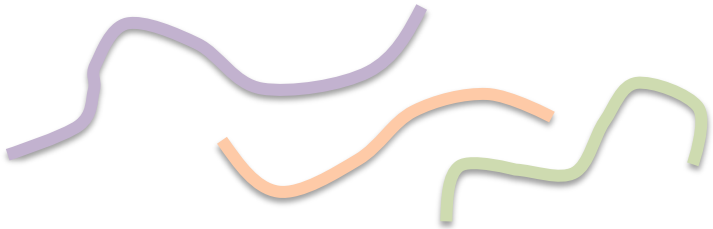
RNA-Seq Empowers Transcriptome Studies

Extract RNA, convert to cDNA

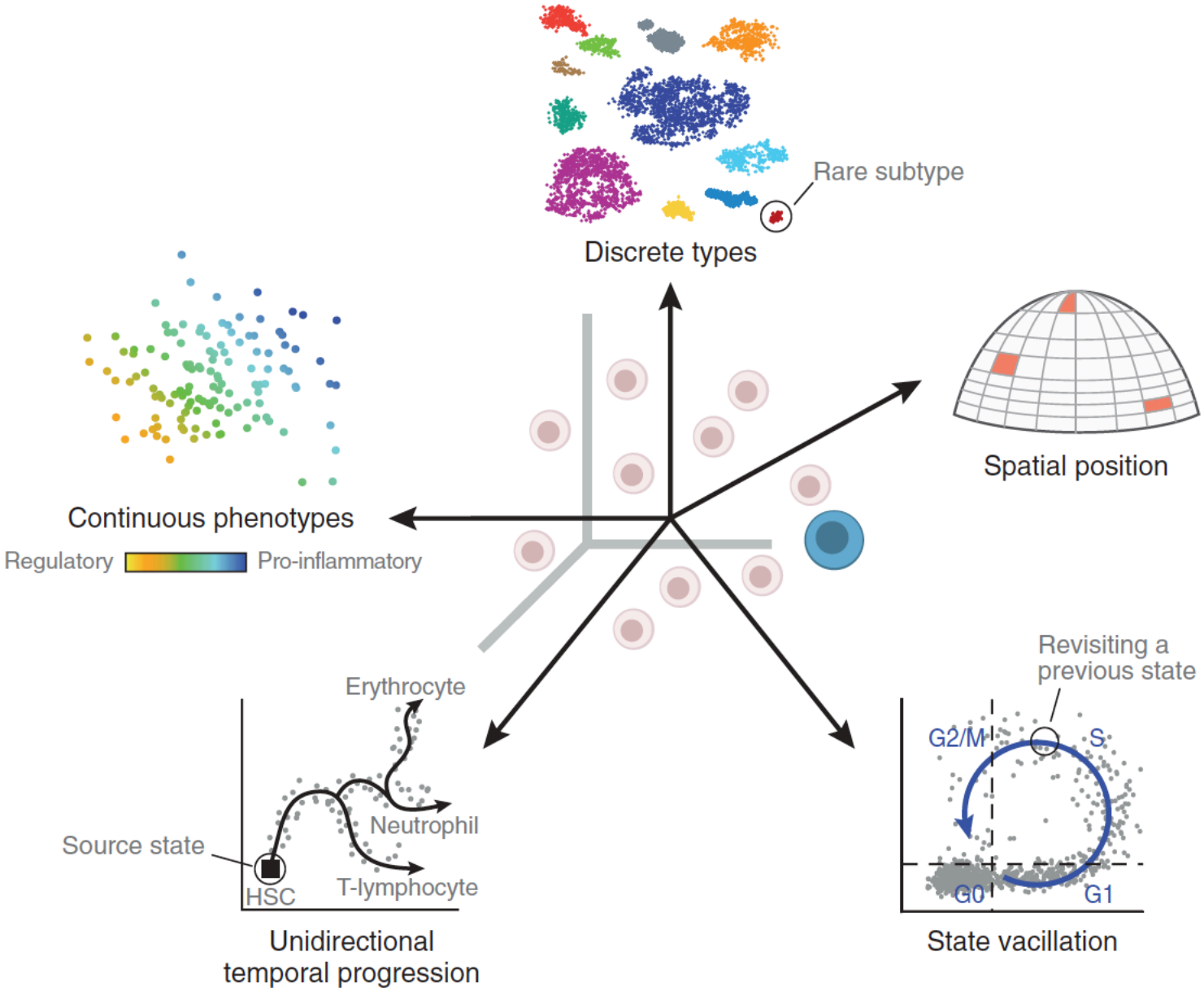


Next-gen Sequencer
(pick your favorite)

RNA-Seq Empowers Many Facets of Biological Investigations

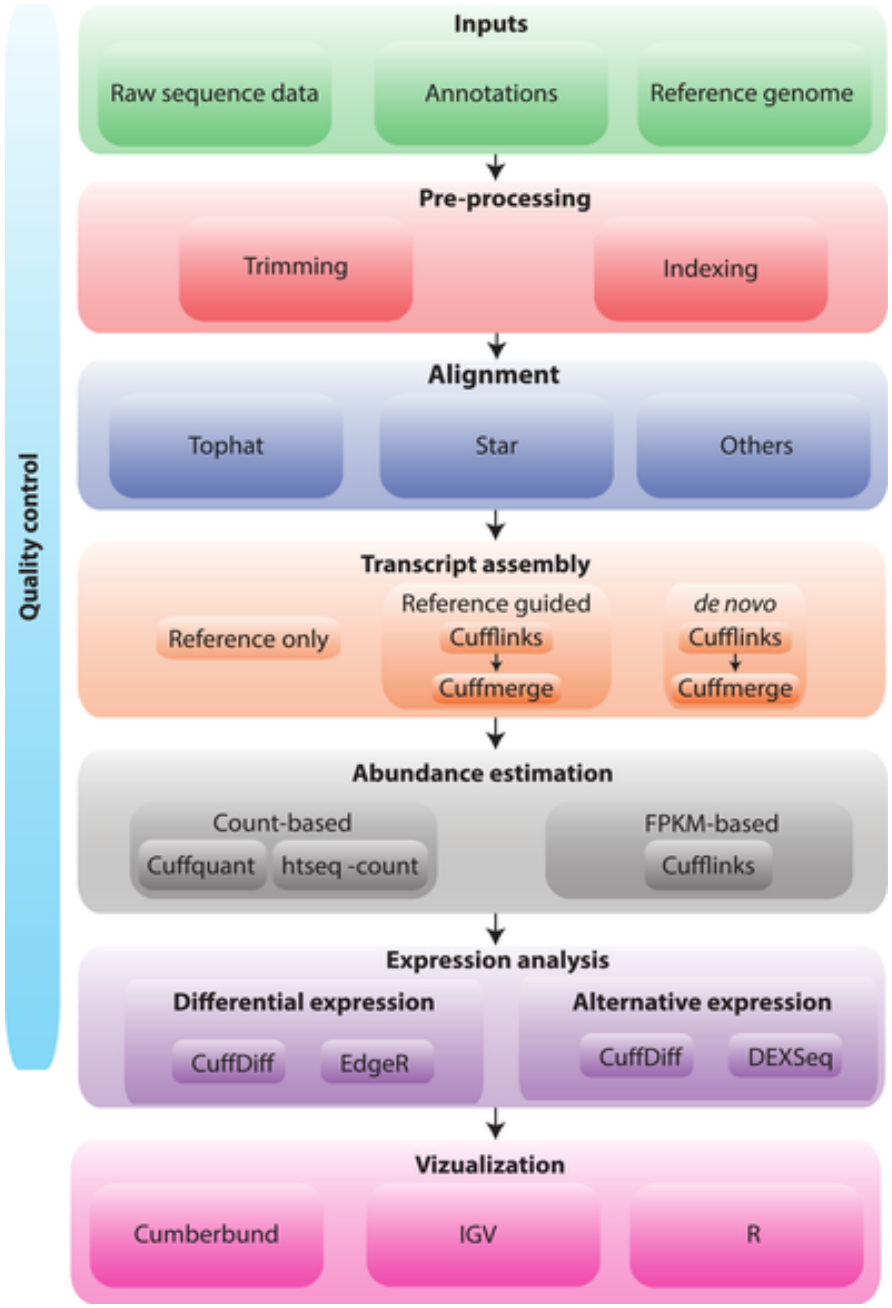
- Transcript identification (ie. which genes active)
- Expression Levels
- Alternative splicing isoforms
- Allelic variants
- Mutations
- Fusion Transcripts
- RNA-editing

RNA-Seq is Empowering Discovery at Single Cell Resolution



Wagner, Regev, and Yosef. NBT 2016

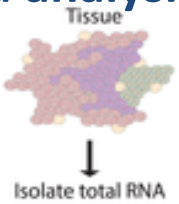
RNA-seq analysis flow chart.



http://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1004393

PLOS | COMPUTATIONAL BIOLOGY

RNA-seq library fragmentation and size selection strategies that influence interpretation and analysis.



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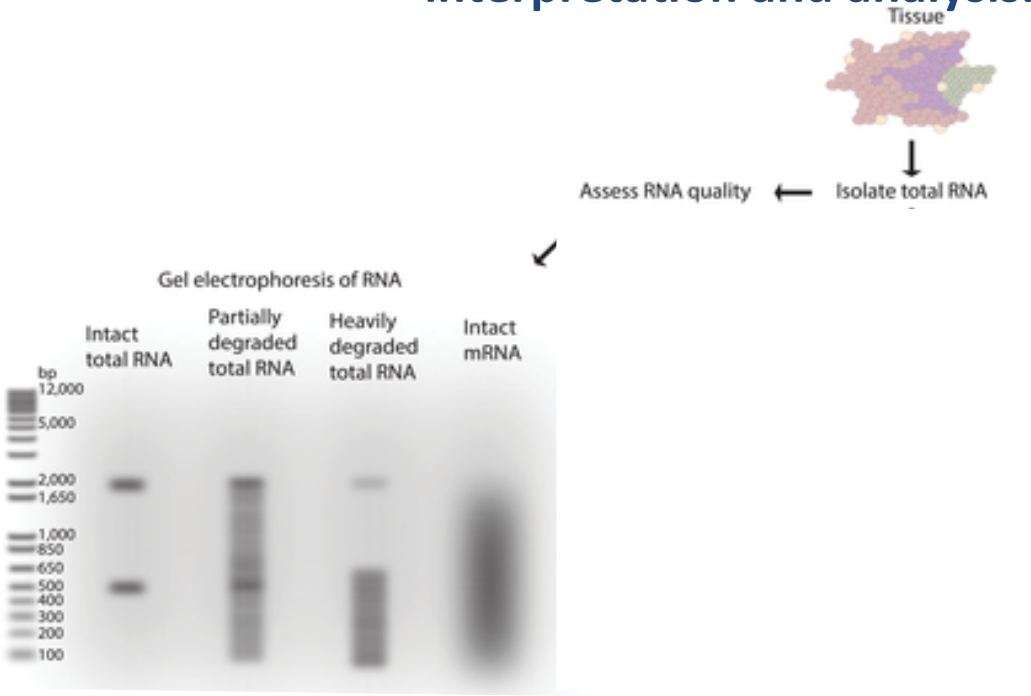

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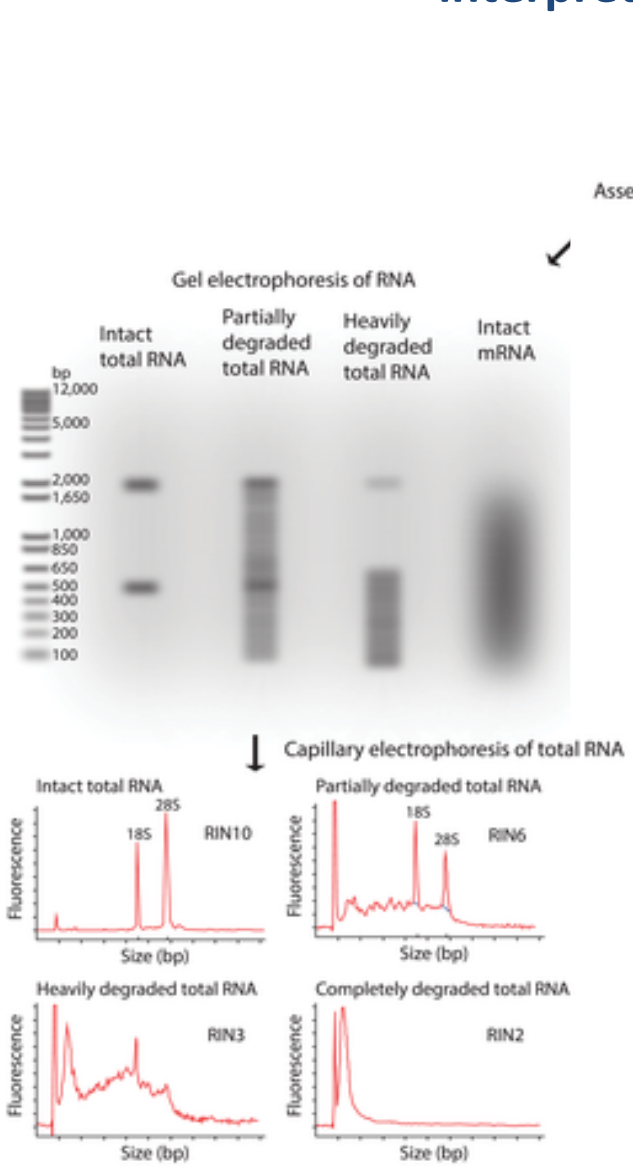

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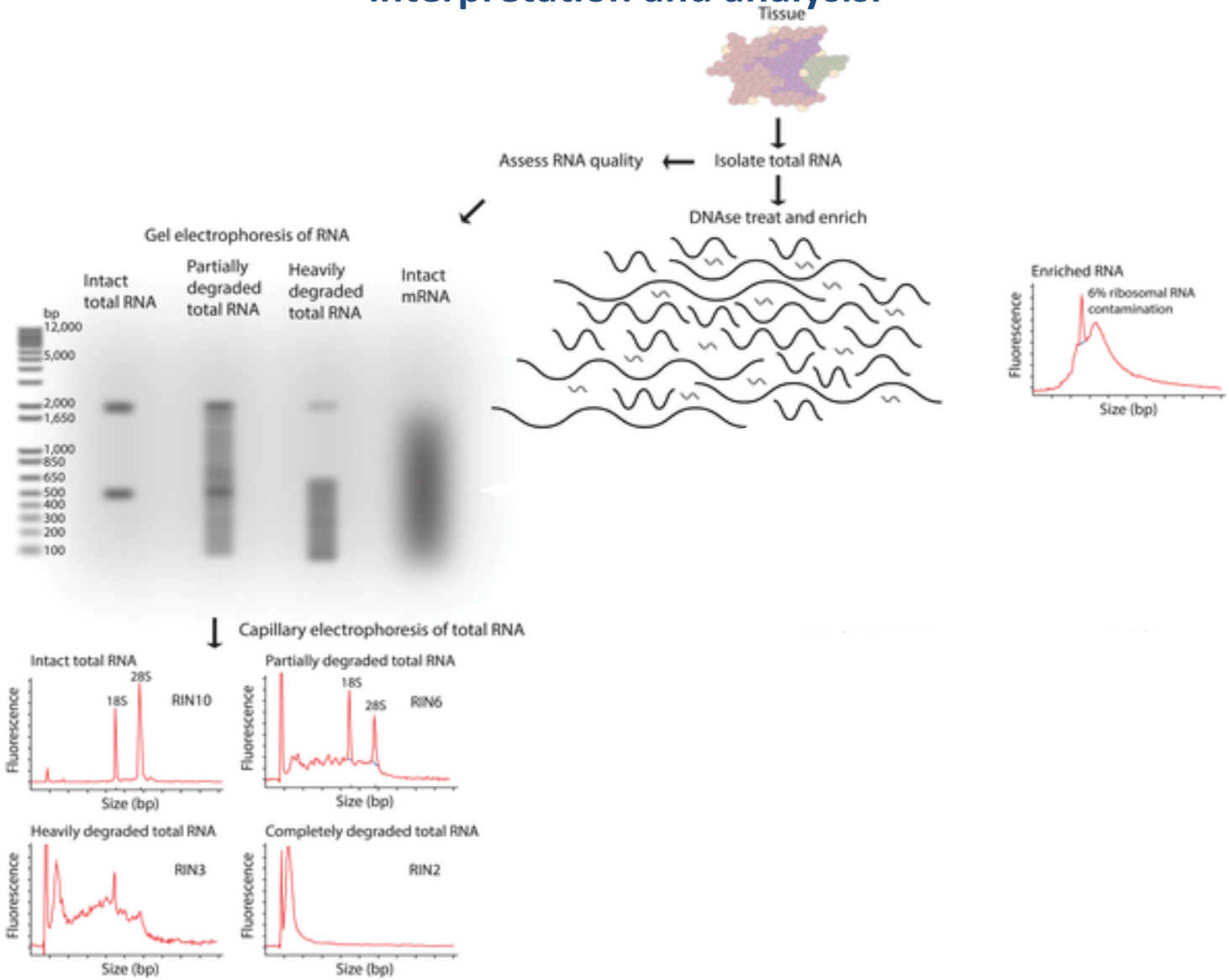

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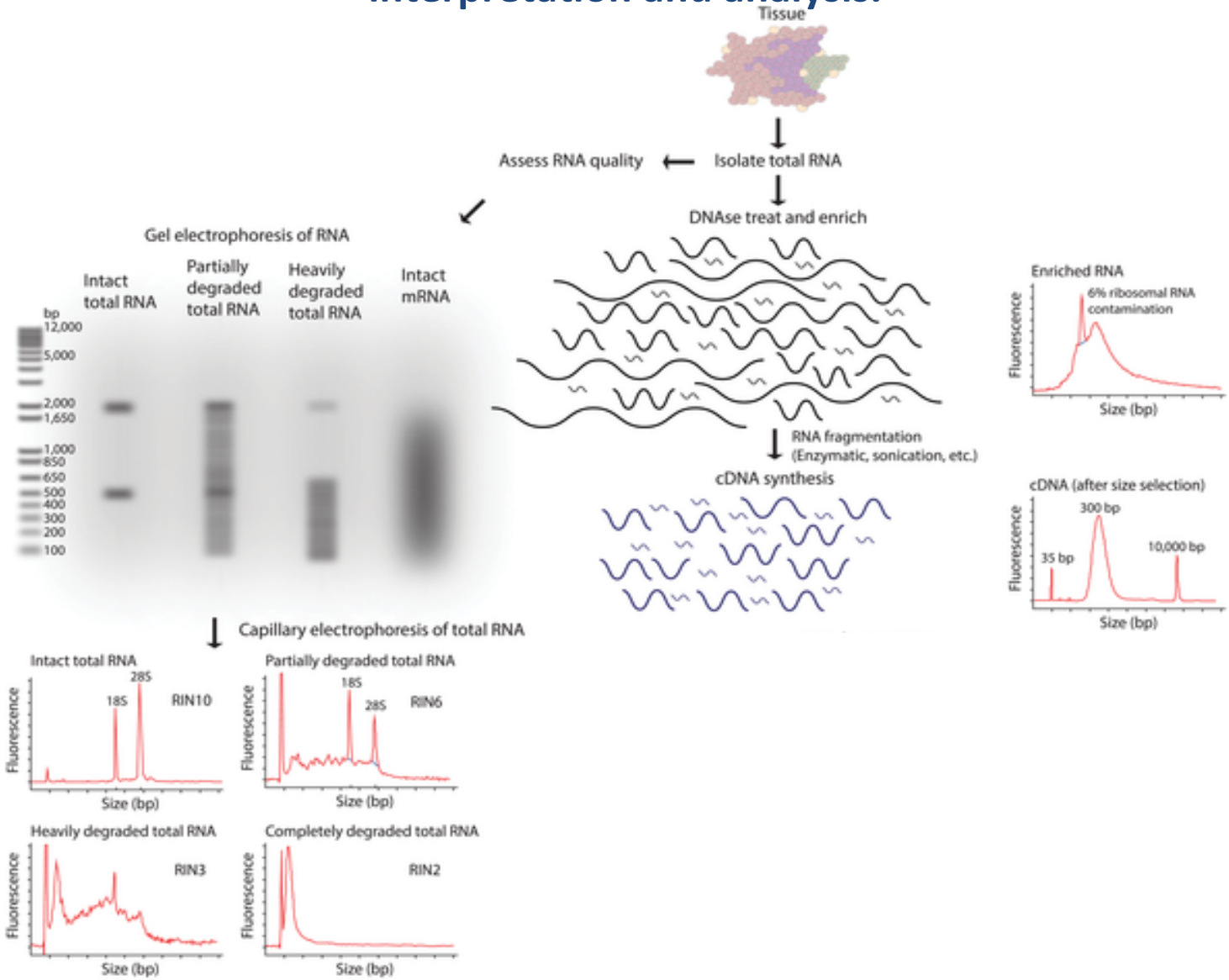
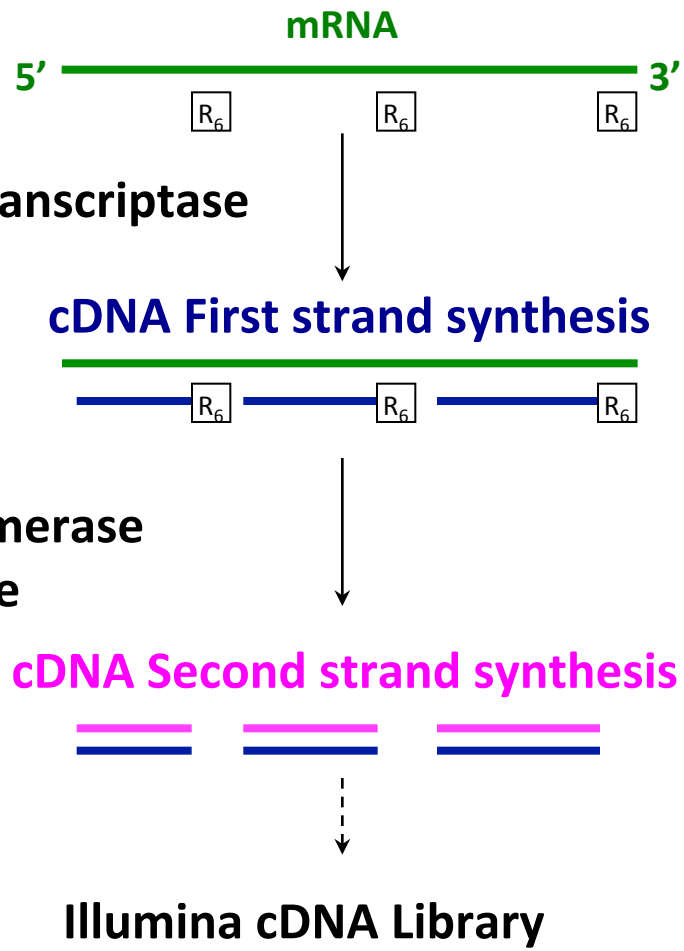


http://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1004393

PLOS | COMPUTATIONAL BIOLOGY

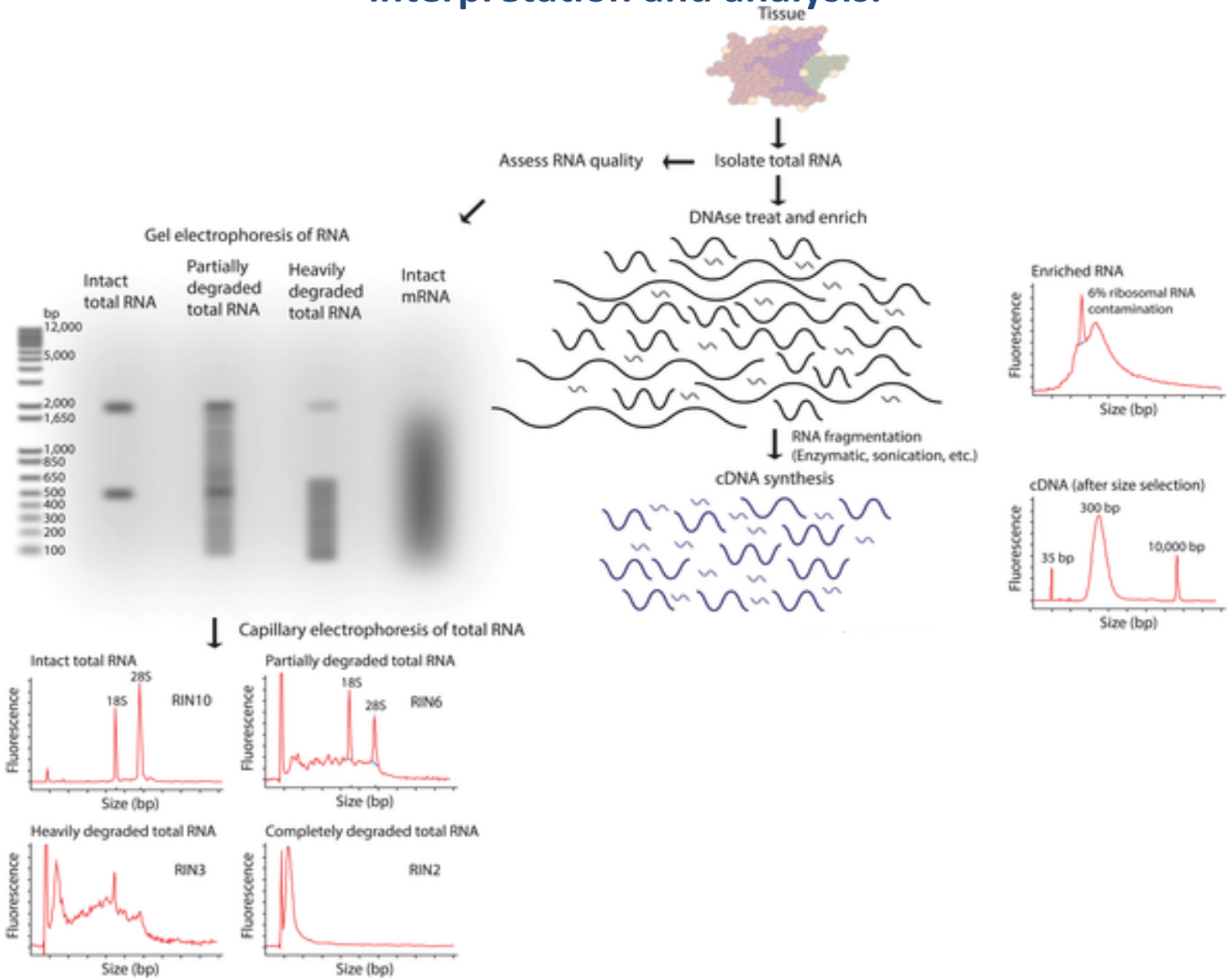
RNA-Seq: How do we make cDNA?

Prime with Random Hexamers (R6)



Slide courtesy of Joshua Levin, Broad Institute.

RNA-seq library fragmentation and size selection strategies that influence interpretation and analysis.



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PLOS | COMPUTATIONAL BIOLOGY

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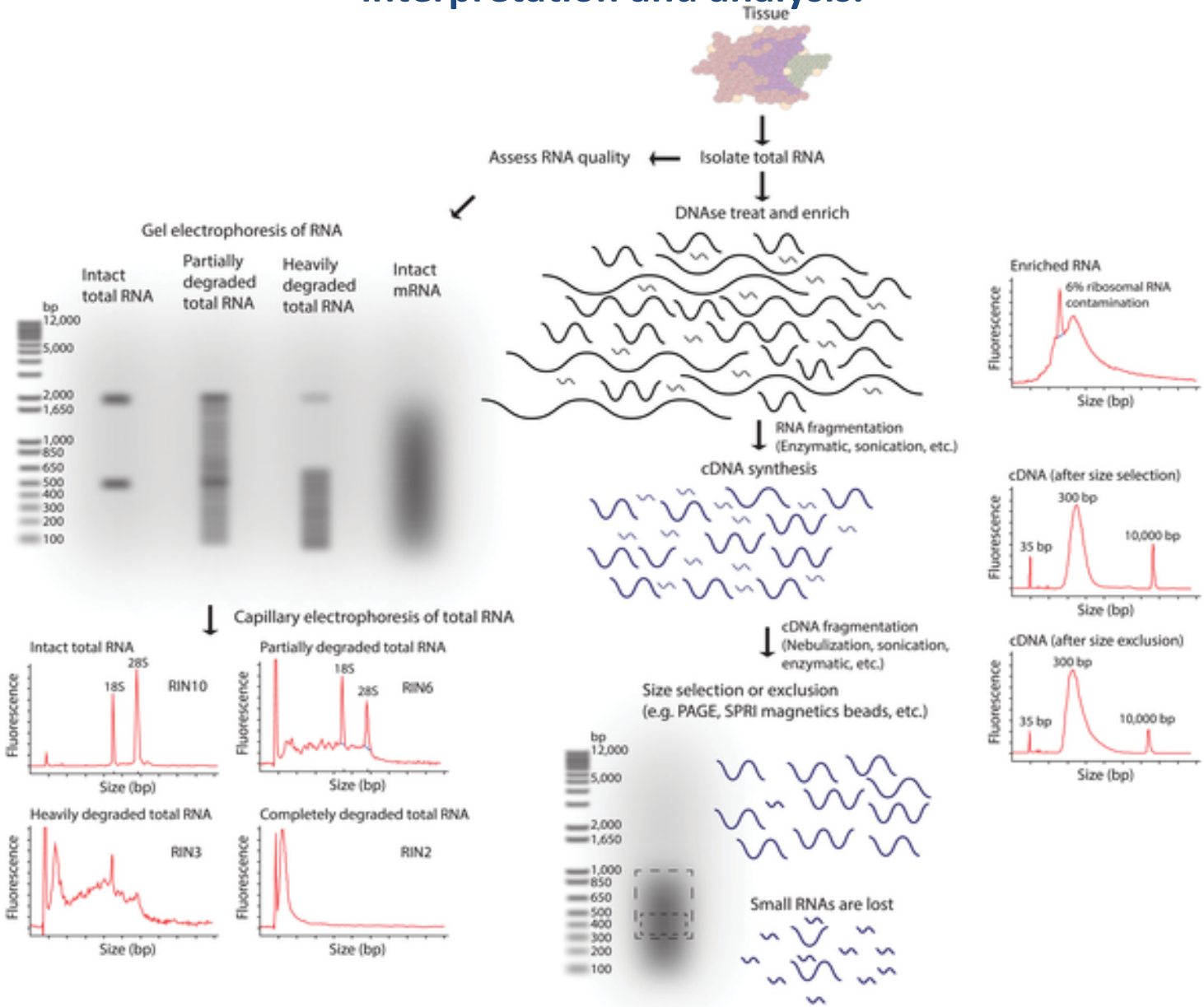

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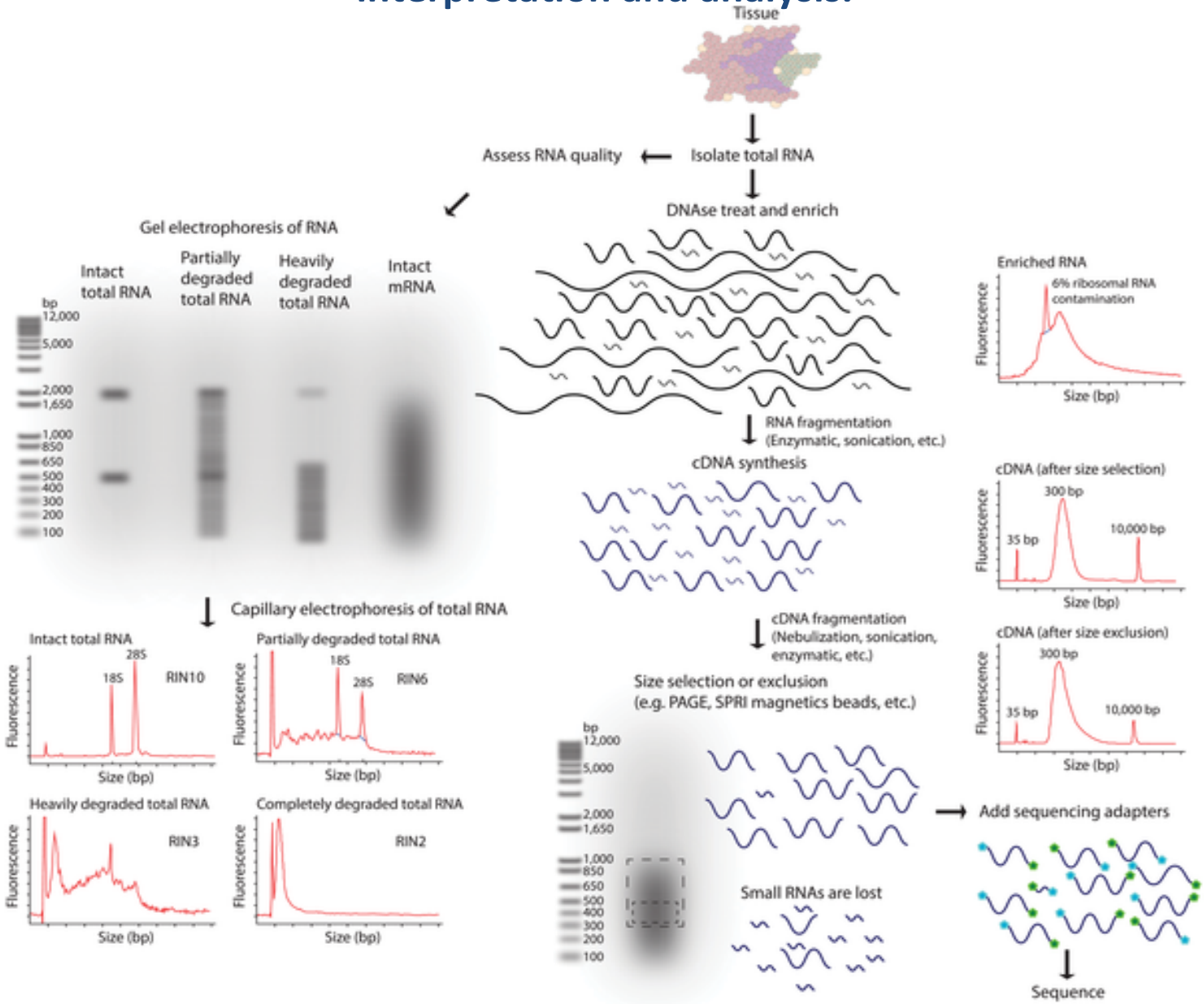

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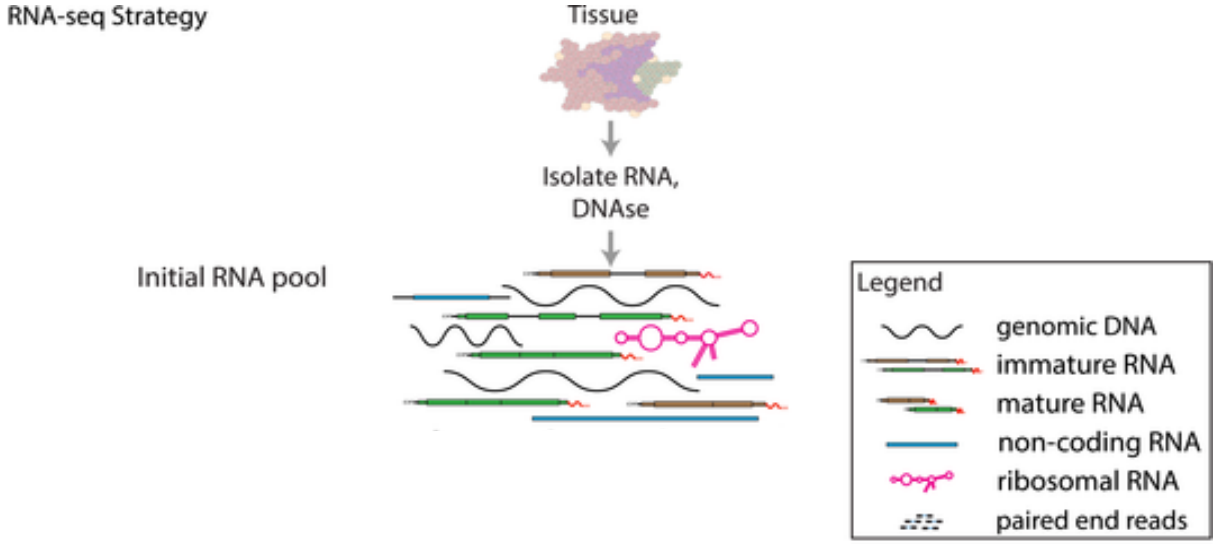
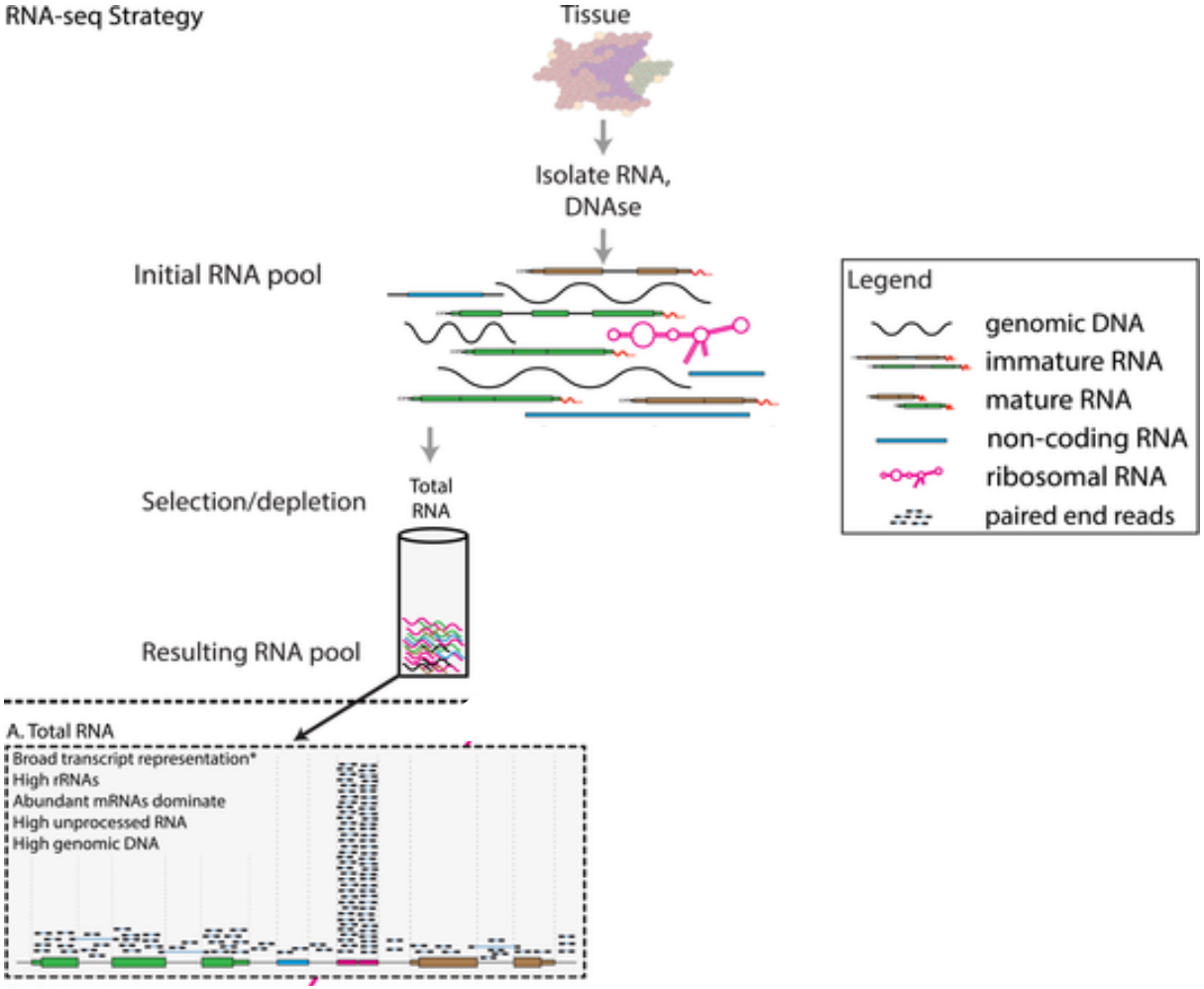
PLOS | COMPUTATIONAL BIOLOGY

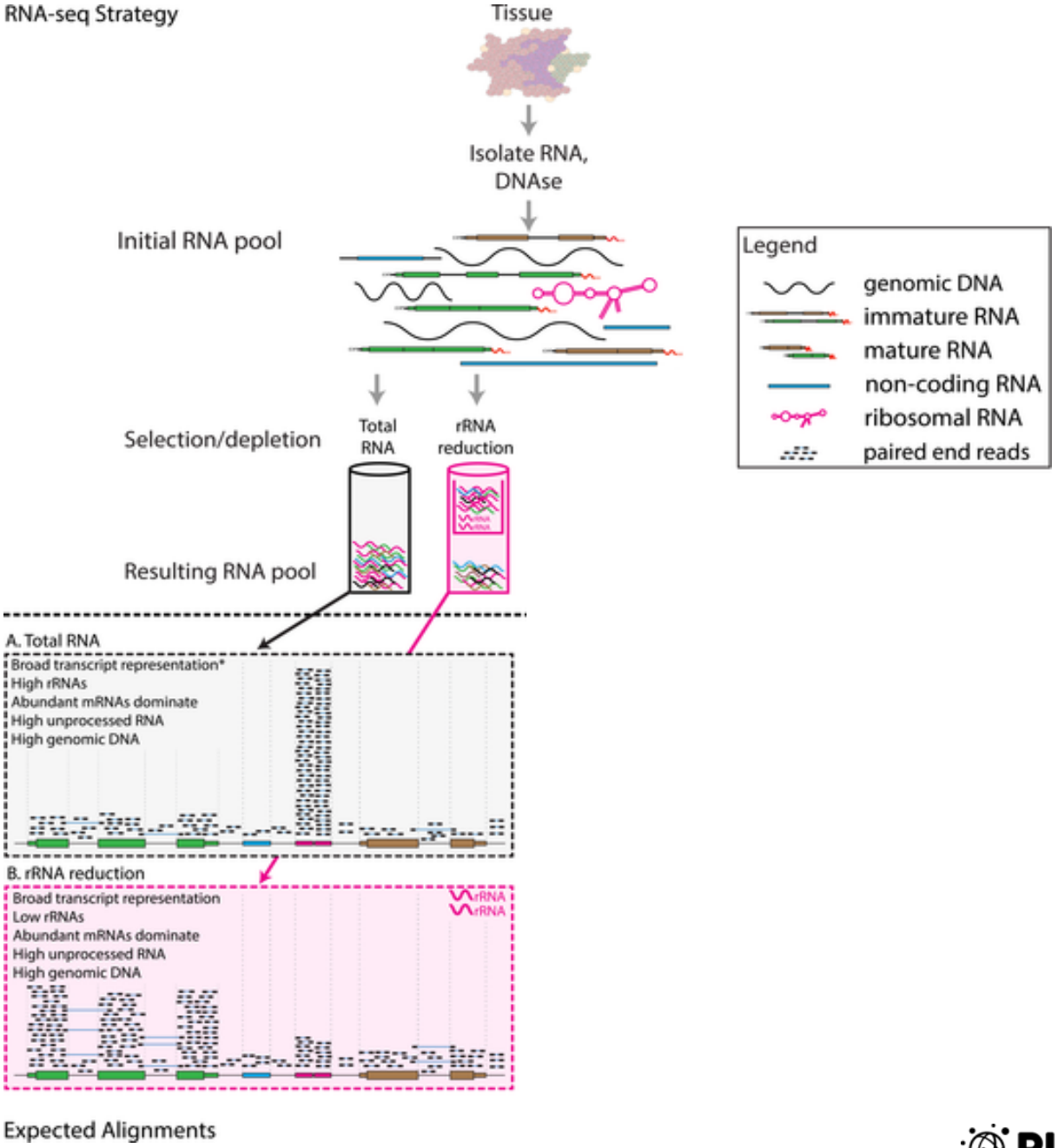
RNA-seq library enrichment strategies that influence interpretation and analysis.



RNA-seq library enrichment strategies that influence interpretation and analysis.



RNA-seq library enrichment strategies that influence interpretation and analysis.



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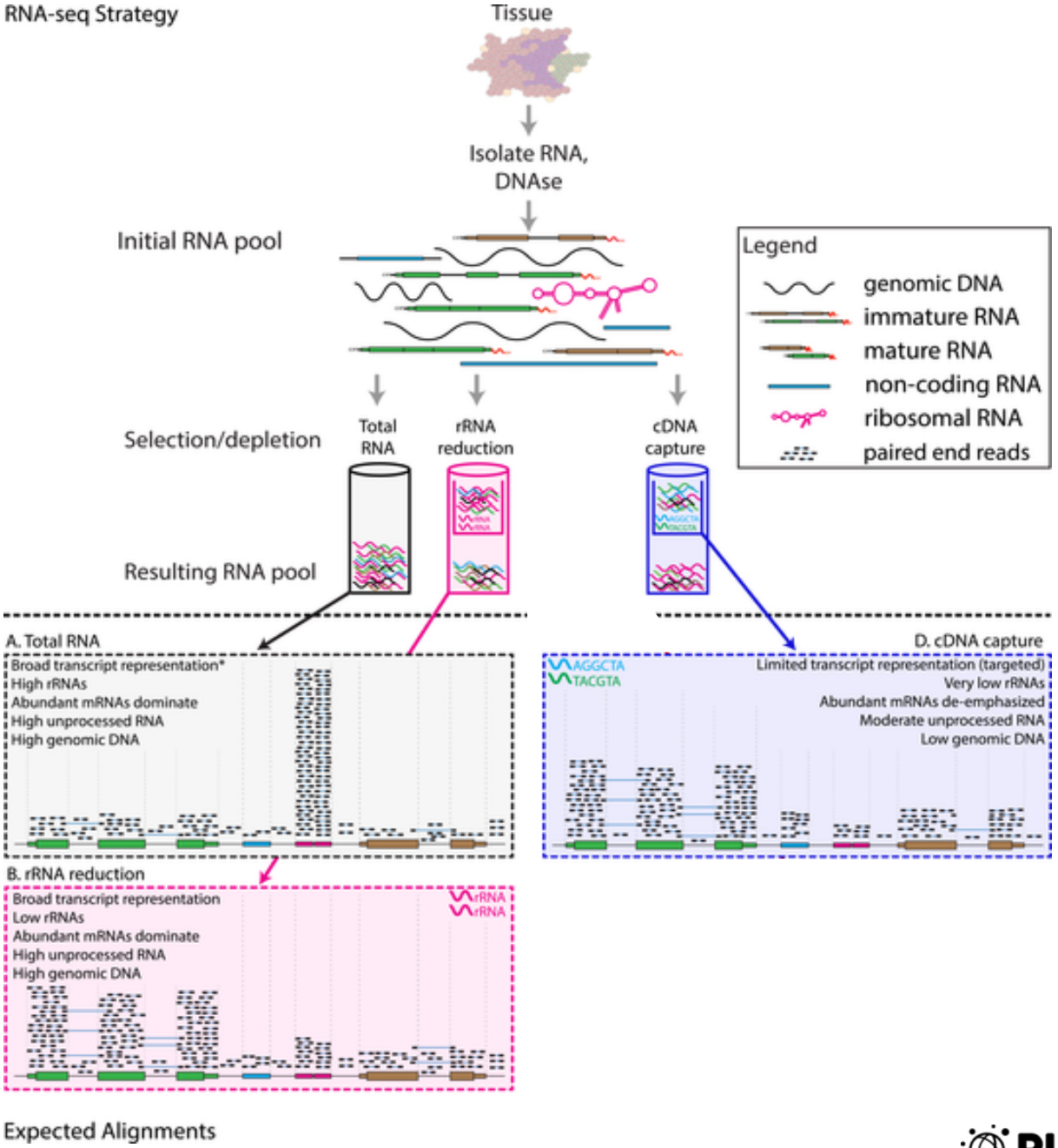

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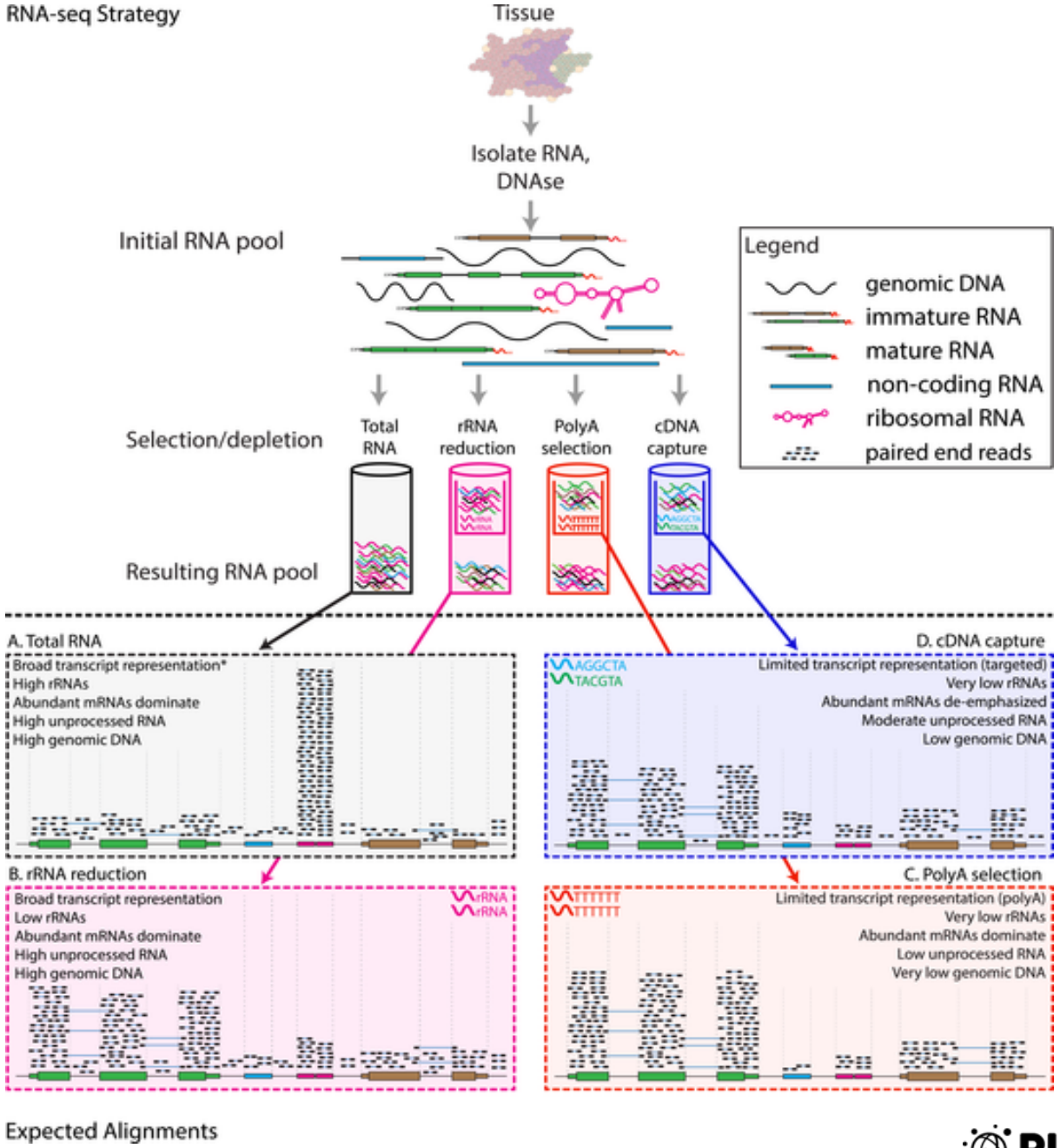


http://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1004393

PLOS | COMPUTATIONAL BIOLOGY

Generating RNA-Seq: *How to Choose?*

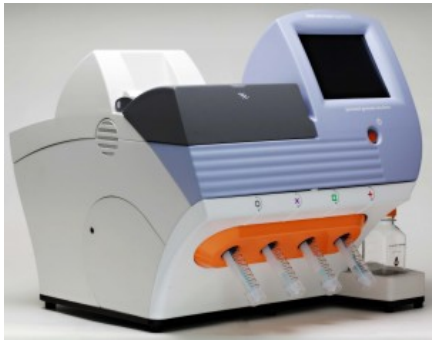
Many different instruments hit the scene in the last decade

Illumina

454

SOLiD

Helicos



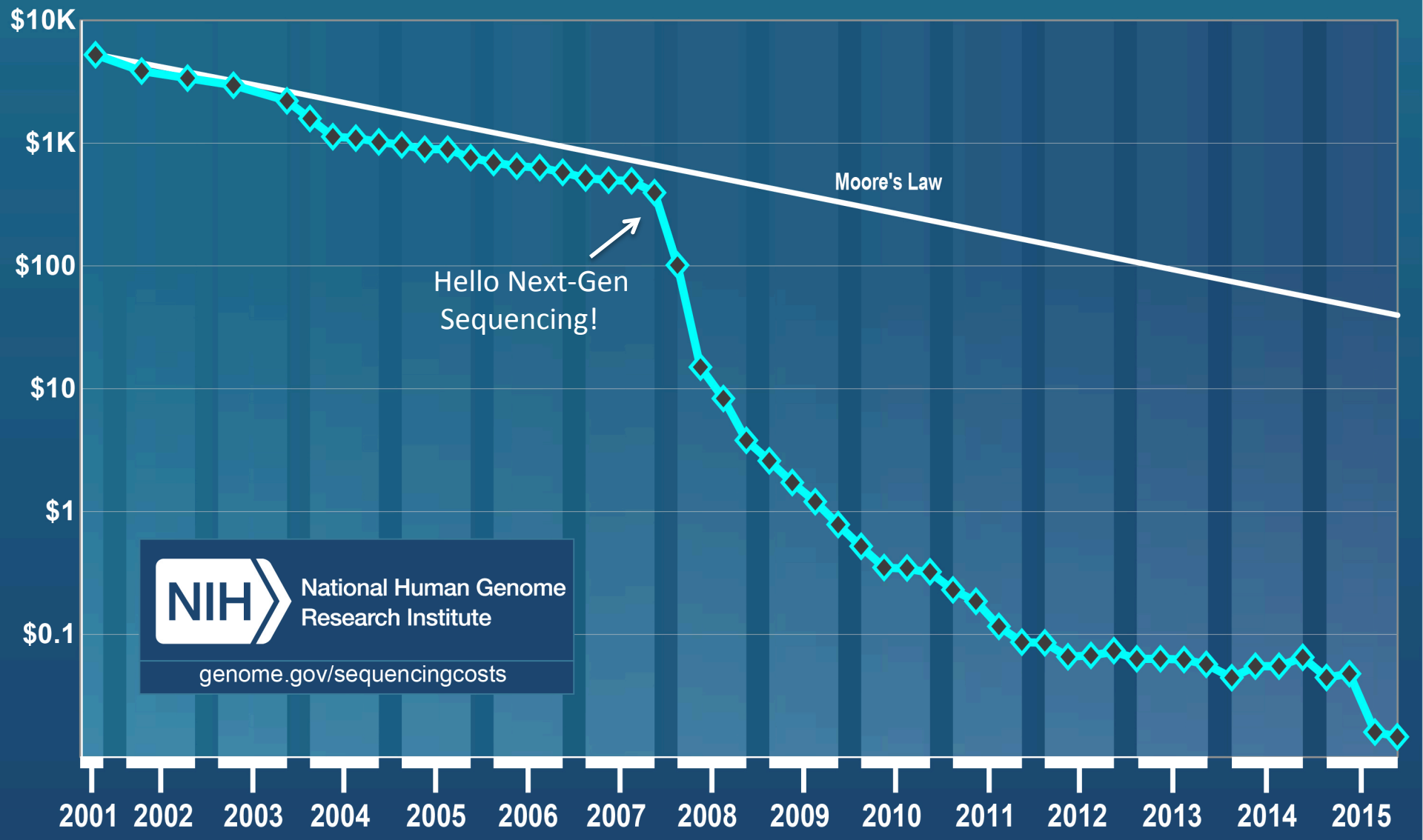
Ion Torrent

Pacific Biosciences

Oxford Nanopore

Slide courtesy of Joshua Levin, Broad Institute.

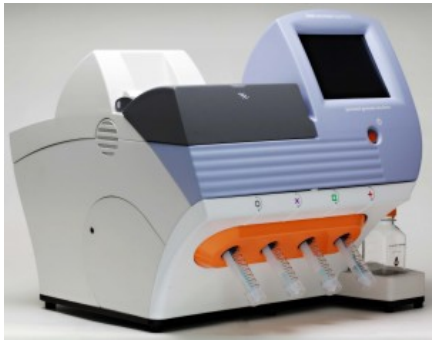
Cost per Raw Megabase of DNA Sequence



From https://www.genome.gov/sequencingcostsdata/

Generating RNA-Seq: *How to Choose?*

Illumina



Ion Torrent

"What I especially like about this baby is this little drawer where I can keep my lunch."

Helicos

Oxford Nanopore

Slide courtesy of Joshua Levin, Broad Institute.

Generating RNA-Seq: *How to Choose?*

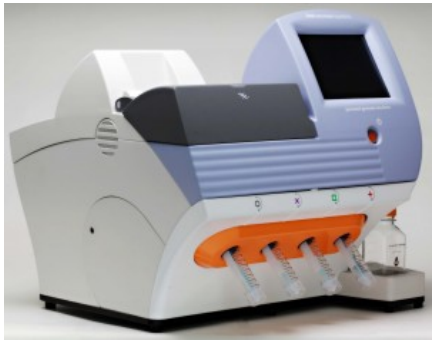
Popular choices for RNA-Seq today

Illumina

454

SOLiD

Helicos



Ion Torrent

Pacific Biosciences

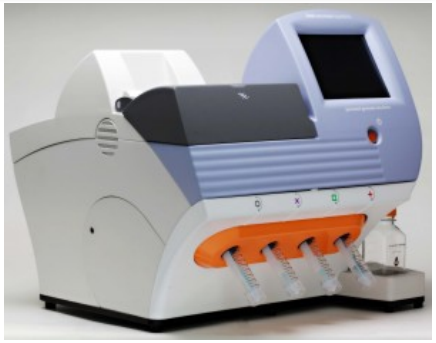
Oxford Nanopore

Generating RNA-Seq: *How to Choose?*

Popular choices for RNA-Seq today

[Current RNA-Seq workhorse]

Illumina



Ion Torrent

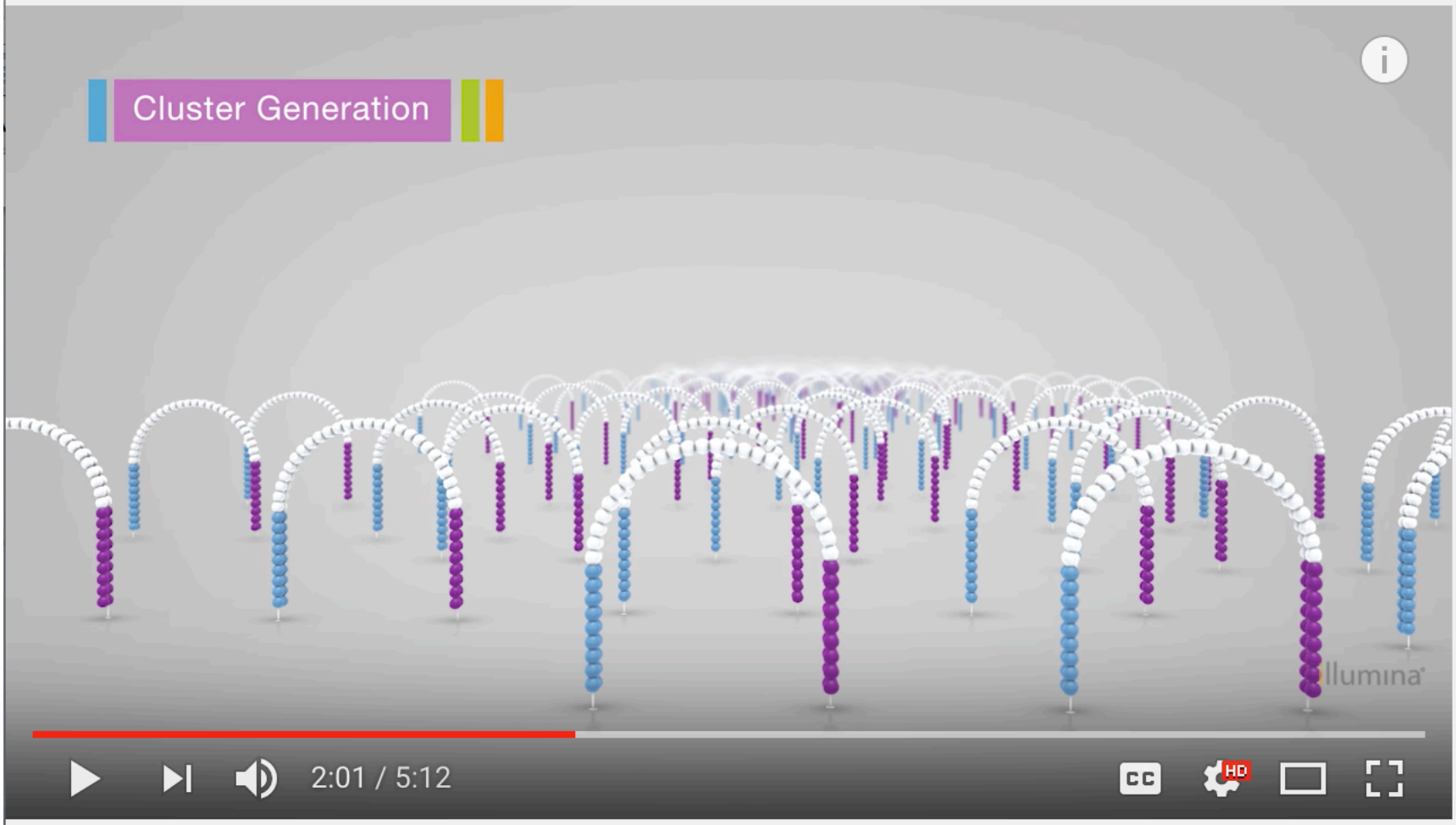
[Full-length single molecule sequencing]

Pacific Biosciences

[Newly emerging technology for full-length single molecule sequencing]

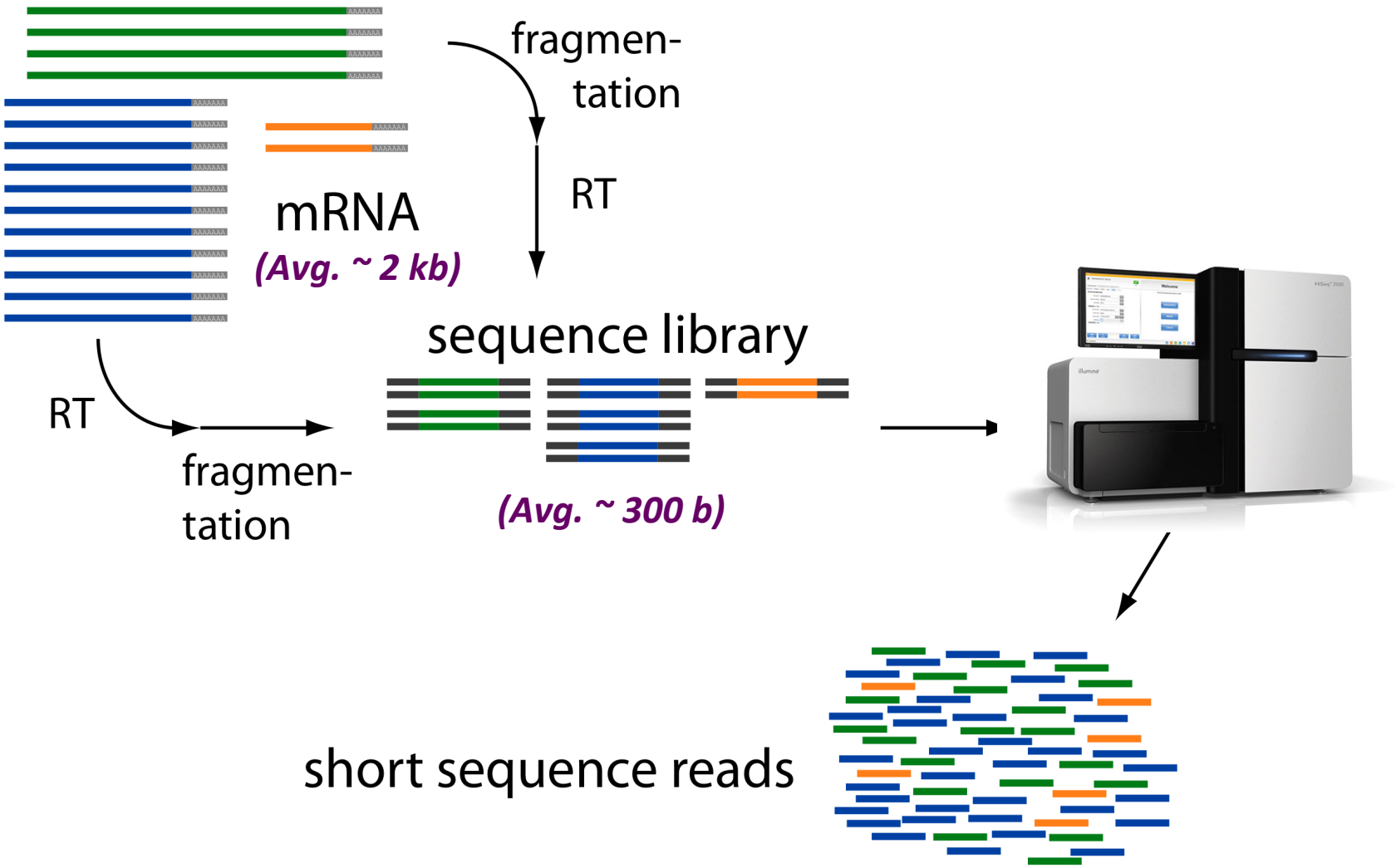
Oxford Nanopore

Illumina Sequencing by Synthesis



Illumina Inc https://www.youtube.com/watch?v=fCd6B5HRaZ8

Millions to Billions of Reads



Adapted from: http://www2.fml.tuebingen.mpg.de/raetsch/members/research/transcriptomics.html

Common Data Formats for RNA-Seq

FASTA format:

```
>61DFRAAXX100204:1:100:10494:3070/1
AAACAACAGGGCACATTGTCACTCTTGTATTTGAAAAACACTTTCCGGCCAT
```

FASTQ format:

```
@61DFRAAXX100204:1:100:10494:3070/1
AAACAACAGGGCACATTGTCACTCTTGTATTTGAAAAACACTTTCCGGCCAT
+
ACCCCCCCCCCCCCCCCCCCCCCCCCCCCCCBC?CCCCCCCCC@@CACCCCCA
```

Read

Quality values

Interpreting Base Quality Values

```
@61DFRAAXX100204:1:100:10494:3070/1
AAACAACAGGGCACATTGTCACTCTTGTATTTGAAAAACACTTTCCGGCCAT
+
ACCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCBC?CCCCCCCCC@@CACCCCCA
```

Read

Quality values

AsciiEncodedQual ('B') = **63**

Phred_Quality_Value = AsciiEncodedQual('B') – 33 = 30

Phred_Quality_Value = -10 * $\log_{10}$(Pwrong('T'))

Pwrong('T') = 10^(30/-10) = 10^-3 = 0.001

Paired-end Sequences

Two FastQ files, read name indicates left (/1) or right (/2) read of paired-end

```
@61DFRAAXX100204:1:100:10494:3070/1
AAACAACAGGGCACATTGTCACTCTTGTATTTGAAAAACACTTTCCGGCCAT
+
ACCCCCCCCCCCCCCCCCCCCCCCCCCCCCCBC?CCCCCCCCC@@CACCCCCA
```

```
@61DFRAAXX100204:1:100:10494:3070/2
CTCAAATGGTTAATTCTCAGGCTGCAAATATTCGTTCAGGATGGAAGAACA
+
C<CCCCCCCACCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCBCCCC
```

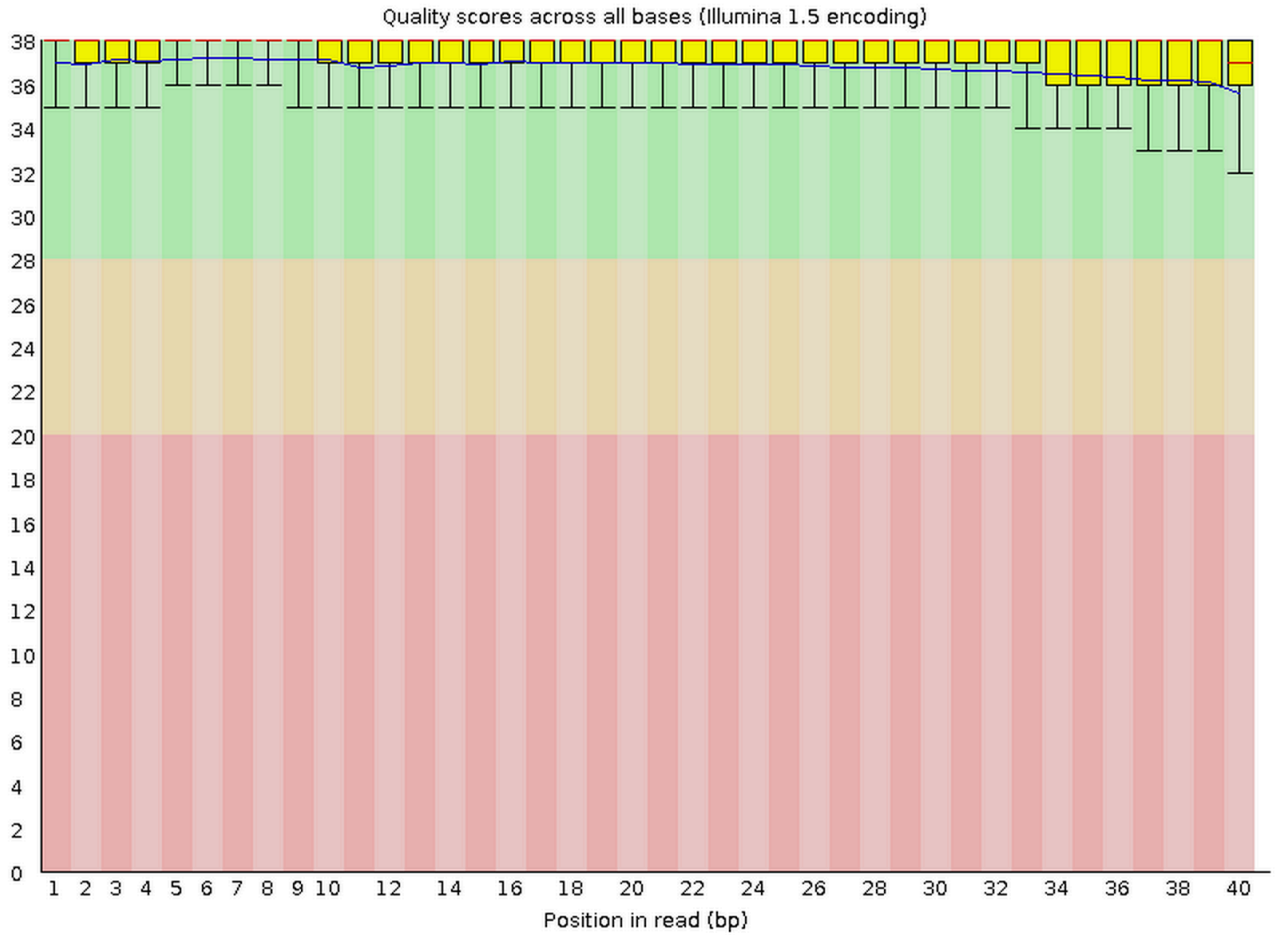
www.bioinformatics.babraham.ac.uk/projects/fastqc/good_sequence_short_fastqc.html#M4

FastQC Report

Wed 25 Mar 2015
good_sequence_short.txt

Summary

Per base sequence quality



Produced by FastQC (version 0.11.3)

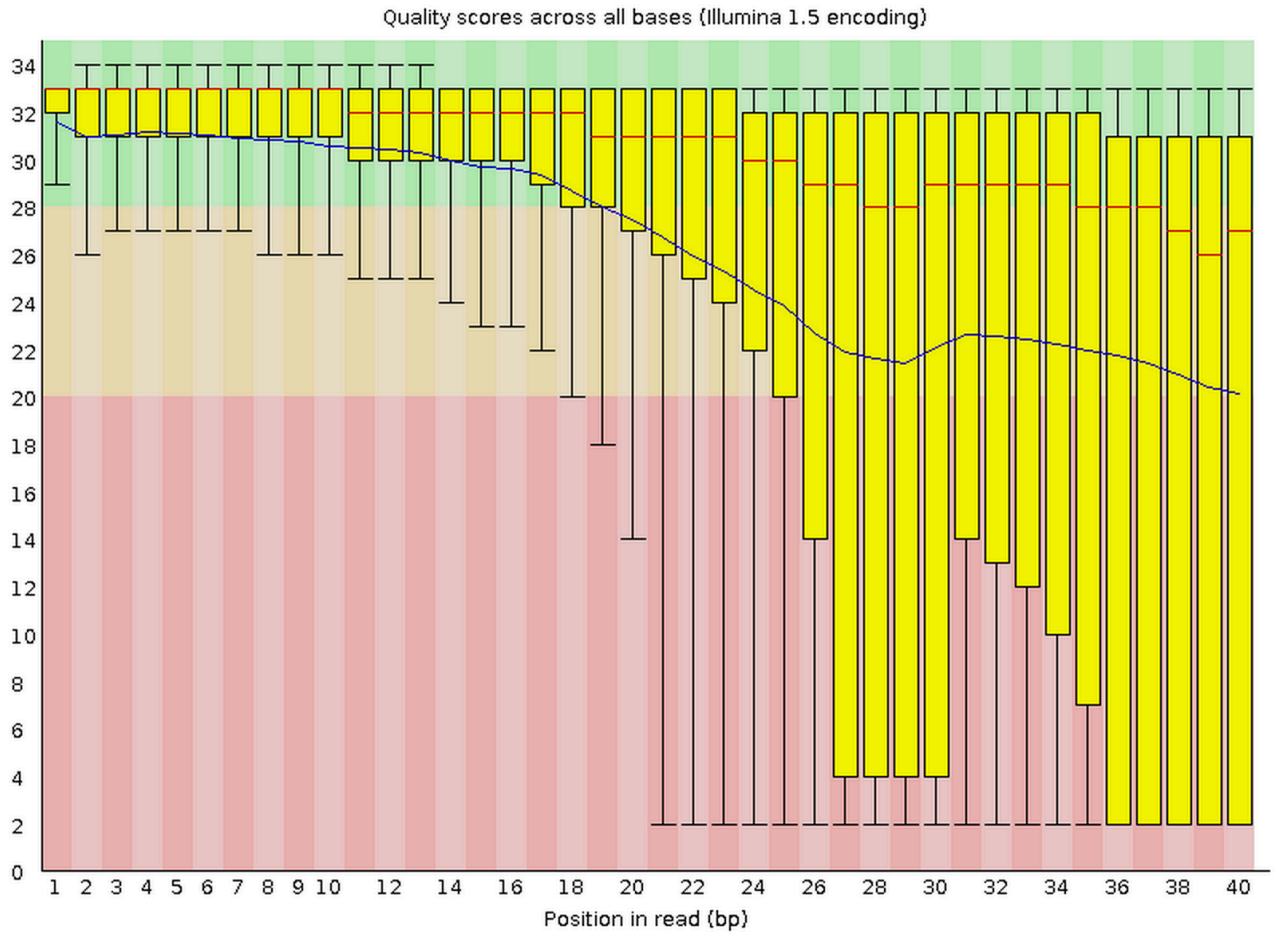
www.bioinformatics.babraham.ac.uk/projects/fastqc/bad_sequence_fastqc.html

FastQC Report

Wed 25 Mar 2015
bad_sequence.txt

Summary

Per base sequence quality



What to do?

- Trim the reads?
- Start over – try sequencing it again?

Trimming low quality regions of reads: Trimmomatic

www.usadellab.org/cms/?page=trimmomatic

USADELLAB.org

Home Research Education Service & Software Publications
Supporting Info About Us NGS, DE and other things

Trimmomatic: A flexible read trimming tool for Illumina NGS data

Citations

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina Sequence Data. *Bioinformatics*, btu170.

Downloading Trimmomatic

Version 0.36: binary, source and manual

Quick start

Paired End:

```
java -jar trimmomatic-0.35.jar PE -phred33 input_forward.fq.gz input_reverse.fq.gz
output_forward_paired.fq.gz output_forward_unpaired.fq.gz
output_reverse_paired.fq.gz output_reverse_unpaired.fq.gz ILLUMINACLIP:TruSeq3-
PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36
```

frontiers in
GENETICS

ORIGINAL RESEARCH ARTICLE
published: 31 January 2014
doi: 10.3389/fgene.2014.00013

On the optimal trimming of high-throughput mRNA sequence data

Matthew D. MacManes [1,2]*

[1] *Department of Molecular, Cellular and Biomedical Sciences, University of New Hampshire, Durham, NH, USA*
[2] *Hubbard Center for Genome Studies, Durham, NH, USA*

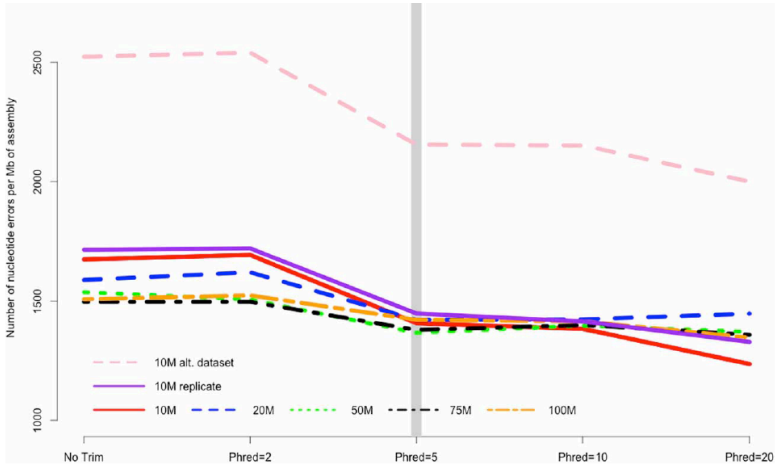
"… researchers interested in assembling transcriptomes *de novo* should elect for a much gentler quality trimming, or no trimming at all."

"… trimming at PHRED=2 or PHRED=5 optimizes assembly quality."

Aggressive Trimming may be harmful, whereas light trimming could be beneficial

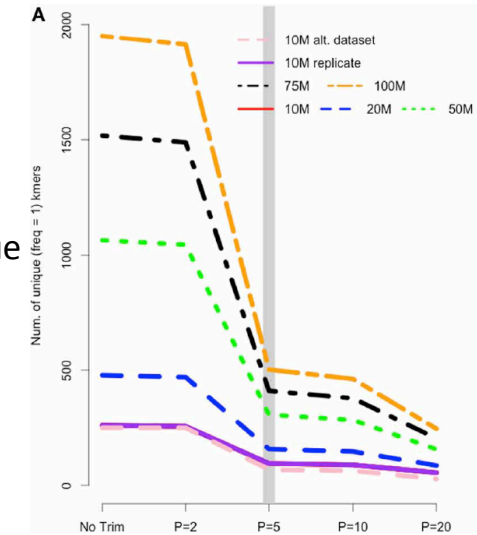
Fewer errors in the assembly

Nucleotide errors / Mb



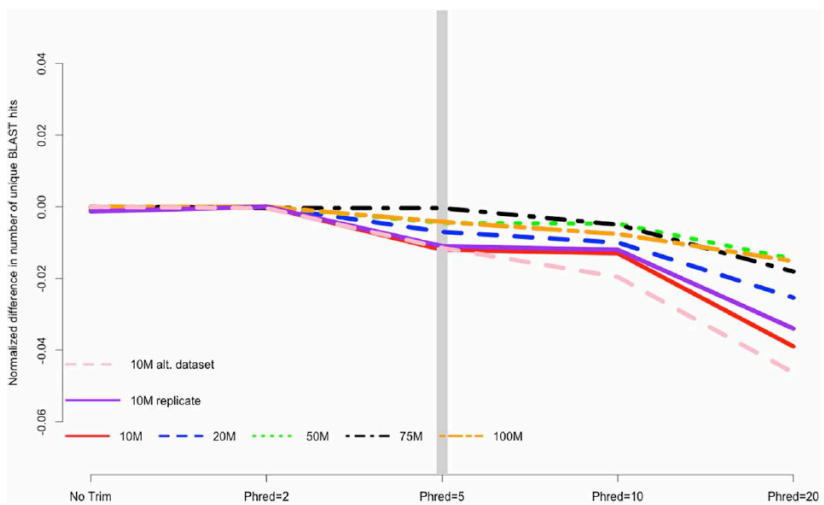
Fewer unique kmers

unique kmers



Light trimming doesn't reduce number of blast matches w/ higher sequencing depths.

Normalized # of blast matches



MacManes 2014

MultiQC - aggregation across all QC on all samples

multiqc.info

Current version: v1.2 | Home | Docs | Plugins | Logo | Example Reports

MultiQC

Aggregate results from bioinformatics analyses across many samples into a single report

MultiQC searches a given directory for analysis logs and compiles a HTML report. It's a general use tool, perfect for summarising the output from numerous bioinformatics tools.

- Introduction to MultiQC *(1:19)*
- Installing MultiQC *(4:33)*
- Running MultiQC *(5:21)*
- Using MultiQC Reports *(6:06)*

GitHub

Python Package Index

Documentation

51 supported tools

Publication / Citation

Get help on Gitter

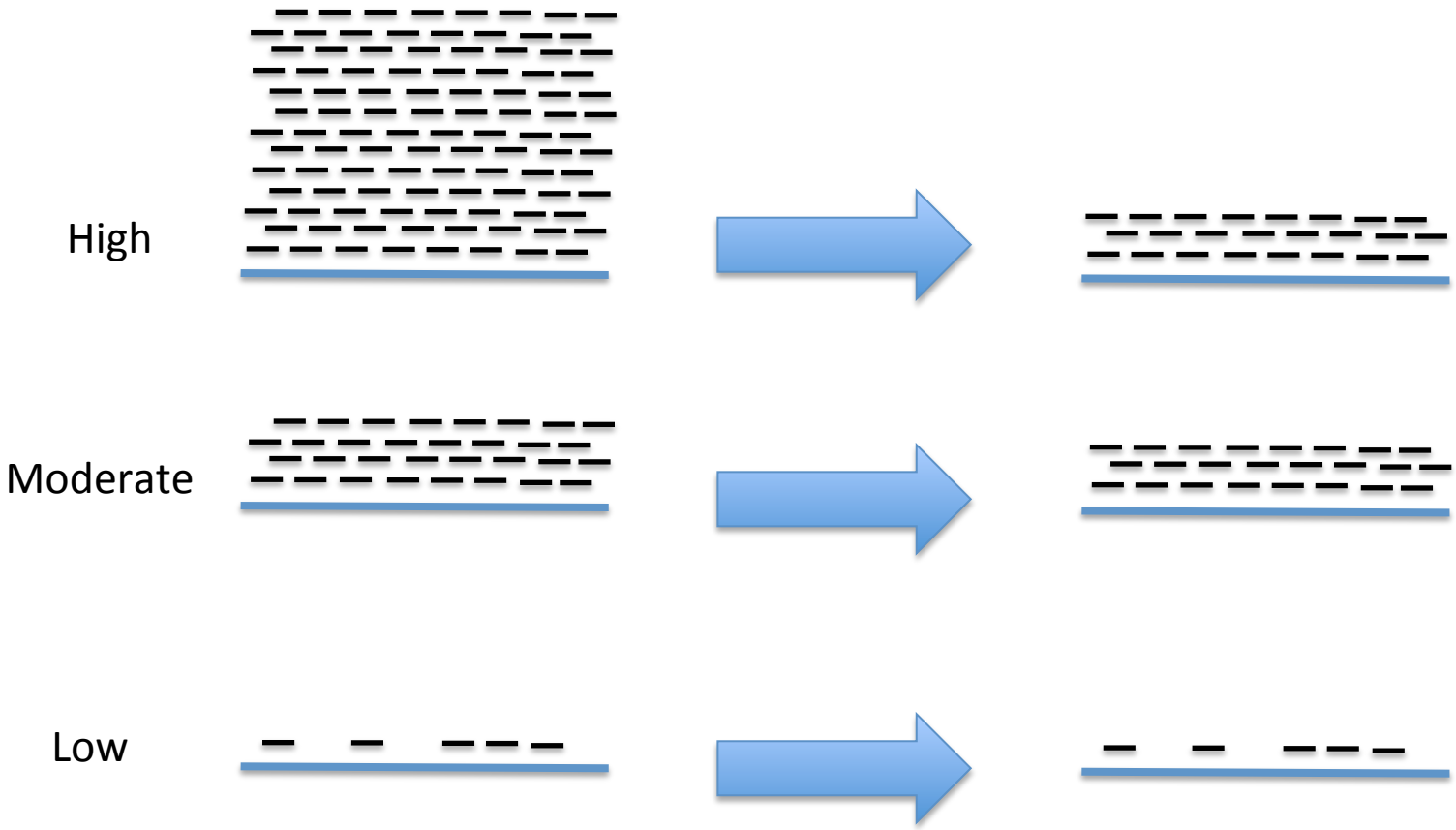
Quick Install

```
pip install multiqc     # Install
multiqc .               # Run
```

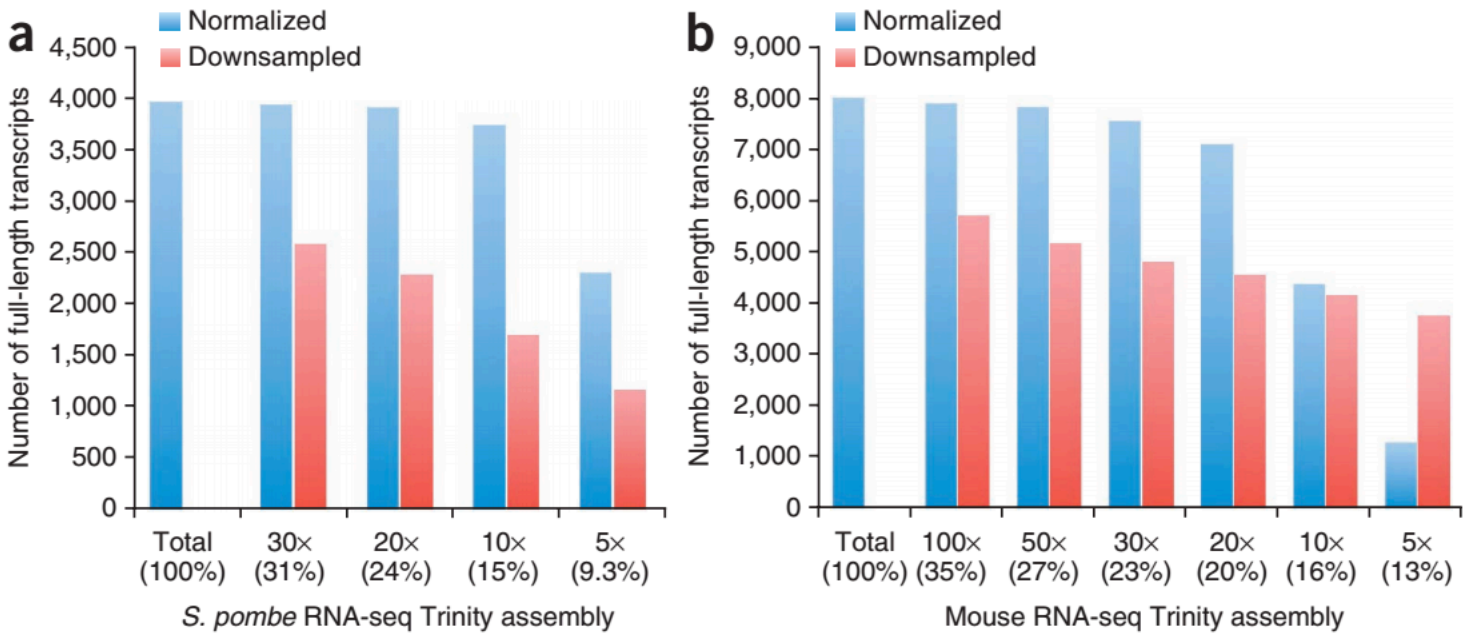
pip | conda | manual

Need a little more help? See the full installation instructions.

In silico normalization of reads



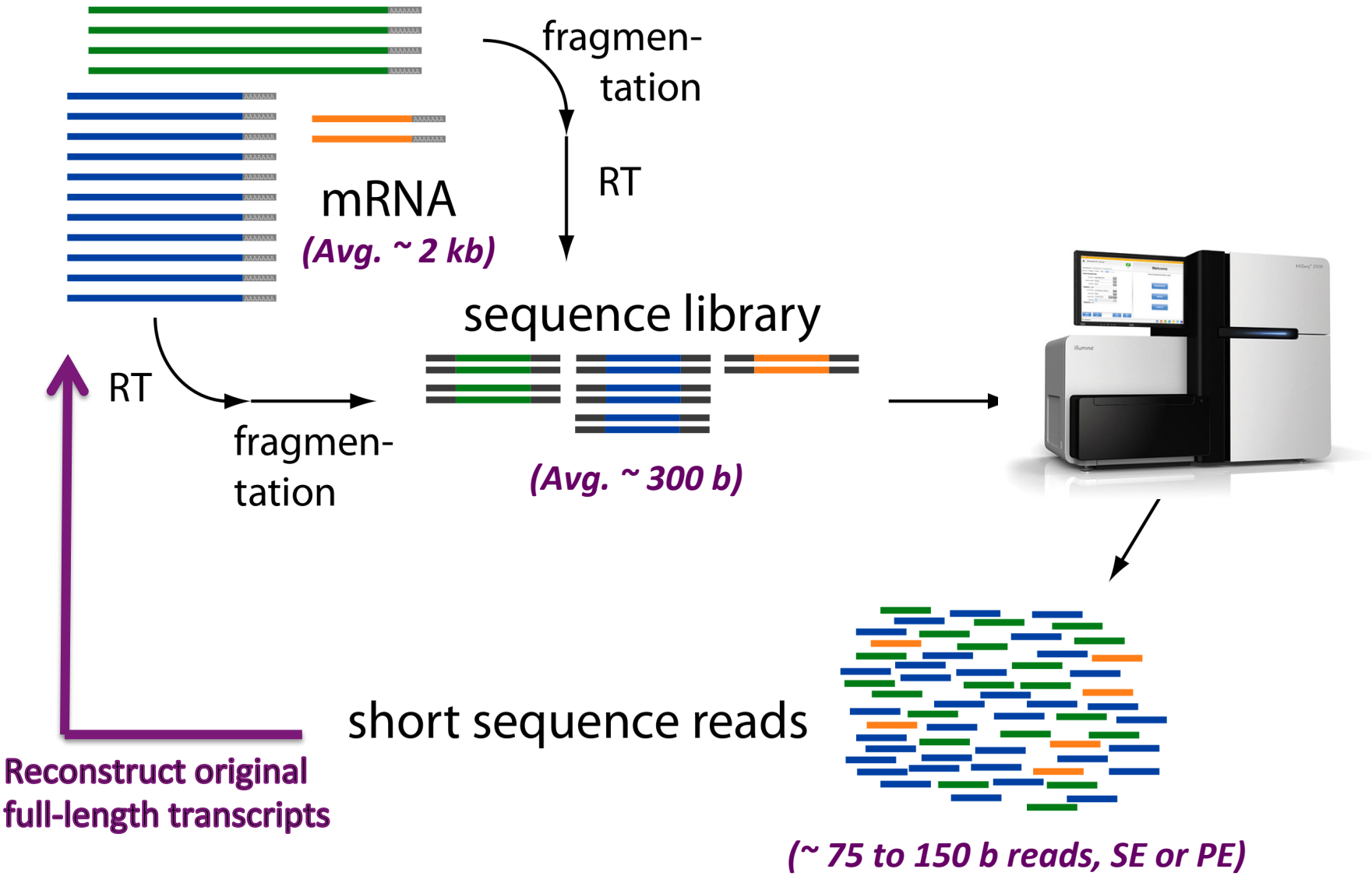
Impact of Normalization on *De novo* Full-length Transcript Reconstruction



Largely retain full-length reconstruction, but use less RAM and assemble much faster.

Haas et al., 2013

RNA-Seq Challenge: Transcript Reconstruction



Adapted from: http://www2.fml.tuebingen.mpg.de/raetsch/members/research/transcriptomics.html

Transcript Reconstruction from RNA-Seq Reads

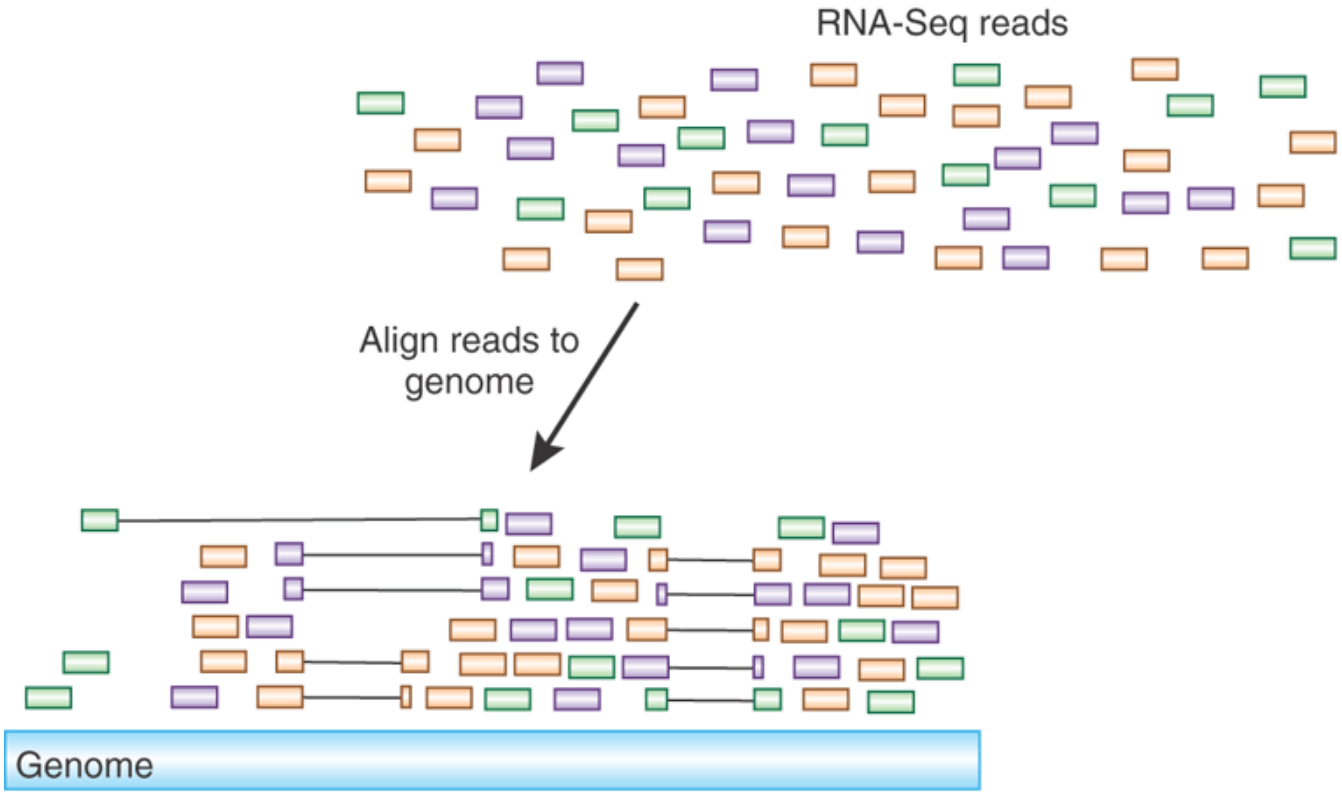
Advancing RNA-Seq analysis

Brian J Haas & Michael C Zody

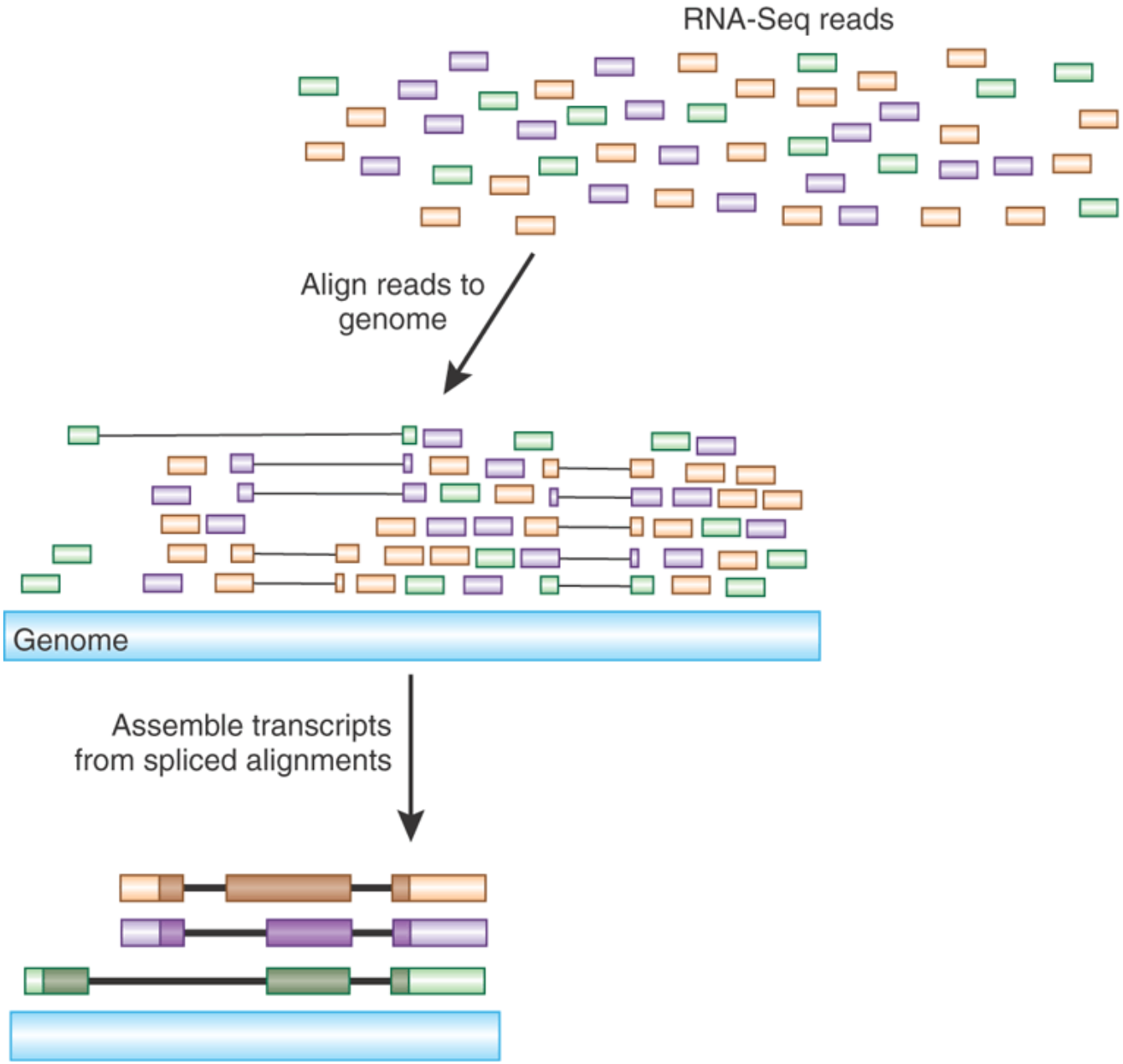
Nature Biotech, 2010

New methods for analyzing RNA-Seq data enable *de novo* reconstruction of the transcriptome.

Transcript Reconstruction from RNA-Seq Reads



Transcript Reconstruction from RNA-Seq Reads



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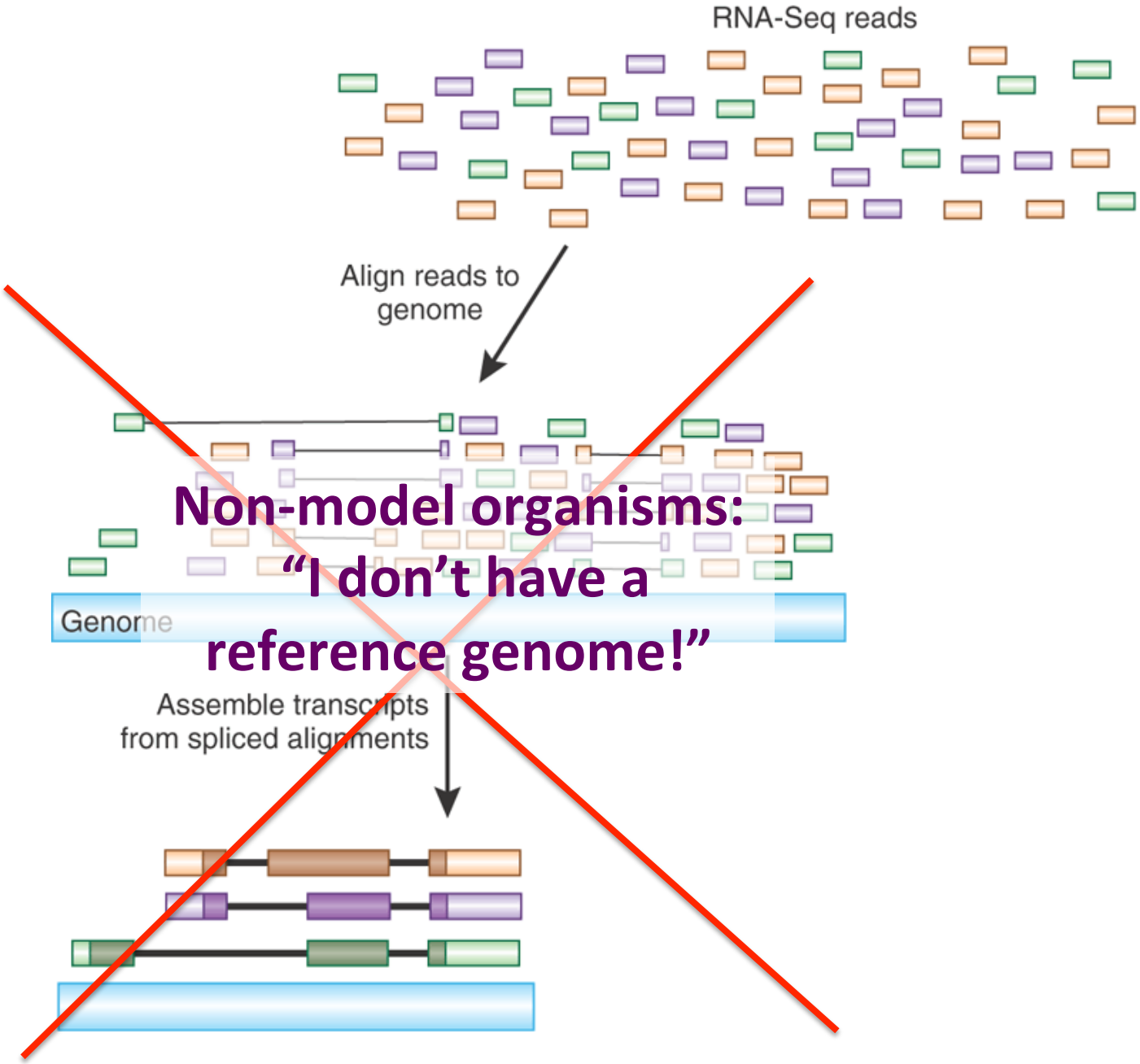
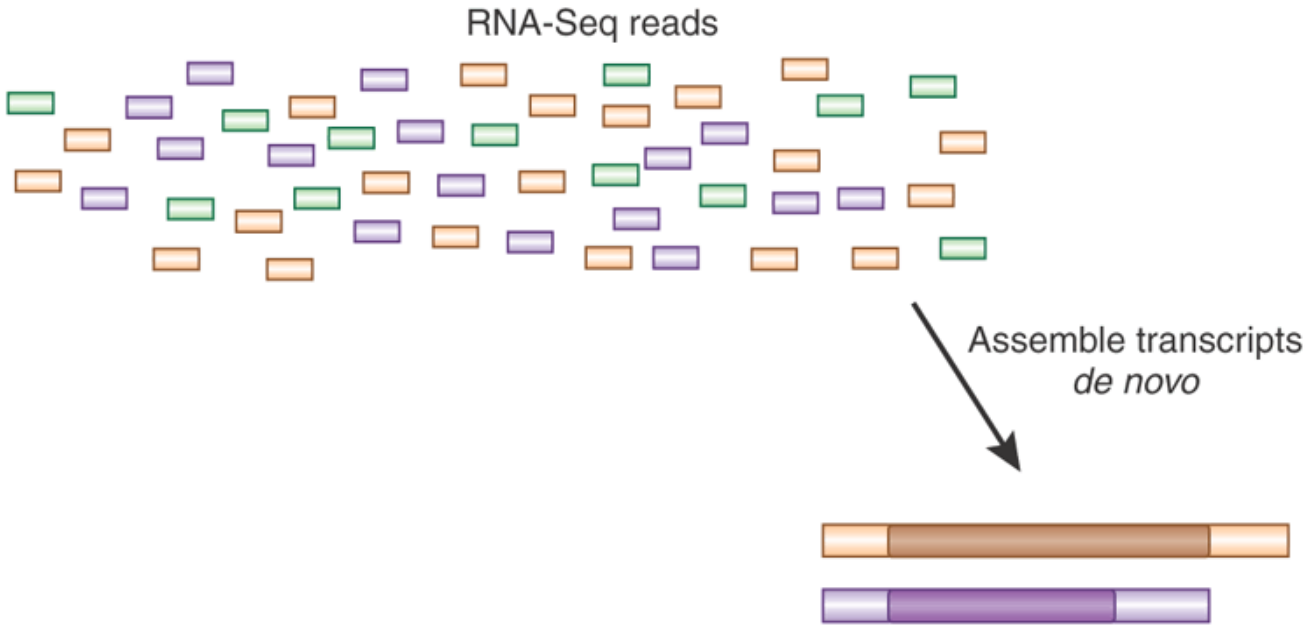
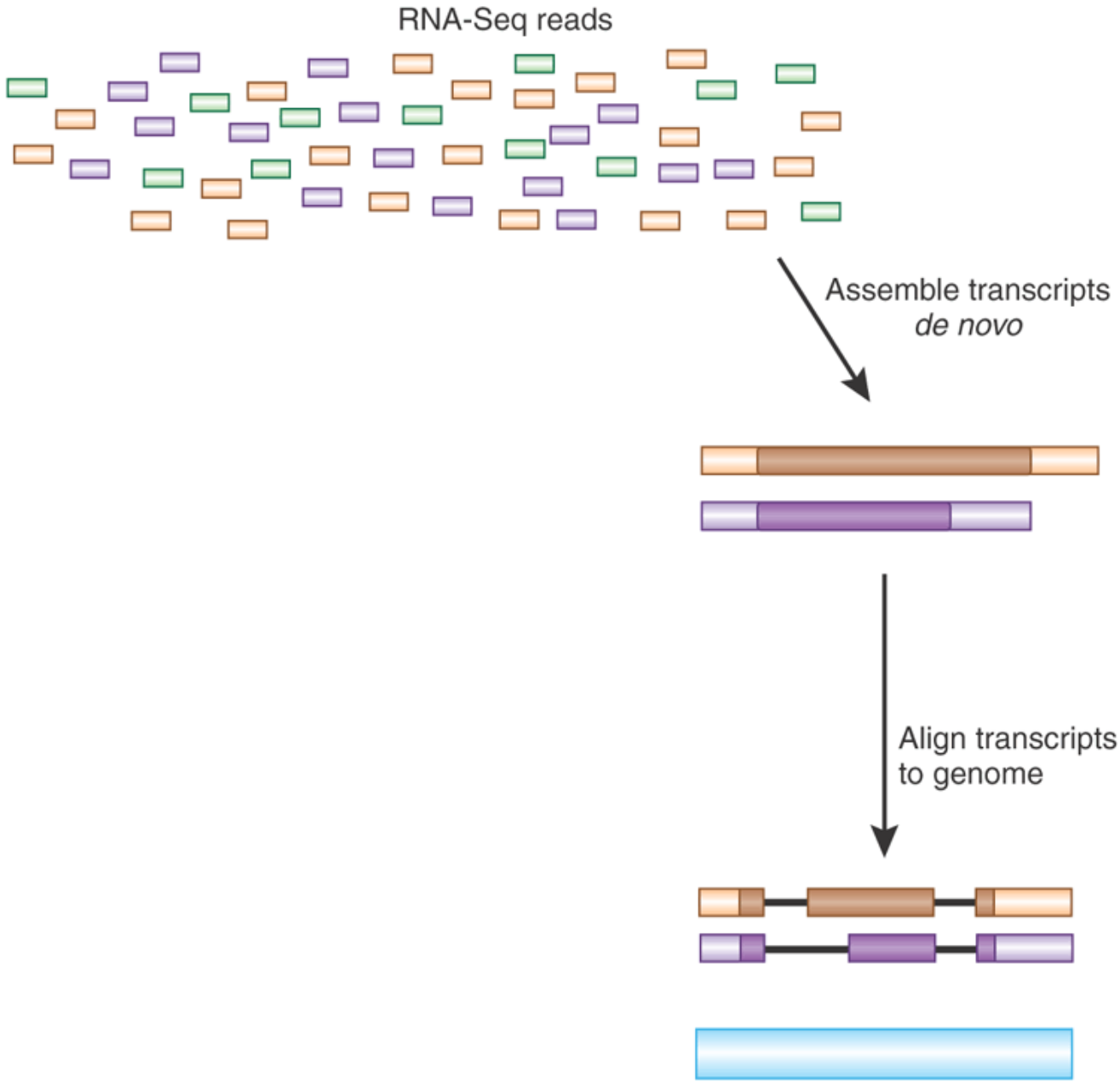


Non-model organisms: "I don't have a reference genome!"

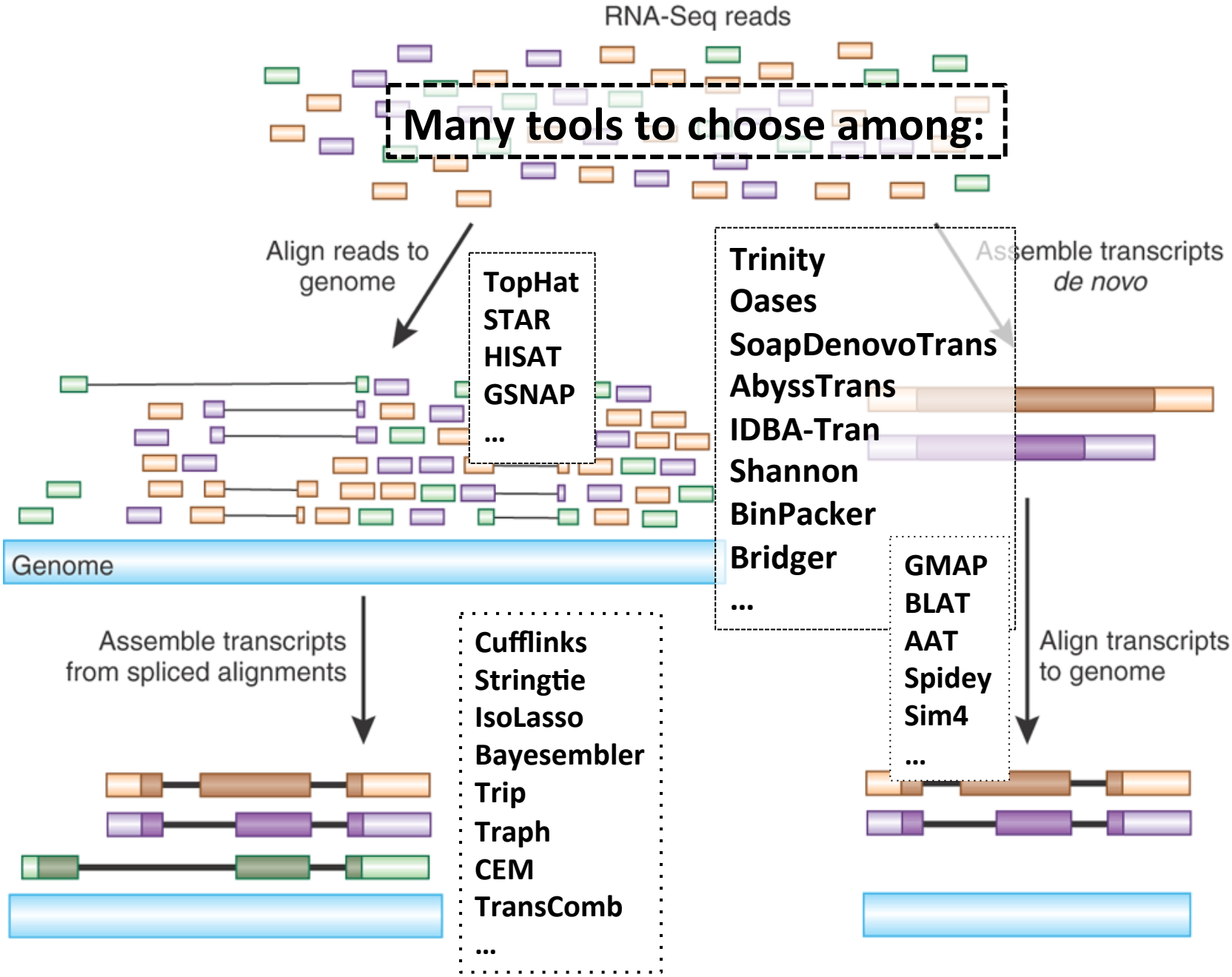
Transcript Reconstruction from RNA-Seq Reads



Transcript Reconstruction from RNA-Seq Reads



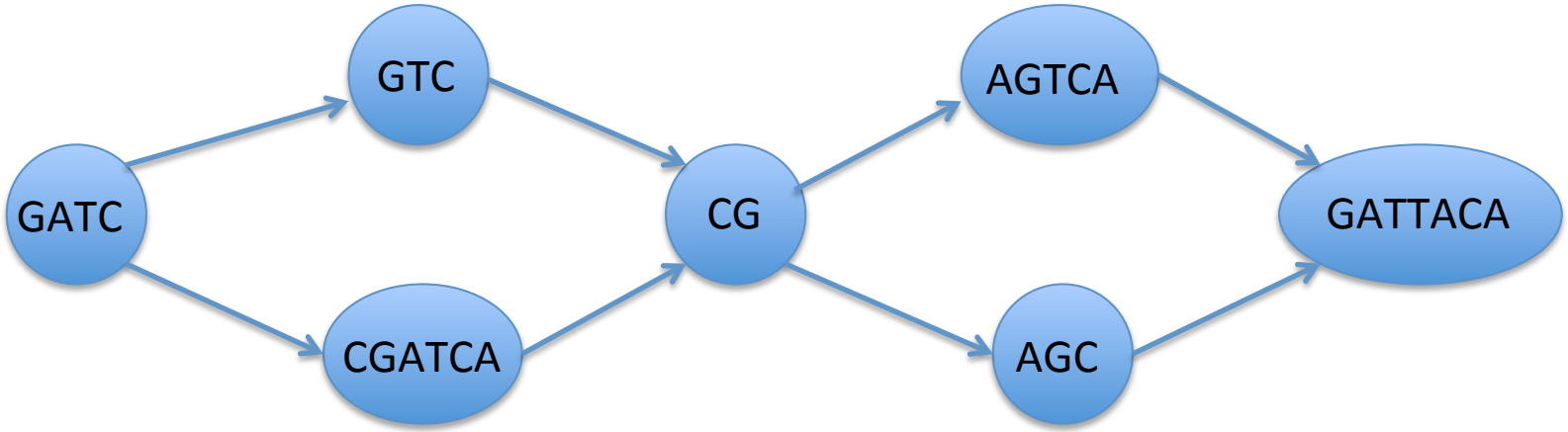
Transcript Reconstruction from RNA-Seq Reads



Graph Data Structures Commonly Used For Assembly

- Sequence
- Order
- Orientation (+, -)
- Overlap

Reads to Graph

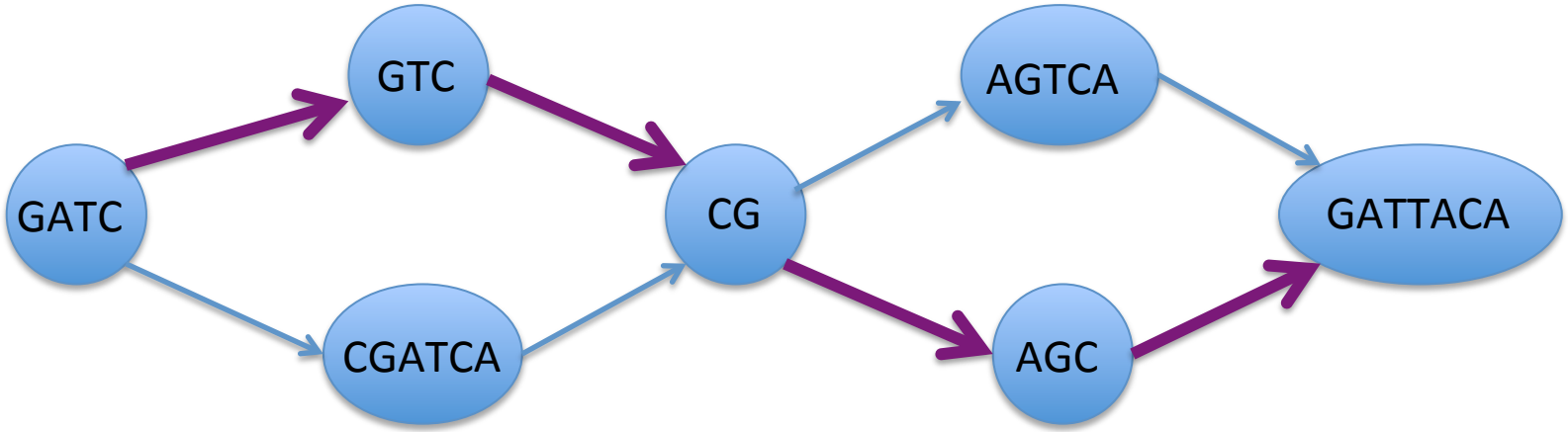


Nodes = sequence (+/-)
Edges = order, overlap

Graph Data Structures Commonly Used For Assembly

- Sequence
- Order
- Orientation (+, -)
- Overlap

Reads to Graph

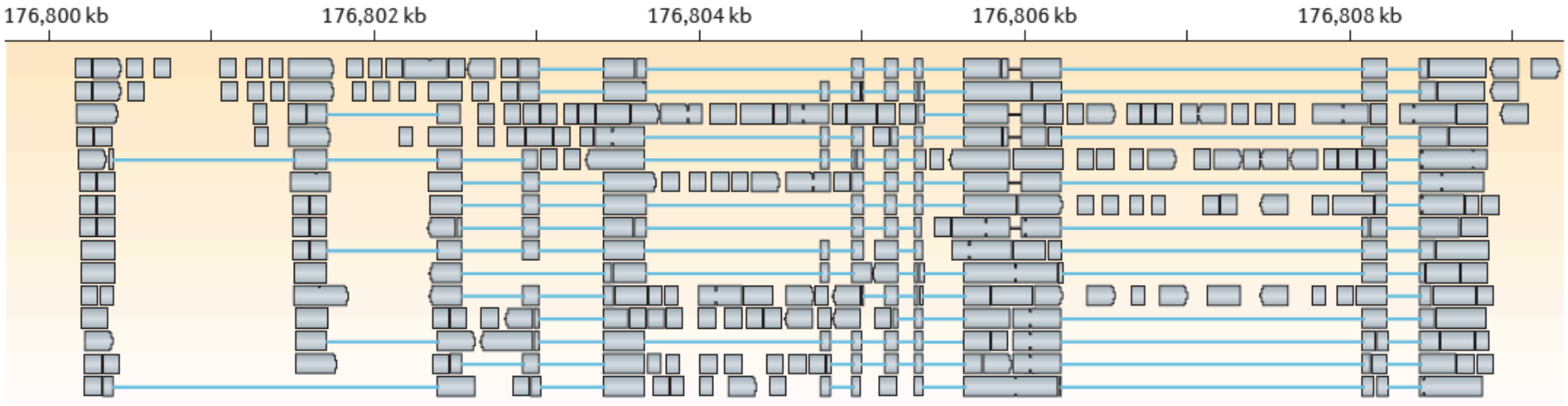


GATCGTCCGAGCGATTACA

Nodes = sequence (+/-)
Edges = order, overlap

Genome-Guided Transcript Reconstruction

Splice-align reads to the genome



From Martin & Wang. Nature Reviews in Genetics. 2011

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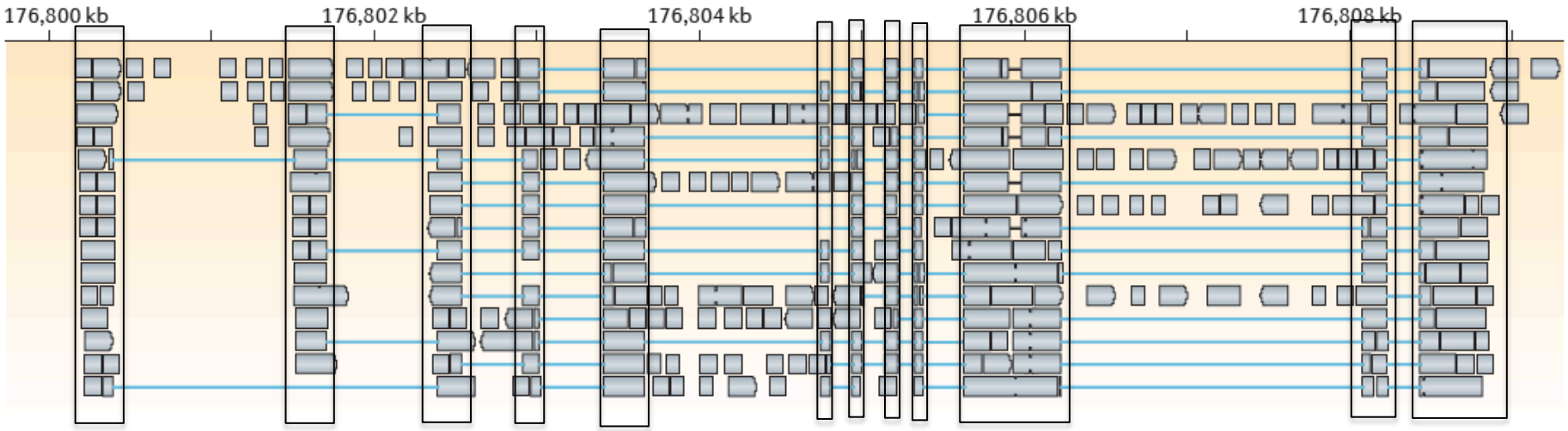
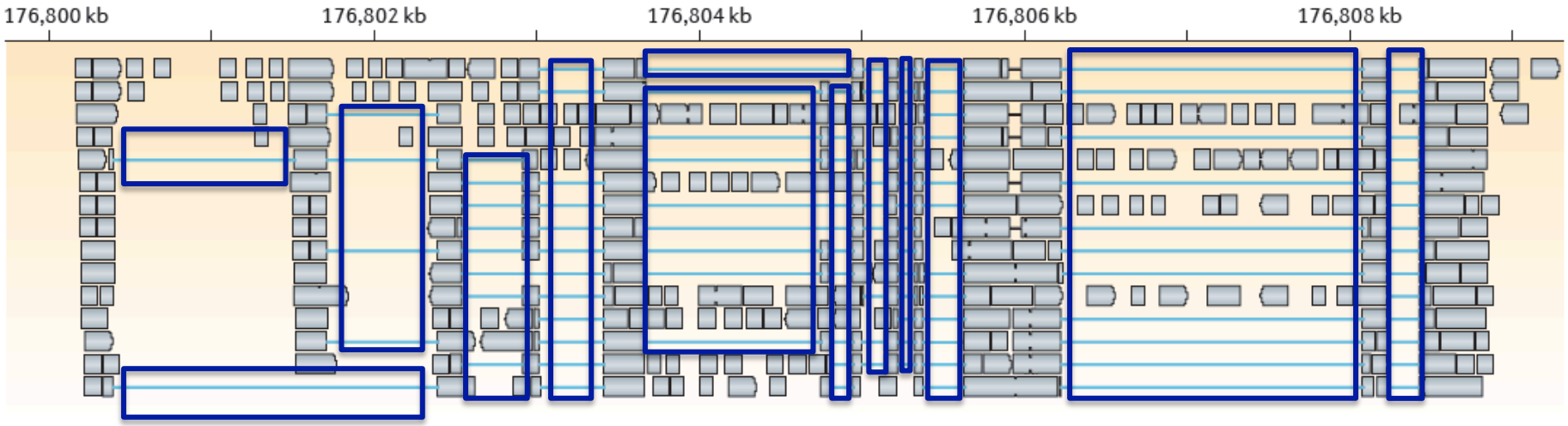


Alignment segment piles => exon regions

From Martin & Wang. Nature Reviews in Genetics. 2011

Genome-Guided Transcript Reconstruction

Splice-align reads to the genome



Large alignment gaps => introns

From Martin & Wang. Nature Reviews in Genetics. 2011

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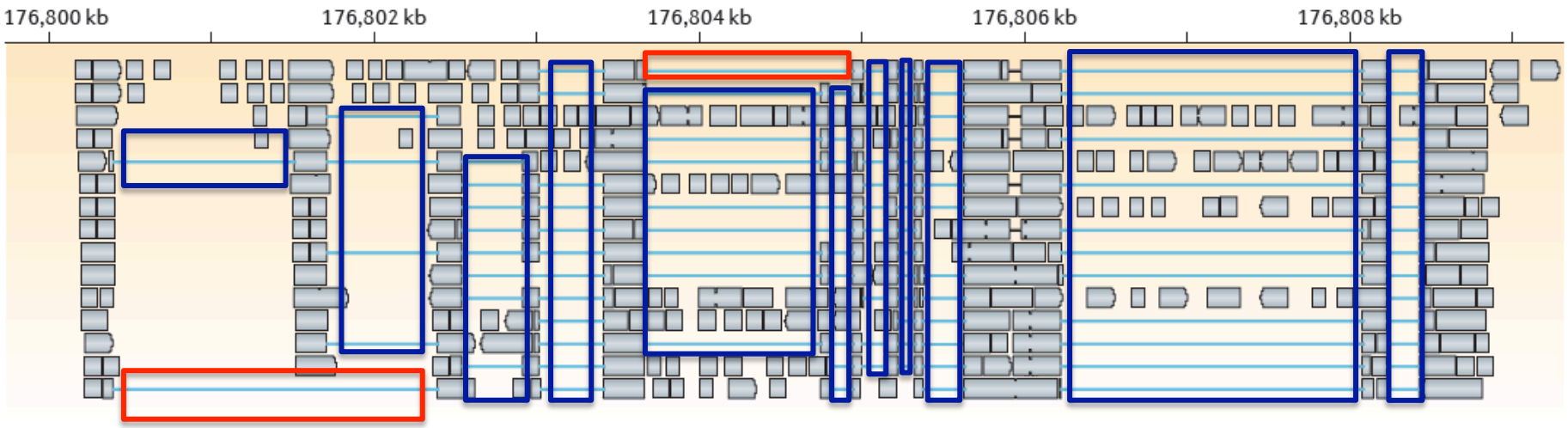
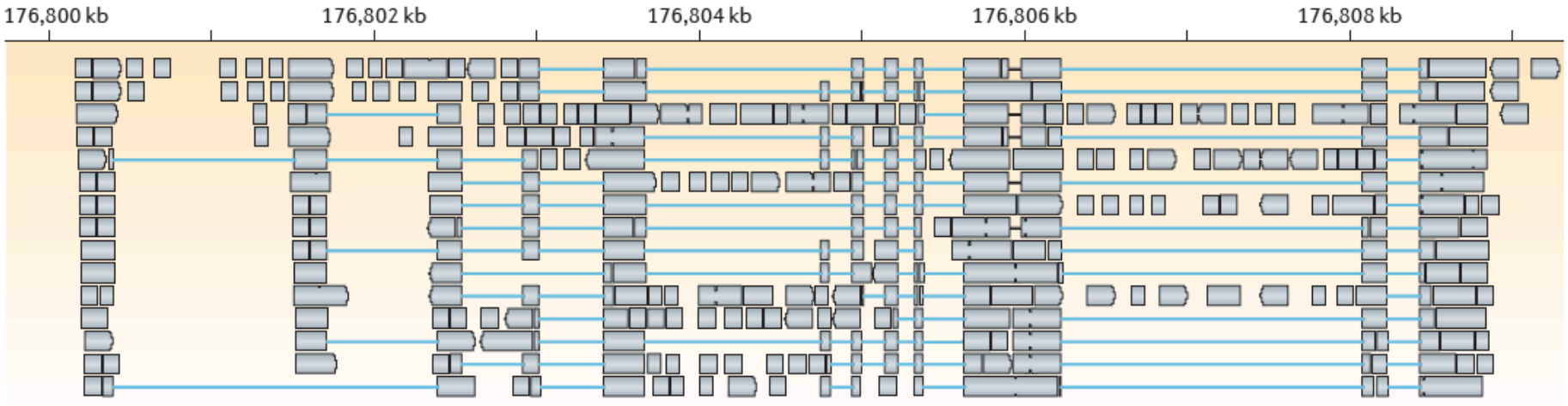


Overlapping but different introns = evidence of alternative splicing

From Martin & Wang. Nature Reviews in Genetics. 2011

Genome-Guided Transcript Reconstruction

Splice-align reads to the genome



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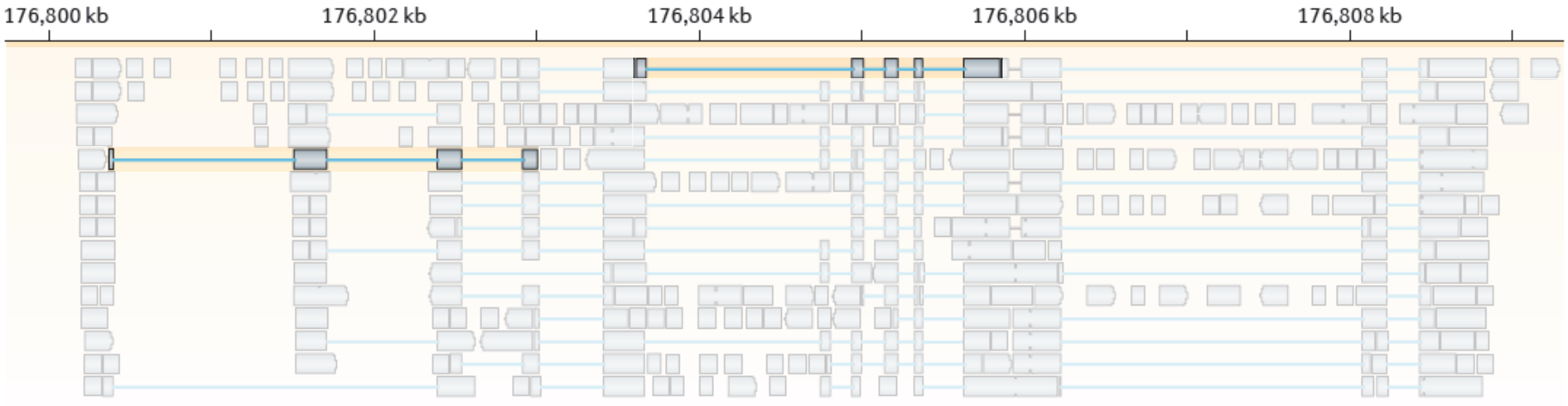
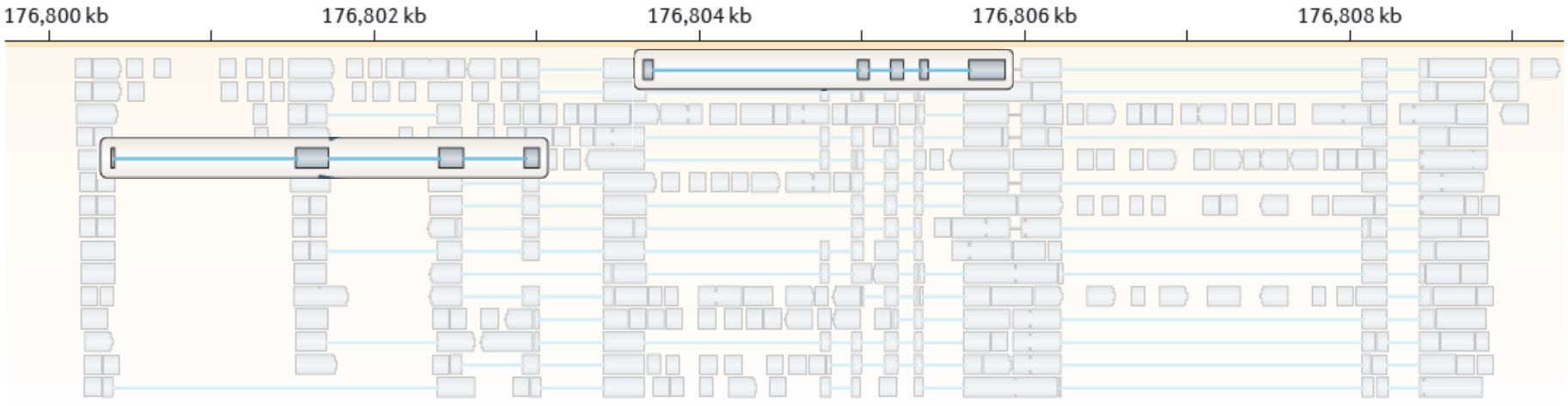


Individual reads can yield multiple exon and intron segments (splice patterns)

From Martin & Wang. Nature Reviews in Genetics. 2011

Genome-Guided Transcript Reconstruction

Splice-align reads to the genome

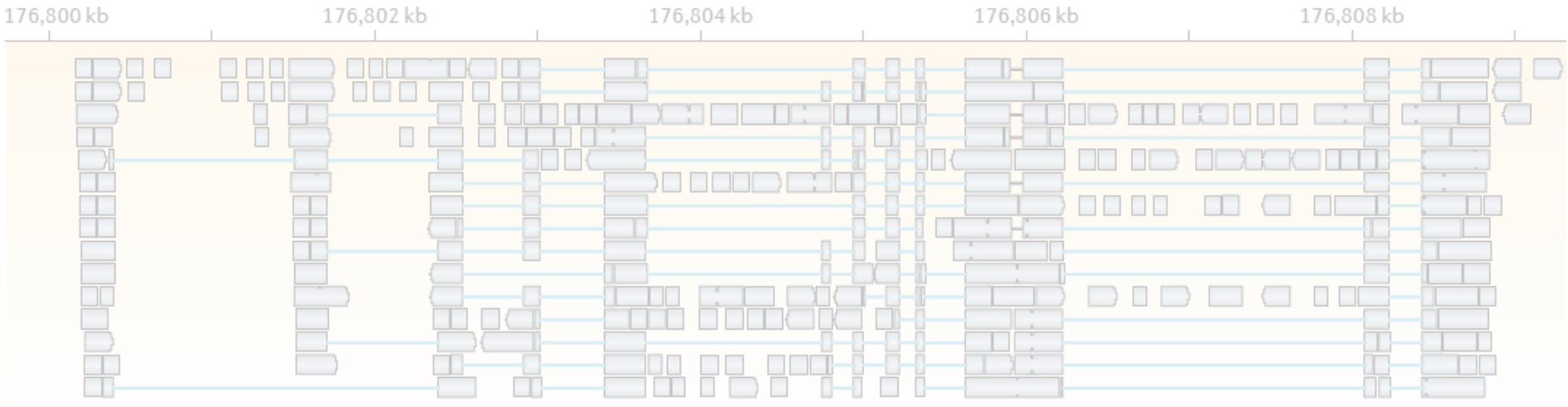


Nodes = unique splice patterns

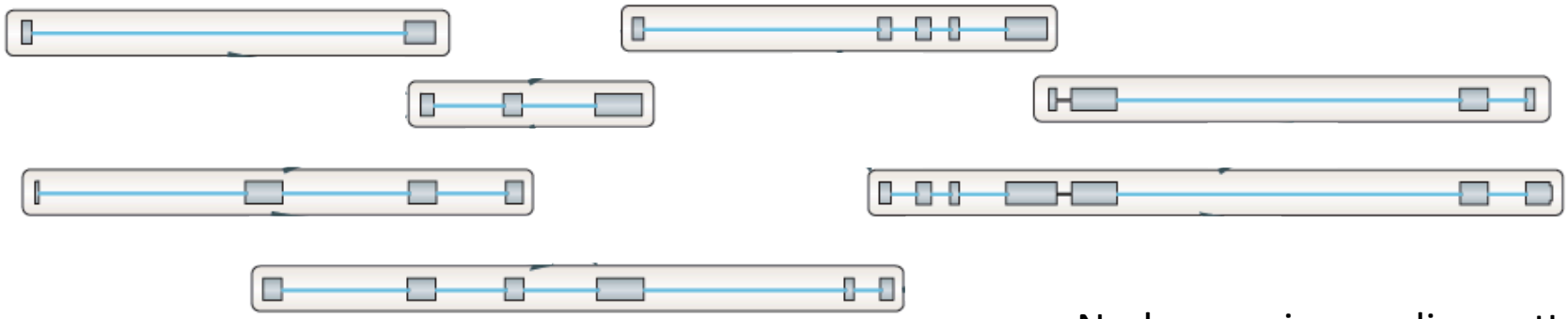
From Martin & Wang. Nature Reviews in Genetics. 2011

Genome-Guided Transcript Reconstruction

Splice-align reads to the genome



Construct graph from unique splice patterns of aligned reads.



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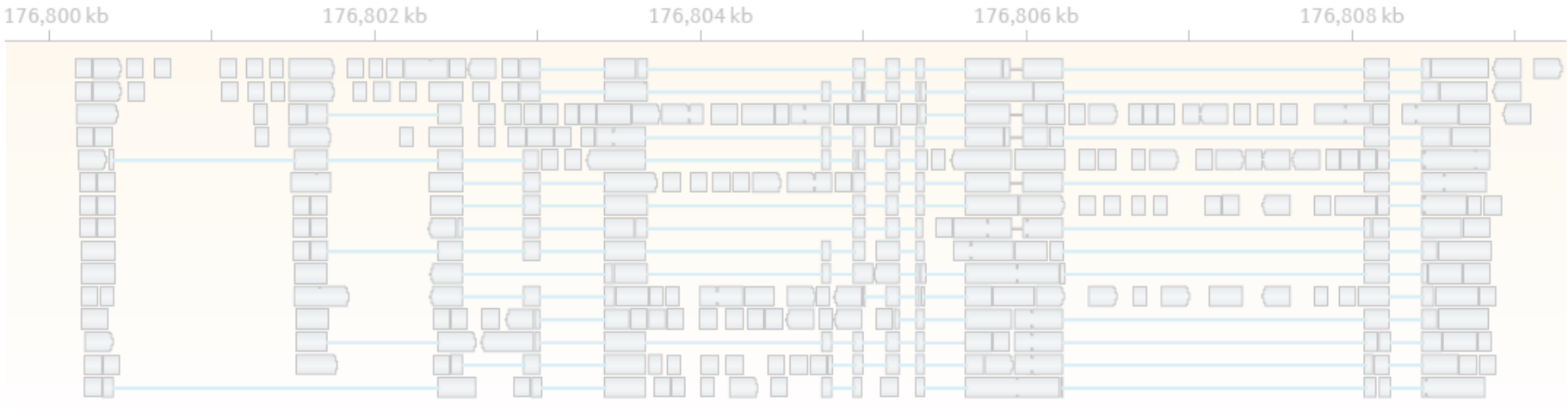

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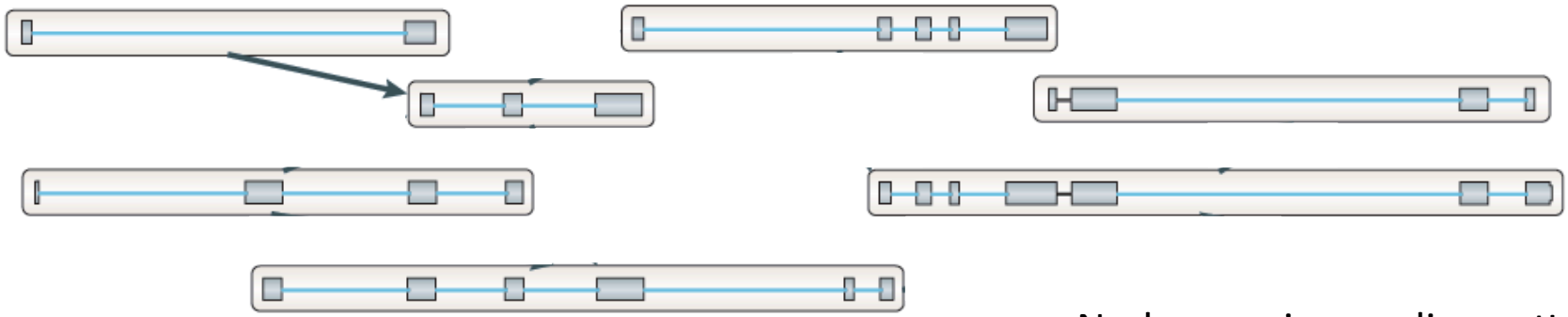

Nodes = unique splice patterns
Edges = compatible patterns

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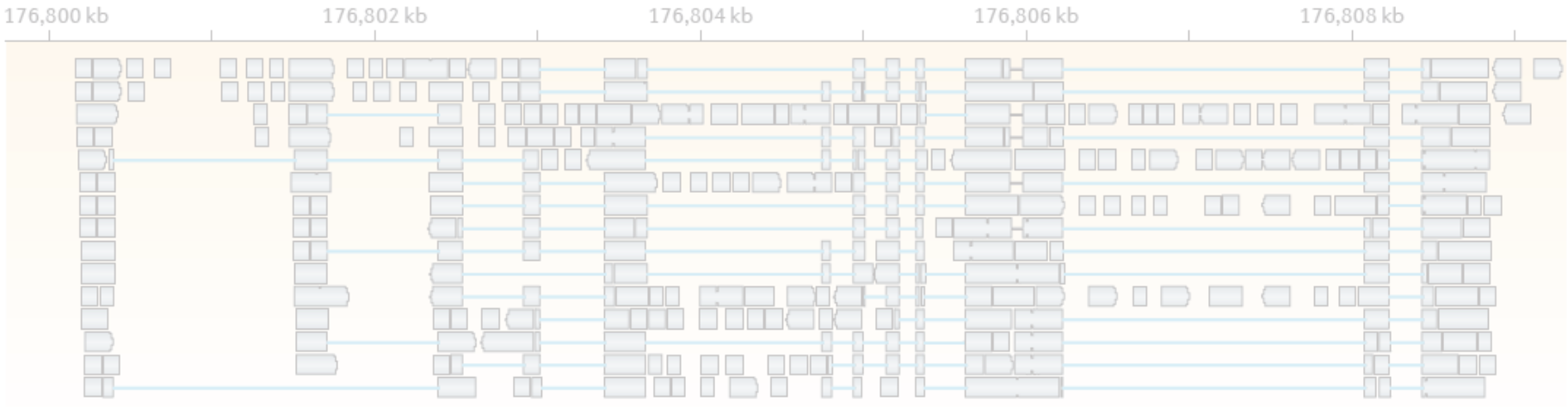

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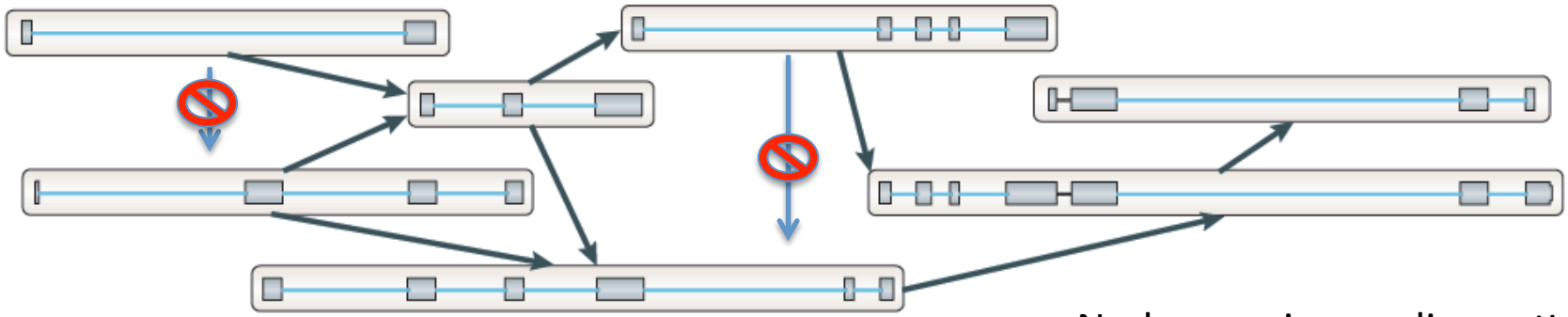

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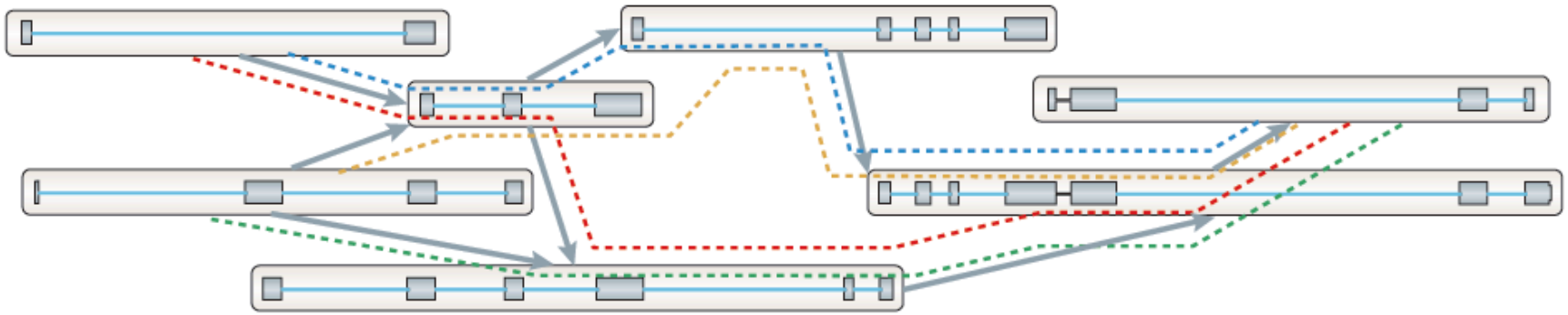
From Martin & Wang. Nature Reviews in Genetics. 2011

Genome-Guided Transcript Reconstruction

Traverse paths through the graph to assemble transcript isoforms



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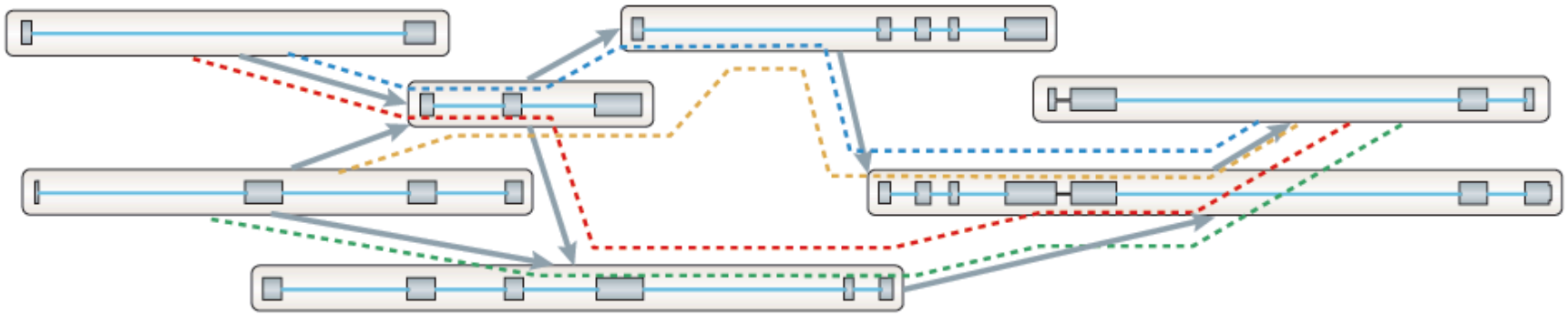
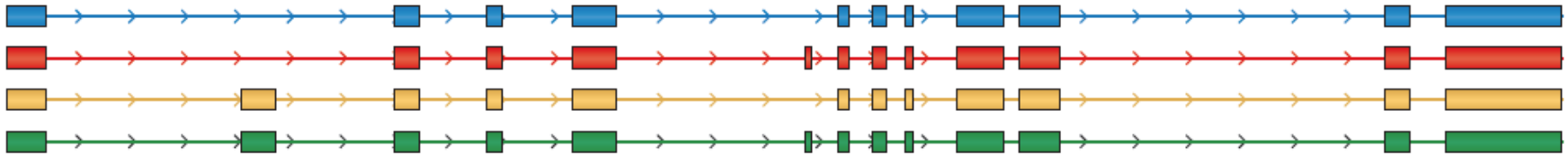


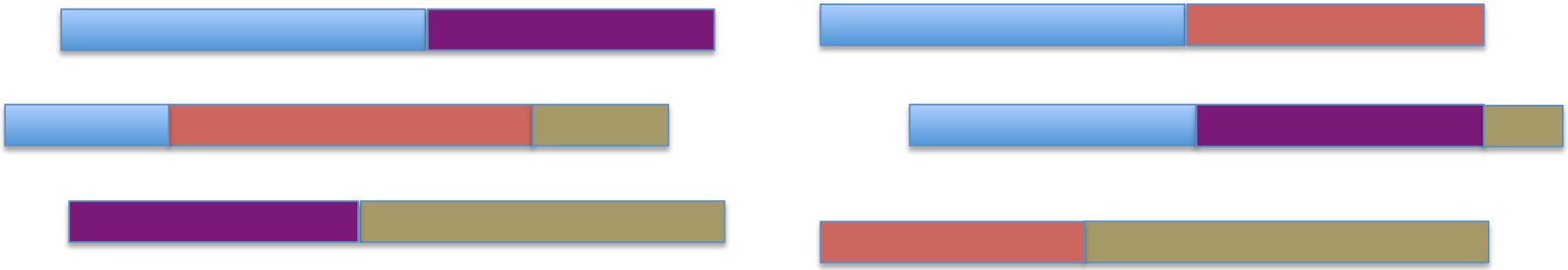
Reconstructed isoforms



From Martin & Wang. Nature Reviews in Genetics. 2011

What if you don’t have a high quality reference genome sequence?

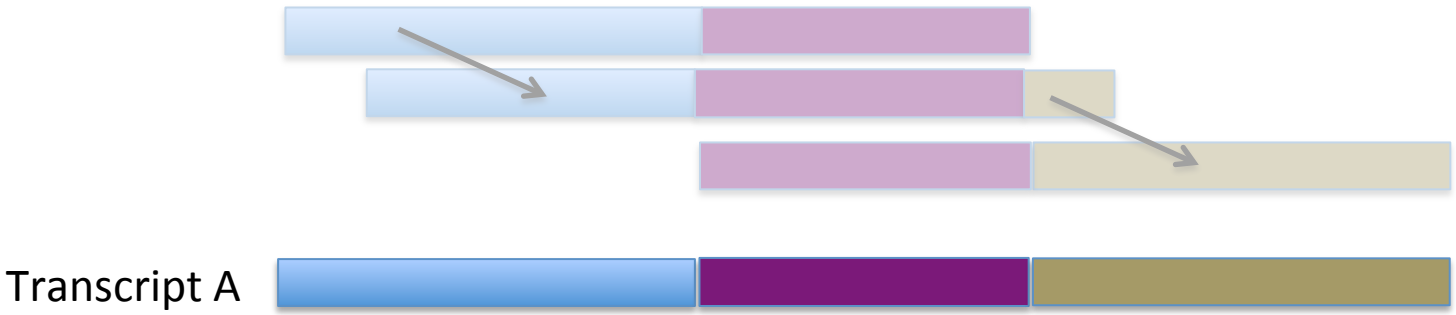
Read Overlap Graph: Reads as nodes, overlaps as edges



Read Overlap Graph: Reads as nodes, overlaps as edges

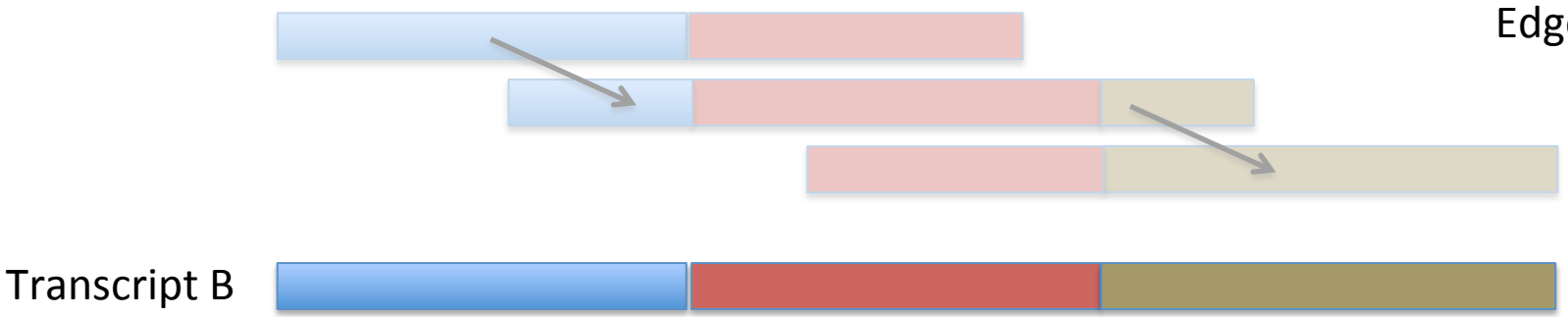
Node = read
Edge = overlap

Read Overlap Graph: Reads as nodes, overlaps as edges

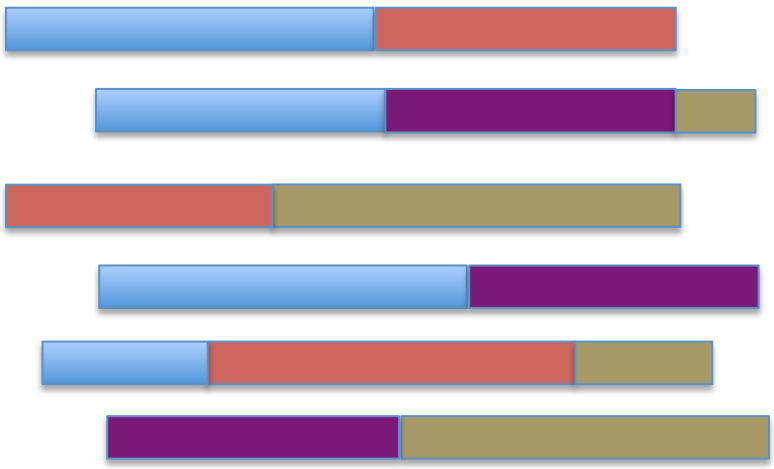


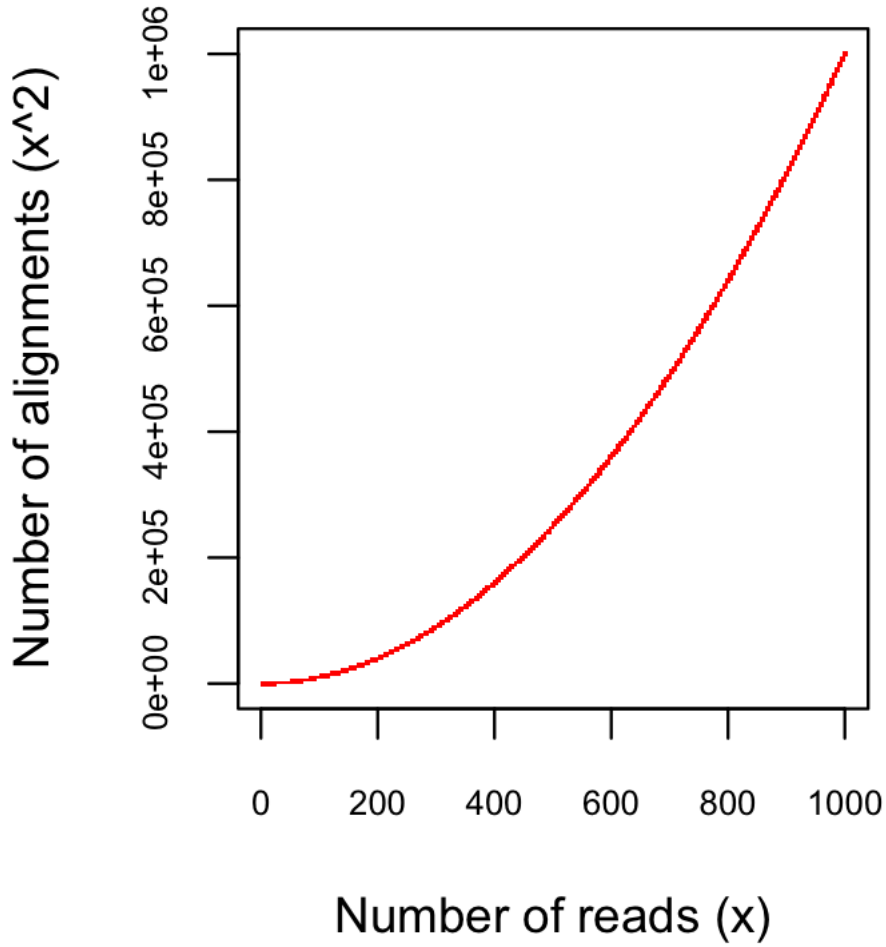
Generate consensus sequence where reads overlap

Node = read
Edge = overlap



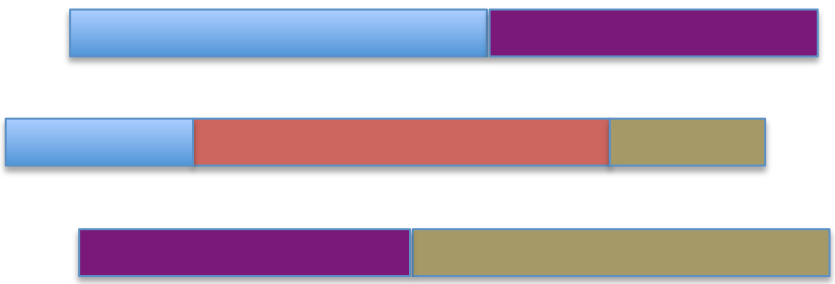
Finding pairwise overlaps between *n* reads involves ~ n^2 comparisons.

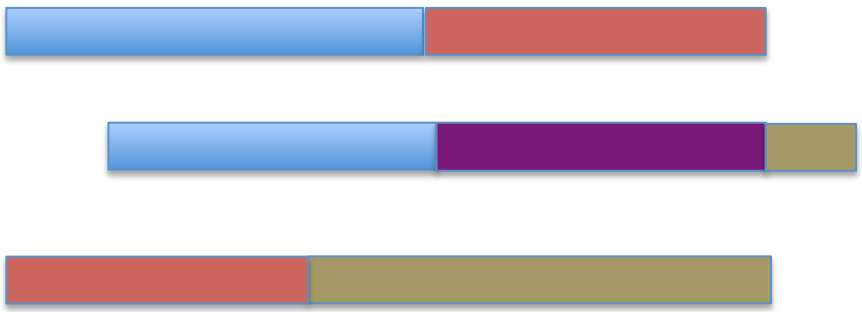




Impractical for typical RNA-Seq data (50M reads)

No genome to align to… **De novo assembly required**



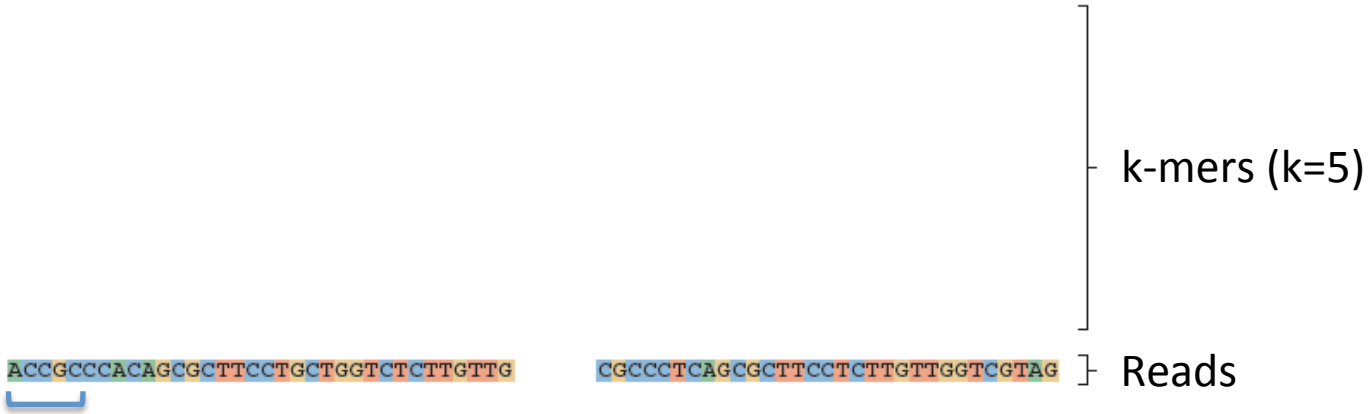


Want to avoid n^2 read alignments to define overlaps

Use a de Bruijn graph

Sequence Assembly via de Bruijn Graphs

Generate all substrings of length k from the reads



From Martin & Wang, Nat. Rev. Genet. 2011

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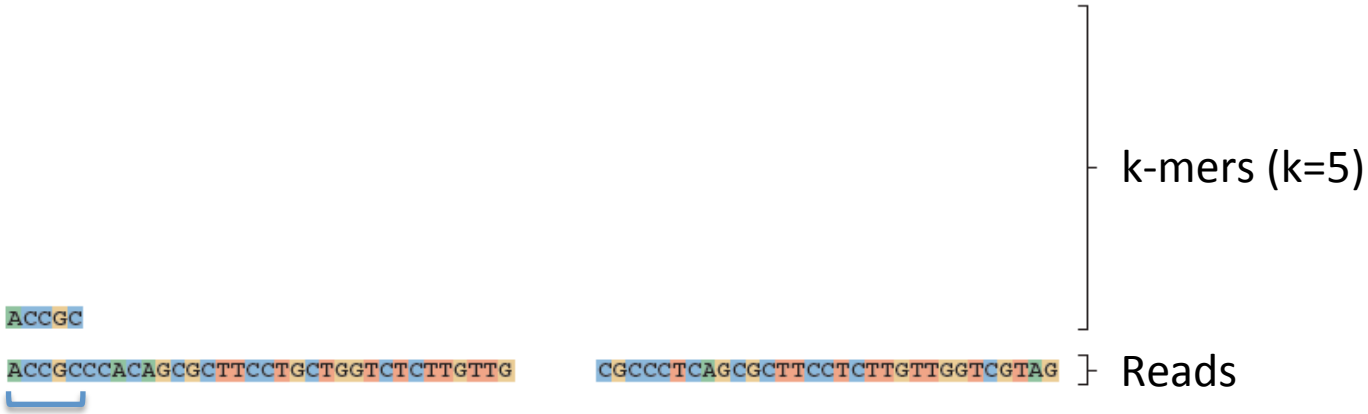

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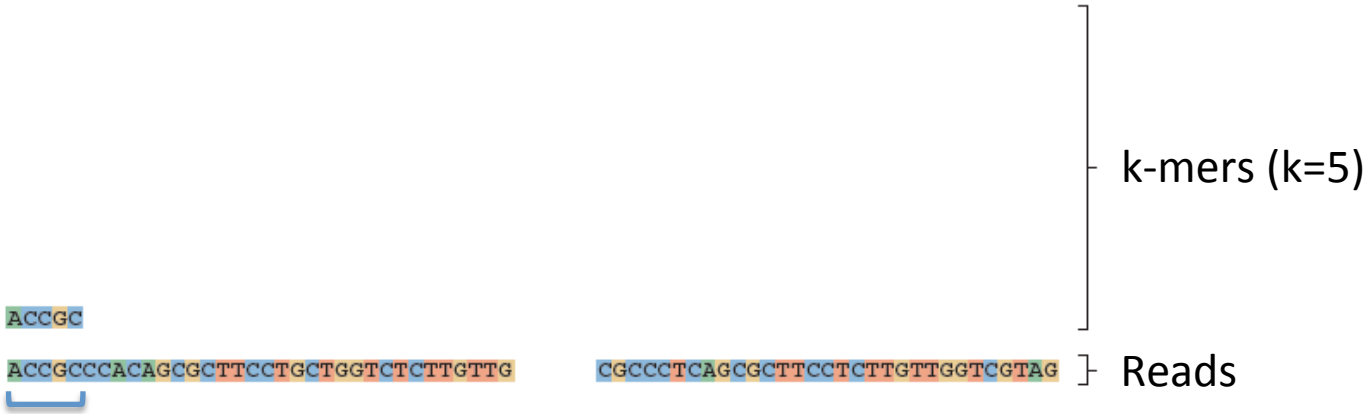

Construct the de Bruijn graph

Nodes = unique k-mers

From Martin & Wang, Nat. Rev. Genet. 2011

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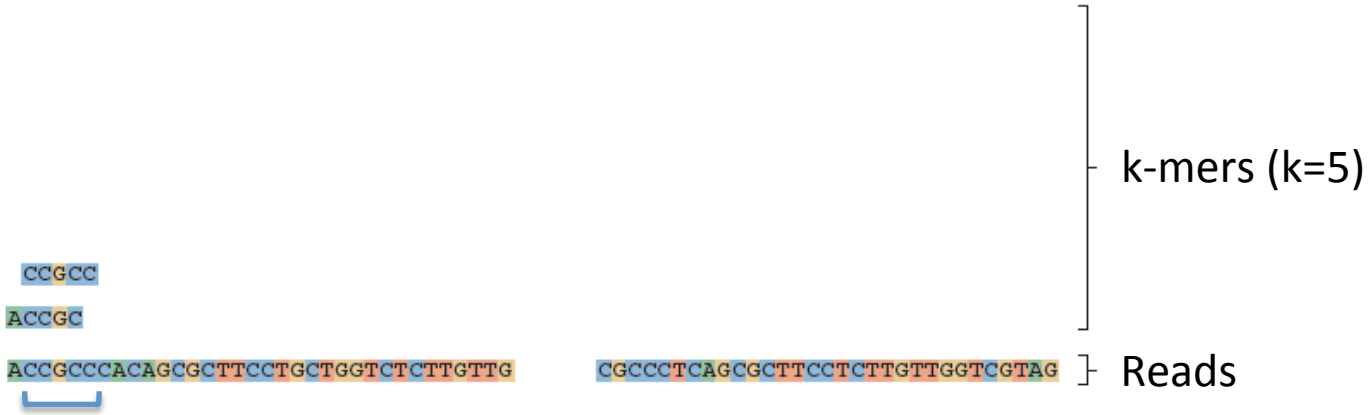

Construct the de Bruijn graph

Nodes = unique k-mers
Edges = overlap by (k-1)

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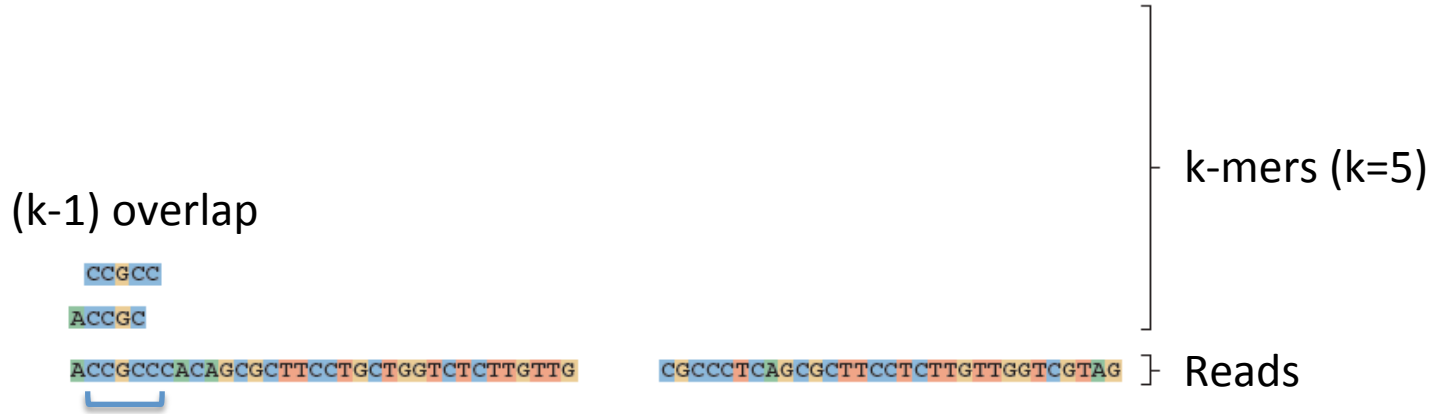

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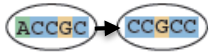

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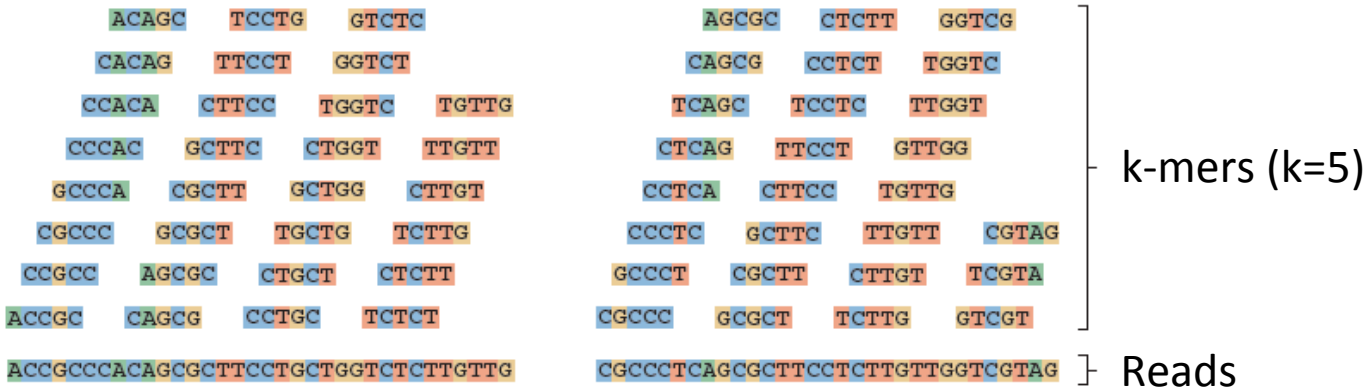

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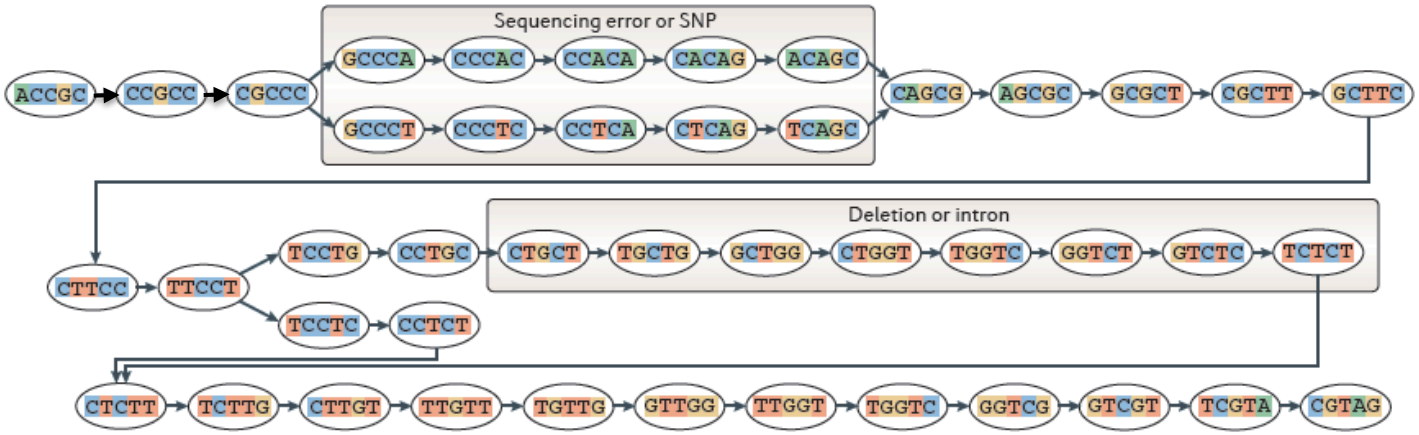

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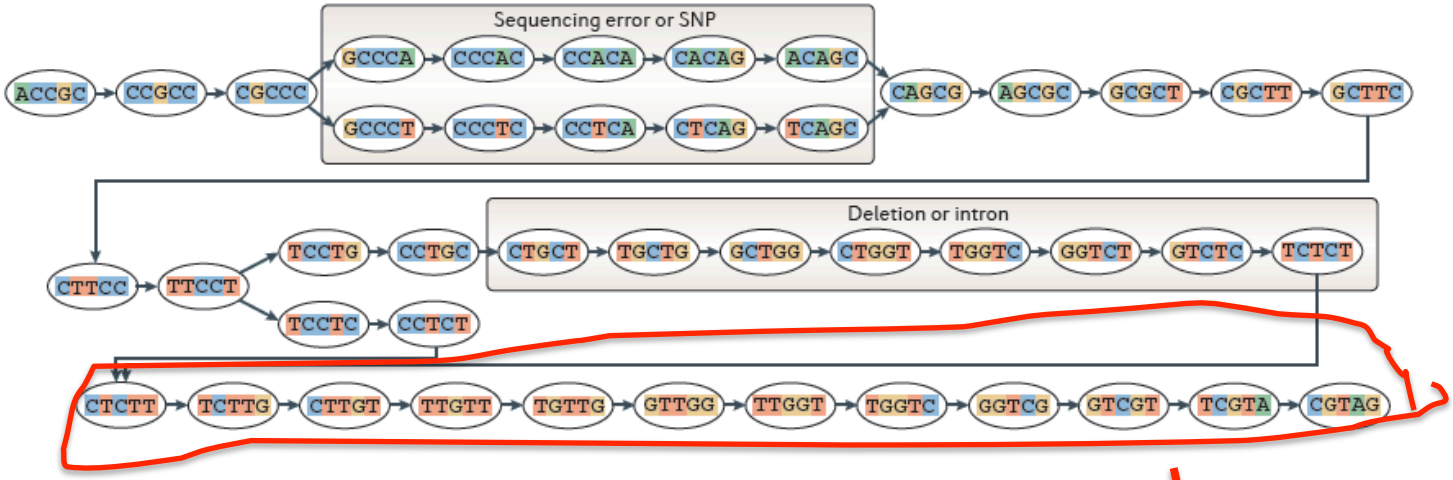
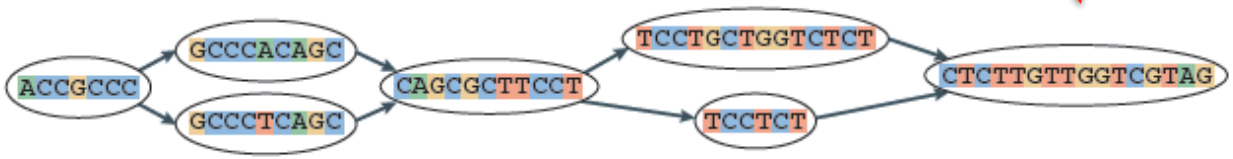


Collapse the de Bruijn graph



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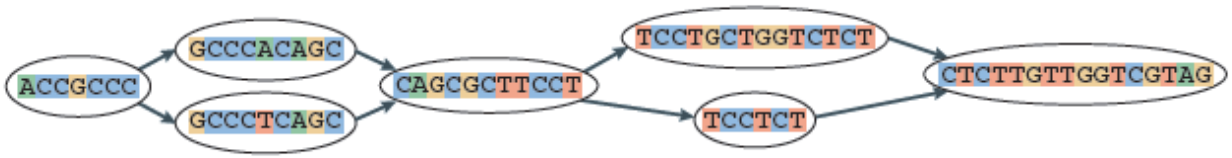
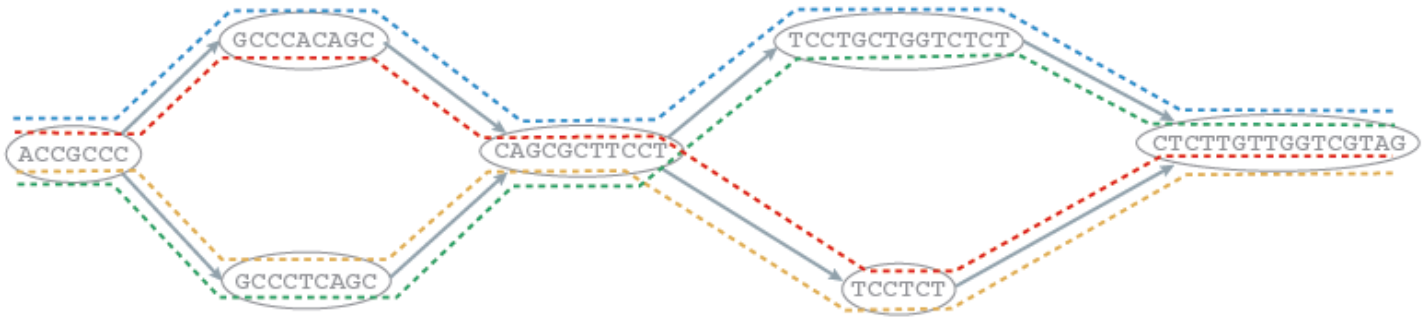


Traverse the graph



Assemble Transcript Isoforms

```
------ ACCGCCCACAGCGCTTCCTGCTGGTCTCTTGTTGGTCGTAG
------ ACCGCCCACAGCGCTTCCT--------CTTGTTGGTCGTAG
------ ACCGCCCTCAGCGCTTCCT--------CTTGTTGGTCGTAG
------ ACCGCCCTCAGCGCTTCCTGCTGGTCTCTTGTTGGTCGTAG
```

From Martin & Wang, Nat. Rev. Genet. 2011

Contrasting Genome and Transcriptome *De novo* Assembly

Genome Assembly

- Uniform coverage
- Single contig per locus
- Assemble small numbers of large Mb-length chromosomes
- Double-stranded data

Transcriptome Assembly

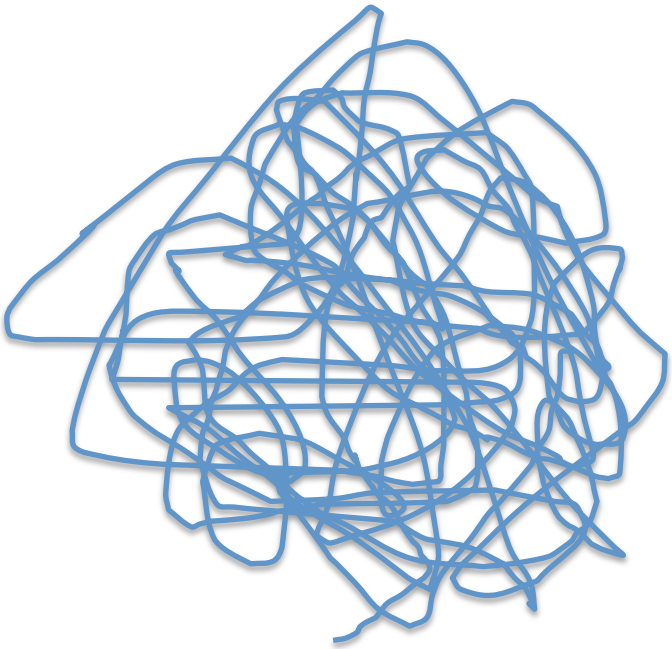
- Exponentially distributed coverage levels
- Multiple contigs per locus (alt splicing)
- Assemble many thousands of Kb-length transcripts
- Strand-specific data available

Trinity

Trinity Aggregates Isolated Transcript Graphs

Genome Assembly

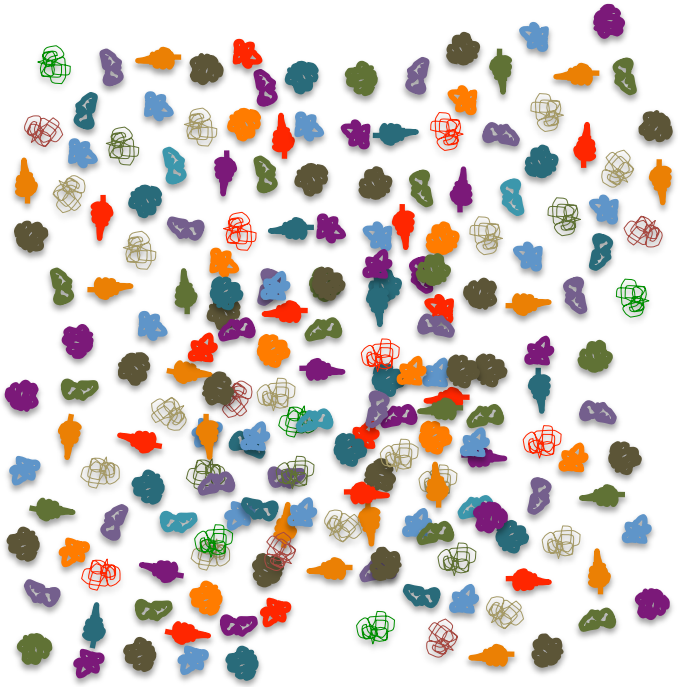
Single Massive Graph



Entire chromosomes represented.

Trinity Transcriptome Assembly

Many Thousands of Small Graphs



Ideally, one graph per expressed gene.

Trinity – How it works:

RNA-Seq reads → **Linear contigs** → **de-Bruijn graphs** → **Transcripts + Isoforms**

Thousands of disjoint graphs

...CTTCGCAA...TGATCGGAT...
...ATTCGCAA...TCATCGGAT...

Inchworm Algorithm

- Decompose all reads into overlapping Kmers => hashtable(kmer, count)

Read: **AATGTGAAAACTGGATTACATGCTGGTATGTC**…

AATGTGA

ATGTGAA Overlapping kmers of length (k)

TGTGAAA

…

Kmer Catalog (hashtable)

Kmer	Count among all reads
AATGTGA	4
ATGTGAA	2
TGTGAAA	1
GATTACA	9

Inchworm Algorithm

- Decompose all reads into overlapping Kmers => hashtable(kmer, count)
- Identify seed kmer as most abundant Kmer, ignoring low-complexity kmers.

GATTACA
9

Kmer Catalog (hashtable)

Kmer	Count among all reads
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TGTGAAA	1
GATTACA	9

Inchworm Algorithm

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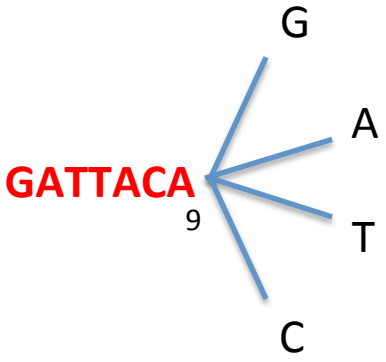
- Identify seed kmer as most abundant Kmer, ignoring low-complexity kmers.
- Extend kmer at 3' end, guided by coverage.



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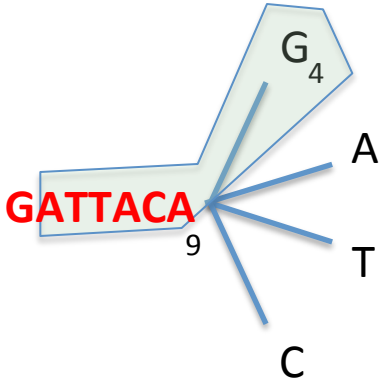

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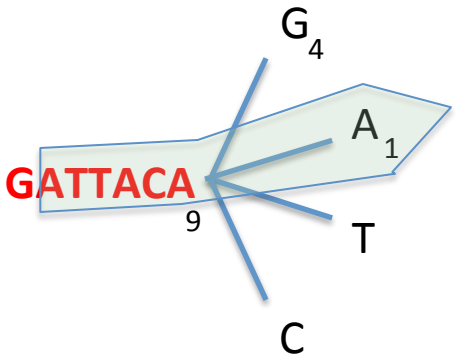
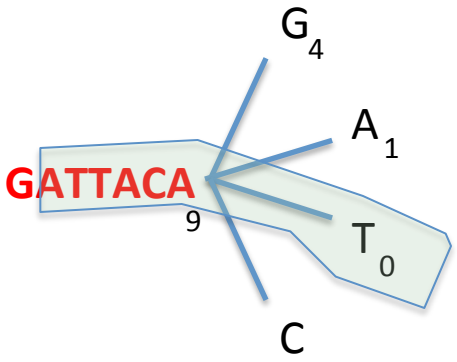


Inchworm Algorithm



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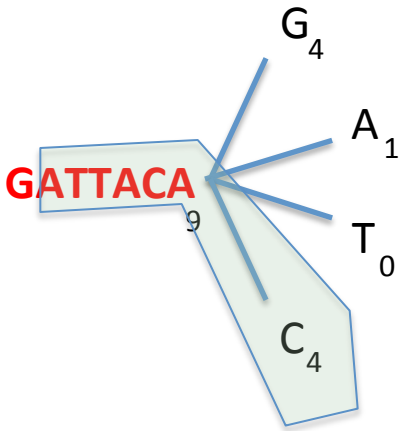
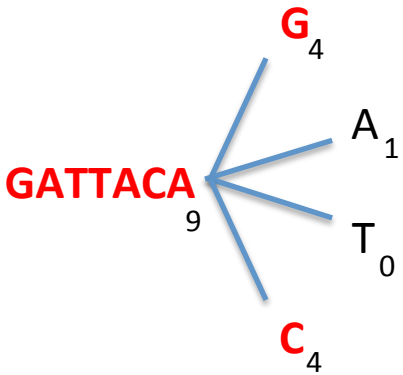
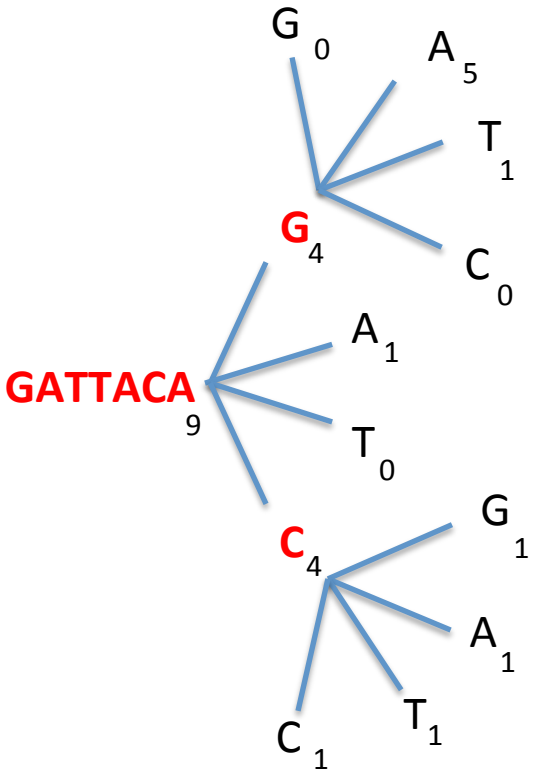


Inchworm Algorithm



Inchworm Algorithm



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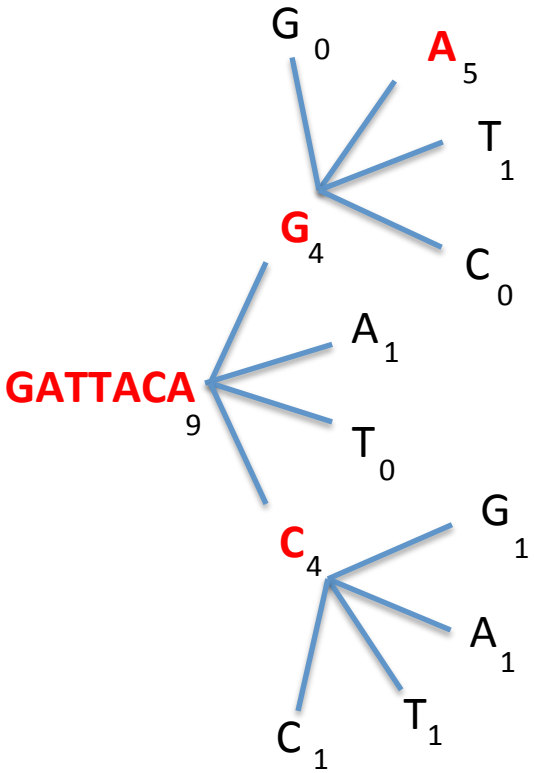

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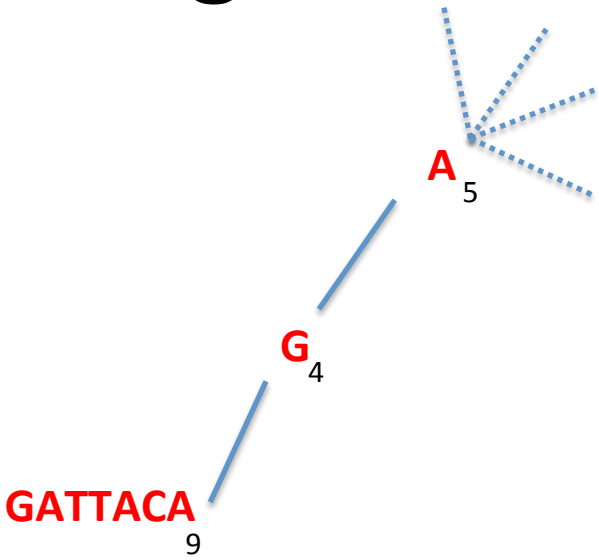

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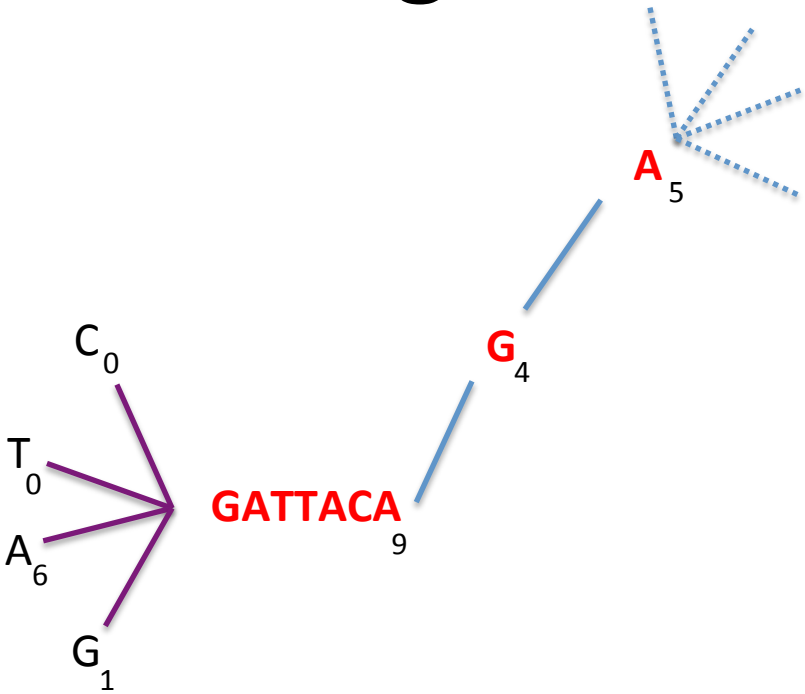

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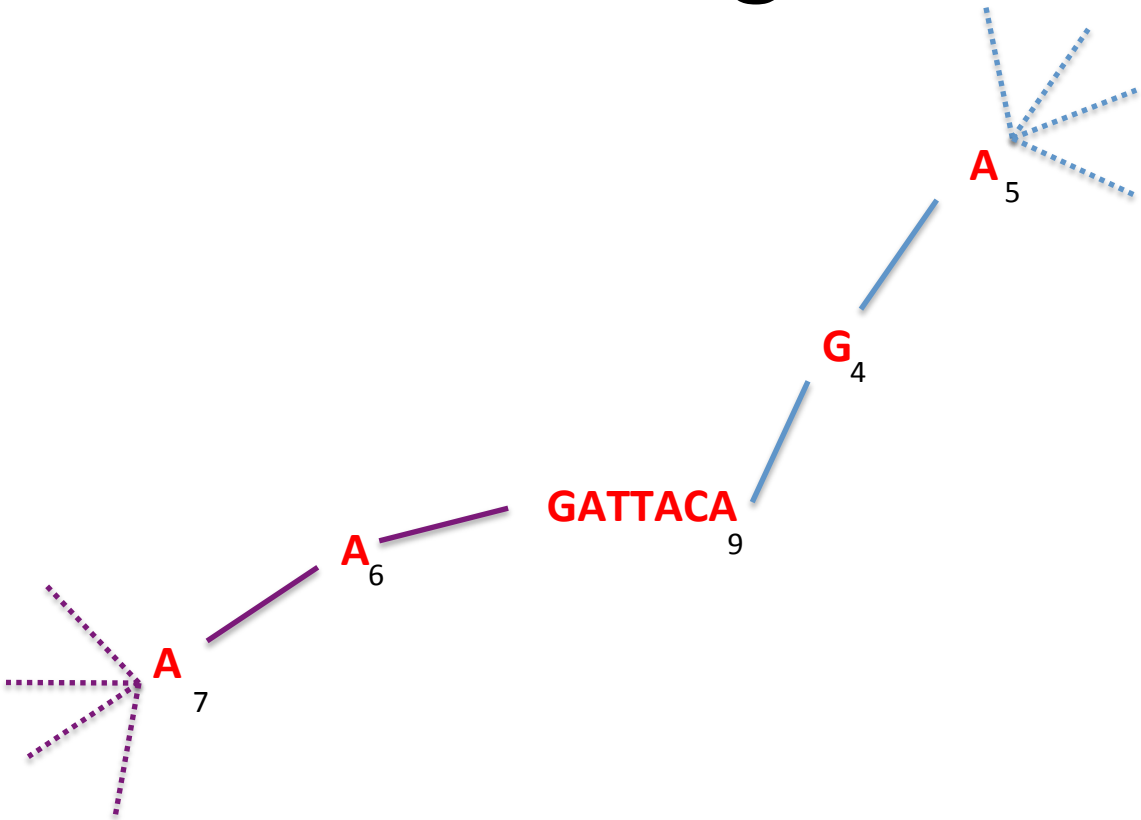


Report contig: ....AAGATTACAGA....

Remove assembled kmers from catalog, then repeat the entire process.

Inchworm Contigs from Alt-Spliced Transcripts

Expressed isoforms

Isoform A

Isoform B

Inchworm Contigs from Alt-Spliced Transcripts

Expressed isoforms

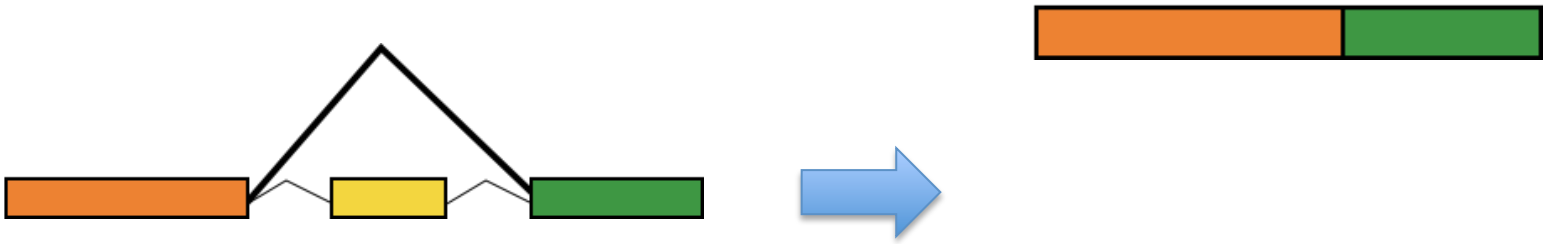
Expression

Isoform A (low)

Isoform B (high)

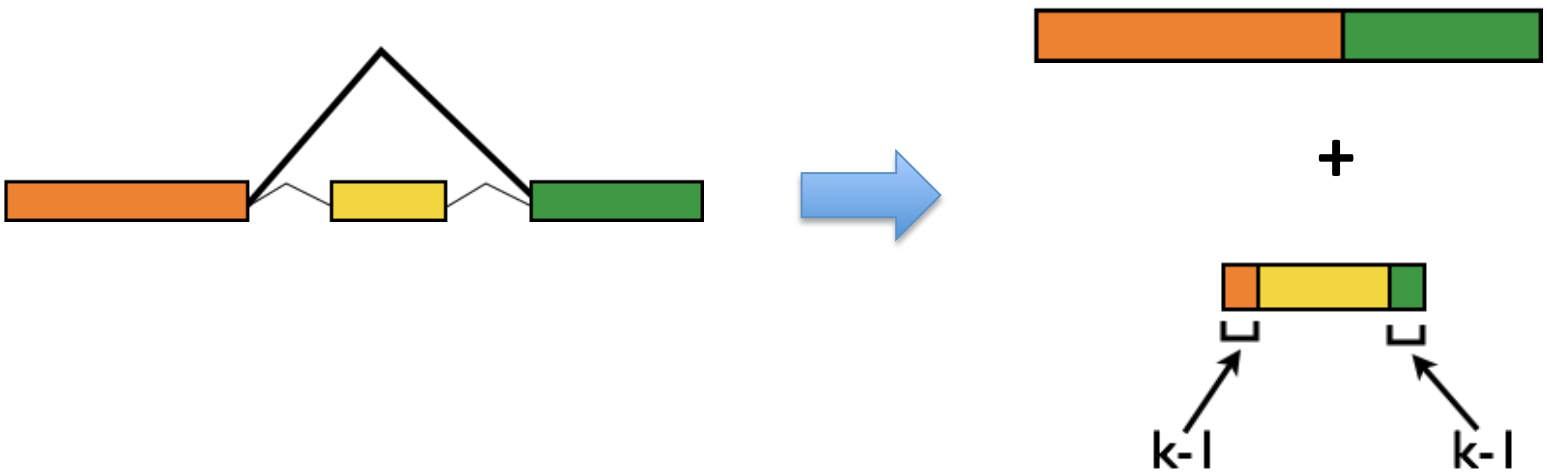
Graphical representation

Inchworm Contigs from Alt-Spliced Transcripts

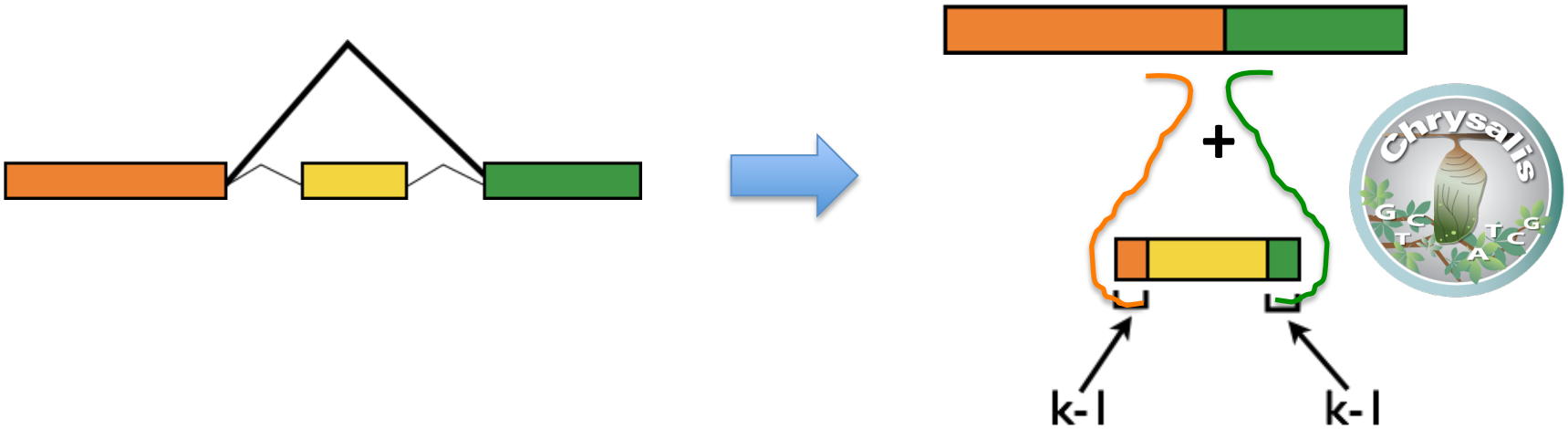


Inchworm Contigs from Alt-Spliced Transcripts

Inchworm Contigs from Alt-Spliced Transcripts

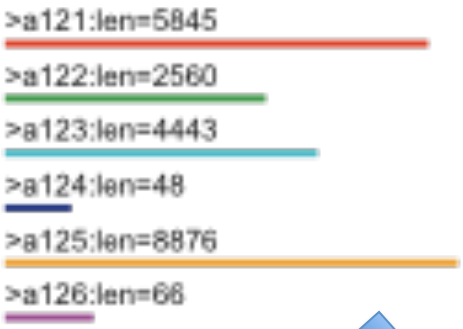


Chrysalis Re-groups Related Inchworm Contigs



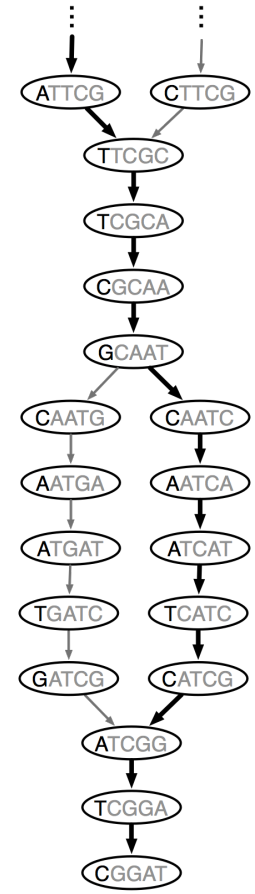
Chrysalis uses (k-1) overlaps and read support to link related Inchworm contigs

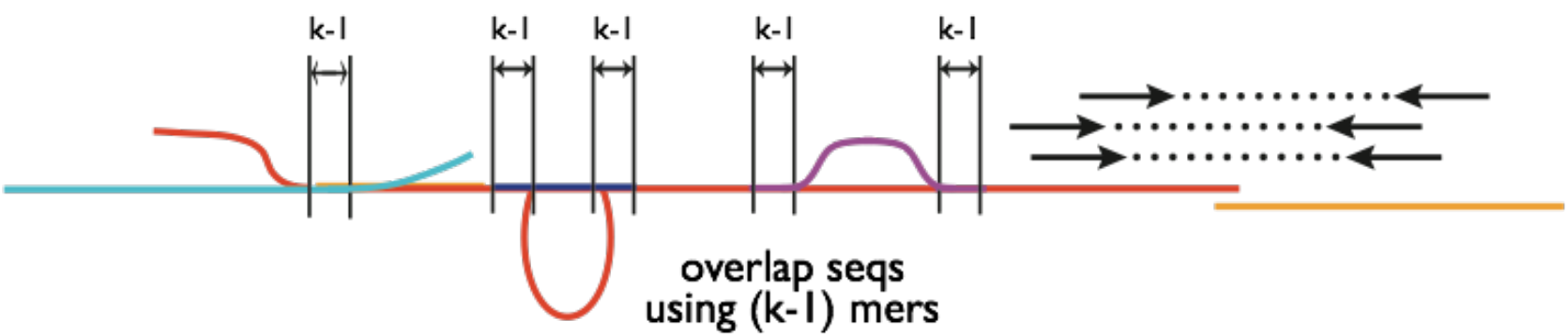
Chrysalis



Integrate isoforms via k-1 overlaps

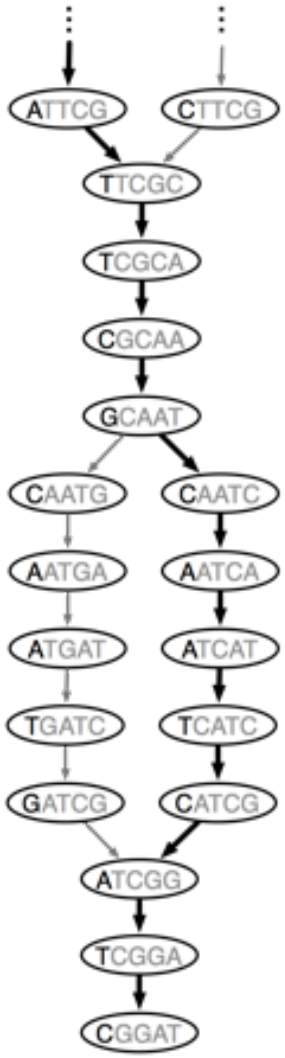
Build de Bruijn Graphs (ideally, one per gene)





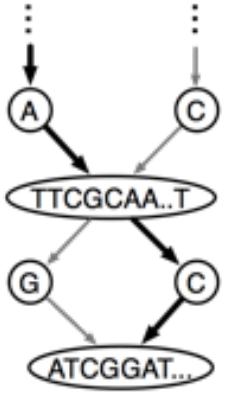

Thousands of Chrysalis Clusters

Butterfly



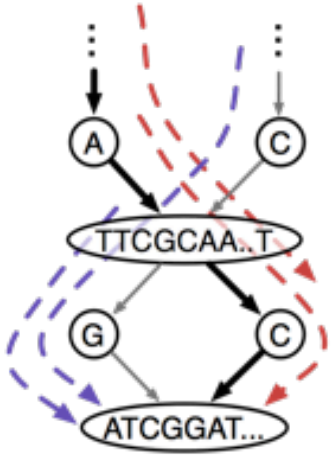
de Bruijn graph

compacting



compact graph

finding paths



compact graph with reads

extracting sequences

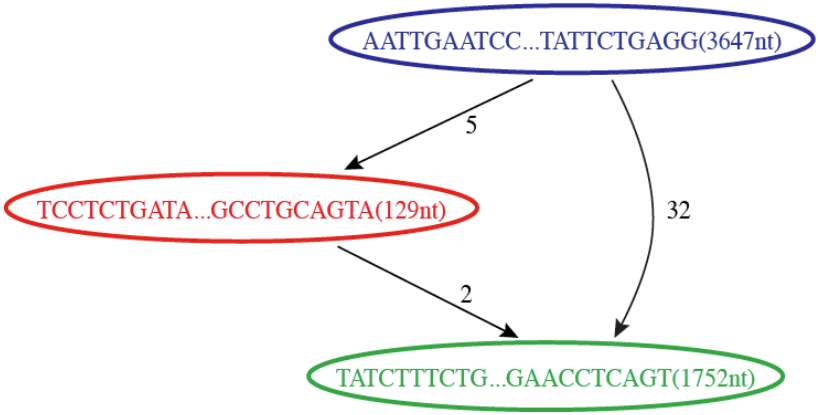
..CTTCGCAA..TGATCGGAT...

..ATTCGCAA..TCATCGGAT...

sequences (isoforms and paralogs)

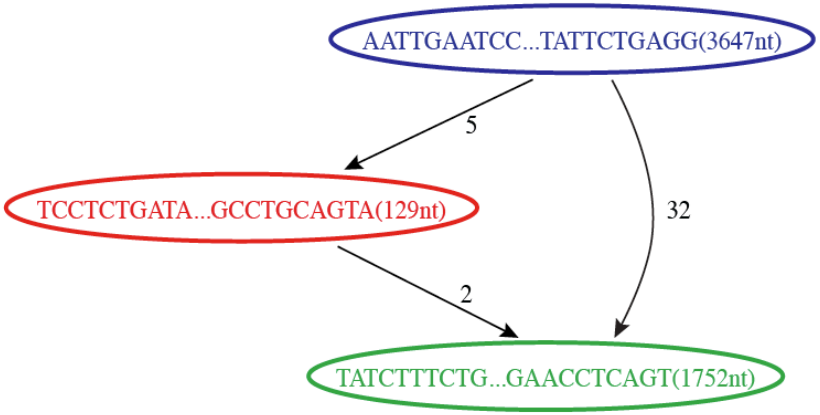
Butterfly Example 1:
Reconstruction of Alternatively Spliced Transcripts

Butterfly's Compacted Sequence Graph



Reconstruction of Alternatively Spliced Transcripts

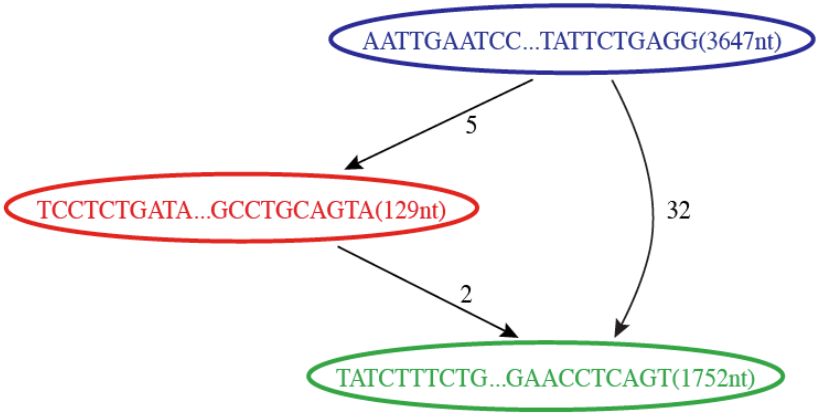
Butterfly's Compacted Sequence Graph



Reconstructed Transcripts

Reconstruction of Alternatively Spliced Transcripts

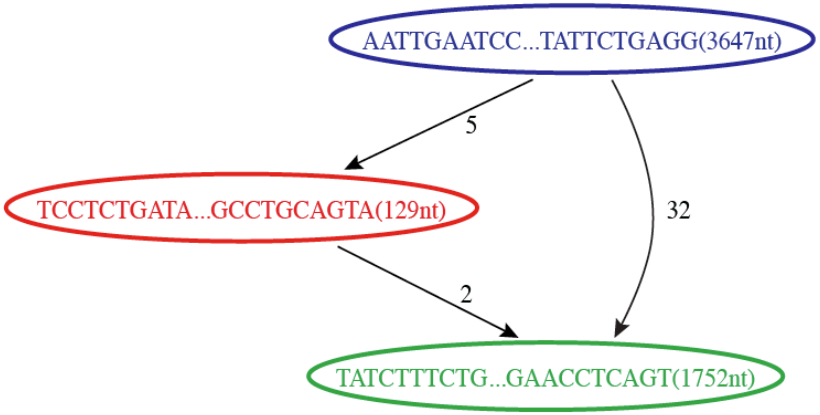
Butterfly's Compacted Sequence Graph



Reconstructed Transcripts

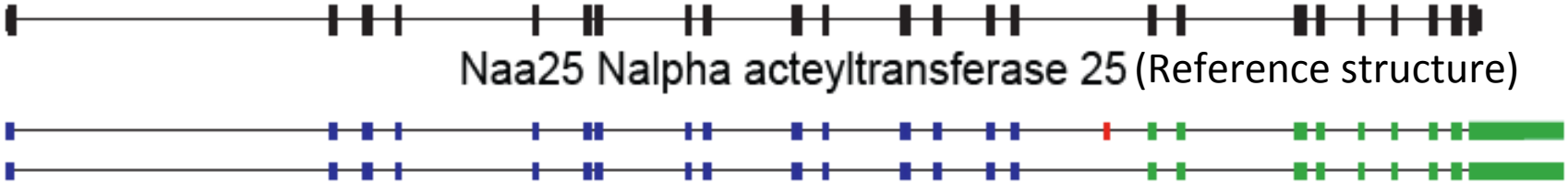
Reconstruction of Alternatively Spliced Transcripts

Butterfly's Compacted Sequence Graph



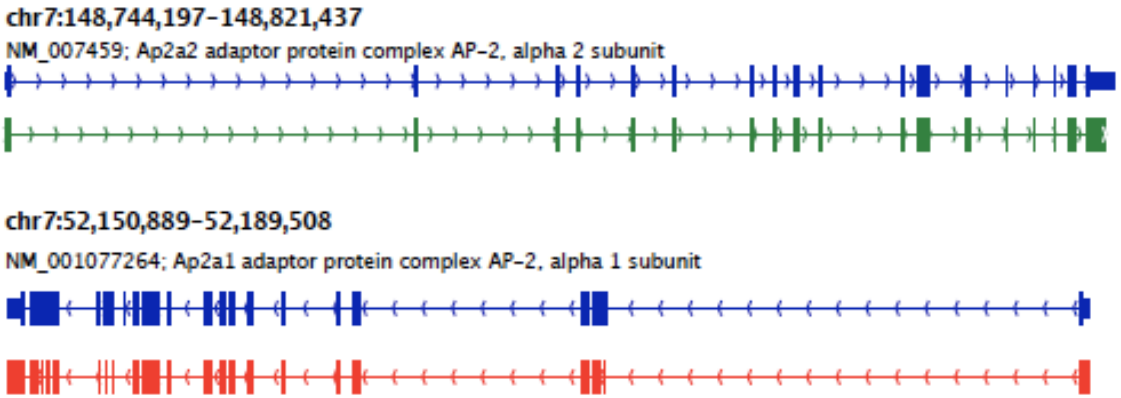
Reconstructed Transcripts

Aligned to Mouse Genome



Butterfly Example 2:
Teasing Apart Transcripts of Paralogous Genes

Teasing Apart Transcripts of Paralogous Genes



Trinity output: A multi-fasta file

```
>comp0_c0_seq1 len=5528 path=[1:0-3646 10775:3647-3775 3648:3776-5527]
```

```
>comp0_c0_seq2 len=5399 path=[1:0-3646 3648:3647-5398]
```

nature protocols

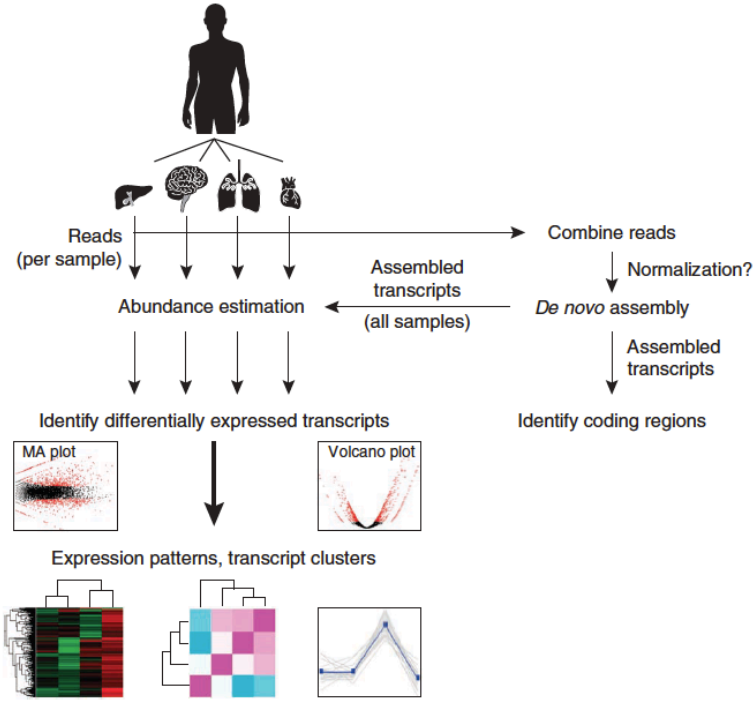
NATURE PROTOCOLS | PROTOCOL

De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis

Brian J Haas, Alexie Papanicolaou, Moran Yassour, Manfred Grabherr, Philip D Blood, Joshua Bowden, Matthew Brian Couger, David Eccles, Bo Li, Matthias Lieber, Matthew D MacManes, Michael Ott, Joshua Orvis, Nathalie Pochet, Francesco Strozzi, Nathan Weeks, Rick Westerman, Thomas William, Colin N Dewey, Robert Henschel, Richard D LeDuc, Nir Friedman & Aviv Regev

Affiliations | Contributions | Corresponding authors

Nature Protocols **8**, 1494–1512 (2013) | doi:10.1038/nprot.2013.084
Published online 11 July 2013



RNA-Seq De novo Assembly Using Trinity

Quick Guide for the Impatient

Trinity assembles transcript sequences from Illumina RNA-Seq data.

Download Trinity here.

Build Trinity by typing 'make' in the base installation directory.

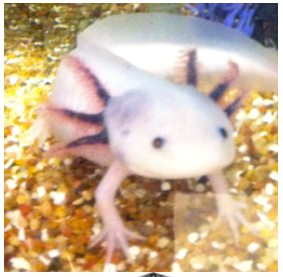
Assemble RNA-Seq data like so:

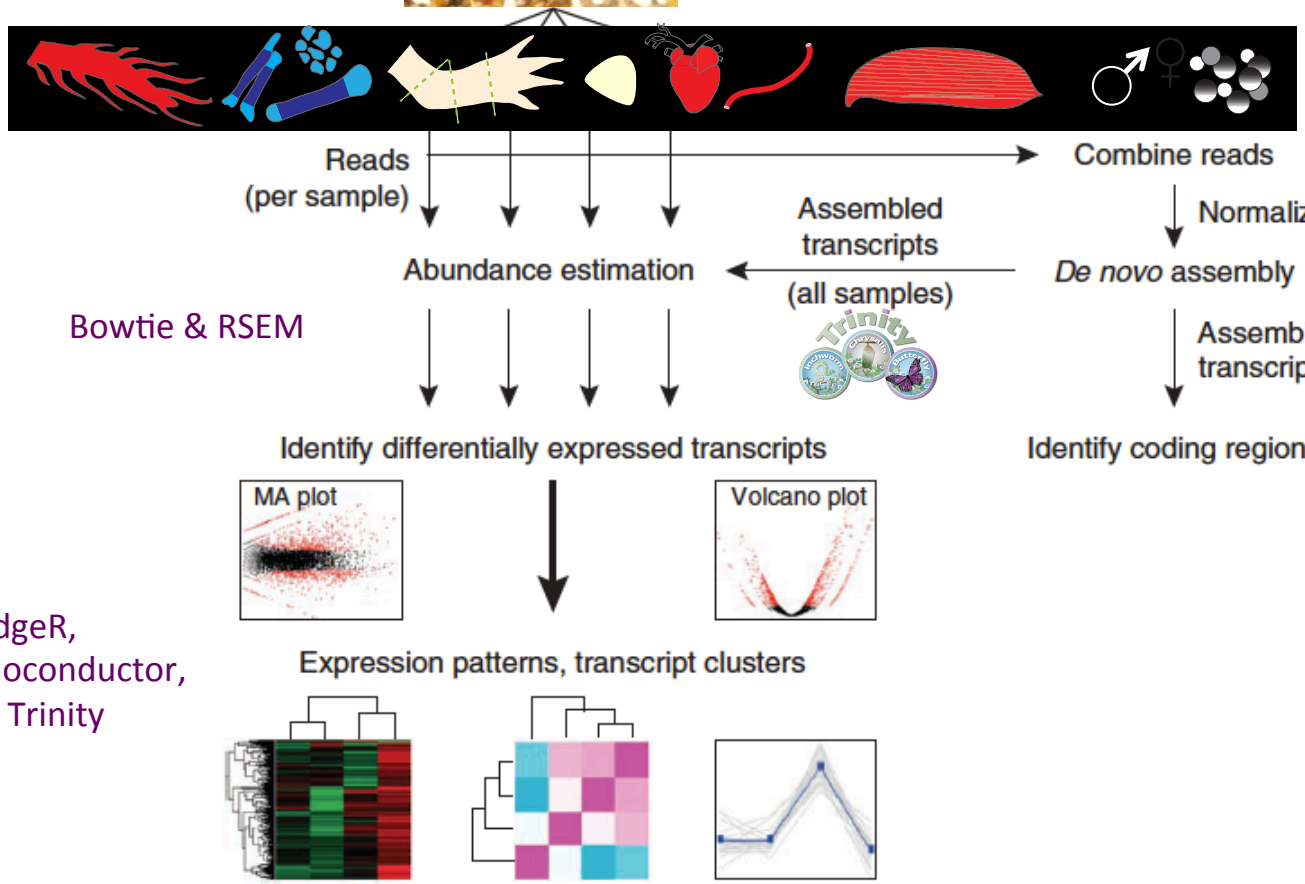
```
Trinity --seqType fq --left reads_1.fq --right reads_2.fq --CPU 6 --max_memory 20G
```

Find assembled transcripts as: 'trinity_out_dir/Trinity.fasta'

Use the documentation links in the right-sidebar to navigate this documentation, and contact our Google group for technical support.

Framework for De novo Transcriptome Assembly and Analysis





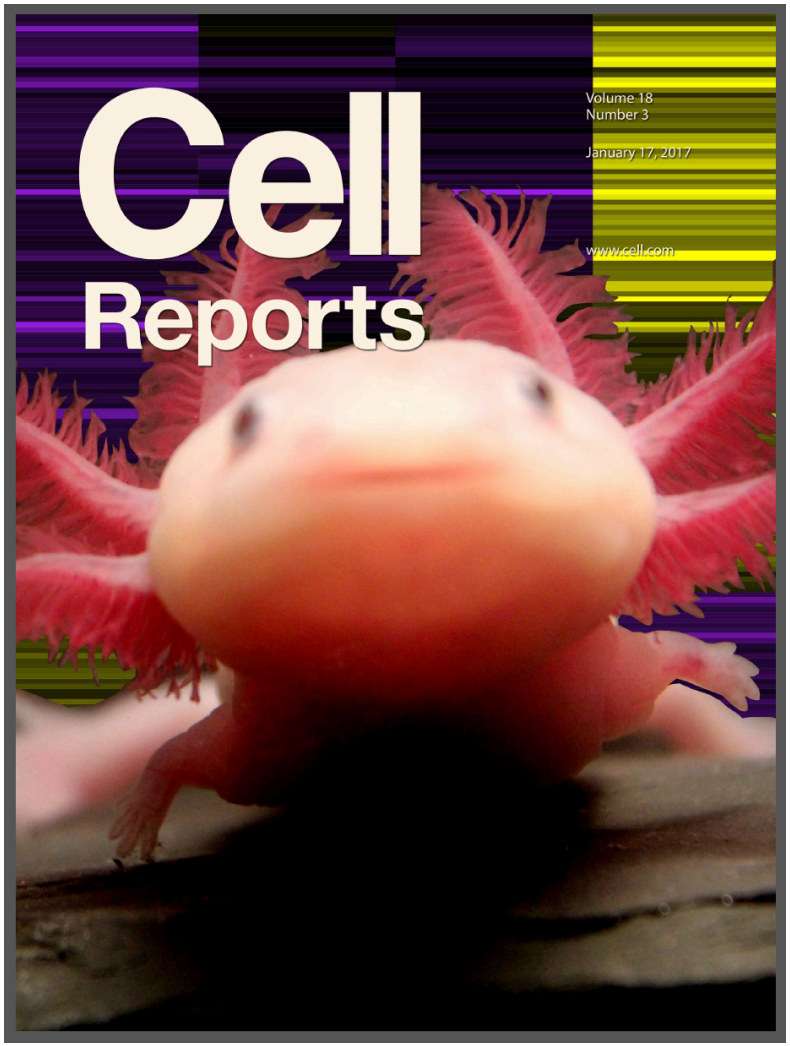
1.3 Billion
Total Reads

86 Million
Normalized Reads

Bowtie & RSEM

EdgeR,
Bioconductor,
& Trinity

Example Applications of the Trinity RNA-Seq Protocol



Resource

A Tissue-Mapped Axolotl De Novo Transcriptome Enables Identification of Limb Regeneration Factors

Donald M. Bryant[1,6], Kimberly Johnson[1,6], Tia DiTommaso[1], Timothy Tickle[2], Matthew Brian Couger[3], Duygu Payzin-Dogru[1], Tae J. Lee[1], Nicholas D. Leigh[1], Tzu-Hsing Kuo[1], Francis G. Davis[1], Joel Bateman[1], Sevara Bryant[1], Anna R. Guzikowski[1], Stephanie L. Tsai[4], Steven Coyne[1], William W. Ye[1], Robert M. Freeman Jr.[5], Leonid Peshkin[5], Clifford J. Tabin[4], Aviv Regev[2], Brian J. Haas[2], Jessica L. Whited[1,7],

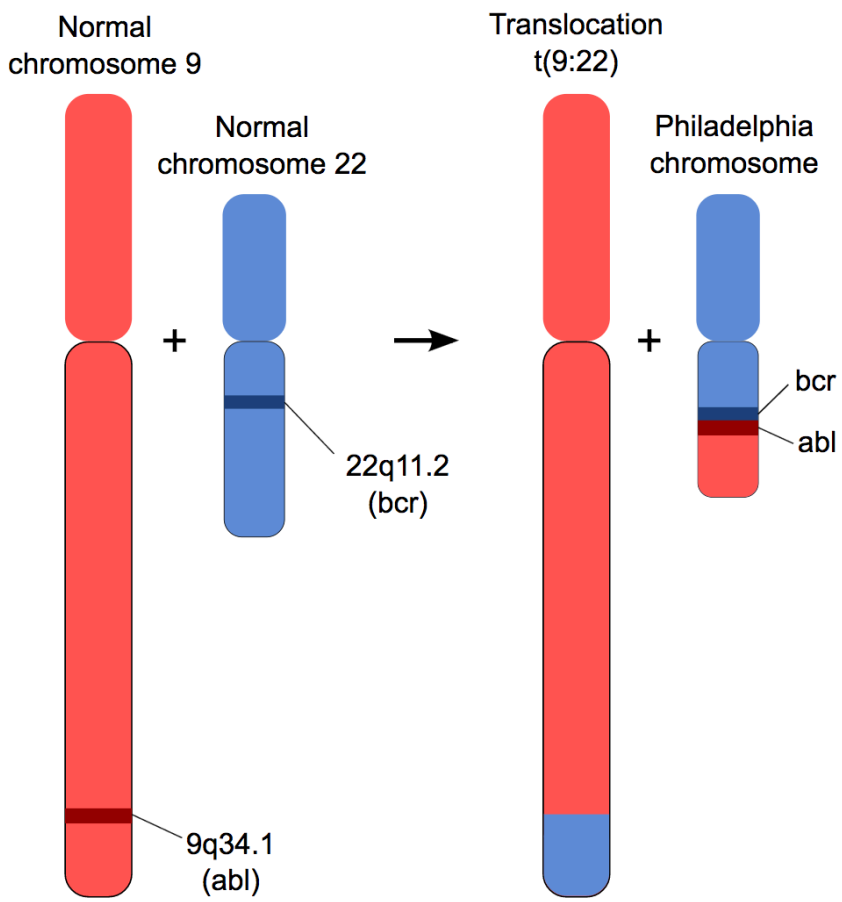
Original Article

Loggerhead sea turtle embryos (*Caretta caretta*) regulate expression of stress response and developmental genes when exposed to a biologically realistic heat stress

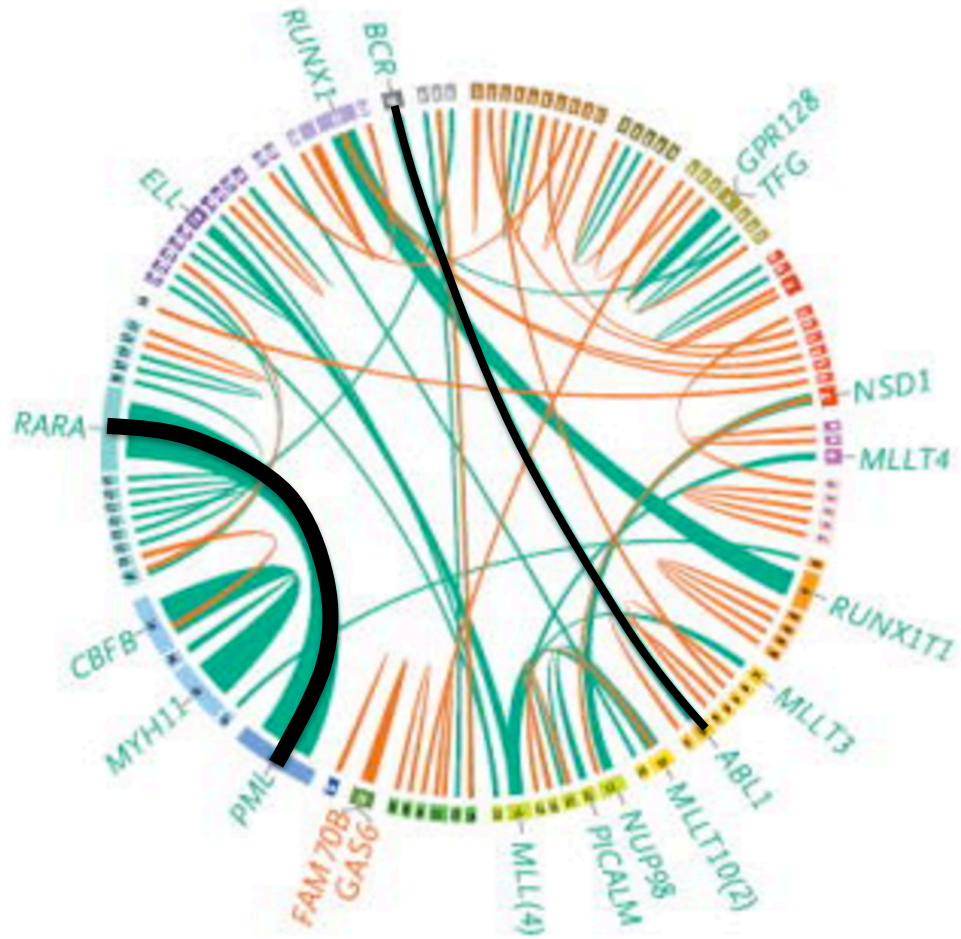
Blair P. Bentley, Brian J. Haas, Jamie N. Tedeschi, Oliver Berry

Biomedical Applications for *de Novo* Transcriptome Assembly

Fusion transcripts in Cancer



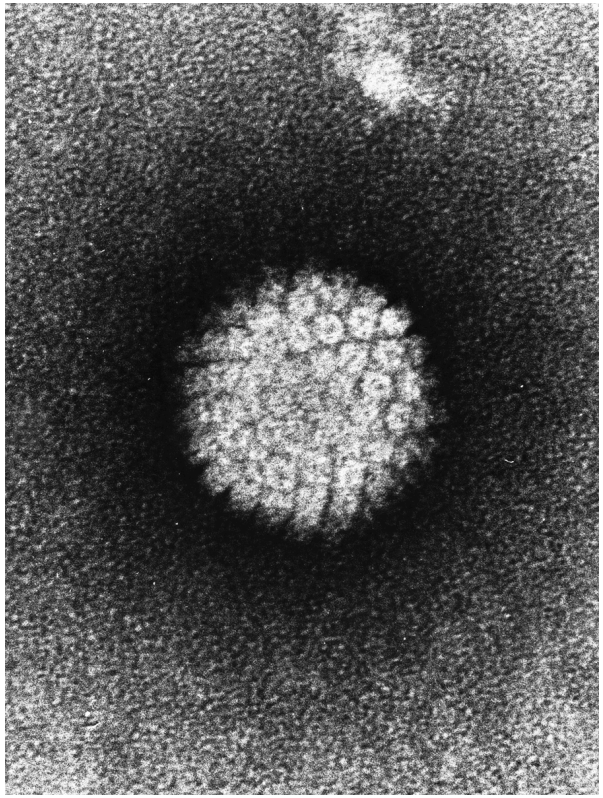
BCR--ABL1 fusion in ~95% of chromic myelogenous leukemias (CML)



Fusions Identified in a cohort of acute myeloid leukemias (AML) using *de novo* transcriptome assembly. *N Engl J Med. 2013 May 30; 368(22)*

Biomedical Applications for *de Novo* Transcriptome Assembly

Detection & Reconstruction of Viral and Microbial Transcripts in Cancer



HPV

Tumor Viruses

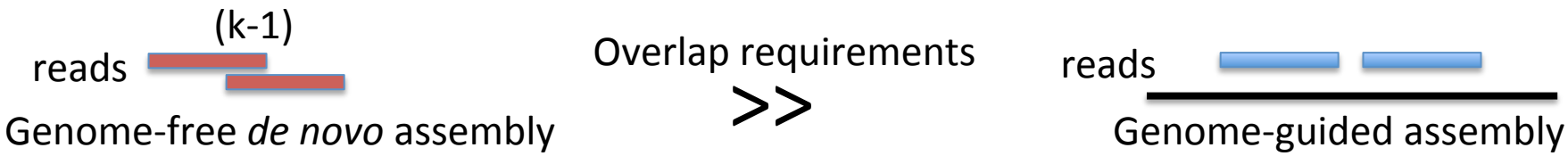
- Human papilloma virus (HPV) in cervical cancer
- Hepatitis B & C in liver cancer
- Eppstein Barr Virus in lymphomas
- T-lymphotrophic virus in adult T-cell leukemia

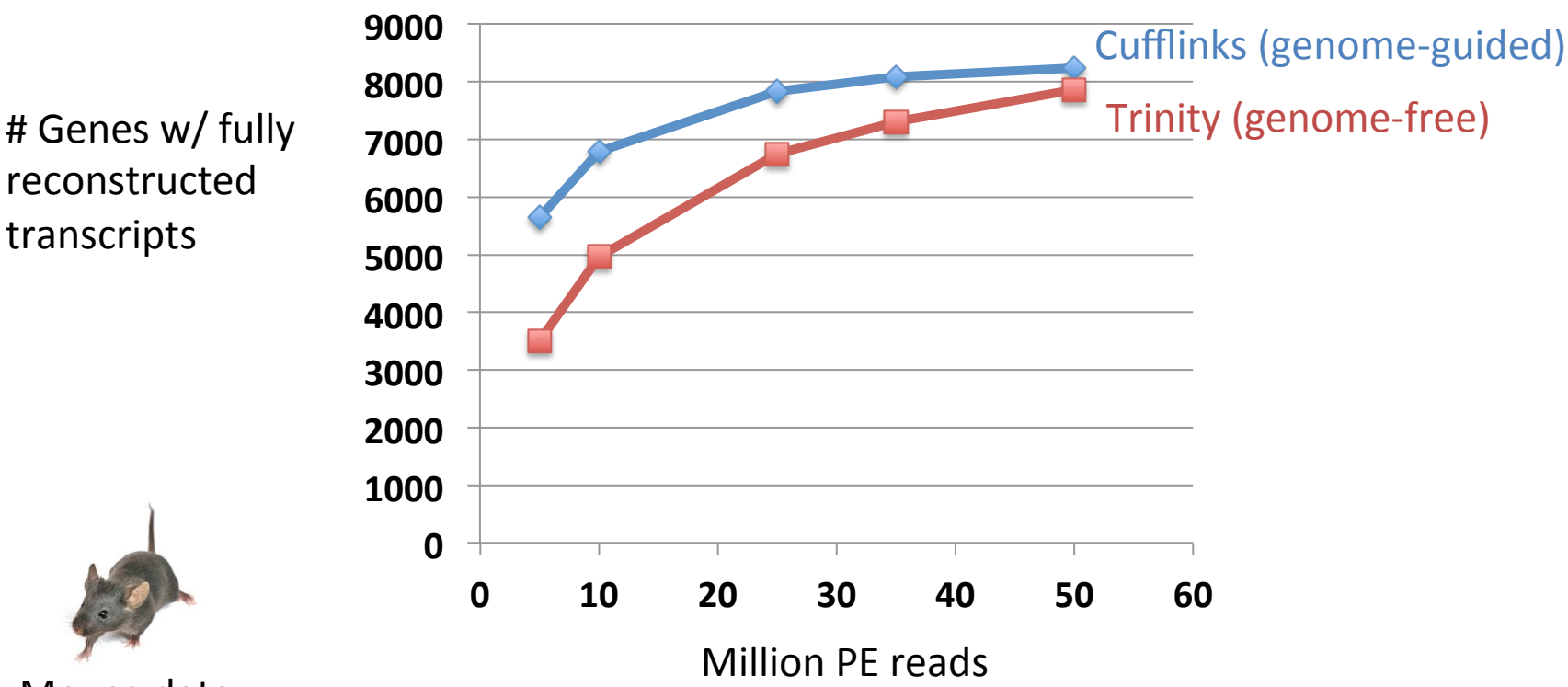
Bacterial / Cancer Associations

- Helicobacter pylori / stomach cancer
- Fusobacterium nucleatum / colon cancer

Contrasting Genome-guided and De novo Assembly

Genome-guided reconstruction is more sensitive than genome-free methods





Mouse data

Summary

- Transcript reconstruction from RNA-Seq data may leverage genome-guided or de novo assembly

- Transcriptome assembly uses directed graph data structures and path traversal

- Advantages and disadvantages to assembly approaches
  - Genome-guided: well-matched samples and very sensitive
  - *De novo*: almost any sample will do, but requires higher depth of read coverage

- Biomedical applications for *de novo* transcriptome assembly
  - Cancer research: fusion transcripts & pathogen detection

Strand-specific RNA-Seq is Preferred

Computationally: fewer confounding graph structures in de novo assembly:

ex. Forward != reverse complement

(GGAA != TTCC)

Biologically: separate sense vs. antisense transcription

NATURE METHODS | VOL.7 NO.9 | SEPTEMBER 2010 |

BROAD INSTITUTE

Comprehensive comparative analysis of strand-specific RNA sequencing methods

Joshua Z Levin[1,6], Moran Yassour[1–3,6], Xian Adiconis[1], Chad Nusbaum[1], Dawn Anne Thompson[1], Nir Friedman[3,4], Andreas Gnirke[1] & Aviv Regev[1,2,5]

Strand-specific, massively parallel cDNA sequencing (RNA-seq) is a powerful tool for transcript discovery, genome annotation

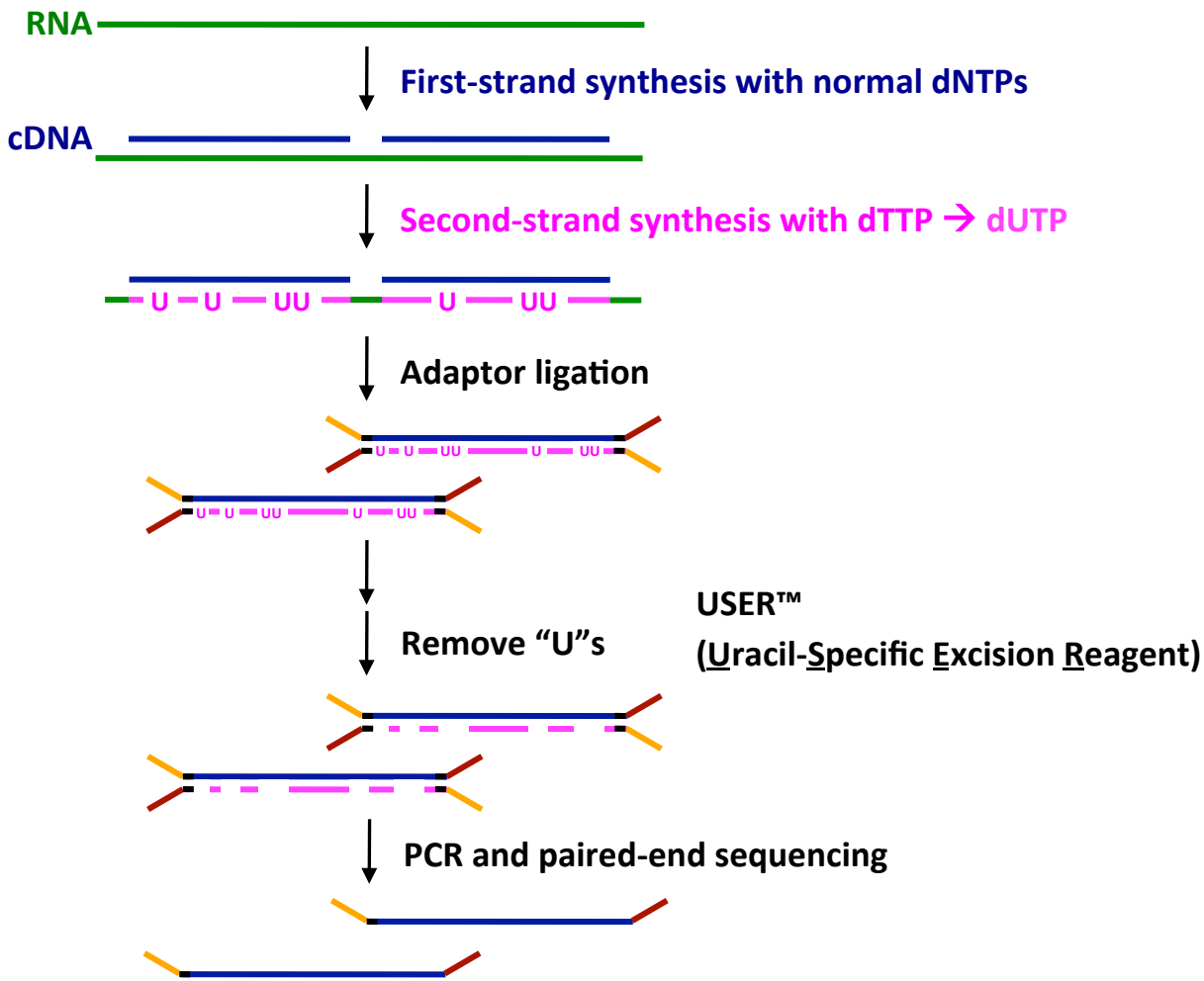
'dUTP second strand marking' identified as the leading protocol

to choose between them. Here we developed a comprehensive computational pipeline to compare library quality metrics from any RNA-seq method. Using the well-annotated *Saccharomyces cerevisiae* transcriptome as a benchmark, we compared seven library-construction protocols, including both published and

Nevertheless, direct information on the originating strand can substantially enhance the value of an RNA-seq experiment. For

transcribed strand of other noncoding RNAs, demarcate the exact boundaries of adjacent genes transcribed on opposite strands and resolve the correct expression levels of coding or noncoding overlapping transcripts. These tasks are particularly challenging in small microbial genomes, prokaryotic and eukaryotic, in which

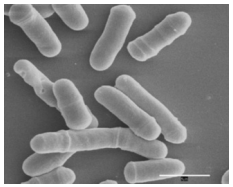
dUTP 2nd Strand Method: Our Favorite



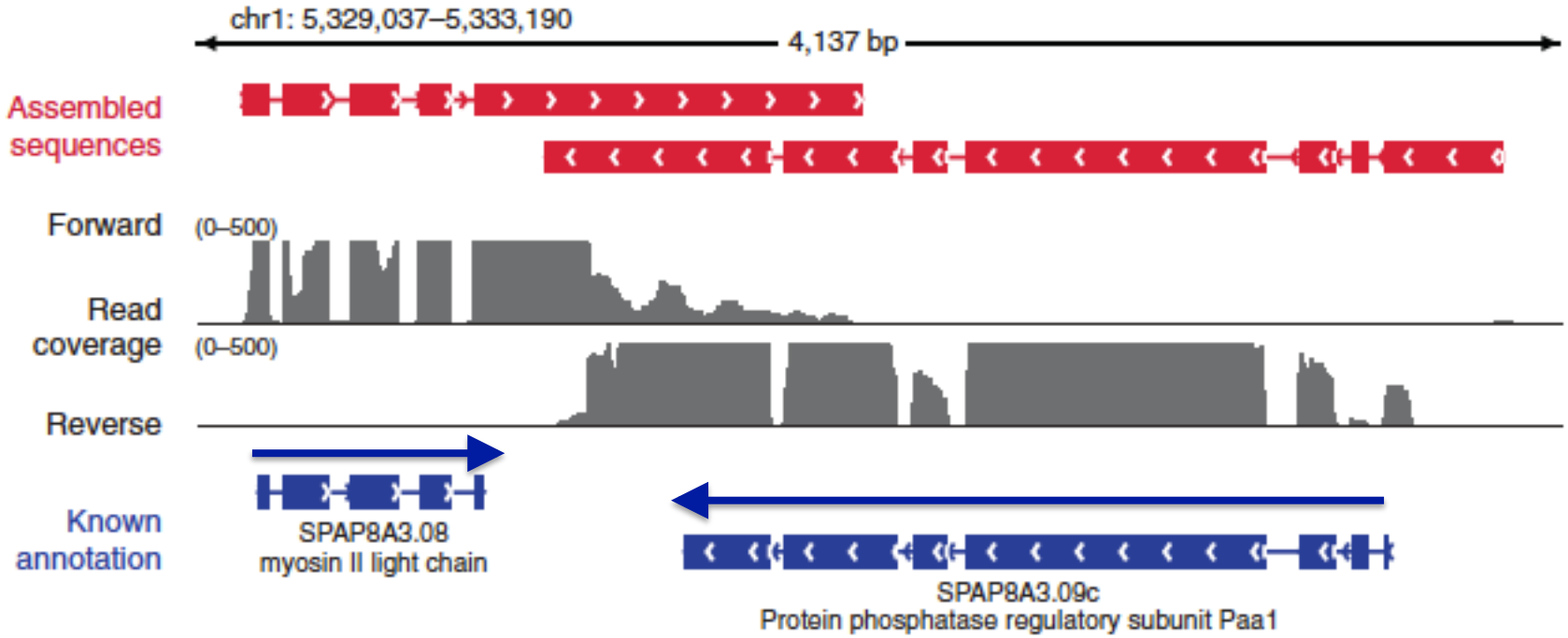
Modified from Parkhomchuk *et al.* (2009) *Nucleic Acids Res.* 37:e123

Slide courtesy of Joshua Levin, Broad Institute.

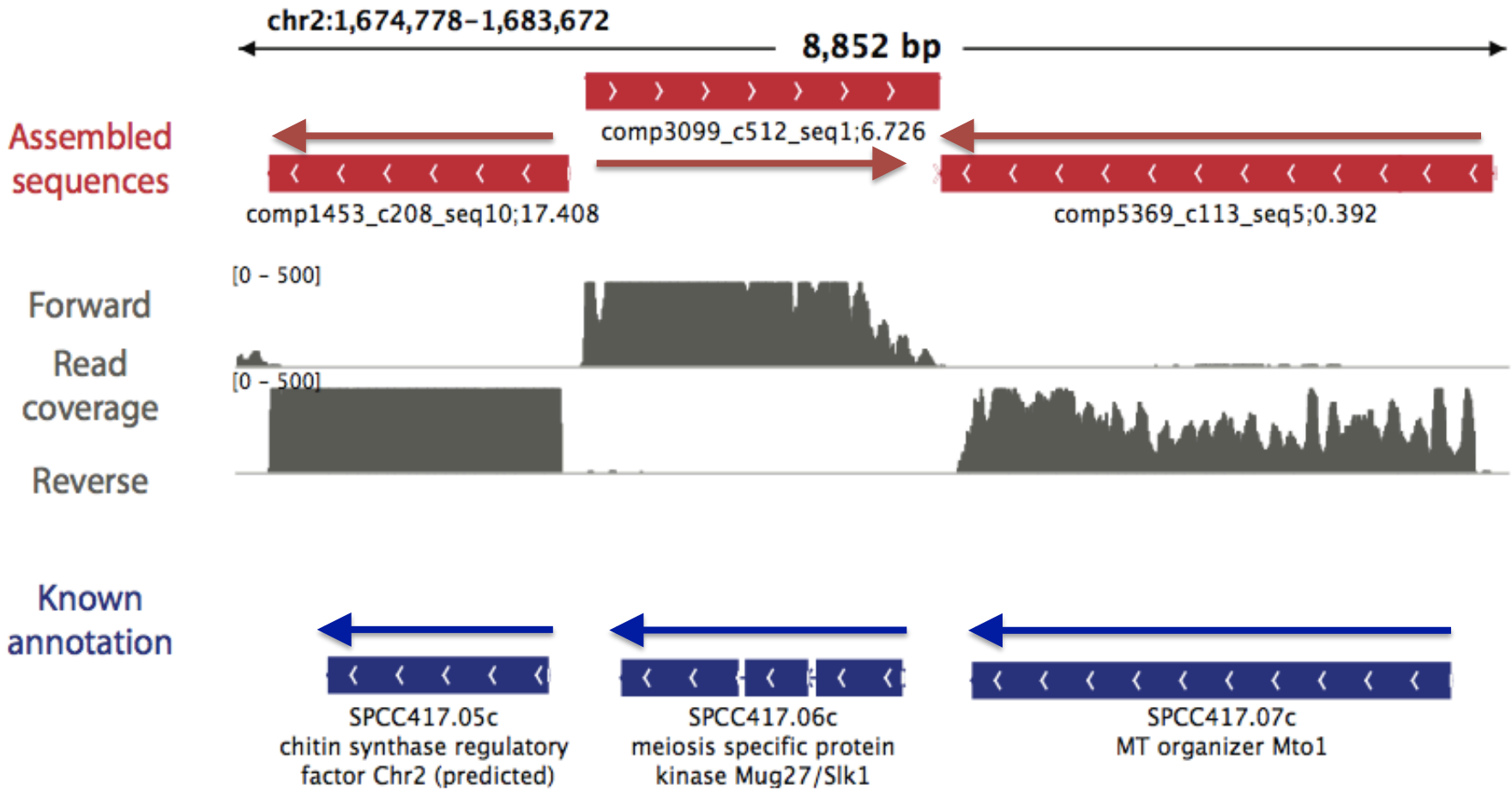
Overlapping UTRs from Opposite Strands

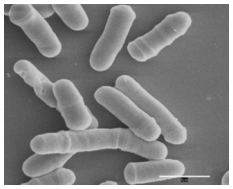


Schizosacharomyces pombe
(fission yeast)



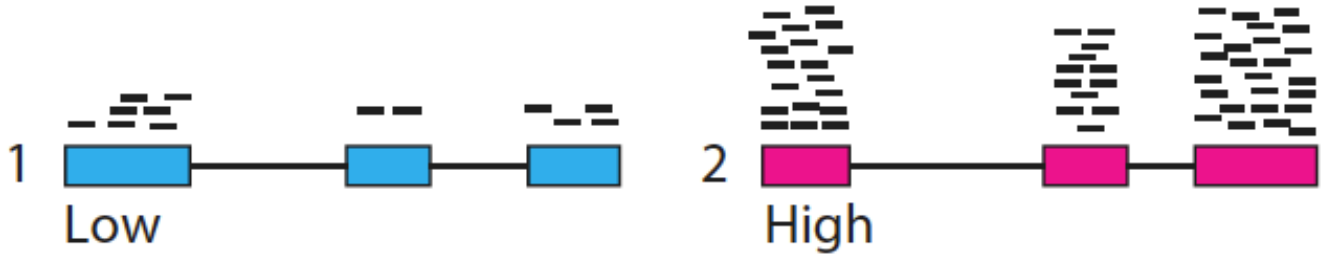
Antisense-dominated Transcription

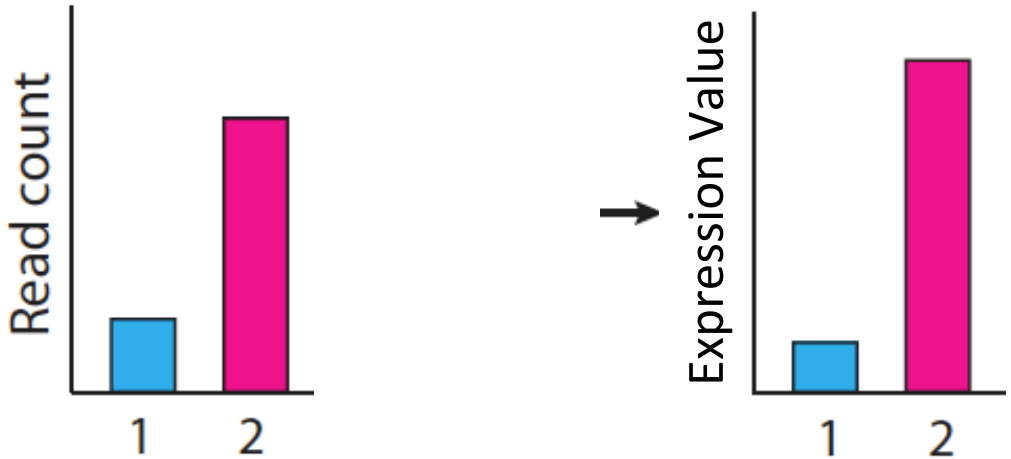




Abundance Estimation
(Aka. Computing Expression Values)

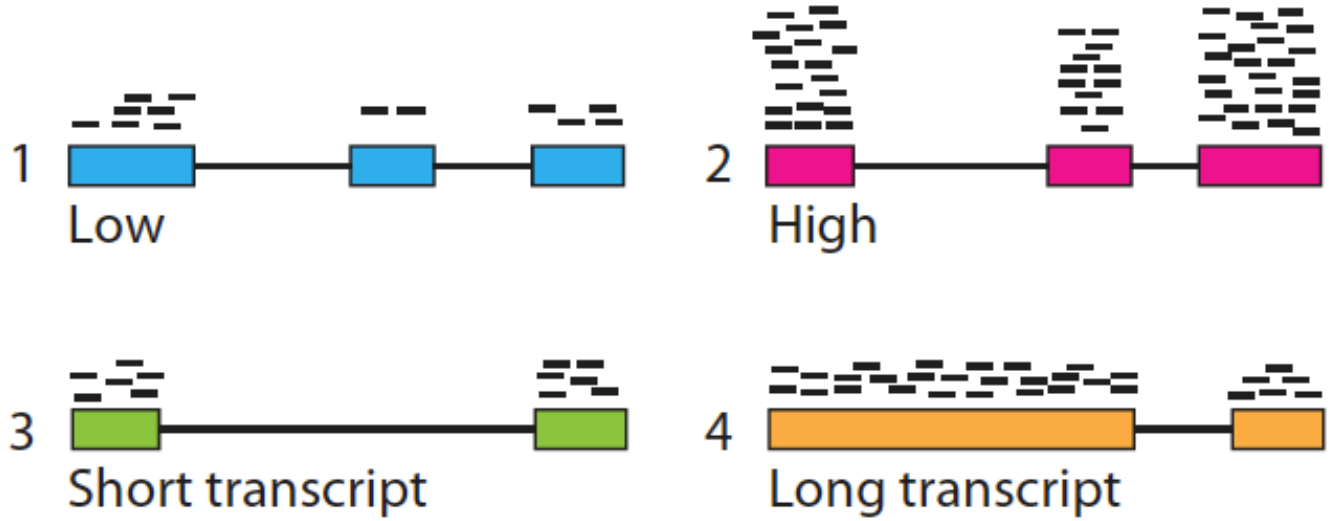
Calculating expression of genes and transcripts

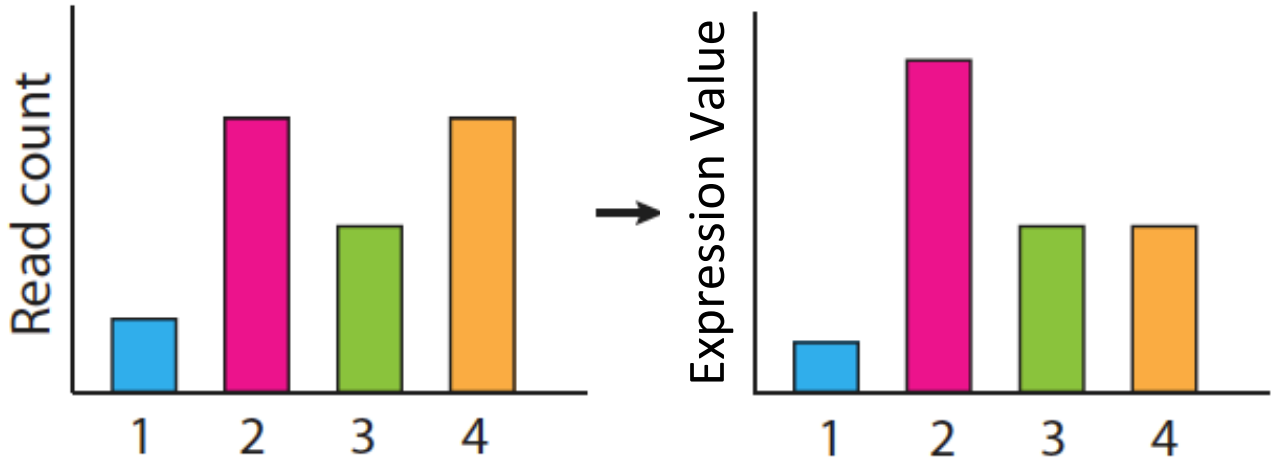




Slide courtesy of Cole Trapnell

Calculating expression of genes and transcripts





Slide courtesy of Cole Trapnell

Normalized Expression Values

- Transcript-mapped read counts are normalized for both length of the transcript and total depth of sequencing.

- Reported as: Number of RNA-Seq **F**ragments
  Per **K**ilobase of transcript
  per total **M**illion fragments mapped

FPKM

RPKM (reads per kb per M) used with Single-end RNA-Seq reads
FPKM used with Paired-end RNA-Seq reads.

Transcripts per Million (TPM)

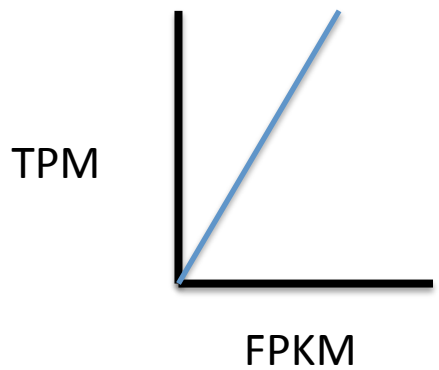
$$TPM_i = \frac{FPKM_i}{\sum_j FPKM} * 1e6$$

Preferred metric for measuring expression

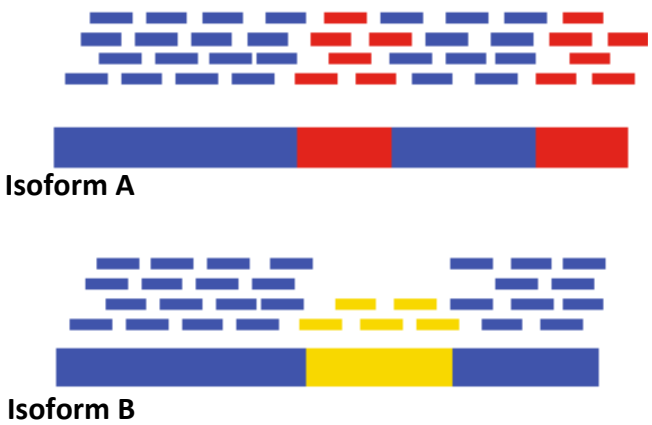
- Better reflects transcript concentration in the sample.
- Nicely sums to 1 million

Linear relationship between TPM and FPKM values.

Both are valid metrics, but best to be consistent.

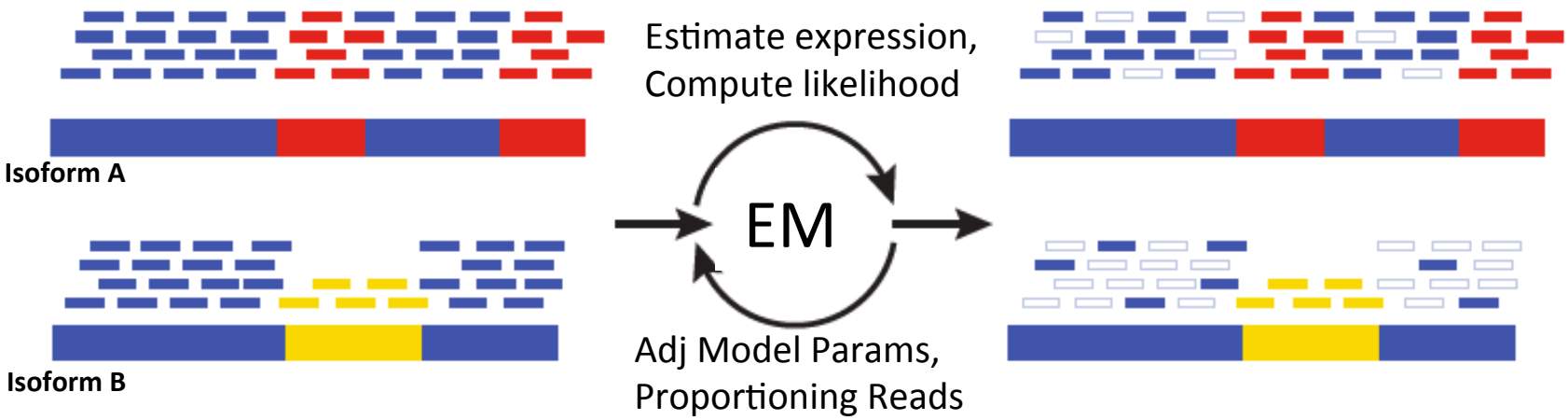


Multiply-mapped Reads Confound Abundance Estimation



Blue = multiply-mapped reads
Red, Yellow = uniquely-mapped reads

Multiply-mapped Reads Confound Abundance Estimation



Blue = multiply-mapped reads
Red, Yellow = uniquely-mapped reads

Use Expectation Maximization (EM) to find the most likely assignment of reads to transcripts.

Performed by:

- Cufflinks, String Tie (Tuxedo)
- RSEM, eXpress (genome-free)
- Kallisto, Salmon (alignment-free)

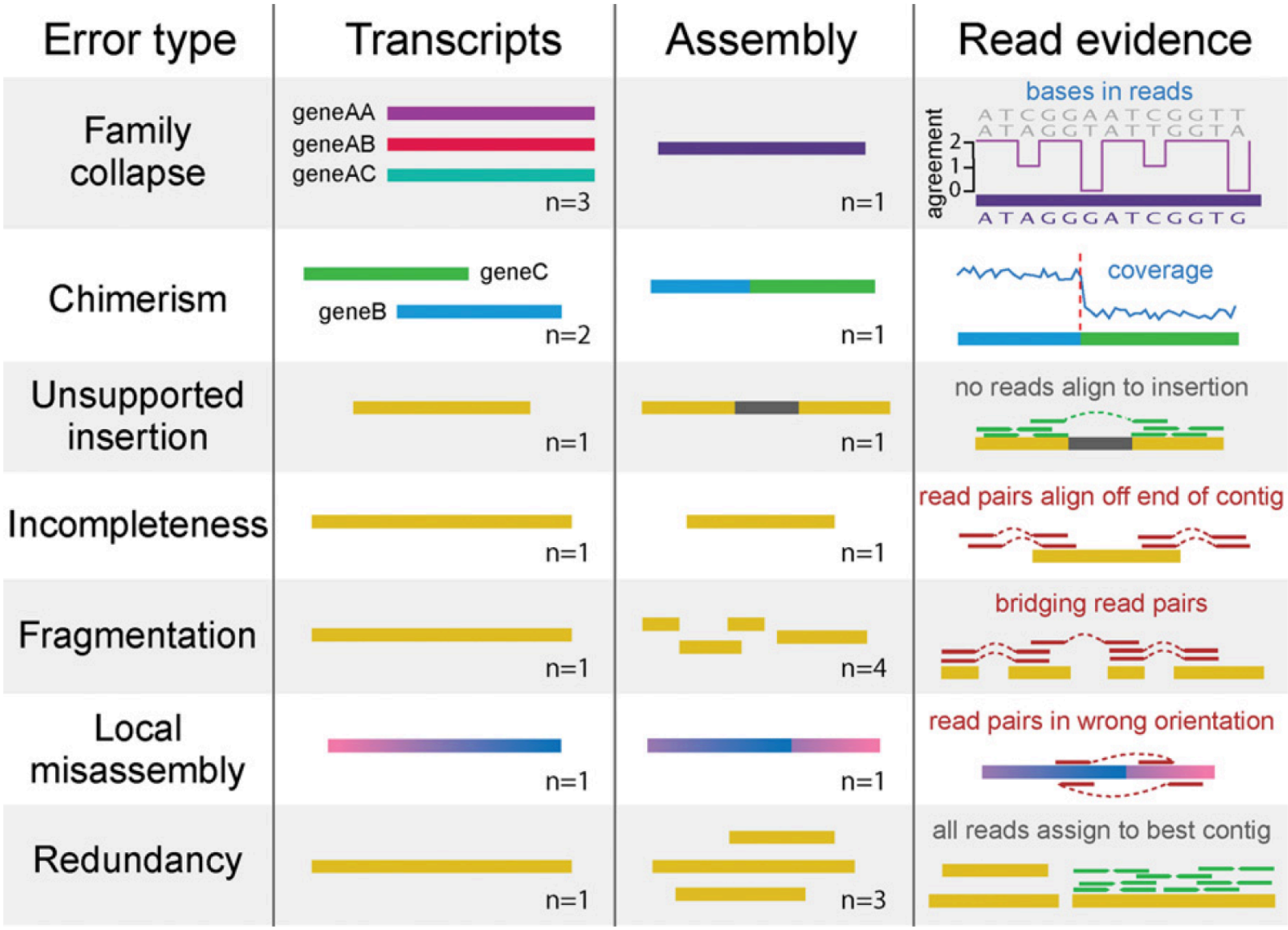
Expression Quantification Results

(ex. from Kallisto)

target_id	length	eff_length	est_counts	tpm
TRINITY_DN10_c0_g1_i1	334	100.489	13	4186.62
TRINITY_DN11_c0_g1_i1	319	87.9968	0	0
TRINITY_DN12_c0_g1_i1	244	38.2208	2	1693.43
TRINITY_DN17_c0_g1_i1	229	30.2382	5	5351.21
TRINITY_DN18_c0_g1_i1	633	384.493	19	1599.2
TRINITY_DN18_c1_g1_i1	289	65.795	1	491.864
TRINITY_DN19_c0_g1_i1	283	61.0618	10	5299.91

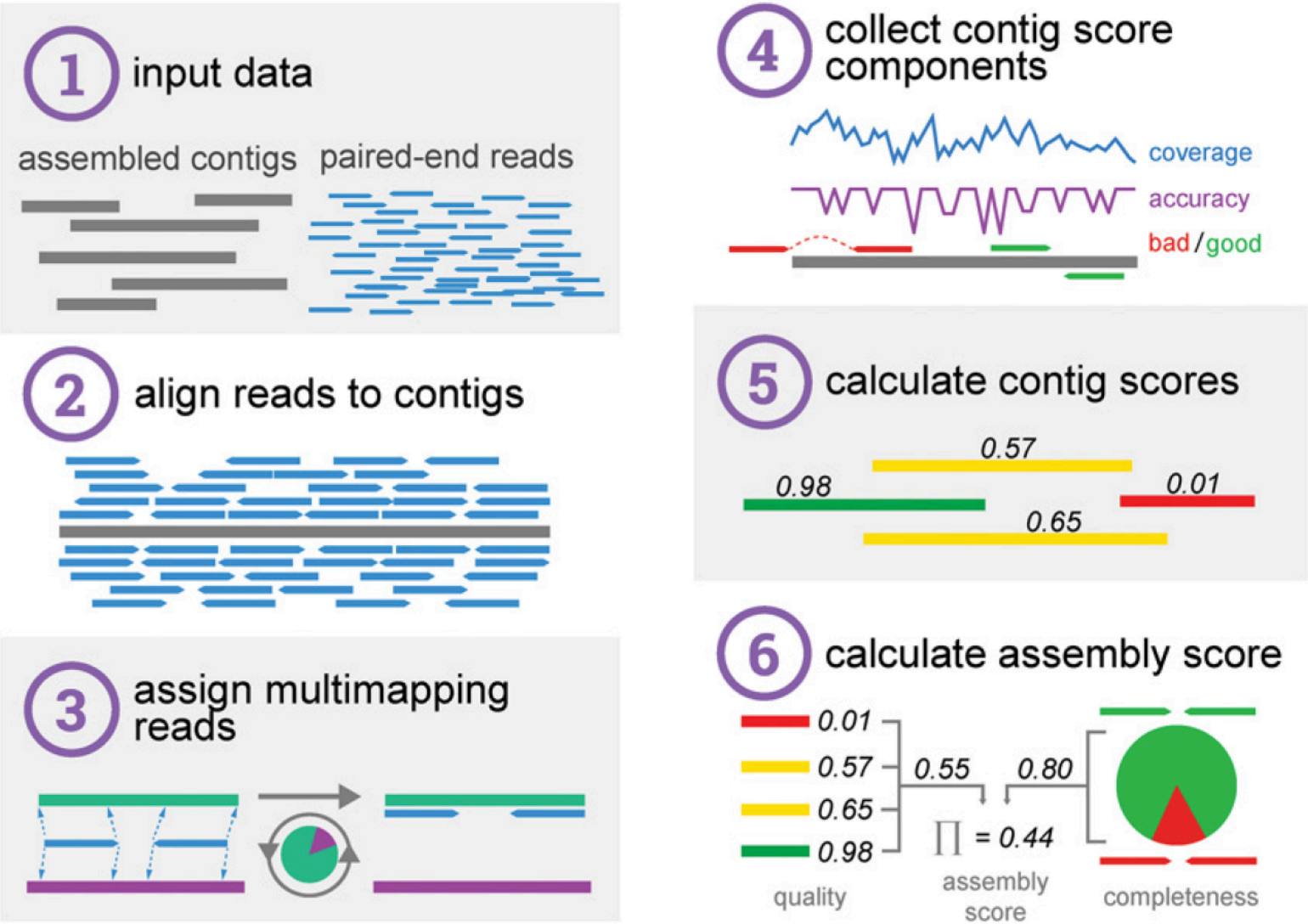
Evaluating the quality of your transcriptome assembly

De novo Transcriptome Assembly is Prone to Certain Types of Errors



Smith-Unna et al. Genome Research, 2016

TransRate



Smith-Unna et al. Genome Research, 2016

Simple Quantitative and Qualitative Assembly Metrics

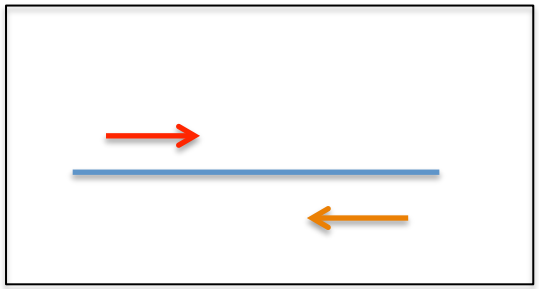
Read representation by assembly

Align reads to the assembled transcripts using Bowtie.
A typical 'good' assembly has ~80 % reads mapping to the assembly and ~80% are properly paired.

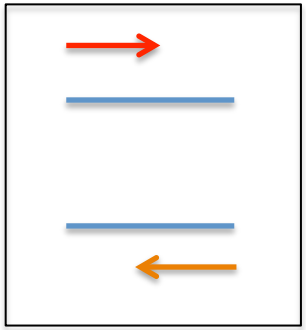
Given read pair: 

Possible mapping contexts in the Trinity assembly are reported:

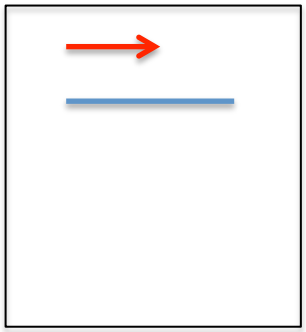
Proper pairs



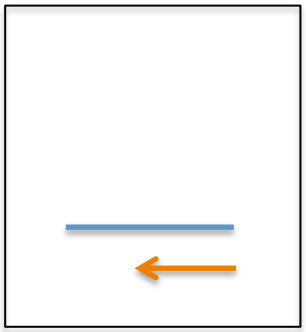
Improper pairs



Left only



Right only



Assembled transcript contig is only as good as its read support.

% samtools tview alignments.bam target.fasta

```
   911       921       931       941       951       961       971       981       991      1001      1011      1021      1031      1041      1051      1061      1071
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IGV

www.broadinstitute.org/igv/

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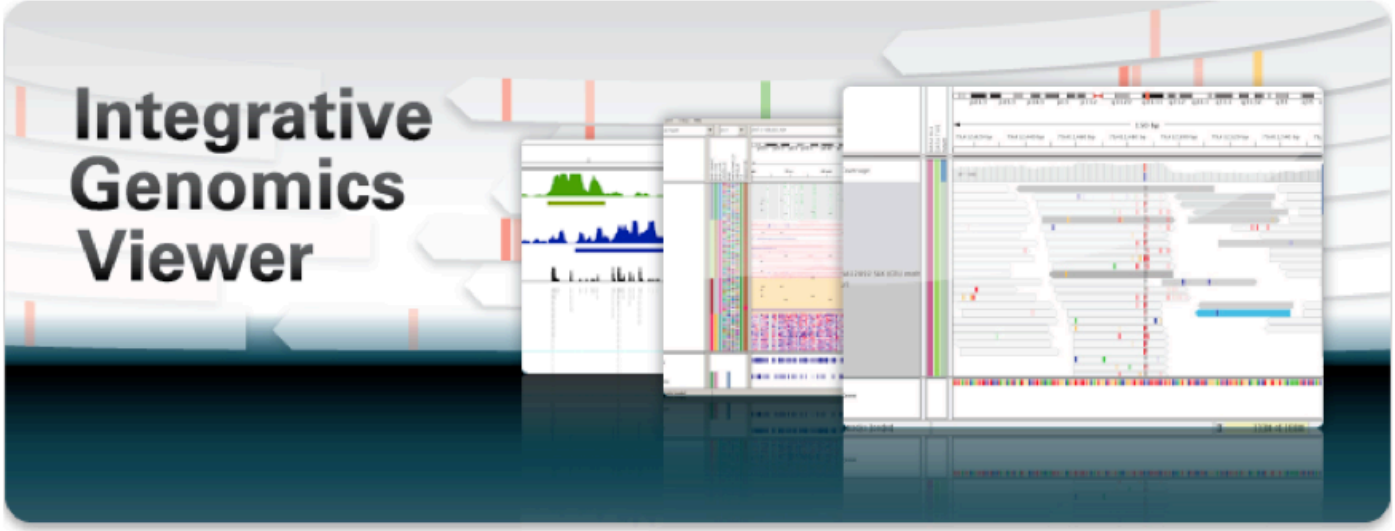


What's New

July 3, 2012. Soybean (Glycine max) and Rat (rn5) genomes have been updated.

April 20, 2012. IGV 2.1 has been released. See the release notes for more details.

April 19, 2012. See our new IGV paper in Briefings in Bioinformatics.

Overview

Citing IGV

To cite your use of IGV in your publication:

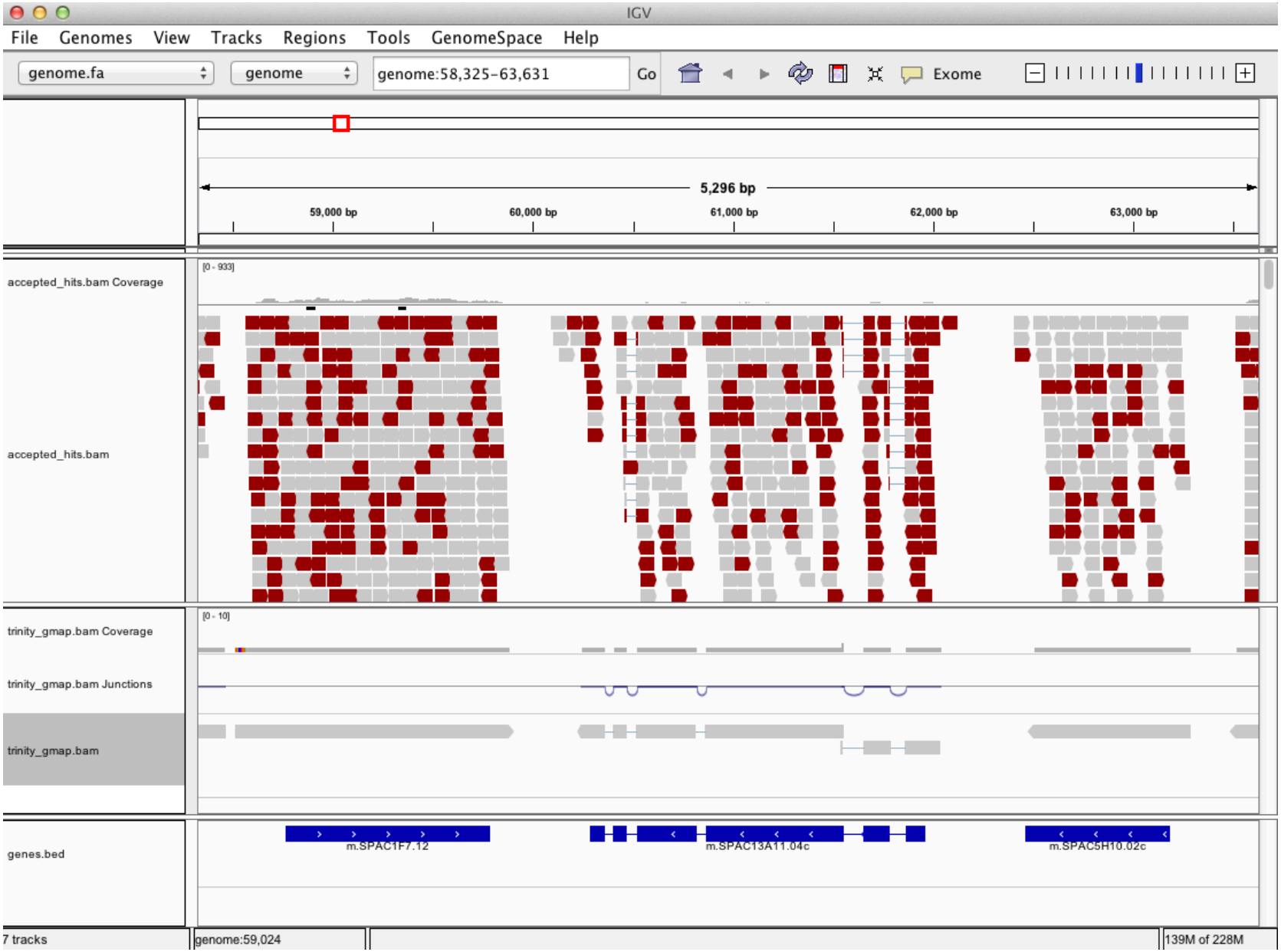
James T. Robinson, Helga Thorvaldsdóttir, Wendy Winckler, Mitchell Guttman, Eric S. Lander, Gad Getz, Jill P. Mesirov. **Integrative Genomics Viewer**. Nature Biotechnology 29, 24–26 (2011), or

Helga Thorvaldsdottir, James T. Robinson, Jill P. Mesirov. **Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration.**

Can Examine Transcript Read Support Using IGV

Can align Trinity transcripts to genome scaffolds to examine intron/exon structures

(Trinity transcripts aligned to the genome using GMAP)

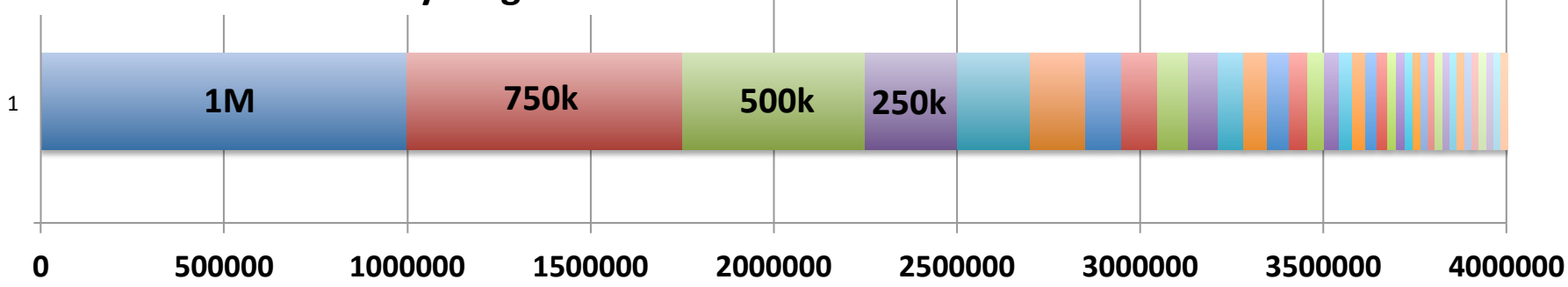


The Contig N50 statistic

"At least half of assembled bases are in contigs that are at least ***N50*** bases in length"

In genome assemblies – used often to judge 'which assembly is better'

Assemblies ordered by length:



N50 contig length = 500k

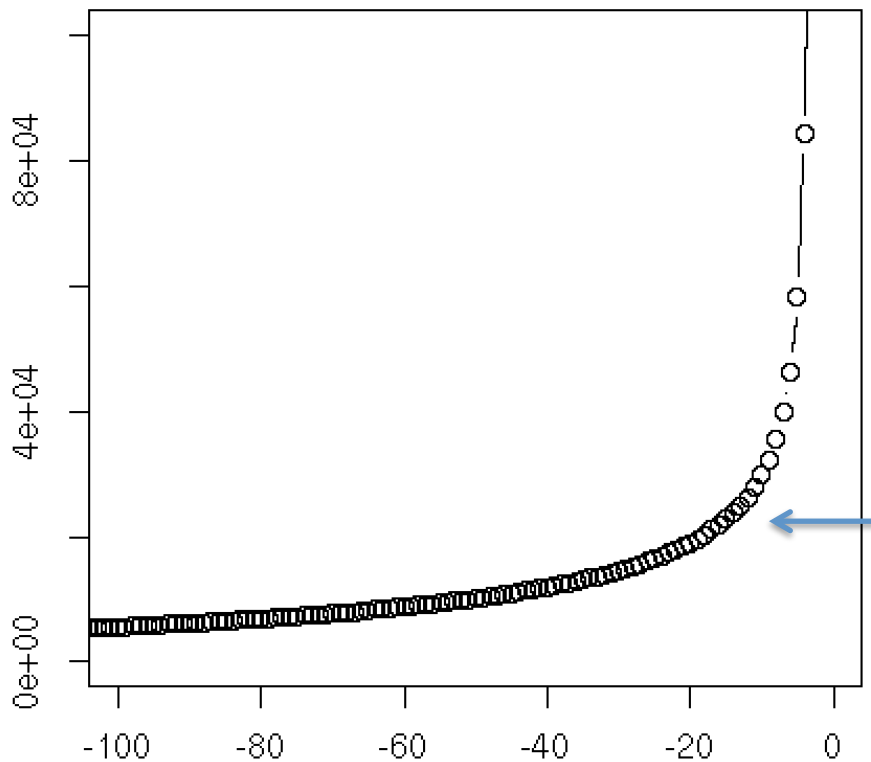
Often, most assembled transcripts are *very* lowly expressed

(How many 'transcripts & genes' are there really?)

1.4 million Trinity transcript contigs

N50 ~ 500 bases

Cumulative # of Transcripts



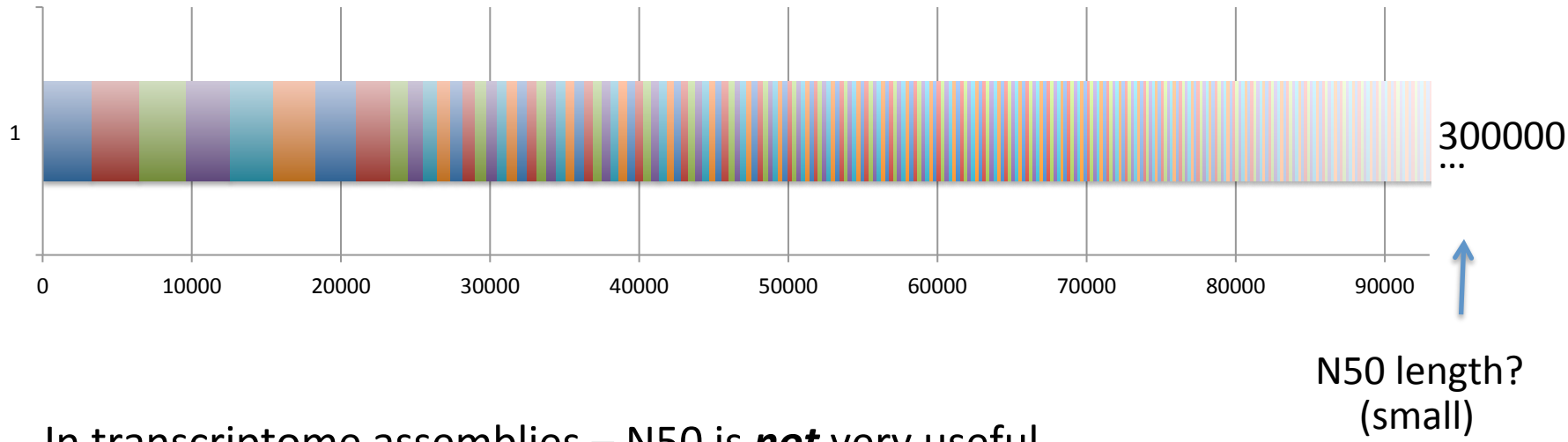
20k transcripts

-1 * minimum TPM

Expression

* Salamander transcriptome

N50 Calculation for *Transcriptome* Assemblies??

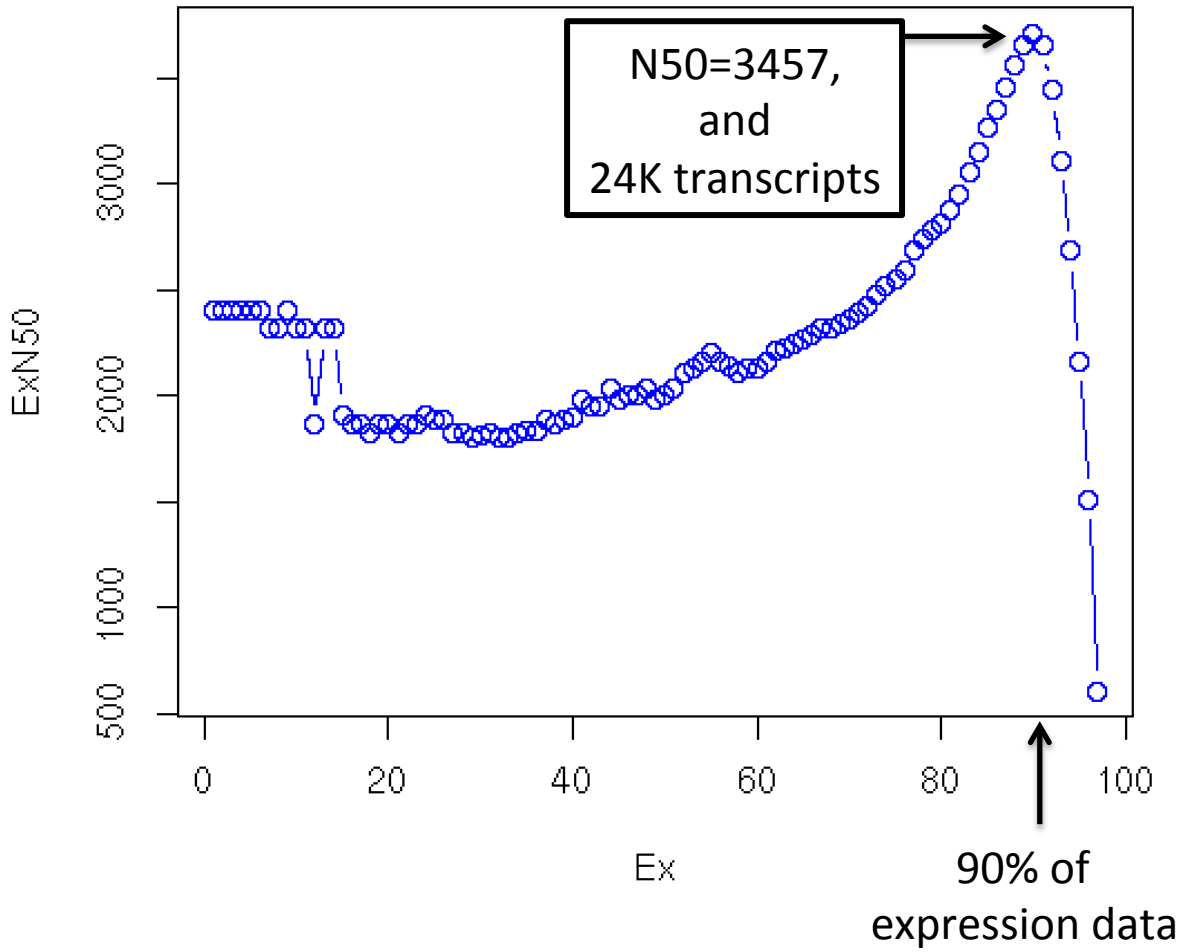


In transcriptome assemblies – N50 is ***not*** very useful.

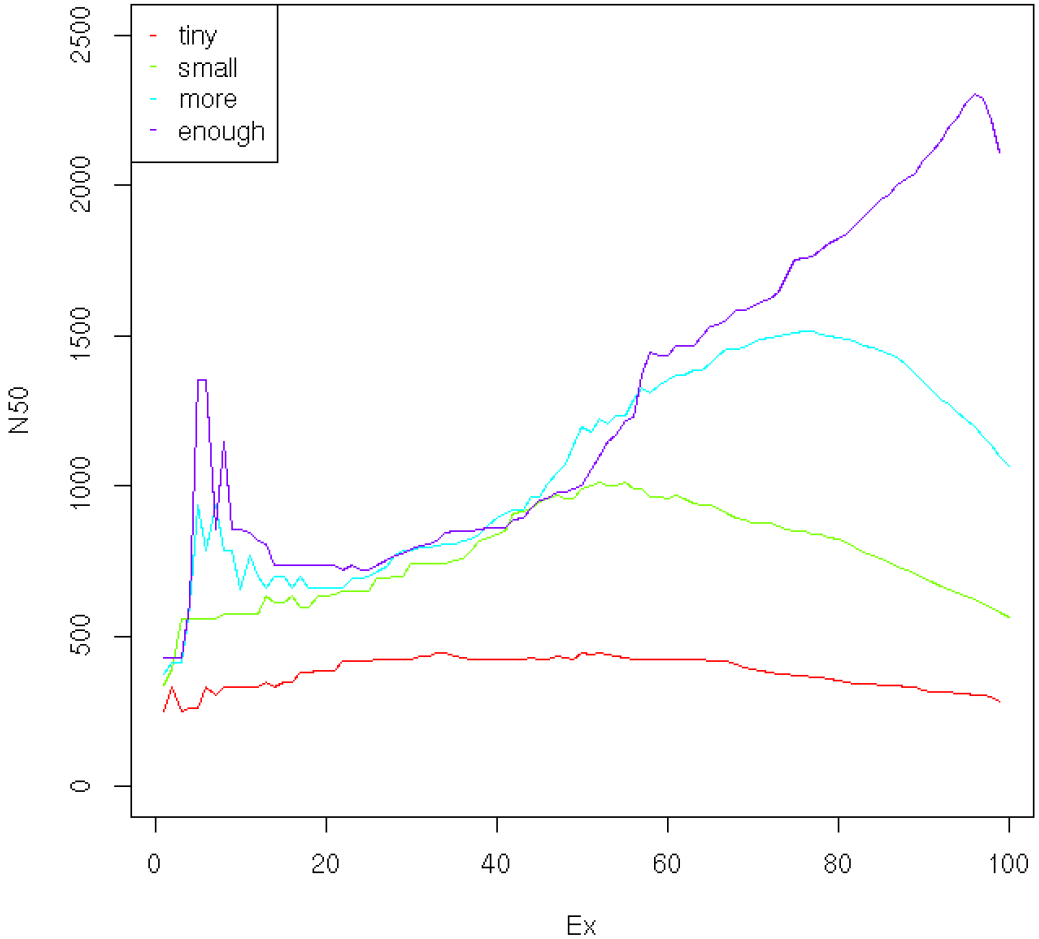
- Overzealous isoform annotation for long transcripts drives higher N50
- Very sensitive reconstruction for short lowly expressed transcripts drives lower N50

Compute N50 Based on the Top-most Highly Expressed Transcripts (ExN50)

- Sort contigs by expression value, descendingly.
- Compute N50 given minimum % total expression data thresholds => ExN50



ExN50 Profiles for Different Trinity Assemblies Using Different Read Depths



Millions of Reads

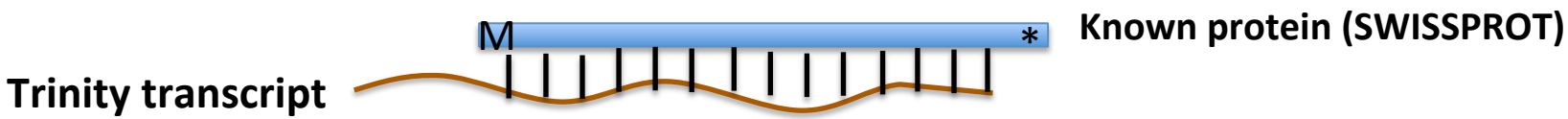
Thousands of Reads

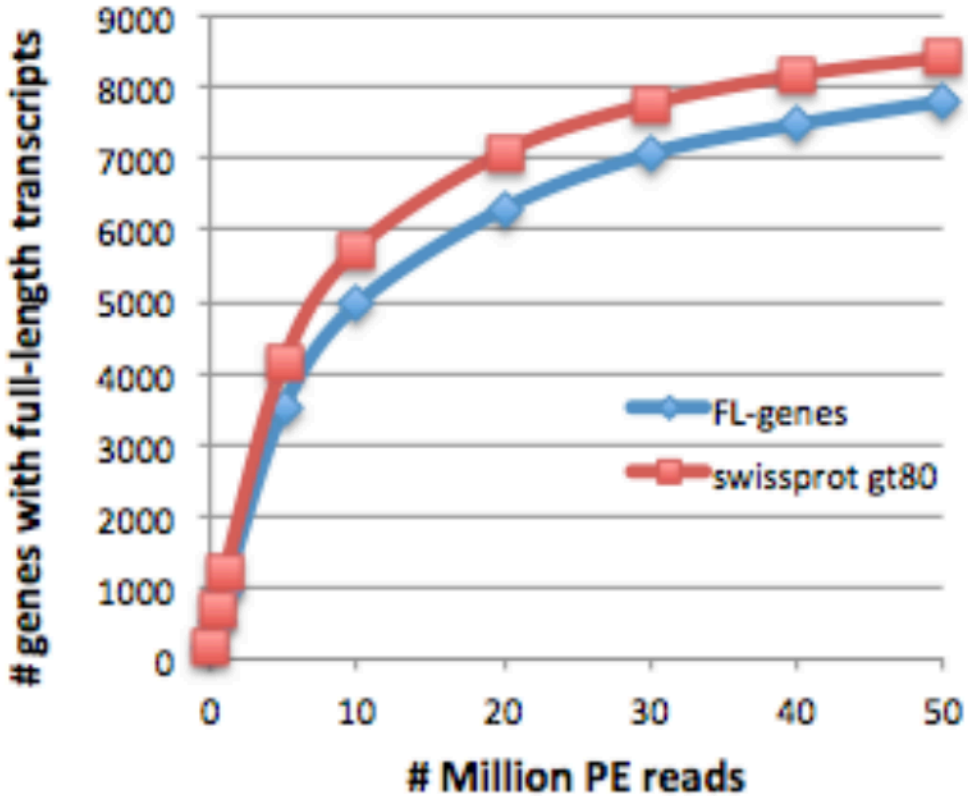
Note shift in ExN50 profiles as you assemble more and more reads.

* Candida transcriptome

Evaluating the quality of your transcriptome assembly

Full-length Transcript Detection via BLASTX





Have you sequenced deeply enough?

Haas et al. Nat. Protoc. 2013

* Mouse transcriptome

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CEGG Home | OrthoDB *v9* | BUSCO *v2*

BUSCO v2

Assessing genome assembly and annotation completeness with **B**enchmarking **U**niversal **S**ingle-**C**opy **O**rthologs

About BUSCO

BUSCO *v2* provides quantitative measures for the assessment of genome assembly, gene set, and transcriptome completeness, based on evolutionarily-informed expectations of gene content from near-universal single-copy orthologs selected from OrthoDB *v9*.

BUSCO assessments are implemented in open-source software, with a large selection of lineage-specific sets of Benchmarking Universal Single-Copy Orthologs. These conserved orthologs are ideal candidates for large-scale phylogenomics studies, and the annotated BUSCO gene models built during genome assessments provide a comprehensive gene predictor training set for use as part of genome annotation pipelines.

busco.ezlab.org

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Zdobnov's Computational Evolutionary Genomics group

CEGG Home | OrthoDB *v9* | BUSCO *v2*

BUSCO v2

Assessing genome assembly and annotation completeness with Benchmarking Universal Single-Copy Orthologs

```
#Summarized BUSCO benchmarking for file: Trinity.fasta
#BUSCO was run in mode: trans

Summarized benchmarks in BUSCO notation:
    C:88%[D:53%],F:4.5%,M:7.3%,n:3023

Representing:
    1045    Complete Single-copy BUSCOs
    1617    Complete Duplicated BUSCOs
    139     Fragmented BUSCOs
    222     Missing BUSCOs
    3023    Total BUSCO groups searched
```

Detonate: Which assembly is better?

"RSEM-EVAL [sic] uses a novel probabilistic model-based method to compute the joint probability of both an assembly and the RNA-Seq data as an evaluation score."

$$\text{score}_{\text{RSEM-EVAL}}(A) = \log P(A, D)$$

"the RSEM-EVAL score of an assembly is defined as the log joint probability of the assembly A and the reads D used to construct it"

$$\begin{aligned}\log P(A, D) &= \log \int_{\Lambda} P(D|A, \Lambda) P(A|\Lambda) P(\Lambda) d\Lambda \\ &\approx \underbrace{\log P(D|A, \Lambda_{\text{MLE}})}_{\text{likelihood}} + \underbrace{\log P(A|\Lambda_{\text{MLE}})}_{\text{assembly prior}} \\ &\quad \underbrace{- \frac{1}{2}(M+1)\log N}_{\text{BIC penalty}},\end{aligned}$$

Li et al. **Evaluation of de novo transcriptome assemblies from RNA-Seq data**, Genome Biology 2014

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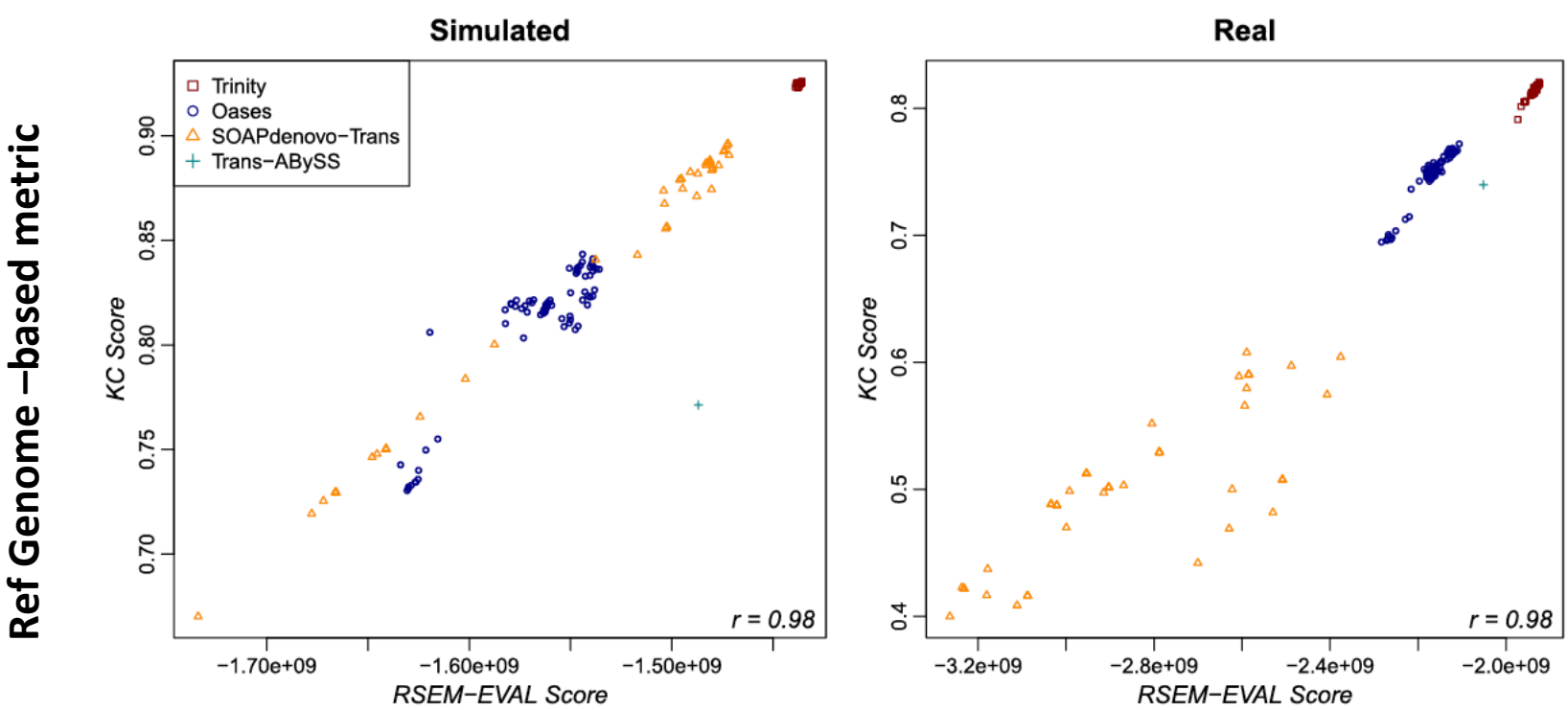
Bigger Score = Better Assembly

Li et al. **Evaluation of de novo transcriptome assemblies from RNA-Seq data**, Genome Biology 2014

Detonate: Which assembly is better?

"RSEM-EVAL [sic] uses a novel probabilistic model-based method to compute the joint probability of both an assembly and the RNA-Seq data as an evaluation score."

Yay Trinity!!

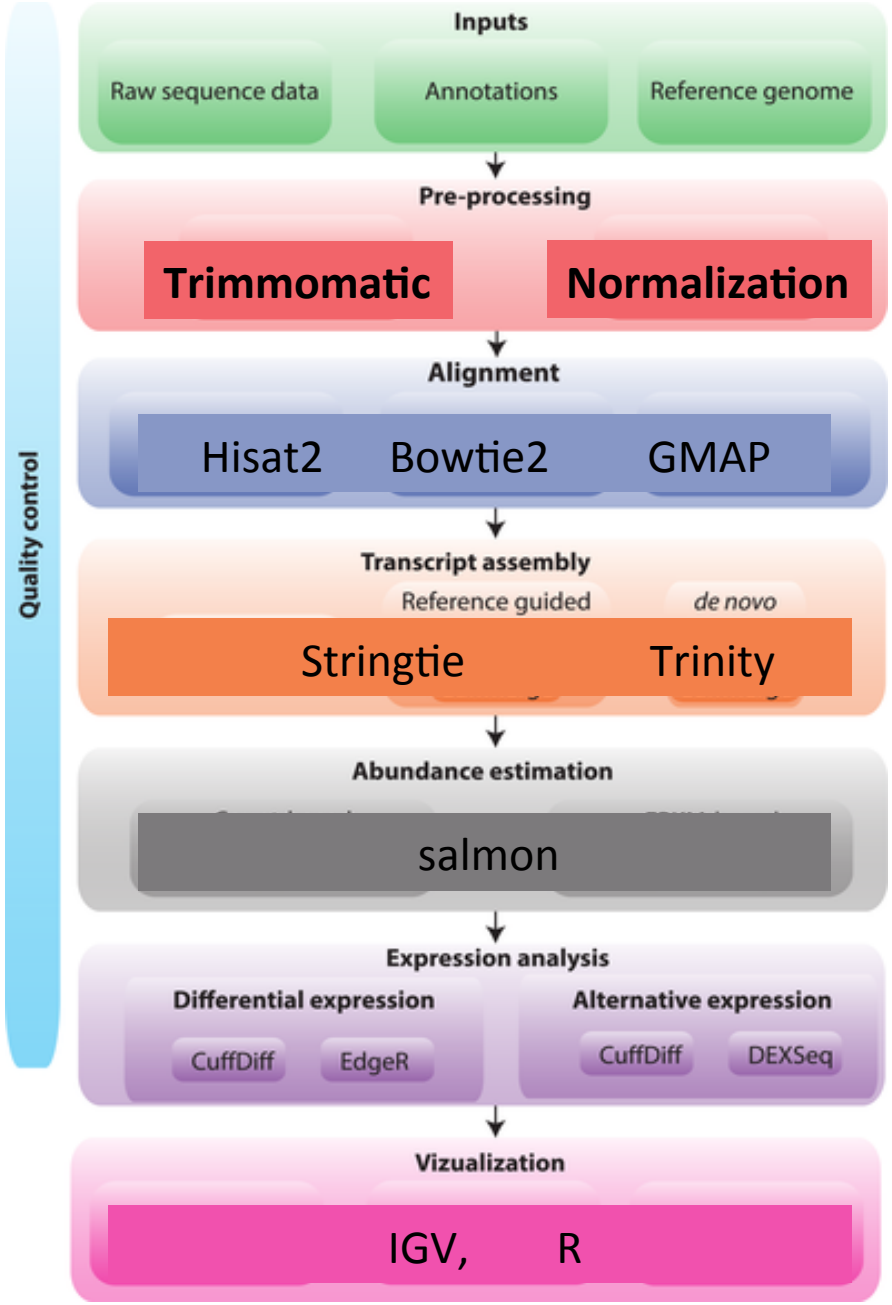


Li et al. **Evaluation of de novo transcriptome assemblies from RNA-Seq data**, Genome Biology 2014

Hands-on Workshop Activities

FastQC, MultiQC

Full-length BlastX, ExN50



(Tomorrow)

http://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1004393

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