

TCC: Differential expression analysis for tag count data with robust normalization strategies

Jianqiang Sun^{1§}, Tomoaki Nishiyama^{2§}, Kentaro Shimizu¹, and Koji Kadota¹

¹ The University of Tokyo, Tokyo, Japan

² Kanazawa University, Kanazawa, Japan

§ Maintainer: Jianqiang Sun (wukong@bi.a.u-tokyo.ac.jp),

Tomoaki Nishiyama (tomoakin@staff.kanazawa-u.ac.jp)

April 12, 2014

Abstract

The R/Bioconductor package, **TCC**, provides users with a robust and accurate framework to perform differential expression (DE) analysis of tag count data. We recently developed a multi-step normalization method (TbT; Kadota et al., 2012) for two-group RNA-seq data. The strategy (called DEGES) is to remove data that are potential differentially expressed genes (DEGs) before performing the data normalization. DEGES in **TCC** is essential for accurate normalization of tag count data, especially when the up- and down-regulated DEGs in one of the groups are extremely biased in their number. A major characteristic of **TCC** is to provide the DEGES-based normalization methods for several kinds of count data (two-group with or without replicates, multi-group, and so on) by virtue of the use of combinations of functions in other sophisticated packages (especially **edgeR**, **DESeq**, and **baySeq**). The appropriate combination provided by **TCC** allows a more robust and accurate estimation to be performed more easily than directly using original packages and **TCC** provides a simple unified interface to perform the robust normalization.

Contents

1	Introduction	4
1.1	Installation	4
1.2	Citations	5
1.3	Quick start	5
2	Preparations	7
2.1	Preparing the count data	7
2.2	Reading the count data	7
2.3	Constructing TCC class object	8
2.4	Filtering low-count genes (optional)	9
3	Normalization	10
3.1	Normalization of two-group count data with replicates	11
3.1.1	DEGES/TbT	11
3.1.2	DEGES/edgeR	13
3.1.3	iDEGES/edgeR	14
3.1.4	DEGES/DESeq	14
3.1.5	DEGES/DESeq2	15
3.2	Normalization of two-group count data without replicates	16
3.2.1	DEGES/edgeR	17
3.2.2	DEGES/DESeq	18
3.3	Normalization of two-group count data without replicates (paired)	19
3.3.1	DEGES/edgeR	19
3.3.2	DEGES/DESeq	20
3.4	Normalization of multi-group count data with replicates	21
3.4.1	DEGES/TbT	22
3.4.2	DEGES/edgeR	23
3.4.3	DEGES/DESeq	24
3.5	Retrieving normalized data	26
3.5.1	Retrieving two-group DEGES/edgeR-normalized data with replicates	27
3.5.2	Retrieving two-group DEGES/DESeq-normalized data with replicates	29
3.5.3	Retrieving two-group DEGES/DESeq-normalized data without replicates	31
3.5.4	Retrieving two-group DEGES/edgeR-normalized data without replicates (paired)	32
3.5.5	Retrieving two-group DEGES/DESeq-normalized data without replicates (paired)	35
3.5.6	Retrieving multi-group iDEGES/edgeR-normalized data with replicates	37
4	Differential expression (DE)	39
4.1	DE analysis for two-group data with replicates	39
4.1.1	edgeR coupled with iDEGES/edgeR normalization	39
4.1.2	DESeq2 coupled with iDEGES/DESeq2 normalization	41
4.2	DE analysis for two-group data without replicates	46
4.2.1	edgeR coupled with iDEGES/edgeR normalization	46
4.2.2	DESeq2 coupled with iDEGES/DESeq2 normalization	49
4.3	DE analysis for two-group data without replicates (paired)	51
4.3.1	edgeR coupled with iDEGES/edgeR normalization	52

4.4	DE analysis for multi-group data with replicates	55
4.4.1	baySeq coupled with DEGES/edgeR normalization	55
4.4.2	edgeR coupled with DEGES/edgeR normalization	56
4.4.3	DESeq coupled with DEGES/edgeR normalization	58
5	Generation of simulation data	60
5.1	Introduction and basic usage	60
5.2	Two-group data without replicates	63
5.3	Multi-group data with and without replicates	64
5.4	Multi-factor data	67
5.5	Other utilities	69
6	Session info	73
7	References	74

1 Introduction

Differential expression analysis based on tag count data has become a fundamental task for identifying differentially expressed genes or transcripts (DEGs). The **TCC** package (Tag Count Comparison; Sun et al., 2013) provides users with a robust and accurate framework to perform differential expression analysis of tag count data. **TCC** provides integrated analysis pipelines with improved data normalization steps, compared with other packages such as **edgeR**, **DESeq**, and **baySeq**, by appropriately combining their functionalities. The package incorporates multi-step normalization methods whose strategy is to remove data that are potential DEGs before performing the data normalization.

Kadota et al. (2012) recently reported that the normalization methods implemented in R packages (such as **edgeR** (Robinson et al., 2010), **DESeq** (Anders and Huber, 2010), and **baySeq** (Hardcastle and Kelly, 2010)) for differential expression (DE) analysis between samples are inadequate when the up- and down-regulated DEGs in one of the samples are extremely biased in their number (i.e., biased DE). This is because the current methods implicitly assume a balanced DE, wherein the numbers of highly and lowly expressed DE entities in samples are (nearly) equal. As a result, methods assuming unbiased DE will not work well on data with biased DE. Although a major purpose of data normalization is to detect such DE entities, their existence themselves consequently interferes with their opportunity to be top-ranked. Conventional procedures for identifying DEGs from tag count data consisting of two steps (i.e., data normalization and identification of DEGs) cannot in principle eliminate the potential DE entities before data normalization.

To normalize data that potentially has various scenarios (including unbiased and biased DE), we recently proposed a multi-step normalization strategy (called TbT, an acronym for the TMM-baySeq-TMM pipeline; Kadota et al., 2012), in which the TMM normalization method (Robinson and Oshlack, 2010) is used in steps 1 and 3 and an empirical Bayesian method implemented in the **baySeq** package (Hardcastle and Kelly, 2010) is used in step 2. Although this multi-step DEG elimination strategy (called "DEGES" for short) can successfully remove potential DE entities identified in step 2 prior to the estimation of the normalization factors using the TMM normalization method in step 3, the **baySeq** package used in step 2 of the TbT method is much more computationally intensive than competing packages like **edgeR** and **DESeq**. While the three-step TbT normalization method performed best on simulated and real tag count data, it is practically possible to make different choices for the methods in each step. A more comprehensive study regarding better choices for DEGES is needed.

This package provides tools to perform multi-step normalization methods based on DEGES and enables differential expression analysis of tag count data without having to worry much about biased distributions of DEGs. The DEGES-based normalization function implemented in **TCC** includes the TbT method based on DEGES for two-group data with or without replicates, much faster method, and methods for multi-group comparison. **TCC** provides a simple unified interface to perform data normalization with combinations of functions provided by **baySeq**, **DESeq**, and **edgeR**. Functions to produce simulation data under various conditions and to plot the data are also provided.

1.1 Installation

This package is available from the Bioconductor website (<http://bioconductor.org/>). To install the package, enter the following command after starting R:

```
> source("http://bioconductor.org/biocLite.R")
> biocLite("TCC")
```

1.2 Citations

This package internally uses many of the functions implemented in the other packages. This is because our normalization procedures consist, in part, of combinations of existing normalization methods and differential expression (DE) methods.

For example, the TbT normalization method (Kadota et al., 2012), which is a particular functionality of the **TCC** package (Sun et al., 2013), consists of the TMM normalization method (Robinson and Oshlack, 2010) implemented in the **edgeR** package (Robinson et al., 2010) and the empirical Bayesian method implemented in the **baySeq** package (Hardcastle and Kelly, 2010). Therefore, please cite the appropriate references when you publish your results.

```
> citation("TCC")
```

1.3 Quick start

Let us begin by showing two examples (Cases 1 and 2) of identifying DEGs between two groups from tag count data consisting of 1,000 genes and a total of six samples (each group has three biological replicates). The hypothetical count data (termed "hypoData") is stored in this package (for details, see section 2.1). We then describe the DE analysis of count data without replicates (i.e., two samples), using the data of the first and the fourth column of hypoData (Case 3). We recommend the use of commands in Cases 2 and 3.

Case 1: DE analysis of two-group count data with replicates by using the exact test (Robinson and Smyth, 2008) in **edgeR** coupled with TbT normalization (termed the TbT-edgeR combination). The **TCC** package was originally designed with the TbT normalization method, and the original study (Kadota et al., 2012) recommended this analysis pipeline. Note that a smaller sampling size (i.e., `samplesize = 100`) is used here to reduce the computation time, but a larger sampling size of around 10,000 (i.e., `samplesize = 10000`) is recommended (Hardcastle and Kelly, 2010). Suggested citations are as follows: **TCC** (Sun et al., 2013), TbT (Kadota et al., 2012), TMM (Robinson and Oshlack, 2010), **baySeq** (Hardcastle and Kelly, 2010), and **edgeR** (Robinson et al., 2010). For details, see section 3.1.1.

```
> library(TCC)
> data(hypoData)
> samplesize <- 100
> group <- c(1, 1, 1, 2, 2, 2)
> tcc <- new("TCC", hypoData, group)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "bayseq",
+                       iteration = 1, samplesize = samplesize)
> tcc <- estimateDE(tcc, test.method = "edgeR", FDR = 0.1)
> result <- getResult(tcc, sort = TRUE)
> head(result)
```

	gene_id	a.value	m.value	p.value	q.value	rank	estimatedDEG
151	gene_151	9.736206	-2.730725	5.750175e-11	5.750175e-08	1	1
39	gene_39	7.110901	-2.438568	1.131188e-09	3.847255e-07	2	1
599	gene_599	5.924266	-3.264398	1.154176e-09	3.847255e-07	3	1
68	gene_68	6.209301	-2.848813	7.905703e-09	1.976426e-06	4	1
144	gene_144	7.588410	-2.108239	1.497165e-08	2.632978e-06	5	1
175	gene_175	7.985570	-2.349050	1.579787e-08	2.632978e-06	6	1

Case 2: DE analysis for two-group count data with replicates by using the exact test coupled with iterative DEGES/edgeR normalization (i.e., the iDEGES/edgeR-edgeR combination). This is an alternative pipeline designed to reduce the runtime (approx. 20 sec.), yet its performance is comparable to the above pipeline. Accordingly, we recommend using this pipeline as a default when analyzing tag count data with replicates. A notable advantage of this pipeline is that the multi-step normalization strategy only needs the methods implemented in the **edgeR** package. The suggested citations are as follows: **TCC** (Sun et al., 2013), **TMM** (Robinson and Oshlack, 2010), the exact test (Robinson and Smyth, 2008), and **edgeR** (Robinson et al., 2010). For details, see section 3.1.3 and 4.1.1.

```
> library(TCC)
> data(hypoData)
> group <- c(1, 1, 1, 2, 2, 2)
> tcc <- new("TCC", hypoData, group)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edger",
+                       iteration = 3, FDR = 0.1, floorPDEG = 0.05)
> tcc <- estimateDE(tcc, test.method = "edger", FDR = 0.1)
> result <- getResult(tcc, sort = TRUE)
> head(result)
```

	gene_id	a.value	m.value	p.value	q.value	rank	estimatedDEG
151	gene_151	9.736785	-2.753816	4.641083e-11	4.641083e-08	1	1
39	gene_39	7.110842	-2.460691	9.115042e-10	3.388777e-07	2	1
599	gene_599	5.927173	-3.282264	1.016633e-09	3.388777e-07	3	1
68	gene_68	6.209395	-2.867694	4.776945e-09	1.194236e-06	4	1
175	gene_175	7.984265	-2.373657	1.082493e-08	1.810608e-06	5	1
144	gene_144	7.588164	-2.130092	1.086365e-08	1.810608e-06	6	1

Case 3: DE analysis for two-group count data without replicates by using the negative binomial (NB) test in **DESeq** coupled with iDEGES/DESeq normalization (i.e., the iDEGES/DESeq-DESeq combination). A procedure using the data of the first and fourth columns of `hypoData` is shown here. Similar to Case 2, this pipeline entirely consists of methods implemented in the **DESeq** package. Suggested citations are as follows: **TCC** (Sun et al., 2013) and **DESeq** (Anders and Huber, 2010). For details, see section 3.2.

```
> library(TCC)
> data(hypoData)
> group <- c(1, 2)
> tcc <- new("TCC", hypoData[,c(1,4)], group)
> tcc <- calcNormFactors(tcc, norm.method = "deseq", test.method = "deseq",
+                       iteration = 3, FDR = 0.1, floorPDEG = 0.05)
> tcc <- estimateDE(tcc, test.method = "deseq", FDR = 0.1)
> result <- getResult(tcc, sort = TRUE)
> head(result)
```

	gene_id	a.value	m.value	p.value	q.value	rank	estimatedDEG
36	gene_36	-0.9988563	-8.525340	0.0001967999	0.1867631	1	0
17	gene_17	5.9635499	-5.470058	0.0061694868	1.0000000	2	0
5	gene_5	3.3084986	-6.499805	0.0175001020	1.0000000	3	0
989	gene_989	-0.9988563	-5.839291	0.0238080262	1.0000000	4	0
187	gene_187	3.6973024	5.507222	0.0305801058	1.0000000	5	0
822	gene_822	-0.9988563	5.436833	0.0496379836	1.0000000	6	0

2 Preparations

2.1 Preparing the count data

Similar to the other packages, **TCC** typically starts the DE analysis with a count table matrix where each row indicates a gene (or transcript), each column indicates a sample (or library), and each cell indicates the number of counts for a gene in a sample. There are many ways to obtain the count data from aligned read files such as BAM (Binary Alignment/Map). This includes the `islandCounts` function in **htSeqTools** (Planet et al., 2012), `summarizeOverlaps` in **GenomicRanges** (Lawrence et al., 2013), `qCount` in **QuasR**, and so on. For Windows end users, we recommend to use the **QuasR** package. It provides a comprehensive workflow for many kinds of NGS data including ChIP-seq, RNA-seq, smallRNA-seq, and BS-seq. The main functionalities are: (1) preprocessing raw sequence reads, (2) aligning reads to the reference genome or transcriptome using **Rbowtie**, and (3) producing count matrix from aligned reads. **TCC** uses the raw count data (a matrix of integer values) as input. As also clearly mentioned in **DESeq2**, the raw count data corresponds to a matrix of integer values. Please DO NOT use any normalized counts such as RPM (reads per million), CPM (counts per million), and RPKM.

2.2 Reading the count data

Here, we assume a hypothetical count matrix consisting of 1,000 rows (or genes) and a total of six columns: the first three columns are produced from biological replicates of Group 1 (G1_rep1, G1_rep2, and G1_rep3) and the remaining columns are from Group 2 (G2_rep1, G2_rep2, and G2_rep3). We start by loading the hypothetical data (`hypoData`) from **TCC** and giving a numeric vector (`group`) indicating which group each sample belongs to.

```
> library(TCC)
> data(hypoData)
> head(hypoData)
```

	G1_rep1	G1_rep2	G1_rep3	G2_rep1	G2_rep2	G2_rep3
gene_1	34	45	122	16	14	29
gene_2	358	388	22	36	25	68
gene_3	1144	919	990	374	480	239
gene_4	0	0	44	18	0	0
gene_5	98	48	17	1	8	5
gene_6	296	282	216	86	62	69

```
> dim(hypoData)
```

```
[1] 1000 6
```

```
> group <- c(1, 1, 1, 2, 2, 2)
```

If you want to analyze another count matrix consisting of nine columns (e.g., the first four columns are produced from biological replicates of G1, and the remaining five columns are from G2), the `group` vector should be indicated as follows.

```
> group <- c(1, 1, 1, 1, 2, 2, 2, 2, 2)
```

2.3 Constructing TCC class object

The `new` function has to be used to perform the main functionalities of **TCC**. This function constructs a **TCC** class object, and subsequent analyses are performed on this class object. The object is constructed from i) a count matrix (`hypoData`) and ii) the corresponding numeric vector (`group`) as follows.

```
> library(TCC)
> data(hypoData)
> group <- c(1, 1, 1, 2, 2, 2)
> tcc <- new("TCC", hypoData, group)
> tcc
```

```
Count:
      G1_rep1 G1_rep2 G1_rep3 G2_rep1 G2_rep2 G2_rep3
gene_1      34      45     122      16      14      29
gene_2     358     388      22      36      25      68
gene_3    1144     919     990     374     480     239
gene_4         0         0      44      18         0         0
gene_5         98         48      17         1         8         5
gene_6        296        282     216         86         62         69
```

```
Sample:
      group norm.factors lib.sizes
G1_rep1     1           1  142177
G1_rep2     1           1  145289
G1_rep3     1           1  149886
G2_rep1     2           1  112100
G2_rep2     2           1  104107
G2_rep3     2           1  101975
```

The count matrix and group vector information can be retrieved from the stored class object by using `tcc$count` and `tcc$group`, respectively.

```
> head(tcc$count)
```

```
      G1_rep1 G1_rep2 G1_rep3 G2_rep1 G2_rep2 G2_rep3
gene_1      34      45     122      16      14      29
gene_2     358     388      22      36      25      68
gene_3    1144     919     990     374     480     239
gene_4         0         0      44      18         0         0
gene_5         98         48      17         1         8         5
gene_6        296        282     216         86         62         69
```

```
> tcc$group
```

```
      group
G1_rep1     1
G1_rep2     1
G1_rep3     1
G2_rep1     2
G2_rep2     2
G2_rep3     2
```


2.4 Filtering low-count genes (optional)

The way to filter out genes with low-count tags across samples depends on the user's philosophy. Although we recommend removing tags with zero counts across samples as a minimum filtering, this effort is optional. The `filterLowCountGenes` function performs this filtering.

```
> library(TCC)
> data(hypoData)
> group <- c(1, 1, 1, 2, 2, 2)
> tcc <- new("TCC", hypoData, group)
> tcc <- filterLowCountGenes(tcc)
> dim(tcc$count)
```

```
[1] 996  6
```

It can be seen that 4(= 1000 – 996) genes were filtered as non-expressed. The same procedure can be performed without the `filterLowCountGenes` function, in which case the filtering is performed before the **TCC** class object is constructed.

```
> filter <- as.logical(rowSums(hypoData) > 0)
> dim(hypoData[filter, ])
```

```
[1] 996  6
```

```
> tcc <- new("TCC", hypoData[filter, ], group)
> dim(tcc$count)
```

```
[1] 996  6
```

3 Normalization

This chapter describes the details of our robust normalization strategy (i.e., DEGES) implemented in **TCC**. Alternative DEGES procedures consisting of functions in other packages (**edgeR**, **DESeq**, and **baySeq**) are also provided, enabling *advanced* users familiar with the existing packages can easily learn what is done in **TCC**. Note that *end* users, who just want to perform robust differential expression analysis using **TCC**, can skip this chapter (3 Normalization) and move from here to, for example, the next chapter (4 Differential expression). Note also that the purpose here is to obtain accurate normalization factors to be used with statistical models (e.g., the exact test or empirical Bayes) for the DE analysis described in the next section (4 Differential expression). **TCC** can manipulate various kinds of experimental designs. Followings are some examples for individual designs. Appropriate sections should be referred for your specific experimental designs.

Table 1: 3.1 unpaired samples (two-group)

Label	Example 1	Example 2	Label	Example 3	Example 4
G1_rep1	G1(mouse_A)	Tumor(patient_A)	G1_rep1	G1(mouse_A)	Tumor(patient_A)
G1_rep2	G1(mouse_B)	Tumor(patient_B)	G1_rep2	G1(mouse_B)	Tumor(patient_B)
G1_rep3	G1(mouse_C)	Tumor(patient_C)	G2_rep1	G2(mouse_D)	Normal(patient_G)
G2_rep1	G2(mouse_D)	Normal(patient_G)	G2_rep2	G2(mouse_E)	Normal(patient_H)
G2_rep2	G2(mouse_E)	Normal(patient_H)			
G2_rep3	G2(mouse_F)	Normal(patient_K)			

Table 2: 3.3 paired samples (two-group)

Label	Example 1	Example 2	Label	Example 3	Example 4
G1_rep1	G1(mouse_A)	Tumor(patient_G)	G1_rep1	G1(mouse_B)	Tumor(patient_J)
G1_rep2	G1(mouse_B)	Tumor(patient_H)	G1_rep2	G1(mouse_C)	Tumor(patient_K)
G1_rep3	G1(mouse_C)	Tumor(patient_K)	G2_rep1	G2(mouse_B)	Normal(patient_J)
G2_rep1	G2(mouse_A)	Normal(patient_G)	G2_rep2	G2(mouse_C)	Normal(patient_K)
G2_rep2	G2(mouse_B)	Normal(patient_H)			
G2_rep3	G2(mouse_C)	Normal(patient_K)			

Table 3: 3.4 unpaired samples (multi-group)

Label	Example 1	Example 2	Label	Example 3	Example 4
G1_rep1	G1(mouse_A)	Kidney(sample_A)	G1_rep1	G1(mouse_A)	Liver(sample_G)
G1_rep2	G1(mouse_B)	Kidney(sample_B)	G1_rep2	G1(mouse_B)	Liver(sample_H)
G1_rep3	G1(mouse_C)	Kidney(sample_C)	G2_rep1	G2(mouse_D)	Brain(sample_Y)
G2_rep1	G2(mouse_D)	Liver(sample_G)	G2_rep2	G2(mouse_E)	Brain(sample_Z)
G2_rep2	G2(mouse_E)	Liver(sample_H)	G3_rep1	G3(mouse_U)	Kidney(sample_B)
G2_rep3	G2(mouse_F)	Liver(sample_K)	G3_rep2	G3(mouse_T)	Kidney(sample_C)
G3_rep1	G3(mouse_G)	Brain(sample_X)			
G3_rep2	G3(mouse_H)	Brain(sample_Y)			
G3_rep3	G3(mouse_I)	Brain(sample_Z)			

3.1 Normalization of two-group count data with replicates

This package provides robust normalization methods based on DEGES proposed by Kadota et al. (2012). When obtaining normalization factors from two-group data with replicates, users can select a total of six combinations (two normalization methods \times three DEG identification methods) coupled with an arbitrary number of iterations ($n = 0, 1, 2, \dots, 100$) in our DEGES-based normalization pipeline. We show some of the practical combinations below.

Since the three-step TbT normalization method was originally designed for normalizing tag count data with (biological) replicates, we will first explain the TbT method (3.1.1 DEGES/TbT). In relation to the other DEGES-based methods, we will call the method "DEGES/TbT" for convenience. As mentioned in the original study, DEGES/TbT needs a long computation time. Accordingly, we present three shorter alternatives (3.1.2 DEGES/edgeR, 3.1.3 iDEGES/edgeR, and 3.1.4 DEGES/DESeq).

3.1.1 DEGES/TbT

The DEGES/TbT (Kadota et al., 2012) with default parameter settings can be performed as follows.

```
> set.seed(1000)
> library(TCC)
> data(hypoData)
> samplesize <- 100
> group <- c(1, 1, 1, 2, 2, 2)
> tcc <- new("TCC", hypoData, group)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "bayseq",
+ iteration = 1, samplesize = samplesize)
```

Note that a smaller sampling size (i.e., `samplesize = 100`) is used here to reduce the computation time when performing the empirical Bayesian method in step 2, but a larger sampling size of around 10,000 (i.e., `samplesize = 10000`) is recommended (Hardcastle and Kelly, 2010). This method estimates an empirical distribution of the parameters of the NB distribution by bootstrapping from the input data. While the sampling size can be made smaller to reduce the computation time (e.g., `samplesize = 40`), the resulting normalization factors will vary from trial to trial. In this vignette, we will call the `set.seed` function for obtaining reproducible results (i.e., the `tcc$norm.factors` values) when using any random

function. The calculated normalization factors and the computation time can be retrieved with the following commands.

```
> tcc$norm.factors

  G1_rep1  G1_rep2  G1_rep3  G2_rep1  G2_rep2  G2_rep3
0.8915273 0.8547597 0.8339354 1.0740765 1.1617617 1.1839393
```

```
> tcc$DEGES$execution.time
```

```
  user  system elapsed
8.240   0.020   8.374
```

Of course, the procedure can be performed by using functions in **edgeR** and **baySeq**, instead of using the **calcNormFactors** function in **TCC**. The **calcNormFactors** function together with the above parameter settings can be regarded as a wrapper function for the following commands.

```
> set.seed(1000)
> library(TCC)
> data(hypoData)
> samplesize <- 100
> group <- c(1, 1, 1, 2, 2, 2)
> ### STEP 1 ###
> d <- DGEList(count = hypoData, group = group)
> d <- calcNormFactors(d)
> norm.factors <- d$samples$norm.factors
> norm.factors <- norm.factors / mean(norm.factors)
> ### STEP 2 ###
> cD <- new("countData", data = hypoData, replicates = group,
+         groups = list(NDE = rep(1, length = length(group)), DE = group),
+         libsizes = colSums(hypoData) * norm.factors)
> cD <- getPriors.NB(cD, samplesize = samplesize, estimation = "QL", cl = NULL)
> cD <- getLikelihoods.NB(cD, pET = "BIC", cl = NULL)
```

```
.
```

```
> is.DE <- as.logical(rank(-cD@posteriors[, "DE"]) <
+                   (nrow(hypoData) * cD@estProps[2]))
> ### STEP 3 ###
> d <- DGEList(count = hypoData[!is.DE, ], group = group)
> d <- calcNormFactors(d)
> norm.factors <- d$samples$norm.factors * colSums(hypoData[!is.DE, ]) /
+                   colSums(hypoData)
> norm.factors <- norm.factors / mean(norm.factors)
> norm.factors
```

```
  G1_rep1  G1_rep2  G1_rep3  G2_rep1  G2_rep2  G2_rep3
0.8915273 0.8547597 0.8339354 1.0740765 1.1617617 1.1839393
```

3.1.2 DEGES/edgeR

Now let us describe an alternative approach that is roughly 200-400 times faster than DEGES/TbT, yet has comparable performance. The TMM-edgeR-TMM pipeline (called DEGES/edgeR) employs the exact test implemented in **edgeR** in step 2. To use this pipeline, we have to provide a reasonable threshold for defining potential DEGs in step 2. We will define the threshold as an arbitrary false discovery rate (FDR) with a floor value of P_{DEG} . The default FDR is < 0.1 , and the default floor P_{DEG} is 5%, but different choices are of course possible. For example, in case of the default settings, $x\%$ ($x > 5\%$) of the top-ranked potential DEGs are eliminated in step 2 if the percentage ($= x\%$) of genes satisfying $\text{FDR} < 0.1$ is over 5%. The DEGES/edgeR pipeline has an apparent advantage over TbT in computation time. It can be performed as follows:

```
> library(TCC)
> data(hypoData)
> group <- c(1, 1, 1, 2, 2, 2)
> tcc <- new("TCC", hypoData, group)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edgeR",
+                       iteration = 1, FDR = 0.1, floorPDEG = 0.05)
> tcc$norm.factors
```

```
  G1_rep1  G1_rep2  G1_rep3  G2_rep1  G2_rep2  G2_rep3
0.8756470 0.8440551 0.8412683 1.0811889 1.1520110 1.2058296
```

```
> tcc$DEGES$execution.time
```

```
  user  system elapsed
0.352   0.004   0.399
```

The normalization factors calculated from the DEGES/edgeR are very similar to those of DEGES/TbT with the default parameter settings (i.e., `samplesize = 10000`). For **edgeR** users, we provide commands, consisting of functions in **edgeR**, to perform the DEGES/edgeR pipeline without **TCC**. The `calcNormFactors` function together with the above parameter settings can be regarded as a wrapper function for the following commands.

```
> library(TCC)
> data(hypoData)
> group <- c(1, 1, 1, 2, 2, 2)
> FDR <- 0.1
> floorPDEG <- 0.05
> d <- DGEList(counts = hypoData, group = group)
> ### STEP 1 ###
> d <- calcNormFactors(d)
> ### STEP 2 ###
> d <- estimateCommonDisp(d)
> d <- estimateTagwiseDisp(d)
> result <- exactTest(d)
> q.value <- p.adjust(result$table$PValue, method = "BH")
> if (sum(q.value < FDR) > (floorPDEG * nrow(hypoData))) {
+   is.DEG <- as.logical(q.value < FDR)
+ } else {
+   is.DEG <- as.logical(rank(result$table$PValue, ties.method = "min") <=
+                         nrow(hypoData) * floorPDEG)
+ }
> ### STEP 3 ###
> d <- DGEList(counts = hypoData[!is.DEG, ], group = group)
```

```

> d <- calcNormFactors(d)
> norm.factors <- d$samples$norm.factors * colSums(hypoData[!is.DEG, ]) /
+               colSums(hypoData)
> norm.factors <- norm.factors / mean(norm.factors)
> norm.factors

```

```

  G1_rep1  G1_rep2  G1_rep3  G2_rep1  G2_rep2  G2_rep3
0.8756470 0.8440551 0.8412683 1.0811889 1.1520110 1.2058296

```

3.1.3 iDEGES/edgeR

Our multi-step normalization can be repeated until the calculated normalization factors converge (Kadota et al., 2012). An iterative version of DEGES/TbT (i.e., iDEGES/TbT) can be described as the TMM-(baySeq-TMM)_n pipeline with $n \geq 2$. Although the iDEGES/TbT would not be practical in terms of the computation time, the TMM-(edgeR-TMM)_n pipeline (iDEGES/edgeR) is potentially superior to both the DEGES/edgeR and the DEGES/TbT. A suggested iDEGES/edgeR implementation ($n = 3$) consists of seven steps, as follows:

```

> library(TCC)
> data(hypoData)
> group <- c(1, 1, 1, 2, 2, 2)
> tcc <- new("TCC", hypoData, group)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edger",
+                       iteration = 3, FDR = 0.1, floorPDEG = 0.05)
> tcc$norm.factors

```

```

  G1_rep1  G1_rep2  G1_rep3  G2_rep1  G2_rep2  G2_rep3
0.8766053 0.8450605 0.8346595 1.0842097 1.1538160 1.2056491

```

```

> tcc$DEGES$execution.time

```

```

  user  system elapsed
1.204   0.000   1.246

```

3.1.4 DEGES/DESeq

The DEGES pipeline can also be performed by using only the functions in the **DESeq** package. Similar to the **edgeR** case above, this DESeq-DESeq-DESeq pipeline (DEGES/DESeq) changes the corresponding arguments of the `norm.method` and `test.method` as follows:

```

> library(TCC)
> data(hypoData)
> group <- c(1, 1, 1, 2, 2, 2)
> tcc <- new("TCC", hypoData, group)
> tcc <- calcNormFactors(tcc, norm.method = "deseq", test.method = "deseq",
+                       iteration = 1, FDR = 0.1, floorPDEG = 0.05)
> tcc$norm.factors

```

```

  G1_rep1  G1_rep2  G1_rep3  G2_rep1  G2_rep2  G2_rep3
0.8885503 0.8810866 0.8298458 1.0698392 1.1513431 1.1793351

```

```
> tcc$DEGES$execution.time
```

```
user  system elapsed
1.016  0.000  1.023
```

For **DESeq** users, we also provide commands, consisting of functions in **DESeq**, to perform the DEGES/DESeq pipeline without **TCC**. The `calcNormFactors` function together with the above arguments can be regarded as a wrapper function for the following commands.

```
> library(TCC)
> data(hypoData)
> group <- c(1, 1, 1, 2, 2, 2)
> FDR <- 0.1
> floorPDEG <- 0.05
> cds <- newCountDataSet(hypoData, group)
> ### STEP 1 ###
> cds <- estimateSizeFactors(cds)
> ### STEP 2 ###
> cds <- estimateDispersions(cds)
> result <- nbinomTest(cds, 1, 2)
> result$pval[is.na(result$pval)] <- 1
> result$padj[is.na(result$padj)] <- 1
> q.value <- result$padj
> if (sum(q.value < FDR) > (floorPDEG * nrow(hypoData))) {
+   is.DEG <- as.logical(q.value < FDR)
+ } else {
+   is.DEG <- as.logical(rank(result$pval, ties.method = "min") <=
+                         nrow(hypoData) * floorPDEG)
+ }
> ### STEP 3 ###
> cds <- newCountDataSet(hypoData[!is.DEG, ], group)
> cds <- estimateSizeFactors(cds)
> norm.factors <- sizeFactors(cds) / colSums(hypoData)
> norm.factors <- norm.factors / mean(norm.factors)
> norm.factors
```

```
  G1_rep1  G1_rep2  G1_rep3  G2_rep1  G2_rep2  G2_rep3
0.8885503 0.8810866 0.8298458 1.0698392 1.1513431 1.1793351
```

3.1.5 DEGES/DESeq2

The DEGES pipeline can also be performed by using only the functions in the **DESeq2** package. Similar to the **DESeq** case above, this DESeq2-DESeq2-DESeq2 pipeline (DEGES/DESeq2) changes the corresponding arguments of the `norm.method` and `test.method` as follows:

```
> library(TCC)
> data(hypoData)
> group <- c(1, 1, 1, 2, 2, 2)
> tcc <- new("TCC", hypoData, group)
> tcc <- calcNormFactors(tcc, norm.method = "deseq2", test.method = "deseq2",
+                       iteration = 1, FDR = 0.1, floorPDEG = 0.05)
> tcc$norm.factors
```

```
  G1_rep1  G1_rep2  G1_rep3  G2_rep1  G2_rep2  G2_rep3
0.8771809 0.8718205 0.8213133 1.0791328 1.1578435 1.1927090
```

```
> tcc$DEGES$execution.time
```

```
user  system elapsed
1.820  0.000  1.844
```

For **DESeq2** users, we also provide commands, consisting of functions in **DESeq2**, to perform the DEGES/DESeq2 pipeline without **TCC**. The `calcNormFactors` function together with the above arguments can be regarded as a wrapper function for the following commands.

```
> library(TCC)
> data(hypoData)
> FDR <- 0.1
> floorPDEG <- 0.05
> group <- factor(c(1, 1, 1, 2, 2, 2))
> cl <- data.frame(group = group)
> design <- formula(~ group)
> dds <- DESeqDataSetFromMatrix(countData = hypoData, colData = cl,
+                               design = design)
> ### STEP 1 ###
> dds <- estimateSizeFactors(dds)
> sizeFactors(dds) <- sizeFactors(dds) / mean(sizeFactors(dds))
> ### STEP 2 ###
> dds <- estimateDispersions(dds)
> dds <- nbinomLRT(dds, reduced = ~ 1)
> result <- results(dds)
> result$pvalue[is.na(result$pvalue)] <- 1
> q.value <- p.adjust(result$pvalue, method = "BH")
> if (sum(q.value < FDR) > (floorPDEG * nrow(hypoData))) {
+   is.DEG <- as.logical(q.value < FDR)
+ } else {
+   is.DEG <- as.logical(rank(result$pvalue, ties.method = "min") <=
+                         nrow(hypoData) * floorPDEG)
+ }
> ### STEP 3 ###
> dds <- DESeqDataSetFromMatrix(countData = hypoData[!is.DEG, ], colData = cl,
+                               design = design)
> dds <- estimateSizeFactors(dds)
> norm.factors <- sizeFactors(dds) / colSums(hypoData)
> norm.factors <- norm.factors / mean(norm.factors)
> norm.factors

      1      2      3      4      5      6
0.8771809 0.8718205 0.8213133 1.0791328 1.1578435 1.1927090
```

3.2 Normalization of two-group count data without replicates

It is important to keep in mind that most R packages (including **edgeR**, **DESeq**, and **baySeq**) are primarily for analyzing data including biological replications because the biological variability has to be accurately estimated to avoid spurious DE calls (Glaus et al., 2012). Similar to 3.1, users can select a total of six combinations (two normalization methods \times three DEG identification methods) when obtaining normalization factors from two-group count data without replicates.

We here describe two DEGES-based normalization pipelines (3.2.1 DEGES/edgeR and 3.2.2 DEGES/DESeq) using the subset of `hypoData` consisting of the first and the fourth columns, i.e.,


```

> library(TCC)
> data(hypoData)
> group <- c(1, 2)
> tcc <- new("TCC", hypoData[, c(1, 4)], group)
> head(tcc$count)

```

```

      G1_rep1 G2_rep1
gene_1      34      16
gene_2     358      36
gene_3    1144     374
gene_4       0      18
gene_5      98       1
gene_6     296      86

```

```

> tcc$group

```

```

      group
G1_rep1    1
G2_rep1    2

```

3.2.1 DEGES/edgeR

The DEGES/edgeR pipeline for two-group data without replicates can be performed as follows:

```

> library(TCC)
> data(hypoData)
> group <- c(1, 2)
> tcc <- new("TCC", hypoData[, c(1, 4)], group)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edger",
+ iteration = 1, FDR = 0.1, floorPDEG = 0.05)
> tcc$norm.factors

```

```

      G1_rep1  G2_rep1
0.9430178 1.0569822

```

For **edgeR** users, we provide commands, consisting of functions in **edgeR**, to perform the DEGES/edgeR pipeline without **TCC**. The `calcNormFactors` function together with the above parameter settings can be regarded as a wrapper function for the following commands.

```

> library(TCC)
> data(hypoData)
> group <- c(1, 2)
> FDR <- 0.1
> floorPDEG <- 0.05
> d <- DGEList(counts = hypoData[, c(1, 4)], group = group)
> ### STEP 1 ###
> d <- calcNormFactors(d)
> ### STEP 2 ###
> d <- estimateGLMCommonDisp(d, method = "deviance", robust = TRUE, subset = NULL)
> result <- exactTest(d)
> q.value <- p.adjust(result$table$PValue, method = "BH")
> if (sum(q.value < FDR) > (floorPDEG * nrow(hypoData))) {
+   is.DEG <- as.logical(q.value < FDR)
+ } else {
+   is.DEG <- as.logical(rank(result$table$PValue, ties.method = "min") <=

```

```

+   nrow(hypoData) * floorPDEG)
+ }
> ### STEP 3 ###
> d <- DGEList(counts = hypoData[!is.DEG, c(1, 4)], group = group)
> d <- calcNormFactors(d)
> norm.factors <- d$samples$norm.factors * colSums(hypoData[!is.DEG, c(1, 4)]) / colSums(hypoData[, c(
> norm.factors <- norm.factors / mean(norm.factors)
> norm.factors

  G1_rep1  G2_rep1
0.9430178 1.0569822

```

3.2.2 DEGES/DESeq

The DEGES/DESeq pipeline for two-group data without replicates can be performed as follows:

```

> library(TCC)
> data(hypoData)
> group <- c(1, 2)
> tcc <- new("TCC", hypoData[, c(1, 4)], group)
> tcc <- calcNormFactors(tcc, norm.method = "deseq", test.method = "deseq",
+ iteration = 1, FDR = 0.1, floorPDEG = 0.05)
> tcc$norm.factors

  G1_rep1  G2_rep1
0.921658 1.078342

```

For **DESeq** users, we provide commands, consisting of functions in **DESeq**, to perform the DEGES/DESeq pipeline without **TCC**. The `calcNormFactors` function together with the above parameter settings can be regarded as a wrapper function for the following commands.

```

> library(TCC)
> data(hypoData)
> group <- c(1, 2)
> FDR <- 0.1
> floorPDEG <- 0.05
> cds <- newCountDataSet(hypoData[, c(1, 4)], group)
> ### STEP 1 ###
> cds <- estimateSizeFactors(cds)
> ### STEP 2 ###
> cds <- estimateDispersions(cds, method = "blind", sharingMode = "fit-only")
> result <- nbinomTest(cds, 1, 2)
> result$pval[is.na(result$pval)] <- 1
> result$padj[is.na(result$padj)] <- 1
> q.value <- result$padj
> if (sum(q.value < FDR) > (floorPDEG * nrow(hypoData))) {
+   is.DEG <- as.logical(q.value < FDR)
+ } else {
+   is.DEG <- as.logical(rank(result$pval, ties.method = "min") <=
+   nrow(hypoData) * floorPDEG)
+ }
> ### STEP 3 ###
> cds <- newCountDataSet(hypoData[!is.DEG, c(1, 4)], group)
> cds <- estimateSizeFactors(cds)
> norm.factors <- sizeFactors(cds) / colSums(hypoData[, c(1, 4)])
> norm.factors <- norm.factors / mean(norm.factors)
> norm.factors

  G1_rep1  G2_rep1
0.921658 1.078342

```

3.3 Normalization of two-group count data without replicates (paired)

edgeR and **DESeq** (and **DESeq2**) employs generalized linear models (GLMs) which can apply to detect DEGs from paired two-group count data. When obtaining normalization factors from paired two group samples, users can select a total of four combinations (two normalization methods \times two DEG identification methods) coupled with an arbitrary number of iterations ($n = 0, 1, 2, \dots, 100$) in our DEGES-based normalization pipeline. The analysis for paired samples can be performed by indicating (1) the pair information when constructing the **TCC** class object and (2) `paired = TRUE` when performing the `calcNormFactors` function. For analyzing paired data, we here use the hypothetical count data (`hypoData`; for details see 2.2) by changing the label information, i.e.,

```
> library(TCC)
> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+                          "N_dogA", "N_dogB", "N_dogC")
> head(hypoData)
```

	T_dogA	T_dogB	T_dogC	N_dogA	N_dogB	N_dogC
gene_1	34	45	122	16	14	29
gene_2	358	388	22	36	25	68
gene_3	1144	919	990	374	480	239
gene_4	0	0	44	18	0	0
gene_5	98	48	17	1	8	5
gene_6	296	282	216	86	62	69

This count data consists of three individuals (or sibships), dogA, dogB, and dogC. "T" and "N" indicate tumor and normal tissues, respectively.

3.3.1 DEGES/edgeR

The DEGES/edgeR pipeline for two-group paired data can be performed as follows:

```
> library(TCC)
> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+                          "N_dogA", "N_dogB", "N_dogC")
> group <- c(1, 1, 1, 2, 2, 2)
> pair <- c(1, 2, 3, 1, 2, 3)
> cl <- data.frame(group = group, pair = pair)
> tcc <- new("TCC", hypoData, cl)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edgeR",
+                          iteration = 1, FDR = 0.1, floorPDEG = 0.05, paired = TRUE)
> tcc$norm.factors
```

T_dogA	T_dogB	T_dogC	N_dogA	N_dogB	N_dogC
0.8836110	0.8470810	0.8344556	1.0724684	1.1508698	1.2115142

For **edgeR** users, we provide commands, consisting of functions in **edgeR**, to perform the DEGES/edgeR pipeline without **TCC**. The `calcNormFactors` function together with the above parameter settings can be regarded as a wrapper function for the following commands.

```
> library(TCC)
> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+                          "N_dogA", "N_dogB", "N_dogC")
> group <- factor(c(1, 1, 1, 2, 2, 2))
```

```

> pair <- factor(c(1, 2, 3, 1, 2, 3))
> design <- model.matrix(~ group + pair)
> coef <- 2
> FDR <- 0.1
> floorPDEG <- 0.05
> d <- DGEList(counts = hypoData, group = group)
> ### STEP 1 ###
> d <- calcNormFactors(d)
> ### STEP 2 ###
> d <- estimateGLMCommonDisp(d, design)
> d <- estimateGLMTrendedDisp(d, design)
> d <- estimateGLMTagwiseDisp(d, design)
> fit <- glmFit(d, design)
> lrt <- glmLRT(fit, coef = coef)
> result <- topTags(lrt, n = nrow(hypoData))
> result <- result$table[rownames(hypoData), ]
> if (sum(result$FDR < FDR) > (floorPDEG * nrow(hypoData))){
+   is.DEG <- as.logical(result$FDR < FDR)
+ } else {
+   is.DEG <- as.logical(rank(result$table$PValue,ties.method = "min") <=
+     nrow(hypoData) * floorPDEG)
+ }
> ### STEP 3 ###
> d <- DGEList(counts = hypoData[!is.DEG, ], group = group)
> d <- calcNormFactors(d)
> norm.factors <- d$samples$norm.factors * colSums(hypoData[!is.DEG, ]) /
+ colSums(hypoData)
> norm.factors <- norm.factors / mean(norm.factors)
> norm.factors

```

```

      T_dogA   T_dogB   T_dogC   N_dogA   N_dogB   N_dogC
0.8836110 0.8470810 0.8344556 1.0724684 1.1508698 1.2115142

```

3.3.2 DEGES/DESeq

The DEGES/DESeq pipeline for two-group paired data can be performed as follows:

```

> library(TCC)
> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+   "N_dogA", "N_dogB", "N_dogC")
> group <- c(1, 1, 1, 2, 2, 2)
> pair <- c(1, 2, 3, 1, 2, 3)
> cl <- data.frame(group = group, pair = pair)
> tcc <- new("TCC", hypoData, cl)
> tcc <- calcNormFactors(tcc, norm.method = "deseq", test.method = "deseq",
+   iteration = 1, FDR = 0.1, floorPDEG = 0.05, paired = TRUE)
> tcc$norm.factors

```

```

      T_dogA   T_dogB   T_dogC   N_dogA   N_dogB   N_dogC
0.8985824 0.8922696 0.8477332 1.0559596 1.1359396 1.1695156

```

For **DESeq** users, we provide commands, consisting of functions in **DESeq**, to perform the DEGES/DESeq pipeline without **TCC**. The `calcNormFactors` function together with the above parameter settings can be regarded as a wrapper function for the following commands.

```

> library(TCC)
> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+                          "N_dogA", "N_dogB", "N_dogC")
> group <- factor(c(1, 1, 1, 2, 2, 2))
> pair <- factor(c(1, 2, 3, 1, 2, 3))
> FDR <- 0.1
> floorPDEG <- 0.05
> cl <- data.frame(group = group, pair = pair)
> fit1 <- count ~ group + pair
> fit0 <- count ~ pair
> ### STEP 1 ###
> cds <- newCountDataSet(hypoData, cl)
> ### STEP 2 ###
> cds <- estimateSizeFactors(cds)
> cds <- estimateDispersions(cds, method = "blind", sharingMode = "fit-only")
> reduced.model <- fitNbinomGLMs(cds, fit0)
.

> full.model <- fitNbinomGLMs(cds, fit1)
.

> p.value <- nbinomGLMTest(full.model, reduced.model)
> p.value[is.na(p.value)] <- 1
> q.value <- p.adjust(p.value, method = "BH")
> if (sum(q.value < FDR) > (floorPDEG * nrow(hypoData))) {
+   is.DEG <- as.logical(q.value < FDR)
+ } else {
+   is.DEG <- as.logical(rank(p.value, ties.method = "min") <=
+                         nrow(hypoData) * floorPDEG)
+ }
> ### STEP 3 ###
> cds <- newCountDataSet(hypoData[!is.DEG, ], group)
> cds <- estimateSizeFactors(cds)
> norm.factors <- sizeFactors(cds) / colSums(hypoData)
> norm.factors <- norm.factors / mean(norm.factors)
> norm.factors

  T_dogA  T_dogB  T_dogC  N_dogA  N_dogB  N_dogC
0.8985824 0.8922696 0.8477332 1.0559596 1.1359396 1.1695156

```

3.4 Normalization of multi-group count data with replicates

Many R packages (including **edgeR**, **DESeq**, and **baySeq**) support DE analysis for multi-group tag count data. **TCC** provides some prototypes of DEGES-based pipelines for such data. Here, we analyze another hypothetical three-group count matrix, the `hypoData_mg` object, provided in **TCC**. It consists of 1,000 genes and a total of nine columns for testing any difference among three groups that each have triplicates.

```

> library(TCC)
> data(hypoData_mg)
> group <- c(1, 1, 1, 2, 2, 2, 3, 3, 3)
> tcc <- new("TCC", hypoData_mg, group)
> tcc

```

```
Count:
      G1_rep1 G1_rep2 G1_rep3 G2_rep1 G2_rep2 G2_rep3 G3_rep1 G3_rep2 G3_rep3
gene_1      63      48      31      15      12      12      24      15      14
gene_2      18       0       7       2       3       8       3       5       2
gene_3     106      66      25       9      14      14      11      11       3
gene_4       4       9       6       1       6       1       0       2       2
gene_5       0       1       2       1       0       1       0       0       1
gene_6      57     100      83      20       5      16      26       7      21
```

```
Sample:
      group norm.factors lib.sizes
G1_rep1     1           1   150490
G1_rep2     1           1   166665
G1_rep3     1           1   199283
G2_rep1     2           1   183116
G2_rep2     2           1   126651
G2_rep3     2           1   131377
G3_rep1     3           1   149828
G3_rep2     3           1   150288
G3_rep3     3           1   141702
```

```
> dim(tcc$count)
```

```
[1] 1000  9
```

Of the 1,000 genes, the first 200 genes are DEGs and the remaining 800 genes are non-DEGs. The breakdowns for the 200 DEGs are as follows: 140, 40, and 20 DEGs are up-regulated in Groups 1, 2, and 3. Below, we show some DEGES-based normalization pipelines for this multi-group data (3.4.1 DEGES/TbT, 3.4.2 DEGES/edgeR, and 3.4.3 DEGES/DESeq).

3.4.1 DEGES/TbT

The DEGES/TbT pipeline for multi-group data is essentially the same as those for two-group data with or without replicates. Note that a smaller sampling size (i.e., `samplesize = 100`) is used here to reduce the computation time, but a larger sampling size of around 10,000 (i.e., `samplesize = 10000`) is recommended (Hardcastle and Kelly, 2010).

```
> set.seed(1000)
> library(TCC)
> data(hypoData_mg)
> samplesize <- 100
> group <- c(1, 1, 1, 2, 2, 2, 3, 3, 3)
> tcc <- new("TCC", hypoData_mg, group)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "bayseq",
+                       iteration = 1, samplesize = samplesize)
> tcc$norm.factors
```

```
      G1_rep1  G1_rep2  G1_rep3  G2_rep1  G2_rep2  G2_rep3  G3_rep1  G3_rep2
1.0369575 0.9165584 0.7861750 0.8229178 1.1755355 1.1965579 1.0224641 1.0227613
      G3_rep3
1.0200725
```

3.4.2 DEGES/edgeR

edgeR employs generalized linear models (GLMs) to find DEGs between any of the groups. The DEGES/edgeR normalization pipeline in **TCC** internally uses functions for the GLM approach that require two models (a full model and a null model). The full model corresponds to a design matrix to describe sample groups. The null model corresponds to the model coefficients. The two models can be defined as follows:

```
> library(TCC)
> data(hypoData_mg)
> group <- c(1, 1, 1, 2, 2, 2, 3, 3, 3)
> tcc <- new("TCC", hypoData_mg, group)
> design <- model.matrix(~ as.factor(group))
> coef <- 2:length(unique(group))
```

The design matrix (`design`) can be constructed by using the `model.matrix` function. For the model coefficients (`coef`), the user should specify all the coefficients except for the intercept term. The DEGES/edgeR pipeline with the two models (`design` and `coef`) can be performed as follows.

```
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edgeR",
+                       iteration = 1, design = design, coef = coef)
> tcc$norm.factors
```

```
  G1_rep1  G1_rep2  G1_rep3  G2_rep1  G2_rep2  G2_rep3  G3_rep1  G3_rep2
1.0375941 0.9040316 0.7825986 0.8327883 1.1751212 1.2029691 1.0140550 1.0325848
  G3_rep3
1.0182572
```

The two models (`design` and `coef`) will automatically be generated when performing the following `calcNormFactors` function if those models are not explicitly indicated. That is

```
> library(TCC)
> data(hypoData_mg)
> group <- c(1, 1, 1, 2, 2, 2, 3, 3, 3)
> tcc <- new("TCC", hypoData_mg, group)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edgeR",
+                       iteration = 1)
> tcc$norm.factors
```

```
  G1_rep1  G1_rep2  G1_rep3  G2_rep1  G2_rep2  G2_rep3  G3_rep1  G3_rep2
1.0375941 0.9040316 0.7825986 0.8327883 1.1751212 1.2029691 1.0140550 1.0325848
  G3_rep3
1.0182572
```

For **edgeR** users, we provide commands, consisting of functions in **edgeR**, to perform the DEGES/edgeR pipeline without **TCC**. The `calcNormFactors` function together with the above parameter settings can be regarded as a wrapper function for the following commands.

```
> library(TCC)
> data(hypoData_mg)
> group <- c(1, 1, 1, 2, 2, 2, 3, 3, 3)
> tcc <- new("TCC", hypoData_mg, group)
> FDR <- 0.1
> floorPDEG <- 0.05
> design <- model.matrix(~ as.factor(group))
> coef <- 2:length(unique(group))
```

```

> d <- DGEList(counts = hypoData_mg, group = group)
> ### STEP 1 ###
> d <- calcNormFactors(d)
> ### STEP 2 ###
> d <- estimateGLMCommonDisp(d, design)
> d <- estimateGLMTrendedDisp(d, design)
> d <- estimateGLMTagwiseDisp(d, design)
> fit <- glmFit(d, design)
> lrt <- glmLRT(fit, coef = coef)
> result <- topTags(lrt, n = nrow(hypoData_mg))
> result <- result$table[rownames(hypoData_mg), ]
> if (sum(result$FDR < FDR) > (floorPDEG * nrow(hypoData_mg))) {
+   is.DEG <- as.logical(result$FDR < FDR)
+ } else {
+   is.DEG <- as.logical(rank(result$PValue, ties.method = "min") <=
+                         nrow(hypoData_mg) * floorPDEG)
+ }
> ### STEP 3 ###
> d <- DGEList(counts = hypoData_mg[!is.DEG, ], group = group)
> d <- calcNormFactors(d)
> norm.factors <- d$samples$norm.factors * colSums(hypoData_mg[!is.DEG, ]) /
+           colSums(hypoData_mg)
> norm.factors <- norm.factors / mean(norm.factors)
> norm.factors

```

```

  G1_rep1  G1_rep2  G1_rep3  G2_rep1  G2_rep2  G2_rep3  G3_rep1  G3_rep2
1.0375941 0.9040316 0.7825986 0.8327883 1.1751212 1.2029691 1.0140550 1.0325848
  G3_rep3
1.0182572

```

3.4.3 DEGES/DESeq

DESeq also employs GLMs for analyzing multi-group experiments. Similar to the **edgeR** package, it requires two models (full model and reduced model). The full model (`fit1`) and reduced model (`fit0`) can be created as follows:

```

> library(TCC)
> data(hypoData_mg)
> group <- c(1, 1, 1, 2, 2, 2, 3, 3, 3)
> tcc <- new("TCC", hypoData_mg, group)
> fit1 <- count ~ condition
> fit0 <- count ~ 1

```

The DEGES/DESeq pipeline with the two models (`fit1` and `fit0`) can be performed as follows.

```

> tcc <- calcNormFactors(tcc, norm.method = "deseq", test.method = "deseq",
+                       iteration = 1, fit1 = fit1, fit0 = fit0)
> tcc$norm.factors

```

```

  G1_rep1  G1_rep2  G1_rep3  G2_rep1  G2_rep2  G2_rep3  G3_rep1  G3_rep2
1.0232434 0.9096572 0.8017352 0.8249186 1.2025101 1.1728534 0.9884002 1.0164697
  G3_rep3
1.0602121

```

The two models (`fit1` and `fit0`) will automatically be generated when performing the following `calcNormFactors` function if those models are not explicitly indicated. That is


```

> library(TCC)
> data(hypoData_mg)
> group <- c(1, 1, 1, 2, 2, 2, 3, 3, 3)
> tcc <- new("TCC", hypoData_mg, group)
> tcc <- calcNormFactors(tcc, norm.method = "deseq", test.method = "deseq",
+                       iteration = 1)
> tcc$norm.factors

```

```

  G1_rep1  G1_rep2  G1_rep3  G2_rep1  G2_rep2  G2_rep3  G3_rep1  G3_rep2
1.0232434 0.9096572 0.8017352 0.8249186 1.2025101 1.1728534 0.9884002 1.0164697
  G3_rep3
1.0602121

```

For **DESeq** users, we provide commands, consisting of functions in **DESeq**, to perform the DEGES/DESeq pipeline without **TCC**. The `calcNormFactors` function together with the above parameter settings can be regarded as a wrapper function for the following commands.

```

> library(TCC)
> data(hypoData_mg)
> group <- c(1, 1, 1, 2, 2, 2, 3, 3, 3)
> FDR <- 0.1
> floorPDEG <- 0.05
> tcc <- new("TCC", hypoData_mg, group)
> fit1 <- count ~ condition
> fit0 <- count ~ 1
> cds <- newCountDataSet(hypoData_mg, group)
> ### STEP 1 ###
> cds <- estimateSizeFactors(cds)
> ### STEP 2 ###
> cds <- estimateDispersions(cds)
> reduced.model <- fitNbinomGLMs(cds, fit0)
.

> full.model <- fitNbinomGLMs(cds, fit1)
.

> p.value <- nbinomGLMTest(full.model, reduced.model)
> p.value[is.na(p.value)] <- 1
> q.value <- p.adjust(p.value, method = "BH")
> if (sum(q.value < FDR) > (floorPDEG * nrow(hypoData_mg))) {
+   is.DEG <- as.logical(q.value < FDR)
+ } else {
+   is.DEG <- as.logical(rank(p.value, ties.method = "min") <=
+                       nrow(hypoData_mg) * floorPDEG)
+ }
> ### STEP 3 ###
> cds <- newCountDataSet(hypoData_mg[!is.DEG, ], group)
> cds <- estimateSizeFactors(cds)
> norm.factors <- sizeFactors(cds) / colSums(hypoData_mg)
> norm.factors <- norm.factors / mean(norm.factors)
> norm.factors

  G1_rep1  G1_rep2  G1_rep3  G2_rep1  G2_rep2  G2_rep3  G3_rep1  G3_rep2
1.0232434 0.9096572 0.8017352 0.8249186 1.2025101 1.1728534 0.9884002 1.0164697
  G3_rep3
1.0602121

```

3.5 Retrieving normalized data

Similar functions for calculating normalization factors are the `calcNormFactors` function in `edgeR` and the `estimateSizeFactors` function in both `DESeq` and `DESeq2`. Note that the terminology used in `DESeq` (i.e., size factors) is different from that used in `edgeR` (i.e., effective library sizes) and ours. The effective library size in `edgeR` is calculated as the library size multiplied by the normalization factor. The size factors in both `DESeq` and `DESeq2` package are comparable to the *normalized* effective library sizes wherein the summary statistics for the effective library sizes are adjusted to one. Our normalization factors, which can be obtained from `tcc$norm.factors`, have the same names as those in `edgeR`. Accordingly, the normalization factors calculated from `TCC` with arbitrary options should be manipulated together with the library sizes when normalized read counts are to be obtained. Since biologists are often interested in such information (Dillies et al., 2012), we provide the `getNormalizedData` function for retrieving normalized data.

Note that the `hypoData` consists of 1,000 genes and a total of six samples (three biological replicates for G1 and three biological replicates for G2); i.e., {G1_rep1, G1_rep2, G1_rep3} vs. {G2_rep1, G2_rep2, G2_rep3}. These simulation data have basically the same conditions as shown in Fig. 1 of the TbT paper (Kadota et al., 2012); i.e., (i) the first 200 genes are DEGs ($P_{\text{DEG}} = 200/1000 = 20\%$), (ii) the first 180 genes of the 200 DEGs are higher in G1 ($P_{\text{G1}} = 180/200 = 90\%$), and the remaining 20 DEGs are higher in G2, and (iii) the level of DE is four-fold. The last 800 genes were designed to be non-DEGs. The different normalization strategies can roughly be evaluated in terms of the similarity of their summary statistics for *normalized* data labeled as non-DEGs in one group (e.g., G1) to those of the other group (e.g., G2). The basic statistics for the non-DEGs are as follows.

```
> library(TCC)
> data(hypoData)
> nonDEG <- 201:1000
> summary(hypoData[nonDEG, ])
```

G1_rep1		G1_rep2		G1_rep3		G2_rep1	
Min. :	0.00	Min. :	0	Min. :	0.00	Min. :	0.0
1st Qu.:	3.00	1st Qu.:	4	1st Qu.:	3.00	1st Qu.:	3.0
Median :	20.50	Median :	20	Median :	20.00	Median :	21.0
Mean :	103.36	Mean :	105	Mean :	104.45	Mean :	113.8
3rd Qu.:	74.25	3rd Qu.:	68	3rd Qu.:	73.25	3rd Qu.:	68.0
Max. :	8815.00	Max. :	9548	Max. :	8810.00	Max. :	9304.0

G2_rep2		G2_rep3	
Min. :	0	Min. :	0.0
1st Qu.:	3	1st Qu.:	3.0
Median :	21	Median :	20.0
Mean :	105	Mean :	104.6
3rd Qu.:	70	3rd Qu.:	70.0
Max. :	9466	Max. :	9320.0

From now on, we will display only the median values for simplicity, i.e.,

```
> apply(hypoData[nonDEG, ], 2, median)
```

G1_rep1	G1_rep2	G1_rep3	G2_rep1	G2_rep2	G2_rep3
20.5	20.0	20.0	21.0	21.0	20.0

In what follows, we show detailed examples using `hypoData`. Note, however, that the basic usage is simple.

```
> normalized.count <- getNormalizedData(tcc)
```

3.5.1 Retrieving two-group DEGES/edgeR-normalized data with replicates

The `getNormalizedData` function can be applied to the TCC class object after the normalization factors have been calculated. The DEGES/edgeR-normalized data can be retrieved as follows.

```
> library(TCC)
> data(hypoData)
> nonDEG <- 201:1000
> group <- c(1, 1, 1, 2, 2, 2)
> tcc <- new("TCC", hypoData, group)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edgeR",
+                       iteration = 1, FDR = 0.1, floorPDEG = 0.05)
> normalized.count <- getNormalizedData(tcc)
> apply(normalized.count[nonDEG, ], 2, median)
```

```
G1_rep1 G1_rep2 G1_rep3 G2_rep1 G2_rep2 G2_rep3
20.23490 20.04158 19.49126 21.29206 21.51732 19.98738
```

```
> range(apply(normalized.count[nonDEG, ], 2, median))
```

```
[1] 19.49126 21.51732
```

The same procedure consisting of functions in **edgeR** is

```
> library(TCC)
> data(hypoData)
> nonDEG <- 201:1000
> group <- c(1, 1, 1, 2, 2, 2)
> FDR <- 0.1
> floorPDEG <- 0.05
> d <- DGEList(counts = hypoData, group = group)
> ### Step 1 ###
> d <- calcNormFactors(d)
> ### Step 2 ###
> d <- estimateCommonDisp(d)
> d <- estimateTagwiseDisp(d)
> result <- exactTest(d)
> q.value <- p.adjust(result$table$PValue, method = "BH")
> if (sum(q.value < FDR) > (floorPDEG * nrow(hypoData))) {
+   is.DEG <- as.logical(q.value < FDR)
+ } else {
+   is.DEG <- as.logical(order(rank(result$table$PValue)) <=
+                         nrow(hypoData) * floorPDEG)
+ }
> ### Step 3 ###
> d <- DGEList(counts = hypoData[!is.DEG, ], group = group)
> d <- calcNormFactors(d)
> norm.factors <- d$samples$norm.factors * colSums(hypoData[!is.DEG, ]) /
+               colSums(hypoData)
> norm.factors <- norm.factors / mean(norm.factors)
> effective.libsizes <- colSums(hypoData) * norm.factors
> normalized.count <- sweep(hypoData, 2,
+                           mean(effective.libsizes) / effective.libsizes, "*")
> apply(normalized.count[nonDEG, ], 2, median)
```

```
G1_rep1 G1_rep2 G1_rep3 G2_rep1 G2_rep2 G2_rep3
20.23490 20.04158 19.49126 21.29206 21.51732 19.98738
```

```
> range(apply(normalized.count[nonDEG, ], 2, median))
```

```
[1] 19.49126 21.51732
```

For comparison, the summary statistics for TMM-normalized data produced using the original normalization method (i.e., TMM) in **edgeR** can be obtained as follows.

```
> library(TCC)
> data(hypoData)
> nonDEG <- 201:1000
> group <- c(1, 1, 1, 2, 2, 2)
> d <- DGEList(count = hypoData, group = group)
> d <- calcNormFactors(d)
> norm.factors <- d$samples$norm.factors
> norm.factors <- norm.factors / mean(norm.factors)
> effective.libsizes <- colSums(hypoData) * norm.factors
> normalized.count <- sweep(hypoData, 2,
+ mean(effective.libsizes) / effective.libsizes, "*")
> apply(normalized.count[nonDEG, ], 2, median)
```

```
G1_rep1 G1_rep2 G1_rep3 G2_rep1 G2_rep2 G2_rep3
19.35893 19.01078 18.59060 22.98591 22.16273 21.00685
```

```
> range(apply(normalized.count[nonDEG, ], 2, median))
```

```
[1] 18.59060 22.98591
```

This is the same as

```
> library(TCC)
> data(hypoData)
> nonDEG <- 201:1000
> group <- c(1, 1, 1, 2, 2, 2)
> cds <- newCountDataSet(hypoData, group)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", iteration = 0)
> normalized.count <- getNormalizedData(tcc)
> apply(normalized.count[nonDEG, ], 2, median)
```

```
G1_rep1 G1_rep2 G1_rep3 G2_rep1 G2_rep2 G2_rep3
19.35893 19.01078 18.59060 22.98591 22.16273 21.00685
```

```
> range(apply(normalized.count[nonDEG, ], 2, median))
```

```
[1] 18.59060 22.98591
```

It is obvious that the summary statistics (ranging from 19.49126 to 21.51732) from DEGES/edgeR-normalized data are closer to the truth (i.e., ranging from 20.0 to 21.0) than those (ranging from 18.59060 to 22.98591 from TMM-normalized data.

3.5.2 Retrieving two-group DEGES/DESeq-normalized data with replicates

Similar to the DEGES/edgeR case, the DEGES/DESeq-normalized data can be retrieved as follows.

```
> library(TCC)
> data(hypoData)
> group <- c(1, 1, 1, 2, 2, 2)
> nonDEG <- 201:1000
> tcc <- new("TCC", hypoData, group)
> tcc <- calcNormFactors(tcc, norm.method = "deseq", test.method = "deseq",
+                       iteration = 1, FDR = 0.1, floorPDEG = 0.05)
> normalized.count <- getNormalizedData(tcc)
> apply(normalized.count[nonDEG, ], 2, median)
```

```
G1_rep1 G1_rep2 G1_rep3 G2_rep1 G2_rep2 G2_rep3
19.98051 19.23724 19.79865 21.56052 21.57241 20.47685
```

```
> range(apply(normalized.count[nonDEG, ], 2, median))
```

```
[1] 19.23724 21.57241
```

The same procedure consisting of functions in **DESeq** is

```
> library(TCC)
> data(hypoData)
> nonDEG <- 201:1000
> group <- c(1, 1, 1, 2, 2, 2)
> FDR <- 0.1
> floorPDEG <- 0.05
> cds <- newCountDataSet(hypoData, group)
> ### Step 1 ###
> cds <- estimateSizeFactors(cds)
> ### Step 2 ###
> cds <- estimateDispersions(cds)
> result <- nbinomTest(cds, 1, 2)
> result$pval[is.na(result$pval)] <- 1
> result$padj[is.na(result$padj)] <- 1
> q.value <- result$padj
> if (sum(q.value < FDR) > (floorPDEG * nrow(hypoData))) {
+   is.DEG <- as.logical(q.value < FDR)
+ } else {
+   is.DEG <- as.logical(rank(result$pval, ties.method = "min") <=
+                         nrow(hypoData) * floorPDEG)
+ }
> ### Step 3 ###
> cds <- newCountDataSet(hypoData[!is.DEG, ], group)
> cds <- estimateSizeFactors(cds)
> norm.factors <- sizeFactors(cds) / colSums(hypoData)
> norm.factors <- norm.factors / mean(norm.factors)
> effective.libsizes <- colSums(hypoData) * norm.factors
> normalized.count <- sweep(hypoData, 2,
+                           mean(effective.libsizes) / effective.libsizes, "*")
> apply(normalized.count[nonDEG, ], 2, median)
```

```
G1_rep1 G1_rep2 G1_rep3 G2_rep1 G2_rep2 G2_rep3
19.98051 19.23724 19.79865 21.56052 21.57241 20.47685
```

```
> range(apply(normalized.count[nonDEG, ], 2, median))
```

```
[1] 19.23724 21.57241
```

For comparison, the summary statistics for DESeq-normalized data produced using the original normalization method in **DESeq** can be obtained as follows.

```
> library(TCC)
> data(hypoData)
> nonDEG <- 201:1000
> group <- c(1, 1, 1, 2, 2, 2)
> cds <- newCountDataSet(hypoData, group)
> cds <- estimateSizeFactors(cds)
> sizeFactors(cds) <- sizeFactors(cds) / mean(sizeFactors(cds))
> normalized.count <- counts(cds, normalized = TRUE)
> apply(normalized.count[nonDEG, ], 2, median)
```

```
G1_rep1 G1_rep2 G1_rep3 G2_rep1 G2_rep2 G2_rep3
19.26777 18.64482 18.88637 22.71530 22.57848 21.05657
```

```
> range(apply(normalized.count[nonDEG, ], 2, median))
```

```
[1] 18.64482 22.71530
```

This is the same as

```
> library(TCC)
> data(hypoData)
> nonDEG <- 201:1000
> group <- c(1, 1, 1, 2, 2, 2)
> cds <- newCountDataSet(hypoData, group)
> tcc <- calcNormFactors(tcc, norm.method = "deseq", iteration = 0)
> normalized.count <- getNormalizedData(tcc)
> apply(normalized.count[nonDEG, ], 2, median)
```

```
G1_rep1 G1_rep2 G1_rep3 G2_rep1 G2_rep2 G2_rep3
19.26777 18.64482 18.88637 22.71530 22.57848 21.05657
```

```
> range(apply(normalized.count[nonDEG, ], 2, median))
```

```
[1] 18.64482 22.71530
```

It is obvious that the summary statistics (ranging from 19.23724 to 21.57241) from DEGES/DESeq-normalized data are closer to the truth (i.e., ranging from 20.0 to 21.0) than those (ranging from 18.64482 to 22.71530) from DESeq-normalized data.

3.5.3 Retrieving two-group DEGES/DESeq-normalized data without replicates

Similar to the case of count data with replicates, the DEGES/DESeq-normalized data without replicates can be retrieved as follows.

```
> library(TCC)
> data(hypoData)
> nonDEG <- 201:1000
> group <- c(1, 2)
> tcc <- new("TCC", hypoData[, c(1, 4)], group)
> tcc <- calcNormFactors(tcc, norm.method = "deseq", test.method = "deseq",
+                       iteration = 1, FDR = 0.1, floorPDEG = 0.05)
> normalized.count <- getNormalizedData(tcc)
> apply(normalized.count[nonDEG, ], 2, median)
```

```
G1_rep1 G2_rep1
19.70555 21.88220
```

The same procedure consisting of functions in **DESeq** is

```
> library(TCC)
> data(hypoData)
> nonDEG <- 201:1000
> group <- c(1, 2)
> FDR <- 0.1
> floorPDEG <- 0.05
> cds <- newCountDataSet(hypoData[,c(1, 4)], group)
> ### Step 1 ###
> cds <- estimateSizeFactors(cds)
> ### Step 2 ###
> cds <- estimateDispersions(cds, method = "blind", sharingMode = "fit-only")
> result <- nbinomTest(cds, 1, 2)
> result$pval[is.na(result$pval)] <- 1
> result$padj[is.na(result$padj)] <- 1
> q.value <- result$padj
> if (sum(q.value < FDR) > (floorPDEG * nrow(hypoData))) {
+   is.DEG <- as.logical(q.value < FDR)
+ } else {
+   is.DEG <- as.logical(rank(result$pval, ties.method = "min") <=
+                         nrow(hypoData) * floorPDEG)
+ }
> ### Step 3 ###
> cds <- newCountDataSet(hypoData[!is.DEG, c(1, 4)], group)
> cds <- estimateSizeFactors(cds)
> norm.factors <- sizeFactors(cds) / colSums(hypoData[, c(1, 4)])
> norm.factors <- norm.factors / mean(norm.factors)
> effective.libsizes <- colSums(hypoData[, c(1, 4)]) * norm.factors
> normalized.count <- sweep(hypoData[, c(1, 4)], 2,
+                           mean(effective.libsizes) / effective.libsizes, "*")
> apply(normalized.count[nonDEG, ], 2, median)
```

```
G1_rep1 G2_rep1
19.70555 21.88220
```

For comparison, the summary statistics for DESeq-normalized data produced using the original normalization method in **DESeq** can be obtained as follows.

```
> library(TCC)
> data(hypoData)
> nonDEG <- 201:1000
> group <- c(1, 2)
```

```

> cds <- newCountDataSet(hypoData[, c(1, 4)], group)
> cds <- estimateSizeFactors(cds)
> sizeFactors(cds) <- sizeFactors(cds) / mean(sizeFactors(cds))
> normalized.count <- counts(cds, normalized = TRUE)
> apply(normalized.count[nonDEG, ], 2, median)

```

```

G1_rep1 G2_rep1
19.43630 22.21582

```

This is the same as

```

> library(TCC)
> data(hypoData)
> nonDEG <- 201:1000
> group <- c(1, 2)
> tcc <- new("TCC", hypoData[, c(1, 4)], group)
> tcc <- calcNormFactors(tcc, norm.method = "deseq", iteration = 0)
> normalized.count <- getNormalizedData(tcc)
> apply(normalized.count[nonDEG, ], 2, median)

```

```

G1_rep1 G2_rep1
19.43630 22.21582

```

It is obvious that the summary statistics (19.70555 and 21.88220) from DEGES/DESeq-normalized data are closer to the truth (i.e., ranging from 20.5 to 21.0) than those (19.43630 and 22.21582) from DESeq-normalized data.

3.5.4 Retrieving two-group DEGES/edgeR-normalized data without replicates (paired)

We here analyze the `hypoData` object provided in **TCC**. As described in 3.3, we regard `hypoData` as a hypothetical paired data. The DEGES/edgeR-normalized data can be retrieved as follows.

```

> library(TCC)
> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+                          "N_dogA", "N_dogB", "N_dogC")
> nonDEG <- 201:1000
> group <- c(1, 1, 1, 2, 2, 2)
> pair <- c(1, 2, 3, 1, 2, 3)
> cl <- data.frame(group = group, pair = pair)
> tcc <- new("TCC", hypoData, cl)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edgeR",
+                          iteration = 1, FDR = 0.1, floorPDEG = 0.05, paired = TRUE)
> normalized.count <- getNormalizedData(tcc)
> head(normalized.count, n = 4)

```

	T_dogA	T_dogB	T_dogC	N_dogA	N_dogB	N_dogC
gene_1	33.25937	44.93456	119.87294	16.35519	14.35977	28.84705
gene_2	350.20163	387.43574	21.61643	36.79917	25.64245	67.64135
gene_3	1119.08006	917.66352	972.73940	382.30247	492.33500	237.73947
gene_4	0.00000	0.00000	43.23286	18.39958	0.00000	0.00000

```

> apply(normalized.count[nonDEG, ], 2, median)

```



```
T_dogA T_dogB T_dogC N_dogA N_dogB N_dogC
20.05345 19.97091 19.65130 21.46618 21.53966 19.89452
```

```
> range(apply(normalized.count[nonDEG, ], 2, median))
```

```
[1] 19.65130 21.53966
```

The same procedure consisting of functions in **edgeR** is

```
> library(TCC)
> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+                          "N_dogA", "N_dogB", "N_dogC")
> nonDEG <- 201:1000
> group <- factor(c(1, 1, 1, 2, 2, 2))
> pair <- factor(c(1, 2, 3, 1, 2, 3))
> design <- model.matrix(~ group + pair)
> coef <- 2
> FDR <- 0.1
> floorPDEG <- 0.05
> d <- DGEList(counts = hypoData, group = group)
> ### STEP 1 ###
> d <- calcNormFactors(d)
> ### STEP 2 ###
> d <- estimateGLMCommonDisp(d, design)
> d <- estimateGLMTrendedDisp(d, design)
> d <- estimateGLMTagwiseDisp(d, design)
> fit <- glmFit(d, design)
> lrt <- glmLRT(fit, coef = coef)
> result <- topTags(lrt, n = nrow(hypoData))
> result <- result$table[rownames(hypoData), ]
> if (sum(result$FDR < FDR) > (floorPDEG * nrow(hypoData))) {
+   is.DEG <- as.logical(result$FDR < FDR)
+ } else {
+   is.DEG <- as.logical(rank(result$table$PValue, ties.method = "min") <=
+     nrow(hypoData) * floorPDEG)
+ }
> ### STEP 3 ###
> d <- DGEList(counts = hypoData[!is.DEG, ], group = group)
> d <- calcNormFactors(d)
> norm.factors <- d$samples$norm.factors * colSums(hypoData[!is.DEG, ]) /
+ colSums(hypoData)
> norm.factors <- norm.factors / mean(norm.factors)
> effective.libsizes <- colSums(hypoData) * norm.factors
> normalized.count <- sweep(hypoData, 2,
+                           mean(effective.libsizes) / effective.libsizes, "*")
> apply(normalized.count[nonDEG, ], 2, median)
```

```
T_dogA T_dogB T_dogC N_dogA N_dogB N_dogC
20.05345 19.97091 19.65130 21.46618 21.53966 19.89452
```

```
> range(apply(normalized.count[nonDEG, ], 2, median))
```

```
[1] 19.65130 21.53966
```

For comparison, the summary statistics for TMM-normalized data produced using the original normalization method (i.e., TMM) in **edgeR** can be obtained as follows.

```

> library(TCC)
> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+                          "N_dogA", "N_dogB", "N_dogC")
> nonDEG <- 201:1000
> group <- factor(c(1, 1, 1, 2, 2, 2))
> d <- DGEList(counts = hypoData, group = group)
> d <- calcNormFactors(d)
> norm.factors <- d$samples$norm.factors
> norm.factors <- norm.factors / mean(norm.factors)
> effective.libsizes <- colSums(hypoData) * norm.factors
> normalized.count <- sweep(hypoData, 2,
+                          mean(effective.libsizes) / effective.libsizes, "*")
> apply(normalized.count[nonDEG, ], 2, median)

```

```

  T_dogA  T_dogB  T_dogC  N_dogA  N_dogB  N_dogC
19.35893 19.01078 18.59060 22.98591 22.16273 21.00685

```

```

> range(apply(normalized.count[nonDEG, ], 2, median))

```

```
[1] 18.59060 22.98591
```

This is the same as

```

> library(TCC)
> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+                          "N_dogA", "N_dogB", "N_dogC")
> nonDEG <- 201:1000
> group <- c(1, 1, 1, 2, 2, 2)
> pair <- c(1, 2, 3, 1, 2, 3)
> cl <- data.frame(group = group, pair = pair)
> tcc <- new("TCC", hypoData, cl)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", iteration = 0, paired = TRUE)
> normalized.count <- getNormalizedData(tcc)
> apply(normalized.count[nonDEG, ], 2, median)

```

```

  T_dogA  T_dogB  T_dogC  N_dogA  N_dogB  N_dogC
19.35893 19.01078 18.59060 22.98591 22.16273 21.00685

```

```

> range(apply(normalized.count[nonDEG, ], 2, median))

```

```
[1] 18.59060 22.98591
```

It is obvious that the summary statistics (ranging from 19.65130 to 21.53966) from DEGES/edgeR-normalized data are closer to the truth (i.e., ranging from 20.0 to 21.0) than those (ranging from 18.59060 to 22.98591) from TMM-normalized data.

3.5.5 Retrieving two-group DEGES/DESeq-normalized data without replicates (paired)

We here analyze the `hypoData` object provided in **TCC**. As described in 3.3, we regard `hypoData` as a hypothetical paired data. The DEGES/DESeq-normalized data can be retrieved as follows.

```
> library(TCC)
> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+                          "N_dogA", "N_dogB", "N_dogC")
> nonDEG <- 201:1000
> group <- c(1, 1, 1, 2, 2, 2)
> pair <- c(1, 2, 3, 1, 2, 3)
> cl <- data.frame(group = group, pair = pair)
> tcc <- new("TCC", hypoData, cl)
> tcc <- calcNormFactors(tcc, norm.method = "deseq", test.method = "deseq",
+                        iteration = 1, FDR = 0.1, floorPDEG = 0.05, paired = TRUE)
> normalized.count <- getNormalizedData(tcc)
> apply(normalized.count[nonDEG, ], 2, median)
```

```
   T_dogA  T_dogB  T_dogC  N_dogA  N_dogB  N_dogC
19.79946 19.03654 19.42212 21.89038 21.91144 20.69270
```

```
> range(apply(normalized.count[nonDEG, ], 2, median))
```

```
[1] 19.03654 21.91144
```

The same procedure consisting of functions in **DESeq** is

```
> library(TCC)
> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+                          "N_dogA", "N_dogB", "N_dogC")
> nonDEG <- 201:1000
> group <- factor(c(1, 1, 1, 2, 2, 2))
> pair <- factor(c(1, 2, 3, 1, 2, 3))
> FDR <- 0.1
> floorPDEG <- 0.05
> cl <- data.frame(group = group, pair = pair)
> fit1 <- count ~ group + pair
> fit0 <- count ~ pair
> ### STEP 1 ###
> cds <- newCountDataSet(hypoData, cl)
> ### STEP 2 ###
> cds <- estimateSizeFactors(cds)
> cds <- estimateDispersions(cds, method = "blind", sharingMode = "fit-only")
> reduced.model <- fitNbinomGLMs(cds, fit0)
```

```
.
```

```
> full.model <- fitNbinomGLMs(cds, fit1)
```

```
.
```

```

> p.value <- nbinomGLMTest(full.model, reduced.model)
> p.value[is.na(p.value)] <- 1
> q.value <- p.adjust(p.value, method = "BH")
> if (sum(q.value < FDR) > (floorPDEG * nrow(hypoData))) {
+   is.DEG <- as.logical(q.value < FDR)
+ } else {
+ is.DEG <- as.logical(rank(p.value, ties.method = "min") <=
+   nrow(hypoData) * floorPDEG)
+ }
> ### STEP 3 ###
> cds <- newCountDataSet(hypoData[!is.DEG, ], group)
> cds <- estimateSizeFactors(cds)
> norm.factors <- sizeFactors(cds) / colSums(hypoData)
> norm.factors <- norm.factors / mean(norm.factors)
> effective.libsizes <- colSums(hypoData) * norm.factors
> normalized.count <- sweep(hypoData, 2,
+   mean(effective.libsizes) / effective.libsizes, "*")
> apply(normalized.count[nonDEG, ], 2, median)

```

```

T_dogA T_dogB T_dogC N_dogA N_dogB N_dogC
19.79946 19.03654 19.42212 21.89038 21.91144 20.69270

```

```

> range(apply(normalized.count[nonDEG, ], 2, median))

```

```

[1] 19.03654 21.91144

```

For comparison, the summary statistics for DESeq-normalized data produced using the original normalization method in **DESeq** can be obtained as follows.

```

> library(TCC)
> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+   "N_dogA", "N_dogB", "N_dogC")
> nonDEG <- 201:1000
> group <- factor(c(1, 1, 1, 2, 2, 2))
> pair <- factor(c(1, 2, 3, 1, 2, 3))
> cl <- data.frame(group = group, pair = pair)
> cds <- newCountDataSet(hypoData, cl)
> cds <- estimateSizeFactors(cds)
> sizeFactors(cds) <- sizeFactors(cds) / mean(sizeFactors(cds))
> normalized.count <- counts(cds, normalized = TRUE)
> apply(normalized.count[nonDEG, ], 2, median)

```

```

T_dogA T_dogB T_dogC N_dogA N_dogB N_dogC
19.26777 18.64482 18.88637 22.71530 22.57848 21.05657

```

```

> range(apply(normalized.count[nonDEG, ], 2, median))

```

```

[1] 18.64482 22.71530

```

This is the same as

```

> library(TCC)
> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+                          "N_dogA", "N_dogB", "N_dogC")
> nonDEG <- 201:1000
> group <- c(1, 1, 1, 2, 2, 2)
> pair <- c(1, 2, 3, 1, 2, 3)
> cl <- data.frame(group = group, pair = pair)
> tcc <- new("TCC", hypoData, cl)
> tcc <- calcNormFactors(tcc, norm.method = "deseq", iteration = 0, paired = TRUE)
> normalized.count <- getNormalizedData(tcc)
> apply(normalized.count[nonDEG, ], 2, median)

```

```

  T_dogA  T_dogB  T_dogC  N_dogA  N_dogB  N_dogC
19.26777 18.64482 18.88637 22.71530 22.57848 21.05657

```

```

> range(apply(normalized.count[nonDEG, ], 2, median))

```

```

[1] 18.64482 22.71530

```

It is obvious that the summary statistics (ranging from 19.03654 to 21.91144) from DEGES/DESeq-normalized data are closer to the truth (i.e., ranging from 20.0 to 21.0) than those (ranging from 18.64482 to 22.71530) from DESeq-normalized data.

3.5.6 Retrieving multi-group iDEGES/edgeR-normalized data with replicates

Here, we analyze another hypothetical three-group count matrix, the `hypoData_mg` object, provided in **TCC**. It consists of 1,000 genes and a total of nine columns for testing any difference among three groups that each have triplicates. Similar to the `hypoData` object, the first 200 genes are DEGs and the remaining 800 genes are non-DEGs. The basic statistics for the non-DEGs are as follows.

```

> library(TCC)
> data(hypoData_mg)
> nonDEG <- 201:1000
> summary(hypoData_mg[nonDEG, ])

```

```

  G1_rep1      G1_rep2      G1_rep3      G2_rep1
Min.   : 0.00  Min.   : 0.0  Min.   : 0.0  Min.   : 0.0
1st Qu.: 2.00  1st Qu.: 2.0  1st Qu.: 2.0  1st Qu.: 2.0
Median : 14.00 Median : 13.0  Median : 14.5 Median : 13.0
Mean   : 135.41 Mean   : 150.5  Mean   : 190.6 Mean   : 199.4
3rd Qu.: 51.25 3rd Qu.: 53.0  3rd Qu.: 55.0 3rd Qu.: 52.0
Max.   :27218.00 Max.   :27987.0  Max.   :66273.0 Max.   :75148.0
  G2_rep2      G2_rep3      G3_rep1      G3_rep2
Min.   : 0.00  Min.   : 0.0  Min.   : 0  Min.   : 0.0
1st Qu.: 2.00  1st Qu.: 2.0  1st Qu.: 2  1st Qu.: 2.0
Median : 13.00 Median : 14.0  Median : 14  Median : 15.0
Mean   : 132.53 Mean   : 138.4  Mean   : 164  Mean   : 166.2
3rd Qu.: 52.25 3rd Qu.: 55.0  3rd Qu.: 52  3rd Qu.: 55.0
Max.   :22381.00 Max.   :24979.0  Max.   :49398  Max.   :49709.0
  G3_rep3
Min.   : 0.0
1st Qu.: 2.0
Median : 15.0
Mean   : 152.1
3rd Qu.: 50.0
Max.   :39299.0

```

From now on, we will display only the median values for simplicity, i.e.,

```
> apply(hypoData_mg[nonDEG, ], 2, median)
```

```
G1_rep1 G1_rep2 G1_rep3 G2_rep1 G2_rep2 G2_rep3 G3_rep1 G3_rep2 G3_rep3
  14.0    13.0    14.5    13.0    13.0    14.0    14.0    15.0    15.0
```

The iDEGES/edgeR-normalized data can be retrieved as follows.

```
> library(TCC)
> data(hypoData_mg)
> nonDEG <- 201:1000
> group <- c(1, 1, 1, 2, 2, 2, 3, 3, 3)
> tcc <- new("TCC", hypoData_mg, group)
> design <- model.matrix(~ as.factor(group))
> coef <- 2:length(unique(group))
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edger",
+                       iteration = 3)
> normalized.count <- getNormalizedData(tcc)
> apply(normalized.count[nonDEG, ], 2, median)
```

```
G1_rep1 G1_rep2 G1_rep3 G2_rep1 G2_rep2 G2_rep3 G3_rep1 G3_rep2
13.88374 13.22872 14.08230 13.01186 13.24126 13.75022 14.22157 14.76685
G3_rep3
15.30593
```

```
> range(apply(normalized.count[nonDEG, ], 2, median))
```

```
[1] 13.01186 15.30593
```

For comparison, the summary statistics for TMM-normalized data produced using the original normalization method (i.e., TMM) in **edgeR** are obtained as follows.

```
> library(TCC)
> data(hypoData_mg)
> nonDEG <- 201:1000
> group <- c(1, 1, 1, 2, 2, 2, 3, 3, 3)
> tcc <- new("TCC", hypoData_mg, group)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", iteration = 0)
> normalized.count <- getNormalizedData(tcc)
> apply(normalized.count[nonDEG, ], 2, median)
```

```
G1_rep1 G1_rep2 G1_rep3 G2_rep1 G2_rep2 G2_rep3 G3_rep1 G3_rep2
13.46685 12.55871 13.42509 13.14068 13.52699 13.84316 14.20297 15.38684
G3_rep3
16.25185
```

```
> range(apply(normalized.count[nonDEG, ], 2, median))
```

```
[1] 12.55871 16.25185
```

It is obvious that the summary statistics (ranging from 13.01186 to 15.30593) from iDEGES/edgeR-normalized data are closer to the truth (i.e., ranging from 13.0 to 15.0) than those (ranging from 12.55871 to 16.25185) from TMM-normalized data.

4 Differential expression (DE)

The particular feature of **TCC** is that it calculates robust normalization factors. Moreover, end users would like to have some accessory functions for subsequent analyses. Here, we provide the `estimateDE` function for identifying DEGs. Specifically, the function internally uses the corresponding functions implemented in three packages. Similar to the usage in the `calcNormFactors` function with the `test.method` argument in **TCC**, those DE methods in **edgeR**, **DESeq**, and **baySeq** can be performed by using the `estimateDE` function with `test.method = "edger"`, `"deseq"`, and `"bayseq"`, respectively. Here, we show some examples of DE analysis for two-group data with replicates (4.1), two-group data without replicates (4.2), paired two-group data without replicates (4.3), and multi-group data with replicates (4.4).

4.1 DE analysis for two-group data with replicates

4.1.1 edgeR coupled with iDEGES/edgeR normalization

We give a procedure for DE analysis using the exact test implemented in **edgeR** together with iDEGES/edgeR normalization factors (i.e., the iDEGES/edgeR-edgeR combination) for the hypothetical two-group count data with replicates (i.e., the `hypoData` object). If the user wants to determine the genes having an FDR threshold of $< 10\%$ as DEGs, one can do as follows.

```
> library(TCC)
> data(hypoData)
> group <- c(1, 1, 1, 2, 2, 2)
> tcc <- new("TCC", hypoData, group)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edger",
+                       iteration = 3, FDR = 0.1, floorPDEG = 0.05)
> tcc <- estimateDE(tcc, test.method = "edger", FDR = 0.1)
```

The results of the DE analysis are stored in the **TCC** class object. The summary statistics for top-ranked genes can be retrieved by using the `getResult` function.

```
> result <- getResult(tcc, sort = TRUE)
> head(result)
```

	gene_id	a.value	m.value	p.value	q.value	rank	estimatedDEG
151	gene_151	9.736785	-2.753816	4.641083e-11	4.641083e-08	1	1
39	gene_39	7.110842	-2.460691	9.115042e-10	3.388777e-07	2	1
599	gene_599	5.927173	-3.282264	1.016633e-09	3.388777e-07	3	1
68	gene_68	6.209395	-2.867694	4.776945e-09	1.194236e-06	4	1
175	gene_175	7.984265	-2.373657	1.082493e-08	1.810608e-06	5	1
144	gene_144	7.588164	-2.130092	1.086365e-08	1.810608e-06	6	1

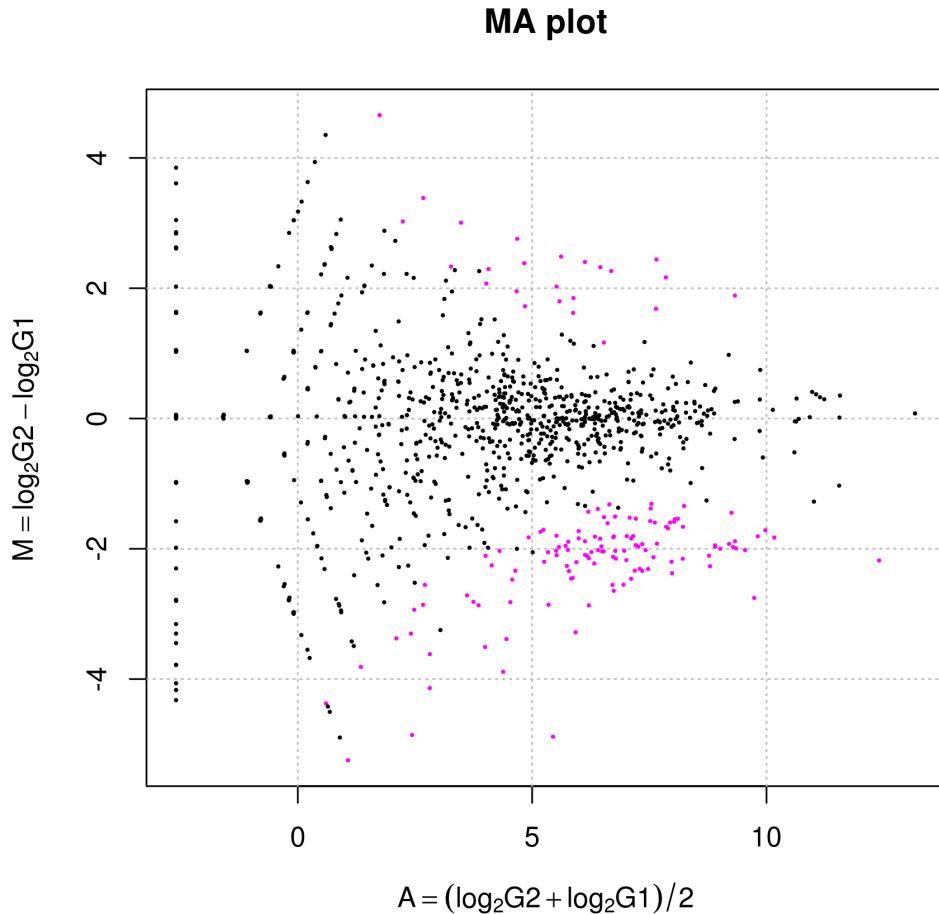
The DE results can be broken down as follows.

```
> table(tcc$estimatedDEG)
```

```
 0  1
854 146
```

This means 854 non-DEGs and 146 DEGs satisfy $FDR < 0.1$. The `plot` function generates an M-A plot, where "M" indicates the log-ratio (i.e., $M = \log_2 G2 - \log_2 G1$) and "A" indicates average read count (i.e., $A = (\log_2 G2 + \log_2 G1)/2$), from the normalized count data. The magenta points indicate the identified DEGs at $FDR < 0.1$.

```
> plot(tcc)
```



Since we know the true information for `hypoData` (i.e., 200 DEGs and 800 non-DEGs), we can calculate the area under the ROC curve (i.e., AUC; $0 \leq AUC \leq 1$) between the ranked gene list and the truth and thereby evaluate the sensitivity and specificity simultaneously. A well-ranked gene list should have a high AUC value (i.e., high sensitivity and specificity).

```
> library(ROC)
> truth <- c(rep(1, 200), rep(0, 800))
> AUC(rocdemo.sca(truth = truth, data = -tcc$stat$rank))
```

```
[1] 0.891775
```

For comparison, the DE results from the original procedure in **edgeR** can be obtained as follows.


```

> library(TCC)
> data(hypoData)
> group <- c(1, 1, 1, 2, 2, 2)
> d <- DGEList(count = hypoData, group = group)
> d <- calcNormFactors(d)
> d <- estimateCommonDisp(d)
> d <- estimateTagwiseDisp(d)
> out <- exactTest(d)
> result <- topTags(out, n = nrow(hypoData), sort.by = "PValue")
> head(result)

```

```

Comparison of groups: 2-1
      logFC  logCPM      PValue      FDR
gene_151 -2.599999 13.268885 2.665007e-10 2.665007e-07
gene_189  2.545357  9.662801 4.175913e-09 2.087957e-06
gene_181  2.311712 11.288422 7.007010e-09 2.139181e-06
gene_599 -3.111937  9.689885 8.556723e-09 2.139181e-06
gene_39  -2.305955 10.556302 1.081319e-08 2.162638e-06
gene_68  -2.718414  9.807876 3.179260e-08 4.580371e-06

```

This is the same as

```

> library(TCC)
> data(hypoData)
> group <- c(1, 1, 1, 2, 2, 2)
> tcc <- new("TCC", hypoData, group)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", iteration = 0)
> tcc <- estimateDE(tcc, test.method = "edger", FDR = 0.1)
> result <- getResult(tcc, sort = TRUE)
> head(result)

```

```

      gene_id a.value  m.value      p.value      q.value rank estimatedDEG
151 gene_151 9.740529 -2.600366 2.665007e-10 2.665007e-07 1 1
189 gene_189 6.127498 2.550316 4.175913e-09 2.087957e-06 2 1
181 gene_181 7.853250 2.312820 7.007010e-09 2.139181e-06 3 1
599 gene_599 5.937099 -3.116273 8.556723e-09 2.139181e-06 4 1
39  gene_39 7.114394 -2.308040 1.081319e-08 2.162638e-06 5 1
68  gene_68 6.209636 -2.723799 3.179260e-08 4.580371e-06 6 1

```

```

> AUC(rocdemo.sca(truth = truth, data = -tcc$stat$rank))

```

```

[1] 0.8752719

```

4.1.2 DESeq2 coupled with iDEGES/DESeq2 normalization

We give a procedure for DE analysis using the likelihood ratio test for GLMs implemented in **DESeq2** together with iDEGES/DESeq2 normalization factors (i.e., the iDEGES/DESeq2-DESeq2 combination) for the hypothetical two-group count data with replicates (i.e., the `hypoData` object). If the user wants to determine the genes having an FDR threshold of < 10% as DEGs, one can do as follows.

```

> library(TCC)
> data(hypoData)
> group <- c(1, 1, 1, 2, 2, 2)
> tcc <- new("TCC", hypoData, group)
> tcc <- calcNormFactors(tcc, norm.method = "deseq2", test.method = "deseq2",
+                       iteration = 3, FDR = 0.1, floorPDEG = 0.05)
> tcc <- estimateDE(tcc, test.method = "deseq2", FDR = 0.1)

```

The results of the DE analysis are stored in the TCC class object. The summary statistics for top-ranked genes can be retrieved by using the `getResult` function.

```
> result <- getResult(tcc, sort = TRUE)
> head(result)
```

	gene_id	a.value	m.value	p.value	q.value	rank	estimatedDEG
168	gene_168	8.902902	-1.967086	4.055000e-25	4.055000e-22	1	1
144	gene_144	7.588630	-2.129300	8.839189e-22	4.419595e-19	2	1
11	gene_11	8.770782	-2.102542	2.039581e-19	6.798604e-17	3	1
115	gene_115	8.902547	-1.945714	1.248352e-18	3.120879e-16	4	1
143	gene_143	9.284093	-1.971059	2.452787e-18	4.905575e-16	5	1
109	gene_109	9.328743	-1.883971	5.952880e-18	9.921467e-16	6	1

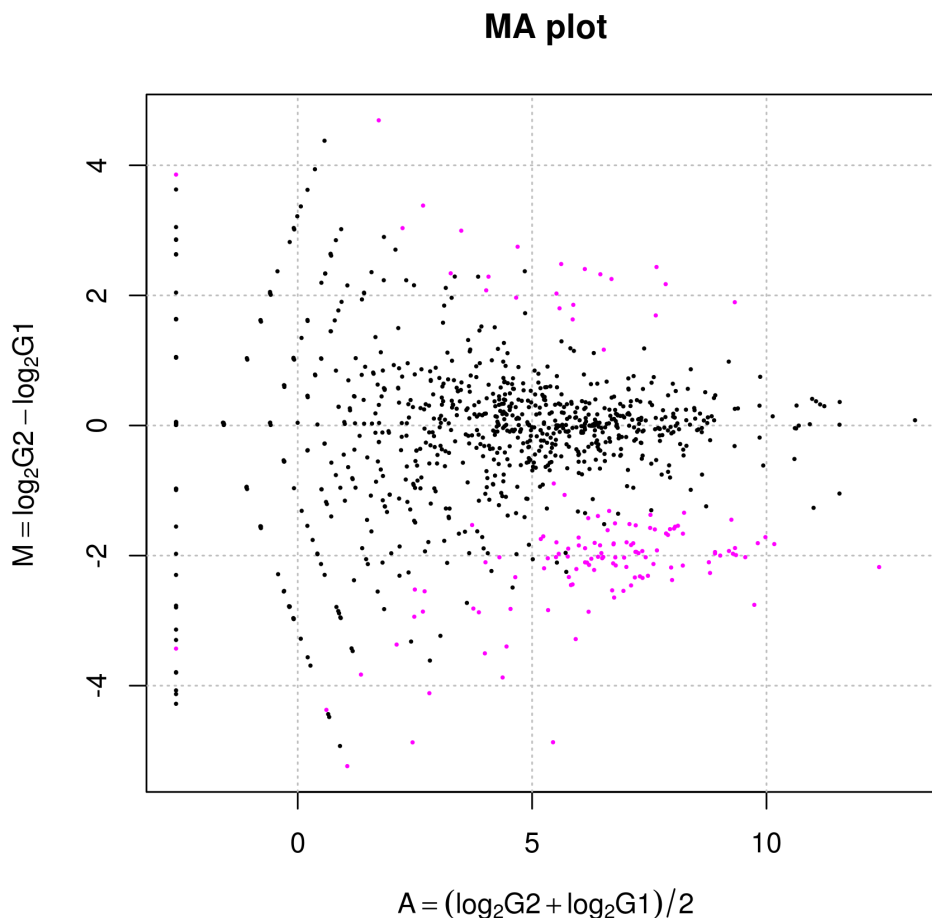
The DE results can be broken down as follows.

```
> table(tcc$estimatedDEG)
```

```
 0  1
861 139
```

This means 861 non-DEGs and 139 DEGs satisfy $FDR < 0.1$. The `plot` function generates an M-A plot, where "M" indicates the log-ratio (i.e., $M = \log_2 G2 - \log_2 G1$) and "A" indicates average read count (i.e., $A = (\log_2 G2 + \log_2 G1)/2$), from the normalized count data. The magenta points indicate the identified DEGs at $FDR < 0.1$.

```
> plot(tcc)
```



Since we know the true information for `hypoData` (i.e., 200 DEGs and 800 non-DEGs), we can calculate the area under the ROC curve (i.e., AUC; $0 \leq \text{AUC} \leq 1$) between the ranked gene list and the truth and thereby evaluate the sensitivity and specificity simultaneously. A well-ranked gene list should have a high AUC value (i.e., high sensitivity and specificity).

```
> library(ROC)
> truth <- c(rep(1, 200), rep(0, 800))
> AUC(rocdemo.sca(truth = truth, data = -tcc$stat$rank))
```

```
[1] 0.8677406
```

For comparison, the DE results from the original procedure in **DESeq2** can be obtained as follows.

```
> library(TCC)
> data(hypoData)
> group <- factor(c(1, 1, 1, 2, 2, 2))
> cl <- data.frame(group = group)
> design <- formula(~ group)
> dds <- DESeqDataSetFromMatrix(countData = hypoData, colData = cl,
+                               design = design)
> dds <- estimateSizeFactors(dds)
> sizeFactors(dds) <- sizeFactors(dds)/mean(sizeFactors(dds))
> dds <- estimateDispersions(dds)
> dds <- nbinomLRT(dds, reduced = ~ 1)
> result <- results(dds)
> head(result[order(result$pvalue),])
```

```
log2 fold change: group 2 vs 1
LRT p-value: '~ group' vs '~ 1'
```

```
DataFrame with 6 rows and 6 columns
```

	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
	<numeric>	<numeric>	<numeric>	<numeric>	<numeric>	<numeric>
gene_168	577.3622	-1.811300	0.1897536	87.19565	9.829961e-21	7.706689e-18
gene_144	239.7483	-1.973040	0.2206407	77.43006	1.375062e-18	5.390243e-16
gene_11	541.2809	-1.946382	0.2316450	66.78489	3.028102e-16	7.913439e-14
gene_143	752.4784	-1.816413	0.2192051	65.19748	6.775613e-16	1.328020e-13
gene_115	574.7956	-1.789796	0.2176106	64.52363	9.538169e-16	1.495585e-13
gene_109	763.2747	-1.726496	0.2155665	61.18879	5.185602e-15	6.775853e-13

```
> library(ROC)
> truth <- c(rep(1, 200), rep(0, 800))
> result$pvalue[is.na(result$pvalue)] <- 1
> AUC(rocdemo.sca(truth = truth, data = -rank(result$pvalue)))
```

```
[1] 0.8495969
```

This is the same as

```
> library(TCC)
> data(hypoData)
> group <- c(1, 1, 1, 2, 2, 2)
> tcc <- new("TCC", hypoData, group)
> tcc <- calcNormFactors(tcc, norm.method = "deseq2", iteration = 0)
> tcc <- estimateDE(tcc, test.method = "deseq2", FDR = 0.1)
> result <- getResult(tcc, sort = TRUE)
> head(result)
```

```

      gene_id a.value  m.value    p.value    q.value rank estimatedDEG
168 gene_168  8.905999 -1.811262 9.829961e-21 9.829961e-18    1          1
144 gene_144  7.591478 -1.973100 1.375062e-18 6.875310e-16    2          1
11  gene_11   8.774276 -1.946228 3.028102e-16 1.009367e-13    3          1
143 gene_143  9.286710 -1.816512 6.775613e-16 1.693903e-13    4          1
115 gene_115  8.905535 -1.789730 9.538169e-16 1.907634e-13    5          1
109 gene_109  9.331890 -1.726446 5.185602e-15 8.642670e-13    6          1

```

```

> library(ROC)
> truth <- c(rep(1, 200), rep(0, 800))
> AUC(rocdemo.sca(truth = truth, data = -tcc$stat$rank))

```

```
[1] 0.8495969
```

```
> tcc$norm.factors
```

```

  G1_rep1  G1_rep2  G1_rep3  G2_rep1  G2_rep2  G2_rep3
0.9271692 0.9147550 0.8753592 1.0217885 1.1069052 1.1540229

```

As described in 3.5, the size factors termed in **DESeq2** and **DESeq** are comparable to the *normalized* effective library sizes termed in **TCC** and **edgeR**. The effective library size in **edgeR** is calculated as the library size multiplied by the normalization factor. The normalization factors and effective library sizes in **DESeq2** can be retrieved as follows.

```

> library(TCC)
> data(hypoData)
> group <- factor(c(1, 1, 1, 2, 2, 2))
> cl <- data.frame(group = group)
> design <- formula(~ group)
> dds <- DESeqDataSetFromMatrix(countData = hypoData, colData = cl,
+                               design = design)
> dds <- estimateSizeFactors(dds)
> size.factors <- sizeFactors(dds)
> size.factors

```

```

      1      2      3      4      5      6
1.0830730 1.0919605 1.0779951 0.9411006 0.9468033 0.9668911

```

```

> hoge <- size.factors / colSums(hypoData)
> norm.factors <- hoge / mean(hoge)
> norm.factors

```

```

      1      2      3      4      5      6
0.9271692 0.9147550 0.8753592 1.0217885 1.1069052 1.1540229

```

```

> ef.libsizes <- norm.factors * colSums(hypoData)
> ef.libsizes

```

```

      1      2      3      4      5      6
131822.1 132903.8 131204.1 114542.5 115236.6 117681.5

```

Note that the following commands should be the simplest procedure provided in **DESeq2**.

```

> library(TCC)
> data(hypoData)
> group <- factor(c(1, 1, 1, 2, 2, 2))
> cl <- data.frame(group = group)
> design <- formula(~ group)
> dds <- DESeqDataSetFromMatrix(countData = hypoData, colData = cl,
+                               design = design)
> dds <- DESeq(dds)
> result <- results(dds)
> head(result[order(result$pvalue),])

```

```

log2 fold change (MAP): group 2 vs 1
Wald test p-value: group 2 vs 1
DataFrame with 6 rows and 6 columns

```

	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
	<numeric>	<numeric>	<numeric>	<numeric>	<numeric>	<numeric>
gene_168	567.1698	-1.795162	0.1883932	-9.528807	1.591014e-21	1.339634e-18
gene_144	235.5159	-1.949344	0.2183256	-8.928606	4.314091e-19	1.816232e-16
gene_11	531.7255	-1.920699	0.2287628	-8.396025	4.618456e-17	1.296247e-14
gene_143	739.1946	-1.794921	0.2167992	-8.279187	1.240188e-16	2.610596e-14
gene_115	564.6485	-1.768912	0.2152987	-8.216083	2.102573e-16	3.540733e-14
gene_109	749.8004	-1.706730	0.2133014	-8.001492	1.229207e-15	1.724986e-13

```

> library(ROC)
> truth <- c(rep(1, 200), rep(0, 800))
> result$pvalue[is.na(result$pvalue)] <- 1
> AUC(rocdemo.sca(truth = truth, data = -rank(result$pvalue)))

```

```
[1] 0.8597
```

The different AUC values (0.8495969 and 0.8597000) caused by the different choices of test methods: the former and the latter procedures used the `nbinomLRT` and the `nbinomWaldTest` functions, respectively. **TCC** internally uses the `nbinomLRT` function in **DESeq2** for this experimental design.

If you want to use the `nbinomWaldTest` function in **TCC**, one can do as follows.

```

> library(TCC)
> data(hypoData)
> group <- c(1, 1, 1, 2, 2, 2)
> tcc <- new("TCC", hypoData, group)
> tcc <- calcNormFactors(tcc, norm.method = "deseq2", iteration = 0)
> tcc <- estimateDE(tcc, test.method = "deseq2", DESeq2.test = "Wald", FDR = 0.1)
> result <- getResult(tcc, sort = TRUE)
> head(result)

```

	gene_id	a.value	m.value	p.value	q.value	rank	estimatedDEG
168	gene_168	8.905999	-1.811262	1.331544e-21	1.331544e-18	1	1
144	gene_144	7.591478	-1.973100	3.636164e-19	1.818082e-16	2	1
11	gene_11	8.774276	-1.946228	4.304073e-17	1.434691e-14	3	1
143	gene_143	9.286710	-1.816512	1.156792e-16	2.891980e-14	4	1
115	gene_115	8.905535	-1.789730	1.932822e-16	3.865645e-14	5	1
109	gene_109	9.331890	-1.726446	1.146622e-15	1.911037e-13	6	1

```

> library(ROC)
> truth <- c(rep(1, 200), rep(0, 800))
> AUC(rocdemo.sca(truth = truth, data = -tcc$stat$rank))

```

```
[1] 0.8597063
```

4.2 DE analysis for two-group data without replicates

It is important to keep in mind that most R packages (including **edgeR**, **DESeq**, **DESeq2** and **baySeq**) are primarily for analyzing data including biological replications because the biological variability has to be accurately estimated to avoid spurious DE calls (Glaus et al., 2012). We here describe two DE analysis pipelines using the subset of **hypoData** consisting of the first and the fourth columns, i.e.,

```
> library(TCC)
> data(hypoData)
> group <- c(1, 2)
> tcc <- new("TCC", hypoData[, c(1, 4)], group)
> head(tcc$count)
```

```
      G1_rep1 G2_rep1
gene_1      34      16
gene_2     358      36
gene_3    1144     374
gene_4       0      18
gene_5      98       1
gene_6     296      86
```

```
> tcc$group
```

```
      group
G1_rep1  1
G2_rep1  2
```

4.2.1 edgeR coupled with iDEGES/edgeR normalization

We give a procedure for DE analysis using the exact test implemented in **edgeR** together with iDEGES/edgeR normalization factors (i.e., the iDEGES/edgeR-edgeR combination) for the hypothetical two-group count data without replicates (i.e., the **hypoData** object). If the user wants to determine the genes having an FDR threshold of $< 10\%$ as DEGs, one can do as follows.

```
> library(TCC)
> data(hypoData)
> group <- c(1, 2)
> tcc <- new("TCC", hypoData[, c(1, 4)], group)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edger",
+                       iteration = 3, FDR = 0.1, floorPDEG = 0.05)
> tcc <- estimateDE(tcc, test.method = "edger", FDR = 0.1)
```

The results of the DE analysis are stored in the TCC class object. The summary statistics for top-ranked genes can be retrieved by using the **getResult** function.

```
> result <- getResult(tcc, sort = TRUE)
> head(result)
```

```
      gene_id  a.value  m.value      p.value  q.value rank estimatedDEG
36  gene_36 -0.9972387 -8.461668 1.001181e-05 0.01001181  1          1
989 gene_989 -0.9972387 -5.775619 1.077045e-03 0.29763751  2          0
17  gene_17  5.9651675 -5.406386 1.176826e-03 0.29763751  3          0
5   gene_5   3.3101162 -6.436133 1.190550e-03 0.29763751  4          0
822 gene_822 -0.9972387  5.500505 2.342792e-03 0.39547725  5          0
730 gene_730 -0.9972387  5.426504 2.630936e-03 0.39547725  6          0
```

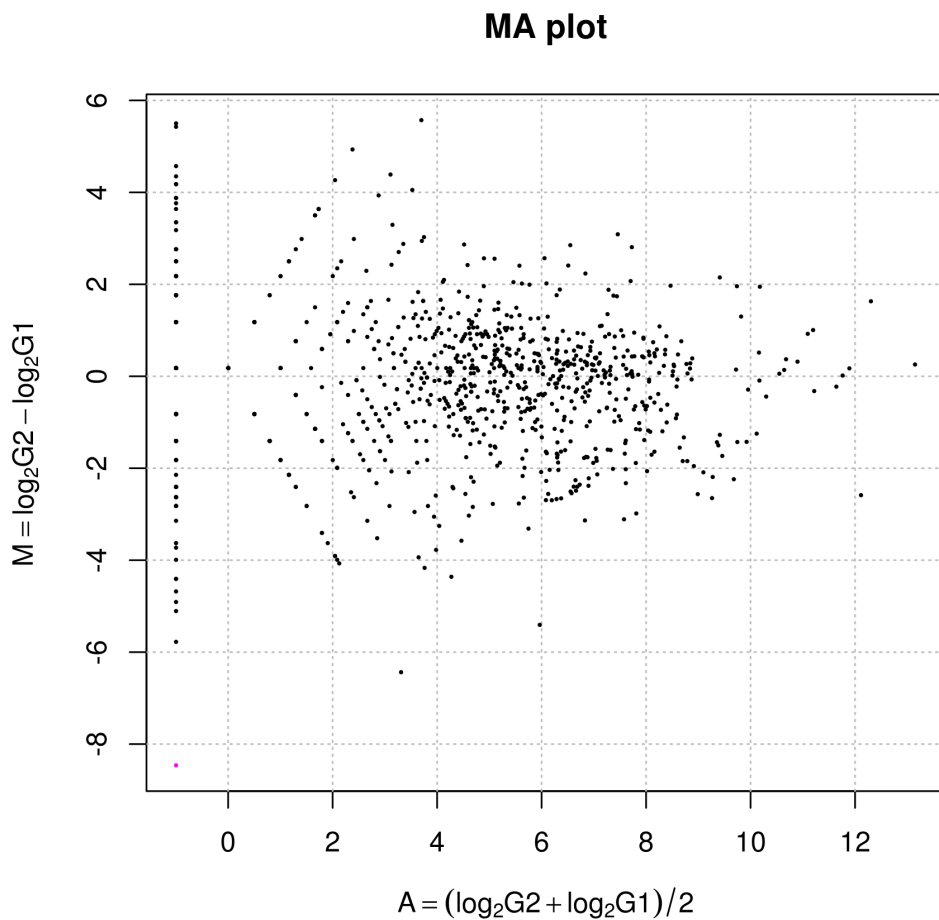
The DE results can be broken down as follows.

```
> table(tcc$estimatedDEG)
```

```
 0  1  
999 1
```

This means 999 non-DEGs and 1 DEGs satisfy $FDR < 0.1$. The `plot` function generates an M-A plot, where "M" indicates the log-ratio (i.e., $M = \log_2 G2 - \log_2 G1$) and "A" indicates average read count (i.e., $A = (\log_2 G2 + \log_2 G1)/2$), from the normalized count data. The magenta points indicate the identified DEGs at $FDR < 0.1$.

```
> plot(tcc)
```



Since we know the true information for `hypoData` (i.e., 200 DEGs and 800 non-DEGs), we can calculate the area under the ROC curve (i.e., AUC; $0 \leq AUC \leq 1$) between the ranked gene list and the truth and thereby evaluate the sensitivity and specificity simultaneously. A well-ranked gene list should have a high AUC value (i.e., high sensitivity and specificity).

```
> library(ROC)  
> truth <- c(rep(1, 200), rep(0, 800))  
> AUC(rocdemo.sca(truth = truth, data = -tcc$stat$rank))
```

```
[1] 0.7628219
```

For comparison, the DE results from the original procedure in **edgeR** can be obtained as follows.

```
> library(TCC)
> data(hypoData)
> group <- c(1, 2)
> d <- DGEList(counts = hypoData[, c(1, 4)], group = group)
> d <- calcNormFactors(d)
> d <- estimateGLMCommonDisp(d, method = "deviance", robust = TRUE, subset = NULL)
> out <- exactTest(d)
> result <- topTags(out, n = nrow(hypoData), sort.by = "PValue")
> head(result)
```

```
Comparison of groups: 2-1
      logFC      logCPM      PValue      FDR
gene_36 -11.538164 10.543868 1.571459e-05 0.01571459
gene_989 -8.854750  7.974963 1.339172e-03 0.39616275
gene_5   -6.245634  8.605105 1.546152e-03 0.39616275
gene_17  -5.353983 10.692250 1.584651e-03 0.39616275
gene_822  8.436518  7.486440 2.671274e-03 0.48648720
gene_730  8.362736  7.417431 2.977998e-03 0.48648720
```

This is the same as

```
> library(TCC)
> data(hypoData)
> group <- c(1, 2)
> tcc <- new("TCC", hypoData[, c(1, 4)], group)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", iteration = 0)
> tcc <- estimateDE(tcc, test.method = "edger", FDR = 0.1)
> result <- getResult(tcc, sort = TRUE)
> head(result)
```

```
      gene_id  a.value  m.value  p.value  q.value rank estimatedDEG
36  gene_36 -0.9960732 -8.427260 1.571459e-05 0.01571459 1 1
989 gene_989 -0.9960732 -5.741211 1.339172e-03 0.39616275 2 0
5   gene_5  3.3112817 -6.401725 1.546152e-03 0.39616275 3 0
17  gene_17 5.9663331 -5.371978 1.584651e-03 0.39616275 4 0
822 gene_822 -0.9960732  5.534913 2.671274e-03 0.48648720 5 0
730 gene_730 -0.9960732  5.460912 2.977998e-03 0.48648720 6 0
```

```
> library(ROC)
> truth <- c(rep(1, 200), rep(0, 800))
> AUC(rocdemo.sca(truth = truth, data = -tcc$stat$rank))
```

```
[1] 0.75835
```


4.2.2 DESeq2 coupled with iDEGES/DESeq2 normalization

We give a procedure for DE analysis using the likelihood ratio test for GLMs implemented in **DESeq2** together with iDEGES/DESeq2 normalization factors (i.e., the iDEGES/DESeq2-DESeq2 combination) for the hypothetical two-group count data without replicates (i.e., the `hypoData` object). If the user wants to determine the genes having an FDR threshold of $< 10\%$ as DEGs, one can do as follows.

```
> library(TCC)
> data(hypoData)
> group <- c(1, 2)
> tcc <- new("TCC", hypoData[, c(1, 4)], group)
> tcc <- calcNormFactors(tcc, norm.method = "deseq2", test.method = "deseq2",
+                       iteration = 3, FDR = 0.1, floorPDEG = 0.05)
> tcc <- estimateDE(tcc, test.method = "deseq2", FDR = 0.1)
```

The results of the DE analysis are stored in the `TCC` class object. The summary statistics for top-ranked genes can be retrieved by using the `getResult` function.

```
> result <- getResult(tcc, sort = TRUE)
> head(result)
```

	gene_id	a.value	m.value	p.value	q.value	rank	estimatedDEG
17	gene_17	5.9635613	-5.469485	0.009533069	1	1	0
989	gene_989	-0.9988449	-5.838719	0.013863931	1	2	0
5	gene_5	3.3085100	-6.499233	0.018050074	1	3	0
822	gene_822	-0.9988449	5.437405	0.023936813	1	4	0
730	gene_730	-0.9988449	5.363405	0.026015943	1	5	0
187	gene_187	3.6973138	5.507795	0.027824550	1	6	0

The DE results can be broken down as follows.

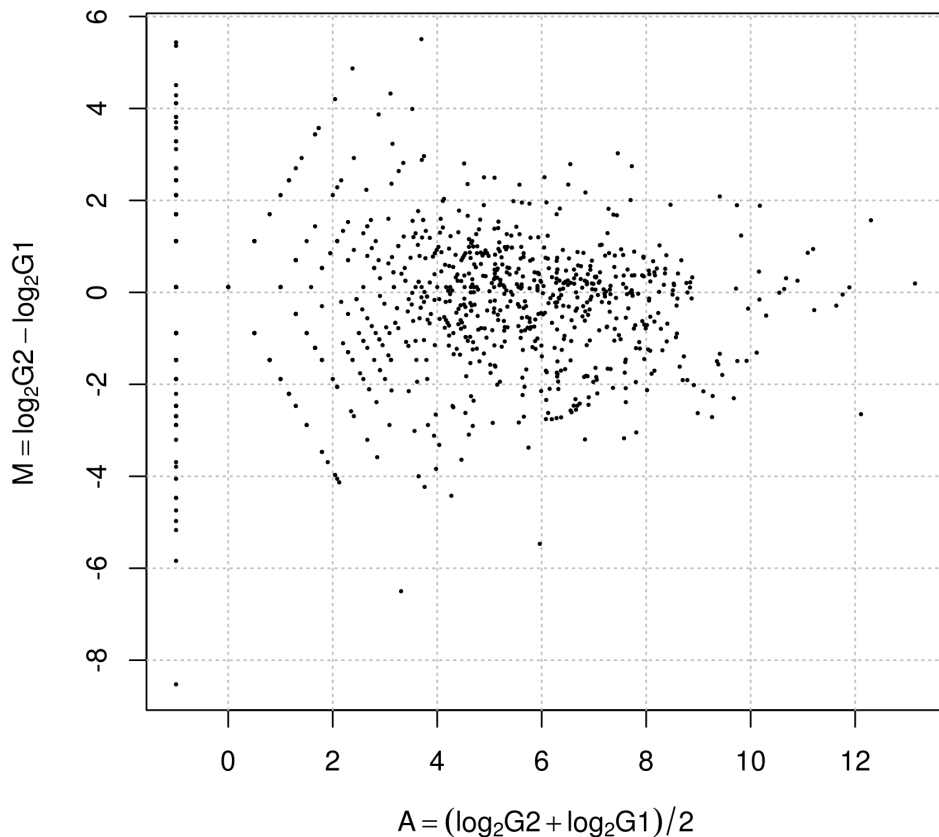
```
> table(tcc$estimatedDEG)
```

```
0
1000
```

This means 1000 non-DEGs and 0 DEGs satisfy $FDR < 0.1$. The `plot` function generates an M-A plot, where "M" indicates the log-ratio (i.e., $M = \log_2 G2 - \log_2 G1$) and "A" indicates average read count (i.e., $A = (\log_2 G2 + \log_2 G1)/2$), from the normalized count data. The magenta points indicate the identified DEGs at $FDR < 0.1$.

```
> plot(tcc)
```

MA plot



Since we know the true information for `hypoData` (i.e., 200 DEGs and 800 non-DEGs), we can calculate the area under the ROC curve (i.e., AUC; $0 \leq \text{AUC} \leq 1$) between the ranked gene list and the truth and thereby evaluate the sensitivity and specificity simultaneously. A well-ranked gene list should have a high AUC value (i.e., high sensitivity and specificity).

```
> library(ROC)
> truth <- c(rep(1, 200), rep(0, 800))
> AUC(rocdemo.sca(truth = truth, data = -tcc$stat$rank))
```

```
[1] 0.7923719
```

For comparison, the DE results from the original procedure in **DESeq2** can be obtained as follows.

```
> library(TCC)
> data(hypoData)
> group <- factor(c(1, 2))
> cl <- data.frame(group = group)
> design <- formula(~ group)
> dds <- DESeqDataSetFromMatrix(countData = hypoData[, c(1, 4)], colData = cl,
+                               design = design)
> dds <- estimateSizeFactors(dds)
> sizeFactors(dds) <- sizeFactors(dds) / mean(sizeFactors(dds))
> dds <- estimateDispersions(dds)
> dds <- nbinomLRT(dds, reduced = ~ 1)
> result <- results(dds)
> head(result[order(result$pvalue),])
```

```
log2 fold change: group 2 vs 1
LRT p-value: '~ group' vs '~ 1'
DataFrame with 6 rows and 6 columns
      baseMean log2FoldChange      lfcSE      stat      pvalue      padj
<numeric> <numeric> <numeric> <numeric> <numeric> <numeric>
gene_17 209.55273 -5.426865 1.762445 6.817655 0.009026103 0.9975203
gene_989 29.39147 -20.818974 246.187769 6.071214 0.013740250 0.9975203
gene_5 46.98644 -6.456588 2.480547 5.620781 0.017748741 0.9975203
gene_822 21.15792 19.787863 189.478585 5.207696 0.022487112 0.9975203
gene_730 20.10003 19.716743 189.638644 5.060354 0.024479234 0.9975203
gene_187 45.37975 5.550362 2.211381 4.976873 0.025688377 0.9975203
```

```
> library(ROC)
> truth <- c(rep(1, 200), rep(0, 800))
> result$pvalue[is.na(result$pvalue)] <- 1
> AUC(rocdemo.sca(truth = truth, data = -rank(result$pvalue)))
```

```
[1] 0.7870344
```

This is the same as

```
> library(TCC)
> data(hypoData)
> group <- c(1, 2)
> tcc <- new("TCC", hypoData[, c(1, 4)], group)
> tcc <- calcNormFactors(tcc, norm.method = "deseq2", iteration = 0)
> tcc <- estimateDE(tcc, test.method = "deseq2", FDR = 0.1)
> result <- getResult(tcc, sort = TRUE)
> head(result)
```

	gene_id	a.value	m.value	p.value	q.value	rank	estimatedDEG
17	gene_17	5.9645700	-5.426894	0.009026103	1	1	0
989	gene_989	-0.9978362	-5.796128	0.013740250	1	2	0
5	gene_5	3.3095187	-6.456641	0.017748741	1	3	0
822	gene_822	-0.9978362	5.479997	0.022487112	1	4	0
730	gene_730	-0.9978362	5.405996	0.024479234	1	5	0
187	gene_187	3.6983225	5.550386	0.025688377	1	6	0

```
> library(ROC)
> truth <- c(rep(1, 200), rep(0, 800))
> AUC(rocdemo.sca(truth = truth, data = -tcc$stat$rank))
```

```
[1] 0.7870344
```

4.3 DE analysis for two-group data without replicates (paired)

edgeR and **DESeq** (and **DESeq2**) employs generalized linear models (GLMs) which can apply to detect DEGs from paired two-group count data. The analysis for paired samples can be performed by indicating (1) the pair information when constructing the TCC class object and (2) `paired = TRUE` when performing the `calcNormFactors` and `estimateDE` functions. For analyzing paired data, we here use the hypothetical count data (`hypoData`; for details see 2.2) by changing the label information, i.e.,

```

> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+                          "N_dogA", "N_dogB", "N_dogC")
> head(hypoData)

```

```

      T_dogA T_dogB T_dogC N_dogA N_dogB N_dogC
gene_1     34     45    122     16     14     29
gene_2    358    388     22     36     25     68
gene_3   1144    919    990    374    480    239
gene_4      0      0     44     18      0      0
gene_5     98     48     17      1      8      5
gene_6    296    282    216     86     62     69

```

This count data consists of three individuals (or sibships), dogA, dogB, and dogC. "T" and "N" indicate tumor and normal tissues, respectively.

4.3.1 edgeR coupled with iDEGES/edgeR normalization

We give a procedure for DE analysis using the likelihood ratio test for GLMs implemented in **edgeR** together with iDEGES/edgeR normalization factors (i.e., the iDEGES/edgeR-edgeR combination) for the paired two-group count data without replicates (i.e., the above `hypoData` object). If the user wants to determine the genes having an FDR threshold of < 10% as DEGs, one can do as follows.

```

> library(TCC)
> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+                          "N_dogA", "N_dogB", "N_dogC")
> group <- c(1, 1, 1, 2, 2, 2)
> pair <- c(1, 2, 3, 1, 2, 3)
> cl <- data.frame(group = group, pair = pair)
> tcc <- new("TCC", hypoData, cl)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edgeR",
+                       iteration = 3, FDR = 0.1, floorPDEG = 0.05, paired = TRUE)
> tcc <- estimateDE(tcc, test.method = "edgeR", FDR = 0.1, paired = TRUE)

```

The results of the DE analysis are stored in the TCC class object. The summary statistics for top-ranked genes can be retrieved by using the `getResult` function.

```

> result <- getResult(tcc, sort = TRUE)
> head(result)

```

```

      gene_id a.value m.value      p.value      q.value rank estimatedDEG
151 gene_151      NA      NA 2.536349e-11 2.536349e-08    1          1
68  gene_68      NA      NA 6.202952e-10 3.101476e-07    2          1
599 gene_599      NA      NA 2.053086e-09 6.843619e-07    3          1
16  gene_16      NA      NA 7.659720e-09 1.696764e-06    4          1
63  gene_63      NA      NA 8.483820e-09 1.696764e-06    5          1
39  gene_39      NA      NA 1.684698e-08 2.807831e-06    6          1

```

The DE results can be broken down as follows.

```

> table(tcc$estimatedDEG)

```

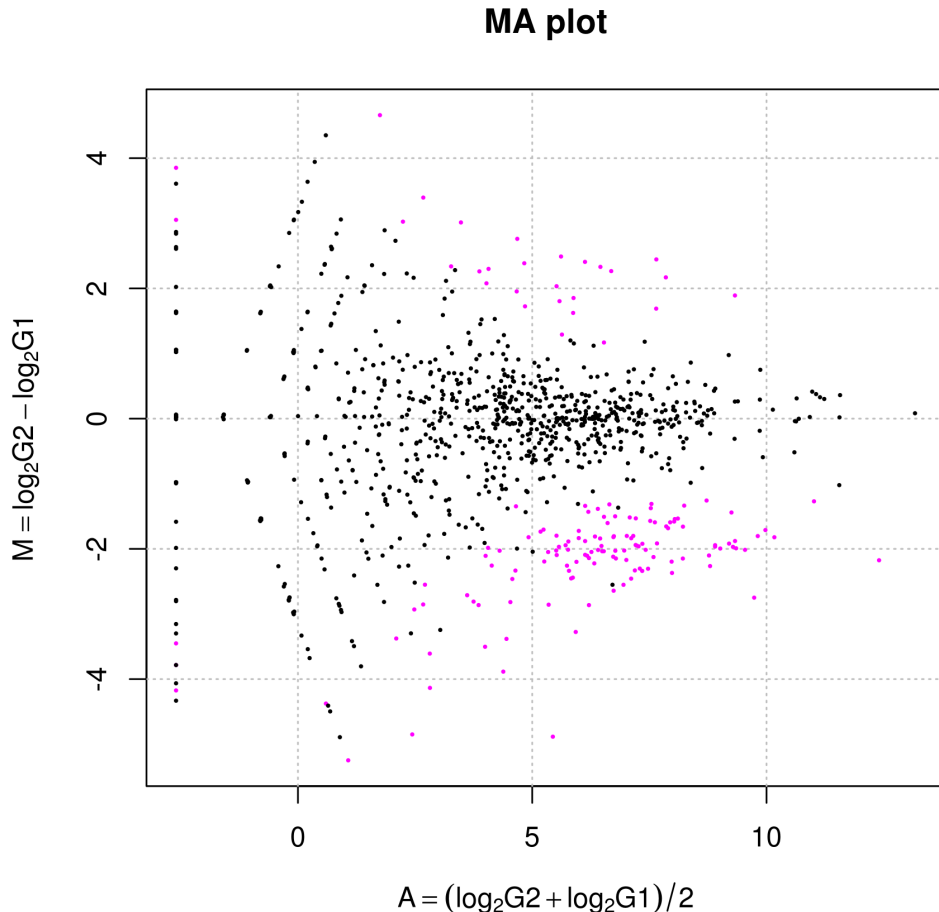
```

0 1
845 155

```

This means 845 non-DEGs and 155 DEGs satisfy $FDR < 0.1$. The `plot` function generates an M-A plot, where "M" indicates the log-ratio (i.e., $M = \log_2 G2 - \log_2 G1$) and "A" indicates average read count (i.e., $A = (\log_2 G2 + \log_2 G1)/2$), from the normalized count data. The magenta points indicate the identified DEGs at $FDR < 0.1$.

```
> plot(tcc)
```



Since we know the true information for `hypoData` (i.e., 200 DEGs and 800 non-DEGs), we can calculate the area under the ROC curve (i.e., AUC; $0 \leq AUC \leq 1$) between the ranked gene list and the truth and thereby evaluate the sensitivity and specificity simultaneously. A well-ranked gene list should have a high AUC value (i.e., high sensitivity and specificity).

```
> library(ROC)
> truth <- c(rep(1, 200), rep(0, 800))
> AUC(rocdemo.sca(truth = truth, data = -tcc$stat$rank))
```

```
[1] 0.8911656
```

For comparison, the DE results from the original procedure in **edgeR** can be obtained as follows.

```

> library(TCC)
> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+                          "N_dogA", "N_dogB", "N_dogC")
> group <- factor(c(1, 1, 1, 2, 2, 2))
> pair <- factor(c(1, 2, 3, 1, 2, 3))
> design <- model.matrix(~ group + pair)
> coef <- 2
> d <- DGEList(counts = hypoData, group = group)
> d <- calcNormFactors(d)
> d <- estimateGLMCommonDisp(d, design)
> d <- estimateGLMTrendedDisp(d, design)
> d <- estimateGLMTagwiseDisp(d, design)
> fit <- glmFit(d, design)
> lrt <- glmLRT(fit, coef = coef)
> topTags(lrt, n = 6)

```

```

Coefficient: group2
      logFC  logCPM      LR      PValue      FDR
gene_151 -2.572265 13.269746 39.50884 3.265780e-10 3.265780e-07
gene_68  -2.765849  9.815239 34.42186 4.437113e-09 2.218557e-06
gene_599 -3.263729  9.698726 32.25283 1.353595e-08 4.511982e-06
gene_181  2.325828 11.285653 30.90053 2.715980e-08 5.872622e-06
gene_196  2.506142  9.956794 30.74916 2.936311e-08 5.872622e-06
gene_16  -2.302686 10.752276 29.95668 4.418075e-08 7.363458e-06

```

```

> p.value <- lrt$table$PValue
> library(ROC)
> truth <- c(rep(1, 200), rep(0, 800))
> AUC(rocdemo.sca(truth = truth, data = -rank(p.value)))

```

```
[1] 0.8763031
```

This is the same as

```

> library(TCC)
> data(hypoData)
> colnames(hypoData) <- c("T_dogA", "T_dogB", "T_dogC",
+                          "N_dogA", "N_dogB", "N_dogC")
> group <- c(1, 1, 1, 2, 2, 2)
> pair <- c(1, 2, 3, 1, 2, 3)
> cl <- data.frame(group = group, pair = pair)
> tcc <- new("TCC", hypoData, cl)
> tcc <- calcNormFactors(tcc, norm.method = "tmm", iteration = 0, paired = TRUE)
> tcc <- estimateDE(tcc, test.method = "edger", FDR = 0.1, paired = TRUE)
> result <- getResult(tcc, sort = TRUE)
> head(result)

```

	gene_id	a.value	m.value	p.value	q.value	rank	estimatedDEG
151	gene_151	NA	NA	3.265726e-10	3.265726e-07	1	1
68	gene_68	NA	NA	4.437078e-09	2.218539e-06	2	1
599	gene_599	NA	NA	1.353585e-08	4.511949e-06	3	1
181	gene_181	NA	NA	2.716017e-08	5.872590e-06	4	1
196	gene_196	NA	NA	2.936295e-08	5.872590e-06	5	1
16	gene_16	NA	NA	4.418125e-08	7.363542e-06	6	1

```

> library(ROC)
> truth <- c(rep(1, 200), rep(0, 800))
> AUC(rocdemo.sca(truth = truth, data = -tcc$stat$rank))

```

```
[1] 0.8763031
```

4.4 DE analysis for multi-group data with replicates

Here, we give three examples of DE analysis coupled with DEGES/edgeR normalization for the hypothetical three-group data with replicates, i.e., the `hypoData_mg` object. The use of the DEGES/edgeR normalization factors is simply for reducing the computation time.

4.4.1 baySeq coupled with DEGES/edgeR normalization

The empirical Bayesian method implemented in **baySeq** after executing the DEGES/edgeR normalization (i.e., the DEGES/edgeR-baySeq combination) can be performed as follows.

```
> library(TCC)
> data(hypoData_mg)
> group <- c(1, 1, 1, 2, 2, 2, 3, 3, 3)
> tcc <- new("TCC", hypoData_mg, group)
> ### Normalization ###
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edgeR",
+                       iteration = 1)
> ### DE analysis ###
> set.seed(1000)
> samplesize <- 100
> tcc <- estimateDE(tcc, test.method = "bayseq",
+                 FDR = 0.1, samplesize = samplesize)
> result <- getResult(tcc, sort = TRUE)
> head(result)
```

	gene_id	a.value	m.value	p.value	q.value	rank	estimatedDEG
27	gene_27	NA	NA	1.089807e-07	1.089807e-07	1	1
179	gene_179	NA	NA	1.377257e-07	1.233532e-07	2	1
134	gene_134	NA	NA	1.999990e-07	1.489018e-07	3	1
194	gene_194	NA	NA	3.274252e-07	1.935327e-07	4	1
169	gene_169	NA	NA	2.120088e-06	5.788437e-07	5	1
74	gene_74	NA	NA	4.835544e-06	1.288294e-06	6	1

```
> table(tcc$estimatedDEG)
```

```
 0  1
896 104
```

It can be seen that the **baySeq** method identified 104 DEGs having $FDR < 0.1$. One can obtain the number of DEGs with another threshold (e.g., $FDR < 0.2$) from the result object as follows.

```
> sum(result$q.value < 0.2)
```

```
[1] 131
```

For **baySeq** users, we provide commands, consisting of functions in **baySeq**, to perform the DEG identification without the function in **TCC**. The `estimateDE` function with `test.method = "bayseq"` can be regarded as a wrapper function for the following commands after the DEGES/edgeR normalization.

```

> set.seed(1000)
> samplesize <- 100
> effective.libsizes <- colSums(tcc$count) * tcc$norm.factors
> groups <- list(NDE = rep(1, length(group)), DE = group)
> cD <- new("countData", data = tcc$count, replicates = group,
+         libsizes = effective.libsizes, groups = groups)
> cD <- getPriors.NB(cD, samplesize = samplesize,
+         estimation = "QL", cl = NULL)
> cD <- getLikelihoods.NB(cD, pET = "BIC", cl = NULL)

```

.

```

> tmp <- topCounts(cD, group = "DE", number = nrow(tcc$count))
> tmp <- tmp[rownames(tcc$count), ]
> p.value <- 1 - tmp$Likelihood
> q.value <- tmp$FDR
> result <- cbind(p.value, q.value)
> rownames(result) <- rownames(tmp)
> head(result)

```

```

      p.value  q.value
gene_1 0.22275288 0.04643278
gene_2 0.97564402 0.62790167
gene_3 0.07028071 0.01413496
gene_4 0.94708066 0.50800029
gene_5 0.99236244 0.77185161
gene_6 0.11824642 0.02784658

```

```

> sum(q.value < 0.1)

```

```
[1] 104
```

```

> sum(q.value < 0.2)

```

```
[1] 131
```

4.4.2 edgeR coupled with DEGES/edgeR normalization

The exact test implemented in **edgeR** after executing the DEGES/edgeR normalization (i.e., the DEGES/edgeR-edgeR combination) can be performed as follows.

```

> library(TCC)
> data(hypoData_mg)
> group <- c(1, 1, 1, 2, 2, 2, 3, 3, 3)
> tcc <- new("TCC", hypoData_mg, group)
> ### Normalization ###
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edgeR",
+         iteration = 1)
> ### DE analysis ###
> tcc <- estimateDE(tcc, test.method = "edgeR", FDR = 0.1)
> result <- getResult(tcc, sort = TRUE)
> head(result)

```


	gene_id	a.value	m.value	p.value	q.value	rank	estimatedDEG
56	gene_56	NA	NA	2.088136e-13	6.378116e-11	1	1
64	gene_64	NA	NA	2.368494e-13	6.378116e-11	2	1
27	gene_27	NA	NA	2.494405e-13	6.378116e-11	3	1
121	gene_121	NA	NA	2.551246e-13	6.378116e-11	4	1
126	gene_126	NA	NA	6.578884e-13	1.315777e-10	5	1
83	gene_83	NA	NA	7.416286e-12	1.236048e-09	6	1

```
> table(tcc$estimatedDEG)
```

```
0 1
829 171
```

Note that these DEGs having $FDR < 0.1$ display DE between any of the groups because the two arguments indicated here (`design` and `coef`) correspond to an AVOVA-like test for any differences provided in **edgeR**, i.e.,

```
> library(TCC)
> data(hypoData_mg)
> group <- c(1, 1, 1, 2, 2, 2, 3, 3, 3)
> design <- model.matrix(~ as.factor(group))
> coef <- 2:length(unique(group))
> tcc <- new("TCC", hypoData_mg, group)
> ### Normalization ###
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edger",
+ iteration = 1)
> ### DE analysis ###
> d <- DGEList(tcc$count, group = group)
> d$samples$norm.factors <- tcc$norm.factors
> d <- estimateGLMCommonDisp(d, design)
> d <- estimateGLMTrendedDisp(d, design)
> d <- estimateGLMTagwiseDisp(d, design)
> fit <- glmFit(d, design)
> lrt <- glmLRT(fit, coef = coef)
> tmp <- topTags(lrt, n = nrow(tcc$count))
> p.value <- tmp$table$PValue
> q.value <- tmp$table$FDR
> result <- cbind(p.value, q.value)
> rownames(result) <- rownames(tmp)
> head(result)
```

	p.value	q.value
gene_56	2.088136e-13	6.378116e-11
gene_64	2.368494e-13	6.378116e-11
gene_27	2.494405e-13	6.378116e-11
gene_121	2.551246e-13	6.378116e-11
gene_126	6.578884e-13	1.315777e-10
gene_83	7.416286e-12	1.236048e-09

```
> sum(q.value < 0.1)
```

```
[1] 171
```

```
> sum(q.value < 0.2)
```

As described in the **edgeR** manual, the second and third columns in the **design** object are relative to the baseline (i.e., Group 1 or G1): **coef** = 2 means G2 vs. G1 and **coef** = 3 means G3 vs. G1. The above procedure with the **coef** object (i.e., `2:length(unique(group))`) indicates the both comparisons (i.e., G2 vs. G1 and G3 vs. G1) and identifies DEGs between any of the three groups. In other words, one can do any two-group comparison of interest from multi-group data with replicates. For example, the DE analysis for G3 vs. G1 together with DEGES/edgeR normalization can be performed as follows.

```
> library(TCC)
> data(hypoData_mg)
> group <- c(1, 1, 1, 2, 2, 2, 3, 3, 3)
> tcc <- new("TCC", hypoData_mg, group)
> ### Normalization ###
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edger",
+                       iteration = 1)
> ### DE analysis ###
> coef <- 3
> tcc <- estimateDE(tcc, test.method = "edger", FDR = 0.1, coef = coef)
> result <- getResult(tcc, sort = TRUE)
> head(result)
```

	gene_id	a.value	m.value	p.value	q.value	rank	estimatedDEG
126	gene_126	NA	NA	1.869059e-10	1.869059e-07	1	1
56	gene_56	NA	NA	5.600579e-10	2.800290e-07	2	1
121	gene_121	NA	NA	1.099674e-09	3.126829e-07	3	1
27	gene_27	NA	NA	1.250732e-09	3.126829e-07	4	1
64	gene_64	NA	NA	2.867107e-09	5.734214e-07	5	1
112	gene_112	NA	NA	8.166477e-09	1.361079e-06	6	1

```
> table(tcc$estimatedDEG)
```

0	1
884	116

4.4.3 DESeq coupled with DEGES/edgeR normalization

The NB test implemented in **DESeq** after executing the DEGES/edgeR normalization (i.e., the DEGES/edgeR-DESeq combination) can be performed as follows.

```
> library(TCC)
> data(hypoData_mg)
> group <- c(1, 1, 1, 2, 2, 2, 3, 3, 3)
> tcc <- new("TCC", hypoData_mg, group)
> ### Normalization ###
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edger",
+                       iteration = 1)
> ### DE analysis ###
> fit1 <- count ~ condition
> fit0 <- count ~ 1
> tcc <- estimateDE(tcc, test.method = "deseq",
+                 FDR = 0.1, fit0 = fit0, fit1 = fit1)
> result <- getResult(tcc, sort = TRUE)
> head(result)
```

	gene_id	a.value	m.value	p.value	q.value	rank	estimatedDEG
126	gene_126	NA	NA	2.338130e-13	2.338130e-10	1	1
63	gene_63	NA	NA	1.410762e-10	7.053808e-08	2	1
27	gene_27	NA	NA	2.279517e-08	7.598390e-06	3	1
176	gene_176	NA	NA	4.695739e-08	1.173935e-05	4	1
121	gene_121	NA	NA	7.462291e-08	1.492458e-05	5	1
64	gene_64	NA	NA	1.096310e-07	1.793916e-05	6	1

```
> table(tcc$estimatedDEG)
```

```
 0  1
871 129
```

For **DESeq** users, we provide commands, consisting of functions in **DESeq**, to perform the DEG identification without the function in **TCC**. The `estimateDE` function with `test.method = "deseq"` can be regarded as a wrapper function for the following commands after the DEGES/edgeR normalization.

```
> library(TCC)
> data(hypoData_mg)
> group <- c(1, 1, 1, 2, 2, 2, 3, 3, 3)
> tcc <- new("TCC", hypoData_mg, group)
> ### Normalization ###
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edger",
+                       iteration = 1)
> ### DE analysis ###
> fit1 <- count ~ condition
> fit0 <- count ~ 1
> cds <- newCountDataSet(tcc$count, group)
> cds <- estimateSizeFactors(cds)
> sizeFactors(cds) <- sizeFactors(cds) / mean(sizeFactors(cds))
> cds <- estimateDispersions(cds)
> reduced.model <- fitNbinomGLMs(cds, fit0)
.
> full.model <- fitNbinomGLMs(cds, fit1)
.
> p.value <- nbinomGLMTest(full.model, reduced.model)
> p.value[is.na(p.value)] <- 1
> q.value <- p.adjust(p.value, method = "BH")
> tmp <- cbind(p.value, q.value)
> rownames(tmp) <- tcc$gene_id
> result <- tmp[order(p.value), ]
> head(result)

      p.value      q.value
gene_126 2.116085e-12 2.116085e-09
gene_63  9.589219e-10 4.794610e-07
gene_176 1.975102e-08 6.583673e-06
gene_83  6.667874e-08 1.666968e-05
gene_27  1.369475e-07 2.738951e-05
gene_56  2.202473e-07 3.482671e-05

> sum(q.value < 0.1)

[1] 123

> sum(q.value < 0.2)

[1] 142
```

5 Generation of simulation data

5.1 Introduction and basic usage

As demonstrated in our previous study (Kadota et al., 2012), the DEGES-based normalization methods implemented in **TCC** theoretically outperform the other normalization methods when the numbers of DEGs (G1 vs. G2) in the tag count data are biased. However, it is difficult to determine whether the up- and down-regulated DEGs in one of the groups are actually biased in their number when analyzing real data (Dillies et al., 2012). This means we have to evaluate the potential performance of our DEGES-based methods using mainly simulation data. The `simulateReadCounts` function generates simulation data under various conditions. This function can generate simulation data analyzed in the TbT paper (Kadota et al., 2012), and that means it enables other researchers to compare the methods they develop with our DEGES-based methods. For example, the `hypoData` object, a hypothetical count dataset provided in **TCC**, was generated by using this function. The output of the `simulateReadCounts` function is stored as a **TCC** class object and is therefore ready-to-analyze.

Note that different trials of simulation analysis generally yield different count data even under the same simulation conditions. We can call the `set.seed` function in order to obtain reproducible results (i.e., the `tcc$count`) with the `simulateReadCounts` function.

```
> set.seed(1000)
> library(TCC)
> tcc <- simulateReadCounts(Ngene = 1000, PDEG = 0.2,
+                           DEG.assign = c(0.9, 0.1),
+                           DEG.foldchange = c(4, 4),
+                           replicates = c(3, 3))
> dim(tcc$count)
```

```
[1] 1000    6
```

```
> head(tcc$count)
```

	G1_rep1	G1_rep2	G1_rep3	G2_rep1	G2_rep2	G2_rep3
gene_1	168	104	62	38	24	35
gene_2	10	8	27	4	5	4
gene_3	64	94	81	15	57	12
gene_4	350	443	472	116	135	108
gene_5	92	263	60	7	15	22
gene_6	561	682	591	19	65	179

```
> tcc$group
```

	group
G1_rep1	1
G1_rep2	1
G1_rep3	1
G2_rep1	2
G2_rep2	2
G2_rep3	2

The simulation conditions for comparing two groups (G1 vs. G2) with biological replicates are as follows: (i) the number of genes is 1,000 (i.e., `Ngene = 1000`), (ii) the first 20% of genes are DEGs (`PDEG = 0.2`), (iii) the first 90% of the DEGs are up-regulated in G1, and the remaining 10% are up-regulated in G2 (`DEG.assign = c(0.9, 0.1)`), (iv) the levels of DE are four-fold in both groups (`DEG.foldchange = c(4, 4)`), and (v) there are a total of six samples (three biological replicates for G1 and three biological replicates for G2) (`replicates = c(3, 3)`). The variance of the NB distribution can be modeled as $V = \mu + \phi\mu^2$. The empirical distribution of the read counts for producing the mean (μ) and dispersion (ϕ) parameters of the model was obtained from *Arabidopsis* data (three biological replicates for each of the treated and non-treated groups) in **NBPSeq** (Di et al., 2011).

The `tcc$count` object is essentially the same as the `hypoData` object of **TCC**. The information about the simulation conditions can be viewed as follows.

```
> str(tcc$simulation)
```

```
List of 4
 $ trueDEG      : num [1:1000] 1 1 1 1 1 1 1 1 1 1 ...
 $ DEG.foldchange: num [1:1000, 1:6] 4 4 4 4 4 4 4 4 4 4 ...
 $ PDEG        : num [1:2] 0.18 0.02
 $ params      : 'data.frame':      1000 obs. of  2 variables:
 ..$ mean: num [1:1000] 32.99 4.13 16.73 103.14 15.57 ...
 ..$ disp: num [1:1000] 0.3868 0.0915 0.1835 0.0503 0.6416 ...
```

Specifically, the entries for 0, 1, and 2 in the `tcc$simulation>trueDEG` object are for non-DEG, DEGs up-regulated in G1, and DEGs up-regulated in G2, respectively. The breakdowns for individual entries are the same as stated above: 800 entries are non-DEGs, 180 DEGs are up-regulated in G1, and 20 DEGs are up-regulated in G2.

```
> table(tcc$simulation>trueDEG)
```

```
 0  1  2
800 180 20
```

This information can be used to evaluate the performance of the DEGES-based normalization methods in terms of the sensitivity and specificity of the results of their DE analysis. A good normalization method coupled with a DE method such as the exact test (Robinson and Smyth, 2008) and the empirical Bayes (Hardcastle and Kelly, 2010) should produce well-ranked gene lists in which the true DEGs are top-ranked and non-DEGs are bottom-ranked when all genes are ranked according to the degree of DE. The ranked gene list after performing the DEGES/edgeR-edgeR combination can be obtained as follows.

```
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edger",
+                       iteration = 1, FDR = 0.1, floorPDEG = 0.05)
> tcc <- estimateDE(tcc, test.method = "edger", FDR = 0.1)
> result <- getResult(tcc, sort = TRUE)
> head(result)
```

	gene_id	a.value	m.value	p.value	q.value	rank	estimatedDEG
74	gene_74	8.095342	-2.542249	1.632080e-10	1.286724e-07	1	1
185	gene_185	7.177367	2.407113	4.534722e-10	1.286724e-07	2	1
181	gene_181	7.861230	2.425154	5.023661e-10	1.286724e-07	3	1
11	gene_11	7.145969	-2.471502	5.786026e-10	1.286724e-07	4	1
113	gene_113	12.197408	-2.233912	6.433622e-10	1.286724e-07	5	1
138	gene_138	9.397467	-2.232690	1.118317e-09	1.863861e-07	6	1

We can now calculate the area under the **ROC** curve (i.e., AUC; $0 \leq \text{AUC} \leq 1$) between the ranked gene list and the truth (i.e., DEGs or non-DEGs) and thereby evaluate the sensitivity and specificity simultaneously. A well-ranked gene list should have a high AUC value (i.e., high sensitivity and specificity). The `calcAUCValue` function calculates the AUC value based on the information stored in the **TCC** class object.

```
> calcAUCValue(tcc)
```

```
[1] 0.8925563
```

This is essentially the same as

```
> AUC(rocdemo.sca(truth = as.numeric(tcc$simulation$trueDEG != 0),
+               data = -tcc$stat$rank))
```

```
[1] 0.8925563
```

The following classic **edgeR** procedure (i.e., the TMM-edgeR combination) make it clear that the DEGES-based normalization method (i.e., the DEGES/edgeR pipeline) outperforms the default normalization method (i.e., TMM) implemented in **edgeR**.

```
> tcc <- calcNormFactors(tcc, norm.method = "tmm", iteration = 0)
> tcc <- estimateDE(tcc, test.method = "edgeR", FDR = 0.1)
> calcAUCValue(tcc)
```

```
[1] 0.8773188
```

The following is an alternative procedure for **edgeR** users.

```
> d <- DGEList(counts = tcc$count, group = tcc$group$group)
> d <- calcNormFactors(d)
> d$samples$norm.factors <- d$samples$norm.factors /
+   mean(d$samples$norm.factors)
> d <- estimateCommonDisp(d)
> d <- estimateTagwiseDisp(d)
> result <- exactTest(d)
> result$table$PValue[is.na(result$table$PValue)] <- 1
> AUC(rocdemo.sca(truth = as.numeric(tcc$simulation$trueDEG != 0),
+               data = -rank(result$table$PValue)))
```

```
[1] 0.8773188
```

As can be expected from the similarity of the normalization factors of DEGES/TbT (3.1.1) and DEGES/edgeR (3.1.2), the AUC value (0.8925563) of DEGES/edgeR is quite similar to the AUC value (0.8931875) of the original TbT method (i.e., DEGES/TbT):

```
> set.seed(1000)
> samplesize <- 100
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "bayseq",
+                       iteration = 1, samplesize = samplesize)
> tcc <- estimateDE(tcc, test.method = "edgeR", FDR = 0.1)
> calcAUCValue(tcc)
```

```
[1] 0.8931875
```

5.2 Two-group data without replicates

Let us generate tag count data without replicates, such as those used in section 3.2 For simplicity, we first generate simulation data whose conditions are essentially the same as those in the previous section (i.e., 5.1), except for the number of replicates in each group: (i) the number of genes is 1,000 (i.e., `Ngene = 1000`), (ii) the first 20% of genes are DEGs (`PDEG = 0.2`), (iii) the first 90% of the DEGs are up-regulated in G1, and the remaining 10% are up-regulated in G2 (`DEG.assign = c(0.9, 0.1)`), (iv) the levels of DE are four-fold in both groups (`DEG.foldchange = c(4, 4)`), and (v) there are a total of two samples (one from G1 and the other from G2) (`replicates = c(1, 1)`).

```
> set.seed(1000)
> library(TCC)
> tcc <- simulateReadCounts(Ngene = 1000, PDEG = 0.2,
+                           DEG.assign = c(0.9, 0.1),
+                           DEG.foldchange = c(4, 4),
+                           replicates = c(1, 1))
> dim(tcc$count)
```

```
[1] 1000  2
```

```
> head(tcc$count)
```

	G1_rep1	G2_rep1
gene_1	168	29
gene_2	10	2
gene_3	64	2
gene_4	350	90
gene_5	92	5
gene_6	561	74

```
> tcc$group
```

	group
G1_rep1	1
G2_rep1	2

Now let us see how the DEGES/DESeq-DESeq combination with the original DESeq-DESeq combination performs. First, we calculate the AUC value for the ranked gene list obtained from the DEGES/DESeq-DESeq combination.

```
> tcc <- calcNormFactors(tcc, norm.method = "deseq", test.method = "deseq",
+                       iteration = 1, FDR = 0.1, floorPDEG = 0.05)
> tcc <- estimateDE(tcc, test.method = "deseq")
> calcAUCValue(tcc)
```

```
[1] 0.7846313
```

Next, we calculate the corresponding value using the original **DESeq** procedure (i.e., the DESeq-DESeq combination).

```
> tcc <- calcNormFactors(tcc, norm.method = "deseq", iteration = 0)
> tcc <- estimateDE(tcc, test.method = "deseq")
> calcAUCValue(tcc)
```

[1] 0.78265

It can be seen that the DEGES/DESeq-DESeq combination outperforms the original procedure under the given simulation conditions. The following is an alternative approach for **DESeq** users.

```
> cds <- newCountDataSet(tcc$count, tcc$group$group)
> cds <- estimateSizeFactors(cds)
> sizeFactors(cds) <- sizeFactors(cds) / mean(sizeFactors(cds))
> cds <- estimateDispersions(cds, method = "blind", sharingMode = "fit-only")
> result <- nbinomTest(cds, 1, 2)
> result$pval[is.na(result$pval)] <- 1
> AUC(rocdemo.sca(truth = as.numeric(tcc$simulation$trueDEG != 0),
+               data = -rank(result$pval)))
```

[1] 0.78265

This procedure is completely the same as the one in **TCC** that gives normalization factors corresponding to those in **edgeR** for different packages. However, the following commands from the **DESeq** manual are of practical value because they give approximately the same AUC value as above.

```
> cds <- newCountDataSet(tcc$count, tcc$group$group)
> cds <- estimateSizeFactors(cds)
> cds <- estimateDispersions(cds, method = "blind", sharingMode = "fit-only")
> result <- nbinomTest(cds, 1, 2)
> result$pval[is.na(result$pval)] <- 1
> AUC(rocdemo.sca(truth = as.numeric(tcc$simulation$trueDEG != 0),
+               data = -rank(result$pval)))
```

[1] 0.78265

5.3 Multi-group data with and without replicates

The `simulateReadCounts` function can generate simulation data with a more complex design. First, we generate a dataset consisting of three groups. The simulation conditions for this dataset are as follows: (i) the number of genes is 1,000 (i.e., `Ngene = 1000`), (ii) the first 30% of genes are DEGs (`PDEG = 0.3`), (iii) the breakdowns of the up-regulated DEGs are respectively 70%, 20%, and 10% in Groups 1-3 (`DEG.assign = c(0.7, 0.2, 0.1)`), (iv) the levels of DE are 3-, 10-, and 6-fold in individual groups (`DEG.foldchange = c(3, 10, 6)`), and (v) there are a total of nine libraries (2, 4, and 3 replicates for Groups 1-3) (`replicates = c(2, 4, 3)`).

```
> set.seed(1000)
> library(TCC)
> tcc <- simulateReadCounts(Ngene = 1000, PDEG = 0.3,
+                          DEG.assign = c(0.7, 0.2, 0.1),
+                          DEG.foldchange = c(3, 10, 6),
+                          replicates = c(2, 4, 3))
> dim(tcc$count)
```

[1] 1000 9


```
> tcc$group
```

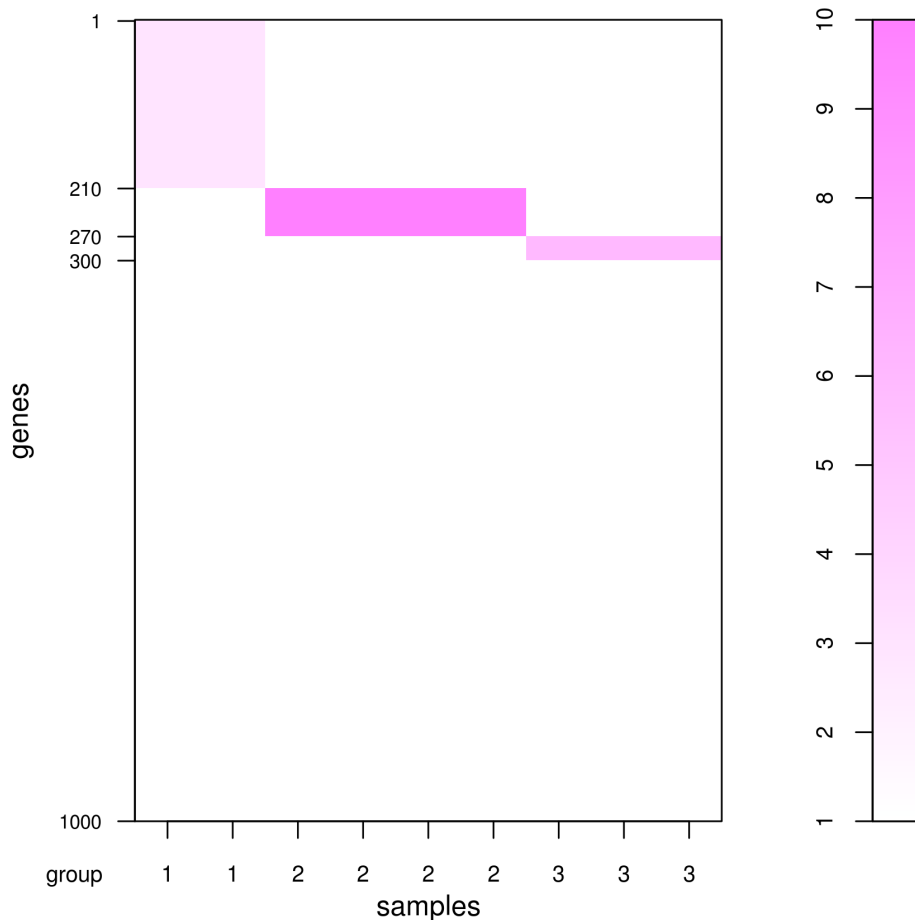
```
      group
G1_rep1  1
G1_rep2  1
G2_rep1  2
G2_rep2  2
G2_rep3  2
G2_rep4  2
G3_rep1  3
G3_rep2  3
G3_rep3  3
```

```
> head(tcc$count)
```

	G1_rep1	G1_rep2	G2_rep1	G2_rep2	G2_rep3	G2_rep4	G3_rep1	G3_rep2	G3_rep3
gene_1	126	86	17	38	24	35	4	19	71
gene_2	7	3	5	4	5	4	1	7	3
gene_3	48	17	3	15	57	12	4	9	10
gene_4	264	331	122	116	135	108	97	83	84
gene_5	69	51	32	7	15	22	4	33	11
gene_6	426	211	58	19	65	179	20	115	88

The pseudo-color image for the generated simulation data regarding the DEGs can be obtained from the `plotFCPseudocolor` function. The right bar (from white to magenta) indicates the degree of fold-change (FC). As expected, it can be seen that the first 210, 60, and 30 genes are up-regulated in G1, G2, and G3, respectively.

```
> plotFCPseudocolor(tcc)
```



Now let us see how the DEGES/edgeR-edgeR combination with the original edgeR-edgeR combination performs. First we calculate the AUC value for the ranked gene list obtained from the DEGES/edgeR-edgeR combination.

```
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edgeR",
+                       iteration = 1)
> tcc <- estimateDE(tcc, test.method = "edgeR", FDR = 0.1)
> calcAUCValue(tcc)
```

```
[1] 0.8748452
```

Next, we calculate the corresponding value using the original **edgeR** procedure for single factor experimental design (i.e., the edgeR-edgeR combination).

```
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edgeR",
+                       iteration = 0)
> tcc <- estimateDE(tcc, test.method = "edgeR", FDR = 0.1)
> calcAUCValue(tcc)
```

```
[1] 0.8692119
```

It can be seen that the DEGES/edgeR-edgeR combination outperforms the original **edgeR** procedure under the given simulation conditions. Note that the `test.method` argument will be ignored when `iteration = 0` is specified.

Next, let us generate another dataset consisting of a total of eight groups. The simulation conditions for this dataset are as follows: (i) the number of genes is 10,000 (i.e., `Ngene = 10000`), (ii) the first 34% of genes are DEGs (`PDEG = 0.34`), (iii) the breakdowns of the up-regulated DEGs are respectively 10%, 30%, 5%, 10%, 5%, 21%, 9%, and 10% in Groups 1-8 (`DEG.assign = c(0.1, 0.3, 0.05, 0.1, 0.05, 0.21, 0.09, 0.1)`), (iv) the levels of DE are 3.1-, 13-, 2-, 1.5-, 9-, 5.6-, 4-, and 2-fold in individual groups (`DEG.foldchange = c(3.1, 13, 2, 1.5, 9, 5.6, 4, 2)`), and (v) there are a total of nine libraries (except for G3, none of the groups have replicates) (`replicates = c(1, 1, 2, 1, 1, 1, 1, 1)`).

```
> set.seed(1000)
> library(TCC)
> tcc <- simulateReadCounts(Ngene = 10000, PDEG = 0.34,
+                          DEG.assign = c(0.1, 0.3, 0.05, 0.1, 0.05, 0.21, 0.09, 0.1),
+                          DEG.foldchange = c(3.1, 13, 2, 1.5, 9, 5.6, 4, 2),
+                          replicates = c(1, 1, 2, 1, 1, 1, 1, 1))
> dim(tcc$count)
```

```
[1] 10000    9
```

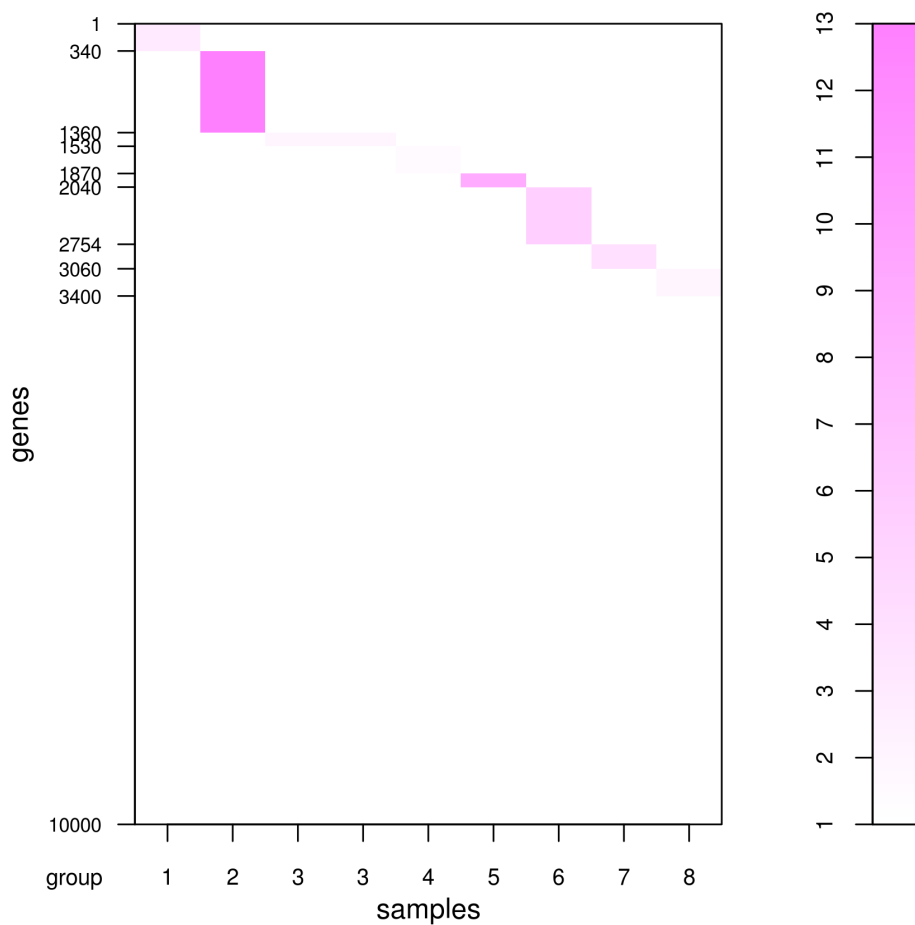
```
> tcc$group
```

```
      group
G1_rep1  1
G2_rep1  2
G3_rep1  3
G3_rep2  3
G4_rep1  4
G5_rep1  5
G6_rep1  6
G7_rep1  7
G8_rep1  8
```

```
> head(tcc$count)
```

	G1_rep1	G2_rep1	G3_rep1	G3_rep2	G4_rep1	G5_rep1	G6_rep1	G7_rep1	G8_rep1
gene_1	253	32	16	14	27	93	25	17	26
gene_2	17	3	4	4	6	7	9	2	2
gene_3	51	17	10	9	9	4	15	10	9
gene_4	289	83	105	84	121	78	76	126	99
gene_5	43	4	4	11	0	19	34	42	15
gene_6	491	99	137	101	55	104	224	105	86

```
> plotFCPseudocolor(tcc)
```



This kind of simulation data may be useful for evaluating methods aimed at identifying tissue-specific (or tissue-selective) genes.

5.4 Multi-factor data

The `simulateReadCounts` function can also generate simulation data in multi-factor experimental design. Different from above single-factor experimental design, the `group` argument should be used instead of `replicates` for specifying sample conditions (or factors) when generating simulation data in multi-factor design. In relation to the `group` specification, the `DEG.foldchange` argument should also be specified as a data frame object.

We generate a dataset consisting of two factors for comparing (i) two Groups (i.e., "WT" vs. "KO") as the first factor, at (ii) two time points (i.e., "1d" vs. "2d") as the second factor, with all samples obtained from independent subjects. There are a total of four conditions ("WT_1d", "WT_2d", "KO_1d", and "KO_2d") each of which has two biological replicates, comprising a total of eight samples. The `group` argument for this experimental design can be described as follows:

```
> group <- data.frame(
+   GROUP = c("WT", "WT", "WT", "WT", "KO", "KO", "KO", "KO"),
+   TIME = c("1d", "1d", "2d", "2d", "1d", "1d", "2d", "2d")
+ )
```

Next, we design the number of types of DEGs and the levels of fold-change by the `DEG.foldchange` argument. We here introduce three types of DEGs: (a) 2-fold up-regulation in the first four samples (i.e., "WT"), (b) 3-fold up-regulation in the last four samples (i.e., "KO"), and (c) 2-fold down-regulation at "2d" in "WT" and 4-fold up-regulation at "2d" in "KO". This implies that the first two types of DEGs are related to the first factor (i.e., "WT" vs. "KO") and the third type of DEG is related to the second factor (i.e., "1d" vs. "2d").

```
> DEG.foldchange <- data.frame(
+   FACTOR1.1 = c(2, 2, 2, 2, 1, 1, 1, 1),
+   FACTOR1.2 = c(1, 1, 1, 1, 3, 3, 3, 3),
+   FACTOR2 = c(1, 1, 0.5, 0.5, 1, 1, 4, 4)
+ )
```

The other simulation conditions for this dataset are as follows: (1) the number of gene is 1,000 (i.e., `Ngene = 1000`), (2) the first 20% of genes are DEGs (i.e., `PDEG = 0.2`), and (3) the breakdowns of the three types of DEGs are 50%, 20%, and 30% (i.e., `DEG.assign = c(0.5, 0.2, 0.3)`).

```
> set.seed(1000)
> tcc <- simulateReadCounts(Ngene = 10000, PDEG = 0.2,
+   DEG.assign = c(0.5, 0.2, 0.3),
+   DEG.foldchange = DEG.foldchange,
+   group = group)
```

Since the first six rows in the dataset corresponds to the first type of DEGs, we can see the 2-fold up-regulation in the first four columns (i.e., WT-related samples) compared to the last four columns (i.e., KO-related samples).

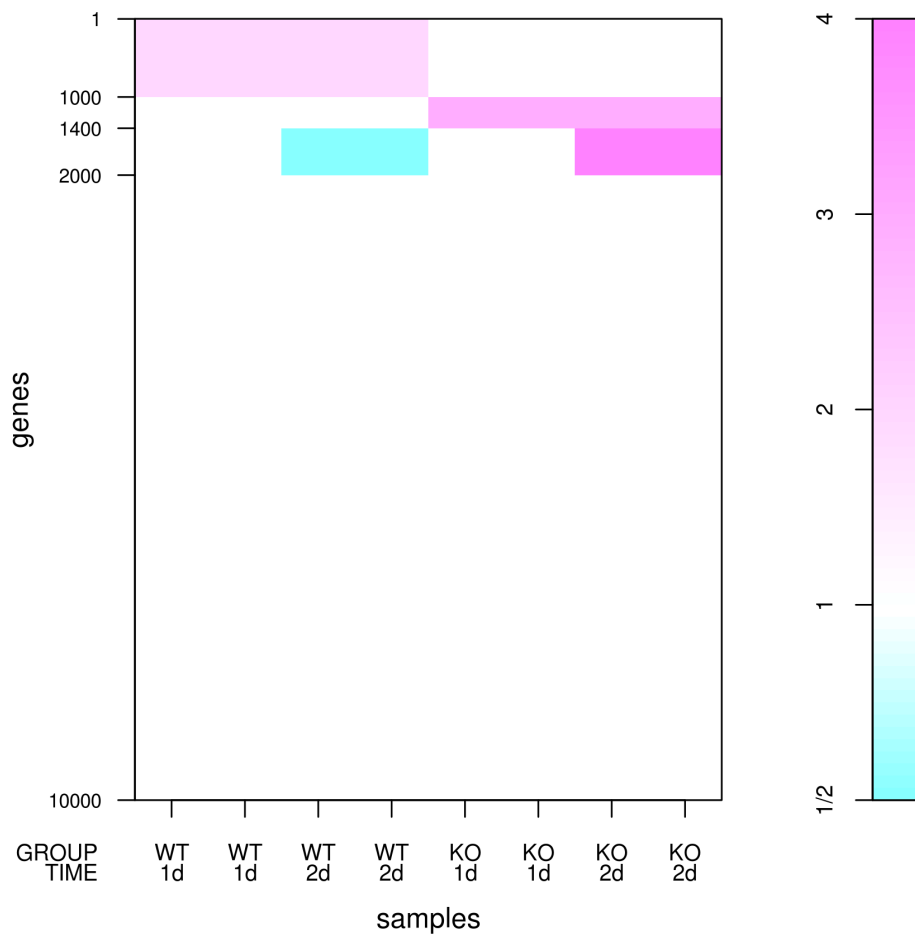
```
> head(tcc$count)
```

	WT1d_rep1	WT1d_rep2	WT2d_rep1	WT2d_rep2	KO1d_rep1	KO1d_rep2	KO2d_rep1
gene_1	162	70	32	31	27	93	25
gene_2	12	5	7	7	6	7	11
gene_3	40	34	20	20	9	4	18
gene_4	210	141	286	163	121	78	52
gene_5	37	8	9	22	0	19	0
gene_6	205	204	43	216	55	104	123
	K02d_rep2						
gene_1	49						
gene_2	3						
gene_3	10						
gene_4	113						
gene_5	3						
gene_6	39						

```
> tcc$group
```

```
      GROUP TIME  
WT1d_rep1  WT   1d  
WT1d_rep2  WT   1d  
WT2d_rep1  WT   2d  
WT2d_rep2  WT   2d  
K01d_rep1  KO   1d  
K01d_rep2  KO   1d  
K02d_rep1  KO   2d  
K02d_rep2  KO   2d
```

```
> plotFCPseudocolor(tcc)
```



5.5 Other utilities

Recall that the simulation framework can handle different levels of DE for DEGs in individual groups, and the shape of the distribution for these DEGs is the same as that of non-DEGs. Let us confirm those distributions by introducing more drastic simulation conditions for comparing two groups (G1 vs. G2) with biological replicates; i.e., (i) the number of genes is 20,000 (i.e., $N_{\text{gene}} = 20000$), (ii) the first 30% of genes are DEGs ($P_{\text{DEG}} = 0.30$), (iii) the first 85% of the DEGs are up-regulated in G1 and the remaining 15% are up-regulated in

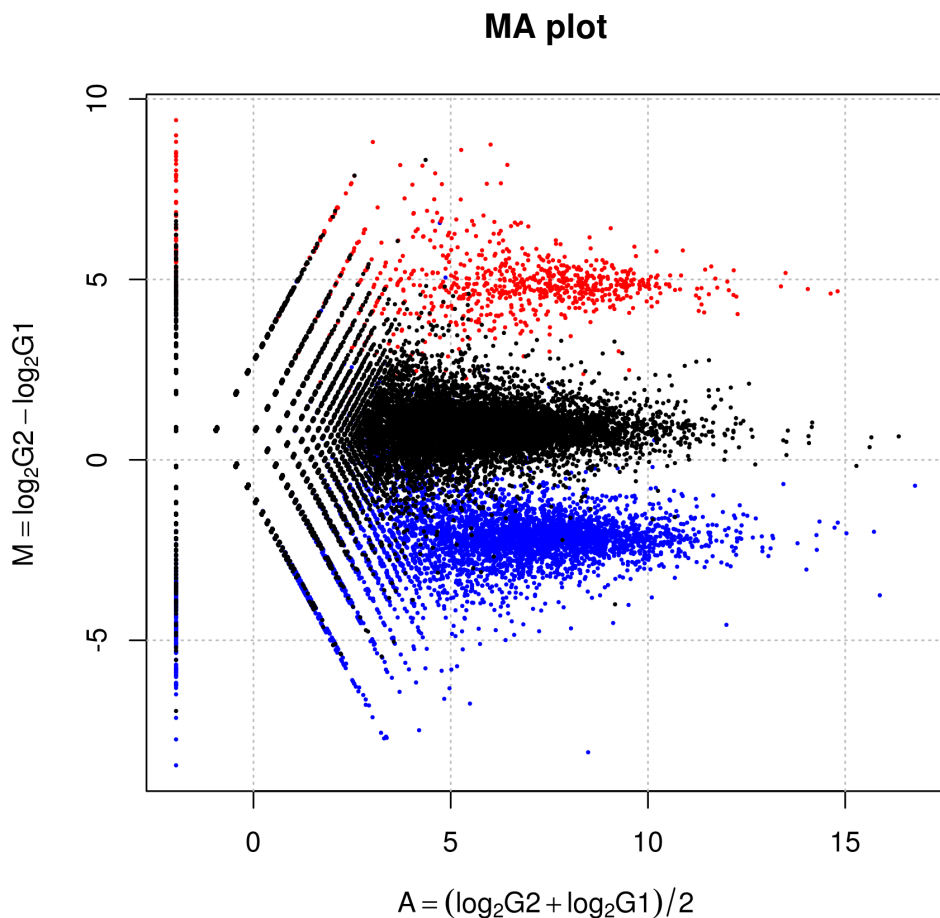
G2 (DEG.assign = c(0.85, 0.15)), (iv) the levels of DE are eight-fold in G1 and sixteen-fold in G2 (DEG.foldchange = c(8, 16)), and (v) there are a total of four samples (two biological replicates for G1 and two biological replicates for G2) (replicates = c(2, 2)).

```
> set.seed(1000)
> library(TCC)
> tcc <- simulateReadCounts(Ngene = 20000, PDEG = 0.30,
+   DEG.assign = c(0.85, 0.15),
+   DEG.foldchange = c(8, 16),
+   replicates = c(2, 2))
> head(tcc$count)
```

	G1_rep1	G1_rep2	G2_rep1	G2_rep2
gene_1	368	243	67	39
gene_2	56	23	9	3
gene_3	140	147	11	18
gene_4	720	932	167	83
gene_5	56	165	50	11
gene_6	504	487	80	133

An M-A plot for the simulation data can be viewed as follows; the points for up-regulated DEGs in G1 and G2 are colored blue and red, respectively. The non-DEGs are in black:

```
> plot(tcc)
```



This plot is generated from simulation data that has been scaled in such a way that the library sizes of each sample are the same as the mean library size of the original data. That is,

```
> normalized.count <- getNormalizedData(tcc)
> colSums(normalized.count)
```

```
G1_rep1 G1_rep2 G2_rep1 G2_rep2
4474229 4474229 4474229 4474229
```

```
> colSums(tcc$count)
```

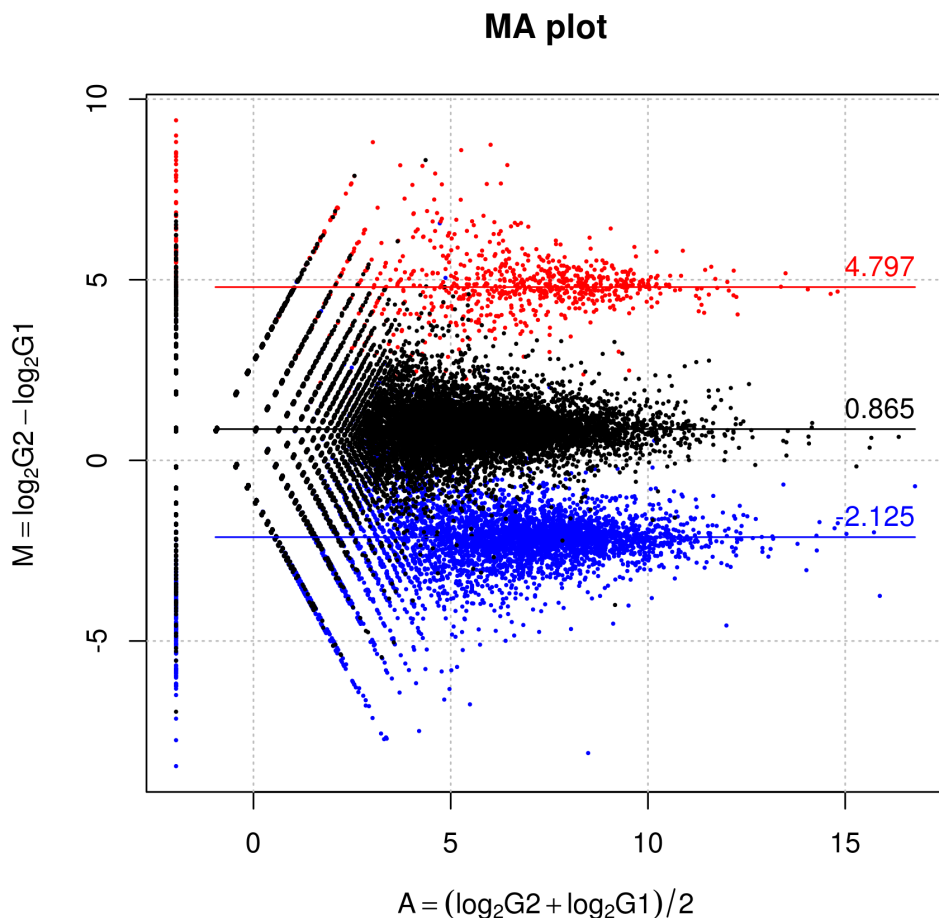
```
G1_rep1 G1_rep2 G2_rep1 G2_rep2
5704644 5813812 3108335 3270126
```

```
> mean(colSums(tcc$count))
```

```
[1] 4474229
```

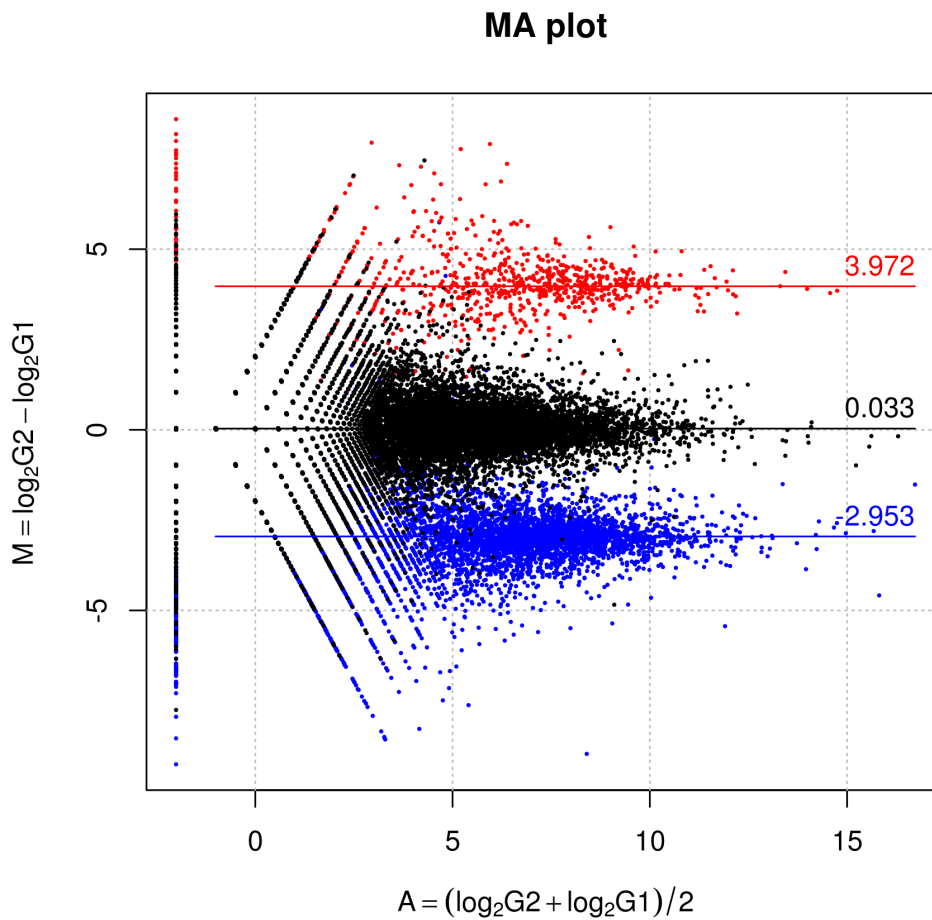
The summary statistics for non-DEGs and up-regulated DEGs in G1 and G2 are upshifted compared with the original intentions of the user (i.e., respective M values of 0, -3, and 4 for non-DEGs and up-regulated DEGs in G1 and G2). Indeed, the median values, indicated as horizontal lines, are respectively 0.865, -2.125, and 4.797 for non-DEGs and up-regulated DEGs in G1 and G2.

```
> plot(tcc, median.lines = TRUE)
```



These upshifted M values for non-DEGs can be modified after performing the iDEGES/edgeR normalization, e.g., the median M value (= 0.033) for non-DEGs based on the iDEGES/edgeR-normalized data is nearly zero.

```
> tcc <- calcNormFactors(tcc, norm.method = "tmm", test.method = "edger",  
+                       iteration = 3, FDR = 0.1, floorPDEG = 0.05)  
> plot(tcc, median.line = TRUE)
```



The comparison of those values obtained from different normalization methods might be another evaluation metric.

6 Session info

```
> sessionInfo()
```

```
R version 3.1.0 (2014-04-10)
```

```
Platform: x86_64-unknown-linux-gnu (64-bit)
```

```
locale:
```

```
[1] LC_CTYPE=en_US.UTF-8      LC_NUMERIC=C
[3] LC_TIME=en_US.UTF-8      LC_COLLATE=C
[5] LC_MONETARY=en_US.UTF-8  LC_MESSAGES=en_US.UTF-8
[7] LC_PAPER=en_US.UTF-8     LC_NAME=C
[9] LC_ADDRESS=C             LC_TELEPHONE=C
[11] LC_MEASUREMENT=en_US.UTF-8 LC_IDENTIFICATION=C
```

```
attached base packages:
```

```
[1] splines  parallel  stats      graphics  grDevices  utils      datasets
[8] methods  base
```

```
other attached packages:
```

```
[1] TCC_1.4.0          ROC_1.40.0         baySeq_1.18.0
[4] edgeR_3.6.0        limma_3.20.0       DESeq2_1.4.0
[7] RcppArmadillo_0.4.200.0 Rcpp_0.11.1        GenomicRanges_1.16.0
[10] GenomeInfoDb_1.0.0 IRanges_1.21.45    DESeq_1.16.0
[13] lattice_0.20-29    locfit_1.5-9.1     Biobase_2.24.0
[16] BiocGenerics_0.10.0
```

```
loaded via a namespace (and not attached):
```

```
[1] AnnotationDbi_1.26.0 DBI_0.2-7          RColorBrewer_1.0-5
[4] RSQLite_0.11.4      XML_3.98-1.1      XVector_0.4.0
[7] annotate_1.42.0      genefilter_1.46.0 geneplotter_1.42.0
[10] grid_3.1.0          samr_2.0           stats4_3.1.0
[13] survival_2.37-7     tools_3.1.0       xtable_1.7-3
```

7 References

- [1] Anders S, Huber W. 2010. Differential expression analysis for sequence count data. *Genome Biol* 11: R106.
- [2] Di Y, Schafer DW, Cumbie JS, Chang JH. 2011. The NBP negative binomial model for assessing differential gene expression from RNA-Seq. *Stat Appl Genet Mol Biol* 10.
- [3] Dillies MA, Rau A, Aubert J, Hennequet-Antier C, Jeanmougin M, Servant N, Keime C, Marot G, Castel D, Estelle J, Guernec G, Jagla B, Jouneau L, Laloë D, Le Gall C, Schaëffer B, Le Crom S, Guedj M, Jaffrézic F; French StatOmique Consortium. 2013. A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis. *Brief Bioinform* 14: 671-683.
- [4] Glaus P, Honkela A, and Rattray M. 2012. Identifying differentially expressed transcripts from RNA-seq data with biological variation. *Bioinformatics* 28: 1721-1728.
- [5] Hardcastle TJ and Kelly KA. 2010. baySeq: empirical Bayesian methods for identifying differential expression in sequence count data. *BMC Bioinformatics* 11: 422.
- [6] Kadota K, Nakai Y, Shimizu K. 2008. A weighted average difference method for detecting differentially expressed genes from microarray data. *Algorithms Mol Biol* 3: 8.
- [7] Kadota K, Nishimura SI, Bono H, Nakamura S, Hayashizaki Y, Okazaki Y, Takahashi K. 2003. Detection of genes with tissue-specific expression patterns using Akaike's Information Criterion (AIC) procedure. *Physiol Genomics* 12: 251-259.
- [8] Kadota K, Nishiyama T, and Shimizu K. 2012. A normalization strategy for comparing tag count data. *Algorithms Mol Biol* 7: 5.
- [9] Kadota K, Ye J, Nakai Y, Terada T, Shimizu K. 2006. ROKU: a novel method for identification of tissue-specific genes. *BMC Bioinformatics* 7: 294.
- [10] McCarthy DJ, Chen Y, Smyth GK. 2012. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res* 40: 4288-4297.
- [11] Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26: 139-140.
- [12] Robinson MD and Oshlack A. 2010. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol* 11: R25.
- [13] Robinson MD and Smyth GK. 2008. Small-sample estimation of negative binomial dispersion, with applications to SAGE data. *Biostatistics* 9: 321-332.
- [14] Sun J, Nishiyama T, Shimizu K, and Kadota K. TCC: an R package for comparing tag count data with robust normalization strategies. *BMC Bioinformatics* 14: 219.
- [15] Ueda T. 1996. Simple method for the detection of outliers. *Japanese J Appl Stat* 25: 17-26.