Cold Spring Harbor Laboratory

# RNA-Seq Module 3
# Abundance Estimation

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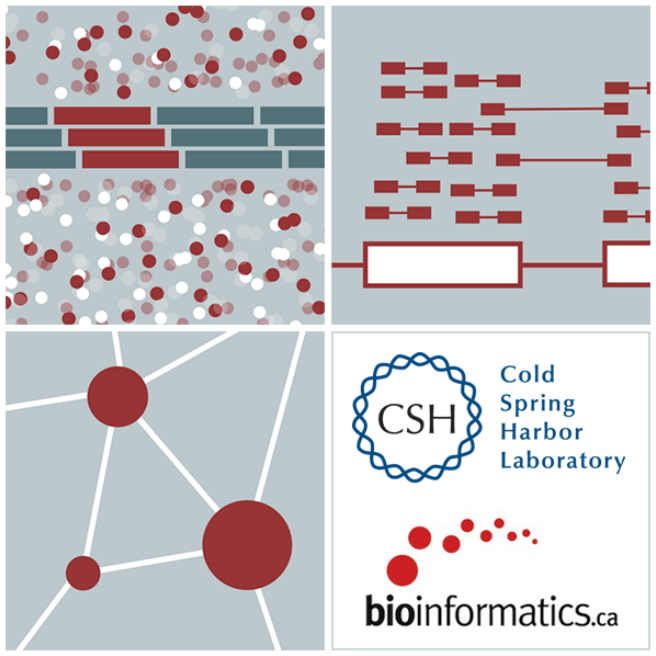
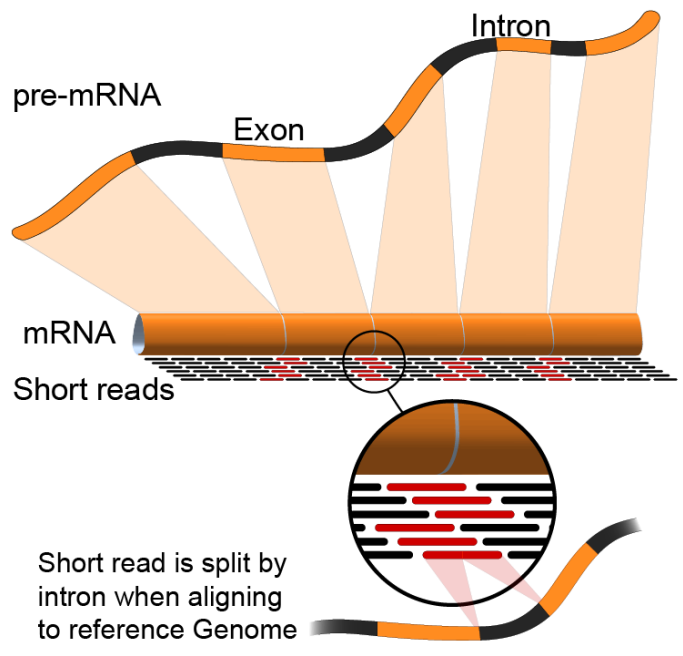
Bfx workshop, October 28, 2024




Washington University in St. Louis
School of Medicine

# To-do

- Open docker desktop app in the background. Then in terminal, type:

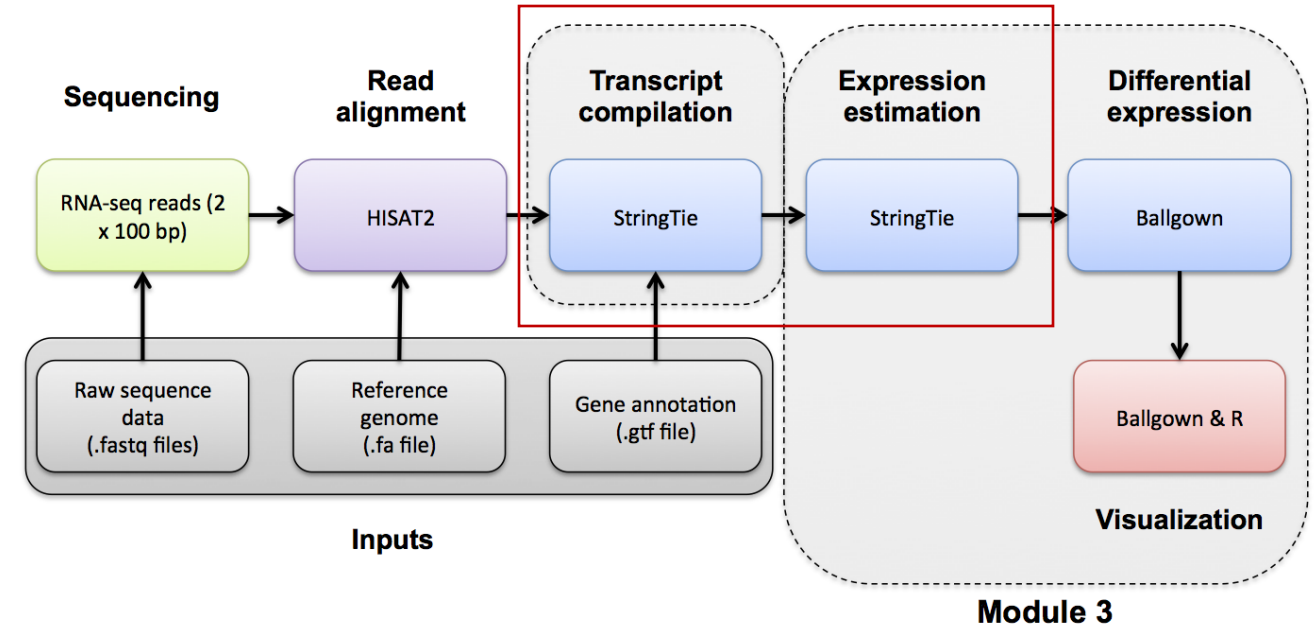
$ docker pull griffithlab/rnabio:0.0.1

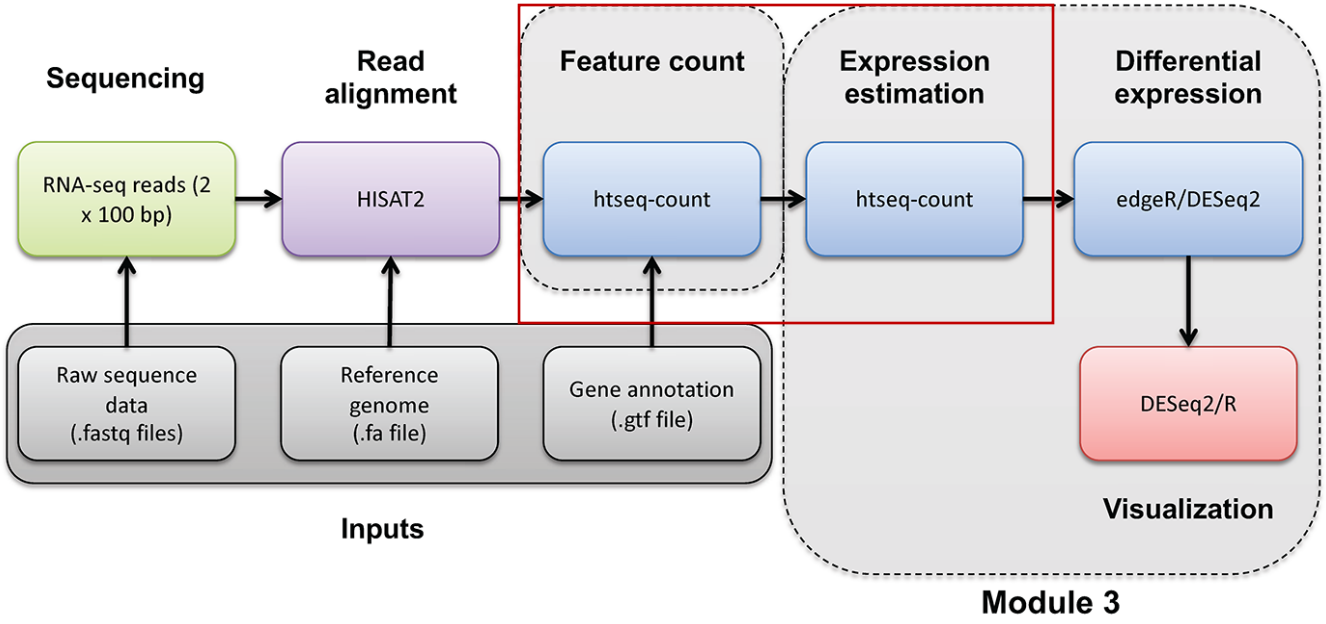
# Overview

Last week: Module 1 + 2
Alignment (HISAT2)

This week: Module 3
Expression estimation (StringTie, htseq count)

… Next week: Module 3 (continued)
Differential expression (Ballgown, edgeR)

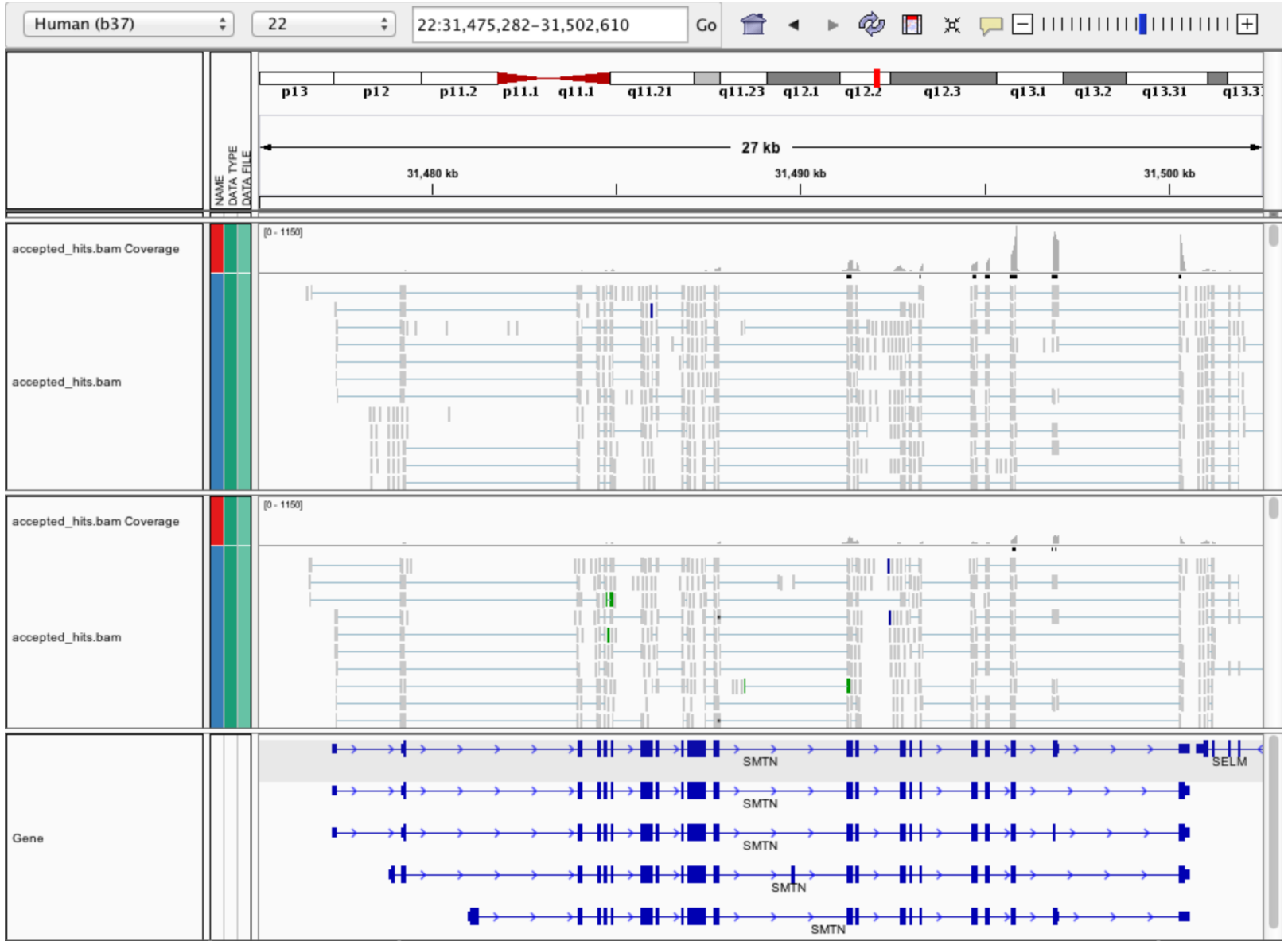


# Learning Objectives of Module 3

- Review basic concepts and popular metrics of abundance estimation:
  - raw counts vs normalized counts
- Discuss normalized count estimation tool: StringTie
- Discuss raw count estimation tool: HTSeq

# Expression estimation for known genes and transcripts




3' bias

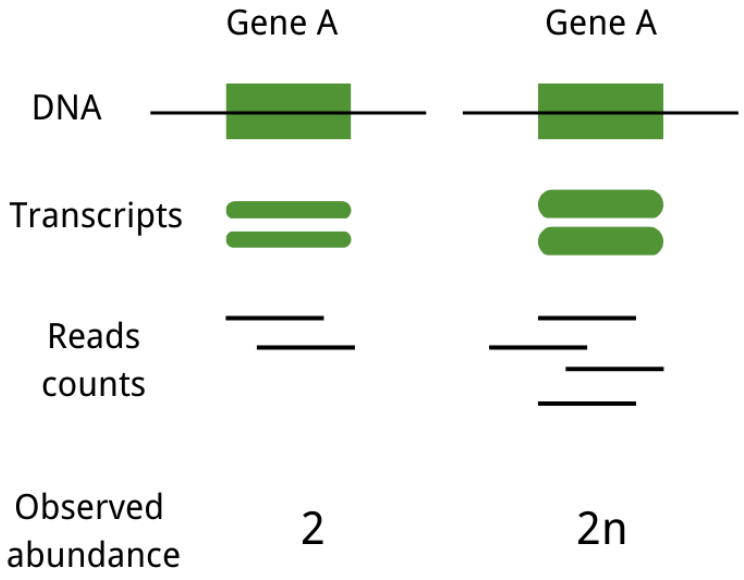
Down-regulated

# Popular metrics for abundance estimation

- Raw counts
- Normalized counts:
  - RPKM, FPKM
  - TPM

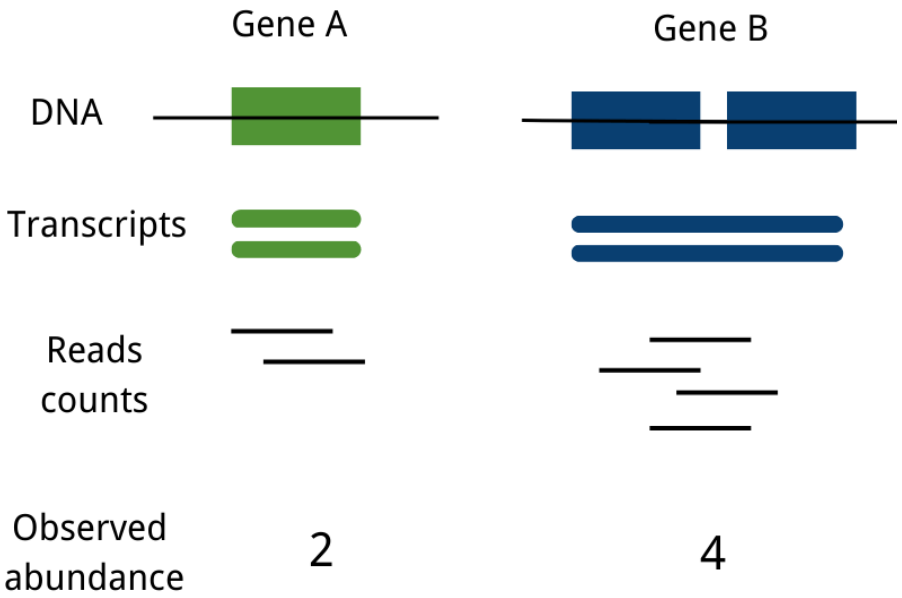
# Normalized counts combat technical biases in sequenced data

- Sequencing depth




Compare expression of 1 gene in 2 samples
Sample with higher sequencing depth has more reads
→ Divided by mapped reads

- Gene length




Compare expression of 2 genes in 1 sample
Longer gene (gene B) has more reads
→ Divided by gene(transcript) length

*Image adapted from novogene*

# What is FPKM (RPKM)?

- RPKM: **Reads** Per Kilobase of transcript per Million mapped reads.
- FPKM: **Fragments** Per Kilobase of transcript per Million mapped reads.
- Similar concept, RPKM is for single-end reads, FPKM is for paired-end reads

# What is FPKM (RPKM)?

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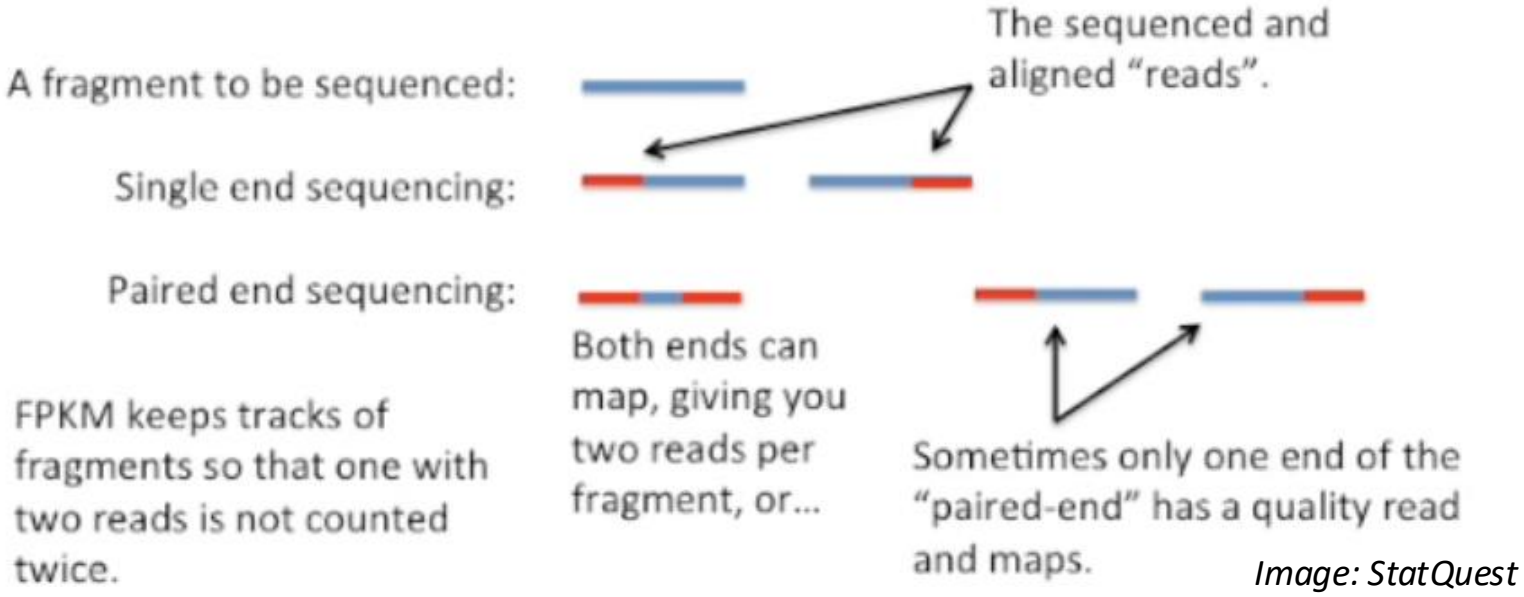
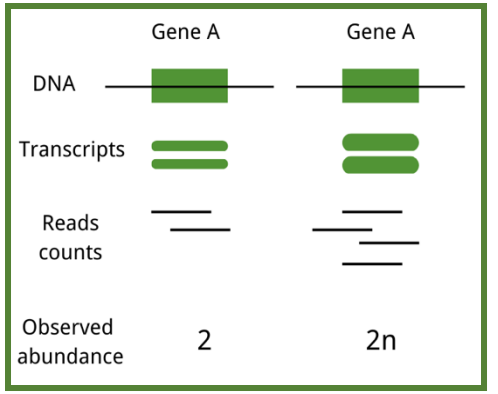
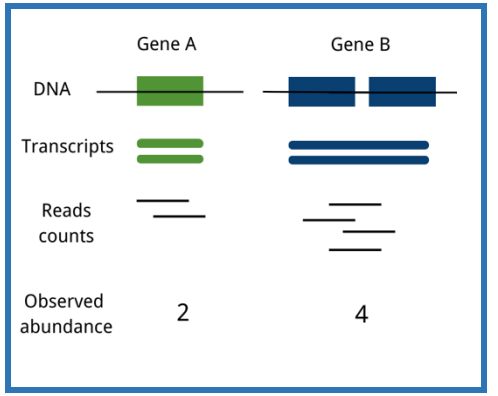



*Image: StatQuest*

# What is FPKM?

- FPKM attempts to normalize for library depth and gene size
  - remember – RPKM is essentially the same!




- C = number of mappable fragments for a gene (transcript)
- N = total number of mappable fragments in the library
- L = number of base pairs in the gene (transcript)
  - FPKM = (C / (N / 1,000,000)) / (L/1000)

    **Per Million mapped reads** **Per Kilobase of transcript**

    = (10^9 x C ) / (N x L)
- More reading:
  - 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 fragment count, divide by 1,000,000 (per Million)

2) Divide each gene/transcript fragment count by #1 (Fragments Per Million)

3) Divide each FPM by length of each gene/transcript in kilobases (FPKM)

Normalize for sequencing depth, then normalize for gene length

**TPM**

1) Divide each gene/transcript fragment count by length of the transcript in kilobases (Fragments Per Kilobase)

2) Sum all FPK values for the sample and divide by 1,000,000 (per Million)

3) Divide #1 by #2 (TPM)

Normalize for gene length, then normalize for sequencing depth

- 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

## RPKM – step 1: normalize for read depth.

| Gene Name | Rep1 Counts | Rep2 Counts | Rep3 Counts |
|---|---|---|---|
| A (2kb) | 10 | 12 | 30 |
| B (4kb) | 20 | 25 | 60 |
| C (1kb) | 5 | 8 | 15 |
| D (10kb) | 0 | 0 | 1 |
| Total reads: | 35 | 45 | 106 |
| Tens of reads: | 3.5 | 4.5 | 10.6 |

For the purpose of this 4 gene example, we're scaling the total read counts by 10 instead of 1,000,000.

## RPKM – step 2: normalize for gene length.

| Gene Name | Rep1 RPM | Rep2 RPM | Rep3 RPM |
|---|---|---|---|
| A (2kb) | 2.86 | 2.67 | 2.83 |
| B (4kb) | 5.71 | 5.56 | 5.66 |
| C (1kb) | 1.43 | 1.78 | 1.42 |
| D (10kb) | 0 | 0 | 0.09 |

Reads are scaled for depth (M) and gene length (K).

| Gene Name | Rep1 RPKM | Rep2 RPKM | Rep3 RPKM |
|---|---|---|---|
| A (2kb) | 1.43 | 1.33 | 1.42 |
| B (4kb) | 1.43 | 1.39 | 1.42 |
| C (1kb) | 1.43 | 1.78 | 1.42 |
| D (10kb) | 0 | 0 | 0.009 |

## TPM – step 1: normalize for gene length

Original data:

| Gene Name | Rep1 Counts | Rep2 Counts | Rep3 Counts |
|---|---|---|---|
| A (2kb) | 10 | 12 | 30 |
| B (4kb) | 20 | 25 | 60 |
| C (1kb) | 5 | 8 | 15 |
| D (10kb) | 0 | 0 | 1 |

RPK – scaled by gene length:

| Gene Name | Rep1 RPK | Rep2 RPK | Rep3 RPK |
|---|---|---|---|
| A (2kb) | 5 | 6 | 15 |
| B (4kb) | 5 | 6.25 | 15 |
| C (1kb) | 5 | 8 | 15 |
| D (10kb) | 0 | 0 | 0.1 |

## TPM – step 2: normalize for sequencing depth

| Gene Name | Rep1 RPK | Rep2 RPK | Rep3 RPK |
|---|---|---|---|
| A (2kb) | 5 | 6 | 15 |
| B (4kb) | 5 | 6.25 | 15 |
| C (1kb) | 5 | 8 | 15 |
| D (10kb) | 0 | 0 | 0.1 |
| Total RPK: | 15 | 20.25 | 45.1 |
| Tens of RPK: | 1.5 | 2.025 | 4.51 |

TPM – scaled by gene length and sequencing depth (M):

| Gene Name | Rep1 TPM | Rep2 TPM | Rep3 TPM |
|---|---|---|---|
| A (2kb) | 3.33 | 2.96 | 3.326 |
| B (4kb) | 3.33 | 3.09 | 3.326 |
| C (1kb) | 3.33 | 3.95 | 3.326 |
| D (10kb) | 0 | 0 | 0.02 |

*Image: StatQuest*

https://www.youtube.com/watch?v=TTUrtCY2k-w

# RPKM vs TPM

RPKM

| Gene Name | Rep1 RPKM | Rep2 RPKM | Rep3 RPKM |
|---|---|---|---|
| A (2kb) | 1.43 | 1.33 | 1.42 |
| B (4kb) | 1.43 | 1.39 | 1.42 |
| C (1kb) | 1.43 | 1.78 | 1.42 |
| D (10kb) | 0 | 0 | 0.009 |
| Total: | 4.29 | 4.5 | 4.25 |

... the sums of each column are very different.

TPM

| Gene Name | Rep1 TPM | Rep2 TPM | Rep3 TPM |
|---|---|---|---|
| A (2kb) | 3.33 | 2.96 | 3.326 |
| B (4kb) | 3.33 | 3.09 | 3.326 |
| C (1kb) | 3.33 | 3.95 | 3.326 |
| D (10kb) | 0 | 0 | 0.02 |
| Total: | 10 | 10 | 10 |

$$TPM = \frac{RPKM}{\sum(RPKM)} \times 10^6$$

RPKM

| Gene Name | Rep1 RPKM | Rep2 RPKM | Rep3 RPKM |
|---|---|---|---|
| A (2kb) | 1.43 | 1.33 | 1.42 |
| B (4kb) | 1.43 | 1.39 | 1.42 |
| C (1kb) | 1.43 | 1.78 | 1.42 |
| D (10kb) | 0 | 0 | 0.009 |
| Total: | 4.29 | 4.5 | 4.25 |

With RPKM, it is harder to compare the proportion of total reads because each replicate has different total (each pie has a different size)

A 1.43 size slice represents a different proportion of reads in in different pies.


Consider 3 pies, each the same size (10).

A 3.33 sized slice is the same in each pie, and is always larger than 3.32.

TPM makes it clear that in Rep1, more of its total reads mapped to gene A than in Rep3.


TPM

| Gene Name | Rep1 TPM | Rep2 TPM | Rep3 TPM |
|---|---|---|---|
| A (2kb) | 3.33 | 2.96 | 3.326 |
| B (4kb) | 3.33 | 3.09 | 3.326 |
| C (1kb) | 3.33 | 3.95 | 3.326 |
| D (10kb) | 0 | 0 | 0.02 |
| Total: | 10 | 10 | 10 |

*Image: StatQuest*

# Summary

- Normalized counts account for sequencing depth and gene length biases
  - RPKM ~ single-end sequencing, FPKM ~ paired-end sequencing
  - The sum of all TPMs in each sample is the same. Easier to compare across samples!

# Learning Objectives of Module 3

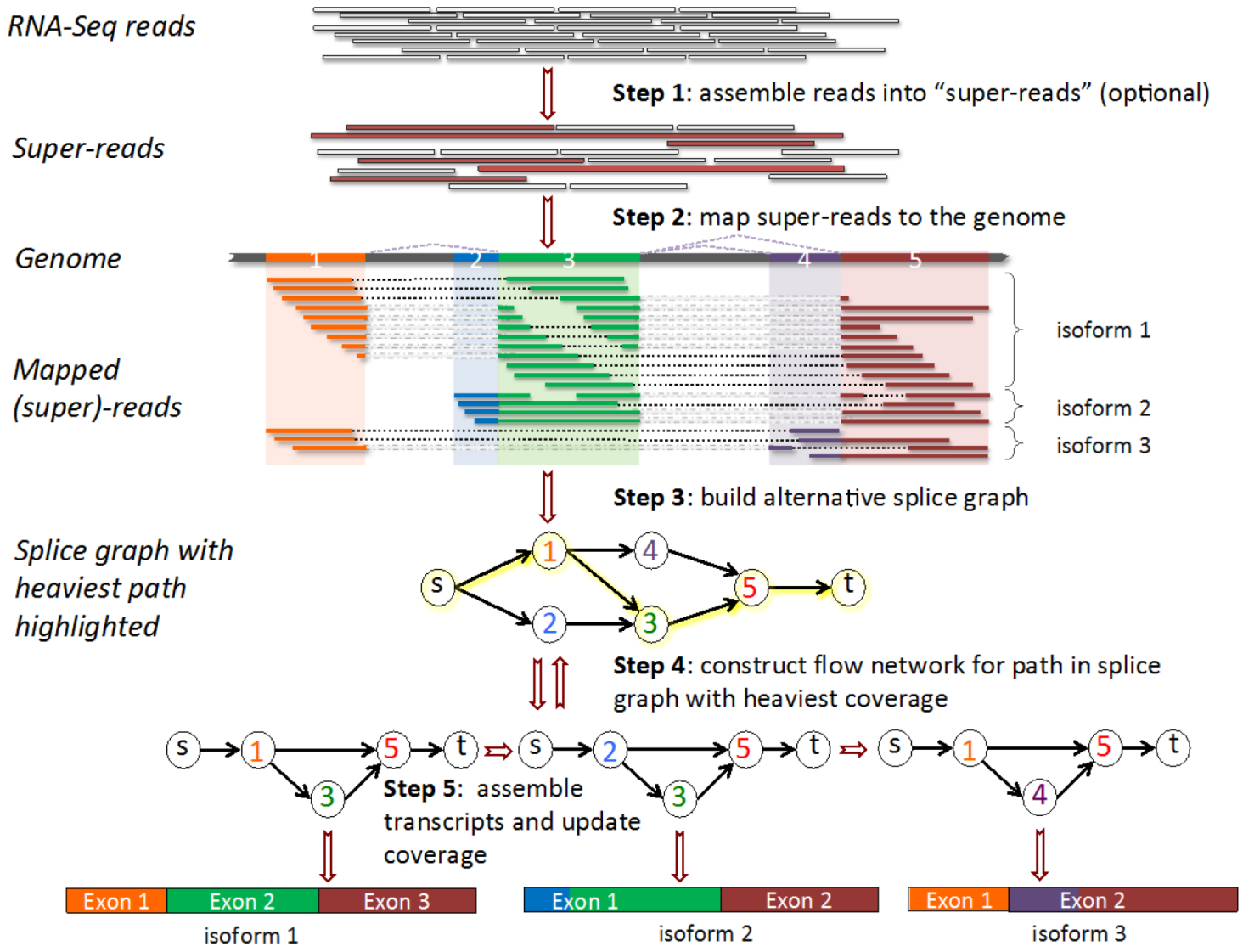
- Review basic concepts and popular metrics of abundance estimation:
  - raw counts vs normalized counts
- Discuss normalized count estimation tool: StringTie
- Discuss raw count estimation tool: HTSeq

# How does StringTie work?

- Align reads to the genome, optionally assemble super-reads and re-align
- Group reads into clusters

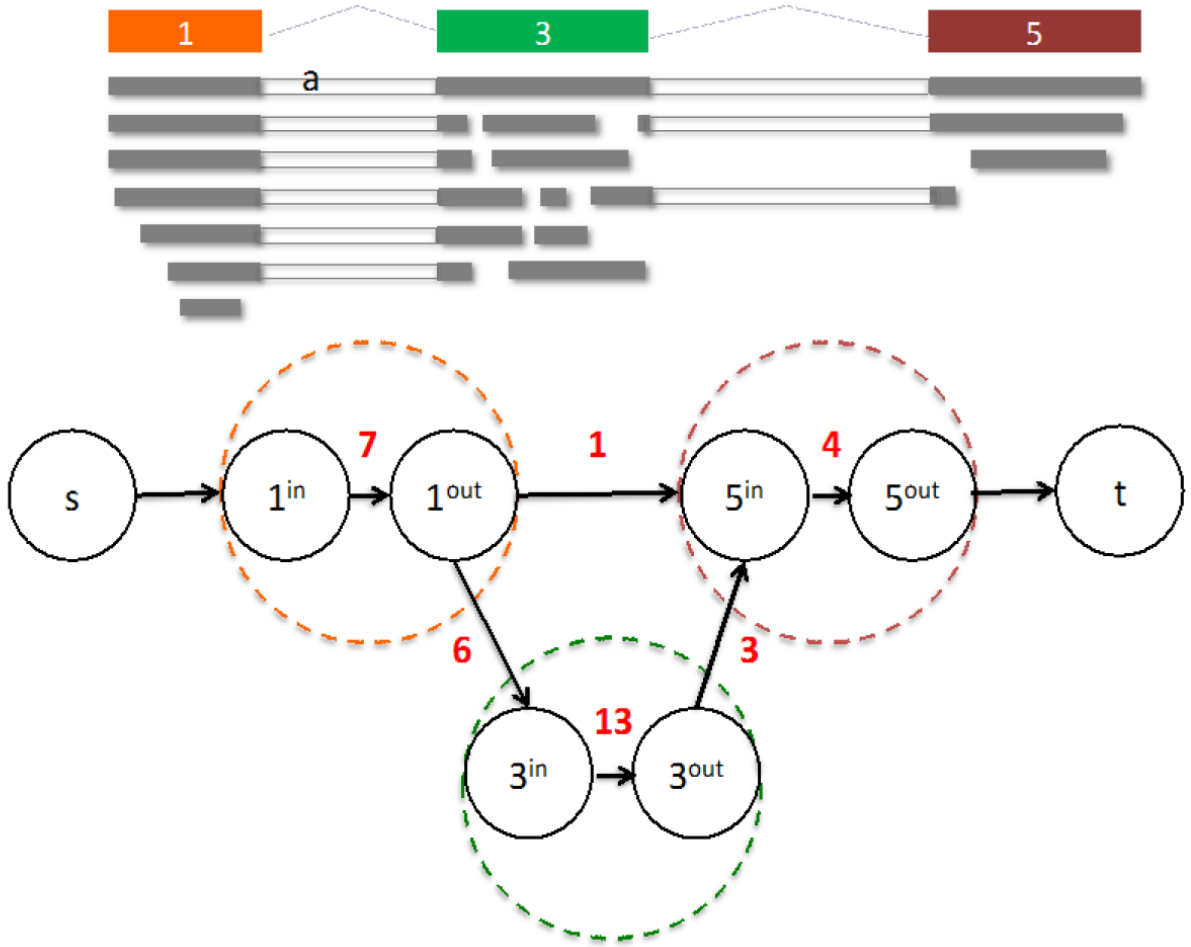
Infer isoforms:

- Build alternative splice graph (ASG)
- Iteratively extract the heaviest path from a splice graph
- construct a flow network
- compute maximum flow to estimate abundance
- update the splice graph by removing reads that were assigned by the flow algorithm
- This process repeats until all reads have been assigned.




Pertea et al. Nature Biotechnology, 2015

# From flow network for each transcript, maximum flow is used to assemble transcript and estimate abundance




StringTie uses basic graph theory (splice graph), custom heuristics (heaviest path), more graph theory (flow network) and optimization theory (maximum flow). See StringTie paper for definitions and math.

# StringTie Modes

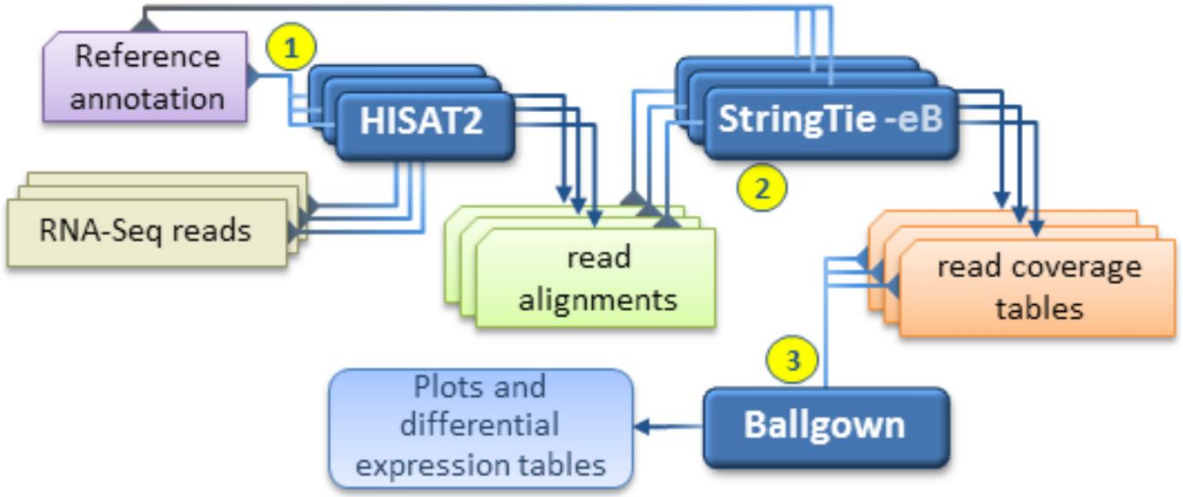
- Expression estimation mode (“Reference Only”) ← What we will use
  - “–G $GTF_File” AND “–e” option
  - no "novel" transcript assemblies (isoforms)
  - read alignments not overlapping reference transcripts ignored
  - Faster, especially when given limited set of reference transcripts
    - Avoids complicated steps of clustering and building alternative splice graph from scratch, skipping straight to abundance estimation
- “Reference guided mode”
  - “–G $GTF_File” WITHOUT “–e” option
  - Both known and novel transcript assemblies
- “De novo” mode
  - NEITHER “–G $GTF_File” NOR “–e” option
  - Novel transcript assemblies only

Pertea et al. Nature Protocols, 2016

# StringTie -merge

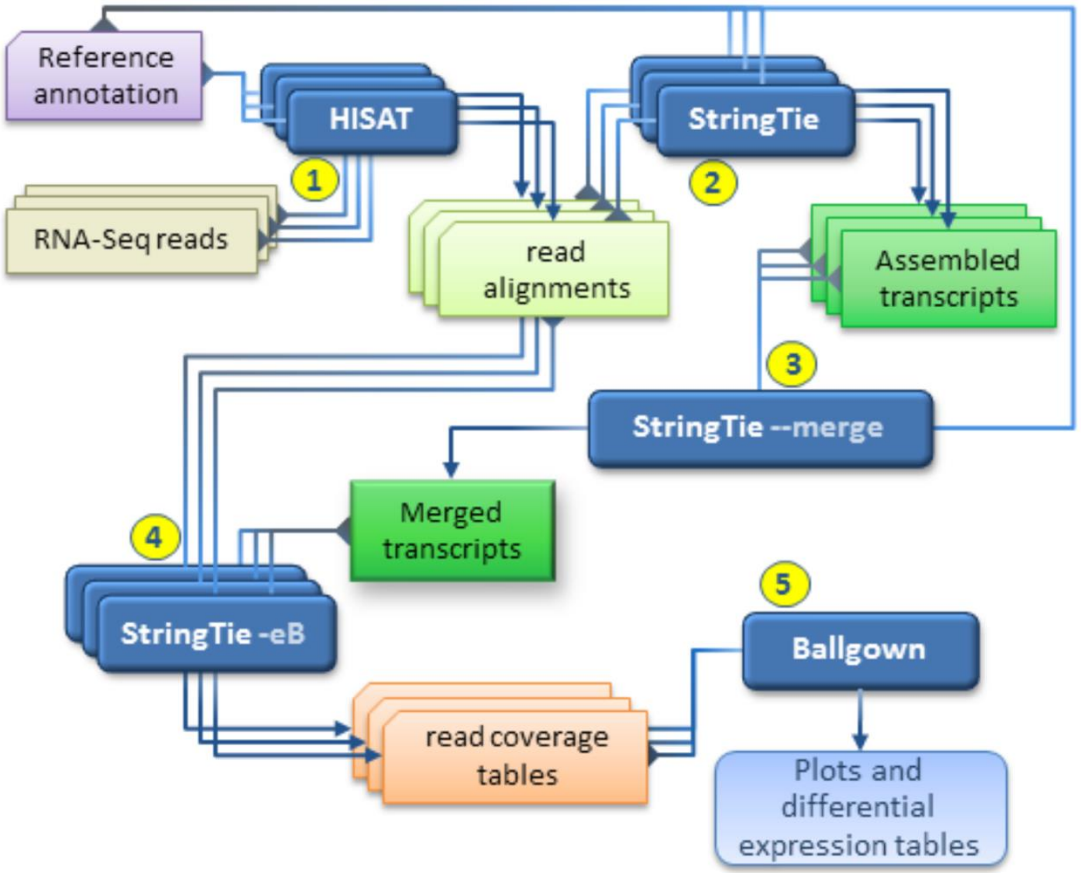
- Merge together all gene structures from all samples
  - Some samples may only partially represent a gene structure
- Incorporates known transcripts with assembled, potentially novel transcripts
- For de novo or reference guided mode, we will rerun StringTie with the merged transcript assembly.

Pertea et al. Nature Protocols, 2016




This is the workflow we use in the exercise:
1 step: use StringTie –G and -e

Expression estimation mode (“Reference Only”)




But in case you want to run Reference-guide or ‘Denovo’ mode, will need 3 steps: run StringTie, then StringTie --merge, then StringTie –e .

https://ccb.jhu.edu/software/stringtie/index.shtml?t=manual

# gffcompare

- gffcompare will compare a merged transcript GTF with known annotation, also in GTF/GFF3 format
- https://ccb.jhu.edu/software/stringtie/gff.shtml#gffcompare

| Priority | Code | Description |
|---|---|---|
| 1 | = | Complete match of intron chain |
| 2 | c | Contained |
| 3 | j | Potentially novel isoform (fragment): at least one splice junction is shared with a reference transcript |
| 4 | e | Single exon transfrag overlapping a reference exon and at least 10 bp of a reference intron, indicating a possible pre-mRNA fragment. |
| 5 | i | A transfrag falling entirely within a reference intron |
| 6 | o | Generic exonic overlap with a reference transcript |
| 7 | p | Possible polymerase run-on fragment (within 2Kbases of a reference transcript) |
| 8 | r | Repeat. Currently determined by looking at the soft-masked reference sequence and applied to transcripts where at least 50% of the bases are lower case |
| 9 | u | Unknown, intergenic transcript |
| 10 | x | Exonic overlap with reference on the opposite strand |
| 11 | s | An intron of the transfrag overlaps a reference intron on the opposite strand (likely due to read mapping errors) |
| 12 | . | (.tracking file only, indicates multiple classifications) |

# Learning Objectives of Module 3

- Review basic concepts and popular metrics of abundance estimation:
  - raw counts vs normalized counts
- Discuss normalized count estimation tool: StringTie
- Discuss raw count estimation tool: HTSeq

# HTseq

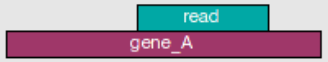
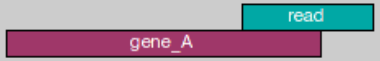
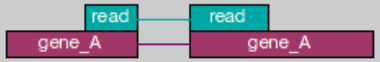
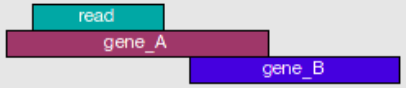
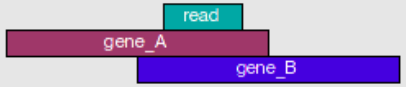
- Raw read counts for differential expression analysis
  - Assign reads/fragments to defined genes/transcripts, get “raw counts”
    - Transcript structures could still be defined by something like Stringtie

- 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 |
|---|---|---|---|
|  | 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 |

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

# Summary

- Normalized counts account for sequencing depth and gene length biases
  - RPKM ~ single-end sequencing, FPKM ~ paired-end sequencing
  - The sum of all TPMs in each sample is the same. Easier to compare across samples!
- Abundance estimation tool that calculates normalized count (FPKM, TPM): StringTie
- Abundance estimation tool that calculates raw count: HTseq

# Set up

- Open docker desktop app in the background. Then in terminal, type:
- $ docker pull griffithlab/rnabio:0.0.1
- $ cd bfx-workshop/rnabio-workspace
- $ docker run -v $PWD/:/workspace:rw -it griffithlab/rnabio:0.0.1 /bin/bash

(or in case it throws error

- $ docker run --platform linux/amd64 -v $PWD/:/workspace:rw -it griffithlab/rnabio:0.0.1 /bin/bash

)

- $ cd workspace
- $ su ubuntu
- $ source ~/.bashrc

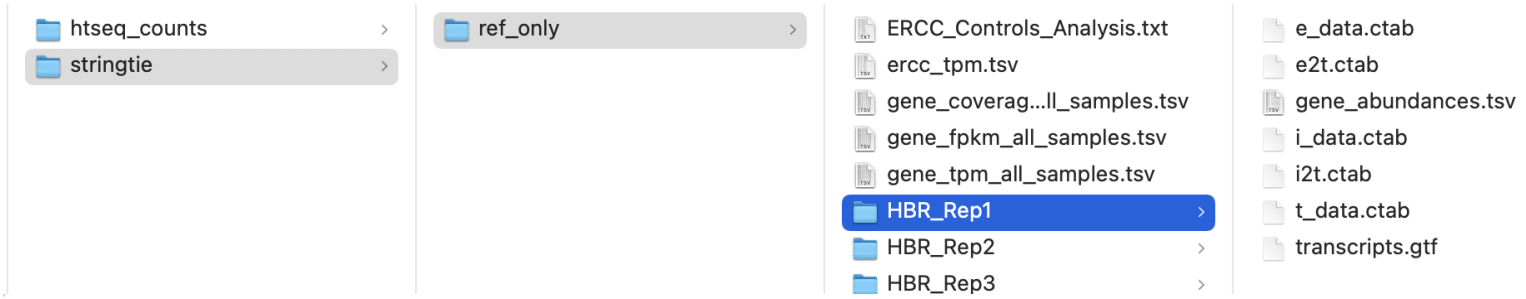
# Stringtie outputs

- Stringtie gives 3 metrics for expression levels: coverage, FPKM, TPM ; for 2 types : transcript and gene.
- Focus on the ‘transcript.gtf’ and ‘gene_abundance.tsv’

+ 'e_data.ctab’ : exon coordinate and corresponding coverage
+ ‘i_data.ctab’: intron and corresponding coverage
+ ‘t_data.ctab’: transcript and corresponding coverage
+ e2t.ctab: table with two columns, e_id and t_id, denoting which exons belong to which transcripts
+ i2t.ctab: table with two columns, i_id and t_id, denoting which introns belong to which transcripts

+ transcripts.gtf : transcripts, the exons made up each transcript, coordinates, gene id/ transcript id, cov / fpkm / tpm

+ gene_abundance.tsv: genes, coordinates, and expr levels




transcripts.gtf

```
# stringtie --rf -p 8 -G /home/ubuntu/workspace/rnaseq/refs/chr22_with_ERCC92.gtf -e -B -o HBR_Rep1/transcripts.gtf -A HBR_Rep1/gene_abundances.tsv /home/ubuntu/workspace/rnaseq/alignments/hisat2/HBR_Rep1.bam
# StringTie version 2.1.6
22      havana  transcript      16869478 16871126 .       +       .       gene_id "ENSG00000237689"; transcript_id "ENST00000442403"; ref_gene_name "AC007064.24"; cov "0.0"; FPKM "0.000000"; TPM "0.000000";
22      havana  exon    16869478 16869626 .       +       .       gene_id "ENSG00000237689"; transcript_id "ENST00000442403"; exon_number "1"; ref_gene_name "AC007064.24"; cov "0.0";
22      havana  exon    16870776 16871126 .       +       .       gene_id "ENSG00000237689"; transcript_id "ENST00000442403"; exon_number "2"; ref_gene_name "AC007064.24"; cov "0.0";
22      havana  transcript      15790709 15791814 .       +       .       gene_id "ENSG00000206195"; transcript_id "ENST00000458623"; ref_gene_name "DUXAP8"; cov "0.0"; FPKM "0.000000"; TPM "0.000000";
22      havana  exon    15790709 15790798 .       +       .       gene_id "ENSG00000206195"; transcript_id "ENST00000458623"; exon_number "1"; ref_gene_name "DUXAP8"; cov "0.0";
22      havana  exon    15791017 15791152 .       +       .       gene_id "ENSG00000206195"; transcript_id "ENST00000458623"; exon_number "2"; ref_gene_name "DUXAP8"; cov "0.0";
22      havana  exon    15791628 15791814 .       +       .       gene_id "ENSG00000206195"; transcript_id "ENST00000458623"; exon_number "3"; ref_gene_name "DUXAP8"; cov "0.0";
```

e_data.ctab

16869478

```
264     22      +       16869478 16869626 0       0       0.00    0.0000  0.0000  0.0000  0.0000
265     22      +       16870776 16871126 2       2       2.00    0.5698  0.5992  0.5698  0.5992
```

t_data.ctab

```
t_id    chr     strand  start   end     t_name  num_exons       length  gene_id gene_name       cov     FPKM
1       22      -       10736171 10736283 ENST00000615943 1       113     ENSG00000277248 U2      0.000000 0.000000
2       22      -       10939388 10961338 ENST00000635667 9       749     ENSG00000283047 FRG1FP  0.000000 0.000000
```

gene_abundances.tsv

```
Gene ID  Gene Name      Reference       Strand  Start   End     Coverage FPKM     TPM
ENSG00000237689  AC007064.24     22      +       16869478 16871126 0.000000 0.000000 0.000000
ENSG00000206195  DUXAP8  22      +       15784959 15829984 0.282236 52.024742        77.325455
```

# Stringtie outputs

- `e_data.ctab` : exon-level expression measurements. One row per exon. Columns are `e_id` (numeric exon id), `chr`, `strand`, `start`, `end` (genomic location of the exon), and the following expression measurements for each sample:
  - `rcount` : reads overlapping the exon
  - `ucount` : uniquely mapped reads overlapping the exon
  - `mrcount` : multi-map-corrected number of reads overlapping the exon
  - `cov` average per-base read coverage
  - `cov_sd` : standard deviation of per-base read coverage
  - `mcov` : multi-map-corrected average per-base read coverage
  - `mcov_sd` : standard deviation of multi-map-corrected per-base coverage
- `i_data.ctab` : intron- (i.e., junction-) level expression measurements. One row per intron. Columns are `i_id` (numeric intron id), `chr`, `strand`, `start`, `end` (genomic location of the intron), and the following expression measurements for each sample:
  - `rcount` : number of reads supporting the intron
  - `ucount` : number of uniquely mapped reads supporting the intron
  - `mrcount` : multi-map-corrected number of reads supporting the intron
- `t_data.ctab` : transcript-level expression measurements. One row per transcript. Columns are:
  - `t_id` : numeric transcript id
  - `chr`, `strand`, `start`, `end` : genomic location of the transcript
  - `t_name` : Cufflinks-generated transcript id
  - `num_exons` : number of exons comprising the transcript
  - `length` : transcript length, including both exons and introns
  - `gene_id` : gene the transcript belongs to
  - `gene_name` : HUGO gene name for the transcript, if known
  - `cov` : per-base coverage for the transcript (available for each sample)
  - `FPKM` : Cufflinks-estimated FPKM for the transcript (available for each sample)
- `e2t.ctab` : table with two columns, `e_id` and `t_id`, denoting which exons belong to which transcripts. These ids match the ids in the `e_data` and `t_data` tables.
- `i2t.ctab` : table with two columns, `i_id` and `t_id`, denoting which introns belong to which transcripts. These ids match the ids in the `i_data` and `t_data` tables.

https://github.com/alyssafrazee/ballgown

# StringTie gene expression = sum of all related transcripts expressions

- Example

gene_abundances.tsv

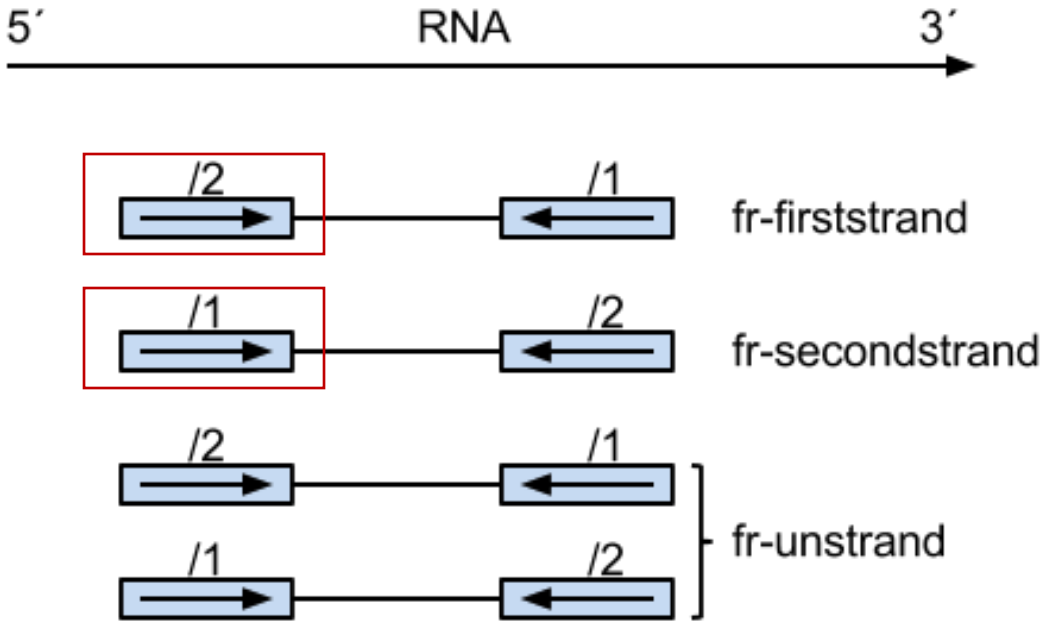
| Gene ID | Gene Name | Reference | Strand | Start | End | Coverage | FPKM | TPM |
|---|---|---|---|---|---|---|---|---|
| ENSG00000206195 | DUXAP8 | 22 | + | 15784959 | 15829984 | 0.282236 | 52.024742 | 77.325455 |

52.024796
=1.711444+3.964907+41.748577
+1.772668+2.827200

Sum of expressions of all related transcripts (transcript.gtf)

# Strandedness

https://rnabio.org/module-09-appendix/0009/12/01/StrandSettings/




The second read (read 2) is from the original RNA strand

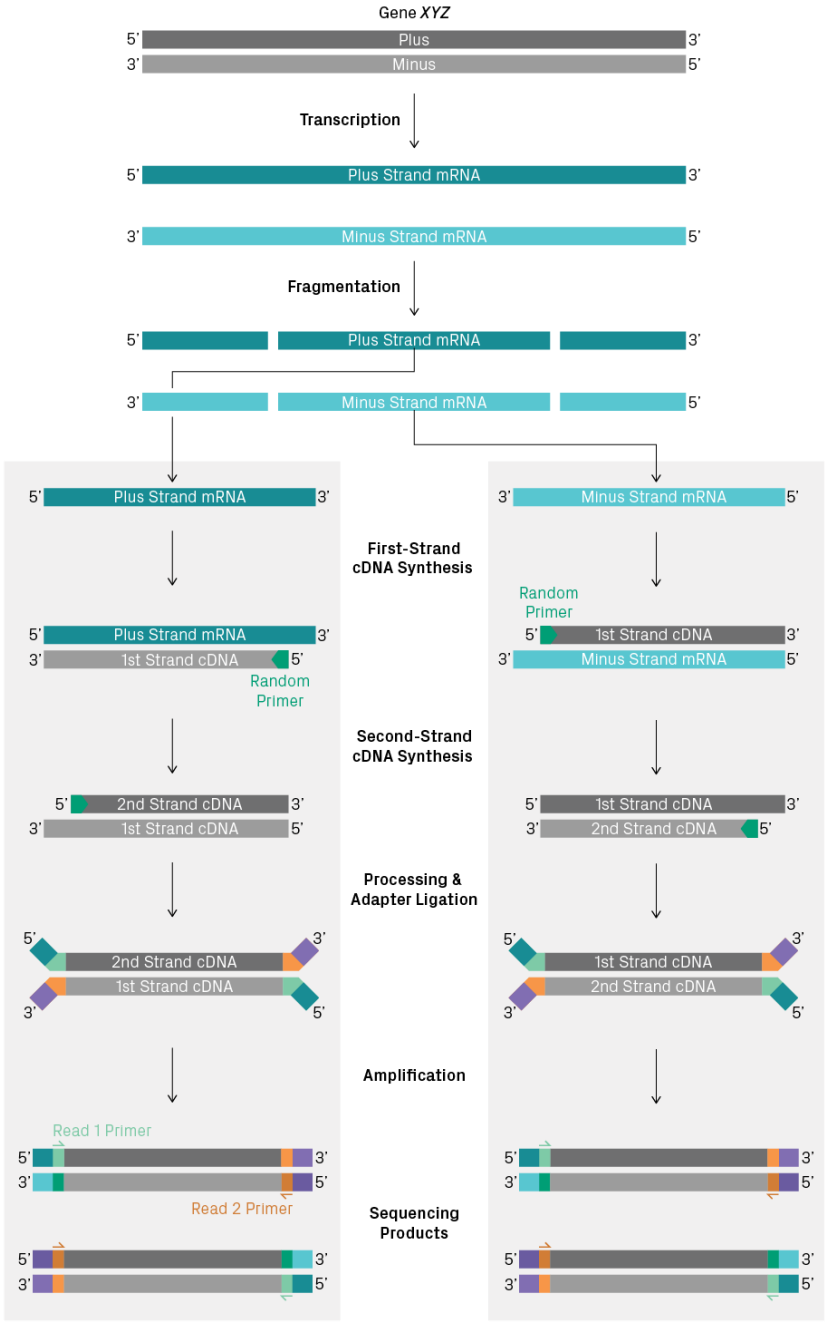
The first read (read 1) is from the original RNA strand

**Why is this so important?**

- If you use wrong directionality parameter in the read counting step with HTSeq, the reads are considered to be from the wrong strand. This means that in the case where there is no gene on that other strand, you won't get any counts, and if there is a gene in the same location on the other strand, your reads are counted for the wrong gene.
- If you use wrong directionality parameter in the reference alignment step, the XS tag in the resulting BAM file will contain wrong strand information. The XS tag is used by transcript assembly programs like Cufflinks and Stringtie, and also Cuffdiff uses it.

https://chipster.csc.fi/manual/library-type-summary.html#:~:text=second%2Dstrand%20%3D%20directional%2C%20where,is%20from%20the%20opposite%20strand.

# Non-Stranded Library Prep




https://www.azenta.com/blog/stranded-versus-non-stranded-rna-seq

# Stranded Library Prep

## Library Preparation Selection Guide

Choosing the right library preparation method depends on several factors, including your experimental objective, budget, and availability of a reference transcriptome for your organism.

The most important consideration is the objective of your experiment. Stranded RNA-Seq is strongly recommended if you're trying to accomplish one or more of the following, as it's important to capture information about transcript directionality:

- Identify antisense transcripts
- Annotate a genome
- Discover novel transcripts

Non-stranded RNA-Seq, on the other hand, is often sufficient for measuring gene expression in organisms with well-annotated genomes. With a reference transcriptome, you can infer orientation for most of the sequencing reads. Since there are fewer steps than stranded library prep, the benefits of this approach are lower cost, simpler execution, and greater recovery of material.

Also, when comparing the results of a new experiment to older ones, many researchers prefer using the same RNA-Seq approach. It enables an apples-to-apples comparison of the data.

## Key Takeaways

- Stranded RNA-Seq enables you to determine RNA orientation from each sequencing read; this information can't be directly obtained from non-stranded approaches
- By differentiating the first and second strands of cDNA, stranded library preparation preserves the directionality of the RNA molecule
- Certain applications require a stranded approach; however, non-stranded RNA-Seq is suitable for many NGS projects

https://www.azenta.com/blog/stranded-versus-non-stranded-rna-seq