Cold Spring Harbor Laboratory

Advanced Sequencing Technologies & Applications

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

Cold Spring Harbor Laboratory

Module 1
Introduction to RNA sequencing (lecture)

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Advanced Sequencing Technologies & Applications

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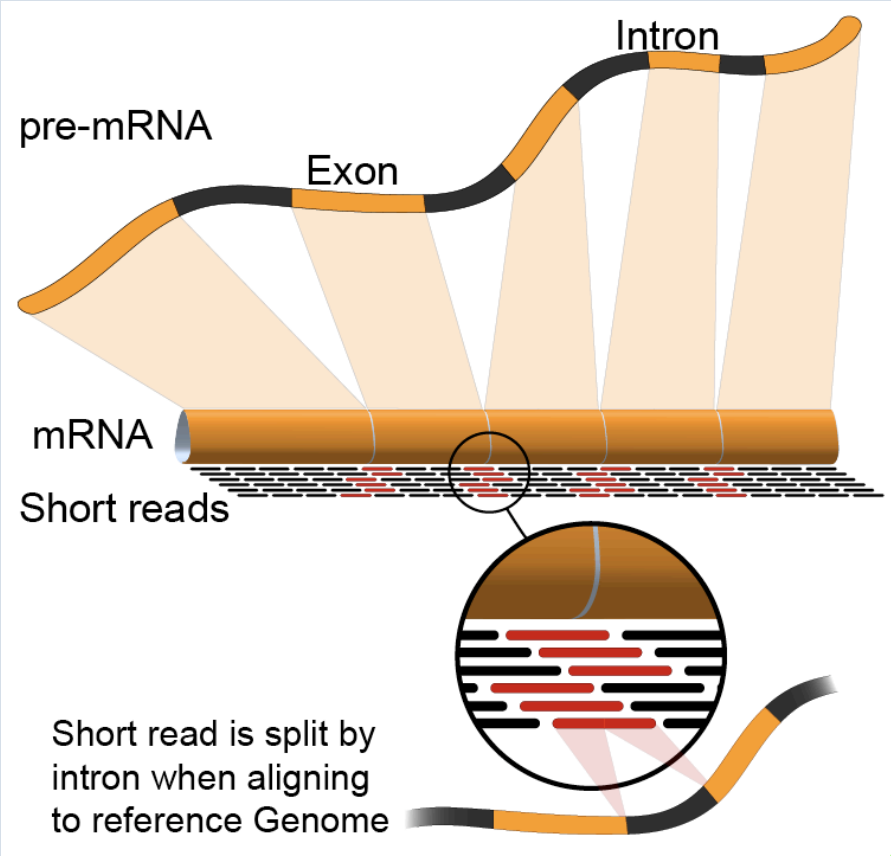


THE GENOME INSTITUTE at Washington University

Learning objectives of the course

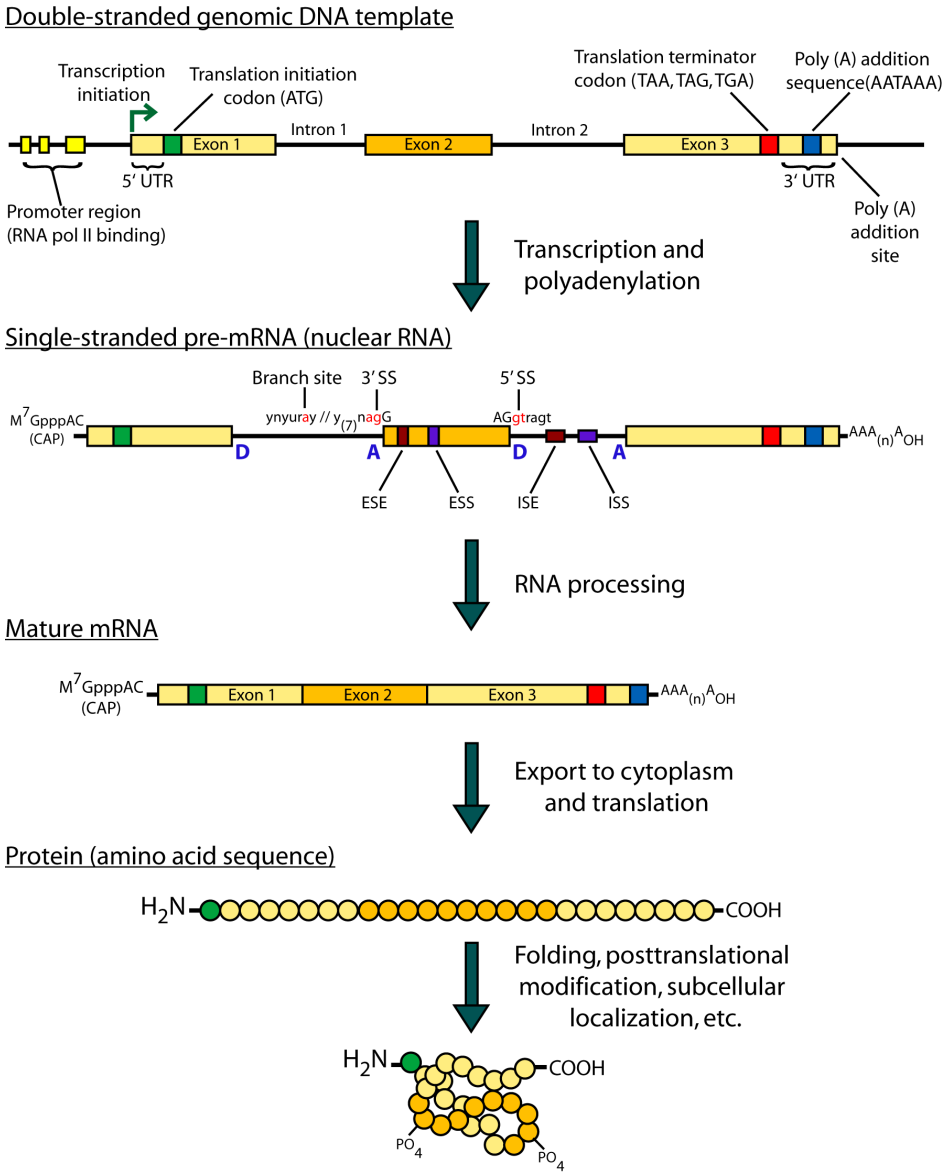
- **Module 1: Introduction to RNA sequencing**
- Module 2: RNA-seq alignment and visualization
- Module 3: Expression and Differential Expression
- Module 4: Isoform discovery and alternative expression

- 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 1

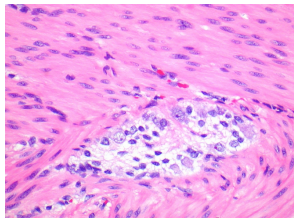
- Introduction to the theory and practice of RNA sequencing (RNA-seq) analysis
  - Rationale for sequencing RNA
  - Challenges specific to RNA-seq
  - General goals and themes of RNA-seq analysis work flows
  - Common technical questions related to RNA-seq analysis
  - Getting help outside of this course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

Gene expression

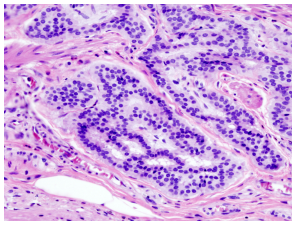


RNA sequencing

Samples of interest

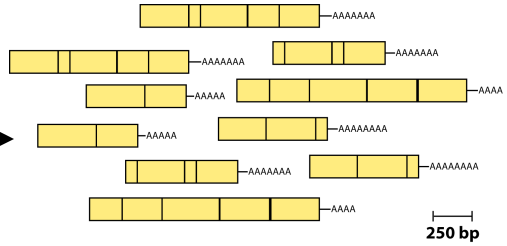


Condition 1
(normal colon)

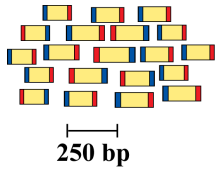


Condition 2
(colon tumor)

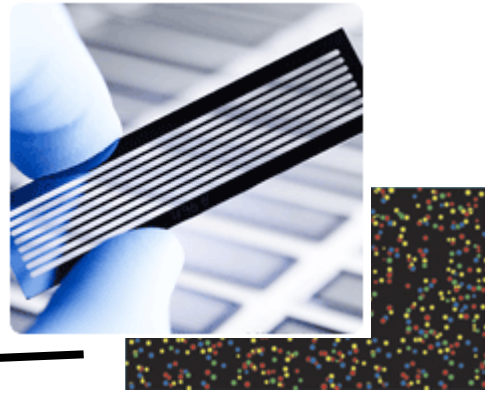
Isolate RNAs



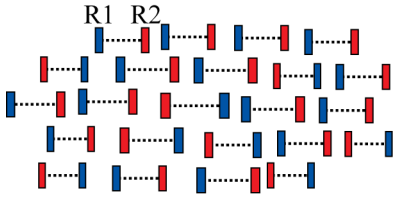
Generate cDNA, fragment, size select, add linkers



Sequence ends



100s of millions of paired reads
10s of billions bases of sequence



Map to genome, transcriptome, and predicted exon junctions

Downstream analysis

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
- Some molecular features can only be observed at the RNA level
  - Alternative isoforms, fusion transcripts, RNA editing
- Predicting transcript sequence from genome sequence is difficult
  - Alternative splicing, RNA editing, etc.

Why sequence RNA (versus DNA)?

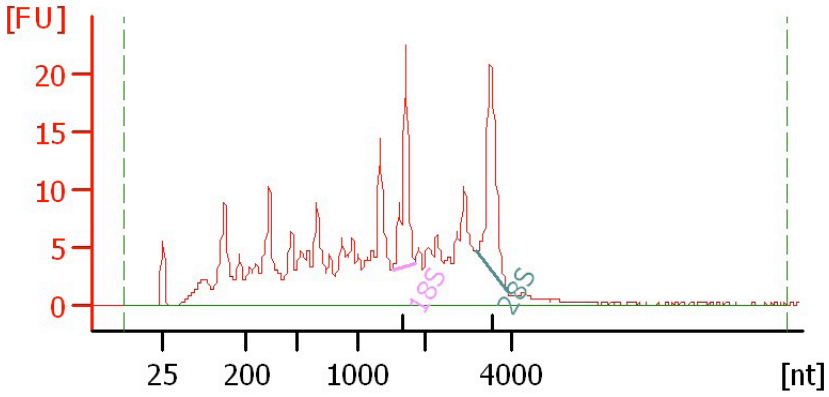
- Interpreting mutations that do not have an obvious effect on protein sequence
  - 'Regulatory' mutations that affect what mRNA isoform is expressed and how much
    - e.g. splice sites, promoters, exonic/intronic splicing motifs, etc.
- Prioritizing protein coding somatic mutations (often heterozygous)
  - 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

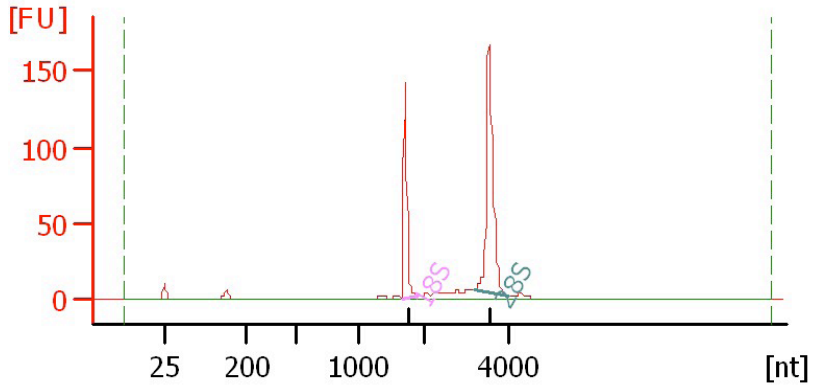
- Sample
  - Purity?, quantity?, quality?
- RNAs consist of small exons that may be separated by large introns
  - Mapping reads to genome is challenging
- The relative abundance of RNAs vary wildly
  - 10^5 – 10^7 orders of magnitude
  - Since RNA sequencing works by random sampling, a small fraction 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

- http://www.alexaplatform.org/courses/2013/cbw/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.

- http://www.alexaplatform.org/courses/2013/cbw/ENCODE_RNAseq_standards_v1.0.pdf

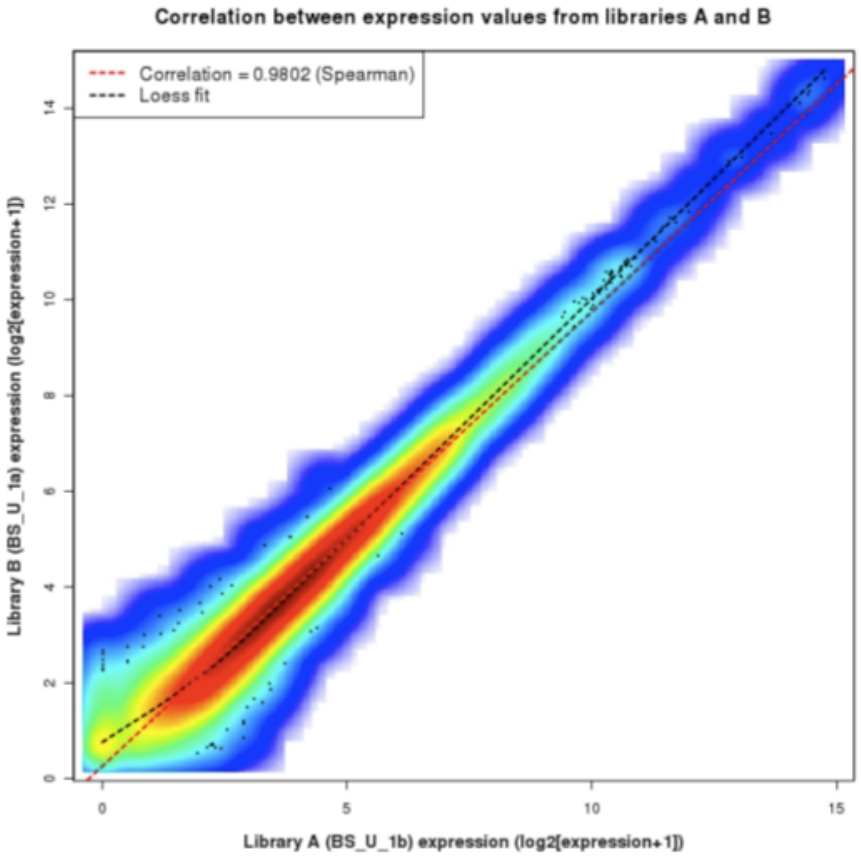
There are many RNA-seq library construction strategies

- 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

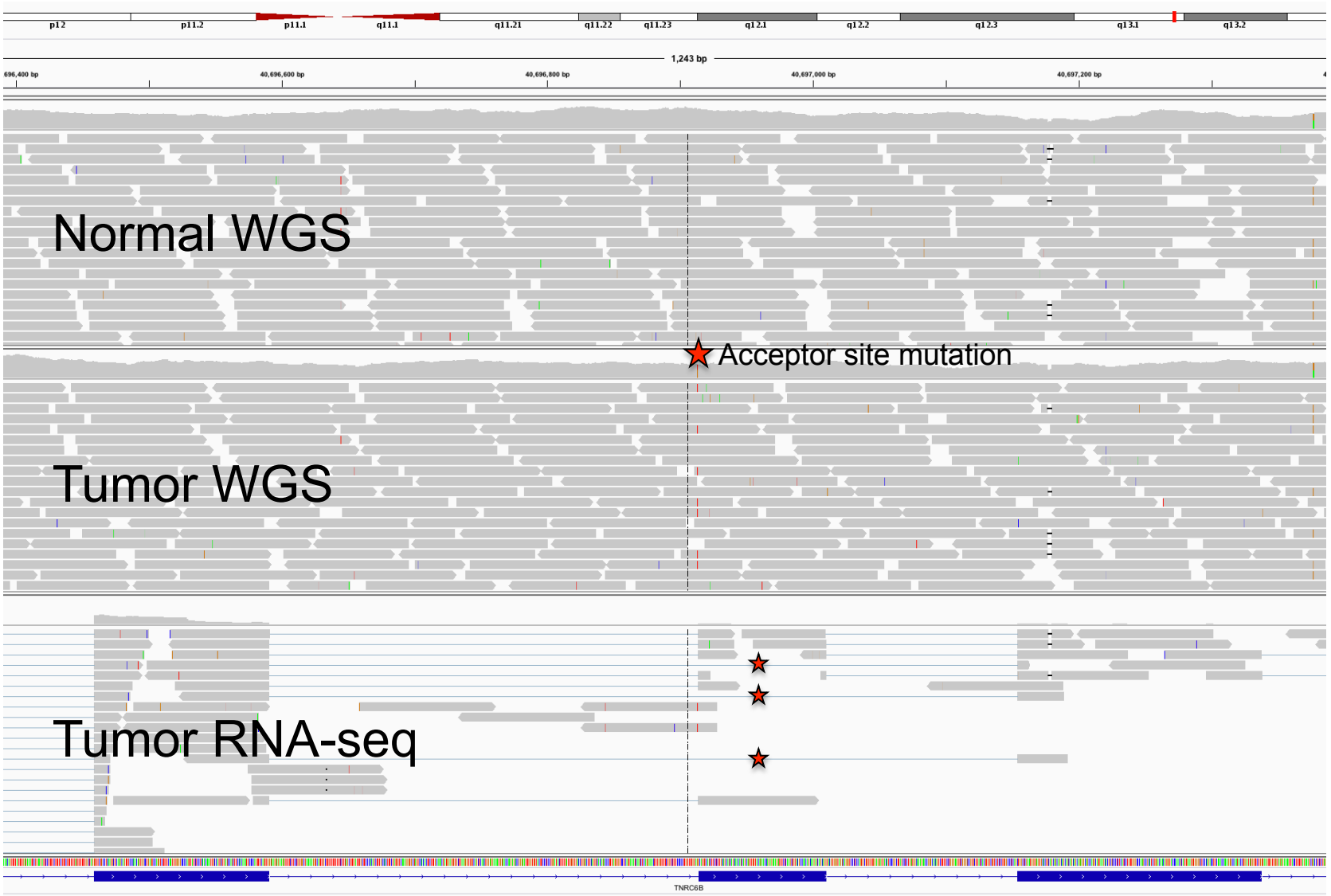
Common questions: How much library depth is needed for RNA-seq?

- 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

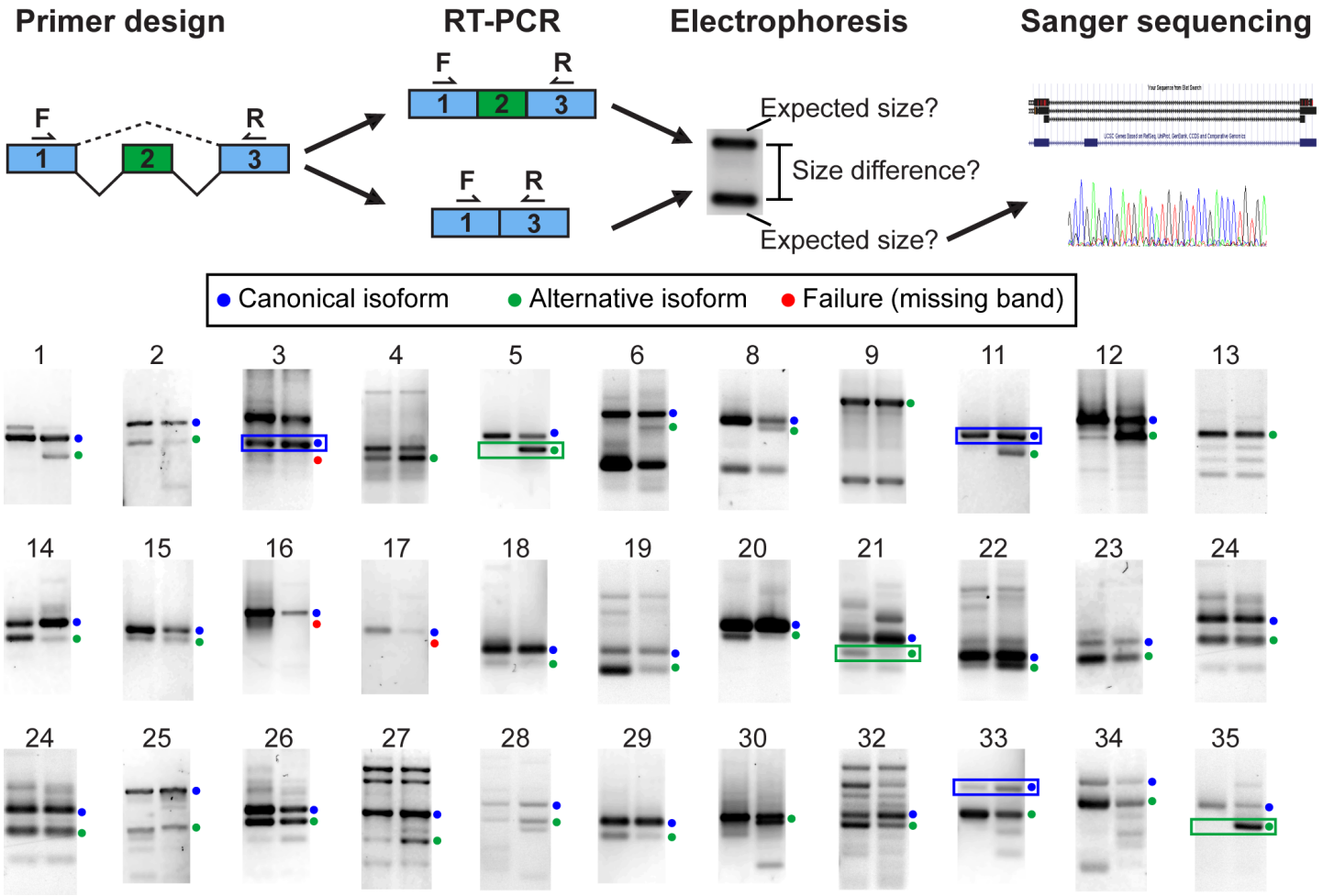


IGV screenshot

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

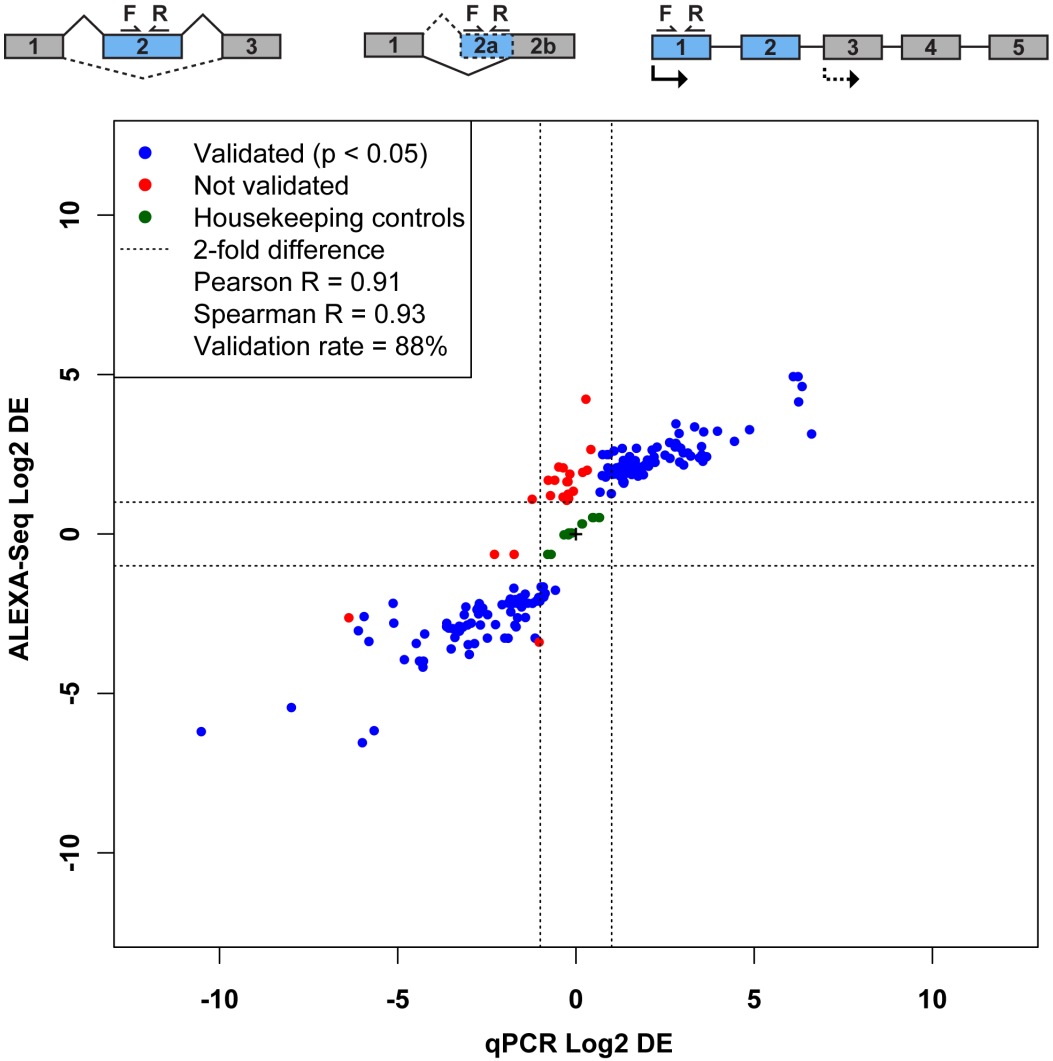
- Are novel exon-exon junctions real?
  - What proportion validate by RT-PCR and Sanger sequencing?
- Are differential/alternative expression changes observed between tissues accurate?
  - How well do DE values correlate with qPCR?
- 384 validations
  - qPCR, RT-PCR, Sanger sequencing
- See ALEXA-Seq 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

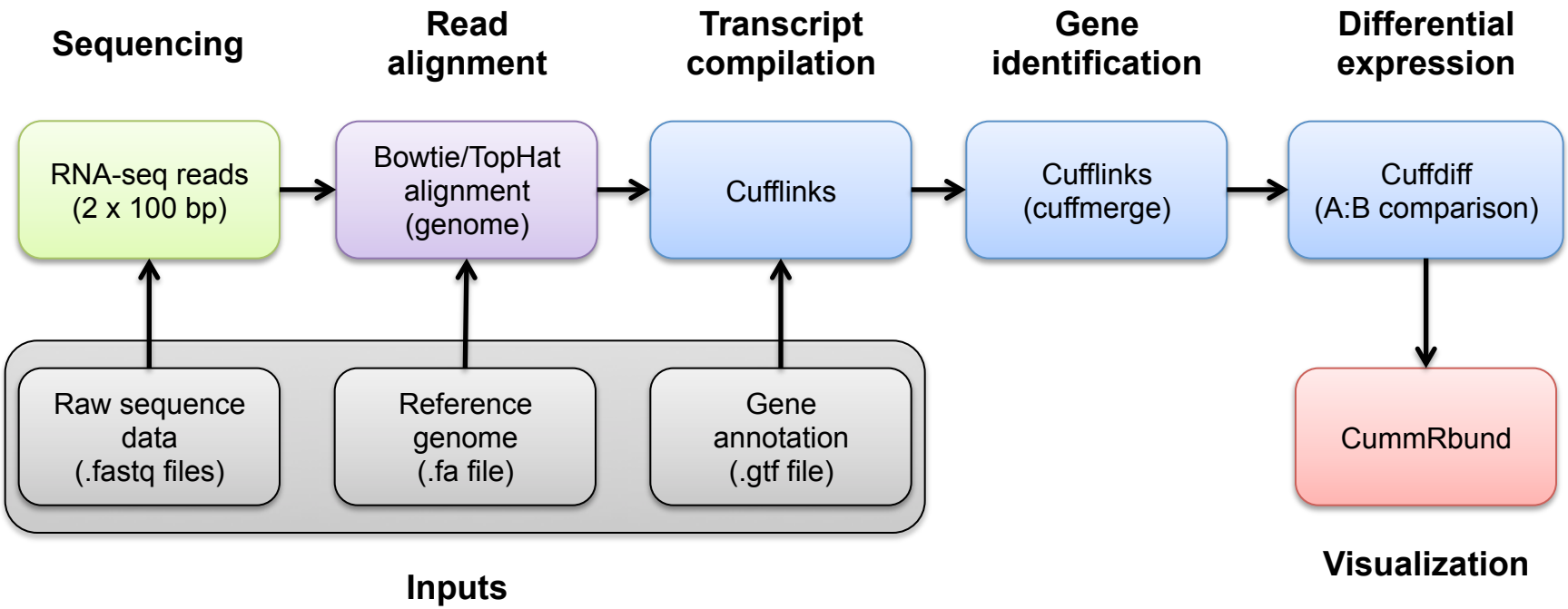
Validation rate = 88%

BioStar exercise

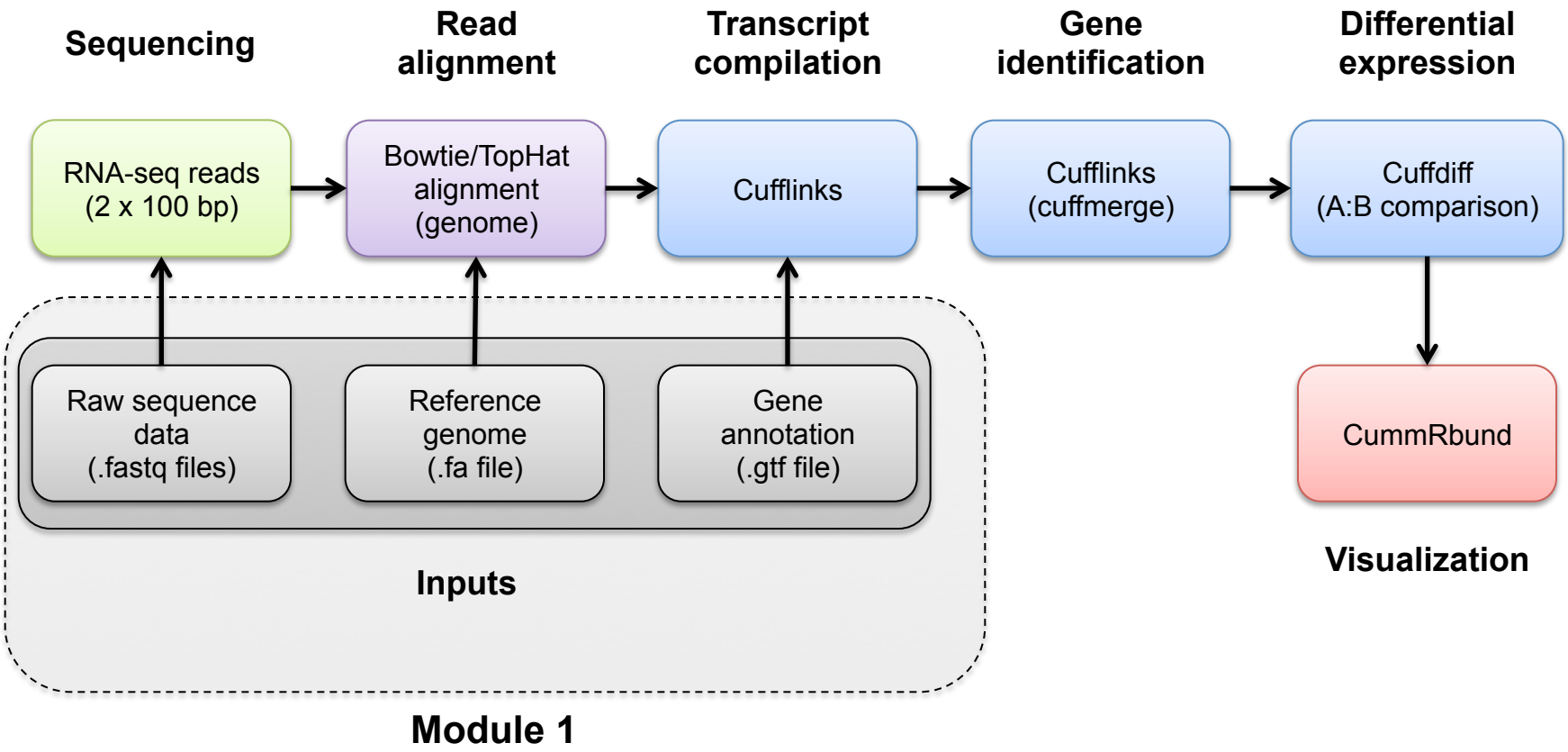
- Go to the BioStar website:
  - http://www.biostars.org/
  - If you do not already have an OpenID (e.g. Google, Yahoo, etc.)
  - Login -> 'get one'
- Login and set up your user profile
- Tasks:
  - Find a question that seems useful and 'vote it up'
  - Answer a question [optional]
  - Search for a topic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

Introduction to tutorial (Module 1)

Bowtie/Tophat/Cufflinks/Cuffdiff RNA-seq Pipeline



Bowtie/Tophat/Cufflinks/Cuffdiff RNA-seq Pipeline



Break