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DIFFERENTIAL EXPRESSION ANALYSIS

The goal of differential expression analysis is to identify genes whose expression differs under different conditions. An important consideration for differential expression analysis is correction for multiple testing.

Load FeatureCountTable into R

```
countdata<- read.table ("/home/mlsi/RNASeq/countTable/featureCounts.txt",header=TRUE, row.names=1)
  class (countdata)

## [1] "data.frame"
```

Edit FeatureCountTable

Deletion of unwanted data and adding data we required in end result.

```
countdata <- countdata[ ,6:ncol(countdata)]
```

Following commands are used to remove .bam or .sam files.

```
colnames(countdata) <- gsub ("\\X.home.mlsi.RNASeq.mapping.","",colnames(countdata))
colnames(countdata) <- gsub ("\\.UHR_[123].bam","",colnames(countdata))
colnames(countdata) <- gsub ("\\.HBR_[123].bam","",colnames(countdata))
colnames(countdata)

## [1] "HBR_1" "HBR_2" "HBR_3" "UHR_1" "UHR_2" "UHR_3"
```

Convert the **data.frame** into a matrix and to do so we uses the followiong command.

```
countdata <- as.matrix(countdata)
class (countdata)

## [1] "matrix"
```

Design coldata

Defining all the characteristics of the sample which we uses and the characteristics we want to compare by performing R.

```
group<- factor(c(rep("HBR",3), rep("UHR",3)))
con<- factor(c(rep("cancer",3), rep("ctrl",3)))
```

Create a coldata frame

Creating coldata frame is a part of colldata design.

```
coldata <- data.frame(row.names=colnames(countdata), group, con)
```

Colors for plots

We can use different colors to represent our data. For this we need RcolorBrewer library.

```
library(RColorBrewer)
mycols <- brewer.pal(11, "Set3")[1:length(unique(group))]
```

Create DESeqDataSet

DESeqDataSet class extends the RangedSummarizedExperiment class of the SummarizedExperiment package.

```
dds<- DESeqDataSetFromMatrix (countData= countdata, colData=coldata, design=
~ con)
```

Counts are displayed.

```
head(counts(dds))

##             HBR_1 HBR_2 HBR_3 UHR_1 UHR_2 UHR_3
## U2              0     0     0     0     0     0
## FRG1FP          0     0     0     0     0     0
## CU104787.1      0     0     0     0     0     0
## BAGE5           0     0     0     0     0     0
## ACTR3BP6        0     0     0     0     0     0
## 5_8S_rRNA       0     0     0     0     0     0
```

To Check the design of the DESEeqDataSets following function is used.

```
design(dds)

## ~con
```

Function to create a data table with read counts normalized to library size.

```
dds <- estimateSizeFactors(dds)
sF<-sizeFactors(dds)
dds_norm_size_factor<- counts(dds, normalized=TRUE)
head(dds_norm_size_factor)

##             HBR_1 HBR_2 HBR_3 UHR_1 UHR_2 UHR_3
## U2              0     0     0     0     0     0
## FRG1FP          0     0     0     0     0     0
## CU104787.1      0     0     0     0     0     0
## BAGE5           0     0     0     0     0     0
## ACTR3BP6        0     0     0     0     0     0
## 5_8S_rRNA       0     0     0     0     0     0

write.table (dds_norm_size_factor, file = "/home/mlsi/RNASeq/analysis/DESeq2/
ddsNormSF.txt", sep = " ", col.names=NA)
```

Pre-Filtering

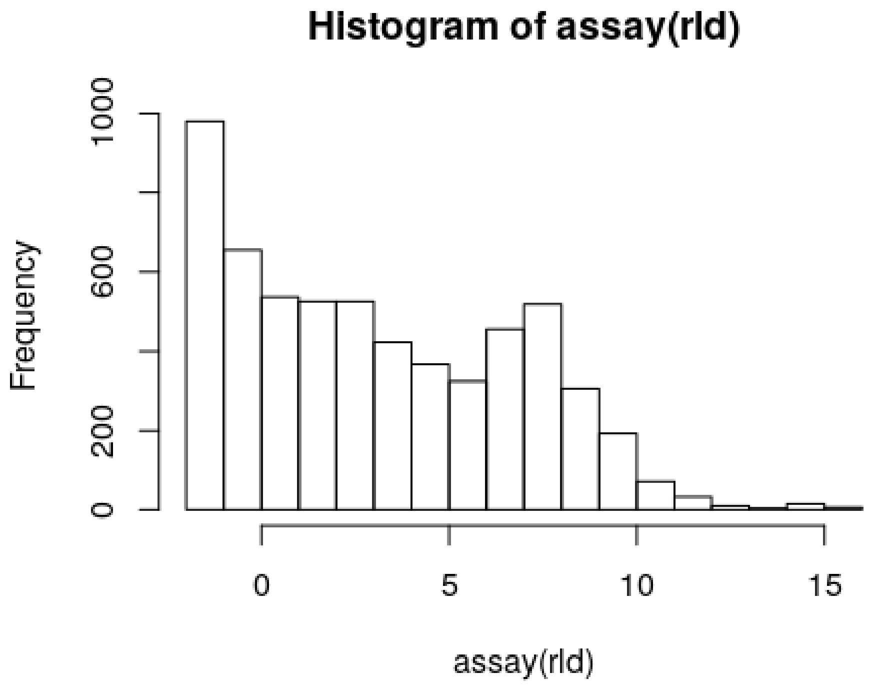
Filtering out low expressed genes to make the data more accurate and workable.

```
dds<- dds [rowSums(counts(dds)) > 1, ]
dim(dds)

## [1] 992   6
```

Rlog Transformation :

This function transforms the count data to the log2 scale in a way which minimizes differences between samples for rows with small counts, and which normalizes with respect to library size.

```
rld<- rlogTransformation(dds)
head(assay(rld))

##                    HBR_1      HBR_2      HBR_3      UHR_1      UHR_2
## LA16c-60D12.1 -0.4166441 -0.4389221 -0.4247142  0.6349742  0.6205201
## LA16c-60D12.2 -0.4904459 -0.5182887 -0.5004650 -0.5680166  1.2020601
## ZNF72P        -1.5452155 -1.5697664 -1.5539134 -1.6178908 -1.1229702
## BNIP3P2       -1.5452155 -1.5697664 -1.5539134 -1.6178908 -1.1229702
## LA16c-60G3.6  -1.5452155 -1.5697664 -1.5539134 -1.6178908 -1.1229702
## ARHGAP42P3    -1.1915067 -1.2311987 -1.2055688 -1.2804190 -0.2088327
##                    UHR_3
## LA16c-60D12.1 -0.4559831
## LA16c-60D12.2  0.1420802
## ZNF72P        -1.5899494
## BNIP3P2       -1.5899494
## LA16c-60G3.6  -1.5899494
## ARHGAP42P3    -1.2638289

hist(assay(rld))
```



Differential Expression Analysis via DESeq2

Explicitly tell results in comparison to make by setting factor levels:

```
design(dds)

## ~con
```

Now we can Run the DESeq-Pipeline for the current condition.

```
dds_con <- DESeq(dds)

## using pre-existing size factors

## estimating dispersions

## gene-wise dispersion estimates

## mean-dispersion relationship

## final dispersion estimates

## fitting model and testing
```

Check factor setting and possible comparisons:

```
resultsNames(dds_con)

## [1] "Intercept"          "con_ctrl_vs_cancer"
```

Create results tables

It is possible to define specific contrast settings.

```
res_con<- results(dds_con, contrast=c("con", "cancer", "ctrl"))
```

A DESeqDataSet object must have an associated design formula. The design formula expresses the variables which will be used in modeling. The formula should be a tilde (~) followed by the variables with plus signs between them (it will be coerced into an formula if it is not already). The design can be changed later, however then all differential analysis steps should be repeated, as the design formula is used to estimate the dispersions and to estimate the log2 fold changes of the model.

You can run the DESeq-Pipeline and results-function with different designs, depending on your defined coldata.

```
summary(res_con)

## 
## out of 992 with nonzero total read count
## adjusted p-value < 0.1
## LFC > 0 (up)       : 182, 18%
## LFC < 0 (down)     : 199, 20%
## outliers [1]       : 0, 0%
## low counts [2]     : 250, 25%
## (mean count < 1)
## [1] see 'cooksCutoff' argument of ?results
## [2] see 'independentFiltering' argument of ?results

mcols(res_con, 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): con cancer vs ctrl
## lfcSE               results         standard error: con cancer vs ctrl
## stat                results         Wald statistic: con cancer vs ctrl
## pvalue              results      Wald test p-value: con cancer vs ctrl
## padj                results                       BH adjusted p-values
```

Change the design of the DESeqDataSet and start a new analysis:

```
design (dds)<- ~group

design(dds)

## ~group

dds_group <- DESeq(dds)

## using pre-existing size factors
## estimating dispersions

## gene-wise dispersion estimates

## mean-dispersion relationship

## final dispersion estimates

## fitting model and testing

resultsNames(dds_group)

## [1] "Intercept"        "group_UHR_vs_HBR"

res_group <- results(dds_group)
```

Exploring and exporting results

Shrinkage

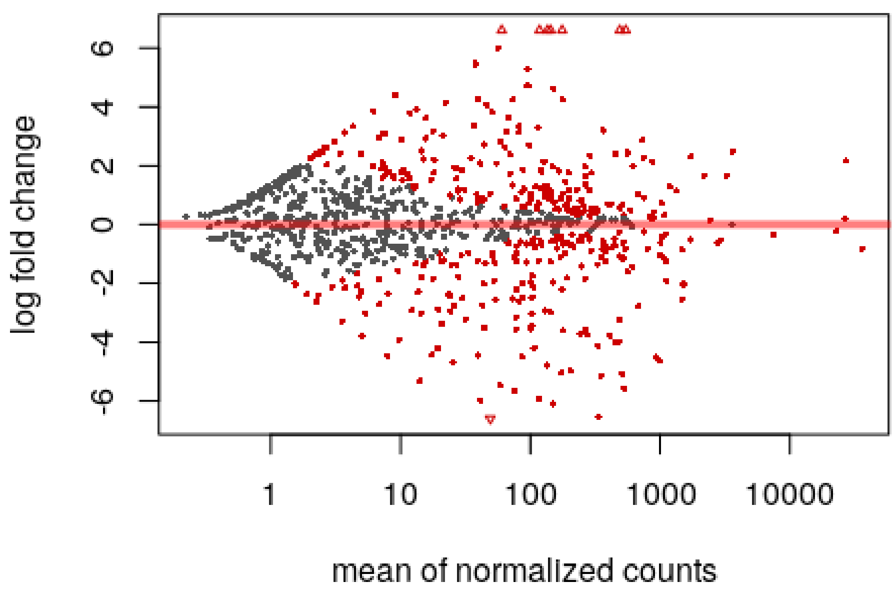
The shrinkage effect is useful for the visualisation as well as the rankinking of different genes.It is more useful visualize the MA-plot for the shrunken log2 fold changes, which remove the noise associated with log2 fold changes from low count genes without requiring arbitrary filtering thresholds. (coef–> check in resultsNames(dds))

```
resLFC_con<- lfcShrink(dds_con,coef=2)
```

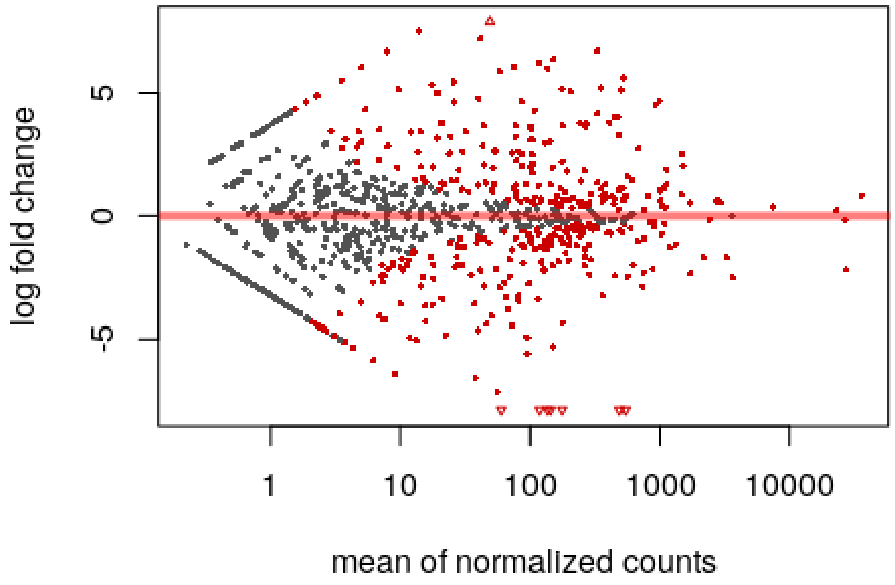
plotMA

A simple helper function that makes a so-called “MA-plot”, i.e. a scatter plot of logarithmic fold changes (on the y-axis) versus the mean of normalized counts (on the x-axis).

```
plotMA(resLFC_con)
```

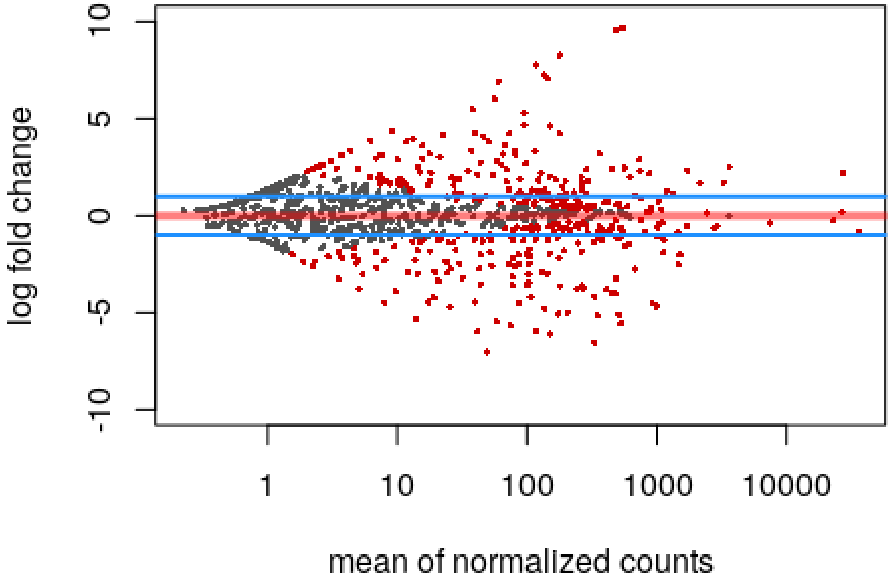


```
plotMA(res_con)
```



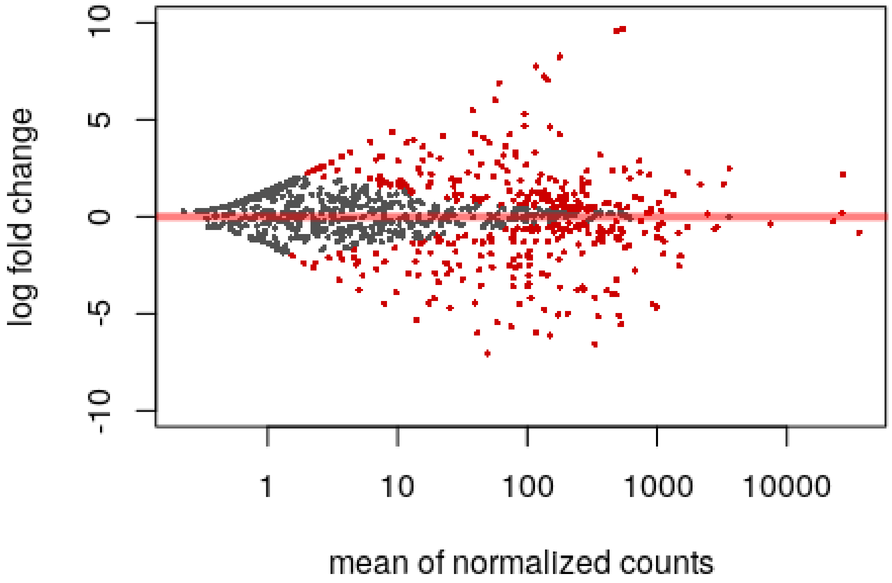
Abline: h : the y-value(s) for horizontal line(s) [v : the x-value(s) for vertical line(s)] a, b : single values specifying the intercept and the slope of the line

```
plotMA(resLFC_con, ylim=c(-10,10))
abline (h=c(-1,1), col="dodgerblue", lwd=2)
```



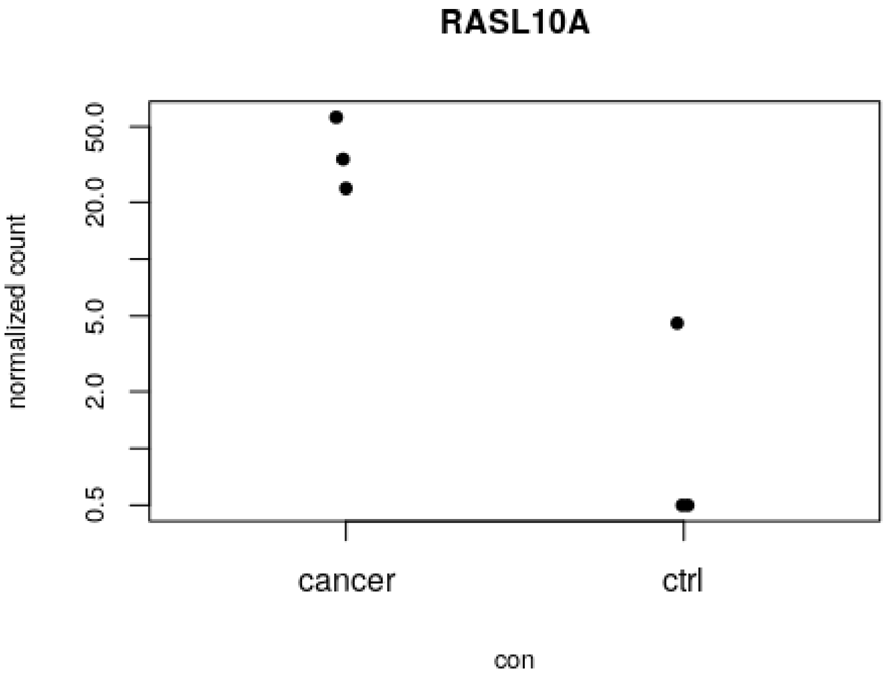
Interactively detect the row number of individual genes by clicking on the plot: (run plotMA and identify in console)

```
plotMA(resLFC_con, ylim=c(-10,10))

idx <- identify(resLFC_con$baseMean, resLFC_con$log2FoldChange)
```



Select the Point you want to identify and press escape after choosing all points . Visualize counts of a single gene of interest via plotCounts:

```
plotCounts(dds_con,gene="RASL10A", intgroup="con", xlab="con",
cex=0.8, pch=19, cex.lab=0.8, cex.sub=0.8, cex.axis=0.8, cex.main=1)
```



Sample Distance Heatmap

A heatmap gives us an overview over similarities and dissimilarities between sampless.

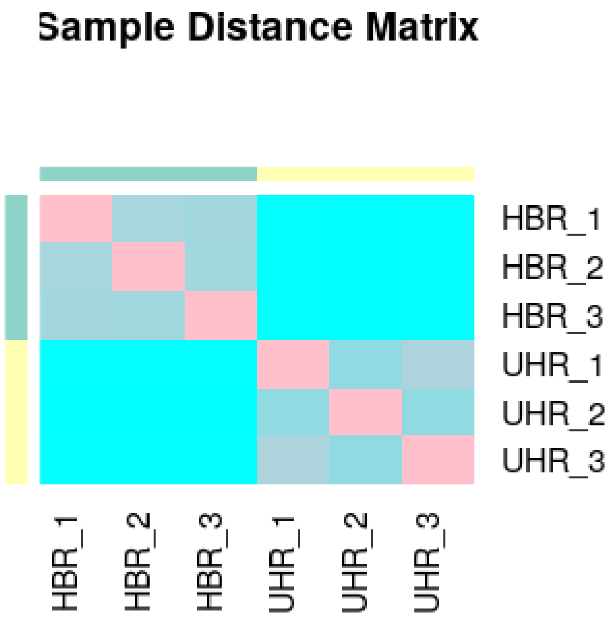
Example 1:

Convert regulized log transferred count data into a sample-dist-matrix:

```
sampleDists <- as.matrix(dist(t(assay(rld))))
```

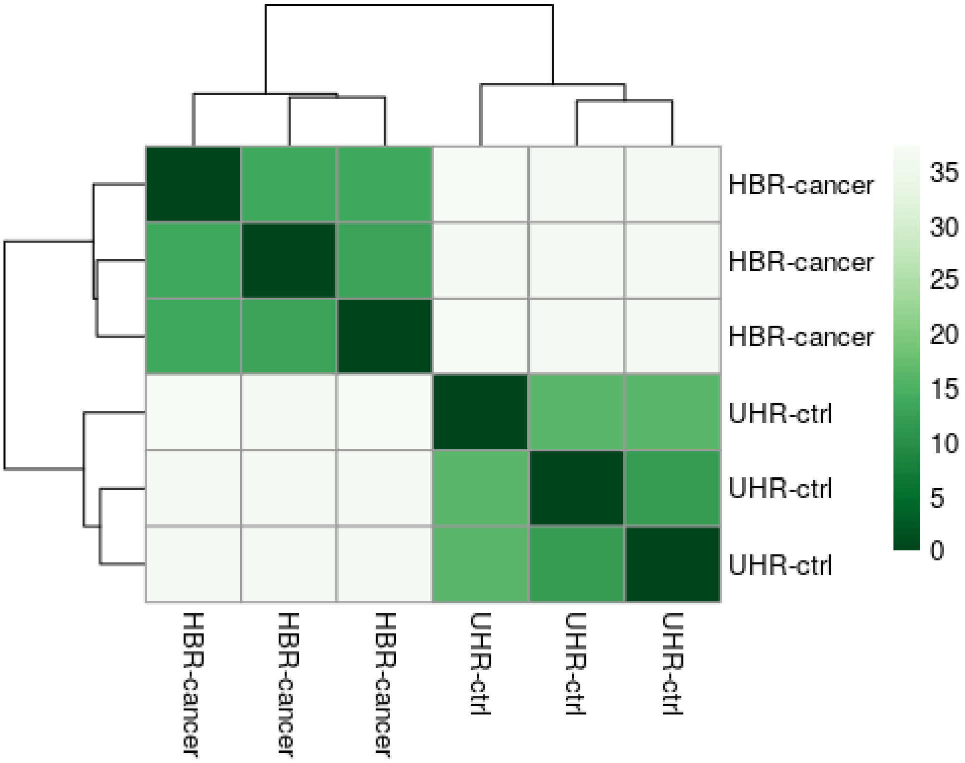
Create the plot:

```
library("gplots")

##
## Attaching package: 'gplots'

## The following object is masked from 'package:IRanges':
##
##     space

## The following object is masked from 'package:S4Vectors':
##
##     space

## The following object is masked from 'package:stats':
##
##     lowess

heatmap.2(as.matrix(sampleDists), key=F, trace="none",Colv = c("cancer", "ctr
l"),Rowv =
"Colv",dendrogram= "none",
        col=colorpanel(100, "pink", "cyan"),
        ColSideColors=mycols[con], RowSideColors=mycols[con],
        margin=c(10, 10), main="Sample Distance Matrix")
```



Example 2:

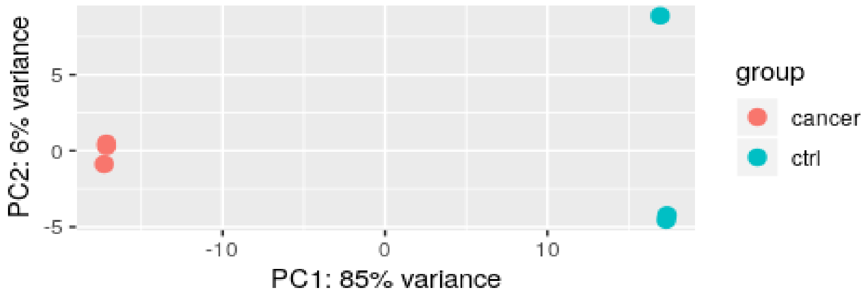
```
library("RColorBrewer")
sampleDists <- dist(t(assay(rld)))
sampleDistMatrix <- as.matrix(sampleDists)
rownames(sampleDistMatrix) <- paste(rld$group, rld$con, sep="-")
colnames(sampleDistMatrix) <- paste(rld$group, rld$con, sep="-")
colors <- colorRampPalette( rev(brewer.pal(9, "Greens")) )(255)
library("pheatmap")
pheatmap(sampleDistMatrix,clustering_distance_rows=sampleDists,
clustering_distance_cols=sampleDists,col=colors)
```



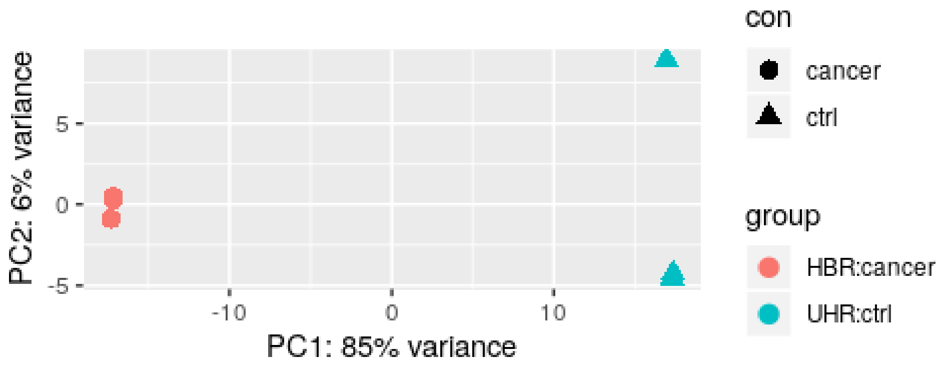
Principal component plot

Principal component analysis is a statistical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal component.

```
library(ggplot2)
plotPCA(rld, intgroup="con")
```



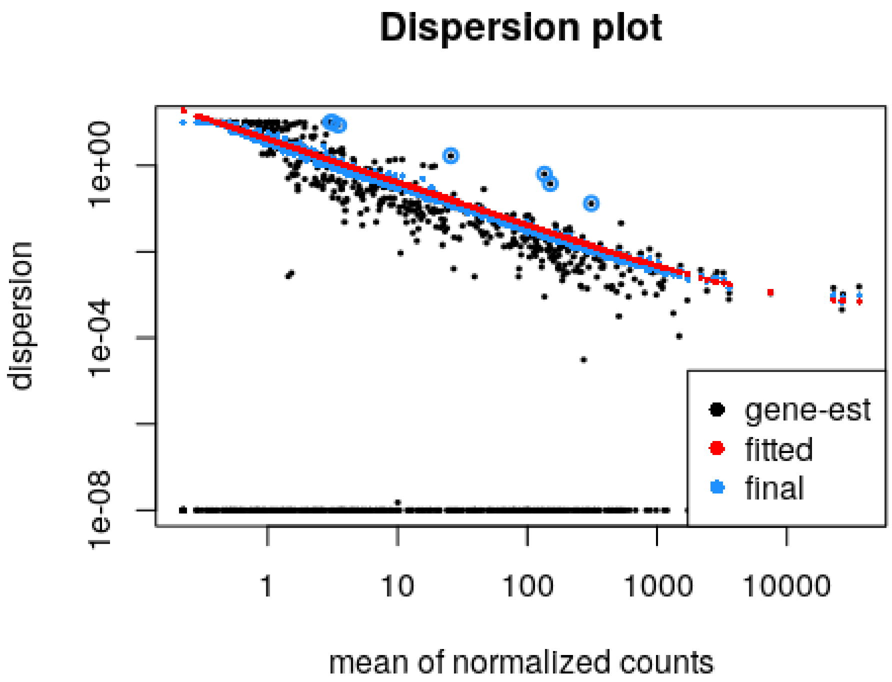
```
pcaData <- plotPCA (rld, intgroup=c("group", "con"), returnData=TRUE)
percentVar <- round(100 * attr(pcaData, "percentVar"))
ggplot(pcaData, aes(PC1, PC2, color=group, shape=con)) +
        geom_point(size=3) +
        xlab(paste0("PC1: ",percentVar[1],"% variance")) +
        ylab(paste0("PC2: ",percentVar[2],"% variance")) +
        coord_fixed()
```



Plot dispersions

DESeq uses a negative binomial distribution. Such distributions have two parameters: mean and dispersion. The dispersion is a parameter describing how much the variance deviates from the mean.

```
plotDispEsts(dds_con, main="Dispersion plot")
```

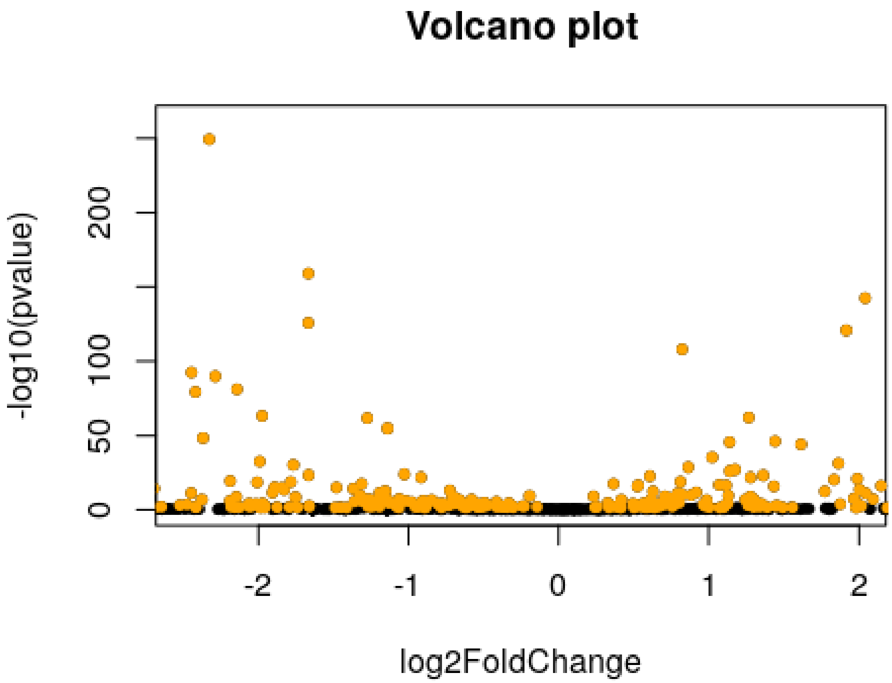


It is some p-values:

```
table(res_con$padj<0.05)

##
## FALSE  TRUE
##   403   339

res_con <- res_con[order(res_con$padj), ]
```

Vulcano Plot

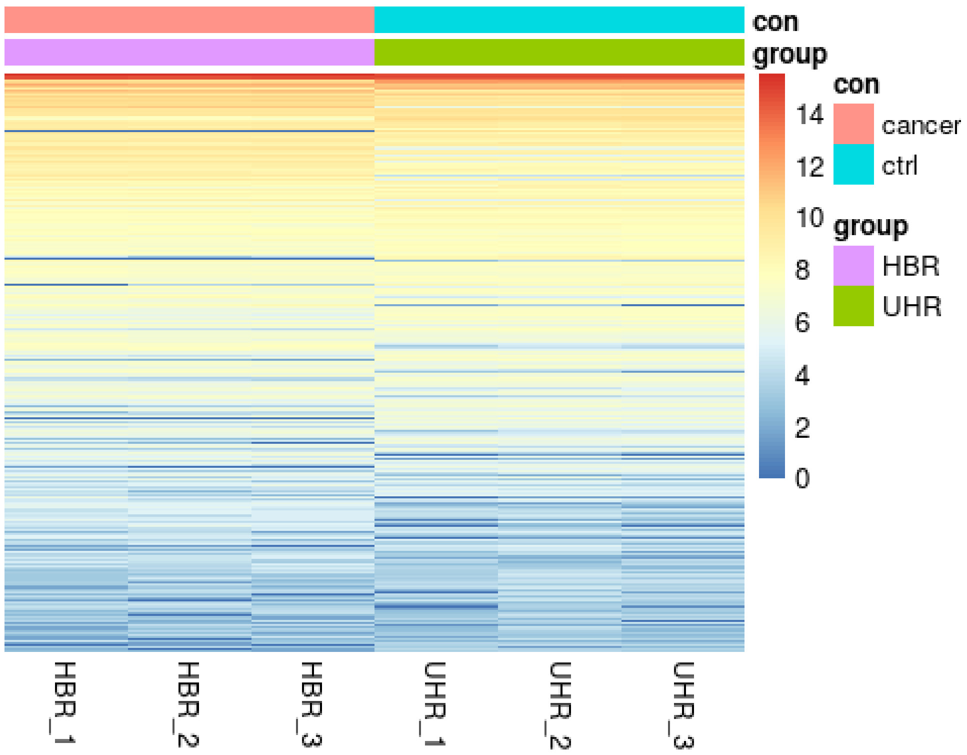
```
with(res_con, plot(log2FoldChange, -log10(pvalue), pch=20, main="Volcano plot
", xlim=c(-2.5,2)))
    with(subset(res_con, padj<.1 ), points(log2FoldChange, -log10(pvalue), pc
h=20, col="orange"))
```



Heatmap of count matrix Version 1:

Heatmap of normalized dds counts

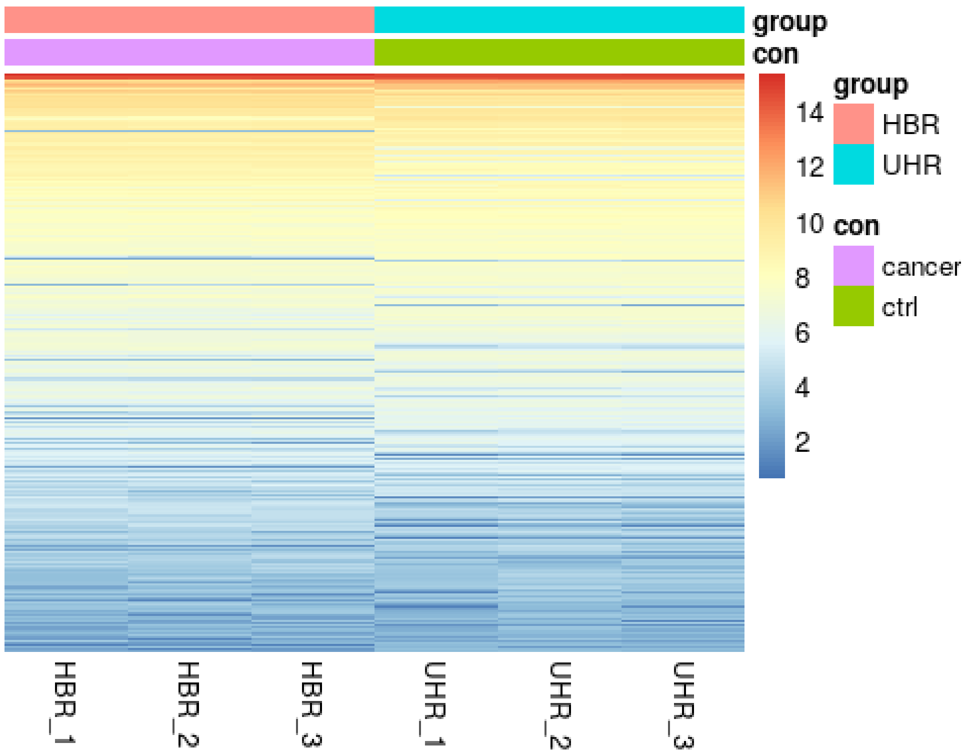
```
library("pheatmap")
select <- order(rowMeans(counts(dds,normalized=TRUE)),decreasing=TRUE) [1:500
]
nt <- normTransform(dds)
log2.norm.counts <- assay(nt)[select,]
df <- as.data.frame(colData(dds)[,c("group","con")])

pheatmap(log2.norm.counts, cluster_rows=FALSE, show_rownames=FALSE,
cluster_cols=FALSE, annotation_col=df, fontsize_row = 5)
```



Version2:

Heatmap of regularized log transformed dds counts

```
df <- as.data.frame(colData(rld)[,c("con","group")])


pheatmap(assay(rld)[select,], cluster_rows=FALSE, show_rownames=F,
cluster_cols=FALSE, annotation_col=df, fontsize_row =8)
```



Merge results with normalized count data

```
resdata <- merge(as.data.frame(res_con), as.data.frame(counts(dds_con, normal
ized=TRUE)), by="row.names", sort=FALSE)
names(resdata)[1] <- "Gene"
head(resdata)

##          Gene   baseMean log2FoldChange      lfcSE      stat        pvalue
## 1      SYNGR1   986.7215       4.662950 0.11914480  39.13683  0.000000e+00
## 2       SEPT3   926.8501       4.527019 0.12032694  37.62266  0.000000e+00
## 3 ERCC-00004  3589.9516      -2.501269 0.05482358 -45.62396  0.000000e+00
## 4 ERCC-00130 27062.3042      -2.175087 0.03596987 -60.46970  0.000000e+00
## 5       YWHAH  1474.2754       2.530765 0.07315383  34.59512 2.991523e-262
## 6 ERCC-00136  1727.3486      -2.326855 0.06889317 -33.77483 4.619251e-250
##             padj     HBR_1      HBR_2     HBR_3       UHR_1       UHR_2
## 1  0.000000e+00 1852.7882  1902.3180 1937.5307    71.74826    94.84367
## 2  0.000000e+00 1710.3648  1728.1621 1890.0422    67.72500    74.44718
## 3  0.000000e+00 1098.3288  1032.5691 1104.1076  5942.36562  6377.98212
## 4  0.000000e+00 9713.0242 10113.4068 9606.9230 44973.42155 42345.15087
## 5 4.439420e-260 2478.9383  2572.1483 2490.7717   431.16013   407.92978
## 6 5.712474e-248  603.0543   585.3286  534.2456  2865.90732  2884.06355
##          UHR_3
## 1     61.09999
## 2     90.35914
## 3   5984.35667
## 4 45621.89887
## 5   464.70414
## 6  2891.49244
```

write.table (resdata, file = “/home/mlsi/RNASeq/analysis/DESeq2/diffexpr-results_RNASeq.txt”, sep = " ", col.names=NA)

resdata_GSEA<- resdata[,-(2:7)] write.table (resdata_GSEA, file = “/home/mlsi/RNASeq/analysis/DESeq2/diffexpr-results_RNASeq_GSEA.txt”, sep = ", col.names=NA)

resdata_GSEA<- read.table (“/home/mlsi/RNASeq/analysis/DESeq2/diffexpr-results_RNASeq_GSEA.txt”, header= TRUE, row.names=2) resdata_GSEA<- resdata_GSEA_2 [,-1] write.table (resdata_GSEA, file = “/home/mlsi/RNASeq/analysis/DESeq2/diffexpr-results_RNASeq_GSEA.txt”, sep = ", col.names=NA)

```

**Next step involved is data visualization using GSEA.**
```