

# EBSeq: An R package for differential expression analysis using RNA-seq data

Ning Leng, John Dawson, and Christina Kendzierski

October 13, 2014

## Contents

<b>1</b>	<b>Introduction</b>	<b>2</b>
<b>2</b>	<b>Citing this software</b>	<b>2</b>
<b>3</b>	<b>The Model</b>	<b>2</b>
3.1	Two conditions . . . . .	2
3.2	More than two conditions . . . . .	4
3.3	Getting a false discovery rate (FDR) controlled list of genes or isoforms . . . . .	4
<b>4</b>	<b>Quick Start</b>	<b>5</b>
4.1	Gene level DE analysis (two conditions) . . . . .	5
4.1.1	Required input . . . . .	5
4.1.2	Library size factor . . . . .	5
4.1.3	Running EBSeq on gene expression estimates . . . . .	5
4.2	Isoform level DE analysis (two conditions) . . . . .	6
4.2.1	Required inputs . . . . .	6
4.2.2	Library size factor . . . . .	7
4.2.3	The $I_g$ vector . . . . .	7
4.2.4	Running EBSeq on isoform expression estimates . . . . .	7
4.3	Gene level DE analysis (more than two conditions) . . . . .	8
4.4	Isoform level DE analysis (more than two conditions) . . . . .	10
<b>5</b>	<b>More detailed examples</b>	<b>13</b>
5.1	Gene level DE analysis (two conditions) . . . . .	13
5.1.1	Running EBSeq on simulated gene expression estimates . . . . .	13
5.1.2	Calculating FC . . . . .	13
5.1.3	Checking convergence . . . . .	14
5.1.4	Checking the model fit and other diagnostics . . . . .	15
5.2	Isoform level DE analysis (two conditions) . . . . .	17
5.2.1	The $I_g$ vector . . . . .	17
5.2.2	Using mappability ambiguity clusters instead of the $I_g$ vector when the gene-isoform relationship is unknown . . . . .	18
5.2.3	Running EBSeq on simulated isoform expression estimates . . . . .	18
5.2.4	Checking convergence . . . . .	19
5.2.5	Checking the model fit and other diagnostics . . . . .	19
5.3	Gene level DE analysis (more than two conditions) . . . . .	24
5.4	Isoform level DE analysis (more than two conditions) . . . . .	29

5.5	Working without replicates . . . . .	34
5.5.1	Gene counts with two conditions . . . . .	34
5.5.2	Isoform counts with two conditions . . . . .	34
5.5.3	Gene counts with more than two conditions . . . . .	35
5.5.4	Isoform counts with more than two conditions . . . . .	35
<b>6</b>	<b>EBSeq pipelines and extensions</b>	<b>36</b>
6.1	RSEM-EBSeq pipeline: from raw reads to differential expression analysis results . . . . .	36
6.2	EBSeq interface: A user-friendly graphical interface for differential expression analysis . . . . .	36
6.3	EBSeq Galaxy tool shed . . . . .	36
<b>7</b>	<b>Acknowledgment</b>	<b>36</b>
<b>8</b>	<b>News</b>	<b>37</b>

# 1 Introduction

EBSeq may be used to identify differentially expressed (DE) genes and isoforms in an RNA-Seq experiment. As detailed in Leng *et al.*, 2013 [3], EBSeq is an empirical Bayesian approach that models a number of features observed in RNA-seq data. Importantly, for isoform level inference, EBSeq directly accommodates isoform expression estimation uncertainty by modeling the differential variability observed in distinct groups of isoforms. Consider Figure 1, where we have plotted variance against mean for all isoforms using RNA-Seq expression data from Leng *et al.*, 2013 [3]. Also shown is the fit within three sub-groups of isoforms defined by the number of constituent isoforms of the parent gene. An isoform of gene  $g$  is assigned to the  $I_g = k$  group, where  $k = 1, 2, 3$ , if the total number of isoforms from gene  $g$  is  $k$  (the  $I_g = 3$  group contains all isoforms from genes having 3 or more isoforms). As shown in Figure 1, there is decreased variability in the  $I_g = 1$  group, but increased variability in the others, due to the relative increase in uncertainty inherent in estimating isoform expression when multiple isoforms of a given gene are present. If this structure is not accommodated, there is reduced power for identifying isoforms in the  $I_g = 1$  group (since the true variances in that group are lower, on average, than that derived from the full collection of isoforms) as well as increased false discoveries in the  $I_g = 2$  and  $I_g = 3$  groups (since the true variances are higher, on average, than those derived from the full collection). EBSeq directly models differential variability as a function of  $I_g$  providing a powerful approach for isoform level inference. As shown in Leng *et al.*, 2013 [3], the model is also useful for identifying DE genes. We will briefly detail the model in Section 3 and then describe the flow of analysis in Section 4 for both isoform and gene-level inference.

# 2 Citing this software

Please cite the following article when reporting results from the software.

Leng, N., J.A. Dawson, J.A. Thomson, V. Ruotti, A.I. Rissman, B.M.G. Smits, J.D. Haag, M.N. Gould, R.M. Stewart, and C. Kendziorski. EBSeq: An empirical Bayes hierarchical model for inference in RNA-seq experiments, *Bioinformatics*, 2013.

# 3 The Model

## 3.1 Two conditions

We let  $X_{g_i}^{C1} = X_{g_i,1}, X_{g_i,2}, \dots, X_{g_i,S_1}$  denote data from condition 1 and  $X_{g_i}^{C2} = X_{g_i,(S_1+1)}, X_{g_i,(S_1+2)}, \dots, X_{g_i,S}$  data from condition 2. We assume that counts within condition  $C$  are distributed as Negative Binomial:



Figure 1: Empirical variance vs. mean for each isoform profiled in the ESCs vs iPSCs experiment detailed in the Case Study section of Leng *et al.*, 2013 [3]. A spline fit to all isoforms is shown in red with splines fit within the  $I_g = 1$ ,  $I_g = 2$ , and  $I_g = 3$  isoform groups shown in yellow, pink, and green, respectively.

$X_{g_i,s}^C | r_{g_i,s}, q_{g_i}^C \sim NB(r_{g_i,s}, q_{g_i}^C)$  where

$$P(X_{g_i,s} | r_{g_i,s}, q_{g_i}^C) = \binom{X_{g_i,s} + r_{g_i,s} - 1}{X_{g_i,s}} (1 - q_{g_i}^C)^{X_{g_i,s}} (q_{g_i}^C)^{r_{g_i,s}} \quad (1)$$

and  $\mu_{g_i,s}^C = r_{g_i,s}(1 - q_{g_i}^C)/q_{g_i}^C$ ;  $(\sigma_{g_i,s}^C)^2 = r_{g_i,s}(1 - q_{g_i}^C)/(q_{g_i}^C)^2$ .

We assume a prior distribution on  $q_{g_i}^C$ :  $q_{g_i}^C | \alpha, \beta^{I_g} \sim \text{Beta}(\alpha, \beta^{I_g})$ . The hyperparameter  $\alpha$  is shared by all the isoforms and  $\beta^{I_g}$  is  $I_g$  specific (note this is an index, not a power). We further assume that  $r_{g_i,s} = r_{g_i,0} l_s$ , where  $r_{g_i,0}$  is an isoform specific parameter common across conditions and  $r_{g_i,s}$  depends on it through the sample-specific normalization factor  $l_s$ . Of interest in this two group comparison is distinguishing between two cases, or what we will refer to subsequently as two patterns of expression, namely equivalent expression (EE) and differential expression (DE):

$$H_0 \text{ (EE)} : q_{g_i}^{C1} = q_{g_i}^{C2} \text{ vs } H_1 \text{ (DE)} : q_{g_i}^{C1} \neq q_{g_i}^{C2}.$$

Under the null hypothesis (EE), the data  $X_{g_i}^{C1,C2} = X_{g_i}^{C1}, X_{g_i}^{C2}$  arises from the prior predictive distribution  $f_0^{I_g}(X_{g_i}^{C1,C2})$ :

$$f_0^{I_g}(X_{g_i}^{C1,C2}) = \left[ \prod_{s=1}^S \binom{X_{g_i,s} + r_{g_i,s} - 1}{X_{g_i,s}} \right] \frac{\text{Beta}(\alpha + \sum_{s=1}^S r_{g_i,s}, \beta^{I_g} + \sum_{s=1}^S X_{g_i,s})}{\text{Beta}(\alpha, \beta^{I_g})} \quad (2)$$

Alternatively (in a DE scenario),  $X_{g_i}^{C1,C2}$  follows the prior predictive distribution  $f_1^{I_g}(X_{g_i}^{C1,C2})$ :

$$f_1^{I_g}(X_{g_i}^{C1,C2}) = f_0^{I_g}(X_{g_i}^{C1}) f_0^{I_g}(X_{g_i}^{C2}) \quad (3)$$

Let the latent variable  $Z_{g_i}$  be defined so that  $Z_{g_i} = 1$  indicates that isoform  $g_i$  is DE and  $Z_{g_i} = 0$  indicates isoform  $g_i$  is EE, and  $Z_{g_i} \sim \text{Bernoulli}(p)$ . Then, the marginal distribution of  $X_{g_i}^{C1,C2}$  and  $Z_{g_i}$  is:

$$(1-p)f_0^{I_g}(X_{g_i}^{C1,C2}) + pf_1^{I_g}(X_{g_i}^{C1,C2}) \quad (4)$$

The posterior probability of being DE at isoform  $g_i$  is obtained by Bayes' rule:

$$\frac{pf_1^{I_g}(X_{g_i}^{C1,C2})}{(1-p)f_0^{I_g}(X_{g_i}^{C1,C2}) + pf_1^{I_g}(X_{g_i}^{C1,C2})} \quad (5)$$

### 3.2 More than two conditions

EBSeq naturally accommodates multiple condition comparisons. For example, in a study with 3 conditions, there are K=5 possible expression patterns (P1,...,P5), or ways in which latent levels of expression may vary across conditions:

$$\begin{aligned} \text{P1: } q_{g_i}^{C1} &= q_{g_i}^{C2} = q_{g_i}^{C3} \\ \text{P2: } q_{g_i}^{C1} &= q_{g_i}^{C2} \neq q_{g_i}^{C3} \\ \text{P3: } q_{g_i}^{C1} &= q_{g_i}^{C3} \neq q_{g_i}^{C2} \\ \text{P4: } q_{g_i}^{C1} &\neq q_{g_i}^{C2} = q_{g_i}^{C3} \\ \text{P5: } q_{g_i}^{C1} &\neq q_{g_i}^{C2} \neq q_{g_i}^{C3} \text{ and } q_{g_i}^{C1} \neq q_{g_i}^{C3} \end{aligned}$$

The prior predictive distributions for these are given, respectively, by:

$$\begin{aligned} g_1^{I_g}(X_{g_i}^{C1,C2,C3}) &= f_0^{I_g}(X_{g_i}^{C1,C2,C3}) \\ g_2^{I_g}(X_{g_i}^{C1,C2,C3}) &= f_0^{I_g}(X_{g_i}^{C1,C2})f_0^{I_g}(X_{g_i}^{C3}) \\ g_3^{I_g}(X_{g_i}^{C1,C2,C3}) &= f_0^{I_g}(X_{g_i}^{C1,C3})f_0^{I_g}(X_{g_i}^{C2}) \\ g_4^{I_g}(X_{g_i}^{C1,C2,C3}) &= f_0^{I_g}(X_{g_i}^{C1})f_0^{I_g}(X_{g_i}^{C2,C3}) \\ g_5^{I_g}(X_{g_i}^{C1,C2,C3}) &= f_0^{I_g}(X_{g_i}^{C1})f_0^{I_g}(X_{g_i}^{C2})f_0^{I_g}(X_{g_i}^{C3}) \end{aligned}$$

where  $f_0^{I_g}$  is the same as in equation 2. Then the marginal distribution in equation 4 becomes:

$$\sum_{k=1}^5 p_k g_k^{I_g}(X_{g_i}^{C1,C2,C3}) \quad (6)$$

where  $\sum_{k=1}^5 p_k = 1$ . Thus, the posterior probability of isoform  $g_i$  coming from pattern  $K$  is readily obtained by:

$$\frac{p_K g_K^{I_g}(X_{g_i}^{C1,C2,C3})}{\sum_{k=1}^5 p_k g_k^{I_g}(X_{g_i}^{C1,C2,C3})} \quad (7)$$

### 3.3 Getting a false discovery rate (FDR) controlled list of genes or isoforms

To obtain a list of DE genes with false discovery rate (FDR) controlled at  $\alpha$  in an experiment comparing two biological conditions, the genes with posterior probability of being DE (PPDE) greater than  $1 - \alpha$  should be used. For example, the genes with PPDE  $\geq 0.95$  make up the list of DE genes with target FDR controlled at 5%. With more than two biological conditions, there are multiple DE patterns (see Section 3.2). To obtain a list of genes in a specific DE pattern with target FDR  $\alpha$ , a user should take the genes with posterior probability of being in that pattern greater than  $1 - \alpha$ . Isoform-based lists are obtained in the same way.

## 4 Quick Start

Before analysis can proceed, the EBSeq package must be loaded into the working space:

```
> library(EBSeq)
```

### 4.1 Gene level DE analysis (two conditions)

#### 4.1.1 Required input

**Data:** The object `Data` should be a  $G \times by \times S$  matrix containing the expression values for each gene and each sample, where  $G$  is the number of genes and  $S$  is the number of samples. These values should exhibit raw counts, without normalization across samples. Counts of this nature may be obtained from RSEM [4], Cufflinks [6], or a similar approach.

**Conditions:** The object `Conditions` should be a Factor vector of length  $S$  that indicates to which condition each sample belongs. For example, if there are two conditions and three samples in each,  $S = 6$  and `Conditions` may be given by  
`as.factor(c("C1", "C1", "C1", "C2", "C2", "C2"))`

The object `GeneMat` is a simulated data matrix containing 1,000 rows of genes and 10 columns of samples. The genes are named `Gene_1`, `Gene_2` ...

```
> data(GeneMat)
> str(GeneMat)

num [1:1000, 1:10] 1879 24 3291 97 485 ...
- attr(*, "dimnames")=List of 2
..$ : chr [1:1000] "Gene_1" "Gene_2" "Gene_3" "Gene_4" ...
..$ : NULL
```

#### 4.1.2 Library size factor

As detailed in Section 3, EBSeq requires the library size factor  $l_s$  for each sample  $s$ . Here,  $l_s$  may be obtained via the function `MedianNorm`, which reproduces the median normalization approach in DESeq [1].

```
> Sizes=MedianNorm(GeneMat)
```

If quantile normalization is preferred,  $l_s$  may be obtained via the function `QuantileNorm`. (e.g. `QuantileNorm(GeneMat, .75)` for Upper-Quantile Normalization in [2])

#### 4.1.3 Running EBSeq on gene expression estimates

The function `EBTest` is used to detect DE genes. For gene-level data, we don't need to specify the parameter `NgVector` since there are no differences in  $I_g$  structure among the different genes. Here, we simulated the first five samples to be in condition 1 and the other five in condition 2, so define:

```
Conditions=as.factor(rep(c("C1", "C2"), each=5))
```

`sizeFactors` is used to define the library size factor of each sample. It could be obtained by summing up the total number of reads within each sample, Median Normalization [1], scaling normalization [5], Upper-Quantile Normalization [2], or some other such approach. These in hand, we run the EM algorithm, setting the number of iterations to five via `maxround=5` for demonstration purposes. However, we note that in practice, additional iterations are usually required. Convergence should always be checked (see Section 5.1.3 for details). Please note this may take several minutes:

```
> EBOut=EBTest(Data=GeneMat,
+ Conditions=as.factor(rep(c("C1", "C2"), each=5)), sizeFactors=Sizes, maxround=5)
```

Removing transcripts with 75 th quantile < = 10  
 950 transcripts will be tested

The posterior probabilities of being DE are obtained as follows, where PP is a matrix containing the posterior probabilities of being EE or DE for each of the 1,000 simulated genes:

```
> PP=GetPPMat(EBOut)
> str(PP)

num [1:950, 1:2] 0 0 0 0 0 ...
- attr(*, "dimnames")=List of 2
 ..$ : chr [1:950] "Gene_1" "Gene_2" "Gene_3" "Gene_4" ...
 ..$ : chr [1:2] "PPEE" "PPDE"

> head(PP)

           PPEE PPDE
Gene_1 0.000000e+00  1
Gene_2 0.000000e+00  1
Gene_3 0.000000e+00  1
Gene_4 0.000000e+00  1
Gene_5 0.000000e+00  1
Gene_6 4.645857e-10  1
```

The matrix PP contains two columns PPEE and PPDE, corresponding to the posterior probabilities of being EE or DE for each gene. PP may be used to form an FDR-controlled list of DE genes with a target FDR of 0.05 as follows:

```
> DEfound=rownames(PP)[which(PP[, "PPDE"]>=.95)]
> str(DEfound)

chr [1:97] "Gene_1" "Gene_2" "Gene_3" "Gene_4" "Gene_5" ...
```

EBSeq found 98 DE genes in total with target FDR 0.05.

## 4.2 Isoform level DE analysis (two conditions)

### 4.2.1 Required inputs

**Data:** The object `Data` should be a  $I - by - S$  matrix containing the expression values for each isoform and each sample, where  $I$  is the number of isoforms and  $S$  is the number of sample. As in the gene-level analysis, these values should exhibit raw data, without normalization across samples.

**Conditions:** The object `Conditions` should be a vector with length  $S$  to indicate the condition of each sample.

**IsoformNames:** The object `IsoformNames` should be a vector with length  $I$  to indicate the isoform names.

**IsosGeneNames:** The object `IsosGeneNames` should be a vector with length  $I$  to indicate the gene name of each isoform. (in the same order as `IsoformNames`.)

`IsoList` contains 1,200 simulated isoforms. In which `IsoList$IsoMat` is a data matrix containing 1,200 rows of isoforms and 10 columns of samples; `IsoList$IsoNames` contains the isoform names; `IsoList$IsosGeneNames` contains the names of the genes the isoforms belong to.

```

> data(IsoList)
> str(IsoList)

List of 3
 $ IsoMat      : num [1:1200, 1:10] 176 789 1300 474 1061 ...
  ..- attr(*, "dimnames")=List of 2
    .. ..$ : chr [1:1200] "Iso_1_1" "Iso_1_2" "Iso_1_3" "Iso_1_4" ...
    .. ..$ : NULL
 $ IsoNames    : chr [1:1200] "Iso_1_1" "Iso_1_2" "Iso_1_3" "Iso_1_4" ...
 $ IsosGeneNames: chr [1:1200] "Gene_1" "Gene_2" "Gene_3" "Gene_4" ...

> IsoMat=IsoList$IsoMat
> str(IsoMat)

num [1:1200, 1:10] 176 789 1300 474 1061 ...
- attr(*, "dimnames")=List of 2
  ..$ : chr [1:1200] "Iso_1_1" "Iso_1_2" "Iso_1_3" "Iso_1_4" ...
  ..$ : NULL

> IsoNames=IsoList$IsoNames
> IsosGeneNames=IsoList$IsosGeneNames

```

#### 4.2.2 Library size factor

Similar to the gene-level analysis presented above, we may obtain the isoform-level library size factors via `MedianNorm`:

```

> IsoSizes=MedianNorm(IsoMat)

```

#### 4.2.3 The $I_g$ vector

While working on isoform level data, EBSeq fits different prior parameters for different uncertainty groups (defined as  $I_g$  groups). The default setting to define the uncertainty groups consists of using the number of isoforms the host gene contains ( $N_g$ ) for each isoform. The default settings will provide three uncertainty groups:

- $I_g = 1$  group: Isoforms with  $N_g = 1$ ;
- $I_g = 2$  group: Isoforms with  $N_g = 2$ ;
- $I_g = 3$  group: Isoforms with  $N_g \geq 3$ .

The  $N_g$  and  $I_g$  group assignment can be obtained using the function `GetNg`. The required inputs of `GetNg` are the isoform names (`IsoformNames`) and their corresponding gene names (`IsosGeneNames`).

```

> NgList=GetNg(IsoNames, IsosGeneNames)
> IsoNgTrun=NgList$IsoformNgTrun
> IsoNgTrun[c(1:3,201:203,601:603)]

```

```

Iso_1_1 Iso_1_2 Iso_1_3 Iso_2_1 Iso_2_2 Iso_2_3 Iso_3_1 Iso_3_2 Iso_3_3
      1       1       1       2       2       2       3       3       3

```

More details could be found in Section 5.2.

#### 4.2.4 Running EBSeq on isoform expression estimates

The `EBTest` function is also used to run EBSeq for two condition comparisons on isoform-level data. Below we use 5 iterations to demonstrate. However, as in the gene level analysis, we advise that additional iterations will likely be required in practice (see Section 5.2.4 for details).

```
> IsoEBOut=EBTest(Data=IsoMat, NgVector=IsoNgTrun,
+ Conditions=as.factor(rep(c("C1","C2"),each=5)),sizeFactors=IsoSizes, maxround=5)
```

Removing transcripts with 75 th quantile < = 10  
1102 transcripts will be tested

```
> IsoPP=GetPPMat(IsoEBOut)
> str(IsoPP)

num [1:1102, 1:2] 0 0 0 0 0 ...
- attr(*, "dimnames")=List of 2
..$ : chr [1:1102] "Iso_1_1" "Iso_1_2" "Iso_1_3" "Iso_1_4" ...
..$ : chr [1:2] "PPEE" "PPDE"
```

```
> head(IsoPP)
```

	PPEE	PPDE
Iso_1_1	0	1
Iso_1_2	0	1
Iso_1_3	0	1
Iso_1_4	0	1
Iso_1_5	0	1
Iso_1_6	0	1

```
> IsoDE=rownames(IsoPP)[which(IsoPP[, "PPDE"]>=.95)]
> str(IsoDE)

chr [1:106] "Iso_1_1" "Iso_1_2" "Iso_1_3" "Iso_1_4" "Iso_1_5" ...
```

We see that EBSeq found 105 DE isoforms at the target FDR of 0.05.

### 4.3 Gene level DE analysis (more than two conditions)

The object `MultiGeneMat` is a matrix containing 500 simulated genes with 6 samples: the first two samples are from condition 1; the second and the third sample are from condition 2; the last two samples are from condition 3.

```
> data(MultiGeneMat)
> str(MultiGeneMat)

num [1:500, 1:6] 411 268 768 1853 878 ...
- attr(*, "dimnames")=List of 2
..$ : chr [1:500] "Gene_1" "Gene_3" "Gene_5" "Gene_7" ...
..$ : NULL
```

In analysis where the data are spread over more than two conditions, the set of possible patterns for each gene is more complicated than simply EE and DE. As noted in Section 3, when we have 3 conditions, there are 5 expression patterns to consider. In the simulated data, we have 6 samples, 2 in each of 3 conditions. The function `GetPatterns` allows the user to generate all possible patterns given the conditions. For example:

```
> Conditions=c("C1","C1","C2","C2","C3","C3")
> PosParti=GetPatterns(Conditions)
> PosParti
```



	C1	C2	C3
Pattern1	1	1	1
Pattern2	1	1	2
Pattern3	1	2	1
Pattern4	1	2	2
Pattern5	1	2	3

where the first row means all three conditions have the same latent mean expression level; the second row means C1 and C2 have the same latent mean expression level but that of C3 is different; and the last row corresponds to the case where the three conditions all have different latent mean expression levels. The user may use all or only some of these possible patterns as an input to `EBMultiTest`. For example, if we were interested in Patterns 1, 2, 4 and 5 only, we'd define:

```
> Parti=PosParti[-3,]
> Parti
```

	C1	C2	C3
Pattern1	1	1	1
Pattern2	1	1	2
Pattern4	1	2	2
Pattern5	1	2	3

Moving on to the analysis, `MedianNorm` or one of its competitors should be used to determine the normalization factors. Once this is done, the formal test is performed by `EBMultiTest`.

```
> MultiSize=MedianNorm(MultiGeneMat)
> MultiOut=EBMultiTest(MultiGeneMat,NgVector=NULL,Conditions=Conditions,
+ AllParti=Parti, sizeFactors=MultiSize, maxround=5)
```

Removing transcripts with 75 th quantile < = 10  
494 transcripts will be tested

The posterior probability of being in each pattern for every gene is obtained by using the function `GetMultiPP`:

```
> MultiPP=GetMultiPP(MultiOut)
> names(MultiPP)
```

```
[1] "PP"      "MAP"      "Patterns"
```

```
> MultiPP$PP[1:10,]
```

	Pattern1	Pattern2	Pattern4	Pattern5
Gene_1	8.533540e-94	0.3986838	5.107954e-72	0.60131622
Gene_3	9.691396e-164	0.9694403	6.680829e-109	0.03055971
Gene_5	6.243738e-26	0.9334990	6.351309e-20	0.06650097
Gene_7	0.000000e+00	0.5564474	0.000000e+00	0.44355263
Gene_9	5.019631e-16	0.9435967	2.013503e-15	0.05640325
Gene_11	1.945436e-11	0.9367403	2.163358e-12	0.06325967
Gene_13	1.426212e-08	0.7295369	6.405126e-10	0.27046313
Gene_15	3.485274e-47	0.9690530	8.526272e-41	0.03094699
Gene_17	3.336485e-184	0.6560285	4.551096e-133	0.34397147
Gene_19	1.744386e-37	0.9042735	1.311200e-24	0.09572651

```
> MultiPP$MAP[1:10]
```

```

      Gene_1      Gene_3      Gene_5      Gene_7      Gene_9      Gene_11      Gene_13
"Pattern5" "Pattern2" "Pattern2" "Pattern2" "Pattern2" "Pattern2" "Pattern2"
      Gene_15      Gene_17      Gene_19
"Pattern2" "Pattern2" "Pattern2"

```

```
> MultiPP$Patterns
```

```

      C1 C2 C3
Pattern1 1 1 1
Pattern2 1 1 2
Pattern4 1 2 2
Pattern5 1 2 3

```

where `MultiPP$PP` provides the posterior probability of being in each pattern for every gene. `MultiPP$MAP` provides the most likely pattern of each gene based on the posterior probabilities. `MultiPP$Patterns` provides the details of the patterns.

#### 4.4 Isoform level DE analysis (more than two conditions)

Similar to `IsoList`, the object `IsoMultiList` is an object containing the isoform expression estimates matrix, the isoform names, and the gene names of the isoforms' host genes. `IsoMultiList$IsoMultiMat` contains 300 simulated isoforms with 8 samples. The first two samples are from condition 1; the second and the third sample are from condition 2; the fifth and sixth sample are from condition 3; the last two samples are from condition 4. Similar to Section 4.2, the function `MedianNorm` and `GetNg` could be used for normalization and calculating the  $N_g$ 's.

```

> data(IsoMultiList)
> IsoMultiMat=IsoMultiList[[1]]
> IsoNames.Multi=IsoMultiList$IsoNames
> IsosGeneNames.Multi=IsoMultiList$IsosGeneNames
> IsoMultiSize=MedianNorm(IsoMultiMat)
> NgList.Multi=GetNg(IsoNames.Multi, IsosGeneNames.Multi)
> IsoNgTrun.Multi=NgList.Multi$IsoformNgTrun
> Conditions=c("C1", "C1", "C2", "C2", "C3", "C3", "C4", "C4")

```

Here we have 4 conditions, there are 15 expression patterns to consider. The function `GetPatterns` allows the user to generate all possible patterns given the conditions. For example:

```

> PosParti.4Cond=GetPatterns(Conditions)
> PosParti.4Cond

```

```

      C1 C2 C3 C4
Pattern1 1 1 1 1
Pattern2 1 1 1 2
Pattern3 1 1 2 1
Pattern4 1 1 2 2
Pattern5 1 2 1 1
Pattern6 1 2 1 2
Pattern7 1 2 2 1
Pattern8 1 2 2 2
Pattern9 1 1 2 3
Pattern10 1 2 1 3
Pattern11 1 2 2 3
Pattern12 1 2 3 1

```

```

Pattern13  1  2  3  2
Pattern14  1  2  3  3
Pattern15  1  2  3  4

```

If we were interested in Patterns 1, 2, 3, 8 and 15 only, we'd define:

```

> Parti.4Cond=PosParti.4Cond[c(1,2,3,8,15),]
> Parti.4Cond

```

```

      C1 C2 C3 C4
Pattern1  1  1  1  1
Pattern2  1  1  1  2
Pattern3  1  1  2  1
Pattern8  1  2  2  2
Pattern15 1  2  3  4

```

Moving on to the analysis, `EBMultiTest` could be used to perform the test:

```

> IsoMultiOut=EBMultiTest(IsoMultiMat,
+ NgVector=IsoNgTrun.Multi,Conditions=Conditions,
+ AllParti=Parti.4Cond, sizeFactors=IsoMultiSize,
+ maxround=5)

```

Removing transcripts with 75 th quantile < = 10  
294 transcripts will be tested

The posterior probability of being in each pattern for every gene is obtained by using the function `GetMultiPP`:

```

> IsoMultiPP=GetMultiPP(IsoMultiOut)
> names(MultiPP)

```

```

[1] "PP"      "MAP"      "Patterns"

```

```

> IsoMultiPP$PP[1:10,]

```

```

      Pattern1  Pattern2  Pattern3  Pattern8  Pattern15
Iso_1_1  3.533233e-32 0.999882138  3.408808e-33  2.143838e-34  1.178620e-04
Iso_1_2  4.231331e-14 0.999826487  1.573392e-16  5.848567e-18  1.735129e-04
Iso_1_3  5.633772e-47 0.992627423  5.963569e-42  5.644910e-50  7.372577e-03
Iso_1_4  4.248398e-35 0.998959777  1.983567e-30  5.054181e-33  1.040223e-03
Iso_1_5  0.000000e+00 1.000000000  0.000000e+00  0.000000e+00  1.584343e-41
Iso_1_6  1.509151e-232 0.002646919  3.147566e-220  6.720686e-188  9.973531e-01
Iso_1_7  2.835263e-138 0.999439469  7.548859e-133  1.613556e-128  5.605313e-04
Iso_1_8  9.654898e-139 0.963893542  3.709303e-105  5.626105e-120  3.610646e-02
Iso_1_9  1.947187e-47 0.957423511  1.073683e-50  3.868129e-46  4.257649e-02
Iso_1_10 7.904509e-08 0.999790300  9.178739e-10  9.386672e-10  2.096196e-04

```

```

> IsoMultiPP$MAP[1:10]

```

```

      Iso_1_1  Iso_1_2  Iso_1_3  Iso_1_4  Iso_1_5  Iso_1_6
"Pattern2"  "Pattern2"  "Pattern2"  "Pattern2"  "Pattern2"  "Pattern15"
      Iso_1_7  Iso_1_8  Iso_1_9  Iso_1_10
"Pattern2"  "Pattern2"  "Pattern2"  "Pattern2"

```

```

> IsoMultiPP$Patterns

```

	C1	C2	C3	C4
Pattern1	1	1	1	1
Pattern2	1	1	1	2
Pattern3	1	1	2	1
Pattern8	1	2	2	2
Pattern15	1	2	3	4

where `MultiPP$PP` provides the posterior probability of being in each pattern for every gene. `MultiPP$MAP` provides the most likely pattern of each gene based on the posterior probabilities. `MultiPP$Patterns` provides the details of the patterns.

## 5 More detailed examples

### 5.1 Gene level DE analysis (two conditions)

#### 5.1.1 Running EBSeq on simulated gene expression estimates

EBSeq is applied as described in Section 4.1.3.

```
> data(GeneMat)
> Sizes=MedianNorm(GeneMat)
> EBOut=EBTest(Data=GeneMat,
+ Conditions=as.factor(rep(c("C1","C2"),each=5)),sizeFactors=Sizes, maxround=5)
> PP=GetPPMat(EBOut)

> str(PP)

num [1:950, 1:2] 0 0 0 0 0 ...
- attr(*, "dimnames")=List of 2
..$ : chr [1:950] "Gene_1" "Gene_2" "Gene_3" "Gene_4" ...
..$ : chr [1:2] "PPEE" "PPDE"

> head(PP)

          PPEE PPDE
Gene_1 0.000000e+00  1
Gene_2 0.000000e+00  1
Gene_3 0.000000e+00  1
Gene_4 0.000000e+00  1
Gene_5 0.000000e+00  1
Gene_6 4.645857e-10  1

> DEfound=rownames(PP)[which(PP[, "PPDE"]>=.95)]
> str(DEfound)

chr [1:97] "Gene_1" "Gene_2" "Gene_3" "Gene_4" "Gene_5" ...
```

EBSeq found 98 DE genes at a target FDR of 0.05.

#### 5.1.2 Calculating FC

The function `PostFC` may be used to calculate the Fold Change (FC) of the raw data as well as the posterior FC of the normalized data. Figure 2 shows the FC vs. Posterior FC on 1,000 gene expression estimates. The genes are ranked by their cross-condition mean (adjusted by the normalization factors). The posterior FC tends to shrink genes with low expressions (small rank); in this case the differences are minor.

```

> GeneFC=PostFC(EBOut)
> str(GeneFC)

List of 3
 $ PostFC   : Named num [1:950] 0.237 0.241 4.127 4.242 3.91 ...
 ..- attr(*, "names")= chr [1:950] "Gene_1" "Gene_2" "Gene_3" "Gene_4" ...
 $ RealFC   : Named num [1:950] 0.237 0.239 4.128 4.28 3.918 ...
 ..- attr(*, "names")= chr [1:950] "Gene_1" "Gene_2" "Gene_3" "Gene_4" ...
 $ Direction: chr "C1 Over C2"

> PlotPostVsRawFC(EBOut, GeneFC)

```

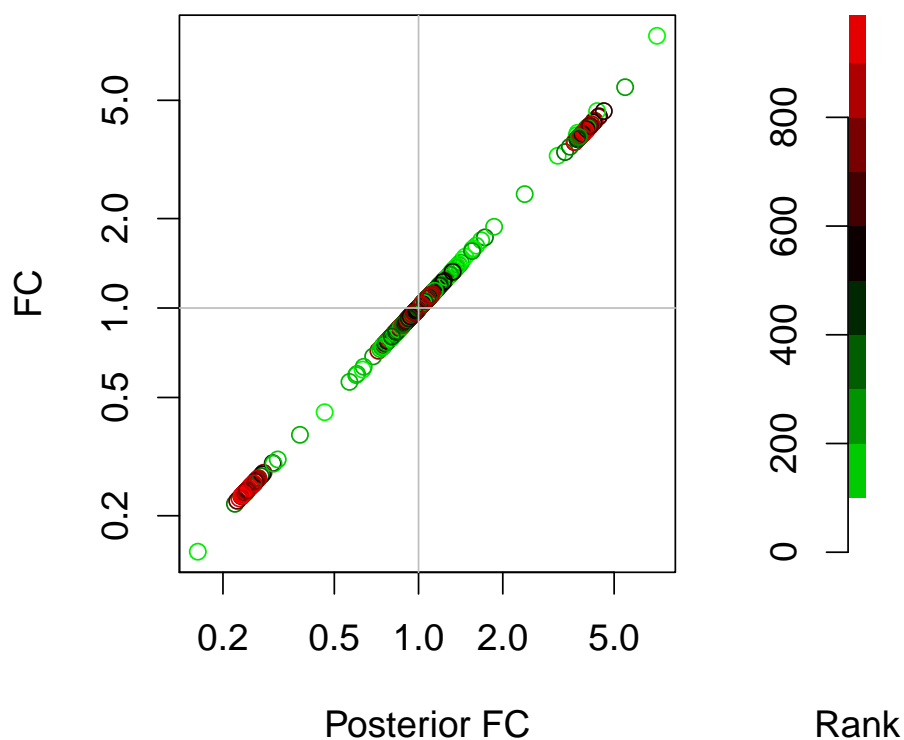


Figure 2: FC vs. Posterior FC for 1,000 gene expression estimates

### 5.1.3 Checking convergence

As detailed in Section 3, we assume the prior distribution of  $q_g^C$  is  $Beta(\alpha, \beta)$ . The EM algorithm is used to estimate the hyper-parameters  $\alpha, \beta$  and the mixture parameter  $p$ . The optimized parameters at each iteration may be obtained as follows (recall we are using 5 iterations for demonstration purposes):

```

> EBOut$Alpha

      [,1]
iter1 0.8313646
iter2 0.8277784
iter3 0.8273499
iter4 0.8264807
iter5 0.8264807

> EBOut$Beta

      Ng1
iter1 1.718970
iter2 1.721066
iter3 1.719731
iter4 1.716137
iter5 1.716137

> EBOut$P

      [,1]
iter1 0.1693038
iter2 0.1338740
iter3 0.1289061
iter4 0.1284153
iter5 0.1284153

```

In this case the differences between the 4th and 5th iterations are always less than 0.01.

#### 5.1.4 Checking the model fit and other diagnostics

As noted in Leng *et al.*, 2013 [3], EBSeq relies on parametric assumptions that should be checked following each analysis. The QQP function may be used to assess prior assumptions. In practice, QQP generates the Q-Q plot of the empirical  $q$ 's vs. the simulated  $q$ 's from the Beta prior distribution with estimated hyperparameters. Figure 3 shows that the data points lie on the  $y = x$  line for both conditions, which indicates that the Beta prior is appropriate.

```
> par(mfrow=c(1,2))
> QQP(EBOut)
```

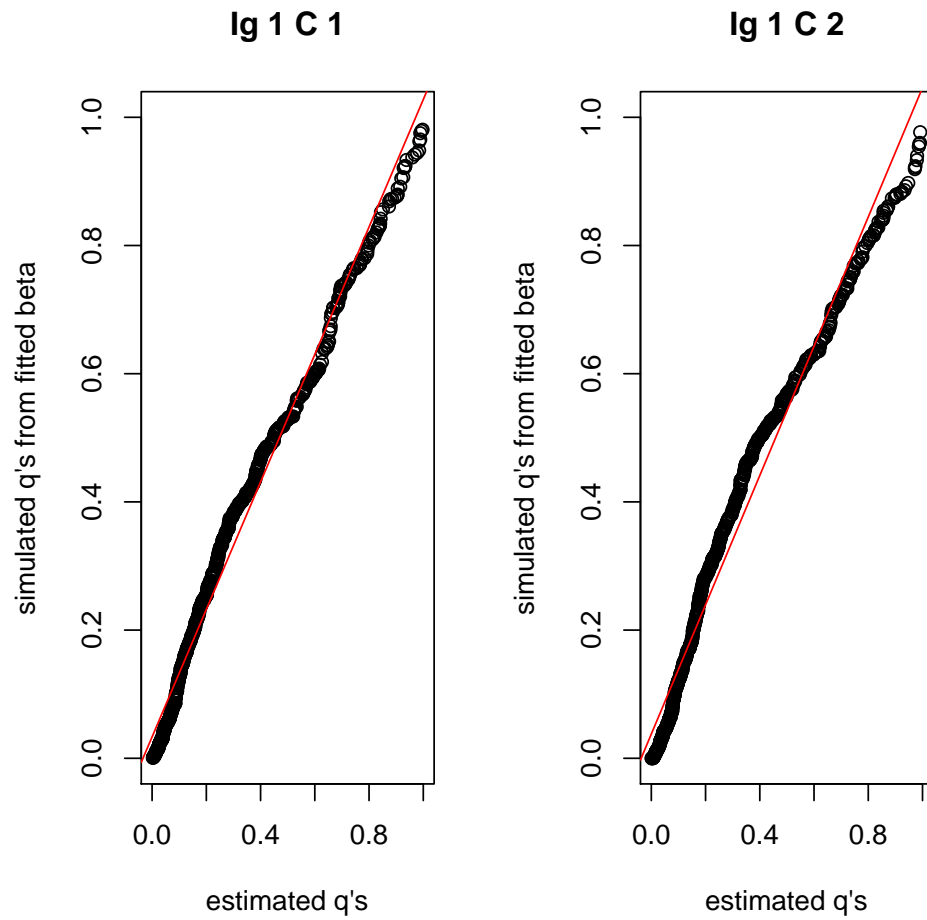


Figure 3: QQ-plots for checking the assumption of a Beta prior (upper panels) as well as the model fit using data from condition 1 and condition 2 (lower panels)

Likewise, the `DenNHist` function may be used to check the density plot of empirical  $q$ 's vs the simulated  $q$ 's from the fitted Beta prior distribution. Figure 4 also shows our estimated distribution fits the data very well.



```
> par(mfrow=c(1,2))
> DenNHist(EBOut)
```

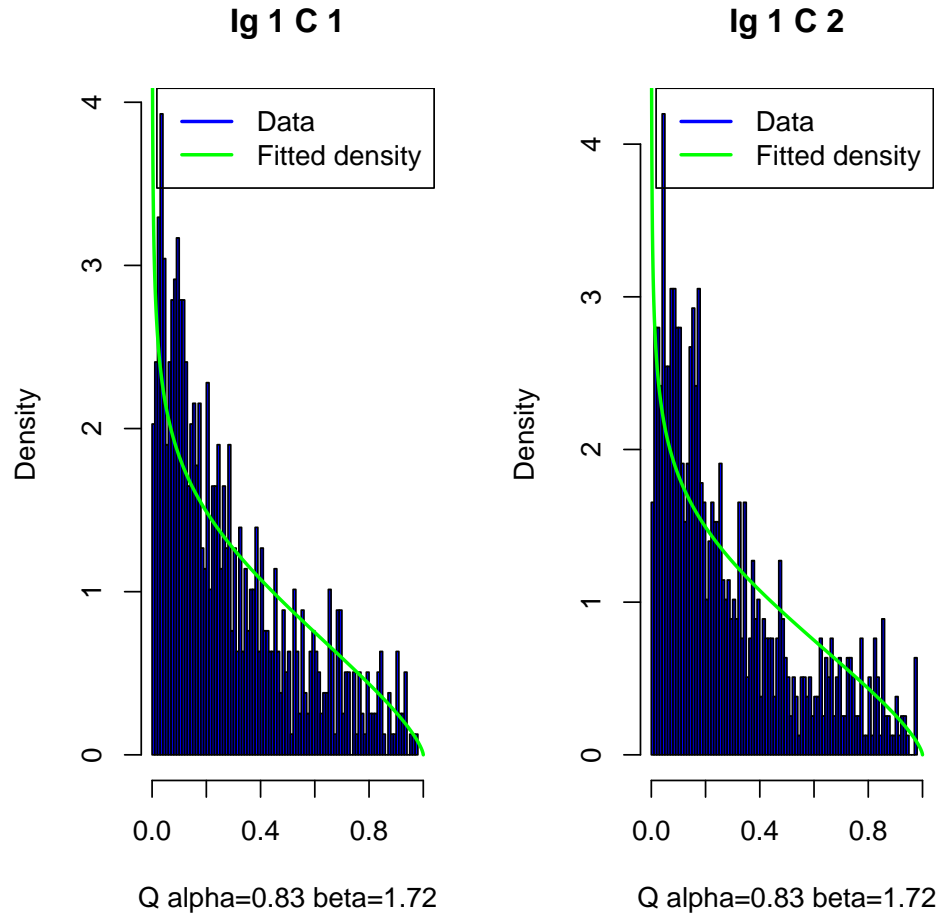


Figure 4: Density plots for checking the model fit using data from condition 1 and condition 2

## 5.2 Isoform level DE analysis (two conditions)

### 5.2.1 The $I_g$ vector

Since EBSeq fits rely on  $I_g$ , we need to obtain the  $I_g$  for each isoform. This can be done using the function `GetNg`. The required inputs of `GetNg` are the isoform names (`IsoformNames`) and their corresponding gene names (`IsosGeneNames`), described above. In the simulated data, we assume that the isoforms in the  $I_g = 1$  group belong to genes `Gene_1`, ..., `Gene_200`; The isoforms in the  $I_g = 2$  group belong to genes `Gene_201`, ..., `Gene_400`; and isoforms in the  $I_g = 3$  group belong to `Gene_401`, ..., `Gene_600`.

```
> data(IsoList)
> IsoMat=IsoList$IsoMat
> IsoNames=IsoList$IsoNames
> IsosGeneNames=IsoList$IsosGeneNames
> NgList=GetNg(IsoNames, IsosGeneNames, TrunThre=3)
```

```
> names(NgList)

[1] "GeneNg"      "GeneNgTrun"  "IsoformNg"   "IsoformNgTrun"

> IsoNgTrun=NgList$IsoformNgTrun
> IsoNgTrun[c(1:3,201:203,601:603)]

Iso_1_1 Iso_1_2 Iso_1_3 Iso_2_1 Iso_2_2 Iso_2_3 Iso_3_1 Iso_3_2 Iso_3_3
      1      1      1      2      2      2      3      3      3
```

The output of `GetNg` contains 4 vectors. `GeneNg` (`IsoformNg`) provides the number of isoforms  $N_g$  within each gene (within each isoform's host gene). `GeneNgTrun` (`IsoformNgTrun`) provides the  $I_g$  group assignments. The default number of groups is 3, which means the isoforms with  $N_g$  greater than 3 will be assigned to  $I_g = 3$  group. We use 3 in the case studies since the number of isoforms with  $N_g$  larger than 3 is relatively small and the small sample size may induce poor parameter fitting if we treat them as separate groups. In practice, if there is evidence that the  $N_g = 4, 5, 6, \dots$  groups should be treated as separate groups, a user can change `TrunThre` to define a different truncation threshold.

### 5.2.2 Using mappability ambiguity clusters instead of the $I_g$ vector when the gene-isoform relationship is unknown

When working with a de-novo assembled transcriptome, in which case the gene-isoform relationship is unknown, a user can use read mapping ambiguity cluster information instead of `Ng`, as provided by RSEM [4] in the output file `output_name.ngvec`. The file contains a vector with the same length as the total number of transcripts. Each transcript has been assigned to one of 3 levels (1, 2, or 3) to indicate the mapping uncertainty level of that transcript. The mapping ambiguity clusters are partitioned via a k-means algorithm on the unmapability scores that are provided by RSEM. A user can read in the mapping ambiguity cluster information using:

```
> IsoNgTrun = scan(file="output_name.ngvec", what=0, sep="\n")
```

Where `"output_name.ngvec"` is the output file obtained from RSEM function `rsem-generate-ngvector`. More details on using the RSEM-EBSeq pipeline on de novo assembled transcriptomes can be found at <http://deweylab.biostat.wisc.edu/rsem/README.html#de>.

Other unmapability scores and other cluster methods (e.g. Gaussian Mixed Model) could also be used to form the uncertainty clusters.

### 5.2.3 Running EBSeq on simulated isoform expression estimates

EBSeq can be applied as described in Section 4.2.4.

```
> IsoSizes=MedianNorm(IsoMat)
> IsoEBOut=EBTest(Data=IsoMat, NgVector=IsoNgTrun,
+ Conditions=as.factor(rep(c("C1","C2"),each=5)),sizeFactors=IsoSizes, maxround=5)
> IsoPP=GetPPMat(IsoEBOut)
> IsoDE=rownames(IsoPP)[which(IsoPP[, "PPDE"]>=.95)]

> str(IsoDE)

chr [1:106] "Iso_1_1" "Iso_1_2" "Iso_1_3" "Iso_1_4" "Iso_1_5" ...
```

We see that EBSeq found 105 DE isoforms at a target FDR of 0.05. The function `PostFC` could also be used here to calculate the Fold Change (FC) as well as the posterior FC on the normalization factor adjusted data.

```
> IsoFC=PostFC(IsoEBOut)
> str(IsoFC)
```

```
List of 3
```

```
$ PostFC : Named num [1:1102] 0.286 0.281 3.553 0.305 3.755 ...
..- attr(*, "names")= chr [1:1102] "Iso_1_1" "Iso_1_2" "Iso_1_3" "Iso_1_4" ...
$ RealFC : Named num [1:1102] 0.285 0.281 3.556 0.305 3.759 ...
..- attr(*, "names")= chr [1:1102] "Iso_1_1" "Iso_1_2" "Iso_1_3" "Iso_1_4" ...
$ Direction: chr "C1 Over C2"
```

### 5.2.4 Checking convergence

For isoform level data, we assume the prior distribution of  $q_{gi}^C$  is  $Beta(\alpha, \beta^{I_g})$ . As in Section 5.1.3, the optimized parameters at each iteration may be obtained as follows (recall we are using 5 iterations for demonstration purposes):

```
> IsoEBOut$Alpha
```

```
      [,1]
iter1 0.7321709
iter2 0.7881646
iter3 0.7885616
iter4 0.7877492
iter5 0.7867663
```

```
> IsoEBOut$Beta
```

```
      Ng1      Ng2      Ng3
iter1 1.667405 2.648591 3.428080
iter2 1.833535 3.081309 4.453516
iter3 1.835056 3.092374 4.464689
iter4 1.838418 3.087711 4.464684
iter5 1.834517 3.082063 4.457264
```

```
> IsoEBOut$P
```

```
      [,1]
iter1 0.2183707
iter2 0.1715496
iter3 0.1601954
iter4 0.1573053
iter5 0.1559198
```

Here we have 3  $\beta$ 's in each iteration corresponding to  $\beta^{I_g=1}, \beta^{I_g=2}, \beta^{I_g=3}$ . We see that parameters are changing less than  $10^{-2}$  or  $10^{-3}$ . In practice, we require changes less than  $10^{-3}$  to declare convergence.

### 5.2.5 Checking the model fit and other diagnostics

In Leng *et al.*, 2013[3], we showed the mean-variance differences across different isoform groups on multiple data sets. In practice, if it is of interest to check differences among isoform groups defined by truncated  $I_g$  (such as those shown here in Figure 1), the function `PolyFitPlot` may be used. The following code generates the three panels shown in Figure 5 (if condition 2 is of interest, a user could change each C1 to C2.):

```

> par(mfrow=c(2,2))
> PolyFitValue=vector("list",3)
> for(i in 1:3)
+   PolyFitValue[[i]]=PolyFitPlot(IsoEBOut$C1Mean[[i]],
+   IsoEBOut$C1EstVar[[i]],5)

```

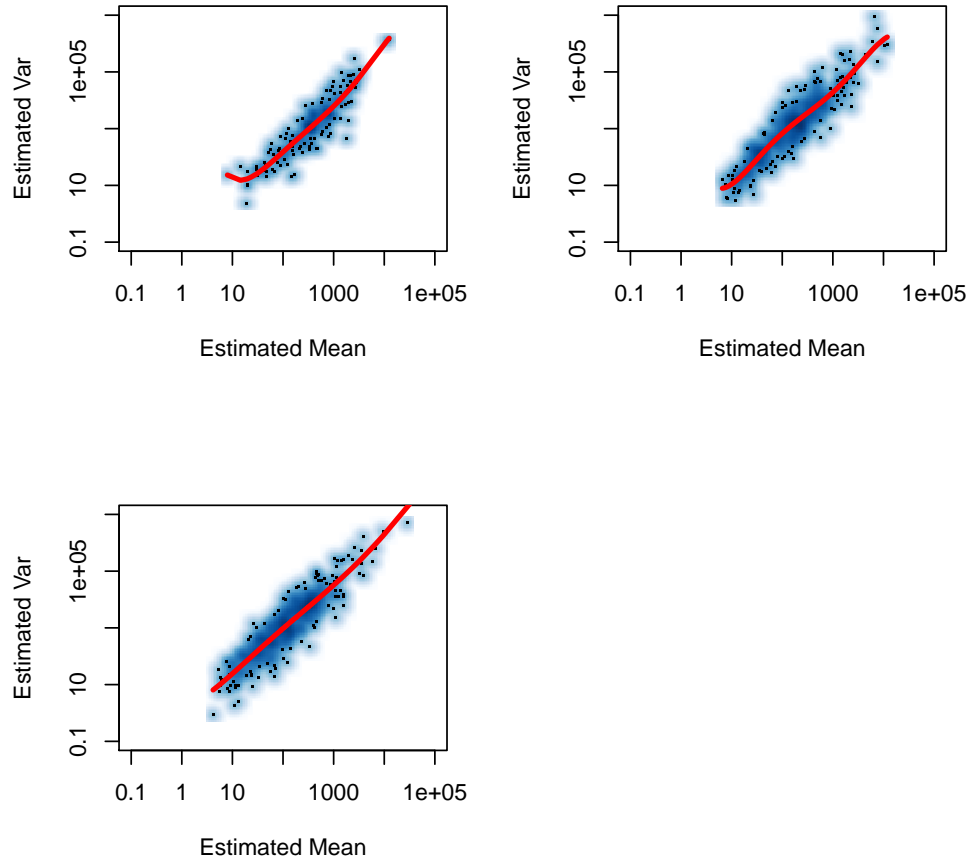


Figure 5: The mean-variance fitting plot for each Ng group

Superimposing all  $I_g$  groups using the code below will generate the figure (shown here in Figure 6), which is similar in structure to Figure 1:

```

> PolyAll=PolyFitPlot(unlist(IsoEBOut$C1Mean), unlist(IsoEBOut$C1EstVar),5)
> lines(log10(IsoEBOut$C1Mean[[1]] [PolyFitValue[[1]]$sort]),
+ PolyFitValue[[1]]$fit [PolyFitValue[[1]]$sort],col="yellow",lwd=2)
> lines(log10(IsoEBOut$C1Mean[[2]] [PolyFitValue[[2]]$sort]),
+ PolyFitValue[[2]]$fit [PolyFitValue[[2]]$sort],col="pink",lwd=2)
> lines(log10(IsoEBOut$C1Mean[[3]] [PolyFitValue[[3]]$sort]),
+ PolyFitValue[[3]]$fit [PolyFitValue[[3]]$sort],col="green",lwd=2)
> legend("topleft",c("All Isoforms", "Ng = 1", "Ng = 2", "Ng = 3"),
+ col=c("red", "yellow", "pink", "green"),lty=1,lwd=3,box.lwd=2)

```

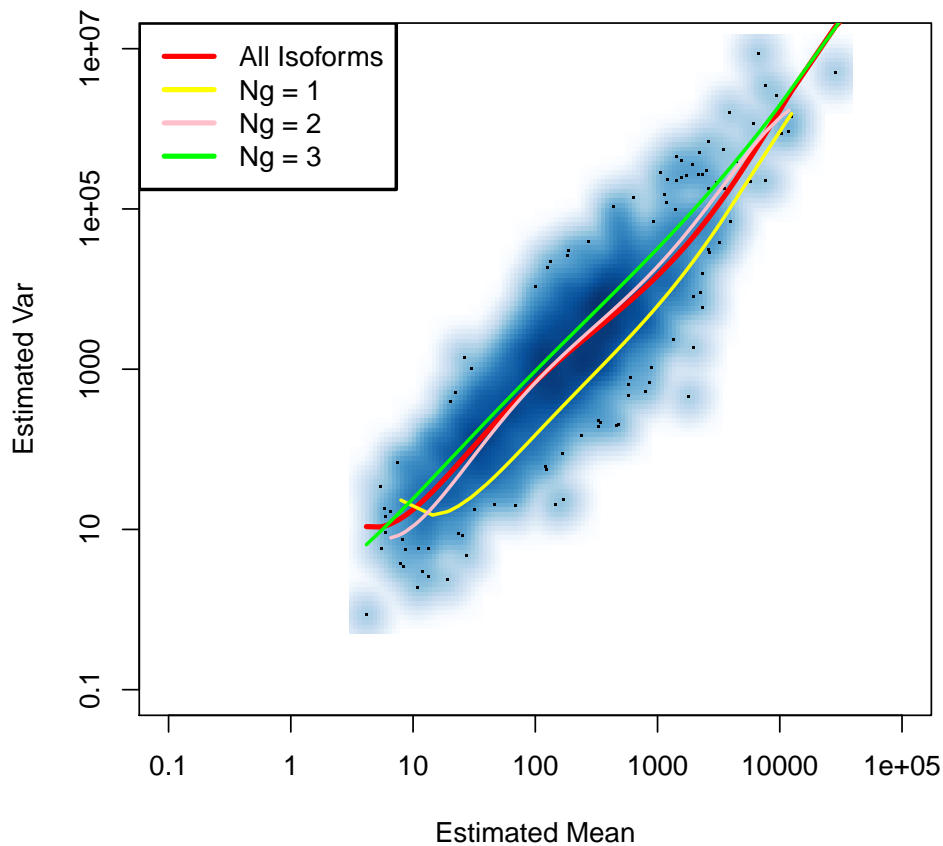


Figure 6: The mean-variance plot for each Ng group

To generate a QQ-plot of the fitted Beta prior distribution and the  $\hat{q}^C$ 's within condition, a user may use the following code to generate 6 panels (as shown in Figure 7).

```
> par(mfrow=c(2,3))
> QQP(IsoEBOut)
```

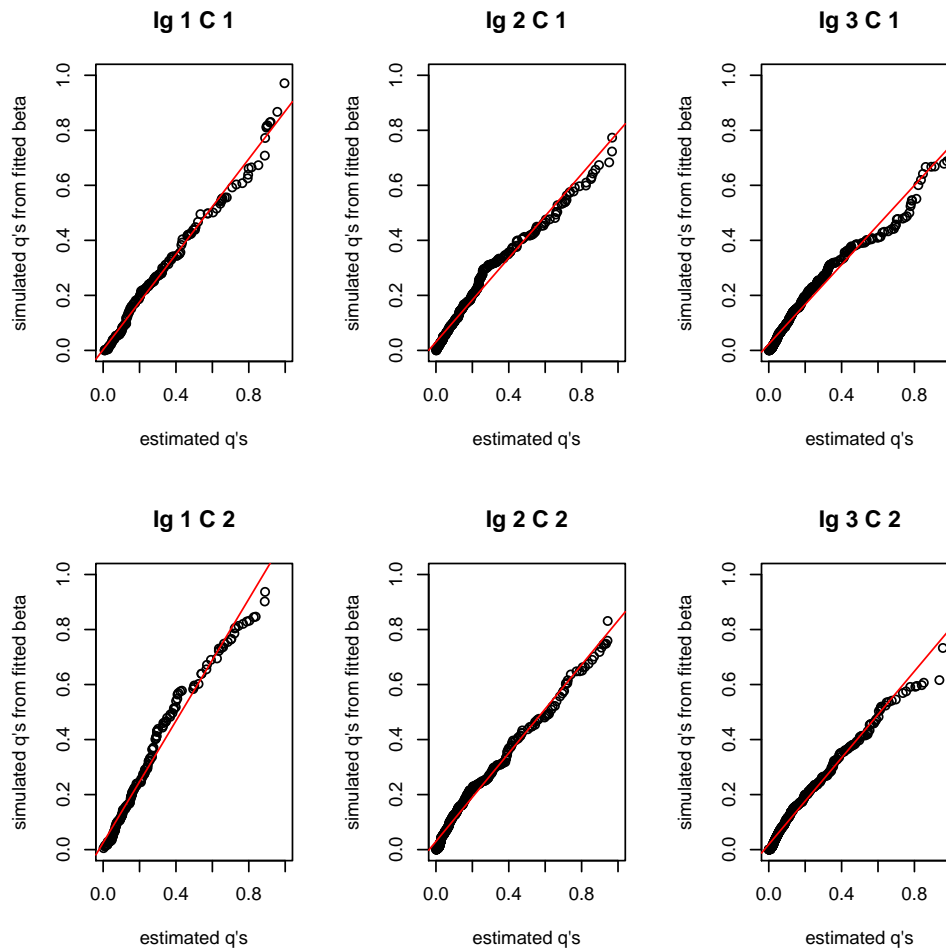


Figure 7: QQ-plots of the fitted prior distributions within each condition and each Ig group

And in order to produce the plot of the fitted Beta prior densities and the histograms of  $\hat{q}^C$ 's within each condition, the following may be used (it generates Figure 8):

```
> par(mfrow=c(2,3))
> DenNHist(IsoEBOut)
```

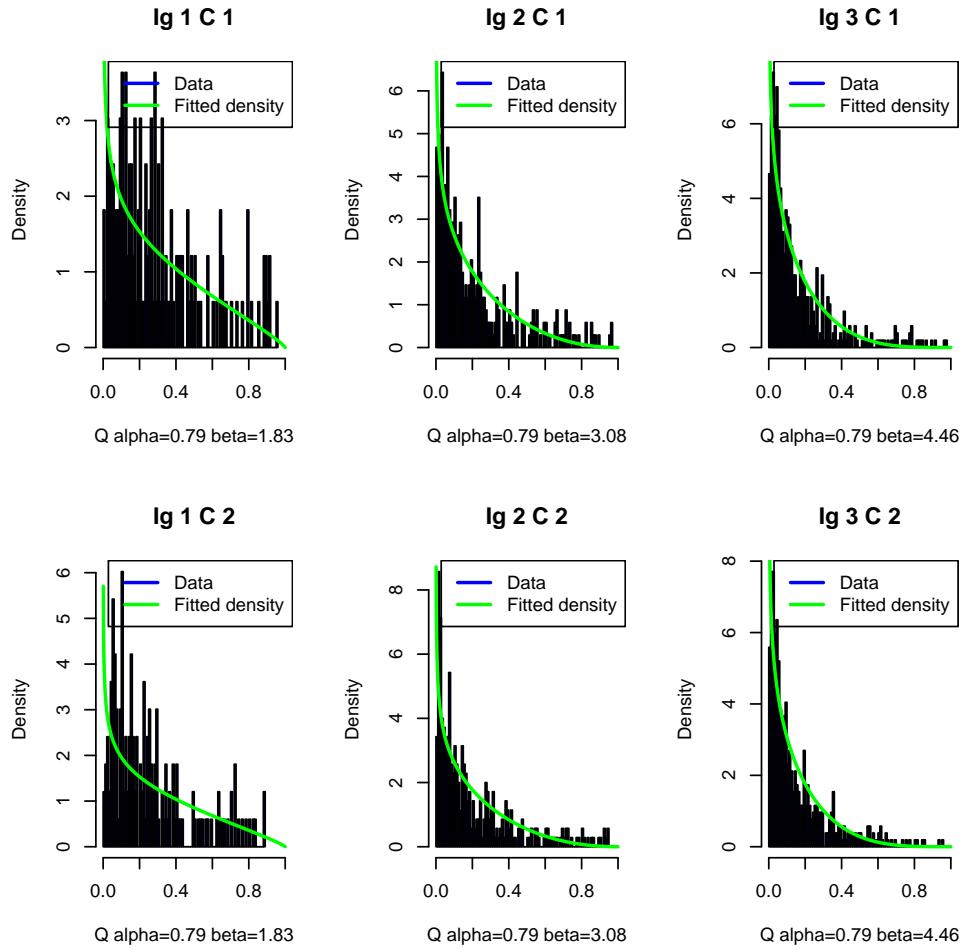


Figure 8: Prior distribution fit within each condition and each Ig group. (Note only a small set of isoforms are considered here for demonstration. Better fitting should be expected while using full set of isoforms.)

### 5.3 Gene level DE analysis (more than two conditions)

As described in Section 4.3, the function `GetPatterns` allows the user to generate all possible patterns given the conditions. To visualize the patterns, the function `PlotPattern` may be used.

```
> Conditions=c("C1","C1","C2","C2","C3","C3")
> PosParti=GetPatterns(Conditions)
> PosParti
```

	C1	C2	C3
Pattern1	1	1	1
Pattern2	1	1	2
Pattern3	1	2	1
Pattern4	1	2	2
Pattern5	1	2	3

```
> PlotPattern(PosParti)
```



Figure 9: All possible patterns



If we were interested in Patterns 1, 2, 4 and 5 only, we'd define:

```
> Parti=PosParti[-3,]
> Parti
```

```
      C1 C2 C3
Pattern1 1 1 1
Pattern2 1 1 2
Pattern4 1 2 2
Pattern5 1 2 3
```

Moving on to the analysis, `MedianNorm` or one of its competitors should be used to determine the normalization factors. Once this is done, the formal test is performed by `EBMultiTest`.

```
> data(MultiGeneMat)
> MultiSize=MedianNorm(MultiGeneMat)
> MultiOut=EBMultiTest(MultiGeneMat,
+ NgVector=NULL, Conditions=Conditions,
+ AllParti=Parti, sizeFactors=MultiSize,
+ maxround=5)
```

The posterior probability of being in each pattern for every gene is obtained using the function `GetMultiPP`:

```
> MultiPP=GetMultiPP(MultiOut)
> names(MultiPP)
```

```
[1] "PP"      "MAP"      "Patterns"
```

```
> MultiPP$PP[1:10,]
```

```
      Pattern1 Pattern2 Pattern4 Pattern5
Gene_1 8.533540e-94 0.3986838 5.107954e-72 0.60131622
Gene_3 9.691396e-164 0.9694403 6.680829e-109 0.03055971
Gene_5 6.243738e-26 0.9334990 6.351309e-20 0.06650097
Gene_7 0.000000e+00 0.5564474 0.000000e+00 0.44355263
Gene_9 5.019631e-16 0.9435967 2.013503e-15 0.05640325
Gene_11 1.945436e-11 0.9367403 2.163358e-12 0.06325967
Gene_13 1.426212e-08 0.7295369 6.405126e-10 0.27046313
Gene_15 3.485274e-47 0.9690530 8.526272e-41 0.03094699
Gene_17 3.336485e-184 0.6560285 4.551096e-133 0.34397147
Gene_19 1.744386e-37 0.9042735 1.311200e-24 0.09572651
```

```
> MultiPP$MAP[1:10]
```

```
      Gene_1      Gene_3      Gene_5      Gene_7      Gene_9      Gene_11      Gene_13
"Pattern5" "Pattern2" "Pattern2" "Pattern2" "Pattern2" "Pattern2" "Pattern2"
      Gene_15      Gene_17      Gene_19
"Pattern2" "Pattern2" "Pattern2"
```

```
> MultiPP$Patterns
```

```
      C1 C2 C3
Pattern1 1 1 1
Pattern2 1 1 2
Pattern4 1 2 2
Pattern5 1 2 3
```

where `MultiPP$PP` provides the posterior probability of being in each pattern for every gene. `MultiPP$MAP` provides the most likely pattern of each gene based on the posterior probabilities. `MultiPP$Patterns` provides the details of the patterns. The FC and posterior FC for multiple condition data can be obtained by the function `GetMultiFC`:

```
> MultiFC=GetMultiFC(MultiOut)
> str(MultiFC)

List of 6
 $ FCMat      : num [1:494, 1:3] 1.217 0.951 1.069 0.923 0.983 ...
  ..- attr(*, "dimnames")=List of 2
  .. ..$ : chr [1:494] "Gene_1" "Gene_3" "Gene_5" "Gene_7" ...
  .. ..$ : chr [1:3] "C10verC2" "C10verC3" "C20verC3"
 $ Log2FCMat  : num [1:494, 1:3] 0.2828 -0.0724 0.0969 -0.1151 -0.0251 ...
  ..- attr(*, "dimnames")=List of 2
  .. ..$ : chr [1:494] "Gene_1" "Gene_3" "Gene_5" "Gene_7" ...
  .. ..$ : chr [1:3] "C10verC2" "C10verC3" "C20verC3"
 $ PostFCMat  : num [1:494, 1:3] 1.216 0.951 1.069 0.923 0.983 ...
  ..- attr(*, "dimnames")=List of 2
  .. ..$ : chr [1:494] "Gene_1" "Gene_3" "Gene_5" "Gene_7" ...
  .. ..$ : chr [1:3] "C10verC2" "C10verC3" "C20verC3"
 $ Log2PostFCMat : num [1:494, 1:3] 0.2819 -0.072 0.0967 -0.115 -0.0251 ...
  ..- attr(*, "dimnames")=List of 2
  .. ..$ : chr [1:494] "Gene_1" "Gene_3" "Gene_5" "Gene_7" ...
  .. ..$ : chr [1:3] "C10verC2" "C10verC3" "C20verC3"
 $ CondMeans   : num [1:494, 1:3] 499 253 813 1843 753 ...
  ..- attr(*, "dimnames")=List of 2
  .. ..$ : chr [1:494] "Gene_1" "Gene_3" "Gene_5" "Gene_7" ...
  .. ..$ : chr [1:3] "C1" "C2" "C3"
 $ ConditionOrder: Named chr [1:3] "C1" "C2" "C3"
  ..- attr(*, "names")= chr [1:3] "Condition1" "Condition2" "Condition3"
```

To generate a QQ-plot of the fitted Beta prior distribution and the  $\hat{q}^C$ 's within condition, a user could also use function `DenNHist` and `QQP`.

```
> par(mfrow=c(2,2))
> QQP(MultiOut)
```

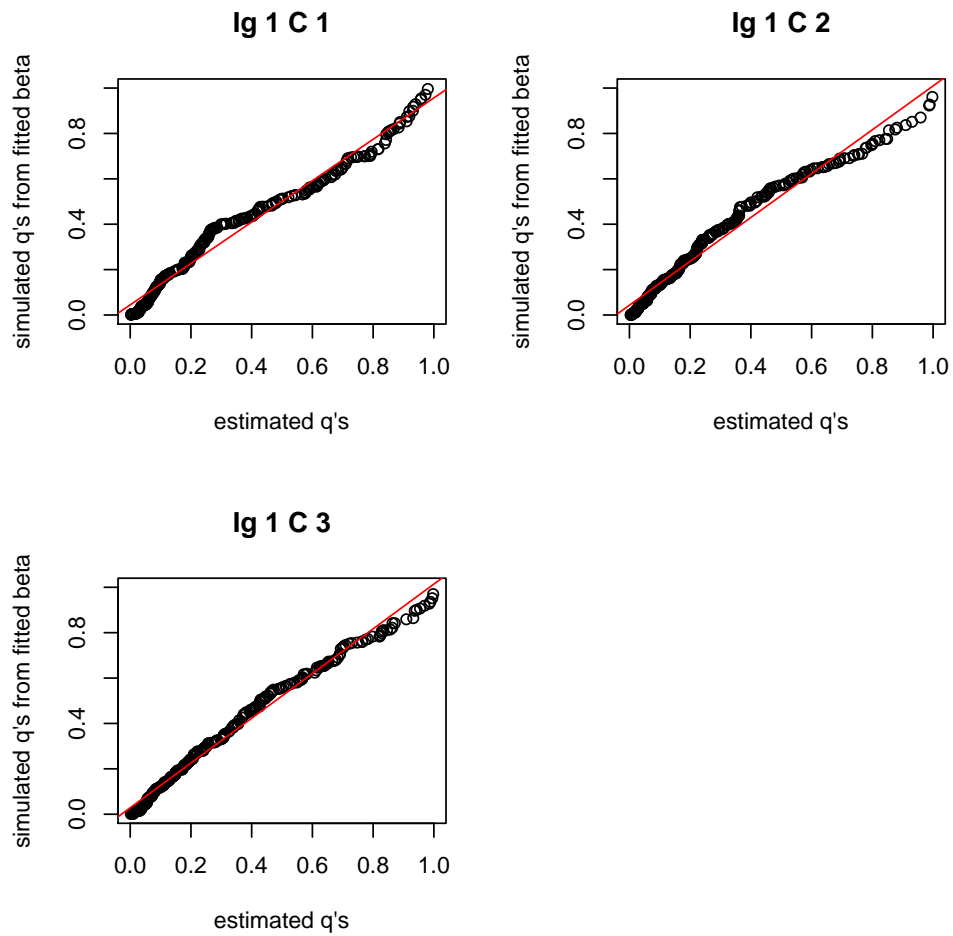


Figure 10: QQ-plots of the fitted prior distributions within each condition and each Ig group

```
> par(mfrow=c(2,2))
> DenNHist(MultiOut)
```

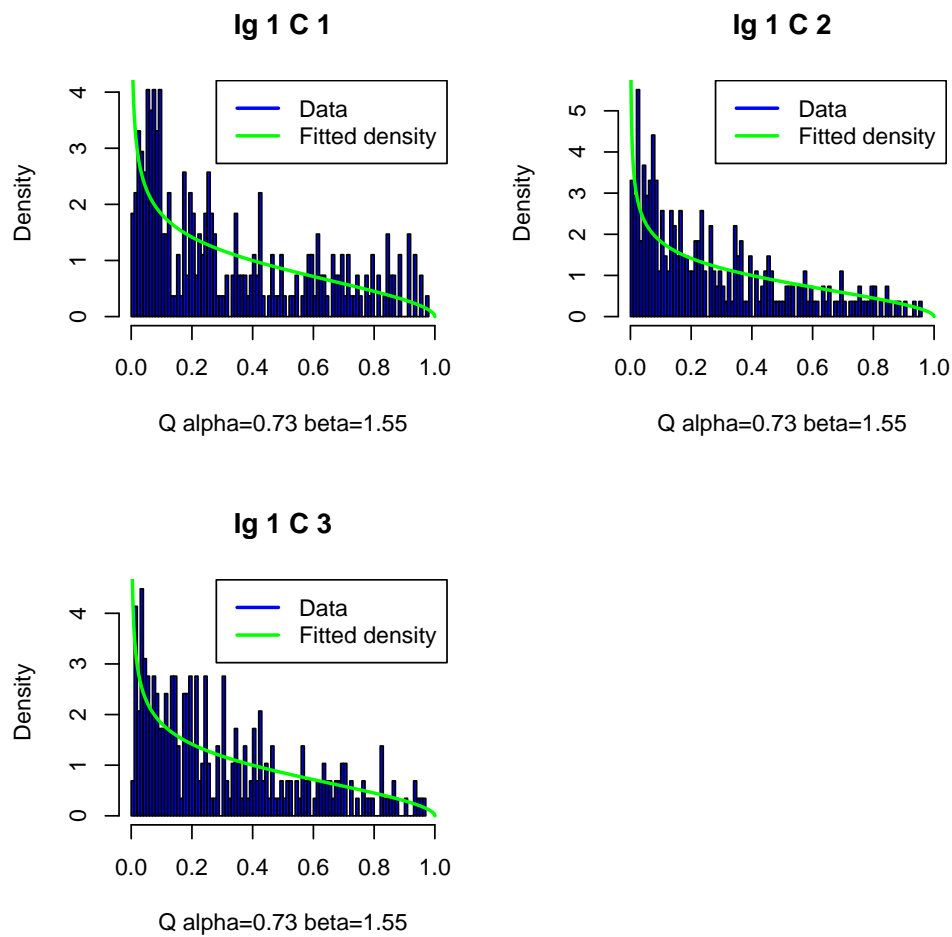


Figure 11: Prior distributions fit within each condition. (Note only a small set of genes are considered here for demonstration. Better fitting should be expected while using full set of genes.)

## 5.4 Isoform level DE analysis (more than two conditions)

Similar to Section 4.3, the function `GetPatterns` allows a user to generate all possible patterns given the conditions. To visualize the patterns, the function `PlotPattern` may be used.

```
> Conditions=c("C1","C1","C2","C2","C3","C3","C4","C4")
> PosParti.4Cond=GetPatterns(Conditions)
> PosParti.4Cond
```

	C1	C2	C3	C4
Pattern1	1	1	1	1
Pattern2	1	1	1	2
Pattern3	1	1	2	1
Pattern4	1	1	2	2
Pattern5	1	2	1	1
Pattern6	1	2	1	2
Pattern7	1	2	2	1
Pattern8	1	2	2	2
Pattern9	1	1	2	3
Pattern10	1	2	1	3
Pattern11	1	2	2	3
Pattern12	1	2	3	1
Pattern13	1	2	3	2
Pattern14	1	2	3	3
Pattern15	1	2	3	4

```

> PlotPattern(PosParti.4Cond)
> Parti.4Cond=PosParti.4Cond[c(1,2,3,8,15),]
> Parti.4Cond

```

	C1	C2	C3	C4
Pattern1	1	1	1	1
Pattern2	1	1	1	2
Pattern3	1	1	2	1
Pattern8	1	2	2	2
Pattern15	1	2	3	4

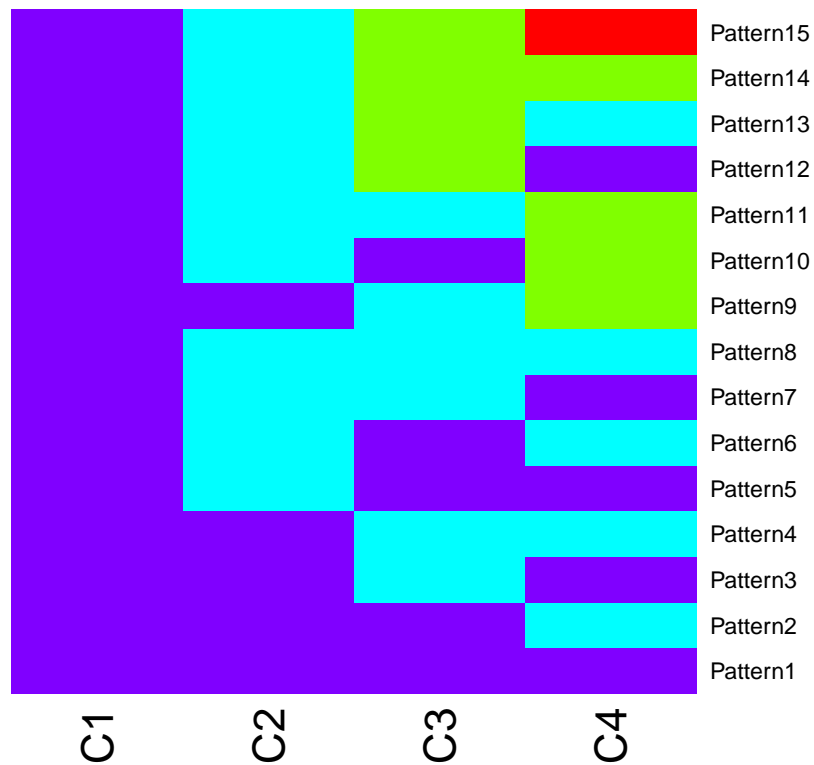


Figure 12: All possible patterns for 4 conditions

```

> data(IsoMultiList)
> IsoMultiMat=IsoMultiList[[1]]
> IsoNames.Multi=IsoMultiList$IsoNames
> IsosGeneNames.Multi=IsoMultiList$IsosGeneNames
> IsoMultiSize=MedianNorm(IsoMultiMat)
> NgList.Multi=GetNg(IsoNames.Multi, IsosGeneNames.Multi)
> IsoNgTrun.Multi=NgList.Multi$IsoformNgTrun
> IsoMultiOut=EBMultiTest(IsoMultiMat,NgVector=IsoNgTrun.Multi,Conditions=Conditions,
+ AllParti=Parti.4Cond,
+ sizeFactors=IsoMultiSize, maxround=5)
> IsoMultiPP=GetMultiPP(IsoMultiOut)

```

```

> names(MultiPP)

```

```

[1] "PP"      "MAP"      "Patterns"

```

```

> IsoMultiPP$PP[1:10,]

```

	Pattern1	Pattern2	Pattern3	Pattern8	Pattern15
Iso_1_1	3.533233e-32	0.999882138	3.408808e-33	2.143838e-34	1.178620e-04
Iso_1_2	4.231331e-14	0.999826487	1.573392e-16	5.848567e-18	1.735129e-04
Iso_1_3	5.633772e-47	0.992627423	5.963569e-42	5.644910e-50	7.372577e-03
Iso_1_4	4.248398e-35	0.998959777	1.983567e-30	5.054181e-33	1.040223e-03
Iso_1_5	0.000000e+00	1.000000000	0.000000e+00	0.000000e+00	1.584343e-41
Iso_1_6	1.509151e-232	0.002646919	3.147566e-220	6.720686e-188	9.973531e-01
Iso_1_7	2.835263e-138	0.999439469	7.548859e-133	1.613556e-128	5.605313e-04
Iso_1_8	9.654898e-139	0.963893542	3.709303e-105	5.626105e-120	3.610646e-02
Iso_1_9	1.947187e-47	0.957423511	1.073683e-50	3.868129e-46	4.257649e-02
Iso_1_10	7.904509e-08	0.999790300	9.178739e-10	9.386672e-10	2.096196e-04

```

> IsoMultiPP$MAP[1:10]

```

Iso_1_1	Iso_1_2	Iso_1_3	Iso_1_4	Iso_1_5	Iso_1_6
"Pattern2"	"Pattern2"	"Pattern2"	"Pattern2"	"Pattern2"	"Pattern15"
Iso_1_7	Iso_1_8	Iso_1_9	Iso_1_10		
"Pattern2"	"Pattern2"	"Pattern2"	"Pattern2"		

```

> IsoMultiPP$Patterns

```

	C1	C2	C3	C4
Pattern1	1	1	1	1
Pattern2	1	1	1	2
Pattern3	1	1	2	1
Pattern8	1	2	2	2
Pattern15	1	2	3	4

```

> IsoMultiFC=GetMultiFC(IsoMultiOut)

```

The FC and posterior FC for multiple condition data can be obtained by the function `GetMultiFC`:  
To generate a QQ-plot of the fitted Beta prior distribution and the  $\hat{q}^C$ 's within condition, a user could also use the functions `DenNHist` and `QQP`.

```

> par(mfrow=c(3,4))
> QQP(IsoMultiOut)
>

```

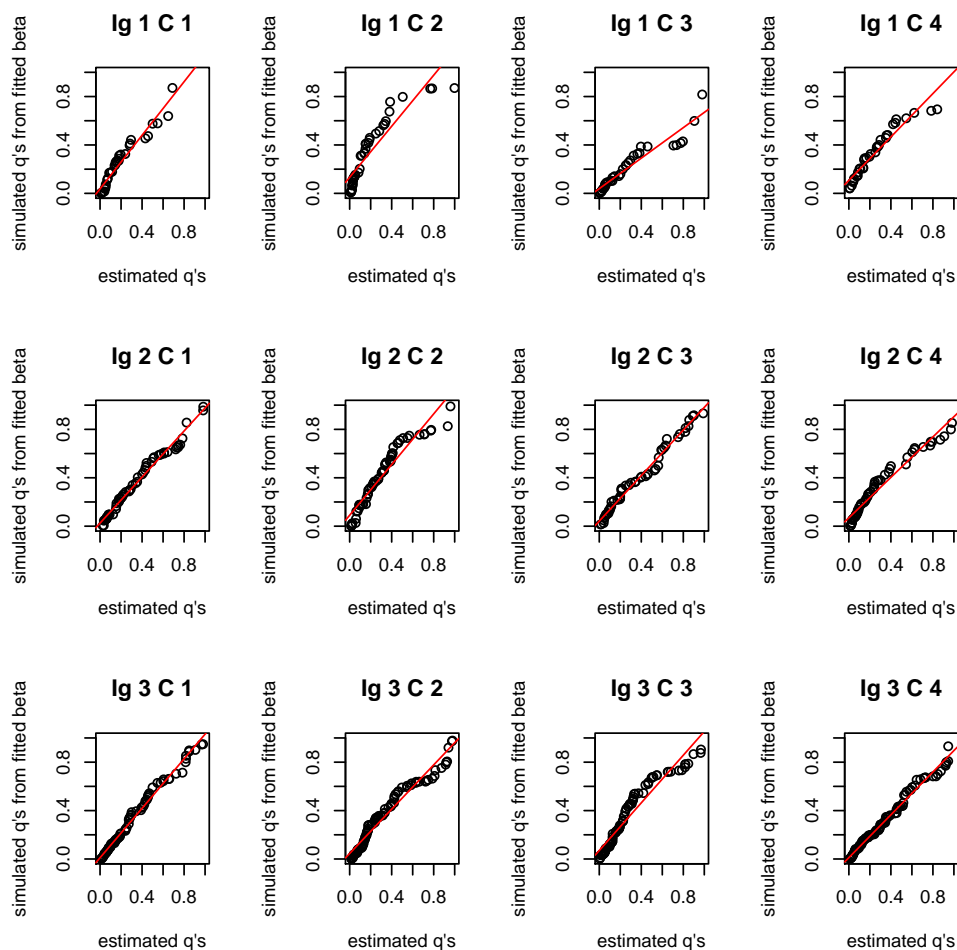


Figure 13: QQ-plots of the fitted prior distributions within each condition and Ig group. (Note only a small set of isoforms are considered here for demonstration. Better fitting should be expected while using full set of isoforms.)



```
> par(mfrow=c(3,4))
> DenNHist(IsoMultiOut)
```

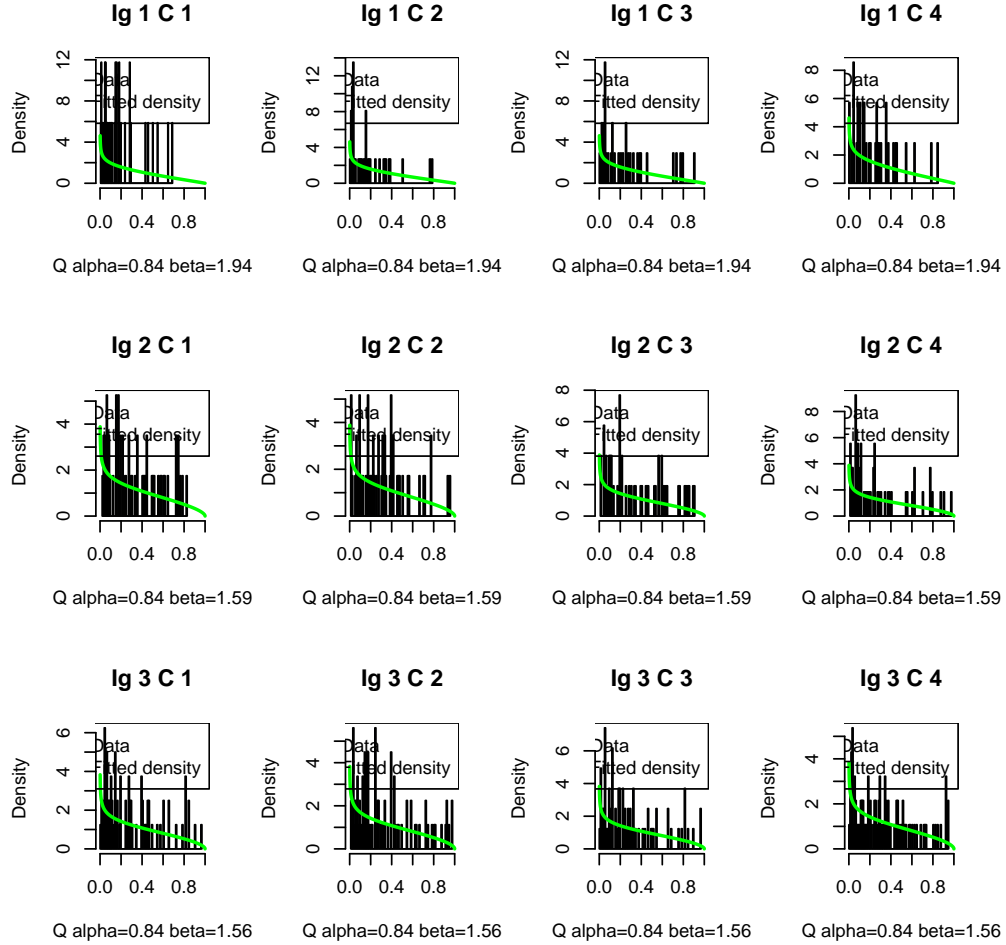


Figure 14: Prior distributions fit within each condition and Ig group. (Note only a small set of isoforms are considered here for demonstration. Better fitting should be expected while using full set of isoforms.)

## 5.5 Working without replicates

When replicates are not available, it is difficult to estimate the transcript specific variance. In this case, EBSeq estimates the variance by pooling similar genes together. Specifically, we take genes with FC in the 25% - 75% quantile of all FC's as candidate genes. By defining `NumBin = 1000` (default in `EBTest`), EBSeq will group genes with similar means into 1,000 bins. For each candidate gene, we use the across-condition variance estimate as its variance estimate. For each bin, the bin-wise variance estimation is taken to be the median of the across-condition variance estimates of the candidate genes within that bin. For each non-candidate gene, we use the bin-wise variance estimate of the host bin (the bin containing this gene) as its variance estimate. This approach works well when there are no more than 50% DE genes in the data set.

### 5.5.1 Gene counts with two conditions

To generate a data set with no replicates, we take the first sample of each condition. For example, using the data from Section 5.1, we take sample 1 from condition 1 and sample 6 from condition 2. Functions `MedianNorm`, `GetPPMat` and `PostFC` may be used on data without replicates.

```
> data(GeneMat)
> GeneMat.norep=GeneMat[,c(1,6)]
> Sizes.norep=MedianNorm(GeneMat.norep)
> EBOut.norep=EBTest(Data=GeneMat.norep,
+ Conditions=as.factor(rep(c("C1","C2"))),
+ sizeFactors=Sizes.norep, maxround=5)
```

Removing transcripts with 75 th quantile < = 10  
938 transcripts will be tested

```
> PP.norep=GetPPMat(EBOut.norep)
> DEfound.norep=rownames(PP.norep)[which(PP.norep[, "PPDE"]>=.95)]
> GeneFC.norep=PostFC(EBOut.norep)
```

### 5.5.2 Isoform counts with two conditions

To generate an isoform level data set with no replicates, we also take sample 1 and sample 6 in the data we used in Section 5.2. Example codes are shown below.

```
> data(IsoList)
> IsoMat=IsoList$IsoMat
> IsoNames=IsoList$IsoNames
> IsosGeneNames=IsoList$IsosGeneNames
> NgList=GetNg(IsoNames, IsosGeneNames)
> IsoNgTrun=NgList$IsoformNgTrun
> IsoMat.norep=IsoMat[,c(1,6)]
> IsoSizes.norep=MedianNorm(IsoMat.norep)
> IsoEBOut.norep=EBTest(Data=IsoMat.norep, NgVector=IsoNgTrun,
+ Conditions=as.factor(c("C1","C2")),
+ sizeFactors=IsoSizes.norep, maxround=5)
```

Removing transcripts with 75 th quantile < = 10  
1088 transcripts will be tested

```
> IsoPP.norep=GetPPMat(IsoEBOut.norep)
> IsoDE.norep=rownames(IsoPP.norep)[which(IsoPP.norep[, "PPDE"]>=.95)]
> IsoFC.norep=PostFC(IsoEBOut.norep)
```

### 5.5.3 Gene counts with more than two conditions

To generate a data set with multiple conditions and no replicates, we take the first sample from each condition (sample 1, 3 and 5) in the data we used in Section 5.3. Example codes are shown below.

```
> data(MultiGeneMat)
> MultiGeneMat.norep=MultiGeneMat[,c(1,3,5)]
> Conditions=c("C1","C2","C3")
> PosParti=GetPatterns(Conditions)
> Parti=PosParti[-3,]
> MultiSize.norep=MedianNorm(MultiGeneMat.norep)
> MultiOut.norep=EBMultiTest(MultiGeneMat.norep,
+ NgVector=NULL,Conditions=Conditions,
+ AllParti=Parti, sizeFactors=MultiSize.norep,
+ maxround=5)
```

Removing transcripts with 75 th quantile < = 10  
492 transcripts will be tested

```
> MultiPP.norep=GetMultiPP(MultiOut.norep)
> MultiFC.norep=GetMultiFC(MultiOut.norep)
```

### 5.5.4 Isoform counts with more than two conditions

To generate an isoform level data set with multiple conditions and no replicates, we take the first sample from each condition (sample 1, 3, 5 and 7) in the data we used in Section 5.4. Example codes are shown below.

```
> data(IsoMultiList)
> IsoMultiMat=IsoMultiList[[1]]
> IsoNames.Multi=IsoMultiList$IsoNames
> IsosGeneNames.Multi=IsoMultiList$IsosGeneNames
> IsoMultiMat.norep=IsoMultiMat[,c(1,3,5,7)]
> IsoMultiSize.norep=MedianNorm(IsoMultiMat.norep)
> NgList.Multi=GetNg(IsoNames.Multi, IsosGeneNames.Multi)
> IsoNgTrun.Multi=NgList.Multi$IsoformNgTrun
> Conditions=c("C1","C2","C3","C4")
> PosParti.4Cond=GetPatterns(Conditions)
> PosParti.4Cond
```

	C1	C2	C3	C4
Pattern1	1	1	1	1
Pattern2	1	1	1	2
Pattern3	1	1	2	1
Pattern4	1	1	2	2
Pattern5	1	2	1	1
Pattern6	1	2	1	2
Pattern7	1	2	2	1
Pattern8	1	2	2	2
Pattern9	1	1	2	3
Pattern10	1	2	1	3
Pattern11	1	2	2	3
Pattern12	1	2	3	1
Pattern13	1	2	3	2

```

Pattern14  1  2  3  3
Pattern15  1  2  3  4

> Parti.4Cond=PosParti.4Cond[c(1,2,3,8,15),]
> Parti.4Cond

      C1 C2 C3 C4
Pattern1  1  1  1  1
Pattern2  1  1  1  2
Pattern3  1  1  2  1
Pattern8  1  2  2  2
Pattern15  1  2  3  4

> IsoMultiOut.norep=EBMultiTest(IsoMultiMat.norep,
+ NgVector=IsoNgTrun.Multi,Conditions=Conditions,
+ AllParti=Parti.4Cond, sizeFactors=IsoMultiSize.norep,
+ maxround=5)

Removing transcripts with 75 th quantile < = 10
293 transcripts will be tested

> IsoMultiPP.norep=GetMultiPP(IsoMultiOut.norep)
> IsoMultiFC.norep=GetMultiFC(IsoMultiOut.norep)

```

## 6 EBSeq pipelines and extensions

### 6.1 RSEM-EBSeq pipeline: from raw reads to differential expression analysis results

EBSeq is coupled with RSEM [4] as an RSEM-EBSeq pipeline which provides quantification and DE testing on both gene and isoform levels.

For more details, see <http://deweylab.biostat.wisc.edu/rsem/README.html#de>

### 6.2 EBSeq interface: A user-friendly graphical interface for differential expression analysis

EBSeq interface provides a graphical interface implementation for users who are not familiar with the R programming language. It takes .xls, .xlsx and .csv files as input. Additional packages need be downloaded; they may be found at [http://www.biostat.wisc.edu/~ningleng/EBSeq\\_Package/EBSeq\\_Interface/](http://www.biostat.wisc.edu/~ningleng/EBSeq_Package/EBSeq_Interface/)

### 6.3 EBSeq Galaxy tool shed

EBSeq tool shed contains EBSeq wrappers for a local Galaxy implementation. For more details, see [http://www.biostat.wisc.edu/~ningleng/EBSeq\\_Package/EBSeq\\_Galaxy\\_toolshed/](http://www.biostat.wisc.edu/~ningleng/EBSeq_Package/EBSeq_Galaxy_toolshed/)

## 7 Acknowledgment

We would like to thank Haolin Xu for checking the package and proofreading the vignette.

## 8 News

2014-1-30: In EBSeq 1.3.3, the default setting of EBTest function will remove low expressed genes (genes whose 75th quantile of normalized counts is less than 10) before identifying DE genes. These two thresholds can be changed in EBTest function. We found that low expressed genes are more easily to be affected by noises. Removing these genes prior to downstream analyses can improve the model fitting and reduce impacts of noisy genes (e.g. genes with outliers).

2014-5-22: In EBSeq 1.5.2, numerical approximations are implemented to deal with underflow. The underflow is likely due to large number of samples.

## References

- [1] S Anders and W Huber. Differential expression analysis for sequence count data. *Genome Biology*, 11:R106, 2010.
- [2] J H Bullard, E A Purdom, K D Hansen, and S Dudoit. Evaluation of statistical methods for normalization and differential expression in mrna-seq experiments. *BMC Bioinformatics*, 11:94, 2010.
- [3] Ning Leng, John A Dawson, James A Thomson, Victor Ruotti, Anna I Rissman, Bart MG Smits, Jill D Haag, Michael N Gould, Ron M Stewart, and Christina Kendzierski. Ebseq: an empirical bayes hierarchical model for inference in rna-seq experiments. *Bioinformatics*, 29(8):1035–1043, 2013.
- [4] B Li and C N Dewey. Rsem: accurate transcript quantification from rna-seq data with or without a reference genome. *BMC Bioinformatics*, 12:323, 2011.
- [5] M D Robinson and A Oshlack. A scaling normalization method for differential expression analysis of rna-seq data. *Genome Biology*, 11:R25, 2010.
- [6] C Trapnell, A Roberts, L Goff, G Pertea, D Kim, D R Kelley, H Pimentel, S L Salzberg, J L Rinn, and L Pachter. Differential gene and transcript expression analysis of rna-seq experiments with tophat and cufflinks. *Nature Protocols*, 7(3):562–578, 2012.