Practical RNA-seq analysis

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Bioinformatics and Research Computing
Whitehead Institute
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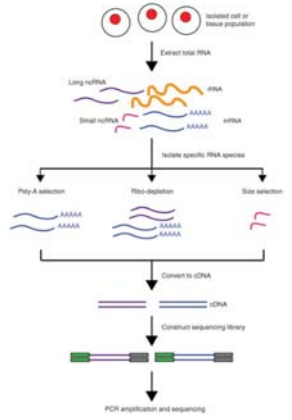
http://barc.wi.mit.edu/hot_topics/

Overview of RNA-seq

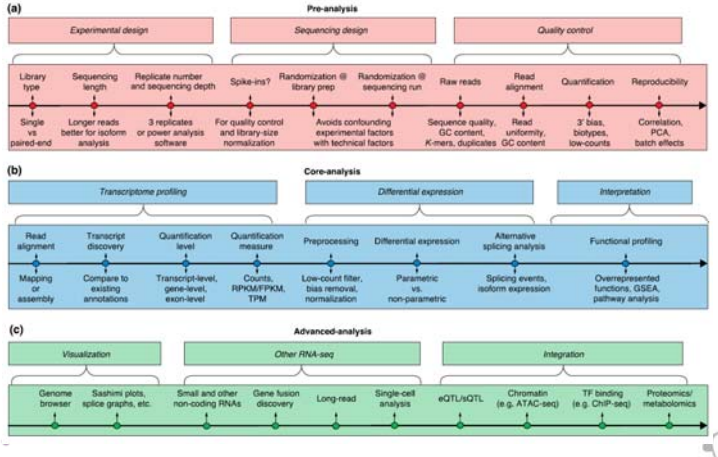


RNA Sequencing and Analysis
K. R. Kukurba and S. B. Montgomery
Cold Spring Harb Protoc. 2015 Nov; 2015(11): 951–969.

Why do RNA-seq?

- RNA-seq includes experiments to
  - **Profile abundance of mRNA and other RNAs**
  - **Identify "differentially expressed" genes**
  - Identify alternated spliced transcript isoforms
  - Assemble transcriptome
  - Identify variants in transcribed regions of genome

RNA-seq Analysis Overview



Conesa, A., et al. *A survey of best practices for RNA-seq data analysis* Genome Biology (2016)

Outline

- Experimental design*
- Quality control
- Sequence preparation*
- Mapping spliced reads
- Counting gene levels
- Normalization and identifying "differentially expressed" genes
- Creating figures and summaries

*not included in the hands-on exercises

Hands-on exercises

- All data is in
  /nfs/BaRC_Public/Hot_Topics/RNA-seq_2020
- Create directories on tak
- Link to data files on BaRC_Public

- See handout for series of commands (step 0)

- Commands can be copied from file
  RNA-seq_2020_commands.txt

Experimental Design

- Replication is essential if results with confidence are desired.

- With the combination of high numbers of reads per sample and multiplexing, the number of Illumina lanes can be much fewer than number of samples.

- Lots of details to think about:
  - Has someone already done an experiment like this?
  - Total RNA or poly(A) RNA or …
  - Number of samples?
  - Read length?
  - Paired or unpaired reads?
  - Number of reads?
  - What reference genome to use?
  - Stranded or unstranded?
  - What reference transcriptome to use?

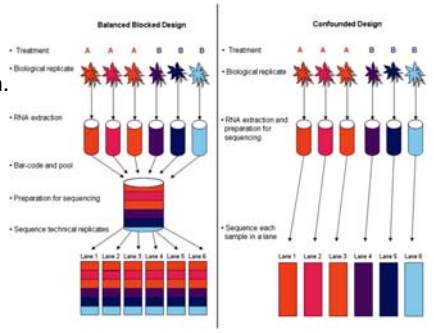
Experimental Design

- Lots of data typically cannot make up for a poor experimental design.

- Look out for bias and confounding.

- Short-read sequencing requires an effectively designed experiment.

- See BaRC about reducing batch effects

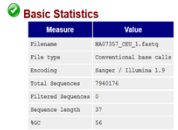


Auer, PL and Doerge, RW *Statistical design and analysis of RNA sequencing data* Genetics (2010)

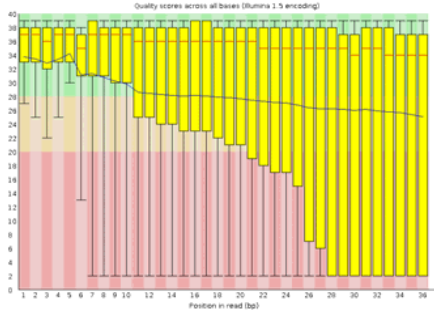
QC Before Alignment

- FastQC, use mulitQC to view
- Check quality of file of raw reads (fastqc_report.html)
- Respond to QC analysis:
  - Filter poor-quality reads
  - Trim poor-quality positions
  - Trim adapter and/or other vector
- Check quality of file of modified reads
- See Hot Topics on quality control Jan 2017
- See handout for fastqc command (step 1)

```
@WIGTC-HISEQ3:1:1212:18183:1997#TTAGGC/1
NCCACAACAGGGCACGGTGCGGAATAGAGAACTATCCCTT
+WIGTC-HISEQ3:1:1212:18183:1997#TTAGGC/1
B[[[[aeaeeaeeeeeeeeeeeeeeeeeeeeeeeeeeeee
```



FastQC: per base sequence quality



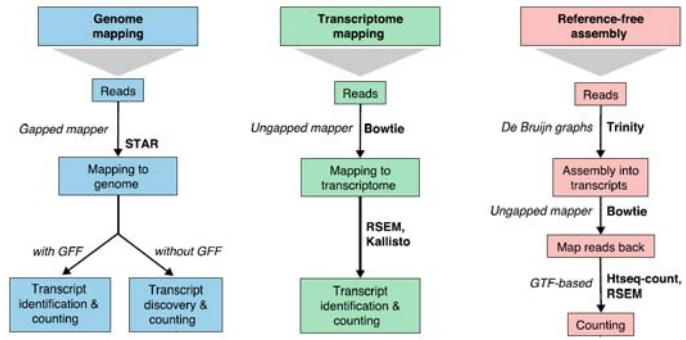
Red: median blue: mean yellow: 25%, 75% whiskers: 10%, 90%

Quality = 10 => error rate = 10% => base call has 90% confidence
Quality = 20 => error rate = 1% => base call has 99% confidence
Quality = 30 => error rate = 0.1% => base call has 99.9% confidence

Responding to Quality Issues

- Method 1:
  - Drop all poor-quality reads
  - Trim poor-quality bases
  - Map only good-quality bases
- Method 2:
  - Keep all reads as is
  - Map as many as possible
  - Current mappers incorporate the read quality score into the mapping quality score

Mapping Considerations



Adapted from Conesa, A., et al. *A survey of best practices for RNA-seq data analysis* Genome Biology (2016)

RNA-Seq Genome Mapping

- Reads can be mapped with a **splice-aware alignment tool** such as STAR (recommended)
- The ideal tool should map to best hit(s), whether to continuous or spliced genome segments
- Look at alignment statistics and mapped reads in a browser (and re-map if needed).

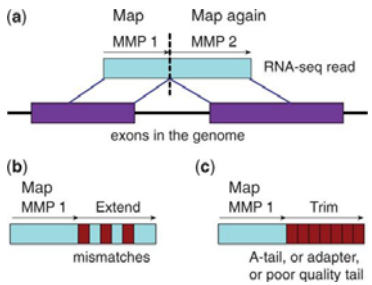
Mapping considerations

- Type of **quality score encoding**.
- Use all or just **canonical** chromosomes?
- Include known splice junctions (in GTF file).
- Do you want to look for novel splice junctions?
- How short of a sub-read should map to an exon boundary?
- How long are your introns? Specify maximum **intron length** allowed.

STAR Aligner

I. Sequential search for Maximal Mappable Prefix (MMP)

II. Stitch together all the seeds that were aligned to the genome from I)



Dobin, A., et al. *STAR: ultrafast universal RNA-seq aligner* Bioinformatics (2012)

Alignment with STAR

- Create genome index using *genomeGenerate,* also see /nfs/genomes

- Run alignment, e.g.

STAR --genomeDir /path/to/GenomeDir --readFilesIn /path/to/read1.fq.gz /path/to/read2.fq.gz --sjdbScore 2 --outFileNamePrefix whateverPrefix --runThreadN 8 --readFilesCommand zcat --outSAMtype BAM SortedByCoordinate

--runMode <alignReads, genomeGenerate>	"alignReads" does the actual mapping. "genomeGenerate" generates the genomeDir required for mapping (default = alignReads).
--genomeDir </path/to/GenomeDir>	Specifies the path to the directory used for storing the genome information created in the genomeGenerate step.
--readFilesIn <read1.fastq read2.fastq>	Specifies the fastq files containing the reads, can be single-end or paired-end.
--sjdbScore <n>	Provides extra alignment score for alignments that cross database junctions (default = 2).
--runThreadN <n>	Specifies the number of threads to use.
--readFilesCommand <cmd>	Specifies the command to uncompress compressed fastq files. For gzipped files (*.gz) use --readFilesCommand zcat.
--outSAMtype <BAM sortingMode>	Specifies the type of BAM file to create. Options: 'BAM Unsorted', 'BAM SortedByCoordinate', 'BAM Unsorted SortedByCoordinate' (to create both unsorted and sorted BAMs)

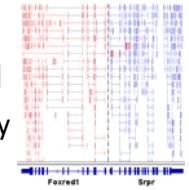
See handout for STAR command (step 2)

Hands on

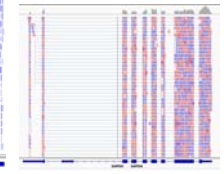
- How does "gene expression" (really: transcript levels) differ between European and Africans?
- European samples (n=2) Montgomery et al., 2010
  - 37nt paired-end reads
- African samples (n=2) Pickrell et al., 2010
  - 46nt single-end reads
- Sample data contains about 10% of total reads
- Is this a good design?

QC after Alignment

- Confirm that reads are stranded or unstranded
  - Run infer_experiment.py (from **RseQC** package)
  - Look at BAM reads in genome browser

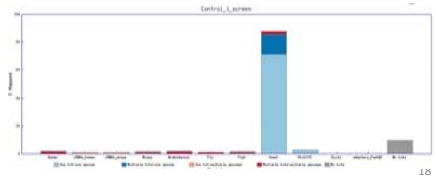


stranded



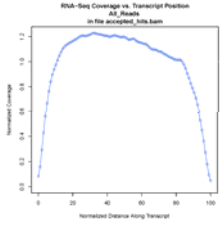
unstranded

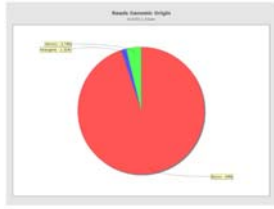
- Contamination?
  - **FastQ Screen**



QC after Alignment

- Do reads cover the length of a typical transcript, or is there 3' or 5' bias?
  - Run **Picard** tool: CollectRnaSeqMetrics
- What fraction of reads map to annotated exons?
  - Run '**qualimap** rnaseq'
- See BaRC SOPs for commands

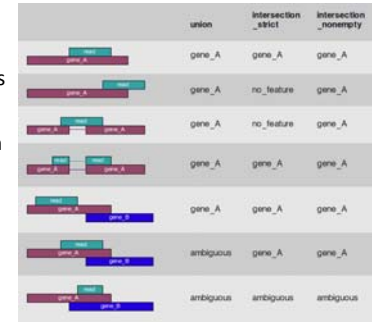




Counting RNA-seq Features

- What features are of interest? Gene, transcript, and/or exon counts?
- How do we want to treat a read maps to multiple places?
- What happens if a read overlaps with multiple features?
- Does the direction of a read need to agree with the direction of the feature? Is RNA stranded, reversed strand or unstranded.

htseq-count "modes"



Counting Methods

- featureCounts (recommended)
  bioinf.wehi.edu.au/featureCounts/
  – Output is raw counts
- htseq-count
  htseq.readthedocs.io/en/master/count.html
  – Output is raw counts
- Cufflinks
  cole-trapnell-lab.github.io/cufflinks/
  – Output is FPKM and related statistics
- Bedtools (intersectBed; coverageBed)
  bedtools.readthedocs.io/
  Output is raw counts (but may need post-processing)

Running featureCounts

Count reads mapping to the specified gene models:

- Usage:

```
featureCounts [options] -a <annotation_file> -o
<output_file> input_file1 [input_file2] ...
```

- Example:

```
#single-end reads (unstranded)
 featureCounts -a gene_anotations.gtf -o
 MySample.featureCounts.txt MySample.bam
#paired-end reads (forward stranded)
 featureCounts –p -s 1 -a gene_anotations.gtf –o
 MySample.featureCounts.txt MySample.sorted.bam
```


Running featureCounts: Options

Option	Description
--minOverlap	Minimum number of overlapping bases in a read that is required for read assignment. 1 by default.
--fracOverlap	Minimum fraction of overlapping bases in a read that is required for read assignment.
-M	Multi-mapping reads will also be counted.
-O	Assign reads to all their overlapping meta-features (or features if -f is specified).
--fraction	Assign fractional counts to features.
-s	Perform strand-specific read counting. Acceptable values: 0 (unstranded), 1 (stranded) and 2 (reversely stranded). 0 by default.

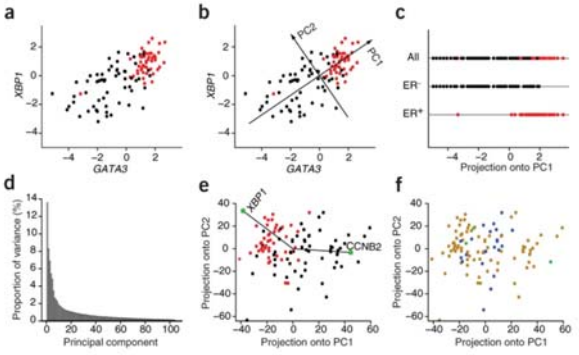
See handout for featureCounts commands (step 3)

Normalization

- Raw counts cannot be compared directly
- Correct for sequencing depth (i.e. library size)
  – CPM: counts per million
  – DESeq: Relative Log Expression (RLE)
  – edgeR: Trimmed Mean M-values (TMM)
  – FPKM*: fragments per kilobase per million mapped reads
  – TPM*: transcripts per million

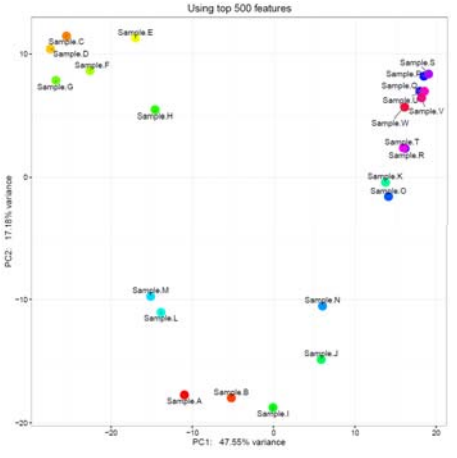
*can be used to compare across genes or transcripts

Using Principal Components Analysis to explore your data



Ringnér, M. What is principal component analysis?. Nat Biotechnol 26, 303–304 (2008). https://doi.org/10.1038/nbt0308-303

Using PCA to explore your data



Are there batch effects on the data?

Talk to BaRC if you want to learn how to remove batch effects.

Differential Expression Methods

- Count-based methods (recommended)
  - Input is matrix of raw counts
  - DESeq2 (R package) -- recommended
  - edgeR (R package)
  - Typically used to compare gene counts
- Accounting for batch effects on count-based methods
  - DESeq2: `dds = DESeqDataSet(se, design = ~ batch + condition)`
  - edgeR: `design = model.matrix(~Batch+Treatment)`

  See http://barcwiki.wi.mit.edu/wiki/SOPs/rna-seq-diff-expressions
- Cufflinks (cuffdiff)
  - Inputs are mapped reads (SAM format)
  - Typically used to compare gene and transcript counts

- See handout for DESeq2 commands (step 4)

Interpreting DESeq2 output

Gene ID (from GTF file)	Mean norm counts	Log2 (fold change)	lfcSE	Wald statistic	Raw p-value	FDR p-value	Raw counts				Normalized counts = raw / (size factor)			
Feature.ID	baseMean	log2(YRI/CEU)	lfcSE	stat	pvalue	padj	CEU_NA07357	CEU_NA11881	YRI_NA18502	YRI_NA19200	CEU_NA07357.norm	CEU_NA11881.norm	YRI_NA18502.norm	YRI_NA19200.norm
ENSG00000251705	114.15	-3.48	0.46	-7.60	2.90E-14	4.48E-11	197	250	8	14	172.84	257.56	5.77	20.42
ENSG00000236552	66.39	-3.86	0.52	-7.47	8.29E-14	1.07E-10	180	96	2	5	157.93	98.9	1.44	7.29
ENSG00000226958	2073.59	-2.45	0.38	-6.52	6.88E-11	7.58E-08	3324	4148	407	556	2916.42	4273.49	293.32	811.14
ENSG00000064886	54.20	3.31	0.54	6.09	1.11E-09	1.05E-06	9	1	84	101	7.9	1.03	60.54	147.35
ENSG00000198786	2006.17	-1.95	0.32	-6.08	1.23E-09	1.05E-06	4601	2391	834	633	4036.84	2463.33	601.05	923.47
ENSG00000100292	58.87	-2.86	0.47	-6.04	1.54E-09	1.19E-06	131	98	13	7	114.94	100.96	9.37	10.21

```
sizeFactors (from DESeq2):

CEU_NA07357 CEU_NA11881 YRI_NA18502 YRI_NA19200
  1.1397535   0.9706359   1.3875763   0.6854579
```

Differential Expression Issues

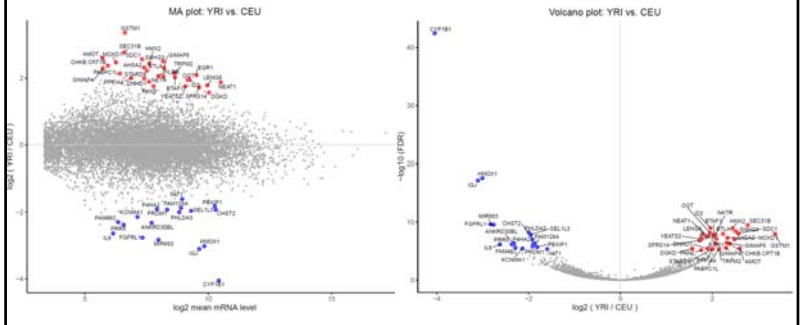
- Given that statistics are
  - based on complex models
  - influenced by even more complex biology

  The p-values may not be accurate but can be very effective at ranking genes
- Statistics don't work very well when one sample has no counts.
- You have to choose appropriate thresholds.

Presenting Results

- What do you want to show?
- All-gene scatterplots can be helpful to
  - See level and fold-change ranges
  - Identify sensible thresholds
  - Hint at data or analysis problems
- Heatmaps are useful if many conditions are being compared but only for gene subsets
- Output normalized read counts with same method used for DE statistics
- Whenever one gene is especially important, look at the mapped reads in a genome browser

MA (ratio-intensity) and volcano plots

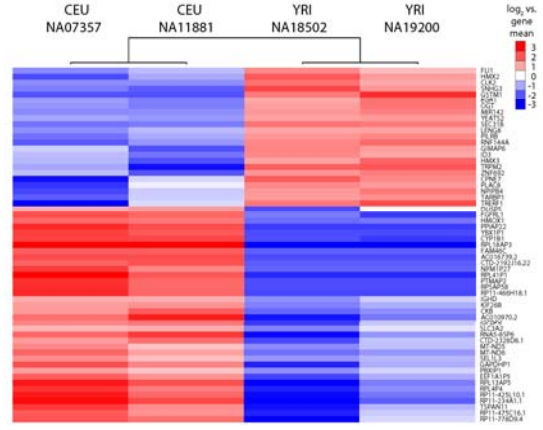


MA (ratio-intensity) plot

Volcano plot

See handout commands step 5

Heatmap example: genes with FDR < 0.2



Excel:
- Add pseudocounts

Cluster 3.0:
- Log-transform
- Mean center
- Cluster

Java TreeView:
- Visualize
- Export

Illustrator
- Assemble

Heatmap

- https://software.broadinstitute.org/morpheus/

Public RNA-seq Datasets

- NCBI GEO
- EBI ArrayExpress
- Broad (e.g. CCLE, GTEx)
- Recount2
- ENCODE
- TCGA
- /nfs/BaRC_datasets

Summary

- Experimental design
- Quality control (fastqc)
- Sequence preparation
- Mapping spliced reads (STAR)
- Counting gene levels (featureCounts)
- Normalization and identifying "differentially expressed" genes (DESeq2 R package)
- Creating figures and summaries
- Save your commands!

Resources

- Previous Hot Topics (http://jura.wi.mit.edu/bio/education/hot_topics/)
- An introduction to R and Bioconductor: A BaRC Short Course
- BaRC SOPs (http://barcwiki.wi.mit.edu/wiki/SOPs)
- Online software manuals
  - STAR, featureCounts, DESeq2, etc.
- Various datasets: **`/nfs/BaRC_datasets`**
- Genome index and GTF files are in **`/nfs/genomes`**

Upcoming Hot Topics

- Single cell RNA-seq: February 27th
- Excel tips and tricks: March 12th
- Dimensionality reduction: March