Introduction to RNA Sequencing

Part 2: Abundance Estimation

Adapted from RNAbio.org

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Learning Objectives of Module 3

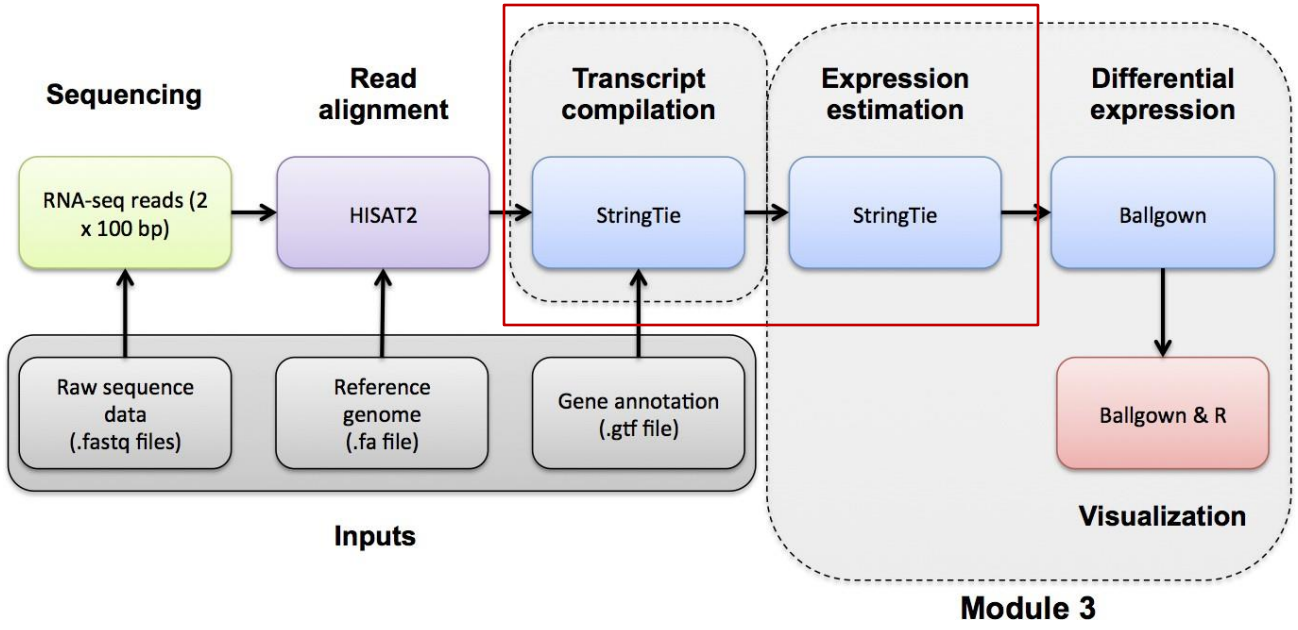
- Review basic concepts and popular metrics of abundance estimation:
  - raw counts vs normalized counts
- Discuss normalized count estimation tool: StringTie
- Discuss raw count estimation tool: HTSeq
- Discuss quantification through pseudo-alignment

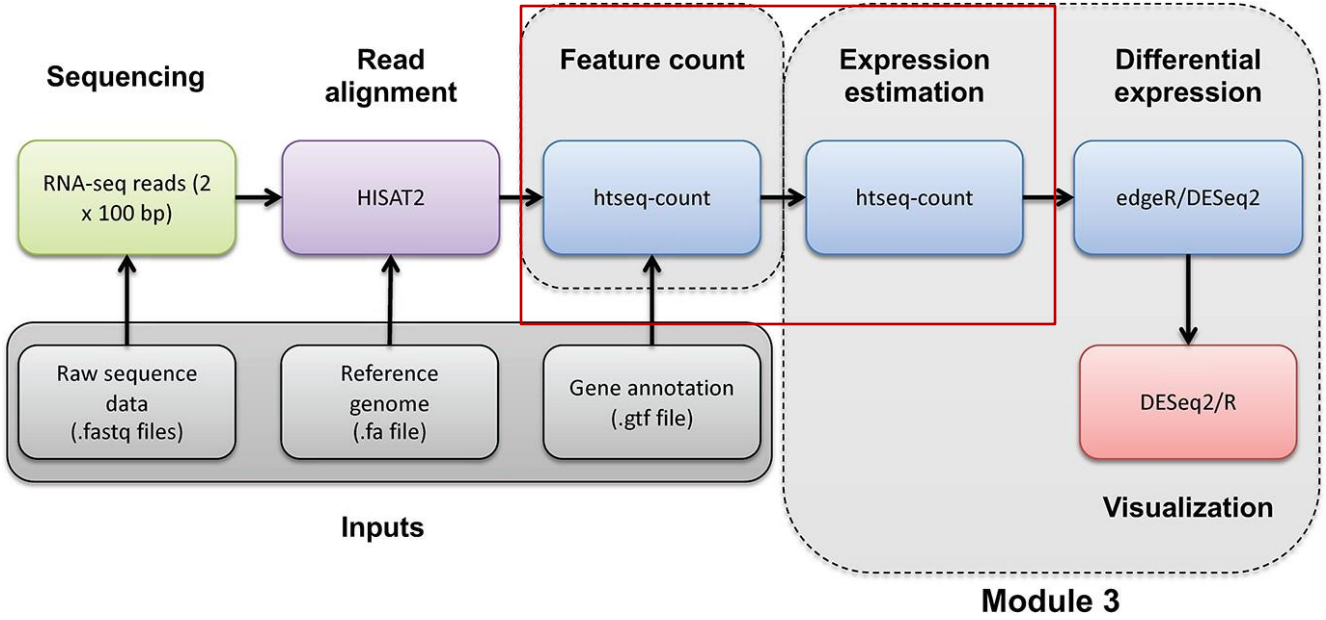
Overview

Last week: Module 1 + 2
Alignment (HISAT2)

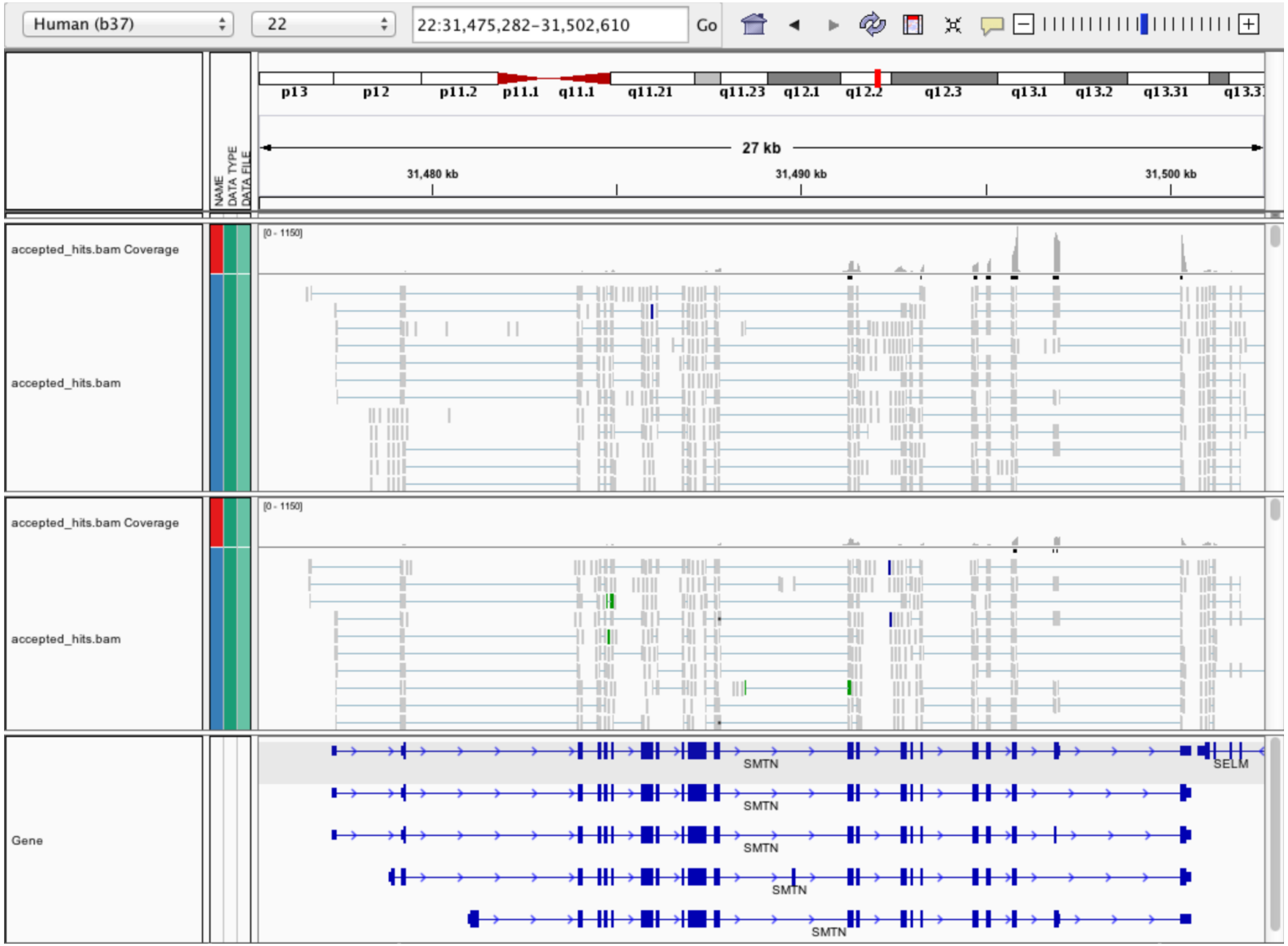
This week: Module 3
Expression estimation (StringTie, htseq count)

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





Expression estimation for known genes and transcripts

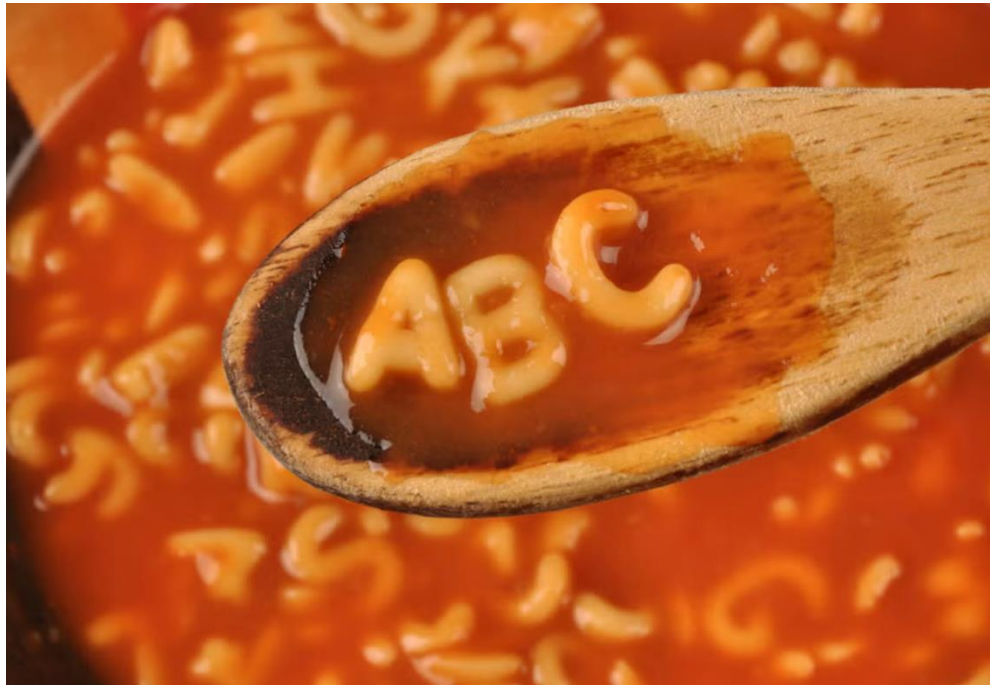


3' bias

Down-regulated

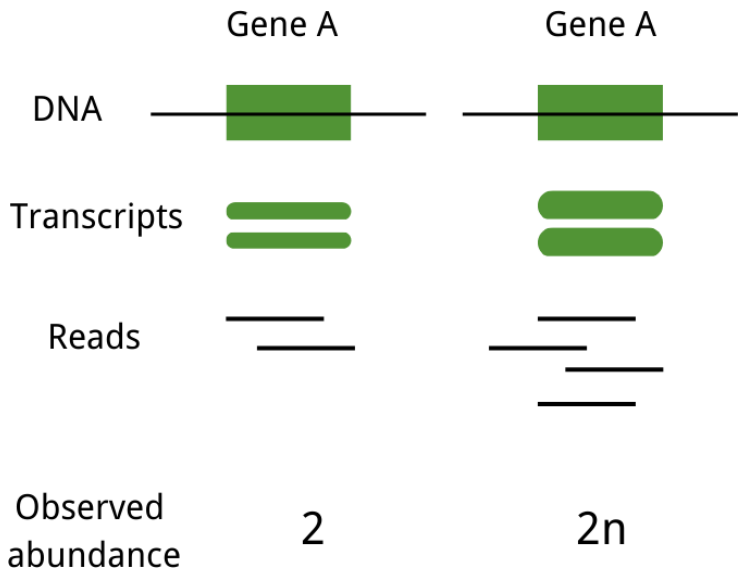
Popular metrics for abundance estimation

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



Normalized counts combat inherent biases in sequenced data

- Sequencing depth

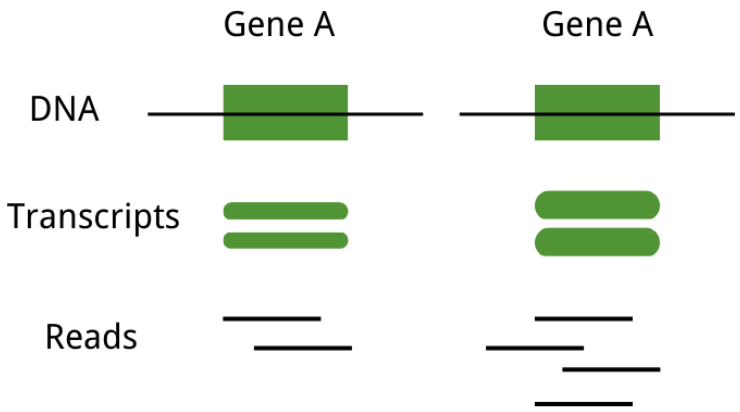


Compare expression of 1 gene in 2 samples
Sample with <u>higher sequencing depth</u> has <u>more reads</u>
→ Divided by mapped reads

Image adapted from novogene

Normalized counts combat inherent biases in sequenced data

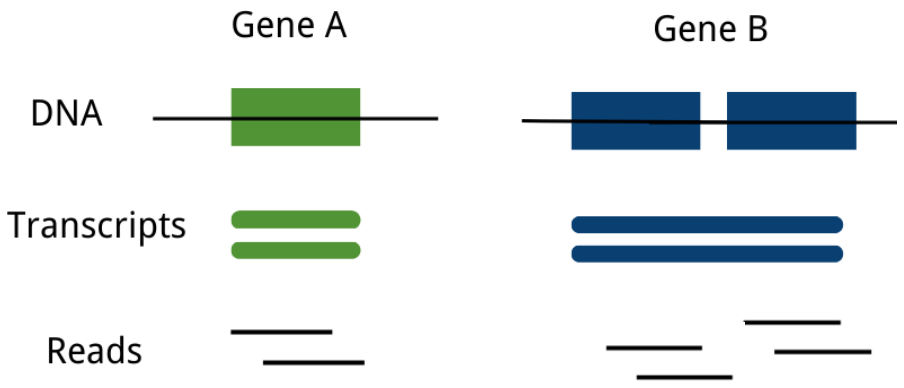
- Sequencing depth



Compare expression of 1 gene in 2 samples
Sample with <u>higher sequencing depth</u> has <u>more reads</u>

→ Divided by mapped reads

- Gene length

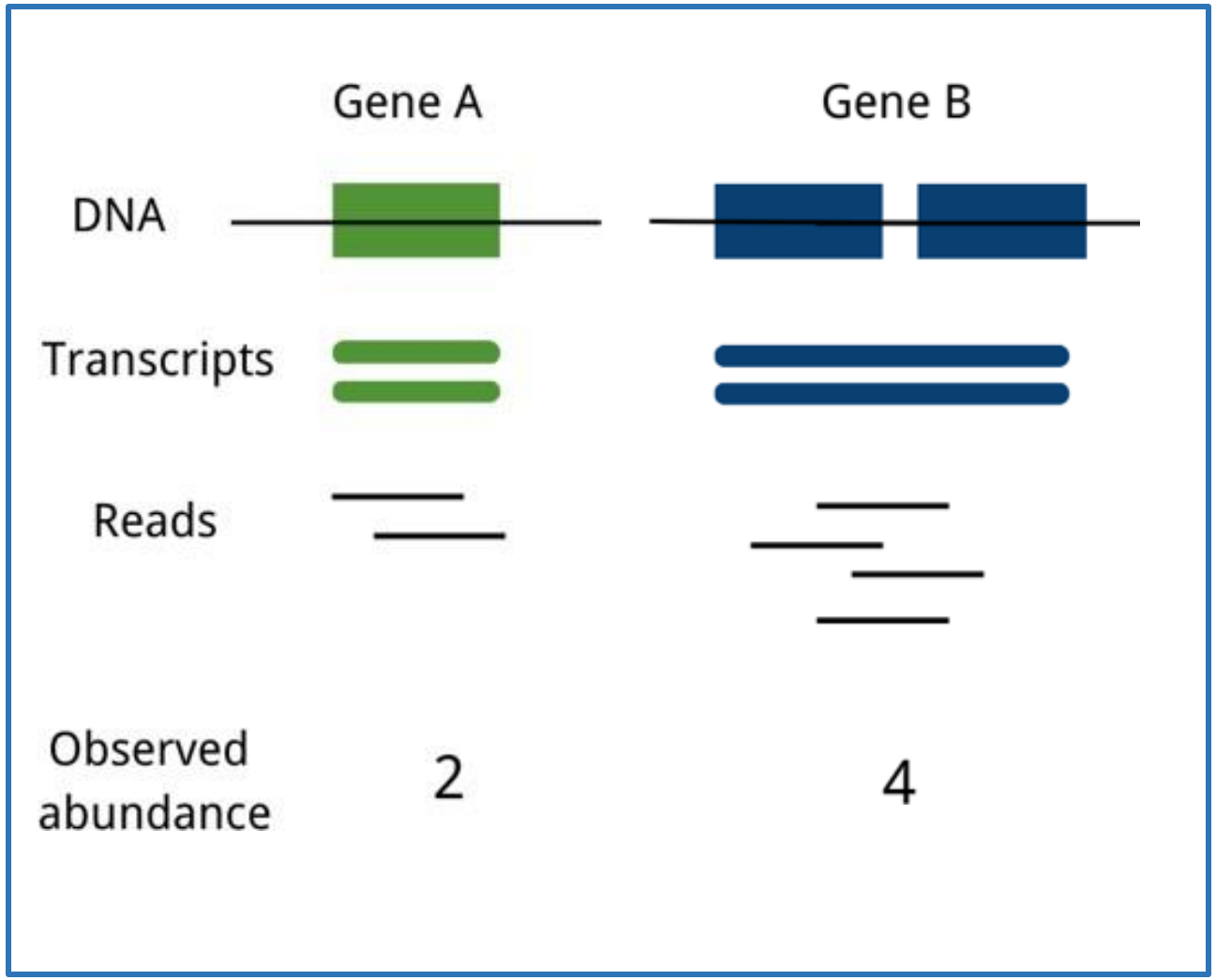


Compare expression of 2 genes in 1 sample
<u>Longer gene</u> (gene B) has <u>more reads</u>

→ Divided by gene(transcript) length

Image adapted from novogene

Raw Counts



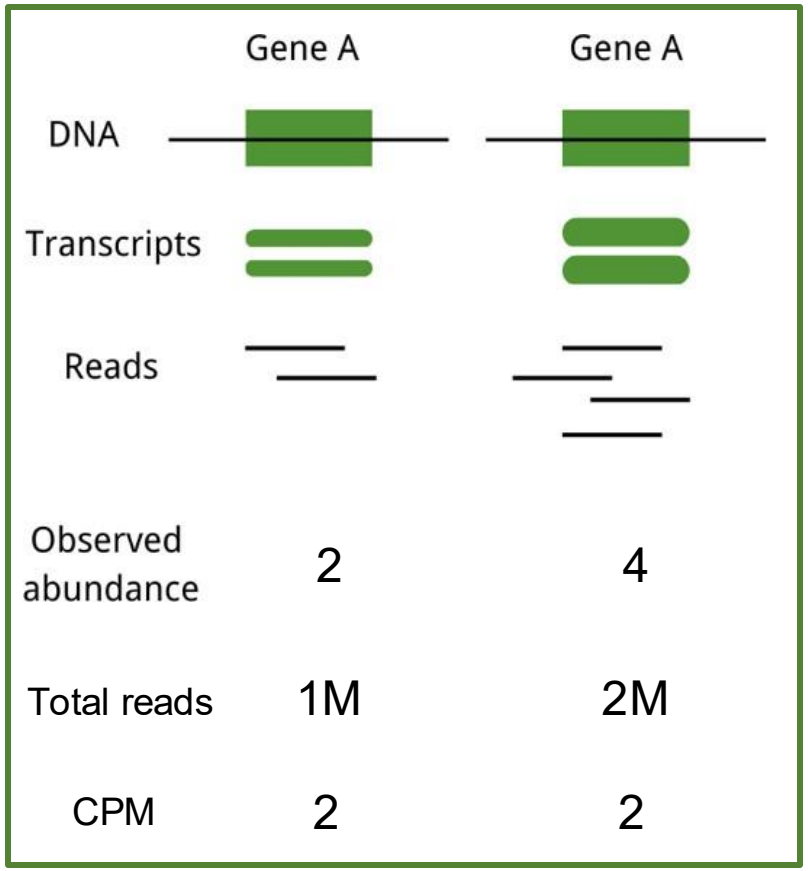
Useful as inputs to algorithms for normalization, differential expression, etc

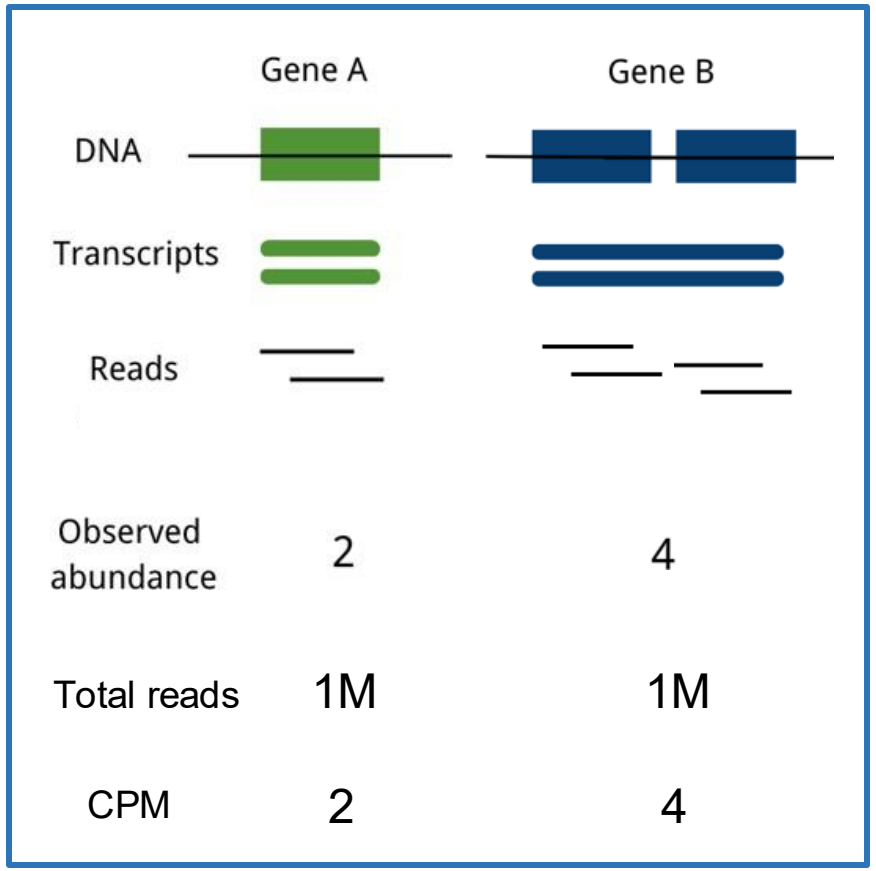
Shouldn't be used as abundance estimates on their own

CPM

Divide counts by total reads (in millions)

Accounts for sequencing depth





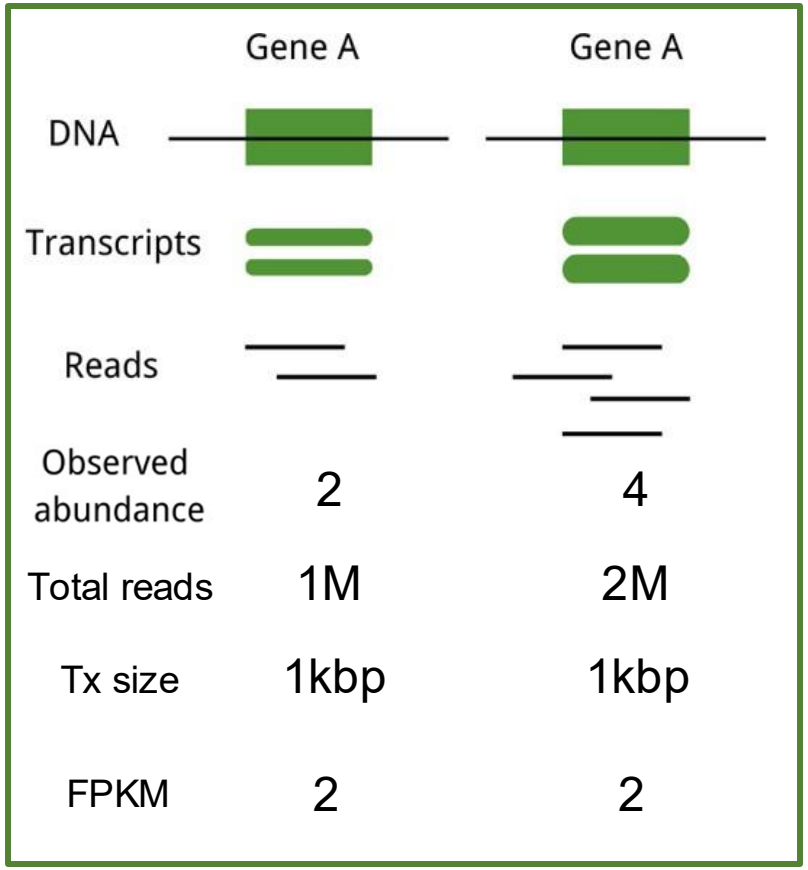
Doesn't account for gene length!

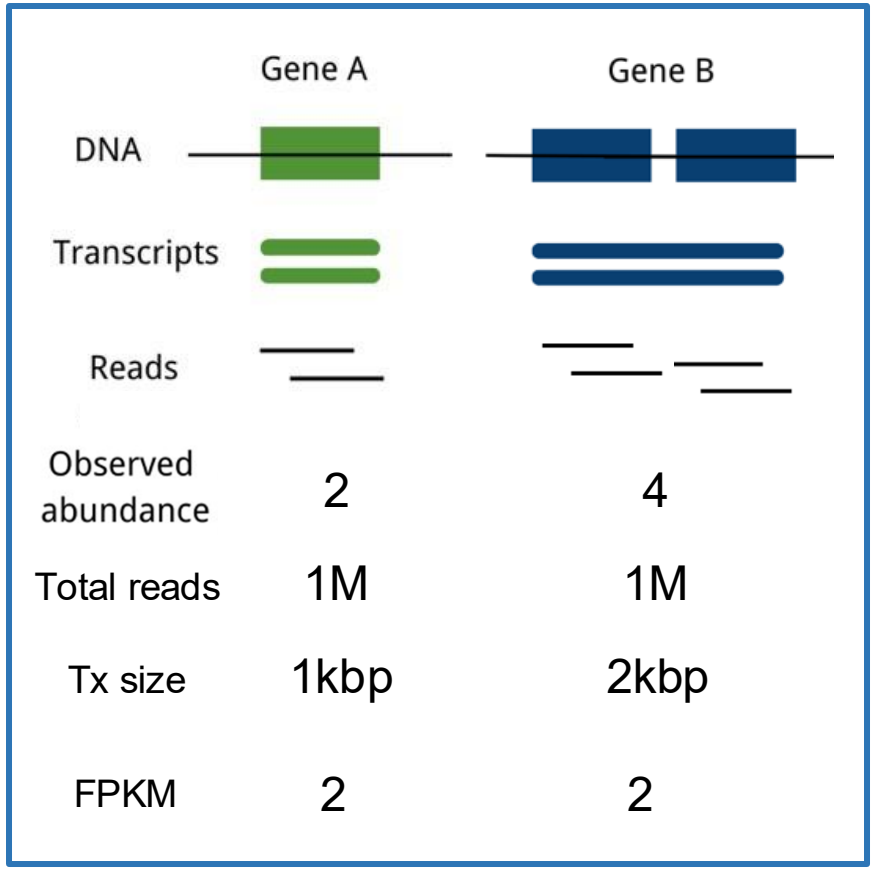
Useful for comparing genes across samples

FPKM

Divide counts by total reads (in millions) and by gene length (in kbp)

Accounts for sequencing depth first





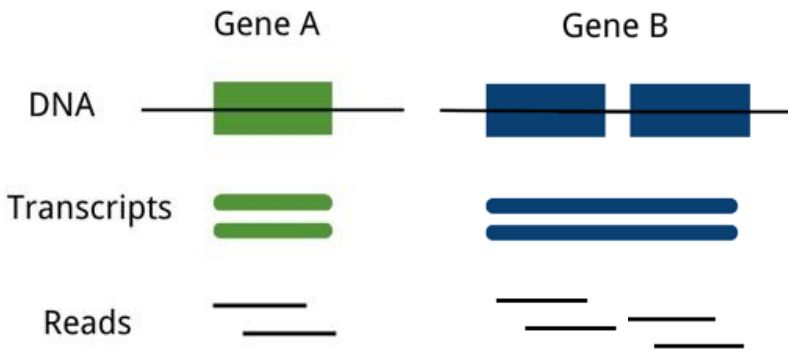
Accounts for gene length second

Useful for comparing genes within samples (and sometimes across samples)

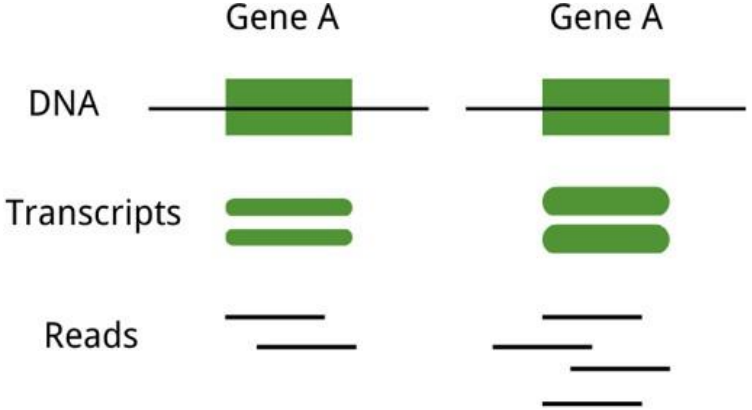
TPM

Divide counts by gene length (in kbp) and sequencing depth (in Mill.)

Accounts for gene length first



	Gene A	Gene B
Observed abundance	2	4
Total reads	1M	1M
Tx size	1kbp	2kbp
FPKM	2	2



	Gene A	Gene A
Observed abundance	2	4
Total reads	1M	2M
Tx size	1kbp	1kbp
FPKM	2	2

Accounts for sequencing depth second

Sum of all TPM values always equals 1 million

Useful for comparing genes within samples (and sometimes across samples)

How do FPKM and TPM differ?

- 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

How do FPKM and TPM differ?

- The order of operations affects the total pool size

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.

FPKM – the sum of all transcript abundance is different

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 – the sum of all transcript abundance is the same: 1M

Comparing abundance values

- Raw counts can't be compared between genes or samples

- If comparing the levels of two genes within a sample, use TPM
  - generally preferred over FPKM

- If comparing a single gene across samples, CPM is fine

- If looking at one or more genes across samples TPM is usually fine
  - provided that the global amount of RNA and the distribution of RNA within the cells is similar.
  - comparing across protocols can be problematic
  - if the fraction of ribosomal or mitochondrial DNA differs wildly, may be misleading

	Raw Counts	CPM	FPKM	TPM
Formula	-	1. Read counts ÷ total reads 2. Multiply by 1,000,000	1. Read counts ÷ total reads (in Mill.) 2. Divide by gene length (kb) 3. Multiply by 1,000	1. Read counts ÷ gene length (kb) 2. Divide by total reads (in Mill.) 3. Multiply by 1,000,000
Sum of transcripts	varies between samples	varies between samples	varies between samples	always 1 million
Corrects for:				
sequencing depth	NO	YES	YES	YES
gene length	NO	NO	YES	YES
Comparing:				
Different Samples	NO	YES	probably*	probably*
Different Genes	NO	NO	YES	YES
Best uses:	differential expression inputs	same-gene comparisons across samples	Legacy data, older tools	comparing across samples
Interpretation	observed counts	adjusting observed counts to account for the total number of bases sequenced	If you were to sequence this pool of RNA again, you expect to see this many fragments for each thousand bases in the feature, for every N/10^6 fragments you've sequenced. (rate of fragments per base)	if you were to sequence one million full length transcripts, TPM is the number of transcripts you would have seen of this type, given the abundances of the other transcripts in your sample
			* can compare across samples if the global amount of RNA in each cell is similar and RNA distribution is similar	* can compare across samples if the global amount of RNA in each cell is similar and RNA distribution is similar

What is RPKM?

Essentially the same as FPKM!

- 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

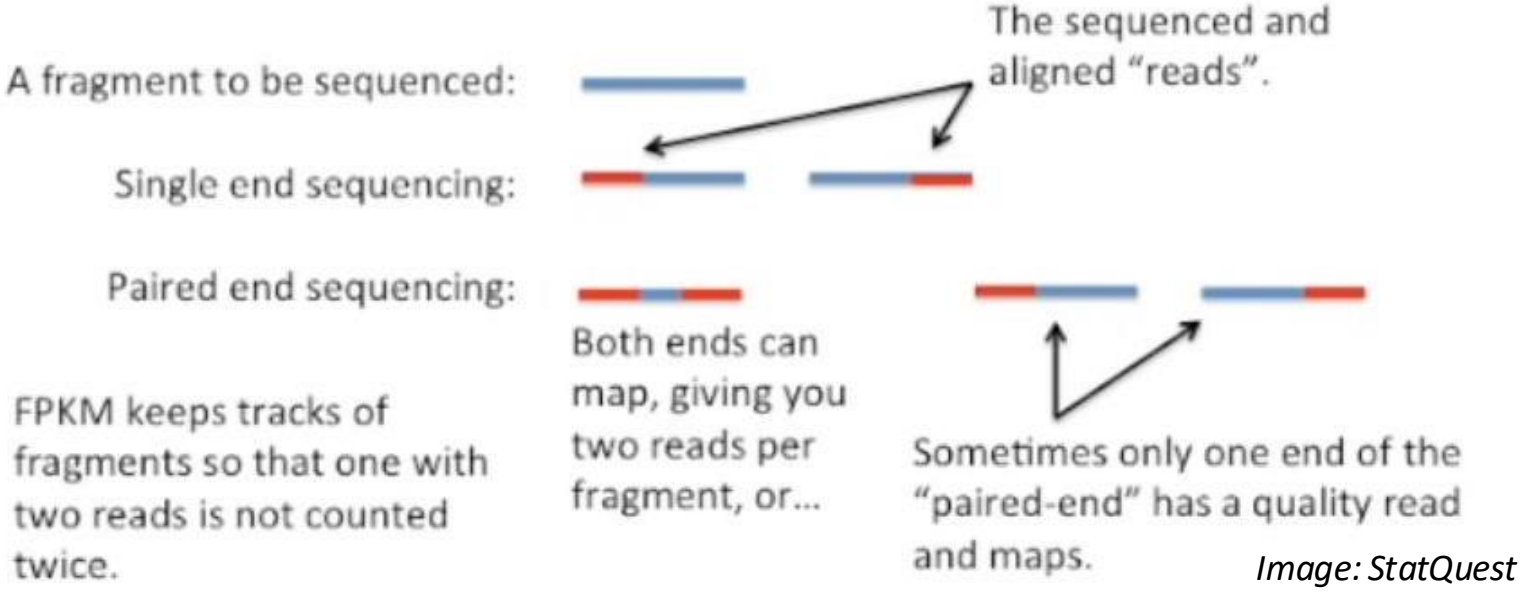


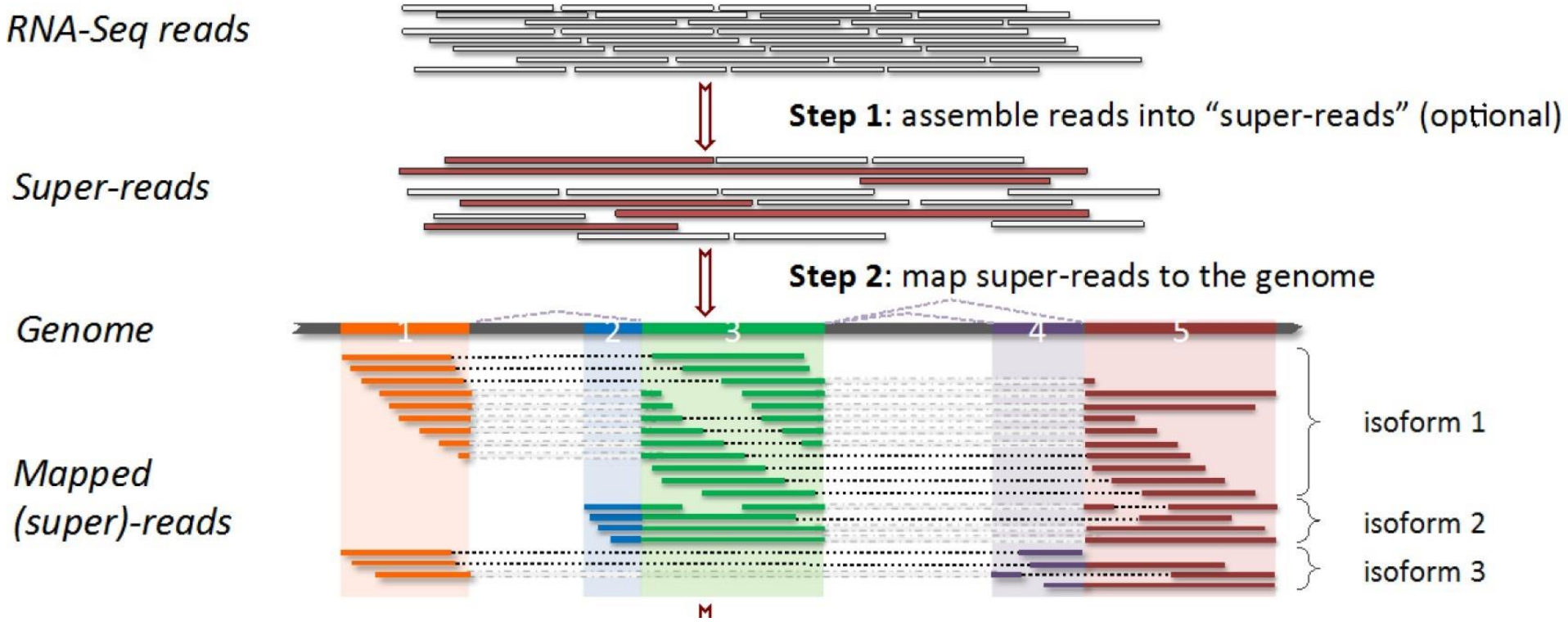
Image: StatQuest

How do I get these abundance values?

- 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

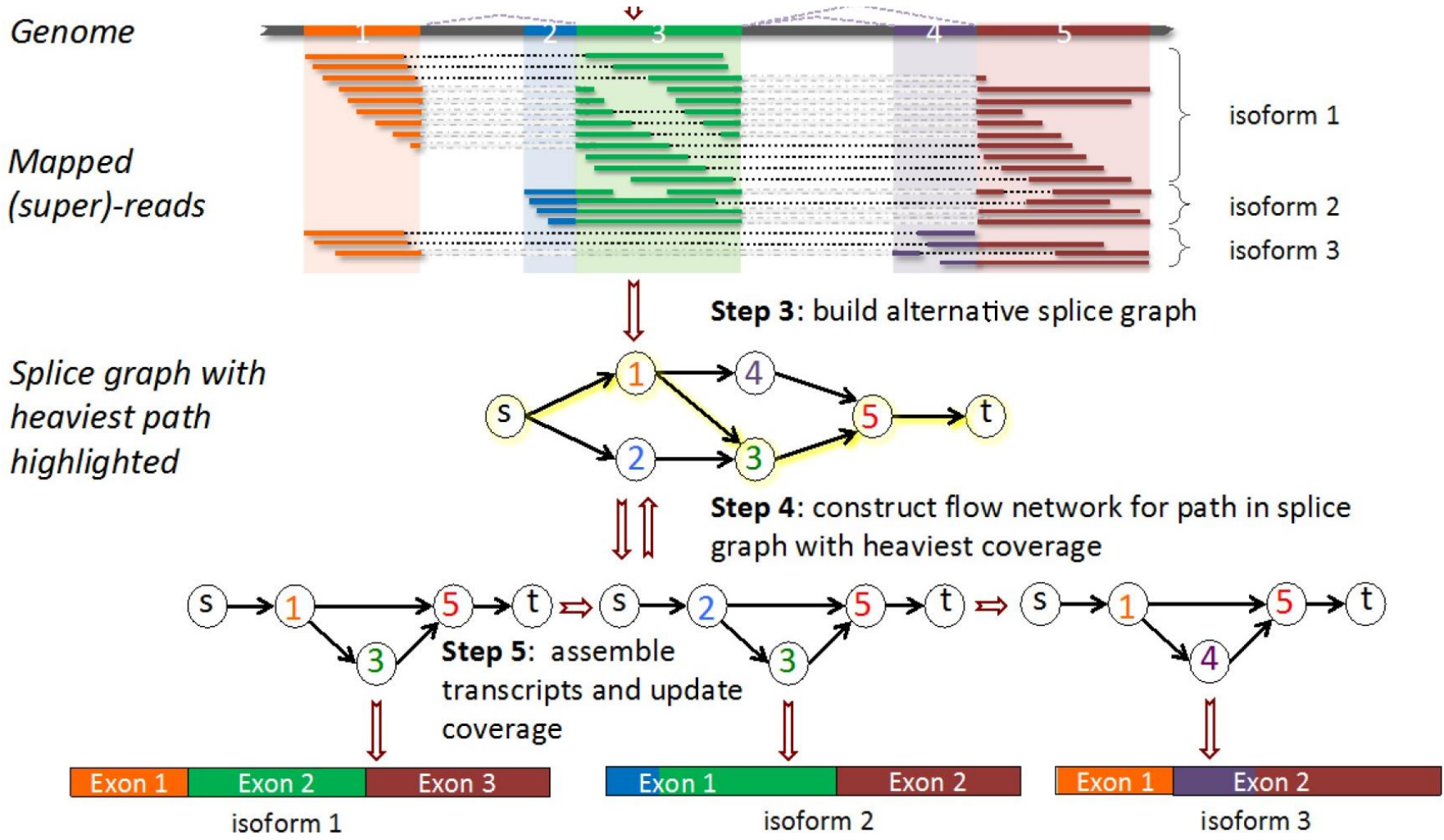


Pertea et al. Nature Biotechnology, 2015

How does StringTie work?

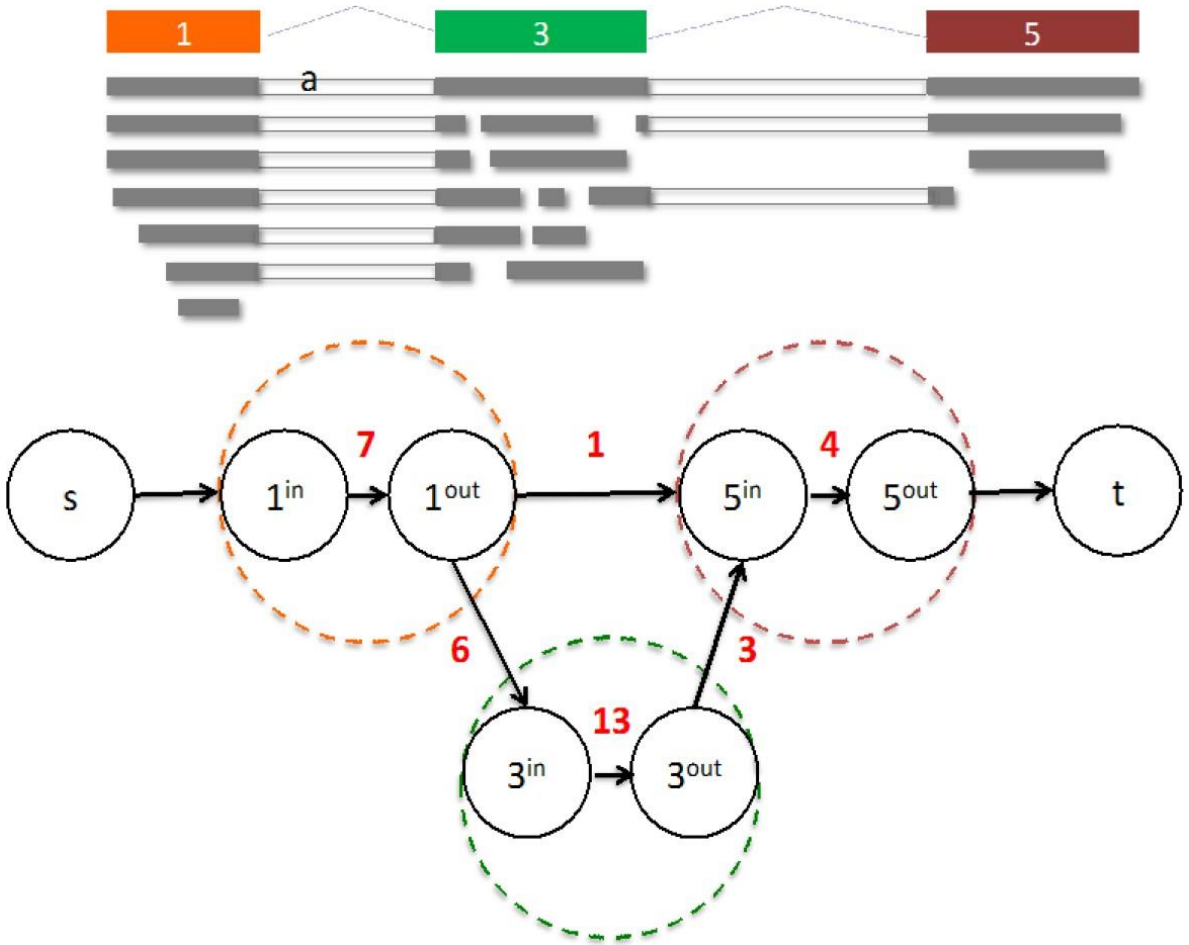
Infer isoforms:

- Build alternative splice graph
- Iteratively extract the heaviest path from 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
- repeat until all reads have been assigned.



Pertea et al. Nature Biotechnology, 2015

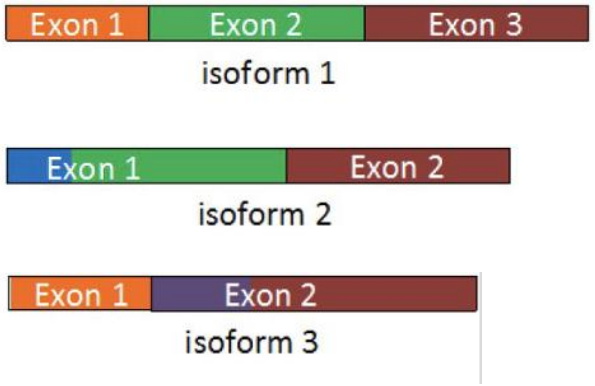
From flow network for each transcript, maximum flow is used to assemble transcript and estimate abundance



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.

Outputs of StringTie

GTF file with: Transcript structure Transcript Quantification



5.34

2.11

0.45

```
chr1  StringTie  transcript  114704469  114716894  1000  -  .  gene_id "ENSG00000213281";
transcript_id "ENST00000369535"; ref_gene_name "NRAS"; cov "111.583565"; FPKM "17.794451"; TPM "34.907570";
```

Gene expression file (summing the transcripts):

Gene ID	Gene Name	Reference	Strand	Start	End	Coverage	FPKM	TPM
ENSG00000213281	NRAS	chr1	-	114704469	114716894	81.372406	21.631533	42.434814

Pertea et al. Nature Biotechnology, 2015

StringTie gene expression = sum of all that gene's transcript expression

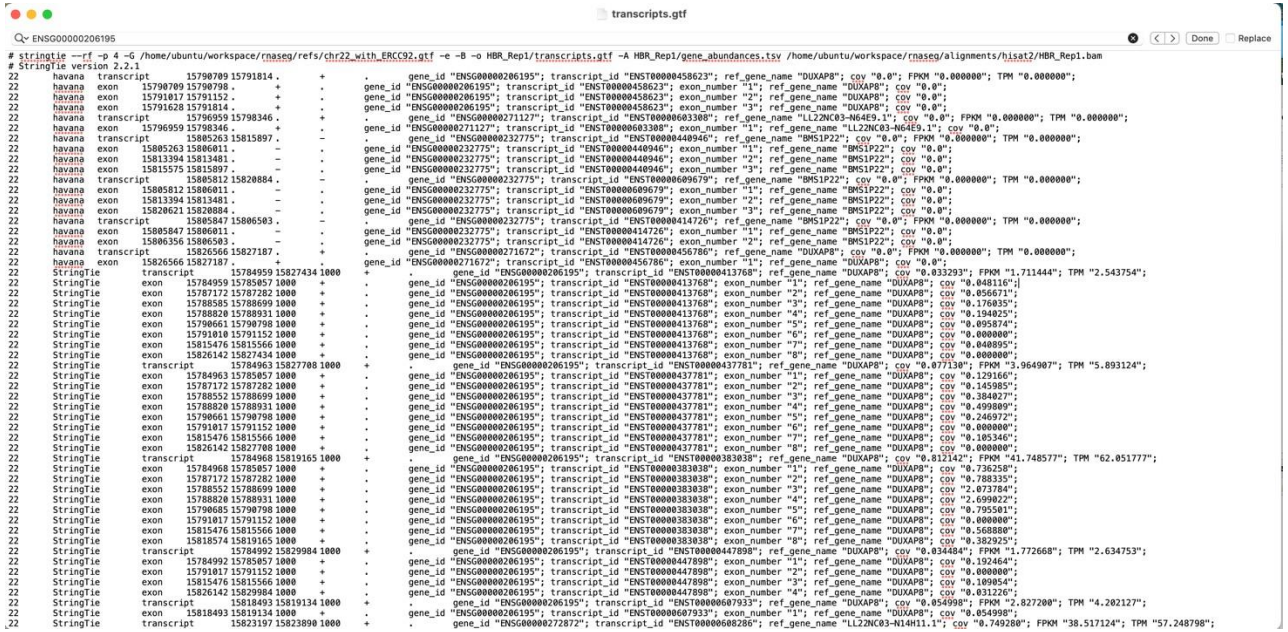
- Example

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

gene_abundances.tsv

52.024796
=1.711444+3.964907+41.748577
+1.772668+2.827200

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



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

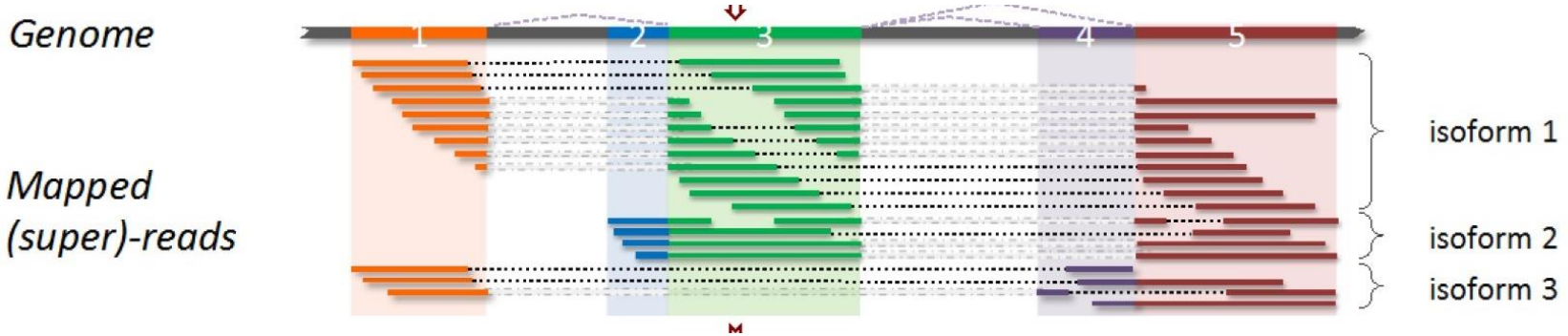
Pertea et al. Nature Protocols, 2016

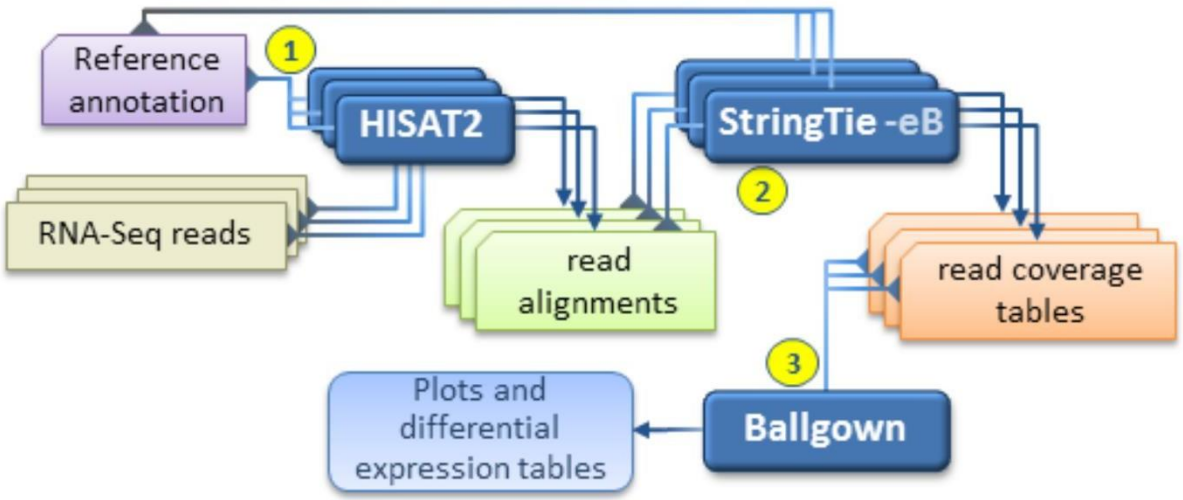
StringTie -merge

- 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

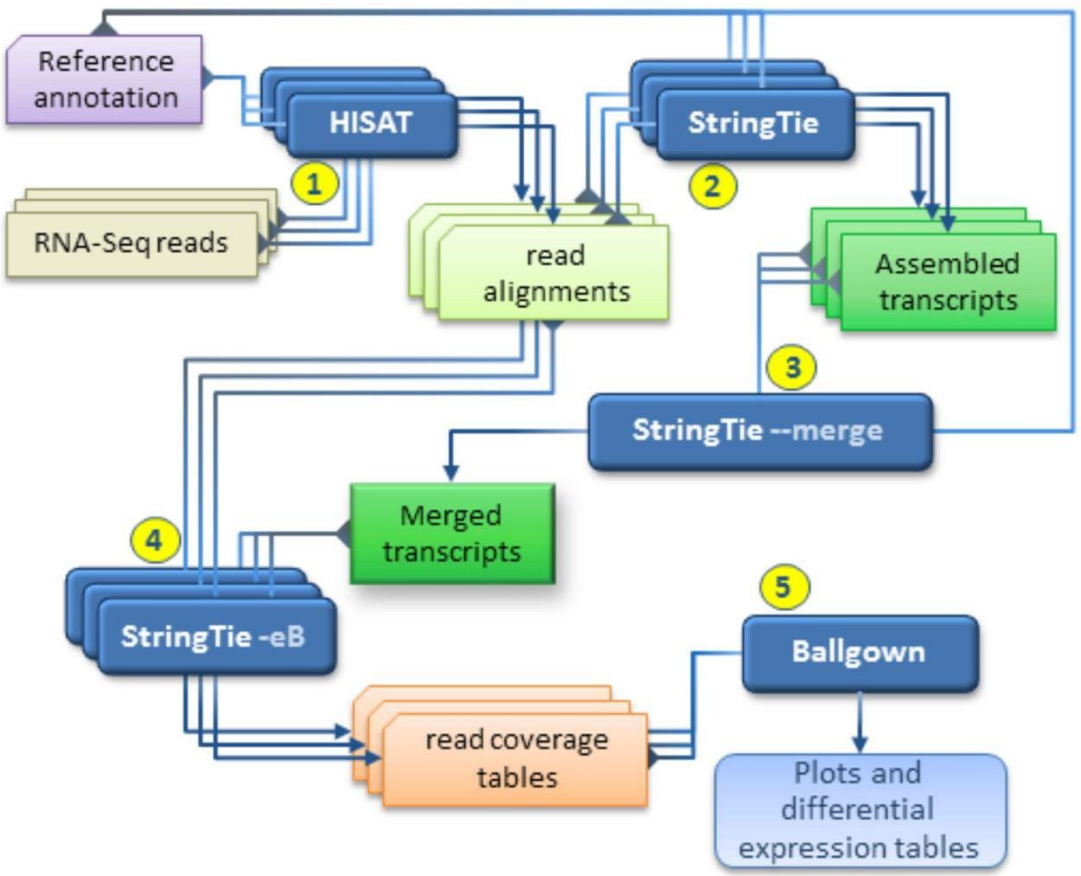
- For de novo or reference guided mode, we will rerun StringTie with the merged transcript assembly.





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

Useful tool: 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)

HTseq

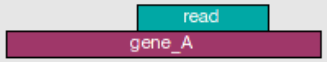
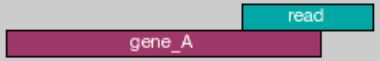
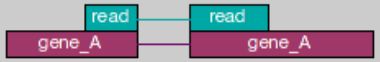
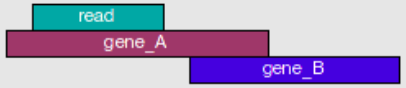
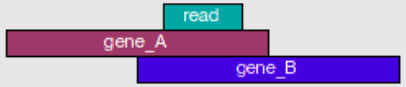
- Raw read counts for differential expression analysis
  - Assign reads/fragments to defined genes/transcripts, get “raw counts”
    - Transcript structures could still be defined by something like Stringtie

- 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. Useful for comparing across samples!

- Abundance estimation tool that calculates normalized count (FPKM, TPM): StringTie
- Abundance estimation tool that calculates raw count: HTseq

Alignment-free quantification

What is a k-mer?

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

1-mer

A
C
G
T

2-mer

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

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

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

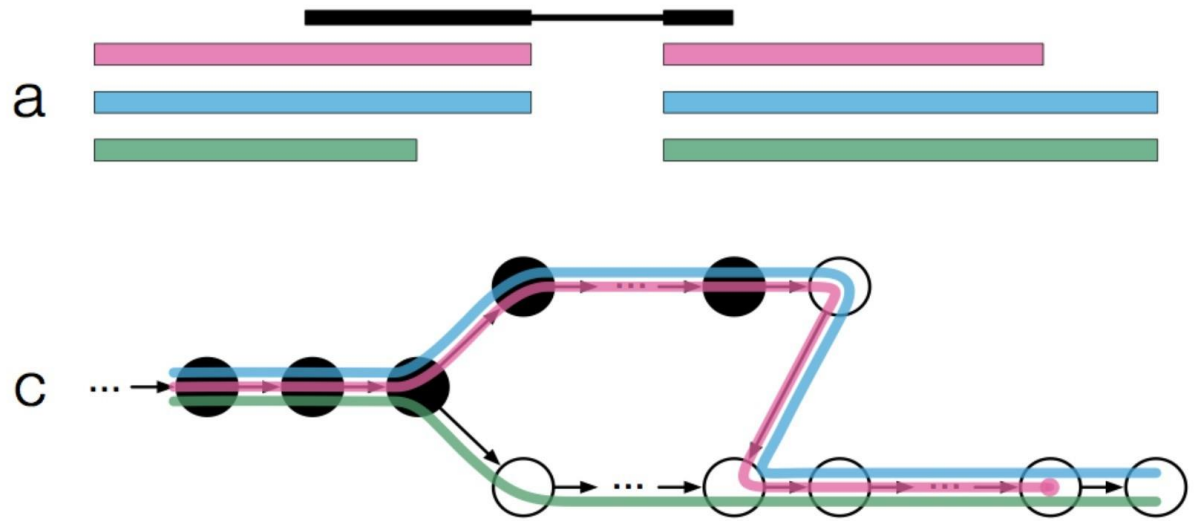
Alignment free approaches for transcript abundance

1. Obtain reference transcript sequences
   - e.g. Ensembl, Refseq, or GENCODE

2. Build a **k-mer index** of all of the k-mers in each transcript sequence
   - Store each k-mer and its position within the transcript. “hashing”

Alignment free approaches for transcript abundance

3. Count number of times each k-mer occurs within each RNAseq 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.



Bray, 2016 doi:10.1038/nbt.3519

https://tinyheero.github.io/2015/09/02/pseudoalignments-kallisto.html

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 (though a pseudo-bam can be created)
  - 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

Extra Info

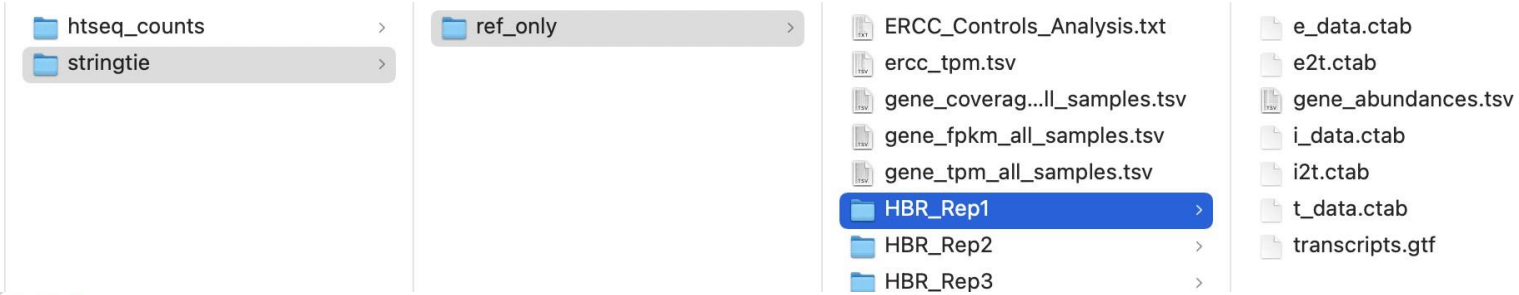
Stringtie outputs

- 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

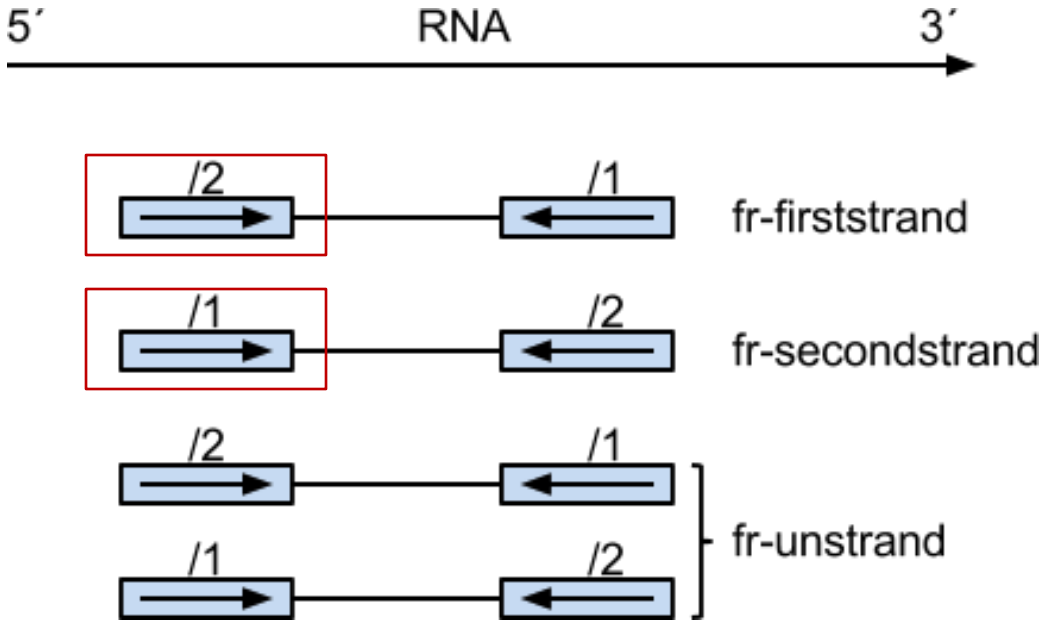
Stringtie outputs

- `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

Strandedness

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



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

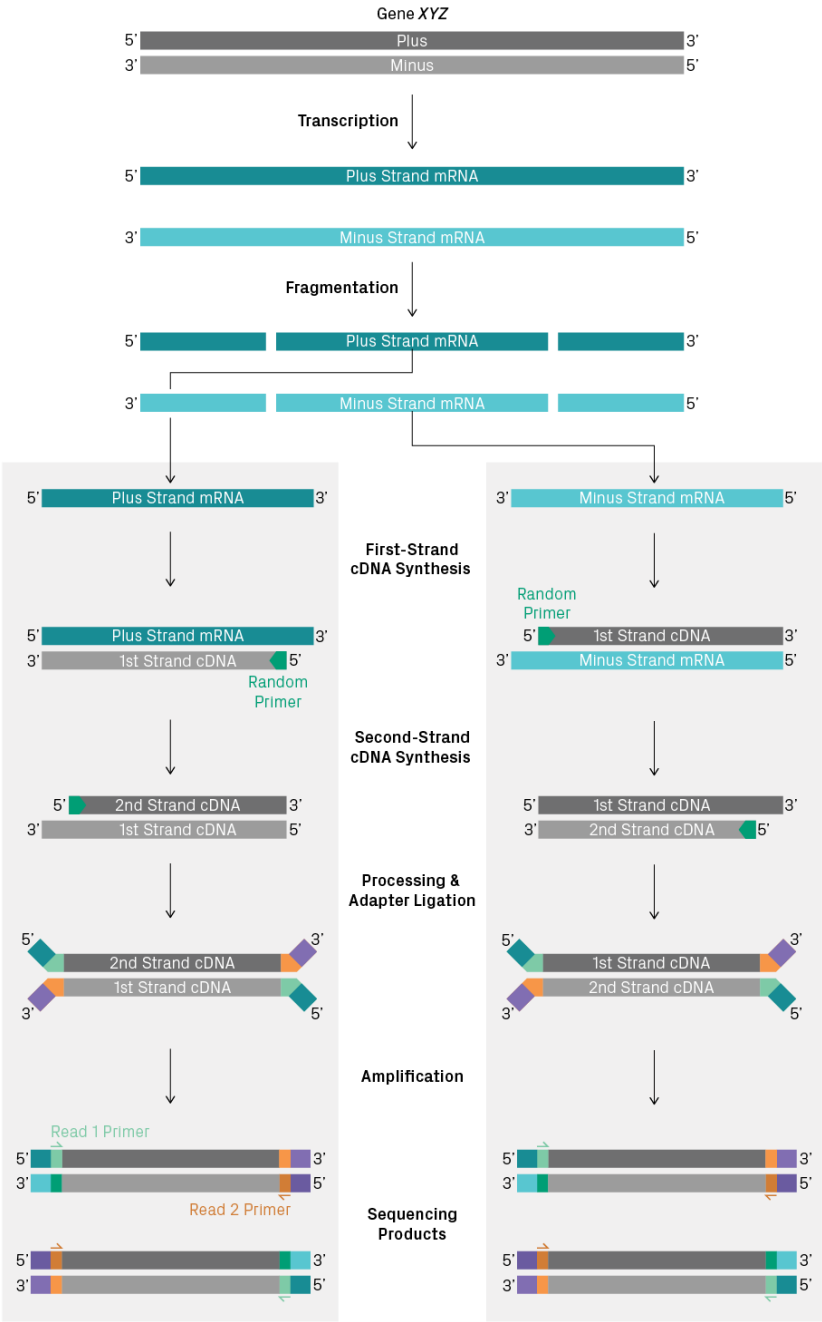
The first read (read 1) is from the original RNA strand

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.

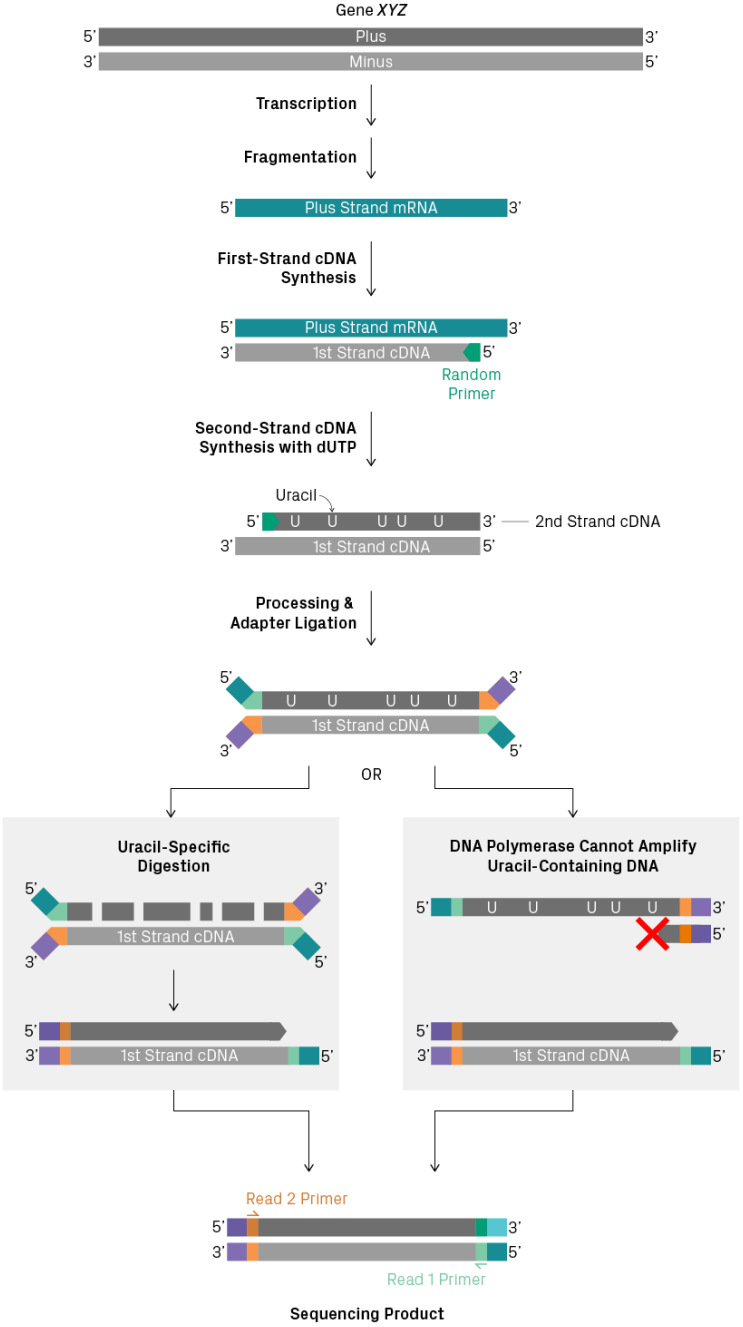
https://chipster.csc.fi/manual/library-type-summary.html#:~:text=second%2Dstrand%20%3D%20directional%2C%20where,is%20from%20the%20opposite%20strand.

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