Quantitative Molecular Neurogenomics (ME.440.825)

Module 6 :

Practical RNA-seq workflow & Enrichment analysis

10/10/2022

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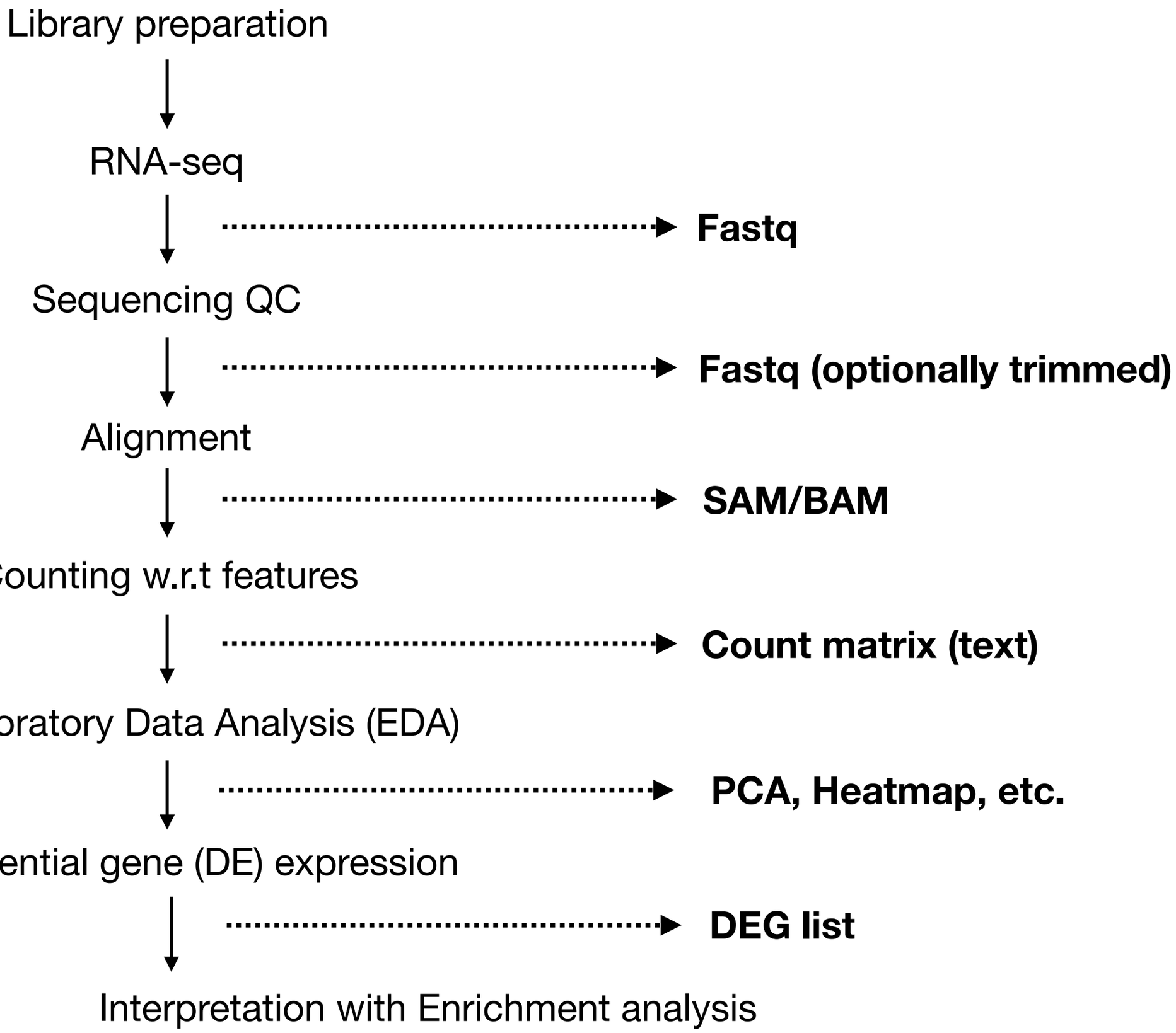
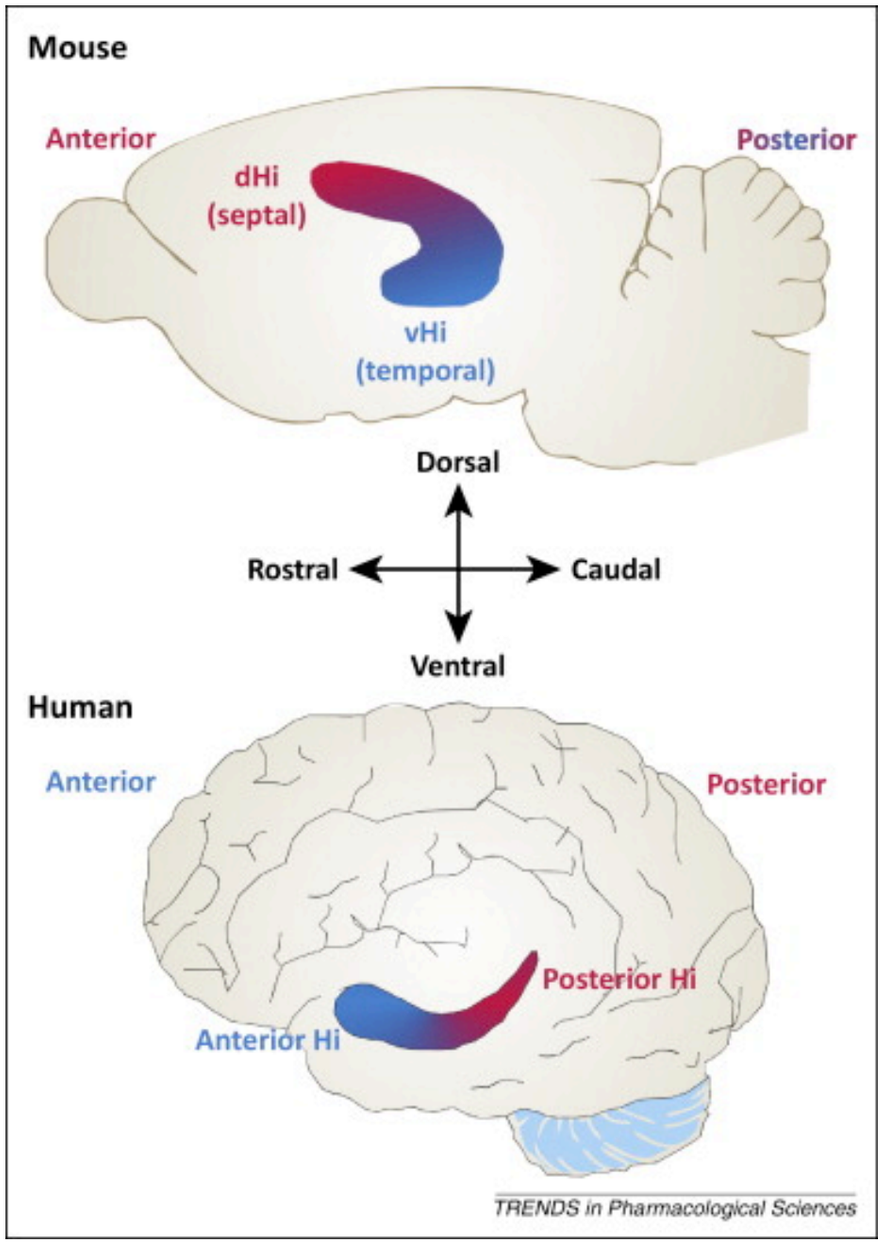
Lieber Institute for Brain Development,
Department of Neurology and Department of Neuroscience

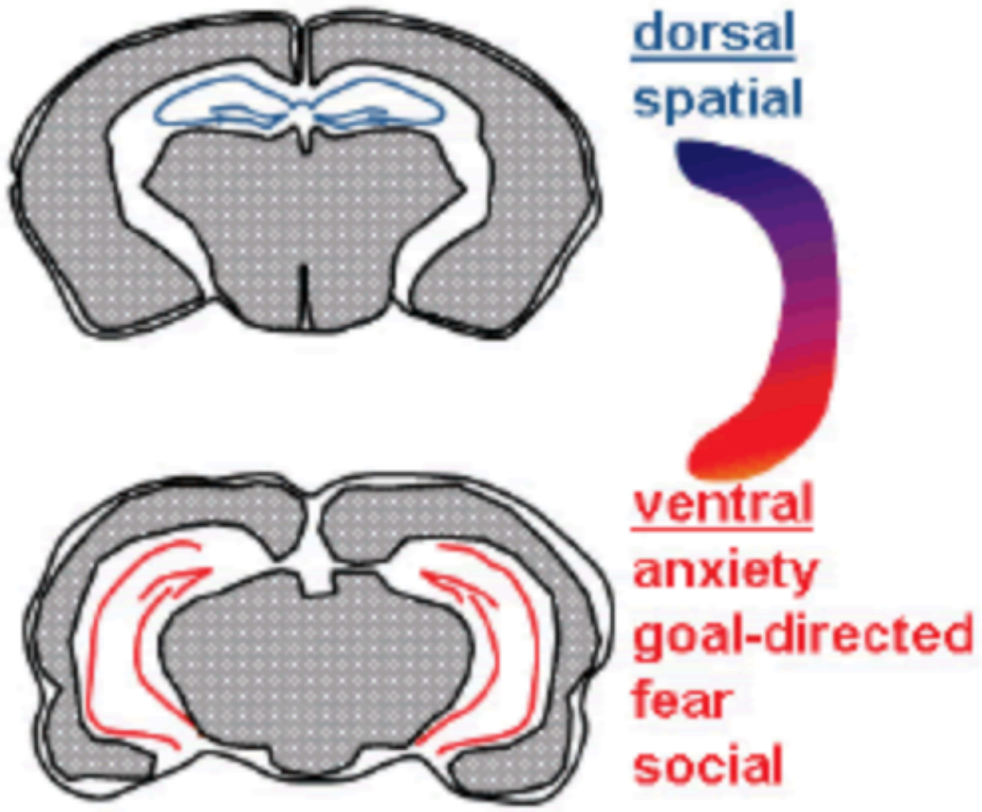
Typical RNA-seq workflow



Sample: mouse hippocampus tissue



The longitudinal axis of the hippocampus



Trends in Pharmacological Sciences, Volume 35, Issue 12, December 2014, 675-687
J Alzheimers Dis Parkinsonism 2018, Vol 8(1): 412

Dataset: RNA-seq of mouse hippocampus

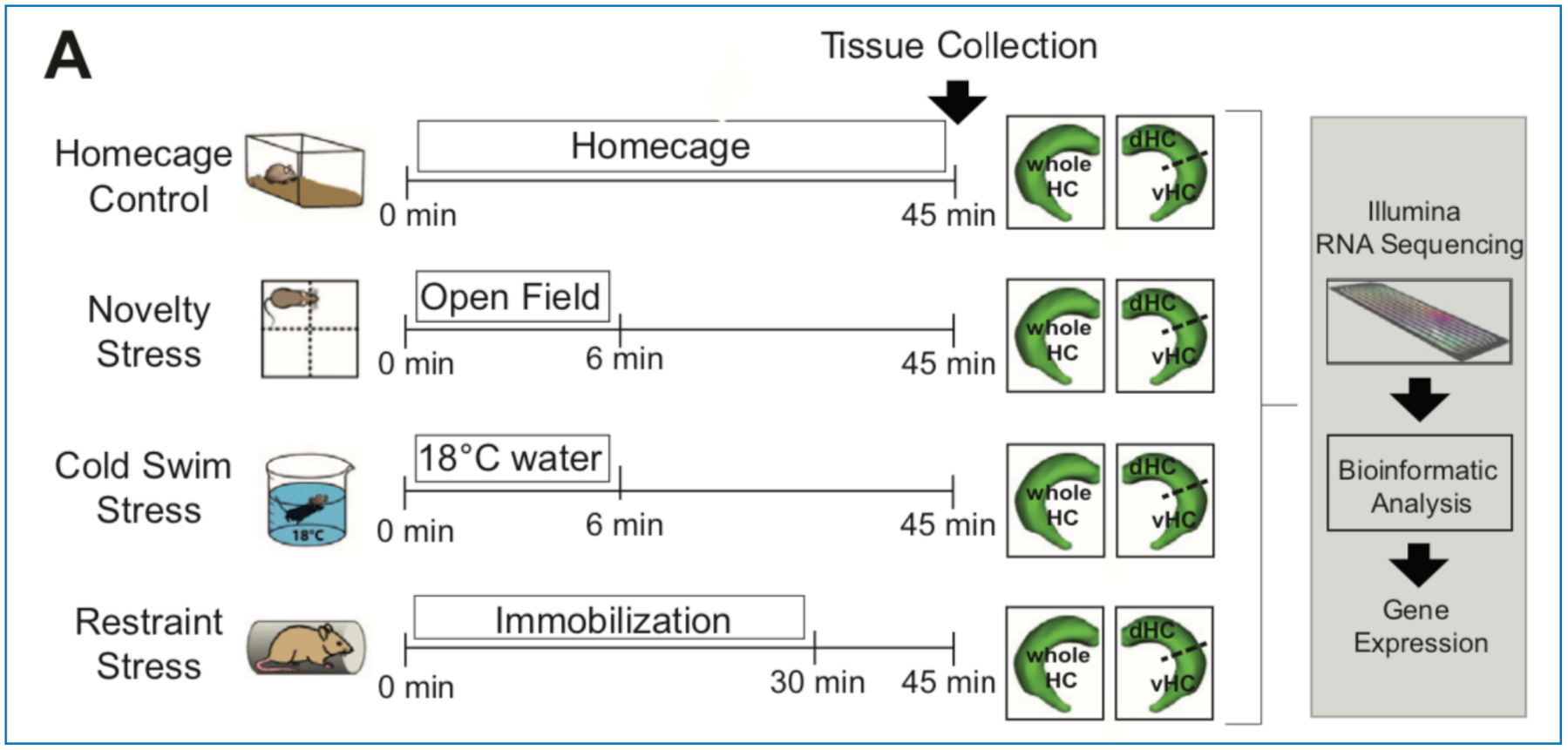
Biological Psychiatry October 1, 2018; 84:531–541

Archival Report

Distinct Proteomic, Transcriptomic, and Epigenetic Stress Responses in Dorsal and Ventral Hippocampus

Amalia Floriou-Servou, Lukas von Ziegler, Luzia Stalder, Oliver Sturman, Mattia Privitera, Anahita Rassi, Alessio Cremonesi, Beat Thöny, and Johannes Bohacek

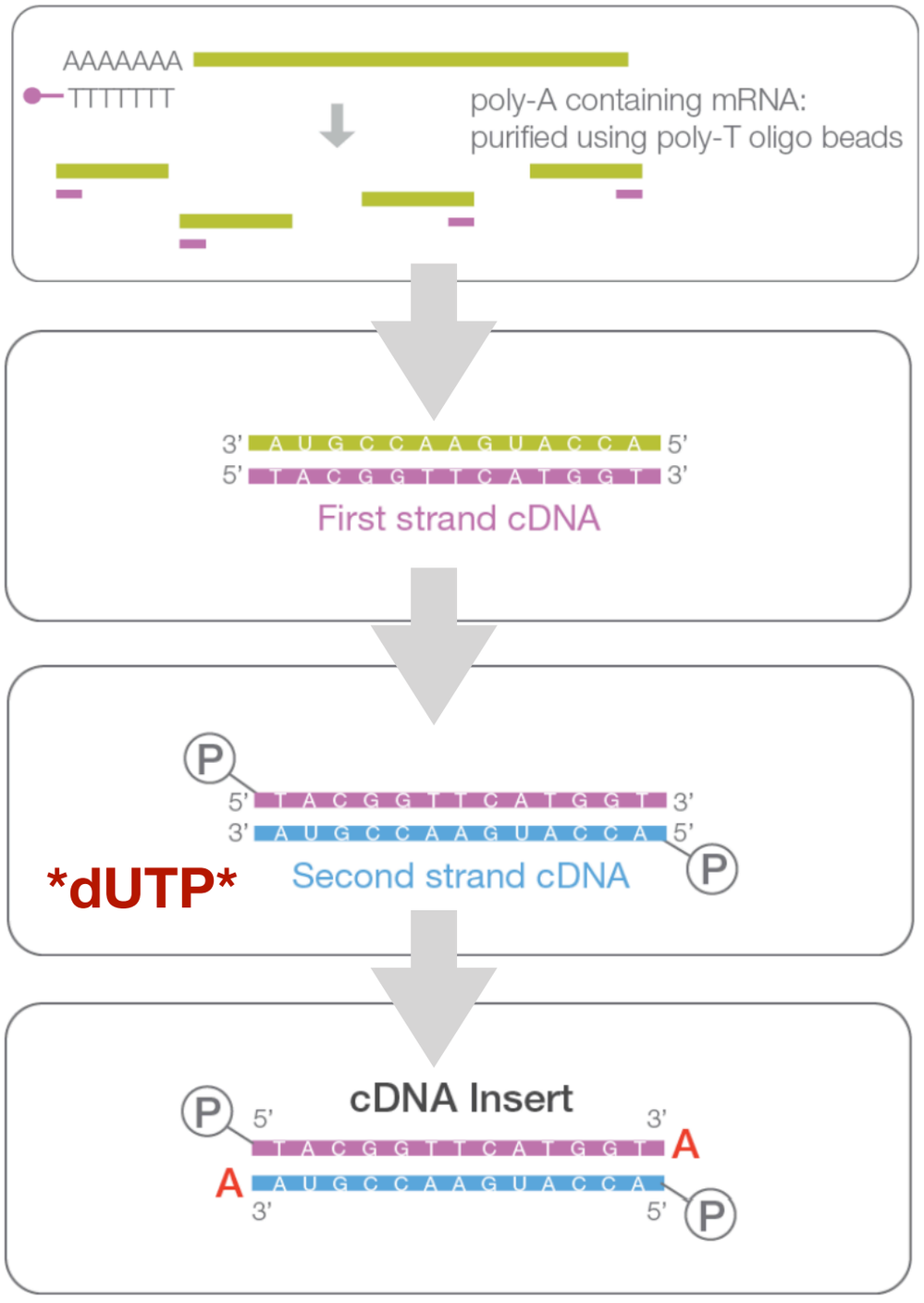
	Sid	Region	Replicate
2	SRR5715043	Dorsal	Rep1
3	SRR5715044	Dorsal	Rep2
4	SRR5715045	Dorsal	Rep3
5	SRR5715063	Ventral	Rep1
6	SRR5715064	Ventral	Rep2
7	SRR5715065	Ventral	Rep3

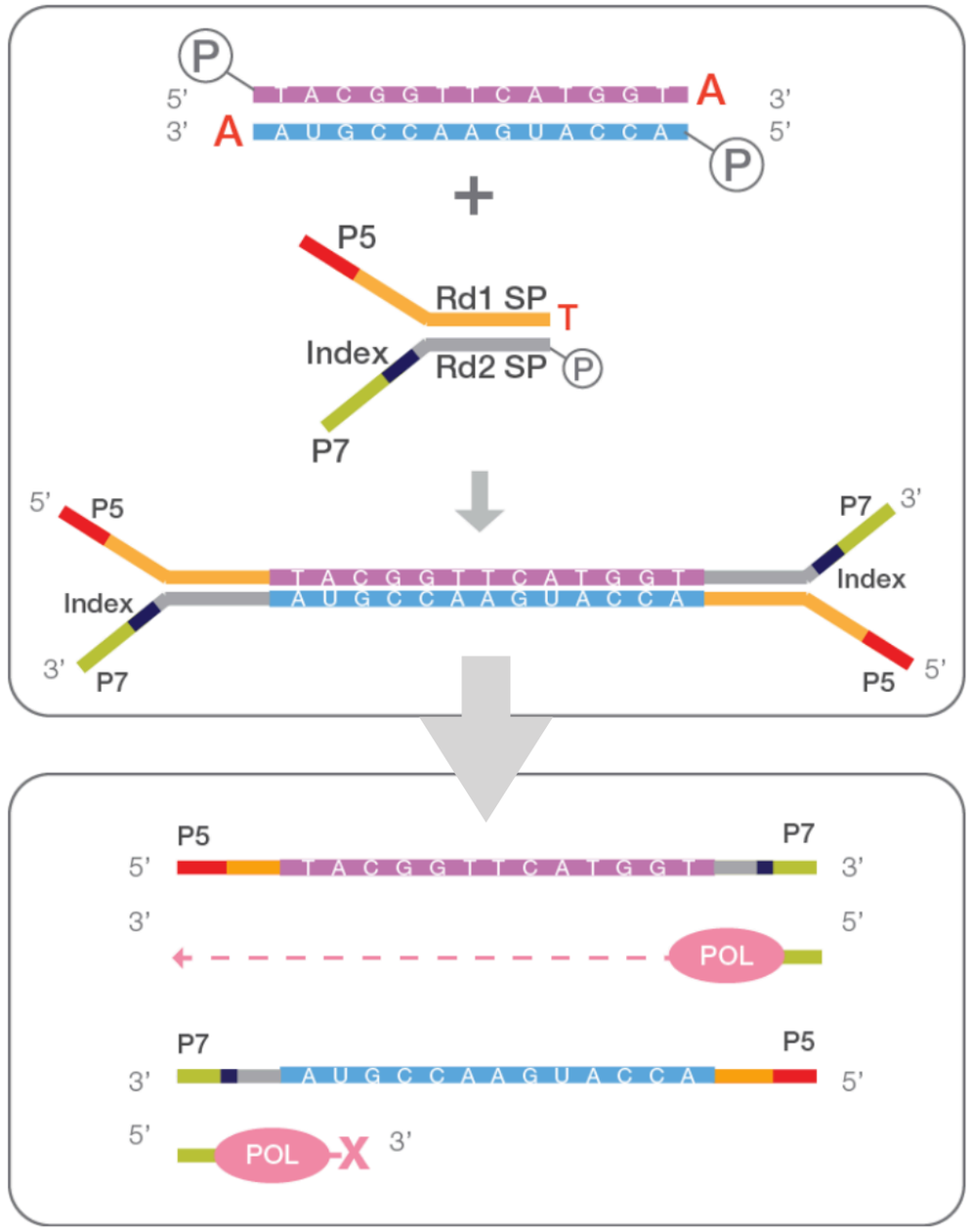


RNA-seq library preparation

Supplementary Method

"For library preparation, the TruSeq stranded RNA kit (Illumina Inc.) was used according to the manufacturer's protocol."





Illumina TruSeq Stranded mRNA Reference Guide Document # 1000000040498 v00

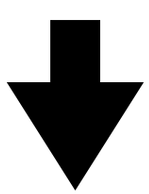
Sequencing QC using Fastqc

```
$ fastqc SRR5715043.fastq.gz
```

FastQC Report

Summary

- Basic Statistics
- Per base sequence quality
- Per sequence quality scores
- Per base sequence content
- Per sequence GC content
- Per base N content
- Sequence Length Distribution
- Sequence Duplication Levels
- Overrepresented sequences
- Adapter Content



No worries

Basic Statistics

Measure	Value
Filename	SRR5715043.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	36027995
Sequences flagged as poor quality	0
Sequence length	126
%GC	49

Per base sequence quality

Fastqc html report: an example

Summary

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Basic Statistics

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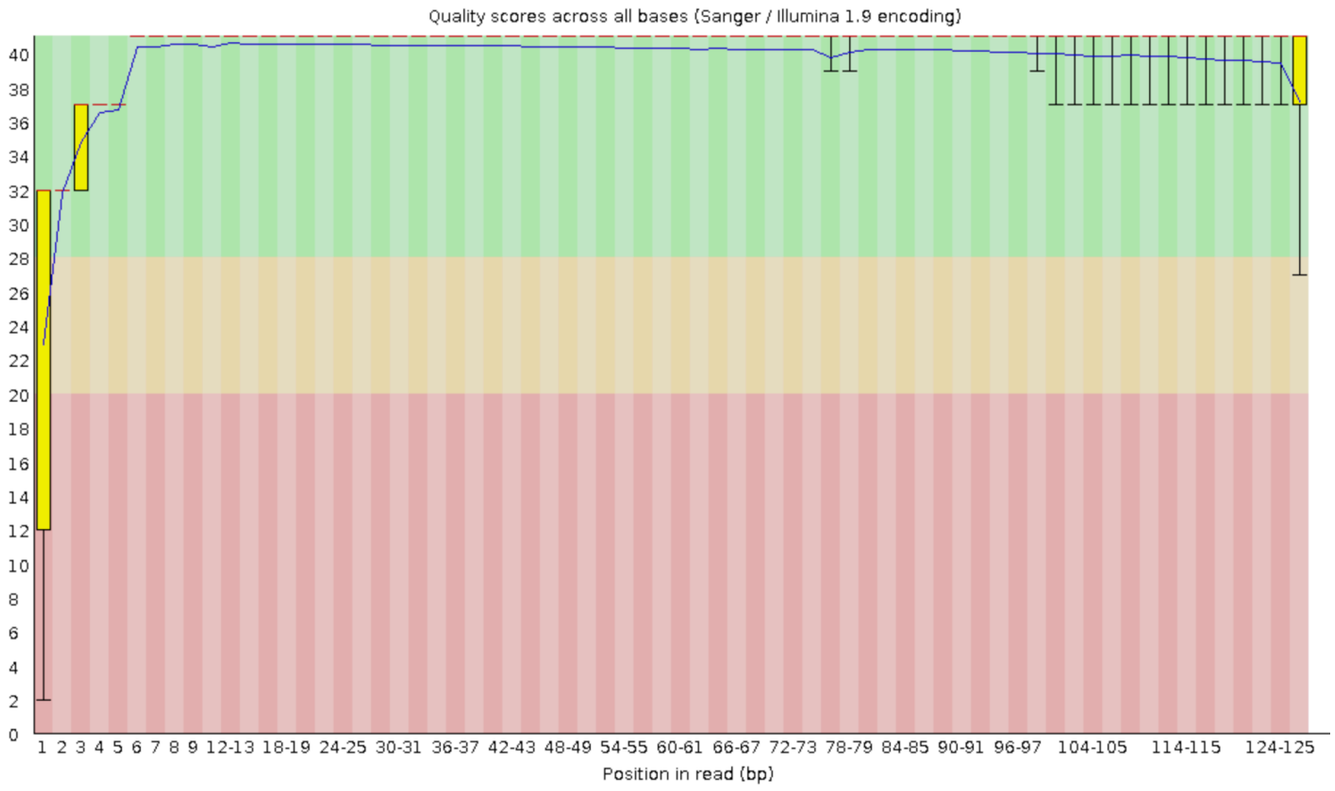
GC % in species

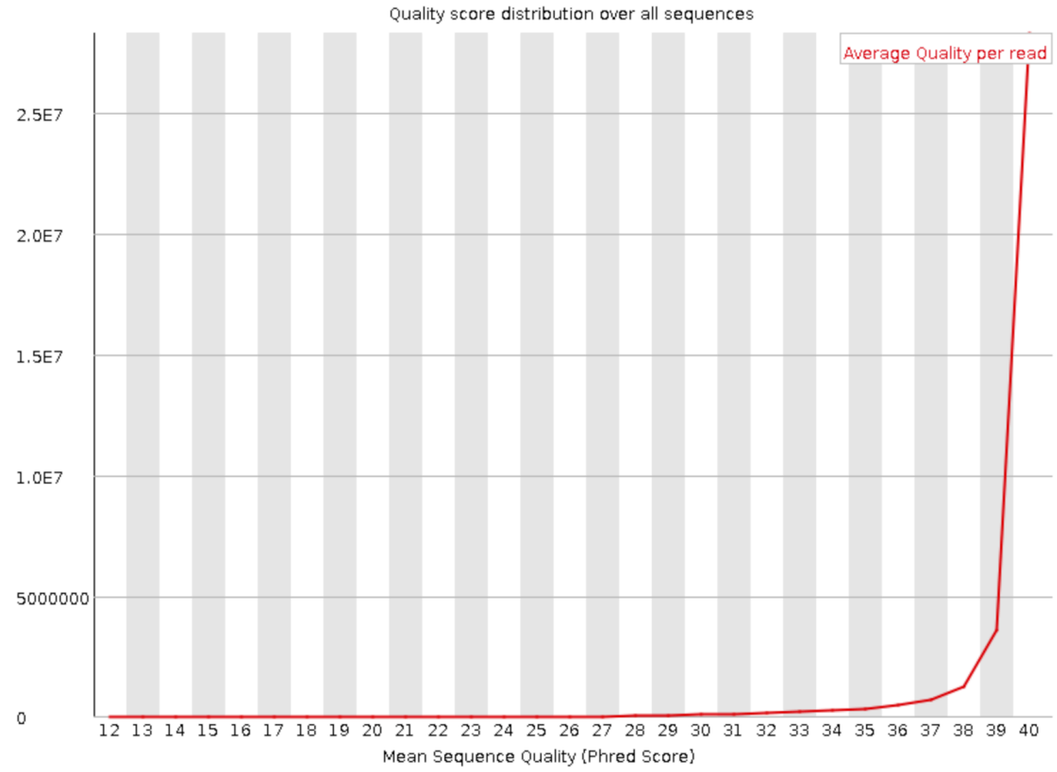
Species	Sequences used (Mb)[1]	CDS count[2]	Exon count[3]	Intron count	Average Exon length (bp)	Average Exon GC (%)	Average Intron length (bp)	Average Intron GC (%)
A. thaliana	119.2	23488	135697	118838	223.7	44.1	163.7	32.7
B. taurus	2434	16829	162223	151199	162.3	52.0	4516.4	46.9
C. elegans	100.3	27123	171102	149895	208.4	43.0	334.7	29.1
C. familiaris	2445	15960	161238	147118	157.2	50.6	3535.5	46.1
D. melanogaster	118.4	8119	43847	40732	370.0	52.7	1530.7	36.5
D. rerio	1547	20256	173438	156972	156.2	48.7	2276.4	34.5
G. gallus	1032	14367	142329	133690	152.1	48.2	2811.8	42.6
H. sapiens	3077	40430	381122	378089	162.7	50.8	5848.7	45.9
M. musculus	2644	30546	254605	243458	170.5	51.1	4683.6	46.0
O. sativa	372.1	41046	178106	144863	306.0	51.0	396.1	37.7
P. troglodytes	3176	30010	296830	287132	156.2	50.7	6003.7	45.3
R. norvegicus	2719	22243	195940	186864	172.9	51.1	4406.4	46.3
T. nigroviridis	217.3	15455	122253	108901	174.3	54.5	599.5	45.4

Patterns of exon-intron architecture variation of genes in eukaryotic genomes. *BMC Genomics* **10**, 47 (2009)

Per base sequence quality

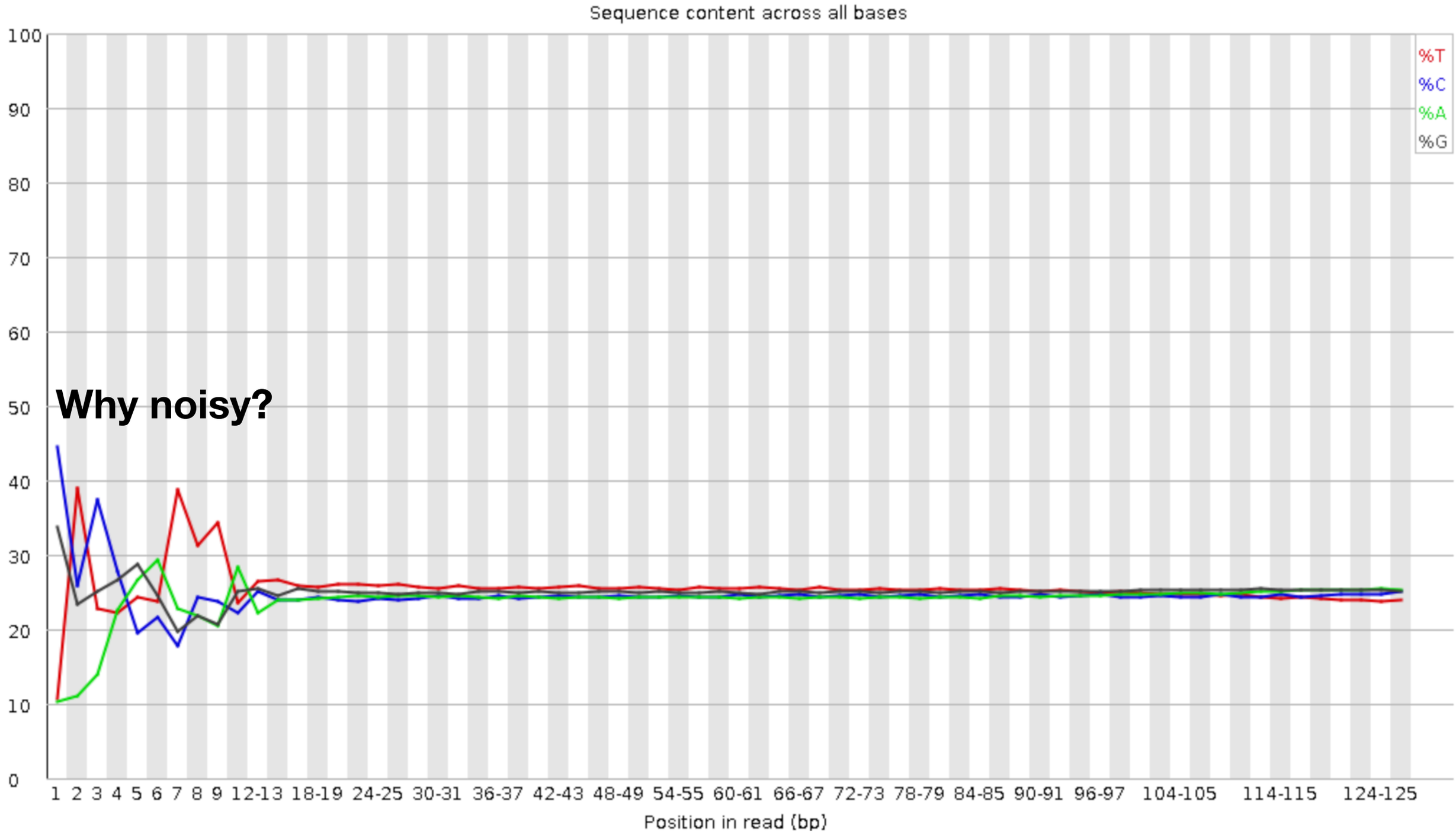


Per sequence quality scores



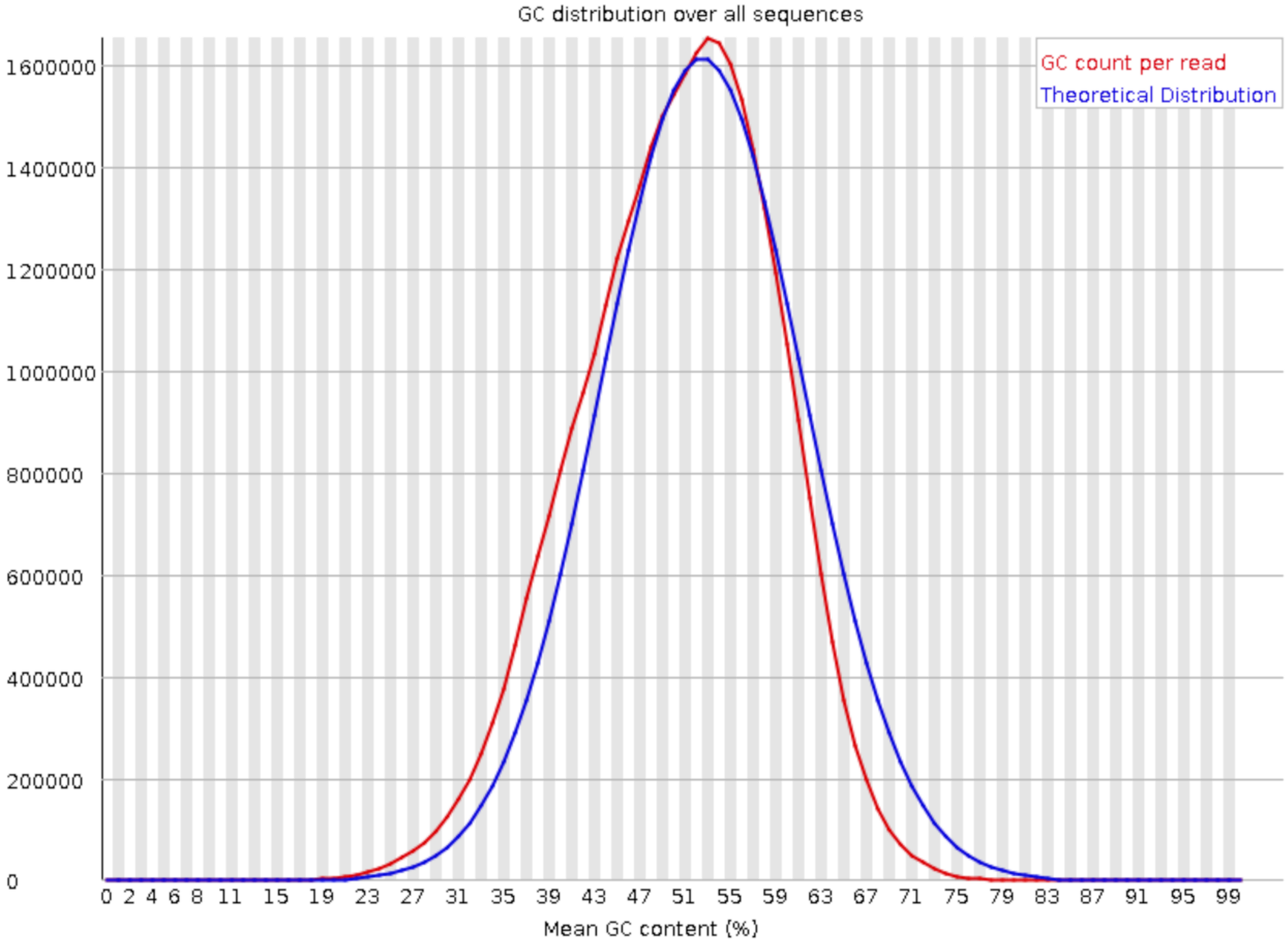
Phred Quality Score	Probability of incorrect base call
10	1 in 10
20	1 in 100
30	1 in 1000
40	1 in 10,000

Per base sequence content

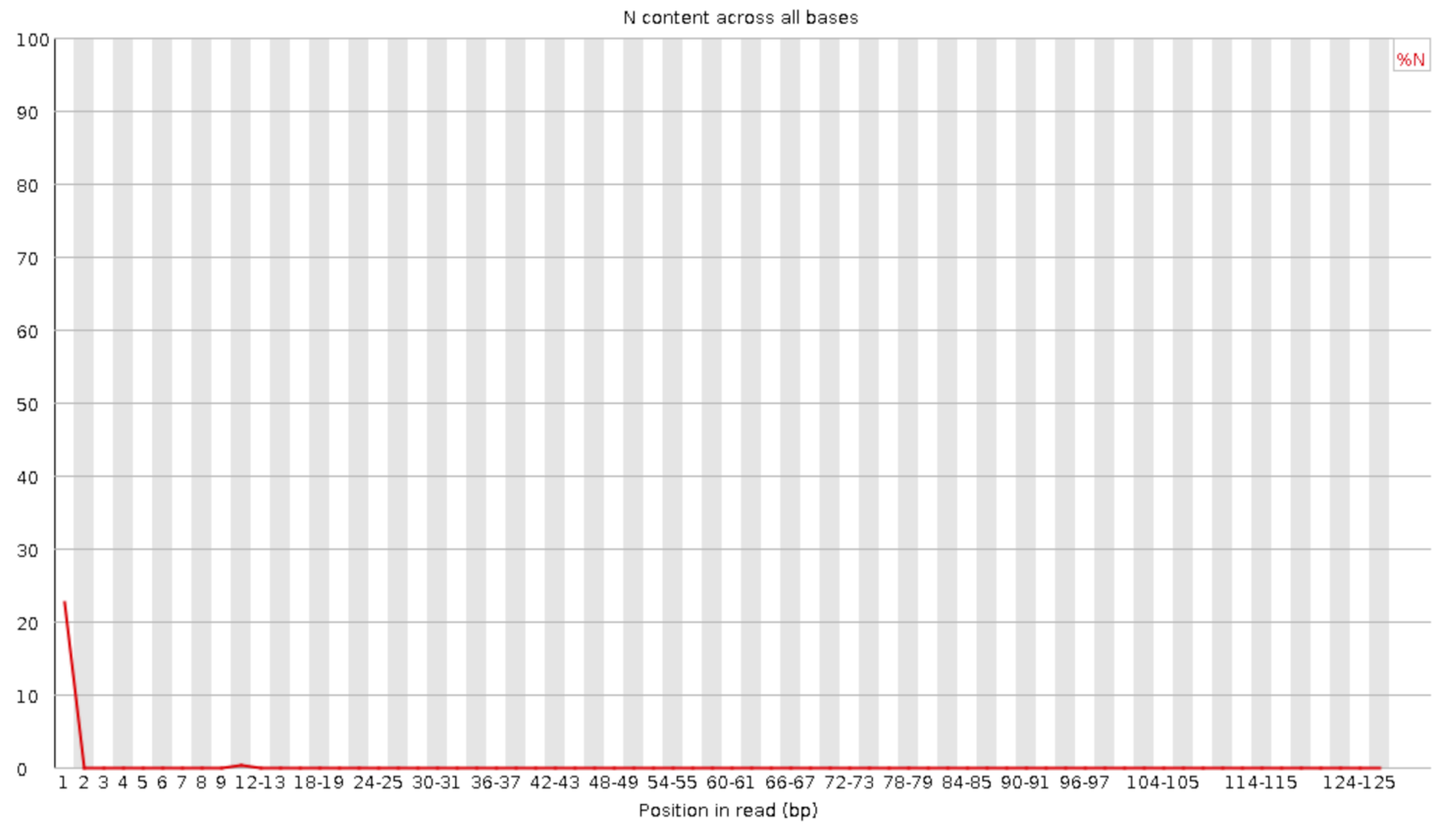


Why noisy?

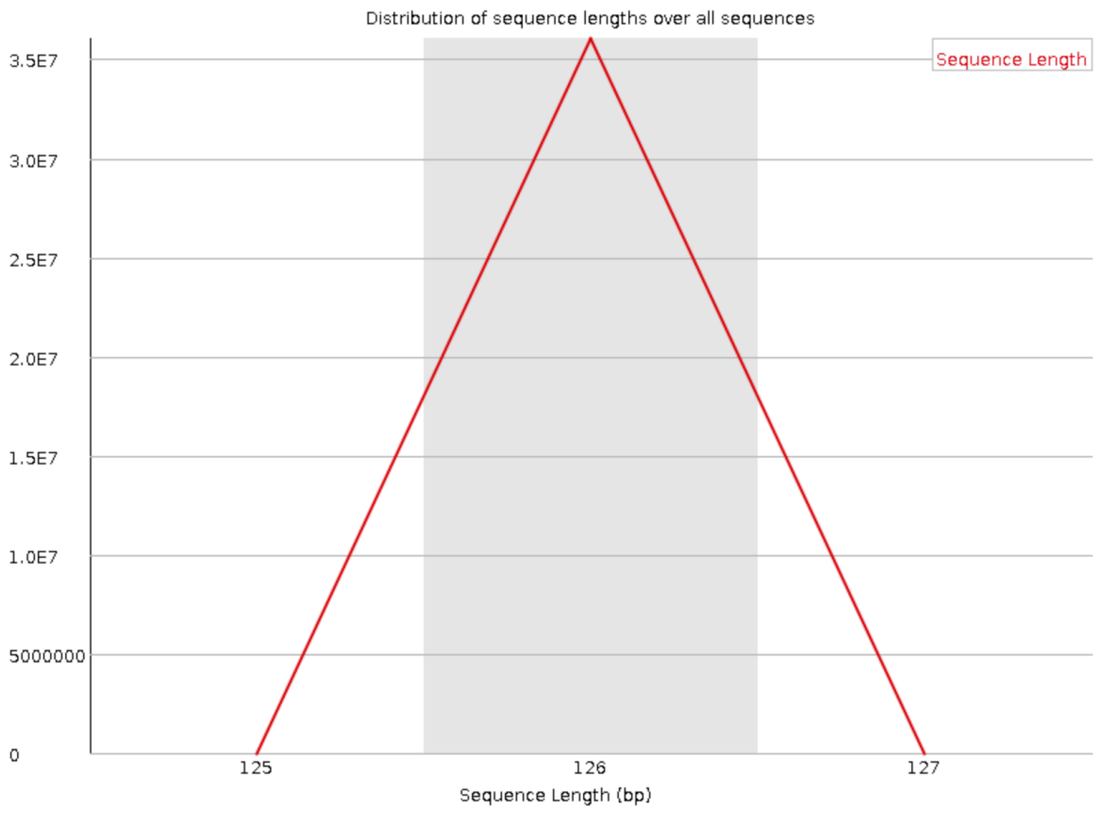
Per sequence GC content



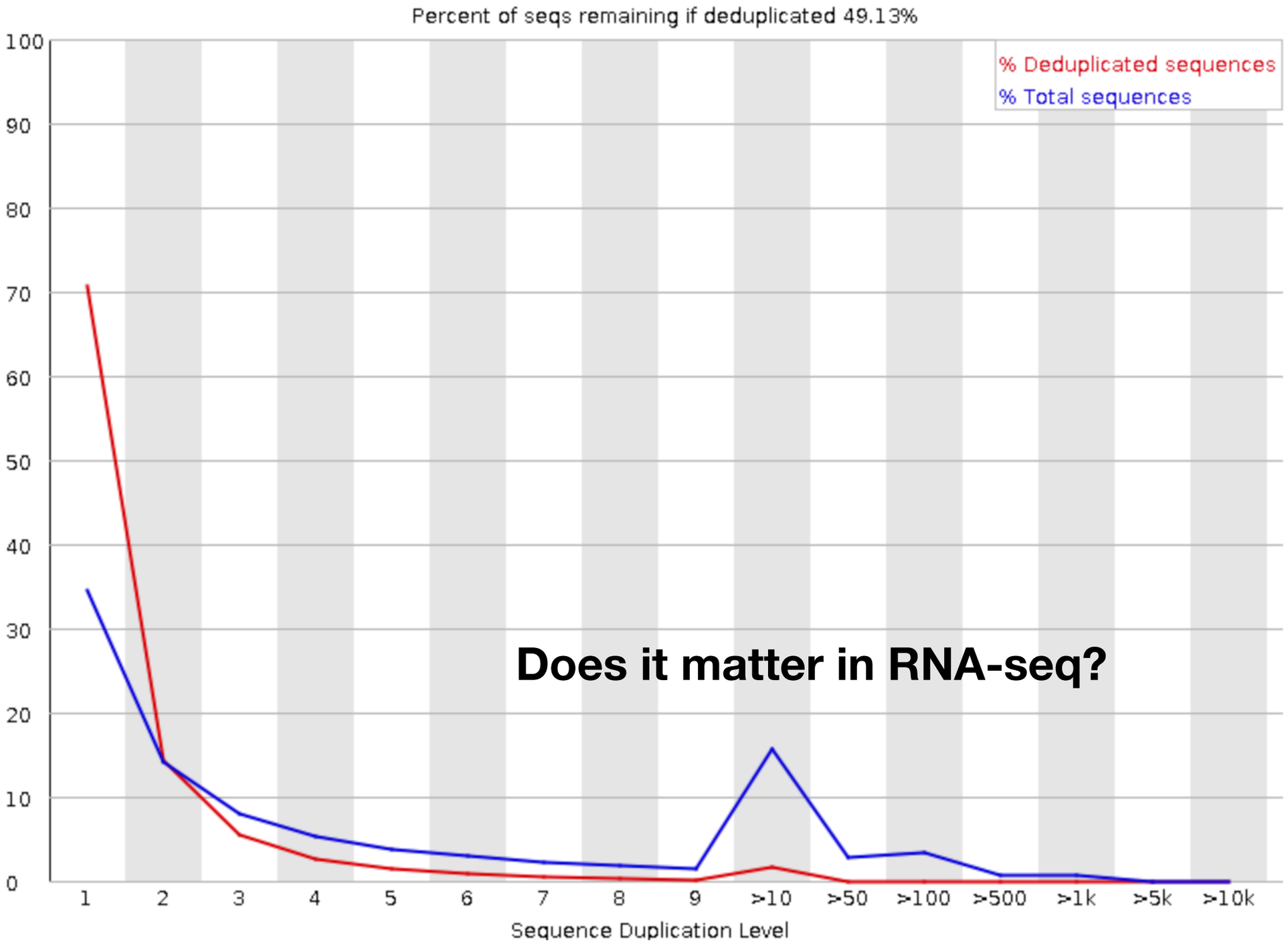
Per base N content



Sequence Length Distribution



Sequence Duplication Levels

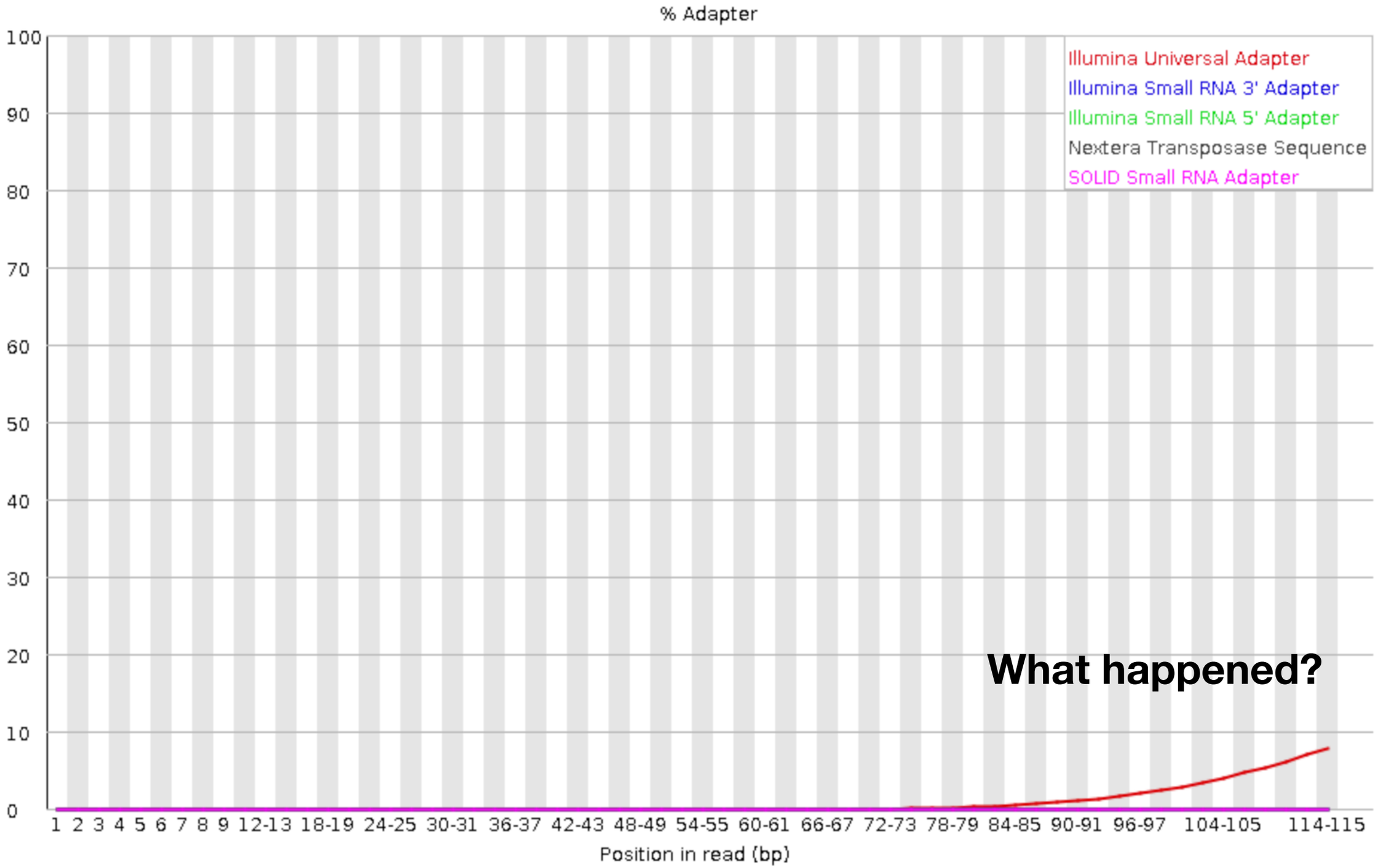


Does it matter in RNA-seq?

Overrepresented sequences

No overrepresented sequences

Adapter Content



Trimming Fastqc using cutadapt

```
$ cutadapt -a AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC -m 20 -
o SRR5715043.trimmed.fastq.gz SRR5715043.fastq.gz
```

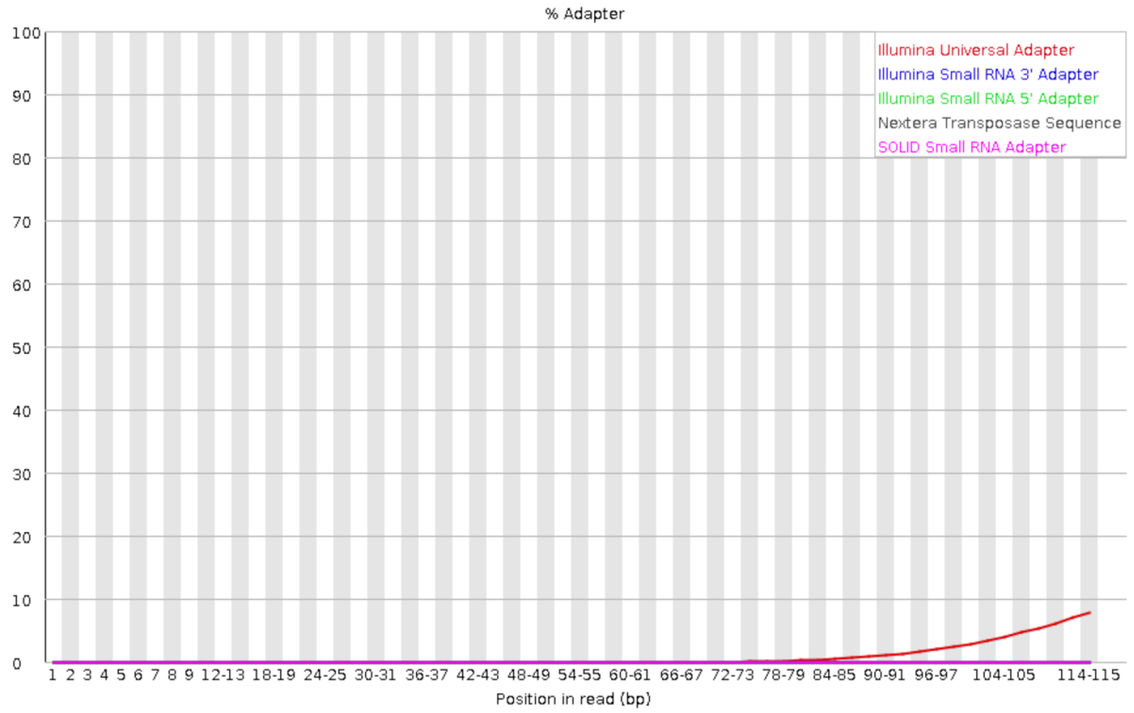
Before

Measure	Value
Filename	SRR5715043.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	36027995
Sequences flagged as poor quality	0
Sequence length	126
%GC	49

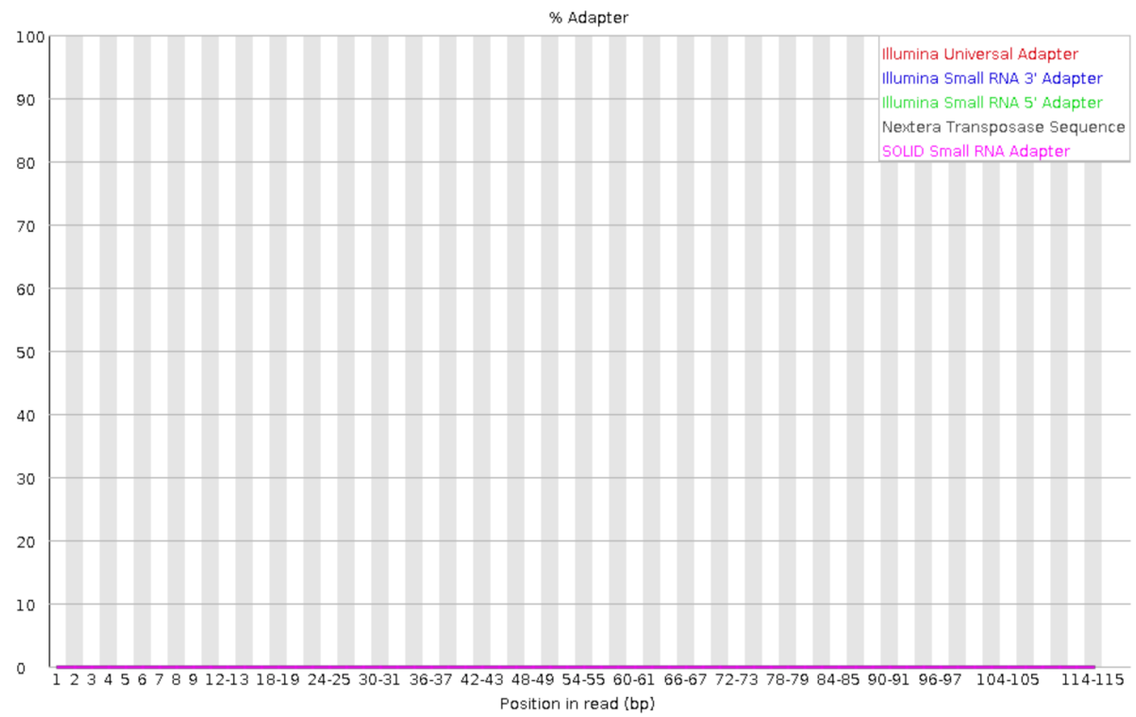
After

Measure	Value
Filename	SRR5715043.trimmed.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	36022775
Sequences flagged as poor quality	0
Sequence length	20-126
%GC	49

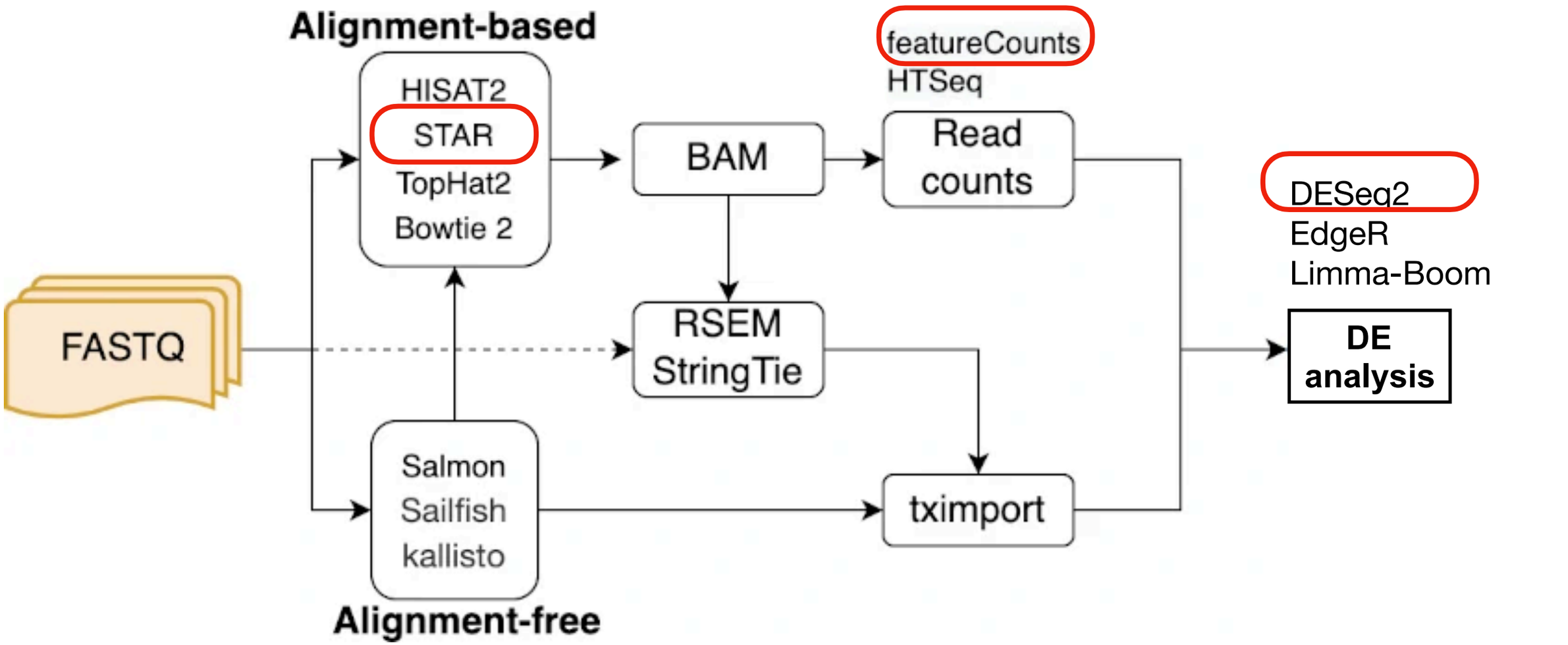
Adapter Content



Adapter Content



Our RNA-seq tools



https://www.reneshbedre.com/blog/deseq2.html

Alignment using STAR

Spliced Transcripts Alignments to a Reference (STAR)

Indexing (only one time per genome+transcript annotation)

```
$STAR --runMode genomeGenerate \
      --runThreadN 6 \
      --genomeFastaFiles genome.fa \
      --sjdbGTFfile annotation.gtf \
      --sjdbOverhang 99 \
      --genomeDir STAR_index
```

Mapping (multiple samples)

```
$STAR --genomeDir ./Genome/STAR_Index \
      --runThreadN 4 \
      --readFilesCommand zcat \
      --readFilesIn SRR5715043.trimmed.fastq.gz \
      --outSAMtype BAM SortedByCoordinate \
      --outFileNamePrefix SRR5715043.
```

A review of module 5

Excerpted from Loyal Goff's slide

Alignment QC - What are we looking for?

- ***Number*** of reads aligned per sample
- ***Percentage*** of reads aligned per sample
- ***Number/percentage*** of reads uniquely mapping
  - % of reads with multiple mapping to reference (ambiguous)
- Paired End read ***concordance***
  - # of PE reads uniquely mapping
  - # of PE reads mapping but not together (discordant)
  - # of PE reads where only one mapped [uniquely]
- ***Consistent*** scores/values across samples
- *Values are often reported after each sample is processed. Useful to try and capture this information in a log file.*

STAR Output example

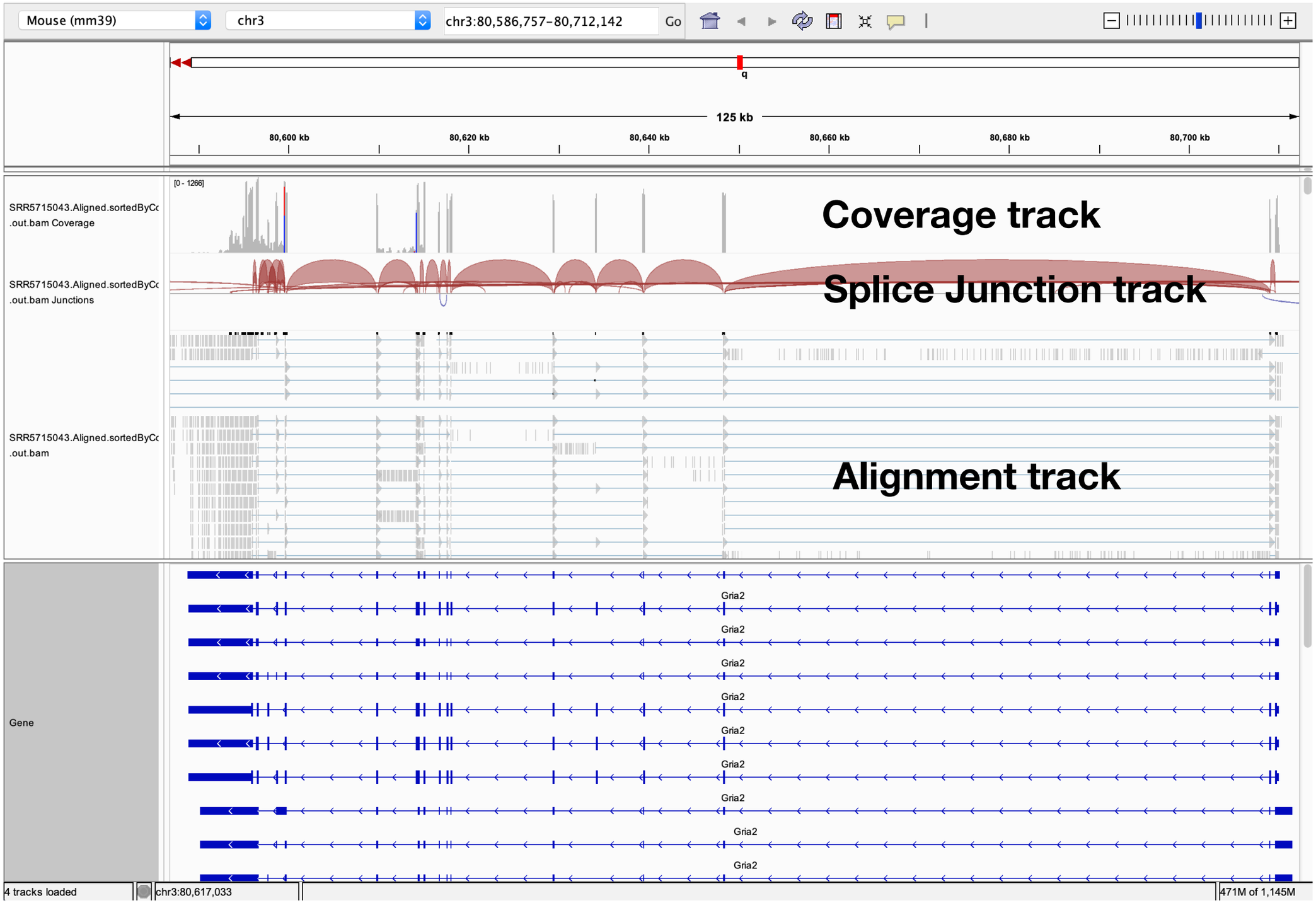
```
$ cat SRR5715043.Log.final.out
```

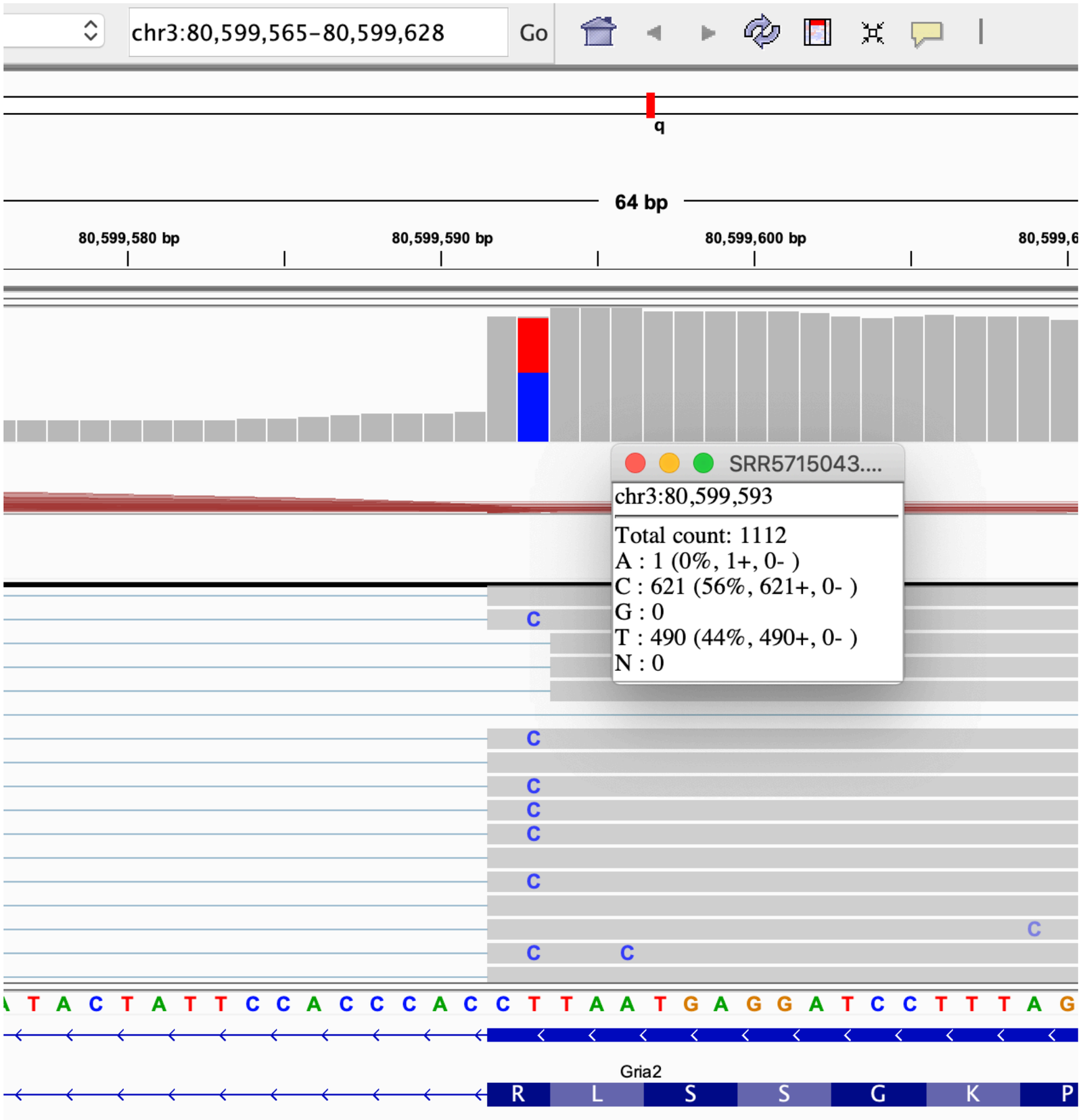
```
                                 Started job on |       Oct 03 17:10:04
                             Started mapping on |       Oct 03 17:10:55
                                    Finished on |       Oct 03 17:16:23
           Mapping speed, Million of reads per hour |       395.37

                               Number of input reads |       36022775
                         Average input read length |       123
                                      UNIQUE READS:
                      Uniquely mapped reads number |       33636271
                           Uniquely mapped reads % |       93.38%
                             Average mapped length |       123.01
                          Number of splices: Total |       11521857
               Number of splices: Annotated (sjdb) |       11418927
                          Number of splices: GT/AG |       11399495
                          Number of splices: GC/AG |       98254
                          Number of splices: AT/AC |       12071
                  Number of splices: Non-canonical |       12037
                         Mismatch rate per base, % |       0.11%
                            Deletion rate per base |       0.01%
                           Deletion average length |       1.96
                          Insertion rate per base |       0.00%
                         Insertion average length |       1.54
                               MULTI-MAPPING READS:
        Number of reads mapped to multiple loci |       1724928
             % of reads mapped to multiple loci |       4.79%
        Number of reads mapped to too many loci |       74776
             % of reads mapped to too many loci |       0.21%
                                  UNMAPPED READS:
Number of reads unmapped: too many mismatches |       0
     % of reads unmapped: too many mismatches |       0.00%
          Number of reads unmapped: too short |       536652
               % of reads unmapped: too short |       1.49%
            Number of reads unmapped: other |       50148
                 % of reads unmapped: other |       0.14%
                               CHIMERIC READS:
                             Number of chimeric reads |       0
                                  % of chimeric reads |       0.00%
```

Visualization with IGV

```
$ samtools index
SRR5715043.Aligned.sortedByCoord.out.bam
```




chr3:80,599,565-80,599,628
Go
q
64 bp
80,599,580 bp
80,599,590 bp
80,599,600 bp
SRR5715043....
chr3:80,599,593
Total count: 1112
A : 1 (0%, 1+, 0-)
C : 621 (56%, 621+, 0-)
G : 0
T : 490 (44%, 490+, 0-)
N : 0
Gria2
R L S S G K P

Counting reads using featureCounts

https://subread.sourceforge.net/featureCounts.html

1. Count the reads for every feature (by default, exon).
2. Sum the feature counts according to meta features (for example, gene).


```
$featureCounts SRR5715043.Aligned.sortedByCoord.out.bam \
-a gencode.vM30.annotation.gtf \
-o SRR5715043.fCounts \
-p                  # Paired-end (not in our case)
-s 2 \              # Strand of read 1 : 0 (unstranded), 1 (stranded), 2 (rc stranded)
-g gene_id          # Meta feature ( a set of features) in GTF
-T 4 \              # Number of threads
```

FeatureCounts Output example

Standard output

```
==========     _____ _    _ ____  _____  ______          _____
=====         / ____| |  | |  _ \|  __ \|  ____|   /\   |  __ \
  =====      | (___ | |  | | |_) | |__) | |__     /  \  | |  | |
    ====      \___ \| |  | |  _ <|  _  /|  __|   / /\ \ | |  | |
      ====    ____) | |__| | |_) | | \ \| |____ / ____ \| |__| |
==========   |_____/ \____/|____/|_|  \_\______/_/    \_\_____/
  v2.0.3

//========================== featureCounts setting ===========================\\
||                                                                            ||
||             Input files : 1 BAM file                                       ||
||                                                                            ||
||                           SRR5715043.Aligned.sortedByCoord.out.bam         ||
||                                                                            ||
||             Output file : SRR5715043.fCounts                               ||
||                 Summary : SRR5715043.fCounts.summary                       ||
||              Paired-end : no                                               ||
||        Count read pairs : no                                               ||
||              Annotation : gencode.vM30.annotation.gtf (GTF)                ||
||      Dir for temp files : ./3.Count                                        ||
||                                                                            ||
||                 Threads : 4                                                ||
||                   Level : meta-feature level                               ||
||       Multimapping reads : not counted                                     ||
||  Multi-overlapping reads : not counted                                     ||
||    Min overlapping bases : 1                                               ||
||                                                                            ||
\\============================================================================//

//================================ Running =================================\\
||                                                                            ||
||  Load annotation file gencode.vM30.annotation.gtf ...                      ||
||     Features : 868862                                                      ||
||     Meta-features : 56691                                                  ||
||     Chromosomes/contigs : 22                                               ||
||                                                                            ||
||  Process BAM file SRR5715043.Aligned.sortedByCoord.out.bam...              ||
||     Strand specific : reversely stranded                                   ||
||     Single-end reads are included.                                         ||
||     Total alignments : 37797275                                            ||
||     Successfully assigned alignments : 28832430 (76.3%)                    ||
||     Running time : 0.16 minutes                                            ||
||                                                                            ||
||  Write the final count table.                                              ||
||  Write the read assignment summary.                                        ||
||                                                                            ||
||  Summary of counting results can be found in file "./3.Count/SRR5715043.fC ||
||  ounts.summary"                                                            ||
||                                                                            ||
\\============================================================================//
```

SRR5715043.fCounts.summary

```
Status  ./2.Alignment/SRR5715043.Aligned.sortedByCoord
Assigned        28832430
Unassigned_Unmapped     0
Unassigned_Read_Type    0
Unassigned_Singleton    0
Unassigned_MappingQuality       0
Unassigned_Chimera      0
Unassigned_FragmentLength       0
Unassigned_Duplicate    0
Unassigned_MultiMapping 4161004
Unassigned_Secondary    0
Unassigned_NonSplit     0
Unassigned_NoFeatures   4381820
Unassigned_Overlapping_Length   0
Unassigned_Ambiguity    422021
```

Exploratory Data Analysis (EDA)

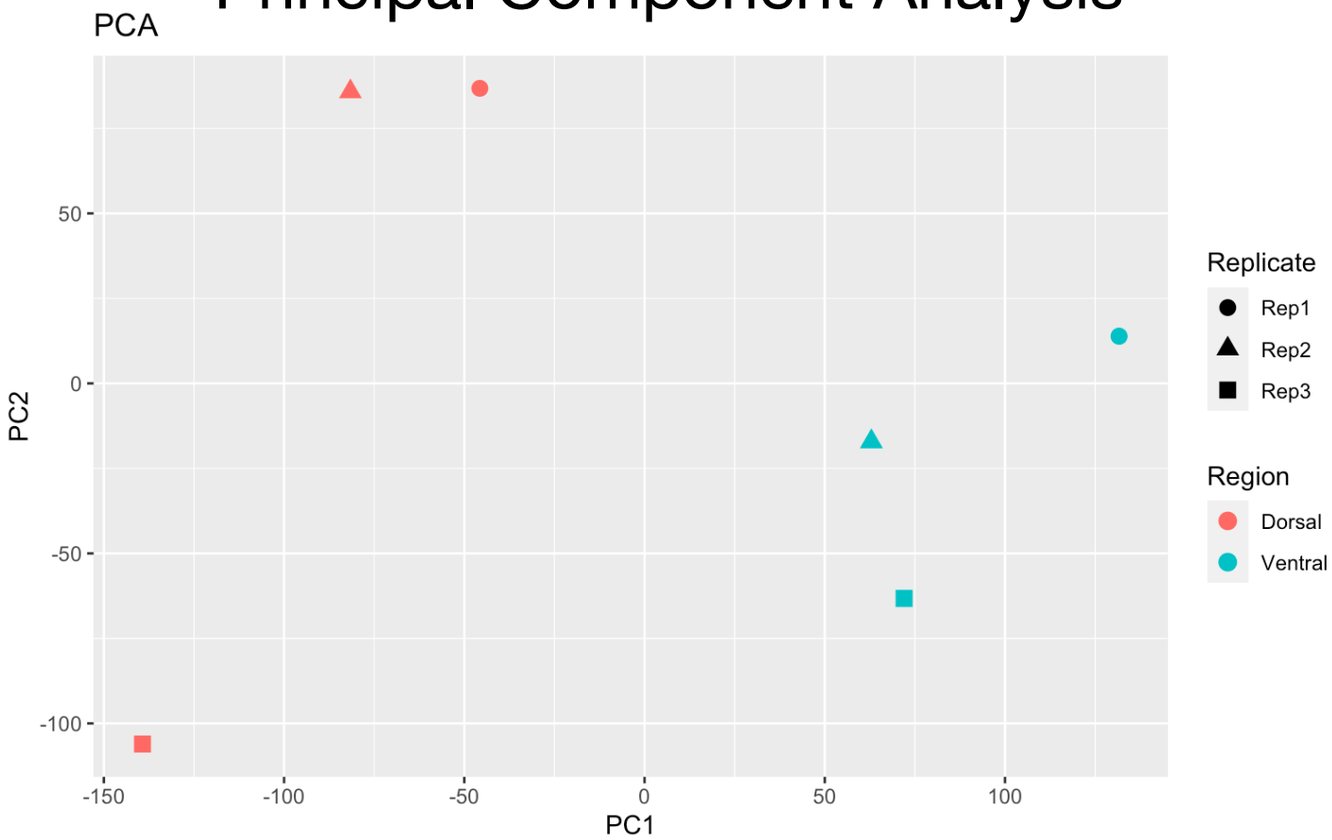
Normalization should be done before any EDA.

Normalization method	Description	Accounted factors	Recommendations for use
CPM (counts per million)	counts scaled by total number of reads	sequencing depth	gene count comparisons between replicates of the same sample group; **NOT for within sample comparisons or DE analysis**
TPM (transcripts per kilobase million)	counts per length of transcript (kb) per million reads mapped	sequencing depth and gene length	gene count comparisons within a sample or between samples of the same sample group; **NOT for DE analysis**
RPKM/FPKM (reads/fragments per kilobase of exon per million reads/fragments mapped)	similar to TPM	sequencing depth and gene length	gene count comparisons between genes within a sample; **NOT for between sample comparisons or DE analysis**
DESeq2's **median of ratios** [1]	counts divided by sample-specific size factors determined by median ratio of gene counts relative to geometric mean per gene	sequencing depth and RNA composition	gene count comparisons between samples and for **DE analysis**; **NOT for within sample comparisons**
EdgeR's **trimmed mean of M values (TMM)** [2]	uses a weighted trimmed mean of the log expression ratios between samples	sequencing depth and RNA composition	gene count comparisons between samples and for **DE analysis**; **NOT for within sample comparisons**

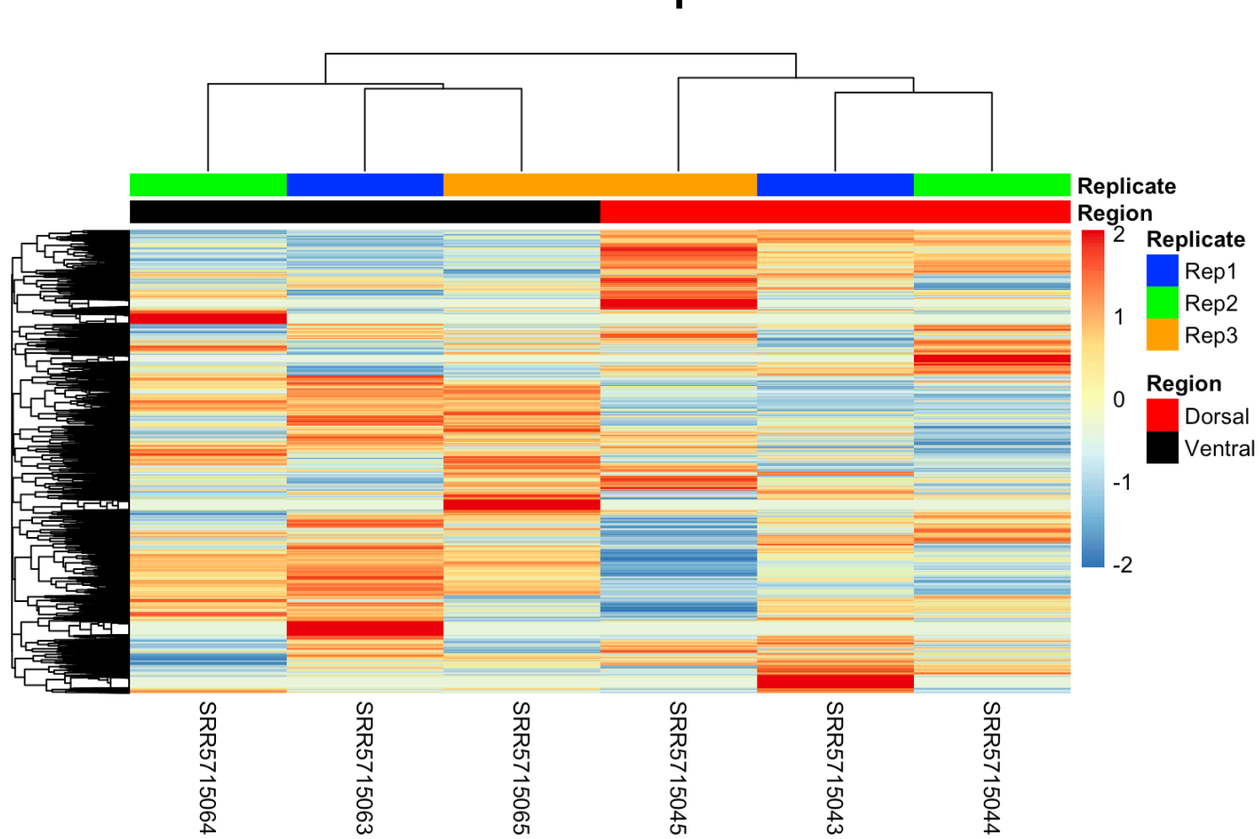
https://github.com/hbctraining/DGE_workshop_salmon_online/blob/master/lessons/02_DGE_count_normalization.md

Exploratory Data Analysis (EDA)

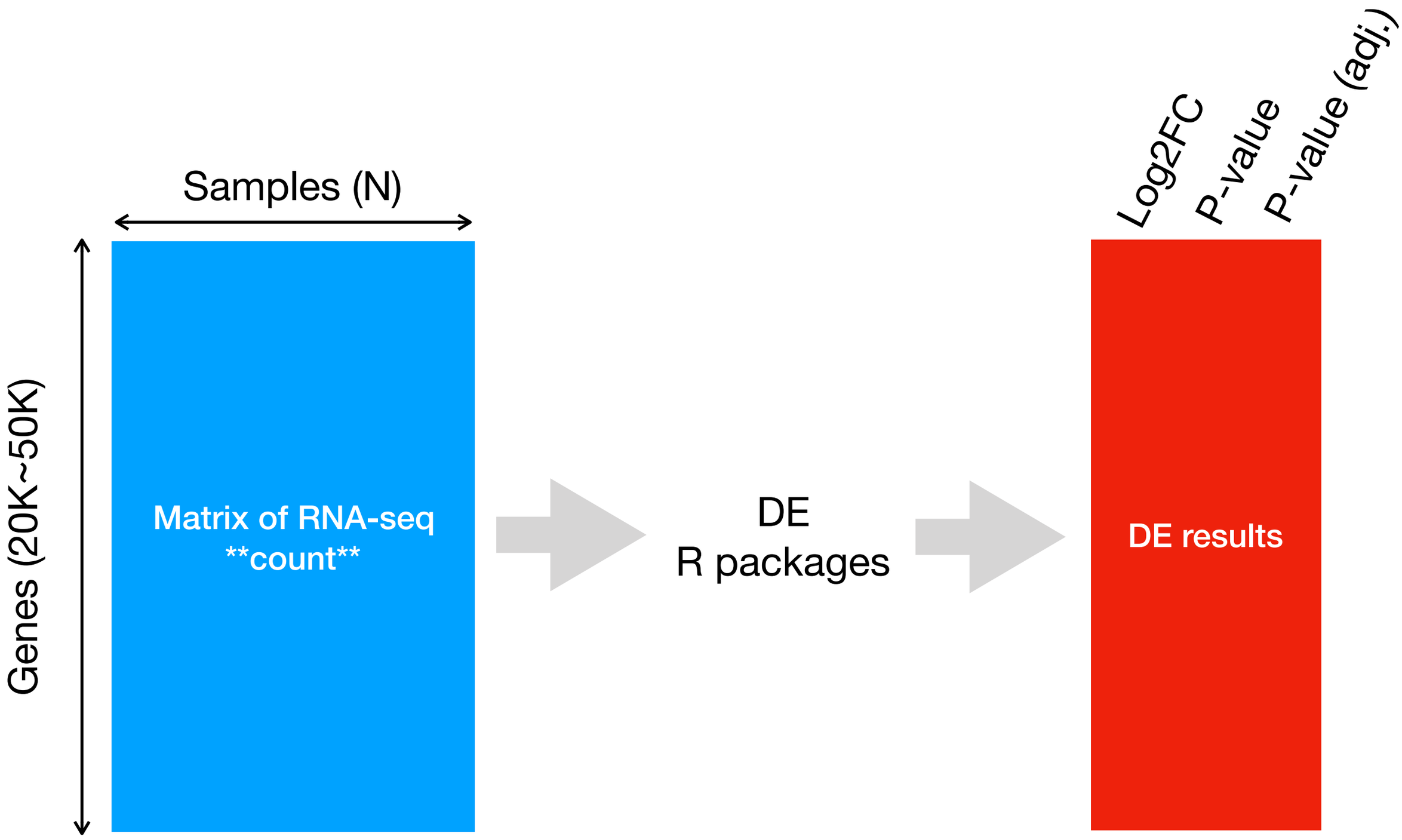
Principal Component Analysis



Heatmap



Differential Expression (DE)

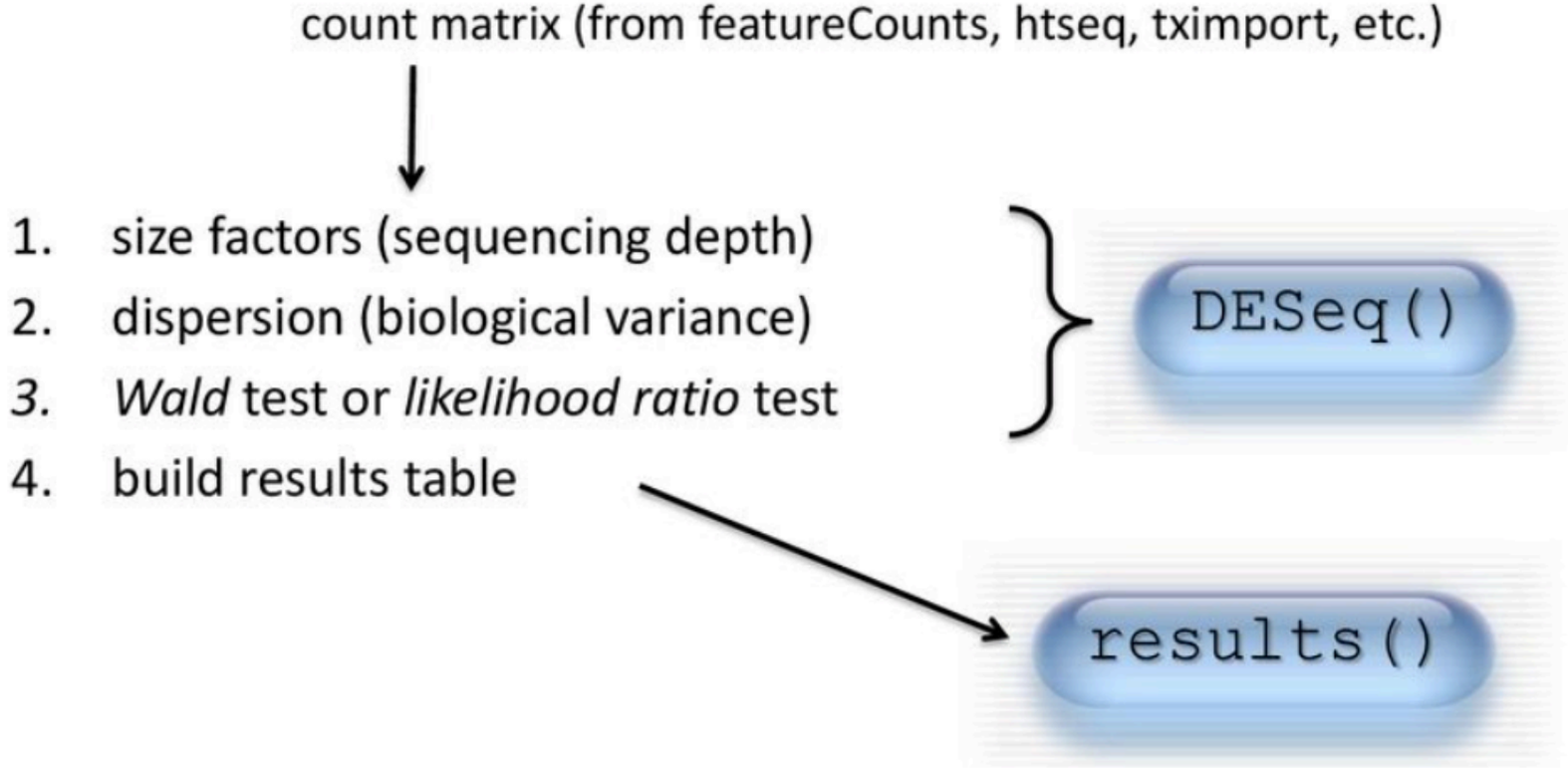


DE R packages

DE tools	Normalization	Count model	Statistical Test (Null: LFC==0)
EdgeR	TMM	Negative Binomial	Exact test or LRT
DESeq2	DESeq's median of ratio	Negative Binomial	Wald test or LRT
Limma-Voom	TMM	Log Normal	Moderated t-test

DESeq2

```
dds <- DESeqDataSetFromMatrix(countM, colData=sampleSheet,
design= ~ Region)
dds <- DESeq(dds)
Res <- results(dds, contrast=c("Region", "Dorsal", "Ventral"))
```



Design formula and contrast

```
dds <- DESeqDataSetFromMatrix(countM, colData=sampleSheet,
design= ~ Region)
dds <- DESeq(dds)
Res <- results(dds, contrast=c("Region", "Dorsal", "Ventral"))
```

- Simple comparison

  design = ~ region

- Controlling covariates

  design = ~ replicate + region

  design = ~ replicate + sex + region

- Investigating interaction effects

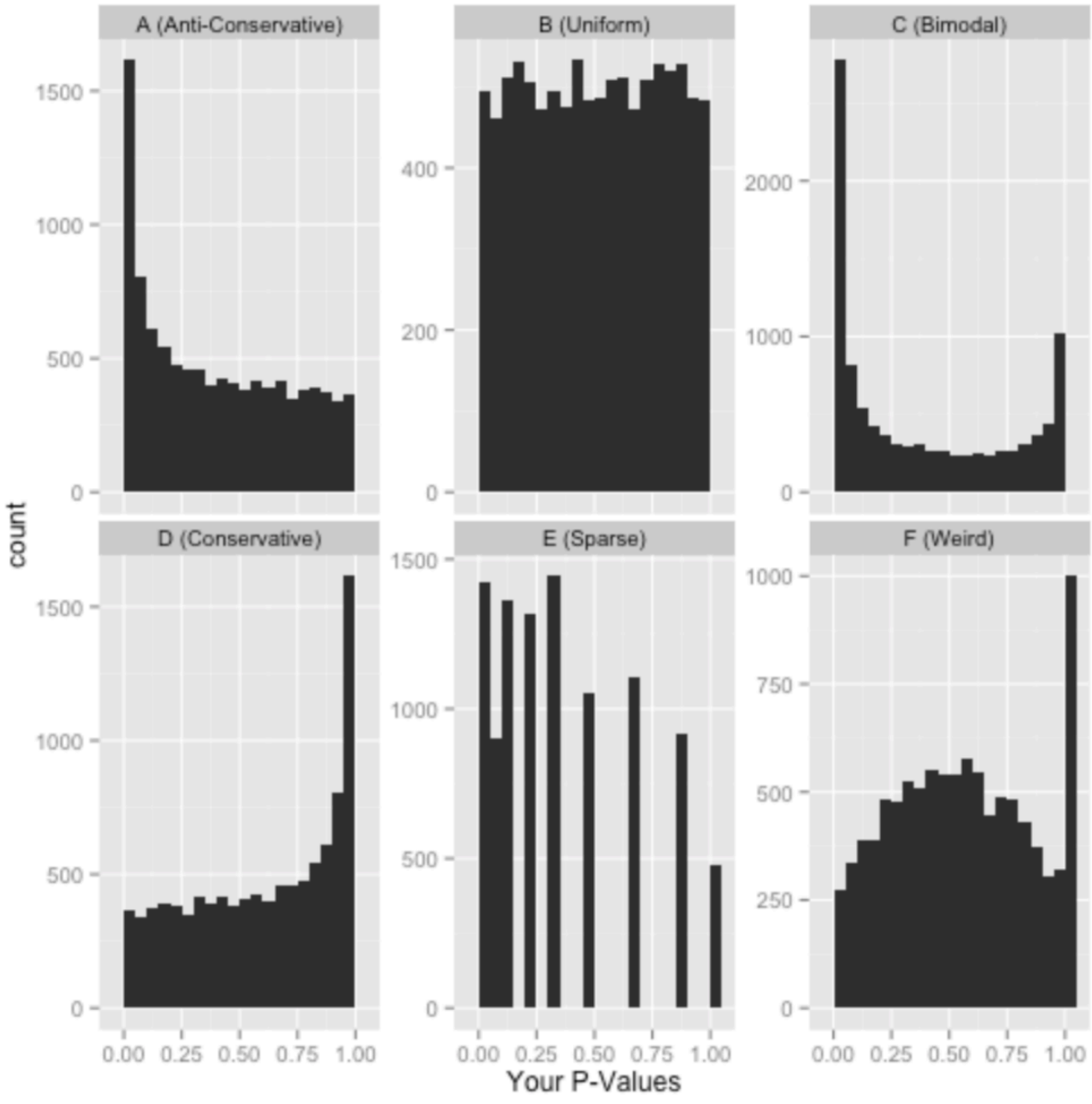
  design = ~ replicate + sex + region + sex : region

sampleSheet

	Sid	Region	Replicate
1	Sid	Region	Replicate
2	SRR5715043	Dorsal	Rep1
3	SRR5715044	Dorsal	Rep2
4	SRR5715045	Dorsal	Rep3
5	SRR5715063	Ventral	Rep1
6	SRR5715064	Ventral	Rep2
7	SRR5715065	Ventral	Rep3

** Check the DESeq2 R package help page **

DE QC: p-value distribution



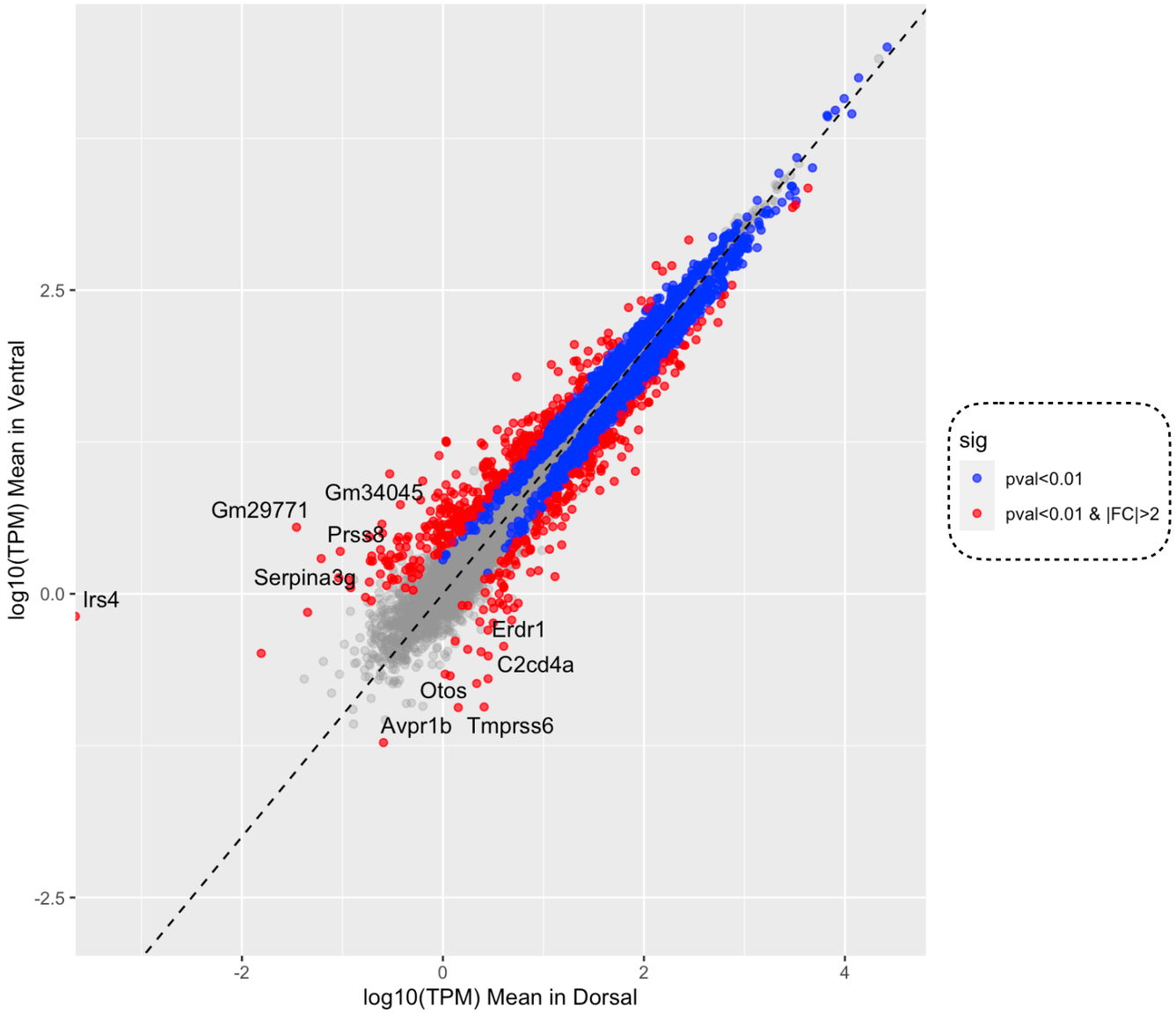
http://varianceexplained.org/statistics/interpreting-pvalue-histogram/

DE Results

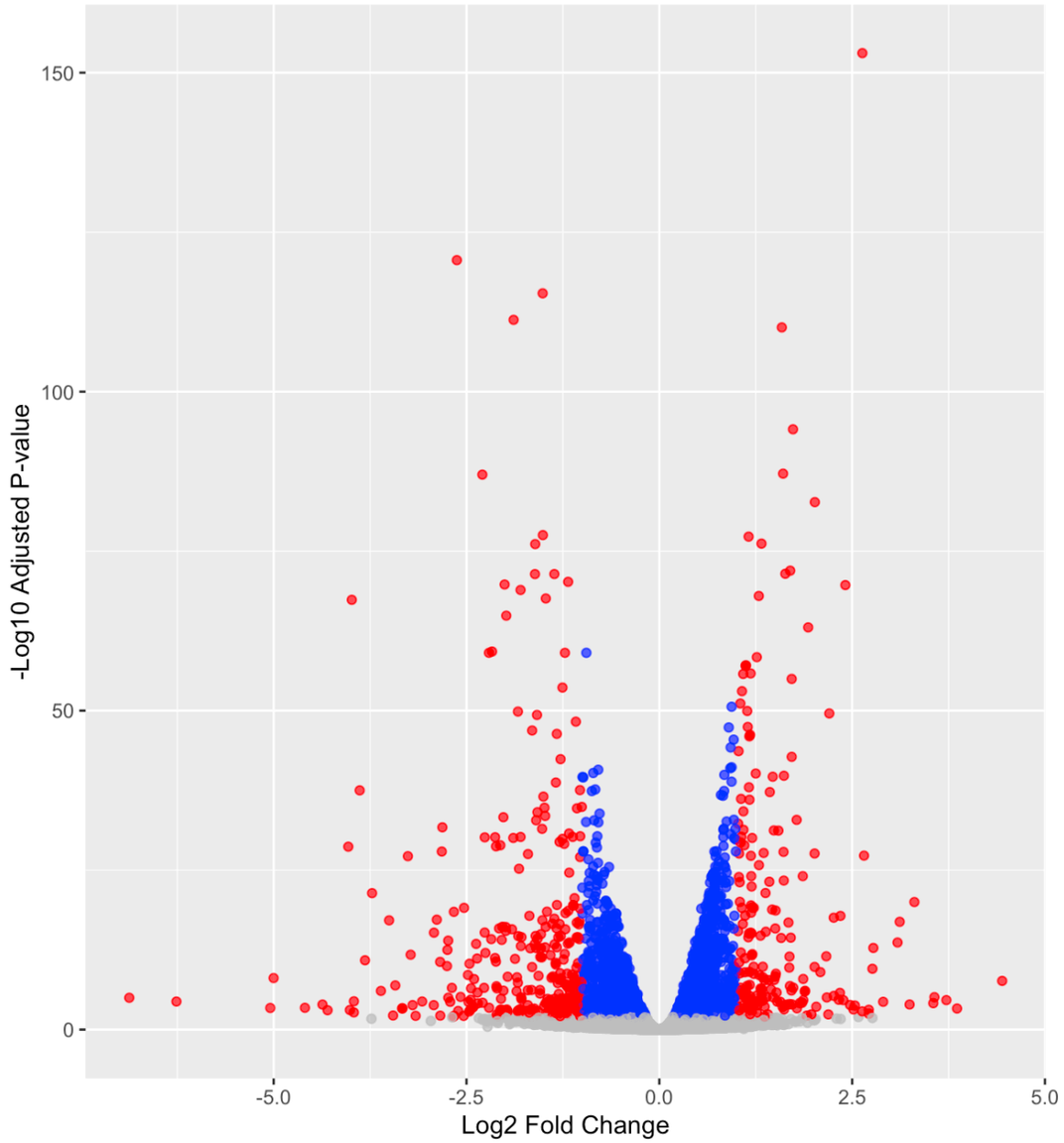
```
> res
log2 fold change (MLE): Region Dorsal vs Ventral
Wald test p-value: Region Dorsal vs Ventral
DataFrame with 56691 rows and 6 columns
                          baseMean     log2FoldChange             lfcSE                stat              pvalue               padj
                         <numeric>          <numeric>         <numeric>           <numeric>           <numeric>          <numeric>
ENSMUSG00000102693.2             0                 NA                NA                  NA                  NA                 NA
ENSMUSG00000064842.3             0                 NA                NA                  NA                  NA                 NA
ENSMUSG00000051951.6   934.374636735581  -0.3552436281259  0.103883171365474  -3.41964558317256 0.000627027707598258 0.0042158466553528
ENSMUSG00000102851.2             0                 NA                NA                  NA                  NA                 NA
ENSMUSG00000103377.2  0.965337795076986  -0.10445910151227  2.64821564471098 -0.0394450888925515   0.968535532124425                 NA
...                             ...               ...               ...                 ...                 ...                ...
ENSMUSG00000064368.1   554.832874720361 -0.268513399642424  0.121424238061778  -2.21136573659874  0.0270105205370121 0.0973450770345984
ENSMUSG00000064369.1   4.70528683513818 -0.229770388610795  1.14361158725448 -0.200916457275861   0.840763899060845                 NA
ENSMUSG00000064370.1   153824.906100841 -0.188788470434184 0.0613087510925515  -3.07930706579212 0.00207482709210156 0.0118964348163462
ENSMUSG00000064371.1   14.6385304120105  0.333703279931054  0.656922522781579  0.507979660246796   0.611467607501022  0.808762996851356
ENSMUSG00000064372.1   29.7519207584107 -0.239430038273013  0.465265290440962  -0.51460971448373   0.606825775339794  0.806035794669969
```

DE result plots

- Scatter plot



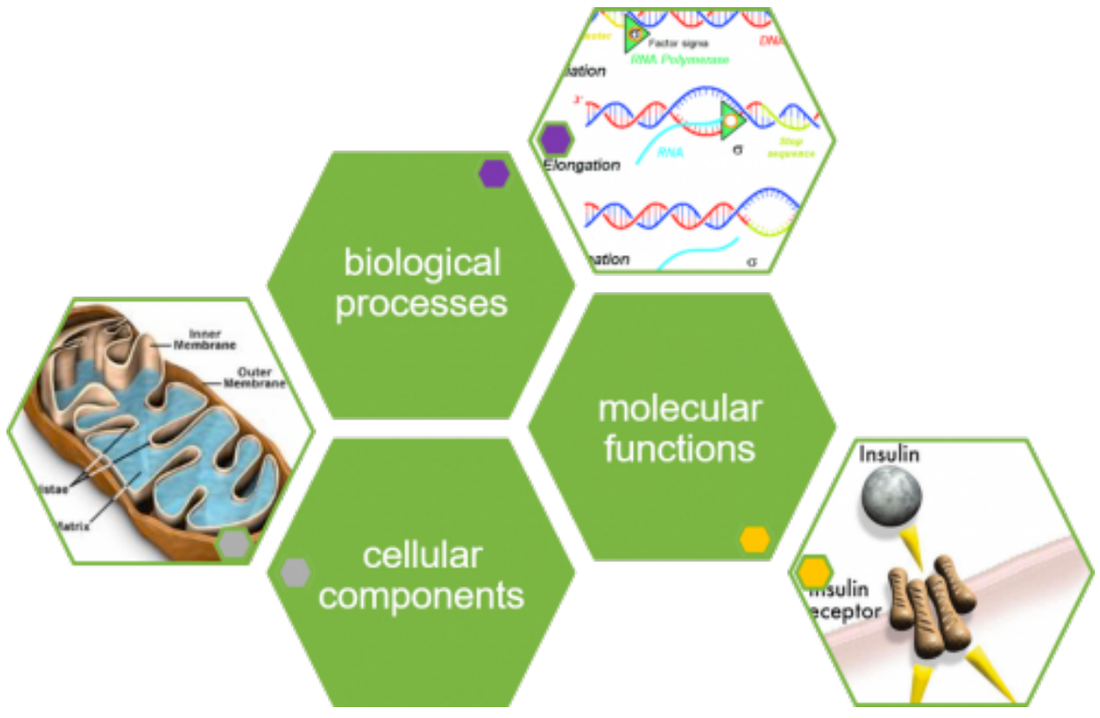
- Volcano plot



Gene Ontology database

GO:1903204
negative regulation of oxidative stress-induced neuron death

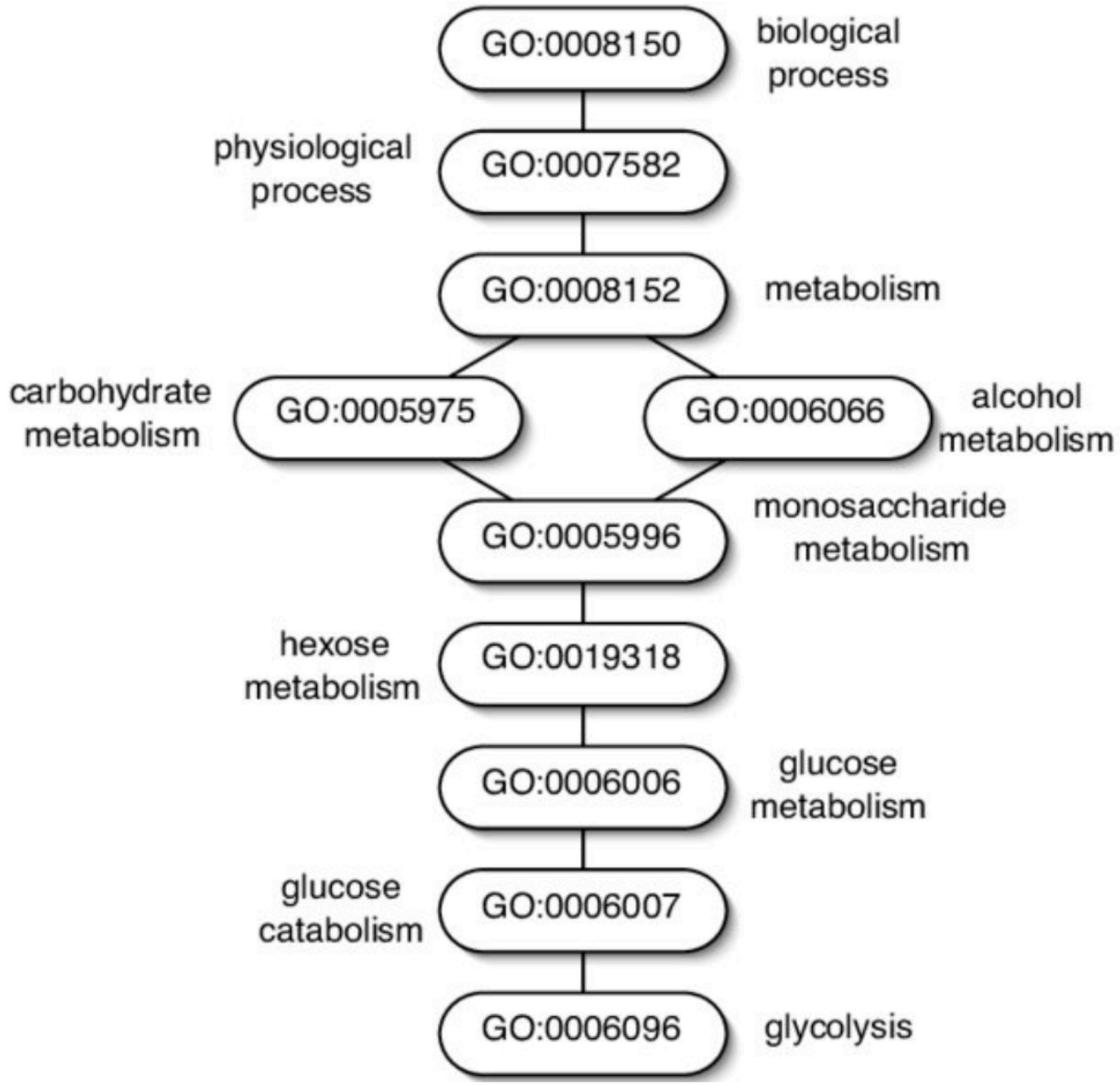
(#18) FBXO7, NONO, PARK7, RACK1, WNT1, PINK1, REST, IL10, NCOA7, MEAK7, HIF1A, SLC7A11, FZD1, GFER, CTNNB1, NR4A3, OXR1, ATF4.



“cytochrome c” can be described by the **molecular function** *oxidoreductase activity*,
the **biological process** *oxidative phosphorylation*, and
the **cellular component** *mitochondrial matrix*.

http://geneontology.org/docs/ontology-documentation/

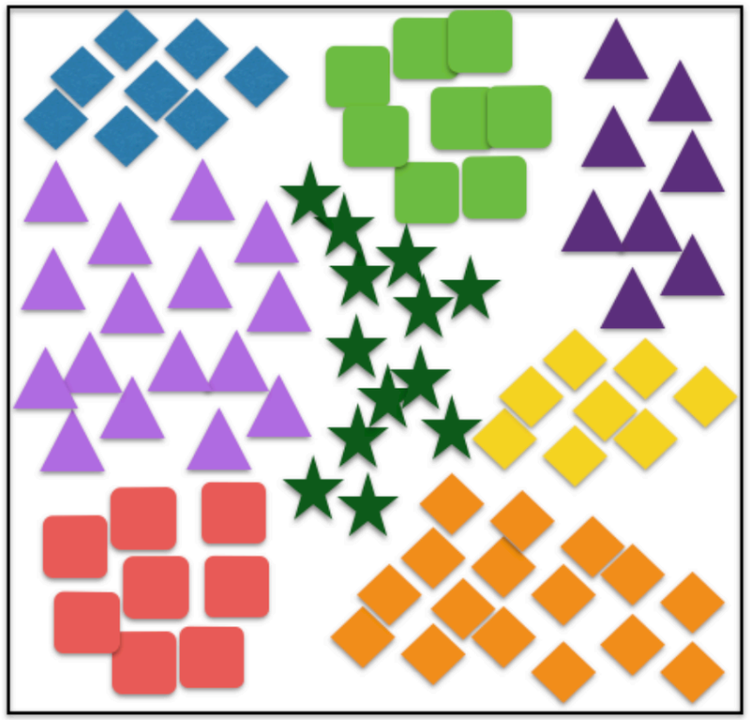
GO: Hierarchical structure



Lecture Notes in Computer Science (2004), vol 3056. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-540-24775-3_82

Enrichment analysis

All known genes in a species
(categorized into groups)



DE analysis

DEGs

Genes categories	Organism-specific Background	DE results	Over-represented?
Functional category 1	35/13000	25/1000	Likely
Functional category 2	56/13000	4/1000	Unlikely
Functional category 3	90/13000	8/1000	Unlikely
Functional category 4	15/13000	10/1000	Likely
…			

https://github.com/hbctraining/DGE_workshop_salmon_online/blob/master/lessons/10_FA_over-representation_analysis.md

Hypergeometic test

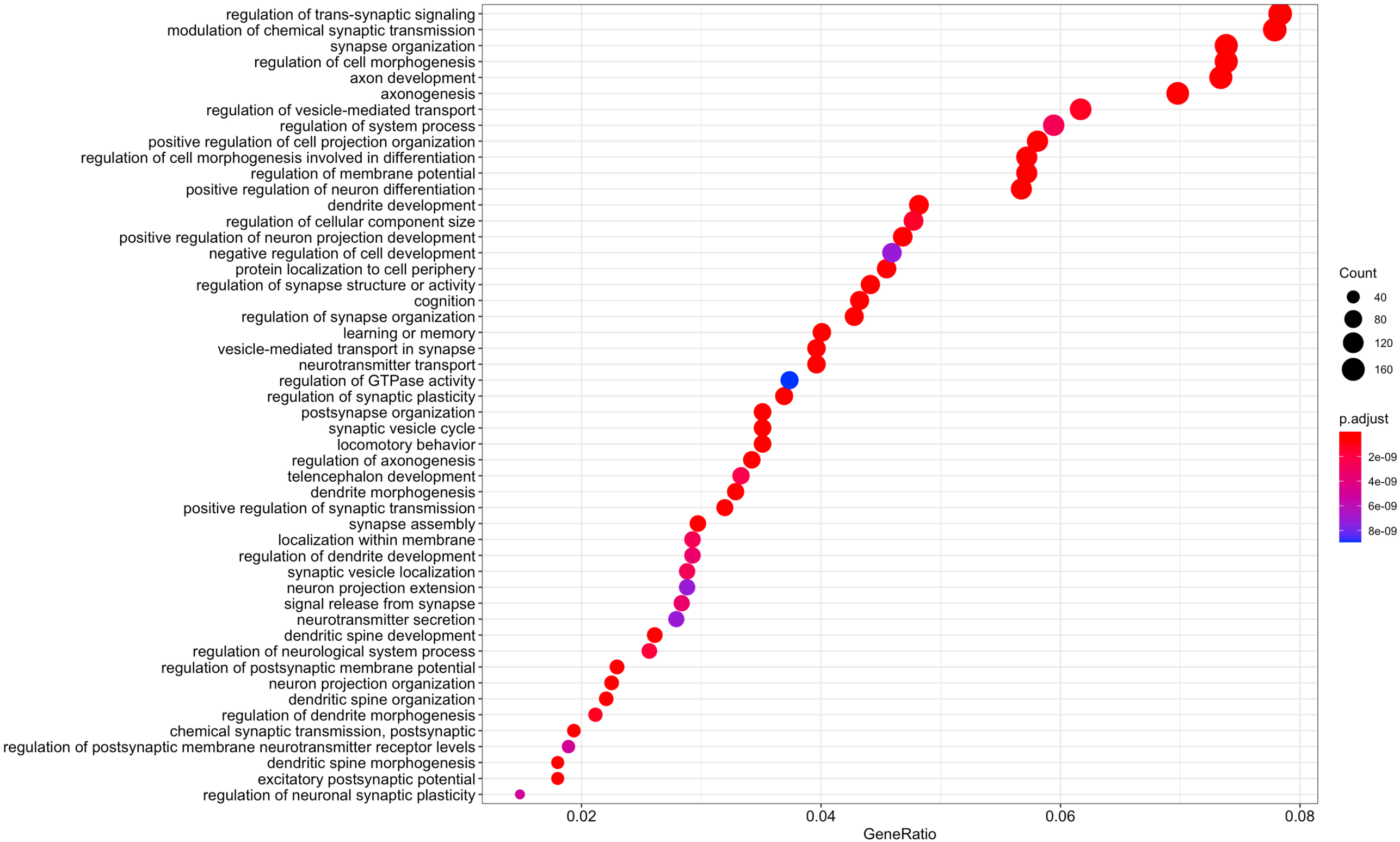
Genes categories	Organism-specific Background	DE results	Over-represented?
Functional category 1	35/13000	25/1000	Likely
Functional category 2	56/13000	4/1000	Unlikely
Functional category 3	90/13000	8/1000	Unlikely
Functional category 4	15/13000	10/1000	Likely
...			

Null distribution: hypergeometric distribution

$$\Pr(X = k) = \frac{\binom{K}{k}\binom{N-K}{n-k}}{\binom{N}{n}}$$

For category 1, N=13000, K=35, n=1000, k=0 to 35
For category 2, N=13000, K=56, n=1000, k=0 to 56

GO terms enriched in DE genes

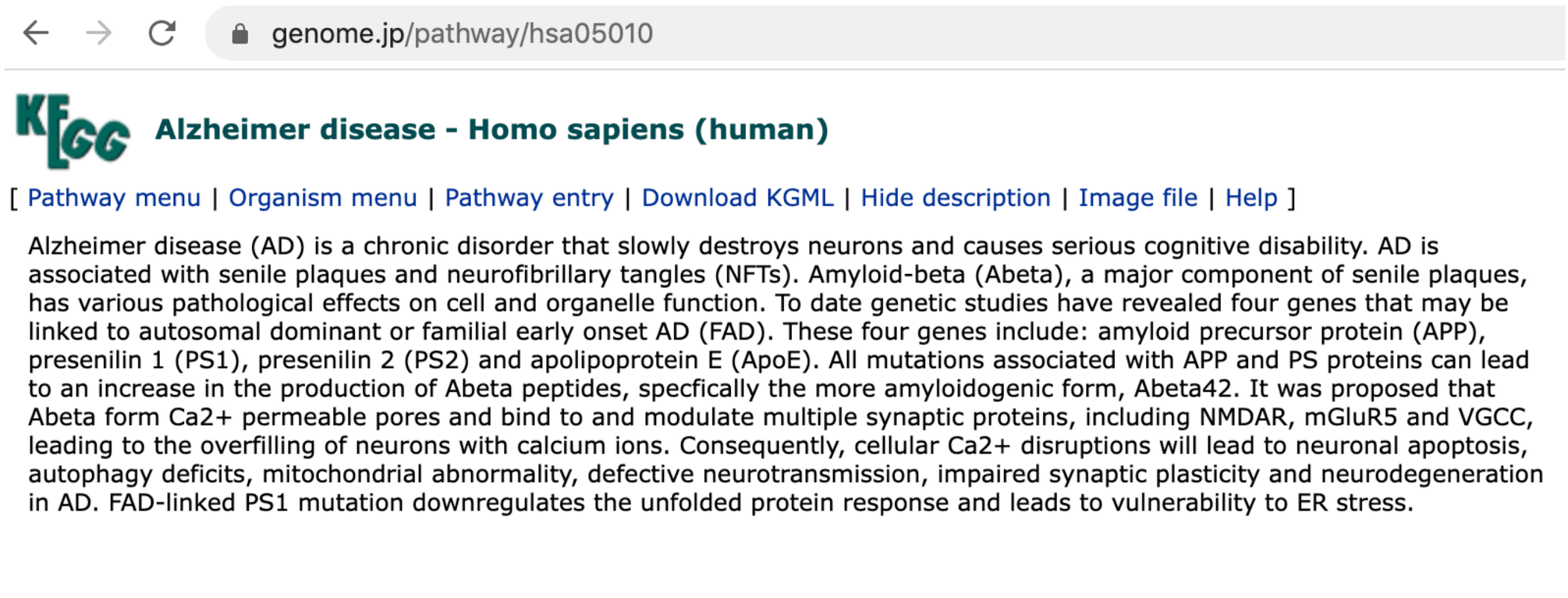


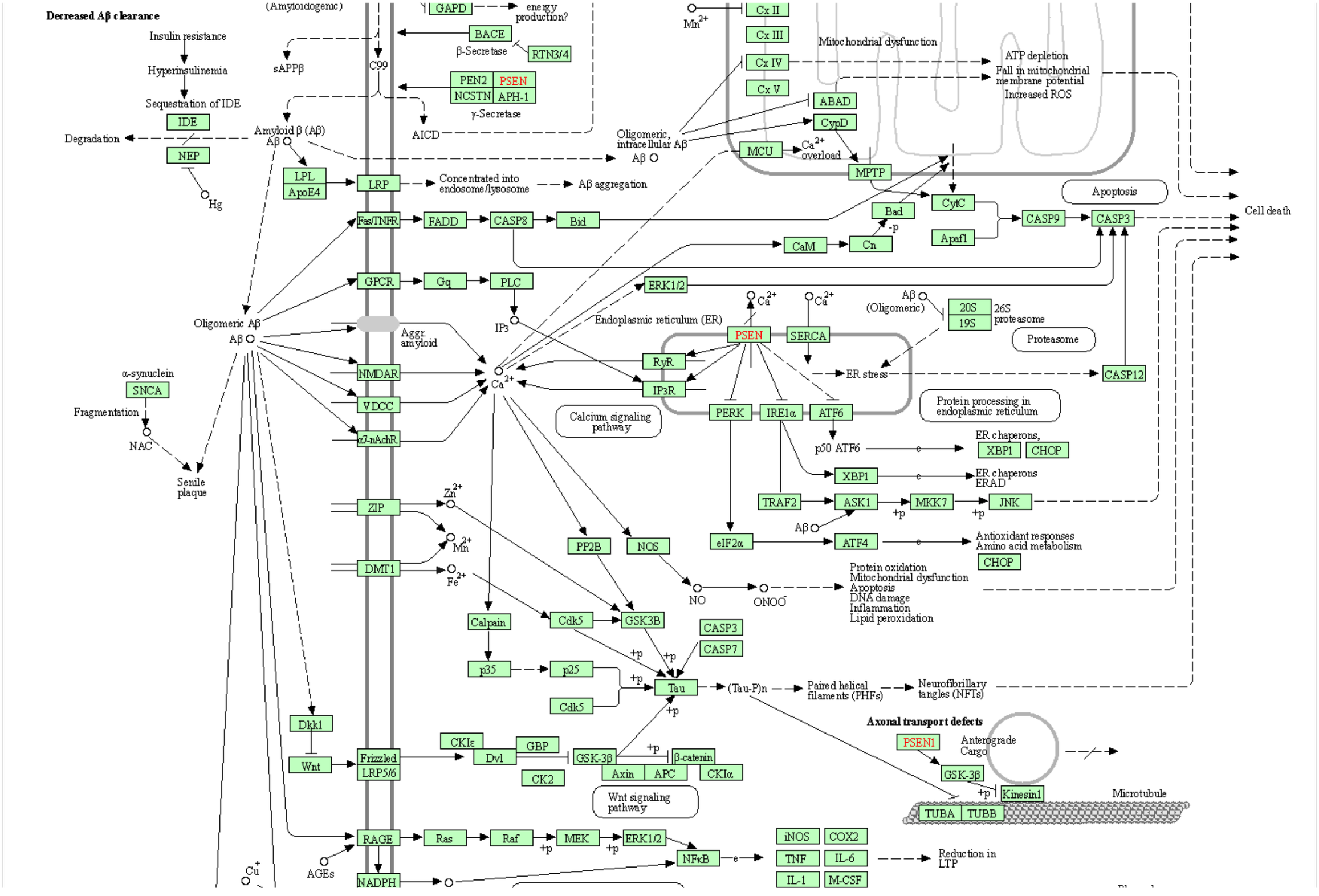
KEGG pathway database

https://www.genome.jp/kegg/pathway.html

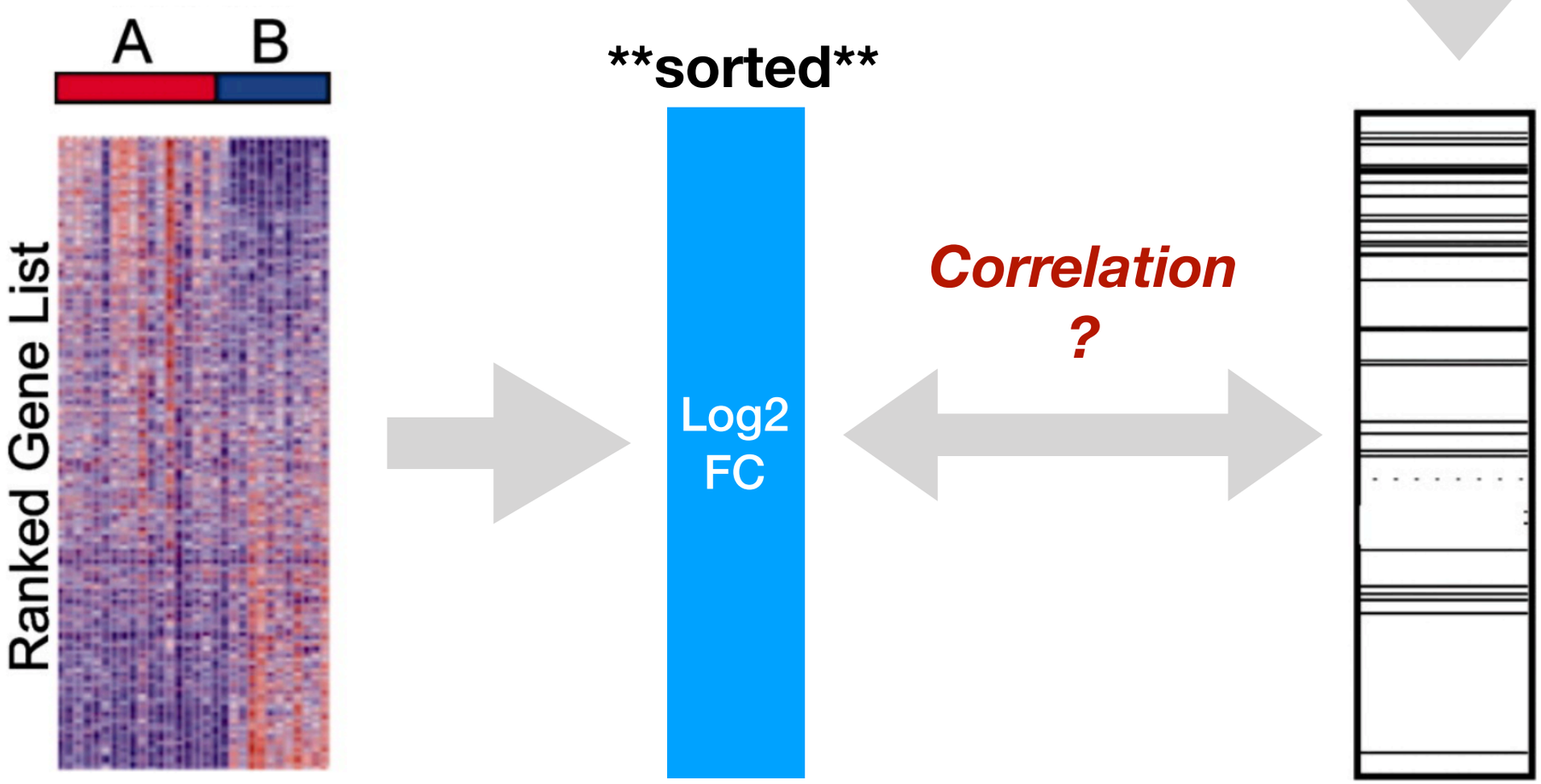
KEGG PATHWAY is a collection of manually drawn pathway maps representing our knowledge of the molecular interaction, reaction and relation networks for:

1. Metabolism
2. Genetic Information Processing
3. Environmental Information Processing
4. Cellular Processes
5. Organismal Systems
6. Human Diseases
7. Drug Development




Decreased Aβ clearance
Insulin resistance
Hyperinsulinemia
Sequestration of IDE
IDE
Degradation
NEP
Hg
(Amyloidogenic)
sAPPβ
C99
AICD
GAPD
energy production?
BACE
β-Secretase
RTN3/4
PEN2
PSEN
NCSTN
APH-1
γ-Secretase
Amyloid β (Aβ)
Aβ
LPL
ApoE4
LRP
Concentrated into endosome/lysosome
Aβ aggregation
Fas/TNFR
FADD
CASP8
Bid
GPCR
Gq
PLC
IP3
Oligomeric Aβ
Aggr. amyloid
NMDAR
VDCC
α7-nAchR
Ca2+
α-synuclein
SNCA
Fragmentation
NAC
Senile plaque
ZIP
Zn2+
Mn2+
DMT1
Fe2+
Cu+
AGEs
RAGE
Ras
Raf
MEK
ERK1/2
NADPH
PP2B
NOS
NO
ONOO-
Protein oxidation
Mitochondrial dysfunction
Apoptosis
DNA damage
Inflammation
Lipid peroxidation
Calpain
Cdk5
GSK3B
CASP3
CASP7
p35
p25
Cdk5
Tau
(Tau-P)n
Paired helical filaments (PHFs)
Neurofibrillary tangles (NFTs)
Dkk1
Wnt
Frizzled
LRP5/6
CKIε
Dvl
GBP
CK2
GSK-3β
Axin
APC
CKIα
β-catenin
Wnt signaling pathway
Axonal transport defects
PSEN1
Anterograde Cargo
GSK-3β
Kinesin1
Microtubule
TUBA
TUBB
NFκB
iNOS
COX2
TNF
IL-6
IL-1
M-CSF
Reduction in LTP
Mn2+
Cx II
Cx III
Cx IV
Cx V
Mitochondrial dysfunction
ATP depletion
Fall in mitochondrial membrane potential
Increased ROS
ABAD
CypD
MCU
Ca2+ overload
MPTP
Oligomeric, intracellular Aβ
Aβ
CytC
Bad
-p
Apaf1
CaM
Cn
Apoptosis
CASP9
CASP3
Cell death
Endoplasmic reticulum (ER)
Ca2+
PSEN
SERCA
RyR
IP3R
Calcium signaling pathway
Aβ (Oligomeric)
20S
19S
26S proteasome
Proteasome
ER stress
CASP12
Protein processing in endoplasmic reticulum
PERK
IRE1α
ATF6
p50 ATF6
XBP1
CHOP
ER chaperons, ERAD
TRAF2
ASK1
MKK7
JNK
Aβ
eIF2α
ATF4
Antioxidant responses
Amino acid metabolism
CHOP

Gene Set Enrichment Analysis (GSEA)



GSEA enrichment score

Ranked Gene List (L)

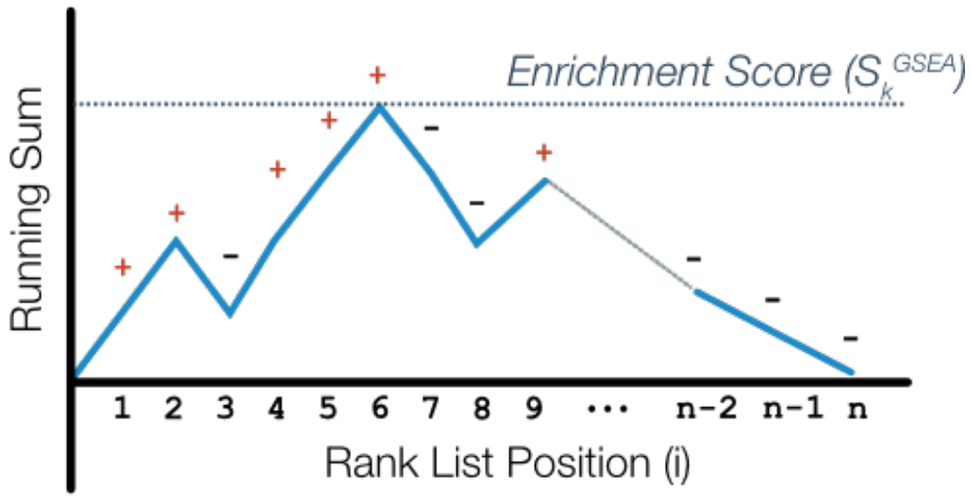
	Gene	Rank Metric (s)
1	*E2f3*	18.63
2	*Myc*	16.05
3	*Laminin*	15.11
4	*E2f2*	15.10
5	*Rb*	14.99
6	*E2f1*	14.77
7	*Acconitase*	14.65
8	*TNF-alpha*	14.26
9	*Cdk4*	13.89
	...	...
i	$gene_i$	s_i
	...	...
n-2	*Tgfb1*	-34.47
n-1	*Nos*	-34.94
n	*Pp2a*	-35.14

Gene Set (G_k)
'Cell Cycle'

	Gene
1	*E2f1*
2	*E2f2*
3	*E2f3*
4	*Rb*
5	*p130*
6	*CyclinD*
7	*CyclinE*
8	*Cdk2*
9	*Cdk4*
	...
j	$gene_j$
	...
n_k-1	*Cdk6*
n_k	*Myc*

Gene set membership

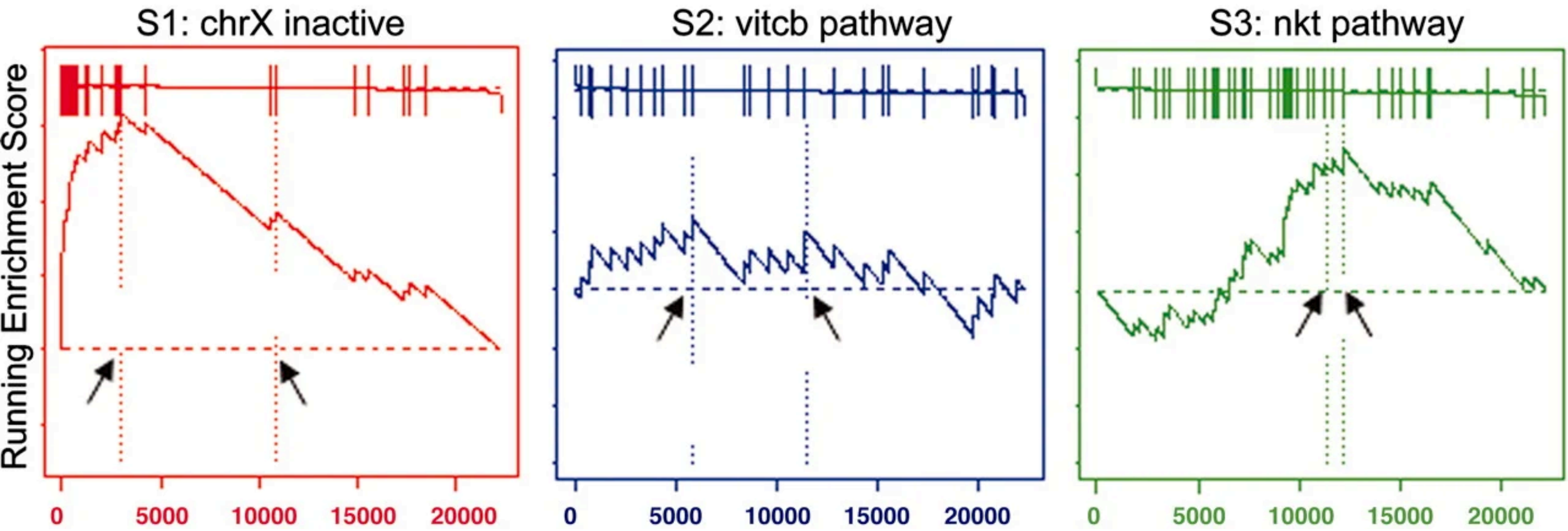
	Gene	Rank Metric (s)
1	***E2f3***	18.63
2	***Myc***	16.05
3	*Laminin*	15.11
4	***E2f2***	15.10
5	***Rb***	14.99
6	***E2f1***	14.77
7	*Acconitase*	14.65
8	*TNF-alpha*	14.26
9	***Cdk4***	13.89
	...	...
i	$gene_i$	s_i
	...	...
n-2	*Tgfb1*	-34.47
n-1	*Nos*	-34.94
n	*Pp2a*	-35.14



https://www.pathwaycommons.org/guide/primers/data_analysis/gsea/

GSEA enrichment examples

"the list of genes in the male/female lymphoblastoid cell line example ranked by their correlation with gender"



Subramanian et PNAS 2005, 102 (43) 15545-15550; DOI: 10.1073/pnas.0506580102

Readings

- **Teaching materials at the Harvard Chan Bioinformatics Core (recommended)**
https://github.com/hbctraining/DGE_workshop_salmon_online/blob/master/schedule/links-to-lessons.md

- **UC-Davis Workshop material**
https://ucdavis-bioinformatics-training.github.io/2019_March_UCSF_mRNAseq_Workshop/data_reduction/alignment.html

- **Galaxy training**
https://training.galaxyproject.org/archive/2019-02-07/topics/transcriptomics/tutorials/ref-based/tutorial.html

- **Read each tool's manual (at least one time, strongly recommended) !!**