מכון ויצמן למדע
WEIZMANN INSTITUTE OF SCIENCE

# Assembly and quantification of transcripts from RNA-Seq data

**Dena Leshkowitz,**

**Introduction to Deep-Sequencing Data Analysis 2018**

**Bioinformatics Unit, LSCF,WIS**

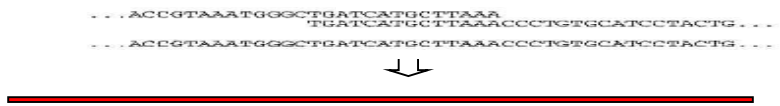
# Main Topics

- RNA-Seq - transcript level analysis
- RNA-Seq pipelines:

  Tophat-Cufflinks-Cuffdiff

  and more…

# From Sequences to Transcriptome Analysis




No Genome or Transcriptome

*De novo* assembly

RNA-Seq

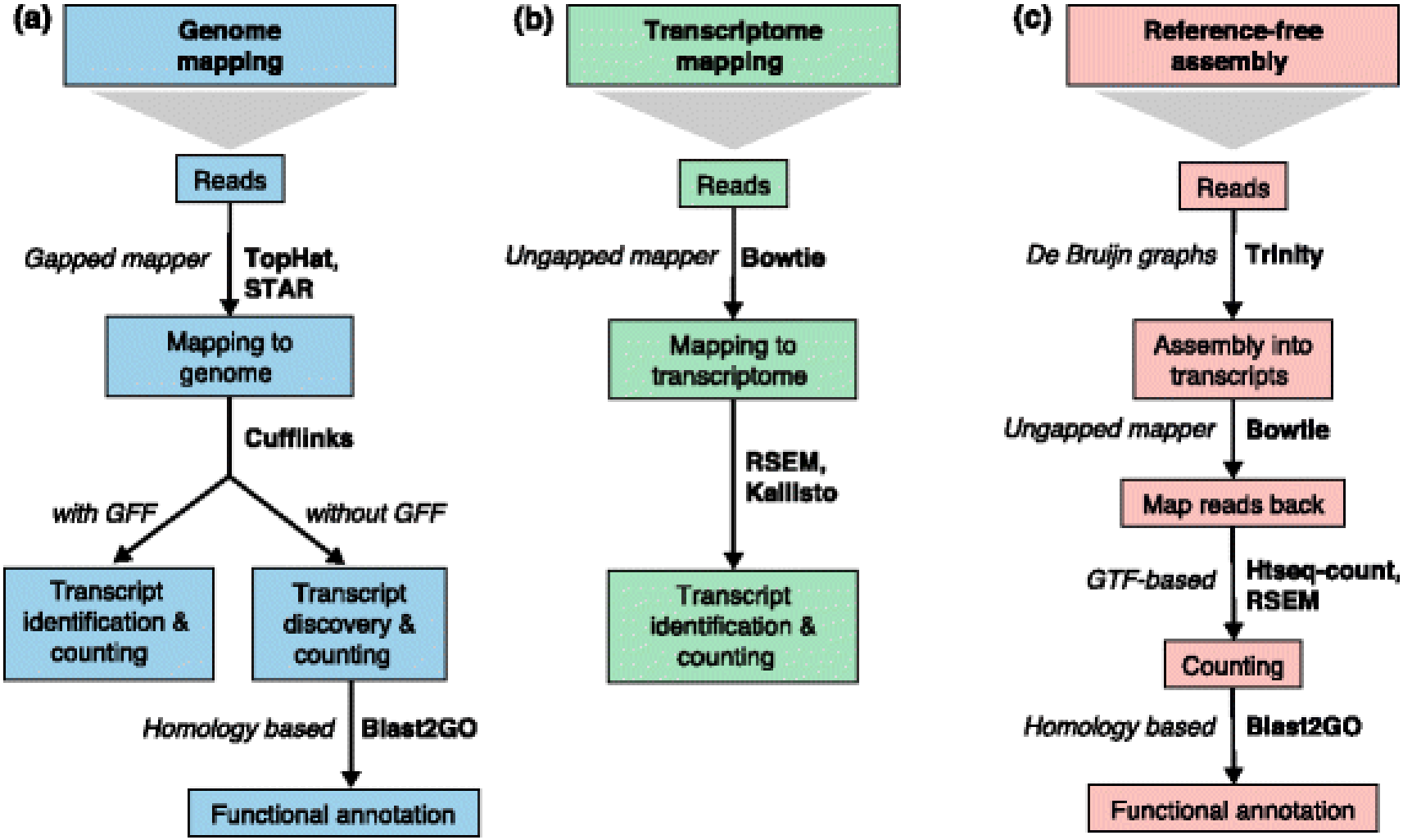
Mapping

Genome

Assemble transcripts

Transcriptome

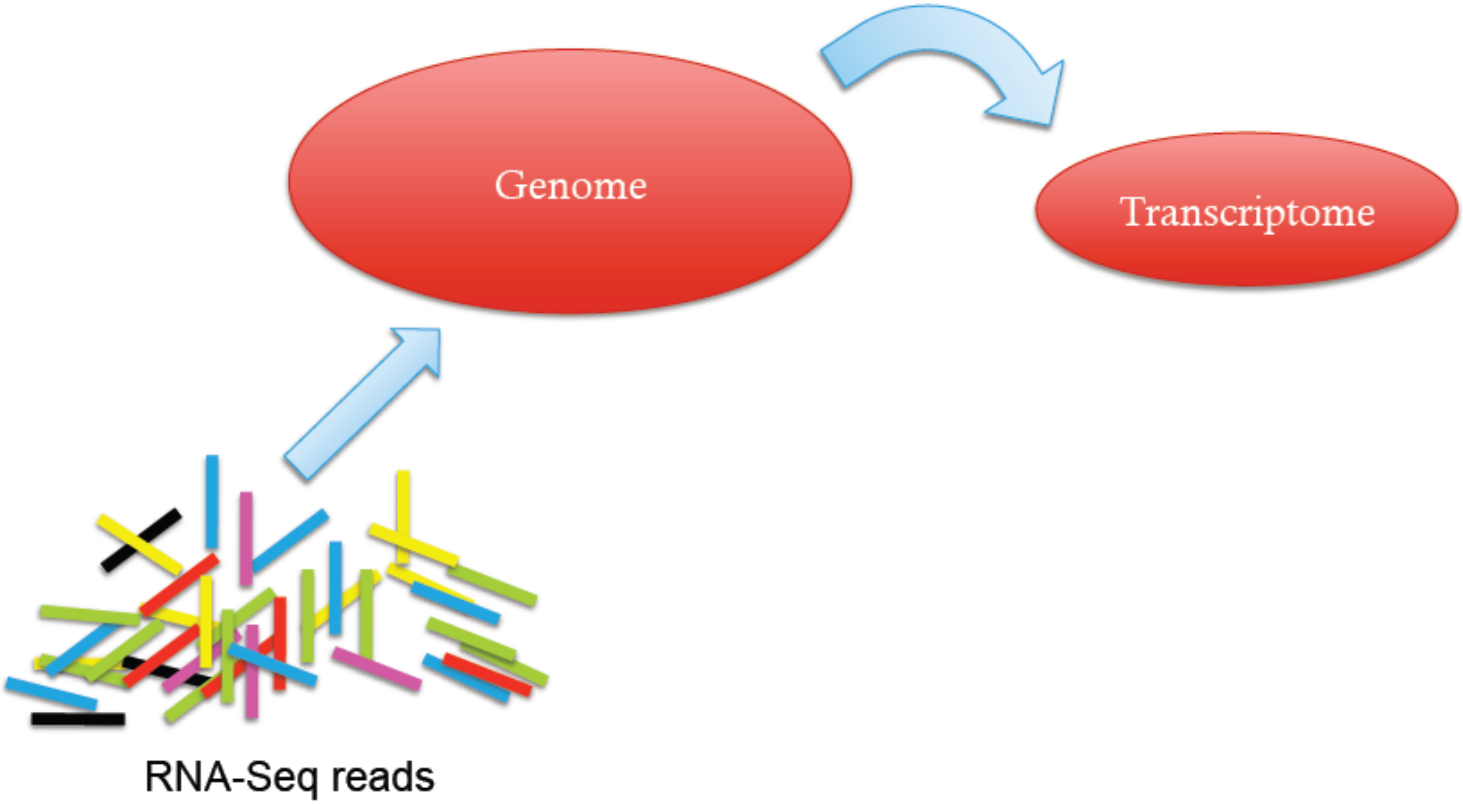
# Three Basic Strategies for Transcript Analysis from RNA-Seq Data




*Genome Biology* 2016 **17**:13

# Mapping Reads to the Genome

Goal: **identify** all transcripts and estimate relative amounts from RNA-Seq data



# The Tuxedo Tools


*NATURE BIOTECHNOLOGY* | RESEARCH | LETTER

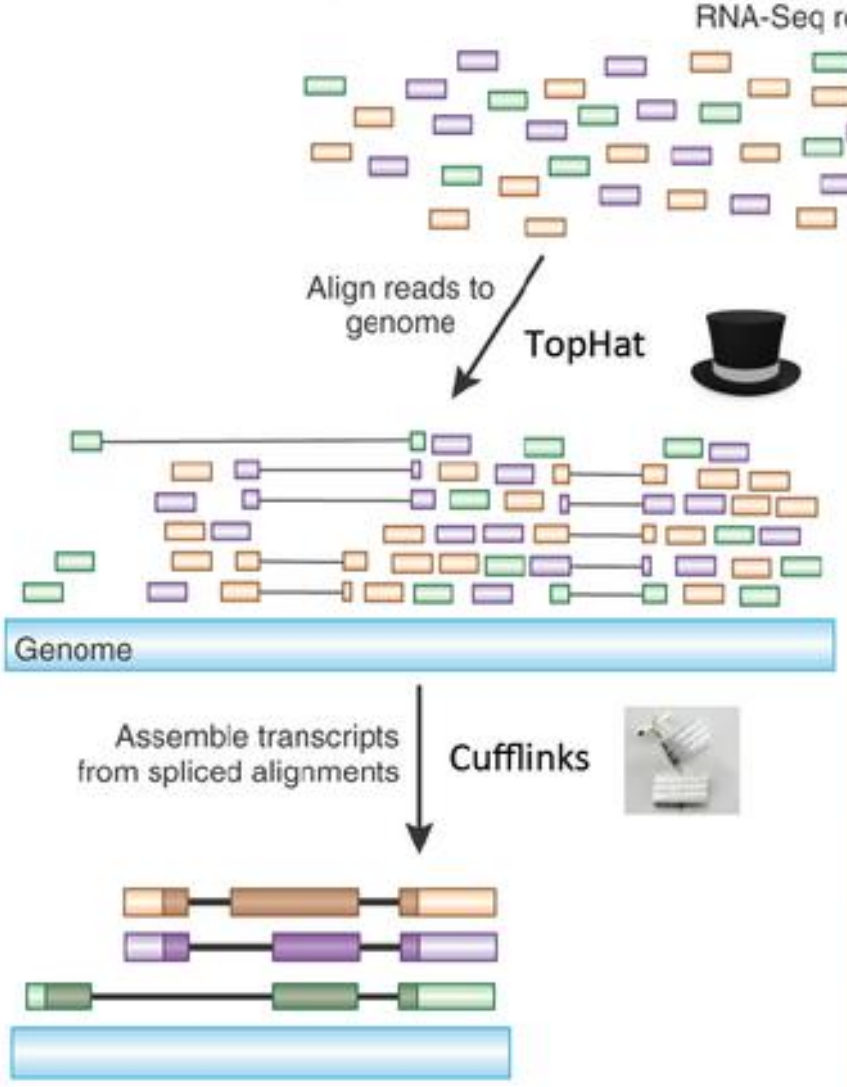
## Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation

**Cole Trapnell, Brian A Williams, Geo Pertea, Ali Mortazavi, Gordon Kwan, Marijke J van Baren, Steven L Salzberg, Barbara J Wold & Lior Pachter**

**Affiliations** | **Contributions** | **Corresponding author**

*Nature Biotechnology* **28**, 511–515 (2010) | doi:10.1038/nbt.1621
Received 02 February 2010 | Accepted 22 March 2010 | Published online 02 May 2010

# Transcript Reconstruction from RNA-Seq Reads




## The Tuxedo Suite:

End-to-end Genome-based RNA-Seq Analysis Software Package

NATURE PROTOCOLS | PROTOCOL

Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks

Cole Trapnell, Adam Roberts, Loyal Goff, Geo Pertea, Daehwan Kim, David R Kelley, Harold Pimentel, Steven L Salzberg, John L Rinn & Lior Pachter

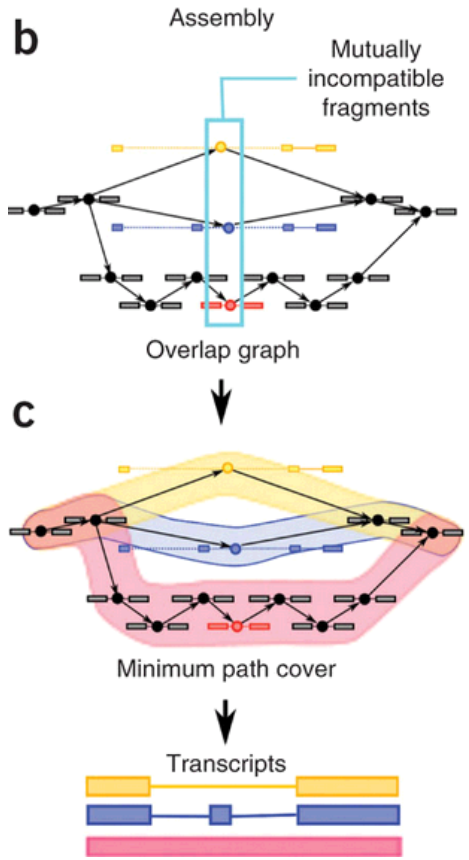
Affiliations | Contributions | Corresponding author

*Nature Protocols* 7, 562–578 (2012) | doi:10.1038/nprot.2012.016
Published online 01 March 2012

# Cufflinks Detects Novel and Known Transcripts

- "To test Cufflinks, we sequenced and analyzed >430 million paired 75-bp RNA-Seq reads from a mouse myoblast cell line over a differentiation time series. We detected 13,692 known transcripts and 3,724 previously unannotated ones, 62% of which are supported by independent expression data or by homologous genes in other species."

# Overview of Cufflinks




- Identify pairs of 'incompatible' fragments that must have originated from distinct spliced mRNA isoforms
- Fragments are connected in an 'overlap graph' when they are compatible and their alignments overlap in the genome
- Find minimum number of transcripts needed to 'explain' all the fragments

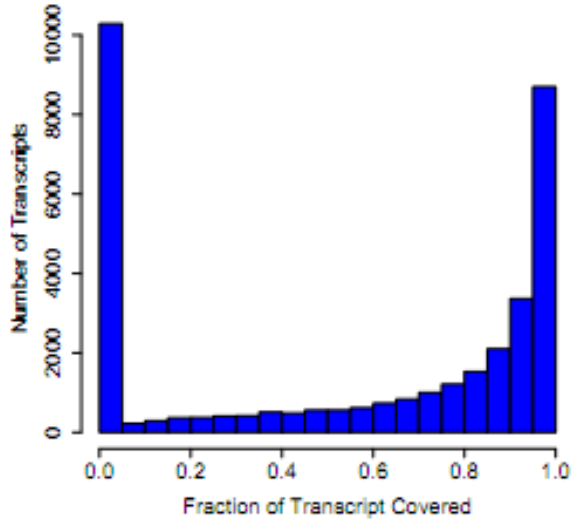

Transcripts and their abundances

*Trapnell et al. Nature Biotechnology* 28, 511-515 (2010)

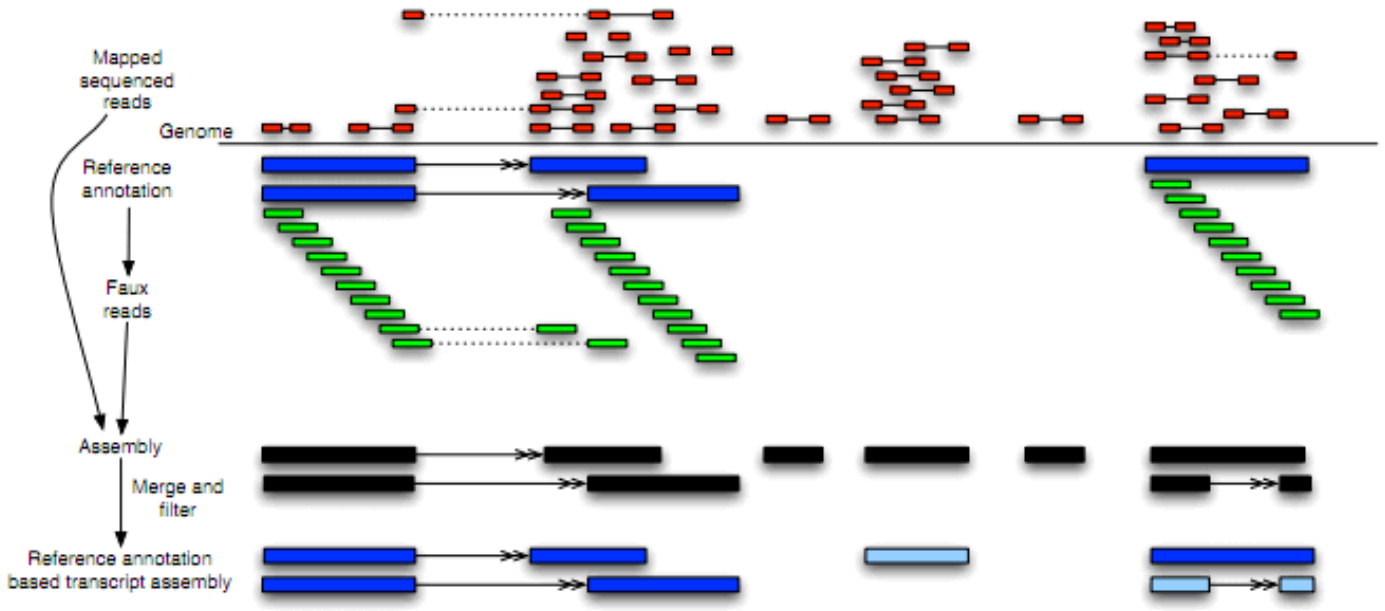
# Cufflinks -RABT

- Transcripts that are expressed in low level are represented by few reads and therefore only partially covered (64%).
- That means that naive assembly methods will fail to construct the majority of the transcripts

Roberts et al. Bioinformatics. 2011 Sep 1;27(17):2325-9.



# RABT: Reference Annotation Based Assembler (-g)




Faux reads tiling the transcripts are added to the real reads by cufflinks algorithm in the process of assembly

# RNA-Seq analysis on the transcript level

- Per sample map Reads to a Genome (using known annotation) (TopHat2)
- Per sample assemble transcripts (Cufflinks)
- **Merge assembled transcripts built for the various samples into one "combined transcripts.gtf" (Cuffmerge)**
- **Per sample quantify merged pool of transcripts (Cuffdiff)**
- **Normalize counts (Cuffdiff)**
- **Detect differentially expressed transcripts (Cuffdiff)**

# Gene transfer format (GTF)

GTF file is used to hold information about gene structure. It is a tab-delimited text format

```
chr1    unknown exon    3214482 3216968 .       -       .       gene_id "Xkr4"; gene_name "Xkr4"; p_id "P15391"; transcript_id "NM_001011874"; tss_id "TSS27105";
chr1    unknown stop_codon      3216022 3216024 .       -       .       gene_id "Xkr4"; gene_name "Xkr4"; p_id "P15391"; transcript_id "NM_001011874"; tss_id "TSS27105";
chr1    unknown CDS     3216025 3216968 .       -       2       gene_id "Xkr4"; gene_name "Xkr4"; p_id "P15391"; transcript_id "NM_001011874"; tss_id "TSS27105";
chr1    unknown CDS     3421702 3421901 .       -       1       gene_id "Xkr4"; gene_name "Xkr4"; p_id "P15391"; transcript_id "NM_001011874"; tss_id "TSS27105";
chr1    unknown exon    3421702 3421901 .       -       .       gene_id "Xkr4"; gene_name "Xkr4"; p_id "P15391"; transcript_id "NM_001011874"; tss_id "TSS27105";
chr1    unknown CDS     3670552 3671348 .       -       0       gene_id "Xkr4"; gene_name "Xkr4"; p_id "P15391"; transcript_id "NM_001011874"; tss_id "TSS27105";
chr1    unknown exon    3670552 3671498 .       -       .       gene_id "Xkr4"; gene_name "Xkr4"; p_id "P15391"; transcript_id "NM_001011874"; tss_id "TSS27105";
chr1    unknown start_codon     3671346 3671348 .       -       .       gene_id "Xkr4"; gene_name "Xkr4"; p_id "P15391"; transcript_id "NM_001011874"; tss_id "TSS27105";
chr1    unknown exon    4290846 4293012 .       -       .       gene_id "Rp1"; gene_name "Rp1"; p_id "P17361"; transcript_id "NM_001195662"; tss_id "TSS6138";
chr1    unknown stop_codon      4292981 4292983 .       -       .       gene_id "Rp1"; gene_name "Rp1"; p_id "P17361"; transcript_id "NM_001195662"; tss_id "TSS6138";
```

## GTF format

GTF (Gene Transfer Format) is a refinement to GFF that tightens the specification. The first eight GTF fields are the same as GFF. The *group* field has been expanded into a list of *attribute* Each attribute consists of a type/value pair. Attributes must end in a semi-colon, and be separated from any following attribute by exactly one space.

The attribute list must begin with the two mandatory attributes:

- **gene_id *value*** - A globally unique identifier for the genomic source of the sequence.
- **transcript_id *value*** - A globally unique identifier for the predicted transcript.

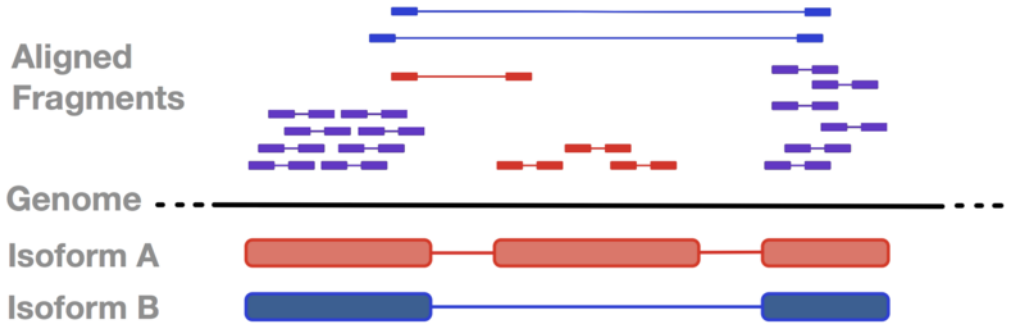
***Example:***
Here is an example of the ninth field in a GTF data line:

```
gene_id "Em:U62317.C22.6.mRNA"; transcript_id "Em:U62317.C22.6.mRNA"; exon_number 1
```

The Genome Browser groups together GTF lines that have the same *transcript_id* value. It only looks at features of type *exon* and *CDS*.

For more information on this format, see http://mblab.wustl.edu/GTF2.html. If you would like to obtain browser data in GTF format, please refer to Genes in gtf or gff format on the wiki.

# Estimate Transcripts Abundance



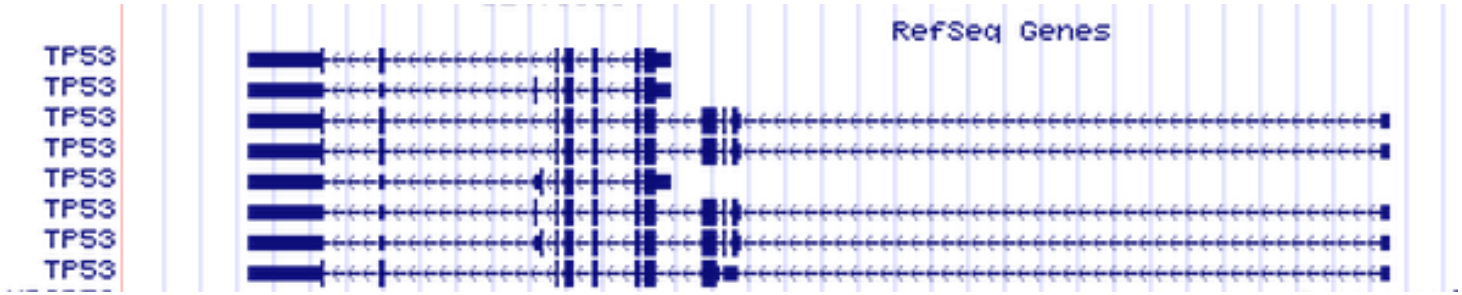

Trapnell et al. Nat Biotechnol. 2010 May;28(5):511-5.

From which transcript did the purple reads originate?

# Align to Transcriptome Quantification Problem

- We encounter the same problem when we align to a trasncriptome
- Counting the number of sequences that map uniquely to transcripts results in false estimates of alternatively spliced transcripts

Human p53 locus

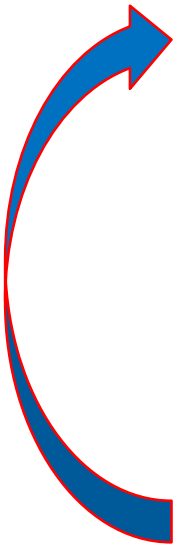



- Computational challenge- use reads that map ambiguously between isoforms and genes (EM algorithm)

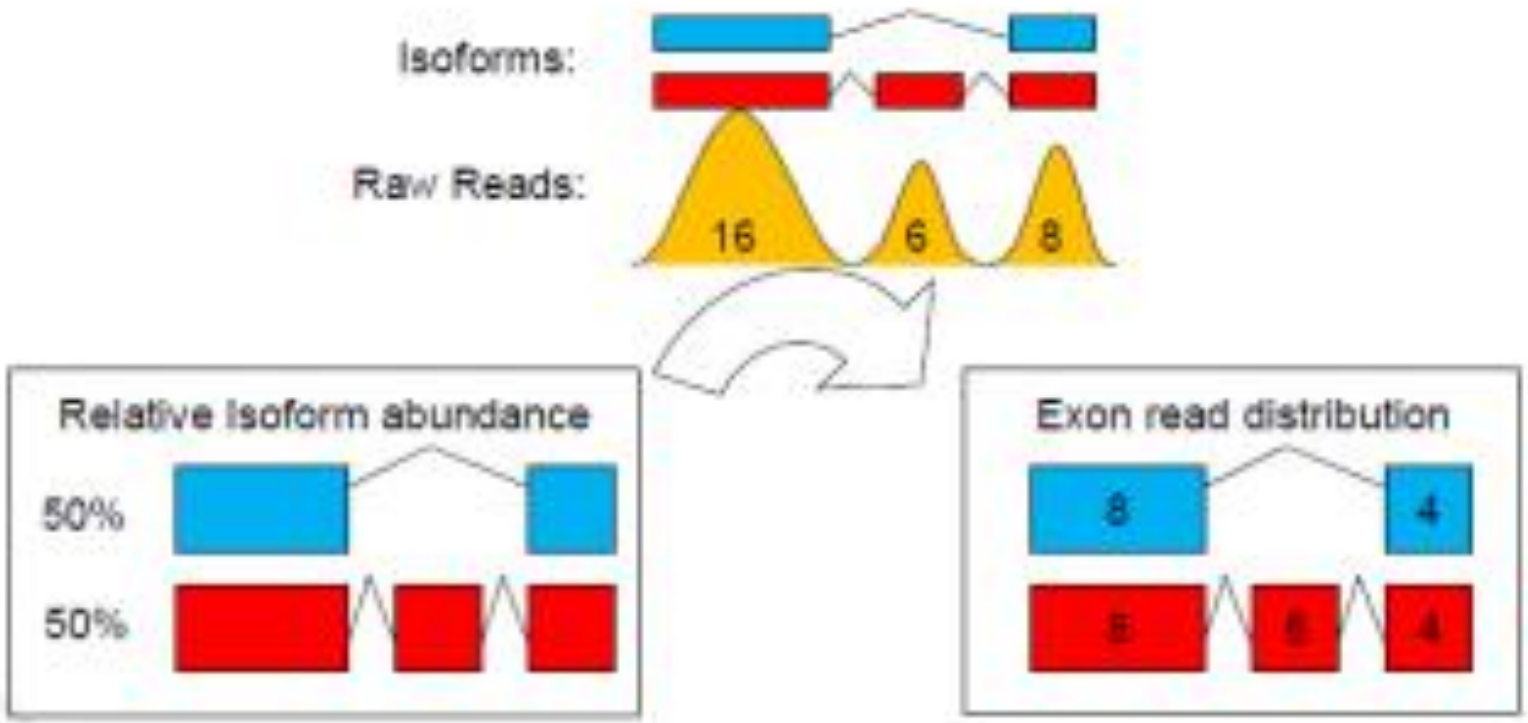
# Isoform Expression Quantification
# Expectation Maximization Algorithm

## Computing relative abundance of transcripts:

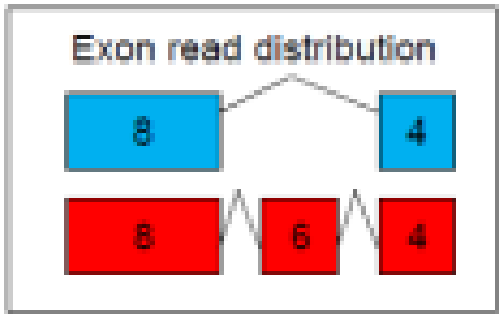
- Step 1- Assume isoforms are equally abundant (in case of 2 transcripts abundance is: $\frac{1}{2}$ and $\frac{1}{2}$)
- Step 2 - Distribute the reads to the isoforms based on the abundance
- Step 3 - Recalculate the isoforms abundance based on the reads counts and isoforms length
- Step 4- If abundance has changed go back to step 2 otherwise stop



# 1st step E/M algorithm



# Calculating Abundance after 1st EM Cycle




| Total reads | Length |
|---|---|
| 12 | 300 |
| 18 | 400 |

Exon length (bp) 200 100 100

The red transcript relative abundance after the first cycle:

$$p_{red} = \frac{counts_{red} / length_{red}}{counts_{red} / length_{red} + counts_{blue} / length_{blue}}$$

$p_{red}$ = 18/400 / (12/300 + 18/400) = 0.53
$p_{blue}$ = 12/300 / (12/300 + 18/400) = 0.47

# Relative Abundance Calculation Using 100 Iterations of EM

| | starting relative proportion (p) | read counts | New proportion after iteration (p) | | starting relative proportion (p) | read counts | New proportion after iteration (p) | | Iteration # |
|---|---|---|---|---|---|---|---|---|---|
| Blue | 0.5 | 12 | 0.470588 | Red | 0.5 | 18 | 0.529412 | | 1 |
| | | 11.29412 | 0.445993 | | | 18.70588 | 0.554007 | | 2 |
| | | 10.70383 | 0.425161 | | | 19.29617 | 0.574839 | | 3 |
| | | 10.20386 | 0.407324 | | | 19.79614 | 0.592676 | | 4 |
| | | 9.775778 | 0.39191 | | | 20.22422 | 0.60809 | | 5 |
| | | 9.405837 | 0.378482 | | | 20.59416 | 0.621518 | | 6 |
| | | 9.083574 | 0.366704 | | | 20.91643 | 0.633296 | | 7 |
| | | 8.800885 | 0.356308 | | | 21.19911 | 0.643692 | | 8 |
| | | 8.551391 | 0.347084 | | | 21.44861 | 0.652916 | | 9 |
| | | 8.330004 | 0.338859 | | | 21.67 | 0.661141 | | 10 |
| | | 6.00743 | 0.25029 | | | 23.99257 | 0.74971 | | 94 |
| | | 6.006965 | 0.250272 | | | 23.99303 | 0.749728 | | 95 |
| | | 6.006529 | 0.250255 | | | 23.99347 | 0.749745 | | 96 |
| | | 6.006121 | 0.250239 | | | 23.99388 | 0.749761 | | 97 |
| | | 6.005738 | 0.250224 | | | 23.99426 | 0.749776 | | 98 |
| | | 6.005379 | 0.25021 | | | 23.99462 | 0.74979 | | 99 |
| | | 6.005042 | **0.2502** | | | 23.99496 | **0.7498** | | 100 |

Blue 25%

Red 75%

# Normalized Expression Values

**Normalize :**

- **Between samples accounting for number of reads**
- **Between genes/transcripts accounting for different length**

$$FPKMi = 10^6 \times 10^3 \times \frac{Ci}{NLi}$$

**Fragments (Reads) Per Kilobase of exon per Million mapped fragments**

**Nat Methods. 2008, Mapping and quantifying mammalian transcriptomes by RNA-Seq. Mortazavi A et al.**

C= The number of fragments mapped onto the transcript exons

N= Total number of (mapped) fragments in the experiment

L= The length of the transcript (sum of exons)

Problem the sum of FPKM for different samples is not necessarily the same

# TPM (Transcripts per million)

- Divide the read counts by the length of each gene in kilobases. This gives you reads per kilobase (RPK).
- Count up all the RPK values in a sample and divide this number by 1,000,000. This is your "per million" scaling factor.
- Divide the RPK values by the "per million" scaling factor. This gives you TPM.

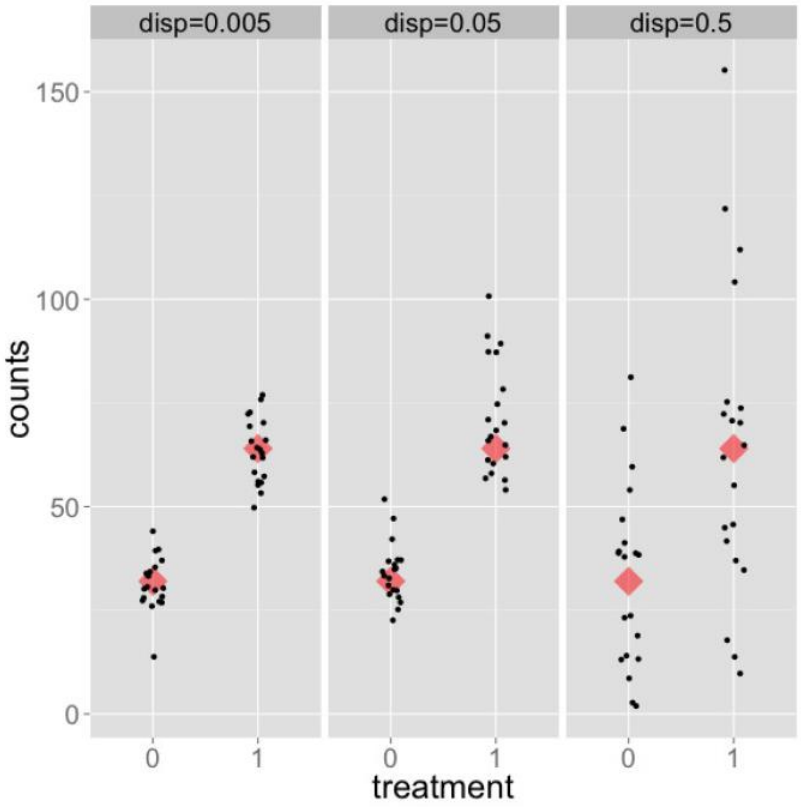
**TPM sum in all samples is a million, better way to normalize.**

# Metrics for quantifying gene expression levels

- RPKM
  - Reads Per Kilobase per Million mapped reads
  - Normalize relative to sequencing depth and gene length
- FPKM
  - Similar to RPKM but count DNA fragments instead of reads
  - Used in paired end RNA-Seq experiments to avoid bias
- TPM
  - Transcripts Per Million
  - Normalize for gene length, then normalize by sequencing depth

# Determining Differentially Expressed Genes and Transcripts

Discover transcripts showing different average expression levels across two groups




http://www.molgen.mpg.de/1242892/rnaseq.pdf

The statistical model for finding differential expressed transcripts or genes depends on whether we have biological replicates. The advantage of having many replicates allows to learn about the biological variation within the conditions tested.

# Negative Binomial Distribution

- Cuffdiff tests for differential expression by the use of negative binomial generalized linear models

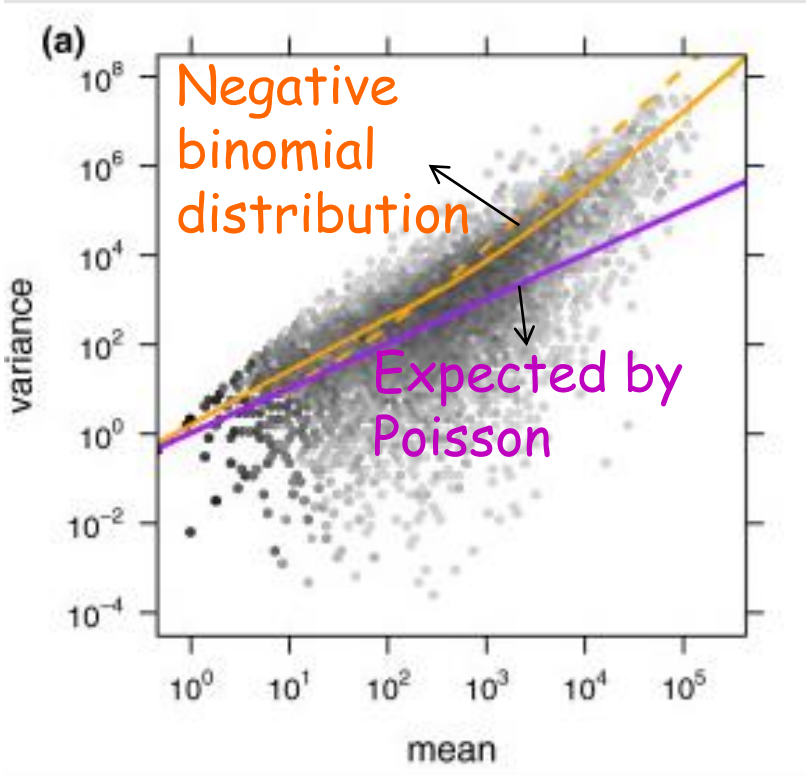
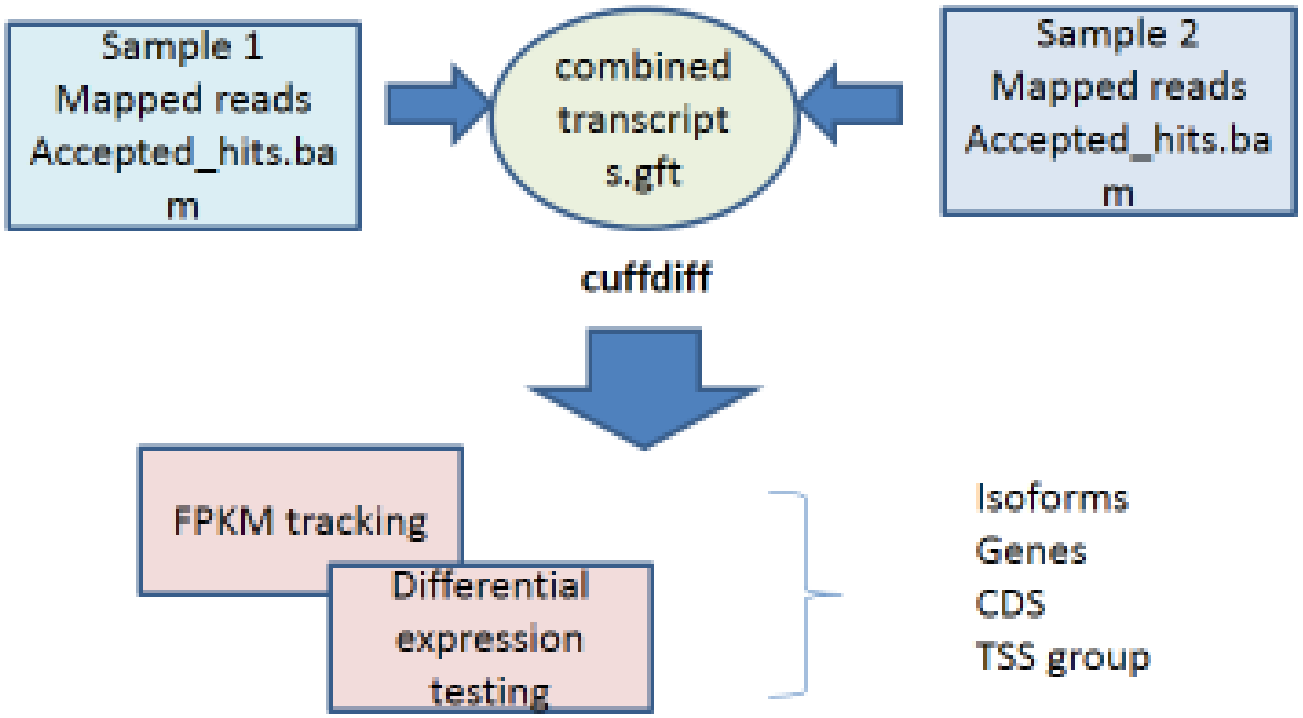



Fig. 1 from Anders & Huber, 2010: Dependence

# Cuffdiff

- Quantifies transcripts and finds significant changes in transcript expression



# The Benefit of Longer and PE Reads

- Reads mapping to junctions
  - With longer reads we will have more reads spanning exons

- Paired end reads

  Knowing both ends of a fragment and an approximation of fragment size we can better determine the transcript from which it was derived.

# Experimental Design

## Mammalian tissue

## Liu Y. et al., 2014; ENCODE 2011 RNA-Seq

| | | |
|---|---|---|
| Differential gene expression profiling | 10-25M | 50 base single-end |
| Alternative splicing | 50-100M | 100 base paired-end |
| Allele specific expression | 50-100M | 100 base paired-end |
| De novo assembly | >100M | 100 base paired-end |

# Assessment of transcript reconstruction methods for RNA-seq

Tamara Steijger, Josep F Abril, Pär G Engström, Felix Kokocinski, The RGASP Consortium, Tim J Hubbard, Roderic Guigó, Jennifer Harrow & Paul Bertone

Affiliations | Contributions

*Nature Methods* **10**, 1177–1184 (2013) | doi:10.1038/nmeth.2714

Received 31 March 2013 | Accepted 23 September 2013 | Published online 03 November 2013

**Example of transcript calls and expression-level estimates**

Results were evaluated from methods based on genome alignments (Augustus, Cufflinks, Exonerate, GSTRUCT, iReckon, mGene, mTim, NextGeneid, SLIDE, Transomics, Trembly and Tromer) as well as *de novo* assembly (Oases and Velvet).
Programs were run without genome annotation, aside from iReckon and SLIDE

No method achieved even 60% accuracy for transcript reconstruction in human

# StringTie enables improved reconstruction of a transcriptome from RNA-seq reads

**Mihaela Pertea, Geo M Pertea, Corina M Antonescu, Tsung-Cheng Chang, Joshua T Mendell & Steven L Salzberg**

**Affiliations | Contributions | Corresponding author**

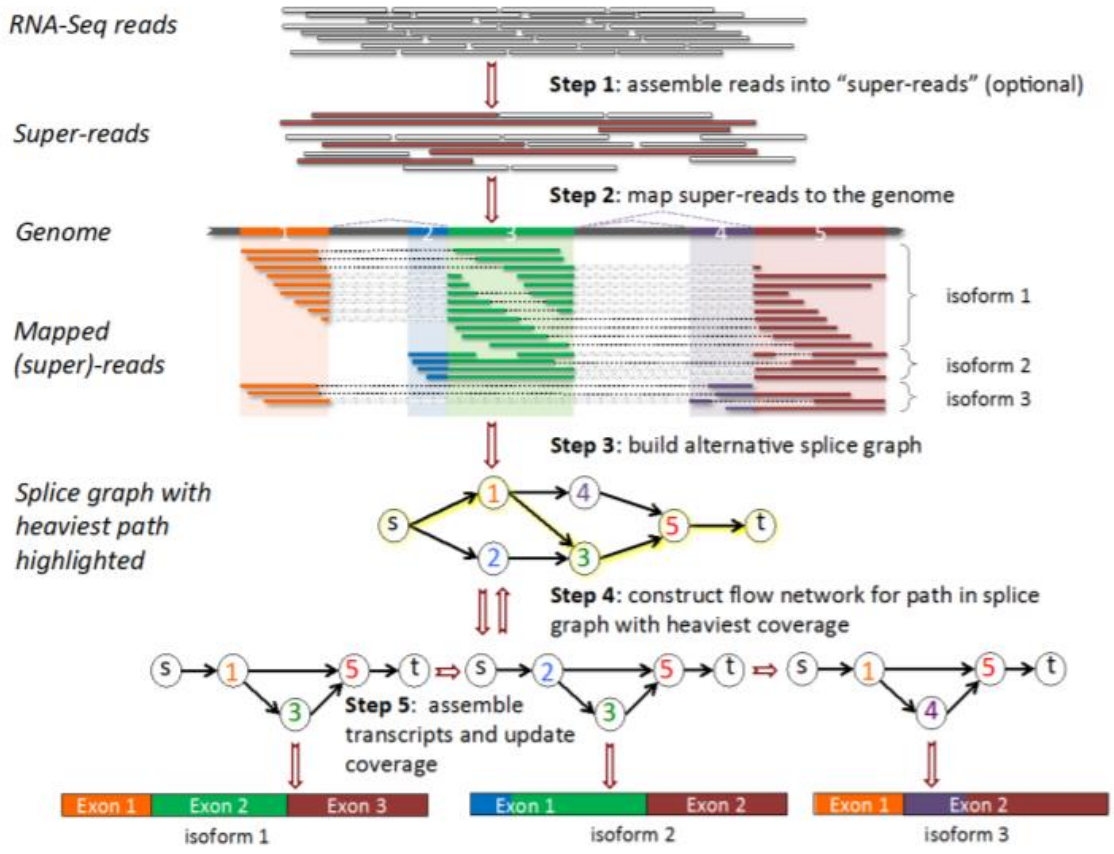
*Nature Biotechnology* **33**, 290–295 (2015) | doi:10.1038/nbt.3122

Received 15 April 2014 | Accepted 09 December 2014 | Published online 18 February 2015

- **Faster**
- **More complete and accurate reconstructions and better estimates of expression levels**

# Stringtie Steps:

1. Pre-assembles paired reads – super reads
2. Uses mapping of reads to the reference genome to build an alternative overlap graph
3. The path with the heaviest coverage is used to build a transcript
4. The assembly of transcripts and estimation of expression level is done simultaneously
5. The assembled transcript is removed from the splice graph and step 3-4 repeated until there is no more transcripts




**Supplementary Figure 12.** The StringTie algorithm: RNA-seq reads are assembled into super-reads (Step 1) and then super-reads plus un-assembled reads are mapped to the genome (Step 2). In Step 3, mapped reads and super-reads are used to build an alternative splice graph. We use the path from source (s) to sink (t) with the heaviest coverage to build a flow network corresponding to the transcript represented by that path (Step 4). The maximum flow in this network represents the coverage of one assembled transcript, which is removed from the splice graph (Step 5). Steps 4 and 5 are repeated until no more transcripts can be assembled.

# the next-generation "Tuxedo" tools

**Bowtie2**

Fast alignment

**HISAT**

Spliced alignment

**Ballgown**

- Differential expression

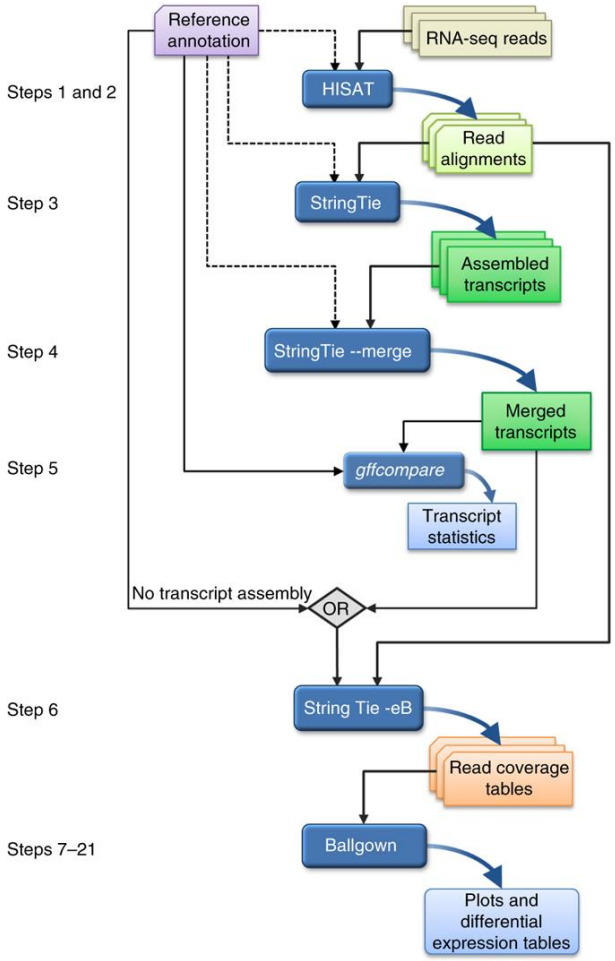
**StringTie**

- Transcript assembly
- Quantitation

Steven Salzberg - Transcriptome Assembly Computational Challenges of Next Generation Sequence Data
https://www.youtube.com/watch?v=2qGiw4MRK3c

# RNA-Seq analysis on the transcript level

- Per sample map reads to a Genome (using known annotation) (STAR, HISAT)
- Per sample assemble transcripts (StringTie)
- Merge assembled transcripts built for the various samples into one "database" (StringTie)
- Per sample quantify merged pool of transcripts (StringTie)
- Normalize counts (Ballgown)
- Detect differentially expressed transcripts (Ballgown)

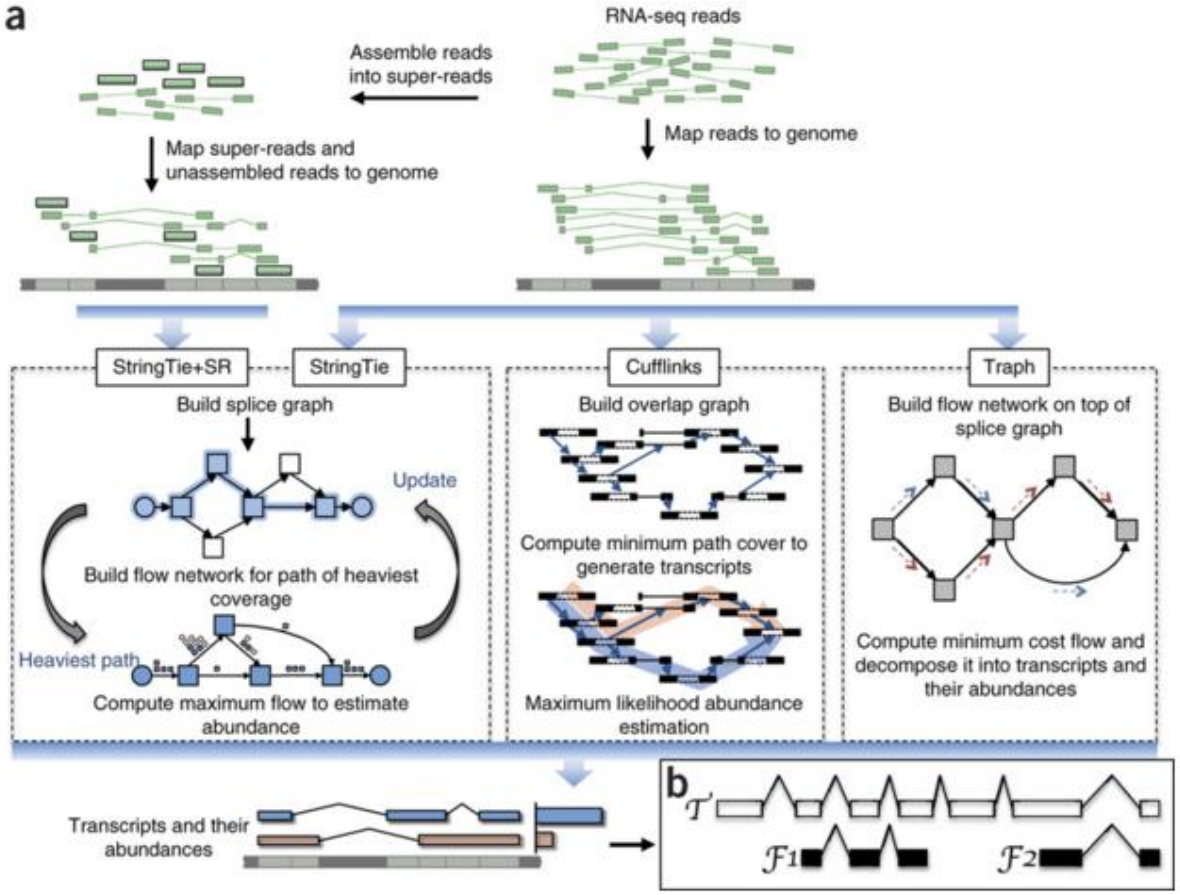


# Todays Exercise

- Run Stringtie
- Use Genome Browser IGV to analyse assembled transcript outputs

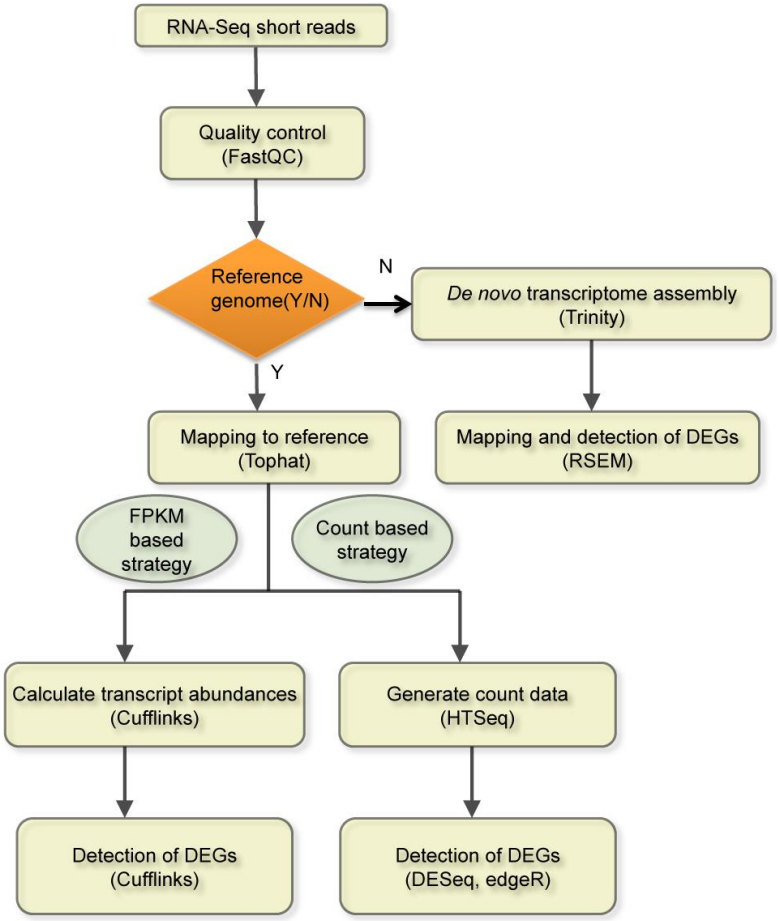
THANKS

Questions???




Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. (2015) **StringTie enables improved reconstruction of a transcriptome from RNA-seq reads.** *Nat Biotechnol* [Epub ahead of print]. [article]

# RNA-Seq pipelines




PLoS ONE 2014 9(8): e103207.

# SAM Alignment Quality

### 1.4 The alignment section: mandatory fields

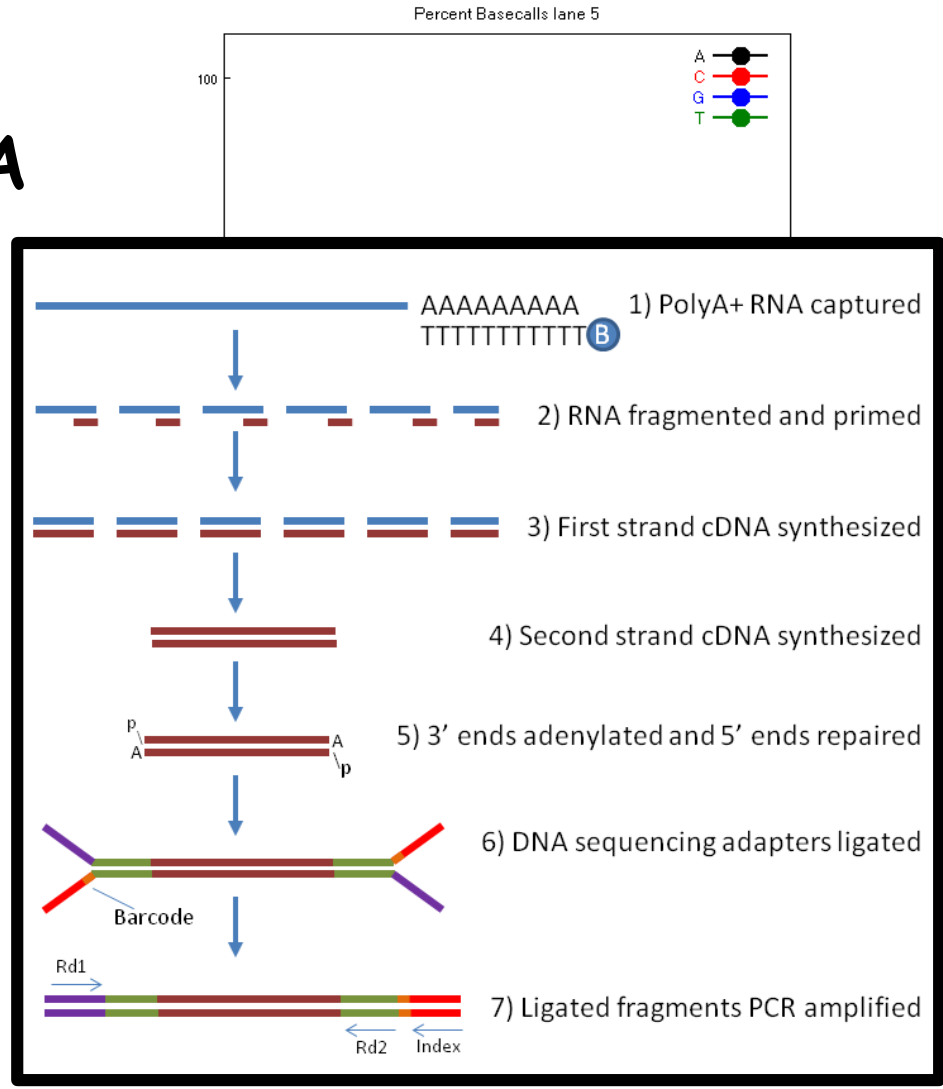
In the SAM format, each alignment line typically represents the linear alignment of a segment. Each line has 11 mandatory fields. These fields always appear in the same order and must be present, but their values can be '0' or '*' (depending on the field) if the corresponding information is unavailable. The following table gives an overview of the mandatory fields in the SAM format:

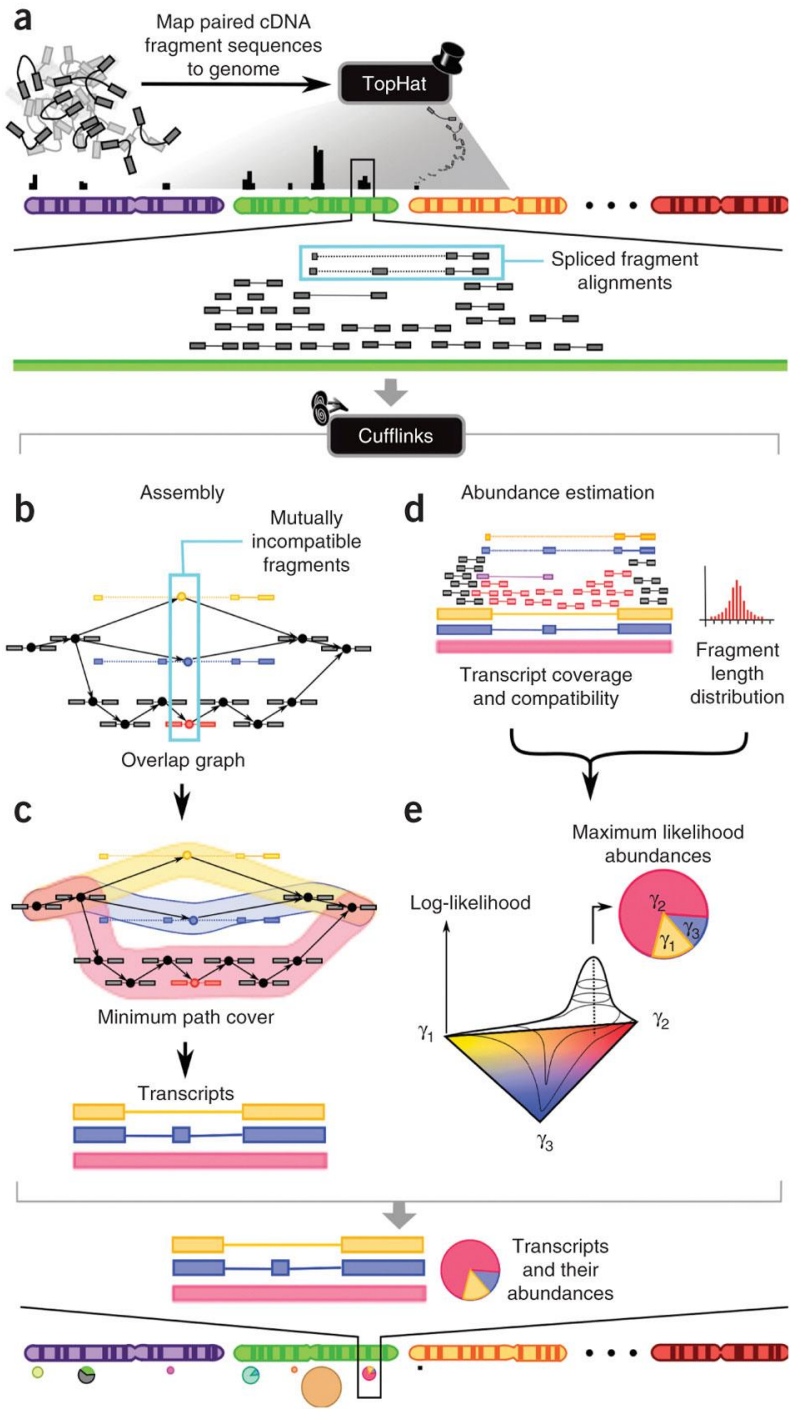
| Col | Field | Type | Regexp/Range | Brief description |
|---|---|---|---|---|
| 1 | QNAME | String | `[!-?A-~]{1,254}` | Query template NAME |
| 2 | FLAG | Int | $[0,2^{16}-1]$ | bitwise FLAG |
| 3 | RNAME | String | `\*|[!-()+-<>-~][!-~]*` | Reference sequence NAME |
| 4 | POS | Int | $[0,2^{31}-1]$ | 1-based leftmost mapping POSition |
| 5 | MAPQ | Int | $[0,2^{8}-1]$ | MAPping Quality |
| 6 | CIGAR | String | `\*|([0-9]+[MIDNSHPX=])+` | CIGAR string |
| 7 | RNEXT | String | `\*|=|[!-()+-<>-~][!-~]*` | Ref. name of the mate/next read |
| 8 | PNEXT | Int | $[0,2^{31}-1]$ | Position of the mate/next read |
| 9 | TLEN | Int | $[-2^{31}+1,2^{31}-1]$ | observed Template LENgth |
| 10 | SEQ | String | `\*|[A-Za-z=.]+` | segment SEQuence |
| 11 | QUAL | String | `[!-~]+` | ASCII of Phred-scaled base QUALity+33 |

- **MAPQ: MAPping Quality.**
- **−10 log10 Pr{mapping position is wrong}** rounded to the nearest integer.
- We say an alignment is unique if it has a much higher alignment score than all the other possible alignments. The bigger the gap between the best alignment's score and the second-best alignment's score, the more unique the best alignment, and the higher its mapping quality should be
- **10 is a common threshold that means that there is 1 to 10 chance that the read originated from somewhere else.**
- **A value 255 indicates that the mapping quality is not available.**

# Cufflinks Bias Correction

The random priming in the process of cDNA creation causes a positional preferred location for sequencing at the beginning of the transcript



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