Differential Gene Expression Workflow

Workshop on RNA-Seq

NBIS | 16-Nov-2020

NBIS, SciLifeLab

Contents

Raw data

- Raw count table

```
##                 Sample_1 Sample_2 Sample_3 Sample_4 Sample_5 Sample_6
## ENSG00000000003      321      303      204      492      455      359
## ENSG00000000005        0        0        0        0        0        0
## ENSG00000000419      696      660      472      951      963      689
## ENSG00000000457       59       54       44      109       73       66
## ENSG00000000460      399      405      236      445      454      374
## ENSG00000000938        0        0        0        0        0        1
```

- Metadata

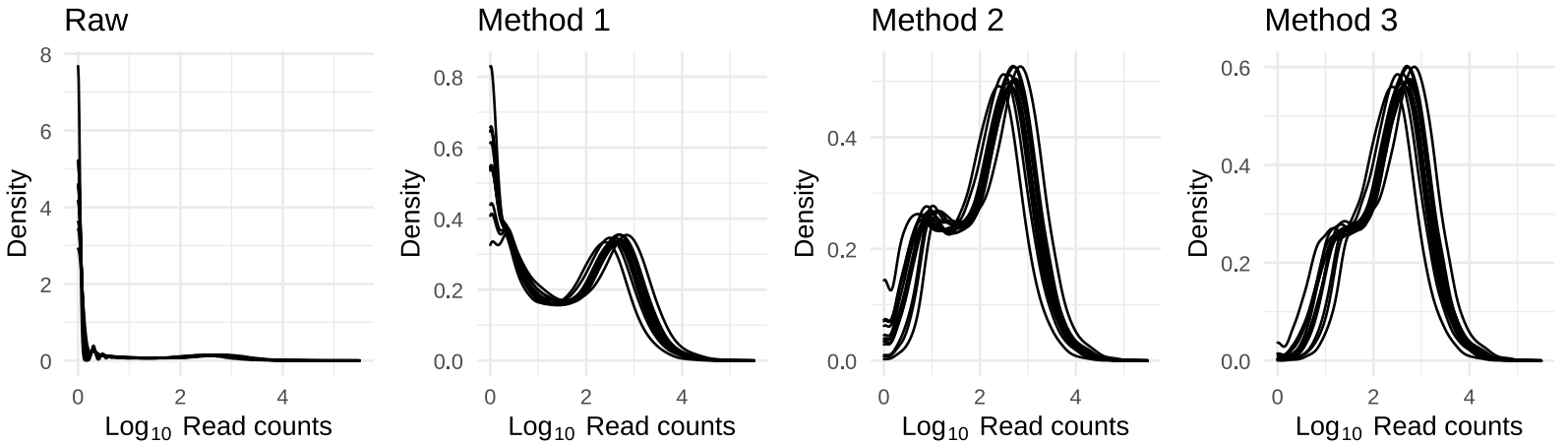
```
##          Sample_ID Sample_Name Time Replicate Cell
## Sample_1  Sample_1        t0_A   t0         A A431
## Sample_2  Sample_2        t0_B   t0         B A431
## Sample_3  Sample_3        t0_C   t0         C A431
## Sample_4  Sample_4        t2_A   t2         A A431
## Sample_5  Sample_5        t2_B   t2         B A431
## Sample_6  Sample_6        t2_C   t2         C A431
```

Preprocessing

- Remove genes and samples with low counts

```
cf1 <- cr[rowSums(cr>0) >= 2, ]
cf2 <- cr[rowSums(cr>5) >= 2, ]
cf3 <- cr[rowSums(edgeR::cpm(cr)>1) >= 2, ]
```

- Inspect distribution

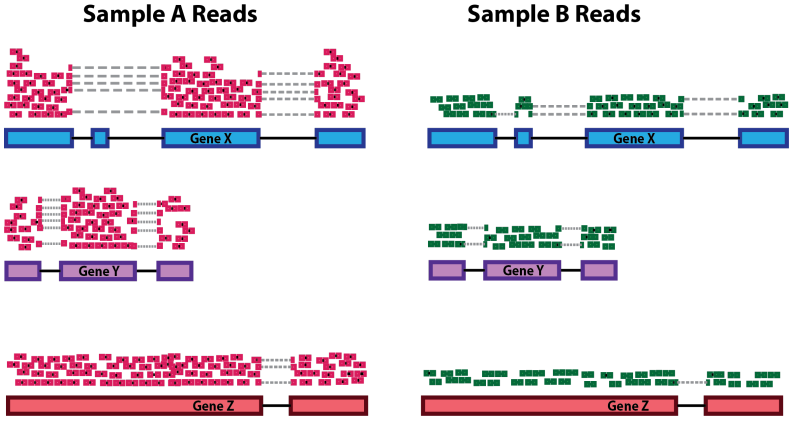


- Inspect the number of rows

```
## Raw: 59573, Method 1: 24194, Method 2: 16519, Method 3: 14578
```

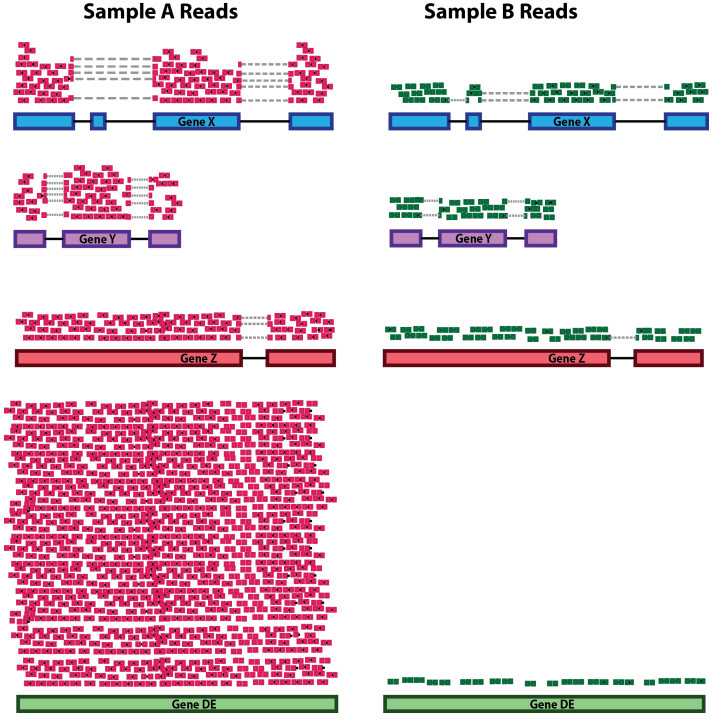
Normalisation

- Make counts comparable across samples
- Control for sequencing depth




```
##   s1 s2 s1_tc s2_tc
## x 20  6  0.33  0.38
## y 25  6  0.42  0.38
## z 15  4  0.25  0.25
```

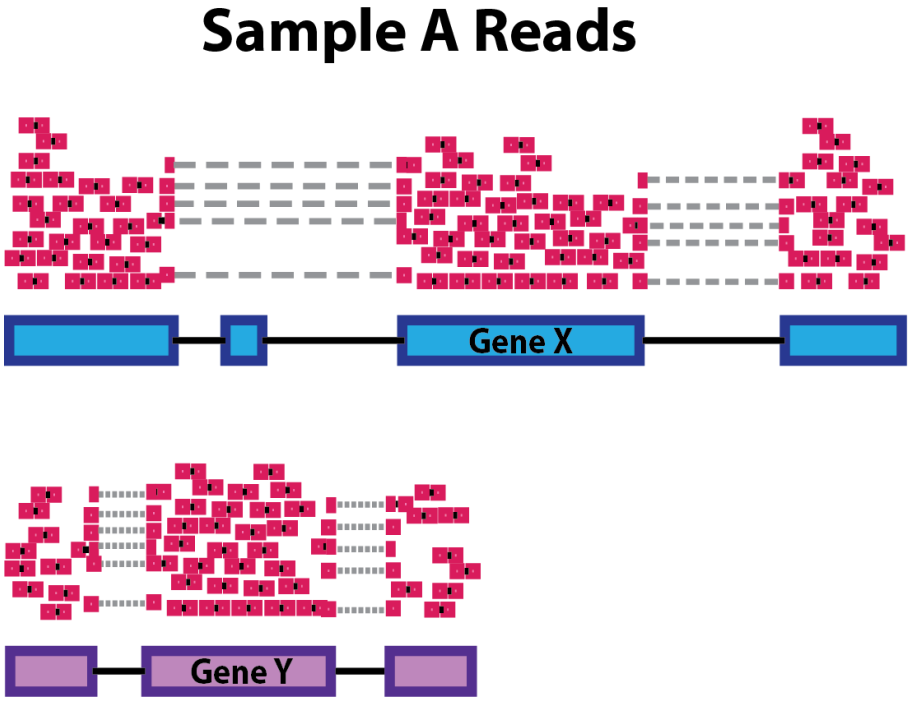
- Control for sequencing bias




```
##     s1 s2 s1_tc s2_tc
## x   20 20  0.12  0.39
## y   25 25  0.16  0.49
## z   15  4  0.09  0.08
## de 100  2  0.62  0.04
```

Normalisation

- Make counts comparable across features (genes)




```
##   counts gene_length norm_counts
## 1     50          10           5
## 2     25           5           5
```

- Bring counts to a human-friendly scale

Normalisation

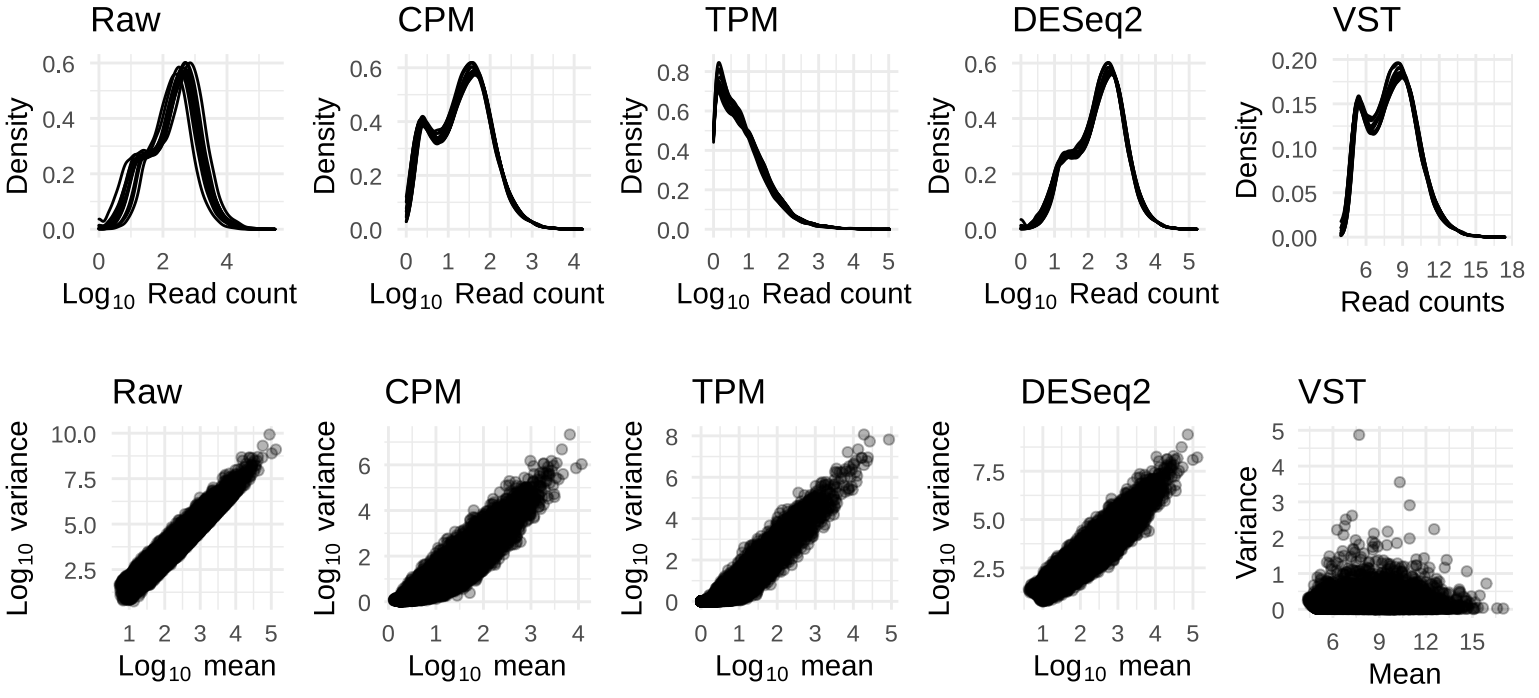
- CPM, RPKM, FPKM, TPM, RLE, MRN, Q, UQ, TMM, VST, RLOG, VOOM ... Too many...
- **CPM**: Controls for sequencing depth when dividing by total count. Not for within-sample comparison or DE.
- **RPKM/FPKM**: Controls for sequencing depth and gene length. Good for technical replicates, not good for sample-sample due to compositional bias. Assumes total RNA output is same in all samples. Not for DE.
- **TPM**: Similar to RPKM/FPKM. Corrects for sequencing depth and gene length. Also comparable between samples but no correction for compositional bias.
- **TMM/RLE/MRN**: Improved assumption: The output between samples for a core set only of genes is similar. Corrects for compositional bias. Used for DE. RLE and MRN are very similar and correlates well with sequencing depth. `edgeR::calcNormFactors()` implements TMM, TMMwzp, RLE & UQ. `DESeq2::estimateSizeFactors` implements median ratio method (RLE). Does not correct for gene length.
- **VST/RLOG/VOOM**: Variance is stabilised across the range of mean values. For use in exploratory analyses. Not for DE. `vst()` and `rlog()` functions from *DESeq2*. `voom()` function from *Limma* converts data to normal distribution.
- geTMM: Gene length corrected TMM.

Dillies, Marie-Agnes, *et al.* "A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis." Briefings in bioinformatics 14.6 (2013): 671-683
Evans, Ciaran, Johanna Hardin, and Daniel M. Stoebel. "Selecting between-sample RNA-Seq normalization methods from the perspective of their assumptions." Briefings in bioinformatics (2017)

Normalisation

Recommendations

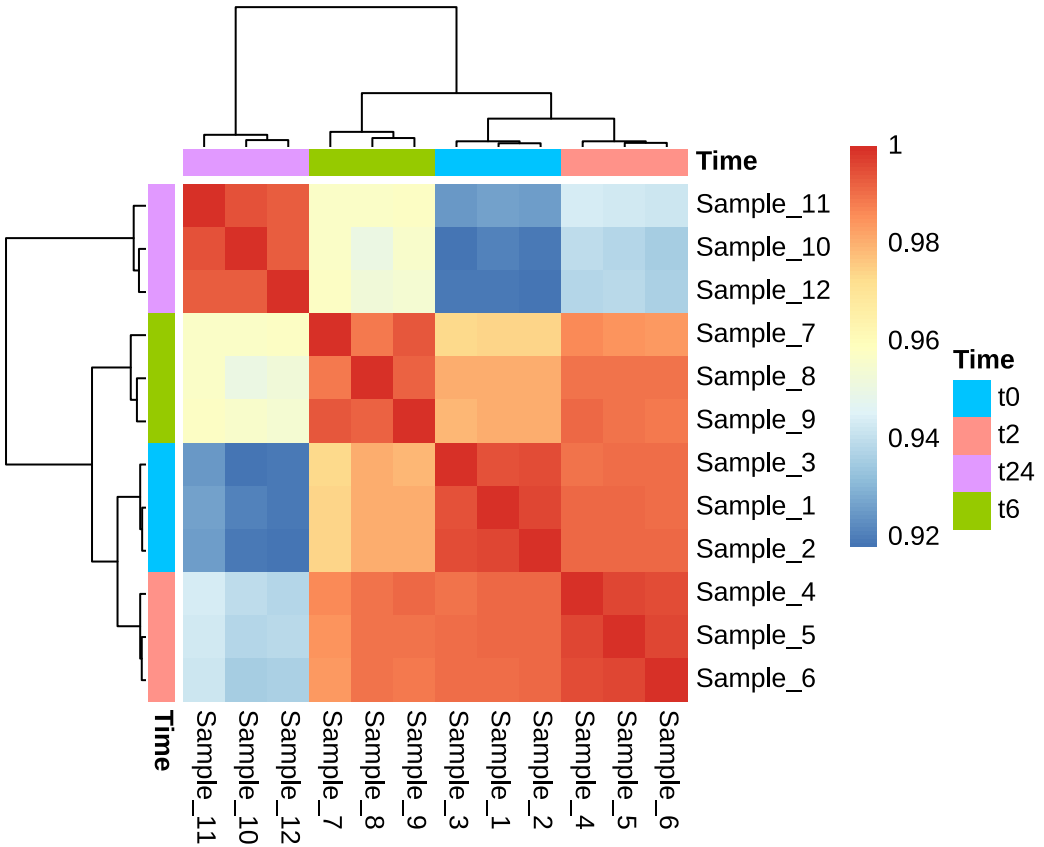
- For DGE using DGE R packages (DESeq2, edgeR, Limma etc), use raw counts
- For visualisation (PCA, clustering, heatmaps etc), use VST or RLOG
- For own analysis with gene length correction, use TPM (maybe geTMM?)
- Other solutions: spike-ins/house-keeping genes



Exploratory | Correlation

- Correlation between samples

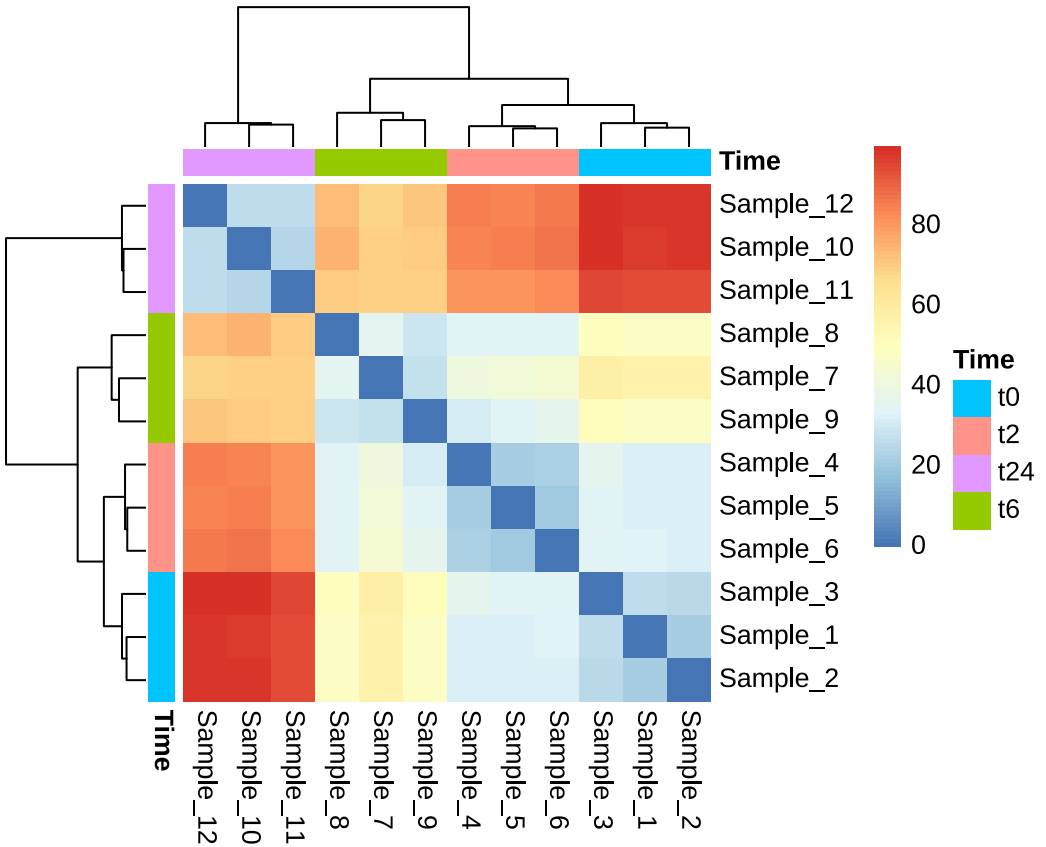
```
dmat <- as.matrix(cor(cv,method="spearman"))
pheatmap::pheatmap(dmat,border_color=NA,annotation_col=mr[,"Time",drop=F],
         annotation_row=mr[,"Time",drop=F],annotation_legend=T)
```



Exploratory | Distance

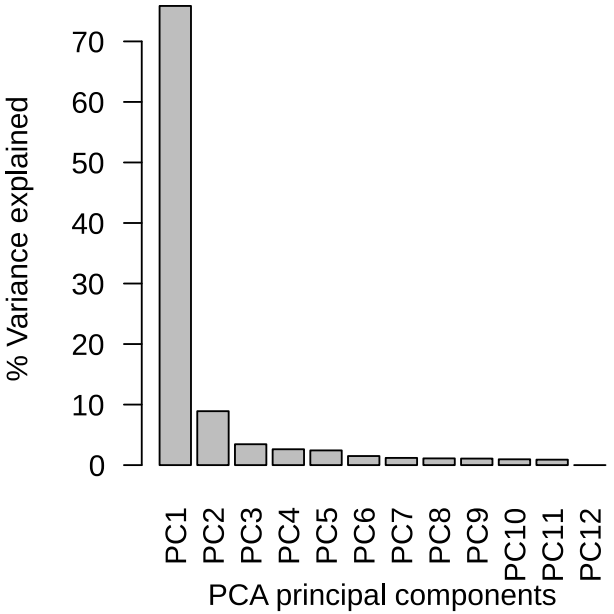
- Similarity between samples

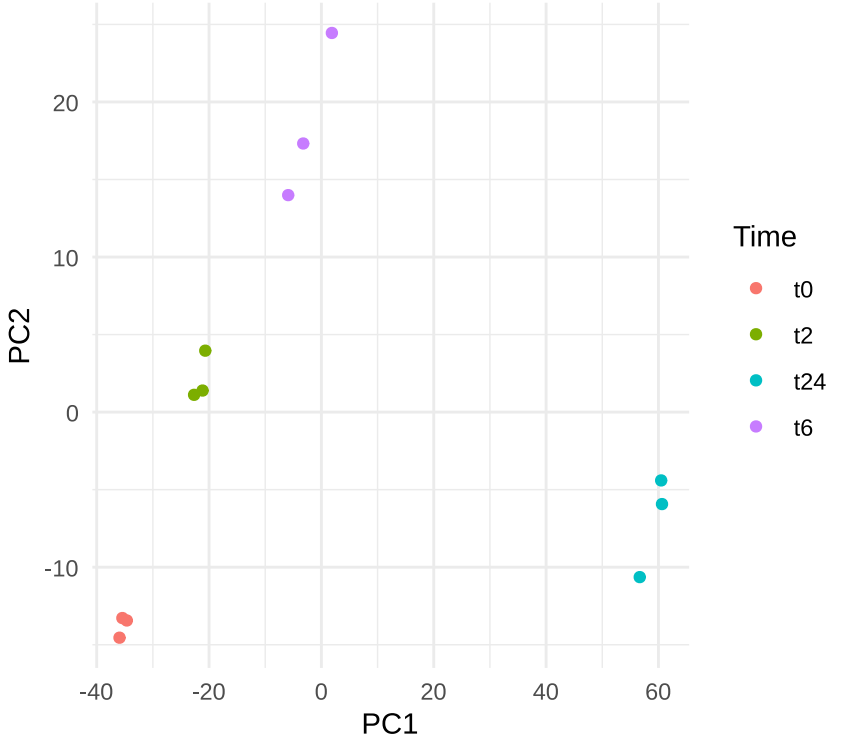
```
dmat <- as.matrix(dist(t(cv)))
pheatmap(dmat,border_color=NA,annotation_col=mr[,"Time",drop=F],
         annotation_row=mr[,"Time",drop=F],annotation_legend=T)
```



Exploratory | PCA

- Relationship between samples





DGE

- Create the DESeq2 object

```
library(DESeq2)
mr$Time <- factor(mr$Time)
d <- DESeqDataSetFromMatrix(countData=cf,colData=mr,design=~Time)
d
```

```
## class: DESeqDataSet
## dim: 14578 12
## metadata(1): version
## assays(1): counts
## rownames(14578): ENSG00000000003 ENSG00000000419 ... ENSG00000266865
##   ENSG00000266876
## rowData names(0):
## colnames(12): Sample_1 Sample_2 ... Sample_11 Sample_12
## colData names(5): Sample_ID Sample_Name Time Replicate Cell
```

- Model must be factors
- `~var`
- `~covar+var`

DGE | Size factors

- Normalisation factors are computed

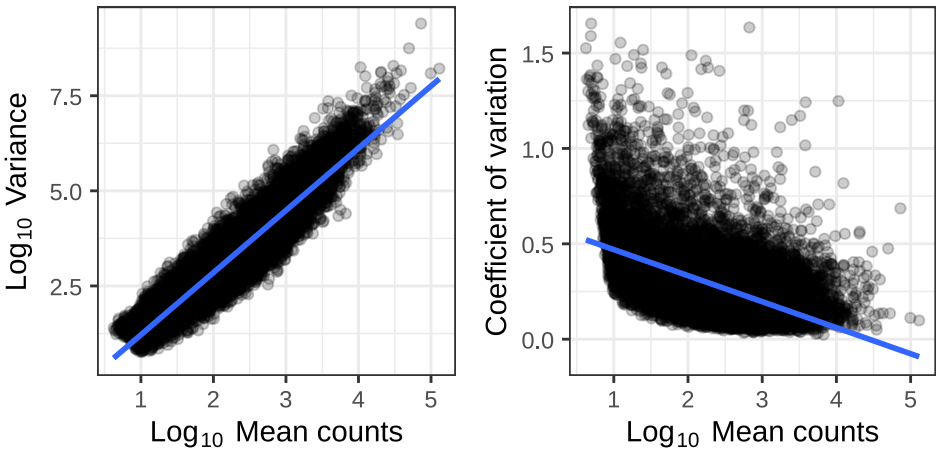
```
d <- DESeq2::estimateSizeFactors(d,type="ratio")
sizeFactors(d)
```

```
##  Sample_1  Sample_2  Sample_3  Sample_4  Sample_5  Sample_6  Sample_7  Sample_8
## 0.9003753 0.8437393 0.5106445 1.1276451 1.0941383 0.8133849 0.7553903 1.1744008
##  Sample_9 Sample_10 Sample_11 Sample_12
## 1.0189325 1.3642797 1.2325485 1.8555904
```

DGE | Dispersion

- We need a measure variability of gene counts

```
dm <- apply(cd,1,mean)
dv <- apply(cd,1,var)
cva <- function(x) sd(x)/mean(x)
dc <- apply(cd,1,cva)
```

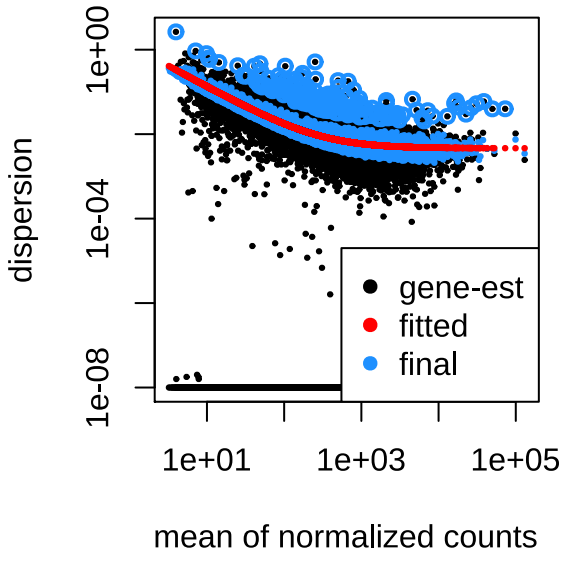


- Dispersion is a measure of variability in gene expression for a given mean

DGE | Dispersion

- D is unreliable for low mean counts
- Genes with similar mean values must have similar dispersion
- Estimate likely (ML) dispersion for each gene based on counts
- Fit a curve through the gene-wise estimates
- Shrink dispersion towards the curve

```
d <- DESeq2::estimateDispersions(d)
{par(mar=c(4,4,1,1))
plotDispEsts(d)}
```



DGE | Test

- Log2 fold changes changes are computed after GLM fitting

```
dg <- nbinomWaldTest(d)
resultsNames(dg)
```

```
## [1] "Intercept"      "Time_t2_vs_t0"  "Time_t24_vs_t0" "Time_t6_vs_t0"
```

- Use `results()` to customise/return results
  - Set coefficients using `contrast` or `name`
  - Filtering by fold change using `lfcThreshold`
  - `cooksCutoff` removes outliers
  - `independentFiltering`
  - `pAdjustMethod`
  - `alpha`

DGE | Test

```
res1 <- results(dg,name="Time_t2_vs_t0",alpha=0.05)
summary(res1)
```

```
##
## out of 14578 with nonzero total read count
## adjusted p-value < 0.05
## LFC > 0 (up)       : 413, 2.8%
## LFC < 0 (down)     : 696, 4.8%
## outliers [1]       : 0, 0%
## low counts [2]     : 2261, 16%
## (mean count < 26)
## [1] see 'cooksCutoff' argument of ?results
## [2] see 'independentFiltering' argument of ?results
```

DGE | Test

```
head(res1)
```

```
## log2 fold change (MLE): Time t2 vs t0
## Wald test p-value: Time t2 vs t0
## DataFrame with 6 rows and 6 columns
##                  baseMean log2FoldChange     lfcSE      stat    pvalue
##                 <numeric>      <numeric> <numeric> <numeric> <numeric>
## ENSG00000000003  490.0172      0.2206198 0.1127611  1.956524 0.0504034
## ENSG00000000419  817.7807      0.0592720 0.1014813  0.584068 0.5591746
## ENSG00000000457   82.0788      0.2077486 0.2204049  0.942577 0.3458972
## ENSG00000000460  356.0716     -0.1291864 0.1151392 -1.122002 0.2618616
## ENSG00000001036  919.6068      0.0288827 0.0851501  0.339198 0.7344609
## ENSG00000001084  529.5940      0.2119648 0.0929811  2.279655 0.0226281
##                      padj
##                 <numeric>
## ENSG00000000003  0.263505
## ENSG00000000419  0.830262
## ENSG00000000457  0.689946
## ENSG00000000460  0.612625
## ENSG00000001036  0.909639
## ENSG00000001084  0.159263
```

- Use `lfcShrink()` to correct fold changes for high dispersion genes

Thank you. Questions?

R version 4.0.3 (2020-10-10)

Platform: x86_64-pc-linux-gnu (64-bit)

OS: Ubuntu 18.04.5 LTS

Built on : 16-Nov-2020 at 18:35:05

2020 • SciLifeLab • NBIS