexamer-primed reverse transcription reactions (References 4 and 17; unpublished data).

Of all the areas and ranges measured for the specified time intervals in the electropherogram, the strongest correlation (Figure 2) with mRNA quality as assessed by the housekeeping score was with the area of expected migration for intact, moderate-to-high molecular weight mRNA [i.e., percent total area from 18S (peak and baseline) up to 28S] (r = 0.41; P = 0.0001). The correlation was also significantly positive for each housekeeping gene considered singly. This finding has not been corrected for multiple comparisons and would need to be confirmed in future studies to be considered a significant result. Furthermore, the parameter highlighted in Figure 2 does not allow the setting of a threshold value on the y-axis, below which all of the mRNA is of poor quality and above which all of the mRNA is of better quality. The same is true for the rRNA ratio parameter in Figure 1.

We were able to identify only one measure that allowed discrimination of the lowest-quality RNAs from the rest of the sample group: the ratio between the 18S maximum (peak height) and the maximum height of the tRNA to 18S baseline (Figure 3). This easily applied method allowed the setting of a threshold value of 2.12, below which the six lowest-quality housekeeping gene values were located and above which all of the housekeeping gene values were better (>95% confidence level). Applying this measure to each housekeeping gene singly, the data for *NEFL* were concordant with six of six of the samples excluded in Figure 3, *GAPDH* and *KYNF* were concordant with five of six, and *β2M* with four of six.

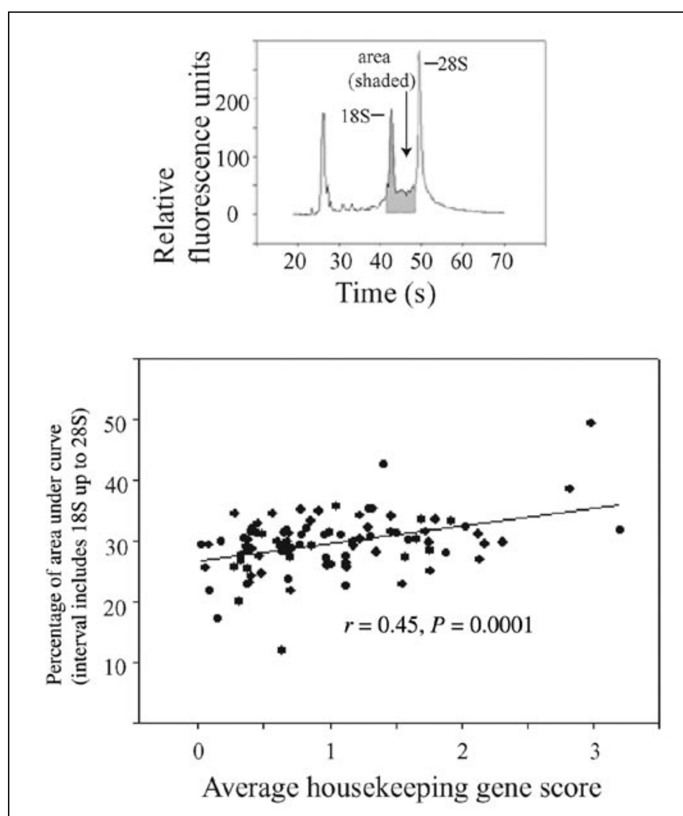

Figure 2. The electropherogram variable identified that correlated most significantly with mRNA quality, as measured by the average real-time RT-PCR score for the mRNA of four housekeeping genes (fold values, *GAPDH, KYNF, NEFL, β2M*). Upper panel: representative electropherogram, illustrating the identified area, approximately 1.9–4.7 kb (shaded) that achieved the most significant correlation with housekeeping gene score. Lower panel: graph and linear regression line illustrating a significant correlation between the plotted variables, but also demonstrating that the criterion (percentage of area under curve, including 18S peak up to 28S peak; complete data available for 95/105 samples) could not be used to screen for mRNA quality. RT-PCR, reverse transcription PCR.

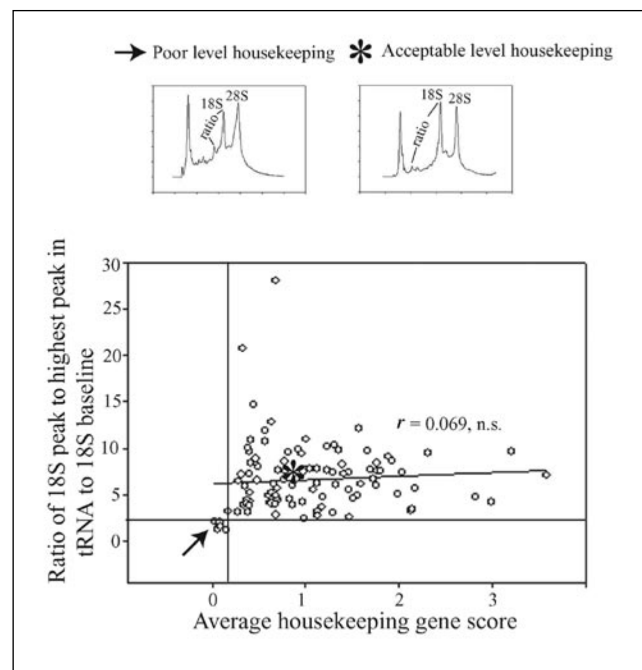

Figure 3. Criterion in the electropherogram that could be effectively applied as a screen for mRNA quality for this type of RNA sample and specific application (random hexamer-primed real-time RT-PCR). Upper panels: illustrate the criterion; that is, the ratio between the 18S peak height and the highest peak in the tRNA to 18S baseline. The left and right panels are good examples of the lack of association between ribosomal ratio and mRNA quality. Lower panel: graph and linear regression line, with examples in the upper panel plotted as an arrow and an asterisk, showing a lack of correlation between the plotted variables, but illustrating the ability to establish a threshold value on the y-axis (2.12) below which the poorest-quality mRNA samples fall. Setting this criterion at 2.12 excludes only those samples that fall below the lower bound of the 95% confidence limit of the average housekeeping gene score (fold values, *GAPDH*, *KYNF*, *NEFL*, *β2M*). RT-PCR, reverse transcription PCR; n.s., nonsignificant.

Auer et al. (24) recently developed a somewhat similar measure to screen RNA for microarray applications; that is, the ratio of the 18S peak to the average peak height in the tRNA to 18S baseline. Although the logical basis for defining the number of baseline peaks is not clear, as the number of peaks is obviously a function of the arbitrary size of the time bin used to collect the data, they demonstrated that the average tRNA to18S baseline peak height significantly correlated mRNA integrity (25) as measured by the 3′ to 5′ signal of two housekeeping genes in several sample sets from cell culture and animal tissue degraded either in situ or in vitro. The shortcoming of their analysis pertains to the results they show for in situ degradation, where an effective screening threshold was not apparent in the data. In particular, for the two sample sets representing human RNA in situ degradation (24), a threshold could not be set that would exclude more than one outlier sample without including samples of lower quality than those excluded.

The potentially relevant difference between the average peak height of the previous method (24) and the measure of maximum height we employed (Figure 3) relates to the degree to which the presumably degraded RNA migrating in the tRNA to 18S baseline migrates as one group. Why such patterns might reflect the status of mRNA is not clear and is certainly worthy of further study. Without a theoretical understanding of these measures, it will remain important to thoroughly evaluate these screening methods for each type of application.

In conclusion, use of the 28S to 18S rRNA ratio would have incorrectly excluded a large portion of our sample set, whereas the measure we have developed (Figure 3) effectively discriminated the RNA that did not amplify well in real-time RT-PCR. This finding is particularly important to studies of postmortem human tissue, which is valuable, difficult to acquire, and necessitates large sample sets to achieve significant results.

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