Biology 644

Old Title: Bioinformatics for Molecular Biologists

Potential New Title: Integrated Bioinformatics Using R for Both Wet and Dry Scientists

RNA-seq

- "Whole Transcriptome Shotgun Sequencing" ("WTSS" or just "WTS") is a technology that uses the capabilities of Next-Generation Sequencing (NGS) to reveal a snapshot of RNA abundance from a genome and tissue-type at a given moment in time
- Provides the ability to look at:
  - Alternative gene-spliced transcripts
  - Post-transcriptional changes
  - Gene fusions
  - mutations/SNPs
  - exon/intron boundaries
  - Changes in:
    - total RNA
    - mRNAs (gene expression)
    - small RNAs (including miRNAs)
    - tRNAs
    - ribosomal RNAs

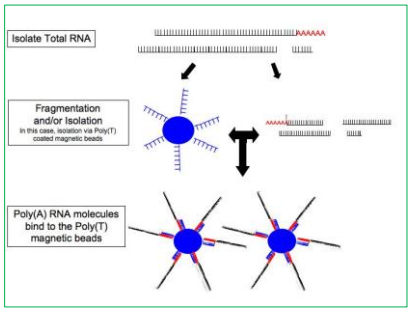
Advantages of RNA-seq

- The main deficiency of microarrays that makes RNA-Seq more attractive has been limited coverage:
  - Arrays target the identification of known common alleles that represent only approximately 500,000 to 2,000,000 SNPs of the more than 10,000,000 in the human genome
  - Microarrays aren't usually available to detect and evaluate rare allele variant transcripts
  - Microarrays are only as good as the SNP databases they're designed from
  - Many cancers are caused by rare <1% mutations and go undetected with microarrays
- The second main deficiency with microarrays is additional noise due to cross-hybridization
  - RNA-seq is more "digital" and has better signal-to-noise ratios
- With RNA-seq, one can sequence to any desired depth in order to get the necessary coverage and no more

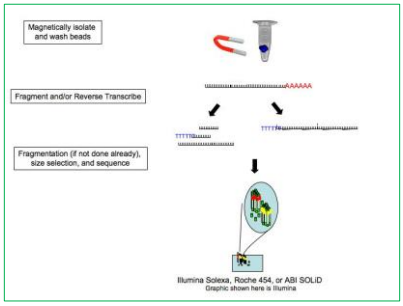
Selecting for mRNA, poly-A RNA, Ribosomal

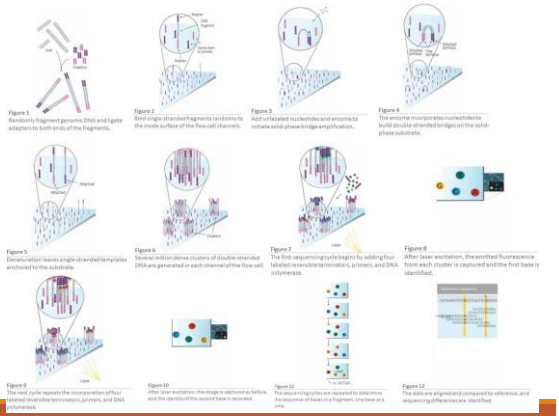
- Frequently, in mRNA analysis the 3' polyadenylated (poly-A) tail is targeted in order to ensure that coding RNA is separated from noncoding RNA.
  - Accomplished simply with poly-T oligos covalently attached to a given substrate. Presently many studies utilize magnetic beads for this step.
  - The flow-through RNA (non-poly-A RNA) contains noncoding RNA
- Probe hybridization with microarrays can separate out Ribosomal RNA
  - Ribosomal RNA represents over 90% of the RNA within a given cell
  - Removing ribosomal RNA before sequencing greatly increases the percentage of the reads that are from the remaining portion of the transcriptome (saves $).
- When sequencing RNA other than mRNA, such as miRNA or other small RNAs, selection is based on the desired size range
  - size exclusion gel
  - size selection magnetic beads

Selecting for mRNA via poly(A) RNA



Selecting for mRNA via poly(A) RNA II





RNA-seq Preparation and Paired-end Sequencing

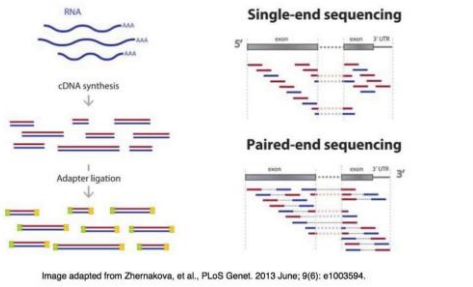
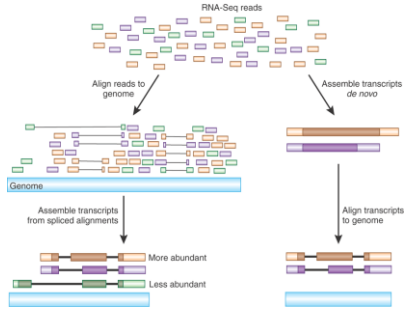


Image adapted from Zhernakova, et al., PLoS Genet. 2013 June; 9(6): e1003594.

RNA-seq "Align & Assemble" Vs. "De Novo"

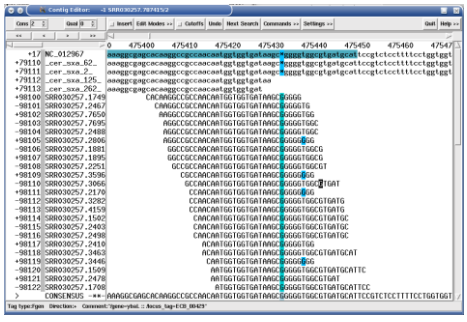


Transcriptome Assembly

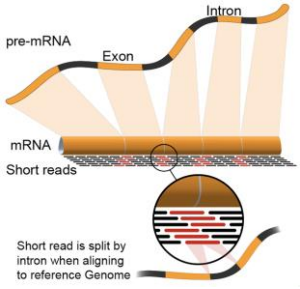
Two different assembly methods are used for producing a transcriptome from raw sequence reads

1. De-novo
   - Does not rely on the presence of a reference genome in order to reconstruct the nucleotide sequence.
   - Requires deep coverage and increased computing power to track all the possible alignments
2. Genome-guided
   - Easier and computationally cheaper approach is aligning the millions of reads to a "reference genome".
   - Several software packages exist for short read alignment, and recently specialized algorithms for transcriptome alignment have been developed
     - Bowtie for RNA-seq short read alignment
     - TopHat for aligning reads to a reference genome to discover splice sites
     - Cufflinks to assemble the transcripts and compare/merge them with others

SNP Detection Using RNA-seq

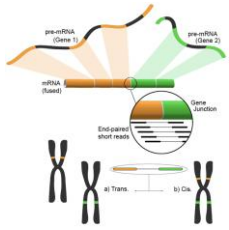


Exon-Intron Boundaries and Isoform Abundance via RNA-seq



Gene Fusion Detection

- Caused by different structural modifications and abnormalities in the genome:
  - Deletions
  - Duplications
  - Translocations
- Fusion genes have gained attention because of their relationship with cancer
- The ability of RNA-seq to analyze a sample's whole transcriptome in an unbiased fashion makes it an attractive tool to find these kinds of common events in cancer



RNA-seq Analysis Workflow

1. Read mapping
2. Counting reads overlapping with genes
3. Analysis of Differentially Expressed Genes (DEGs)
4. Clustering of co-expressed genes
5. Gene set/GO term enrichment analysis

Fastq Raw Reads File

- A FASTQ file normally uses 4 lines per sequence.
  - Line 1 begins with a '@' character and is followed by a sequence identifier and an optional description (like a FASTA title line).
  - Line 2 is the raw sequence calls.
  - Line 3 begins with a '+' character and is optionally followed by the same sequence identifier (and any description) again.
  - Line 4 encodes the phred quality scores for the sequence in Line 2, and must contain the same number of symbols as nucleotide calls in the sequence.
- Example:

```
@SEQ_ID
GATTTGGGGTTCAAAGCAGTATCGATCAAATAGTAAATCCATTTGTTCAACTCACAGTTT
+
!''*((((***+))%%%++)(%%%%).1***-+*''))**55CCF>>>>>>CCCCCCC65
```

- The character '!' represents the lowest quality while '~' is the highest.
- Here are the phred quality value characters in left-to-right increasing order of quality (ASCII):

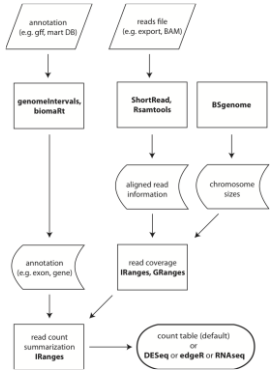
```
!"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
```

SAMTools

- Set of utilities for interacting with and post-processing of short, DNA-sequence read alignments in the SAM/BAM format
- SAM/BAM files are generated as output by short read aligners like BWA, Bowtie, etc.
- Supports complex tasks like variant calling and alignment viewing as well as sorting, indexing, data extraction and format conversion.
- SAM files can be very large (10s of Gigabytes is common), so compression into BAM is used to save space.
- SAM files are human-readable text files, while BAM files are simply the binary equivalent.
- BAM files more efficient for software to work with because the files are much smaller
- SAMtools makes it possible to work directly with a compressed BAM file, without having to uncompress to a SAM file.
- SAM/BAM files are complex - containing reads, references, alignments, quality information, and user-specified annotations

easyRNASeq

- package to ease the processing of RNA-seq data in R/Bioconductor.
- The main function of the easyRNASeq package is easyRNASeq:
  - should be the only processing method you need to know about when using the package.
  - It is essentially a wrapper around other functions performing the different tasks
  - The lower-level functions which are all exported too, if you feel you need to have a look at them



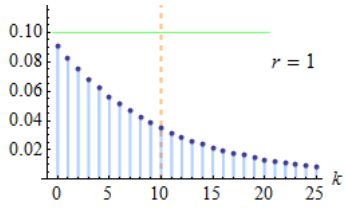
Package 'edgeR'

- "Empirical analysis of Digital Gene Expression data in R"
- A package for the analysis of digital gene expression data arising from RNA sequencing technologies such as SAGE (single-end), CAGE (5'-Cap), Tag-seq (single-end)or RNA-seq (paired-end), with emphasis on testing for differential expression.
- Particular strengths of the package include the ability to estimate biological variation between replicate libraries, and to conduct exact tests of significance which are suitable for small counts.
- The package is able to make maximal use of replicates
- Differential expression analysis of RNA-seq and digital gene expression profiles with biological replication.
- Uses empirical Bayes estimation and exact tests based on the negative binomial distribution.
- Also useful for differential signal analysis with other types of genome-scale count data.

Package 'DESeq'

- "Differential gene Expression analysis of Sequencing data based on the negative binomial distribution"
- Estimates variance-mean dependence in count data from high-throughput sequencing assays and tests for differential expression based on a model using the negative binomial distribution
- Negative binomial distribution is a discrete probability distribution of the number of successes in a sequence of independent and identically distributed Bernoulli trials before a specified (non-random) number of r failures
  - 6-sided die, define a "1" as failure, and all "non-1"s as successes
  - Roll a die repeatedly until the 3rd time "1" appears (r = 3 failures)
  - The probability distribution of the number of "non-1"s that had appeared will be negative binomially distributed

Negative Binomial Probability Mass Function



- The orange line represents the mean, which is equal to 10 in each of these plots
- The green line shows the standard deviation.

Wig and bigWig

- Wig (Wiggle)
  - Older format for display of dense, continuous data
    - GC percent, probability scores, and transcriptome data
  - Data elements must be equally sized
  - wiggle data is compressed and stored internally in 128 unique bins
  - This compression causes a minor loss of precision when data is exported from a wiggle track
  - If your data is sparse or contains elements of varying size, use the bedGraph or bigBed format instead of the wiggle format
- bigWig
  - Recommended format for almost all graphing track needs data elements must be equally sized
  - Indexed binary format
  - Main advantage of the bigWig files is that only the portions of the files needed to display a particular region are transferred and loaded into browser
  - For large data sets bigWig is considerably faster than regular wiggle files
  - Only the portion that is needed for the chromosomal position you are currently viewing is locally cached as a "sparse file"

Wiggle Format

- Example 1

```
variableStep chrom=chr2
300701 12.5
300702 12.5
300703 12.5
300704 12.5
300705 12.5
```

- Example 2 (same result)

```
variableStep chrom=chr2 span=5
300701 12.5
```

- Example 3

```
fixedStep chrom=chr3 start=400601 step=100 span=5
11
22
33
```

  - causes the values 11, 22, and 33 to be displayed as 5-base regions on chromosome 3 at positions 400601-400605, 400701-400705, and 400801-400805, respectively.

BED Files

- Provides a flexible way to define the data lines that are displayed in an annotation track
- 3 required fields and 9 additional optional fields
- The number of fields per line must be consistent throughout any single set of data in an annotation track
- The order of the optional fields is binding: lower-numbered fields must always be populated if higher-numbered fields are used

- The first 3 required BED fields are:
  1. chrom
  2. chromStart
  3. chromEnd

- The 9 additional optional BED fields are:
  4. name - Defines the name of the BED line
  5. score - A score between 0 and 1000
  6. strand - Defines the strand - either '+' or '-'
  7. thickStart - The starting position at which the feature is drawn thickly
  8. thickEnd - The ending position at which the feature is drawn thickly
  9. itemRgb - An RGB value of the form R,G,B (e.g. 255,0,0)
  10. blockCount - The number of blocks (exons) in the BED line
  11. blockSizes - A comma-separated list of the block sizes
  12. blockStarts - A comma-separated list of block starts

BedGraph and BigBed

- BedGraph
  - Allows display of continuous-valued data in track format
  - Useful for probability scores and transcriptome data
  - Similar to the wiggle (WIG) format, but unlike the wiggle format, data exported in the bedGraph format are preserved in their original state (no rounding)

- BigBed
  - Stores annotation items that can either be simple, or a linked collection of exons, much as BED files do
  - Indexed binary format
  - The main advantage of the bigBed files is that only the portions of the files needed to display a particular region are transferred and loaded into browser
  - For large data sets bigBed is considerably faster than regular BED files
  - The bigBed file remains on the web accessible server (http, https, or ftp)
    - not on the UCSC server
  - Only the portion that is needed for the chromosomal position you are currently viewing is locally cached as a "sparse file"

GFF (General Feature Format) Files

- Based on the Sanger GFF2 specification.
- 9 required fields that must be tab-separated.
  - If the fields are separated by spaces instead of tabs, the track will not display correctly

- GFF fields:
  1. seqname - The name of the sequence. Must be a chromosome or scaffold.
  2. source - The program that generated this feature.
  3. feature - The name of this type of feature. Some examples of standard feature types are "CDS", "start_codon", "stop_codon", and "exon".
  4. start - The starting position of the feature in the sequence. The first base is numbered 1.
  5. end - The ending position of the feature (inclusive)
  6. score - A score between 0 and 1000. If the track line useScore attribute is set to 1 for this annotation data set, the score value will determine the level of gray in which this feature is displayed (higher numbers = darker gray).
  7. strand - Valid entries include '+', '-', or '.' (for don't know/don't care).
  8. frame - If the feature is a coding exon, frame should be a number between 0-2 that represents the reading frame of the first base. If the feature is not a coding exon, the value should be '.'.
  9. group - All lines with the same group are linked together into a single item.

GTF (Gene Transfer Format) Files

- Refinement to GFF that tightens the specification.

- First 8 GTF fields are the same as GFF

- The 9th (Group) field has been expanded into a list of attributes
  - Each attribute consists of a type/value pair
  - Attributes must end in a semi-colon, and be separated from any following attribute by exactly one space

- The attribute list must begin with the two mandatory attributes:
  - gene_id - A globally unique identifier for the genomic source of the sequence
  - transcript_id - A globally unique identifier for the predicted transcript

  - Attribute list Example:
    - gene_id "Em:U62317.C22.6.mRNA"; transcript_id "Em:U62317.C22.6.mRNA"; exon_number 1

  - The UCSC Genome Browser groups together GTF lines that have the same transcript_id value
    - It only looks at features of type exon and CDS.