Multiple freeze-thaw cycles lead to a loss of consistency in poly(A)-enriched RNA sequencing

Benjamin P. Kellman[1,2,*], Hratch M. Baghdassarian[1,2,*], Tiziano Pramparo[3], Isaac Shamie[1,2],

Vahid Gazestani[1,3], Arjana Begzati[4], Shengzhong Li[1,6], Srinivasa Nalabolu[3], Sarah Murray[5],

Linda Lopez[3], Karen Pierce[3], Eric Courchesne[3], Nathan E. Lewis[1,6,7]

[1] Department of Pediatrics, University of California, San Diego

[2] Bioinformatics and Systems Biology Program, University of California San Diego

[3] Autism Center of Excellence, Department of Neuroscience, University of California San Diego

[4] Department of Medicine, University of California San Diego

[5] Department of Pathology, University of California San Diego
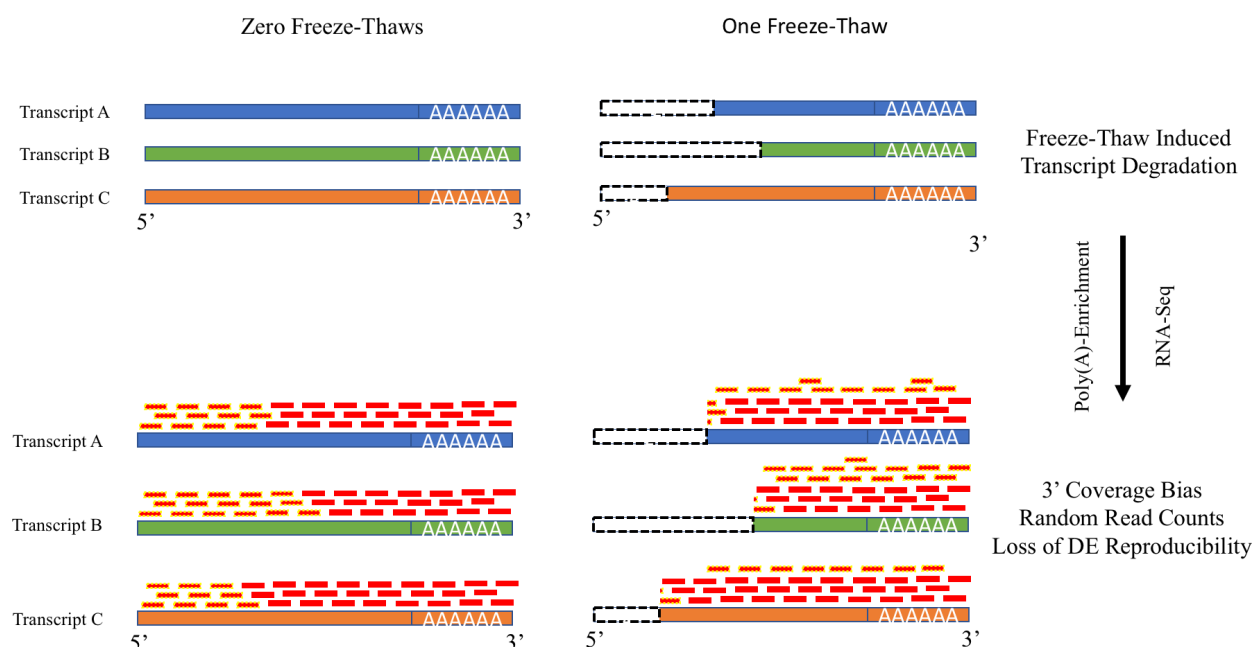
[6] Department of Bioengineering, University of California San Diego

[7] Novo Nordisk Foundation Center for Biosustainability, University of California San Diego

[*] These authors contributed equally

Correspondence to: Nathan E. Lewis, nlewisres@ucsd.edu

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Abstract

19 RNA-Seq is ubiquitous, but depending on the study, sub-optimal sample handling may be

20 required, resulting in repeated freeze-thaw cycles. However, little is known about how each cycle

21 impacts downstream analyses, due to a lack of study and known limitations in common RNA

22 quality metrics, e.g., RIN, at quantifying RNA degradation following repeated freeze-thaws.

23 Here we quantify the impact of repeated freeze-thaw on the reliability of downstream RNA-Seq

24 analysis. To do so, we developed a method to estimate the relative noise between technical

25 replicates independently of RIN. Using this approach we inferred the effect of both RIN and the

26 number of freeze-thaw cycles on sample noise. We find that RIN is unable to fully account for

27 the change in sample noise due to freeze-thaw cycles. Additionally, freeze-thaw is detrimental to

28 sample quality and differential expression (DE) reproducibility, approaching zero after three

29 cycles for poly(A)-enriched samples, wherein the inherent 3' bias in read coverage is more

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30   exacerbated by freeze-thaw cycles, while ribosome-depleted samples are less affected by freeze-

31   thaws. The use of poly(A)-enrichment for RNA sequencing is pervasive in library preparation of

32   frozen tissue, and thus, it is important during experimental design and data analysis to consider

33   the impact of repeated freeze-thaw cycles on reproducibility.

Introduction

35   RNA sequencing (RNA-Seq) is a ubiquitous technology, used to answer a wide range of

36   biological questions. Methods for aligning, quantifying, normalizing and analyzing expression

37   data are available through popular packages such as Tophat, STAR, cufflinks, SVA, RUV,

38   Combat, DESeq2, edgeR, Kallisto, Salmon, BWA-MEM, and many others[1–11]. Each method

39   aims to accommodate and mitigate the unique challenges presented by RNA-Seq data. Some

40   approaches attempt to account for characterized variability in RNA-Seq measurements due to

41   factors such as sequencing depth, gene length, and transcripts' physical characteristics (e.g., GC

42   content). Others account for "unwanted variance" due to technical, batch, or experimental

43   variation. Yet, the influence of sample processing, such as tissue lysis and processing time[12–14], is

44   not sufficiently characterized such that it can be explicitly controlled. It is important to

45   adequately characterize noise introduced to RNA-Seq measurements by sample processing steps

46   to optimize sample quality, account for transcript degradation, and improve the accuracy and

47   reproducibility of sequencing.

48   Transcript degradation continues after sample acquisition and affects data quality. Sample

49   storage conditions (e.g. temperature and the use of stabilizing reagents) affect sample quality via

50   RNA degradation[14,15]. Yet, varying sources of degradation can impact RNA-Seq in different

51   manners[16]. Degradation introduces variability in signal and can be impacted by sample handling.

52   Non-uniformity in degradation across genes and samples causes inaccurate normalization and

53   transcript quantification[17]. Poly(A)-enrichment methods are commonly used to separate mRNA

54   from other highly abundant RNA molecules (e.g., rRNA, tRNA, snoRNAs, etc.), but variable

55   degradation directly impacts read counts by causing non-uniform transcript coverage[18]. Of

56   particular interest, freeze-thaw can induce 20% degradation of spike-in standards per cycle, a

57   factor that may be generalizable to mRNA transcripts[19]. Freeze-thaw cycles increase RNA

58   degradation by disrupting lysosomes which store RNases, freeing the enzymes to promiscuously

59   catalyze nuclease activity[20]. Furthermore, partially defrosted crystals create uneven cleaving

60   pressure on mRNA strands[21,22]. Despite these observations, the extent to which freeze-thaw

61   negatively impacts count and differential expression in RNA-Seq analyses has not been

62   comprehensively characterized.

63   Standard sample quality control often relies on RNA integrity number (RIN), which quantifies

64   the 28S to 18S rRNA ratio[23]. RIN-based quality control approaches rely on a heuristic threshold

65   to assess sufficient quality[24,25]. RIN-based metrics have known confounders such as transcript

66   level, and thus have been called into question as an appropriate quality metric[26]. For example,

67   RIN failed to indicate a decrease in sample quality in lung cancer tissue samples that underwent

68   five freeze-thaw cycles[27] and, in statistical analyses, failed to correct for the effects of

69   degradation[28]. Despite this, many studies rely on RIN to correct for and assess sample quality

70   confounders[16,29,30]. This is especially problematic in the case of transcript degradation because

71   RIN scores are based on entire samples, while degradation effects can be transcript-

72   specific[17,3132]. Furthermore, existing studies on degradation are not simply generalizable to

73   freeze-thaw, which has distinct and independent effects on sample quality and must be fully
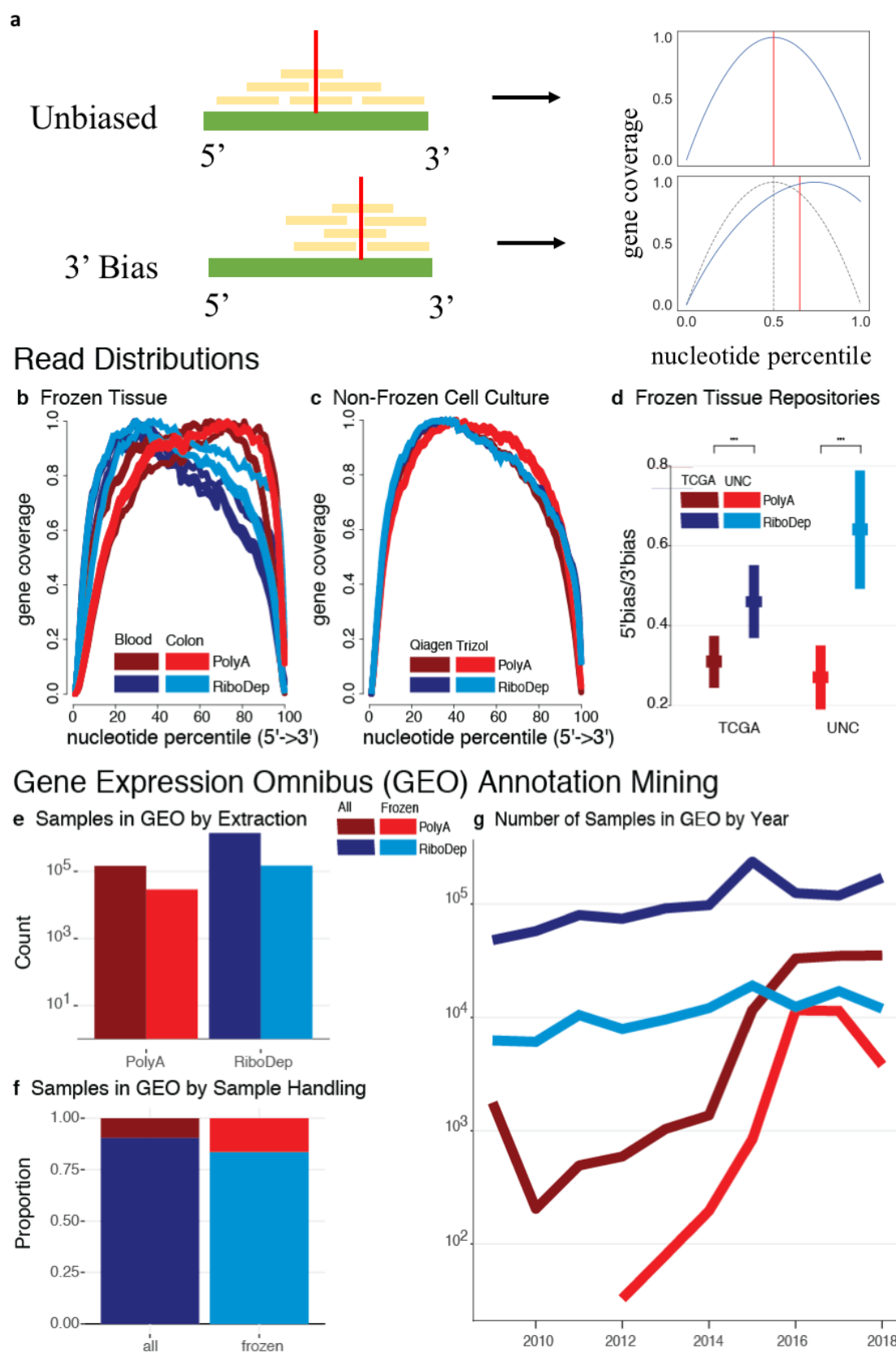
74   explored as such[16,33].

75   Here, we tested the susceptibility of poly(A)-enriched RNA-Seq results after multiple freeze-

76   thaw cycles. We assessed sample quality independently of RIN by simulating read count

77   variability to capture the noise between technical replicates. We found that each additional

78   freeze-thaw cycle increased the random counts between technical replicates by approximately

79   4%. Subsequently, differential expression reproducibility approached zero after three freeze-

80   thaw cycles. These effects are not captured by RIN. We find that these effects are reflected in

81   increasing 3' bias in read coverage when combining poly(A)-extraction with freeze-thaw, a

82   phenomena that appears to be generalizable to publically available datasets.

Results

3' bias in read coverage of public datasets is associated to poly(A)-extraction and freeze-thaw

86   To examine the prevalence of bias from repeated freeze-thaw cycles, we search for the presence

87   of RNA-seq distortion in publically available datasets. Specifically, we analyze the gene-

88   coverage distribution in samples prepared with either poly(A)-extraction or ribosomal depletion.

89   Since freeze-thaw enhances transcript degradation and poly(A)-enriched samples select mRNA

90   by hybridization to the poly(A)-tail, we expect increased read coverage on the 3' end of

91   transcripts--3' bias--when these two factors are combined. To test this expectation, we compared

92   gene body coverage from the 5' to 3' end between poly(A)-enrichment and ribosomal depletion

93   prepared samples with and without freezing. Specifically, we examined the median coverage

94   percentile, the percentile-normalized nucleotide at which median cumulative coverage for a

95   given sample is achieved (**Fig. 1a**).

5

96   We analyzed the gene-coverage distribution in three studies: (1) RNA extraction in previously

97   frozen solid and liquid tissues, (2) RNA extracted immediately after lysis of cultured cells

98   without freezing, and (3) RNA extraction in previously frozen tissue from important public tissue

99   resources.

Read Distributions

Gene Expression Omnibus (GEO) Annotation Mining

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7

101 **Figure 1**: **3' Bias is Exacerbated in Frozen, Poly(A)-extracted Samples Across Multiple Studies:** *(a)*

102 *Demonstration for determining median coverage percentile (red vertical line). When coverage is*

103 *unbiased, reads (yellow) are distributed throughout the entire body of the transcript (green). In the*

104 *absence of read bias and observing coverage as a function of the nucleotide percentile, we see that*

105 *cumulative coverage along the transcript reaches 50% half-way through the gene body, at the 50th*

106 *percentile nucleotide. In contrast, given a 3' read bias, there is a shift in the distribution of reads and*

107 *cumulative coverage reaches 50% at, for example, the 60th percentile nucleotide. This results in a*

108 *rightward shift in median coverage percentile towards the 3' end of the transcript. In the middle row,*

109 *gene coverage (y-axis) at the ith nucleotide percentile from 5' to 3' (x-axis) is displayed for samples that*

110 *were extracted using either poly(A)-enrichment or ribosomal depletion. Gene body coverage distributions*

111 *were calculated for (b) for tissue samples that underwent an unspecified number of freeze-thaw cycles*

112 *and (c) cell-culture samples samples that underwent no freeze-thaw cycles. (d) Comparison of 5' to 3'*

113 *bias ratio (y-axis) of samples from the TCGA and UNC tissue repositories (x-axis) between extraction*

114 *methods (two-sample t-test). Quantifying human RNA samples listed in GEO from 2008-2018, and*

115 *stratifying by those annotated as "frozen", we observe (d) the number of samples prepared with poly(A)-*

116 *extraction or ribosomal depletion (x-axis) (gray), (f) the proportion of samples extracted using either*

117 *method, and (g) the change in the number of samples over time.*

118 In the first study[34], a comparison of the performance of poly(A) and ribosomal depletion in liquid

119 and solid frozen tissue, we see a significant (one-sided Wilcoxon test, p = 0.01591) shift in the

120 median gene coverage percentile towards the 3' end in the poly(A)-extracted samples (**Fig. 1b**).

121 Using generalized linear regression, we found that the median coverage percentile of poly(A)-

122 extracted samples was 3.88% higher (Wald p = 0.011) than comparable ribosomal depletion

123 extracted samples. In the second study[35], cells were not frozen before extraction and there was a

124 small and insignificant difference in the 3' bias associated with library preparation (one-sided

125 Wilcoxon test, p = 0.13, **Fig. 1c**). While the first two studies extract RNA from tissue and cell-
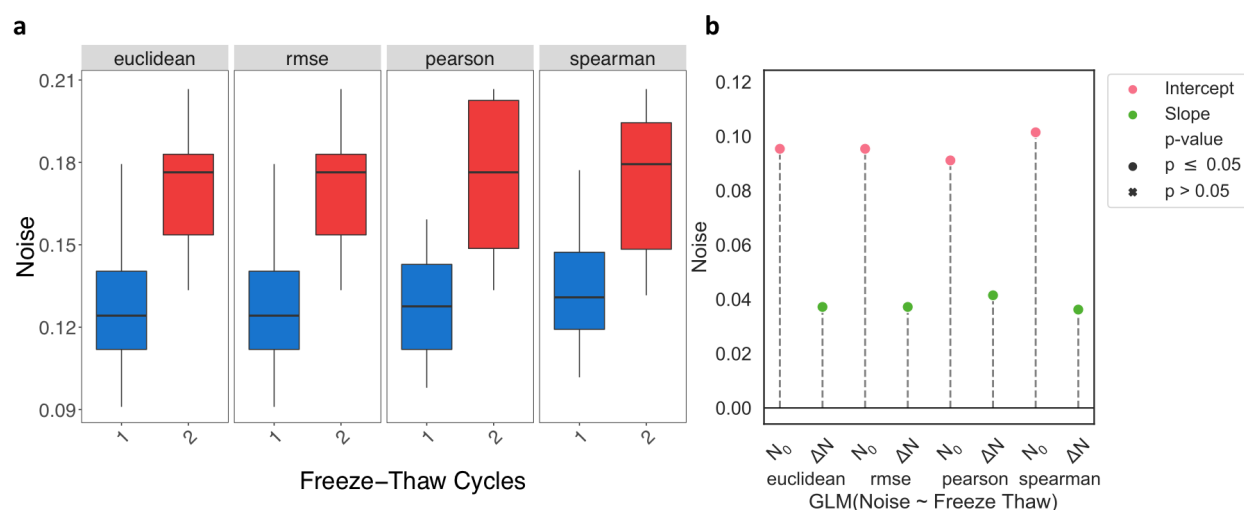
126   culture respectively--tissue extraction is typically lower quality, they are internally controlled

127   and therefore comparable. Finally, the third study[36] examines the impact of RNA extraction in

128   frozen tissue from the UNC and TCGA tumor tissue repositories. We found a significant (two-

129   sample t-test, $p<1e16$) decrease in the 5'-to-3' coverage ratio in poly(A)-extracted samples

130   compared to ribosomal depletion (**Fig. 1d**), indicating an increase in 3' bias. 3' bias was

131   consistent across RNA extracted from tissue frozen at either repository.

132   Next, we explored how widespread the impact of these observations may be by quantifying the

133   prevalence of poly(A)-extraction from frozen tissue by examining metadata in the Gene

134   Expression Omnibus (GEO). With GEOmetadb [37], we queried all human RNA samples between

135   2008 and 2018 using either poly(A)-extraction or ribosomal depletion. There are tens to hundred

136   of thousands of samples annotated as "frozen" for both total and poly(A)-extraction methods

137   (**Fig. 1e**). We note that this is an order of magnitude less than the total query, a value potentially

138   diminished by the complexity of the metadata. In samples annotated as "frozen", the frequency

139   of poly(A) extraction increases from less than 10% to over 25% (**Fig. 1f**) suggesting that the

140   problematic combination is prevalent and apparently preferred. Finally, stratifying this trend over

141   time, we see that poly(A)-extraction, as well as the relative proportion of poly(A)-extracted

142   frozen samples, is increasing in popularity relative to total RNA extraction, where usage has

143   remained fairly consistent (**Fig. 1g**).Taken together, these results indicate a potential, widespread

144   distortion in RNA-seq associated with a deleterious interaction between poly(A)-extraction and

145   freeze-thaw. To explore this potential more formally, the remainder of our analyses focus on a

146   specific experiment to address this question. Specifically we subjected whole-blood extracted

147   leukocyte samples--with technical replicates--from autistic or typically developing toddlers to a

148   varying number of freeze-thaw cycles, which we record along with other sample quality metrics

149   such as RIN.

An Additional Freeze-Thaw Cycle Increases Random Read Counts 1.4-Fold

151   To address the scarcity in quantification of loss in sample quality due to freeze-thaw, we

152   compare changes in sample quality between technical replicates. We first note that neither RIN

153   nor TIN capture significant (one-sided Wilcoxon test) decreases in sample quality due to

154   increased freeze-thaw (**Fig. S1**). Given previous indications that these metrics may not

155   sufficiently address transcript degradation, we instead measure the introduction of noise—the

156   randomness in read counts between technical replicates—to samples by freeze-thaw. We

157   simulated this randomness to reflect the dissimilarity between technical replicates. Since noise

158   does not rely on RIN, we could compare freeze-thaw and RIN-effects independently.

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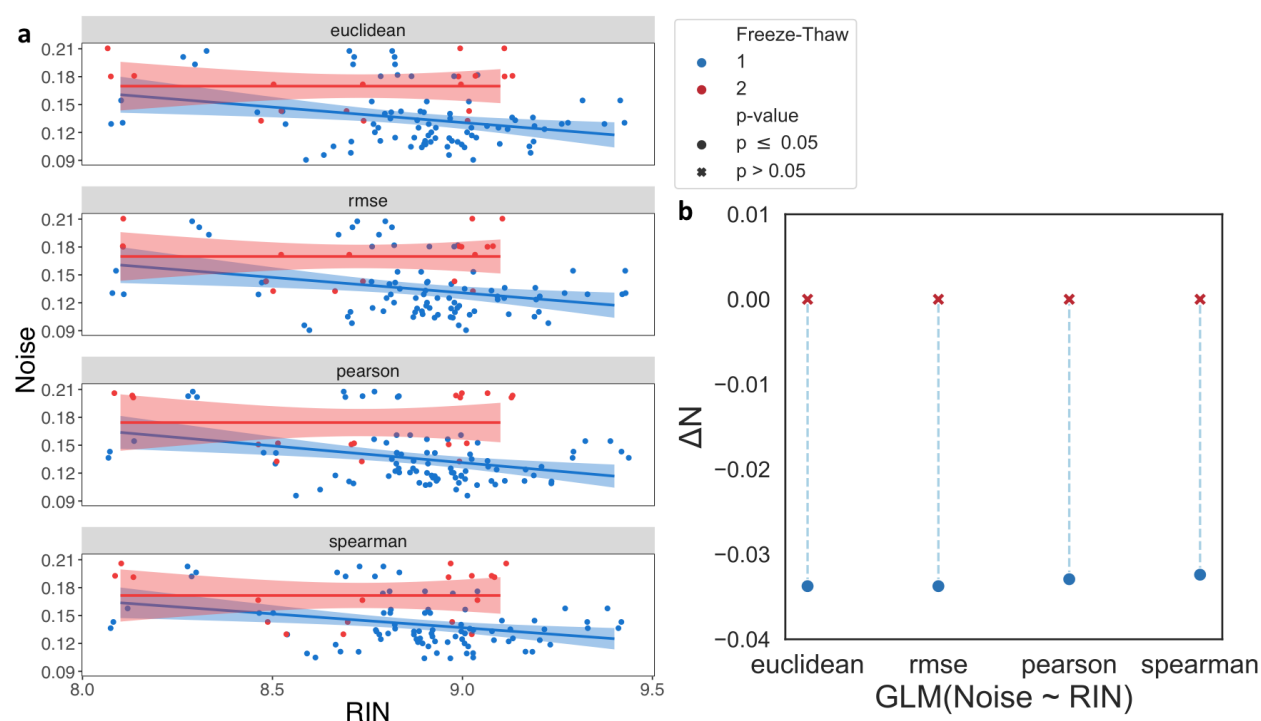160   **Figure 2**: **Higher Noise in Samples with More Freeze-Thaw Cycles.** *From left to right, noise—*

161   *the randomness in read counts between technical replicates—is estimated using Euclidean distance,*

162   *RMSE, Pearson correlation,and Spearman correlation. (a) Box plots of noise for samples that underwent*

163   *either one or two freeze-thaws. (b) A generalized linear model was used to determine the expected noise*

164   *at one freeze-thaw (N_0, pink) and the expected change in noise with each additional freeze-thaw (ΔN,*

165   *green). All estimates are significant ($p \leq 0.05$).*

166

167   Median noise increased 1.4-fold from one to two freeze-thaw cycles (one-sided Mann-Whitney

168   U test, $p \leq 0.007$) on average across all measures (**Fig. 2a**). Noise between technical replicates

169   when samples have only undergone one freeze-thaw was estimated to be 9.11-10.15% (Wald

170   test, $p \leq 5.77e-7$). The expected increase in noise per additional freeze-thaw cycle was estimated

171   to be 3.6-4.1 percentage points (Wald test, $p \leq 8.12e-3$) (**Fig. 2b**).

RIN Does Not Predict Additional Noise After One Freeze-Thaw Cycle

173   To follow up on our observations that RIN does not sufficiently capture changes in sample

174   quality due to freeze-thaw, we asked whether RIN can reflect the differences in sample quality as

175   measured by noise.

176

177 **Figure 3**: **Discrepancy in the Relationship Between Noise and RIN due to additional Freeze-**

178 **Thaw.** *Examining the relationship between noise, calculated by Euclidean distance, RMSE, pearson,*

179 *and spearman correlation, for samples that underwent either one (blue) or two (red) freeze-thaw cycles.*

180 *(a) Scatter plots comparing noise (y-axis) to RIN (x-axis). The solid lines show a linear regression fit and*

181 *the shaded regions is the 95% confidence interval for this fit. (b) The expected change in noise due to a*

182 *one point increase in RIN (ΔN, y-axis) estimated by a generalized linear model. Significant estimates ($p \leq$*

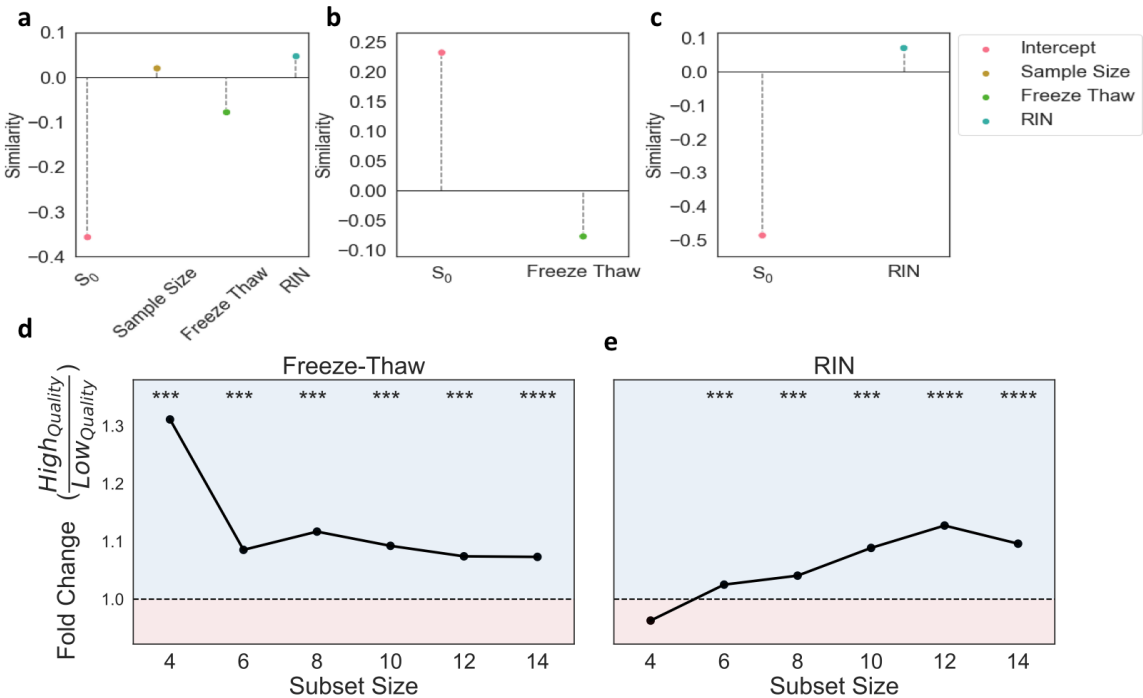183 *0.05) are marked by a circle and insignificant estimates are marked by a cross.*

184 When only considering samples that underwent one freeze-thaw, each unit increase in RIN

185 decreases noise by 3.24-3.38 percentage points for all metrics (Wald test, $p \leq 6.3e\text{-}3$) (Fig. **3a-b**).

186 Yet, when only accounting for samples that underwent two freeze-thaw cycles, noise does not

187 significantly change as RIN increases. Taken together, these results indicate that while RIN can

188 be a good measure of noise for samples that underwent one freeze-thaw, it does not capture the

189 loss in sample quality induced by two freeze-thaw cycles.

190 ## Differential Expression Similarity Increases 10.3% in High Quality Samples

191 Next, we investigated how the introduction of technical variation, noise, impacts downstream

192 RNA-Seq analysis, specifically, differential expression (DE) analysis. As such, we assessed the

193 reproducibility of DE results on combinations of samples with varying sample quality. We

194 compared DE results between subsets of various sizes (4 - 14 samples). We measure

195 reproducibility using similarity or discordance, based on correlation and dispersion, respectively.

196 Higher similarity and lower discordance each represent higher reproducibility. We use these

197 measures to assess differences that arise between subsets consisting of high quality (low freeze-

198 thaw or high RIN) and low quality (high freeze-thaw or low RIN) samples.

12

199   We held two expectations regarding the effect of sample quality on DE reproducibility in the

200   context of similarity: 1) the reproducibility between subsets with high quality samples should be

201   higher than those with low quality samples, and 2) both subset size and sample quality should

202   interact to increase the reproducibility of DE analysis; the increase in stability is reflected by a

203   higher rate of increase in reproducibility with respect to subset size.

204   As expected, similarity increases with subset size. This is reflected in the upward shift in the

205   similarity distribution with increasing subset size (**Fig. S10**) and the estimated 0.02 (Wald test, p

206   = 2.2e-5) increase in similarity per additional sample (**Fig. 4a**); thus, expected similarity would

207   increase by 0.20 in a subset with 14 samples over a subset with 4 samples. Regression results for

208   each model predicting similarity are reported in **Supplementary Table 4**.

209

211   **Figure 4**: **Freeze-Thaw and RIN Both Demonstrate Higher Similarity with Increased**

212   **Quality.** *Top panels summarize generalized linear models used to quantify the change in similarity per*

213   *unit increase in (a) sample size, number of freeze-thaws and RIN combined, (b) only the number of*

214   *freeze-thaws, and (c) only RIN. S_0 represents the intercept estimate and sample size, freeze-thaw, and RIN*

215   *represent coefficient estimates. All estimates are significant. Bottom panels demonstrate fold-change in*

216   *median similarity of high quality subsets with respect to low quality subsets at each subset size. The*

217   *region shaded in blue (fold-change > 1) indicates instances where the median similarity for high quality*

218   *is larger than that of low quality. The region shaded in red (fold-change < 1) indicates instances where*

219   *the median similarity for low quality is larger than that of high quality. Average (d) freeze-thaw or (e)*

220   *RIN are used to place subset pairs into high or low quality sample bins. Significance (one-sided Mann-*

221   *Whitney U test) of comparisons in similarity distributions between high and low quality subset pairs are*

222   *displayed above each subset size.*

223   We tested our first expectation by placing subset pairs into high and low sample quality bins,

224   defined by either RIN or freeze-thaw, for each subset size and comparing their similarity values.

225   Regardless of sample quality, DE similarity increases with subset size. Yet, for nearly all subset

226   sizes, higher quality bins have significantly (one-sided Mann-Whitney U test, $p \leq 2.8e\text{-}17$)

227   higher similarity than low quality bins (**Fig. 4e-f**). Across subset sizes, we observed an average

228   1.13-fold and 1.06-fold increase in similarity from low to high quality samples for freeze-thaw

229   and RIN, respectively.

230   Similarity significantly (Wald test, $p \leq 9.2e\text{-}3$) decreases with the number of freeze-thaws and

231   increases with RIN when accounting for the effects of sample size (**Fig. 4a-c**), validating our

232   second expectation. Similarity decreases by 0.077 per additional freeze-thaw cycle (Wald test, p

233    $= 8.77e\text{-}4$). Given the estimated similarity of 0.23 for samples that have not undergone freeze-

234   thaw, this implies that DE reproducibility will approach zero after approximately three freeze-

235   thaw cycles (**Fig. 4b**). Even when accounting for subset size and the effects of RIN, the

14

236   estimated decrease in similarity from freeze-thaw is nearly the same--0.078 (Wald test, p =

237   8.77e-4); this further corroborated that RIN alone cannot capture the changes in sample quality

238   due to freeze-thaw. Taken together, these results indicate that higher sample quality increases DE

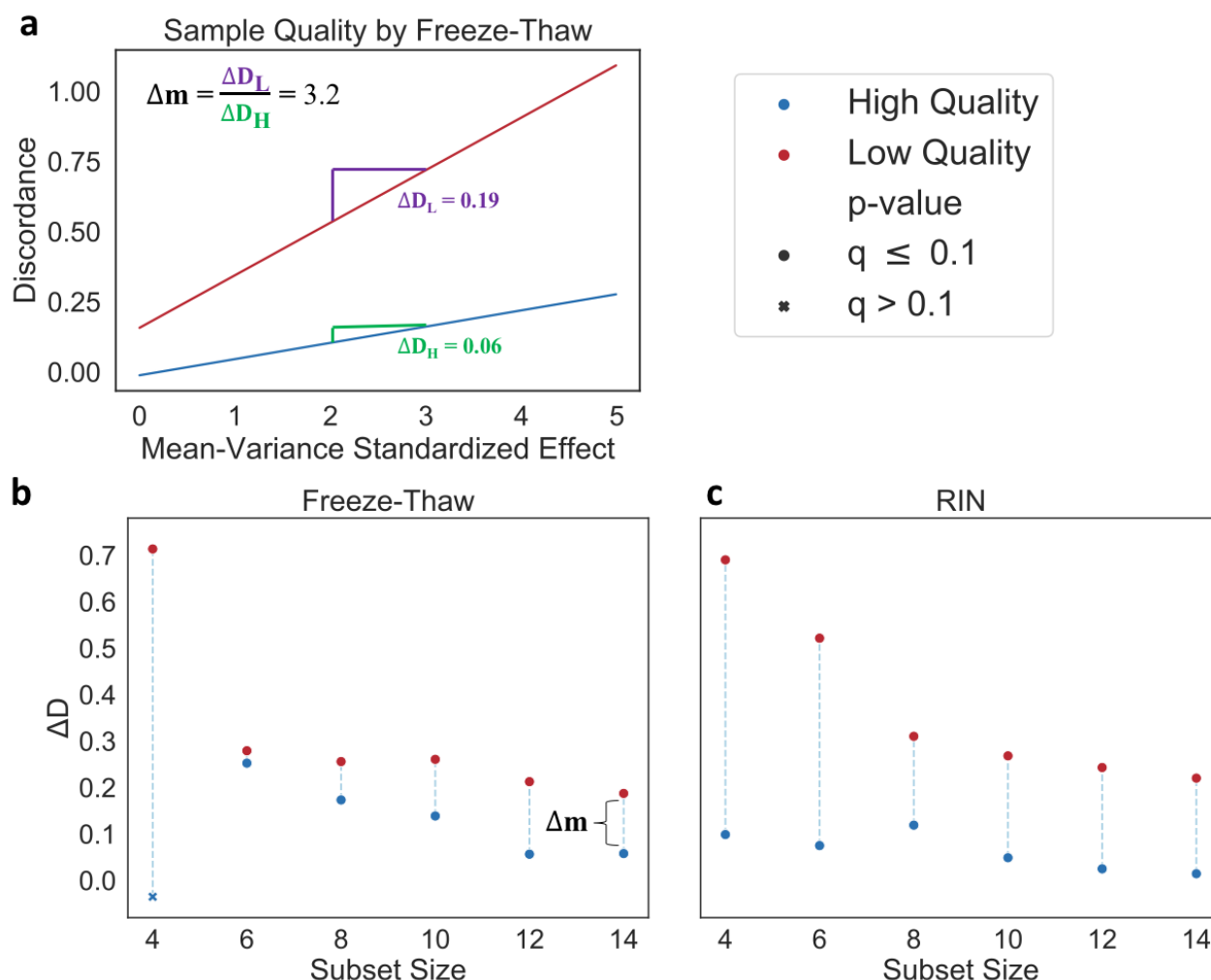239   reproducibility as measured by similarity.

Discordance Decreases ~5-fold In High Quality Samples

241   We further investigated the relationship between DE reproducibility and sample quality using an

242   effect size sensitive measure of discordance. Specifically, we explored how sample quality

243   affects the relationship between discordance and the mean-variance standardized effect at each

244   subset size. In this context, we expected 1) discordance at any given effect size to be lower in

245   high-quality subsets and 2) the rate of increase in discordance to be lower in high quality subsets

246   relative to low quality subsets.

247   Corresponding to the regression models used for this analysis, we label our expected discordance

248   when effect size is zero as D_0 and the change in discordance per unit increase in effect size as

249   ΔD. We observed a significant (Wald test, $p \leq 9.45e-141$) decreasing trend in both D_0 and ΔD

250   with increasing subset size (**Fig. S11, Supplementary Table 5**).

251   As expected, independent of sample quality, ΔD demonstrates an overall decreasing trend with

252   respect to subset size for both RIN and freeze-thaw. Given freeze-thaw, at a subset size of 6,

253   there is a 1.1-fold decrease in the value of ΔD from low quality subsets to high quality subsets.

254   The disparity in ΔD between high and low sample quality ($\Delta m = \Delta D_{Low\ Quality} / \Delta D_{High\ Quality}$)

255   increases nearly monotonically through to the subset size of 14, at which point there is a 3.2-fold

256   decrease; the monotonicity is an indication of the stability of this relation between discordance

257   and sample quality. This causes notable differences in discordance values, even at low effect

15

258   sizes (**Fig. 5a**).

259

Figure 5: Higher Sample Quality has Lower Discordance at Each Subset Size. *GLM estimates*

of discordance predicted from effect size and subset size at high and low quality subsets at each subset

size. High sample quality (blue) is compared to low sample quality (red). ΔD values represent the change

in discordance per unit increase in effect size. (a) The predicted discordance with respect to the mean-

variance standardized effect at a subset size of 14; sample quality is assessed by freeze-thaw. The

disparity (Δm) between the change in discordance per unit increase in effect size for high (ΔD$_H$) and low

(ΔD$_H$) quality subsets is also displayed. Summary of results for each subset size (x-axis) for sample

quality represented by either (b) freeze-thaw or (c) RIN. Significant estimates (Wald test, Benjamini-

16

268 *Hochberg FDR correction, q ≤ 0.1) are marked by a circle and insignificant estimates are marked by a*

269 *cross. For freeze-thaw, Δm corresponding to panel A is also displayed.*

270 We estimate discordance with respect to effect size at each subset size and for subsets of either

271 high and low quality (**Fig. 5a**). Consistent with our expectations, D_0 and ΔD are consistently

272 lower for high quality subsets as compared to low quality subsets for both freeze-thaw and RIN

273 across all subset sizes (**Fig. 5b-c**). Nearly all estimates are significant after multiple test

274 correction (Wald test, Benjamini-Hochberg FDR correction, $q \leq 0.07$), with the exception of

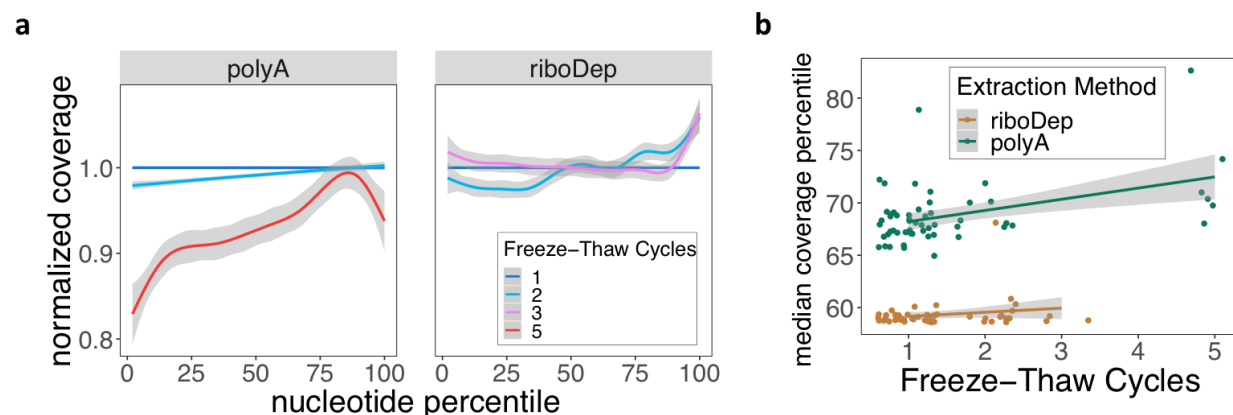275 those for the smallest subset size for freeze-thaw.

276 Taken together, these results indicate that higher sample quality increases DE reproducibility as

277 measured by discordance.

Additional freeze-thaw cycles show increased 3' bias in poly(A)-enriched but not ribosomal depletion samples

280 Finally, we asked whether repeated freeze-thaw cycles can induce a 3' bias, consistent with the

281 induction of random reads and the loss of DE reproducibility as well as our initial observation in

282 the public datasets.

283 Using the median coverage percentile, we found a shift in mRNA coverage towards the 3' end in

284 the poly(A)-enriched samples relative to ribosomal depletion (**Fig. S13a**). Specifically, the

285 median coverage percentile for poly(A)-enriched samples is significantly (one-sided Wilcoxon

286 test, $p < 2.2e-16$) larger than that of ribosome depletion (**Fig. S13b**). Samples prepared with

287 poly(A)-extraction have more 3' coverage bias compared to ribosomal depletion in both one

288 (one-sided Wilcoxon test, $p = 3.5e-15$) and two (one-sided Wilcoxon test, $p = 1.4e-4$) freeze-

289 thaw cycles **(Fig. S13d)**. Altogether, this indicates an overall 3' bias of poly(A)-enriched

290   samples, even independently of freeze-thaw **(Fig. S13b)**.

291

292   **Figure 6**: **Freeze-Thaw Cycles Exacerbate 3' Bias in poly(A)-enriched samples.** *(a) Gene coverage (y-*

293   *axis) at the ith nucleotide percentile (x-axis) for samples that underwent 1-5 freeze-thaws and were*

294   *extracted using either poly(A)-enrichment or ribosome depletion. Coverage is normalized to samples that*

295   *underwent one freeze-thaw. For each sample, coverage is averaged across all genes; samples are*

296   *aggregated using generalized additive model smoothing, with shaded regions representing 95%*

297   *confidence intervals. (b) Linear model fits comparing the change in median coverage percentile to the*

298   *number of freeze-thaw cycles for ribosome depletion (orange) or poly(A)-extraction (green).*

299   Crucially, this 3' bias is accentuated when samples are stratified by the number of freeze-thaw

300   cycles **(Fig. 6a).** We observe a significant increase (Wald test, p = 8.3e-4) in median coverage

301   percentile due to the number of freeze-thaw cycles in poly(A)-enrichment. The increase was not

302   maintained when was isolated using ribosome depletion (Wald test, p = 0.155) **(Fig. 6b,**

303   **Supplementary Table 7)**. For poly(A)-enriched samples, median coverage percentile increases

304   3.3 percentage points per root freeze-thaw cycle; the square-root of freeze-thaw was used to

305   stabilize variance. We further demonstrate a dependency of 3' bias on freeze-thaw cycles by

306   showing that median coverage percentile significantly increases with freeze-thaw in poly(A)-

307   enriched samples (Kruskal-Wallis test, p = 0.01). This 3' bias is particularly apparent after five

308   freeze-thaw cycles (one-sided Wilcoxon test, p = 2.4e-3). In contrast, we observe that median

309   coverage percentile does not significantly change across freeze-thaw cycle counts in ribosomal

310   depletion (Kruskal-Wallis test, p = 0.084) **(Fig. S13c)**.

311   Taken together, these analyses indicate that poly(A)-enrichment inherently introduces a 3' bias

312   in coverage as compared to ribosome depletion, and that this bias is exclusively exacerbated in

313   poly(A)-enriched samples due to freeze-thaw cycles. Thus, 3' bias may indicate the severity of

314   freeze-thaw induced signal degradation in poly(A)-extracted samples. If this 3' bias is the root

315   cause of freeze-thaw induced instability in absolute and differential RNA-seq quantification,

316   such instabilities may be subverted by substituting poly(A)-selection for ribosomal depletion

317   during library preparation; such instabilities may be an unnecessary and avoidable

318   inconvenience.

Discussion

320   Despite the utility and ubiquity of RNA-Seq, many of the confounding elements associated with

321   the technology are still being characterized. In this work, we demonstrated how one such

322   confounder--sample freeze-thaw--impacts sample quality and downstream analyses. We

323   highlighted biases in publically available datasets, and observed an increased 3' bias when both

324   freeze-thaw and poly(A)-extraction are features of a sample.

325   To gain a comprehensive understanding of this effect, we first simulated technical replication to

326   measure the noise between technical replicates with different treatments. This allowed us to

327   examine the impact of freeze-thaw cycles and the ability of RIN to capture those impacts. Next,

328   we examined the impact of freeze-thaw cycles on the robustness and reproducibility of

329   differential expression analysis. We found freeze-thaw cycles were substantially detrimental to

330   the stability of gene expression analysis. By our estimates and at these subset sizes,

331   reproducibility of a differential expression signature approaches zero after three freeze-thaw

332   cycles (**Supplementary Table 4**). Finally, we demonstrated that poly(A)-enriched samples

333   demonstrate substantial 3' bias in read coverage with increased freeze-thaw cycles. Our results

334   have implications with regards to technical variation due to sample handling, the sensitivity of

335   differential gene expression analysis for frozen tissues and samples, and the utility of RIN.

336   Technical variation in RNA-Seq is substantial and can be attributed to a variety of factors,

337   including read coverage, mRNA sampling fraction, library preparation batch, GC content, and

338   sample handling[38,39]. As such, accounting for technical variation has been a major research area

339   of focus for the past decade[1,2,5,39,40]. Degradation in combination with poly(A)-enrichment is a

340   known source of variation in RNA-Seq. Yet, before technical variation can be accounted for, it

341   must be characterized. While studies have looked into the effect of degradation on RNA-Seq,

342   each mode of degradation impacts sample quality differently, and direct connections between

343   freeze-thaw and sample quality has mainly been assessed via RIN[16,41,42].

344   Our simulation of technical replicates helps delineate technical variation due to sample handling-

345   -specifically, freeze-thaw. Furthermore, the resulting noise provides an estimate for the number

346   of random read counts associated with a gene. For example, given an average 25 million reads

347   sequenced per sample, our approximate 4 percentage points difference in noise between one and

348   two freeze-thaw cycles gives an expected stochasticity in 1 million of those reads; approximating

349   the number of protein-coding genes in the human genome to be 20-25 thousand[43], we can expect

350   a difference of ~40-50 random counts per gene to exist between technical replicates due to a

351   freeze-thaw cycle (**Supplementary Methods, Fig. S15**). Thus, each freeze-thaw introduces a

352   non-negligible level of noise to the quantification of gene expression and differential expression

20

353   of such genes.

354   To check for the possibility that there is a signature which can help correct for freeze-thaw

355   distortion of RNA-Seq, we attempt to find a group of common DE genes across various DE

356   methods. We find no such signature (**Supplementary Results**). This is expected, given that a

357   major source of reduced sample quality due to freeze-thaw is mRNA degradation, which occurs

358   randomly for each transcript and sample. A possible path forward is to correct for sample

359   degradation. Several methods have been proposed for this. While some of these methods rely on

360   RIN or similar metrics (e.g. mRIN, TIN, etc.)[16,44], others have implemented statistical

361   frameworks which account for gene-specific biases. DegNorm, for example, accounts for the

362   gene-specific relative randomness in degradation in its correction approach[17]. Quality surrogate

363   variable analysis (qSVA) specifically improves differential expression by identifying transcript

364   features associated with RNA degradation for its correction[28]. Furthermore, there are recent

365   methods which only assay the 3' end of a transcript and therefore claim robustness in degraded

366   samples[45]. While these approaches could help account for noise introduced by RNA degradation

367   during repeated freeze-thaw cycles, they cannot necessarily remedy the associated loss of signal.

368   The effect of freeze-thaw and resultant degradation on RNA-Seq is particularly concerning when

369   considering differential gene expression analysis. It has been observed that RNA degradation can

370   induce the apparent differential expression in as many as 56% of genes[44]. To this end, we

371   quantified this loss of DE reproducibility by measuring similarity and discordance in the context

372   of sample quality. We found a decrease in reproducibility with both decreasing RIN and

373   increasing freeze-thaw. Interestingly, for most reproducibility assessments, we observed a

374   monotonic or near monotonic increase in disparity between low and high quality subsets with

375   respect to subset size. Similarity demonstrated a larger average magnitude of disparity for freeze-

376 thaw, whereas discordance demonstrated a larger average magnitude of disparity for RIN.

377 Based on our analysis, the utility of RIN in assessing quality when samples undergo freeze-thaw

378 is questionable. The non-uniformity in mRNA degradation[46–49] due to freeze-thaw sheds light on

379 these challenges, since RIN cannot quantify quality at the individual gene level[23]. This is

380 reflected in the fact that samples with RIN > 8 demonstrate degradation[32]. Furthermore, results

381 assessing the effect of freeze-thaw cycles on RIN are inconclusive. While some studies claim

382 RIN can be used to account for degradation effects in RNA-Seq[16], others suggest it does not

383 sufficiently capture the effects of degradation on sample quality[26,28]. When directly observing the

384 effect of freeze-thaw on RIN, studies have found a negligible effect[12] or can only detect an

385 effect after numerous cycles[27,50].

386 As such, we re-examined the utility of RIN as a measure of sample quality in relation to our

387 noise estimation of random reads per sample[23]. We found that while noise increases with both

388 decreasing RIN and increasing freeze-thaw, RIN may be an insufficient indicator of quality for

389 samples that have undergone two or more freeze-thaws. Given these results, RIN may not

390 always be a good metric to quantify the difference between technical replicates that have

391 undergone variable sample handling[17,26–28]. We validate noise by confirming that it does not

392 change with input RNA concentration, excepting outliers **(Fig. S12)**. Therefore, in the future,

393 noise could be a useful supplement to RIN when technical replicates are present.

394 The fact that our predicted decrease in similarity due to freeze-thaw does not change when

395 incorporating RIN into our model further indicates that RIN alone cannot capture the changes in

396 sample quality due to freeze-thaw. Despite this, RIN is a good indicator of sample quality, if not

397 specifically for freeze-thaw. This is reflected in the fact that RIN validates our expectations for

398 DE reproducibility analysis and the comparable range of noise, similarity, and discordance

22

399   values between freeze-thaw and RIN assessments.

400   Finally, to confirm our expectation that freeze-thaw decreases sample quality[18–22] and to further

401   characterize the underlying mechanism, we validated the presence of a 3' bias in coverage. This

402   builds on our and others' observations that a lower percentage of poly(A)-enriched transcripts

403   are covered[42]. We compared coverage to ribosome depleted RNA-Seq data, which does not use

404   3' hybridization to retain transcripts. We find that poly(A)-enrichment does in fact introduce a

405   strong 3' bias in coverage as compared to ribosome depletion. This bias is further exacerbated

406   with additional freeze-thaw cycles in poly(A)-enriched but not ribosome depleted samples. This

407   implies that degradation due to freeze-thaw does not impact RNA-sequencing of ribosome

408   depleted samples to the extent that it does in poly(A)-enriched samples. In light of our

409   demonstrations that 3' bias is associated with a substantial increase in noise and a decrease in DE

410   reproducibility, these findings suggest that RNA-seq from samples that have both been poly(A)-

411   extracted and undergone freeze-thaw cycles likely has unknown, diminished stability. While not

412   all studies have technical replicates to estimate noise, the presence of exaggerated 3' bias when

413   poly(A)-extraction is combined with freeze-thaw can be a simple indicator of RNA-seq

414   distortion.

415   # Conclusion

416   Altogether, these results indicate that transcriptomics quality control steps cannot rely on RIN

417   alone for samples that have undergone poly(A)-enrichment and multiple freeze-thaws.

418   Furthermore, accounting for the effect of freeze-thaw on poly(A)-enriched RNA sequencing is

419   crucial. poly(A)-enrichment is prevalent for RNA-sequencing, and, in parallel, samples that

420   undergo multiple freeze-thaws are common in many protocols, especially rare tissues, e.g.,

421   postmortem neural tissue. Yet, there is no clear recommendation to avoid poly(A)-enrichment

422   following multiple freeze-thaws. Together, these results indicate that ribosomal depletion could

423   be a better alternative when freeze-thaw is necessary.

Declarations

Ethics Approval and Consent to Participate

426   In this study, we performed transcriptomics analyses of blood samples drawn from male toddlers

427   with the age range of 1–4 years. Research procedures were approved by the Institutional Review

428   Board of the University of California, San Diego. Parents of toddlers underwent informed

429   consent procedures with a psychologist or study coordinator at the time of their child's

430   enrollment. Additional details for the recruitment protocol are executed as described in Gazestani

431   et al.[51]

Author contributions

433   B.P.K., N.E.L., T.P., S.M., and E.C. designed and planned the experiments. T.P., S.N., K.P.,

434   S.M. and L.L. collected the samples, managed diagnostics, conducted transcriptome assays and

435   managed the data. B.P.K, H.M.B. and V.G. planned and conducted analyses. I.S. performed

436   RNA-seq QC. A.B. performed functional enrichment analyses. S.L. wrote the RNA-Seq

437   processing pipeline. B.P.K., H.M.B, and N.E.L wrote the manuscript. N.E.L. supervised the

438   project.

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Availability of Data and Materials

445   The datasets supporting the conclusions of this article are available in the NCBI Sequence Read

446   Archive repository (PRJNA627540,

447   https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA627540&o=acc_s%3Aa). Associated

448   metadata is available in Supplementary Tables 1-2.

Competing Interests

450   There are no competing interests in this study.

Methods

Sample Collection and Storage

453   Blood samples drawn from male toddlers with the age range of 1-4 years were usually taken at

454   the end of the clinical evaluation sessions. To monitor health status, the temperature of each

455   toddler was monitored using an ear digital thermometer immediately preceding the blood draw.

456   The blood draw was scheduled for a different day when the temperature was higher than 37 °C.

457   Moreover, blood draw was not taken if a toddler had some illness (for example, cold or flu), as

458   observed by us or stated by parents. We collected 4–6 ml blood into EDTA-coated tubes from

459   each toddler. Blood leukocytes were captured using LeukoLOCK filters (Ambion). After rinsing

460   the LeukoLOCK filters with PBS, the filters were flushed with RNAlater (Invitrogen) to stabilize

25

461   RNA within the intact leukocytes. After RNA stabilization, the LeukoLOCK filters were

462   immediately placed in a −20 °C freezer. Additional RNA standards were sourced from normal

463   human peripheral leukocytes pooled from 39 Asian individuals, ages 18 to 47 (Takara/ClonTech:

464   636592). The RNA standards underwent 1-5 simulated freeze-thaw cycles; 24hrs frozen at

465   −80 °C and 1hr defrosting on ice.

RNA Extraction, Sequencing and Quantification

467   For 47 samples (from 16 individuals), mRNA was extracted using polyA selection with the

468   TruSeq Stranded mRNA library preparation kit (Illumina). Ribosomal depletion was used to

469   prepare an additional 52 samples. Relevant metadata regarding polyA-enriched and ribosomal

470   depleted samples can be found in **Supplementary Table 1-2**. Ribosome depletion prepared

471   samples used the TruSeq Stranded Total RNA with RiboZero Gold library preparation kit

472   (Illumina). RNA Integrity Numbers (RIN) were measured using a NanoDrop ND-1000

473   (ThermoFisher). Poly-A selected samples were sequenced using 50-base pair single end

474   sequencing on a HiSeq4000 (Illumina) to a depth of 25M reads. The ribo-depletion prepared

475   libraries were sequenced using 100-base pair paired end sequencing on a HiSeq4000 (Illumina)

476   to a depth of 50M reads.

477   Fastq files for each sample underwent quality control using FastQC (v0.33). PolyA and adaptor-

478   trimming were conducted using Trimmomatic [52]. Reads were aligned to the gencode annotated

479   (v25) human reference genome (GRCh38) using STAR (v2.4.0) [7]. Alignments were processed to

480   sorted SAM files using SAMtools (v1.7) [53]. Finally, HTSeq using default settings (v0.6.1) was

481   used to quantify reads [53,54].

Estimation of noise between technical replicates

To estimate the noise between technical replicates of the same individual blood samples, we simulate random loss and gain of reads **(Fig. S2)**. One technical replicate was chosen as the "reference" replicate, making the other technical replicate the "target" replicate. Specifically, we designated replicates that have undergone one freeze-thaw as the reference, and those that underwent two freeze-thaw cycles as the target. The dissimilarity between replicates is measured by one of four metrics (Euclidean distance, RMSE, Pearson correlation, and Spearman correlation). We iteratively add and remove random reads to the reference replicate until the dissimilarity between the simulated replicate and the reference replicate was equal to the dissimilarity between a target replicate and the reference replicate **(Fig. S3, Figure S2)**. We define the noise between the reference and target replicate as the fraction of reads added or removed per total reads in the reference replicate to achieve the aforementioned level of dissimilarity. We represent this as a percentage, e.g. 5% noise between a reference and target replicate can be interpreted as 5% randomness between their reads. For additional details on noise simulation, see Supplementary Methods.

Measuring the Effect of Sample Quality on Noise

To measure the association between noise and sample quality metrics (number of freeze-thaw cycles, input RNA concentrations, and RNA integrity number), we used a generalized linear model (GLM). The significance of the model parameters is determined by the Wald test. All results are reported in **Supplementary Table 3**.

For each model, to mitigate the contribution of potential confounding variables, samples with input RNA concentrations in the top and bottom 5% ($|z| \geq 1.645$) were removed, decreasing the

27

504   total number of samples from 47 to 41. For noise prediction from concentration, samples with

505   more than one freeze-thaw were also excluded, decreasing the total number of samples to 35.

506   Noise prediction from RIN was run separately for samples that had undergone one freeze-thaw

507   and samples that had undergone two freeze-thaws.

508   **Differential Expression Analysis**

509   We assess whether the observed sample qualities (freeze-thaw and RIN) have an impact on

510   differential expression (DE) reproducibility using a bootstrapping approach. DE was run on

511   random sample subsets of varying sizes (**Fig. S4**). Before subsetting, we filtered our expression

512   matrix for genes with an average count $\leq$ 20 across all samples. This reduced the number of

513   genes from 10,028 to 4,520. The total number of samples considered was 46 when disregarding

514   samples that were industry standards, were not assigned to either an ASD or TD indication, or

515   did not have a recorded sample quality (RIN or freeze-thaw) value.

516   We generated subsets containing N = 4-14 samples. For each subset size N, we generated 2,000

517   unique subsets. Each subset had an equal number of TD or ASD samples. Additionally, only one

518   replicate from each blood sample could be included. These requirements limited our subset size

519   to a maximum of 14 samples.

520   DE between ASD and TD subjects was conducted using DESeq2[1]. **Fig. S10** summarizes DE

521   results for all subsets. To account for potential confounders, we used RUV[5]. Specifically, we use

522   a set of "in-silico empirical" negative control genes, including all but the top 5,000 differentially

523   expressed genes as described in section 2.4 of the documentation for RUVseq

524   (http://bioconductor.org/packages/release/bioc/vignettes/RUVSeq/inst/doc/RUVSeq.pdf). We

525   check that RUV produces consistent results with previous Autism leukocyte gene expression

526 signatures[51,55], see **Supplementary Results.**

Similarity to Assess Differential Expression Reproducibility

528 To assess DE reproducibility, we measure the similarity in log-fold-change (LFC) values

529 between DE runs. Similarity is calculated as the pairwise spearman correlation of LFC between

530 all subsets of the same size **(Fig. S6)**. Genes with a median base mean (the mean of counts of all

531 samples, normalizing for sequencing depth) or median LFC in the bottom 10th percentile across

532 all subsets were excluded, filtering for low magnitude effects **(Fig. S5)**.

533 Average RIN and freeze-thaw were measured for all subset pairs. Resulting distributions for all

534 collected values from similarity analyses are displayed in **Fig. S7**.

535 Next, subsets of each size were split into two quantile bins for each quality metric separately.

536 High sample quality bins (low freeze-thaw or high RIN) were compared to low sample quality

537 bins. High sample quality subsets were tested for higher similarity than low sample quality bins

538 using a one-sided Mann-Whitney U test.

539 Additionally, three generalized linear models (GLMs) were fit to quantify the contribution of

540 sample quality metrics to the change in similarity for DE results across subsets. We fit one model

541 to predict similarity from freeze-thaw and RIN, while also accounting for the improvement in

542 reproducibility due to increase in subset size (Similarity ~ Freeze-Thaw + RIN + Subset Size).

543 We also fit two models predicting similarity from freeze-thaw or RIN alone.

Discordance to Assess Differential Expression Reproducibility

545 We adapted a measure of concordance to measure discordance, or the lack of reproducibility,

546 between differential expression results[56]. Average RIN and freeze-thaw were calculated for each

547 subset **(Fig. S8-9)**. Subsets for each subset size were split into two quantile bins for either quality

548 metric (RIN and freeze-thaw). Genes with a median base mean across all subsets in the bottom

549 tenth percentile were excluded from the analysis **(Fig. S5)**.

550 We do not use the original concordance at the top (CAT) metric because we are not comparing

551 our results to a gold standard dataset. Instead, we use gene-wise LFC standard deviation across

552 subsets as a measure of discordance. Thus, the average LFC for each gene across DE runs is

553 analogous to the gold standard, and the dispersion from this average indicates a lack of

554 reproducibility. At each combination of subset size and quality bins, we calculate discordance

555 and compare it to the gene-wise median effect size **(Fig. S8)**. We measure effect size as the

556 mean-variance standardized effect[1]. This and two additional effect size metrics (Cohen's d and

557 absolute median LFC) we use are further described in **Fig. S9**. Results for all three effect size

558 metrics reflect similar trends and can be found in **Supplementary Tables 5-6.**

559 We used a GLM to predict discordance from effect size at each subset size. Additionally, in a

560 separate GLM, we account for the interaction between effect size and sample quality

561 (Discordance ~ Effect Size x Sample Quality) at each subset size. Here, sample quality is a

562 dummy variable, assuming a value of 0 for low quality and 1 for high quality. We did not

563 include a term for subset size because regressions were fit within each subset size.

Read Coverage Bias

565 The distribution of read coverage over each gene body was measured using

566 *geneBody_coverage.py* from the *RSeQC* (v3.0.0) package [57]. We measure this coverage ranging

567 from the 0th percentile (5' end) to the 100th percentile (3' end) nucleotide. The ith percentile

568 nucleotide is calculated as $nucleotide_i / length_{gene}$. Coverage at the ith percentile nucleotide is

569   normalized across all genes within a sample.

570   For a given sample, the median coverage percentile is defined as the nucleotide percentile at

571   which median cumulative coverage is achieved; cumulative coverage is aggregated from the 5'

572   end to the 3' end. The larger the median coverage percentile value, the larger the 3' bias in

573   coverage. We include 9 industry standards to our analysis--six of which had undergone five

574   freeze-thaw cycles and three of which had undergone one freeze-thaw cycle--to explore the

575   impact at higher freeze-thaw counts. We also include ribosomal depletion extracted samples as a

576   negative control.

577   We extended this read coverage bias to three additional datasets, all of which contained both

578   poly(A)-extracted and ribosomal depletion extracted samples. The first dataset (PRJNA427184)

579   contains samples for liquid and solid frozen tissue [34]. The second (PRJEB4197) contains

580   HEK293 samples that explicitly never underwent freeze-thaw cycles [35]. The third

581   (phs000676.v1.p1) contains frozen tissue samples from the UNC and TCG tumor tissue

582   repositories [36]. For the first two datasets, fastq files were aligned using STAR and gene body

583   coverage was calculated from alignments using RSeQC as previously described. We did not

584   directly analyze the raw files from TCGA or UNC, but instead reanalyzed the reported 5' to 3'

585   bias ratios. This ratio is similar to the inverse of the median coverage percentile: the smaller it is,

586   the larger the 3' bias in read coverage.

587

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