

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
## ENSMUSG000000102693	0	0	0	0	0	0
## ENSMUSG000000064842	0	0	0	0	0	0
## ENSMUSG000000051951	0	1	2	0	3	2
## ENSMUSG000000102851	0	0	0	0	0	0
## ENSMUSG000000103377	0	0	0	0	0	0
## ENSMUSG000000104017	0	0	0	0	0	0

- Metadata

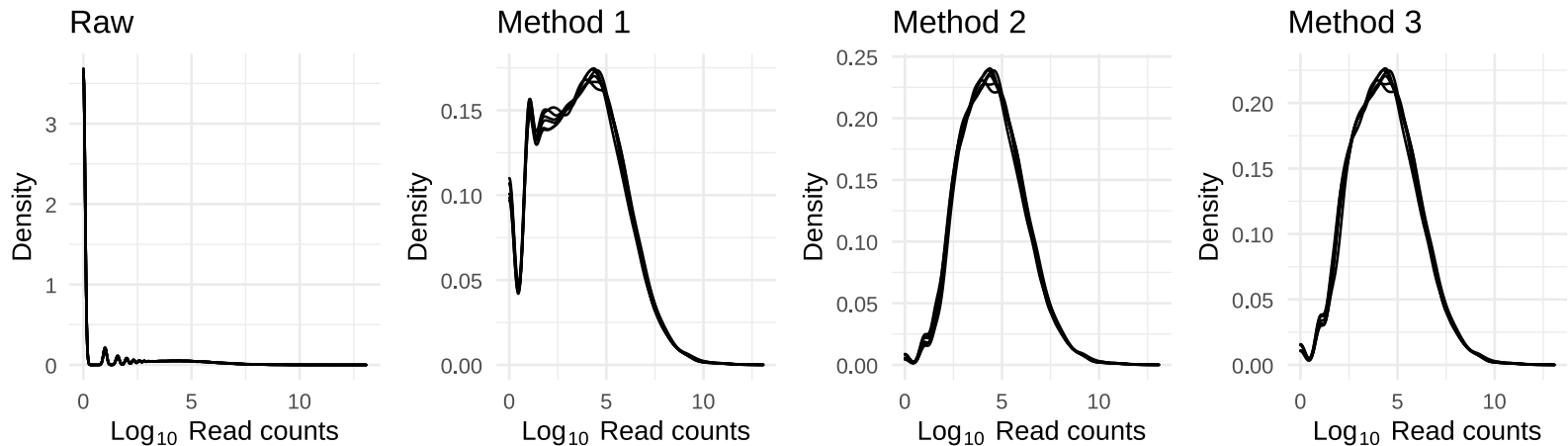
##	SampleName	SampleID	No	Model	Day	Group	Replicate
## DSSd00_1	DSSd00_1	KI_PC1606_01	1	DSS	0	day00	1
## DSSd00_2	DSSd00_2	KI_PC1606_02	2	DSS	0	day00	2
## DSSd00_3	DSSd00_3	KI_PC1606_03	3	DSS	0	day00	3
## DSSd07_1	DSSd07_1	KI_PC1606_13	13	DSS	7	day07	1
## DSSd07_2	DSSd07_2	KI_PC1606_14	14	DSS	7	day07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

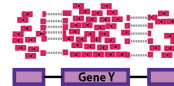
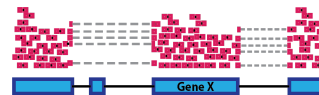
```
## Raw: 55487, Method 1: 16099, Method 2: 11783, Method 3: 12496
```

Normalisation

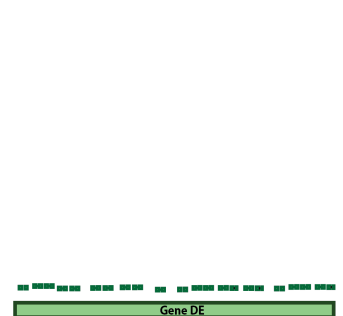
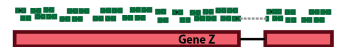
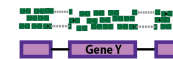
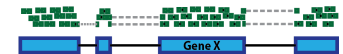
- Removing technical biases in sequencing data (e.g. sequencing depth and gene length)
- Make counts comparable across samples

- Control for compositional bias

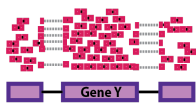
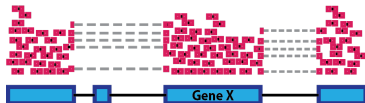
Sample A Reads



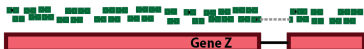
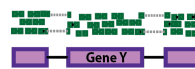
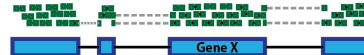
Sample B Reads



Sample A Reads



Sample B Reads



```
##      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
```

```
##      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)

Sample A Reads



##	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_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 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
```

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, VROOM
- For use in exploratory analyses. Not for DE.
- `vst()` and `rlog()` functions from *DESeq2*
- `voom()` function from *Limma* converts data to normal distribution

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



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 🕒 09:02:17

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