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# PMBIO Module 06

## **RNAseq.** RNA-sequence analysis

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Introduction to bioinformatics for DNA and RNA sequence analysis (IBDR01)
29 October – 2 November, 2018
Glasgow

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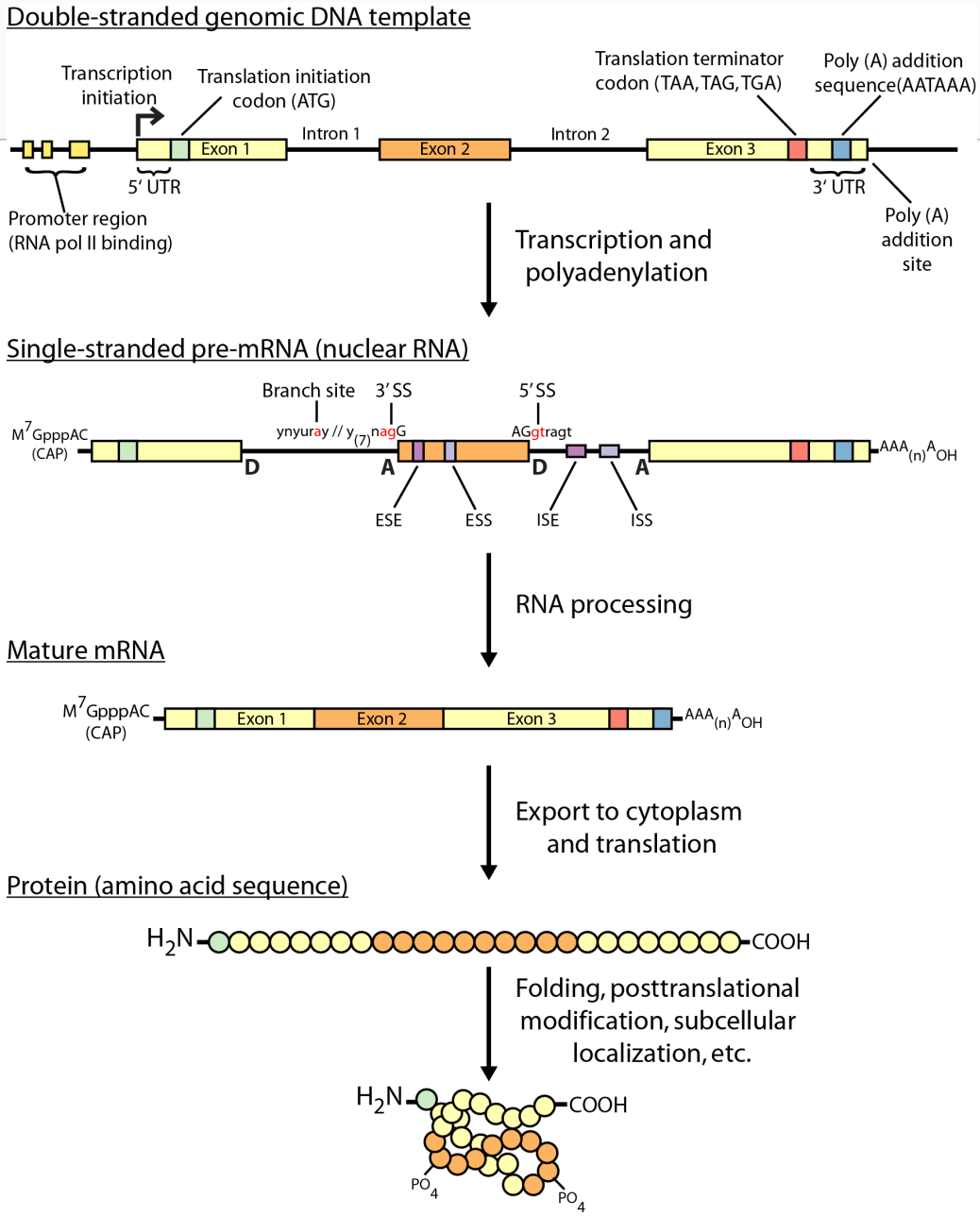
# Learning objectives of module 06: RNAseq

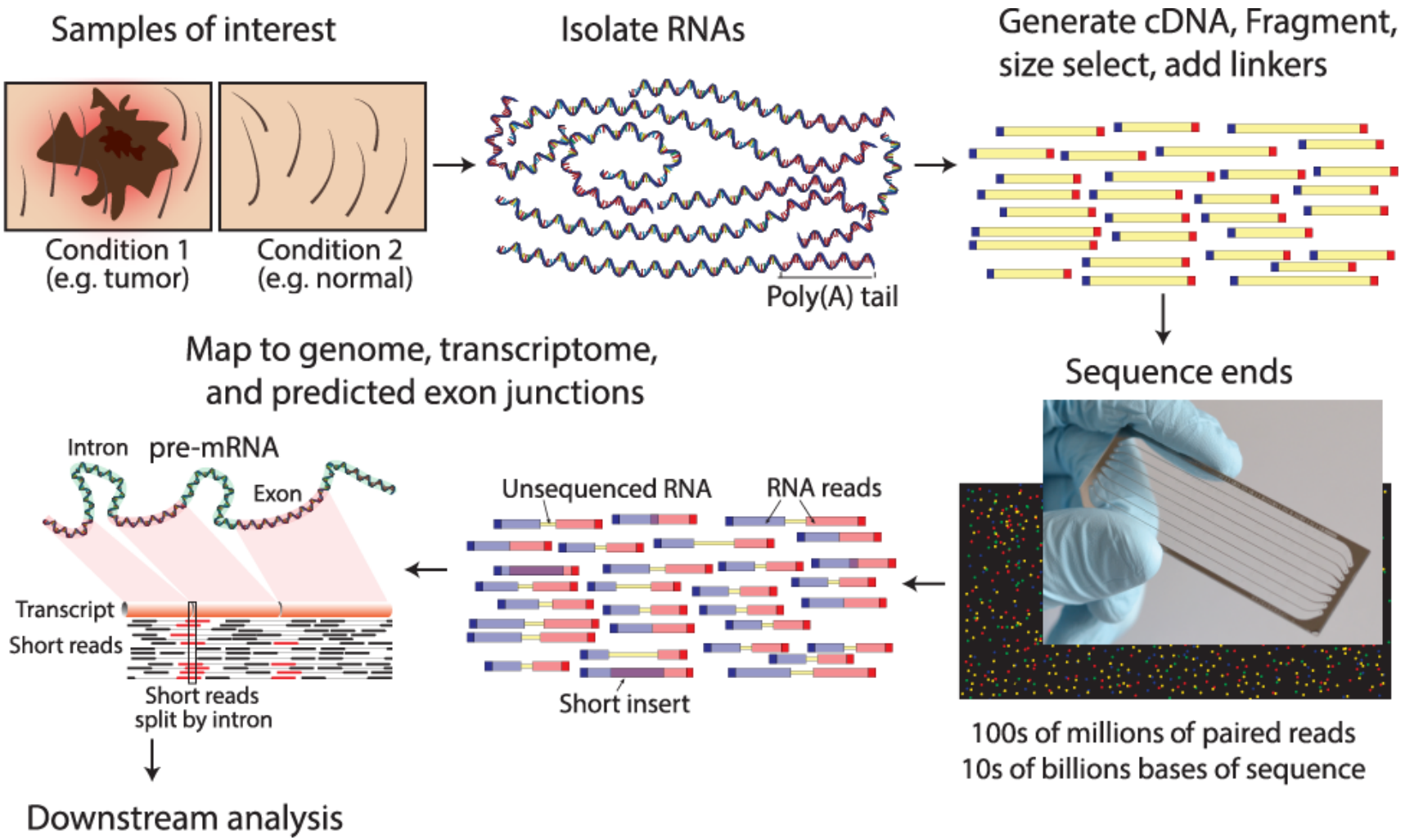
- **Key concepts**: RNA-seq, library strategies, RNA splicing, genes and transcripts, transcription strand, gene/transcript abundance estimation, FPKM and TPM, differential expression (DE) analysis, normalization, batch effects

- Perform alignment of RNA-seq data and a basic QC analysis of the resulting alignments
- Obtain gene and transcript abundance estimates
- Perform a reference-free alignment and abundance estimation and contrast the results with the reference based approach
- Basic differential expression analysis
- Perform an expression outlier analysis
- Run an RNA fusion detection tool and parse the results for interesting candidates
- Assess expression of specific gene markers of relevance (e.g. HER2, ER, PR)
- Assess the expression of specific variants identified in previous sections.
- Identify an example of allele specific expression

# Gene expression



# RNA sequencing

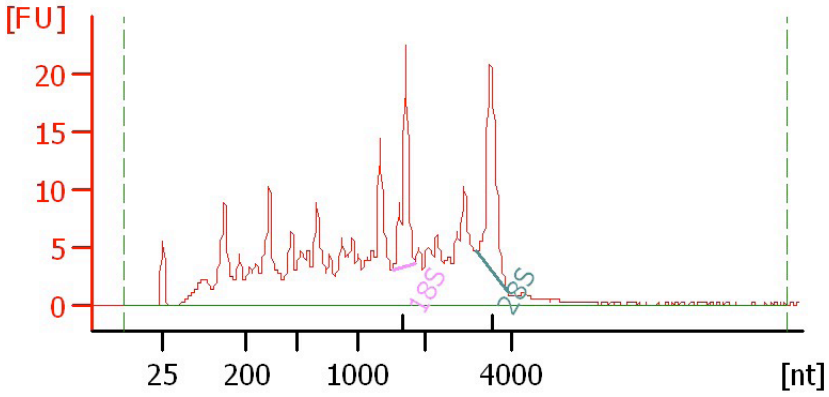


# Challenges

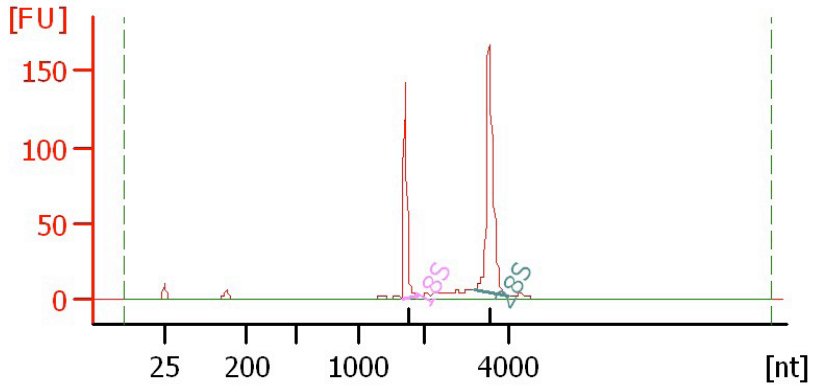
- Sample
  - Purity?, quantity?, quality?
- RNAs consist of small exons that may be separated by large introns
  - Mapping reads to genome is challenging
- The relative abundance of RNAs vary wildly
  - $10^5$ – $10^7$ orders of magnitude
  - Since RNA sequencing works by random sampling, a small fraction of highly expressed genes may consume the majority of reads
  - Ribosomal and mitochondrial genes
- 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)

# Agilent example / interpretation

- https://goo.gl/uC5a3C
- ‘RIN’ = RNA integrity number
  - 0 (bad) to 10 (good)




RIN = 6.0



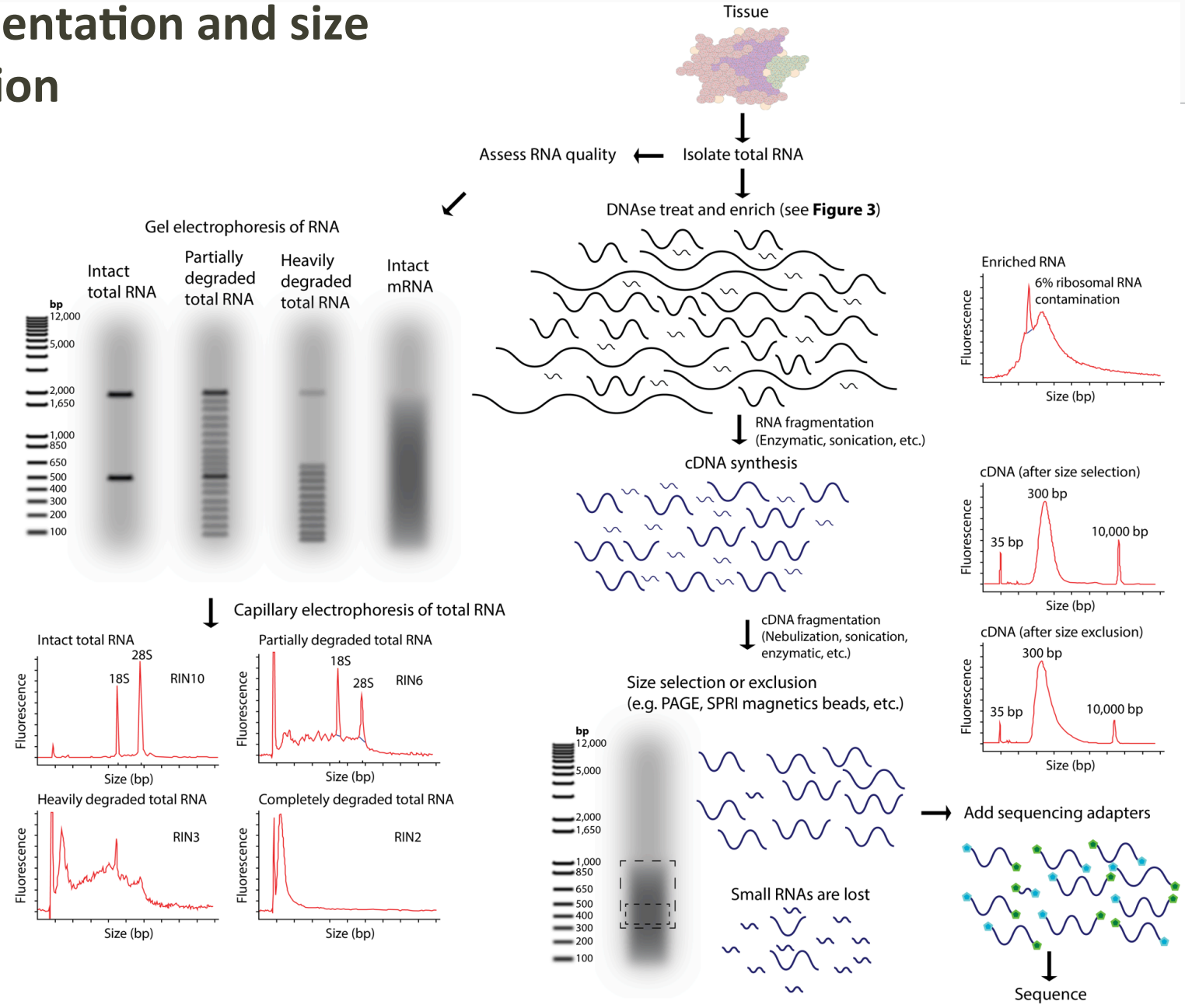

RIN = 10

# There are many RNA-seq library construction strategies

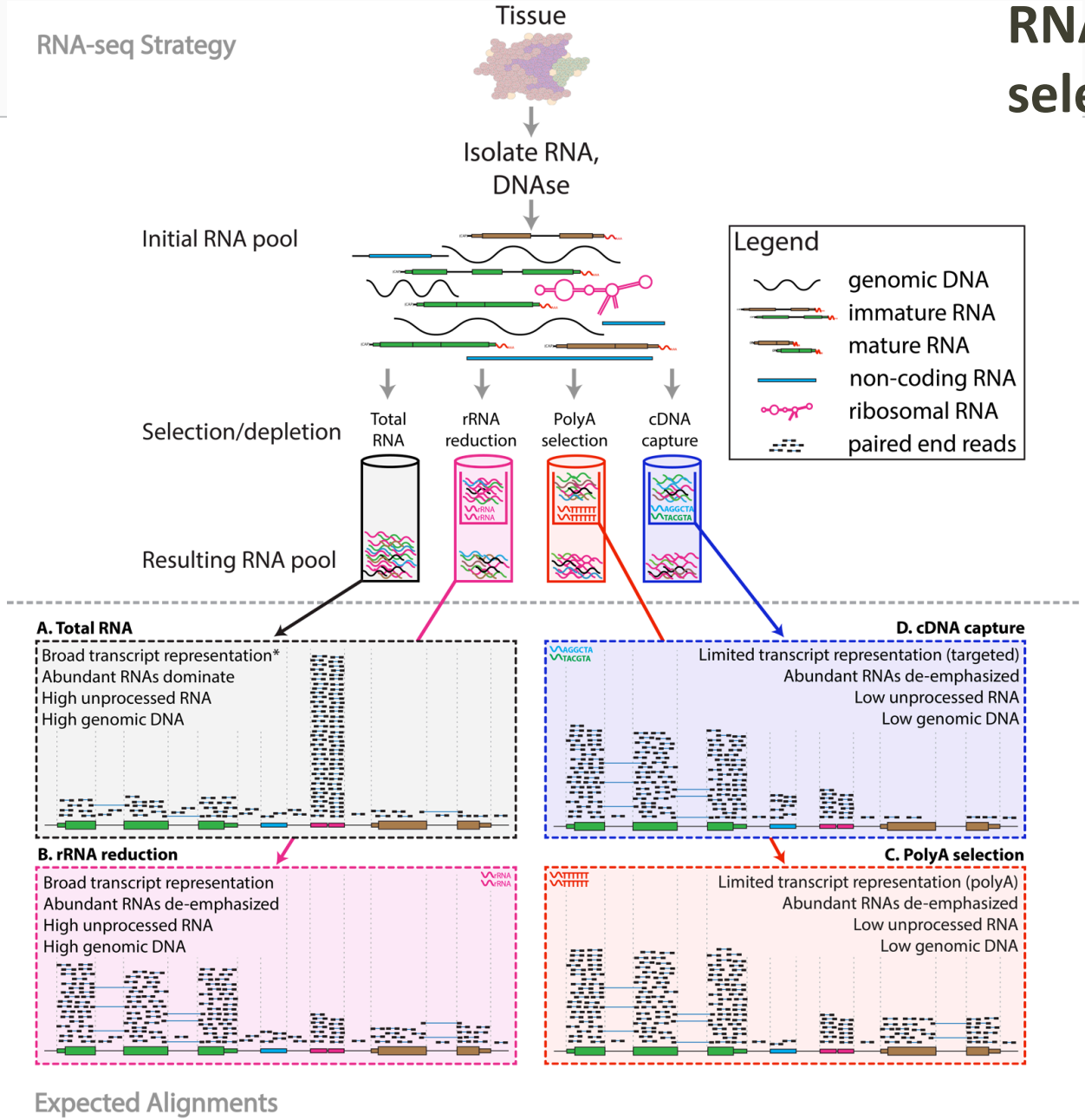
- Total RNA versus polyA+ RNA?
- Ribo-reduction?
- Size selection (before and/or after cDNA synthesis)
  - Small RNAs (microRNAs) vs. large RNAs?
  - A narrow fragment size distribution vs. a broad one?
- Linear amplification?
- Stranded vs. un-stranded libraries
- Exome captured vs. un-captured
- Library normalization?

- These details can affect analysis strategy
  - Especially comparisons between libraries

# Fragmentation and size selection

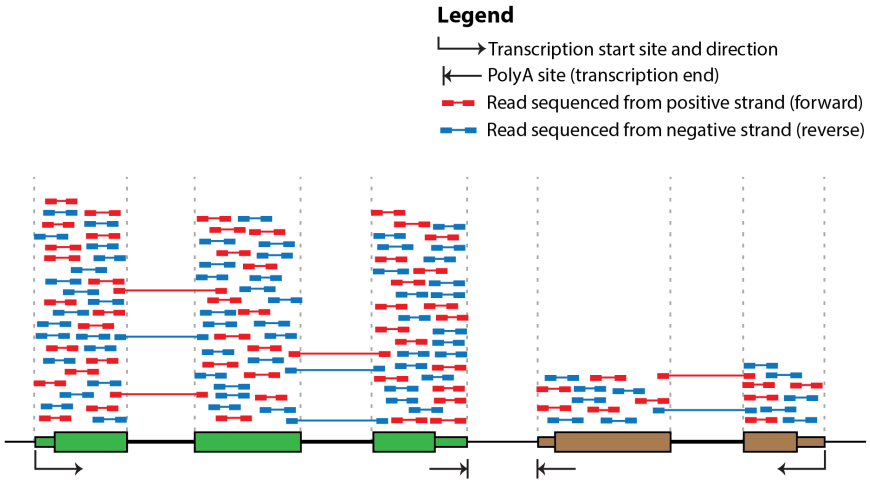


# RNA sequence selection/depletion

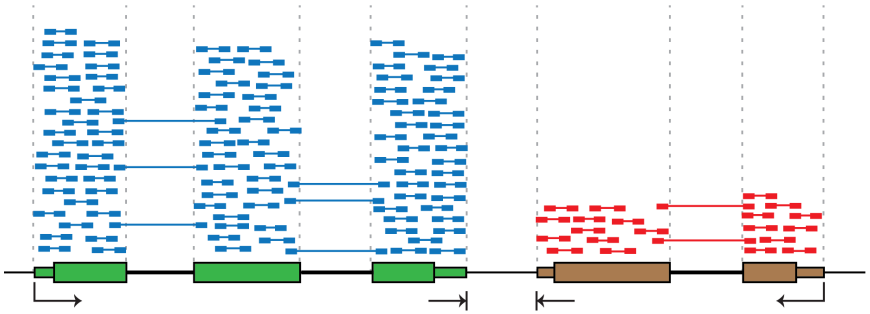


# Stranded vs. unstranded

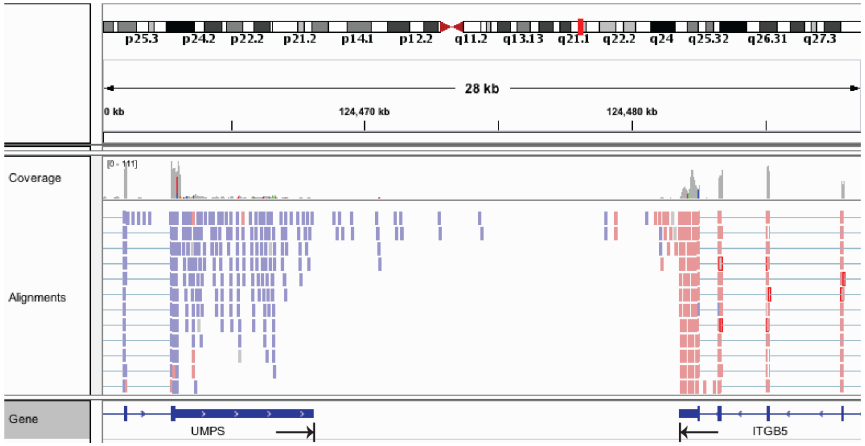
## A. Depiction of cDNA fragments from an unstranded library




## B. Depiction of cDNA fragments from an stranded library

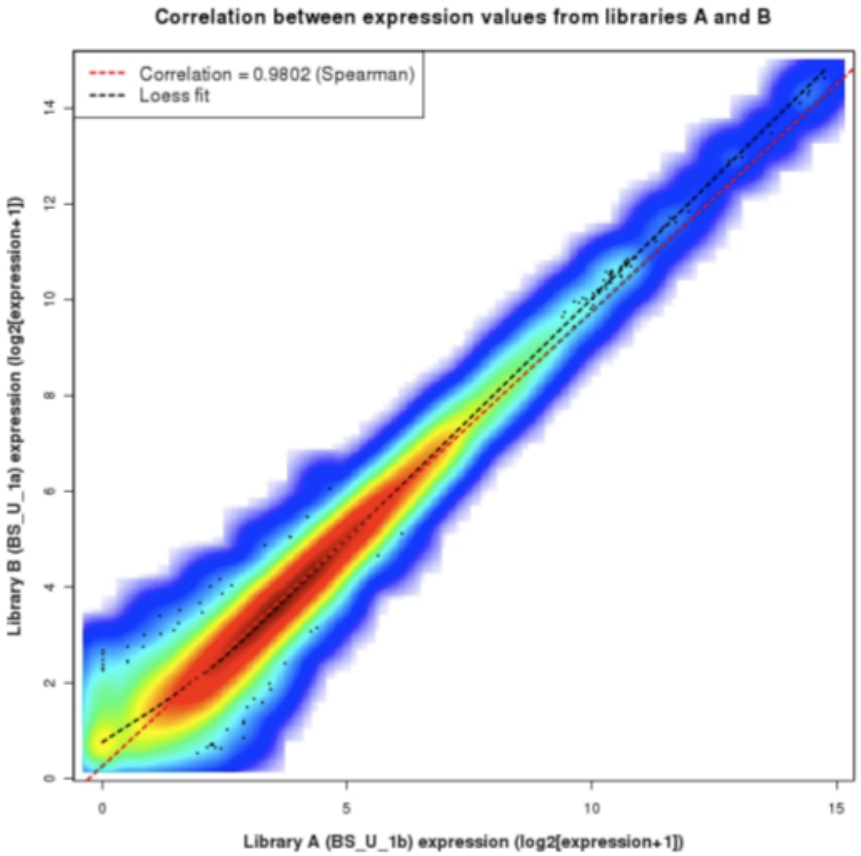


## C. Viewing strand of aligned reads in IGV



# Replicates

- Technical Replicate
  - Multiple instances of sequence generation
    - Flow Cells, Lanes, Indexes
- Biological Replicate
  - Multiple isolations of cells showing the same phenotype, stage or other experimental condition
  - Some example concerns/ challenges:
    - Environmental Factors, Growth Conditions, Time
  - Correlation Coefficient 0.92-0.98

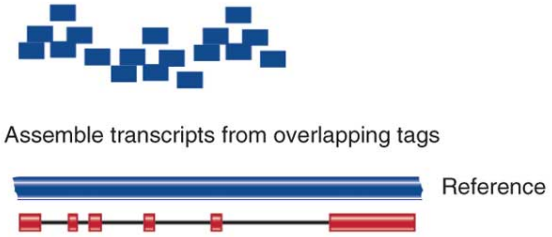


# Common analysis goals of RNA-Seq  analysis (what can you ask of the data?)

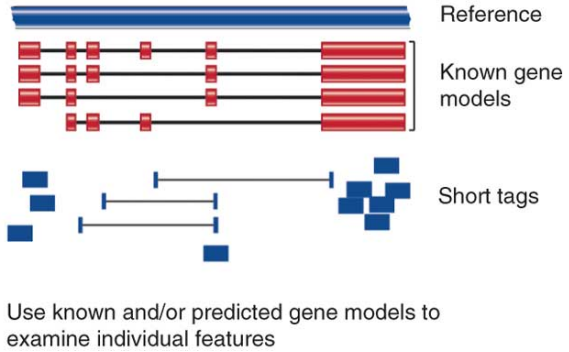
- Gene expression and differential expression
- Alternative expression analysis
- Transcript discovery and annotation
- Allele specific expression
  - Relating to SNPs or mutations
- Mutation discovery
- Fusion detection
- RNA editing

# Three RNA-seq mapping strategies

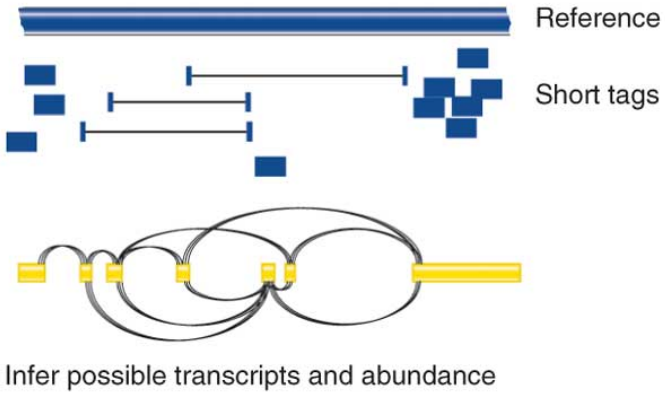
## De novo assembly




## Align to transcriptome

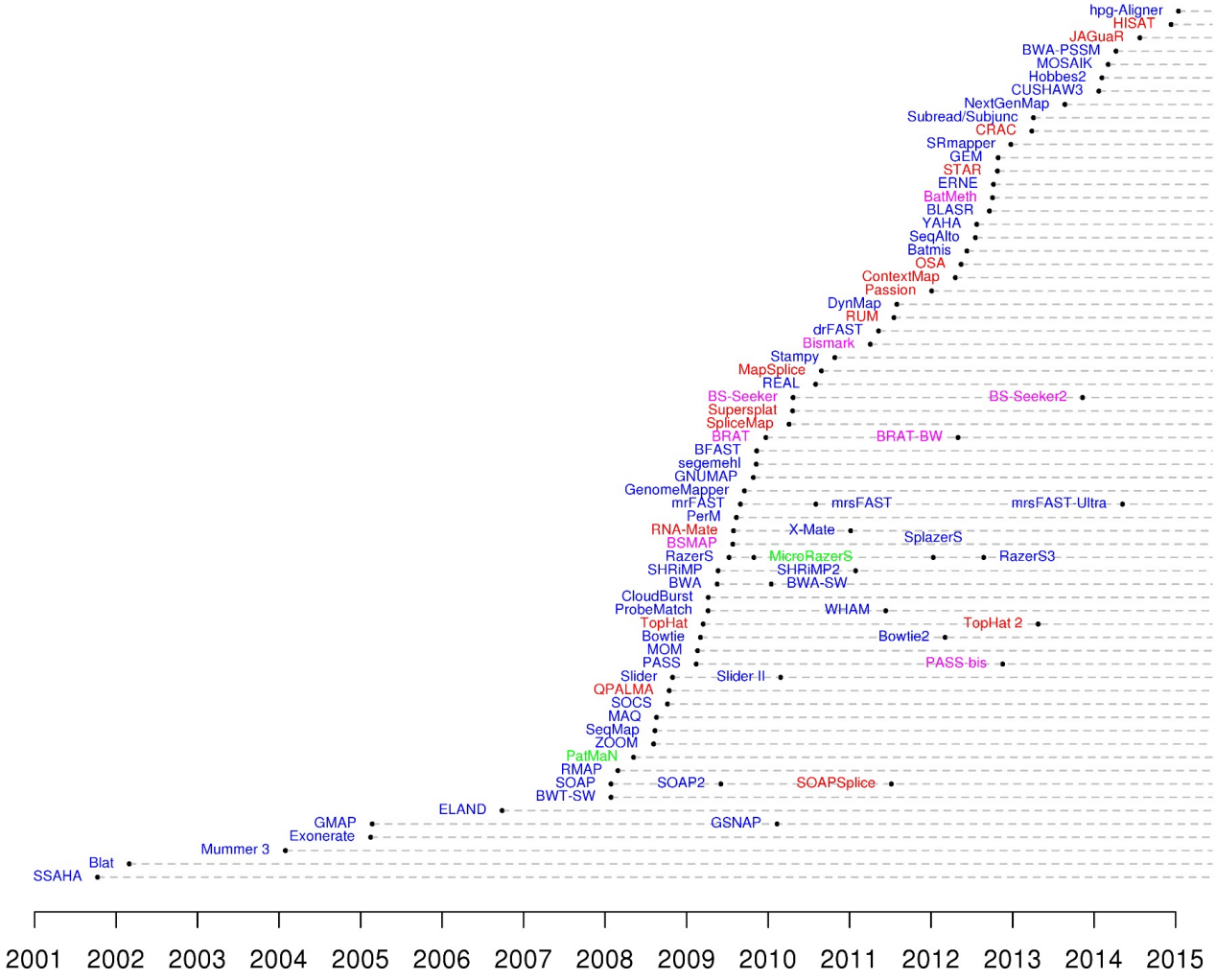



## Align to reference genome




Diagrams from Cloonan & Grimmond, Nature Methods 2010

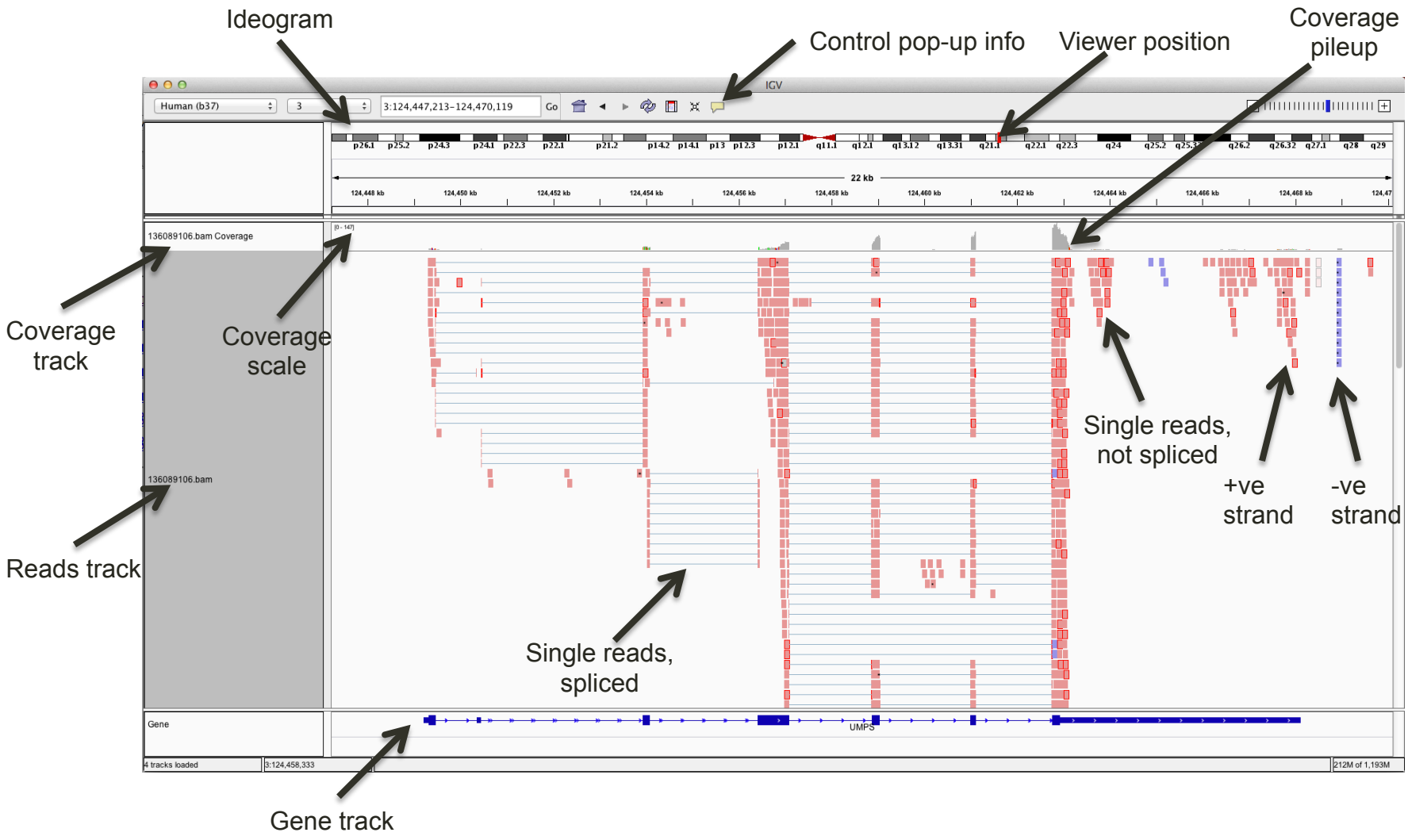
# Which read aligner should I use?



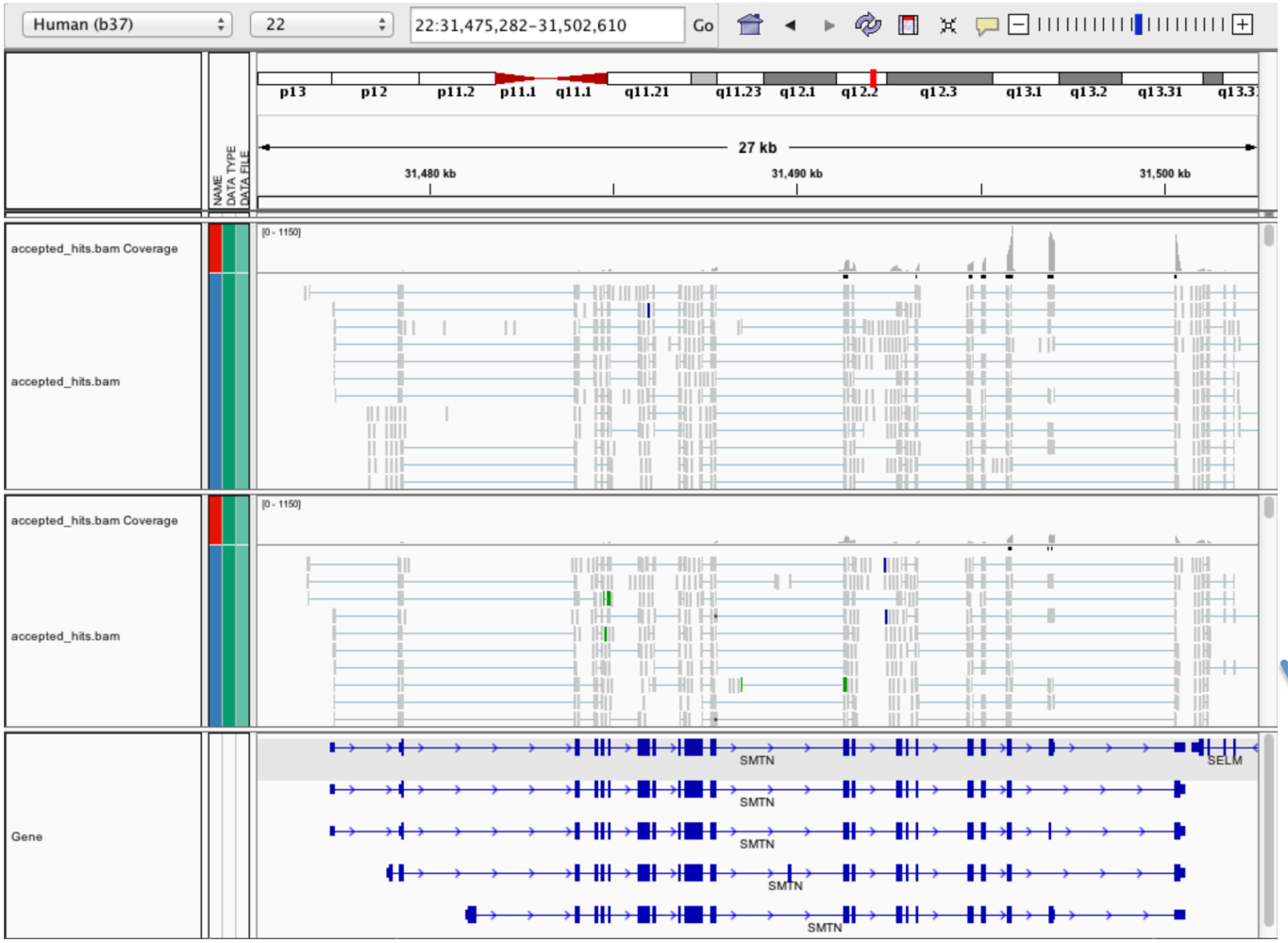

RNA
Bisulfite
DNA
microRNA

http://wwwdev.ebi.ac.uk/fg/hts_mappers/

# Visualization of RNA-seq alignments in IGV browser



# Expression estimation for known genes and transcripts




3' bias

Down-regulated

# What is FPKM (RPKM)

- RPKM: Reads Per Kilobase of transcript per Million mapped reads.
- FPKM: Fragments Per Kilobase of transcript per Million mapped reads.
- In RNA-Seq, the relative expression of a transcript is proportional to the number of cDNA fragments that originate from it. However:
  - The number of fragments is also biased towards larger genes
  - The total number of fragments is related to total library depth
- FPKM (RPKM) attempt to normalize for gene size and library depth

- FPKM (RPKM) = (10^9 * C) / (N * L)
  - C = number of mappable reads/fragments for a gene/transcript/exon/etc
  - N = total number of mappable reads/fragments in the library
  - L = number of base pairs in the gene/transcript/exon/etc

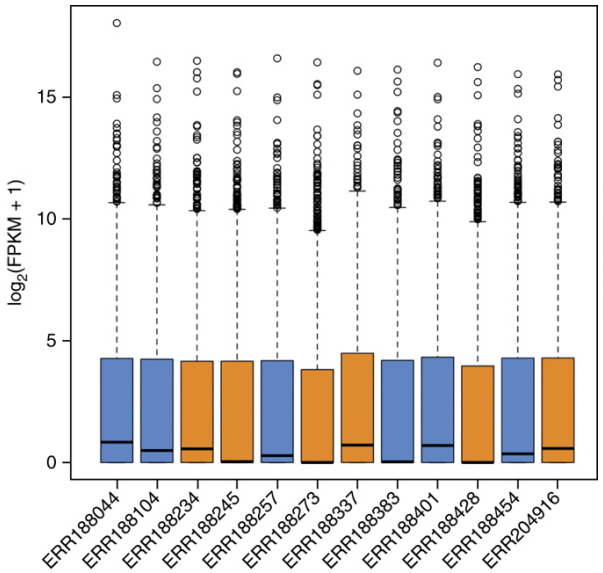
- http://www.biostars.org/p/11378/
- http://www.biostars.org/p/68126/

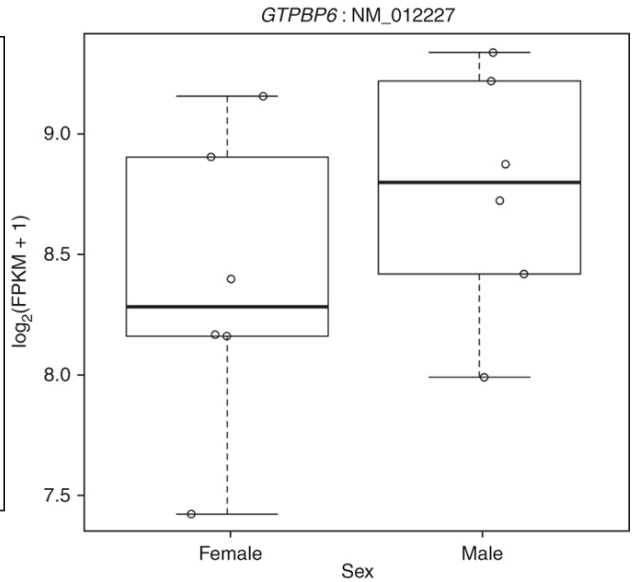
# How do FPKM and TPM differ?

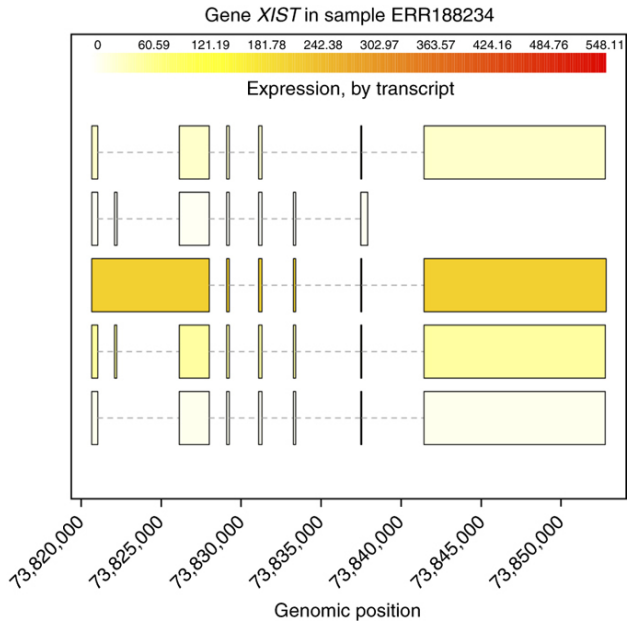
- TPM: Transcript per Kilobase Million
- The difference is in the order of operations:
  - FPKM
    - 1) Determine total library/sample fragment count and divide by 1,000,000
      - "per million" scaling factor
    - 2) Divide each gene/transcript fragment count by #1
      - fragments per million, FPM
    - 3) Divide each FPM by length of each gene/transcript in kilobases (FPKM)
  - TPM
    - 1) Divide each gene/transcript fragment count by length of each gene/transcript in kilobases
      - fragments per kilobase, FPK
    - 2) Sum all FPK values for the sample and divide by 1,000,000
      - "per million" scaling factor
    - 3) Divide #1 by #2 (TPM)

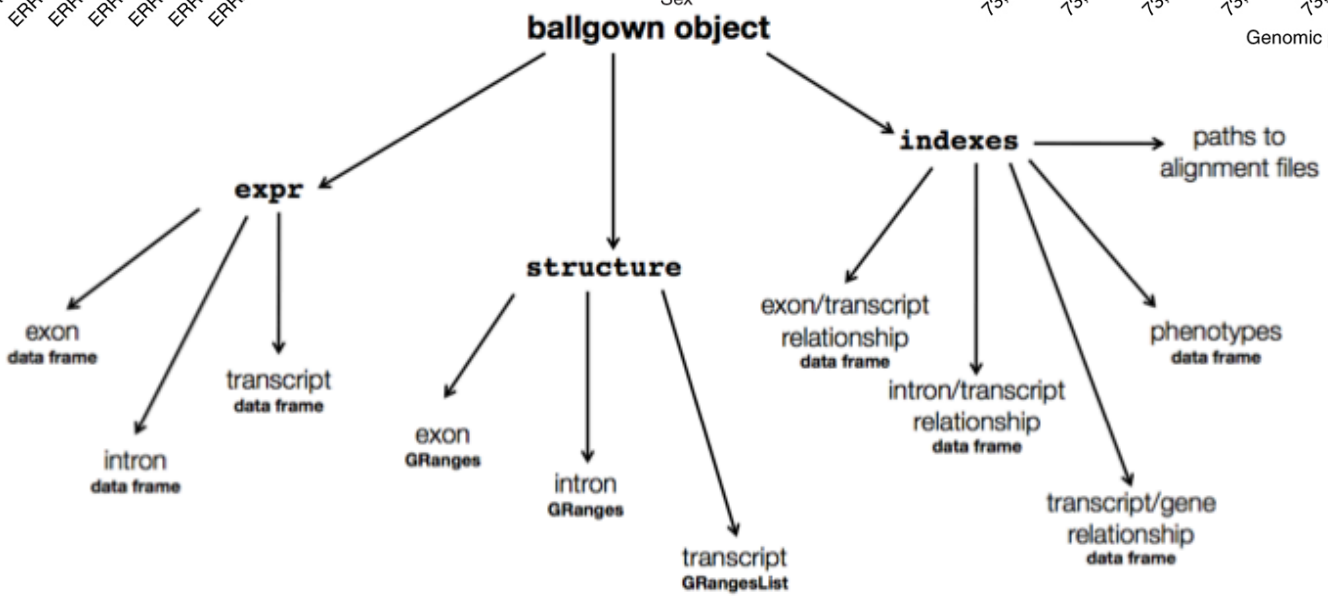
- The sum of all TPMs in each sample is the same. Easier to compare across samples!
- http://www.rna-seqblog.com/rpkm-fpkm-and-tpm-clearly-explained/
- https://www.ncbi.nlm.nih.gov/pubmed/22872506

# Ballgown for Visualization with R



# Learning objectives of module 4

- Alignment free estimation of transcript abundance

- Introduction to k-mers
- Alignment free tools
  - Sailfish, RNA-Skim, Kallisto, Salmon
- Abundance estimation and differential expression analysis with Kallisto and Sleuth

# What is a k-mer?

- A fixed sized (***K***) sequence

- A string of length ***N*** contains ***N-K+1*** k-mers

1-mer

| |
|---|
| A |
| C |
| G |
| T |

2-mer

| | | | |
|---|---|---|---|
| AA | AC | AG | AT |
| CA | CC | CG | CT |
| GA | GC | GG | GT |
| TA | TC | TG | TT |

ATTCGACAGTAGCCATGACTGG

...

- One can build *K*-mer index to represent a string

| 7-mer | iD | N |
|---|---|---|
| ATTCGAC | 1 | 1 |
| TTCGACA | 2 | 1 |
| TCGACAG | 3 | 1 |
| ... | | |

Sailfish: Alignment-free Isoform Quantification from RNA-seq Reads using Lightweight Algorithms Rob Patro, Stephen M. Mount, and Carl Kingsford. *Manuscript Submitted* (2013) http://www.cs.cmu.edu/~ckingsf/class/02714-f13/Lec05-sailfish.pdf

https://www.slideshare.net/duruofei/cmsc702-project-final-presentation

# Basic concept of alignment free approaches for transcript abundance

1. Obtain reference transcript sequences (e.g. Ensembl, Refseq, or GENCODE)
2. Build a **k-mer index** of all of the k-mers occurring in each transcript sequence
   - Store each k-mer and its position within the transcript. “hashing”
3. Parse all RNA-seq reads and count how many times each k-mer occurs within each read
   - Model relationship between RNA-seq read k-mers and the transcript k-mer index.
   - What transcript is the most likely source for each read?
   - Called “pseudoalignment” , “quasi-mapping”, etc.
4. Handle sequencing errors, isoforms, ambiguity, and determine abundance estimates
   - Transcriptome de Bruijn graphs, likelihood function, expectation maximization, etc.

# Advantages/disadvantages of alignment free approaches

- Advantages
  - Very fast and efficient
    - Similar accuracy to alignment based approach but with much, much shorter run time.
  - Do not need a reference genome, only a reference transcriptome

- Disadvantages
  - You don’t get a proper BAM file
  - Information in reads with sequence errors may be ignored
  - Limited potential for transcript discovery, variant calling, fusion detection, etc.

# Common alignment free tools

- Sailfish
  - “Sailfish enables alignment-free isoform quantification from RNA-seq reads using lightweight algorithms.” 2014
  - https://www.ncbi.nlm.nih.gov/pubmed/24752080
- RNA-Skim
  - “RNA-Skim: a rapid method for RNA-Seq quantification at transcript level.” 2014
  - https://www.ncbi.nlm.nih.gov/pubmed/24931995
- Kallisto
  - “Near-optimal probabilistic RNA-seq quantification.” 2016
  - https://www.ncbi.nlm.nih.gov/pubmed/27043002
- Salmon
  - “Salmon provides fast and bias-aware quantification of transcript expression.” 2017
  - https://www.ncbi.nlm.nih.gov/pubmed/28263959

# Which is best?

- Somewhat controversial …
- https://liorpachter.wordpress.com/2017/08/02/how-not-to-perform-a-differential-expression-analysis-or-science/

- Various sources suggest that Salmon, Kallisto, and Sailfish results are quite comparable
- Usability, documentation, and supporting downstream tools could be used to decide

# HISAT2/StringTie/Ballgown RNA-seq Pipeline