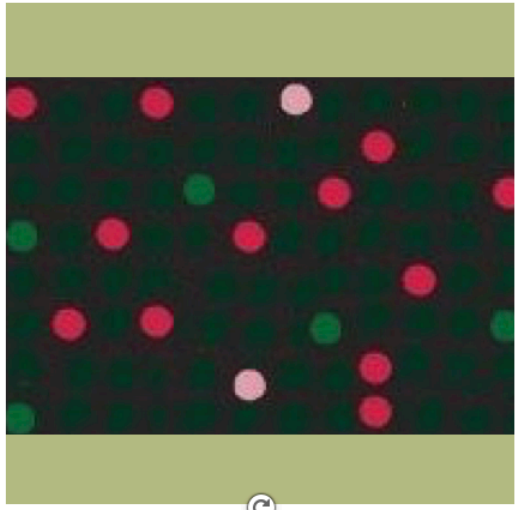
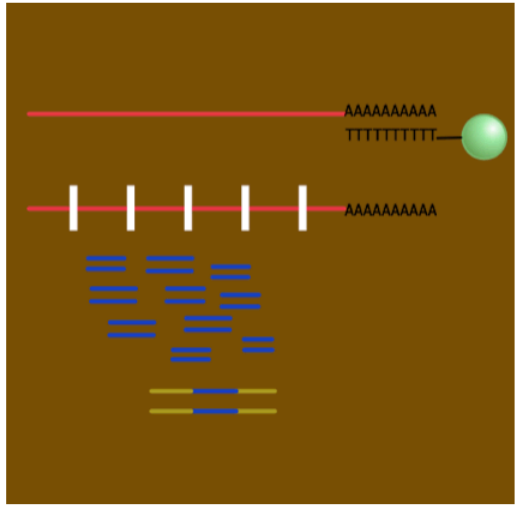
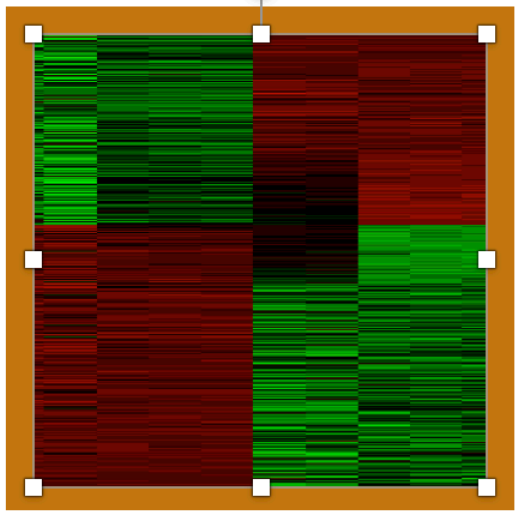
Whole Transcriptomic Analysis

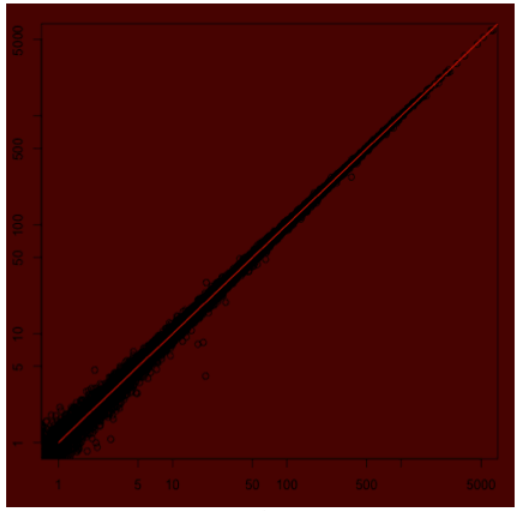
Manjula Thimma, AlaguRaj Veluchamy, Arun P Nagarajan, Robert Lehmann, Octavio

RNAseq for differential gene expression analysis





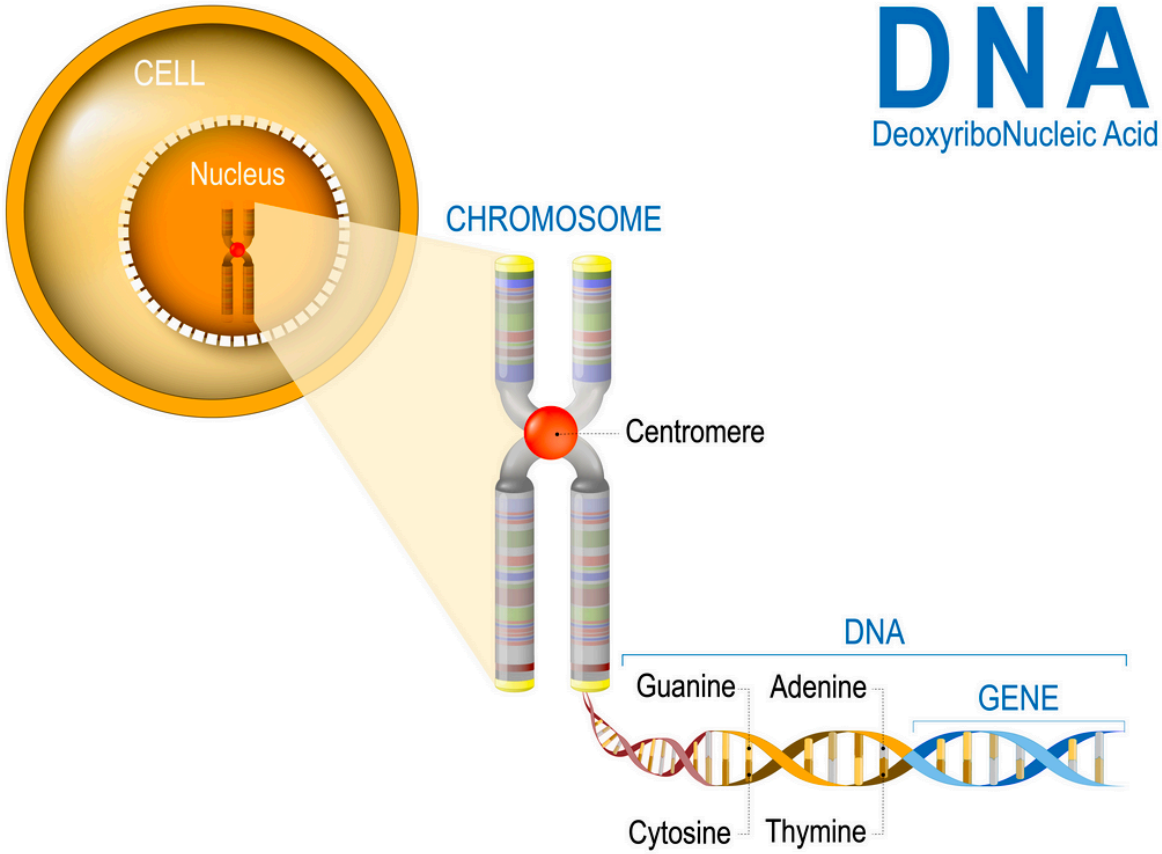




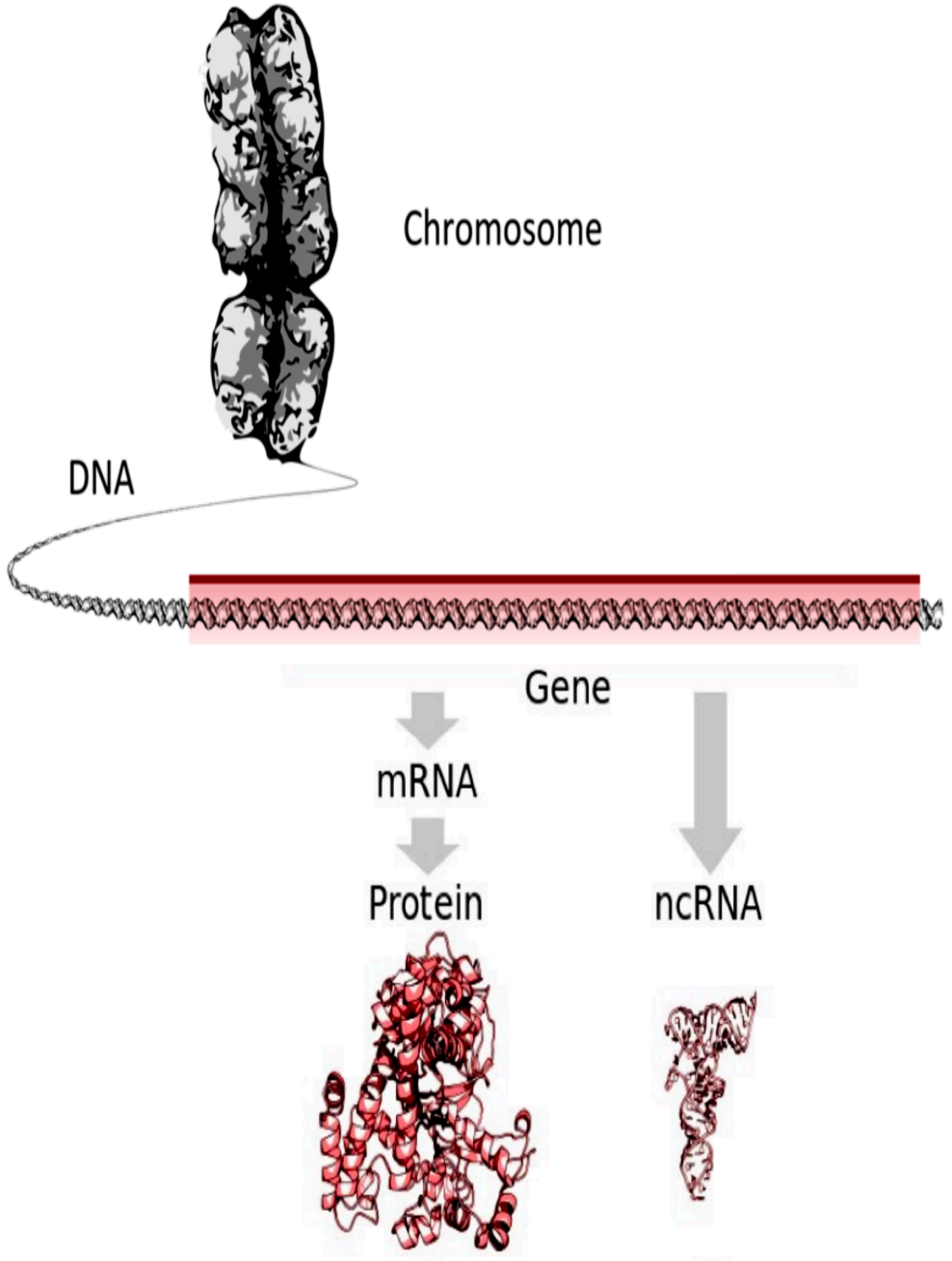
Manjula Thimma

Prof. Jesper Tegner

DNA

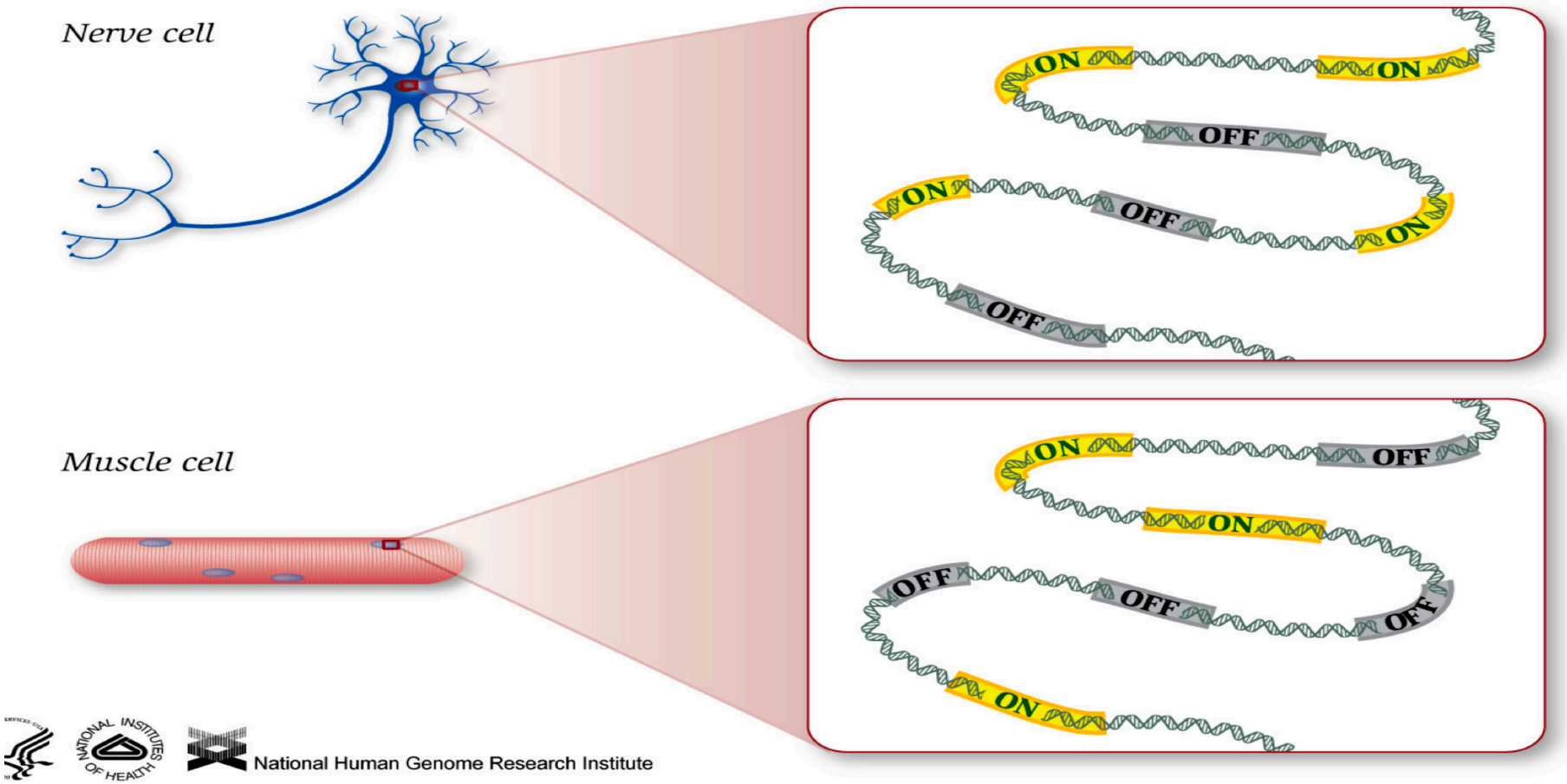


DNA
DeoxyriboNucleic Acid

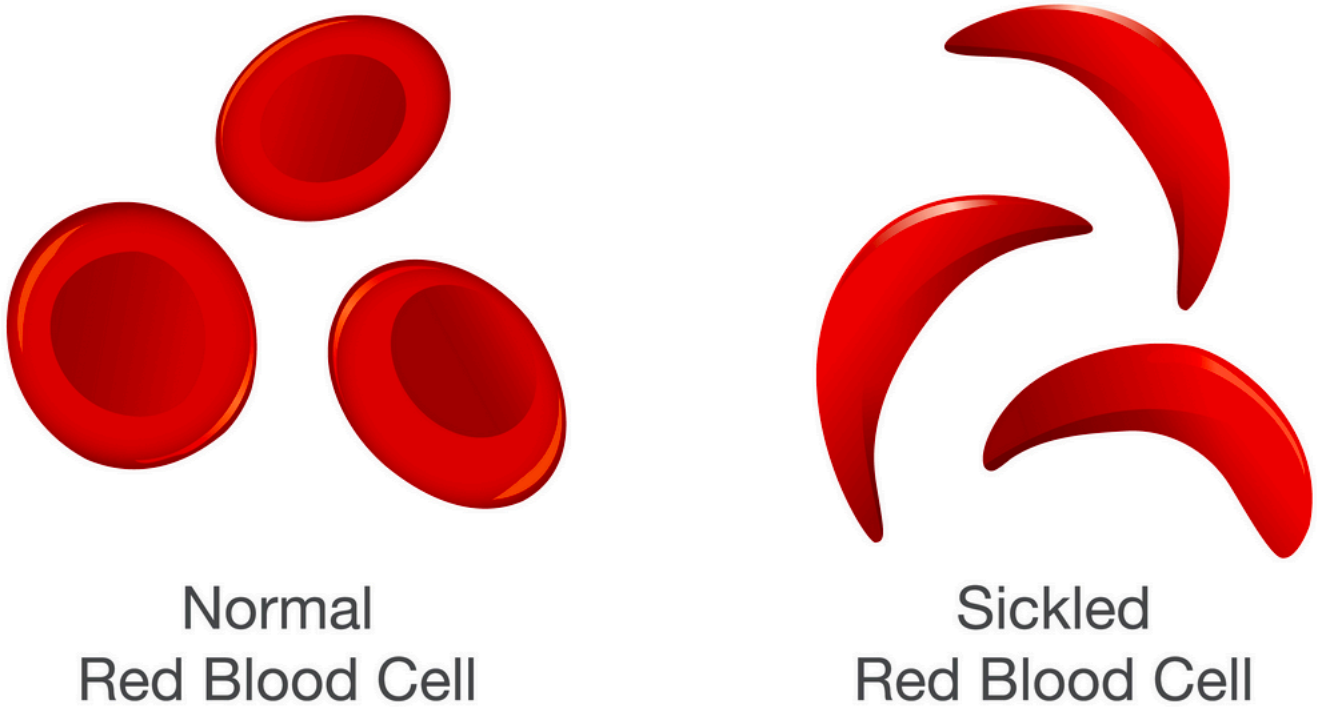


All organism contain instruction for their life in the genome; genome contains DNA ie nucleotides A. C. G, T.

Transcriptomes specific to cells



Disease specific transcriptomes



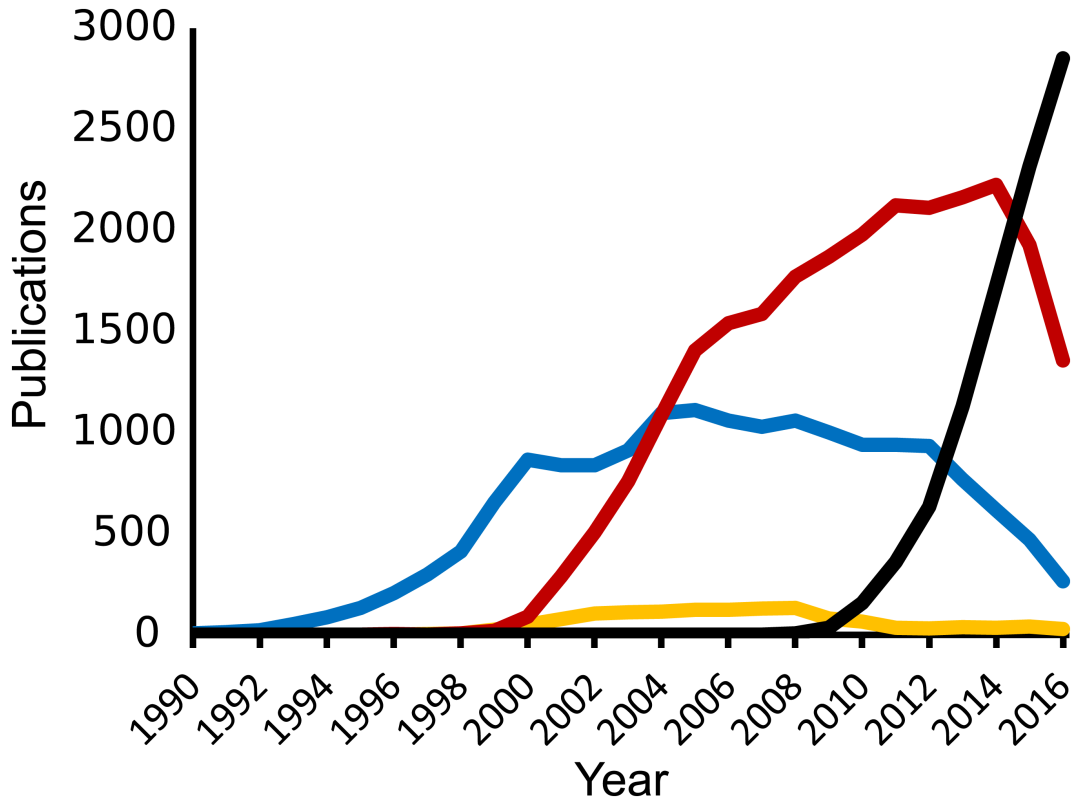
Disease can turn certain genes on or off, resulting in difference in quantity of RNA produced.

RNA-seq can help to observe whether there are significant difference in gene expression in different conditions.

RNA-Seq questions

- What genes are differentially expressed between sample groups?
- Are there any trends in gene expression over time or across conditions.
- Which groups of genes change similarly over time or across conditions.
- What processes or pathways are important for my condition of interest?

Transcriptomic methods used over time



Published papers since 1990, referring to

RNA sequencing (black)

RNA microarray (red)

expressed sequence tag (blue)

serial/cap analysis of gene expression (yellow)

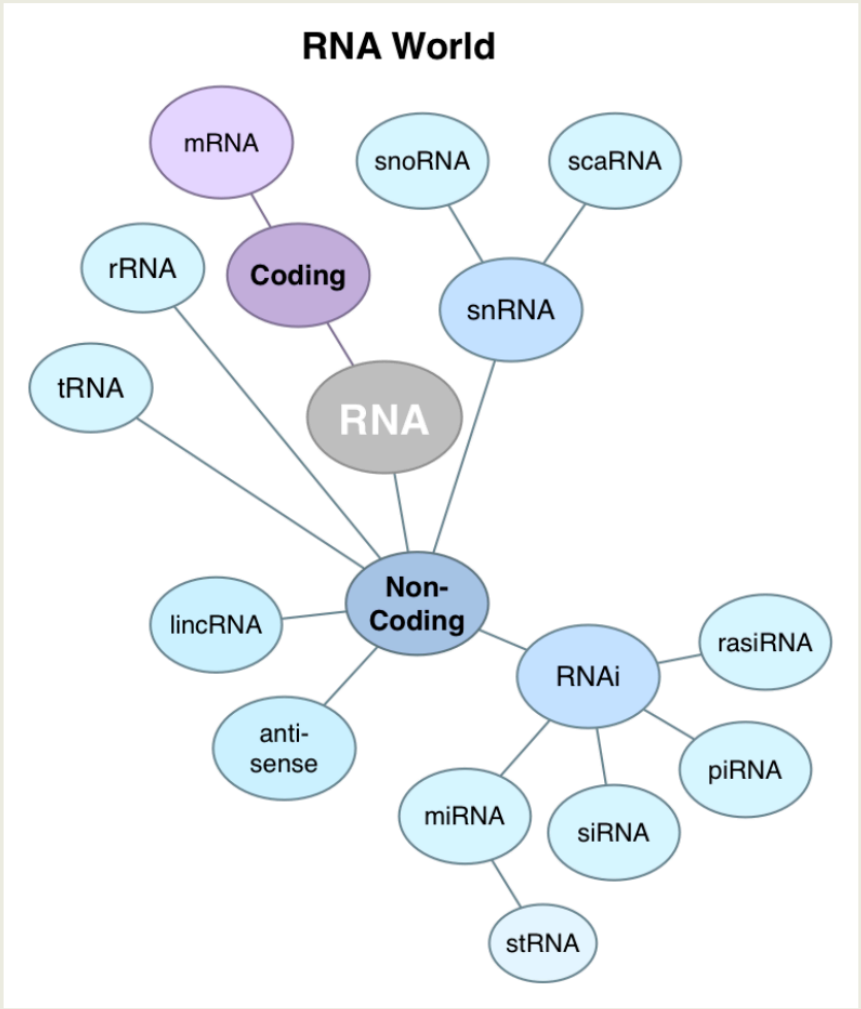
Rohan Lowie et al, **Transcriptomics technologies,** PLOS computational biology, 2017
http://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1005457

RNA-seq vs. microarray

- RNA-seq can be used to characterize **novel transcripts** and **splicing variants** as well as to profile the expression levels of **known transcripts** (but hybridization-based techniques are limited to **detect transcripts** corresponding to known genomic sequences)
- RNA-seq has **higher resolution** than whole genome tiling array analysis
  - In principle, mRNA can achieve single-base resolution, where the resolution of tiling array depends on the density of probes
- RNA-seq can apply the **same experimental protocol** to various purposes, whereas specialized arrays need to be designed in these cases
  - Detecting single nucleotide polymorphisms (needs SNP array otherwise)
  - Mapping exon junctions (needs junction array otherwise)
  - Detecting gene fusions (needs gene fusion array otherwise)

– Next-generation sequencing (NGS) technologies are now challenging microarrays as the tool of choice for genome analysis.

Not all types of RNA encode information



The bulk (~95%) of cellular RNA is rRNA and tRNA.

tp://finchtalk.blogspot.com/2009/05/small-rnas-get-smaller.html

Types of RNA-Seq

Types of RNA-Seq analysis

- Gene expression analysis
- Single cell RNA-Seq (scRNA-Seq)
- Small RNA-Seq (miRNA-Seq)
- Analysis of RNA-protein/RNA-RNA-interaction

Applications of RNA-Seq experiments

- Differential expression
- Gene fusion (arising due to translocation, deletion, chromosomal inversion)
- Alternative splicing
- Novel transcribed regions
- Allele-specific expression
- RNA editing
- Transcriptome for non-model organisms

Experimental planning is essential

RNA-Seq Workflow: RNA-Seq Experimental Design

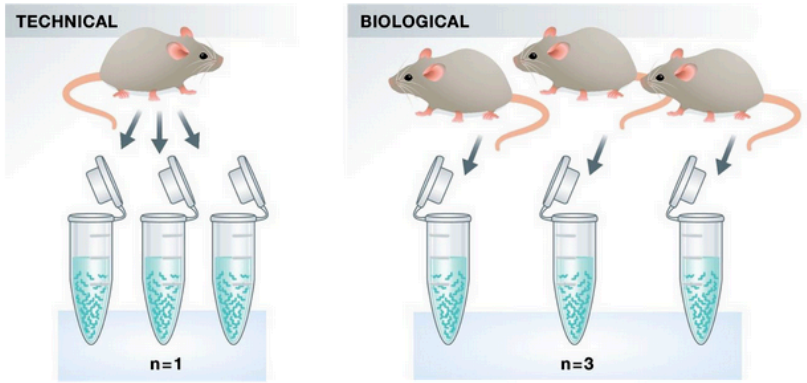
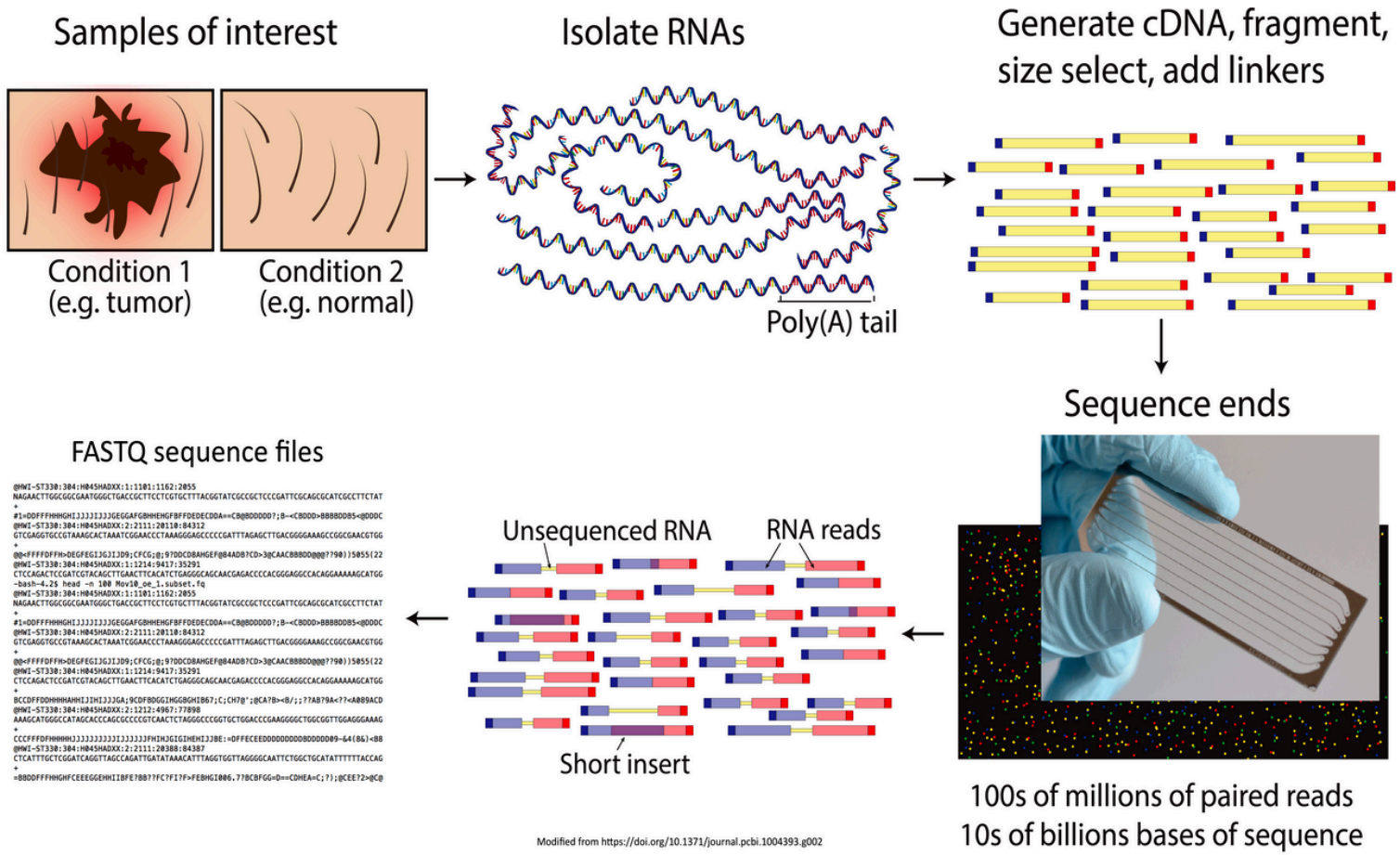


Image adapted from: Klaus B., EMBO J (2015) 34: 2727–2730

- **Technical replicates:** Generally low technical variation, so unnecessary.
- **Biological replicates:** Crucial to the success of RNA-Seq differential expression analyses. The more replicates the better, but at the very least have 3.
- **Batch effects:** Avoid as much as possible and note down all experimental variables.

- Try to avoid batch effect either by processing all samples in one batch or distribute all of your samples in different batches.
- Avoid confounding variations like using only male mice as control and female mice as treated/samples.

Biological sample/library preparation



Samples are harvested. RNAs isolated, DNA contamination removed, rRNAs removed, mature RNAs are selected by their polyA tails.
RNA to cDNA, fragmented, size selected, adapters ligated to get RNA-seq library to be sequenced.
Ends of fragments sequenced are READS.

Sequencing strategies

- Which library preparation protocol to use?
- How many replicates?
- What is the optimal library size (sequencing depth)?
- Paired end or single end?
- Which data analysis pipeline to use?

DESIGN OF EXPERIMENT

Design Of Experiment is as important as performing it !

- Performing RNASeq is not just about → crush cells → extract RNA →prepare library → sequence → Analysis
- Gene expression is highly sensitive to conditions setup
- Nature of experiment, variability involved in each step can introduce confounding effects
- What to expect from the data ? – detection level (low, medium, high expression levels)
- What is the minimum number of replicates required to achieve statistical power over data ?
- Does the budget allow the current configuration ?

Why is a good experimental design vital?

A typical RNA-seq experiment aims to find differentially expressed genes between two conditions (e.g. up and down-regulated genes in knock-out mice compared to wild-type mice)

RNASeq produces high dimensional data. i.e produces huge number of observations from small N of samples

Each measurement is comprised of a mix of biological signal and unwanted noise. Hence carefully design the experiment.

Ask the following questions before you start:

Why do you expect to find differentially expressed genes in the particular tissue?
What types of genes do you expect to find differentially expressed?
What are the sources of variability from your samples?
Where do you expect most of your variation to come from?

Variability: A measure of how much data is spread. A larger variance means it is harder to identify DEGs

Covariate: Factor- property of the sample which may have some influence on gene expression and should be represented in the RNA-seq model – categorical (sex, batch, condition), continuous (time points, age)

Confounding variable: Nuisance variable – e.g. All wild-type is sampled in morning, all knock-out sampled in evening. Here the time of sample collection is confounding factor

The amount of variance between your biological replicates will affect the outcome of your analysis. Ideally, you aim to have minimal variability between samples so you only measure the effect of the condition of interest.

Too much variability between samples can drown out the signal of truly differentially expressed genes.

Strategies to minimize variation between samples and to control confounding variables include:

- choosing organisms from the same litter,
- choosing organisms of the same sex if possible,
- using a constant sample collection time,
- having the same laboratory technician perform each library prep,
- randomizing samples to prevent a confounding batch effect if all samples can't be processed at one time.

Power analysis can help make a decision

- Here, we will not focus on the maths *per se* ! (*sigh*), but discuss about outcomes and interpretation of results

- Required package “PROPER” in R: PROspective Power Evaluation for RNASeq

Expected % genes that are DE

# of genes in org.of.interest

Data to estimate parameters -> can be your own data (*ex*. count table)

```
library(PROPER)
sim.opts.Gilad = RNAseq.SimOptions.2grp(ngenes = 29269, p.DE=0.10, lOD="gilad", lBaselineExpr="gilad")
simres = runSims(Nreps = c(3, 4, 5, 7, 10), sim.opts=sim.opts.Gilad, DEmethod="DESeq2", nsims=20)
powers = comparePower(simres, alpha.type="fdr", alpha.nominal=0.1, stratify.by="expr", delta=0.5)
summaryPower(powers)
plotAll(powers)
power.seqDepth(simres, powers)
```

Simulate for different number of replicates Nreps

Power detection you wish to achieve (i.e 0.5 fold, 1 fold, 2 fold..)

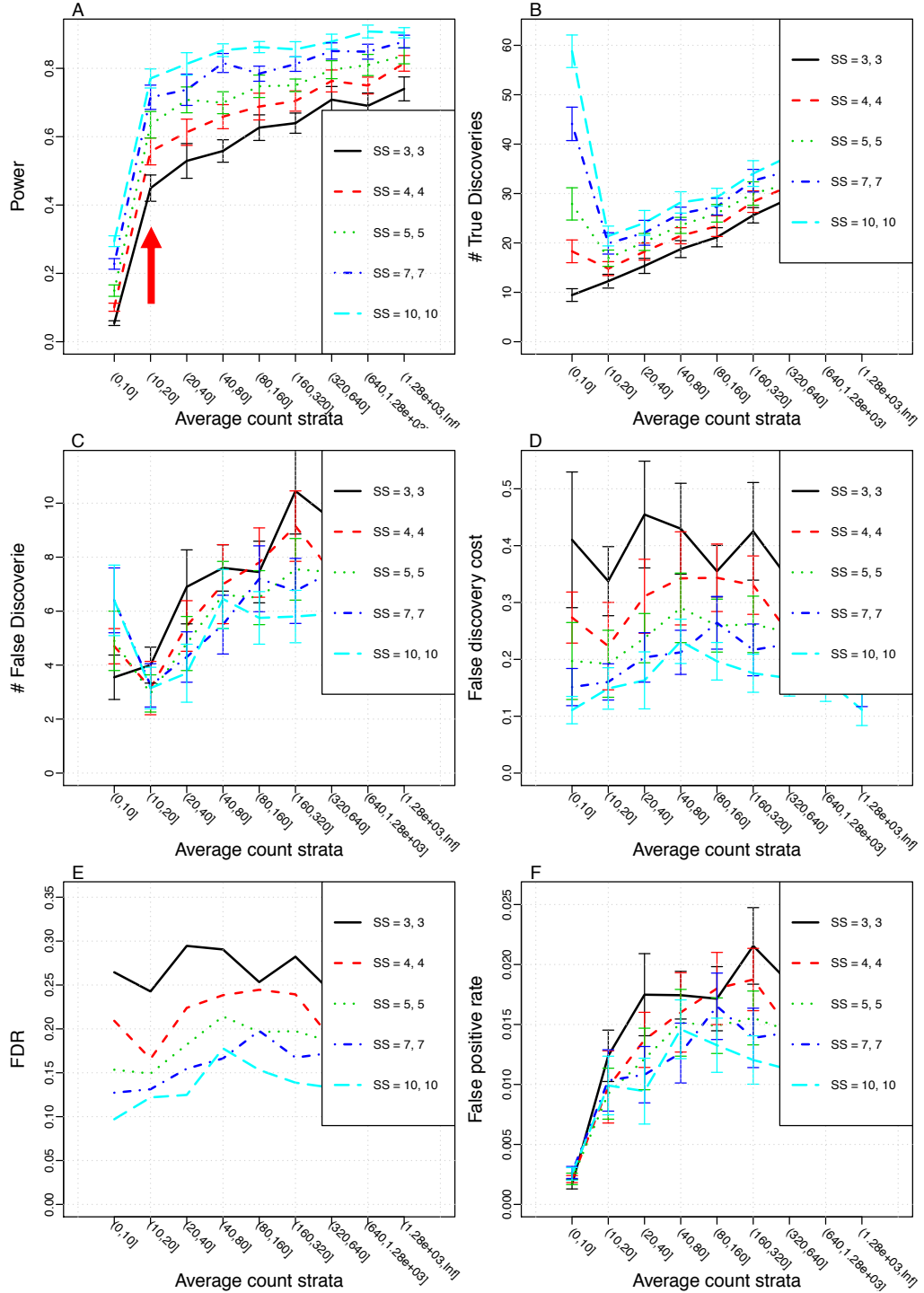
delta = 0.5 fold (lfc) ngenes = 29269, p.DE=0.10

SS2	Nominal FDR	Actual FDR	Marginal Power	Avg of TD	Avg of SD	FDC
3	0.1	0.25	0.42	190	66	0.34
4	0.1	0.21	0.48	220	60	0.27
5	0.1	0.18	0.52	250	54	0.22
7	0.1	0.15	0.58	280	53	0.19
10	0.1	0.13	0.63	310	47	0.15

power.seqDepth(simres, powers)

	SS=3,3	SS=4,4	SS=5,5	SS=7,7	SS=10,10
0.2	0.31	0.37	0.42	0.49	0.55
0.5	0.37	0.43	0.48	0.54	0.6
1	0.42	0.48	0.52	0.58	0.63
2	0.46	0.52	0.56	0.62	0.67
5	0.52	0.58	0.62	0.67	0.71
10	0.56	0.62	0.66	0.71	0.75

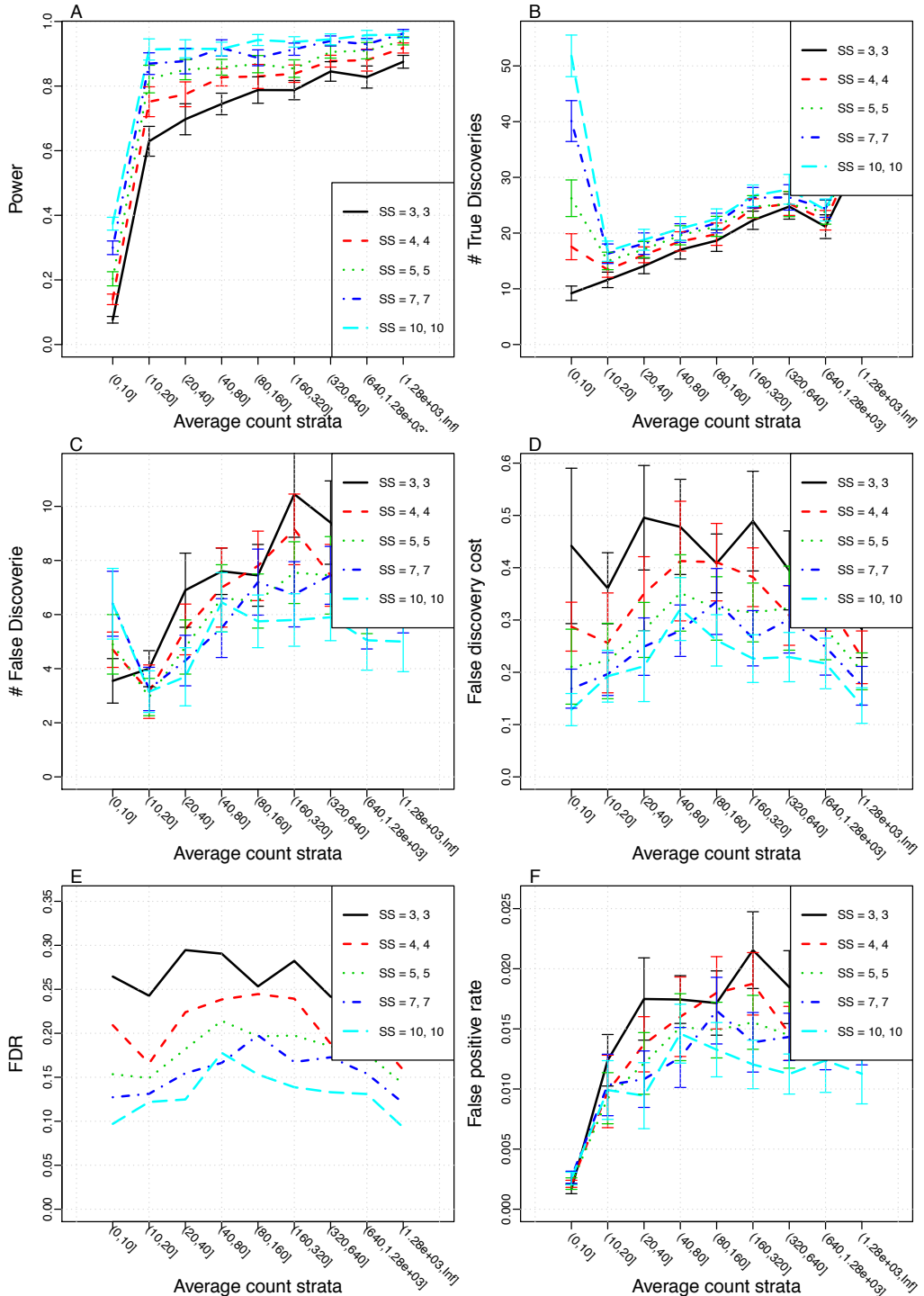
- Simulated with dataset accounting for large biological variation between 2 groups
- Maximum power achieved with 4 replicates was 0.48, by increasing sequencing depth – reach 0.52 or increasing sample size
- Sensitive for 0.5 fold (log-fold change)
- Power rapidly increases with 10-20 count per gene



delta = 1 fold (lfc)

SS2	Nominal FDR	Actual FDR	Marginal Power	Avg of TD	Avg of SD	FDC
3	0.1	0.25	0.53	170	66	0.38
4	0.1	0.21	0.58	190	60	0.31
5	0.1	0.18	0.63	210	54	0.26
7	0.1	0.15	0.68	230	53	0.23
10	0.1	0.13	0.71	250	47	0.19

	SS=3,3	SS=4,4	SS=5,5	SS=7,7	SS=10,10
0.2	0.41	0.47	0.52	0.59	0.64
0.5	0.48	0.54	0.58	0.64	0.68
1	0.53	0.58	0.63	0.68	0.71
2	0.58	0.63	0.67	0.72	0.75
5	0.64	0.69	0.72	0.77	0.79
10	0.69	0.74	0.77	0.8	0.82



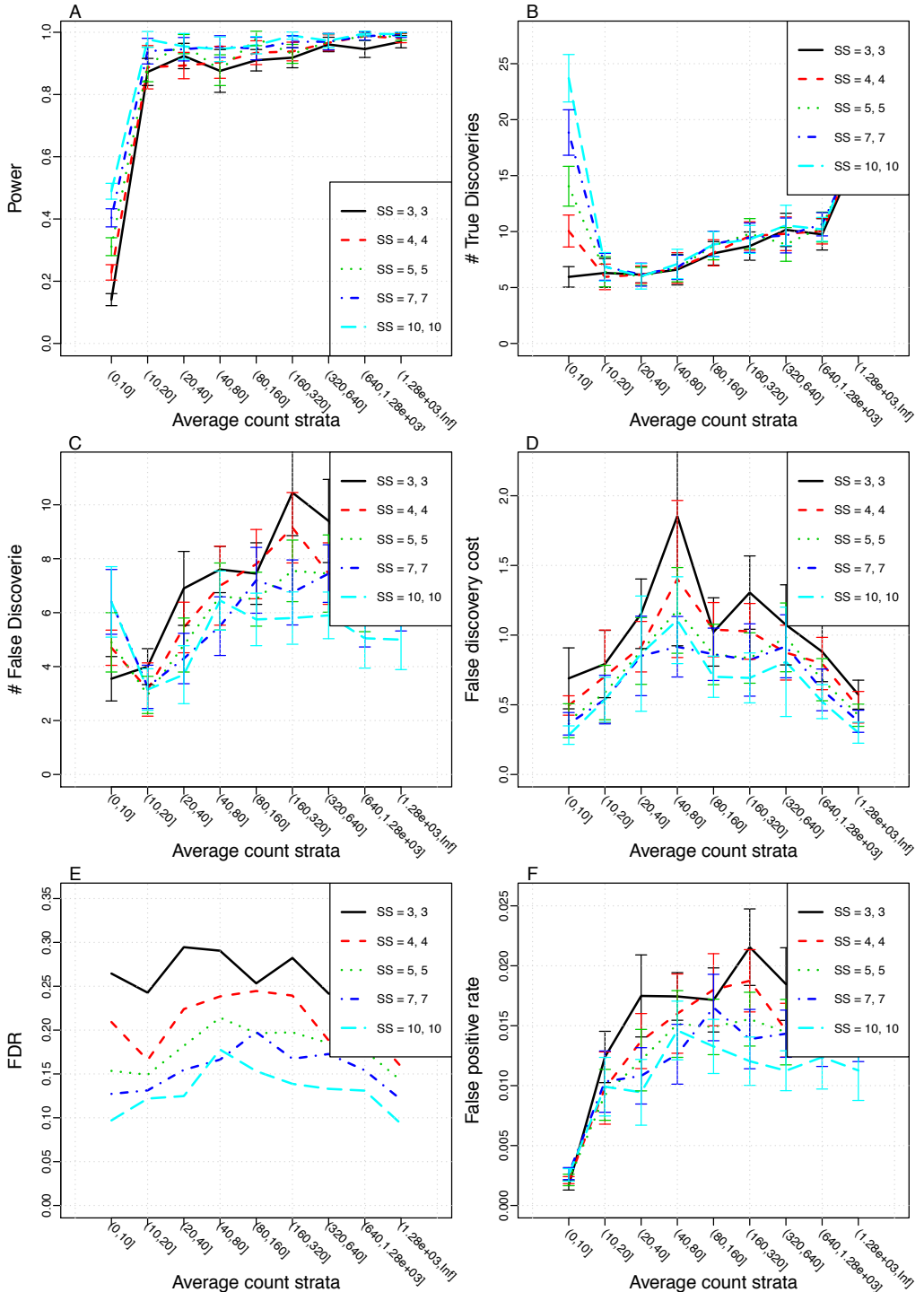
delta = 2 fold (lfc)

SS2	Nominal FDR	Actual FDR	Marginal Power	Avg of TD	Avg of SD	FDC
3	0.1	0.25	0.65	79	66	0.84
4	0.1	0.21	0.69	84	60	0.71
5	0.1	0.18	0.72	89	54	0.61
7	0.1	0.15	0.76	95	53	0.56
10	0.1	0.13	0.79	100	47	0.47

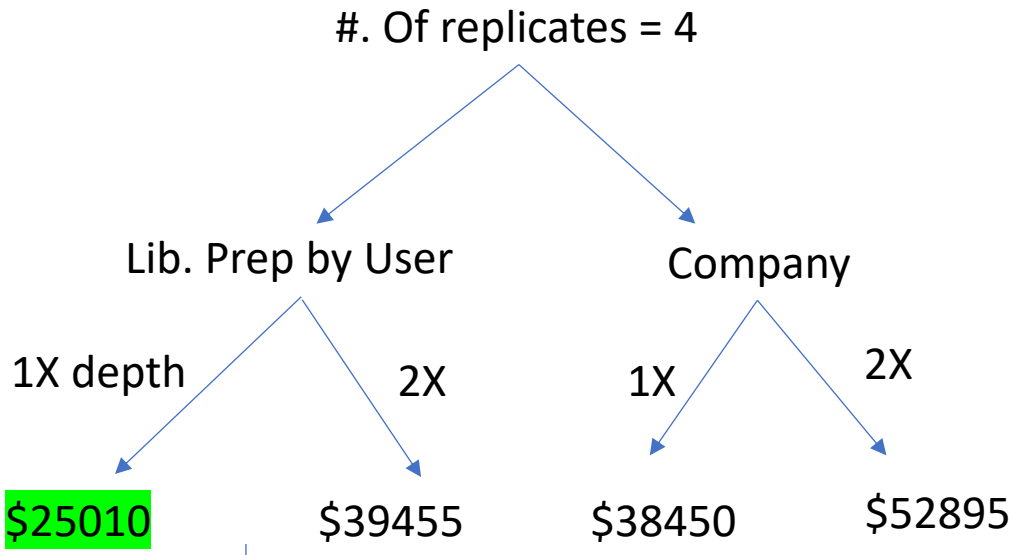
- Studies aim for power = 0.8
- Fishing for genes with 2 fold change can yield good power for as low as 3 replicates and 10-20 counts
- Housekeeping genes have basal level expression…to capture them, reduce the fold change

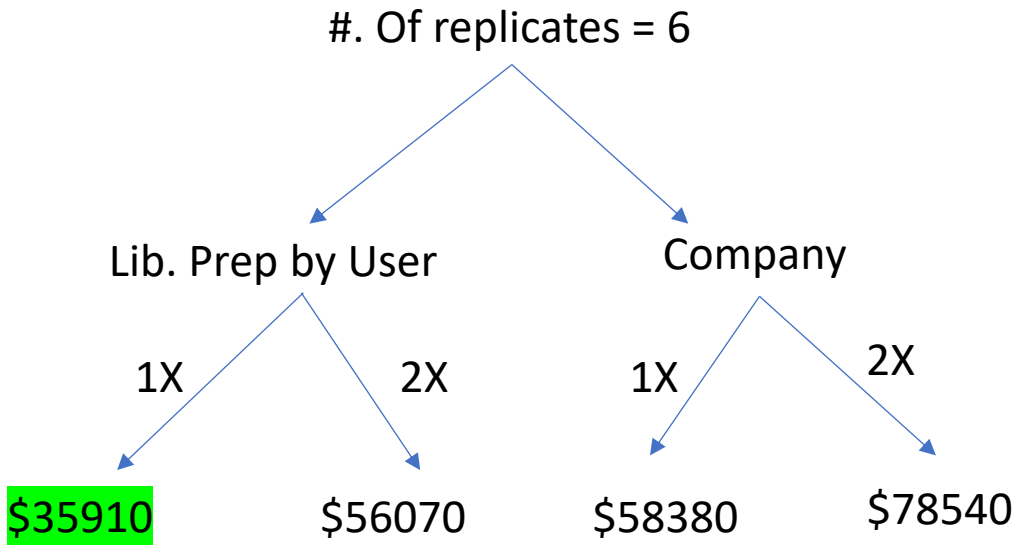
Combination:

- less replicates -> high fold change -> 10-20 count per gene -> normal sequencing depth -> more power
- More replicates-> normal sequencing depth -> 10-20 count per gene -> more power
- Less replicates -> less fold change -> high count per gene -> high sequencing depth -> more power



Decision table





Power achieved for delta = 0.5

	SS=3,3	SS=4,4	SS=5,5	SS=6,6	SS=7,7	SS=10,10
0.2	0.65	0.71	0.74	0.77	0.79	0.84
0.5	0.74	0.79	0.82	0.84	0.85	0.88
1	0.8	0.84	0.86	0.88	0.89	0.91
2	0.85	0.88	0.9	0.91	0.92	0.94
5	0.9	0.92	0.94	0.94	0.95	0.96
10	0.93	0.95	0.96	0.96	0.97	0.98

Power achieved for delta = 1

	SS=3,3	SS=4,4	SS=5,5	SS=6,6	SS=7,7	SS=10,10
0.2	0.76	0.80	0.83	0.85	0.87	0.90
0.5	0.83	0.86	0.88	0.90	0.91	0.93
1	0.88	0.90	0.91	0.93	0.94	0.95
2	0.92	0.93	0.94	0.95	0.96	0.97
5	0.95	0.96	0.97	0.97	0.98	0.98
10	0.97	0.98	0.98	0.98	0.99	0.99

Power achieved for delta = 2

	SS=3,3	SS=4,4	SS=5,5	SS=6,6	SS=7,7	SS=10,10
0.2	0.87	0.90	0.91	0.93	0.94	0.96
0.5	0.91	0.93	0.94	0.95	0.96	0.97
1	0.94	0.95	0.96	0.97	0.97	0.98
2	0.96	0.97	0.97	0.98	0.98	0.99
5	0.98	0.98	0.99	0.99	0.99	0.99
10	0.99	0.99	0.99	0.99	0.99	1.00

Power achieved even with 4 biological replicates (out of 6) of actual 25deg & 32deg data

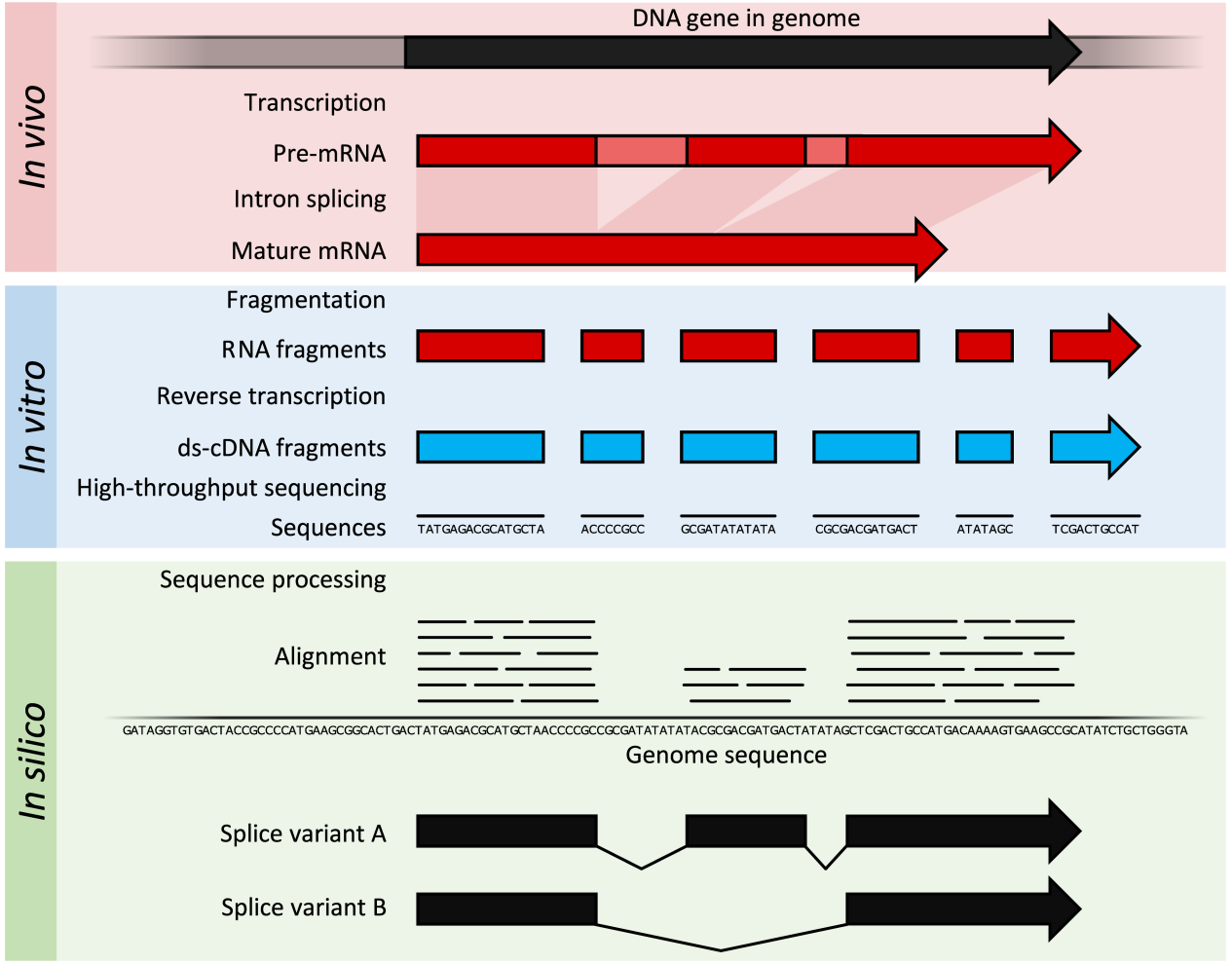
Further Reading:

Yuwen Liu, Jie Zhou, Kevin P. White, RNA-seq differential expression studies: more sequence or more replication?, *Bioinformatics*, Volume 30, Issue 3, 1 February 2014, Pages 301–304, https://doi.org/10.1093/bioinformatics/btt688

End of Experiment Design

Heading to Sequencing

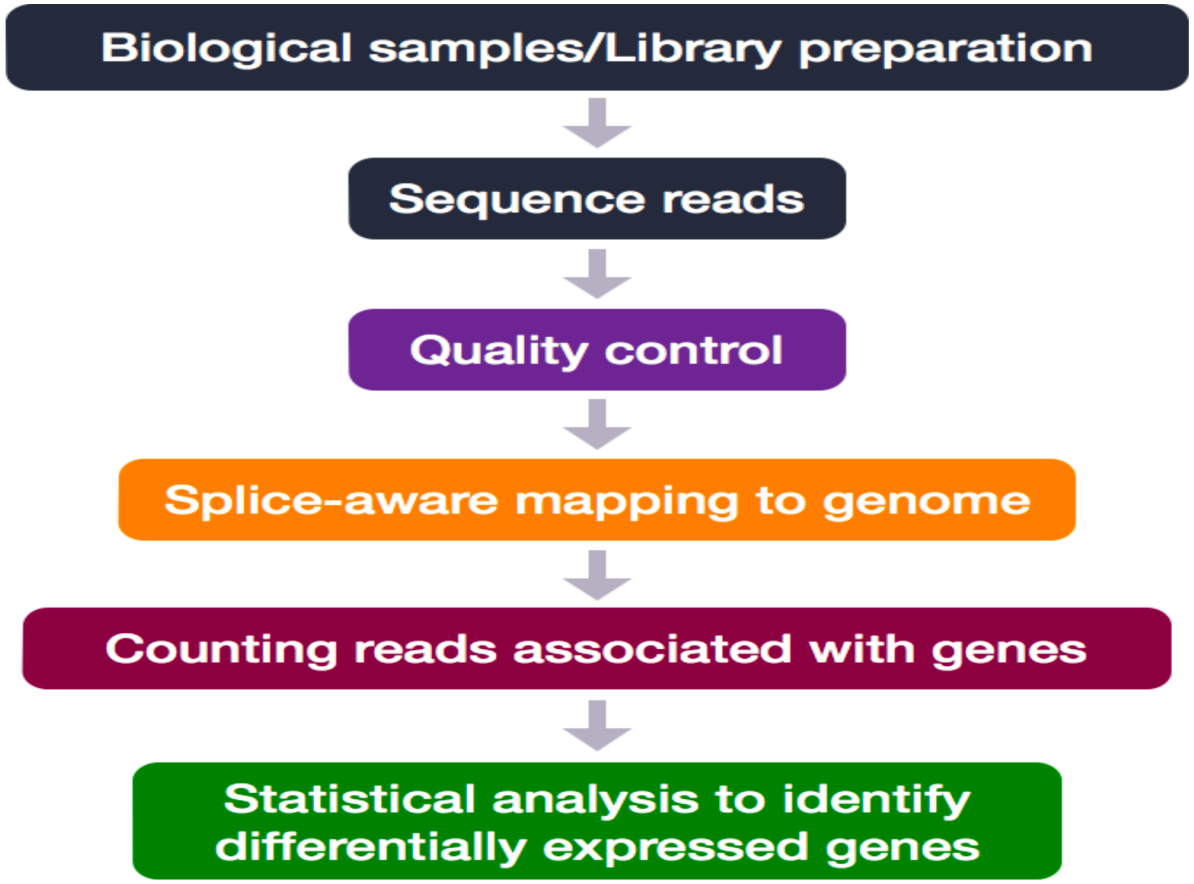
Bulk RNA-Seq Process



Within the organisms, genes are transcribed and spliced (in eukaryotes) to produce mature mRNA transcripts (red). The mRNA is extracted from the organism, fragmented and copied into stable double-stranded–cDNA (ds-cDNA; blue). The ds-cDNA is sequenced using high-throughput, short-read sequencing methods. These sequences can then be aligned to a reference genome sequence to reconstruct which genome regions were being transcribed. These data can be used to annotate where expressed genes are, their relative expression levels, and any alternative splice variants.

Rohan Lowie et al, **Transcriptomics technologies, PLOS computational biology, 2017**

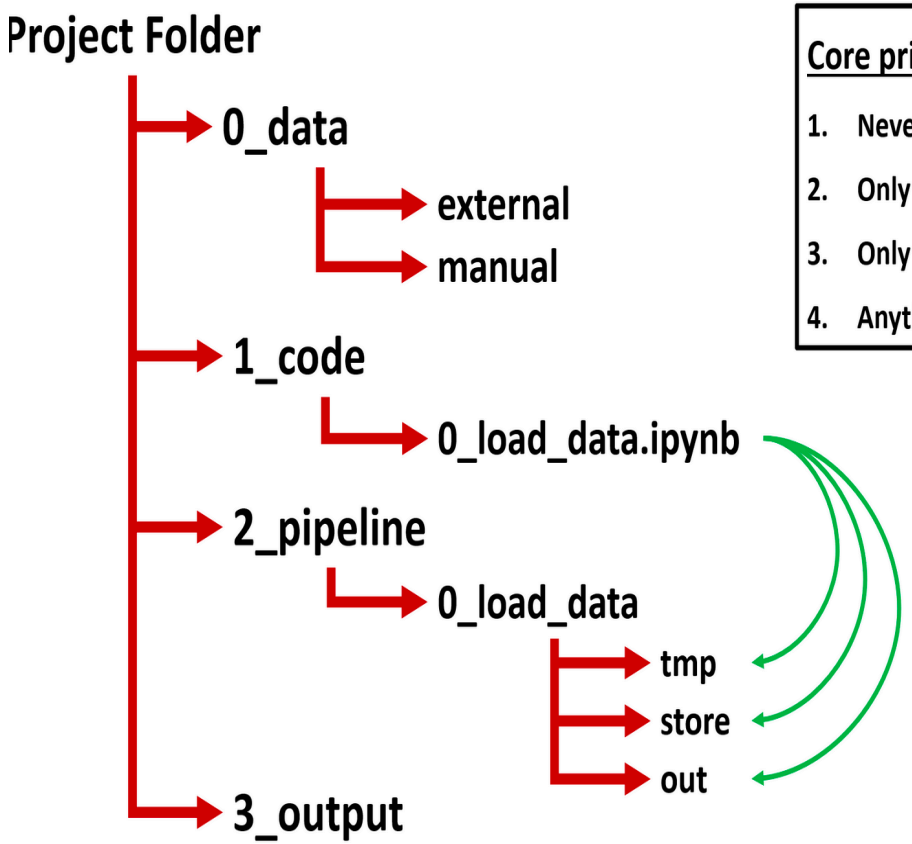
RNA-Seq Analysis Workflow



Pipeline for RNA-Seq data analysis

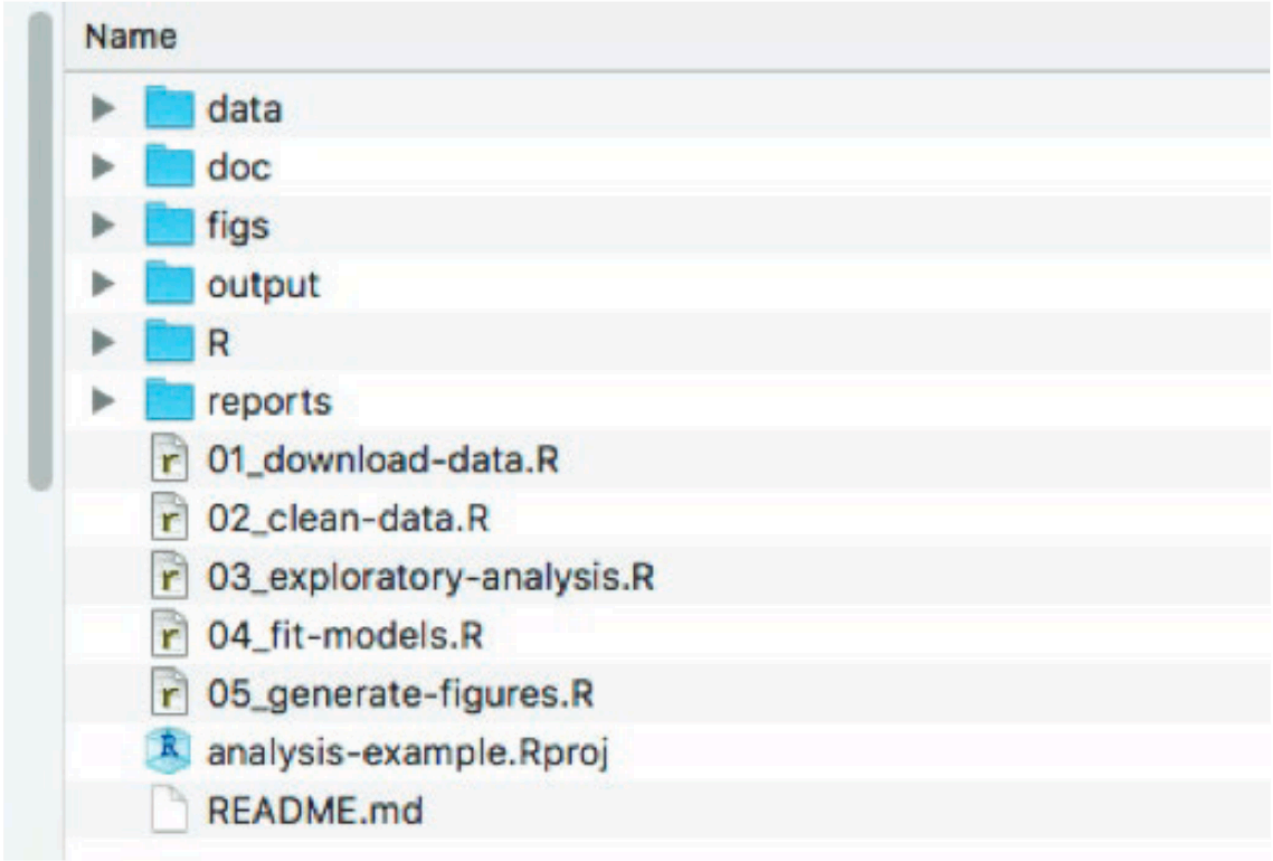
Input data → quality filtering → Mapping → Counting → DEG → Funcational Analysis → Alternative splicing → data validation → publication (Bingo!!!)

Folder structure might help project organisation



Core principles:

1. **Never modify** 0_data
2. **Only save to** pipeline **folder**
3. **Only load from** 0_data **or** out
4. **Anything in** tmp **can be deleted**



How we organized our working

```
1_SMOC_RAWDATA
2_FASTQC
3_MULTIQC
4_TRIMMING
5_TRIMMEDQC
6_TRIMMEDMULTIQC
7_MAPPING
8_COUNTING
9_DifferentialExpression
CommandUsed.txt
CountTablesMerging.R
Data_cleaning.log
RunTophat.py
samplenames.txt
scripts
temp
Testing
WT_NORMAL1_509.fq.gz
WT_NORMAL1_509_nonempty_count_table.txt
WT_NORMAL1_509_tophathout
WT_NORMAL1_509_Trimmed.fastq.gz
WT_NORMAL1_Trimm_Summary.txt
```

Modules in this course

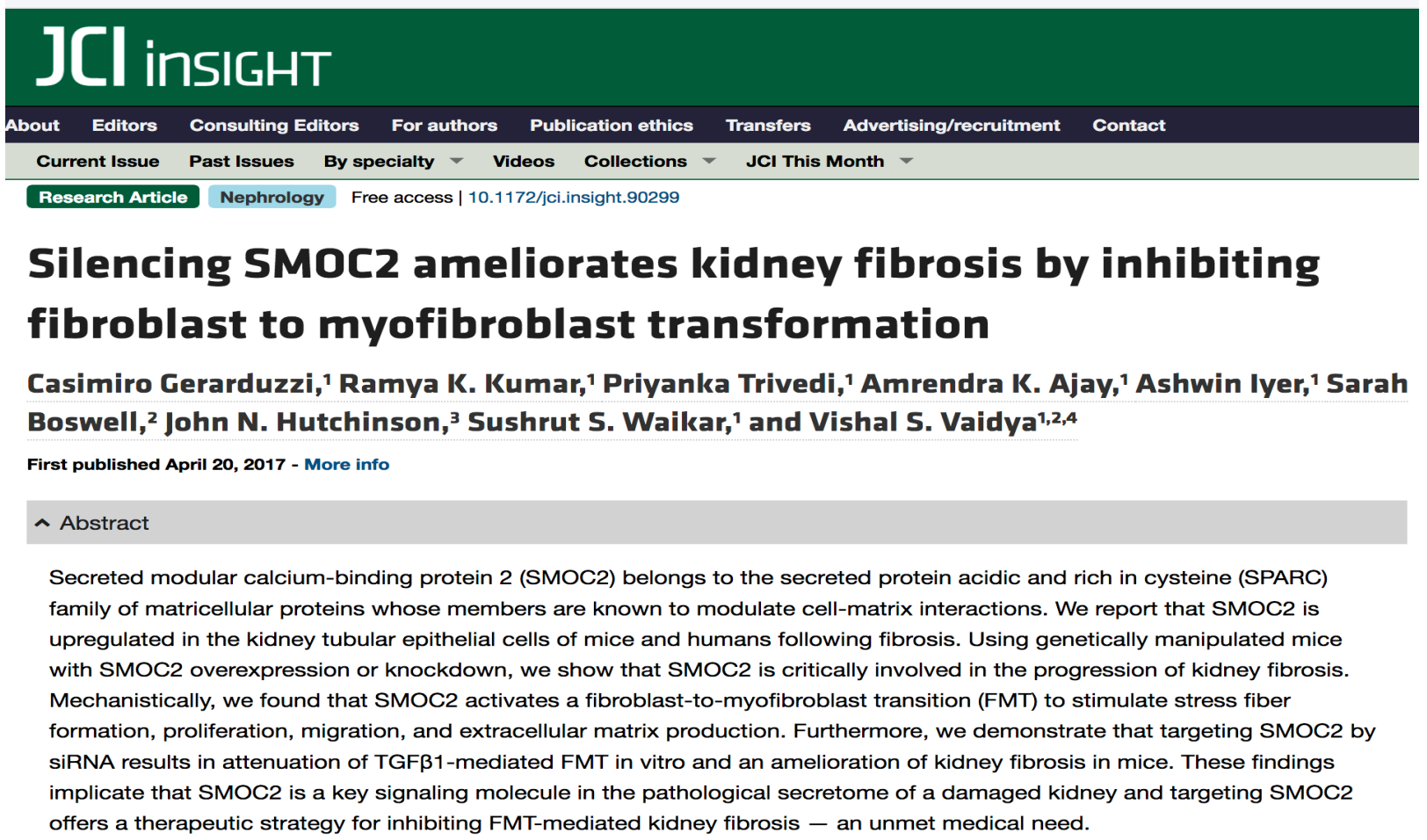
- Data download
- Quality assessment
- Data filtering
- Post filtering qc
- Mapping
- Mapped qc
- Count table
- DEG / Visualisation
- Functional Analysis
- Alternative splicing study

Module 1 : Input Data

Data Downloading and Cleaning

The data source can be from your own project or collaboration or even from the open databases like SRA/ENA/GEO etc.

Here we have used publicly available data from an article, as shown below.



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Silencing SMOC2 ameliorates kidney fibrosis by inhibiting fibroblast to myofibroblast transformation

Casimiro Gerarduzzi,[1] Ramya K. Kumar,[1] Priyanka Trivedi,[1] Amrendra K. Ajay,[1] Ashwin Iyer,[1] Sarah Boswell,[2] John N. Hutchinson,[3] Sushrut S. Waikar,[1] and Vishal S. Vaidya[1,2,4]

First published April 20, 2017 - More info

Abstract

Secreted modular calcium-binding protein 2 (SMOC2) belongs to the secreted protein acidic and rich in cysteine (SPARC) family of matricellular proteins whose members are known to modulate cell-matrix interactions. We report that SMOC2 is upregulated in the kidney tubular epithelial cells of mice and humans following fibrosis. Using genetically manipulated mice with SMOC2 overexpression or knockdown, we show that SMOC2 is critically involved in the progression of kidney fibrosis. Mechanistically, we found that SMOC2 activates a fibroblast-to-myofibroblast transition (FMT) to stimulate stress fiber formation, proliferation, migration, and extracellular matrix production. Furthermore, we demonstrate that targeting SMOC2 by siRNA results in attenuation of TGFβ1-mediated FMT in vitro and an amelioration of kidney fibrosis in mice. These findings implicate that SMOC2 is a key signaling molecule in the pathological secretome of a damaged kidney and targeting SMOC2 offers a therapeutic strategy for inhibiting FMT-mediated kidney fibrosis — an unmet medical need.

Seek for the accession

fluorometer, Agilent TapeStation 2200, and qPCR using the Kapa Biosystems library quantification kit according to manufacturer's protocols. Uniquely indexed libraries were pooled in equimolar ratios and sequenced on a single Illumina NextSeq500 run with single-end 75 bp reads by the Dana-Farber Cancer Institute Molecular Biology Core Facilities (Boston, MA). STAR aligner was used to map sequenced reads to build 9 of the mus musculus genome (mm9) genome assembly and to quantify gene level expression. The full dataset is available in the NCBI GEO database with the accession number GSE85209.

Usually publications are accompanied with data submission accession details. They can be either GEO or SRP or other database.

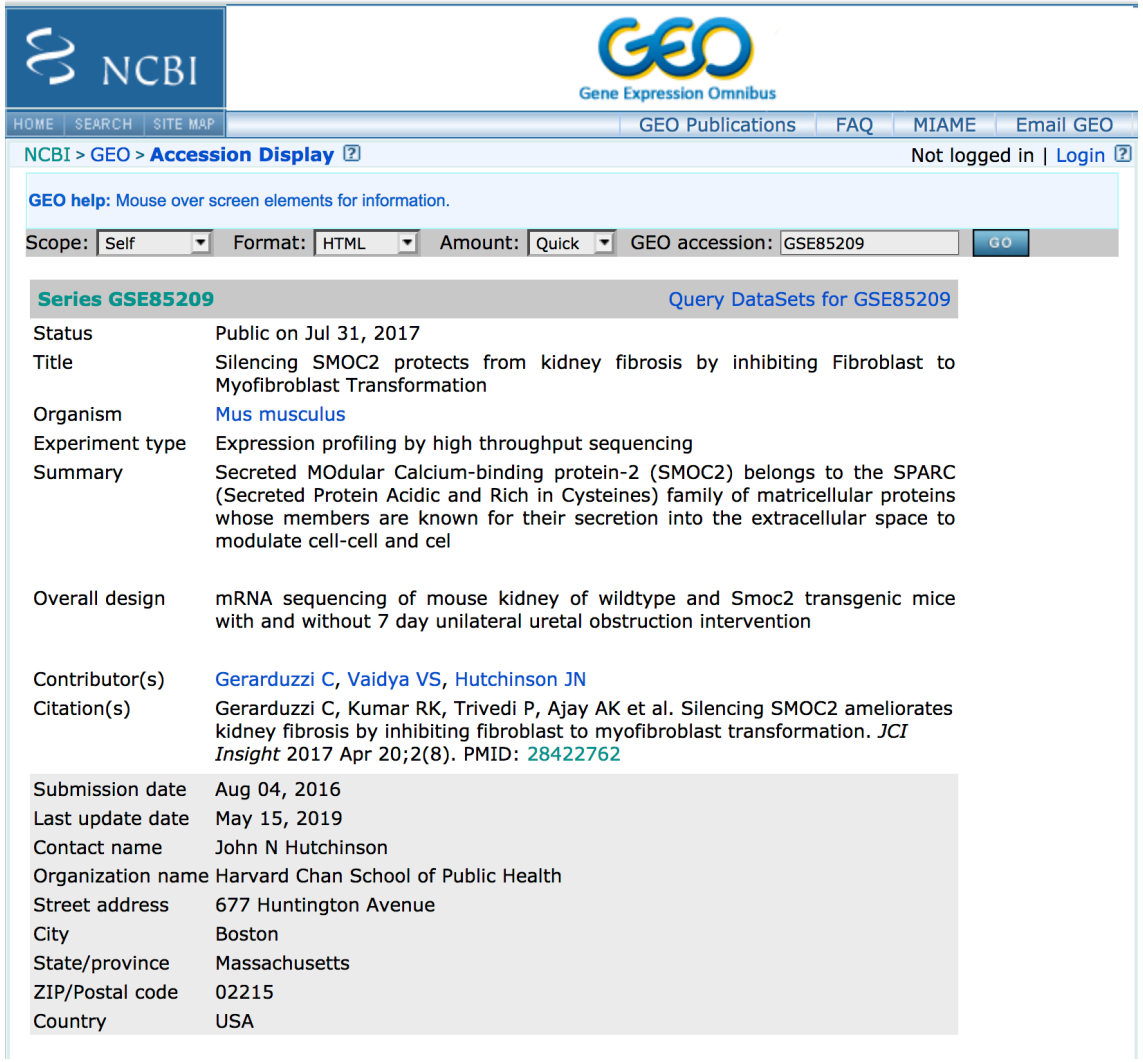
Skim for those accession numbers and reach their source.

Here we could see the data accession is GSE85209

Data for analysis

1. Can come from your own experiment
2. From your collaborators' lab
3. From public domain
4. Ways to get them could be either manual down or automatic download (using scripts/web interface scratching)
5. Next few slides will take you through manual download.

Data Acquisition



NCBI

Gene Expression Omnibus

HOME | SEARCH | SITE MAP | GEO Publications | FAQ | MIAME | Email GEO

NCBI > GEO > **Accession Display** Not logged in | Login

GEO help: Mouse over screen elements for information.

Scope: Self Format: HTML Amount: Quick GEO accession: GSE85209 GO

Series GSE85209 Query DataSets for GSE85209

Status	Public on Jul 31, 2017
Title	Silencing SMOC2 protects from kidney fibrosis by inhibiting Fibroblast to Myofibroblast Transformation
Organism	Mus musculus
Experiment type	Expression profiling by high throughput sequencing
Summary	Secreted MOdular Calcium-binding protein-2 (SMOC2) belongs to the SPARC (Secreted Protein Acidic and Rich in Cysteines) family of matricellular proteins whose members are known for their secretion into the extracellular space to modulate cell-cell and cel
Overall design	mRNA sequencing of mouse kidney of wildtype and Smoc2 transgenic mice with and without 7 day unilateral uretal obstruction intervention
Contributor(s)	Gerarduzzi C, Vaidya VS, Hutchinson JN
Citation(s)	Gerarduzzi C, Kumar RK, Trivedi P, Ajay AK et al. Silencing SMOC2 ameliorates kidney fibrosis by inhibiting fibroblast to myofibroblast transformation. *JCI Insight* 2017 Apr 20;2(8). PMID: 28422762
Submission date	Aug 04, 2016
Last update date	May 15, 2019
Contact name	John N Hutchinson
Organization name	Harvard Chan School of Public Health
Street address	677 Huntington Avenue
City	Boston
State/province	Massachusetts
ZIP/Postal code	02215
Country	USA

- Make sure have reached the right accession number.
- Is it linked to the paper you were interested by confirming with the title, authors, journal and year.
- Get the list of samples used in the experiment.

Biosamples of this project

Platforms (1)	GPL19057 Illumina NextSeq 500 (Mus musculus)
Samples (14)	GSM2260466 SMOC2_normal_1
Less...	GSM2260467 SMOC2_normal_3
	GSM2260468 SMOC2_normal_4
	GSM2260469 SMOC2_UUO_1
	GSM2260470 SMOC2_UUO_2
	GSM2260471 SMOC2_UUO_3
	GSM2260472 SMOC2_UUO_4
	GSM2260473 WT_normal_1
	GSM2260474 WT_normal_2
	GSM2260475 WT_normal_3
	GSM2260476 WT_UUO_1
	GSM2260477 WT_UUO_2
	GSM2260478 WT_UUO_3
	GSM2260479 WT_UUO_4

Relations

BioProject	PRJNA336552
SRA	SRP080947

Download family	Format
SOFT formatted family file(s)	SOFT
MINiML formatted family file(s)	MINiML
Series Matrix File(s)	TXT

Supplementary file	Size	Download	File type/resource
GSE85209_RAW.tar	2.9 Mb	(http)(custom)	TAR (of TXT)

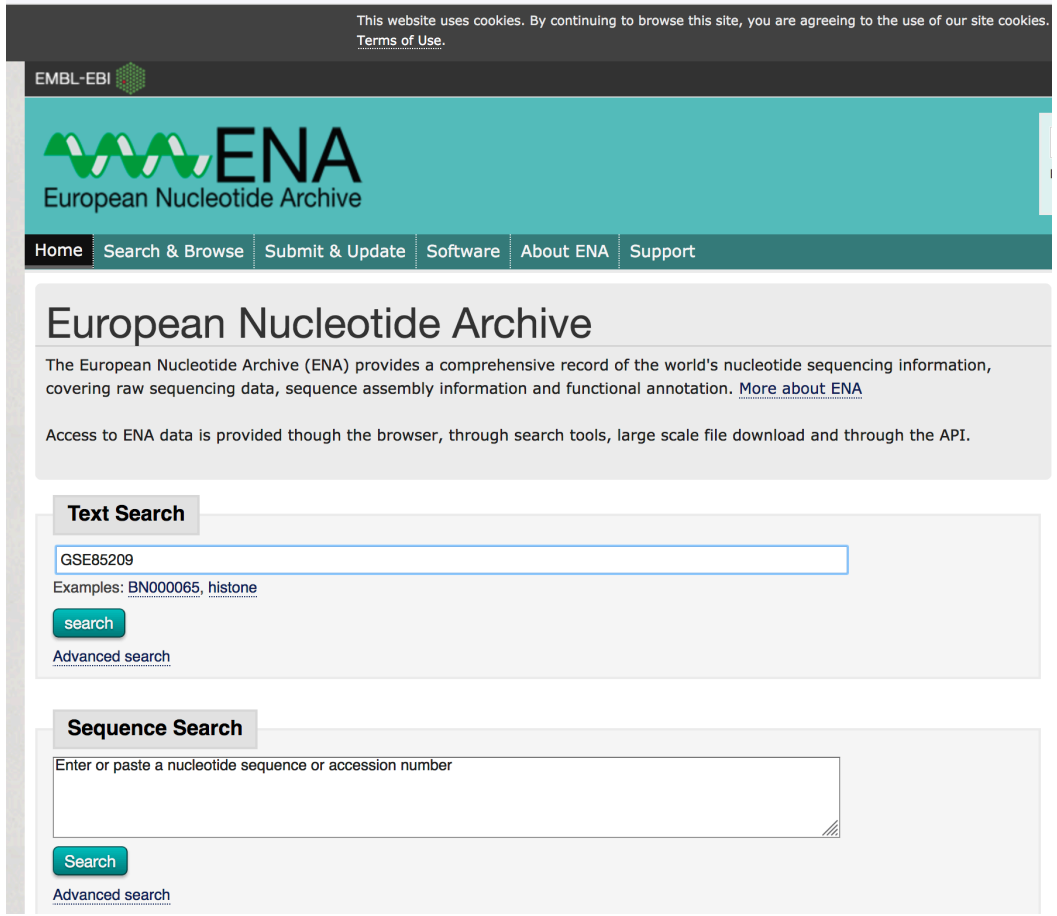
SRA Run Selector

The link for supplementary file has raw counts from each of the 14 samples.

Those who are eager to start from raw count, can use this file and their analysis.

But we wanted you to be more self dependent, hence teaching you how to start from fastq files!

Getting data from ENA



Click on the SRA448488 link to get the data

Downloading fastq files

Download: 1 - 14 of 14 results in TEXT

Select columns

Showing results 1 - 10 of 14 results

Study accession	Sample accession	Secondary sample accession	Experiment accession	Run accession	Tax ID	Scientific name	Instrument model	Library layout	FASTQ files (FTP)	FASTQ files (Galaxy)	Submitted files (FTP)	Submitted files (Galaxy)	NCBI SRA file (FTP)	NCBI SRA file (Galaxy)	CRAM Index files (FTP)	CRAM Index files (Galaxy)
PRJNA336552	SAMN05513160	SRS1601477	SRX2000833	SRR4000502	10090	Mus musculus	NextSeq 500	SINGLE	File 1	File 1			File 1	File 1		
PRJNA336552	SAMN05513159	SRS1601476	SRX2000834	SRR4000503	10090	Mus musculus	NextSeq 500	SINGLE	File 1	File 1			File 1	File 1		
PRJNA336552	SAMN05513147	SRS1601480	SRX2000835	SRR4000504	10090	Mus musculus	NextSeq 500	SINGLE	File 1	File 1			File 1	File 1		
PRJNA336552	SAMN05513149	SRS1601479	SRX2000836	SRR4000505	10090	Mus musculus	NextSeq 500	SINGLE	File 1	File 1			File 1	File 1		
PRJNA336552	SAMN05513148	SRS1601478	SRX2000837	SRR4000506	10090	Mus musculus	NextSeq 500	SINGLE	File 1	File 1			File 1	File 1		
PRJNA336552	SAMN05513158	SRS1601482	SRX2000838	SRR4000507	10090	Mus musculus	NextSeq 500	SINGLE	File 1	File 1			File 1	File 1		
PRJNA336552	SAMN05513157	SRS1601481	SRX2000839	SRR4000508	10090	Mus musculus	NextSeq 500	SINGLE	File 1	File 1			File 1	File 1		
PRJNA336552	SAMN05513156	SRS1601483	SRX2000840	SRR4000509	10090	Mus musculus	NextSeq 500	SINGLE	File 1	File 1			File 1	File 1		
PRJNA336552	SAMN05513155	SRS1601484	SRX2000841	SRR4000510	10090	Mus musculus	NextSeq 500	SINGLE	File 1	File 1			File 1	File 1		
PRJNA336552	SAMN05513154	SRS1601485	SRX2000842	SRR4000511	10090	Mus musculus	NextSeq 500	SINGLE	File 1	File 1			File 1	File 1		

Hands on

- create a folder to download data
- download the data
- Go to ENA, enter the GSE id, download fastq file
- copy the link ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR400/002/SRR4000502/SRR4000502.fastq.gz to your folder.
- wget <the link> .

```
-rw-r--r--  1 thimmamp g-thimmamp 1364481388 Jul 11 09:40 SMOC2_UUO2_506.fastq.gz
-rw-r--r--  1 thimmamp g-thimmamp 1320058249 Jul 11 09:51 WT_UUO4_515.fastq.gz
-rw-r--r--  1 thimmamp g-thimmamp 1706110238 Jul 11 10:01 WT_UUO3_514.fastq.gz
-rw-r--r--  1 thimmamp g-thimmamp 1715144354 Jul 11 10:12 SMOC2_UUO1_505.fastq.gz
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-rw-r--r--  1 thimmamp g-thimmamp 1538261110 Jul 11 10:30 SMOC2_UUO3_507.fastq.gz
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-rw-r--r--  1 thimmamp g-thimmamp 1414014416 Jul 11 10:47 SMOC2_NORMAL3_503.fastq.gz
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-rw-r--r--  1 thimmamp g-thimmamp 1352399657 Jul 11 11:04 SMOC2_UUO4_508.fastq.gz
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-rw-r--r--  1 thimmamp g-thimmamp 1421404436 Jul 11 11:23 WT_NORMAL2_510.fastq.gz
-rw-r--r--  1 thimmamp g-thimmamp 1665959039 Jul 11 11:33 WT_NORMAL1_509.fastq.gz
drwxr-xr-x  2 thimmamp g-thimmamp       4096 Jul 11 11:33 .
-rw-r--r--  1 thimmamp g-thimmamp 1879360748 Jul 11 11:47 WT_NORMAL3_511.fastq.gz
drwxr-xr-x 14 thimmamp g-thimmamp       4096 Aug  3 06:49 ..
```

End of data download

- Do the same for all the files
- DATA DOWNLOAD is done!
- Next Module is about data quality

Module 2 : Quality control of the data

- We need FastQC installed in our computers/servers.
- https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
- It is both command line and GUI tool.
- Download and install
- Open our data file using FastQC.
- Let us understand more on the quality and to clean the bad data in the following slides

Data Quality and Cleaning

Factors influencing sequencing outcomes

- Human-independent factors
    - Ability to measure fluorescence signal intensities accurately
    - Loss of efficacy of reagents over time – duration of the sequencing run
- Human Errors
    - Excessive fragmentation of the input DNA
    - Contaminants

Factors influencing sequencing outcomes

- Human-independent factors
    - Ability to measure fluorescence signal intensities accurately
    - Loss of efficacy of reagents over time – duration of the sequencing run
- Human Errors
    - Excessive fragmentation of the input DNA
    - Contaminants

Your RNA-Seq file looks like this

1. FASTA
2. **RNA-seq data (FASTQ)**
3. GFF3/GTF
4. SAM/BAM

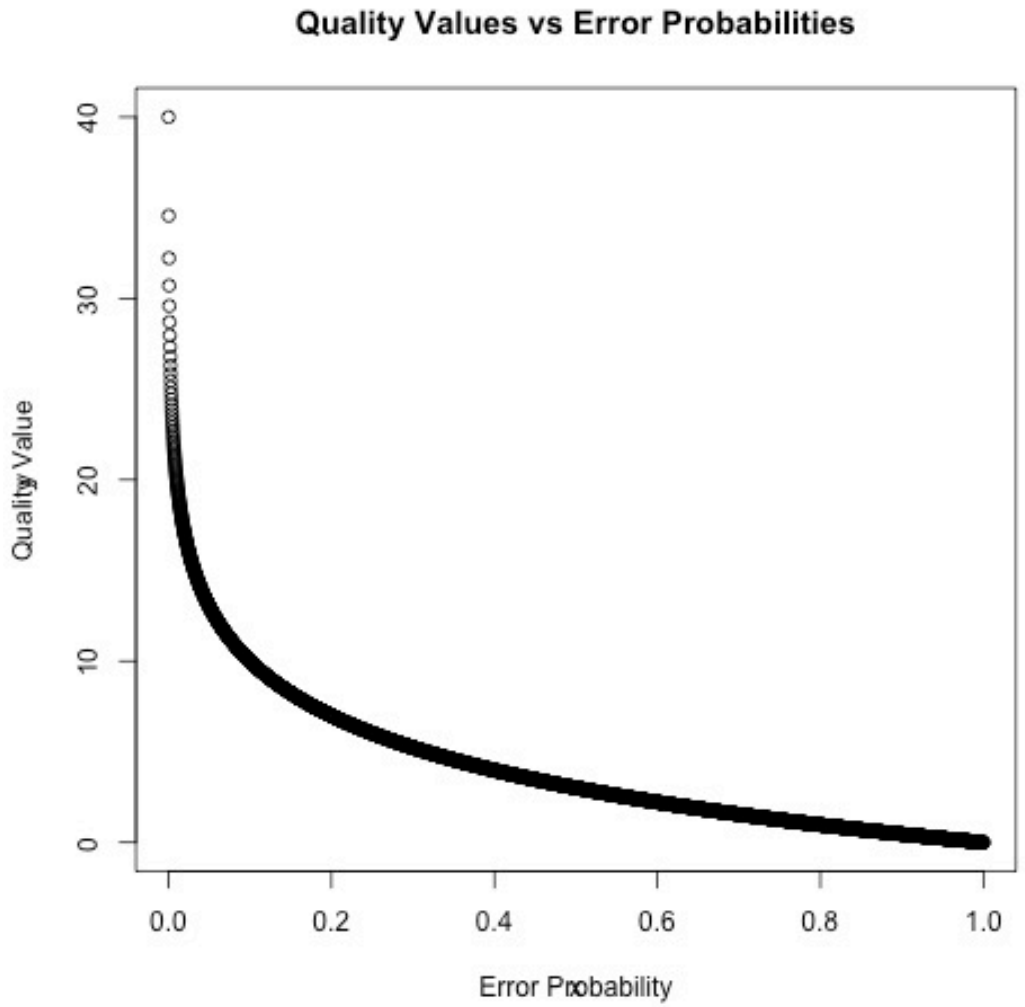
```
@HWUSI-EAS525:2:1:13336:1129#0/1
GTTGGAGCCGGCGAGCGGGACAAGGCCCTTGTCCA
+
ccacacccacccccccccc[[cccc_ccaccbbb_
@HWUSI-EAS525:2:1:14101:1126#0/1
GCCGGGACAGCGTGTTGGTTGGCGCGCGGTCCCTC
+
BBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBB
@HWUSI-EAS525:2:1:15408:1129#0/1
CGGCCTCATTCTTGGCCAGGTTCTGGTCCAGCGAG
+
cghhchhgchehhdffccgdgh]gcchhcahWcea
@HWUSI-EAS525:2:1:15457:1127#0/1
CGGAGGCCCCCGCTCCTCTCCCCCGCGCCCGCGCC
+
^BBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBB
@HWUSI-EAS525:2:1:15941:1125#0/1
TTGGGCCCTCCTGATTTCATCGGTTCTGAAGGCTG
+
SUIF\_XYWW]VaOZZZ\V\bYbb_]ZXTZbbb_b
@HWUSI-EAS525:2:1:16426:1127#0/1
GCCCGTCCTTAGAGGCTAGGGGACCTGCCCGCCGG
```

Data quality and its relevance

- Significance
- How NGS data quality is quantified?
- Phred quality scores

$Q = -10 \log_{10} P$

where P is the probability that the base called is incorrect



Quality score function and its significance

- $Q = -10 \log_{10} P$, where P is the probability that the base called is incorrect

- Significance

P	% Error	% Reliability	*Q*
1/10	10	90	10
1/100	1	99	20
1/1000	0.1	99.9	30
1/10000	0.01	99.99	40

and so on…

Illumina's Sequence Quality Encoding

Symbol	ASCII Code	Q-Score	Symbol	ASCII Code	Q-Score
!	33	0	6	54	21
"	34	1	7	55	22
#	35	2	8	56	23
$	36	3	9	57	24
%	37	4	:	58	25
&	38	5	;	59	26
'	39	6	<	60	27
(	40	7	=	61	28
)	41	8	>	62	29
*	42	9	?	63	30
+	43	10	@	64	31
,	44	11	A	65	32
-	45	12	B	66	33
.	46	13	C	67	34
/	47	14	D	68	35
0	48	15	E	69	36
1	49	16	F	70	37
2	50	17	G	71	38
3	51	18	H	72	39
4	52	19	I	73	40
5	53	20			

How to decipher the Sequence Quality string?

Sequence: GTGTATG

Quality string: ?A>F@JI

Nucleotide	Character from Quality string	ASCII value	Quality Value
G	?	63	63-33 = 30
T	A	65	65-33 = 32
G	>	62	62-33 = 29
T	F	70	70-33 = 37
A	@	64	64-33 = 31
T	J	74	74-33 = 41
G	I	73	73-33 = 40

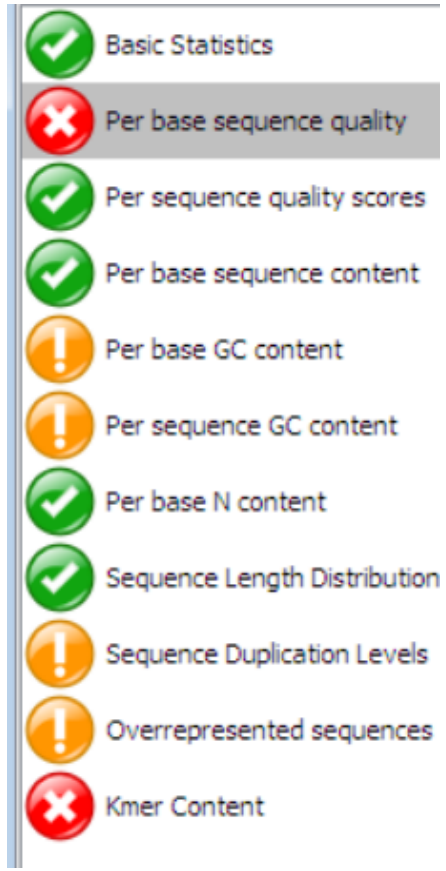
Why FastQC?

- Large sequence throughput
- Need to check quality before any analysis aimed at biological inference
- Limitations of quality report from the sequencer

Highlights of the FastQC tool

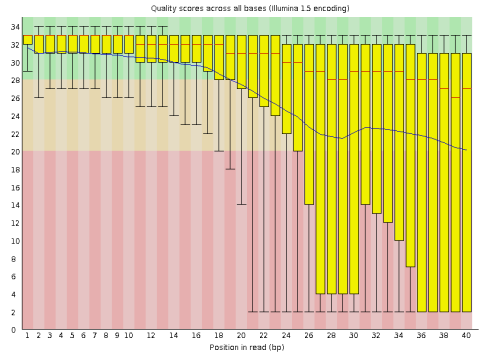
- FastQC is a program written in Java
- Runs on all standard software platforms – Linux, Mac, Windows
- Easy to install
- “Light weight” on its compute requirements
- Well-maintained and regularly updated
- Well-documented
- Widely used and discussed in the community
- Can be run in either standalone interactive / non-interactive (batch) mode

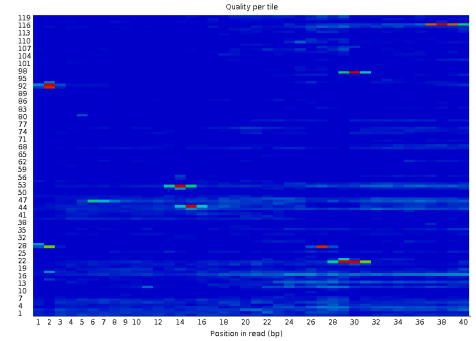
Reports generated by FastQC

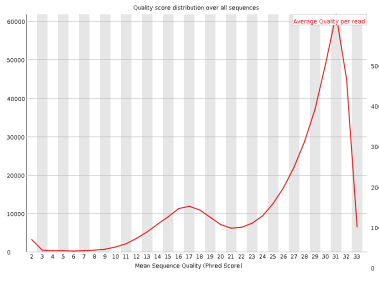


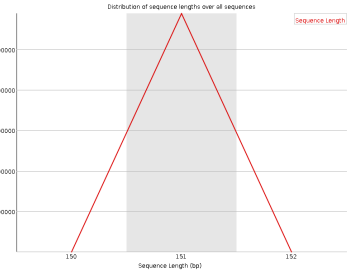
Basic Statistics

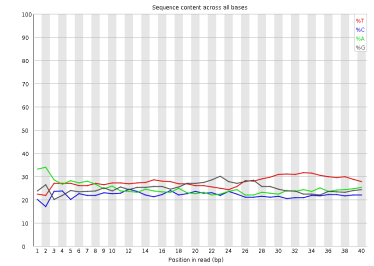
Measure	Value
Filename	M_19_0108_AM7962_AD004_L003_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	5885062
Sequences flagged as poor quality	0
Sequence length	151
%GC	51

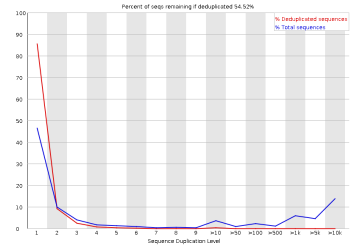


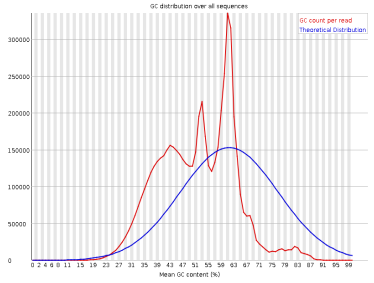












Overrepresented sequences

Sequence	Count	Percentage	Possible Source
CTGGAGTGCAGTGGCTATTCACAGCCCGATCCCACTACTGATCAGCACC	81075	1.377640541425052	No Hit
CCAGCCTGCAGTGCAGTGCCTATTCACAGCCCGATCCCACTACTCATCA	61843	1.0508470429028616	No Hit
CTGGAGTCCTGGAAGCTTGACTACCCTACCTTCTCCTACAAAATGGACCTT	57843	0.9828783452068983	No Hit
GGCTGGAGTGCAGTGGCTATTCACAGCCCGATCCCACTACTGATCAGCA	56821	0.9655123429455799	No Hit
GGCAACCTGCTGGTCCCCCCCTCCCCGGAGGTCACCATATTGATCCCAA	54501	0.9260904982819211	No Hit
CAGCCTGCAGTGCAGTGGCTATTCACAGCCCGATCCCACTACTGATCAG	47865	0.8133304288043184	No Hit
CCTAGGCAACCTGGTGGTCCCCCCCTCCCCGGAGGTCACCATATTGATCC	47157	0.8012999693121329	No Hit
CCTTAGGCAACCTGGTGGTCCCCCCCTCCCCGGAGGTCACCATATTGATC	41412	0.703679927246306	No Hit
CTCAGGCCTGGAGTGCAGTGGCTATTCACAGCCCGATCCCACTACTGATC	39707	0.6747082698534017	No Hit
CACAAATTATGCAGTCGAGTTTCCCACATTTGGGGAAATCGCAGGGGTCA	35622	0.6052952373313993	No Hit

Explanation of FastQC modules – Basic Statistics

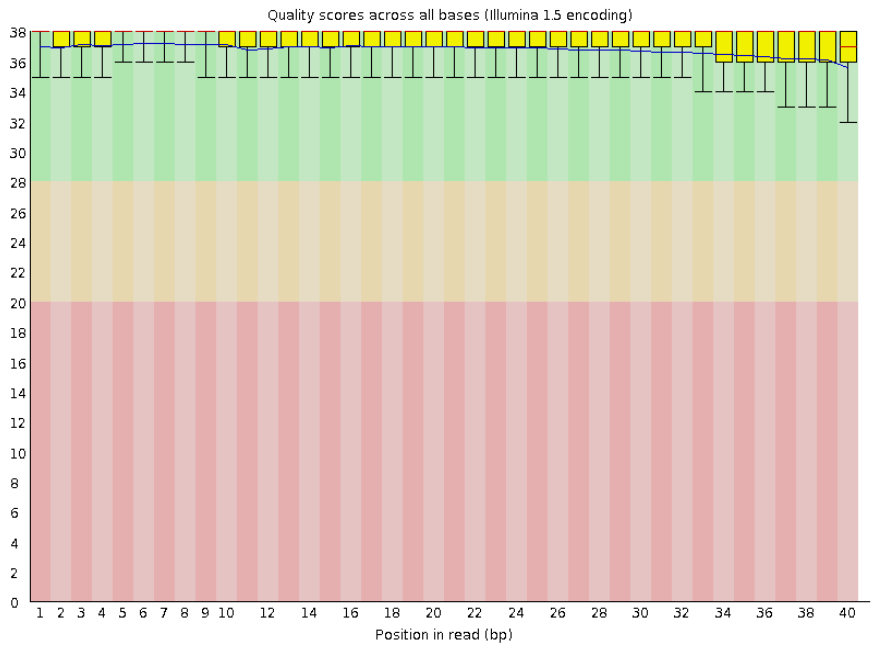
Basic Statistics

Measure	Value
Filename	M_19_0108_AM7962_AD004_L003_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	5885062
Sequences flagged as poor quality	0
Sequence length	151
%GC	51

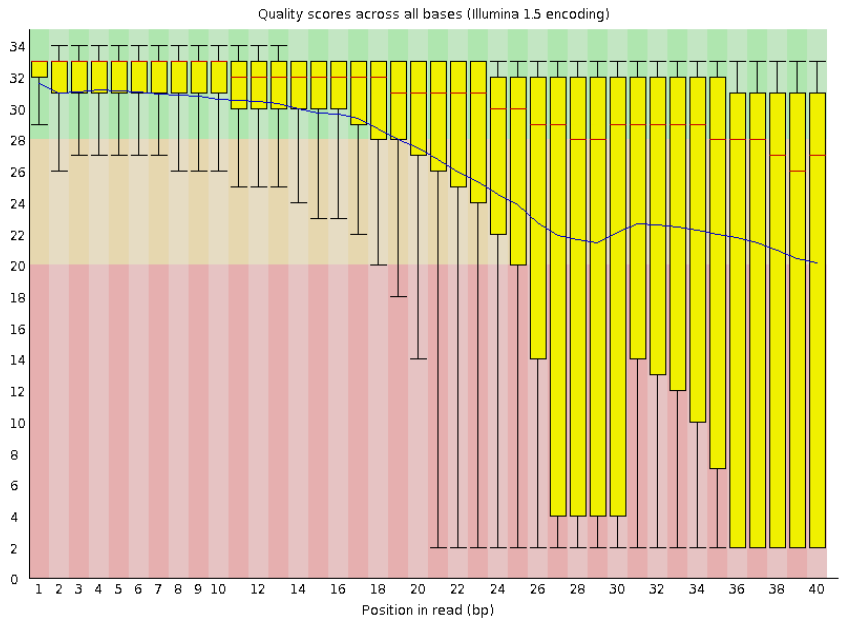
Explanation of FastQC modules – Per Base Sequence Quality

Per base sequence quality

Good data



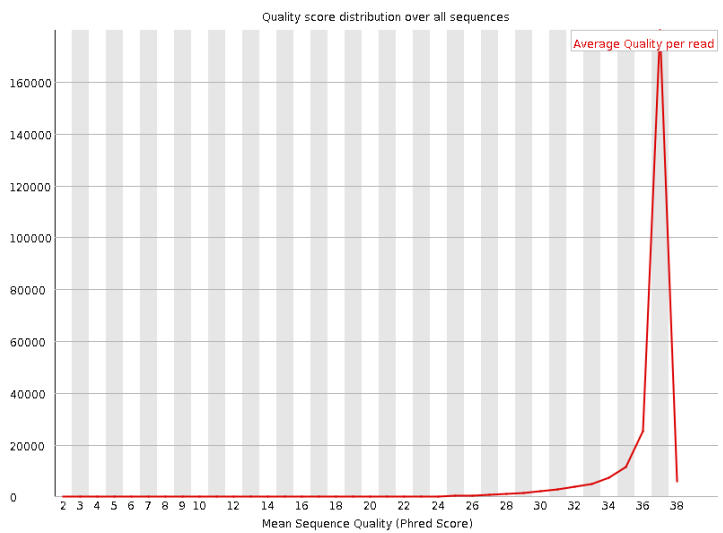
Bad data



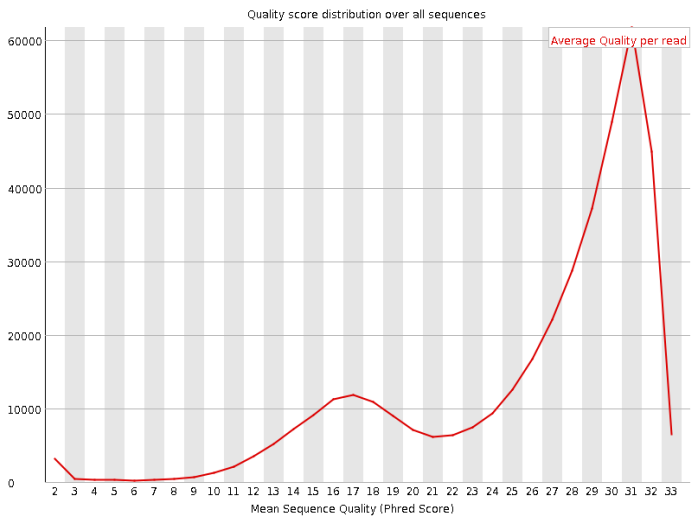
Explanation of FastQC modules – Per Sequence Quality Scores

Per sequence quality scores

Good data



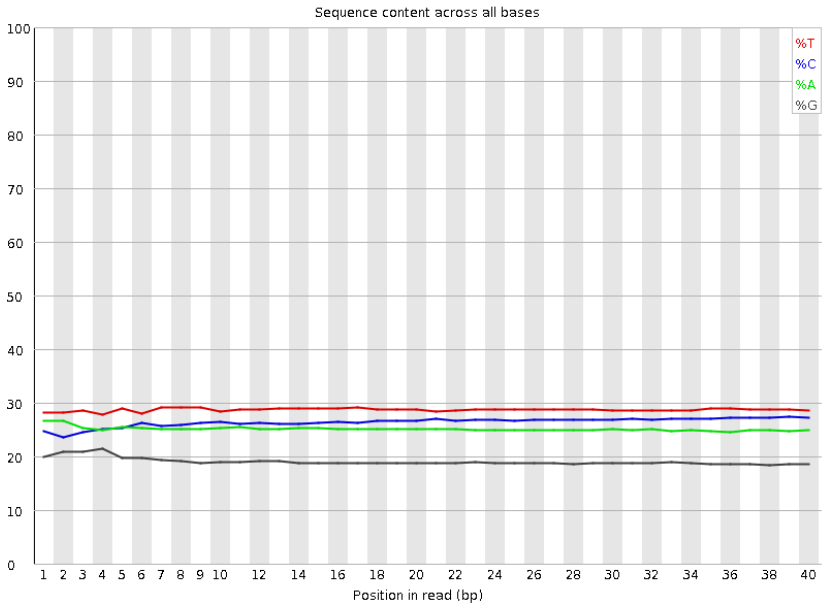
May need to be understood



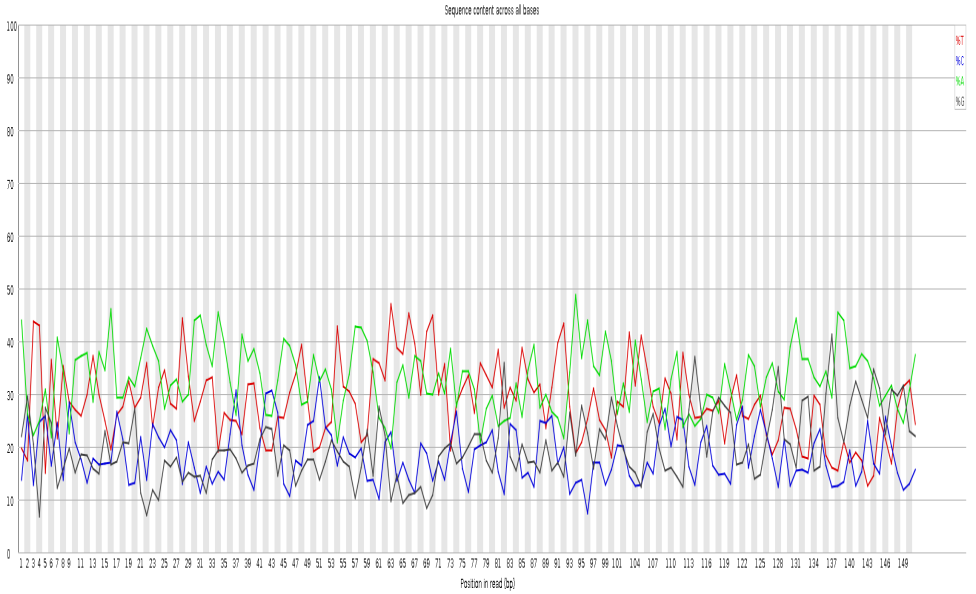
Explanation of FastQC modules – Per Base Sequence Content

Per base sequence content

Good data



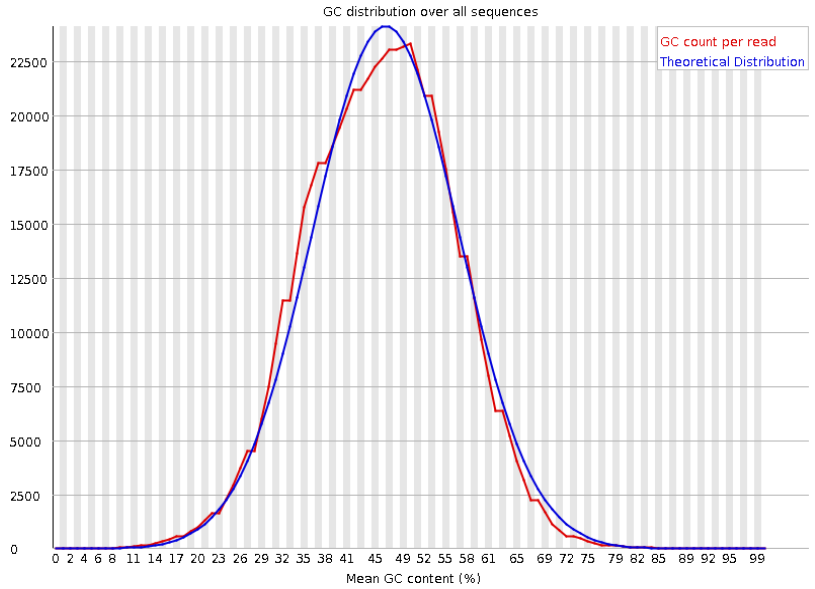
Bad data



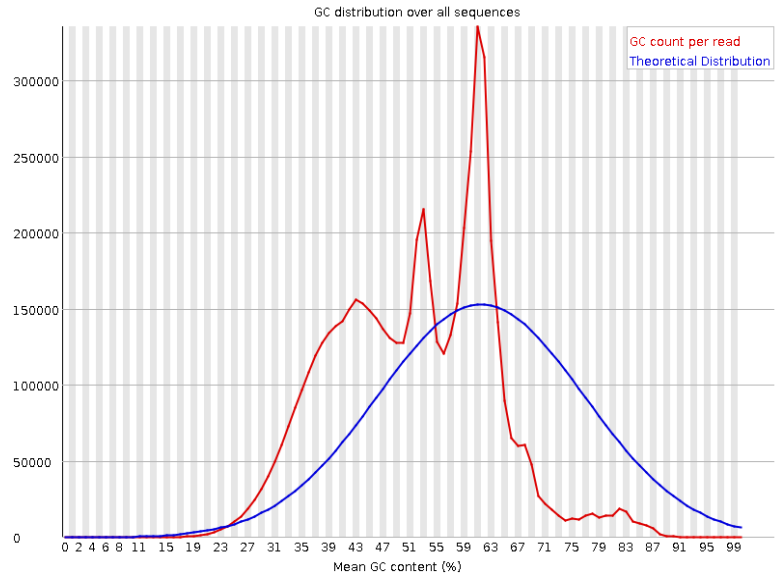
Explanation of FastQC modules – Per Sequence GC Content

Per sequence GC content

Good data



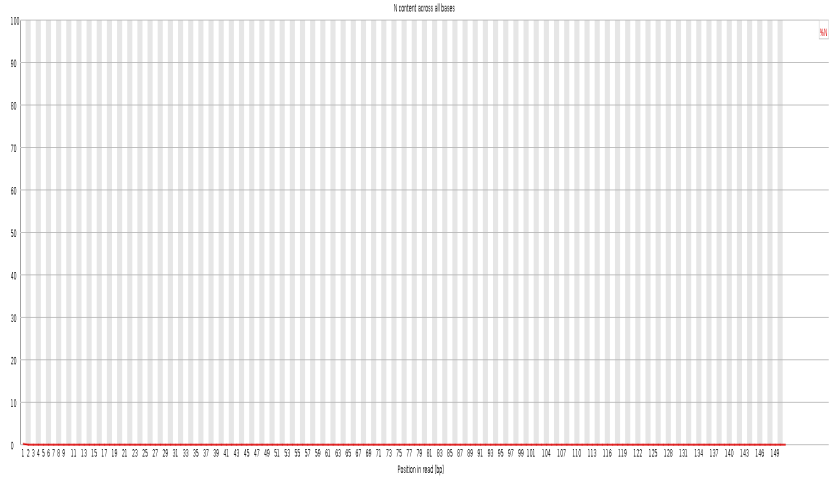
Needs investigation



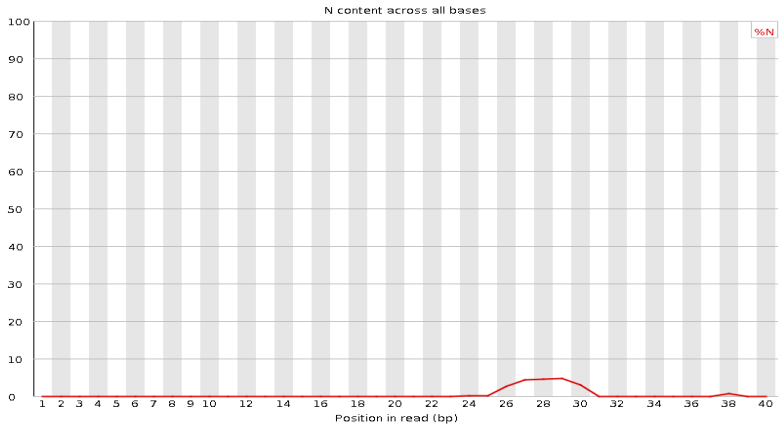
Explanation of FastQC modules – Per Base N Content

Per base N content

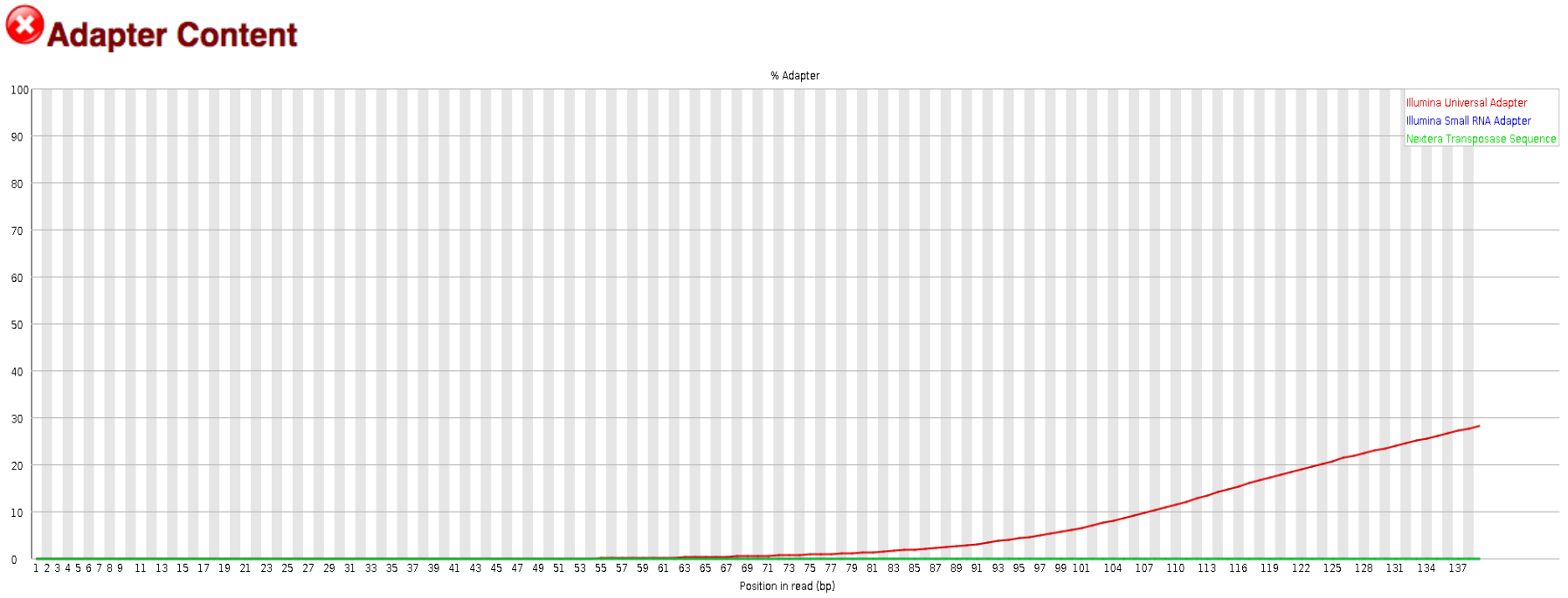
Good data



Few reads have N bases

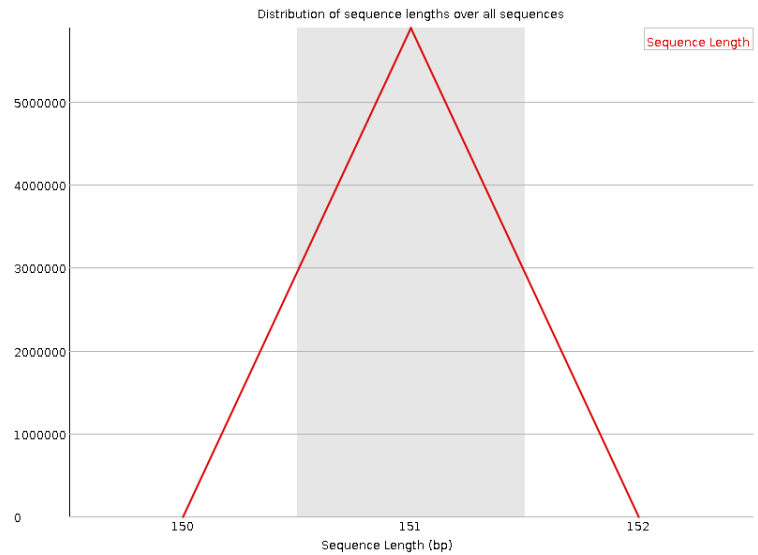


Explanation of FastQC modules – Adapter Content



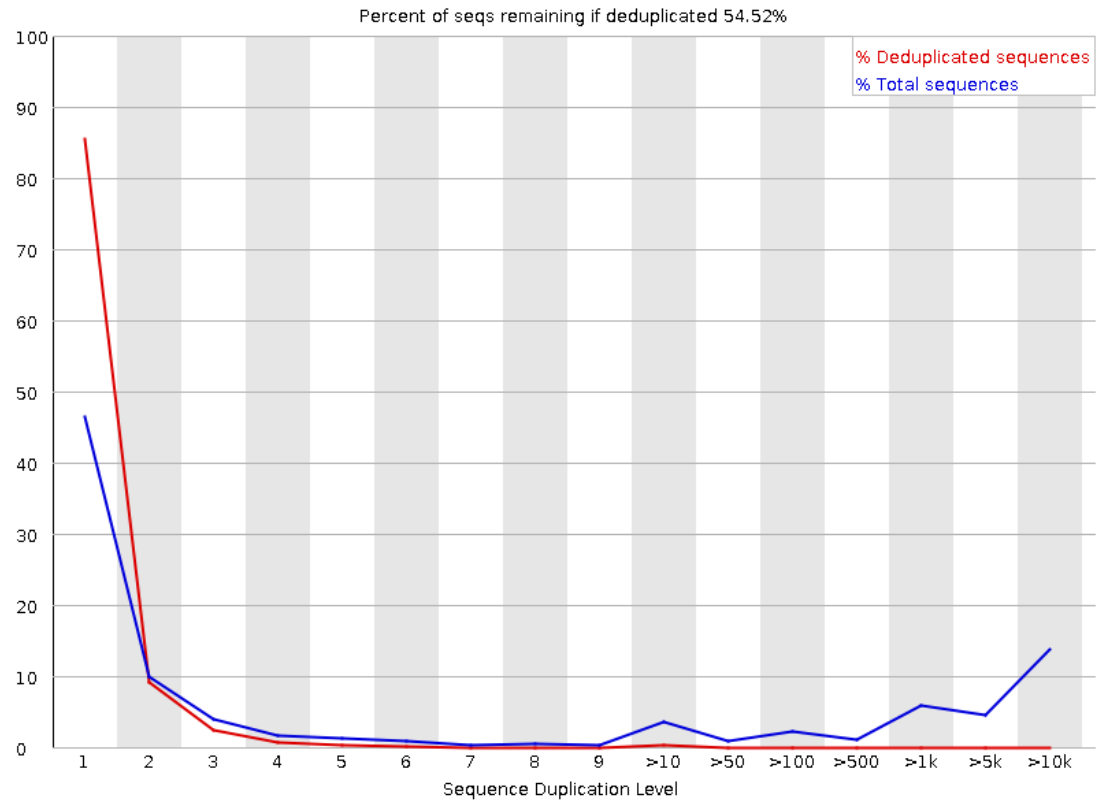
Explanation of FastQC modules – Sequence Length Distribution

Sequence Length Distribution



Explanation of FastQC modules – Sequence Duplication Levels

Sequence Duplication Levels

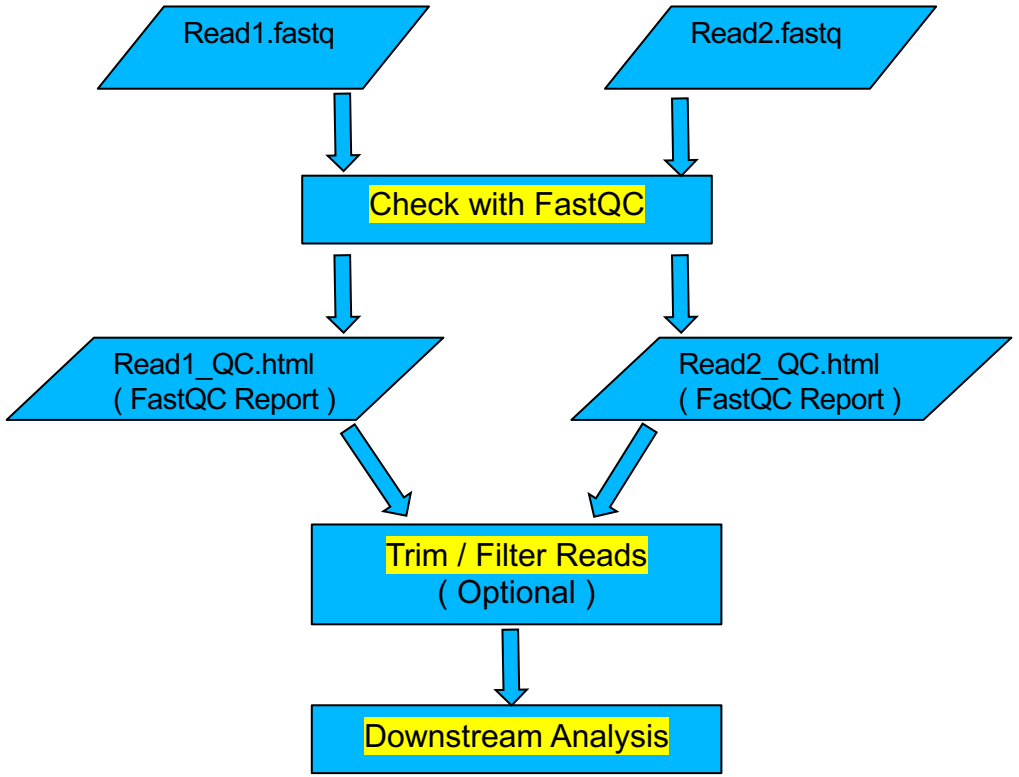


Explanation of FastQC modules – Overrepresented Sequences

Overrepresented sequences

Sequence	Count	Percentage	Possible Source
CTGGAGTGCAGTGGCTATTCACAGGCGCGATCCCACTACTGATCAGCACG	81075	1.377640541425052	No Hit
CCAGGCTGGAGTGCAGTGGCTATTCACAGGCGCGATCCCACTACTGATCA	61843	1.0508470429028616	No Hit
CTGGAGTCTTGGAAGCTTGACTACCCTACGTTCTCCTACAAATGGACCTT	57843	0.9828783452068983	No Hit
GGCTGGAGTGCAGTGGCTATTCACAGGCGCGATCCCACTACTGATCAGCA	56821	0.9655123429455799	No Hit
GGCAACCTGGTGGTCCCCCGCTCCCGGGAGGTCACCATATTGATGCCGAA	54501	0.9260904982819211	No Hit
CAGGCTGGAGTGCAGTGGCTATTCACAGGCGCGATCCCACTACTGATCAG	47865	0.8133304288043184	No Hit
CTTAGGCAACCTGGTGGTCCCCCGCTCCCGGGAGGTCACCATATTGATGC	47157	0.8012999693121329	No Hit
CCTTAGGCAACCTGGTGGTCCCCCGCTCCCGGGAGGTCACCATATTGATG	41412	0.703679927246306	No Hit
CTCAGGCTGGAGTGCAGTGGCTATTCACAGGCGCGATCCCACTACTGATC	39707	0.6747082698534017	No Hit
CACAAATTATGCAGTCGAGTTTCCCACATTTGGGGAAATCGCAGGGGTCA	35622	0.6052952373313993	No Hit

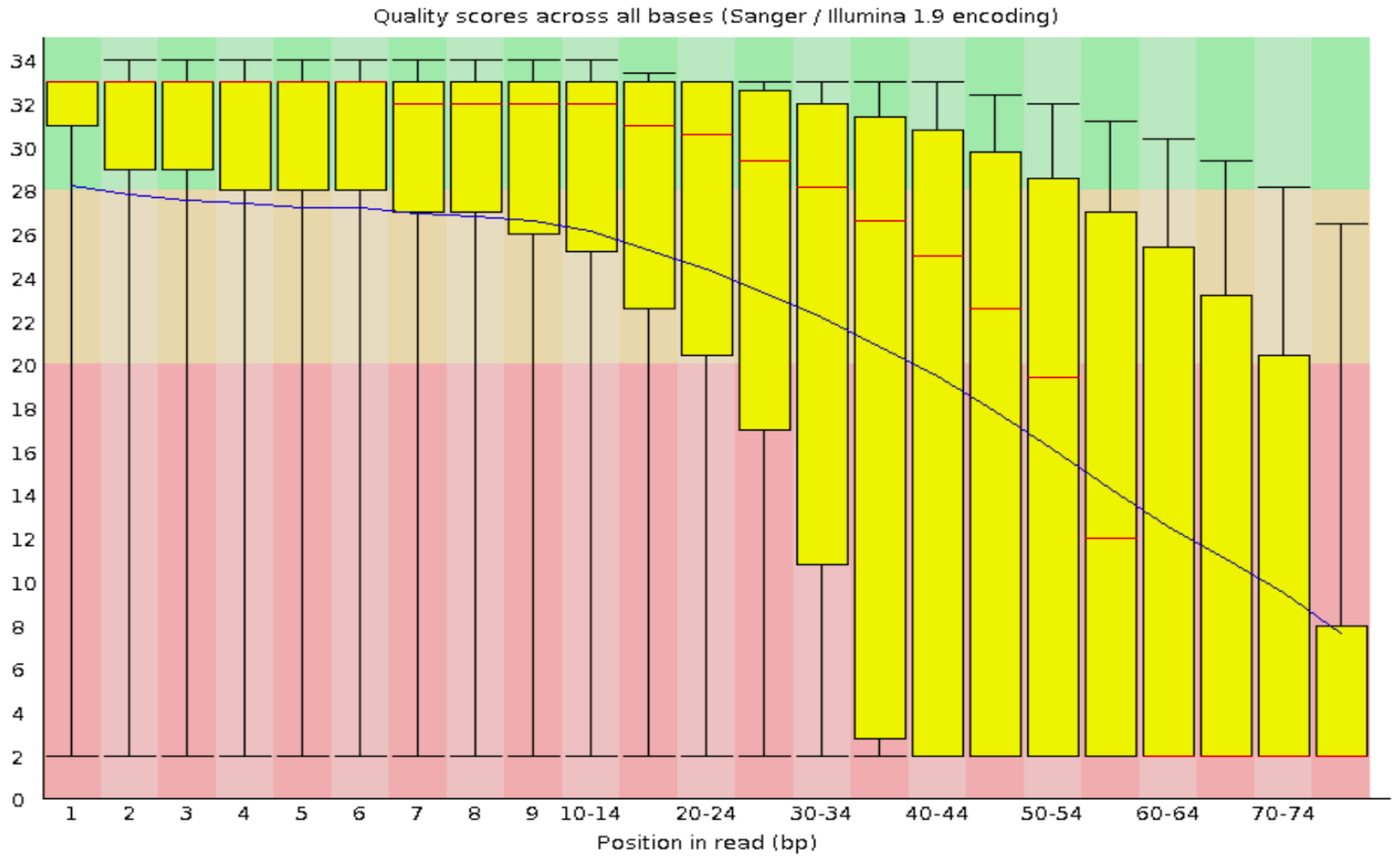
Representative QC workflow



Quality Control of RNA-Seq Reads

Step 1. Quality Control (QC) using FASTQC Software

1. Sequencing quality score

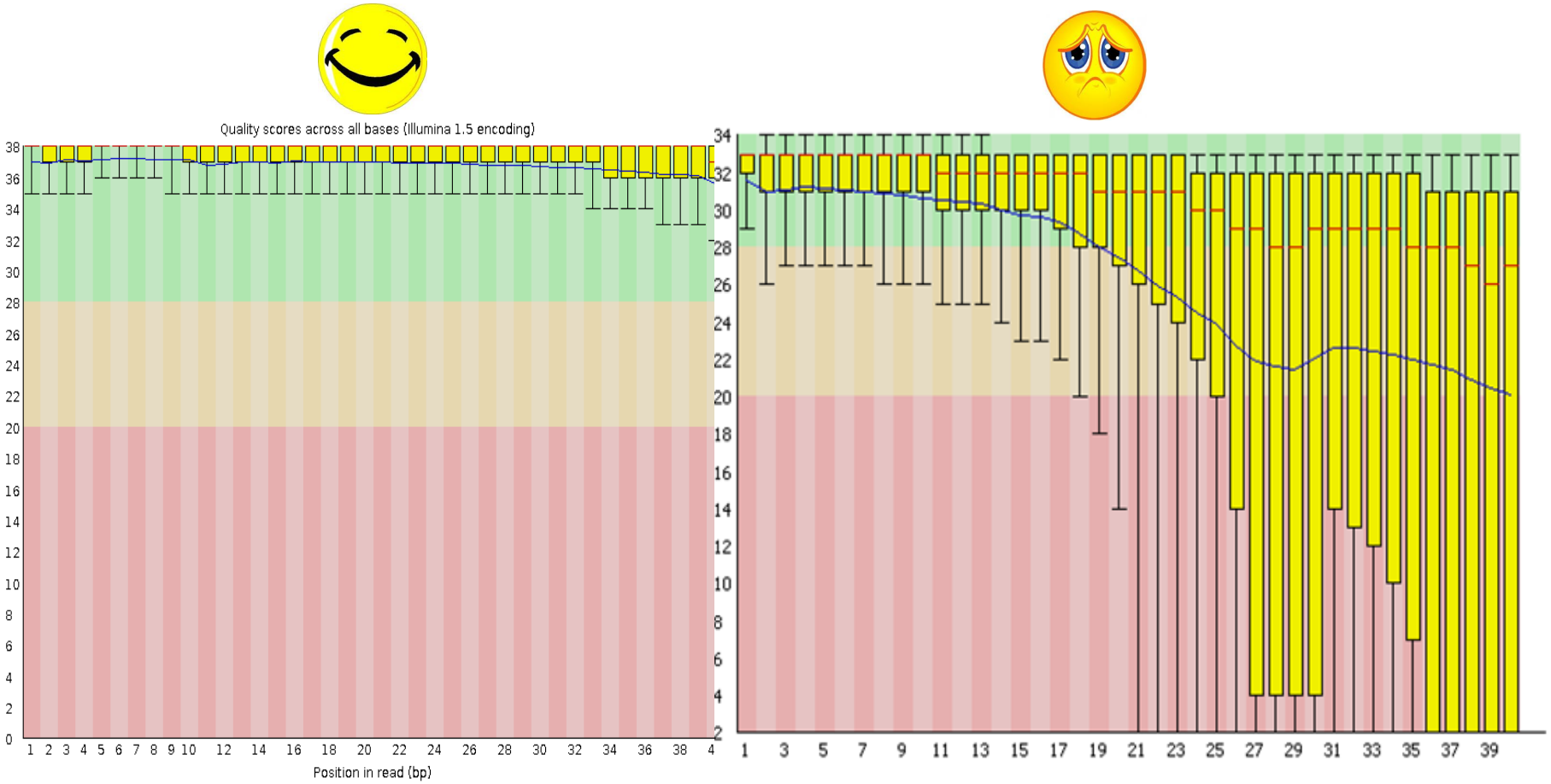


Look for any erroneous sequences, bacterial contamination, remove adapters and ribosomal RNAs

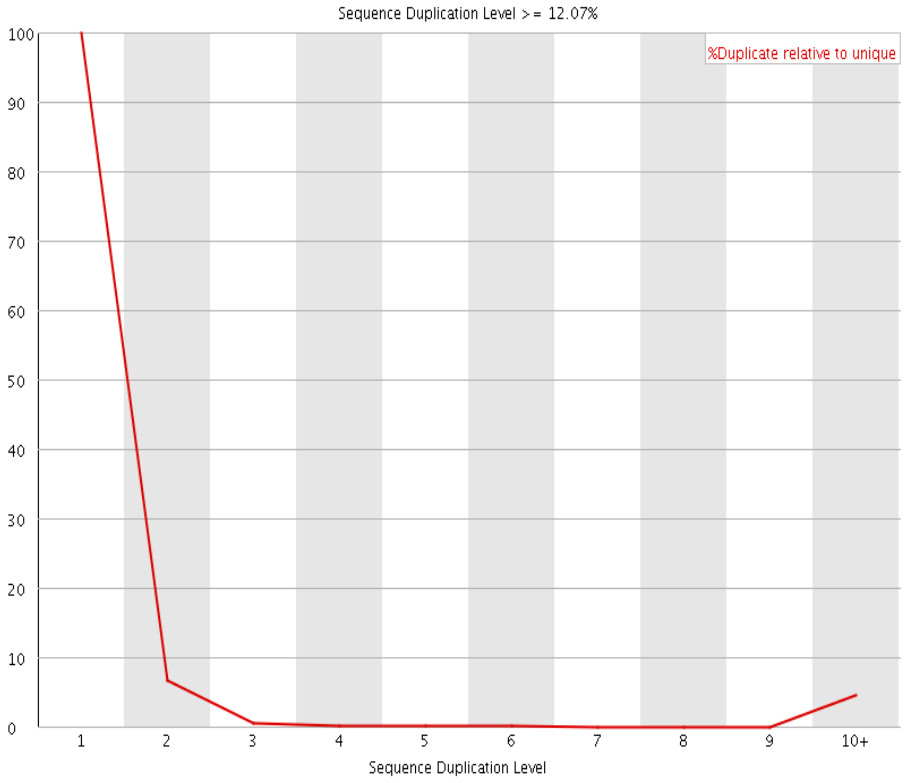
Overrepresented sequences

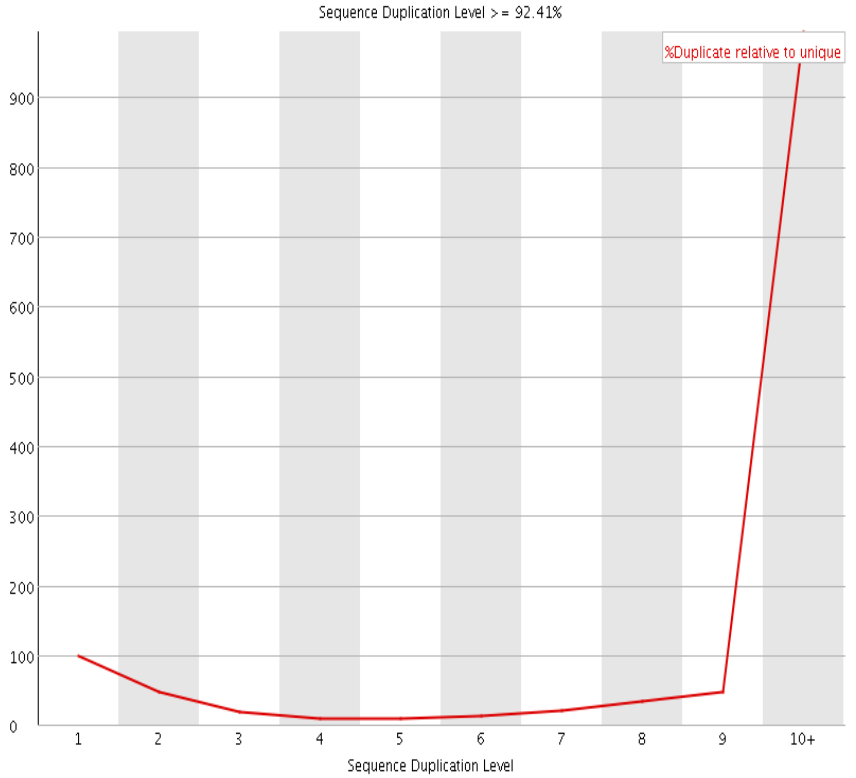
Sequence	Count	Percentage	Possible Source
CTGCTATGGCCACCAGACTCTCAGGCTCCATGCAGTGGCCAGCCTCATCG	2554	0.8349133703824779	No Hit
CAGCGGTCTAGTTTGAAGAACCTGACCCGAGTCTTGGTGACGAAGGCCAG	2463	0.8051650866296176	No Hit
GTTTGAAGAACCTGACCCGAGTCTTGGTGACGAAGGCCAGATTTGCGATC	1920	0.6276560967636483	No Hit
CCACAGGGTCCCAGGTCATGGGTACCGAGTCCAGGTCATAGTGCCGGATG	1219	0.39849624060150374	No Hit
GAAGAACCTGACCCGAGTCTTGGTGACGAAGGCCAGATTTGCGATCTTCA	1186	0.3877084014383786	No Hit
GGCAGGTGGACCCGGAGCCGCTGACAGAGGAGGTCAGCCCCTGAGTTGGA	1111	0.3631905851585486	No Hit
CACAGGGTCCCAGGTCATGGGTACCGAGTCCAGGTCATAGTGCCGGATGT	1079	0.35272965021248776	No Hit

Per base sequence quality



Duplication level





Summary of take-home messages (A short re-cap)

- We saw about the nature of NGS data – massively parallel sequencing and its challenges in terms of quality inference.
- A peek in to some concepts related to quality encoding.
- An example of how we could obtain the quality of a base from its corresponding quality string character.
- Introduced the FastQC tool.
- Visited the various modules in FastQC.
- **Importantly!** Always, evaluate and understand the quality of your sequenced data BEFORE ANY analysis.

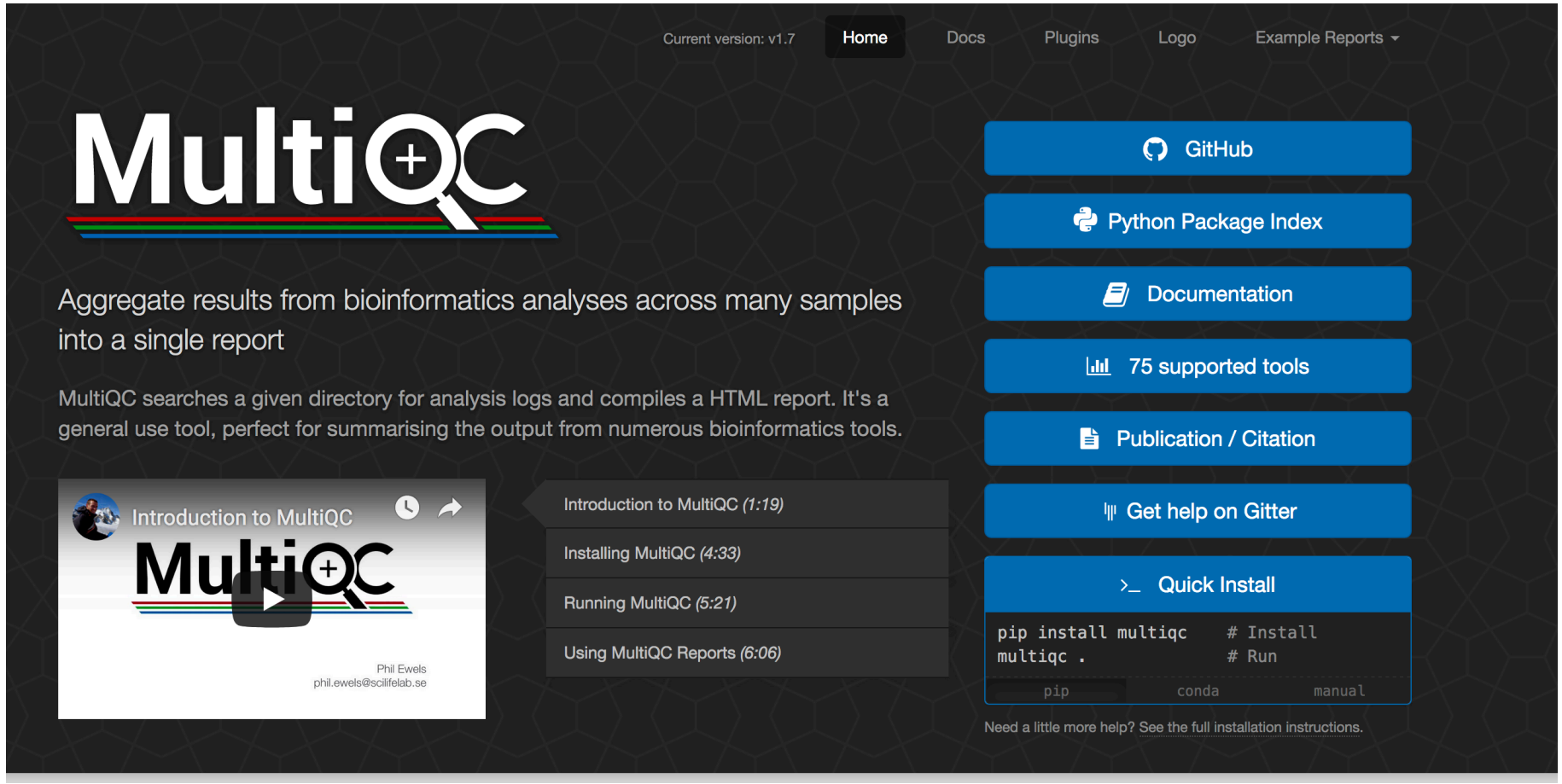
Hands on for FastQC

- conda create -n fastqc
- conda install fastqc
- conda install multiqc
- time fastqc ./SMOC_DATA/*.fastq.gz -o ./QC/ -t 14
- time multiqc ./QC/

Output of batch fastqc run looks like this

```
total 20392
-rw-r--r--  1 thimmamp g-thimmamp 710102 Jul 11 11:47 WT_UUO2_513_fastqc.html
-rw-r--r--  1 thimmamp g-thimmamp 710188 Jul 11 11:47 WT_NORMAL1_509_fastqc.html
-rw-r--r--  1 thimmamp g-thimmamp 775921 Jul 11 11:47 WT_UUO1_512_fastqc.zip
-rw-r--r--  1 thimmamp g-thimmamp 779912 Jul 11 11:47 WT_UUO3_514_fastqc.zip
-rw-r--r--  1 thimmamp g-thimmamp 770059 Jul 11 11:47 SMOC2_UUO3_507_fastqc.zip
-rw-r--r--  1 thimmamp g-thimmamp 709380 Jul 11 11:47 SMOC2_UUO1_505_fastqc.html
-rw-r--r--  1 thimmamp g-thimmamp 774530 Jul 11 11:47 SMOC2_UUO1_505_fastqc.zip
-rw-r--r--  1 thimmamp g-thimmamp 712398 Jul 11 11:47 WT_UUO1_512_fastqc.html
-rw-r--r--  1 thimmamp g-thimmamp 776084 Jul 11 11:47 SMOC2_NORMAL3_503_fastqc.zip
-rw-r--r--  1 thimmamp g-thimmamp 773657 Jul 11 11:47 WT_UUO2_513_fastqc.zip
-rw-r--r--  1 thimmamp g-thimmamp 704944 Jul 11 11:47 SMOC2_UUO2_506_fastqc.html
-rw-r--r--  1 thimmamp g-thimmamp 780460 Jul 11 11:47 SMOC2_NORMAL1_502_fastqc.zip
-rw-r--r--  1 thimmamp g-thimmamp 775004 Jul 11 11:47 WT_NORMAL2_510_fastqc.zip
-rw-r--r--  1 thimmamp g-thimmamp 775873 Jul 11 11:47 WT_NORMAL3_511_fastqc.zip
-rw-r--r--  1 thimmamp g-thimmamp 717080 Jul 11 11:47 SMOC2_NORMAL1_502_fastqc.html
-rw-r--r--  1 thimmamp g-thimmamp 766597 Jul 11 11:47 SMOC2_UUO2_506_fastqc.zip
-rw-r--r--  1 thimmamp g-thimmamp 779011 Jul 11 11:47 SMOC2_UUO4_508_fastqc.zip
-rw-r--r--  1 thimmamp g-thimmamp 709184 Jul 11 11:47 SMOC2_NORMAL3_503_fastqc.html
-rw-r--r--  1 thimmamp g-thimmamp 771708 Jul 11 11:47 WT_NORMAL1_509_fastqc.zip
-rw-r--r--  1 thimmamp g-thimmamp 711619 Jul 11 11:47 WT_NORMAL3_511_fastqc.html
-rw-r--r--  1 thimmamp g-thimmamp 712082 Jul 11 11:47 WT_UUO4_515_fastqc.html
-rw-r--r--  1 thimmamp g-thimmamp 714440 Jul 11 11:47 SMOC2_UUO4_508_fastqc.html
-rw-r--r--  1 thimmamp g-thimmamp 716267 Jul 11 11:47 WT_UUO3_514_fastqc.html
-rw-r--r--  1 thimmamp g-thimmamp 710867 Jul 11 11:47 WT_NORMAL2_510_fastqc.html
-rw-r--r--  1 thimmamp g-thimmamp 779924 Jul 11 11:47 WT_UUO4_515_fastqc.zip
-rw-r--r--  1 thimmamp g-thimmamp 710352 Jul 11 11:47 SMOC2_NORMAL4_504_fastqc.html
-rw-r--r--  1 thimmamp g-thimmamp 774038 Jul 11 11:47 SMOC2_NORMAL4_504_fastqc.zip
drwxr-xr-x  2 thimmamp g-thimmamp   4096 Jul 11 11:47 .
-rw-r--r--  1 thimmamp g-thimmamp 706128 Jul 11 11:47 SMOC2_UUO3_507_fastqc.html
drwxr-xr-x 14 thimmamp g-thimmamp   4096 Aug  3 06:49 ..
```

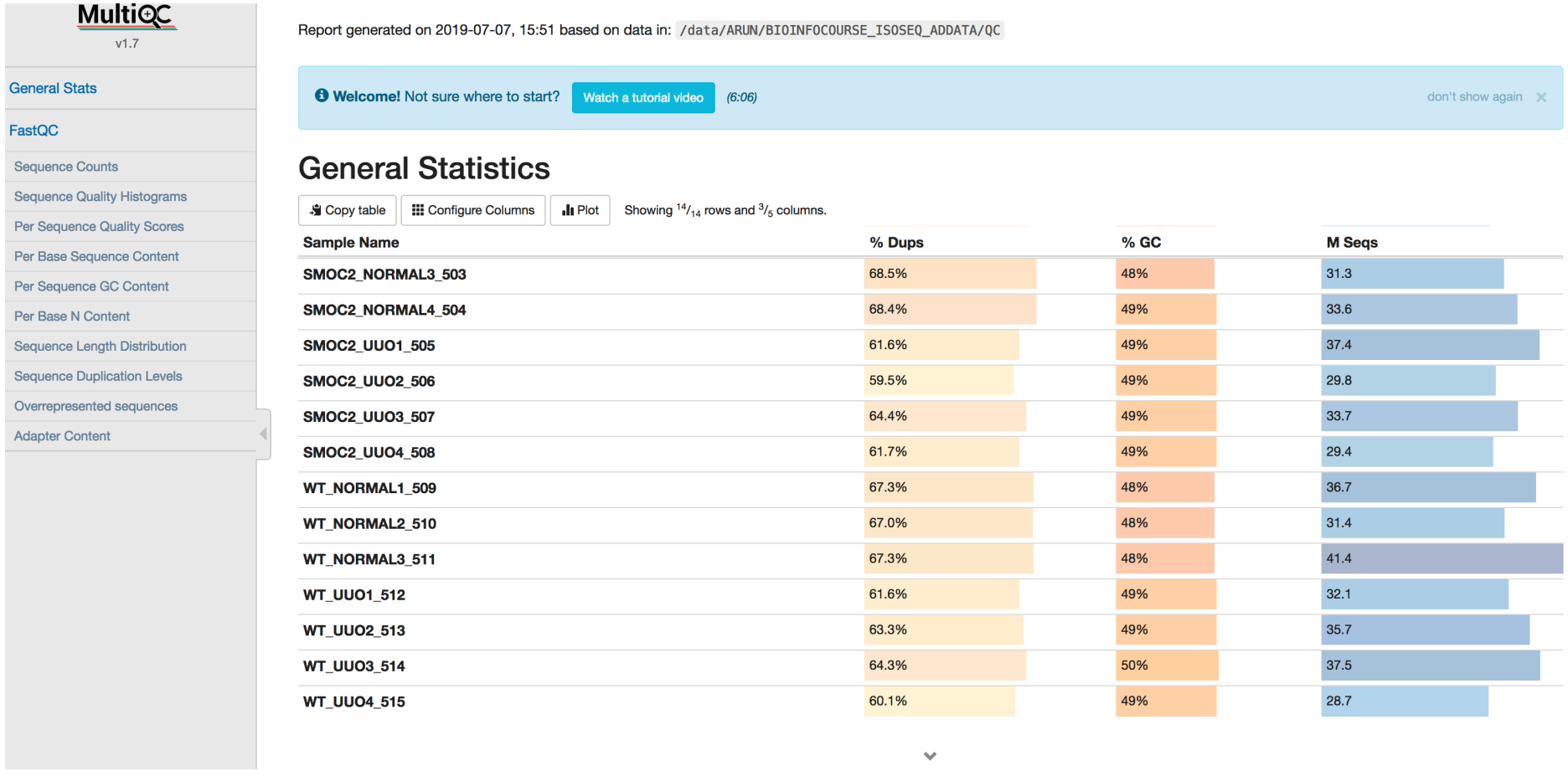
MultiQC



To look at the summary qc report for all your samples.

Command to run is
> Multiqc <your qc folder with fastqc output>

Summary of All samples QC : multiqc output



MultiQC
v1.7

General Stats

FastQC

- Sequence Counts
- Sequence Quality Histograms
- Per Sequence Quality Scores
- Per Base Sequence Content
- Per Sequence GC Content
- Per Base N Content
- Sequence Length Distribution
- Sequence Duplication Levels
- Overrepresented sequences
- Adapter Content

Report generated on 2019-07-07, 15:51 based on data in: `/data/ARUN/BIOINFOCOURSE_ISOSEQ_ADDATA/QC`

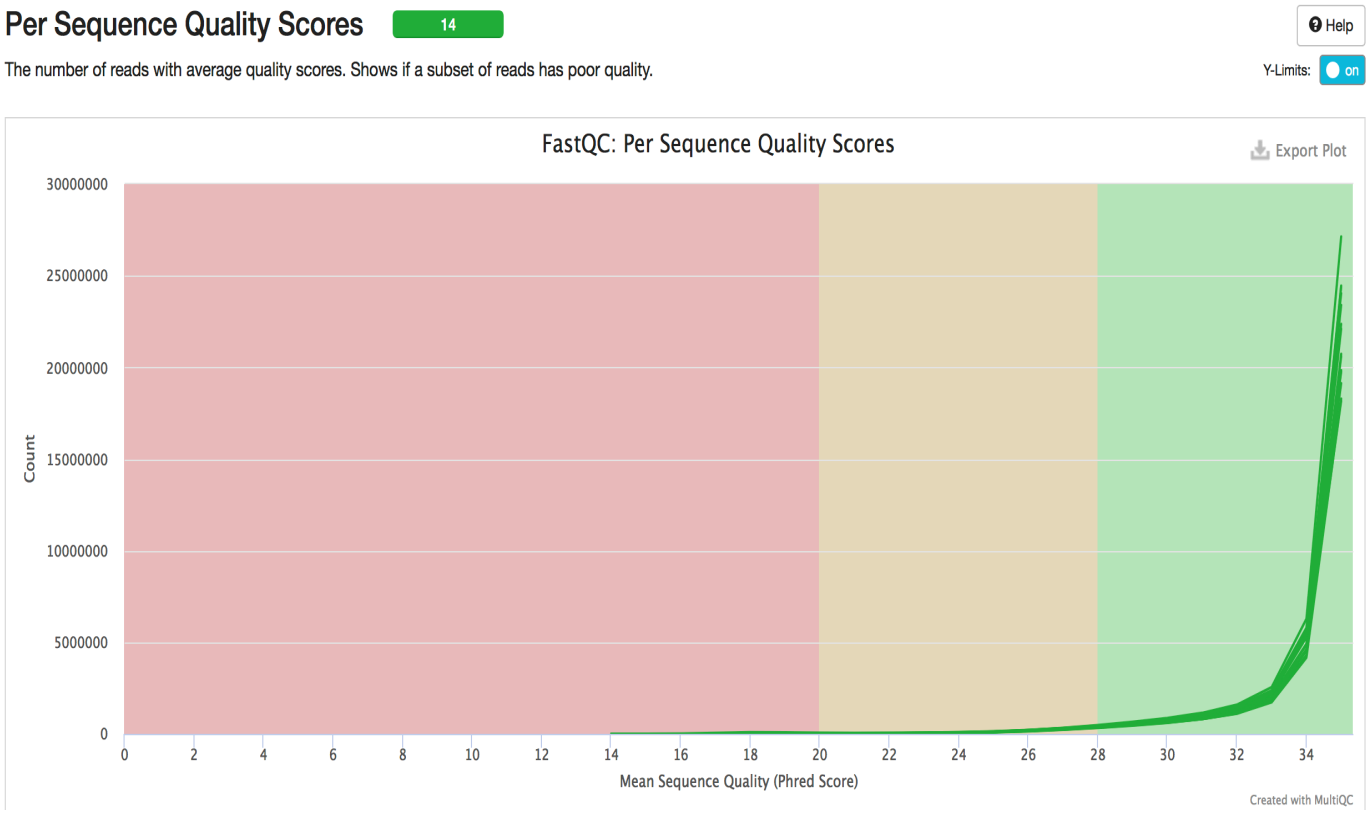
Welcome! Not sure where to start? Watch a tutorial video *(6:06)* don't show again

General Statistics

Copy table | Configure Columns | Plot | Showing 14/14 rows and 3/5 columns.

Sample Name	% Dups	% GC	M Seqs
SMOC2_NORMAL3_503	68.5%	48%	31.3
SMOC2_NORMAL4_504	68.4%	49%	33.6
SMOC2_UUO1_505	61.6%	49%	37.4
SMOC2_UUO2_506	59.5%	49%	29.8
SMOC2_UUO3_507	64.4%	49%	33.7
SMOC2_UUO4_508	61.7%	49%	29.4
WT_NORMAL1_509	67.3%	48%	36.7
WT_NORMAL2_510	67.0%	48%	31.4
WT_NORMAL3_511	67.3%	48%	41.4
WT_UUO1_512	61.6%	49%	32.1
WT_UUO2_513	63.3%	49%	35.7
WT_UUO3_514	64.3%	50%	37.5
WT_UUO4_515	60.1%	49%	28.7

MultiQC Summary output



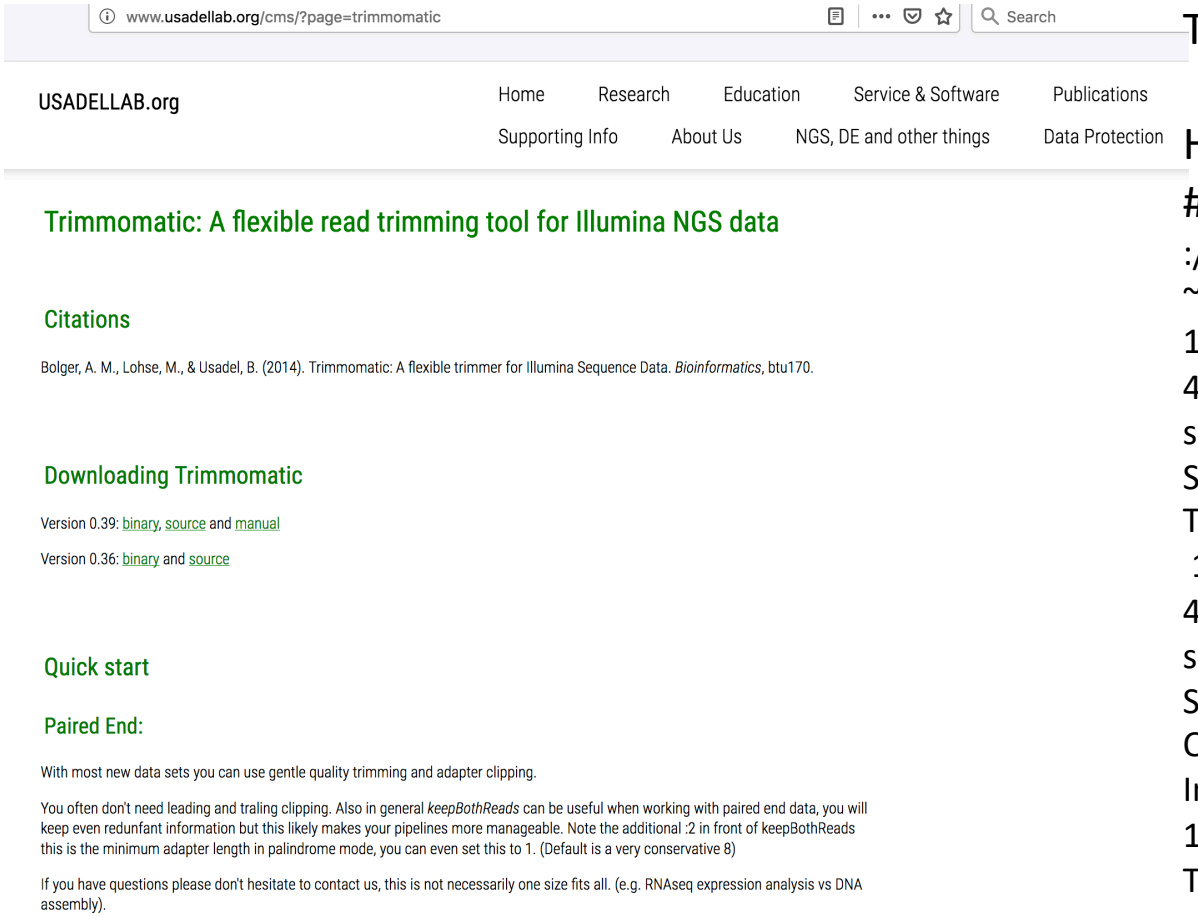
Quality of all 14 samples shown here together.

End of Module 2 QC

Module 3 : Data Trimming

- After scanning through the quality of your raw data, it is time to tailor your data cleaning strategy
- Remove low quality bases
- Trim off adapter and primer sequences
- make sure minimal read length to be carried for further down stream analysis.

Trimmomatic



This is java based tool, to trim your reads.

How to run:

####Trimming

:/data1/BESE_COURSE2019$ time java -jar ~/bioinformatics_tools/Trimmomatic-0.38/trimmomatic-0.38.jar SE 1_SMOC_RAWDATA/WT_NORMAL1_509.fastq.gz 4_TRIMMING/WT_NORMAL1_509_Trimmed.fastq.gz -threads 12 -summary WT_NORMAL1_Trimm_Summary.txt LEADING:3 TRAILING:3 SLIDINGWINDOW:4:30 MINLEN:75

TrimmomaticSE: Started with arguments:
1_SMOC_RAWDATA/WT_NORMAL1_509.fastq.gz 4_TRIMMING/WT_NORMAL1_509_Trimmed.fastq.gz -threads 12 -summary WT_NORMAL1_Trimm_Summary.txt LEADING:3 TRAILING:3 SLIDINGWINDOW:4:30 MINLEN:75

Quality encoding detected as phred33

Input Reads: 36746448 Surviving: 22973692 (62.52%) Dropped: 13772756 (37.48%)

TrimmomaticSE: Completed successfully

real 5m12.718s

user 8m43.928s

sys 0m36.734s

Hands on

- Download and install Trimmomatic
- Run Trimmomatic on your fastq files
- Observe the output
- Make sure you have good read files by observing throught FastQC and MultiQC, once again!

Trimmed output

```
-rw-rw-r--  1 thimmamp g-thimmamp        128 Jul 12 13:01 WT_UUO3_514_Trim_Summary.txt
-rw-rw-r--  1 thimmamp g-thimmamp 1548042804 Jul 12 13:01 WT_UUO3_514_Trimmed.fastq.gz
-rw-rw-r--  1 thimmamp g-thimmamp        128 Jul 12 13:10 WT_UUO2_513_Trim_Summary.txt
-rw-rw-r--  1 thimmamp g-thimmamp 1493210406 Jul 12 13:10 WT_UUO2_513_Trimmed.fastq.gz
-rw-rw-r--  1 thimmamp g-thimmamp        128 Jul 12 13:17 SMOC2_NORMAL4_504_Trim_Summary.txt
-rw-rw-r--  1 thimmamp g-thimmamp 1389811794 Jul 12 13:17 SMOC2_NORMAL4_504_Trimmed.fastq.gz
-rw-rw-r--  1 thimmamp g-thimmamp        128 Jul 12 13:24 SMOC2_UUO4_508_Trim_Summary.txt
-rw-rw-r--  1 thimmamp g-thimmamp 1235838466 Jul 12 13:24 SMOC2_UUO4_508_Trimmed.fastq.gz
-rw-rw-r--  1 thimmamp g-thimmamp        128 Jul 12 13:31 SMOC2_NORMAL3_503_Trim_Summary.txt
-rw-rw-r--  1 thimmamp g-thimmamp 1289922014 Jul 12 13:31 SMOC2_NORMAL3_503_Trimmed.fastq.gz
-rw-rw-r--  1 thimmamp g-thimmamp        128 Jul 12 13:38 WT_UUO1_512_Trim_Summary.txt
-rw-rw-r--  1 thimmamp g-thimmamp 1353778191 Jul 12 13:38 WT_UUO1_512_Trimmed.fastq.gz
-rw-rw-r--  1 thimmamp g-thimmamp        128 Jul 12 13:45 SMOC2_UUO2_506_Trim_Summary.txt
-rw-rw-r--  1 thimmamp g-thimmamp 1247090279 Jul 12 13:45 SMOC2_UUO2_506_Trimmed.fastq.gz
-rw-rw-r--  1 thimmamp g-thimmamp        128 Jul 12 13:54 SMOC2_UUO1_505_Trim_Summary.txt
-rw-rw-r--  1 thimmamp g-thimmamp 1565973159 Jul 12 13:54 SMOC2_UUO1_505_Trimmed.fastq.gz
-rw-rw-r--  1 thimmamp g-thimmamp        128 Jul 12 14:03 WT_NORMAL3_511_Trim_Summary.txt
-rw-rw-r--  1 thimmamp g-thimmamp 1720206958 Jul 12 14:03 WT_NORMAL3_511_Trimmed.fastq.gz
-rw-rw-r--  1 thimmamp g-thimmamp        128 Jul 12 14:11 SMOC2_UUO3_507_Trim_Summary.txt
-rw-rw-r--  1 thimmamp g-thimmamp 1407673668 Jul 12 14:11 SMOC2_UUO3_507_Trimmed.fastq.gz
-rw-rw-r--  1 thimmamp g-thimmamp        128 Jul 12 14:18 WT_NORMAL2_510_Trim_Summary.txt
-rw-rw-r--  1 thimmamp g-thimmamp 1299420702 Jul 12 14:18 WT_NORMAL2_510_Trimmed.fastq.gz
-rw-rw-r--  1 thimmamp g-thimmamp        128 Jul 12 14:26 WT_NORMAL1_509_Trim_Summary.txt
-rw-rw-r--  1 thimmamp g-thimmamp 1519606187 Jul 12 14:26 WT_NORMAL1_509_Trimmed.fastq.gz
-rw-rw-r--  1 thimmamp g-thimmamp        128 Jul 12 14:33 WT_UUO4_515_Trim_Summary.txt
-rw-rw-r--  1 thimmamp g-thimmamp 1182502044 Jul 12 14:33 WT_UUO4_515_Trimmed.fastq.gz
drwxrwxr-x  2 thimmamp g-thimmamp       4096 Jul 12 14:33 .
-rw-rw-r--  1 thimmamp g-thimmamp        128 Jul 12 14:39 SMOC2_NORMAL1_502_Trim_Summary.txt
-rw-rw-r--  1 thimmamp g-thimmamp 1196965868 Jul 12 14:39 SMOC2_NORMAL1_502_Trimmed.fastq.gz
```

Ensure good quality reads after trimming

```
(base) thimmamp@kw60284:/data1/BESE_COURSE2019$ ls -altr 5_TRIMMEDQC/
total 12932
-rw-rw-r--  1 thimmamp g-thimmamp 669484 Jul 14 09:42 WT_UUO4_515_Trimmed_fastqc.zip
-rw-rw-r--  1 thimmamp g-thimmamp 266988 Jul 14 09:42 WT_UUO4_515_Trimmed_fastqc.html
-rw-rw-r--  1 thimmamp g-thimmamp 672991 Jul 14 09:42 SMOC2_NORMAL1_502_Trimmed_fastqc.zip
-rw-rw-r--  1 thimmamp g-thimmamp 269897 Jul 14 09:42 SMOC2_NORMAL1_502_Trimmed_fastqc.html
-rw-rw-r--  1 thimmamp g-thimmamp 673070 Jul 14 09:42 SMOC2_UUO4_508_Trimmed_fastqc.zip
-rw-rw-r--  1 thimmamp g-thimmamp 267993 Jul 14 09:42 SMOC2_UUO4_508_Trimmed_fastqc.html
-rw-rw-r--  1 thimmamp g-thimmamp 669827 Jul 14 09:42 SMOC2_UUO2_506_Trimmed_fastqc.zip
-rw-rw-r--  1 thimmamp g-thimmamp 266057 Jul 14 09:42 SMOC2_UUO2_506_Trimmed_fastqc.html
-rw-rw-r--  1 thimmamp g-thimmamp 675974 Jul 14 09:42 SMOC2_NORMAL3_503_Trimmed_fastqc.zip
-rw-rw-r--  1 thimmamp g-thimmamp 268436 Jul 14 09:42 SMOC2_NORMAL3_503_Trimmed_fastqc.html
-rw-rw-r--  1 thimmamp g-thimmamp 674693 Jul 14 09:42 WT_NORMAL2_510_Trimmed_fastqc.zip
-rw-rw-r--  1 thimmamp g-thimmamp 267983 Jul 14 09:42 WT_NORMAL2_510_Trimmed_fastqc.html
-rw-rw-r--  1 thimmamp g-thimmamp 672270 Jul 14 09:43 WT_UUO1_512_Trimmed_fastqc.zip
-rw-rw-r--  1 thimmamp g-thimmamp 268423 Jul 14 09:43 WT_UUO1_512_Trimmed_fastqc.html
-rw-rw-r--  1 thimmamp g-thimmamp 671939 Jul 14 09:43 SMOC2_UUO3_507_Trimmed_fastqc.zip
-rw-rw-r--  1 thimmamp g-thimmamp 267285 Jul 14 09:43 SMOC2_UUO3_507_Trimmed_fastqc.html
-rw-rw-r--  1 thimmamp g-thimmamp 672269 Jul 14 09:43 SMOC2_NORMAL4_504_Trimmed_fastqc.zip
-rw-rw-r--  1 thimmamp g-thimmamp 267990 Jul 14 09:43 SMOC2_NORMAL4_504_Trimmed_fastqc.html
-rw-rw-r--  1 thimmamp g-thimmamp 673169 Jul 14 09:43 WT_UUO2_513_Trimmed_fastqc.zip
-rw-rw-r--  1 thimmamp g-thimmamp 267936 Jul 14 09:43 WT_UUO2_513_Trimmed_fastqc.html
-rw-rw-r--  1 thimmamp g-thimmamp 678017 Jul 14 09:43 WT_NORMAL1_509_Trimmed_fastqc.zip
-rw-rw-r--  1 thimmamp g-thimmamp 271753 Jul 14 09:43 WT_NORMAL1_509_Trimmed_fastqc.html
-rw-rw-r--  1 thimmamp g-thimmamp 670277 Jul 14 09:43 SMOC2_UUO1_505_Trimmed_fastqc.zip
-rw-rw-r--  1 thimmamp g-thimmamp 265757 Jul 14 09:43 SMOC2_UUO1_505_Trimmed_fastqc.html
-rw-rw-r--  1 thimmamp g-thimmamp 674604 Jul 14 09:43 WT_UUO3_514_Trimmed_fastqc.zip
-rw-rw-r--  1 thimmamp g-thimmamp 270375 Jul 14 09:43 WT_UUO3_514_Trimmed_fastqc.html
-rw-rw-r--  1 thimmamp g-thimmamp 671115 Jul 14 09:43 WT_NORMAL3_511_Trimmed_fastqc.zip
drwxrwxr-x  2 thimmamp g-thimmamp   4096 Jul 14 09:43 .
-rw-rw-r--  1 thimmamp g-thimmamp 265895 Jul 14 09:43 WT_NORMAL3_511_Trimmed_fastqc.html
```

Run FastQC on the trimmed data and make sure you got good quality reads to carry on for next step of analysis!

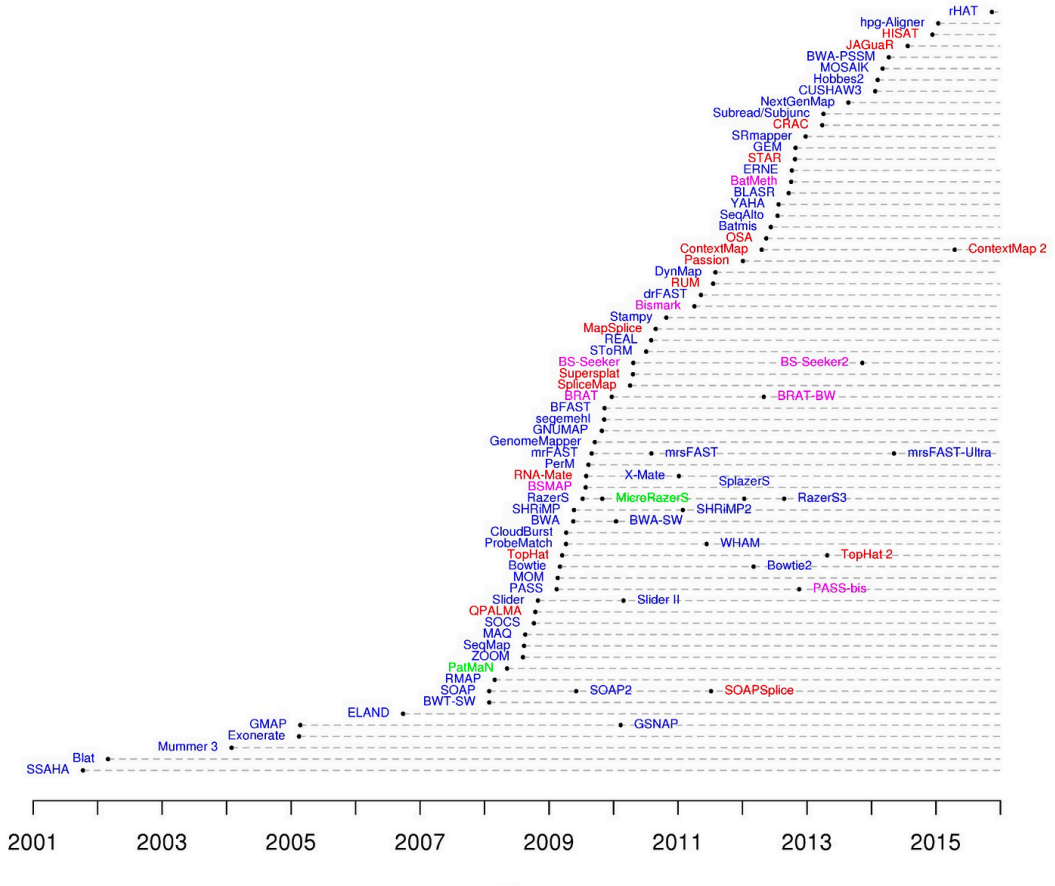
Module 4 : Mapping/aligning

- The Clean reads are next mapped to the reference genome of interest to get the quantification of genes/transcripts.

- We are going to use Tophat for this task.

- TopHat can identify splicing junctions without relying on database of known splice junctions.

Many Aligners available out there



How to compare read alignment software?

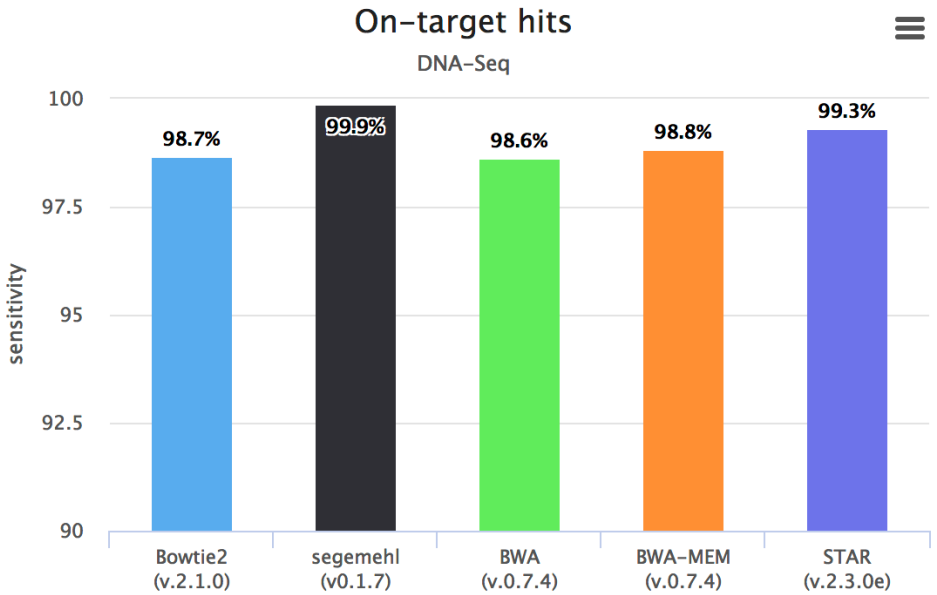
Every time a new read alignment software is developed and officially presented in a peer reviewed journal, the authors are asked to provide a comparison to existing tools. This is typically done in a benchmark where certain aspects of a software tool are assessed (ideally) in a scientifically sound manner. You can then compare these benchmarks and use them to decide on the optimal tool for your case. However, this procedure has its limitations: only a small set of the many aspects - typically things like mapping rate, sensitivity, speed - can be assessed in a short paper. And only certain program versions, parameter settings and data can be assessed.

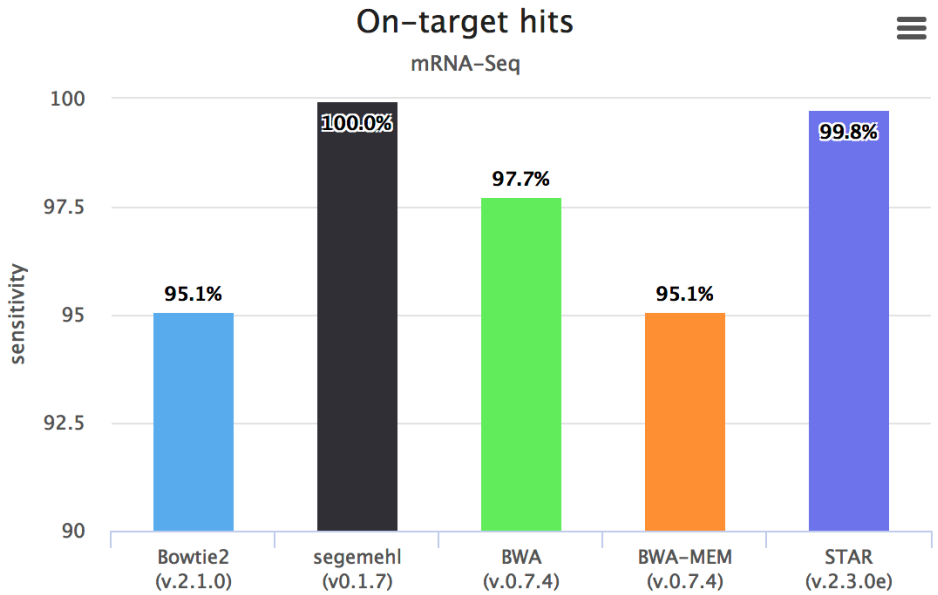
Comparing performance of aligners

Here, we provide detailed performance comparisons of NGS read aligners. In light of heated debates, we would like to stress that benchmarks only measure specific aspects and may not be used to claim any universal superiority or inferiority of a particular tool.

In order to compare different short read aligners, we use a published real-life paired-end DNA/RNA-Seq dataset. All optimal alignments (also multiple mapping loci) of 100,000 read pairs of each sample were obtained by RazerS 3 (full sensitivity mapping tool). In the benchmark shown below, we measured the performance in finding all optimal hits of different NGS mappers with default parameters. True positives are reads with up to 10 multiple mapping loci, allowing up to 10 errors (mismatches and indels).

Note that we explicitely want to find all multiple mapping loci in this benchmark and not only unique mapping loci or just one random hit of several. We believe that reads mapping multiple times should not be discarded since gene duplications and repeat regions are known to be biologically relevant.





Reference: https://www.ecseq.com/support/benchmark

How to decide on the best alignment software?

Assume you have a benchmark of your favorite alignment tools, what aspects should you look for? In general, you should try to answer the following questions:

1. What kind of sequences/experiment do you have? Do you have fragmented DNA inserts or spliced sequences from total RNA-seq? Is there a special protocol, DNA treatment or enrichment involved? What species is it and how is the quality of its genome assembly?
2. What sequencing platform do you have? Do you deal with Illumina,Ion Torrent reads or PacBio? Each machine has its characteristic read length and error types and not all mapping tools can handle them.
3. What kind of further analysis do you plan to carry out with the alignments? Do subsequent tools maybe depend on the reported alignment types and formats.
4. What kind of infrastructure do you have? Can you run your computations on a high performance computing cluster or does it need to run on your desktop computer?

Note that besides this “hard” benchmark there are also other factors to consider: is the output or input format of the program usable for you? Does the software have special features relevant for you? Is the program easy to use? Are there special license requirements or fees associated with it?

And the answer is...

...there is no best read aligner. It really depends on your goals and the specific case. What is the application? What sequencing technology has been used? What is the species? What are the computational constraints, etc.? You need to take into account the answer to those questions and then decide on the best read mapper according to the performance in the aspects important to you as well as in the software's features.

Alignment to reference genome

Alignment to reference genome/transcriptome

- **Goal is to find out where a read originated from**
  - Challenge: variants, sequencing errors, repetitive sequence
- **Mapping to**
  - transcriptome allows you to count hits to known transcripts
  - genome allows you to find new genes and transcripts
- **Many organisms have introns, so RNA-seq reads map to genome non-contiguously → spliced alignments needed**
  - Difficult because sequence signals at splice sites are limited and introns can be thousands of bases long

Old Tuxedo tools

NATURE PROTOCOLS | PROTOCOL

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

Cole Trapnell, Adam Roberts, Loyal Goff, Geo Pertea, Daehwan Kim, David R Kelley, Harold Pimentel, Steven L Salzberg, John L Rinn & Lior Pachter

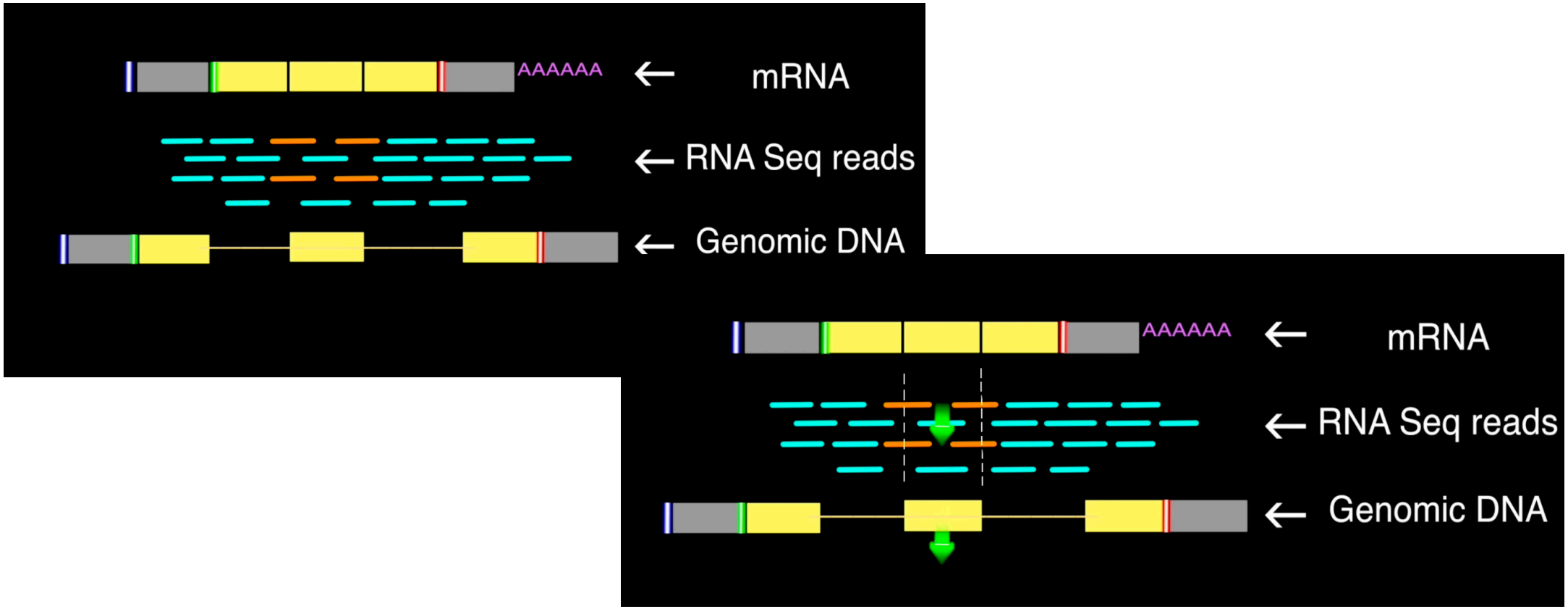
Affiliations | Contributions | Corresponding author

Nature Protocols **7**, 562–578 (2012) | doi:10.1038/nprot.2012.016
Published online 01 March 2012

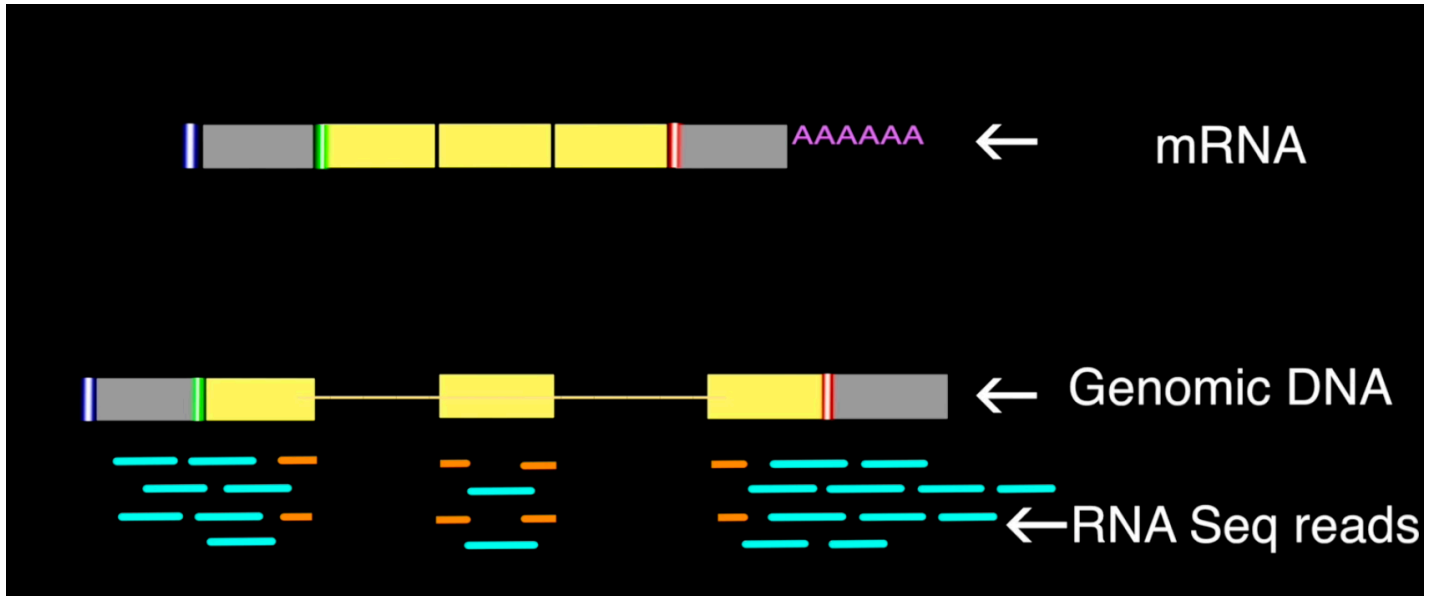
Abstract

Abstract · Introduction · Materials · Procedure · Troubleshooting · Timing · Anticipated results · Accession codes · References · Acknowledgments · Author information

Recent advances in high-throughput cDNA sequencing (RNA-seq) can reveal new genes and splice variants and quantify expression genome-wide in a single assay. The volume and complexity of data from RNA-seq experiments necessitate scalable, fast and mathematically principled analysis software. TopHat and Cufflinks are free, open-source software tools for gene discovery and comprehensive expression analysis of high-throughput mRNA sequencing (RNA-seq) data. Together, they allow biologists to identify new genes and new splice variants of known ones, as


AAAAAA
mRNA
RNA Seq reads
Genomic DNA
AAAAAA
mRNA
RNA Seq reads
Genomic DNA

Reads containing splicing junctions



TopHat builds the database of splice junctions from the alignment output.

By this method, TopHat can identify novel splice junctions as well.

Dr. Alagu will focus on alternative splicing on a exclusive session.

Splice-aware aligners

- **TopHat, HISAT (use Bowtie aligner internally)**
- **STAR**
- **GSNAP**
- **RUM**
- **MapSplice**

...

Systematic evaluation of spliced alignment programs for RNA-seq data

Pär G Engström[1,13], Tamara Steijger[1], Botond Sipos[1], Gregory R Grant[2,3], André Kahles[4,5], The RGASP Consortium[6], Gunnar Rätsch[4,5], Nick Goldman[1], Tim J Hubbard[7], Jennifer Harrow[7], Roderic Guigó[8,9] & Paul Bertone[1,10–12]

Nature methods 2013 (10:1185)

Mapping quality

- **Confidence in read's point of origin**
- **Depends on many things, including**
  - uniqueness of the aligned region in the genome
  - length of alignment
  - number of mismatches and gaps

- **Expressed in Phred scores, like base qualities**
  - $Q = -10 * \log_{10}$ (probability that mapping location is wrong)

- **TopHat mapping qualities**
  - 50 = unique mapping
  - 3 = maps to 2 locations
  - 1 = maps to 3-4 locations
  - 0 = maps to 5 or more locations

Mapping with Tophat
(more parameters than you care to care about)

- What do you absolutely need to specify for each run?

  - The genome index file (just the prefix)
  - The reads files, one for each paired end
  - The transcript files (gtf format)
  - The insert size (-r). This is the size of the fragment minus reads length
  - The library type (stranded or not, illumina-like or not)

Mapping with Tophat

… more parameters than you'd care to care about.

You need to give Tophat a reference genome for mapping your reads.

We actually give it an "index file" which is a compressed map of the genome.
There are 6 genome index files.
If you built these with bowtie1-build, they look like this

Just specify the prefix, e.g.: hg19

The genome index file (just the prefix)
The reads files, one for each paired end
The transcript files (gtf format)
The insert size (-r). This is the size of the fragment minus reads length
The library type (stranded or not, illumina-like or not)

Mapping with Tophat

… more parameters than you'd care to care about.

Next, we give Tophat your actual sequenced reads.

These are FastQ files, which contain both sequence information as well as quality scores for each read.
For paired end reads, we tell Tophat about both of them, (pair the reads) so it can try to map them together.

Example: First WT replicate (rep1); Read 1 paired with Read 2

wt-rep1_R1.fq.gz
wt-rep1_R2.fq.gz

The genome index file (just the prefix)
The reads files, one for each paired end
The transcript files (gtf format)
The insert size (-r). This is the size of the fragment minus reads length
The library type (stranded or not, illumina-like or not)

Mapping with Tophat

… more parameters than you'd care to care about.

Tophat works much better if you give it the exon/intron structure of known genes, i.e., a gtf file.

You can still choose to find novel transcripts later, but providing this file makes it easier to find reads that span known introns, which makes the search faster.

Example: the human file is hg19_refseq_genes.gtf

****Be careful – tophat is very picky about the format of this file. Either download one from their website, or get someone to give you one that plays nicely with tophat.**

The genome index file (just the prefix)
The reads files, one for each paired end
The transcript files (gtf format)
The insert size (-r). This is the size of the fragment minus reads length
The library type (stranded or not, illumina-like or not)

Mapping with Tophat

… more parameters than you'd care to care about.

Mate Inner Distance

Let's say your RNAseq library prep kit preferentially fragments the RNA into 500 bp fragments. Then, you did a PE100bp run.

Your "insert size" is 500 – 100 – 100 = **300**.

You did a PE300 run? Okay, your insert size is 0.

Why does Tophat care? Tophat judges how well the two ends of a paired-end segment mapped by whether the inferred distance between them is consistent with the expected insert size. This is part of "mapping quality."

The genome index file (just the prefix)

The reads files, one for each paired end

The transcript files (gtf format)

The mate inner distance (-r). This "insert size" is the size of the fragment minus reads length

The library type (stranded or not, illumina-like or not)

Mapping with Tophat

… more parameters than you'd care to care about.

In general, an Illumina Tru-seq Stranded RNA-seq library prep kit should use **fr-firststrand.** That means the library is stranded and the complementary strand is the first one sequenced. In other words, all your reads are the reverse-complement of the transcriptome!

The other options:

fr-unstranded	common for non-stranded libraries
fr-secondstrand	used for ABI Solid libraries – not common

The genome index file (just the prefix)
The reads files, one for each paired end
The transcript files (gtf format)
The insert size (-r). This is the size of the fragment minus reads length
The library type (stranded or not, illumina-like or not)

Mapping with Tophat

What other options might you care to change?

Preset default options

-N 2 number of mismatches per read (default is 2)

-g 2 maximum number of alignments per read to report (default is 2)

--suppress-hits set this if you want to suppress reads that map more than max (2)

--no-mixed don't report an alignment if you can't map both ends of the fragment

--no-novel-juncs don't look for novel splice junctions – just use the ones in the GTF file
*(if you will be skipping CuffLinks, you must run TopHat Advanced and choose this option to **not** look for novel junction)*

Mapping with Tophat

What should your output look like?

A typical tophat output directory should have these files:
(The output files will be in your Project folder in the Data Store)

accepted_hits.bam deletions.bed junctions.bed **logs**
insertions.bed left_kept_reads.info right_kept_reads.info

The bam file is your main output – the aligned reads.
It's in binary sam format.
Here is an example of what one line of a bam file looks like:

read name | map flags | map position chr/start | map quality | other PE read is mapped to same chr (=) at pos 709587, which is 699060 bp away

```
MENDEL_0001_FC61FR7AAXX7:69:18748:7104#0        81          chr1        10563       255         36M         =
            709587      699060      CGCAGCTCCGCCCTCGCGGTGCTCTCCGGGTCTGTG
            CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC                NM:i:0      NH:i:1
```

read sequence | read qualities | tophat flags

NM:i:0 = 0 mismatches
NH:i:1 = aligns uniquely

Genome Annotation and output bam file

```
chr12   unknown exon    96066054        96067770        .       +       .
gene_id "PGAM1P5"; gene_name "PGAM1P5"; transcript_id "NR_077225"; tss_id
"TSS14770";
chr12   unknown CDS     96076483        96076598        .       -       1
gene_id "NTN4"; gene_name "NTN4"; p_id "P12149"; transcript_id
"NM_021229"; tss_id "TSS6395";
chr12   unknown exon    96076483        96076598        .       -       .
gene_id "NTN4"; gene_name "NTN4"; p_id "P12149"; transcript_id
"NM_021229"; tss_id "TSS6395";
chr12   unknown CDS     96077274        96077487        .       -       2
gene_id "NTN4"; gene_name "NTN4"; p_id "P12149"; transcript_id
"NM_021229"; tss_id "TSS6395";
chr12   unknown exon    96077274        96077487        .       -       .
gene_id "NTN4"; gene_name "NTN4"; p_id "P12149"; transcript_id
"NM_021229"; tss_id "TSS6395";
chr12   unknown CDS     96104219        96104407        .       -       2
gene_id "NTN4"; gene_name "NTN4"; p_id "P12149"; transcript_id
"NM_021229"; tss_id "TSS6395";
chr12   unknown exon    96104219        96104407        .       -       .
gene_id "NTN4"; gene_name "NTN4"; p_id "P12149"; transcript_id
"NM_021229"; tss_id "TSS6395";
```

```
HWUSI-EAS525_0042_FC:6:23:10200:18582#0/1    16    1    10    40    35M
*    0    0    AGCCAAAGATTGCATCAGTTCTGCTGCTATTTCCT
agafgfaffcfdf[fdcffcggggccfdffagggg    MD:Z:35 NH:i:1  HI:i:1  NM:i:0  SM:i:40
XQ:i:40 X2:i:0
HWUSI-EAS525_0042_FC:3:28:18734:20197#0/1    16    1    10    40    35M
*    0    0    AGCCAAAGATTGCATCAGTTCTGCTGCTATTTCCT
hghhghhhhhhhhhhhhhhhhghhhhhghhfhhh    MD:Z:35 NH:i:1  HI:i:1  NM:i:0
SM:i:40 XQ:i:40 X2:i:0
HWUSI-EAS525_0042_FC:3:94:1587:14299#0/1    16    1    10    40    35M
*    0    0    AGCCAAAGATTGCATCAGTTCTGCTGCTATTTCCT
hfhghhhhhhhhhhghhhhhhhhhhhhhhhhhhhg    MD:Z:35 NH:i:1  HI:i:1  NM:i:0
SM:i:40 XQ:i:40 X2:i:0
D3B4KKQ1:227:D0NE9ACXX:3:1305:14212:73591    0    1    11    40    51M
*    0    0    GCCAAAGATTGCATCAGTTCTGCTGCTATTTCCTCCTATCATTCTTTCTGA
CCCFFFFFFGFFHHJGIHHJJJFGGJJGIIIIIIGJJJJJJJJJIJJJJJE    MD:Z:51 NH:i:1  HI:i:1
NM:i:0  SM:i:40 XQ:i:40 X2:i:0
HWUSI-EAS525_0038_FC:5:35:11725:5663#0/1    16    1    11    40    35M
*    0    0    GCCAAAGATTGCATCAGTTCTGCTGCTATTTCCTC
hhehhhhhhhhhghghhhhhhhhhhhhhhhhhhh    MD:Z:35 NH:i:1  HI:i:1  NM:i:0
SM:i:40 XQ:i:40 X2:i:0
```

Hands on For Mapping

time tophat2 -p 24 –o WT_NORMAL1_509_tophathout /ibex/scratch/projects/c2024/BESE_COURSE2019/mm10/Sequence/Bowtie2Index/genome /ibex/scratch/projects/c2024/BESE_COURSE2019/4_TRIMMING/WT_NORMAL1_509_Trimmed.fastq.gz

Output looks like this:

[2019-07-12 16:14:43] Beginning TopHat run (v2.1.1)

[2019-07-12 16:14:43] Checking for Bowtie

Bowtie version: 2.2.6.0

[2019-07-12 16:14:43] Checking for Bowtie index files (genome)..

[2019-07-12 16:14:43] Checking for reference FASTA file

[2019-07-12 16:14:43] Generating SAM header for /home/thimmamp/References/mm10/Mus_musculus/UCSC/mm10/Sequence/Bowtie2Index/genome

[2019-07-12 16:15:11] Preparing reads

left reads: min. length=75, max. length=75, 34183396 kept reads (5258 discarded)

[2019-07-12 16:20:16] Mapping left_kept_reads to genome genome with Bowtie2

[2019-07-12 16:32:56] Mapping left_kept_reads_seg1 to genome genome with Bowtie2 (1/3)

[2019-07-12 16:34:08] Mapping left_kept_reads_seg2 to genome genome with Bowtie2 (2/3)

[2019-07-12 16:35:21] Mapping left_kept_reads_seg3 to genome genome with Bowtie2 (3/3)

[2019-07-12 16:36:31] Searching for junctions via segment mapping

[2019-07-12 16:38:46] Retrieving sequences for splices

[2019-07-12 16:39:56] Indexing splices

Building a SMALL index

[2019-07-12 16:40:07] Mapping left_kept_reads_seg1 to genome segment_juncs with Bowtie2 (1/3)

[2019-07-12 16:40:43] Mapping left_kept_reads_seg2 to genome segment_juncs with Bowtie2 (2/3)

[2019-07-12 16:41:17] Mapping left_kept_reads_seg3 to genome segment_juncs with Bowtie2 (3/3)

[2019-07-12 16:41:47] Joining segment hits

[2019-07-12 16:43:47] Reporting output tracks

Mapping QC

Information we need to check

- Percentage of reads properly mapped or uniquely mapped
- Among the mapped reads, the percentage of reads in exon, intron, and intergenic regions.
- 5' or 3' bias
- The percentage of expressed genes

Genome analysis **2012, Bioinformatics** Advance Access publication April 25, 2012

RNA-SeQC: RNA-seq metrics for quality control and process optimization

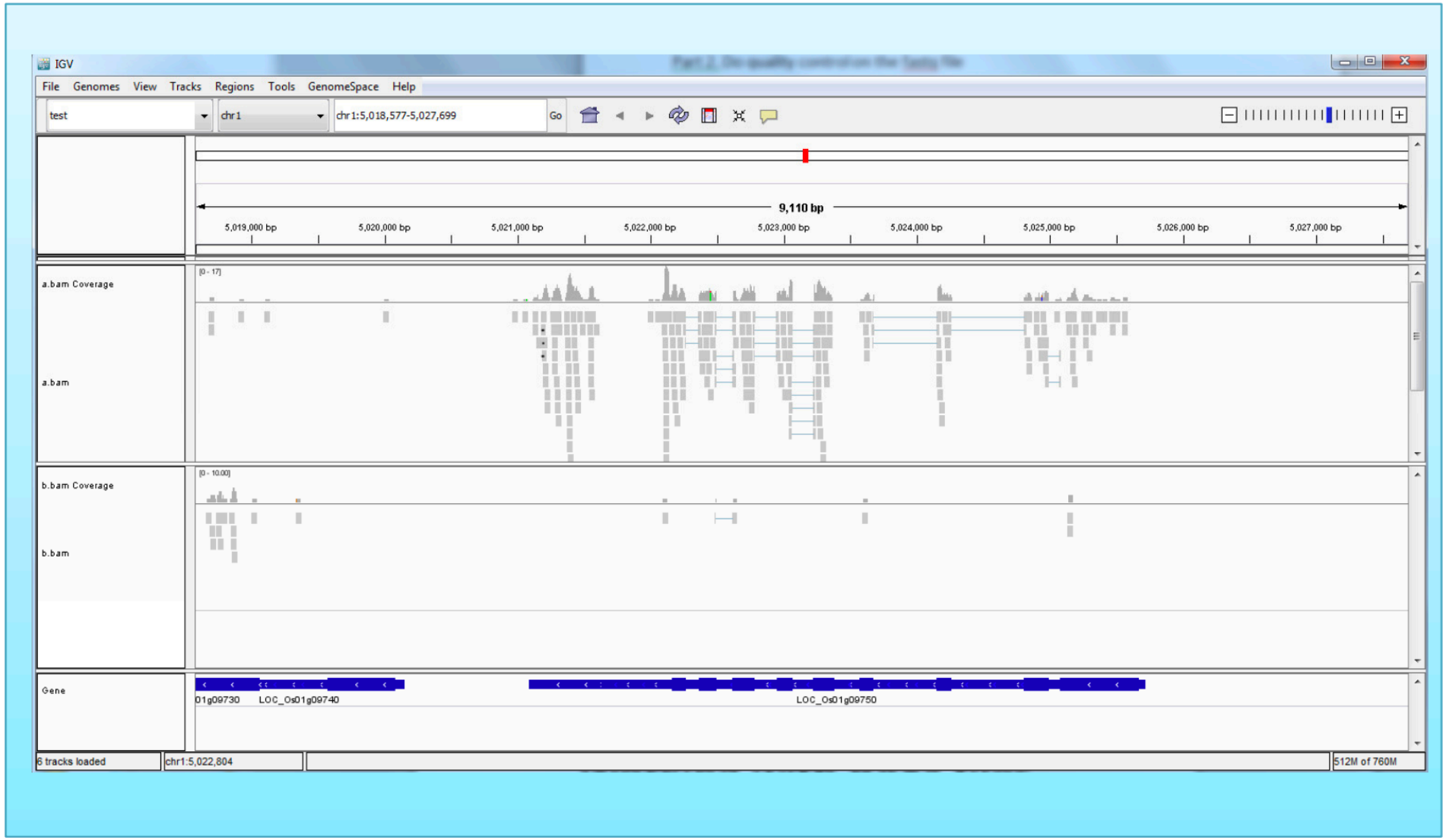
David S. DeLuca*, Joshua Z. Levin, Andrey Sivachenko, Timothy Fennell,
Marc-Danie Nazaire, Chris Williams, Michael Reich, Wendy Winckler and Gad Getz*
The Broad Institute of MIT and Harvard, Cambridge, MA, USA

- Read Metrics
  - Total, unique, duplicate reads
  - Alternative alignment reads
  - Read Length
  - Fragment Length mean and standard deviation
  - Read pairs: number aligned, unpaired reads, base mismatch rate for each pair mate, chimeric pairs
  - Vendor Failed Reads
  - Mapped reads and mapped unique reads
  - rRNA reads
  - Transcript-annotated reads (intragenic, intergenic, exonic, intronic)
  - Expression profiling efficiency (ratio of exon-derived reads to total reads sequenced)
  - Strand specificity
- Coverage
  - Mean coverage (reads per base)
  - Mean coefficient of variation
  - 5'/3' bias
  - Coverage gaps: count, length
  - Coverage Plots
- Downsampling
- GC Bias
- Correlation:
  - Between sample(s) and a reference expression profile
  - When run with multiple samples, the correlation between every sample pair is reported

https://confluence.broadinstitute.org/display/CGATools/RNA-SeQC

Visualizing BAM files with IGV

* Before using IGV, the BAM files need to be indexed with “samtools index”, which creates a .bai file.



Quality of mapping

Mapping quality

- **Confidence in read's point of origin**
- **Depends on many things, including**
  - uniqueness of the aligned region in the genome
  - length of alignment
  - number of mismatches and gaps

- **Expressed in Phred scores, like base qualities**
  - $Q = -10 * \log_{10}$ (probability that mapping location is wrong)

- **TopHat mapping qualities**
  - 50 = unique mapping
  - 3 = maps to 2 locations
  - 1 = maps to 3-4 locations
  - 0 = maps to 5 or more locations

How to look at the quality of mapping

```
(base) thimmamp@kw60284:/data1/BESE_COURSE2019$ samtools view 7_MAPPING/WT_NORMAL1_509_tophathout/accepted_hits.bam | grep "
NH:i:1" | wc -l
28843809
(base) thimmamp@kw60284:/data1/BESE_COURSE2019$ samtools view 7_MAPPING/WT_NORMAL1_509_tophathout/accepted_hits.bam | grep "
NH:i:1" | head
SRR4000509.26291084     0       chr1    3031449 0       75M     *       0       0       GCCAGAGTGCAAGAAGCAAGAGAGCAAGAAGCAAGA
GAGAGAGAAAACGAAACCCCGTCCCTATTAAGGAGAATT AAAAAEEAEEEEEEEEEEEEEAEEEEEEEEEEEEEEEEEEEEEEEEEAA/EEEEEEEEEEEEEEEEEEE/E     AS:i
:0      XN:i:0  XM:i:0  XO:i:0  XG:i:0  NM:i:0  MD:Z:75 YT:Z:UU NH:i:17 CC:Z:=  CP:i:181263616  HI:i:0
SRR4000509.6408277      272     chr1    3053490 0       75M     *       0       0       GTGCTCGCCTCCTAGCCCTTACTGAAGATTTTTTAG
AAAGCTCCCAATCGCCATCTCACAAAAGCAAAACTAAGC E/EAAEAEEEEEEEEAEEEEAEEEEEEEEEEEAAEEEEEEEEEEEAEEEEEEEEEEAEEEEEEEEEEEEEAAAA6     AS:i
:0      XN:i:0  XM:i:0  XO:i:0  XG:i:0  NM:i:0  MD:Z:75 YT:Z:UU NH:i:13 CC:Z:chr15      CP:i:9225541    HI:i:0
SRR4000509.19622941     272     chr1    3053490 0       75M     *       0       0       GTGCTCGCCTCCTAGCCCTTACTGAAGATTTTTTAG
AAAGCTCCCAATCGCCATCTCACAAAAGCAAAACTAAGC A/EAAEEEAEEEEEEEEEEEEEE<EAEEEEEEEEEEEEEEEEEEEEEEEEEEE6EEEEEEEEEAEAAAAA     AS:i
:0      XN:i:0  XM:i:0  XO:i:0  XG:i:0  NM:i:0  MD:Z:75 YT:Z:UU NH:i:13 CC:Z:chr15      CP:i:9225541    HI:i:0
SRR4000509.16564974     272     chr1    3054452 0       75M     *       0       0       CTCCCACTATTCGGCCATTTCCCTTATATGAAAGAG
GAGTGAGGACCTTCCTCCCTGGGATCCTCAGAAGTCTAC EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEAEEEEEAAAAA     AS:i
:-5     XN:i:0  XM:i:1  XO:i:0  XG:i:0  NM:i:1  MD:Z:39C35      YT:Z:UU NH:i:16 CC:Z:=  CP:i:16833476   HI:i:0
SRR4000509.26549395     272     chr1    3054480 0       75M     *       0       0       TGAAAGAGGAGTGAGGACCTTCCTCCCTGGGATCCT
CAGAAGTCTACTGGGCACAAAAAATAACTGATTCATGGC E<EEAEEEEEE/EEEEE<EEEEEEEEEEEEEEEEAEEEEEEEEEEEEEEEEEEEEEE6EEEEEEEEEAAAAA     AS:i
:-8     XN:i:0  XM:i:2  XO:i:0  XG:i:0  NM:i:2  MD:Z:11C43G19   YT:Z:UU NH:i:15 CC:Z:=  CP:i:16833448   HI:i:0
SRR4000509.6304667      272     chr1    3056106 0       75M     *       0       0       ATAGCTATAAAAAGTAAAAGTCTTTTATTGATCTAA
ATTTATAAGATTCGTGAAGCCACTTCATTTGGTTTTTGA EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEAAAAA     AS:i
:-5     XN:i:0  XM:i:1  XO:i:0  XG:i:0  NM:i:1  MD:Z:25A49      YT:Z:UU NH:i:18 CC:Z:chr12      CP:i:23701234   HI:i:0
SRR4000509.34245684     16      chr1    3057761 50      75M     *       0       0       ACCATGGGCTGCAGCAAGGAGTACCAACCTGGTGCC
AACTTAAAATAAAAACAACTAAAAAAGCTGACTCCTGAG EEEEEEEEEEEEEEEEEEEEEEEAEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEAAAAA     AS:i
:-5     XN:i:0  XM:i:1  XO:i:0  XG:i:0  NM:i:1  MD:Z:70G4       YT:Z:UU NH:i:1
SRR4000509.28556553     272     chr1    3058013 0       75M     *       0       0       TTCTTGTCAATTAACTAATTGTGTAGATCCTAATTT
AGATAAGGAATCTACTGTTATGTTTTTGTAAGAGACCTG EEEEEEEEEEAE<EEAEE/EEEAEEEEAEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEAAAAA     AS:i
:0      XN:i:0  XM:i:0  XO:i:0  XG:i:0  NM:i:0  MD:Z:75 YT:Z:UU NH:i:11 CC:Z:chr3       CP:i:24237730   HI:i:0
SRR4000509.27411499     256     chr1    3066281 0       75M     *       0       0       CCATTTGTCAATTCTCGATCTTACAGCACAAGCCAT
TACTGTTCTGTTCAGGAATTTTTCCCCTGTGCCCATATC AAAAAEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE/EEEEEEEEEEEEEEEEEEEEEEEEEEE<EEEEEE     AS:i
:0      XN:i:0  XM:i:0  XO:i:0  XG:i:0  NM:i:0  MD:Z:75 YT:Z:UU NH:i:13 CC:Z:chr10      CP:i:65670410   HI:i:0
SRR4000509.22830477     256     chr1    3087813 0       75M     *       0       0       GTCCTCTCTTTCCAGTGATGGCCGACTAGGCCATCT
TTTGATACATATGCAGCTAGAGTCAAGAGCTCCGGGGTA AAAAAEEEEAEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE     AS:i
:0      XN:i:0  XM:i:0  XO:i:0  XG:i:0  NM:i:0  MD:Z:75 YT:Z:UU NH:i:17 CC:Z:=  CP:i:94587835   HI:i:0
```

```
(base) thimmamp@kw60284:/data1/BESE_COURSE2019$ cat 7_MAPPING/WT_NORMAL1_509_tophathout/align_summary.txt
Reads:
          Input     :  34188654
           Mapped   :  32668795 (95.6% of input)
            of these:   4936701 (15.1%) have multiple alignments (320 have >20)
95.6% overall read mapping rate.
```

Counting rules

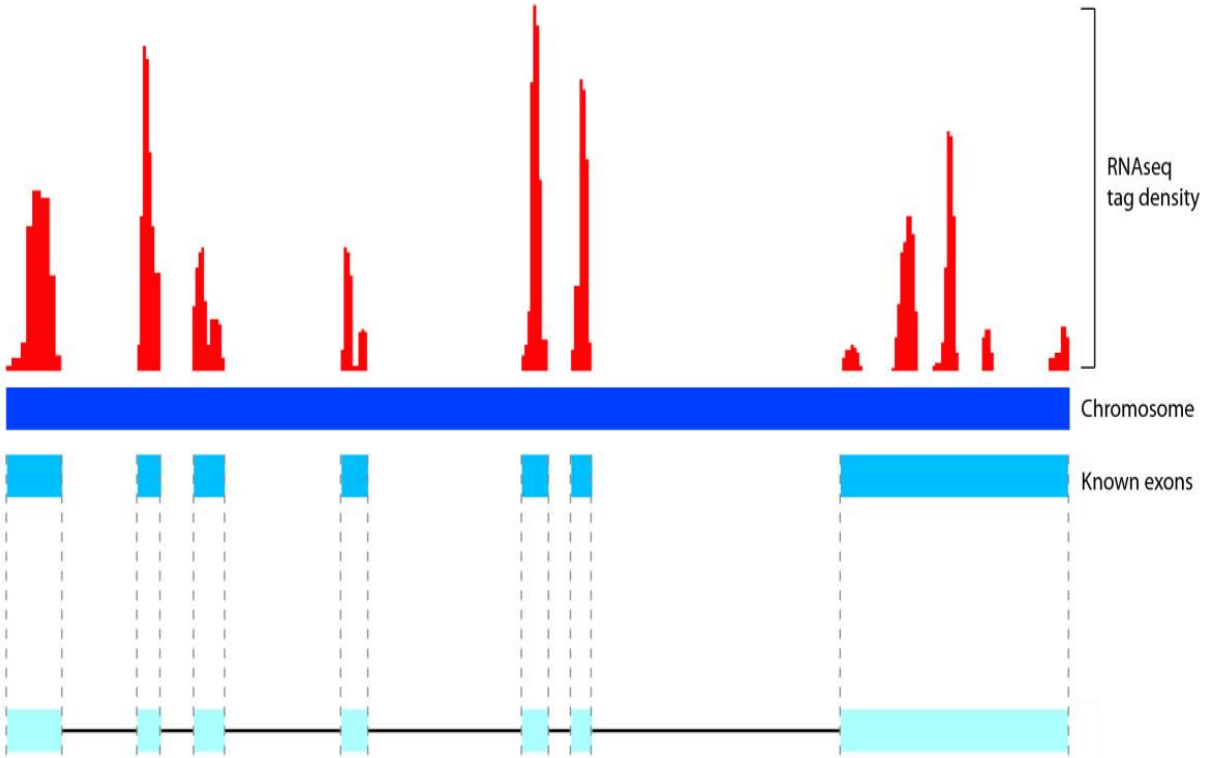
- Count reads, not base-pairs
- Count each read at most once.
- Discard a read if
  - it cannot be uniquely mapped
  - its alignment overlaps with several genes
  - the alignment quality score is bad
  - (for paired-end reads) the mates do not map to the same gene

Expression quantification

FPKM /RPKM

- Count data
  - Summarized mapped reads to CDS, gene or exon level

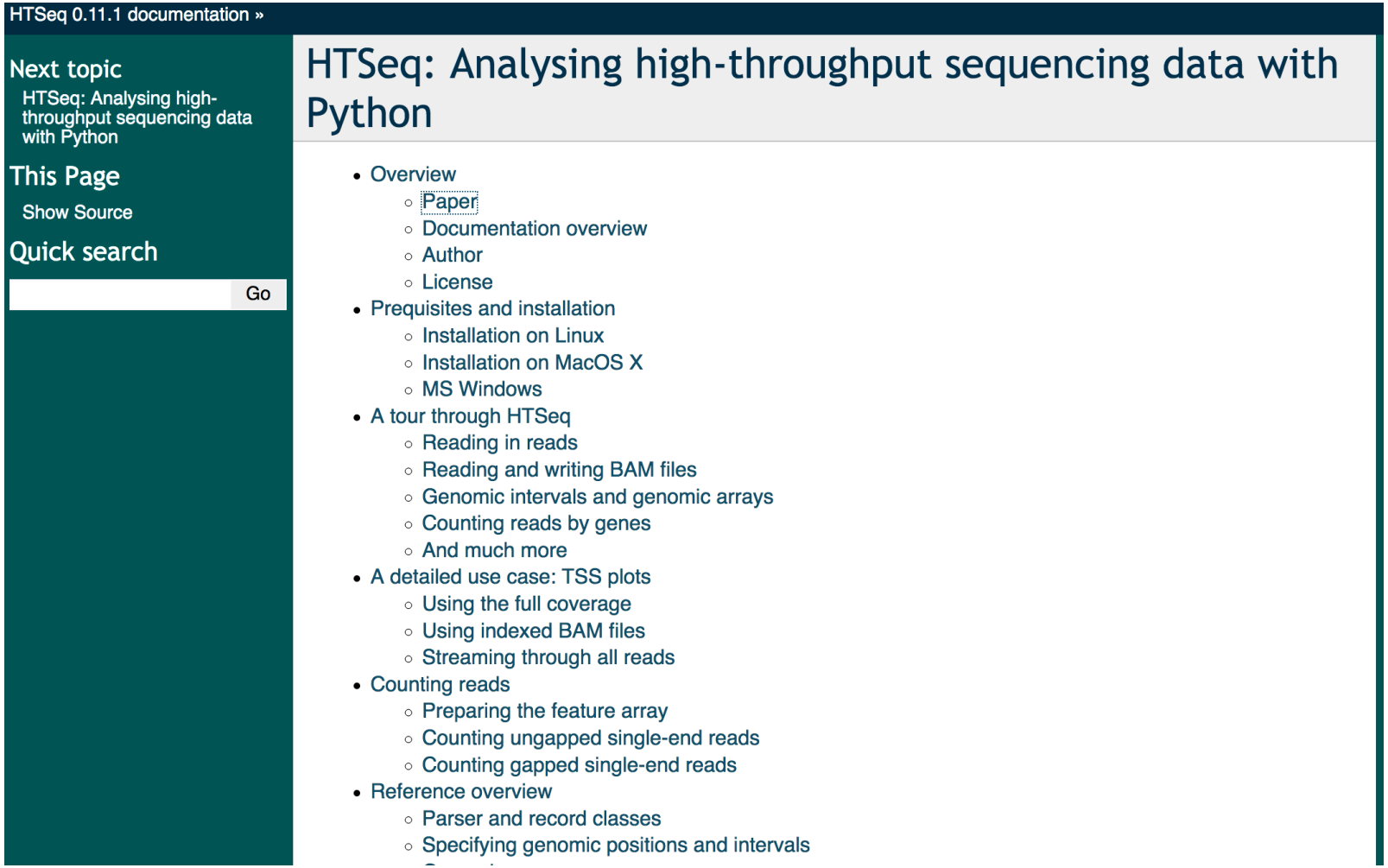
$$FPKM = \frac{Counts\ of\ mapped\ fragments}{Total\ mapped\ fragments\ (million) \times Exon\ length\ of\ transcript\ (KB)}$$



End of Module 4 : Mapping

Next Module 5: Quantification

Module 5: Quantification using HTSEQ-Count



HTSeq 0.11.1 documentation »

Next topic

HTSeq: Analysing high-throughput sequencing data with Python

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HTSeq: Analysing high-throughput sequencing data with Python

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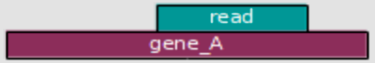
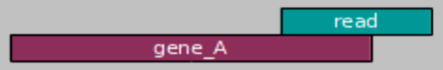
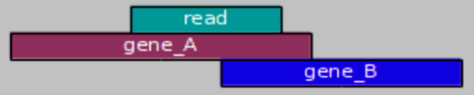
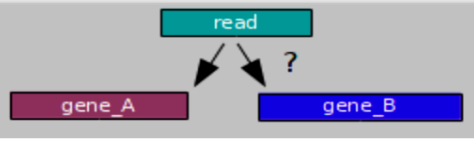
- A tour through HTSeq
  - Reading in reads
  - Reading and writing BAM files
  - Genomic intervals and genomic arrays
  - Counting reads by genes
  - And much more
- A detailed use case: TSS plots
  - Using the full coverage
  - Using indexed BAM files
  - Streaming through all reads
- Counting reads
  - Preparing the feature array
  - Counting ungapped single-end reads
  - Counting gapped single-end reads
- Reference overview
  - Parser and record classes
  - Specifying genomic positions and intervals

How to assign reads to features

The following figure illustrates the effect of these three modes and the `--nonunique` option:

	union	intersection_strict	intersection_nonempty
	gene_A	gene_A	gene_A
	gene_A	no_feature	gene_A
	gene_A	no_feature	gene_A
	gene_A	gene_A	gene_A
	gene_A	gene_A	gene_A
	ambiguous (both genes with --nonunique all)	gene_A	gene_A
	ambiguous (both genes with --nonunique all)		
	alignment_not_unique (both genes with --nonunique all)		

Install HTSeq python module

Prequisites and installation

HTSeq is available from the Python Package Index (PyPI):

To use HTSeq, you need Python 2.7 or 3.4 or above (3.0-3.3 are not supported), together with:

- NumPy, a commonly used Python package for numerical calculations
- Pysam, a Python interface to samtools.
- To make plots you will need matplotlib, a plotting library.

At the moment, HTSeq supports Linux and OSX but not Windows operating systems, because one of the key dependencies, Pysam, lacks automatic support and none of the HTSeq authors have access to such a machine. However, it *might* work with some work, if you need support for this open an issue on our Github page.

HTSeq follows install conventions of many Python packages. In the best case, it should install from PyPI like this:

```
pip install HTSeq
```

If this does not work, please open an issue on Github and also try the instructions below.

Installation on Linux

You can choose to install HTSeq via your distribution packages or via *pip*. The former is generally recommended but might be updated less often than the *pip* version.

Distribution package manager

- Ubuntu (e.g. for Python 2.7):

```
sudo apt-get install build-essential python2.7-dev python-numpy python-matplotlib python-pysam py
```

- Arch (e.g. using `aura`, you can grab the AUR packages otherwise):

```
sudo pacman -S python python-numpy python-matplotlib
sudo aura -A python-pysam python-htseq
```

Counting with htseq-count

Counting reads in features with `htseq-count`

Given a file with aligned sequencing reads and a list of genomic features, a common task is to count how many reads map to each feature.

A feature is here an interval (i.e., a range of positions) on a chromosome or a union of such intervals.

In the case of RNA-Seq, the features are typically genes, where each gene is considered here as the union of all its exons. One may also consider each exon as a feature, e.g., in order to check for alternative splicing. For comparative ChIP-Seq, the features might be binding region from a pre-determined list.

Special care must be taken to decide how to deal with reads that align to or overlap with more than one feature. The `htseq-count` script allows to choose between three modes. Of course, if none of these fits your needs, you can write your own script with HTSeq. See the chapter A tour through HTSeq for a step-by-step guide on how to do so. See also the FAQ at the end, if the following explanation seems too technical.

The three overlap resolution modes of `htseq-count` work as follows. For each position *i* in the read, a set *S(i)* is defined as the set of all features overlapping position *i*. Then, consider the set *S*, which is (with *i* running through all position within the read or a read pair)

- the union of all the sets *S(i)* for mode `union`. This mode is recommended for most use cases.
- the intersection of all the sets *S(i)* for mode `intersection-strict`.
- the intersection of all non-empty sets *S(i)* for mode `intersection-nonempty`.

If *S* contains precisely one feature, the read (or read pair) is counted for this feature. If *S* is empty, the read (or read pair) is counted as `no_feature`. If *S* contains more than one feature, `htseq-count` behaves differently based on the `--nonunique` option:

- `--nonunique none` (default): the read (or read pair) is counted as `ambiguous` and not counted for any features. Also, if the read (or read pair) aligns to more than one location in the reference, it is scored as `alignment_not_unique`.
- `--nonunique all`: the read (or read pair) is counted as `ambiguous` and is also counted in all features to which it was assigned. Also, if the read (or read pair) aligns to more than one location in the reference, it is scored as `alignment_not_unique` and also separately for each location.

Notice that when using `--nonunique all` the sum of all counts will not be equal to the number of reads (or read pairs), because those with multiple alignments or overlaps get scored multiple times.

The following figure illustrates the effect of these three modes and the `--nonunique` option:

Run HTSeq on our bam file

Hands on

htseq-count -i gene_id --mode=union --nonunique=none --format=bam <bam file> <reference_genome_gtf_file> > samplename_count_table.txt

Output:

- head 8_COUNTING/WT_NORMAL2_510_count_table.txt
- Gene WT_NORMAL2_510
- 0610005C13Rik 3
- 0610007P14Rik 25
- 0610009B22Rik 0
- 0610009L18Rik 2
- 0610009O20Rik 12
- 0610010B08Rik 0
- 0610010F05Rik 0
- 0610010K14Rik 5
- 0610011F06Rik 2

Merge the counts from all samples into one file

- You need to know how to write python or R script to merge all the count files from individual samples into one single master count file!

```
smoc2_norm1 <- read.table(file = "SMOC2_NORMAL1_502_count_table.txt", header=TRUE,sep = "\t")
smoc2_norm3 <- read.table(file = "SMOC2_NORMAL3_503_count_table.txt", header=TRUE, sep = "\t")
smoc2_norm4 <- read.table(file = "SMOC2_NORMAL4_504_count_table.txt", header=TRUE,sep = "\t")
smoc2_uuo1 <- read.table(file = "SMOC2_UUO1_505_count_table.txt", header=TRUE,sep = "\t")
smoc2_uuo2 <- read.table(file = "SMOC2_UUO2_506_count_table.txt", header=TRUE,sep = "\t")
smoc2_uuo3 <- read.table(file = "SMOC2_UUO3_507_count_table.txt", header=TRUE,sep = "\t")
smoc2_uuo4 <- read.table(file = "SMOC2_UUO4_508_count_table.txt", header=TRUE,sep = "\t")
wt_norm1 <- read.table(file = "WT_NORMAL1_509_count_table.txt", header=TRUE,sep = "\t")
wt_norm2 <- read.table(file = "WT_NORMAL2_510_count_table.txt", header=TRUE,sep = "\t")
wt_norm3 <- read.table(file = "WT_NORMAL3_511_count_table.txt", header=TRUE,sep = "\t")
wt_uuo1 <- read.table(file = "WT_UUO1_512_count_table.txt", header=TRUE,sep = "\t")
wt_uuo2 <- read.table(file = "WT_UUO2_513_count_table.txt", header=TRUE,sep = "\t")
wt_uuo3 <- read.table(file = "WT_UUO3_514_count_table.txt", header=TRUE,sep = "\t")
wt_uuo4 <- read.table(file = "WT_UUO4_515_count_table.txt", header=TRUE,sep = "\t")
mergeCol = c("Gene")
 #<- merge(smoc2_norm1, smoc2_norm3, by=mergeCol, all = TRUE)
final <- Reduce(function(x, y) merge(x, y, by=mergeCol, all=TRUE), list(smoc2_norm1, smoc2_norm3,
                                                                        smoc2_norm4, smoc2_uuo1, smoc2_uuo2, smoc2_uuo3, sm
oc2_uuo4,
                                                                        wt_norm1, wt_norm2, wt_norm3,
                                                                        wt_uuo1, wt_uuo2, wt_uuo3, wt_uuo4))


mydf <- final[, -1] # all sample columns except gene name
rownames(mydf) <- final[,1] # now get gene column
head(mydf)
dataForDE <- mydf[rowSums(mydf)>0, ] # filter out genes with 0 for all samples
write.table(dataForDE, file = "Samples_Merged.txt", sep = "\t")
```

End of Quantification

Next Mopdule 6: DE Analysis


THANK YOU!
QUESTIONS?