Introduction to Bulk RNAseq data analysis

Differential Expression of RNA-seq data - Part 1

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Contents

Load the data

In the previous session we read the results from Salmon into R and created a `txi` object, which we then saved into an "rds" file. We can now load the txi from that file to start the differential expression analysis. We will also need the sample meta data sheet

First load the packages we need.

```
library(DESeq2)
library(tidyverse)
```

Now load the data from the earlier session.

```
txi <- readRDS("RObjects/txi.rds")
sampleinfo <- read_tsv("data/samplesheet_corrected.tsv", col_types="cccc")
```

It is important to be sure that the order of the samples in rows in the sample meta data table matches the order of the columns in the data matrix - `DESeq2` will **not** check this. If the order does not match you will not be running the analyses that you think you are.

```
all(colnames(txi$counts)==sampleinfo$SampleName)
```

```
## [1] TRUE
```

The model formula and design matrices

Now that we are happy that the quality of the data looks good, we can proceed to testing for differentially expressed genes. There are a number of packages to analyse RNA-Seq data. Most people use DESeq2 (Love, Huber, and Anders 2014) or edgeR (Robinson, McCarthy, and Smyth 2010; McCarthy, Chen, and Smyth 2012). There is also the option to use the limma package and transform the counts using its `voom` function .They are all equally valid approaches (Ritchie et al. 2015). There is an informative and honest blog post here by Mike Love, one of the authors of DESeq2, about deciding which to use.

We will use **DESeq2** for the rest of this practical.

Create a DESeqDataSet object with the raw data

Creating the design model formula

First we need to create a design model formula for our analysis. `DESeq2` will use this to generate the model matrix, as we have seen in the linear models lecture.

We have two variables in our experiment: "Status" and "Time Point."

We will fit two models under two assumptions: no interaction and interaction of these two factors, however, to demonstrate the how `DESeq2` is used we will start with a simple model which considers Status but ignores Time Point.

First, create a variable containing the model using standard R 'formula' syntax.

```
simple.model <- as.formula(~ Status)
```

What does this look like as a model matrix?

```
model.matrix(simple.model, data = sampleinfo)
```

```
##    (Intercept) StatusUninfected
## 1            1                0
## 2            1                0
## 3            1                0
## 4            1                0
## 5            1                1
## 6            1                0
## 7            1                1
## 8            1                1
## 9            1                1
## 10           1                1
## 11           1                0
## 12           1                1
## attr(,"assign")
## [1] 0 1
```

```
## attr(,"contrasts")
## attr(,"contrasts")$Status
## [1] "contr.treatment"
```

The intercept has been set automatically to the group in the factor that is alphabetically first: `Infected`.

It would be nice if `Uninfected` were the base line/intercept. To get R to use `Uninfected` as the intercept we need to use a `factor`. Let's set factor levels on Status to use `Uninfected` as the intercept.

```
sampleinfo <- mutate(sampleinfo, Status = fct_relevel(Status, "Uninfected"))
model.matrix(simple.model, data = sampleinfo)
```

```
##    (Intercept) StatusInfected
## 1            1              1
## 2            1              1
## 3            1              1
## 4            1              1
## 5            1              0
## 6            1              1
## 7            1              0
## 8            1              0
## 9            1              0
## 10           1              0
## 11           1              1
## 12           1              0
## attr(,"assign")
## [1] 0 1
## attr(,"contrasts")
## attr(,"contrasts")$Status
## [1] "contr.treatment"
```

Build a DESeq2DataSet

We don't actually need to pass `DESeq2` the model matrix, instead we pass it the design formula and the `sampleinfo` it will build the matrix itself.

```
# create the DESeqDataSet object
ddsObj.raw <- DESeqDataSetFromTximport(txi = txi,
                                       colData = sampleinfo,
                                       design = simple.model)
```

```
## using counts and average transcript lengths from tximport
```

When we summarised the counts to gene level, `tximport` also calculated an average transcript length for each gene for each sample. For a given gene the average transcript length may vary between samples if different samples are using alternative transcripts. `DESeq2` will incorporate this into its "normalisation."

Filter out the unexpressed genes

Just as we did in session 7, we should filter out genes that uninformative.

```
keep <- rowSums(counts(ddsObj.raw)) > 5
ddsObj.filt <- ddsObj.raw[keep,]
```

Differential expression analysis with DESeq2

The DESeq2 work flow

The main DESeq2 work flow is carried out in 3 steps:

estimateSizeFactors

First, Calculate the "median ratio" normalisation size factors for each sample and adjust for average transcript length on a per gene per sample basis.

```
ddsObj <- estimateSizeFactors(ddsObj.filt)
```

```
## using 'avgTxLength' from assays(dds), correcting for library size
```

Let's have a look at what that did DESeq2 has calculated a normalizsation factor for each gene for each sample.

```
normalizationFactors(ddsObj.filt)
```

```
## NULL
```

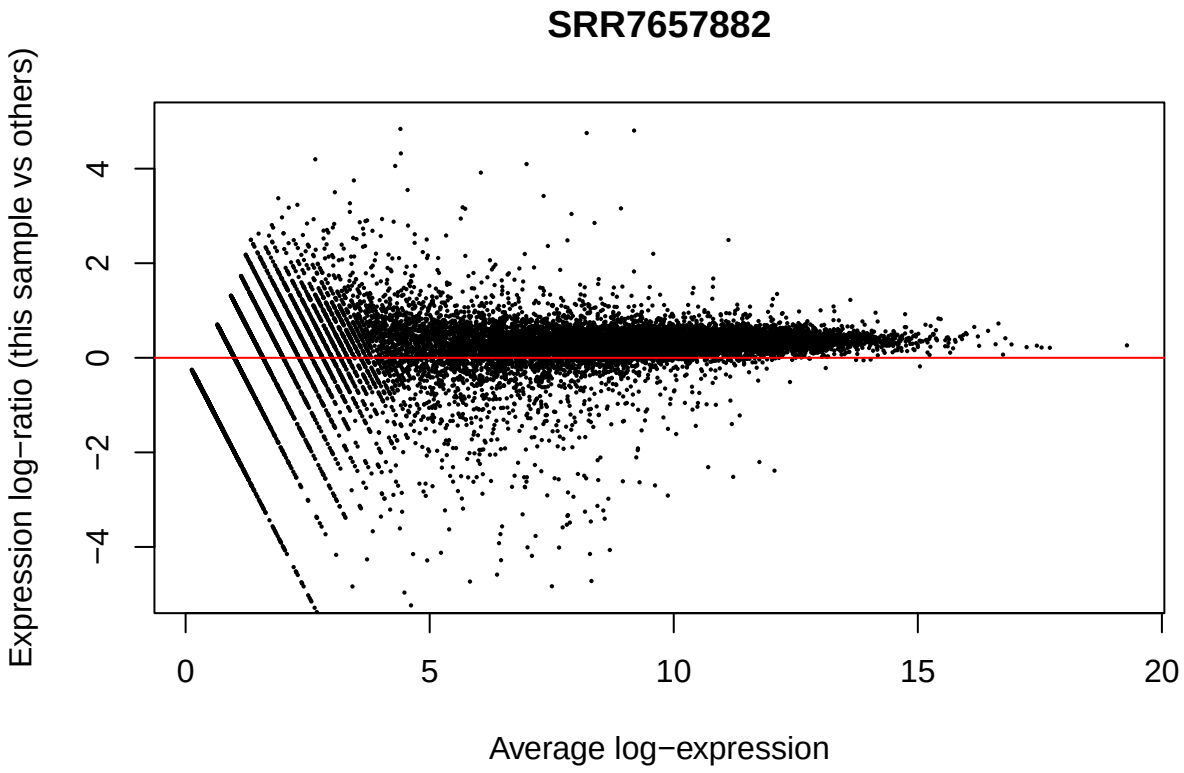
```
normalizationFactors(ddsObj)
```

```
##                    SRR7657878 SRR7657881 SRR7657880 SRR7657874 SRR7657882
## ENSMUSG00000000001  0.9650391 0.96894491 0.94927826  0.9018189 1.20992201
## ENSMUSG00000000028  1.1565743 1.00859533 0.94221210  0.8798304 1.22726399
## ENSMUSG00000000037  0.9372735 1.11991330 0.52624731  1.0355843 0.77648936
##                    SRR7657872 SRR7657877 SRR7657876 SRR7657879 SRR7657883
## ENSMUSG00000000001  0.9615574  1.1078271 1.01950314 0.95990882  0.9028895
## ENSMUSG00000000028  1.0086842  1.2535886 0.95400723 0.77315414  0.7550835
## ENSMUSG00000000037  0.8718128  2.2493164 0.77345594 0.67601042  0.5549084
##                    SRR7657873 SRR7657875
## ENSMUSG00000000001  1.0472683 1.04743598
## ENSMUSG00000000028  1.0609078 1.12777706
## ENSMUSG00000000037  1.8562184 2.13169578
##  [ reached getOption("max.print") -- omitted 20088 rows ]
```

We can use plotMA from limma to look at the of these normalisation factors on data in an MA plot. Let's look at **SRR7657882**, the fifth column, which has the largest normalisation factors.

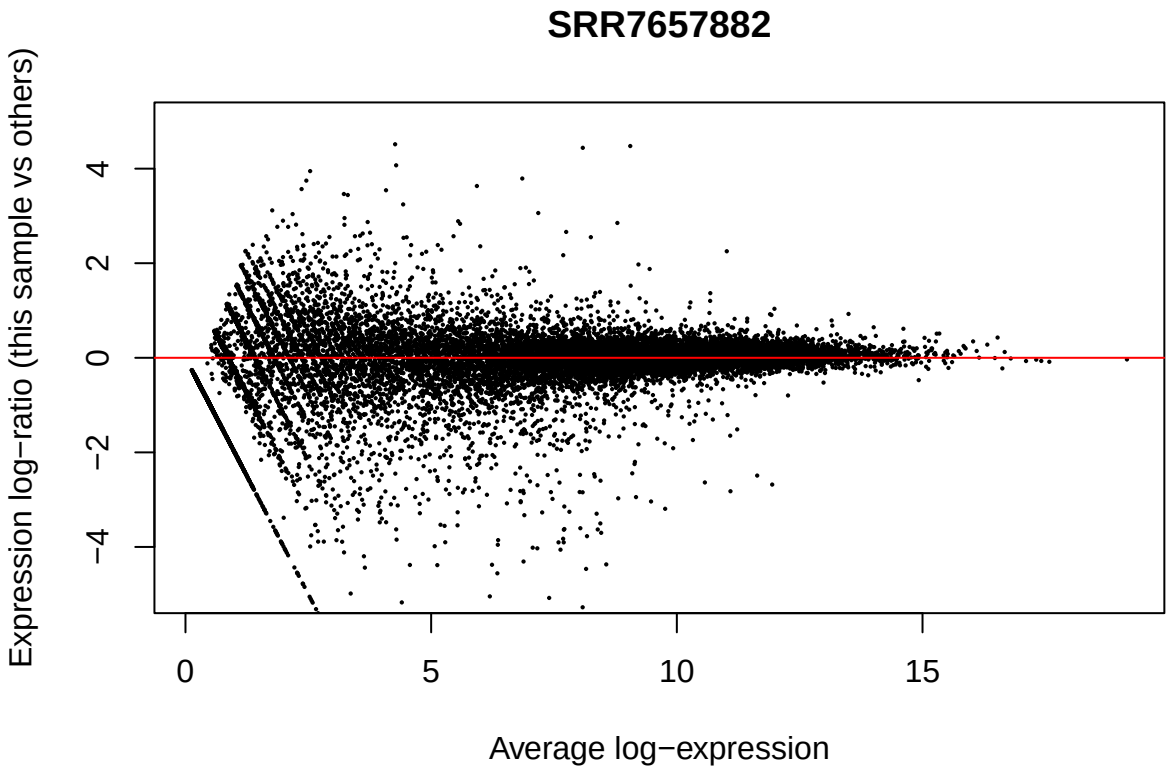
```
logcounts <- log2(counts(ddsObj, normalized=FALSE)  + 1)

limma::plotMA(logcounts, array = 5, ylim =c(-5, 5))
abline(h=0, col="red")
```




```
logNormalizedCounts <- log2(counts(ddsObj, normalized=TRUE)  + 1)

limma::plotMA(logNormalizedCounts, array=5, ylim =c(-5, 5))
abline(h=0, col="red")
```



DESeq2 doesn't actually normalise the counts, it uses raw counts and includes the normalisation factors in the modeling as an "offset." Please see the DESeq2 documentation if you'd like more details on exactly how they are incorporated into the algorithm. For practical purposes we can think of it as a normalisation.

estimateDispersions

Next we need to estimate the dispersion parameters for each gene.

```
ddsObj <- estimateDispersions(ddsObj)
```

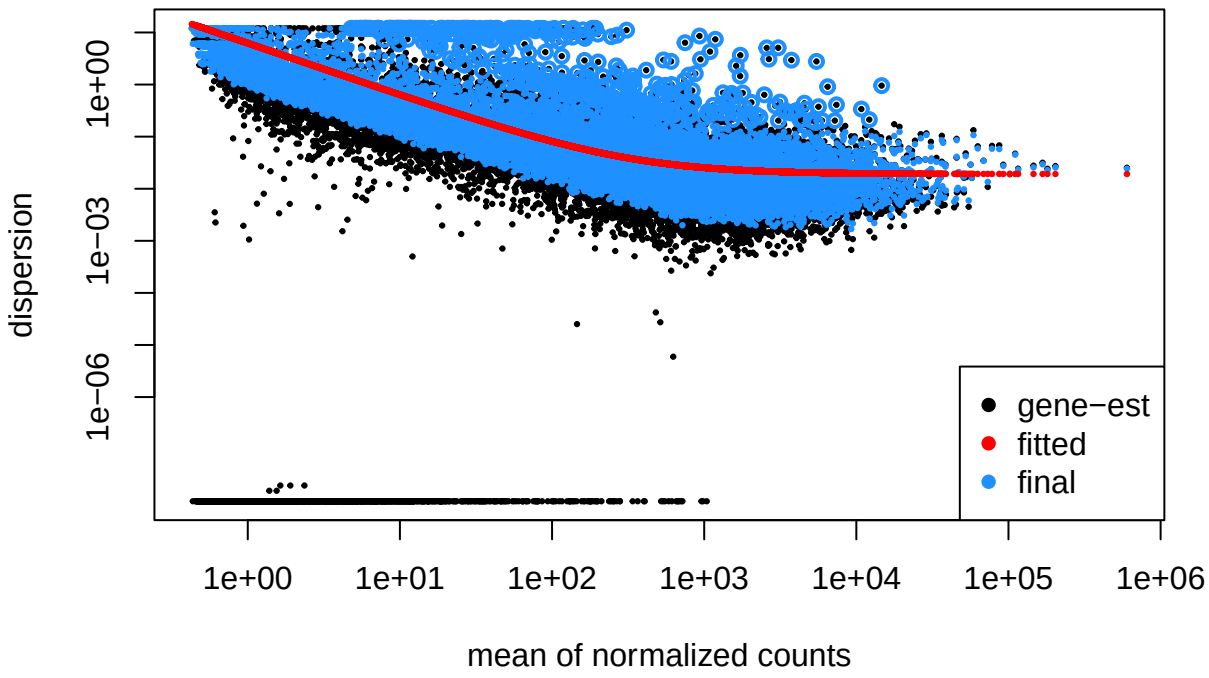
```
## gene-wise dispersion estimates

## mean-dispersion relationship

## final dispersion estimates
```

We can plot all three sets of dispersion estimates. It is particularly important to do this if you change any of the default parameters for this step.

```
plotDispEsts(ddsObj)
```



nbinomWaldTest

Finally, apply Negative Binomial GLM fitting and calculate Wald statistics.

```
ddsObj <- nbinomWaldTest(ddsObj)
```

The DESeq command

In practice the 3 steps above can be performed in a single step using the `DESeq` wrapper function. Performing the three steps separately is useful if you wish to alter the default parameters of one or more steps, otherwise the `DESeq` function is fine.

```
ddsObj <- DESeq(ddsObj.filt)
```

```
## estimating size factors

## using 'avgTxLength' from assays(dds), correcting for library size

## estimating dispersions

## gene-wise dispersion estimates

## mean-dispersion relationship
```

```
## final dispersion estimates

## fitting model and testing
```

Generate a results table

We can generate a table of differential expression results from the DDS object using the `results` function of DESeq2.

```
results.simple <- results(ddsObj, alpha=0.05)
results.simple
```

```
## log2 fold change (MLE): Status Infected vs Uninfected
## Wald test p-value: Status Infected vs Uninfected
## DataFrame with 20091 rows and 6 columns
##                      baseMean log2FoldChange     lfcSE       stat      pvalue
##                     <numeric>      <numeric> <numeric>  <numeric>   <numeric>
## ENSMUSG00000000001 1102.56094    -0.00802952  0.102877  -0.078050    0.937788
## ENSMUSG00000000028   58.60055     0.30498077  0.254312   1.199239    0.230435
## ENSMUSG00000000037   49.23586    -0.05272685  0.416862  -0.126485    0.899348
## ENSMUSG00000000049    7.98789     0.38165132  0.644869   0.591827    0.553966
## ENSMUSG00000000056 1981.00402    -0.16921845  0.128542  -1.316449    0.188024
##                          padj
##                     <numeric>
## ENSMUSG00000000001    0.975584
## ENSMUSG00000000028    0.480598
## ENSMUSG00000000037    0.961314
## ENSMUSG00000000049    0.772123
## ENSMUSG00000000056    0.426492
##  [ reached getOption("max.print") -- omitted 6 rows ]
```

Exercise 1

> Now we have made our results table using our simple model, let have a look at which genes are changing and how many pass our 0.05 threshold. Why might this not be straigtforward?
>
> a) how many genes are significantly (with an FDR < 0.05) up-regulated?
>
> b) how many genes are significantly (with an FDR < 0.05) down-regulated?

Independent filtering

From `DESeq2` manual: "The results function of the `DESeq2` package performs independent filtering by default using the mean of normalized counts as a filter statistic. A threshold on the filter statistic is found which optimizes the number of adjusted p values lower than a [specified] significance level."

The default significance level for independent filtering is `0.1`, however, you should set this to the FDR cut off you are planning to use. We will use `0.05` - this was the purpose of the `alpha` argument in the previous command.

Remember in Session 7 we said that there is no need to pre-filter the genes as DESeq2 will do this through a process it calls 'independent filtering.' The genes with `NA` are the ones `DESeq2` has filtered out.

Love, Michael I., Wolfgang Huber, and Simon Anders. 2014. "Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2." *Genome Biology* 15: 550. https://doi.org/10.1186/s13059-014-0550-8.

McCarthy, Davis J, Yunshun Chen, and Gordon K Smyth. 2012. "Differential Expression Analysis of Multifactor RNA-Seq Experiments with Respect to Biological Variation." *Nucleic Acids Research* 40 (10): 4288–97. https://doi.org/10.1093/nar/gks042.

Ritchie, Matthew E, Belinda Phipson, Di Wu, Yifang Hu, Charity W Law, Wei Shi, and Gordon K Smyth. 2015. "limma Powers Differential Expression Analyses for RNA-Sequencing and Microarray Studies." *Nucleic Acids Research* 43 (7): e47. https://doi.org/10.1093/nar/gkv007.

Robinson, Mark D, Davis J McCarthy, and Gordon K Smyth. 2010. "edgeR: A Bioconductor Package for Differential Expression Analysis of Digital Gene Expression Data." *Bioinformatics* 26 (1): 139–40. https://doi.org/10.1093/bioinformatics/btp616.