



Cold
Spring
Harbor
Laboratory

Advanced Sequencing Technologies & Applications

<http://meetings.cshl.edu/courses.html>

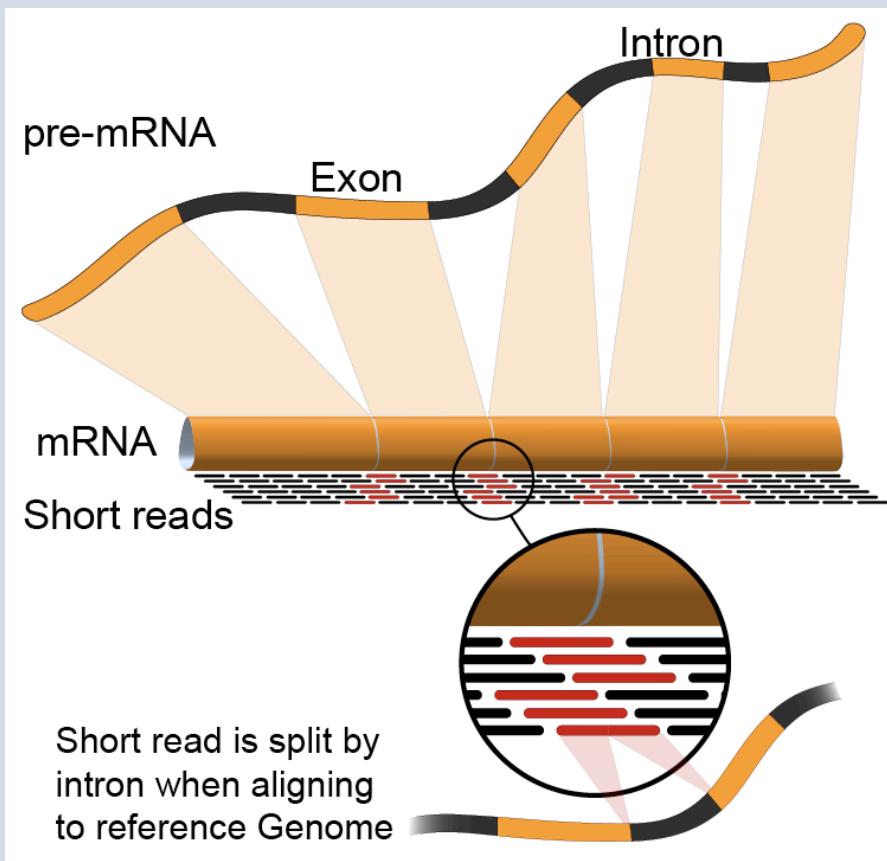


Cold Spring Harbor Laboratory

Module 2

Introduction to RNA sequencing (lecture)

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Advanced Sequencing Technologies & Applications
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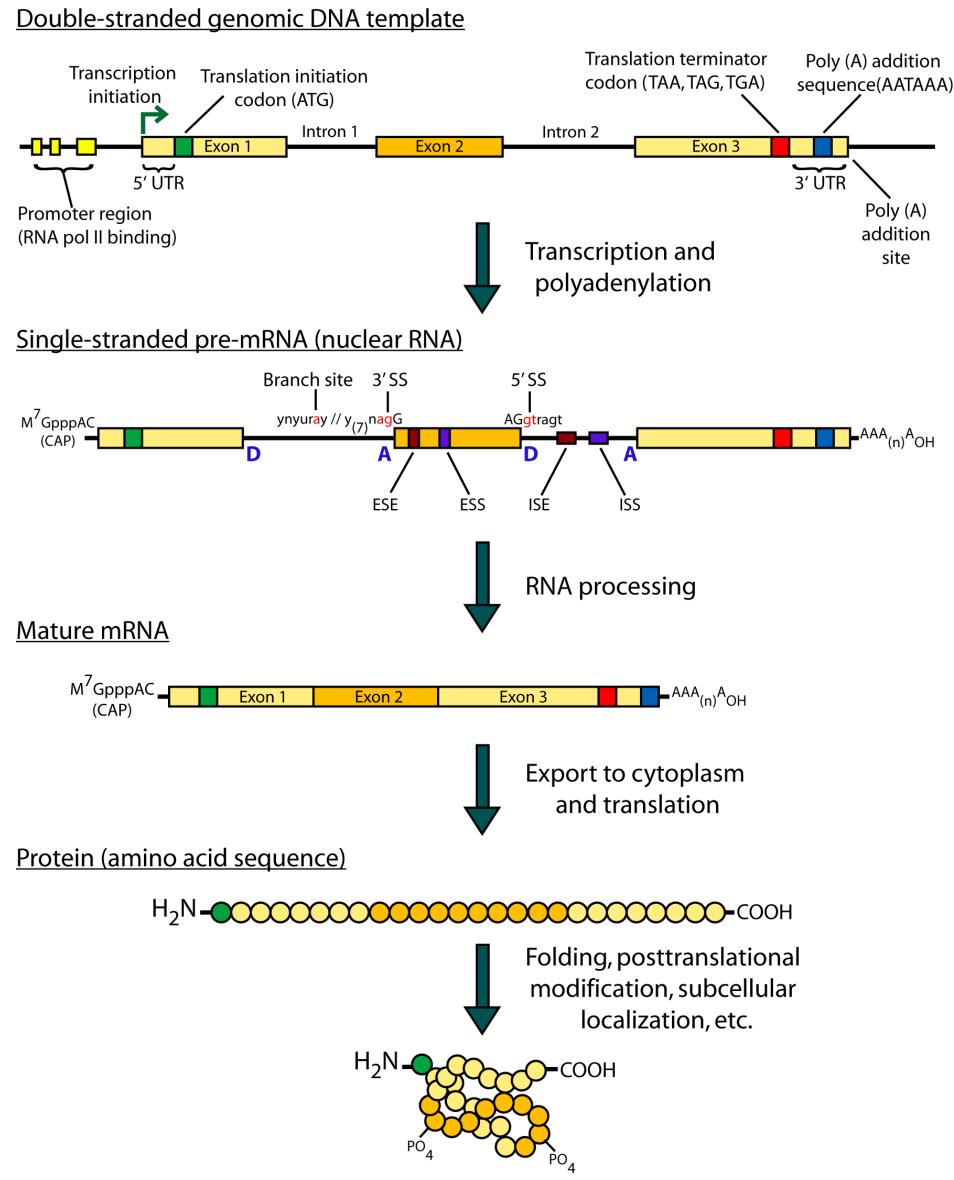
Learning objectives of the course

- Module 1: Introduction to cloud computing
- **Module 2: Introduction to RNA sequencing**
- Module 3: RNA-seq alignment and visualization
- Module 4: Expression and Differential Expression
- Module 5: Isoform discovery and alternative expression
- Tutorials
 - Provide a working example of an RNA-seq analysis pipeline
 - Run in a ‘reasonable’ amount of time with modest computer resources
 - Self contained, self explanatory, portable

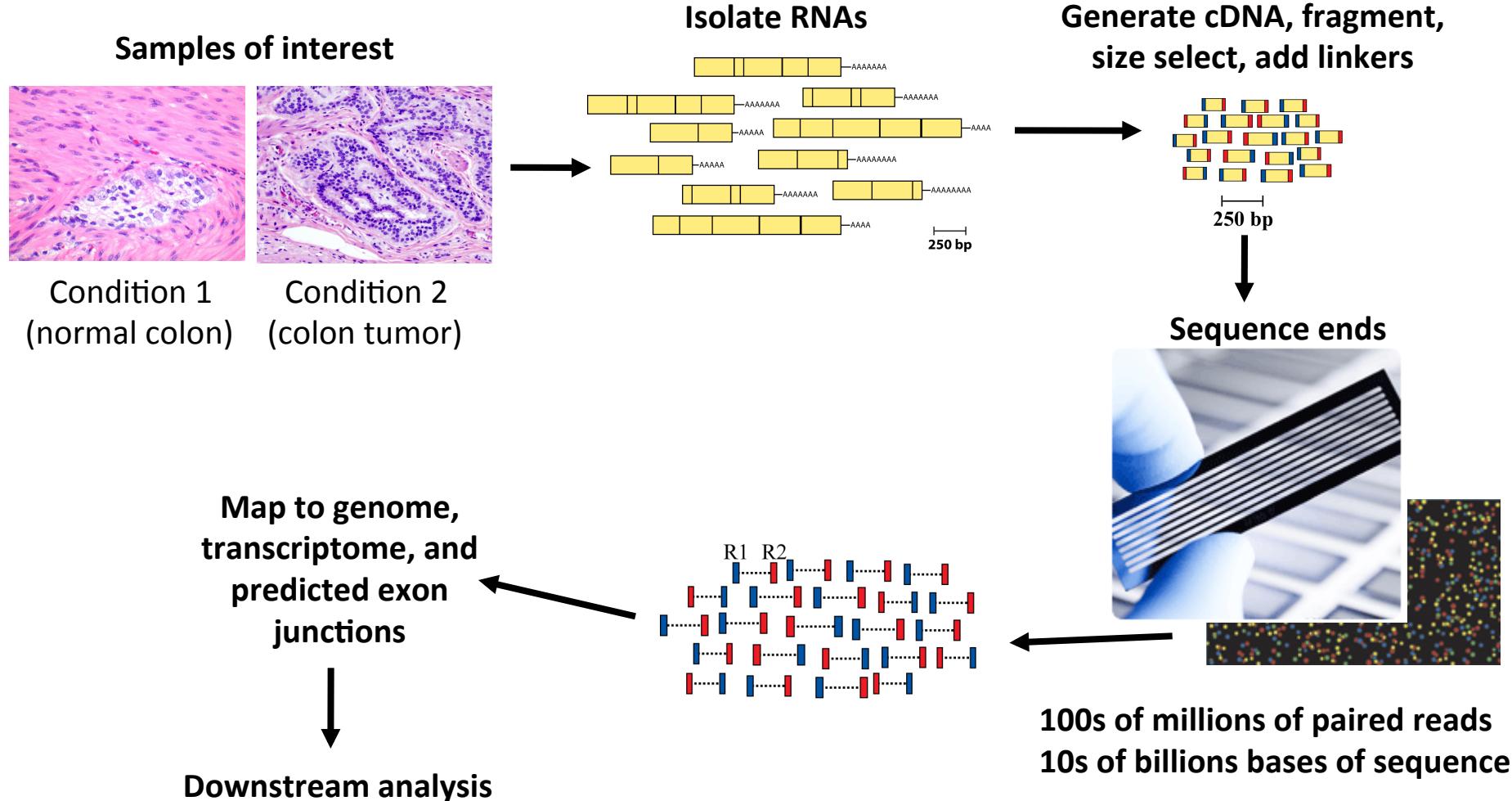
Learning objectives of module 2

- Introduction to the theory and practice of RNA sequencing (RNA-seq) analysis
 - Rationale for sequencing RNA
 - Challenges specific to RNA-seq
 - General goals and themes of RNA-seq analysis work flows
 - Common technical questions related to RNA-seq analysis
 - Getting help outside of this course
 - Introduction to the RNA-seq hands on tutorial

Gene expression



RNA sequencing



Why sequence RNA (versus DNA)?

- Functional studies
 - Genome may be constant but an experimental condition has a pronounced effect on gene expression
 - e.g. Drug treated vs. untreated cell line
 - e.g. Wild type versus knock out mice
- Predicting transcript sequence from genome sequence is difficult
 - Gene annotation is revolutionized by RNA-seq
- Some molecular features can only be observed at the RNA level
 - Alternative isoforms, fusion transcripts, RNA editing

Why sequence RNA (versus DNA)?

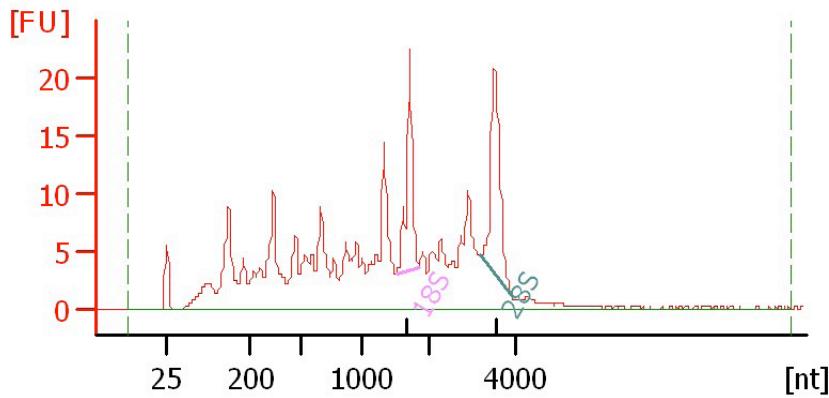
- Interpreting mutations that do not have an obvious effect on protein sequence
 - ‘Regulatory’ mutations that affect what mRNA isoform is expressed and how much
 - e.g. splice sites, promoters, exonic/intronic splicing motifs, etc.
- Prioritizing protein coding somatic mutations (often heterozygous)
 - If the gene is not expressed, a mutation in that gene would be less interesting
 - If the gene is expressed but only from the wild type allele, this might suggest loss-of-function (haploinsufficiency)
 - If the mutant allele itself is expressed, this might suggest a candidate drug target

Challenges

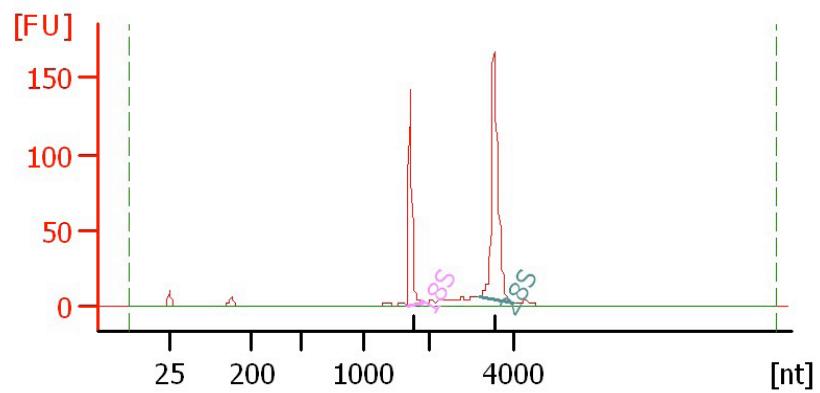
- Sample
 - Purity?, quantity?, quality?
- RNAs consist of small exons that may be separated by large introns
 - Mapping reads to genome is challenging
- The relative abundance of RNAs vary wildly
 - $10^5 - 10^7$ orders of magnitude
 - Since RNA sequencing works by random sampling, a small fraction of highly expressed genes may consume the majority of reads
 - Ribosomal and mitochondrial genes
- RNAs come in a wide range of sizes
 - Small RNAs must be captured separately
 - PolyA selection of large RNAs may result in 3' end bias
- RNA is fragile compared to DNA (easily degraded)

Agilent example / interpretation

- https://github.com/griffithlab/rnaseq_tutorial/wiki/Resources/Agilent_Trace_Examples.pdf
- ‘RIN’ = RNA integrity number
 - 0 (bad) to 10 (good)



RIN = 6.0



RIN = 10

Design considerations

- Standards, Guidelines and Best Practices for RNA-seq
 - The ENCODE Consortium
 - Download from the Course Wiki
 - Meta data to supply, replicates, sequencing depth, control experiments, reporting standards, etc.
- https://github.com/griffithlab/rnaseq_tutorial/wiki/Resources/ENCODE_RNAseq_standards_v1.0.pdf

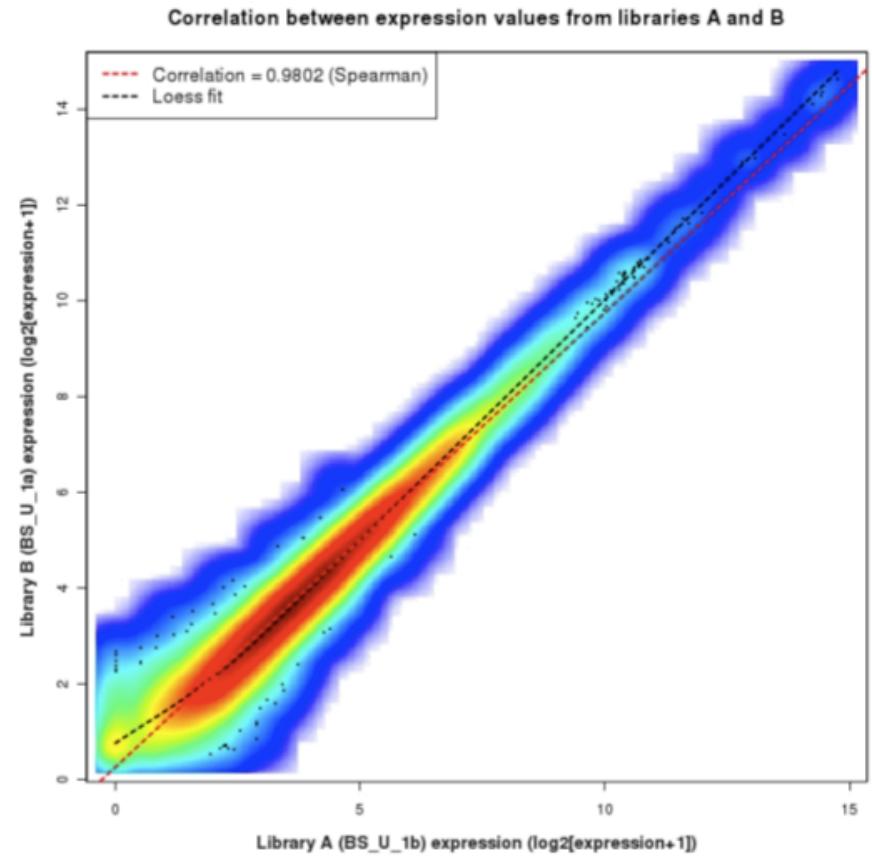
There are many RNA-seq library construction strategies

- Total RNA versus polyA+ RNA?
- Ribo-reduction?
- Size selection (before and/or after cDNA synthesis)
 - Small RNAs (microRNAs) vs. large RNAs?
 - A narrow fragment size distribution vs. a broad one?
- Linear amplification?
- Stranded vs. un-stranded libraries
- Exome captured vs. un-captured
- Library normalization?

- These details can affect analysis strategy
 - Especially comparisons between libraries

Replicates

- Technical Replicate
 - Multiple instances of sequence generation
 - Flow Cells, Lanes, Indexes
- Biological Replicate
 - Multiple isolations of cells showing the same phenotype, stage or other experimental condition
 - Some example concerns/challenges:
 - Environmental Factors, Growth Conditions, Time
 - Correlation Coefficient 0.92-0.98



Common analysis goals of RNA-Seq analysis (what can you ask of the data?)

- Gene expression and differential expression
- Alternative expression analysis
- Transcript discovery and annotation
- Allele specific expression
 - Relating to SNPs or mutations
- Mutation discovery
- Fusion detection
- RNA editing

General themes of RNA-seq workflows

- Each type of RNA-seq analysis has distinct requirements and challenges but also a common theme:
 1. Obtain raw data (convert format)
 2. Align/assemble reads
 3. Process alignment with a tool specific to the goal
 - e.g. ‘cufflinks’ for expression analysis, ‘defuse’ for fusion detection, etc.
 4. Post process
 - Import into downstream software (R, Matlab, Cytoscape, Ingenuity, etc.)
 5. Summarize and visualize
 - Create gene lists, prioritize candidates for validation, etc.

Tool recommendations

- Alignment
 - BWA (PMID: 20080505)
 - Align to genome + junction database
 - Tophat (PMID: 19289445), STAR (PMID: 23104886), MapSplice (PMID: 20802226), hmmSplicer (PMID: 21079731)
 - Spliced alignment to genome
- Expression, differential expression alternative expression
 - Cufflinks/Cuffdiff (PMID: 20436464), ALEXA-seq (PMID: 20835245), RUM (PMID: 21775302)
- Fusion detection
 - Tophat-fusion (PMID: 21835007), ChimeraScan (PMID: 21840877), Defuse (PMID: 21625565), Comrad (PMID: 21478487)
- Transcript assembly
 - Trinity (PMID: 21572440), Oases (PMID: 22368243), Trans-ABySS (PMID: 20935650)
- Visit the ‘SeqAnswers’ or ‘BioStar’ forums for more recommendations and discussion
 - <http://seqanswers.com/>
 - <http://www.biostars.org/>

SeqAnswers exercise

- Go to:
 - <http://seqanswers.com/>
- Click the ‘Wiki’ link
 - <http://seqanswers.com/wiki/SEQanswers>
- Visit the ‘Software Hub’
 - <http://seqanswers.com/wiki/Software>
- Browse the software that has been added
 - <http://seqanswers.com/wiki/Special:BrowseData>
- Use the tag cloud to identify tools related to your area of interest. e.g. RNA-seq alignment

Common questions: Should I remove duplicates for RNA-seq?

- Maybe... more complicated question than for DNA
- Concern.
 - Duplicates may correspond to biased PCR amplification of particular fragments
 - For highly expressed, short genes, duplicates are expected even if there is no amplification bias
 - Removing them may reduce the dynamic range of expression estimates
- Assess library complexity and decide...
- If you do remove them, assess duplicates at the level of paired-end reads (fragments) not single end reads

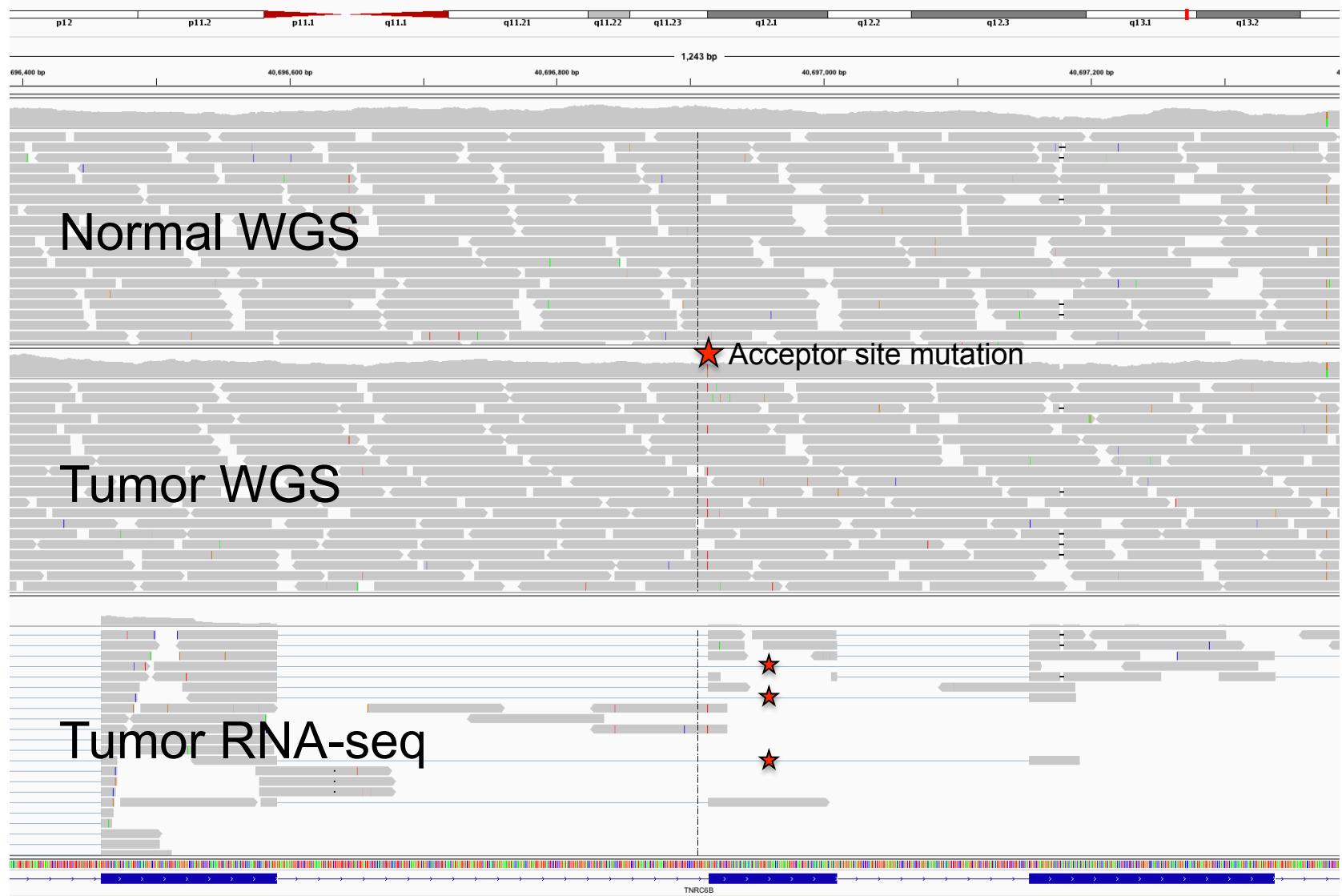
Common questions: How much library depth is needed for RNA-seq?

- Depends on a number of factors:
 - Question being asked of the data. Gene expression? Alternative expression? Mutation calling?
 - Tissue type, RNA preparation, quality of input RNA, library construction method, etc.
 - Sequencing type: read length, paired vs. unpaired, etc.
 - Computational approach and resources
- Identify publications with similar goals
- Pilot experiment
- Good news: 1-2 lanes of recent Illumina HiSeq data should be enough for most purposes

Common questions: What mapping strategy should I use for RNA-seq?

- Depends on read length
- < 50 bp reads
 - Use aligner like BWA and a genome + junction database
 - Junction database needs to be tailored to read length
 - Or you can use a standard junction database for all read lengths and an aligner that allows substring alignments for the junctions only (e.g. BLAST ... slow).
 - Assembly strategy may also work (e.g. Trans-ABySS)
- > 50 bp reads
 - Spliced aligner such as Bowtie/TopHat

Visualization of spliced alignment of RNA-seq data

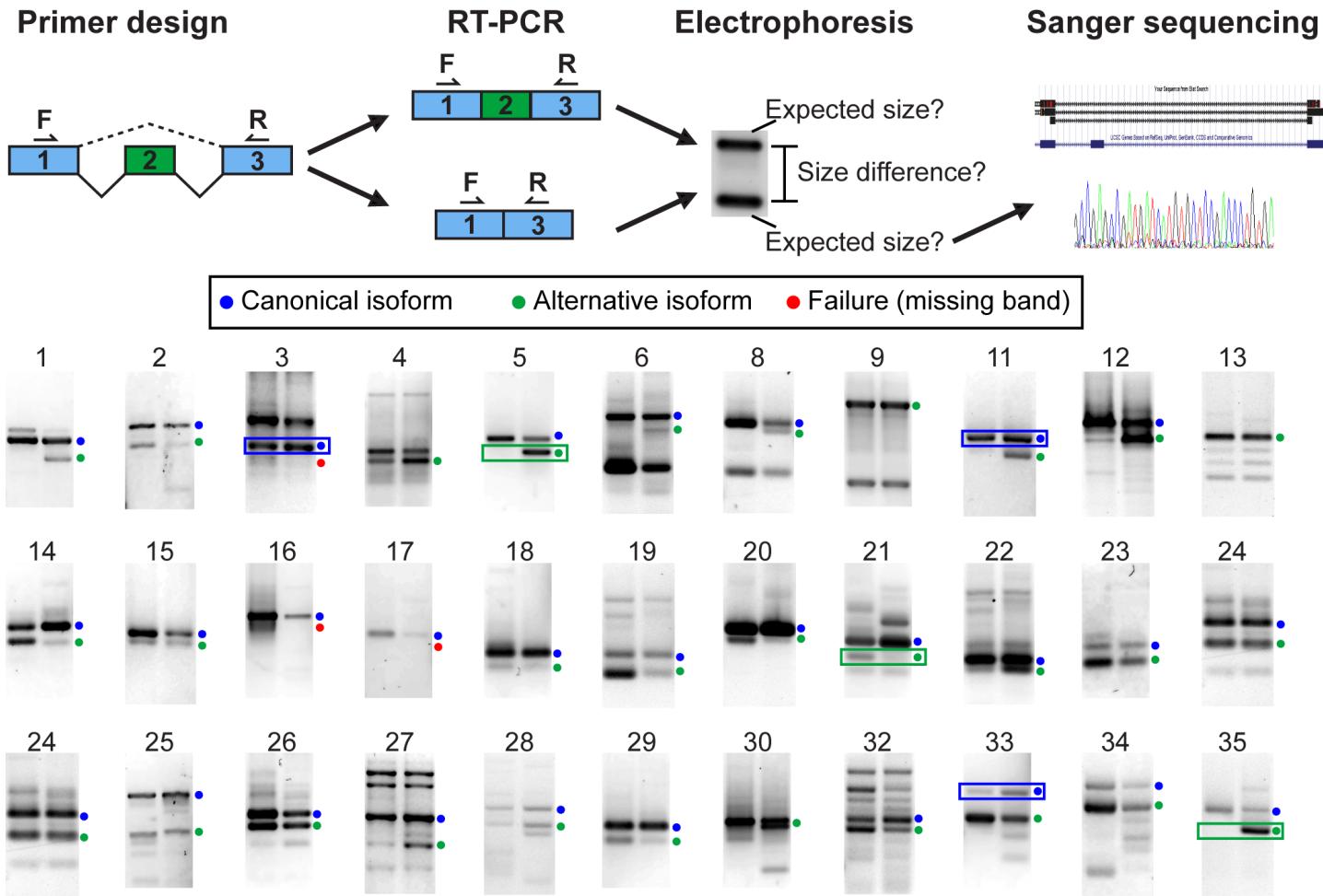


IGV screenshot

Common questions: how reliable are expression predictions from RNA-seq?

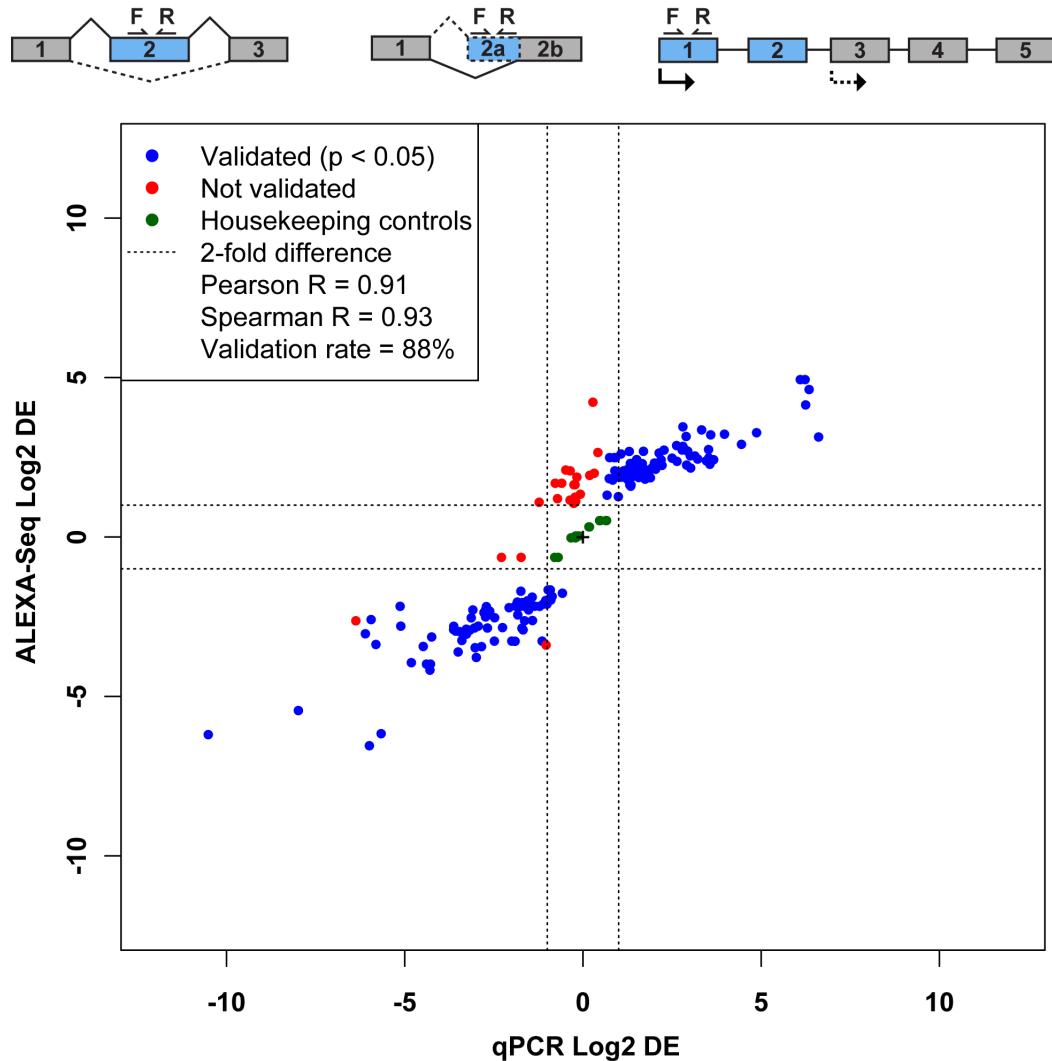
- Are novel exon-exon junctions real?
 - What proportion validate by RT-PCR and Sanger sequencing?
- Are differential/alternative expression changes observed between tissues accurate?
 - How well do DE values correlate with qPCR?
- 384 validations
 - qPCR, RT-PCR, Sanger sequencing
- See ALEXA-Seq publication for details:
 - Also includes comparison to microarrays
 - Griffith et al. *Alternative expression analysis by RNA sequencing*. Nature Methods. 2010 Oct;7(10):843-847.

Validation (qualitative)



33 of 192 assays shown. Overall validation rate = 85%

Validation (quantitative)



qPCR of 192
exons identified
as alternatively
expressed by
ALEXA-Seq

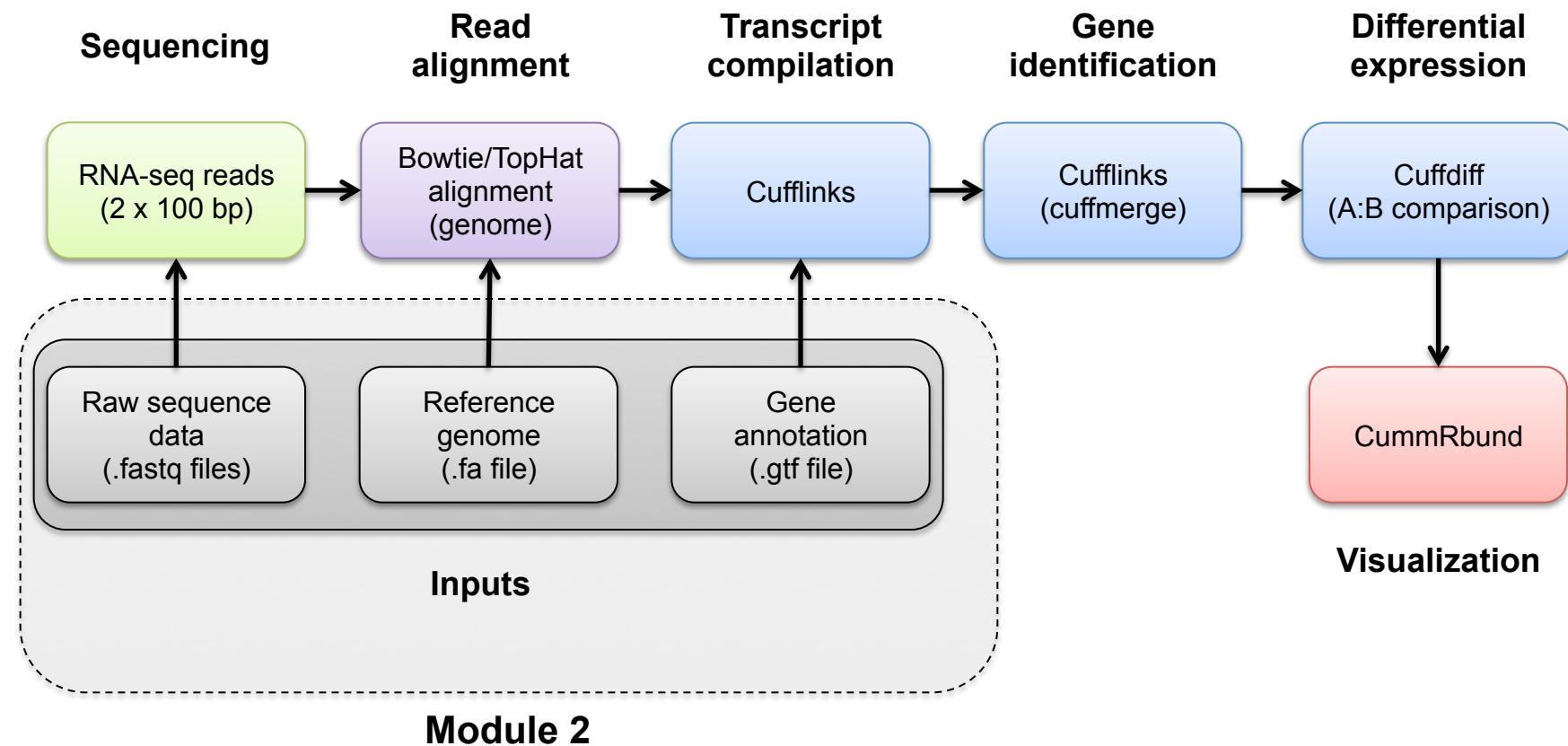
Validation rate = 88%

BioStar exercise

- Go to the BioStar website:
 - <http://www.biostars.org/>
 - If you do not already have an OpenID (e.g. Google, Yahoo, etc.)
 - Login -> ‘get one’
- Login and set up your user profile
- Tasks:
 - Find a question that seems useful and ‘vote it up’
 - Answer a question [optional]
 - Search for a topic area of interest and ask a question that has not already been asked [optional]

Introduction to tutorial (Module 2)

Bowtie/Tophat/Cufflinks/Cuffdiff RNA-seq Pipeline



Break