

Analysis of bulk RNA-Seq data

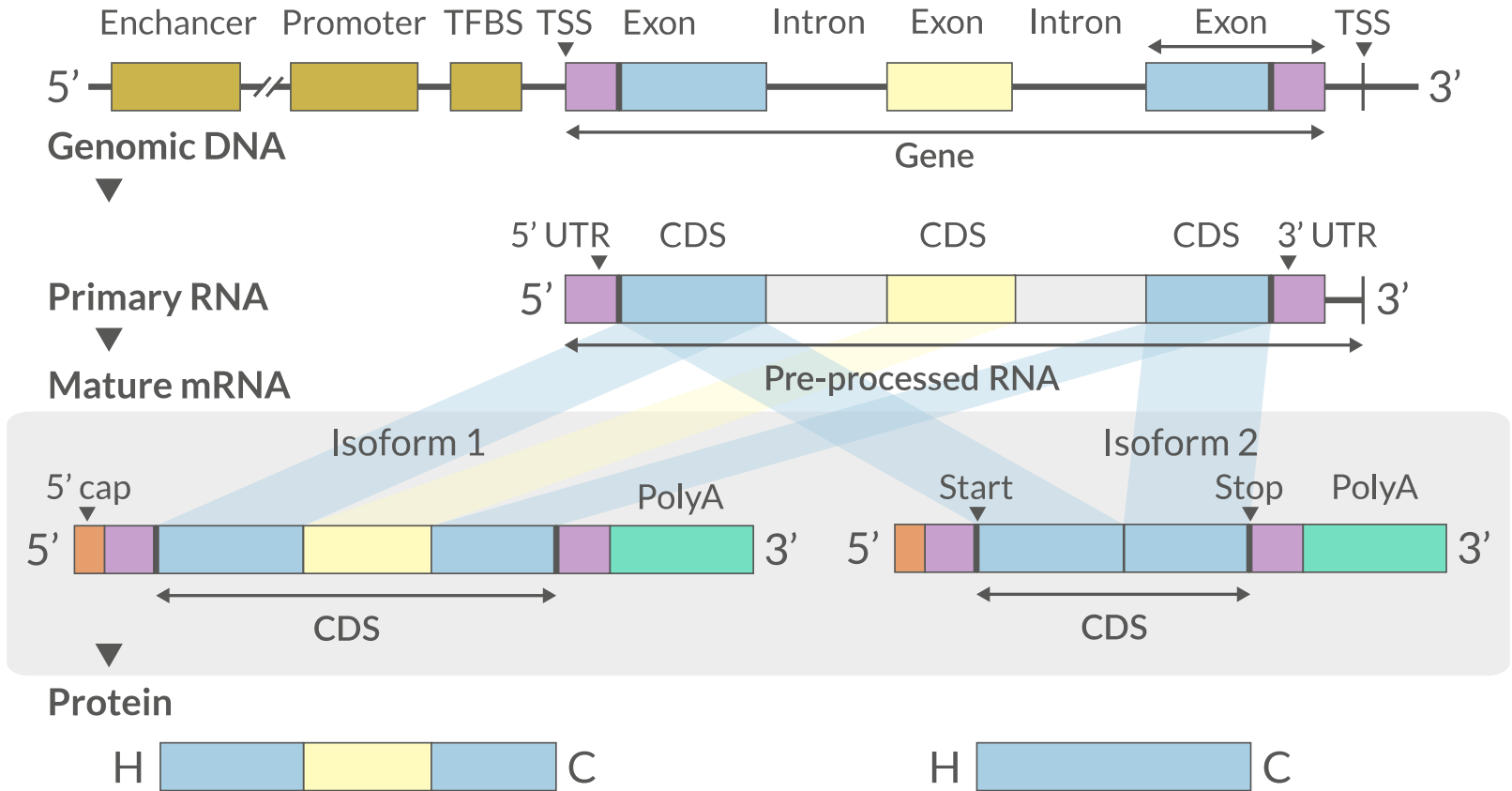
Introduction To Bioinformatics Using NGS Data

26-Mar-2023

Contents

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

What is RNA?

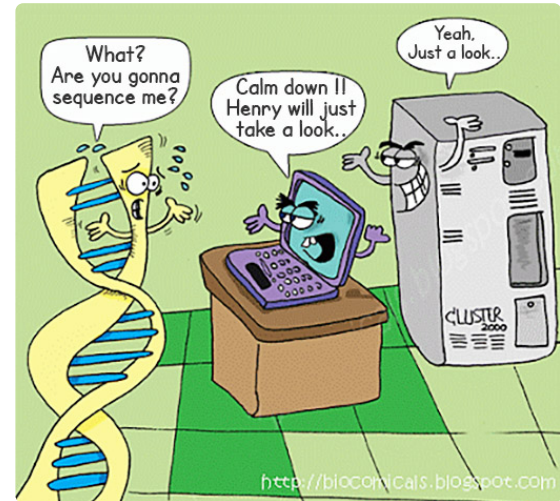
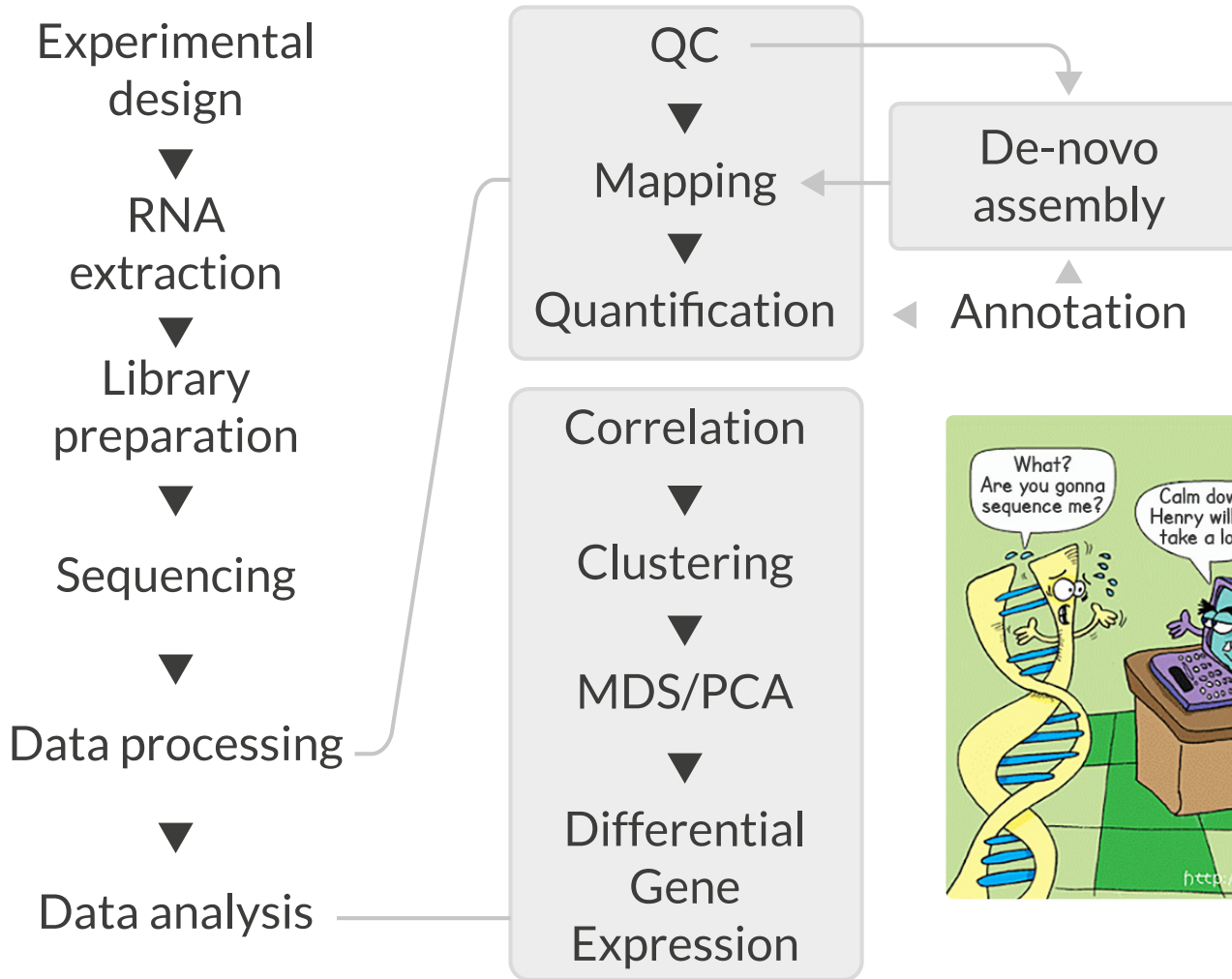


- The transcriptome is spatially and temporally dynamic
- Data comes from functional units (coding regions)
- Only a tiny fraction of the genome

Applications

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

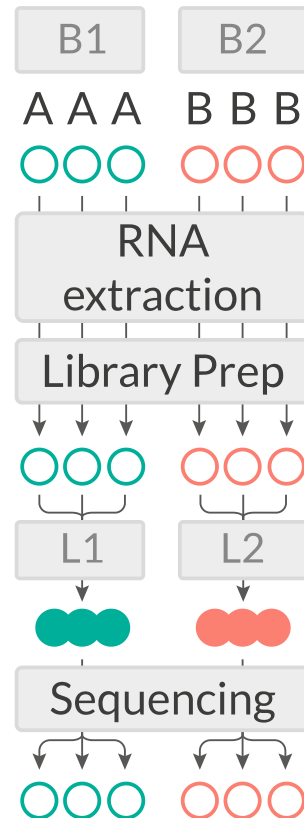
Workflow



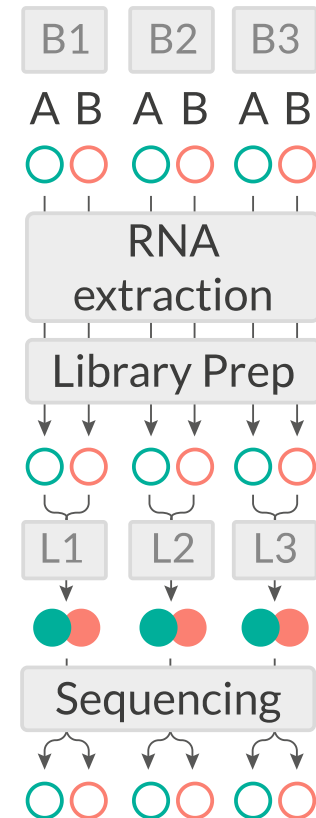
Experimental design

- Biological replicates: 6 - 12 [Schurch et al, 2016](#)
- Sample size estimation [Hart et al, 2013](#)
- Power analysis [RNASeqPower](#) [RNASeqPower web app](#)
- Balanced design to avoid batch effects
[experDesign](#) [DeclareDesign](#)
- RIN values have strong effect [Romero et al, 2014](#)

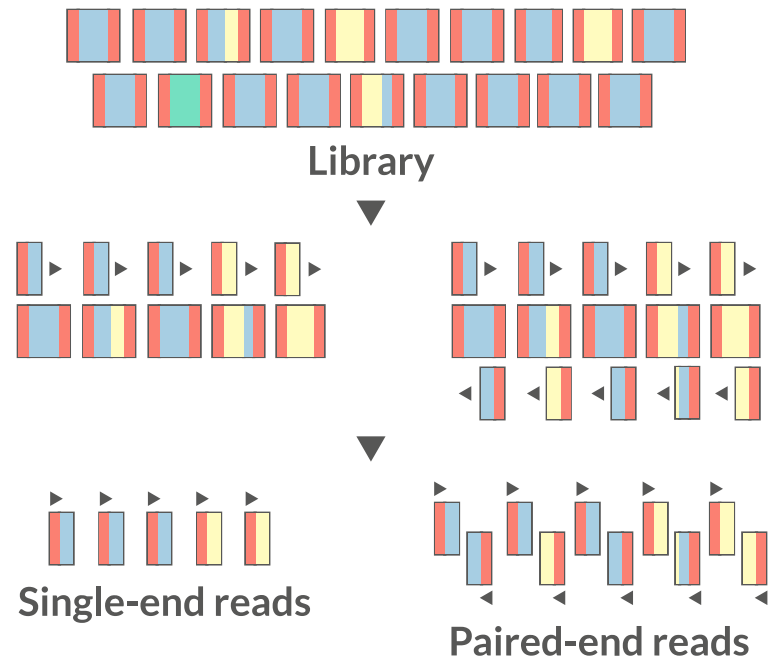
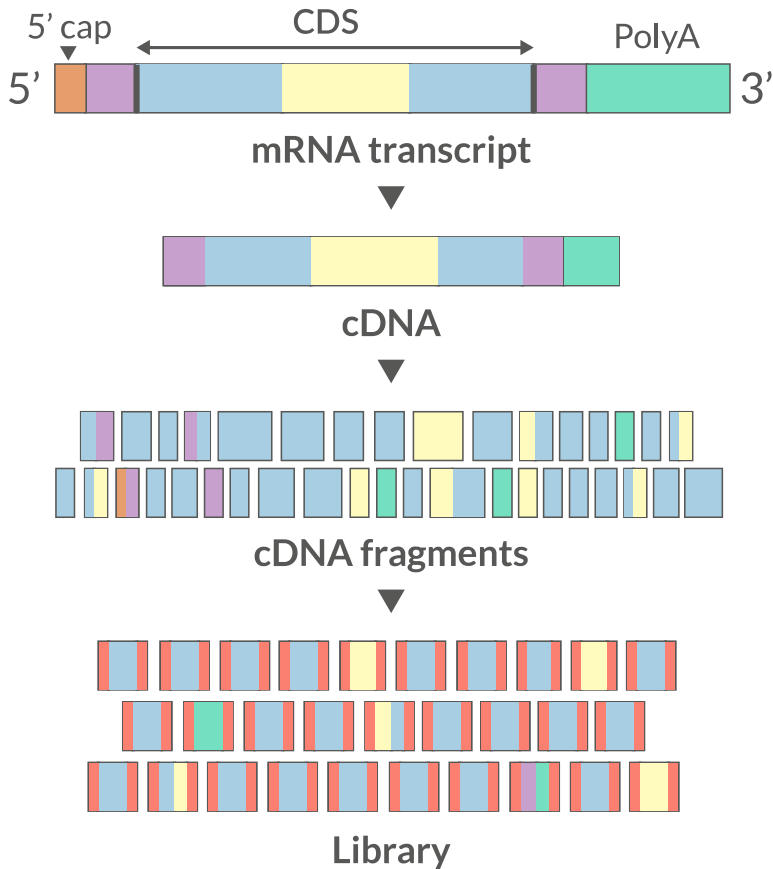
Confounding



Balanced



Library & Sequencing



- polyA selection/Ribosomal RNA depletion
- single-end/Paired-end

Workflow • DGE

Reads

FastQ

FastQ

FastQ



Mapping

STAR

HiSat2

[Kallisto/
Salmon]



Quantification

featureCounts

StringTie



Differential
gene expression

DESeq2/
edgeR/
Limma

Ballgown

Sleuth

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



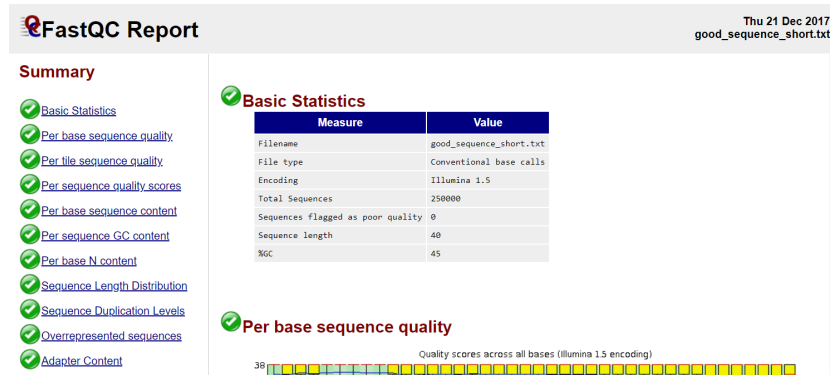
FastQC MultiQC

<https://sequencing.qcfail.com/>

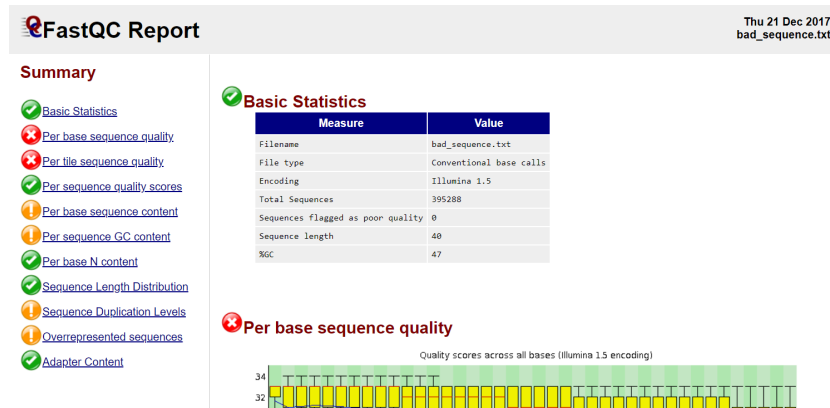
 **QCFAIL.com**

Articles about common next-generation
sequencing problems

Good quality

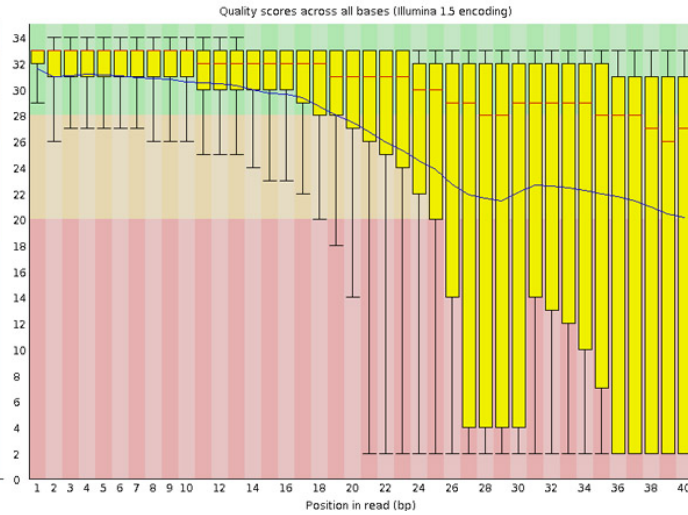
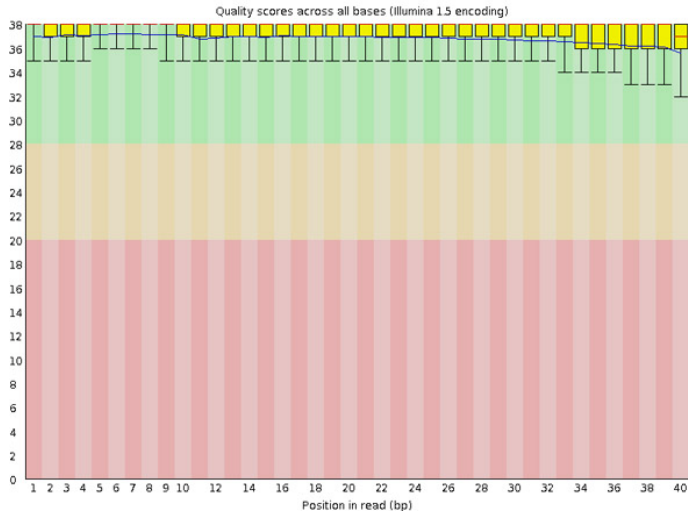


Poor quality

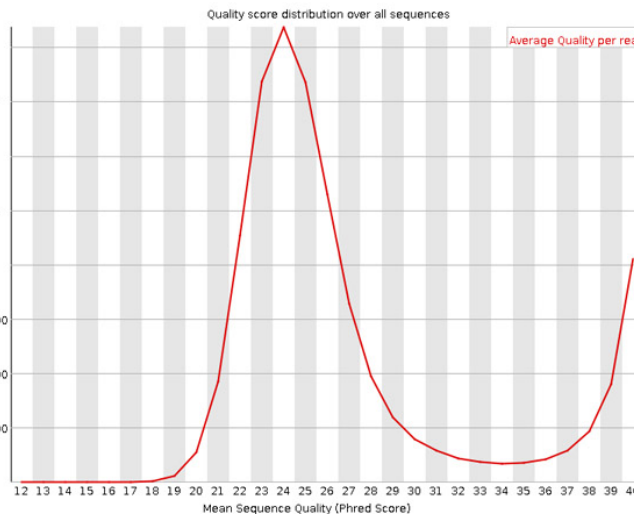
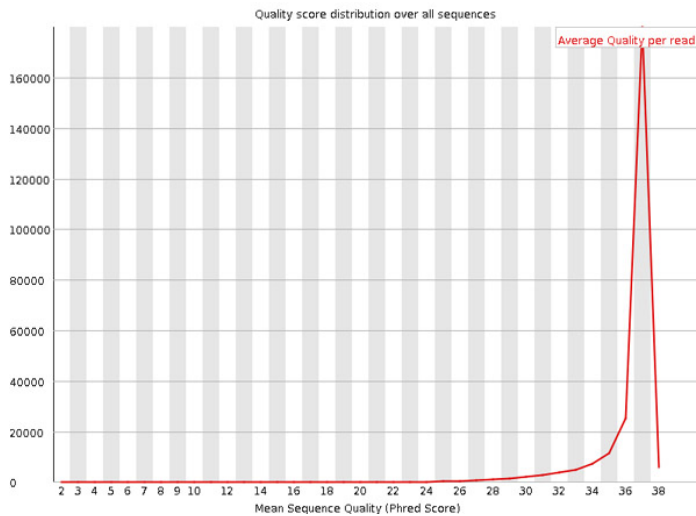


Read QC • PBSQ, PSQS

Per base sequence quality



Per sequence quality scores



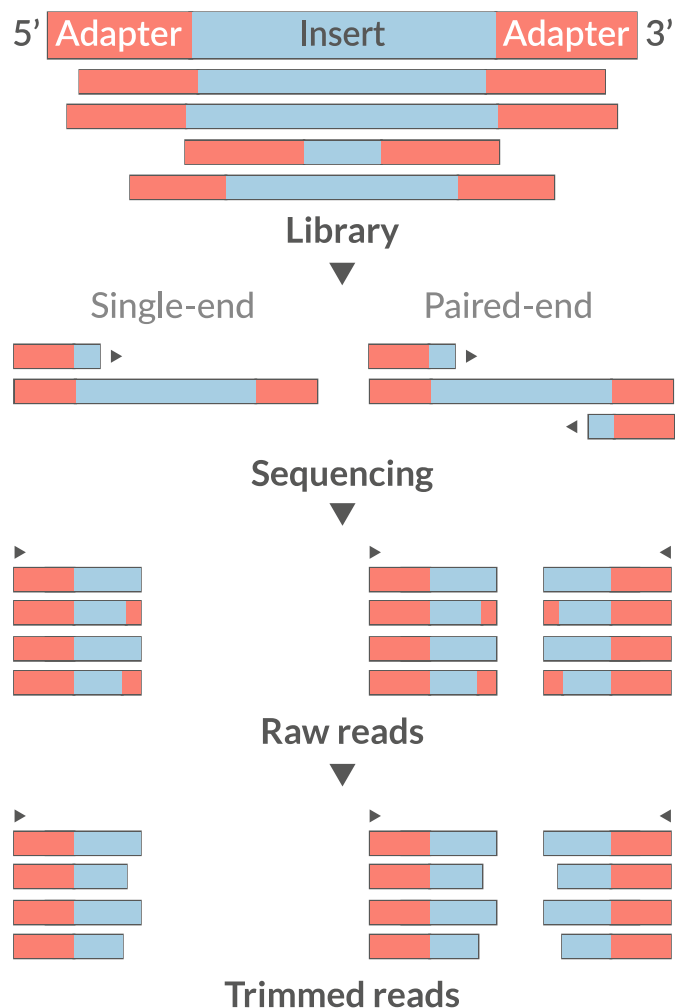
Trimming

- Trimming reads to remove adapter/readthrough or low quality bases
- Related options are hard clipping, filtering reads
- Sliding window trimming
- Filter by min/max read length
 - Remove reads less than ~18nt
- Demultiplexing/Splitting

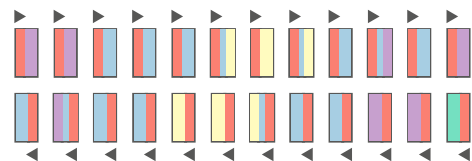
When to avoid trimming?

- Read trimming may not always be necessary [Liao et al, 2020](#)
- Fixed read length may sometimes be more important
- Expected insert size distribution may be more important for assemblers

Cutadapt
 fastp
 Prinseq

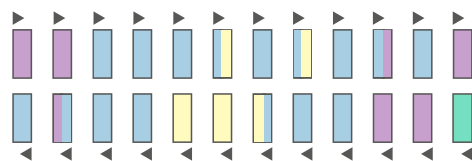


Mapping



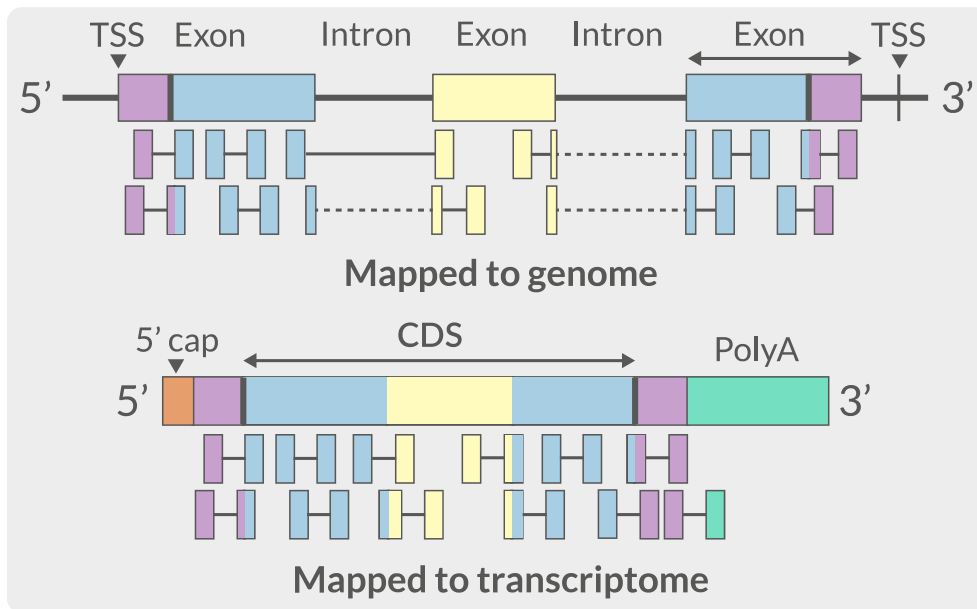
Paired-end reads

Adapter trimming



Trimmed reads

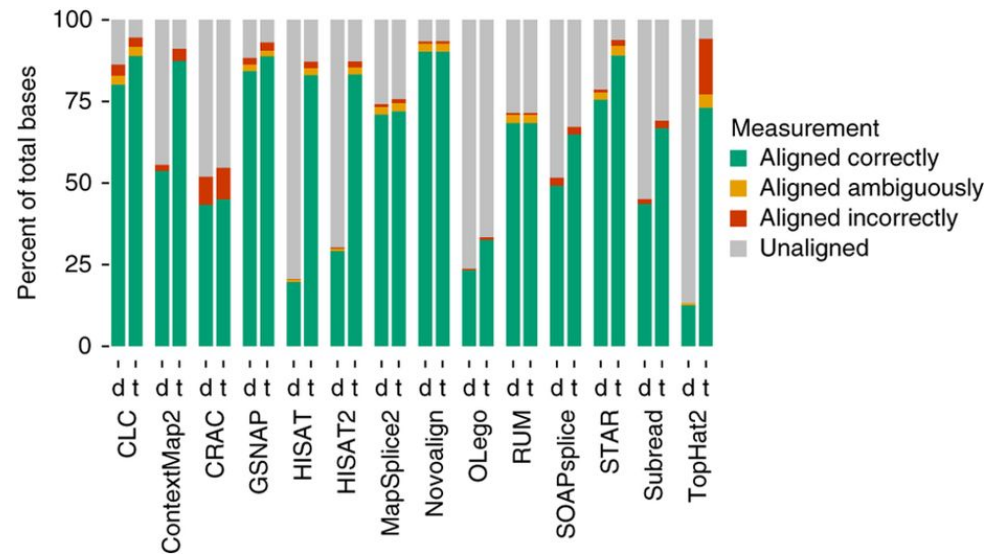
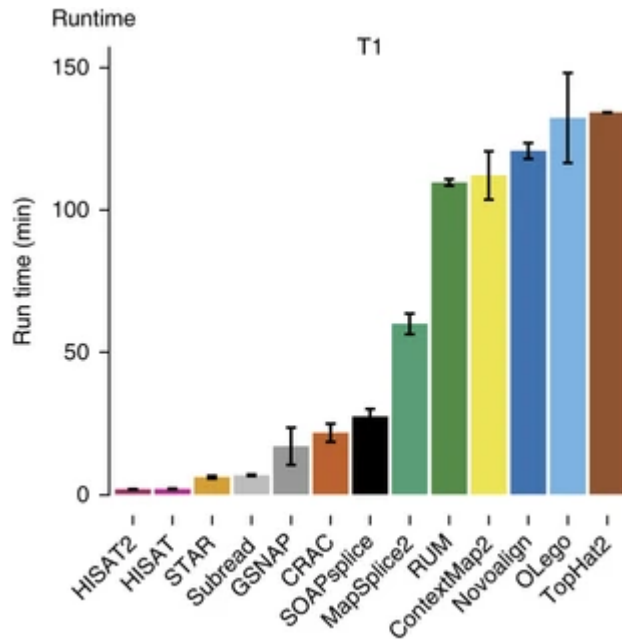
Mapping



- Aligning reads back to a reference sequence
- Mapping to genome vs transcriptome
- Splice-aware alignment (genome) (STAR, HISAT2 etc)

STAR
 HiSat2
 Baruzzo et al, 2017

Aligners • Metrics



Baruzzo et al, 2017

Increasing Accuracy

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

Mapping

- Reads (FASTQ)

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

```
@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
CAAATTAATTTAGCCAGAGGCGCACAAACATACGACCTCTAAAAAAGGTGCTGTAACATG
```

- Annotation (GTF/GFF)

```
#!genome-build GRCz10
#!genebuild-last-updated 2016-11
4 ensembl_havana gene 6732 52059 . - . gene_id "ENSDARG000
```

```
seq source feature start end score strand frame attribute
```

▣ Illumina FASTQ format

▣ GTF format

Alignment

- SAM/BAM (Sequence Alignment Map format)

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

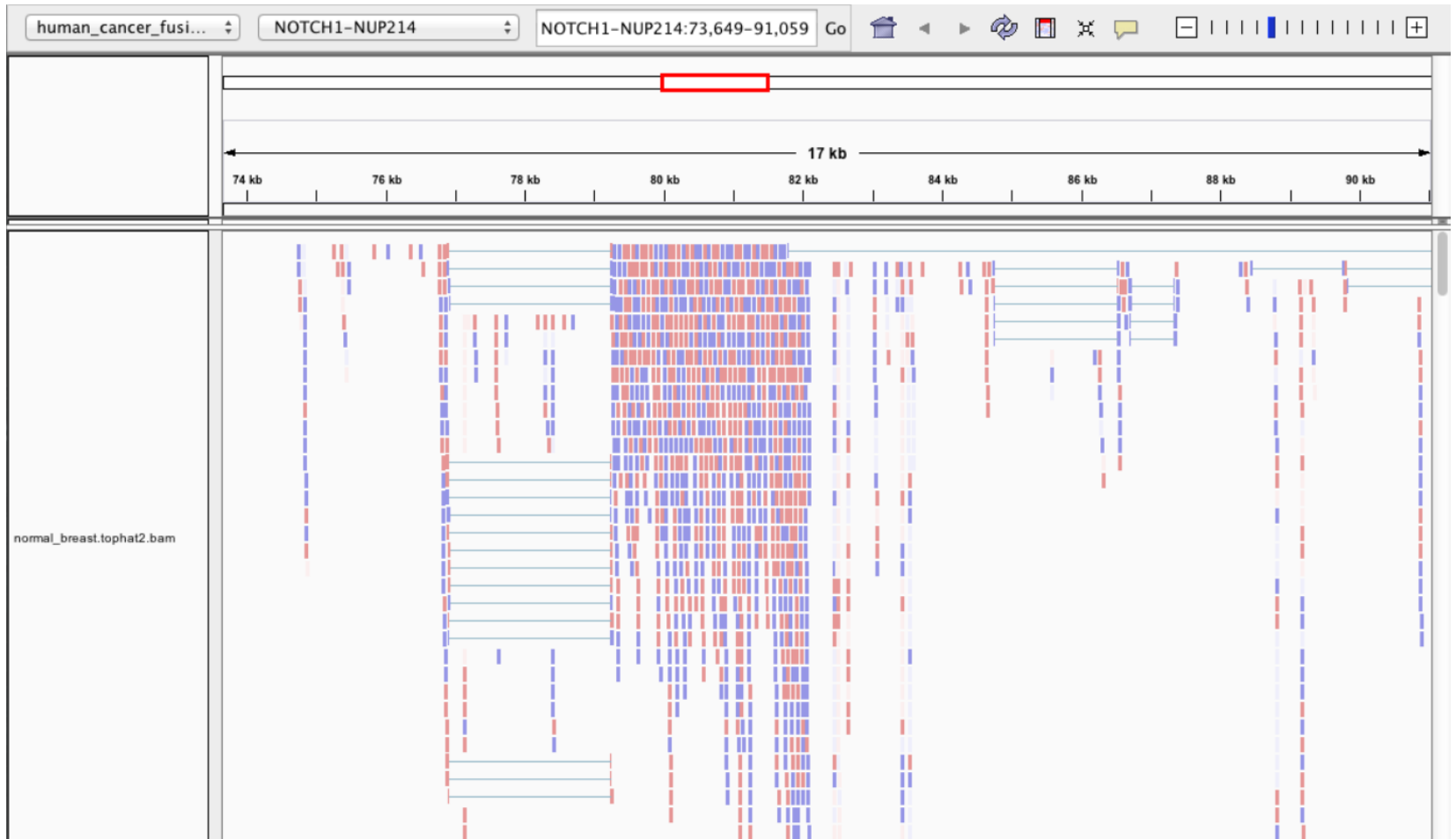
```
query flag ref pos mapq cigar mrnm mpos tlen seq qual opt
```

Never store alignment files in raw SAM format. Always compress it!

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 format

Visualisation • IGV



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

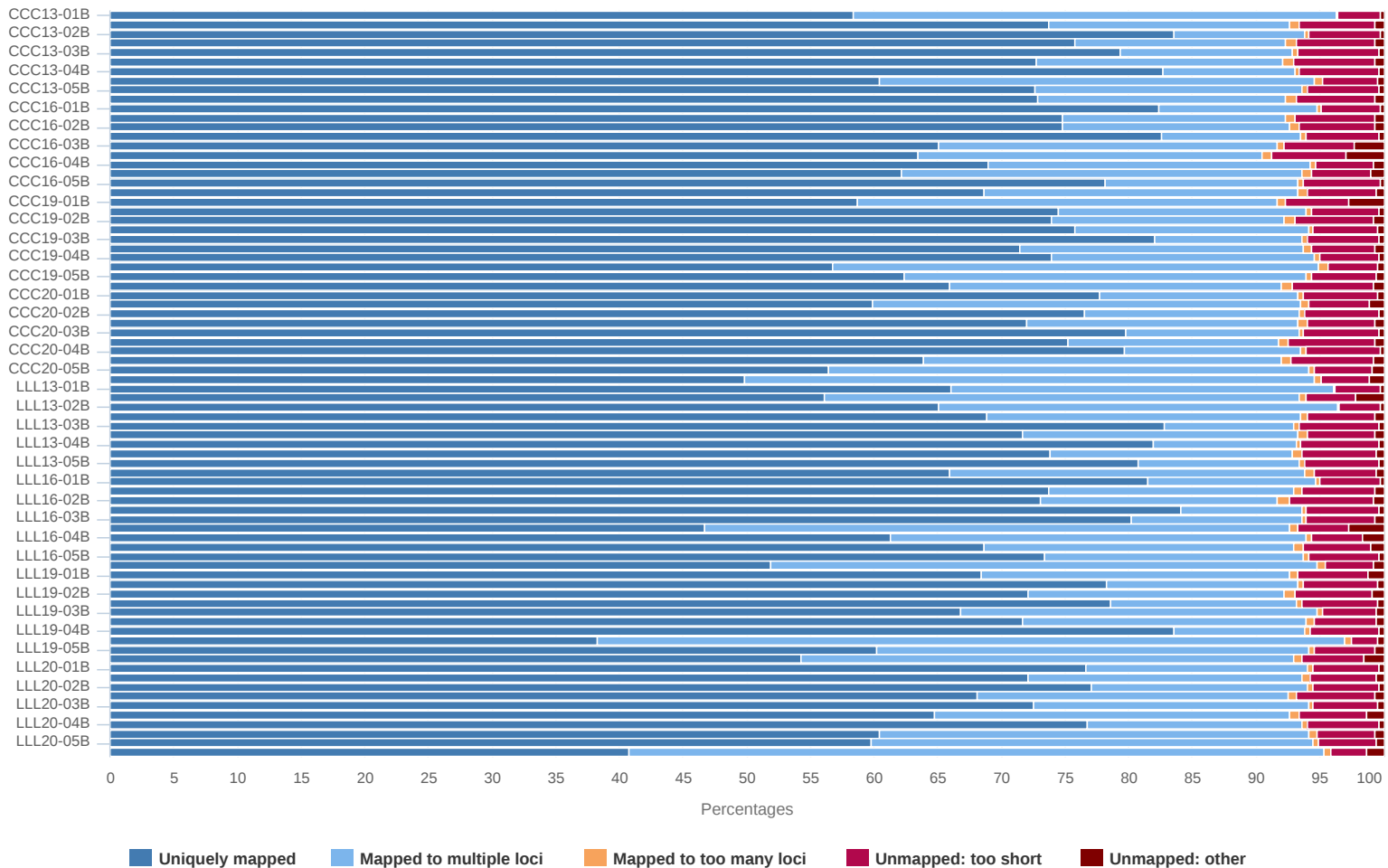
[STAR \(final log file\)](#)[samtools stats](#)[bamtools stats](#)[QoRTs](#)[RSeQC](#)[Qualimap](#)

Alignment QC • STAR Log



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

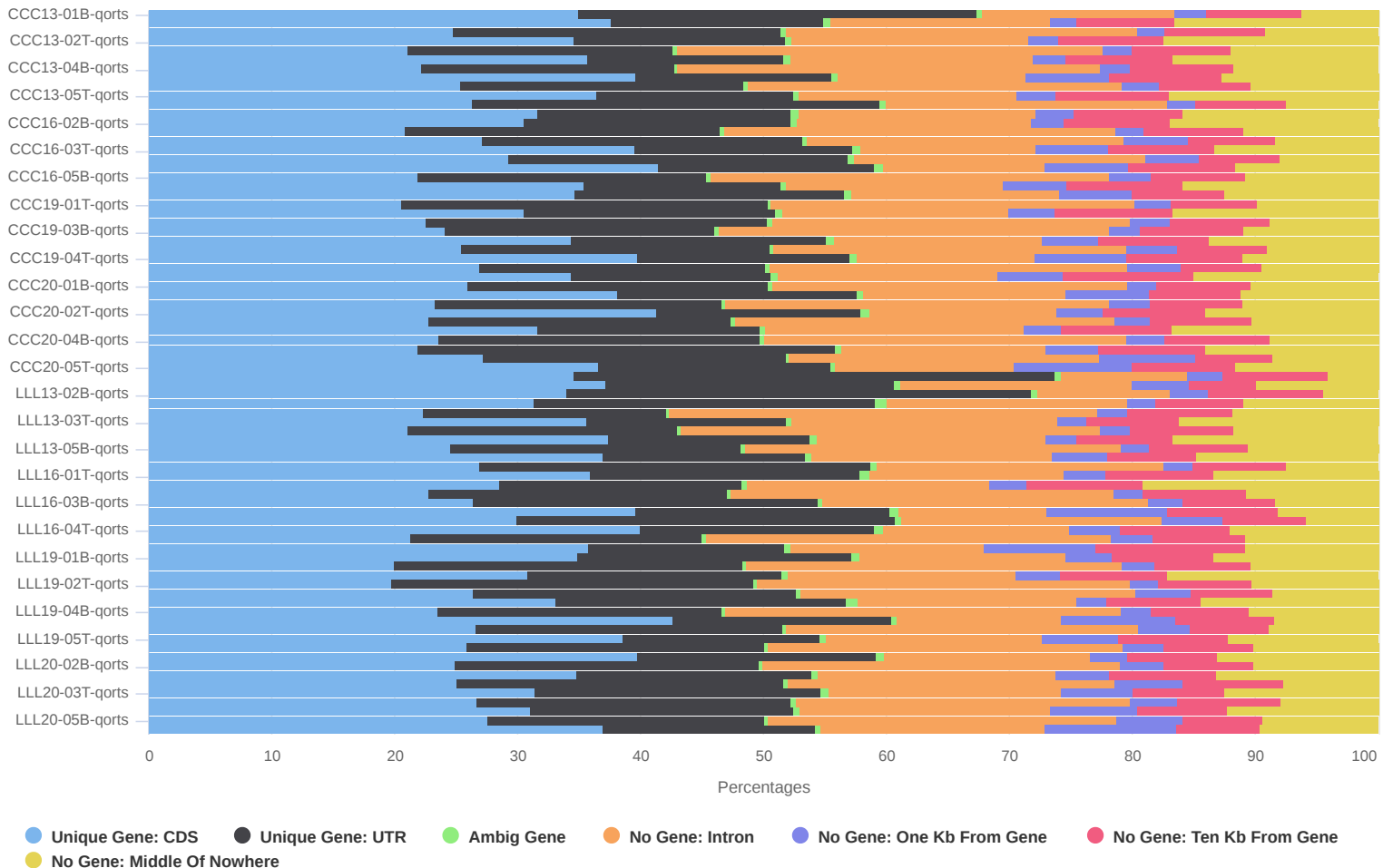
STAR Alignment Scores



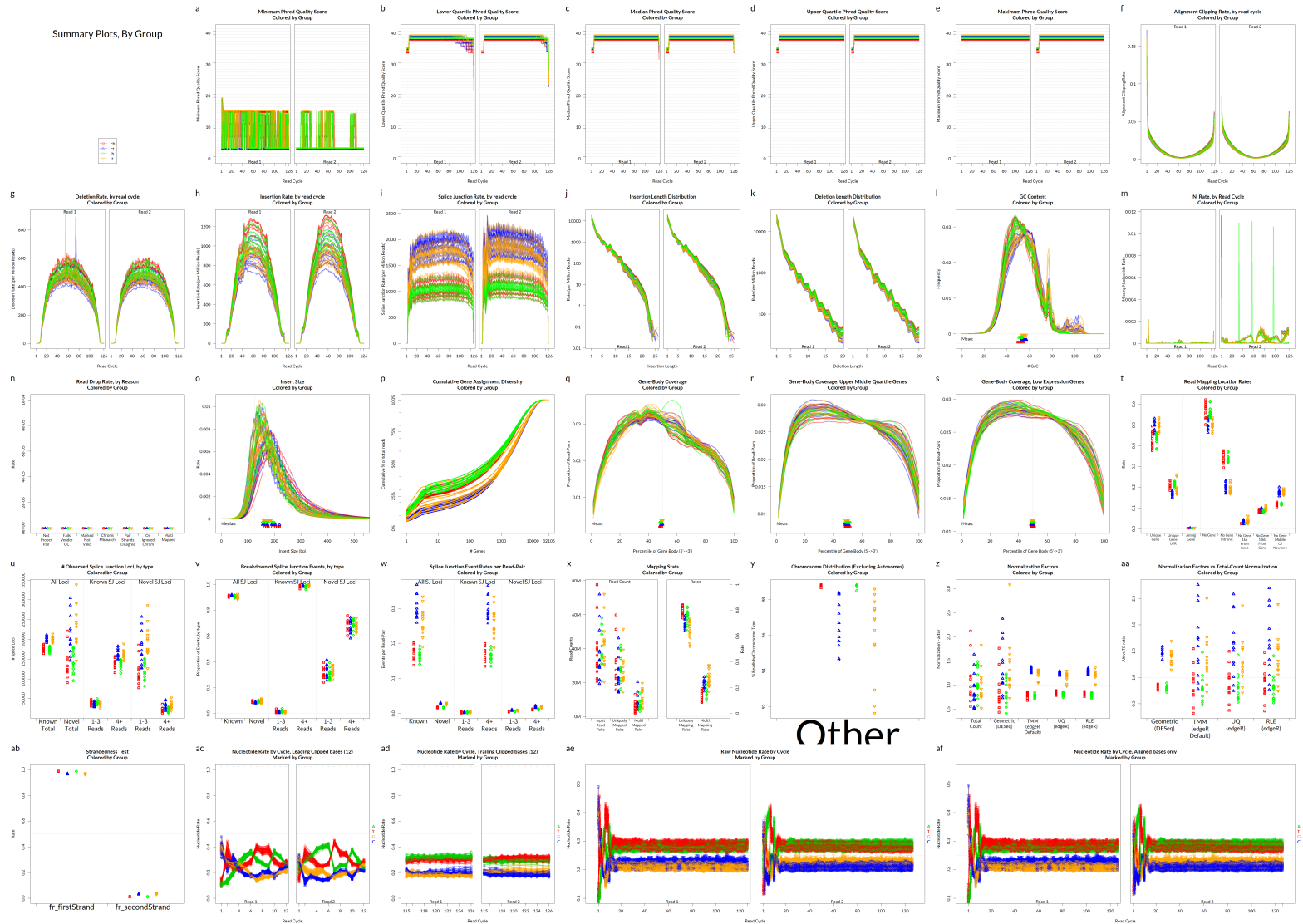
Alignment QC • Features

QoRTs was run on all samples and summarised using MultiQC.

QoRTs: Alignment Locations

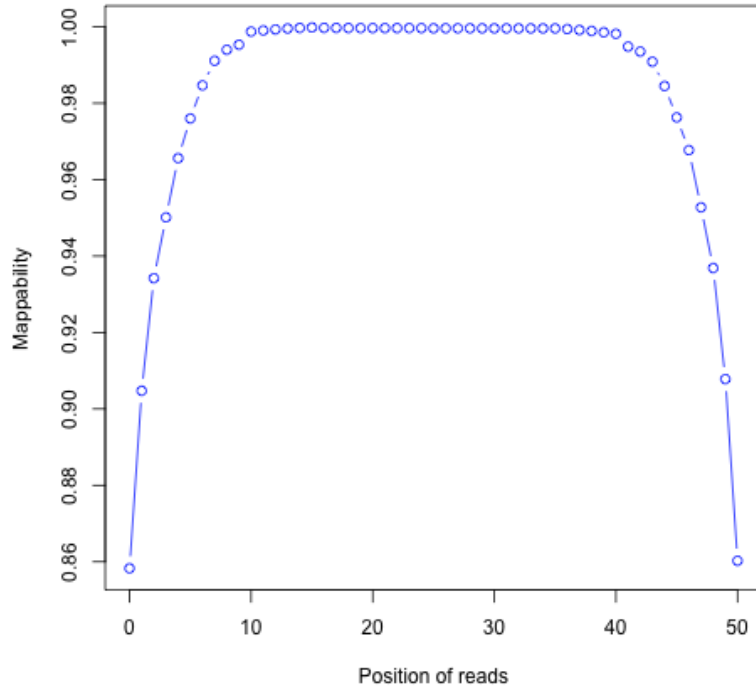


Alignment QC • QoRTs

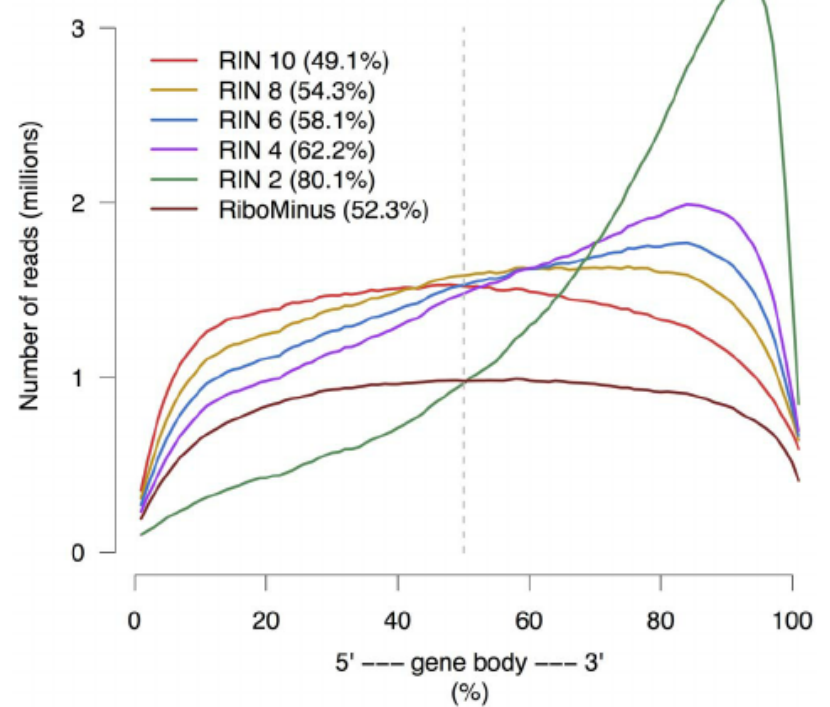


Alignment QC • Examples

Read mapping profile



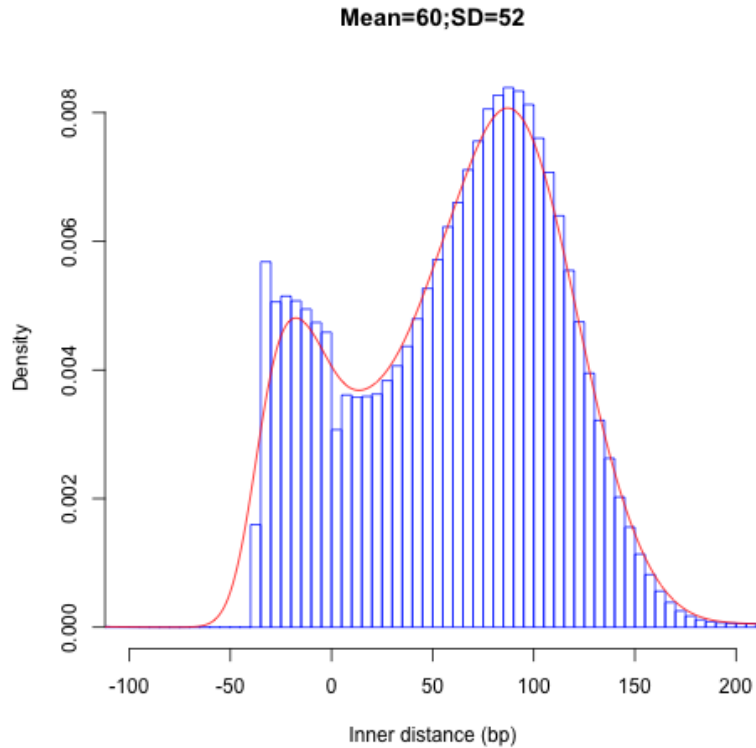
Gene body coverage



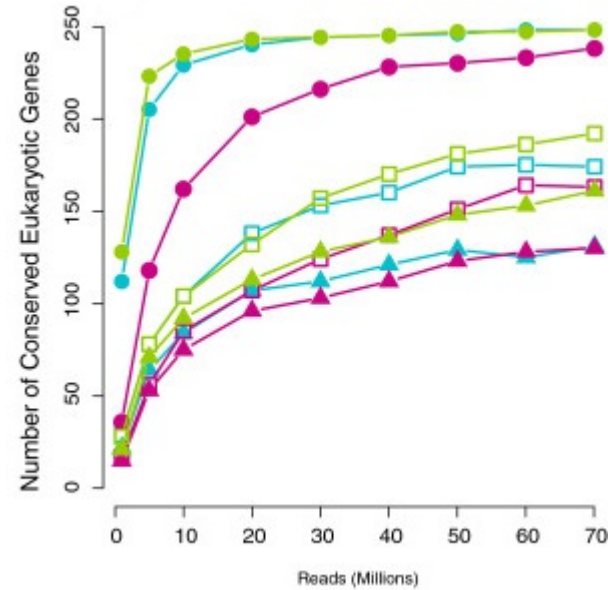
■ Sigurgeirsson et al, 2014

Alignment QC • Examples

Insert size



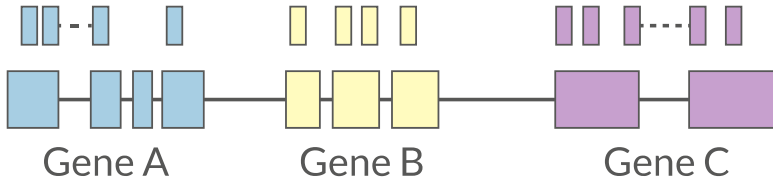
Saturation curve



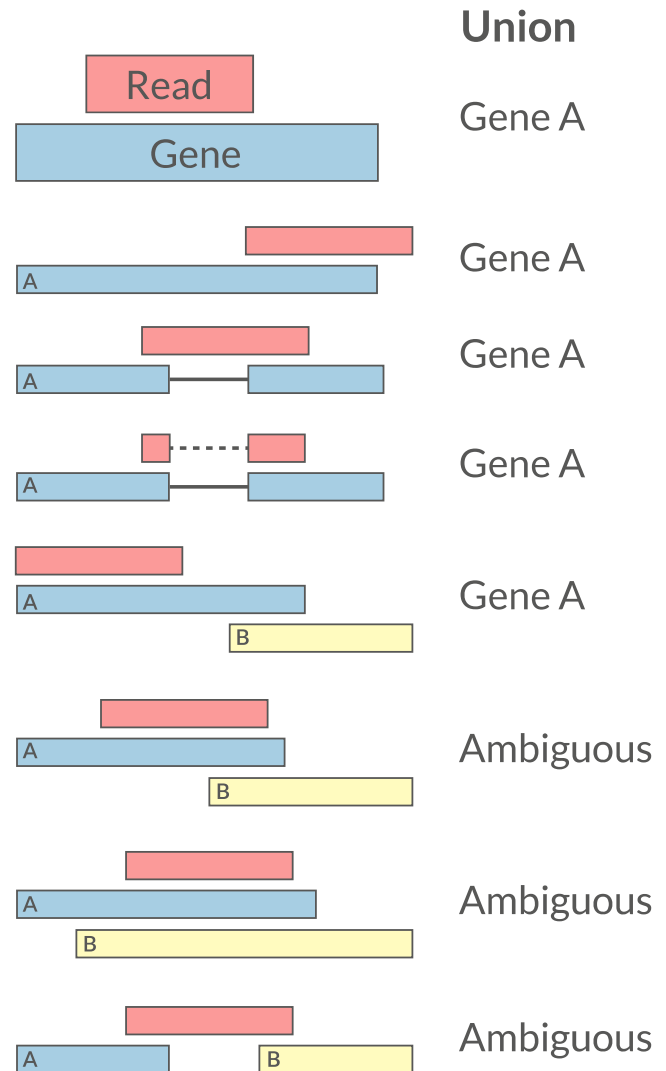
Francis et al, 2013

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 HT Seq



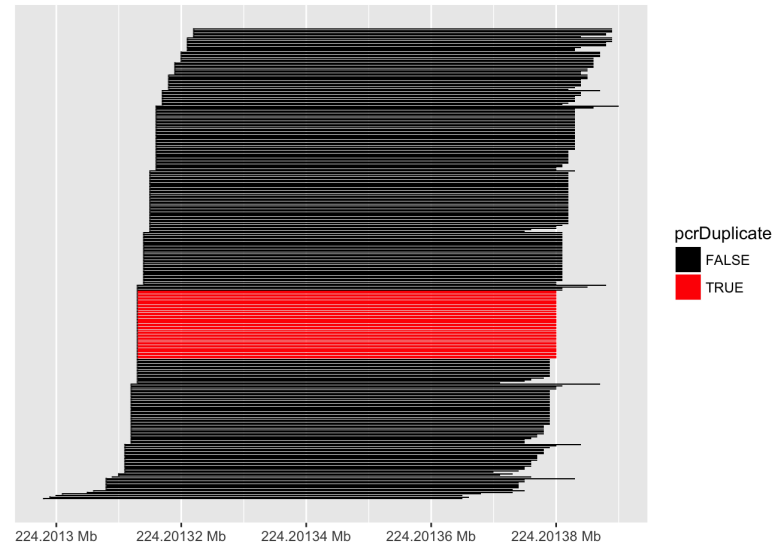
Quantification

PCR duplicates

- Computational deduplication not recommended [Klepikova et al, 2017](#) [Parekh et al, 2016](#)
- Use PCR-free library-prep kits
- Use UMIs during library-prep [Fu et al, 2018](#)

Multi-mapping

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



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

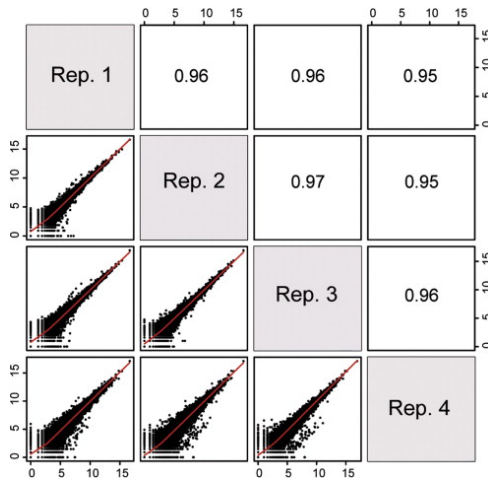
- Ultra-fast & alignment-free
- Bootstrapping & quantification confidence
- Transcript-level counts
- Transcript-level estimates improves gene-level estimates [Soneson et al, 2015](#) [tximport](#)
- Evaluation and comparison of isoform quantification tools [Zhang et al, 2017](#)

[RSEM](#) [Kallisto](#) [Salmon](#)

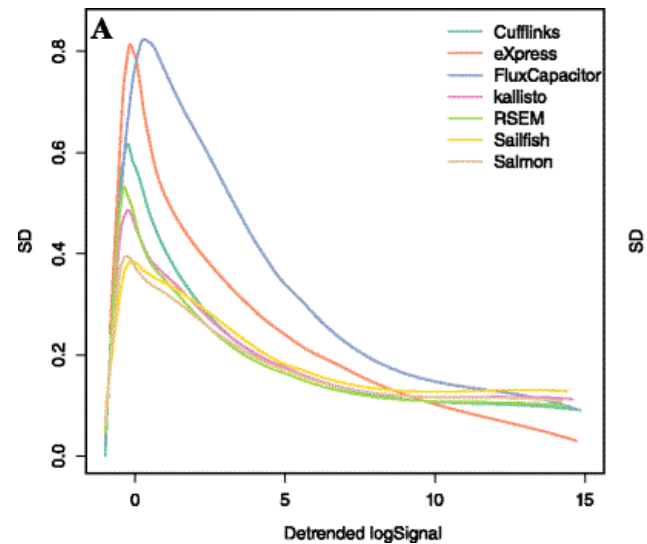
Quantification QC

ENSG000000000003	140	242	188	143	287	344	438	280	253
ENSG000000000005	0	0	0	0	0	0	0	0	0
ENSG000000000419	69	98	77	55	52	94	116	79	69
ENSG000000000457	56	75	104	79	157	205	183	178	153
ENSG000000000460	33	27	23	19	27	42	69	44	40
ENSG000000000938	7	38	13	17	35	76	53	37	24
ENSG000000000971	545	878	694	636	647	216	492	798	323
ENSG000000001036	79	154	74	80	128	167	220	147	72

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



- Count QC using RNASeqComp



- 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

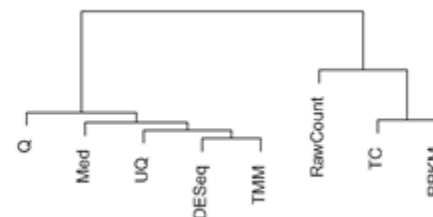
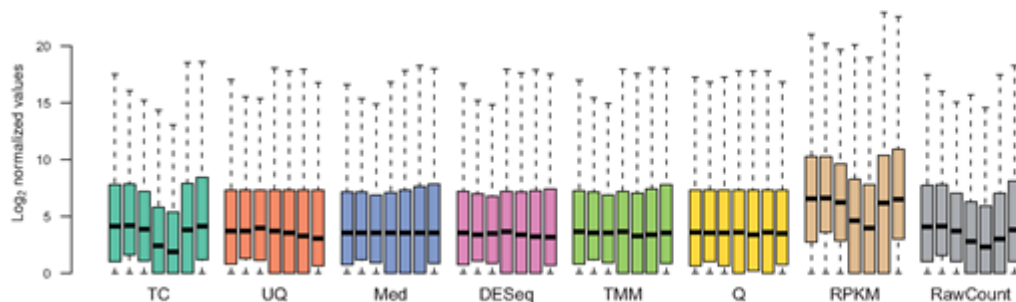
Showing 8 rows and 10 columns.

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

Toolbox
 Pin
 A
 Refresh
 Download
 Home
 Help

Normalisation

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

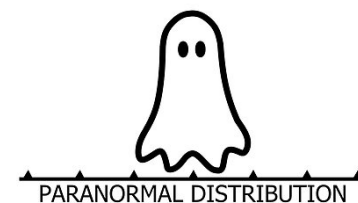
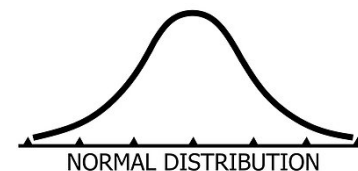


- For DGE using DGE packages, use raw counts
- For clustering, heatmaps etc use VST, VROOM or RLOG
- For own analysis, plots etc, use TPM
- Other solutions: spike-ins/house-keeping genes

▣ Dillies et al, 2013

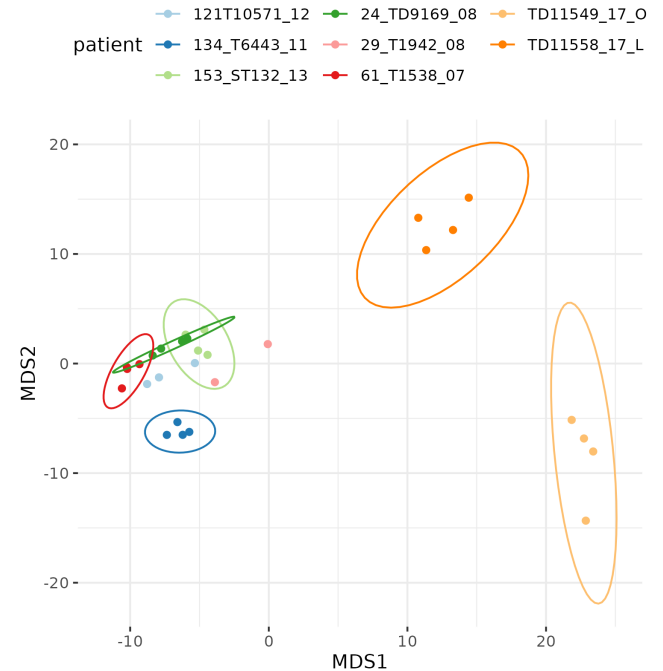
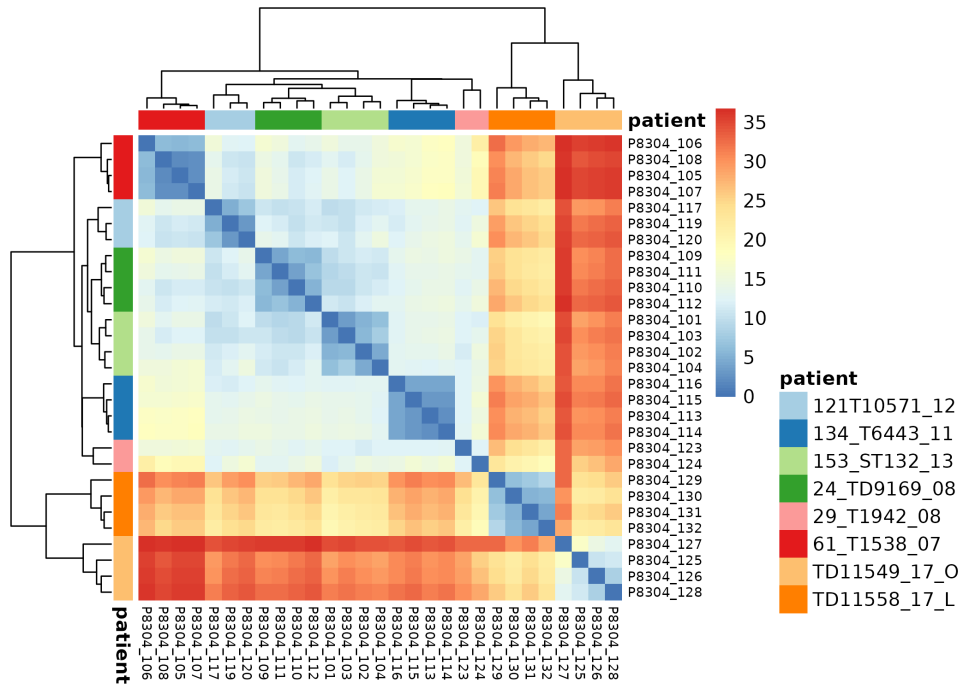
▣ Evans et al, 2017

▣ Wagner et al, 2012



Exploratory

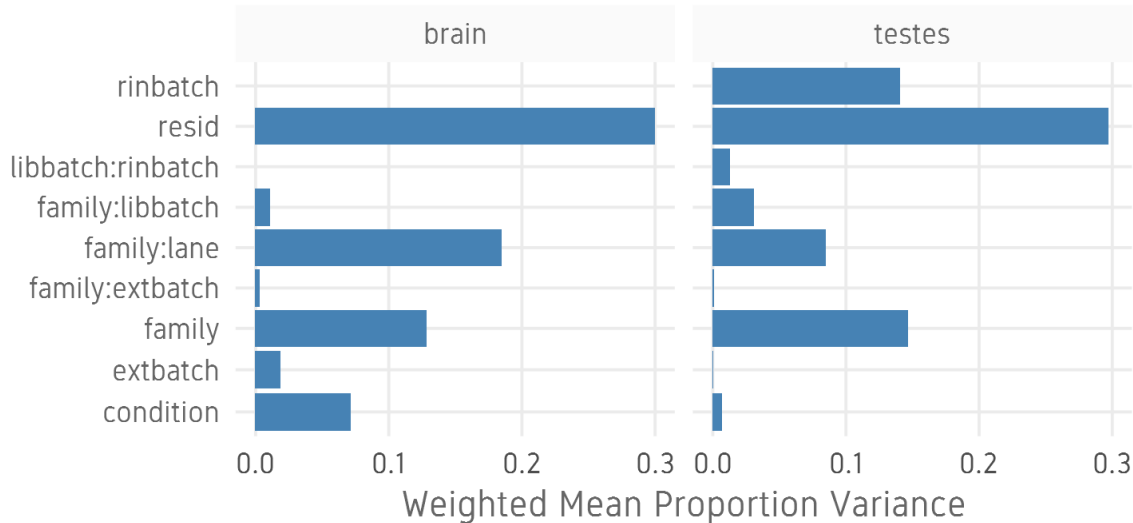
- Remove lowly expressed genes
- Transform raw counts to VST, VOOM, RLOG, TPM etc
- Heatmaps, MDS, PCA etc.



pheatmap







Batch correction

- Estimate variation explained by variables (PVCA) [PVCA](#)



- Find confounding effects as surrogate variables (SVA) [SVA](#)
- Model known batches in the LM/GLM model
- Correct known batches (ComBat from SVA)(Only if you are desperate! [Zindler et al, 2020](#))
- Interactively evaluate batch effects and correction (BatchQC) [Manimaran et al, 2016](#) [BatchQC](#)

Differential expression

		Gene A	Gene B	...	Gene N
Group 1	Sample 1 	12	54
	Sample 2 	8	47
	Sample 3 	13	48
Group 2	Sample 1 	22	50
	Sample 2 	18	48
	Sample 3 	25	41

- Univariate testing gene-by-gene
- More descriptive, less predictive

- 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>
ENSG000000000003	242.307796723287	-0.932926089608546	0.114285150312285
	stat	pvalue	padj
	<numeric>	<numeric>	<numeric>
ENSG000000000003	-8.16314356729037	3.26416150242775e-16	1.36240609998527e-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%

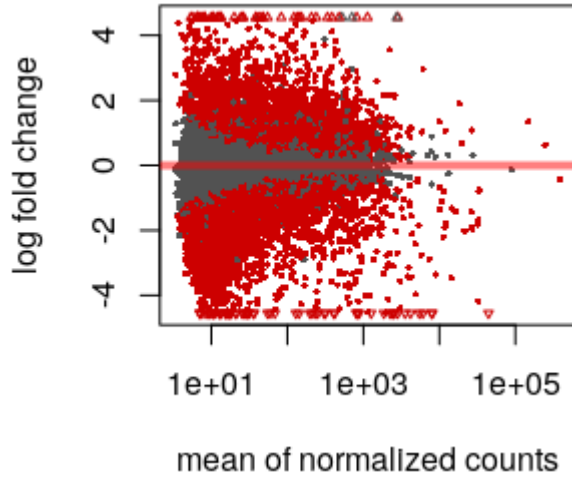
(mean count < 3)

[1] see '`cooksCutoff`' argument of `?results`

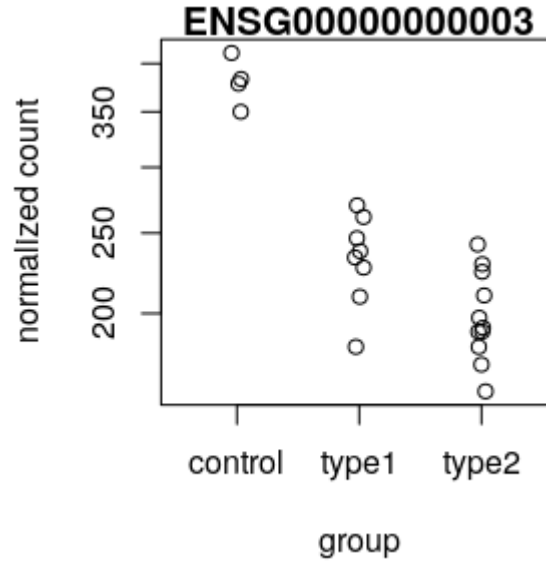
[2] see '`independentFiltering`' argument of `?results`

DGE

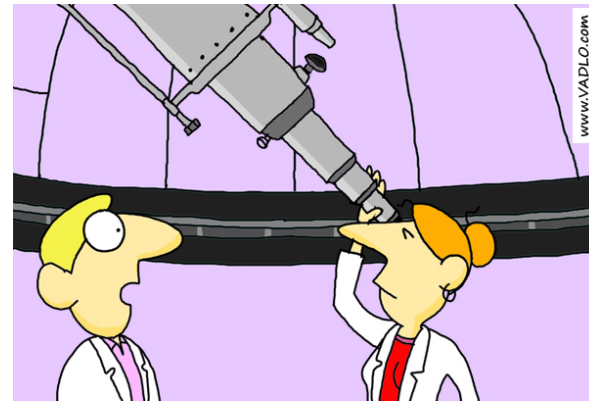
- MA plot `plotMA()`



- Normalised counts `plotCounts()`



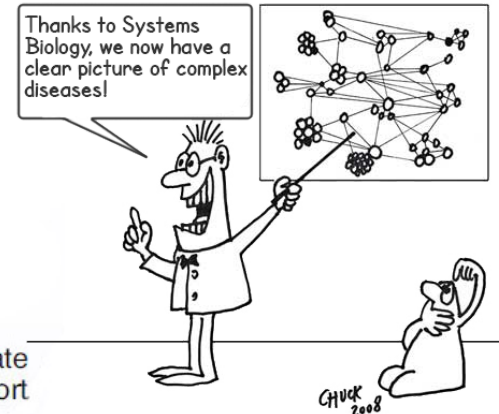
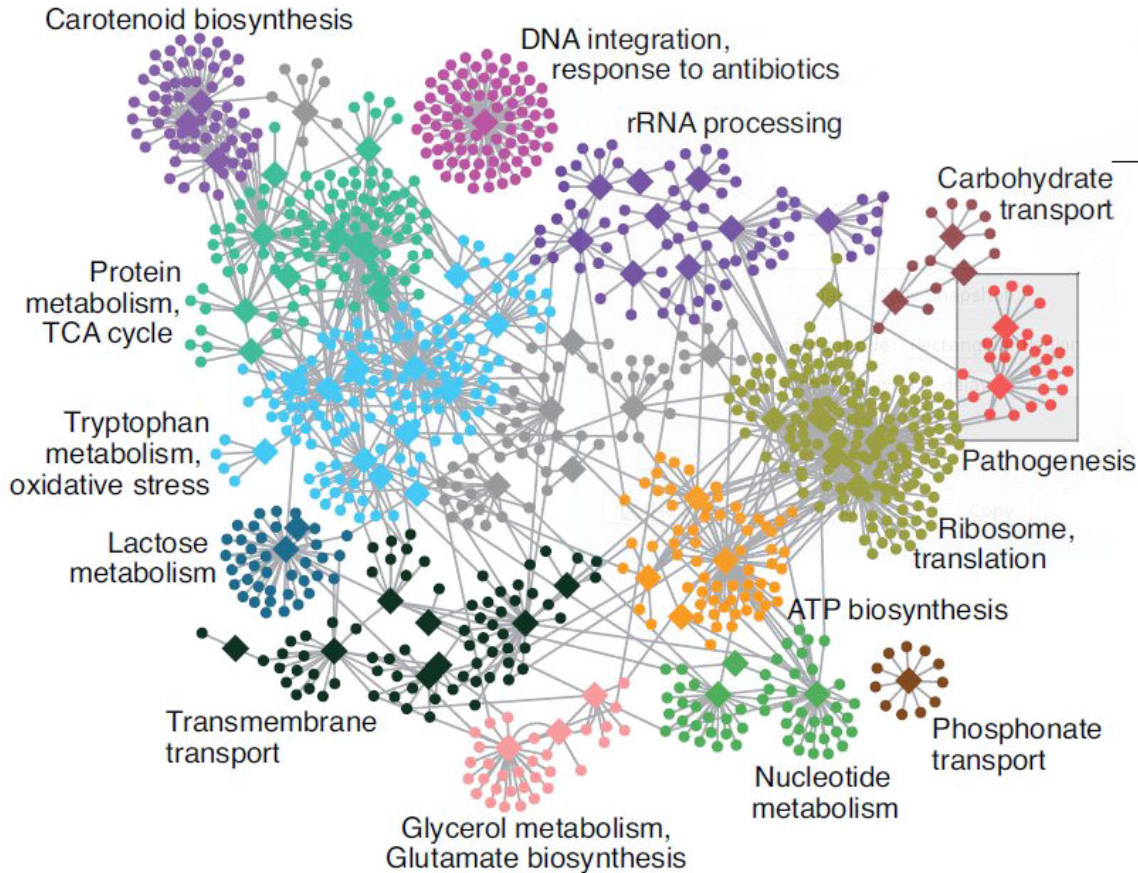
- Volcano plot



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

Functional analysis • GO

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



Summary

- Sound experimental design to avoid confounding
- Plan carefully about lib prep, sequencing etc based on experimental objective
- For DGE, biological replicates may be more important than other considerations (paired-end, sequencing depth, long reads etc)
- 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, A., Madrigal, P., Tarazona, S., Gomez-Cabrero, D., Cervera, A., McPherson, A., ... & Mortazavi, A. (2016). A survey of best practices for RNA-seq data analysis. *Genome biology*, 17(1), 1-19.

Thank you. Questions?



Graphics from  freepik.com

Created: 26-Mar-2023 • SciLifeLab • NBIS • NGI

Hands-On tutorial

Main exercise

- 01 Check the quality of the raw reads with **FastQC**
- 02 Map the reads to the reference genome using **HISAT2**
- 03 Assess the post-alignment quality using **QualiMap**
- 04 Count the reads overlapping with genes using **featureCounts**
- 05 Find differentially expressed genes using **DESeq2** in R

Bonus exercises

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

Data: `/sw/courses/ngsintro/rnaseq/`

Work: `/proj/snic2022-22-1124/nobackup/user/rnaseq/`

Hands-On tutorial

- Course data directory

```
/sw/courses/ngsintro/rnaseq/
```

```
rnaseq/  
+-- bonus/  
| +-- assembly/  
| +-- exon/  
| +-- funannot/  
| +-- plots/  
+-- documents/  
+-- main/  
    +-- 1_raw/  
    +-- 2_fastqc/  
    +-- 3_mapping/  
    +-- 4_qualimap/  
    +-- 5_dge/  
    +-- 6_multiqc/  
    +-- reference/  
    | +-- mouse_chr19_hisat2/  
    +-- scripts/
```

- Your work directory

```
/proj/snic2022-22-  
1124/nobackup/user/rnaseq/
```

```
[user]/  
rnaseq/  
    +-- 1_raw/  
    +-- 2_fastqc/  
    +-- 3_mapping/  
    +-- 4_qualimap/  
    +-- 5_dge/  
    +-- 6_multiqc/  
    +-- reference/  
    | +-- mouse_chr19_hisat2/  
    +-- scripts/  
    +-- funannot/  
    +-- plots/
```