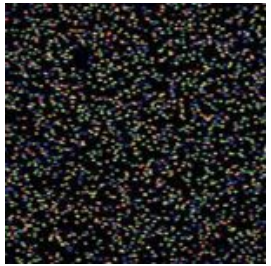
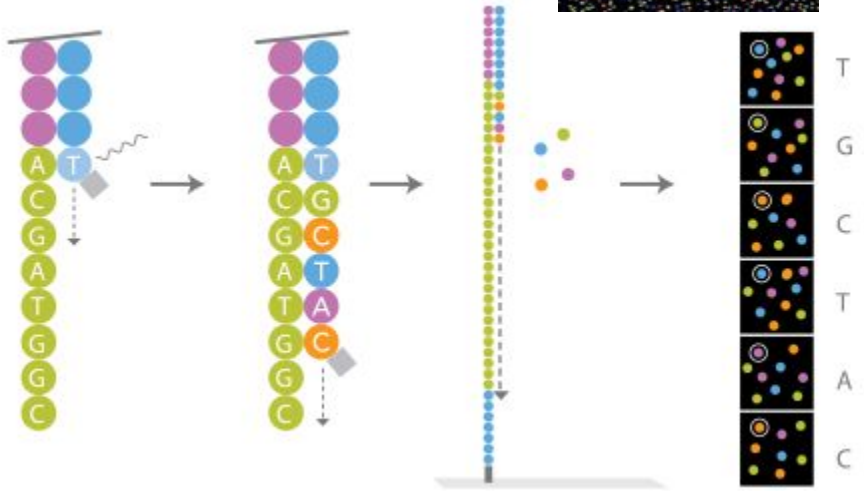
RNA-seq

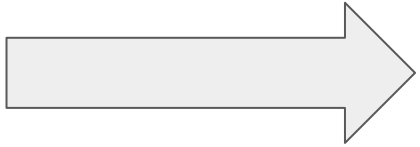
From sequencing to counts

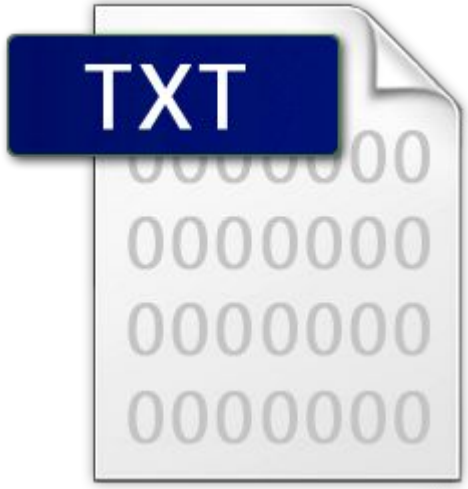
Sequence Data: Raw Reads

Demultiplexing: from image to fastq







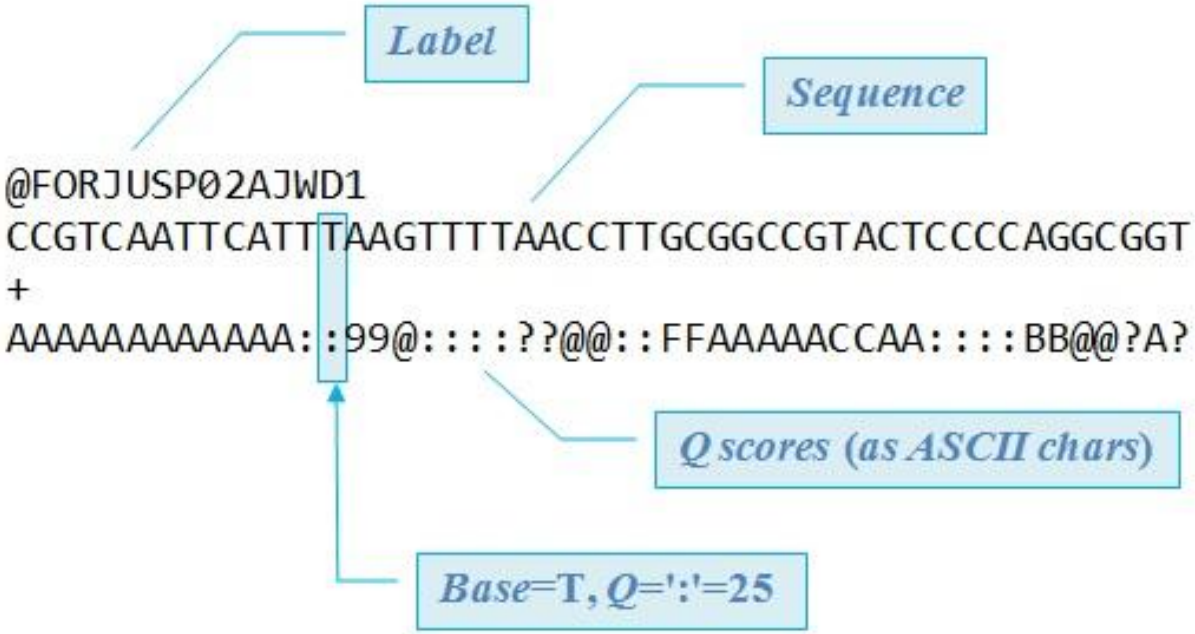


Fastq

Fastq is a text based format containing the reads (sequences) that came from the machine.

Often this file is several MB to GB in size, therefore it is gzipped (.fastq.gz).

Fastq: File format

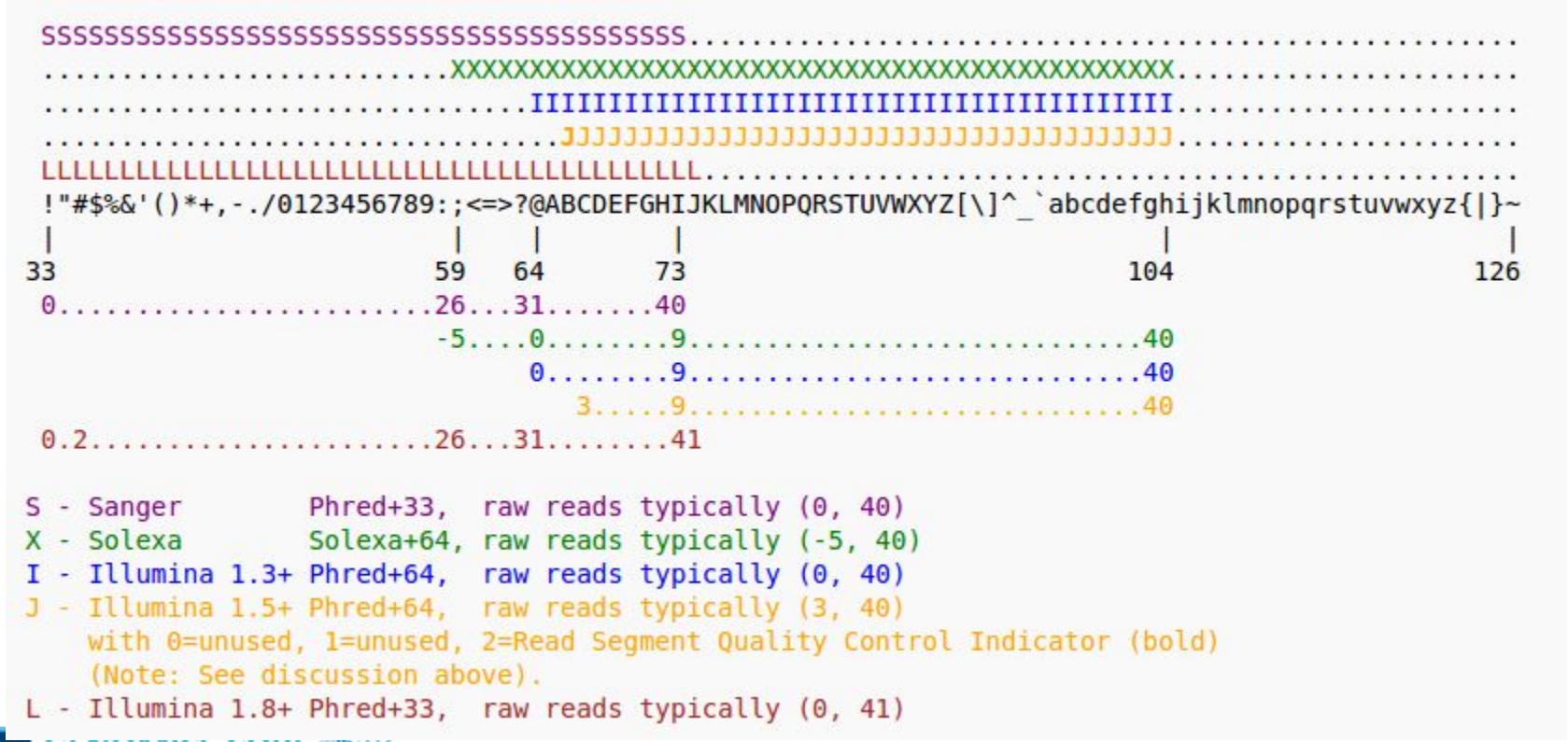


Fastq: The label

```
@EAS139:136:FC706VJ:2:2104:15343:197393 1:Y:18:ATCACG
```

EAS139:136	Machine and run ID
FC706VJ	Flowcell ID
2	Lane
2104	Tile number in the lane
15343:197393	Coordinate of the cluster within the tile
1	Member of the pair (1 or 2)
Y:18	Past filter information of the Illumina software
ATCACG	The Illumina index/barcode

Fastq: Scores



Fastqc: Quality control of fastq files

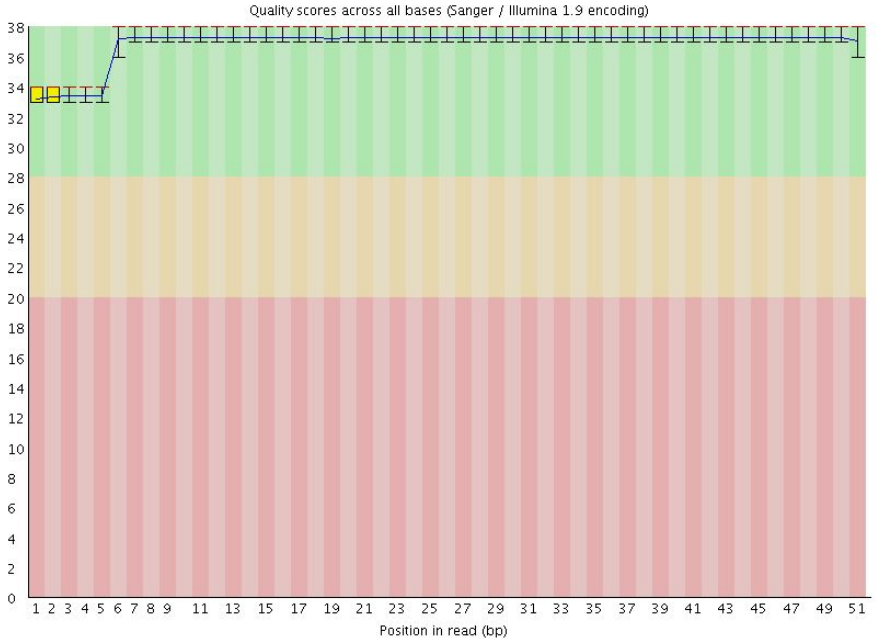
Some important stats for RNA-seq:

- Number of reads
- Base quality
- Length of reads

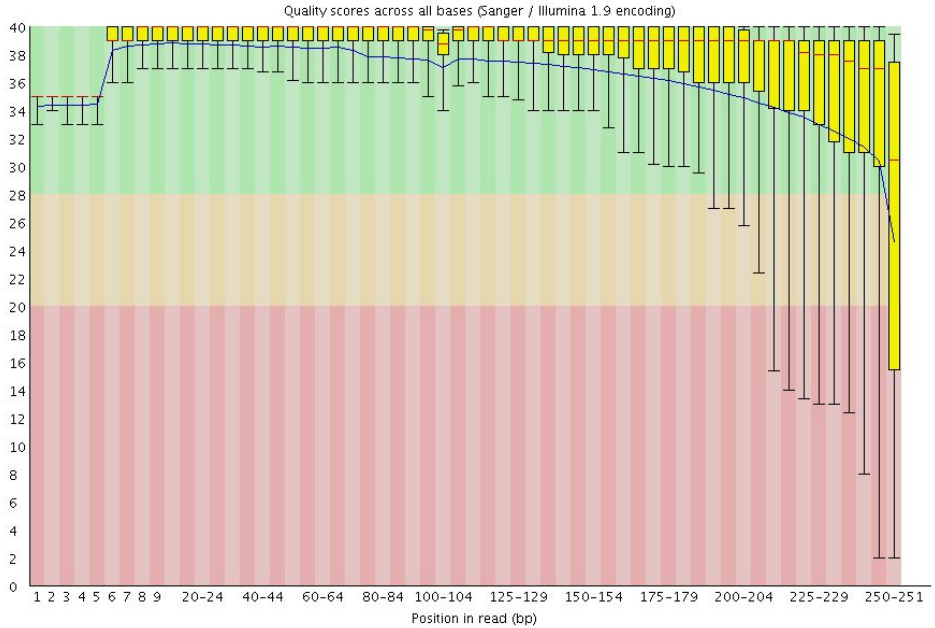
Open Fastqc

Fastqc: Base Quality

Per base sequence quality

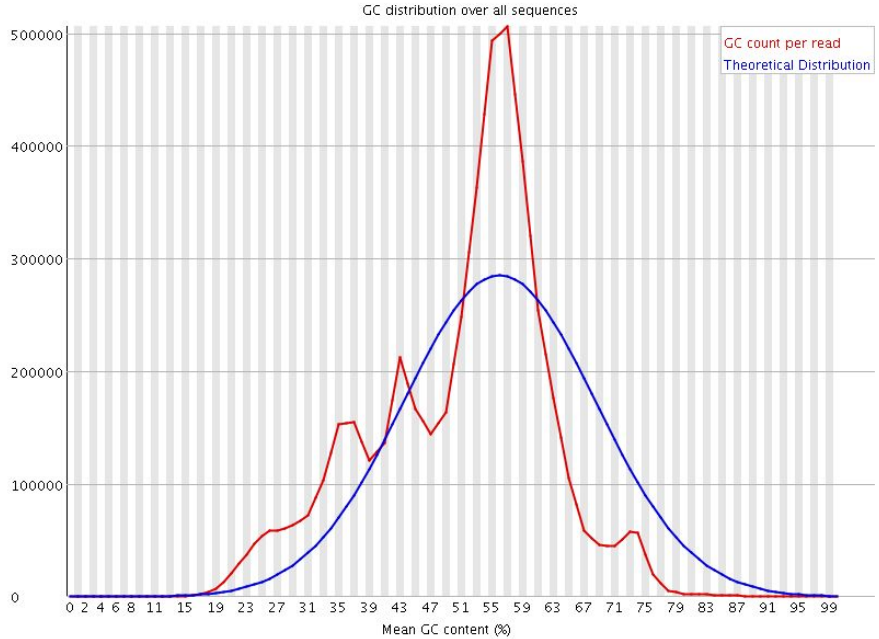


Per base sequence quality

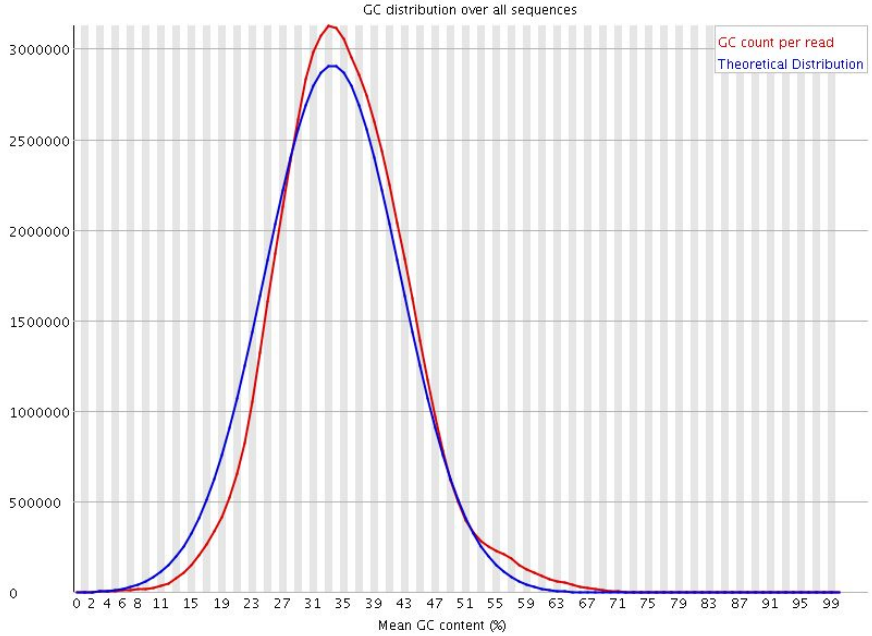


Fastqc: GC content

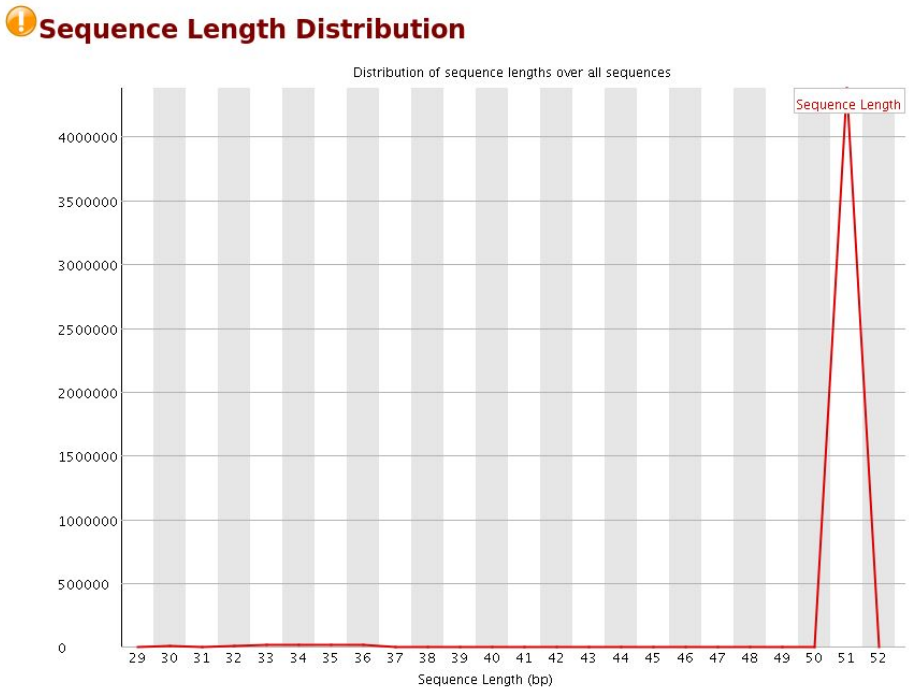
Per sequence GC content

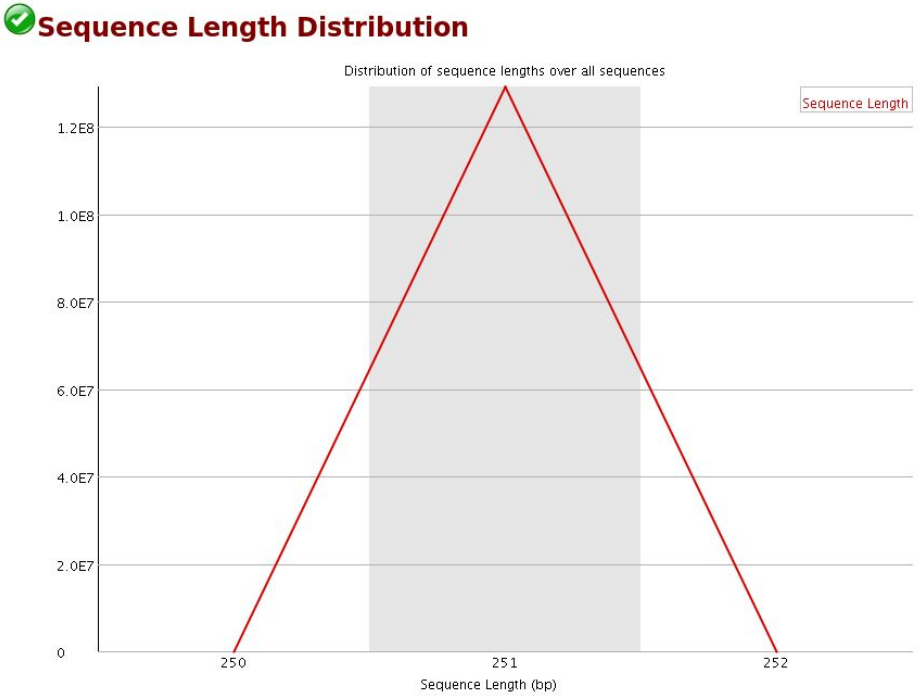


Per sequence GC content



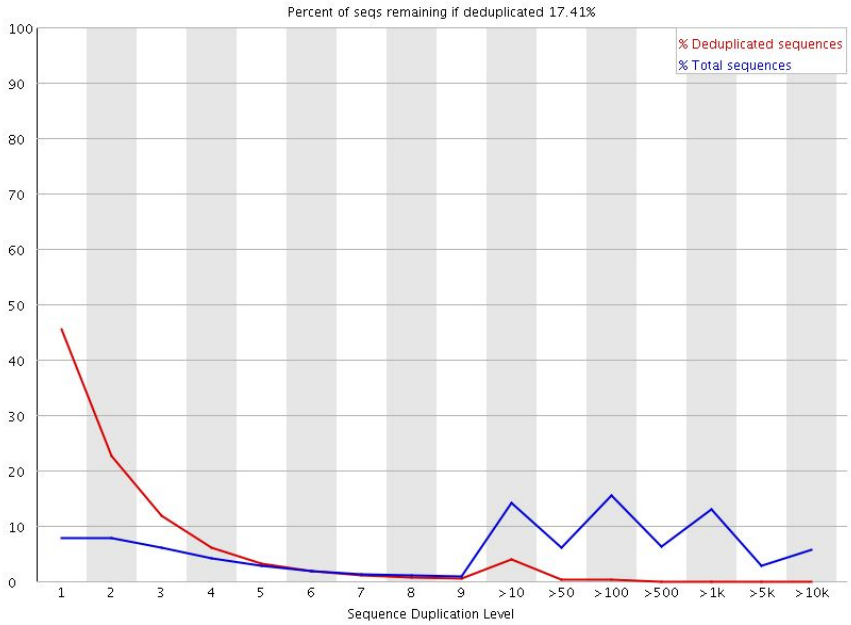
Fastqc: Read length



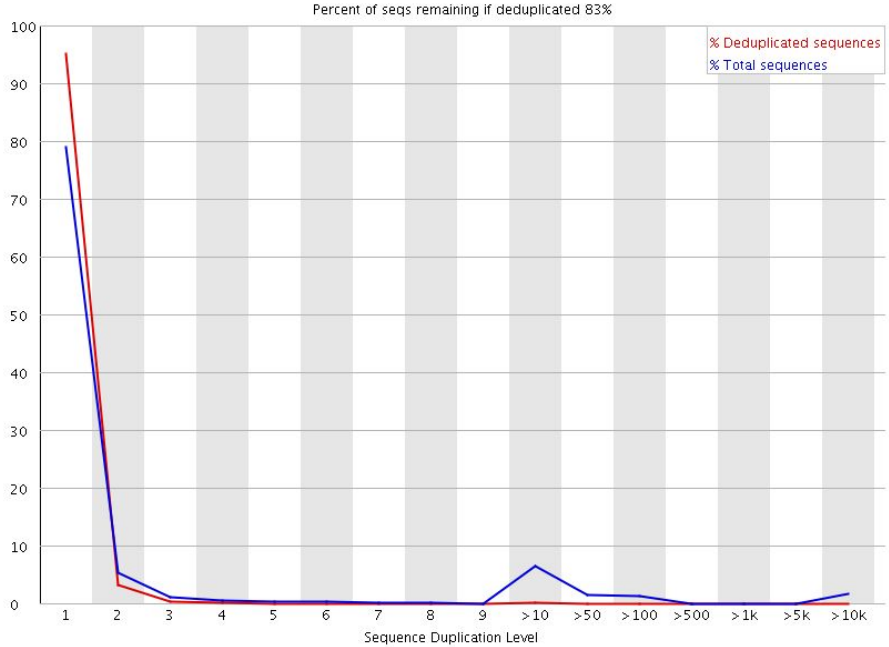


Fastqc: duplication levels

Sequence Duplication Levels

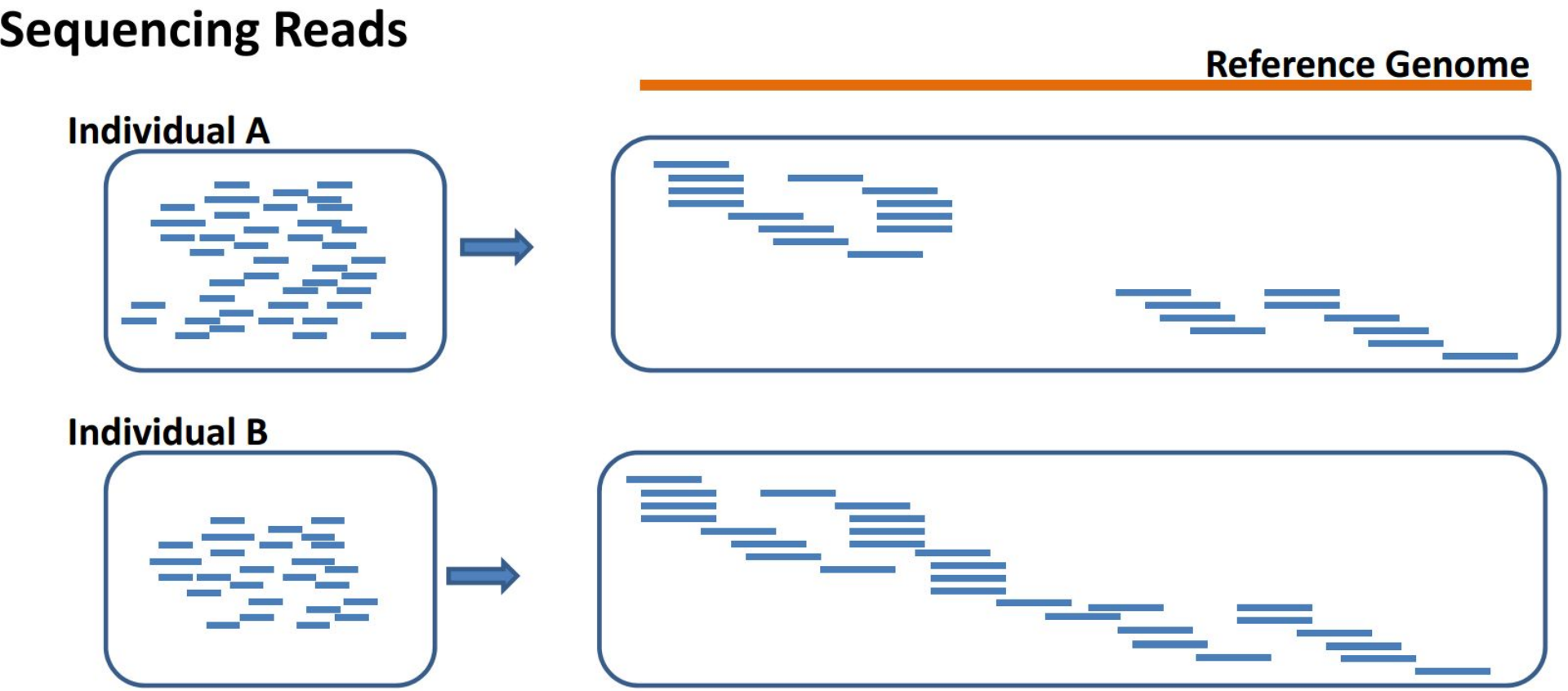


Sequence Duplication Levels



Finding the Location of the Reads in the Reference

Mapping


Sequencing Reads
Reference Genome
Individual A
Individual B

Mapping: Where to start?

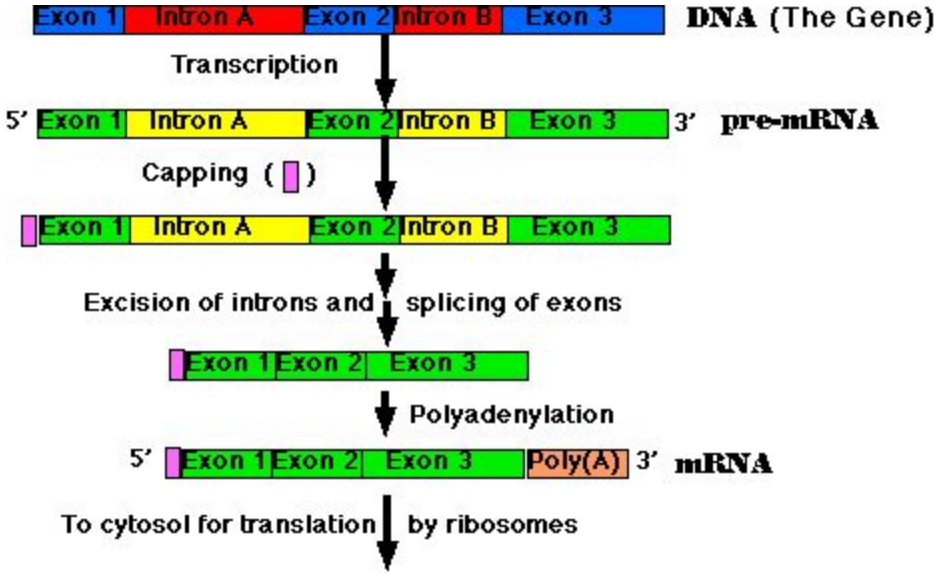
Needed information:

1. The dataset in fastq format (1 or 2 files, depends on single reads or paired end data)
2. A reference
   a. The reference is species dependent.
   b. It can have any quality (from 1k+ contigs, to chromosome level)
   c. It can be the genome or the transcriptome
   d. The version is tracked using a certain build. Make sure the build you use has an annotation!

Mapping

Possible References:

- Transcriptome:
  Created from mRNA, does only include exons.
  All positions in the reference are possible to be in the expressed dataset
- Genome:
  Created from the DNA, does include introns, and other ‘junk’
  Only a small part of the reference will be in the expressed dataset



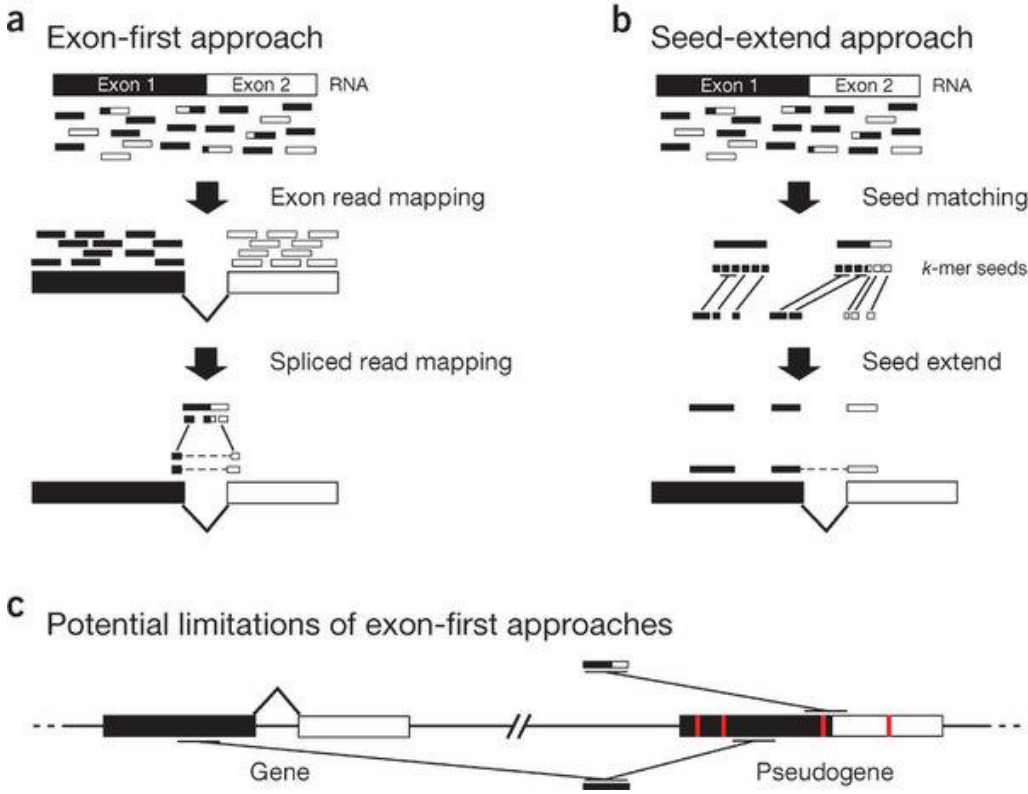
Split Read Mapping

Exon-first approach

1. map read to transcriptome
2. if mapped, map to genome
3. if read was mapped on a known splicing location, splice.

Seed-extend approach

1. Find seed (k-mer) in genome
2. Extend seed to maximum length
3. Repeat for multiple seed in read
4. Combine the found positions



Mapping: Output format: SAM and BAM format

- SAM
  Sequence Alignment/Map format
  Header starts with @
  Mapped Reads are tab-delimited lines
  1-based system (includes SAM, VCF, GFF, GTF)
- BAM
  Binary Alignment/Map format
  Is the binary form of the SAM format, so reduces storage
  Contains exact the same information as the SAM format
  0-based system (includes BAM, BED)

Mapping: Output format: SAM and BAM format

```
>gene1:3-53     0       chr0    22      255     50M     *       0       0       TTTGTTCATGCGTATTTTTCTACAGTCGGGTAGCAAAGTATAACTGGATT      AAAAAA5AFFFFFFFFF@FFFFFFFF7FFFFFFFFFFFFFFF7FFFFFF      NH:i:1  HI:i:1  AS:
i:49 nM:i:0
>gene1:4-54     0       chr0    23      255     50M     *       0       0       TTGTTCATGCGTATTTTTCTACAGTCGAGTAGCAAAGTATAACTGGATTT      AAA@AAAAFFFFFFFFFFFFFFFFF@FFFFFFFFFFFFFFF7FFFFFFF      NH:i:1  HI:i:1  AS:
i:47 nM:i:1
>gene1:6-56     0       chr0    25      255     50M     *       0       0       GTTCATGCGTATTTTTCTACAGTCGGGTAGCAAAGTATAACTGGATTTAA      AAAAAAAA>FFFFFF>FFFFFFFFFFFFF<FFFF+FFFFFFFF@:FFF      NH:i:1  HI:i:1  AS:
i:49 nM:i:0
>gene1:8-58     0       chr0    27      255     50M     *       0       0       TCATGCGTATTTTTCTACAGTCGGGTAGCAAAGTATAACTGGATTTAATT      AAAAAAAAFFFFF8+F<FFFFFFFFFFFFFFFFFF5FFFFFFFFFFFF      NH:i:1  HI:i:1  AS:
i:49 nM:i:0
>gene1:14-64    0       chr0    33      255     50M     *       0       0       GTATTTTTCTACAGTCGGGTAGCAAAGTATAACTGGATTTAATTTAGAAA      AA6AAAA8FFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFF      NH:i:1  HI:i:1  AS:
i:49 nM:i:0
>gene1:17-67    0       chr0    37      255     1S49M   *       0       0       ATTTTCTACAGTCGGGTAGCAAAGTATAACTGGATTTAATTTAGAAAAAA      AAAAAAAA86FFFFFFFFFFFFFFFFFFFF@FFFFFFFFFFFFFFFF      NH:i:1  HI:i:1  AS:
i:48 nM:i:0
>gene1:19-69    0       chr0    38      255     50M     *       0       0       TTTCTACAGTCGGGTAGCAAAGTATAACTGGATTTAATTTAGAAAAAATA      AAAAAAAAFFFFFFFFFF>FFFFFF5F<FF@FFFFFFFFF@FFFFFFF>      NH:i:1  HI:i:1  AS:
i:49 nM:i:0
>gene1:19-69    0       chr0    38      255     50M     *       0       0       TTTCTACAGTCGGATAGCAAAGTATAACTGGATTTAATTTAGAAAAAATA      AAAAAA7AFFF5FFFFF:FFFFFFFFF@FFFFFFFFFFFFFFFFFFFF      NH:i:1  HI:i:1  AS:
i:47 nM:i:1
>gene1:26-76    0       chr0    45      255     50M     *       0       0       AGTCGGGTAGCAAAGTATAACTGGATTTAATTTAGAAAAAATACAGGTGT      A+AAAAAAFFFFFFFFF+FFFFFFFFFFFFFFFFFF8FFFFFFFFFFFF      NH:i:1  HI:i:1  AS:
i:49 nM:i:0
>gene1:26-76    0       chr0    45      255     50M     *       0       0       AGTCGGGTAGCAAAGTATAACTGGATTTAATTTAGAAAAAATACAGGTGT      AA>AAAAAFF7FFFF@FFFFFFFFFFFFFFFFFF8FFFFFFFF>FF>FF      NH:i:1  HI:i:1  AS:
i:49 nM:i:0
>gene1:28-78    0       chr0    47      255     50M     *       0       0       TCGGGTAGCAAAGTATAACTGGATTTAATTTAGAAAAAATACAGGTGTTG      8AA@AAAAFFFFFFFFF:FFF7FFFFF5F<FFF<F:F5FFFFF<@FFFFF      NH:i:1  HI:i:1  AS:
i:49 nM:i:0
>gene1:33-83    0       chr0    52      255     50M     *       0       0       TAGCAAAGTATAACTGGATTTAATTTAGAAAAAATACAGGTGTTGGTTTC      A6AAAAAAFFFF7FFFFFFFFFFF:FFFFFFFFFFFFF+FFFFFFFF      NH:i:1  HI:i:1  AS:
i:49 nM:i:0
>gene1:33-83    0       chr0    52      255     50M     *       0       0       TAGCAAAGTATAACTGGATTTAATTTAGAAAAAATACAGGTGTTGGTTTC      :AAAAAAAFFFFFF5FFFF<>FFFFFFFFFFFFFFFFFFFFFFFFF>      NH:i:1  HI:i:1  AS:
i:49 nM:i:0
>gene1:39-89    0       chr0    58      255     50M     *       0       0       AGTATAACTGGATTTAATTTAGAAAAAATACAGGTGTTGATTTCTAATTA      AA:AAAAAFFFF@FFFFFFFF8FFFFFF+FFFFFF>FFFFFFFFFFFFF      NH:i:1  HI:i:1  AS:
i:47 nM:i:1
>gene1:45-95    0       chr0    64      255     50M     *       0       0       ACTGGATTTAATTTAGAAAAAATACAGGTGTTGGTTTCTAATTAGTCGGC      AAAA<A7<FFFFFFFFFF8FFFF@FFFFFFFFFFFFFFFFFFFF:FFFF      NH:i:1  HI:i:1  AS:
i:49 nM:i:0
>gene1:45-95    0       chr0    64      255     50M     *       0       0       ACTGGATTTAATTTAGAAAAAATACAGGTGTTGGTTTCTAATTAGTCGGC      A>AAAAAAFFFFFFFFFFFFFFFFFFFFFFFFFFFFFF6FF7FFFFFF      NH:i:1  HI:i:1  AS:
i:49 nM:i:0
>gene1:57-107   0       chr0    76      255     50M     *       0       0       TTAGAAAAAATACAGGTGTTGGTTTCTAATTAGTCGGCGTACGGCCGTTA      AAAAAAAAFFFFFFFFFFFFFFFFFFFF7FF5F6FFFFF>FFFFFFF6FF      NH:i:1  HI:i:1  AS:
i:49 nM:i:0
>gene1:60-110   0       chr0    79      255     50M     *       0       0       GAAAAAATACAGGTGTTGGTTTCTAATTAGTCGGCGTACGGCCGTTACAT      AAAAAAAA:FFFF+FFF6FFFF+FF+FFFFFFFFFFFFFFFFFFFFFF      NH:i:1  HI:i:1  AS:
i:49 nM:i:0
>gene1:66-116   0       chr0    85      255     50M     *       0       0       ATACAGGTGTTGGTTTCTAATTAGTCGGCGTACGGCCGTTACATTATTCG      AAAAAAAAFFFFFFF8FFFFFFF<FFFFF8FFFF8FFFFFFFFFFFF8F      NH:i:1  HI:i:1  AS:
i:49 nM:i:0
>gene1:67-117   0       chr0    86      255     50M     *       0       0       TACAGGTGTTGGTTTCTAATTAGTCGGCGTACGGCCGTTACATTATTCGT      AAAAAAAAFFFFF>FFFFFFFFF5:6FFFFFFFFFFFFFFFFFFFFFF      NH:i:1  HI:i:1  AS:
i:49 nM:i:0
>gene1:67-117   0       chr0    86      255     48M2S   *       0       0       TACAGGTGTTGGTTTCTAATTAGTCGGCGTACGGCCGTTACATTATTCAT      AAAAAA:AFFFFFFFFF8FFFFFFFF>FFFFFFF:FF+FFF@FFF5F      NH:i:1  HI:i:1  AS:
i:47 nM:i:0
>gene1:68-118   0       chr0    87      255     50M     *       0       0       ACAGGTGTAGGTTTCTAATTAGTCGGCGTACGGCCGTTACATTATTCGTG      AAAAAAAAFFFFF>FFFFFFF>F7FFFFFFFFF6FFFFFF:FF@FFFFFF      NH:i:1  HI:i:1  AS:
i:47 nM:i:1
>gene1:70-120   0       chr0    89      255     49M1S   *       0       0       AGGTGTTGGTTTCTAATTAGTCGGCGTACGGCCGTTACATTATTCGTGTA      AAAA5AAAF:FFFFFFFFFFFFF8FFFFF8FFFFFFFFFFFFF5FFFFF      NH:i:1  HI:i:1  AS:
i:48 nM:i:0
```

Mapping: Output format: SAM and BAM format

```
>gene1:3-53     0       chr0    22      255     50M     *       0       0       TTTGTTCATGCGTATTTTTCTACAGTCGGGTAGCAAAGTATAACTGGATT
AAAAAA5AFFFFFFFFF@FFFFFFF7FFFFFFFFFFFFFFFF7FFFFF      NH:i:1  HI:i:1  AS:i:49 nM:i:0
```

>gene1:3-53	Name of the sequence
0	The flag (containing information about mapped/unmapped, forward/reverse, paired, info of the paired read)
chr0	The chromosome
22	Start position
255	Mapping quality
50M	CIGAR string (info about the alignment)
*	Chromosome of the paired read (no pair here)
0	Start position of the paired read
0	Template length (calculated from the start from the first read, to the end of the second read)
TTTTT….	Sequence
AAAAA….	Quality

Mapping: view in IGV

- Open IGV
- Load reference file

  Genomes > Load Genome from File
- Index the bam file, if no index available (.bai)

  Tools > Run IGVtools > Select Index
- Open 2 bam files

  File > Load from File
- Load gtf file

  File > Load from File

Mapping: view in IGV

- Adjust view:
  - change read view
    Right mouse button on Track > Collapsed/Expended
  - sort per read strand
    Right mouse button on Track > Group alignments by > read strand
  - show soft clipped
    View > Preferences > Alignment > Show soft-clipped bases
  - Remove low quality reads
    View > Preferences > Alignment > Mapping quality threshold
- How to search for a position/gene
  The position track
- How to take screenshot
  File > Save Image

Counting the Reads per Feature

Counting: Where to start?

Needed information:

1. The mapped data
   => BAM file
2. Information about the annotation.
   Where are the genes?
   => GTF file
   GTF files are linked with the used species, version and build. These files also has there own versions (often used are the Ensembl versions)

Counting: GTF file

```
1 transcribed_unprocessed_pseudogene  gene        11869 14409 . + . gene_id "ENSG00000223972"; gene_name "DDX11L1"; gene_source "havana"; gene_biotype "
1 processed_transcript                transcript  11869 14409 . + . gene_id "ENSG00000223972"; transcript_id "ENST00000456328"; gene_name "DDX11L1"; gen
```

seqname	Name of the chromosome/contig
source	The database or program that generated this feature
feature	Feature type: e.g. gene, transcript, exon, ...
start	Start position of the feature (1-based)
end	End position of the feature
score	A floating point value (. when not available)
strand	Forward (+) or reverse (-)
frame	0, 1 or 2. Indicates the start of a codon
attribute	Semicolon separated tag-value pairs, like gene, gene_id, gene_name, ...

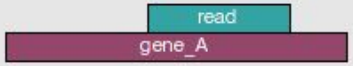
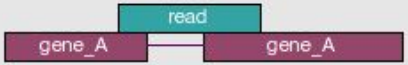
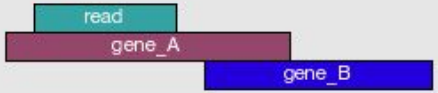
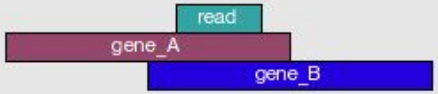
Counting

- **Simple**

  Combine exons, analyse at gene level
  Simple, powerful, sometimes inaccurate
  HTSeq-count *STANDARD*

- **Complex**

  Quantitate transcripts and merge to gene level
  Potentially cleaner, more powerful signal
  High degree of uncertainty
  Cuffquant of Cufflinks 2.2.1

	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

Counting

Example of a file (first lines):

A4GNT	0
AA06	0
AAAS	0
AACS	2
AACSP1	0
AADAC	0
AADACL2	0
AADACL2-AS1	0
AADACL3	0
AADACL4	0
AADACP1	0
AADAT	0
AAED1	0
AAGAB	3
AAK1	0
AAMDC	0
AAMP	4
AANAT	0
AAR2	0
AARD	0
AARS	0
AARS2	0
AARSD1	0
AARSP1	0
AASDH	0
AASDHPPT	0

Example of a file (last lines):

snoZ6	0
snosnR66	0
uc_338	0
yR211F11.2	0
_no_feature	357
_ambiguous	5007
_too_low_aQual	47490
_not_aligned	10470
_alignment_not_unique	0

_no_feature	Reads map to location outside known genes
_ambiguous	Reads map to an overlap of multiple genes
_too_low_aQual	Reads whom the mapping quality read is to low
_not_aligned	Reads that are not mapped
_alignment_not_unique	Reads that mapped to multiple locations

These output count files can be opened with any text editor or spreadsheet.

Beware if opened with a spreadsheet, the gene names can be automatically changed:

Gene name errors are widespread in the scientific literature

Mark Ziemann, Yotam Eren and Assam El-Osta

Genome Biology 2016 **17**:177 | **DOI:** 10.1186/s13059-016-1044-7 | © The Author(s). 2016

Published: 23 August 2016

Abstract

The spreadsheet software Microsoft Excel, when used with default settings, is known to convert gene names to dates and floating-point numbers. A programmatic scan of leading genomics journals reveals that approximately one-fifth of papers with supplementary Excel gene lists contain erroneous gene name conversions.