RNA-Seq

Bioinformatics Applications (PLPTH813)

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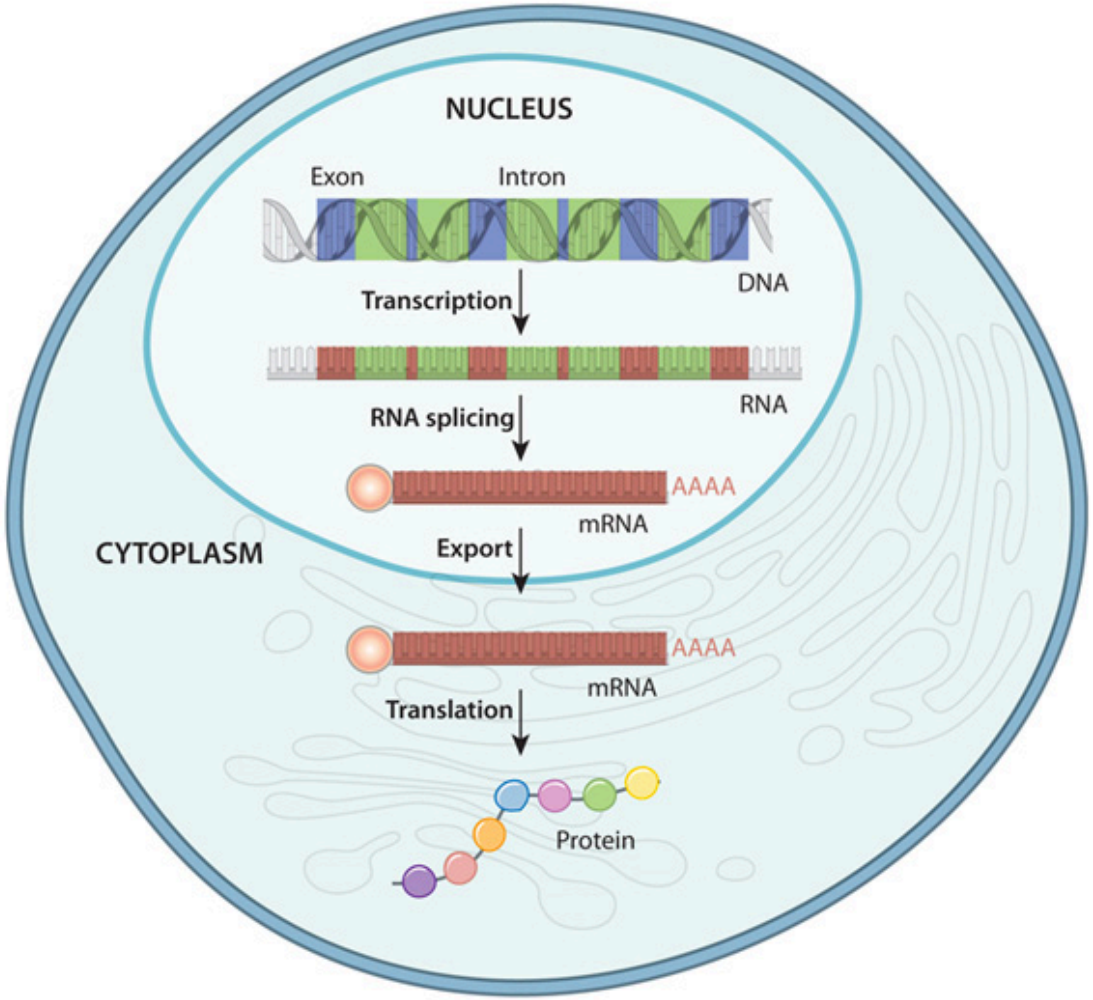
4/13/2021

Outline

- Introduction of RNA-Seq
- RNA-Seq procedure
- Reference guided assembly
- RNA-Seq *de novo* assembly
- PacBio Iso-Seq
- Nanopore RNA-Seq

Transcriptome



DNA to protein in eukaryote

nature.com/scitable/topicpage/gene-expression-14121669

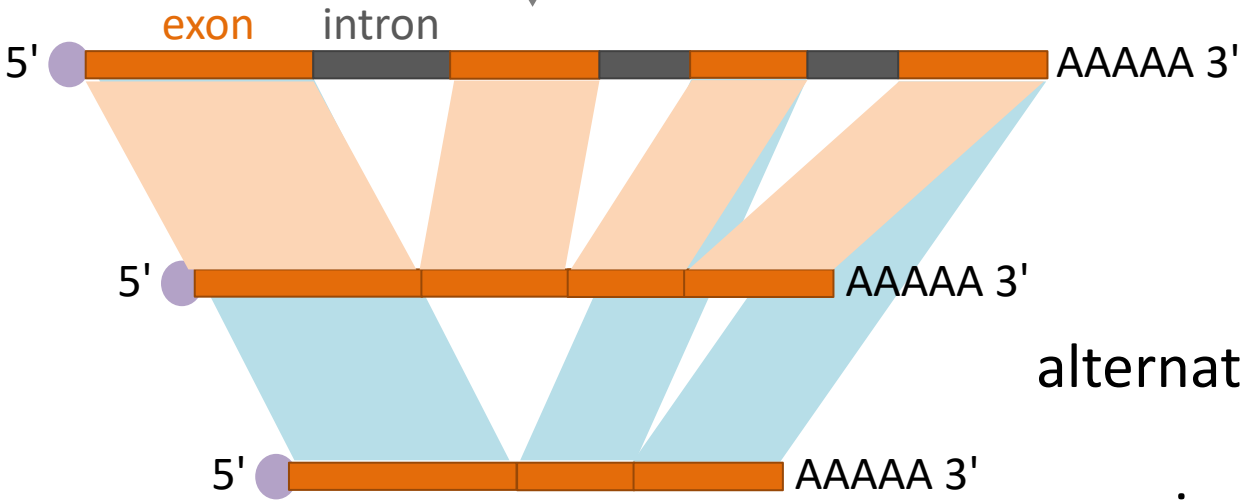
Alternative splicing

Genome

transcription

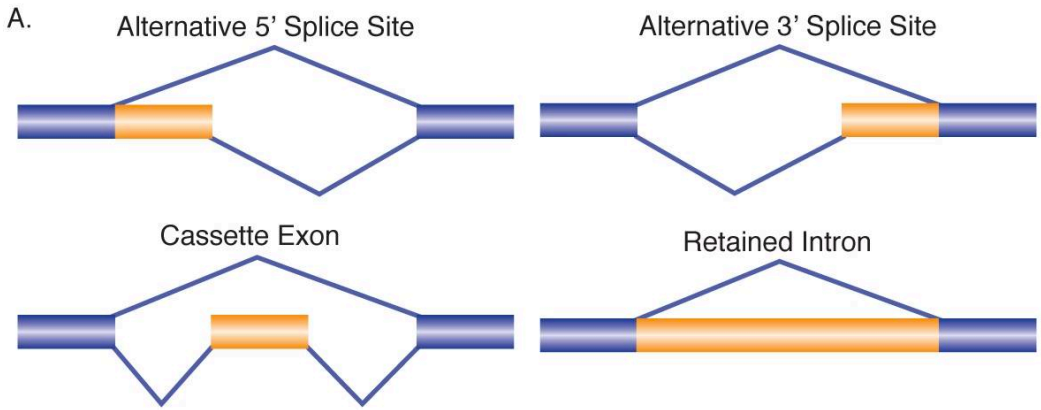
Pre-mRNA

mRNA transcript



alternative splicing

isoforms



Curr Opin Genet Dev. 21(4): 373–379 (2011)

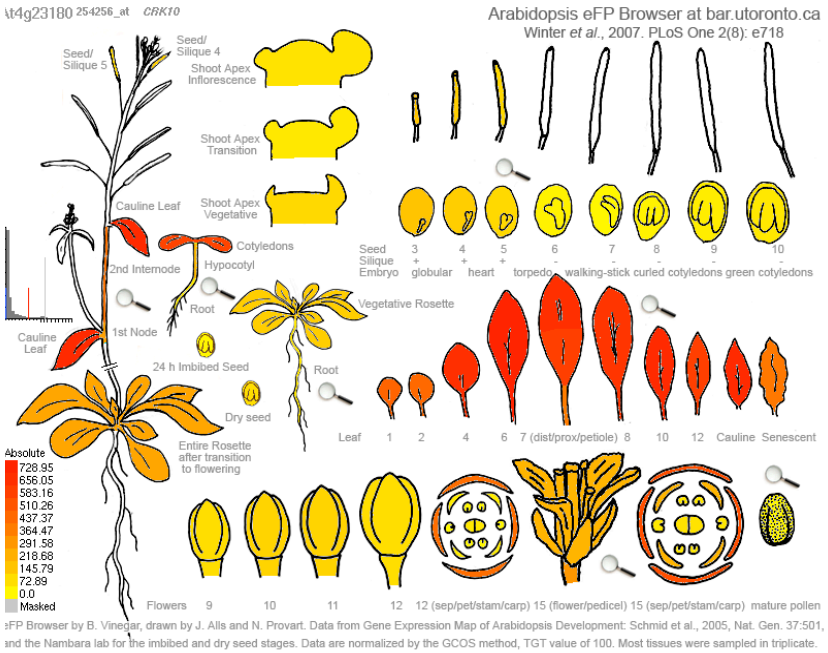
Complexity of transcriptome

In many eukaryotic organisms, the majority of genes are alternatively spliced to produce multiple transcript isoforms.

In humans, for example, there is evidence for alternative splicing of more than 95% of genes, with an average of more than five isoforms per gene.

- Tilgner et al. (2014) PNAS

Transcriptome analysis



Expression profiles in different tissues

Response to biotic stress

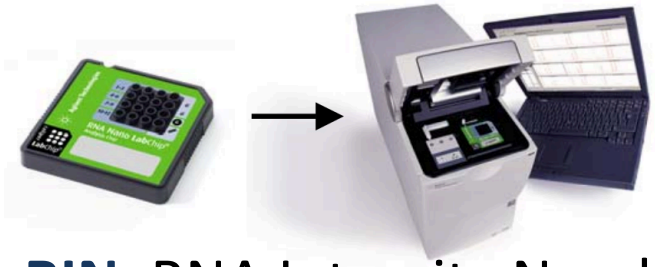
1. What are sequences of transcripts?
2. What is the expression level of each transcript?

RNA-Seq well addresses both questions

total RNA

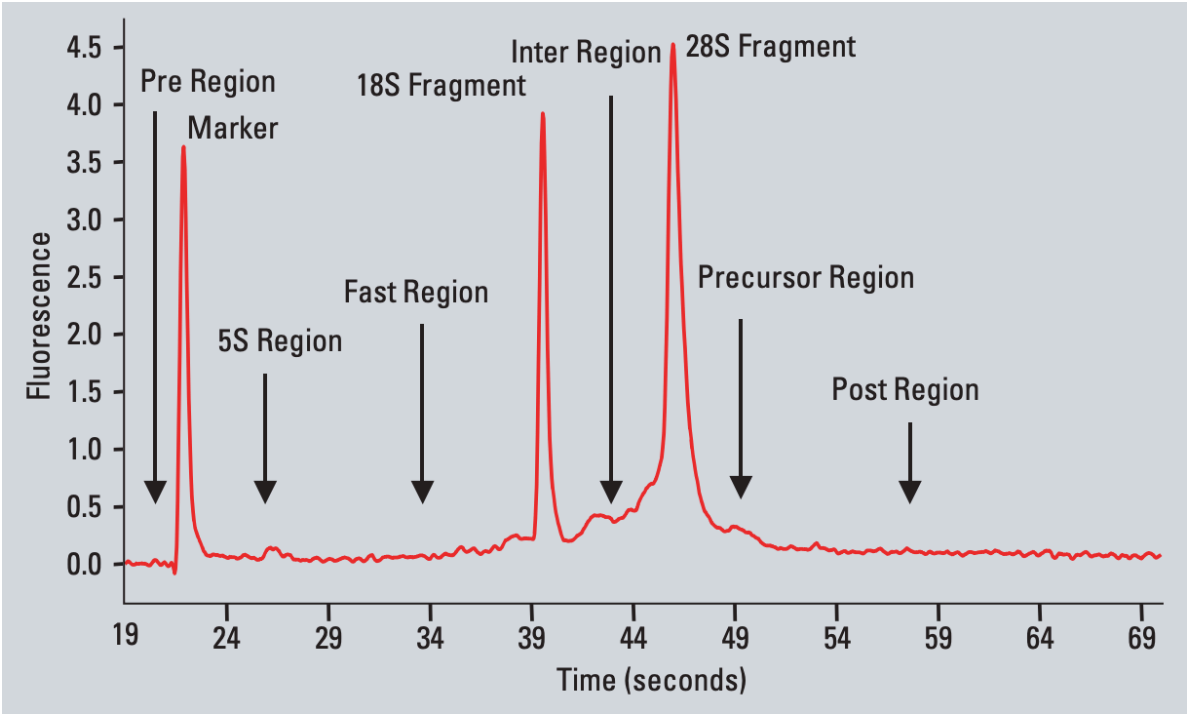
RNA-Seq

- total RNA
- mRNA
- DNA library
- sequencing
- reads



RIN: RNA Integrity Number

a value from 1 to 10, with 10 being the least degraded.



https://www.agilent.com/cs/library/applications/5989-1165EN.pdf

total RNA

RNA-Seq

- total RNA
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- DNA library
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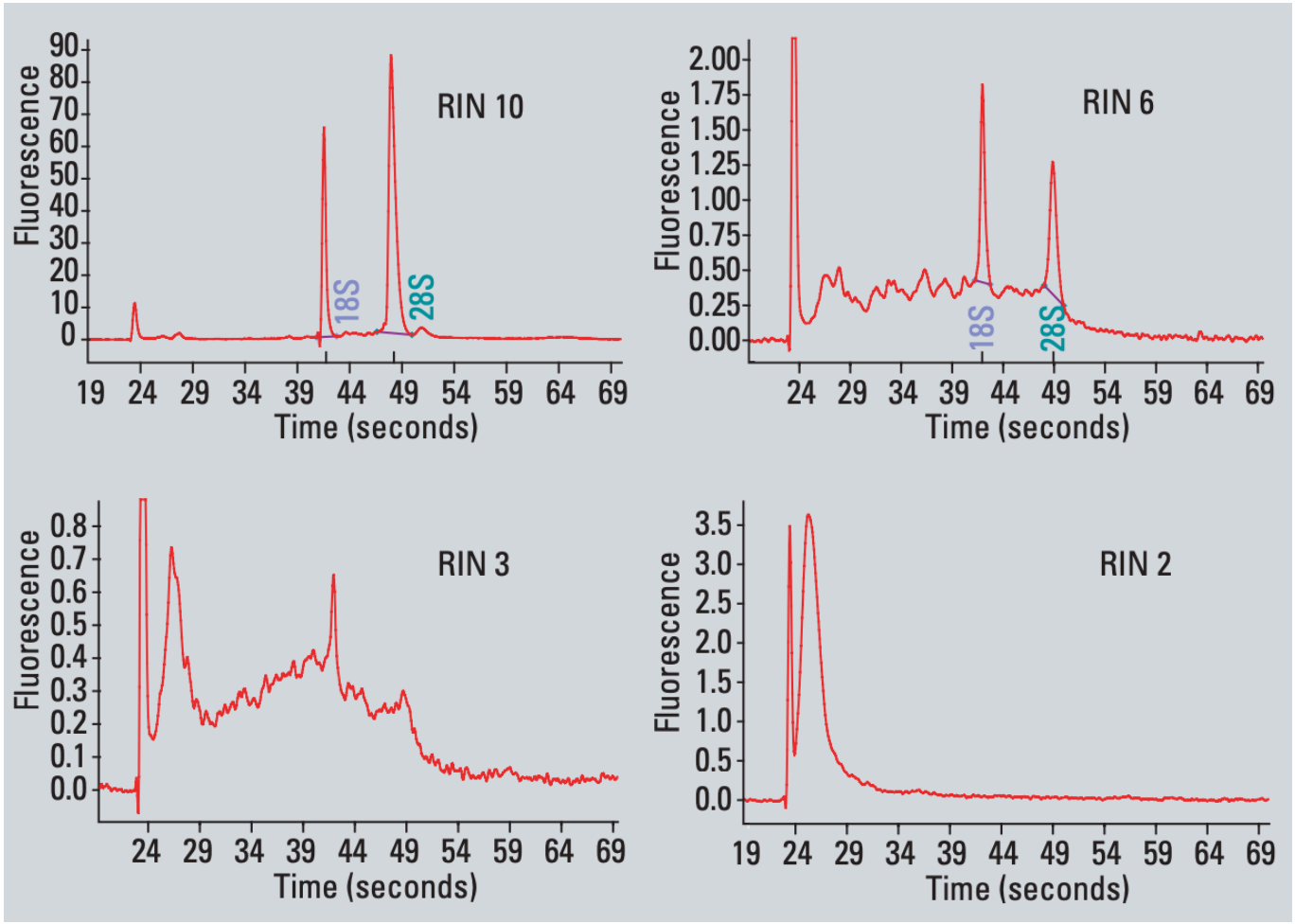

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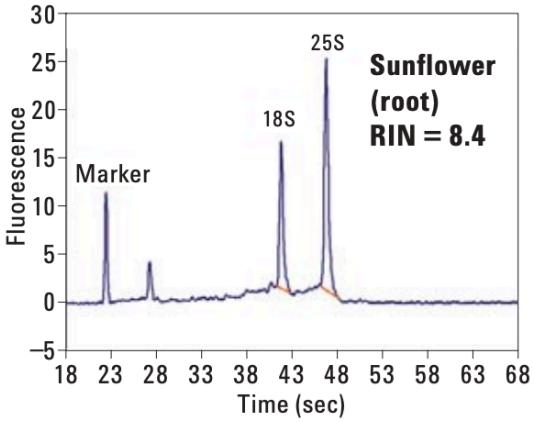
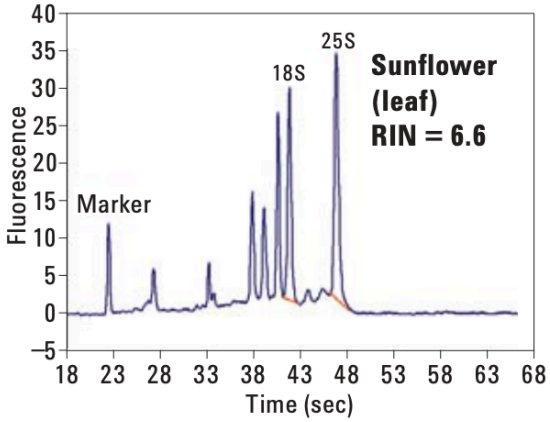
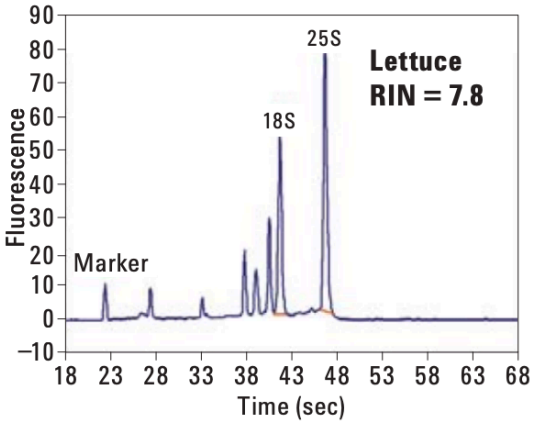
total RNA of plant tissues

Result of plant total RNA using the RNA 6000 Nano Kit







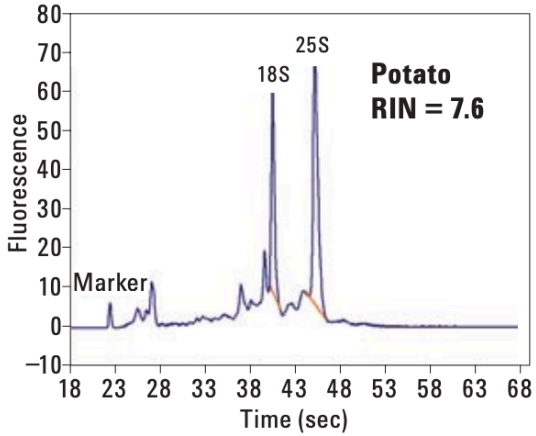
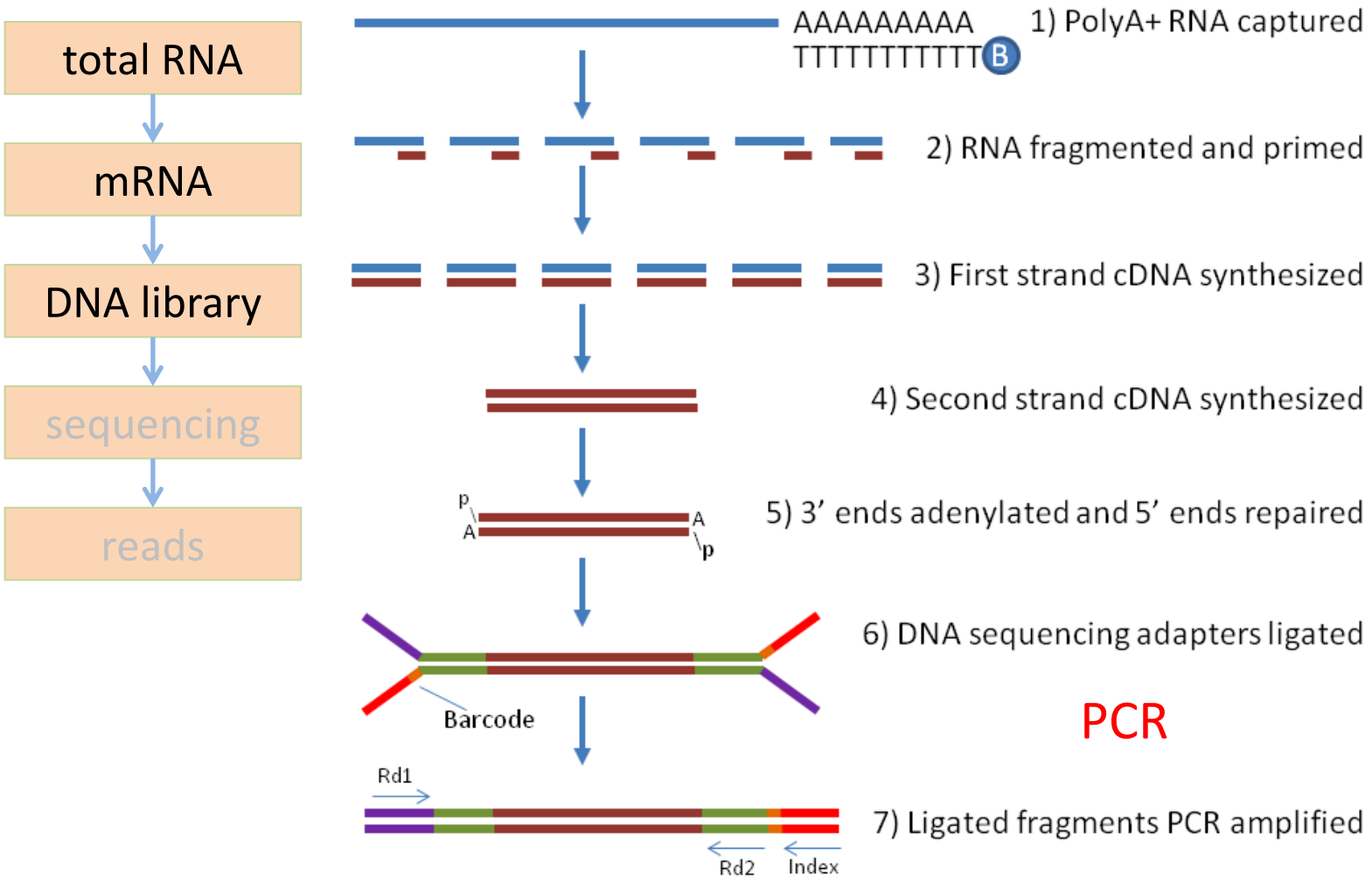


Table. Plant total RNA

Ribosomal RNA	Types of RNA
Cytosolic ribosomes	
Larger subunit	25S, 8S, 5S
Smaller subunit	18S
Chloroplasts ribosomes	
Larger subunit	23S, 5S
Smaller subunit	16S
Mitochondrial ribosomes	
Larger subunit	24S, 5S
Smaller subunit	18S, 5S

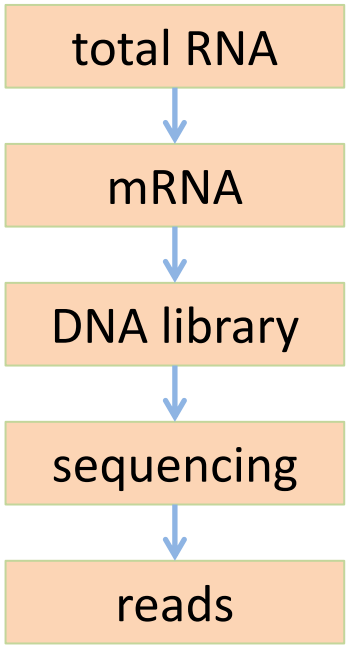
https://www.agilent.com/cs/library/applications/5990-8850EN.pdf

Illumina RNA-Seq library preparation



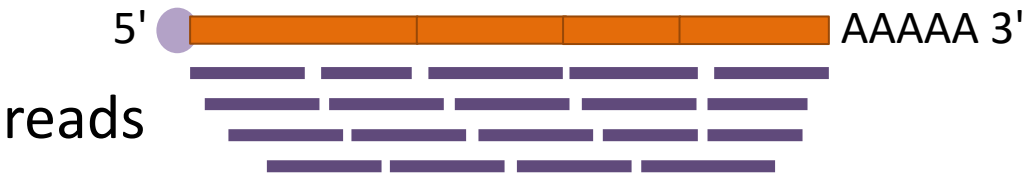
labome.com/method/RNA-seq-Using-Next-Generation-Sequencing.html
review paper: doi.org/10.1093/nar/gky167

Illumina sequencing



Single-end reads

Paired-end reads

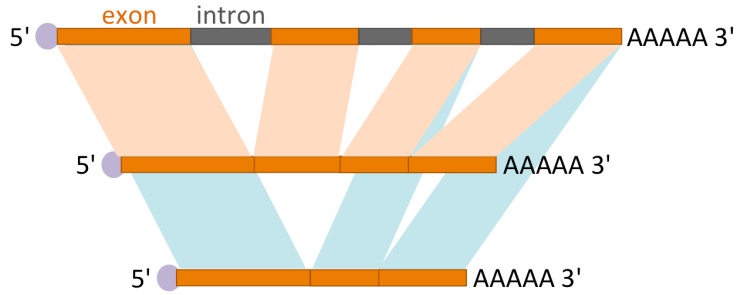


How to reconstruct full-length transcripts from short reads?

Challenges of reconstruction of full-length transcripts (transcriptome assembly)

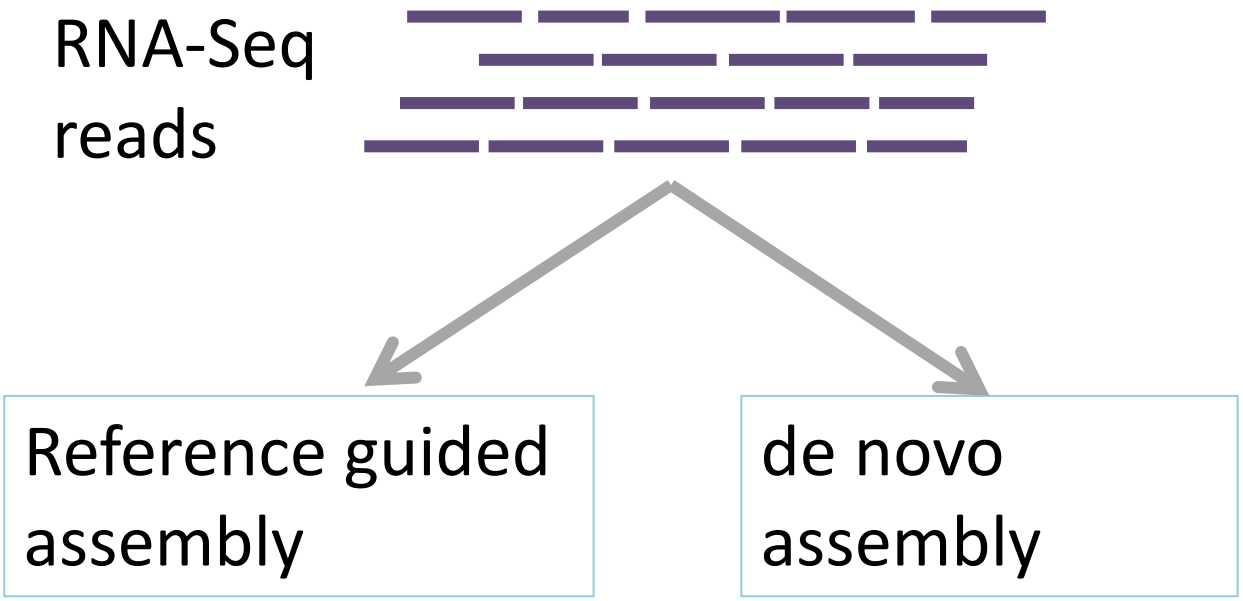
1. Sequencing errors
2. Repeats in different genes

3. Various coverage on different transcripts
4. Read coverage may be uneven across the transcript's length, owing to sequencing biases
5. Alternative splicing greatly complicates transcriptome assembly



Nature Biotechnology 29, 644–652 (2011)

Two main strategies for transcriptome assembly



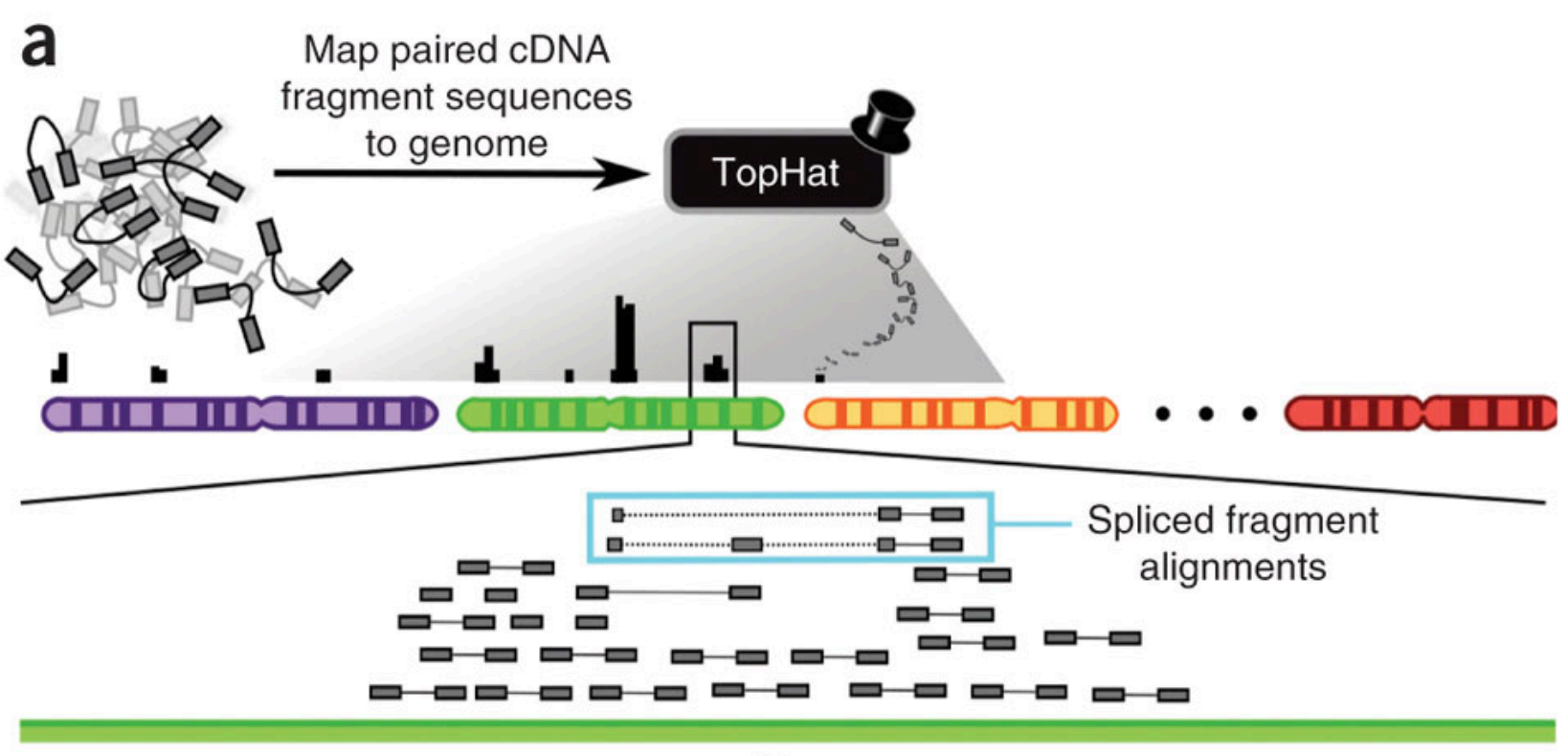
Reference-guided transcriptome assembly

RNA-Seq reads

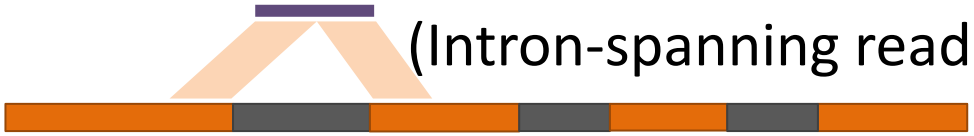
Reference guided assembly (Cufflinks)
1. alignment to the reference genome
2. assembly based on alignments

Question: Using a genome as the reference, what difference between RNA-Seq alignments and DNA-Seq alignments?

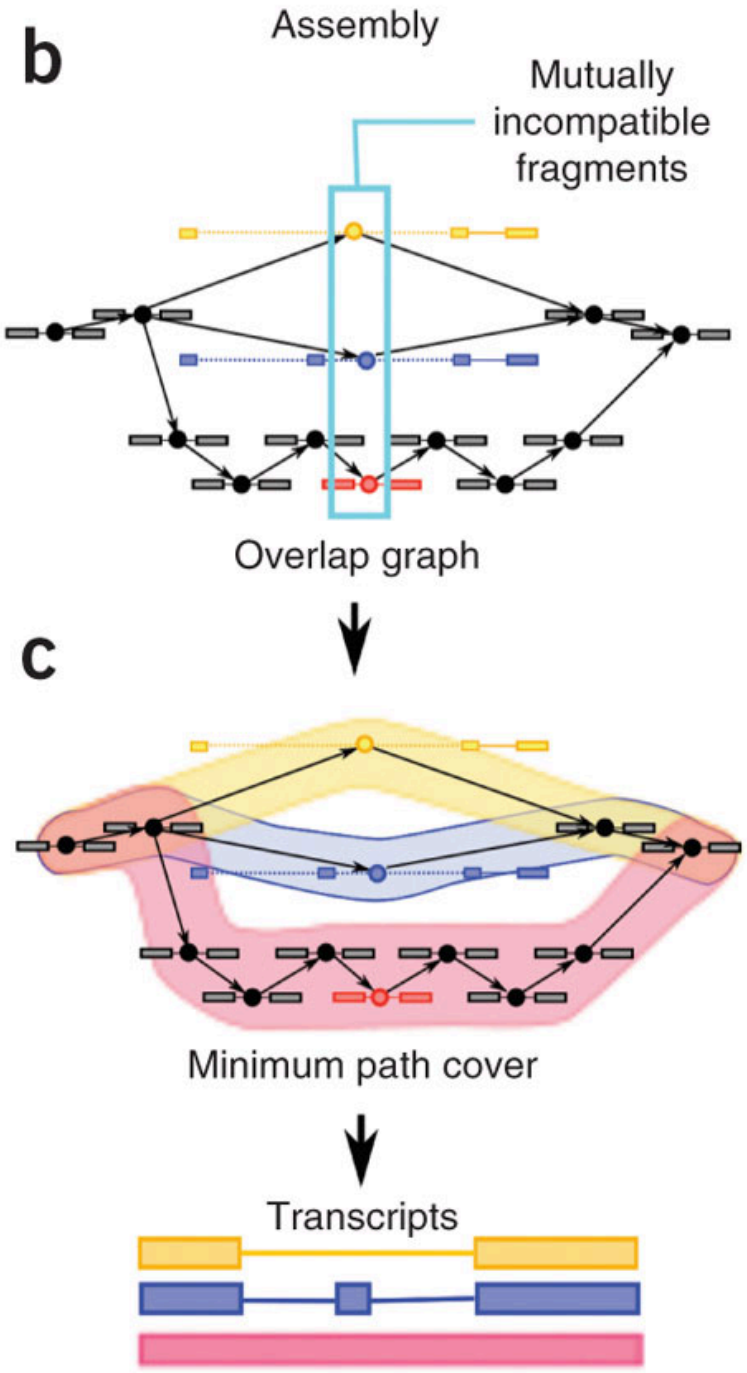
Cufflinks - spliced alignments



split read
(Intron-spanning read)



Nature Biotechnology 28, 511–515 (2010)



Cufflinks - assembly

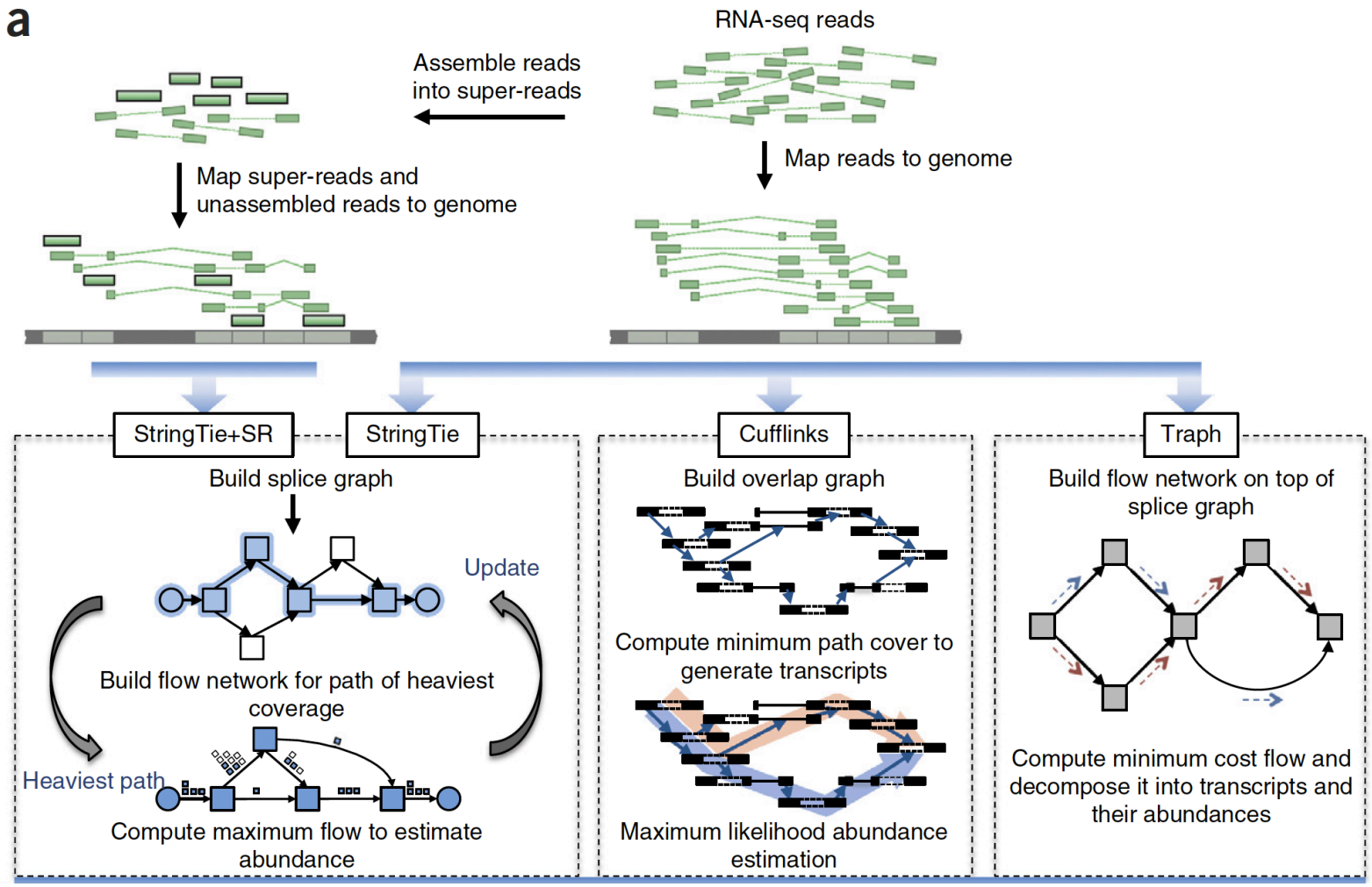
Overlapping graph is made up with compatible fragments (nodes) and alignments overlap in the genome

Incompatible fragments are fragments originated from distinct spliced mRNA isoforms

Isoforms are assembled from the overlap graph

Nature Biotechnology 28, 511–515 (2010)

StringTie



StringTie produces more complete and accurate reconstructions of genes and better estimates of expression levels, compared with Cufflinks, IsoLasso, Scripture and Traph.

Pros and cons of reference-guided assembly

Theoretically, reference-guided assembly promises **maximum sensitivity**

The quality of assembly is **depended on the accuracy of read-to-reference alignments**, which are complicated by splicing, sequencing errors and the lack or incompleteness of many reference genomes

Alignments are even more complicated when **the species used to construct a reference genome is distant from the species** that is used for constructing a reference genome

De novo assembly

***De novo* transcriptome assembly** is to perform an assembly from scratch, which does not rely on read-reference alignments.

De novo transcriptome assembly is important when the genomic sequence is not available, gapped, highly fragmented, or substantially different with the reference genome.

Trinity

step 1: *Inchworm* assembles reads into unique sequences of transcripts (**reads to contigs**)

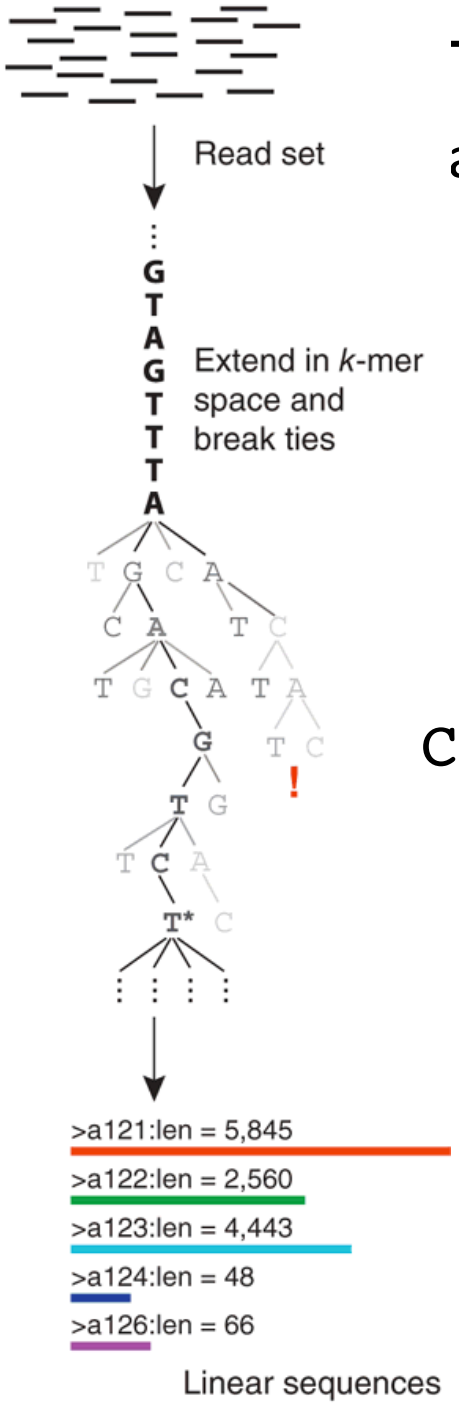
step 2: *Chrysalis* clusters related contigs (**contigs to clusters**)

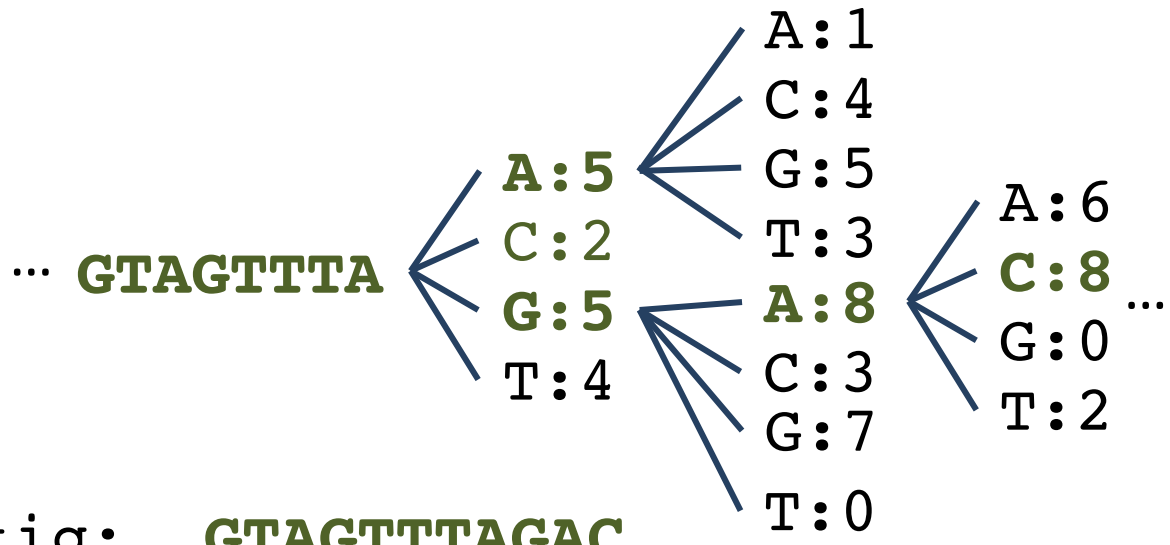
step 3: *Butterfly* analyzes the paths taken by reads in the context of the corresponding de Bruijn graph and reports all plausible transcripts (**cluster to transcripts**)

Nature Biotechnology 29, 644–652 (2011)

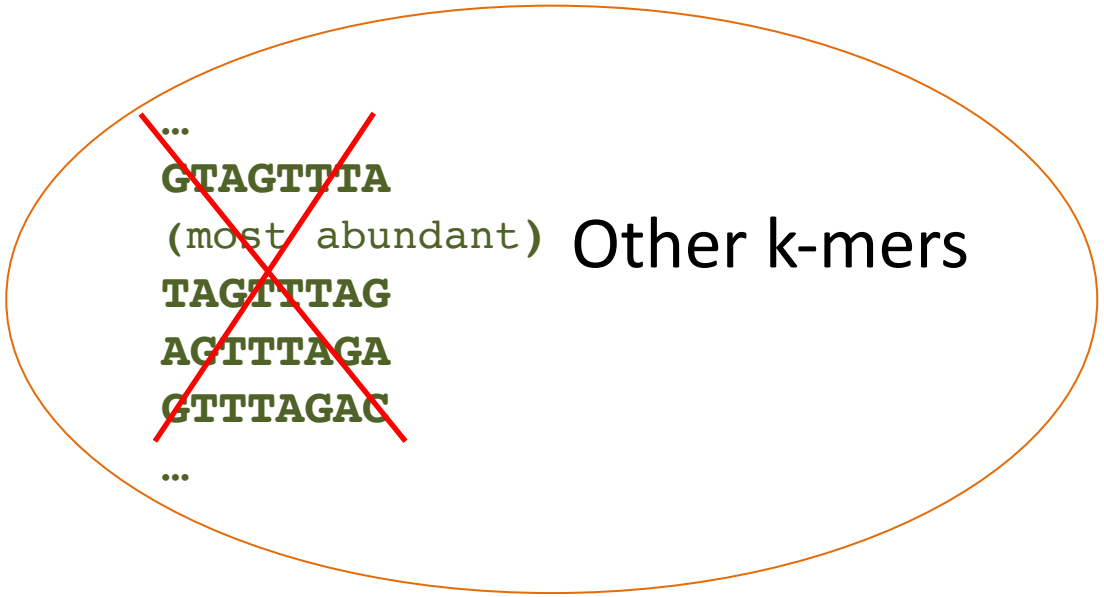
Trinity – Inchworm

a k-mer–based approach for transcript assembly

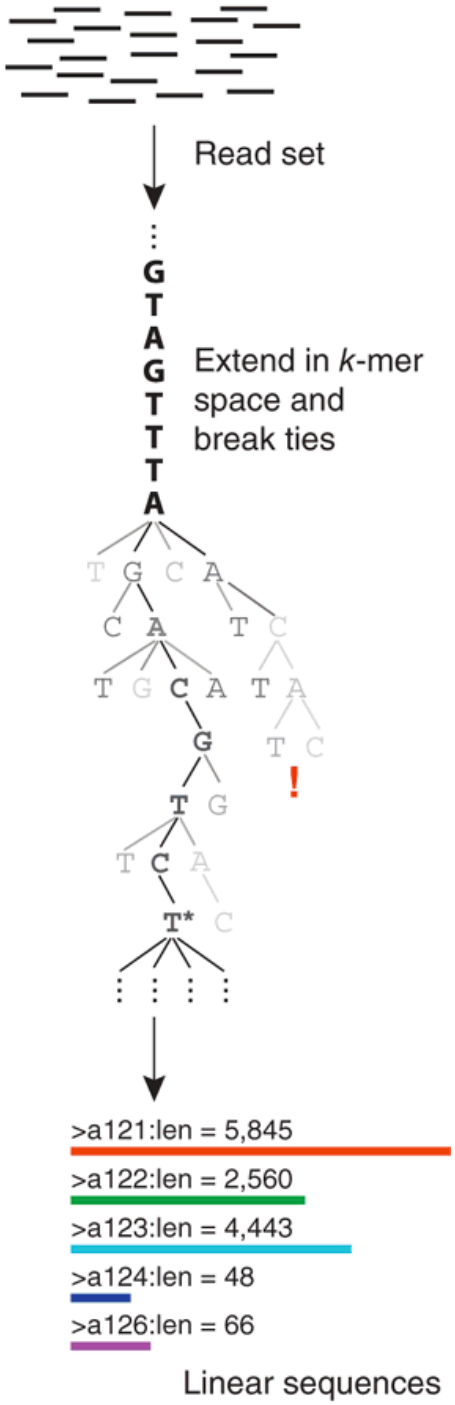




Contig: …GTAGTTTAGAC…

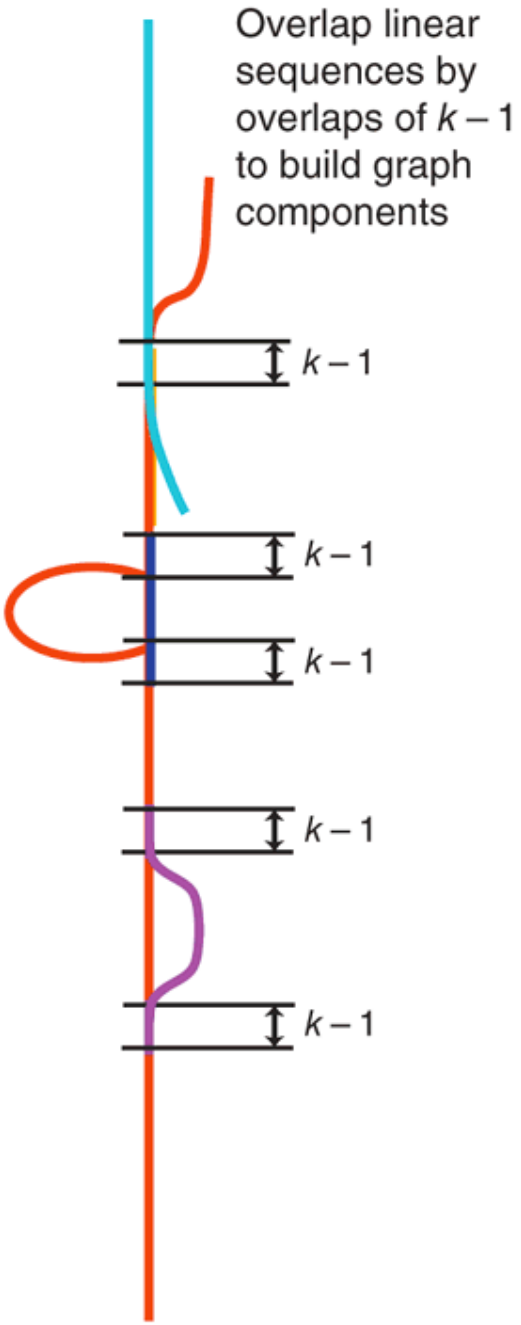


Trinity - Inchworm



Inchworm assembles reads into the unique sequences of transcripts (contigs), recovering only a single (best) representative for a set of alternative variants that share k-mers.

The contigs alone do not capture the full complexity of the transcriptome

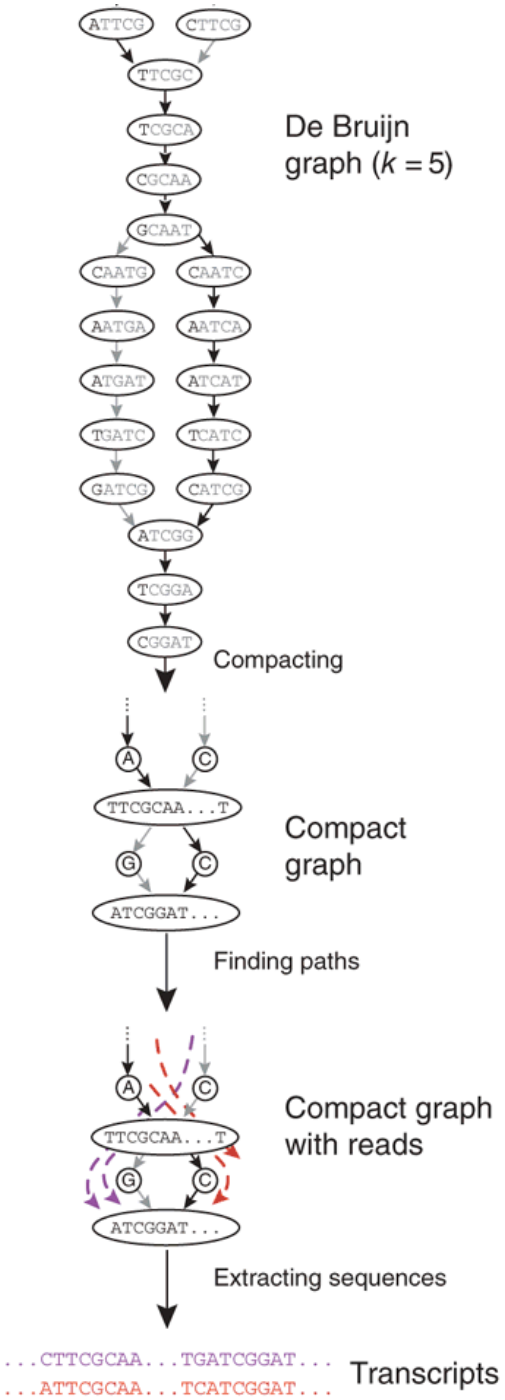


Trinity - Chrysalis

step 2: **Chrysalis clusters related contigs**

Chrysalis then **constructs a de Bruijn graph** for each cluster of related contigs

Determine **components.** Each component defines a collection of contigs that are likely to be derived from alternative splice forms or closely related paralogs.

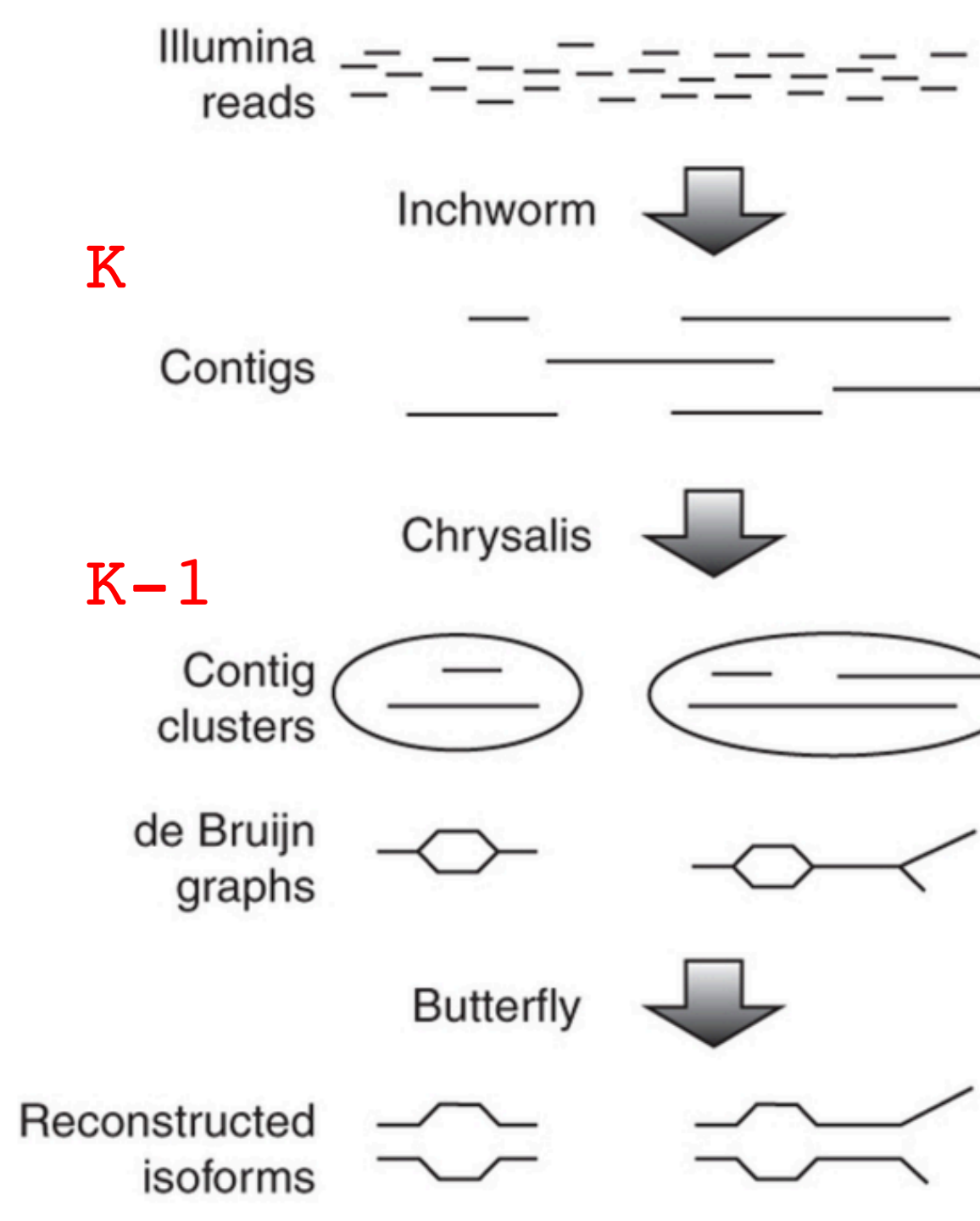


Trinity - Butterfly

step 3: Butterfly reconstructs plausible, full-length, linear transcripts by reconciling the individual de Bruijn graphs generated by Chrysalis **with the original reads and paired ends**.

Butterfly resolves alternatively spliced isoforms and transcripts derived from paralogous genes.

Trinity summary

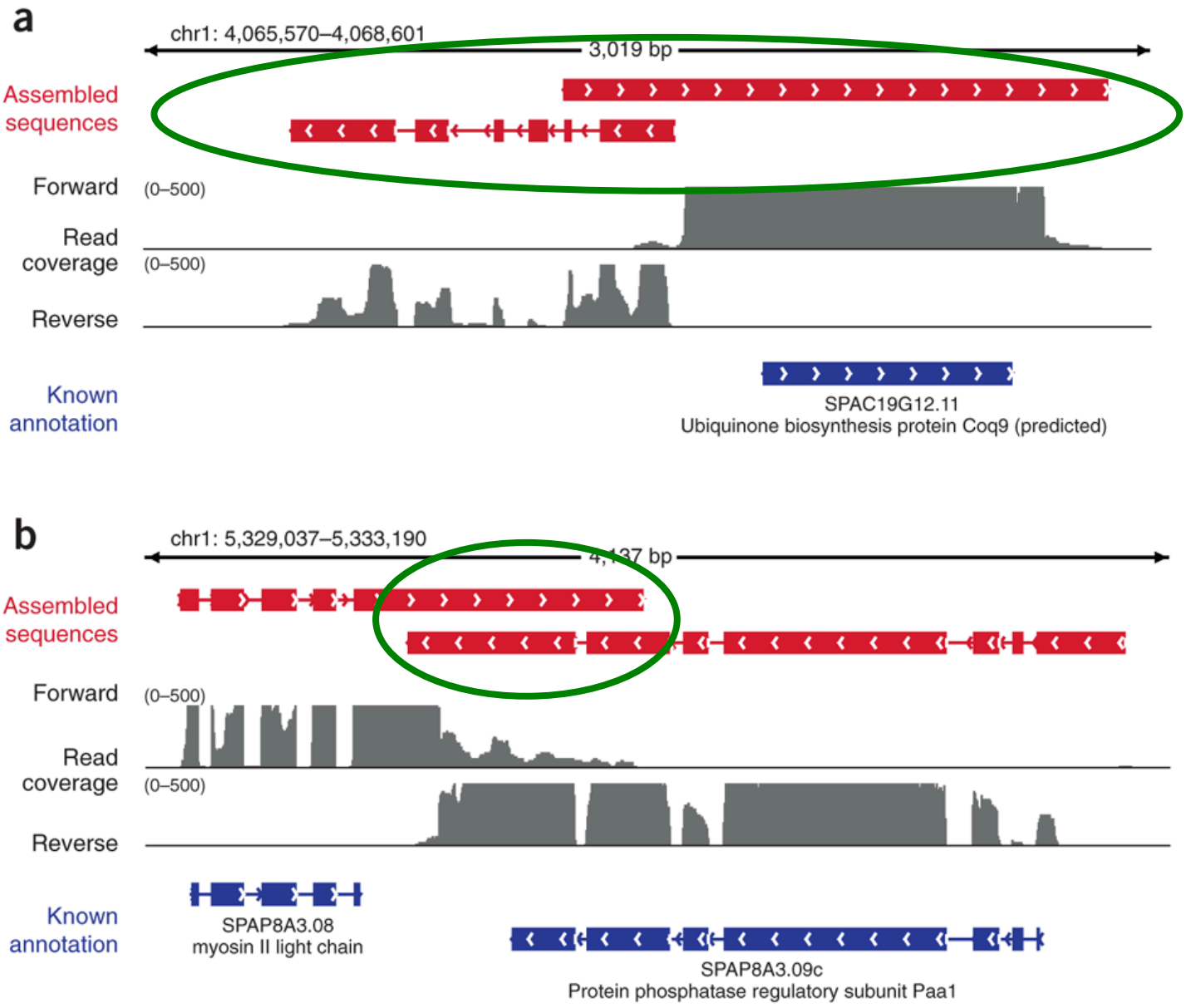


reads to contigs

contigs to cluster/graph

graph to transcripts

De novo assembly could correct wrong annotations



Note

Trinity also provides an option to perform reference-guided transcriptome assembly,

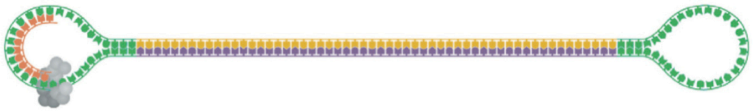
https://github.com/trinityrnaseq/trinityrnaseq/wiki/Genome-Guided-Trinity-Transcriptome-Assembly

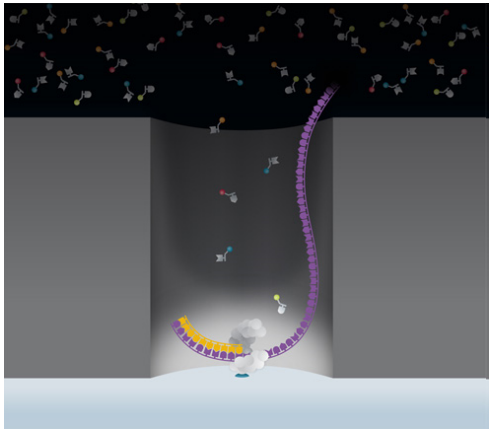
Outline

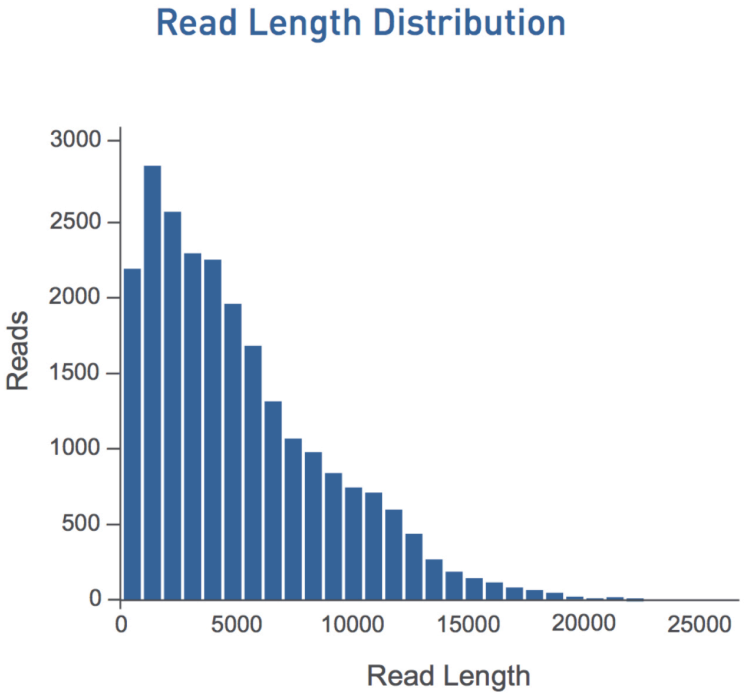
- Introduction of RNA-Seq
- RNA-Seq procedure
- Reference guided assembly
- RNA-Seq *de novo* assembly
- PacBio Iso-seq

PacBio long reads for RNA sequencing (Iso-Seq)

- Iso-Seq for analyzing full-length transcripts
- High-quality, single-molecule, circular-consensus (CCS)

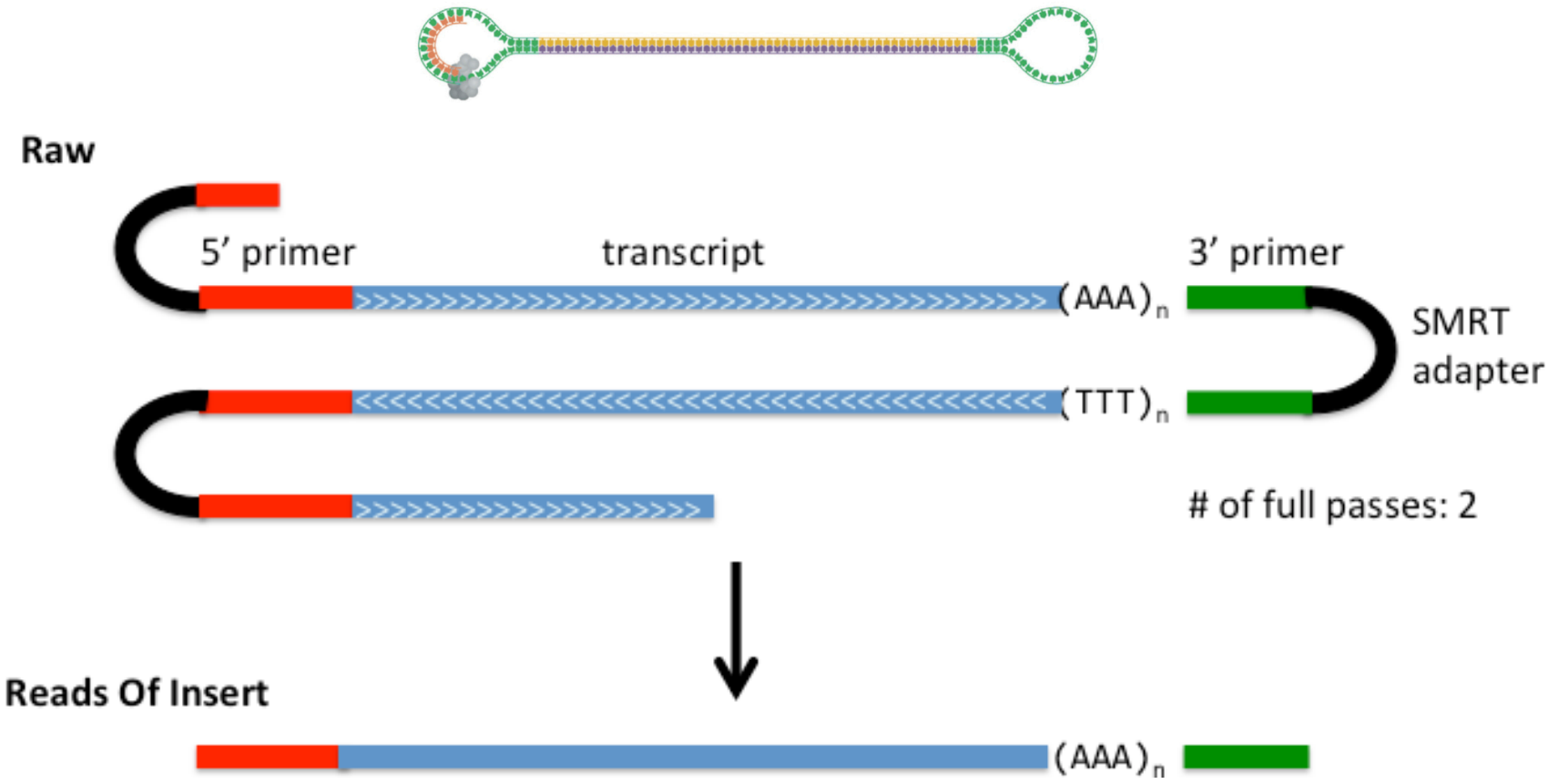






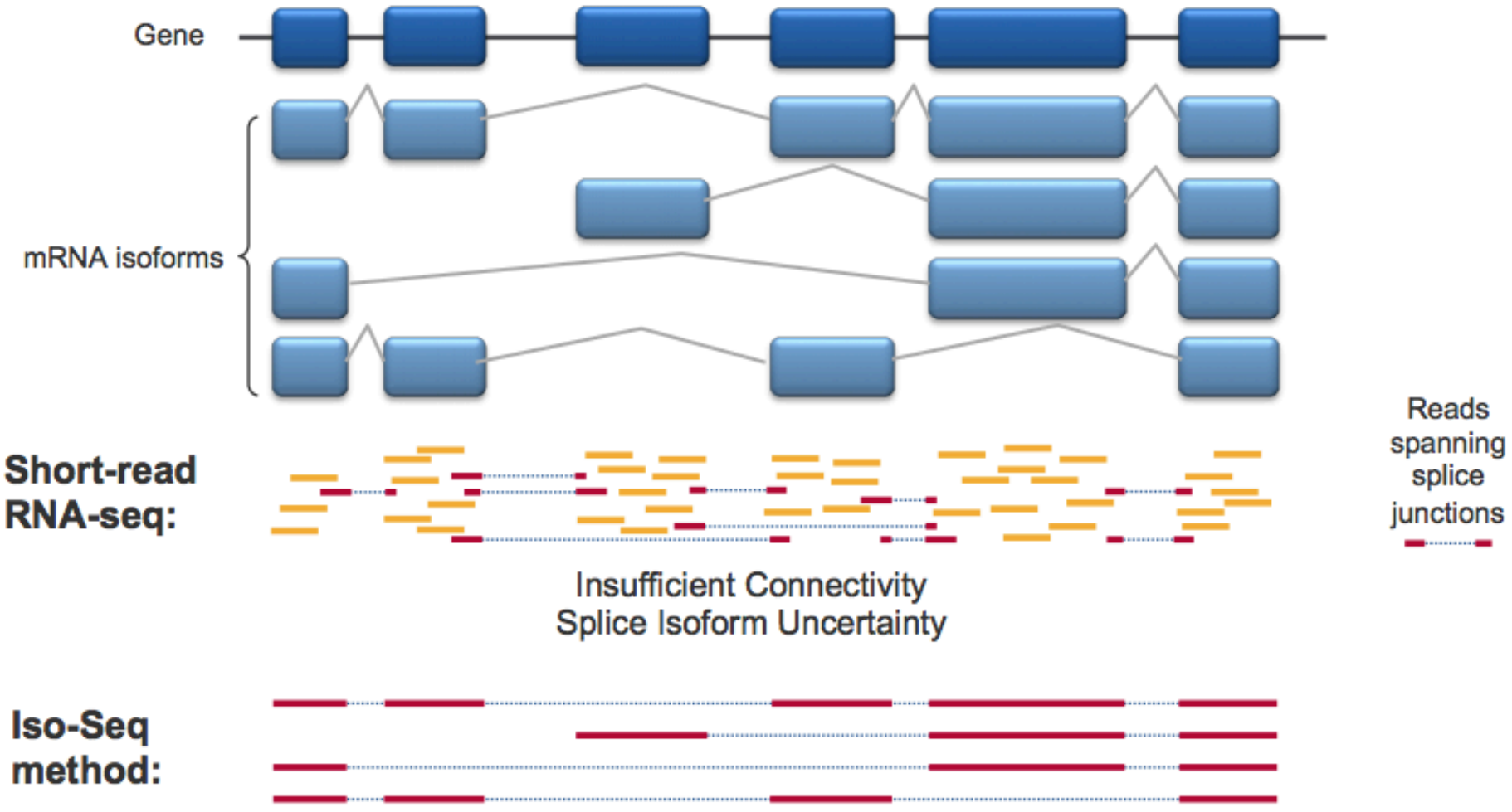
http://flxlexblog.wordpress.com/2013/02/11/applications-for-pacbio-circular-consensus-sequencing/

Multiple passes improve sequence quality



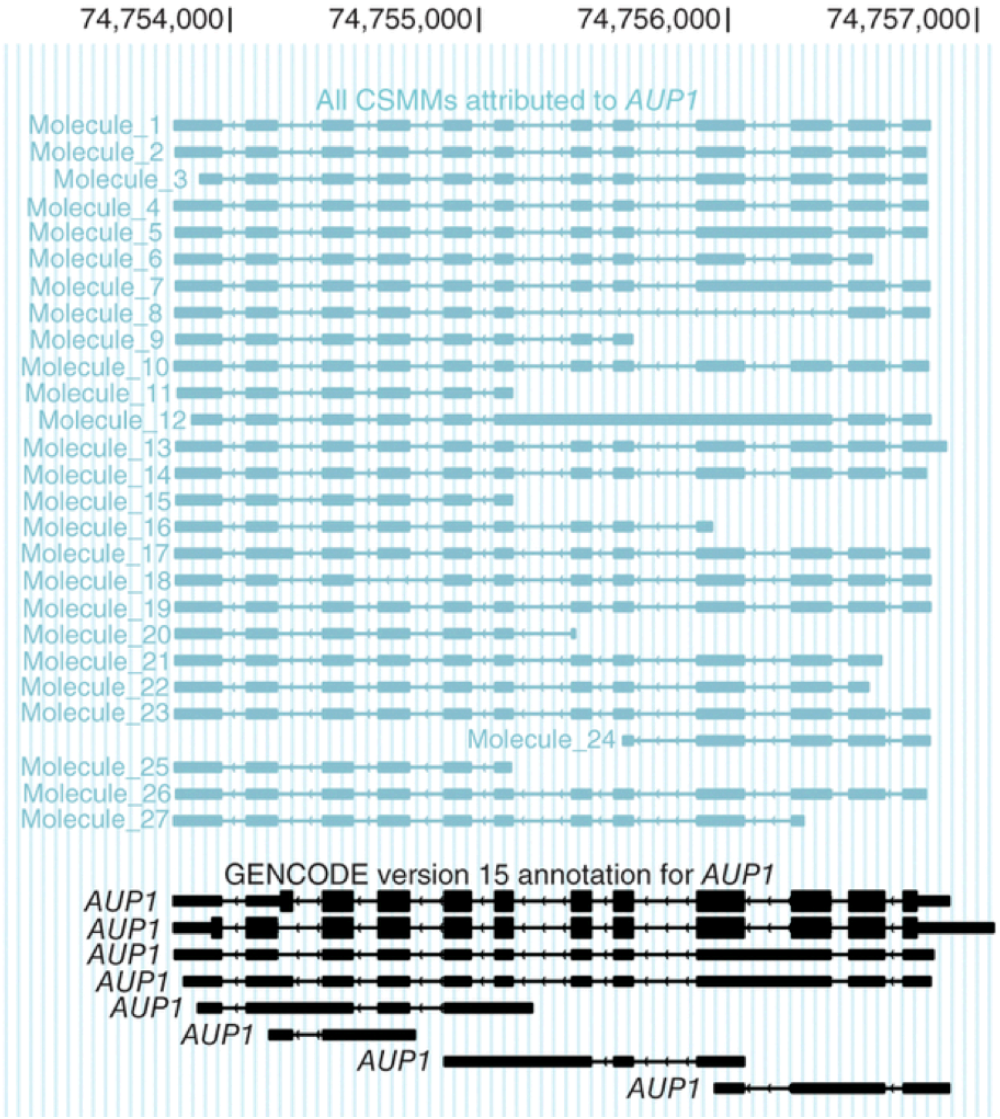
https://github.com/PacificBiosciences/cDNA_primer/wiki/Understanding-PacBio-transcriptome-data

Long-read, full-length, no assembly required



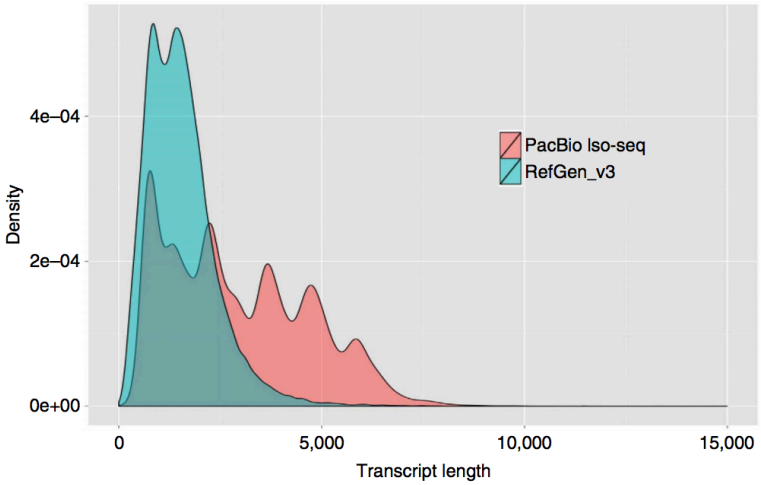
Long reads to sequence full-length cDNA

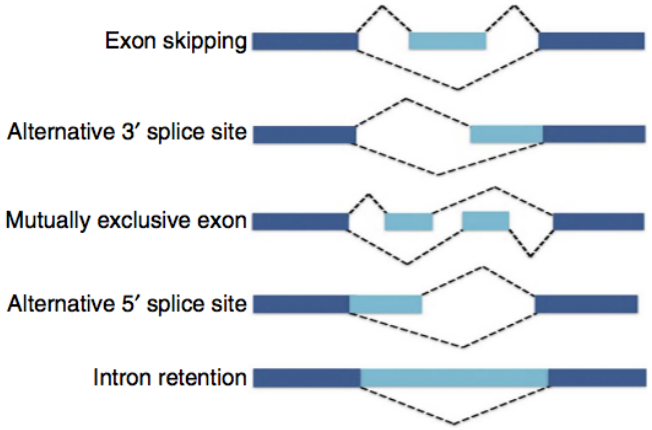
- The majority of reads represent all splice sites of original transcripts
- Isoforms can be monitored at a single-molecule level without amplification or fragmentation

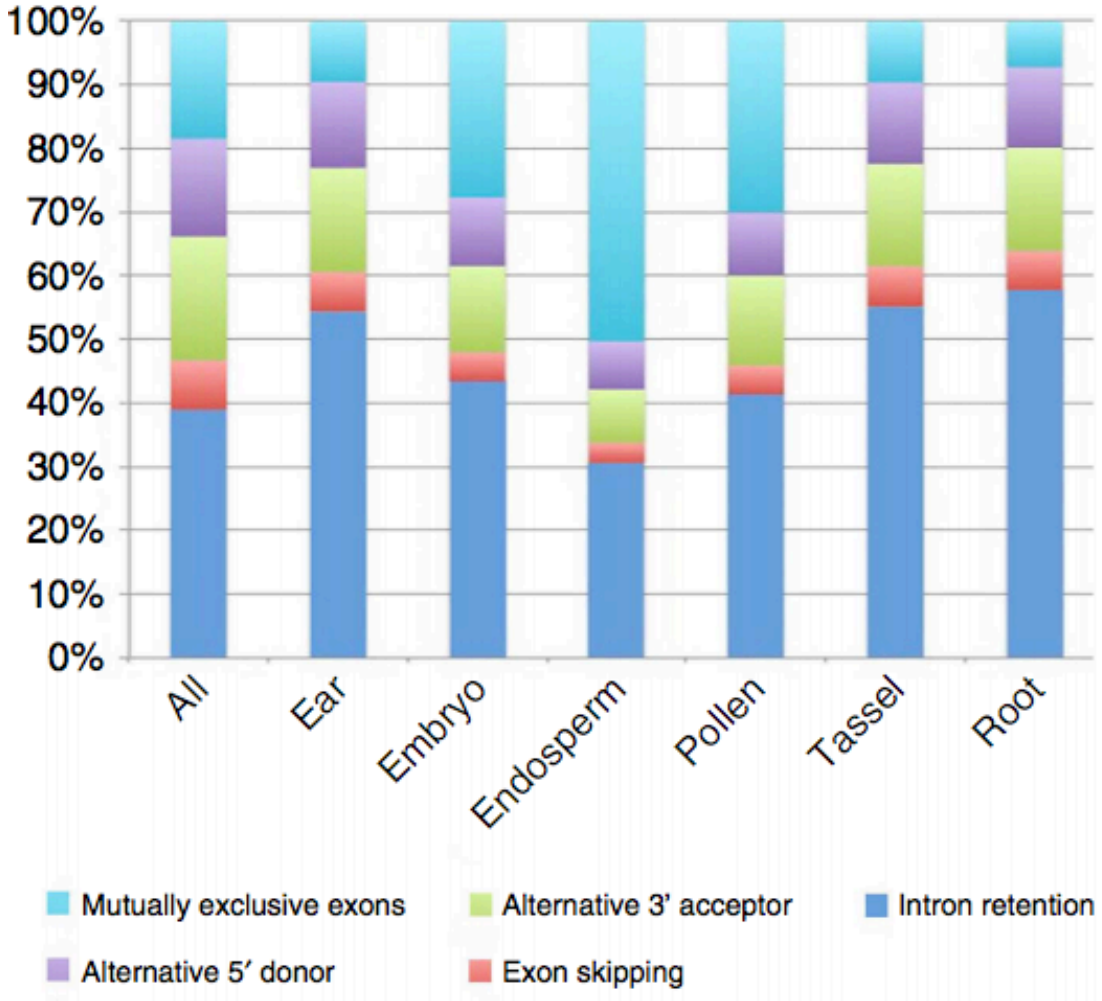


Sharon et al. Nature Biotechnology 31, 1009–1014 (2013)

maize – Iso-Seq





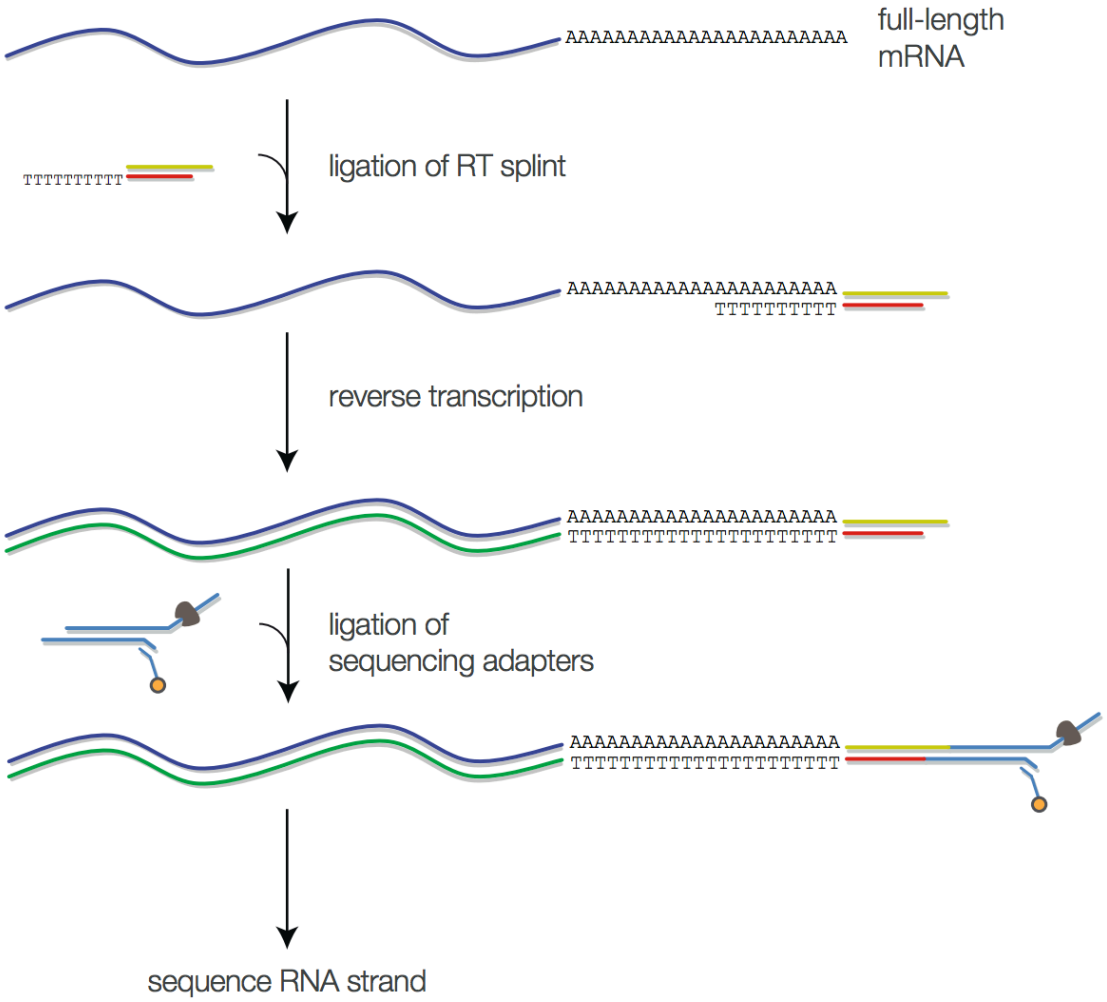


Wang et al., Nature Communications, 2016, 7:11708

Nanopore provides long RNA-Seq reads

	Direct RNA Sequencing	cDNA-PCR Sequencing	Direct cDNA Sequencing
Input	500 ng RNA (polyA)	1 ng RNA (polyA)	100 ng RNA (polyA)
RT required	Optional	Yes	Yes
PCR required	No	Yes	No
Read length	Equal to RNA length	Enriched for full-length cDNA	Enriched for full-length cDNA
Typical # reads (MinION)	1 million	7 - 12 million	5 - 10 million

Nanopore – Direct RNA Sequencing (DRS)



http://biorxiv.org/content/early/2016/08/12/068809

Application of Nanopore DRS

1. Nanopore DRS detects long, complex mRNAs and short, structured non-coding RNAs
2. Spurious antisense reads are rare or absent in nanopore DRS (strand specific RNA)
3. Nanopore DRS confirms sites of RNA 3’ end formation and estimates poly(A) tail length
4. DRS detect m^6A modification of RNA

eLife 2020;9:e49658

References

Cufflinks paper: Trapnell C et al., Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol. 2010, 28, 511–5.

Tophat-Cufflinks-Cuffdiff-CummeRbund protocol: Trapnell C et al., Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protocols. 2012, 7, 562–578.

Trinity paper: Grabherr MG et al., Full-length transcriptome assembly from RNA-seq data without a reference genome. Nat Biotechnol. 2011, 29:644-52.

Protocol for using Trinity: Haas BJ et al., De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat Protoc. 2013, 8:1494-512.

Performance tuning of Trinity: Henschel R et al., Trinity RNA-Seq assembler performance optimization. XSEDE 2012 Proceedings of the 1st Conference of the Extreme Science and Engineering Discovery Environment: Bridging from the eXtreme to the campus and beyond.