Introduction to RNA-seq

Overview & Alignment

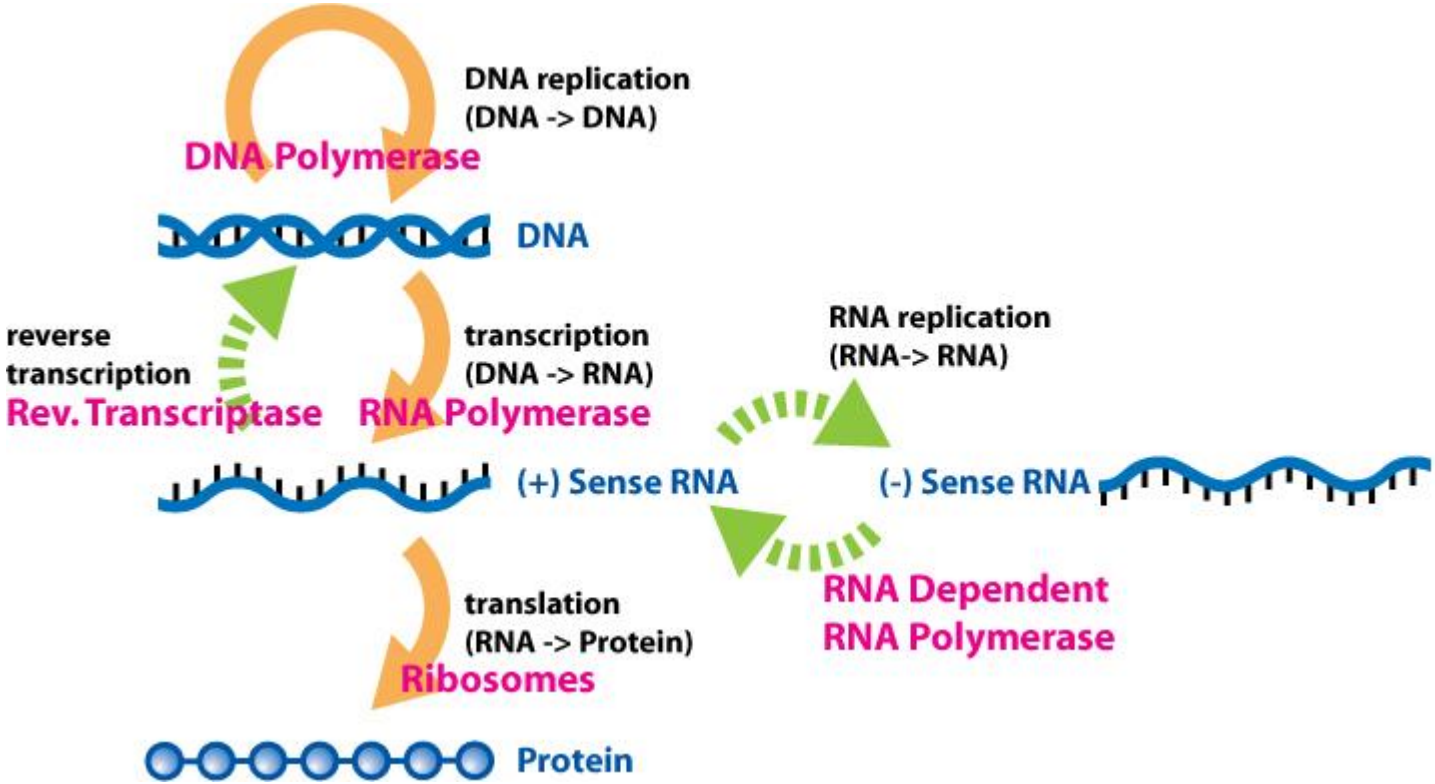
Why do RNA-seq?

- Mapping from genotype-to-phenotype
- Annotation of genes and transcripts
- Tissue biology
- Gene regulatory networks

Count Matrix

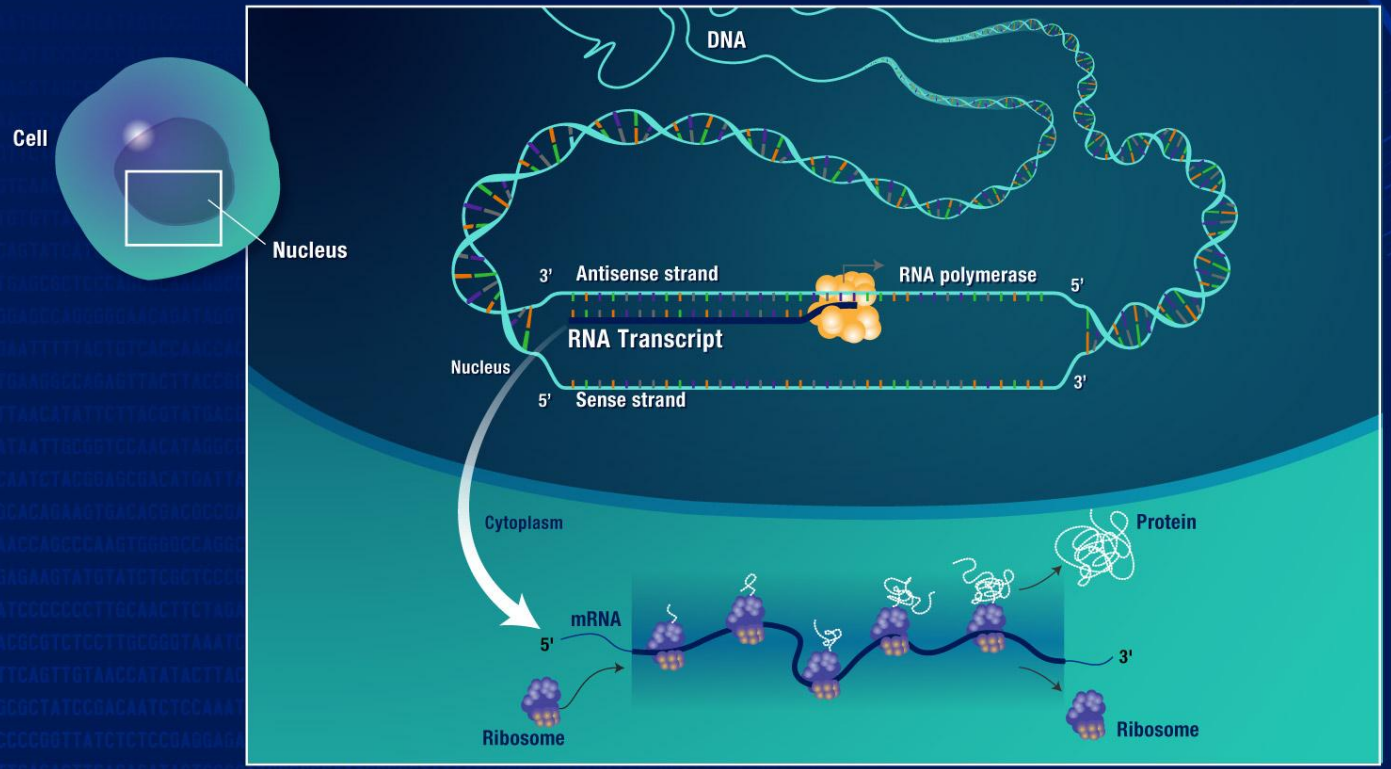
	WT1	**treatment1**	...
gene1	50	15	
gene2	20	87	
...			

Central dogma



Transcriptome

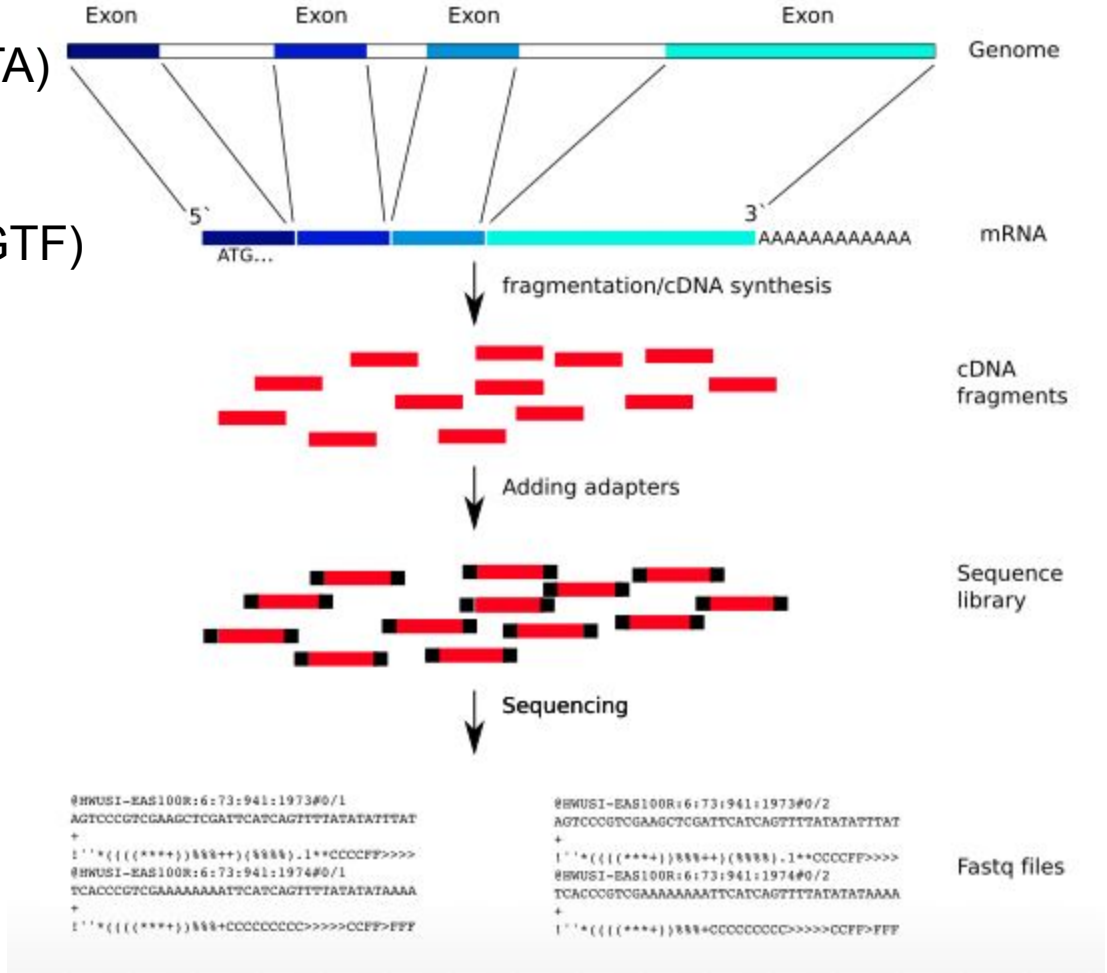
NHGRI FACT SHEETS
genome.gov



NIH National Human Genome Research Institute

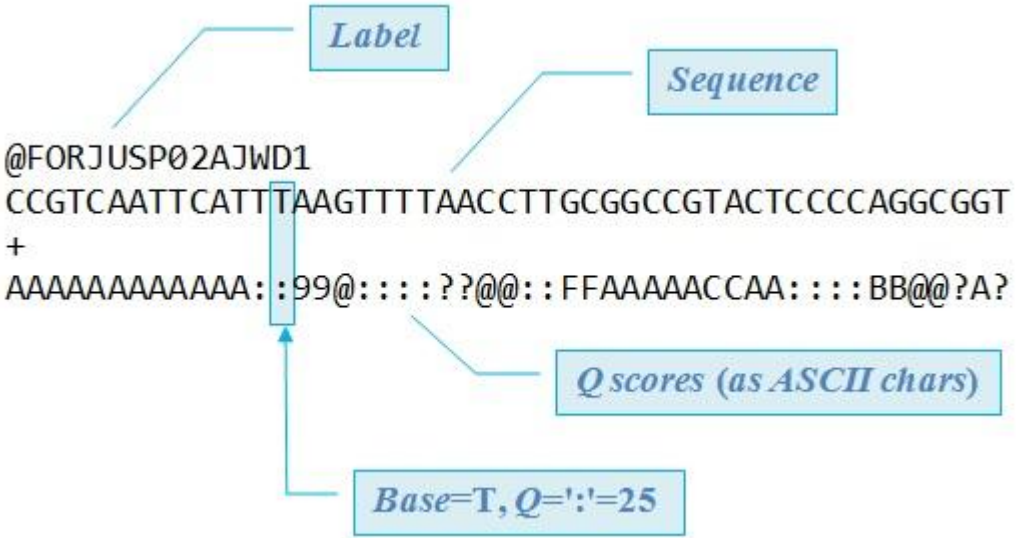
Reference (FASTA)

Annotation (GTF)



FASTQ format

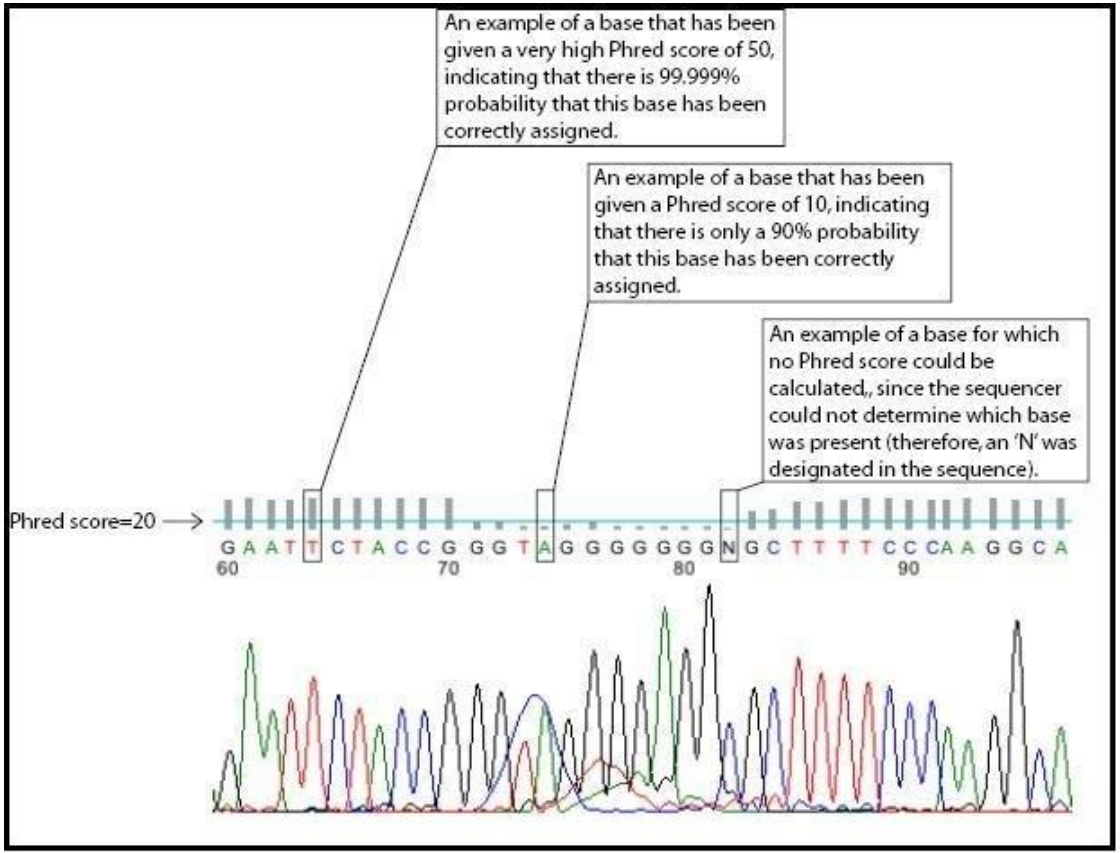
1. Read ID + properties
2. Read sequence
3. (space)
4. Base qualities



Phred quality score

$Q = -10 \cdot \log_{10}(P)$

- P is the probability of incorrect base call
- Q is the measure for probability of correct base call



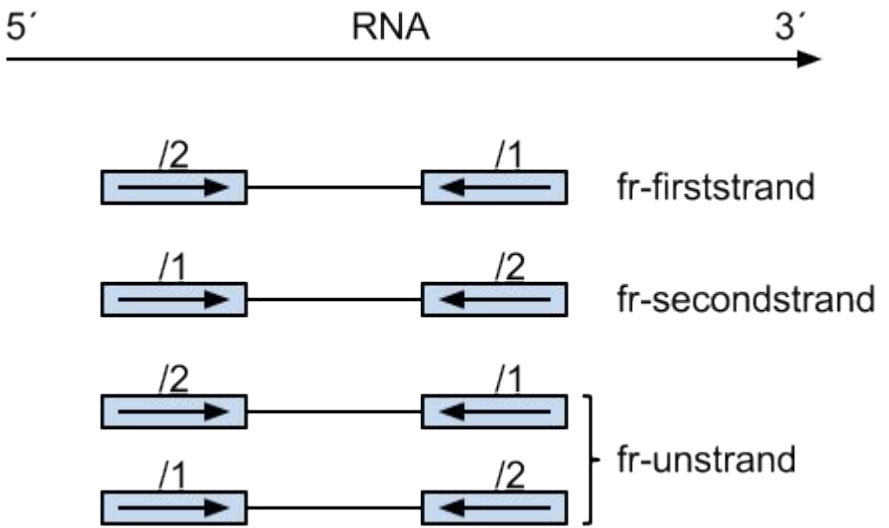
Strandness: single-end vs paired-end

Single-end

- F: sense orientation
- R: antisense orientation

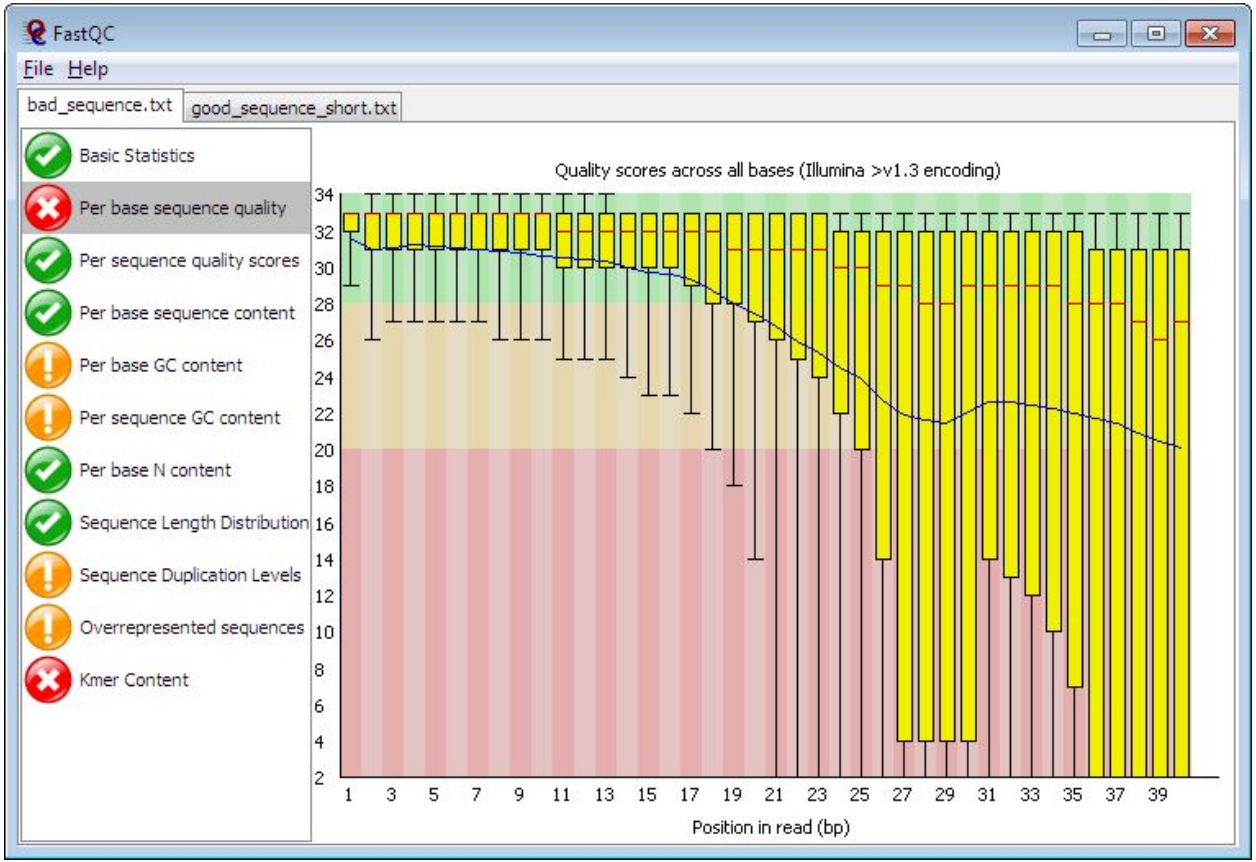
Paired-end

- RF: first read is R, second F
- FR: first read is F, second R



Quality control

- FastQC offers a visual check on raw sequence data
- Per base sequence quality plot
- Distribution of quality scores at each read position across all reads



Read filtering

Trimmomatic and SOAPnuke are commonly used for filtering poor quality reads

- Sequence adapters
- Low quality base rate
- Unknown (N) base rate

Alignment

Types of alignment

Reference-based analysis

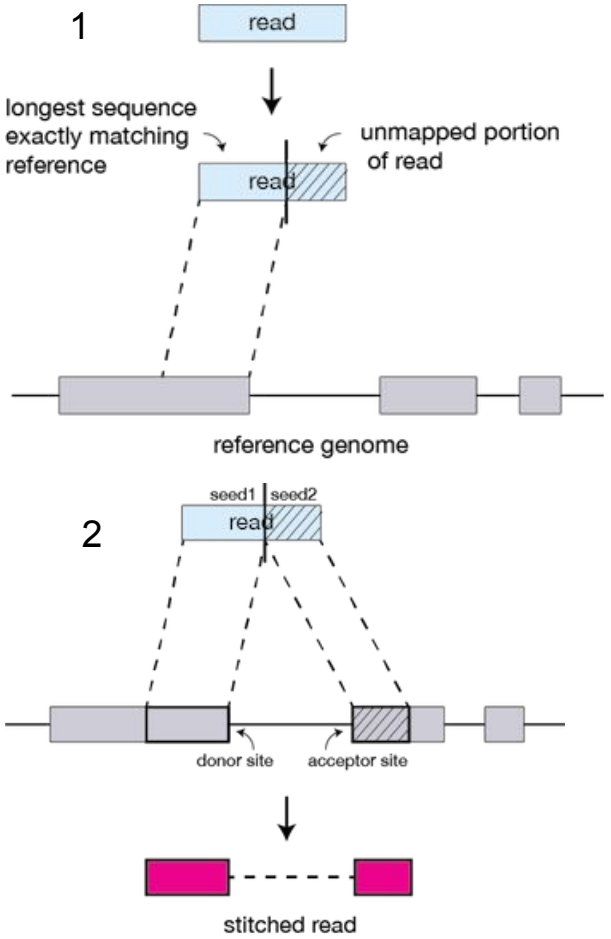
- Mapping to a reference genome
  - Aligners: find the location of reads on the genome
    e.g. STAR and HISAT2
  - “pseudo-alignment” quantifiers: find transcript counts based on approximate location
    e.g. Salmon and Kallisto

de-novo assembly

- Building a reference transcriptome
- Contigs resemble RNA transcripts

STAR

1. Seed searching: find longest sequence that matches 1+ locations on reference genome
2. Clustering, stitching, and scoring: seeds are grouped together by anchor and then by score (mismatch, indel, gap)



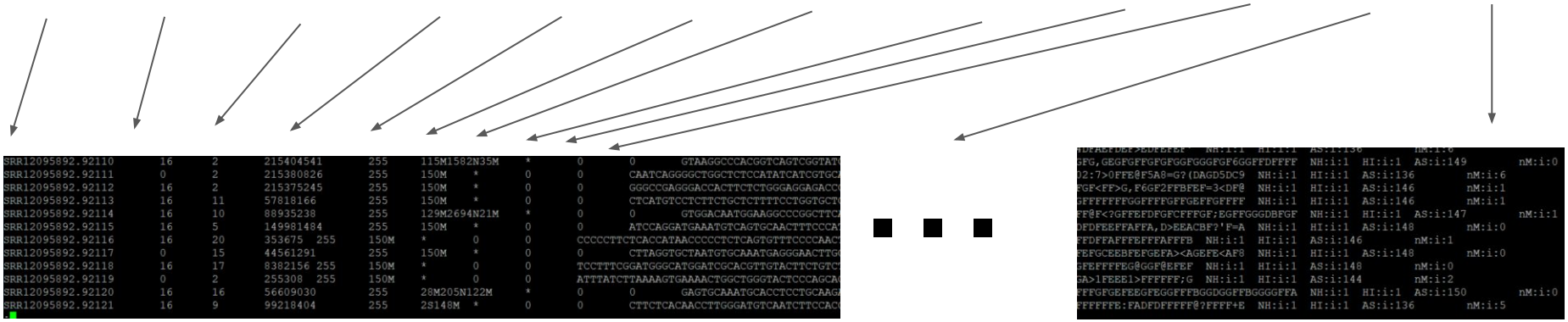
Sample STAR command

```
STAR --genomeDir genome_index \
--runThreadN 4 \
--readFilesIn sample1.fastq \
--quantMode GeneCounts \
--outFileNamePrefix STAR/aligned \
--outSAMattributes Standard \
```

Sample STAR output

SAM file format

QNAME	FLAG	RNAME	POS	MAPQ	CIGAR	RNEXT	RNEXT	PNEXT	TLEN	SEQ	QUAL



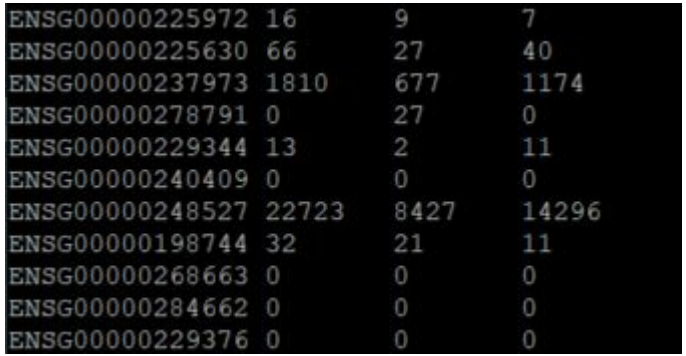
Summary statistics

- Uniquely mapped reads
- Mismatch rate
- Deletion rate
- Insertion rate

Mapping speed, Million of reads per hour	1688.14
Number of input reads	45017142
Average input read length	150
UNIQUE READS:	
Uniquely mapped reads number	43729271
Uniquely mapped reads %	97.14%
Average mapped length	149.50
Number of splices: Total	23787911
Number of splices: Annotated (sjdb)	23610473
Number of splices: GT/AG	23580970
Number of splices: GC/AG	134444
Number of splices: AT/AC	17558
Number of splices: Non-canonical	54939
Mismatch rate per base, %	0.39%
Deletion rate per base	0.01%
Deletion average length	1.85
Insertion rate per base	0.01%
Insertion average length	1.76
MULTI-MAPPING READS:	
Number of reads mapped to multiple loci	1005151
% of reads mapped to multiple loci	2.23%
Number of reads mapped to too many loci	8267
% of reads mapped to too many loci	0.02%
UNMAPPED READS:	
Number of reads unmapped: too many mismatches	0
% of reads unmapped: too many mismatches	0.00%
Number of reads unmapped: too short	268027
% of reads unmapped: too short	0.60%
Number of reads unmapped: other	6426
% of reads unmapped: other	0.01%

Gene counts

- STAR can generate gene counts
  - Have to strip genes and left-most counts for each aligned FASTQ file and then merge into count matrix
- The more general approach is to use featureCounts from Subread
  - SAM or BAM files as input



ENSG00000225972	16	9	7
ENSG00000225630	66	27	40
ENSG00000237973	1810	677	1174
ENSG00000278791	0	27	0
ENSG00000229344	13	2	11
ENSG00000240409	0	0	0
ENSG00000248527	22723	8427	14296
ENSG00000198744	32	21	11
ENSG00000268663	0	0	0
ENSG00000284662	0	0	0
ENSG00000229376	0	0	0

Other resources

- RNA-seqlopedia
  https://rnaseq.uoregon.edu

- Gene ontology reference
  http://geneontology.org