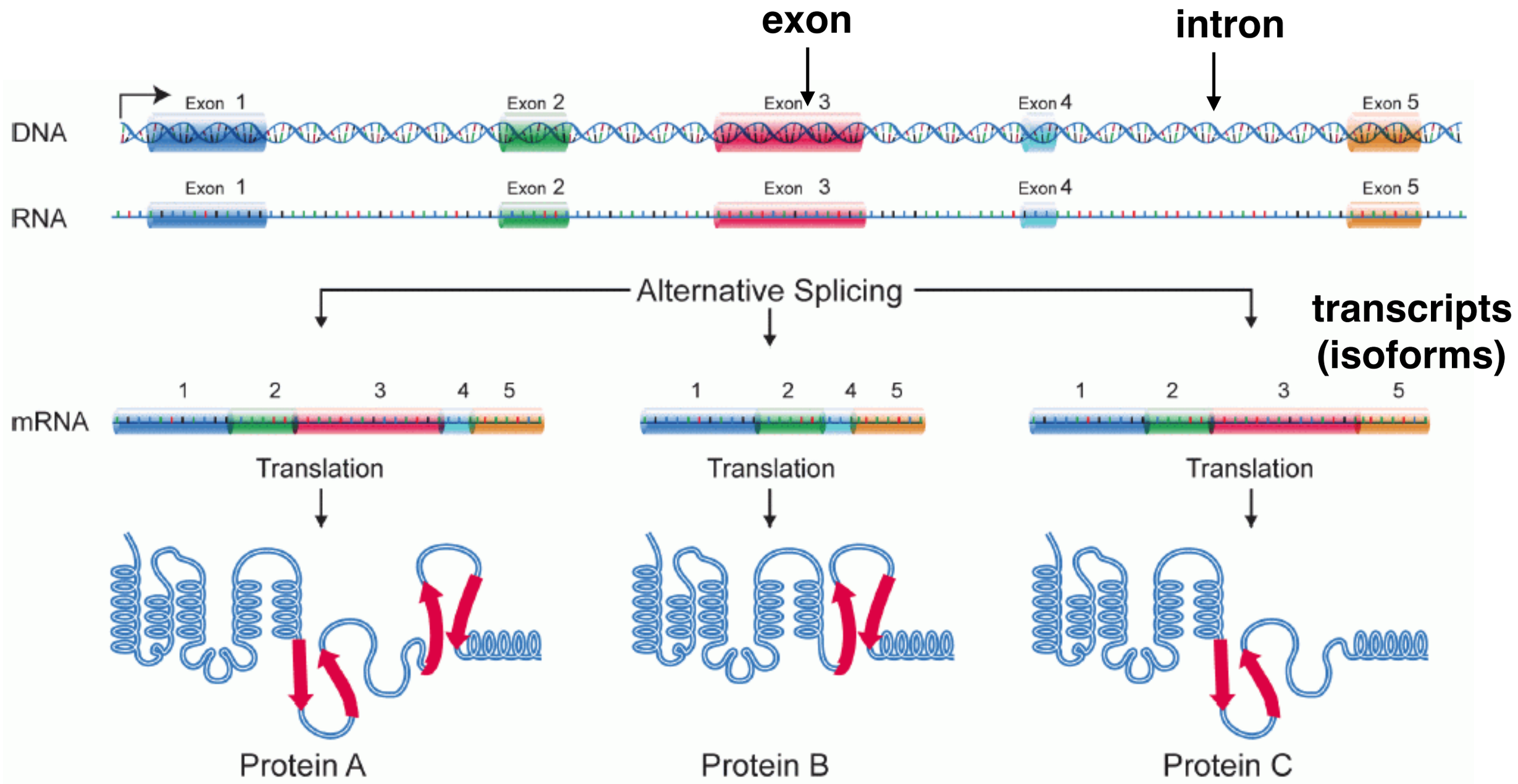
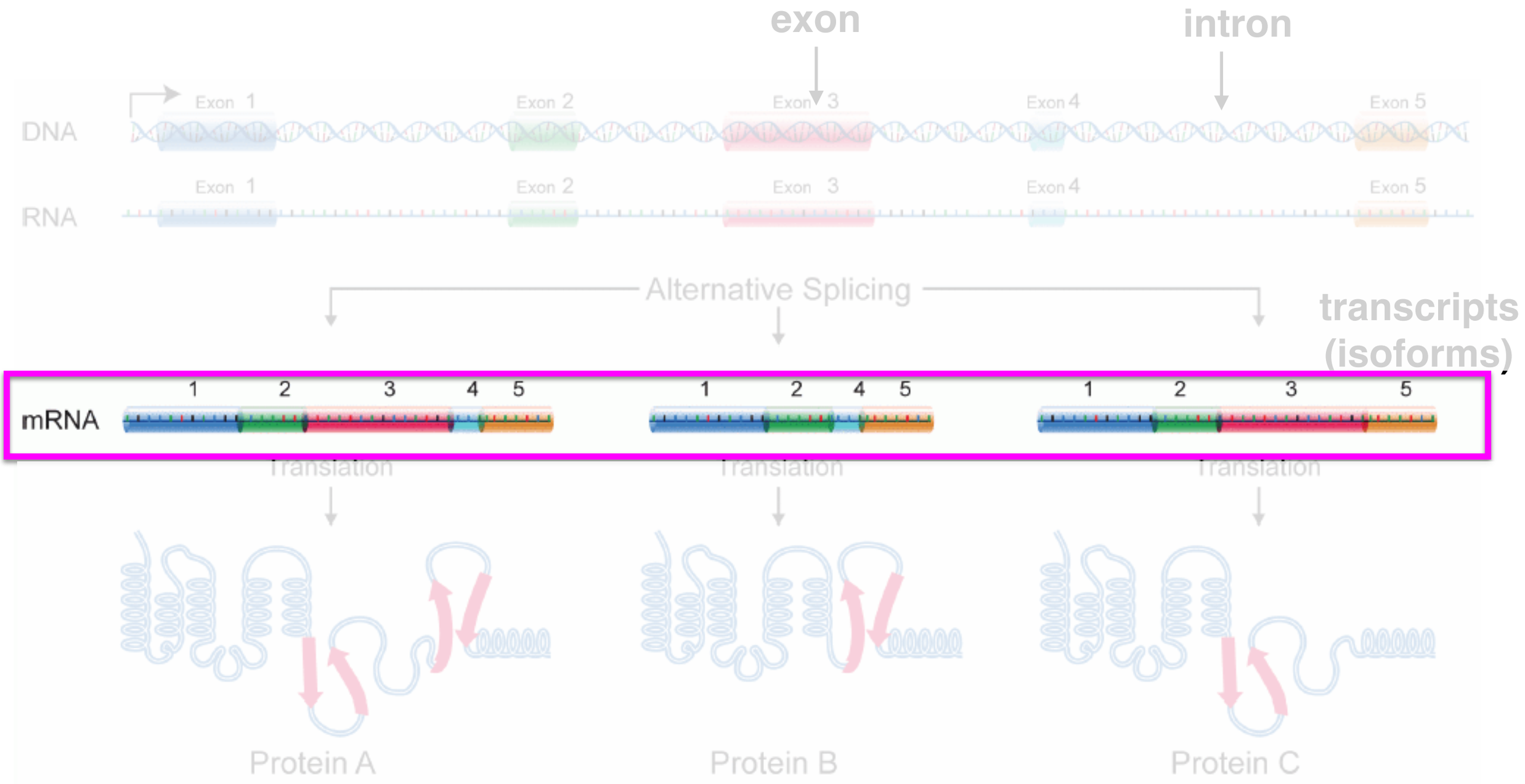
# An RNA-seq workflow

Charlotte Soneson

2018-10-29


exon
intron
DNA
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
RNA
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
Alternative Splicing
transcripts (isoforms)
mRNA
1 2 3 4 5
1 2 4 5
1 2 3 5
Translation
Translation
Translation
Protein A
Protein B
Protein C

Wikipedia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Wikipedia

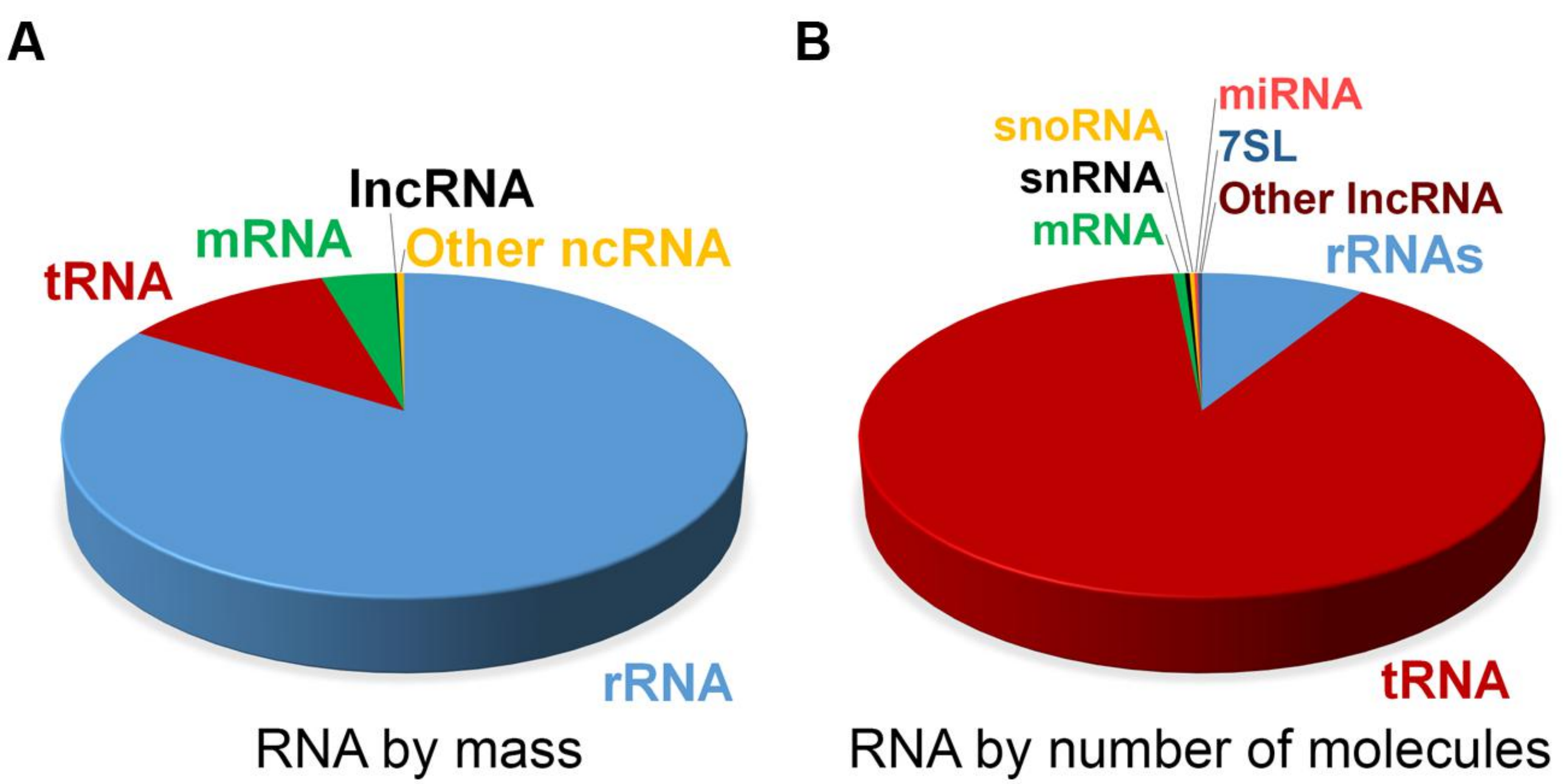



FIGURE 1. Estimate of RNA levels in a typical mammalian cell. Proportion of the various classes of RNA in mammalian somatic cells by total mass (A) and by absolute number of molecules (B). Total number of RNA molecules is estimated at roughly $10^7$ per cell. Other ncRNAs in (A) include snRNA, snoRNA, 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

# Differential analysis types for RNA-seq

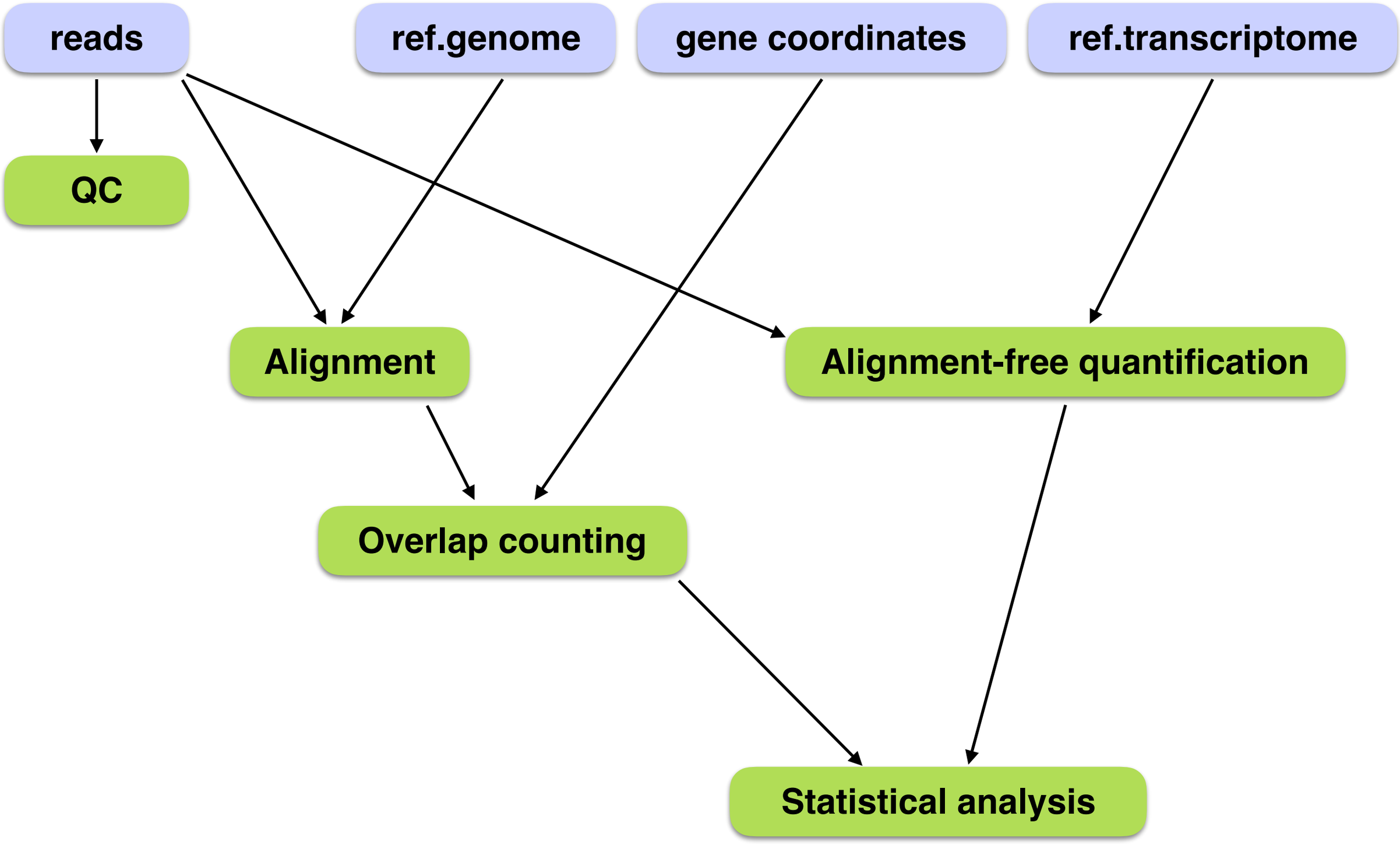
- Does the total output of a gene change between conditions? **D**ifferential **G**ene **E**xpression

- Does the expression of individual transcripts change? **D**ifferential **T**ranscript **E**xpression

- Does *any* isoform of a given gene change? **DTE+G**

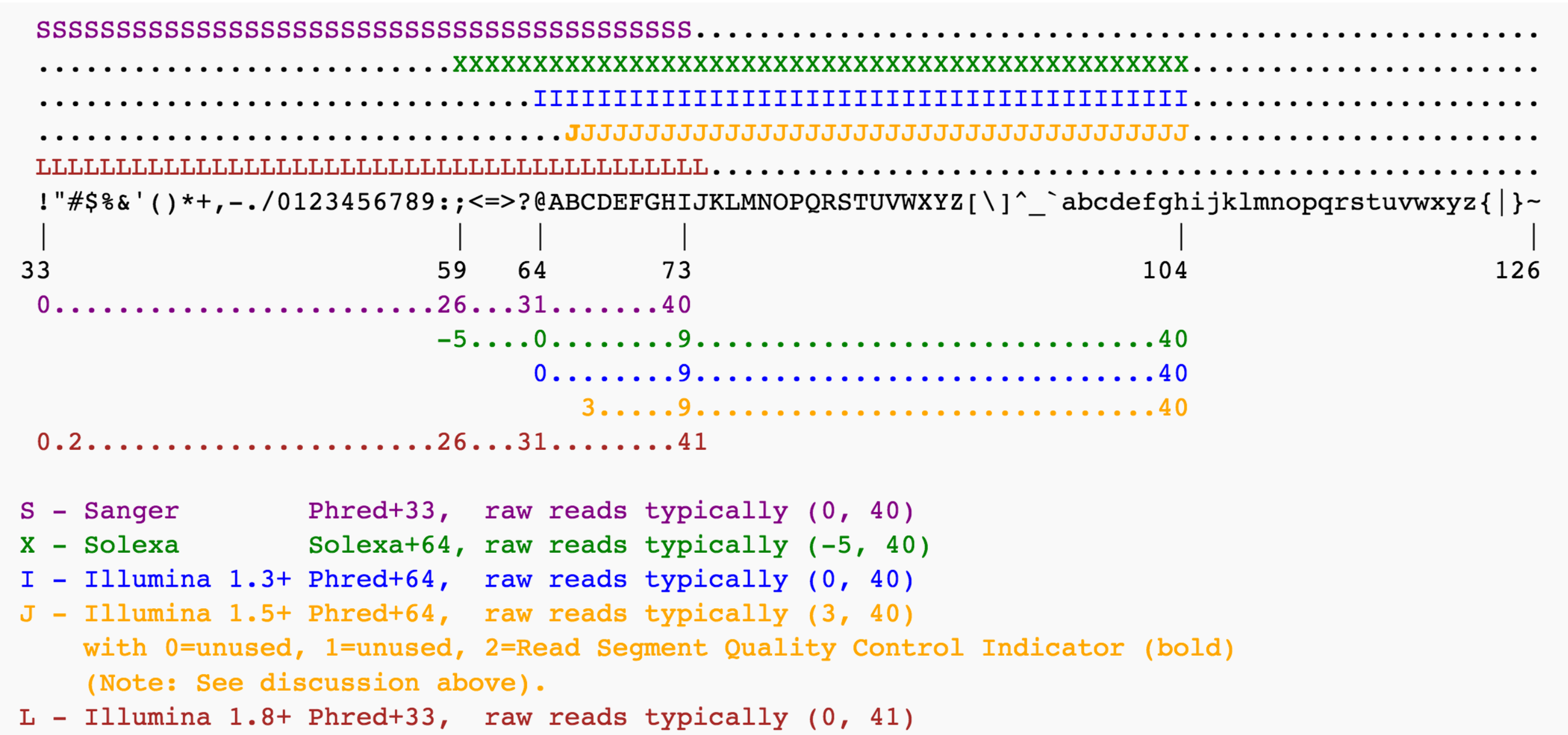
- Does the isoform composition for a given gene change? **D**ifferential **T**ranscript **U**sage**/D**ifferential **E**xon **U**sage

- need **different** computational approaches (quantifications + tests)


reads
ref.genome
gene coordinates
ref.transcriptome
QC
Alignment
Alignment-free quantification
Overlap counting
Statistical analysis

# Raw data

- FASTQ files: sequence + base quality (phred score)
- quality encoding:



# DEMO

# Reference files

- Ensembl: http://www.ensembl.org/info/data/ftp/index.html
- Gencode (human & mouse): https://www.gencodegenes.org/
- UCSC: http://hgdownload.cse.ucsc.edu/downloads.html
- iGenome: http://support.illumina.com/sequencing/sequencing_software/igenome.html
- **Be consistent!**
- Different chromosome identifiers

# Reference files

- Reference genomes and annotations are continuously refined, extended and improved

- Keep track of version and be consistent!

| SPECIES | UCSC VERSION | RELEASE DATE | RELEASE NAME | STATUS |
|---|---|---|---|---|
| **MAMMALS** | | | | |
| Human | hg38 | Dec. 2013 | Genome Reference Consortium GRCh38 | Available |
| | hg19 | Feb. 2009 | Genome Reference Consortium GRCh37 | Available |
| | hg18 | Mar. 2006 | NCBI Build 36.1 | Available |
| | hg17 | May 2004 | NCBI Build 35 | Available |
| | hg16 | Jul. 2003 | NCBI Build 34 | Available |
| | hg15 | Apr. 2003 | NCBI Build 33 | Archived |
| | hg13 | Nov. 2002 | NCBI Build 31 | Archived |
| | hg12 | Jun. 2002 | NCBI Build 30 | Archived |
| | hg11 | Apr. 2002 | NCBI Build 29 | Archived (data only) |
| | hg10 | Dec. 2001 | NCBI Build 28 | Archived (data only) |
| | hg8 | Aug. 2001 | UCSC-assembled | Archived (data only) |
| | hg7 | Apr. 2001 | UCSC-assembled | Archived (data only) |
| | hg6 | Dec. 2000 | UCSC-assembled | Archived (data only) |
| | hg5 | Oct. 2000 | UCSC-assembled | Archived (data only) |
| | hg4 | Sep. 2000 | UCSC-assembled | Archived (data only) |
| | hg3 | Jul. 2000 | UCSC-assembled | Archived (data only) |

# Ex: Ensembl, GRCh38.94

- ftp://ftp.ensembl.org/pub/release-94/fasta/homo_sapiens/dna/

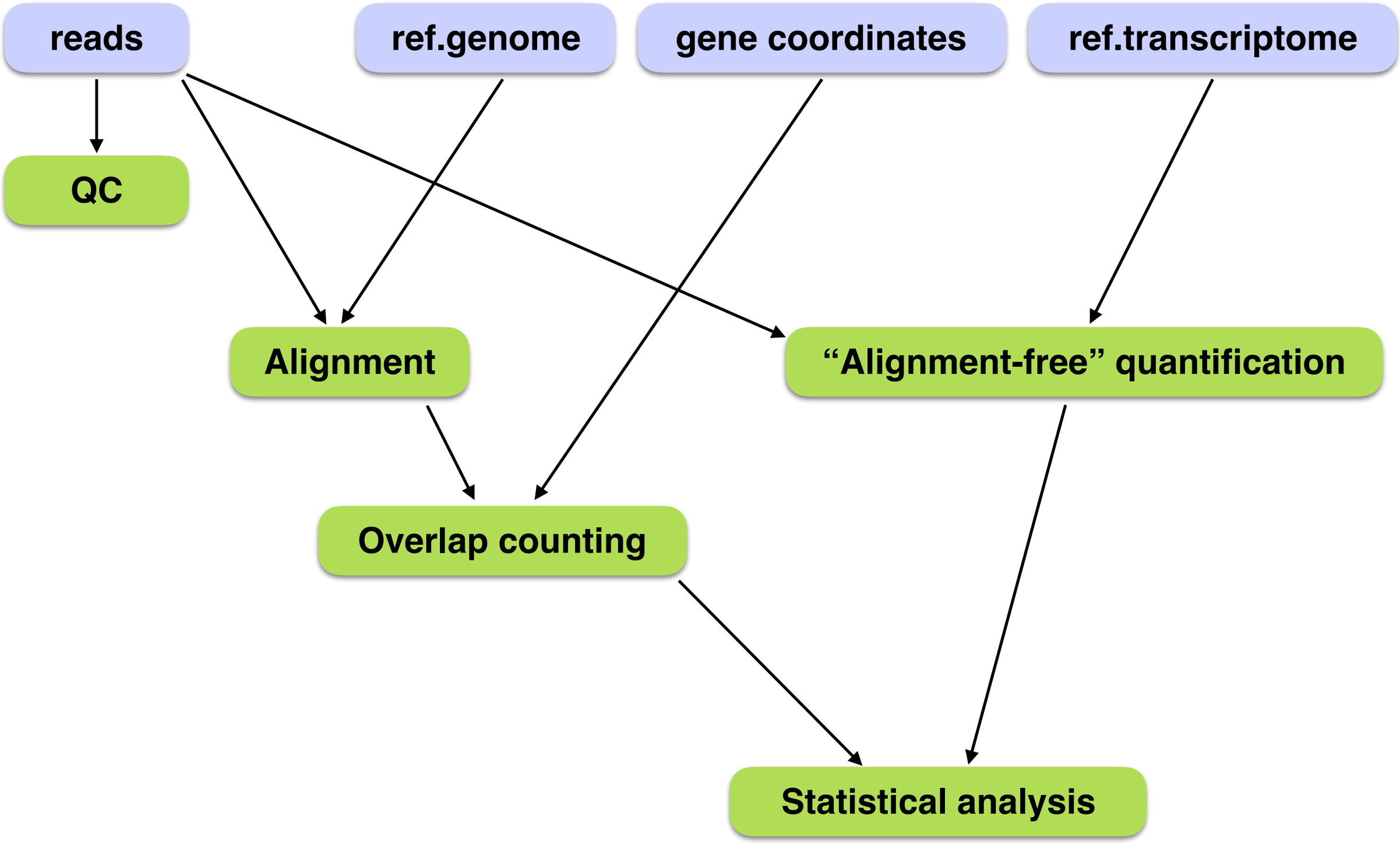
```
---------
TOPLEVEL
---------
These files contains all sequence regions flagged as toplevel in an Ensembl
schema. This includes chromsomes, regions not assembled into chromosomes and
N padded haplotype/patch regions.


-----------------
PRIMARY ASSEMBLY
-----------------
Primary assembly contains all toplevel sequence regions excluding haplotypes
and patches. This file is best used for performing sequence similarity searches
where patch and haplotype sequences would confuse analysis.
```

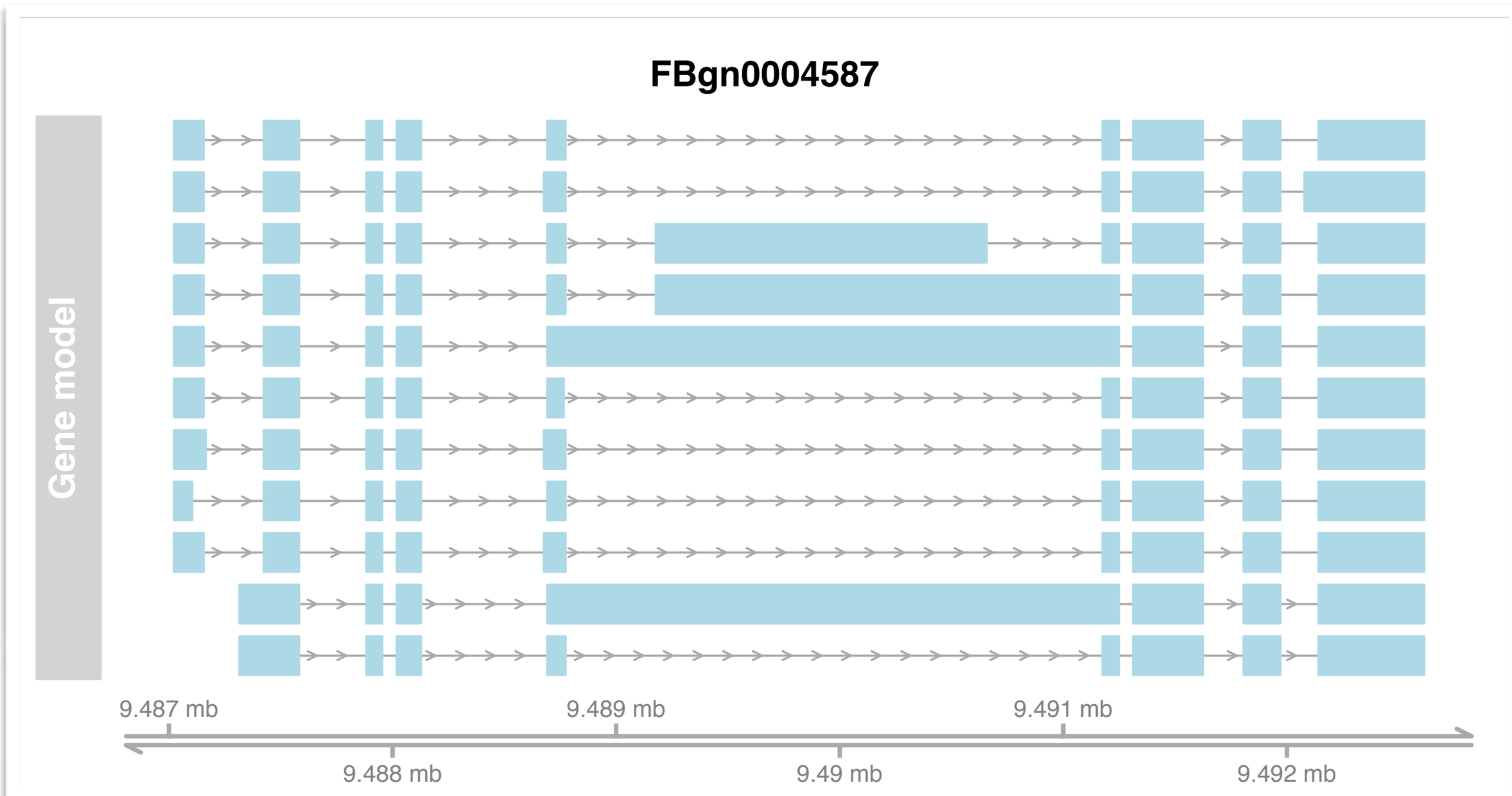
# DEMO

# Preprocessing

- We often want to compare abundance (expression) of genes or other features between conditions
- Data come as sequencing reads
- Preprocessing turns these into an abundance table


reads
ref.genome
gene coordinates
ref.transcriptome
QC
Alignment
“Alignment-free” quantification
Overlap counting
Statistical analysis

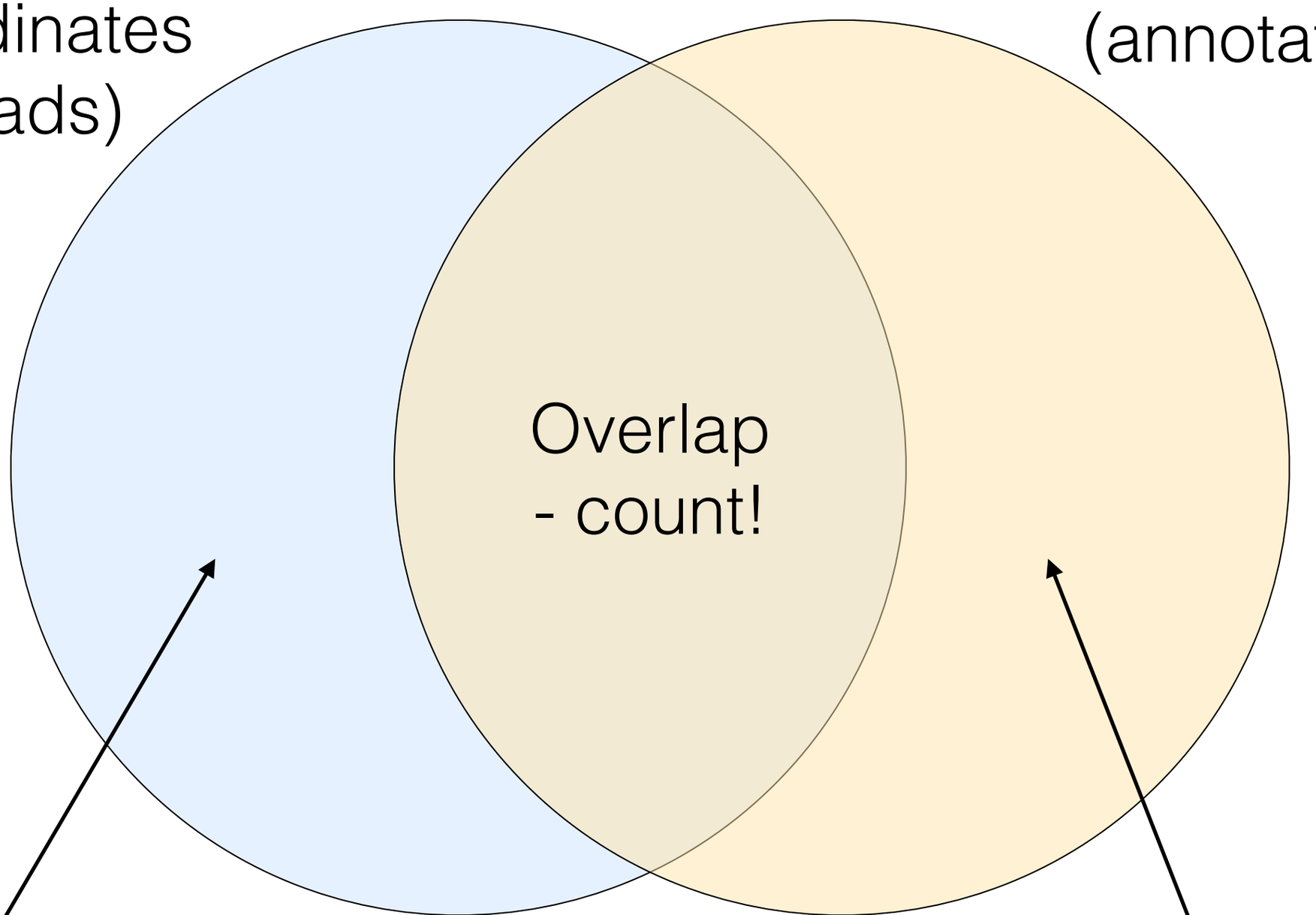
# Genome vs transcriptome alignment



# DEMO

Alignment coordinates (reads)

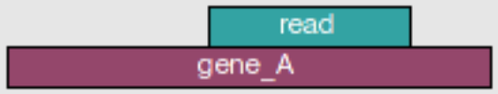
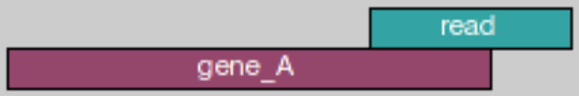
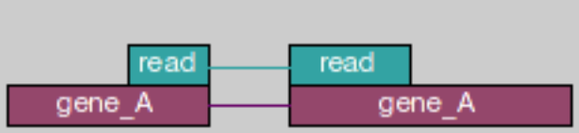
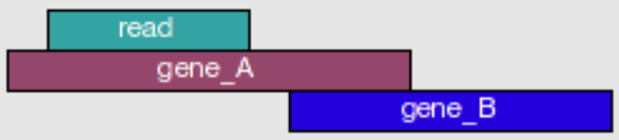
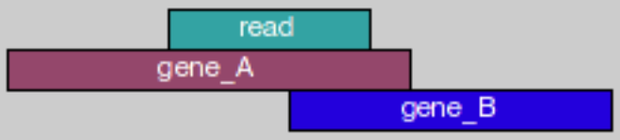
Gene coordinates (annotation)




Reads from “unannotated” genes

Non-expressed genes

**The annotation matters!**

| | 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 |

# The annotation matters!

# DEMO

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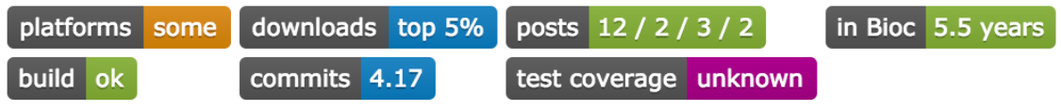
- Italy, 14 minutes ago
- Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, 35

https://bioconductor.org/packages/release/bioc/html/Rsubread.html

# Rsubread




## Subread sequence alignment for R

Bioconductor version: Release (3.3)

Provides powerful and easy-to-use tools for analyzing next-gen sequencing read data. Includes quality assessment of sequence reads, read alignment, read summarization, exon-exon junction detection, fusion detection, detection of short and long indels, absolute expression calling and SNP calling. Can be used with reads generated from any of the major sequencing platforms including Illumina GA/HiSeq/MiSeq, Roche GS-FLX, ABI SOLiD and LifeTech Ion PGM/Proton sequencers.

Author: Wei Shi and Yang Liao with contributions from Jenny Zhiyin Dai and Timothy Triche, Jr.

Maintainer: Wei Shi <shi at wehi.edu.au>

Citation (from within R, enter `citation("Rsubread")`):

> Liao Y, Smyth GK and Shi W (2013). "The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote." *Nucleic Acids Research*, **41**, pp. e108.

### Installation

To install this package, start R and enter:

```
## try http:// if https:// URLs are not supported
source("https://bioconductor.org/biocLite.R")
biocLite("Rsubread")
```

### Documentation

To view documentation for the version of this package installed in your system, start R and enter:

```
browseVignettes("Rsubread")
```

| | | |
|---|---|---|
| PDF | R Script | Rsubread Vignette |
| PDF | | Reference Manual |
| Text | | NEWS |

# DEMO

Homo_sapiens.GRC... | 18 | 18:47,006,705-47,019,802 | Go

13 kb

47,008 kb | 47,010 kb | 47,012 kb | 47,014 kb | 47,016 kb | 47,018 kb

SRR1039508_chr18_hisat2.bam
rage

[0 - 6912]

SRR1039508_chr18_hisat2.bam

Homo_sapiens.GRCh37.75.chr18

ENST00000579820
ENST00000581232
RPL17
ENST00000318240
ENST00000410758
ENST00000584364
ENST00000582782
ENST00000584895
ENST00000607313
ENST00000582392
C18orf32
ENST00000580261
ENST00000332968
SNORD58B
ENST00000577910

# DEMO

# Gene identifiers (ex: BCL2)

- Ensembl ID: ENSG00000171791
- Entrez ID: 596
- Vega ID: OTTHUMG00000132791
- HGNC ID: 990
- RefSeq ID: NG_009361.1
- UCSC ID: uc002liu.2
- Official symbol: BCL2
- Synonyms: PPP1R50, Bcl-2
- …

**Typically, no 1-1 mapping between different ID types**

# Gene symbols can change over time

| ensembl_gene_id | may2009_hgnc_symbol | may2012_hgnc_symbol | dec2013_hgnc_symbol |
|---|---|---|---|
| ENSG00000162825 | KIAA1245 | | NBPF8 |

| feb2014_hgnc_symbol | aug2014_hgnc_symbol | oct2014_hgnc_symbol | dec2014_hgnc_symbol |
|---|---|---|---|
| NBPF8 | NBPF8 | NBPF8 | NBPF8 |

| mar2015_hgnc_symbol | may2015_hgnc_symbol | jul2015_hgnc_symbol | sep2015_hgnc_symbol |
|---|---|---|---|
| NBPF8 | NBPF8 | NBPF20 | NBPF20 |

| dec2015_hgnc_symbol | mar2016_hgnc_symbol | jul2016_hgnc_symbol | oct2016_hgnc_symbol |
|---|---|---|---|
| NBPF20 | NBPF20 | NBPF20 | NBPF20 |

| ensembl_gene_id | may2009_hgnc_symbol | may2012_hgnc_symbol | dec2013_hgnc_symbol |
|---|---|---|---|
| ENSG00000179412 | | | HNRNPCP5 |

| feb2014_hgnc_symbol | aug2014_hgnc_symbol | oct2014_hgnc_symbol | dec2014_hgnc_symbol |
|---|---|---|---|
| HNRNPCP5 | HNRNPCL2 | HNRNPCL2 | HNRNPCL4 |

| mar2015_hgnc_symbol | may2015_hgnc_symbol | jul2015_hgnc_symbol | sep2015_hgnc_symbol |
|---|---|---|---|
| HNRNPCL4 | HNRNPCL4 | HNRNPCL4 | HNRNPCL4 |

| dec2015_hgnc_symbol | mar2016_hgnc_symbol | jul2016_hgnc_symbol | oct2016_hgnc_symbol |
|---|---|---|---|
| HNRNPCL4 | HNRNPCL4 | HNRNPCL4 | HNRNPCL4 |

# DEMO

# Normalization

- Observed counts depend on:
  - abundance
  - gene length
  - sequencing depth
  - sequencing biases
  - …
- “As-is”, not directly comparable across samples

# Normalization

raw count for gene *i* in sample *j*

relative abundance

normalization factor

$$C_{ij} \sim NB(\mu_{ij} = s_{ij} q_{ij}, \theta_i)$$

dispersion

- $s_{ij}$ is a normalization factor (or offset) in the model
- counts are not explicitly scaled
  - important exception: voom/limma (followed by explicit modeling of mean-variance association)

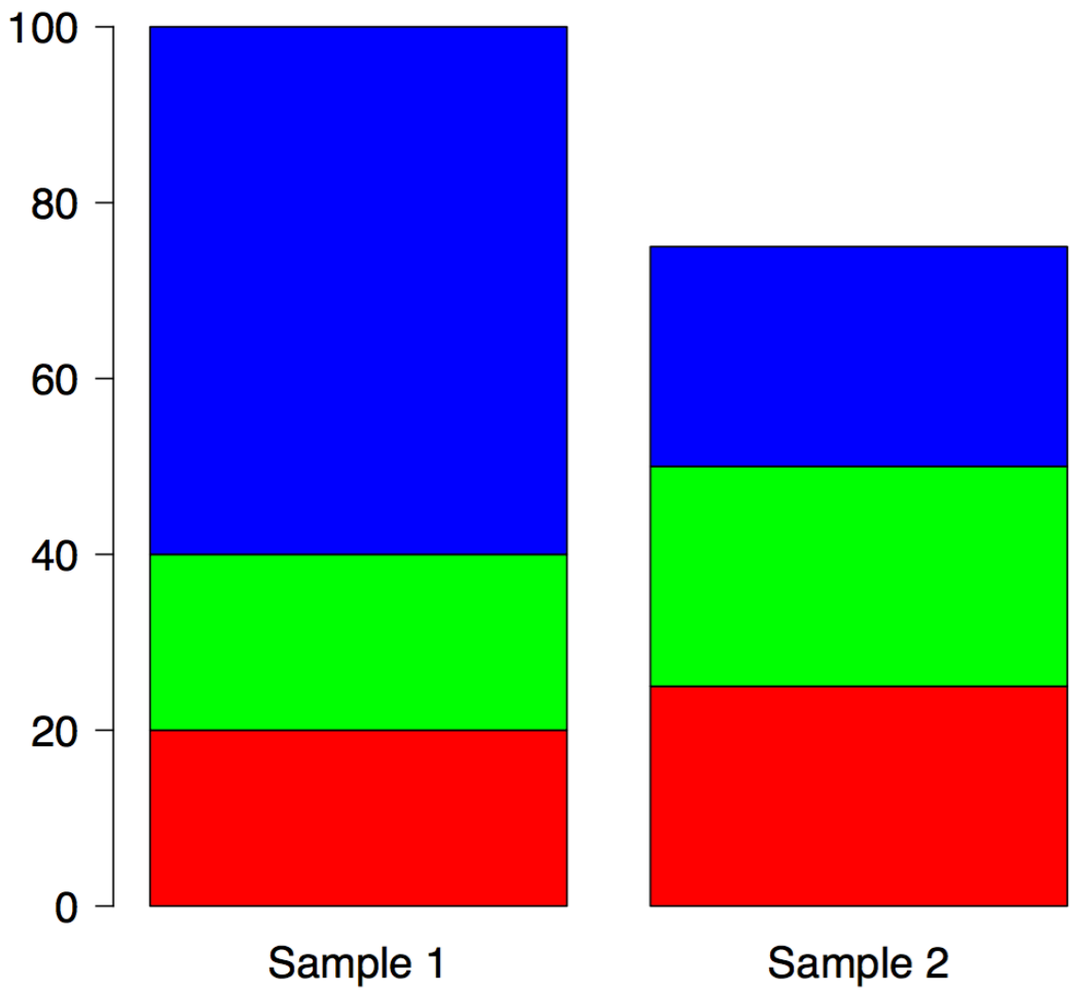
# How to calculate normalization factors?

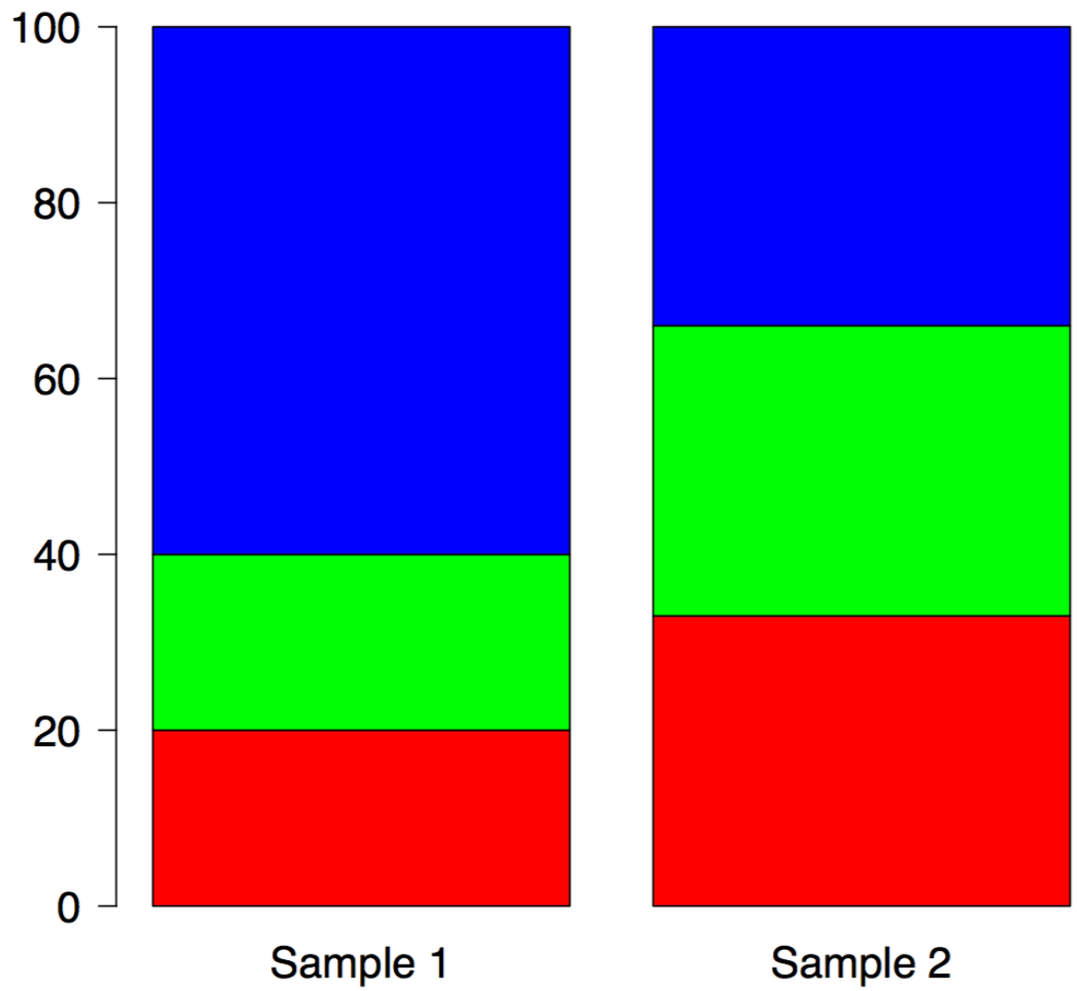
- Attempt 1: **total count** (library size)
  - Define a reference sample (one of the observed samples or a “pseudo-sample”) - gives a “target library size”
- Normalization factor for sample *j* is defined by

$$\frac{\text{total count in sample } j}{\text{total count in reference sample}}$$

# The influence of RNA composition

- Observed counts are relative
- High counts for some genes are “compensated” by low counts for other genes



# How to calculate normalization factors?

- Attempt 2: total count (library size) * compensation for differences in composition

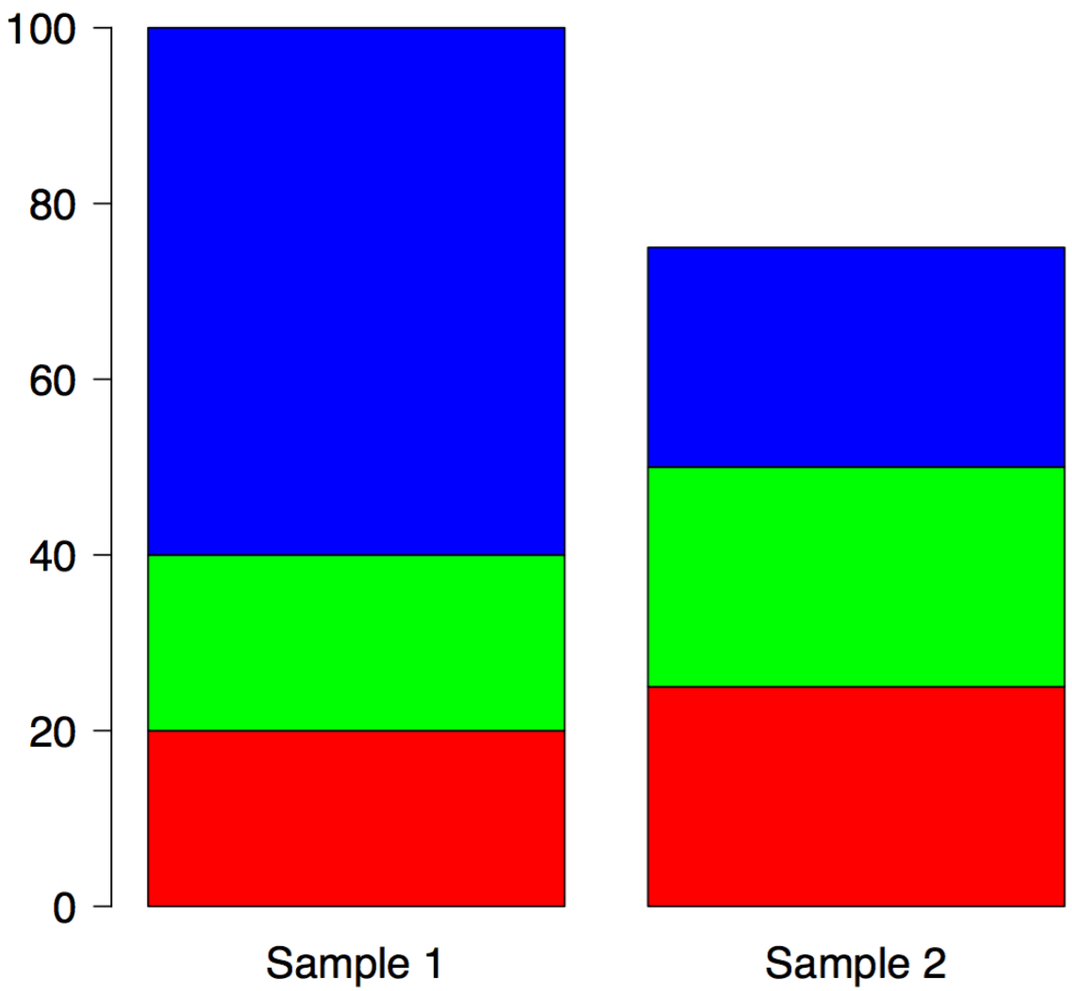
- Idea: use only non-differentially expressed genes to compute the normalization factor

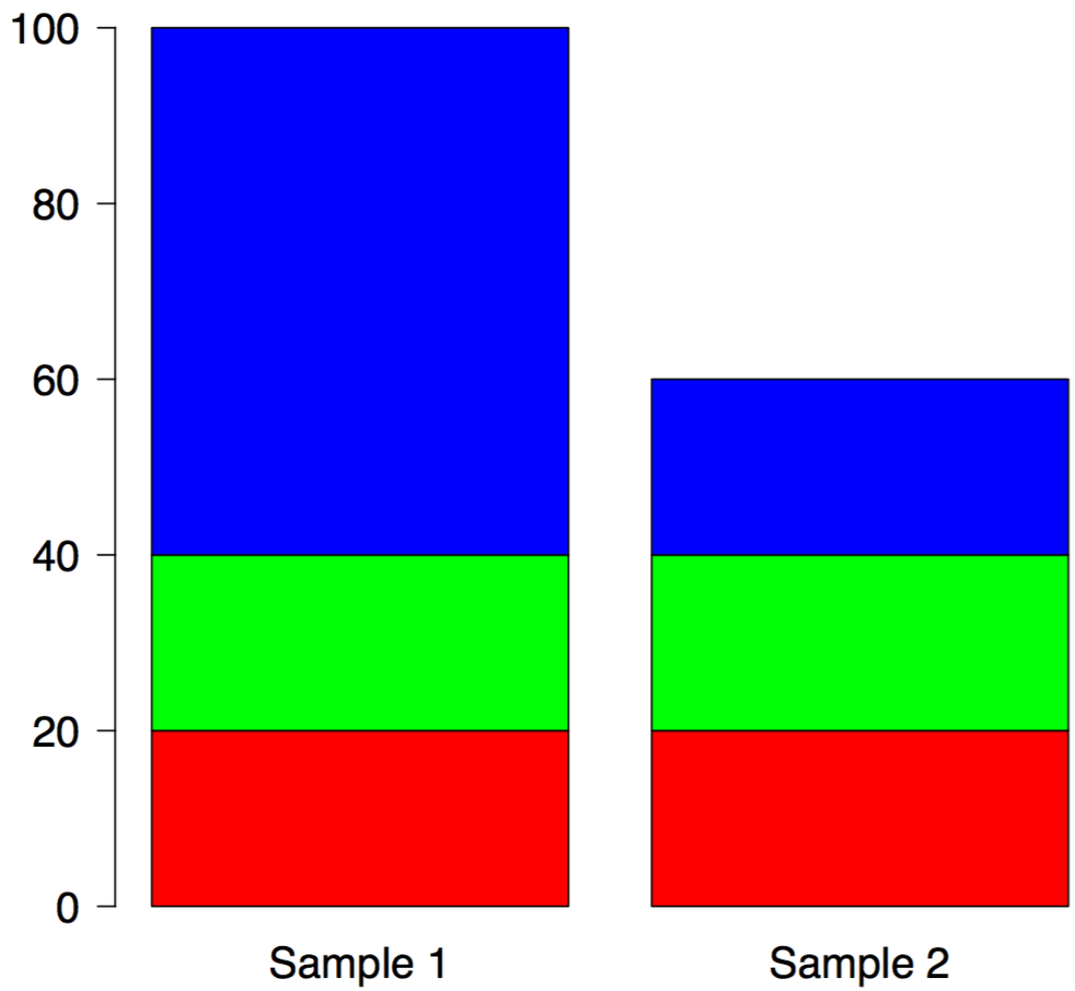
- Implemented by both edgeR (TMM) and DESeq2 (median count ratio)

- Both these methods assume that most genes are not differentially expressed

# How to calculate normalization factors?

- Attempt 2: total count (library size) * compensation for differences in composition



# DEMO

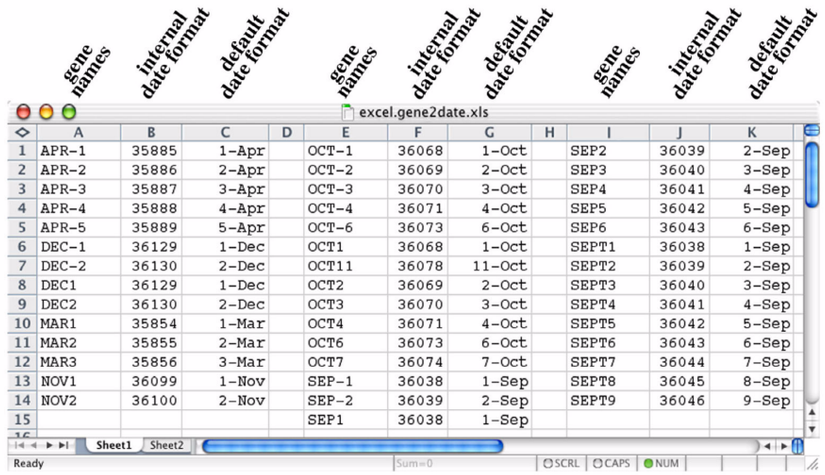
# Be careful when importing identifiers in Excel

| gene names | internal date format | default date format | gene names | internal date format | default date format | gene names | internal date format | default date format |
|---|---|---|---|---|---|---|---|---|
| APR-1 | 35885 | 1-Apr | OCT-1 | 36068 | 1-Oct | SEP2 | 36039 | 2-Sep |
| APR-2 | 35886 | 2-Apr | OCT-2 | 36069 | 2-Oct | SEP3 | 36040 | 3-Sep |
| APR-3 | 35887 | 3-Apr | OCT-3 | 36070 | 3-Oct | SEP4 | 36041 | 4-Sep |
| APR-4 | 35888 | 4-Apr | OCT-4 | 36071 | 4-Oct | SEP5 | 36042 | 5-Sep |
| APR-5 | 35889 | 5-Apr | OCT-6 | 36073 | 6-Oct | SEP6 | 36043 | 6-Sep |
| DEC-1 | 36129 | 1-Dec | OCT1 | 36068 | 1-Oct | SEPT1 | 36038 | 1-Sep |
| DEC-2 | 36130 | 2-Dec | OCT11 | 36078 | 11-Oct | SEPT2 | 36039 | 2-Sep |
| DEC1 | 36129 | 1-Dec | OCT2 | 36069 | 2-Oct | SEPT3 | 36040 | 3-Sep |
| DEC2 | 36130 | 2-Dec | OCT3 | 36070 | 3-Oct | SEPT4 | 36041 | 4-Sep |
| MAR1 | 35854 | 1-Mar | OCT4 | 36071 | 4-Oct | SEPT5 | 36042 | 5-Sep |
| MAR2 | 35855 | 2-Mar | OCT6 | 36073 | 6-Oct | SEPT6 | 36043 | 6-Sep |
| MAR3 | 35856 | 3-Mar | OCT7 | 36074 | 7-Oct | SEPT7 | 36044 | 7-Sep |
| NOV1 | 36099 | 1-Nov | SEP-1 | 36038 | 1-Sep | SEPT8 | 36045 | 8-Sep |
| NOV2 | 36100 | 2-Nov | SEP-2 | 36039 | 2-Sep | SEPT9 | 36046 | 9-Sep |
| | | | SEP1 | 36038 | 1-Sep | | | |



Zeeberg et al., BMC Bioinformatics 2004

# There is a solution




| | A | B | C | D | E | F | G | H | I | J | K |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | APR-1 | 35885 | 1-Apr | | OCT-1 | 36068 | 1-Oct | | SEP2 | 36039 | 2-Sep |
| 2 | APR-2 | 35886 | 2-Apr | | OCT-2 | 36069 | 2-Oct | | SEP3 | 36040 | 3-Sep |
| 3 | APR-3 | 35887 | 3-Apr | | OCT-3 | 36070 | 3-Oct | | SEP4 | 36041 | 4-Sep |
| 4 | APR-4 | 35888 | 4-Apr | | OCT-4 | 36071 | 4-Oct | | SEP5 | 36042 | 5-Sep |
| 5 | APR-5 | 35889 | 5-Apr | | OCT-6 | 36073 | 6-Oct | | SEP6 | 36043 | 6-Sep |
| 6 | DEC-1 | 36129 | 1-Dec | | OCT1 | 36068 | 1-Oct | | SEPT1 | 36038 | 1-Sep |
| 7 | DEC-2 | 36130 | 2-Dec | | OCT11 | 36078 | 11-Oct | | SEPT2 | 36039 | 2-Sep |
| 8 | DEC1 | 36129 | 1-Dec | | OCT2 | 36069 | 2-Oct | | SEPT3 | 36040 | 3-Sep |
| 9 | DEC2 | 36130 | 2-Dec | | OCT3 | 36070 | 3-Oct | | SEPT4 | 36041 | 4-Sep |
| 10 | MAR1 | 35854 | 1-Mar | | OCT4 | 36071 | 4-Oct | | SEPT5 | 36042 | 5-Sep |
| 11 | MAR2 | 35855 | 2-Mar | | OCT6 | 36073 | 6-Oct | | SEPT6 | 36043 | 6-Sep |
| 12 | MAR3 | 35856 | 3-Mar | | OCT7 | 36074 | 7-Oct | | SEPT7 | 36044 | 7-Sep |
| 13 | NOV1 | 36099 | 1-Nov | | SEP-1 | 36038 | 1-Sep | | SEPT8 | 36045 | 8-Sep |
| 14 | NOV2 | 36100 | 2-Nov | | SEP-2 | 36039 | 2-Sep | | SEPT9 | 36046 | 9-Sep |
| 15 | | | | | SEP1 | 36038 | 1-Sep | | | | |

- Import properly: http://www.genenames.org/help/importing-gene-symbol-data-into-excel-correctly

- The *HGNChelper* R package can help identify misrepresented gene symbols

# DEMO

# Downloading public data

- Search for data sets by keywords in GEO (https://www.ncbi.nlm.nih.gov/geo/) or ArrayExpress (https://www.ebi.ac.uk/arrayexpress/)

- Download fastq files from the European Nucleotide Archive (ENA): http://www.ebi.ac.uk/ena

- Note the number of files per sample (single- vs paired-end, one sample can have multiple *runs*)

# Assignment

- Choose **one** of the following:

  - Download the fastq file(s) for one publicly available sample. Briefly describe the sample, run FastQC on the file(s) and comment on the results.

  - Download the fastq file(s) for one publicly available sample. Briefly describe the sample, determine the appropriate parameters for running Salmon or kallisto, build a transcriptome index and quantify the transcript abundances.

  - Choose one organism and characterize its annotated transcripts. E.g., give the total number of transcripts and genes, plot the distribution of transcripts per gene, plot the distribution of transcript lengths, … [tip: look at the R packages rtracklayer and Biostrings].

# Resources

csoneson / **rnaseqworkflow**

Unwatch 4 | Star 11 | Fork 5

Code | Issues 9 | Pull requests 0 | Projects 0 | Wiki | Insights | Settings

Light-weight Snakemake workflow for preprocessing and statistical analysis of RNA-seq data

Edit

rna-seq | workflow | Manage topics

123 commits | 5 branches | 1 release | 5 contributors | MIT

https://peerj.com/preprints/27283/

## RNA sequencing data: hitchhiker's guide to expression analysis

Literature review | Bioinformatics | Computational Biology | Genomics | Data Science

Koen Van Den Berge *[1], Katharina Hembach *[2], Charlotte Soneson *[2,3], Simone Tiberi *[2], Lieven Clement[1], Michael I Love[4], Rob Patro[5], Mark Robinson[2]

October 17, 2018

# Some suggestions for further reading

- Robinson et al.: edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26(1):139-140 (2010) - **edgeR**
- Love et al.: Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15:550 (2014) - **DESeq2**
- Law et al.: voom: precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biology 15:R29 (2014) - **voom**
- Patro et al.: Accurate, fast, and model-aware transcript expression quantification with Salmon. bioRxiv http://dx.doi.org/10.1101/021592 (2015) - **Salmon**
- Bray et al.: Near-optimal probabilistic RNA-seq quantification. Nature Biotechnology 34(5):525-527 (2016) - **kallisto**
- Patro et al.: Sailfish enables alignment-free isoform quantification from RNA-seq reads using lightweight algorithms. Nature Biotechnology 32:462-464 (2014) - **Sailfish**
- Pimentel et al.: Differential analysis of RNA-Seq incorporating quantification uncertainty. bioRxiv http://dx.doi.org/10.1101/058164 (2016) - **sleuth**
- Wagner et al.: Measurement of mRNA abundance using RNA-seq data: RPKM measure is inconsistent among samples. Theory in Biosciences 131:281-285 (2012) - **TPM vs FPKM**
- Soneson et al.: Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. F1000Research 4:1521 (2016) - **ATL offsets (tximport package)**
- Li et al.: RNA-seq gene expression estimation with read mapping uncertainty. Bioinformatics 26(4):493-500 (2010) - **TPM, RSEM**
- Soneson, Matthes et al.: Isoform prefiltering improves performance of count-based methods for analysis of differential transcript usage. Genome Biology 17:12 (2016)
- Schurch et al.: How many biological replicates are needed in an RNA-seq experiment and which differential expression tool should you use? RNA 22:839-851 (2016)
- Dillies et al.: A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis. Briefings in Bioinformatics 14(6):671-683 (2013)
- Soneson & Delorenzi: A comparison of methods for differential expression analysis of RNA-seq data. BMC Bioinformatics 14:91 (2013)
- Anders et al.: Detecting differential usage of exons from RNA-seq data. Genome Research 22(10):2008-2017 (2012) - **DEXSeq**
- Goeman & Bühlmann: Analyzing gene expression data in terms of gene sets: methodological issues. Bioinformatics 23(8): 980-987 (2007) - **competitive vs self-contained gene set tests**