Effect of *de novo* transcriptome assembly on transcript quantification

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Abstract

Background: Correct quantification of transcript expression is essential to understand the functional products of the genome in different physiological conditions and developmental stages. Recently, the development of high-throughput RNA sequencing (RNA-Seq) allows the researchers to perform transcriptome analysis for the organisms without the reference genome and transcriptome. For such projects, *de novo* transcriptome assembly must be carried out prior to quantification. However, a large number of erroneous contigs produced by the assemblers might result in unreliable estimation on the abundance of transcripts. In this regard, this study comprehensively investigates how assembly quality affects the performance of quantification for RNA-Seq analysis based on *de novo* transcriptome assembly.

Results: Several important factors that might seriously affect the accuracy of the RNA-Seq analysis were thoroughly discussed. First, we found that the assemblers perform comparatively well for the transcriptomes with lower biological complexity. Second, we examined the over-extended and incomplete contigs, and then demonstrated that assembly completeness has a strong impact on the estimation of contig abundance. Lastly, we investigated the behavior of the quantifiers with respect to sequence ambiguity which might be originally present in the transcriptome or accidentally produced by assemblers. The results suggest that the quantifiers

often over-estimate the expression of family-collapse contigs and under-estimate the expression of duplicated contigs.

Conclusions: In summary, we explicated the behavior of quantifiers when erroneous contigs are present and we outlined the potential problems that the assemblers might cause for the downstream analysis of RNA-Seq. We anticipate the analytic results conducted in this study provides valuable insights for future development of transcriptome assembly and quantification.

46 **Background**

47 Quantification and comparison of transcript expression are essential to understanding the role
48 of RNA in different physiological conditions or developmental stages. Such experiments and
49 analyses are widely used in the studies of molecular biology. Over the past decades, several
50 biological technologies have been developed to quantify the abundance of transcripts, such as
51 expression microarray [1] and high-throughput RNA sequencing (RNA-Seq) [2]. For organisms
52 with sufficient genomic information, the design of microarray provides a high throughput and
53 cost-effective solution to examine transcript expression. On the other hand, RNA-Seq is
54 superior in delivering lower background signals and larger dynamic ranges [3]. Despite the fact
55 that many genome sequencing projects have been carried out, such as Genome10K [4], 5000
56 arthropod genomes initiative (i5K) [5] and Bird10K [6], whole genome studies are still
57 demanding efforts for many research groups because of the immense cost and time-consuming
58 process. For non-model organisms, the expression microarray needs to rely on cross-species
59 hybridization [7]. On the contrary, RNA-Seq is more suitable owing to its capability of
60 detecting novel transcripts without additional genomic information [3].

61 When the reference genome and transcriptome are not available, RNA-Seq reads are first
62 used to reconstruct the transcriptome [8, 9]. Nowadays, many programs have been developed

63 for *de novo* transcriptome assembly, such as Oases [10], rnaSPAdes [11], SOAPdenovo-Trans
64 [12], Trans-ABySS [13] and Trinity [14]. After transcriptome sequences are reconstructed,
65 quantification methods including BitSeq [15], Kallisto [16], RSEM [17] and Salmon [18] can
66 be applied. These methods are able to inference the abundance of expression without the need
67 of genomic sequences, using the number of RNA-Seq reads that overlap with the assembled
68 contigs [9]. Nevertheless, quantification is much more challenging without reliable reference
69 sequences because of the erroneous contigs produced by the assemblers, which often result
70 from sequencing errors, insufficient sequencing depth and biological variability [19]. To
71 address these problems, a great number of comparative studies have been published recently.
72 While many studies evaluated transcriptome assembly [19-21] or quantification [22, 23]
73 programs independently, few have discussed how transcriptome assembly influences the
74 downstream quantification analysis. In 2013, Vijay, N., *et al*. performed an *in silico* assessment
75 of RNA-Seq experiments. That study examined the impact of various aspects of sequencing
76 reads on transcriptome assembly and differentially expressed genes (DEG) analysis [7], but the
77 effect of redundant contigs and multiple-mapping reads on quantification was not well
78 discussed. Another study conducted by Wang, S. and M. Gribskov evaluated the quality of
79 assembled contigs and their effects on DEG analysis [24]. However, their study mainly focused

80 on the evaluation of entire workflow from assembly, quantification, to DEG analysis, which
81 makes it obscure to unravel how the erroneous contigs affect the authenticity of downstream
82 analysis.

83 This study aims comprehensively discussing the effect of biological complexity on
84 transcriptome assembly, as well as the influence of incomplete and over-extended contigs on
85 quantification. We used both *in silico* simulated and authentic RNA-Seq data from three species
86 (yeast, dog, and mouse) to examine the effect of biological complexity on *de novo* transcriptome
87 assembly. Three state-of-the-art assemblers, namely rnaSPAdes, Trinity and Trans-ABySS,
88 were evaluated under different biological complexities based on TransRate [19] scores, which
89 were previously proposed to assess the quality of *de novo* transcriptome assemblies using the
90 alignments of sequencing reads to the assembled sequences. After *de novo* assembly, the
91 reference transcripts were assigned to assembled contigs according to the BLASTn [25]
92 alignments. Each transcript-contig alignment was then categorized based on accuracy, recovery
93 and sequence ambiguity. Subsequently, we thoroughly examined the impact of erroneous
94 contigs on the quantifiers Kallisto, RSEM and Salmon. By exploring the interplay between each
95 stage in RNA-Seq analysis workflow, this study provides valuable insights into conducting
96 RNA-Seq analysis and we anticipate these discoveries would be useful in the future

97 development of assembly or quantification algorithms.

98

99 Materials and Methods

100 Datasets

101 Three experimental and three simulated RNA-Seq datasets were used in this study. Both
102 experimental and simulated data included three species: yeast (*Saccharomyces cerevisiae*), dog
103 (*Canis lupus familiaris*) and mouse (*Mus musculus*). The experimental datasets were collected
104 from the Sequence Read Archive (SRA). The yeast dataset (SRR453566) was from the study
105 of Nookaew *et al.* [26], comprising 5.5 million non-stranded paired-end reads cultivated under
106 the batch condition. The dog dataset (SRR882109) was produced by Liu, *et al.* [27], comprising
107 20.8 million non-stranded paired-end reads sampled from normal mammary gland tissues of
108 domestic dogs. Finally, the mouse dataset (SRR203276) was collected from the study of
109 Grabherr, *et al* [14], containing 43.4 million stranded paired-end reads extracted from dendritic
110 cells. For the simulated datasets, Flux Simulator (ver. 1.2.1) [28] was adopted to synthesize
111 RNA reads for yeast, dog and mouse, respectively, based on the genomic sequences and
112 annotations from the Ensembl database [29]. To facilitate the analysis, only the transcripts
113 annotated as messenger RNA (mRNA) and with over 500 nucleotides in length were extracted.

114 The parameters used for the simulation are shown in Additional File 1: Table S1. In total, 81.7
115 million non-stranded paired-end reads were generated for the assembly and quantification
116 analysis. The quality of both experimental and simulated datasets was examined using FastQC
117 (ver. 0.11.5) [30] and the low-quality subsequences of the reads were trimmed using
118 Trimmomatic (ver. 0.36) [31] with parameters *SLIDINGWINDOW:4:20 MINLEN:30*. The
119 resultant RNA reads that were unable to maintain the paired relation were discarded. The
120 detailed information of the processed RNA reads is provided in Additional File 1: Table S2
121 (The mean and standard deviation of the insert sizes were estimated based on the alignments
122 produced by Burrows-Wheeler Aligner [32]).
123
124 **Expression Metrics**
125 In order to evaluate the performance of transcript quantification, the ground truth of expression
126 abundance for each transcript must be first determined. For simulated datasets, the numbers of
127 the generated RNA reads for each transcript was recorded during the simulation process. Since
128 transcriptome assemblers sometimes generate duplicated, incomplete or over-extended contigs,
129 the metrics we use for quantifying expression must consider the normalization with respect to
130 both sequence length and the number of total nucleotides. In this regard, the number of

131 generated RNA reads was transformed into a simplified version of Transcripts per Million

132 (TPM) [33] using the following equation:

133 $$\text{Ground Truth TPM}_i = \frac{f_i/l_i}{\sum_{k=1}^{n} f_k/l_k} \times 10^6,$$

134 where n is the total number of transcripts, f_i is the number of RNA reads generated from

135 transcript i and l_i is the effective length of transcript i. In contrast, because the ground truth

136 abundance for each RNA molecule is unknown for experimental datasets, we calculated the

137 average TPM inferred by Kallisto (ver. 0.43.0), RSEM (ver. 1.2.31) (default parameters) and

138 Salmon (ver. 0.8.2) for the reference transcript as the ground truth expression when evaluating

139 the abundance of an assembled contig. Although the estimated expression might not perfectly

140 reflect the real number of RNA molecules in a biological sample, it still provides valuable

141 information when comparing the performance of quantification before and after *de novo*

142 transcriptome assembly.

143

144 ***de novo* Transcriptome Assembly and Quantification**

145 The processed RNA-Seq reads were assembled into contigs using the following three programs:

146 (1) rnaSPAdes (ver. 3.11.1), (2) Trans-ABySS (ver. 1.5.5) along with ABySS (ver. 1.5.2) [34]

147 and (3) Trinity (ver. 2.4.0) with default parameters. To minimize the effect of fragmented

contigs, only the contigs with over 500 nucleotides in length were kept for the quantification analysis. The assemblies were evaluated based on the length of contigs, the number of recovered transcripts, the number of erroneous contigs and the evaluation scores provided by TransRate. The TransRate scores that we used in this study are the *score of bases covered*, *score of good mapping*, *score of not segmented* and *overall score*. The score of bases covered represents the proportion of nucleotide bases in a contig that are covered by reads. The score of good mapping represents the proportion of read pairs of which both reads are aligned in the correct orientation on a single contig. The score of not segmented represents the proportion of contigs that might be a chimera of multiple transcripts. Subsequently, the expression abundance for each contig was estimated using one alignment-based and two alignment-free quantifiers, namely (1) Bowtie2 (ver. 2.3.0) [35] (*--dpad 0 --gbar 99999999 --mp 1,1 --np 1 --score-min L,0,-0,1 -k 200 --sensitive --no-mixed --no-discordant*) followed by RSEM (ver. 1.2.31) (default parameter*s*); (2) Kallisto (ver. 0.43.0) (indexing with *-k 31* and quantifying with default parameters); and (3) Salmon (ver. 0.8.2) (indexing with *-k 31* and quantifying with default parameters).

165 **Transcript Assignment**

166 For the purpose of comparing the estimated abundance of contigs with the ground truth
167 expression from the corresponding transcripts, we assigned the reference transcripts to
168 assembled contigs based on BLASTn (2.5.0+) [25] alignments. Here, only the high scoring
169 pairs (HSPs) with identity over 70% and E-value smaller than 1E−5 were considered. We
170 integrated the remained HSPs onto the coordinates of both transcript and contig to obtain the
171 global alignment. Similar to a previous study [7], we calculated the *recovery* and *accuracy* for
172 each global alignment, which refer to the proportion of matched nucleotides on the transcript
173 and the proportion of correctly matched nucleotides on the contig respectively (Additional File
174 2: Fig. S1). Furthermore, we defined the overall *alignment score* as $\sqrt{recovery \times accuracy}$. A
175 transcript is assigned to a contig if either *accuracy* or *recovery* of the global alignment between
176 them is above 90%. In this manner, we were able to identify all the corresponding transcripts
177 for each contig. Note that it is possible that a contig can be associated with multiple transcripts,
178 and a transcript can assign to multiple contigs as well. We considered multiple assignments here
179 in order to understand the impact of redundant sequences on the quantification. Once the
180 transcripts have been assigned to the contigs, we used the following equation to calculate the
181 relative error of expression, in order to evaluate the quality of transcript quantification for each

182 transcript-contig pair:

183 $$\text{Relative Error}_{(i,\,j)} = \frac{TPM_i^{est} - TPM_j^{g.t}}{TPM_i^{est} + TPM_j^{g.t}} \times 100\%,$$

184 where the TPM_i^{est} is the expression estimated by quantifiers for $contig_i$, and the $TPM_j^{g.t}$ is the

185 ground truth expression for $transcript_j$ given ($contig_i$, $transcript_j$) is a valid global alignment

186 (either *accuracy* or *recovery* of the global alignment between them is above 90%).

187

188 **Sequence Ambiguity**

189 To determine the origin of the RNA-Seq reads that can be mapped to multiple transcripts is an

190 important issue for the development of quantification algorithms. In this regard, it is of interest

191 to understand the impact of sequence ambiguity on transcript quantification. We performed

192 pairwise sequence alignment on both transcripts and contigs using BLASTn, respectively. Here,

193 only the HSPs with identity over 70% and E-value smaller than 1E−5 were considered as

194 potential ambiguity. In addition, to better explicate the relation between sequences that share

195 similar subsequences, we build a connected component graph, where two sequences were

196 grouped into the same connected component if the proportion of identical nucleotides between

197 them is over 90% of the either sequence (Fig. 1). The size of a connected component is defined

198 as the number of sequence members inside. We call the sequences in a connected component

199 containing only one sequence as *unique sequence*. Furthermore, we used the proportion of

200 estimated abundance to investigate the behavior of quantifier while ambiguous sequences are

201 presented. Given n contigs $c_1, c_2, c_3 \ldots c_n$ in the same connected component C. The proportion

202 of estimated abundance for each contig is defined as follow:

203 $$\text{Proportion of Estimated Abundance}_i = \frac{\text{Allocated Read Count for } c_i}{\sum_{j=1}^{n} \text{Allocated Read Count for } c_j}.$$

204 If the highest proportion of estimated abundance in the connected component is close to 1, then

205 it suggests that the quantifiers allocate the all reads derive from the connected component to

206 one specific contigs. In contrast, if the highest proportion of estimated abundance in the

207 connected component is close to $1/n$, then it suggests that the quantifiers tend to allocate the

208 reads evenly in the connected component.

209

210 **Contig Categories**

211 The assembled contigs are categorized into five particular categories in this study: (1) *full-*

212 *length*, (2) *incompleteness*, (3) *over-extension*, (4) *family-collapse* and (5) *duplication* (Fig. 2).

213 The analysis of the first three categories were not affected by the factor of sequence ambiguity,

214 allowing us to investigate the impact of assembly completeness on quantification independently.

215 Given the length of contig l_c and the length of the corresponding transcript l_t, the

completeness of a contig was examined through the difference in length:

$$\text{Difference in Length}_{(c,\,t)} = \frac{l_c - l_t}{l_c + l_t} \times 100\%.$$

In contrast, *family-collapse*, *duplication* and *multiple-alignment* focused on the contigs that completely recovered the transcripts (*recovery* $\geq$ 90) but considered the influence of sequence ambiguity. To be more specific, *family-collapse* represents contigs which are assigned with multiple transcripts and *duplication* stands for the multiple contigs assigned by a single transcript. By examining these contigs, the problems caused by the assemblers that fail to distinguish similar transcripts from each other or generate a large number of redundant contigs are investigated. The detailed definitions for contig categories are provided in Additional File 1: Table S3.

Results

de novo Transcriptome Assembly

Based on pairwise BLASTn, yeast has the simplest transcriptome, with 94.11% of the transcripts sharing no similar subsequences with others. We call these sequences unique transcripts in the transcriptome. On the other hand, 66.29% of the dog transcripts are unique, while only 28.45% of the mouse transcripts are unique (Additional File 1: Table S4). First, we

233 performed transcriptome assembly on the RNA reads of the three species with different
234 biological complexities. We adopted three assemblers, rnaSPAdes, Trans-ABySS and Trinity,
235 to construct contigs for both experimental and simulated RNA-Seq reads. Next, we built
236 connected components for the assembled contigs. The statistics of sequence length and
237 sequence ambiguity for the assembled contigs are shown in Additional File 1: Table S5, while
238 the numbers of contigs that were categorized in each contig category are shown in Additional
239 File 1: Table S6. The proportion of recovered transcripts (*recovery* $\geq$ 90) and accurate contigs
240 (*accuracy* $\geq$ 90) is shown in Additional File 2: Fig. S2, and finally, the TransRate scores for
241 each assembly are shown in Additional File 2: Fig. S3.

242 In general, rnaSPAdes constructed the least amount of contigs with the highest overall
243 TransRate score for most of the datasets (5 among 6 datasets). As shown in Additional File 1:
244 Table S5, rnaSPAdes also delivered the lowest average size of the connected components across
245 species, suggesting that the contigs generated by rnaSPAdes are less redundant when compared
246 to those from the other two assemblers. Trinity outperformed other assemblers in terms of N50
247 and the proportion of the recovered transcripts in all the simulated datasets. Despite the fact that
248 Trinity generated longer contigs, the overall TransRate scores and the proportions of accurate
249 contigs from Trinity assembly are marginally lower. Trans-ABySS constructed the contigs with

250 comparatively high accuracy, with 66.37% of the contigs aligned with at least one transcript
251 that show accuracy higher than 0.90. Nonetheless, Trans-ABySS constructed smaller numbers
252 of unique and long contigs relatively. In other words, Trans-ABySS generated shorter contigs
253 with more redundancy. The summary of the assemblies also demonstrates that the proportion
254 of recovered transcripts are significantly higher in the datasets of yeast than that of dog or
255 mouse. With higher complexity, it appears to become more difficult for the assemblers to
256 properly reconstruct the transcriptome. For the estimated abundance of assembled contigs, the
257 estimation made by quantifiers RSEM, Kallisto and Salmon shows considerably high
258 consistency (Additional File 2: Fig. S4), with both Pearson's and Spearman's correlation
259 coefficients higher than 0.95 between any of the two quantifiers.
260

261 **Impact of Assembly Completeness**

262 In this study, the influence of *de novo* transcriptome assembly on expression quantification is
263 mainly discussed with respect to two major issues: assembly completeness and sequence
264 ambiguity. We would like to primarily look into the impact of assembly completeness on
265 quantification in this section. In order to reduce the possible effect of sequence ambiguity
266 generated by the assemblers, only the unique contigs (contigs in a connected component

containing only itself) that are assigned with a single transcript were examined here. The unique contigs were further categorized into *full-length*, *incompleteness* and *over-extension* (see Methods 2.6 for detailed definitions). The reliability of quantification was examined based on the relative error for contigs with different extent of assembly completeness (Fig. 3).

In summary, full-length contigs show the lowest relative error of quantification, with the medians of error smaller than $\pm 10\%$. The scatter plots and correlation coefficients also suggest that the estimated abundance of full-length contigs is highly reliable, with Pearson's and Spearman's correlation coefficient between the estimated and ground truth abundance both larger than 0.97 in all the datasets (Fig. 4, Fig. 5). The incomplete contigs yield slight over-estimation on the expression abundance. Overall, the quantification errors gradually increased as the assembly completeness decreased. This phenomenon can be observed more obviously on the dog and mouse datasets. When compared with the full-length contigs, the correlation coefficients are comparatively lower, ranging from 0.70 to 0.94 (Fig. 4, Fig. 5). Lastly, for the category of over-extended contigs, the quantifiers underestimated the expression abundance and the correlation coefficients slightly dropped (Fig. 4, Fig. 5). Nevertheless, the number of over-extended contigs is much fewer than those of other categories (Additional File 1: Table S6), which indicates that the assemblers did not overly extend the assembled contigs in most of

284 the cases. In other words, only a limited number of contigs in the quantification will be affected
285 in the practical RNA-Seq analysis. Although the TPM metrics has already been normalized for
286 sequence length and total nucleotides, researchers might still need to be aware of the length
287 bias caused by incomplete or over-extended contigs while using TPM as the metrics to estimate
288 the expression of contigs.
289
290 **Impact of Sequence Ambiguity**
291 In this section, the impact of sequence ambiguity on quantification was thoroughly discussed.
292 Similarly, to reduce the compound effect from assembly completeness, only the accurately
293 assembled contigs (*accuracy* $\geq$ 90) were examined here. In the first part of this sub-section,
294 we looked through the reliability of quantification when the assemblers report only one contig
295 for many similar transcripts, denoted as *family-collapse*. In the second part, we examined the
296 impact of contigs with similar sequence content which are assigned with the same transcript,
297 denoted as *duplication* (see Methods 2.6 for detailed definition). By using these contigs, we
298 examined the behavior of the quantification algorithms while sequence ambiguity is present in
299 the assembly.
300 For the contigs categorized as family-collapse, it is much more difficult to analyze the

301 accuracy of quantification because of multiple transcripts being assigned to a contig. Based on
302 our observation, there are in average 2 to 3.16 transcripts being assigned to a family-collapse
303 contig across the six datasets. Since there is only one contig that were assigned by multiple
304 transcripts, we would like to find out of which transcript expression delivers the estimated
305 abundance of the contig actually reflects. To our surprise, the estimated abundance is closer to
306 the transcript with the highest expression rather than the one with highest alignment score (Fig.
307 6, Additional File 2: Fig. S5).

308 In contrast with family-collapse, duplication represents the redundant contigs which are
309 clustered into the same connected component and are assigned with a single transcript. Here,
310 we use the maximum estimated abundance in the connected component to investigate the
311 behavior of quantifiers (see Methods 2.5 for detailed definition). We observed that
312 quantification algorithms tend to allocate most of the RNA reads to a single contig within the
313 connected component in most of the cases (15 out of 18 of the datasets show that over 50% of
314 the connected component has one contig with the proportion of estimated abundance over 75%)
315 (Additional File 2: Fig. S6). Furthermore, we would like to understand which estimated
316 abundance of the contig in the connected component can accurately reflect the expression of
317 corresponding transcript. Here, we used three approach to select the estimated abundance of

318 the contig in the connected component: (1) the contigs with the highest alignment score, (2) the
319 contigs that are allocated with the most RNA reads and (3) the total expression of connected
320 component of the contigs. Consequently, we found that the estimated abundance of contigs that
321 were allocated with the most amount of RNA reads in the connected component show
322 significantly low quantification error with the transcript expression. However, in the cases when
323 the quantifiers distribute the RNA reads evenly to the duplicated contigs, the ground truth
324 expression for the transcripts cannot be accurately represented by the estimated abundance of
325 contigs. (Fig. 7). To address this problem, it is advisable to use the total expression of the
326 connected component for duplicated contigs to measure the expression of corresponding
327 transcripts.
328

329 ## Discussions

330 **Component-level Quantification**

331 Based on the observation discussed in the previous section, we first discovered that once the
332 assemblers failed to distinguish the RNA reads generated from similar transcripts and reported
333 one single contig, the estimated abundance of family-collapse contigs often reflect the
334 expression of the transcripts attracting the most amount of RNA reads. In contrast, to estimate
335 the expression of transcripts corresponding to the duplicated contigs, researchers might need to

336 use to abundance of the contigs that are allocated with the majority of the RNA-reads in order
337 to get accurate estimation. Nevertheless, these conclusions are drawn from the strictly defined
338 scenario. In most of the practical RNA-Seq analysis, the information of the transcriptome
339 sequence is not available for non-model organisms. Therefore, it is essential to understand
340 which approach is the best suited to select the contigs and the corresponding transcripts with
341 the closest estimation on abundance. Here, we looked through all the valid assignment of
342 transcripts for each contig and we demonstrated four different methods: (1) selecting the
343 transcripts with the highest alignment score for each contig, followed by selecting the contig
344 with the highest alignment score for each transcript, (2) selecting the transcripts with the highest
345 alignment score for each contig, followed by selecting the contig with the highest estimated
346 abundance in the connected component, (3) selecting the transcripts with the highest expression
347 in the connected component, followed by selecting the contig with the highest alignment score
348 for each transcript, and (4) selecting the transcripts with the highest expression in the connected
349 component, followed by selecting the contig with the highest estimated abundance in the
350 connected component. As a result, it seems to be more appropriate to use the maximum
351 abundance in the contig connected component to estimate the expression of transcripts (Fig. 8).
352 Despite the fact that this strategy abandon the concept of quantification for individual

353 transcripts, many advantages emerge such as higher accuracy on the quantification, reliable
354 read inference, robust statistical performance and clear interpretation of the data [36]. More
355 importantly, this strategy does not have to deal with the thorny problem resulting from multiple
356 transcript assignment in contig annotation.
357
358 **The Selection of Programs**
359 We argue that the difficulties discussed in this research emerge mainly because the goals for
360 each step of the analysis is not specifically designed to suit *de novo* RNA-Seq analysis. Most
361 of the assemblers are optimized to reconstruct the whole transcriptome, which sometimes leads
362 to many false predictions on SNPs or isoforms. These artificial or incorrect contigs thereafter
363 deteriorate the accuracy of quantifiers because most of the quantification algorithms infer the
364 expression based on the overlapping relation between RNA-Seq reads and the given sequences
365 (transcripts or contigs). Furthermore, the annotation step often utilizes programs that excel in
366 sequence alignment without considering the abundance of expression. This observation
367 demonstrates that the selection of the programs has a strong impact on the *de novo* RNA-Seq
368 analysis. For instance, to minimize the effect of assembly completeness on quantification,
369 assemblers that construct the sequences with appropriate length are preferable. On the other

370 hand, to reduce the problem in contig annotation resulting from sequence ambiguity, the
371 assemblers that report less false prediction for SNPs or isoforms are better choices. The
372 performance for the quantifiers we analyzed throughout this study demonstrates high
373 consistency in terms of accuracy of quantification. Therefore, we recommended to use the
374 alignment independent quantifiers such as Kallisto and Salmon for significantly lower
375 computational time [16, 18]. The selection of programs for contig annotation is relatively
376 irrelevant because the bottleneck mainly lies in the insufficient transcript information. Since
377 there are no reference transcripts available in the practical *de novo* RNA-Seq analysis, most of
378 the research utilizes the protein sequences from proximate species with small genetic changes
379 because of comparatively higher conservation in protein sequences. However, to align the
380 contigs with proteins of other species might reduce the precision and accuracy of sequence
381 alignment, which makes it more difficult to find the correct annotation for the assembled contigs.
382

383 **Other Important Factors in RNA-Seq Analysis**

384 Although we mainly discussed the impact of assembly completeness and sequence ambiguity
385 on quantification and annotation throughout this study, there are many other factors that might
386 as well result in unreliable experimental results in the practical *de novo* RNA-Seq analysis. For

387 instance, the read length or the fragment size directly determine the maximum length of the
388 nucleotides that overlap with the reference sequences for each read pair. Therefore, the number
389 of RNA-Seq reads that can be aligned to multiple origins of the transcripts reduces when longer
390 reads are adopted, which mitigates the problem of sequence ambiguity on the inference of the
391 origin of the reads [33, 37]. The strand specificity provides the information for the strand of the
392 RNA-Seq reads, which improves the precision of sequence alignment and quantification [38].
393 Last but not least, the sequence-specific and positional bias derived from library construction
394 might lead to RNA-Seq reads that over- or under-represent the number of transcripts in the
395 molecules In this regard, it is important to model the fragment bias in the process of
396 quantification [39]. However, considering all the combined effects of the factors mentioned
397 above will significantly increases the complexity of this research. Therefore, we generated non-
398 stranded RNA-Seq with uniform read length, and the fragment bias for the reads were excluded.
399 Moreover, the start coordinates of the RNA-Seq reads uniformly distributed. By this means, we
400 excluded the potential impact of these factors and focused only on the effect of assembly
401 completeness and sequence ambiguity. But the importance of these factors should not be
402 ignored in the practical *de novo* RNA-Seq analysis.
403

404 **Sequencing Technologies**

405 In this section, we briefly highlight two sequencing technologies that provide another new
406 perspective and mitigate the problems in RNA-Seq analysis: single-cell RNA sequencing
407 (scRNA-Seq) and long-read RNA sequencing. By isolating individual cell prior to sequencing,
408 scRNA-Seq technology allows the researchers to extract mRNA from a single cell. The
409 development of scRNA-Seq allows the researchers to identify uncharacterized cell types, to
410 observe the stochastic nature of gene expression, and to compare the expression profiles for the
411 individual cells within the population [9, 40]. Nevertheless, the number of reads generated using
412 scRNA-Seq is relatively small since only a small number of mRNA molecules are expressed in
413 a single cell. This drawback makes it difficult to reconstruct the transcriptome for each cell
414 [40]. Moreover, assembly algorithms that take the advantages of unique molecular identifiers
415 (UMIs) for each read are still in demand. In the *de novo* RNA-Seq analysis, it is advisable to
416 aggregate the scRNA-Seq from all cells as the bulk RNA-Seq reads to reconstruct the
417 transcriptome and subsequently estimate the abundance of each cell subsequently. The
418 restriction makes the exploitation of scRNA-Seq mainly limit to species with highly-curated
419 transcript sequences available.

420 Another technology that developed with increasing interests recently is long-read RNA
421 sequencing. Unlike next-generation RNA-Seq technology, long-read RNA sequencing
422 generates a single read for each mRNA molecule in real-time, which results in considerably
423 longer RNA-Seq data that allows the full-length reconstruction for transcripts without the need
424 of assembly [9]. Although the demanding cost for sequencing in higher coverage makes it hard
425 to be considered for quantification at this moment, this technology still provides an
426 extraordinary breakthrough for identifying the transcriptome in non-model organisms [41]. If
427 the research expenditure is sufficient, we recommend combining the long-read technology with
428 scRNA-Seq reads for the identification and quantification of the novel transcriptome
429 respectively.
430

431 **Conclusion**

432 While most of the related studies focused on optimizing the quantification or assembly
433 algorithms independently, few studies have discussed how the erroneous contigs generated by
434 the assemblers affect the downstream analysis of RNA-Seq. In this study, we comprehensively
435 examined the impact of biological complexity, assembly completeness and sequence ambiguity.
436 We comparatively evaluated the performance of rnaSPAdes, Trans-ABySS and Trinity for *de*

437 *novo* transcriptome assembly under three transcriptomes with different complexities. All of the
438 selected assemblers showed a lower proportion of the fully-reconstructed transcripts as the
439 complexity of transcriptome increases. In general, rnaSPAdes constructed the least number of
440 contigs with the highest TransRate score, Trinity produced the longer contigs, and Trans-
441 ABySS generated the contigs with the higher accuracy. As for quantification, we measured the
442 reliability of RSEM, Kallisto and Salmon. The estimation made by three algorithms shows
443 marginal differences. For each erroneous contig, the incomplete or over-extended contigs lead
444 to unreliable estimation of the abundance of contigs. Moreover, we have found that if the
445 redundant contigs are present in the assembly, the quantifiers tended to allocate the RNA-Seq
446 reads to one of the duplicated contig. However, in rare cases, the quantifiers distributed the
447 reads evenly to the contigs that share similar sequence content. On the contrary, the quantifiers
448 tended to over-estimate the contigs that were assigned with multiple transcripts since the
449 assemblers failed to distinguish the difference of these transcripts and reported only a single
450 contig. To circumvent these issues, it is advisable to estimate the abundance on gene-level rather
451 than for individual transcripts using tools such as Corset. By exploring how these factors
452 deteriorate the reliability of *de novo* RNA-Seq analysis, we provided valuable insights for the
453 interplay between transcriptome assembly, quantification and sequence annotation. We

454 anticipated these discoveries will be useful in the future development of assembly or

455 quantification programs.

456

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The analysis pipelines used in this study are available and can be downloaded from GitHub repository: https://github.com/dn070017/QuantEval. The RNA reads supporting the results of this article are available in the NCBI Short Read Archive, with the accession numbers SRR453566 (yeast) and SRR882109 (dog), SRR203276 (mouse).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Ping-Han Hsieh performed the analysis and wrote the manuscript. Chien-Yu Chen and Yen-Jen Oyang commented on the draft and revised the manuscript. All authors read and approved the final manuscript.

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596

Figure Legends

Figure 1: Construction of Ambiguity Network. The diagram illustrates pairwise alignments in a contig set and how the ambiguity network was constructed. The connected component was first initialized given the contig sequences, creating a single cluster for each sequence. By analyzing the global alignment between contigs (the blue dot lines), the cluster expanded by union two contig cluster at a time if the alignment length between the sequence is over 90% of the length for either sequence. Note that the ambiguity network was constructed for both contig and transcript set. For the purpose of simplicity, we only illustrated the scenario for contigs in this figure.

Figure 2: Examples of Contig Categories. The diagram gives examples for each contig category we analyzed throughout this study. The middle column shows an example for each category, while the right column portrays the relation of contigs and transcripts in network representation. The sequence nodes are connected together in solid line if they are in the same ambiguity cluster. On the other hand, the blue dot arrow represents the transcript assignment for the contigs. The analysis of contigs labeled as full-length, incompleteness and over-extended exclude the factor of sequence ambiguity. On the contrary, family-collapse and duplication remove the potential effect of assembly completeness, focusing only on the impact of redundant or duplicated sequences.

Figure 3: Quantification Error for Unique Sequences. The box plots illustrate the relative quantification error for unique contigs for simulated dataset. The estimation of contig abundance is made by Kallisto based on Trinity assembly. The contigs are separated by the extent of assembly completeness, the numbers on the X-axis represents the lower bound of difference in length. For instance, the contigs locate on -10 means that the percentage of difference in length is in the range of $[-10, 0)$. The data is color coded based on the contig categories. The box plots suggest that the estimation made on full-length contigs yield the smallest relative error, while the incomplete contigs show over-estimation and over-extended contigs show under-estimation on quantification

Figure 4: Scatter plots of Estimated Abundance and Ground Truth Expression for Unique Sequences. The scatter plots illustrate the estimated and ground truth abundance for contigs categorized as full-length, incompleteness and over-extension of simulated dog dataset. The

630 estimation of contig abundance is made by Kallisto based on Trinity assembly. The metrics are
631 recorded in $log_2(TPM + 1)$. The data points are color coded based on the relative
632 quantification error, with blue represents under-estimation and orange for over-estimation. In
633 general, the estimation on expression for full-length contigs is highly reliable. There are some
634 incomplete contigs with over-estimated abundance. Moreover, the correlation coefficients for
635 the estimation of incomplete contigs are also relatively lower than that of full-length contigs.
636 As for over-extended contigs, a marginal under-estimation in quantification can be observed.
637
638 **Figure 5. Correlation Coefficients between Estimated Abundance and Ground Truth**
639 **Expression for Unique Sequences.** The figures illustrate the Pearson's and Spearman's
640 correlation coefficients between estimated abundance and ground truth expression. In general,
641 the estimation based on full-length contigs have considerably high correlation with the ground
642 truth expression of corresponding transcripts. In contrast, the incomplete and over-extended
643 contigs show relatively lower correlation coefficients. There are significantly low correlation
644 coefficients in the rnaSPAdes assembly based on simulated yeast data; however, due to a small
645 number of data (n = 11), it should not be used to draw conclusion on this dataset.
646
647 **Figure 6: Scatter plots of Estimated Abundance and Ground Truth Expression for**
648 **Family-Collapse Sequences.** The scatter plots illustrate the estimated and ground truth
649 abundance for contigs categorized as family-collapse of simulated dog dataset. The estimation
650 of contig abundance is made by Kallisto based on Trinity assembly. The metrics are recorded
651 in $log_2(TPM + 1)$. The data points are color coded based on the relative quantification error,
652 with blue represents under-estimation and orange for over-estimation. Since there are more than
653 one transcripts correspond to one contig, we categorize the expression of corresponding
654 transcript into (1) transcript with the maximum alignment score, (2) transcript with the highest
655 expression, and (3) others. In general, the estimated abundance of the contig actually reflect to
656 the transcript with the highest expression.
657
658 **Figure 7: Box Plots for the Relative Error of Duplicated Contigs.** The box plots illustrate
659 the relative quantification error for duplicated contigs of experimental mouse dataset. The
660 contigs are separated by the proportion of maximum estimated abundance in the connected
661 component, the numbers on the X-axis represents the lower bound of the proportion. For
662 instance, the contigs locate on 0.45 means that the percentage of difference in length is in the
663 range of $[0.45, 0.50)$. Since there are more than one contigs which are assigned with the same

transcripts. We would like to find out which contigs' estimated abundance can accurately reflect the expression of the transcripts. Here, we categorize the quantification error into three categories: (1) based on the contigs with the highest alignment score, (2) based on the contigs that are allocated with the most RNA reads and (3) the total expression of connected component of the contigs. The box plots suggest that contigs with the highest alignment score or the highest estimation made within the connected component have considerably lower quantification error if most of the reads are assigned to one specific contig. However, when the quantifiers allocate the RNA reads evenly to the contigs within the connected component, it is advisable to use the total expression of connected component in order to get the accurate estimation upon the expression of transcripts.

Figure 8: Scatter plots of Estimated Abundance and Ground Truth Expression for All Sequences. The scatter plots illustrate the estimated and ground truth abundance for all the contigs of simulated dog dataset. The estimation of contig abundance is made by Kallisto based on Trinity assembly. The metrics are recorded in $log_2(TPM + 1)$. The data points are color coded based on the relative quantification error, with blue represents under-estimation and orange for over-estimation. We used two different methods to select the contig in interest: (1) the contigs with highest alignment score, and (2) the contigs with highest number of allocated reads in the connected component. We would like to understand whether the estimated abundance for both methods can be used to accurately estimate the expression of transcript. In general, the estimated abundance of the contig with the highest number of allocated reads in the connected component yield a more accurate estimation.

Additional Files

Additional File 1. Title: Supplementary Tables. Description of data: Table S1 to Table S6.

Additional File 2. Title: Supplementary Figures. Description of data: Fig. S1 to Fig S??.

sequences

contig a	TTACACAATCGTAGCATGTATG
contig b	TACACAATCGTAGCATGTATGG
contig c	AATCGTAG
contig d	CAATCGTAGCATGTATGG
contig e	GACCTTTGTTCACGTTTGCCCAT

alignments

```
contig a   TTACACAATCGTAGCATGTATG
           |||||||||||||||||||||
contig b   TACACAATCGTAGCATGTATGG
```

alignment length ≥ 90% length of both sequences

```
contig c   AATCGTAG
           ||||||||
contig d   CAATCGTAGCATGTATGG
```

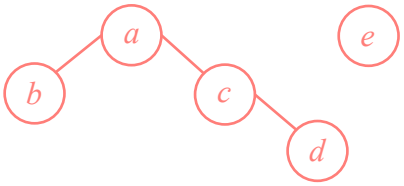
alignment length ≥ 90% length of either sequence

```
contig a   TTACACAATCGTAGCATGTATG
               ||||||||
contig c       AATCGTAG
```

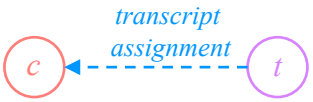
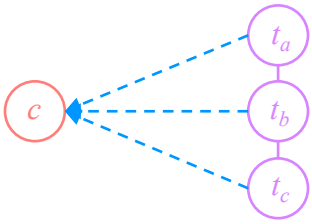
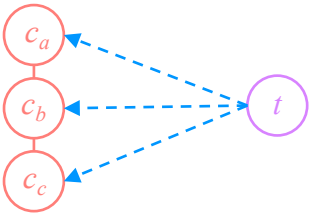
alignment length ≥ 90% length of either sequence

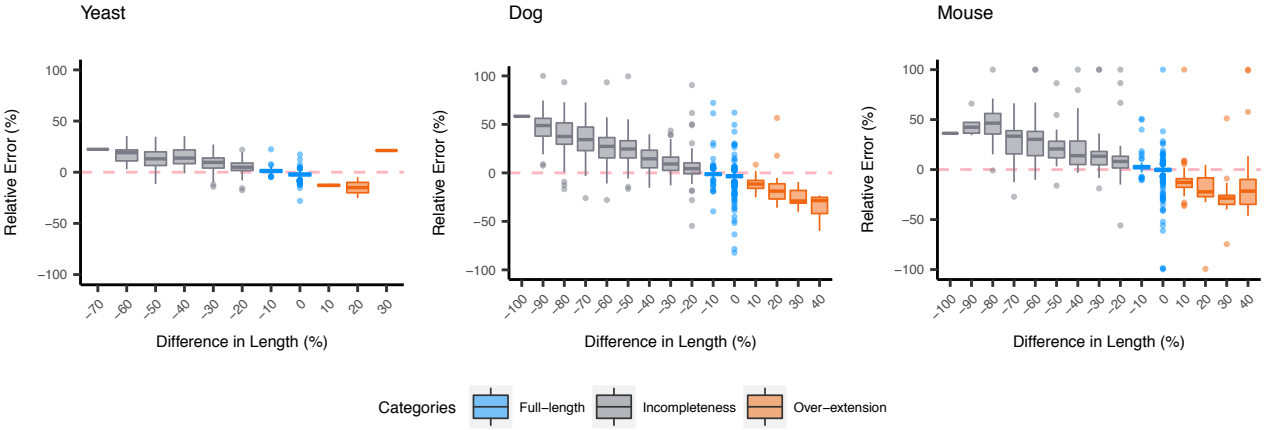
network initialization

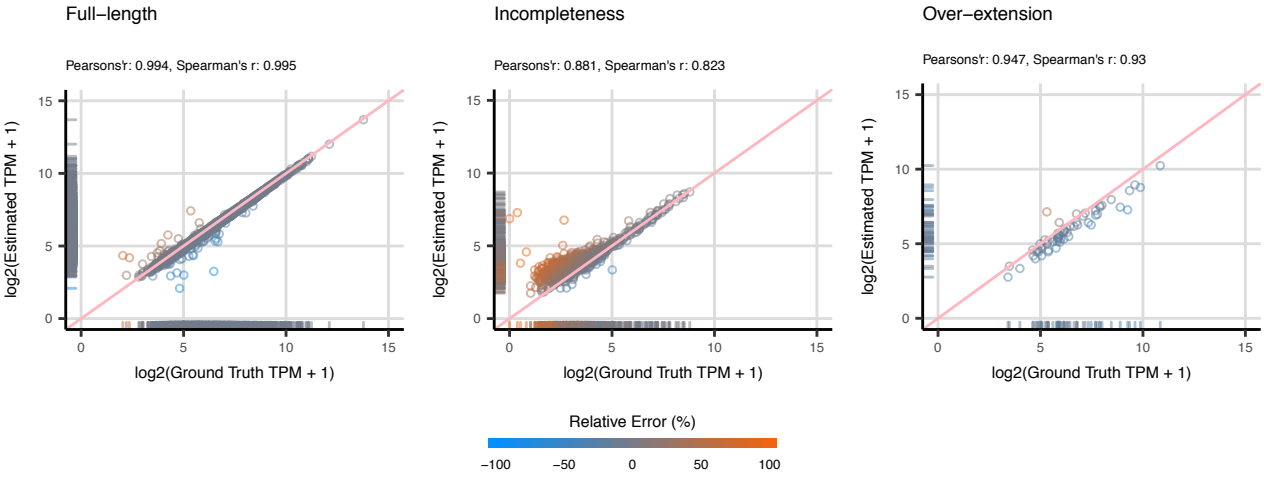
network operation

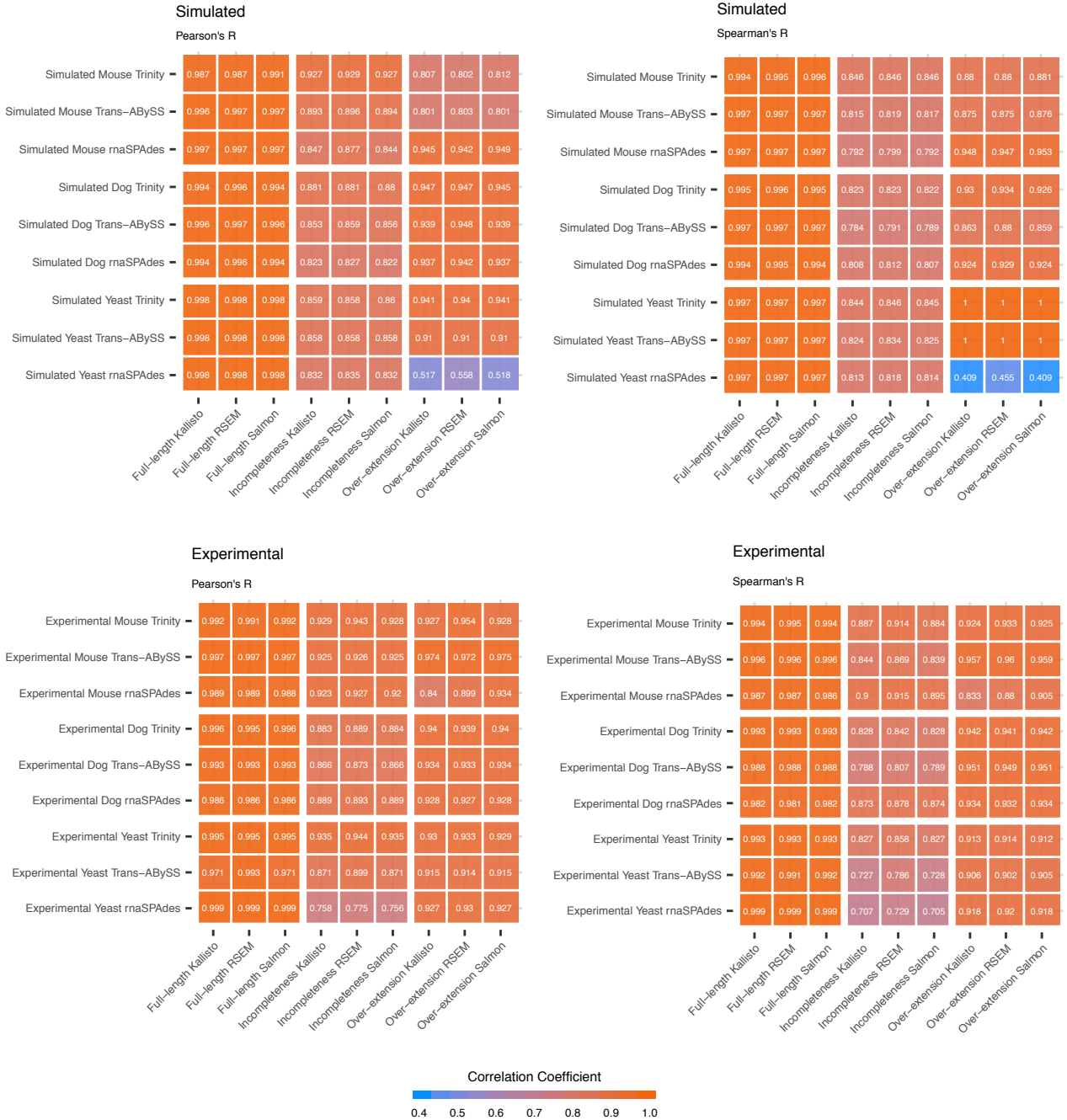


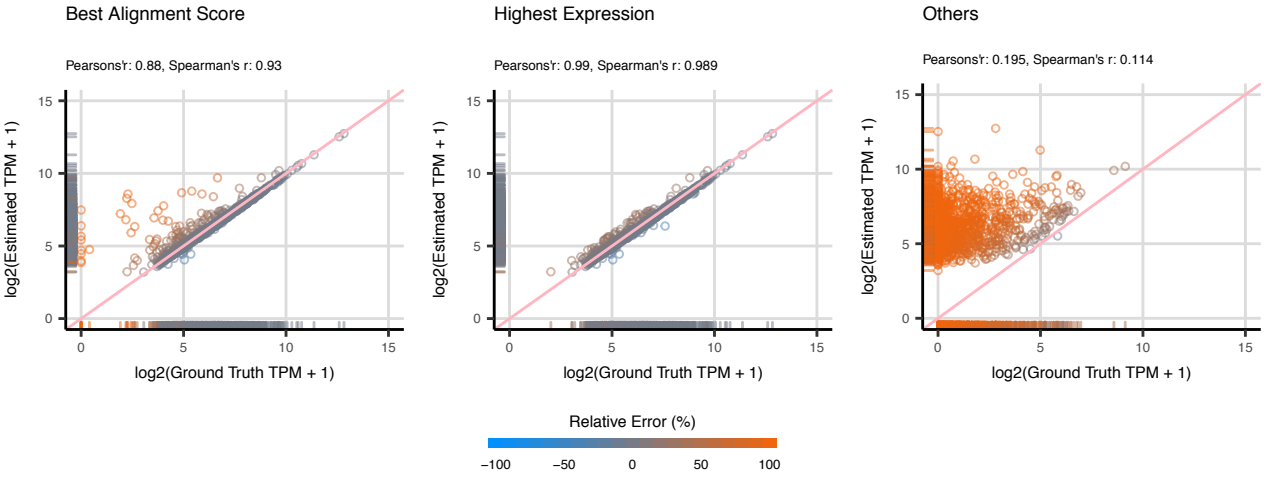
union(a, c) *union*(a, b) *union*(c, d)

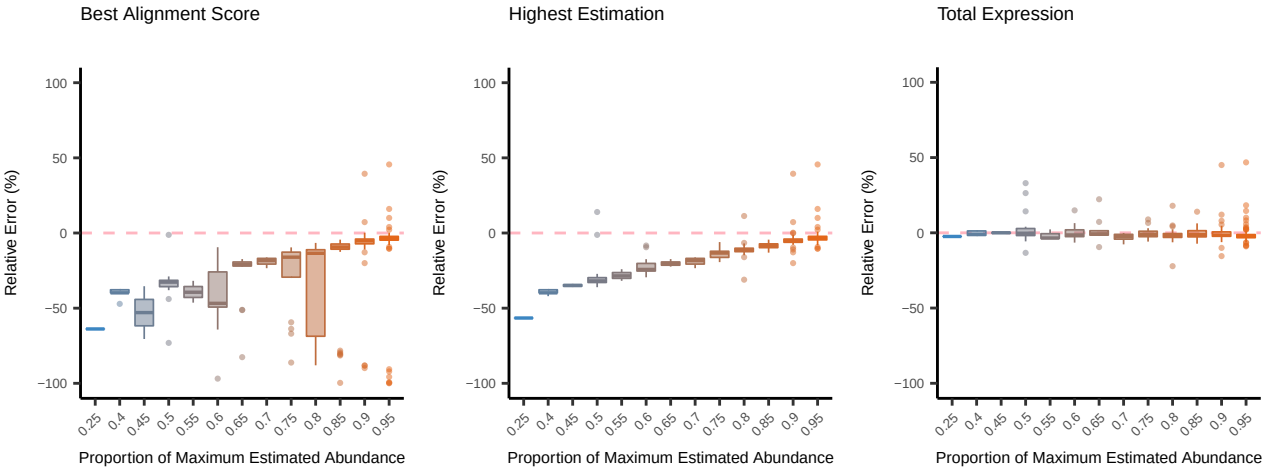
Full-length	*contig* *transcript*	TTACACAATCGTAG TACACAATCGTAGC	
Incompleteness	*contig* *transcript*	TACA TACACAATCGTAGC	
Over-extended	*contig* *transcript*	TTACACAATCGTAG CACA	
Family-collapse	*contig* *transcript a* *transcript b* *transcript c*	TTACACAATCGTAG TACACAATCGTAGC TTACACGATCGTA CTACACAATGGTAG	
Duplication	*contig a* *contig b* *contig c* *transcript*	TTACACAATCGTAG TACACAATCGTAGC TTACACGATCGTA CTACACAATGGTAG	

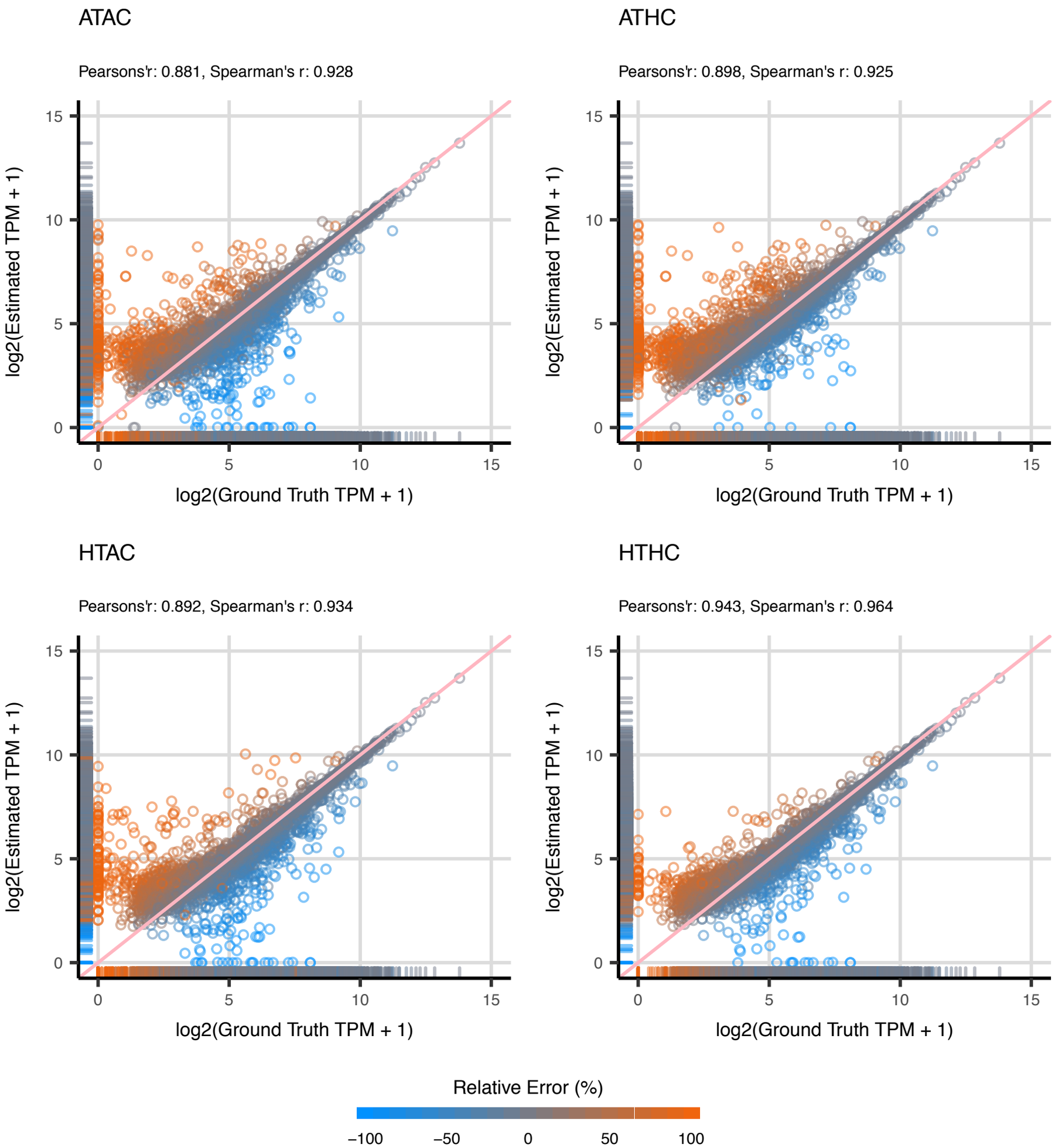

Yeast
Dog
Mouse
Relative Error (%)
Difference in Length (%)
Categories
Full−length
Incompleteness
Over−extension


Full−length
Pearsons'r: 0.994, Spearman's r: 0.995
Incompleteness
Pearsons'r: 0.881, Spearman's r: 0.823
Over−extension
Pearsons'r: 0.947, Spearman's r: 0.93
log2(Estimated TPM + 1)
log2(Ground Truth TPM + 1)
0
5
10
15
Relative Error (%)
−100
−50
0
50
100


Simulated
Pearson's R
Simulated Mouse Trinity 0.987 0.987 0.991 0.927 0.929 0.927 0.807 0.802 0.812
Simulated Mouse Trans−ABySS 0.996 0.997 0.997 0.893 0.896 0.894 0.801 0.803 0.801
Simulated Mouse rnaSPAdes 0.997 0.997 0.997 0.847 0.877 0.844 0.945 0.942 0.949
Simulated Dog Trinity 0.994 0.996 0.994 0.881 0.881 0.88 0.947 0.947 0.945
Simulated Dog Trans−ABySS 0.996 0.997 0.996 0.853 0.859 0.856 0.939 0.948 0.939
Simulated Dog rnaSPAdes 0.994 0.996 0.994 0.823 0.827 0.822 0.937 0.942 0.937
Simulated Yeast Trinity 0.998 0.998 0.998 0.859 0.858 0.86 0.941 0.94 0.941
Simulated Yeast Trans−ABySS 0.998 0.998 0.998 0.858 0.858 0.858 0.91 0.91 0.91
Simulated Yeast rnaSPAdes 0.998 0.998 0.998 0.832 0.835 0.832 0.517 0.558 0.518
Full−length Kallisto
Full−length RSEM
Full−length Salmon
Incompleteness Kallisto
Incompleteness RSEM
Incompleteness Salmon
Over−extension Kallisto
Over−extension RSEM
Over−extension Salmon
Simulated
Spearman's R
Simulated Mouse Trinity 0.994 0.995 0.996 0.846 0.846 0.846 0.88 0.88 0.881
Simulated Mouse Trans−ABySS 0.997 0.997 0.997 0.815 0.819 0.817 0.875 0.875 0.876
Simulated Mouse rnaSPAdes 0.997 0.997 0.997 0.792 0.799 0.792 0.948 0.947 0.953
Simulated Dog Trinity 0.995 0.996 0.995 0.823 0.823 0.822 0.93 0.934 0.926
Simulated Dog Trans−ABySS 0.997 0.997 0.997 0.784 0.791 0.789 0.863 0.88 0.859
Simulated Dog rnaSPAdes 0.994 0.995 0.994 0.808 0.812 0.807 0.924 0.929 0.924
Simulated Yeast Trinity 0.997 0.997 0.997 0.844 0.846 0.845 1 1 1
Simulated Yeast Trans−ABySS 0.997 0.997 0.997 0.824 0.834 0.825 1 1 1
Simulated Yeast rnaSPAdes 0.997 0.997 0.997 0.813 0.818 0.814 0.409 0.455 0.409
Full−length Kallisto
Full−length RSEM
Full−length Salmon
Incompleteness Kallisto
Incompleteness RSEM
Incompleteness Salmon
Over−extension Kallisto
Over−extension RSEM
Over−extension Salmon
Experimental
Pearson's R
Experimental Mouse Trinity 0.992 0.991 0.992 0.929 0.943 0.928 0.927 0.954 0.928
Experimental Mouse Trans−ABySS 0.997 0.997 0.997 0.925 0.926 0.925 0.974 0.972 0.975
Experimental Mouse rnaSPAdes 0.989 0.989 0.988 0.923 0.927 0.92 0.84 0.899 0.934
Experimental Dog Trinity 0.996 0.995 0.996 0.883 0.889 0.884 0.94 0.939 0.94
Experimental Dog Trans−ABySS 0.993 0.993 0.993 0.866 0.873 0.866 0.934 0.933 0.934
Experimental Dog rnaSPAdes 0.986 0.986 0.986 0.889 0.893 0.889 0.928 0.927 0.928
Experimental Yeast Trinity 0.995 0.995 0.995 0.935 0.944 0.935 0.93 0.933 0.929
Experimental Yeast Trans−ABySS 0.971 0.993 0.971 0.871 0.899 0.871 0.915 0.914 0.915
Experimental Yeast rnaSPAdes 0.999 0.999 0.999 0.758 0.775 0.756 0.927 0.93 0.927
Full−length Kallisto
Full−length RSEM
Full−length Salmon
Incompleteness Kallisto
Incompleteness RSEM
Incompleteness Salmon
Over−extension Kallisto
Over−extension RSEM
Over−extension Salmon
Experimental
Spearman's R
Experimental Mouse Trinity 0.994 0.995 0.994 0.887 0.914 0.884 0.924 0.933 0.925
Experimental Mouse Trans−ABySS 0.996 0.996 0.996 0.844 0.869 0.839 0.957 0.96 0.959
Experimental Mouse rnaSPAdes 0.987 0.987 0.986 0.9 0.915 0.895 0.833 0.88 0.905
Experimental Dog Trinity 0.993 0.993 0.993 0.828 0.842 0.828 0.942 0.941 0.942
Experimental Dog Trans−ABySS 0.988 0.988 0.988 0.788 0.807 0.789 0.951 0.949 0.951
Experimental Dog rnaSPAdes 0.982 0.981 0.982 0.873 0.878 0.874 0.934 0.932 0.934
Experimental Yeast Trinity 0.993 0.993 0.993 0.827 0.858 0.827 0.913 0.914 0.912
Experimental Yeast Trans−ABySS 0.992 0.991 0.992 0.727 0.786 0.728 0.906 0.902 0.905
Experimental Yeast rnaSPAdes 0.999 0.999 0.999 0.707 0.729 0.705 0.918 0.92 0.918
Full−length Kallisto
Full−length RSEM
Full−length Salmon
Incompleteness Kallisto
Incompleteness RSEM
Incompleteness Salmon
Over−extension Kallisto
Over−extension RSEM
Over−extension Salmon
Correlation Coefficient
0.4 0.5 0.6 0.7 0.8 0.9 1.0


Best Alignment Score
Pearsons'r: 0.88, Spearman's r: 0.93
Highest Expression
Pearsons'r: 0.99, Spearman's r: 0.989
Others
Pearsons'r: 0.195, Spearman's r: 0.114
log2(Estimated TPM + 1)
log2(Ground Truth TPM + 1)
0
5
10
15
Relative Error (%)
−100
−50
0
50
100


Best Alignment Score
Highest Estimation
Total Expression
Relative Error (%)
100
50
0
−50
−100
0.25
0.4
0.45
0.5
0.55
0.6
0.65
0.7
0.75
0.8
0.85
0.9
0.95
Proportion of Maximum Estimated Abundance


ATAC
Pearsons'r: 0.881, Spearman's r: 0.928
ATHC
Pearsons'r: 0.898, Spearman's r: 0.925
HTAC
Pearsons'r: 0.892, Spearman's r: 0.934
HTHC
Pearsons'r: 0.943, Spearman's r: 0.964
log2(Estimated TPM + 1)
log2(Ground Truth TPM + 1)
0
5
10
15
Relative Error (%)
−100
−50
0
50
100