Data Preprocessing

Workshop on RNA-Seq

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NBIS, SciLifeLab

Raw data

- Raw count table

```
##         DSSd00_1 DSSd00_2 DSSd00_3 DSSd07_1 DSSd07_2 DSSd07_3
## mt-Tp          0        0        0        0        0        0
## mt-Tt          0        0        0        0        0        0
## mt-Cytb        0        1        2        0        3        2
## mt-Te          0        0        0        0        0        0
## mt-Nd6         0        0        0        0        0        0
## mt-Nd5         0        0        0        0        0        0
```

- Metadata

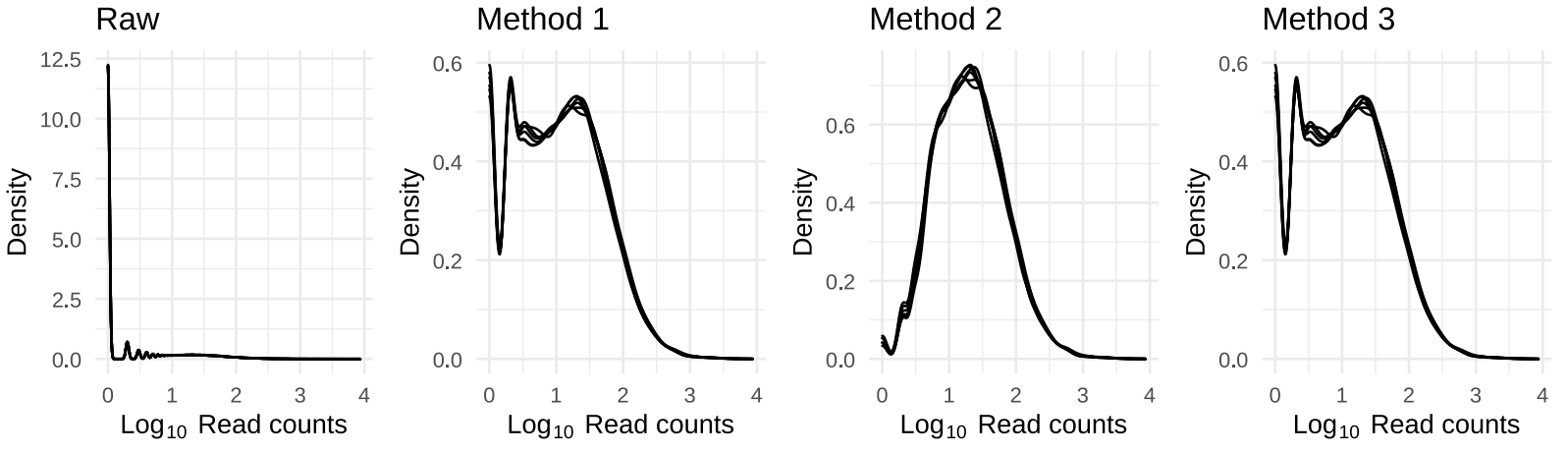
```
##          SampleName     SampleID No Model Day Group Replicate
## DSSd00_1   DSSd00_1 KI_PC1606_01  1   DSS   0 day00         1
## DSSd00_2   DSSd00_2 KI_PC1606_02  2   DSS   0 day00         2
## DSSd00_3   DSSd00_3 KI_PC1606_03  3   DSS   0 day00         3
## DSSd07_1   DSSd07_1 KI_PC1606_13 13   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
```

Preprocessing

- Remove genes and samples with low counts

```
cf1 <- cr[rowSums(cr>0) >= 2, ]
cf2 <- cr[rowSums(cr>3) >= 2, ]
cf3 <- cr[rowSums(edgeR::cpm(cr)>1) >= 2, ]
```

- Inspect distribution

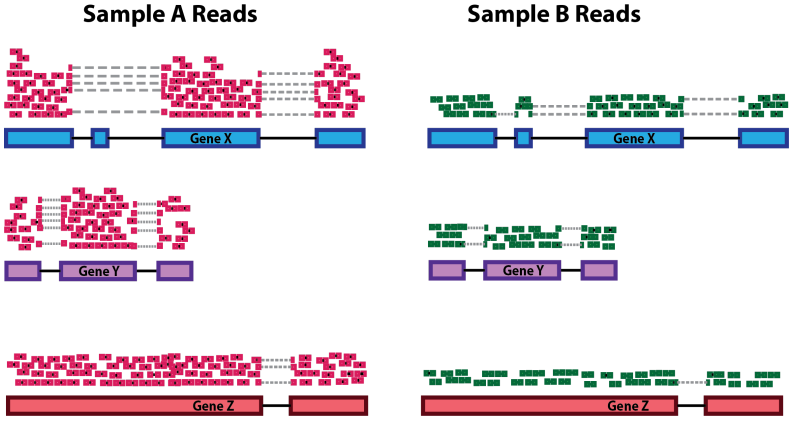


- Inspect the number of rows (genes)

```
## Raw: 55367, Method 1: 17515, Method 2: 12473, Method 3: 17515
```

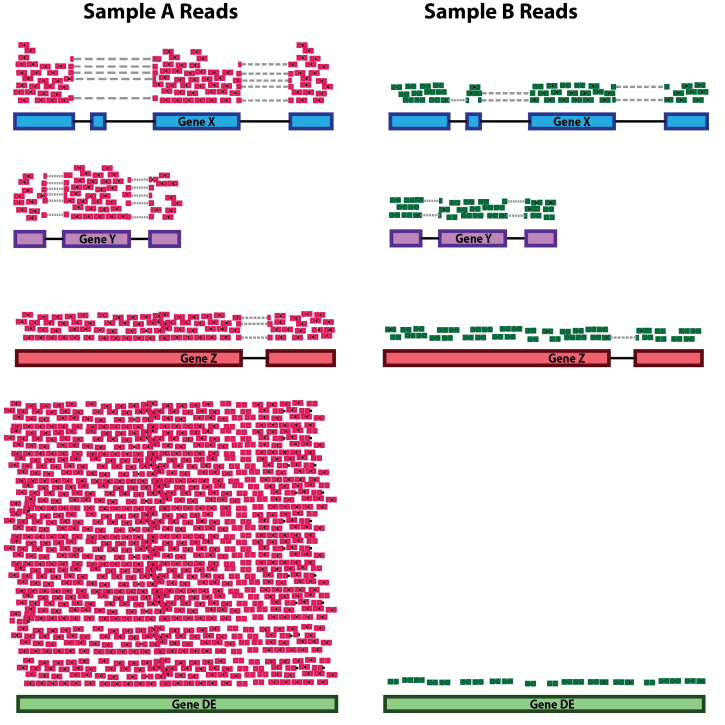
Normalisation

- Make counts comparable across samples
- Control for sequencing depth




```
##    A B A_tc B_tc
## x 20 6  Inf  Inf
## y 25 6  Inf  Inf
## z 15 4  Inf  Inf
```

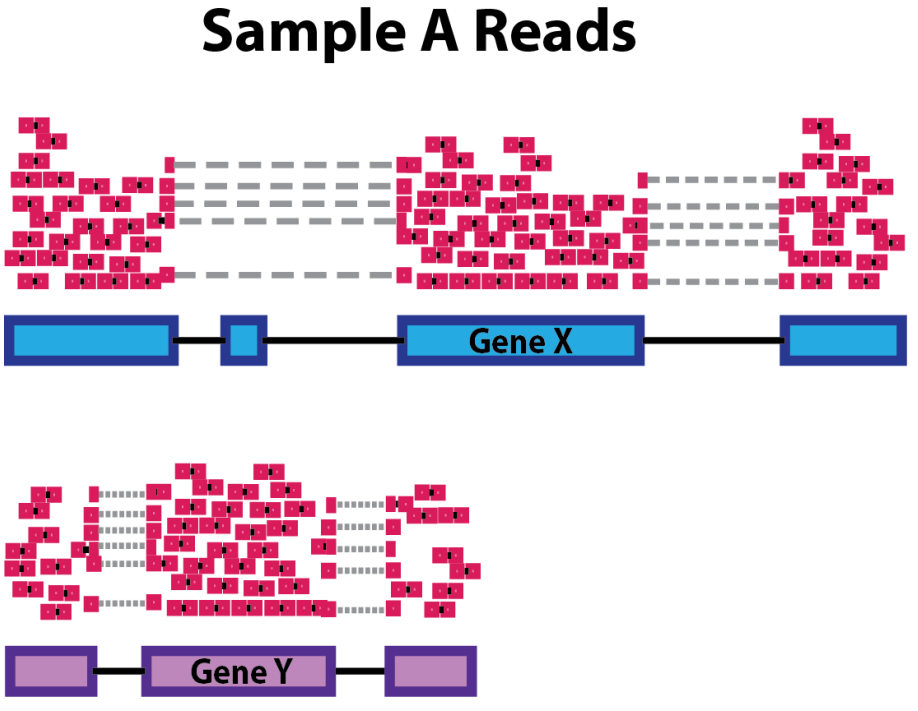
- Control for compositional bias




```
##      A  B A_tc B_tc
## x    0 20  NaN  Inf
## y   25 25  Inf  Inf
## z   15  4  Inf  Inf
## de 100  2  Inf  Inf
```

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
- TPM enables better comparison between samples and between experiments

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

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

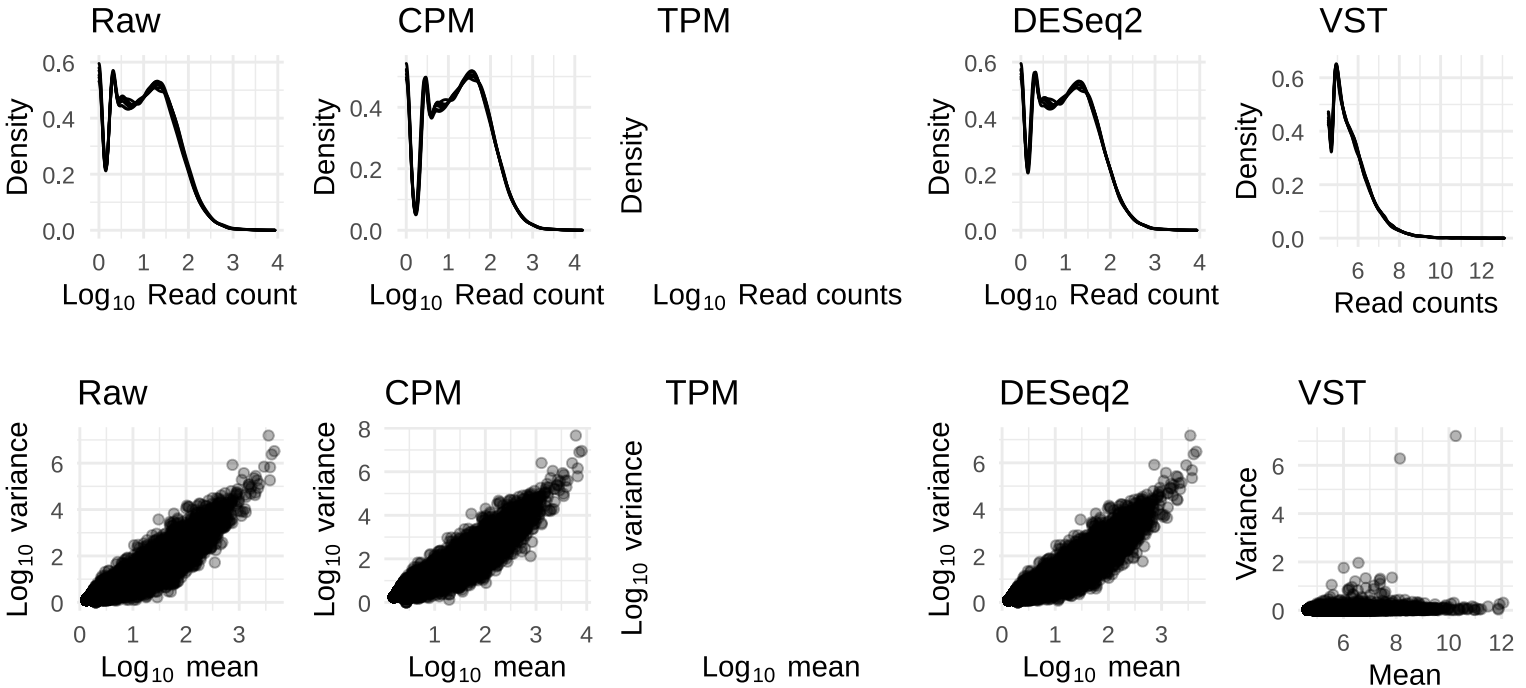
Stabilizing variance

- Variance is stabilised across the range of mean values
- Mwthods 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

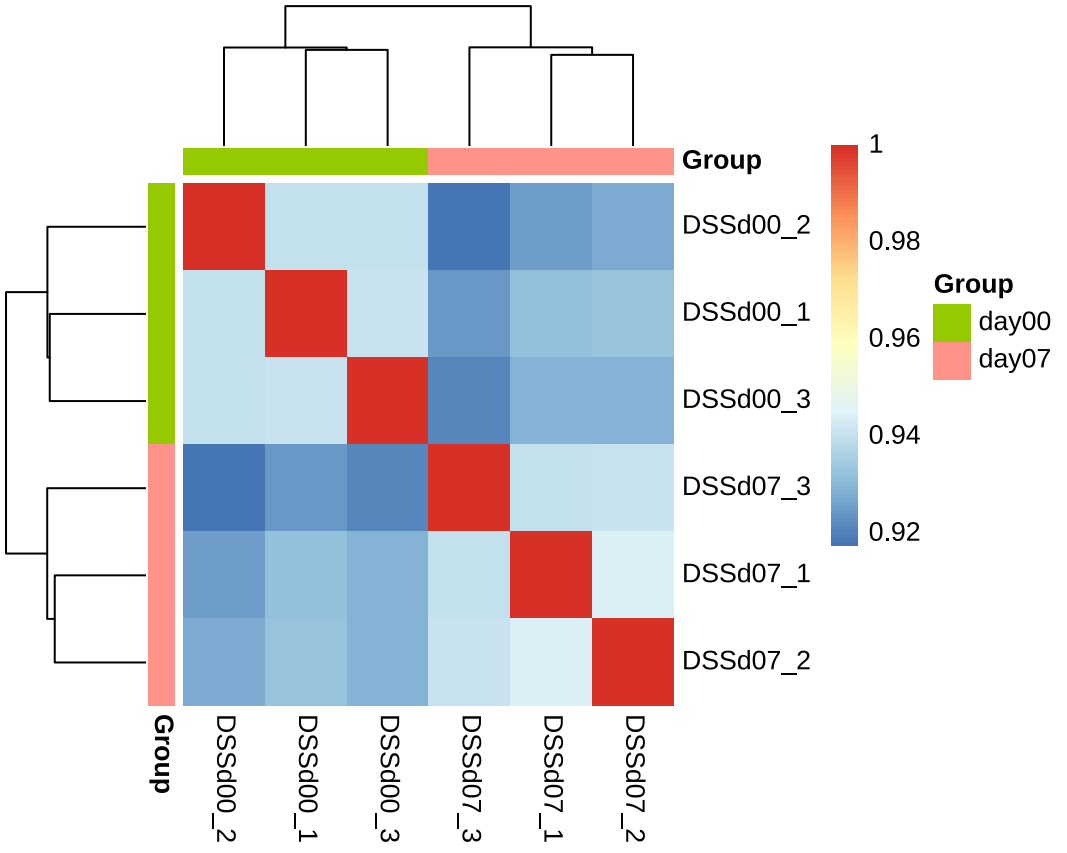


Dillies, Marie-Agnes, *et al.* "A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis." Briefings in bioinformatics 14.6 (2013): 671-683

Exploratory | Correlation

- Correlation between samples

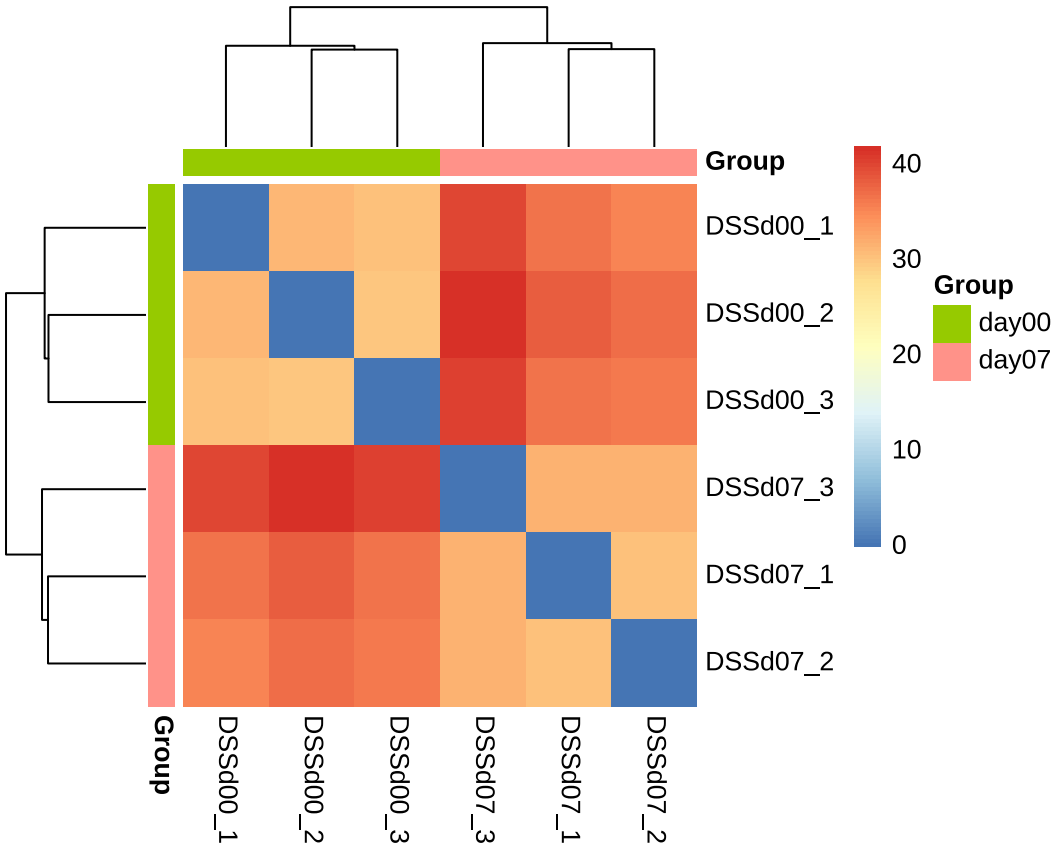
```
dmat <- as.matrix(cor(cv,method="spearman"))
pheatmap::pheatmap(dmat,border_color=NA,annotation_col=mr[,"Group",drop=F],
         annotation_row=mr[,"Group",drop=F],annotation_legend=T)
```



Exploratory | Distance

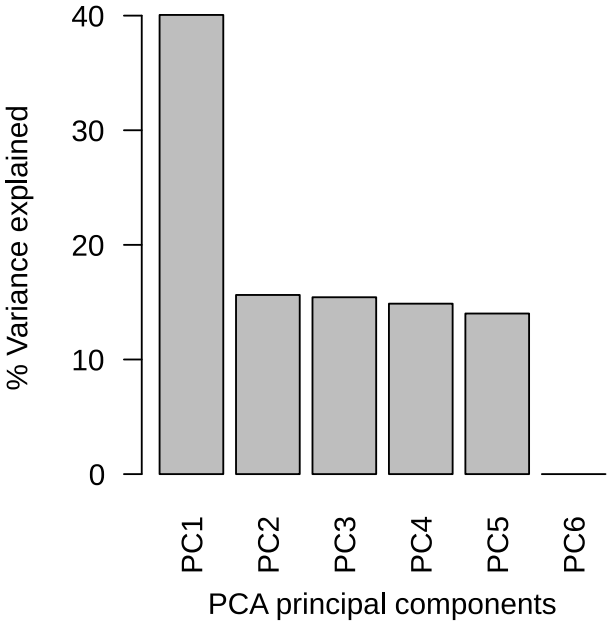
- Similarity between samples

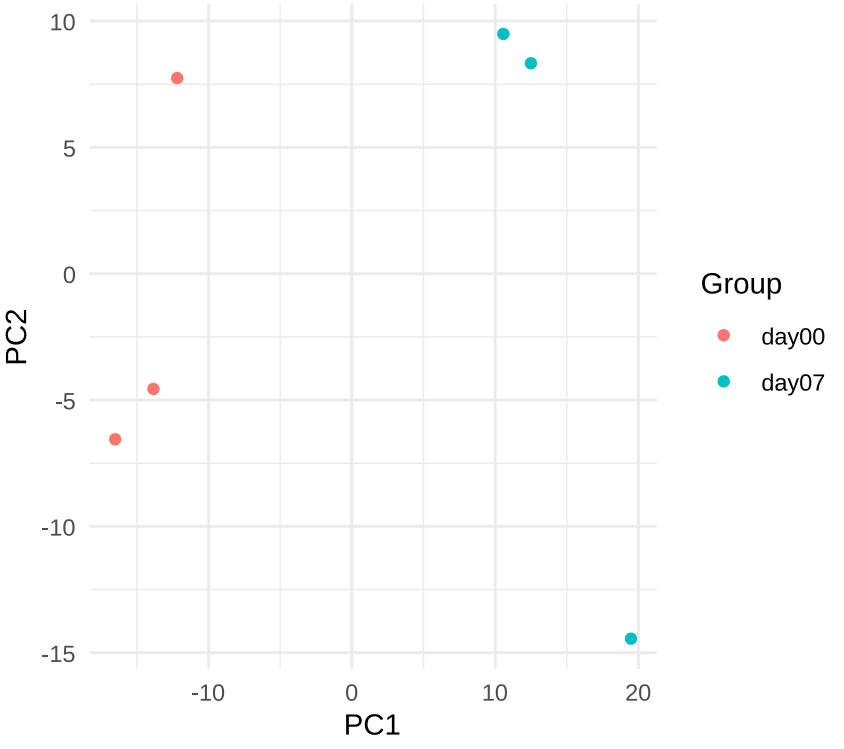
```
dmat <- as.matrix(dist(t(cv)))
pheatmap(dmat,border_color=NA,annotation_col=mr[,"Group",drop=F],
         annotation_row=mr[,"Group",drop=F],annotation_legend=T)
```



Exploratory | PCA

- Relationship between samples





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 : 26-Nov-2020 at 16:14:35

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