

# INTRODUCTION TO OMICS DATA ANALYSIS RNA-seq

Bioinformatics Course UEB-VHIR

June, 2022

**Esther Camacho**<sup>1</sup>, Mireia Ferrer<sup>1</sup>, Àlex Sánchez<sup>1,2</sup>, Angel Blanco<sup>1,2</sup>, Berta Miró<sup>1</sup>

<sup>1</sup> Unitat d'Estadística i Bioinformàtica (UEB) VHIR <sup>2</sup> Departament de Genètica Microbiologia i Estadística, UB

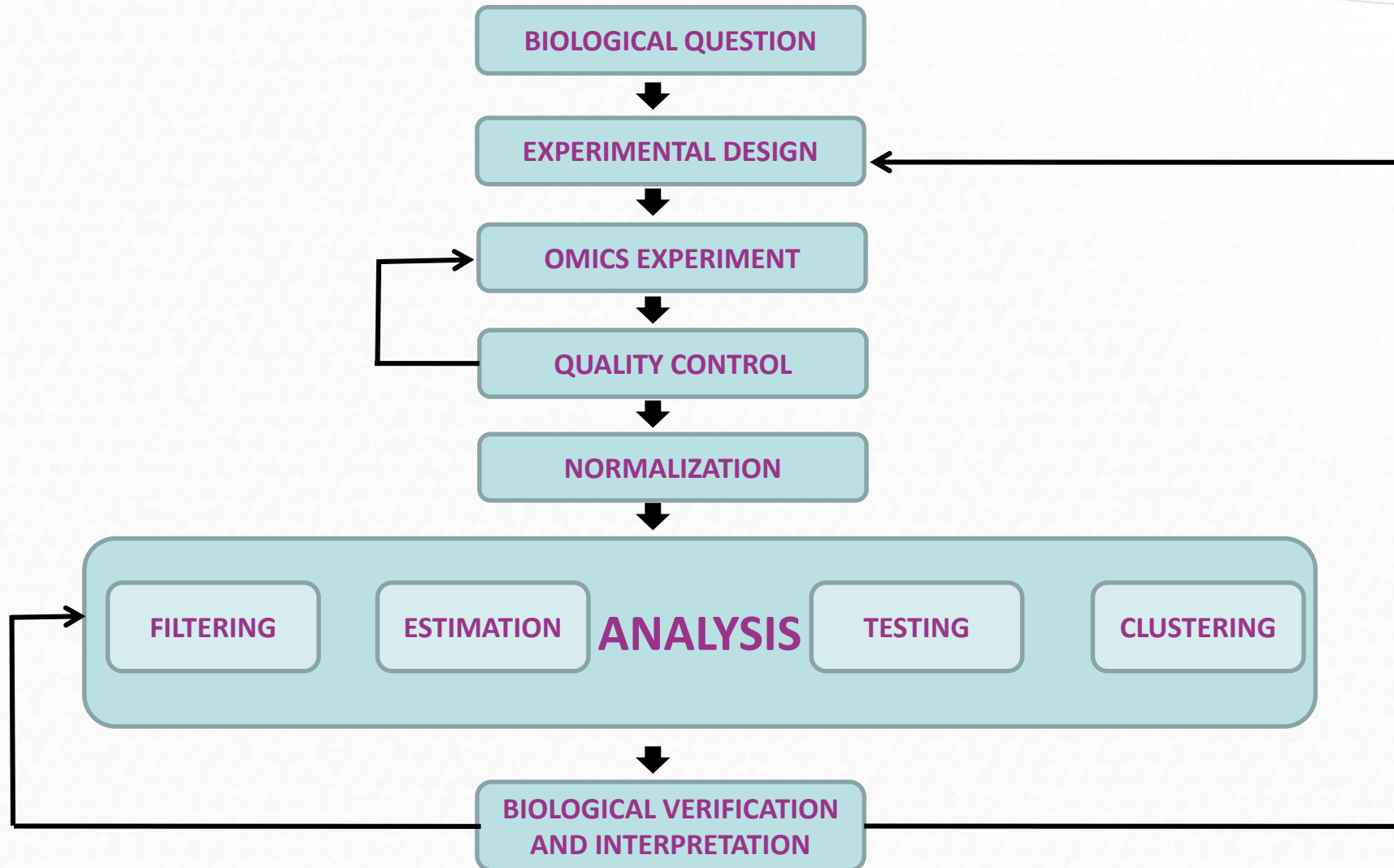
- 1. Introduction to omics data analysis**
- 2. An example of omics data analysis. RNA-seq**
  - 1. What is RNA-seq**
  - 2. Basic key concepts**
  - 3. Main challenges in RNA-seq**
  - 4. RNA-seq vs Microarrays**
  - 5. RNA-seq analysis pipeline(s)**
  - 6. Alignment**
  - 7. Transcript assembly**
  - 8. DEG Analysis**
  - 9. Visualization**

# **1. Introduction to omics data analysis**

## **2. An example of omics data analysis. RNA-seq**

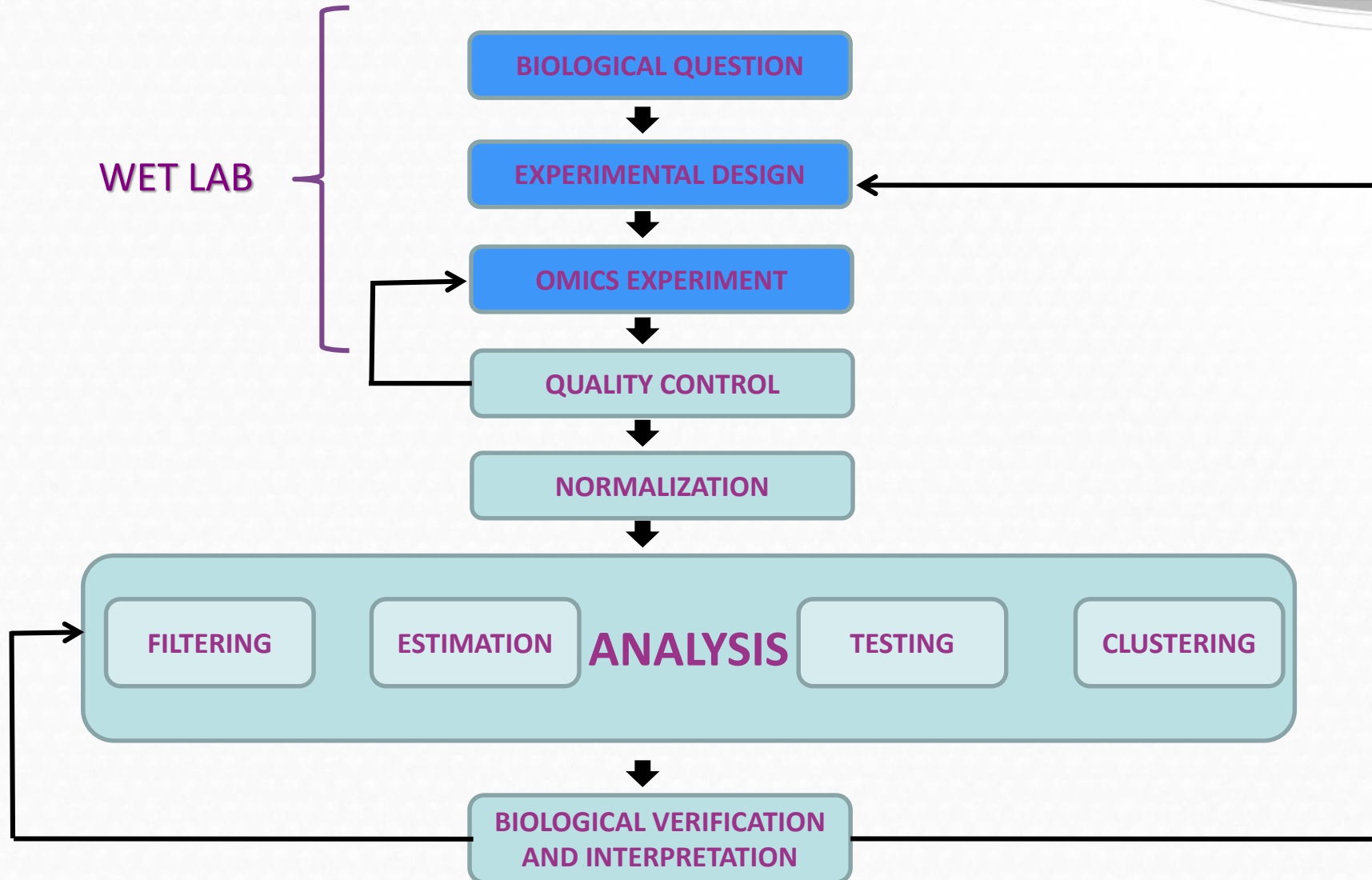
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# 1. Introduction to omic data analysis

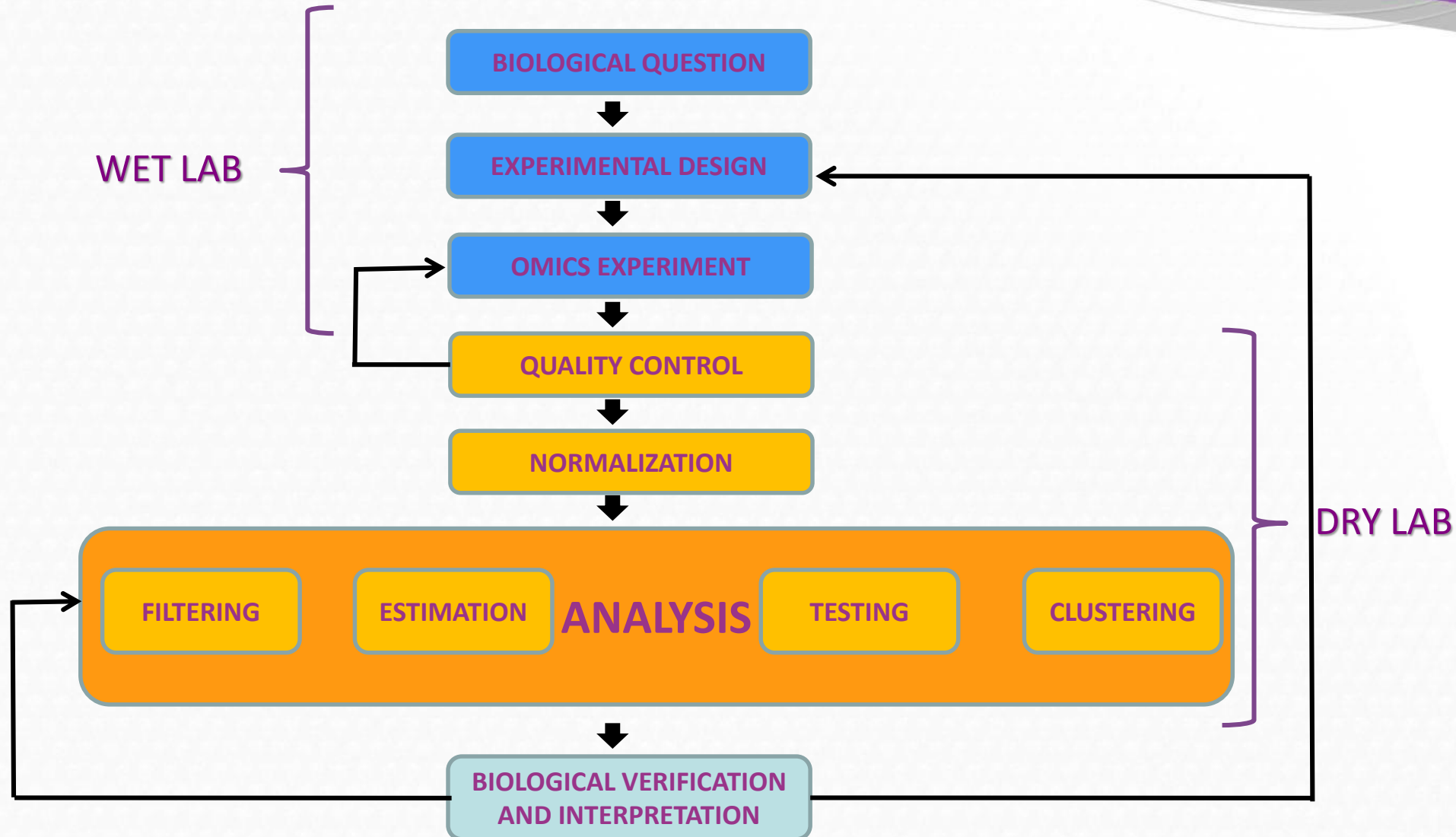




# 1. Introduction to omic data analysis



# 1. Introduction to omic data analysis



# **1. Introduction to omics data analysis**

## **2. An example of omics data analysis. RNA-seq**

### **1. What is RNA-seq**

### **2. Basic key concepts**

### **3. Main challenges in RNA-seq**

### **4. RNA-seq vs Microarrays**

### **5. RNA-seq analysis pipeline(s)**

### **6. Alignment**

### **7. Transcript assembly**

### **8. DEG Analysis**

### **9. Visualization**

## 2.1 What is RNA-seq?

### RNA-Seq

- Sequencing technique of **NGS**.
- It reveals the **presence and quantity of RNA** in a sample.
- It lets to the determination/analysis of the **transcriptome**.
- This sample is sequenced in a **particular moment**, so the transcriptome obtained is limited to this precise moment.



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RNA-seq enables the finding of:

- Alternative gene spliced transcripts,
- Post-transcriptional modifications,
- Gene fusion,
- Mutations/SNPs,
- Changes in gene expression

## 2.1 What is RNA-seq?

- Functional studies
  - **Genome may be constant** but an **experimental condition has a pronounced effect on gene expression**
    - ✓ e.g. Drug treated vs. untreated cell line
    - ✓ e.g. Wild type versus knock out mice
- Some **molecular features** can only be observed at the RNA level
  - Alternative isoforms, fusion transcripts, RNA editing
- Predicting transcript sequence from genome sequence is **difficult**
  - Alternative splicing, RNA editing, etc.

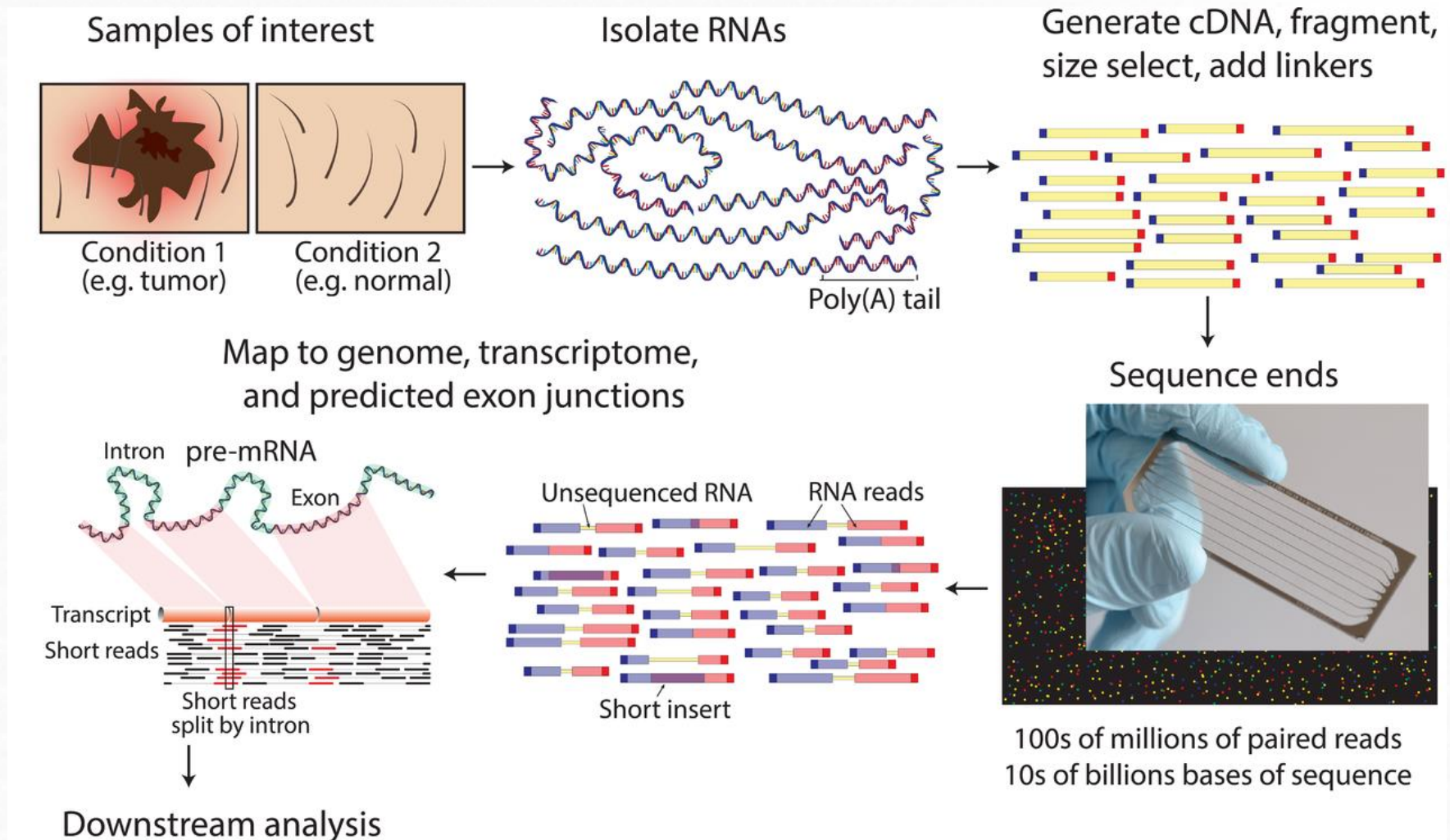
## 2.1 What is RNA-seq?

- RNA-seq is the high throughput sequencing of **cDNA** using NGS technologies
- RNA-seq works by **sequencing every RNA molecule** and profiling the expression of a particular gene by **counting** the number of time its transcripts have been sequenced.
- The summarized RNA-seq data is widely known as *count table*

	Condition A			Condition B		
Gene1	4	0	2	12	14	13
Gene2	0	23	50	47	22	0
Gene3	0	2	6	13	11	15
...	...	...	...	...	...	...
GeneG	156	238	37	129	51	118

## 2.1 What is RNA-seq?

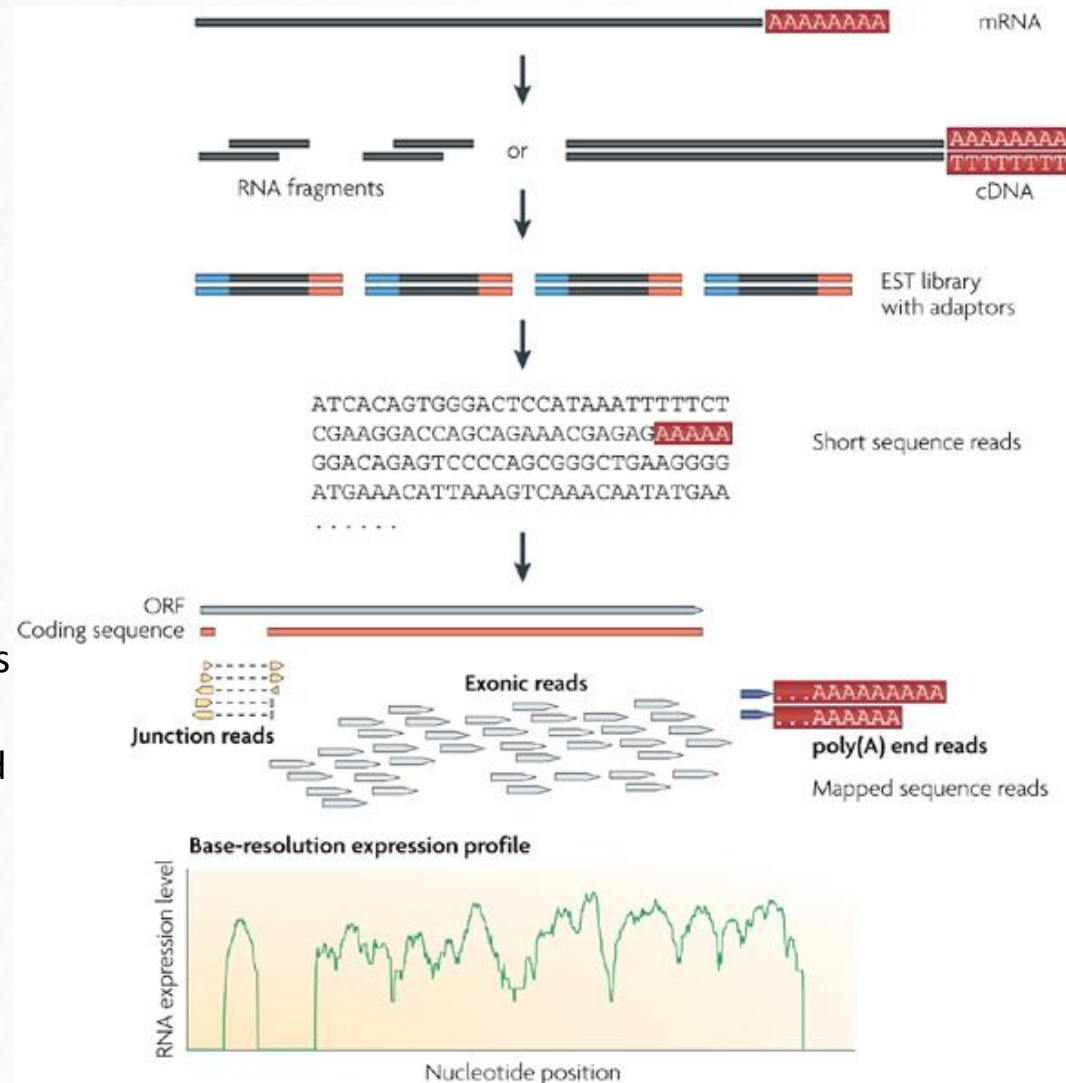
### RNA-seq (SGS)





## 2.1 What is RNA-seq?

- Long RNAs are first converted into a library of **cDNA** fragments through either RNA fragmentation or DNA fragmentation.
- Sequencing **adaptors (blue)** are added to each cDNA fragment and a short sequence is obtained from each cDNA using high-throughput sequencing technology.
- The resulting sequence reads are **aligned** with the reference genome or transcriptome, and classified as three types: exonic reads, junction reads and poly(A) end-reads. These three types are used to generate a **base-resolution expression profile** for each gene, as illustrated at the bottom; a yeast ORF with one intron is shown.



## 2.1 What is RNA-seq?

### Classes of RNA Molecules in Human Cells

#### Ribosomal RNA – rRNA

~80% of total RNA

- 28 S
- 18 S
- 5S and 5.8 S

#### Noncoding RNA - ncRNA

- tRNA
- snoRNA
- lincRNA
- miRNA
- Many, many others...

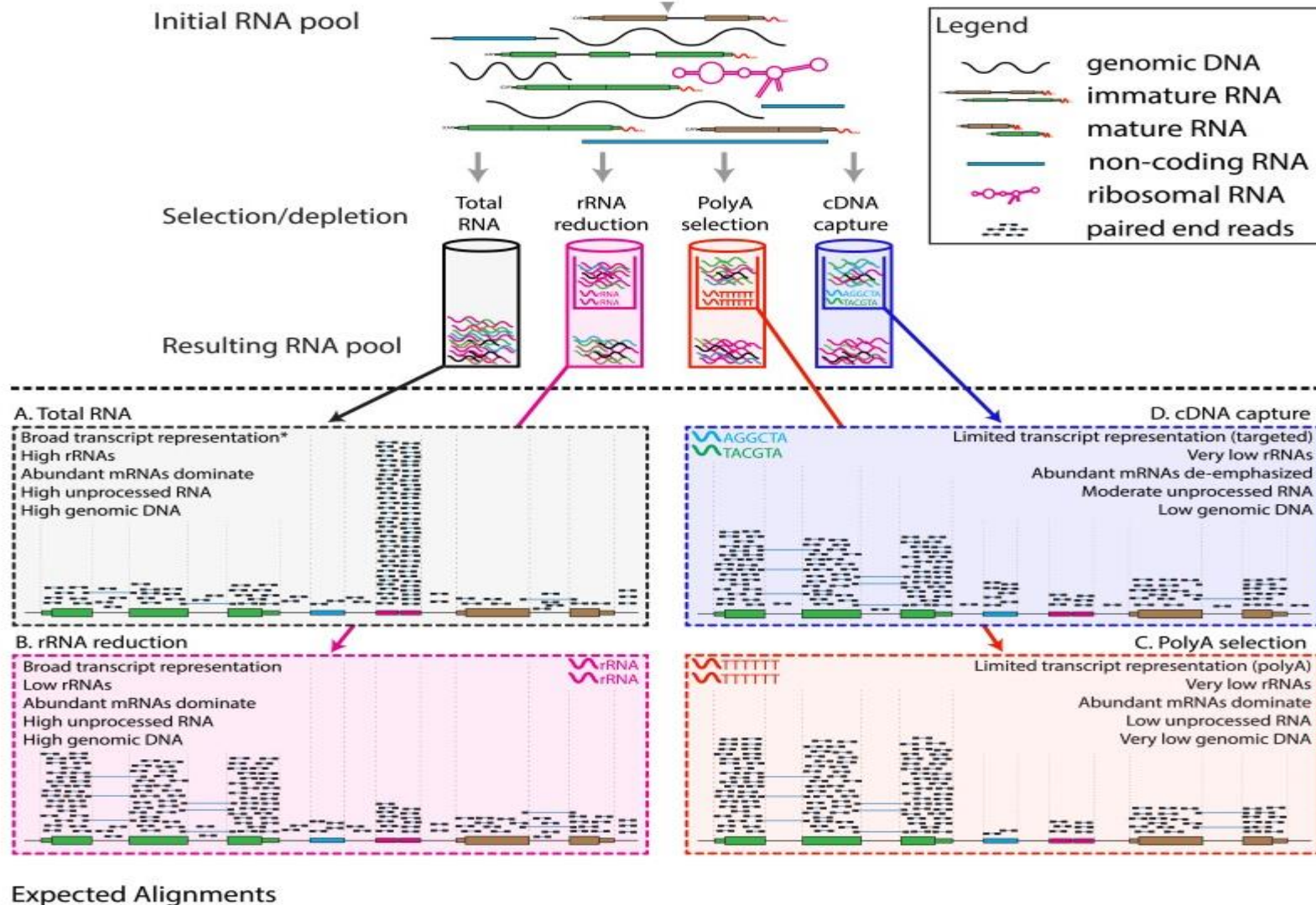
#### Mitochondrial RNA - mtRNA

#### Messenger RNA – mRNA

1-3% of Total RNA

- Highly expressed transcripts (>10,000 copies per cell)
- Rarely expressed transcripts (~1 copy per cell)

### 3.3 Main challenges in RNA-seq





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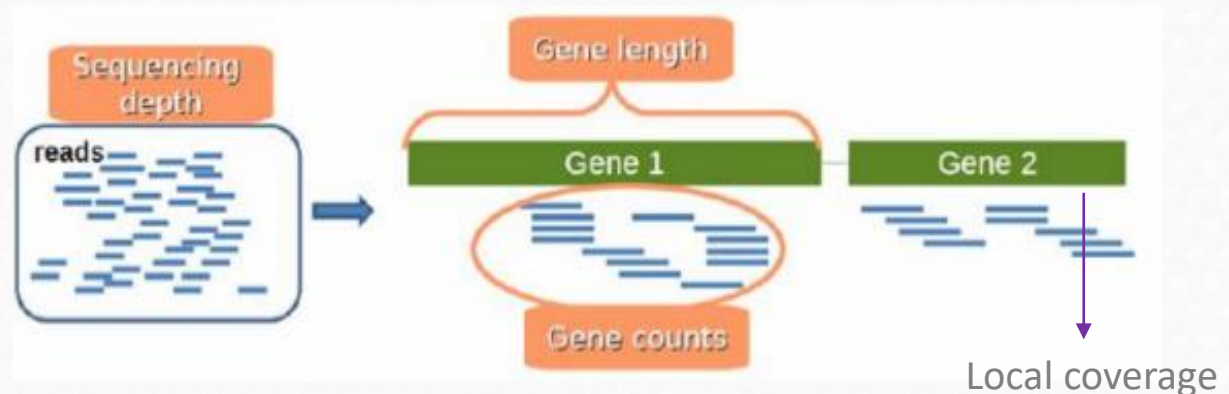
## 2.2 Basic key concepts?

**Sequencing depth:** Total number of reads mapped to the genome. (**Library size**) Could also be applied to samples.

**Coverage:** Number of reads mapped to a specific region (average of them if we are talking about the whole genome...)

**Gene length:** Number of bases that a gene has.

**Gene counts:** Number of reads mapping to that gene (expression measurement)



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### 3.3 Main challenges in RNA-seq

- **Sample**
  - Purity? Quantity? Quality?
- **RNAs consist of small exons that may be separated by large introns**
  - Mapping reads to genome is challenging
  - **Non-uniformity coverage** of the genome

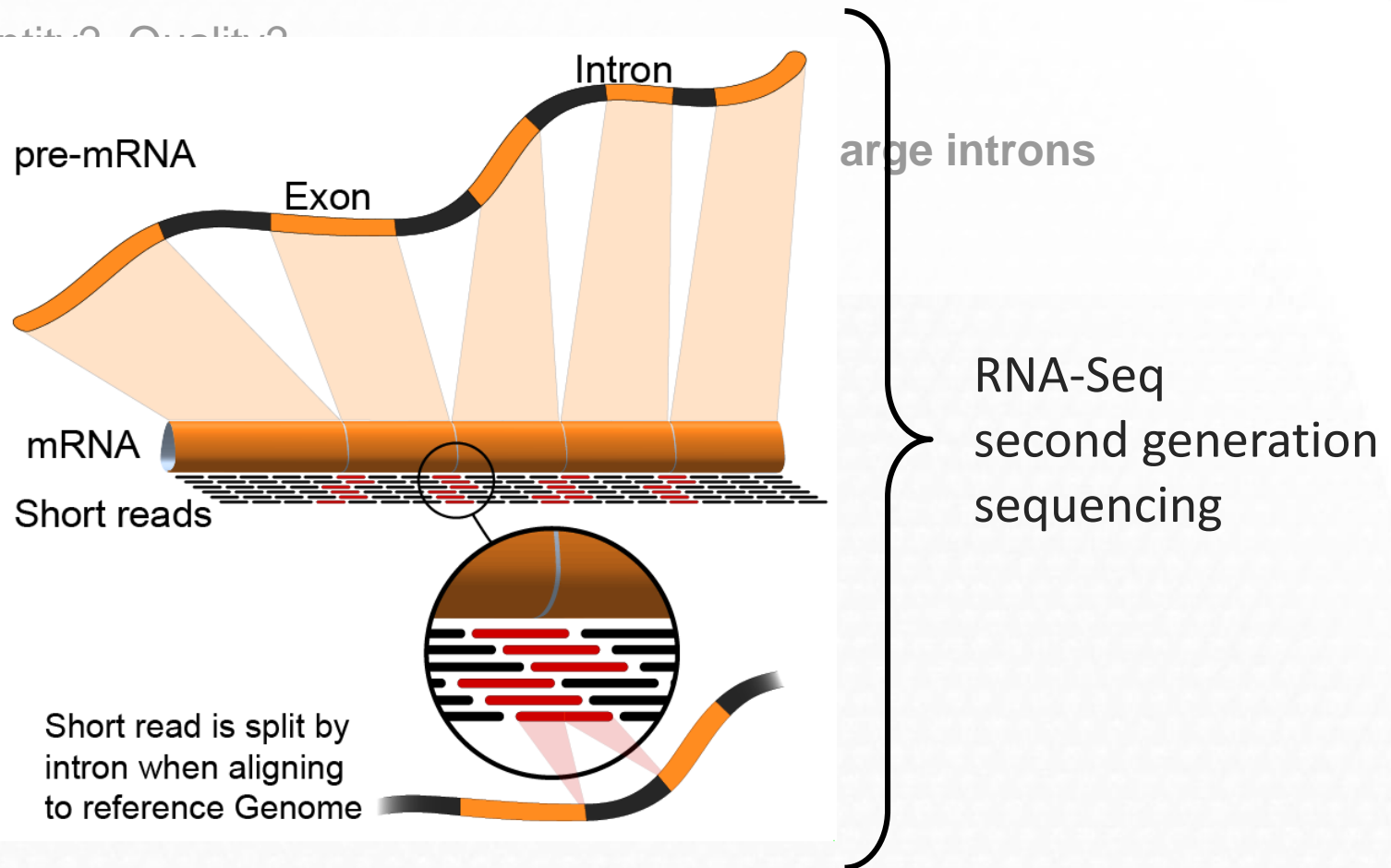
### 3.3 Main challenges in RNA-seq

- **Sample**

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- **RNAs consist of** pre-mRNA

- Mapping reads
- Non-uniform





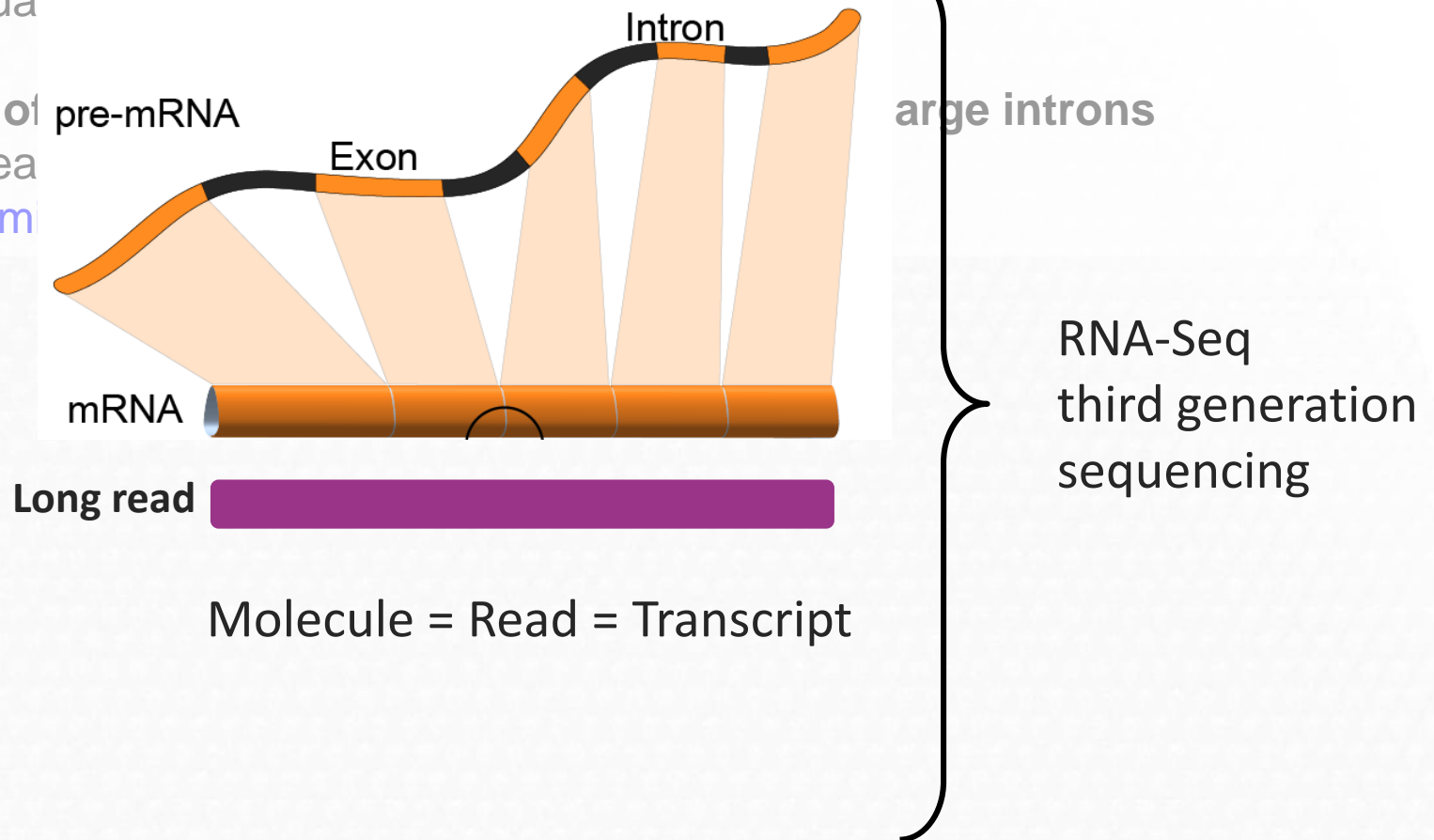
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- **Sample**

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- **RNAs consist of** pre-mRNA



- Mapping reads
- Non-uniform



### 3.3 Main challenges in RNA-seq

- **Sample**
  - Purity? Quantity? Quality?
- **RNAs consist of small exons that may be separated by large introns**
  - Mapping reads to genome is challenging
  - **Non-uniformity coverage** of the genome
- **The **relative abundance of RNAs vary wildly****
  - $10^5 - 10^7$  orders of magnitude
  - Since RNA sequencing works by random sampling, a small fraction of highly expressed genes may consume the majority of reads (rRNA)
- **RNAs come in a **wide range of sizes****
  - Small RNAs must be captured separately
  - PolyA selection of large RNAs may result in 3' end bias
- **RNA is fragile** compared to DNA (easily degraded)
- **Library** preparation

### 3.3 Main challenges in RNA-seq

- **Capture of less abundant RNAs**
- **Avoid the conversion to cDNA prior to library construction**
- **RNA-Seq TGS**  **Increasing the yield**  
 **Avoid degradation at 5' end**

### 3.3 Main challenges in RNA-seq

- Independently of the software used, one needs to think about

#### DATA STORAGE & MANAGEMENT!!



1 Illumina Flow Cell equals up to

- 1.5 Bn individual Clusters
- = 3 Bn Reads
- = 300 Gbases raw sequence
- = 2.5 TByte of disk space (raw data)
- > 100 GByte of disk space (fastq data)



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## 2.4 RNA-seq vs Microarrays

Published online 15 October 2008 | *Nature* 455, 847 (2008) |  
doi:10.1038/455847a

News

### The death of microarrays?

High-throughput gene sequencing seems to be stealing a march on microarrays. Heidi Ledford looks at a genome technology facing intense competition.

#### Announcing the death of the Micro-array

30 August 2010 by Anthony Fejes, posted in Uncategorized



| Anthony Fejes  
| About the blog  
| Blog homepage

- reproducibility
- only show you what you're looking for
- what about 'indels', inversions, translocations...
- accuracy
- sensitivity

## 2.4 RNA-seq vs Microarrays

[PLoS One](#). 2013 Aug 20;8(8):e71462. doi: 10.1371/journal.pone.0071462. eCollection 2013.

### Large scale comparison of gene expression levels by microarrays and RNAseq using TCGA data.

[Guo Y<sup>1</sup>](#), [Sheng Q](#), [Li J](#), [Ye F](#), [Samuels DC](#), [Shyr Y](#).

#### Abstract

RNAseq and microarray methods are frequently used to measure gene expression level. While similar in purpose, there are fundamental differences between the two technologies. Here, we present the largest comparative study between microarray and RNAseq methods to date using The Cancer Genome Atlas (TCGA) data. **We found high correlations between expression data obtained from the Affymetrix one-channel microarray and RNAseq (Spearman correlations coefficients of ~0.8).** We also observed that the low abundance genes had poorer correlations between microarray and RNAseq data than high abundance genes. As expected, due to measurement and normalization differences, Agilent two-channel microarray and RNAseq data were poorly correlated (Spearman correlations coefficients of only ~0.2). By examining the differentially expressed genes between tumor and normal samples we observed reasonable concordance in directionality between Agilent two-channel microarray and RNAseq data, although a small group of genes were found to have expression changes reported in opposite directions using these two technologies. Overall, RNAseq produces comparable results to microarray technologies in term of expression profiling. The RNAseq normalization methods RPKM and RSEM produce similar results on the gene level and reasonably concordant results on the exon level. Longer exons tended to have better concordance between the two normalization methods than shorter exons.

## 2.4 RNA-seq vs Microarrays

### Comparison of RNA-Seq and Microarray in Transcriptome Profiling of Activated T Cells

Shanrong Zhao , Wai-Ping Fung-Leung, Anton Bittner, Karen Ngo, Xuejun Liu 

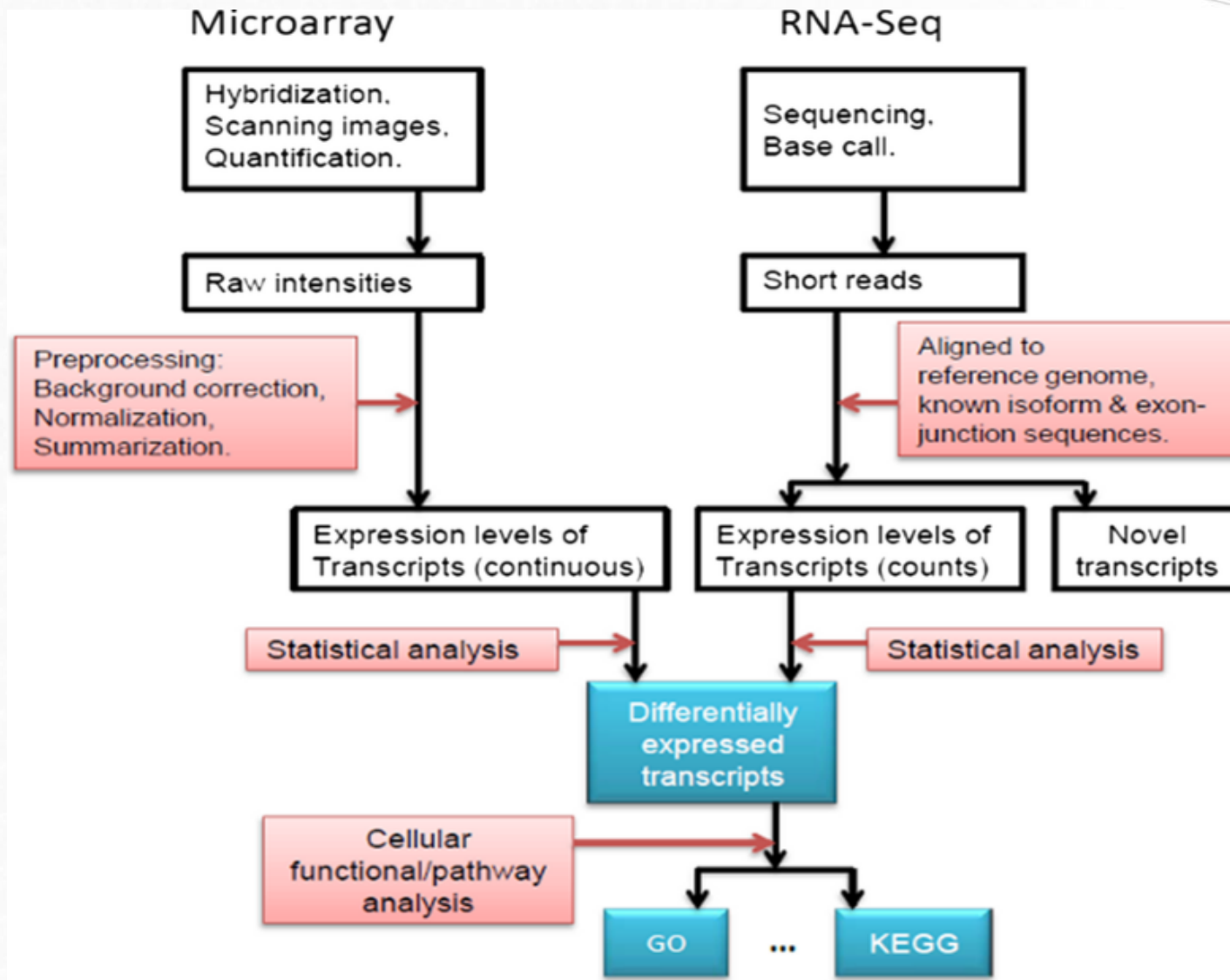
Published: January 16, 2014 • DOI: 10.1371/journal.pone.0078644

- RNA-Seq was **superior in detecting low abundance** transcripts
- also **better detecting differentiating biologically isoforms**
- RNA-Seq demonstrated a **broader dynamic range** than microarray.
- RNA-Seq **avoid problems inherent to microarray probe performance**

!!!The study try to demonstrate the benefits of RNA-Seq over microarray in transcriptome profiling



## 2.4 RNA-seq vs Microarrays



## 2.4 RNA-seq vs Microarrays

### Pros and cons of both technologies

#### Microarrays

- 😊 Costs,
- 😊 well established methods,  
small data
- 😞 Hybridization bias,
- 😞 sequence must be known

#### RNA-seq

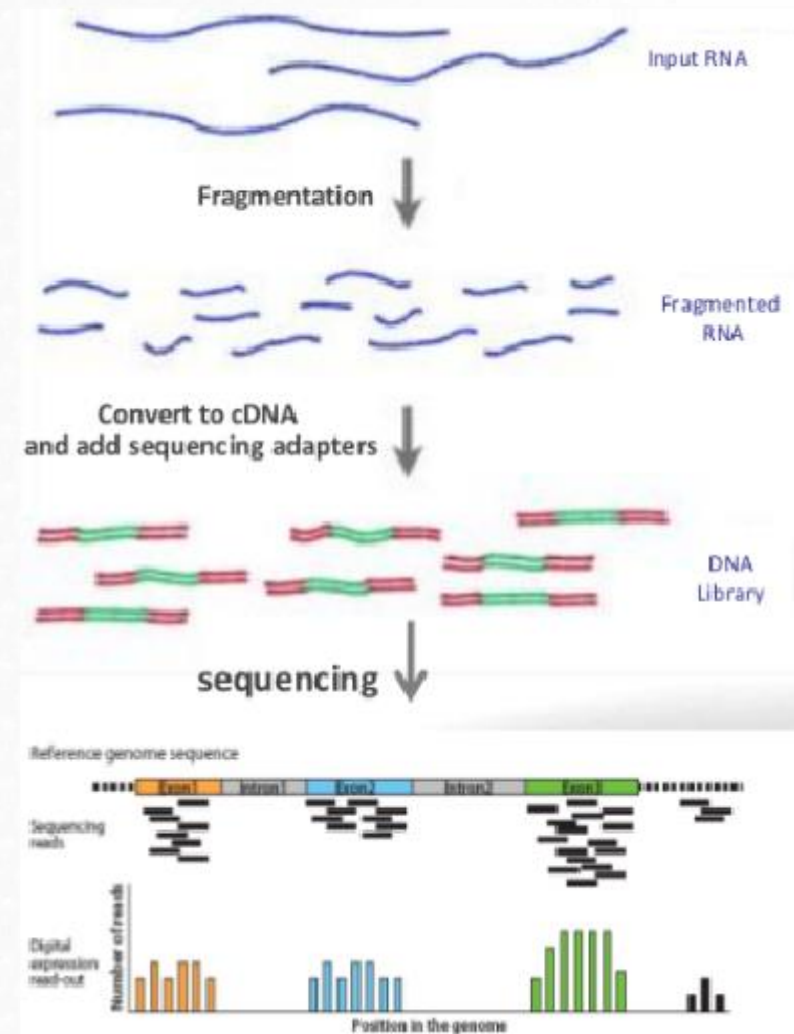
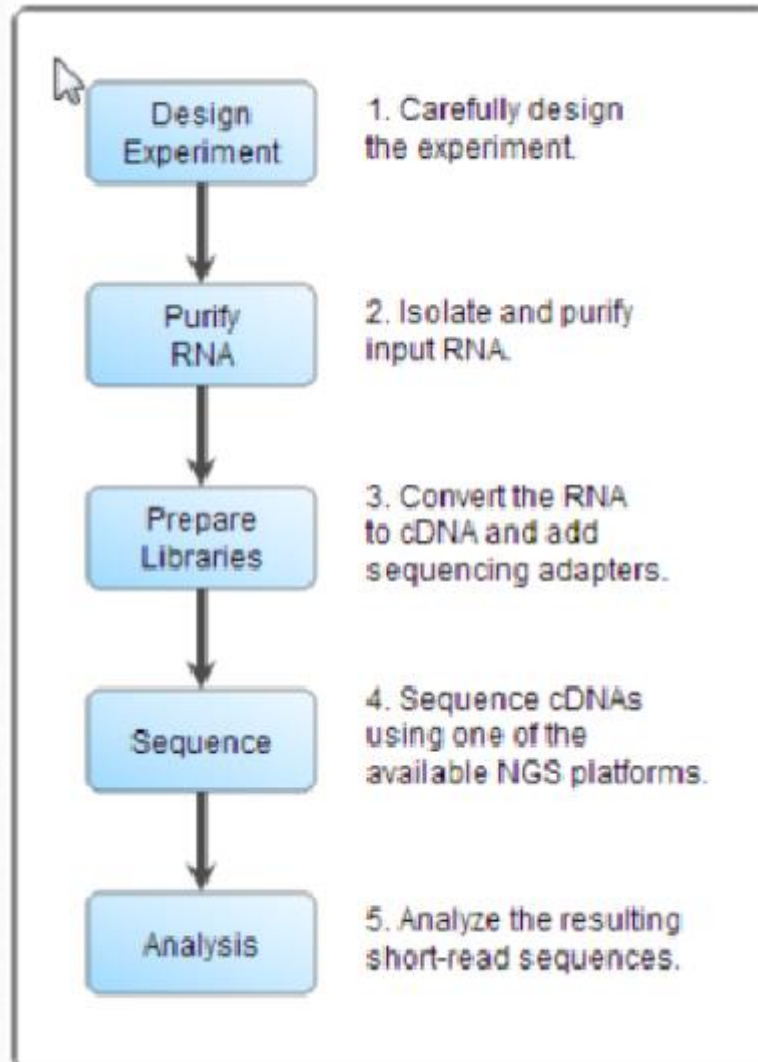
- 😊 High reproducibility,
- 😊 not limited to expression
- 😞 Costs,
- 😞 complexity of analysis

“High correlation between gene expression profiles generated by the two platforms.”

“RNA-Seq sequencing technology is new to most researchers, more expensive than microarray, data storage is more challenging and analysis is more complex.”

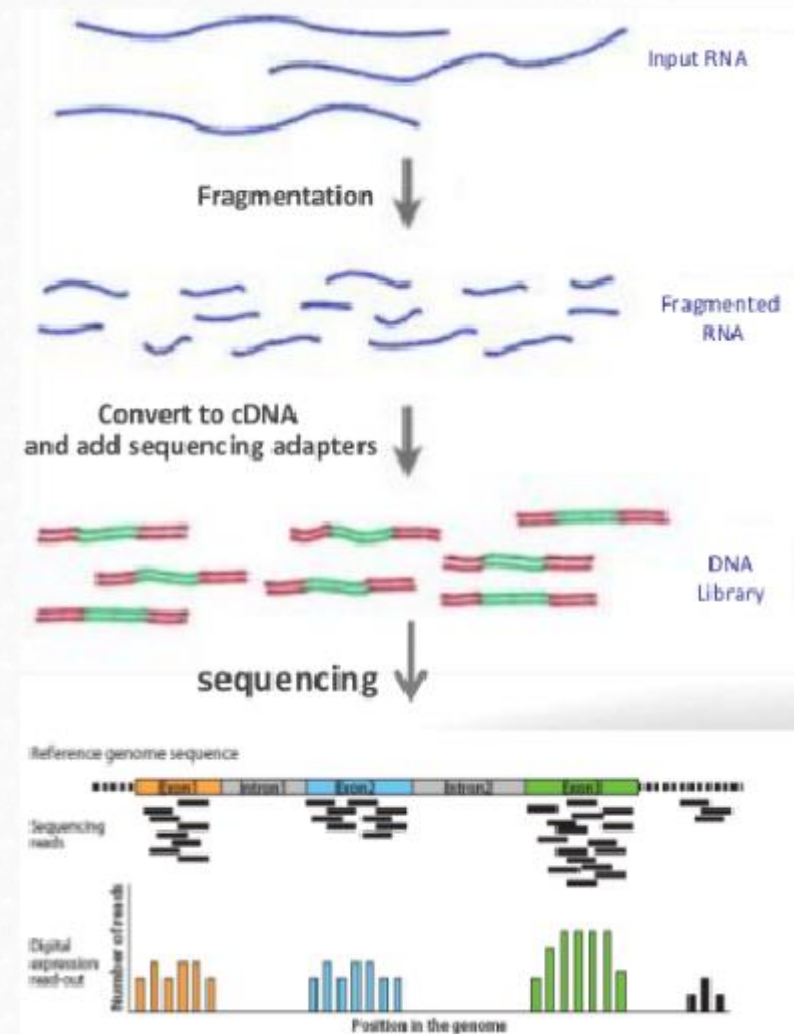
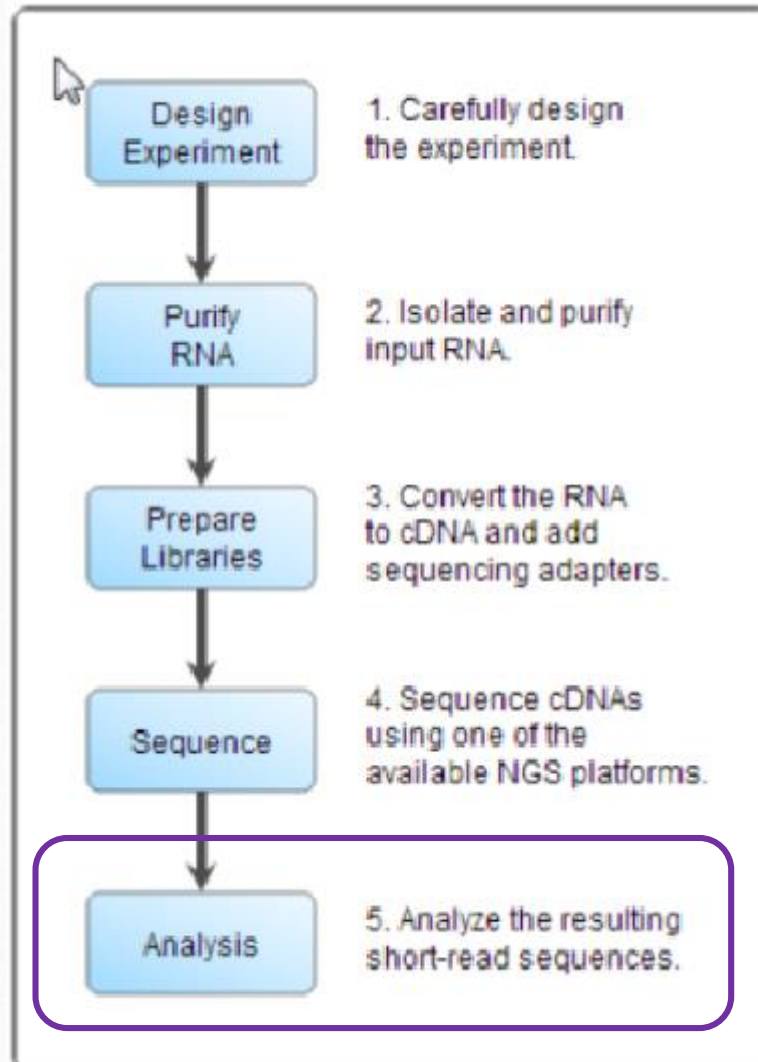
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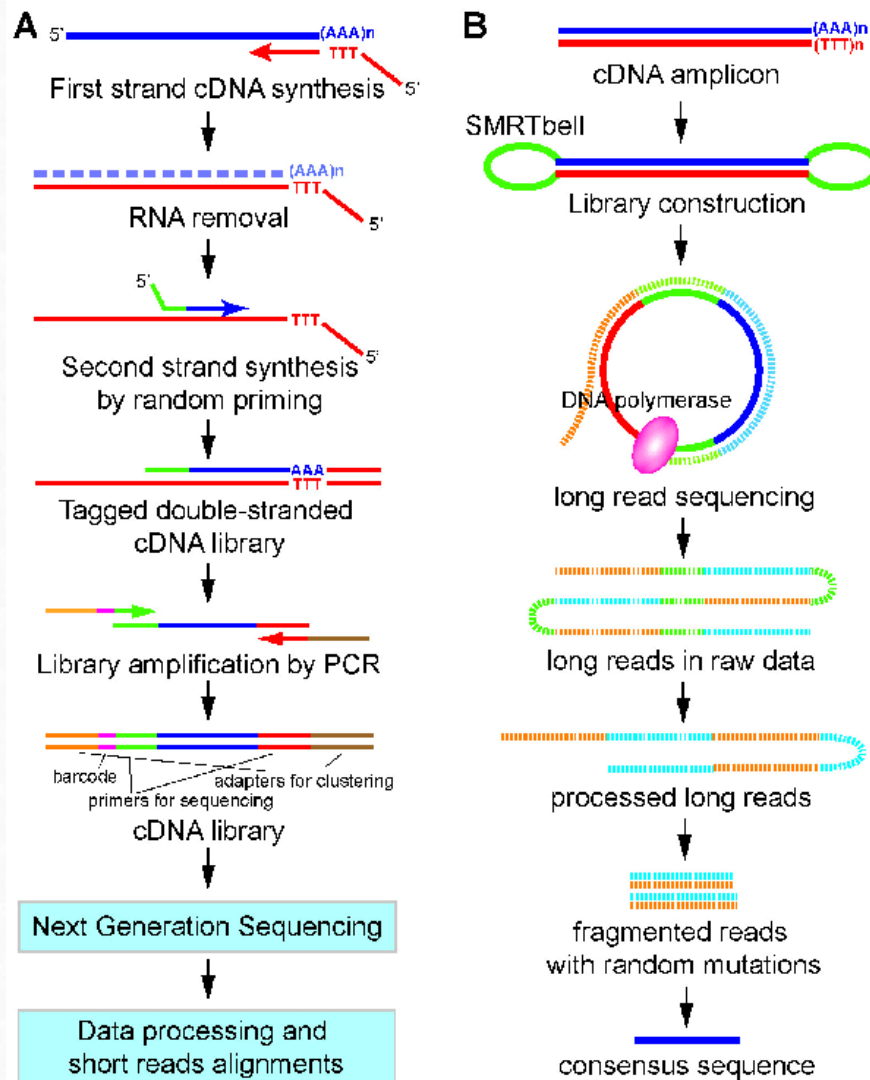




## 2.5 RNA-seq analysis pipeline(s)



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### RNA-Seq workflow

A= RNA-Seq (SGS)

B= RNA-Seq (TGS) -> Iso-Seq (PacBio)

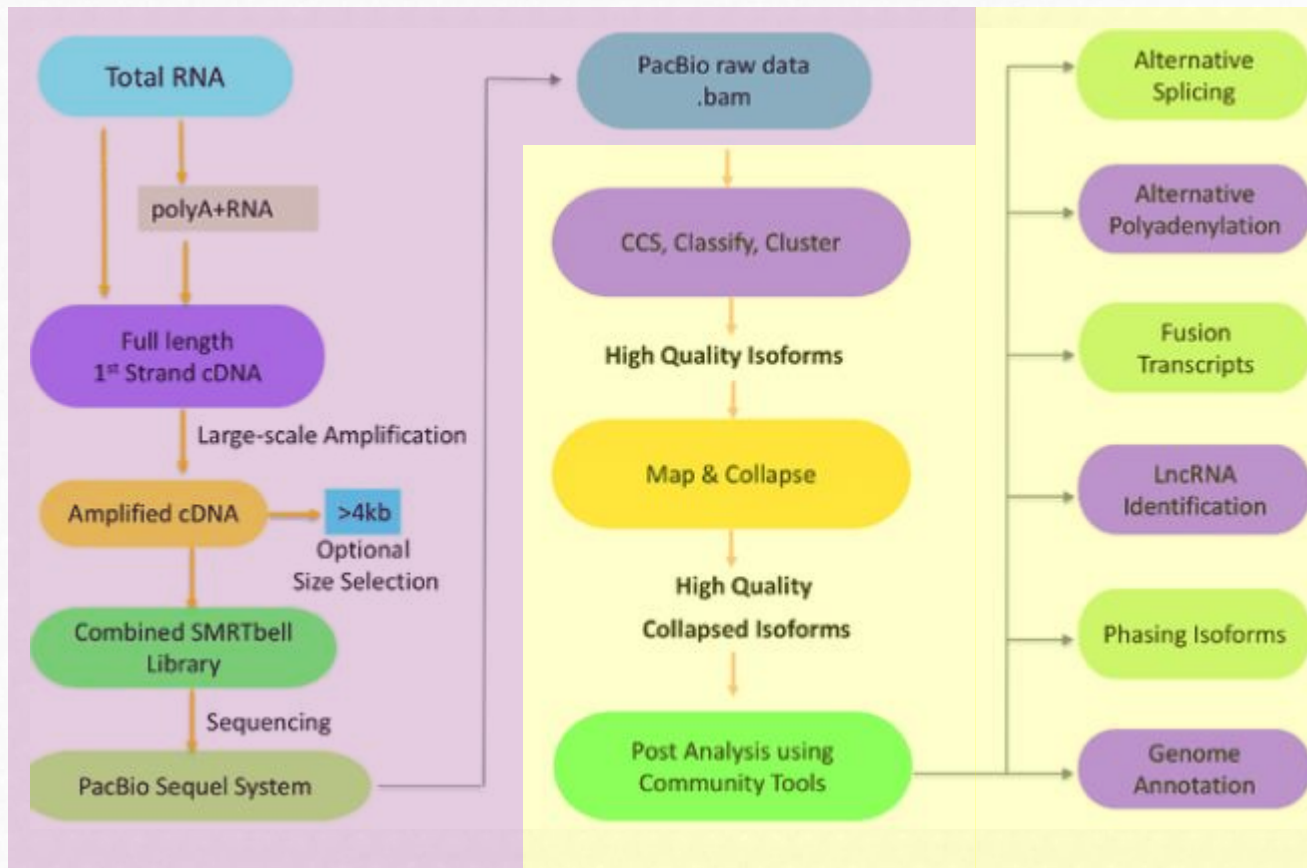


<https://pubmed.ncbi.nlm.nih.gov/28148393/>

Analysis

## 2.5 RNA-seq analysis pipeline(s)

RNA-Seq (TGS) pipeline exemple: **Iso-Seq**

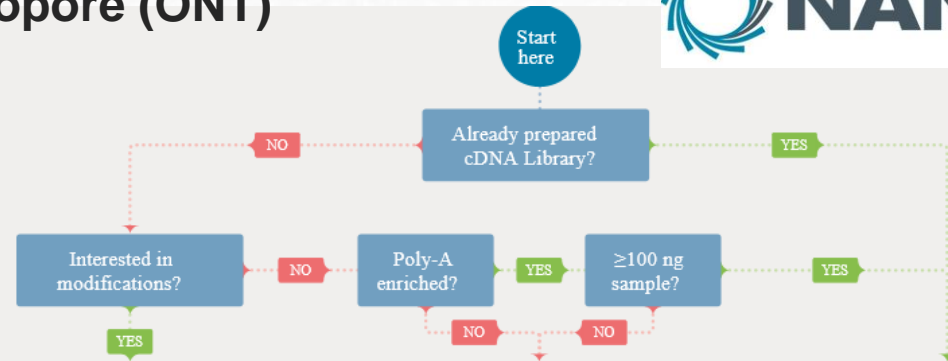


## 2.5 RNA-seq analysis pipeline(s)

### RNA-Seq (TGS) : Oxford nanopore (ONT)

Tenen 3 kits!

<https://nanoporetech.com/applications/rna-sequencing>



	Direct RNA Sequencing Kit	cDNA-PCR Sequencing Kit	Direct cDNA Sequencing Kit
Preparation time	105 mins	165 mins	275 mins
Input requirement	500 ng RNA (poly-A <sup>+</sup> )	1 ng RNA (poly-A <sup>+</sup> )	100 ng RNA (poly-A <sup>+</sup> )
RT required	Optional	Yes	Yes
PCR required	No	Yes	No
Read length	Equal to RNA length	Enriched for full-length cDNA	Enriched for full-length cDNA
Typical throughput	● ○ ○	● ● ●	● ● ○
Typical number of reads (MinION, 1 kb avg. length)	1 million	7 - 12 million	5 - 10 million
Typical number of reads (PromethION, 1 kb avg. length)	8 million	> 60 million	30 - 60 million
Multiplexing options	In development	PCR Barcoding Kit	Native Barcoding Kit

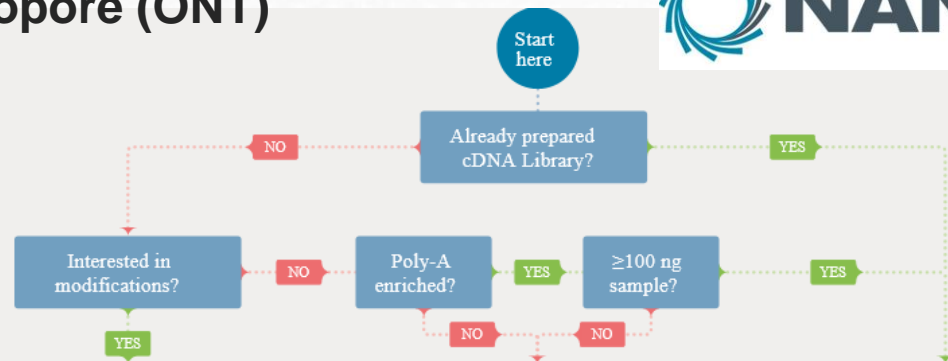


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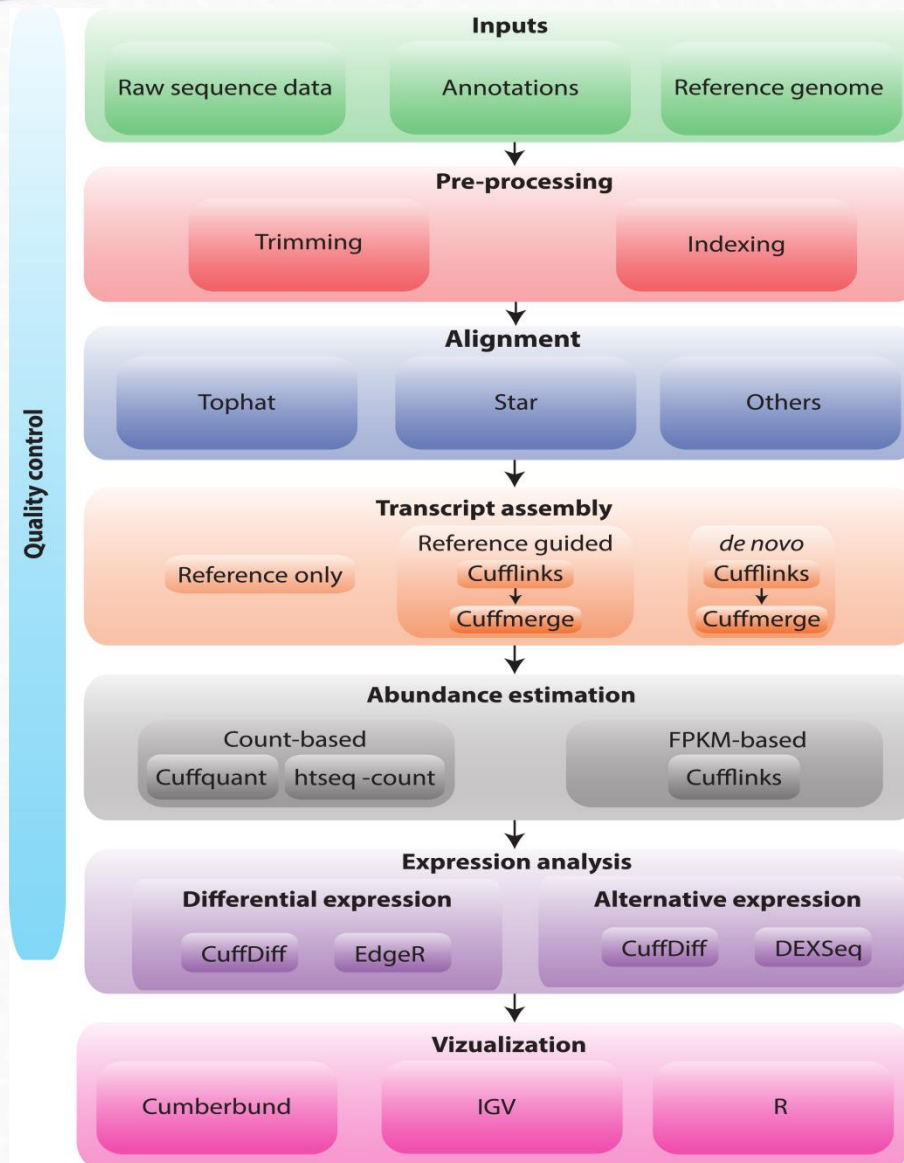
## 2.5 RNA-seq analysis pipeline(s)

**RNA-Seq (SGS)**



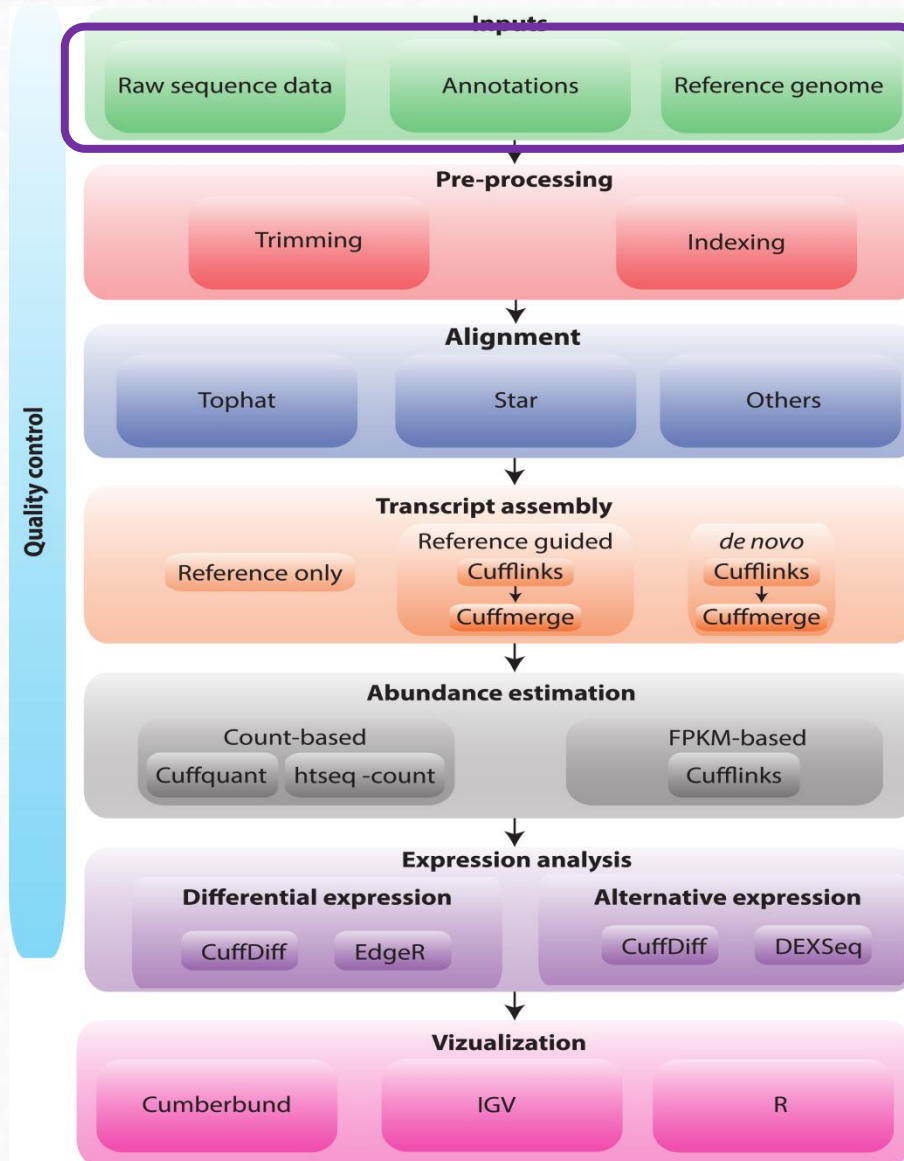
Steps Standard pipeline

## 2.5 RNA-seq analysis pipeline(s)



RNA-Seq (SGS)

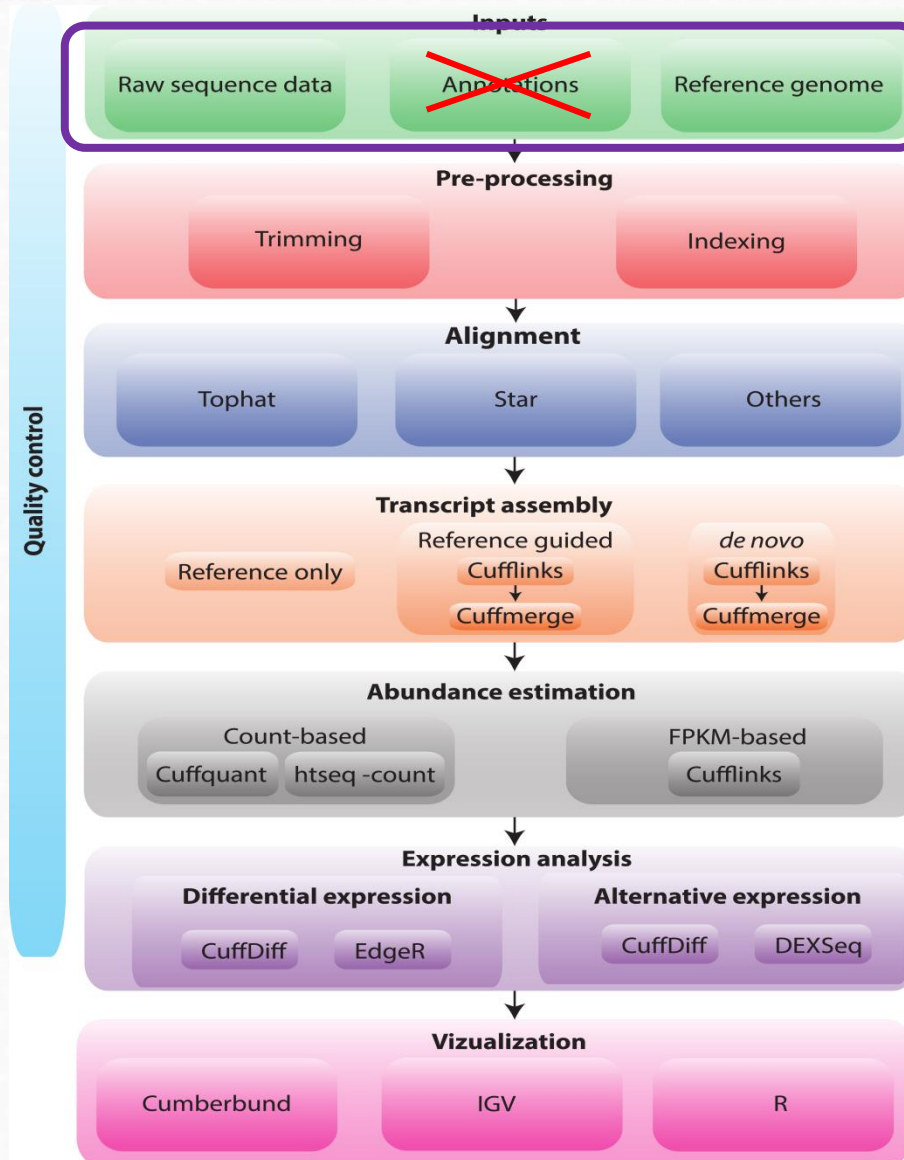
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- Fastq files
- FASTQC (quality control)

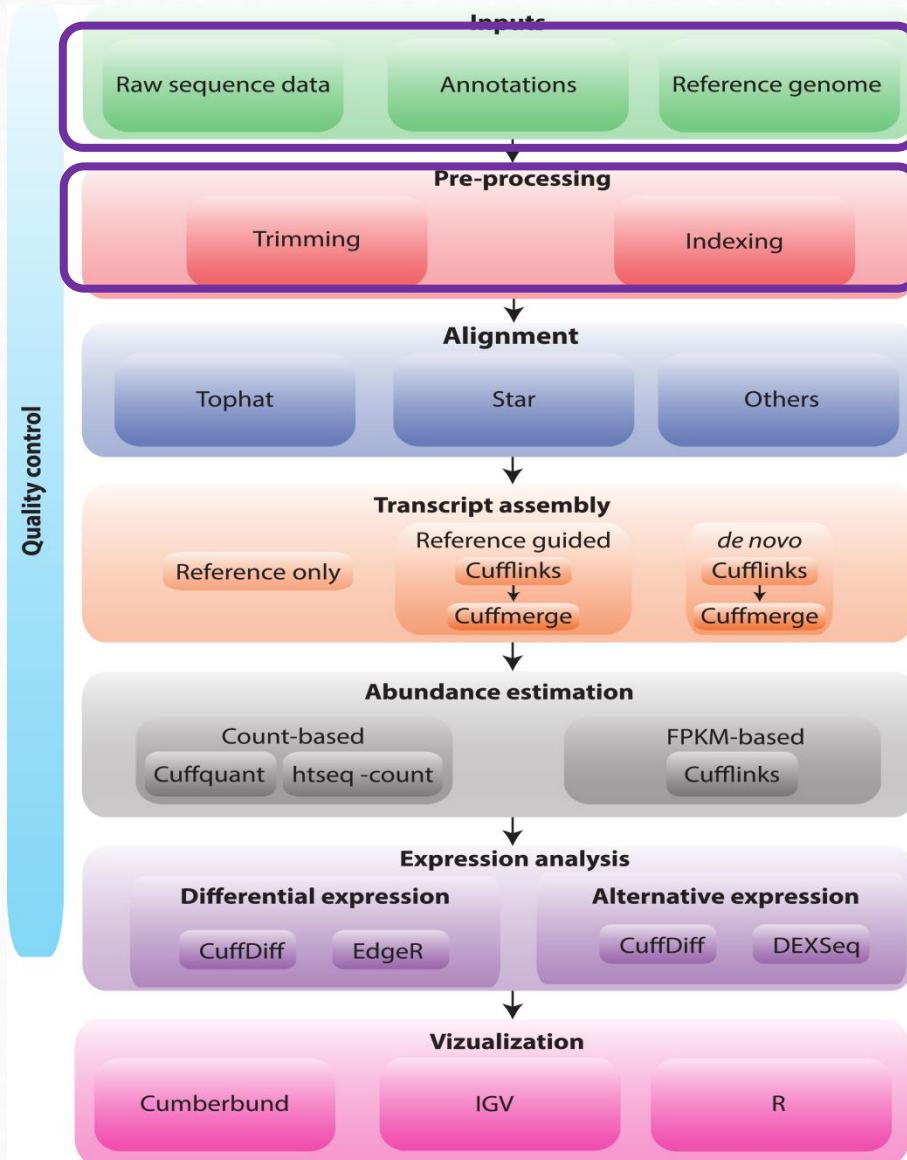


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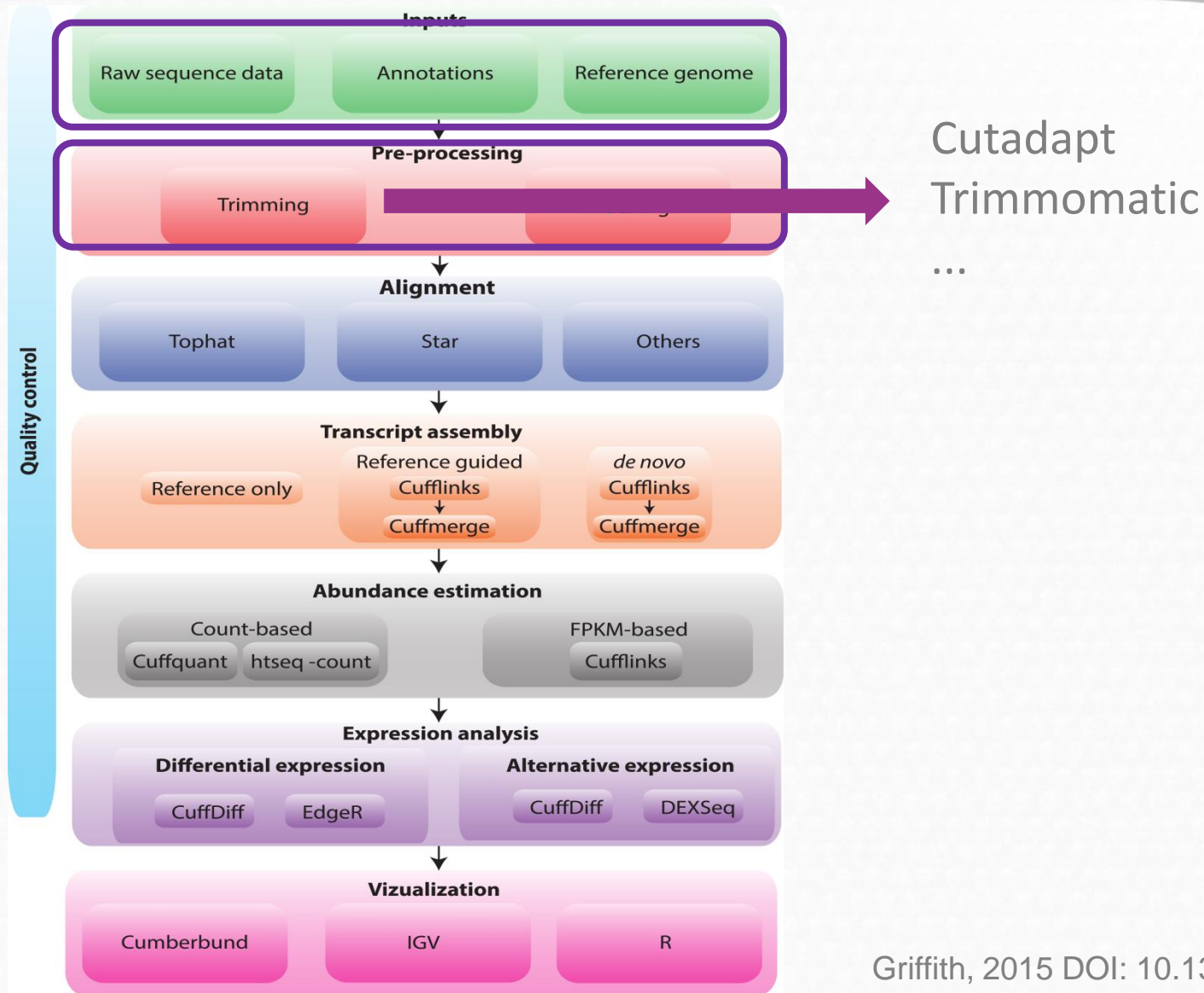


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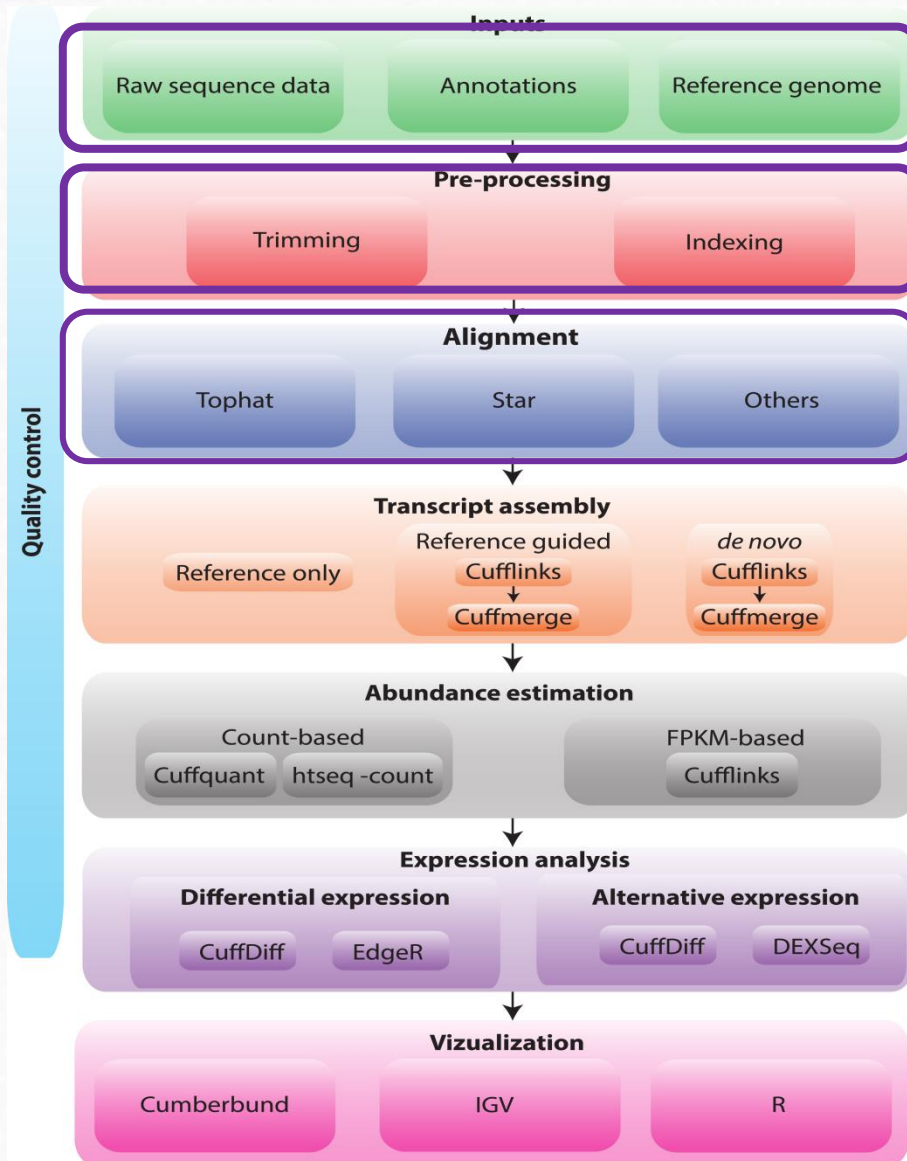
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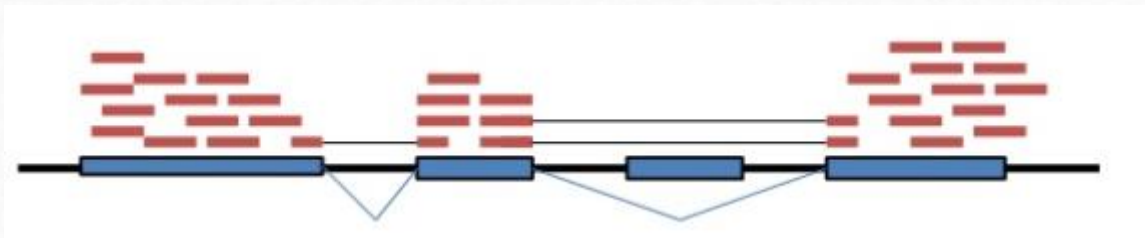


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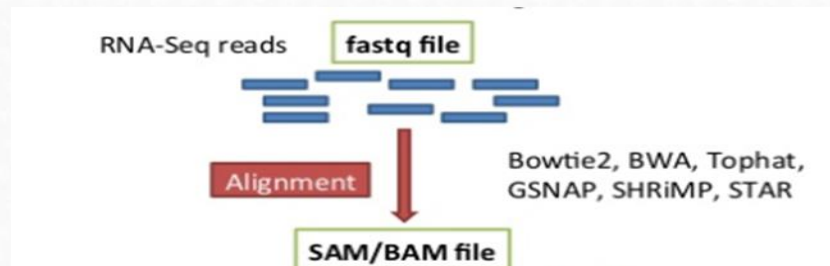
## 2.6 Alignment

### What to map to?

Map to the genome, with knowledge of transcript annotations



- **Well annotated genome reference is required.**
- To effectively map to exon junctions, you need a **mapping algorithm** that can divide the sequencing reads and map portions independently.
- Identifying **alternative transcript isoforms** involves complex algorithms



### Which sequence mappers to use?

- RNASeq Alignment algorithm must be
  - **Fast**
  - Able to **handle SNPs, indels, and sequencing errors**
  - Maintain **accurate quantification**
  - Allow for introns for reference genome alignment (spliced alignment detection)

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  - Maintain **accurate quantification**
  - Allow for introns for reference genome alignment (spliced alignment detection)
- **Burrows-Wheeler Transform (BWT) mappers**
  - **Fast**
  - **Limited mismatches** allowed (<3)
  - **Limited indel detection** ability
  - Examples: **Bowtie2, BWA, Tophat, HISAT2**
  - Use cases: **large and conserved genome and transcriptomes**
- **Hash Table mappers**
  - Require **large amount of RAM** for indexing
  - **More mismatches** allowed
  - **Indel detection**
  - Examples: **GSNAP, SHRiMP, STAR**
  - Use case: **highly variable or smaller genomes, transcriptomes**



### Which sequence mappers to use?

- RNASeq Alignment algorithm must be
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  - Able to **handle SNPs indels and sequencing errors**

[Front Genet.](#) 2018; 9: 35.

PMCID: PMC5834436

Published online 2018 Feb 26. doi: [\[10.3389/fgene.2018.00035\]](https://doi.org/10.3389/fgene.2018.00035)

PMID: [29535759](https://pubmed.ncbi.nlm.nih.gov/29535759/)

### Comparison of Burrows-Wheeler Transform-Based Mapping Algorithms Used in High-Throughput Whole-Genome Sequencing: Application to Illumina Data for Livestock Genomes<sup>1</sup>

[Brittney N. Keel\\*](#) and [Warren M. Snelling](#)

- Hash Table mappers
  - Require **large amount of RAM** for indexing
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  - Examples: [GSNAP](#), [SHRiMP](#), [STAR](#)
  - Use case: highly **variable or smaller genomes**, transcriptomes

## 2.6 Alignment

### Steps with TopHat

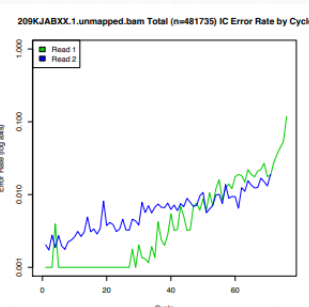
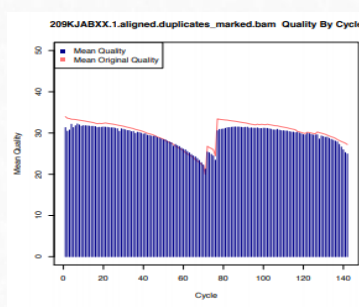
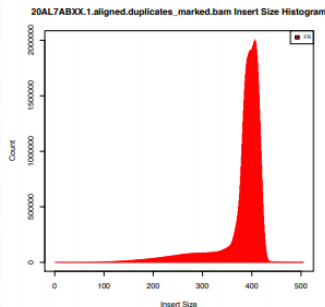
1. **Unspliced reads** are mapped to locate exons (with [Bowtie](#))
2. Unmapped reads are then split and aligned independently to identify exon



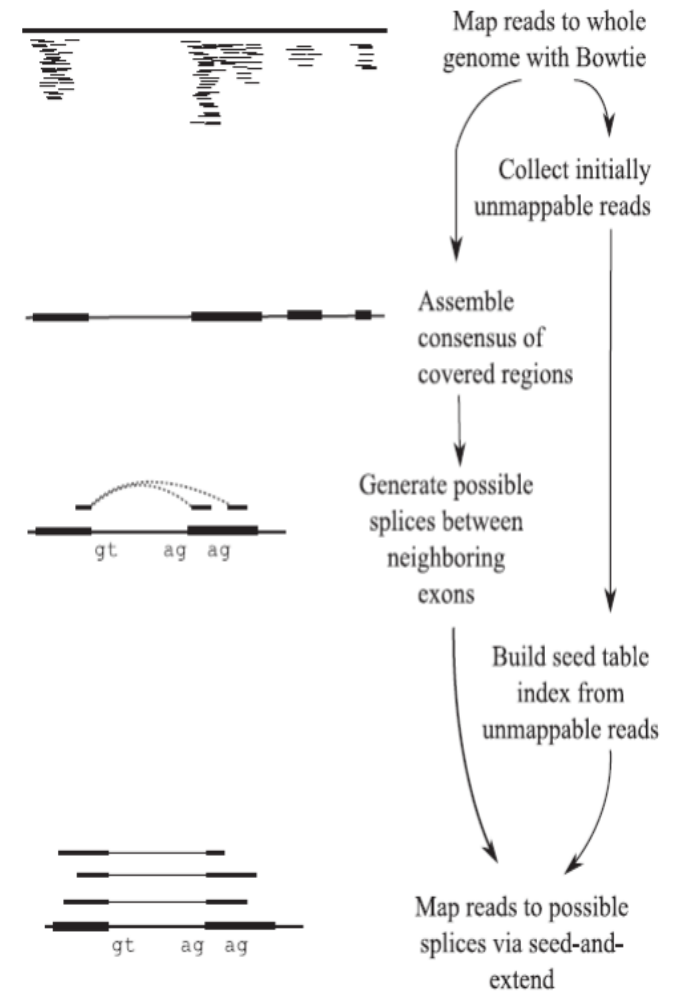
Important to **check the quality** of mapping process  
(percentage of mapped reads)



Picard can be used for quality control of mapping



### TopHat Pipeline



### Alignment-independent quantification for RNA-Seq

- Alignment steps are **computationally heavy** and can be very **time-consuming** even with multi-threading.
- In 2014, **Sailfish** method, demonstrated that it was not necessary to actually **align each read to the genome** in order to obtain accurate transcript each read.

Brief Communication | Published: 20 April 2014

#### Sailfish enables alignment-free isoform quantification from RNA-seq reads using lightweight algorithms

Rob Patro, Stephen M Mount & Carl Kingsford 

*Nature Biotechnology* **32**, 462–464 (2014) | [Download Citation](#) 

### Alignment-independent quantification for RNA-Seq

- Alignment steps are **computationally heavy** and can be very **time-consuming** even with multi-threading.
- In 2014, **Sailfish** method, demonstrated that it was not necessary to actually **align each read to the genome** in order to obtain accurate transcript each read.
- All you actually need to do is establish the **most likely transcript** for each read



1. shredding the transcriptome and reads into **kmers** (short overlapping sequences)
2. **matching the transcriptome and read kmers** (is a very fast and low memory usage)



### Alignment-independent quantification for RNA-Seq

- Nowadays there exist various tools:
  - Salmon, Kallisto, Sailfish

#### PROS:

- Extremely Fast & Lightweight (can quantify 20 million reads in five minutes on a laptop computer)
- Easy to use

### Alignment-independent quantification

- Nowadays there exist various tools for

➤ Salmon

### Limitations of alignment-free tools in total RNA-seq quantification

Douglas C. Wu<sup>1,2</sup> , Jun Yao<sup>1,2</sup>, Kevin S. Ho<sup>1,2</sup>, Alan M. Lambowitz<sup>1,2</sup> and Claus O. Wilke<sup>1,3\*</sup>

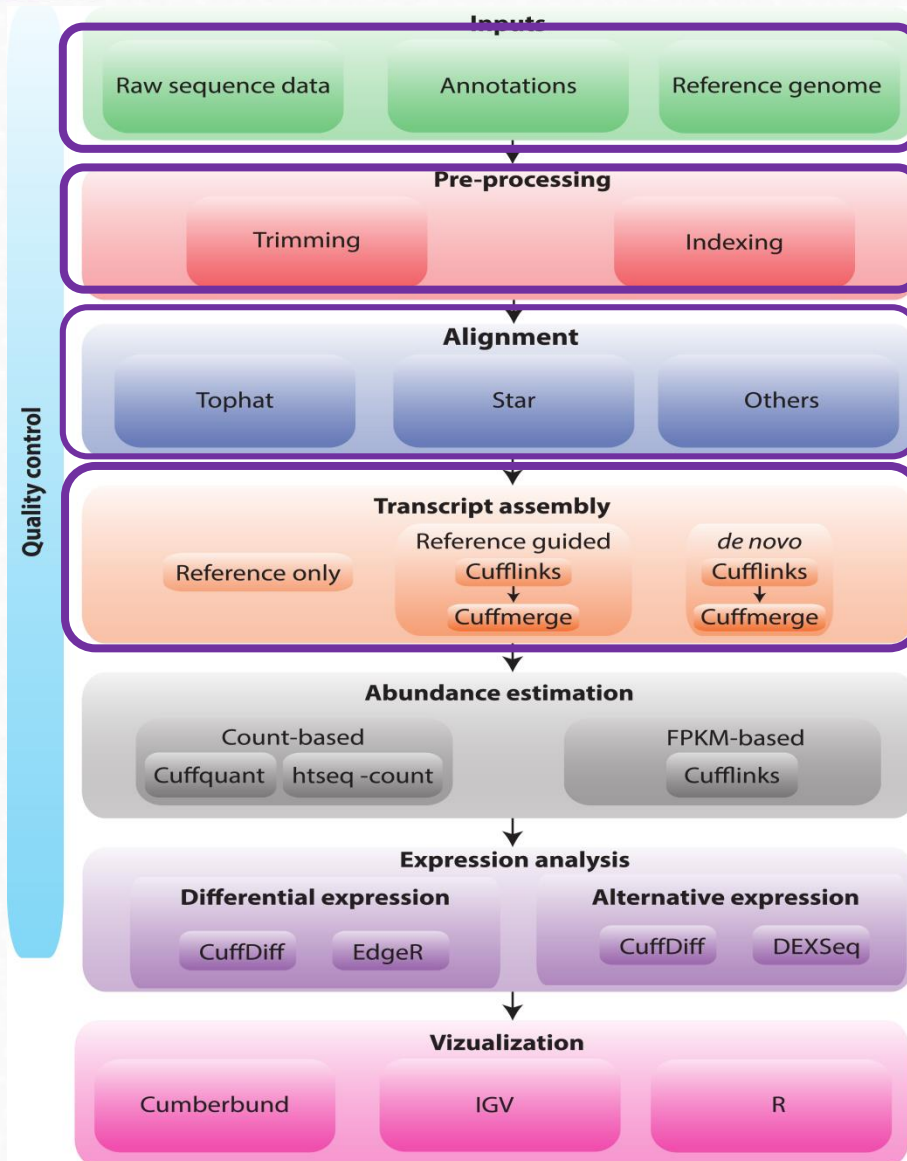
Wu et al. BMC Genomics (2018) 19:510  
<https://doi.org/10.1186/s12864-018-4869-5>

**Conclusion:** We have shown that alignment-free and traditional alignment-based quantification methods perform similarly for common gene targets, such as protein-coding genes. However, we have identified a potential pitfall in analyzing and quantifying lowly-expressed genes and small RNAs with alignment-free pipelines, especially when these small RNAs contain biological variations.

... in five minutes on a laptop

- 1. Introduction to omics data analysis**
- 2. An example of omics data analysis. RNA-seq**
  - 1. What is RNA-seq**
  - 2. Basic key concepts**
  - 3. Main challenges in RNA-seq**
  - 4. RNA-seq vs Microarrays**
  - 5. RNA-seq analysis pipeline(s)**
  - 6. Alignment**
  - 7. Transcript assembly**
  - 8. DEG Analysis**
  - 9. Vizualization**

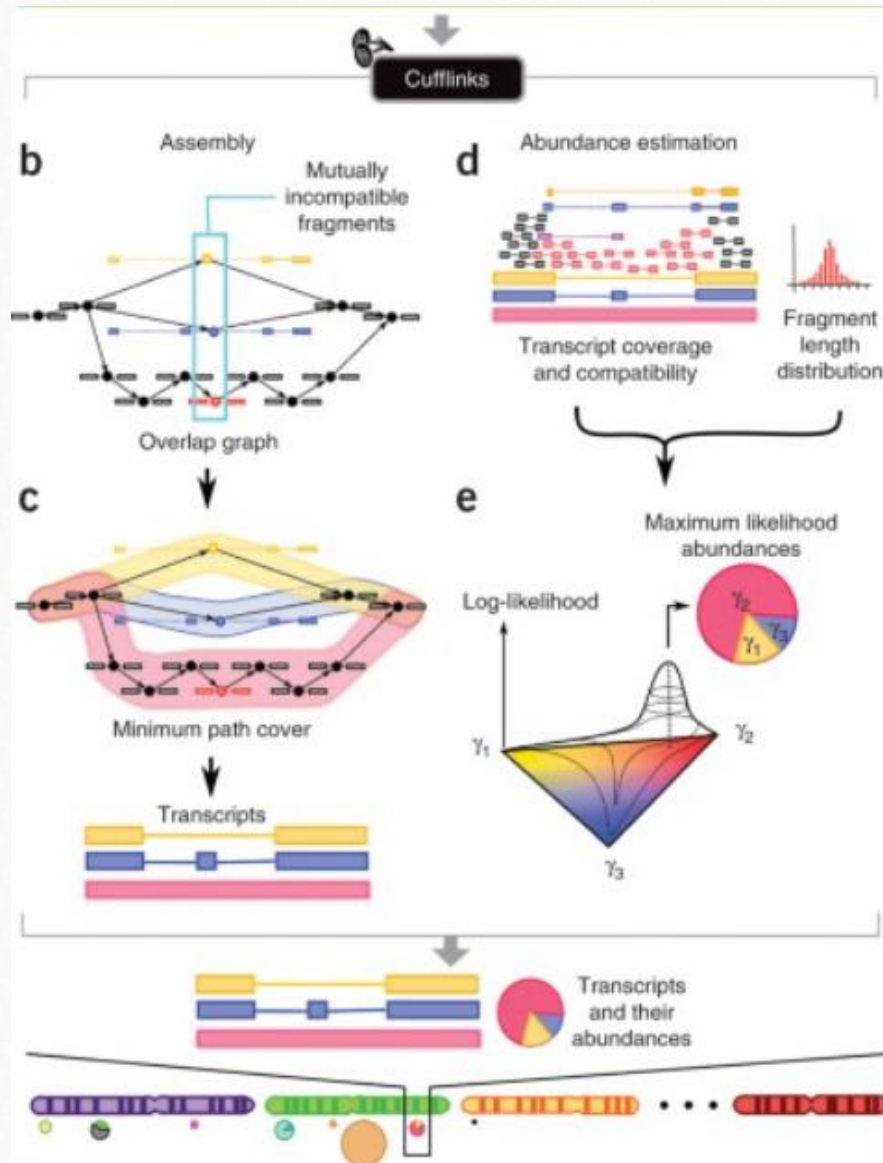
## 2.5 RNA-seq analysis pipeline(s)





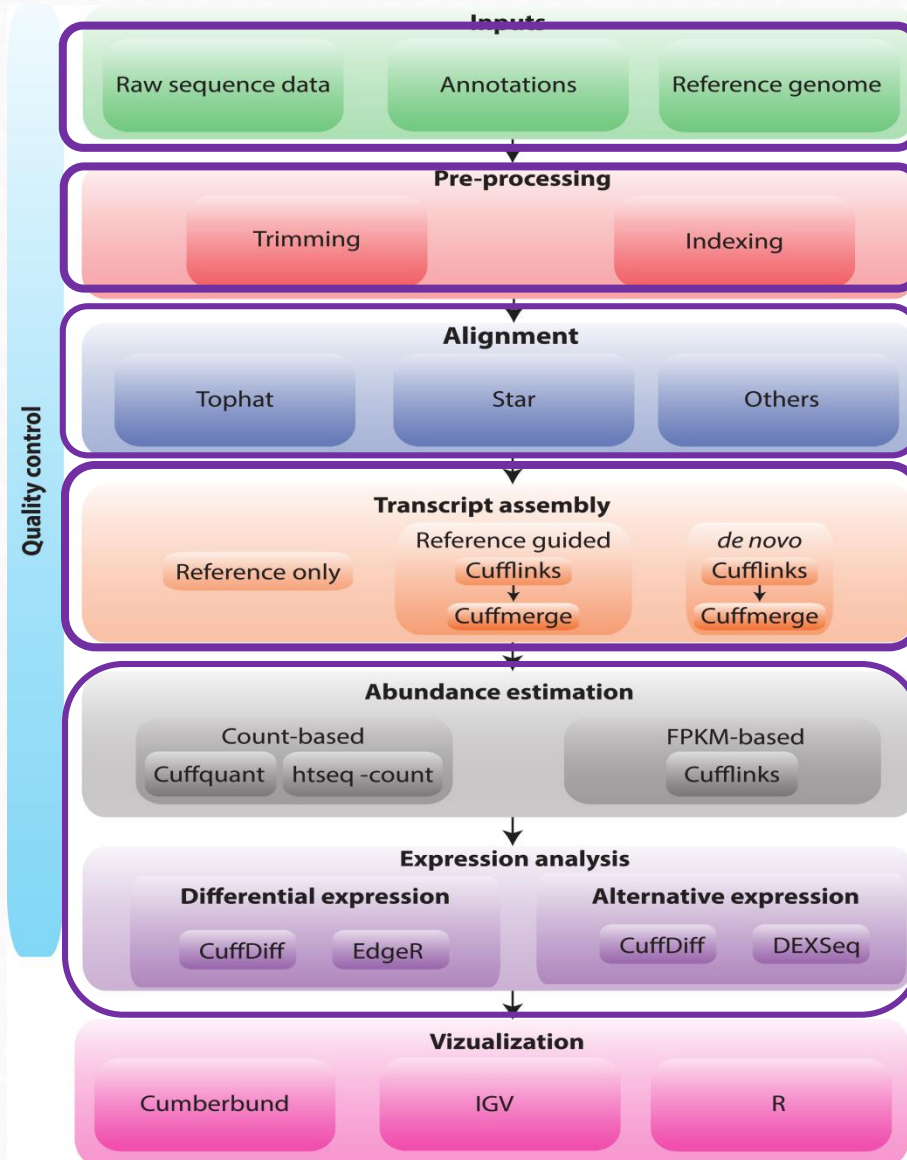
## 2.5 RNA-seq analysis pipeline(s)

### TopHat alignment

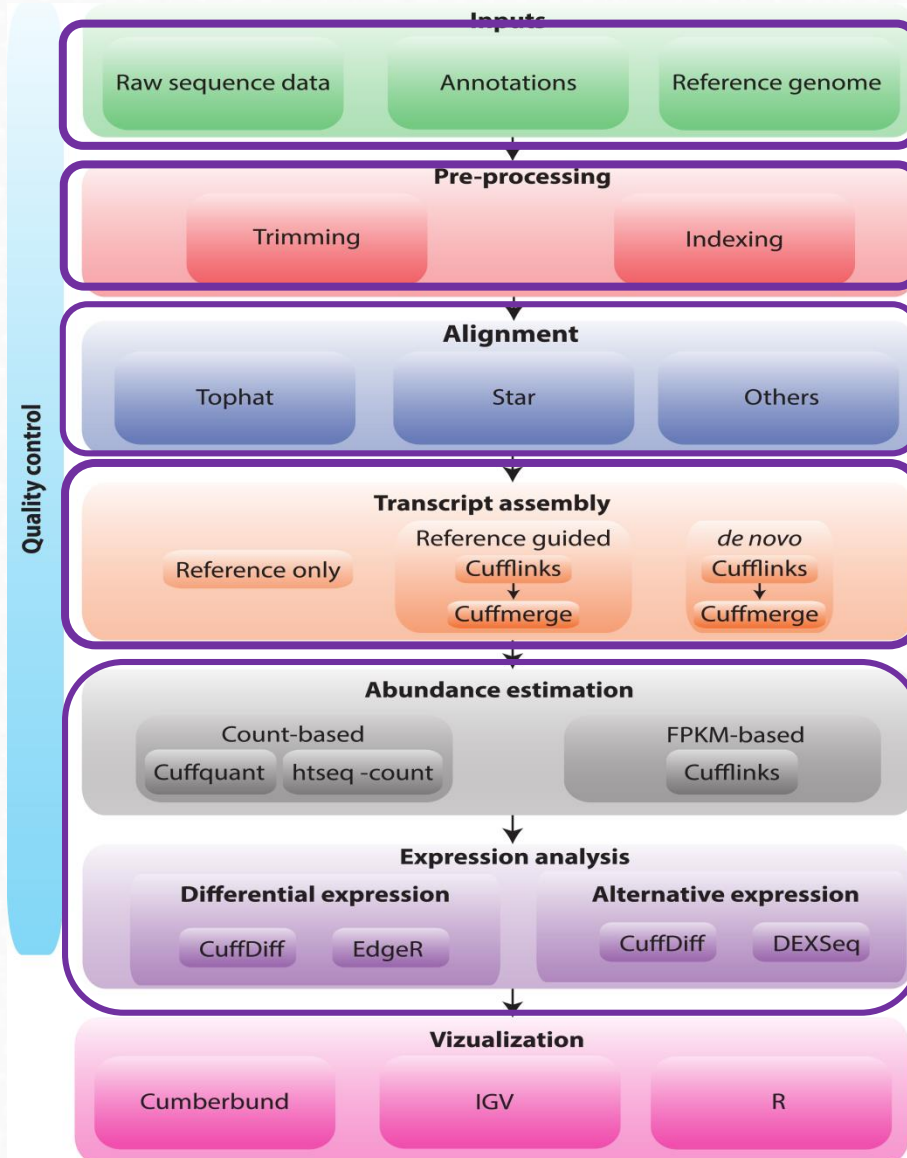


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## 2.5 RNA-seq analysis pipeline(s)



## 2.5 RNA-seq analysis pipeline(s)



### Count-based methods:

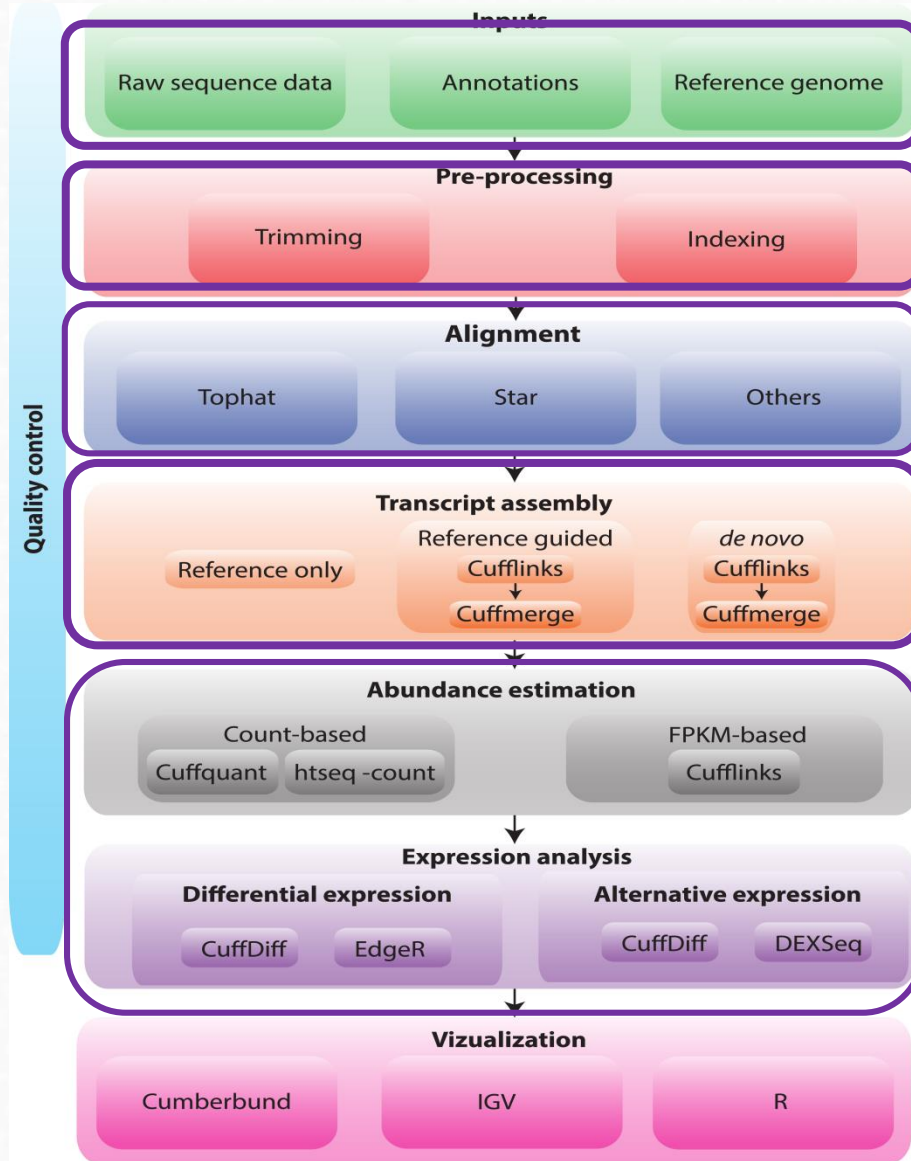
assign the reads to transcripts directly.

### Abundance based methods:

assign abundance of each transcript with a probabilistic model that makes use of info such as fragment length distribution etc. Many software tools want raw counts for input because they do their own normalization.



## 2.5 RNA-seq analysis pipeline(s)



Griffith, 2015 DOI: 10.1371/journal.pcbi.1004393

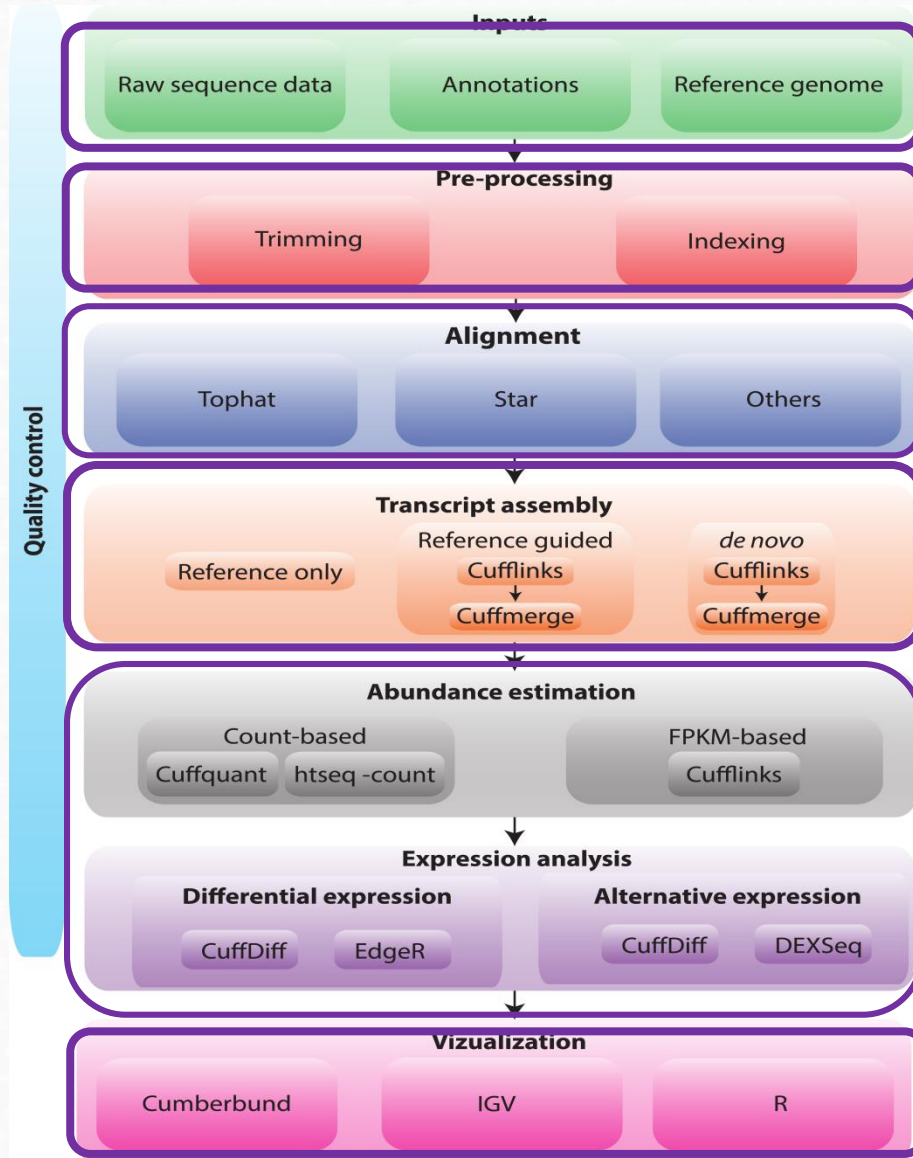
### Differential expression:

taking the normalized read count data and performing statistical analysis to discover quantitative changes in expression levels between experimental groups.

**Alternative expression:** analysis of RNA-seq data to catalog transcripts and assess alternative expression of known and predicted mRNA isoforms in cells and tissues

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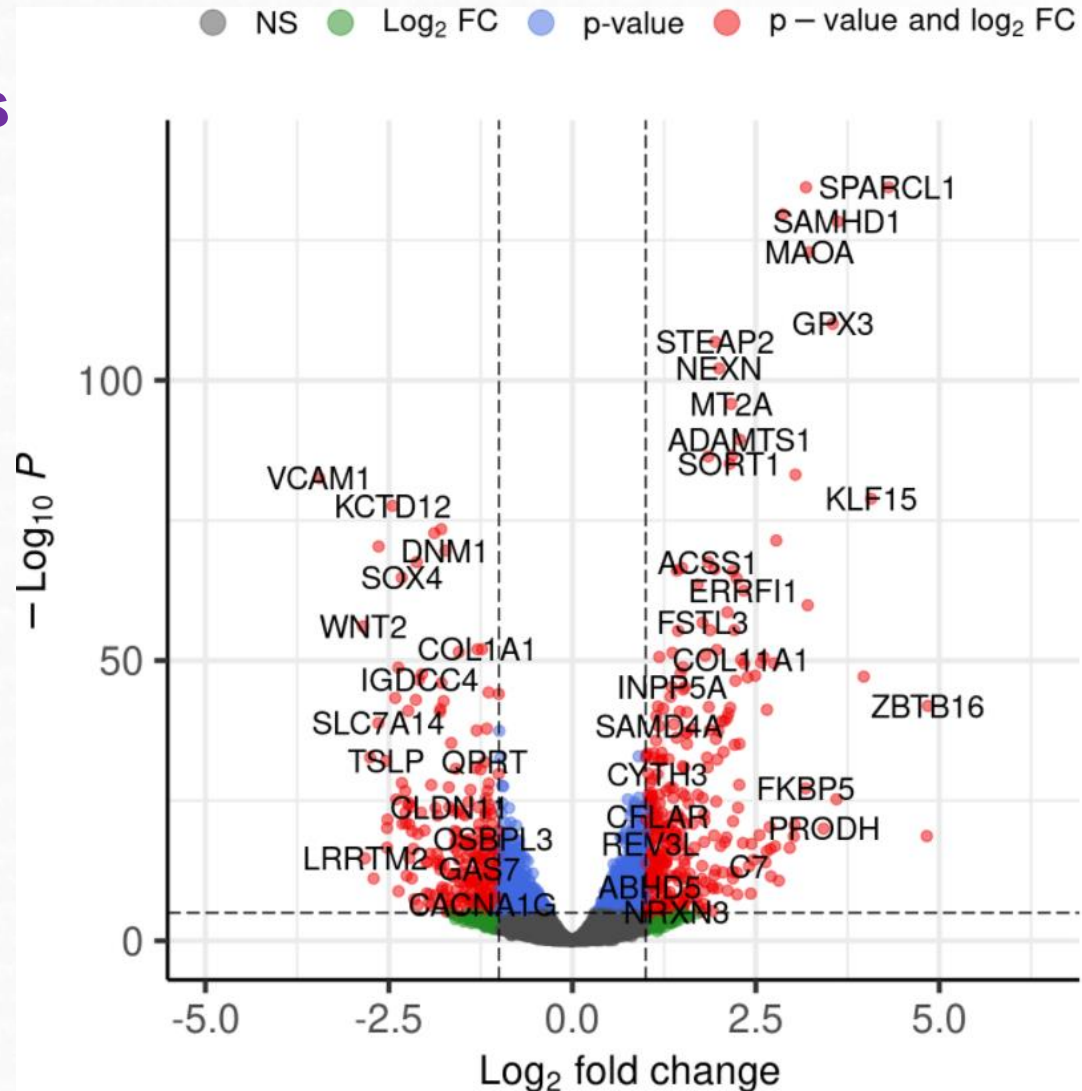
## 2.5 RNA-seq analysis pipeline(s)



Griffith, 2015 DOI: 10.1371/journal.pcbi.1004393

## 2.10 Vizualization

### Volcano plots

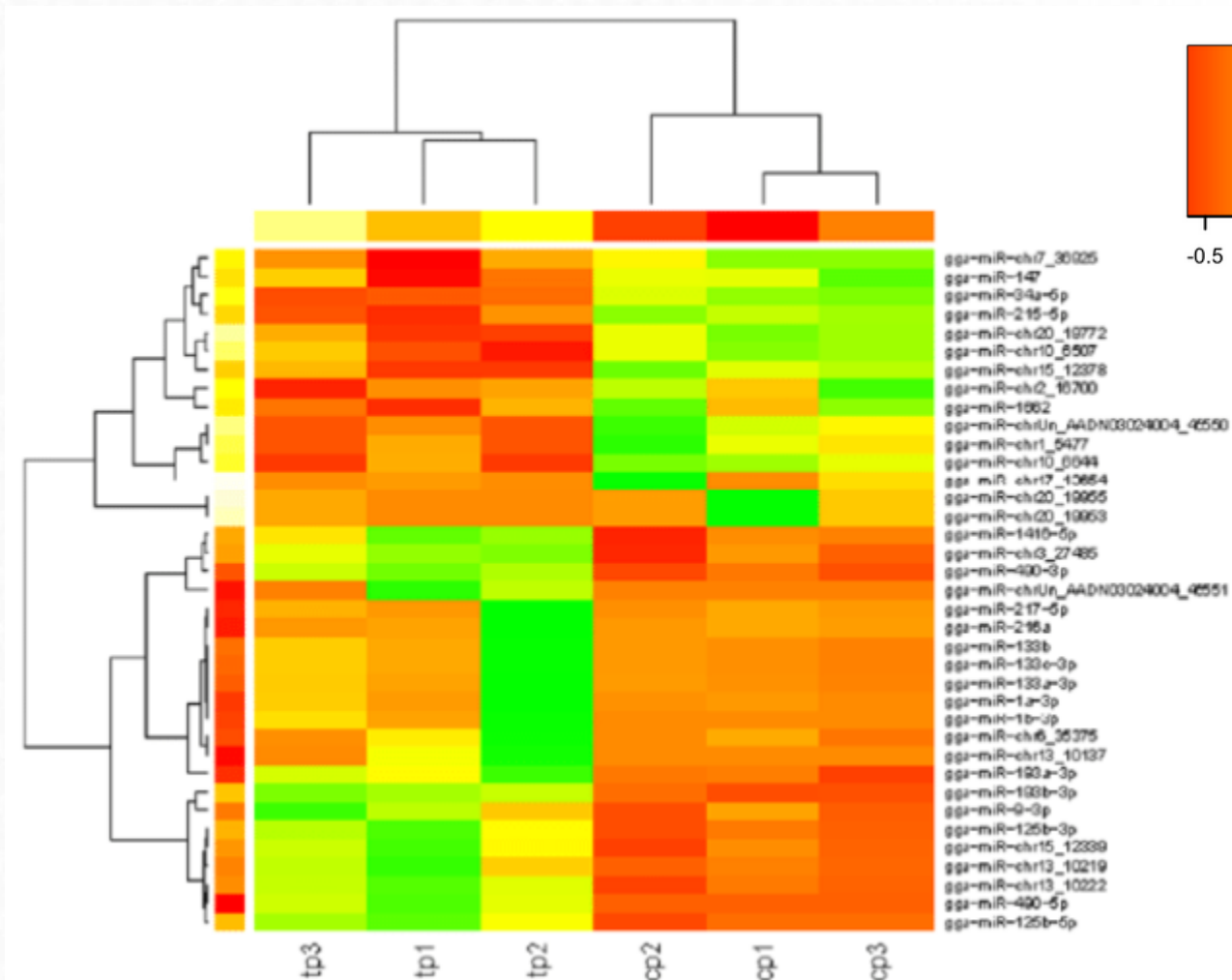


<https://www.bioconductor.org/packages/release/bioc/vignettes/EnhancedVolcano/inst/doc/EnhancedVolcano.html>



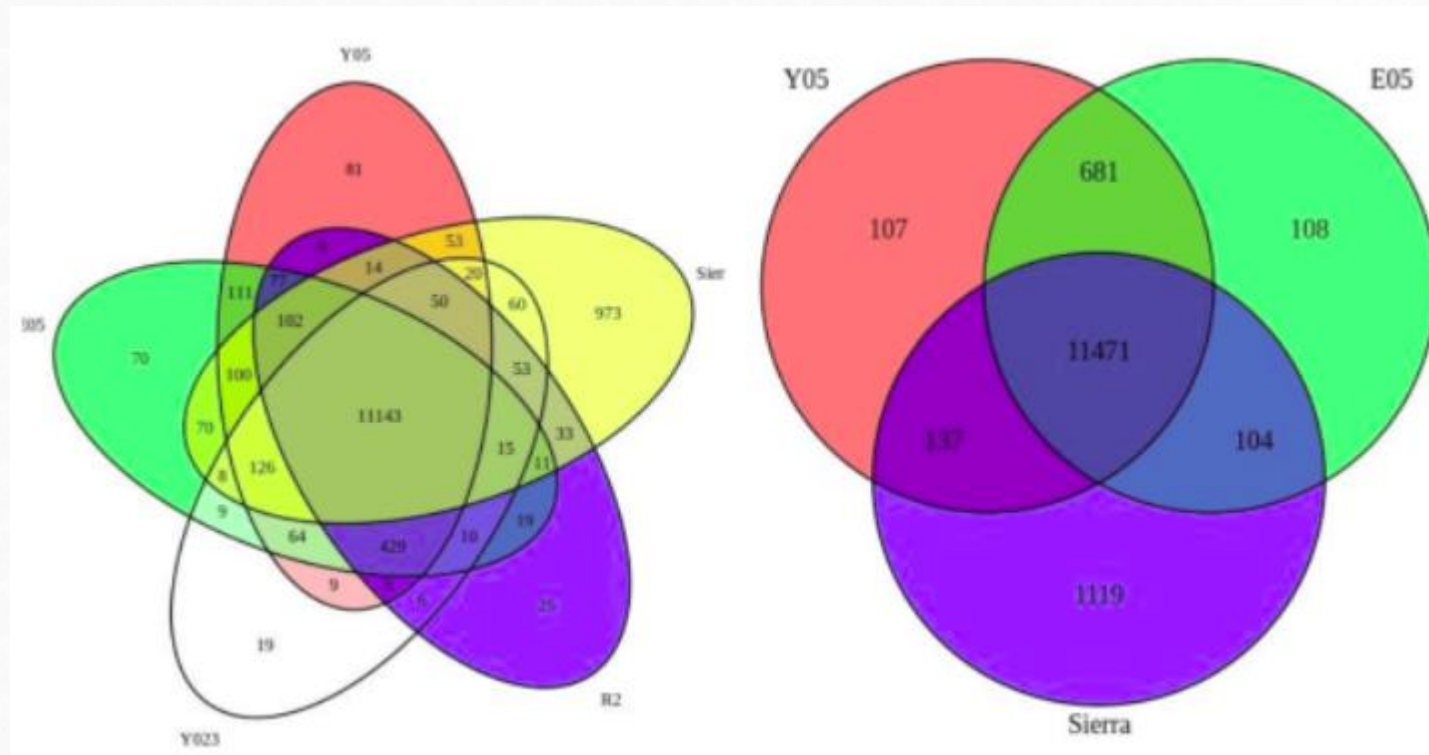
## 2.10 Vizualization

### Heatmap



## 2.10 Vizualization

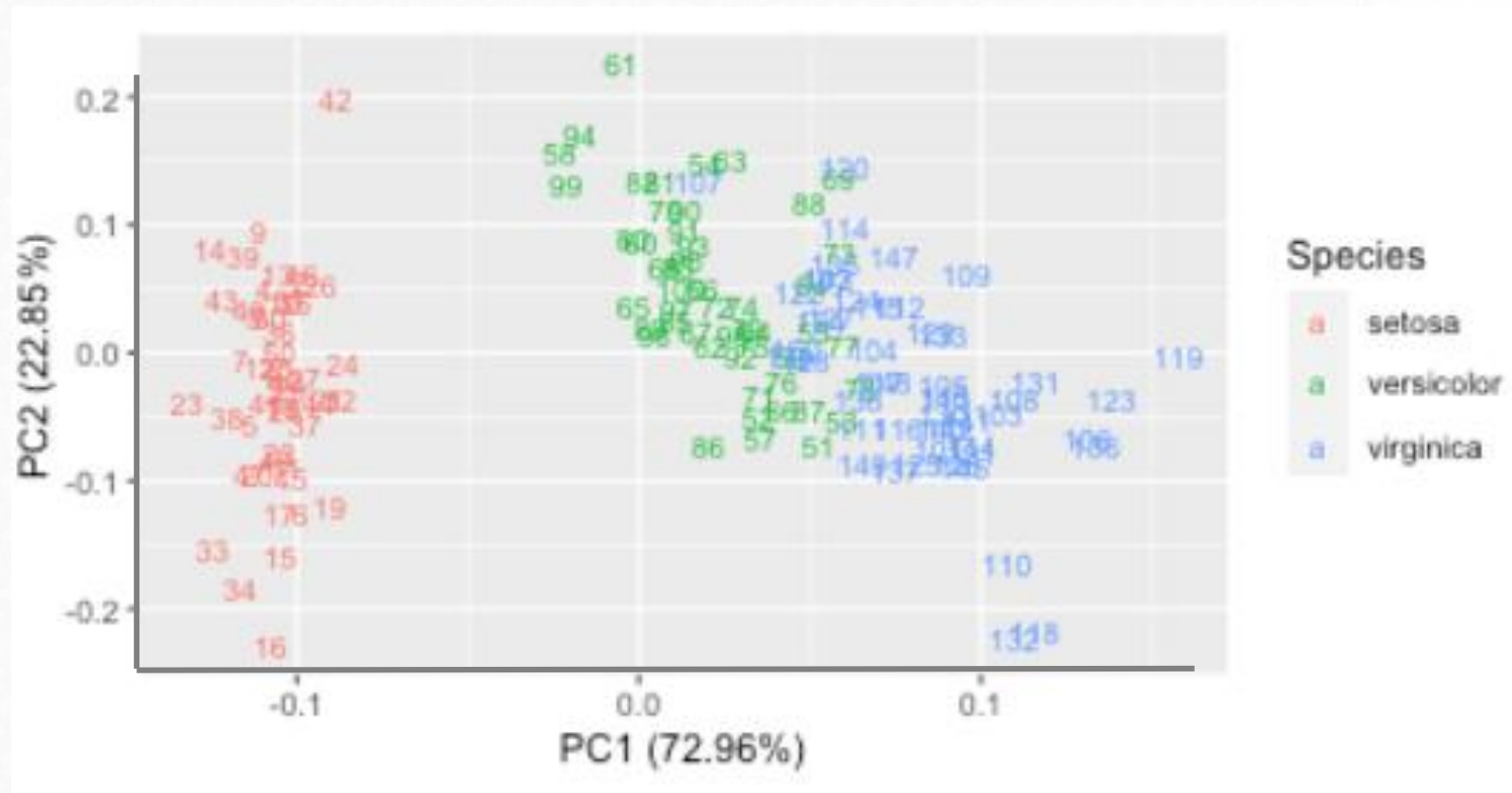
### Venn diagrams



<https://www.r-bloggers.com/2020/08/comparing-data-sets-with-venn-diagrams/>

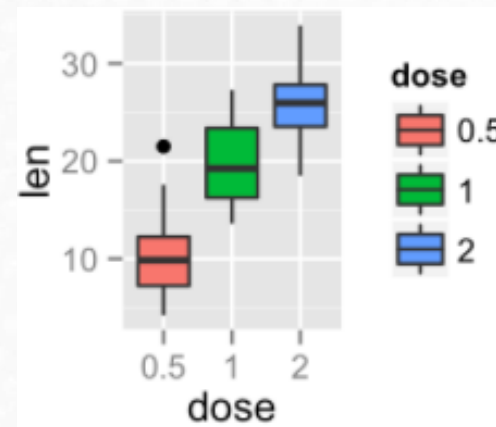
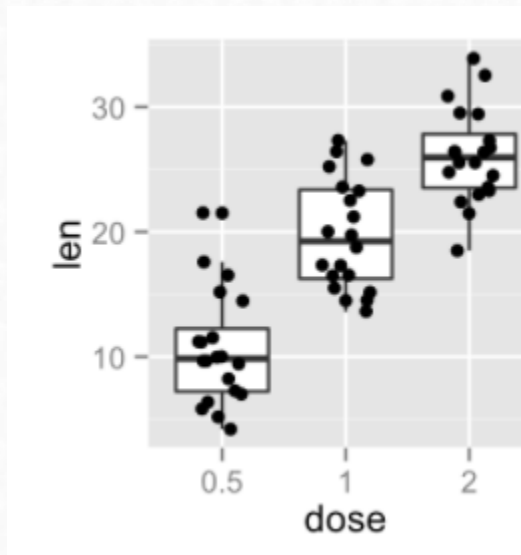
## 2.10 Vizualization

### PCA



## 2.10 Vizualization

### Boxplot

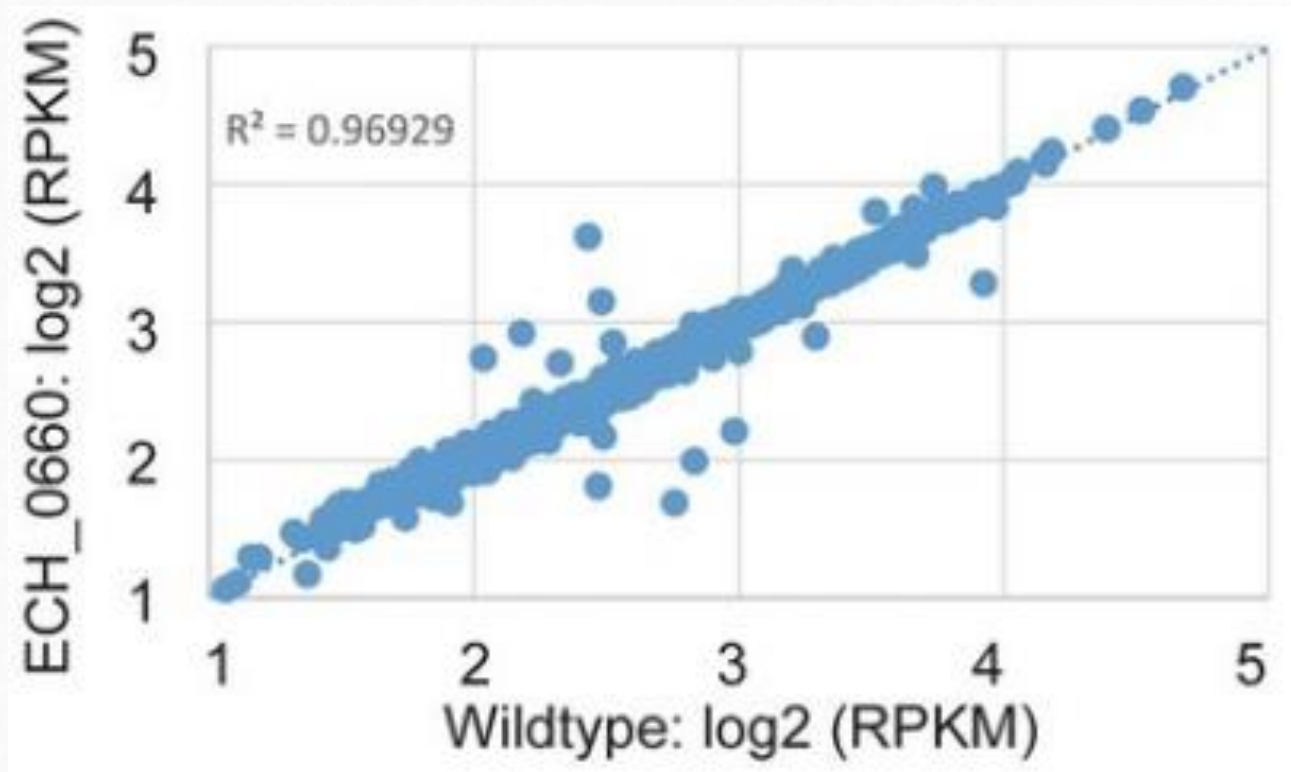


<http://www.sthda.com/english/wiki/ggplot2-box-plot-quick-start-guide-r-software-and-data-visualization>



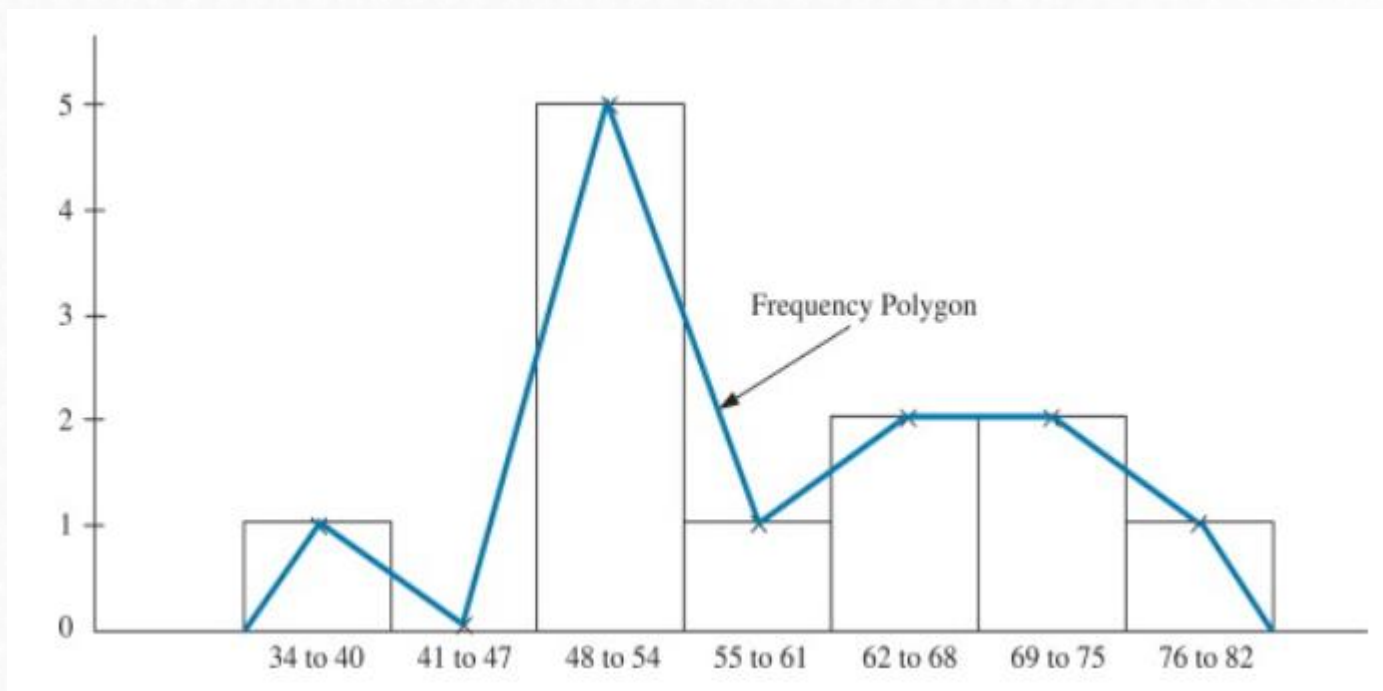
## 2.10 Vizualization

### Scatter plot



## 2.10 Vizualization

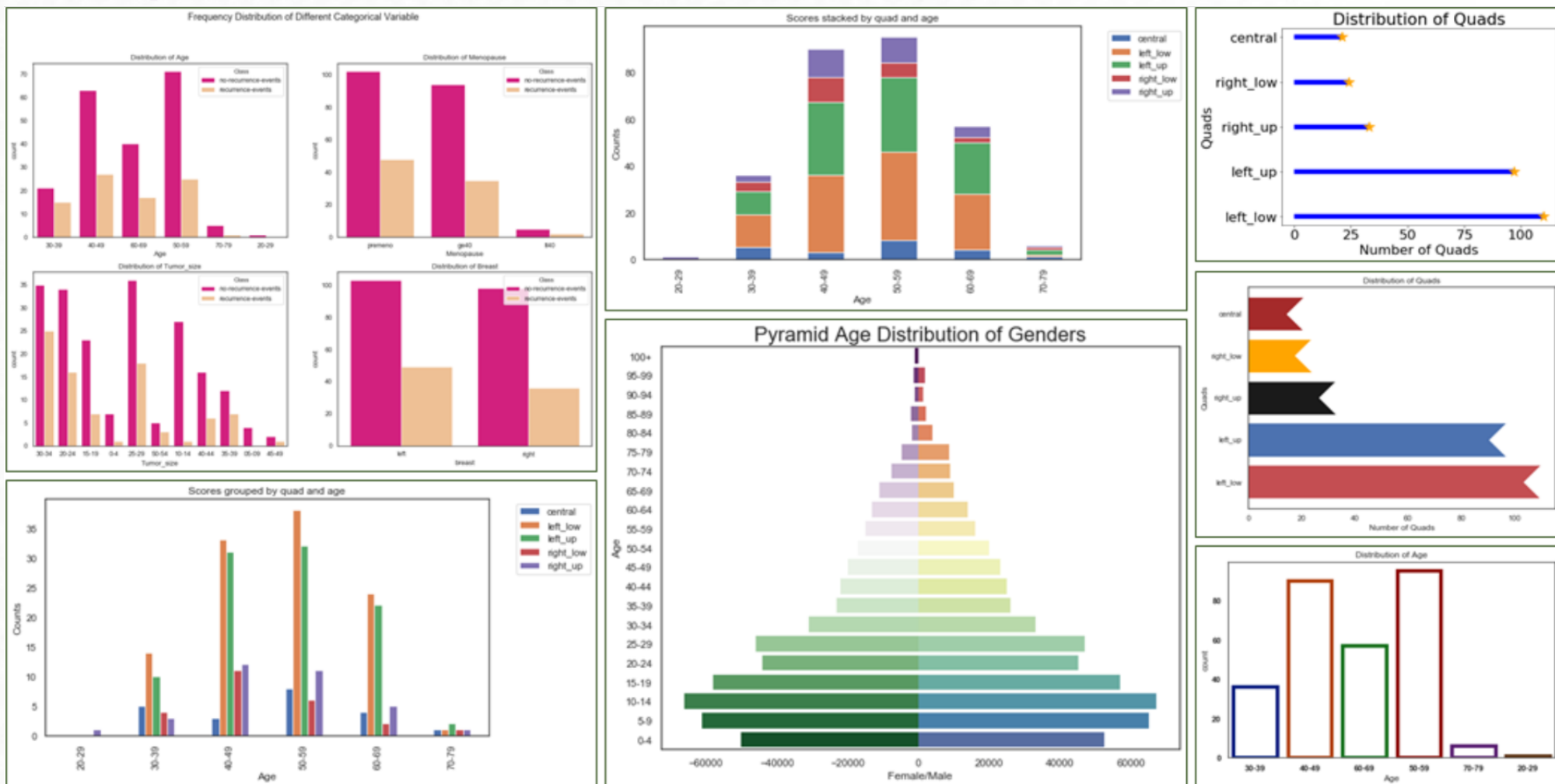
### Frequency plots



# 2.10 Vizualization

## Barplots

<https://towardsdatascience.com/different-bar-charts-in-python-6d984b9c6b17>

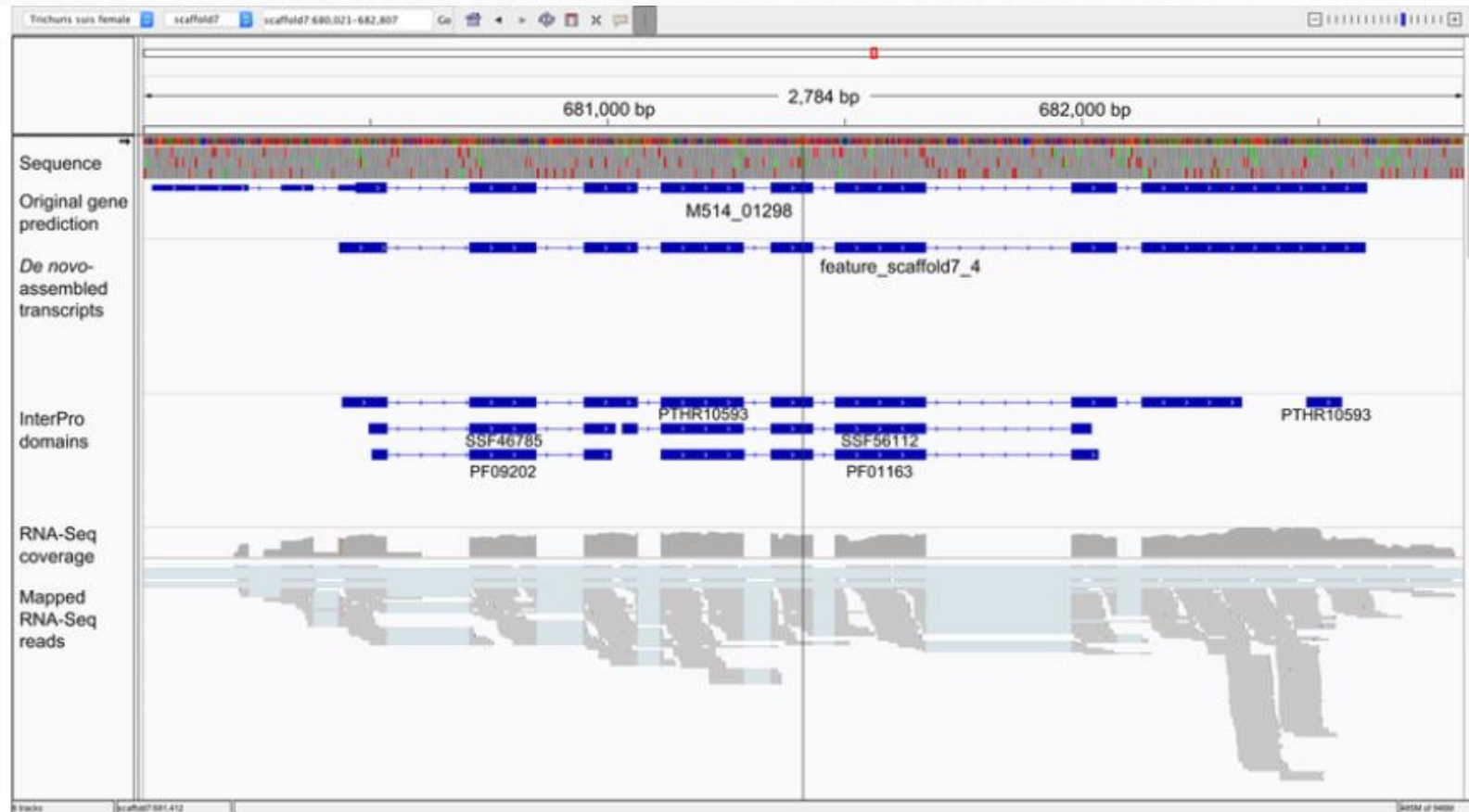


## 2.10 Vizualization

### Viewers

UCSC genome browser, **IGV**...

<https://pubmed.ncbi.nlm.nih.gov/29717207/>





## 2.10 Vizualization

### RNA-seq paired and stranded reads



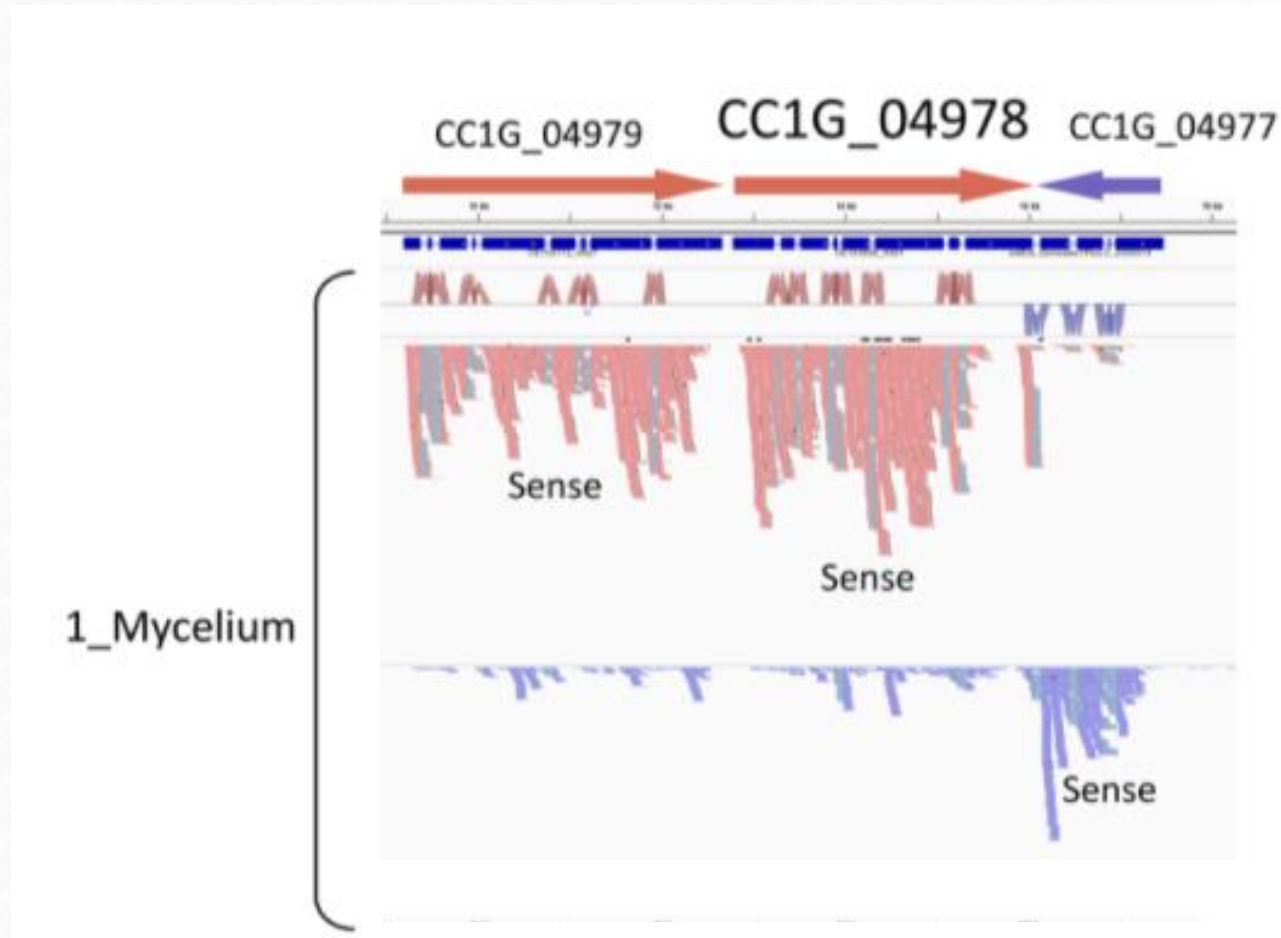
## 2.10 Vizualization

### RNA-seq paired and stranded reads



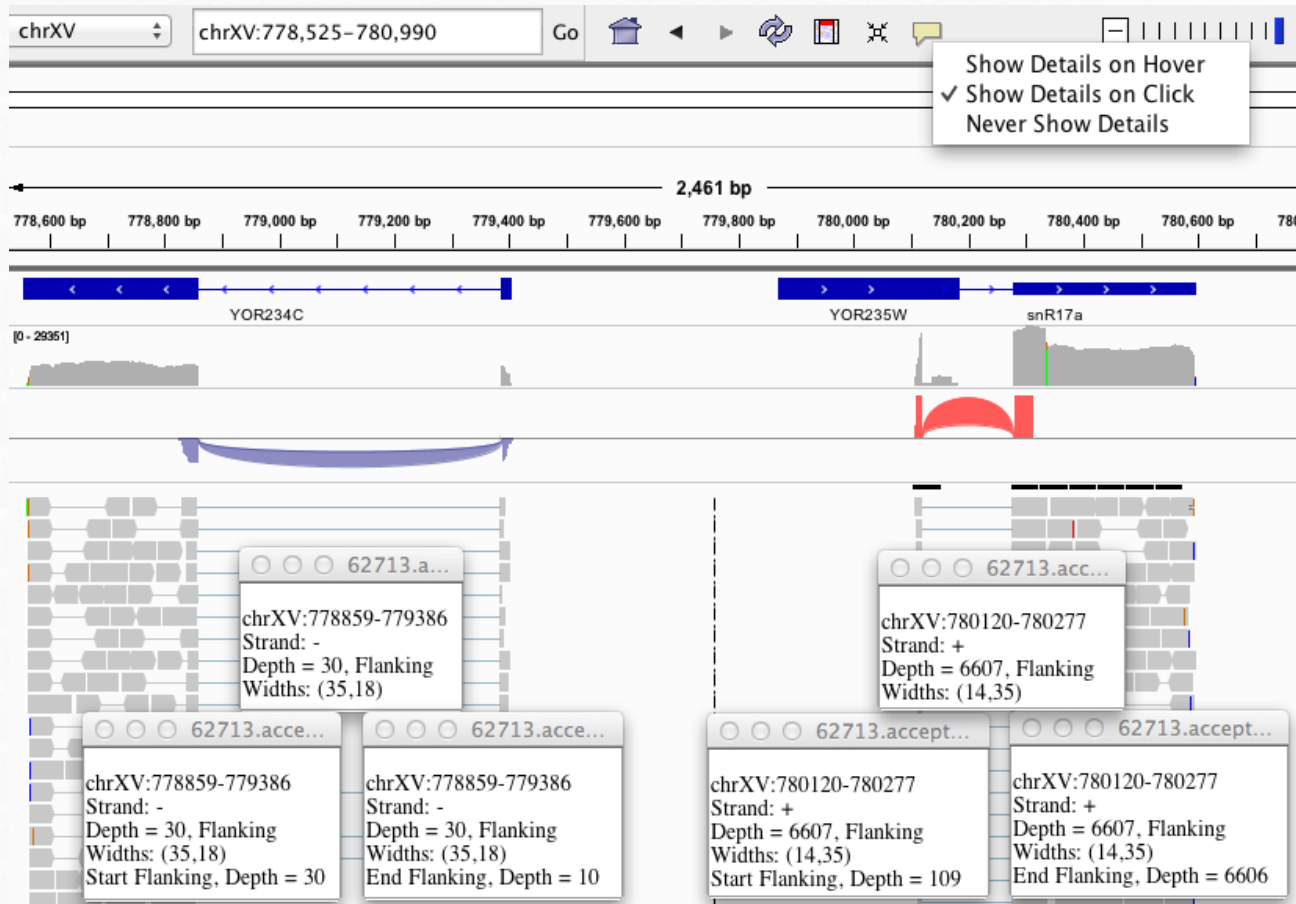
## 2.10 Vizualization

RNA-seq paired and stranded reads



## 2.10 Vizualization

### Junctions detected with TopHat



[https://software.broadinstitute.org/software/igv/splice\\_junctions](https://software.broadinstitute.org/software/igv/splice_junctions)



**A**

BAK1

chr6(p21.31)

Iso-Seq alignments

33,574 kb 33,576 kb 33,578 kb 33,580 kb

13 kb

PB.4024.2

231-WT

231-PTX

33,574 kb 33,576 kb 33,578 kb 33,580 kb

13 kb

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