An analysis of RNA quality metrics in human brain tissue

Abbreviated title: Analysis of RNA quality metrics in brain tissue

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Abstract

Human brain tissue studies have historically used a range of metrics to assess RNA quality. However, few large-scale cross-comparisons of pre-sequencing quality metrics with RNA-seq quality have been published. Here, we analyze how well metrics gathered before RNA sequencing (post-mortem interval (PMI) and RNA integrity number RIN) relate to analyses of RNA quality after sequencing (Percent of counts in Top Ten genes (PTT), 5' bias, and 3' bias) as well as with individual gene counts across the transcriptome. We conduct this analysis across four different human cortical brain tissue collections sequenced with varying library preparation protocols. PMI and RIN have a low inverse correlation, and both PMI and RIN show consistent and opposing correlations with PTT. Unlike PMI, RIN shows strong consistent correlations with measurements of 3' and 5' bias, and RIN also correlates with 3,933 genes across datasets, in comparison to 138 genes for PMI. Neuronal and immune response genes correlate positively and negatively with RIN respectively, suggesting that different gene sets have divergent relationships with RIN in brain tissue. In summary, these analyses suggest that conventional metrics of RNA quality have varying degrees of value, and that PMI has an overall minimal but reproducible effect on RNA quality.

51 **Introduction:**

52 Brain tissue is routinely collected and stored at academic hospitals for a variety of research

53 programs (1, 2). A major determinant of tissue quality is the post-mortem interval (PMI), which

54 is often assumed to be relevant for RNA quality. For RNA studies, an RNA integrity number

55 (RIN), as measured by a bioanalyzer, is frequently used as a more direct metric.

56

57 The relationship between RIN and PMI on the one hand and RNA quality on the other has been

58 extensively studied by multiple groups. While RIN has been shown to consistently predict RNA

59 quality (3-6), the literature on PMI is more mixed. Several groups have found little to no

60 relationship between PMI and RNA quality (4, 6-9), while others have found that PMI does affect

61 the expression of some genes (3, 10). Notably, comparison of RIN and PMI to RNA quality is

62 mostly limited to qPCR studies (3, 5-9, 11, 12), and some microarray data (7, 13). With RNA-

63 seq now ubiquitous as a technology (14-17), there is a need for a more thorough comparison of

64 these pre-sequencing quality metrics with commonly used QC metrics relevant for sequencing-

65 based transcriptomic analysis in brain tissue, as well as an analysis of how RIN and PMI relate

66 to gene expression across the entire transcriptome.

67

68 Here, we analyze how well PMI and RIN relate to analyses of RNA quality in cortical brain tissue

69 after RNA-seq. We do this across four different datasets of RNA-seq data, including 106

70 surgical specimens with poly-A selection, 534 autopsy specimens with poly-A selection, and two

71 different autopsy datasets with ribosomal RNA depletion and no poly-A selection, with 513 and

72 365 specimens in each dataset. We relate PMI and RIN to a range of post-sequencing QC

73 metrics and show that RIN is predictive of overall RNA quality, while PMI has a reproducible but

74 overall minimal impact. We also identify genes that correlate most reliably with RIN across all

75 four datasets, which has implications for research where these genes are relevant. In summary,

76 our work is the most extensive analysis to date of the effects of RIN and PMI in human brain

77   tissue and has implications for the relative importance that brain banks should place on these

78   parameters.

79

80

81   **Materials and Methods:**

82   *Data Acquisition:* All four datasets used in this study were generated for other studies. The

83   NPH dataset is a collection of cortical biopsies taken during ventricular shunt placement at

84   Columbia University Irving Medical Center. Biopsies were taken from frontal cortex in 2/3 of the

85   subjects and parietal cortex in 1/3 of the subjects in our original cohort. As reported in (16),

86   RNA was extracted from biopsy samples using miRNeasy Mini Kit (QIAGEN; Cat

87   No./ID: 217004), and samples with RIN values ≥ 6 were selected for sequencing. RNAs were

88   prepared for sequencing using the Illumina TruSeq mRNA library prep kit, and samples

89   underwent single-end sequencing to 30M read depth.

90

91   New York Brain Bank (NYBB) RNA-seq data from dorsolateral/dorsomedial prefrontal cortex

92   was also generated at Columbia University (18). Total RNA was used to make cDNA using

93   Kapa Total library prep, which removes ribosomal RNA and is optimized for degraded RNA. Our

94   QC threshold for using RNA was based on RIN, trace degradation, and presence of ribosomal

95   RNA subunits (some samples had a RQN generated on a fragment analyzer instead of RIN on a

96   bioanalyzer, and we use "RIN" to refer to both RIN and RQN values from this dataset given the

97   interchangeability of these two numbers). RNA samples that had RIN above 4 passed. Samples

98   that had RIN values between 2.5-3.9 were evaluated and passed for sequencing based on

99   visual inspection of trace degradation and ribosomal subunit presence.

100

101   Religious Orders Study and Rush Memory and Aging Project (ROSMAP) RNA-seq data from

102   dorsolateral prefrontal cortex (DLPFC) was obtained from the Rush Alzheimer's Disease Center

103   (19, 20); ROSMAP RNA-seq data is available via the AMP-AD data portal through Synapse

104   (https://www.synapse.org/#!Synapse:syn3219045) and the RADC Research Resource Sharing

105   Hub (https://www.radc.rush.edu). Of the 899 ROSMAP samples in this study, 534 were

106   sequenced using poly-A selection, while 365 used KAPA Stranded RNA-Seq Kit with RiboErase

107   (kapabiosystems); these two sets are analyzed separately in this manuscript.

108

109   All FASTQ files were aligned to the GRCh38 genome (Ensembl Release 101) using STAR

110   (v2.7.6a) in 2-pass mapping mode with standard ENCODE parameters. Gene counts were

111   quantified from STAR-aligned BAM files using featureCounts (v2.0.1). RNA sequencing metrics

112   were collected using Picard by generating a ribosomal interval list and a ref_flat file from

113   annotated genomic data. The median 5' and 3' bias of the 1000 most highly expressed

114   transcripts in each biopsy sample were extracted for further analysis.

115

116   *Correlation Analysis on Datasets*. Correlation analysis was conducted on NPH (n = 106), NYBB

117   (n = 513), and ROSMAP (n = 534, 365) datasets to examine relationships among metrics.

118   Spearman's correlation analysis was performed using the corr.test function from the psych

119   library in R, with p-values adjusted for false discovery rate (FDR) at a threshold of 0.05. The

120   metrics analyzed included RNA Integrity Number (RIN), the proportion of the top 10 gene counts

121   to total gene expression (PTT), Post-Mortem Interval (PMI), DV200, median 5' bias, median 3'

122   bias, and the median 5' to 3' ratio, where applicable. For Tables 1 and 2, each panel (a, b, c,

123   etc.) has p-values FDR adjusted across that panel.

124

125   *Single-gene Correlation Analysis.* Spearman's correlation analysis was performed to examine

126   the relationship between RNA Integrity Number (RIN) and Post-Mortem Interval (PMI) for NYBB

127   and ROSMAP samples, and RIN for NPH biopsies. Genes with fewer than 5 counts in at least

128   90% of samples were filtered out from the raw count matrix. The filtered counts were then

129   normalized using variance stabilizing transformation (VST) with the

130   *varianceStabilizingTransformation* function from the DESeq2 R package. RIN and PMI for each

131   sample were correlated with the normalized gene counts of each gene across all samples.

132   Ontology analysis on biological processes was performed using Enrichr (21).

133

134   **Results:**

135   We analyzed bulk RNA-seq data from neocortex across four different groups: a set of 106

136   biopsy specimens from patients with normal pressure hydrocephalus (16), a cohort of 899

137   dorsolateral prefrontal cortex specimens from the ROSMAP cohort (19, 20) (divided into two

138   groups, see below), and a group of 513 dorsolateral/dorsomedial prefrontal cortex specimens

139   from the New York Brain Bank (NYBB) at Columbia University. The NPH and 534 of the

140   ROSMAP specimens underwent poly-A selection and subsequent RNA-seq, while the NYBB

141   specimens and 365 of the ROSMAP specimens underwent enzymatic depletion of ribosomal

142   RNA and did not employ poly-A selection. All samples had RIN values generated (see Methods),

143   and for NYBB samples a DV200 value (the percentage of RNA fragments > 200 nucleotides)

144   was also generated. Post-mortem interval (relevant for the three autopsy cohorts) was also

145   recorded for all samples. After bulk RNA-seq, the percent of counts in the top ten genes (PTT)

146   was calculated for each sample. In addition, the percent of counts in the 5' or 3' end of each

147   transcript normalized by counts over the entire transcript was calculated for the two datasets

148   with poly-A selection, given that this technique is sensitive to 3' bias with degraded RNA (22).

149   The goal of this study was to examine how all of these metrics relate to each other across these

150   four different datasets and to determine how well pre-sequencing RNA quality metrics predict

151   RNA quality measured post-sequencing. For all autopsy datasets, we performed our analysis in

152   both the full dataset and in a subset of samples with RIN values restricted to the range in the

153   NPH data (RIN of 6 and higher) to evaluate if lower RIN scores in the autopsy tissue is

154   influencing any differences with the surgical tissue set.

155

156 *RIN and PMI show similar and divergent relationships with post-sequencing QC metrics*

157 The set of 106 surgical specimens is free of post-mortem artifact by definition, and there is no

158 associated PMI. In this dataset, RIN correlates strongly and inversely with PTT (r = -0.55) as

159 expected, but has no significant correlation with measures of 5' or 3' bias (Table 1A). One

160 caveat to this analysis is that the correlation of 3' bias with RIN (r = -0.18) has an adjusted p-

161 value of 0.091. The ROSMAP dataset (discussed below) has a similar correlation between RIN

162 and 3' bias (r = -0.2) for higher RIN samples that is significant. The ROSMAP cohort is several

163 fold larger than the NPH sample set, so one could speculate that the lack of significance in the

164 NPH samples is primarily a power issue. Although 5' and 3' have no significant relationship with

165 RIN in the NPH data, they are relevant RNA quality metrics in this dataset, as indicated by the

166 fact that 5' bias, 3' bias, and the ratio of 5' to 3' reads all correlate strongly and consistently with

167 PTT.

168

169 Next, we examined the NYBB set of RNA-seq data (Table 1B). Measurements of 5' and 3' bias

170 are irrelevant in this dataset given the fact that poly-A selection was not used. Thus, for post-

171 sequencing measurements we only have PTT. However, for pre-sequencing measurements,

172 the DV200 was also recorded for these samples. Comparing all these available metrics to one

173 another, we observed that RIN negatively correlates with PTT (r = -0.53), similarly to the NPH

174 dataset. RIN and DV200 are closely aligned (r = 0.72), and RIN has a weak negative

175 correlation with PMI (r = -0.11). Of note, PMI positively correlates with PTT in this dataset (r =

176 0.19), suggesting that PMI does influence RNA quality post-sequencing. When we restrict NYBB

177 samples to the range of RIN values seen in the NPH surgical tissue dataset (RIN > 6), we see

178 similar trends, although we lose significance for some correlations, likely due to lower power

179 (Table 1C).

180

181   Both the NPH and NYBB RNA-seq data were generated from samples at Columbia University.

182   To further generalize our results, we next analyzed pre- and post-sequencing RNA quality

183   metrics in ROSMAP autopsy tissue (Table 2). The ROSMAP samples sequenced with poly-A

184   selection demonstrated consistent and significant correlations between RIN and all other

185   measured metrics in this dataset. PMI correlates significantly with PTT (r = 0.22). The

186   relationship of RIN and PMI to 5' and 3' bias is more complex. RIN shows consistent and

187   significant correlations with these metrics, although 3' bias isn't significant in the full dataset but

188   is when we restrict our analyses to samples to the range of RIN values seen in the NPH surgical

189   tissue dataset (RIN of 6 or higher). PMI also shows a complex relationship with 3' bias, as it

190   shows a significant negative correlation with 3' bias in the full dataset (the opposite of what

191   would be predicted), and this relationship is no longer significant when samples are restricted to

192   RIN of 6 and higher. Although it is somewhat difficult to interpret this data, both 3' and 5' bias do

193   have strong and consistent relationships with PTT for both the full and restricted datasets,

194   suggesting that these measurements of RNA quality are performing as expected. At minimum,

195   PMI does not relate to 3' or 5' bias in a coherent way, while RIN has an overall consistent trend

196   that may depend on additional factors for reproducibility.

197

198   In the ROSMAP subjects that were sequenced with ribosomal RNA depletion, RIN correlates

199   with PTT in the full dataset, and this is lost after restricting the samples to RIN of 6 or higher,

200   possibly due to lower statistical power. In summary, PMI negatively correlates with RIN in two of

201   the three autopsy datasets we examined. Both RIN and PMI correlate with PTT in all but the

202   final dataset (ROSMAP with ribosomal RNA depletion), where only RIN correlates with PTT.

203   The major area of divergence in predictive power of RIN vs. PMI is in measurements of 3' and 5'

204   bias, where RIN significantly correlates with these measurements in the autopsy cohort and

205   approaches significance in the NPH data, whereas PMI shows no predictive power.

206

207   *RIN and PMI show divergent effects at the individual gene level*

208   Finally, we analyzed RNA-seq data for genes that correlate with RIN or PMI. Spearman's

209   correlations with PMI and RIN were calculated for all genes in the transcriptome for all four

210   datasets, and significance was set at FDR < 0.05. We then determined how many genes

211   positively correlate with RIN in all four datasets and found 2,512 genes that commonly overlap

212   (see Supplemental Data for genes that commonly correlate with RIN and PMI in the four

213   datasets). Notably, 1,421 genes negatively correlate with RIN in all four datasets, suggesting a

214   complex relationship between RIN and gene expression values. We next determined how many

215   genes positively and negatively correlate with PMI in the three autopsy cohorts. Interestingly,

216   far fewer genes correlate with PMI across these datasets, with 138 positively correlating genes

217   and no negatively correlating genes passing FDR of 0.05. To understand whether there are

218   common biological processes that characterize the sets of genes that correlate with RIN and

219   PMI, we performed ontology analysis on the genes in common (Table 3; See Supplemental

220   Data for full ontology analysis). Genes that positively correlate with RIN were significantly

221   enriched for ontology categories characterized by neuronal function, whereas genes that

222   negatively correlate with RIN are characterized by the immune response. Given that these

223   tissue collections are from neurodegeneration studies, we asked whether this gene signature

224   was capturing neurodegenerative change in any way. While RIN mildly correlates with Braak

225   stage in the ROSMAP poly-A dataset ($r = -0.11$, $p = 0.01$), RIN does not correlate with Braak

226   stage in the ROSMAP ribosomal RNA depletion dataset ($r = -0.091$, $p = 0.15$) or the NYBB

227   dataset ($r = -0.052$, $p = 0.318$). We have previously quantified β-amyloid and tau pathology in

228   the NPH biopsies (16), and RIN does not correlate with either of these metrics (β-amyloid $r = -$

229   0.119, $p = 0.386$; tau $r = -0.046$, $p = 0.808$). Thus, it is unlikely that neurodegenerative disease

230   is indirectly driving these changes in gene expression. Instead, these ontology themes suggest

231   that inflammation may be related to RNA degradation in brain tissue more broadly (see

232   Discussion). The more limited sets of genes that correlate with PMI did not show significant

233   ontology categories. In summary, the relative abundance of genes that correlate with RIN vs.

234   PMI and the correlation of RIN with measurements of 3' and 5' bias point to ways that RIN is

235   uniquely capturing RNA fragmentation in brain tissue, although PMI may also relate to some

236   aspects of RNA degradation.

237

238

239   **Discussion:**

240   RNA quality in brain autopsy tissue may be influenced by a variety of factors, including peri-

241   mortem conditions that influence pH (4, 6, 7, 12, 13). Here, we conduct a study across multiple

242   datasets relating RIN and PMI to post-sequencing RNA-seq quality control metrics and find that

243   PMI is minimally useful for predicting RNA quality using metrics derived from RNA-seq data.

244   The majority of the prior work that has been done using non-sequencing based analysis

245   suggests that PMI has no to minimal value for predicting RNA quality (4, 6-9), although some

246   publications suggest that PMI does matter to varying degrees (3, 10). Notably, Birdsill et al.

247   found that 4 genes (ADAM9, LPL, PRKCG, and SERPINA3) negatively correlated with PMI in

248   human brain tissue (out of 85 tested) (3), and Durrenberger et al. found that 18S rRNA

249   negatively correlated with PMI (out of 7 reference genes tested) (11). At the individual gene

250   level, we find that PMI positively correlates with 138 genes in common across the 3 autopsy

251   datasets. While this is a small number in comparison to RIN, it is also interesting that all of

252   these genes positively associate with PMI. While no ontology categories are significant, one

253   could speculate that these genes may represent post-mortem changes in transcription

254   associated with dying tissue, which is different from the RNA degradation that is normally

255   assumed to the primary artifact associated with PMI.

256

257   Indeed, PMI also does not correlate with 3' or 5' bias, which is a common measurement of RNA

258   degradation. This is particularly striking in the ROSMAP dataset with poly-A selection, where 3'

259   and 5' bias correlate with RIN, but do not correlate with PMI. However, PMI does consistently

260   correlate with PTT in two out of the three autopsy datasets, suggesting that reduced

261   transcriptomic complexity relates to PMI in some fashion. We also note that PTT is a general

262   barometer of RNA health, correlating with both RIN and 3' and 5' bias consistently. This makes

263   PTT is the broadest QC metric we analyzed, as PTT captures variation in RNA quality that is

264   observed in RIN, DV200, and measurements of 3' and 5' bias. PMI also significantly correlates

265   with RIN in two of the three autopsy datasets, which suggests that RIN may be modulated by

266   variables that in turn are not significantly correlating with many of the post-sequencing RNA

267   quality metrics associated with RIN.

268

269   In contrast to PMI, RIN correlates with 3' and 5' bias, as well as with several thousand genes

270   across all four datasets. Of particular interest is the fact that neuronal function ontology groups

271   positively correlate with RIN, while immune response ontology groups negatively correlate with

272   RIN. The fact that genes negatively correlate with RIN at all is itself counterintuitive, given that

273   declining RIN scores and accompanying RNA fragmentation would be predicted to lead to loss

274   of genes, not increases in genes that negatively correlate with these phenomena. Although

275   speculative, one possible way to think about this is to not assume that RIN is driving these

276   changes, but perhaps the other way around. As noted earlier, peri-mortem factors (for example

277   sepsis) influence RNA quality (4, 6, 7, 12, 13). While this explanation obviously doesn't apply to

278   the NPH biopsies that also show these changes (as well as autopsy cases that didn't die with

279   sepsis), a more general phenomenon may be that brain inflammation, regardless of cause,

280   contributes to lower RNA integrity. While it remains unclear exactly why inflammatory

281   transcriptomic changes are elevated in brain tissue with lower RIN scores, the fact that these

282   changes are found in common across four datasets suggests this is a reproducible

283   phenomenon that is worth exploring in future work.

284

285   In summary, we have comprehensively assessed the impact of PMI and RIN on post-

286   sequencing quality control metrics in brain tissue. Our results are overall consistent with prior

287   work that has been done in this area with qPCR and microarray analysis, and give a broader

288   assessment of the marginal but nevertheless reproducible impact of PMI on RNA quality. In

289   addition, we also identify genes that are most likely to be impacted by RIN in RNA-seq studies.

290   These findings will be of interest to researchers using human brain tissue for transcriptomic

291   analysis, and also of potential interest to brain bank staff banking cases of interest with longer

292   PMIs.

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295   **Acknowledgements:**

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Table 1: Correlations of QC Metrics in NPH Biopsy Data and NYBB Autopsy Data

(a) NPH (n = 106)

	1	2	3	4	5
1. RIN	1.00				
2. PTT	-.55**	1.00			
3. median 5' bias	-.025	-.54**	1.00		
4. median 3' bias	-.18	.57**	-.75**	1.00	
5. median 5' to 3' bias ratio	.028	-.58**	.98**	-.78**	1.00

(b) NYBB (n = 513)

	1	2	3	4
1. RIN	1.00			
2. PMI	-.11*	1.00		
3. PTT	-.53**	.19**	1.00	
4. DV200	.72**	-.19**	-.60**	1.00

(c) NYBB (RIN ≥ 6, n = 160)

	1	2	3	4
1. RIN	1.00			
2. PMI	-.10	1.00		
3. PTT	-.35**	.21*	1.00	
4. DV200	.58**	-.11	-.41**	1.00

*FDR-adjusted p-value < 0.05, ** FDR-adjusted p-value < 0.001*

Table 2: Correlations of QC Metrics in ROSMAP Autopsy Data

(a) ROSMAP poly-A Selection Library Preparation (n = 534)

	1	2	3	4	5	6
1. RIN	1.00					
2. PMI	-.26**	1.00				
3. PTT	-.68**	.22**	1.00			
4. median 5' bias	.47**	.051	-.60**	1.00		
5. median 3' bias	-.029	-.11*	.32**	-.46**	1.00	
6. median 5' to 3' bias ratio	.18**	.11*	-.46**	.73**	-.92**	1.00

(b) ROSMAP poly-A Selection Library Preparation (RIN ≥ 6.0, n = 441)

	1	2	3	4	5	6
1. RIN	1.00					
2. PMI	-.26**	1.00				
3. PTT	-.67**	.24**	1.00			
4. median 5' bias	.49**	.024	-.60**	1.00		
5. median 3' bias	-.20**	-.061	.42**	-.54**	1.00	
6. median 5' to 3' bias ratio	.29**	.066	-.51**	.76**	-.93**	1.00

(c) ROSMAP Ribosomal Depletion Library Preparation (n = 365)

	1	2	3
1. RIN	1.00		
2. PMI	-.088	1.00	
3. PTT	-.54**	.026	1.00

(d) ROSMAP Ribosomal Depletion Library Preparation (RIN ≥ 6.0, n = 103)

	1	2	3
1. RIN	1.00		
2. PMI	-.0050	1.00	
3. PTT	-.063	.071	1.00

*FDR-adjusted p-value < 0.05, ** FDR-adjusted p-value < 0.001*

Table 3: Ontology Categories of Commonly Correlating Genes in the NPH, NYBB, and both ROSMAP datasets

(a) Representative Ontology Categories (RIN Positively Correlating Genes)

Ontology Categories	FDR-adjusted p-value	Odds Ratio
Chemical Synaptic Transmission (GO:0007268)	1.55E-23	4.43
Anterograde Trans-Synaptic Signaling (GO:0098916)	1.34E-17	4.50
Vesicle-Mediated Transport (GO:0016192)	4.42E-08	2.29
Neurotransmitter Secretion (GO:0007269)	6.33E-07	7.35
Modulation Of Chemical Synaptic Transmission (GO:0050804)	7.51E-07	3.52
Neuron Projection Development (GO:0031175)	7.78E-07	2.84
Signal Release From Synapse (GO:0099643)	1.07E-06	7.79
Regulation Of Neuron Projection Development (GO:0010975)	1.07E-06	2.93
Neuron Development (GO:0048666)	3.49E-06	3.02
Axon Development (GO:0061564)	1.57E-05	3.51

(b) Representative Ontology Categories (RIN Negatively Correlating Genes)

Ontology Categories	FDR-adjusted p-value	Odds Ratio
Response To Cytokine (GO:0034097)	9.02E-06	4.20
Regulation Of I-kappaB kinase/NF-kappaB Signaling (GO:0043122)	9.56E-05	2.86
Defense Response To Symbiont (GO:0140546)	6.20E-04	3.23
Positive Regulation Of I-kappaB kinase/NF-kappaB Signaling (GO:0043123)	0.00125	2.86
Defense Response To Virus (GO:0051607)	0.00201	2.70
Positive Regulation Of Cytokine-Mediated Signaling Pathway (GO:0001961)	0.0133	4.94
Regulation Of NIK/NF-kappaB Signaling (GO:1901222)	0.0173	3.40
Cellular Response To Cytokine Stimulus (GO:0071345)	0.0186	2.04
Positive Regulation Of Defense Response To Virus By Host (GO:0002230)	0.0186	6.23
Cellular Response To Mechanical Stimulus (GO:0071260)	0.0195	4.39

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