Characterizing transcriptomes using NGS data

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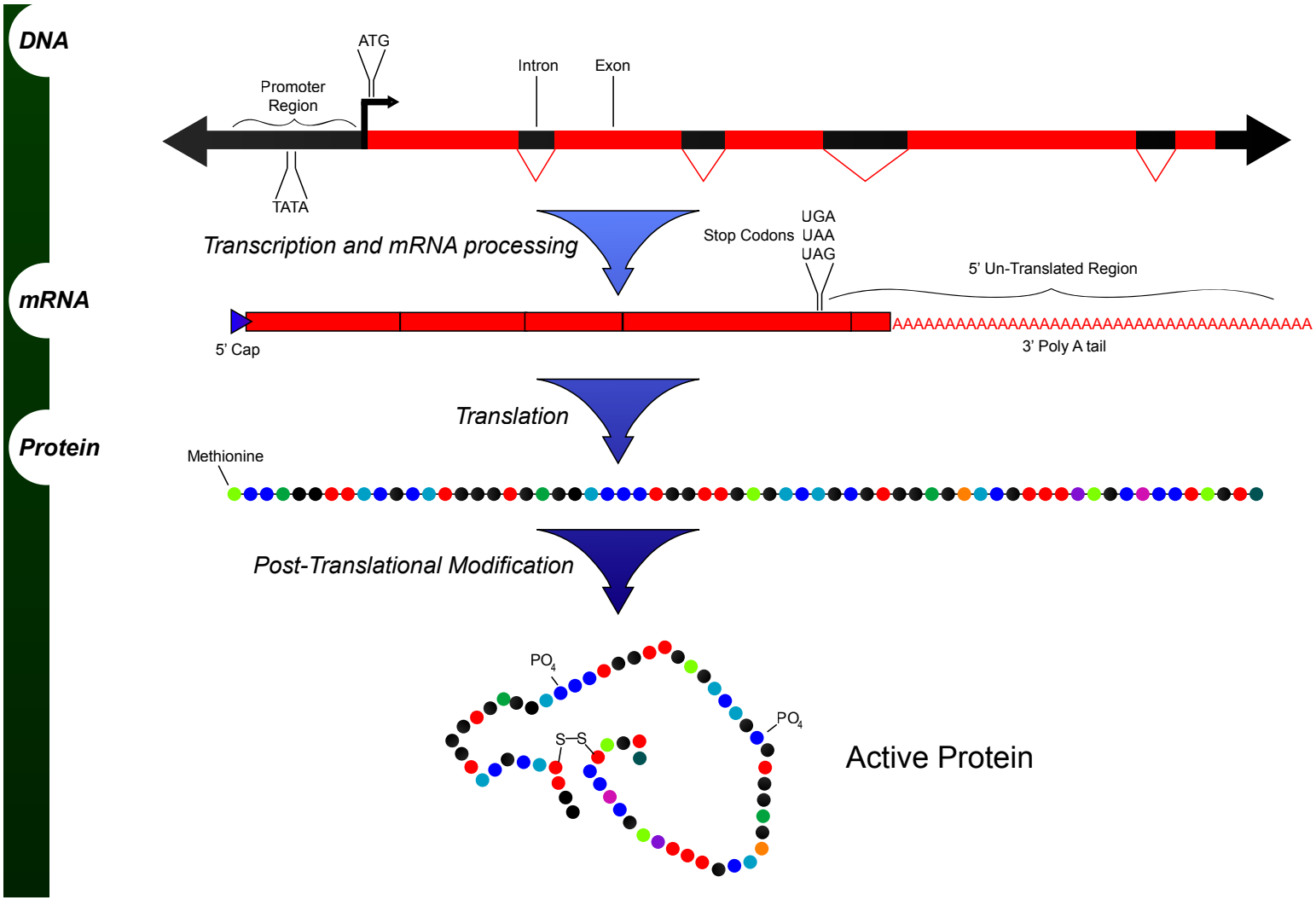
Outline

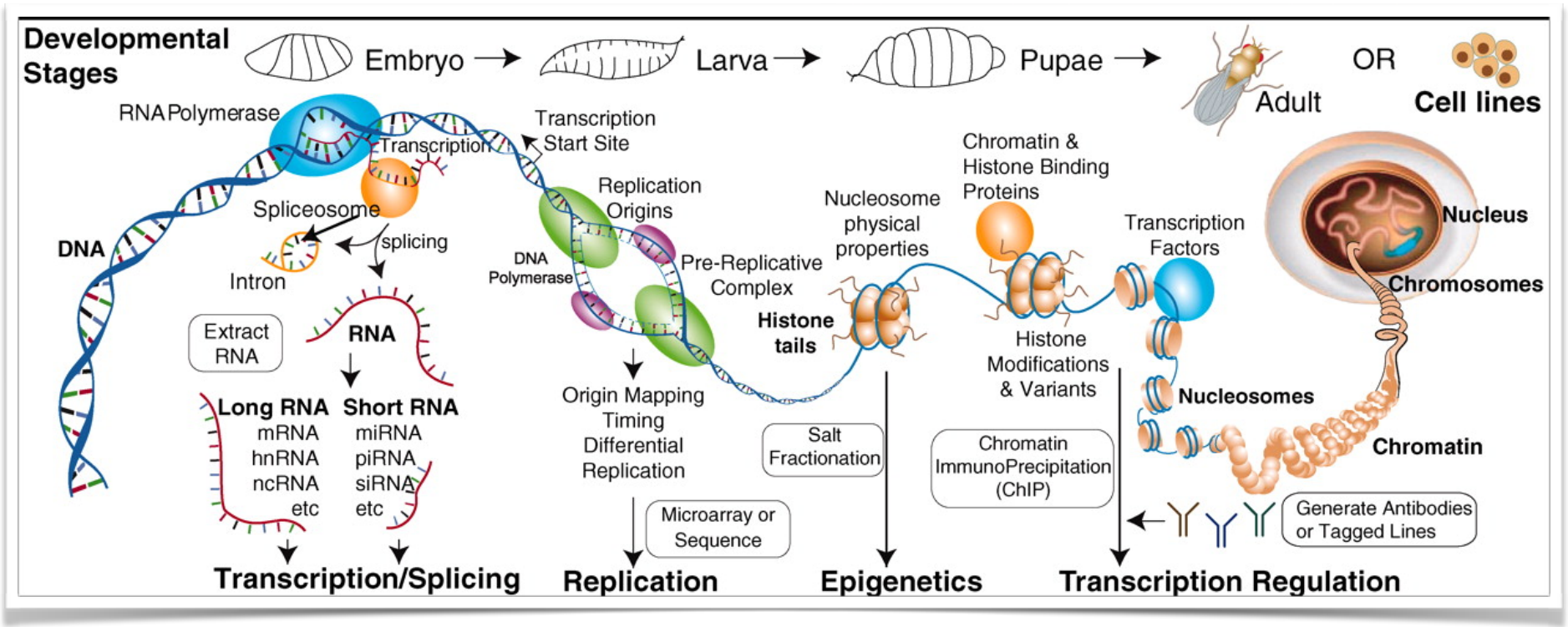
1. Why study transcriptome?
2. Overview of RNA-seq work flow
3. Understanding sequencing output
4. RNA-seq data analysis
   - Mapping based approach
   - Transcriptome assembly
5. Exercises

Why study transcriptome?

The Central Dogma



A more complex view



Advantages and Applications

Transcriptomes are

- **dynamic**, that is not the same over tissues and time points
- **directly derived** from functional genomics elements, mostly protein-coding genes, providing a useful functionally relevant subset of the genome, that is **smaller sequence space**

Transcriptomes enable

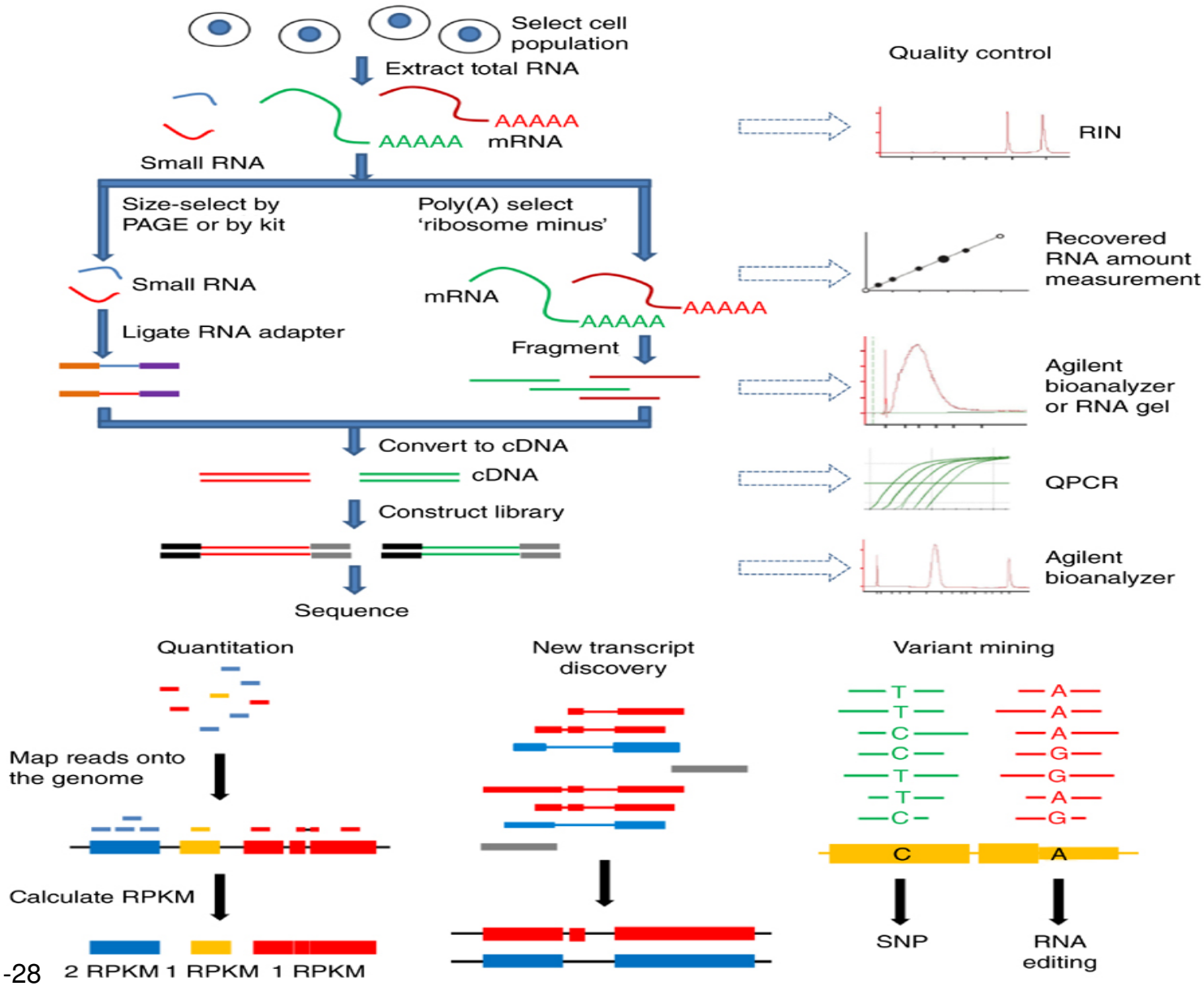
- to investigate **differences in gene expression** patterns
- to distinguish different **isoforms** and allelic expression
- to explore gene functions
- to analyze single nucleotide variants, fusion genes and co-expression networks

Overview of RNA-seq work flow

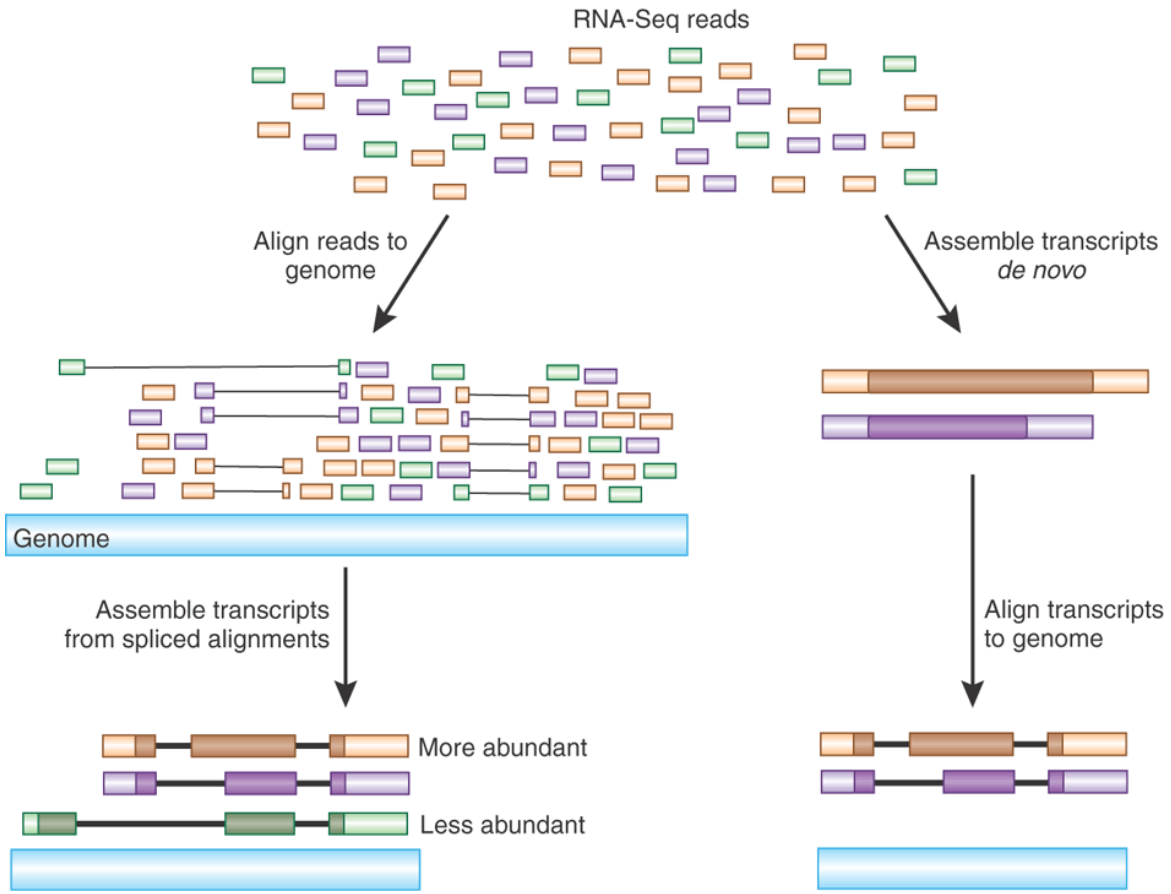
High level work flow overview

- Experimental design (biology, medicine, statistics)
- RNA extraction (biology, biotechnology)
- Library preparation (biology, biotechnology)
- High throughput sequencing (engineering, biology, chemistry, biotechnology, bioinformatics)
- Data processing (bioinformatics)
- Data analysis (bioinformatics & biostatistics)

More detailed work flow overview

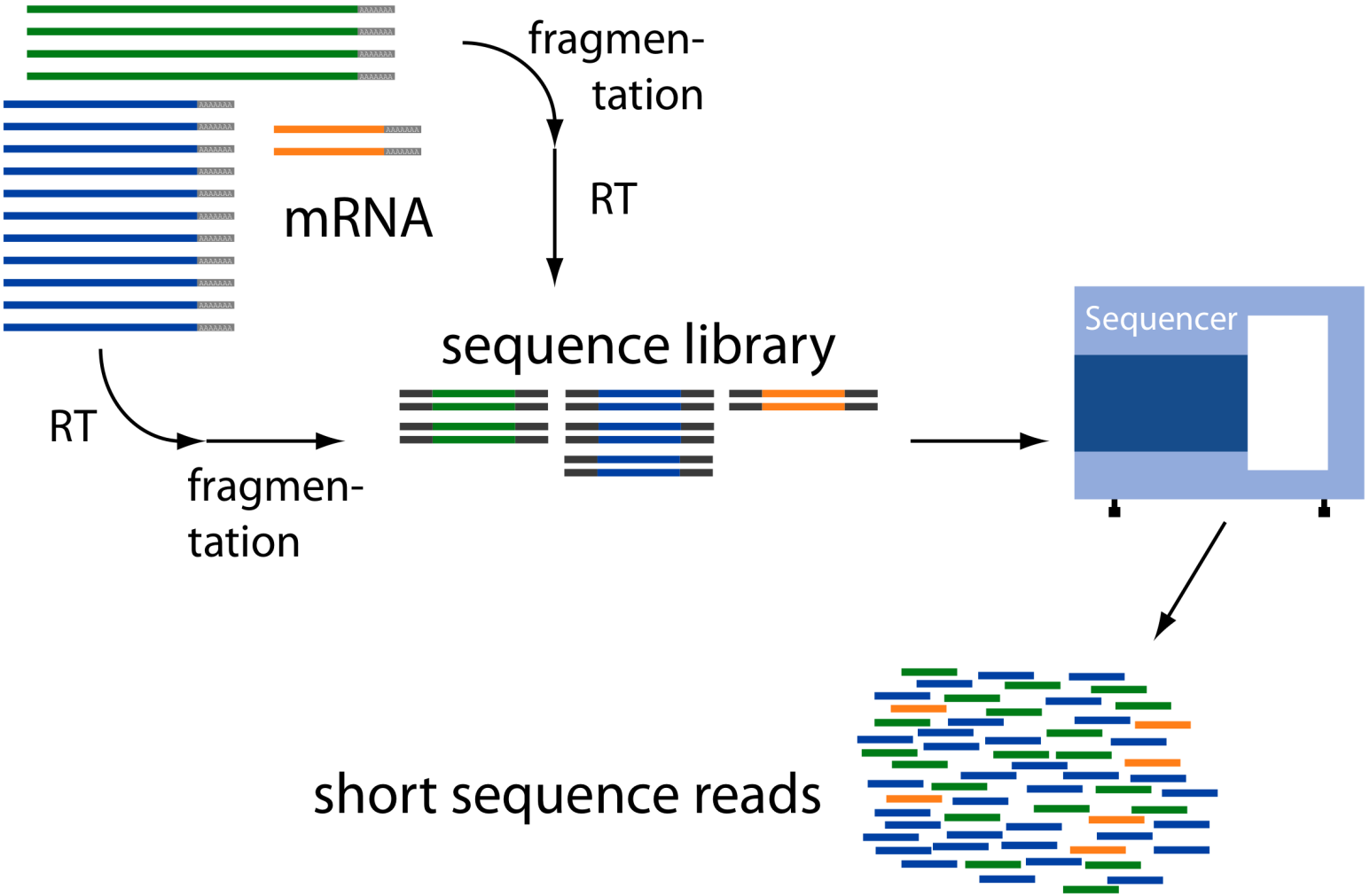


Two main bioinformatics routes

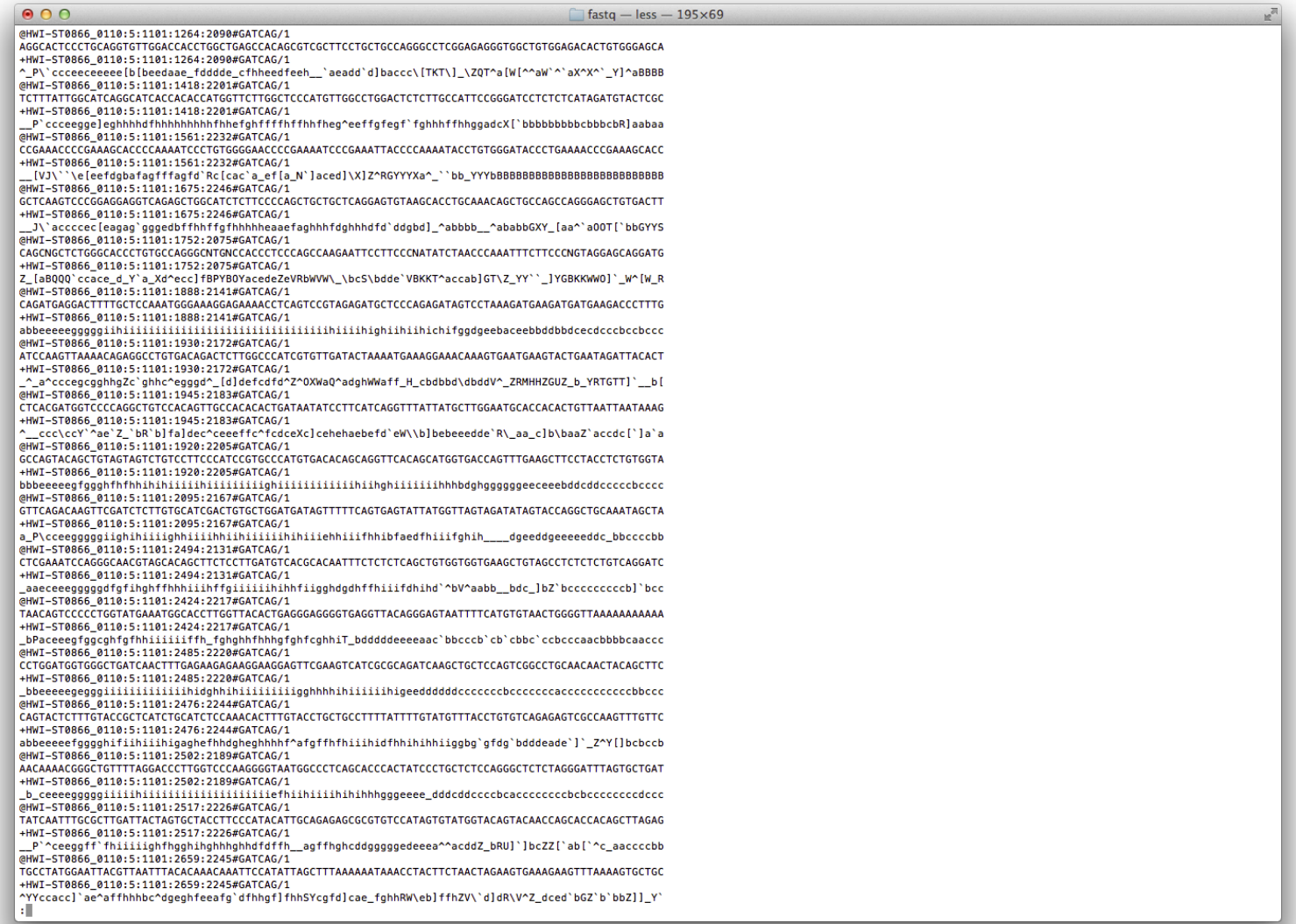


Understanding sequencing output

NGS data


fragmen-
tation
RT
mRNA
sequence library
RT
fragmen-
tation
Sequencer
short sequence reads

.fastq Machine output



.fastq Machine output

- Line 1 begins with a '@' character and is followed by a sequence identifier and an optional description
- Line 2 is the raw sequence letters
- Line 3 begins with a '+' character and is optionally followed by the same sequence identifier (and any description) again
- Line 4 encodes the quality values for the sequence in Line 2, and must contain the same number of symbols as letters in the sequence

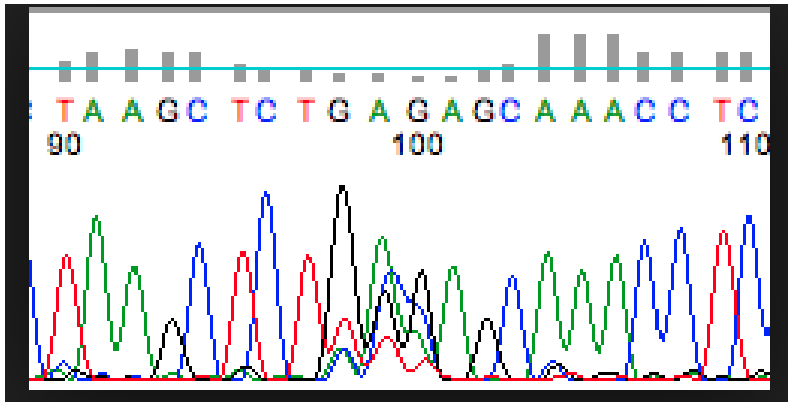
.fastq

```
@MISEQ:233:000000000-AGJP2:1:1101:15260:1358
CTGTAAATTGCCTGACTTGCTAATTGTGATTAACTTAGTTT
+
BBBBBFFFFFFFGGGGGGGGGGHFFFHGHHGFFHHHHHAG
```

Sequence quality

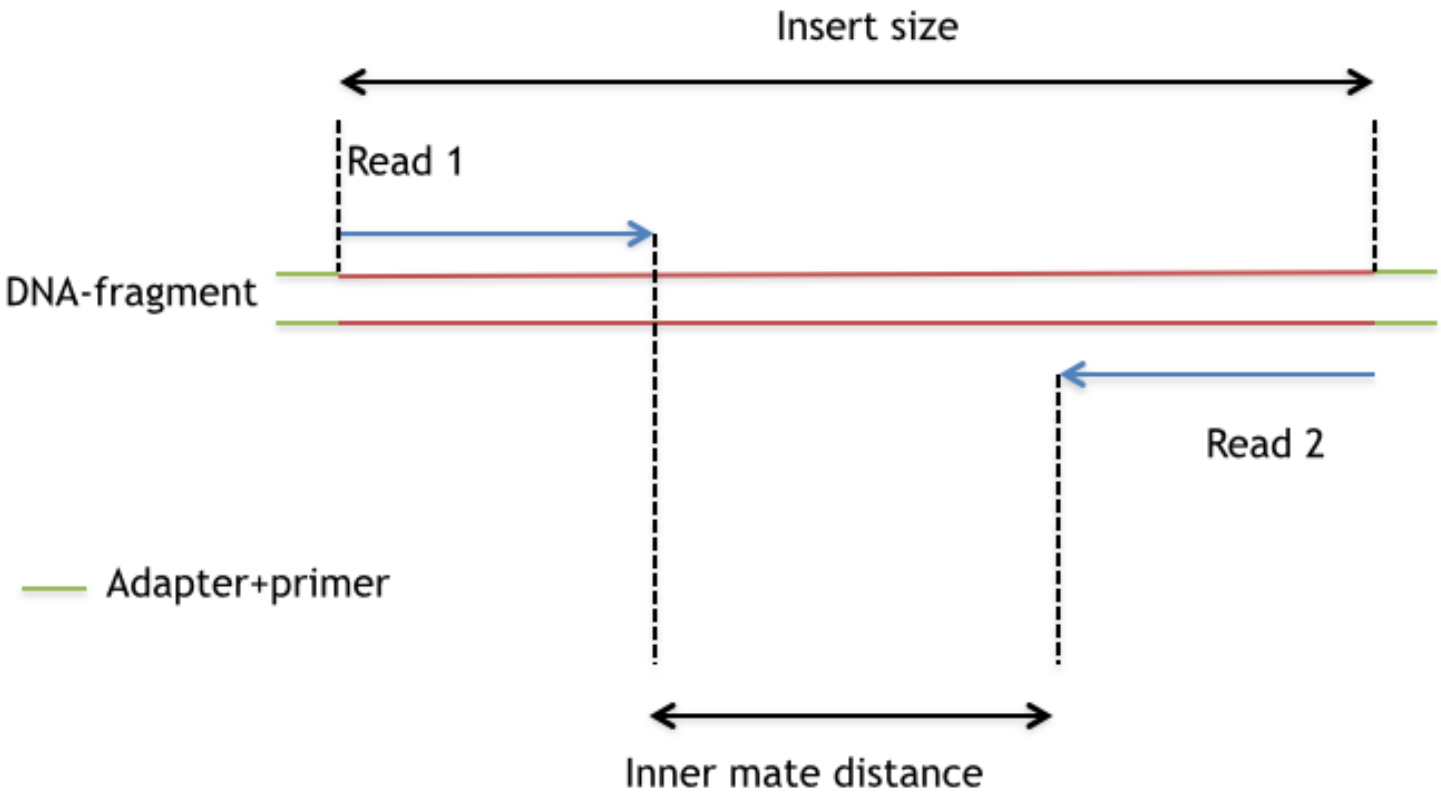
Phred Quality Score

- Q = -10 x log P
- where:
  - P, probability of base calling being incorrect
  - High Q = high probability of the base being correct



- A Phred quality score of 10 to a base, means that the base is called incorrectly in 1 out of 10 times.
- A Phred quality score of 20 to a base, means that the base is called incorrectly in 1 out of 100 times.
- A Phred quality score of 30 to a base, means that the base is called incorrectly in 1 out of 1000 times.
- etc.

Paired-end (PE) reads



Paired-end (PE) reads

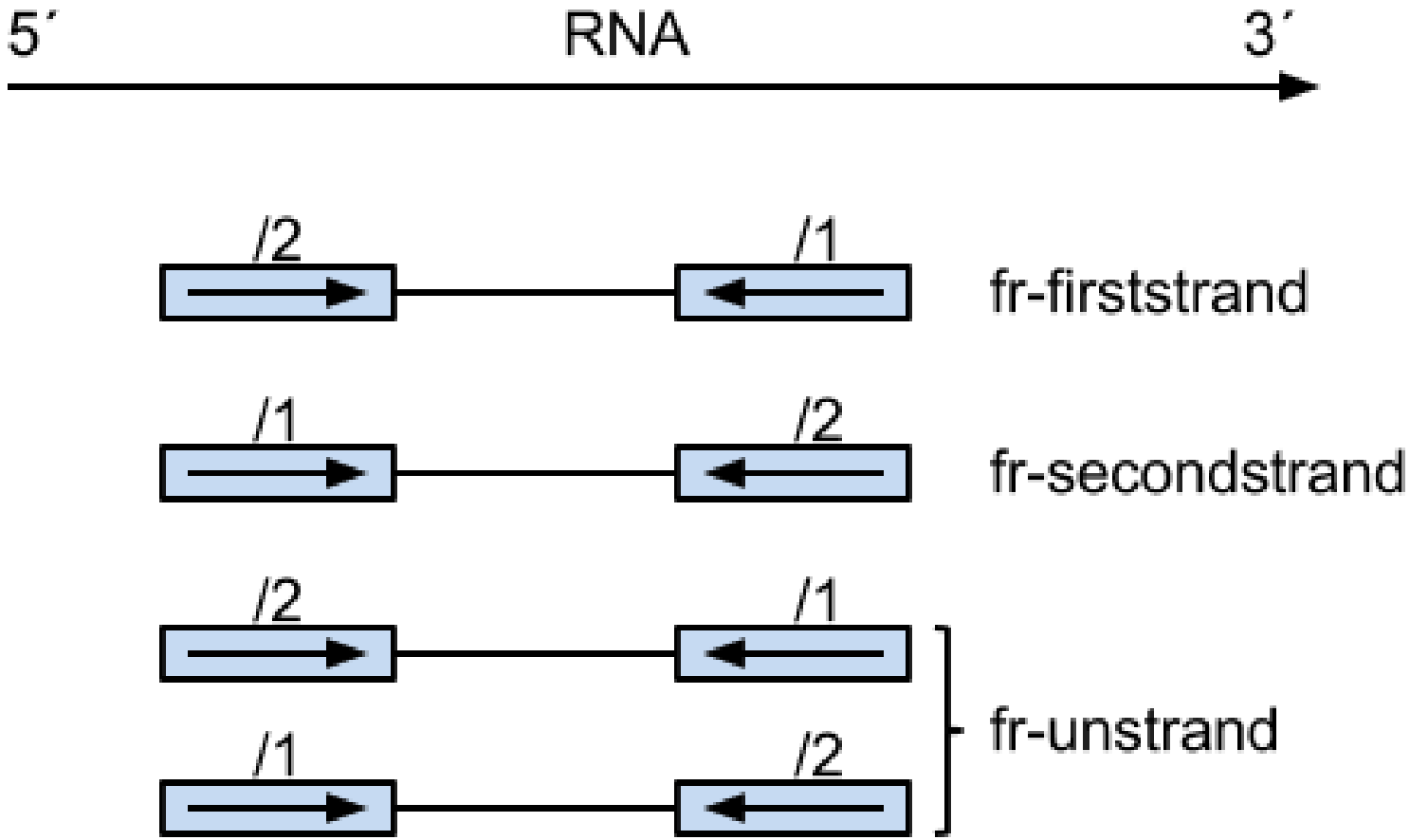
File format

- Two files are created
- The order in files identical and naming of reads are the same with the exception of the end
- The way of naming reads are changing over time so the read names depend on software version

```
@61DFRAAXX100204:1:100:10494:3070/1
AAACAACAGGGCACATTGTCACTCTTGTATTTGAAAAACACTTTCCGGCCAT
+
ACCCCCCCCCCCCCCCCCCCCCCCCCCCCCBC?CCCCCCCCC@@CACCCCCA
```

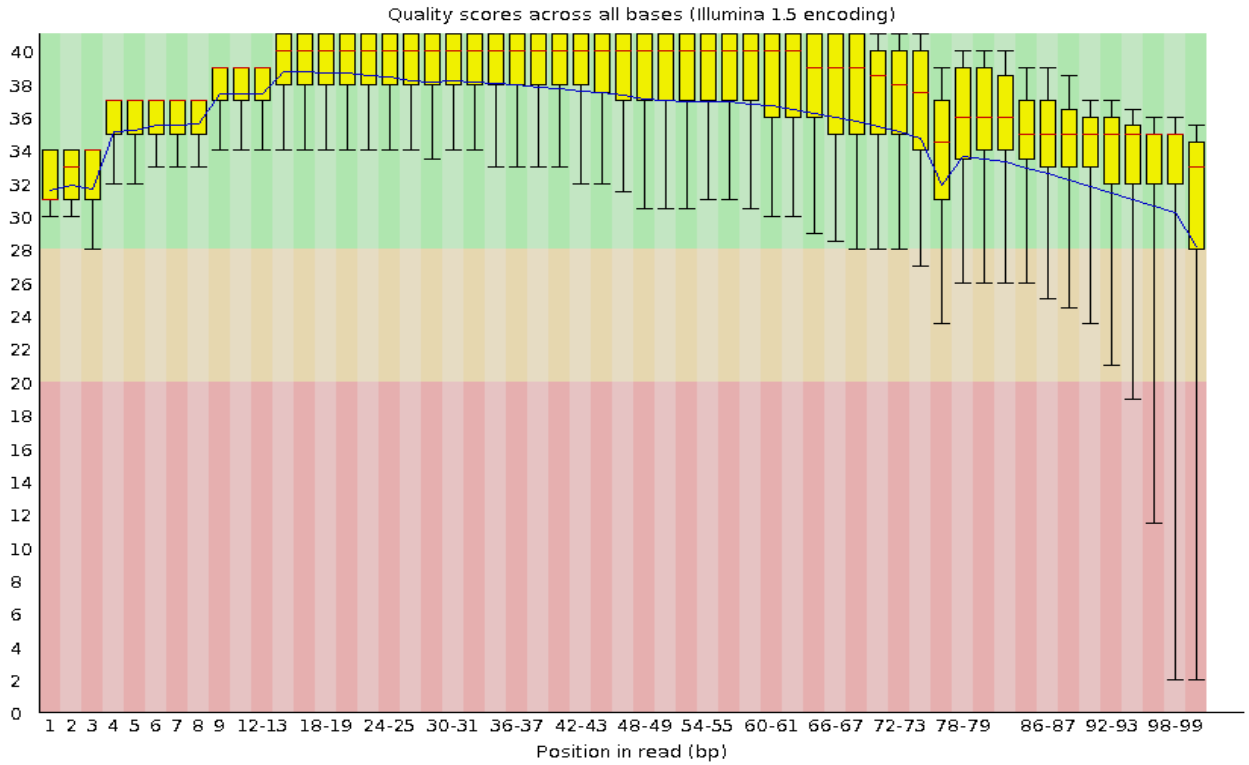
```
@61DFRAAXX100204:1:100:10494:3070/2
ATCCAAGTTAAAACAGAGGCCTGTGACAGACTCTTGGCCCATCGTGTTGATA
+
_^_a^cccegcgghhgZc`ghhc^egggd^_[d]defcdfd^Z^OXWaQ^ad
```

Strandness


5´
RNA
3´
/2
/1
fr-firststrand
/1
/2
fr-secondstrand
/2
/1
/1
/2
fr-unstrand

RNA-seq data analysis
Mapping based approach

Quality control of raw reads



Available tools

FastQC, PRINSEQ

Raw reads filtering and trimming

- filtering reads for quality score, e.g. with avg. quality below 20 defined within 4-base wide sliding window
- filtering reads for read length, e.g. reads shorter than 36 bases
- removing artificial sequences, e.g. adapters

Available tools

TRIMMOMATIC, FastX, PRINSEQ, Cutadapt

Reference genome and annotations

.fasta (download reference genome FASTA file)

```
>1 dna:chromosome chromosome:GRCm38:1:1:195471971:1 REF
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
```

.gtf (download the corresponding genome annotation in GTF or GFF)

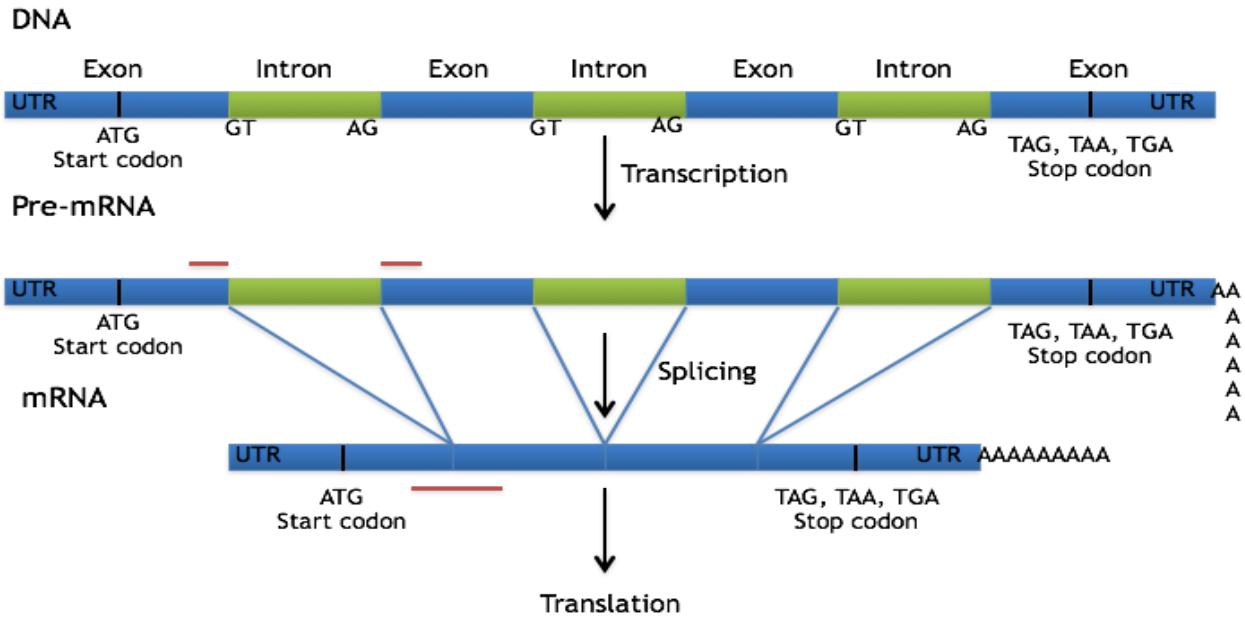
```
#!genome-build GRCm38.p4
#!genome-version GRCm38
#!genome-date 2012-01
#!genome-build-accession NCBI:GCA_000001635.6
#!genebuild-last-updated 2015-07
1       havana  gene    3073253 3074322 .       +       .       gene_id "ENSMUSG00000102693"; gene_version "1"; gene_name "493340
1J01Rik"; gene_source "havana"; gene_biotype "TEC"; havana_gene "OTTMUSG00000049935"; havana_gene_version "1";
1       havana  transcript      3073253 3074322 .       +       .       gene_id "ENSMUSG00000102693"; gene_version "1"; transcrip
t_id "ENSMUST00000193812"; transcript_version "1"; gene_name "4933401J01Rik"; gene_source "havana"; gene_biotype "TEC"; havana_ge
ne "OTTMUSG00000049935"; havana_gene_version "1"; transcript_name "4933401J01Rik-001"; transcript_source "havana"; transcript_bio
type "TEC"; havana_transcript "OTTMUST00000127109"; havana_transcript_version "1"; tag "basic"; transcript_support_level "NA";
```

Source

ENSEMBL, NCBI

Mapping reads to the genome

- Choose adequate aligner
- Use annotations and allow for spliced mapping



Available tools

Star, Tophat, Subread and many more...

Mapping reads to the genome: QC

Reads should mostly map to known genes

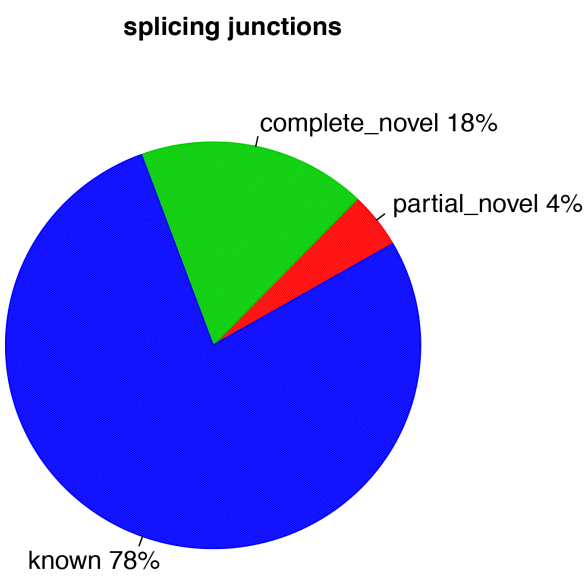
```
read_distribution.py  -i Pairend_StrandSpecific_51mer_Human_hg19.bam -r hg19.refseq.bed12
```

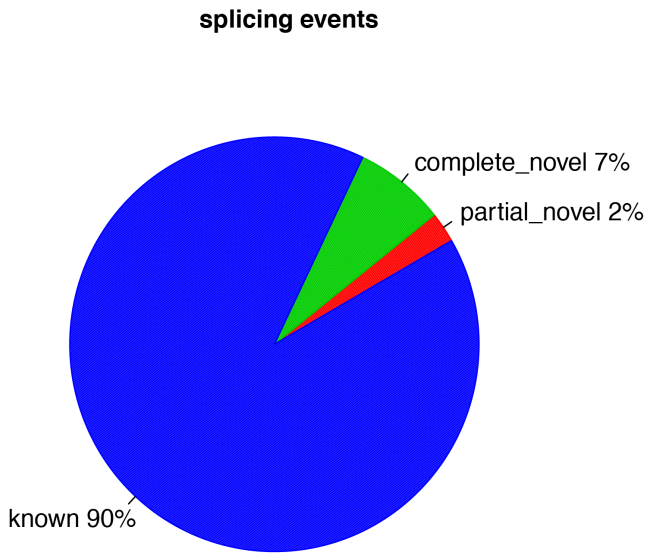
Output:

Group	Total_bases	Tag_count	Tags/Kb
CDS_Exons	33302033	20002271	600.63
5'UTR_Exons	21717577	4408991	203.01
3'UTR_Exons	15347845	3643326	237.38
Introns	1132597354	6325392	5.58
TSS_up_1kb	17957047	215331	11.99
TSS_up_5kb	81621382	392296	4.81
TSS_up_10kb	149730983	769231	5.14
TES_down_1kb	18298543	266161	14.55
TES_down_5kb	78900674	729997	9.25
TES_down_10kb	140361190	896882	6.39

Mapping reads to the genome: QC

Most splice event should be known and canonical (GU-AG)

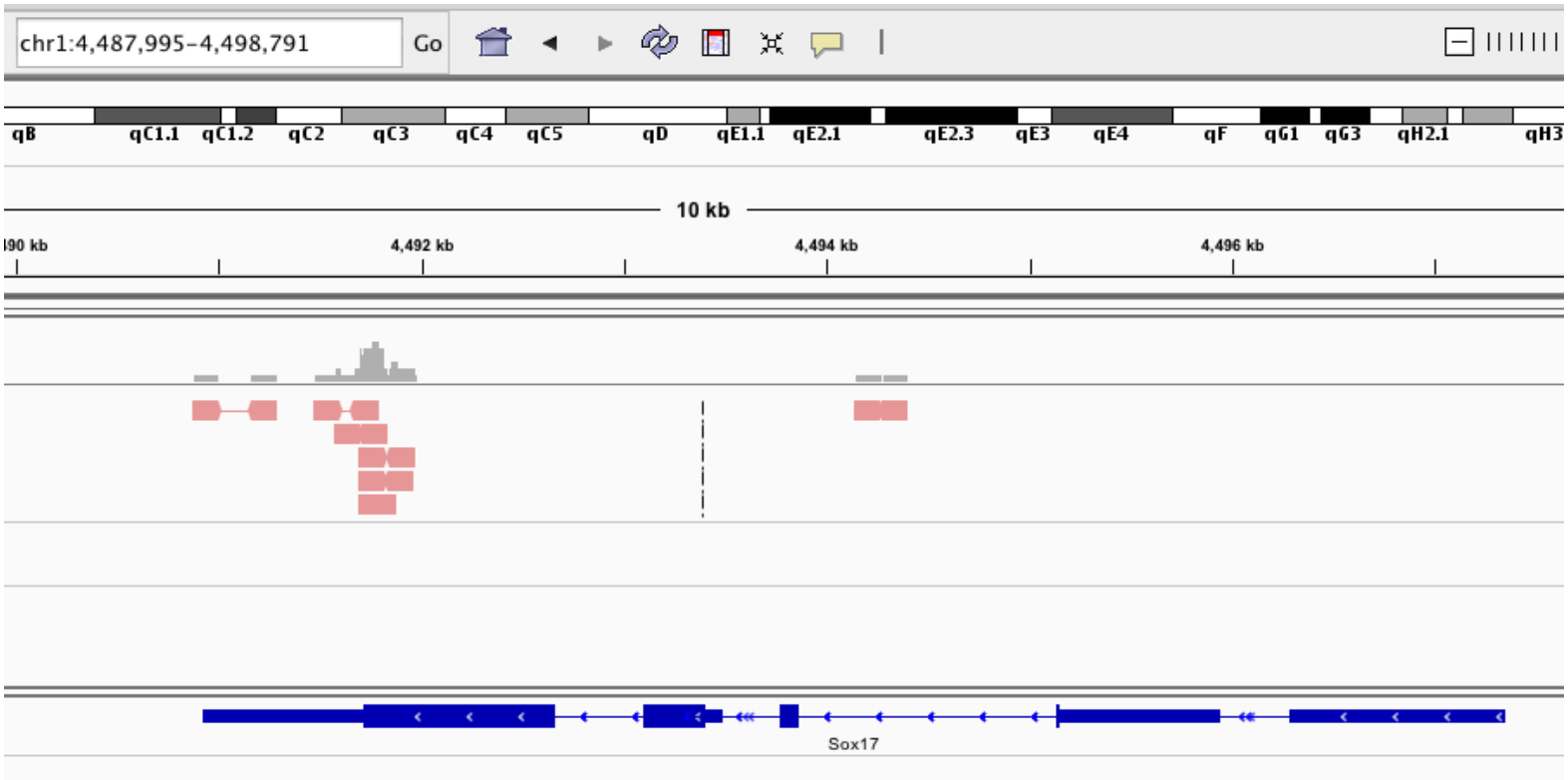




Available tools

RseQC, Picard, QualiMap

Counting reads



Available tools

HTSeq, featureCounts, R

Counting reads

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	Transcript	P1822_1	P1822_2	P1822_3	P1822_4	P1822_5	P1822_6	P1822_7	P1822_8	P1822_9	P1822_10	P1822_11	P1822_12	P1822_13	P1822_14	P1822_15	P1822_16
2	ENSMUSG00000102693	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30	ENSMUSG00000088000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
31	ENSMUSG00000103265	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
32	ENSMUSG00000103922	7	7	7	4	1	12	3	6	14	3	9	3	9	7	9	7
33	ENSMUSG00000033845	972	860	878	1085	1058	1009	992	1143	947	1059	970	1147	801	837	1042	927
34	ENSMUSG00000102275	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
35	ENSMUSG00000025903	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
36	ENSMUSG00000104217	16	13	17	16	22	17	12	27	11	5	12	15	8	9	9	12
37	ENSMUSG00000033813	2560	2581	2937	3904	2975	3100	3027	3417	2272	2801	2266	3294	2491	2578	2554	2806
38	ENSMUSG00000062588	3	1	1	1	0	1	0	3	3	0	4	0	2	1	0	0
39	ENSMUSG00000103280	1	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0
40	ENSMUSG00000002459	7	10	5	7	4	6	3	8	2	5	7	8	1	5	4	1
41	ENSMUSG00000091305	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
42	ENSMUSG00000102653	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
43	ENSMUSG00000085623	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
44	ENSMUSG00000091665	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
45	ENSMUSG00000033793	3682	3757	4414	5978	3774	4102	3815	4250	4193	4962	4240	5694	3565	3757	3849	4094
46	ENSMUSG00000104352	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
47	ENSMUSG00000104046	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
48	ENSMUSG00000102907	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
49	ENSMUSG00000025905	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
50	ENSMUSG00000103936	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
51	ENSMUSG00000093015	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
52	ENSMUSG00000103519	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
53	ENSMUSG00000033774	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
54	ENSMUSG00000103090	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
55	ENSMUSG00000025907	1816	2087	2088	2820	2012	2236	2065	2727	2586	2931	2813	3667	2410	2739	2479	2745
56	ENSMUSG00000090031	43	58	55	73	38	38	57	96	89	107	98	123	76	93	66	69

Available tools

HTSeq, featureCounts, R

Normalization: from counts to gene expression

RPKM & FPKM

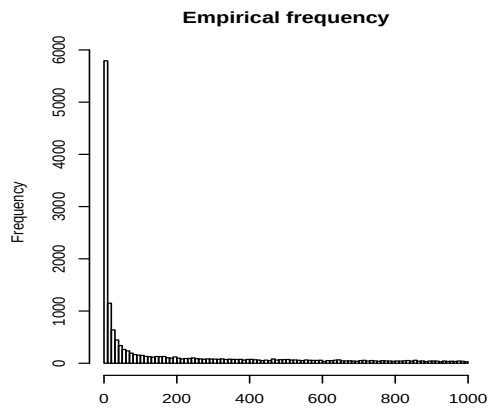
- Reads or Fragments Per Kilobase per Milion
- Correct for: differences in sequencing depth and transcript length

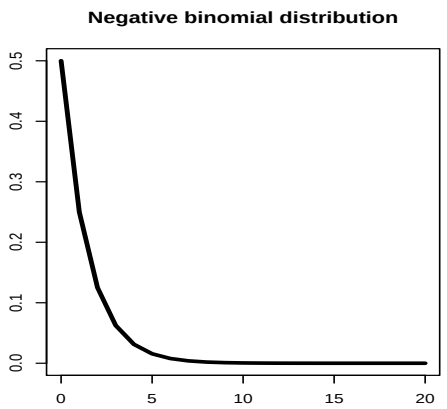
Other

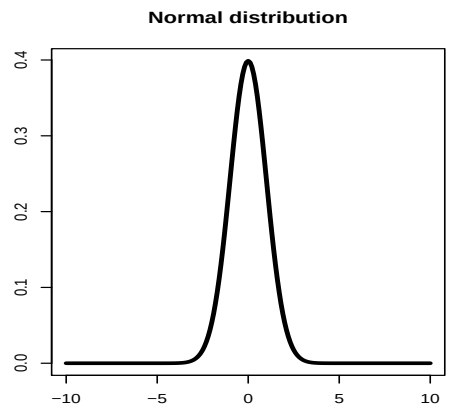
- TMM: correct for differences in transcript pool composition
- TPM: correct for transcript length distribution in RNA pool
- Voom: removing dependence of variance on the mean

Differential expression

- Reads counts do not follow normal distribution
- Typically low number of replicates or samples per group
- Recommend to use statistical packages prepared specifically for the statistical analyses of count data







Available tools

Cuffdiff, edgeR (R), limma (R), DESeq (R)

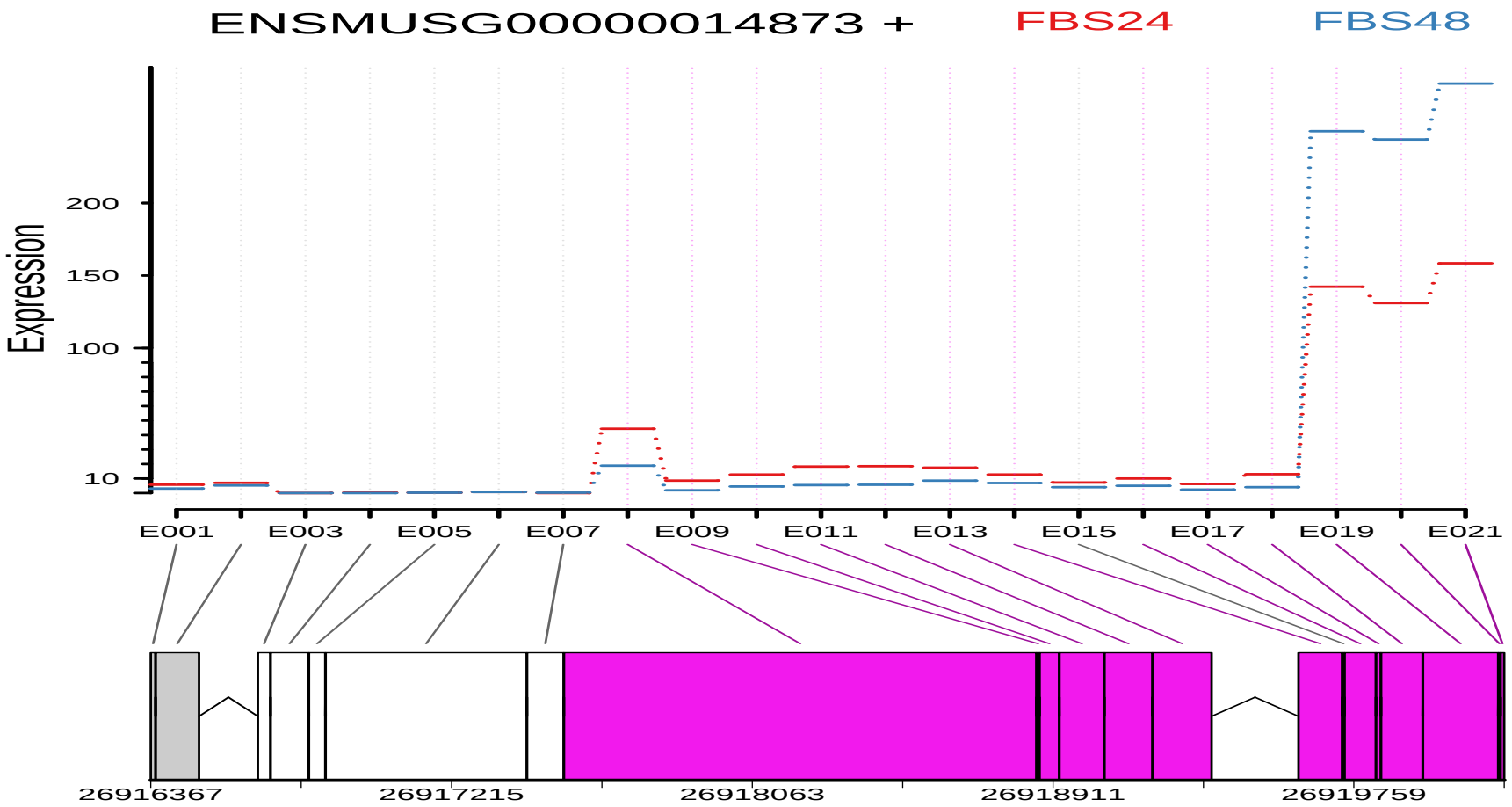
Differential expression

	A	B	C	D	E	F	G	H	I	J
1	ensembl_gene_id	ensembl_transcript_id	chromosome_name	mgi_symbol	description	logFC	logCPM	LR	PValue	FDR
2	ENSMUSG00000028328	ENSMUST00000107773	4	Tmod1	tropomodulin 1 [Source:MGI Symbol;Acc:MGI:98775]	1.971089	5.958225	581.2916	1.96E-128	2.79E-124
3	ENSMUSG00000066705	ENSMUST00000085939	9	Fxyd6	FXYD domain-containing ion transport regulator 6 [Source:MGI Symbol;Acc	3.18062	5.916499	553.8787	1.80E-122	1.28E-118
4	ENSMUSG00000049112	ENSMUST00000053306	6	Oxtr	oxytocin receptor [Source:MGI Symbol;Acc:MGI:109147]	3.820952	3.423774	375.1689	1.40E-83	6.65E-80
5	ENSMUSG00000017446	ENSMUST00000124861	11	C1qtnf1	C1q and tumor necrosis factor related protein 1 [Source:MGI Symbol;Acc:M	1.484213	7.145099	345.7577	3.56E-77	1.26E-73
6	ENSMUSG00000029123	ENSMUST00000094836	5	Stk32b	serine/threonine kinase 32B [Source:MGI Symbol;Acc:MGI:1927552]	3.453001	2.321613	338.7155	1.22E-75	3.46E-72
7	ENSMUSG00000009378	ENSMUST00000009522	19	Slc16a12	solute carrier family 16 (monocarboxylic acid transporters), member 12 [Sc	4.173029	3.89466	335.706	5.50E-75	1.30E-71
8	ENSMUSG00000025355	ENSMUST00000026411	10	Mmp19	matrix metallopeptidase 19 [Source:MGI Symbol;Acc:MGI:1927899]	1.940915	8.973932	328.4969	2.04E-73	4.15E-70
9	ENSMUSG00000029671	ENSMUST00000128245	6	Wnt16	wingless-type MMTV integration site family, member 16 [Source:MGI Syml	2.339149	5.673738	315.6779	1.27E-70	2.25E-67
10	ENSMUSG00000042190	ENSMUST00000047936	5	Cmklr1	chemokine-like receptor 1 [Source:MGI Symbol;Acc:MGI:109603]	2.518748	3.540638	305.0157	2.66E-68	4.20E-65
11	ENSMUSG00000028035	ENSMUST00000134701	3	Dnajb4	DnaJ (Hsp40) homolog, subfamily B, member 4 [Source:MGI Symbol;Acc:M	1.417856	7.292192	297.1316	1.39E-66	1.98E-63
12	ENSMUSG00000048960	ENSMUST00000027056	1	Prex2	phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2	1.706461	6.676335	283.7963	1.12E-63	1.44E-60
13	ENSMUSG00000002289	ENSMUST00000002360	17	Angptl4	angiopoietin-like 4 [Source:MGI Symbol;Acc:MGI:1888999]	-1.73049	7.972378	282.7705	1.87E-63	2.22E-60

Available tools

Cuffdiff, edgeR (R), limma (R), DESeq2 (R)

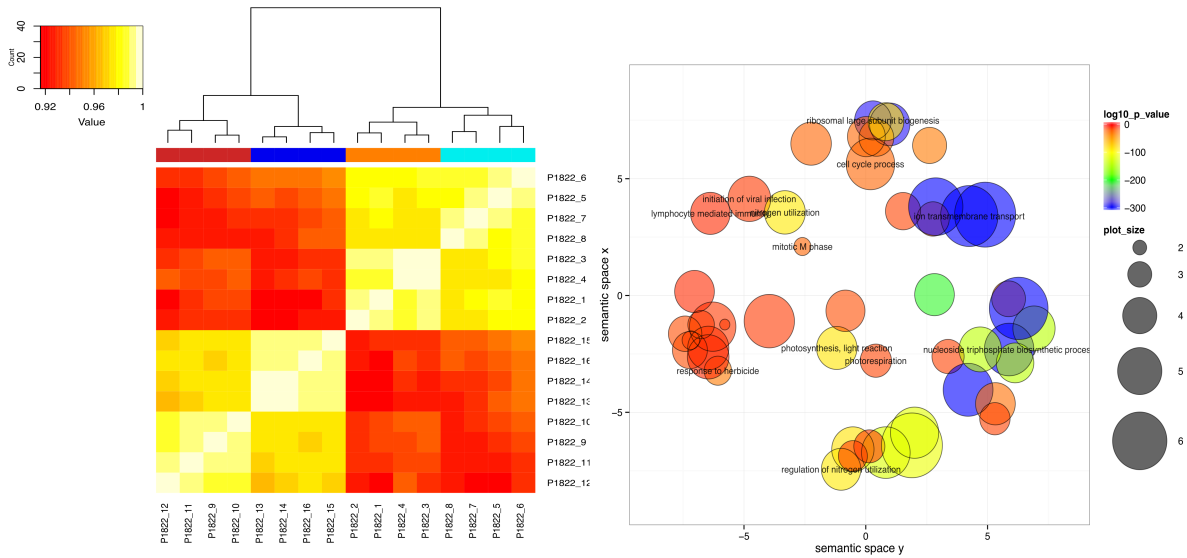
Differential expression



Available tools

Cuffdiff, edgeR (R), limma (R), DESeq2 (R)

Beyond differential expression



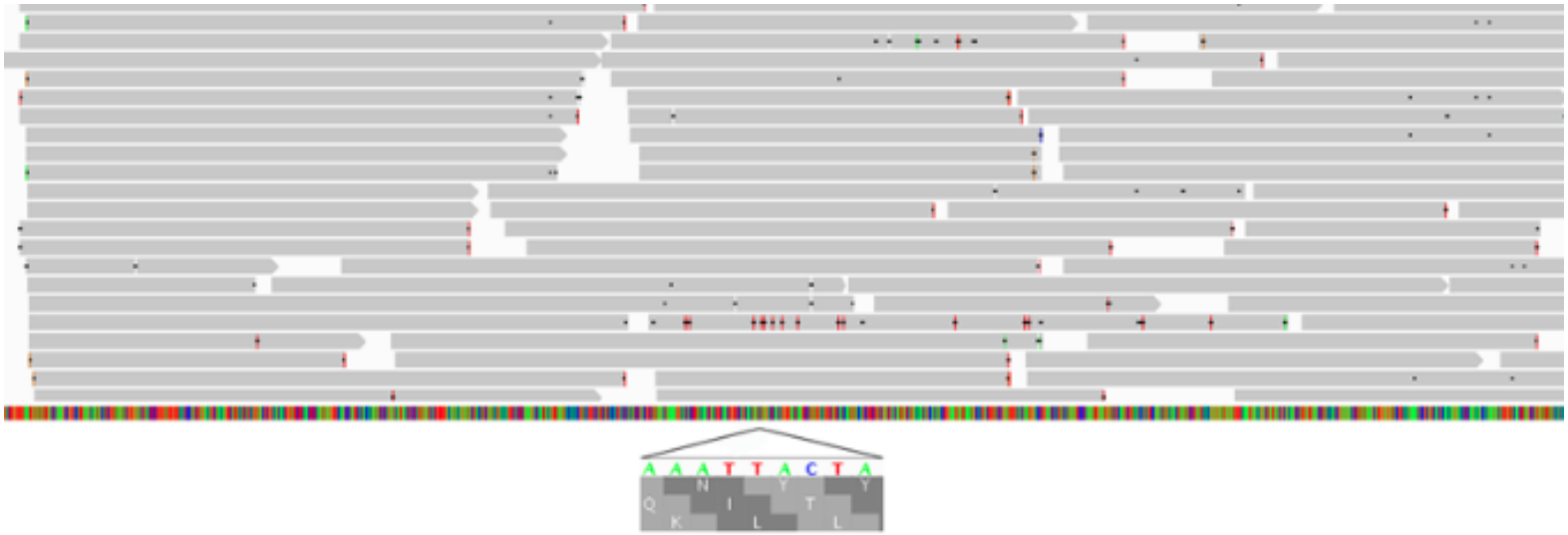
- Annotating the results e.g. with gene symbols, GO terms
- Visualising the results, e.g. Volcano plots
- Gene set analysis etc...

Available tools

bioMart (R), DAVID, GOrilla, REVIGO, ClustVis...

RNA-seq data analysis
Transcriptome assembly

Transcriptome assembly



- The goals is to reconstruct full-length transcript based on the sequence reads
- This is done via algorithms using the small overlapping reads fragments
  - If the reference genome is known: genome guided
  - If the reference genome is unknown *de novo* assembly

Transcriptome assembly

Challenges

- Genes show different levels of gene expression, hence uneven coverage among genes
- More sequencing depth is needed to represent less abundant genes and rare events
- In order to balance the abundance differences between genes, laboratory procedures for library normalisation

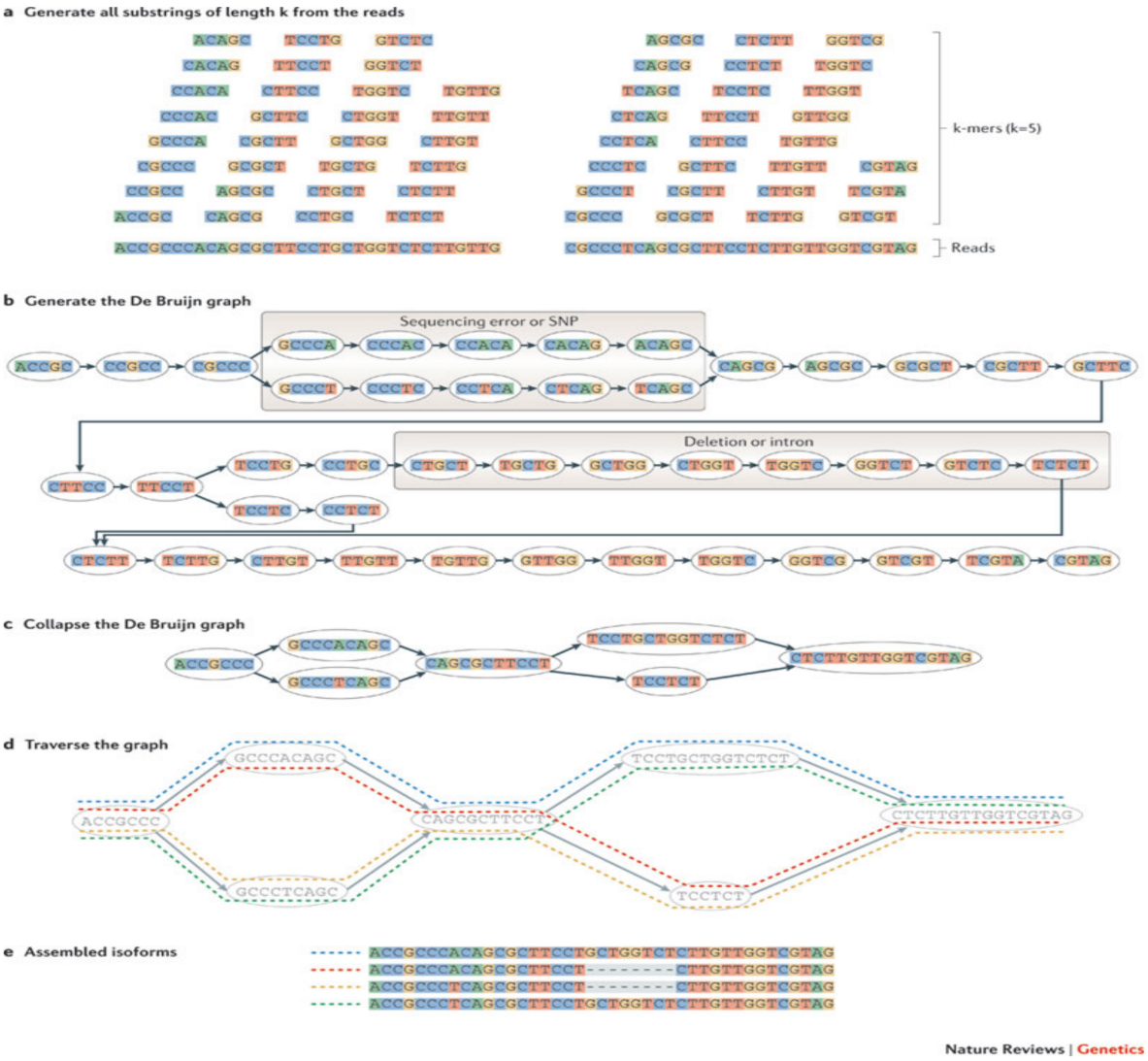
Transcriptome assembly

Available tools

- SOAP-denovo TRANS
- Oases
- Trans-ABYSS
- Trinity

All of them uses de Bruijn graphs to cope with the data and many of them have been developed from a genome assembly program

Trinity


a Generate all substrings of length k from the reads
k-mers (k=5)
Reads
b Generate the De Bruijn graph
Sequencing error or SNP
Deletion or intron
c Collapse the De Bruijn graph
d Traverse the graph
e Assembled isoforms
Nature Reviews | Genetics

Summary

- Many different expertises needed for RNA-seq experiment
  - Think ahead, plan wisely, ask for help
  - If your experimental design is wrong nothing will help
- Assess and try to improve the quality of raw reads
  - use QC tools and talk to sequencing centre
- If reference genome is available
  - get a corresponding genome annotation
  - align your reads using spliced alignment
  - in well-annotated genomes most reads should map to known genes
  - use tools designed for statistical analyses of sequencing count data (bioconductor)

Summary

- If interested in transcriptome assembly
  - use reference genome to guide it, if available
  - send lots of time in assessing the results e.g. by comparing related species, looking at ORFs
  - consider merging with other data sources
  - consider trying different assembler
- Ensure that your experimental design allows addressing the question of interest
  - More replicates translates into more power for differential gene expression and easier publication process

Exercises

Exercises

Exercise 1

- Tophat: Align reads to reference genome, using genome annotations & allowing for spliced read mapping
- Cufflinks: Extract transcripts from spliced read alignments
- Cuffmerge: Merge results from multiple Cufflinks runs
- Cuffdiff: Detect differentially expressed genes

Exercise 2

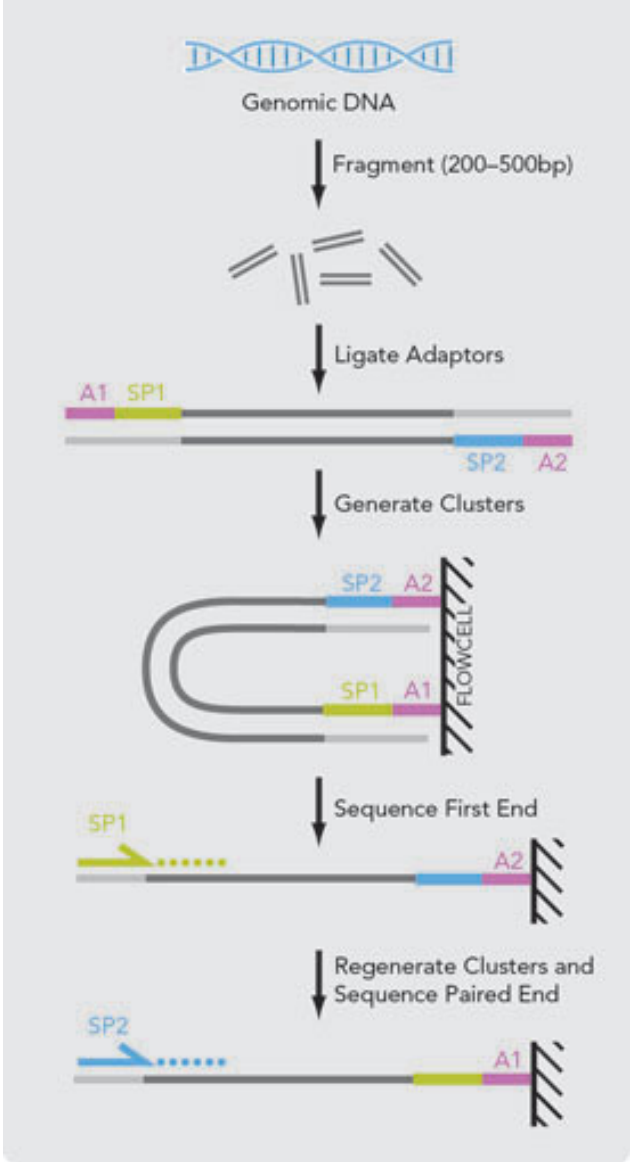
- Reconstruct the transcriptome (Trinity)
- Explore the transcriptome (command line)

Questions?

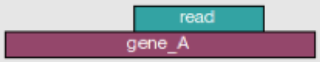
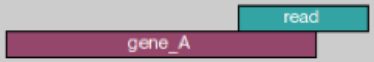
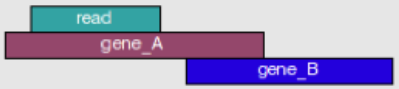
.fastq Machine output

```
@SRR038845.3 HWI-EAS038:6:1:0:1938 length=36
CAACGAGTTCACACCTTGGCCGACAGGCCCGGGTAA
+SRR038845.3 HWI-EAS038:6:1:0:1938 length=36
BA@7>B=>:>>7@7@>>9=BAA?;>52;>:9=8.=A
@SRR038845.41 HWI-EAS038:6:1:0:1474 length=36
CCAATGATTTTTTTCCGTGTTTCAGAATACGGTTAA
+SRR038845.41 HWI-EAS038:6:1:0:1474 length=36
BCCBA@BB@BBBBAB@B9B@=BABA@A:@693:@B=
@SRR038845.53 HWI-EAS038:6:1:1:360 length=36
GTTCAAAAAGAACTAAATTGTGTCAATAGAAAACTC
+SRR038845.53 HWI-EAS038:6:1:1:360 length=36
BBCBBBBBB@@BAB?BBBBCBC>BBBAA8>BBBAA@
```

.fastq Machine output: Paired-end (PE) sequencing

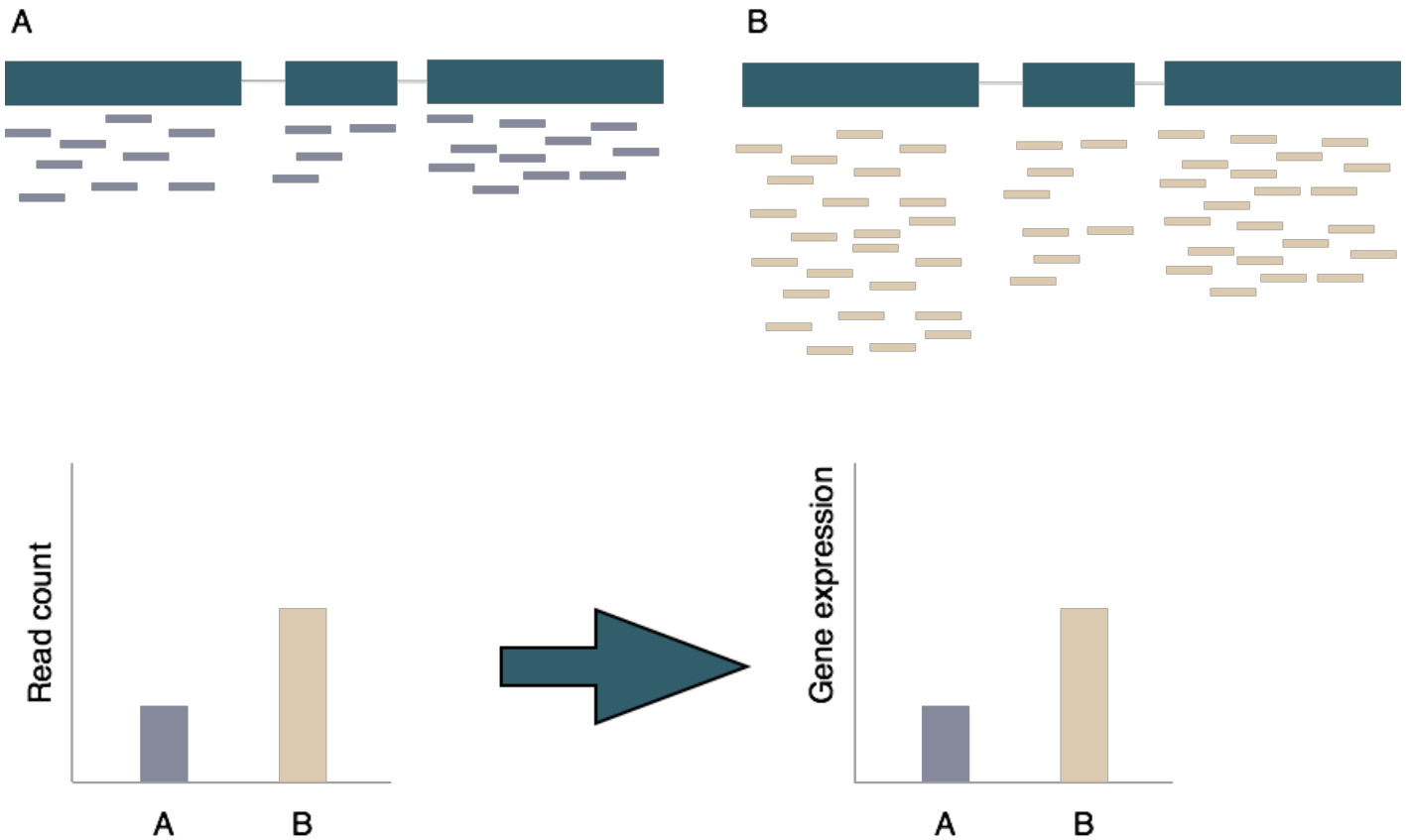


Counting reads

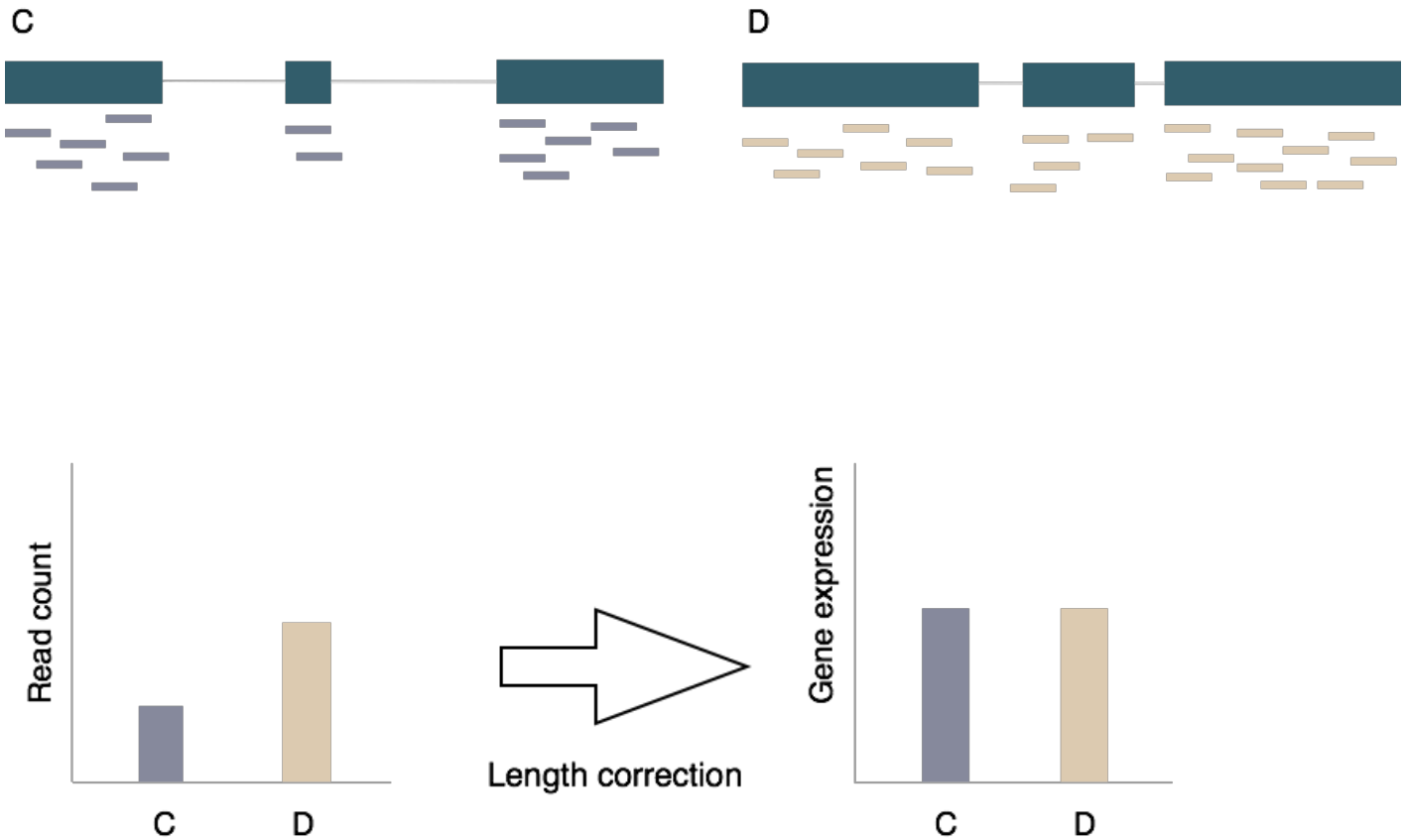
	union	intersection _strict	intersection _nonempty
	gene_A	gene_A	gene_A
	gene_A	no_feature	gene_A
	gene_A	no_feature	gene_A
	gene_A	gene_A	gene_A
	gene_A	gene_A	gene_A
	ambiguous	gene_A	gene_A
	ambiguous	ambiguous	ambiguous

from: http://www-huber.embl.de/users/anders/HTSeq/doc/count.html

From counts to gene expression



From counts to gene expression



Experimental design

- Count reads (convert to RPKM/FPKM?)
- Small number of reads (= low RPKM/FPKM values) often non-significant
- Remember that Fold change is not the same as significance

	Condition 1	Condition 2	Fold_Change	Significant?
Gene A	1	2	2-fold	No
Gene B	100	200	2-fold	Yes