أكاديمية كاوست
KAUST ACADEMY

Gene expression analysis & Enrichment

Day 03

The KAUST Academy & The Bioinformatics Platform

4-7 Feb 2026

Evaluation

1. Theoretical Exam

- **No coding required**
  - Questions will evaluate your conceptual understanding in bioinformatics, the process of genomics data analysis, etc.
- **Weight:** 75% of the total grade
- **Exam Duration:** 2 hours
- **Question style:**
  - Conceptual, Comprehension & Familiarity with Bioinformatics Analysis
  - Designed to test **your understanding**, not memorization
- **Number of questions > 50**

2a. Project Presentation & Report

- **One report per group**
- **Number of presenters:** flexible (up to the group)
- **Presentation format:** no strict format required we look into:
  - Quality of the analysis
  - Clear explanation of main steps and relevance of the results
  - Problem solving skills
- **Time allocation: 5 minutes** presentation per group

2b. Delivery

Each group is responsible for uploading:

•One report

•One presentation

📂 Upload both files (**Report & PPT**) to the **Report & Presentation** folder

⏰ **Deadline: 9:00 AM, February 7**

Agenda – Day 03

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Morning Session

9-9:15	S1: Recap of Day 2
9:15-10	S2: Differential Expression Concepts
10-11	*L1:* Perform differential expression analysis, inspect DE results
11-11:30	S3: Visualization & Reporting
11:30-12	L2: Visualization & Integrated QC

Afternoon Session

2-2:45	S4: Pathway & Enrichment Analysis
2:45-3:30	*L3: Hands-on Enrichment Analysis*
3:30-4	S5: Extended Applications of RNA-seq
4-5	S6: Project Work

Recap

- Quality Control
- Reference Genome Alignment
- Pseudo Alignment: Gene Quantification

Agenda – day 3

01

Differential Expression

Normalization, statistical modeling,
hypothesis testing, DESeq2 workflow

02

Visualization

PCA, volcano plots, MA plots,
heatmaps, quality assessment

03

Enrichment Analysis

Gene Ontology, pathway analysis

04

Extended Applications of RNA-seq

Variant detection, single cell, spatial transcriptomics

Agenda – Day 03

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2:45-3:30	*L3: Hands-on with Transcriptomics Data*
3:30-4	S5: Extended Applications of RNA-seq
4-5	S6: Team Formation & Project Overview

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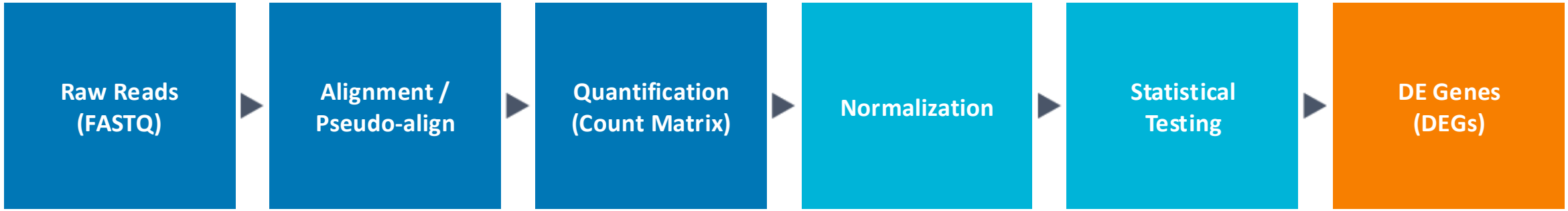
Differential Expression Concepts

Day 03 – Session 02

Differential Expression

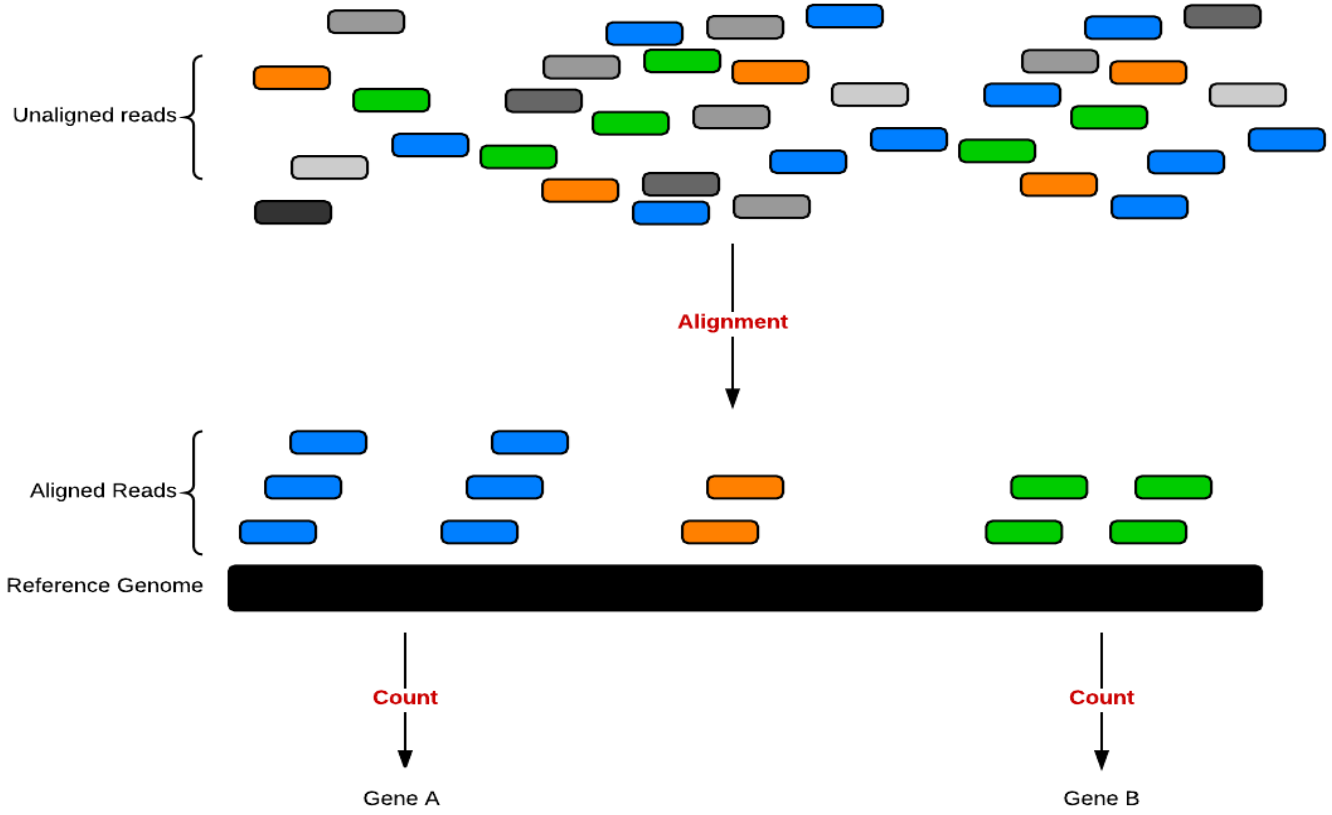
The goal of DE testing is to determine which genes are expressed at statistically different levels between experimental conditions.



This session covers steps from the count matrix through DE identification

*Key tools: **DESeq2**, edgeR, limma-voom | References: Love et al. 2014, Robinson et al. 2010, Law et al. 2014*

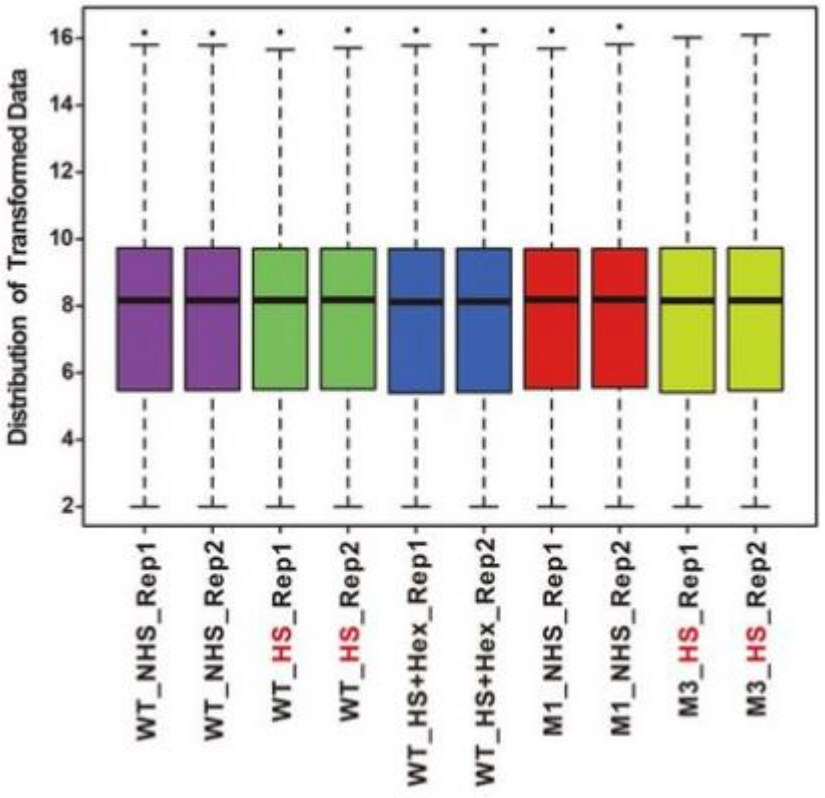
Differential Expression

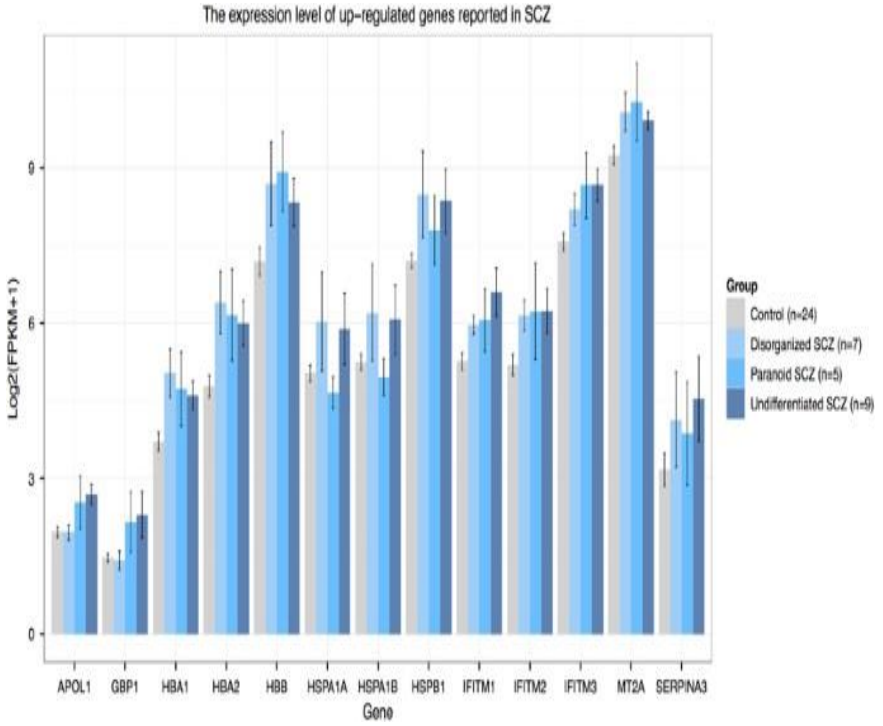


Differential expression (DE) identifies genes whose expression changes **beyond random noise** between conditions.

The goal of differential expression testing is to determine which genes are expressed at different levels between conditions.

Differential Expression





Key ideas:

- Counts vary naturally
- Replicates are essential
- Statistics separate signal from noise
- Even identical samples won't have identical counts.
- We must model **biological + technical variability**.

Count Matrix

RNA-seq quantification produces a matrix of integer read counts: rows = genes, columns = samples.

Gene	Ctrl_1	Ctrl_2	Ctrl_3	Treat_1	Treat_2	Treat_3
BRCA1	523	612	498	1247	1189	1356
TP53	3042	2987	3156	3201	3089	3245
MYC	876	923	845	412	378	445
GAPDH	15234	14876	15567	15012	14923	15345

Raw counts are influenced by technical factors and must be normalized before comparison.

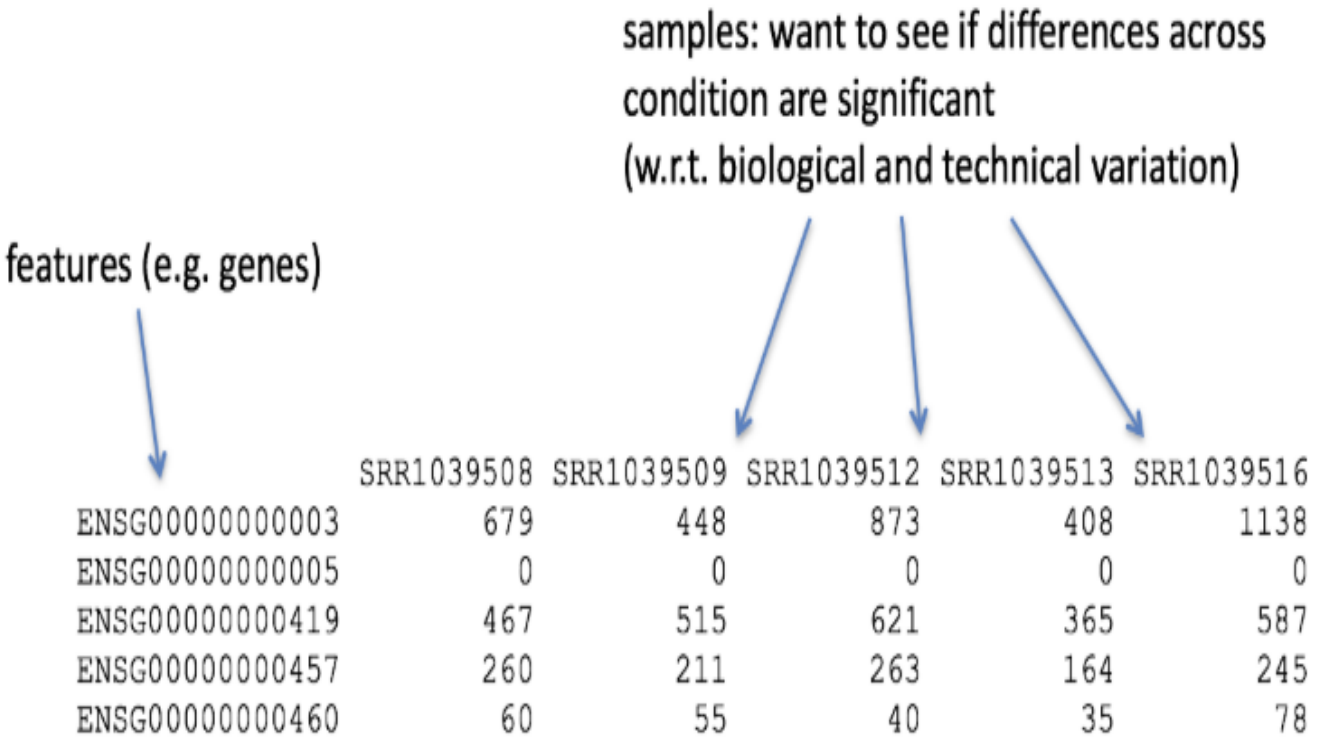
Higher counts ≠ higher expression without normalization!

Count Matrix (Chr11 count matrix)

Filter

	KO_1	KO_2	KO_3	WT_1	WT_2	WT_3
ENSG00000002330	894.001	945.225	828.000	709.835	784.982	676.000
ENSG00000005801	1112.096	1195.335	1372.418	2031.598	2640.474	1953.730
ENSG00000006071	6.976	8.123	6.126	5.052	5.156	6.898
ENSG00000006118	2359.351	2656.001	2388.003	3827.001	3935.999	3365.999
ENSG00000006534	2154.000	2106.336	2144.988	5160.683	5213.987	4869.684
ENSG00000006611	56.999	63.999	50.000	98.000	84.999	56.001
ENSG00000007372	692.885	645.840	724.059	942.793	914.968	848.283
ENSG00000011347	901.000	948.018	894.999	412.999	401.999	388.000
ENSG00000011405	5696.433	6079.254	5782.191	6658.913	7492.005	6401.650
ENSG00000013725	1.000	1.000	0.000	1.000	1.000	2.000
ENSG00000014138	1949.034	1974.479	1942.497	2357.336	2592.020	2295.673
ENSG00000014216	3296.001	3477.000	3288.059	3344.152	3406.679	3258.001
ENSG00000019102	0.000	1.000	0.000	0.000	0.000	0.000
ENSG00000019144	2172.117	2415.428	2487.230	2119.817	2210.612	1997.139
ENSG00000019485	1035.000	1096.037	966.000	1696.001	1734.000	1482.000
ENSG00000019505	4.220	10.315	11.003	4.148	2.043	2.789
ENSG00000020922	2921.648	3050.366	3095.713	4064.267	4674.470	3831.009
ENSG00000021300	1.000	0.000	0.000	0.000	2.000	0.000
ENSG00000021762	1888.003	2091.558	1903.999	2534.285	2633.878	2115.262
ENSG00000023171	3899.036	4222.015	3920.074	3614.194	3667.034	3122.008

Count Matrix cnt.



	SRR1039508	SRR1039509	SRR1039512	SRR1039513	SRR1039516
ENSG00000000003	679	448	873	408	1138
ENSG00000000005	0	0	0	0	0
ENSG00000000419	467	515	621	365	587
ENSG00000000457	260	211	263	164	245
ENSG00000000460	60	55	40	35	78

What does the count data actually represent?

The count data used for differential expression analysis represents the number of sequence reads that originated from a particular gene.

The higher the number of counts, the more reads associated with that gene, and the assumption that there was a higher level of expression of that gene in the sample.

Try to interpret me :)

	KO_1	KO_2	KO_3	WT_1	WT_2	WT_3
ENSG00000177830	149.999	141.000	144.000	1644.998	1761.001	1692.999
ENSG00000284057	43.565	33.351	0.000	245.576	263.860	277.515
ENSG00000151364	14.000	21.000	19.340	111.990	110.693	114.346
ENSG00000175592	190.000	267.000	157.000	537.001	544.999	422.000
ENSG00000165905	108.000	106.000	114.001	299.999	261.001	241.999
ENSG00000172927	44.000	45.000	31.001	74.000	111.001	106.000
ENSG00000006534	2154.000	2106.336	2144.988	5160.683	5213.987	4869.684
ENSG00000243964	329.668	477.683	1603.927	1934.557	1905.867	1830.601
ENSG00000259112	22.501	40.467	48.717	74.813	99.510	91.088
ENSG00000110514	1439.999	1575.443	1489.000	3438.018	3840.000	3066.998
ENSG00000204529	41.000	45.007	29.000	74.146	108.027	85.723
ENSG00000148926	254.000	306.001	226.001	504.001	677.000	482.001
ENSG00000214756	32.000	26.000	34.000	75.129	59.000	54.000

Data structure – Experiment Design

countData

	ctrl_1	ctrl_2	exp_1	exp_1
geneA	10	11	56	45
geneB	0	0	128	54
geneC	42	41	59	41
geneD	103	122	1	23
geneE	10	23	14	56
geneF	0	1	2	0
…	…	…	…	…
…	…	…	…	…
…	…	…	…	…

colData

	treatment	sex
ctrl_1	control	male
ctrl_2	control	female
exp_1	treatment	male
exp_2	treatment	female

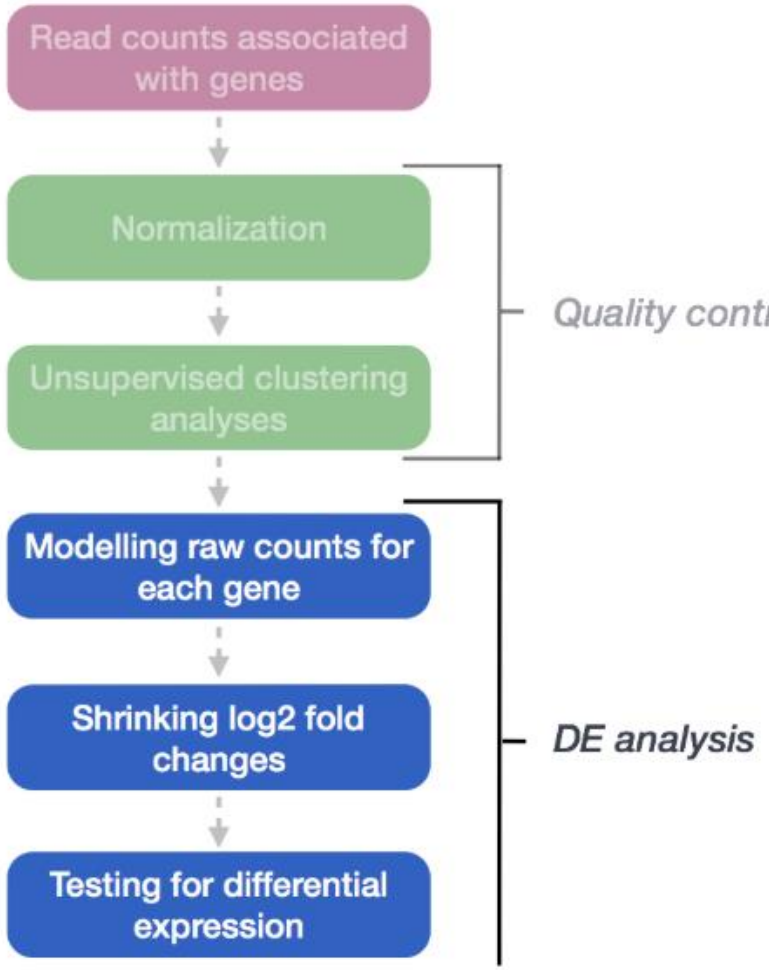
Sample names:
ctrl_1, ctrl_2, exp_1, exp_2

countData is the count matrix
(number of reads mapping to each gene for each sample)

colData describes metadata about the *columns* of countData

colnames(countData) == rownames(colData)

DEG pipeline



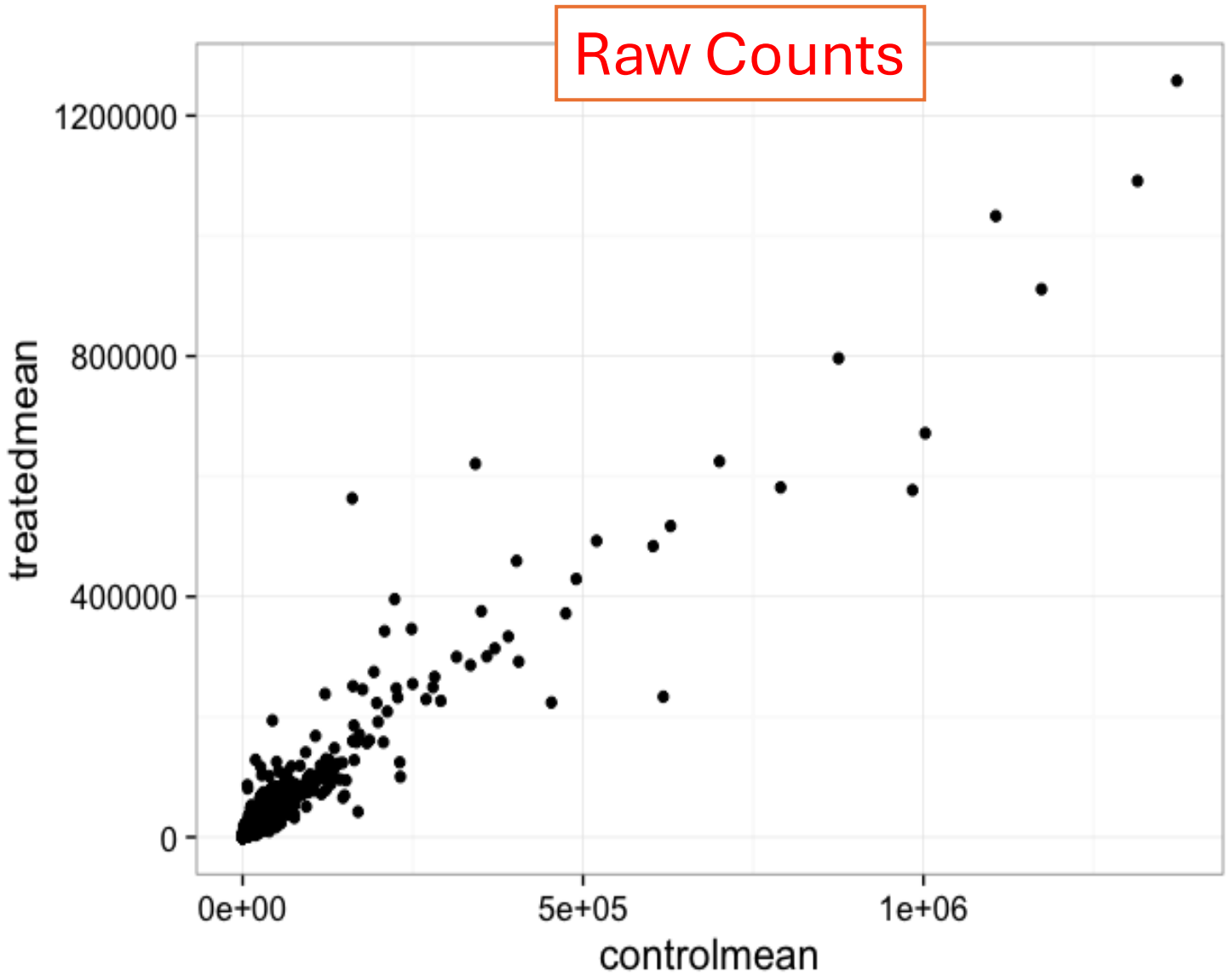
Why this steps is important?

Why do we need normalization?

Raw counts are influenced by technical artifacts that must be removed before comparing expression between samples.

Sequencing Depth	Different total reads per sample. A sample sequenced 2x deeper will have ~2x counts for every gene.
Gene Length	Longer genes capture more fragments. Important for within-sample comparisons (RPKM/TPM), NOT needed for DE of same gene across conditions.
RNA Composition	A few highly expressed genes consume disproportionate reads, making other genes appear under-expressed. Library-size normalization alone cannot fix this.

Why Are Raw Counts Not Comparable

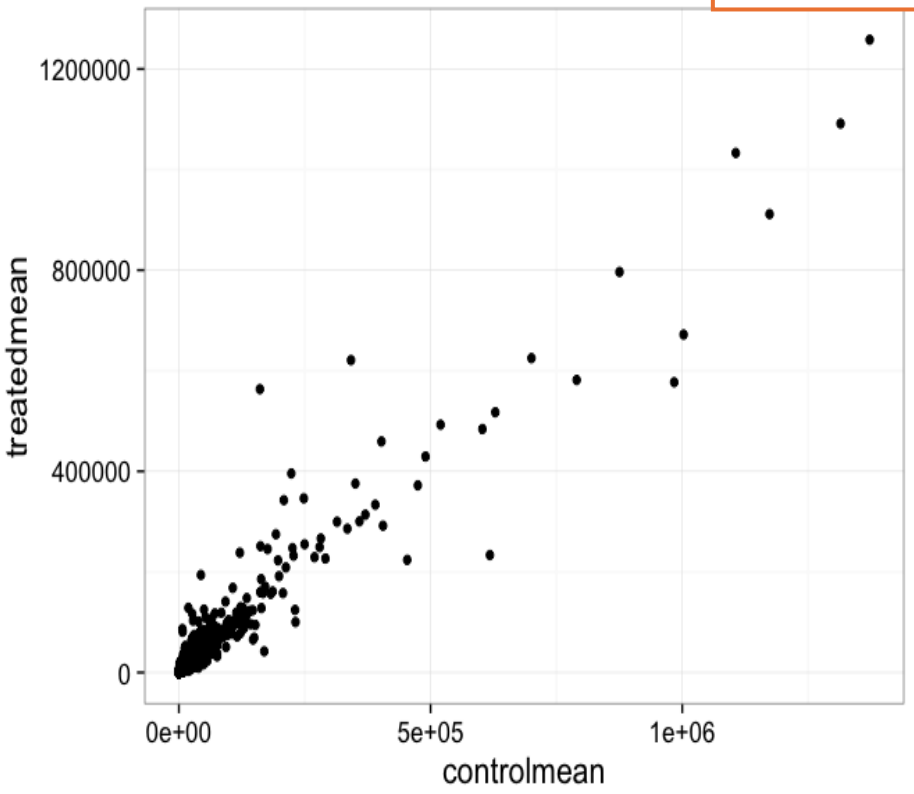


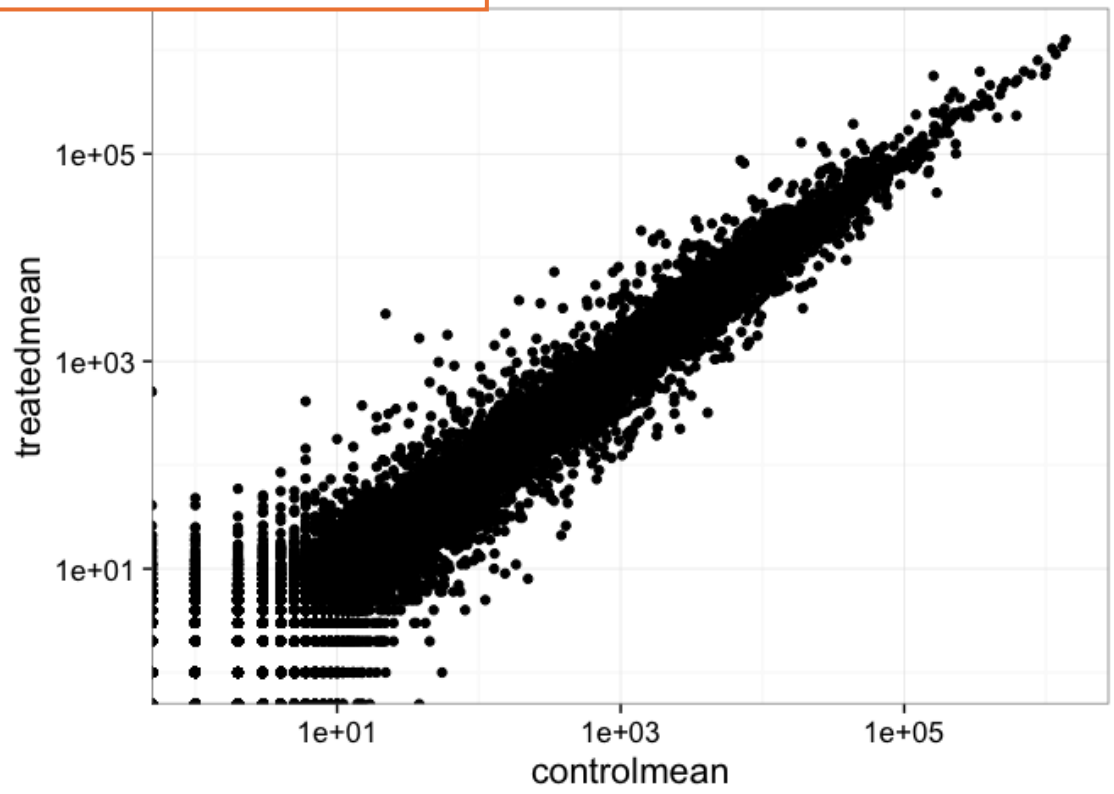
1.Create a scatter plot showing the mean of the treated samples against the mean of the control samples.

2.Wait a sec. There are 60,000-some rows in this data, but we only seeing a few dozen dots at most outside of the big clump around the origin.

Why Are Raw Counts Not Comparable

Transformed Counts



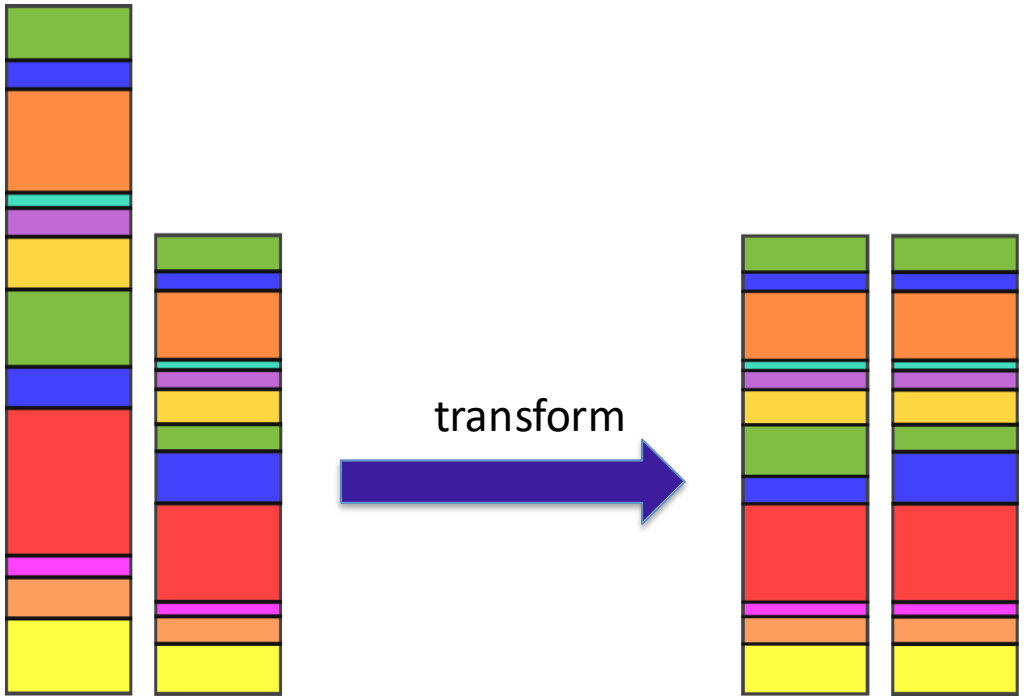


1.Try plotting both axes on a log scale (*hint*: `... + scale_..._log10( )`)

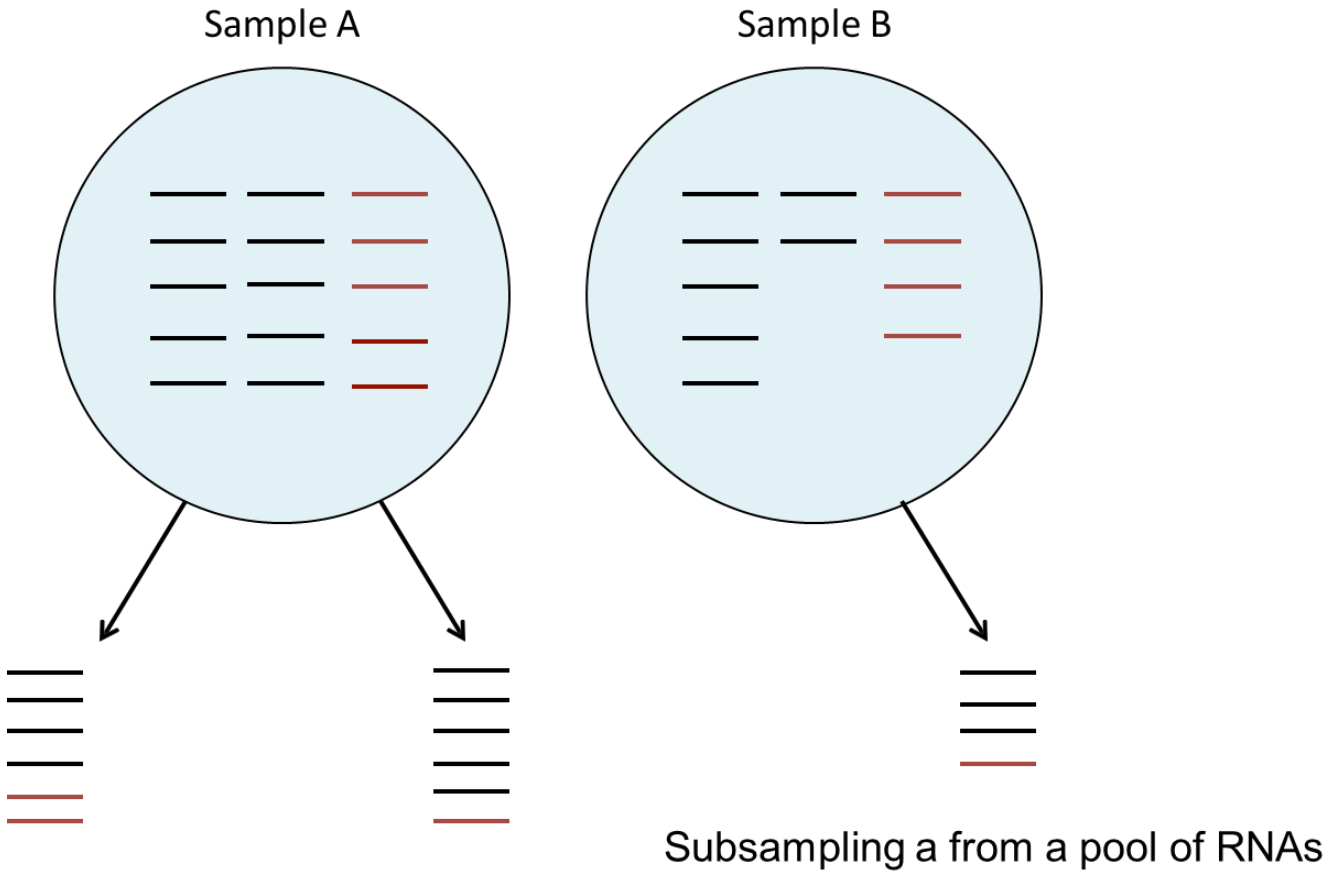
Transformation

Total Count

- Normalise each sample by total number of reads sequenced.
- Can also use another statistic similar to total count; eg. median, upper quartilex



Source of variation: Sampling Bias

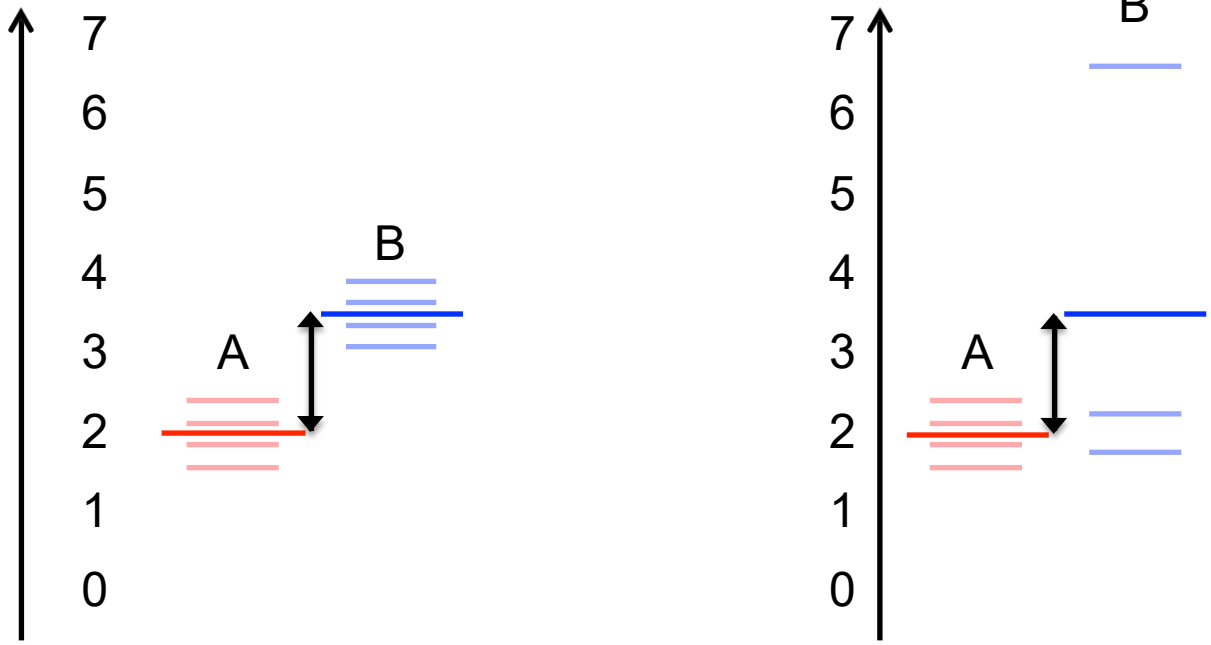


Subsampling a from a pool of RNAs

Necessary to make accurate comparisons of gene expression between samples.

Source of variation: Variations in replicates

- Simple difference in means



Differential expression (DE) identifies genes whose expression changes **beyond random noise** between conditions.

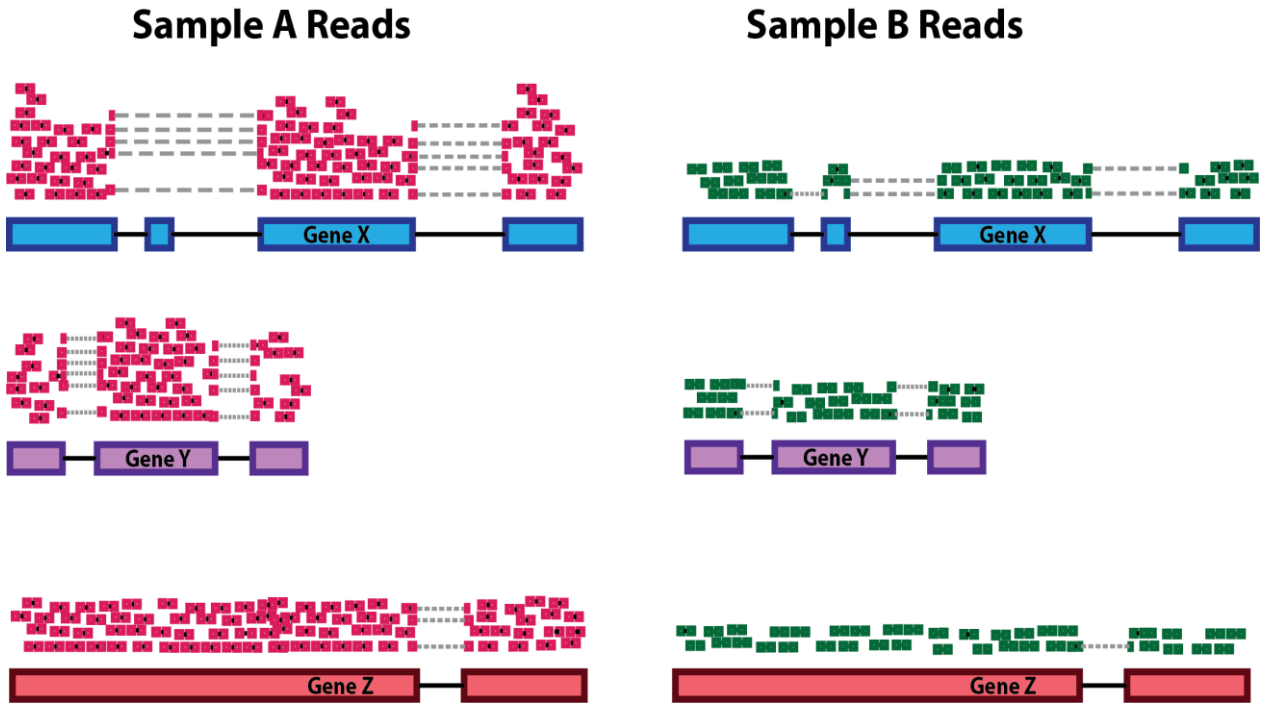
Key ideas:

- Counts vary naturally
- Replicates are essential
- Statistics separate signal from noise

- Replication introduces variance

Source of variation: Sequencing Depth

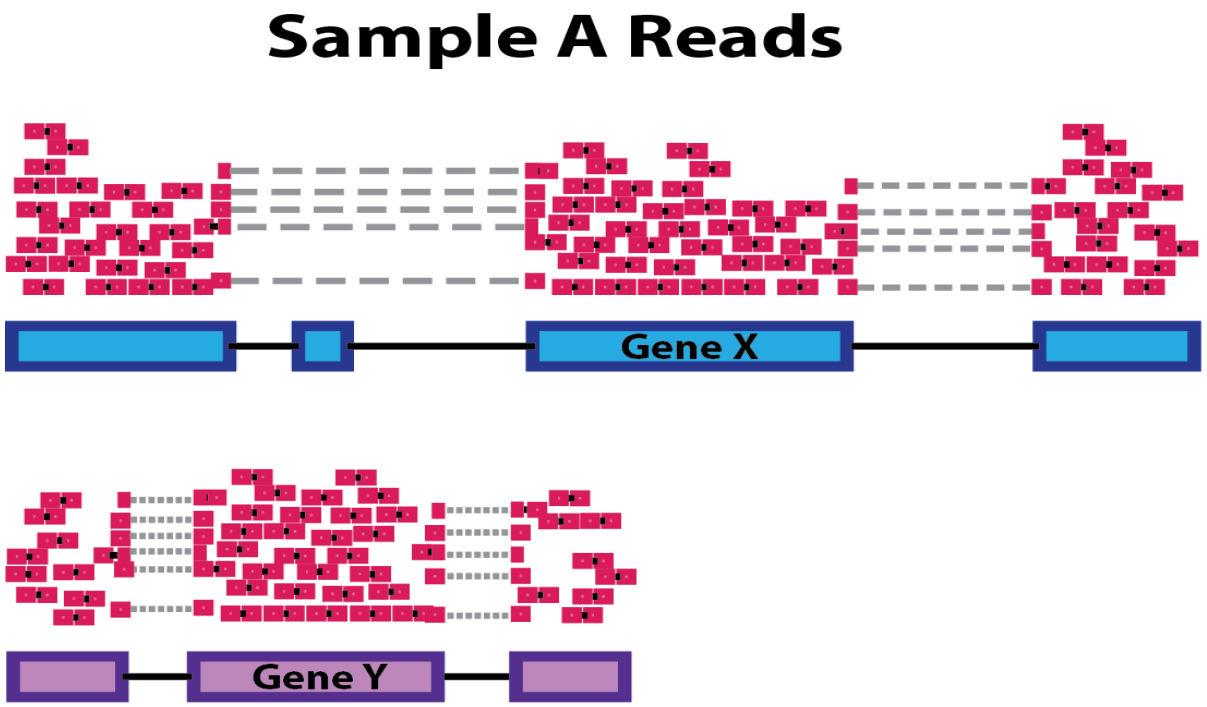
The main factors often considered during normalization are:



1. **Sequencing depth:** Accounting for sequencing depth is necessary for comparison of gene expression between samples. In the example below, each gene appears to have doubled in expression in *Sample A* relative to *Sample B*, however this is a consequence of *Sample A* having double the sequencing depth.

Source of variation: Gene Length

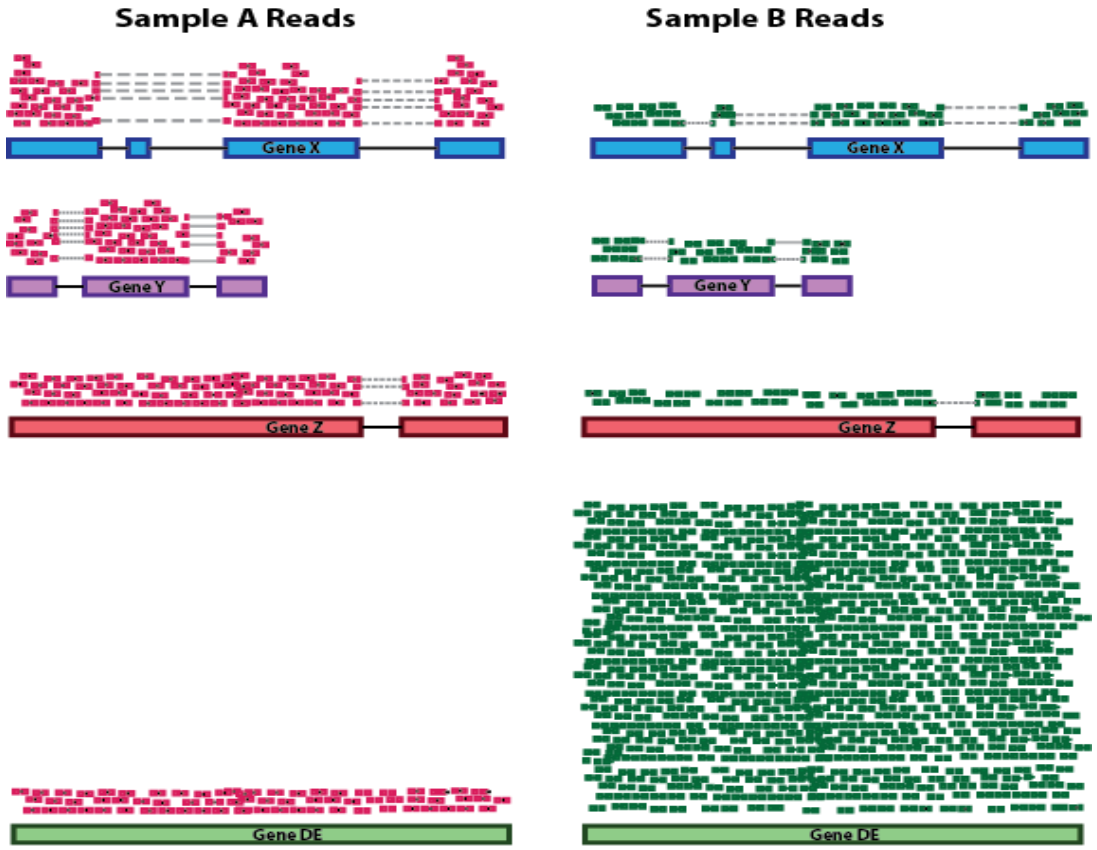
The main factors often considered during normalization are:



2. Gene length:
Accounting for gene length is necessary for comparing expression between different genes within the same sample. In the example, *Gene X* and *Gene Y* have similar levels of expression, but the number of reads mapped to *Gene X* would be many more than the number mapped to *Gene Y* because *Gene X* is longer.

Source of variation: RNA Composition

The main factors often considered during normalization are:



3. RNA composition: A few highly differentially expressed genes between samples, differences in the number of genes expressed between samples, or presence of contamination can skew some types of normalization methods. Accounting for RNA composition is recommended for accurate comparison of expression between samples, and is particularly important when performing differential expression analyses.

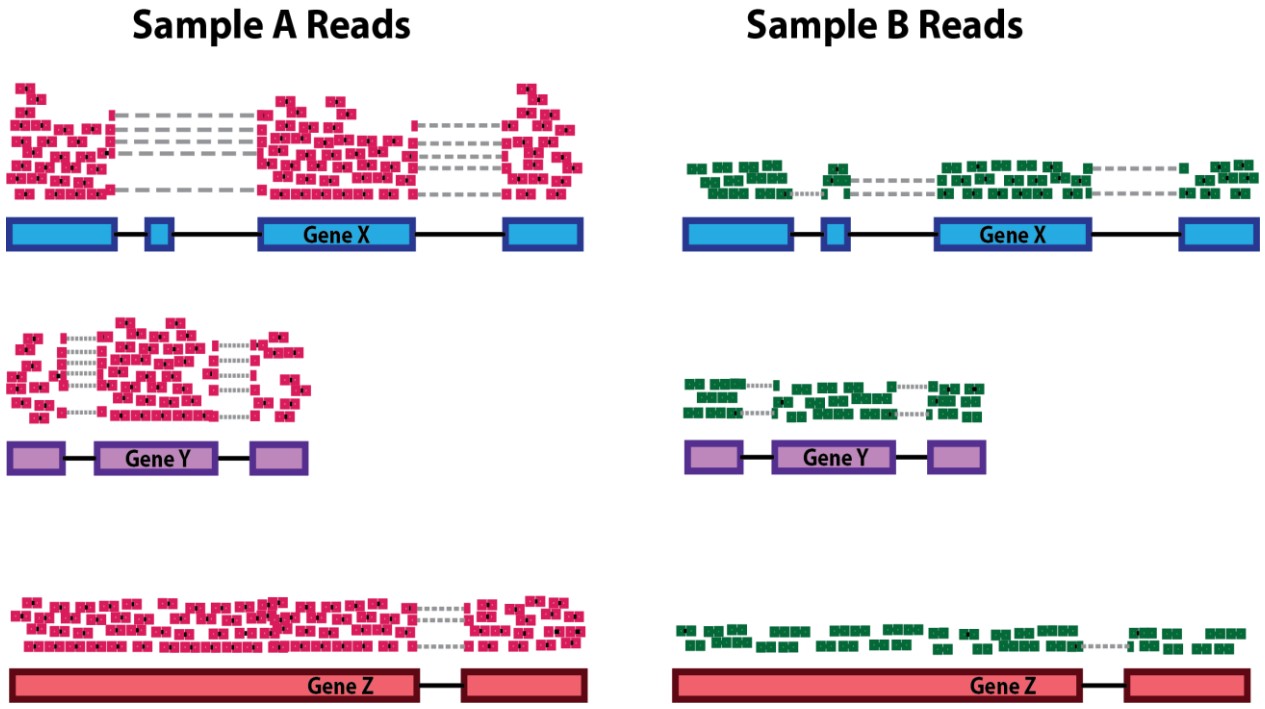
Normalization Methods Comparison

Method	Accounts For	Suitable For	Limitations
CPM	Sequencing depth	Quick exploratory analysis	Ignores RNA composition
RPKM / FPKM	Depth + gene length	Within-sample comparisons	Not comparable across samples
TPM	Depth + gene length	Cross-sample gene comparison	No RNA composition correction
DESeq2 (Median of Ratios)	Depth + RNA composition	Differential expression	Assumes most genes not DE
TMM (edgeR)	Depth + RNA composition	Differential expression	May over-trim in extremes

For DE analysis, use DESeq2 or TMM — NOT RPKM/FPKM/TPM
For Visualization, use TPM

Normalization: Counts per million (CPM)

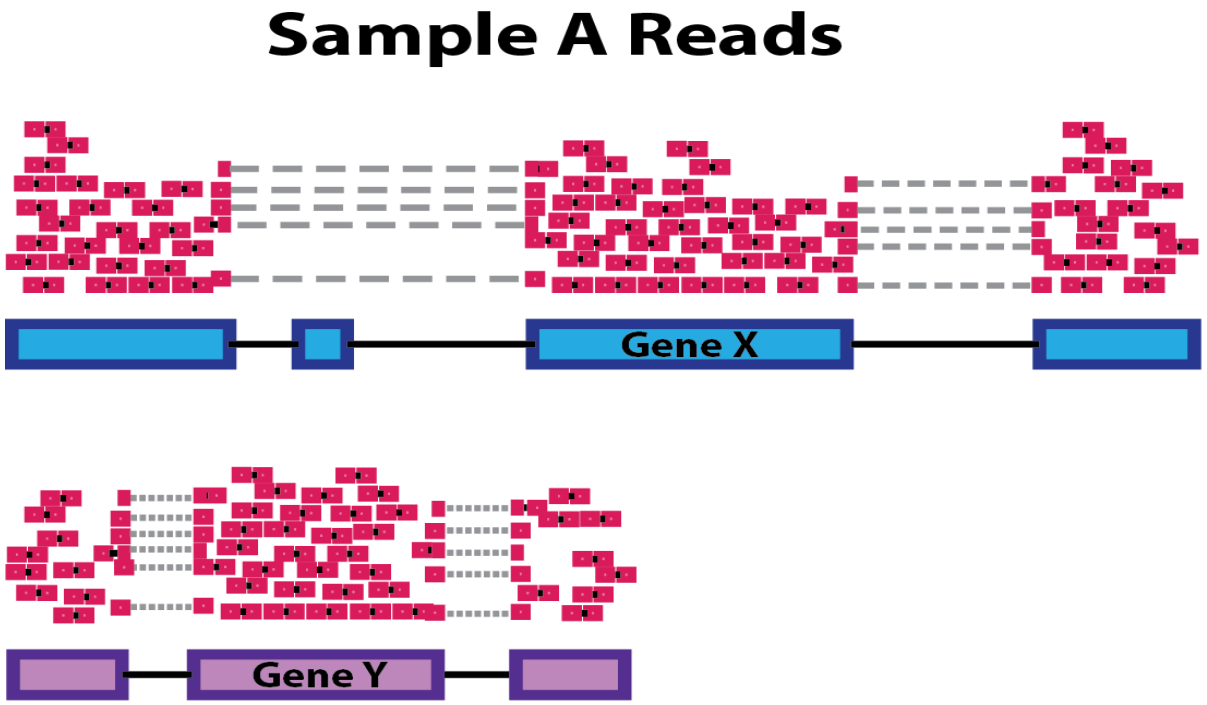
The main factors often considered during normalization are:



$$\text{CPM} = \left(\frac{\text{Raw Counts}}{\text{Total Mapped Reads}} \right)$$

- Counts per million (CPM) mapped reads are the number of raw reads mapped to a transcript, scaled by the number of sequencing reads in your sample, multiplied by a million.
- It normalizes RNA-seq data for sequencing depth but not gene length.
- Therefore, although it is a within sample normalization approach, CPM normalization is unsuitable for within sample comparisons of gene expression.
- Between sample comparisons can be made when CPMis used alongside ‘within a dataset’ normalization methods.

Normalization: Transcripts per Million (TPM)



The calculation involves two main steps, often described as normalizing for gene length first, then sequencing depth:

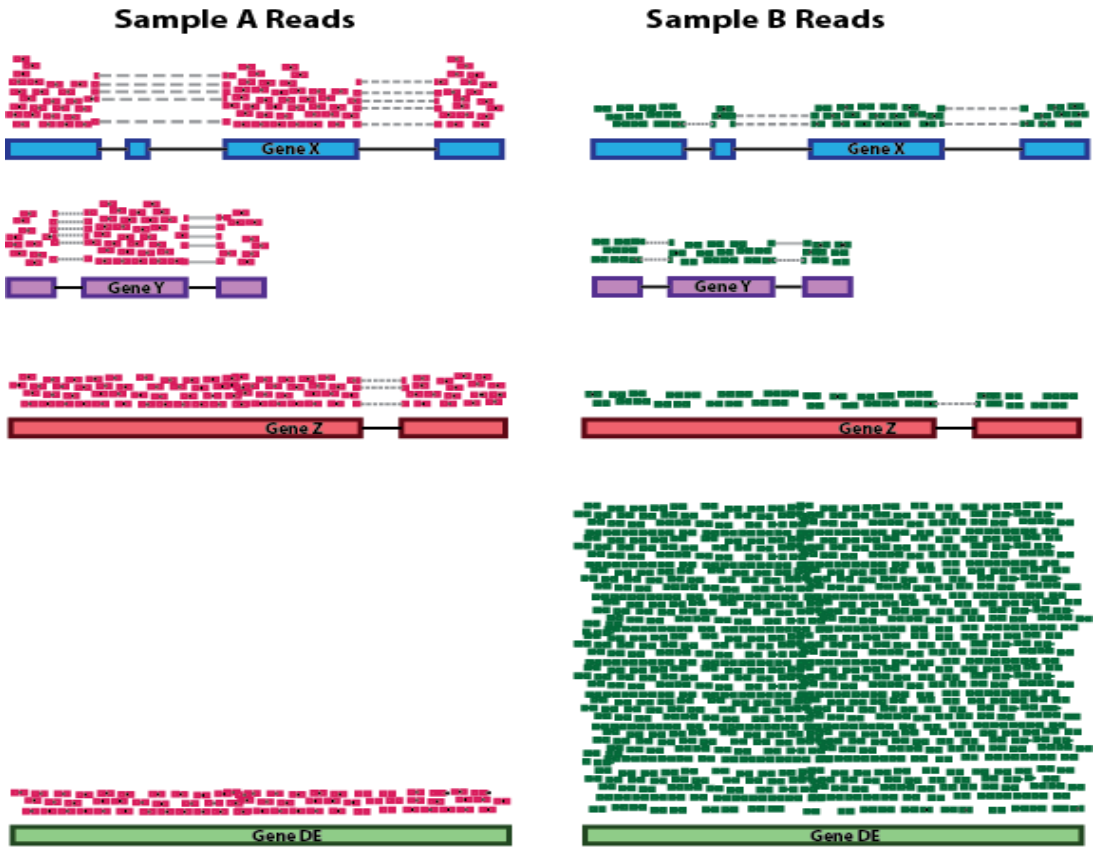
1. **Calculate Reads Per Kilobase (RPK)** for each gene (i):

$$\mathrm{RPK}_i = \frac{\text{Mapped Reads}_i}{\text{Length of gene } i \text{ in kb}}$$

2. **Calculate TPM** for gene (i):

$$\mathrm{TPM}_i = \left(\frac{\mathrm{RPK}_i}{\sum_j \mathrm{RPK}_j} \right) \times 10^6$$

Normalization: RPKM



RPKM

- Reads per kilobase per million =

$$\frac{\text{reads for gene } A}{\text{length of gene } A \text{ X Total number of reads}}$$

Oshlack, A. & Wakefield, M.J. (2009) *Biology Direct*

Normalization: DESeq2: Median of Ratios Normalization

DESeq2 uses the "median of ratios" method (Anders & Huber, 2010), accounting for sequencing depth and RNA composition.

1. **Pseudo-reference**
   For each gene, compute geometric mean across all samples = pseudo-reference.

2. **Calculate ratios**
   For each gene per sample: ratio = count / geometric_mean. Most genes aren't DE, so ratios reflect technical scaling.

3. **Median = size factor**
   For each sample, take median of all gene ratios. This is the size factor (s_j), robust to DE outliers.

4. **Normalize**
   Divide each raw count by its sample's size factor: normalized_ij = count_ij / s_j

Key Assumption: The majority of genes are NOT differentially expressed between conditions.

Normalization answers:

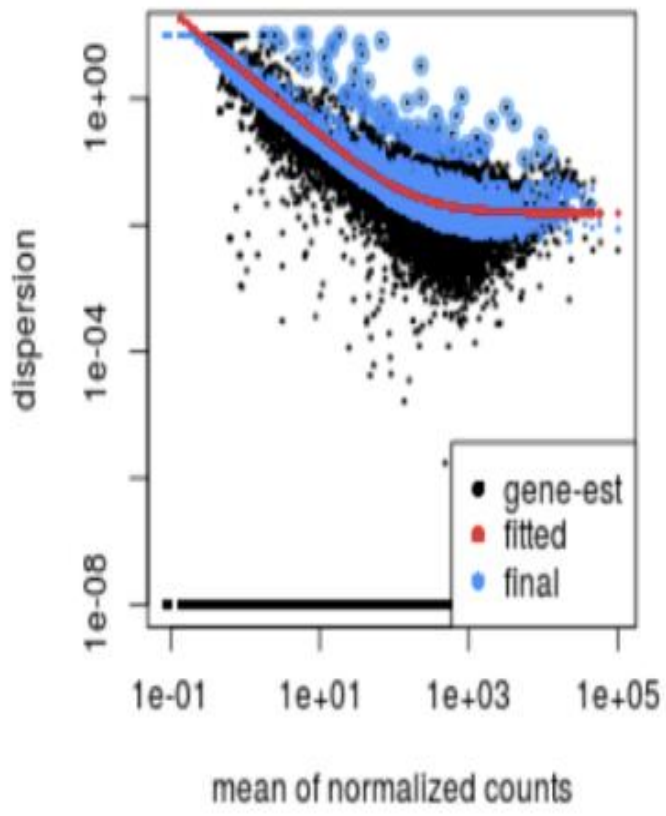
“Are samples comparable?”

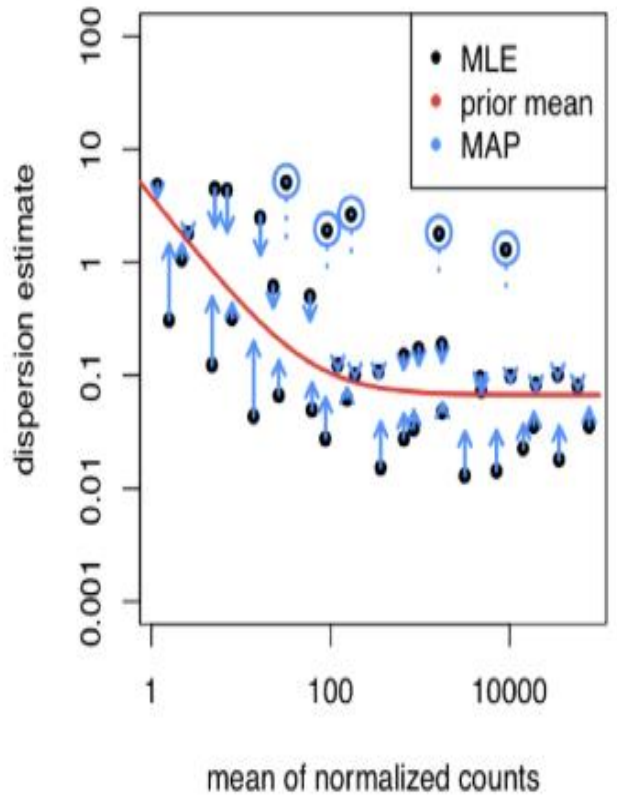
Differential expression answers:

“Which genes actually change between conditions?”

“Now the data are comparable… how do we detect biology?”

We Need Special Statistical Models

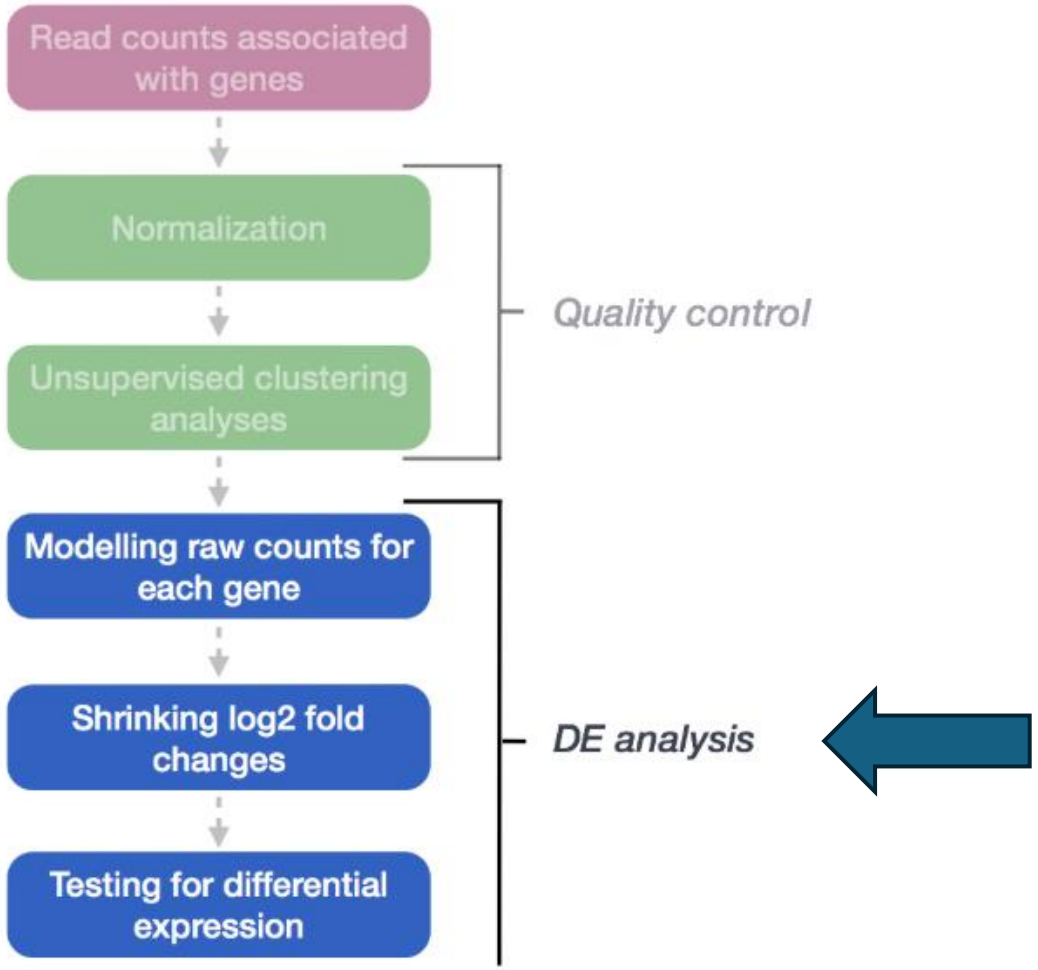




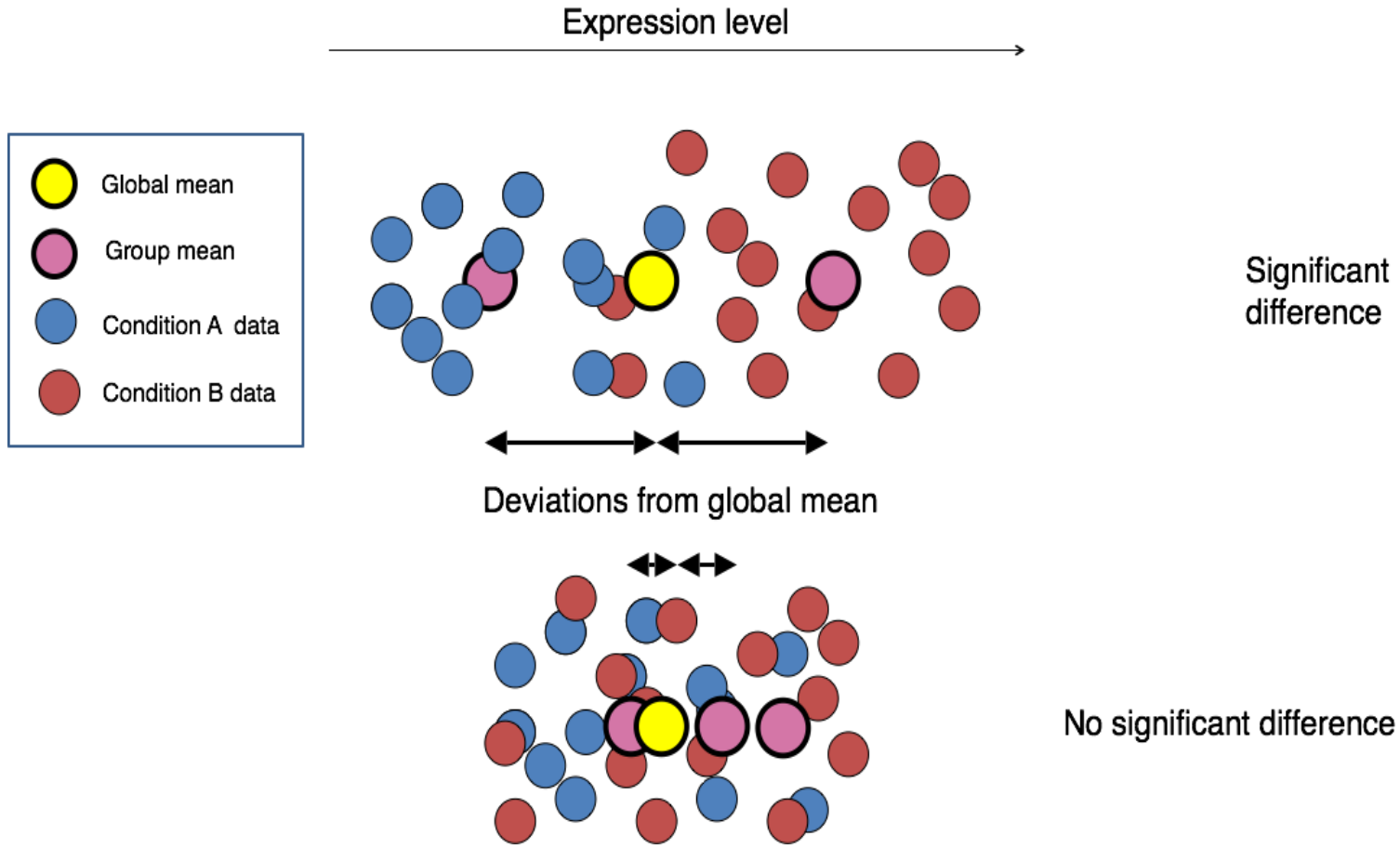
RNA-seq data

- Discrete counts
- Over-dispersed
- Not normally distributed

DEG pipeline



Differential expression analysis with DESeq2



Model RNA-seq count data using a Negative Binomial distribution to account for biological variability, then statistically test each gene to determine whether its expression differs significantly between experimental conditions.

We model how RNA-seq counts behave statistically.

Confounding factors: complex design

	sex	age	litter	treatment	treat_sex
sample1	M	11	1	Ctrl	CtrlM
sample2	M	13	2	Ctrl	CtrlM
sample3	M	11	1	Treat	TreatM
sample4	M	13	1	Treat	TreatM
sample5	F	11	1	Ctrl	CtrlF
sample6	F	13	1	Ctrl	CtrlF
sample7	F	11	1	Treat	TreatF
sample8	F	13	2	Treat	TreatF

DESeq2 also allows for the analysis of complex designs.

Example: we can explore the effect of sex on the treatment

```
design <- ~ sex + age + treatment + sex:treatment
```

Key Considerations for DE Analysis

Biological replicates essential	At least 3 per condition. Technical replicates do not substitute.
Use raw integer counts	Never use TPM/FPKM as input. DE tools normalize internally.
Filter lowly expressed genes	Remove genes with <10 total counts. Reduces testing burden.
Check for batch effects	Include batches in design formula. Use PCA to detect confounders.
Do NOT use fold-change alone	Large FC without stats is meaningless. Always report padj.
Do NOT use t-tests on counts	Violates normality. Use NB-based or voom-transformed methods.

Interpreting DE Results Table

Understanding each column of the DESeq2 results table is critical for proper interpretation.

Column	Description
`baseMean`	Average normalized count across all samples. Overall expression level.
`log2FoldChange`	Effect size. Positive = upregulated. log2FC=1 means 2-fold change.
`lfcSE`	Standard error of log2FC. Larger for lowly expressed genes.
`stat`	Wald statistic = log2FC / lfcSE. Distance from zero in SE units.
`pvalue`	Raw p-value. NOT corrected for multiple testing.
`padj`	BH-adjusted p-value (FDR). USE THIS for calling DE. Threshold: <0.05.

Typical DE criteria: padj < 0.05 AND |log2FC| > 1 (2-fold change)

DESeq2: Size of change: Log2FC

$$\log2FC = \log_2\left(\frac{\text{Expression in Condition B}}{\text{Expression in Condition A}}\right)$$

Gene	baseMean	log2FC	SE	p-value	FDR
GeneA	120	1.5	0.3	1e-6	1e-4
GeneB	15	−0.8	0.6	0.12	0.4

DESeq2 is an R package for analyzing count-based NGS data like RNA-seq.

Input

- Raw count matrix

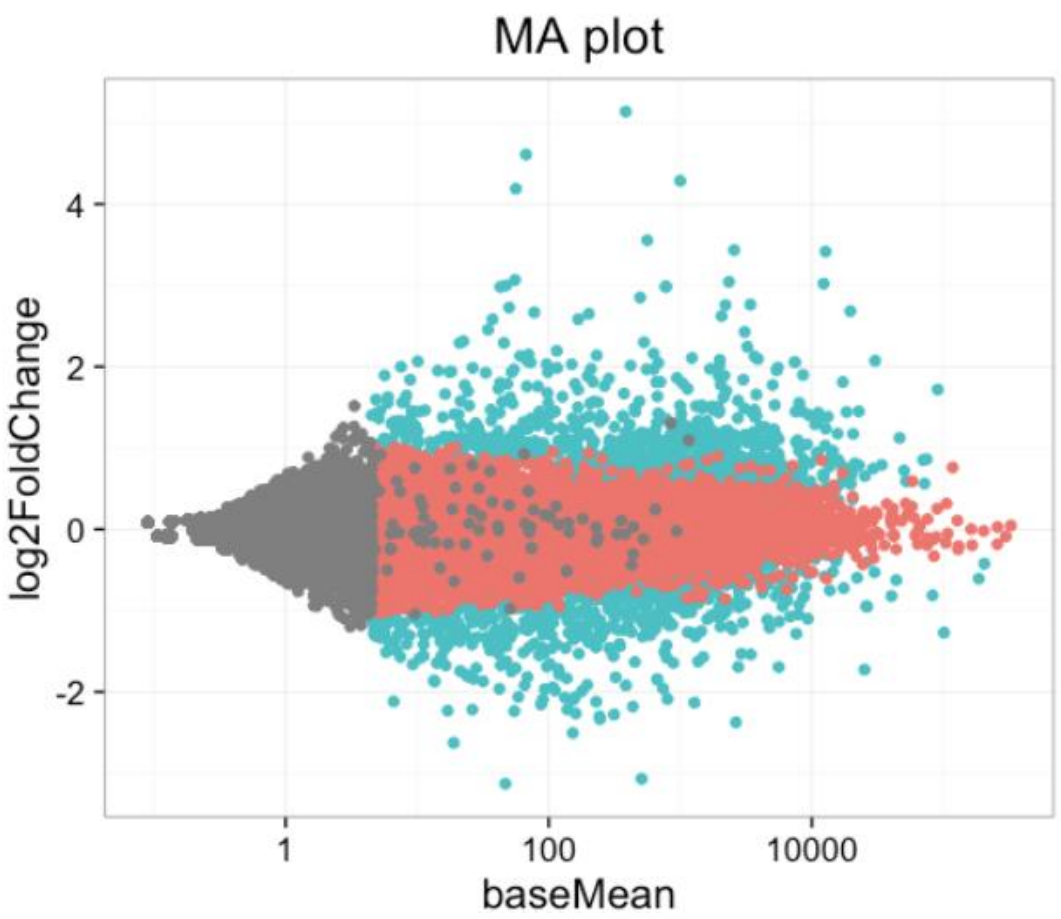
Output

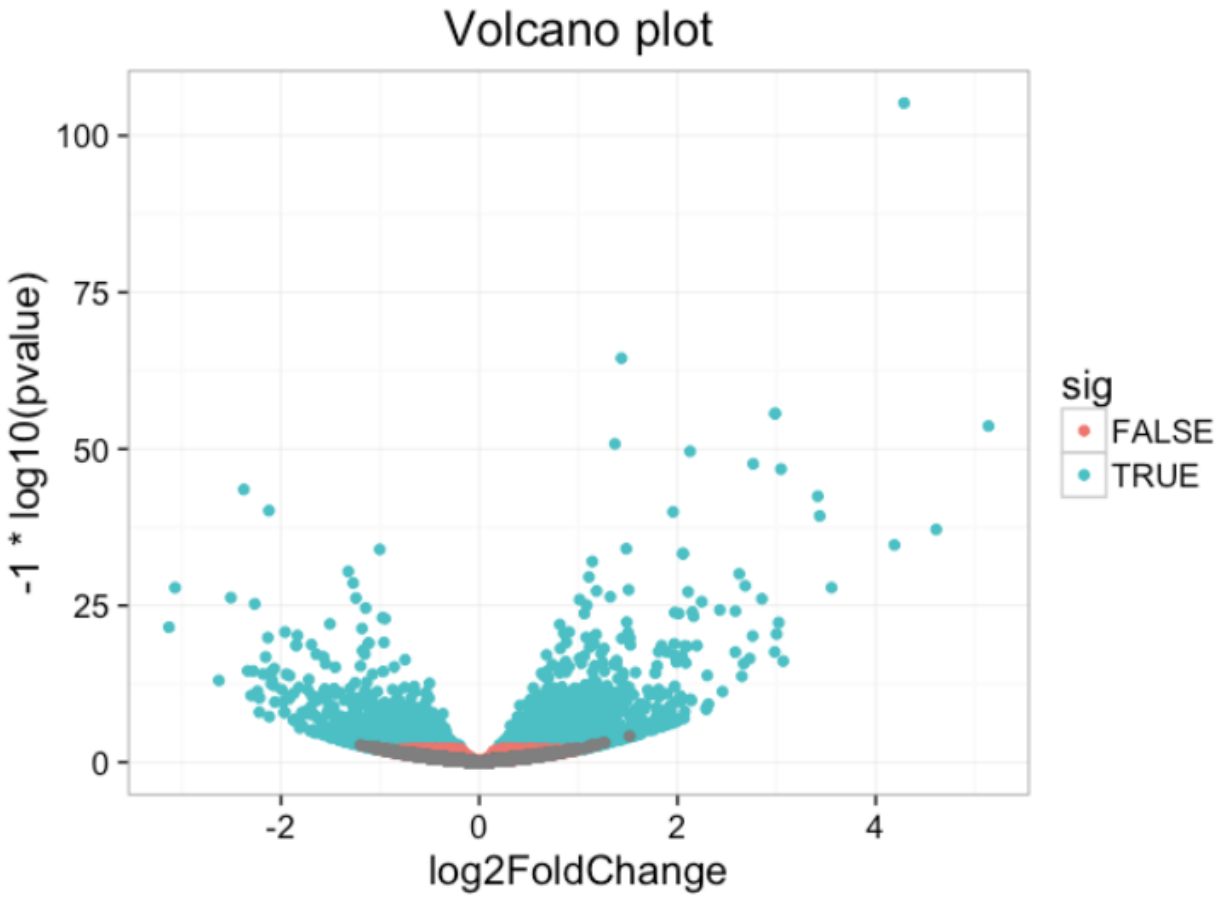
- Log2 Fold Change(effect size)
- p-value (statisticalevidence)

A gene is considered DE if:

- **|log2FC| ≥ 1**
- **padj ≤ 0.05**

Visualize the result:





Agenda – Day 03

أكاديمية كاوست
KAUST ACADEMY

Morning Session

9-9:15	S1: Recap of Day 2
9:15-10	S2: Differential Expression Concepts
10-11	*L1:* Perform differential expression analysis, inspect DE results
11-11:30	S3: Visualization & Reporting
11:30-12	L2: Visualization & Integrated QC

Afternoon Session

2-2:45	S4: Pathway & Enrichment Analysis
2:45-3:30	*L3: Hands-on with Transcriptomics Data*
3:30-4	S5: Extended Applications of RNA-seq
4-5	S6: Team Formation & Project Overview