

# Explicit Scale Simulation for analysis of RNA-sequencing count data with ALDEX2

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## Introduction

- 1 High-throughput sequencing (HTS) is a ubiquitous tool used to explore many biological phenomenon such  
2 as gene expression (single-cell sequencing, RNA-sequencing, meta-transcriptomics), microbial community  
3 composition (16S rRNA gene sequencing, shotgun metagenomics) and differential enzyme activity (selex,  
4 CRISPR killing). HTS proceeds by taking a sample from the environment, making a library, multiplexing  
5 (merging) multiple libraries together, and then applying a sample of the multiplexed library to a flow cell. Each  
6 of these steps is a compositional sampling step as only a fixed-size subsample of nucleic acid is carried over to  
7 subsequent steps. Thus, with each sampling step the connection between the actual number of molecules in  
8 the sampled DNA pool and the environmental scale (e.g., total number of molecules, microbial load, or total  
9 gene expression) of the measured biological system is degraded or lost. In the end, the information contained  
10 in the data relates only to relative abundances and has an arbitrary scale imposed by the sequencing process  
11 (1–3).
- 12 The analysis of HTS data suffers from several known problems that can be traced, in whole or in part, to  
13 misspecification of scale in the output data. The first issue is poor control of the false discovery rate (FDR)  
14 (4–8), exhibited as dataset-dependent FDR control which is observed as a disconnect between statistical and  
15 biological significance (8, 9). In current practice, this issue is addressed by a dual-filtering method, whereby  
16 both a low p-value (or equivalently a low q-value following FDR correction (10)) and a large difference between  
17 groups is used to identify transcripts or genes of interest for follow-up analysis (9, 11). This double-filtering  
18 approach is graphically exemplified by the volcano plot (11), but is known to not appropriately control  
19 the FDR (12, 13). Since there is no standard way of determining what fold-change cutoff should be used  
20 researchers have unlimited degrees of freedom which is known to lead to unreliable inference (14). In particular  
21 Li et al. (8) used patient or clinical-derived transcriptome datasets and found that many methods suffer from  
22 an extremely high false positive rate. The second issue is poor performance when analyzing data where the  
23 mean change between groups is non-zero (3). Such asymmetric data can arise when a gene set is expressed in  
24 one group but not the other, or when one group contains different gene content from the other. This type of  
25 data frequently arises in in-vitro selection experiments (SELEX), transcriptome analysis, and microbiome  
26 analysis (15). The third issue is that the actual scale of the environment is often a major confounding variable  
27 during analysis (3, 16). This has long been known in transcriptome analysis and was a major driver for  
28 the development of normalization factors (17) and the use of molecular spike-ins to provide a reference set  
29 of known counts (18–21) to estimate scale. While often useful, spike-in methods only provide information  
30 downstream of the step in the sample preparation protocol where the intervention was made and introduce  
31 an additional source of variation that must be accounted for (19). In the microbiome field, a recent landmark

32 paper showed that biological scale was a major unacknowledged confounder in many human analyses (16).  
33 These authors built a machine learning model to uncover the biological variation in scale and including this  
34 information was useful despite exhibiting only a modest correlation (estimated to be 0.6) with the scale of  
35 data external to the training set (16). The final issue is that all the above problems become more pronounced  
36 as more samples are collected; that is, more information results in optimizing for a precise but inaccurate  
37 analysis (3, 8, 22).

38 The four problems were recently shown by Nixon et al. (3) to be a result of a mismatch between the underlying  
39 size or scale of the system and the assumptions of the normalizations used for the analysis of HTS. Biological  
40 variation in scale often represents an important unmeasured confounder in HTS analyses (15, 16, 19, 23). For  
41 example, cells transformed by the cMyc oncogene have about 3 times the amount of mRNA and about twice  
42 the rRNA content than non-transformed cells (24), and this dramatically skews transcriptome analysis unless  
43 spike-in approaches are used (19). In addition, wild-type and mutant strains of cell lines, yeast or bacteria  
44 have different growth rates and RNA contents under different conditions, which affect our ability to identify  
45 truly differentially abundant genes (25–27). As another example, the total bacterial load of the vaginal  
46 microbiome differs by 1–2 orders of magnitude in absolute abundance between the healthy and bacterial  
47 vaginosis states (28), and the cell and RNA composition between these states is dramatically different (29,  
48 30). Thus, a full description of any of these systems includes both relative change (composition) and absolute  
49 abundance (scale). Current methods access only the compositional information yet make implicit assumptions  
50 about the scale (22).

51 Nixon et al. (3) showed that the challenge of non-biological variation in sequencing depth could be explained  
52 as a problem of partially-identified models. They showed that *all* normalizations make some assumption about  
53 scale but these implicit assumptions are often inappropriate and difficult to interpret. As a result, different  
54 normalizations provide different outputs when applied to the same dataset (4, 6, 17, 31, 32). Intuitively,  
55 normalizations in widespread use assume that either all samples have the same scale, e.g. proportions,  
56 rarefaction (33), RPKM (34, 35), etc; or that a subset of features in one sample can be chosen as a reference  
57 to which the others are scaled e.g. the TMM (36), the LVHA (15) qPCR (37), the additive log-ratio (38);  
58 or that different sub-parts of each sample maintain a constant scale across samples e.g. the RLE (39); or  
59 that the geometric mean of the parts is appropriate e.g. the CLR (40) and its derivatives. Notably when the  
60 assumption of similar scale across samples is not violated, or violated only weakly, any method will provide a  
61 reasonable analysis. The problem arises when this assumption is violated, in which case all tools fail without  
62 warning.

63 The original scale-naive ALDEx2 (41) model unwittingly made a strict assumption about scale through  
64 the CLR normalization and we found that the CLR was very sensitive to violations of the assumption  
65 of scale identity between groups (15). Moreover, when the assumption of identity was not true the CLR  
66 used by ALDEx2 could be outperformed by other normalizations in simulation studies (42). As illustrated  
67 graphically in Figure 1, Nixon et al. (3) showed through simulation that introducing uncertainty in the scale  
68 assumptions, and in extreme cases altering the location of the scale assumption, resulted in more reproducible  
69 data analysis including better control of both false positive and false negative results. We modified ALDEx2  
70 to explicitly model uncertainty in scale over a range of reasonable normalization parameters, and showed  
71 significant improvements in performance in microbiome and in-vitro selection experiments (22) and in a  
72 vaginal metatranscriptome analysis (43). Here, we briefly review these modifications and show how adding  
73 scale uncertainty can greatly improve modeling in transcriptome and meta-transcriptome datasets to provide  
74 substantially more robust and reproducible results.

## 75 Methods

76 Formal and expanded descriptions of the concepts that follow are given in (3, 22). To be concrete, we let  $\mathbf{Y}$   
77 denote the *measured*  $D \times N$  matrix of sequence counts with elements  $\mathbf{Y}_{dn}$  indicating the number of measured  
78 DNA molecules mapping to feature  $d$  (e.g., a taxon, transcript or gene) in sample  $n$ . Likewise, we can denote  
79  $\mathbf{W}_{dn}$  as the *true* amount of class  $d$  in the biological system from which sample  $n$  was obtained. We can think  
80 of  $\mathbf{W}$  as consisting of two parts, the scale  $\mathbf{W}^{Tot}$  (e.g., totals) and the composition  $\mathbf{W}^{Comp}$  (i.e., proportions).  
81 That is,  $\mathbf{W}^{Tot}$  is a  $N$ -vector with elements  $\mathbf{W}_n^{Tot} = \sum_d \mathbf{W}_{dn}$  while  $\mathbf{W}^{Comp}$  is a  $D \times N$  matrix with elements

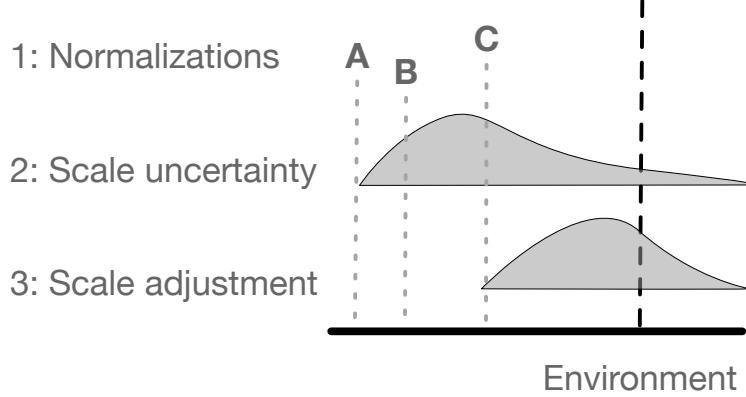


Figure 1: Mismatches between estimated scale and true scale lead to poor estimation of high throughput sequencing data. All normalizations used for differential abundance analysis make some strict assumption about the scale of the environment as shown in line 1. In this example all normalizations produce a biased estimate of the environmental scale, but estimate C is the closest to the truth. Adding uncertainty to normalization C as represented by the distribution in the line 2 leads to less bias and now includes the actual environmental scale in the assumption. As shown in line 3 in some cases it may be useful to adjust the centre of the scale uncertainty estimate if the initial normalizations give very poor estimates of the underlying environment.

<sup>82</sup>  $\mathbf{W}_{dn}^{Comp} = \mathbf{W}_{dn}/\mathbf{W}_n^{Tot}$ . Note that with these definitions  $\mathbf{W}$  can be written as the element-wise combination  
<sup>83</sup> of scale and composition:  $\mathbf{W}_{dn} = \mathbf{W}_{dn}^{Comp}\mathbf{W}_n^{Tot}$ , or as the logarithm  $\log \mathbf{W}_{dn} = \log \mathbf{W}_{dn}^{Comp} + \log \mathbf{W}_n^{Tot}$ .  
<sup>84</sup> Many of the normalizations used in tools such as DESeq2 (44), edgeR (36), metagenomeSeq (45) ALDEx2  
<sup>85</sup> (46) can be stated as ratios of the form  $\hat{\mathbf{W}}_{dn} \approx \mathbf{Y}_{dn}/f(\mathbf{Y})$ , where the denominator is determined by some  
<sup>86</sup> function of the observation. We use the hat notation (^) to indicate that the output is an estimate of the true  
<sup>87</sup> value. The technical variation in sequencing depth, which is often called “library size” has no relationship  
<sup>88</sup> with the actual number of molecules in the sampled environment (1). In other words ( $\mathbf{Y}_n^{Tot} = \sum_d \mathbf{Y}_{dn}$ )  
<sup>89</sup> the observed data  $\mathbf{Y}$  provides us with information about the system composition  $\mathbf{W}^{Comp}$  but little to no  
<sup>90</sup> information in the system scale  $\mathbf{W}^{Tot}$  (Lovell et al. 2011).

## <sup>91</sup> Adding Scale Uncertainty in ALDEx2

<sup>92</sup> The ALDEx2 R package (41, 46) is a general purpose toolbox to model the uncertainty of HTS data and  
<sup>93</sup> to use that model to estimate the significance of the underlying LFC (log-fold change). At a high-level,  
<sup>94</sup> ALDEx2 has three connected components to estimate the uncertainty inherent in HTS datasets. First, the  
<sup>95</sup> tool accounts for the uncertainty of the sequencing counts using Dirichlet multinomial sampling to build  
<sup>96</sup> a probabilistic model of the data; i.e.,  $\hat{\mathbf{W}}^{Comp} \approx \text{Dir}(\mathbf{Y})$ . Secondly, ALDEx2 uses the centred log-ratio  
<sup>97</sup> transformation to scale the data (41). It was this step that was modified to account for scale uncertainty and  
<sup>98</sup> misspecification (22) explained with more details in (3, 22) and summarized in the next paragraph. Finally, a  
<sup>99</sup> standard null-hypothesis test and a non-parametric estimate of mean standardized difference are used to  
<sup>100</sup> report on the finite sample variation. These sources of uncertainty and variation are combined via reporting  
<sup>101</sup> the expected values from a Monte-Carlo simulation framework. For simplicity, we use the term ‘difference’ to  
<sup>102</sup> refer to the absolute difference between groups, and ‘dispersion’ to refer to the within-condition difference or  
<sup>103</sup> pooled variance as defined in (41). These are calculated on a  $\log_2$  scale. For more details on ALDEx2 see (3,  
<sup>104</sup> 22, 41, 46).

<sup>105</sup> Scale models were incorporated into ALDEx2, turning the ALDEx2 model into a specialized type of statistical  
<sup>106</sup> model which Nixon et al. (3) term a *Scale Simulation Random Variable* (SSRV). To do this, Nixon et al.  
<sup>107</sup> (3) generalized the concept of normalizations by introducing the concept of a *scale model* to account for  
<sup>108</sup> potential error in the centred log-ratio normalization step. They did this by including a model for  $\hat{\mathbf{W}}_n^{Tot}$ .  
<sup>109</sup> The CLR normalization used by ALDEx2 makes the assumption  $\hat{\mathbf{W}}_n^{Tot} = 1/G_n$ , where  $G_n$  is the geometric

mean of the counts (or the corresponding proportions) of each part in sample  $n$ , which while being a random variable, is essentially constant across each Monte-Carlo replicate, but that differs between samples. With this modification, ALDEx2 can be generalized by considering probability models for the scale  $\hat{\mathbf{W}}_n^{Tot}$  that have mean  $1/G_n$ . For example, the following scale model generalizes the CLR:

$$\log \hat{\mathbf{W}}_n^{Tot} = -\log G_n + \Lambda x_n \quad \Lambda \sim N(\mu, \gamma^2).$$

This formulation is quite flexible (3, 22). In the simple or ‘default’ configuration,  $\mu = 0$  and  $\gamma$  is a tunable parameter drawn from a log-Normal distribution (3). Adding scale uncertainty with the  $\gamma$  parameter (as shown in Figure 1:1) controls only the degree of uncertainty of the CLR assumption for the  $x_n$  binary condition indicator (e.g.,  $x_n = 1$  denotes case and  $x_n = 0$  denotes control). In the advanced or ‘informed’ configuration,  $\mu$  takes different values for each group and controls the location of the LFC assumption (as shown in Figure 1:2); combining  $\mu$  with a  $\gamma$  estimate allows for uncertainty in both the location and the scale. In the manuscript where the idea of scale was originally derived, Nixon et al. (3) conducted extensive simulations showing that both the default and informed approaches exhibit increased sensitivity and specificity (22). In this report we show that these approaches also work well to control the FDR in transcriptome and metatranscriptome datasets. We also provide some additional insights into how this is achieved. These modifications are instantiated in ALDEx2 which is the first software package designed for SSRV-based inference.

## Results

### Adding scale uncertainty replaces the need for dual significance cutoffs.

Gierliński et al. (47) generated a highly replicated yeast transcriptome dataset to compare gene expression between a wild-type strain and a *snf2* gene knockout,  $\Delta snf2$ . This dataset of 86 samples (44 and 42 per group) is an example of technical growth replicate experiments common in the literature. The dataset was used to test several RNA-seq tools for their power to detect the set of differentially abundant transcripts identified in the full dataset when the data was subset (9). In this original study each tool had its own ‘gold standard’ set of transcripts with different tools identifying between 65% to >80% of all transcripts as being significantly different. Since the majority of transcripts were significantly different, the authors suggested that it was more appropriate to apply a dual cutoff composed of both a Benjamini-Hochberg (48) corrected p-value (q-value) plus a difference cutoff to limit the number of identified transcripts to a much smaller fraction of the total. In other datasets, Nixon et al. (22) showed that adding even a small amount of scale uncertainty with ALDEx2 dramatically reduced the number of significant transcripts identified, removing the need for the dual cutoff approach in this dataset and others.

We start with the assumption that not all statistically significant differences are biologically relevant (49), and that a result where the majority of transcripts are significant breaks the necessary assumption for DA/DE expression that most parts be invariant (31). Transcriptomic analysis commonly uses a dual cutoff approach graphically exemplified by volcano plots (9, 11). Using either DESeq2 or ALDEx2, a majority of transcripts are statistically significantly different between groups with a q-value cutoff of  $\leq 0.05$ ; i.e. 4636 (79%, DESeq2) or 4172 (71%, ALDEx2) of the 5891 transcripts. These values are in line with those observed by (9). Such large numbers of statistically significant transcripts seems biologically unrealistic. That 118 transcripts are identified by ALDEx2 and not DESeq2, while DESeq2 identifies 582 transcripts that ALDEx2 does not, suggests that the choice of normalization plays a role in which results are returned as significant and that some, if not the majority, are driven by technical differences in the analysis (8, 31) or are false positives.

The Volcano plots in Figure 2 A and B show that adding scale uncertainty increases the minimum q-value and increases the concordance between the q-value and the difference between groups (compare panels A and B). The effect plots (50) in Figure 2C shows that the majority of significant transcripts (red, orange) have negligible differences between groups and very low dispersion. We suggest that this low dispersion is driven by the experimental design which is actually a technical wet lab replication rather than a true biological replication design (47). Scale uncertainty can be incorporated using the `gamma` parameter that controls the

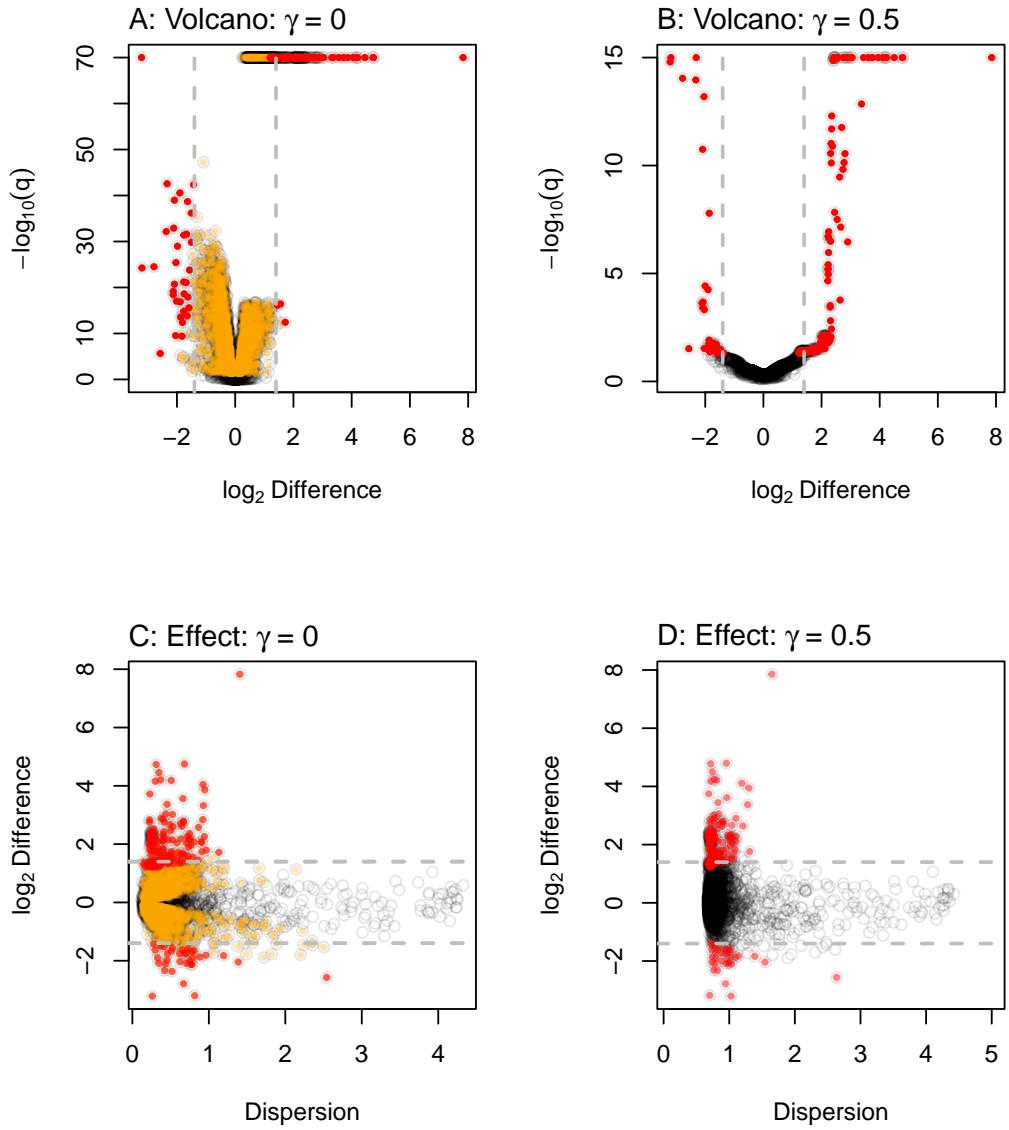


Figure 2: Volcano and effect plots for unscaled and scaled transcriptome analysis. ALDEX2 was used to conduct a differential expression (DE) analysis on the yeast transcriptome dataset. The results were plotted to show the relationship between difference and dispersion using effect plots or difference and the q-values using volcano plots. Panels A,C are for the naive analyses, and Panels B,D are for the default analyses that include scale uncertainty. Each point represents the values for one transcript, with the color indicating if that transcript was significant in the both analyses (red) or in the naive analysis only (orange). Points in grey are not statistically significantly different under any condition. The horizontal dashed lines represent a  $\log_2$  difference of  $\pm 1.4$ .

amount of noise added to the CLR mean assumption when we call either `aldex()`, or `aldex.clr()`. Figure 2 B,D shows that setting  $\gamma = 0.5$  now results in 205 which is far fewer statistically significant transcripts than in the naive analysis and we observe that the minimum dispersion increases from 0.12 ( $\gamma = 0$ ) to 0.67 ( $\gamma = 0.5$ ).

It is common practice to use a dual-cutoff by choosing transcripts based on thresholds for both q-values and fold-changes (9), and these were first proposed for microarray experiments through volcano plots (11). Note that there is considerable variation in recommended cutoff values (9), and that this controversy has persisted ever since fold-change was suggested (51). Unfortunately, universal cutoff fold-change values cannot be identified in part because different tools have intrinsically different variance in their log<sub>2</sub>-fold change ranges (52). This has led to the widespread practice of applying a post-hoc fold-change cutoff to reduce the number of positive identifications to a manageable proportion of the whole dataset. Here, we applied the dual-cutoff method using a fold-change of at least a  $2^{1.4}$  fold change that reduces the number of significant outputs to 193 for DESeq2 and to 186 for ALDEx2. This cutoff was chosen for convenience and is mid-way between the high and low fold-change recommendations of (9). These limits are shown by the dashed grey lines in Figure 2 and we can see that a  $2^{1.4}$  fold change (~ 2.6 fold) cutoff identifies a similar number of transcripts as does ALDEx2 using  $\gamma = 0.5$  which identifies 205 transcripts.

Supplementary Figures 1 shows an example of the `aldex.senAnalysis()` function to identify those transcripts that are very sensitive to scale uncertainty in this dataset. Here we see that adding a very small amount of scale  $\gamma = 0.1$  reduces the number of significant transcripts by more than half in the yeast dataset. This allows the analyst to ignore those low-dispersion transcripts that were significant only because of an absence of scale uncertainty.

We next examined how adding scale would alter the analysis in a real dataset to which synthetically generated true positive counts had been added. We show results from the anti-PD-1 therapy RNA-seq dataset (53) which examined changes in gene expression when cells were exposed or not to a cell-cycle checkpoint inhibitor. This dataset was used by Li et al. (8) as an example of the dangers of relying on tools with high false positive error rates when analyzing clinical or clinically-related transcriptome samples. Indeed, in the benchmarking analysis done by this group, they found that parameter based methods such as DESeq2 and edgeR that on reported p-value and fold-change cutoffs often led to conclusions in the original dataset that were indistinguishable from permutations of the dataset. In other words, that the analysis of transcriptome datasets from patient-derived samples often exhibited many false-positive identifications.

Figure 3 shows the results of ten permutations of this dataset while simulating 5% of the transcripts to be true positives where the difference from no change was derived from a Normal distribution (54). This simulation was conducted with the `seqgendiff` R package (54) to both permute the dataset and to add true positive features. We did ten permutations and kept track of the number of true and false positive identifications. Figure 3A shows the actual false discovery rate for ALDEx2 with and without the addition of scale uncertainty and for DESeq2 with or without a fold-change cutoff of  $2^{0.5}$ . When the modeled difference was greater than 0, ALDEx2 exhibited a FDR of  $\gamma = 0$ : 0.002,  $\gamma = 0.2$ : 0, and  $\gamma = 0.5$ : 0, while DESeq2 with no fold-change cutoff had an FDR of 0.32 and with a 0.5 fold-change cutoff an FDR of 0.31. Figure 3 also plots these results as a function of the minimum modelled fold change of the true positive transcripts. First, we can see that this analysis recapitulates the observations of Li et al (8) in that DESeq2 has very poor false positive control at a nominal FDR of 0.05. The FDR is not controlled any better when a fold-change cutoff is applied, and this agrees with previous work showing that fold-change cutoffs do not materially improve FDR control in high throughput datasets (12, 13). Figure 3B shows that DESeq2 has higher sensitivity than does ALDEx2, and not surprisingly this sensitivity increases as the difference between groups increases. For the case where the mean difference is 0 or greater, ALDEx2 exhibited a sensitivity of  $\gamma = 0$ : 0.66,  $\gamma = 0.2$ : 0.54, and  $\gamma = 0.5$ : 0.35, while DESeq2 with no fold-change cutoff had a sensitivity of 0.76 and with a 0.5 fold-change cutoff a sensitivity of 0.73. In this example, scale-naive ALDEx2 has near perfect FDR control and reasonable sensitivity at low modeled difference between groups. However, when the modeled difference between groups becomes large, then the scale-naive version of ALDEx2 begins to exhibit unacceptable rates of false positives and the false positive rate for DESeq2 also increases. Adding in even small amounts of scale uncertainty drops the true FDR rate to 0, but at the expense of sensitivity. The major contributor to the increase in FDR with larger modeled differences is that the tools are identifying as positives those transcripts

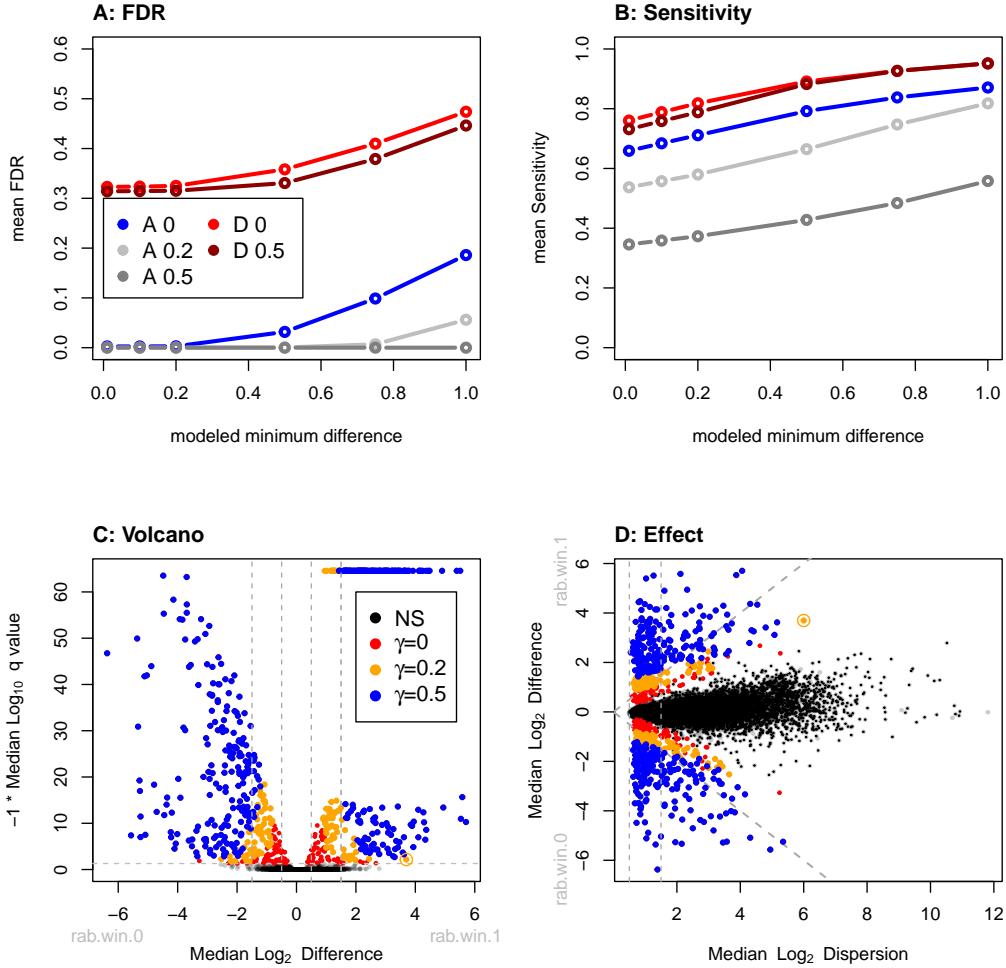


Figure 3: Results of modeling true positive (TP) differences in the PD-1 dataset. For this the data were shuffled and 5% of the transcripts were modeled to have TP differences between groups where the differences were drawn from a Normal distribution with a mean difference of 0 and a sd of 2. Panel A shows the mean FDR of 10 instances for scale-naive ALDEx2 (A 0), and ALDEx2 with  $\gamma = 0.2$  (A 0.2) or  $\gamma = 0.5$  (A 0.5), and where significant features were identified by DESeq2 without (D 0), or with (D 0.5) a 0.5-fold change difference. The x-axis shows how the FDR changes for each tool as a function of the estimated modeled difference between groups. Panel B shows the mean sensitivity (TP found / all TP). Panels C and D show volcano and effect plots for the ALDEx2 output with the transcripts identified as significant at each scale setting as colored points, and non-significant transcripts in black.

208 that are modeled to have differences just below the threshold. We need to recognize that there is no such  
209 thing as a statistical free lunch; the analyst can have high sensitivity but low confidence that any individual  
210 transcript is truly different, or have lower sensitivity but have very high confidence that the difference is real.  
211 In other words, the sensitivity of a method is directly tied to how much error the investigator is willing to  
212 tolerate.

213 Examination of the volcano plot (11) and effect plot (50) in Figure 3C and D provides some insight into  
214 why adding scale uncertainty provides better FDR control than does the approach of using a p-value and a  
215 fold-change cutoff. In the volcano plot, adding scale uncertainty differentially excludes transcripts with a  
216 combination of marginal p-values and low difference between groups, and that this becomes more pronounced  
217 with a larger scale value. This effect is also seen in Figure 2 but is more nuanced. In contrast, the fold-change  
218 cutoff does not incorporate the magnitude of the p-value and so transcripts with large differences, but marginal  
219 p-values are retained. The effect plot in Figure 3D shows that the transcripts with marginal p values and  
220 large differences that are excluded when scale uncertainty is added are those that have a large dispersion.

221 As a concrete example consider the point that is circled in panels C and D with a marginal p-value, with  
222 a difference between of nearly 4 and a dispersion of greater than 6. This transcript is no longer significant  
223 when  $\gamma = 0.2$ , but would require a very large fold-change cutoff to be excluded by the standard approach.  
224 In addition, transcripts with very small dispersion and very small differences are also excluded when scale  
225 uncertainty is added. Thus, the addition of scale uncertainty achieves the desired outcome of lowering  
226 the FDR for those transcripts that are either marginally differentially abundant, or where the underlying  
227 dispersion—and hence the uncertainty in measurement—is very high, or for transcripts that fit both criteria.

228 Supplementary Figure 2 shows a second permuted dataset with the addition of modeled differences between  
229 groups. Here we used another real dataset with over 200 biological replicates of BRCA1 tumor and control  
230 tissue samples from Li et al. (8). This Supplementary Figure shows that the FDR control of DESeq2 is  
231 somewhat better than in the PD-1 dataset, although still much greater than the the anticipated 5%. Further,  
232 a fold-change cutoff reduces the FDR of DESeq2 from about 30% to just over 20% with a power of over  
233 80%. However, we can see that scale-naive ALDEx2 performs substantially better with a negligible FDR and  
234 comparable power. Adding scale uncertainty again improves FDR even for those transcripts modeled to have  
235 larger differences and the power is substantially better than in the PD-1 dataset, reaching the same power as  
236 DESeq2 or scale-naive ALDEx2 when the modeled difference is large. As before, this is driven by removing  
237 from consideration those transcripts with either a small difference between or a marginal p-value, or both.

238 Supplementary Figures 3 shows that effect of applying  $\gamma = 0.5$  to this dataset results in reducing the number  
239 of positive transcripts from being  $\sim 70\%$  of the whole dataset to less than 10% of the dataset and that this is  
240 largely because of a reduction in significance of those transcripts with low dispersion. Supplementary Figures  
241 4 shows a sensitivity analysis of the BRCA1 dataset showing that different scale uncertainty amounts alter  
242 the number of significant transcripts in a biological replicate experiment similarly to a technical replicate  
243 experiment. Supplementary Figures 5 and 6 delve into how adding scale uncertainty affects the variace-  
244 abundance relationship in subtle ways, and may help readers to understand the observations seen in Figure 3  
245 and supplementary Figure 2.

246 Together the results in this section show that adding scale uncertainty has the desirable effect of altering the  
247 transcripts identified as significantly different between groups in a way that exhibits better control of FDR  
248 albeit with a corresponding reduction in sensitivity. Those parts that were statistically significantly different  
249 only because of low dispersion or that had marginal p-values or both, are now preferentially excluded from  
250 statistical significance. In practice, we suggest that a `gamma` parameter of between 0.2 and 0.5 is realistic for  
251 most experimental designs (22) regardless if the replication is technical or biological.

## 252 Housekeeping genes and functions to guide scale model choices.

253 Dos Santos et al. (55) used a vaginal metatranscriptome dataset to compare the gene expression in bacteria  
254 collected from healthy (H) and bacterial vaginosis (BV) affected women. This dataset is derived from two  
255 publicly available datasets composed of a set of 20 non-pregnant women from London, Ontario Canada  
256 (56) and a subset of 22 non-pregnant women collected from German women who underwent metronidazole  
257 treatment for BV (57). Batch effects for these two groups were removed with ComBat-seq (58) and the two

258 datasets were merged into one, giving a total of 16 H and 26 BV samples. In the Dos Santos paper, all results  
 259 from this initial analysis were replicated in a much larger dataset derived from the MOMS-PI study (59)

260 In this vaginal environment, both the relative abundance of species between groups and the gene expression  
 261 level within a species is different (60). Additionally, prior research suggests that the total number of bacteria  
 262 is about 10 times more in the BV than in the H condition (28). Thus, these are extremely challenging datasets  
 263 in which to determine differential abundance as there are both compositional and scale changes between  
 264 conditions. The usual method to analyze vaginal metratranscriptome data is to do so on an organism-by-  
 265 organism basis (57, 59, 60) because the scale confounding of the environment is less pronounced. One attempt  
 266 at system-wide analysis returned several housekeeping functions as differentially expressed between groups  
 267 (57); a result likely due to a disconnect between the assumptions of the normalization used and the actual  
 268 scale of the environment (15).

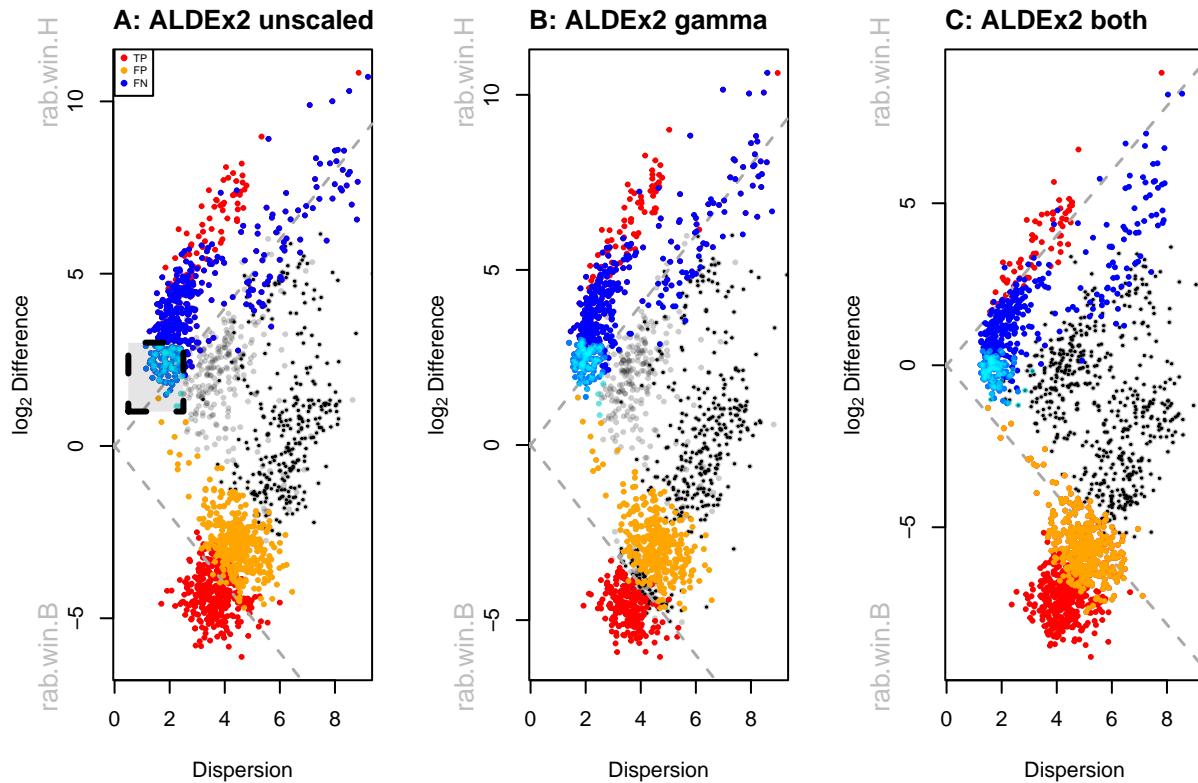


Figure 4: Analysis of vaginal transcriptome data aggregated at the Kegg Orthology (KO) functional level. Panel A shows an effect plot for the default analysis where the functions that are elevated in the healthy individuals have positive values and functions that are elevated in BV have negative values. Highliged in the box are KOs that are almost exclusively housekeeping functions; these and are colored cyan. These housekeeping functions should be located on the midline of no difference. Panel B shows the same data scaled with  $\gamma = 0.5$ , which increase the minimum dispersion as before. Panel C shows the same data scaled with  $\gamma = 0.5$  and a 0.14 fold difference in dispersion applied to the BV samples relative to the H samples. In these plots statistically significant ( $q\text{-value} < 0.01$ ) functions in the informed model are in red, false positive functions are in blue, non-significant functions in black and false negative functions are in orange.

269 In this example, we show how to specify and interpret a user defined or *informed* scale model that can explicitly  
 270 account for some of these modeling difficulties (22) even in a difficult to analyze dataset. An informed scale  
 271 model can control for both the mean difference of scale between groups (e.g., directly incorporate information  
 272 on the differences in total number of bacteria between the BV and H conditions) as well as the uncertainty  
 273 of that difference as illustrated in Figure 1:3. To specify a user-defined scale model, we can pass a matrix  
 274 of scale values instead of an estimate of just the scale uncertainty to `aldex.clr()`. This matrix should

275 have the same number of rows as the of Monte-Carlo Dirichlet samples, and the same number of columns  
276 as the number of samples. While this matrix can be computed from scratch by the analyst, there is an  
277 `aldex.makeScaleModel()` function that can be used to simplify this step in most cases. This encodes the  
278 scale model as  $\Lambda \sim N(\log_2 \mu_n, \gamma^2)$ , where  $\mu_n$  represents the scale value for each sample or group and *gamma*  
279 is the uncertainty as before. The scale estimate can be a measured value (cell count, nucleic acid input,  
280 spike-ins, etc) or an estimate. Nixon et al. (3, 22) showed that only the ratio of the means are important  
281 when providing values for  $\mu_n$ ; i.e., the ratio between the  $\log_2 \mu_i$  and  $\log_2 \mu_j$  values. See the supplement to  
282 Nixon et al. (22) for more information.

283 Figure 4A shows an effect plot of the data where reads are grouped by homologous function regardless of the  
284 organism of origin. Each point represents one of 3728 KEGG functions (61). There are many more functions  
285 represented in the BV group (bottom) than in the healthy group (top). This is because the *Lactobacilli* that  
286 dominate a healthy vaginal microbiome have reduced genome content relative to the anaerobic organisms  
287 that dominate in BV, because there is a greater diversity of organisms in BV than in H samples, and because  
288 the BV condition has about an order of magnitude more bacteria than does the H condition.

289 The naive scale model appears to be reflecting the bacterial load as observed by calculating the mean scale  
290 value for each group. Using a negligible scale value; i.e.,  $\gamma = 1e - 3$  exposes the naive scale estimate for  
291 samples in the `@scaleSamps` slot from the `aldex.clr` output. The naive scale estimate for the healthy group  
292 is 17.41 and for the BV group is 14.59 for a difference of 2.82. This is interpreted as the scale of the H group  
293 of samples being 7.06 greater than the BV group.

294 Applying the default scale model of  $\gamma = 0.5$  increases the dispersion slightly but does not move the  
295 housekeeping functions toward the midline. This is as expected; the mean of the default scale model is based  
296 on the CLR normalization so no shift in location would be expected over the scale-naive ALDEx2 model.  
297 Nevertheless, about 30% of the housekeeping functions are no longer statistically significantly different. Note  
298 that this change is simple to conduct, has no additional computational complexity and requires only a slight  
299 modification for the analyst.

300 There are 101 functions with low dispersion that appear to be shared by both groups (boxed area in Figure  
301 3A, and colored in cyan). Inspection shows that these largely correspond to core metabolic functions such as  
302 transcription, translation, ribosomal functions, glycolysis, replication, chaperones, etc (Supplementary file  
303 `housekeeping.txt`). The transcripts of many of these are commonly used as invariant reference sequences in wet  
304 lab experiments (62) and so are not be expected to contribute to differences in ecosystem behaviour. Because  
305 we expect housekeeping functions to be nearly invariant in their expression and to occur in all organisms, the  
306 average location of these should be centred on 0 difference to represent an internal reference set. However,  
307 with the naive scale model, the mean of these housekeeping functions is approximately located at 2.3. Thus,  
308 we desire a scale model that approximately centres the housekeeping functions and an appropriate scale in  
309 this dataset for functional analysis will place these functions closer to 0 than does the naive estimate. One  
310 way to choose an appropriate value for  $\mu_n$  is to use the `aldex.clr` function on only the presumed invariant  
311 functions setting  $\gamma > 0$ , and then accessing the `@scaleSamps` slot as before. Doing so suggests that the  
312 difference in scale should be about 14%. A second approach would be to identify the functions used as the  
313 denominator with the `denom="lvha"` option (15) for the `aldex.clr` function, and then to use these values as  
314 before. This approach suggests a 5% difference in scale, and is potentially less subject to user interpretation.

315 For the purposes of this example, if we assume a 14% difference in scale, we can set  $\mu_i = 1$  and  $\mu_j = 1.14$  using  
316 the `makeScaleMatrix` function. This function uses a logNormal distribution to build a scale matrix given a  
317 user-specified mean difference between groups and uncertainty level. Applying a per-group relative differential  
318 scale of 0.14 moves the housekeeping functions close to the midline of no difference (Figure 3C, assuming 14%  
319 mean difference = -0.24, assuming a 5% mean difference = -0.34), and applying a gamma of 0.5 provides the  
320 same dispersion as in panel B of Figure 3. Note that now a significant number of functions are differentially up  
321 in BV that were formerly classed as not different without the full scale model (orange), or when only a default  
322 scale was applied. Inspection of the functions shows that these are largely missing from the *Lactobacillus*  
323 species and so should actually be captured as differentially abundant in the BV group. Supplementary Figure  
324 7 shows that the using either the 5% or the 14% scale difference give imperceptibly different results suggesting  
325 that an informed scale model does not have to precisely estimate the scale difference to be useful. Nixon

326 et al, (22) also found that multiple reasonable estimates for the  $\mu_n$  part of the informed scale model were  
327 similarly useful in microbiome data.

328 Thus, applying an informed scale allows us to distinguish between both false positives (housekeeping functions  
329 in cyan, and others in blue) and false negatives (orange functions) even in a very difficult to analyze dataset.  
330 We used this scale model to uncover hiter-to-now unknown differences in microbiome functional activity  
331 between the Healthy and BV cohorts that were missed in previos analyses and that explain important clinical  
332 differences between them (55). The remarkable improvements in biological interpretation afforded by an  
333 informed scale model, and the transferrability of it between sample cohorts of the same condition is outlined in  
334 dos Santos et al. (55). We suggest that the default scale model is sufficient when the data are approximately  
335 centred but that an informed model is more appropriate with datasets are not well centred or when the  
336 investigator has prior information about the underlying biology.

## 337 Discussion

338 Scale estimates affect two parts of the analysis. Modeling uncertainty in scale prevents false certainty in  
339 the precision of estimation and controls false positive identification. Modeling between-group scale relaxes  
340 the assumption of identity between the sizes of the environments and allows better control of false negative  
341 identification. The scale estimates can be derived from the total number of molecules in the environment or  
342 from other estimates of input size (cell counts, initial concentrations, spike-ins, growth rates, etc).

343 Biological systems are both predictably variable and stochastic (63) and systems biology experiments  
344 show that there are transcripts with approximately constant concentrations in the cell and those with large  
345 variability under different growth conditions (25). Current measurement methods that rely on high throughput  
346 sequencing fail to capture all of the variation, particularly variation due to scale (3, 22). In the absence  
347 of external information (19, 20, 64) sequencing depth normalisation methods cannot recapture the scale  
348 information (19, 23), and can only normalize for the technical variation due to sequencing depth. Here we  
349 demonstrated that even approximate estimates of the true system scale and the uncertainty of measuring it  
350 can aid in the interpretation of RNA-sequencing experiments.

351 Nixon et al. (3) introduced the idea of explicitly modeling the scale of a HTS dataset, and showed how to  
352 incorporate these models in the analysis of microbiome and other datasets (22). They demonstrated that  
353 many tools commonly used to analyze HTS datasets had substantial Type 1 and Type 2 error rates in line  
354 with recent findings by others (5, 7, 8). A version of ALDEx2 with the ability to include scale uncertainty was  
355 shown to be able to correct for high Type 1 error rate for that tool, albeit with some loss of sensitivity. Finally,  
356 they showed that incorporating an informed scale model incorporating both location and scale uncertainty  
357 estimates could both control for Type 1 and Type 2 error rates (22, 43).

358 The process of choosing the parameters are experiment-specific and can be anchored in known information  
359 such as cell counts, spike-ins, information from the literature or similar (3, 22, 65). In the metatranscriptome  
360 example used in this report [(43);] the choice of parameter was driven by the assumption inherent in the biology  
361 that core housekeeping functions would serve as an appropriate standard (37). The choice of parameters must  
362 be guided by the experimental question and other approaches in the literature which suggest normalizing the  
363 transcript levels to the bacterial metagenomic levels (66) could be used to set the scale parameters, but in  
364 this case are more granular at the individual organism level rather than at the systemic functional level.

365 Building and using a scale model thus has substantial benefits relative to the dual cutoff approach that is  
366 advocated for many gene expression experiments (9, 11). In particular, the dual cutoff approach has long been  
367 known to not control for Type 1 errors (12, 13), and the frequent lack of concordance between tools when  
368 benchmarked on transcriptomes (5, 7–9, 17, 67) and microbiomes (4, 6, 32, 42, 68, 69) suggests poor control  
369 of Type 2 errors as well (5, 8). Thus, incorporating a scale model during the analysis of HTS data promises  
370 the best of both worlds. A default scale model can control for Type 1 errors with minimal prior knowledge of  
371 the environment and this can be done with essentially no additional computational overhead. Furthermore,  
372 this work and previous (22, 43) show that even minimal information about the underlying environment can  
373 be used to build a relatively robust informed scale model that controls for both Type 1 and 2 error rates. It  
374 is important to note that the approach advocated here is distinct from that suggested by Zhang et al. (66,

70) where the DNA amount for a gene is a covariate in the model for transcriptomic differential abundance.  
375 In our analysis we grouped all the transcript information to functional level regardless of organism, instead  
376 of modeling per-organism gene abundances. In the future we anticipate being able to build more complex  
377 models similar to those used by Zhang et al. with the additional information of uncertainty in the underlying  
378 gene count.

380 In the analysis of HTS data it is often observed that larger datasets converge on the majority of parts  
381 being significantly different (3, 8, 9). Li et al. (8) conducted a permutation-based benchmarking study and  
382 found that widely used tools performed worse than simple Wilcoxon rank-sum tests coupled with the TPM  
383 normalization in controlling the FDR when sample sizes became large. Li et al. suggested that the presence  
384 of outliers were one of the factors driving the extreme FDR in some tests. We found that when the Wilcoxon  
385 test was used within the ALDEx2 framework that it had essentially the same outputs as did the t-test. For  
386 example, in the PD-1 dataset where  $\gamma = 0$  the ALDEx2 t-test exhibited a mean FDR of 0.2% and mean  
387 sensitivity of 65.9% while the corresponding values from the ALDEx2 Wilcoxon test were 0.3% and 68.9%.  
388 This result again supports that the assumptions of the normalizations are as important or more important  
389 than the statistical test. Brooks et al. (71) suggested that inappropriate choice of benchmarking methods  
390 are also a major contributing factor and that better objective standards of truth are needed. In this report  
391 we generated semi-synthetic test data used binomial thinning which produces data that more closely mimic  
392 the properties of real high throughput sequencing data, and so can more rigorously test different tools (54).  
393 From the perspective of our work the disagreement between tools can be explained by the observation that  
394 different analytic approaches produce different parameter estimates for either location or scale, or for both,  
395 as suggested in Figure 1. Thus, more data produces worse estimates because the additional data simply  
396 increases the precision of a flawed estimate (3, 72).

397 Scale simulation is now built into ALDEx2 (22) and in this report we suggest that there are two main root  
398 causes to common HTS data pathologies. The first contributing factor is the observed very low dispersion  
399 estimate for many features that is a by-product of some experimental designs and normalizations (Figure  
400 2). Supplying additional uncertainty alleviates many FP but in a way that more appropriately controls the  
401 FDR as shown in Figure 3. The second contributing factor is unacknowledged asymmetry in many datasets  
402 (15); i.e., different gene content or a directional change in the majority of features. In the case of asymmetry,  
403 the use of a user-specified scale model can be very useful for otherwise difficult-to-analyze datasets such  
404 as meta-transcriptomes and in-vitro selection datasets where the majority of features can change as shown  
405 in Figure 4. We showed two ways of estimating the scale difference between groups and found that any  
406 reasonable estimate is an improvement over the naive approach and also over the default scale model. This  
407 is in line with the observations by Nixon et al (22) in a 16S rRNA gene sequencing dataset. While we  
408 acknowledge that some prior information is needed that this information is widely available and is already  
409 used when performing the gold-standard quantitative PCR test of differential abundance (73, 74).

410 Beyond concerns of fidelity and rigor, scale models also enhance the reproducibility and transparency of HTS  
411 analyses. The development of HTS and the associated problems of very high dimensional data that was not  
412 always statistically well-behaved led to many different proposed solutions including multiple normalizations  
413 and moderated test statistics. That these perform poorly in real data is shown by the simulation data in this  
414 report and elsewhere where both DESeq2 (which we used) and edgeR (which uses moderated statistical tests)  
415 performed poorly in controlling the FDR (8). While these approaches often work in many datasets they fail to  
416 address the underlying problems of information that is missing in the data which is what is supplied by adding  
417 uncertainty around the information we have about that data. The addition of scale uncertainty directly  
418 addresses the missing information by testing the model over a range of normalizations (3). In doing so, the  
419 scale-based approach removes the need for moderated statistics and can replace the consensus approach that  
420 has been proposed by some groups (6, 75) with no additional computational overhead. Thus, an advantage of  
421 incorporating scale is that analyses can be made much more robust such that actual or potential differences  
422 in scale can be tested and accounted for explicitly. While it is beyond the scope of the present article, we  
423 note that there are many ways of building scale models that enhance the interpretability of the parameters  
424 and assumptions and a detailed description of these points is describe elsewhere (3).

425 In summary, we supply a toolkit that makes incorporating scale uncertainty and location information simple  
426 to incorporate for transcriptomes or indeed any type of HTS dataset. While the underlying scale of the

427 system is generally inaccessible, the effect of scale uncertainty on the analysis outcomes can be modelled  
428 and can help explain some of the underlying biology. Adding scale information to the analysis allows for  
429 more robust inference because the features that are sensitive to scale can be identified and their impact on  
430 conclusions weighted accordingly. The use of informed scale models permits difficult to analyze datasets  
431 to be examined in a robust and principled manner even when the majority of features are asymmetrically  
432 distributed or expressed (or both) in the groups (55). Thus, using and reporting scale uncertainty should  
433 become a standard practice in the analysis of HTS datasets.

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