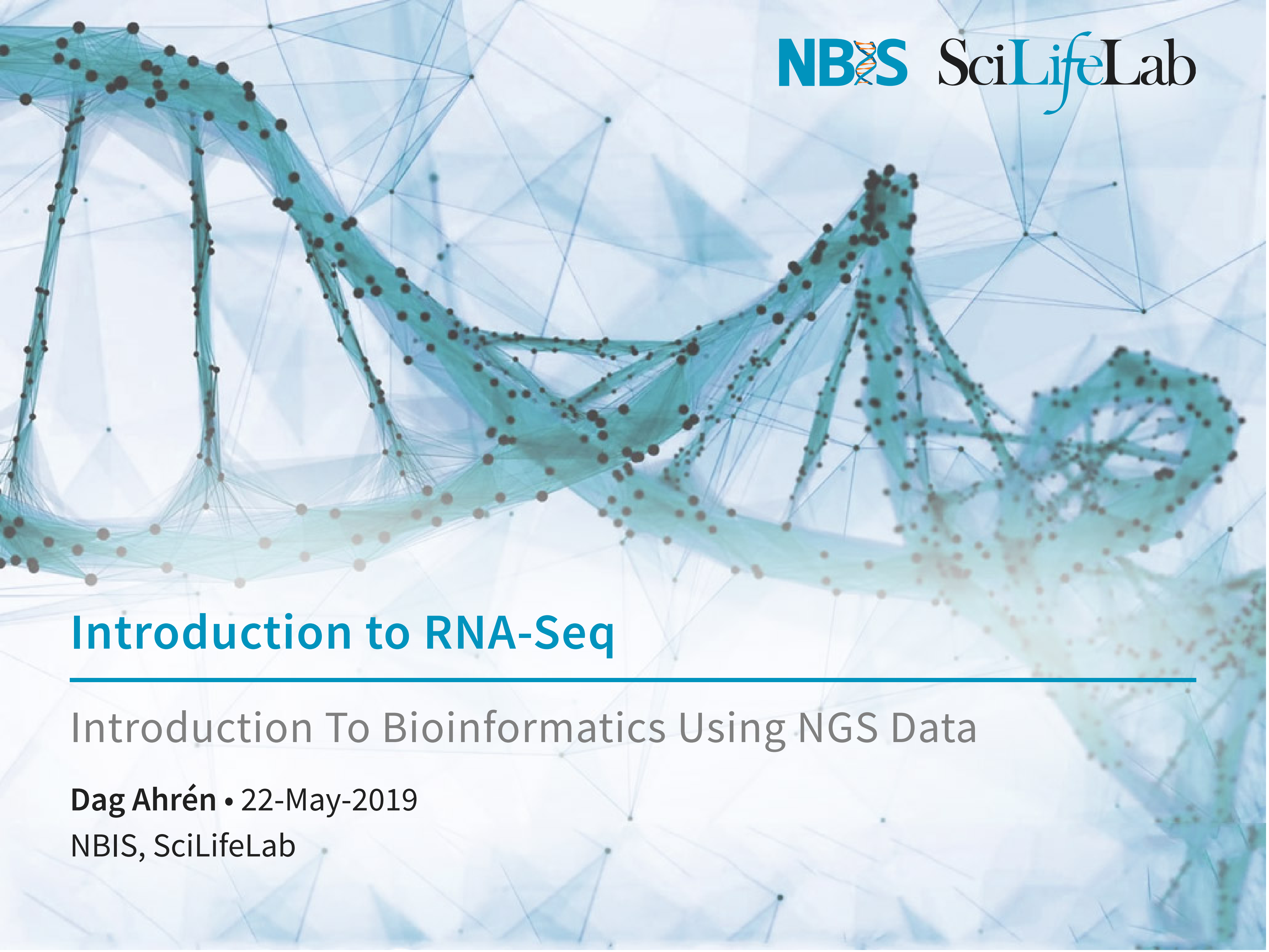


Introduction to RNA-Seq

Introduction To Bioinformatics Using NGS Data

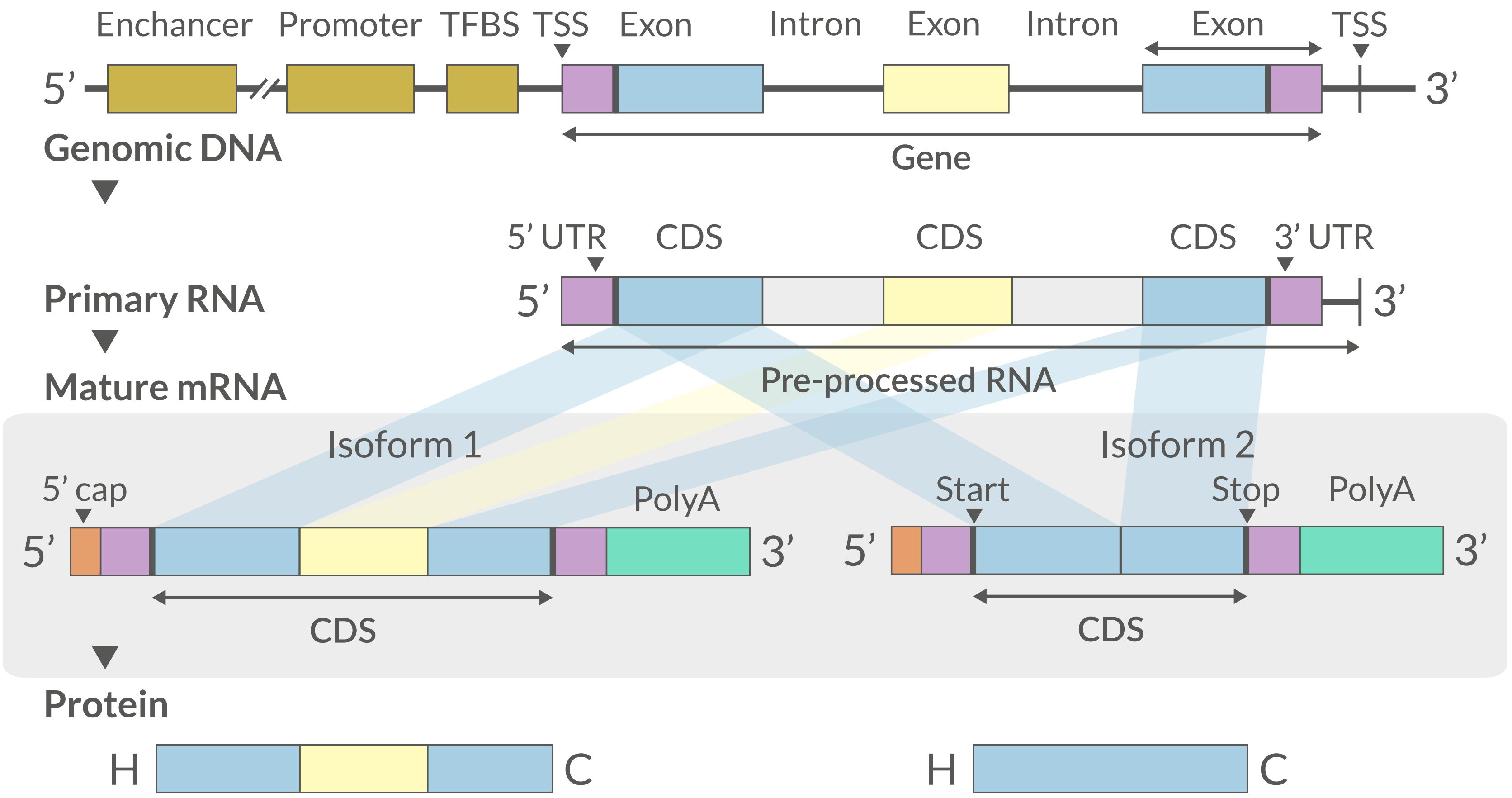
Dag Ahrén • 22-May-2019

NBIS, SciLifeLab

Contents

- RNA Sequencing
- Workflow
- DGE Workflow
- ReadQC
- Mapping
- Alignment QC
- Quantification
- Normalisation
- Exploratory
- DGE
- Functional analyses
- Summary
- Help

RNA Sequencing



- The transcriptome is spatially and temporally dynamic
- Data comes from functional units (coding regions)
- Only a tiny fraction of the genome

How many do RNASeq?

How many of you have/will have RNASeq as a component in your research?

- Raise of hands

Menti.com

Applications

- Identify gene sequences in genomes
- Learn about gene function
- Differential gene expression
- Explore isoform and allelic expression
- Understand co-expression, pathways and networks
- Gene fusion
- RNA editing
- Phylogeny
- Gene discovery
- Other

Workflow

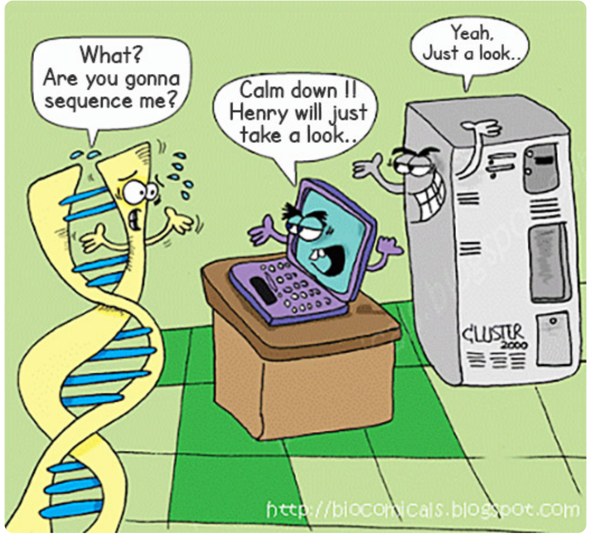
Experimental design
▼
RNA extraction
▼
Library preparation
▼
Sequencing
▼
Data processing
▼
Data analysis

QC
▼
Mapping
▼
Quantification

De-novo assembly

Annotation

Correlation
▼
Clustering
▼
MDS/PCA
▼
Differential Gene Expression

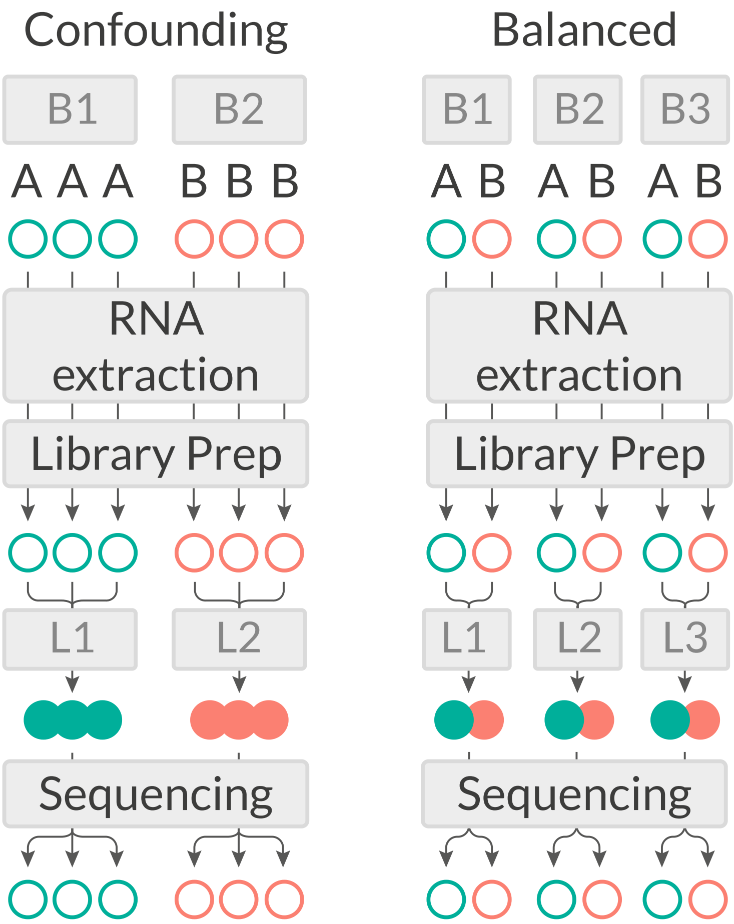

What? Are you gonna sequence me?
Calm down !! Henry will just take a look..
Yeah, Just a look..
CLUSTER 2000
http://biocomicals.blogspot.com

Conesa, Ana, *et al.* "A survey of best practices for RNA-seq data analysis." Genome biology 17.1 (2016): 13

Experimental design

- Balanced design
- Technical replicates not necessary (Marioni *et al.*, 2008)
- Biological replicates: 6 - 12 (Schurch *et al.*, 2016)
- ENCODE consortium
- Previous publications
- Power analysis

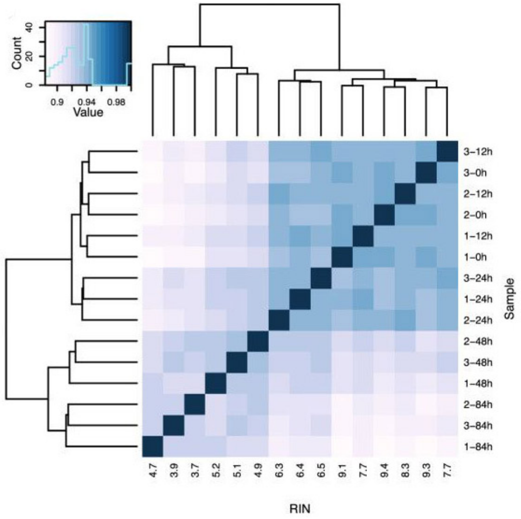
🧰 RnaSeqSampleSize (Power analysis), Scotty (Power analysis with cost)

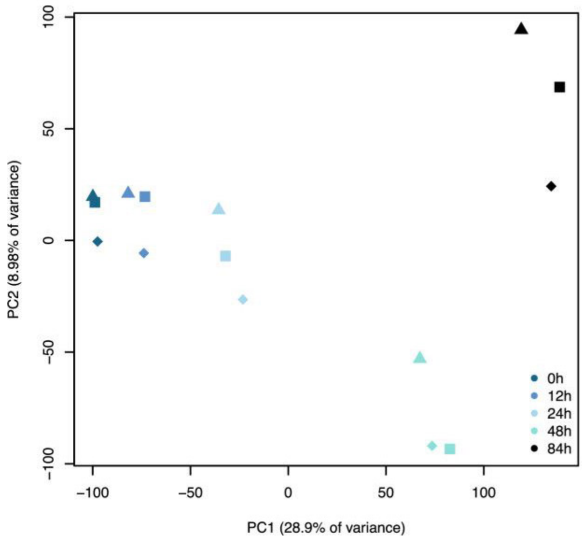


🔗 Busby, Michele A., *et al.* "Scotty: a web tool for designing RNA-Seq experiments to measure differential gene expression." Bioinformatics 29.5 (2013): 656-657
🔗 Marioni, John C., *et al.* "RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays." Genome research (2008)
🔗 Schurch, Nicholas J., *et al.* "How many biological replicates are needed in an RNA-seq experiment and which differential expression tool should you use?." Rna (2016)
🔗 Zhao, Shilin, *et al.* "RnaSeqSampleSize: real data based sample size estimation for RNA sequencing." BMC bioinformatics 19.1 (2018): 191

RNA extraction

- Sample processing and storage
- Total RNA/mRNA/small RNA
- DNAse treatment
- Quantity & quality
- RIN values (Strong effect)
- Batch effect
- Extraction method bias (GC bias)

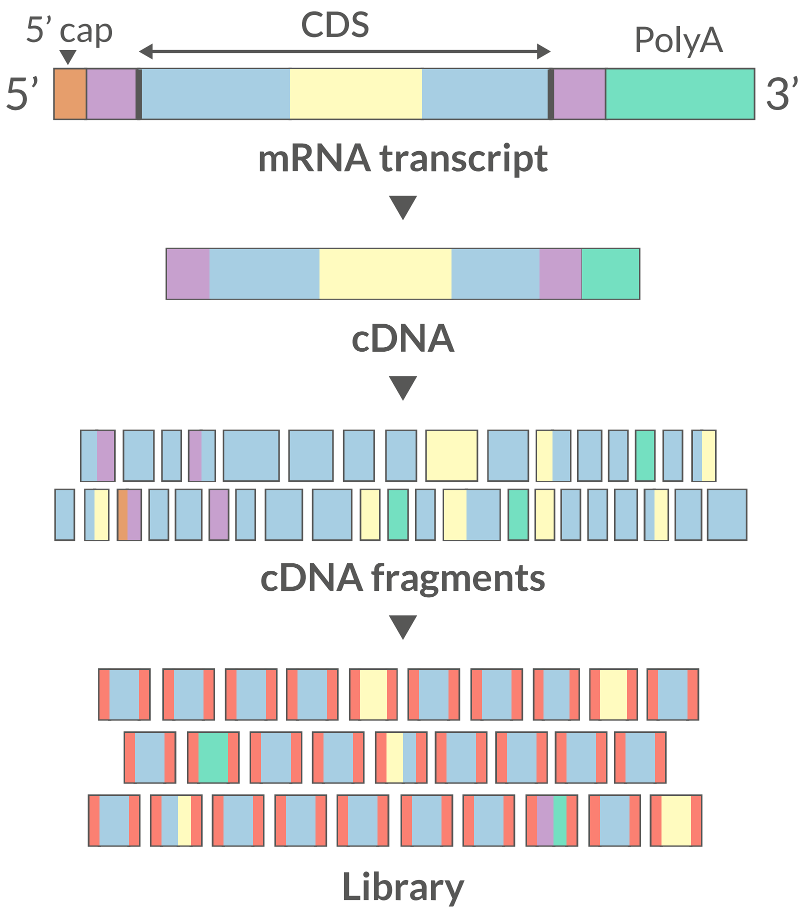




🔗 Romero, Irene Gallego, *et al*. "RNA-seq: impact of RNA degradation on transcript quantification." BMC biology 12.1 (2014): 42
🔗 Kim, Young-Kook, *et al*. "Short structured RNAs with low GC content are selectively lost during extraction from a small number of cells." Molecular cell 46.6 (2012): 893-89500481-9).

Library prep

- PolyA selection
- rRNA depletion
- Size selection
- PCR amplification (See section PCR duplicates)
- Stranded (directional) libraries
    - Accurately identify sense/antisense transcript
    - Resolve overlapping genes
- Exome capture
- Library normalisation
- Batch effect

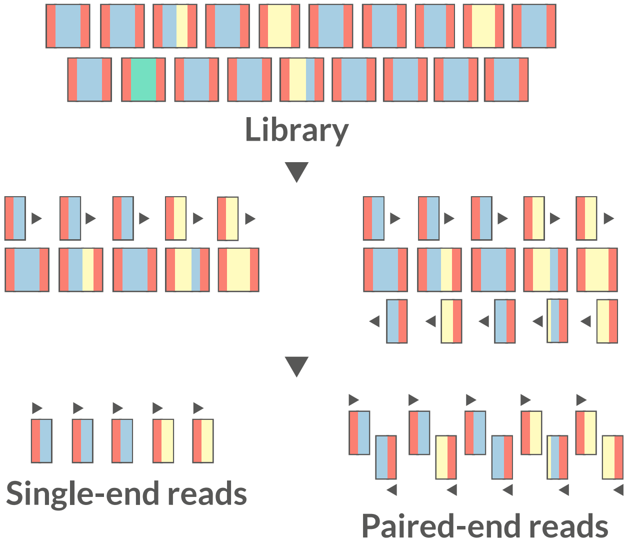


Zhao, Shanrong, et al. "Comparison of stranded and non-stranded RNA-seq transcriptome profiling and investigation of gene overlap." BMC genomics 16.1 (2015): 675

Levin, Joshua Z., et al. "Comprehensive comparative analysis of strand-specific RNA sequencing methods." Nature methods 7.9 (2010): 709

Sequencing

- Sequencer (Illumina/PacBio)
- Read length
  - Greater than 50bp does not improve DGE
  - Longer reads better for isoforms
- Pooling samples
- Sequencing depth (Coverage/Reads per sample)
- Single-end reads (Cheaper)
- Paired-end reads
  - Increased mappable reads
  - Increased power in assemblies
  - Better for structural variation and isoforms
  - Decreased false-positives for DGE



🔗 Chhangawala, Sagar, et al. "The impact of read length on quantification of differentially expressed genes and splice junction detection." Genome biology 16.1 (2015): 131
🔗 Corley, Susan M., et al. "Differentially expressed genes from RNA-Seq and functional enrichment results are affected by the choice of single-end versus paired-end reads and stranded versus non-stranded protocols." BMC genomics 18.1 (2017): 399
🔗 Liu, Yuwen, Jie Zhou, and Kevin P. White. "RNA-seq differential expression studies: more sequence or more replication?." Bioinformatics 30.3 (2013): 301-304 🔗 Comparison of PE and SE for RNA-Seq, SciLifeLab

Workflow • DGE

Reads	FastQ	FastQ	FastQ
	▼	▼	▼
Mapping	STAR	HiSat2	[Kallisto/ Salmon]
	▼	▼	
Quantification	featureCounts	StringTie	
	▼	▼	▼
Differential gene expression	DESeq2/ edgeR/ Limma	Ballgown	Sleuth

De-Novo assembly

- When no reference genome available
- To identify novel genes/transcripts/isoforms
- Identify fusion genes
- Assemble transcriptome from short reads
- Access quality of assembly and refine
- Map reads back to assembled transcriptome

Trinity, SOAPdenovo-Trans, Oases, rnaSPAdes

Hsieh, Ping-Han *et al.*, "Effect of de novo transcriptome assembly on transcript quantification" 2018 bioRxiv 380998
Wang, Sufang, and Michael Gribskov. "Comprehensive evaluation of de novo transcriptome assembly programs and their effects on differential gene expression analysis." Bioinformatics 33.3 (2017): 327-333

Read QC

- Number of reads
- Per base sequence quality
- Per sequence quality score
- Per base sequence content
- Per sequence GC content
- Per base N content
- Sequence length distribution
- Sequence duplication levels
- Overrepresented sequences
- Adapter content
- Kmer content

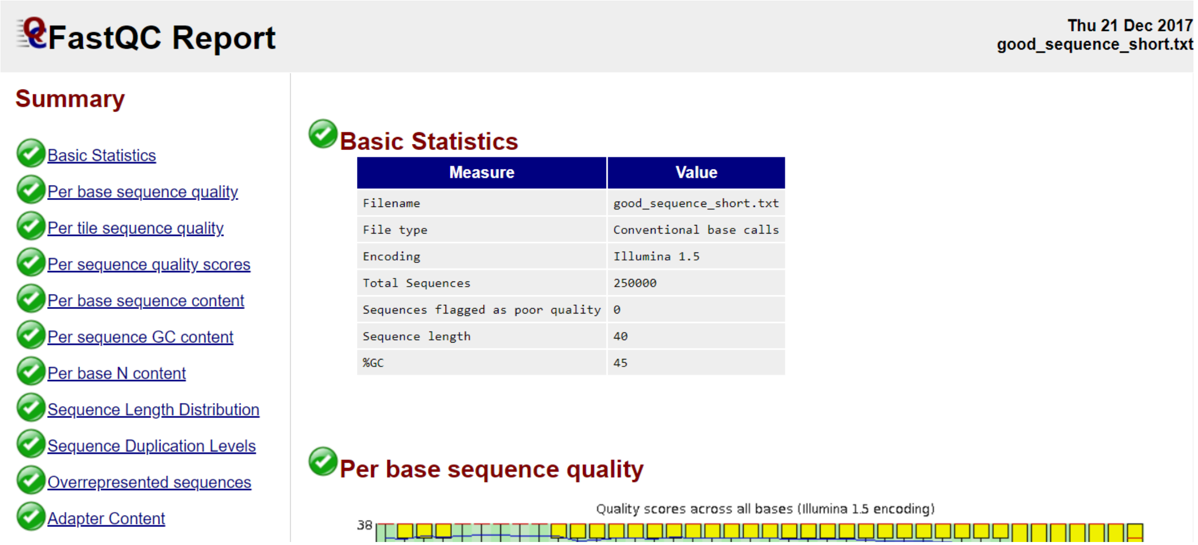
🧰 FastQC, MultiQC

https://sequencing.qcfail.com/

QCFAIL.com

Articles about common next-generation sequencing problems

FastQC



FastQC Report

Thu 21 Dec 2017
good_sequence_short.txt

Summary

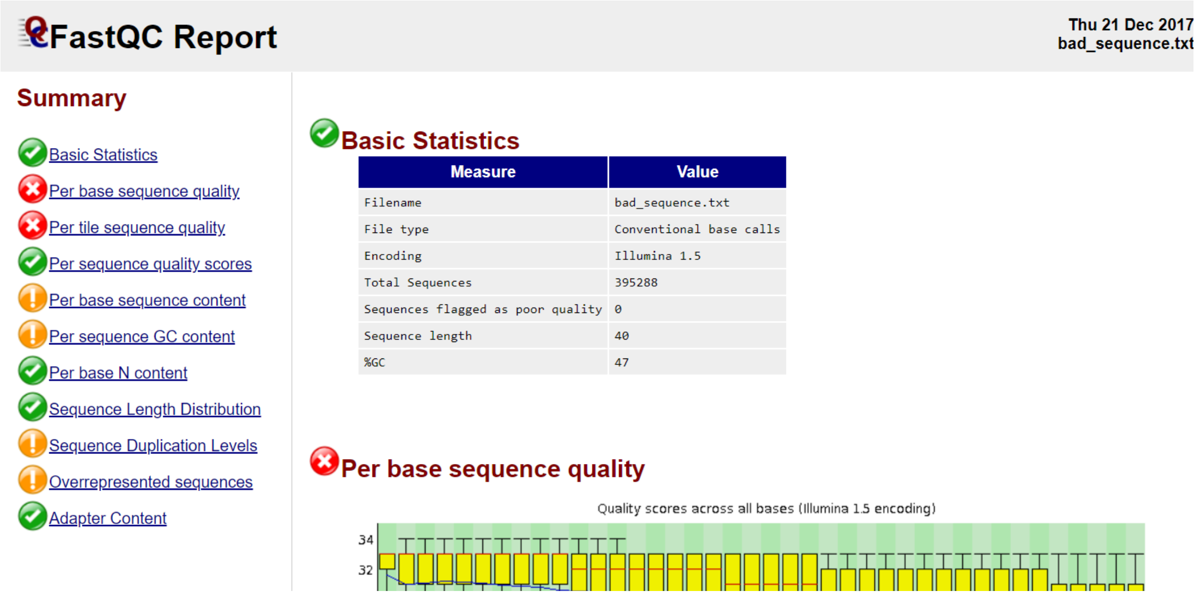
- ✓ Basic Statistics
- ✓ Per base sequence quality
- ✓ Per tile sequence quality
- ✓ Per sequence quality scores
- ✓ Per base sequence content
- ✓ Per sequence GC content
- ✓ Per base N content
- ✓ Sequence Length Distribution
- ✓ Sequence Duplication Levels
- ✓ Overrepresented sequences
- ✓ Adapter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

✓ **Basic Statistics**

Measure	Value
Filename	good_sequence_short.txt
File type	Conventional base calls
Encoding	Illumina 1.5
Total Sequences	250000
Sequences flagged as poor quality	0
Sequence length	40
%GC	45

✓ **Per base sequence quality**

Quality scores across all bases (Illumina 1.5 encoding)



FastQC Report

Thu 21 Dec 2017
bad_sequence.txt

Summary

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

✓ **Basic Statistics**

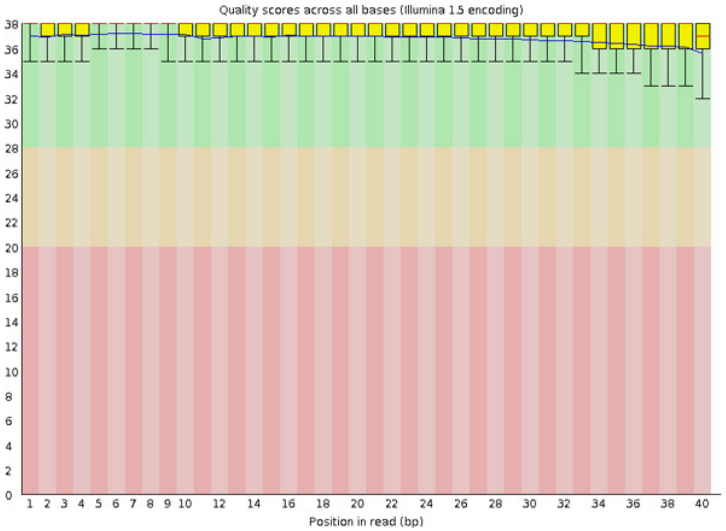
Measure	Value
Filename	bad_sequence.txt
File type	Conventional base calls
Encoding	Illumina 1.5
Total Sequences	395288
Sequences flagged as poor quality	0
Sequence length	40
%GC	47

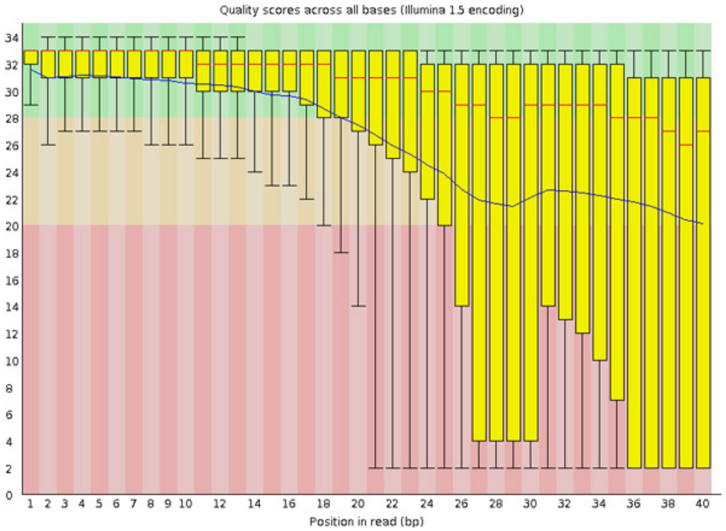
✗ **Per base sequence quality**

Quality scores across all bases (Illumina 1.5 encoding)

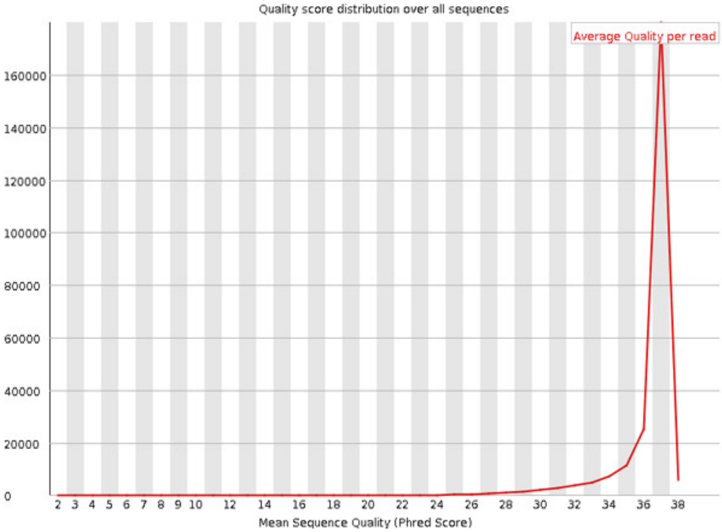
Read QC • PBSQ, PSQS

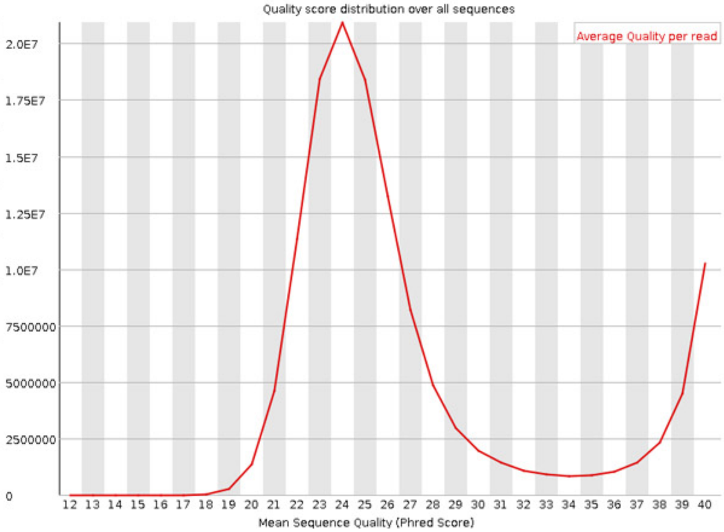
Per base sequence quality





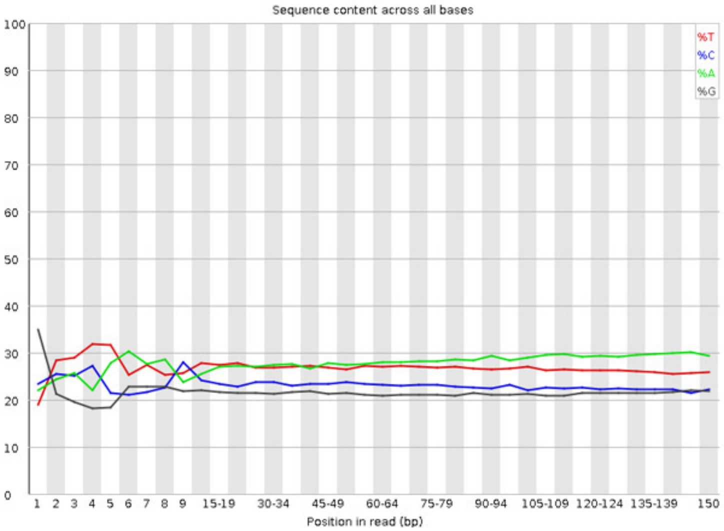
Per sequence quality scores

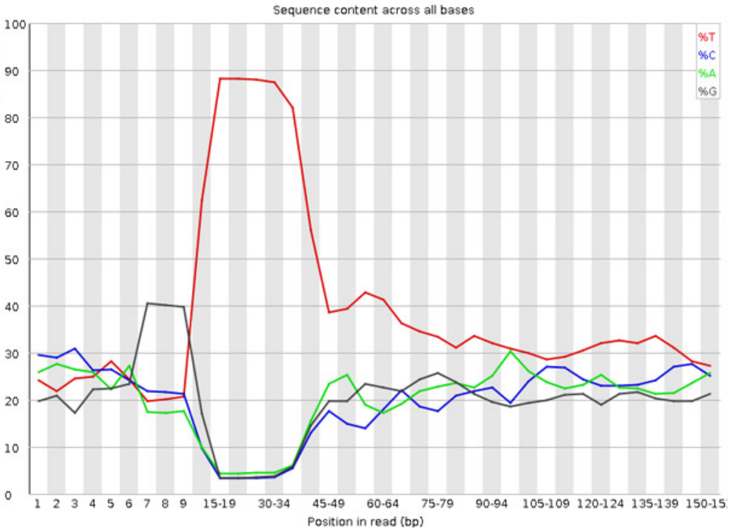




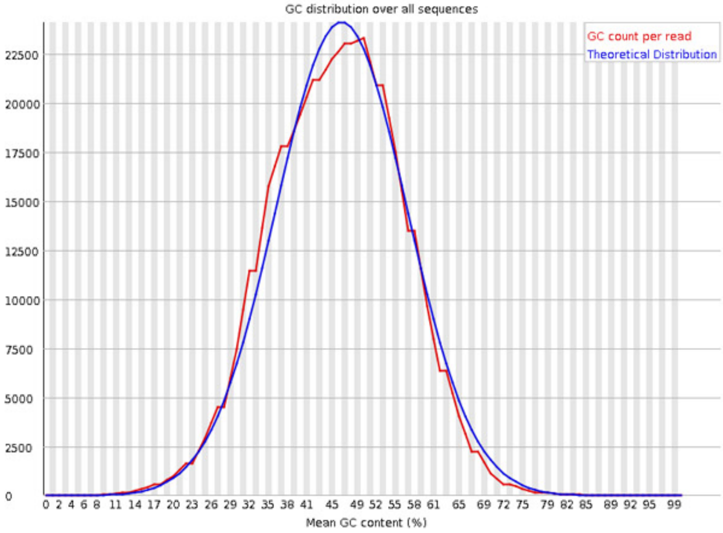
Read QC • PBSC, PSGC

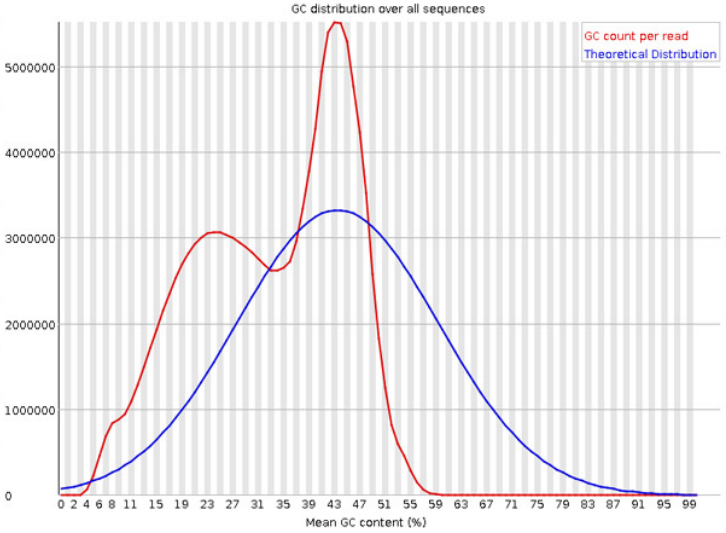
Per base sequence content





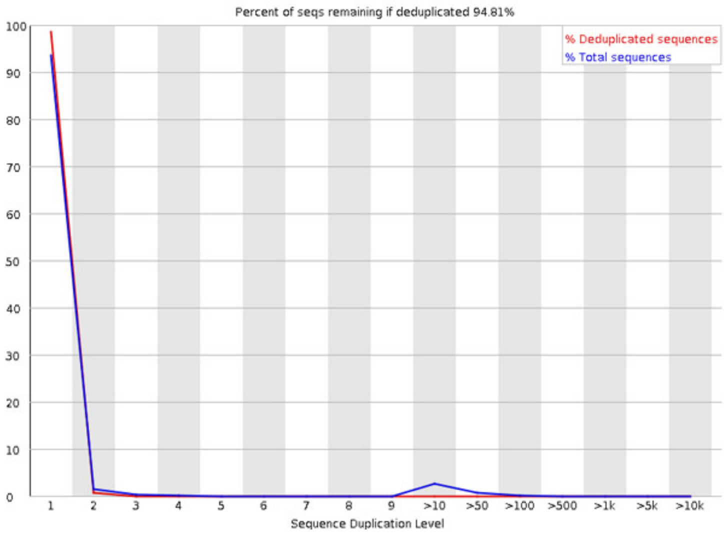
Per sequence GC content

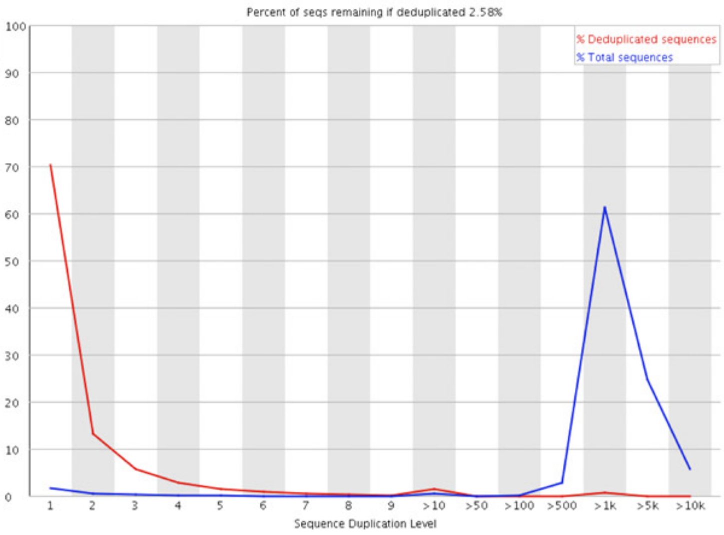




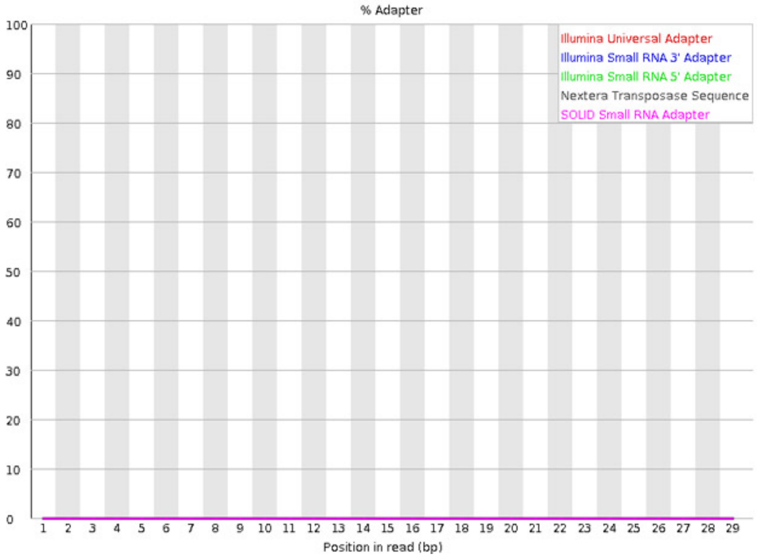
Read QC • SDL, AC

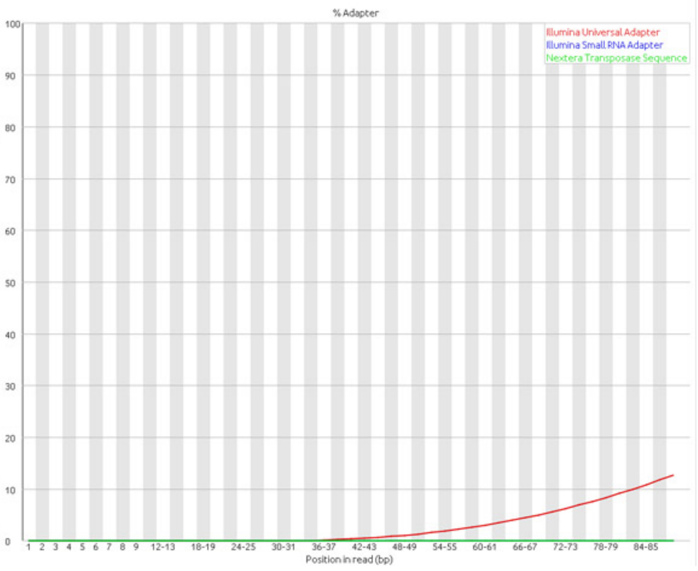
Sequence duplication level





Adapter content





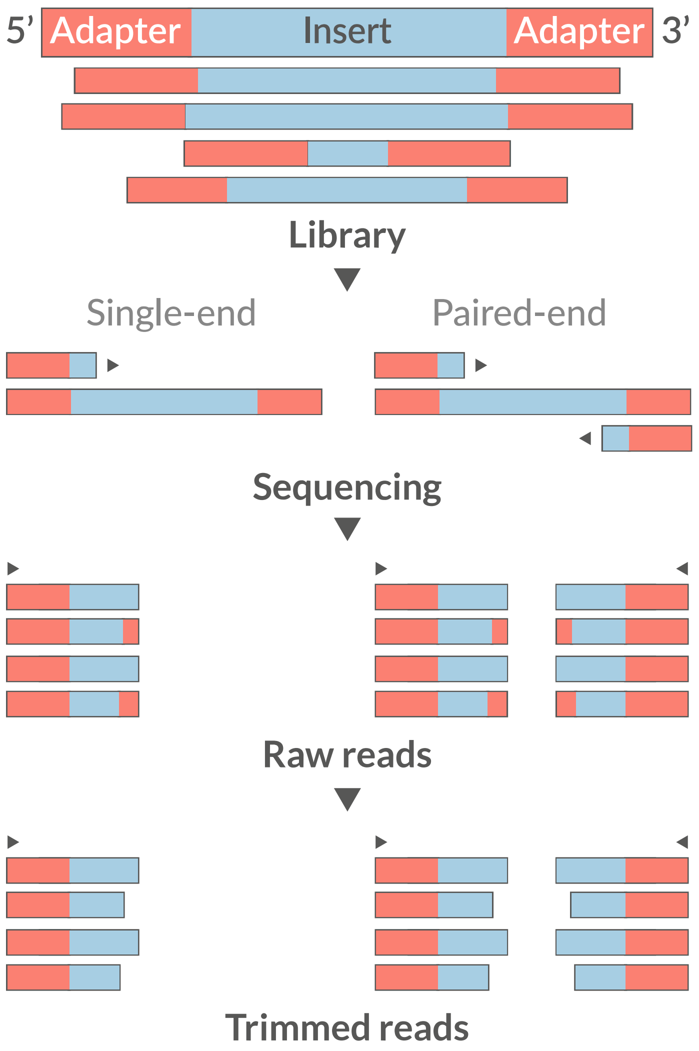
Trimming

- Trim IF necessary
  - Synthetic bases can be an issue for SNP calling
  - Insert size distribution may be more important for assemblers

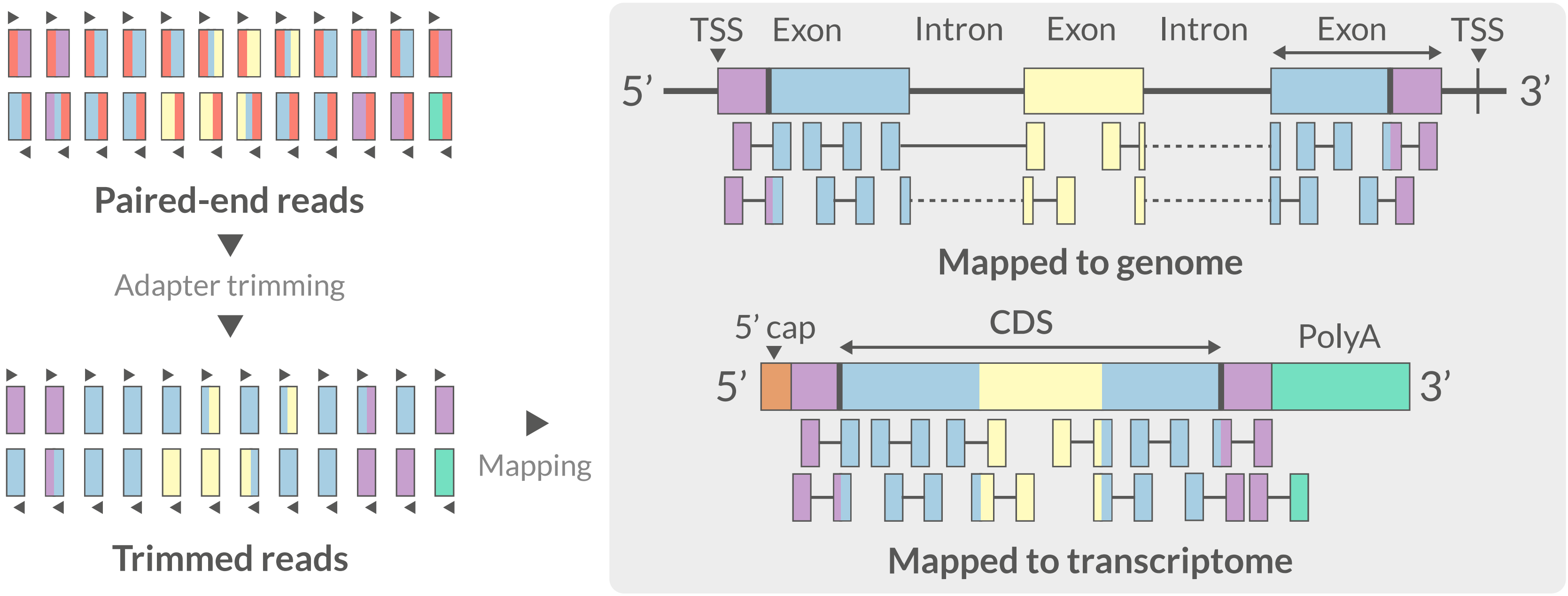
- Trim/Clip/Filter reads
- Remove adapter sequences
- Trim reads by quality
- Sliding window trimming
- Filter by min/max read length
  - Remove reads less than ~18nt

- Demultiplexing/Splitting

🧰 Cutadapt, fastp, Skewer, Prinseq



Mapping



Paired-end reads

Adapter trimming

Trimmed reads

Mapping

Mapped to genome

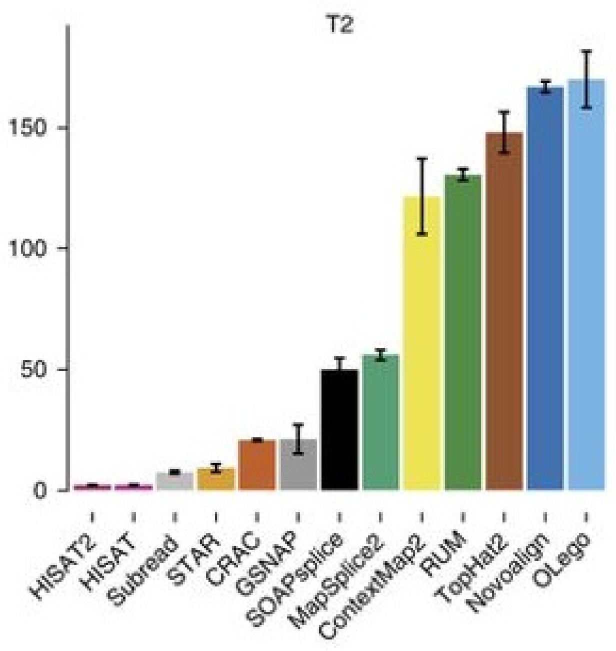
Mapped to transcriptome

- Aligning reads back to a reference sequence
- Mapping to genome vs transcriptome
- Splice-aware alignment (genome)

STAR, HiSat2, GSNAP, Novoalign (Commercial)

Baruzzo, Giacomo, *et al*. "Simulation-based comprehensive benchmarking of RNA-seq aligners." Nature methods 14.2 (2017): 135

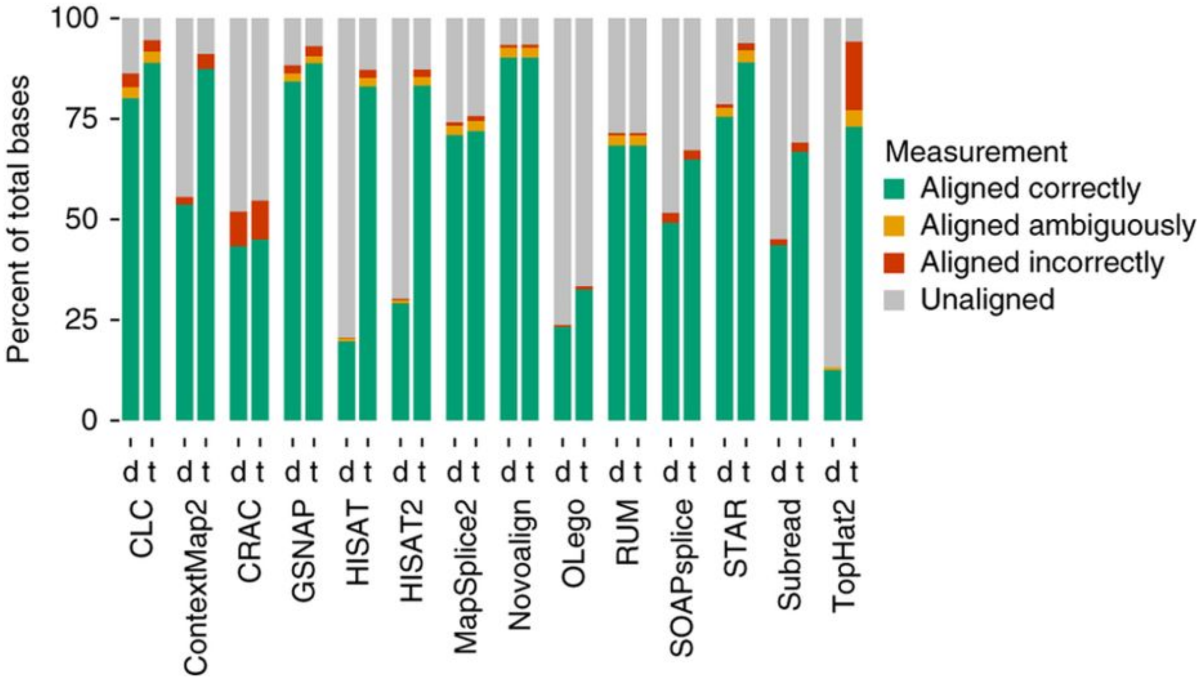
Aligners • Speed



Program	Time_Min	Memory_GB
HISATx1	22.7	4.3
HISATx2	47.7	4.3
HISAT	26.7	4.3
STAR	25	28
STARx2	50.5	28
GSNAP	291.9	20.2
TopHat2	1170	4.3

Baruzzo, Giacomo, *et al*. "Simulation-based comprehensive benchmarking of RNA-seq aligners." Nature methods 14.2 (2017): 135

Aligners • Accuracy



Increasing Accuracy

- Novel variants / RNA editing
- Allele-specific expression
- Genome annotation
- Gene and transcript discovery
- Differential expression

STAR, HiSat2, GSNAP, Novoalign (Commercial)

Baruzzo, Giacomo, *et al*. "Simulation-based comprehensive benchmarking of RNA-seq aligners." Nature methods 14.2 (2017): 135

Mapping

- Reads (FASTQ)

```
@ST-E00274:179:HHYMLALXX:8:1101:1641:1309 1:N:0:NGATGT
NCATCGTGGTATTTGCACATCTTTTCTTATCAAATAAAAAGTTTAACCTACTCAGTTATGCGCATACGTTTTTTGATGGCATTTCCATAAACCGATTTTTTTTTTA
+
#AAAFAFA<-AFFJJJAFA-FFJJJJFFFAJJJJ-<FFJJJ-A-F-7--FA7F7-----FFFJFA<FFFFJ<AJ--FF-A<A-<JJ-7-7-<FF-FFFJAFFAA-
```

`@instrument:runid:flowcellid:lane:tile:xpos:ypos read:isfiltered:controlnumber:sampleid`

- Reference Genome/Transcriptome (FASTA)

```
>1 dna:chromosome chromosome:GRCz10:1:1:58871917:1 REF
GATCTTAAACATTTATTCCCCCTGCAAACATTTTCAATCATTACATTGTCATTTCCCCTC
CAAATTAAATTTAGCCAGAGGCGCACAACATACGACCTCTAAAAAAGGTGCTGTAACATG
```

- Annotation (GTF/GFF)

```
#!genome-build GRCz10
#!genebuild-last-updated 2016-11
4       ensembl_havana  gene    6732    52059   .       -       .       gene_id "ENSDARG00000104632"; gene
```

`seq source feature start end score strand frame attribute`

Illumina read name format, GTF format

Alignment

- SAM/BAM (Sequence Alignment Map format)

```
ST-E00274:188:H3JWNCCXY:4:1102:32431:49900      163      1       1       60       8S139M4S      =       385
```

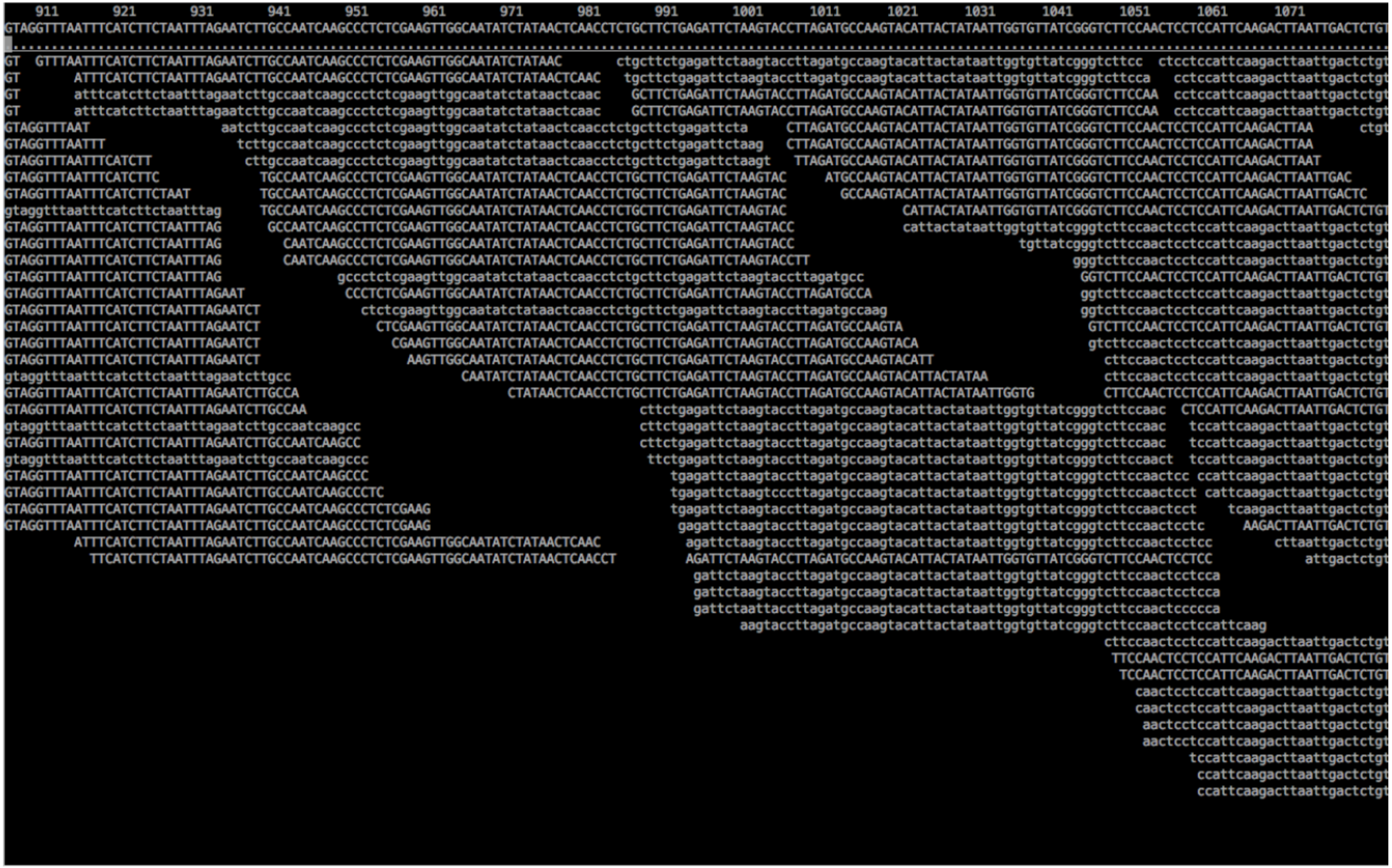
```
query flag ref pos mapq cigar mrnm mpos tlen seq qual opt
```

Format	Size_GB
SAM	7.4
BAM	1.9
CRAM lossless Q	1.4
CRAM 8 bins Q	0.8
CRAM no Q	0.26

SAM file format

Visualisation • `tview`

```
samtools tview alignment.bam genome.fasta
```



```
    911       921       931       941       951       961       971       981       991       1001      1011      1021      1031      1041      1051      1061      1071
GTAGGTTTAATTTCATCTTCTAATTTAGAATCTTGCCAATCAAGCCCTCTCGAAGTTGGCAATATCTATAACTCAACCTCTGCTTCTGAGATTCTAAGTACCTTAGATGCCAAGTACATTACTATAATTGGTGTTATCGGGTCTTCCAACTCCTCCATTCAAGACTTAATTGACTCTGT
...................................................................................................................................................................................
GT  GTTTAATTTCATCTTCTAATTTAGAATCTTGCCAATCAAGCCCTCTCGAAGTTGGCAATATCTATAAC       ctgcttctgagattctaagtaccttagatgccaagtacattactataattggtgttatcgggtcttcc  ctcctccattcaagacttaattgactctgt
GT       ATTTCATCTTCTAATTTAGAATCTTGCCAATCAAGCCCTCTCGAAGTTGGCAATATCTATAACTCAAC   tgcttctgagattctaagtaccttagatgccaagtacattactataattggtgttatcgggtcttcca   cctccattcaagacttaattgactctgt
GT       atttcatcttctaatttagaatcttgccaatcaagccctctcgaagttggcaatatctataactcaac    GCTTCTGAGATTCTAAGTACCTTAGATGCCAAGTACATTACTATAATTGGTGTTATCGGGTCTTCCAA  cctccattcaagacttaattgactctgt
GT       atttcatcttctaatttagaatcttgccaatcaagccctctcgaagttggcaatatctataactcaac    GCTTCTGAGATTCTAAGTACCTTAGATGCCAAGTACATTACTATAATTGGTGTTATCGGGTCTTCCAA  cctccattcaagacttaattgactctgt
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GTAGGTTTAATTT                 tcttgccaatcaagccctctcgaagttggcaatatctataactcaacctctgcttctgagattctaag   CTTAGATGCCAAGTACATTACTATAATTGGTGTTATCGGGTCTTCCAACTCCTCCATTCAAGACTTAA
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GTAGGTTTAATTTCATCTTCTAAT         TGCCAATCAAGCCCTCTCGAAGTTGGCAATATCTATAACTCAACCTCTGCTTCTGAGATTCTAAGTAC       GCCAAGTACATTACTATAATTGGTGTTATCGGGTCTTCCAACTCCTCCATTCAAGACTTAATTGACTC
gtaggtttaatttcatcttctaatttag     TGCCAATCAAGCCCTCTCGAAGTTGGCAATATCTATAACTCAACCTCTGCTTCTGAGATTCTAAGTAC               CATTACTATAATTGGTGTTATCGGGTCTTCCAACTCCTCCATTCAAGACTTAATTGACTCTGT
GTAGGTTTAATTTCATCTTCTAATTTAG      GCCAATCAAGCCTTCTCGAAGTTGGCAATATCTATAACTCAACCTCTGCTTCTGAGATTCTAAGTACC              cattactataattggtgttatcgggtcttccaactcctccattcaagacttaattgactctgt
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GTAGGTTTAATTTCATCTTCTAATTTAGAATCTTGCCA                           CTATAACTCAACCTCTGCTTCTGAGATTCTAAGTACCTTAGATGCCAAGTACATTACTATAATTGGTG         CTTCCAACTCCTCCATTCAAGACTTAATTGACTCTGT
GTAGGTTTAATTTCATCTTCTAATTTAGAATCTTGCCAA                                           cttctgagattctaagtaccttagatgccaagtacattactataattggtgttatcgggtcttccaac  CTCCATTCAAGACTTAATTGACTCTGT
gtaggtttaatttcatcttctaatttagaatcttgccaatcaagcc                                    cttctgagattctaagtaccttagatgccaagtacattactataattggtgttatcgggtcttccaac   tccattcaagacttaattgactctgt
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gtaggtttaatttcatcttctaatttagaatcttgccaatcaagccc                                    ttctgagattctaagtaccttagatgccaagtacattactataattggtgttatcgggtcttccaact  tccattcaagacttaattgactctgt
GTAGGTTTAATTTCATCTTCTAATTTAGAATCTTGCCAATCAAGCCC                                       tgagattctaagtaccttagatgccaagtacattactataattggtgttatcgggtcttccaactcc ccattcaagacttaattgactctgt
GTAGGTTTAATTTCATCTTCTAATTTAGAATCTTGCCAATCAAGCCCTC                                     tgagattctaagtcccttagatgccaagtacattactataattggtgttatcgggtcttccaactcct cattcaagacttaattgactctgt
GTAGGTTTAATTTCATCTTCTAATTTAGAATCTTGCCAATCAAGCCCTCTCGAAG                               tgagattctaagtaccttagatgccaagtacattactataattggtgttatcgggtcttccaactcct    tcaagacttaattgactctgt
GTAGGTTTAATTTCATCTTCTAATTTAGAATCTTGCCAATCAAGCCCTCTCGAAG                                gagattctaagtaccttagatgccaagtacattactataattggtgttatcgggtcttccaactcctc     AAGACTTAATTGACTCTGT
         ATTTCATCTTCTAATTTAGAATCTTGCCAATCAAGCCCTCTCGAAGTTGGCAATATCTATAACTCAAC           agattctaagtaccttagatgccaagtacattactataattggtgttatcgggtcttccaactcctcc        cttaattgactctgt
           TTCATCTTCTAATTTAGAATCTTGCCAATCAAGCCCTCTCGAAGTTGGCAATATCTATAACTCAACCT         AGATTCTAAGTACCTTAGATGCCAAGTACATTACTATAATTGGTGTTATCGGGTCTTCCAACTCCTCC            attgactctgt
                                                                                         gattctaagtaccttagatgccaagtacattactataattggtgttatcgggtcttccaactcctcca
                                                                                         gattctaagtaccttagatgccaagtacattactataattggtgttatcgggtcttccaactcctcca
                                                                                         gattctaattaccttagatgccaagtacattactataattggtgttatcgggtcttccaactccccca
                                                                                               aagtaccttagatgccaagtacattactataattggtgttatcgggtcttccaactcctccattcaag
                                                                                                                                              cttccaactcctccattcaagacttaattgactctgt
                                                                                                                                               TTCCAACTCCTCCATTCAAGACTTAATTGACTCTGT
                                                                                                                                                TCCAACTCCTCCATTCAAGACTTAATTGACTCTGT
                                                                                                                                                  caactcctccattcaagacttaattgactctgt
                                                                                                                                                  caactcctccattcaagacttaattgactctgt
                                                                                                                                                   aactcctccattcaagacttaattgactctgt
                                                                                                                                                   aactcctccattcaagacttaattgactctgt
                                                                                                                                                         tccattcaagacttaattgactctgt
                                                                                                                                                          ccattcaagacttaattgactctgt
                                                                                                                                                          ccattcaagacttaattgactctgt
```
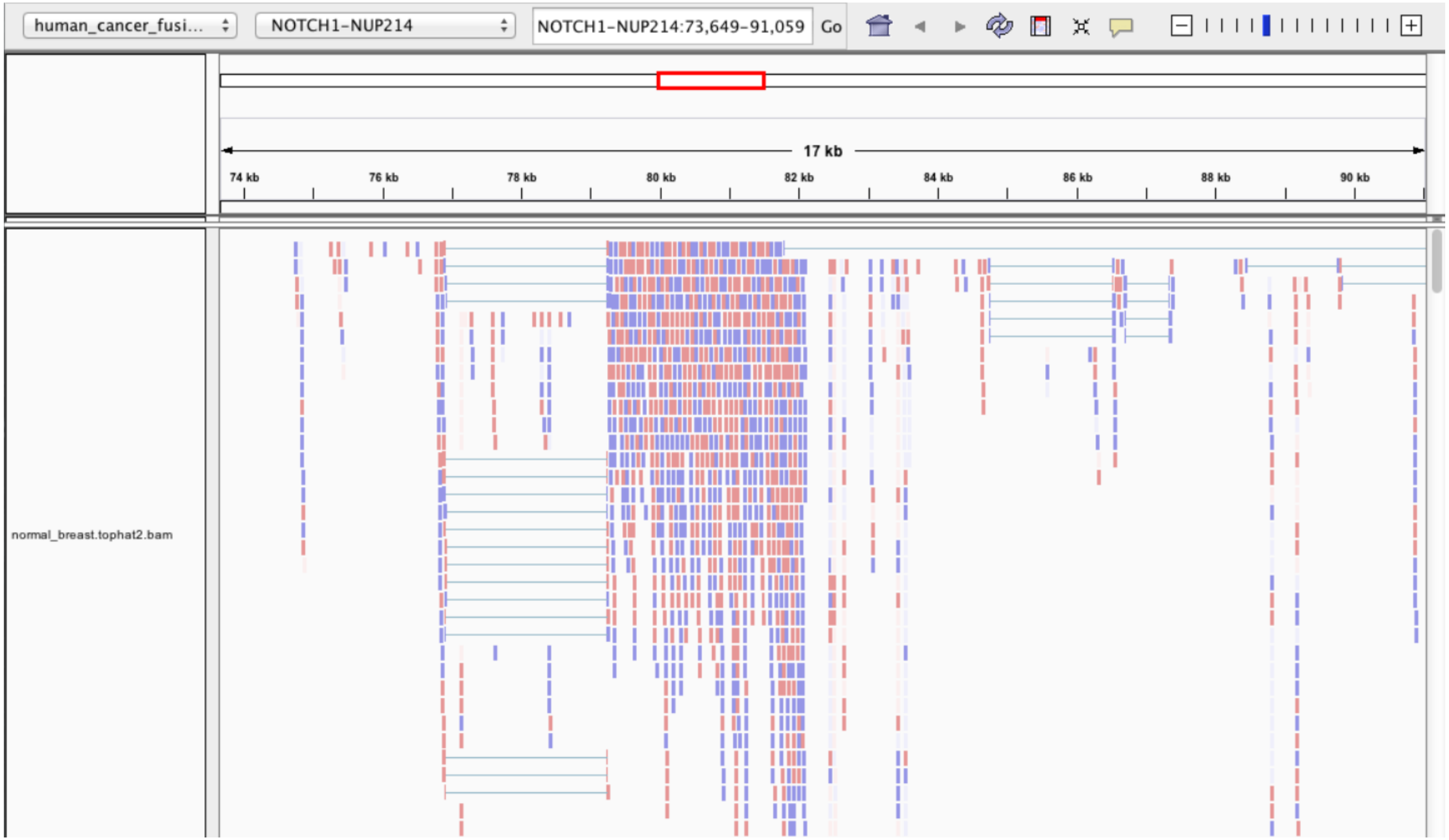
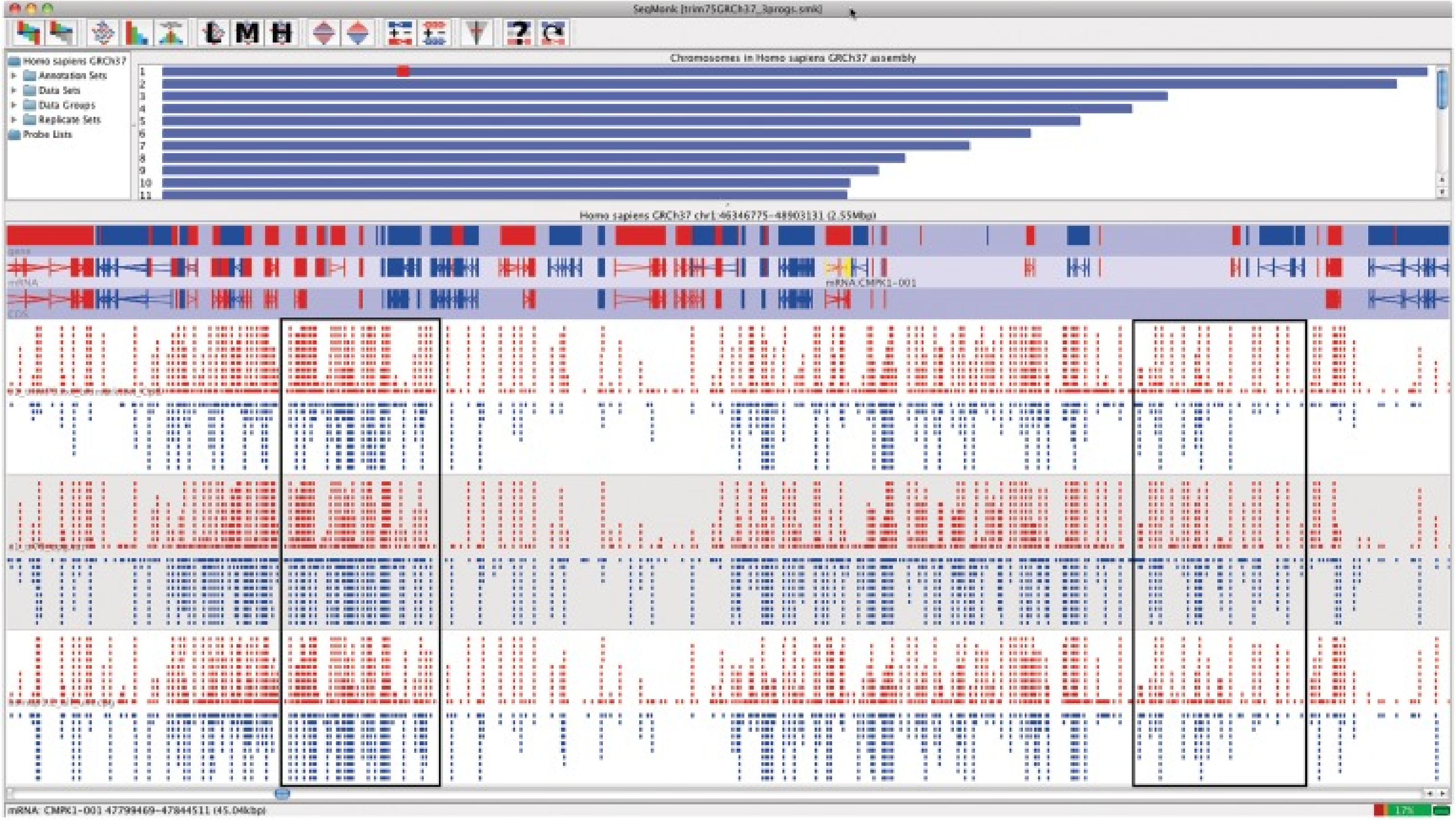

Visualisation • IGV



IGV, UCSC Genome Browser

Visualisation • SeqMonk


SeqMonk [trim75GRCh37_3progs.smk]
Homo sapiens GRCh37
Annotation Sets
Data Sets
Data Groups
Replicate Sets
Probe Lists
Chromosomes in Homo sapiens GRCh37 assembly
1
2
3
4
5
6
7
8
9
10
11
Homo sapiens GRCh37 chr1:46346775-48903131 (2.55Mbp)
gene
mRNA
CDS
mRNA CMPK1-001
mRNA: CMPK1-001 47799469-47844511 (45.04kbp)
17%

SeqMonk

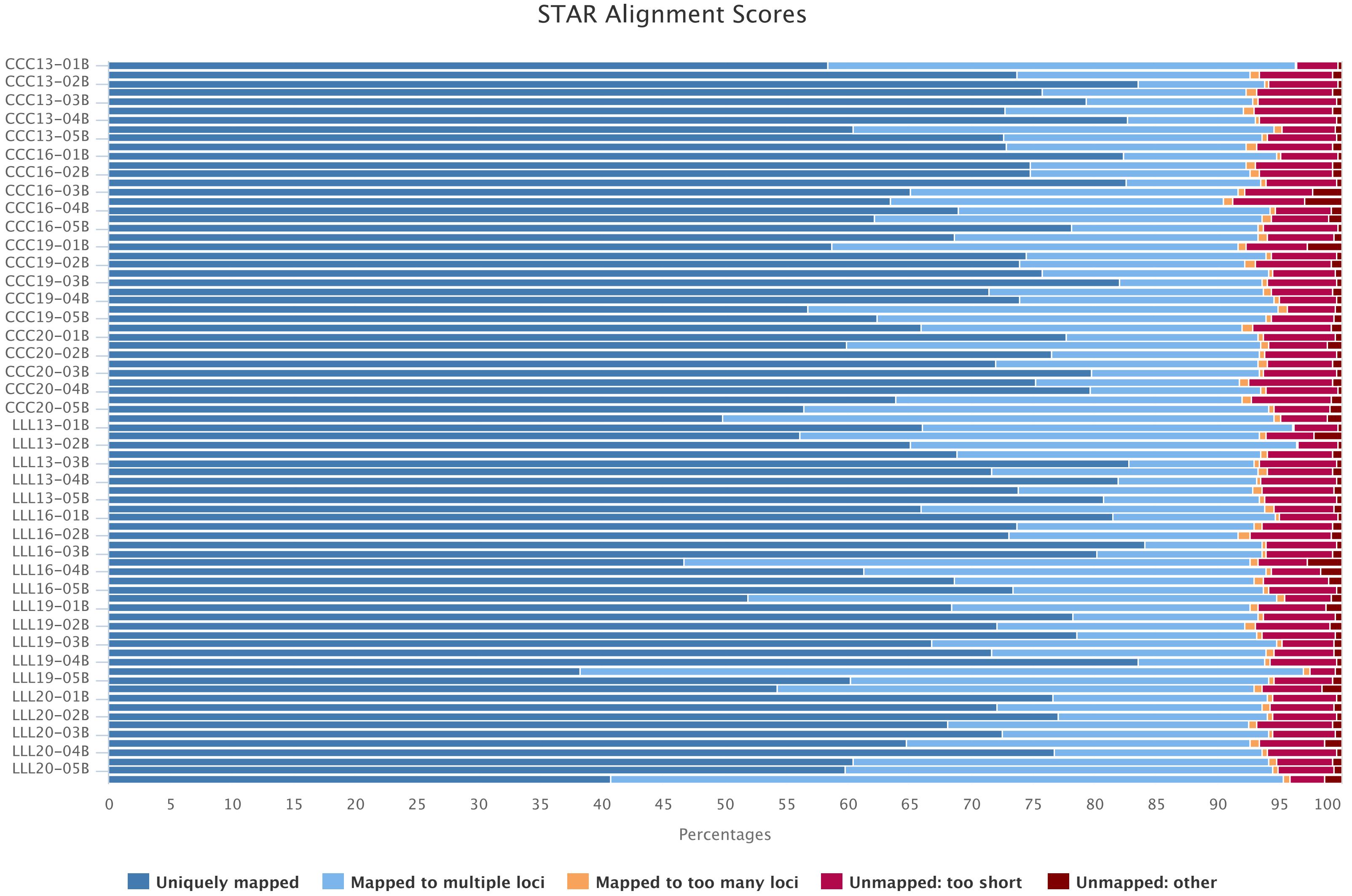
Alignment QC

- Number of reads mapped/unmapped/paired etc
- Uniquely mapped
- Insert size distribution
- Coverage
- Gene body coverage
- Biotype counts / Chromosome counts
- Counts by region: gene/intron/non-genic
- Sequencing saturation
- Strand specificity

🧰 STAR (final log file), samtools > stats, bamtools > stats, QoRTs, RSeQC, Qualimap

Alignment QC • STAR Log

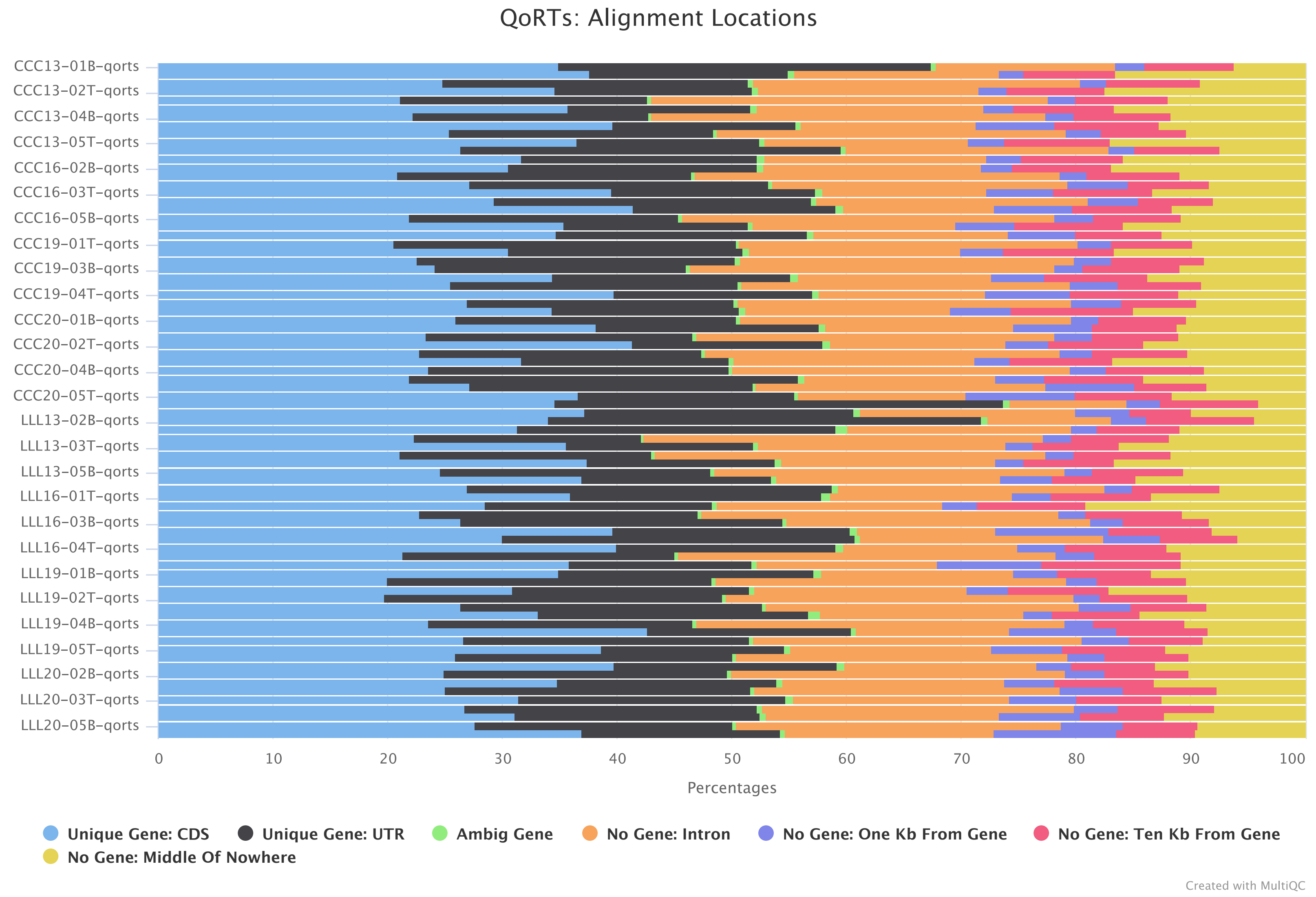
MultiQC can be used to summarise and plot STAR log files.



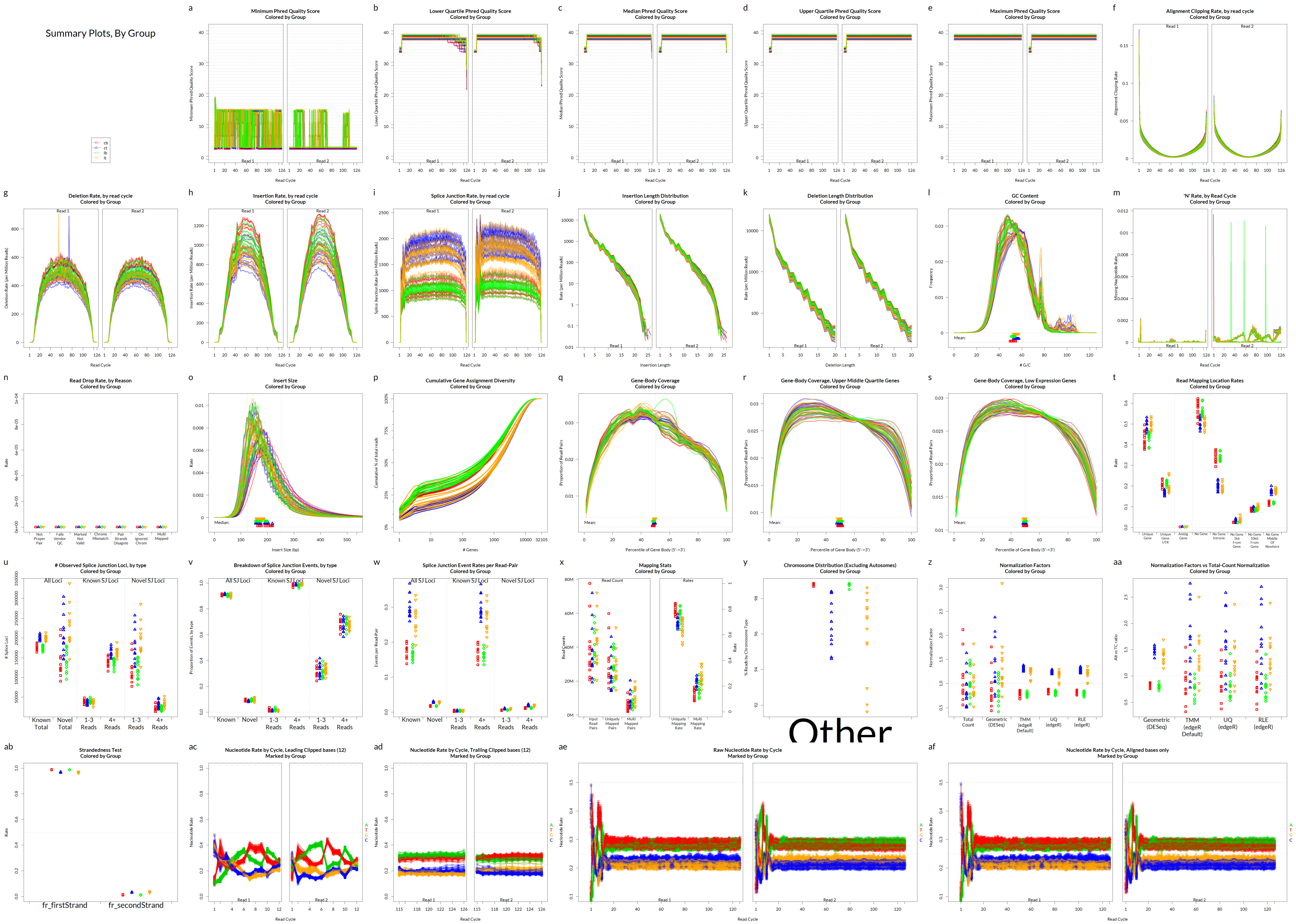
Created with MultiQC

Alignment QC • Features

QoRTs was run on all samples and summarised using MultiQC.



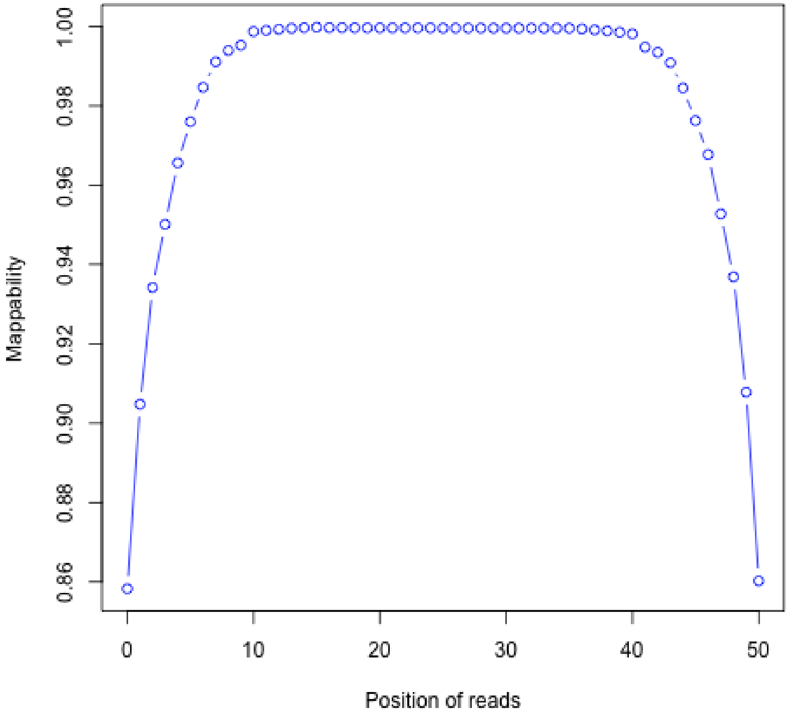
QoRTs


Summary Plots, By Group
a Minimum Phred Quality Score Colored by Group
b Lower Quartile Phred Quality Score Colored by Group
c Median Phred Quality Score Colored by Group
d Upper Quartile Phred Quality Score Colored by Group
e Maximum Phred Quality Score Colored by Group
f Alignment Clipping Rate, by read cycle Colored by Group
g Deletion Rate, by read cycle Colored by Group
h Insertion Rate, by read cycle Colored by Group
i Splice Junction Rate, by read cycle Colored by Group
j Insertion Length Distribution Colored by Group
k Deletion Length Distribution Colored by Group
l GC Content Colored by Group
m 'N' Rate, by Read Cycle Colored by Group
n Read Drop Rate, by Reason Colored by Group
o Insert Size Colored by Group
p Cumulative Gene Assignment Diversity Colored by Group
q Gene-Body Coverage Colored by Group
r Gene-Body Coverage, Upper Middle Quartile Genes Colored by Group
s Gene-Body Coverage, Low Expression Genes Colored by Group
t Read Mapping Location Rates Colored by Group
u # Observed Splice Junction Loci, by type Colored by Group
v Breakdown of Splice Junction Events, by type Colored by Group
w Splice Junction Event Rates per Read-Pair Colored by Group
x Mapping Stats Colored by Group
y Chromosome Distribution (Excluding Autosomes) Colored by Group
z Normalization Factors Colored by Group
aa Normalization Factors vs Total-Count Normalization Colored by Group
ab Strandedness Test Colored by Group
ac Nucleotide Rate by Cycle, Leading Clipped bases (12) Marked by Group
ad Nucleotide Rate by Cycle, Trailing Clipped bases (12) Marked by Group
ae Raw Nucleotide Rate by Cycle Marked by Group
af Nucleotide Rate by Cycle, Aligned bases only Marked by Group
Read 1
Read 2
Read Cycle
All SJ Loci
Known SJ Loci
Novel SJ Loci
Known Total
Novel Total
1-3 Reads
4+ Reads
Known
Novel
Read Count
Rates
Total Count
Geometric (DESeq)
TMM (edgeR Default)
UQ (edgeR)
RLE (edgeR)
fr_firstStrand
fr_secondStrand
Insertion Length
Deletion Length
% G/C
Insert Size (bp)
# Genes
Percentile of Gene Body (5'->3')

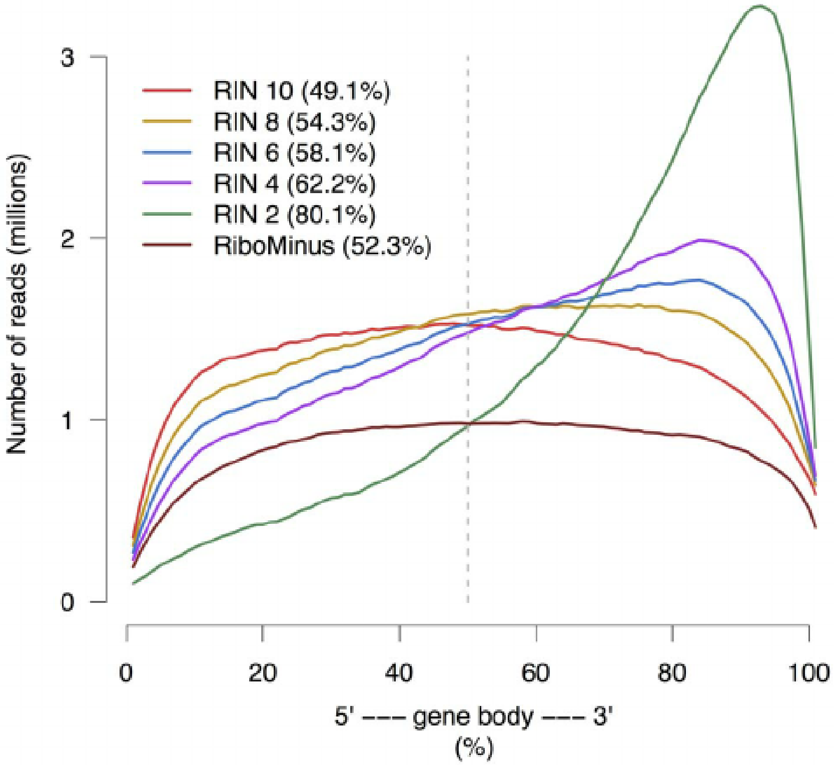
Other

Alignment QC

Soft clipping

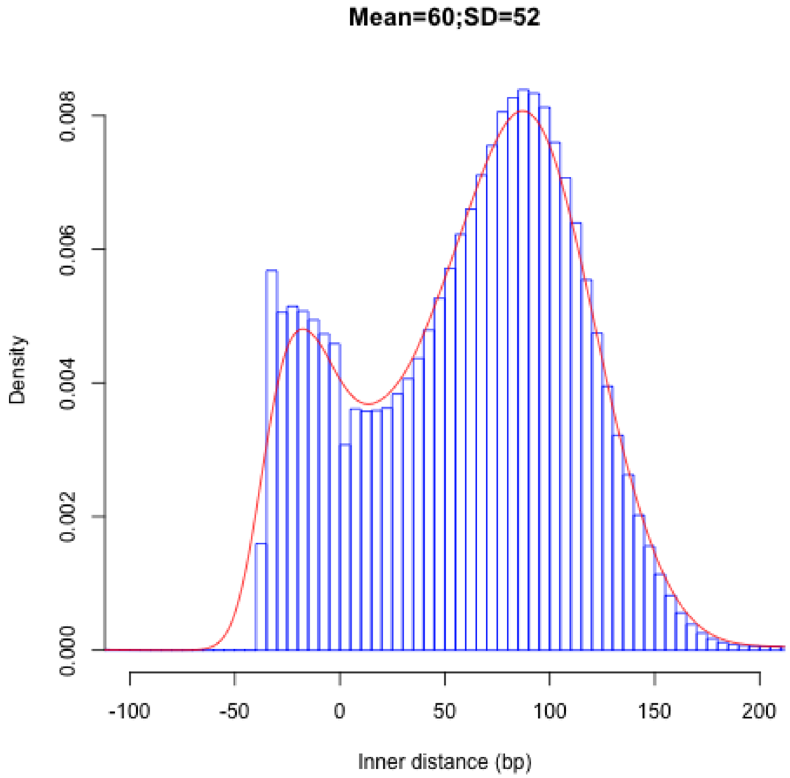


Gene body coverage

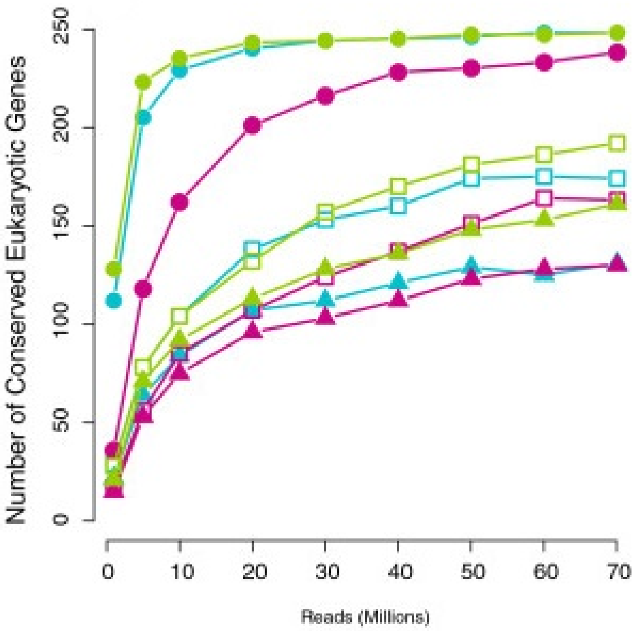


Alignment QC

Insert size

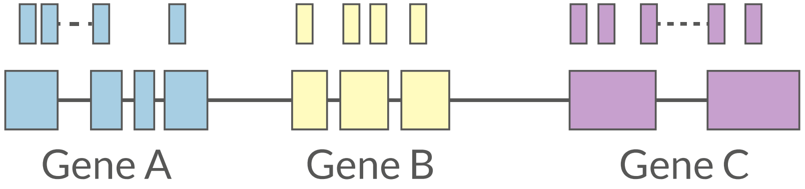


Saturation curve



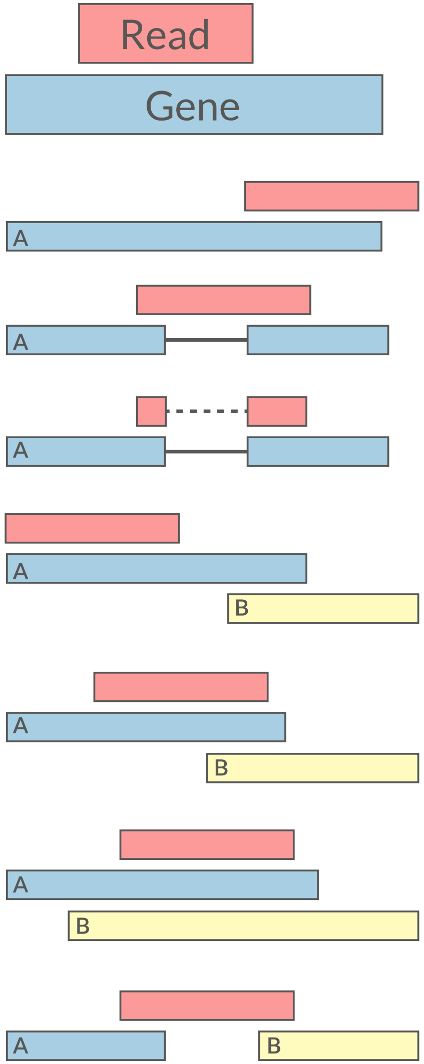
Quantification • Counts

- Read counts = gene expression
- Reads can be quantified on any feature (gene, transcript, exon etc)
- Intersection on gene models
- Gene/Transcript level



featureCounts, HTSeq

Union



Gene A

Gene A

Gene A

Gene A

Gene A

Ambiguous

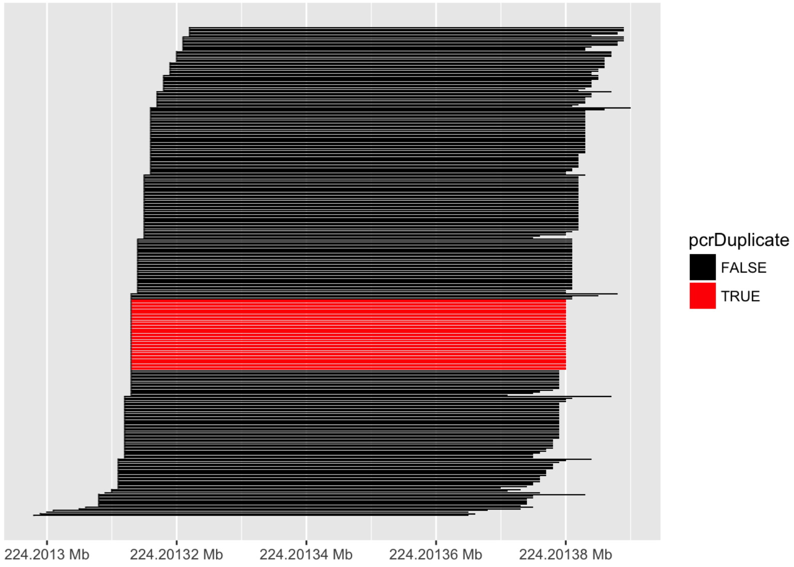
Ambiguous

Ambiguous

Quantification

PCR duplicates

- Ignore for RNA-Seq data
- Computational deduplication (Don't!)
- Use PCR-free library-prep kits
- Use UMIs during library-prep



Multi-mapping

- Added (BEDTools multicov)
- Discard (featureCounts, HTSeq)
- Distribute counts (Cufflinks)
- Rescue
  - Probabilistic assignment (Rcount, Cufflinks)
  - Prioritise features (Rcount)
  - Probabilistic assignment with EM (RSEM)

Fu, Yu, *et al*. "Elimination of PCR duplicates in RNA-seq and small RNA-seq using unique molecular identifiers." BMC genomics 19.1 (2018): 531

Parekh, Swati, *et al*. "The impact of amplification on differential expression analyses by RNA-seq." Scientific reports 6 (2016): 25533

Klepikova, Anna V., *et al*. "Effect of method of deduplication on estimation of differential gene expression using RNA-seq." PeerJ 5 (2017): e3091

Quantification • Abundance

- Count methods
  - Provide no inference on isoforms
  - Cannot accurately measure fold change
- Probabilistic assignment
  - Deconvolute ambiguous mappings
  - Transcript-level
  - cDNA reference

Kallisto, Salmon

- Ultra-fast & alignment-free
- Subsampling & quantification confidence
- Transcript-level estimates improves gene-level estimates
- Kallisto/Salmon > transcript-counts > `tximport()` > gene-counts

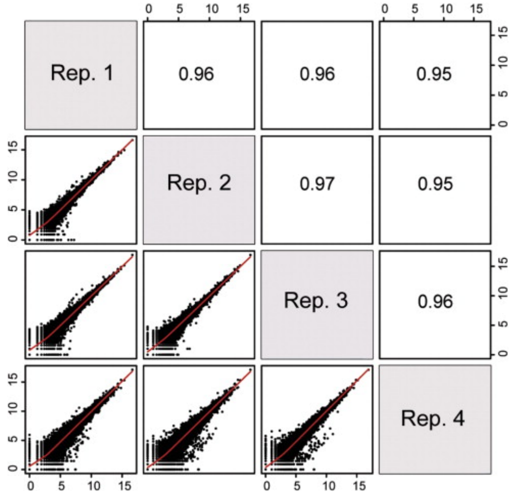
🧰 RSEM, Kallisto, Salmon, Cufflinks2

🔗 Soneson, Charlotte, *et al*. "Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences." F1000Research 4 (2015)
🔗 Zhang, Chi, *et al*. "Evaluation and comparison of computational tools for RNA-seq isoform quantification." BMC genomics 18.1 (2017): 583

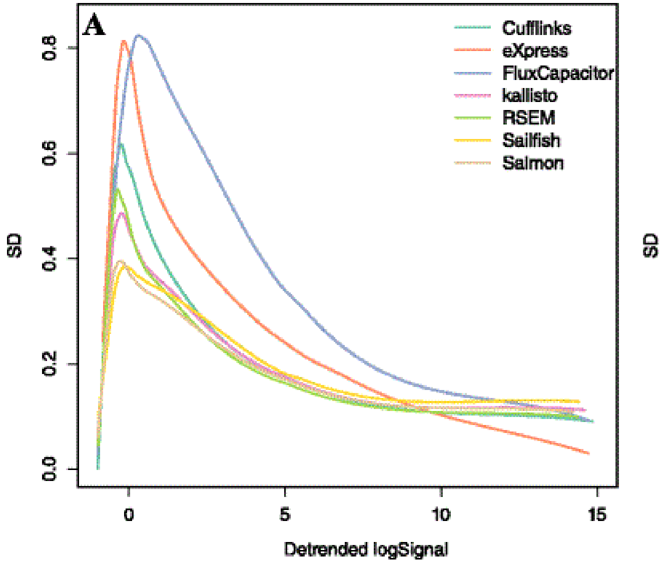
Quantification QC

```
ENSG00000000003    140   242   188   143   287   344   438   280   253
ENSG00000000005    0     0     0     0     0     0     0     0     0
ENSG00000000419    69    98    77    55    52    94    116   79    69
ENSG00000000457    56    75    104   79    157   205   183   178   153
ENSG00000000460    33    27    23    19    27    42    69    44    40
ENSG00000000938    7     38    13    17    35    76    53    37    24
ENSG00000000971    545   878   694   636   647   216   492   798   323
ENSG00000001036    79    154   74    80    128   167   220   147   72
```

- Pairwise correlation between samples must be high (>0.9)



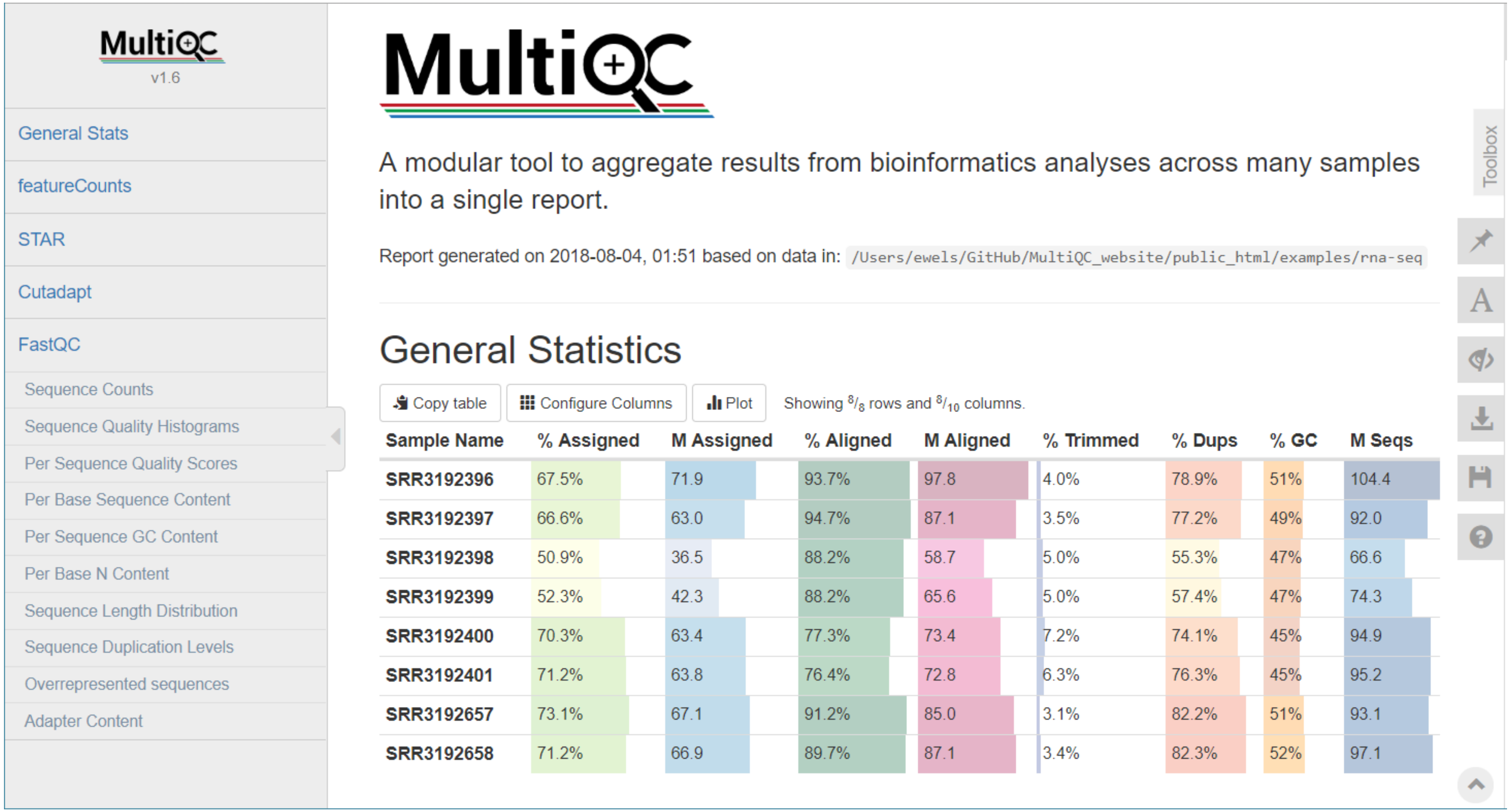
- Count QC using RNASeqComp



RNASeqComp

Teng, Mingxiang, *et al.* "A benchmark for RNA-seq quantification pipelines." Genome biology 17.1 (2016): 74

MultiQC



MultiQC v1.6

- General Stats
- featureCounts
- STAR
- Cutadapt
- 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

MultiQC

A modular tool to aggregate results from bioinformatics analyses across many samples into a single report.

Report generated on 2018-08-04, 01:51 based on data in: `/Users/ewels/GitHub/MultiQC_website/public_html/examples/rna-seq`

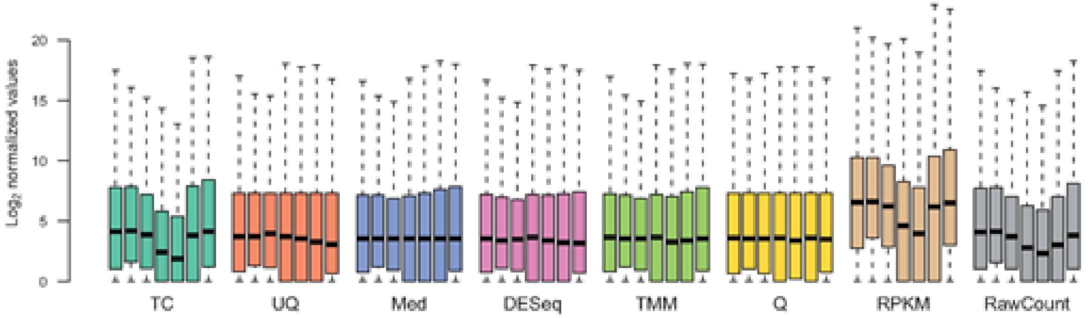
General Statistics

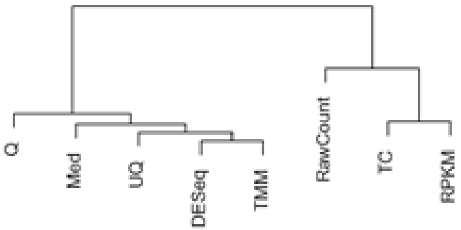
Showing 8/8 rows and 8/10 columns.

Sample Name	% Assigned	M Assigned	% Aligned	M Aligned	% Trimmed	% Dups	% GC	M Seqs
SRR3192396	67.5%	71.9	93.7%	97.8	4.0%	78.9%	51%	104.4
SRR3192397	66.6%	63.0	94.7%	87.1	3.5%	77.2%	49%	92.0
SRR3192398	50.9%	36.5	88.2%	58.7	5.0%	55.3%	47%	66.6
SRR3192399	52.3%	42.3	88.2%	65.6	5.0%	57.4%	47%	74.3
SRR3192400	70.3%	63.4	77.3%	73.4	7.2%	74.1%	45%	94.9
SRR3192401	71.2%	63.8	76.4%	72.8	6.3%	76.3%	45%	95.2
SRR3192657	73.1%	67.1	91.2%	85.0	3.1%	82.2%	51%	93.1
SRR3192658	71.2%	66.9	89.7%	87.1	3.4%	82.3%	52%	97.1

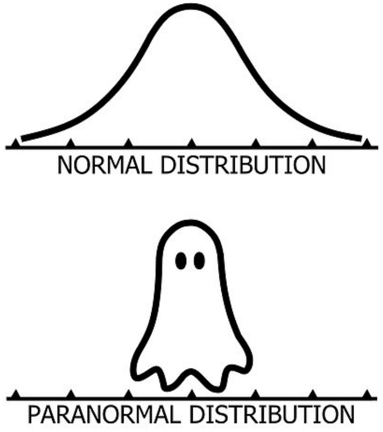
Normalisation

- Control for Sequencing depth & compositional bias
- Median of Ratios (DESeq2) and TMM (edgeR) perform the best





- For DGE using DGE packages, use raw counts
- For clustering, heatmaps etc use VST, VOOM or RLOG
- For own analysis, plots etc, use TPM
- Other solutions: spike-ins/house-keeping genes



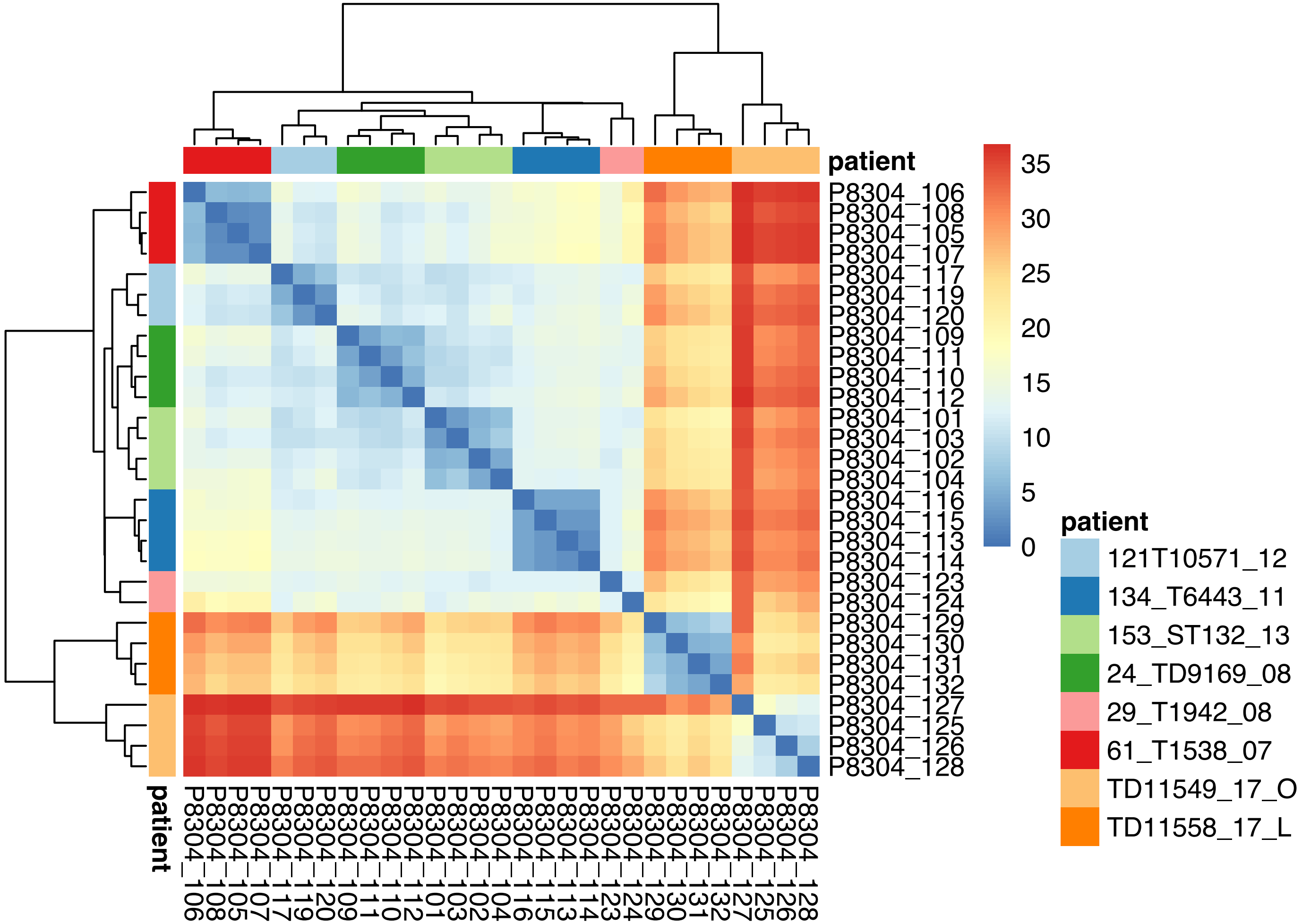
Dillies, Marie-Agnes, *et al*. "A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis." Briefings in bioinformatics 14.6 (2013): 671-683

Evans, Ciaran, Johanna Hardin, and Daniel M. Stoebel. "Selecting between-sample RNA-Seq normalization methods from the perspective of their assumptions." Briefings in bioinformatics (2017)

Wagner, Gunter P., Koryu Kin, and Vincent J. Lynch. "Measurement of mRNA abundance using RNA-seq data: RPKM measure is inconsistent among samples." Theory in biosciences 131.4 (2012): 281-285

Exploratory • Heatmap

- Remove lowly expressed genes
- Transform raw counts to VST, VOOM, RLOG, TPM etc
- Sample-sample clustering heatmap



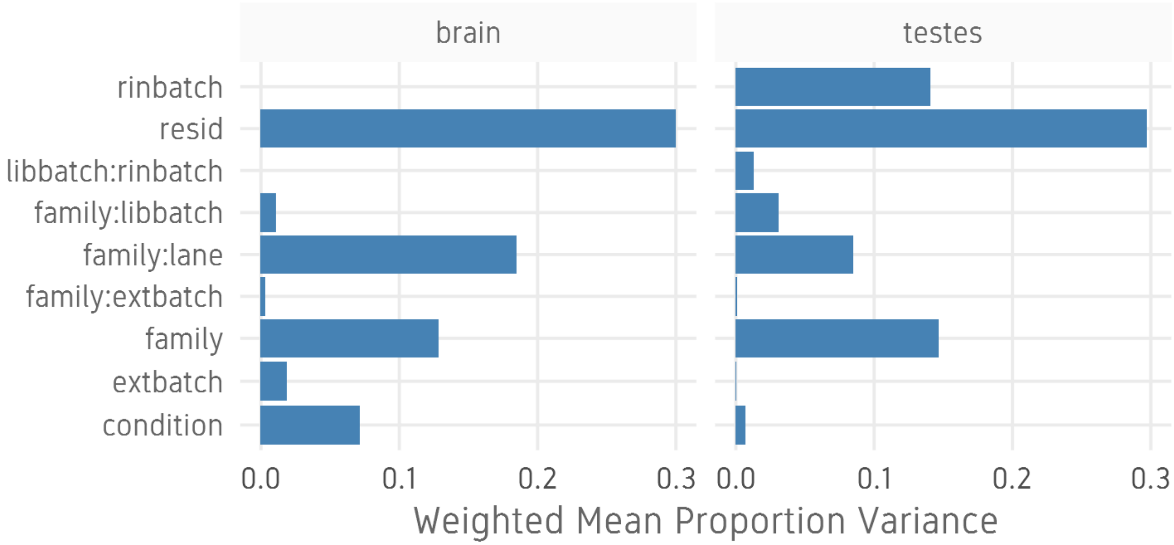
Exploratory • MDS

- ● 121T10571_12
- ● 134_T6443_11
- ● 153_ST132_13
- ● 24_TD9169_08
- ● 29_T1942_08
- ● 61_T1538_07
- ● TD11549_17_O
- ● TD11558_17_L

`cmdscale()`, plotly

Batch correction

- Estimate variation explained by variables (PVCA)



- Find confounding effects as surrogate variables (SVA)
- Model known batches in the LM/GLM model
- Correct known batches (ComBat)(Harsh!)
- Interactively evaluate batch effects and correction (BatchQC)

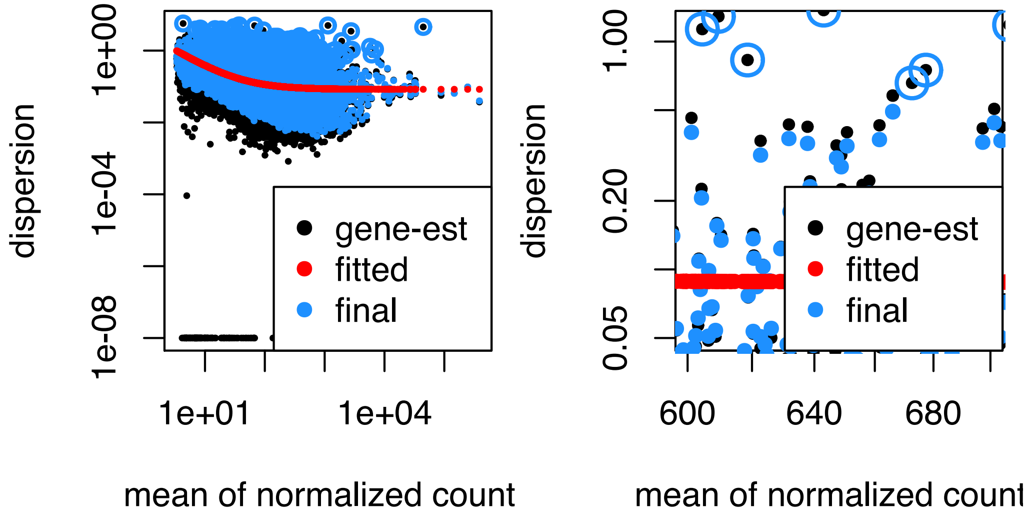
SVA, PVCA, BatchQC

Liu, Qian, and Marianthi Markatou. "Evaluation of methods in removing batch effects on RNA-seq data." Infectious Diseases and Translational Medicine 2.1 (2016): 3-9

Manimaran, Solaiappan, et al. "BatchQC: interactive software for evaluating sample and batch effects in genomic data." Bioinformatics 32.24 (2016): 3836-3838

DGE

- DESeq2, edgeR (Neg-binom > GLM > Test), Limma-Voom (Neg-binom > Voom-transform > LM > Test)
- DESeq2 `~age+condition`
  - Estimate size factors `estimateSizeFactors()`
  - Estimate gene-wise dispersion `estimateDispersions()`
  - Fit curve to gene-wise dispersion estimates
  - Shrink gene-wise dispersion estimates
  - GLM fit for each gene
  - Wald test `nbinomWaldTest()`



DESeq2, edgeR, Limma-Voom

Seyednasrollah, Fatemeh, *et al*. "Comparison of software packages for detecting differential expression in RNA-seq studies." Briefings in bioinformatics 16.1 (2013): 59-70

DGE

- Results `results()`

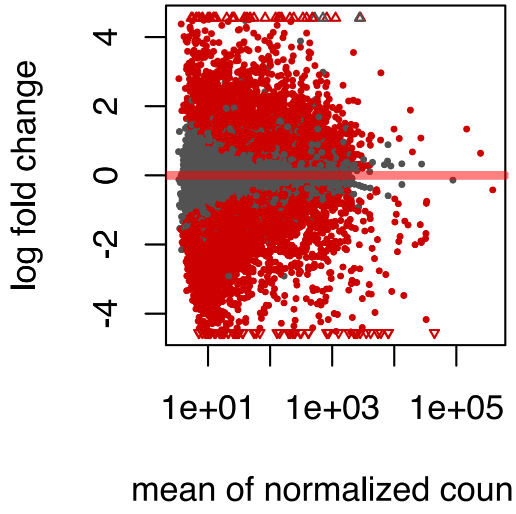
```
## log2 fold change (MLE): type type2 vs control
## Wald test p-value: type type2 vs control
## DataFrame with 1 row and 6 columns
##                         baseMean    log2FoldChange            lfcSE
##                        <numeric>         <numeric>        <numeric>
## ENSG00000000003 242.307796723287 -0.93292608960856 0.11428515031257
##                              stat               pvalue
##                         <numeric>            <numeric>
## ENSG00000000003 -8.16314356727017 3.26416150297406e-16
##                                 padj
##                            <numeric>
## ENSG00000000003 1.36240610021329e-14
```

- Summary `summary()`

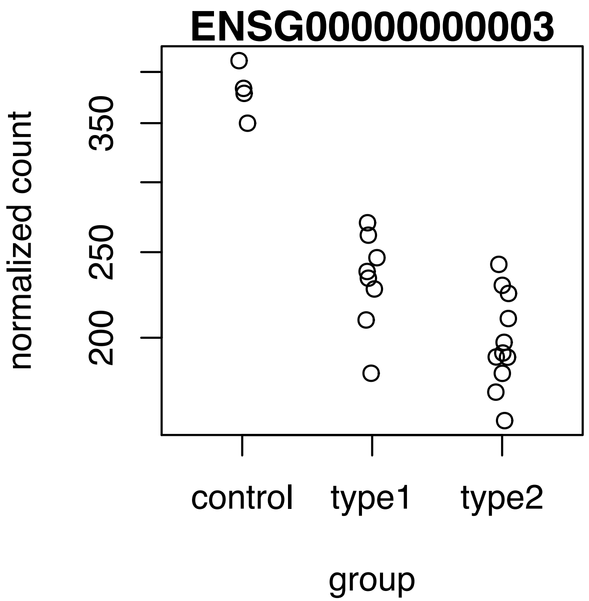
```
##
## out of 17889 with nonzero total read count
## adjusted p-value < 0.1
## LFC > 0 (up)       : 4526, 25%
## LFC < 0 (down)     : 5062, 28%
## outliers [1]       : 25, 0.14%
## low counts [2]     : 0, 0%
## (mean count < 3)
## [1] see 'cooksCutoff' argument of ?results
## [2] see 'independentFiltering' argument of ?results
```

DGE

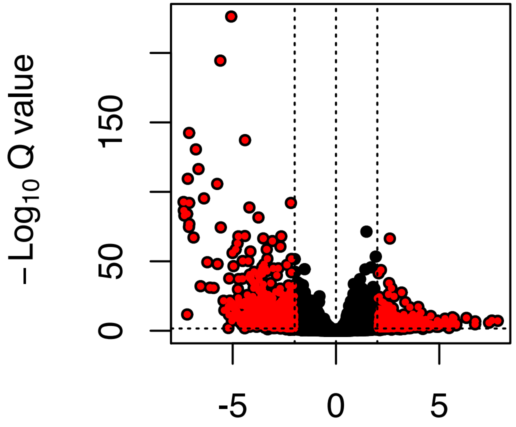
- MA plot `plotMA()`

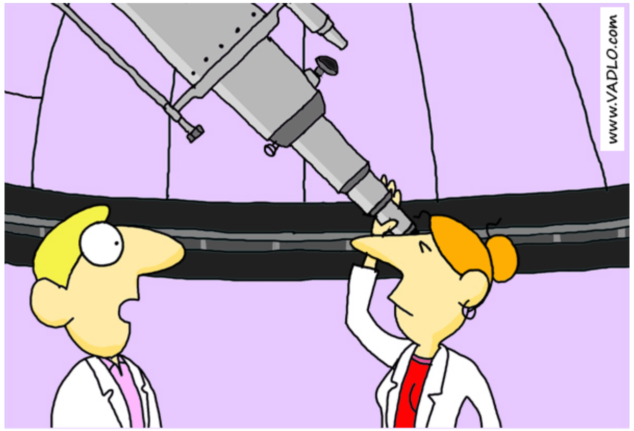


- Normalised counts `plotCounts()`



- Volcano plot

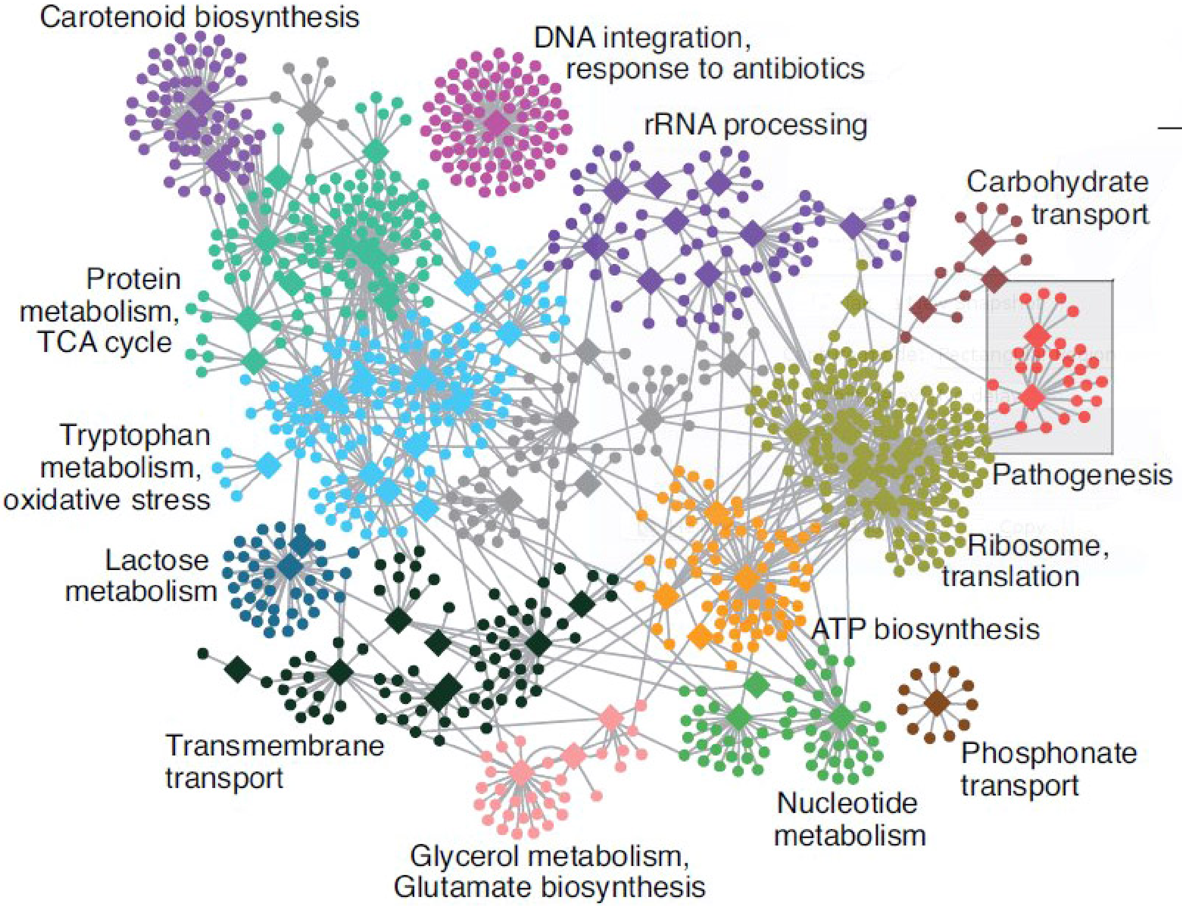


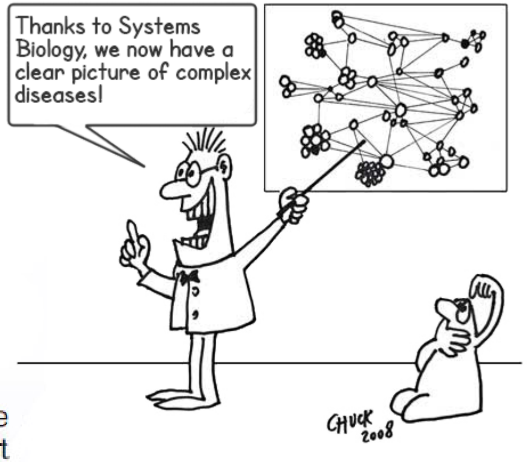


“Can you see the upper points of my scatter plot?”

Functional analysis • GO

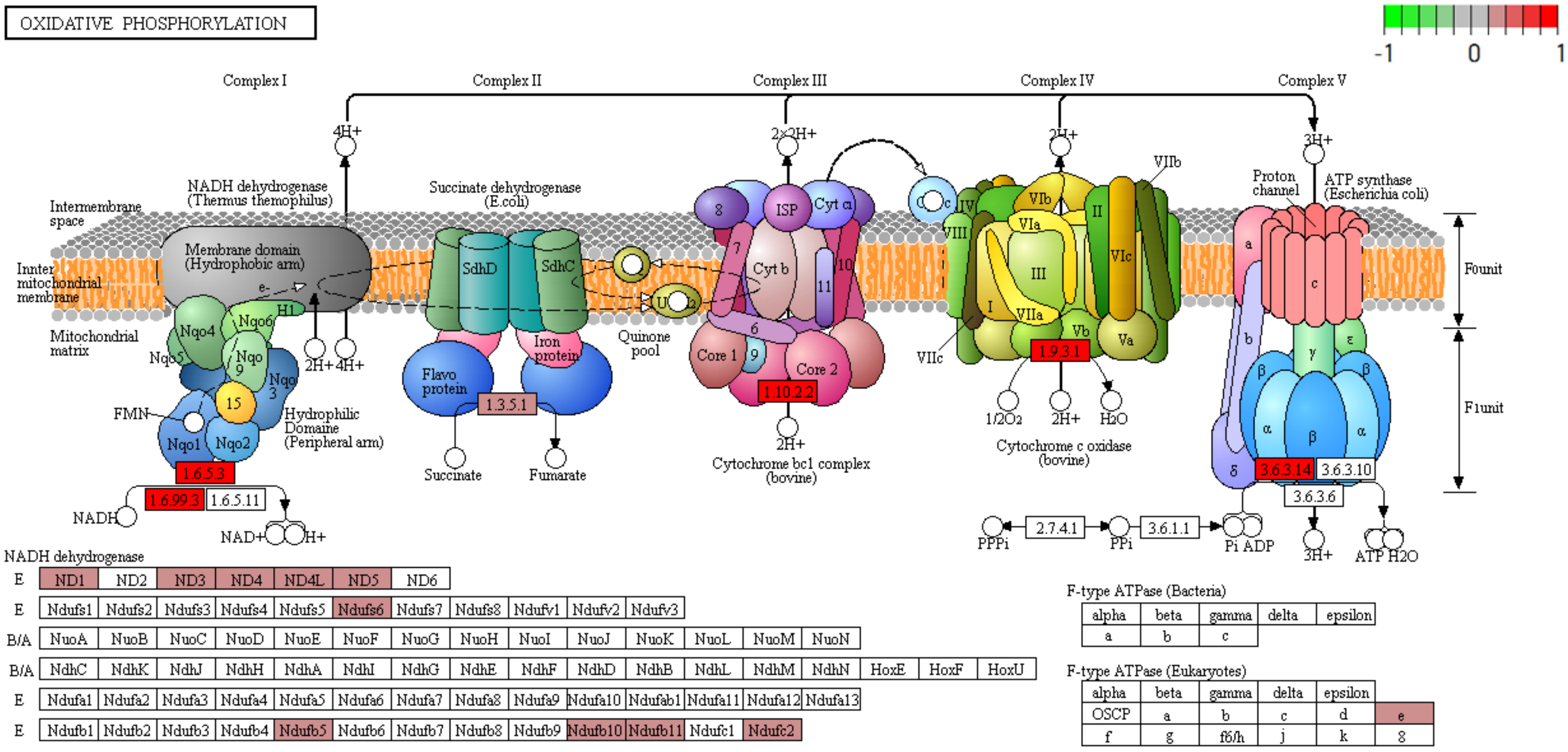
- Gene enrichment analysis
- Gene set enrichment analysis (GSEA)
- Gene ontology / Reactome databases




Thanks to Systems Biology, we now have a clear picture of complex diseases!
CHUCK 2008

Functional analysis • Kegg

- Pathway analysis (Kegg)



NADH dehydrogenase

E	ND1	ND2	ND3	ND4	ND4L	ND5	ND6										
E	Ndufs1	Ndufs2	Ndufs3	Ndufs4	Ndufs5	Ndufs6	Ndufs7	Ndufs8	Ndufv1	Ndufv2	Ndufv3						
B/A	NuoA	NuoB	NuoC	NuoD	NuoE	NuoF	NuoG	NuoH	NuoI	NuoJ	NuoK	NuoL	NuoM	NuoN			
B/A	NdhC	NdhK	NdhJ	NdhH	NdhA	NdhI	NdhG	NdhE	NdhF	NdhD	NdhB	NdhL	NdhM	NdhN	HoxE	HoxF	HoxU
E	Ndufa1	Ndufa2	Ndufa3	Ndufa4	Ndufa5	Ndufa6	Ndufa7	Ndufa8	Ndufa9	Ndufa10	Ndufab1	Ndufa11	Ndufa12	Ndufa13			
E	Ndufb1	Ndufb2	Ndufb3	Ndufb4	Ndufb5	Ndufb6	Ndufb7	Ndufb8	Ndufb9	Ndufb10	Ndufb11	Ndufc1	Ndufc2				

F-type ATPase (Bacteria)

alpha	beta	gamma	delta	epsilon
a	b	c		

F-type ATPase (Eukaryotes)

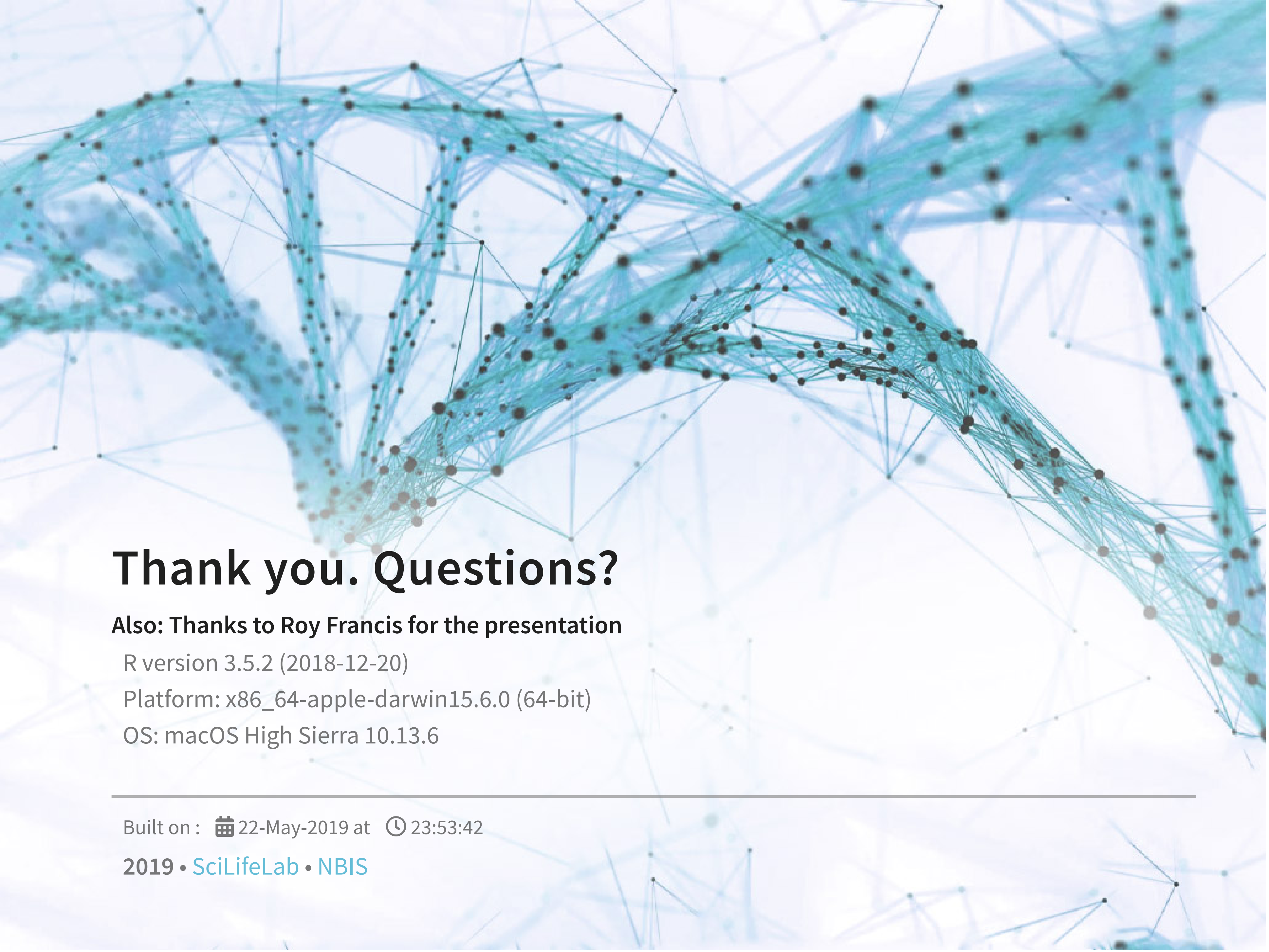
alpha	beta	gamma	delta	epsilon	
OSCP	a	b	c	d	e
f	g	f6/h	j	k	8

DAVID, clusterProfiler, ClueGO, ErmineJ, pathview

Summary

- Sound experimental design to avoid confounding
- Plan carefully about lib prep, sequencing etc based on experimental objective
- Biological replicates may be more important than paired-end reads or long reads
- Discard low quality bases, reads, genes and samples
- Verify that tools and methods align with data assumptions
- Experiment with multiple pipelines and tools
- QC! QC everything at every step

🔗 Conesa, Ana, *et al.* "A survey of best practices for RNA-seq data analysis." Genome biology 17.1 (2016): 13



Thank you. Questions?

Also: Thanks to Roy Francis for the presentation

R version 3.5.2 (2018-12-20)
Platform: x86_64-apple-darwin15.6.0 (64-bit)
OS: macOS High Sierra 10.13.6

Built on : 📅 22-May-2019 at 🕒 23:53:42

2019 • SciLifeLab • NBIS

Hands-On tutorial

Main exercise

- 01 Check the quality of the raw reads with **FastQC**
- 02 Map the reads to the reference genome using **Star**
- 03 Assess the post-alignment quality using **QualiMap**
- 04 Count the reads overlapping with genes using **featureCounts**
- 05 Find DE genes using **edgeR** in R

Bonus exercises

- 01 Functional annotation of DE genes using **GO/Reactome/Kegg** databases
- 02 Visualisation of RNA-seq BAM files using **IGV** genome browser
- 03 RNA-Seq figures and plots using **R**
- 04 De-novo transcriptome assembly using **Trinity**

Data: `/sw/courses/ngsintro/rnaseq/`
Work: `/proj/g2019007/nobackup/<user>/rnaseq/`

Hands-On tutorial

- Course data directory

`/sw/courses/ngsintro/rnaseq/`

```
rnaseq/
+-- bonus/
|   +-- assembly/
|   +-- exon/
|   +-- funannot/
|   +-- visual/
+-- documents/
+-- main/
|   +-- 1_raw/
|   +-- 2_fastqc/
|   +-- 3_mapping/
|   +-- 4_qualimap/
|   +-- 5_dge/
|   +-- 6_multiqc/
+-- reference/
|   +-- mouse/
|   +-- mouse_chr11/
+-- scripts/
```

- Your work directory

`/proj/g2019007/nobackup/[user]/`

```
[user]/
rnaseq/
  +-- 1_raw/
  +-- 2_fastqc/
  +-- 3_mapping/
  +-- 4_qualimap/
  +-- 5_dge/
  +-- 6_multiqc/
  +-- reference/
  |   +-- mouse/
  |   +-- mouse_chr11/
  +-- scripts/
  +-- funannot/
  +-- assembly/
```