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

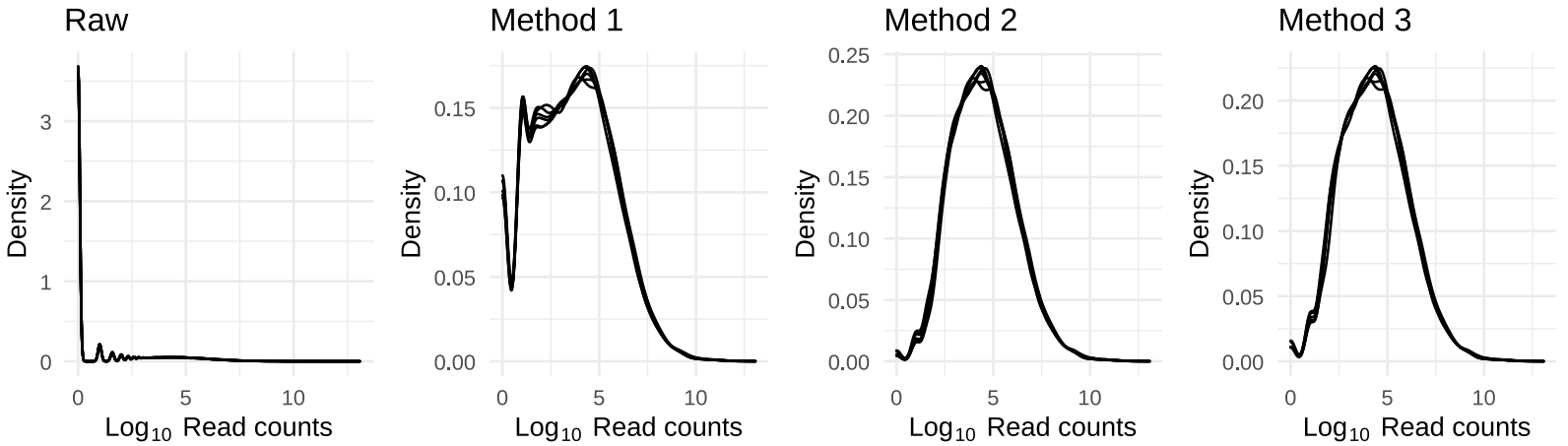
```
##          SampleName     SampleID No Model Day Group Replicate
## DSSd00_1   DSSd00_1 KI_PC1606_01  1   DSS   0 day00         1
## DSSd00_2   DSSd00_2 KI_PC1606_02  2   DSS   0 day00         2
## DSSd00_3   DSSd00_3 KI_PC1606_03  3   DSS   0 day00         3
## DSSd07_1   DSSd07_1 KI_PC1606_13 13   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
cf2 <- cr[rowSums(cr>3) >= 3, ] # Keep rows/genes that have at least three reads
cf3 <- cr[rowSums(edgeR::cpm(cr)>5) >= 3, ] # need at least  three samples to have cpm >
```

- Inspect distribution

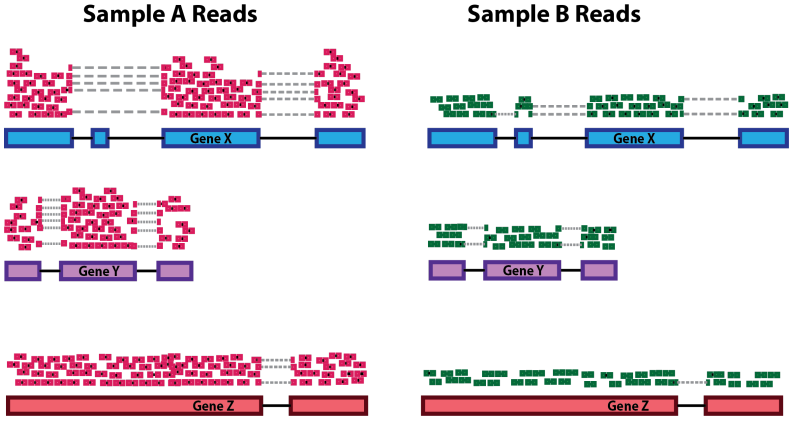


- Inspect the number of rows (genes) available after filtering

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

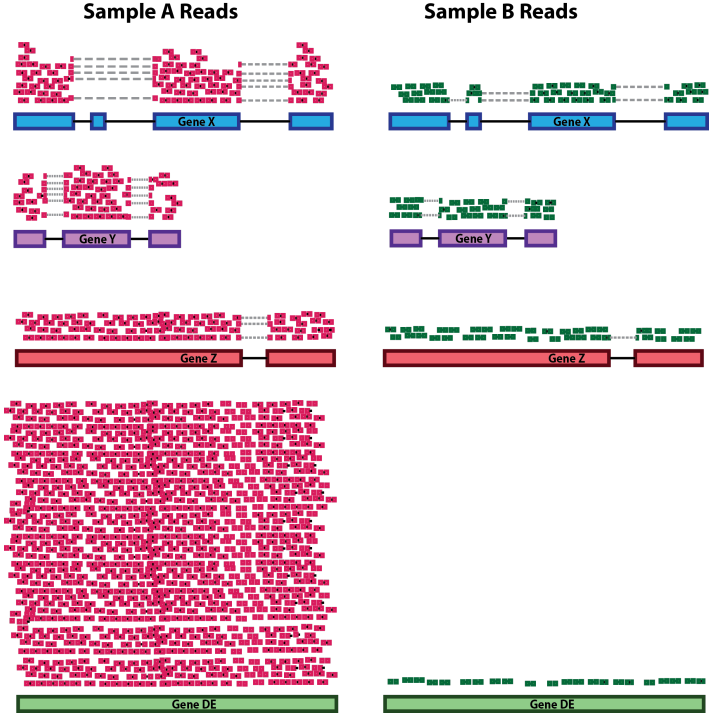
Normalisation

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

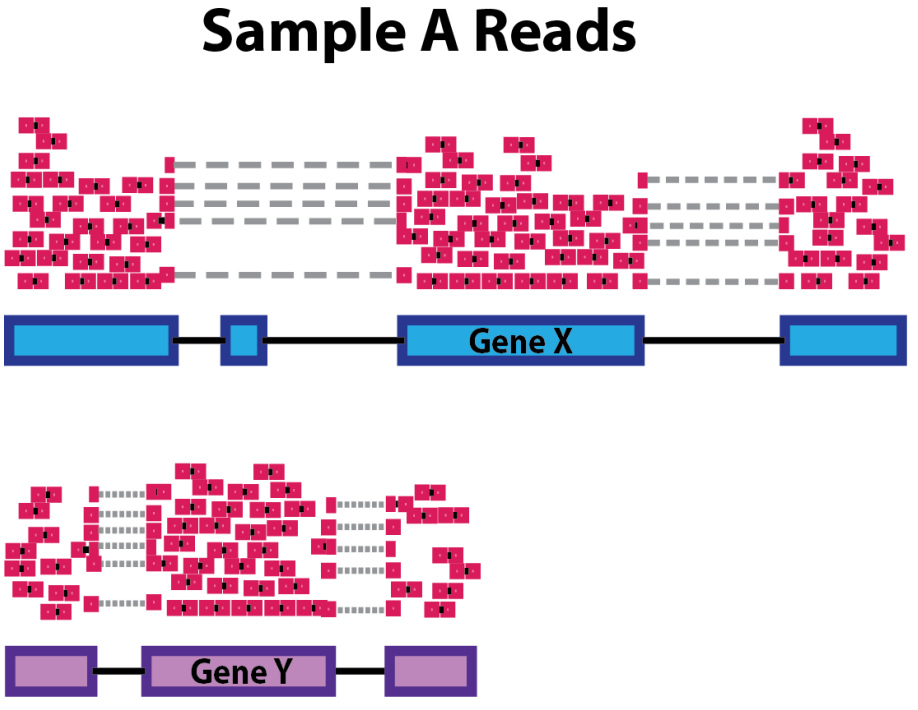
- Control for compositional bias




```
##      A  B A_tc B_tc
## x   10 20 0.07 0.39
## y   25 25 0.17 0.49
## z   15  4 0.10 0.08
## de 100  2 0.67 0.04
```

Normalisation

- Make counts comparable across features (genes)




```
##   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 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 20000  408163  222222  20.41  11.11 0.001000 0.00030  493827  153846
## y   25  6 40000  510204  222222  12.76   5.56 0.000625 0.00015  308642   76923
## z    4 15 10000   81633  555556   8.16  55.56 0.000400 0.00150  197531  769231
## sum 49 27 70000 1000000 1000000  41.33  72.23 0.002025 0.00195 1000000 1000000
```

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, TMMwzp, RLE & UQ
- `DESeq2::estimateSizeFactors()` implements median ratio method (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 20000 10.95    1.83    0.55 10.928962 10.90909
## y 25  6 40000 12.25    2.04    0.49 13.661202 10.90909
## z  4 15 10000  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?

R version 4.0.5 (2021-03-31)
Platform: x86_64-pc-linux-gnu (64-bit)
OS: Ubuntu 18.04.6 LTS

Built on : 01-Nov-2021 at 13:24:00

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