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1 Introduction

Transcriptome analysis can measure the whole gene expression at the same time and space, so detecting gene expression differences in different tissues or different time points is the most basic and cost-effective method of molecular biology. MGI MegaBOLT RNA-seq pipeline can quickly analyze and detect all gene expression in a sample (normally stored as FPKM file), and this App-note introduces how to use MegaBOLT expression results to do the analysis.

Usually, we recommend differential expression analysis on replicates (e.g., triplicated samples), so that we can calculate the statistics p-value to prove that the difference between the two samples is valid and not caused by sampling error. However, in some special cases (such as sparse samples), we can only perform a single sample sequencing without repetition. This situation is also included in this app-note.

Time series analysis is a very important field in data science. It interprets the development process of individuals or tissues by counting gene expression at different times at the same spatial location, usually time series data will include parts with patterns and irregular parts, and what we need to do is to eliminate the irregular parts and find the pattern, then make a prediction.

The Scripts and test data are available at GitHub:

https://github.com/MGI-APAC-FBS/App-Note_RNA-seq

2 Differential expression for replicated samples (DESeq2)

Normally people use DESeq2 for he replicated samples analysis. In this case, an example with a batch of triplicated samples would be used for the analysis.

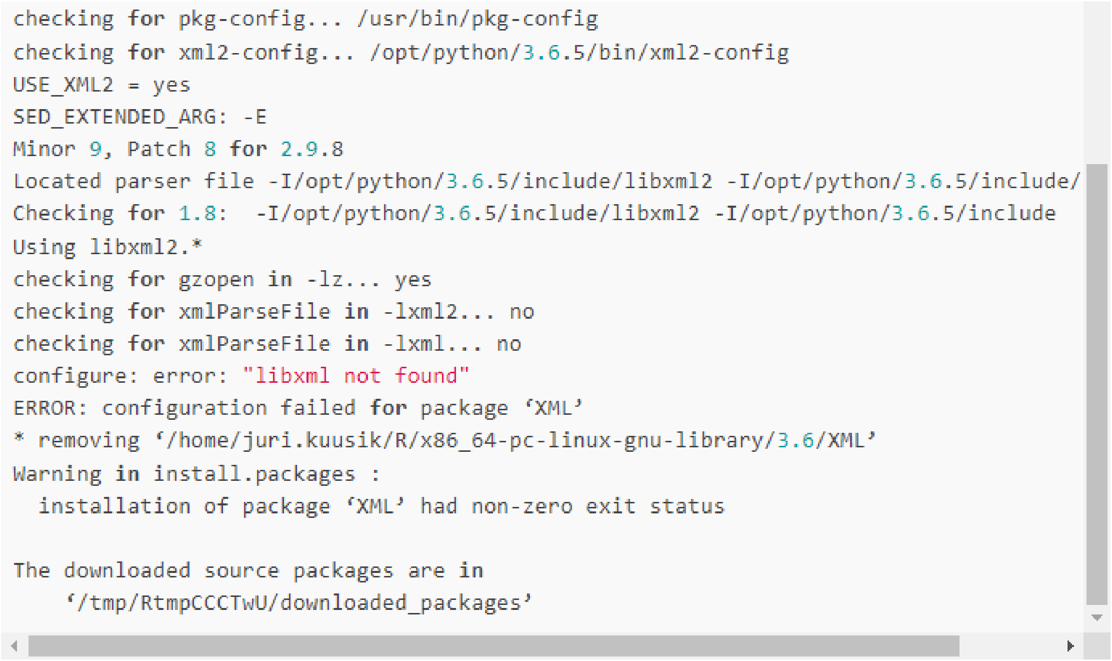
2.1 Installation

The easiest way is to use Bioconductor for the installation. We just need to find an appropriate Bioconductor version for your R system, i.e., Bioconductor 3.14 is only for R4.1, and 3.10 is for R3.6.

In this case we will use R3.6, which is widely used in most laboratories. Please refer to the following linkage for the installation:

https://bioconductor.org/packages/release/bioc/html/DESeq2.html

In some cases, there maybe some software missed in your operating system, i.e., you cannot install XML package on R-Studio Pro, which gives an error as:



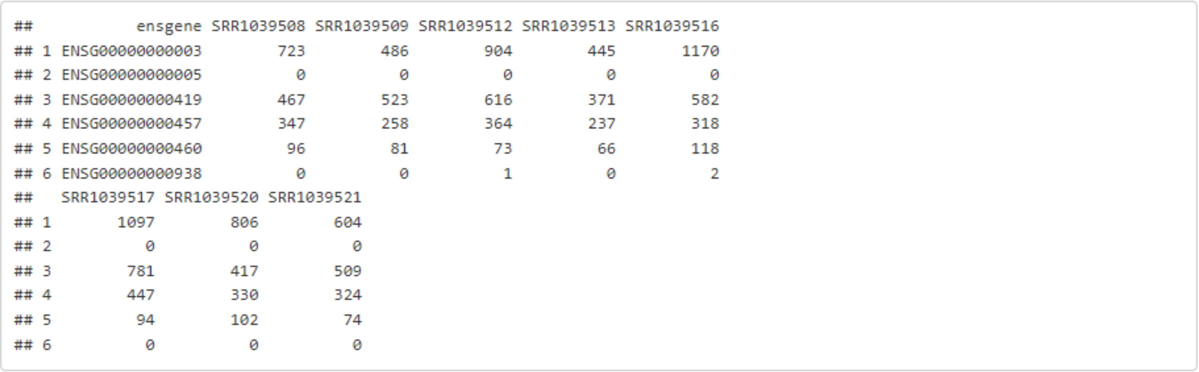
```
checking for pkg-config... /usr/bin/pkg-config
checking for xml2-config... /opt/python/3.6.5/bin/xml2-config
USE_XML2 = yes
SED_EXTENDED_ARG: -E
Minor 9, Patch 8 for 2.9.8
Located parser file -I/opt/python/3.6.5/include/libxml2 -I/opt/python/3.6.5/include/
Checking for 1.8:  -I/opt/python/3.6.5/include/libxml2 -I/opt/python/3.6.5/include
Using libxml2.*
checking for gzopen in -lz... yes
checking for xmlParseFile in -lxml2... no
checking for xmlParseFile in -lxml... no
configure: error: "libxml not found"
ERROR: configuration failed for package ‘XML’
* removing ‘/home/juri.kuusik/R/x86_64-pc-linux-gnu-library/3.6/XML’
Warning in install.packages :
  installation of package ‘XML’ had non-zero exit status

The downloaded source packages are in
    ‘/tmp/RtmpCCCTwU/downloaded_packages’
```

It means the libxml2 is missing in your operating system, so what you should do is to install it separately, if the system you are using is CentOS, you should install it in command as:

```
yum install libxml2
```

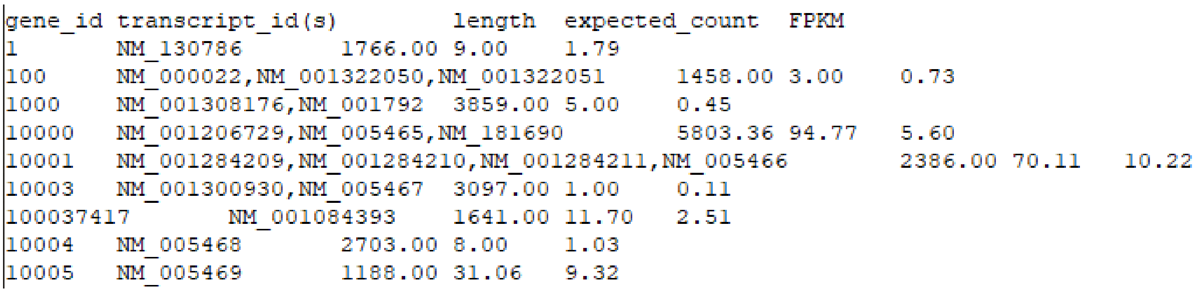
2.2 Preparing count matrix

The input file for DESeq2 is in the form of a matrix of integer values. The first column is genes id, and the following ones are the expression for different samples, which **MUST BE** raw counts of sequencing reads. For example:



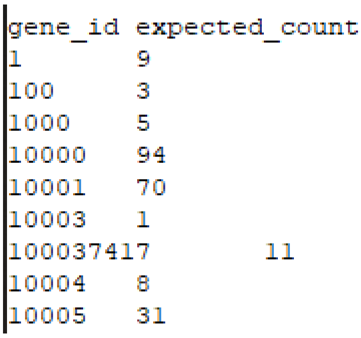
```
##            ensgene SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516
## 1 ENSG00000000003        723        486        904        445       1170
## 2 ENSG00000000005          0          0          0          0          0
## 3 ENSG00000000419        467        523        616        371        582
## 4 ENSG00000000457        347        258        364        237        318
## 5 ENSG00000000460         96         81         73         66        118
## 6 ENSG00000000938          0          0          1          0          2
##   SRR1039517 SRR1039520 SRR1039521
## 1       1097        806        604
## 2          0          0          0
## 3        781        417        509
## 4        447        330        324
## 5         94        102         74
## 6          0          0          0
```

To use MegaBOLT data in DESeq2, we need to extract the reads counts for different samples and store them in a single file. For example, the format of FPKM result in MegaBOLT is from RSEM:



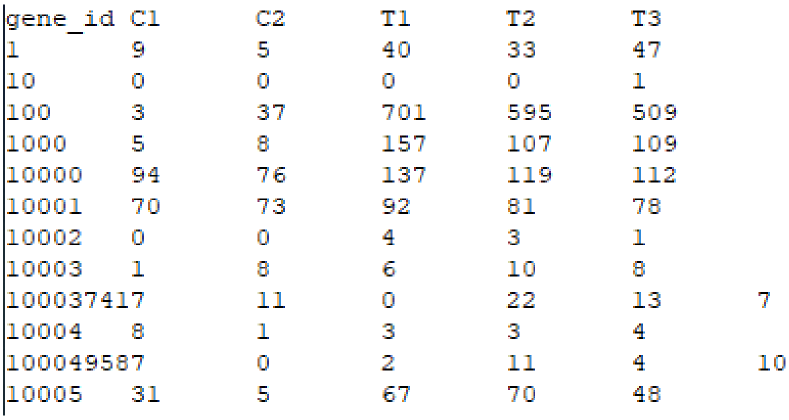
```
gene_id transcript_id(s)        length  expected_count  FPKM
1       NM_130786       1766.00 9.00    1.79
100     NM_000022,NM_001322050,NM_001322051     1458.00 3.00    0.73
1000    NM_001308176,NM_001792  3859.00 5.00    0.45
10000   NM_001206729,NM_005465,NM_181690        5803.36 94.77   5.60
10001   NM_001284209,NM_001284210,NM_001284211,NM_005466        2386.00 70.11   10.22
10003   NM_001300930,NM_005467  3097.00 1.00    0.11
100037417       NM_001084393    1641.00 11.70   2.51
10004   NM_005468       2703.00 8.00    1.03
10005   NM_005469       1188.00 31.06   9.32
```

The data we need for each sample should be from the 1st and 4th column (need to change to integer format):



```
gene_id expected_count
1       9
100     3
1000    5
10000   94
10001   70
10003   1
100037417       11
10004   8
10005   31
```

By doing so for all samples, the Metrix would be:



```
gene_id C1      C2      T1      T2      T3
1       9       5       40      33      47
10      0       0       0       0       1
100     3       37      701     595     509
1000    5       8       157     107     109
10000   94      76      137     119     112
10001   70      73      92      81      78
10002   0       0       4       3       1
10003   1       8       6       10      8
100037417       11      0       22      13      7
10004   8       1       3       3       4
100049587       0       2       11      4       10
10005   31      5       67      70      48
```

There is a script for the generating of expression matrix available in GitHub, you can go to the folder ‘01_extract_Exp_From_RSEM’ in the following website:

https://github.com/MGI-APAC-FBS/App-Note_RNA-seq

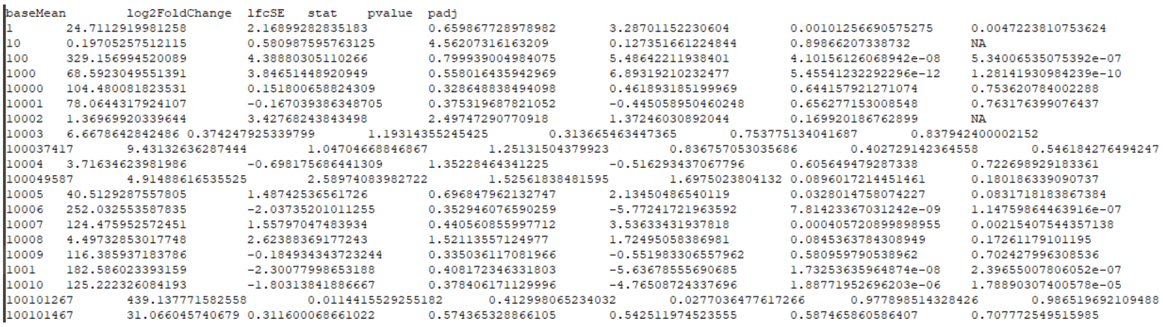
2.3 Run DESeq2

In this step, you can use the input file generated in last step to do the analysis. The analysis need to be done on R system, so you need to prepare a R script first:

```
library("DESeq2")
countdata <- read.table("./all_exp.txt",sep="\t",header=T) ## input file

## set gene names as the row names
len <- length(countdata)
rownames(countdata) <- countdata[,1]
countdata <- countdata[,2:len]

## 2 controls + 3 treatments
type <- c("Control","Control","Treat","Treat","Treat")
coldata <- data.frame(type)

## execute DESeq2
dds <- DESeqDataSetFromMatrix(countData=countdata, colData=coldata, design = ~ type)
dds <- DESeq(dds)
result <- results(dds)
write.csv(result, file="./DiffGeneExp_DESeq2.csv") ## output csv file, can be opened by Excel

## extract differentially expressed genes, by setting 'padj < 0.05 && log2FoldChange > 1 or < -1'
filter_deseq2 <- subset(result, padj < 0.05 & (log2FoldChange > 1 | log2FoldChange < -1))
write.csv(filter_deseq2, file="./DiffGeneExp_DESeq2_Filter.csv") ## output csv file, can be opened by Excel
```

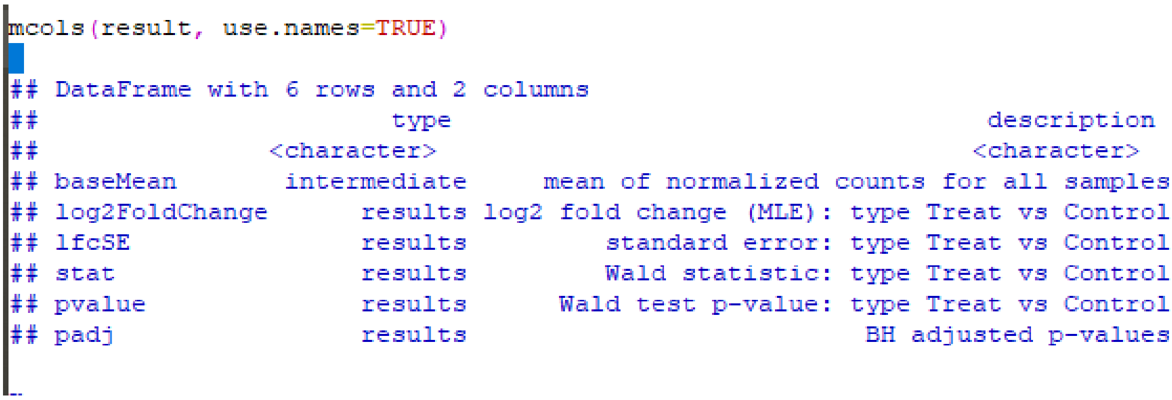
2.4 Output file of DESeq2

The output file contails the information for differentially expressed genes. The format for the output file is:



```
baseMean        log2FoldChange  lfcSE   stat    pvalue  padj
1       24.7112919981258        2.16899282835183        0.659867728978982       3.28701152230604        0.00101256690575275     0.0047223810753624
10      0.19705257512115        0.580987595763125       4.56207316163209        0.127351661224844       0.89866207338732        NA
100     329.156994520089        4.38880305110266        0.799939004984075       5.48642211938401        4.10156126068942e-08    5.34006535075392e-07
1000    68.5923049551391        3.84651448920949        0.558016435942969       6.89319210232477        5.45541232292296e-12    1.28141930984239e-10
10000   104.480081823531        0.151800658824309       0.328648838494098       0.461893185199969       0.644157921271074       0.753620784002288
10001   78.0644317924107        -0.167039386348705      0.375319687821052       -0.445058950460248      0.656277153008548       0.763176399076437
10002   1.36969920339644        3.42768243843498        2.49747290770918        1.37246030892044        0.169920186762899       NA
10003   6.6678642842486 0.374247925339799       1.19314355245425        0.313665463447365       0.753775134041687       0.837942400002152
100037417       9.43132636287444        1.04704668846867        1.25131504379923        0.836757053035686       0.402729142364558       0.546184276494247
10004   3.71634623981986        -0.698175686441309      1.35228464341225        -0.516293437067796      0.605649479287338       0.722698929183361
100049587       4.91488616535525        2.58974083982722        1.52561838481595        1.6975023804132 0.0896017214451461      0.180186339090737
10005   40.5129287557805        1.48742536561726        0.696847962132747       2.13450486540119        0.0328014758074227      0.0831718183867384
10006   252.032553587835        -2.03735201011255       0.352946076590259       -5.77241721963592       7.81423367031242e-09    1.14759864463916e-07
10007   124.475952572451        1.55797047483934        0.440560855997712       3.53633431937818        0.000405720899898955    0.00215407544357138
10008   4.49732853017748        2.62388369177243        1.52113557124977        1.72495058386981        0.0845363784308949      0.17261179101195
10009   116.385937183786        -0.184934343723244      0.335036117081966       -0.551983306557962      0.580959790538962       0.702427996308536
1001    182.586023393159        -2.30077998653188       0.408172346331803       -5.63678555690685       1.73253635964874e-08    2.39655007806052e-07
10010   125.222326084193        -1.80313841886667       0.378406171129996       -4.76508724337696       1.88771952696203e-06    1.78890307400578e-05
100101267       439.137771582558        0.0114415529255182      0.41299806523403       0.0277036477617266      0.977898514328426       0.986519692109488
100101467       31.066045740679 0.311600068661022       0.574365328866105       0.542511974523555       0.587465860586407       0.707772549515985
```

You can get information on the meaning of the columns by checking the ‘result’, which is a DataFram object:



```
mcols(result, use.names=TRUE)

## DataFrame with 6 rows and 2 columns
##                        type                                 description
##                 <character>                                 <character>
## baseMean       intermediate      mean of normalized counts for all samples
## log2FoldChange      results log2 fold change (MLE): type Treat vs Control
## lfcSE               results         standard error: type Treat vs Control
## stat                results         Wald statistic: type Treat vs Control
## pvalue              results      Wald test p-value: type Treat vs Control
## padj                results                       BH adjusted p-values
```

The DESeq2 also provide many types of analysis, such as Summary, plot Counts, volcano plot and PCA. You can find the instruction in:

https://lashlock.github.io/compbio/R_presentation.html

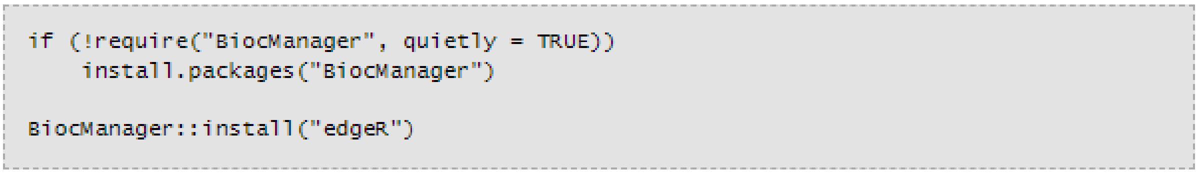
https://bioc.ism.ac.jp/packages/2.14/bioc/vignettes/DESeq2/inst/doc/beginner.pdf

3 Differential expression for non-replicated samples (edgeR)

For some samples which is not suitable to do replicated sequencing, the log2foldchange should be considered, which can be done directly by comparing the FPKM result from 2 samples. There is also a software ‘edgeR’ can be used for this type of comparison. In edgeR, the p-value can be generated, but the p-value is of less reference.

3.1 Installation

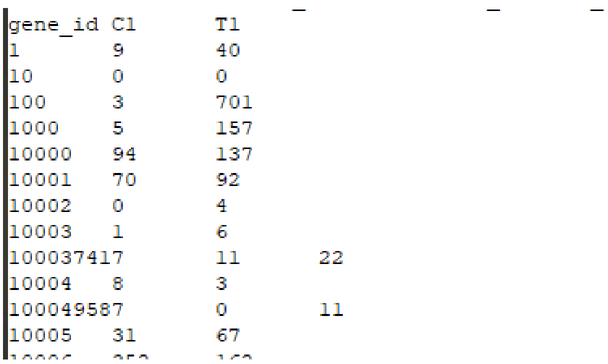
Like DESeq2, the edgeR can be installed in using of Bioconductor. The way of installation is:



```
if (!require("BiocManager", quietly = TRUE))
    install.packages("BiocManager")

BiocManager::install("edgeR")
```

3.2 Preparing count matrix

Since there is no replicated samples being sequenced, the matrix should contains 3 columns (this can be done by using the same scripts in 2.2). In this case, we use C1 and T1 data prepared for the 2.2, which is:



```
gene_id C1      T1
1       9       40
10      0       0
100     3       701
1000    5       157
10000   94      137
10001   70      92
10002   0       4
10003   1       6
100037417       11      22
10004   8       3
100049587       0       11
10005   31      67
```

3.3 Run edgeR

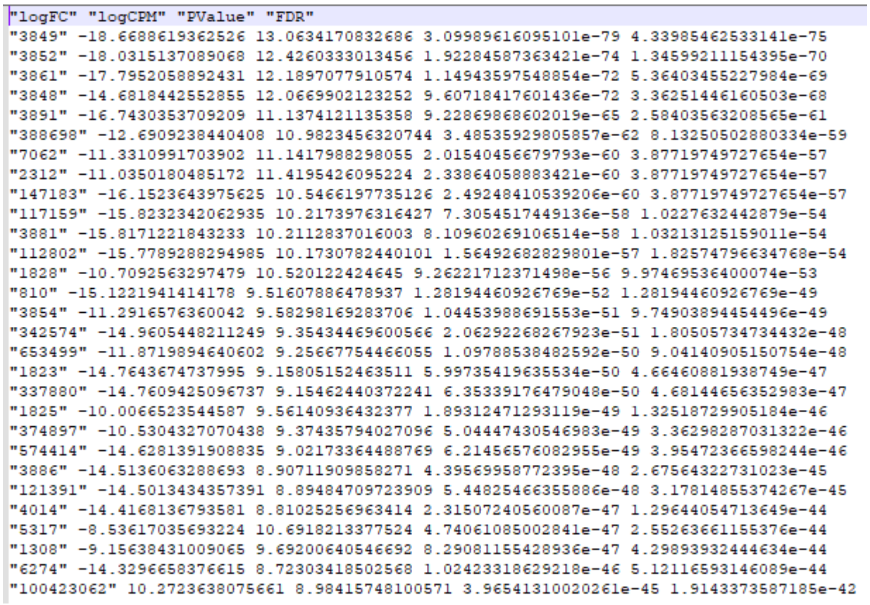
Like DESeq2, to run the edgeR, you need to prepare a R script:

```
library(edgeR)

## input count matrix
countdata <- read.table("./all_exp.txt",sep="\t",header=T) ## input file

## set gene names as the row names
len <- length(countdata)
rownames(countdata) <- countdata[,1]
countdata <- countdata[,2:len]

## make DGEList
type <- c("Control","Treat") ## one control, one treatment
y<-DGEList(counts=countdata,group=type)

## filter  genes with low expression
keep = filterByExpr(y)
y = y[keep,,keep.lib.sizes = FALSE]

## standardization
y = calcNormFactors(y)

## differential expressed genes calculation, use a loose BCV value
y_bcv <- y
bcv <- 0.2
diffExp <- exactTest(y_bcv,dispersion = bcv ^ 2)

## extract result
result = topTags(diffExp, n =90000)
write.csv(result, file="./DiffGeneExp_edgeR.csv") ## output csv file, can be opened by Excel

## extract differentially expressed genes, by setting 'FDR < 0.05 && logFC > 1 or < -1'
result<- as.data.frame(result)
result_DEG = subset(result, FDR < 0.05 & (logFC > 1 | logFC < -1))
write.csv(result_DEG, file="./DiffGeneExp_edgeR_Filter.csv") ## output csv file, can be opened by Excel
```

3.4 Output file of edgeR

The output file of edgeR includes the log2foldchange and other information.

Apart from the widely used value (logFC, pValue, FDR), there is also a value named logCPM, which is the log2 counts-per-million, which can be understood as measuring expression level.



```
"logFC" "logCPM" "PValue" "FDR"
"3849" -18.6688619362526 13.0634170832686 3.09989616095101e-79 4.33985462533141e-75
"3852" -18.0315137089068 12.4260333013456 1.92284587363421e-74 1.34599211154395e-70
"3861" -17.7952058892431 12.1897077910574 1.14943597548854e-72 5.36403455227984e-69
"3848" -14.6818442552855 12.0669902123252 9.60718417601436e-72 3.36251446160503e-68
"3891" -16.7430353709209 11.1374121135358 9.22869868602019e-65 2.58403563208565e-61
"388698" -12.6909238440408 10.9823456320744 3.48535929805857e-62 8.13250502880334e-59
"7062" -11.3310991703902 11.1417988298055 2.01540456679793e-60 3.87719749727654e-57
"2312" -11.0350180485172 11.4195426095224 2.33864058883421e-60 3.87719749727654e-57
"147183" -16.1523643975625 10.5466197735126 2.49248410539206e-60 3.87719749727654e-57
"117159" -15.8232342062935 10.2173976316427 7.3054517449136e-58 1.0227632442879e-54
"3881" -15.8171221843233 10.2112837016003 8.10960269106514e-58 1.03213125159011e-54
"112802" -15.7789288294985 10.1730782440101 1.56492682829801e-57 1.82574796634768e-54
"1828" -10.7092563297479 10.520122424645 9.26221712371498e-56 9.9746953640007 4e-53
"810" -15.1221941414178 9.51607886478937 1.28194460926769e-52 1.28194460926769e-49
"3854" -11.2916576360042 9.58298169283706 1.04453988691553e-51 9.74903894454496e-49
"342574" -14.9605448211249 9.35434469600566 2.06292268267923e-51 1.80505734734432e-48
"653499" -11.8719894640602 9.25667754466055 1.09788538482592e-50 9.04140905150754e-48
"1823" -14.7643674737995 9.15805152463511 5.99735419635534e-50 4.66460881938749e-47
"337880" -14.7609425096737 9.15462440372241 6.35339176479048e-50 4.68144656352983e-47
"1825" -10.0066523544587 9.56140936432377 1.89312471293119e-49 1.32518729905184e-46
"374897" -10.5304327070438 9.37435794027096 5.04447430546983e-49 3.36298287031322e-46
"574414" -14.6281391908835 9.02173364488769 6.21456576082955e-49 3.95472366598244e-46
"3886" -14.513606328869 3 8.90711909858271 4.39569958772395e-48 2.67564322731023e-45
"121391" -14.501343435739 1 8.89484709723909 5.44825466355886e-48 3.17814855374267e-45
"4014" -14.4168136793581 8.81025256963414 2.31507240560087e-47 1.29644054713649e-44
"5317" -8.53617035693224 10.6918213377524 4.74061085002841e-47 2.55263661155376e-44
"1308" -9.15638431009065 9.69200640546692 8.29081155428936e-47 4.29893932444634e-44
"6274" -14.3296658376615 8.72303418502568 1.02423318629218e-46 5.12116593146089e-44
"100423062" 10.272363807566 1 8.98415748100571 3.96541310020261e-45 1.914337358718 5e-42
```

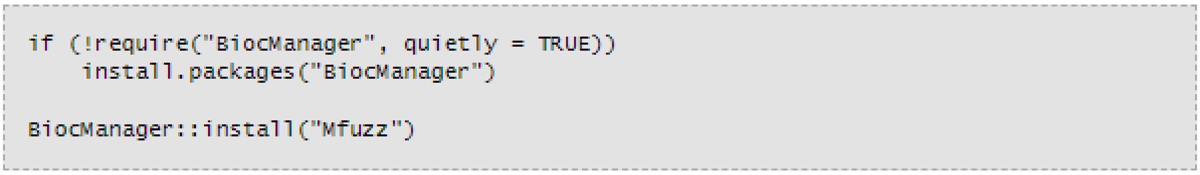
For more information, please refer to the instruction in Bioconductor:

https://bioconductor.org/packages/release/bioc/vignettes/edgeR/inst/doc/edgeRUsersGuide.pdf

4 Time series analysis (Mfuzz)

The common way for time series analysis is to use gene expression of time series sample to build the expression pattern, normally heatmap. The most classical way is Mfuzz, which is a soft clustering function based on fuzzy c-means. It groups genes based on the Euclidean distance and the c-means objective function which is a weighted square error function. Each gene is assigned a membership value between 0 and 1 for each cluster. Hence, genes can be assigned to different clusters in a gradual manner.

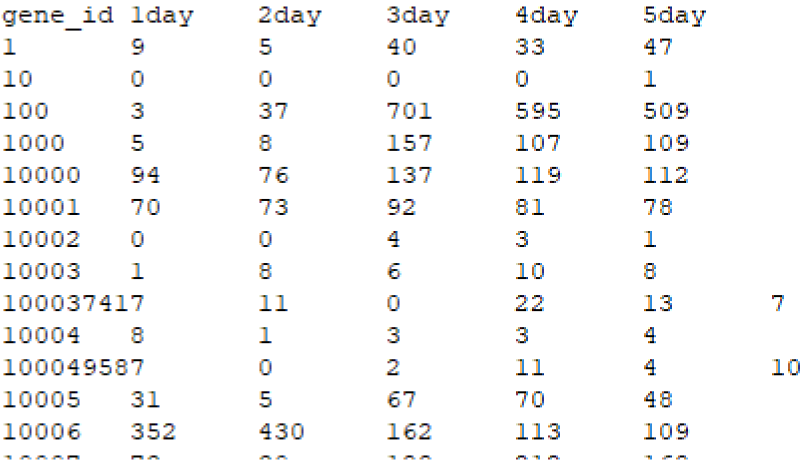
4.1 Installation



```
if (!require("BiocManager", quietly = TRUE))
    install.packages("BiocManager")

BiocManager::install("Mfuzz")
```

4.2 Preparing count matrix

In this case, we prepared samples from 5 time point (this can be done by using the same scripts in 2.2). The first part of the file should be like:



```
gene_id 1day    2day    3day    4day    5day
1       9       5       40      33      47
10      0       0       0       0       1
100     3       37      701     595     509
1000    5       8       157     107     109
10000   94      76      137     119     112
10001   70      73      92      81      78
10002   0       0       4       3       1
10003   1       8       6       10      8
100037417       11      0       22      13      7
10004   8       1       3       3       4
100049587       0       2       11      4       10
10005   31      5       67      70      48
10006   352     430     162     113     109
```

4.3 Run Mfuzz

The Mfuzz is run on R system, so you need to prepare a R script.

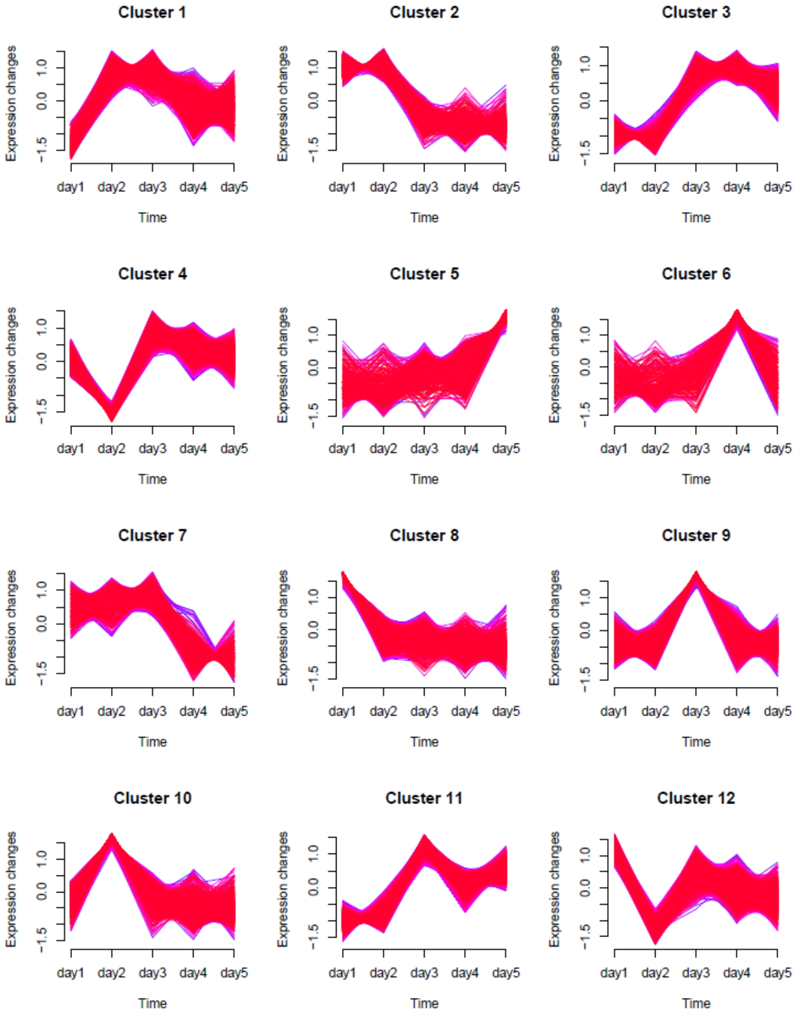
```
library("Mfuzz")

## input matrix
data<-table2eset("./all_exp.txt") ## input file

## data triming, get rid of odd number
data.r <- filter.NA(data, thres=0.25)
data.m <- fill.NA(data.r,mode="mean")
data.f <- filter.std(data.m,min.std=0.05,visu=F)

## standardization
data.s <- standardise(data.f)

## cluster based on fuzzy c-means
cl <- mfuzz(data.s,c=12,m=1.25)

## plot
pdf("TimeSeries.mfuzz.plot.pdf",width=7,height=9) ## output cluster figure
mfuzz.plot2(data.s,cl=cl,mfrow=c(4,3),min.mem=0.75,time.labels=c("day1","day2","day3","day4","day5"),x11 = FALSE)
dev.off()
pdf("TimeSeries.mfuzz.plot.split.pdf",width=7,height=9) ## another cluster figure, one figure per page
mfuzz.plot2(data.s,cl=cl,mfrow=c(1,1),min.mem=0.75,time.labels=c("day1","day2","day3","day4","day5"),x11 = FALSE)

## output
cluster<-cl$cluster
expStandard<-exprs(data.s)
write.table(cluster,file="TimeSeries.mfuzz.cluster") ## output cluster information
write.table(expStandard,file="TimeSeries.mfuzz.expStandard") ## out up/down regulation across different samples
dev.off()
```

4.4 Output files for Mfuzz

4.4.1 output cluster figure

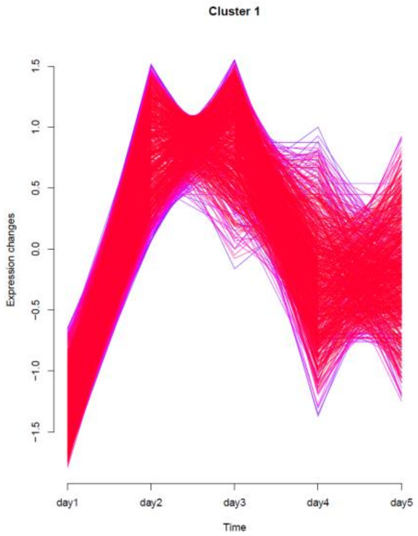
In this case, we have 2 figures for the cluster, the first one is for the summary and another one gives detailed information for each cluster. Here is an example for the cluster result:

RNA-seq - Differential expressed genes analysis



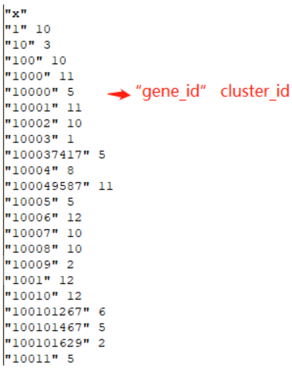
From this figure we can see that 12 clusters was generated and each cluster represent one expression pattern.

The second figure gives amplified clusters information, for example:



4.4.2 output cluster information

If you want to have more information for a single gene, you can go to the file timeSeries.cluster which contains the linkage between gene and cluster:

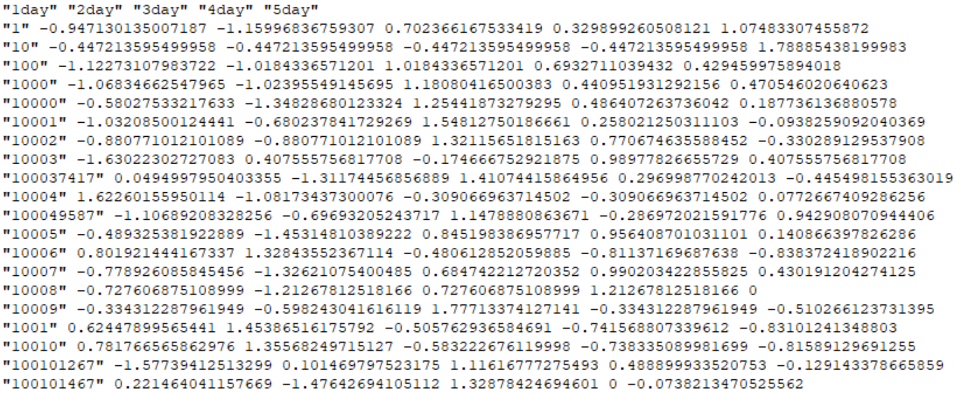


```
"x"
"1" 10
"10" 3
"100" 10
"1000" 11
"10000" 5
"10001" 11
"10002" 10
"10003" 1
"100037417" 5
"10004" 8
"100049587" 11
"10005" 5
"10006" 12
"10007" 10
"10008" 10
"10009" 2
"1001" 12
"10010" 12
"100101267" 6
"100101467" 5
"100101629" 2
"10011" 5
```

"gene_id" cluster_id

4.4.3 output expression

This file gives more information about the expression pattern:

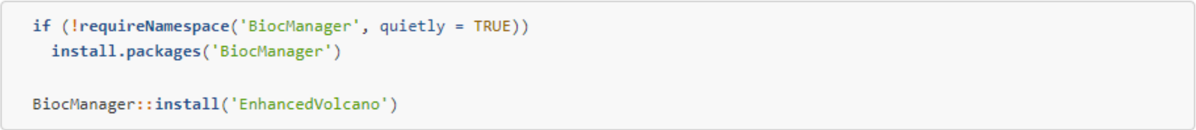


```
"1day" "2day" "3day" "4day" "5day"
"1" -0.947130135007187 -1.15996836759307 0.702366167533419 0.329899260508121 1.07483307455872
"10" -0.447213595499958 -0.447213595499958 -0.447213595499958 -0.447213595499958 1.78885438199983
"100" -1.12273107983722 -1.0184336571201 1.0184336571201 0.6932711039432 0.429459975894018
"1000" -1.06834662547965 -1.02395549145695 1.18080416500383 0.440951931292156 0.470546020640623
"10000" -0.58027533217633 -1.34828680123324 1.25441873279295 0.486407263736042 0.187736136880578
"10001" -1.03208500124441 -0.680237841729269 1.54812750186661 0.258021250311103 -0.0938259092040369
"10002" -0.880771012101089 -0.880771012101089 1.32115651815163 0.770674635588452 -0.330289129537908
"10003" -1.63022302727083 0.407555756817708 -0.174666752921875 0.98977826655729 0.407555756817708
"100037417" 0.0494997950403355 -1.31174456856889 1.41074415864956 0.296998770242013 -0.445498155363019
"10004" 1.62260155950114 -1.08173437300076 -0.309066963714502 -0.309066963714502 0.0772667409286256
"100049587" -1.10689208328256 -0.69693205243717 1.1478880863671 -0.286972021591776 0.942908070944406
"10005" -0.489325381922889 -1.45314810389222 0.845198386957717 0.956408701031101 0.140866397826286
"10006" 0.801921444167337 1.32843552367114 -0.480612852059885 -0.81137169687638 -0.838372418902216
"10007" -0.778926085845456 -1.32621075400485 0.684742212720352 0.990203422855825 0.430191204274125
"10008" -0.727606875108999 -1.21267812518166 0.727606875108999 1.21267812518166 0
"10009" -0.334312287961949 -0.598243041616119 1.77713374127141 -0.334312287961949 -0.510266123731395
"1001" 0.62447899565441 1.45386516175792 -0.505762936584691 -0.741568807339612 -0.83101241348803
"10010" 0.781766565862976 1.35568249715127 -0.583222676119998 -0.738335089981699 -0.81589129691255
"100101267" -1.57739412513299 0.101469797523175 1.11616777275493 0.488899933520753 -0.129143378665859
"100101467" 0.221464041157669 -1.47642694105112 1.32878424694601 0 -0.0738213470525562
```

5 Volcano plot

The volcano plot would be easier for the interpretation of DEG result. There are many software and methods to generate this figure, in this case, we will use the R packages ‘EnhancedVolcano’ for the plotting.

5.1 Installation



```
if (!requireNamespace('BiocManager', quietly = TRUE))
  install.packages('BiocManager')

BiocManager::install('EnhancedVolcano')
```

5.2 Run EnhancedVolcano

The inputs can be directly from the output of DESeq2 or edgeR, to make a volcano plot, you need to prepare a R script first:

5.2.1 R script for result from DESeq2:

```
library(EnhancedVolcano)

## input the result from DESeq2
inputFileName="./DiffGeneExp_DESeq2.csv"
exp_data <- read.csv(inputFileName, header=TRUE)

## set gene names as the row names
len <- length(exp_data)
rownames(exp_data) <-exp_data[,1]
exp_data <- exp_data[,2:len]

## change values to numeric
exp_data$log2FoldChange=as.numeric(exp_data$log2FoldChange)
exp_data$padj=as.numeric(exp_data$padj)

## plot the figure, for log2FlodChange and padj.
pdf("Volcano_For_DESeq2.pdf")
EnhancedVolcano(exp_data,
lab = rownames(exp_data),
    x = 'log2FoldChange',
    y = 'padj')
```

5.2.2 R script for result from edgeR:

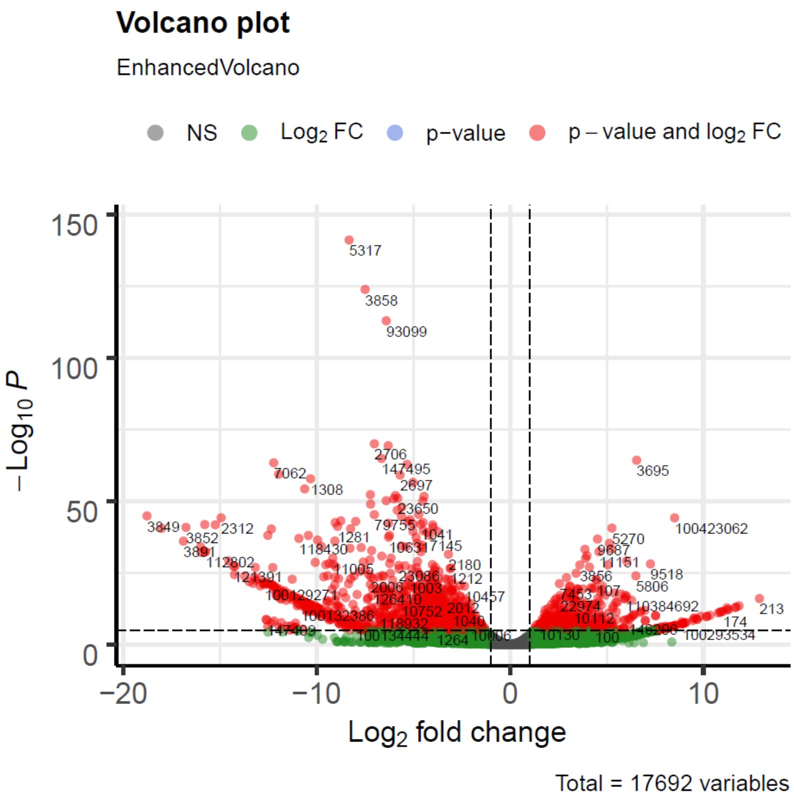
```
library(EnhancedVolcano)

## input the result from edgeR
inputFileName="./DiffGeneExp_edgeR.csv"
exp_data <- read.csv(inputFileName, header=TRUE)

## set gene names as the row names
len <- length(exp_data)
rownames(exp_data) <-exp_data[,1]
exp_data <- exp_data[,2:len]

## change values to numeric
exp_data$logFC=as.numeric(exp_data$logFC)
exp_data$FDR=as.numeric(exp_data$FDR)

## plot the figure, for log2FlodChange and FDR.
pdf("Volcano_For_edgeR.pdf")
EnhancedVolcano(exp_data,
lab = rownames(exp_data),
    x = 'logFC',
    y = 'FDR')
```

5.3 Output files

This case shows the most basic volcano plot, for more functions, please visit the Bioconductor:

https://bioconductor.org/packages/release/bioc/vignettes/EnhancedVolcano/inst/doc/EnhancedVolcano.html

In the following picture, the default cut-off for log2FC is >|2|; the default cut-off for P value is 10e-6.

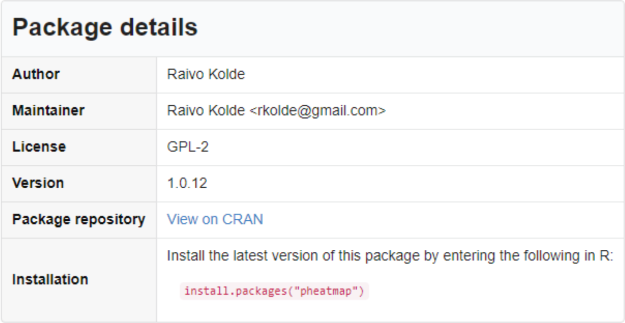


6 Heatmap

After we got the differentially expressed genes, a heatmap would be more intuitive and effective for the display of these genes. In this case, the R package 'pheatmap' would be used for the picture generating.

6.1 Installation

For a typical installation, you just need to open R and install the packages by:

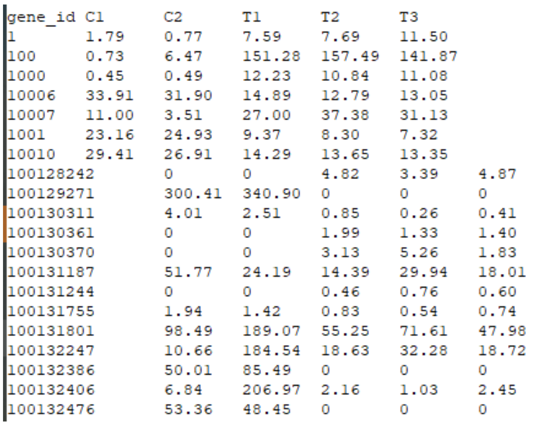


Package details

Author	Raivo Kolde
Maintainer	Raivo Kolde <rkolde@gmail.com>
License	GPL-2
Version	1.0.12
Package repository	View on CRAN
Installation	Install the latest version of this package by entering the following in R: `install.packages("pheatmap")`

6.2 Preparing count matrix

The only thing needed for the input is the expression for all samples. Normally we will use the differentially expressed genes for the plot, so the subset of the whole data should be used, the format for it is:



```
gene_id C1      C2      T1      T2      T3
1       1.79    0.77    7.59    7.69    11.50
100     0.73    6.47    151.28  157.49  141.87
1000    0.45    0.49    12.23   10.84   11.08
10006   33.91   31.90   14.89   12.79   13.05
10007   11.00   3.51    27.00   37.38   31.13
1001    23.16   24.93   9.37    8.30    7.32
10010   29.41   26.91   14.29   13.65   13.35
100128242       0       0       4.82    3.39    4.87
100129271       300.41  340.90  0       0       0
100130311       4.01    2.51    0.85    0.26    0.41
100130361       0       0       1.99    1.33    1.40
100130370       0       0       3.13    5.26    1.83
100131187       51.77   24.19   14.39   29.94   18.01
100131244       0       0       0.46    0.76    0.60
100131755       1.94    1.42    0.83    0.54    0.74
100131801       98.49   189.07  55.25   71.61   47.98
100132247       10.66   184.54  18.63   32.28   18.72
100132386       50.01   85.49   0       0       0
100132406       6.84    206.97  2.16    1.03    2.45
100132476       53.36   48.45   0       0       0
```

6.3 Run the pheatmap

To run the pheatmap, a R script should be prepared:

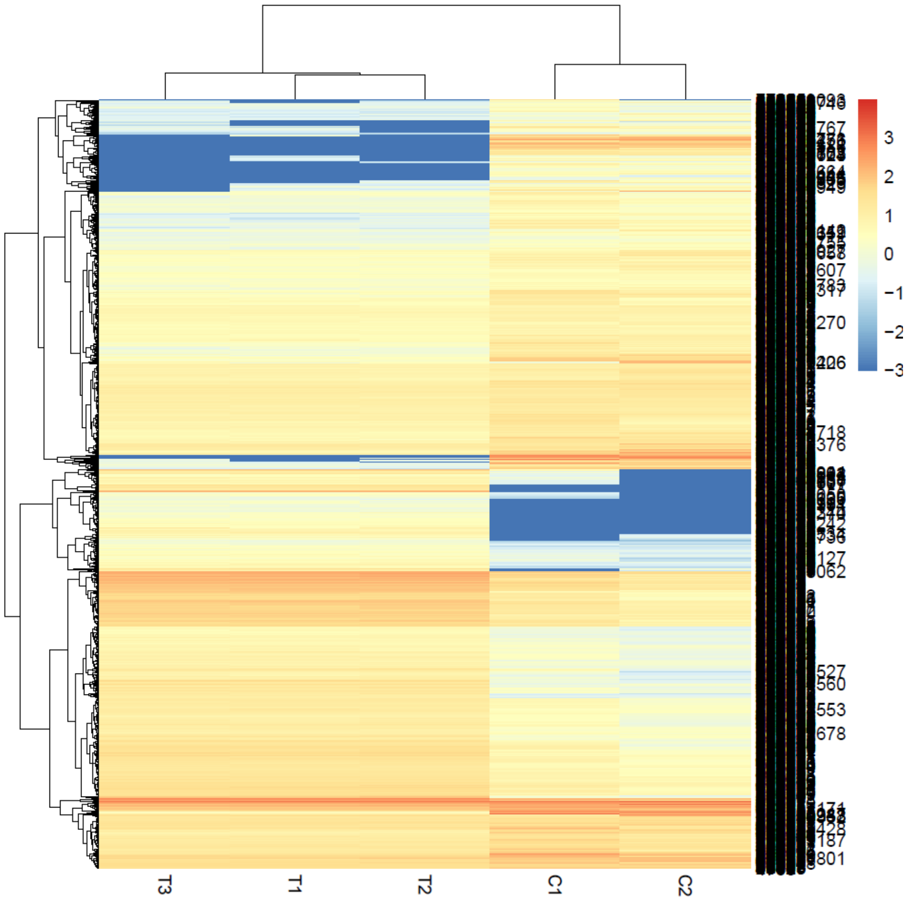
```
library(pheatmap)

## input count matrix
countdata <- read.table("./all_exp.txt",sep="\t",header=T) ## input file

## set gene names as the row names
len <- length(countdata)
rownames(countdata) <- countdata[,1]
countdata <- countdata[,2:len]

## doing log10 for all values. All number 0 should be changed to 0.001 before that.
countdata[countdata == 0] <- 0.001
lcountdata <- log(countdata)/log(10)

# Heatmap
pdf("./pheatmap_Diff_Exp.pdf")
pheatmap(lcountdata)
```

6.4 Output files

The result is a heatmap with upgrade or downgrade information, and clustering for both genes and samples. For more information, you could visit:

https://r-charts.com/correlation/pheatmap/

RNA-seq - Differential expressed genes analysis



-End-