

ABSSeq: a new RNA-Seq analysis method based on modelling absolute expression differences

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May 1, 2024

1 Introduction

This vignette is intended to give a brief introduction of the `ABSSeq` R package by analyzing the simulated data from Soneson et al. [4]. For details about the approach, consult Yang [3]. Currently, `ABSSeq` integrates two modules: basic model for pairwise comparison and linear model for complex design.

RNA-Seq quantifies gene expression with reads count, which usually consists of conditions (or treatments) and several replicates for each condition. The expected expression of each gene is estimated from number of read count, proportional to the expectation value of the true concentration of count. As a result, a normalization method need to be apply on the original counts. The normalized counts usually have enormous variation across genes and compared conditions. The reliable identification of differential expression (DE) genes from such data requires a probabilistic model to account for ambiguity caused by sample size, biological and technical variations, levels of expression and outliers.

`ABSSeq` infers differential expression directly by the counts difference between conditions. It assumes that the sum counts difference between conditions follow a Negative binomial distribution with mean μ (proportional to expression level) and dispersion factor r (size). The μ and r is determined by variation in the experiment, i.e., biological variation, sequencing and mapping biases. Typically, the number of replicates in a study is small and not enough to reveal all variation. To overcome this problem, a common solution is to borrow information across genes. Here, we use local regression to smooth dispersion across genes. The smoothed dispersions are then used to produce pseudocounts in the μ estimation to accounts for dynamic dispersions across expression levels, which in turn moderates the fold-change.

`ABSSeq` tests counts difference directly against a baseline estimated from the data set (μ), and therefore reports p-values related to magnitude of difference (fold-change). In addition, `ABSSeq` moderates the fold-changes by two steps: the expression level and gene-specific dispersion, that might facilitate the gene ranking by fold-change and visualization (Heatmap). New alternative approach (named `aFold`) was introduced, which calls DE genes via log fold-change (see second Section for example). `aFold` uses a polynomial function to model the uncertainty (or variance) of read count, and thus takes into consideration the variance of expression levels across treatments and genes. `aFold` produces accurate estimation of fold changes. In combination with the linear model from

limma [2], aFold is capable to analyze data set with complex experimental design (see last Section for example).

2 Pairwise study

We firstly import the ABSSeq package.

```
> library(ABSSeq)
```

Then, we load a simulated data set. It is a list object and contains three elements: the counts matrix, denoted by 'counts', the groups, denoted by 'groups' and differential expression genes, denoted by 'DEs'.

```
> data(simuN5)
> names(simuN5)
```

```
[1] "counts" "groups" "DEs"
```

simuN5 is simulated from Negative binomial distribution with means and variances from Pickrell's data [5] and added outliers randomly [4]. simuN5 includes group information.

```
> simuN5$groups
```

```
[1] 0 0 0 0 0 1 1 1 1 1
```

An ABSDataSet object is required for ABSSeq and could be constructed with the ABSDataSet function by providing counts matrix and defined groups. Here, we can also initiate a paired comparison for specific samples, such as data for cancer and normal tissue from same individuals, by setting the paired parameter in ABSDataSet object.

```
> obj <- ABSDataSet(simuN5$counts, factor(simuN5$groups))
> pairedobj <- ABSDataSet(simuN5$counts, factor(simuN5$groups), paired=TRUE)
```

The ABSDataSet function also includes the parameter for the normalization method, which has a default as qtotal. qtotal assesses the influence of DE on data structure to normalize the data. Additional choices of normalization methods are also provided, that are, total by total reads count, geometric from DESeq [6], quantile by reads count in the first three quartiles from baySeq [?], TMM from edgeR [1] and user through size factors provided by users. The normalization method can be showed and revised by normMethod.

```
> obj1 <- ABSDataSet(simuN5$counts, factor(simuN5$groups),
+                   normMethod="user", sizeFactor=runif(10,1,2))
> normMethod(obj1)
```

```
[1] "user"
```

```
> normMethod(obj1) <- "geometric"
> normMethod(obj1)
```

```
[1] "geometric"
```

The size factors could be estimated from an `ABSDataSet` object via the function `normalFactors` and retrieved by the function `sFactors`.

```
> obj <- normalFactors(obj)
> sFactors(obj)
```

```
[1] 1.2701723 1.1188592 0.6950348 1.1697439 1.1474554 0.9574766 0.9201882
[8] 1.1359601 0.8054707 0.7796388
```

Reads count after normalization could be retrieved by the function `counts`.

```
> head(counts(obj,norm=TRUE))
```

| | [,1] | [,2] | [,3] | [,4] | [,5] | [,6] |
|---|--------------|--------------|--------------|-------------|-------------|-------------|
| 1 | 58.25981 | 14.300280 | 48.91842 | 0.854888 | 1.742987 | 51.17618 |
| 2 | 1451.77156 | 967.950211 | 1256.05230 | 2067.119167 | 2926.475379 | 6327.04741 |
| 3 | 2662.63092 | 2245.143980 | 2474.69640 | 1502.038204 | 1938.201680 | 3469.53640 |
| 4 | 25.19343 | 9.831443 | 12.94899 | 17.952648 | 18.301365 | 17.75500 |
| 5 | 1491.13630 | 3674.278225 | 3630.03432 | 2285.970493 | 5126.125128 | 12830.60043 |
| 6 | 847.12917 | 832.097550 | 546.73525 | 130.797863 | 1342.100084 | 1438.15521 |
| | [,7] | [,8] | [,9] | [,10] | | |
| 1 | 6.520405 | 0.000000 | 7.449061 | 35.91407 | | |
| 2 | 57776.223615 | 4703.510143 | 12370.407060 | 12392.91781 | | |
| 3 | 3163.483249 | 3104.862488 | 30017.232227 | 43420.10493 | | |
| 4 | 190.178485 | 4.401563 | 24.830203 | 61.56697 | | |
| 5 | 8308.082941 | 18284.092397 | 15852.843010 | 17635.08870 | | |
| 6 | 949.805689 | 1277.333561 | 1443.876296 | 1602.02384 | | |

With the size factors, we can calculate the absolute counts difference between conditions, mean (μ), size factor (r) and moderate \log_2 of fold-change for each gene. It can be done by function `callParameter` as

```
> obj=callParameter(obj)
```

If we want to see correlation between the absolute \log_2 fold-change (with or without moderation) and expression level in same conditions, we can use function `plotDiffToBase`.

```
> obj <- callDEs(obj)
> plotDiffToBase(obj)
```

In the end, we model the counts differences with Negative binomial distribution and calculate the pvalue for each gene. DE calling is performed by the function `callDEs`, which reports pvalues as well as adjusted pvalues, that can be accessed by `results` with names of `pvalue` and `adj.pvalue`. Noticely, this function also provides fold-change moderation according to gene-specific dispersion by utilizing `aFold`, which will report fold-changes closer to gene's dispersion. In the end, `ABSSeq` produces three kinds fold-changes: the original (denoted by 'rawFC'), corrected by expression level (denoted by 'lowFC') and moderated by expression level and gene-specific dispersion (denoted by 'foldChange'), which are stored in the `ABSDataSet` object and could be also retrieved by `results`.

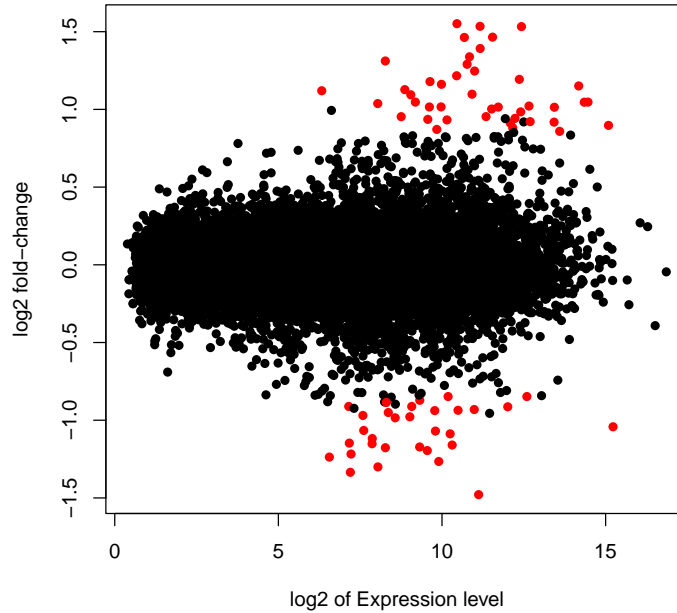


Figure 1: 'Absolute log2 fold-change against expression level'-plot for count data. We show the fitted and raw data with different colors.

```
> obj <- callDEs(obj)
> head(results(obj, c("rawFC", "lowFC", "foldChange", "pvalue", "adj.pvalue")))
```

| | rawFC | lowFC | foldChange | pvalue | adj.pvalue |
|---|------------|------------|-------------|--------------|------------|
| 1 | -0.1824058 | -0.1030012 | -0.04125768 | 7.682173e-01 | 1.00000000 |
| 2 | 2.9174817 | 2.6816216 | 0.36224008 | 4.230884e-02 | 1.00000000 |
| 3 | 2.0016381 | 0.8968301 | 0.50537531 | 2.661240e-02 | 0.86631330 |
| 4 | 0.8868678 | 0.6140338 | 0.21658502 | 1.460386e-01 | 1.00000000 |
| 5 | 2.2377865 | 2.1213283 | 1.02150694 | 4.989514e-05 | 0.01516204 |
| 6 | 1.1772042 | 0.9992554 | 0.43023062 | 6.939515e-02 | 1.00000000 |

The `results` function can be used to access all information in an `ABSDataSet`.

```
> head(results(obj))
```

| | Amean | Bmean | baseMean | absD | Variance | rawFC | lowFC |
|---|-----------|-----------|-------------|-------|--------------|------------|------------|
| 1 | 3.562610 | 3.380204 | 62.16317 | 23 | 1.220177e+03 | -0.1824058 | -0.1030012 |
| 2 | 10.650049 | 13.567531 | 23642.68938 | 84901 | 4.895532e+08 | 2.9174817 | 2.6816216 |
| 3 | 11.052189 | 13.053827 | 5514.05983 | 10509 | 2.181432e+06 | 2.0016381 | 0.8968301 |
| 4 | 4.093067 | 4.979935 | 115.68473 | 215 | 5.799681e+03 | 0.8868678 | 0.6140338 |
| 5 | 11.539225 | 13.777011 | 18473.15191 | 56703 | 1.873455e+07 | 2.2377865 | 2.1213283 |
| 6 | 9.192270 | 10.369474 | 1796.40395 | 3012 | 2.590491e+05 | 1.1772042 | 0.9992554 |

| | foldChange | pvalue | adj.pvalue | trimmed |
|---|-------------|--------------|------------|---------|
| 1 | -0.04125768 | 7.682173e-01 | 1.00000000 | 0 |
| 2 | 0.36224008 | 4.230884e-02 | 1.00000000 | 0 |
| 3 | 0.50537531 | 2.661240e-02 | 0.86631330 | 2 |
| 4 | 0.21658502 | 1.460386e-01 | 1.00000000 | 0 |
| 5 | 1.02150694 | 4.989514e-05 | 0.01516204 | 0 |
| 6 | 0.43023062 | 6.939515e-02 | 1.00000000 | 0 |

In addition to a step-by-step analysis in above, DE calling could be simply performed by the function `ABSSeq`, which runs a default analysis by calling above functions in order and returns a `ABSDataset` object with all information.

```
> data(simuN5)
> obj <- ABSDataSet(simuN5$counts, factor(simuN5$groups))
> obj <- ABSSeq(obj)
> res <- results(obj, c("Amean", "Bmean", "foldChange", "pvalue", "adj.pvalue"))
> head(res)
```

| | Amean | Bmean | foldChange | pvalue | adj.pvalue |
|---|-----------|-----------|-------------|--------------|------------|
| 1 | 3.562610 | 3.380204 | -0.04125768 | 7.682173e-01 | 1.00000000 |
| 2 | 10.650049 | 13.567531 | 0.36224008 | 4.230884e-02 | 1.00000000 |
| 3 | 11.052189 | 13.053827 | 0.50537531 | 2.661240e-02 | 0.86631330 |
| 4 | 4.093067 | 4.979935 | 0.21658502 | 1.460386e-01 | 1.00000000 |
| 5 | 11.539225 | 13.777011 | 1.02150694 | 4.989514e-05 | 0.01516204 |
| 6 | 9.192270 | 10.369474 | 0.43023062 | 6.939515e-02 | 1.00000000 |

Moreover, `ABSSeq` also allows testing on user-defined baseline for counts difference by giving a same value to `minRates` and `maxRates` as

```
> data(simuN5)
> obj <- ABSDataSet(simuN5$counts, factor(simuN5$groups), minRates=0.2, maxRates=0.2)
> #or by slot functions
> #minRates(obj) <- 0.2
> #maxRates(obj) <- 0.2
> obj <- ABSSeq(obj)
> res <- results(obj, c("Amean", "Bmean", "foldChange", "pvalue", "adj.pvalue"))
> head(res)
```

| | Amean | Bmean | foldChange | pvalue | adj.pvalue |
|---|-----------|-----------|-------------|--------------|--------------|
| 1 | 3.562610 | 3.380204 | -0.04125768 | 7.038358e-01 | 1.000000000 |
| 2 | 10.650049 | 13.567531 | 0.36224008 | 2.192792e-02 | 0.4006852443 |
| 3 | 11.052189 | 13.053827 | 0.50537531 | 4.337366e-03 | 0.1594078122 |
| 4 | 4.093067 | 4.979935 | 0.21658502 | 8.852225e-02 | 0.6238114781 |
| 5 | 11.539225 | 13.777011 | 1.02150694 | 7.752227e-07 | 0.0002099674 |
| 6 | 9.192270 | 10.369474 | 0.43023062 | 1.754797e-02 | 0.3604790716 |

`ABSSeq` penalizes the dispersion estimation by adding a common dispersion value to the observed dispersion for each gene, which is obtained by quantile estimation on observed dispersions. This penalized dispersion could be provided by user as

```

> data(simuN5)
> obj <- ABSDataSet(simuN5$counts, factor(simuN5$groups),minDispersion=0.1)
> #or by slot functions
> #minimalDispersion(obj) <- 0.2
> obj <- ABSSeq(obj)
> res <- results(obj,c("Amean","Bmean","foldChange","pvalue","adj.pvalue"))
> head(res)

```

| | Amean | Bmean | foldChange | pvalue | adj.pvalue |
|---|-----------|-----------|-------------|------------|------------|
| 1 | 3.562610 | 3.380204 | -0.04125768 | 0.75314495 | 1.0000000 |
| 2 | 10.650049 | 13.567531 | 0.36224008 | 0.04397104 | 1.0000000 |
| 3 | 11.052189 | 13.053827 | 0.50537531 | 0.04324281 | 1.0000000 |
| 4 | 4.093067 | 4.979935 | 0.21658502 | 0.14923425 | 1.0000000 |
| 5 | 11.539225 | 13.777011 | 1.02150694 | 0.00043097 | 0.1732082 |
| 6 | 9.192270 | 10.369474 | 0.43023062 | 0.09085401 | 1.0000000 |

In addition, ABSSeq provides special parameter estimation for data set without replicates. It firstly treats the two groups as replicates and separates genes into two sets according to fold-change cutoff (depends on expression level). The set with fold-change under cutoff is used to estimate the dispersion for each gene by local regression as well as fold-change moderation. Here is the example, which replaces the callParameter by callParameterwithoutReplicates.

```

> data(simuN5)
> obj <- ABSDataSet(simuN5$counts[,c(1,2)], factor(c(1,2)))
> obj <- ABSSeq(obj)
> res <- results(obj,c("Amean","Bmean","foldChange","pvalue","adj.pvalue"))
> head(res)

```

| | Amean | Bmean | foldChange | pvalue | adj.pvalue |
|---|-----------|-----------|-------------|-------------|------------|
| 1 | 6.141438 | 4.176475 | -1.30085645 | 0.003116490 | 0.01707491 |
| 2 | 10.760856 | 10.176462 | -0.56268049 | 0.153013673 | 0.35063405 |
| 3 | 11.635516 | 11.389556 | -0.23985467 | 0.845037624 | 1.00000000 |
| 4 | 4.958562 | 3.671725 | -0.73879630 | 0.089056224 | 0.23482571 |
| 5 | 10.799432 | 12.100000 | 1.25952601 | 0.001112932 | 0.00740706 |
| 6 | 9.984289 | 9.958485 | -0.02476103 | 0.999999092 | 1.00000000 |

3 Detecting DE via aFold

Recently, ABSSeq integrates a new method for DE detection: aFold. aFold utilizes a polynomial function to model the uncertainty of observed reads count and moderate the fold change calculation. aFold takes into account variations among samples and genes and reports DE and fold changes in a reliable way. The fold changes produced by aFold may help the experimentalist to avoid arbitrary choice of cut-off thresholds and may enhance subsequent downstream functional analyses. Here is the example for how to use aFold in ABSSeq.

```

> data(simuN5)
> obj <- ABSDataSet(counts=simuN5$counts, groups=factor(simuN5$groups))
> obj <- ABSSeq(obj, useaFold=TRUE)

```

```
> res <- results(obj,c("Amean","Bmean","foldChange","pvalue","adj.pvalue"))
> head(res)
```

| | Amean | Bmean | foldChange | pvalue | adj.pvalue |
|---|-----------|-----------|-------------|--------------|--------------|
| 1 | 3.562610 | 3.380204 | -0.04125768 | 7.757687e-01 | 9.410283e-01 |
| 2 | 10.650049 | 13.567531 | 0.36224008 | 1.238933e-02 | 1.665142e-01 |
| 3 | 11.052189 | 13.053827 | 0.50537531 | 4.847530e-04 | 1.531717e-02 |
| 4 | 4.093067 | 4.979935 | 0.21658502 | 1.348430e-01 | 5.603766e-01 |
| 5 | 11.539225 | 13.777011 | 1.02150694 | 1.759256e-12 | 5.345993e-10 |
| 6 | 9.192270 | 10.369474 | 0.43023062 | 2.975533e-03 | 6.158168e-02 |

4 PCA analysis via aFold

aFold model also stabilizes variances across expression levels, which could be used for principal component analysis (PCA). Here is an example. Noticeably, the group information is not necessary for the `ABSDataSet` object under PCA analysis.

```
> data(simuN5)
> obj <- ABSDataSet(counts=simuN5$counts)
> ##as one group
> cond <- as.factor(rep("hex",ncol(simuN5$counts)))
> ##normalization
> cda <- counts(obj,T)
> ##variance stabilization
> sds <- genAFold(cda,cond)
> ##sds is list vector, which contains variance stabilized read counts in 3rd element
> ##or expression level adjusted counts in 4th element. 3rd element is more sensitive
> ##to difference between samples than the 4th one. Here we use the 4th element for a
> ##PCA analysis.
> ## log transformation
> ldat <- log2(sds[[4]])
> ## PCA analysis
> PCA <- prcomp(t(ldat), scale = F)
> ## Percentage of components
> percentVar <- round(100*PCA$sdev^2/sum(PCA$sdev^2),1)
> ## plotting
> pc1=PCA$x[,1]
> pc2=PCA$x[,2]
> #plot(pc1,pc2,main="",pch=16,col="black",xlab="PC1",ylab="PC2",cex=1.2)
```

5 DE analysis with complex design

In combination with linear model from `limma` [2], aFold is capable to analyze data set with complex experimental design, which is performed by the function `ABSSeqlm`. Here is an example.

```
> data(simuN5)
> groups<-factor(simuN5$groups)
```

```

> obj <- ABSDataSet(counts=simuN5$counts)
> design <- model.matrix(~0+groups)
> res <- ABSSeqlm(obj,design,condA=c("groups0"),condB=c("groups1"))
> head(res)

```

| | basemean | logFC | pvalue | p.adj |
|---|-----------|-------------|--------------|--------------|
| 1 | 4.690153 | -0.03870026 | 8.051044e-01 | 9.610751e-01 |
| 2 | 14.191909 | 0.36116377 | 2.129451e-02 | 4.435846e-01 |
| 3 | 14.022025 | 0.35511690 | 2.356381e-02 | 4.705514e-01 |
| 4 | 5.924728 | 0.20662942 | 1.876926e-01 | 8.335799e-01 |
| 5 | 13.832014 | 1.00860220 | 1.270311e-10 | 4.796003e-08 |
| 6 | 10.391500 | 0.41516516 | 8.120320e-03 | 2.443746e-01 |

Noticely, the parameters `condA` and `condB` could contain multiple conditions (factors) to run a comparison between multiple condntions. The function `ABSSeqlm` could be also used for analysis of variance (ANOVA) across conditions. To run ANOVA, all conditions (factors) are imported to the parameter `condA` in the function `ABSSeqlm` (without `condB`).

```

> res <- ABSSeqlm(obj,design,condA=c("groups0","groups1"))
> head(res)

```

| | basemean | logFC | pvalue | p.adj |
|---|-----------|------------|--------------|------------|
| 1 | 4.690153 | 0.02736522 | 8.631600e-01 | 0.99995335 |
| 2 | 14.191909 | 0.25538135 | 1.077354e-01 | 0.99995335 |
| 3 | 14.022025 | 0.25110556 | 1.137579e-01 | 0.99995335 |
| 4 | 5.924728 | 0.14610906 | 3.574518e-01 | 0.99995335 |
| 5 | 13.832014 | 0.71318946 | 7.060844e-06 | 0.00266579 |
| 6 | 10.391500 | 0.29356610 | 6.446461e-02 | 0.99995335 |

The linear model is performed by `lmFit` from `limma` [2], which could be suppressed via the parameter `lmodel` as

```

> res <- ABSSeqlm(obj,design,condA=c("groups0"),condB=c("groups1"),lmodel=FALSE)
> head(res)

```

| | basemean | logFC | pvalue | p.adj |
|---|-----------|-------------|--------------|--------------|
| 1 | 4.690153 | -0.03870026 | 8.051044e-01 | 9.610751e-01 |
| 2 | 14.191909 | 0.36116377 | 2.129451e-02 | 4.435846e-01 |
| 3 | 14.022025 | 0.35511690 | 2.356381e-02 | 4.705514e-01 |
| 4 | 5.924728 | 0.20662942 | 1.876926e-01 | 8.335799e-01 |
| 5 | 13.832014 | 1.00860220 | 1.270311e-10 | 4.796003e-08 |
| 6 | 10.391500 | 0.41516516 | 8.120320e-03 | 2.443746e-01 |

References

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