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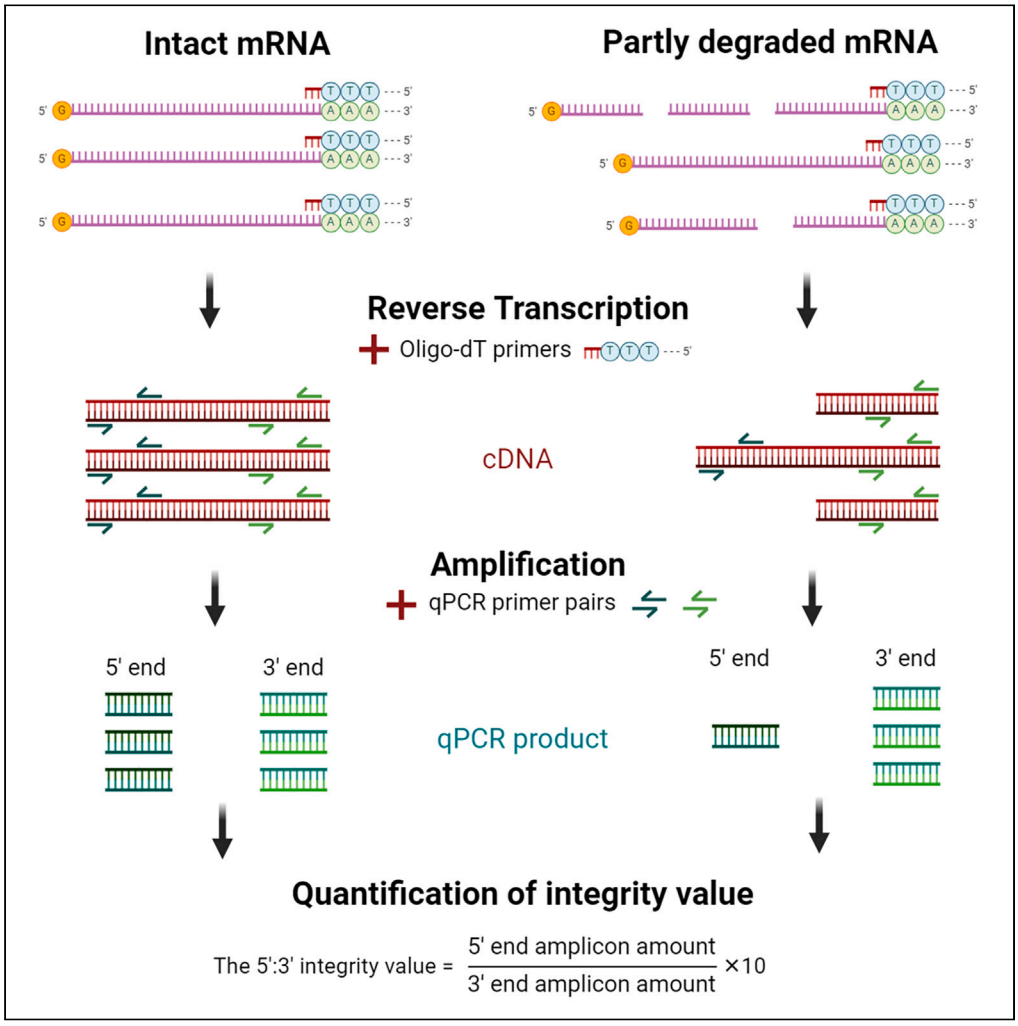

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Highlights

5′:3′ assay measures mRNA integrity directly with RT-qPCR

Integrity score accuracy is ensured by a mathematical correction of qPCR results

5′:3′ integrity value represents the mRNA integrity better than RIN

5′:3′ assay allows mRNA integrity evaluation in subcellular samples lacking rRNA

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Integrity assay for messenger RNA in mouse and human brain samples and synaptosomal preparations

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SUMMARY

Traditionally, RNA integrity evaluation is based on ribosomal RNAs (rRNAs). Nevertheless, gene expression studies are usually focused on protein-coding messenger RNAs (mRNAs). Here, we present an RT-qPCR-based assay, which estimates mRNA integrity by comparing the abundance of 3′ and 5′ mRNA fragments. The assay was validated using plasmids with cloned 3′- and 5′-ends of the cDNA reflecting different ratios of 3′ and 5′ cDNA amplicons in partially degraded RNA samples. The accuracy of integrity value was ensured by including primer efficiency. We used 5′:3′ assay to quantify RNA degradation in heat- and enzyme-degraded mouse and human brain tissue RNA as well as in clinical human brain RNA samples. In addition, the 5′:3′ assay was suitable for assessing mRNA integrity in synaptosomal preparations that lack rRNAs. We concluded that the 5′:3′ assay can be used as a reliable method to evaluate mRNA integrity in tissue and subcellular preparations.

INTRODUCTION

Good RNA quality is essential for acquiring reliable and replicable data from any gene expression analysis.[1] Purified RNA is quite sensitive to environmental factors and is prone to degradation due to its single-stranded structure and abundant RNases in the environment. Therefore, measuring RNA integrity is established as a ubiquitous step to check RNA quality before any further downstream analysis. The first methods to test RNA integrity were based on RNA electrophoresis, where some of the RNA sample was run on denaturing agarose gel, and RNA integrity was evaluated by comparing the intensity of visible small 18S and large 28S ribosomal RNA (rRNA) bands.[2] However, this method required at least a few hundred nanograms of RNA, was difficult to standardize, and the conclusions heavily depended on subjective visual interpretation. Since 2006, microfluidic capillary electrophoresis has become a gold standard for RNA integrity measurements assigning an RNA integrity number (RIN) to an assayed RNA sample.[3] However, both RNA gel and capillary electrophoresis predominantly evaluate rRNA integrity, assuming that it reflects the integrity of all other sample RNAs, including messenger RNA (mRNA).

Meanwhile, gene expression studies are mainly concerned with the quality of mRNA and not rRNA. It has been shown that because of structural and functional differences between them, rRNA cannot represent the integrity of mRNA accurately enough.[1,4,5] In the cells, rRNAs are compact and contain many post-transcriptional modifications to form complex and functional ribosomes, which grants them exceptional stability.[6] In contrast, mRNA has a more linear structure and is more prone to degradation.[7] Therefore, RNases and any environmental factors such as temperature affect the stability and degradation rates of rRNA and mRNA differently.[5] Moreover, rRNA-based methods have not been used to evaluate RNA integrity in subcellular extracts or purified organelles, most likely because rRNA content is reduced or completely lacking.

The integrity of mRNA in RNA samples can be measured directly using real-time quantitative reverse-transcription PCR (RT-qPCR). This approach, called 3′:5′ assay, was first proposed in 2006 by Nolan and colleagues.[8] The 3′:5′ assay measures the integrity of the ubiquitously expressed mRNA of choice, independently of rRNAs. By now a few versions of the 3′:5′ assay have been published that used different template mRNAs for different species.[8–11] However, the 3′:5′ assay's accuracy has not been sufficiently explored, as the few existing studies validate the assay only by comparing 3′:5′ assay's integrity values to RIN values. There has been no validation modeling different availability of 3′ and 5′ binding sites to show how the integrity values of 3′:5′ assay represent degraded mRNA. Furthermore, recent studies demonstrated that

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even minor differences in primer efficiencies result in significant under- or overestimation of mRNA levels, which should be accounted for in a qPCR-based assay.[12]

Therefore, to have a convenient and reliable method for direct mRNA integrity evaluation, we developed and optimized a *PGK1*-transcript-based 5′:3′ assay for human and mice brain tissue. To adjust for any inevitable differences in the efficiencies of qPCR primer pairs used in the assay, we refined the integrity calculation by using efficiency correction. Moreover, we aligned 5′:3′ integrity value calculation to other existing RNA integrity evaluation systems, where integrity values close to 10 indicate very good quality samples and decrease when RNA is degraded. We demonstrated that the 5′:3′ assay can be confidently used as an alternative method to measure RNA integrity in mouse and human brain tissue samples. Finally, we showed that this approach can be successfully used to evaluate RNA integrity in subcellular fractions that lack rRNA. As a model of such a fraction, we used synaptosomes, which are isolated synaptic terminals from the neurons. Synaptosomes are scarce of rRNA and therefore cannot be assessed by conventional rRNA-based RNA integrity methods.

RESULTS

5′:3′ assay based on *PGK1* cDNA as a template

Transcript integrity can be estimated by evaluating the length of reverse transcription products obtained from one of the transcript's termini. Poor sample integrity is reflected in mRNA breaks that lead to shortened cDNAs, the length of which can be assayed by quantitative PCR, comparing the amounts of 5′ and 3′ fragments of assayed mRNA. The straightforward approach to reverse transcription of mRNA from one of its termini is to use oligo-dT primers that bind to the polyadenylated end of mature mRNA. Therefore, the 5′:3′ approach is based on using oligo-dT primers for reverse transcription and two sets of primers for qPCR to measure the relative expression of two amplicons located on the 5′ and 3′ regions of a long constitutively expressed mRNA of choice and then comparing the relative amounts of 5′- and 3′-ends of the transcript (Figure 1). If mRNA in the sample is intact, reverse transcription from poly(A) tails goes on uninterrupted, generating full-length cDNA. Therefore, successive qPCR generates similar levels of 5′- and 3′-end products. In a partly degraded mRNA sample, cDNA synthesis from the poly(A) tail of fragmented mRNA leads to the truncated cDNA at the site of mRNA cleavage. This truncation depletes the binding sites for 5′-end primer pair, resulting in a reduced amount of 5′-end product. The 5′:3′ integrity value for a sample is then quantified by dividing the amount of 5′-end amplicon amount by that of 3′-end amplicon and multiplying by 10 to get an integrity score from 10 (intact mRNA) to 0 (totally degraded mRNA), in line with other RNA integrity assays that use 1–10 scale.[3]

The mRNA template for 5′:3′ assay has to be carefully selected and ideally should be a long transcript with a stable and abundant expression in the tissue of interest with as few pseudogenes as possible to ensure reliable results. The choice of a long transcript ensures that there is sufficient distance between 5′ and 3′ primer pair binding sites to capture the breaks in the mRNA caused by degradation. Here, we used mouse and human *PGK1* cDNA as a template for the 5′:3′ assay. *PGK1* is a ubiquitously expressed housekeeping gene that produces a relatively long transcript with a well-characterized exon-intron structure well suited for this assay.[13] Its product is an enzyme called phosphoglycerate kinase, which is involved in glycolysis.[14] Due to its high and stable expression, *PGK1* has been recommended as a reference gene for quantitative measurements of gene expression in RNA samples isolated from human blood.[15] Moreover, the *PGK1* gene is highly conserved and homologous between rats, mice, and humans. In mice and humans, there are two *PGK* isozymes encoded by different genes,*PGK1* and *PGK2*, but only *PGK1* is expressed ubiquitously.[16] *PGK1* transcripts have been previously used to measure mRNA integrity in rat toxicology studies[10] and horse samples.[11] Here, we designed the qPCR primers for 5′:3′ assay based on mouse and human *PGK1* cDNA to measure mRNA integrity in mouse and human RNA samples (Table 1). To ensure that any remaining genomic DNA in the mRNA sample does not interfere with the assay, we treated samples with DNase I and designed the primer pairs to be exclusively specific to mRNA. In particular, one primer in each primer pair used in this assay binds to an exon-exon junction to avoid any unwanted remnant genomic DNA amplification (Figure S1).

The integrity value of 5′:3′ assay is corrected to reflect primer pair amplification efficiency

The 5′:3′ integrity assay relies heavily on the prerequisite that both 5′- and 3′-end fragments are amplified with equal efficiency. Therefore, it is important to assess the amplification efficiency of used primer pairs. To calculate the efficiency, we used plasmids with cloned fragments of mice (55–1342 bp) and human *PGK1* (21–1270 bp) cDNA as a template for qPCR (Figures S2A and S2C). Acquired Ct values of both 5′- and 3′-end amplicons were plotted against the corresponding plasmid copy number, and amplification efficiency (*E*) was calculated from the slope of the linear regression equation. For both mouse and human *PGK1*, the primer pair for the 5′-end had lower amplification efficiency (Figures 2A and 2B). The same pattern was observed when amplification efficiency was assessed in total RNA samples from mouse and human brains (Figures 2C and 2D).

Even a slight difference in the amplification efficiency between the primer pairs results in shifted integrity values of 5′:3′ assay. Therefore, we applied a recently published STAR protocol for qPCR data analysis that corrects the relative expression of the gene(s) of interest according to primer amplification efficiency.[12] This protocol uses a mathematical transformation to calculate the amplification factor from the amplification efficiency (slope) for each primer pair that can be expressed as presented in equation (*1*). The amplification factor is then used to correct the qPCR data, by defining a linear form of the original gene expression (*2*). We integrated this approach to obtain adjusted 5′:3′ integrity values. To assess the outcome of this correction, we compared uncorrected and corrected 5′:3′ integrity values to RIN defined in the same set of RNA samples, isolated from surgically resected human brain tissue (Table S1). Paired t test revealed that without the correction, the integrity values were significantly lower than RIN values ($p < 0.0001$, $n = 16$), whereas 5′:3′ integrity values after correction were comparable to RIN values ($p = 0.029$, $n = 16$) (Table S2).

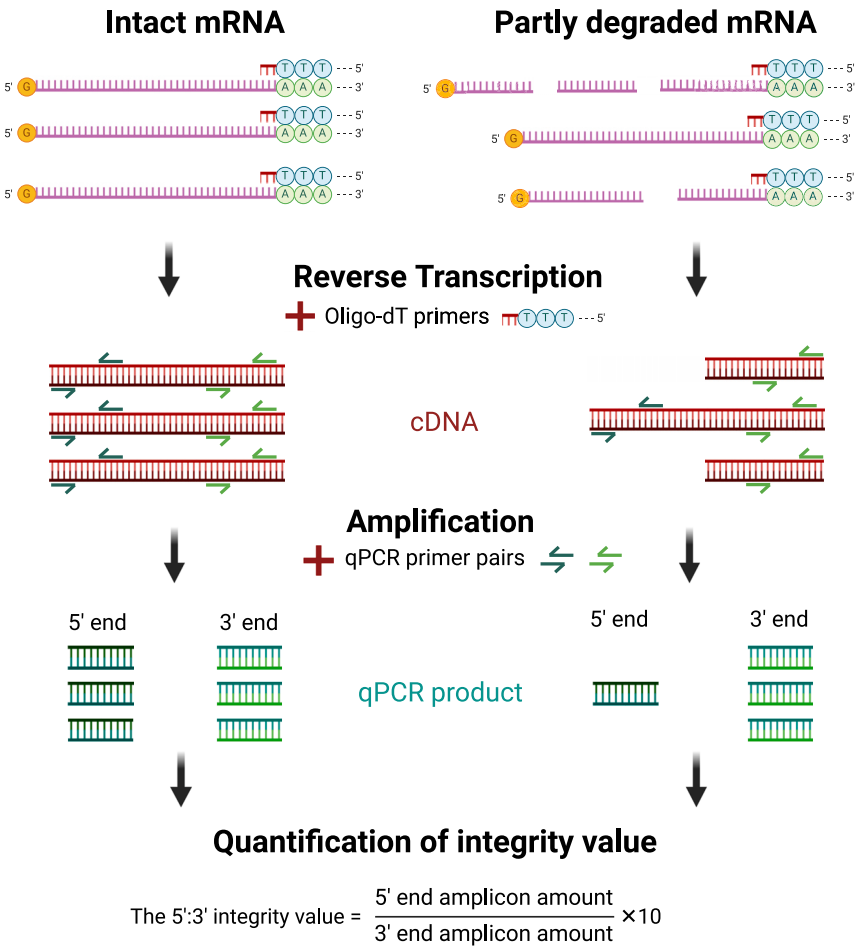


Figure 1. The principle of 5′:3′ assay for mRNA integrity evaluation

Reverse transcription reaction used oligo-dT primers to generate cDNA. In RNA samples of good quality, mRNA is intact and full-length cDNA is synthesized. In this case, both 5′and 3′ pairs of qPCR primers have the same number of sites to attach and produce comparable amounts of 5′- and 3′-end PCR products. In contrast, in poor-quality RNA samples with partly degraded mRNA, shorter cDNA is synthesized because of the breaks in mRNA. On truncated cDNA, PCR primer pair for the 5′-end of mRNA has lower number of binding sites compared to the 3′-end, close to the poly(A) tail of mRNA. After qPCR amplification, the 5′:3′ integrity value is calculated by dividing the amount of the 5′-end amplicon by that of the 3′-end amplicon and multiplying by 10, allowing the quantitative evaluation of mRNA quality in the sample. Created with Biorender.com.

(1) $Amplification\ factor = Dilution\ factor^{\left(-\frac{1}{slope}\right)}$

(2) $Corrected\ expression = Amplification\ factor^{-Ct}$

The integrity value of 5′:3′ assay accurately represents different ratios of primer binding sites

To demonstrate that the calculated integrity value translates to the actual abundance of 5′ and 3′ binding sites, we cloned plasmids with truncated mouse and human *PGK1* cDNA. These plasmids contained 3′ half of the cDNA (675–1342 bp of mouse *Pgk1* cDNA and 608–1270 bp of human *PGK1* cDNA) (Figures S2B and S2D). By mixing different quantities of plasmids with cloned full-length *PGK1* and truncated 3′-end *PGK1* fragment, we prepared samples with known 5′- and 3′-end ratios to model variable cDNA sets resulting from RNA samples degraded to different extents. The 5′:3′ assay of these samples demonstrated a linear relationship between 5′- and 3′-end ratios and obtained integrity value ($R^2 = 0.93$, $p < 0.0001$ for mouse *Pgk1* and $R^2 = 0.89$, $p < 0.0001$ for human *PGK1*, Pearson correlation) (Figure 3). These results showed that the 5′:3′ assay's integrity value can accurately represent the difference in the abundance of existing binding sites for created primer pairs.

5′:3′ integrity value and RIN value correlate well in heat-treated RNA samples

Traditionally, RNA integrity is evaluated qualitatively by inspecting the intensities of the 28S and 18S ribosomal RNA (rRNA). This principle is used by the currently most popular approach to assess RNA integrity—the Agilent Bioanalyzer system, which assigns RIN to RNA sample. To assess the capacity of the 5′:3′ assay to detect the degradation in RNA samples compared to RIN value, we used heat-degraded total RNA

Table 1. Primer pair sequences for 5′:3′ assay in mouse and human RNA samples

Species	Primer name	Primer sequence (5′–3′)	Amplicon size (bp)
Mouse	mPgk1_5′For	TCGTGATGAGG/GTGGACTT	109
	mPgk1_5′Rev	GCTCCATTGTCCAAGCAGA	
	mPgk1_3′For	TGGGGTATTTGAATGGGAAG	107
	mPgk1_3′Rev	TGTCTCCACC/ACCTATGATAGT	
Human	hPGK1_5′For	GGTCGTTATGAG/AGTCGACTTC	109
	hPGK1_5′Rev	GCTCCATTGTCCAAGCAGA	
	hPGK1_3′For	TGGGGGTATTTGAATGGGAAG	107
	hPGK1_3′Rev	TGTCTCCACC/ACCTATGATGG	

/: Exon-exon junction.

samples from mouse cortex. The samples were incubated for 1–15 min at 90°C and then analyzed using mouse 5′:3′ RNA integrity assay and Agilent Bioanalyzer. Both the 5′:3′ integrity values and the RIN values gradually decreased with prolonged heat treatment (Figure 4A). RIN values and 5′:3′ integrity values correlated well (R^2 = 0.67, p < 0.0001, Pearson correlation), and samples that had lower RIN values had lower 5′:3′ integrity values and vice versa (Figure 4B). A similar but lower correlation was observed when samples were incubated with RNase A (R^2 = 0.62, p < 0.007, Pearson correlation) (Figure S3). However, when RNAse A was used, the slope of the regression line was more than twice steeper compared to the slope from heat-degraded mouse cortical RNA samples (slope = 2.3 for RNAse-A-degraded samples; slope = 1.1 for heat-degraded samples), indicating that mRNA is more sensitive to enzymatic degradation than rRNA. In primary not heated samples, the values calculated with both methods were comparable (7.2–8.5 for RIN and 7.6–9.7 for 5′:3′ integrity values). However, with prolonged heating, 5′:3′ assay's integrity values dropped faster than RIN values (Figures 4C and 4D). Therefore, RIN cutoff value of 7.0, which is commonly used as a cutoff for good-quality RNA samples suitable for further analysis, is equivalent to 5′:3′ assay's integrity value of approximately 6.

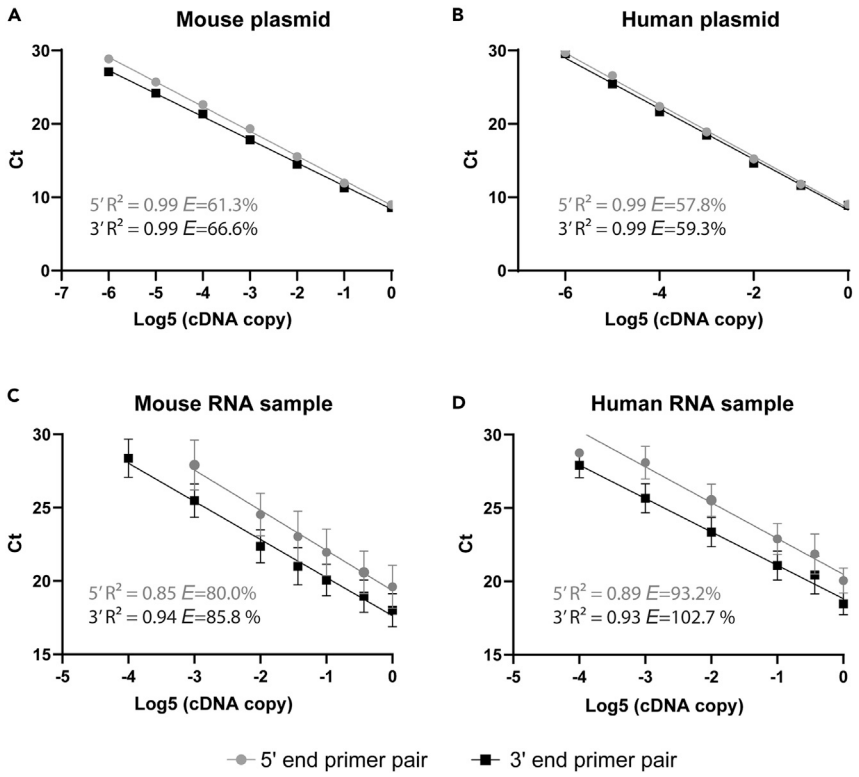


Figure 2. The amplification efficiency of the 5′- and 3′-end primer pairs differs for both mouse and human 5′:3′ assay

Plasmids with cloned mouse (A) and human *PGK1* (B) cDNA or cDNA from mouse (C, n = 2) and human (D, n = 5) RNA samples were used for serial dilutions and following qPCR to determine primer amplification efficiency E.

Data in (C and D) presented as mean ± SEM.

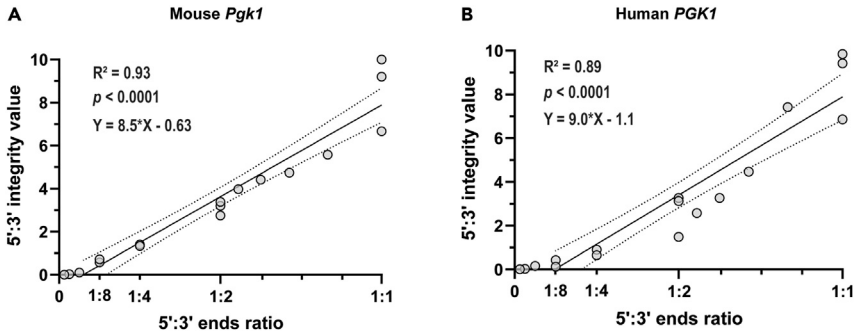


Figure 3. The calculated 5′:3′ integrity value represents the ratios of the binding sites for the used primer pairs

The different 5′:3′ end ratios were acquired by mixing plasmids with cloned full-length and truncated mouse and human *PGK1* cDNA to mimic a wide range of mRNA degradation. The 5′:3′ integrity value was calculated and plotted against the end ratio for (A) mouse and (B) human assays. A linear relationship was observed for 5′:3′ end ratios and integrity values for both mouse and human assays (Pearson correlation, each dot represents a separate experiment). Linear regression line is shown with 95% confidence interval of the true best-fit line.

Next, we applied the same principle to assess 5′:3′ RNA integrity assay with human brain RNA samples. Three total RNA samples from surgically resected human brain tissue were heat-degraded for 1–10 min at 90°C and then analyzed using the Agilent Bioanalyzer and human 5′:3′ RNA integrity assay. As seen with mouse RNA samples, both 5′:3′ integrity values and RIN values decreased with longer duration of sample heating (Figure 5A). There was a very strong correlation between 5′:3′ RNA integrity values and RIN values ($R^2 = 0.86$, $p < 0.0001$, Pearson correlation), showing that both values reflect RNA degradation in samples comparatively (Figure 5B). As with mouse RNA samples, heat degradation decreased human 5′:3′ integrity values faster than RIN values (Figures 5A, 5C, and 5D).

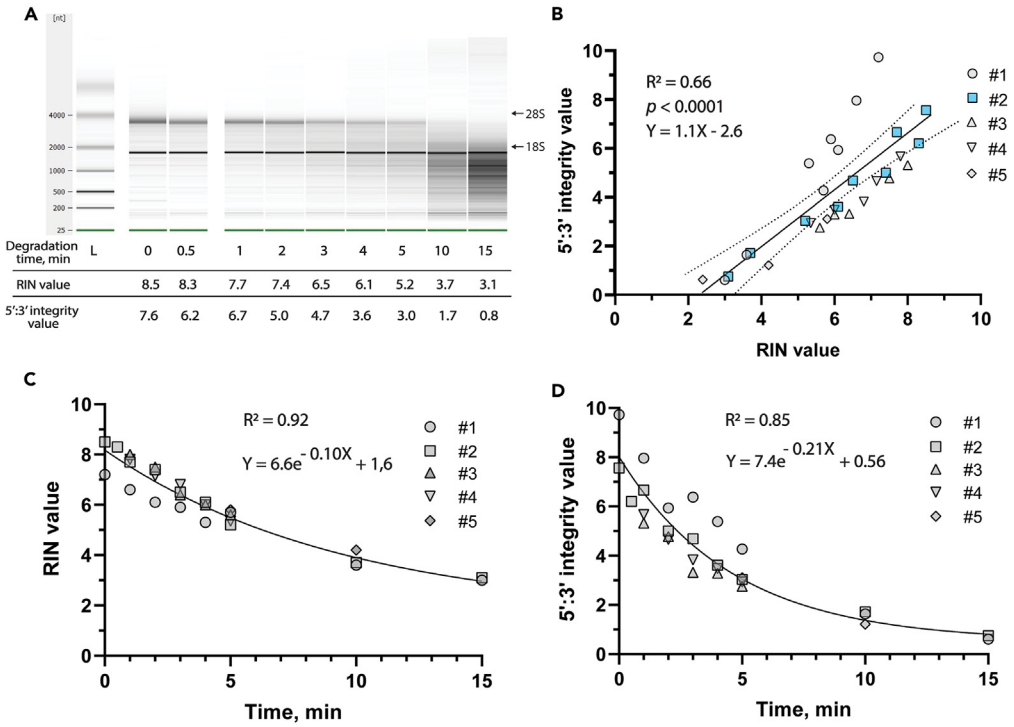


Figure 4. Evaluating RNA integrity with 5′:3′ assay versus RIN values using heat-degraded mouse RNA samples

(A) Representative image of Agilent 2100 Bioanalyzer RNA Pico Chip gel of mouse brain sample #2 total RNA heat-degraded at 90°C for 1, 2, 3, 4, 5, 10, and 15 min. At the bottom: RIN values and obtained 5′:3′ values. The displayed values are marked in blue in (B).

(B) There was a strong linear correlation between RIN value and 5′:3′ integrity value (Pearson correlation, each dot represents a separate experiment, $n = 5$, individual mouse cortex samples). Linear regression line is shown with 95% confidence interval of the true best-fit line.

(C and D) Both RIN values (C) and 5′:3′ integrity values (D) decreased with longer heating duration. The lines in (C) and (D) represent one-phase exponential decay of the data.

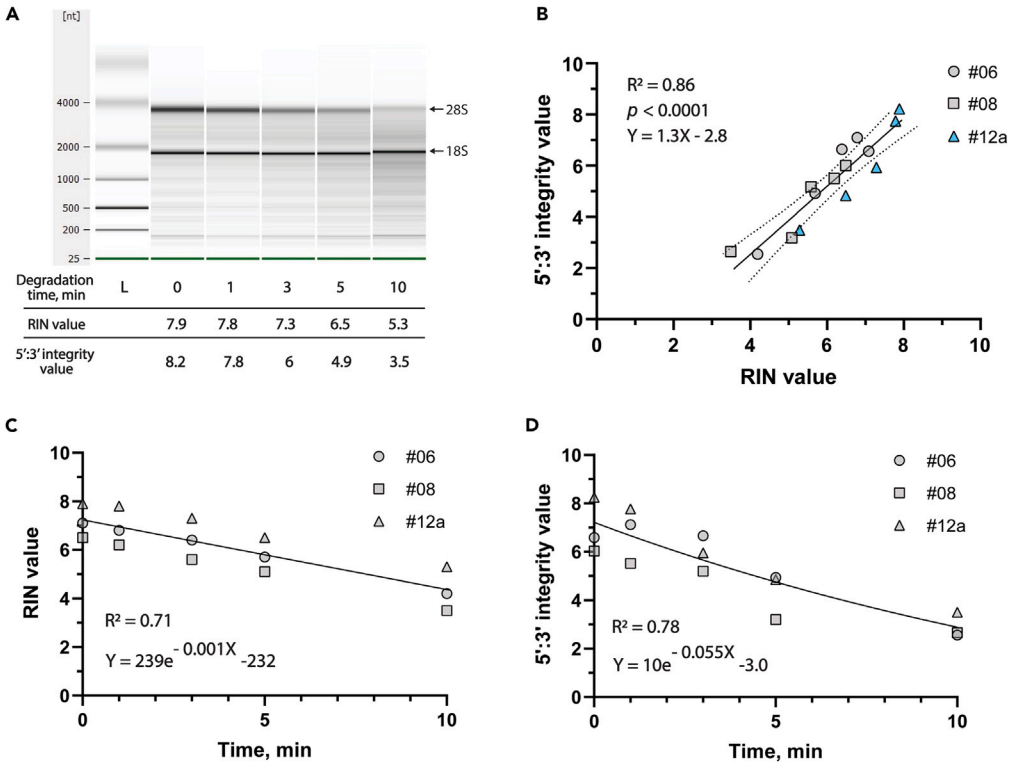


Figure 5. Evaluating RNA integrity with human 5′:3′ assay versus RIN values using heat-degraded human brain RNA samples

(A) Representative image of Agilent 2100 Bioanalyzer RNA Pico Chip gel of human brain sample #12a RNA heat-degraded at 90°C for 1, 3, 5, and 10 min. (Bottom) RIN values and obtained 5′:3′ values. The displayed values are marked in blue in (B).

(B) Three total RNA samples from surgically resected human brain tissue were used to define 5′:3′ integrity values and RIN values (#06, #08, #12a, Table S1). There was a strong linear correlation between RIN values and 5′:3′ integrity values in these samples (Pearson correlation, each dot represents a separate experiment). Linear regression line is shown with 95% confidence bands of the true best-fit line.

(C and D) Both RIN values (C) and 5′:3′ integrity values (D) decreased with longer heating duration. The lines in (C) and (D) represent one-phase exponential decay of the data.

In human post-surgical brain tissue samples, 5′:3′ integrity value represents the RNA integrity better than RIN

RT-qPCR-based RNA integrity evaluation method can be applied as an RNA quality control method for most medical laboratories working with human tissues. The increasing use of human tissue in research emphasizes the importance of developing appropriate and easy-to-use methods for tissue quality assessment. For that reason, we applied human 5′:3′ assay to evaluate RNA integrity in human brain samples, obtained from the glioma or epilepsy surgery. RIN values were measured in the same samples for comparison. The surgically resected brain tissue was kept viable during transportation in cold artificial cerebrospinal fluid (aCSF) and then dissected immediately for homogenization, RNA extraction, and both RNA integrity assays (Figure 6A). Obtained 5′:3′ integrity values varied from 3.6 to 9.7, whereas RIN values varied from 4.6 to 7.8 in these samples. There was a significant moderate correlation between 5′:3′ integrity assay and RIN values (R^2 = 0.59, p = 0.0005, Pearson correlation) (Figure 6B). This further supports our proposition that the 5′:3′ integrity value could be used to reliably evaluate RNA quality in clinical samples as an alternative to RIN.

For gene expression studies, it is important to specifically define the integrity of mRNA in particular rather than total RNA or rRNA, which is assessed by RIN. It is known that RNA integrity determines acquired Ct values in qPCR reactions for gene expression studies.[1] For gene expression studies using qPCR, random hexamer primers are usually used to synthetize cDNA. These primers bind to random parts of mRNA independently of the distance of primer binding site from either 5′- or 3′-end. As RNA degradation lowers the cDNA quantity, length, and abundance of the binding sites for qPCR primers, it leads to higher Ct values in partially degraded mRNA samples compared to intact RNA samples. Therefore, we compared 5′:3′ integrity values and RIN values to the expression of a housekeeping gene *GAPDH* in the same clinical samples, as defined by real-time qPCR using Maxima First Strand cDNA Synthesis Kit with random hexamer primers and TaqMan system. The 5′:3′ integrity value correlated better with *GAPDH* Ct values than RIN values, which further supported our assertion that 5′:3′ assay reflected transcript quality in RNA samples better than RIN value (R^2 = 0.48, p = 0.0063 for 5′:3′ integrity value and R^2 = 0.34, p = 0.0283 for RIN value, Pearson correlation) (Figures 6C and 6D). Importantly, when another highly expressed *CMAS* gene was used for comparison, only 5′:3′ integrity value correlated significantly with the results of defined expression of *CMAS* transcripts (Figure S4A), confirming that direct evaluation of mRNA integrity is more reliable for RNA quality assessment than other methods. Better correlation between Ct and 5′:3′ integrity

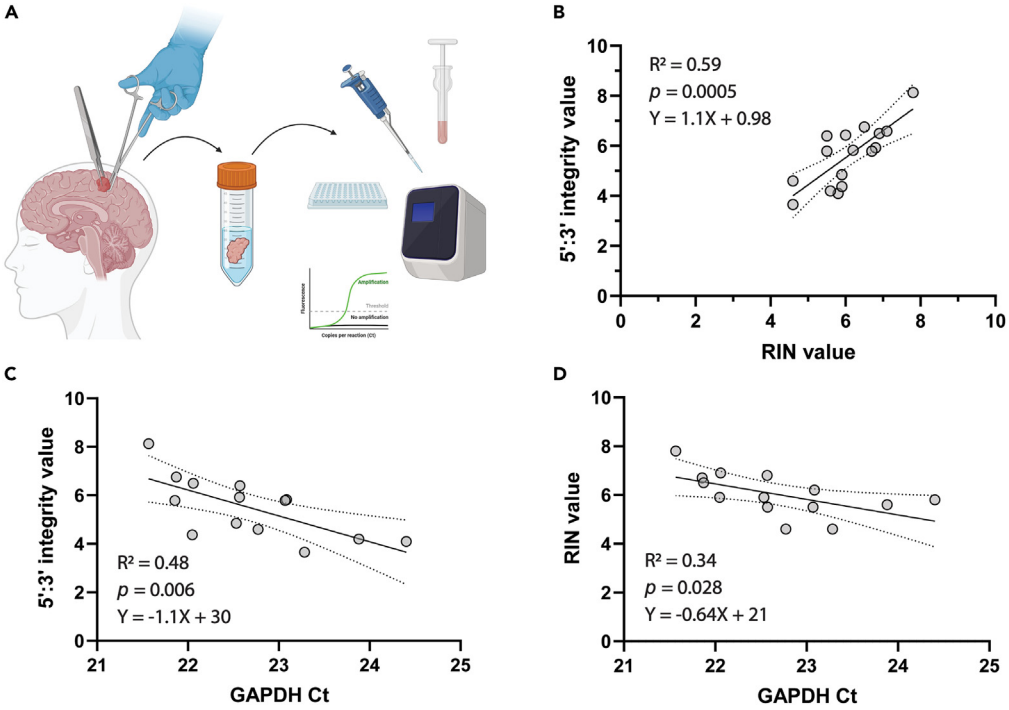


Figure 6. RNA integrity in total RNA samples from surgically resected human brain tissue

(A) Human post-surgical brain tissue from the glioma or epilepsy surgery was kept viable during transportation in artificial cerebrospinal fluid (aCSF). The sample was then immediately dissected and homogenized for RNA extraction and RNA integrity assays. Created with Biorender.com.

(B) Relationship between 5′:3′ integrity value and RIN in RNA samples from human brain tissue (Pearson correlation, each dot represents a separate experiment, $n = 16$). Linear regression line in (B) is shown with 95% confidence interval of the true best-fit line.

(C) There was a moderate linear correlation between the human brain tissue RNA sample 5′:3′ integrity values and Ct values from qPCR for *GAPDH* expression, while the correlation with RIN values was weak (D) ($n = 14$).

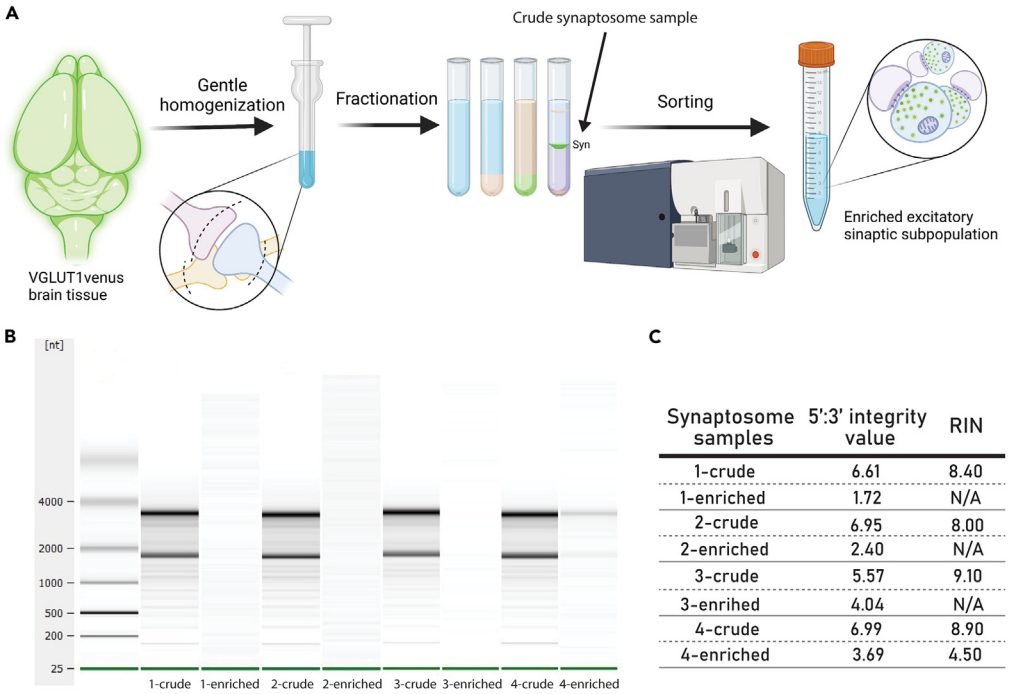
value rather than the RIN was independent of transcript length (Figures S5B–S5D). This shows the consistency and dependability of 5′:3′ integrity values, demonstrating that this assay can reliably predict the integrity of both shorter and longer transcripts.

3′:5′ assay can be used to measure RNA integrity in synaptosomal preparations

Measuring RNA integrity in subcellular fraction samples presents further challenges, as such samples usually lack sufficient quantities of ribosomal RNA to determine RIN values. The examples of such fractions are various extracellular vesicles or synaptosomes—isolated neuronal synaptic terminals. Pure synaptosome sample can be obtained from the brain of genetically engineered mouse, in which presynaptic terminals are tagged by fluorescent protein. Such synapses can be collected using fluorescence-activated synaptosome sorting (FASS) and then used for the determination of local synaptic transcriptome. It has been shown that because of its ubiquitous expression, *PGK1* is abundant in synaptosomes.[17] Therefore, to test whether 5′:3′ integrity assay can be used to evaluate RNA integrity in subcellular fractions, we used RNA from eight matched synaptosomal preparations: four crude and four FASS-enriched synaptosome samples. Crude synaptosome samples were prepared by gently homogenizing vGLUT1^{mVenus} mouse[18] cortical tissue and fractionating synaptosomes in sucrose gradient. To enrich the samples for excitatory synaptosomes, we sorted out mVenus^{+} synaptosomes from crude synaptosomal sample by FASS[19] (Figure 7A). After the sorting, 60%–90% of all the particles were mVenus+ compared to ~20% in crude preparations, indicating significant excitatory synapse enrichment (Figure S5). Crude synaptosome samples were a mixture of similar size and density particles still containing some rRNA. Therefore, RNA integrity analysis of crude synaptosome samples demonstrated comparable values for 5′:3′ integrity assay and RIN, consistent with the linear regression trend observed in mouse cortical samples (Figures 4B and S6). However, in the enriched synaptosomal samples, there was almost no ribosomal RNA, thus there were no detected rRNA bands in electrophoresis lanes, and RIN values could not be determined. In contrast, 5′:3′ assay, which is independent of rRNA, was suitable to evaluate mRNA integrity in enriched synaptosome samples despite low amounts of RNA (Figures 7B and 7C; Table S3), demonstrating its applicability for the samples containing subcellular fractions.

DISCUSSION

In healthy cells, RNA degradation is strictly regulated.[7] However, during sample collection, this homeostatic regulation is disrupted, and RNA degradation is enhanced. The degradation of RNA samples undermines the accuracy of gene expression analysis by qPCR or RNA



C

Synaptosome samples	5′:3′ integrity value	RIN
1-crude	6.61	8.40
1-enriched	1.72	N/A
2-crude	6.95	8.00
2-enriched	2.40	N/A
3-crude	5.57	9.10
3-enrihed	4.04	N/A
4-crude	6.99	8.90
4-enriched	3.69	4.50

Figure 7. Evaluating RNA integrity in synaptosomes

(A) VGLUT1^{mVenus} mouse brain tissue was homogenized, and the homogenate was serially fractionated to obtain the particles that have the size and the density corresponding to the synaptosomes. This crude synaptosome sample was then sorted according to mVenus fluorescence to get enriched excitatory synaptosome subpopulation. Created with Biorender.com.

(B) Representative image of Agilent 2100 Bioanalyzer RNA Pico Chip gel of total RNA samples from crude and enriched mouse synaptosomal samples.

(C) 5′:3′ integrity value and RIN value of crude and enriched mouse synaptosomal samples.

sequencing.[1] RNA sequencing is particularly sensitive to mRNA degradation, as mRNAs are usually captured by poly-dT probes in order to deplete overabundant rRNA. When an mRNA sample is degraded, only a few full-length transcripts are captured, which leads to the 3′ bias of RNA sequencing results.[20,21] Due to this 3′ bias, the reads are predominantly limited to the last exon of mRNA, which distorts gene expression profiles compared to not degraded RNA samples.[20] Therefore, to obtain reliable and reproducible gene expression data, it is crucial to take into account the integrity of sample mRNA. Conventionally, RNA integrity is measured by RNA-electrophoresis-based methods. However, these methods rely on the assumption that rRNA integrity represents the mRNA integrity well enough, even though it has been shown not to be the case.[4,5] Therefore, we developed a direct mRNA integrity evaluation assay that does not require any specialized equipment and is based on the comparison of the abundance of 5′ and 3′ transcripts of housekeeping genes in mouse and human RNA samples.

This mouse and human 5′:3′ assay is based on *PGK1* mRNA, as it is a constitutively expressed long transcript of a highly conserved gene.[16] The *PGK1* RNA was previously successfully used for designing 3′:5′ assay primers for rat and horse RNA samples.[10,11] Several other genes have also been tested in the past. The 3′:5′ assay was first published based on human *GAPDH* gene, as it is a popular choice for endogenous control.[8] There was also a variant of this method based on *β-actin* mRNA optimized for sea bass larvae.[9] However, other reports have demonstrated high-expression variability of *GAPDH*.[22] It is also well known that *GAPDH* and *β-actin* have many pseudogenes.[23–25] This means that the results obtained using these genes as constitutive controls may not translate very well across different tissues or conditions and will be more susceptible to errors caused by genomic DNA contamination.

Having an easily understandable scale for 5′:3′ assay was one of our goals to make it convenient for users. Commercial systems designed for the assessment of RNA integrity usually give values that span from 1 (totally degraded RNA) to 10 (intact RNA). All of the previously published 3′:5′ assay protocols used the opposite scale, in which high-quality RNA samples are represented by integrity values close to 1. This was unnecessarily confusing for the researchers and provided challenges when comparing different RNA integrity assays. Therefore, we established a reversed 5′:3′ integrity value to have the same scale as RIN.

An important improvement of 5′:3′ assay was the correction of the integrity score according to the amplification efficiency of used primer pairs. Primer amplification efficiency is known to fluctuate between different primer pairs. This cannot be completely avoided during primer design process and highly depends on the qPCR machine, the choice of reagents for PCR, the presence of various inhibitors in the sample, and even the sample volume used for the dilutions.[26] Therefore, it is important to correct the real-time PCR data using primer amplification efficiency to enhance the analytic accuracy.[12] We added the correction to the integrity value calculations ensuring a robust method for RNA

integrity assessment. This will help to successfully establish this method in any other laboratory, as the correction eliminates the bias introduced by using different primers, equipment, or reagents.

To validate 5′:3′ assay's ability to reflect the RNA integrity status, we chose to compare our results with widely used RIN value. 5′:3′ assay integrity value and RIN values were very comparable in heat-degraded mouse and human RNA samples (Figures 4B and 5B). There was a higher variability in the RNA integrity estimation from heat-degraded mouse RNA samples but no outliers were identified. We speculate that the higher values of mouse sample #1 were caused by variation between the independent experiments. In clinical samples the correlation was lower (Figure 6B) compared to the mouse and human heat-degraded RNA samples, suggesting that enzymatic RNA degradation, which occurs during sample collection and preparation, might affect messenger and ribosomal RNA differently. Heat-induced RNA degradation does not depend on RNA structure and thus affects both rRNA and mRNA similarly. However, mRNA is significantly more sensitive to enzymatic degradation by nuclease activity compared to rRNA.[4] In clinical samples or any other RNA samples, where RNA degradation is caused by endogenous nucleases, the results from rRNA-based methods can overestimate the RNA integrity. As sample collection in clinical settings and field studies are prone to rapid tissue necrosis, which is associated with enzymatic degradation of nucleic acids, 5′:3′ assay would be a better indicator of RNA quality in such samples.

Ribosomal-RNA-based methods for RNA integrity evaluation cannot be applied for various subcellular samples, such as synaptosomes or extracellular vesicles. Such samples share similar features that affect the assessment of the expression of their local transcripts: prolonged processing of the sample, low yields of RNA, and most importantly, lack of rRNA.[27] Despite the accumulating evidence on local protein synthesis and the presence of both ribosomal and messenger RNAs in synaptosomes, the RNA quality of synaptosomal preparations has not been previously reported, possibly due to the lack of suitable methods. Ribosomal RNA quantity in the synaptosomes is far lower than in general tissue RNA samples and not sufficient to calculate RIN even when the same amount of RNA is used (Figure 7B). Messenger RNAs are very different in lengths and do not form distinct bands; therefore, mRNAs are not observed as a trail in capillary electrophoresis gels and cannot be used to calculate RIN. Therefore, RIN could not be determined in subcellular samples, but mRNA-based 5′:3′ assay was suitable to measure the RNA integrity (Figure 7C). This shows a significant advantage and potential of this method to be used to check RNA integrity on any sample that lacks high quantities of rRNA, making the application of common RNA integrity assays impossible. The analysis of subcellular samples requires a careful choice of reference transcript for 5′:3′ assay due to the compartmentalization of mRNAs within the cell. For example, previous studies have found that the transcriptome in the synaptosomes is selectively enriched for specific mRNAs and therefore differs from total cortical transcriptome.[17] In this study, we chose *Pgk1* transcript for 5′:3′ assay, as it is ubiquitously expressed throughout neurons, including their presynaptic compartments. However, different subcellular samples may need other reference mRNAs, and it is advisable to confirm their localization in advance.

In conclusion, the 5′:3′ assay is a promising method for assessing mRNA integrity for gene expression studies, offering advantages over conventional techniques. The assay, based on *PGK1* mRNA, provides a user-friendly assessment system, aligning the integrity score with the widely used RIN values. Its correction for primer amplification efficiency enhances analytical accuracy, addressing the variations in qPCR conditions. While validation against RIN values showed strong correlation in heat-degraded samples, a slight disparity of correlation in clinical samples emphasizes the likely impact of the differences in enzymatic degradation of mRNA and rRNA. Despite acknowledged limitations, the adaptability of the 5′:3′ assay by designing primers for specific transcripts holds high potential for tailored assessments, offering a step forward in refining mRNA integrity evaluation.

Limitations of the study

5′:3′ assay holds a potential as an easily applicable method with improved accuracy for estimating RNA degradation for gene expression studies. However, there are a few limitations worth mentioning. All RNA integrity evaluation strategies are based on generalized assumptions about a very diverse nucleic acid pool. For example, it has been shown that the degree of degradation varies among different transcripts, and a substantial fraction of the variation can be explained by functional and structural features of different transcripts.[20] Also, 5′:3′ assay based on *PGK1* transcript might slightly overestimate the integrity of short transcripts, as it is known that shorter transcripts degrade faster than longer ones.[28] Therefore, more research is needed to understand natural mRNA degradation process and how it affects different transcripts. Nevertheless, one of the advantages of 5′:3′ assay is that it can be easily tailored by designing new qPCR primers for a specific transcript, which is then taken as a representative of mRNA pool. Thus, such transcript can be chosen to represent not all but a fraction of mRNAs with certain length, structure, or function of interest. Such adaptation of 5′:3′ assay to reflect a certain group of similar transcripts could improve the accuracy of mRNA integrity evaluation even further.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110419.

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AUTHOR CONTRIBUTIONS

D.B. carried out the experiments, performed data analysis and visualization, wrote the original draft of the article. K.M. and U.K. prepared and analyzed human RNA samples. J.D. and E.B. provided the equipment and expertise for FASS experiments. S.K. designed and cloned plasmids. G.L. and S.R provided surgically resected human brain tissue. U.N. supervised the study and wrote the manuscript. All the authors approved the final version of the manuscript for submission.

DECLARATION OF INTERESTS

The authors declare no competing interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
DH10B Competent *E. coli* cells	Thermo Scientific	Cat#EC0113
Biological samples		
C57BL/6J mice	Provided by Life Scineces Center. Available for purchase at The Jackson laboratory https://www.jax.org/strain/000664	Strain #:000664
VGLUT1^{mVenus} mice	Provided by the University of Bordeaux in 2018 and later bred and kept at the animal facility of the Life Sciences Center. https://doi.org/10.1523/JNEUROSCI.2073-11.2011	N/A
Human brain tissue samples	Human brain tissue specimens were obtained at Vilnius University Hospital Santaros Klinikos with informed consent as requested by the regional ethics committee (Nr.2/2020 02 18).	N/A
Chemicals, peptides, and recombinant proteins		
Trizol	ThermoFisher Scientific	Cat#15596026
D-glucose	ROTH	Cat#CN72.2 CAS No. 3671-99-6
HEPES	ROTH	Cat#9105.3 CAS No. 7365-45-9
Magnesium sulfate	ROTH	Cat#2278.3 CAS No. 7487-88-9
Sodium phosphate monobasic monohydrate	Sigma-Aldrich	Cat#10049-21-5
Calcium chloride (dehydrate)	Sigma-Aldrich	Cat#10035-04-8
N-methyl-d-glucamine chloride (NMDG-Cl)	Sigma-Aldrich	Cat#6284-40-8
Potassium chloride	ROTH	Cat#6781.1 CAS No. 7447-40-7
Sodium bicarbonate	Sigma-Aldrich	Cat#144-55-8
Sodium L-ascorbate	Sigma-Aldrich	Cat# 134-03-2
Sodium pyruvate	Gibco™	Cat# 11360070
Thiourea	Sigma-Aldrich	Cat#62-56-6
Sucrose (RNAse, DNAse free)	ROTH	Cat# 9097.1 CAS No. 57-50-1
RiboLock RNase Inhibitor	Thermo Scientific™	Cat#EO0381
SynaptoRed C2	Tocris Bioscience	Cat#5118
Anchored Oligo-dT primers	Invitrogen	Cat#12577-011
Maxima Reverse Transcriptase	Thermo Scientific	Cat#MAN0012044
Maxima SYBR Green/ROX qPCR Master Mix	Thermo Scientific	Cat#K0221
dNTP Mix	Thermo Scientific	Cat#R0191
Critical commercial assays		
RNA Purification Kit and Phasemaker Tubes Complete System	Invitrogen	Cat#A33254
TURBO DNA-free Kit	Invitrogen	Cat#AM1907

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Agilent RNA 6000 Pico Kit	Agilent Technologies	Cat#5067-1513
Maxima™ First Strand cDNA Synthesis Kit	Thermo Scientific	Cat#K1671
TaqMan assay for human GAPDH	TaqMan	Cat#4331182 Hs02758991_g1
TaqMan assay for human CMAS	TaqMan	Cat#4331182 Hs00218814_m1
TaqMan assay for human NEU1	TaqMan	Cat#4331182 Hs00166421_m1
TaqMan assay for human NEU3	TaqMan	Cat#4351372 Hs05025667_m1
Deposited data		
Raw data from Figures 2, 3, 4, 5, 6, S3, and S4 were deposited on Mendeley Data at https://doi.org/10.17632/293bp5jn6t.1	This paper	N/A
Oligonucleotides		
See Table 1 for a list of primers for 5′:3′ assay	This paper	N/A
Pgk1_For_MMPCR: 5′-GGAGGCCCGGCATTCTGCAC-3′	This paper	N/A
PGK1_Rev_HSPCR: 5′-CCACCCCCAGTGCTCACATG-3′	This paper	N/A
PGK1_For_HSPCR: 5′-GGCAGTCGGCTCCCTCGTTG-3′	This paper	N/A
Pgk1_Rev_MMPCR: 5′-ACCGCCCCCAGTGCTCACATG-3′	This paper	N/A
Recombinant DNA		
Plasmid with cloned mouse *Pgk1* cDNA	This paper. Sequence available at Mendeley Data https://doi.org/10.17632/293bp5jn6t.1	N/A
Plasmid with truncated mouse *Pgk1* cDNA	This paper Sequence available at Mendeley Data https://doi.org/10.17632/293bp5jn6t.1	N/A
Plasmid with cloned human *PGK1* cDNA	This paper Sequence available at Mendeley Data https://doi.org/10.17632/293bp5jn6t.1	N/A
Plasmid with truncated human *PGK1* cDNA	This paper Sequence available at Mendeley Data https://doi.org/10.17632/293bp5jn6t.1	N/A
Software and algorithms		
GraphPad Prism 8	GraphPad Software	https://www.graphpad.com/
Adobe Illustrator	Adobe	https://www.adobe.com/
Biorender	Biorender	https://app.biorender.com

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Urte Neniskyte (urte.neniskyte@gmc.vu.lt).

Materials availability

Plasmids generated in this study and raw data from Figures 2, 3, 4, 5, 6, S3, and S4 have been deposited to Mendeley Data: https://doi.org/10.17632/293bp5jn6t.1.

Data and code availability

- The authors declare that all data supporting the findings of the present study are publicly available in the article, its supplemental figures, tables and Mendeley Data repository. DOIs are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mouse brain tissue samples

C57BL/6J mice were obtained from the local Life Sciences Center colonies. VGLUT1^{mVenus} mice[18] were used in the homozygous state. Animal studies were conducted in accordance with the requirements of the Directive 2010/63/EU and were approved by the Lithuanian State Food and Veterinary Service (permit No. G2-92). All mice were bred and kept at the animal facility of the Life Sciences Center of Vilnius University.

For total brain RNA extraction, mouse brains were harvested from adult C57BL/6 mouse after cervical dislocation. The cortex was immediately dissected, homogenized in Trizol and stored at −80°C until RNA isolation. VGLUT1^{mVenus} mice aged P21, P28 or P35 were used for synaptosomal preparations.

Human brain tissue samples

Permission to use human brain tissue was obtained from Vilnius Regional Biomedical Research Ethics Committee (Approval No. 2020/2-1202-687). Human brain tissue specimens were obtained with the informed consent as requested by the Regional Ethics Committee (No. 2/2020 02 18).

Human neocortical access tissue (n = 16) was sampled from either glioma tumor resection surgery (n = 6), using distant cortex without tumor infiltration, or access cortex tissue from epilepsy surgery (n = 5) obtained during the resection of epileptic foci. Human hippocampus tissue (n = 5) were samples resected during epilepsy surgery. Detailed information regarding the donors is provided in Table S1.

METHOD DETAILS

Human brain tissue preparation

Tissue was immersed in 4°C Artificial Cerebrospinal Fluid (aCSF) immediately post-resection and transferred for homogenization in Trizol (#15596026, ThermoFisher Scientific). Homogenized samples were stored at −80°C until further procedures.

ACSF used for tissue transport was prepared as previously reported.[29] Shortly, the solution contained 0.5 mM calcium chloride (dehydrate), 25 mM D-glucose, 20 mM HEPES, 10 mM magnesium sulfate, 1.2 mM sodium phosphate monobasic monohydrate, 92 mM N-methyl-d-glucamine chloride (NMDG-Cl), 2.5 mM potassium chloride, 30 mM sodium bicarbonate, 5 mM sodium L-ascorbate, 3 mM sodium pyruvate, and 2 mM thiourea. Prior to use, the solution was equilibrated with 95% O_2, 5% CO_2. Osmolality was verified to be between 295 and 305 mOsm/kg, pH was adjusted to 7.3 using HCl.

Plasmids

Mouse and human *PGK1* cDNA fragments were copied and amplified by PCR using Thermo Scientific Phusion High-Fidelity DNA polymerase from mouse or human brain total RNA sample. The following primers were used: *Pgk1_For_MMPCR*: 5′- GGAGGCCCGGCATTCTGCAC-3′ and *Pgk1_Rev_MMPCR*: 5′- ACCGCCCCCAGTGCTCACATG-3′ for mouse gene and *PGK1_For_HSPCR*: 5′- GGCAGTCGGCTCCCTCGTTG-3′, *PGK1_Rev_HSPCR*: 5′- CCACCCCCAGTGCTCACATG-3′ for human gene. The PCR fragments were then ligated into pJET1.2/blunt plasmid using CloneJET PCR cloning Kit (Thermo Scientific). To generate plasmids containing 3′-end of *PGK1* cDNA 5′ part of *PGK1* was excised by NcoI from previously generated full-length *PGK1* cDNA plasmids. All pasmids were linearized by XhoI for experiments.

Crude synaptosome sample preparation

Fresh mouse brain was cleaned and cooled in ice-cold PBS. All the following steps were carried on ice. Mouse visual cortex was dissected and placed into a 2 cm clean ice-cold glass-Teflon potter. The tissue was then homogenized in 1 mL of ice-cold 0.32 M sucrose buffer (0.32 M sucrose, 4 mM HEPES, 4 U/μL Ribolock, pH 7.4) at 900 rpm for 15s moving the pestle up and down. The homogenate was centrifuged at 1000×g for 5 min at 4°C. The supernatant was then centrifuged at 12,500×g for 8 min at 4°C. The second supernatant was discarded and the pellet was carefully resuspended in ice-cold 0.3 mL 0.32 M sucrose buffer. Discontinuous sucrose gradient was prepared in 5 mL ultracentrifuge tubes (Beckman Coulter, C14279) with 2 mL of 1.2 M sucrose solution (1.2 M sucrose, 4 mM HEPES, 0.2 U/μL Ribolock, pH 7.4) at the bottom and 2 mL 0.8 M sucrose solution (0.8 M sucrose, 4 mM HEPES, 0.2 U/μL Ribolock, pH 7.4) at the top. The resuspended pellet was then carefully laid over the prepared gradient. The tubes were ultracentrifuged at 24,000 rpm for 45 min at 4°C. After ultracentrifugation two layers of particles and a pellet appeared. The middle layer containing synaptic particles was collected with a syringe by piercing through the tube

wall. Collected crude synaptosome sample was then diluted in 8 mL of ice-cold 0.22 μm filtered PBS. This sample was then split and part of it was collected onto a 0.1 μm polycarbonate filter and washed with Trizol reagent for RNA extraction. The second part was used for Fluorescence Activated Synaptosome Sorting (FASS) to prepare matching excitatory synaptosome-enriched samples.

Fluorescence Activated Synaptosome Sorting (FASS)

The prepared crude synaptosome sample was diluted in PBS and stained with SynaptoRed C2 (1 μg/ml, Tocris Bioscience) to label all plasma membrane-containing particles to increase synaptosome detection sensitivity. The dilution was optimized every time to reach around 15 000 events per second at the flow rate between 3 and 4. A new sorting sample was prepared every 45 min and the collection tube was changed at the same time. Synaptosome sorting was performed as described previously.[19] For sorting we used BD FACSAria III Cell Sorter set as follows: 70 μm Nozzle, sample shaking at 300 rpm, sample temperature at 4°C, FSC neutral density filter 1.0, 488 nm Laser on, Area Scaling 1.18, Window Extension 0.0, FSC Area Scaling 0.96, Sort Precision: 0-16-0. Thresholding on SynaptoRed C2 with a threshold value of 800. Synaptosomes were sorted into a 5 mL polystyrene round-bottom tubes (BD Falcon) and kept on ice. The synaptosomes were sorted and collected for at least 6 h. Then synaptosomes were collected onto a 0.1 μm polycarbonate filter and washed with Trizol reagent for RNA extraction.

RNA extraction

Total RNA from mouse cortex, crude and enriched synaptosomal preparations and surgically resected human brain samples was extracted using TRIzol Plus RNA Purification Kit and Phasemaker Tubes Complete System (Invitrogen, cat. no. A33254). The remaining gDNA was removed using TURBO DNA-free Kit (Invitrogen, cat. no. AM1907) following the manufacturer's protocols.

Preparation of heat-degraded RNA samples

Mouse cortex RNA samples were diluted to 100 ng/μL. An aliquot (12 μL) of each diluted RNA sample was taken into separate tubes. While one tube served as the control (heated at 70°C for 2 min to denature RNA), the other tubes were exposed to 90°C heat for 1, 2, 3, 4, 5, 10, or 15 min in a thermocycler. The same procedure was applied to human brain total RNA samples, which were kept at 90°C for 1, 3, 5, or 10 min.

Preparation of RNAse A-degraded RNA samples

Mouse cortex RNA samples were diluted to 300 ng/μL and aliquoted to 10 μL samples. Enzymatic degradation of RNA was induced by the treatment with 10 ng/mL of RNAse A (Thermo Scientific) for 5, 10, 15 or 30 min at 37°C. Enzymatic reaction was stopped by adding 2 U/μL Ribolock to each of the samples.

Evaluation of RNA concentration and rRNA integrity

A NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA) was used to measure the absorbance at 260 nm to evaluate RNA concentration. rRNA integrity was assessed by an Agilent 2100 Bioanalyzer system, using the Agilent RNA 6000 Pico Kit (Part Number: 5067-1513) (Agilent Technologies, Mississauga, ON, Canada) following the manufacturer's protocols.

cDNA synthesis and RT-qPCR

The reverse transcription reactions were performed in a total volume of 20 μL using 1 μg of total RNA (heated at 70 C for 2 min to denature RNA), 2.5 μg Anchored Oligo-dT primers (Invitrogen), 0.5 mM dNTP Mix (Thermo Scientific) and 200 U Thermo Scientific Maxima Reverse Transcriptase (Thermo Scientific) following manufacturer's first-strand cDNA synthesis protocol. All cDNA samples were diluted with nuclease-free water to the final cDNA concentration of 20 ng/μL.

Quantitative PCR was performed with The StepOne Real-Time PCR System or QuantStudio 3 System (Applied Biosystems) using 5 μL diluted cDNA, 0.3 μM of the forward and reverse (5 μL) primer mix (designed primer pairs targeting either 3′ or 5′ *Pgk1* cDNA ends are presented in Table 1), and 12.5 μL 2× Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) and 2.5 μL of nuclease-free water in a reaction volume of 25 μL. The RT-qPCR reaction mix was denatured at 95°C for 10 min and then subjected to 40 amplification cycles (15 s denaturation at 95°C, 30 s annealing at 60°C and 30 s extension at 72°C) following manufacturer's protocols for the SYBR Green Master Mix. The reactions were run in triplicates. The specificity of the qPCR products was assessed by melting-curve analysis. No-template controls (NTC) did not record any positive Ct values. Maxima First Strand cDNA Synthesis Kit was used for reverse transcription followed by TaqMan PCR assay in accordance with manufacturer's first-strand cDNA synthesis protocol. The Ct values of *CMAS* and *GAPDH* in surgically resected human brain tissue RNA samples were defined by TaqMAN qPCR assay. The following primers were used: *CMAS* (Hs00218814_m1, #4331182, Thermo Fisher Scientific), *GAPDH* (Hs02758991_g1, #4331182, Thermo Fisher Scientific), *NEU1* (Hs00166421_m1, #4331182, Thermo Fisher Scientific) and *NEU3* (Hs05025667_m1, #4351372, Thermo Fisher Scientific).

Integrity value correction

To estimate amplification efficiency, seven 5-fold dilutions were prepared using constructed linearized plasmids or prepared mouse or human cDNA and RNAse/DNAse free water. For plasmid dilution series, the highest concentration was 1.04×10^8 plasmids per reaction. For cDNA dilution series the highest concentration was 170 ng of cDNA per 25 μL q-PCR reaction. RT-PCR was performed as described above. Acquired mean Ct values were plotted against concentration logarithm by 5 (the number of dilution factor) using Microsoft Excel Spreadsheet Software.

Next, a linear regression curve was generated and the slope of the trend line was calculated. Amplification efficiency was estimated using the following equation:

$$Amplification\ efficiency = \left(5^{\frac{-1}{Slope}} - 1\right) \times 100$$

Then the amplification factor was calculated and corrections for the expression were made as described by Damgaard and Treebak.[12]

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism 8 software was used for statistical analysis. R-squared test was applied to determine the strength of the relationship between the linear model and the dependent variables. Prism's linear regression analysis was used to compare the slopes and the intercepts of two linear regression lines. Concordance between datasets was estimated through Pearson correlation. Outliers were defined as exceeding three interquartile ranges and removed from the analysis. Shapiro-Wilk test was used to assess the normality of the datasets and all data was confirmed to have normal distribution except data from Figure 3B ($p = 0.011$). Paired t-test was used to compare RIN to 5′:3′ integrity values.

The amplification efficiency was calculated from two different human or mouse RNA samples. RNA samples from five mouse cortices were used for RIN and 5′:3′ integrity score evaluation. RNA samples from postsurgical brain tissue of three patients were used for RIN and 5′:3′ integrity score evaluation. Four crude and sorted synaptosome samples from four different animals were used for mRNA integrity study of subcellular samples.