

Validation of methods for Low-volume RNA-seq

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Abstract

1
2 Recently, a number of protocols extending RNA-sequencing to the
3 single-cell regime have been published. However, we were concerned that
4 the additional steps to deal with such minute quantities of input sam-
5 ple would introduce serious biases that would make analysis of the data
6 using existing approaches invalid. In this study, we performed a critical
7 evaluation of several of these low-volume RNA-seq protocols, and found
8 that they performed slightly less well in metrics of interest to us than a
9 more standard protocol, but with at least two orders of magnitude less
10 sample required. We also explored a simple modification to one of these
11 protocols that, for many samples, reduced the cost of library preparation
12 to approximately \$20/sample.

13 1 Introduction

14 Second-generation sequencing of RNA (RNA-seq) has proven to be a sensitive
15 and increasingly inexpensive approach for a number of different experiments,
16 including annotating genes in genomes, quantifying gene expression levels in a
17 broad range of sample types, and determining differential expression between
18 samples. As technology improves, transcriptome profiling has been able to be
19 applied to smaller and smaller samples, allowing for more powerful assays to
20 determine transcriptional output. For instance, our lab has used RNA-seq on
21 single *Drosophila* embryos to measure zygotic gene activation [18] and medium-
22 resolution spatial patterning [4]. Further improvements will allow an even
23 broader array of potential experiments on samples that were previously too
24 small.

25 For instance, over the past few years, a number of groups have published de-
26 scriptions of protocols to perform RNA-seq on single cells (typically mammalian

27 cells) [25, 23, 24, 11, 14]. A number of studies, both from the original authors
28 of the single-cell RNA-seq protocols and from others, have assessed various as-
29 pects of these protocols, both individually and competitively [2, 27, 19]. One
30 particularly powerful use of these approaches is to sequence individual cells in
31 bulk tissues, revealing different states and cellular identities [3, 26].

32 However, we felt that published descriptions of single-cell and other low-
33 volume protocols did not adequately address whether a change in concentration
34 of a given RNA between two samples would result in a proportional change
35 in the FPKM (or any other measure of transcriptional activity) between those
36 samples. While there are biases inherent to any protocol, we were concerned
37 that direct amplification of the mRNA would select for PCR compatible genes
38 in difficult to predict, and potentially non-linear ways. For many of the pub-
39 lished applications of single cell RNA-seq, this is not likely a critical flaw, since
40 the clustering approaches used are moderately robust to quantitative changes.
41 However, to measure spatial and temporal activation of genes across an embryo,
42 it is important that the output is monotonic with respect to concentration, and
43 ideally linear.

44 While it is possible to estimate absolute numbers of cellular RNAs from an
45 RNAseq experiment, doing so requires spike-ins of known concentration and
46 estimates of total cellular RNA content [21, 17]. However, many RNA-seq ex-
47 periments do not do these controls, nor are such controls strictly necessary un-
48 der reasonable, though often untested, assumptions of approximately constant
49 RNA content. While ultimately absolute concentrations will be necessary to
50 fully predict properties such as noise tolerance of the regulatory circuits [9, 8],
51 many current modeling efforts rely only on scaled concentration measurements,
52 often derived from *in situ*-hybridization experiments [7, 13, 12]. Given that, we
53 felt it was not important that different protocols should necessarily agree on
54 any particular expression value for a given gene, nor are we fully convinced that
55 absolute expression of any particular gene can truly reliably be predicted in a
56 particular experiment.

57 In order to convince ourselves that data generated from limiting samples
58 would be suitable for our purposes, we evaluated several protocols for perform-
59 ing RNA-seq on extremely small samples. We also investigated a simple modifi-
60 cation to one of the protocols that reduced sample preparation cost per library
61 by more than 2-fold. Finally, we evaluated the effect of read depth on quality of
62 the data. This study provides a single, consistent comparison of these diverse
63 approaches, and shows that in fact all data from the low-volume protocols we
64 examined are usable in similar contexts to the earlier bulk approach.

65 2 Results

66 2.1 Experiment 1: Evaluation of Illumina TruSeq

67 In our hands, the Illumina TruSeq protocol has performed extremely reliably
68 with samples on the scale of 100ng of total RNA, the manufacturer recom-

69 mended lower limit of the protocol. However, attempts to create libraries from
70 much smaller samples yielded low complexity libraries, corresponding to as much
71 as 30-fold PCR duplication of fragments. Anecdotally, less than 5% of libraries
72 made with at least 90ng of total RNA yielded abnormally low concentrations,
73 which we observed correlated with low complexity (Data not shown). To deter-
74 mine the lower limit of input needed to reliably produce libraries, we attempted
75 to make libraries from 40, 50, 60, 70, and 80 ng of *Drosophila* total RNA, each
76 in triplicate.

Table 1: Total TruSeq cDNA library yields made with a given amount of input total RNA. Yields measured by Nanodrop of cDNA libraries resuspended in 25 μ L of EB. The italicized samples were unusually low, and when analyzed with a Bioanalyzer, showed abnormal size distribution of cDNA fragments.

| Amount Input RNA | Replicate A | Replicate B | Replicate C |
|------------------|---------------|-------------|-------------|
| 40 ng | <i>57 ng</i> | 425 ng | 672 ng |
| 50 ng | 435 ng | 768 ng | 755 ng |
| 60 ng | <i>115 ng</i> | 663 ng | 668 ng |
| 70 ng | 300 ng | 593 ng | 653 ng |
| 80 ng | 468 ng | 550 ng | 840 ng |

77 We considered the two libraries with lower than usual concentration to be
78 failures. While a failure rate of approximately 1 in 3 might be acceptable for
79 some purposes, we ultimately wanted to perform RNA sequencing on precious
80 samples, where a failure in any one of a dozen or more libraries would neces-
81 sitate regenerating all of the libraries. Furthermore, due to the low sample
82 volumes involved (less than approximately 500pg of poly-adenylated mRNA),
83 common laboratory equipment is not able to determine the particular point in
84 the protocol where the failures occurred.

85 Thus, we consider 70 ng of total RNA to be the conservative lower limit to
86 the protocol. While this is about 30% smaller than the manufacturer suggests, it
87 is still several orders of magnitude larger than we needed it to be. We therefore
88 considered using other small-volume and “single-cell” RNA-seq kits, which we
89 had less experience with and less faith in the data.

90 2.2 Experiment 2: Competitive Comparison of Low-volume 91 RNAseq protocols

92 We first sought to determine whether the low-volume RNAseq protocols avail-
93 able faithfully recapitulate linear changes in abundance of known inputs. We
94 generated synthetic spike-ins by combining *D. melanogaster* and *D. virilis* total
95 RNA in known, predefined proportions of 0, 5, 10, and 20% *D. virilis* RNA. For
96 each of the low-volume protocols, we used 1ng of total RNA as input, whereas
97 for the TruSeq protocol we used 100ng.

98 Although pre-defined mixes of spike-in controls have been developed and are
99 commercially available [15], we felt it was important to ensure that a given pro-
100 tocol would function reproducibly with natural RNA, which almost certainly has
101 a different distribution of 6-mers, which could conceivably affect random cDNA
102 priming and other amplification effects. Furthermore, our spike-in sample more
103 densely covers the approximately 10^5 fold coverage typical of RNA abundances.
104 It should be noted, however, that our sample is not directly comparable to any
105 other standards, nor is the material of known strandedness. We assumed that
106 the majority of each sample is from the standard annotated transcripts, but did
107 not verify this prior to library construction and sequencing.

108 The different protocols had a variation in yield of libraries from between
109 6 fmole (approximately 3.6 trillion molecules) and 2,400 femtomoles, with the
110 TruSeq a clear outlier at the high end of the range, and the other protocols all
111 below 200 fmole (Table 2.2). All of these quantities are sufficient to generate
112 hundreds of millions of reads—far more than is typically required for an RNA-
113 seq experiment. We pooled the samples, attempting equimolar fractions in the
114 final pool; however, due to a pooling error, we generated significantly more reads
115 than intended for the TruSeq protocol, and correspondingly fewer in the other
116 protocols. Unless otherwise noted, we therefore sub-sampled the mapped reads
117 to the lowest number of mapped reads in any sample in order to provide a fair
118 comparison between protocols.

119 We were interested in the fold-change of each *D. virilis* gene across the four
120 samples, rather than the absolute abundance of any particular gene. Therefore,
121 after mapping and gene quantification, we normalized the abundance A_{ij} of
122 every gene i across the $j = 4$ samples by a weighted average of the quantity Q_j
123 of *D. virilis* in sample j , as show in equation 1. Thus, within a given gene, a
124 linear fit of \hat{A}_{ij} vs Q_j should have a slope of one and an intercept of zero.

$$125 \quad \hat{A}_{ij} = A_{ij} \div \frac{\sum_j Q_j A_{ij}}{\sum_j (Q_j)^2} \quad (1)$$

126 We filtered the *D. virilis* genes for those with at least 20 mapped fragments
127 in the sample with 20% *D. virilis*, then calculated an independent linear re-
128 gression for each of those genes. As expected, for every protocol, the mean
129 slope was 1 (t -test, $p < 5 \times 10^{-7}$ for all protocols). Similarly, the average in-
130 tercepts for all protocols was 0 (t -test, $p < 5 \times 10^{-7}$ for all protocols). Also
131 unsurprisingly, the TruSeq protocol had a noticeably higher mean correlation
132 coefficient (0.98 ± 0.02) than any of the other protocols (0.95 ± 0.06 , 0.92 ± 0.09 ,
133 and 0.95 ± 0.06 for Clontech, TotalScript, and SMART-seq2, respectively). The
134 mean correlation coefficient was statistically and practically indistinguishable
135 between the Clontech samples and the SMART-seq2 samples (t -test $p = .11$,
136 Figure 2.2).

137 Indeed, the only major differentiator we could find between the low-volume
138 protocols we measured was cost. For only a handful of libraries, the kit-based
139 all inclusive model of the Clontech and TotalScript kits could be a significant
140 benefit, allowing the purchase of only as much of the reagents as required. By

141 contrast, the Smart-seq2 protocol requires the a la carte purchase of a number
142 of reagents, some of which are not available or more expensive per unit for
143 smaller quantities. Furthermore, there could potentially be a “hot dogs and
144 buns” problem, where reagents are sold in non-integer multiples of each other,
145 leading to leftovers. Many of these reagents are not single-purpose, however, so
146 leftovers could in principle be repurposed in other experiments.

Table 2: Summary of protocols used in experiments 2 and 3. Cost is estimated per sample assuming a large number of libraries at US catalog prices as of May 2014, and includes RNA extraction.

| Protocol | Shorthand | Cost/library |
|-------------------------------|-----------|--------------|
| TruSeq | TruS | \$45 |
| Clontech | CT | \$105 |
| TotalScript | TotS | \$115 |
| Smart-seq2, standard protocol | SS | \$55 |
| Smart-seq2, 2.5 fold dilution | SS—2.5x | \$28 |
| Smart-seq2, 5 fold dilution | SS—5x | \$20 |

147 **2.3 Experiment 3: Further modifications to the SMART-** 148 **seq2 protocol**

149 Although the SMART-seq2 was the cheapest of the protocols, we wondered
150 whether it could be performed even more cheaply without compromising data
151 quality. This would enable us to include more biological replicates in the future
152 experiments for which we are evaluating these protocols. In the original protocol,
153 we noticed that roughly 60% of the cost came from the Nextera XT reagents.
154 Thus, reducing the cost of tagmentation was the obvious goal to target.

155 We made additional libraries, again starting with 1ng of total RNA. We
156 amplified a single set of spike-in samples with 0, 5, 10, and 20% *D. virilis*
157 total RNA as in experiment 2, and made a single an additional sample with
158 1% *D. virilis* RNA. Starting at the point in the SMART-seq2 protocol where
159 tagmentation was started, we performed reactions in volumes 2.5× and 5×
160 smaller, using proportionally less cDNA as well. Due to the low total yield, we
161 increased the number of enrichment cycles from 6 to 8 (see methods).

162 When normalized to the same number of reads as in experiment 2, the
163 protocols with diluted Nextera reagents performed effectively identically: for
164 instance, the mean correlation coefficients were in both cases 0.96 ± 0.05 (Fig.
165 2 and Table 4). This is despite the additional cycles of enrichment, which
166 improved yield.

167 Because we used a common set of pre-amplified cDNA samples that was
168 performed in a distinct pre-amplification from experiment 2, we can estimate
169 the contribution of that pre-amplification to the overall variation. If, in fact, the
170 pre-amplification is a major contributor to the variation, then we would expect
171 to find that the correlation between, for instance, the slopes of two runs of the

| Experiment | Protocol | % <i>D. virilis</i> | Yield (fmole) | Reads | Mapped |
|------------|----------|---------------------|---------------|------------|------------|
| 2 | CT | 0 | 6.5 | 3,803,843 | 3,374,520 |
| 2 | " | 5 | 15.7 | 4,372,738 | 4,164,781 |
| 2 | " | 10 | 47.4 | 10,013,087 | 9,527,023 |
| 2 | " | 20 | 17.8 | 4,781,463 | 4,317,101 |
| 2 | TotS | 0 | 176.8 | 3,281,134 | 2,930,058 |
| 2 | " | 5 | 170.2 | 2,498,134 | 2,237,330 |
| 2 | " | 10 | 102.5 | 5,777,523 | 5,424,366 |
| 2 | " | 20 | 119.9 | 6,068,996 | 5,740,496 |
| 2 | TruS | 0 | 2,401.0 | 67,560,511 | 64,024,881 |
| 2 | " | 5 | 2,001.1 | 23,370,854 | 22,589,083 |
| 2 | " | 10 | 2,174.2 | 39,454,390 | 38,093,763 |
| 2 | " | 20 | 2,379.2 | 35,265,536 | 34,304,792 |
| 2 | SS2 | 0 | 34.3 | 2,439,518 | 2,297,087 |
| 2 | " | 5 | 59.6 | 2,550,023 | 2,419,889 |
| 2 | " | 10 | 67.9 | 2,534,628 | 2,444,568 |
| 2 | " | 20 | 39.8 | 2,504,340 | 2,389,850 |
| 3 | SS2—2.5x | 0 | 104.4 | 15,769,915 | 14,393,959 |
| 3 | " | 1 | 124.7 | 21,349,748 | 20,084,131 |
| 3 | " | 5 | 113.0 | 17,047,120 | 16,329,641 |
| 3 | " | 10 | 103.5 | 23,762,232 | 22,372,562 |
| 3 | " | 20 | 123.8 | 20,809,781 | 20,041,548 |
| 3 | SS2—5x | 0 | 59.4 | 19,214,155 | 17,324,598 |
| 3 | " | 1 | 58.6 | 23,832,274 | 22,364,220 |
| 3 | " | 5 | 65.4 | 18,149,452 | 17,157,450 |
| 3 | " | 10 | 28.8 | 15,821,419 | 14,869,864 |
| 3 | " | 20 | 57.2 | 22,466,345 | 21,620,603 |

Table 3: Sequencing summary statistics for samples. Protocols are the short-hands used in table 2. Reads indicates the total number of reads, and Mapped the total number of reads that mapped at least once to either genome. Experiments 2 and 3 were run in a single HiSeq lane each.

Table 4: Distribution of fit parameters. A simple linear fit, $\hat{A}_{ij} = m \cdot Q_j + b$ was computed for each gene i , and a correlation coefficient r calculated. For brevity, \bar{x} is the mean of some variable x , and σ_x is its standard deviation.

| Protocol | $\bar{m} \pm \sigma_m$ | $b \pm \sigma_b$ | $\bar{r} \pm \sigma_r$ |
|-------------------------------|------------------------|------------------|------------------------|
| TruSeq | 1.01±0.0698 | -0.108±1.05 | 0.98±0.019 |
| Clontech | 1.01±0.12 | -0.217±1.79 | 0.95±0.061 |
| TotalScript | 0.952±0.129 | 0.715±1.93 | 0.93±0.094 |
| Smart-seq2 | 1.03±0.121 | -0.506±1.82 | 0.95±0.057 |
| Smart-seq2, 2.5 fold dilution | 0.996±0.111 | 0.0623±1.67 | 0.96±0.053 |
| Smart-seq2, 5 fold dilution | 1.01±0.111 | -0.173±1.66 | 0.96±0.049 |

172 same experiment with different pre-amplifications would be significantly lower
173 than the correlation between the slopes of two runs using the same pre-amplified
174 cDNA pools.

175 Unsurprisingly, the sets of samples that used the same preamplification were
176 more correlated with each other than with the set of samples that used a separate
177 pre-amplification (Fig. 3). By analogy to dual-reporter expression studies[6], we
178 term variation along the diagonal “extrinsic noise” ($\eta_{ext} = \text{std}(m_1 + m_2)$), and
179 variation perpendicular to the diagonal “intrinsic noise” ($\eta_{int} = \text{std}(m_1 - m_2)$),
180 being intrinsic to the pre-amplification step. Using that metric, the intrinsic
181 noise is lower for the samples with the same pre-amplification ($\eta_{int} = 0.09$)
182 than for the samples with different pre-amplifications ($\eta_{int} = 0.16$). Somewhat
183 surprisingly, the extrinsic noise is higher for the samples with the same pre-
184 amplification ($\eta_{ext} = 0.20$ vs $\eta_{ext} = 0.16$), perhaps due to the 2 additional
185 cycles of PCR enrichment.

186 3 Discussion

187 When sample size is not the limiting factor, it is clear that using well-established
188 protocols that involve minimal sequence-specific manipulation of the sample
189 yields the best results, both in terms of reproducibility and linearity of response.
190 However, if it is not practical to collect such relatively large samples, we believe
191 that any of the “single-cell” protocols we have tested should perform similarly,
192 and can be used as a drop-in replacement. While preamplification steps do
193 introduce some detectable variance, it is not vastly detrimental to the data
194 quality, and does not introduce obvious sequence-specific biases.

195 Such methods should be strongly preferred if it is feasible to collect a suit-
196 ably homogenous sample. While bulk tissues may be a mixture of multiple
197 distinct cell types, this may or may not affect the particular research question
198 an RNAseq experiment is designed to answer. In our hands, the lower limit
199 of reliable library construction using the Illumina TruSeq kit is approximately
200 70ng of total RNA; with non precious samples, the practical limit is likely to

201 be even lower. Although we believe there is significant user-to-user variation, it
202 seems unreasonable to expect order-of-magnitude improvements are possible in
203 techniques for precious samples. We suggest that this limit may be related to
204 cDNA binding to tubes or purification beads, but since the quantities are lower
205 than the detection threshold of many standard quality control approaches, we
206 cannot directly verify this, nor do we believe that knowing the precise cause is
207 likely to suggest remediation techniques.

208 Compared to the regimes these protocols were designed for, we used a rel-
209 atively large amount of input RNA—1 ng of total RNA—corresponding to ap-
210 proximately 50 nuclei of a mid-blastula transition *Drosophila* embryo. Previous
211 studies have shown that this amount of RNA is well above the level where
212 stochastic variation in the number of mRNAs per cell will strongly affect the
213 measured expression of a vast majority of genes [19]. It is nevertheless a small
214 enough quantity to be experimentally relevant. For instance, we have previously
215 dissected single embryos into approximately 12 sections, yielding approximately
216 10ng per section[4], and one could conceivably perform similar experiments on
217 imaginal discs or antennal structures, which contain a similar amount of cells
218 [16, 10].

219 One of the more striking results is that costs can be significantly reduced by
220 simply performing smaller reactions, without noticeably degrading data quality.
221 We do not suspect this will be true for arbitrarily small samples, such as from
222 single cells. Instead, it is likely only true for samples near the high end of the
223 effective range of the protocol. We have not explored where this result breaks
224 down, and strongly caution others to verify this independently using small pilot
225 experiments before scaling up.

226 4 Methods

227 4.1 RNA Extraction, Library Preparation, and Sequenc- 228 ing

229 We performed RNA extraction in TRIzol (Life Technologies, Grand Island, NY)
230 according to manufacturer instructions, except with a higher concentration of
231 glycogen as carrier (20 ng) and a higher relative volume of TRIzol to the ex-
232 pected material (1 mL, as in [18] and [4]). We quantified RNA concentrations
233 using a fluorometric Qubit RNA HS assay (Life Technologies).

234 TruSeq libraries were prepared with the “TruSeq RNA Sample Preparation
235 Kit v2” (Illumina Cat.#RS-122-2001) according to manufacturer instructions,
236 except for the following modifications. All reactions were performed in half
237 the volume of reagents. We find that this increases the effective concentration
238 of RNA and cDNA. We performed all reactions and cleanups in 8-tube PCR
239 strip tubes, which allowed us to reduce the volume of Resuspension Buffer to
240 minimize volume left behind after each cleanup.

241 Clontech libraries were prepared with the “Low Input Library Prep Kit”
242 (Clontech Cat.#634947). We generated cDNA by using TruSeq reagents until

243 the cDNA synthesis step. Then, we used the Low Input Library Prep Kit to
244 modify the cDNA into sequencing-competent libraries. We believe that a similar
245 cDNA synthesis could be performed using oligo dT Dynabeads, RNA fragmen-
246 tation reagents, and Superscript II (Life Technologies), for an approximate cost
247 per sample of \$15.

248 TotalScript libraries were prepared with the “TotalScript RNA-Seq Kit” and
249 “TotalScript Index Kit” (Epicentre Cat.#TSRNA1296 and TSIDX12910). We
250 followed the manufacturer’s instructions, and used the oligo dT priming option.
251 We performed the mixed priming option in parallel, which yielded approximately
252 4-fold more library, but did not sequence them due to concerns of ribosomal
253 contamination.

254 SMARTseq2 libraries were prepared according to the protocol in Picelli *et*
255 *al.*(2014) [22]. Because we had already extracted and mixed the RNA, we began
256 at step 5 with 3.7 μ L of dNTPs and 1 μ L of 37 μ M oligo dT primer, yielding the
257 same concentration of primer and oligo as originally reported. We used 18 cycles
258 for the preamplification PCR in step 14, added 1ng of cDNA to the Nextera XT
259 reactions in step 28, and used 6 and 8 cycles for the final enrichment in step 33
260 (experiments 2 and 3, respectively).

261 Libraries were quantified using a combination of Qubit High Sensitivity
262 DNA (Life Technologies) and Bioanalyzer (Agilent Technologies, Sunnyvale,
263 CA) readings, then pooled to equalize index concentration. Due to a pooling
264 error in experiment 2, the TruSeq libraries were included at much higher abun-
265 dance. Pooled libraries were then submitted to the Vincent Coates Genome
266 Sequencing Laboratory for 50bp single-end sequencing according to standard
267 protocols for the Illumina HiSeq 2500. Bases were called using HiSeq Control
268 Software v1.8 and Real Time Analysis v2.8.

269 4.2 Mapping and Quantification

270 Reads were mapped using STAR [5] to a combination of the FlyBase reference
271 genome version 5.54 for *D. melanogaster* and *D. virilis* [20]. We randomly sam-
272 pled the mapped reads to use an equal number in each sample compared. We
273 used HTSeq (command line options “htseq-count --idattr='gene_name' --stranded=no --sorted=pos”
274 to count absolute read abundance per gene [1].

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276 6 Additional Information and Declarations

277 6.1 Competing Interests

278 The authors declare no competing interests exist.

279 **6.2 Author Contributions**

280 Peter A. Combs conceived and designed the experiments, analyzed the data,
281 and wrote the paper.

282 Michael B. Eisen conceived and designed the experiments and wrote the
283 paper.

284 **6.3 Data Deposition**

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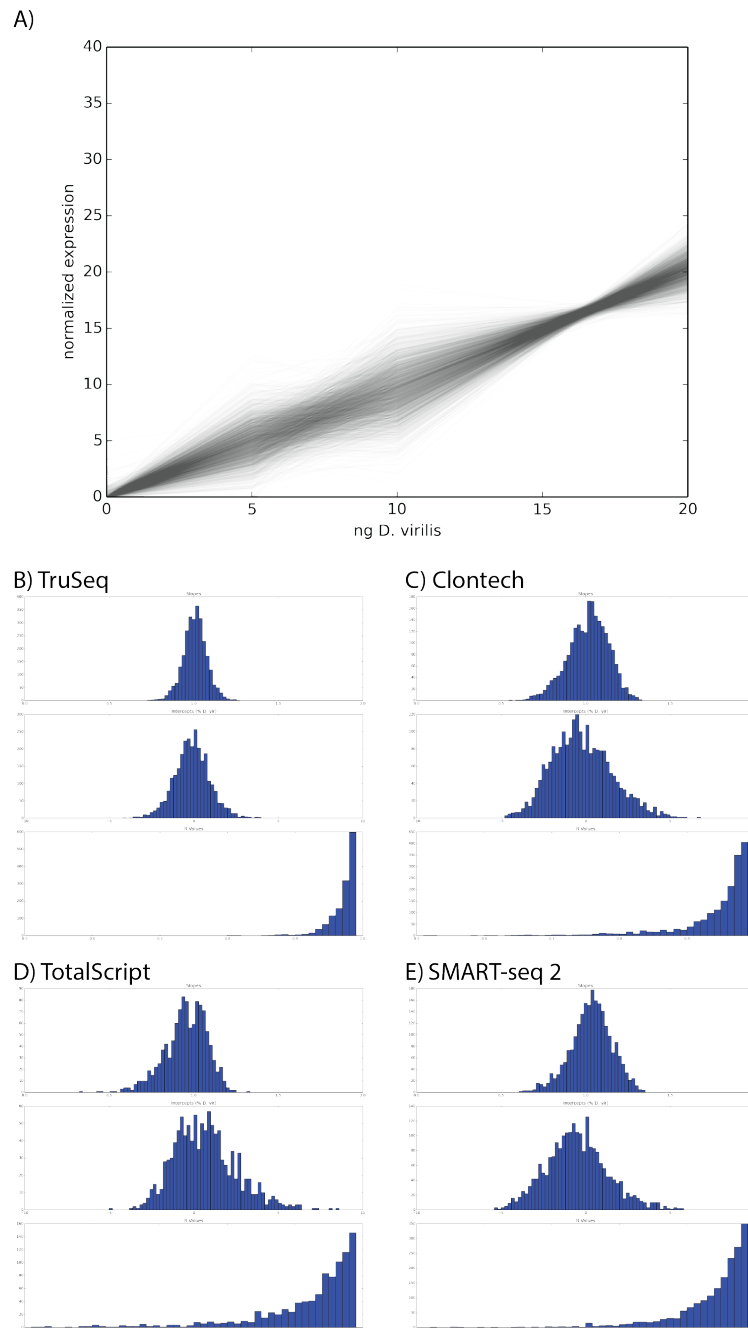


Figure 1: **Comparison of linearity between different RNA-seq protocols.** A) Normalized levels of gene expression \hat{A} across samples using the TruSeq protocol, where each line is for a different gene. B-E) Distributions of slopes, intercepts, and correlation coefficient for linear regressions of the data, as in panel A.

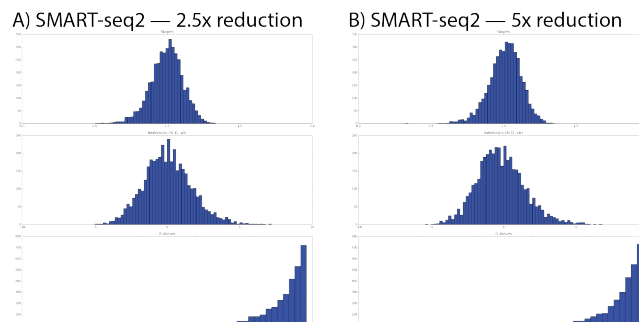


Figure 2: Distributions of slopes, intercepts, and correlation coefficients for experiment 3. Nextera XT reactions were reduced in volume by the indicated amount.

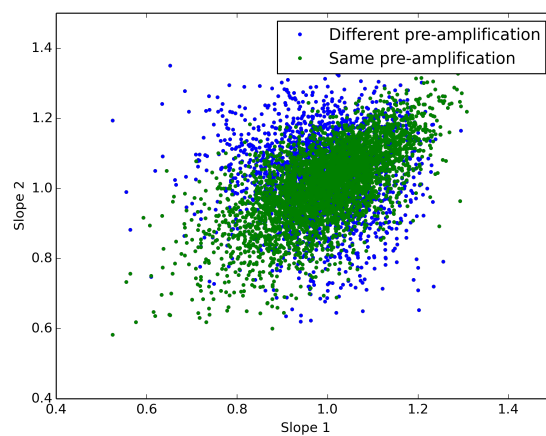


Figure 3: **Estimating the source of preamplification noise.** Plotted are the estimated slopes for each of the 3 samples. The 2.5 \times and 5 \times dilution samples used the same preamplified cDNA, but different tagmentation reactions, whereas the Full Size sample used different preamplification and tagmentation reactions.