RNA-seq data analysis:
How to find differentially expressed genes?

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What will I learn?

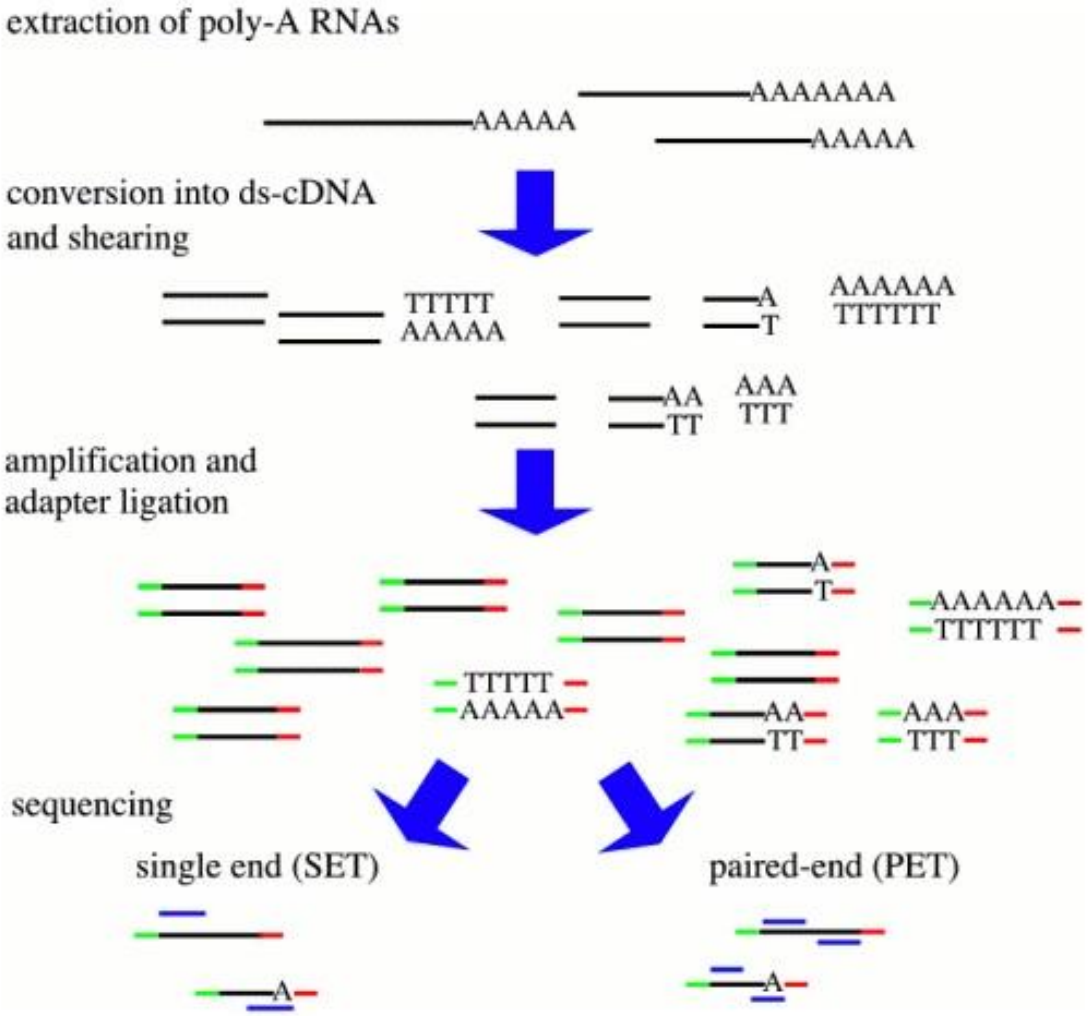
- **Introduction to RNA-seq**
- **How to operate the Chipster software used in the exercises**
- **Differential gene expression analysis**
  - Central concepts
  - Analysis steps
  - File formats

Introduction to RNA-seq

What can I investigate with RNA-seq?

- **Differential gene expression**
- **Isoform switching**
- **New transcripts (and genes)**
- **New transcriptomes**
- **Variants**
- **Allele-specific expression**
- **Etc etc**

How was your data produced?



PolyA purification

cDNA generation
& fragmentation

Library construction

Size selection

http://cmb.molgen.mpg.de/2ndGenerationSequencing/Solas/RNA-seq.html

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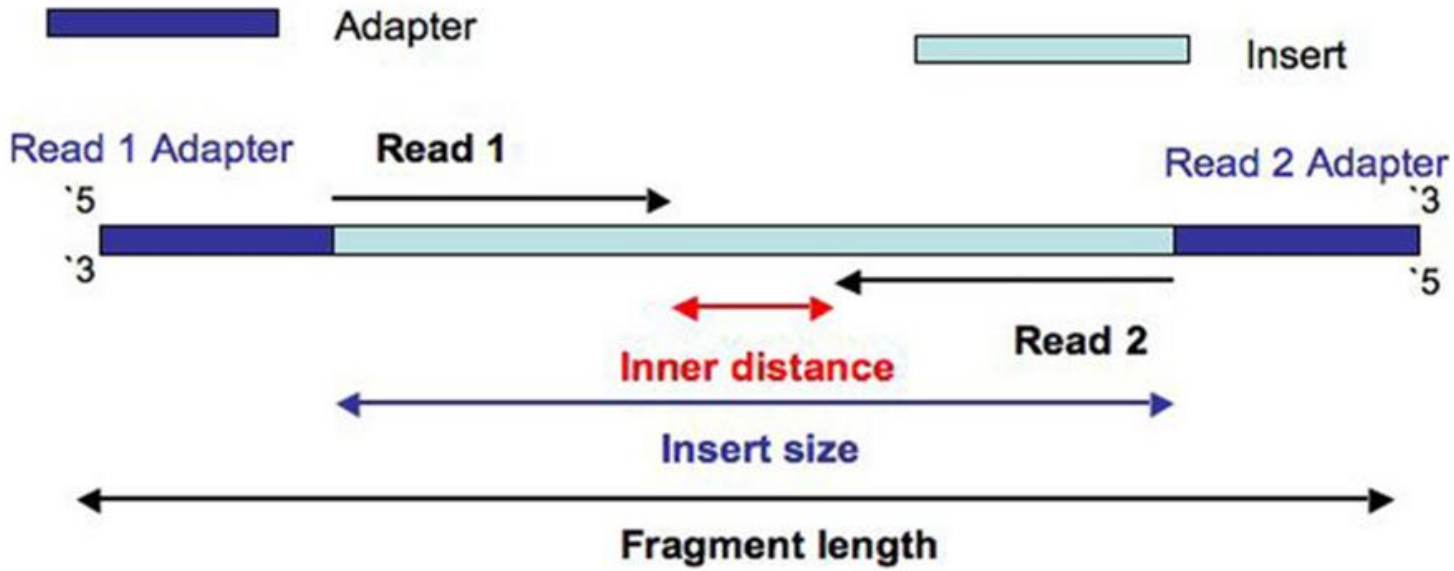
Differently sized fragments & inner distance

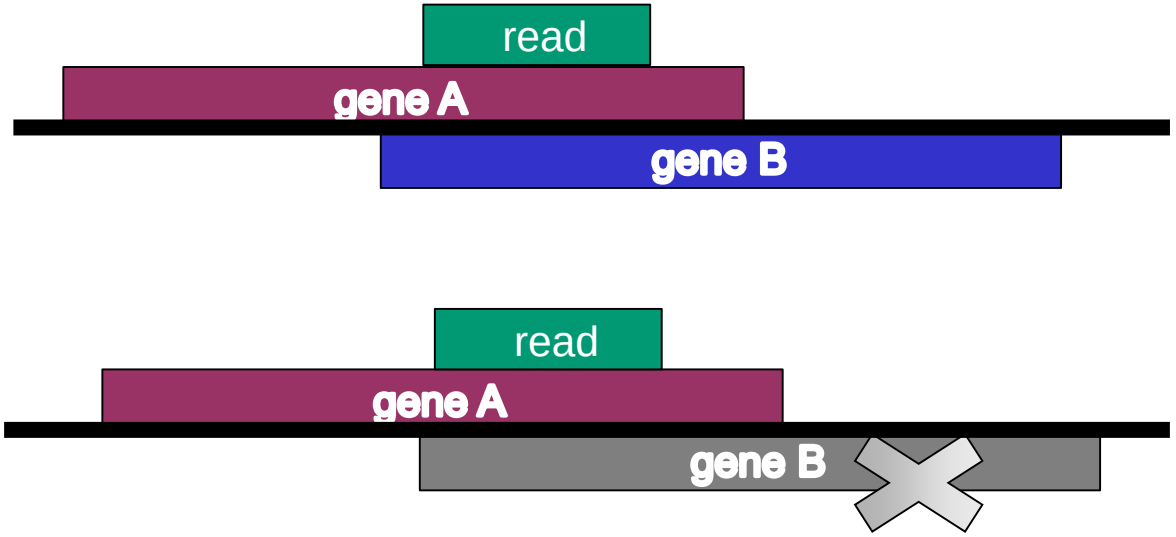
- **Illumina reads are always of same length**
- **But the size of the initial RNA fragment (=insert) may vary**



https://www.ecseq.com/support/ngs/why-do-the-reads-all-have-the-same-length-when-sequencing-differently-sized-fragments

Stranded RNA-seq data

- **Tells if a read maps to the same strand where the parental gene is, or to the opposite strand**
  - Useful information when a read maps to a genomic location where there is a gene on both strands
- **Several lab methods, you need to know which one was used**
  - TruSeq stranded, NEB Ultra Directional, Agilent SureSelect Strand-Specific…



Unstranded data: Does the read come from geneA or geneB?

Stranded data → the read comes from geneA

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Differential gene expression analysis

Differential expression analysis types

- **DGE (differential gene expression): has the expression of a gene changed overall?**
- **DTE (differential transcript expression): has the expression of an individual transcript changed?**
- **DTU (differential transcript usage): has the relative expression of the different transcript isoforms of a gene changed?**

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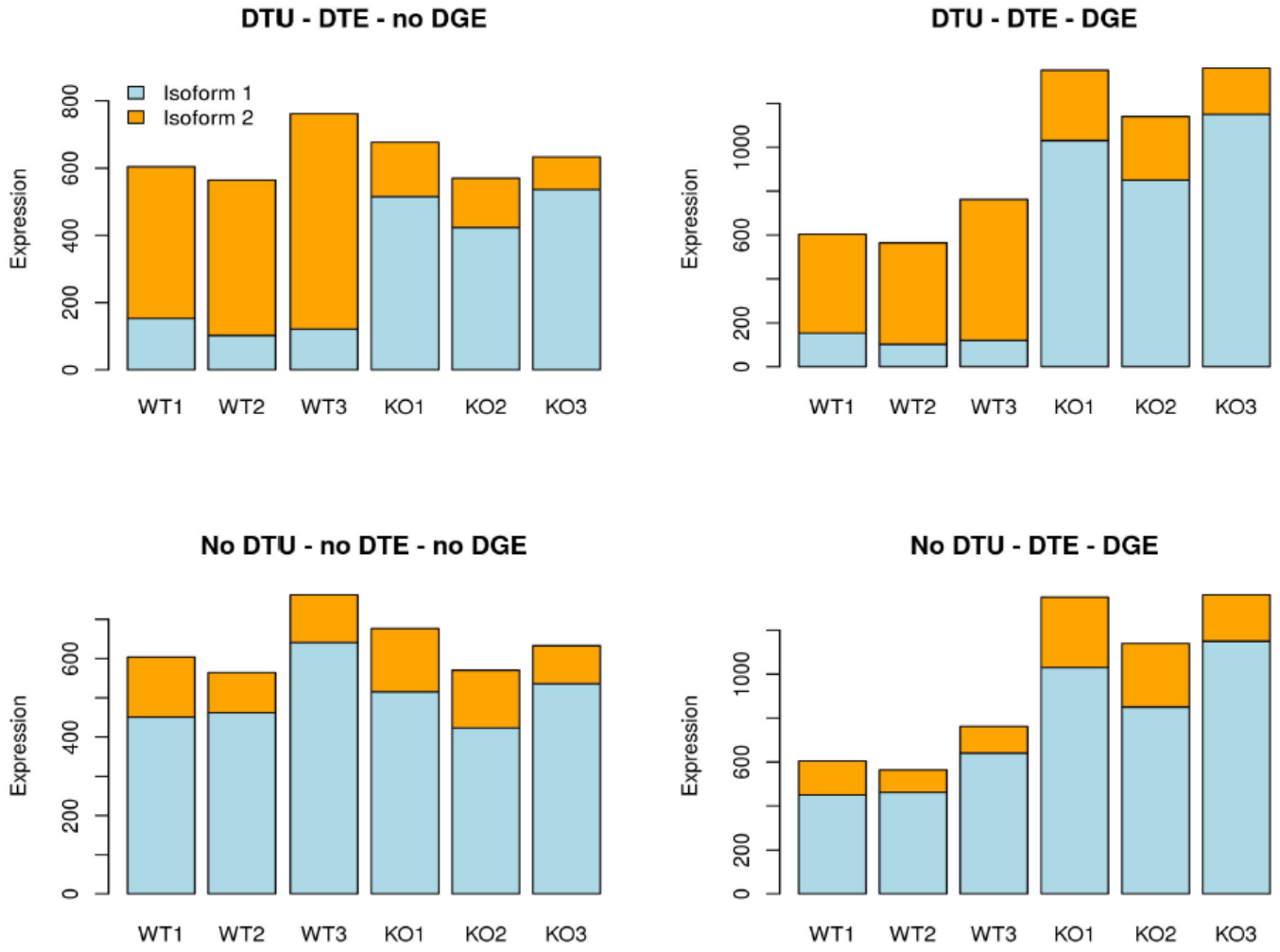
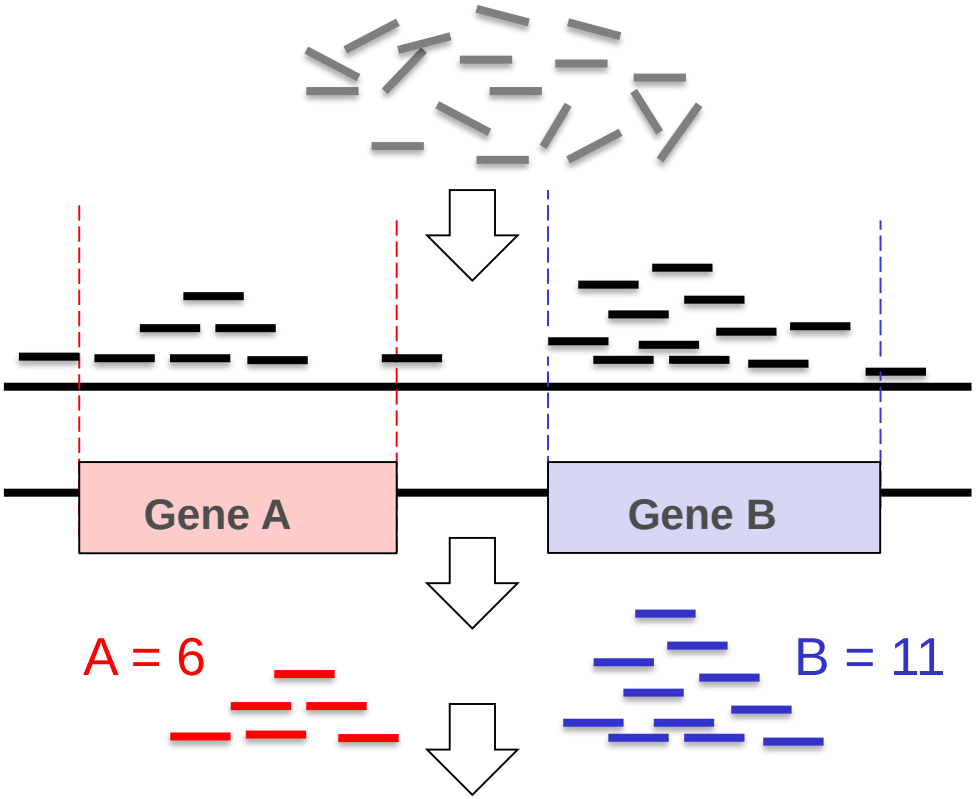
DTE, DGE & DTU



Institut Pasteur

DGE analysis: typical steps



Raw data (reads)

Align reads to reference genome

Match alignment positions with known gene positions

Count how many reads each gene has

	Control 1	Control 2	Control 3	Sample 1	Sample 2	Sample 3
Gene A	6	5	7	170	100	110
Gene B	11	11	10	3	4	2
Gene C	200	150	355	50	1	3
Gene D	0	1	0	2	0	1

Compare sample groups: differential expression analysis

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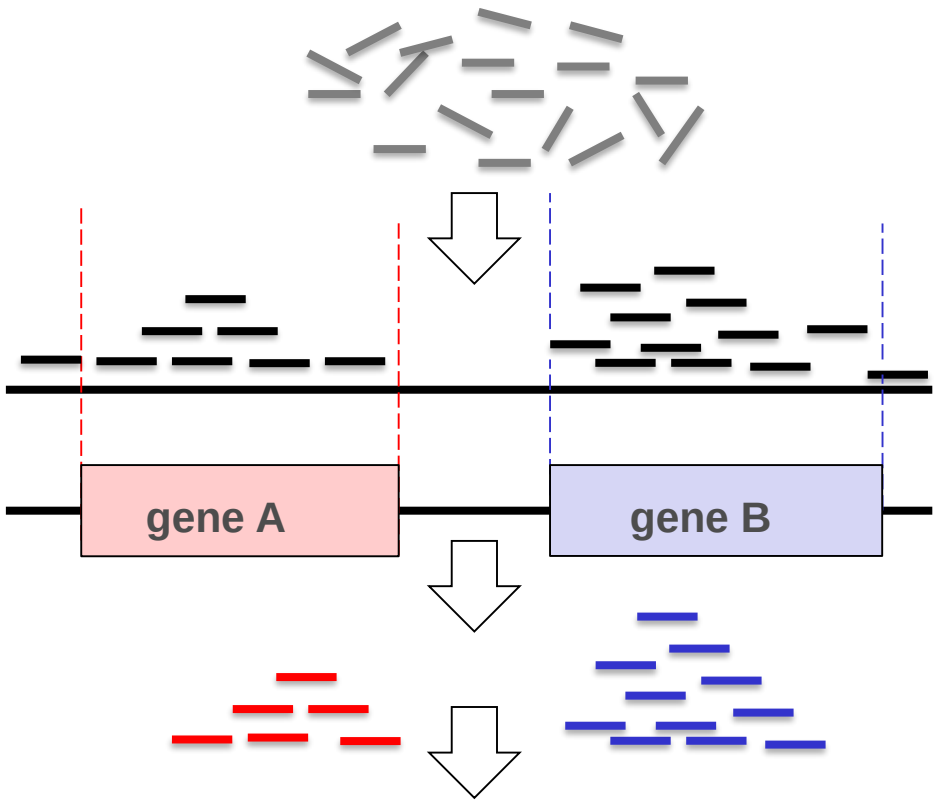
DGE analysis: steps, tools and files



	Control 1	Control 2	Control 3	Sample 1	Sample 2	Sample 3
Gene A	6	5	7	170	100	110
Gene B	11	11	10	3	4	2
Gene C	200	150	355	50	1	3
Gene D	0	1	0	2	0	1

STEP	TOOL	FILE
Quality control	FastQC	FASTQ
Pre-processing	Trimmo-matic	FASTQ
Alignment	HISAT2	BAM
Quality control	RSeQC	
Quantitation	HTSeq	Read count file (TSV)
Combine count files to table	Define NGS experiment	Read count table (TSV)
Quality control	PCA, clustering	
Differential expression analysis	DESeq2, edgeR	Gene lists (TSV)

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Data analysis workflow

- **Quality control of raw reads**
- **Preprocessing if needed**
- **Alignment to reference genome**
- **Alignment level quality control**
- **Quantitation**
- **Experiment level quality control**
- **Differential expression analysis**
- **Annotation**
- **Pathway analysis**

Data analysis workflow

- **Quality control of raw reads**
- Preprocessing if needed
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What and why?

- **Potential problems**
  - low confidence bases, Ns
  - sequence specific bias, GC bias
  - adapters
  - sequence contamination
  - …

Knowing about potential problems in your data allows you to

- **correct for them before you spend a lot of time on analysis**
- **take them into account when interpreting results**

Software packages for quality control

- **FastQC**
- **MultiQC**
- **FastX**
- **TagCleaner**
- **...**

Raw reads: FASTQ file format

- **Four lines per read:**

  @read name

  GATTTGGGGTTCAAAGCAGTATCGATCAAATAGTAAATCCATTTGTTCAACTCACAGTTT

  + read name

  !''*((((***+))%%%++)(%%%%).1***-+*''))**55CCF>>>>>>CCCCCCC65

- **http://en.wikipedia.org/wiki/FASTQ_format**

- **Attention: Do not unzip FASTQ files**
  - Chipster's analysis tools can cope with zipped files (.gz)

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Base qualities

- **If the quality of a base is 20, the probability that it is wrong is 0.01.**
  - **Phred quality score** Q = -10 * $\log_{10}$ (probability that the base is wrong)

```
T  C  A  G  T  A  C  T  C  G
40 40 40 40 40 40 40 40 37 35
```

- **”Sanger” encoding: numbers are shown as ASCII characters**
  - Note that older Illumina data uses different encoding

Phred Quality Score	Probability of Incorrect Base Call	Base Call Accuracy	ASCII coding in FASTQ file
10	1 in 10	90%	+
20	1 in 100	99%	5
30	1 in 1,000	99.9%	?
40	1 in 10,000	99.99%	I

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Base quality encoding systems

```
  SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS.....................................

  !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmr
  |                          |    |        |                                    |
 33                         59   64       73                                  104
  0.........................26...31.......40

S - Sanger        Phred+33,  raw reads typically (0, 40)
```

http://en.wikipedia.org/wiki/FASTQ_format

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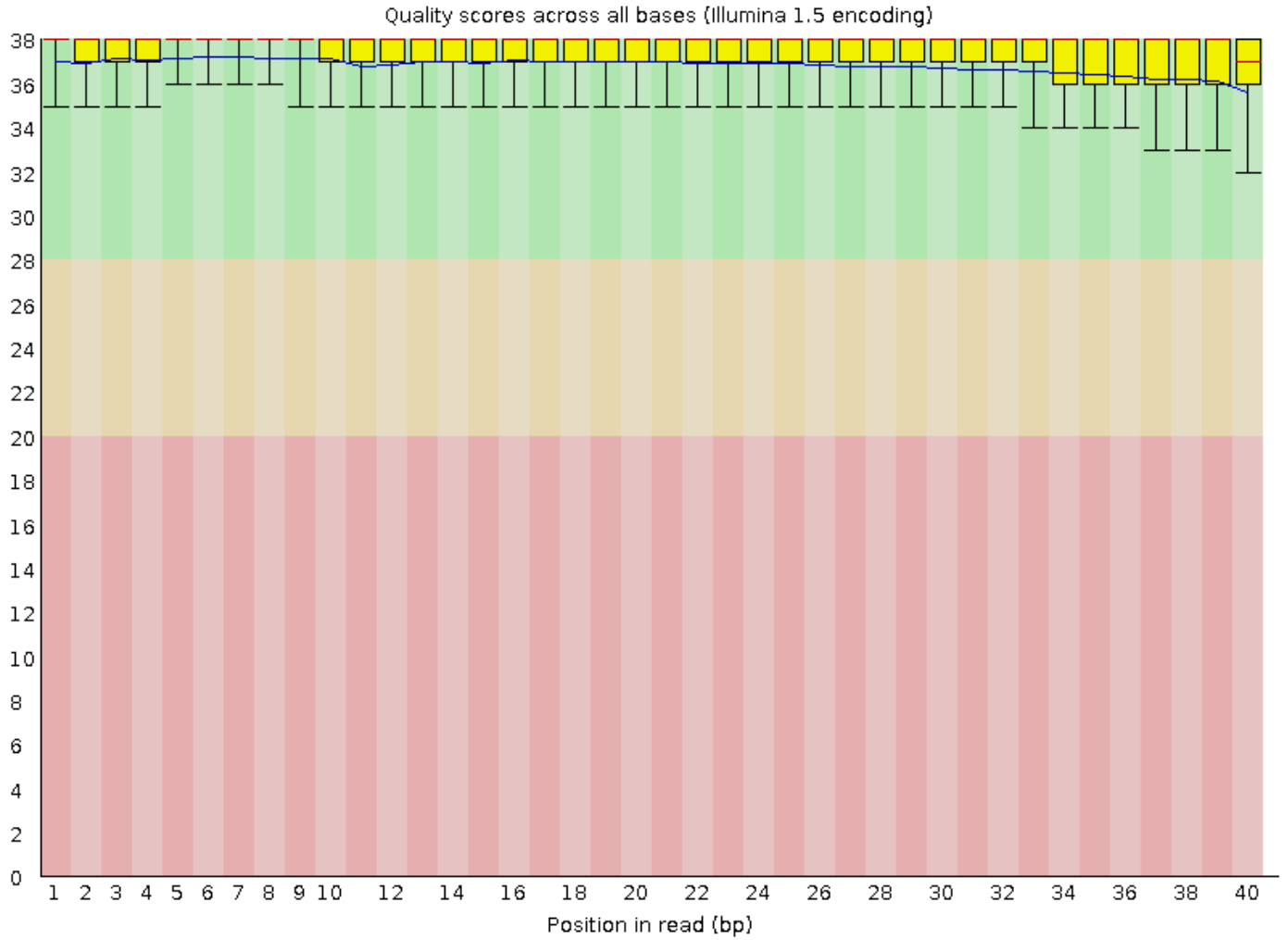
How to check read quality?

- **You can use FastQC either directly or via MultiQC**
  - If you have many samples, MultiQC is handier
- **Reports many things, including**
  - base quality
  - base composition
  - duplication
  - Ns
  - k-mers
  - adaptors

MultiQC

- **Can combine info from many tools.**
  - In Chipster it uses FastQC
- **Features**
  - Interactive plots
  - Traffic lights (they might not be suitable for your data!)
- **Toolbox (click on the right side panel), allows you to**
  - Highlight samples
  - Show only selected samples
  - Download plots
  - Rename samples
- **Good tutorial video**
  - https://www.youtube.com/watch?v=qPbIlO_KWN0

Per position base quality (FastQC)



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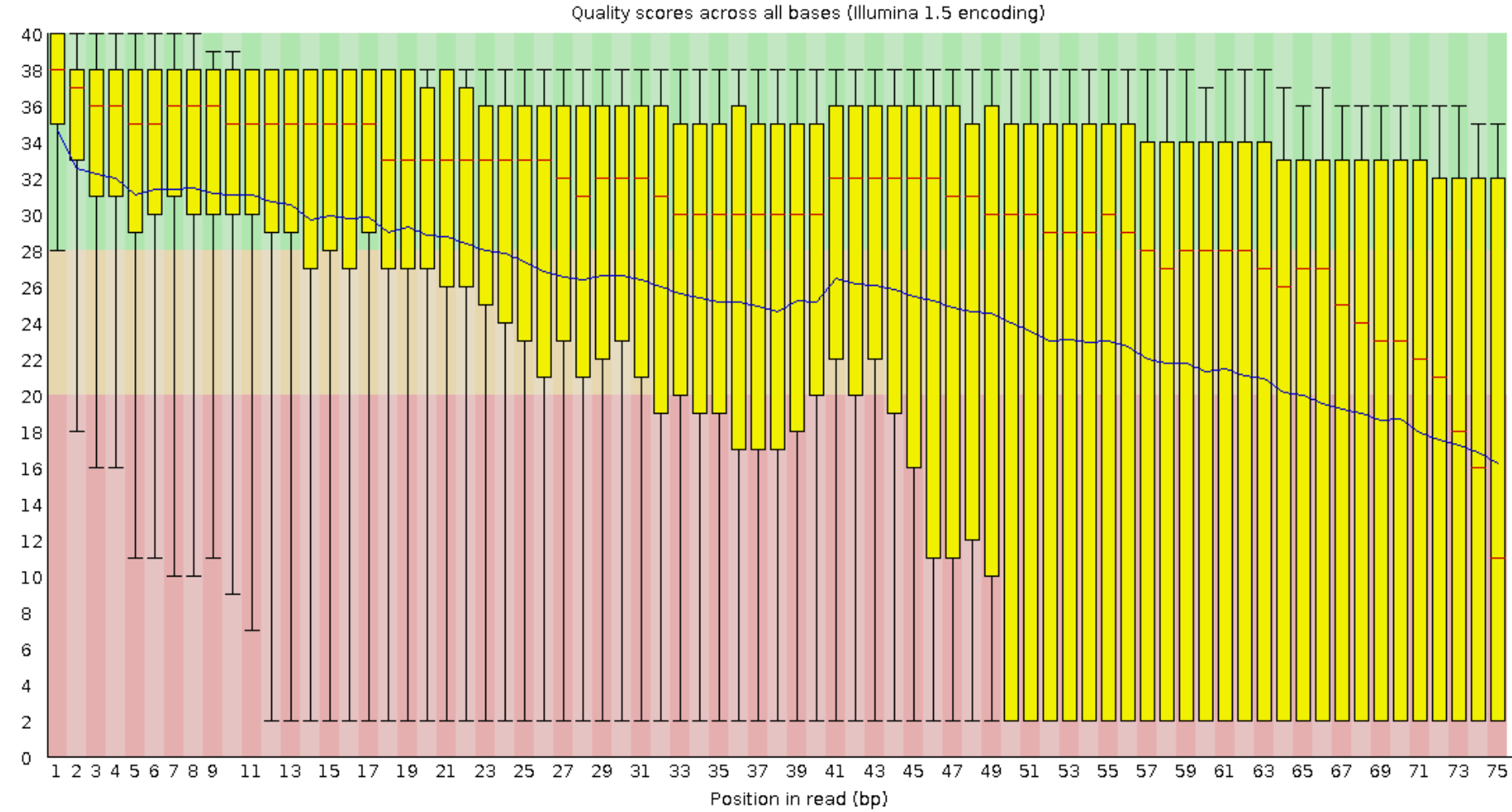
Per position base quality (FastQC)



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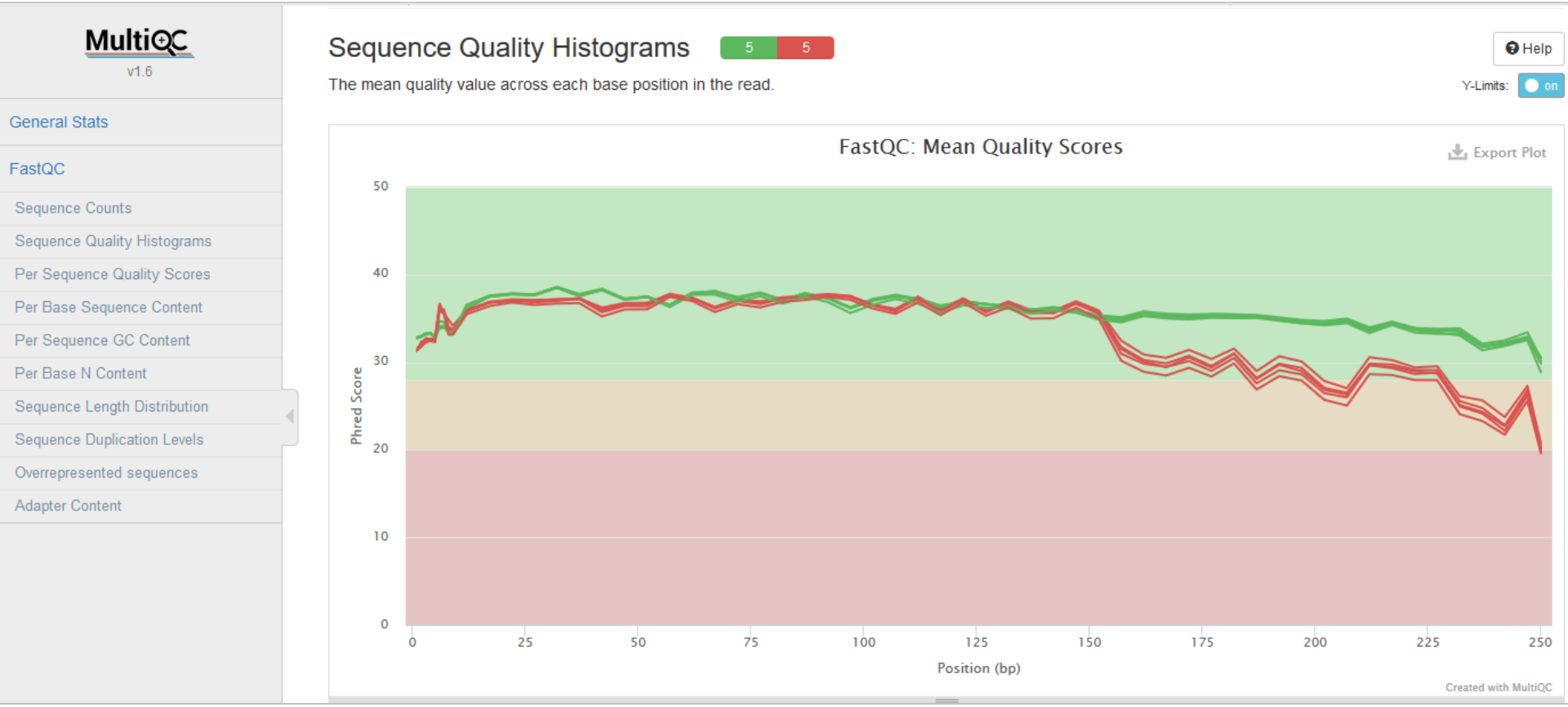
Per position base quality (MultiQC)



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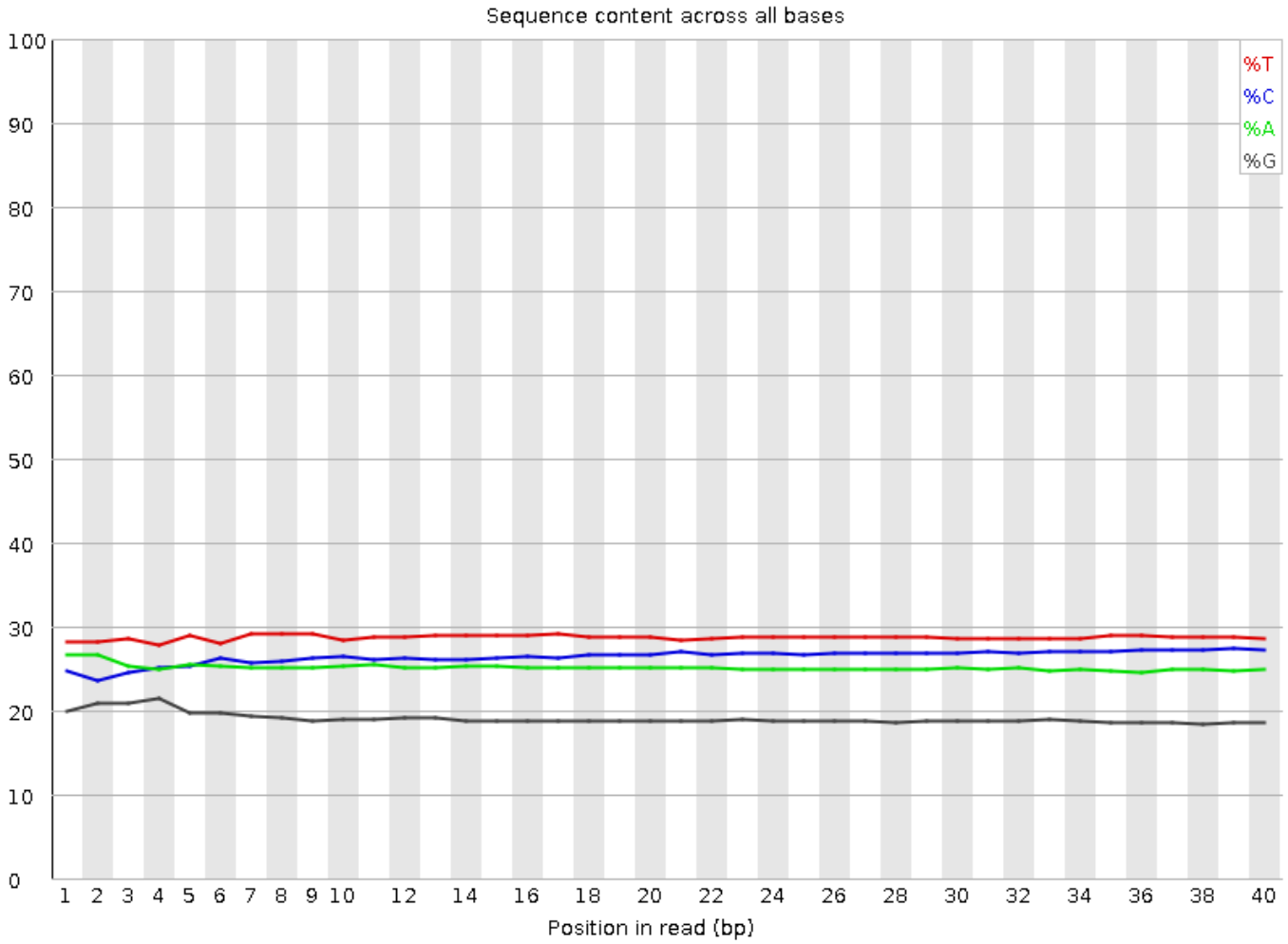
Per position sequence content (FastQC)



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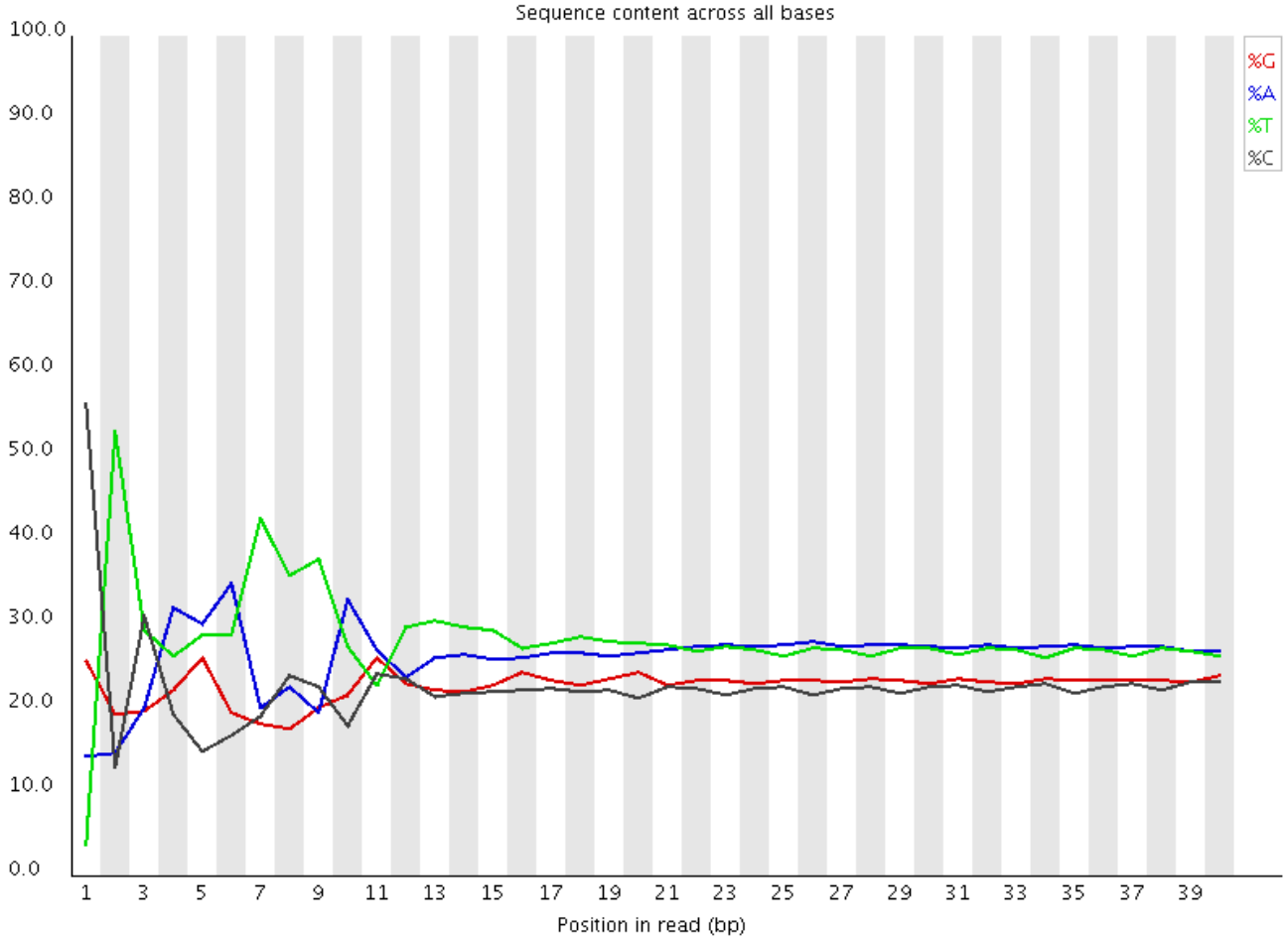
Per position sequence content (FastQC)



- **Enrichment of k-mers at the 5’ end due to use of random hexamers or transposases in the library preparation**
- **Typical for RNA-seq data**
- **Can’t be corrected, doesn’t usually effect the analysis**

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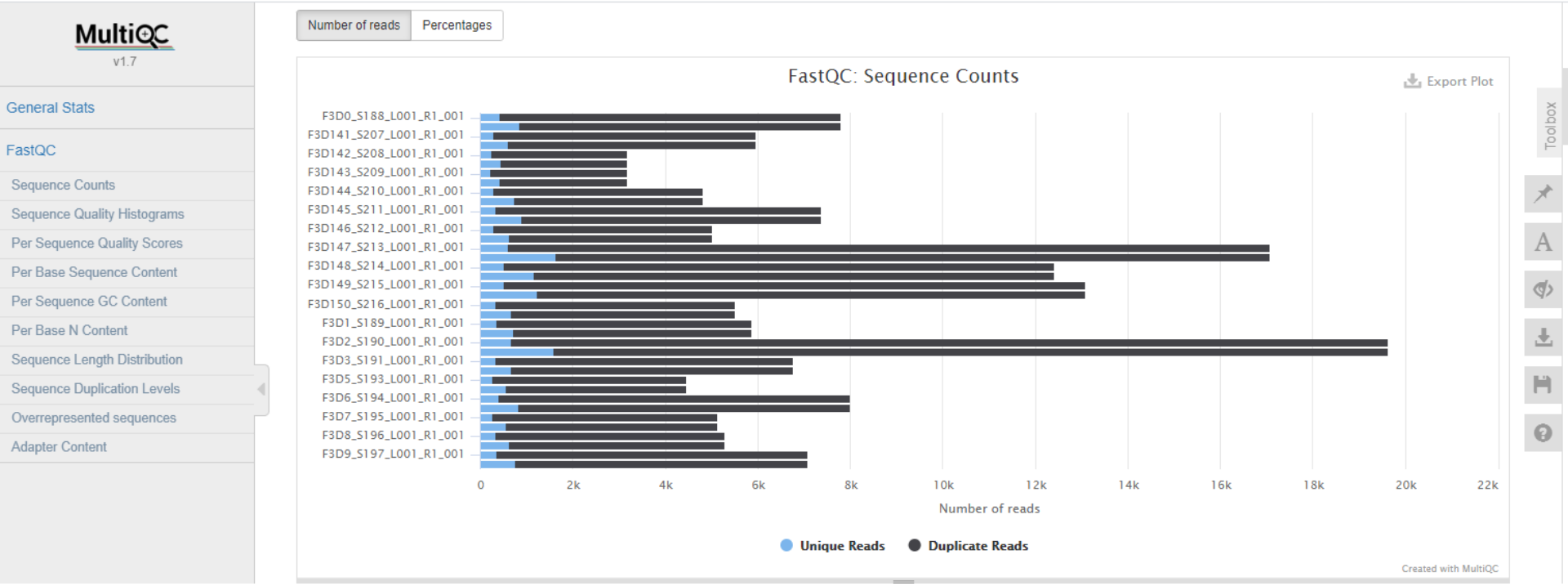
Sequence counts (MultiQC)



Was your data made with stranded protocol?

- **You need to indicate it when**
  - aligning reads to genome (e.g. HISAT2)
  - counting reads per genes (e.g. HTSeq)

- **If you don’t know which stranded sequencing protocol was used, you can check it**
  - Select your FASTQ file and run the tool Quality control / RNA-seq strandedness inference with RSeQC
  - Aligns a subset of the reads to genome and compares the locations to reference annotation

- **For more info please see the manual**
  - http://chipster.csc.fi/manual/library-type-summary.html

RSeQC strandedness report

experiment_data.txt •••

Text Details

File size 468.0 bytes.

```
This is SingleEnd Data
Fraction of reads failed to determine: 0.0433
Fraction of reads explained by "++,--": 0.9498
Fraction of reads explained by "+-,-+": 0.0069

It seems the data is stranded. Read is always on the same strand as the gene.

Corresponding parameters are:
TopHat, Cufflinks and Cuffdiff: library-type fr-secondstrand
HISAT2: RNA-strandness: F
HTSeq: stranded -- yes
RSeQC: ++,--

Input files were assigned as follows:
Read 1 file: hESC.fastq
```

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Data analysis workflow

- Quality control of raw reads
- **Preprocessing (trimming / filtering) if needed**
- Alignment to reference genome
- Alignment level quality control
- Quantitation
- Experiment level quality control
- Differential expression analysis
- Annotation
- Pathway analysis

Filtering vs trimming

- **Filtering removes the entire read**
- **Trimming removes only the bad quality bases**
  - It can remove the entire read, if all bases are bad
- **Trimming makes reads shorter**
  - This might not be optimal for some applications
- **Paired end data: the matching order of the reads in the two files has to be preserved**
  - If a read is removed, its pair has to removed as well

What base quality threshold should be used?

- **No consensus**
- **Trade-off between having good quality reads and having enough sequence**
- **Start with gentle trimming and check with FastQC**

An Extensive Evaluation of Read Trimming Effects on Illumina NGS Data Analysis

Cristian Del Fabbro[1☯], Simone Scalabrin[2☯], Michele Morgante[1], Federico M. Giorgi[1,3*]

1 Institute of Applied Genomics, Udine, Italy, 2 IGA Technology Services, Udine, Italy, 3 Center for Computational Biology and Bioinformatics, Columbia University, New York, New York, United States of America

frontiers in GENETICS

ORIGINAL RESEARCH ARTICLE
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doi: 10.3389/fgene.2014.00013

On the optimal trimming of high-throughput mRNA sequence data

*Matthew D. MacManes[1,2]**

[1] Department of Molecular, Cellular and Biomedical Sciences, University of New Hampshire, Durham, NH, USA
[2] Hubbard Center for Genome Studies, Durham, NH, USA

Software packages for preprocessing

- **Trimmomatic**
- **FastX**
- **TagCleaner**
- **...**

Trimmomatic options in Chipster

- **Adapters**
- **Minimum quality**
  - Per base, one base at a time or in a sliding window, from 3’ or 5’ end
  - Per base adaptive quality trimming (balance length and errors)
  - Minimum (mean) base quality
- **Trim x bases from left/ right**
- **Minimum read length after trimming**
- **Copes with paired end data**

Data analysis workflow

- Quality control of raw reads
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- **Alignment to reference genome**
- Alignment level quality control
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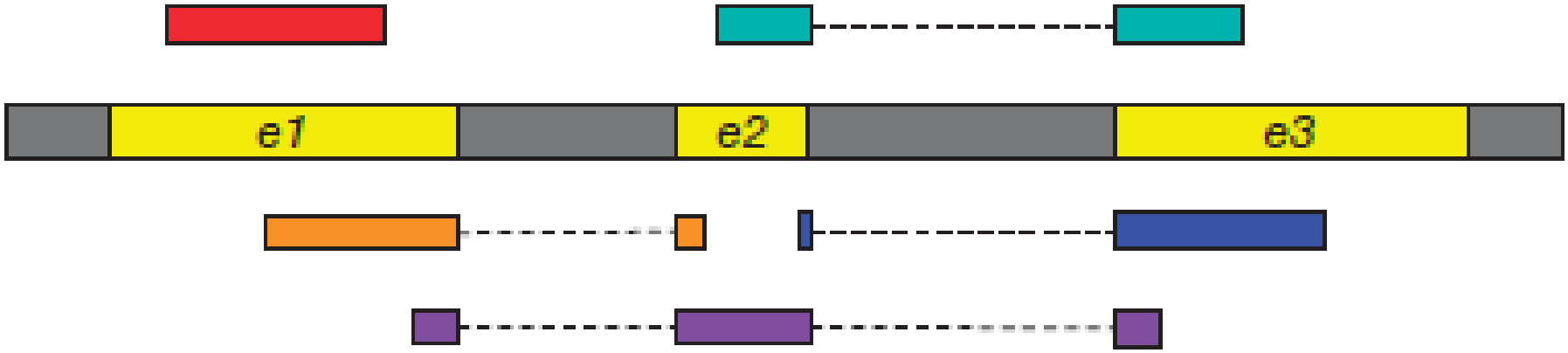
Aligning reads to reference genome

- **The goal is to find the location where the read originated from**
- **Challenges**
  - Reads contain genomic variants and sequencing errors
  - Genomes contain non-unique sequence and introns
- **RNA-seq aligner needs to be able to align splice junction spanning reads to genome non-contiguously**
  - Spliced alignments are difficult because sequence signals at splice sites are limited, and introns can be thousands of bases long



Modified from Kim et al (2015) Nature methods 12:358

Alignment programs

- **Many aligners have been developed over the years**
  - Convert genome fasta file to a data structure which is faster to search (e.g. BWT index or suffix array)
  - Differ in speed, memory requirements, accuracy and ability to deal with spliced alignments
- **Use splice-aware aligner for mapping RNA-seq reads, for example**
  - STAR (fast and accurate, needs a lot of memory)
  - HISAT2 (fast and accurate, creating the genomic index needs a LOT of memory)
  - TopHat2 (slower, needs less memory)

Splice-aware aligners in Chipster

- **STAR**
  - Human, mouse & rat genomes available
- **HISAT2**
  - Many genomes available
  - You can also supply own reference genome if it is small
- **TopHat2**
  - You can supply own reference genome

- **Output files**
  - BAM = contains the read alignments
  - bai = index file for BAM, required by genome browsers etc
  - log = useful information about the alignment run

HISAT2

- **HISAT = Hierarchical Indexing for Spliced Alignment of Transcripts**
- **Fast spliced aligner with low memory requirement**
- **Reference genome is (BWT FM) indexed for fast searching**
  - Chipster provides many reference genomes
  - You can provide own (small) reference genome in fasta format
- **Uses two types of indexes**
  - A global index: used to anchor a read in genome (28 bp is enough)
  - Thousands of small local indexes, each covering a genomic region of 56 Kbp: used for rapid extension of alignments (good for spliced reads with short anchors)
- **Uses splice site information found during the alignment of earlier reads in the same run**

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HISAT2 parameters

Parameters

Genome Genome or transcriptome that you would like to align your reads against.	Homo_sapiens.GRCh38.95
RNA-strandness Specify strand-specific information. FR means read 1 is always on the same strand as the gene. RF means read 2 is always on the same strand as the gene. The default is unstranded.	unstranded
Base quality encoding used Quality encoding used in the fastq file.	Sanger - Phred+33
How many hits to report per read Instructs HISAT2 to report up to this many alignments to the reference for a given read.	5
Minimum intron length Sets minimum intron length. Default: 20	20
Maximum intron length Sets maximum intron length. Default: 500000	500000
Disallow soft-clipping Is soft-cliping used. By default HISAT2 may soft-clip reads near their 5' and 3' ends.	Use soft-clipping
Require long anchor lengths for subsequent assembly With this option, HISAT2 requires longer anchor lengths for de novo discovery of splice sites. This leads to fewer alignments with short-anchors, which helps transcript assemblers improve significantly in computation and memory usage.	Don't require

Input files

Reads to align	G1Esubset_R1.fq.gz G1Esubset_R2.fq.gz
List of read 1 files	
List of read 2 files	

- Remember to set the strandedness correctly!
- Require long anchors (> 16 bp) if you are going to do transcript assembly
- Soft-clipping = read ends don’t need to align, if this maximizes the alignment score

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STAR

- **STAR = Spliced Transcripts Alignment to a Reference**
- **Reference genome fasta is converted to a suffix array for fast searching**
- **2-pass alignment process**
  - splice junctions found during the 1st pass are inserted into the genome index, and all reads are re-aligned in the 2nd mapping pass
  - this doesn't increase the number of detected novel junctions, but it allows more spliced reads aligning to novel junctions.
- **Maximum alignments per read -parameter sets the maximum number of loci the read is allowed to map to**
  - Alignments (all of them) will be output only if the read maps to no more loci than this. Otherwise no alignments will be output.
- **Chipster offers an Ensembl GTF file to detect annotated splice junctions**
  - you can also give your own, e.g. GENCODE GTF

If ***one*** sample has more than two FASTQ files

- **Illumina NextSeq produces eight FASTQ files for one sample**
  - Four R1 FASTQ files
  - Four R2 FASTQ files
- **Use the tool Utilities / Merge FASTQ**
  - Merge the four R1 FASTQ files → one big R1 FASTQ file
  - Merge the four R2 FASTQ files → one big R2 FASTQ file
- **Select both big FASTQ files and run the aligner**

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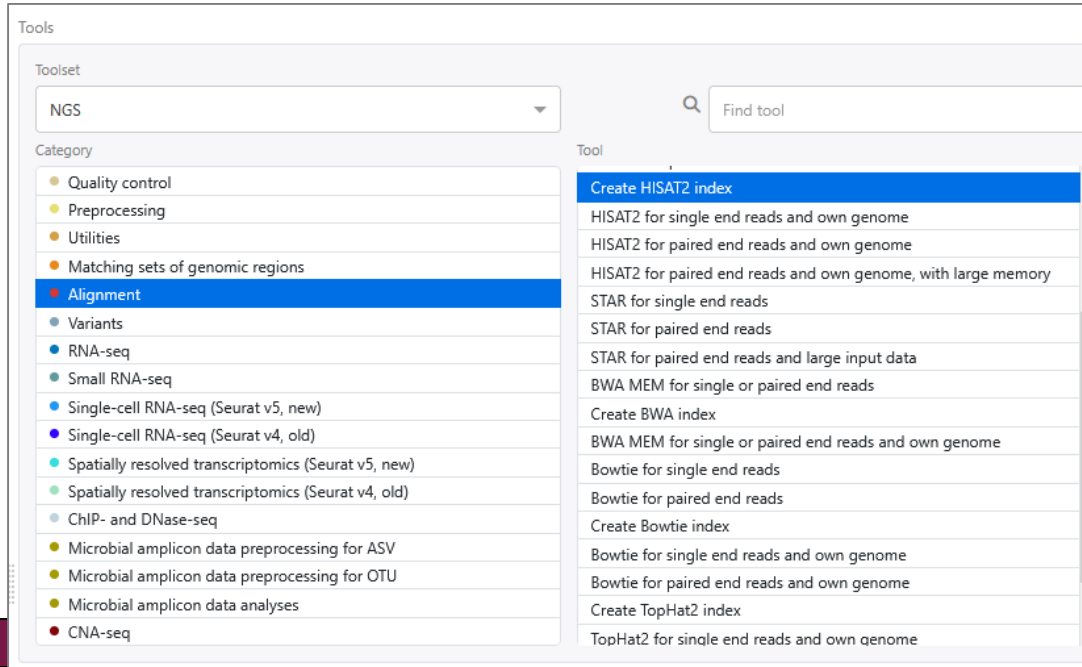
Aligning to own reference genome

- **Create genome aligner index for the genome you want to use**
  - Provide the genome in fasta format
  - Provide GTF if you have one
  - The tool produces a genome index as a tar package
- **Run the aligner with the following input files**
  - Reads to be aligned (FASTQ files)
  - Aligner genome index (tar package)



File format for mapped reads: BAM/SAM

- BAM is a compact binary file containing aligned reads. SAM (Sequence Alignment/Map) contains the same information in tab-delimited text.
- You can view BAM as text by running the tool Create a preview for BAM.

lymphnode4a_R1.prev.sam •••

Text Details

File size 66.9 kB. Full Screen

```
@HD     VN:1.0  SO:coordinate
@SQ     SN:1    LN:248956422
@SQ     SN:2    LN:242193529
@SQ     SN:3    LN:198295559
@SQ     SN:4    LN:190214555
@SQ     SN:5    LN:181538259
@SQ     SN:6    LN:170805979
@SQ     SN:7    LN:159345973
@SQ     SN:8    LN:145138636
@SQ     SN:9    LN:138394717
@SQ     SN:10   LN:133797422
@SQ     SN:11   LN:135086622
@SQ     SN:12   LN:133275309
@SQ     SN:13   LN:114364328
@SQ     SN:14   LN:107043718
@SQ     SN:15   LN:101991189
@SQ     SN:16   LN:90338345
@SQ     SN:17   LN:83257441
@SQ     SN:18   LN:80373285
@SQ     SN:19   LN:58617616
@SQ     SN:20   LN:64444167
@SQ     SN:21   LN:46709983
@SQ     SN:22   LN:50818468
@SQ     SN:X    LN:156040895
@SQ     SN:Y    LN:57227415
@SQ     SN:MT   LN:16569
@PG     ID:hisat2       PN:hisat2       VN:2.1.0        CL:"/opt/chipster/tools/hisat2/hisat2-align-s --wrapper basic-0 --phred33 --min-intronlen 20 --max-intronlen 500000 -x Homo_sapiens.GRCh38.
ERR315371.8524369       409     1       12808   1       101M    =       12808   0       GATGCCCCTCCACACCCTCTTGATCTTCCCTGTGATGTCATCTGGAGCCCTGCTGCTTGCGGTGGCCTATAAAGCCTCCTGGTCTGGCTCCAAGGCCTGGC    BB7
ERR315371.5736647       163     1       14453   1       101M    =       14519   167     CTTAAGAACACTGTGGCGCAGGCTGGGTGGAGCCGTCCCCCCATGGAGCACAGGCAGACAGAAGTCCCCGCCCCAGCTGTGTGGCCTCAAGCCAGCCTTCC    BBB
ERR315371.5736647       419     1       14453   1       101M    =       185040  150229  CTTAAGAACACTGTGGCGCAGGCTGGGTGGAGCCGTCCCCCCATGGAGCACAGGCAGACAGAAGTCCCCGCCCCAGCTGTGTGGCCTCAAGCCAGCCTTCC    BBB
ERR315371.5736647       83      1       14519   1       101M    =       14453   -167    CCCGCCCCAGCTGTGTGGCCTCAAGCCAGCCTTCCGCTCCTTGAAGCTGGTCTCCACACAGTGCTGGTTCCGTCACCCCCTCCCAAGGAAGTAGGTCTGAG    FBB
ERR315371.8571896       355     1       14554   1       101M    =       185209  150297  GCTCCTTGAAGCTGGTCTCCACACAGTGCTGGTTCCGTCACCCCCTCCCAAGGAAGTAGGTCTGAGCAGCTTGTCCTGGCTGTGTCCATGTCAGAGCAACG    BBB
ERR315371.2401177       99      1       14701   0       87M14S  =       185222  150149  ATTCCCAGTCGTCCTCGTCCTCCTCTGCCTGTGGCTGCTGCGGTGGCGGCAGAGGAGGGGATGGAGTCTGACACGCGGGCAAAGGCTCAGATCGGAAGAGCA    BBB
ERR315371.2401177       355     1       14701   0       87M14S  =       14701   -115    ATTCCCAGTCGTCCTCGTCCTCCTCTGCCTGTGGCTGCTGCGGTGGCGGCAGAGGAGGGGATGGAGTCTGACACGCGGGCAAAGGCTCAGATCGGAAGAGCA    BBB
```

BAM header

alignment information: one line per read alignment, containing 11 mandatory fields, followed by optional tags

Fields in BAM/SAM files

- **read name** HWI-EAS229_1:2:40:1280:283
- **flag** 272
- **reference name** 1
- **position** 18506
- **mapping quality** 0
- **CIGAR** 49M6183N26M
- **mate name** *
- **mate position** 0
- **insert size** 0
- **sequence**
  AGGGCCGATCTTGGTGCCATCCAGGGGGCCTCTACAAGGAT
  AATCTGACCTGCTGAAGATGTCTCCAGAGACCTT
- **base qualities**
  ECC@EEF@EB:EECFEECCCBEEEE;>5;2FBB@FBFEEFCF@F
  FFFCEFFFFEE>FFEFC=@A;@>1@6.+5/5
- **tags** MD:Z:75 NH:i:7 AS:i:-8 XS:A:-

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)
- **Values differ in different aligners. E. g. unique mapping is**
  - 60 in HISAT2
  - 255 in STAR
  - 50 in TopHat
  - https://sequencing.qcfail.com/articles/mapq-values-are-really-useful-but-their-implementation-is-a-mess/

CIGAR string

- M = match or mismatch
- I = insertion
- D = deletion
- N = intron (in RNA-seq read alignments)
- S = soft clip (ignore these bases)
- H = hard clip (ignore and remove these bases)

- Example:

  @HD VN:1.3 SO:coordinate

  @SQ SN:ref LN:45

  r001 163 ref 7 30 8M2I4M1D3M = 37 39 TTAGATAAAGGATACTG *

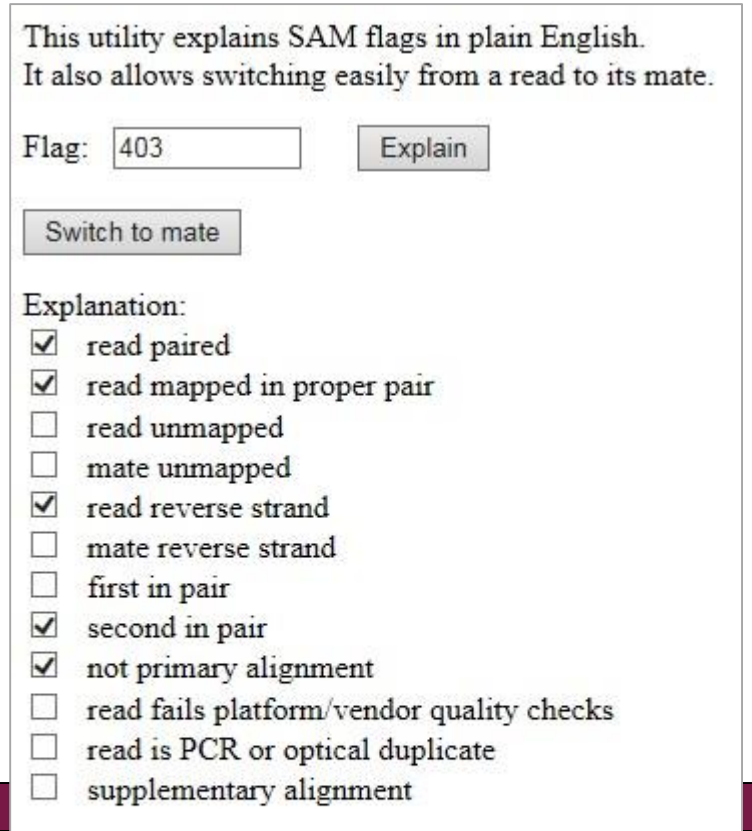
  - The corresponding alignment

```
Ref   AGCATGTTAGATAA**GATAGCTGTGCTAGTAGGCAGTCAGCGCCAT
r001        TTAGATAAAGGATA*CTG
```

CSC

Flag field in BAM

- **Read's flag number is a sum of values**
  - E.g. 4 = unmapped, 1024 = duplicate
  - Explained in detail at http://samtools.github.io/hts-specs/SAMv1.pdf
  - You can interpret them at http://broadinstitute.github.io/picard/explain-flags.html



CSC

How did the alignment go? Check the log file

- **How many reads aligned to the reference genome?**
  - How many of them aligned uniquely?
- **How many read pairs aligned to the reference genome?**
  - How many pairs aligned concordantly?
- **What was the overall alignment rate?**

Visualisation

View text

```
25354832 reads; of these:
  25354832 (100.00%) were paired; of these:
    6098272 (24.05%) aligned concordantly 0 times
    18567284 (73.23%) aligned concordantly exactly 1 time
    689276 (2.72%) aligned concordantly >1 times
    ----
    6098272 pairs aligned concordantly 0 times; of these:
      724806 (11.89%) aligned discordantly 1 time
    ----
    5373466 pairs aligned 0 times concordantly or discordantly; of these:
      10746932 mates make up the pairs; of these:
        8812069 (82.00%) aligned 0 times
        1800817 (16.76%) aligned exactly 1 time
        134046 (1.25%) aligned >1 times
82.62% overall alignment rate
```

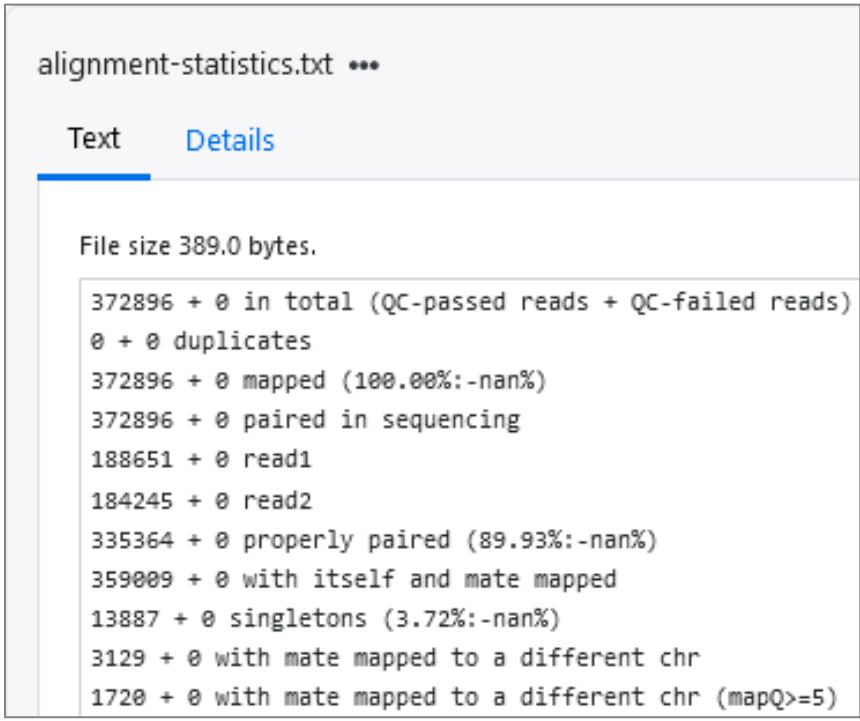
CSC

Log file by STAR

```
                                 Started job on |   Feb 17 12:38:11
                             Started mapping on |   Feb 17 12:47:47
                                    Finished on |   Feb 17 12:52:32
       Mapping speed, Million of reads per hour |   320.27

                           Number of input reads |   25354832
                       Average input read length |   202
                                     UNIQUE READS:
                    Uniquely mapped reads number |   20409554
                         Uniquely mapped reads % |   80.50%
                           Average mapped length |   197.39
                        Number of splices: Total |   12378576
             Number of splices: Annotated (sjdb) |   12378175
                        Number of splices: GT/AG |   12272618
                        Number of splices: GC/AG |   89423
                        Number of splices: AT/AC |   9589
                    Number of splices: Non-canonical |   6946
                       Mismatch rate per base, % |   0.39%
                          Deletion rate per base |   0.01%
                         Deletion average length |   1.75
                         Insertion rate per base |   0.01%
                        Insertion average length |   1.36
                           MULTI-MAPPING READS:
        Number of reads mapped to multiple loci |   970016
             % of reads mapped to multiple loci |   3.83%
        Number of reads mapped to too many loci |   11610
             % of reads mapped to too many loci |   0.05%
                               UNMAPPED READS:
       % of reads unmapped: too many mismatches |   0.00%
                 % of reads unmapped: too short |   15.55%
                     % of reads unmapped: other |   0.08%
                               CHIMERIC READS:
                     Number of chimeric reads |   0
                          % of chimeric reads |   0.00%
```

Other tools for checking BAM files

- **Count alignments in BAM**
  - How many alignments does the BAM contain.
  - Includes an optional mapping quality filter.
- **Count alignments per chromosome in BAM**
- **Count alignment statistics for BAM**
- **Collect multiple metrics for BAM**



Tools for manipulating BAM files

- **Make a subset of BAM**
  - Retrieve alignments for a given chromosome/region, e.g. chr1:100-1000
  - Can filter based on mapping quality
- **Index BAM**
- **Convert SAM to BAM, sort and index BAM**
  - ”Preprocessing” when importing SAM/BAM, runs on your computer.
  - The tool available in the ”Utilities” category runs on the server
- **Create a preview for BAM**
  - Creates a SAM file containing the BAM header and the first 200 alignments. SAM is a text file so you can view it in Chipster.

Full alignment or lightweight mapping?

- **Aligning reads to reference genome is slow → many quantitation tools offer now lightweight ”mapping”. Different flavors:**
  - selective alignment (Salmon)
  - quasi-mapping (Sailfish, Salmon)
  - pseudoalignment (kallisto)
- **These tools match reads to transcripts and report transcripts that a read is compatible with (no base-to-base alignments)**
  - Difficult to assign reads to isoforms because they share exons, and technical biases cause non-uniform coverage
  - Need complete transcriptome

- **Srivastava et al 2019: Alignment and mapping methodology influence transcript abundance estimation**
  - Quantification accuracy is better when using traditional alignments

CSC

Data analysis workflow

- Quality control of raw reads
- Preprocessing (trimming / filtering) if needed
- Alignment to reference genome
- **Alignment level quality control**
- Quantitation
- Experiment level quality control
- Differential expression analysis
- Annotation
- Pathway analysis

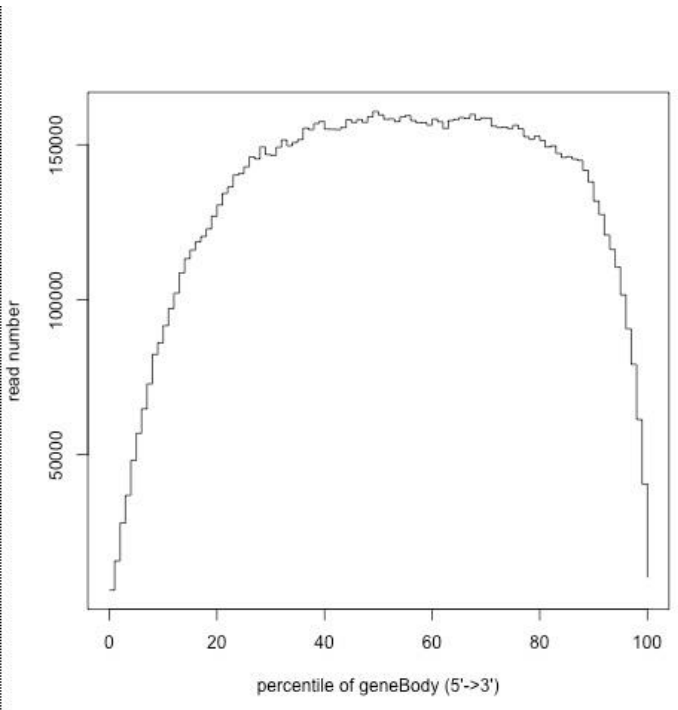
Annotation-based quality metrics

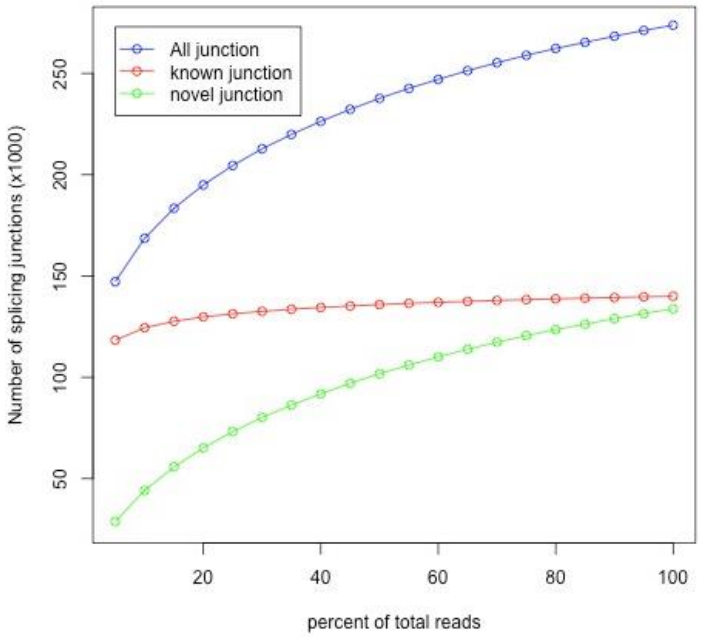
- **Saturation of sequencing depth**
  - Would more sequencing detect more genes and splice junctions?
- **Read distribution between different genomic features**
  - Exonic, intronic, intergenic regions
  - Coding, 3’ and 5’ UTR exons
  - Protein coding genes, pseudogenes, rRNA, miRNA, etc
- **Is read coverage uniform along transcripts?**
  - Biases introduced in library construction and sequencing
    - polyA capture and polyT priming can cause 3’ bias
    - random primers can cause sequence-specific bias
    - GC-rich and GC-poor regions can be under-sampled
  - Genomic regions have different mappabilities (uniqueness)

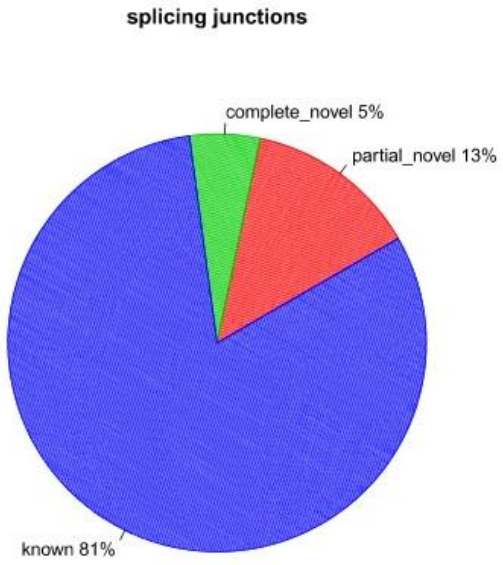
CSC

Quality assessment with RSeQC

- **Checks coverage uniformity, saturation of sequencing depth, novelty of splice junctions, read distribution between different genomic regions, etc.**
- **Takes a BAM file and a BED file**
  - Chipster has BED files available for several organisms
  - You can also use your own BED if you prefer







CSC

BED file format

- **BED (Browser extensible data) file format is used for reporting location of features (e.g. genes and exons) in a genome**
- **5 obligatory columns: chr, start, end, name, score**
- **0-based, like BAM**

column0	column1	column2	column3	column4
chr22	21022480	21024796	JUNC00000001	1
chr19	201609	201783	JUNC00000002	5
chr19	281478	282180	JUNC00000003	3
chr19	282242	282811	JUNC00000004	21
chr19	282751	287541	JUNC00000005	37
chr19	287705	288084	JUNC00000006	6
chr19	288105	291354	JUNC00000007	18
chr19	307484	308600	JUNC00000008	1
chr19	308603	308858	JUNC00000009	2
chr19	308868	311907	JUNC00000010	13
chr19	311872	312256	JUNC00000011	26
chr19	312205	313558	JUNC00000012	22
chr19	313575	325706	JUNC00000013	68

CSC

Own BED? Check chromosome names

- **RSeQC needs the same chromosome naming in BAM and BED**
- **Chromosome names in BED files can have the prefix “chr”**
  - e.g. chr1
- **Chipster BAM files are Ensembl-based and don’t have the prefix**
  - If you use your own BED (e.g. from UCSC Table browser) you need to remove the prefix (chr1 → 1)
- **Use the tool Utilities / Modify text with the following parameters:**
  - Operation = Replace text
  - Search string = chr
  - Input file format = BED

QC tables by RSeQC

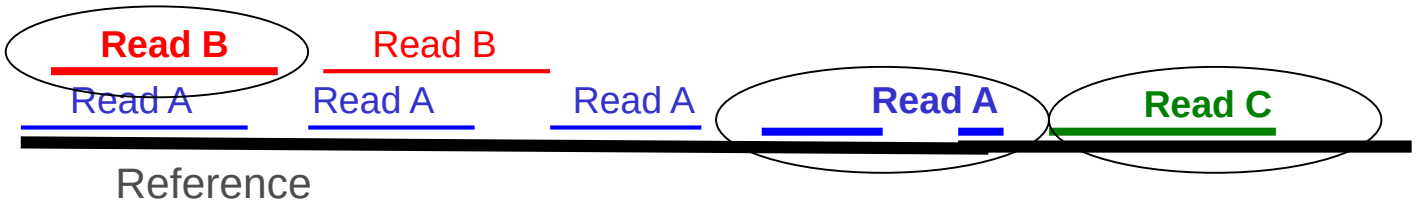
```
#==================================================
#All numbers are READ count
#==================================================

Total records:                          103284

QC failed:                              0
Optical/PCR duplicate:                  0
Non primary hits                        18476
Unmapped reads:                         0
mapq < mapq_cut (non-unique):           4208
mapq >= mapq_cut (unique):              80600
Read-1:                                 0
Read-2:                                 0
Reads map to '+':                       48292
Reads map to '-':                       32308
Non-splice reads:                       50919
Splice reads:                           29681
Reads mapped in proper pairs:           0
Proper-paired reads map to different chrom:0
```

(alignment, actually…)

Default=30

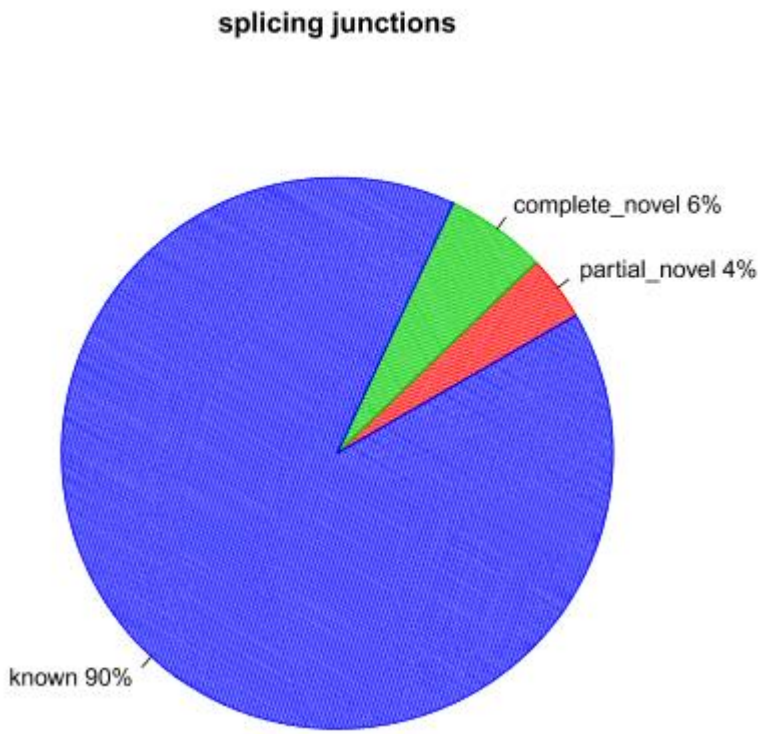
```
read_distribution:

Total Reads                     84808
Total Tags                      116738
Total Assigned Tags             111352
=====================================================================
Group           Total_bases     Tag_count       Tags/Kb
CDS_Exons       2211343         90961           41.13
5'UTR_Exons     529860          1662            3.14
3'UTR_Exons     1415234         12423           8.78
Introns         25801210        5349            0.21
TSS_up_1kb      1295771         31              0.02
TSS_up_5kb      5332522         321             0.06
TSS_up_10kb     8804879         584             0.07
TES_down_1kb    1292506         217             0.17
TES_down_5kb    5108821         344             0.07
TES_down_10kb   8282641         373             0.05
=====================================================================
```

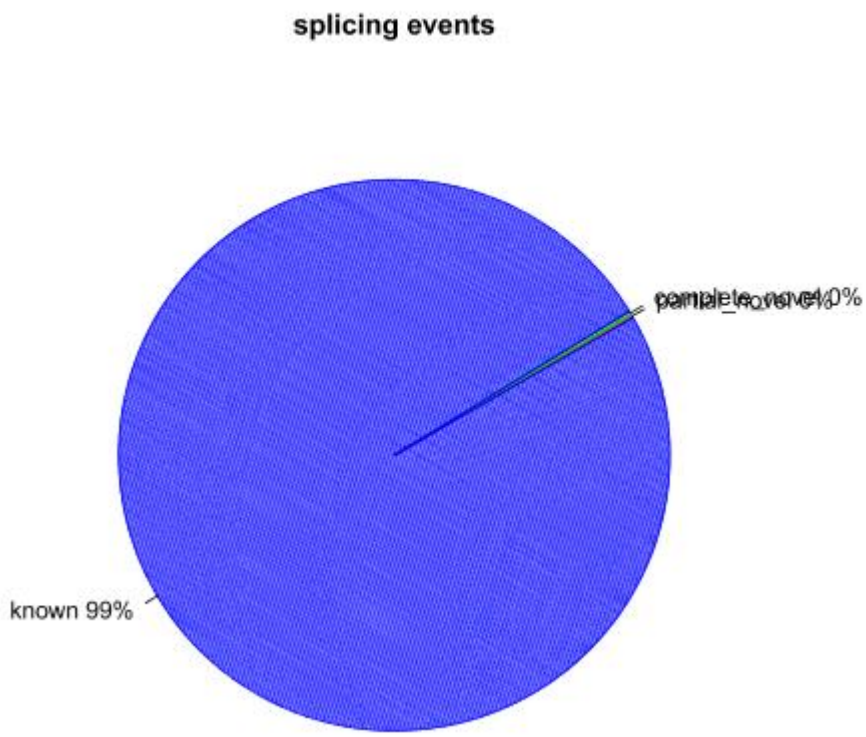
Total records:	7
Non primary hits:	4
Total reads:	3
Total tags:	8



CSC

Splicing graphs by RSeQC





- **Splicing junction = exon-exon junction covered by one or more reads**
- **Splicing event = a read is split across a splice junction**

CSC

Data analysis workflow

- Quality control of raw reads
- Preprocessing (trimming / filtering) if needed
- Alignment to reference genome
- Alignment level quality control
- **Quantitation**
- Describing the experiment with phenodata
- Experiment level quality control
- Differential expression analysis
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- Pathway analysis

Software for counting reads per genes or transcripts

- **HTSeq**

- **StringTie**
- **Cufflinks**

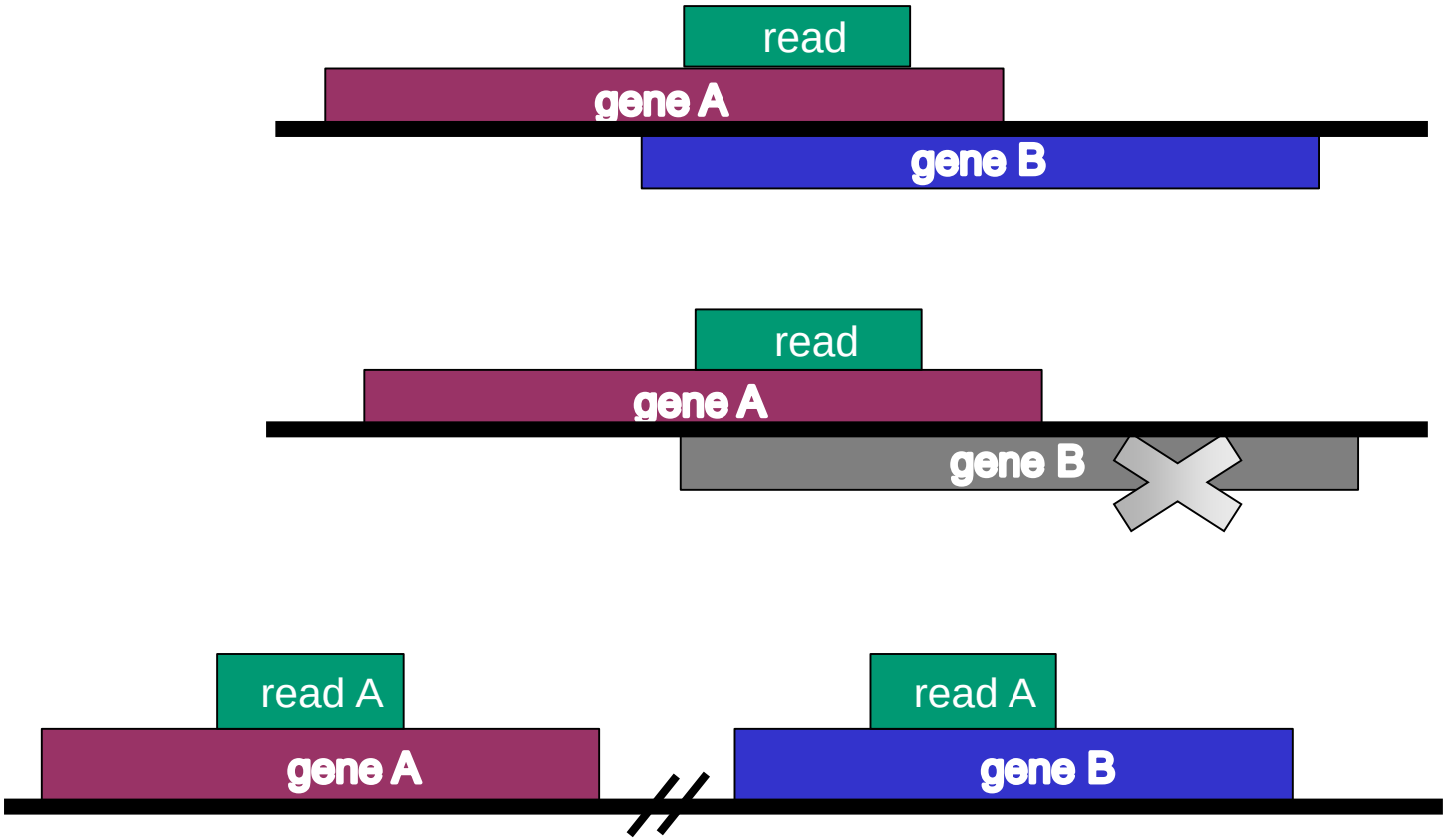
- **Salmon**
- **Kallisto**

Counting reads per genes with HTSeq

- **Given a BAM file and a GTF file with gene locations, counts how many reads map to each gene.**
  - A gene is considered as the union of all its exons.
  - Reads can be counted also per exons.
- **Chipster provides Ensembl GTF files, but you can give your own**
  - Note that GTF and BAM must use the same chromosome naming
  - All exons of a gene must have the same gene_id (avoid UCSC GTFs)
- **Multimapping reads and ambiguous reads are not counted**
- **3 modes to handle reads which overlap several genes**
  - Union (default), Intersection-strict, Intersection-nonempty
- **Attention: was your data made with stranded protocol?**
  - You need to select the right counting mode!

CSC

Not unique or ambiguous?



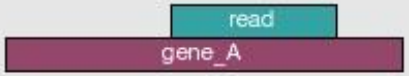
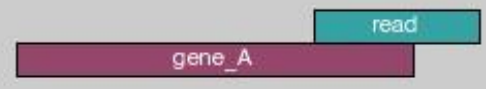
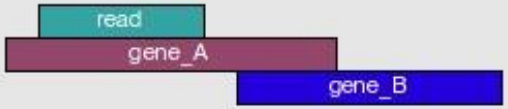
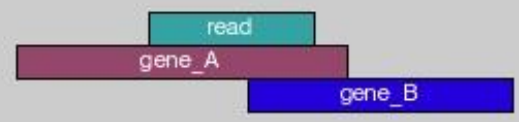
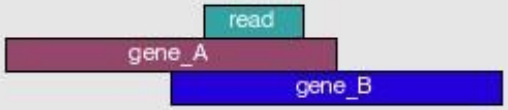
Ambiguous

Stranded data
→ Not ambiguous

Multimapping
(not unique)

CSC

HTSeq count modes

	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	gene_A	gene_A
	ambiguous	ambiguous	ambiguous

CSC

HTSeq result files: summary info and counts

htseq-count-info.txt •••

Text Details

File size 150.0 bytes.

```
__no_feature    149620
__ambiguous     28344
__too_low_aQual 0
__not_aligned   0
__alignment_not_unique  55693

not_counted     233657
counted 401694
total   635351
```

hESC2_chr18.tsv

Showing all 63677 rows.

id	chr	start	end	len	strand	count
ENSG00000152234	chr18	43664109	43684300	20191	-	28474
ENSG00000235552	chr18	6462142	6463014	872	-	15356
ENSG00000074657	chr18	56529831	56653712	123881	+	9285
ENSG00000133313	chr18	72163050	72188366	25316	+	8325
ENSG00000046604	chr18	29078005	29128971	50966	+	7373
ENSG00000134440	chr18	55267887	55289445	21558	-	7196
ENSG00000175886	chr18	36914835	36915639	804	-	6102
ENSG00000235297	chr18	72057118	72057532	414	-	6009
ENSG00000101680	chr18	6941742	7117813	176071	-	5647
ENSG00000177426	chr18	3411605	3458409	46804	+	5024
ENSG00000265273	chr18	29542140	29543581	1441	+	4900
ENSG00000118680	chr18	3261906	3278282	16376	+	4603
ENSG00000176014	chr18	12307667	12344319	36652	+	4538
ENSG00000134759	chr18	33709406	33757909	48503	+	4202
ENSG00000134779	chr18	34359986	34409158	49172	-	4067
ENSG00000081913	chr18	60382671	60647666	264995	+	3997
ENSG00000101608	chr18	3247478	3256234	8756	+	3962
ENSG00000141401	chr18	11981023	12030876	49853	+	3816
ENSG00000101544	chr18	77866914	77905406	38492	+	3687
ENSG00000167088	chr18	19192227	19210417	18190	+	3392
ENSG00000167315	chr18	47309868	47340330	30462	-	3290
ENSG00000141425	chr18	33564349	33647539	83190	-	3165

GTF file format

- **9 obligatory columns: chr, source, name, start, end, score, strand, frame, attribute**
- **1-based**
- **For HTSeq to work, all exons of a gene must have the same gene_id**
  - Use GTFs from Ensembl, avoid UCSC

```
chr1    unknown    exon    14362    14829    .    -    .    gene_id "WASH7P"; gene_name "WASH7P"; transcript_id "NR_024540"; tss_id "TSS7245";
chr1    unknown    exon    14970    15038    .    -    .    gene_id "WASH7P"; gene_name "WASH7P"; transcript_id "NR_024540"; tss_id "TSS7245";
chr1    unknown    exon    15796    15947    .    -    .    gene_id "WASH7P"; gene_name "WASH7P"; transcript_id "NR_024540"; tss_id "TSS7245";
chr1    unknown    exon    16607    16765    .    -    .    gene_id "WASH7P"; gene_name "WASH7P"; transcript_id "NR_024540"; tss_id "TSS7245";
chr1    unknown    exon    16858    17055    .    -    .    gene_id "WASH7P"; gene_name "WASH7P"; transcript_id "NR_024540"; tss_id "TSS7245";
chr1    unknown    exon    17233    17368    .    -    .    gene_id "WASH7P"; gene_name "WASH7P"; transcript_id "NR_024540"; tss_id "TSS7245";
chr1    unknown    exon    17606    17742    .    -    .    gene_id "WASH7P"; gene_name "WASH7P"; transcript_id "NR_024540"; tss_id "TSS7245";
chr1    unknown    exon    17915    18061    .    -    .    gene_id "WASH7P"; gene_name "WASH7P"; transcript_id "NR_024540"; tss_id "TSS7245";
chr1    unknown    exon    18268    18366    .    -    .    gene_id "WASH7P"; gene_name "WASH7P"; transcript_id "NR_024540"; tss_id "TSS7245";
chr1    unknown    exon    24738    24891    .    -    .    gene_id "WASH7P"; gene_name "WASH7P"; transcript_id "NR_024540"; tss_id "TSS7245";
chr1    unknown    exon    29321    29370    .    -    .    gene_id "WASH7P"; gene_name "WASH7P"; transcript_id "NR_024540"; tss_id "TSS7245";
```

Isoform switching can confound DGE analysis

- **The number of reads obtained from an expressed gene depends on the transcript length**
  - Longer transcripts produce more fragments and hence more reads
- **If a gene switches from a long transcript isoform to a short one, this can confound DGE analysis**

Expression level of gene A is the same in both samples, but cancer cells express the shorter isoform

CSC

Isoform switching can confound DGE analysis

- **The number of reads obtained from an expressed gene depends on the transcript length**
  - Longer transcripts produce more fragments and hence more reads
- **If a gene switches from one transcript isoform to another one, this can confound DGE analysis**

We get twice as many reads from the control sample
→ is gene A down-regulated in cancer?

Is isoform switching a major problem?

- **The magnitude of the effect depends on**
  - the extent of differential transcript usage (DTU)
  - the difference in length between the differentially expressed isoforms.
    - If the longer isoform is < 34% longer, false positives are controlled ok
    - Among all human transcript pairs in which both transcripts belong to the same gene, the median length ratio is 1.85
    - For one third of such pairs the longer isoform is < 38% longer
- **Many human genes express mainly one, dominant isoform**
  - → the global impact of isoform switching is relatively small in many real datasets (as opposed to simulated ones)

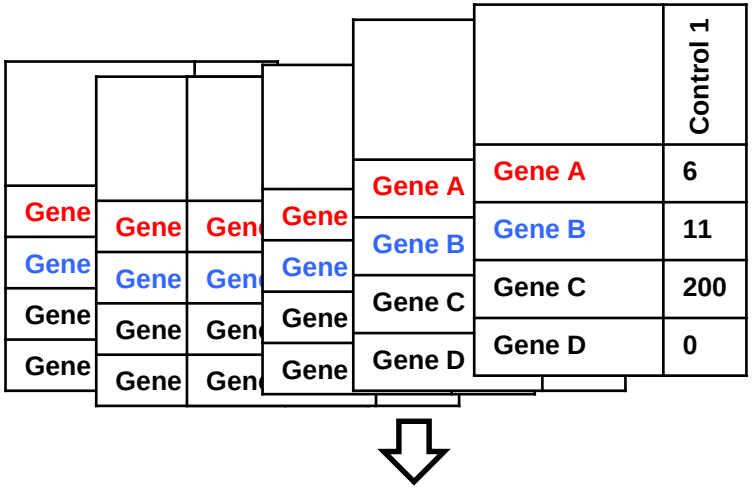
Soneson et al. F1000 Research 2016

Data analysis workflow

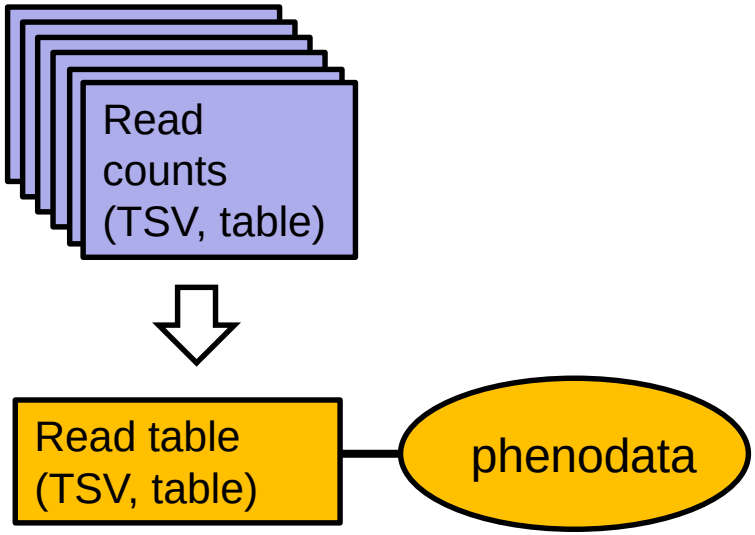
- Quality control of raw reads
- Preprocessing (trimming / filtering) if needed
- Alignment to reference genome
- Alignment level quality control
- Quantitation
- **Describing the experiment with phenodata**
- Experiment level quality control
- Differential expression analysis
- Annotation
- Pathway analysis

Combine individual count files into a count table

- **Select all the count files and run “Utilities / Define NGS experiment”**
- **This creates a table of counts and a phenodata file, where you can describe experimental groups**



	Control 1	Control 2	Control 3	Sample 1	Sample 2	Sample 3
Gene A	6	5	7	17	10	11
Gene B	11	11	10	3	4	2
Gene C	200	150	355	50	1	3
Gene D	0	1	0	2	0	1



CSC

Phenodata file: describe the experiment

- **Describe experimental groups, time, pairing etc <u>with numbers</u>**
  - e.g. 1 = control, 2 = cancer
- **Define sample names for visualizations in the Description column**

sample	original_name	description	patient	group	treatment	time	hours
ngs001.tsv	SRR479052	1_C_24	1	1	Control	1	24h
ngs002.tsv	SRR479053	1_C_48	1	1	Control	2	48h
ngs003.tsv	SRR479054	1_DP_24	1	2	DPN	1	24h
ngs004.tsv	SRR479055	1_DP_48	1	2	DPN	2	48h
ngs007.tsv	SRR479058	2_C_24	2	1	Control	1	24h
ngs008.tsv	SRR479059	2_C_48	2	1	Control	2	48h
ngs009.tsv	SRR479060	2_DP_24	2	2	DPN	1	24h
ngs011.tsv	SRR479062	2_DP_48	2	2	DPN	2	48h
ngs015.tsv	SRR479066	3_C_24	3	1	Control	1	24h
ngs016.tsv	SRR479067	3_C_48	3	1	Control	2	48h
ngs017.tsv	SRR479068	3_DP_24	3	2	DPN	1	24h
ngs018.tsv	SRR479069	3_DP_48	3	2	DPN	2	48h

CSC

Data analysis workflow

- Quality control of raw reads
- Preprocessing (trimming / filtering) if needed
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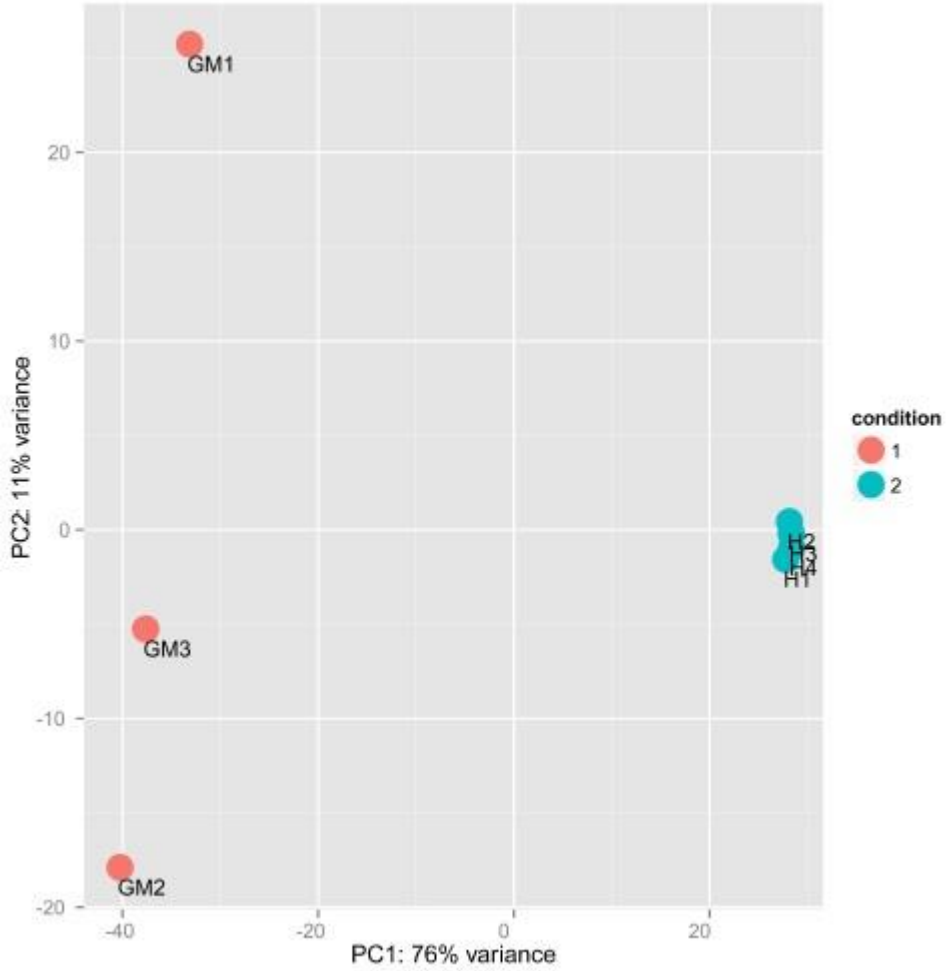
- **Experiment level quality control**
- Differential expression analysis
- Annotation
- Pathway analysis

Experiment level quality control

- **Getting an overview of similarities and dissimilarities between samples allows you to check**
  - Do the experimental groups separate from each other?
  - Is there a confounding factor (e.g. batch effect) that should be taken into account in the statistical analysis?
  - Are there sample outliers that should be removed?

- **Several methods available**
  - MDS (multidimensional scaling)
  - PCA (principal component analysis)
  - Clustering

PCA plot by DESeq2



- **The first two principal components, calculated after variance stabilizing transformation**
- **Indicates the proportion of variance explained by each component**
  - If PC2 explains only a small percentage of variance, it can be ignored

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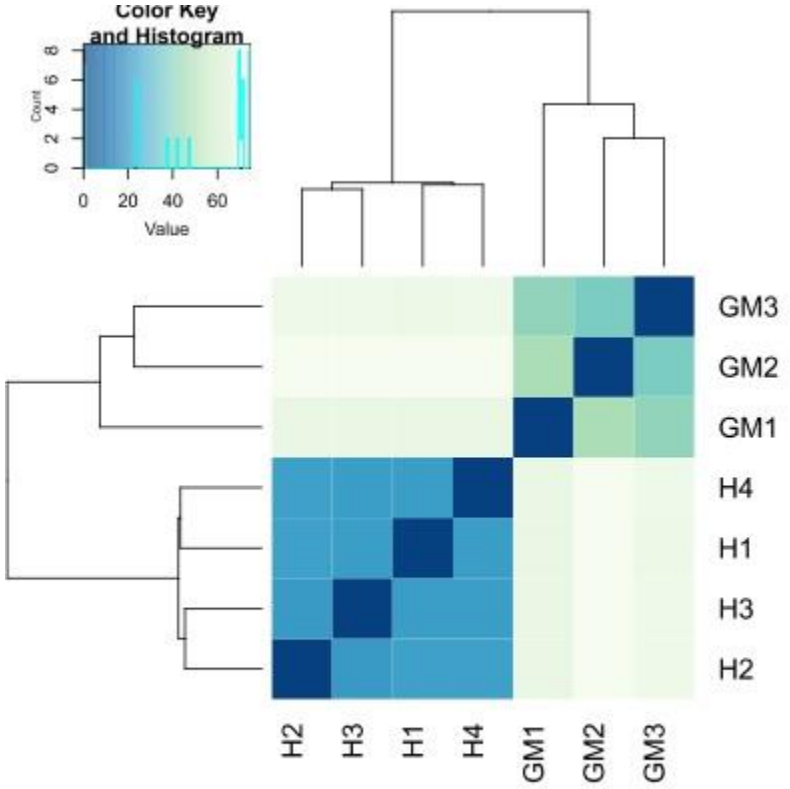
Sample heatmap by DESeq2



- **Euclidean distances between the samples, calculated after variance stabilizing transformation**

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What if you detect a problem?

- **Removing an outlier sample**
  - Create a new column called ”keep” in phenodata
  - Enter 1 for the samples to be kept and 0 for the outlier
  - Run the tool Utilities / Remove samples from a table

- **Taking a confounding factor (e.g. batch effect) taken into account in the statistical analysis**
  - Create a column for it in the phenodata and mark batches with numbers
  - DESeq2: second experimental factor
  - edgeR:

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Data analysis workflow

- Quality control of raw reads
- Preprocessing (trimming / filtering) if needed
- Alignment to reference genome
- Alignment level quality control
- Quantitation
- Experiment level quality control
- **Differential expression analysis**
- Annotation
- Pathway analysis

Software packages for DE analysis

- **edgeR**
- **DESeq2**
- **Sleuth**
- **DRIMSeq**
- **DEXSeq**
- **Cuffdiff, Ballgown**
- **Limma + voom, limma + vst**
- **...**

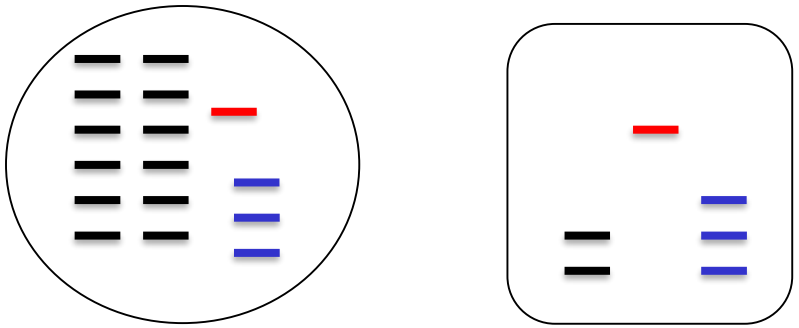
Differential gene expression analysis

- **Normalization**
- **Dispersion estimation**
- **Log fold change estimation**
- **Statistical testing**
- **Filtering**
- **Multiple testing correction**

Differential expression analysis:
Normalization

Normalization

- **For comparing gene expression <u>between (groups of) samples</u>, normalize for**
  - Library size (number of reads obtained)
  - RNA composition effect



- **The number of reads for a gene is also affected by transcript length and GC content**
  - When studying differential gene expression we *assume that they stay the same*

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Normalization by edgeR and DESeq

- **Aim to make normalized counts for non-differentially expressed genes similar between samples**
  - Do not aim to adjust count distributions between samples
- **Assume that**
  - Most genes are not differentially expressed
  - Differentially expressed genes are divided equally between up- and down-regulation
- **Do not transform data, but use normalization factors within statistical testing**

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Normalization by edgeR and DESeq – how?

- **DESeq(2)**
  - Take geometric mean of gene's counts across all samples
  - Divide gene's counts in a sample by the geometric mean
  - Take median of these ratios → sample's normalization factor (applied to read counts)
- **edgeR**
  - Select as reference the sample whose upper quartile is closest to the mean upper quartile
  - Log ratio of gene's counts in sample vs reference → M value
  - Take weighted trimmed mean of M-values (TMM) → normalization factor (applied to library sizes)
    - Trim: Exclude genes with high counts or large differences in expression
    - Weights are from the delta method on binomial data

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edgeR and DESeq2 expect raw read counts

- **Raw counts are needed to assess the quantification uncertainty**
- **Uncertainty information is lost if counts are transformed to FPKM**
  - FPKM = fragments per kilobase per million mapped reads.
  - Normalizes for gene length and library size. Example:
    - 20 kb transcript has 400 counts, library size is 20 million reads: FPKM = (400/20) / 20
    - 0.5 kb transcript has 10 counts, library size is 20 million reads: FPKM = (10/0.5) / 20
      → in both cases FPKM =1, but it is less likely to get 400 reads just by chance

- **The negative binomial assumption of edgeR and DESeq2 is flexible enough to deal with gene-level counts summarized from Salmon's transcript-level abundance estimates**

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Differential expression analysis:
Dispersion estimation

Dispersion

- **When comparing gene's expression levels between groups, it is important to know also its within-group variability**

- **Dispersion = $(BCV)^2$**
  - BCV = gene's biological coefficient of variation
  - E.g. if gene's expression typically differs from replicate to replicate by 20% (so BCV = 0.2), then this gene's dispersion is $0.2^2 = 0.04$

- **Note that the variability seen in counts is a sum of 2 things:**
  - Sample-to-sample variation (dispersion)
  - Uncertainty in measuring expression by counting reads

How to estimate dispersion reliably?

- **We cannot typically afford tens or hundreds of biological replicates**

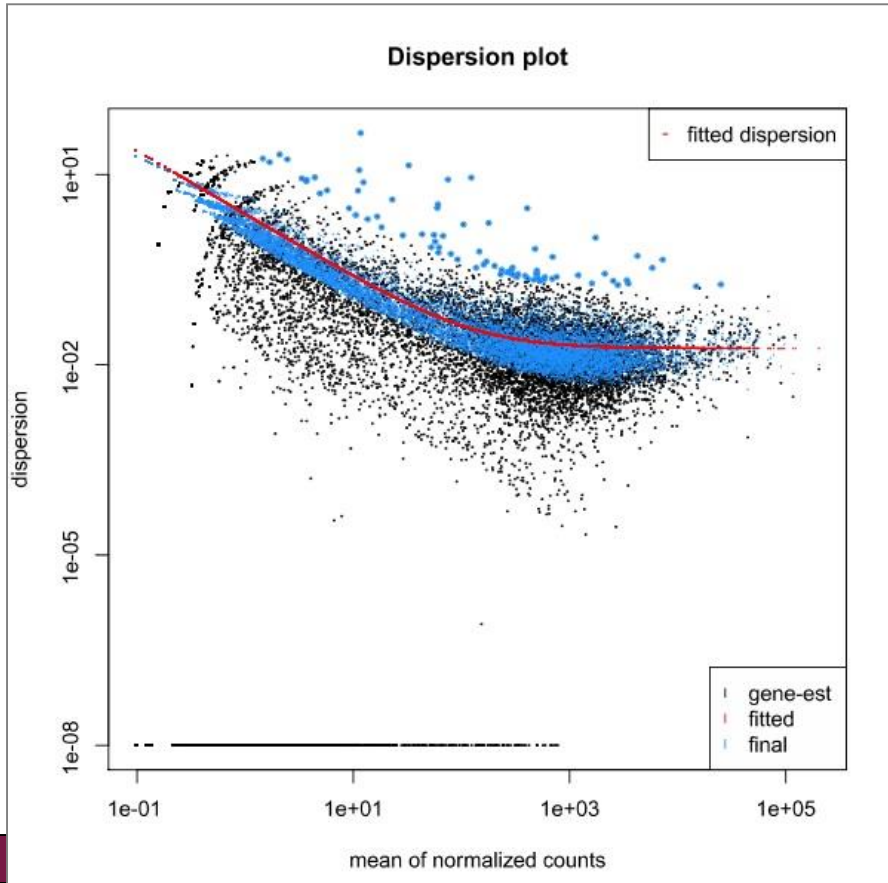
  → it is difficult to estimate within-group variability

- **Solution: pool information across genes which are expressed at similar level**
  - assumes that genes of similar average expression strength have similar dispersion

- **Different approaches**
  - edgeR
  - DESeq2

Dispersion estimation by DESeq2

- **Estimates genewise dispersions using maximum likelihood**
- **Fits a curve to capture the dependence of these estimates on the average expression strength**
- **Shrinks genewise values towards the curve using an empirical Bayes approach**
  - The amount of shrinkage depends on several things including sample size
  - Genes with high gene-wise dispersion estimates are dispersion outliers (blue circles above the cloud) and they are not shrunk



Differential expression analysis:
Statistical testing

Generalized linear models

- **Model the expression of each gene as a linear combination of explanatory factors (eg. group, time, patient)**
  - $y = a + (b \cdot group) + (c \cdot time) + (d \cdot patient) + e$

    y = gene's expression

    a, b, c and d = parameters estimated from the data

    a = intercept (expression when factors are at reference level)

    e = error term

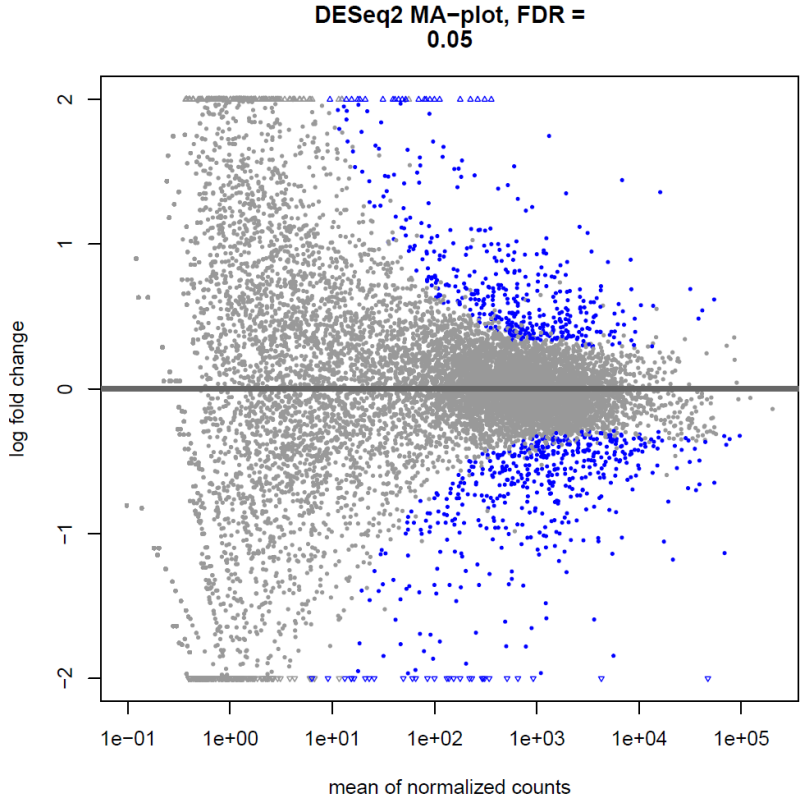
- **<u>Generalized</u> linear model (GLM) allows the expression value distribution to be different from normal distribution**
  - Negative binomial distribution used for count data

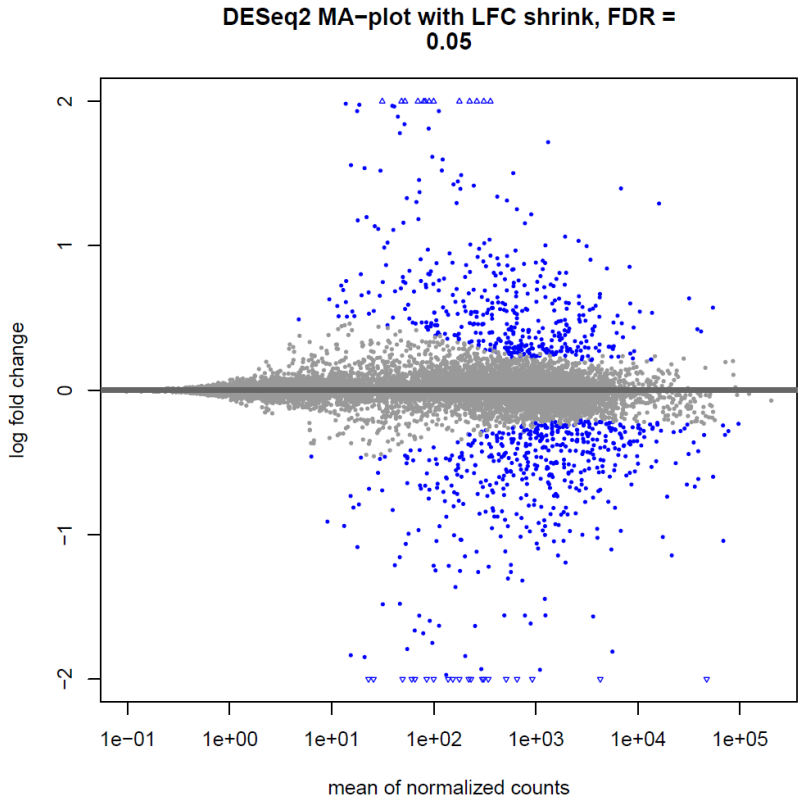
Statistical testing

- **DESeq2**
  - Generalized linear model, Wald test for significance
    - Log fold change is divided by its standard error and the resulting z statistic is compared to a standard normal distribution
- **edgeR**
  - Two group comparisons
    - Exact test for negative binomial distribution
  - Multifactor experiments
    - Generalized linear model, likelyhood ratio test

DESeq2 shrinks log2 fold changes estimates

- **uses the ashr (Adaptive SHRinkage) method**
  - the amount of shrinkage is determined from the data
  - measurements with high standard error will undergo more shrinkage
  - https://github.com/stephens999/ashr





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Multiple testing correction

- **We test thousands of genes, so it is possible that some genes get good p-values just by chance**
  - This problem is much bigger, if you test transcripts (DTE)

- **To control this problem of false positives, p-values need to be corrected for multiple testing**

- **Several methods are available, the most popular one is the Benjamini-Hochberg correction (BH)**
  - largest p-value is not corrected
  - second largest p = (p *n)/ (n-1)
  - third largest p = (p * n)/(n-2)
  - …
  - smallest p = (p * n)/(n- n+1) = p * n

- **The adjusted p-value is FDR (false discovery rate)**

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Filtering

- **Reduces the severity of multiple testing correction by removing some genes (makes n smaller)**

- **Filter out genes which have little chance of showing evidence for significant differential expression**
  - genes which are not expressed
  - genes which are expressed at very low level (low counts are unreliable)

- **Should be independent**
  - do not use information on what group the sample belongs to

- **DESeq2 selects filtering threshold automatically**

edgeR result table

- **logFC = log2 fold change**
- **logCPM = average log2 counts per million**
- **Pvalue = raw p-value**
- **FDR = false discovery rate (Benjamini-Hochberg adjusted p-value)**

de-list-edger.tsv

Showing all 757 rows.

	chip.treat	chip.trea	chip.trea	chip.unt	chip.un	chip.un	chip.unti	logFC	logCPM	PValue	FDR
FBgn0039155	56	62	74	1756	2238	1081	1229	-4.69899422081019	6.03560818295738	7.20308463504266e-135	6.22058389082284e-131
FBgn0029167	1649	1624	1175	6810	9182	4877	4919	-2.23433823802528	8.24793917660261	1.99034107006072e-67	8.59429274052218e-64
FBgn0034736	35	44	29	358	593	295	283	-3.49985798252498	4.04448825313873	6.11382510741391e-60	1.75996645425422e-56
FBgn0035085	179	212	172	1075	1292	749	861	-2.52597752387173	5.53857558363739	4.25001852624925e-54	9.17578999817213e-51
FBgn0000071	775	822	645	78	151	86	96	2.74080535171334	4.6808323300972	2.25722664890845e-48	3.89868186799468e-45
FBgn0029896	192	156	123	704	1274	595	611	-2.43819206791615	5.18811150378514	1.46605619431662e-45	2.11014354901973e-42
FBgn0039827	27	20	46	588	656	471	501	-4.27016967435994	4.60377905047843	3.03506500413406e-42	3.74440305367167e-39
FBgn0033764	196	184	155	16	21	12	10	3.48503218393543	2.5488391163408	2.526432524815e-41	2.7272839105378e-38
FBgn0034434	17	13	20	226	299	219	237	-4.01870183550384	3.44909319532712	2.01715887090541e-39	1.93557600101545e-36
FBgn0051092	349	384	331	64	91	49	44	2.4232155517234	3.68036599989021	4.54650866609765e-37	3.92636488404193e-34
FBgn0011260	650	455	466	84	139	81	75	2.35185375445073	4.24174827367645	7.37076473305667e-35	5.78672038497067e-32

DESeq2 result table

- baseMean = mean of counts (divided by size factors) taken over all samples
- **log2FoldChange = log2 of the ratio meanB/meanA**
- lfcSE = standard error of log2 fold change
- stat = Wald statistic
- pvalue = raw p-value
- **padj = Benjamini-Hochberg adjusted p-value**

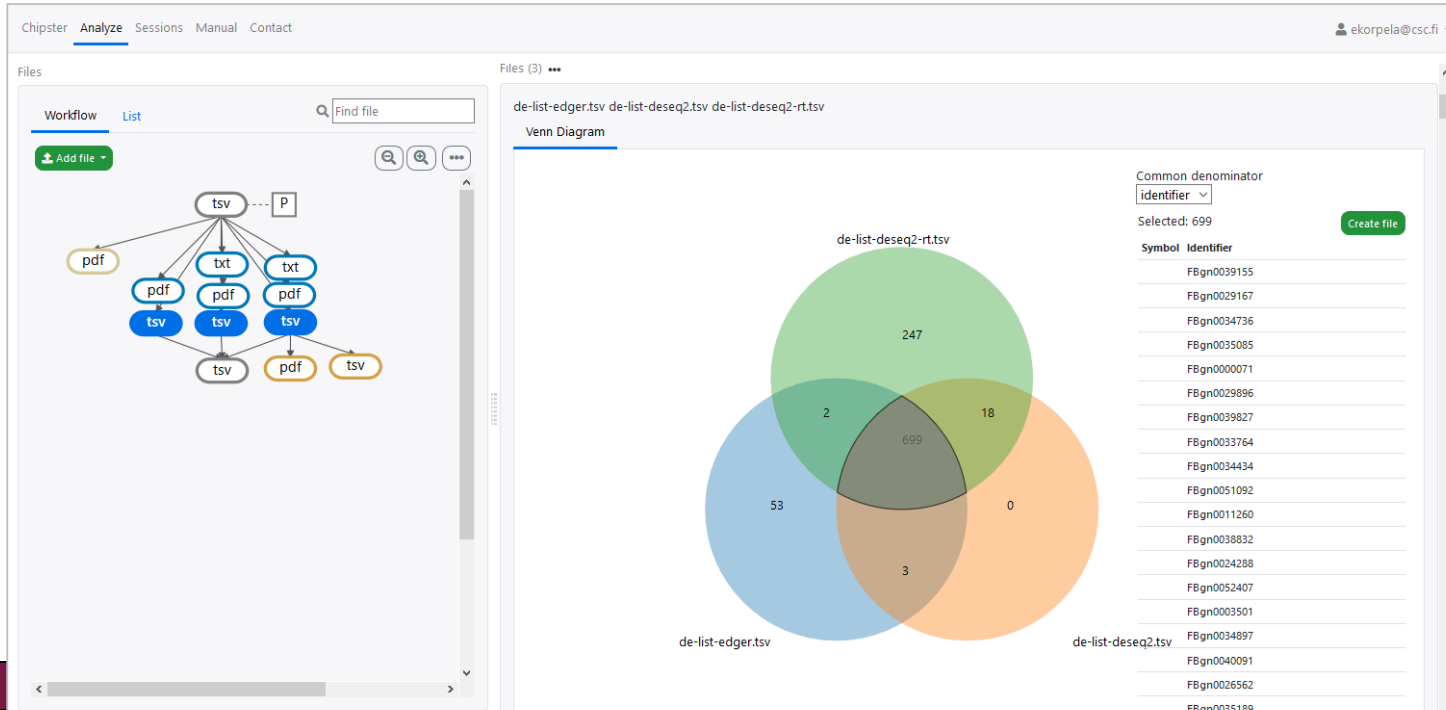
de-list-deseq2-rt.tsv

Showing all 973 rows.

	chip.treate	chip.treat	chip.trea	chip.unt	chip.unt	chip.unt	chip.un	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
FBgn0039155	56	62	74	1756	2238	1081	1229	924.27	-4.17	0.15	-28.66	1.246e-180	9.941e-177
FBgn0026562	13037	17789	14377	61534	87864	55454	72147	47282.42	-2.32	0.09	-24.84	3.333e-136	1.330e-132
FBgn0029167	1649	1624	1175	6810	9182	4877	4919	4287.44	-2.13	0.1	-21.79	3.121e-105	8.300e-102
FBgn0039827	27	20	46	588	656	471	501	342.77	-3.49	0.18	-19.48	1.660e-84	3.311e-81
FBgn0035085	179	212	172	1075	1292	749	861	654.94	-2.36	0.12	-19.01	1.332e-80	2.126e-77
FBgn0034736	35	44	29	358	593	295	283	231.7	-2.93	0.17	-17.25	1.119e-66	1.488e-63
FBgn0000071	775	822	645	78	151	86	96	359.53	2.42	0.14	16.76	4.495e-63	5.123e-60
FBgn0034434	17	13	20	226	299	219	237	153.84	-3.11	0.19	-16.25	2.071e-59	2.065e-56

Interactive Venn diagram

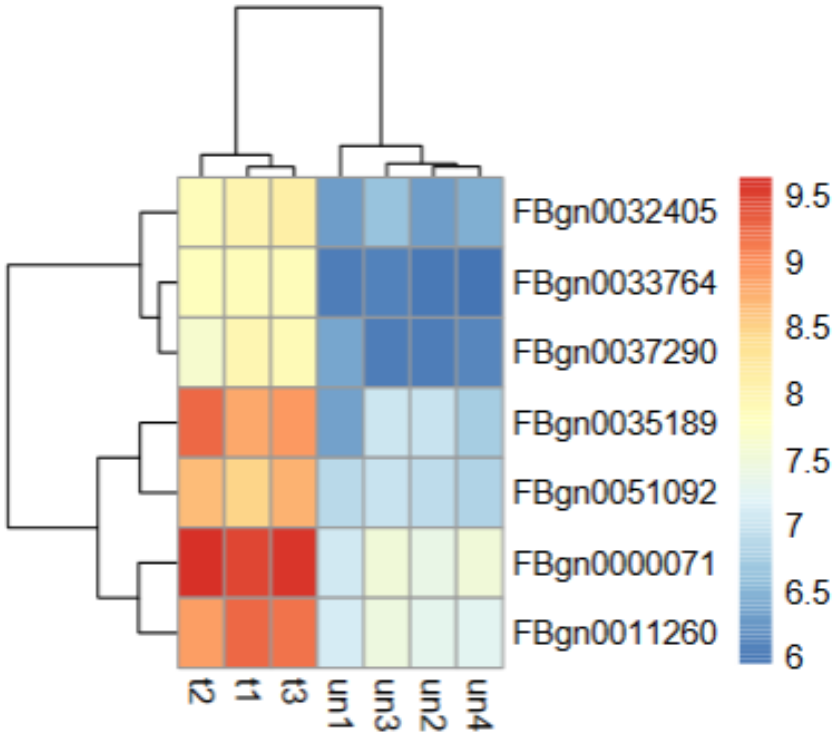
- **You can compare result files, e.g. were the same genes found**
  - Select 2-3 tsv files and click Draw
- **Make a new gene list**
  - Click on the image to select an area (e.g. the intersection) and click Create file → new gene list appears
  - If the files have columns with the same name (e.g. padj), the values to the new gene list are taken from the input file that you selected first



Heatmap of differentially expressed genes

- **Use the tool Heatmap for RNA-seq results**
- **Counts are transformed using variance stabilization transformation**
  - calculated using the experiment-wide trend of variance over mean

→ You need to give 2 input files: the original count table and the list of differentially expressed genes. Check that they are correctly assigned!



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What if I have several experimental factors?

- **The tool Differential expression using edgeR for multivariate experiments can cope with 3 main effects and pairing**
- **Main effects can be treated as**
  - Linear = is there a trend towards higher numbers?
  - Factor = are there differences between the levels?

  If the main effect has only two levels (e.g. control and cancer), selecting linear or factor gives the same result

- **Note that the result table contains all the genes, so in order to get the differentially expressed genes you have to filter it**
  - Use the tool **Utilities / Filter using a column value**
  - Select the FDR column that corresponds to the comparison of your interest

PValue-as.factor(group)2	FDR-as.factor(group)2	logFC-as.factor(time)2	logCPM-as.factor(time)2	LR-as.factor(time)2	PValue-as.factor(time)2	FDR-as.factor(time)2	logFC-as.factor(patient)2	l
4.4e-05	0.01647	-1.583315	5.782999	308.077737	0	0	-2.002844	5
0.000102	0.027761	1.473723	-0.397191	12.823652	0.000342	0.003208	-0.696252	-
0	0.000276	0.037768	6.612959	0.328104	0.566777	0.746462	0.287009	6
0.000182	0.037215	0.56783	7.896608	84.211177	0	0	0.667624	7
0.000245	0.044683	0.319444	7.146574	27.072628	0	5e-06	-0.029676	7
8e-06	0.004906	-0.087083	9.060264	0.78067	0.376936	0.592923	0.216045	9
0.000285	0.049417	-0.073242	7.146943	1.895127	0.168625	0.35584	-0.445068	7

Analyzing differential gene expression: things to take into account

- **Biological replicates are important!**
- **Normalization is required in order to compare expression between samples**
  - Different library sizes
  - RNA composition bias caused by sampling approach
- **Raw counts are needed to assess measurement precision**
  - Counts are the ”the units of evidence” for expression
  - Gene-level counts summarized from Salmon’s transcript-level estimates seem to be ok
- **Multiple testing problem**

Data analysis workflow

- Quality control of raw reads
- Preprocessing (trimming / filtering) if needed
- Alignment to reference genome
- Alignment level quality control
- Quantitation
- Experiment level quality control
- Differential expression analysis
- **Annotation of gene identifiers**
- Pathway analysis

Add gene symbols and descriptions to data

- **Tool Utilities / Annotate Ensembl identifiers**
  - Ensembl IDs can be
    - in the first column, with or without a title
    - in the middle of the file if the column title is ensembl_id
  - Fetches annotations from the EBI
    - Max100 000 Ensembl IDs can be annotated in one job

annotated.tsv

Showing all 3600 rows.

	symbol	description	chr	start	end	length	sequence	chip.sample001.ts
ENSG00000064042	LIMCH1	LIM and calponin homology domains 1 [Source:HGNC Symbol;Acc:HGNC:29191]	4	41359606	41700044	340438	NA	1948
ENSG00000185499	MUC1	mucin 1, cell surface associated [Source:HGNC Symbol;Acc:HGNC:7508]	1	155185823	155192916	7093	NA	630
ENSG00000198722	UNC13B	unc-13 homolog B [Source:HGNC Symbol;Acc:HGNC:12566]	9	35161991	35405338	243347	NA	851
ENSG00000013588	GPRC5A	G protein-coupled receptor class C group 5 member A [Source:HGNC Symbol;Acc:HGNC:9836]	12	12890781	12917937	27156	NA	3009
ENSG00000131400	NAPSA	napsin A aspartic peptidase [Source:HGNC Symbol;Acc:HGNC:13395]	19	50358476	50365830	7354	NA	3532
ENSG00000112782	CLIC5	chloride intracellular channel 5 [Source:HGNC Symbol;Acc:HGNC:13517]	6	45898450	46080395	181945	NA	1436
ENSG00000170017	ALCAM	activated leukocyte cell adhesion molecule [Source:HGNC Symbol;Acc:HGNC:400]	3	105366908	105576900	209992	NA	1019
ENSG00000111319	SCNN1A	sodium channel epithelial 1 subunit alpha [Source:HGNC Symbol;Acc:HGNC:10599]	12	6346842	6377730	30888	NA	494
ENSG00000122852	SFTPA1	surfactant protein A1 [Source:HGNC Symbol;Acc:HGNC:10798]	10	79610938	79615455	4517	NA	28321

Summary of DGE analysis steps and files

- **Quality control / Read quality with MultiQC → html report**
- (Preprocessing / Trim reads with Trimmomatic → FASTQ)
- (Utilities / Make a list of file names → txt)
- **Alignment / HISAT2 or STAR → BAM**
- **Quality control / RNA-seq quality metrics with RSeQC → pdf**
- **RNA-seq / Count aligned reads per genes with HTSeq → tsv**
- **Utilities / Define NGS experiment → tsv**
- **Quality control / PCA and heatmap of samples with DESeq2 → pdf**
- **RNA-seq / Differential expression using DESeq2 → tsv**
- **Utilities / Annotate Ensembl identifiers → tsv**

Data analysis workflow

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- Annotation
- **Pathway analysis**

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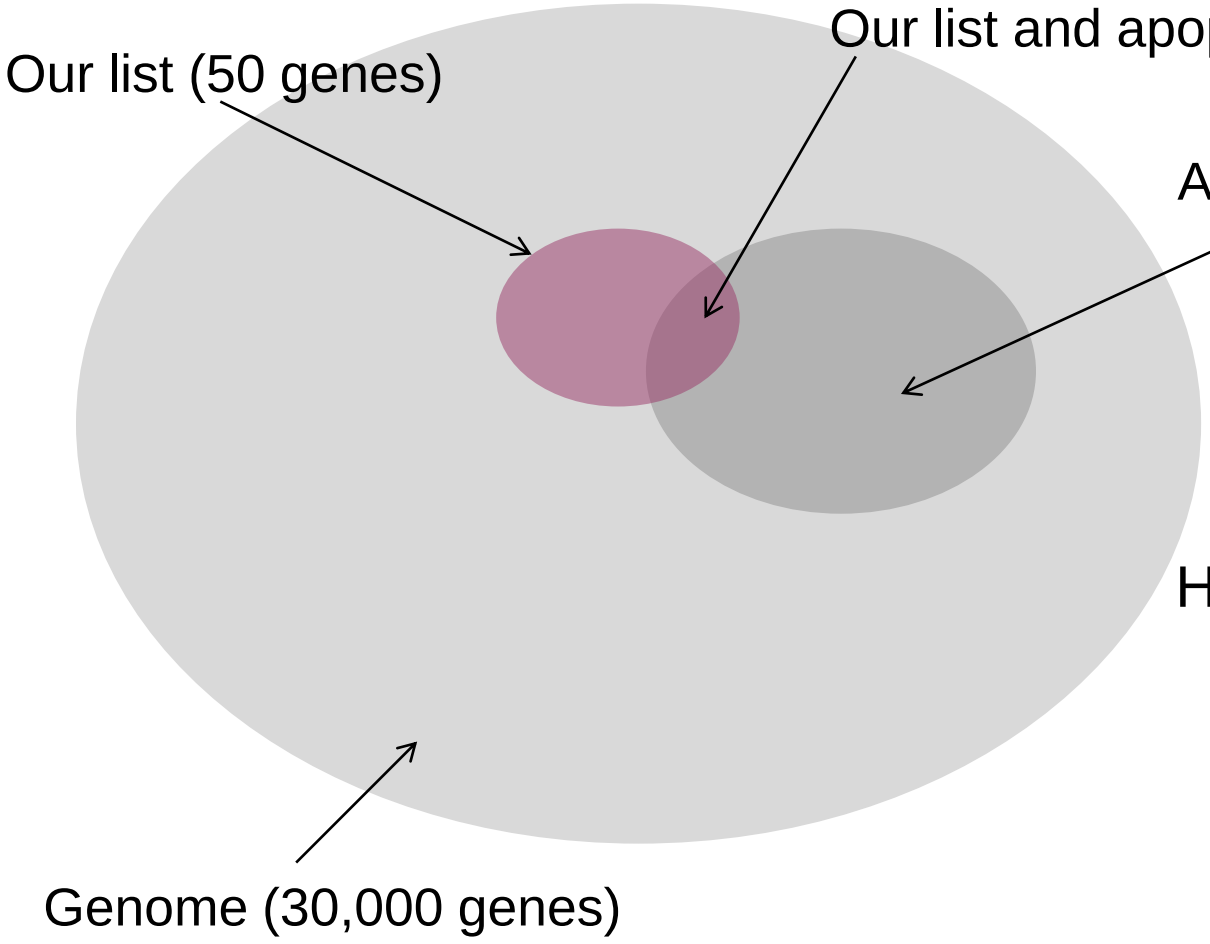
Pathway analysis – why?

- **Statistical tests can yield thousands of differentially expressed genes**
- **It is difficult to make ”biological” sense out of the result list**
- **Looking at the bigger picture can be helpful, e.g. which pathways are differentially expressed between the experimental groups**
- **Databases such as KEGG, GO, Reactome and ConsensusPathDB provide grouping of genes to pathways, biological processes, molecular functions, etc**

Gene set enrichment analysis

1. **Perform a statistical test to find differentially expressed genes**
2. **Check if the list of differentially expressed genes is ”enriched” for some pathways**



$$H_0 : \frac{\frac{10}{50}}{\frac{200}{30000}} = 30 >> 1$$

ConsensusPathDB

- **One-stop shop: Integrates pathway information from 32 databases covering**
  - biochemical pathways
  - protein-protein, genetic, metabolic, signaling, gene regulatory and drug-target interactions

- **Developed by Ralf Herwig's group at the Max-Planck Institute in Berlin**

- **ConsensusPathDB over-representation analysis tool is integrated in Chipster**
  - runs on the MPI server in Berlin

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