# Transcriptome-based Study Designs

*Emerging Approaches For Tumor Analyses in Epidemiological Studies*

NIH NATIONAL CANCER INSTITUTE

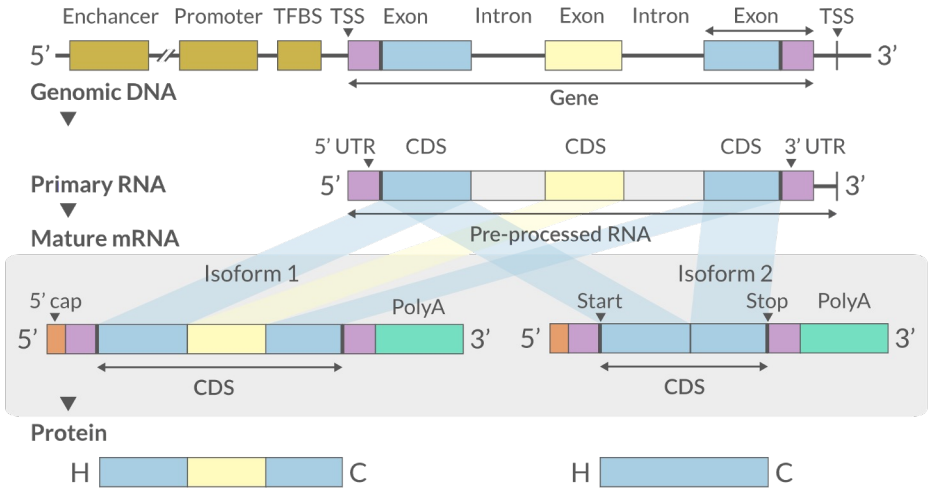
April 26, 2023
9:30am - 12:00pm

# Session overview

- Why RNA-seq
- Challenges
- Study design
  - Goal of study
  - Experimental strategy
- RNA-seq analysis pipeline
  - Alignment and Quality Control (QC)
  - Visualization
  - Quantification

# Why RNA-seq

# Why RNA-seq – RNA vs DNA-seq




The transcriptome

- is dynamic.
- represents only a small fraction of the genome (<5%).
- is indicative of gene activity.

# Why RNA-seq – RNA vs DNA-seq

- Evaluate the functional consequences of genomic changes
  - Difficult to infer from DNA sequences
  - ‘Regulatory’ mutations that do not directly affects the protein sequences
- Regulation at RNA level
  - Detection of alternative splicing, RNA editing, gene fusions
- Prioritize the genomic alterations that are more likely to be relevant
  - Mutations in expressed genes are more likely to be functionally relative
  - Allele-specific expression of the wild type or mutant allele.

# Why RNA-seq – RNA vs DNA-seq

- Understand the interactions between genes/pathways
  - Gene-gene interaction could be indicative of the activity of transcription factors that orchestrate gene expression.
- Identify the consequence of experimental perturbation
  - E.g. measuring the changes in gene expression in response to drug treatment

# Why RNA-seq – other related technologies/platforms

| | RNA-seq | microarray | Nanostring nCounter (array-based) | Single-cell/nucleus RNA-seq | Spatial transcriptomics |
|---|---|---|---|---|---|
| Identify new RNA sequences? | Yes | No | No | Yes | No |
| Resolution | Non-single-cell<br>Can be as low as 100 cells | Non-single-cell | Non-single-cell<br>Less starting material than bulk RNA-seq | Single-cell | Ranges from single-cell level to small spots/regions of interest. |
| Sensitivity | High | Low | High | Low | Low |
| Dynamic range | High | Low | High | Low | Low |
| Sample types | Fresh, Fresh-frozen, FFPE | Fresh, Fresh-frozen, FFPE | Fresh, Fresh-frozen, FFPE | Fresh, Fresh-frozen (compromised performance) | Fresh, Fresh-frozen, FFPE |
| # transcripts/genes | Whole transcriptome | Thousands to 50,000 | Up to 800 | Whole transcriptome | Targeted or whole transcriptome |
| Output | Characterizes the sequences and measures the average gene expression of cell populations. | Measures the average gene expression of preselected genes of cell populations. | Measures the average gene expression of preselected genes of cell populations. | Measures the gene expression of individual cells. | Quantify the gene expression within the spatial context of tissues. Usually integrated with scRNA-seq analysis. |

# Challenges

# Challenges

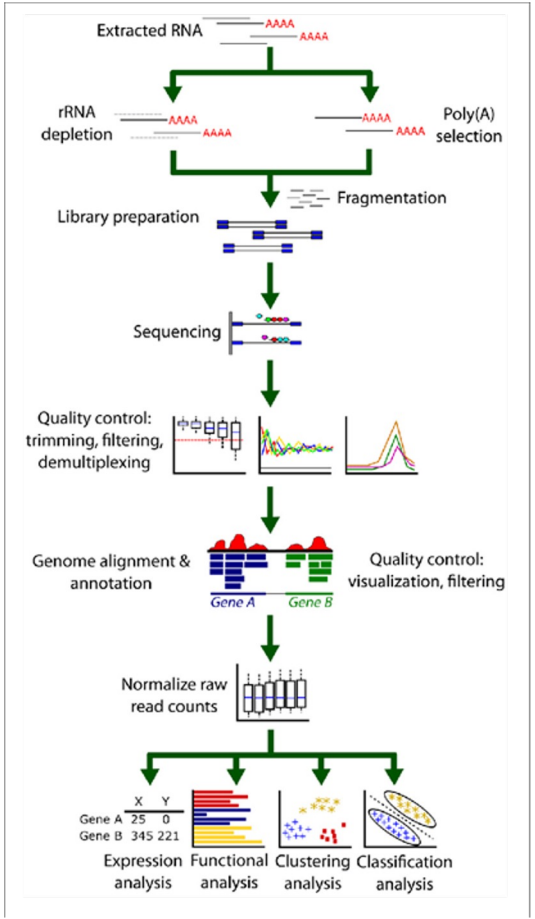
- Compared to DNA, RNAs
  - Are more fragile
  - Have tissue-specificity
  - Vary in orders of magnitudes in quantity
- Sample – quality and quantity of samples (purity, clinical sample)
- Types of RNA (rRNA, miRNA) should be removed or processed separately
- Technical bias in mapping and quantification due to degradation.
- Specific gene structure can be challenging (e.g. large intron)

# Study design

# Study design – Goal of study

- Characterization of the gene expression pattern
- Differential gene expression across samples
- Identification of novel transcripts
- Alternative splicing analysis
- Detection of gene fusion events
  - Explore the functional consequences of structural variants
- Discovery or validation of mutation
  - Allele-specific expression analysis
- Identification of RNA editing

# Study design – Generic pipeline of RNA-seq studies






Namjoshi SV, Raab-Graham KF. Front Mol Neurosci 2017;10:45.

# Study design – Experimental strategy

- General best practices for RNA-seq experiments
- Goal-driven experimental variables
- RNA Quality considerations

# General best practices for RNA-Seq

- Use of replicates
- Importance of batching, randomization during processing
- RNA-sequencing best practices (short read)

# Replicates

Experimental replicates can be performed as **technical replicates** or **biological replicates**.

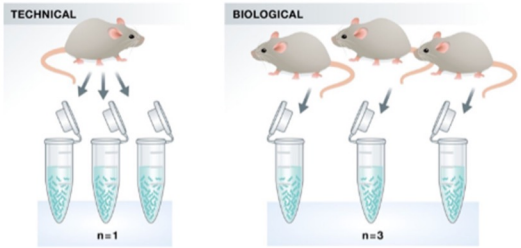



*Image credit: Klaus B., EMBO J (2015) **34**: 2727-2730*

- **Technical replicates:** use the same biological sample to repeat the technical or experimental steps in order to accurately measure technical variation and remove it during analysis.
- **Biological replicates** use different biological samples of the same condition to measure the biological variation between samples.

- What is the difference between technical, biological replicates?
- For RNA-seq, technical replicates are not generally necessary
- Biological replicates are essential; 6-12 biological replicates generally recommended

https://hbctraining.github.io/Intro-to-rnaseq-hpc-salmon/lessons/experimental_planning_considerations.html#:~:text=Technical%20replicates%3A%20use%20the%20same,the%20biological%20variation%20between%20samples.

# Batch effects

- More likely to impact RNA-seq than DNA sequencing
- When, where, method, reagents
- Were all study samples:
  - Extracted on the same day?
  - Libraries prepared on the same day?
  - Was the same method used for extraction, library prep, sequencing, at the same facility?
  - Was all sequencing performed on the same run?
  - If any of the answers are “no”, then you have batches….
- Batch effects can sometimes be addressed during experimental design

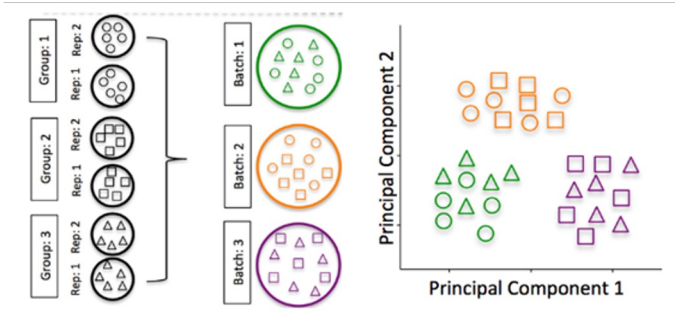
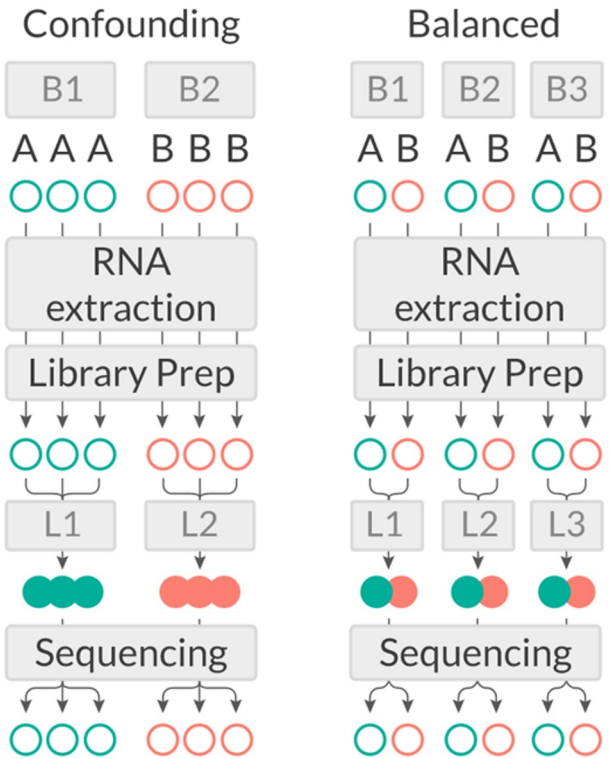



*Image credit: Hicks SC, et al., bioRxiv (2015)*

Are the differences in expression observed in the data due to biological differences in your treatment groups? Or due to processing variables between batches of samples?

https://hbctraining.github.io/Intro-to-rnaseq-hpc-salmon/lessons/experimental_planning_considerations.html#:~:text=Technical%20replicates%3A%20use%20the%20same,the%20biological%20variation%20between%20samples.

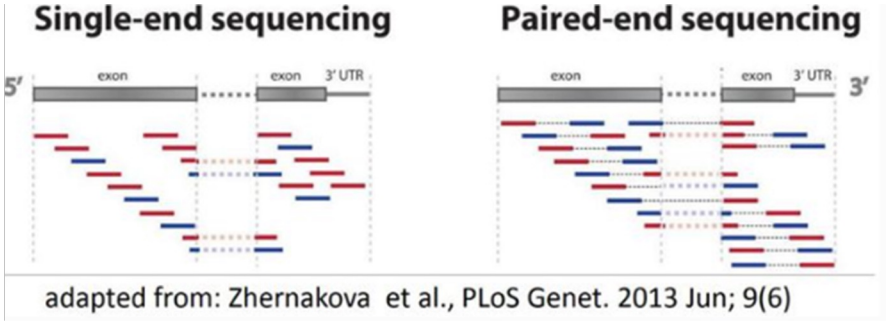
# Best practices for batches

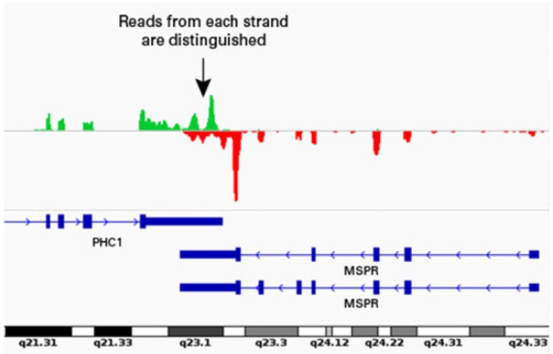



- Avoid confounding your experiment by batch
- Reduce processing variables between treatment groups (A, B at left)
- When batches are unavoidable, split biological replicates across batches
- Track batch metadata for downstream analysis

# RNA-seq best practices (short read)

- Paired End (PE) sequencing generally preferable to Single End (SE) sequencing
  - Improved mappability
  - Read pairs more likely to span exons
- Stranded RNA-seq preferable to non-stranded
  - Better resolution for reads from overlapping genes transcribed on opposite strands
  - Better resolution for bi-directional transcription
- Both strategies improve the accuracy of gene expression quantification




http://www.takara.co.kr/file/manual/634836.html

# Goal-driven experimental design variables

- Sequencing depth
- RNA Library prep methods
- Sequencing platform

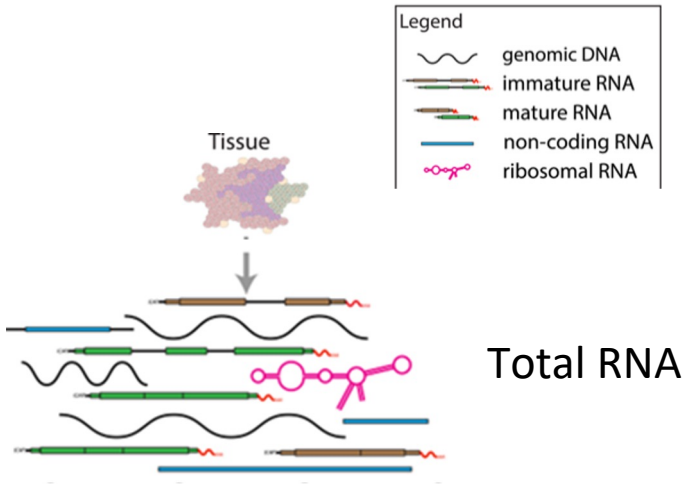
# Sequencing depth

- Highly dependent on goals
- Why it's difficult to answer the question "How much does RNA-seq cost"?

| Analysis Goal | Reads Recommended |
|---|---|
| Differential expression of highly expressed genes | 5-25 million |
| Differential expression across transcriptome, isoform level | 30-60 million |
| Alternative splicing | 80-100 million |
| Assembly of new transcripts | 100-200 million |

# RNA Library prep methods

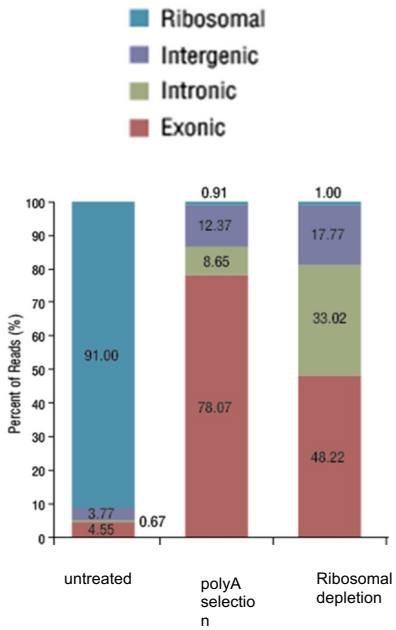
- Total RNA is 80-95% rRNA (ribosomal RNA)
- In order to focus on the transcriptome, we have to reduce the rRNA in total RNA during Library prep
- Two methods:
  - Poly A selection
  - Ribosomal depletion
  - Selection depends on goals, RNA quality




https://journals.plos.org/ploscompbiol/article/figure?id=10.1371/journal.pcbi.1004393.g004

# RNA Library prep methods

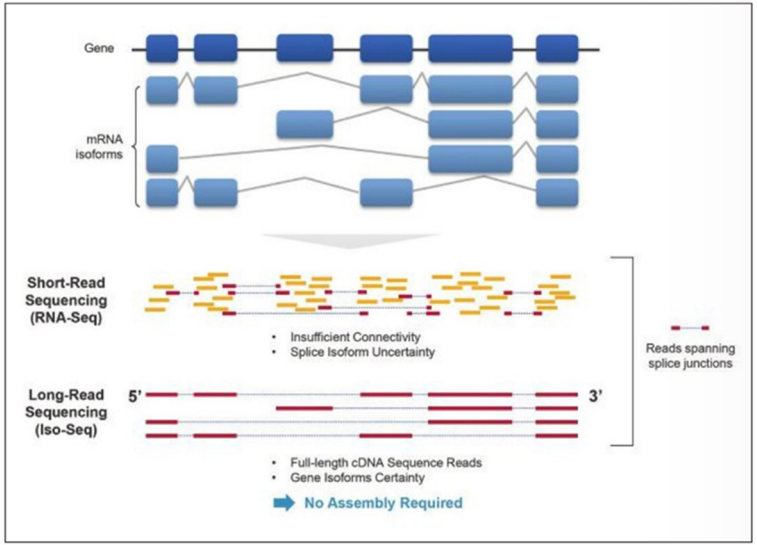
- polyA (mRNA) selection
  - Selects for only mRNAs with a polyA tail
  - Enriched for protein coding only reads
  - Very little pre-mRNA, lincRNA, etc.
  - Doesn't work well with low quality, degraded RNA (bias)
  - **Bottom line:** more efficient if all you're interested in is coding RNA, and your RNA is very high quality
- Ribosomal depletion
  - Removes rRNA with probes
  - Leaves everything else to be sequenced
  - Diverse RNA sequences
  - Not as enriched for exonic reads
  - Effective for degraded RNA
  - **Bottom line:** good for large studies where RNA quality might be variable; or non-coding RNAs are of interest




https://www.neb.com/products/e6310-nebnext-rrna-depletion-kit-human-mouse-rat#Product%20Information

# Sequencing platform

- Short-read vs Long-read
  - Short-read is by far the most common approach for RNA-seq, with broad analytical utility
  - RNA-seq can also be performed using long-read technologies (PacBio, Oxford Nanopore)
  - Long-read RNA-seq often called “Iso-Seq”
    - Not geared towards Differential Expression
    - Entire transcripts spanned by single reads
    - Isoform Discovery, resolution of complex splicing/fusion events
    - Disambiguates Isoforms that short reads can’t resolve
    - Requires VERY high quality RNA






chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://www.pacb.com/wp-content/uploads/2018-10-NA-UGM-Iso-Seq-Method.pdf

# RNA Quality considerations

- RNA MUCH more susceptible to degradation than DNA
  - During processing, handling
  - Depending on storage temperature
  - Freeze/Thaw cycles
- How intact or degraded your RNA is can impact feasibility of
  - Experiments that can be performed
  - Reliability of analysis

# RNA Quality considerations

- Quality of RNA should be assessed, reported after extraction, before library prep
- Often reported as the “RIN”, or RNA Integrity Number
- Quality score from 1-10
- Virtually all FFPE RNA will be “Low” quality

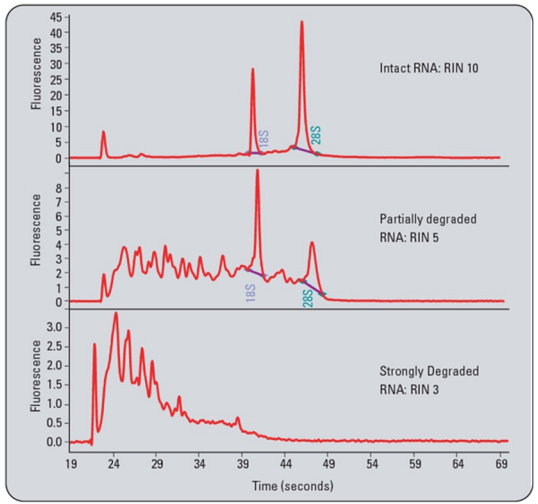


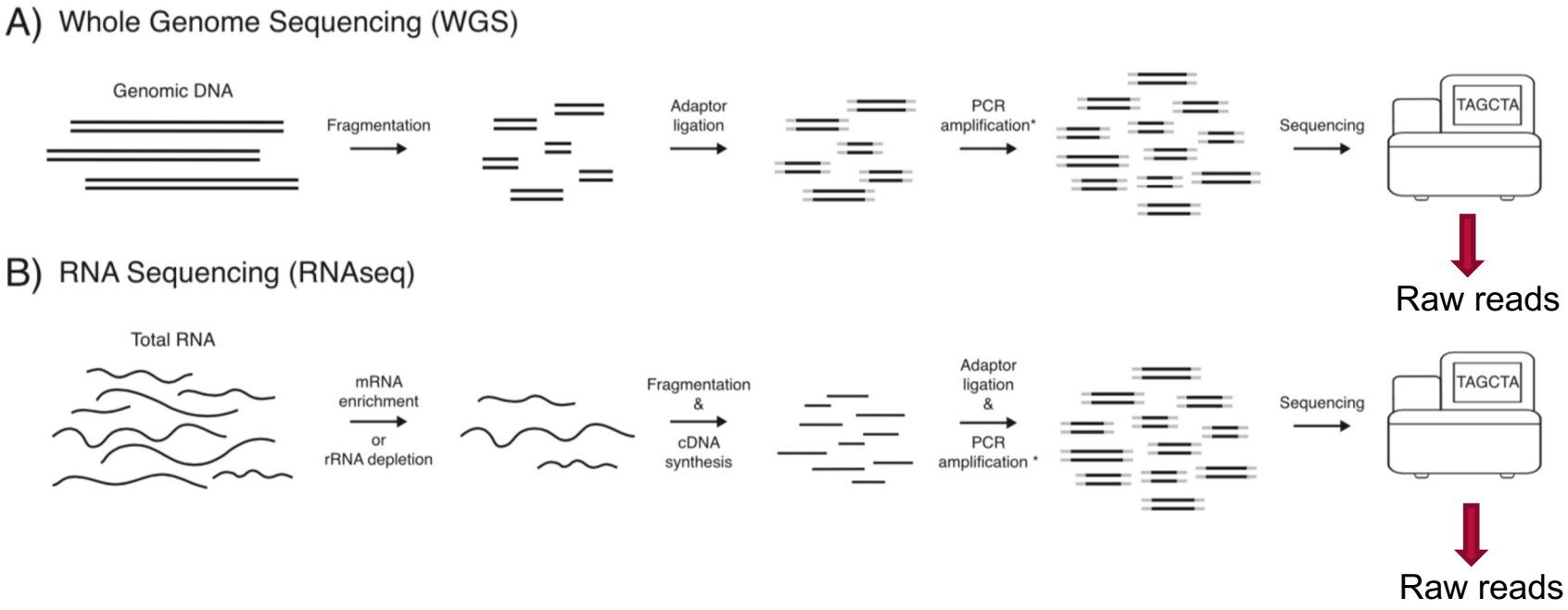

Figure 6

| RNA Quality | RIN Score | Limitations |
|---|---|---|
| High | 8-10 | |
| Medium | 4-7 | No long-read, no polyA selection |
| Low | 1-3 | No long-read, no polyA selection, RNA-seq may be piloted to assess suitability |

https://blog.genohub.com/2017/12/24/rin-numbers-how-theyre-calculated-what-they-mean-and-why-theyre-important/

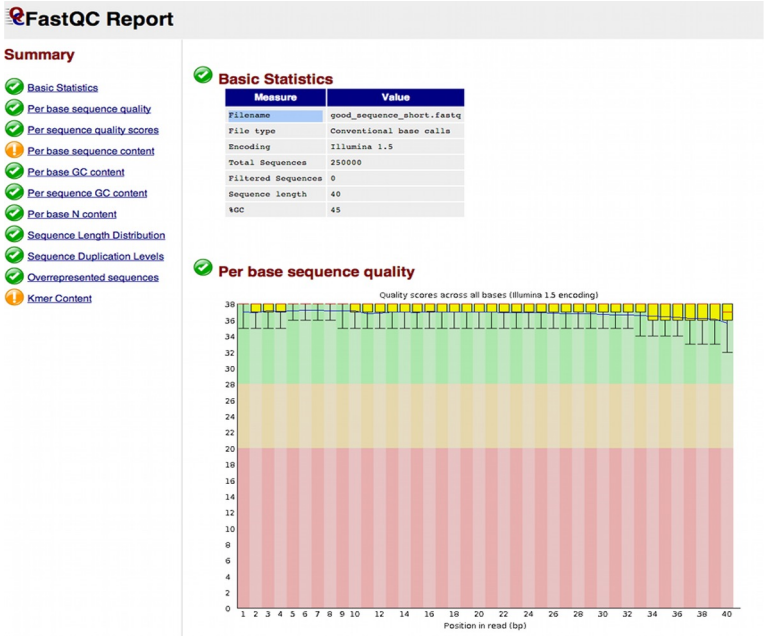
# RNA-seq analysis pipeline

# Sequencing

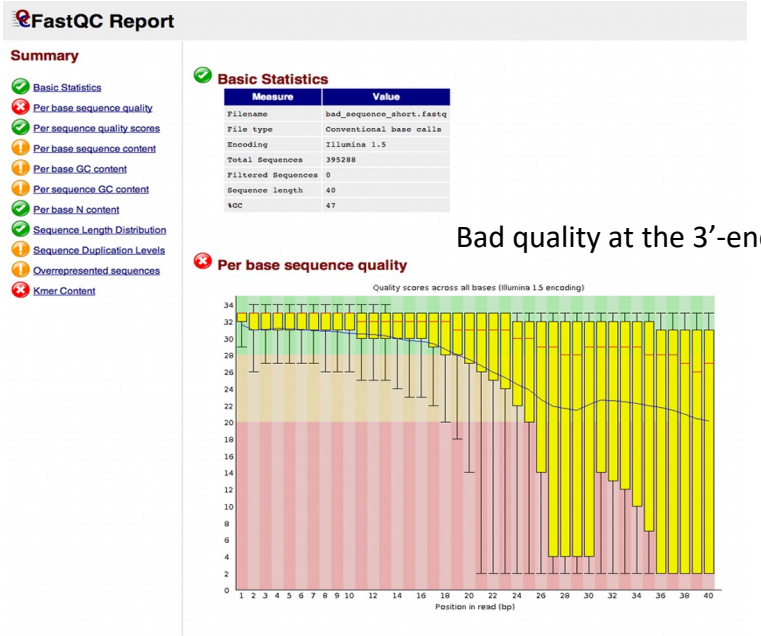

A) Whole Genome Sequencing (WGS)
Genomic DNA
Fragmentation
Adaptor ligation
PCR amplification*
Sequencing
TAGCTA
Raw reads
B) RNA Sequencing (RNAseq)
Total RNA
mRNA enrichment
or
rRNA depletion
Fragmentation & cDNA synthesis
Adaptor ligation & PCR amplification *
Sequencing
TAGCTA
Raw reads

# Quality Control (QC) of Raw Reads

- FastQC
  - Good example




  - Bad example



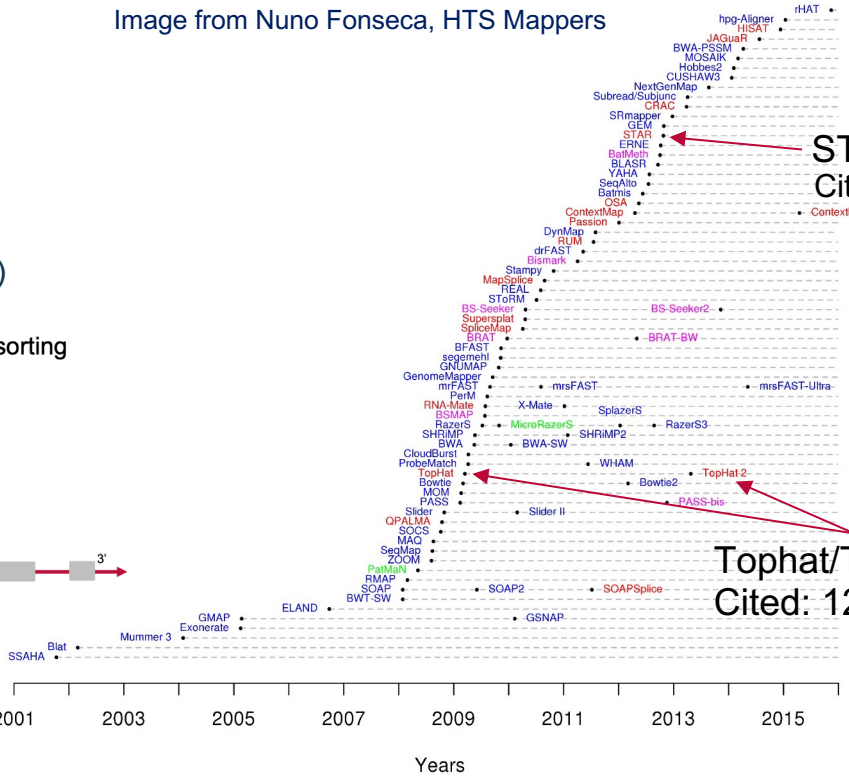

Bad quality at the 3’-end

# Splice-aware aligners

- Tophat/Tophat2
- STAR
- MapSplice
- SpliceMap

## Timeline of NGS read aligners

Image from Nuno Fonseca, HTS Mappers

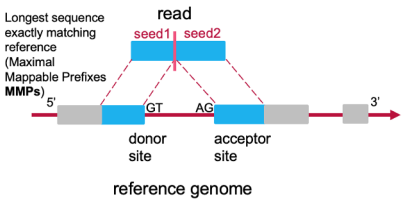



STAR
Cited: 30,158

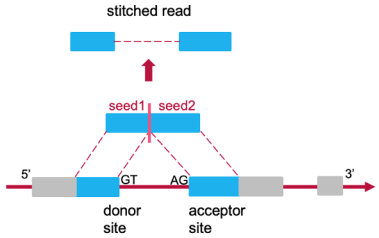
Tophat/Tophat2
Cited: 12,727/12,346

### STAR (**S**pliced **T**ranscripts **A**lignment to a **R**eference)

- Seed searching



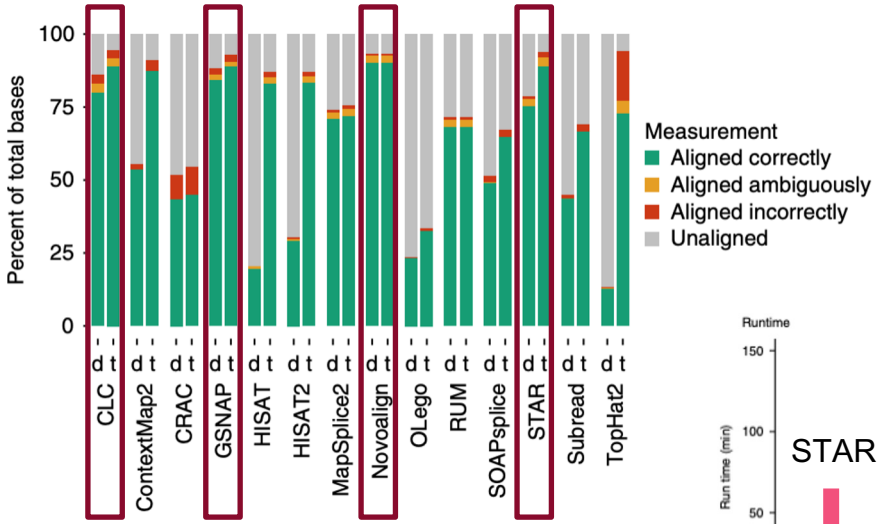

- Clustering, stitching, and sorting




PMID: 23104886

# Benchmark of RNA-seq aligners

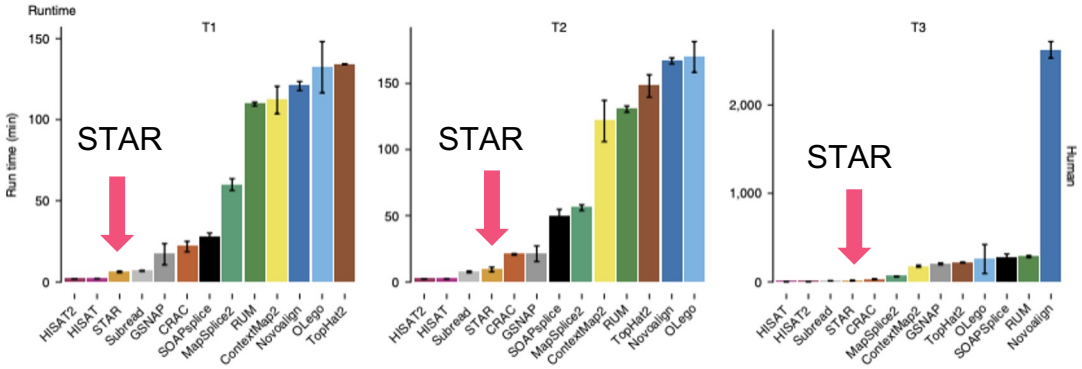
**Accuracy performance**




d: default parameters
t: tunned parameters

STAR is the winner in terms of performance and runtime. The only issue is memory intensive. HPC is needed.

**Runtime performance on human dataset**

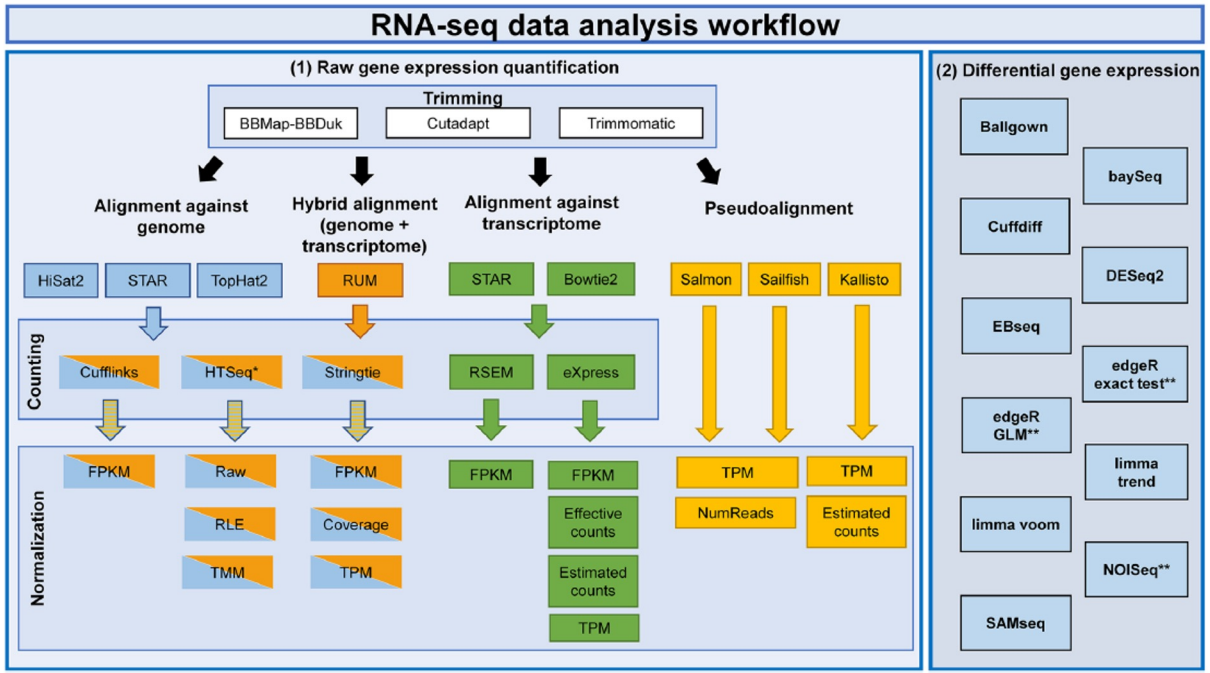



PMID: 27941783

# RNA-seq analysis pipeline

- No standard pipeline, many tool combinations, reference based

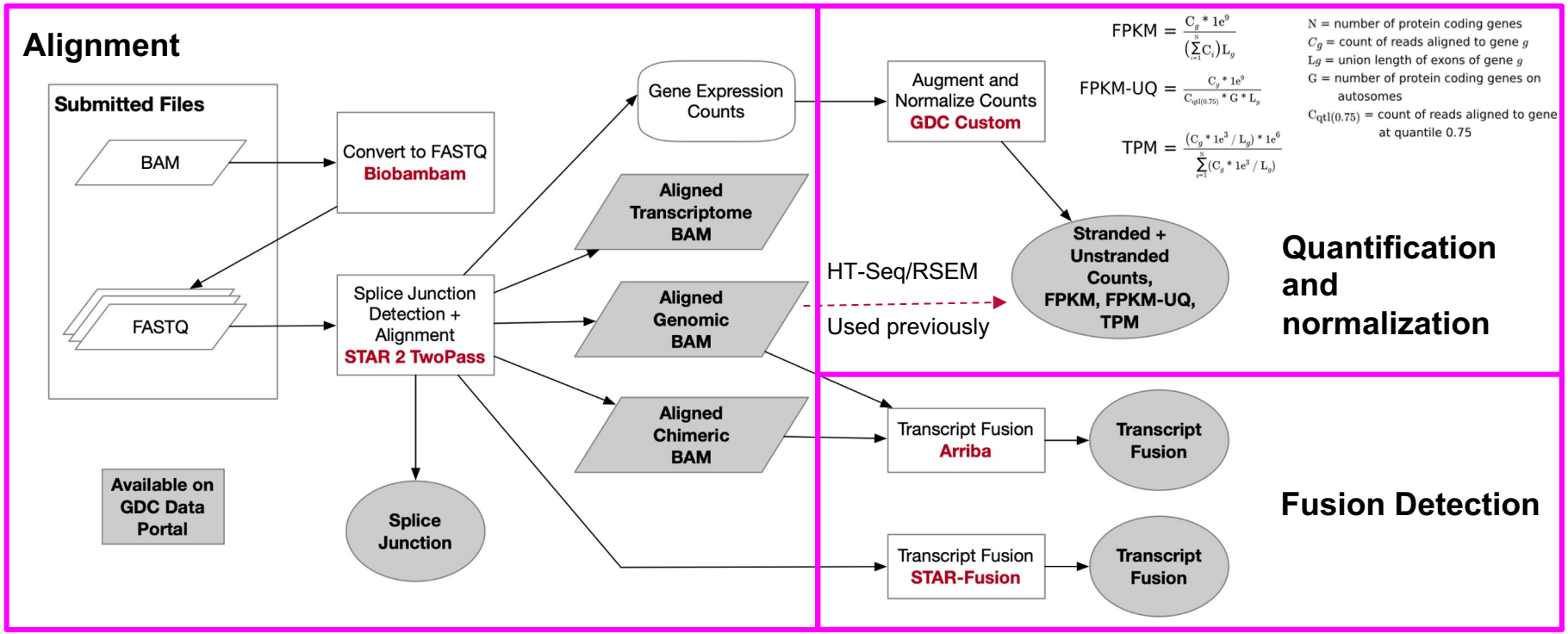
1. Alignment
2. Quantification and normalization
3. Downstream analysis




Corchete et al. Sci Reports, 2020.
https://doi.org/10.1038/s41598-020-76881-x

# RNA-seq analysis pipeline

## TCGA RNA-seq pipeline as an example




ICGC has a very similar pipeline.

https://docs.gdc.cancer.gov/Data/Bioinformatics_Pipelines/Expression_mRNA_Pipeline/

# RPKM, FPKM, FPKM-UQ and TPM

- RPKM (single-end read)
  Reads per Kilobase of transcript per Million mapped

- FPKM (pair-end reads)
  Fragments per Kilobase of transcript per Million mapped

- FPKM-UQ (pair-end reads)
  Upper quartile normalized FPKM

- TPM (pair-end reads)
  Transcripts per Million mapped

$$RPKM = \frac{ExonMappedReads * 10^9}{TotalMappedReads * ExonLength}$$

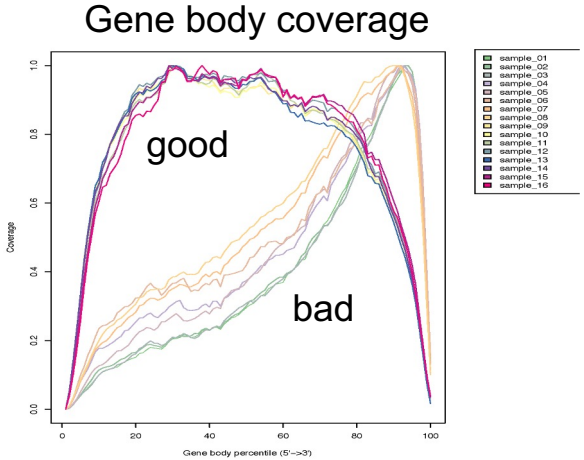
$$FPKM = \frac{ExonMappedFragments * 10^9}{TotalMappedFragments * ExonLength}$$

$$TPM = \frac{Ni/Li * 10^6}{sum(N1/L1 + N2/L2 + \ldots + Nn/Ln)}$$

Ni is the number of reads compared to the i-th exon; Li is the length of the i-th exon; sum(N1/L1+N2/L2+.....+Nn/Ln) is the sum of the values of all (n) exons after normalization by length.

# QC After Alignment

- %mapped reads/uniquely mapped reads (aka, sequencing depth)
- Gene body coverage (e.g. 5’-to-3’ bias)
- Quality distribution
- Average insert size
- PCR duplicates
- Distribution across exon/intron/intergenic regions
- Strand-specificity
- Sequencing saturation

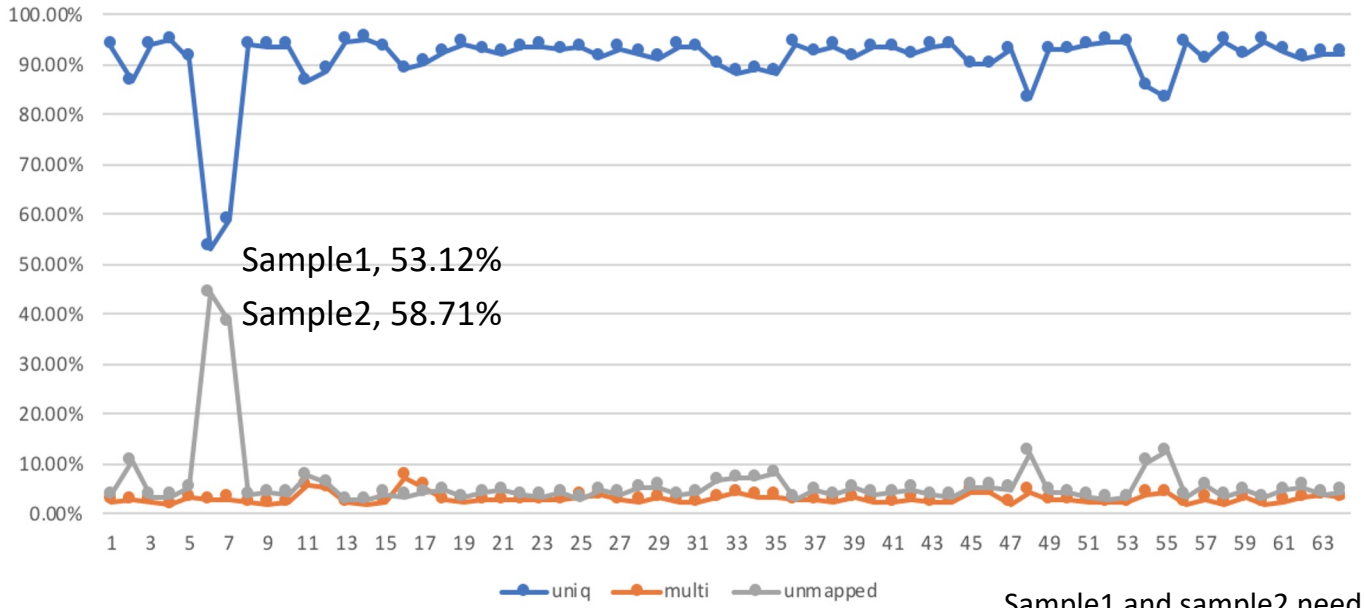



RSeQC and
RNA-SeQC2

PMID: 22743226
PMID: 33677499

# QC After Alignment

## Mapping rate to the Human Genome (hg19): An example

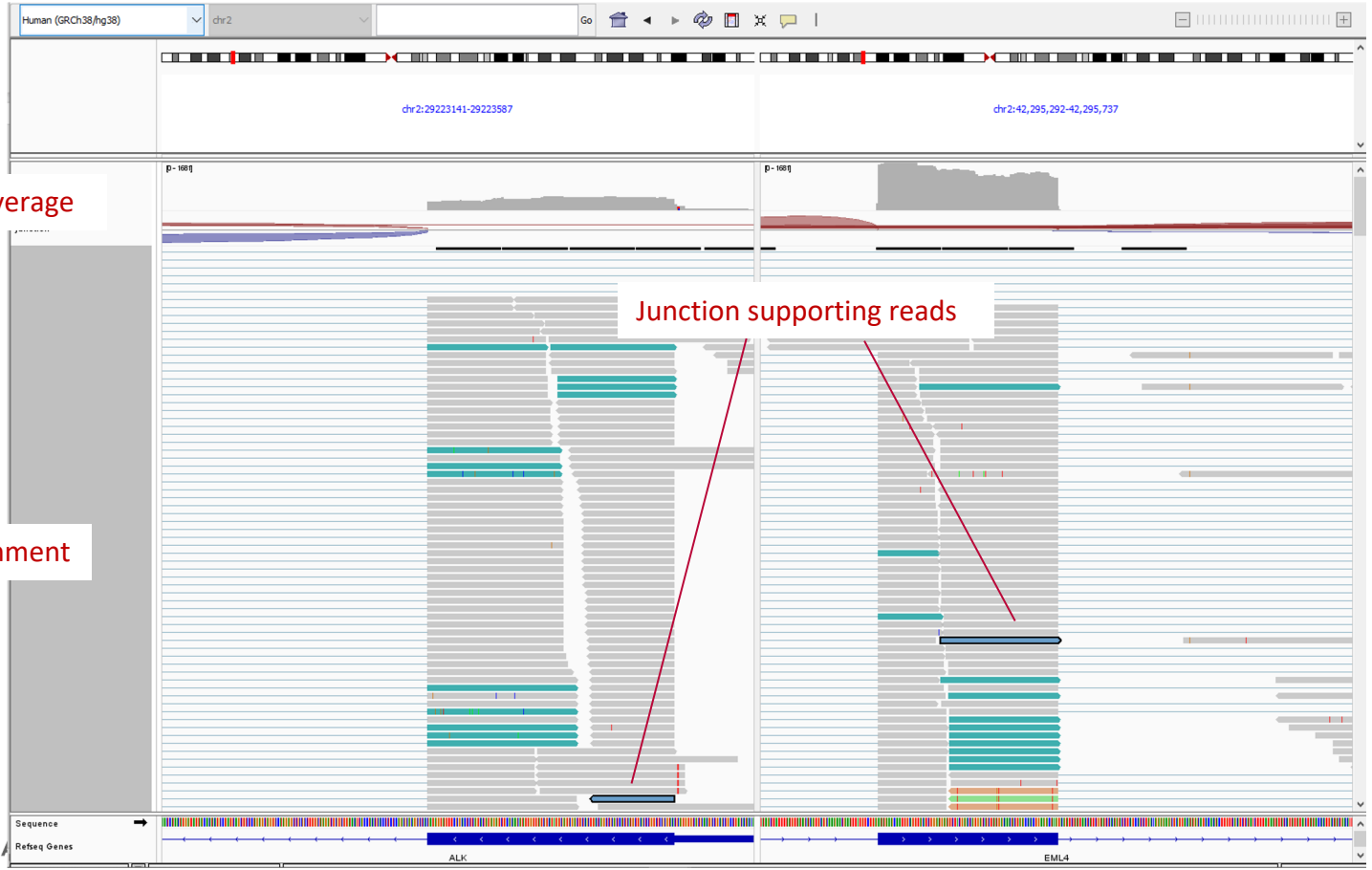



Sample1 and sample2 need to be removed for further analysis.

# Visualization

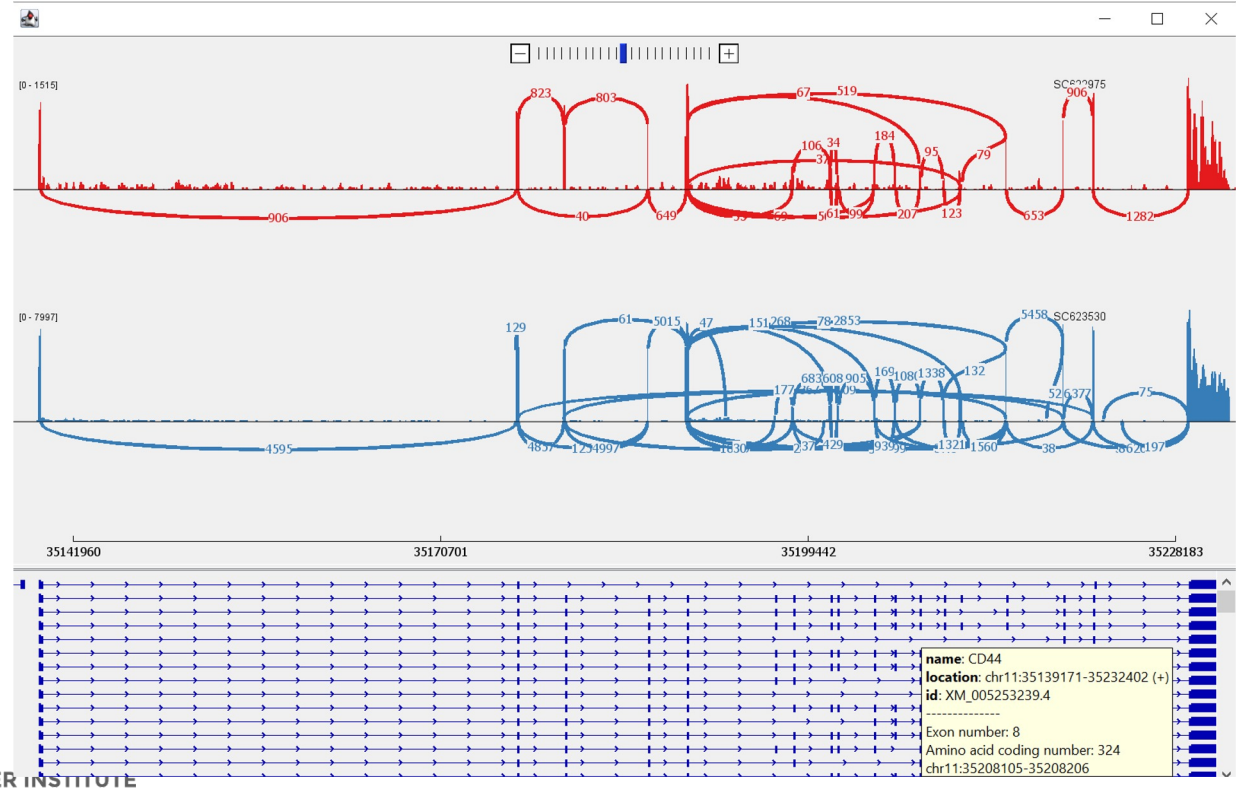
- Plot types
  - Pileup plot
  - Sashimi plot
- Tools
  - Best overall: Integrative Genomics Viewer/IGV
  - Alternative: samtools tview (for quick check), Tablet (support more format, high-quality graphs), Integrated Genome Browser/IGB
  - More information:
    - https://github.com/cmdcolin/awesome-genome-visualization
    - http://jermdemo.blogspot.com/2010/08/ngs-viewers-reviewed.html

# Visualization – IGV



# Visualization – IGV

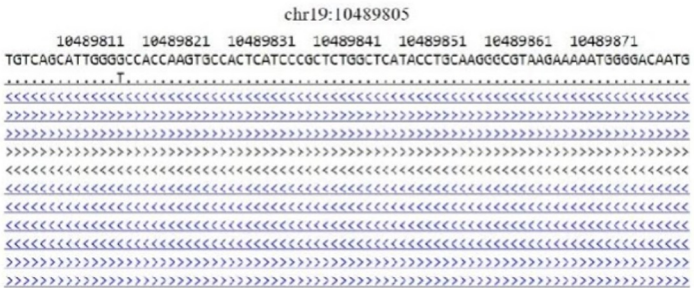
- Sashimi plots visualize junctions from alignments.



# Visualization – Samtools

- Samtools tview

samtools tview –p chr:pos –d H alignment.bam genome.fasta

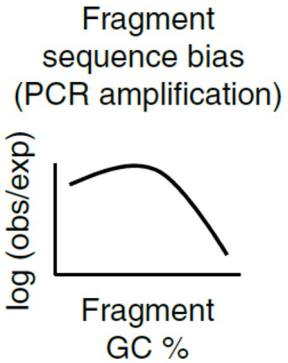


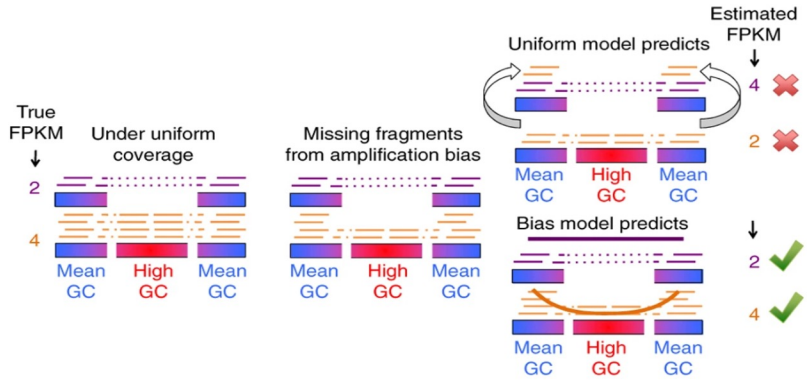

# Quantification

- Counting mapped reads as a measure of expression
- Reads can be summarized and aggregated over any biologically meaningful features (e.g. genes, transcripts, exons, etc.)
- Intersection on gene models

# Quantification – artifacts and biases

- GC bias
  - Samples processed in the same batch with the same protocol usually would show similar GC bias. Then the GC bias correction can be skipped for comparisons across samples.
  - Can be examined computationally using tools like FastQC.
  - If significant difference in GC content exist, can be corrected using tools like EDASeq or alpine.



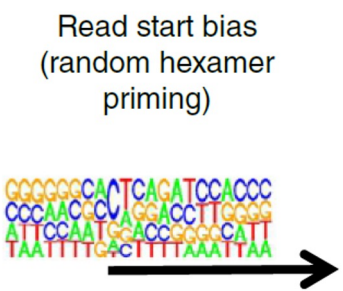



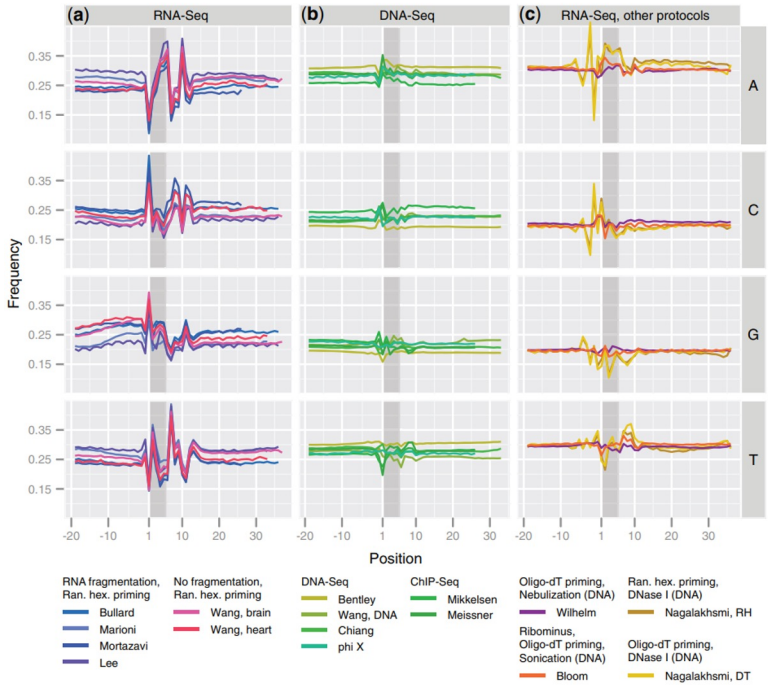



Love, M., et. al. *Nat Biotechnol* **34**, 1287–1291 (2016).

# Quantification – artifacts and biases

- Random hexamer bias
  - a bias in the nucleotide composition at the start of sequencing reads
  - no significant pattern in DNA and Chip-seq
  - some tools (e.g. Cufflinks) would correct the bias. Usually doesn't need correction separately.

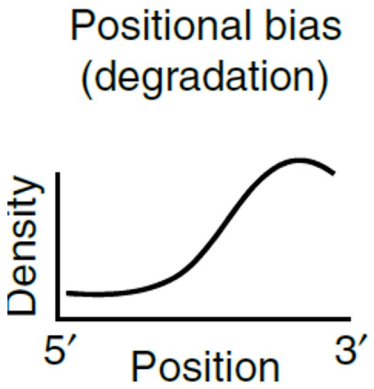



Love, M., et. al. *Nat Biotechnol* **34**, 1287–1291 (2016).
Hansen, K, et. al. *Nucleic Acids Res.* **38**(12): e131 (2010)

# Quantification – artifacts and biases

- Positional bias: 5’-to-3’ bias
  - Mostly due to the poly-A selection and partial degradation of RNA
  - Correlated with RIN score.
  - If the bias is similar across samples, usually doesn’t need to make adjustment.
  - Some mathematical models are developed to neutralize the effect of positional bias.
  - Alternatively, RIN scores can be used as a covariate.





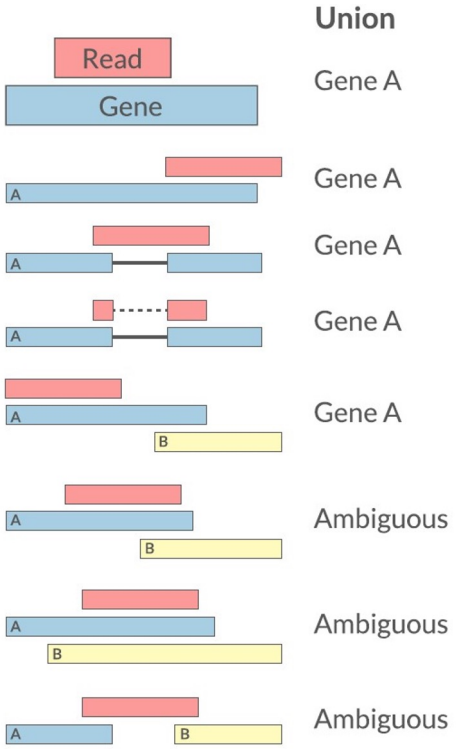

Love, M., et. al. *Nat Biotechnol* **34**, 1287–1291 (2016).

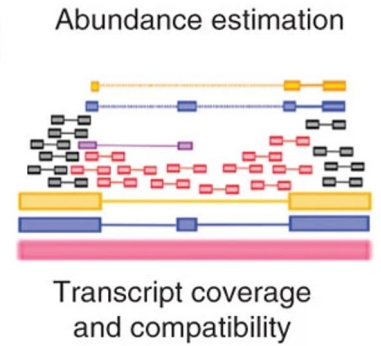
# Quantification – artifacts and biases

- PCR duplicates
  - Most RNA-seq pipelines do NOT include computational deduplication.
  - De-duplication computationally is carried out by sequence of comparison or aligned coordinates.
  - Short transcripts and very highly expressed transcripts (common in some species) will contribute the last majority of biological ‘duplicates’.
  - The fraction of identified duplicates is correlated with the number of aligned reads.
  - Use UMIs in case of very low input sample or very deep sequencing library.
  - More information: https://www.biostars.org/p/55648/
- Mapping errors

# Quantification – artifacts and biases

- Ambiguous alignment
  - Overlapping genes
  - Alternate splicing



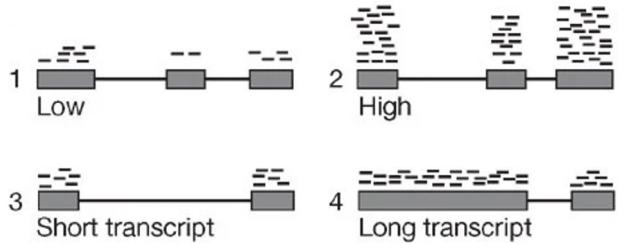




https://scilifelab.github.io/courses/ngsintro/1905/slides/rnaseq/presentation.html#33

Trapnell, C. et. al. *Nat Biotechnol* **28**, 511–515 (2010). https://doi.org/10.1038/nbt.1621

# Quantification – artifacts and biases

- Sequencing depth
- Gene length bias



# Quantification – Common software

| Methods | Quantification features | Output | Ambiguous reads | Confidence level | Notes |
|---|---|---|---|---|---|
| **HTSeq** | Gene | Counts | Ignore | NA | |
| STAR geneCounts | Gene | Counts | Ignore | NA | |
| Cufflinks | Transcript | RPKM | Split equally, rescue | NA | Assembly |
| featureCounts | Gene | Counts | Ignore, count all, split equally | NA | |
| **RSEM** | Gene, transcript, Exon | Counts, RPKM, TPM | Expectation-Maximization (EM) | 95% confidence intervals | |
| **StringTie** | Transcript | FPKM, TPM | Flow network | NA | Assembly |
| eXpress | Gene, transcript | Counts, FPKM, TPM | EM | 95% confidence intervals | |
| Kallisto | Transcript | TPM | EM | Bootstrap | Pseudoalignment* |
| Salmon | Transcript | Counts, TPM | EM | Bootstrap | Pseudoalignment* |

*Pseudoalignment: Reads are mapped to a reference transcriptome, and are judged on compatibility with transcripts, not aligned.

# Resources

- NCI BTEP Bioinformatics for beginners: https://btep.ccr.cancer.gov/docs/b4b/
- UC Davis Bioinformatics workshop RNA-seq analysis: https://ucdavis-bioinformatics-training.github.io/2021-September-RNA-Seq-Analysis/



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