NBIS

Data Preprocessing

Workshop on RNA-Seq

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Raw data

- Raw count table

```
##                    DSSd00_1 DSSd00_2 DSSd00_3 DSSd07_1 DSSd07_2 DSSd07_3
## ENSMUSG00000102693        0        0        0        0        0        0
## ENSMUSG00000064842        0        0        0        0        0        0
## ENSMUSG00000051951        0        1        2        0        3        2
## ENSMUSG00000102851        0        0        0        0        0        0
## ENSMUSG00000103377        0        0        0        0        0        0
## ENSMUSG00000104017        0        0        0        0        0        0
```

- Metadata

```
##          SampleName     SampleID No Model Day Group Replicate
## DSSd00_1   DSSd00_1 KI_PC1606_01  1   DSS   0 day00         1
## DSSd00_2   DSSd00_2 KI_PC1606_02  2   DSS   0 day00         2
## DSSd00_3   DSSd00_3 KI_PC1606_03  3   DSS   0 day00         3
## DSSd07_1   DSSd07_1 KI_PC1606_13 13   DSS   7 day07         1
## DSSd07_2   DSSd07_2 KI_PC1606_14 14   DSS   7 day07         2
## DSSd07_3   DSSd07_3 KI_PC1606_15 15   DSS   7 day07         3
```

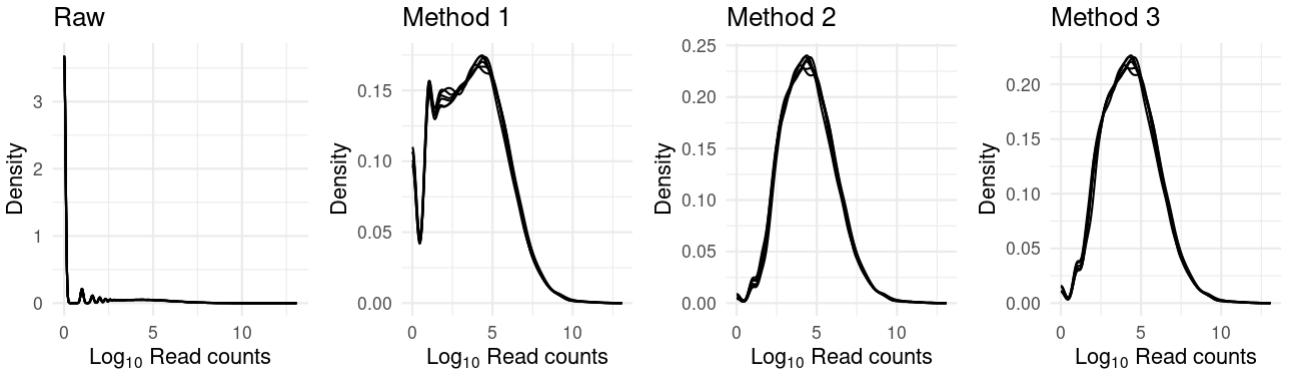
Filtering

- Remove genes and samples with low counts

```
cf1 <- cr[rowSums(cr>0) >= 3, ] # Keep rows/genes that have at least one read in +3 samples
cf2 <- cr[rowSums(cr>3) >= 3, ] # Keep rows/genes that have at least three reads in +3 samp
cf3 <- cr[rowSums(edgeR::cpm(cr)>5) >= 3, ] # need at least  three samples to have cpm > 5.
```

count/read per million (cpm/rpm): a normalized value for sequencing depth.

- Inspect distribution

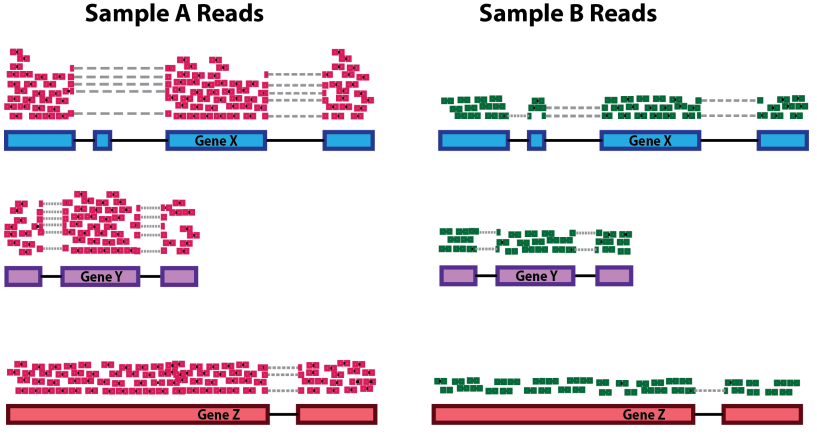


- Inspect the number of rows (genes) available after filtering

```
## Raw: 55487, Method 1: 16099, Method 2: 11783, Method 3: 12496
```

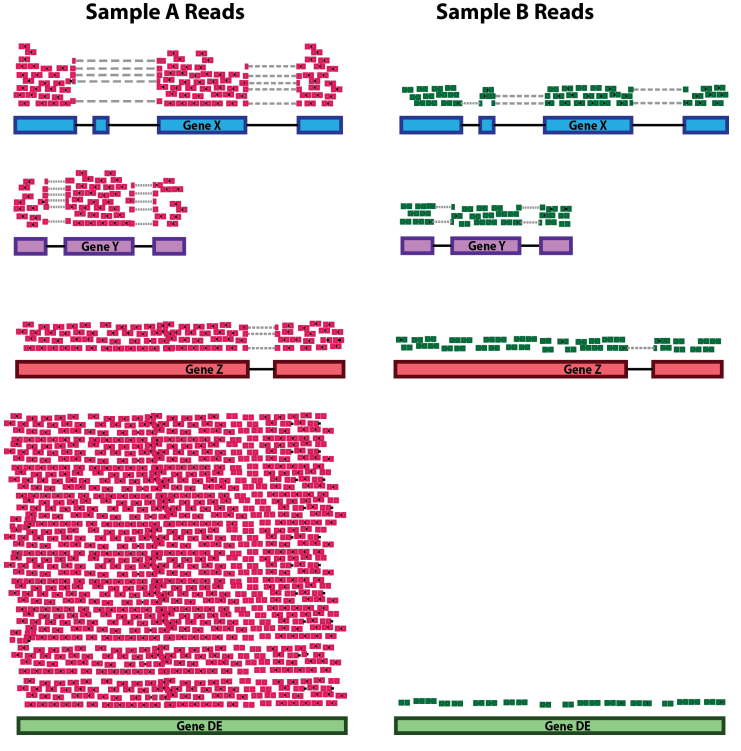
Normalisation

- Removing technical biases in sequencing data (e.g. sequencing depth and gene length)
- Make counts comparable across features (genes).
- Make counts comparable across samples




```
##    A B A_tc B_tc
## x 20 6 0.33 0.38
## y 25 6 0.42 0.38
## z 15 4 0.25 0.25
```

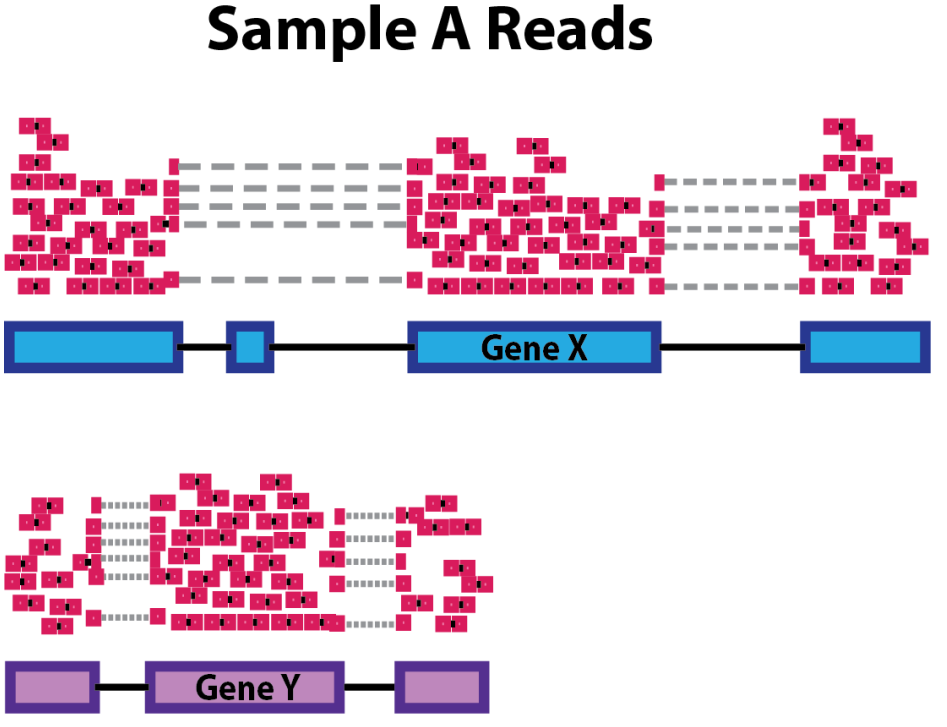
- Control for compositional bias




```
##      A B A_tc B_tc
## x   20 6 0.12 0.33
## y   25 6 0.16 0.33
## z   15 4 0.09 0.22
## de 100 2 0.62 0.11
```

Normalisation

- Controlling for gene length: It can be useful for gene to gene comparisons.




```
##   counts gene_length norm_counts
## x     50          10           5
## y     25           5           5
```

- Bring counts to a human-friendly scale

Normalisation

Normalisation by library size

- Assumes total expression is the same under different experimental conditions
- Methods include TC, RPKM, FPKM, TPM
- RPKM, FPKM and TPM control for sequencing depth and gene length
- Total number of RPKM/FPKM normalized counts for each sample will be different, therefore, you cannot compare the normalized counts for each gene equally between samples.
- TPM is proportional to RPKM and enables better comparison between samples because total per sample sums to equal value

```
##      A  B  len   A_rpm   B_rpm   A_rpkm   B_rpkm A_rpk B_rpk   A_tpm   B_tpm
## x   20  6 2000  408163  222222 204081.5 111111.0 10.00   3.0  493827  153846
## y   25  6 4000  510204  222222 127551.0  55555.5  6.25   1.5  308642   76923
## z    4 15 1000   81633  555556  81633.0 555556.0  4.00  15.0  197531  769231
## sum 49 27 7000 1000000 1000000 413265.5 722222.5 20.25  19.5 1000000 1000000
```

rpm = cpm.

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

Normalisation by distribution

- Assumes technical effects are same for DE and non-DE genes
- Assumes number of over and under-expressed genes are roughly same across conditions
- Corrects for compositional bias
- Methods include Q, UQ, M, RLE, TMM, MRN
- `edgeR::calcNormFactors()` implements TMM, TMMwsp, RLE & UQ
- `DESeq2::estimateSizeFactors()` implements relative log expression (RLE)
- Does not correct for gene length
- **geTMM** is gene length corrected TMM

```
##    A  B  len   ref A_ratio B_ratio     A_mrn    B_mrn
## x 20  6 2000 10.95    1.83    0.55 10.928962 10.90909
## y 25  6 4000 12.25    2.04    0.49 13.661202 10.90909
## z  4 15 1000  7.75    0.52    1.94  2.185792 27.27273
```

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

Normalisation by testing

- A more robust version of normalisation by distribution
- A set of non-DE genes are detected through hypothesis testing
- Tolerates a larger difference in number of over and under expressed genes between conditions
- Methods include PoissonSeq, DEGES

Normalisation using Controls

- Assumes controls are not affected by experimental condition and technical effects are similar to all other genes
- Useful in conditions with global shift in expression
- Controls could be house-keeping genes or spike-ins
- Methods include RUV, CLS

Stabilizing variance

- Variance is stabilised across the range of mean values
- Methods include VST, RLOG, VOOM
- For use in exploratory analyses. Not for DE.
- `vst()` and `rlog()` functions from *DESeq2*
- `voom()` function from *Limma* converts data to normal distribution

Normalisation

Recommendations

- Most tools use a mix of many different normalisations
- 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?)
- Custom solutions: spike-ins/house-keeping genes

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

Thank you. Questions?

Graphics from freepik.com
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