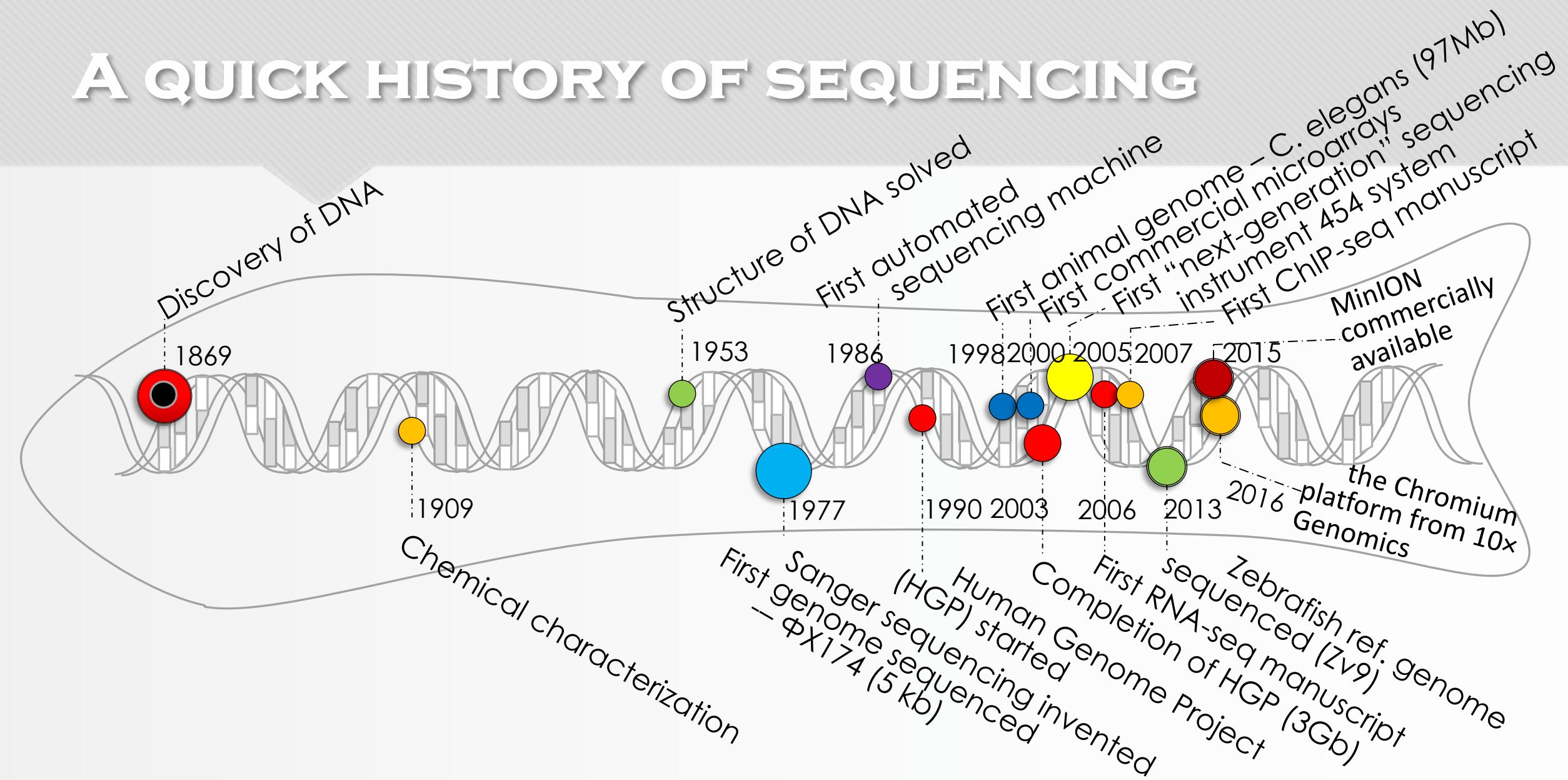


RNA-SEQ, CHIP-SEQ, AND ATAC-SEQ ANALYSIS PRIMER

Jianhong Ou @ DUKE, 2022



A QUICK HISTORY OF SEQUENCING



3 MAIN STEPS FOR RNA-SEQ AND CHIP-SEQ

01

Experimental design

Library type / strategy
Sequencing depth
Number of replicates
Batch effect

Consult a data analyst or bioinformatician to make sure robust experimental design

02

Bench work

RNA/DNA extraction
Library preparation
Sequencing & imaging

Double check
1. Library prep batch
2. Layout of library on lane/
flow cell

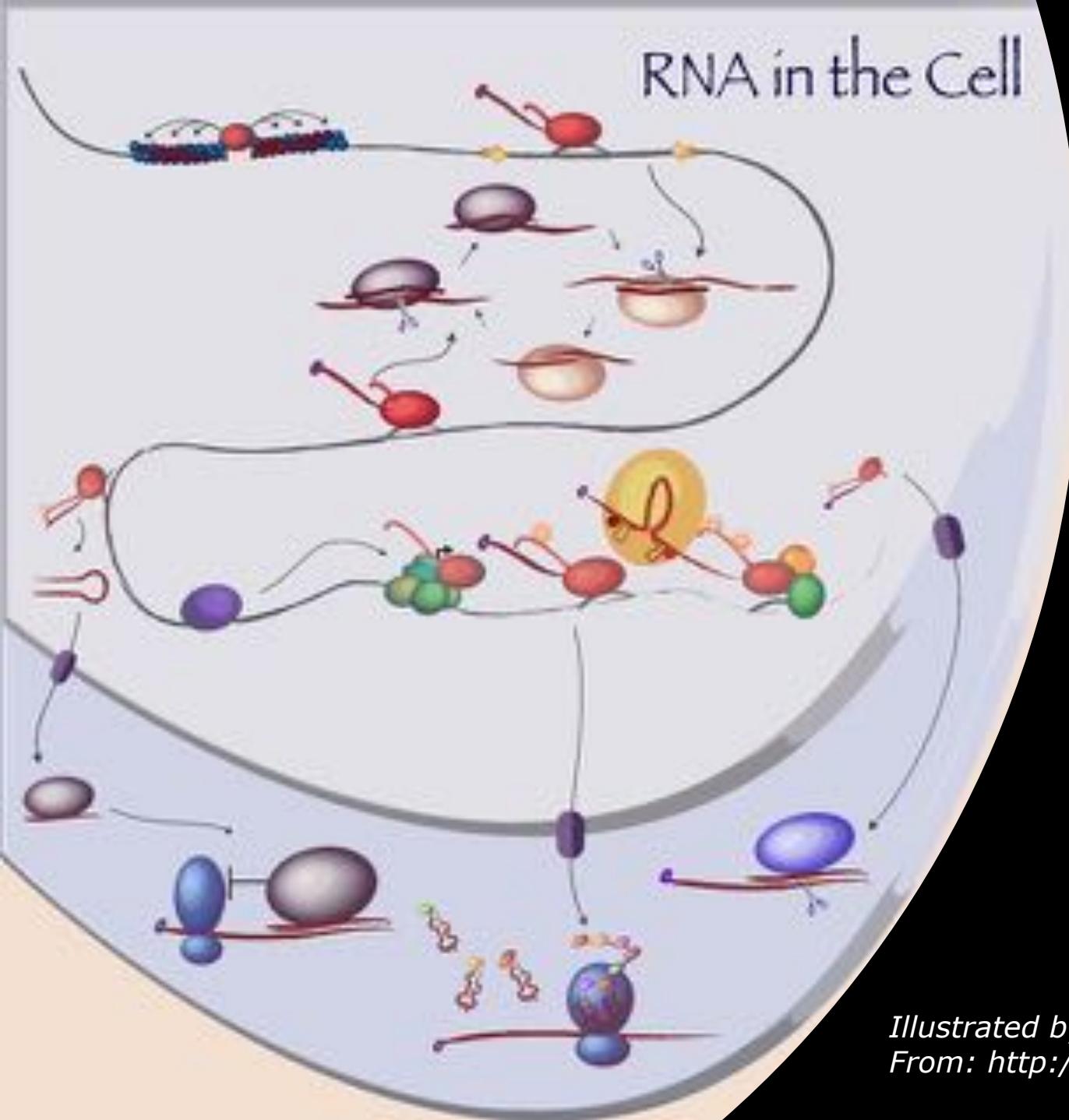
03

Data analysis

Quality control
Alignment & Quantification
Differential Expression test
Functional profiling

Discuss and optimize the analysis to meet your experiment design
1. Blind analysis to avoid the bias
2. Discuss in time to optimize the analysis

RNA-SEQ



THE TRANSCRIPTOME IS VERY COMPLEX

*Illustrated by Mary Lindstrom; designed by M.L. and Amy White
From: <http://web.mit.edu/sharplab/researchsummary.html>*

coding RNA

mRNA

Most are poly-adenylated

Non-coding RNA

Small RNAs

- siRNA
- miRNA
- snoRNA
- snRNA

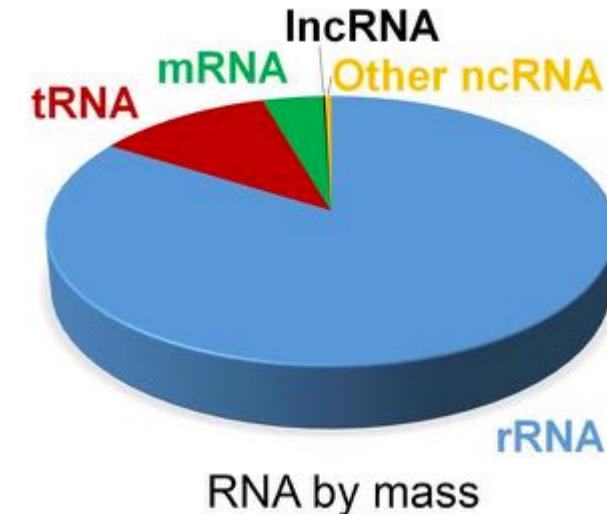
Transcriptional RNAs

- rRNA
- tRNA

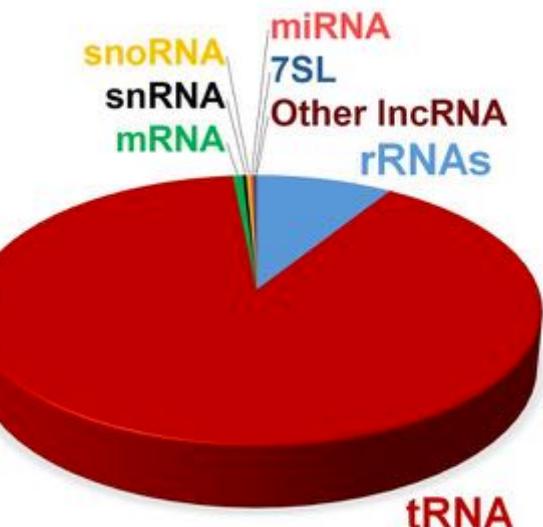
lncRNAs

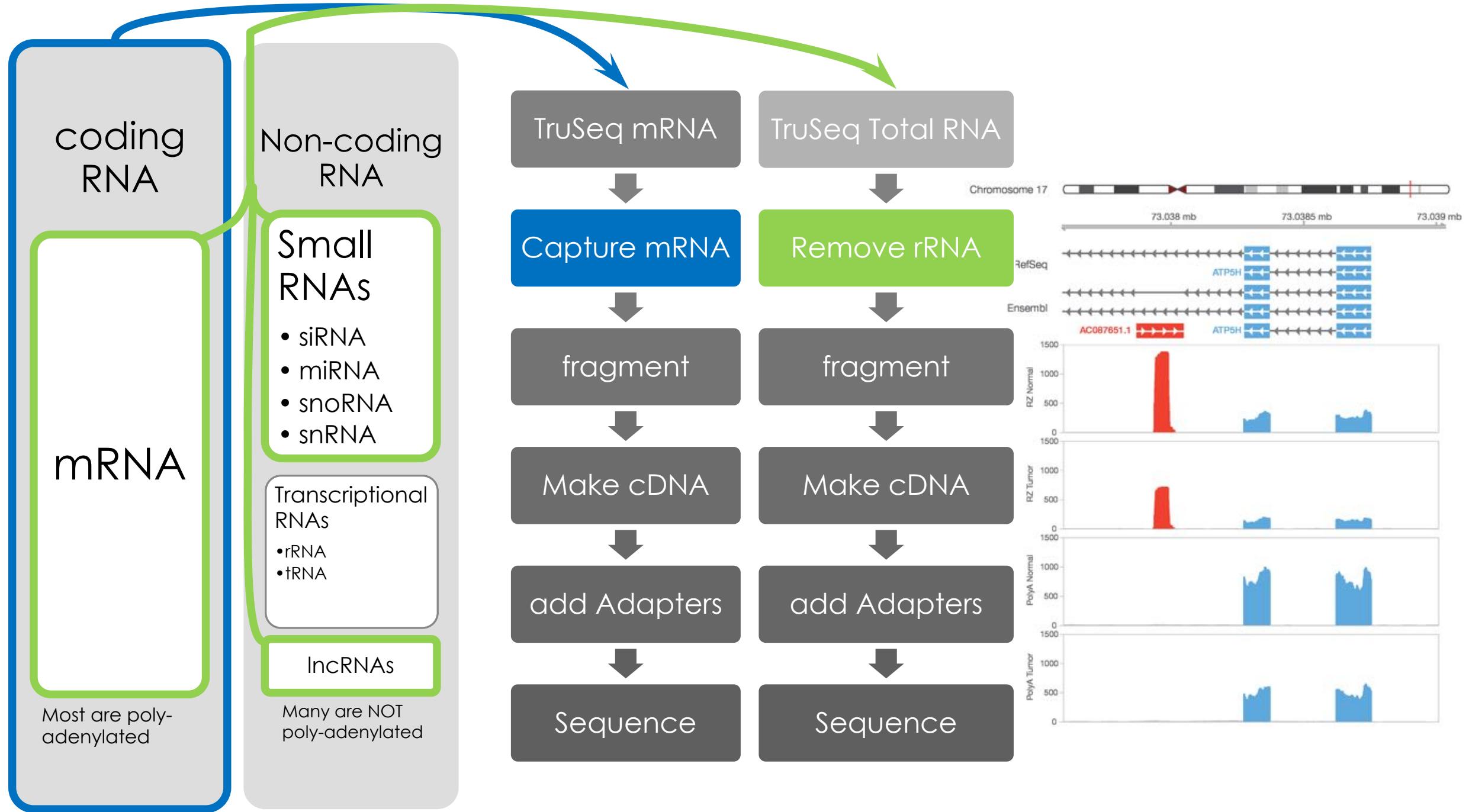
Many are NOT poly-adenylated

A

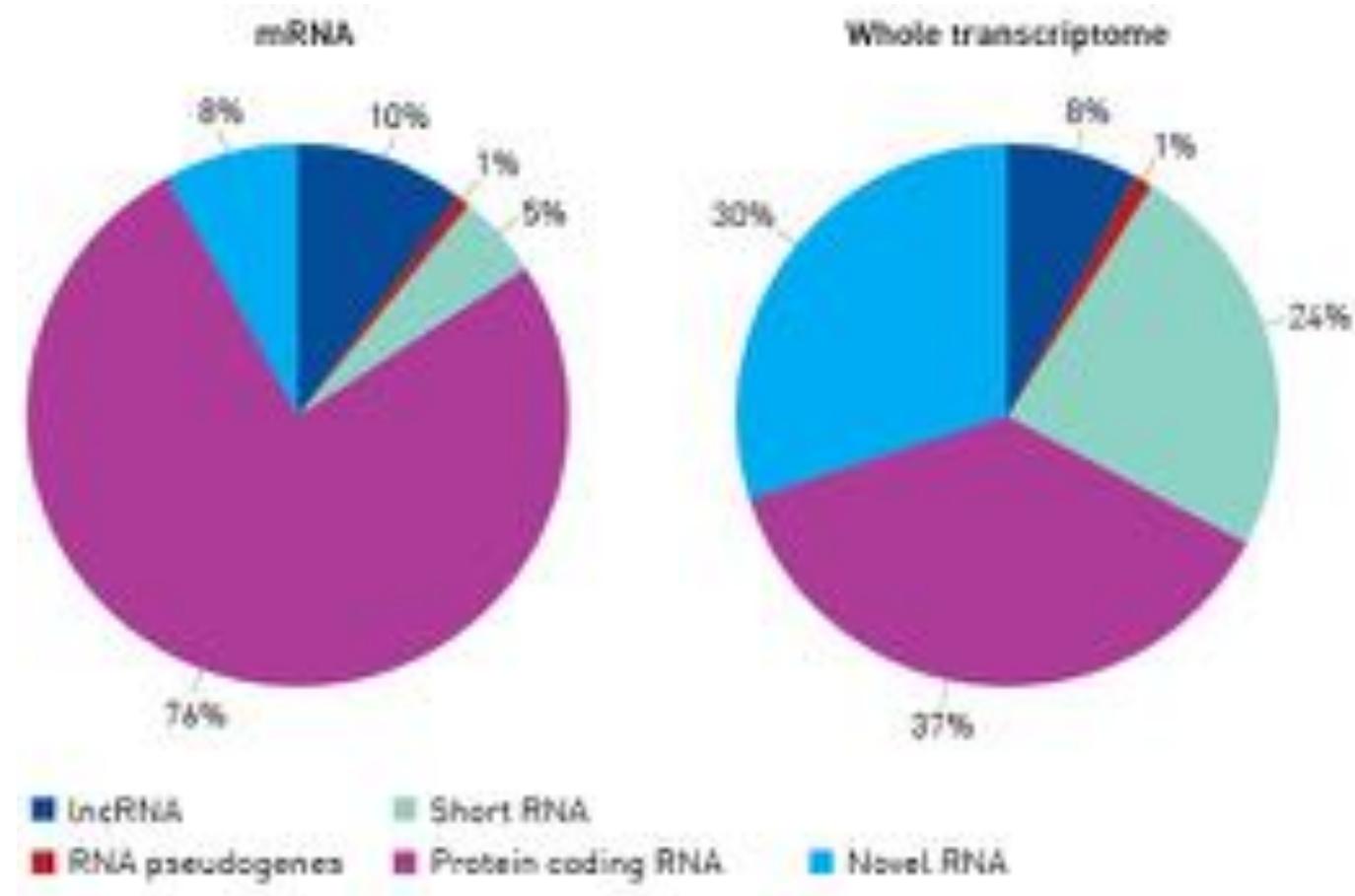


B

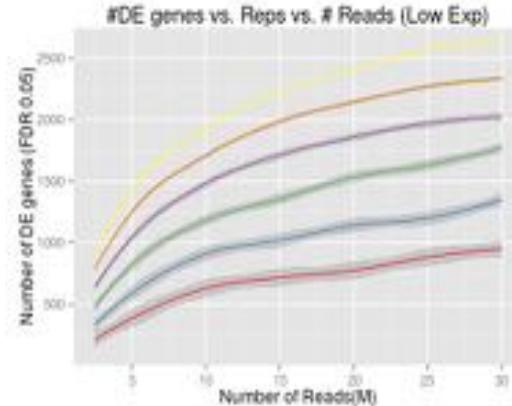
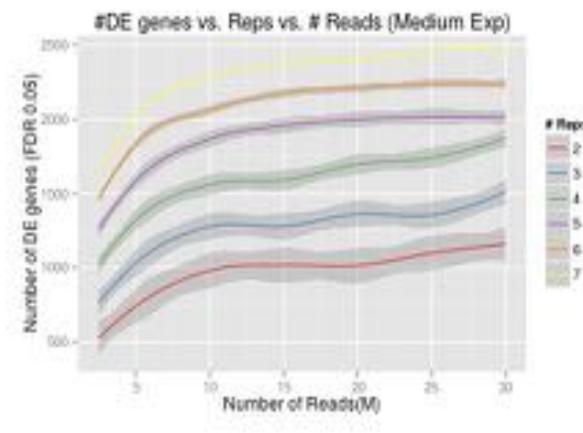
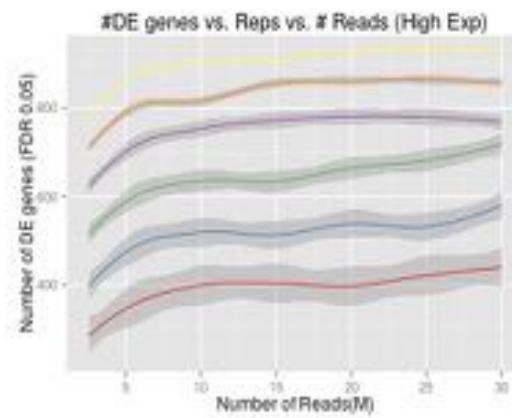




Distribution of RNA species in NGS samples after poly-A enrichment (mRNA) and rRNA depletion (whole transcriptome) sequencing



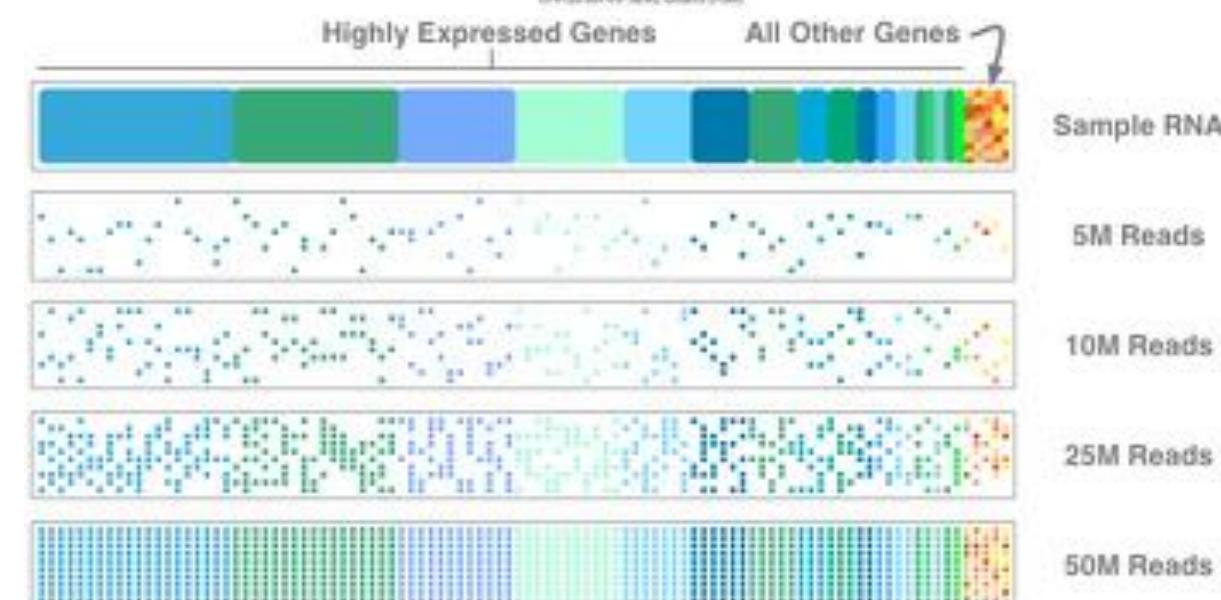
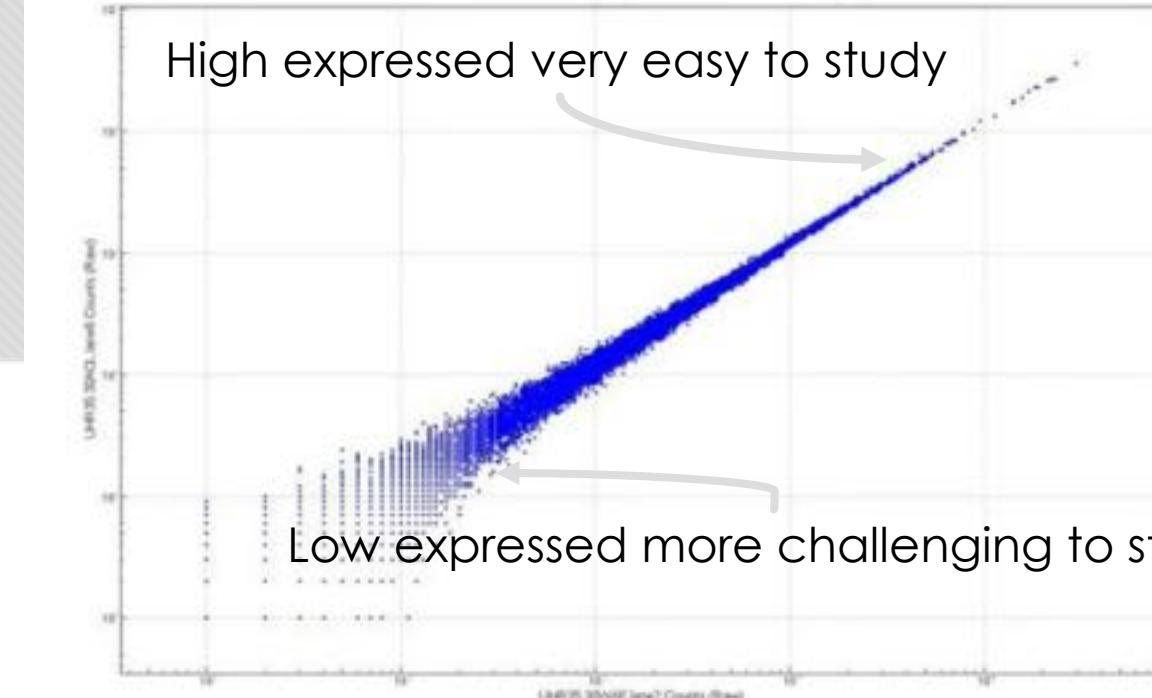
How many reads?



In most cases, increasing replicates is more beneficial than increasing sequencing depth.

High expressed very easy to study

Low expressed more challenging to study



Single End vs Paired End

- Paired end (PE) sequencing improves mapping
 - to repeat sequences in the genome
 - across exon-exon junctions
- Single-end (SE) sequencing is cheaper than PE.

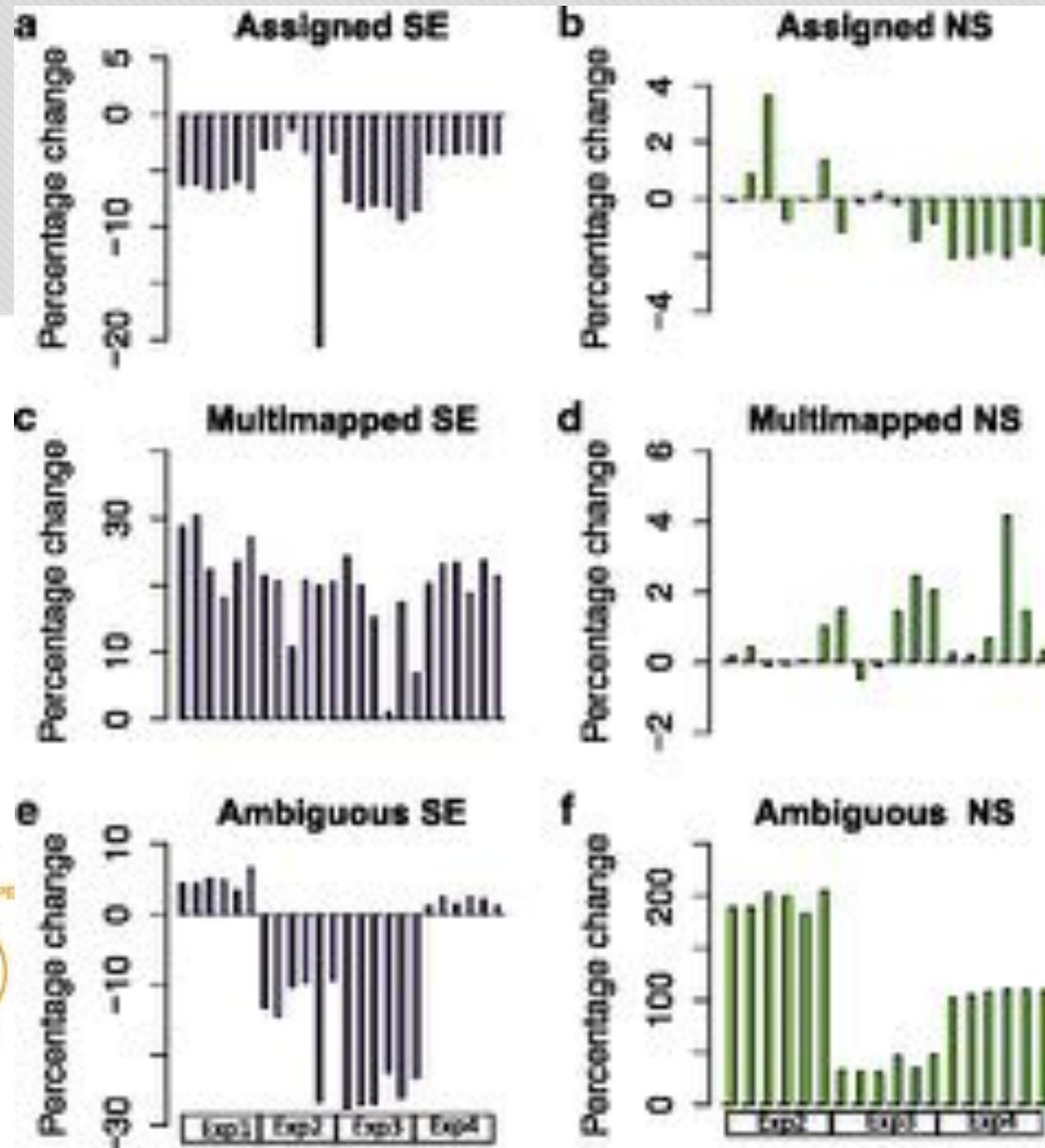
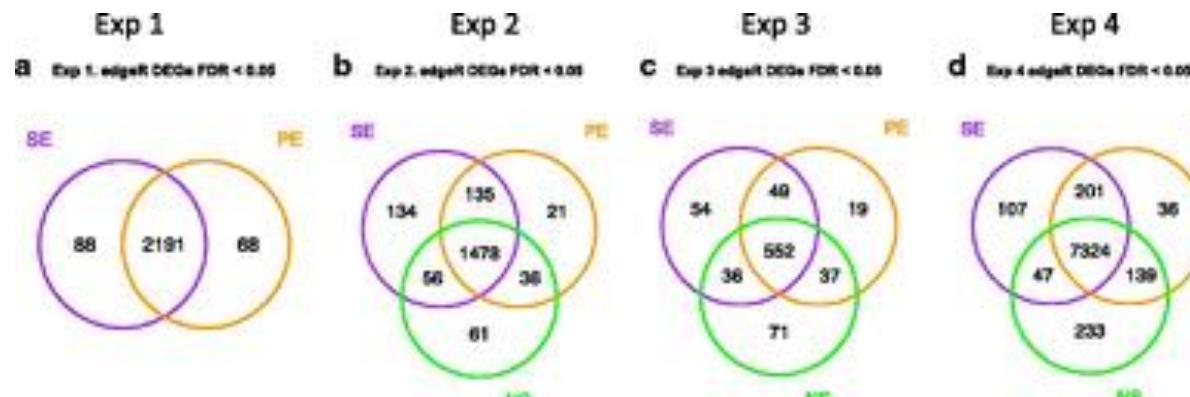


Table 1: Recommended sequencing depths for typical RNA-seq experiments for different genome sizes (Genohub, 2015). DGE = differential gene expression, SR = single read, PE = paired-end.

	Small (bacteria)	Intermediate (fruit fly, worm)	Large (mouse, hu- man)
No. of reads for DGE ($\times 10^6$)	5 SR	10 SR	20–50 SR
No. of reads for <i>de novo</i> transcriptome assembly ($\times 10^6$)	30–65 PE	70–130 PE	100–200 PE
Read length (bp)	50	50–100	>100

For a basic RNA-seq differential expression experiment, 10M to 20M single end reads per sample is usually enough

VARIANCE AND BLOCKING

Good experimental designs mitigate experimental error and the impact of factors not under study.

Krzywinski et.al., 2014 doi: 10.1038/nmeth.3005

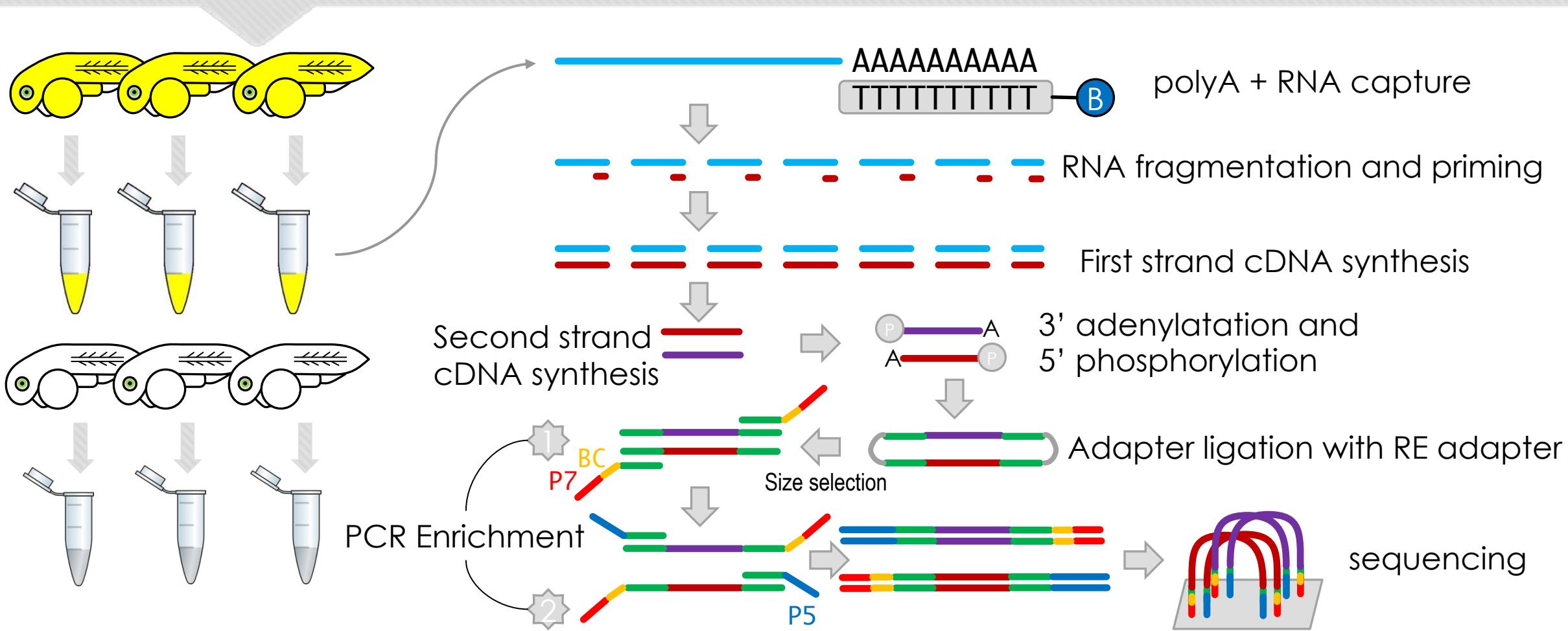
DO

- Choosing organisms of **similar genetic background** (littermates)
- Choosing organisms of the **same sex** if possible
- Using a **constant sample collection time and sampling sites on tissues**.
- Having the **same laboratory technician** perform RNA prep and library prep, same **lots of reagents, limit processing time range**
- If variation between samples can not be removed, use **balanced blocking design** and **modeling the block effect** during analysis
- Randomizing samples to prevent a **confounding batch** effect if all samples can't be processed at one time.

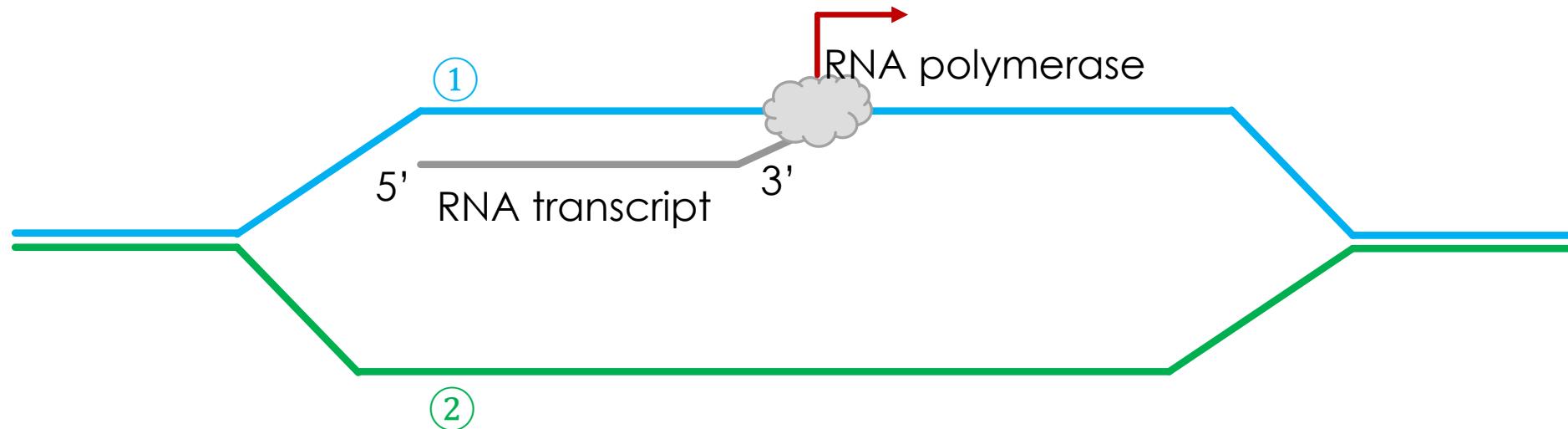
DON'T

- Do all controls in one day and all exp another day.
- Use different reagents or kits within one experiment batch

Illumina TruSeq RNA-seq protocol



Quiz



Which one is sense strand?

Non-stranded vs. stranded

5'-AUGCCAAGUACCA-3'

3'-TACGGTTCATGGT-5'
5'-AUGCCAAGUACCA-3'

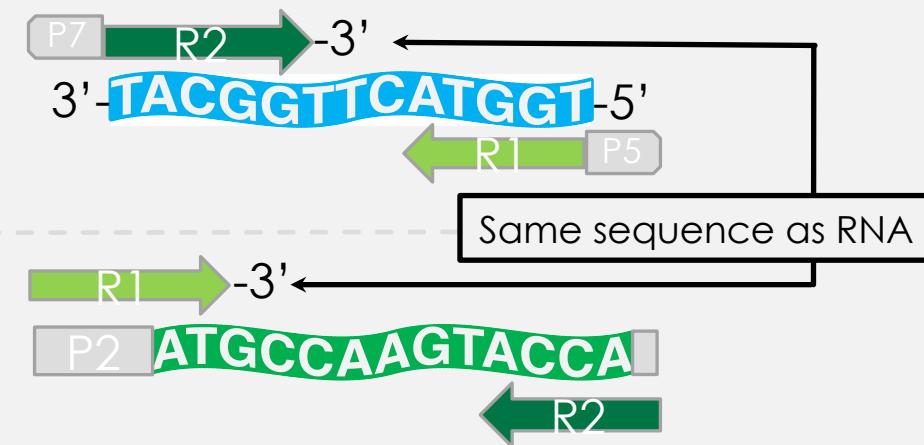
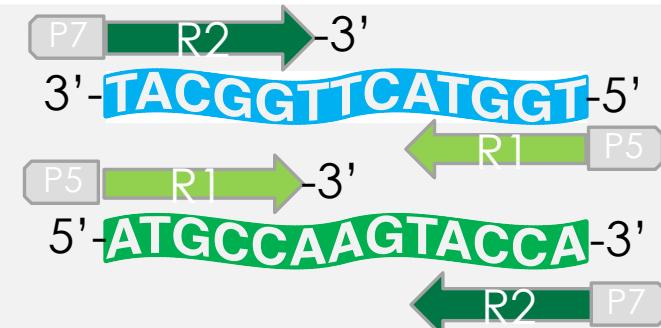
Non-stranded

3'-TACGGTTCATGGT-5'
5'-ATGCCAAGTACCA-3'

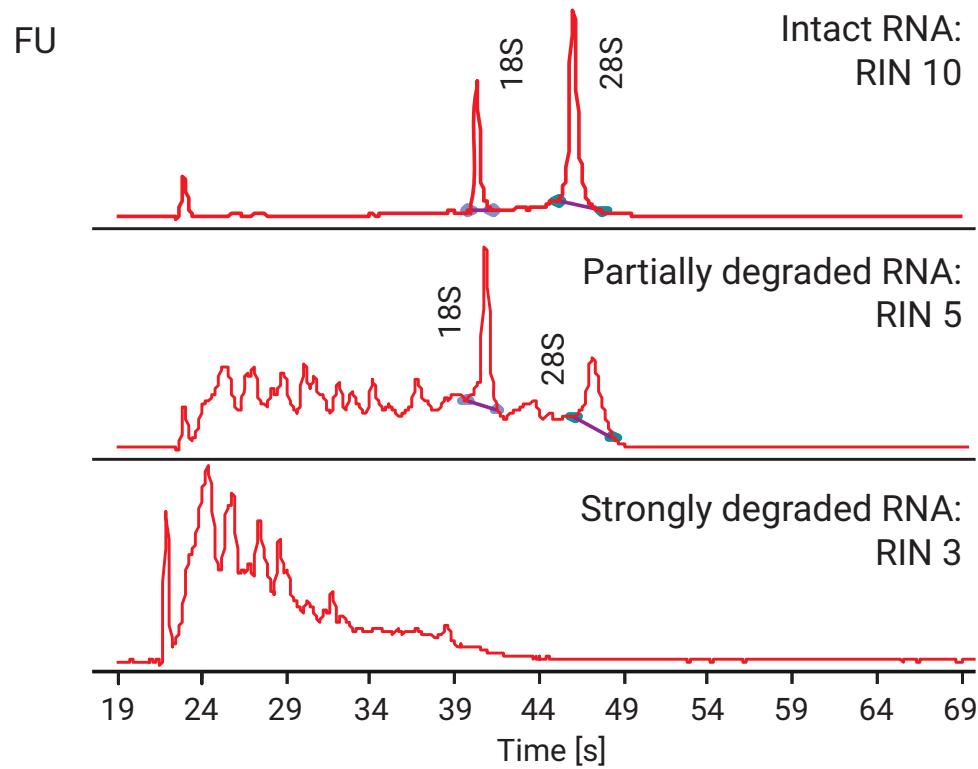
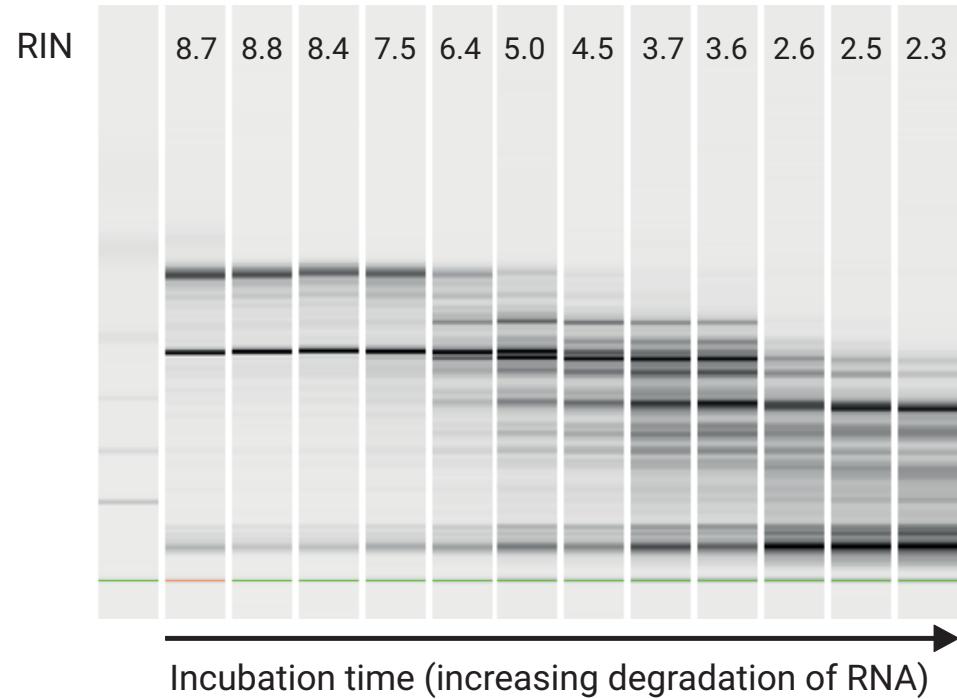
stranded

3'-TACGGTTCATGGT-5'
5'-AUGCCAAGUACCA-3'

P1 TACGGTTCATGGT
P2 ATGCCAAGTACCA



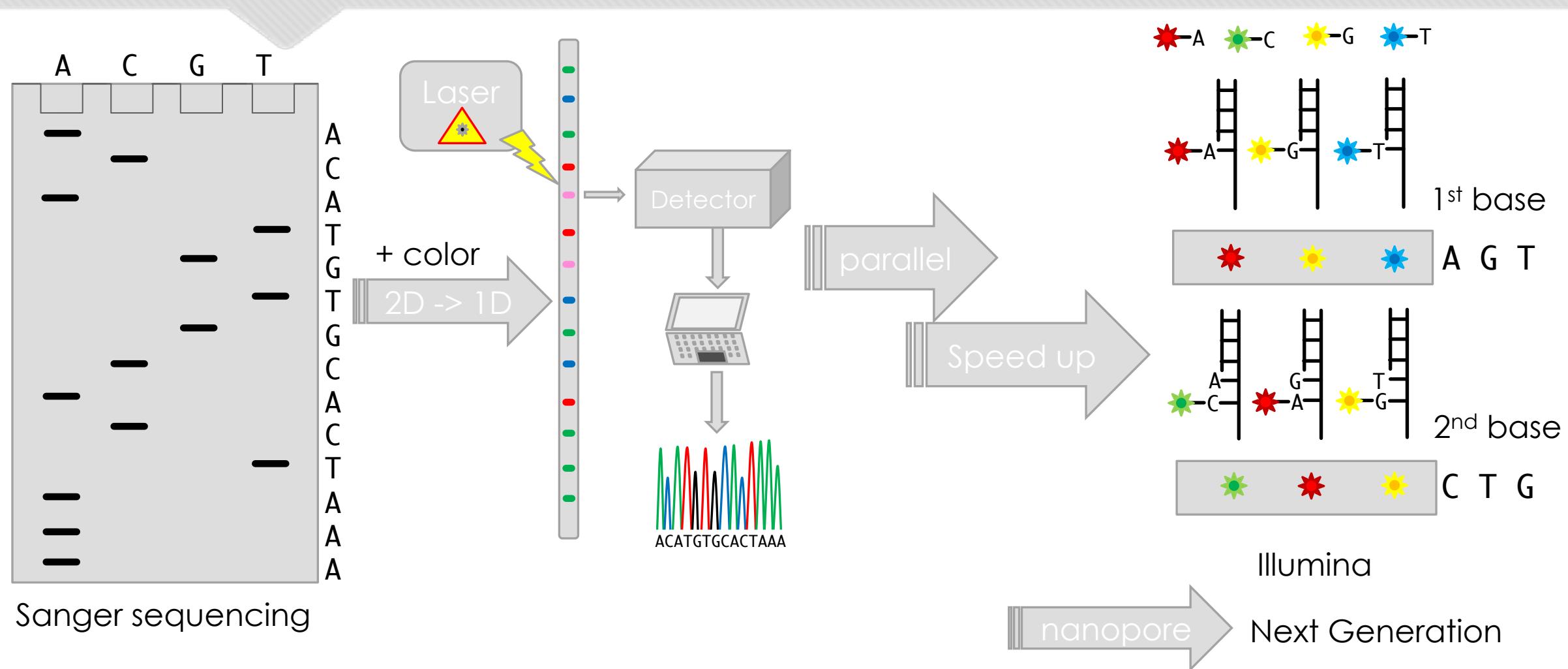
RNA QUANTITY AND QUALITY (RIN)



https://www.agilent.com/cs/library/applications/5991-8974EN_Bioanalyzer-TapeStation_appcompendium.pdf

Schroeder et al., 2006. doi:10.1186/1471-2199-7-3

Next Generation Sequencing (NGS)



RNA-seq data analysis pipeline

Reads

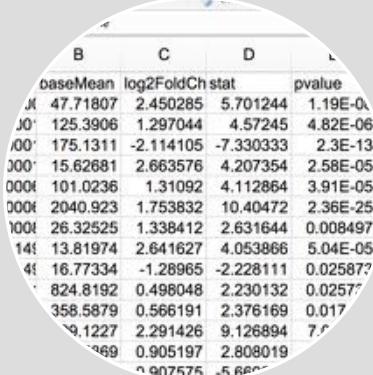


Alignments

Read counts

Gene	Read Count
a4a	11
oca5	1065
abcb3l1	1000
abcb4	3
abcb6a	144
abcb8	748
bcb9	217
cc2	237
1	8443

Differential Expression



PREPROCESS



Download data from Duke Data Service

Hello xxxxxxx,

You have accepted delivery of project xxxx.

You may access it via the following

link: <https://dataservice.duke.edu/#/project/xxxxxxxxxxxxxxxx>

You can download your data using the ddsclient command line tool. For setup and download instructions

see <http://seqweb.gcb.duke.edu/DataDelivery/NGSequencingCoreDataDownloadInstructionTitlePage.html>

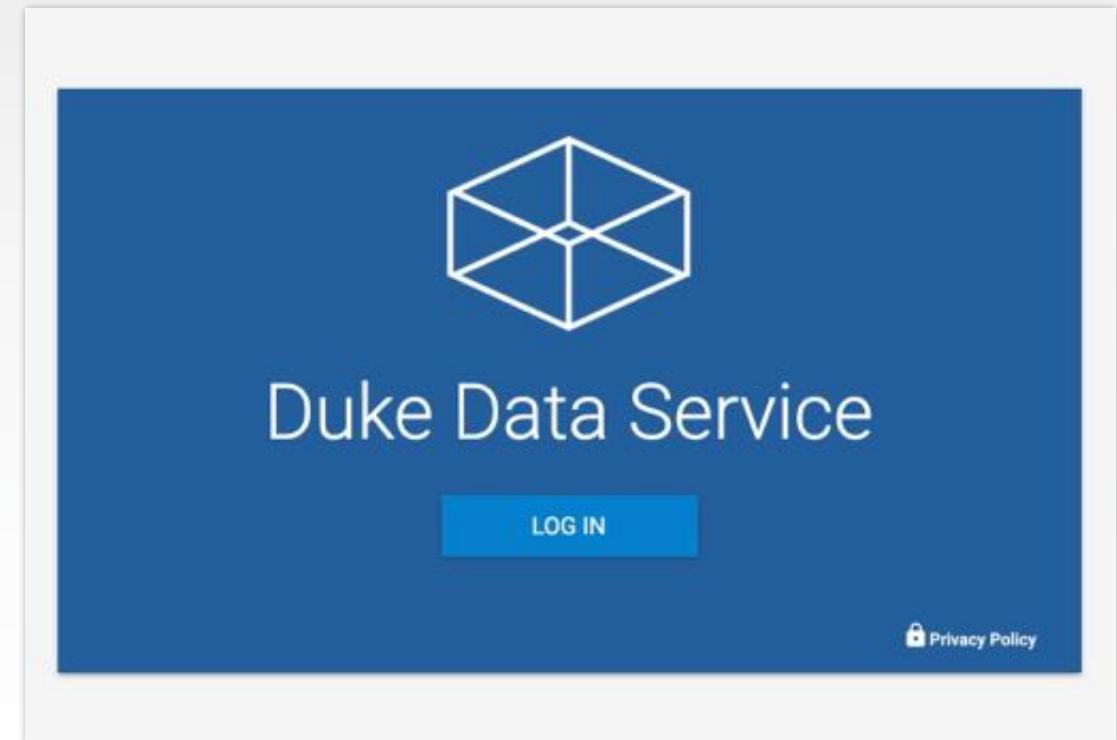
If you would like someone else to access (i.e download) the data, you can use the DDS Website (<https://dataservice.duke.edu>). Select the project you would like to share, click the triple dot icon and choose "Add Project Members". You can then enter a name and select "File Downloader" under Project Role. Click "ADD TEAM MEMBER" to commit the changes. You can repeat this if you'd like to share the project with other colleagues.

Warning: There is a known (and unfixed) bug in the DDS Website where you are unable add users that have ", Ph.D." at the end of their name.

Due to limited freezer space, all samples and libraries related to this order will be disposed of in 3 months. If you would like those samples or libraries back, please notify us by emailing sequencing@duke.edu

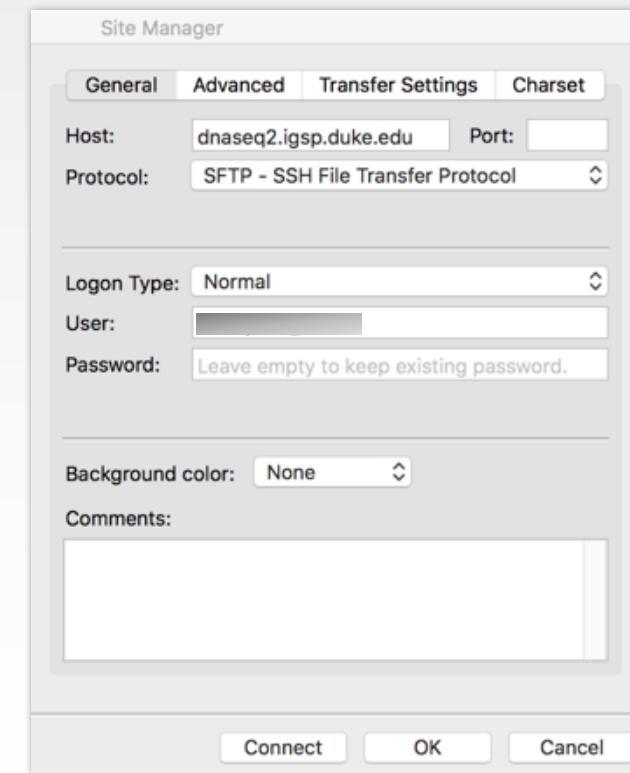
Please, let us know if you have any questions or concerns.

Best regards,



Install FileZilla and download a file From FTP

- Goto <https://software.duke.edu/>
- NetID Login
- Search for software: FileZilla
- Click FileZilla
- Click download here
- Download FileZilla Client
- Install FileZilla Client



Download data from aws s3 storage

- S3 console
- Rclone
- Cyberduck
- ExpanDrive



Sign in as IAM user

Account ID (12 digits) or account alias

IAM user name

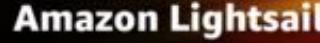
Password

Remember this account

Sign in

[Sign in using root user email](#)

[Forgot password?](#)



Amazon Lightsail

Lightsail is the easiest way
to get started on AWS

[Learn more »](#)



File formats: FASTA and FASTQ

○ FASTA file:

- Description line: each sequence started with a ">" symbol, and with or without sequence name
- Following the initial line is the actual sequences

○ FASTQ file:

- Line1: begins with a '@' character and is followed by a sequence identifier and an optional description
- Line2: is the raw sequence letters
- Line3: begins with a '+' character and is *optionally* followed by the same sequence identifier (and any description) again
- Line4: encodes the quality values for the sequence in Line 2, and must contain the same number of symbols as letters in the sequence

```
>seq1
ACGTACGTACGT
ACGTACGTACGT
>seq2
TGCATGCATGCA

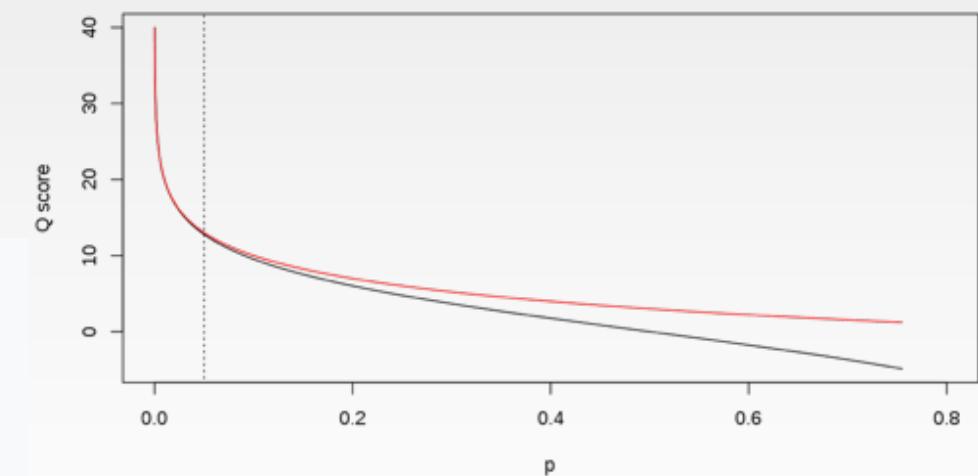
@seq1
ACGTACGTACGTACGTACGT
+
ABCDEFGHI!#%1234567890+-
@seq2
TGCATGCATGCA
+
ABCDEFGHI!#%
```

FASTQ: quality score

Quality

$$Q_{sanger} = -10 \log_{10} p \quad ; \quad Q_{solexa} = -10 \log_{10} \frac{p}{1-p}$$

Encoding



Relationship between Q and p using the Sanger (red) and Solexa (black) equations (described above). The vertical dotted line indicates $p = 0.05$, or equivalently, $Q \approx 13$.

Submitting high-throughput sequence data to Gene Expression Omnibus (GEO)

This screenshot shows the 'Raw Files' section of the GEO Metadata Template. It includes a table with columns for 'file name', 'file type', and 'file checksum'. The data is as follows:

file name	file type	file checksum
Run123abc.csfasta	solid_native_csfasta	6cc6ee3cce10b970e
Run123abc_QV.qual	solid_native_qual	88ceb0e0d056dda92
2011_01_gfh_qseq.txt	Illumina_native_qseq	95cf1d1fa509d871b2
DS18389-7_1.fastq	fastq	95cf1d1fa509d871b2
DS18389-7_2.fastq	fastq	0wd6ee3cce10b970e

This screenshot shows the 'Instrument Model' and 'Read Length' section of the GEO Metadata Template. It includes a table with columns for 'instrument model', 'read length', and 'single or paired-end'. The data is as follows:

instrument model	read length	single or paired-end
AB SOLiD System 3.0	50	single
AB SOLiD System 3.0	50	single
Illumina HiSeq 2000	72	single
Illumina HiSeq 2000	50	paired-end
Illumina HiSeq 2000	50	paired-end

File formats: md5 and compressed files

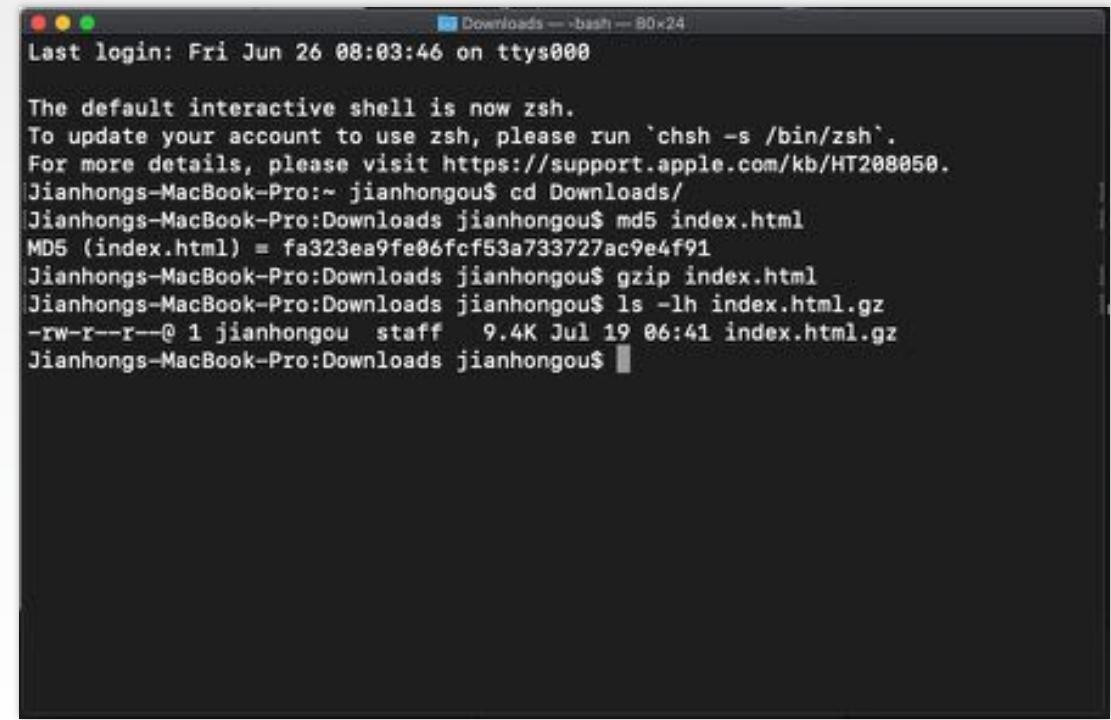
○ **md5**

- **Unix:** md5sum <file>
- **OS X:** md5 <file>
- **Windows:** Application required. Many are available for free download.
Eg: FCIV -md5 <file>; certUtil -hashfile <file> MD5

- **Data File Compression:** Individual files can be compressed to speed transfer, but this is not required. Acceptable compression formats are gzip and bzip2 (i.e. files ending with a .gz or .bz2 extension). Never compress binary files (e.g., BAM, bigWig, bigBed), and DO NOT upload ZIP archives (files with a .zip extension).

Exercises

- Get checksum for a file
- Compress and decompress a file



A screenshot of a Mac OS X terminal window titled "Downloads -- bash -- 80x24". The window shows a login message and then a series of shell commands. The commands include changing the default shell to zsh, navigating to the Downloads directory, calculating the MD5 checksum of "index.html", compressing it with gzip, and listing files with ls.

```
Last login: Fri Jun 26 08:03:46 on ttys000
The default interactive shell is now zsh.
To update your account to use zsh, please run `chsh -s /bin/zsh`.
For more details, please visit https://support.apple.com/kb/HT208050.
Jianhongs-MacBook-Pro:~ jianhongou$ cd Downloads/
Jianhongs-MacBook-Pro:Downloads jianhongou$ md5 index.html
MD5 (index.html) = fa323ea9fe06fcf53a733727ac9e4f91
Jianhongs-MacBook-Pro:Downloads jianhongou$ gzip index.html
Jianhongs-MacBook-Pro:Downloads jianhongou$ ls -lh index.html.gz
-rw-r--r--@ 1 jianhongou staff 9.4K Jul 19 06:41 index.html.gz
Jianhongs-MacBook-Pro:Downloads jianhongou$ █
```

Backup your data

- Keep two copies in physically different storage spaces with their checksum.
 - Keep it in the cloud: Duke's Box (50G), OneDrive (1TB)
 - External Hard Drive
 - Burn it to a Blu-ray Disc
 - Put it on a USB Flash Drive
 - Save it to Network Attached Storage (NAS) Device

FASTQC

Function

A quality control tool for high throughput sequence data.

Language

Java

Requirements

A suitable Java Runtime Environment

Code Maturity

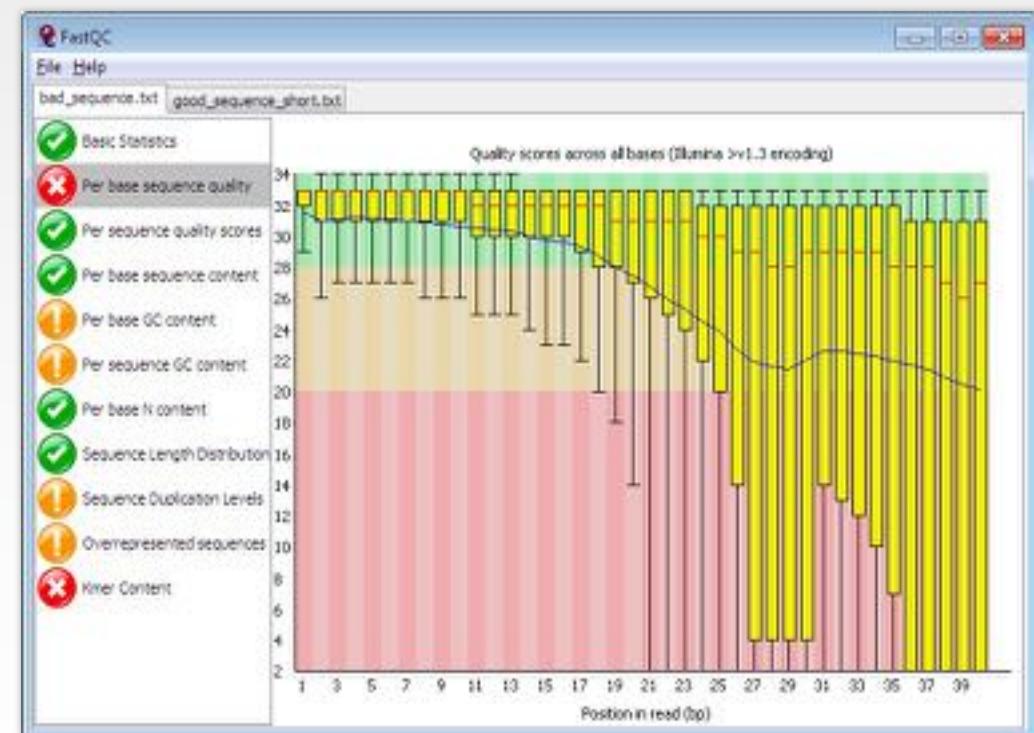
Stable.

Code Released

Yes, under GPL v3 or later.

Initial Contact

Simon Andrews

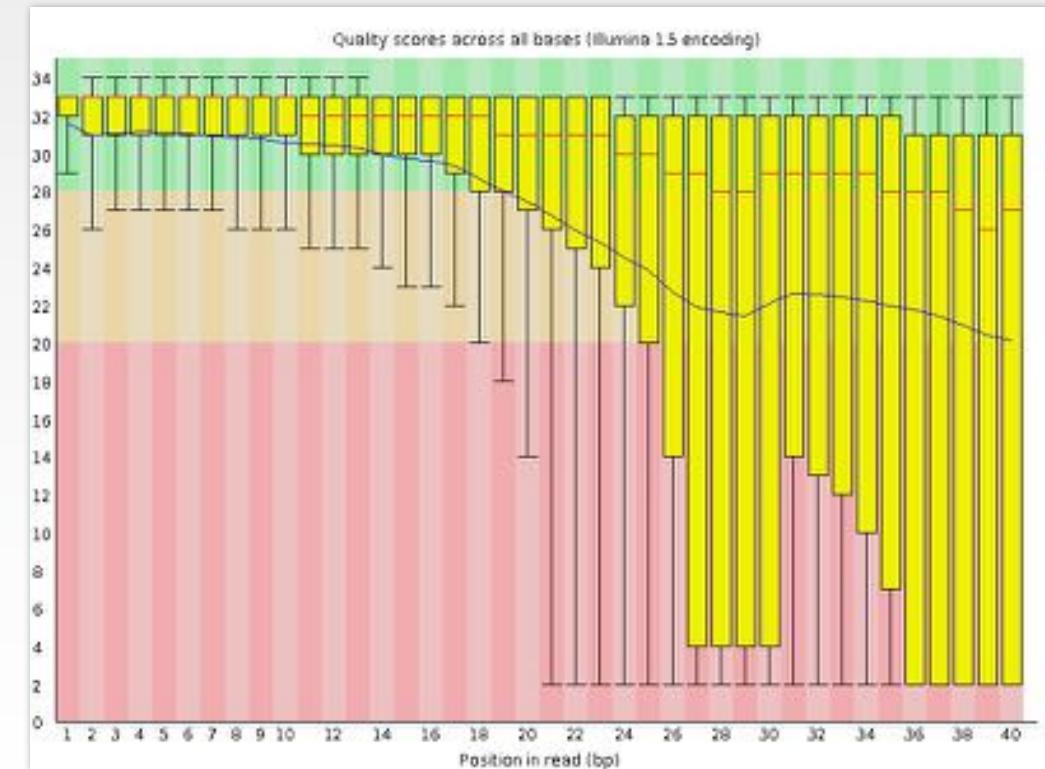
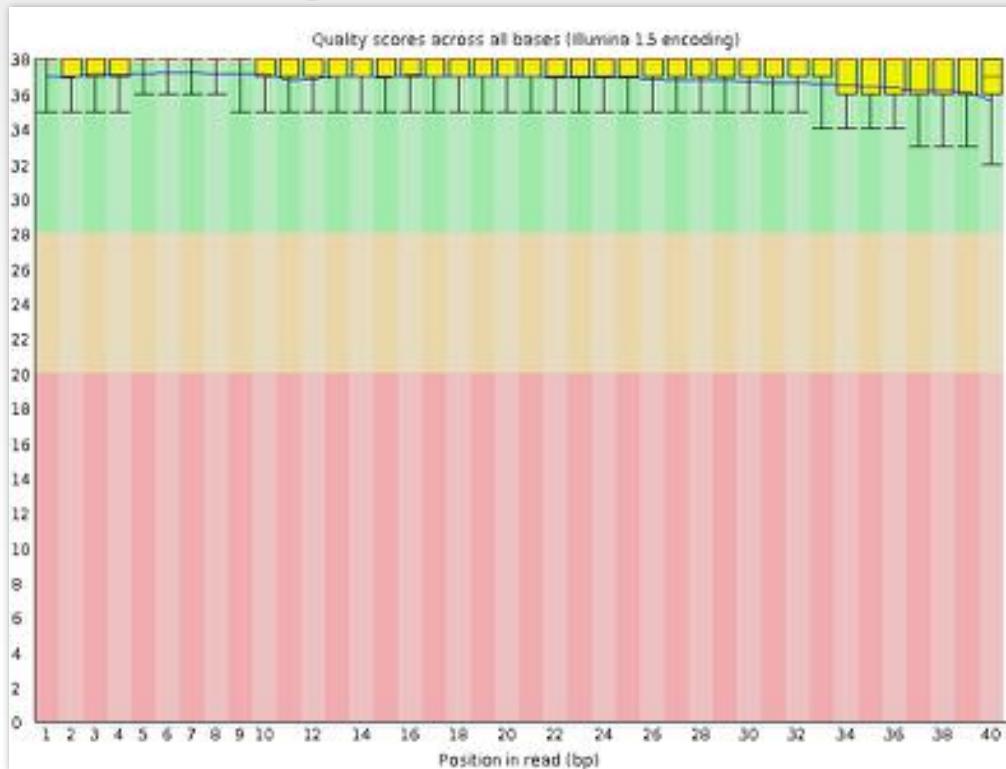


FASTQC: Basic Statistics

Measure	Value	Description
Filename	good_sequence_short.txt	The original filename of the file which was analysed
File type	Conventional base calls	Says whether the file appeared to contain actual base calls or colorspace data which had to be converted to base calls
Encoding	Illumina 1.5	Says which ASCII encoding of quality values was found in this file
Total Sequences	250000	A count of the total number of sequences processed
Sequences flagged as poor quality	0	A count of the number of sequences flagged as poor quality
Sequence length	40	Provides the length of the shortest and longest sequence in the set
%GC	45	The overall %GC of all bases in all sequences

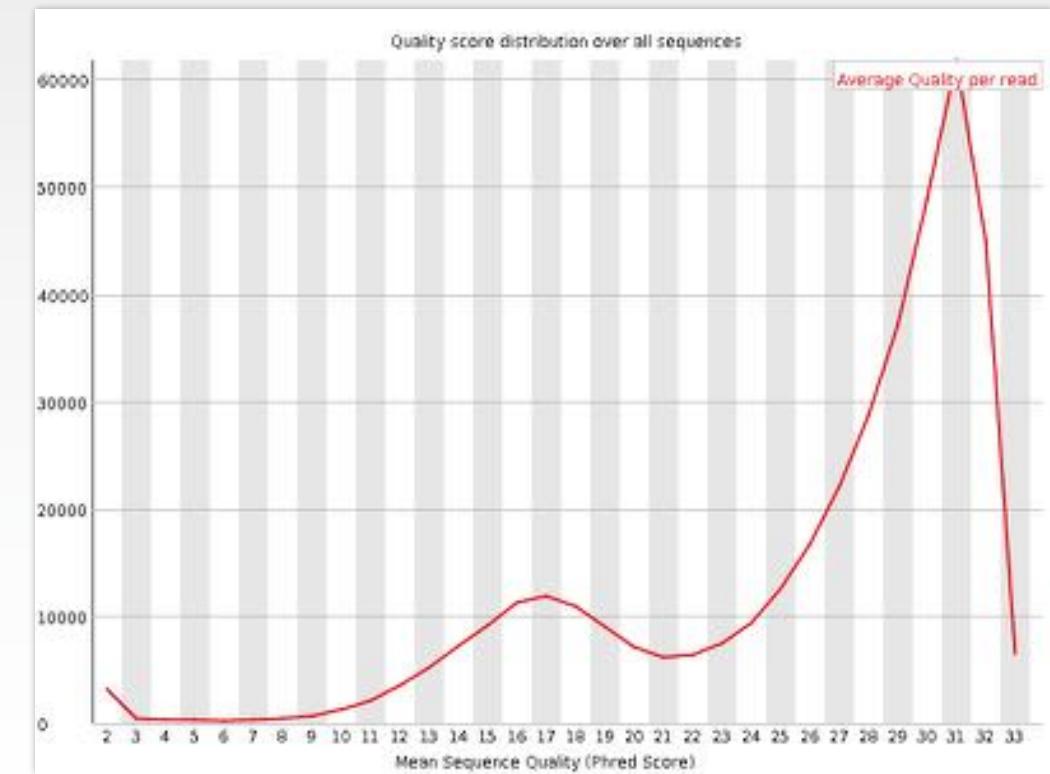
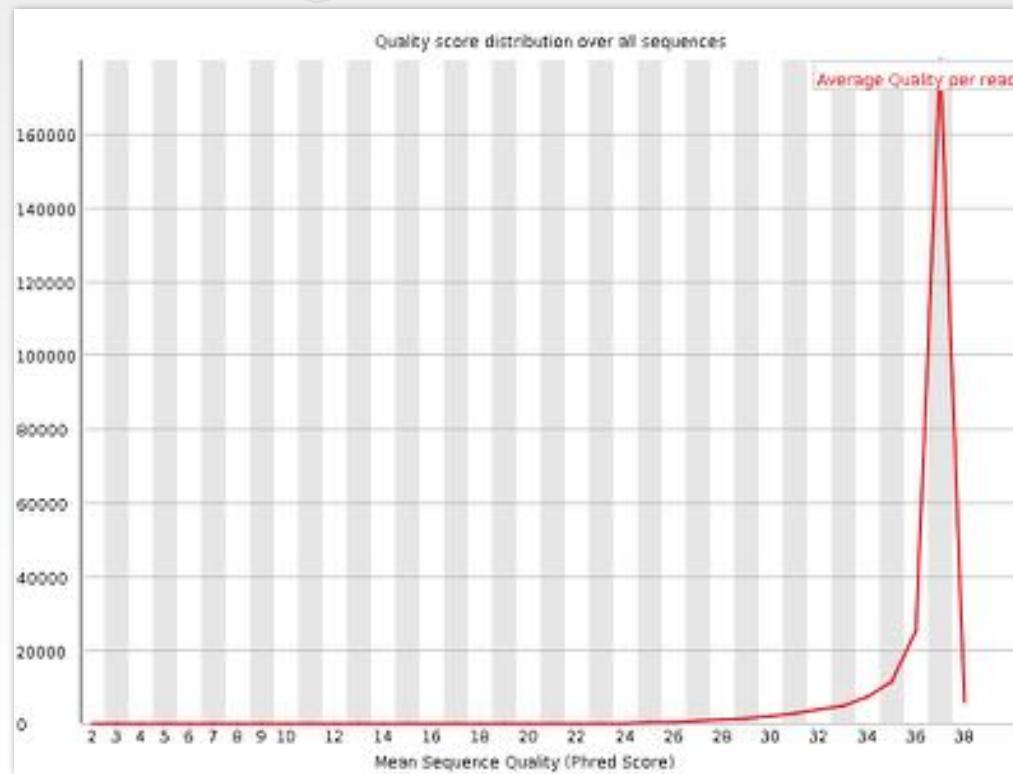
FASTQC: Per base sequence quality

$$Q_{sanger} = -10 \log_{10} p \quad ; \quad Q_{solexa} = -10 \log_{10} \frac{p}{1-p}$$



Warning: A warning will be issued if the lower quartile for any base is less than 10, or if the median for any base is less than 25.
Failure: This module will raise a failure if the lower quartile for any base is less than 5 or if the median for any base is less than 20.

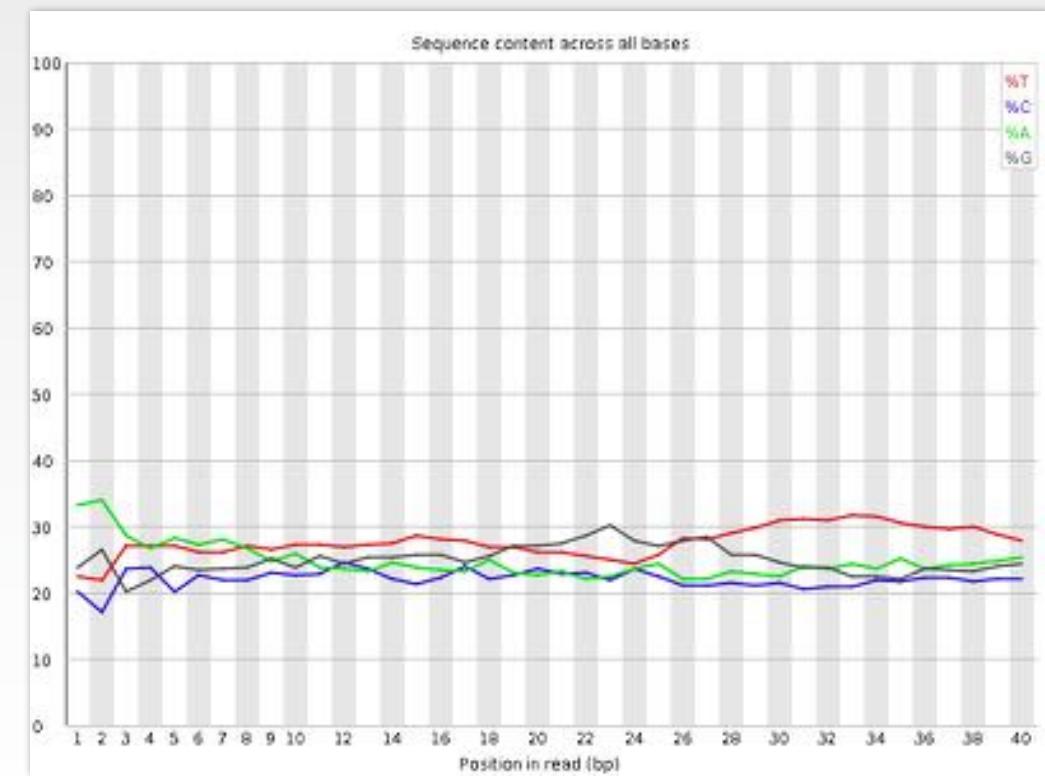
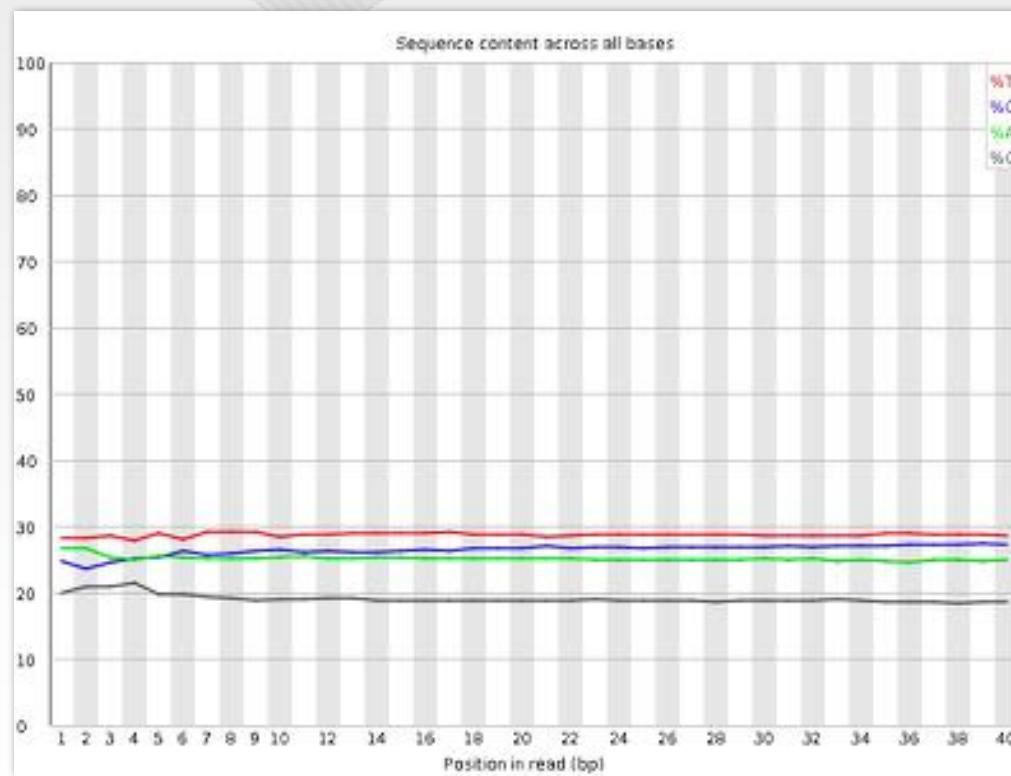
FASTQC: Per sequence quality scores



Warning: A warning is raised if the most frequently observed mean quality is below 27 - this equates to a 0.2% error rate.

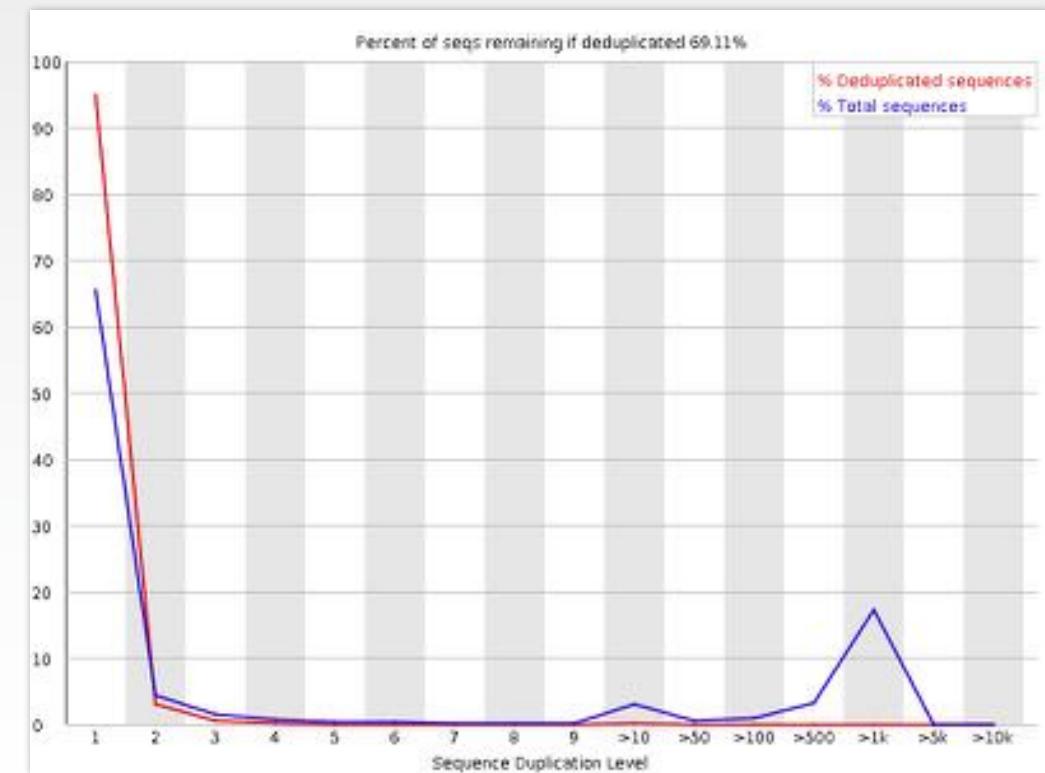
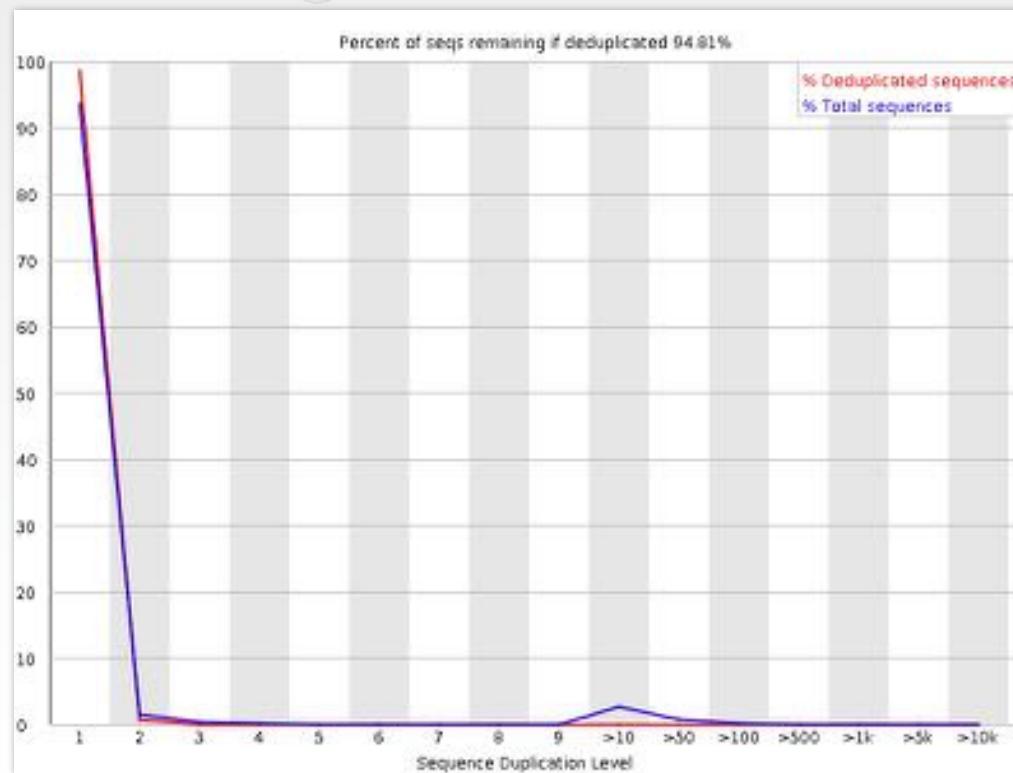
Failure: An error is raised if the most frequently observed mean quality is below 20 - this equates to a 1% error rate.

FASTQC: Per base sequence content



Warning: This module issues a warning if the difference between A and T, or G and C is greater than 10% in any position.
Failure: This module will fail if the difference between A and T, or G and C is greater than 20% in any position.

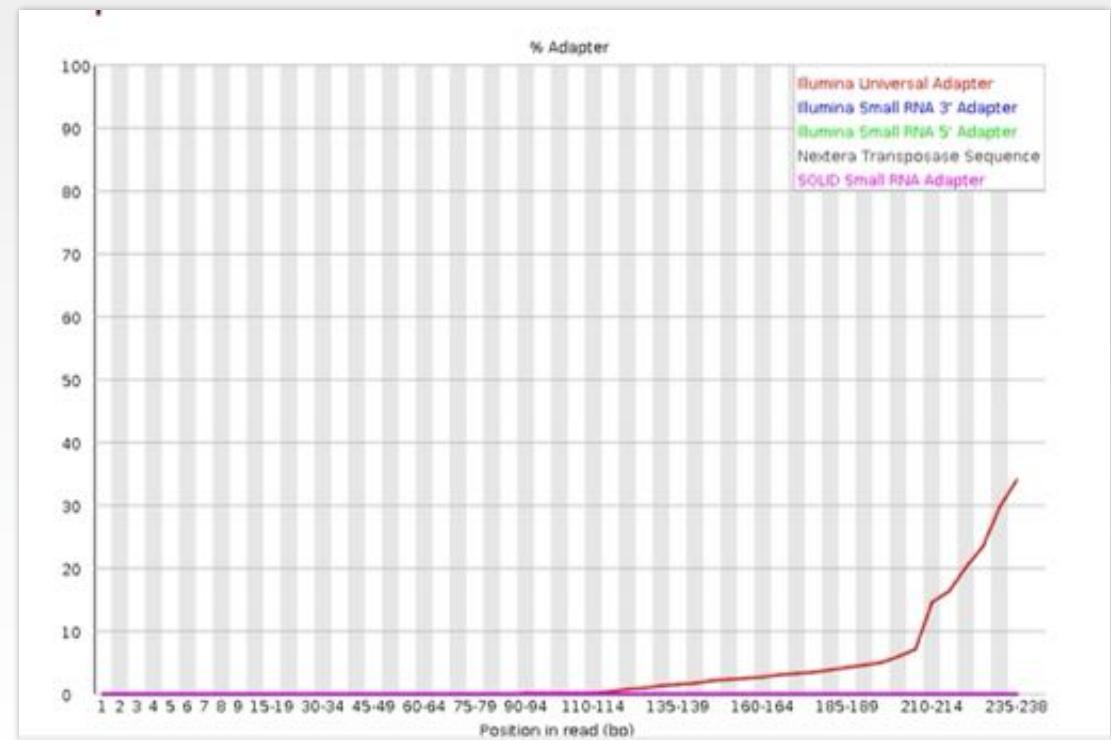
FASTQC: Sequence Duplication Levels



Warning: This module will issue a warning if non-unique sequences make up more than 20% of the total.
Failure: This module will issue an error if non-unique sequences make up more than 50% of the total.

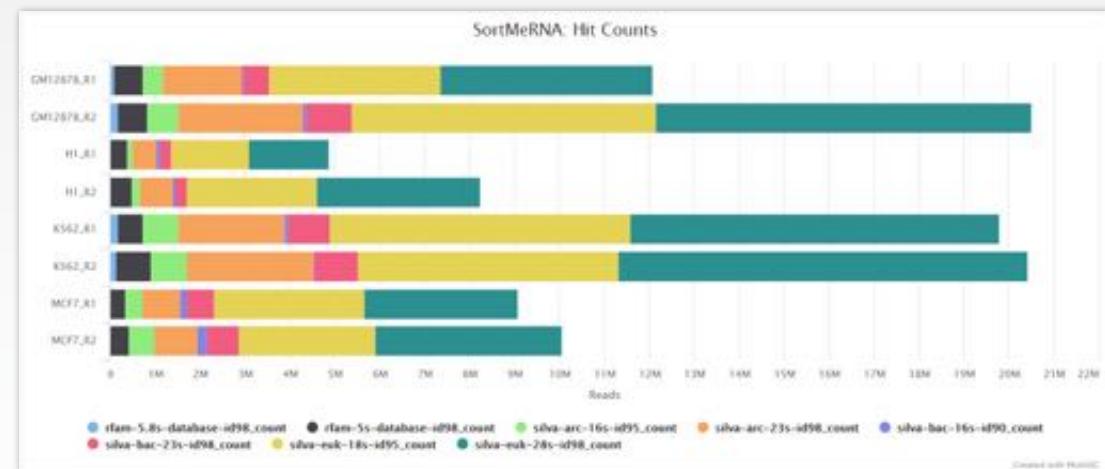
Trimming low-quality bases and adapters

- Cutadapt, Trim Galore!
- Trimmomatic
 - Using relaxed trimming thresholds
 - Improve mappability
 - Reduce mapping artifacts



Remove ribosomal RNA by SortMeRNA

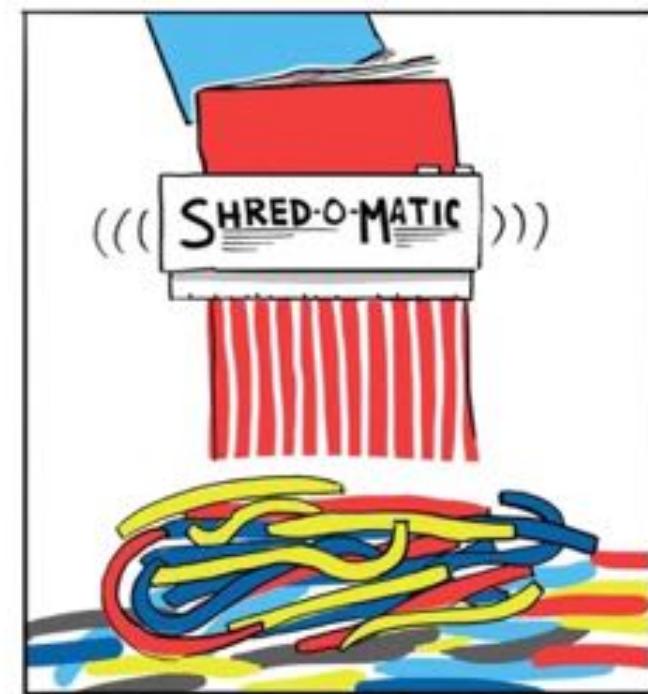
- SortMeRNA is a local sequence alignment tool for filtering, mapping and clustering. The main application of SortMeRNA is filtering rRNA from metatranscriptomic data.
- rRNA database defined in the SortMeRNA:
https://github.com/biocore/sortmerna/tree/master/data/rRNA_databases



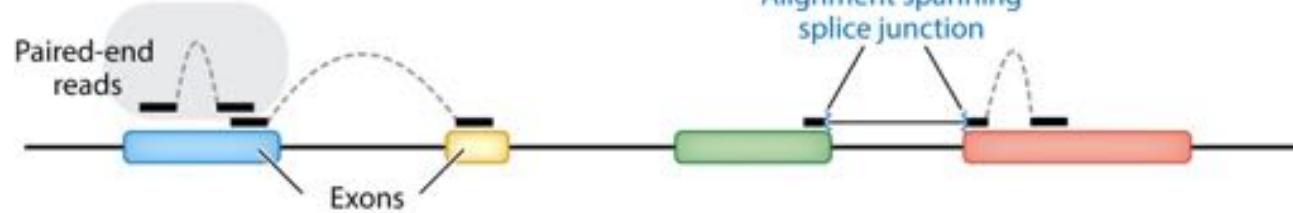
A large red 3D puzzle piece stands out from a background of white puzzle pieces. The red piece is positioned centrally and is slightly tilted, symbolizing a missing or unique element in a process.

ALIGNMENT AND QUANTIFICATION

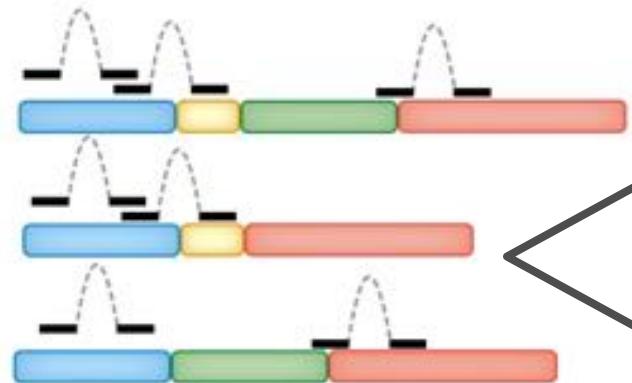
Genome reconstruction—akin to reassembling magazine articles after they have been through a paper shredder.



a Spliced alignment against genome using splice-aware aligner: STAR, HISAT2, GMAP, Tophat2



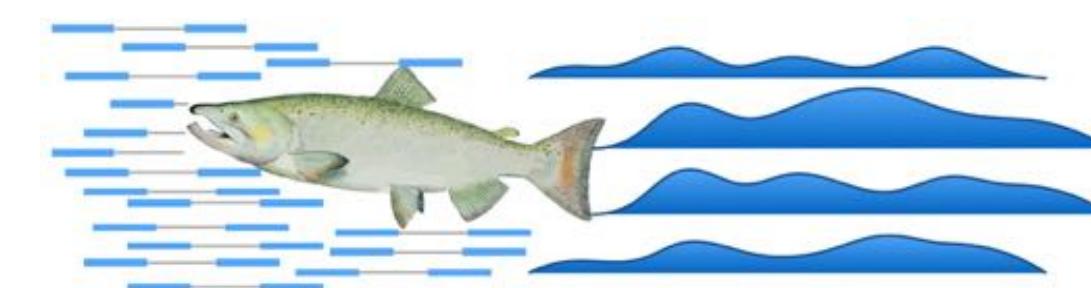
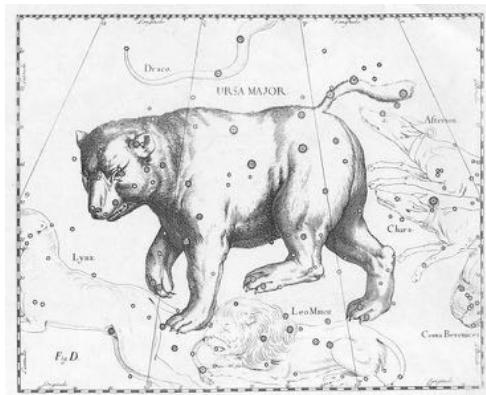
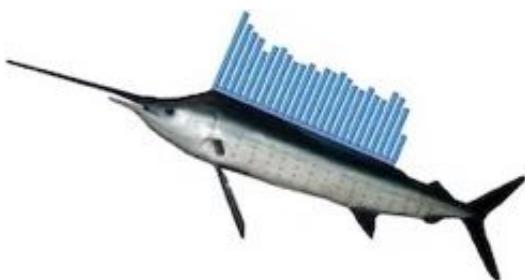
b Unspliced alignment against transcriptome using ungapped aligner: Bowtie, bwa



well
annotated
reference
genome

Van den Berge K, et al. 2019.
Annu. Rev. Biomed. Data Sci. 2:139–73

Alignment free or Pseudo-alignment against transcriptome: *Sailfish*: k -mer-based approaches
Salmon, Sailfish, Kallisto



Bray et al., 2016. doi: 10.1038/nbt.3519

tximport + DESeq2/edgeR/limma-voom

- STAR (Spliced Transcripts Alignment to a Reference)
- RSEM(RNA-Seq by Expectation-Maximization)
- StringTie(downstream followed by Ballgown)
- =====
- Sailfish (Patro, Mount, and Kingsford 2014)
- Kallisto (Bray et al. 2016)
- Salmon (Patro et al. 2017)

Sailfish enables alignment-free isoform quantification from RNA-seq reads using lightweight algorithms

Fleximer: Accurate uantification of RNA-Seq via Variable-Length k-mers

Near-optimal probabilistic RNA-seq quantification

Salmon provides fast and bias-aware quantification of transcript expression



“Lightweight”
approaches

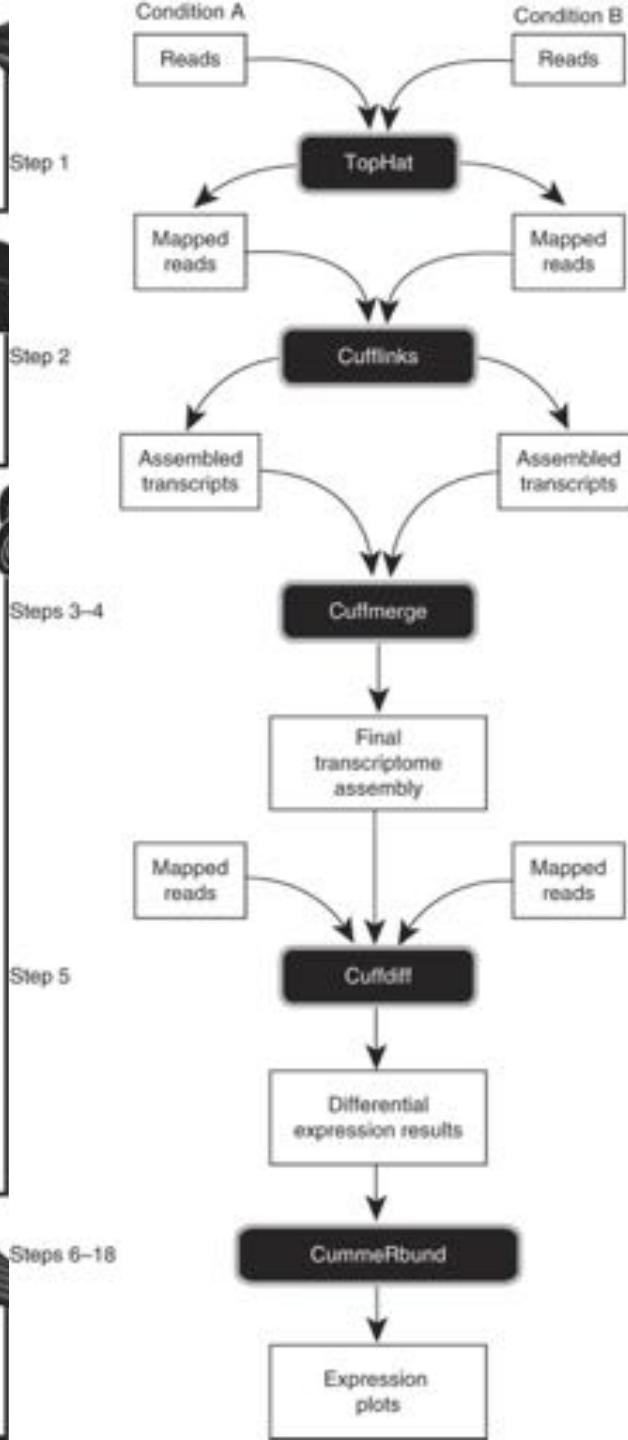
Bowtie
Extremely fast, general purpose short read aligner

TopHat
Aligns RNA-Seq reads to the genome using Bowtie
Discovers splice sites

Cufflinks package

- Cufflinks Assembles transcripts
- Cuffcompare Compares transcript assemblies to annotation
- Cuffmerge Merges two or more transcript assemblies
- Cuffdiff Finds differentially expressed genes and transcripts
Detects differential splicing and promoter use

CummeRbund
Plots abundance and differential expression results from Cuffdiff



PROTOCOL

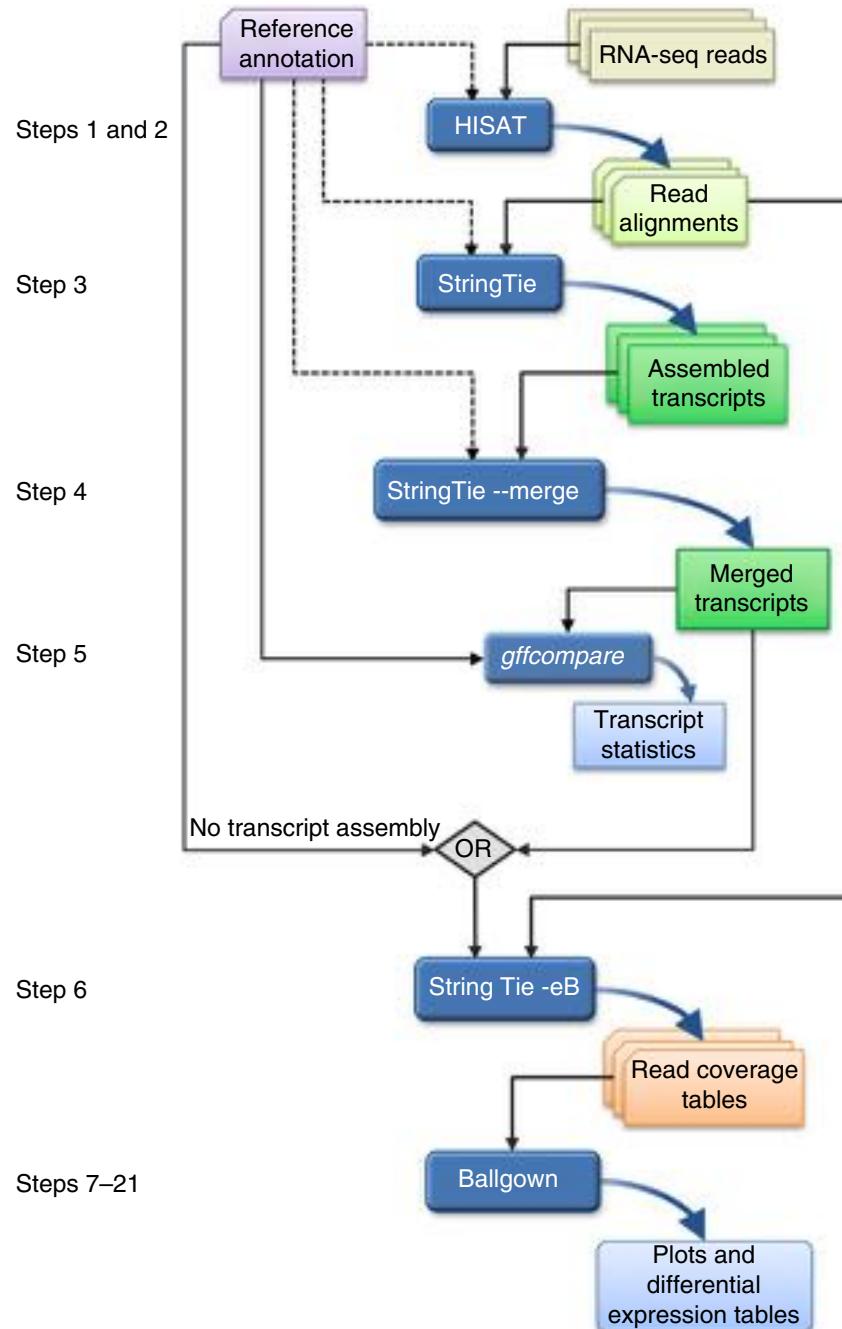
Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks

Cole Trapnell^{1,2}, Adam Roberts³, Loyal Goff^{1,2,4}, Geo Pertea^{5,6}, Daehwan Kim^{5,7}, David R Kelley^{1,2}, Harold Pimentel³, Steven L Salzberg^{5,6}, John L Rinn^{1,2} & Lior Pachter^{3,8,9}

¹Broad Institute of MIT and Harvard, Cambridge, Massachusetts, USA. ²Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, Massachusetts, USA. ³Department of Computer Science, University of California, Berkeley, California, USA. ⁴Computer Science and Artificial Intelligence Lab, Department of Electrical Engineering and Computer Science, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA. ⁵Department of Medicine, McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. ⁶Department of Biostatistics, Johns Hopkins University, Baltimore, Maryland, USA. ⁷Center for Bioinformatics and Computational Biology, University of Maryland, College Park, Maryland, USA. ⁸Department of Mathematics, University of California, Berkeley, California, USA. ⁹Department of Molecular and Cell Biology, University of California, Berkeley, California, USA. Correspondence should be addressed to C.T. (cole@broadinstitute.org).

Published online 1 March 2012; corrected after print 7 August 2014; doi:10.1038/nprot.2012.016

- ❖ Reads are mapped directly to reference genome allowing for splice junctions across exons
- ❖ Methods require large amount of memory and CPU time
- ❖ Can only handle simple experiment design



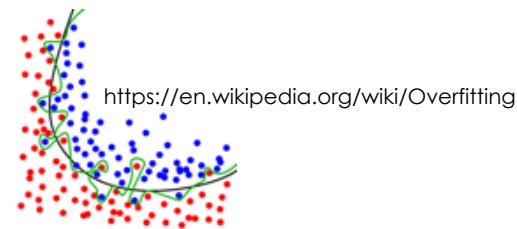
PROTOCOL

Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown

Mihaela Perteal^{1,2}, Daehwan Kim¹, Geo M Perteal¹, Jeffrey T Leek³ & Steven L Salzberg¹⁻⁴

¹Center for Computational Biology, McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins School of Medicine, Baltimore, Maryland, USA. ²Department of Computer Science, Whiting School of Engineering, Johns Hopkins University, Baltimore, Maryland, USA. ³Department of Biostatistics, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland, USA. ⁴Department of Biomedical Engineering, Johns Hopkins University, Baltimore, Maryland, USA. Correspondence should be addressed to S.L.S. (salzberg@jhu.edu). 

Published online 11 August 2016; doi:10.1038/nprot.2016.095

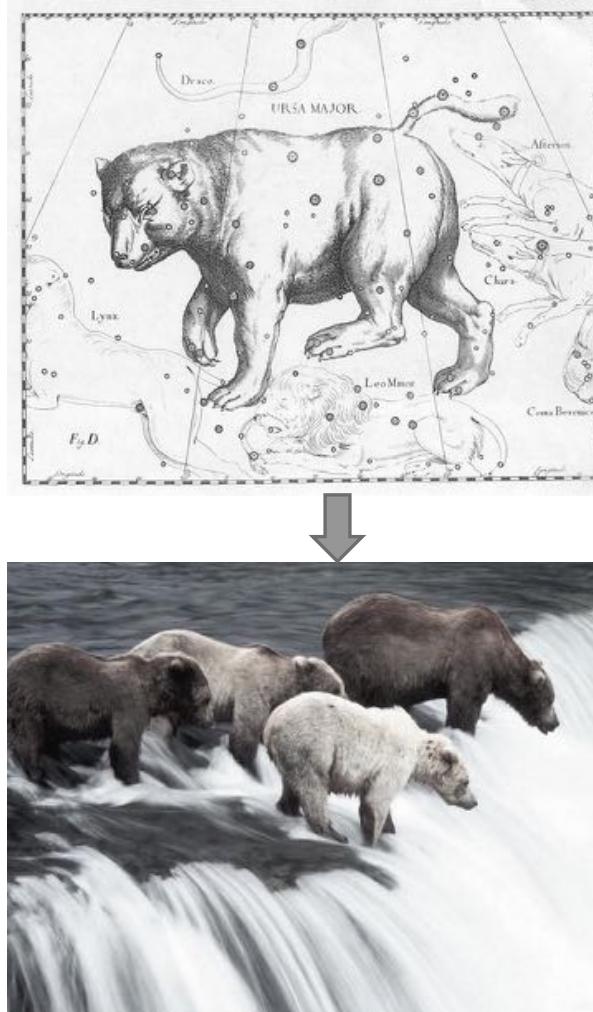


Note that Ballgown's statistical test is a standard linear model-based comparison. **For small sample sizes ($n < 4$ per group), it is often better to perform regularization.** This can be done using the limma³³ package in Bioconductor. Other regularized methods such as DESeq²³ and edgeR²⁰ can be applied to gene or exon counts, but they are not appropriate for direct application to FPKM abundance estimates. The statistical test uses a cumulative upper quartile normalization³⁴.

KALLISTO, SLEUTH PIPELINE

- ❖ considerably faster than traditional alignment + counting
- ❖ Transcripts level estimates rather than gene level. Transcripts level estimates can be aggregated to gene level, but abundance estimates for lowly expressed transcripts are highly variable and should be interpreted with caution.
- ❖ No precise alignments (no alignment files for visualization in genome browser)

<https://scilifelab.github.io/courses/rnaseq/labs/kallisto>



Near-optimal probabilistic RNA-seq quantification

Nicolas L Bray¹, Harold Pimentel², Pál Melsted³
& Lior Pachter^{2,4,5}

We present kallisto, an RNA-seq quantification program that is two orders of magnitude faster than previous approaches and achieves similar accuracy. Kallisto pseudoaligns reads to a reference, producing a list of transcripts that are compatible with each read while avoiding alignment of individual bases. We use kallisto to analyze 30 million unaligned paired-end RNA-seq reads in <10 min on a standard laptop computer. This removes a major computational bottleneck in RNA-seq analysis.

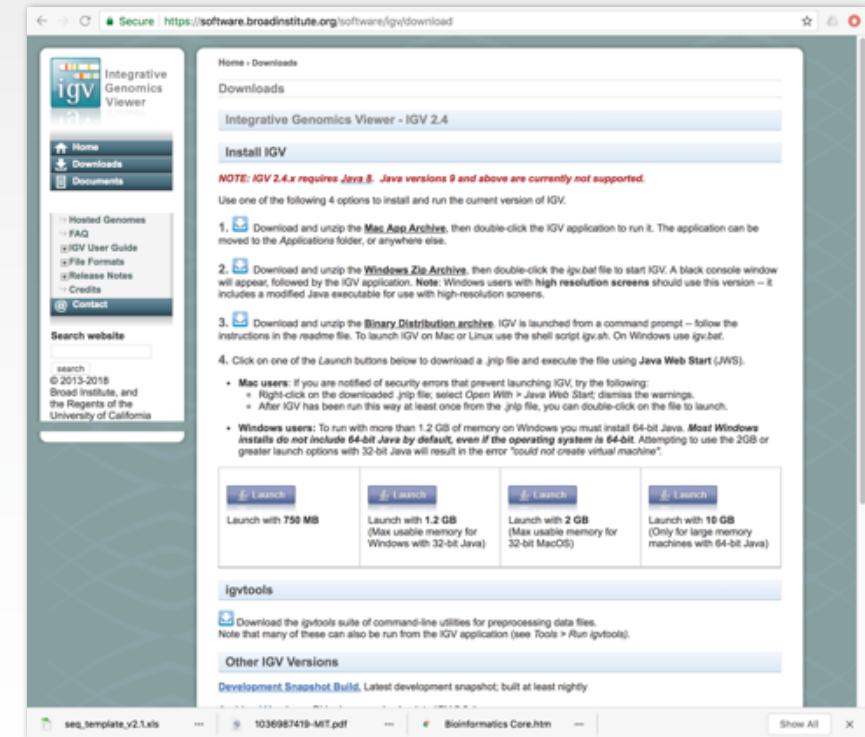
Differential analysis of RNA-seq incorporating quantification uncertainty

Harold Pimentel¹, Nicolas L Bray², Suzette Puente³,
Pál Melsted⁴ & Lior Pachter⁵

We describe sleuth (<http://pachterlab.github.io/sleuth>), a method for the differential analysis of gene expression data that utilizes bootstrapping in conjunction with response error linear modeling to decouple biological variance from inferential variance. sleuth is implemented in an interactive shiny app that utilizes kallisto quantifications and bootstraps for fast and accurate analysis of data from RNA-seq experiments.

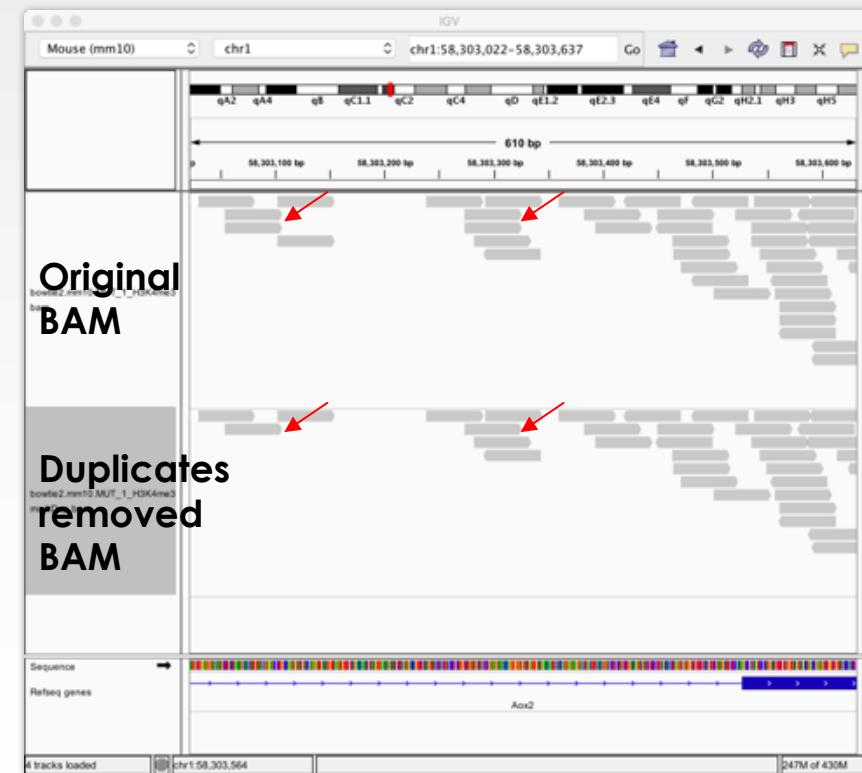
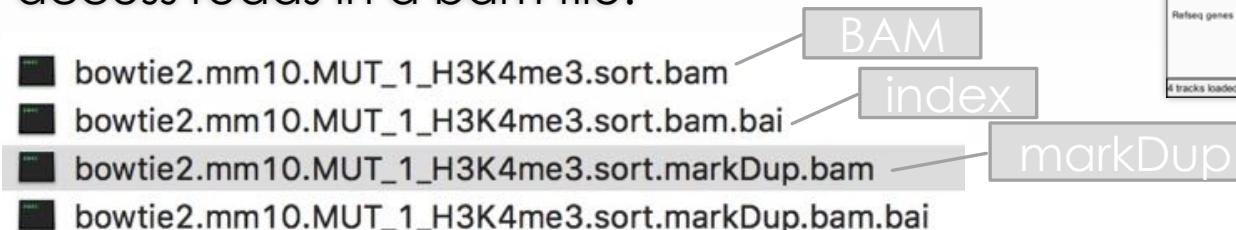
IGV: Integrative Genomics Viewer

- ➊ Goto
<https://software.broadinstitute.org/software/igv/>
- ➋ Click Downloads icon
- ➌ Download IGV



File formats: sam/bam

- Sam/bam: alignment file contain mapping information.
- markDup: output of MarkDuplicates function of picard tools. Remove the duplicated reads for DNA-seq.
- Sort: sort the bam files by name or coordinates.
- Index: Bam index file for more efficiently access reads in a bam file.



Exercises



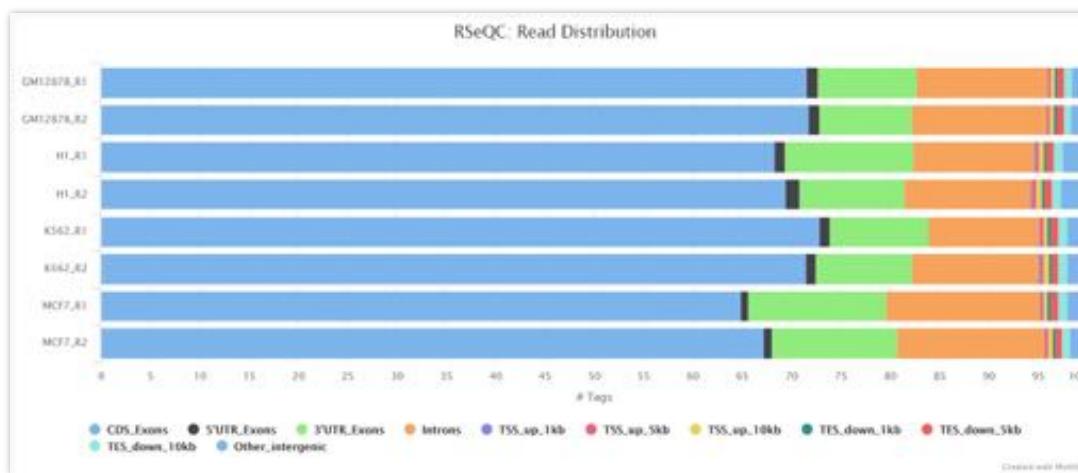
Visualizing data in
Integrative Genomics
Viewer



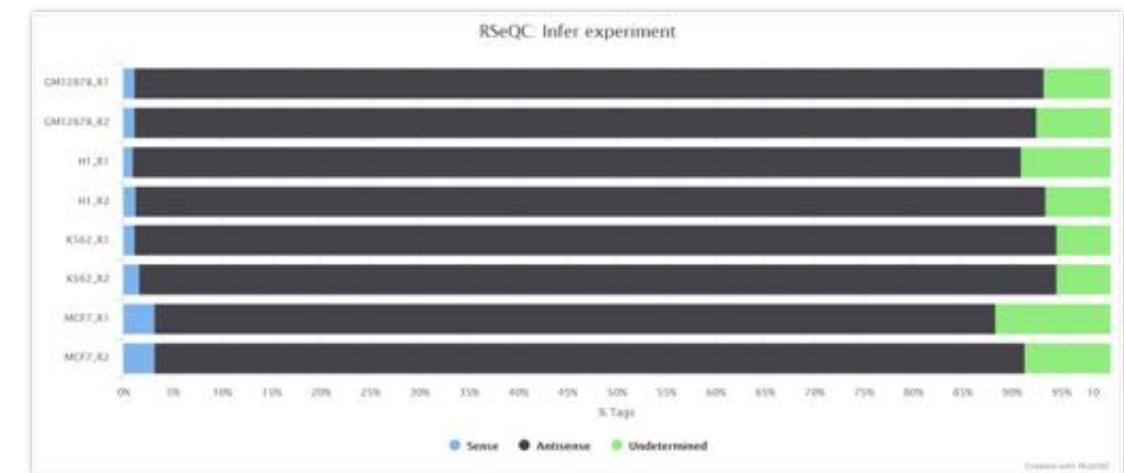
Visualizing data in UCSC
genome browser

Quality control of post-alignment

Read distribution by RSeQC

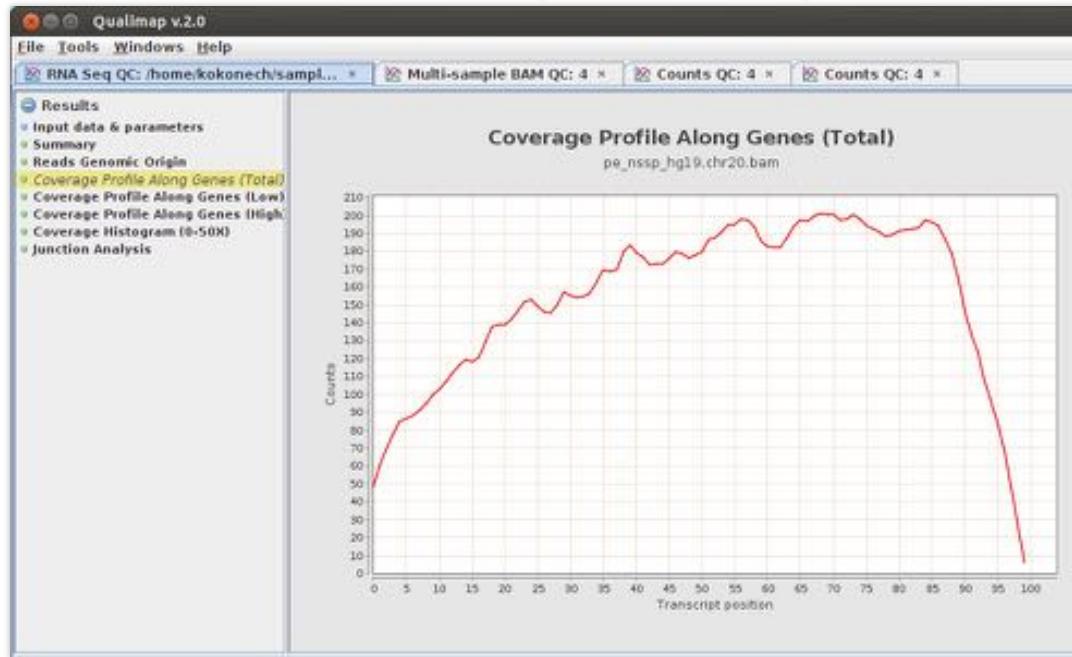


Strand information by RSeQC

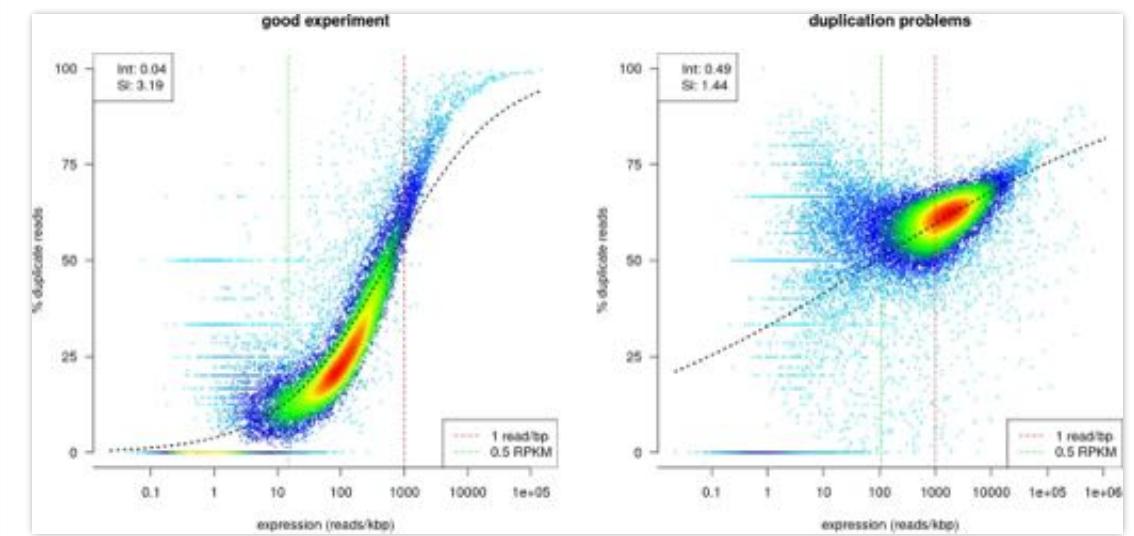


Quality control of post-alignment

Mapping coverage relative to gene features by Qualimap



Library complexity analysis by dupRadar

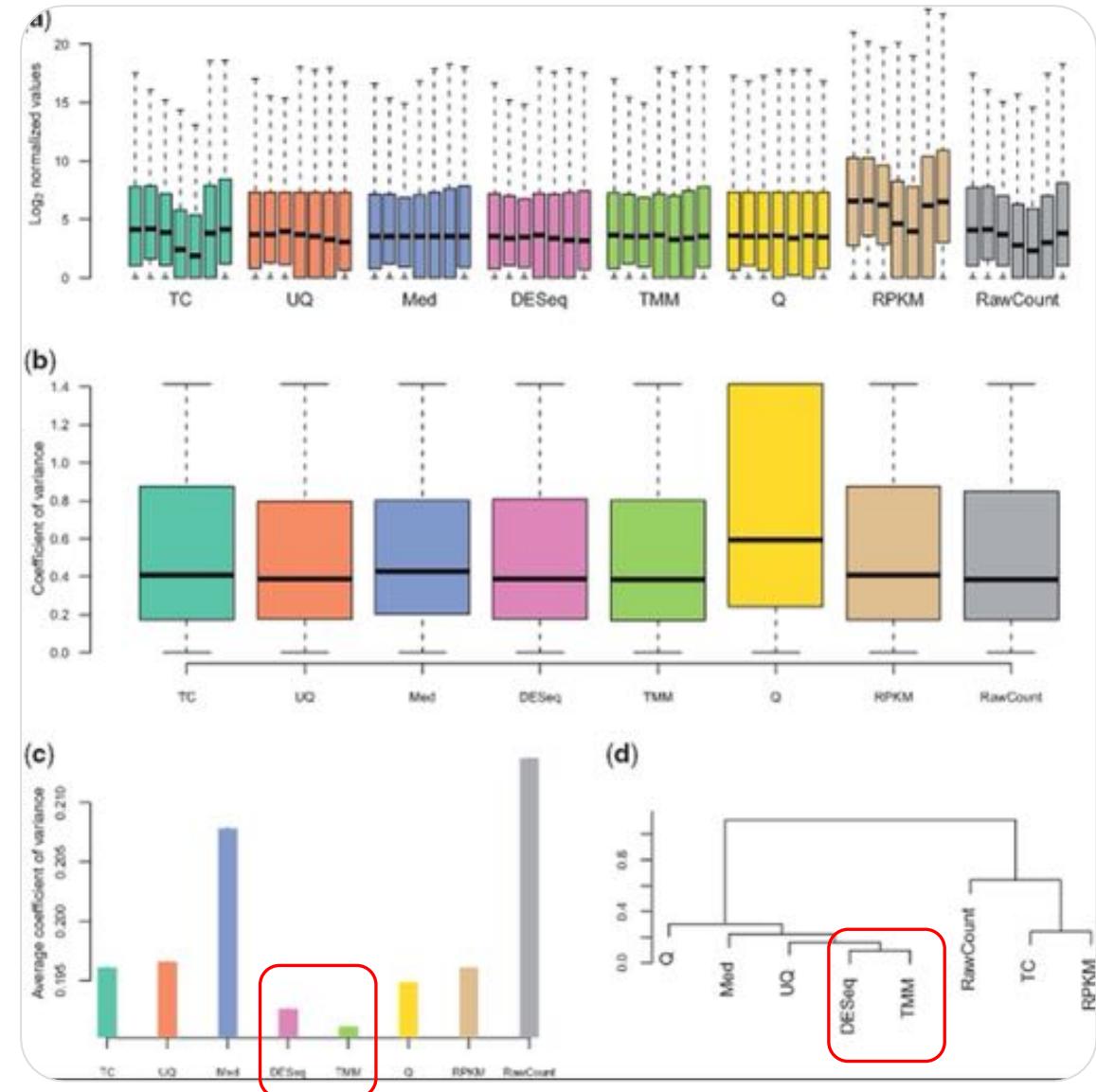


COUNT TABLE

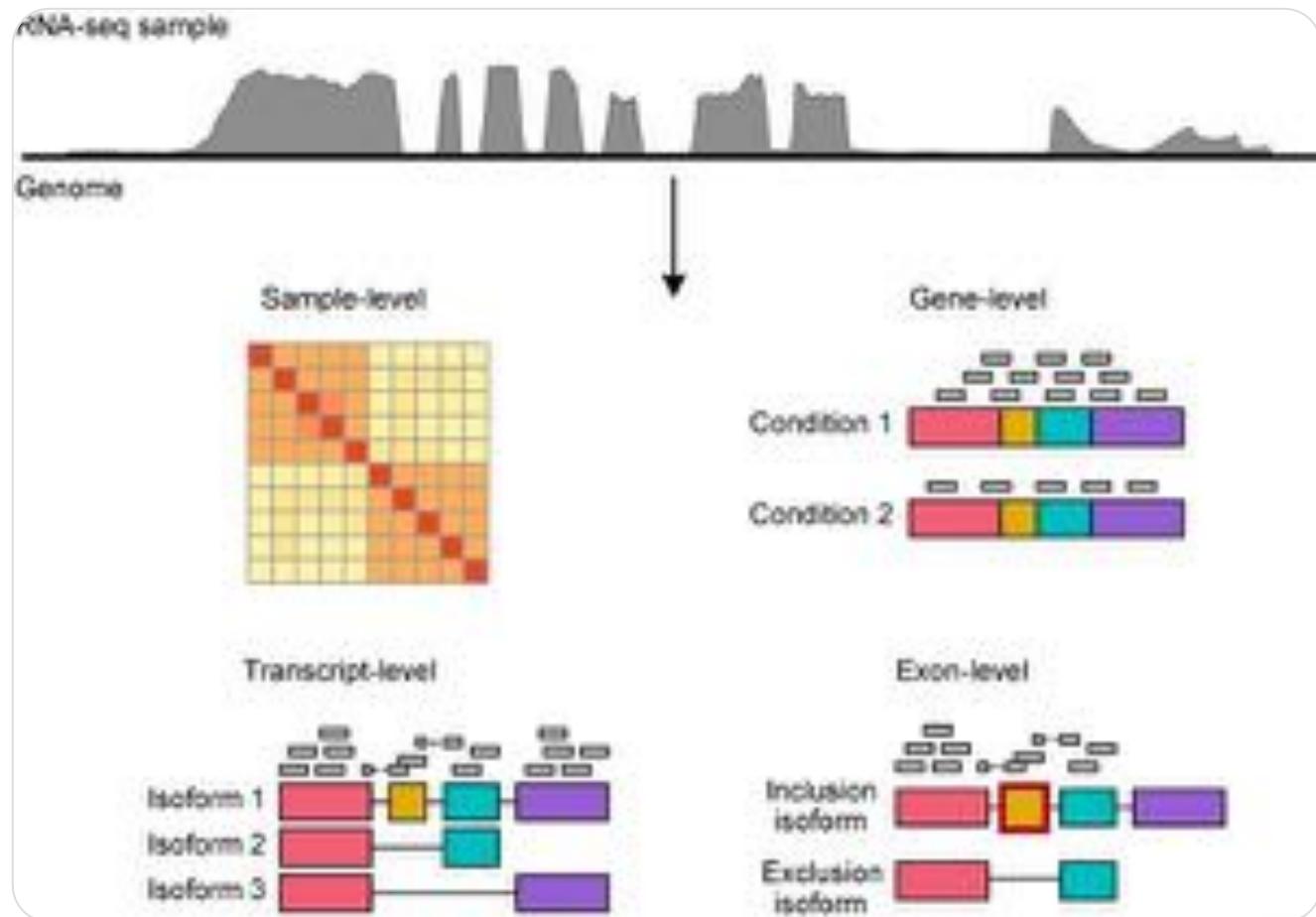
Gene Symbol	WT_rep1	WT_rep2	WT_rep3	KD_rep1	KD_rep2	KD_rep3
ackr4b	30	28	42	5	66	7
aclya	24	48	56	77	89	108
acot14	0	0	0	0	0	0
acsbg1	5	9	7	123	98	70
actr8	3536	3639	4586	225	545	898

Normalization: most genes are not differential expressed

- TMM (Trimmed weighted mean)
- DESeq (median of log expression ratio)

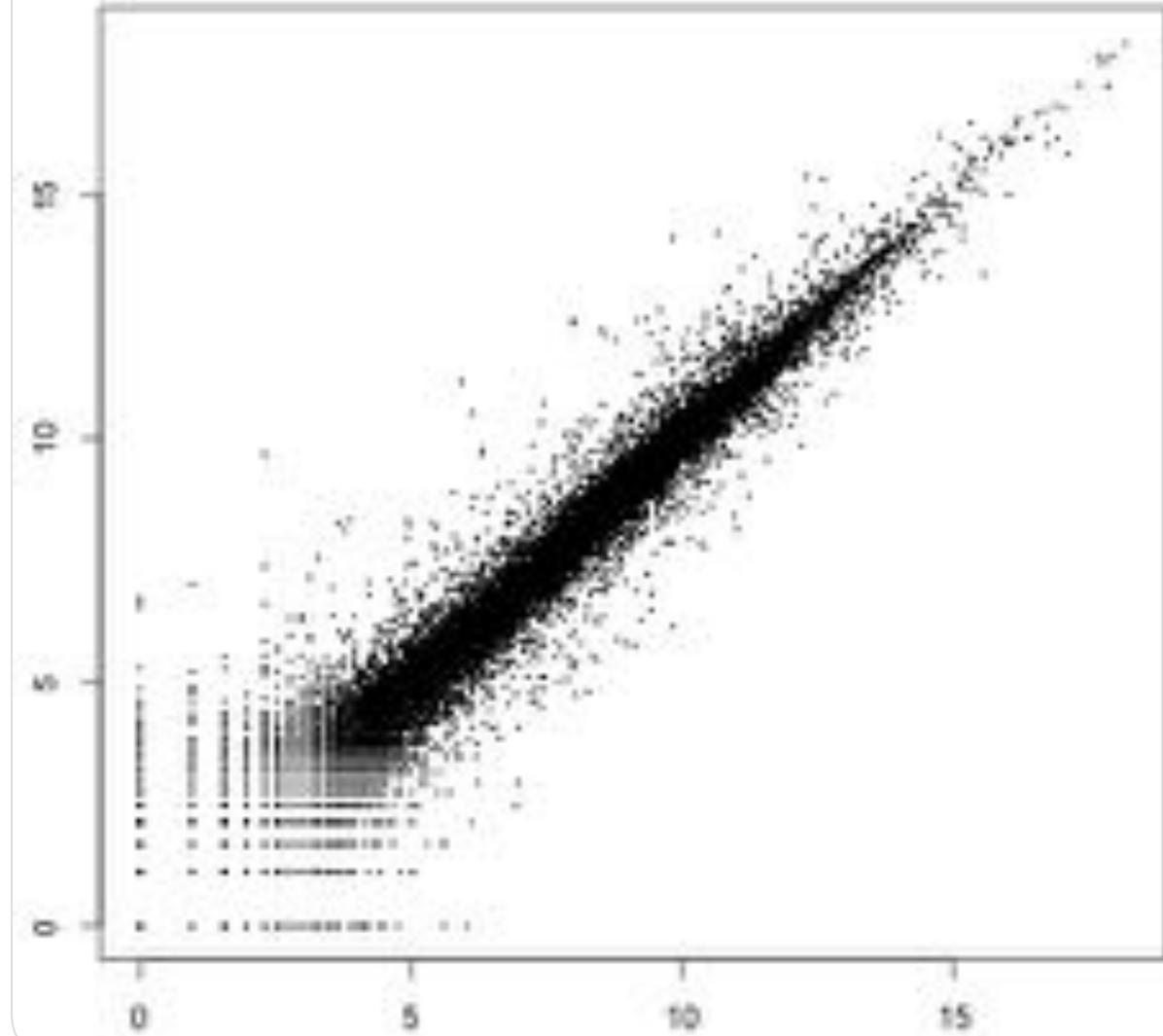
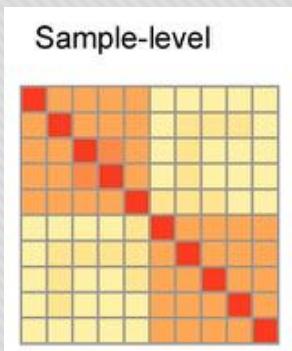


RNA-seq analyses at four different levels: sample-level, gene-level, transcript-level, and exon-level.

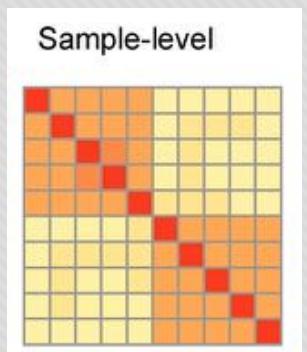


Li et.al., 2018. doi: 10.1007/s40484-018-0144-7

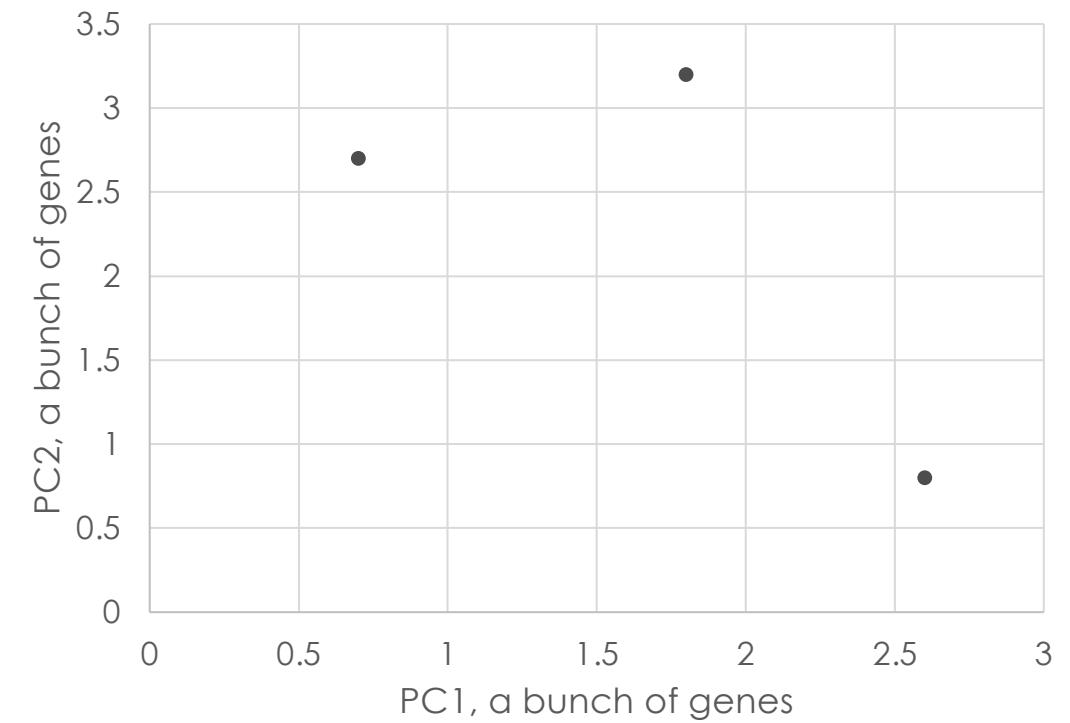
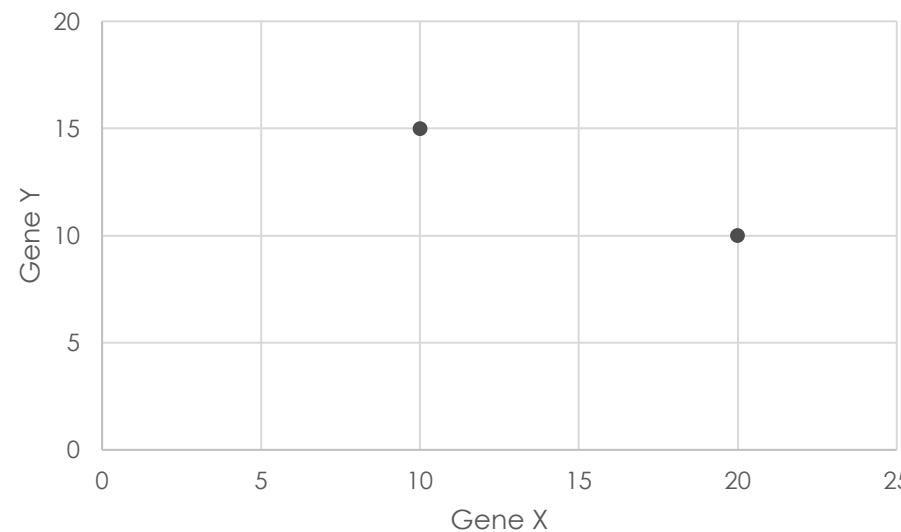
Scatter plot for replicates



Principal component analysis (PCA)

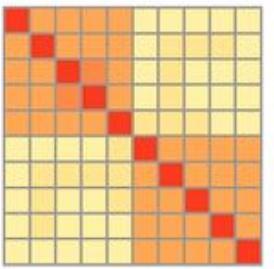


Gene	Sample1	Sample2
X	10	20
Y	15	10

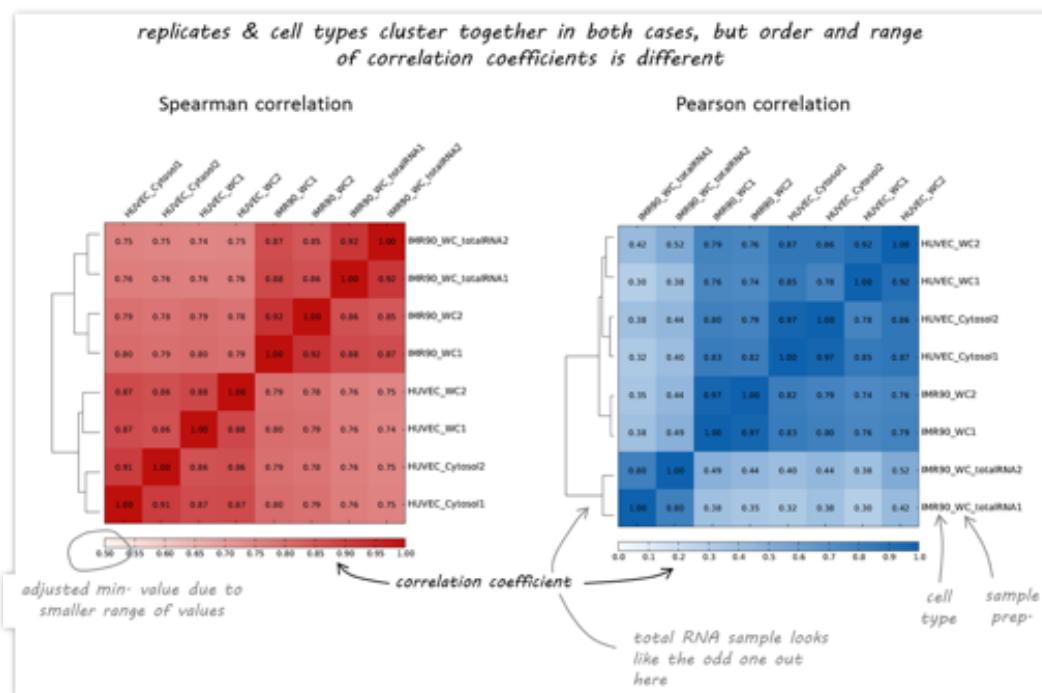


PCA reduces the complexity of expression data to show relationships on two axes

Sample-level

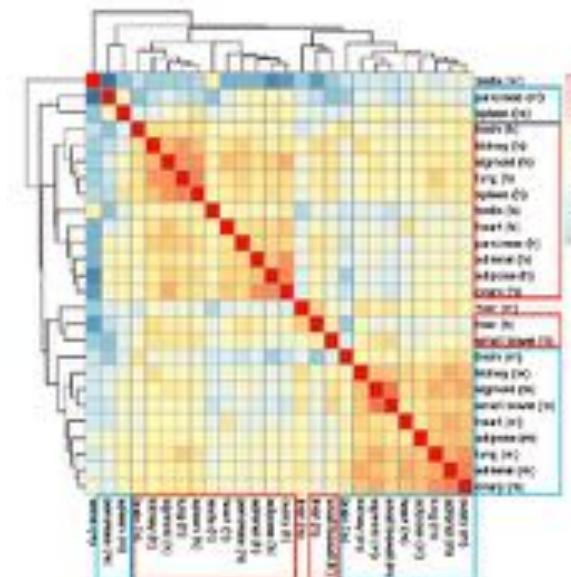


Sample-sample distance plot

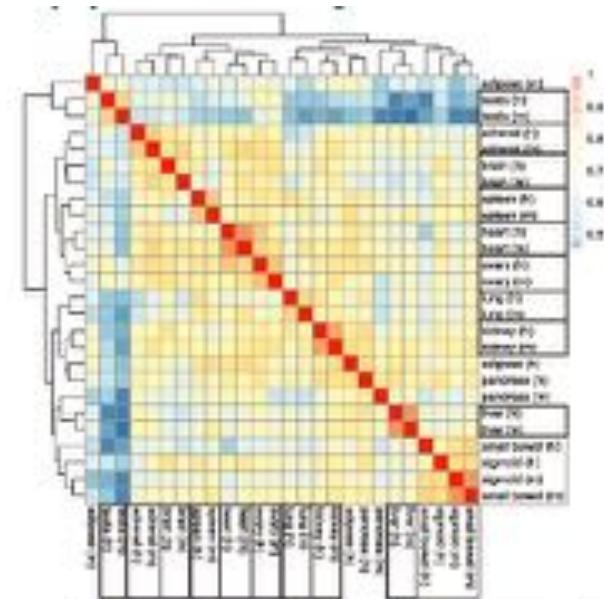


<https://deeptools.readthedocs.io/en/develop/content/tools/plotCorrelation.html>

Sample-level analysis is affected by batch effect



"Overall, our results indicate that there is considerable RNA expression diversity between humans and mice, well beyond what was described previously, likely reflecting the fundamental physiological differences between these two organisms."



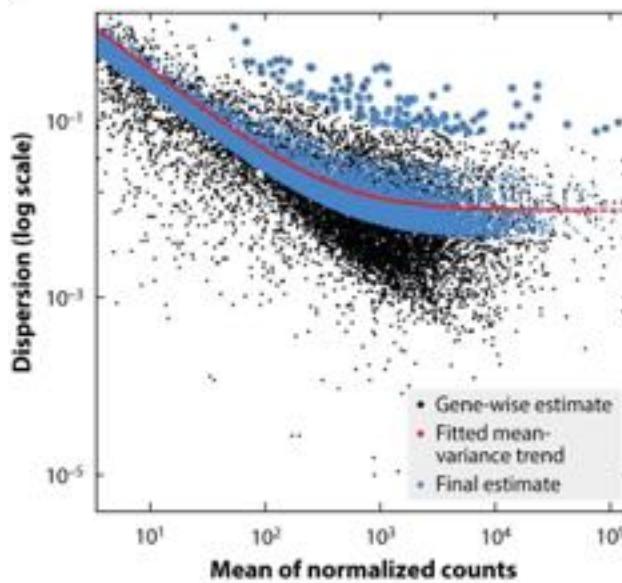
"Once we accounted for the batch effect (...), the comparative gene expression data no longer clustered by species, and instead, we observed a clear tendency for clustering by tissue."

Summary of suitable analysis approaches for the three types of comparative analyses

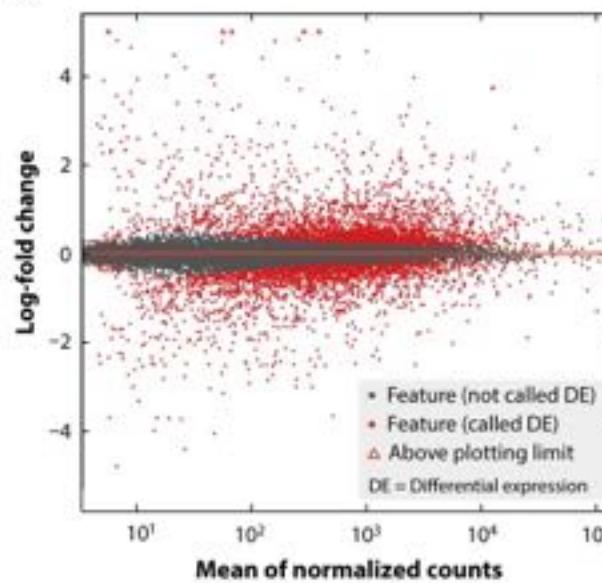
Task	Input data	Software (examples)
differential gene expression (DGE)	Gene counts	DESeq2, edgeR, voom/limma
differential transcript usage (DTU)	Transcript counts	DESeq2, edgeR, sleuth, voom/limma
differential exon usage (DEU)	Exon counts	DEXSeq, voom/limma, MISO, IsoformSwitchAnalyzeR

Differential Expression

a

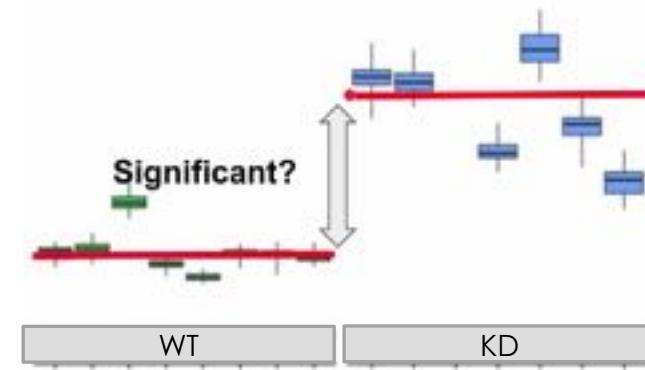


b



$$\text{Total } CV^2 = \text{Biological } CV^2 + \text{Technical } CV^2$$

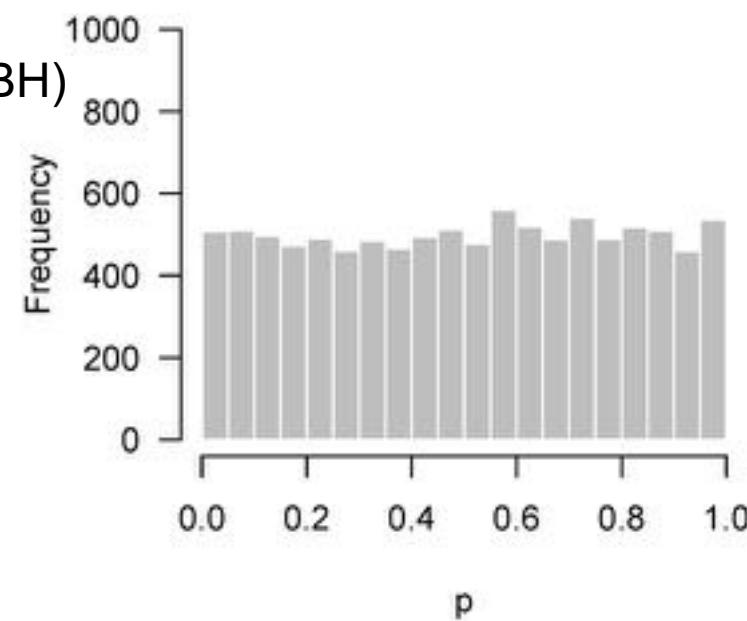
Testing:
the variation **between** groups >
the variation **within** groups



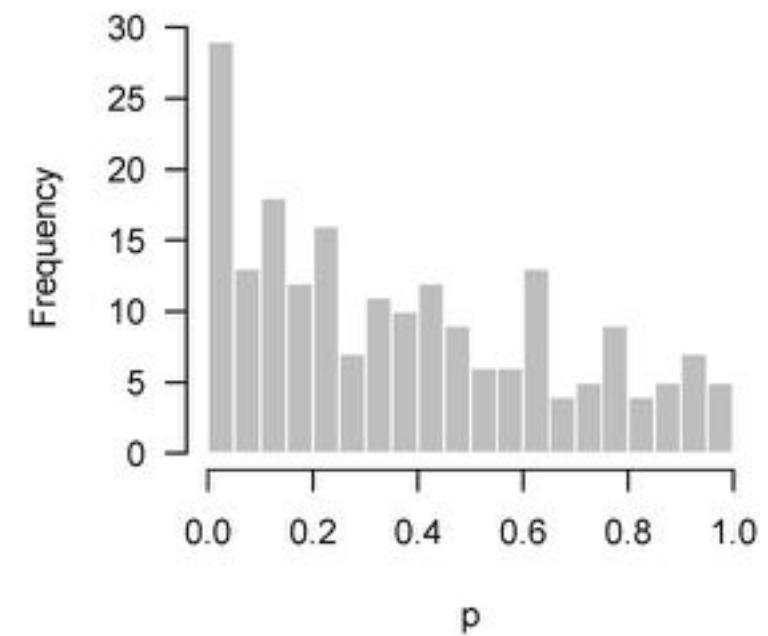
Multiple comparisons

Benjamini-Hochberg Correction (BH)
false-discovery rate (FDR)

From a uniform random number generator



From a sufficiently powered experiment



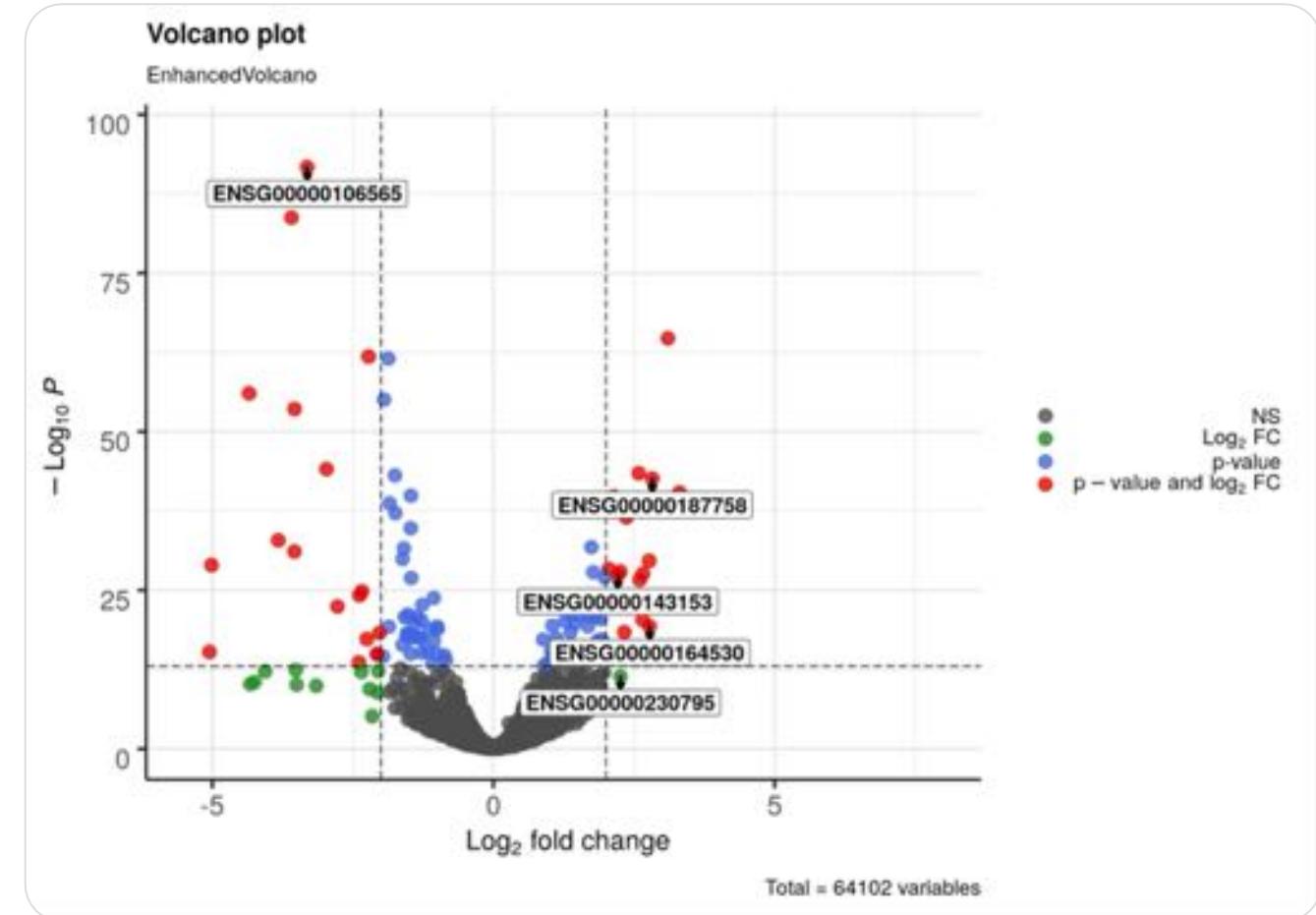
Results

baseMean	log2FoldChange	IfcSE	stat	pvalue	padj	gene_name	ensembl_id
569.7493	-2.51313	0.352548	-7.12847	1.01E-12	6.32E-12	aacs	ENSDARG00000012468
228.7958	-2.64457	0.241543	-10.9487	6.74E-28	1.41E-26	aamdc	ENSDARG00000103957
3299.677	-1.25607	0.237625	-5.28595	1.25E-07	4.81E-07	aamp	ENSDARG00000045019
151.558	1.228695	0.449503	2.733449	0.006267	0.012898	aarsd1	ENSDARG00000015747
423.9228	-1.38447	0.159835	-8.66182	4.64E-18	4.53E-17	aass	ENSDARG00000051816
2594.372	1.402565	0.149554	9.37834	6.7E-21	8.28E-20	abca1b	ENSDARG00000079009
1215.935	-1.7795	0.223888	-7.94816	1.89E-15	1.52E-14	abcb3l1	ENSDARG00000036787
208.7542	-2.28293	0.19053	-11.982	4.42E-33	1.35E-31	abcb6a	ENSDARG00000063297
206.3376	-1.2775	0.346485	-3.68701	0.000227	0.000587	abcb9	ENSDARG00000056200
223.5531	2.194671	0.430981	5.092271	3.54E-07	1.29E-06	abcc2	ENSDARG00000014031

Visualization of DEGs



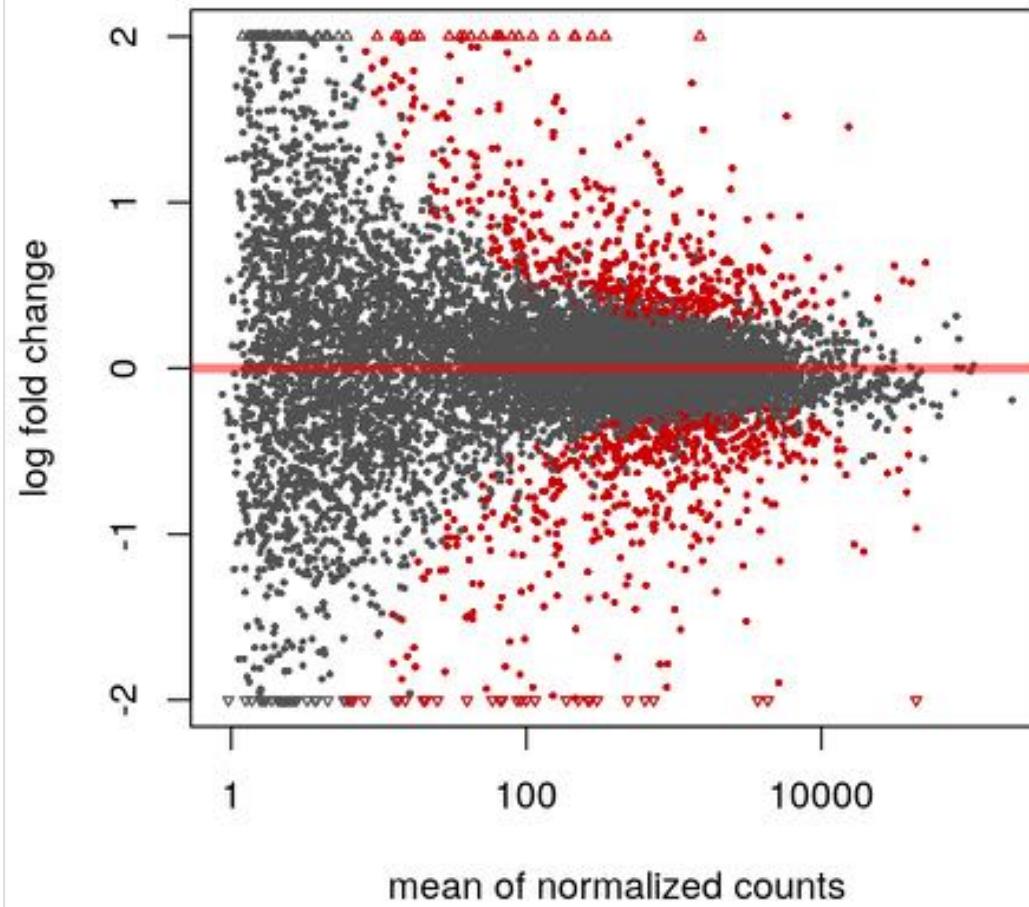
Volcano plot



<https://bioconductor.org/packages/release/bioc/vignettes/EnhancedVolcano/inst/doc/EnhancedVolcano.html>

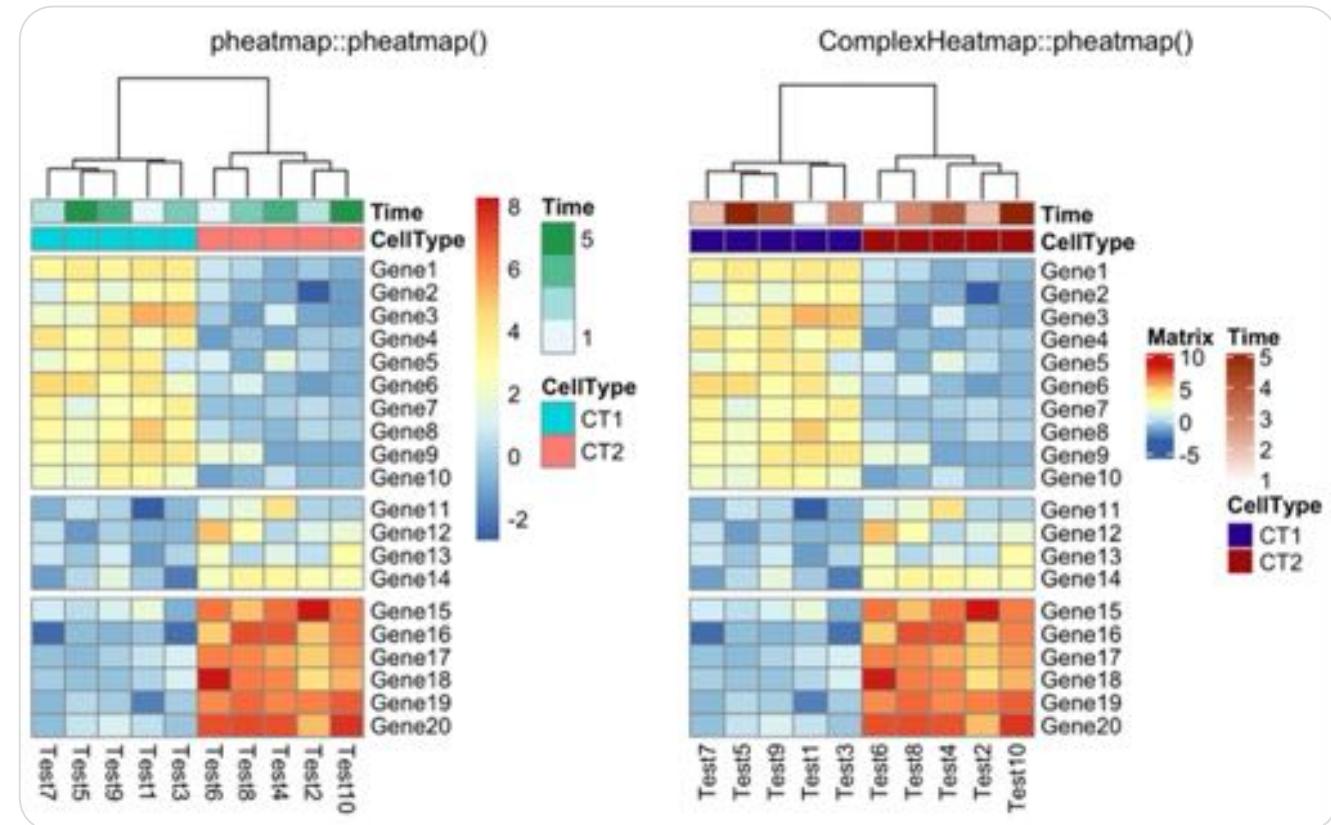
MA plot

M (log ratio) and A (mean average) scales



Heatmap

- Heatmap is a graphical reorientation of 3D data in 2D figure. It uses a system of color-coding to represent different values.
- Packages: pheatmap, ggplot2, ComplexHeatmap ...



<https://CRAN.R-project.org/package=pheatmap>

<https://bioconductor.org/packages/ComplexHeatmap/>

<https://jokergoo.github.io/2020/05/06/translate-from-pheatmap-to-complexheatmap/>

Functional annotation of DE genes

Gene ontology
(GO) enrichment
analysis

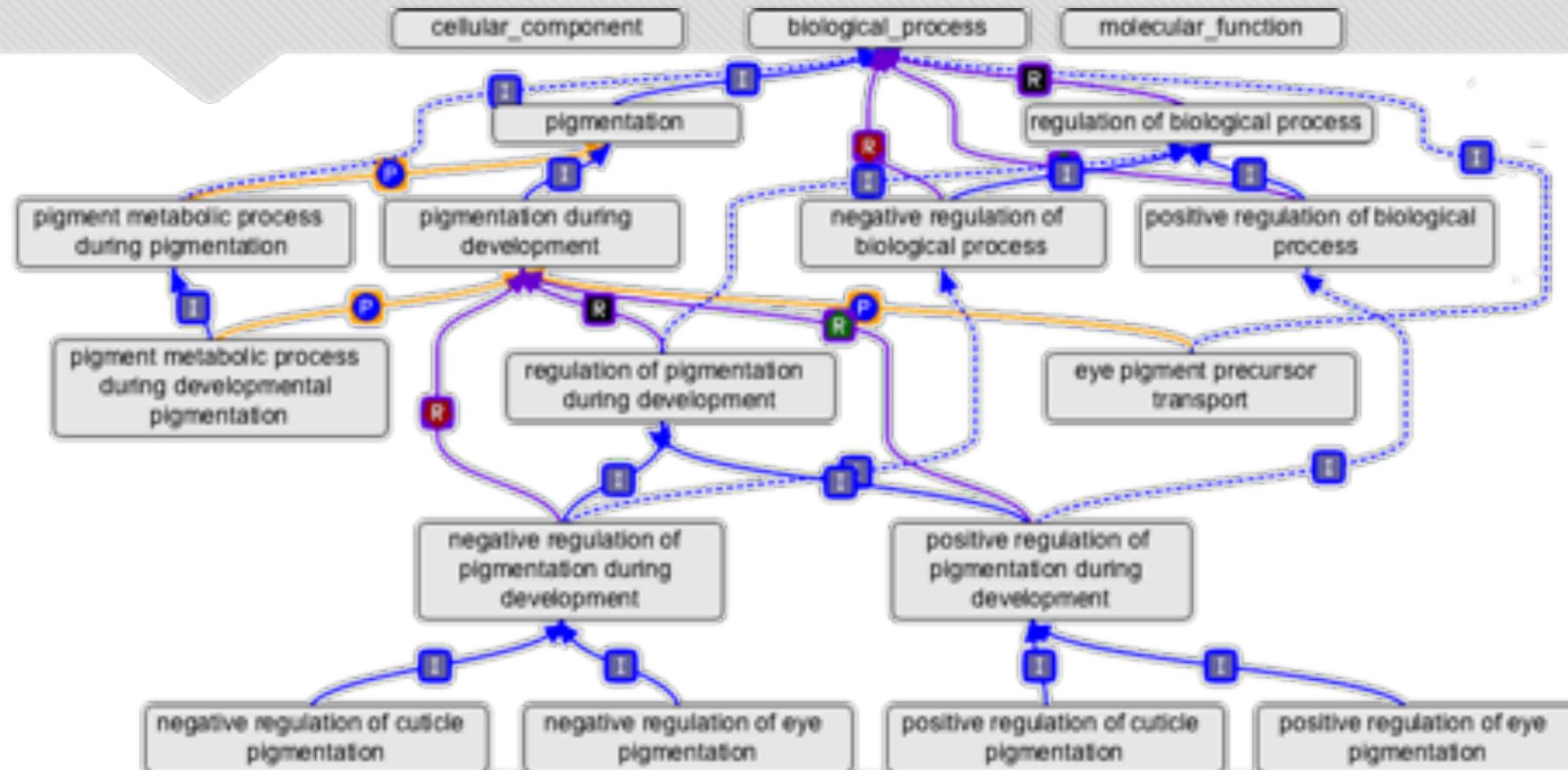
Kyoto Encyclopedia
of Genes and
Genomes (KEGG)
pathway
enrichment analysis

Gene set
enrichment analysis
(GSEA)

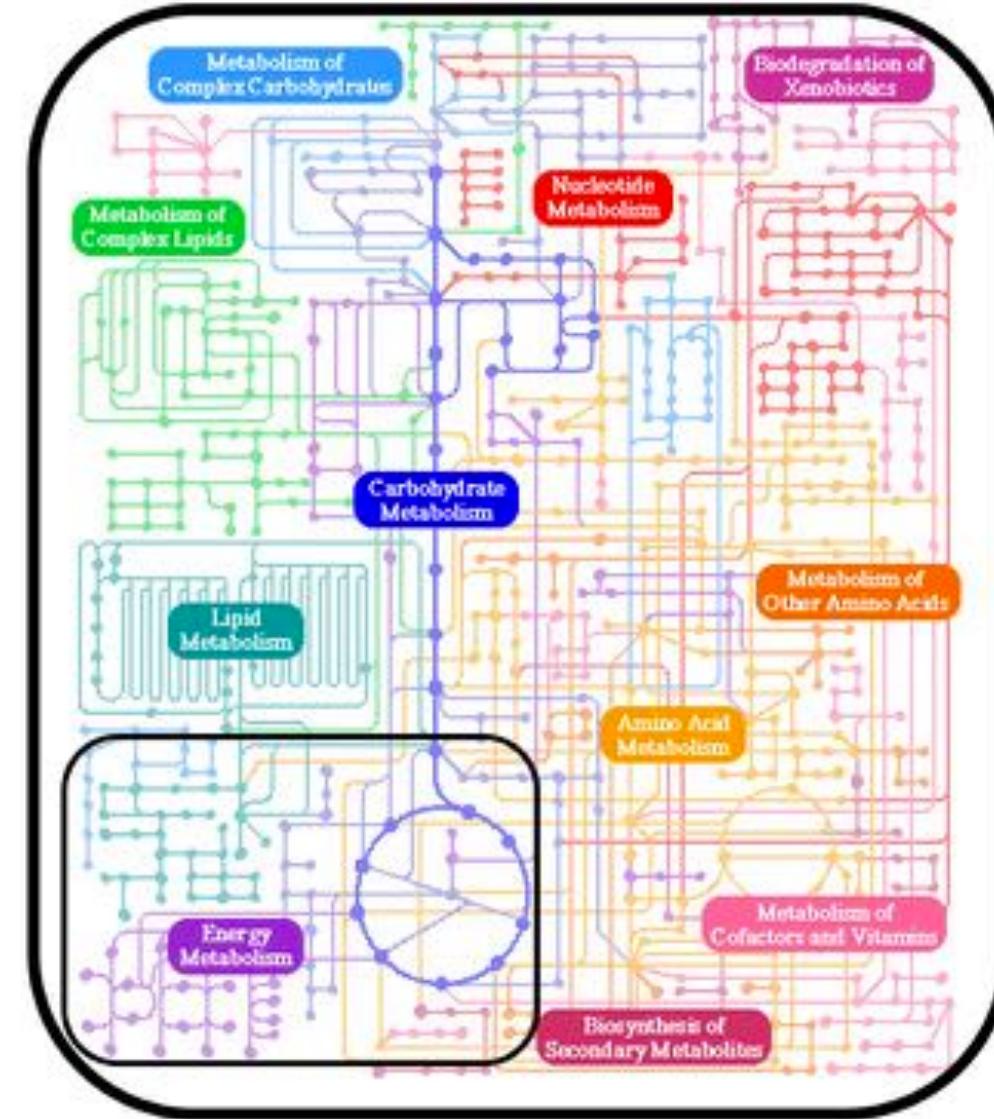
QIAGEN Ingenuity
Pathway Analysis
(IPA)

A gene set over-represented comparing to random sampling

Gene ontology

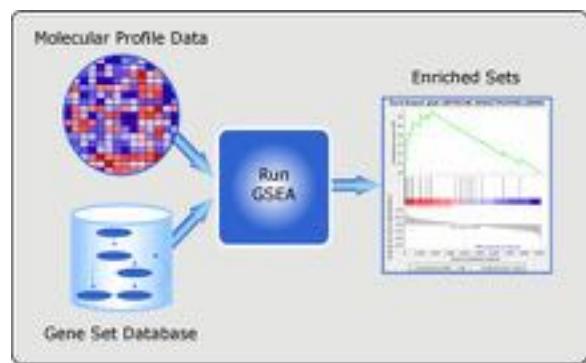


KEGG pathway enrichment analysis



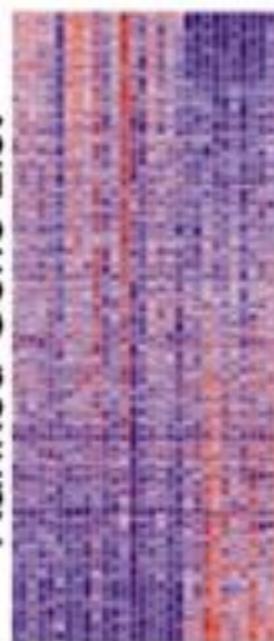
<https://www.genome.jp/kegg/pathway.html>

GSEA



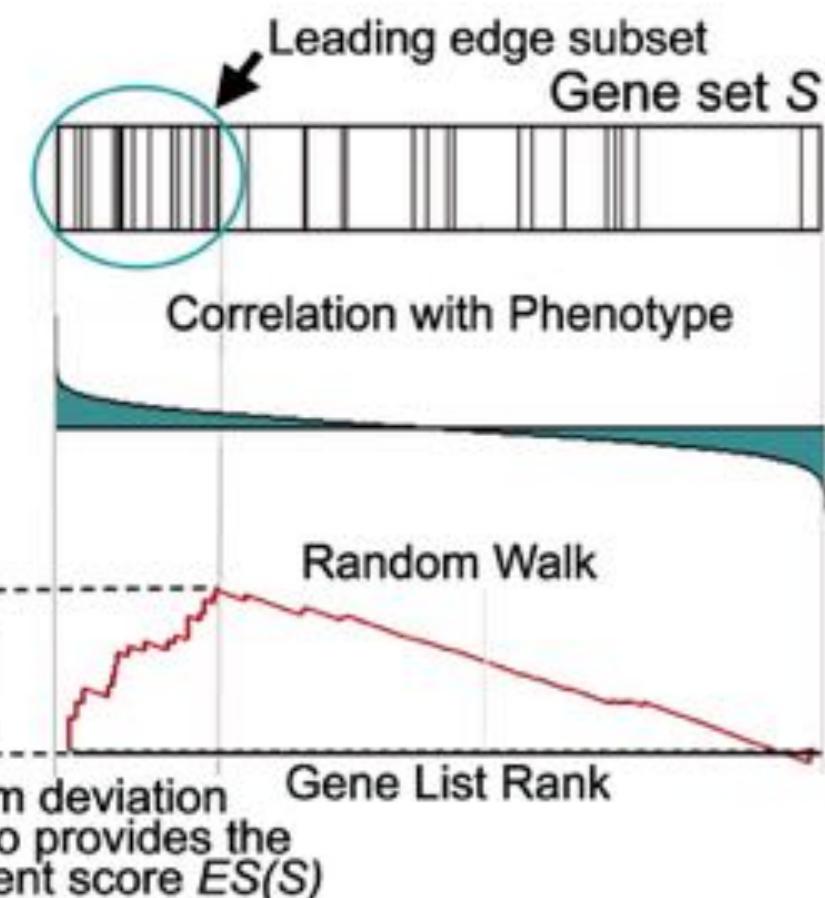
A Phenotype Classes
A B

Ranked Gene List

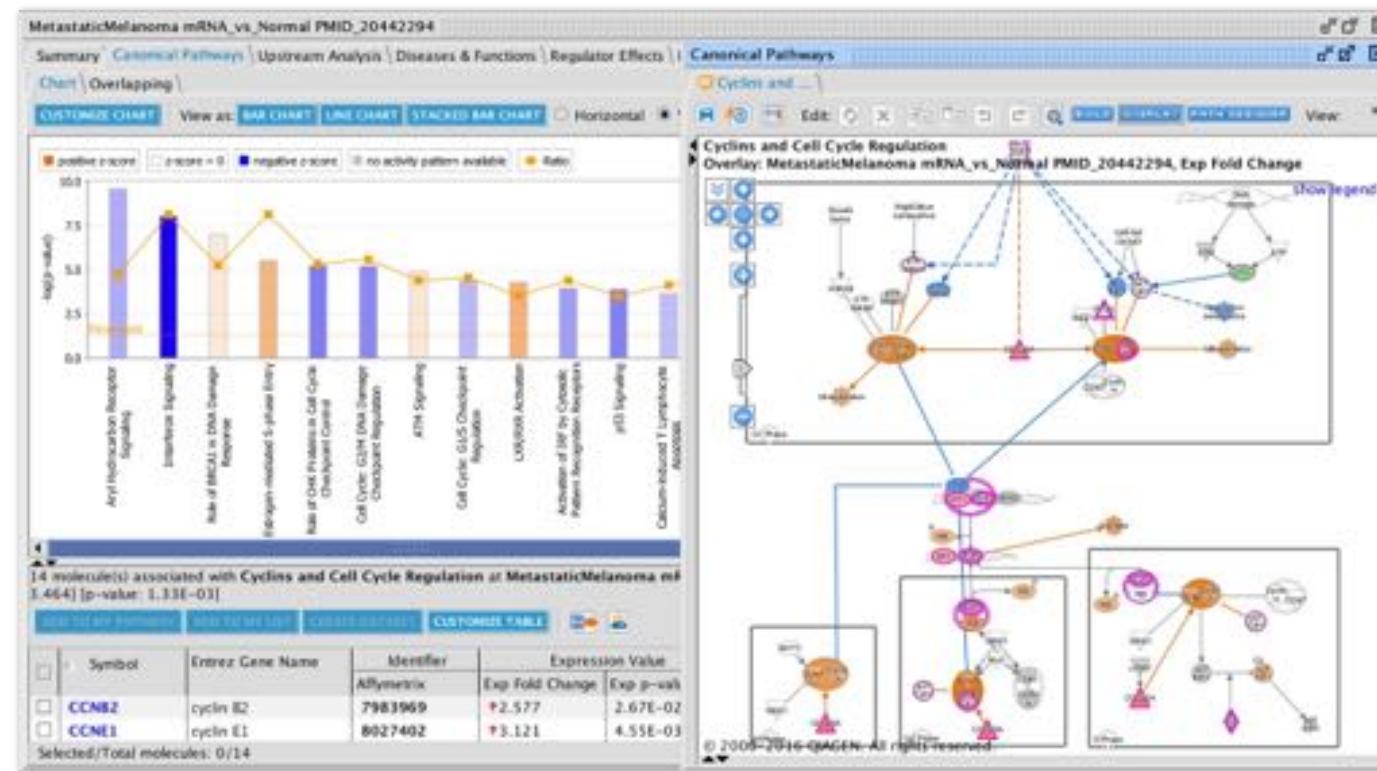


B

Gene set S

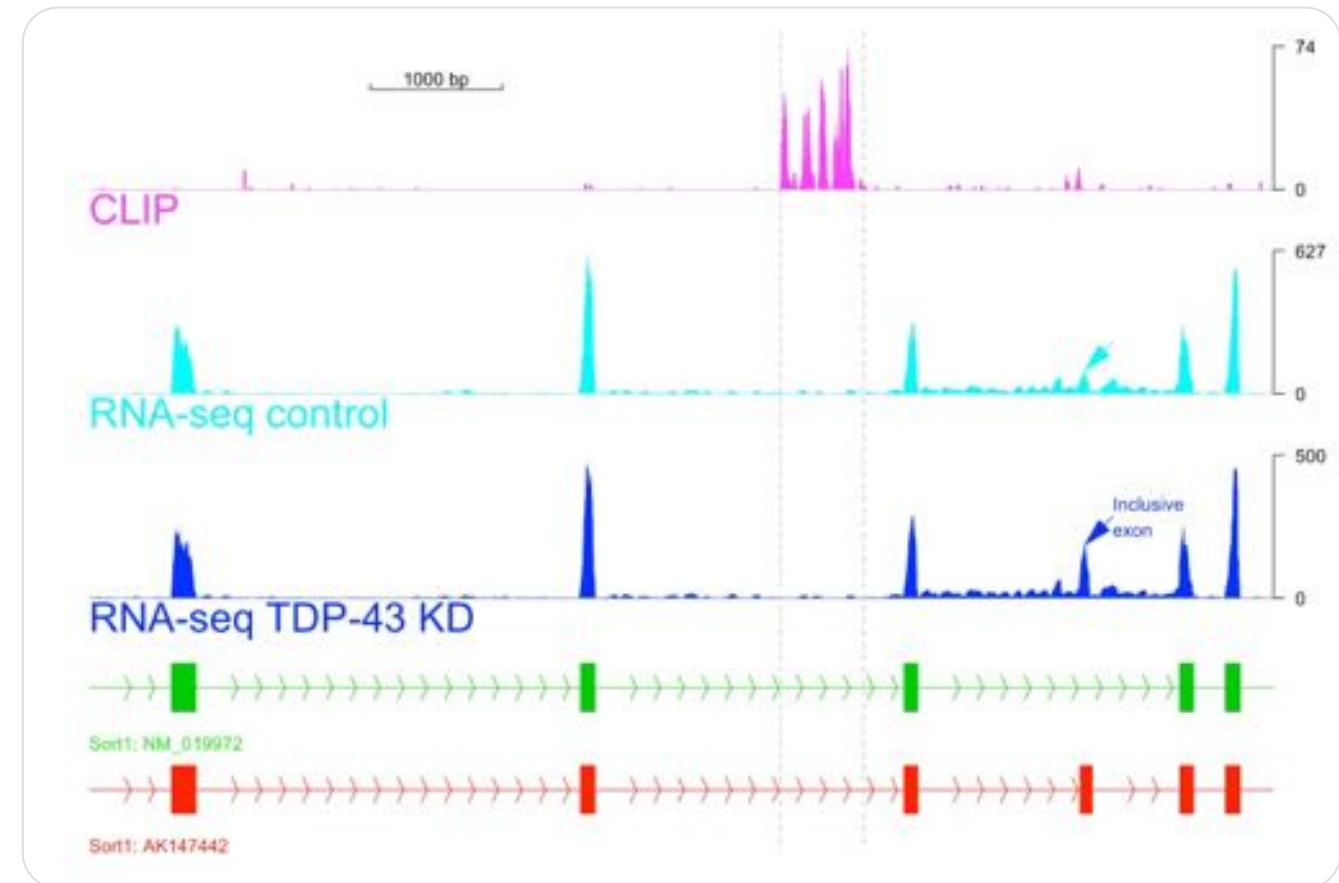


IPA



<https://digitalinsights.qiagen.com/products/features/>

View tracks by trackViewer



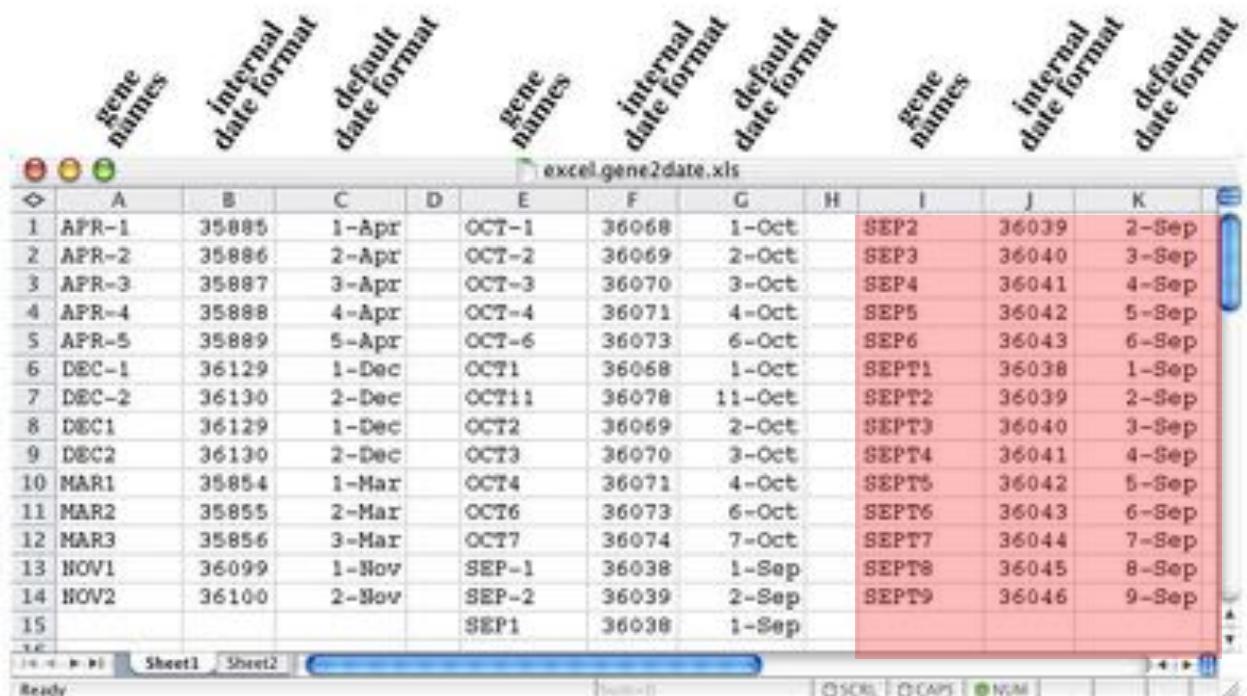
Ou et.al., 2019. doi: 10.1038/s41592-019-0430-y

SAVE RESULTS

The results can be saved in XLS file format using [WriteXLS](#) package to avoid the gene name errors that can be inadvertently introduced when opened by Excel.

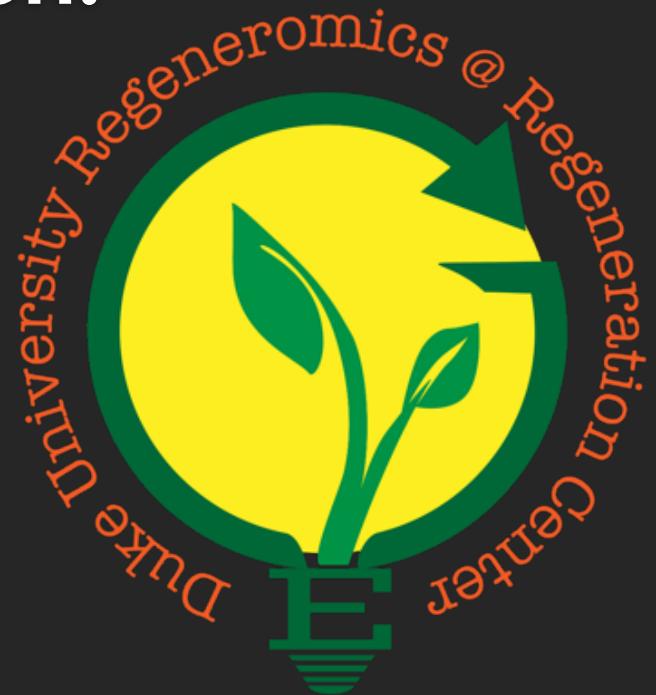


```
library(WriteXLS)  
WriteXLS(a_data_frame, "output.xls")
```



	A	B	C	D	E	F	G	H	I	J	K
1	APR-1	35885	1-Apr	OCT-1	36068	1-Oct	SEP2	36039	2-Sep		
2	APR-2	35886	2-Apr	OCT-2	36069	2-Oct	SEP3	36040	3-Sep		
3	APR-3	35887	3-Apr	OCT-3	36070	3-Oct	SEP4	36041	4-Sep		
4	APR-4	35888	4-Apr	OCT-4	36071	4-Oct	SEP5	36042	5-Sep		
5	APR-5	35889	5-Apr	OCT-6	36073	6-Oct	SEP6	36043	6-Sep		
6	DEC-1	36129	1-Dec	OCT1	36068	1-Oct	SEPT1	36038	1-Sep		
7	DEC-2	36130	2-Dec	OCT11	36078	11-Oct	SEPT2	36039	2-Sep		
8	DEC1	36129	1-Dec	OCT2	36069	2-Oct	SEPT3	36040	3-Sep		
9	DEC2	36130	2-Dec	OCT3	36070	3-Oct	SEPT4	36041	4-Sep		
10	MAR1	35854	1-Mar	OCT4	36071	4-Oct	SEPT5	36042	5-Sep		
11	MAR2	35855	2-Mar	OCT6	36073	6-Oct	SEPT6	36043	6-Sep		
12	MAR3	35856	3-Mar	OCT7	36074	7-Oct	SEPT7	36044	7-Sep		
13	HOV1	36099	1-Nov	SEP-1	36038	1-Sep	SEPT8	36045	8-Sep		
14	HOV2	36100	2-Nov	SEP-2	36039	2-Sep	SEPT9	36046	9-Sep		
15				SEP1	36038	1-Sep					

Regeneromics Shared Resource can help your research!



We do: experimental design, bioinformatics analysis, manuscript preparation, grant applications



Jianhong Ou, Ph.D.

Email: regeneromics@duke.edu

<https://sites.duke.edu/dukeregenerationcenter/jobsrni/>

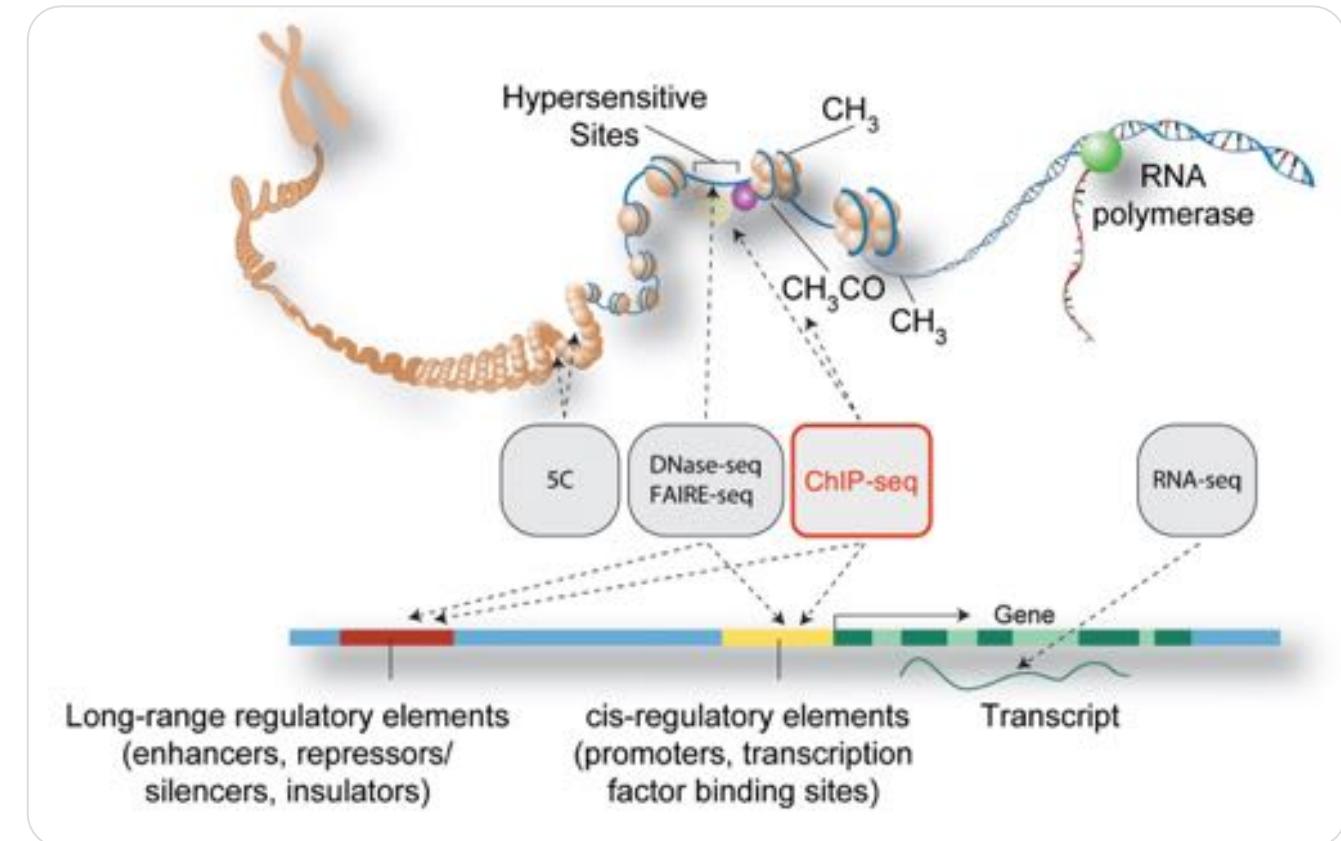
CHIP-SEQ

<https://jianhong.github.io/genomictools/index.html>

ChIP-seq technology

Chromatin immunoprecipitation followed by sequencing (ChIP-seq) is a technique for genome-wide profiling of DNA-binding proteins, histone modifications or nucleosomes. – Peter J. Park

Park, 2009. doi:10.1038/nrg2641



The ENCODE Project Consortium 2011. doi: 10.1371/journal.pbio.1001046

Uses

- Determine the DNA targets for transcription factors (TFs) in genome-wide
- Capture the histone modifications across the entire genome
- Define the TF binding motifs
- Reveal gene regulatory networks in combination with RNA sequencing and methylation analysis

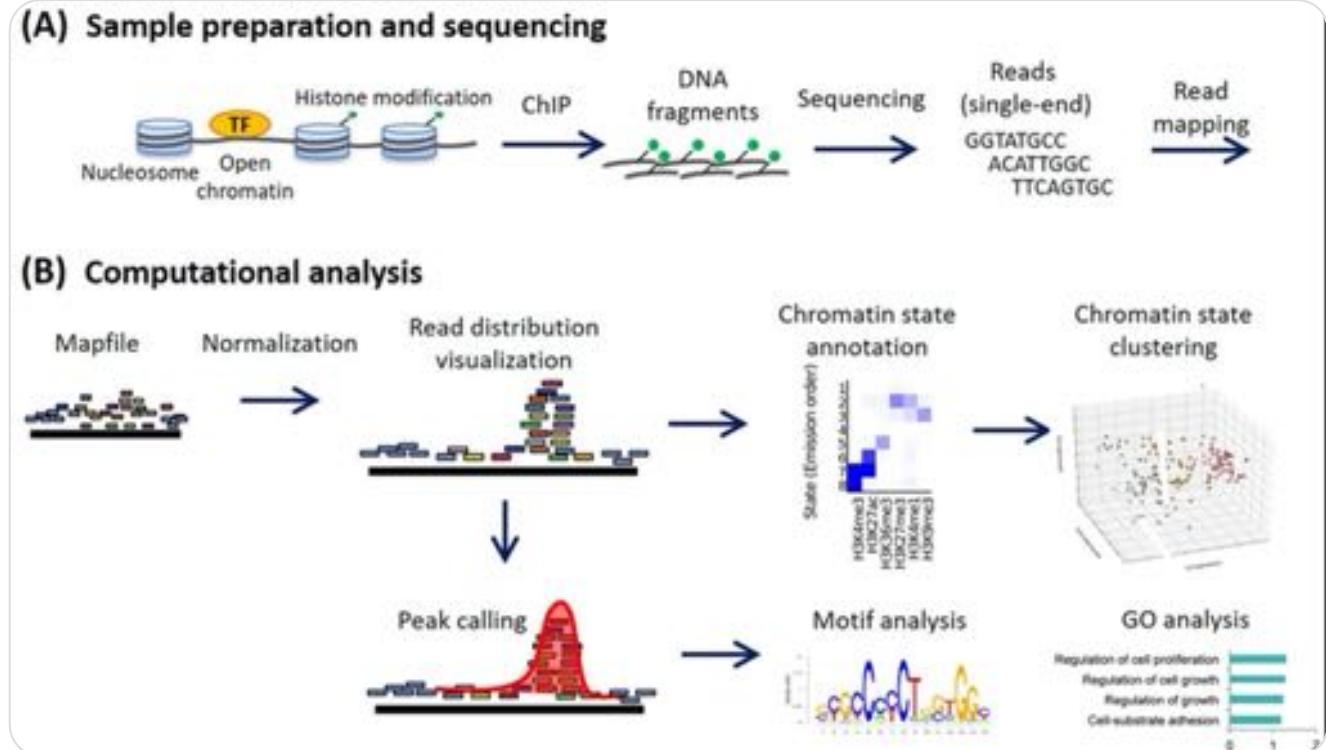
<https://www.illumina.com/techniques/sequencing/dna-sequencing/chip-seq.html>

ENCODE and Epigenomics Roadmap

The collage consists of three main panels:

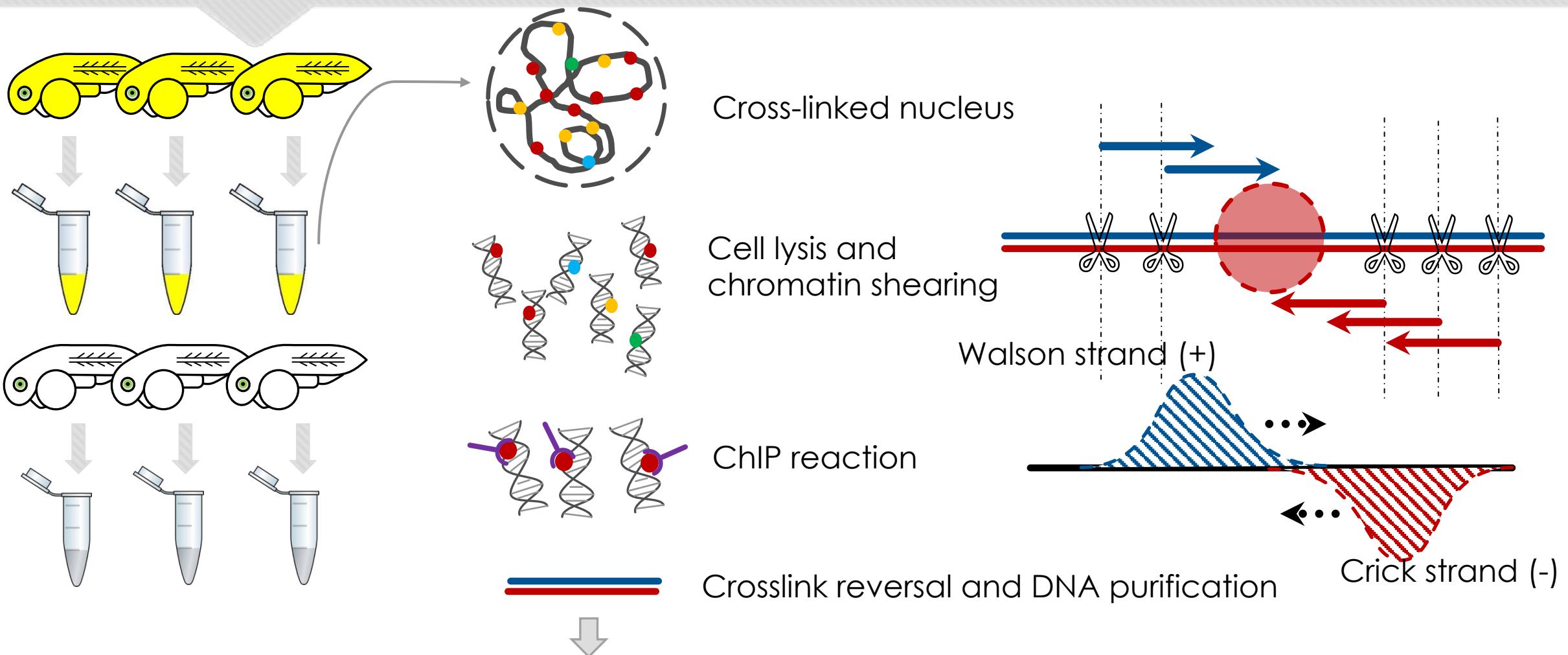
- Left Panel (nature magazine cover):** The cover of the September 6, 2012, issue of *nature*. It features a large circular graphic titled "GUIDEBOOK TO THE HUMAN GENOME" with "ENCODE" in the center. Below it, the text "The ENCODE project in print and online" and "PAGE 45" is visible.
- Middle Panel (ENCODE and NIH Roadmap websites):**
 - ENCODE Portal:** A screenshot of the ENCODE Encyclopedia of DNA Elements portal. It shows a diagram of epigenetic marks (H3K4me1, H3K27me3, H3K36me3, H3K9me3, H3K27ac, H3K4me3, H3K9me2, H3K36me2) and their relationship to transcription factors, chromatin, and genes. Below the diagram are search and browse interfaces for the ENCODE Encyclopedia and candidate cis-regulatory elements, with tabs for Human GRCh38 and Mouse mm10.
 - NIH Roadmap Epigenomics Mapping Consortium:** A screenshot of the NIH Roadmap Epigenomics Mapping Consortium website. It features a diagram of a cell showing chromatin, genes, and RNA. Below the diagram is a section titled "NIH Roadmap Epigenomics Mapping Consortium".
- Right Panel (nature magazine cover):** The cover of the February 14, 2013, issue of *nature*. It features a colorful illustration of a futuristic city with flying cars, a hot air balloon, and a bridge. The title "nature" is at the top, and the subtitle "THE INTERNATIONAL WEEKLY JOURNAL OF SCIENCE" is below it. The main headline is "EPIGENOME ROADMAP Functional regulatory elements in genomes from human tissues PAGE 213". Other news items include "LAST RAYS OF THE SUN" (PAGE 29 & 124), "PALAEONTOLOGY HARNESSING FOSSIL POWER" (PAGE 22), "TOXICOLOGY RETHINK ON RISK DATA" (PAGE 27), "SPECTRUM OF CONFUSION" (PAGE 298), "CREATIVE ACCOUNTING" (PAGE 296), and "LITHIUM IN THE STARS" (PAGE 297 & 298). A barcode is in the bottom right corner.

ChIP-seq analysis workflow



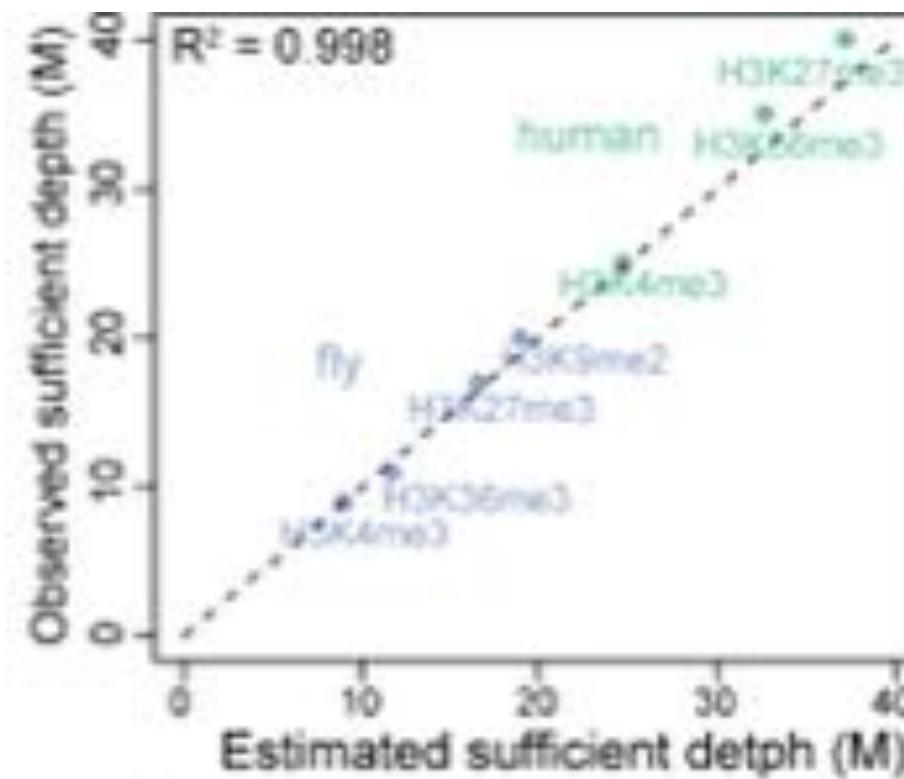
Nakato et.al., 2020. doi: 10.1016/j.ymeth.2020.03.005

ChIP-seq protocol



Sequencing Depth

- Sharp peaks (TFs)
 - > 10M reads human/mouse genomes
- Broad Peaks (Histones)
 - > 20M reads human/mouse genomes



Single end or paired end

- Paired end (PE) sequencing
 - improved efficiency of alignment to repetitive regions
 - Greater ability to detect fragment sizes
- Single-end (SE) sequencing is cheaper than PE.

Control sample

Input DNA

- Controls for CNVs, sequencing biases, fragmentation and shearing biases

IgG

- As with input but also controls for non-specific binding

Replicates

- Biological replicate experiments are necessary.
- Use different antibodies are recommended.

Short reads alignment for ChIP-seq



Quality Assessment of sequencing:
FastQC

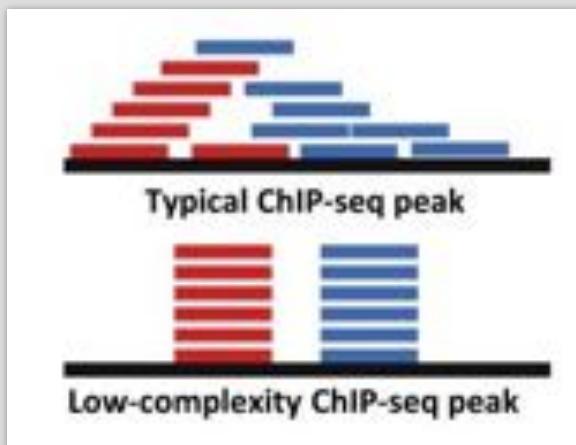


Mapping reads to reference genome:
bowtie/2, bwa, Rsubread, STAR



Remove potential PCR duplicates:
Picard software suite::MarkDuplicates,
Samtools::rmdup

Library complexity (Duplicates)

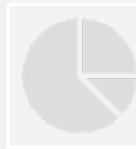


Landt et.al., 2012. doi: 10.1101/gr.136184.111



Duplication rates are a useful QC metric

(Duplicate reads/Total Mapped Reads) *100
Expected to be low



Non-Redundant Fraction (NRF)

ENCODE guidelines: NRF >= 0.8 for 10M reads

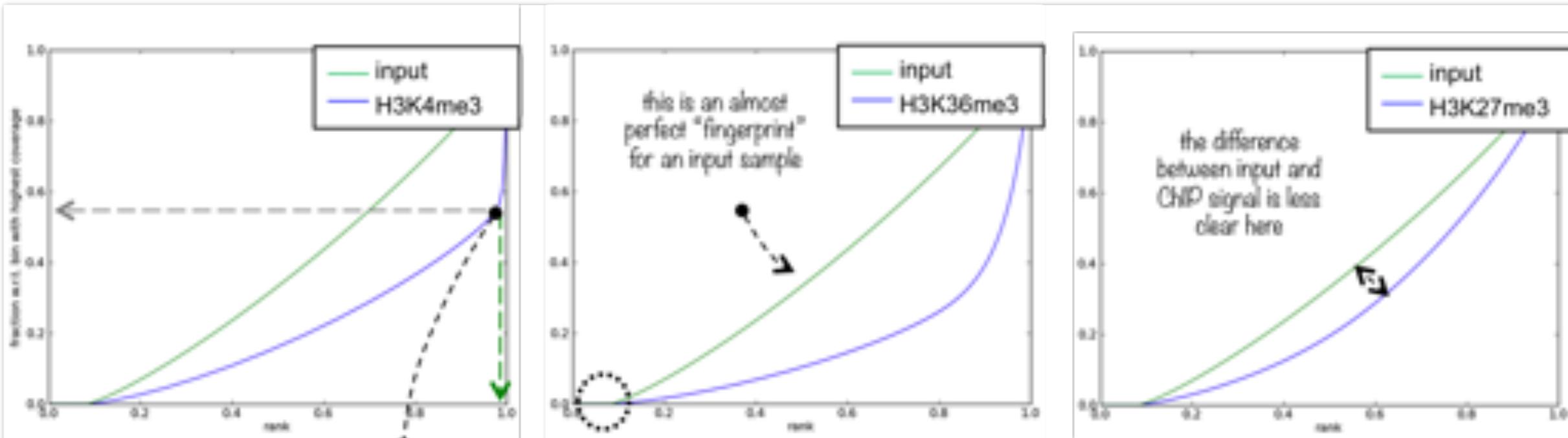


PCR Bottleneck Coefficient (PBC)

A measure of library complexity. High-quality ChIP-seq data set should have PBC values >= 0.9

<https://genome.ucsc.edu/ENCODE/qualityMetrics.html>

CUMULATIVE CURVE



when counting the reads contained in 97% of all genomic bins, only ca. 55% of the maximum number of reads are reached, i.e. 3% of the genome contain a very large fraction of reads!

→ this indicates very localized, very strong enrichments!
(as every biologist hopes for in a ChIP for H3K4me3)

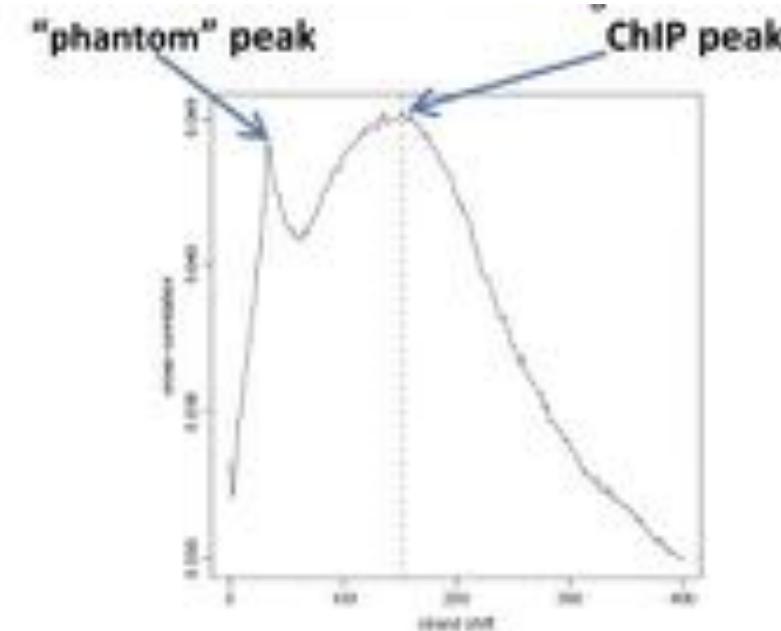
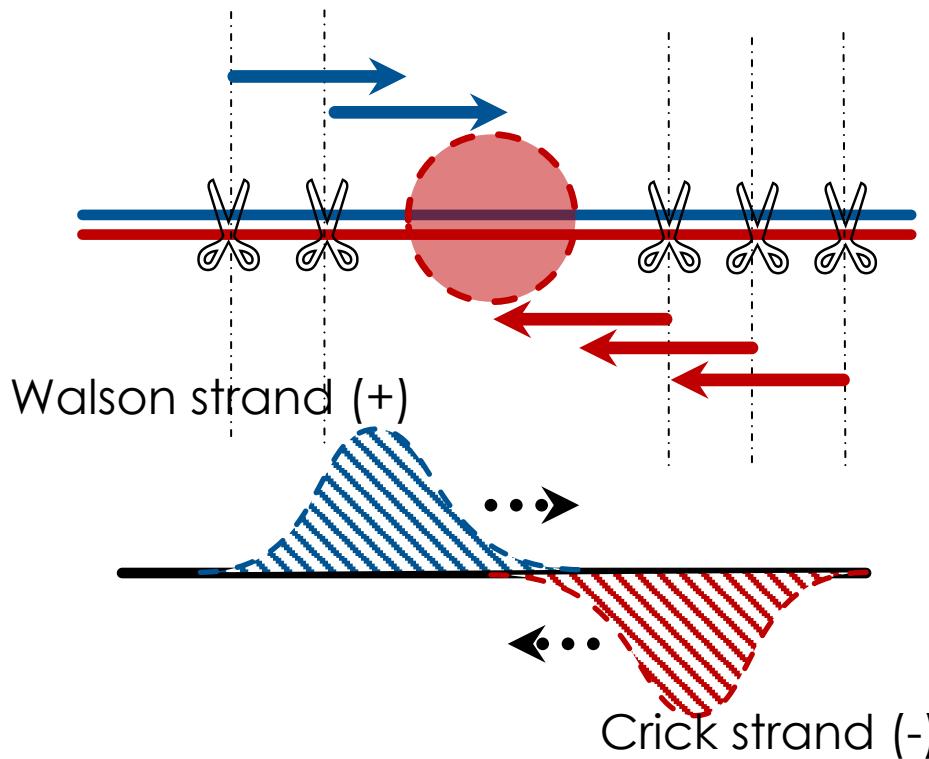
pay attention to where the curves start to rise – this already gives you an assessment of how much of the genome you have not sequenced at all (i.e. bins containing zero reads – for this example, ca. 10% of the entire genome do not have any read)

H3K27me3 is a mark that yields broad domains instead of narrow peaks



it is more difficult to distinguish input and ChIP, it does not mean, however, that this particular ChIP experiment failed

Cross-correlation



Strand cross-correlation is computed as the Pearson correlation between the positive and the negative strand profiles at different strand shift distances

Quality Control

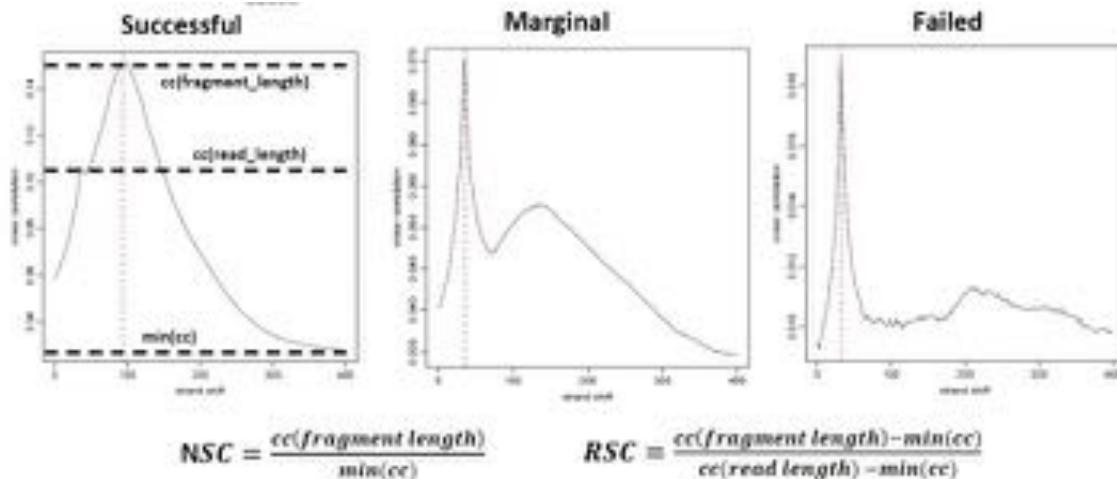


Normalized Strand Cross-correlation coefficient (NSC)
High-quality ChIP-seq data sets should have NSC values ≥ 1.05 .



Relative Strand Cross-correlation coefficient (RSC)
High-quality ChIP-seq data sets should have RSC values ≥ 0.8

<https://genome.ucsc.edu/ENCODE/qualityMetrics.html>



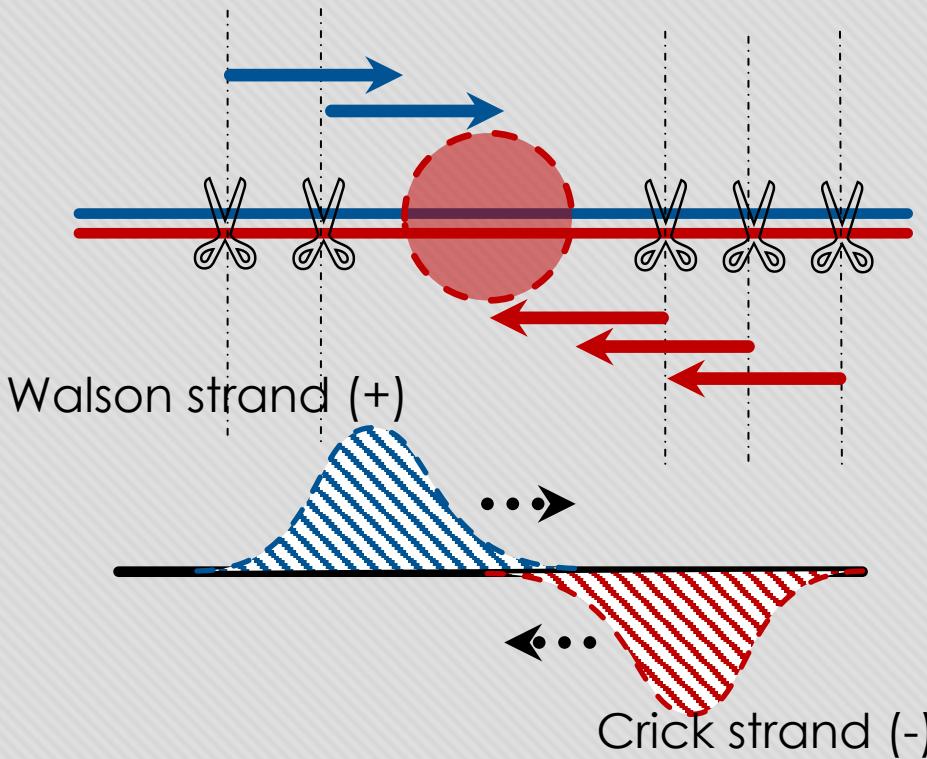
Landt et.al., 2012. doi: 10.1101/gr.136184.111

Calculate NSC and RSC by phantompeaktools

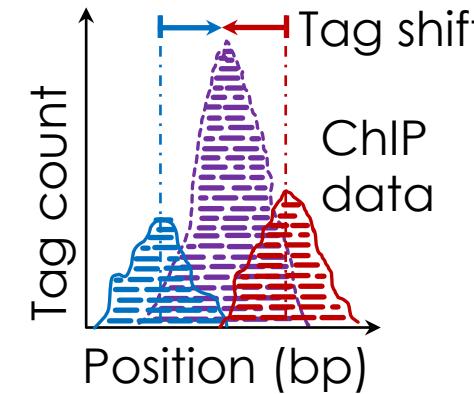
Rscript run_spp.R -c=<tagAlign/BAMfile> -savp -out=<outFile>

<https://github.com/kundajelab/phantompeakqualtools>

Call peaks

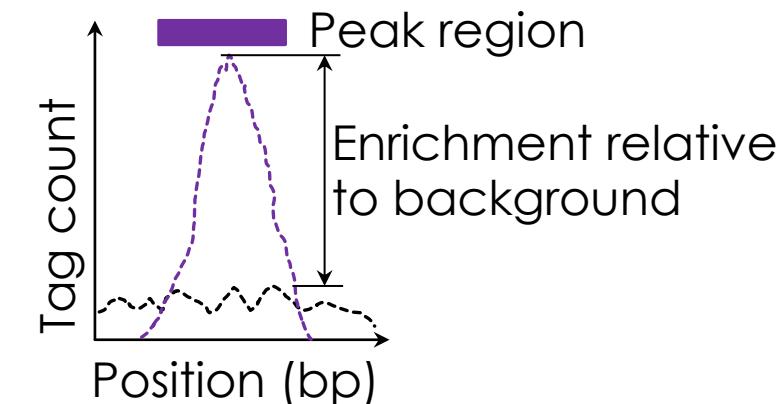
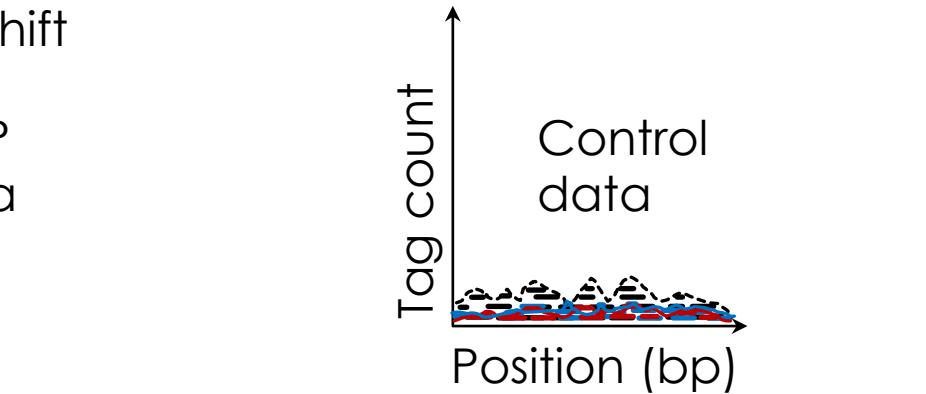


Generate signal profile
along each chromosome



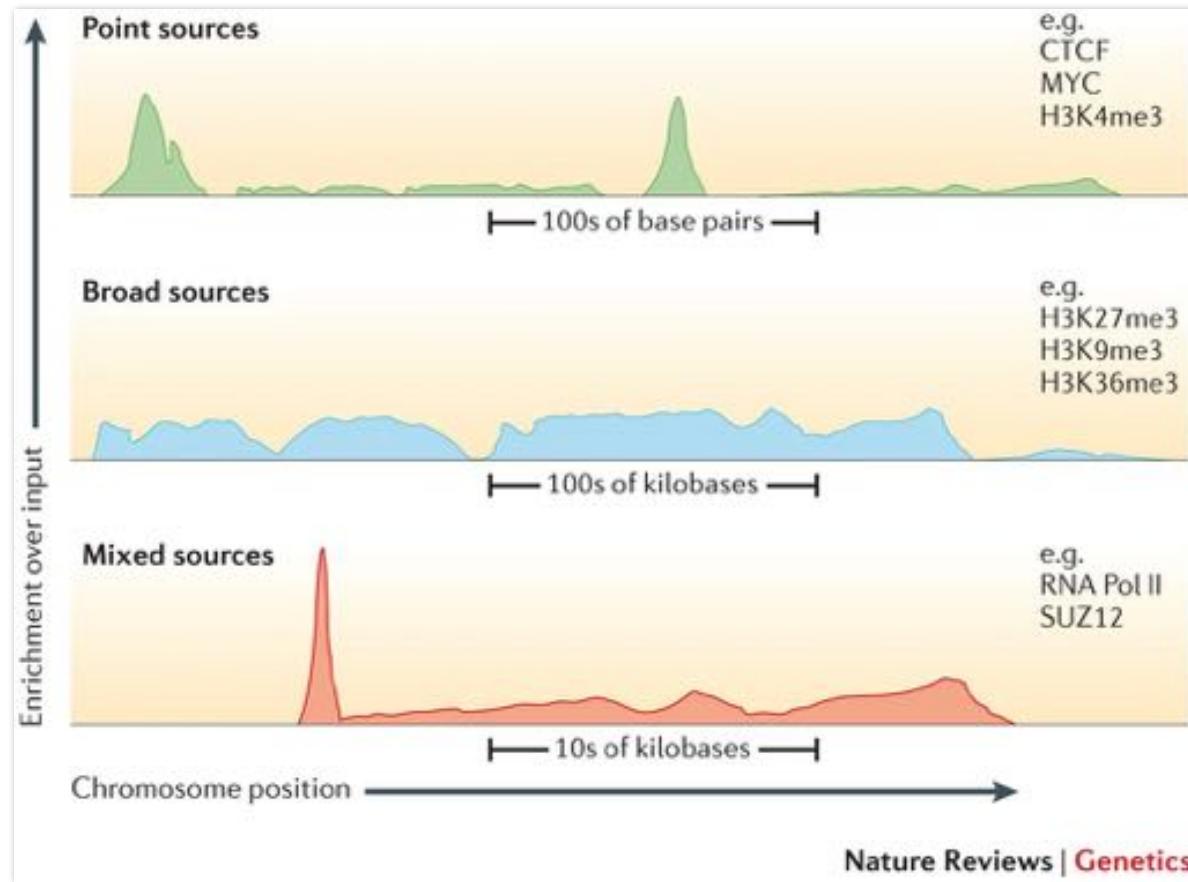
Identify
peaks in
ChIP
signal

Define background
(model or data)

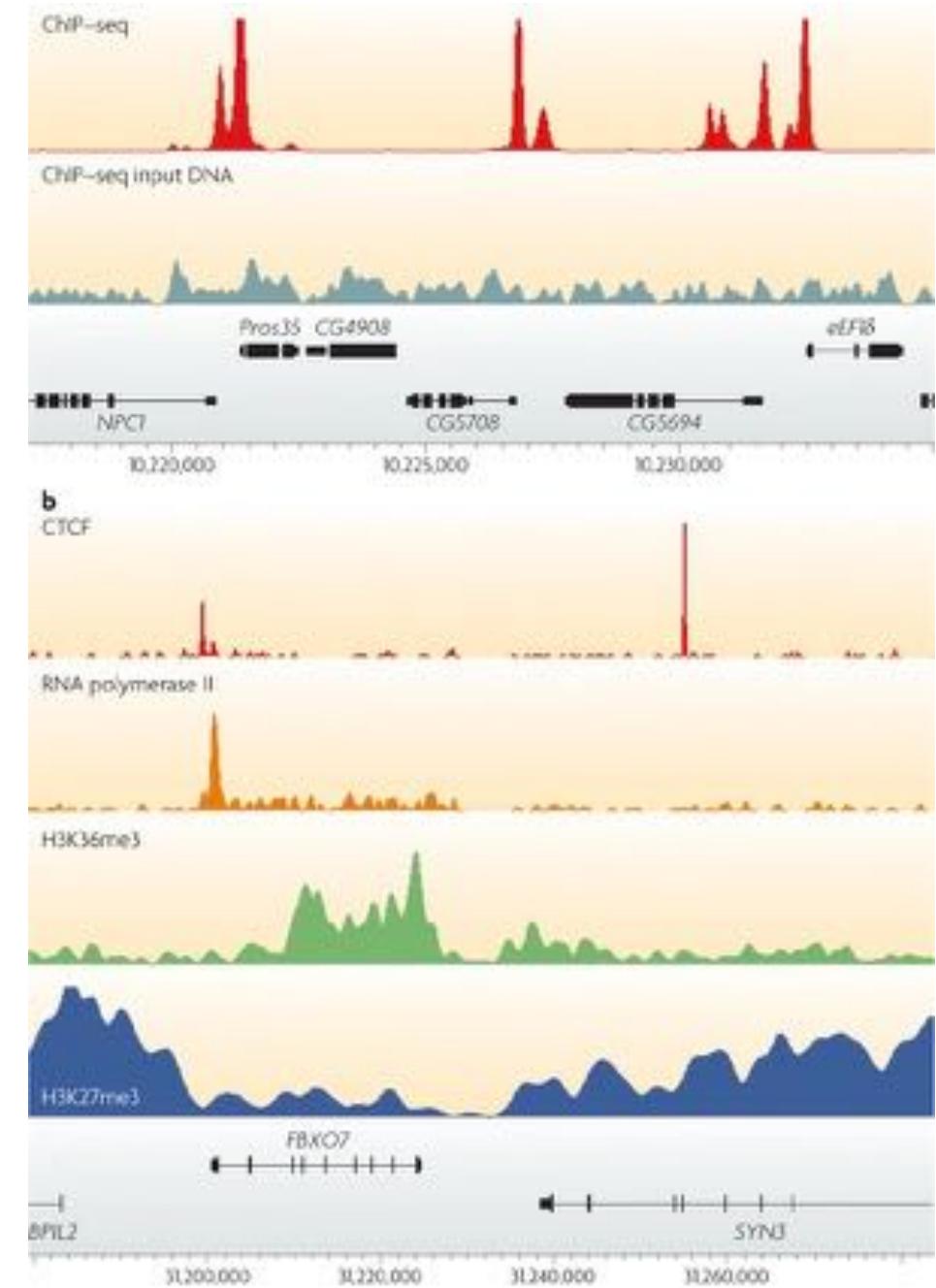


(Modified from [Nature Methods doi:10.1038/nmeth.1371](https://doi.org/10.1038/nmeth.1371),
S.Pepke)

THREE DIFFERENT TYPES OF PEAKS



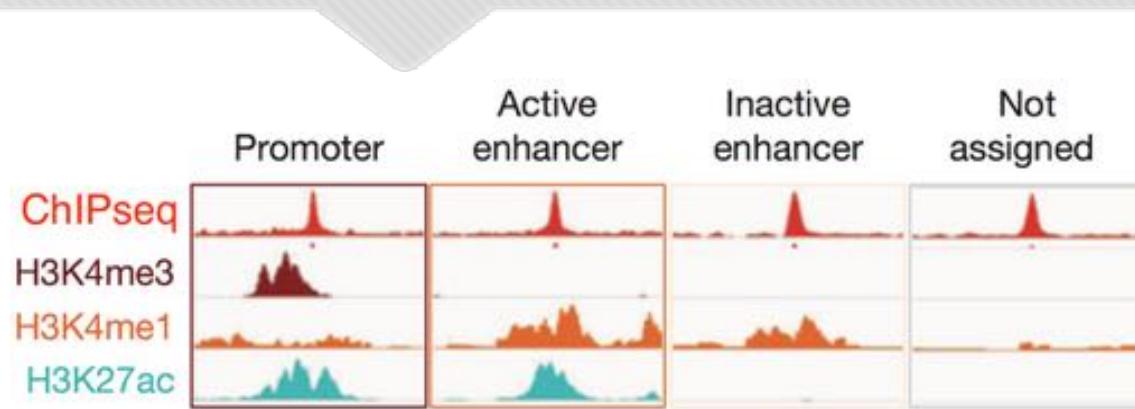
Sims et.al., 2014. doi: 10.1038/nrg3642



Park, 2009. doi:10.1038/nrg2641,
P Park

Nature Reviews | Genetics

Histone modifications



- H3K27ac <--> active enhancers and promoters
- H3K4me3 <--> active promoter
- H3K4me1 <--> active enhancers
- H3K27me3 <--> silenced genes

Broad peaks	Narrow peaks
H3K27me3	H3ac
H3K36me3	H3K27ac
H3K4me1	H3K4me2
H3K79me2	H3K4me3
H3K79me3	H3K9ac
H3K9me1	
H3K9me2	
H4K20me1	

Zanconato et.al., 2015. doi:10.1038/ncb3216

<https://www.cellsignal.com/contents/resources-reference-tables/histone-modification-table/science-tables-histone>

Peak caller

Transcription factor peaks:

- MACS
- Homer

Histone marks:

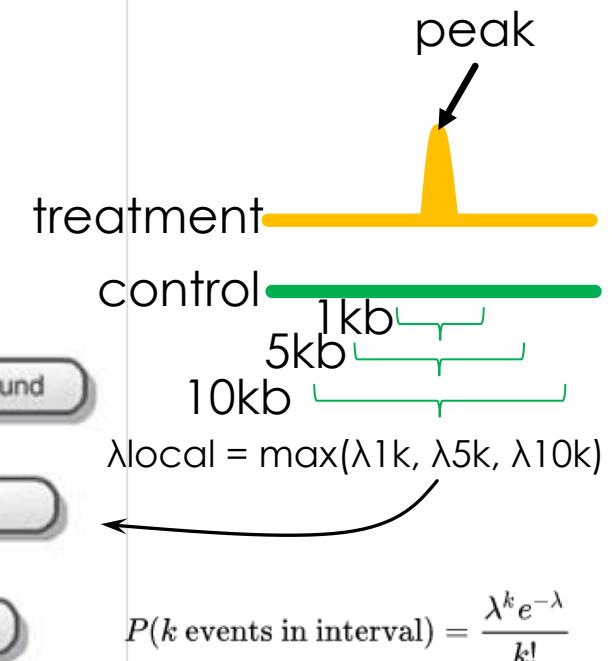
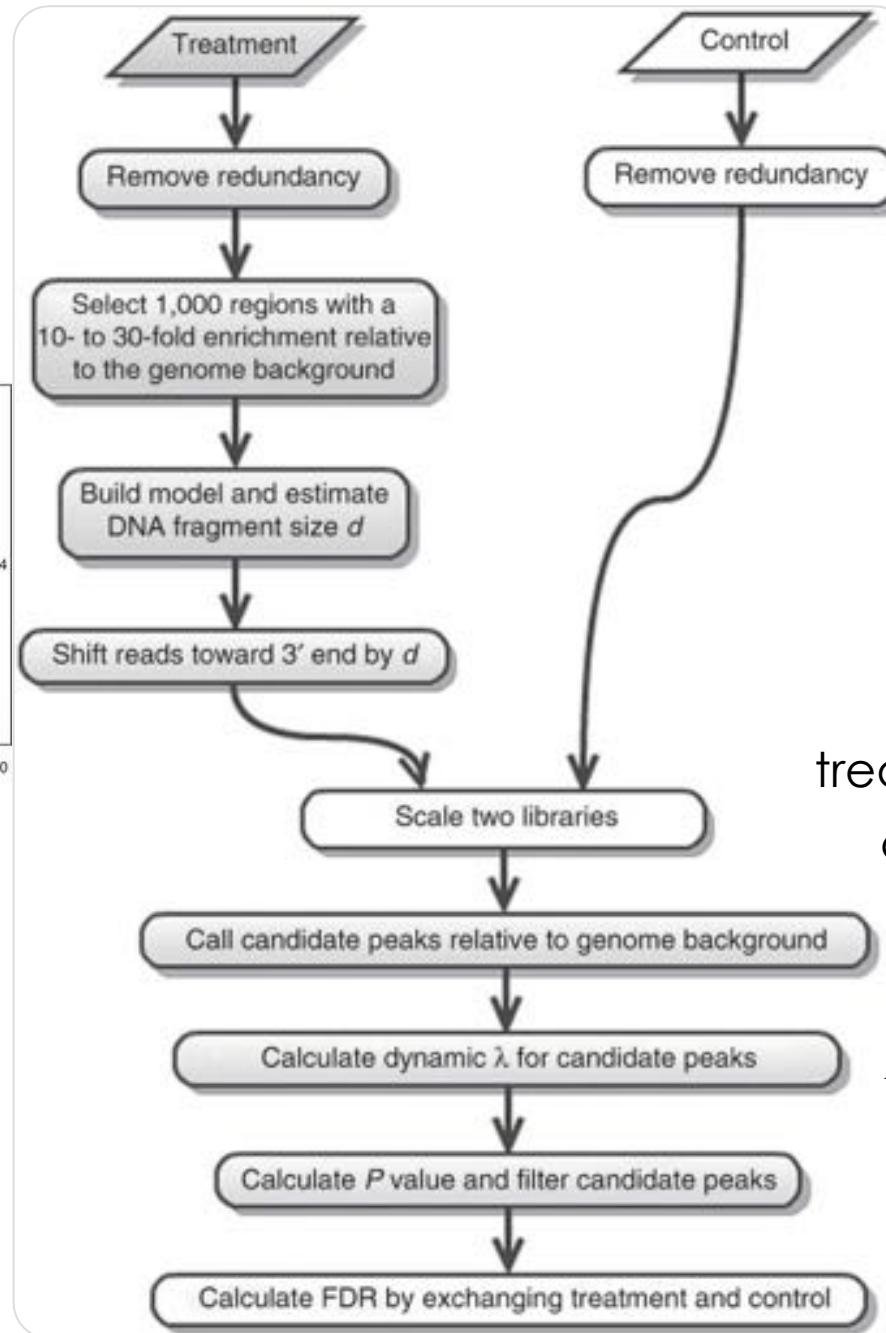
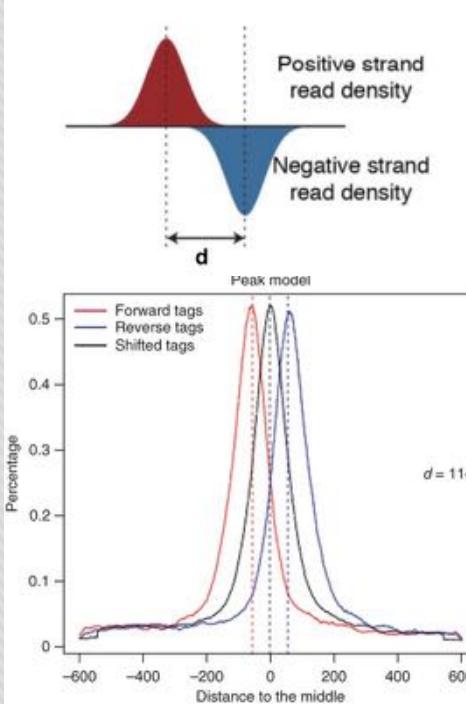
- SICER2
- SPP+MACS
- Homer

Program	Reference	Version	Graphical user interface?	Window-based scan	Tag clustering	Gaussian kernel density estimator	Strand-specific scoring	Peak height or fold enrichment (FEE)	Background subtraction	Compensates for genomic duplications or deletions	False Discovery Rate	Compare to normalized control data (FEE)	Compare to statistical model fitted with control data	Statistical model or test
CisGenome	28	1.1	X*	X			X	X		X		X		conditional binomial model
Minimal ChIPSeq Peak Finder	16	2.0.1			X			X			X			
E-RANGE	27	3.1			X			X			X	X		chromosome scale Poisson dist.
MACS	13	1.3.5		X				X				X		local Poisson dist.
QuEST	14	2.3				X		X			X**		X	chromosome scale Poisson dist.
HPeak	29	1.1		X				X				X		Hidden Markov Model
Sole-Search	23	1	X	X				X		X		X		One sample t-test
PeakSeq	21	1.01			X			X				X		conditional binomial model
SiSSRS	32	1.4		X			X				X			
spp package (wid & mtc)	31	1.7		X			X		X	X'	X			

X* = Windows-only GUI or cross-platform command line interface
X** = optional if sufficient data is available to split control data
X' = method excludes putative duplicated regions, no treatment of deletions

Generating density profiles Peak assignment Adjustments w. control data Significance relative to control data

MACS/2 (Model-based Analysis of ChIP-seq)



Running MACS2

Sharp peaks:

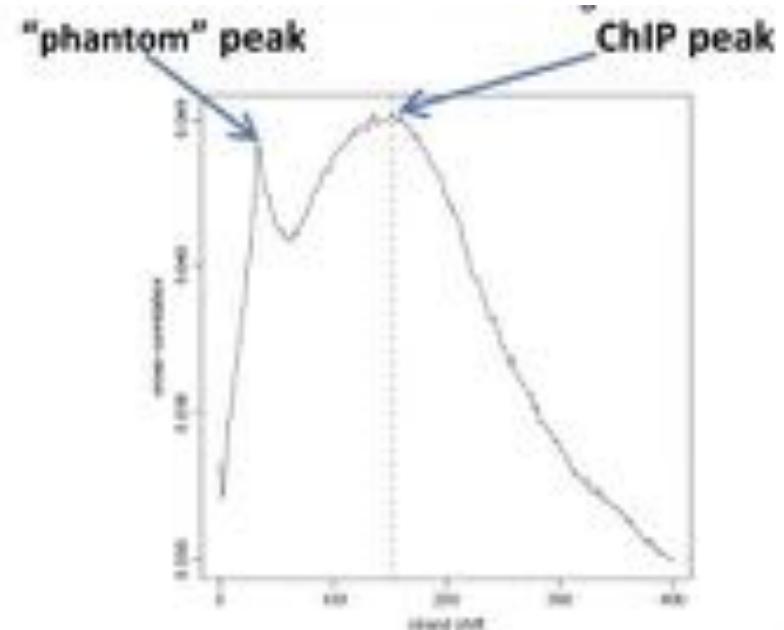
```
macs2 callpeak -t treatment.bam -c input.bam \
-f BAM -g hs -n sampleName --outdir outputFolder \
-q 0.01
```

Broad peaks:

```
macs2 callpeak -t treatment.bam -c input.bam \
-f BAM -g hs -n sampleName --outdir outputFolder \
--broad --broad-cutoff 0.1
```

Running MACS2 with user-defined model

```
macs2 callpeak -t treamtment.bam -c input.bam \
-f BAM -g hs -n sampleName \
--outdir outputFolder \
--nomodel --shift 80 --extsize 160
```



Calculate cross-correlation by phantompeaktools

Rscript run_spp.R -c=<tagAlign/BAMfile> -savp -out=<outFile>

<https://github.com/kundajelab/phantompeakqualtools>

Output of MACS2

- NAME_peaks.xls
- NAME_peaks.narrowPeak
- NAME_summits.bed
- chromosome name
- start position of peak
- end position of peak
- length of peak region
- absolute peak summit position
- pileup height at peak summit
- $-\log_{10}(\text{pvalue})$ for the peak summit (e.g. pvalue = $1e-10$, then this value should be 10)
- fold enrichment for this peak summit against random Poisson distribution with local lambda,
- $-\log_{10}(\text{qvalue})$ at peak summit

File formats: bed, bedGraph, wig, bigwig, narrowPeak, broadPeak, ...

- Help documentation:
<https://genome.ucsc.edu/FAQ/FAQformat.html>
- BED format:
 - chrom chromStat chromEnd name score strand ...
 - Sample file:

```
chr22 1000 5000 cloneA 960 +
chr22 2000 6000 cloneB 900 -
```
- WIG (Wiggle Track) format: (no strand information)
 - **variableStep format**

```
variableStep chrom=chrN
[span=windowSize]
chromStartA minValueA
chromStartB minValueB
... etc ... etc ...
```
 - **fixedStep format**

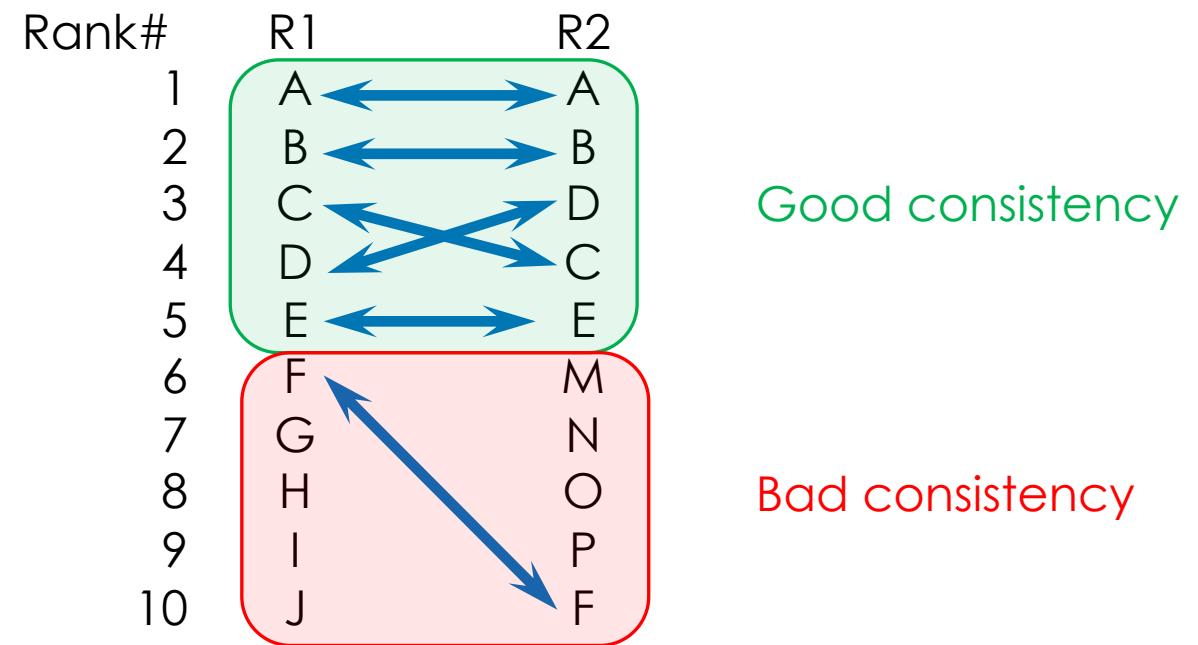
```
fixedStep chrom=chrN
start=position step=stepInterval
[span=windowSize]
minValue1
minValue2
... etc ...
```

Blacklist/Hotspots

- Blacklisted regions are genomic regions with anomalous, unstructured, high signal or read counts in NGS experiments, independent of cell type or experiment.
- Where to download: <https://sites.google.com/site/anshulkundaje/projects/blacklists>

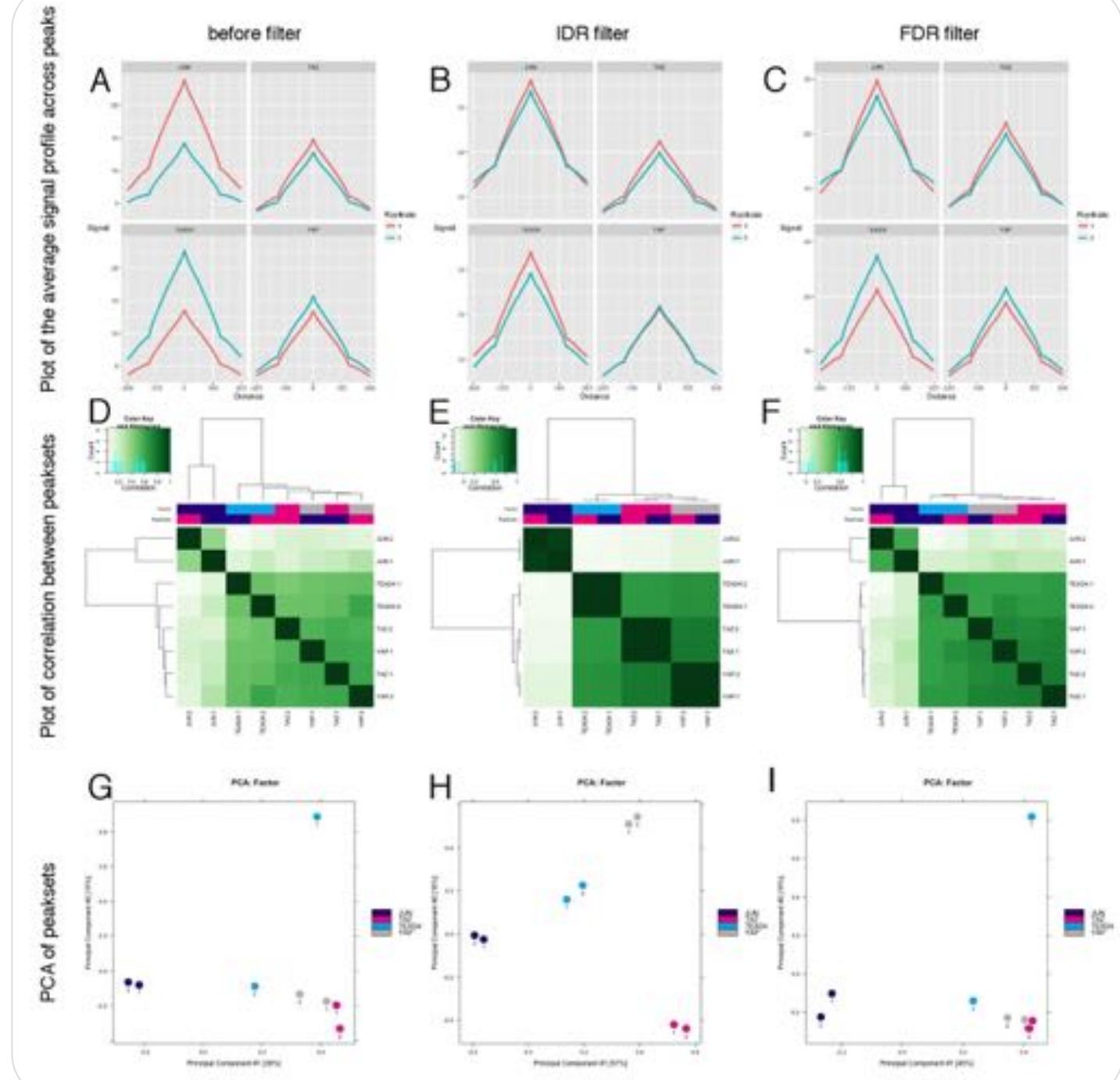
Irreproducibility Discovery Rate (IDR) analysis

The IDR framework to filter the low reproducible peaks. The IDR framework is a unified approach to measure the reproducibility of findings identified from replicate experiments and provide highly stable thresholds based on reproducibility. ([Landt et al., 2012](#))



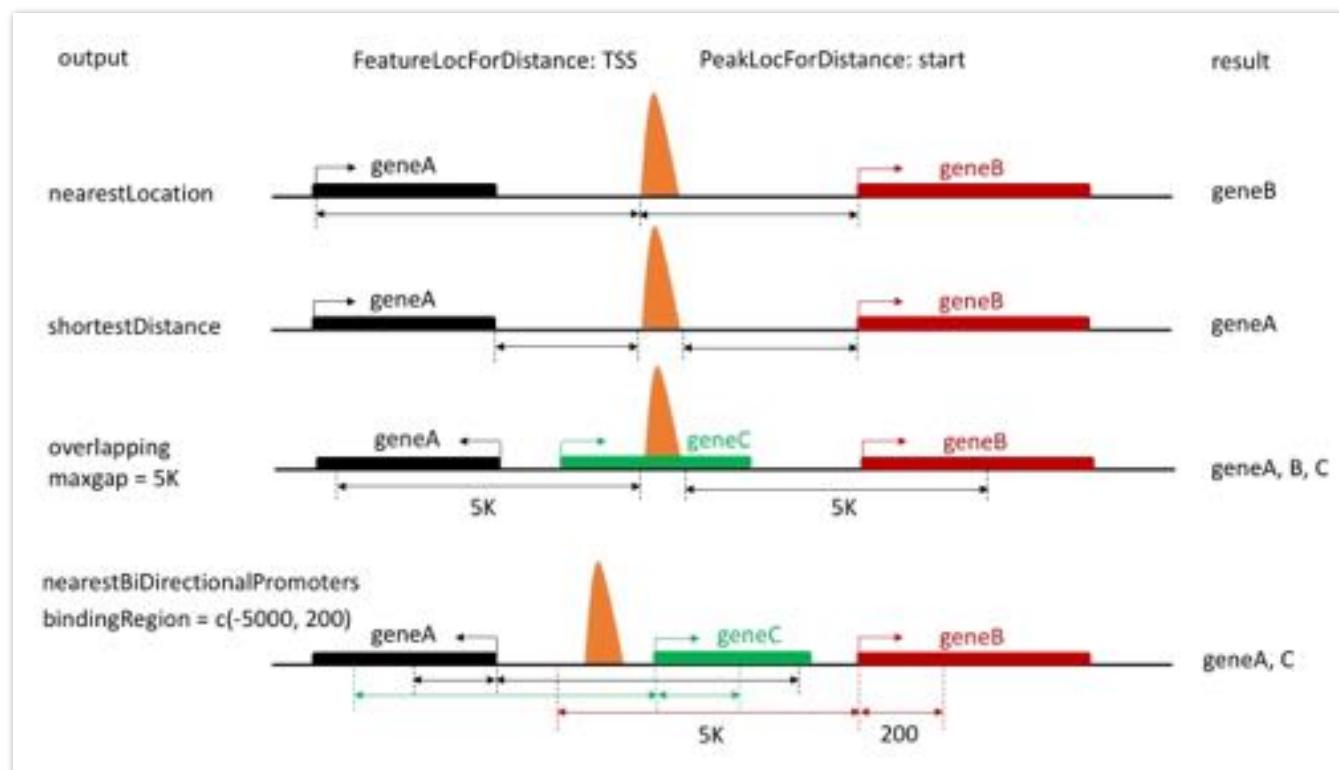
<https://arxiv.org/abs/1110.4705>
<https://github.com/nboley/idr>

The effect of IDR

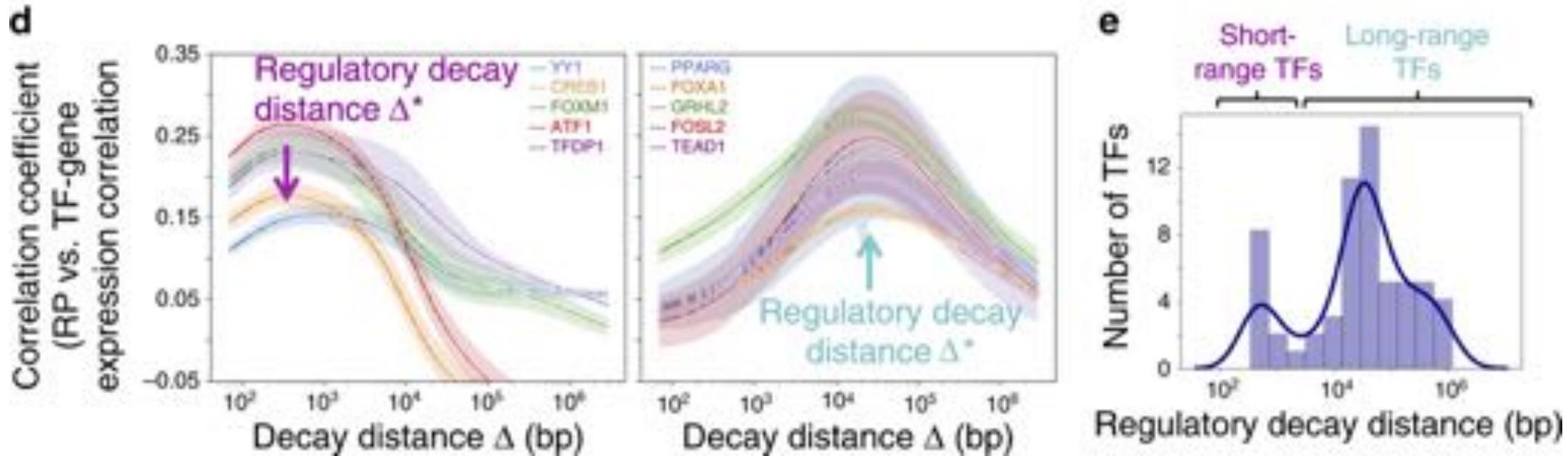


Annotation by ChIPpeakAnno

The *annotatePeakInBatch* and *annoPeaks* functions in [ChIPpeakAnno](#) package can be used to annotate the peaks. Depend on the experiment, we can annotate the peaks in multiple ways.

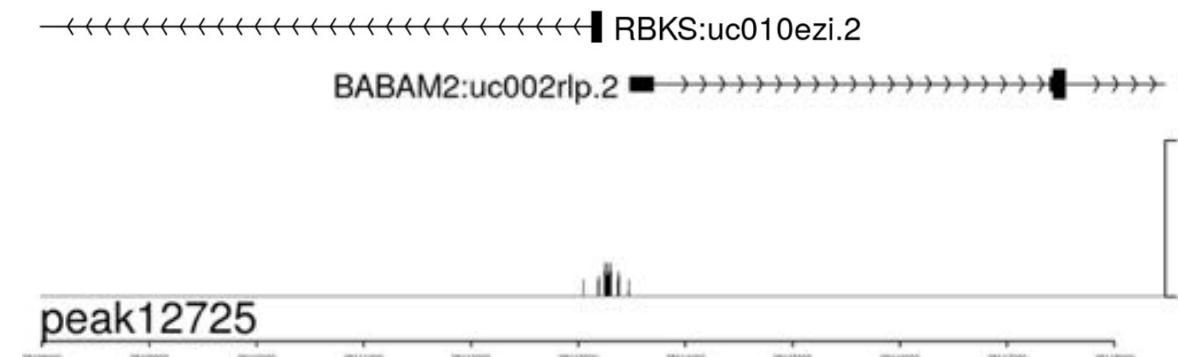


Two distinct TF classes: short-range and long-range



Bidirectional promoters

- Bidirectional promoters are the DNA regions located between the 5' ends of two adjacent genes coded on opposite strands. The two adjacent genes are transcribed to the opposite directions, and often co-regulated by this shared promoter region(Robertson et al., 2007).



Functional annotation of DE genes

Gene ontology
(GO) enrichment
analysis

Kyoto Encyclopedia
of Genes and
Genomes (KEGG)
pathway
enrichment analysis

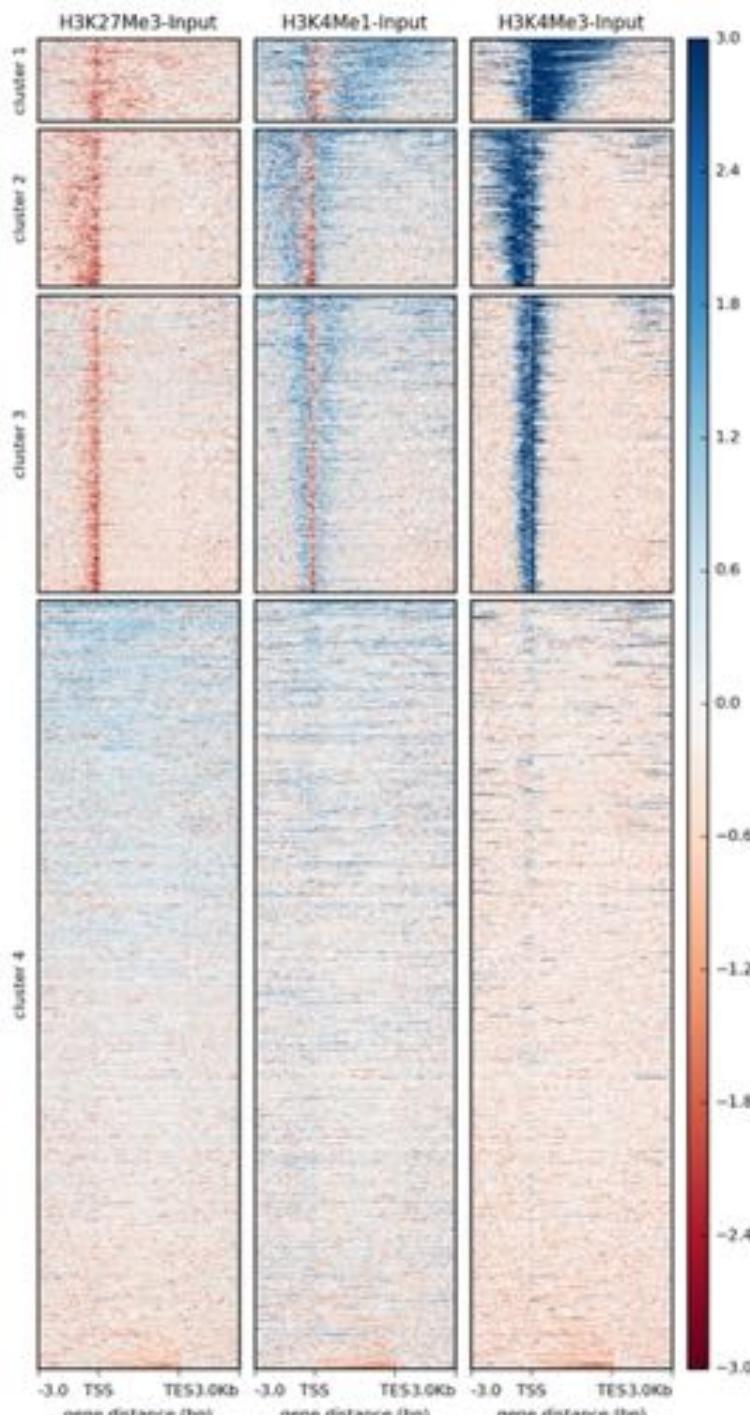
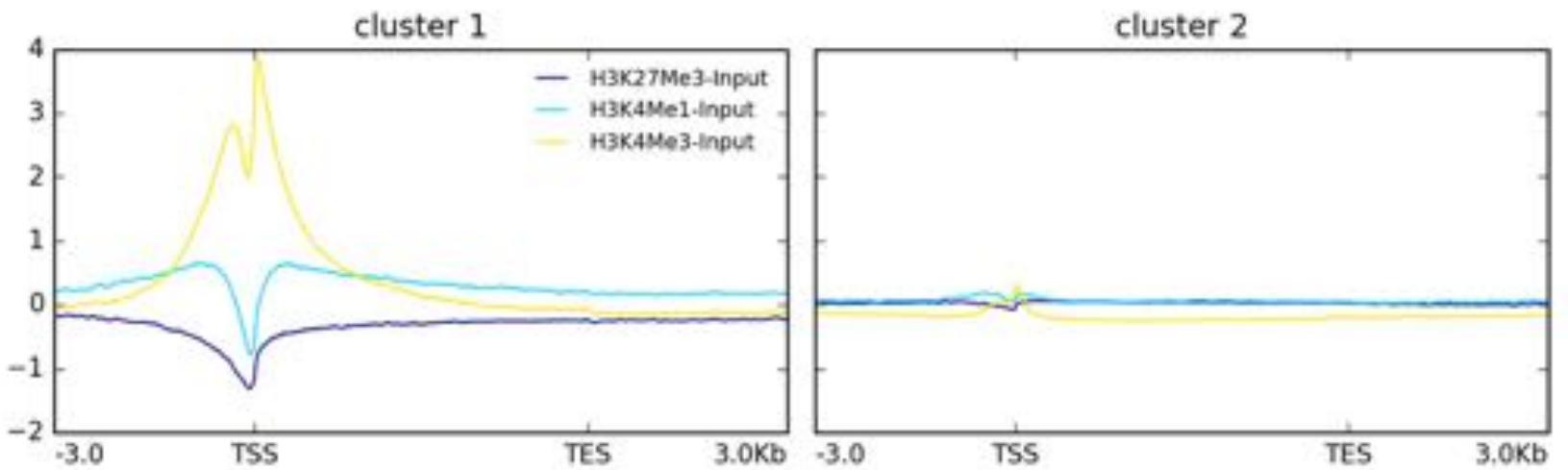
Gene set
enrichment analysis
(GSEA)

QIAGEN Ingenuity
Pathway Analysis
(IPA)

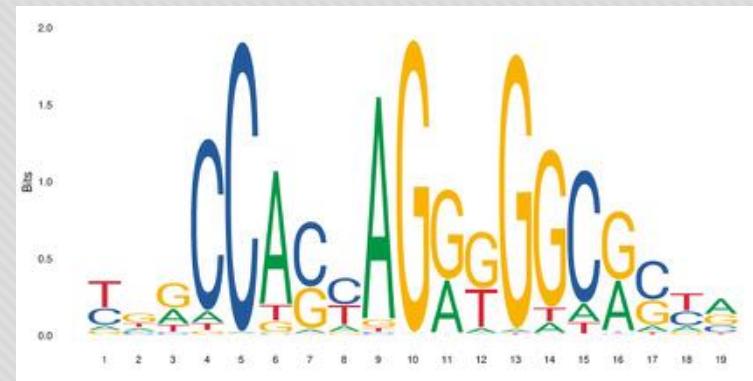
A gene set over-represented comparing to random sampling

Metagene analysis

- A metagene analysis is an average of quantitative data over more than one genomic regions. The interested genomic regions include promoters, transcription factor binding sites, enhancers, etc.



Motif search



- Sequence motif represents conserved characteristics such as DNA binding sites, where transcription factors bind, and catalytic sites in enzymes.
- A sequence logo is a graphical representation of sequence conservation in multiple sequences.
- To search an enriched motifs, multiple softwares could be used, such as [the MEME Suite](#)(Bailey et al., 1994), [Consensus](#)(Hertz et al., 1990), [rGADEM](#), [Homer](#), and et. al.

Build Regulation Network: Integrating TF binding with transcriptomic data

GeneNetworkBuilder (GNB) can be used to facilitate identification of the complex regulatory network of TFs and how indirect targets are inter-connected.

Issue: hairball – too much information

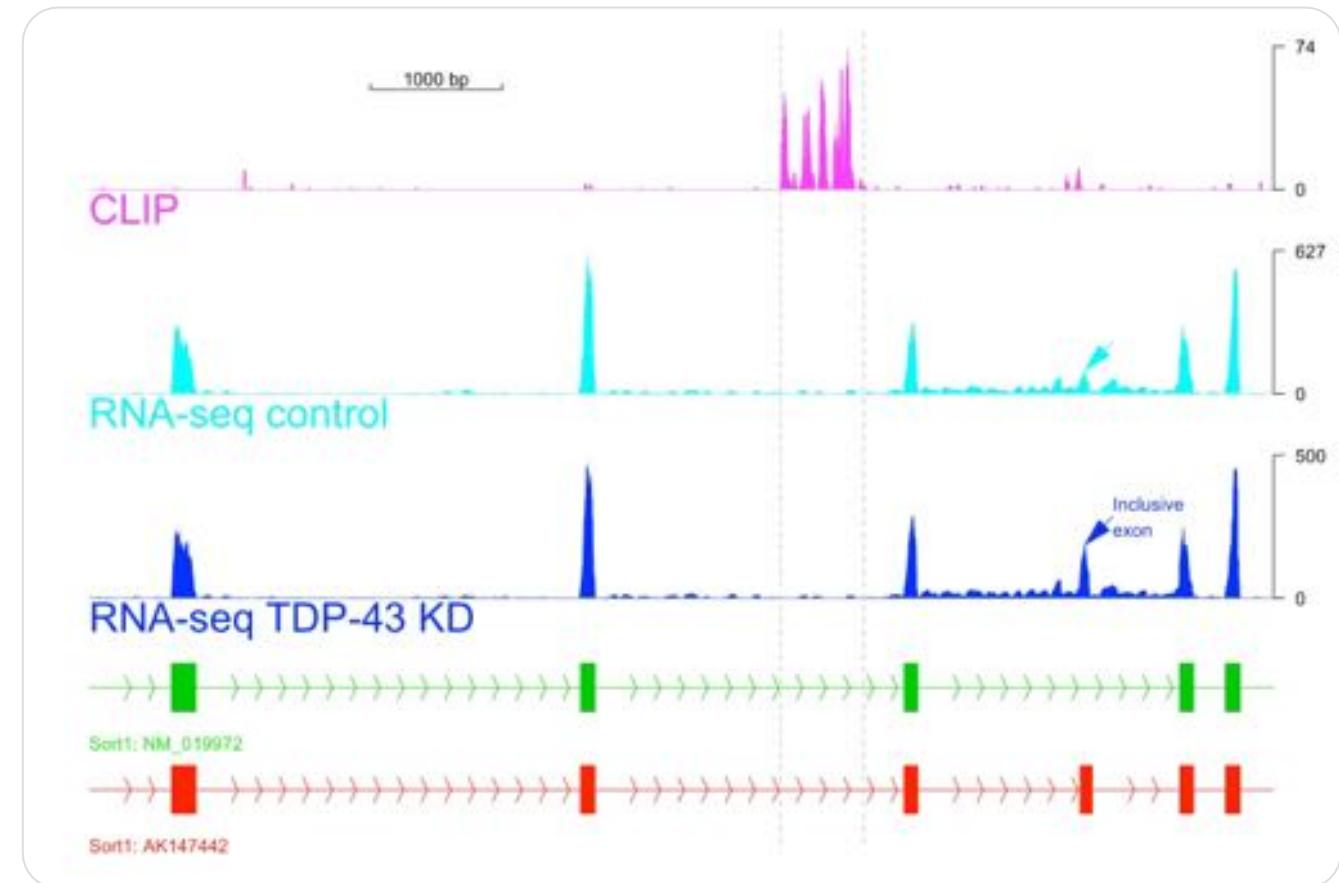
filter data by enrichment analysis: ClueGO

filter the interaction database by evidence.

Focus on a subset network

<https://bioconductor.org/packages/GeneNetworkBuilder/>

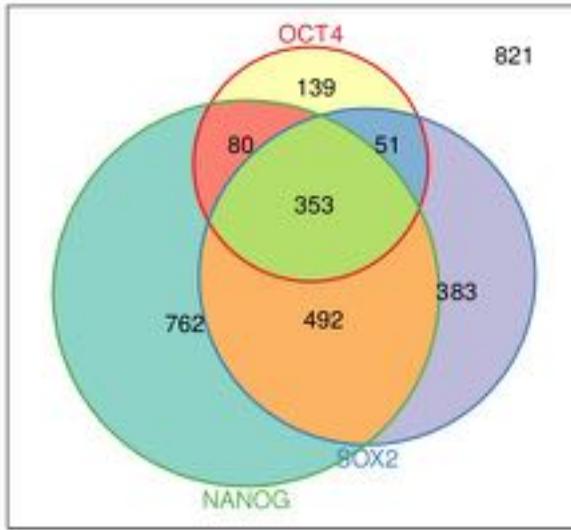
View tracks by trackViewer



Ou et.al., 2019. doi: 10.1038/s41592-019-0430-y

Association among different sets of peaks

Venn diagram and upset plot



<https://github.com/js229/Vennerable>

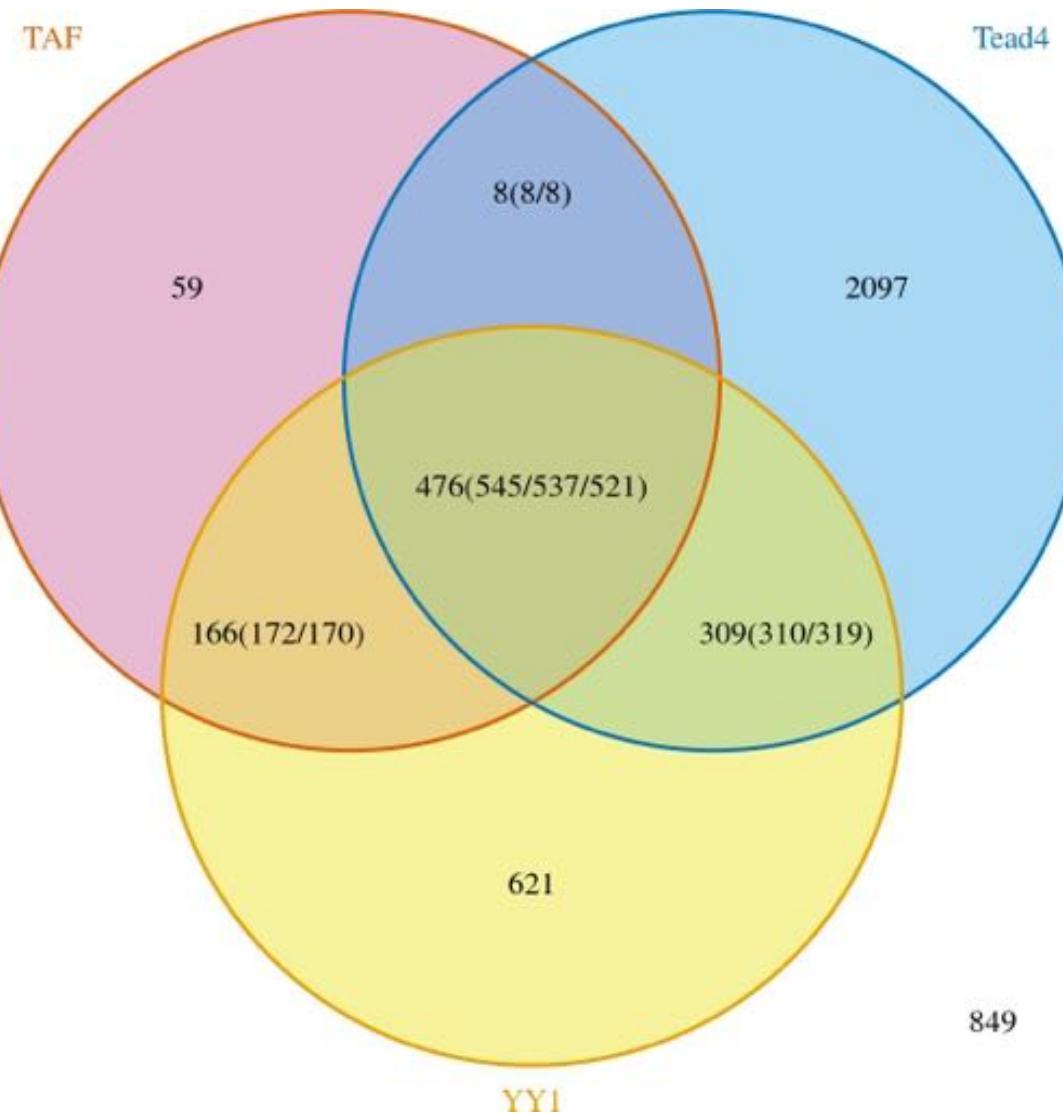


<http://vcg.github.io/upset/>

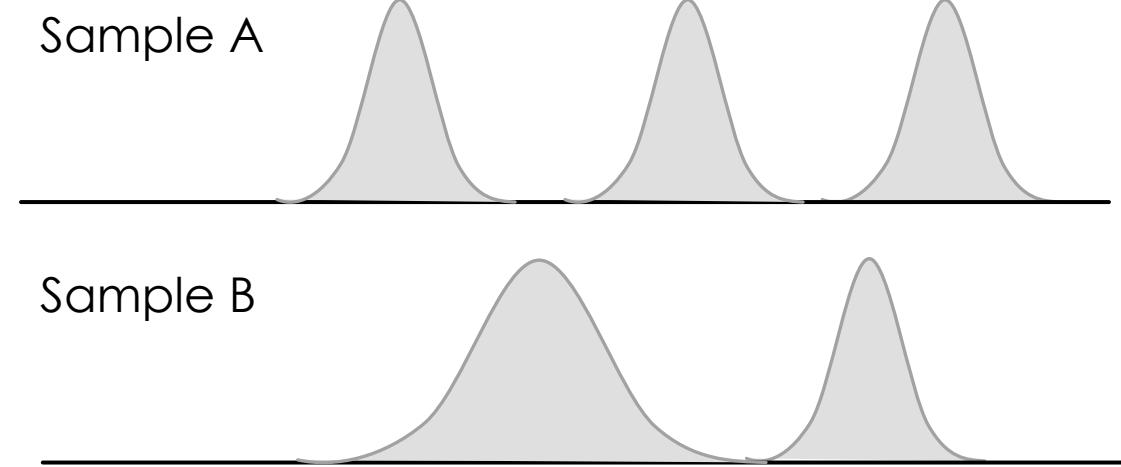
<http://caleydo.org/tools/upset/>



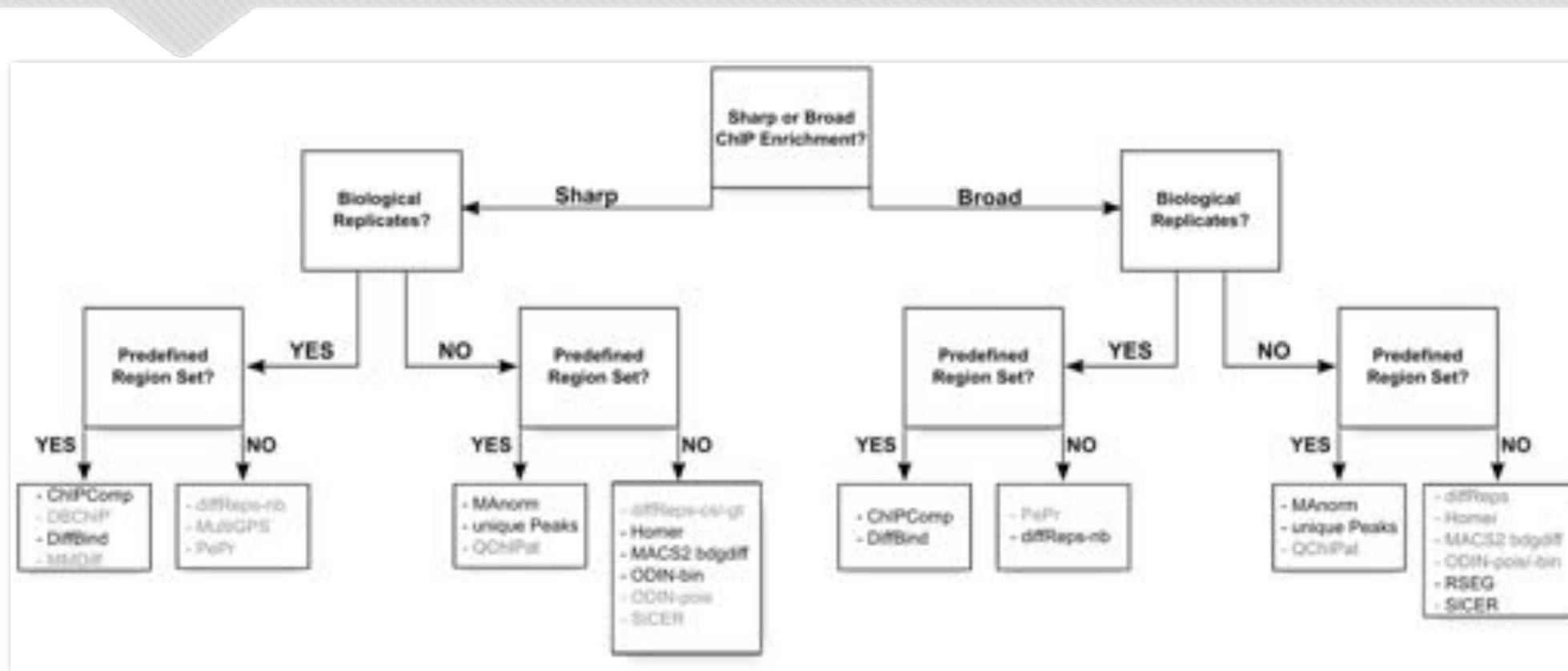
FIND OVERLAPS FOR REPLICATES, *findOverlapsOfPeaks*



849



Differential binding sites analysis



Differential binding sites analysis



DiffBind vs.
csaw

Peak
based vs.
window
based



Using control or not

Tools for ChIP-seq analysis

- Mapping: [bowtie1/2](#), [Rsubread](#), [bwa](#)
- Peak calling: [CCAT](#), [SICER](#), [MACS](#), [ZINBA](#), [BayesPeak](#), [chipseq](#), [ChIPseqR](#), [CSAR](#), [csaw](#), [GenoGAM](#), [iSeq](#), [PICS](#)
- Quality assessment: [ChIPQC](#)
- Analysis of differential binding: [DiffBind](#), [csaw](#), [ChIPDiff](#), [DBChIP](#), [MMDiff](#)
- Peak annotation and pathway analysis: [ChIPpeakAnno](#), [Homer](#), [PAVIS](#), [GREAT](#), [ChIPseeker](#), [clusterProfiler](#), [GOstats](#)
- Gene Network Building: [**GeneNetworkBuilder**](#), [ChIPXpress](#)
- Visualization: [trackViewer](#), [IGV](#), [UCSC genome browser](#), [rtracklayer](#)
- Motif enrichment analysis: [The MEME Suite](#), [homer](#)

Regeneromics Shared Resource can help your research!



We do: experimental design, bioinformatics analysis, manuscript preparation, grant applications

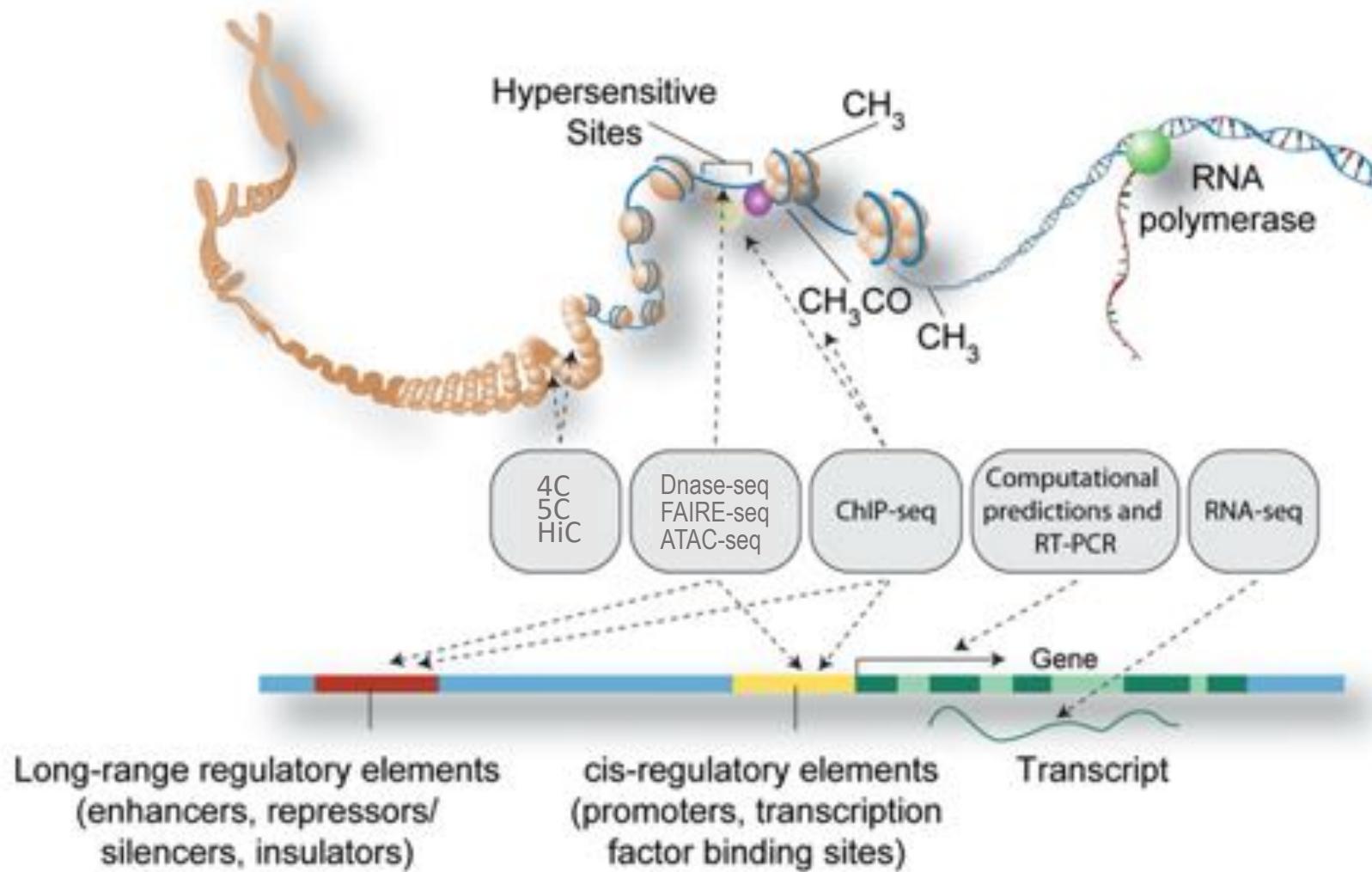


Jianhong Ou, Ph.D.

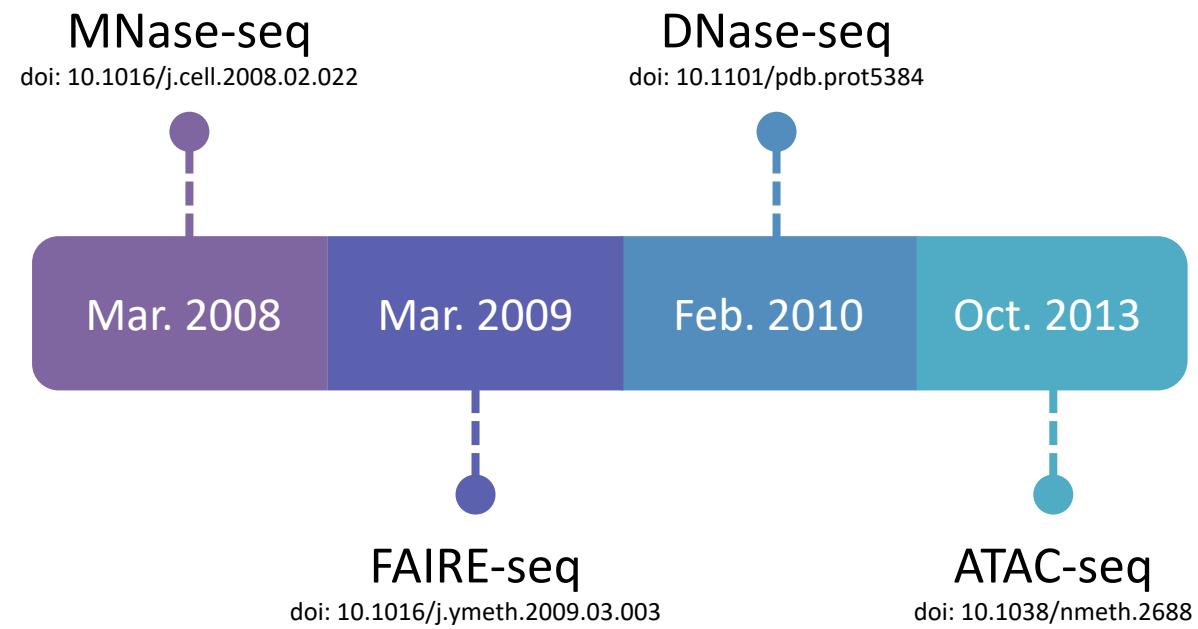
Email: regeneromics@duke.edu

<https://sites.duke.edu/dukeregenerationcenter/jobsrni/>

ATAC-SEQ



PUBLISH TIME



THE ATAC-SEQ DATASETS SUBMITTED TO GEO

FAIRE-seq 116

DNase-Seq 4076

MNase-Seq 6647

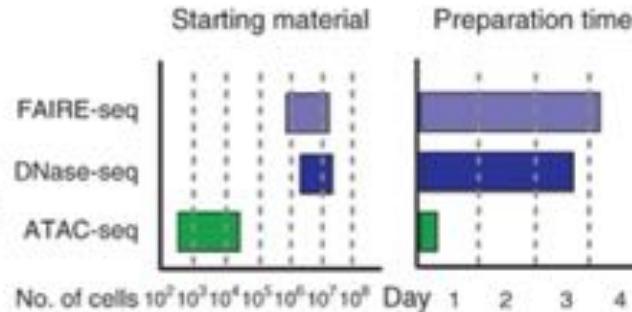
ATAC-seq 21236

```
library(SRAdb)
sqlfile <- getSRAdbFile()
sra_con <- dbConnect(SQLite(),sqlfile)
(sra_tables <- dbListTables(sra_con))
dbListFields(sra_con,"sra")
library_strategy <-
  dbGetQuery(sra_con,
  'SELECT library_strategy,
  COUNT(*) AS count
  FROM sra
  GROUP BY library_strategy')
subs <- library_strategy[
  library_strategy$library_strategy %in%
  c("ATAC-seq", "FAIRE-seq",
  "MNase-Seq", "DNase-Hypersensitivity"), ]
barplot(subs$count)
```

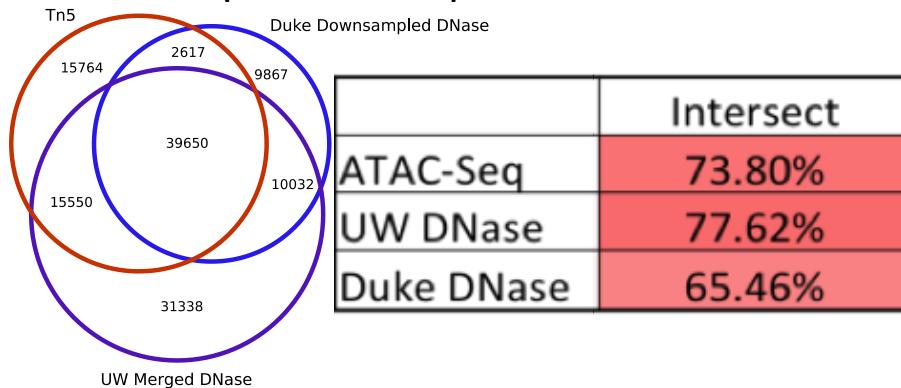
METHODS FOR PROFILING CHROMATIN ACCESSIBILITY

Why ATAC-seq

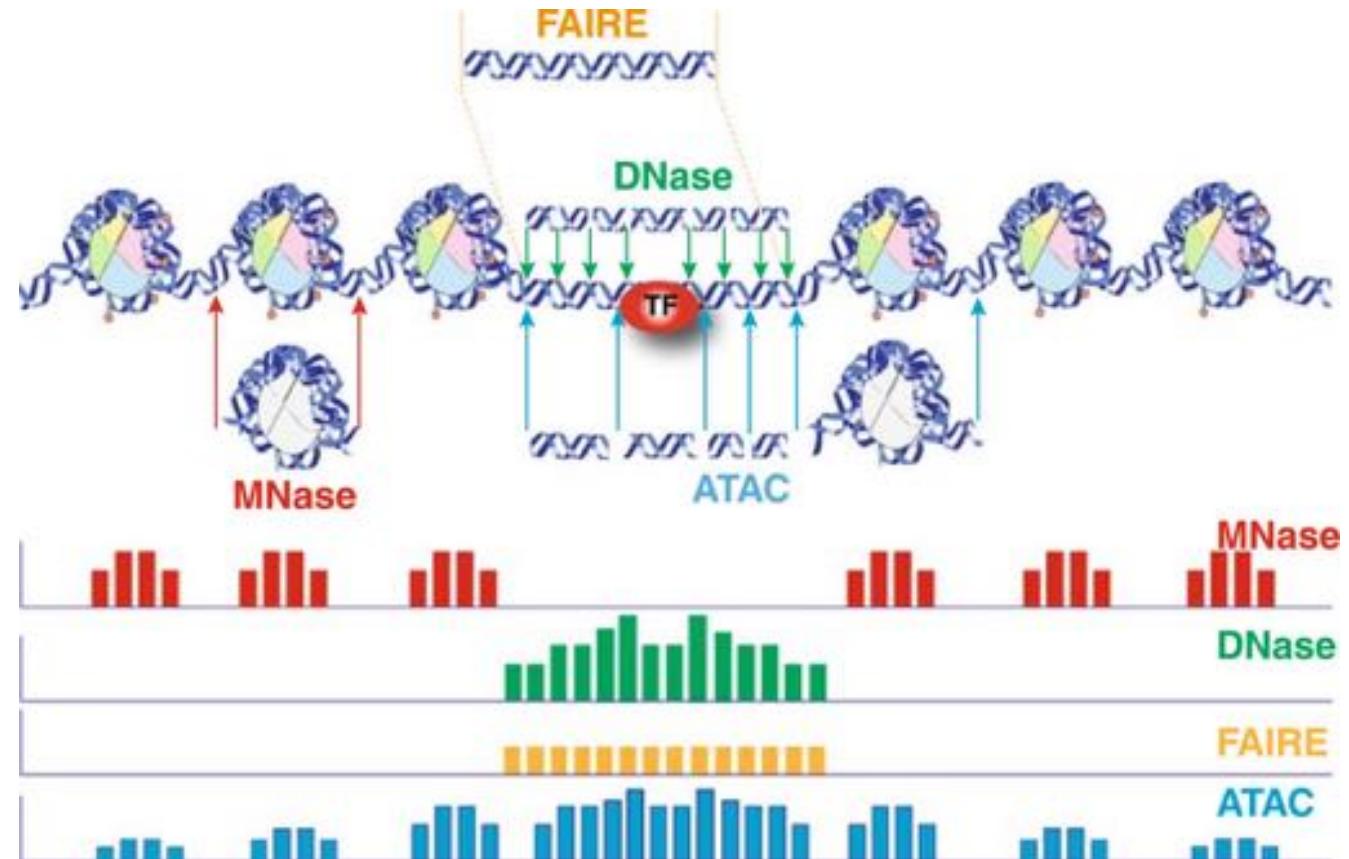
- ✿ rapid and sensitive



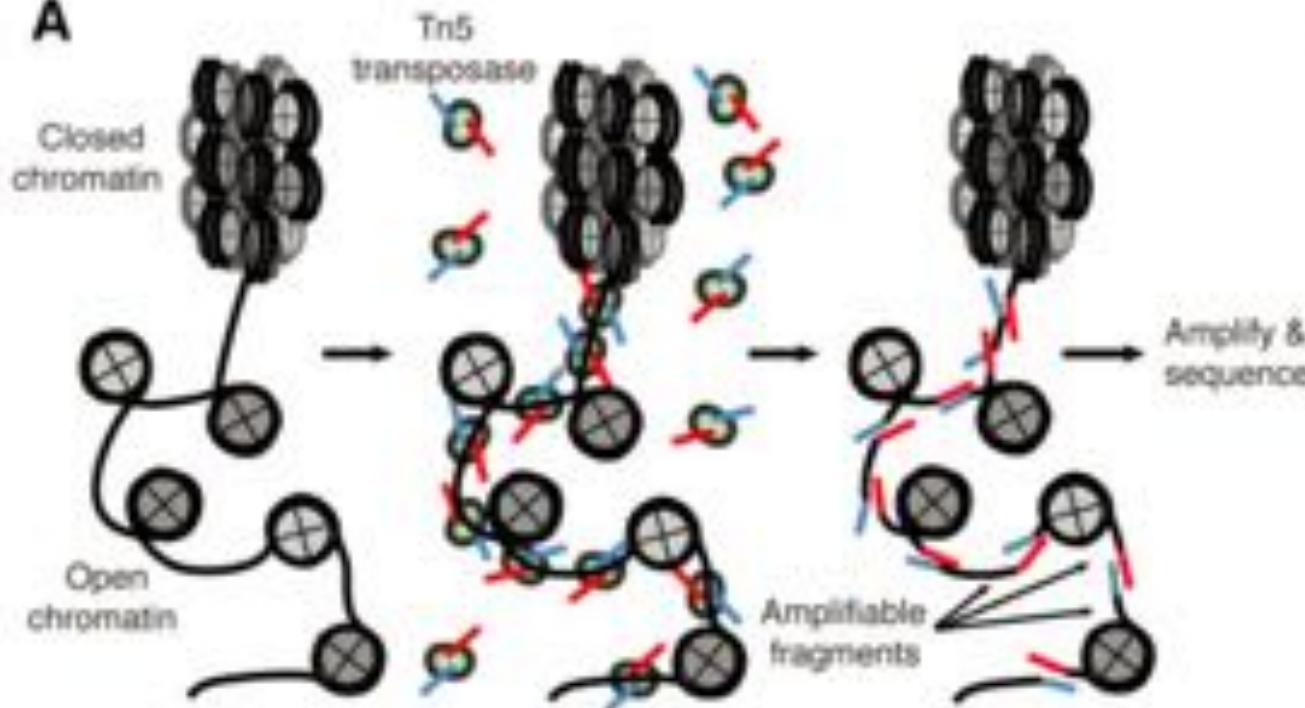
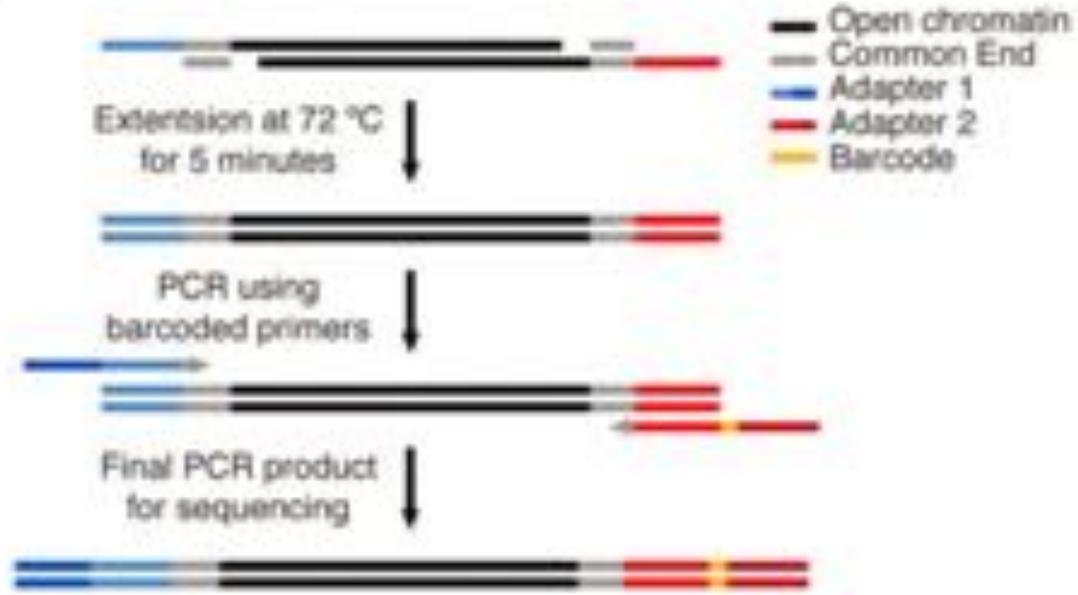
- ✿ comprehensive profile



Ref: Buenrostro et.al., 2013. doi: 10.1038/nmeth.2688



Ref: Tsompana and Buck. 2014. doi: 10.1186/1756-8935-7-33

A**B**

Ref: Buenrostro et.al., 2015. doi: 10.1002/0471142727.mb2129s109

ATAC-SEQ IS A SIMPLE TWO-STEP PROTOCOL

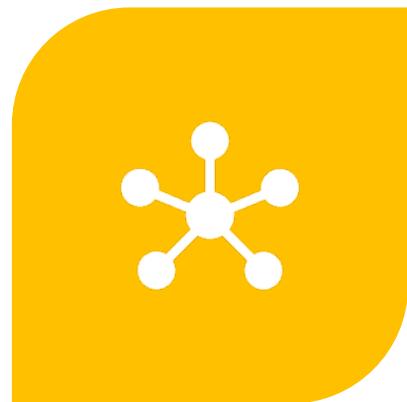
1. Insertion of Tn5 transposase with adaptors
2. PCR amplification

PREPARE SAMPLE DATA SET

Sample data can be downloaded from <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM1155959>



TRIM

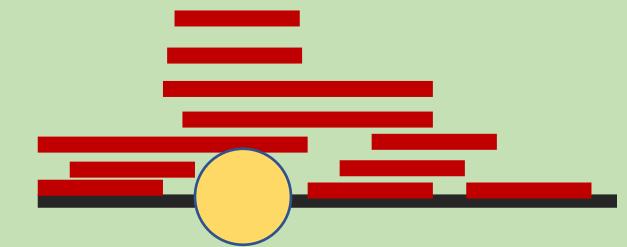
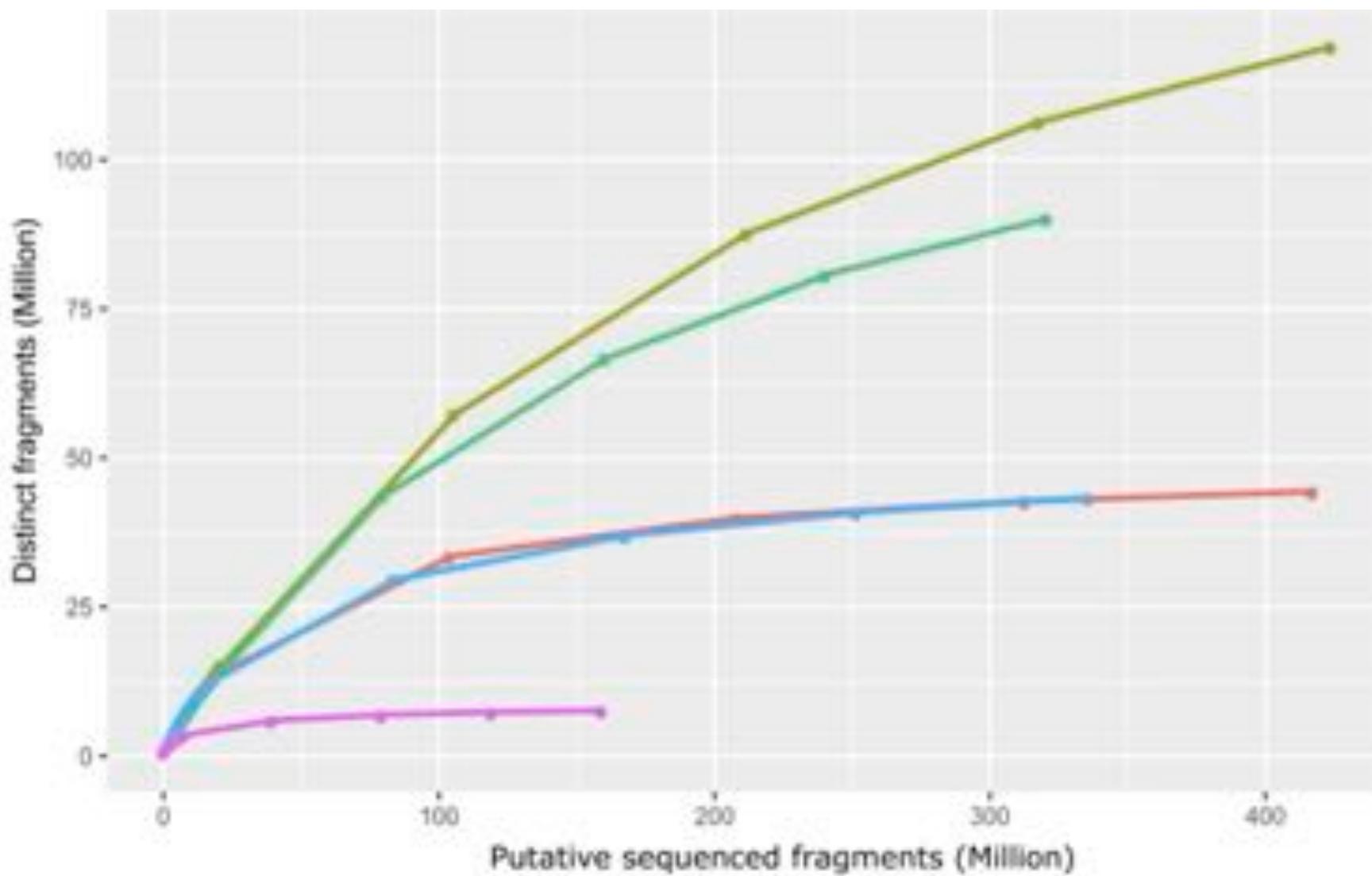


ALIGN

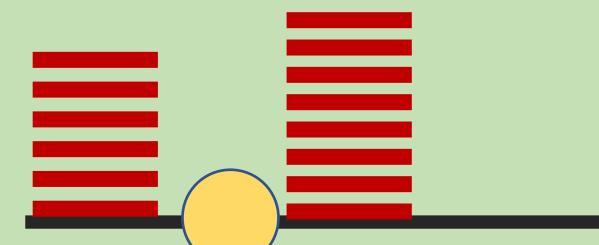


FILTER

LIBRARY COMPLEXITY



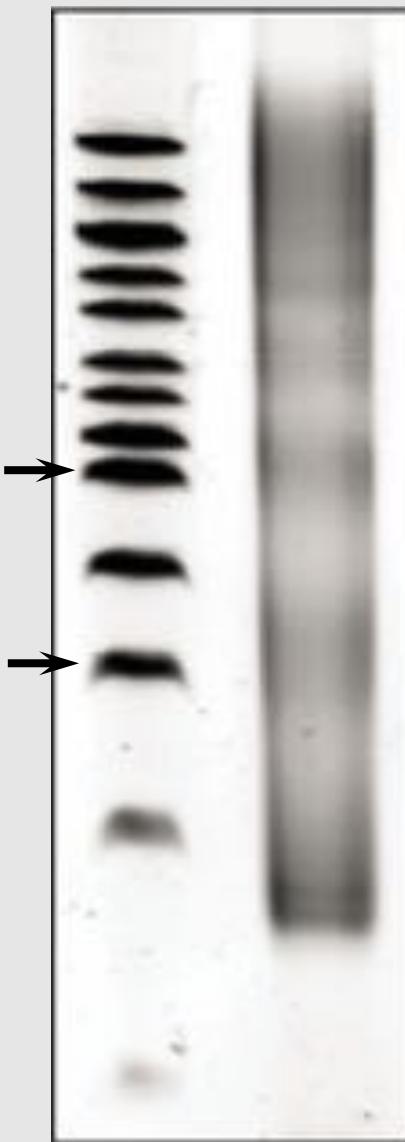
Typical ATAC-seq peak



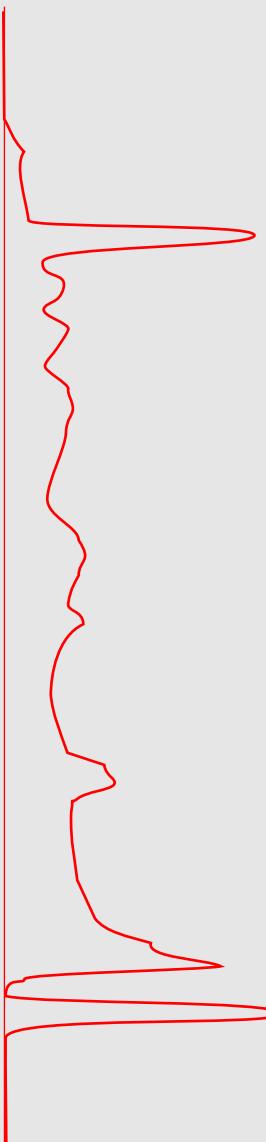
Low-complexity ATAC-seq peak

Library fragment size

Agarose Gel

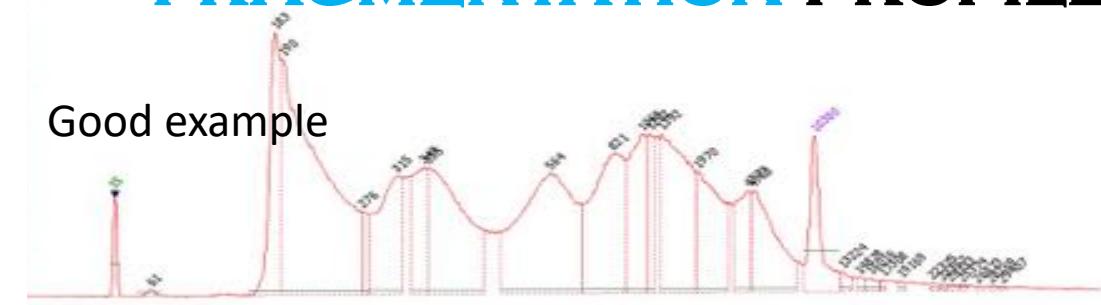


Bioanalyzer



ATAC-SEQ LIBRARY FRAGMENTATION PROFILE

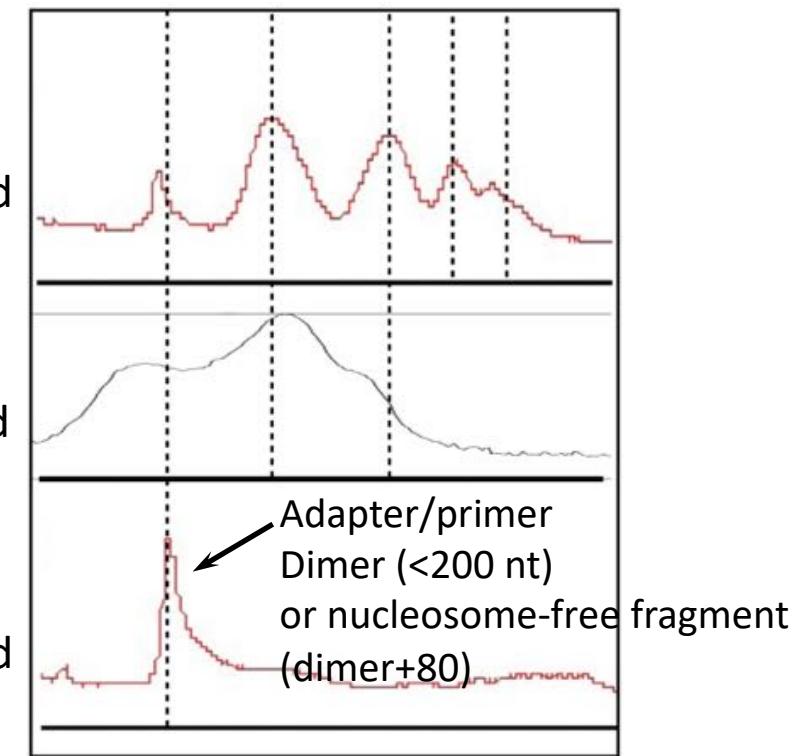
Good example



Good

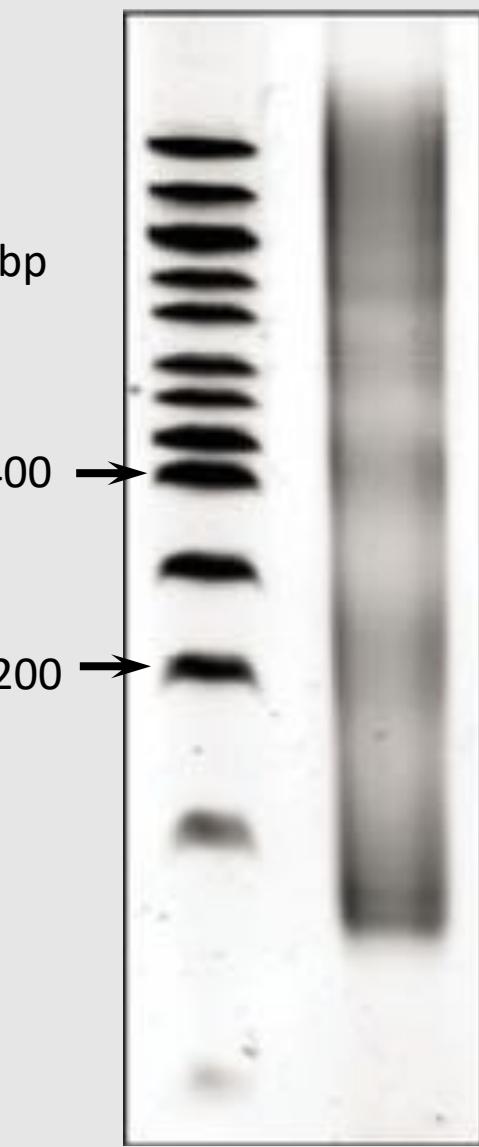
Need to be improved

bad



Library fragment size

Agarose Gel



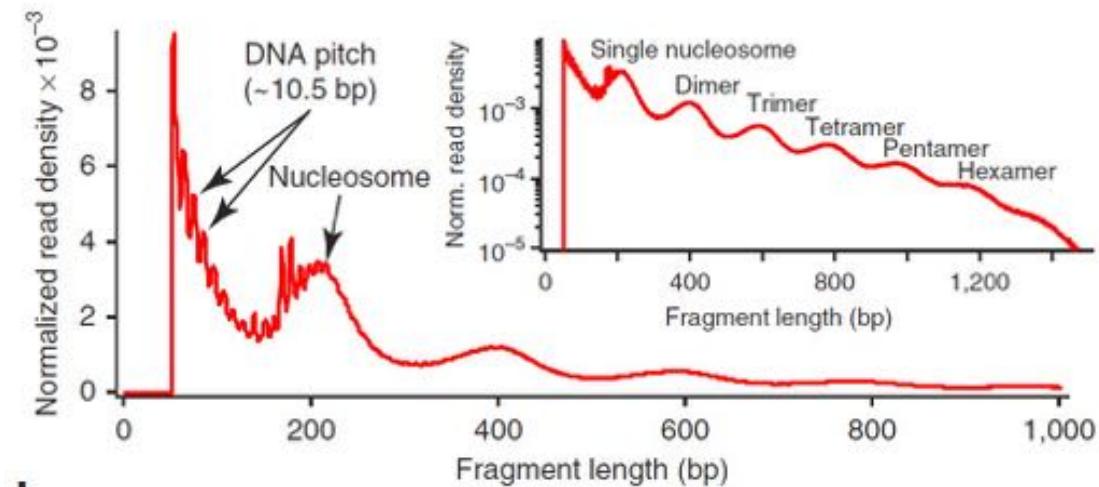
Bioanalyzer



Ref: Buenrostro et.al., 2015. doi: 10.1002/0471142727.mb2129s109

ATAC-SEQ LIBRARY INSERTION PROFILE

Library insert size (=size in gel – adapter/primer size)



Ref: Buenrostro et.al., 2013. doi: 10.1038/nmeth.2688

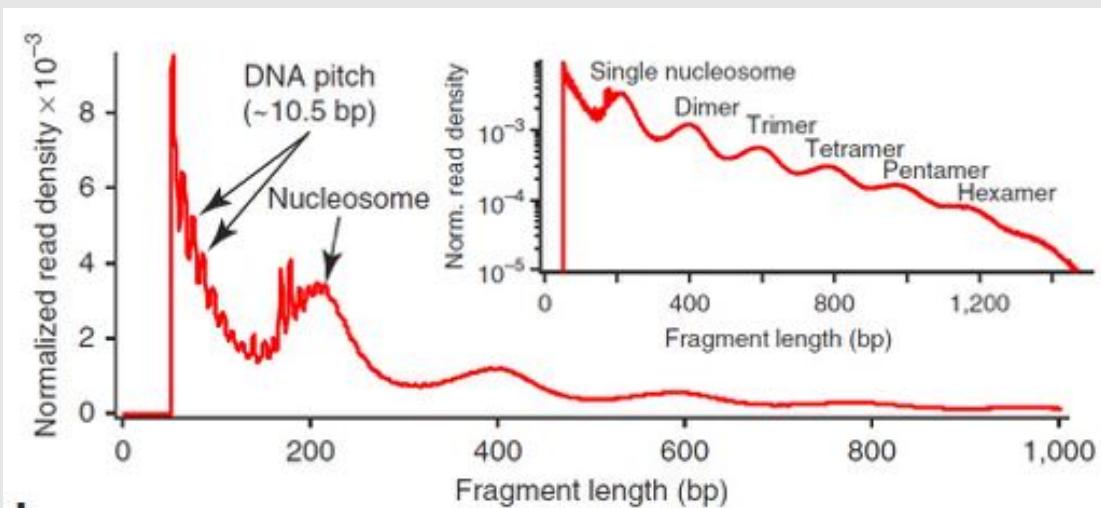
Must be Paired End reads



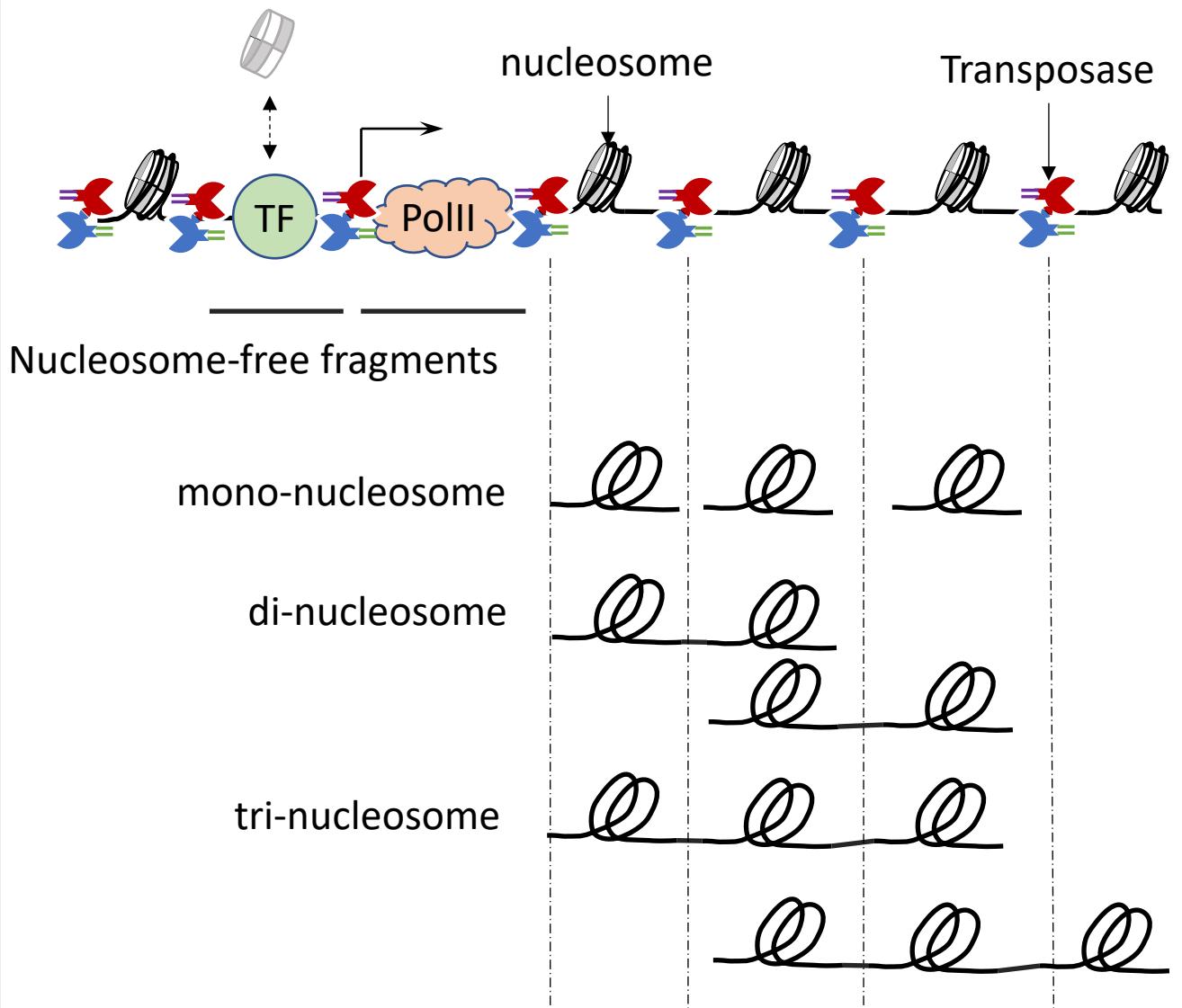
fragSizeDist(bamfile, bamfile.labels)

Function in ATACseqQC

ATAC-SEQ LIBRARY FRAGMENTATION PROFILE

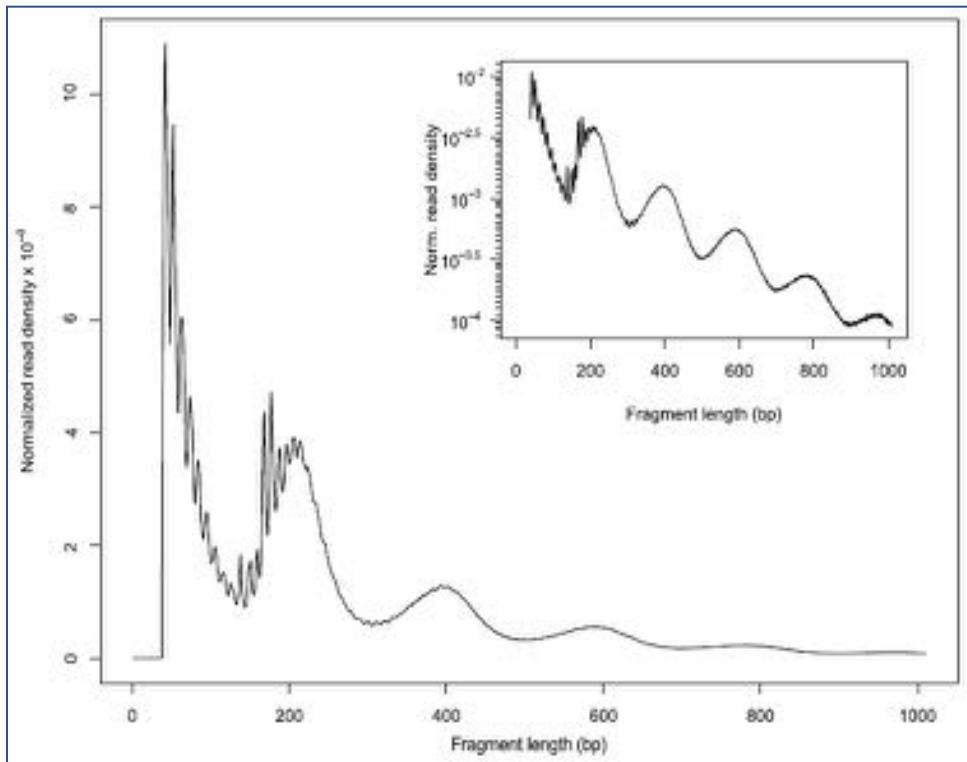


Ref: Buenrostro et.al., 2015. doi: 10.1002/0471142727.mb2129s109

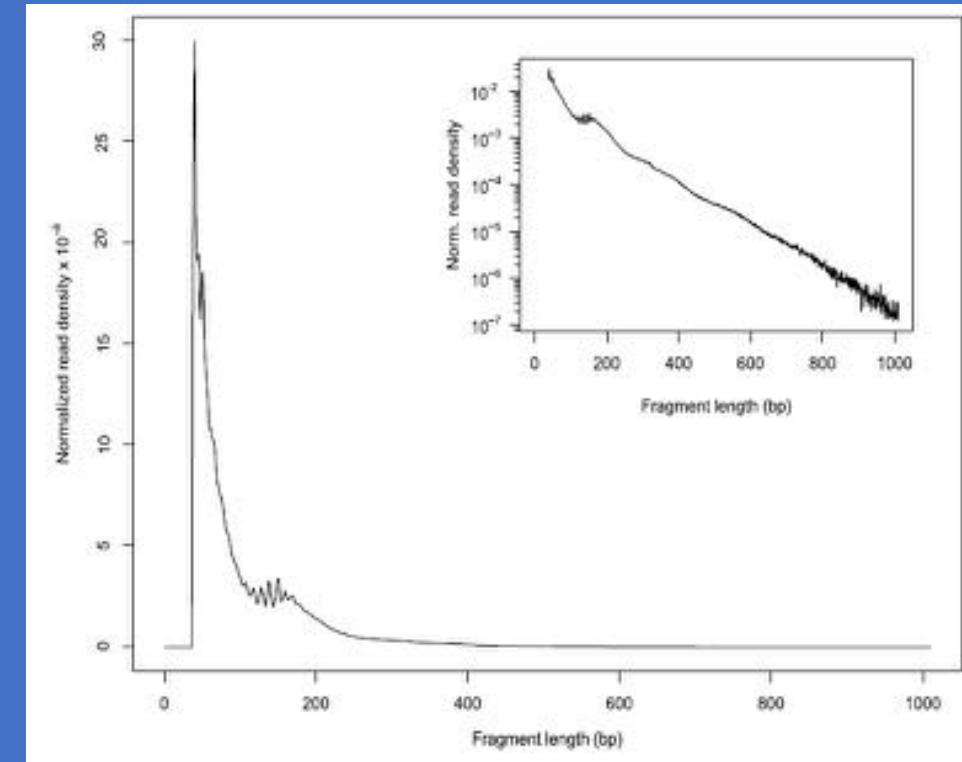


ASSESSING INSERT SIZE DISTRIBUTION

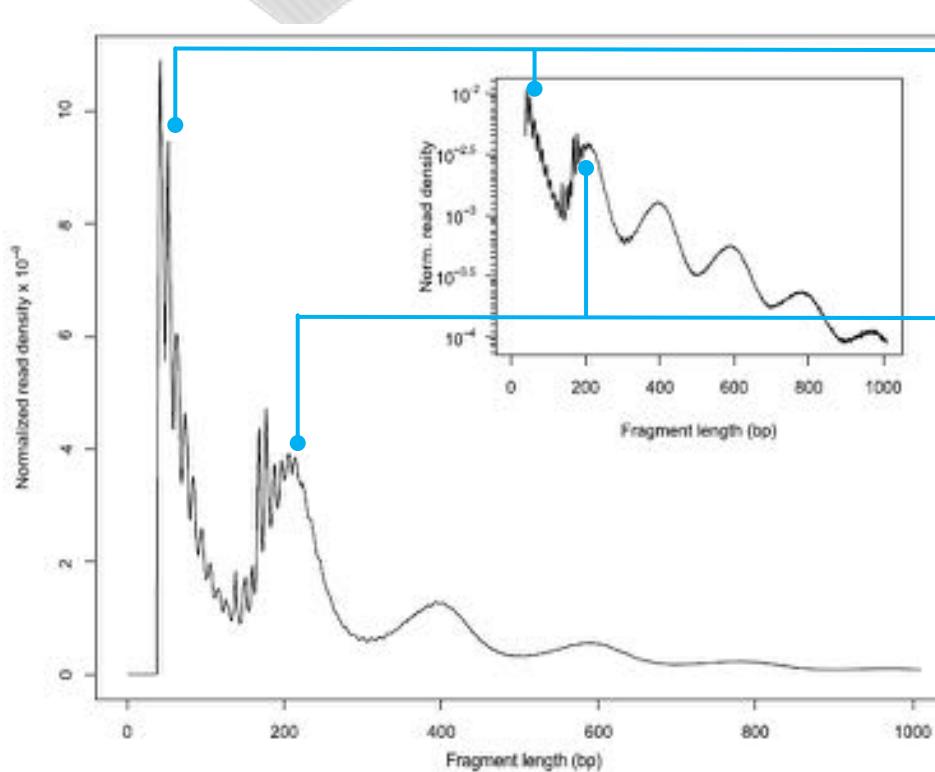
A (High quality)



B (Poor quality)



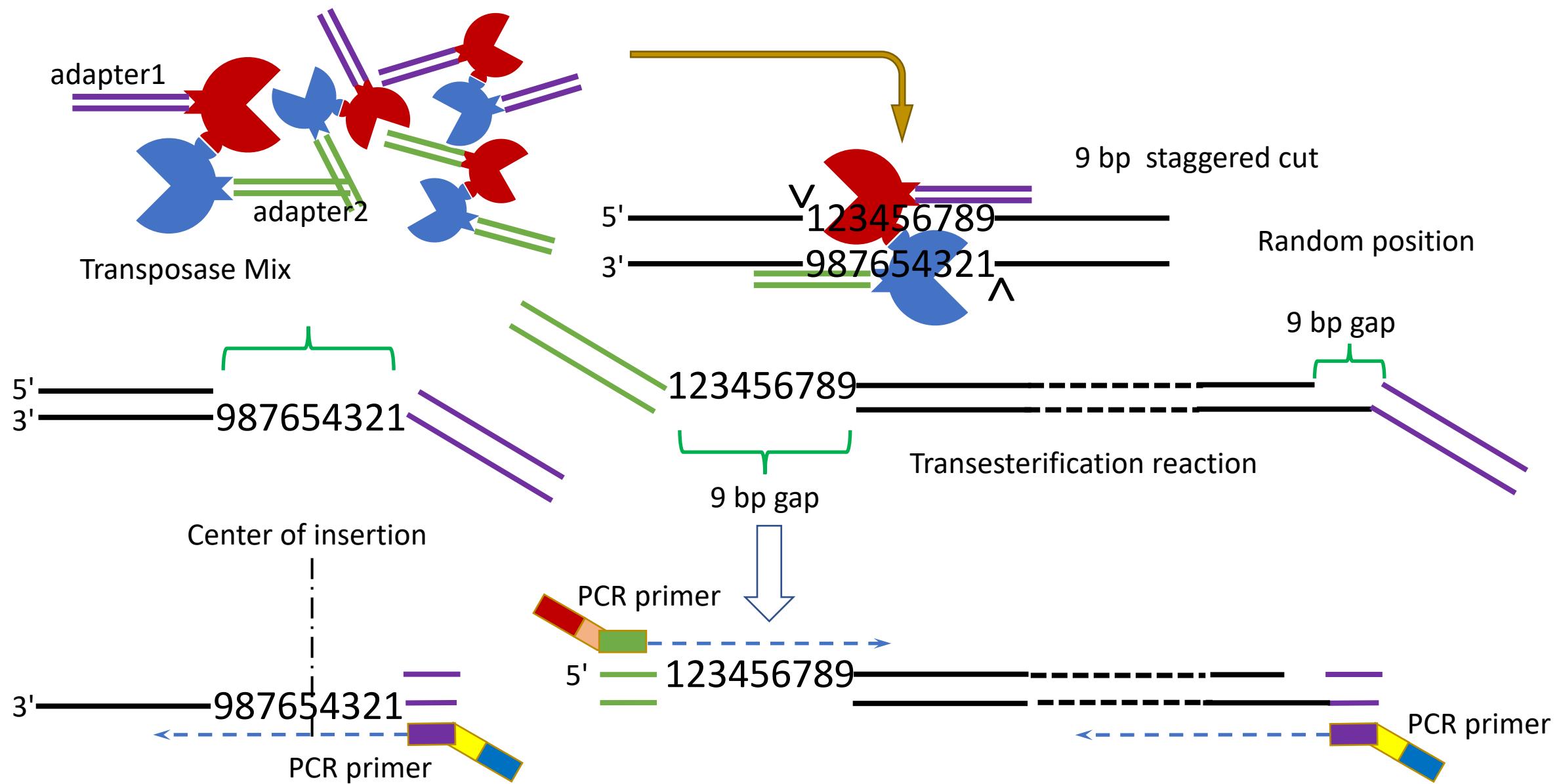
MINIMAL CRITERIA FOR INSERT SIZE DISTRIBUTION



- A nucleosome-free region (NFR) peak
- A mono-nucleosome region peak (147~294 bp).

First, there should be a large proportion of reads with less than 100 bp, which represents the nucleosome-free region. Second, the fragment size distribution should have a clear periodicity, which is evident in the inset figure, indicative of nucleosome occupancy (present in integer multiples).

SHIFTING ALIGNED READS



SHIFTING ALIGNED READS

```
## bamfile tags to be read in
possibleTag <- combn(LETTERS, 2)
possibleTag <- c(paste0(possibleTag[1, ], possibleTag[2, ]),
                paste0(possibleTag[2, ], possibleTag[1, ]))
library(Rsamtools)
## use top 100 reads to test possible tags
bamTop100 <- scanBam(BamFile(bamfile, yieldSize = 100),
                      param = ScanBamParam(tag=possibleTag))[[1]]$tag
tags <- names(bamTop100)[lengths(bamTop100)==100]
tags
## [1] "AS" "MD" "PG" "XG" "NM" "XM" "XN" "XO" "XS" "YS" "YT"
## files will be output into outPath
outPath <- "splited"
dir.create(outPath)
## shift the coordinates of 5'ends of alignments in the bam file
library(BSgenome.Hsapiens.UCSC.hg38)
seqlev <- "chr1" ## subsample data for quick run
which <- as(seqinfo(Hsapiens)[seqlev], "GRanges")
gal <- readBamFile(bamfile, tag=tags, which=which,
                   asMates=TRUE, bigFile=TRUE)
shiftedBamfile <- file.path(outPath, "shifted.bam")
gal1 <- shiftGAlignmentsList(gal, outbam=shiftedBamfile)
```

SAM file example

```
@HD VN:1.6 SO:coordinate
@SQ SN:ref LN:45
r001 99 ch1 7 30 8M2I4M = 37 39 TTAGATAAAGGCTG *
r002 0 ch1 9 30 3S6M. * 0 0 AAAAGATAAGGATA *
r003 0 ch1 9 30 5S6M * 0 0 GCCTAAGCTAA * SA:Z:ref,29,-,6H5M,17,0;
r004 0 ch1 16 30 6M14M * 0 0 ATAGCTTCAGC *
r003 2064 ch1 29 17 6H5M * 0 0 TAGGC * SA:Z:ref,9,+,5S6M,30,1;
r001 147 ch1 37 30 9M. = 7 -39 CAGCGGCAT * NM:i:1
```

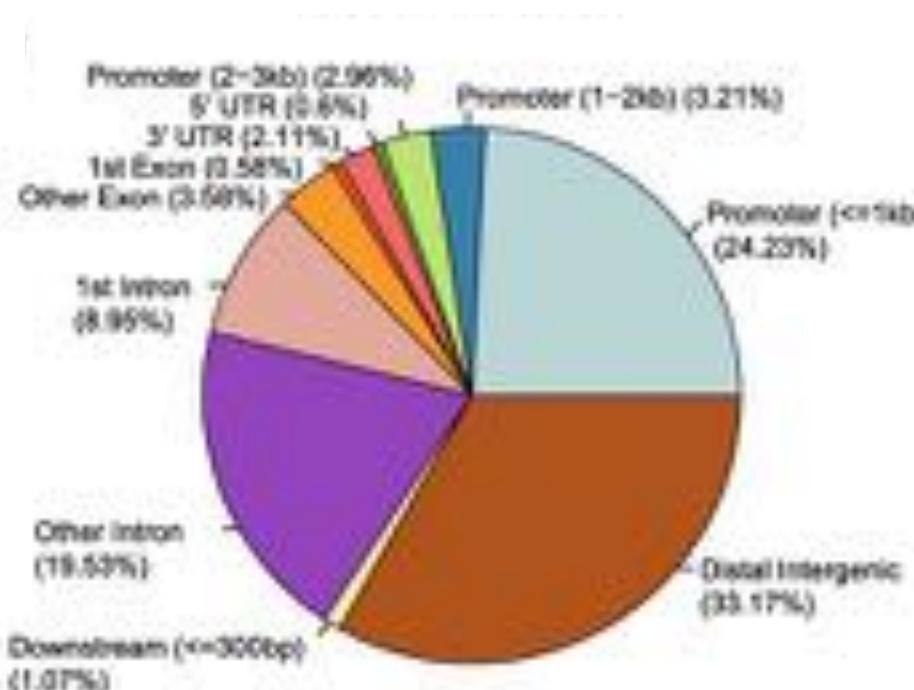
The alignment section: mandatory fields

Col	Field	Type	Brief description
1	QNAME	String	Query template
2	FLAG	Int	bitwise FLAG
3	RNAME	String	Reference sequence NAME
4	POS	Int	1-based leftmost mapping POSition
5	MAPQ	Int	MAPping Quality
6	CIGAR	String	CIGAR string
7	RNEXT	String	Reference name of the mate/next read
8	PNEXT	Int	Position of the mate/next read
9	TLEN	Int	observed Template LENGTH
10	SEQ	String	segment SEQuence
11	QUAL	String	ASCII of Phred-scaled base QUALity+33

The alignment section: optional fields

All optional fields follow the TAG:TYPE:VALUE format where TAG is a two-character string that matches /[A-Za-z][A-Za-z0-9]/. Each TAG can only appear once in one alignment line.

ENRICHMENT IN PROMOTERS



Typical peak annotation pie chart

Ref: Yan et.al., 2020. doi: 10.1186/s13059-020-1929-3

PT score

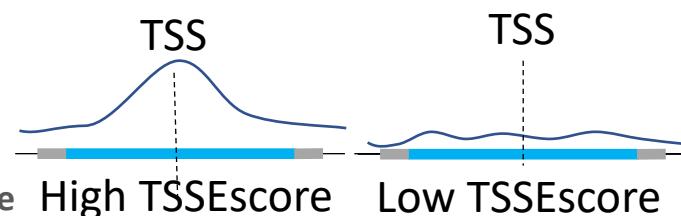
Promoter/Transcript body score



PT score is calculated as the coverage of promoter divided by the coverage of its transcript body. PT score will show if the signal is enriched in promoters.

TSSE score

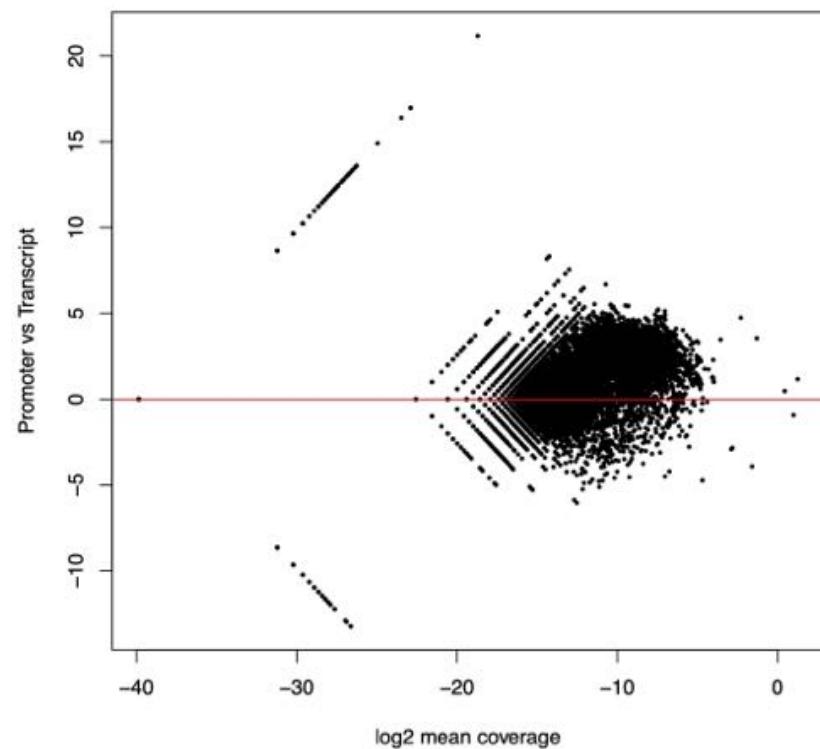
Transcription Start Site (TSS) Enrichment Score



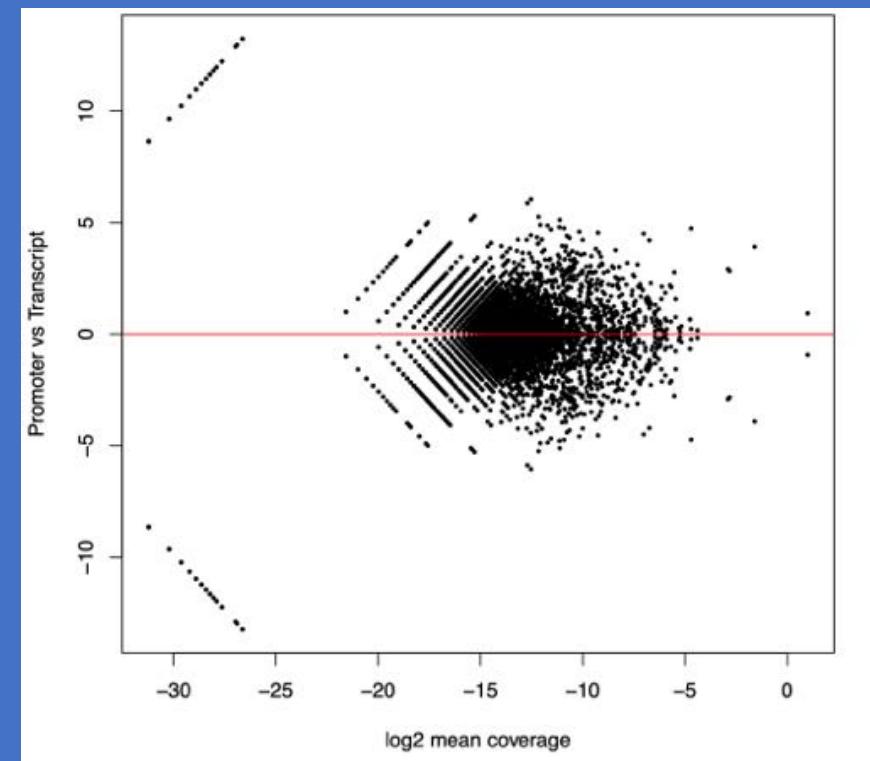
TSS enrichment score is a ratio between aggregate distribution of reads centered on TSSs and that flanking the corresponding TSSs. TSS score = the depth of TSS (1000 bp each side) / the depth of end flanks (100bp each end).

PT SCORE

A (High quality)

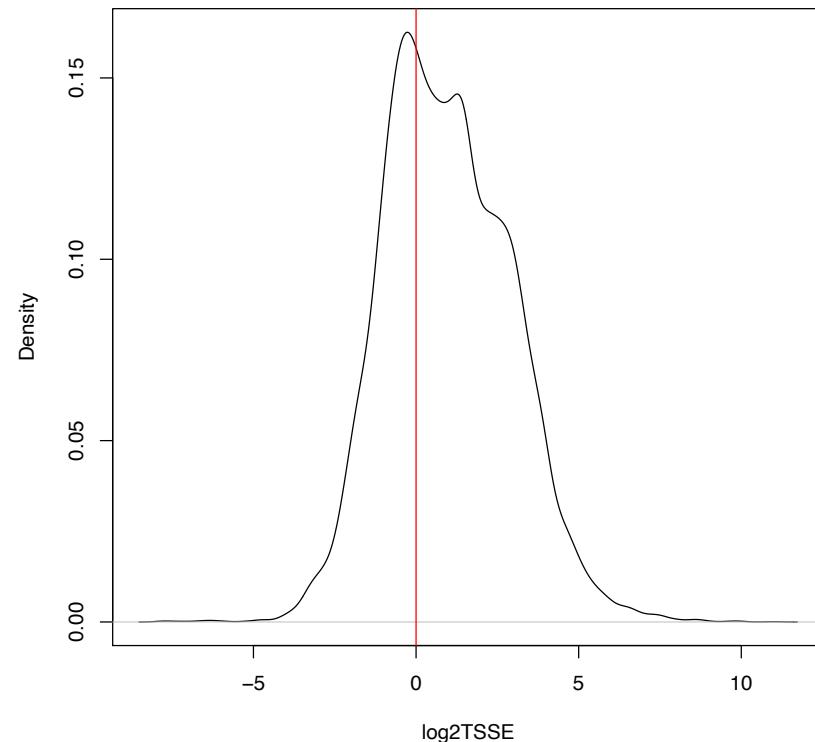


B (Poor quality)

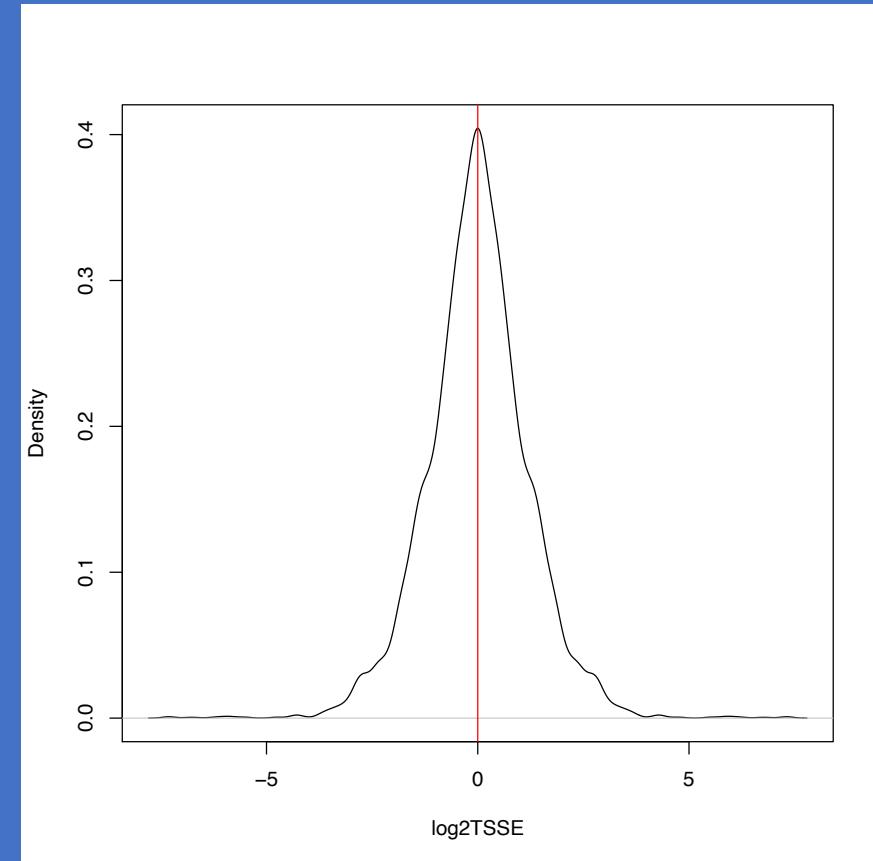


TSSE SCORE

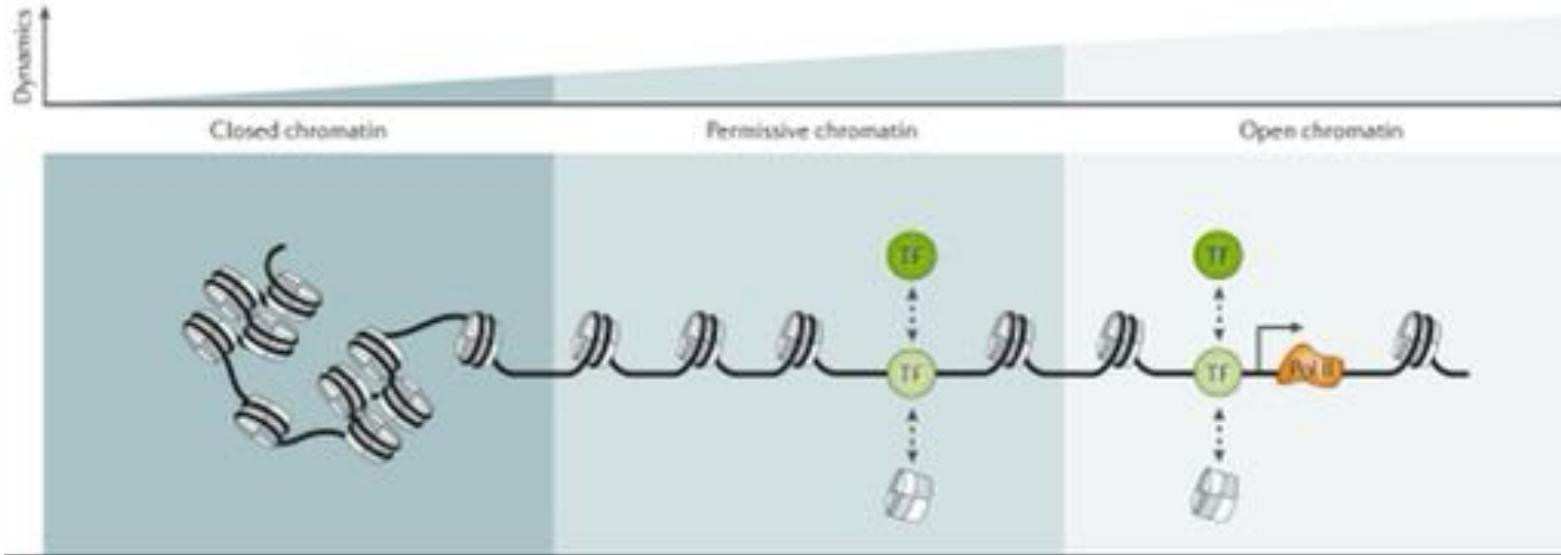
A (High quality)



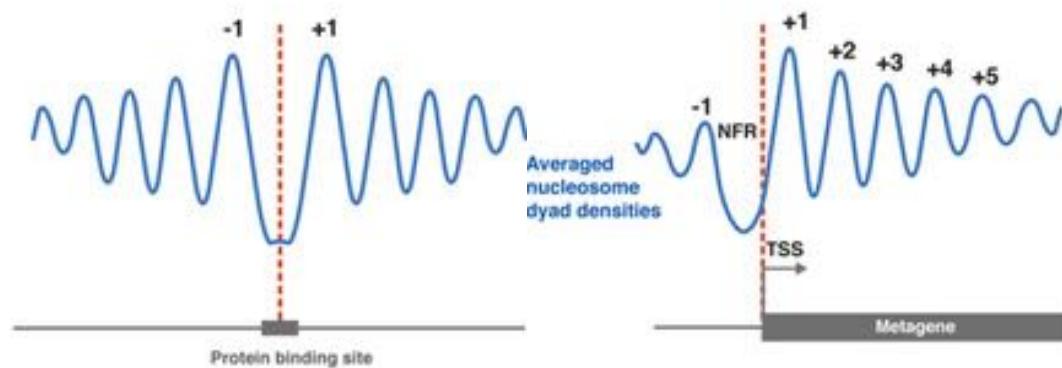
B (Poor quality)



NUCLEOSOME POSITIONING AROUND TSSs AND TF-BOUND SITES



Ref: Klemm et.al., 2019. doi: 10.1038/s41576-018-0089-8

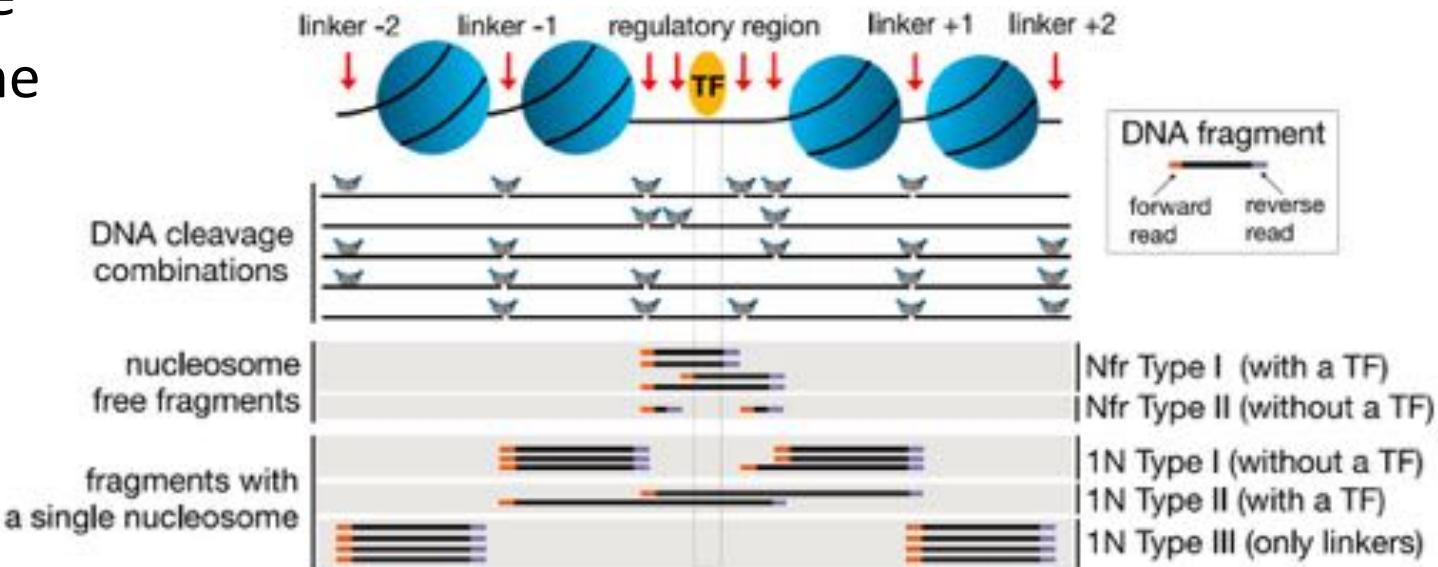


Ref: Baldi. 2018 doi: 10.1042/EBC20180058

SPLITTING BAM FILES

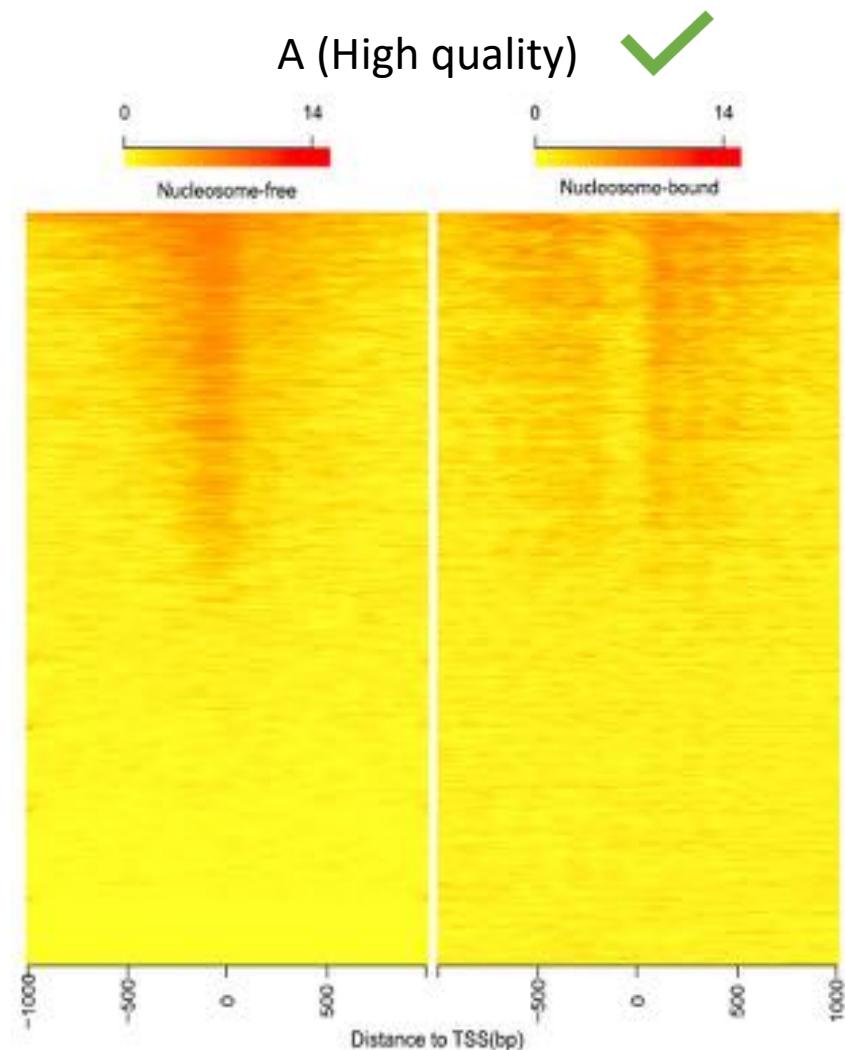
split aligned reads into different bins:

- Nucleosome-free
 - Mononucleosome
 - Dinucleosome
 - Trinucleosome

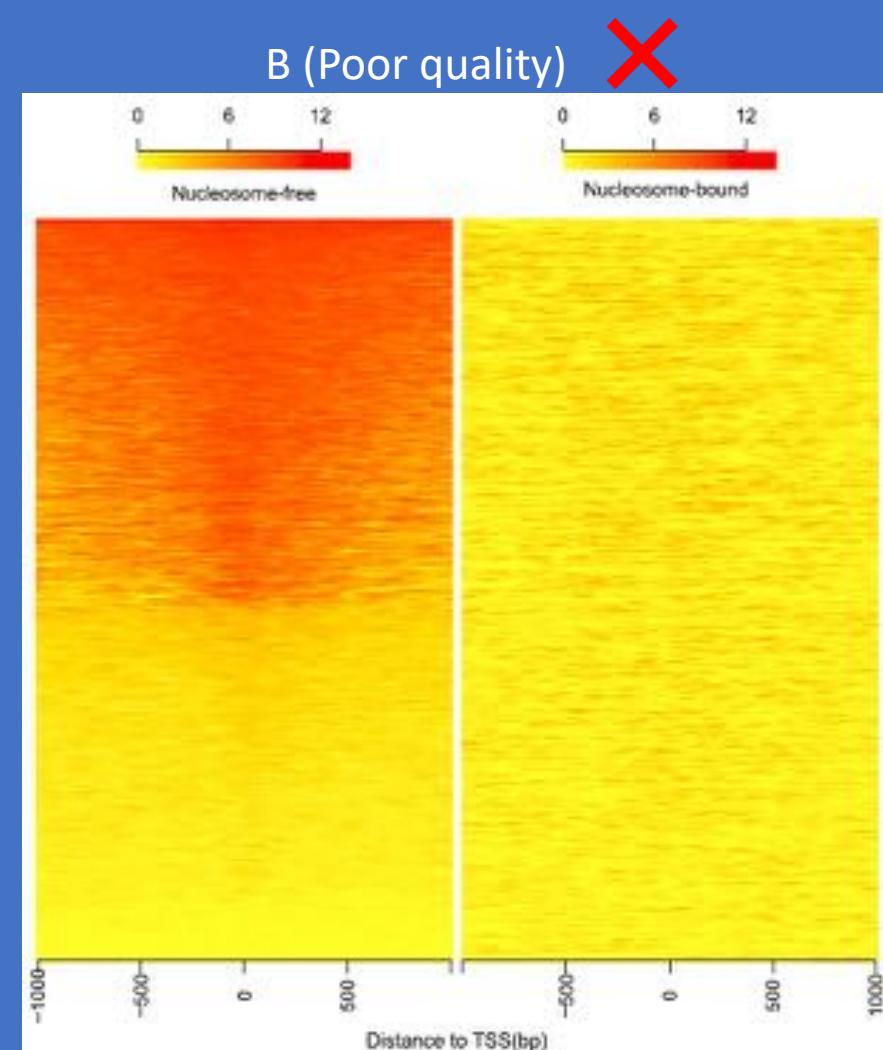


Li et al. 2019. doi: 10.1186/s13059-019-1642-2

HEATMAPS SHOWING FROM DIFFERENT BINS

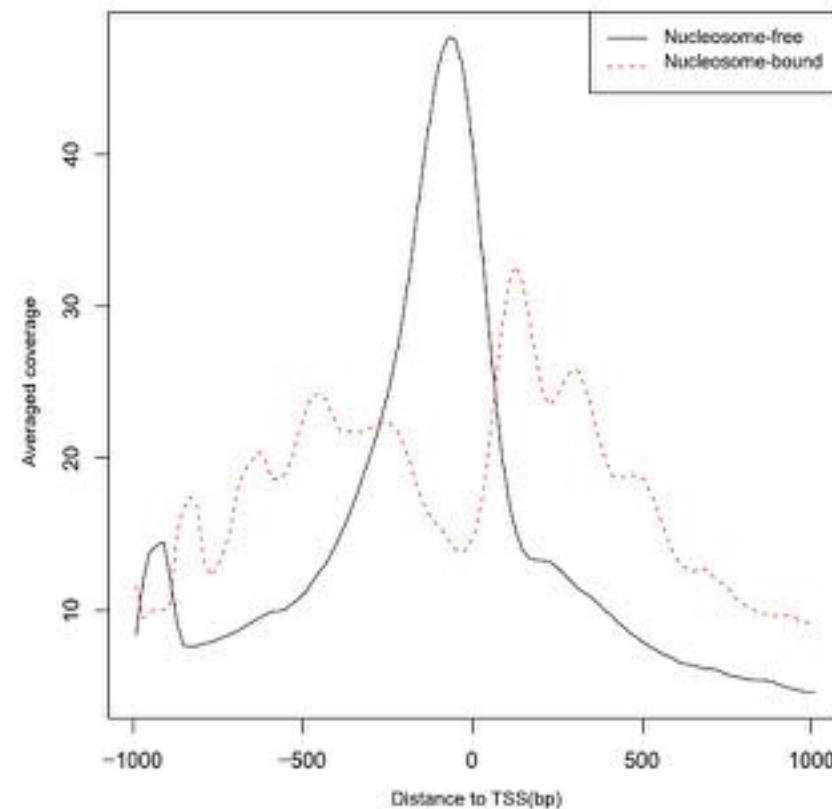


DISTRIBUTION OF READS AROUND TSSs



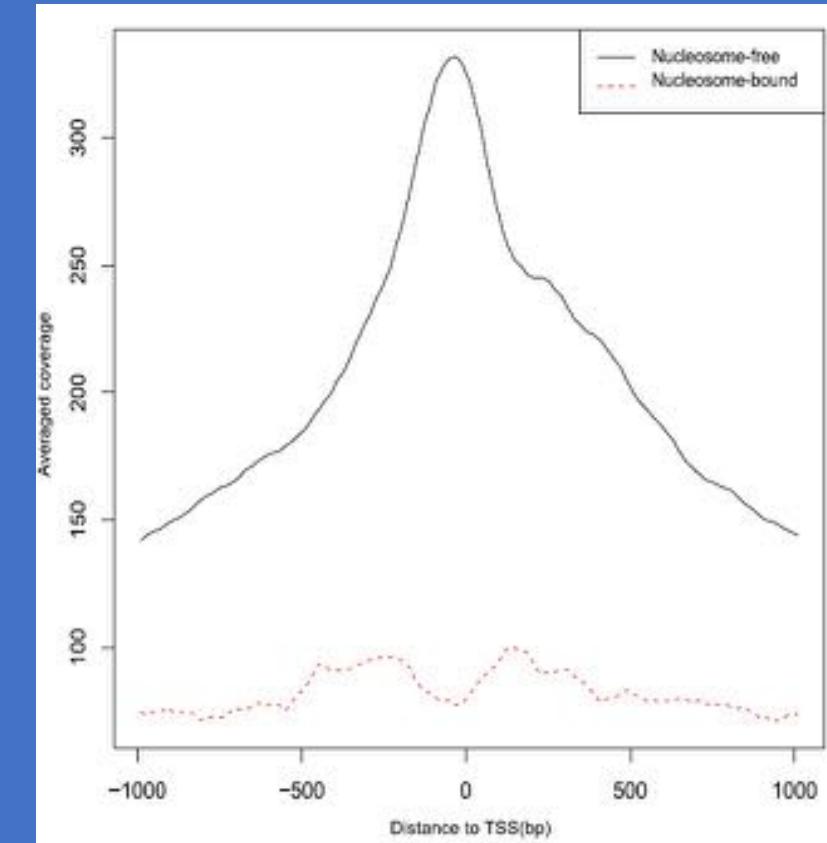
DENSITY PLOTS SHOWING READS FROM DIFFERENT

A (High quality)

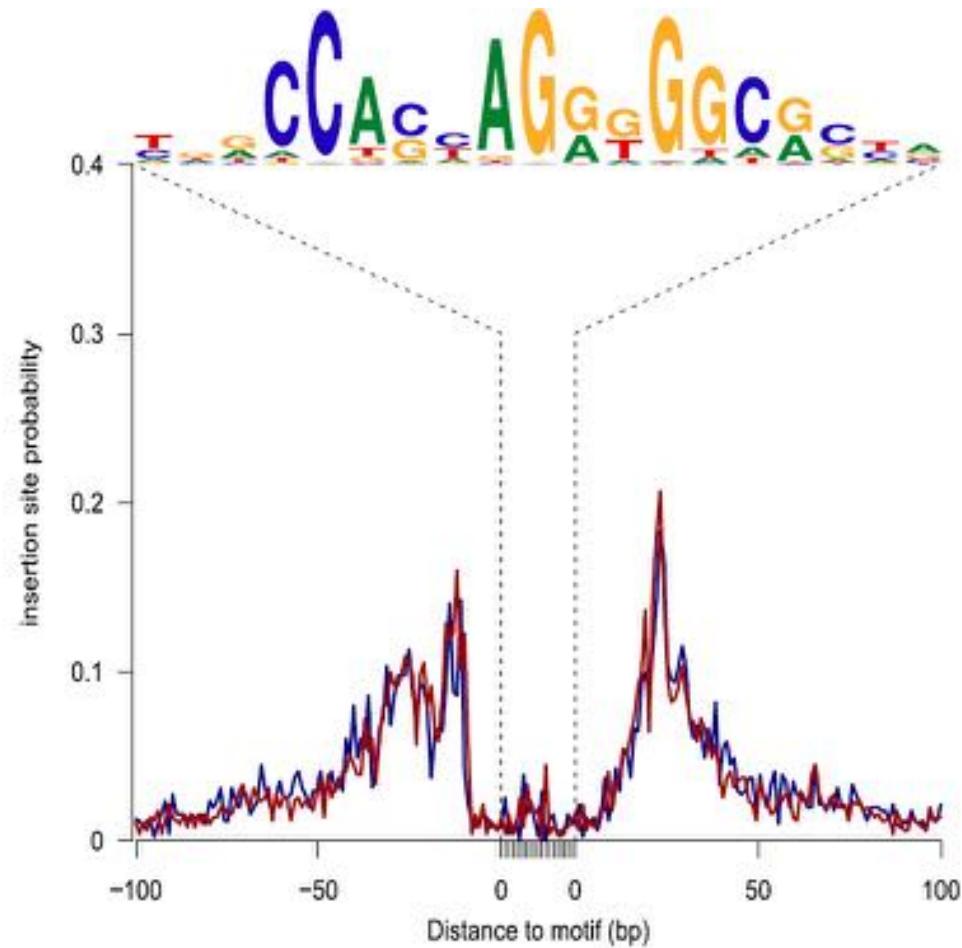


DISTRIBUTION OF BINS AROUND TSSs

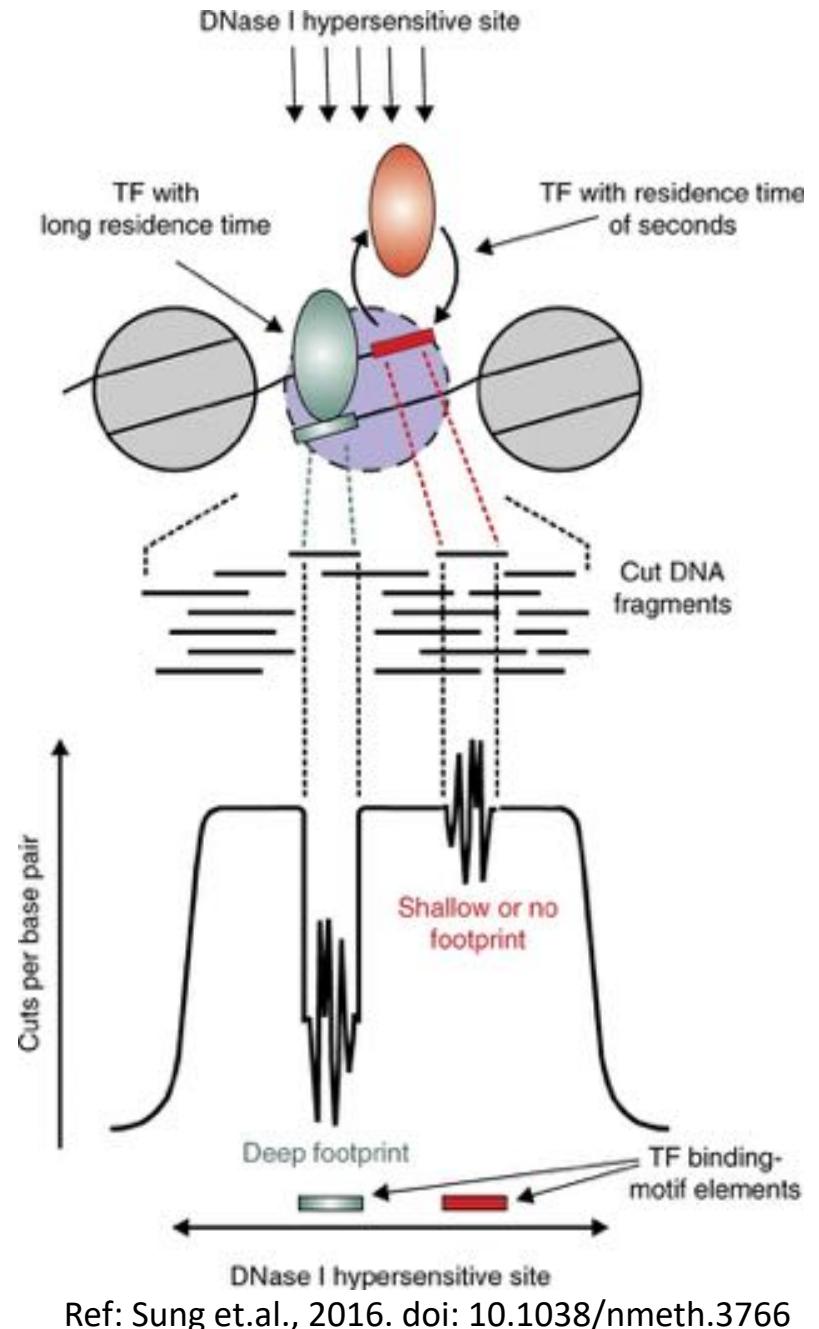
B (Poor quality)



ASSESSING FOOTPRINTS OF DNA-BINDING FACTORS



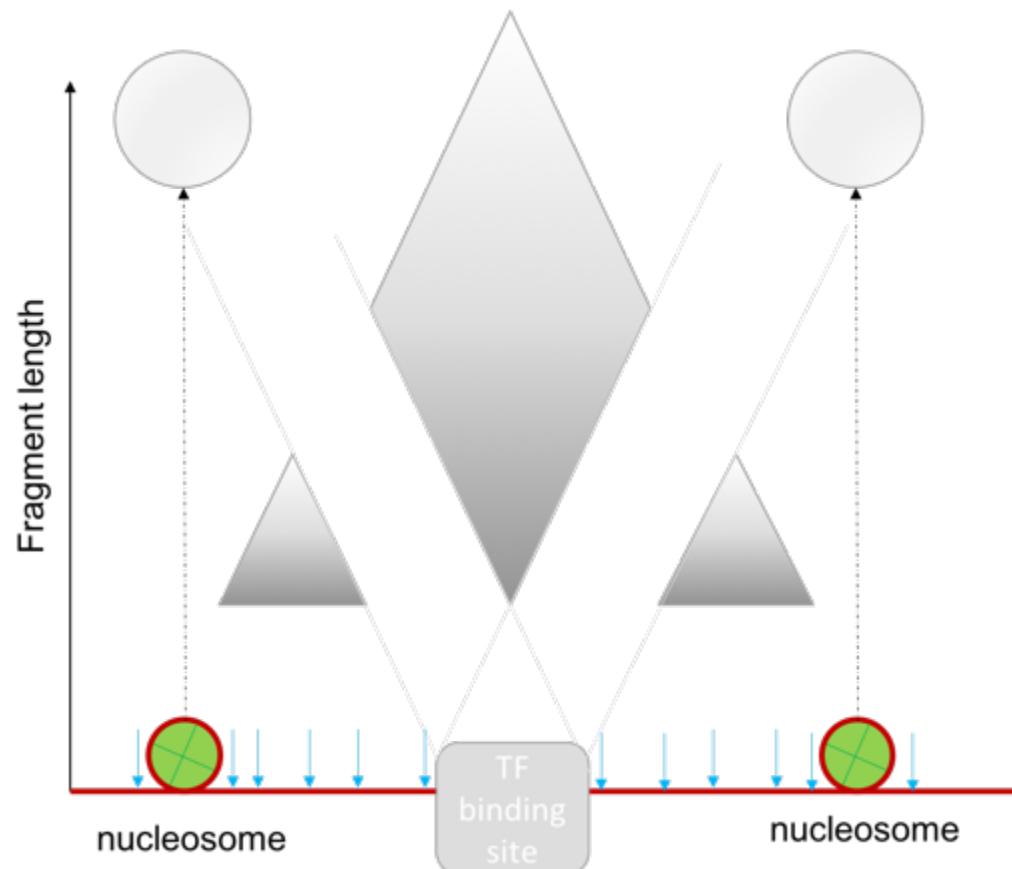
Ref: Ou et.al., 2018. doi: 10.1186/s12864-018-4559-3



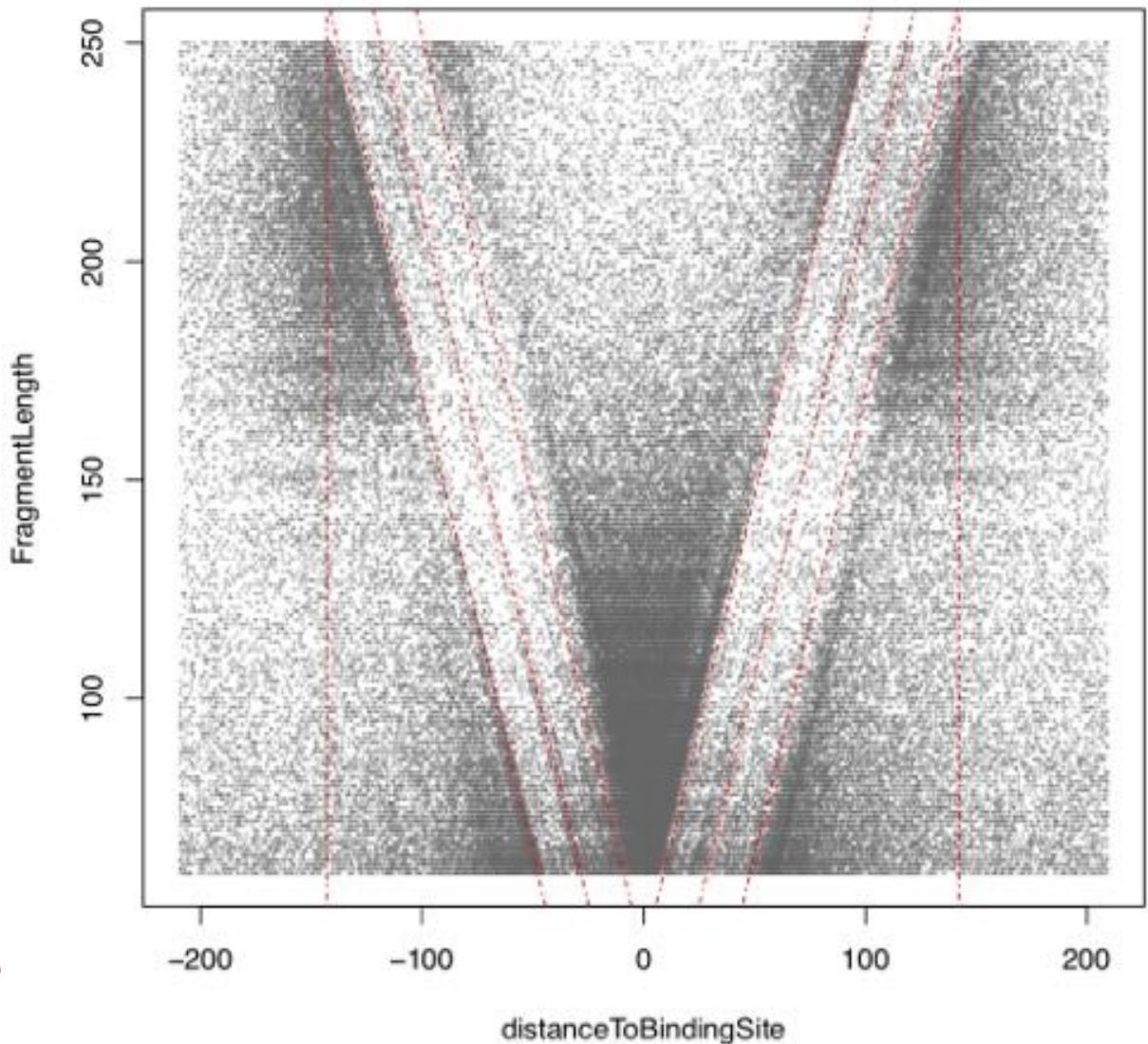
Ref: Sung et.al., 2016. doi: 10.1038/nmeth.3766

vPLOT

V-plot is a plot to visualize fragment midpoint vs length for a given transcription factors.



distanceNucl=285bp, CTCFbindingWidth=41bp



Tools for ATAC-seq analysis

- Mapping: [bowtie1/2](#), [Rsubread](#), [bwa](#)
- Quality assessment and reads shift: ATACseqQC, ataqv
- Peak calling: [MACS](#)
- Analysis of differential binding: [DiffBind](#), [csaw](#)
- Peak annotation and pathway analysis: [ChIPpeakAnno](#), [Homer](#), [PAVIS](#), [GREAT](#), [ChIPseeker](#), [clusterProfiler](#), [GOstats](#)
- Gene Network Building: [GeneNetworkBuilder](#), [ChIPXpress](#)
- Visualization: [trackViewer](#), [IGV](#), [UCSC genome browser](#), [rtracklayer](#)
- Motif enrichment analysis: [The MEME Suite](#), [homer](#)

Regeneromics Shared Resource can help your research!



We do: experimental design, bioinformatics analysis, manuscript preparation, grant applications



Jianhong Ou, Ph.D.

Email: regeneromics@duke.edu

<https://sites.duke.edu/dukeregenerationcenter/jobsrni/>

HANDS-ON

Questions will be answered

- Prepare your system
- Basics of shell script
- Introduction of R programming language

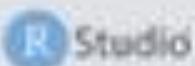
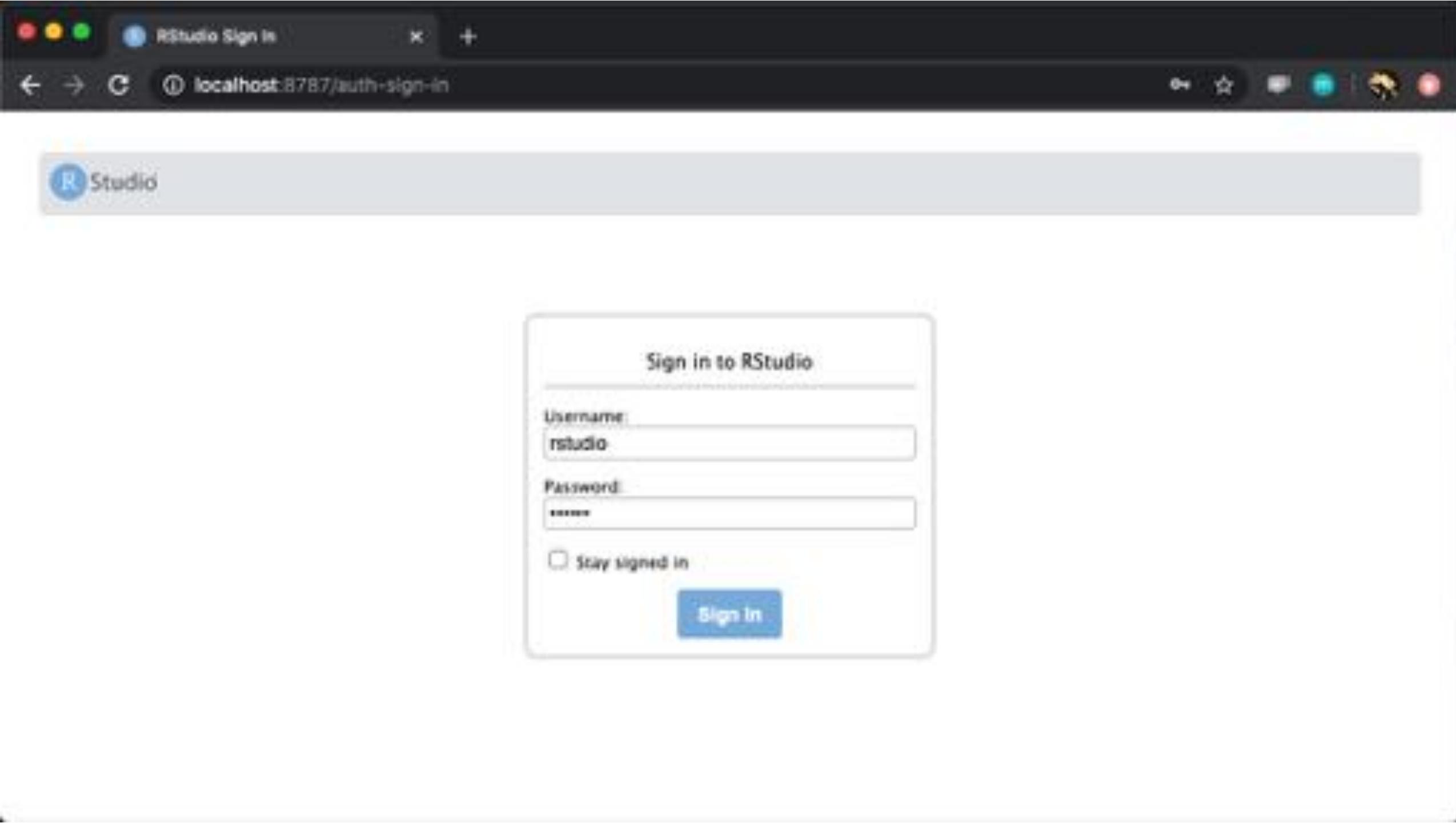
Set up your computing environment (m1)

- BEST Way: Install from source

Set up your computing environment (m2)

- Install Docker (<https://www.docker.com/get-started>) (30min)
- Check docker installed: docker --version
- docker pull jianhong/genomictools:latest
- cd ~
- mkdir tmp4genomictools
- docker run -e PASSWORD=123456 -p 8787:8787 -v \${PWD}/tmp4genomictools:/home/rstudio jianhong/genomictools:latest
 - localhost:8787 by username: rstudio password:123456

<https://github.com/jianhong/genomictools>



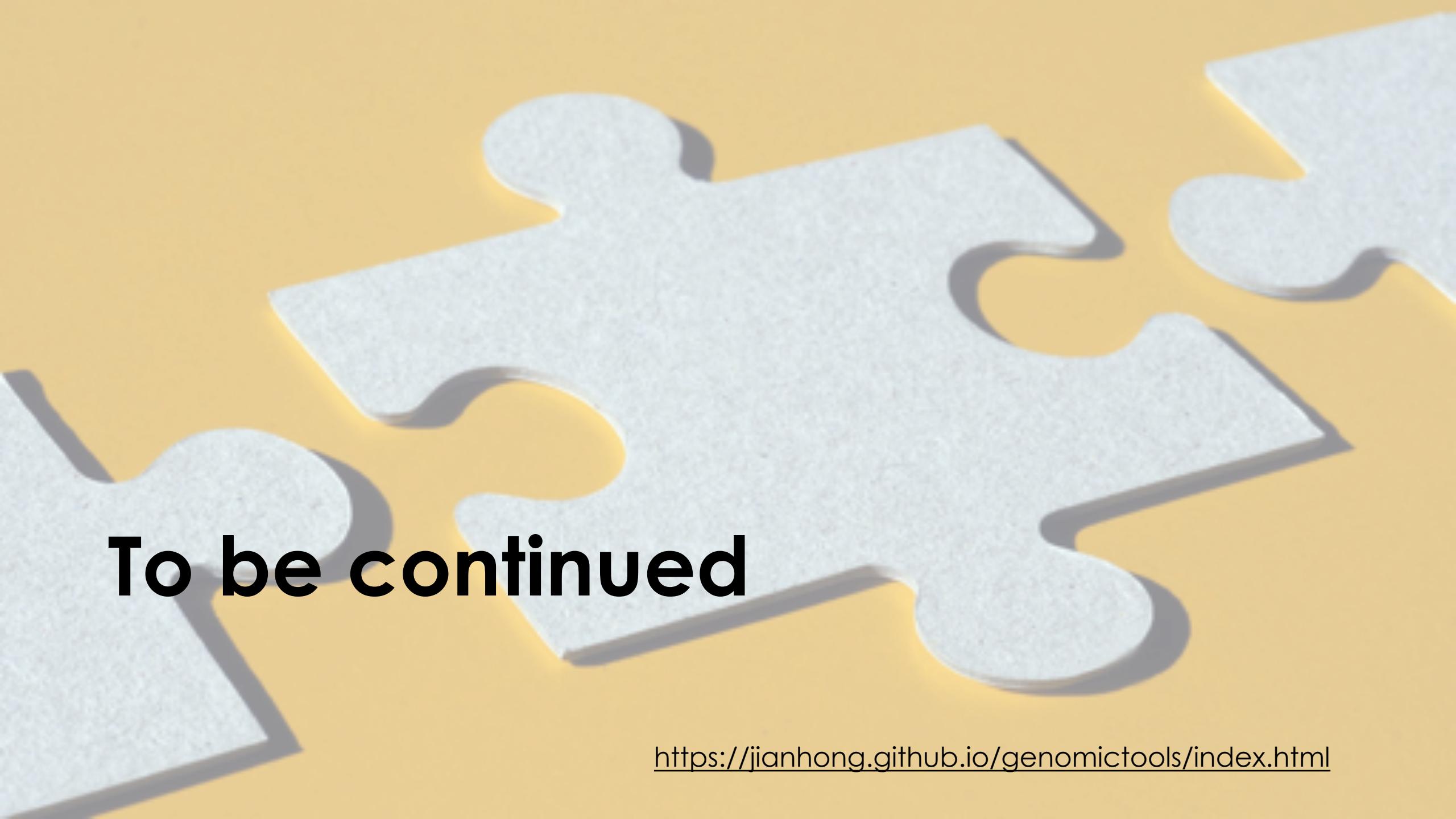
Sign in to RStudio

Username:

Password:

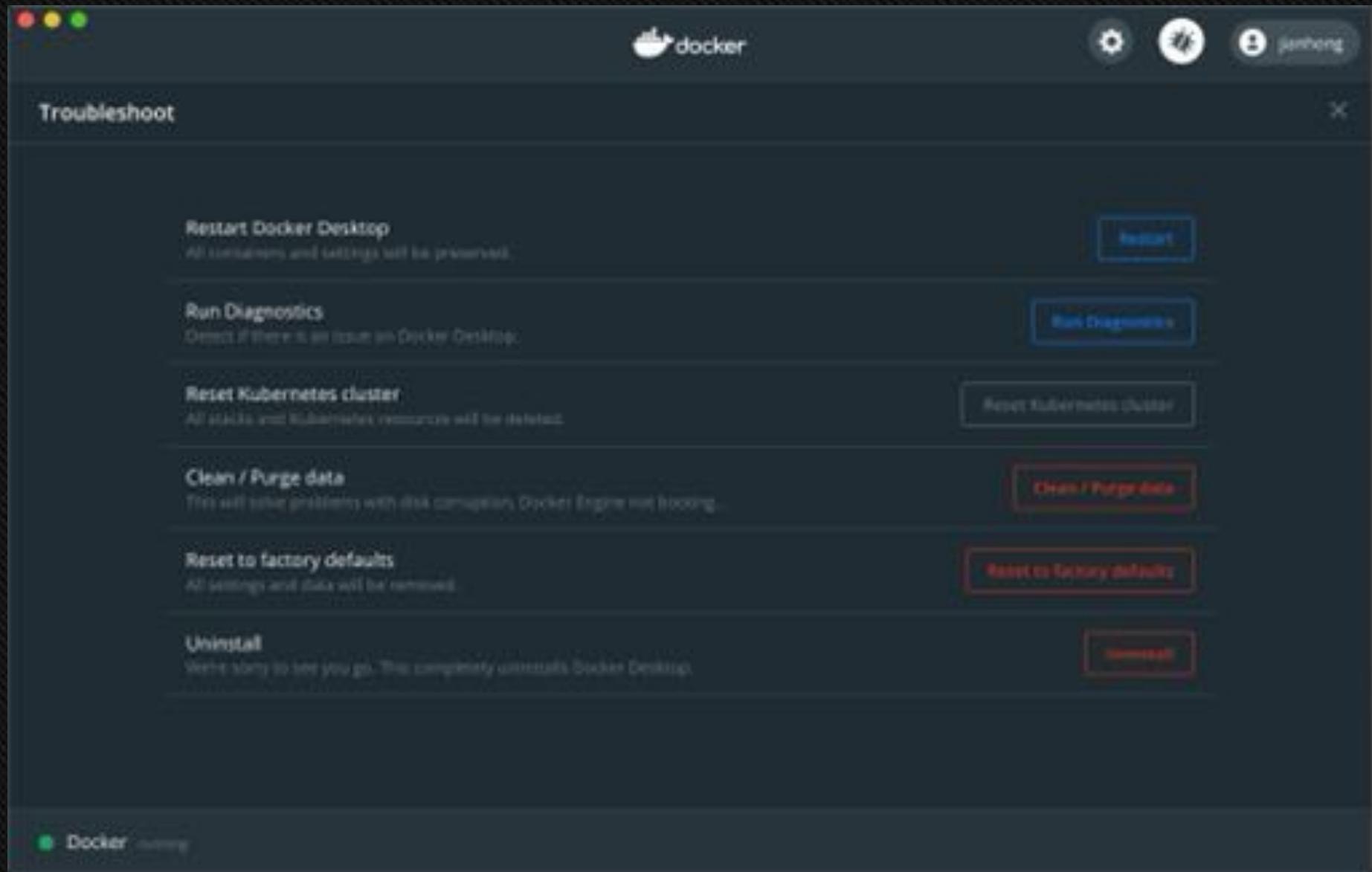
Stay signed in

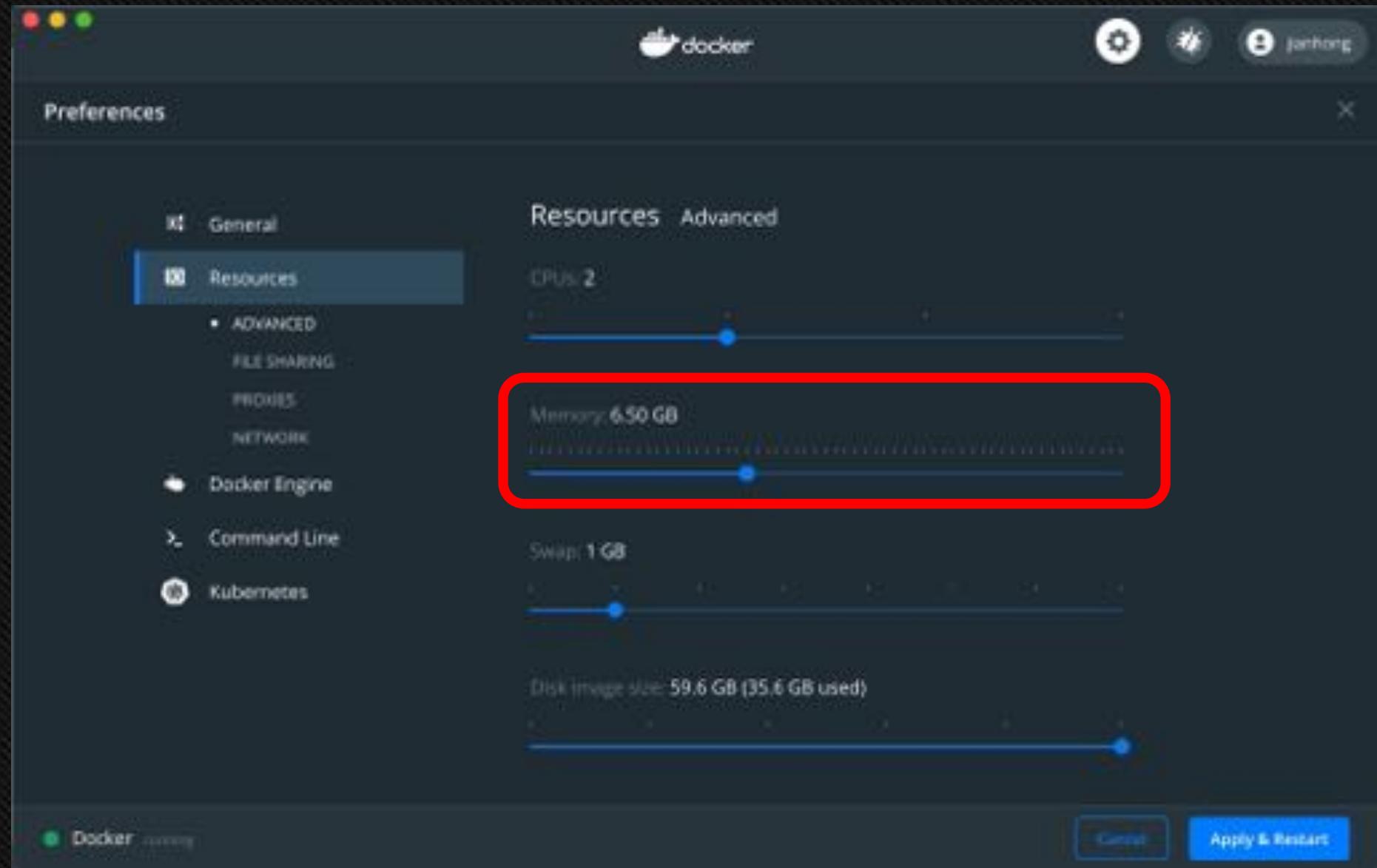
Sign In



To be continued

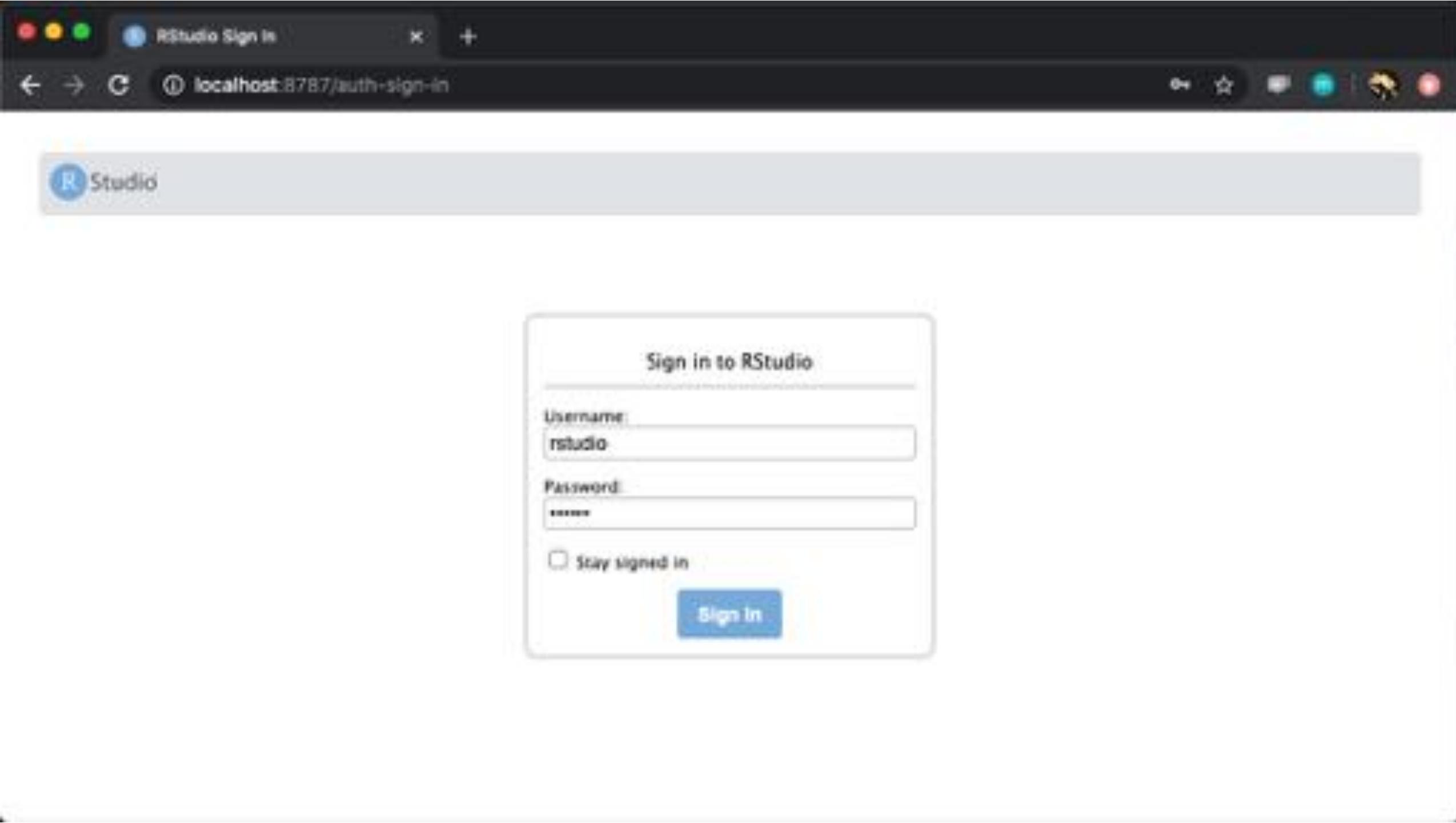
<https://jianhong.github.io/genomictools/index.html>





Start your docker

```
cd ~  
mkdir tmp4genomictools  
docker run -e PASSWORD=123456 -p 8787:8787 \  
-v ${PWD}/tmp4genomictools:/home/rstudio \  
jianhong/genomictools :latest
```



Studio

Sign in to RStudio

Username:

rstudio

Password:

 Stay signed in

Sign In

Basics of shell scripts

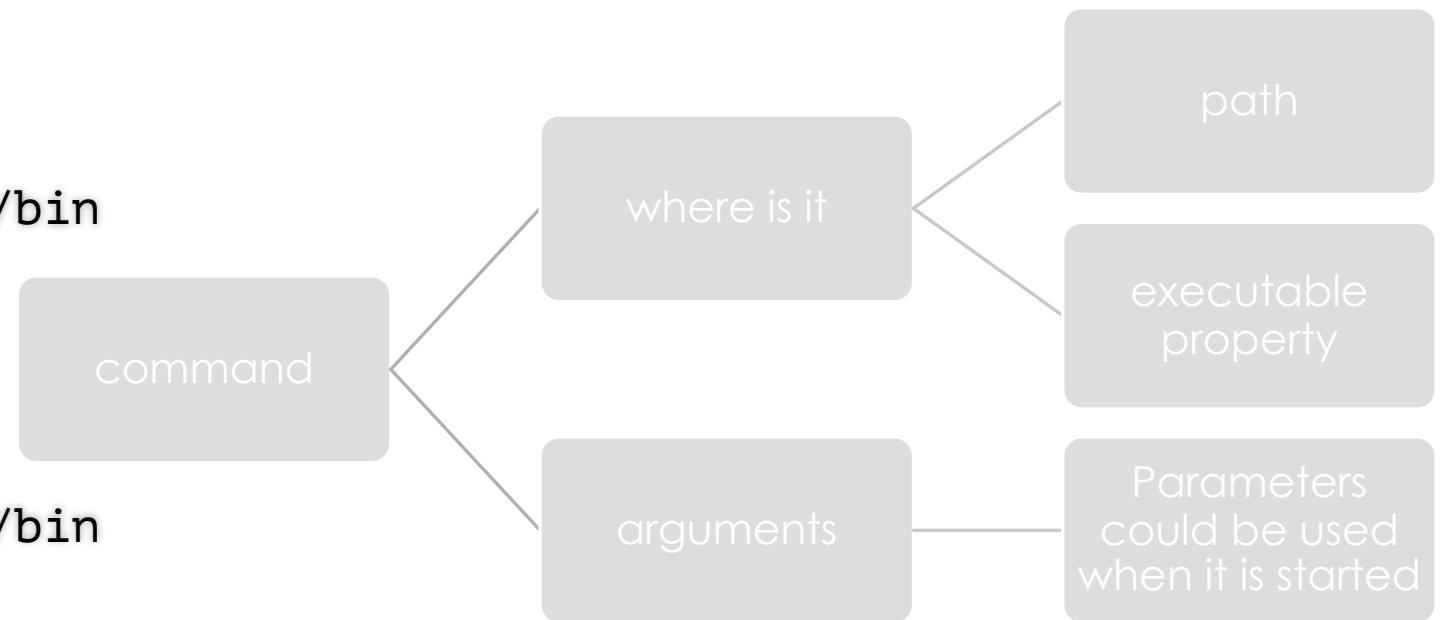
- Unix-like operating systems: “behave” like the original Unix operating system and comply(at least partially) with POSIX (portable operating system interface) standards.
 - Examples: Linux, OS X, Free BSD.
- A terminal: is a software that emulates a teletype writer terminal used in the early days of Unix.
- A shell: is a software that runs inside a terminal and interprets and executes user commands. One of the most popular shells being used today is called BASH (Bourne Again Shell)

Hello Bash

```
command          Options or arguments
riboimager:bin jianhongou$ echo Hello Bash
Hello Bash
prompt          arguments
riboimager:bin jianhongou$ ssh user@dcc-slogin.oit.duke.edu
#####
command
#####
# You are about to access a Duke University computer network that is intended #
# for authorized users only. You should have no expectation of privacy in      #
# your use of this network. Use of this network constitutes consent to          #
# monitoring, retrieval, and disclosure of any information stored within the    #
# network for any purpose including criminal prosecution.                      #
#####
user@dcc-slogin.oit.duke.edu's password: user@dcc-slogin-03 ~ $ whoami
user
command
```

command line

```
## to see working directory  
$ pwd  
/Users/jianhongou/miniconda3/bin  
$ which pwd  
/bin/pwd  
$ /bin/pwd  
/Users/jianhongou/miniconda3/bin  
$ ls -lh bowtie2  
-rwxrwxr-x 2 jianhongou staff 18K Jan 22 2017 bowtie2
```



Getting help

```
## try to get documentation of bowtie
$ ./bowtie2 -h
## get help of `ls`
$ man ls
```

Download transcript fasta

		Show 10 entries	Show/hide columns		Filter		
★	Species	DNA (FASTA)	cDNA (FASTA)	CDS (FASTA)	ncRNA (FASTA)	Protein sequence (FASTA)	
Y	Human <i>Homo sapiens</i>	FASTA					
Y	Mouse <i>Mus musculus</i>	FASTA					
Y	Zebrafish <i>Danio rerio</i>	FASTA					

<http://useast.ensembl.org/info/data/ftp/index.html>

```
curl -0 ftp://ftp.ensembl.org/pub/release-100/fasta/danio_rerio/cdna/Danio_rerio.GRCz11.cdna.all.fa.gz
```

Step1: build transcriptome index

kallisto

```
kallisto index -i danRer.GRCz11_transcrits.idx Danio_rerio.GRCz11.cdna.all.fa.gz
```

Salmon

```
salmon index -i danRer.GRCz11_transcrits.salmon.idx -t Danio_rerio.GRCz11.cdna.all.fa.gz
```

Step 2: quantify

kallisto

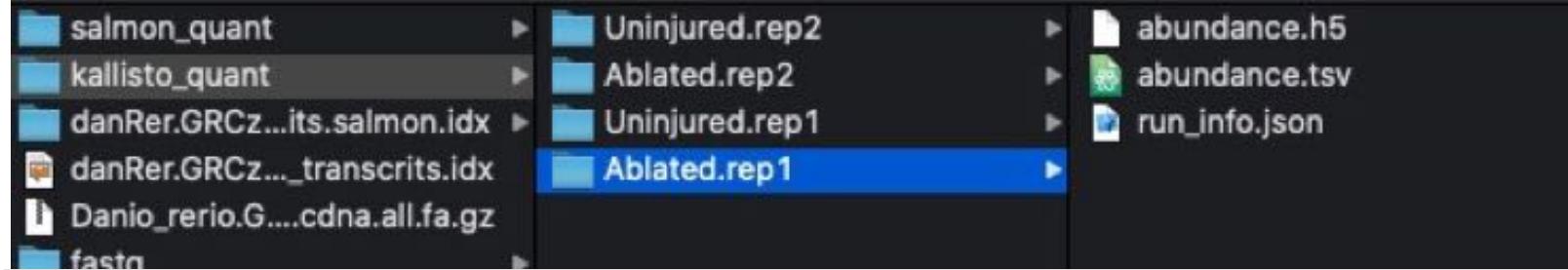
```
kallisto quant -i danRer.GRCz11_transcrits.idx \
-o kallisto_quant/$cond.rep$rep \
-b 30 -t 2 fastq/$cond.rep$rep.fastq.gz \
--single -l 200 -s 50
```

Salmon

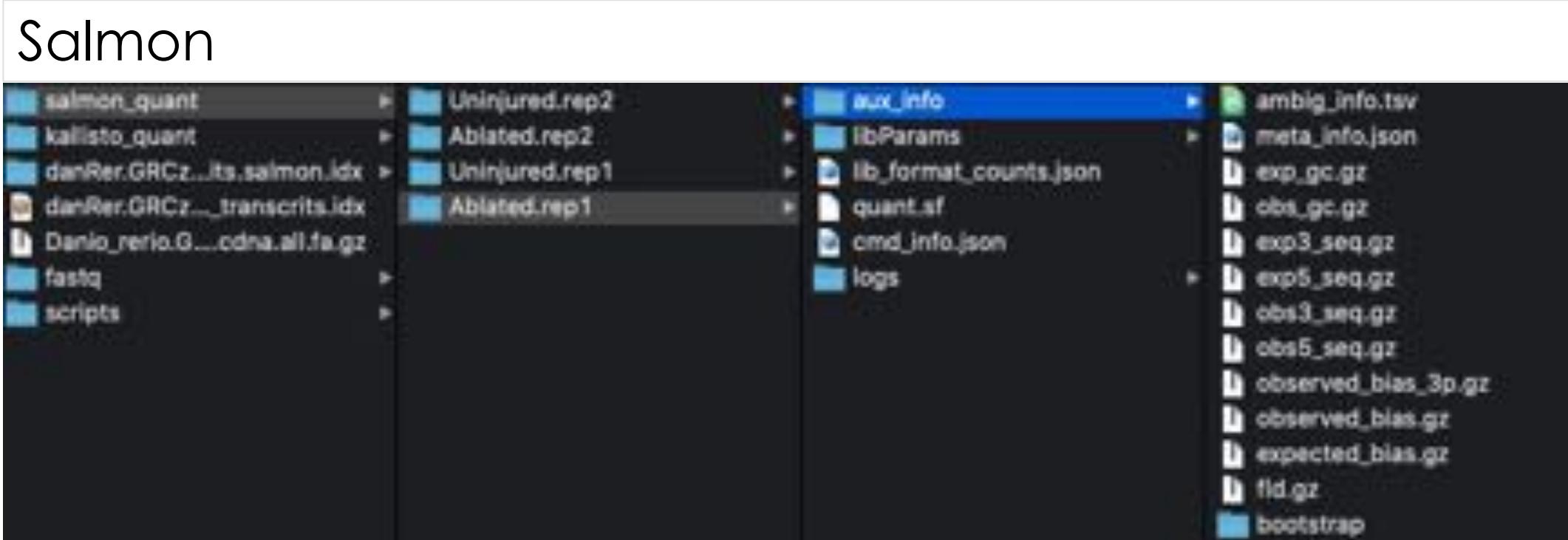
```
salmon quant -i danRer.GRCz11_transcrits.salmon.idx -l A \
-r fastq/$cond.rep$rep.fastq.gz \
--validateMappings -p 2 \
-o salmon_quant/$cond.rep$rep \
--numBootstraps 30 --seqBias --gcBias
```

output

kallisto



Salmon



output

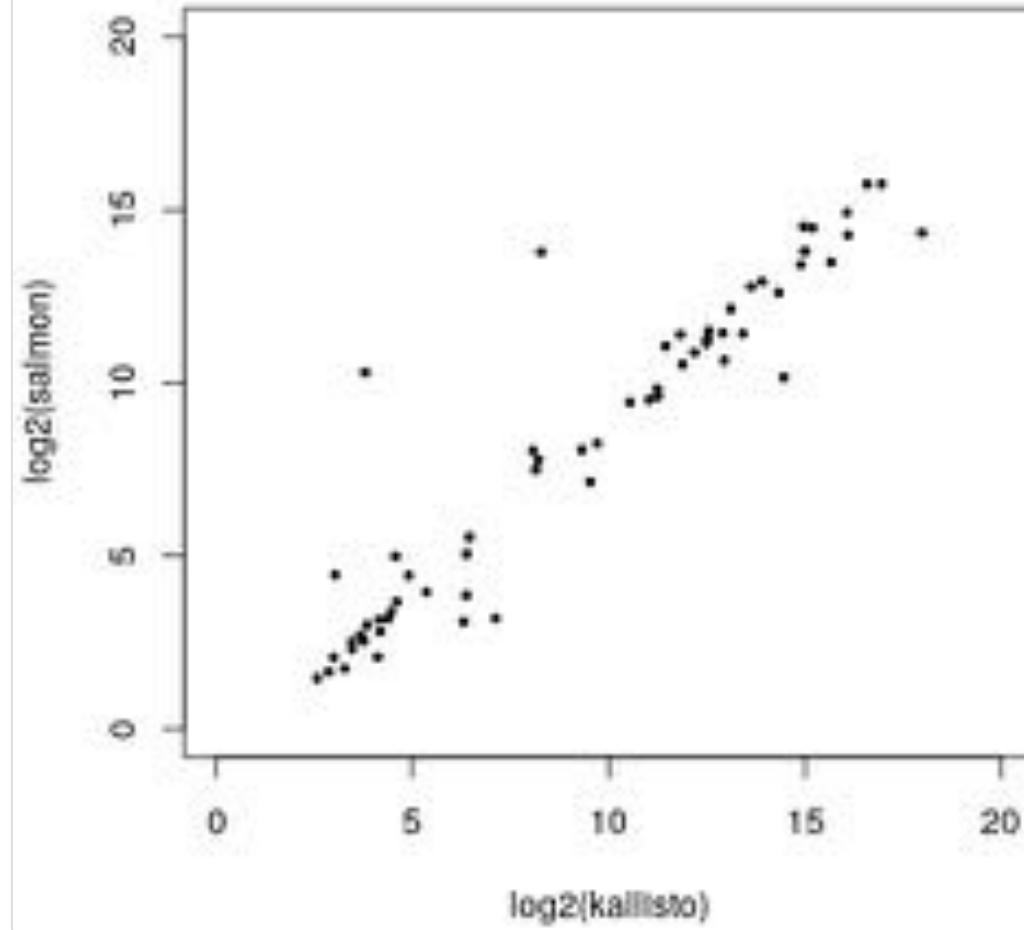
kallisto

	target_id	length	eff_length	est_counts	tpm
256	ENSDART00000146315.3	2622	2422.990	260.627000	5793.73000
257	ENSDART0000021121.8	1181	981.994	200.373000	10990.60000
279	ENSDART00000140081.2	6779	6579.990	1.760960	14.41500
281	ENSDART00000138182.3	6809	6609.990	0.239042	1.94789
330	ENSDART00000169072.2	8455	8255.990	1178.350000	7687.70000
331	ENSDART00000147947.2	8535	8335.990	719.649000	4650.01000

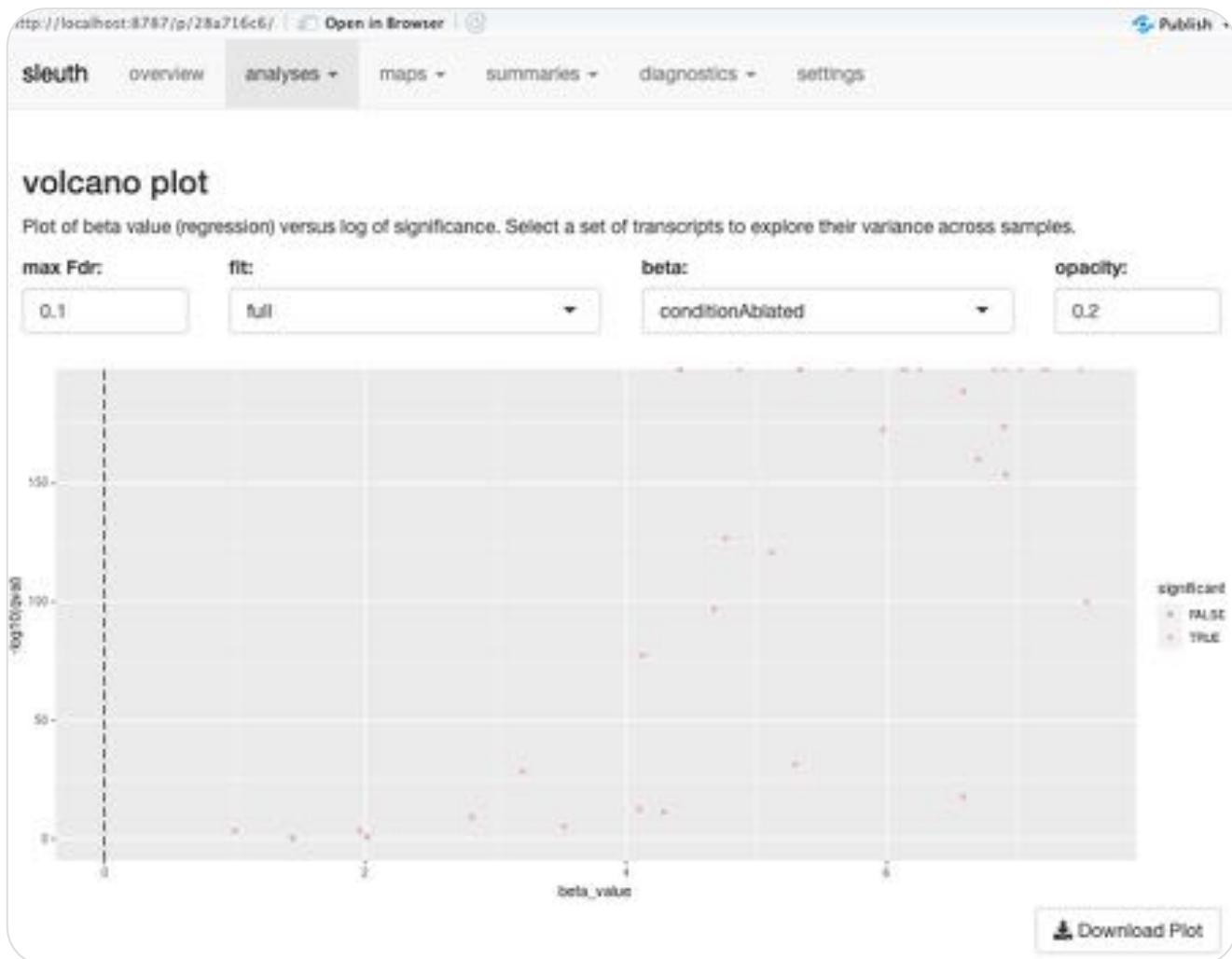
Salmon

	Name	Length	EffectiveLength	TPM	NumReads
256	ENSDART00000146315.3	2622	2838.809	2434.698251	292.406
257	ENSDART0000021121.8	1181	1526.830	2749.368836	177.594
279	ENSDART00000140081.2	6779	5857.133	8.071238	2.000
330	ENSDART00000169072.2	8455	9663.830	2781.720176	1137.280
331	ENSDART00000147947.2	8535	9769.232	1884.152025	778.720
339	ENSDART0000060594.6	2004	1621.171	14.579657	1.000

Kallisto and
Salmon
gene
counts
overall
similar



Sleuth_live



Introduction of R programming language

```
    .operation == "MIRROR_X":  
    mirror_mod.mirror_object =  
    mirror_mod.use_x = True  
    mirror_mod.use_y = False  
    mirror_mod.use_z = False  
    .operation == "MIRROR_Y":  
    mirror_mod.use_x = False  
    mirror_mod.use_y = True  
    mirror_mod.use_z = False  
    .operation == "MIRROR_Z":  
    mirror_mod.use_x = False  
    mirror_mod.use_y = False  
    mirror_mod.use_z = True
```

```
#Selection at the end -add to  
#ob.select= 1  
#ter_ob.select=1  
#context.scene.objects.active  
#("Select "+ str(modifier)  
#) for modifier in  
#["X", "Y", "Z"]:  
#    if ob.select == 1:  
#        ob.select = 0  
#        data.objects[one_name].select = 1  
#        break
```

```
print("please select exactly one object. CLASSES -----")
```

```
types.Operator):  
    " X mirror to the selected object.mirror_mirror_X"  
    " mirror_X"
```

Install R/Bioconductor/Rstudio

- <https://cloud.r-project.org/>



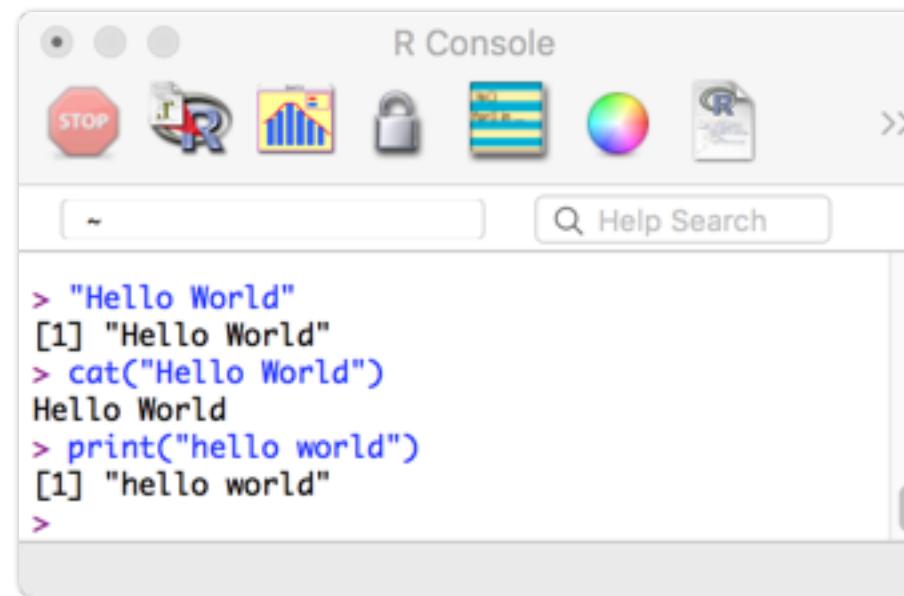
- <https://www.rstudio.com/products/rstudio/download/>



- <https://Bioconductor.org/>



Exercise: Hello world



The image shows a screenshot of an R console window. The title bar reads "R Console". The interface includes standard Mac OS X window controls (minimize, maximize, close) and a toolbar with icons for Stop, Help, and other functions. Below the toolbar is a search bar labeled "Help Search". The main area displays the following R session:

```
> "Hello World"
[1] "Hello World"
> cat("Hello World")
Hello World
> print("hello world")
[1] "hello world"
>
```

Where to start?

- From vignettes of a package
<https://bioconductor.org/packages/4.0/bioc/vignettes/trackViewer/inst/doc/trackViewer.html#lolliplot>
- From help documents
- From a book

Grammar of R

```
hairLength <- 25
waistHeight <- 110
height <- 168
marryMe <- FALSE
wait <- 100 * 365
for(day in 1:wait){
  if(hairLength >= height - waistHeight){
    marryMe <- TRUE
    break
  }else{
    if(runif(1) < 0.5){
      next
    }
    hairLength <- hairLength + .1
  }
}
day
```

- 待我长发及腰, When my hair is longer enough to make hair bun
- 少年娶我可好? Will you give me your promise ring?
- 待你青丝绾正, When you get your driver license,
- 铺十里红妆可愿? Will you roll out the red carpet for me?
- 却怕长发及腰, I am afraid before my hair grew till my waist,
- 少年倾心他人。 You will falling in love with another woman.
- 待你青丝绾正, I am afraid when you get your driver license,
- 笑看君怀她笑颜。 I see another woman burying herself in your arms.
- ——何晓道 《十里红妆女儿梦》

Data structure of R

Dimension	Homogeneous	Heterogeneous
1d	Atomic vector	List
2d	Matrix	Data frame
Nd	Array	

Atomic vector: character, integer, logical, numeric, factor

Matrix, Array: atomic vector with dimension. Matrix is an array.

List: a chain for different kinds of data structures, including list.

Data frame: 2D version of List; All lists have same length of elements.

Exercises

- What is the class of `c('A', TRUE, 1L, 1, factor(letters[1:3]))`
- What is the class of `c(TRUE, 1L, 1, factor(letters[1:3]))`
- What is the class of `c(TRUE, 1L, factor(letters[1:3]))`
- What is the class of `c(TRUE, factor(letters[1:3]))`
- ```
m <- array(1:18, dim=c(3, 3, 2))
i <- 1; j <- 3; k <- 2;
m[i, j, k]
m[(k-1)*(nrow(m)*ncol(m)) + (j-1)*nrow(m) + i]
```
- What is the difference between `as.numeric(as.character(factor(c(3, 5, 1))))` and `as.numeric(factor(c(3, 5, 1)))`
- What is the difference between `as.list(m)` and `as.list(as.data.frame(m))`

# How to get help

- `help(functionName)` or `?functionName` or `??functionName` : `help(help); help("for")`
- `browseVignettes("grid")`
- Google
- R mailing list: <https://www.r-project.org/mail.html#instructions>

# How to install a package

- Install R packages: `install.packages("BiocManager", repos="https://cloud.r-project.org")`
- Install Bioconductor packages:  
`BiocManager::install(c("org.Dr.eg.db", "TxDb.Drerio.UCSC.danRer10.refGene"))`

# Merge tables

- Question: how could we merge two excel sheet by gene symbols?

- Code:

```
m <- data.frame(symbol=letters[1:5], foldChange=runif(5))
n <- data.frame(gene=sample(letters[1:10], size=6), foldChange=runif(n=6))
z <- merge(m, n, by.x="symbol", by.y="gene", suffixes=c(".m", ".n"), all.x=TRUE, all.y=TRUE)
z
```

# Set operations

- Question: how to find the shared/unique genes from 2 lists of genes?

- Code:

```
A <- sample(letters[1:10], size=8)
B <- sample(letters[1:10], size=5)
intersect(A, B) ## shared genes
setdiff(A, B) ## only in A
setdiff(B, A) ## only in B
```

# How to read data from a file

- Tab-delimited file (tsv): `read.delim("file.txt")`
- Comma-delimited file (csv): `read.csv("file.csv")`
- Excel sheet (xls, xlsx): `library(gdata); read.xls("file.xlsx", sheet=1)`

# How to save a table

- Save to a csv file: `write.csv(data, "file.csv")`
- Save to a xls file: `library(WriteXLS); WriteXLS(dataframe, "file.xls")`

# Basics of R plot

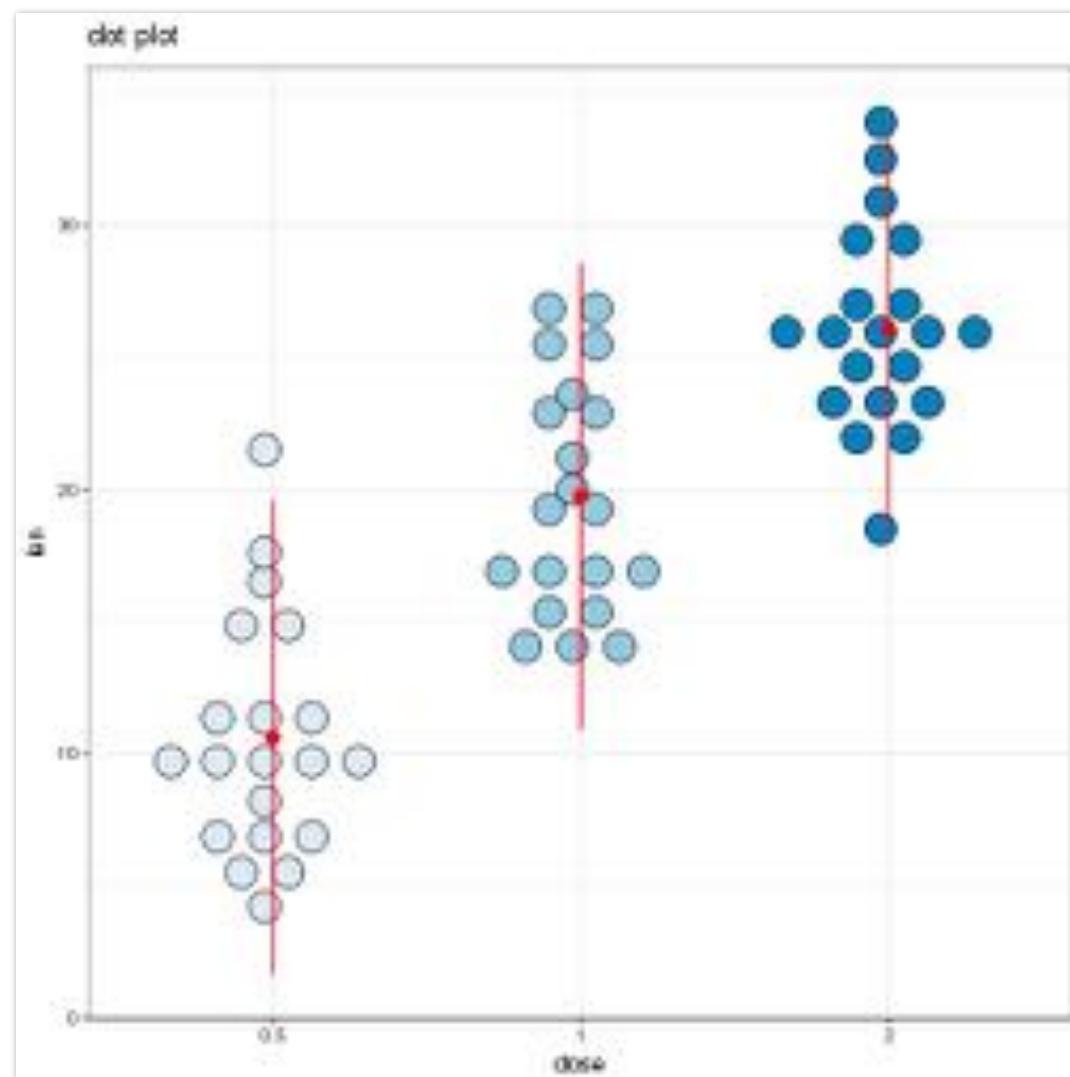
- Dots plot: `plot(x=mtcars$wt, y=mtcars$mpg)`
- Bar plot: `counts <- table(mtcars$vs, mtcars$gear); barplot(counts)`
- Histogram: `hist(mtcars$vs)`
- Pie: `x<-table(mtcars$gear); pie(x)`
- Boxplot: `boxplot(mpg~cyl,data=mtcars)`

# ggplot2

Based on the grammar of graphics

```
library(ggplot2)
data("ToothGrowth")
ToothGrowth$dose <- factor(ToothGrowth$dose)

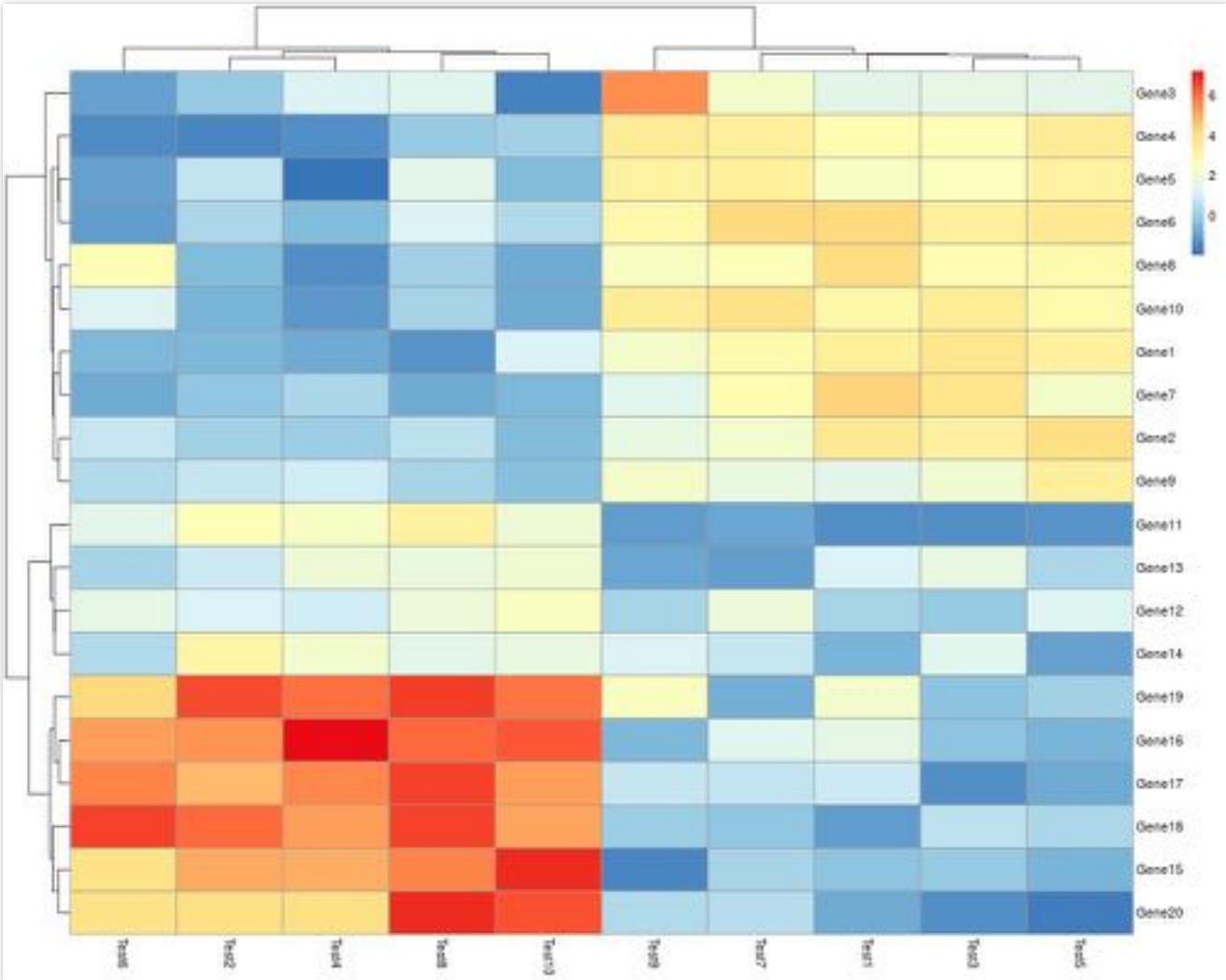
ggplot(ToothGrowth, aes(x=dose, y=len, fill=dose)) +
 geom_dotplot(binaxis='y', stackdir='center',
 stackratio=1.5, dotsize=1.2) +
 stat_summary(fun.data=mean_sdl, mult=1,
 geom="pointrange", color="red") +
 scale_fill_brewer(palette="Blues", guide=FALSE) +
 theme_bw() + labs(title="dot plot")
```



# pheatmap

## Pretty heatmap

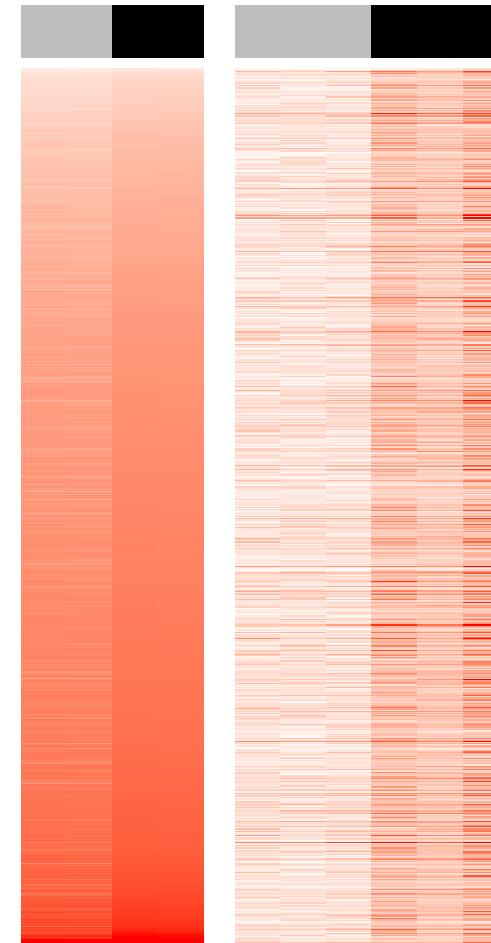
```
library(BiocManager)
install("pheatmap")
library(pheatmap)
test <- matrix(rnorm(200), 20, 10)
test[1:10, seq(1, 10, 2)] <- test[1:10, seq(1, 10, 2)] + 3
test[11:20, seq(2, 10, 2)] <- test[11:20, seq(2, 10, 2)] + 2
test[15:20, seq(2, 10, 2)] <- test[15:20, seq(2, 10, 2)] + 4
colnames(test) <- paste("Test", 1:10, sep = "")
rownames(test) <- paste("Gene", 1:20, sep = "")
pheatmap(test)
```



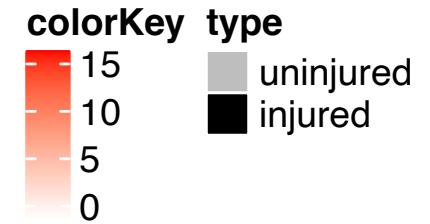
## ComplexHeatmap

Plot multiple heatmaps in one canvas  
rowAnnotation and colAnnotation

RNAseq ATACseq



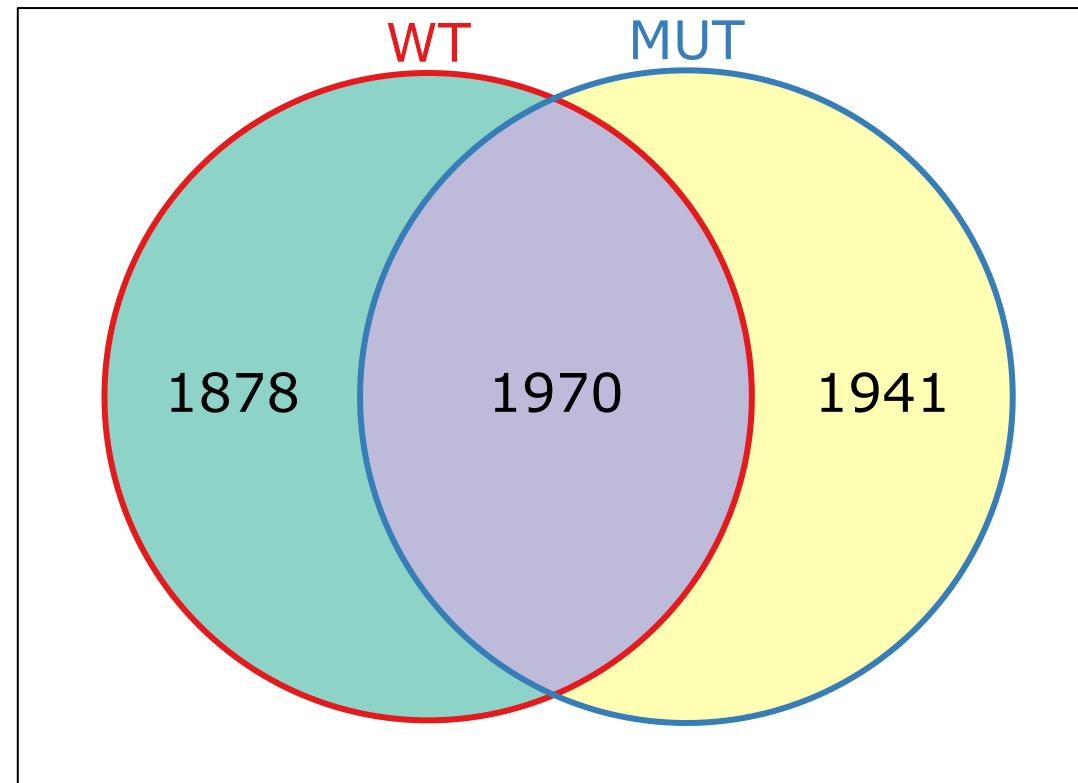
— fgf20a



— marcks1a  
— cx43  
— mdka

## Venn Diagram: Venerable

displays Venn and Euler diagrams



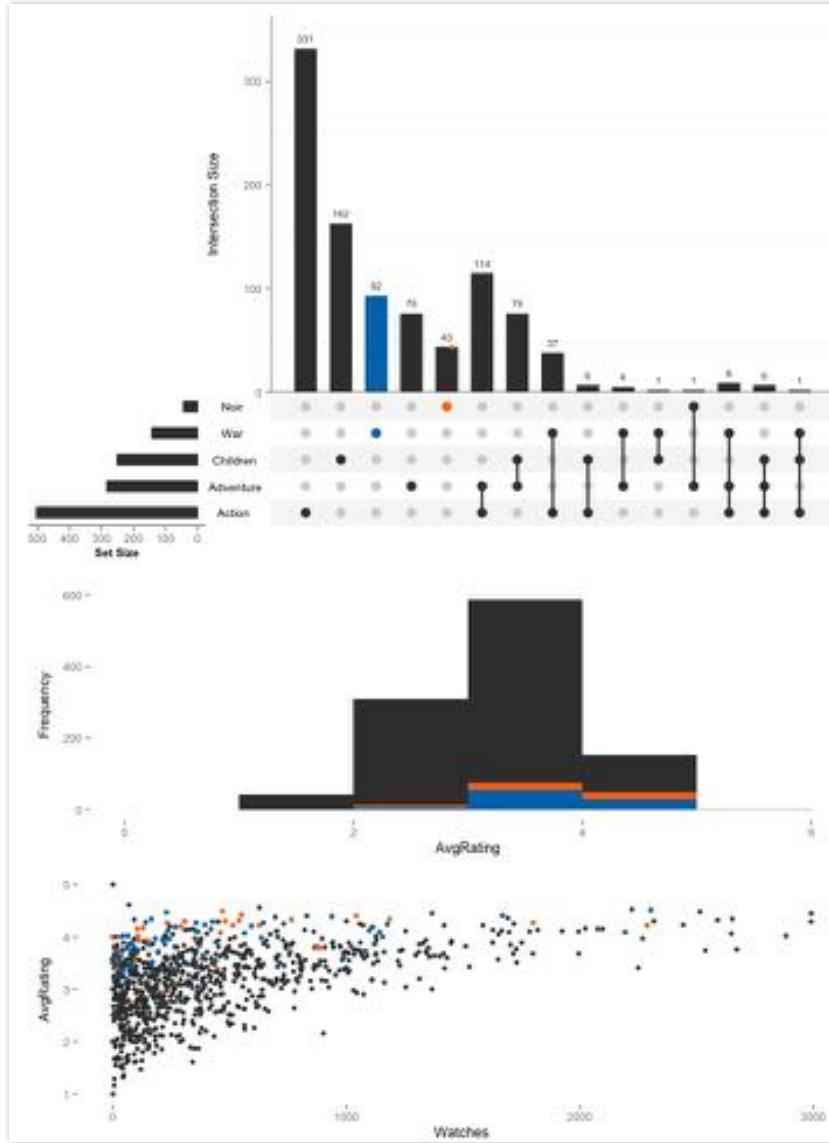
# UpSetR

visualizes set intersections in a matrix layout

```
install("UpSetR")
library(UpSetR)

movies <- read.csv(
 system.file("extdata", "movies.csv",
 package = "UpSetR"), header=TRUE,
 sep=";")

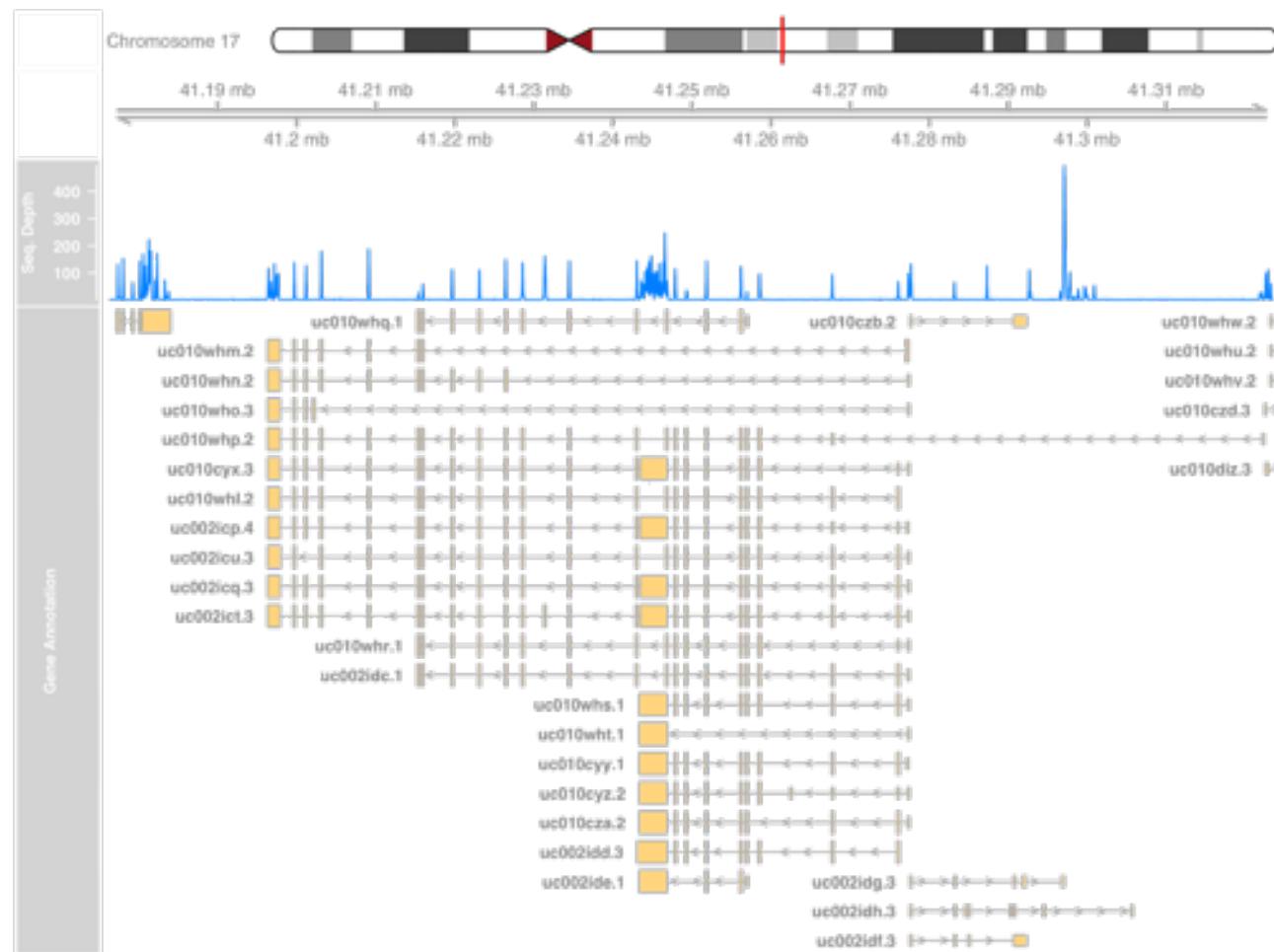
upset(movies, nsets = 7, nintersects =
 30, mb.ratio = c(0.5, 0.5), order.by =
 c("freq", "degree"), decreasing =
 c(TRUE, FALSE))
```



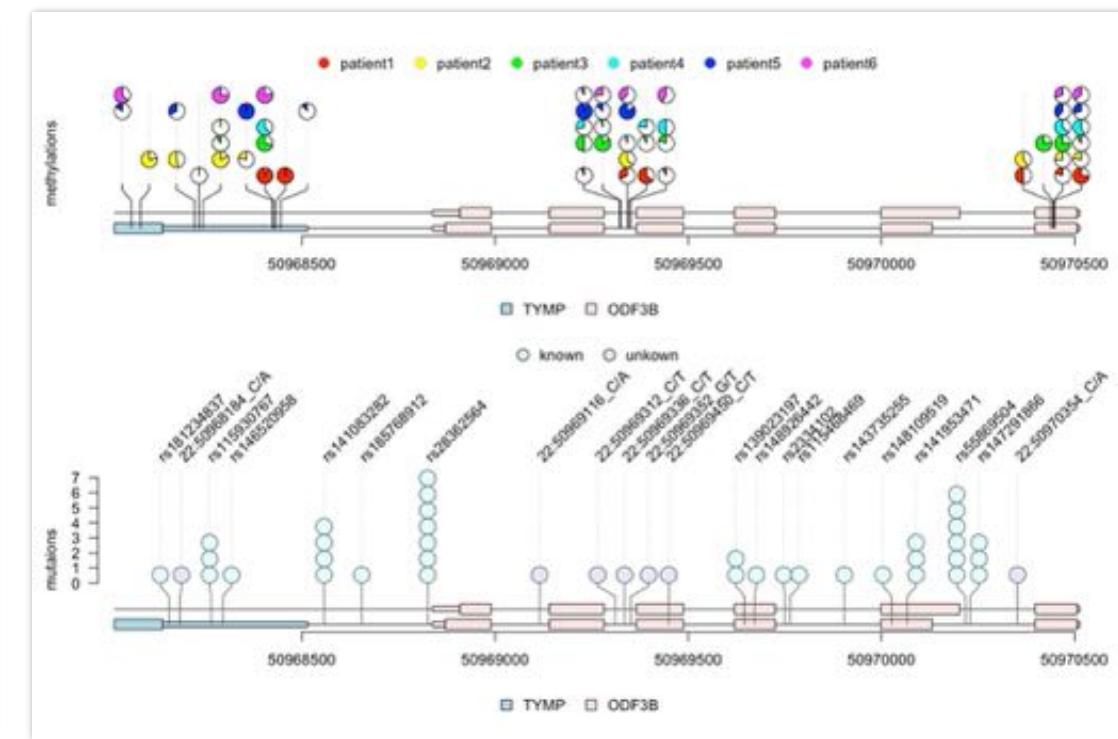
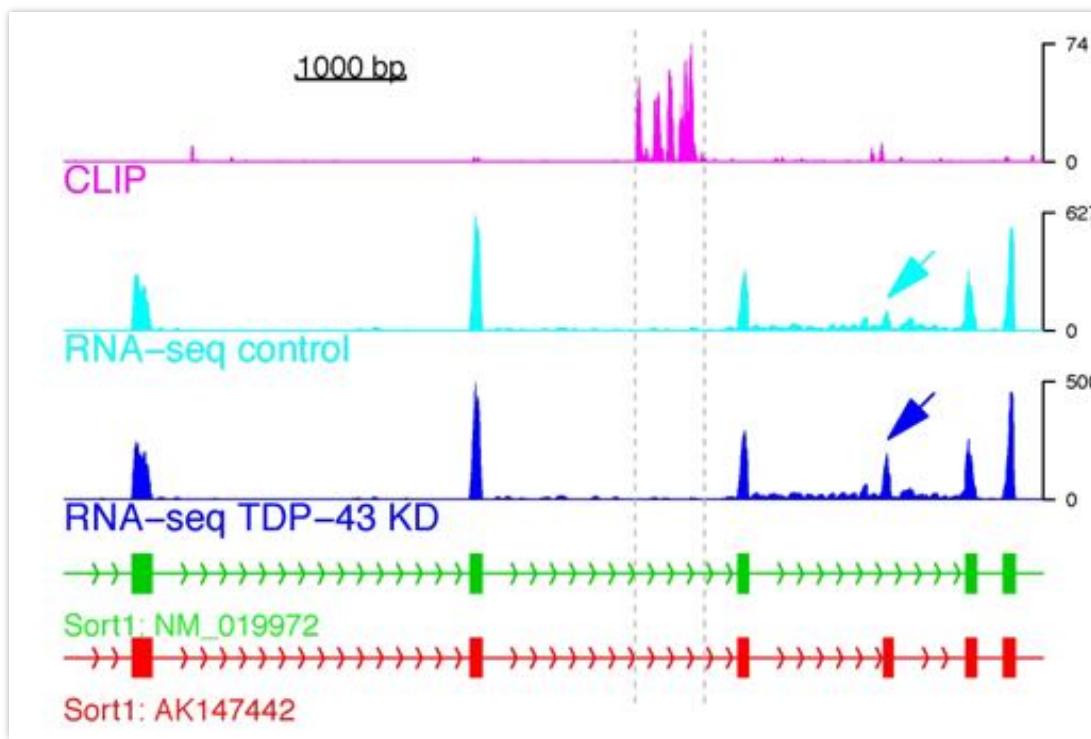
# Gviz

Plotting data and annotation information along genomic coordinates

```
library(Gviz)
library(GenomicRanges)
data(cpgIslands)
chr <- as.character(unique(seqnames(cpgIslands)))
gen <- genome(cpgIslands)
atrack <- AnnotationTrack(cpgIslands,
 name="CpG")
gtrack <- GenomeAxisTrack()
data(geneModels)
grtrack <- GeneRegionTrack(geneModels,
 genome=gen, chromosome=chr, name="Gene
 Model")
plotTracks(list(gtrack, atrack, grtrack))
```

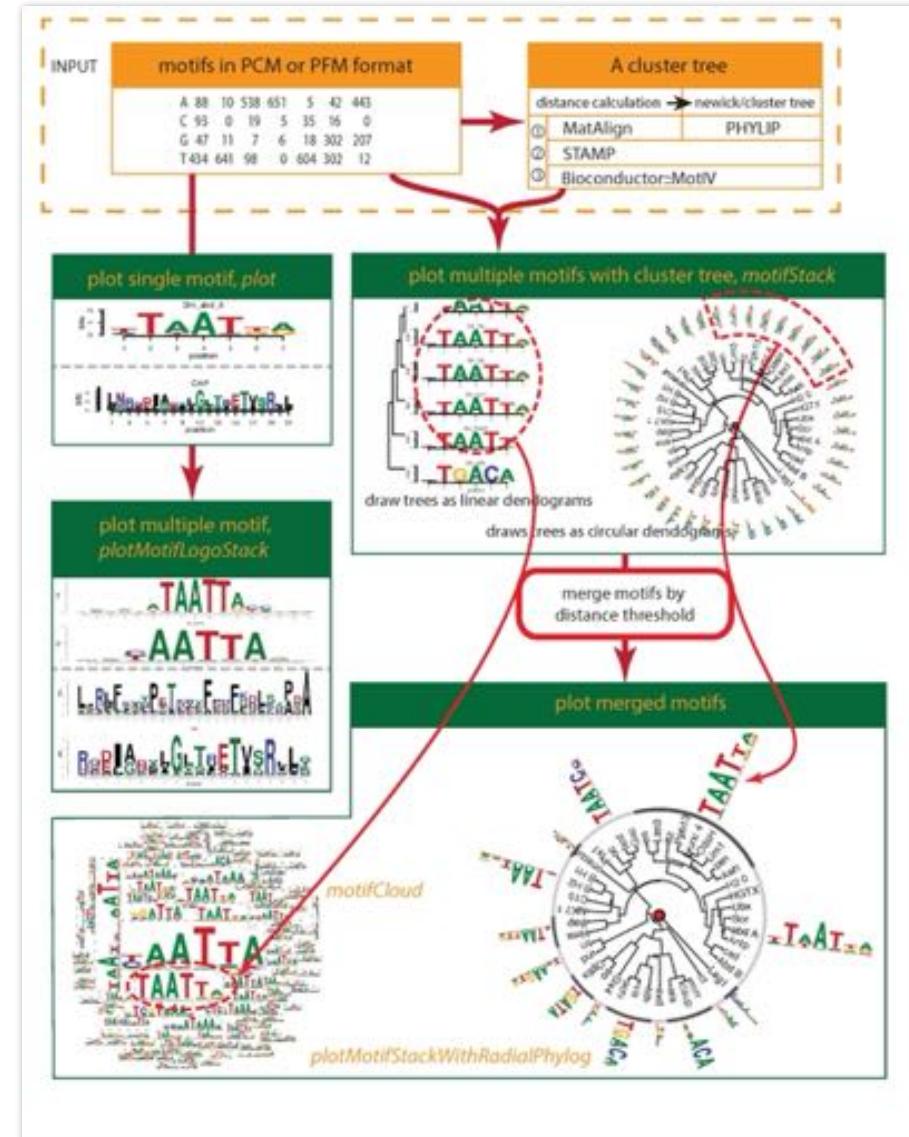


# trackViewer



# motifStack

Plot stacked logos for single or multiple DNA, RNA and amino acid sequence

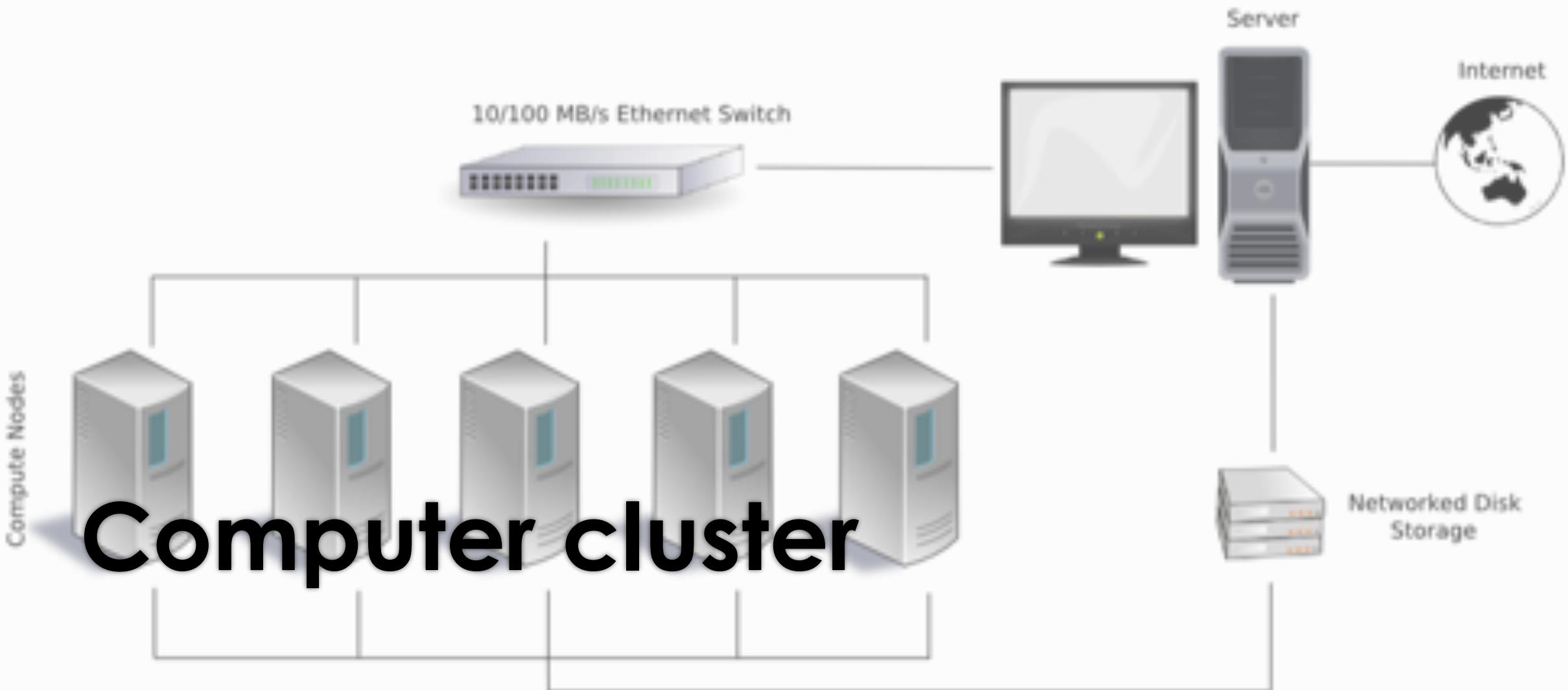


# Basics of Bioconductor objects

- GenomicRanges::GRanges:  
gr0 <- GRanges(Rle(c("chr2", "chr2", "chr1", "chr3"), c(1, 3, 2, 4)), IRanges(1:10, width=10:1))  
gr0
- SummarizedExperiment::RangedSummarizedExperiment:  
nrows <- 200; ncols <- 6  
counts <- matrix(runif(nrows \* ncols, 1, 1e4), nrows)  
rowRanges <- GRanges(rep(c("chr1", "chr2"), c(50, 150)),  
IRanges(floor(runif(200, 1e5, 1e6)), width=100),  
strand=sample(c("+", "-"), 200, TRUE),  
feature\_id=sprintf("ID%03d", 1:200))  
colData <- DataFrame(Treatment=rep(c("ChIP", "Input"), 3),  
row.names=LETTERS[1:6])  
rse <- SummarizedExperiment(assays=SimpleList(counts=counts),  
rowRanges=rowRanges, colData=colData)  
rse

# Basics of annotation

- annotationDBi: include ChipDb, OrgDb, GODb, InparanoidDb and ReactomeDb
- BSgenome: The BSgenome class is a container for storing the full genome sequences of a given organism.
- GenomicFeatures: The GenomicFeatures package retrieves and manages transcript-related features from the UCSC Genome Bioinformatics and BioMart data resources.



# Cluster software

- Oracle Grid Engine, previously known as Sun Grid Engine (SGE):`qsub`
- IBM Platform Load Sharing Facility (LSF):`bsub`
- Slurm workload manager:`sbatch`
- More: [https://en.wikipedia.org/wiki/Comparison\\_of\\_cluster\\_software](https://en.wikipedia.org/wiki/Comparison_of_cluster_software)

# The Duke compute cluster

- Website: <https://rc.duke.edu/dcc/>
- Next class: **8/4 OR 9/1**, <https://rc.duke.edu/dcc-training/>
- DCC use slurm workload manager.

# Accessing the DCC

- Login: ssh NetID@dcc-slogin.oit.duke.edu
- Copy files: scp, rsync
  - scp -r sourceFolder netid@dcc-slogin.oit.duke.edu:/work/netid/targetFolder
  - rsync -av sourceFolder netid@dcc-slogin.oit.duke.edu:/work/netid/targetFolder

# DCC file systems

- /dscrhome: 250 GB group quota
- /work: temporary storage
- /datacommons: archival storage; available for \$80/TB/year

# module

- module avail
- module load
  - which STAR
  - module load STAR/2.5.3a
  - which STAR
  - echo \$PATH

# Submit a job

- **srun:**
  - Interactive mode: srun --pty bash -i
  - Run a script: srun --mem 1G /bin/hostname
- **sbatch:**
  - `#!/bin/bash`
  - `#SBATCH -J mapping #jobname`
  - `#SBATCH --array=1-2 # job array, can be 1,3,5,7 or 1-7:2 '%' will limit the number of job`
  - `#SBATCH -o pindel.out.%A_%a.txt # %A == $SLURM_ARRAY_JOB_ID #log folder must be there`
  - `#SBATCH -e pindel.err.%A_%a.txt # %a == $SLURM_ARRAY_TASK_ID`
  - `#SBATCH --mem-per-cpu=5G #5G`
  - `#SBATCH -c 1 # cpus-per-task`
  - `#SBATCH --partition=common`
  - `i=$((($SLURM_ARRAY_TASK_ID-1))`
  - `echo $i > job.id.txt`

# DCC partitions

- **common**, for jobs that will run on the DCC core nodes (up to 64 GB RAM).
- **common-large**, for jobs that will run on the DCC core nodes (64-240 GB RAM).
- **gpu-common**, for jobs that will run on DCC GPU nodes.
- **Group partitions** (partition name varies), for jobs that will run on lab-owned nodes

# Slurm commands

- `sbatch` – Submit a batch job
- `squeue` – show list of jobs
- `scancel` – delete one or more jobs



## Additional information

# Basic tools

- [Bedtools](#)
- [Samtools](#)
- [Picard](#)
- [Ucsc genomic tools](#)

# Samtools

- SAM, BAM file convert:
  - samtools view -h file.bam > file.sam
  - samtools view -b -S file.sam > file.bam
- Sorting BAM
  - samtools sort file.bam -o outputbam
- Create BAM index
  - samtools index file.bam

# Online resources

- [cBio Cancer Genomics Portal](#) (cBioPortal) & package [cgdsr](#)
- [The Cancer Genome Atlas \(TCGA\)](#) & package [TCGAbiolinks](#), [RTCGAToolbox](#)
- [Gene Expression Omnibus \(GEO\)](#) & package [GEOquery](#), [SRAdb](#)
- [Encyclopedia of DNA Elements \(ENCODE\)](#) & package [ENCODEExplorer](#)
- [recount2](#) & package [recount](#)

# Use cgdsr package download data

```
● BiocManager::install("cgdsr")
● library(cgdsr)
● # Create CGDS object
● mycgds <- CGDS("https://www.cbioportal.org/")
● # Test the connection
● test(mycgds)
● # Get list of cancer studies at server
● getStudies(mycgds)[, 1:2]
● # Get available case lists (collection of samples) for a
 given cancer study
● (mycancerstudy <- getStudies(mycgds)[2,1])
● getCaseLists(mycgds, mycancerstudy)
● mycaselist <- getCaseLists(mycgds, mycancerstudy)[1, 1]
● # Get available genetic profiles
● getGeneticProfiles(mycgds, mycancerstudy)
● (mygeneticprofile <-
 getGeneticProfiles(mycgds, mycancerstudy)[2,1])
● # Get data slices for a specified list of genes, genetic
 profile and case list
● getDataSlices(mycgds, c('BRCA1', 'BRCA2'), mygeneticprofile,
 mycaselist)
● # Get clinical data for the case list
● myclinicaldata <- getClinicalData(mycgds, mycaselist)
● dim(myclinicaldata)
● head(myclinicaldata)
```

# Download expression data from recount2

```
in shell apt-get update && apt-get install libxml2-dev
BiocManager::install("recount")
library('recount')
Find a project of interest
project_info <- abstract_search('GSE32465')
Explore info
project_info
Download the gene-level RangedSummarizedExperiment data
download_study(project_info$project)
Load the data
load(file.path(project_info$project, 'rse_gene.Rdata'))
Delete it if you don't need it anymore
unlink(project_info$project, recursive = TRUE)
rse_gene
the sample phenotype data provided by the recount project
head(colData(rse_gene))
the sample feature data provided by the recount project
rowData(rse_gene)
```

- ## Scale counts by taking into account the total coverage per sample
- rse <- scale\_counts(rse\_gene)
- (title <- colData(rse)\$title)
- (group <- ifelse(grepl("uninduced", title), "uninduced", "induced"))
- (target <- sub("^.\*targeting (.\*) gene.\*\$", "\\\1", title))
- ## Add sample information for DE analysis
- colData(rse)\$group <- factor(group)
- colData(rse)\$gene\_target <- factor(target)
- library(DESeq2)
- ## Specify design and switch to DESeq2 format
- dds <- DESeqDataSet(rse, ~ gene\_target + group)
- ## Perform DE analysis
- dds <- DESeq(dds, test = 'LRT', reduced = ~ gene\_target, fitType = 'local')
- res <- results(dds)
- ## Explore results
- head(res)
- library(WriteXLS)
- WriteXLS(as.data.frame(res), "DE.results.GSE32465.xls")

# DESeq2

```
library(DESeq2)
load(url("http://qiubio.com/bioconductor/RNA-seq/ds1.Rdata"))
ls()
head(counts[, 1:7], 3)
grp <- as.factor(substr(colnames(counts), 1, 2)) ##assign group
dds <- DESeqDataSetFromMatrix(counts,
 colData=data.frame(grp),
 design=formula(~1+grp))
design(dds)
dds <- DESeq(dds)
res <- results(dds)
res
```

```
mcols(res, use.names=TRUE)
plotMA(res)
plotDispEsts(dds)
save results
library(WriteXLS)
WriteXLS(as.data.frame(res), "results.xls")
try comparison of other groups
results(dds, contrast = c("grp", "PE", "DE"))
question, how to compare group PE vs FE
```

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<https://sites.duke.edu/dukeregenerationcenter/jobsrni/>