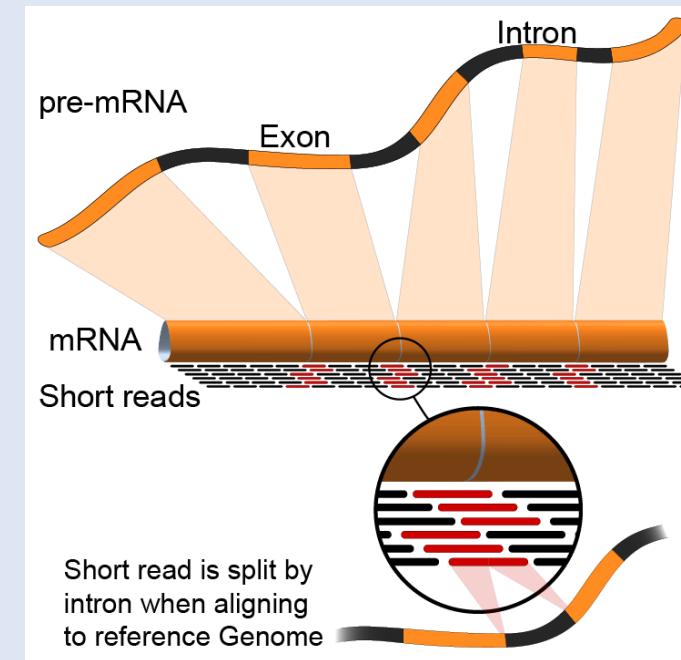
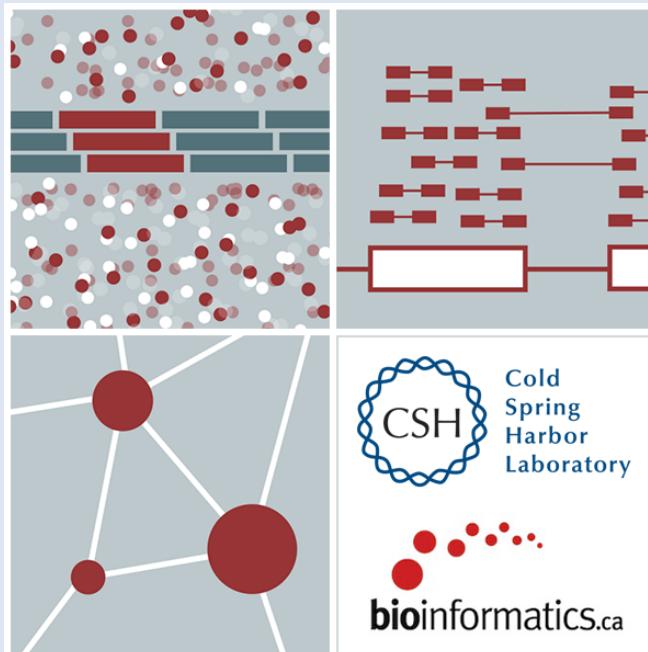




Cold
Spring
Harbor
Laboratory



RNA-Seq Module 3

Abundance Estimation and Differential Expression

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Advanced Sequencing Technologies & Bioinformatics Analysis November 10-21, 2025

Washington University in St. Louis
SCHOOL OF MEDICINE

Alternatives to FPKM

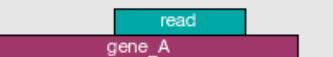
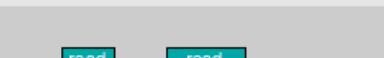
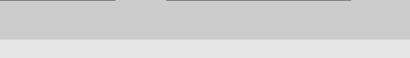
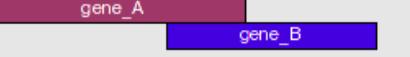
- Raw read counts for differential expression analysis
 - Assign reads/fragments to defined genes/transcripts, get “raw counts”
 - Transcript structures could still be defined by something like Stringtie

- HTSeq (htseq-count)
 - <https://htseq.readthedocs.io/>

```
htseq-count --mode intersection-strict --stranded no --minaqual 1 --type exon --idattr transcript_id  
accepted_hits.sam chr22.gff > transcript_read_counts_table.tsv
```

- Caveats of ‘transcript’ analysis by htseq-count:
 - Designed for genes - ambiguous reads from overlapping transcripts may not be handled!
 - <http://seqanswers.com/forums/showthread.php?t=18068>

HTSeq-count basically counts reads supporting a feature (exon, gene) by assessing overlapping coordinates

	union	intersection _strict	intersection _nonempty
 read gene_A	gene_A	gene_A	gene_A
 gene_A read	gene_A	no_feature	gene_A
 read gene_A gene_A	gene_A	no_feature	gene_A
 read gene_A gene_A	gene_A	gene_A	gene_A
 read gene_A gene_B	gene_A	gene_A	gene_A
 read gene_A gene_B	ambiguous	gene_A	gene_A
 read gene_A gene_B	ambiguous	ambiguous	ambiguous

Note, if gene_A and gene_B on opposite strands, sequence data is stranded, and correct HTSeq parameter set then this read may not be ambiguous

Whether a read is counted depends on the nature of overlap and “mode” selected

We are on a Coffee Break & Networking Session