# RNA-Seq

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# What is RNA-Seq

- Massively parallel sequencing method for transcriptome analyses
- Complementary DNA (cDNA) generated from RNA are sequenced using next-generation “short read” technologies
- Reads are aligned to a reference genome and a transcriptome map is constructed

# Transcriptome

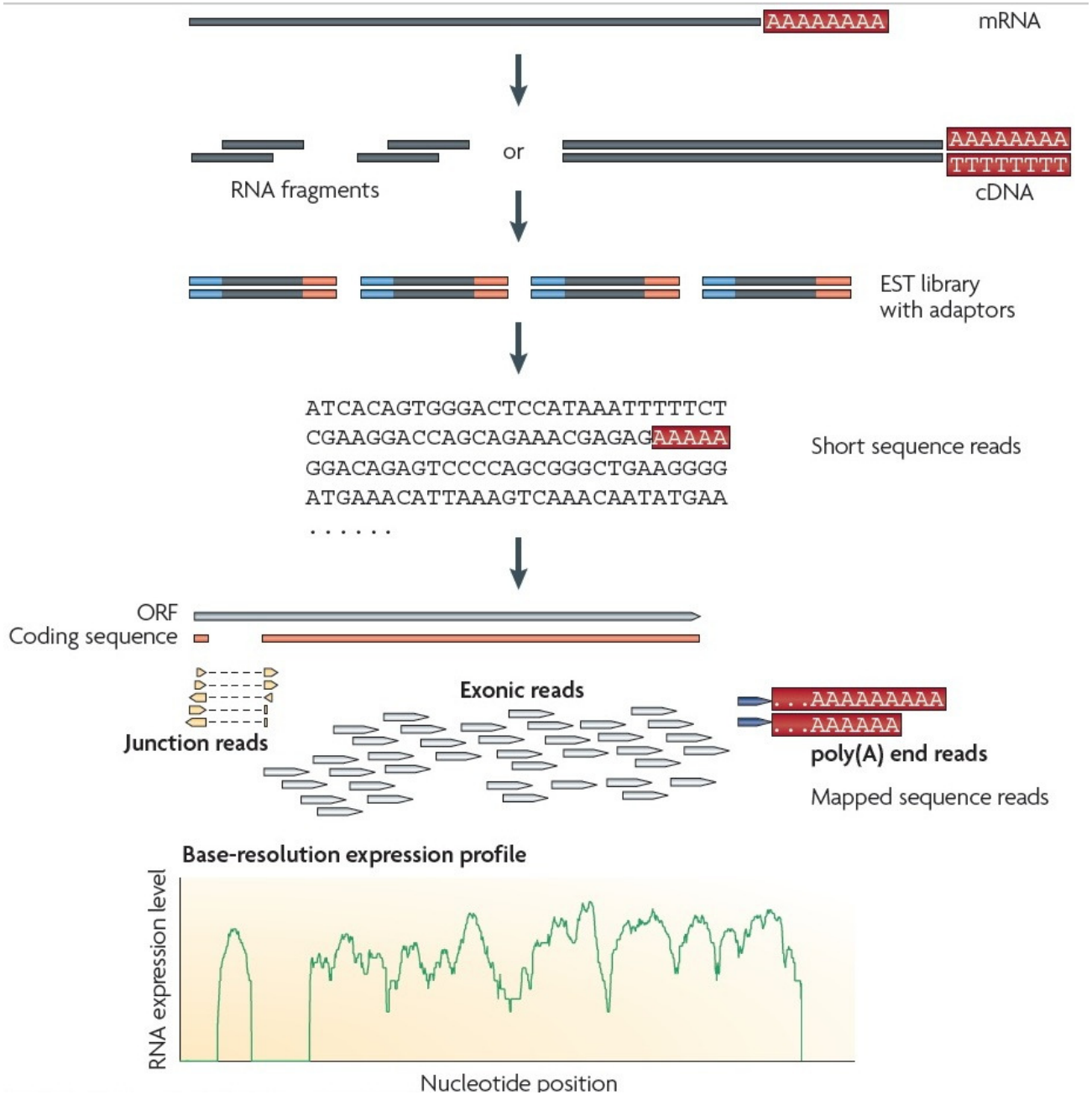
- The transcriptome is the complete set of transcripts in a cell, and their quantity, for a specific developmental stage or physiological condition
- Understanding the transcriptome is essential for
  - interpreting the functional elements of the genome
  - revealing the molecular constituents of cells, tissues
  - understanding development and disease

RNA-Seq: a revolutionary tool for transcriptomics Nat Rev Genet. 2009 Jan;10(1):57-63.

# Aims of RNA-Seq

- To quantify mRNA abundance
- To determine the transcriptional structure of genes: start sites, 5′ and 3′ ends, splicing patterns
- To quantify the changing expression levels of each transcript during development and under different conditions

RNA-Seq: a revolutionary tool for transcriptomics Nat Rev Genet. 2009 Jan;10(1):57-63.

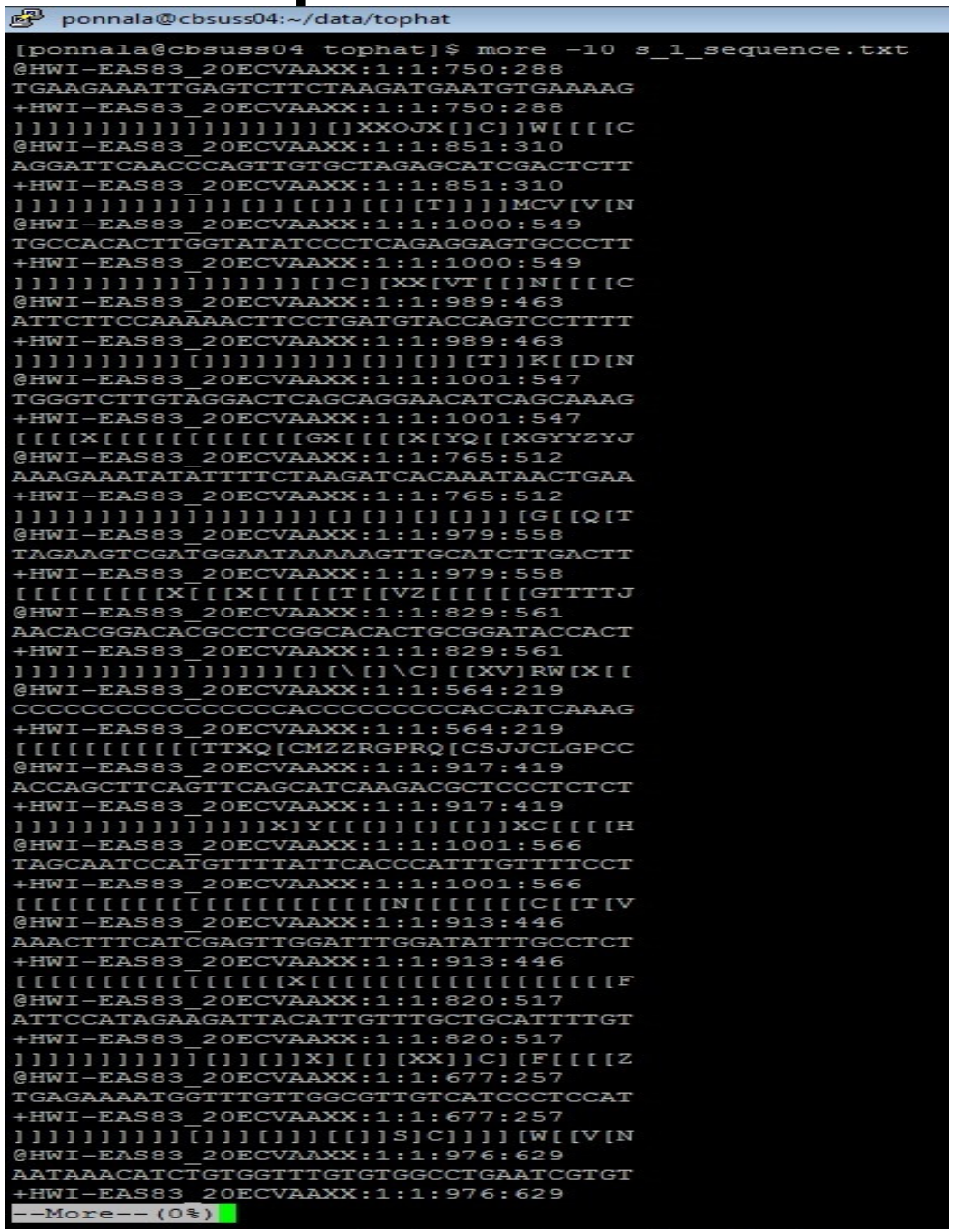



RNA-Seq: a revolutionary tool for transcriptomics Nat Rev Genet. 2009 Jan;10(1):57-63.

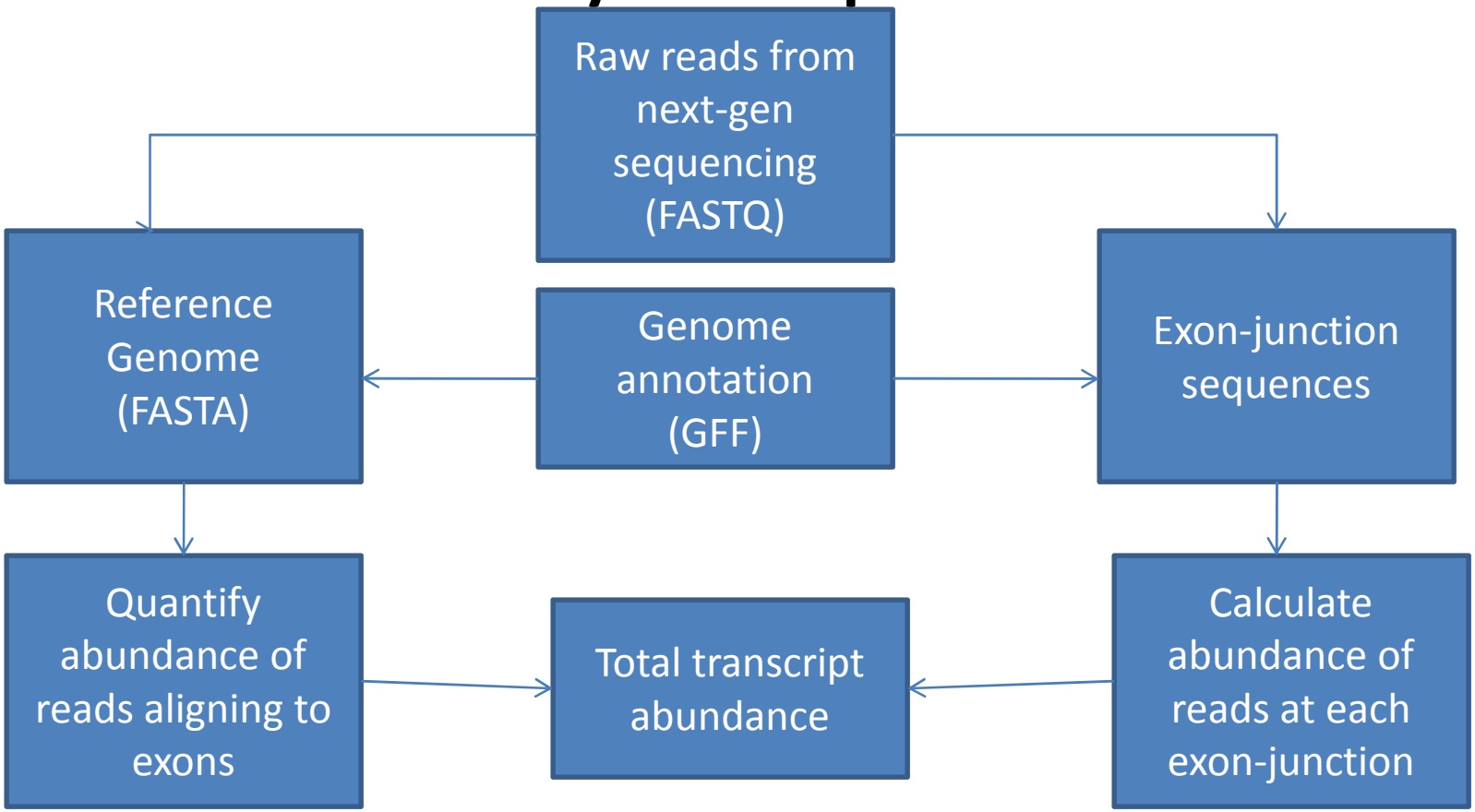
# Technology

- Single-end, paired-end
- Typically 30-400bp reads
- Popular platforms: Illumina, 454, SOLID
- >10 million reads in a single “lane”
- Alignment tools: Bowtie, BWA, Eland etc
- Additional step: align to exon-junctions
- Automated pipeline for RNA-Seq:
  - **Tophat** : for alignment
  - **Cufflinks** : for calculating expression levels

# Sequence data



# Analysis Pipeline



# Alignment Issues

- Exon Boundaries
  - What if exon is shorter than read?
- Multiple matches
  - Simple weighting
  - Evidence-based weighting

# Units of measurement

- RPKM : Reads per kilobase per million mapped reads

  1kb transcript with 1000 alignments in a sample of 10 million reads (out of which 8 million reads can be mapped) will have

  $$RPKM = 1000/(1 * 8) = 125$$

- FPKM : for paired-end sequencing
  - A pair of reads constitute one fragment

# Tophat

- Aligns sequences to the whole genome AND to exon-junctions
- Uses Bowtie, an ultrafast, memory-efficient short read aligner
- Output reported in SAM format
- Independently aligns segments of each read (default 25bp) allowing up to 2 mismatches
- Does not support indels / gapped alignments

http://tophat.cbcb.umd.edu/index.html

# Tophat : junctions

- From supplied annotation file (GFF) or list of junction coordinates
- Without reference annotation
  - Sets of coverage islands : high coverage regions
  - Paired end reads: using genomic distance between mates
  - Segments of same read mapped far apart: “GT-AG” introns

# Running Tophat

- Index the genome:

  bowtie-build maize_pseudo.fa maize_pseudo

- Run tophat:

  tophat -o zero -G annot.gff --no-novel-juncs maize_pseudo s_1_sequence.txt

- Output files:
  - accepted_hits.sam
  - annot.juncs
  - junctions.bed

Maize genome: http://maizesequence.org/ Illumina data: Tom Brutnell, BTI

# Viewing the alignments (IGV)

samtools faidx maize_pseudo.fa
samtools view -bt maize_pseudo.fa.fai -o accepted_hits.bam accepted_hits.sam
samtools index accepted_hits.bam


http://samtools.sourceforge.net/ http://www.broadinstitute.org/igv/

# Cufflinks

- can estimate the abundances of the isoforms present in the sample, using either:
  - a known "reference" annotation
  - an ab-initio assembly of the transcripts
- constructs a set of transcripts that "explain" the reads observed in an RNA-Seq experiment
- Input: alignments in SAM format, annotation in GTF (optional)
- Output: assembled transfrags, genes

http://cufflinks.cbcb.umd.edu/index.html

# Cufflinks

- Command line:

cufflinks -G annot.gtf accepted_hits.sam

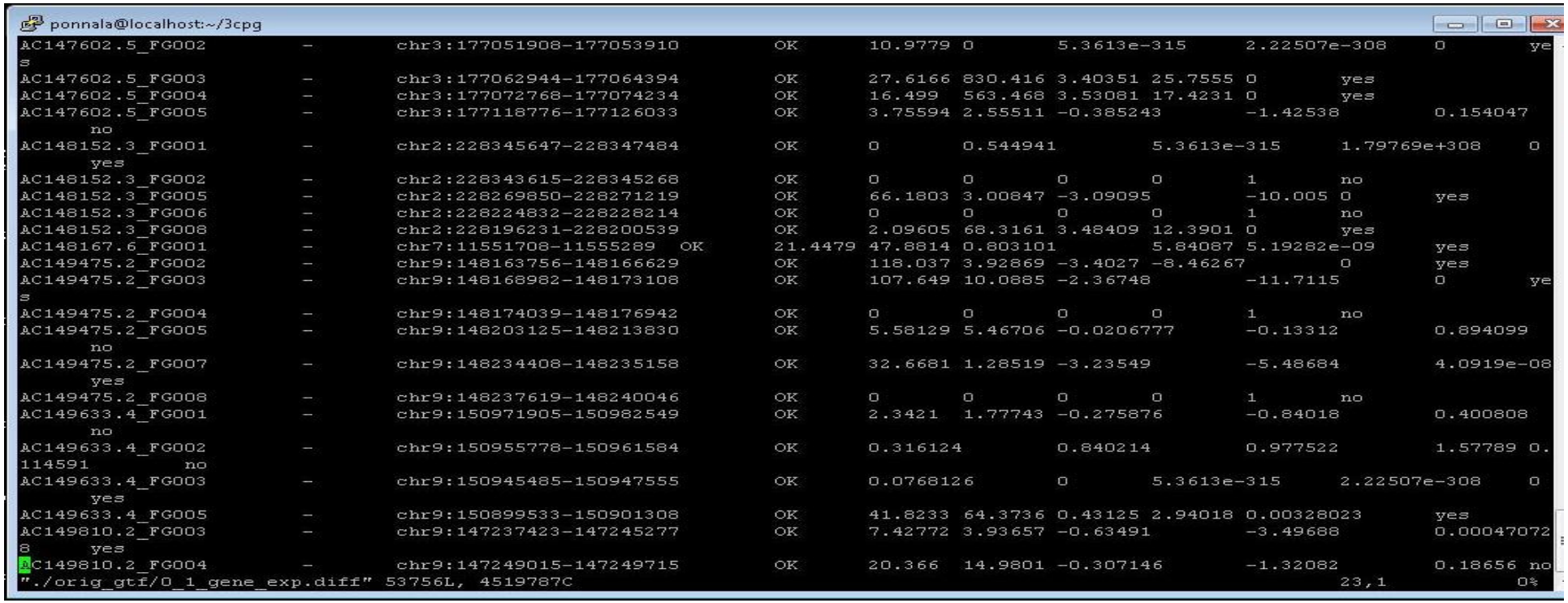
- Output files:
  - transcripts.gtf
  - transcripts.expr
  - genes.expr

# Cuffdiff

- Differential expression at the transcript “isoform” level and at the gene level

cuffdiff annot.gtf ./zero/accepted_hits.sam ./one/accepted_hits.sam

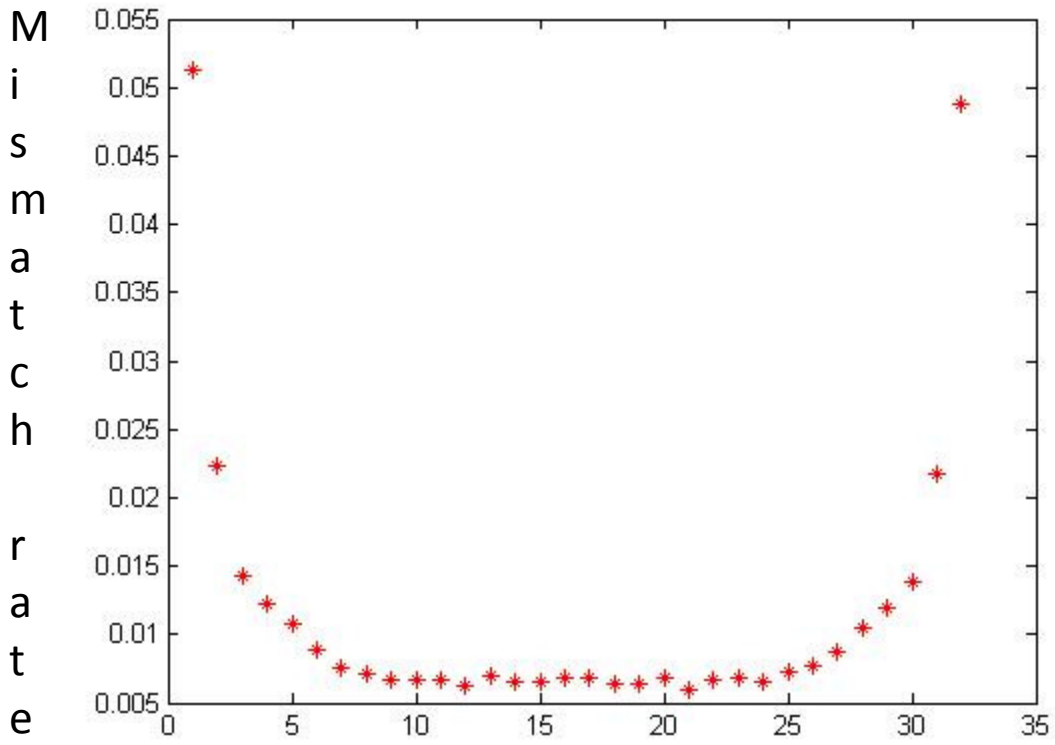
Examine output file: 0_1_gene_exp.diff



# Advantages of RNA-Seq

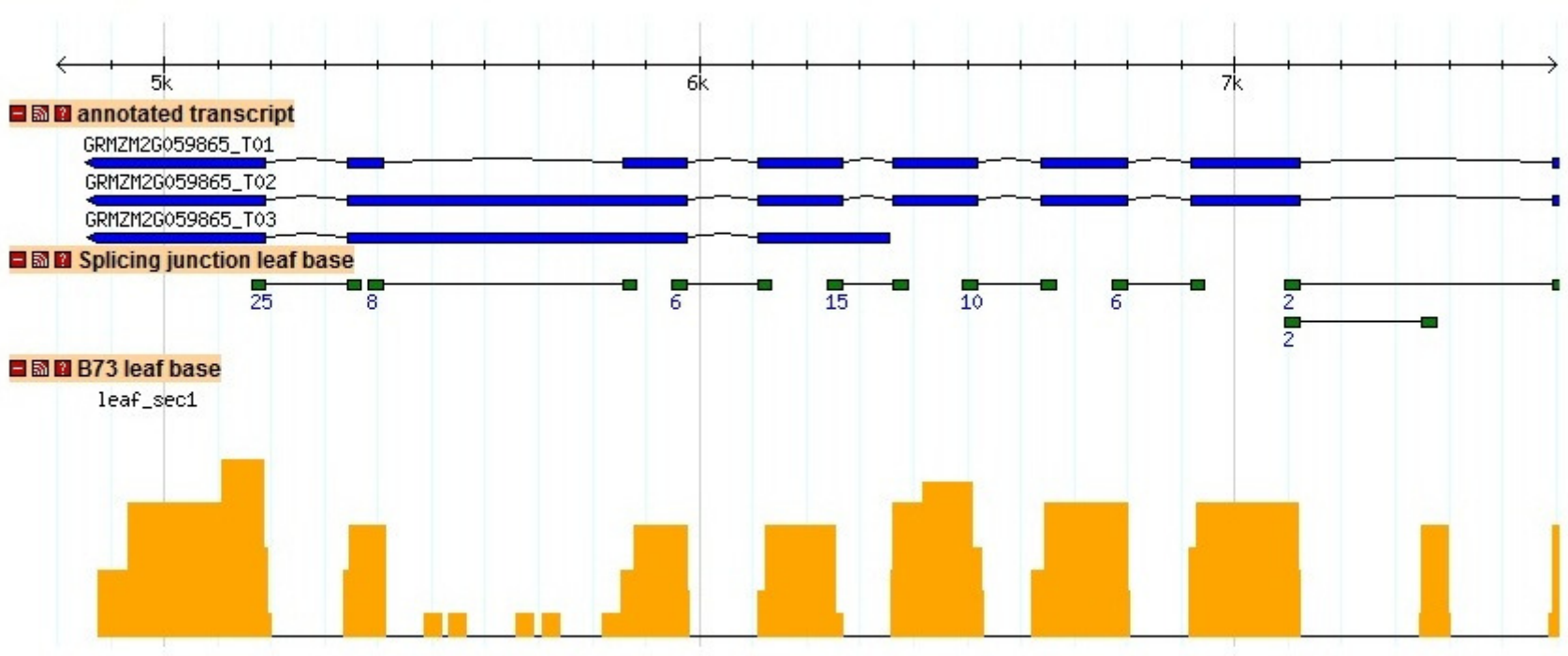
- Does not require existing genomic sequence
  - Unlike hybridization approaches
- Very low background noise
  - Reads can be unabmiguously mapped
- Resolution
  - Up to 1 bp
- High-throughput
  - Better than Sanger sequencing of cDNA or EST libraries
- Cost
  - Lower than traditional sequencing
- Can reveal sequence variations (SNPs)

# Issues


Mismatch rate
0.055
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
5
10
15
20
25
30
35
base position

# Issues

- Depth of coverage depends on "sequenceability" of the genomic region



# Conclusion

RNA-Seq

- Offers high-throughput quantitative measurement of transcript abundance
- Expression levels correlate well with qPCR
- Costs continue to fall due to multiplexing
- Expected to replace microarrays for transcriptomic studies
- Automated pipeline (Tophat/Cufflinks)

# References


- Wang Z, Gerstein M, Snyder M. *RNA-Seq: a revolutionary tool for transcriptomics*. Nat Rev Genet. 2009 Jan;10(1):57-63.
- Nagalakshmi U, Waern K, Snyder M. *RNA-Seq: A Method for Comprehensive Transcriptome Analysis.* Curr Protoc Mol Biol. 2010 Jan;Chapter 4:Unit 4.11.1-13