

RNAseq practical

Contents

Introduction	1
Experimental Data	1
Exploratory analysis and visualization	2
Loading and Exploring the data	2
The <i>DESeqDataSet</i> object, sample information and the design formula	9
Clustering and PCA	10
Differential expression analysis	13
Running the differential expression pipeline	13
Multiple testing	16
visualizing results	17
Reference	19
PLEASE WRITE DOWN THE ANSWERS TO THE QUESTIONS IN A SEPARATED DOCUMENT!	

Introduction

This practical is based on the BioConductors' *RNA-seq workflow: gene-level exploratory analysis and differential expression*; a comprehensive workflow that describes how to go from FASTQ-files to perform a differential expression analysis and annotating results. Here, we will only explore a few steps and focus on the differential expression analysis. The full workflow is described here. The workflow also appeared as a F1000 paper (Love et al. 2015) and a slightly shorter version is available as the DESeq2 vignette.

Experimental Data

The data used in this workflow is stored in the **airway** package that summarizes an RNA-seq experiment wherein airway smooth muscle cells were treated with dexamethasone, a synthetic glucocorticoid steroid with anti-inflammatory effects (Himes et al. 2014). Glucocorticoids are used, for example, by people with asthma to reduce inflammation of the airways. In the experiment, four primary human airway smooth muscle cell lines were treated with 1 micromolar dexamethasone for 18 hours. For each of the four cell lines, we have a treated and an untreated sample. For more description of the experiment see the PubMed entry 24926665 and for raw data see the GEO entry GSE52778.

Exploratory analysis and visualization

Loading and Exploring the data

The airway-package is available from BioConductor as a data-package and contains both the gene expression counts as well as metadata on the experiment and samples. This prepared dataset is what we will use in the practical.

We won't go into the details of how to construct such a dataset or object but it is good to know that many BioConductor package use specialized objects to ease various analyses, for example, later we will see an *DESeqDataSet* which we will use specifically for doing differential expression analysis using the DESeq2-package.

Use the following code to figure out how many samples and genes are in the dataset, i.e. what is the dimension of the object?

```
library(airway)           #loading the airway library
data("airway")           #loading the airway data
se <- airway              #for ease of typing shorter name
se
```

```
## class: RangedSummarizedExperiment
## dim: 64102 8
## metadata(1): ''
## assays(1): counts
## rownames(64102): ENSG000000000003 ENSG000000000005 ... LRG_98 LRG_99
## rowData names(0):
## colnames(8): SRR1039508 SRR1039509 ... SRR1039520 SRR1039521
## colData names(9): SampleName cell ... Sample BioSample
```

```
colData(se)
```

```
## DataFrame with 8 rows and 9 columns
##      SampleName      cell      dex      albut      Run avgLength
##      <factor> <factor> <factor> <factor> <factor> <integer>
## SRR1039508 GSM1275862 N61311 untrt untrt SRR1039508      126
## SRR1039509 GSM1275863 N61311 trt untrt SRR1039509      126
## SRR1039512 GSM1275866 N052611 untrt untrt SRR1039512      126
## SRR1039513 GSM1275867 N052611 trt untrt SRR1039513       87
## SRR1039516 GSM1275870 N080611 untrt untrt SRR1039516      120
## SRR1039517 GSM1275871 N080611 trt untrt SRR1039517      126
## SRR1039520 GSM1275874 N061011 untrt untrt SRR1039520      101
## SRR1039521 GSM1275875 N061011 trt untrt SRR1039521       98
##      Experiment      Sample      BioSample
##      <factor> <factor> <factor>
## SRR1039508 SRX384345 SRS508568 SAMN02422669
## SRR1039509 SRX384346 SRS508567 SAMN02422675
## SRR1039512 SRX384349 SRS508571 SAMN02422678
## SRR1039513 SRX384350 SRS508572 SAMN02422670
## SRR1039516 SRX384353 SRS508575 SAMN02422682
## SRR1039517 SRX384354 SRS508576 SAMN02422673
## SRR1039520 SRX384357 SRS508579 SAMN02422683
## SRR1039521 SRX384358 SRS508580 SAMN02422677
```

Use the following code to see which are the three most abundant gene biotypes.

```
library(EnsDb.Hsapiens.v75)
listGenebiotypes(EnsDb.Hsapiens.v75)
```

```
## [1] "protein_coding"      "pseudogene"
## [3] "processed_transcript" "antisense"
## [5] "lincRNA"             "polymorphic_pseudogene"
## [7] "IG_V_pseudogene"     "IG_V_gene"
## [9] "sense_overlapping"   "sense_intronic"
## [11] "TR_V_gene"           "misc_RNA"
## [13] "snRNA"               "miRNA"
## [15] "snoRNA"              "rRNA"
## [17] "Mt_tRNA"             "Mt_rRNA"
## [19] "IG_C_gene"           "IG_J_gene"
## [21] "TR_J_gene"           "TR_C_gene"
## [23] "TR_V_pseudogene"     "TR_J_pseudogene"
## [25] "IG_D_gene"           "IG_C_pseudogene"
## [27] "TR_D_gene"           "IG_J_pseudogene"
## [29] "3prime_overlapping_ncrna" "processed_pseudogene"
## [31] "LRG_gene"
```

```
gene_length <- lengthOf(EnsDb.Hsapiens.v75) #extract gene lengths
annotation <- genes(EnsDb.Hsapiens.v75)     #extract gene annotation
annotation$gene_length <- gene_length         #add gene lengths
mid <- match(rownames(se), names(annotation)) #matching to se object
annotation[mid, ]                           #verify match
```

```
## GRanges object with 64102 ranges and 7 metadata columns:
##           seqnames           ranges strand |           gene_id
##           <Rle>             <IRanges> <Rle> |           <character>
## ENSG000000000003           X [ 99883667, 99894988] - | ENSG000000000003
## ENSG000000000005           X [ 99839799, 99854882] + | ENSG000000000005
## ENSG000000000419          20 [ 49551404, 49575092] - | ENSG000000000419
## ENSG000000000457           1 [169818772, 169863408] - | ENSG000000000457
## ENSG000000000460           1 [169631245, 169823221] + | ENSG000000000460
##           ...           ...           ...           ...
##           LRG_94          10 [72357104, 72362531] - |           LRG_94
##           LRG_96          15 [55495792, 55582001] - |           LRG_96
##           LRG_97          22 [37621310, 37640305] - |           LRG_97
##           LRG_98          11 [36589563, 36601312] + |           LRG_98
##           LRG_99          11 [36613493, 36619812] - |           LRG_99
##           gene_name   entrezid   gene_biotype seq_coord_system
##           <character> <character> <character> <character>
## ENSG000000000003   TSPAN6       7105 protein_coding chromosome
## ENSG000000000005   TNMD        64102 protein_coding chromosome
## ENSG000000000419   DPM1         8813 protein_coding chromosome
## ENSG000000000457   SCYL3        57147 protein_coding chromosome
## ENSG000000000460   C1orf112      55732 protein_coding chromosome
##           ...           ...           ...           ...
##           LRG_94   LRG_94        5551     LRG_gene chromosome
##           LRG_96   LRG_96        5873     LRG_gene chromosome
##           LRG_97   LRG_97        5880     LRG_gene chromosome
```

```
##          LRG_98      LRG_98      5896      LRG_gene      chromosome
##          LRG_99      LRG_99      5897      LRG_gene      chromosome
##              symbol gene_length
##          <character> <integer>
## ENSG000000000003      TSPAN6      106
## ENSG000000000005      TNMD      415
## ENSG000000000419      DPM1      412
## ENSG000000000457      SCYL3      6928
## ENSG000000000460      C1orf112      9865
##          ...      ...      ...
##          LRG_94      LRG_94      4554
##          LRG_96      LRG_96      1640
##          LRG_97      LRG_97      981
##          LRG_98      LRG_98      1682
##          LRG_99      LRG_99      6813
## -----
## seqinfo: 273 sequences from GRCh37 genome
```

```
head(rownames(se))
```

```
## [1] "ENSG000000000003" "ENSG000000000005" "ENSG000000000419" "ENSG000000000457"
## [5] "ENSG000000000460" "ENSG000000000938"
```

```
tail(rownames(se))
```

```
## [1] "LRG_93" "LRG_94" "LRG_96" "LRG_97" "LRG_98" "LRG_99"
```

```
rowRanges(se) <- annotation[mid,]      #add to se object
rowRanges(se)                          #inspect annotation
```

```
## GRanges object with 64102 ranges and 7 metadata columns:
```

```
##          seqnames      ranges strand |          gene_id
##          <Rle>          <IRanges> <Rle> |          <character>
## ENSG000000000003      X [ 99883667, 99894988] - | ENSG000000000003
## ENSG000000000005      X [ 99839799, 99854882] + | ENSG000000000005
## ENSG000000000419     20 [ 49551404, 49575092] - | ENSG000000000419
## ENSG000000000457      1 [169818772, 169863408] - | ENSG000000000457
## ENSG000000000460      1 [169631245, 169823221] + | ENSG000000000460
##          ...      ...      ...      ...
##          LRG_94      10 [72357104, 72362531] - |          LRG_94
##          LRG_96      15 [55495792, 55582001] - |          LRG_96
##          LRG_97      22 [37621310, 37640305] - |          LRG_97
##          LRG_98      11 [36589563, 36601312] + |          LRG_98
##          LRG_99      11 [36613493, 36619812] - |          LRG_99
##          gene_name      entrezid      gene_biotype      seq_coord_system
##          <character> <character>      <character>      <character>
## ENSG000000000003      TSPAN6      7105      protein_coding      chromosome
## ENSG000000000005      TNMD      64102      protein_coding      chromosome
## ENSG000000000419      DPM1      8813      protein_coding      chromosome
## ENSG000000000457      SCYL3      57147      protein_coding      chromosome
## ENSG000000000460      C1orf112      55732      protein_coding      chromosome
##          ...      ...      ...      ...
```

```
##          LRG_94      LRG_94      5551      LRG_gene      chromosome
##          LRG_96      LRG_96      5873      LRG_gene      chromosome
##          LRG_97      LRG_97      5880      LRG_gene      chromosome
##          LRG_98      LRG_98      5896      LRG_gene      chromosome
##          LRG_99      LRG_99      5897      LRG_gene      chromosome
##              symbol gene_length
##              <character> <integer>
## ENSG000000000003      TSPAN6      106
## ENSG000000000005      TNMD      415
## ENSG000000000419      DPM1      412
## ENSG000000000457      SCYL3      6928
## ENSG000000000460      C1orf112      9865
##          ...          ...          ...
##          LRG_94      LRG_94      4554
##          LRG_96      LRG_96      1640
##          LRG_97      LRG_97      981
##          LRG_98      LRG_98      1682
##          LRG_99      LRG_99      6813
## -----
## seqinfo: 273 sequences from GRCh37 genome
```

```
rowData(se)
```

```
## DataFrame with 64102 rows and 7 columns
##          gene_id  gene_name  entrezid  gene_biotype
##          <character> <character> <character> <character>
## 1 ENSG000000000003      TSPAN6      7105 protein_coding
## 2 ENSG000000000005      TNMD      64102 protein_coding
## 3 ENSG000000000419      DPM1      8813 protein_coding
## 4 ENSG000000000457      SCYL3      57147 protein_coding
## 5 ENSG000000000460      C1orf112      55732 protein_coding
## ...          ...          ...          ...
## 64098      LRG_94      LRG_94      5551      LRG_gene
## 64099      LRG_96      LRG_96      5873      LRG_gene
## 64100      LRG_97      LRG_97      5880      LRG_gene
## 64101      LRG_98      LRG_98      5896      LRG_gene
## 64102      LRG_99      LRG_99      5897      LRG_gene
##          seq_coord_system      symbol gene_length
##          <character> <character> <integer>
## 1      chromosome      TSPAN6      106
## 2      chromosome      TNMD      415
## 3      chromosome      DPM1      412
## 4      chromosome      SCYL3      6928
## 5      chromosome      C1orf112      9865
## ...          ...          ...          ...
## 64098      chromosome      LRG_94      4554
## 64099      chromosome      LRG_96      1640
## 64100      chromosome      LRG_97      981
## 64101      chromosome      LRG_98      1682
## 64102      chromosome      LRG_99      6813
```

```
table(rowData(se)$gene_biotype)
```

```
##
```

```
## 3prime_overlapping_ncrna      antisense      IG_C_gene
##          24          5485          23
##      IG_C_pseudogene      IG_D_gene      IG_J_gene
##          11          64          24
##      IG_J_pseudogene      IG_V_gene      IG_V_pseudogene
##          6          178          255
##          lincRNA      LRG_gene      miRNA
##      7340          425          3361
##          misc_RNA      Mt_rRNA      Mt_tRNA
##      2174          2          22
## polymorphic_pseudogene      processed_pseudogene      processed_transcript
##          53          1          819
##      protein_coding      pseudogene      rRNA
##      22810          15583          566
##      sense_intronic      sense_overlapping      snoRNA
##          767          208          1549
##          snRNA      TR_C_gene      TR_D_gene
##      2067          6          3
##      TR_J_gene      TR_J_pseudogene      TR_V_gene
##          82          4          150
##      TR_V_pseudogene
##          40
```

What is the library-size or sequencing depth of each sample/run?

```
counts <- assay(se)
colnames(counts) <- paste( se$dex, se$cell, sep = " - " )
colSums(counts)
```

```
## untrt - N61311      trt - N61311 untrt - N052611      trt - N052611
##      20637971      18809481      25348649      15163415
## untrt - N080611      trt - N080611 untrt - N061011      trt - N061011
##      24448408      30818215      19126151      21164133
```

```
round(colSums(counts)/1e6 , 1) #this is often represented as counts
```

```
## untrt - N61311      trt - N61311 untrt - N052611      trt - N052611
##      20.6          18.8          25.3          15.2
## untrt - N080611      trt - N080611 untrt - N061011      trt - N061011
##      24.4          30.8          19.1          21.2
```

#per million

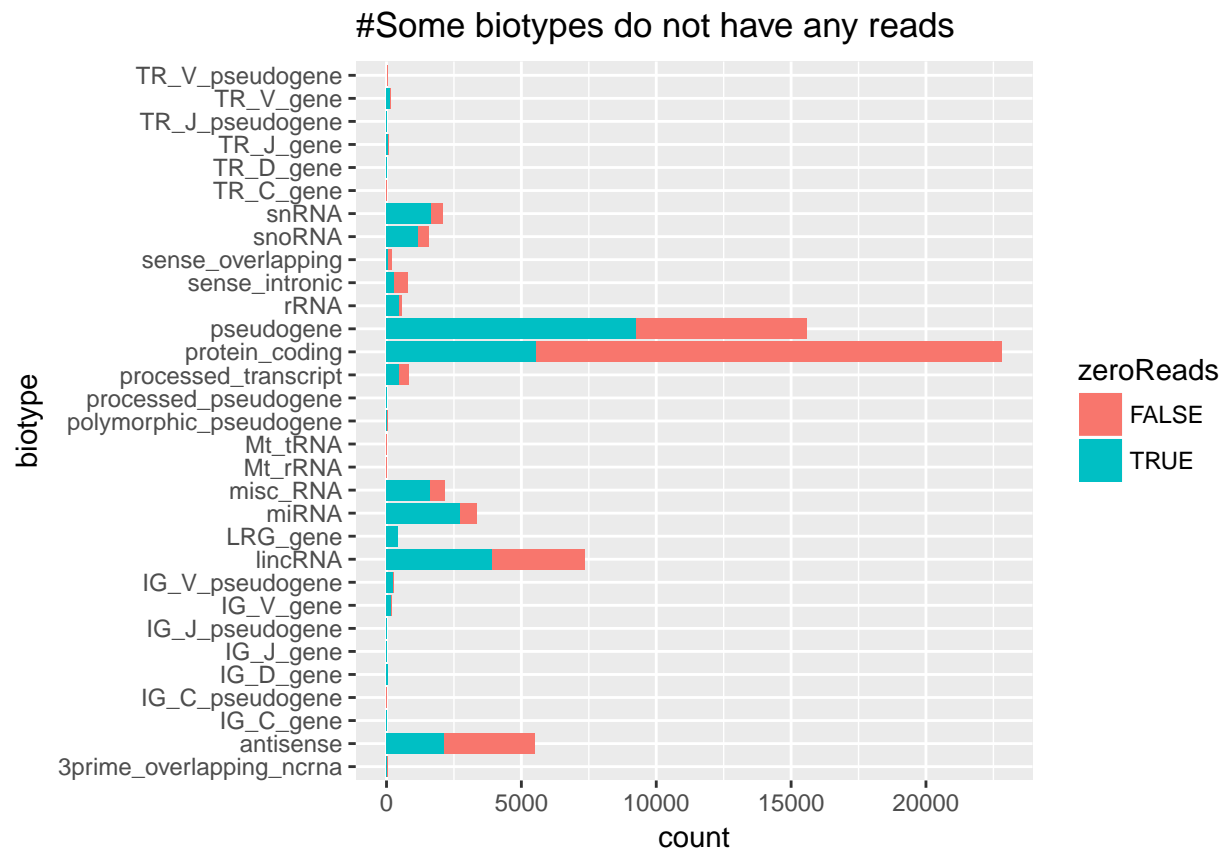
How many genes are there without any reads?

To which biotypes belong these genes; give a few examples?

```
zeroReads <- rowSums(counts) == 0 #identify genes with zero number of reads across all samples
table(zeroReads) #how many genes are there
```

```
## zeroReads
## FALSE TRUE
## 33469 30633
```

```
data <- data.frame(zeroReads = zeroReads,
                   biotype = rowData(se)$gene_biotype)
gp <- ggplot(data, aes(biotype, fill=zeroReads))
gp + geom_bar() + coord_flip() + ggtitle("#Some biotypes do not have any reads")
```



For example, microRNA are overrepresented among the genes with zero reads.

Could you give an explanation for this?

Actually we can formally test this using the Fisher exact test.

```
miRNAs <- rowData(se)$gene_biotype == "miRNA"
table(miRNAs, zeroReads)
```

```
##          zeroReads
## miRNAs  FALSE  TRUE
##   FALSE 32861 27880
##   TRUE   608   2753
```

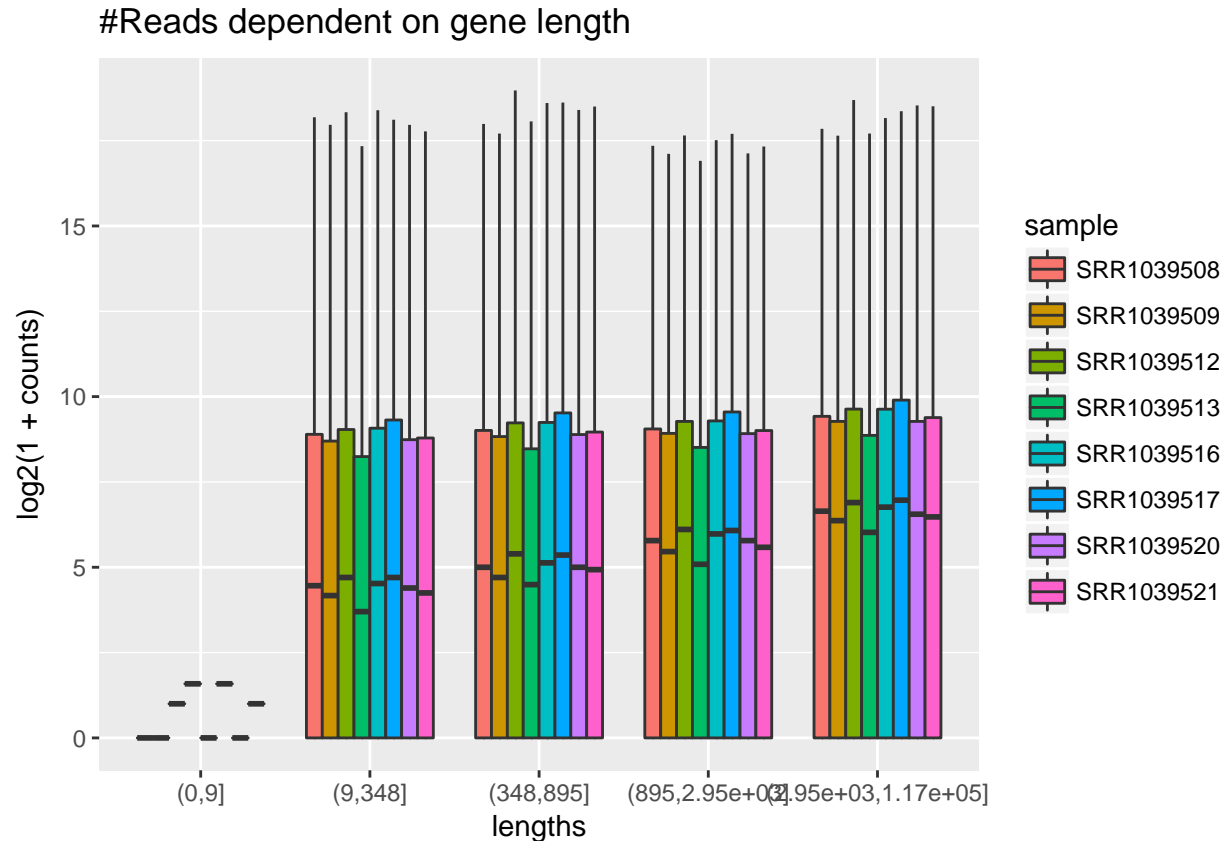
```
fisher.test(table(miRNAs, zeroReads))
```

```
##
## Fisher's Exact Test for Count Data
##
## data:  table(miRNAs, zeroReads)
## p-value < 2.2e-16
## alternative hypothesis: true odds ratio is not equal to 1
## 95 percent confidence interval:
##  4.879031 5.845114
## sample estimates:
## odds ratio
##  5.337562
```

Tests for enrichment of a certain group of genes are performed frequently in downstream analysis of gene expression data.

The number of reads for a given gene depends on the expression level of the gene but also on the length. This is because mRNA (or actually the cDNA) is fragmented into reads and long genes/transcripts produce more reads.

```
pc <- se[rowData(se)$gene_biotype == "protein_coding",]
qs <- c(0, quantile(rowData(pc)$gene_length, 0:4/4))
lengths <- cut(rowData(pc)$gene_length, qs)
data <- data.frame(lengths = rep(lengths, ncol(pc)),
                   counts = as.vector(assay(pc)),
                   sample = rep(colnames(pc), each=nrow(pc)))
gp <- ggplot(data, aes(lengths, log2(1 + counts), fill=sample))
gp + geom_boxplot() + ggtitle("#Reads dependent on gene length")
```

Actually, for differential expression analysis this is not a concern since the interest is on comparisons among samples, e.g. dexamethasone treated versus untreated and not among genes. However, tests for enrichment of a certain group of genes should take this in account (Young et al. 2010).

The *DESeqDataSet* object, sample information and the design formula

For the differential expression analysis we will use the DESeq2-package (Love, Huber, and Anders 2014).

Here we will construct a *DESeqDataSet* from the airway data and add the design formula containing the covariates on which we will perform the differential expression analysis.

```
library(DESeq2)
## it is preferred in R that the first level of a factor be the
## reference level (e.g. control, or untreated samples), so we need to
## relevel the dex factor
se$dex <- relevel(se$dex, "untrt")
dds <- DESeqDataSet(se, design = ~ cell + dex) #add formula
dds

## class: DESeqDataSet
## dim: 64102 8
## metadata(2): ' version
## assays(1): counts
## rownames(64102): ENSG00000000003 ENSG00000000005 ... LRG_98 LRG_99
## rowData names(7): gene_id gene_name ... symbol gene_length
## colnames(8): SRR1039508 SRR1039509 ... SRR1039520 SRR1039521
```

```
## colData names(9): SampleName cell ... Sample BioSample
```

Our *DESeqDataSet* contains many rows with only zeros, and additionally many rows with only a few fragments total. In order to reduce the size of the object, and to increase the speed of our functions, we can remove the rows that have no or nearly no information about the amount of gene expression. Here we apply the most minimal filtering rule: removing rows of the *DESeqDataSet* that have no counts, or only a single count across all samples. Still some ambiguous biotypes are present using this mild filter rule.

```
nrow(dds)
```

```
## [1] 64102
```

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

```
## [1] 29391
```

Clustering and PCA

A useful first step in an RNA-seq analysis is often to assess overall similarity between samples: Which samples are similar to each other, which are different? Does this fit to the expectation from the experiment's design?

We use the R function `dist` to calculate the Euclidean distance between samples. To ensure we have a roughly equal contribution from all genes, we use it on the rlog-transformed data. We need to transpose the matrix of values using `t`, because the `dist` function expects the different samples to be rows of its argument, and different dimensions (here, genes) to be columns.

```
library(vsn)  
rld <- rlog(dds, blind = FALSE)  
sampleDists <- dist(t(assay(rld)))  
sampleDists
```

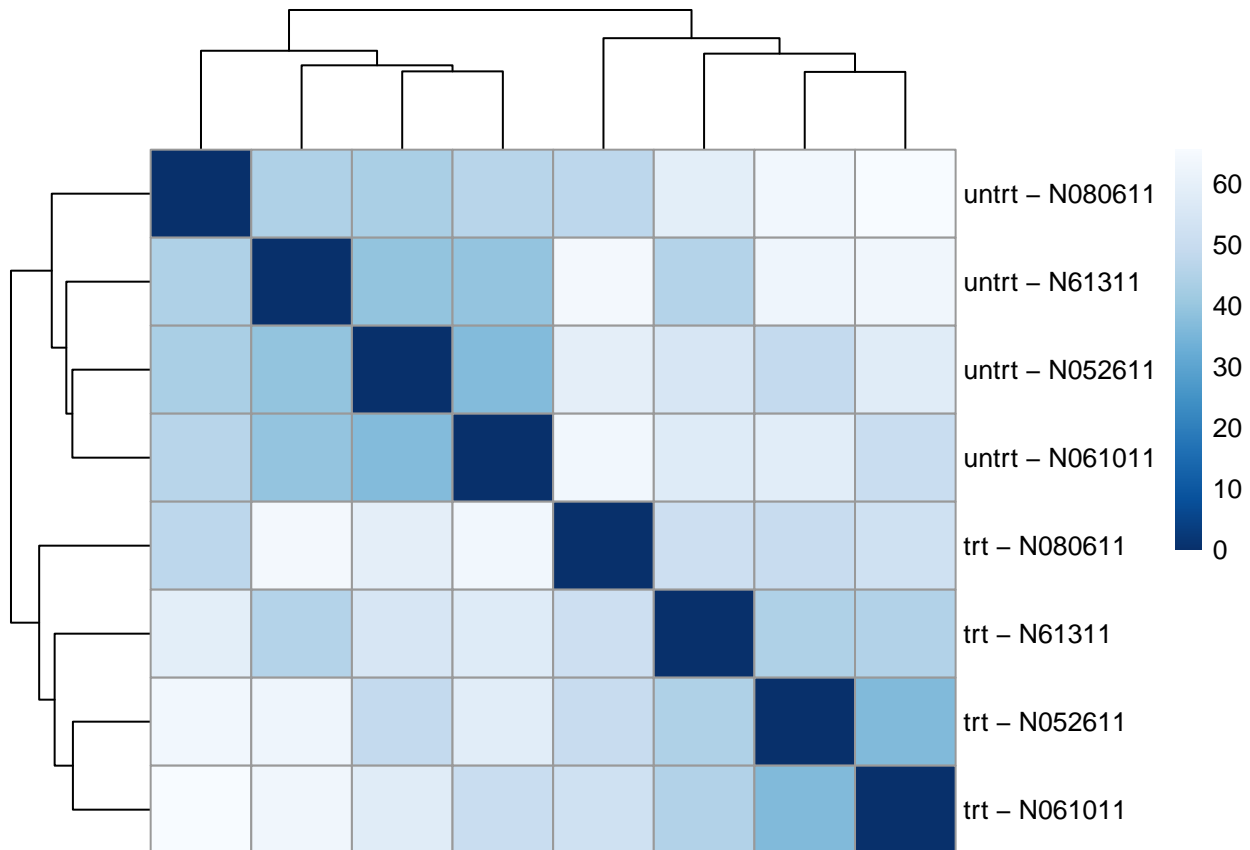
```
##           SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516  
## SRR1039509    45.69859  
## SRR1039512    39.25239    54.90828  
## SRR1039513    62.63201    44.52740    48.72579  
## SRR1039516    44.50557    59.06364    43.57856    63.74275  
## SRR1039517    64.49410    51.44882    59.22962    49.87992    47.48200  
## SRR1039520    39.57693    57.46259    36.74434    58.49014    46.40786  
## SRR1039521    63.36124    45.05732    57.87616    36.49484    65.54600  
##           SRR1039517 SRR1039520  
## SRR1039509  
## SRR1039512  
## SRR1039513  
## SRR1039516  
## SRR1039517  
## SRR1039520    63.59942  
## SRR1039521    52.31695    50.13430
```

We visualize the distances in a heatmap in a figure below, using the function `pheatmap` from the `pheatmap` package.

```

library(pheatmap)
library(RColorBrewer)
sampleDistMatrix <- as.matrix( sampleDists )
rownames(sampleDistMatrix) <- paste( rld$dex, rld$cell, sep = " - " )
colnames(sampleDistMatrix) <- NULL
colors <- colorRampPalette( rev(brewer.pal(9, "Blues")) )(255)
pheatmap(sampleDistMatrix,
          clustering_distance_rows = sampleDists,
          clustering_distance_cols = sampleDists,
          col = colors)

```



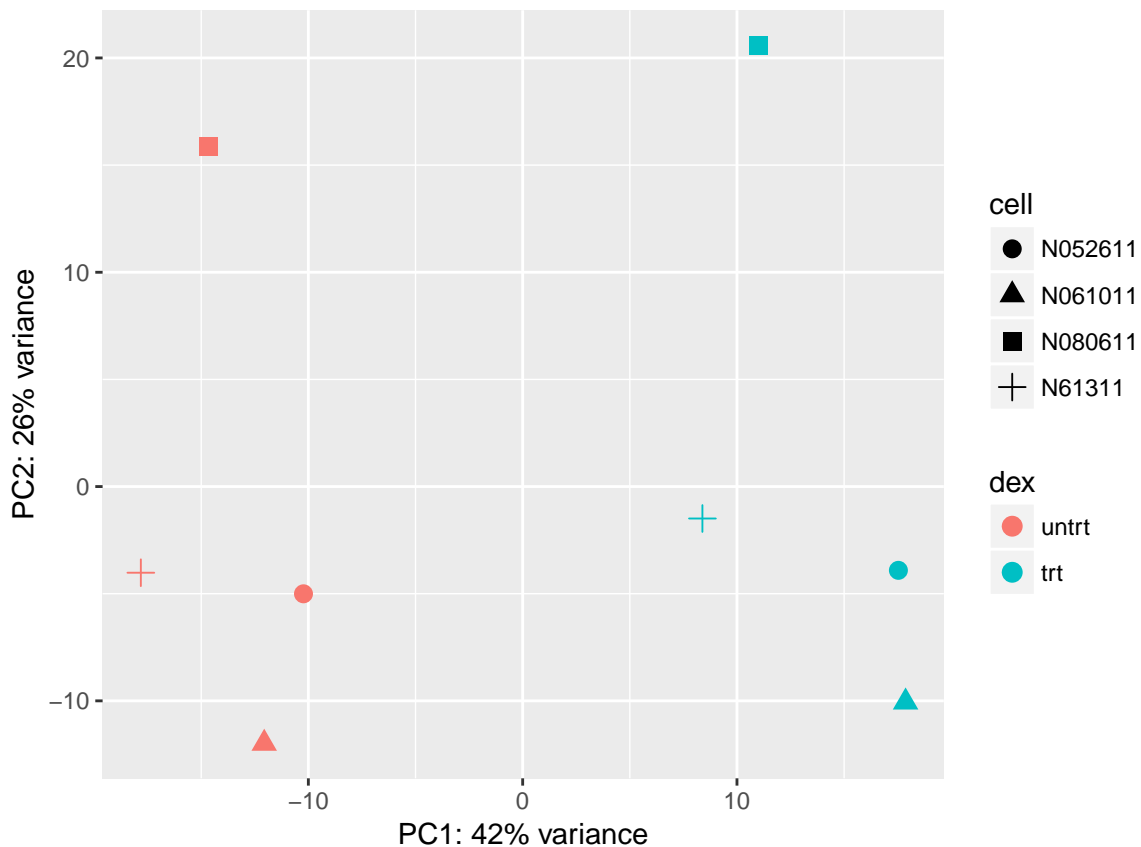
Which samples are more similar to each other?

Another way to visualize sample-to-sample distances is a principal components analysis (PCA). In this ordination method, the data points (here, the samples) are projected onto the 2D plane such that they spread out in the two directions that explain most of the differences (figure below). The x-axis is the direction that separates the data points the most. The values of the samples in this direction are written PC1. The y-axis is a direction (it must be orthogonal to the first direction) that separates the data the second most. The values of the samples in this direction are written PC2. The percent of the total variance that is contained in the direction is printed in the axis label. Note that these percentages do not add to 100%, because there are more dimensions that contain the remaining variance (although each of these remaining dimensions will explain less than the two that we see).

```
pcaData <- plotPCA(rld, intgroup = c( "dex", "cell"), returnData = TRUE)
pcaData
```

```
##           PC1          PC2      group  dex   cell      name
## SRR1039508 -17.81773  -4.020836  untrt : N61311 untrt  N61311 SRR1039508
## SRR1039509  8.38790  -1.490805    trt : N61311   trt  N61311 SRR1039509
## SRR1039512 -10.22735  -5.004069  untrt : N052611 untrt  N052611 SRR1039512
## SRR1039513  17.53277  -3.909890    trt : N052611   trt  N052611 SRR1039513
## SRR1039516 -14.67169  15.873239  untrt : N080611 untrt  N080611 SRR1039516
## SRR1039517  10.98782  20.598625    trt : N080611   trt  N080611 SRR1039517
## SRR1039520 -12.06035 -11.985876  untrt : N061011 untrt  N061011 SRR1039520
## SRR1039521  17.86863 -10.060389    trt : N061011   trt  N061011 SRR1039521
```

```
percentVar <- round(100 * attr(pcaData, "percentVar"))
ggplot(pcaData, aes(x = PC1, y = PC2, color = dex, shape = cell)) +
  geom_point(size = 3) +
  xlab(paste0("PC1: ", percentVar[1], "% variance")) +
  ylab(paste0("PC2: ", percentVar[2], "% variance")) +
  coord_fixed()
```



Which samples are more similar to each other? What can you say about the impact of different cell-lines?

Differential expression analysis

Running the differential expression pipeline

As we have already specified an experimental design when we created the `DESeqDataSet`, we can run the differential expression pipeline on the raw counts with a single call to the function `DESeq`:

```
dds <- DESeq(dds)
```

```
## estimating size factors
```

```
## estimating dispersions
```

```
## gene-wise dispersion estimates
```

```
## mean-dispersion relationship
```

```
## final dispersion estimates
```

```
## fitting model and testing
```

This function will print out a message for the various steps it performs. These are described in more detail in the manual page for `DESeq`, which can be accessed by typing `?DESeq`. Briefly these are: - the estimation of size factors (controlling for differences in the sequencing depth of the samples), - the estimation of dispersion values for each gene, -and fitting a generalized linear model.

A `DESeqDataSet` is returned that contains all the fitted parameters within it, and the following section describes how to extract out results tables of interest from this object.

```
res <- results(dds)
```

```
res
```

```
## log2 fold change (MAP): dex trt vs untrt
```

```
## Wald test p-value: dex trt vs untrt
```

```
## DataFrame with 29391 rows and 6 columns
```

```
##           baseMean log2FoldChange      lfcSE      stat
##           <numeric>      <numeric> <numeric> <numeric>
## ENSG000000000003 708.6021697   -0.37415193 0.09884432 -3.7852648
## ENSG000000000419 520.2979006    0.20206144 0.10974240  1.8412340
## ENSG000000000457 237.1630368    0.03616620 0.13834538  0.2614196
## ENSG000000000460  57.9326331   -0.08446385 0.24990676 -0.3379815
## ENSG000000000938  0.3180984   -0.08413904 0.15133427 -0.5559814
## ...           ...           ...           ...           ...
## ENSG00000273485   1.2864477    0.03398815 0.2932360  0.1159071
## ENSG00000273486 15.4525365   -0.09560732 0.3410333 -0.2803460
## ENSG00000273487  8.1632350    0.55007412 0.3725061  1.4766847
## ENSG00000273488  8.5844790    0.10515293 0.3683834  0.2854442
## ENSG00000273489  0.2758994    0.06947900 0.1512520  0.4593591
##           pvalue      padj
##           <numeric> <numeric>
## ENSG000000000003 0.000153545 0.00128686
```

```
## ENSG00000000419 0.065587276 0.19676183
## ENSG00000000457 0.793768939 0.91372953
## ENSG00000000460 0.735377161 0.88385059
## ENSG00000000938 0.578223585 NA
## ...
## ENSG00000273485 0.9077261 NA
## ENSG00000273486 0.7792120 0.9062268
## ENSG00000273487 0.1397602 0.3389275
## ENSG00000273488 0.7753038 0.9039857
## ENSG00000273489 0.6459763 NA
```

```
res[order(res$padj),]
```

```
## log2 fold change (MAP): dex trt vs untrt
## Wald test p-value: dex trt vs untrt
## DataFrame with 29391 rows and 6 columns
##      baseMean log2FoldChange lfcSE      stat
##      <numeric>      <numeric> <numeric> <numeric>
## ENSG00000152583    997.4398      4.313968 0.1721375    25.06117
## ENSG00000165995    495.0929      3.186818 0.1281563    24.86665
## ENSG00000101347  12703.3871      3.618751 0.1489433    24.29616
## ENSG00000120129    3409.0294      2.871488 0.1182491    24.28338
## ENSG00000189221    2341.7673      3.230386 0.1366745    23.63562
## ...
## ENSG00000273474    1.5868550      0.006251418 0.3008329    0.02078037
## ENSG00000273476    0.5334215      0.081543999 0.1636397    0.49831434
## ENSG00000273483    2.6895651      0.162556697 0.3311375    0.49090397
## ENSG00000273485    1.2864477      0.033988148 0.2932360    0.11590715
## ENSG00000273489    0.2758994      0.069479001 0.1512520    0.45935911
##      pvalue      padj
##      <numeric>      <numeric>
## ENSG00000152583 1.319002e-138 2.373412e-134
## ENSG00000165995 1.708334e-136 1.536988e-132
## ENSG00000101347 2.152388e-130 1.291002e-126
## ENSG00000120129 2.937637e-130 1.321496e-126
## ENSG00000189221 1.659454e-123 5.972044e-120
## ...
## ENSG00000273474    0.9834209      NA
## ENSG00000273476    0.6182625      NA
## ENSG00000273483    0.6234944      NA
## ENSG00000273485    0.9077261      NA
## ENSG00000273489    0.6459763      NA
```

Calling results without any arguments will extract the estimated log2 fold changes and p values for the last variable in the design formula. If there are more than 2 levels for this variable, results will extract the results table for a comparison of the last level over the first level. The comparison is printed at the top of the output: dex trt vs untrt.

The first column, baseMean, is a just the average of the normalized count values, divided by the size factors, taken over all samples in the DESeqDataSet. The remaining four columns refer to a specific contrast, namely the comparison of the trt level over the untrt level for the factor variable dex. We will find out below how to obtain other contrasts.

The column log2FoldChange is the effect size estimate. It tells us how much the gene's expression seems to have changed due to treatment with dexamethasone in comparison to untreated samples. This value

is reported on a logarithmic scale to base 2: for example, a log2 fold change of 1.5 means that the gene's expression is increased by a multiplicative factor of $2^{1.5} \sim 2.82$.

Of course, this estimate has an uncertainty associated with it, which is available in the column `lfcSE`, the standard error estimate for the log2 fold change estimate. We can also express the uncertainty of a particular effect size estimate as the result of a statistical test. The purpose of a test for differential expression is to test whether the data provides sufficient evidence to conclude that this value is really different from zero. DESeq2 performs for each gene a hypothesis test to see whether evidence is sufficient to decide against the null hypothesis that there is zero effect of the treatment on the gene and that the observed difference between treatment and control was merely caused by experimental variability (i.e., the type of variability that you can expect between different samples in the same treatment group). As usual in statistics, the result of this test is reported as a p value, and it is found in the column `pvalue`. Remember that a p value indicates the probability that a fold change as strong as the observed one, or even stronger, would be seen under the situation described by the null hypothesis.

We can also summarize the results with the following line of code, which reports some additional information.

```
summary(res)

##
## out of 29391 with nonzero total read count
## adjusted p-value < 0.1
## LFC > 0 (up)      : 2617, 8.9%
## LFC < 0 (down)    : 2204, 7.5%
## outliers [1]      : 0, 0%
## low counts [2]    : 11397, 39%
## (mean count < 5)
## [1] see 'cooksCutoff' argument of ?results
## [2] see 'independentFiltering' argument of ?results
```

Note that there are many genes with differential expression due to dexamethasone treatment at the FDR level of 10%. This makes sense, as the smooth muscle cells of the airway are known to react to glucocorticoid steroids. However, there are two ways to be more strict about which set of genes are considered significant:

lower the false discovery rate threshold (the threshold on `padj` in the results table)

raise the log2 fold change threshold from 0 using the `lfcThreshold` argument of `results`

If we lower the false discovery rate threshold, we should also inform the `results()` function about it, so that the function can use this threshold for the optimal independent filtering that it performs:

```
res.05 <- results(dds, alpha = 0.05)
table(res.05$padj < 0.05)
```

```
##
## FALSE  TRUE
## 12841  4014
```

If we want to raise the log2 fold change threshold, so that we test for genes that show more substantial changes due to treatment, we simply supply a value on the log2 scale. For example, by specifying `lfcThreshold = 1`, we test for genes that show significant effects of treatment on gene counts more than doubling or less than halving, because $2^1 = 2$.

```
resLFC1 <- results(dds, lfcThreshold=1)
table(resLFC1$padj < 0.1)
```

```
##
## FALSE TRUE
## 18368 196
```

What is the effect of treatment with dexamethasone on the top differentially expressed gene?

How strong is this effect in fold-change comparing treated vs untreated?

Multiple testing

In high-throughput biology, we are careful to not use the p values directly as evidence against the null, but to correct for multiple testing. What would happen if we were to simply threshold the p values at a low value, say 0.05? There are 5676 genes with a p value below 0.05 among the 29391 genes for which the test succeeded in reporting a p value:

```
sum(res$pvalue < 0.05, na.rm=TRUE)
```

```
## [1] 5648
```

```
sum(!is.na(res$pvalue))
```

```
## [1] 29391
```

Now, assume for a moment that the null hypothesis is true for all genes, i.e., no gene is affected by the treatment with dexamethasone. Then, by the definition of the p value, we expect up to 5% of the genes to have a p value below 0.05. This amounts to 1470 genes. If we just considered the list of genes with a p value below 0.05 as differentially expressed, this list should therefore be expected to contain up to $1470 / 5676 = 26\%$ false positives.

DESeq2 uses the Benjamini-Hochberg (BH) adjustment (Benjamini and Hochberg 1995) as implemented in the base R `p.adjust` function; in brief, this method calculates for each gene an adjusted p value that answers the following question: if one called significant all genes with an adjusted p value less than or equal to this gene's adjusted p value threshold, what would be the fraction of false positives (the false discovery rate, FDR) among them, in the sense of the calculation outlined above? These values, called the BH-adjusted p values, are given in the column `padj` of the `res` object.

The FDR is a useful statistic for many high-throughput experiments, as we are often interested in reporting or focusing on a set of interesting genes, and we would like to put an upper bound on the percent of false positives in this set.

Hence, if we consider a fraction of 10% false positives acceptable, we can consider all genes with an adjusted p value below $10\% = 0.1$ as significant. How many such genes are there?

```
sum(res$padj < 0.1, na.rm=TRUE)
```

```
## [1] 4821
```

We subset the results table to these genes and then sort it by the log2 fold change estimate to get the significant genes with the strongest down-regulation:


```
resSig <- subset(res, padj < 0.1)
head(resSig[ order(resSig$log2FoldChange), ])
```

```
## log2 fold change (MAP): dex trt vs untrt
## Wald test p-value: dex trt vs untrt
## DataFrame with 6 rows and 6 columns
##           baseMean log2FoldChange    lfcSE      stat      pvalue
##           <numeric>    <numeric> <numeric> <numeric>    <numeric>
## ENSG00000162692 508.17023      -3.449451 0.1767133 -19.520040 7.418019e-85
## ENSG00000105989 333.21469      -2.847367 0.1763077 -16.149989 1.135822e-58
## ENSG00000146006  46.80760      -2.828103 0.3377002  -8.374596 5.541346e-17
## ENSG00000214814 243.27698      -2.753580 0.2235524 -12.317379 7.302919e-35
## ENSG00000267339  26.23357      -2.704529 0.3519704  -7.683967 1.542363e-14
## ENSG00000013293 244.49733      -2.641033 0.1992865 -13.252442 4.367569e-40
##           padj
##           <numeric>
## ENSG00000162692 9.534273e-82
## ENSG00000105989 5.839423e-56
## ENSG00000146006 2.702195e-15
## ENSG00000214814 1.228119e-32
## ENSG00000267339 5.904954e-13
## ENSG00000013293 8.830342e-38
```

... and with the strongest up-regulation:

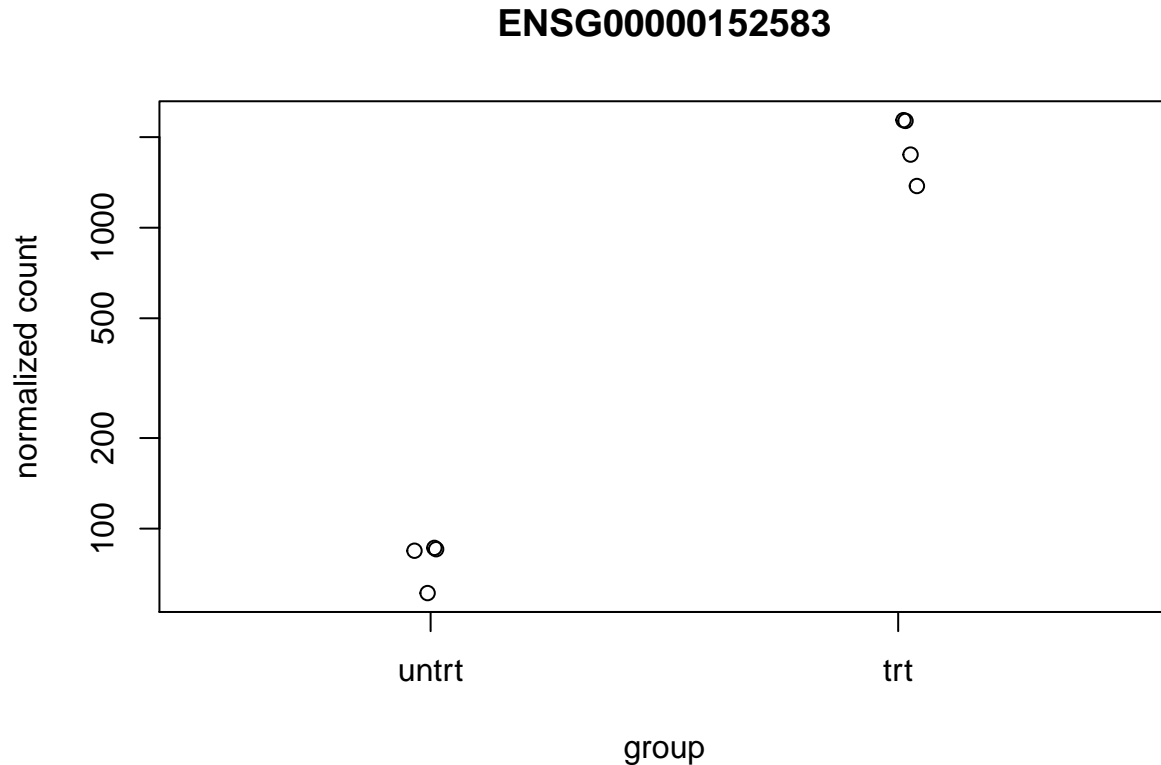
```
head(resSig[ order(resSig$log2FoldChange, decreasing = TRUE), ])
```

```
## log2 fold change (MAP): dex trt vs untrt
## Wald test p-value: dex trt vs untrt
## DataFrame with 6 rows and 6 columns
##           baseMean log2FoldChange    lfcSE      stat      pvalue
##           <numeric>    <numeric> <numeric> <numeric>    <numeric>
## ENSG00000109906 385.07103       4.847146 0.3313650  14.62781 1.866877e-48
## ENSG00000179593  67.24305       4.830826 0.3314188  14.57620 3.980821e-48
## ENSG00000152583 997.43977       4.313968 0.1721375  25.06117 1.319002e-138
## ENSG00000163884 561.10717       4.074334 0.2104702  19.35824 1.737077e-83
## ENSG00000250978  56.31819       4.054730 0.3294741  12.30667 8.339021e-35
## ENSG00000168309 159.52692       3.977125 0.2558468  15.54495 1.721597e-54
##           padj
##           <numeric>
## ENSG00000109906 5.893437e-46
## ENSG00000179593 1.193848e-45
## ENSG00000152583 2.373412e-134
## ENSG00000163884 1.953560e-80
## ENSG00000250978 1.389373e-32
## ENSG00000168309 7.744606e-52
```

visualizing results

A quick way to visualize the counts for a particular gene is to use the `plotCounts` function that takes as arguments the `DESeqDataSet`, a gene name, and the group over which to plot the counts (figure below).

```
topGene <- rownames(res)[which.min(res$padj)]
plotCounts(dds, gene = topGene, intgroup=c("dex"))
```



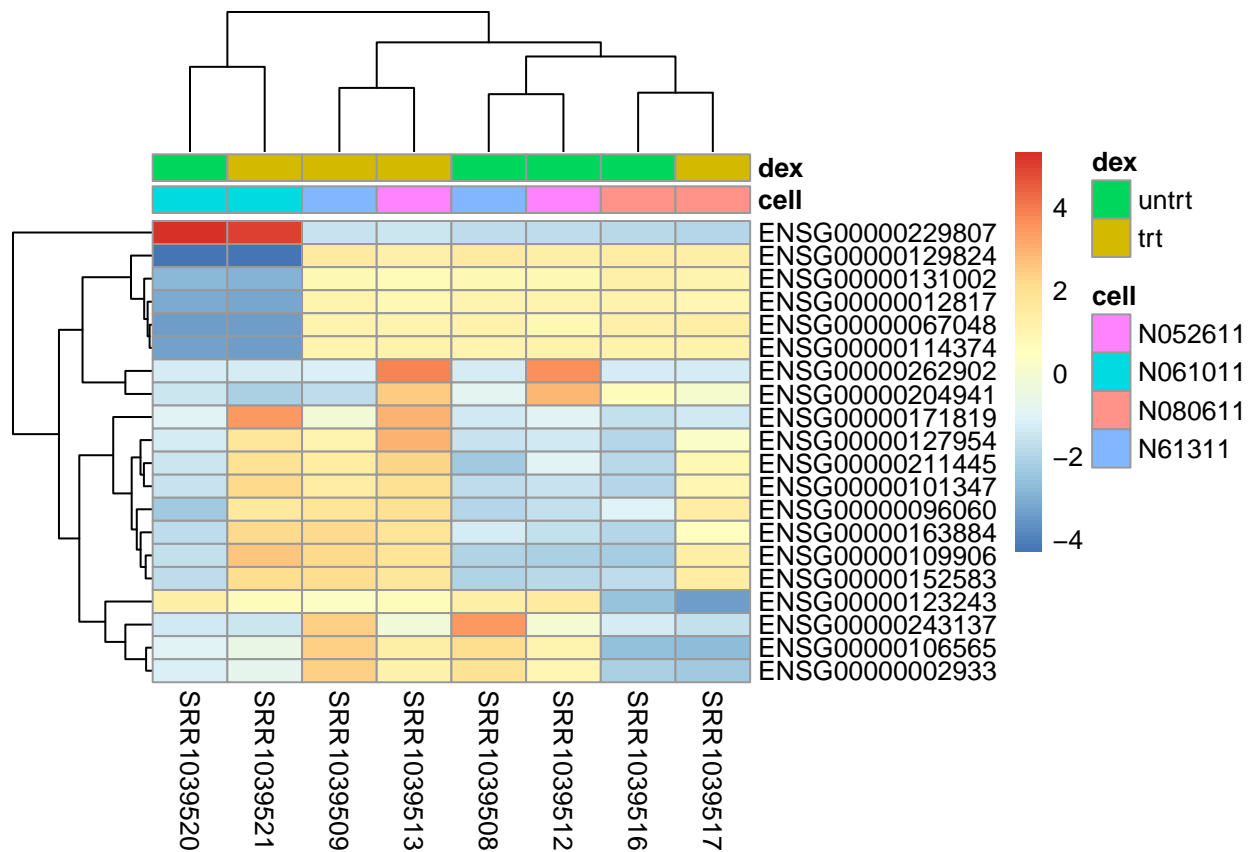
Did you find the same gene as top differentially expressed and in the same direction as the previous two questions?

What is the gene symbol of this top gene?

In the sample distance heatmap made previously, the dendrogram at the side shows us a hierarchical clustering of the samples. Such a clustering can also be performed for the genes. Since the clustering is only relevant for genes that actually carry a signal, one usually would only cluster a subset of the most highly variable genes. Here, for demonstration, let us select the 20 genes with the highest variance across samples. We will work with the rlog transformed counts:

The heatmap becomes more interesting if we do not look at absolute expression strength but rather at the amount by which each gene deviates in a specific sample from the gene's average across all samples. Hence, we center each genes' values across samples, and plot a heatmap (figure below). We provide a data.frame that instructs the pheatmap function how to label the columns.

```
library(genefilter)
topVarGenes <- head(order(rowVars(assay(rld))), decreasing = TRUE), 20)
mat <- assay(rld)[ topVarGenes, ]
mat <- mat - rowMeans(mat)
anno <- as.data.frame(colData(rld)[, c("cell","dex")])
pheatmap(mat, annotation_col = anno)
```



What are the gene symbols of these genes?

Repeat the analysis but now keeping genes with at least 1 count per million in at least for samples!

Reference

Himes, B. E., X. Jiang, P. Wagner, R. Hu, Q. Wang, B. Klanderman, R. M. Whitaker, et al. 2014. "RNA-Seq transcriptome profiling identifies CRISPLD2 as a glucocorticoid responsive gene that modulates cytokine function in airway smooth muscle cells." *PLoS ONE* 9 (6): e99625.

Love, M. I., S. Anders, V. Kim, and W. Huber. 2015. "RNA-Seq workflow: gene-level exploratory analysis and differential expression." *F1000Res* 4: 1070.

Love, M. I., W. Huber, and S. Anders. 2014. "Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2." *Genome Biol.* 15 (12): 550.

Young, M. D., M. J. Wakefield, G. K. Smyth, and A. Oshlack. 2010. "Gene ontology analysis for RNA-seq: accounting for selection bias." *Genome Biol.* 11 (2): R14.