B@G 2018

Theory and methods of RNA-seq studies

Part I
protocol, quality control, alignment and quantification

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About me

- 2010 BSc in Statistics at The Sapienza University of Rome.
- 2012 MSc in Statistics at The University of Padua.
- 2017 PhD in Statistics at The University of Warwick: Bayesian modelling for systems biology data.
- 2016-present Postdoc (institute of molecular life sciences) at The University of Zurich: Bayesian methods for differential analyses via RNA-seq data (differential splicing).

Overview of the day

- Three lectures in class:
  - Part I, introduction about RNA-seq: protocol, quality control, alignment and quantification;
  - Part II, the data and gene-level analyses: normalization, exploratory plots and gene level analyses (differential gene expression);
  - Part III, transcript level analyses (differential splicing) and general topics: p.value correction for multiple testing, assessing the performance of a method (in simulations) and recent developments (e.g. long reads and single cell RNA-seq).
- Four tutorials we will go through together:
  - ex. I, quality control (via FastQC and MultiQC), alignment, quantification and loading the output in R;
  - ex. II, exploratory plots and differential gene expression analyses (via edgeR, DESeq and DESeq2);
  - ex. III, differential splicing (via DRIMSeq and DEXSeq);
  - ex. IV, compare the performance of three methods.

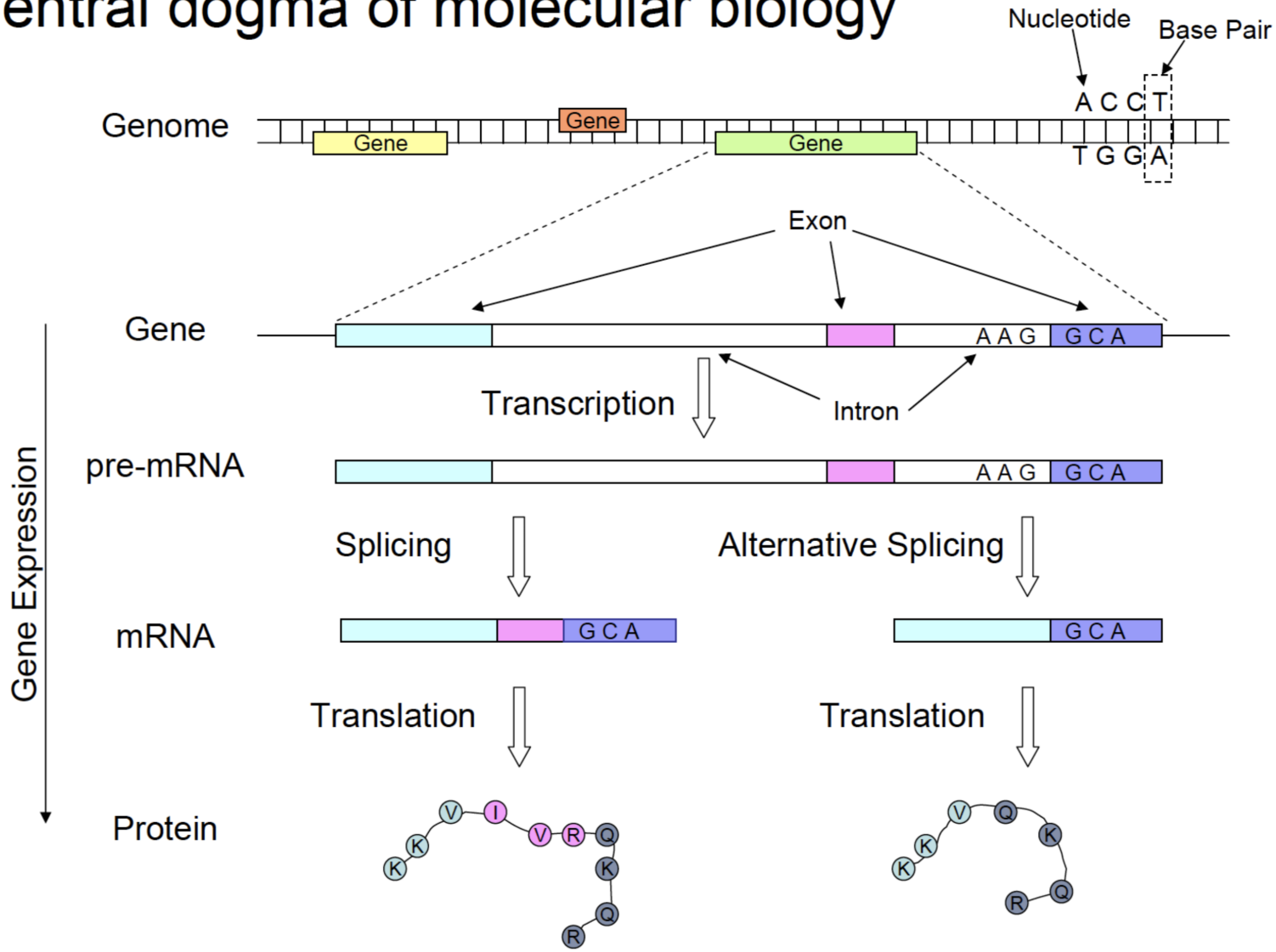
Acknowledgements

- Most of the material of today's course was taken or inspired from the work of:
  - Charlotte Soneson, University of Zurich;
  - Hubert Rehrauer, ETH Zurich;
  - Mark D Robinson, University of Zurich.

Introduction

- The focus moves from DNA to mRNA: RNA-seq data represents gene expression levels, i.e. the population of mRNA molecules.
- Most of the genome does not appear in RNA-seq data: in humans, genes only constitute approx. 3% of the genome.
- Typically, we study how the expression or splicing patterns change over two or more conditions, e.g. healthy vs. disease, via differential analyses.
- We can also study how different isoforms/trascripts, and hence proteins, are produced from a single gene.
- Changes in expression and splicing patterns are mostly studied related to diseases, but they are also ways of adapting and can be studied in the context of adaptation, for instance by comparing species or sub-populations of the same species in different environments.

Central dogma of molecular biology



92–94% of human genes undergo alternative splicing,

Hubert Rehrauer, ETH Zurich

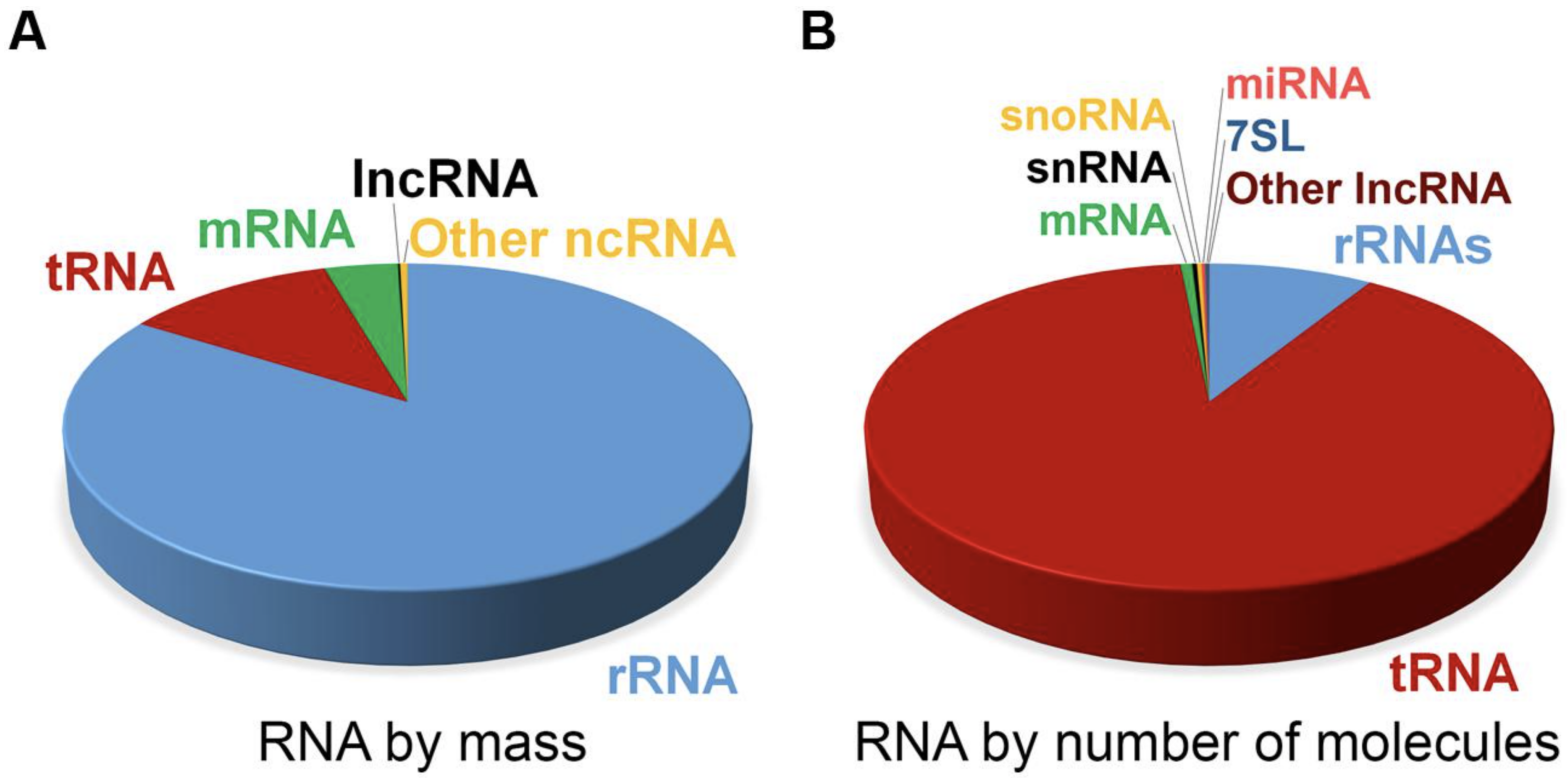


FIGURE 1. Estimate of RNA levels in a typical mammalian cell. Proportion of the various classes of RNA in mammalian somatic cells by total mass (A) and by absolute number of molecules (B). Total number of RNA molecules is estimated at roughly 10^7 per cell. Other ncRNAs in (A) include snRNA, snoRNA, and miRNA. Note that due to their relatively large sizes, rRNA, mRNA, and lncRNAs make up a larger proportion of the mass as compared to the overall number of molecules.

Palazzo & Lee, Front. Genet. 2015

Overview of sequencing technologies

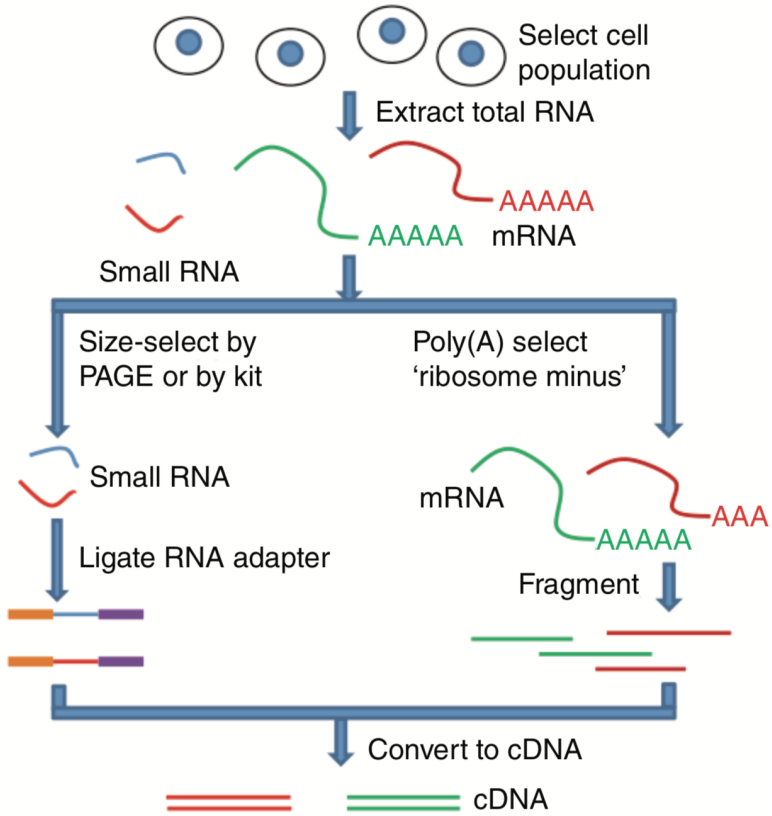
- First generation: microarrays (mostly replaced by RNA-seq).
- **Next generation sequencing or second generation: RNA-seq (mostly Illumina).**
- Third generation: long-reads (mostly PacBio and Oxford nanopore). It is growing in parallel, mostly useful for de-novo assembly, new transcript discoveries and transcript level analyses.
- Recent developments of RNA-seq: single-cell RNA-seq (scRNA-seq). Similar to "bulk RNA-seq" (standard RNA-seq) but with a single-cell resolution, i.e. observations are available for single cells instead of aggregating expression over many cells.

cDNA

- Note that RNA-seq technologies don't sequence mRNA directly: first, they reverse transcribe mRNA to obtain a string of complementary DNA (cDNA), then, they sequence the string of cDNA.
- Recently, Oxford Nanopore proposed a technology to sequence (for the 1st time) directly RNA, without reverse transcription or amplification steps:
  Garalde et al. (2018). Highly parallel direct RNA sequencing on an array of nanopores, Nature methods.

Illumina reads I

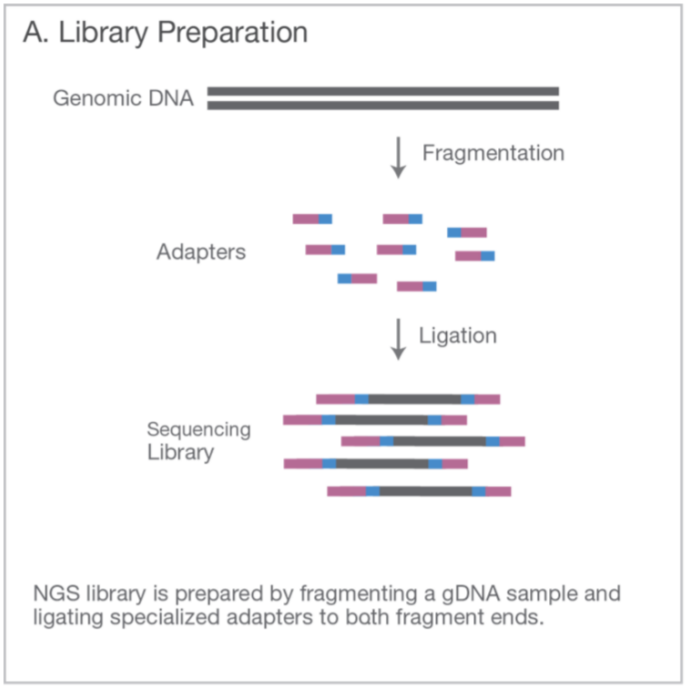
- The total RNA is extracted from a population of cells.
- The mRNAs are selected (typically via poly-A selection) and the other types of RNAs are filter out.
- The selected mRNAs are reverse transcribed into cDNAs (gDNA in the next slide).

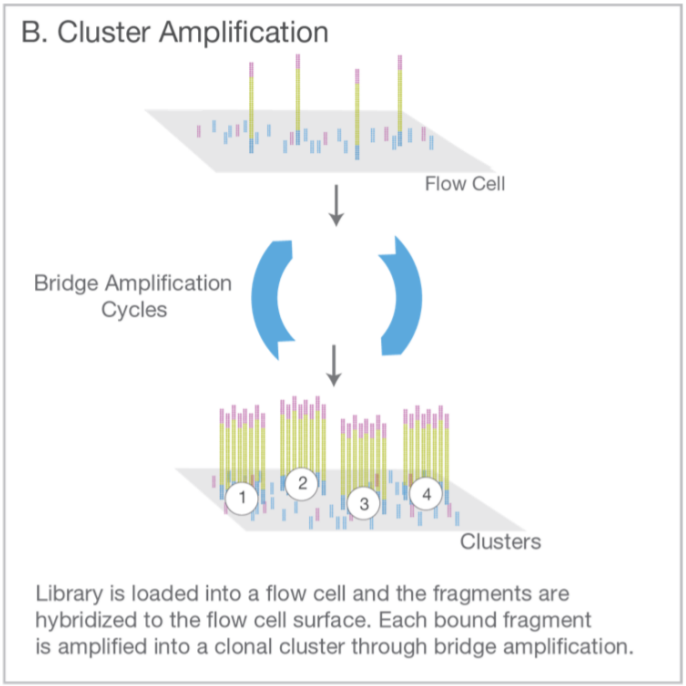


Zeng et al. (2012), Nature Immunology.

Illumina reads II

- The strings of cDNA are fragmented.
- Adapters are ligated.
- Fragments are hybridized on a flow cell and amplified via bridge amplification.

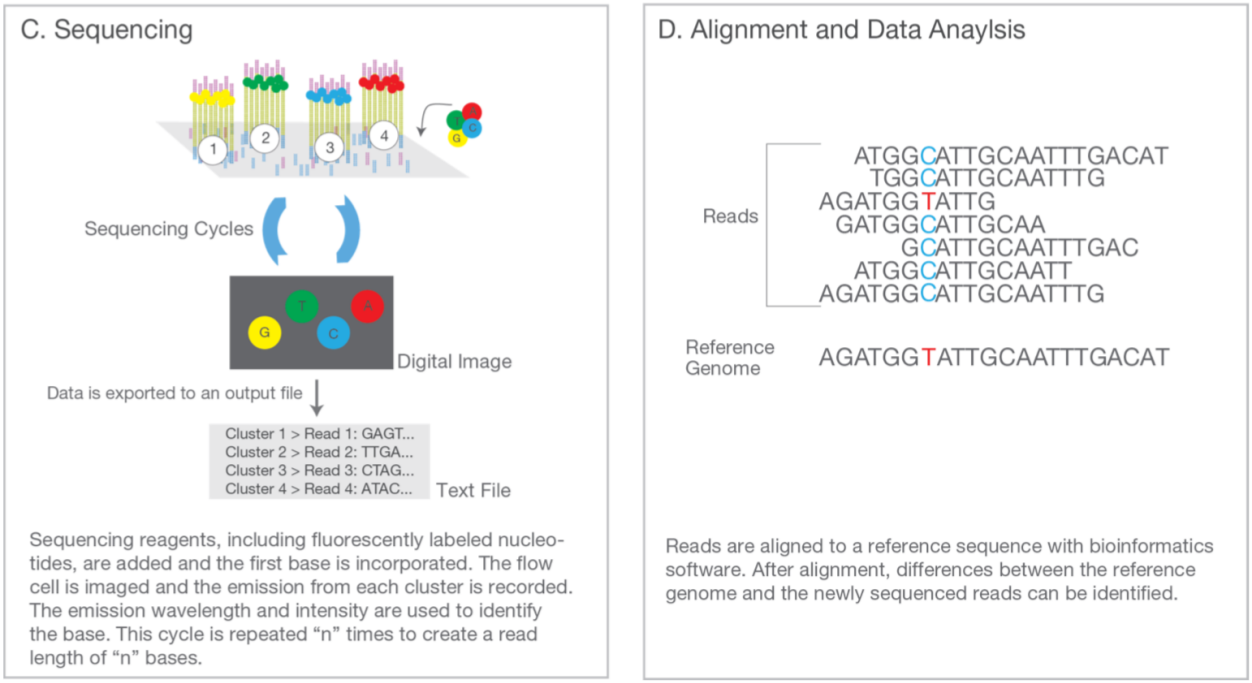




illumina, An introduction to Next-Generation Sequency Technology

Illumina reads III

- The fragments are fluorescently labelled: each colour corresponds to a base.
- The light each cluster emits is recorded n times to create a read of n bases.
- Reads are typically aligned to a reference...we'll see it later in detail.



illumina, An introduction to Next-Generation Sequency Technology

Single-end vs. paired-end reads

- Single-end:
  - reads are sequenced from one end only;
  - cheaper and faster;
  - reads are approx. 100 base pairs (bp) long.
- Paired-end (fragments):
  - reads are sequenced from both ends;
  - more expensive but the most popular protocol nowadays;
  - fragments are approx. 250-800 (bp) long, i.e. approx. 100 at each end plus a gap in between (of known length);
  - more accurate as it reduces the number of multi-mapping reads.

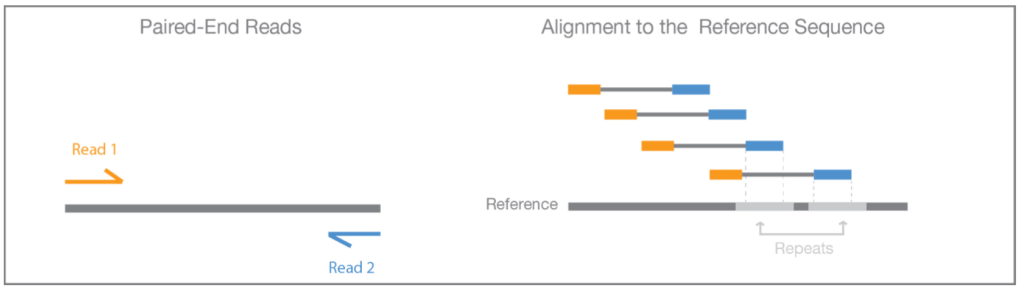


Figure 4: Paired-End Sequencing and Alignment—Paired-end sequencing enables both ends of the DNA fragment to be sequenced. Because the distance between each paired read is known, alignment algorithms can use this information to map the reads over repetitive regions more precisely. This results in better alignment of reads, especially across difficult-to-sequence, repetitive regions of the genome.

illumina, An introduction to Next-Generation Sequency Technology

Stranded vs. unstranded

- Unstranded:
  - we don't know what strand reads come from.
- Stranded:
  - we know what strand reads come from:
  - it decreases the number of ambiguous reads, when reads fall in regions overlapping between multiple genes.

FASTQ file

- The raw reads are stored in a FASTQ file.
- Each read is represented in 4 lines:
  - 1) a header for the read (starting with @);
  - 2) the sequence itself;
  - 3) a header for the quality (starting with +);
  - 4) a quality score as long as the sequence: each base of the sequence is associated to a score.

```
@HWI-ST1034:40:C08PJACXX:2:1101:20681:1994 1:N:0:ATCACG
CTCGNAGACTGGCAACTTGTTCTGGTTTACTGCACCTTCTTTTAAAGGCAGAAAGGC
+
CCCF#2ADHHHGHJJJIJJHIIIJJHIJJJJJJJJJBGIIJJJJJJJJJJJJJJIJ
```

Phred score

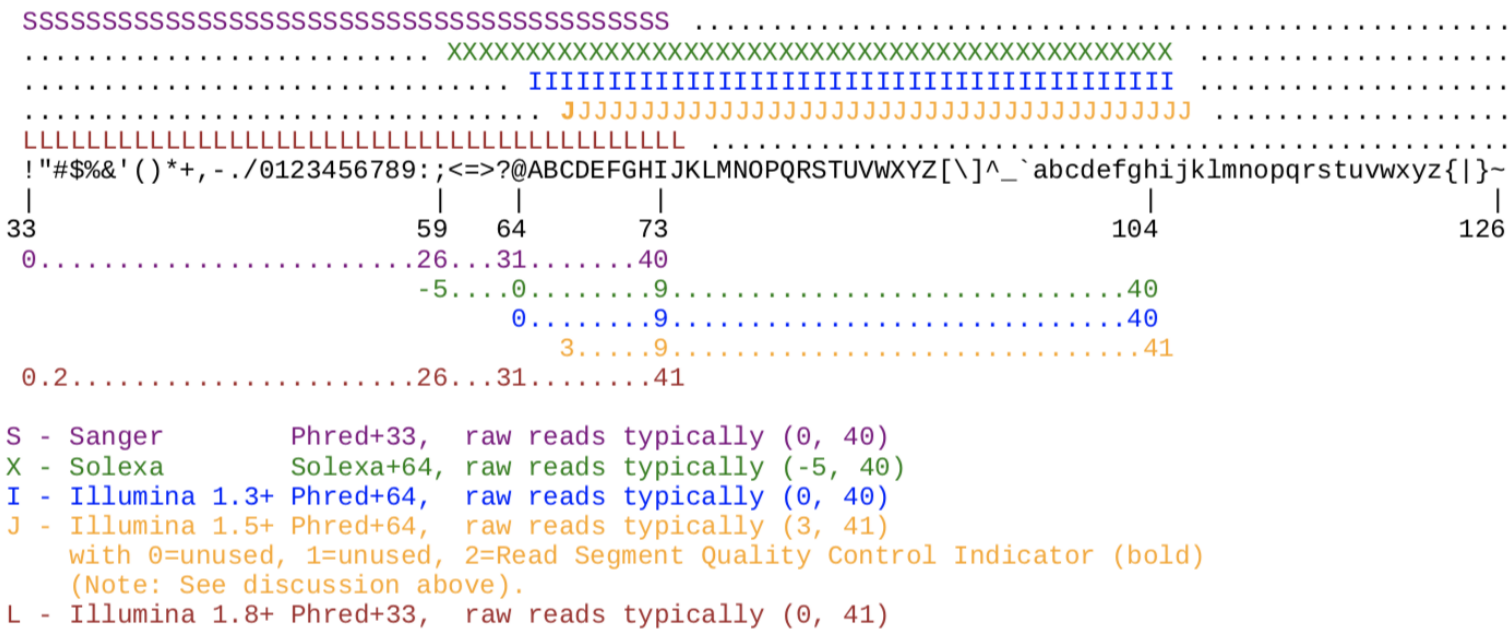
- The Phred score, Q, associates each base with the probability that the base is called incorrectly, P.
- Phred score: $Q = -10log_{10}P$.
- A Phred score of 30 or more is considered to be good enough: the error probability for a single base $< 0.1\%$.

Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%
60	1 in 1,000,000	99.9999%

Wikipedia

Phred score

- The association between the Phred score and the characters of the FASQT file changes with the technology.
- For recent Illumina technologies, capital letters have good quality: error probability for a single base $< 0.1\%$.



Wikipedia

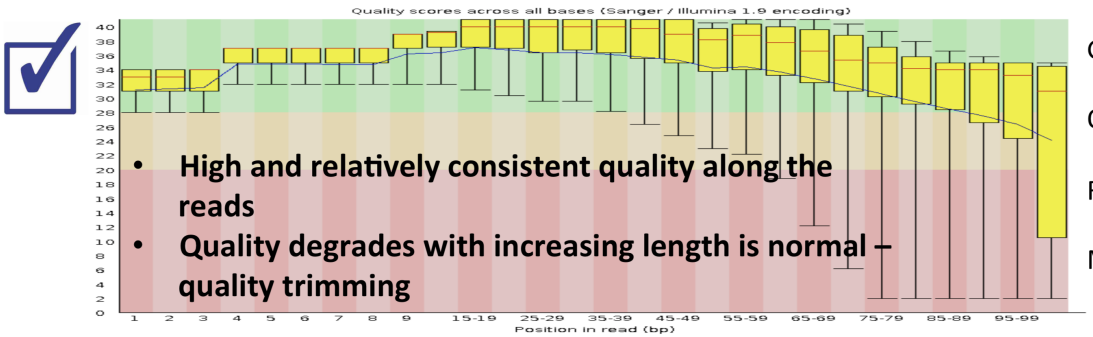
Assessing the quality of reads

- We can assess the quality of the raw reads, before aligning them to the genome/transcriptome, via **FastQC** (see the 1st tutorial).
- FastQC outputs one file per sample for single-end reads and two files for paired-end reads.
- To jointly visualize the output of FastQC for multiple samples, you can use **MultiQC** (see the 1st tutorial).
- In next slides we will inspect the output from FastQC in individual samples.
- Nice interpretation of FastQC output in:
  `https://www.youtube.com/watch?v=GnWSXwQeJ_U`
  How to Check the Quality of Illumina Sequencing Reads with FastQC (Part 2).
- Interpretation of MultiQC output in:
  `https://www.youtube.com/watch?v=qPbIlO_KWNO`.
  Using MultiQC Reports.

FastQC

Per base sequence quality - FastQC

- Range of quality values across all bases at each position

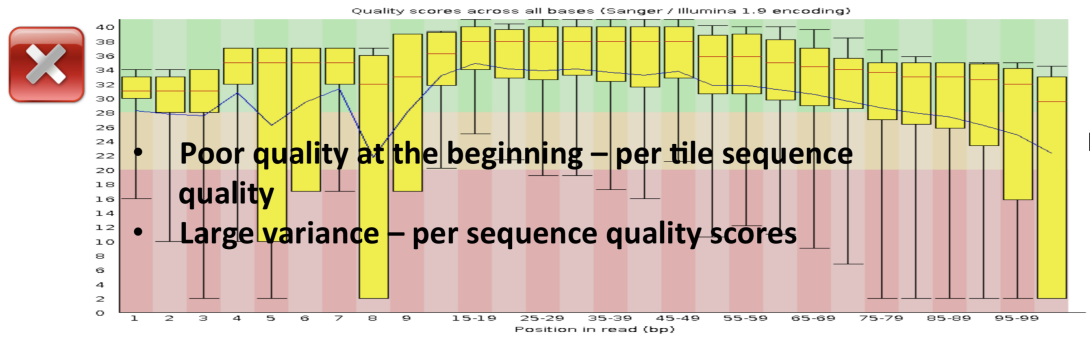


Green: >Q28, good

Orange: >Q20, reasonable

Red:<Q20, poor

Median > Q25



Median < Q20

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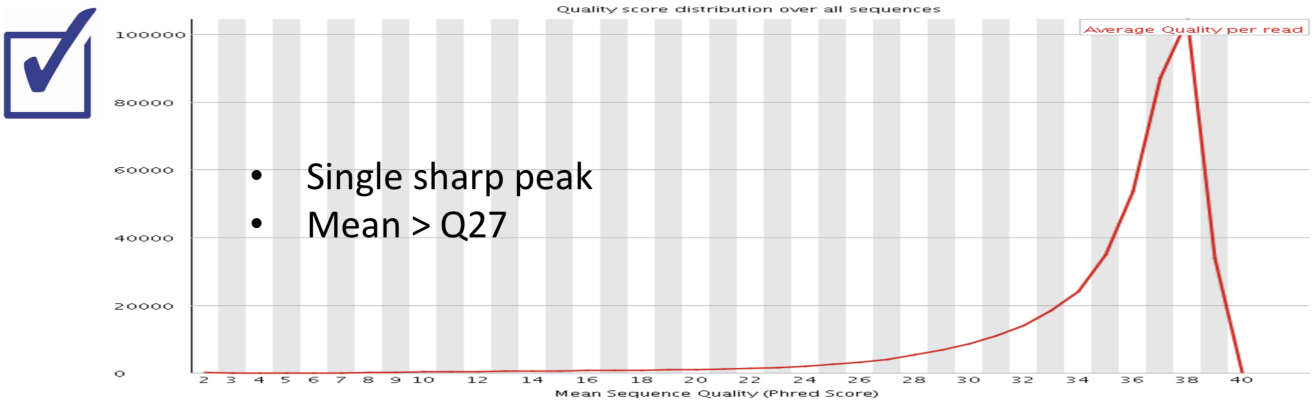
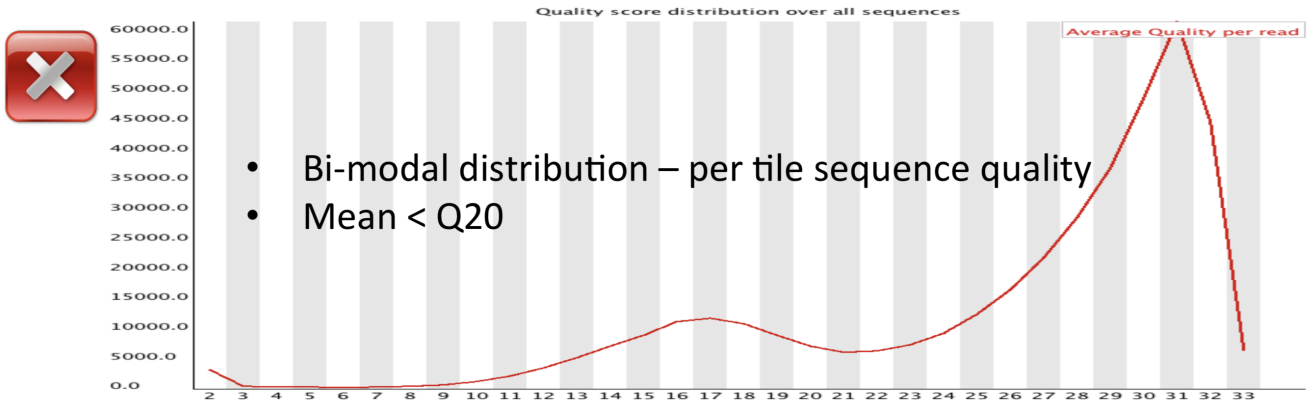
FastQC

Per sequence quality scores - FastQC

- Subset of sequences with universally low quality values





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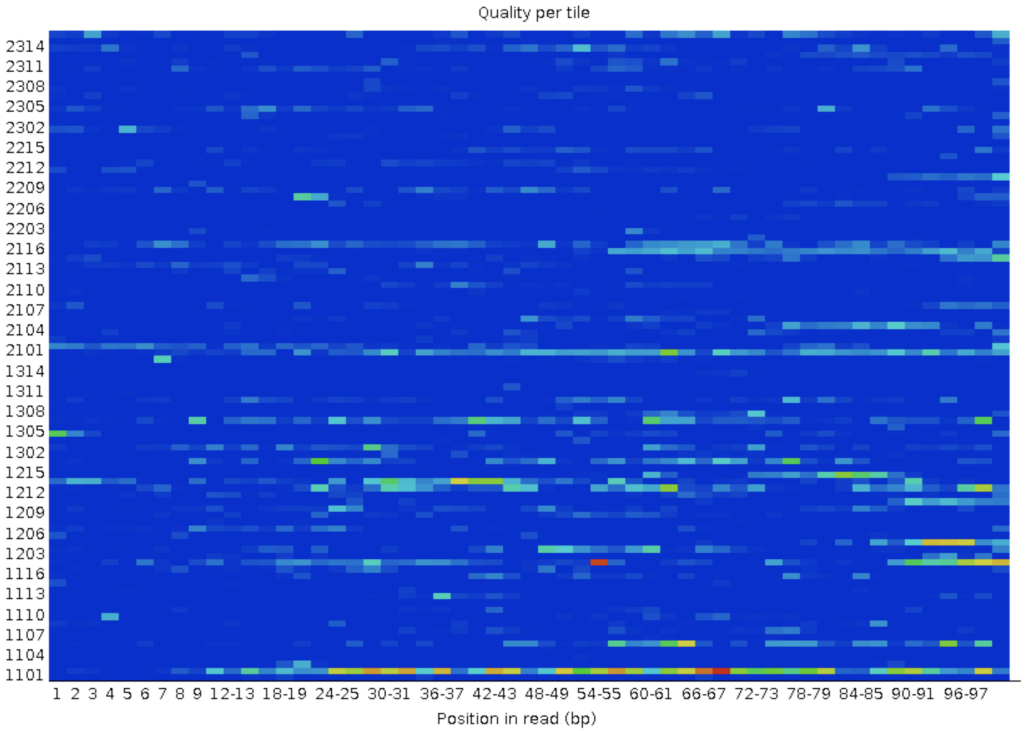
FastQC

Per tile sequence quality - FastQC

- Quality scores from each tile across all bases - loss in quality associated with only one part of the flowcell



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Deviation from average quality

Cold colors: ≥ average

Hotter color: worse quality

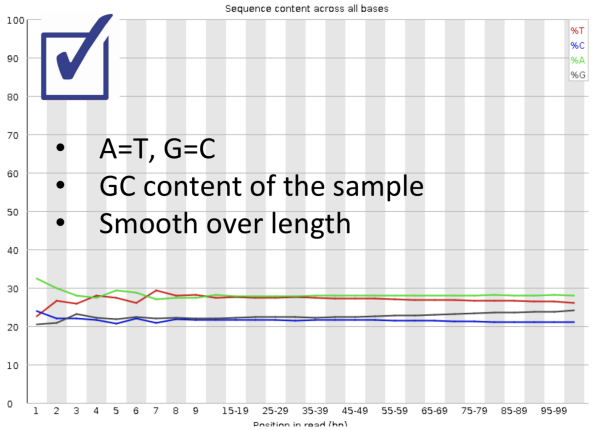
Good: universal blue

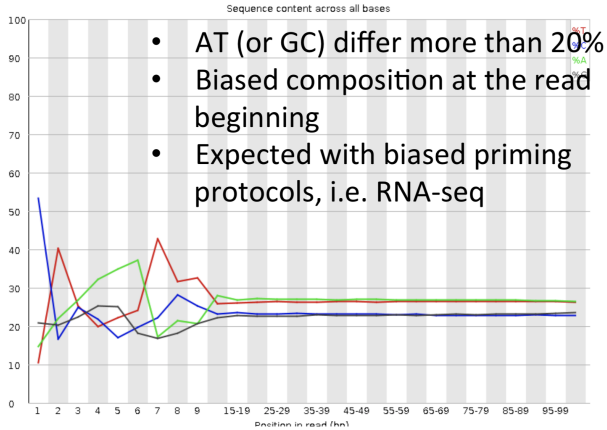
Failure: < average - 5

FastQC

Per base sequence content - FastQC

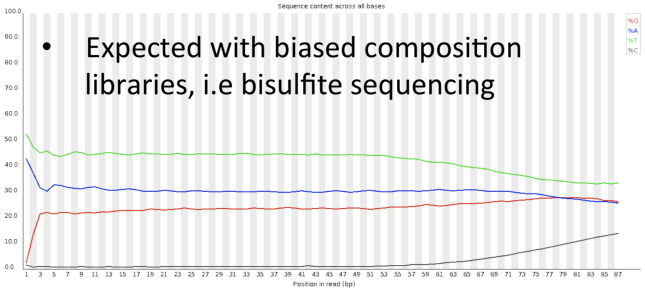
- The portion of A, T, G, and C at each position





Biases in Illumina transcriptome sequencing caused by random hexamer priming

Kasper D. Hansen[1,*], Steven E. Brenner[2] and Sandrine Dudoit[1,3]



Treatment of DNA with bisulfite converts cytosine to uracil, but leaves methylated cytosine unaffected. Therefore, DNA that has been treated with bisulfite retains only methylated cytosines.

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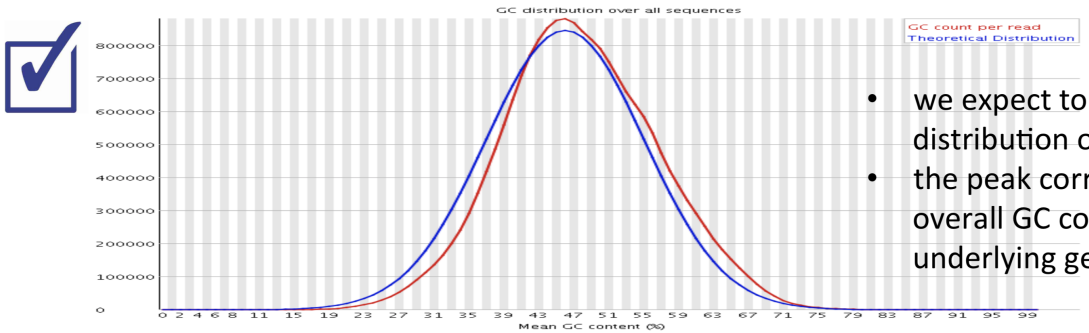
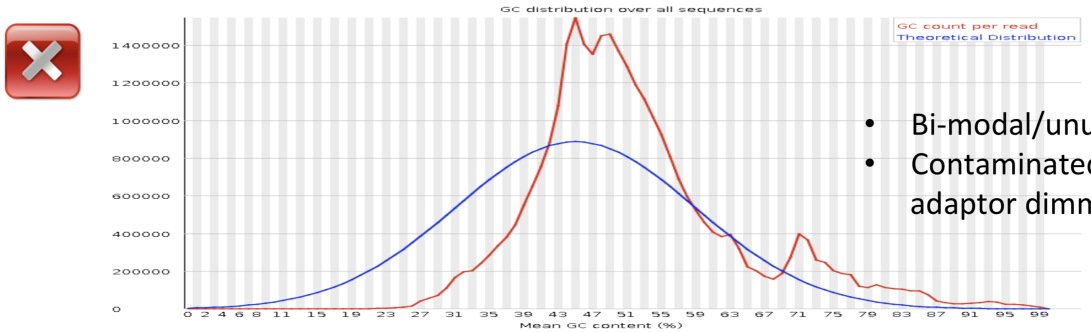
FastQC

Per sequence GC content - FastQC

- Distribution of average GC in all reads



- we expect to see a roughly normal distribution of GC content
- the peak corresponds to the overall GC content of the underlying genome



- Bi-modal/unusual distribution
- Contaminated/biased subset, i.e. adaptor dimmers, rRNA etc

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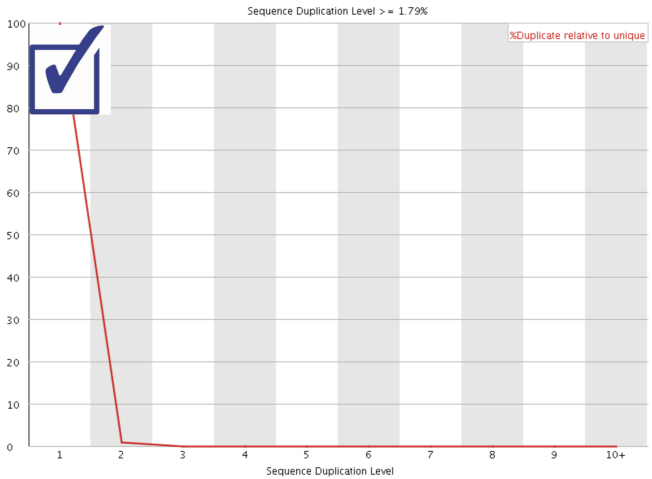
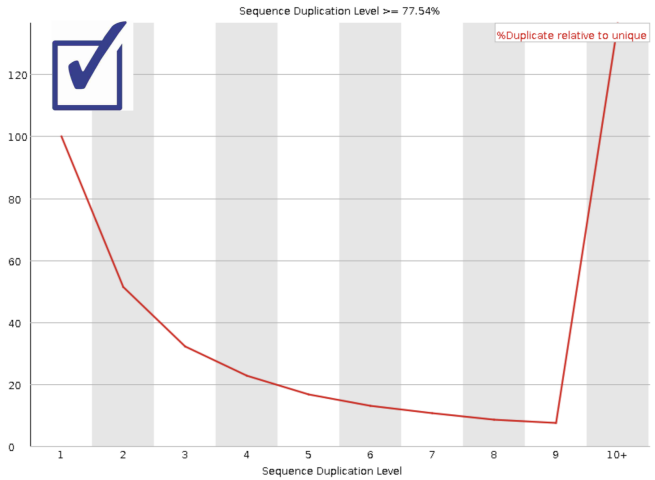
FastQC

Sequence duplication - FastQC

- Relative number of sequences with different degrees of duplication





- Essentially no duplication

High duplication levels:

- DNA-seq: PCR over amplification, too little input material
- Normal in RNA-seq: high expression

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FastQC

Overrepresented sequences - FastQC

- Sequences make up >0.1 % of the total
- Compare those with a contamination database for finding contamination (i.e. adaptor dimmers)

Overrepresented sequences

Sequence	Count	Percentage	Possible Source
GGAAGAGCACACGTCTGAACTCCAGTCACCAGATCATCTCGTATGCCGTC	75874	1.5613887498682963	TruSeq Adapter, Index 7 (100% over 50bp)
GGAAGAGCACACGTCTGAACTCCAGTCACCGATGTATCTCGTATGCCGTC	7636	0.15713900010536297	TruSeq Adapter, Index 2 (100% over 50bp)
GGAAGAGCACACGTCTGAACTCCAGTCACACAGTGATCTCGTATGCCGTC	7539	0.1551428656095248	TruSeq Adapter, Index 5 (100% over 50bp)
GGAAGAGCACACGTCTGAACTCCAGTCACGCCAATATCTCGTATGCCGTC	5117	0.10530123933199874	TruSeq Adapter, Index 6 (100% over 50bp)

- Can be normal and biologically meaningful
  - highly expressed transcripts
  - high copy number repeats
  - Less diverse library (amplicons)

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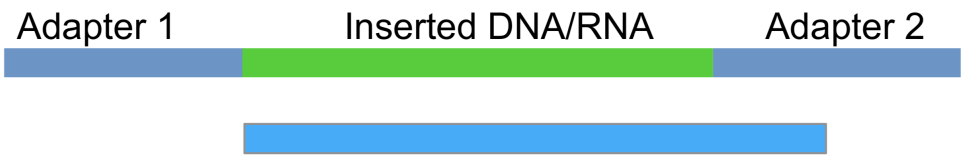
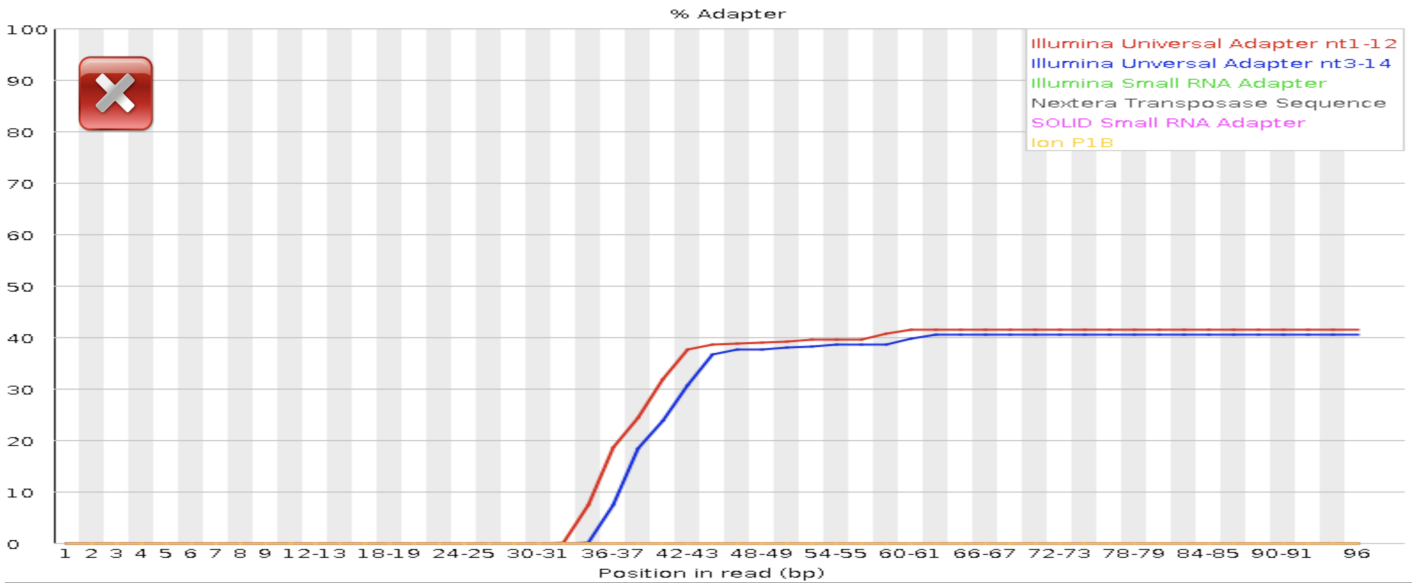
FastQC

Adapter Content - FastQC





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FastQC

Data preprocessing common tasks

1. Trimming: remove bad bases from (end(s) of) reads
   - Adaptor sequence
   - Low quality bases
2. Filtering: remove bad reads
   - Low quality reads
   - Contaminating sequences
   - Low complexity reads (repeats)
   - Short (<20bp) reads – they slow down mapping software

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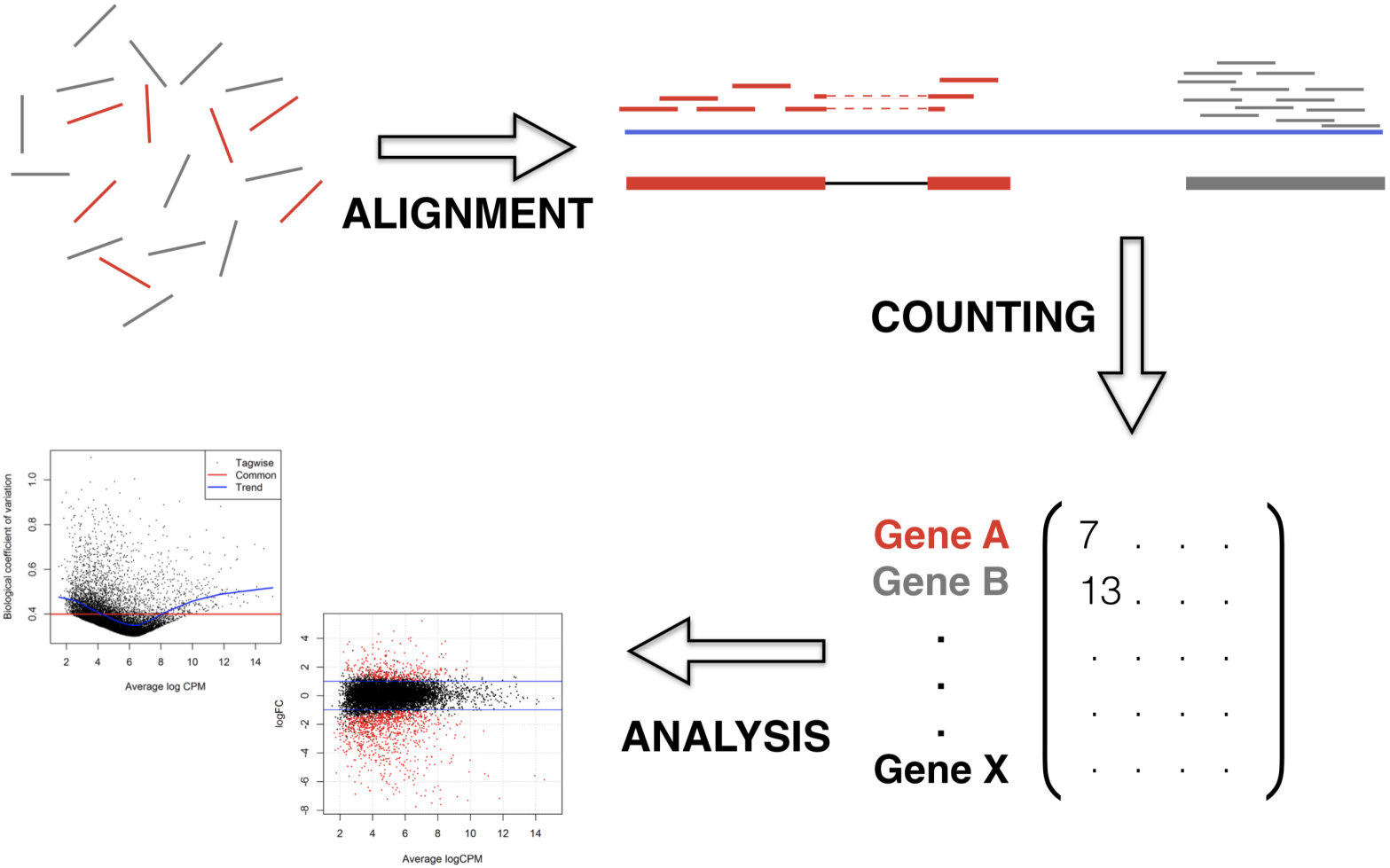
- All reads have increasing error rates towards the end of the read.
- If needed, we can use **trimmomatic** to trim reads (we will not use it in the tutorials).
- We can cut the end of the reads when they fall below a minimum quality or we can trim after a fixed number of bases.

Alignment



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Genome alignment

- In most analyses, after checking the quality of our reads, we align them to a reference.
- We can use a full aligner to align reads to a reference genome (**STAR**, **TopHat**, ...).
- Full aligners attempt to align the entire read to a reference.
- To align our reads to the genome we need:
  - a reference genome (DNA), usually in a fasta format;
  - a gene transfer format (GTF) file which contains the location of genes on the reference genome.
- Both can be downloaded from `http://www.ensembl.org/info/data/ftp/index.html` under the DNA and Gene sets columns.

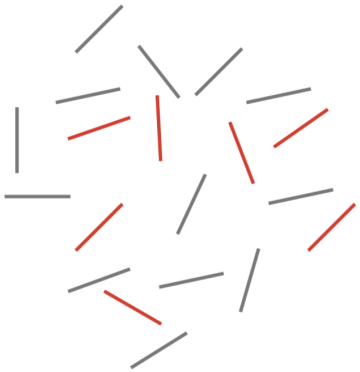
Transcriptome alignment

- Alternatively, we can align our reads directly to a reference transcriptome.
- Most transcript aligners are pseudo/quasi aligners (**Salmon**, **kallisto**, ...).
- Pseudo/quasi aligners don't align the full reads, instead they use a low cost pseudo alignment:
  - from each read they create many k-mers (substrings of the read of k base pairs);
  - they map the k-mers to the reference transcriptome and check their compatibility with the transcripts.
- To align our reads to the transcriptome we need a reference transcriptome (cDNA) alone, which can be downloaded from `http://www.ensembl.org/info/data/ftp/index.html` under the cDNA column.

Considerations on the alignment

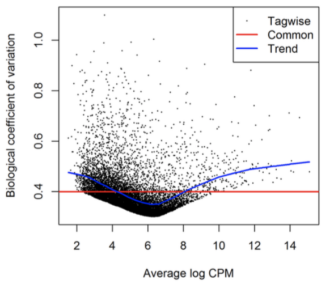
- Aligners take into account mismatches with respect to the reference, due to sequencing error (approx. 1/500) or mutations (approx. 1/10,000 in humans).
- A read could align in multiple positions of the reference genome/transcriptome, with the same or similar alignment scores. The output is represented by both unique aligning reads and multi mapping reads. Some reads (a minority) remain unmapped because they don't align well enough to any location of the genome.
- Alignment is an optimization problem: for every read, it looks for the alignment with the highest score.
- Aligners do not run a complete search of all possible alignments of all read, the optimization is heuristic, not optimal (yet very sophisticated).

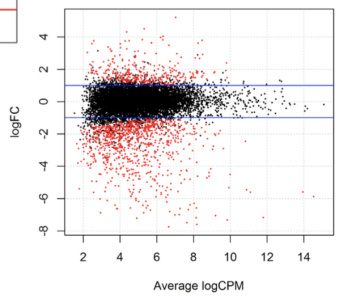
Counting



ALIGNMENT

COUNTING





ANALYSIS

Gene A
Gene B
.
.
.
Gene X

$$\begin{pmatrix} 7 & \cdot & \cdot & \cdot \\ 13 & \cdot & \cdot & \cdot \\ & \cdot & \cdot & \cdot \\ & \cdot & \cdot & \cdot \\ & \cdot & \cdot & \cdot \end{pmatrix}$$

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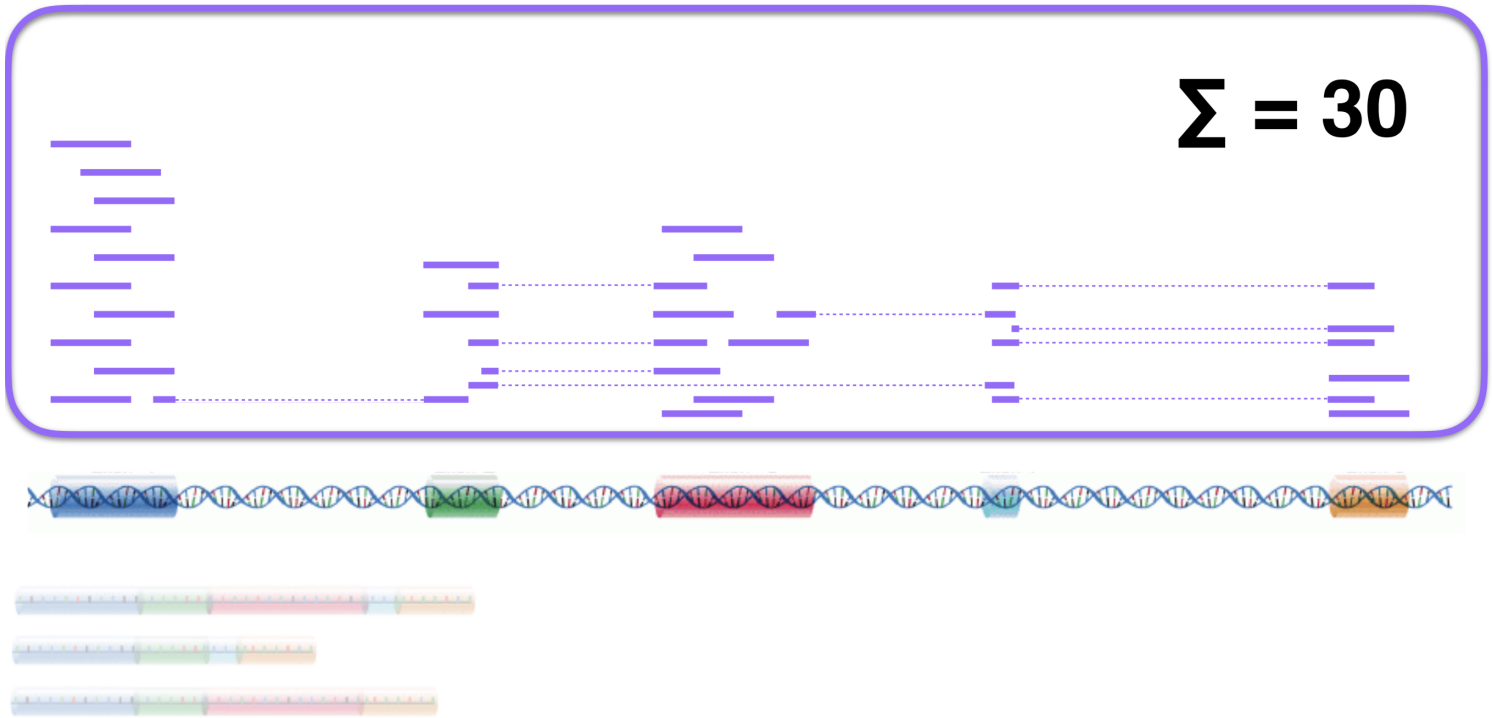
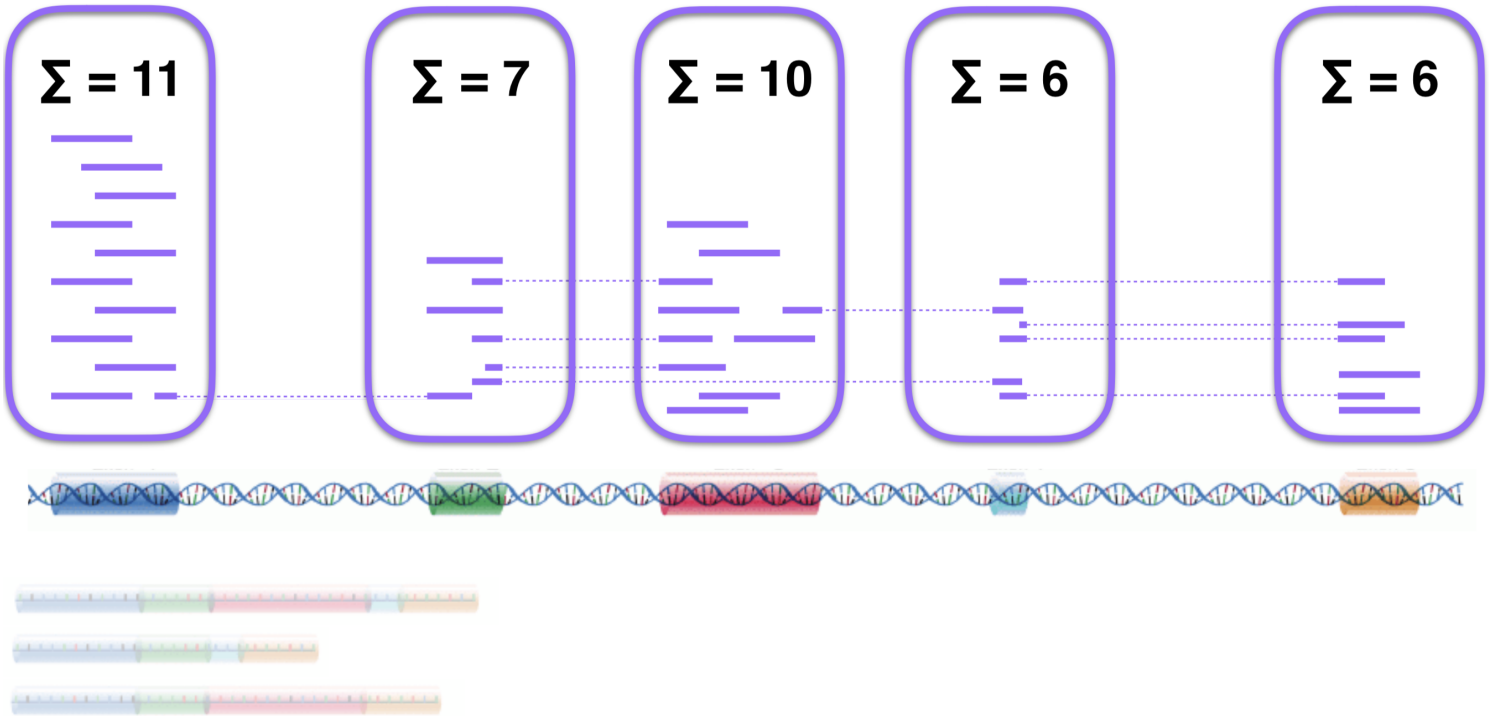
Quantification with genome aligners: gene counts

- Once the reads are aligned to the genome, we need to sort them (for STAR use the *SortedByCoordinate* option).
- We can then count how many reads overlap each gene.
- **STAR** and **TopHat** are the most popular genome aligners (we will see STAR in the 1st tutorial).
- Remember that RNA-seq reads only map to exons!



Quantification with genome aligners: exon counts

- The counts of each individual transcript cannot be obtained due to the overlapping regions between transcripts.
- To study the transcript, the attention sometimes shifts towards the exons, for which we can observe the counts.



Charlotte Soneson, UZH

Quantification with genome aligners: junction counts

- An alternative is to only consider junction counts: counts of reads that span over two exons (the ones with the dotted lines in the previous image).
- Pros:
  - we know what exons reads connect;
  - useful for discovering new transcripts with non annotated exon junctions (STAR also outputs non-annotated junction reads).
- Cons:
  - we still miss what transcript each read maps to (there could be > 1 transcripts associated to the two exons the reads spans over);
  - we only use a sub-set of the data.
- STAR outputs junction counts in the SJ.out.tab file.

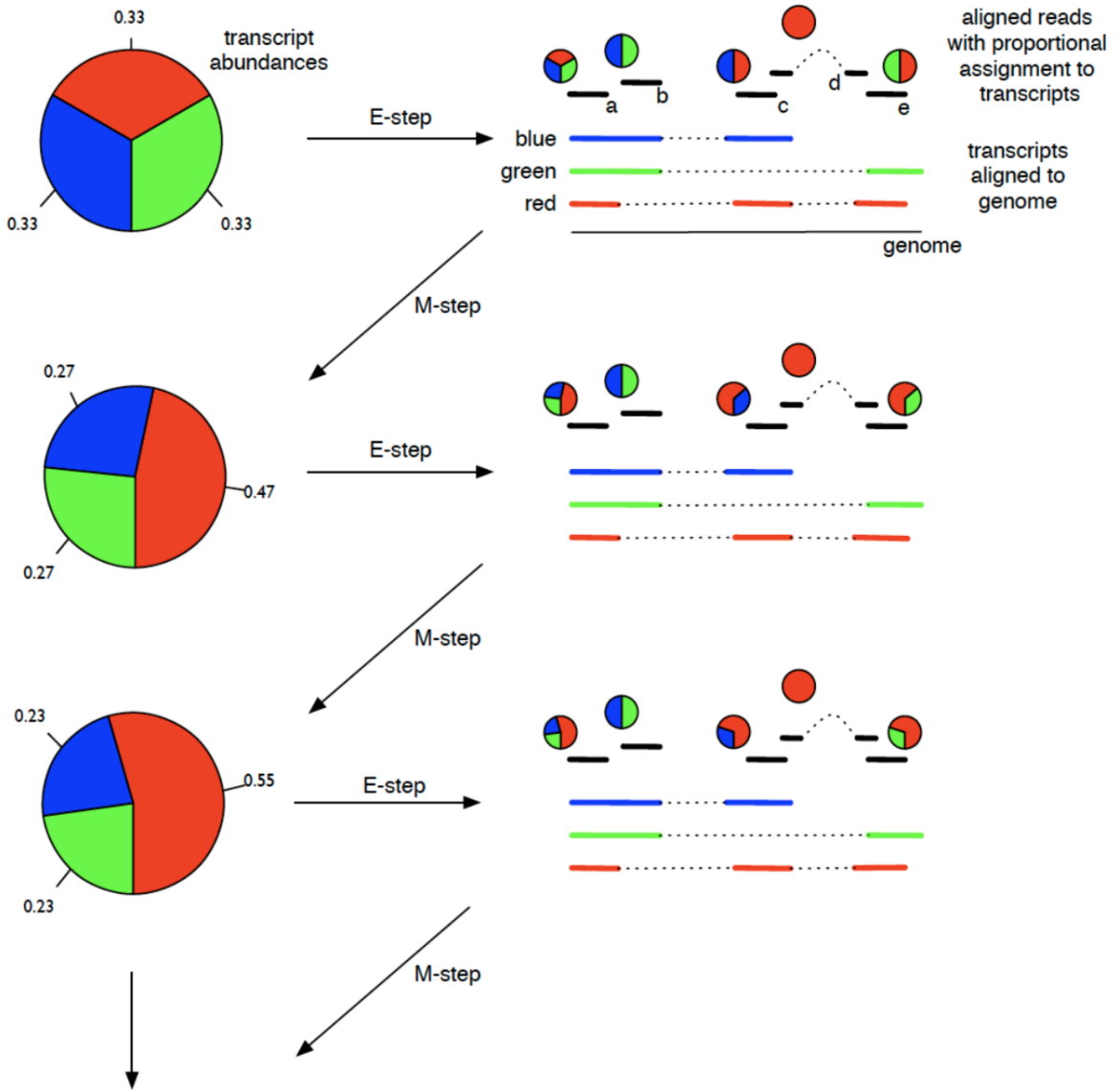
Quantification with transcriptome aligners

- Reads can be mapped directly to the transcriptome, skipping genome mapping.
- A well annotated transcriptome is required: if a transcript is missing in the reference, the reads coming from that transcript will either be mapped to other transcripts (if compatible), or remain unmapped.
- Multi-mapping reads are a lot more frequent when mapping to the transcriptome: for every exon, there can be multiple transcripts that contain it.
- Pseudo aligners don't actually map the full reads, but k-mers built from the reads.
- **Salmon** and **kallisto** are the most popular transcript aligners (we will use Salmon in the 1st tutorial).

Quantification with transcriptome aligners

- After mapping reads to transcripts, an expectation-maximization (EM) algorithm is used to estimate the expected number of reads mapping each transcript.
- The algorithm outputs estimated transcript level counts, from which we can easily obtain gene level counts.
- Note that these numbers are estimates, not real counts.
- Salmon and kallisto provide bootstrap replicates to measure the uncertainty in the estimated counts.

Quantification with transcriptome aligners



Reference-free assembly

- An alternative to genome and transcript mapping is to use a reference-free approach and assemble the reads into transcripts, build a reference transcriptome and then map them back to the reference.
- Very challenging to reconstruct full transcripts from Illumina short reads, even if paired-end reads.
- **Trinity** is the most popular tool for reference-free assembly: Grabherr et al. (2011). Trinity: reconstructing a full-length transcriptome without a genome from RNA-Seq data, Nature Biotechnology.
- Rarely used in well studied organisms.

Importing the counts

- For each sample, the main output of the quantification will be a matrix of genes/transcripts and respective counts, i.e. the number of reads mapping to a gene/transcript.
- Remember that counts are discrete, not continuous.
- The estimated counts from Salmon or kallisto are continuous though because they are expected counts.
- We can import the counts from STAR/TopHat output (stored in a .bam or .sam file) in R via featureCounts (**Rsubread**), see the 1st tutorial.
- We can import the counts from Salmon/kallisto output (stored in a .bam or .sam file) in R via tximport (**tximport**), see the 1st tutorial.

References

- Overview paper: Conesa at el. (2016). A survey of best practices for RNA-seq data analysis, Genome Biology.
- **STAR**: Dobin et al. (2013). STAR: ultrafast universal RNA-seq aligner, Bioinformatics.
- **TopHat**: Trapnell et al. (2009). TopHat: discovering splice junctions with RNA-Seq, Bioinformatics.
- **Salmon**: Patro et al. (20017). Salmon provides fast and bias-aware quantification of transcript expression, Nature methods.
- **kallisto**: Bray et al. (2016). Near-optimal probabilistic RNA-seq quantification, Nature biotechnology.
- **Trinity**: Grabherr et al. (2011). Trinity: reconstructing a full-length transcriptome without a genome from RNA-Seq data, Nature Biotechnology.
- **tximport**: Soneson et al. (2016). Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences, F1000Research.

Questions?