

So you want to do a: RNAseq Experiment Differential Expression Analysis

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Disclaimer

- This talk/workshop is full of opinion, there are as many different ways to perform analysis as there are Bioinformaticians.
- My opinion is based on over a decade of experience and spending a considerable amount of time to understand data, how it's created and how it relates to the biological question.
- Each experiment is unique, this workshop is a starting place and should be adapted to the specific characteristics of your experiment.

What is Differential Expression

Differential expression analysis means taking the normalized sequencing fragment count data and performing statistical analysis to discover quantitative changes in gene expression levels between experimental groups.

For example, we use statistical testing to decide whether, for a given gene, an overserved difference in fragment counts between groups is significant, that is, whether it is greater than what would be expected just due to random biological variation.

Goals

- Focus on differential **gene** expression studies
- Discussion of considerations from sample collection through analysis and analysis output.

The Bioinformatics Core

So you want to do a:

RNASeq Experiment – Differential Expression Analysis

The **mission** of the Bioinformatics Core facility is to facilitate outstanding omics- scale research through these activities:

Data Analysis

The Bioinformatics Core promotes experimental design, advanced computation and informatics analysis of omics scale datasets that drives research forward.

Research Computing

Maintain and make available high-performance computing hardware and software necessary for todays data-intensive bioinformatic analyses.

Training

The Core helps to educate the next generation of bioinformaticians through highly acclaimed training workshops, seminars and through direct participation in research activities.

UC Davis Bioinformatics Core in the Genome Center

Core Facility Manager

Dr. Matthew Settles

Faculty Advisor

Dr. Ian Korf

Data Analysis Group

Genomics Bioinformatics

Dr. Joseph Fass
Dr. Monica Britton
Nikhil Joshi

Proteomics Bioinformatics

Dr. Jessie Li

Metabolomics Bioinformatics

Biostatistics

Dr. Blythe Durbin-Johnson

Research Computing Group

System Administration

Michael Casper Lewis
Richard Feltstykket

Database/Web Programming

Adam Schaal

Undergraduate Assistant

I-San Stephanie Chang

Training

Microbial Community Analysis (amplicon community profiling) May 2018 @ UCB

May 21, 2018, 9:30 a.m. - May 23, 2018, 4:30 p.m.

Organizer - UCB-QB3 Genomics Sequencing Facility

Contact - UC Davis Bioinformatics Core, training.bioinformatics@ucdavis.edu

Bioinformatics: RNA-Seq Workshop 2018

June 18, 2018, 9:30 a.m. - June 22, 2018, 4:30 p.m.

Organizer - Bioinformatics Core

Contact - UC Davis Bioinformatics Core, training.bioinformatics@ucdavis.edu

Date(s)	Event title	Organizer
Apr 18, 2018	Dovetail Genomics Lunch & Learn Seminar April 18 2018	DNA Technologies Core
Mar 26 - Mar 28, 2018	Bioinformatics Prerequisites Workshop	Bioinformatics Core
Mar 19 - Mar 21, 2018	Bioinformatics: Single Cell RNA-Seq Workshop @ UCSF	Bioinformatics Core
Mar 15, 2018	Lexogen Lunch & Learn Seminar March 15 2018	DNA Technologies Core
Feb 12, 2018	New Product Seminar: 3' RNAseq for gene expression from single cells, low input and population based analysis	DNA Technologies Core

<http://registration.genomecenter.ucdavis.edu/>

What do you get when you collaborate with the Bioinformatics Core

- ‘Borrow’ our computational resources
 - High throughput data requires a lot of storage and computing power
- Immediate access to our experience and expertise
 - Ensures you’ll get high quality results and in a format you can actually use
- We know how to select the optimal analysis
 - This results in a much quicker path to interpretation
- Collaborating with the Bioinformatics core makes the project far more likely to succeed.
 - The sequencing experts contribute their expertise, the biologist contributes theirs, and the bioinformaticians contribute theirs

Fee for service based on hourly rate of \$97/hour most projects 20-50 hours can do partial FTE for large grants

Diving in to Bioinformatics

- Know and Understand the experiment
 - “The Question of Interest”
- Build a set of assumptions/expectations
 - Mix of technical and biological
 - Spend your time testing your assumptions/expectations
 - Don’t spend your time finding the “best” software
- Don’t under-estimate the time Bioinformatics may take
- Be prepared to accept ‘failed’ experiments

Introduction to Sequencing

So you want to do a:

RNASeq Experiment – Differential Expression Analysis

'Next' Generation

- 2005 – 454 life Sciences (later acquired by Roche), Pyrosequencing 1.5Gb/day, ~500bp reads

Discontinued



- 2006 – Solexa (later acquired by Illumina). Now the dominant platform with >75% market share of sequencers and estimated >90% of all bases sequenced are from Illumina machines, Sequencing by Synthesis > 1600Gb/day, 2x150bp reads.



The ‘Next, Next’ Generation Sequencers (3rd Generation)

- 2009 – Single Molecule Read Time sequencing by Pacific Biosystems, most successful third generation sequencing platforms, RSII ~2Gb/day, newer Pac Bio Sequel ~14Gb/day, near 100Kb reads.



Iso-seq on Pac Bio, transcriptome without ‘assembly’

- 2015 – Another 3rd generation sequencer, founded in 2005 and currently in beta testing. The sequencer uses nanopore technology developed in the 90’s to sequence single molecules. Throughput is about 500Mb per flowcell, capable of near 200kb reads.

**Fun to play with but results
are highly variable**

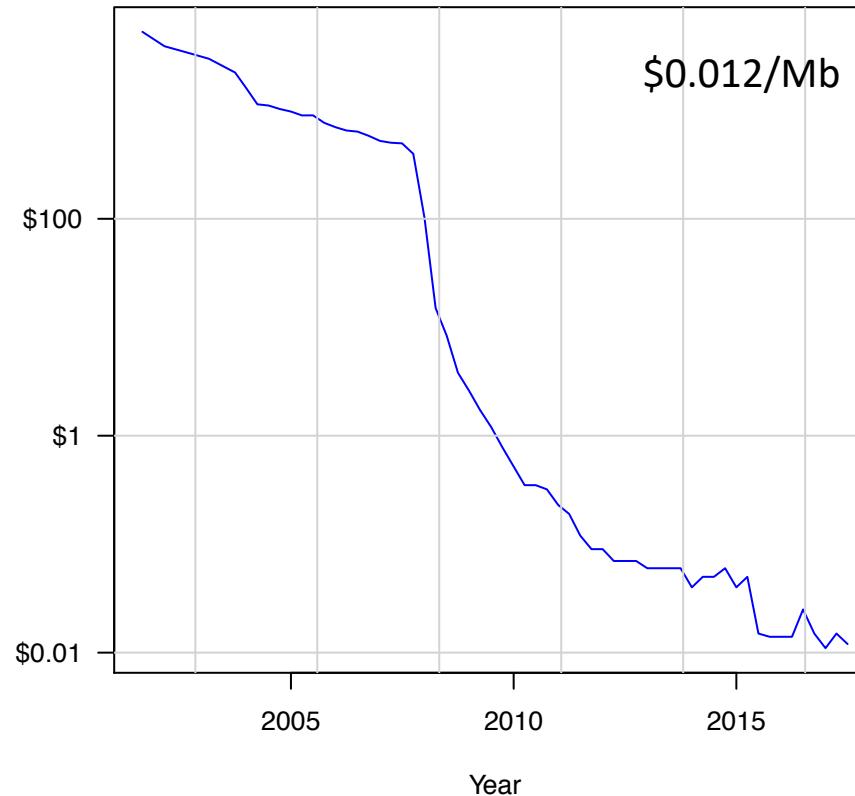


FYI: 4th generation sequencing is being described as In-situ sequencing

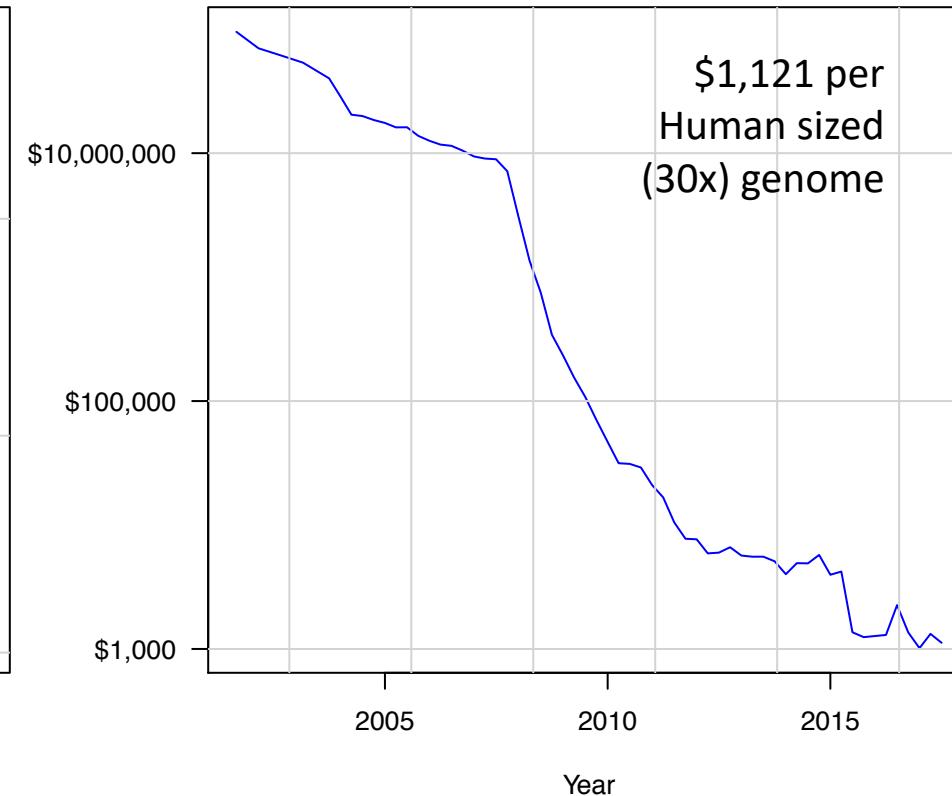
Sequencing Costs

July 2017

Cost per Megabase of Sequence



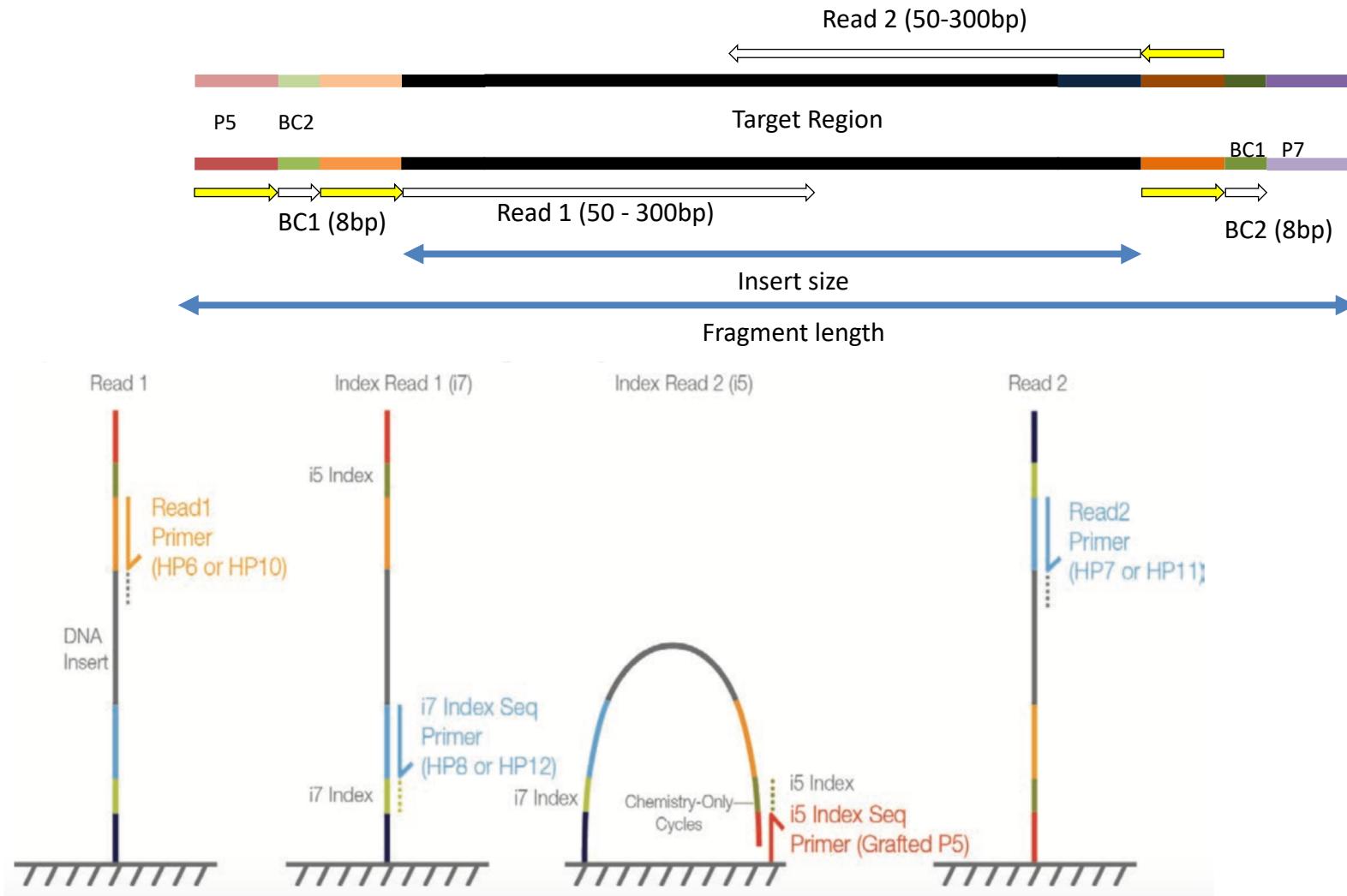
Cost per Human Sized Genome @ 30x



- Includes: labor, administration, management, utilities, reagents, consumables, instruments (amortized over 3 years), informatics related to sequence productions, submission, indirect costs.
- <http://www.genome.gov/sequencingcosts/>

Illumina sequencing sequencing by synthesis

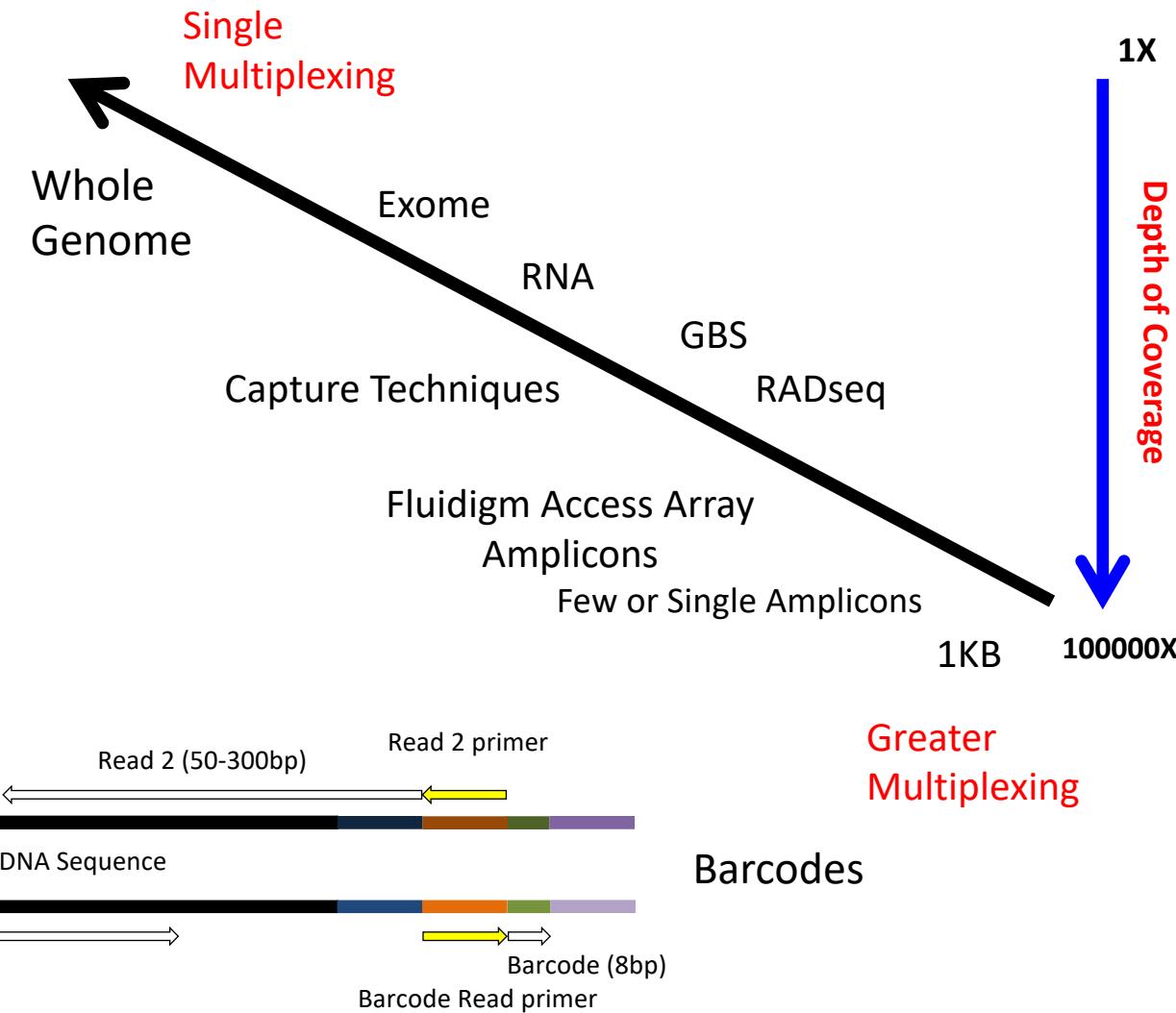
Illumina SBS



Flexibility – short but not too short

Genomic reduction allows for greater coverage and multiplexing of samples.

You can fine tune your depth of coverage needs and sample size with the reduction technique



Sequencing Libraries : MLA-seq

DNA-seq	DNase-seq	tagRNA-seq	EnD-seq
RNA-seq	ATAC-seq	PAT-seq	Pool-seq
Amplicons	MNase-seq	Structure-seq	G&T-seq
CHiP-seq	FAIRE-seq	MPE-seq	Tn-Seq
MeDiP-seq	Ribose-seq	STARR-seq	BrAD-seq
RAD-seq	smRNA-seq	Mod-seq	SLAF-seq
ddRAD-seq			

[Mol Cell](#). 2018 Apr 18. pii: S1097-2765(18)30217-X. doi: 10.1016/j.molcel.2018.03.014. [Epub ahead of print]

CapZyme-Seq Comprehensively Defines Promoter-Sequence Determinants for RNA 5' Capping with NAD.

Vvedenskaya IO¹, Bird JG², Zhang Y³, Zhang Y⁴, Jiao X⁵, Barvík I⁶, Krásný L⁷, Kiledjian M⁵, Taylor DM⁸, Ebright RH⁹, Nickels BE¹⁰.

Experimental Design and Libraries

So you want to do a:

RNASeq Experiment – Differential Expression Analysis

Treating Bioinformatics as a Data Science

Seven stages to data science

1. Define the question of interest
2. Get the data
3. Clean the data
4. Explore the data
5. Fit statistical models
6. Communicate the results
7. Make your analysis reproducible

Data science done well looks easy and
that's a big problem for data scientists

simplystatistics.org
March 3, 2015 by Jeff Leek

Designing Experiments

Beginning with the question of interest (and working backwards)

- The final step of a DE analysis is the application of a linear model to each gene in your dataset.

Traditional statistical considerations and basic principals of statistical design of experiments apply.

- **Control** for effects of outside variables, avoid/consider possible biases, avoid confounding variables in sample preparation.
- **Randomization** of samples, plots, etc.
- **Replication** is essential (triplicates are THE minimum)
- You should know your final (De) model and comparison contrasts before beginning your experiment.

Three outcomes Goldilocks and the three bears

- Technical and/or biological variation exceeds that of experimental variation, results in 0 differentially expressed genes.
- Experiment induces an extreme phenotype with cascading effects and/or little to no biological variation between replication (think cell lines), results in 1000s of differentially expressed genes. Some of which are due directly to the experiment; however, many due to downstream cascading effects.
- Technical artifacts are controlled. Biological variation is induced in the experiment, and cascading effects are controlled, or accounted for, results in 10s to 100s of differentially expressed genes directly applicable to the question of interest.

General rules for preparing samples

- Prepare more samples than you are going to need, i.e. expect some will be of poor quality, or fail
- Preparation stages should occur across all samples at the same time (or as close as possible) and by the same person
- Spend time practicing a new technique to produce the highest quality product you can, reliably
- Quality should be established using Fragment analysis traces (pseudo-gel images, RNA RIN > 7.0)
- DNA/RNA should not be degraded
 - 260/280 ratios for RNA should be approximately 2.0 and 260/230 should be between 2.0 and 2.2. Values over 1.8 are acceptable
- Quantity should be determined with a Fluorometer, such as a Qubit.

Sample preparation

In high throughput biological work (Microarrays, Sequencing, HT Genotyping, etc.), what may seem like small technical details introduced during sample extraction/preparation can lead to large changes, or technical bias, in the data.

Not to say this doesn't occur with smaller scale analysis such as Sanger sequencing or qRT-PCR, but they do become more apparent (seen on a global scale) and may cause significant issues during analysis.

Be Consistent

BE CONSISTENT ACROSS ALL SAMPLES!!!

Generating RNA-seq libraries

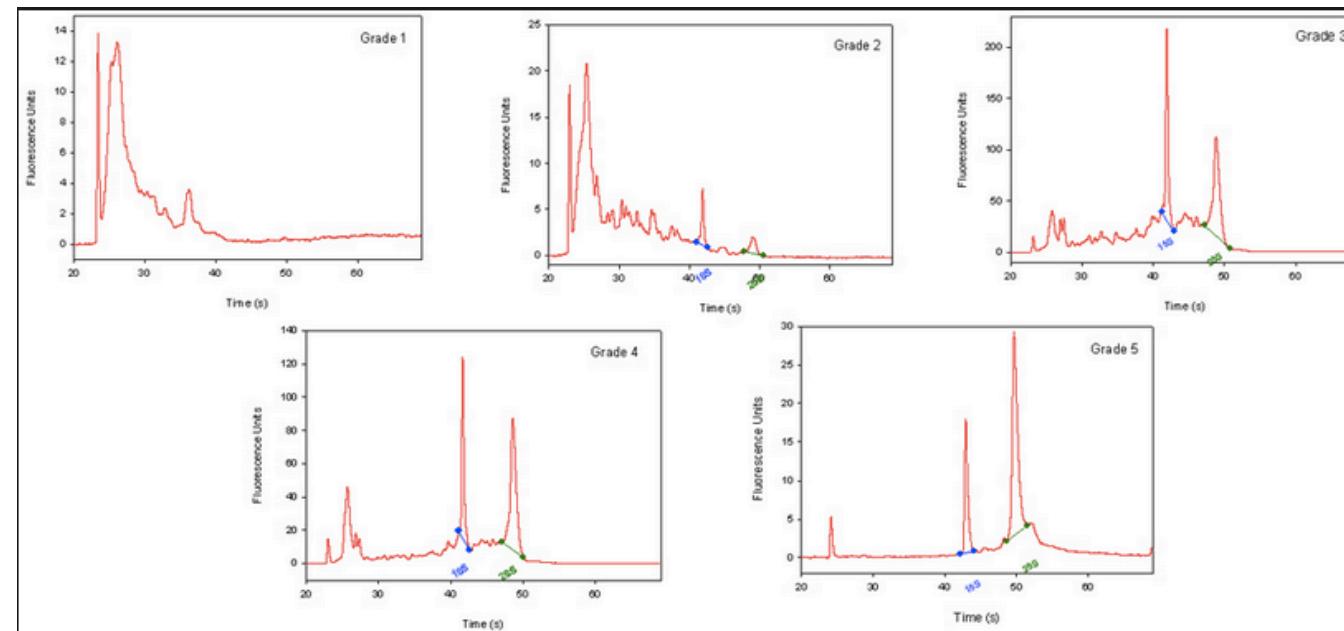
Considerations

- QA/QC of RNA samples
- What is the RNA of interest
- Library Preparation
 - Stranded Vs. Unstranded
- Size Selection/Cleanup
 - Final QA

QA/QC of RNA samples

RNA Quality and RIN (RQN on AATI Fragment Analyzer)

- RNA sequencing begins with high-quality total RNA, only an Agilent BioAnalyzer (or equivalent) can adequately determine the quality of total RNA samples. RIN values between 7 and 10 are desirable.



BE CONSISTANT!!!

RNA of interest

- From “total RNA” we extract “RNA of interest”. Primary goal is to NOT sequence 90% (or more) ribosomal RNAs, which are the most abundant RNAs in the typical sample. There are two main strategies for enriching your sample for “RNA of interest”.
 - polyA selection. Enrich mRNA (those with polyA tails) from the sample by oligo dT affinity.
 - rRNA depletion. rRNA knockdown using RiboZero (or Ribominus) is mainly used when your experiment calls for sequencing non-polyA RNA transcripts and non-coding RNA (ncRNA) populations. This method is also usually more costly.

rRNA depletion will result in a much larger proportion of reads align to intergenic and intronic regions of the genome.

Library Preparation

- Some library prep methods first require you to generate cDNA, in order to ligate on the Illumina barcodes and adapters.
 - cDNA generation using oligo dT (3' biased transcripts)
 - cDNA generation using random hexomers (less biased)
 - full-length cDNAs using SMART cDNA synthesis method
- Also, can generate strand specific libraries, which means you only sequence the strand that was transcribed.
 - This is most commonly performed using dUDP rather than dNTPs in cDNA generation and digesting the “rna” strand.
 - Can also use a RNA ligase to attach adapters and then PCR the second strand and remainder of adapters.

Size Selection/Cleanup/qA

Final insert size optimal for DE are ~ 150bp

- Very important to be consistent across all samples in an experiment on how you size select your final libraries. You can size select by:
 - Fragmenting your RNA, prior to cDNA generation.
 - Chemically heat w/magnesium
 - Mechanically (ex. ultra-sonicator)
- Cleanup/Size select after library generation using SPRI beads or (gel cut)
- QA the samples using an electrophoretic method (Bioanalyzer) and quantify with qPCR.

Most important thing is to be consistent!!!

[SUMMARY] Generating RNA-seq libraries

Considerations

- QA/QC of RNA samples [Consistency across samples is most important.]
- What is the RNA of interest [polyA extraction is recommended.]
- Library Preparation
 - Stranded Vs. Unstranded [Standard stranded library kits]
- Size Selection/Cleanup [Target mean 150bp or kit recommendation]
 - Final QA [Consistency across samples is most important.]

Sequencing Depth

- Coverage is determined differently for "Counting" based experiments (RNAseq, amplicons, etc.) where an expected number of reads per sample is typically more suitable.
- The first and most basic question is how many reads per sample will I get
Factors to consider are (per lane):
 1. Number of reads being sequenced
 2. Number of samples being sequenced
 3. Expected percentage of usable data

$$\frac{\text{reads}}{\text{sample}} = \frac{\text{reads. sequenced} * 0.8}{\text{samples. pooled}}$$

- Read length, or SE vs PE, does not factor into sequencing depth.

Sequencing

Characterization of transcripts, or differential gene expression

Factors to consider are:

- Read length needed depends on likelihood of mapping uniqueness, but generally longer is better and paired-end is better than single-end. (2 x >75bp is best)
- Interest in measuring genes expressed at low levels (<< level, the >> the depth and necessary complexity of library)
- The fold change you want to be able to detect (< fold change more replicates, more depth)
- Detection of novel transcripts, or quantification of isoforms requires >> sequencing depth

The amount of sequencing needed for a given sample/experiment is determined by the goals of the experiment and the nature of the RNA sample.

Barcodes and Pooling samples for sequencing

- Best to have as many barcodes as there are samples
 - Can purchase barcodes from vendor, generate them yourself from IDT dna (example), or consult with the DNA technologies core.
- Best to pool all samples into one large pool, then sequence multiple lanes
- IF you cannot generate enough barcodes, or pool into one large pool, RANDOMIZE samples into pools.
 - Bioinformatics core can produce a randomization scheme for you.
 - **This must be consider/determined PRIOR to library preparation**

Cost Estimation

- RNA extraction and QA/QC (Per sample)
- Enrichment of RNA of interest + library preparation (Per sample)
 - Library QA/QC (Bioanalyzer and Qubit)
 - Pooling [If you do your own libraries]
- Sequencing (Number of lanes)
- Bioinformatics (General rule is to estimate the same amount as data generation, i.e. double your budget)

Illumina sequencing costs

I use **350M** fragments per lane

	HiSeq 3000 System	HiSeq 4000 System
No. of Flow Cells per Run	1	1 or 2
Data Yield - 2 × 150 bp	650–750 Gb	1300–1500 Gb
Data Yield - 2 × 75 bp	325–375 Gb	650–750 Gb
Data Yield - 1 × 50 bp	105–125 Gb	210–250 Gb
Clusters Passing Filter (8 lanes per flow cell)	up to 2.5B single reads or 5B paired end reads	up to 5B single reads or 10B PE reads
Quality Scores - 2 × 50 bp	≥ 85% bases above Q30	≥ 85% bases above Q30
Quality Scores - 2 × 75 bp	≥ 80% bases above Q30	≥ 80% bases above Q30
Quality Scores - 2 × 150 bp	≥ 75% bases above Q30	≥ 75% bases above Q30
Daily Throughput	> 200 Gb	> 400 Gb
Run Time	< 1–3.5 days	< 1–3.5 days
Human Genomes per Run*	up to 6	up to 12
Exomes per Run†	up to 48	up to 96
Transcriptomes per Run‡	up to 50	up to 100

<http://www.illumina.com/systems/hiseq-3000-4000/specifications.html>

Cost Estimation

Example: 12 samples, ribo-depletion libraries, target 30M reads per sample, Hiseq 4000 (2x100).

- 12 Samples
 - QA Bioanalyzer = \$98 for all 12 samples
 - Library Preparation (ribo-depletion) = \$383/sample = \$4,596
- Sequencing = \$2346 per lane
 - 2.1 - 2.5 Billion reads per run / 8 lanes = Approximately 300M reads per lane
 - Multiplied by a 0.8 buffer equals 240M expected good reads
 - Divided by 12 samples in the lane = 20M reads per sample per lane.
 - Target 30M reads means 2 lanes of sequencing $\$2346 \times 2 = \4692
- Bioinformatics, simple pairwise comparison design, DE only \$2000
 - Double your data generation budget
- Total = $\$98 + \$4596 + \$4692 = \$9,386$ w/Bioinformatics ~\$18K

Approximately \$950 per sample @ 40M reads per sample

Overview of RNASeq Analysis

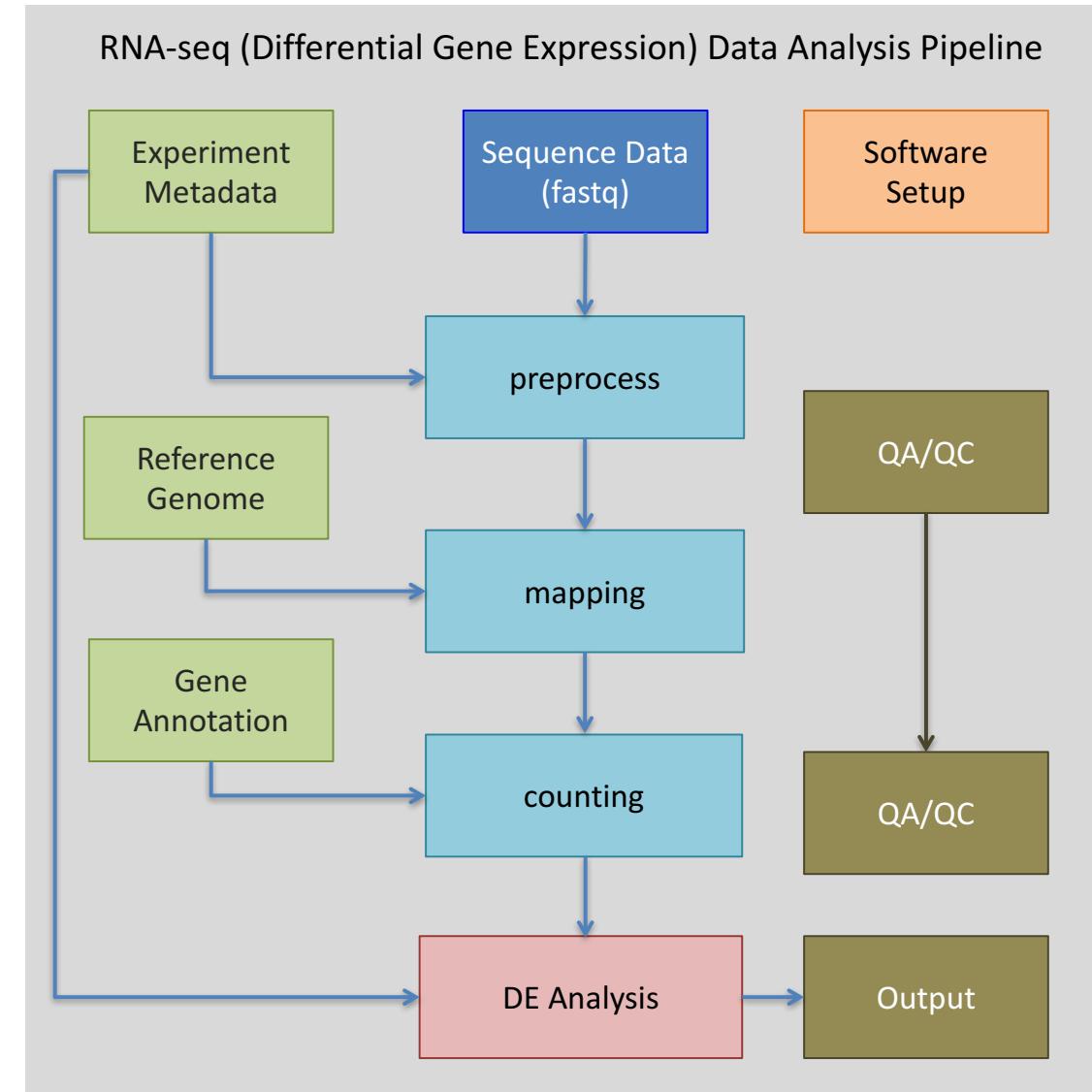
So you want to do a:

RNASeq Experiment – Differential Expression Analysis

Prerequisites

- Access to a multi-core (24 cpu or greater), ‘high’ memory 64Gb or greater Linux server.
- Familiarity with the ‘command line’ and at least one programming language.
- Basic knowledge of how to install software
- Basic knowledge of R (or equivalent) and statistical programming
- Basic knowledge of Statistics and model building

RNA-seq pipeline overview



Preprocessing

So you want to do a:

RNASeq Experiment – Differential Expression Analysis

Why Preprocess reads

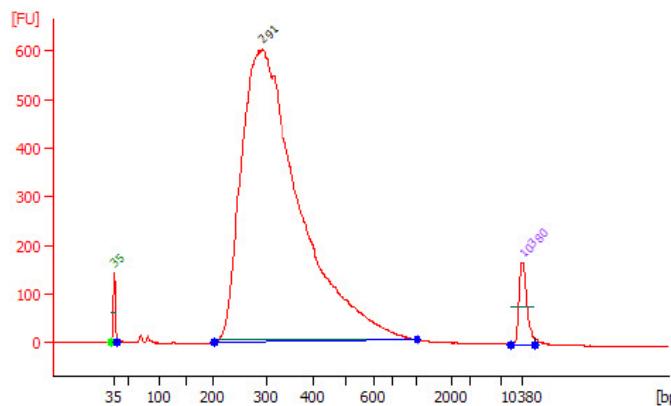
- We have found that aggressively “cleaning” and processing reads can make a large difference to the **speed** and **quality** of assembly and mapping results. Cleaning your reads means, removing reads/bases that are:
 - other unwanted sequence (polyA tails in RNA-seq data)
 - artificially added onto sequence of primary interest (vectors, adapters, primers)
 - join short overlapping paired-end reads
 - low quality bases
 - originate from PCR duplication
 - not of primary interest (contamination)
- Preprocessing also produces a number of statistics that are technical in nature that should be used to evaluate “experimental consistency”

Ribosomal RNA

- Ribosomal RNA makes up 90% or more of a typical total RNA sample.
 - Library prep methods reduce the rRNA representation in a sample
 - oligoDt only binds to polyA tails to enrich a sample for mRNA
 - Ribo-depletion binds rRNA sequences
- Neither technique is 100% efficient

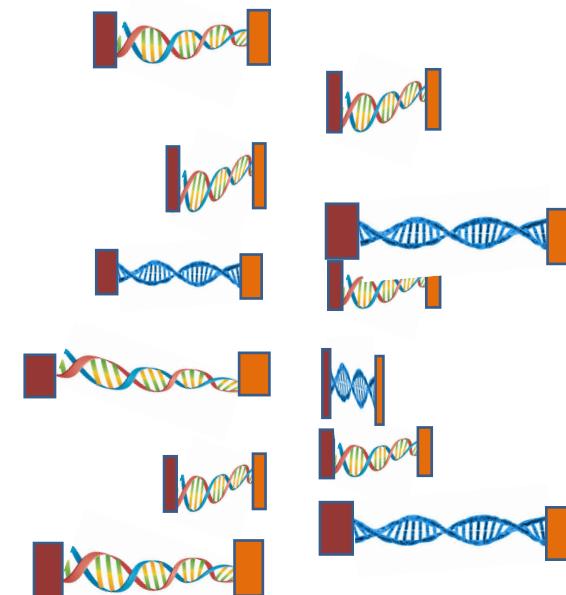
Can screen (map reads to rRNA sequences) to determine rRNA efficiency and potentially remove those reads.

DNA/RNA, could contain 'contamination'



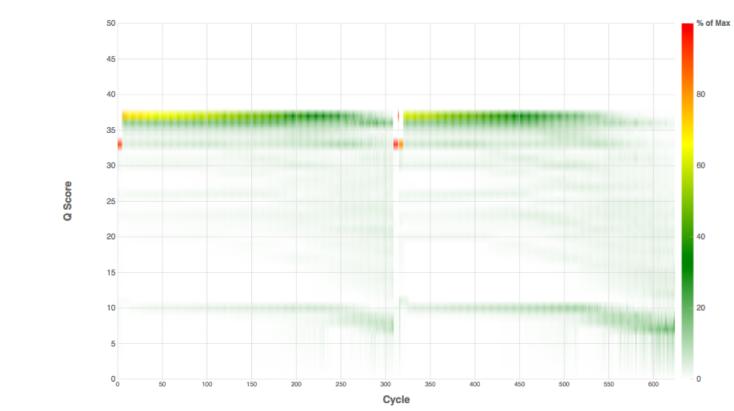
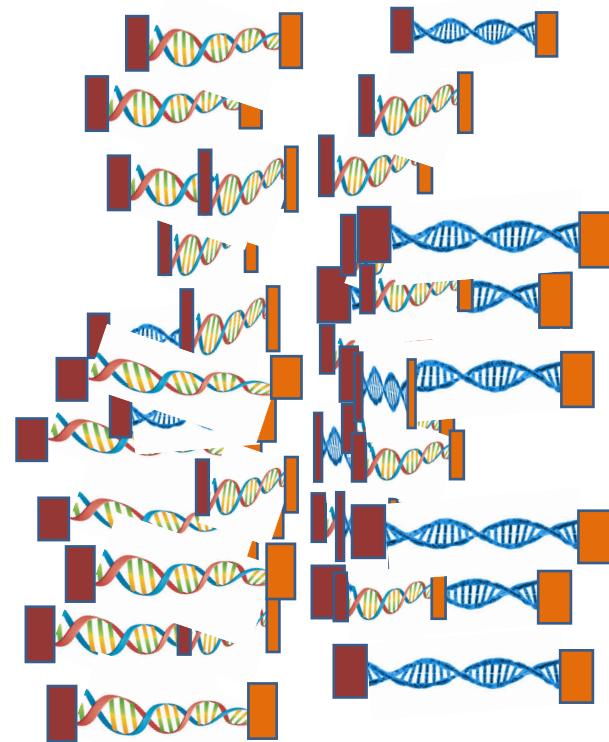
Final Library, size distribution

Library prep, fragmentation,
adapter addition



Possible addition of phiX

PCR enrichment



Sequencing Characteristics/ Quality

Preprocessing

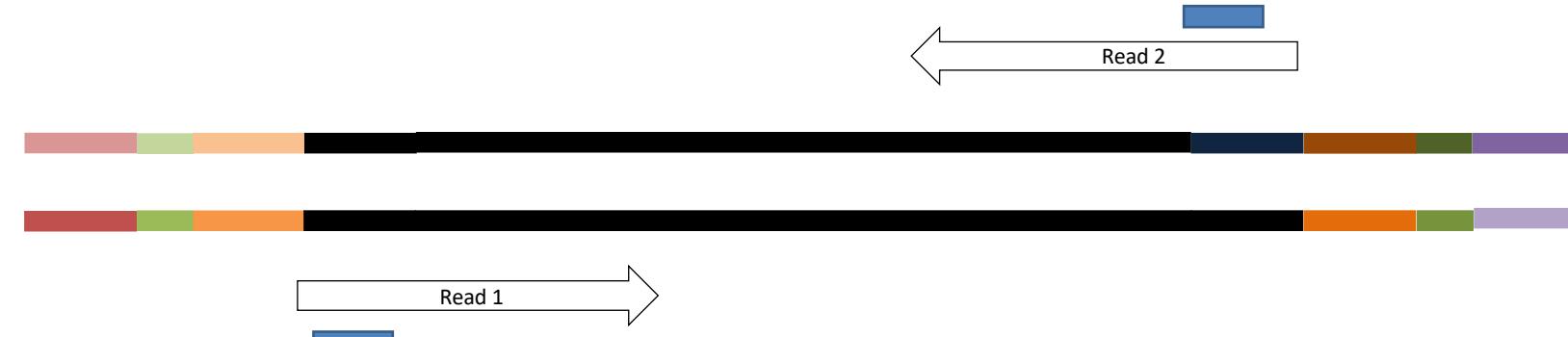
- Map reads to contaminants/PhiX and extract unmapped reads [bowtie2 --local]
 - Remove contaminants (at least PhiX), uses bowtie2 then extracts all reads (pairs) that are marked as unmapped.
- Super-Deduper [PE reads only]
 - Remove PCR duplicates (we use bases 10-35 of each paired read)
- FLASH2 [PE reads only]
 - Join and extend, overlapping paired end reads
 - If reads completely overlap they will contain adapter, remove adapters
 - Identify and remove any adapter dimers present
- Scythe [SE Reads only]
 - Identify and remove adapter sequence
- Sickle
 - Trim sequences (5' and 3') by quality score (I like Q20)
- cleanup
 - Run a polyA/T trimmer
 - Remove any reads that are less than the minimum length parameter
 - Produce preprocessing statistics

New application: HTStream
<https://github.com/ibest/HTStream>

Why Screen for PhiX

- PhiX is a common control in Illumina runs, facilities rarely tell you if/when PhiX has been spiked in
 - Does not have a barcode, so in theory should not be in your data
- **However**
 - When I know PhiX has been spiked in, I find sequence every time
 - When I know PhiX has not been spiked in, I **do not** find sequence
- Better safe than sorry and screen for it.

Super Deduper



Data	Alignment Algorithm	MarkDuplicates	Rmdup	Super Deduper	FastUniq	Fulcrum	Total # of Reads
PhiX	BWA MEM	1,048,278 (0.25%)	1,011,145 (1.05%)	1,156,700 (13.7%)	4,202,526	3,092,155	4,750,299
	Bowtie 2 Local	1,054,725 (6.62%)	948,784 (10.2%)	1,166,936 (14.0%)	4,236,647	3,103,872	4,790,972
	Bowtie 2 Global	799,524 (0%)	800,868 (0.12%)	896,487 (9.92%)	3,768,641	2,704,114	4,293,787
Acropora digitifera	BWA MEM	5,132,111 (2.26%)	6,906,634 (44.5%)	5,133,339 (10.2%)	12,968,469	2,103,567	54,108,240
	Bowtie 2 Local	4,688,809 (4.03%)	5,931,862 (38.9%)	3,971,743 (9.32%)	9,893,903	4,259,619	41,728,154
	Bowtie 2 Global	1,457,865 (3.62%)	1,512,966 (24.2%)	1,185,838 (11.4%)	3,014,498	1,286,031	11,600,847

Super Deduper

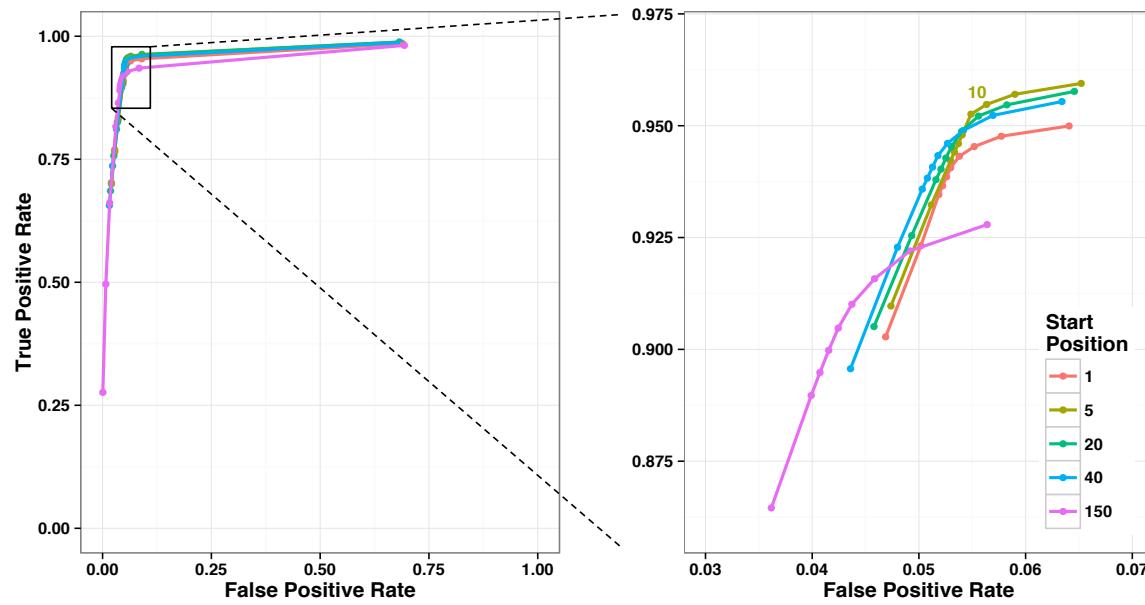
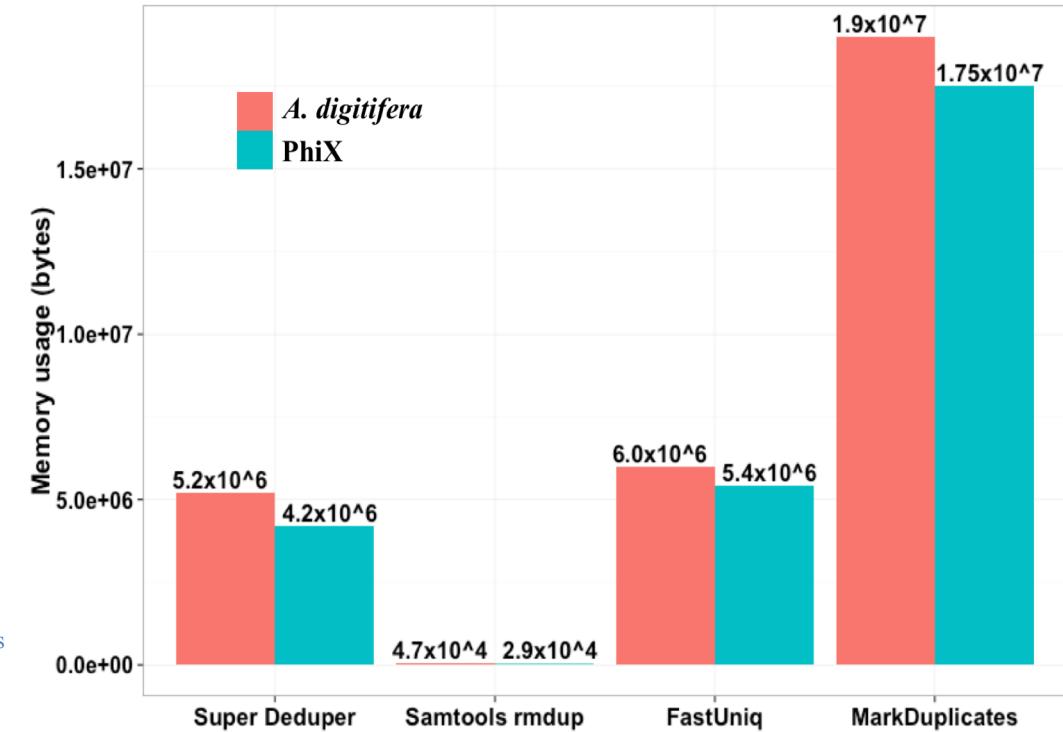


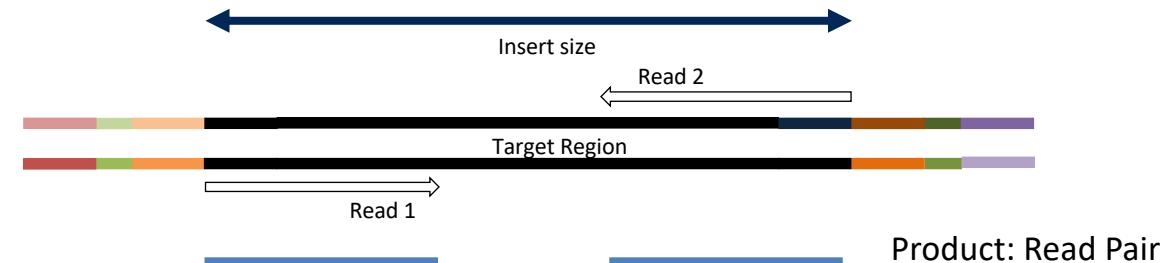
Figure 1: ROC curves. Only a representative subset of the different start positions is shown. The image on the left shows the full ROC curves and the image on the left is a zoomed in view of corner of the curves. Each curve represents a start position and each point represents a length. The labeled point in the image on the right is the default start and length for Super Deduper.



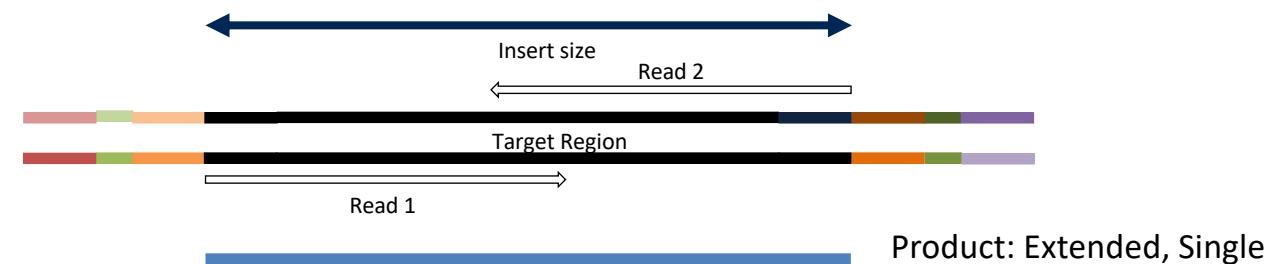
We calculated the Youden Index for every combination tested and the point that acquired the highest index value (as compared to Picard MarkDuplicates) occurred at a start position of 5bp and a length of 10bps (20bp total over both reads)

Flash2 – overlapping of reads and adapter removal in paired end reads

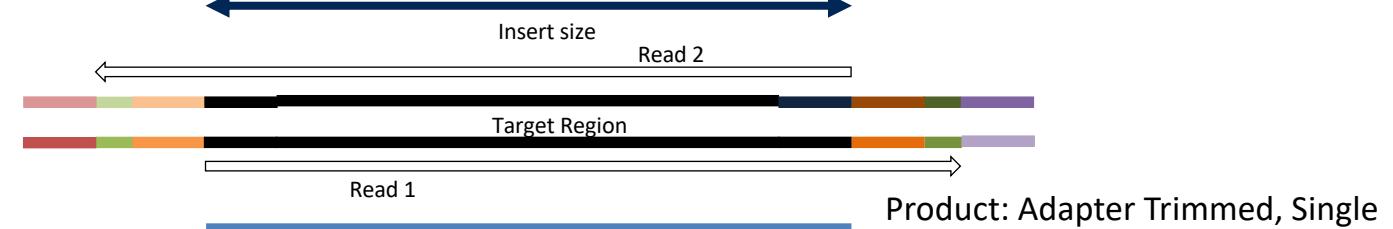
Insert size > length of the number of cycles



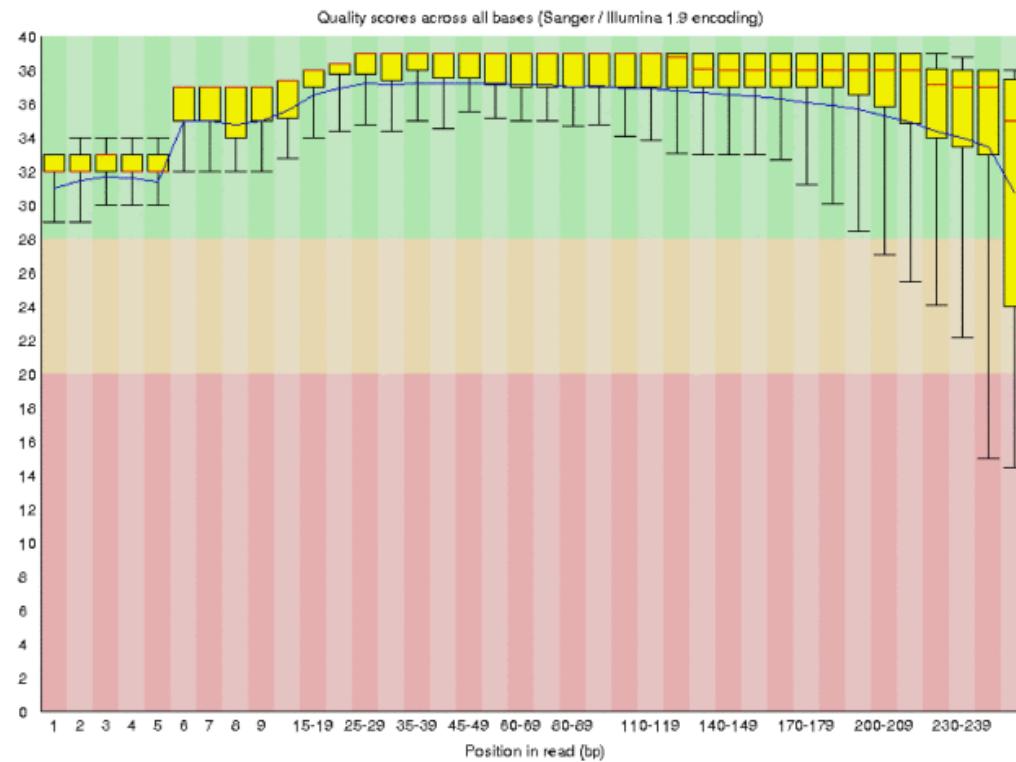
Insert size < length of the number of cycles (10bp min)



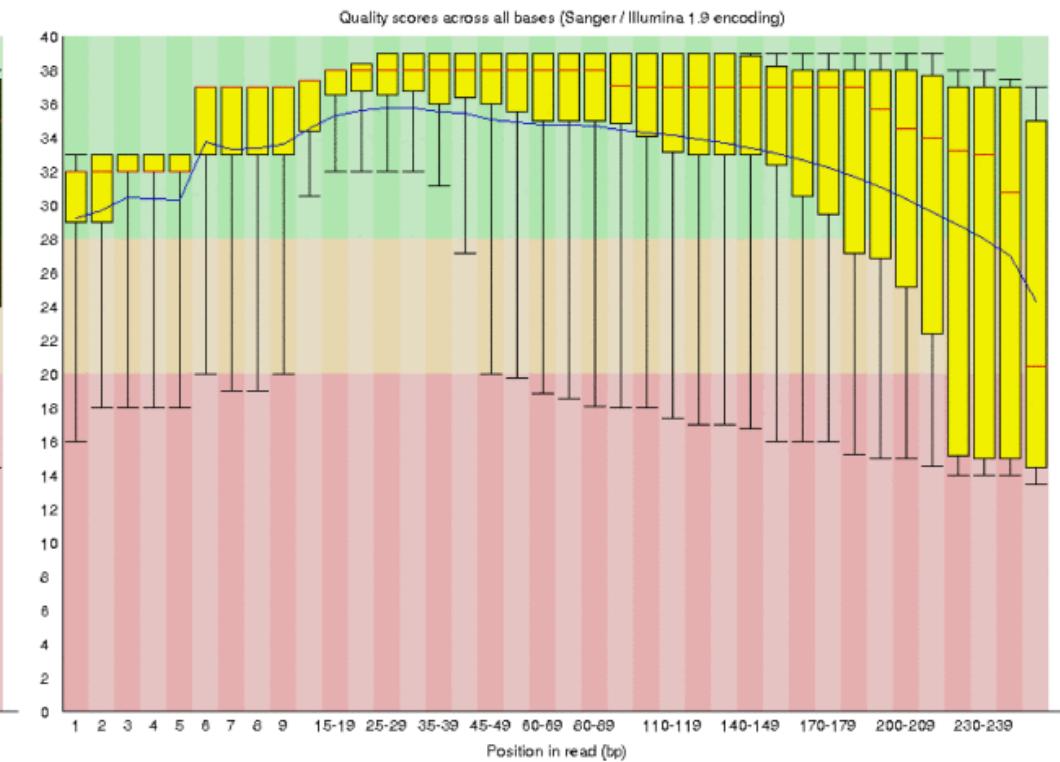
Insert size < length of the read length



Quality Trimming - Sickle



Forward reads



Reverse reads

Remove “poor” quality sequence from both the 5’ and 3’ ends

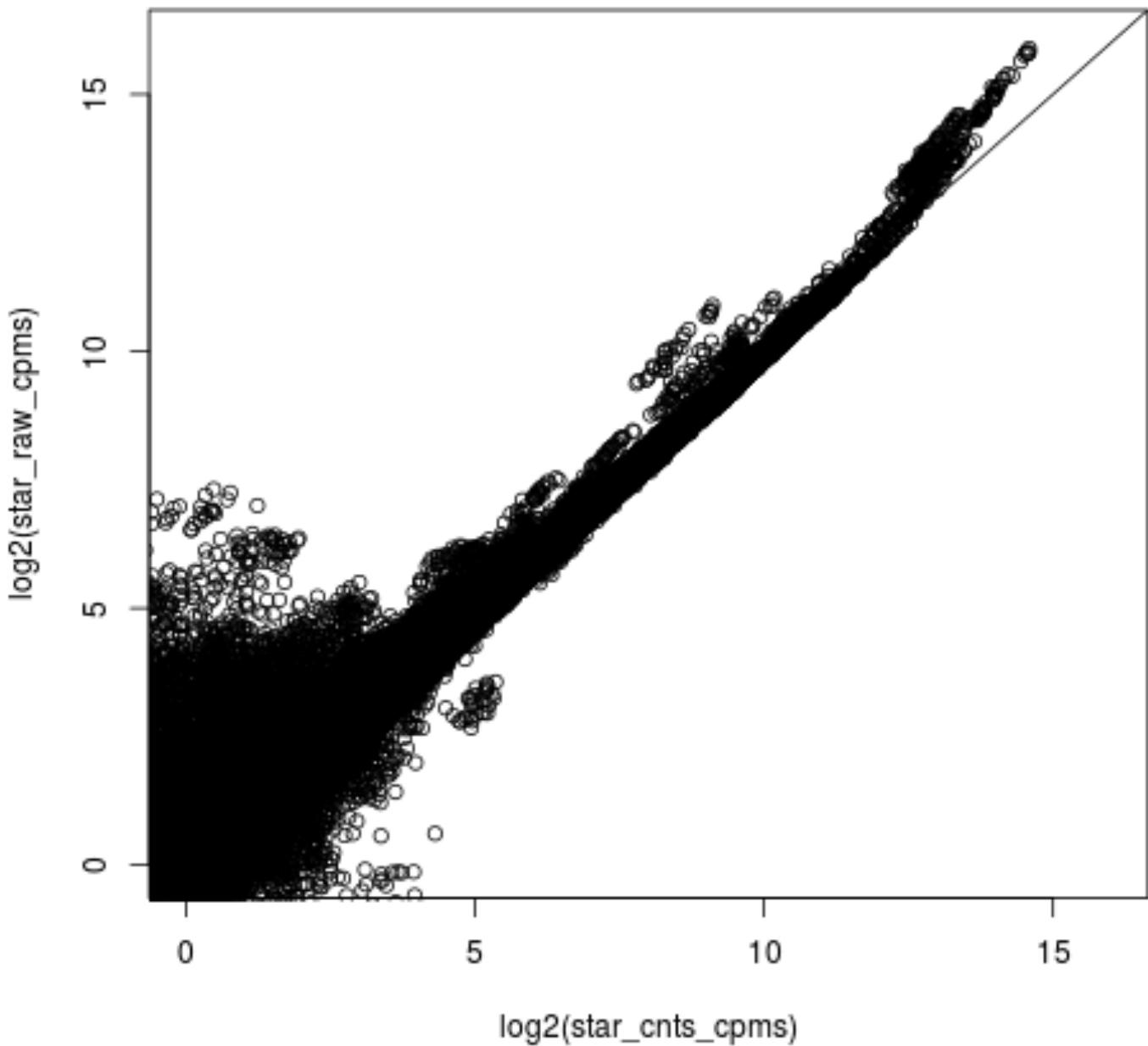
QA/QC

- Beyond generating ‘better’ data for downstream analysis, cleaning statistics also give you an idea as to the quality of the sample, library generation, and sequencing quality used to generate the data.
- This can help inform you of what you might do in the future.
- I’ve found it best to perform QA/QC on both the run as a whole (poor samples can affect other samples) and on the samples themselves as they compare to other samples **(REMEMBER, BE CONSISTANT).**
 - Reports such as Basespace for Illumina, are great ways to evaluate the runs as a whole.
 - PCA/MDS plots of the preprocessing summary are a great way to look for technical bias across your experiment

Comparing Mapping Raw vs Preprocessed with star

pe	se	FALSE	TRUE
FALSE		16352	18
TRUE		2542	13971

raw	prep	FALSE	TRUE
FALSE		15545	591
TRUE		160	16587



Sequence Read Mapping

So you want to do a:

RNASeq Experiment – Differential Expression Analysis

Mapping vs Assembly

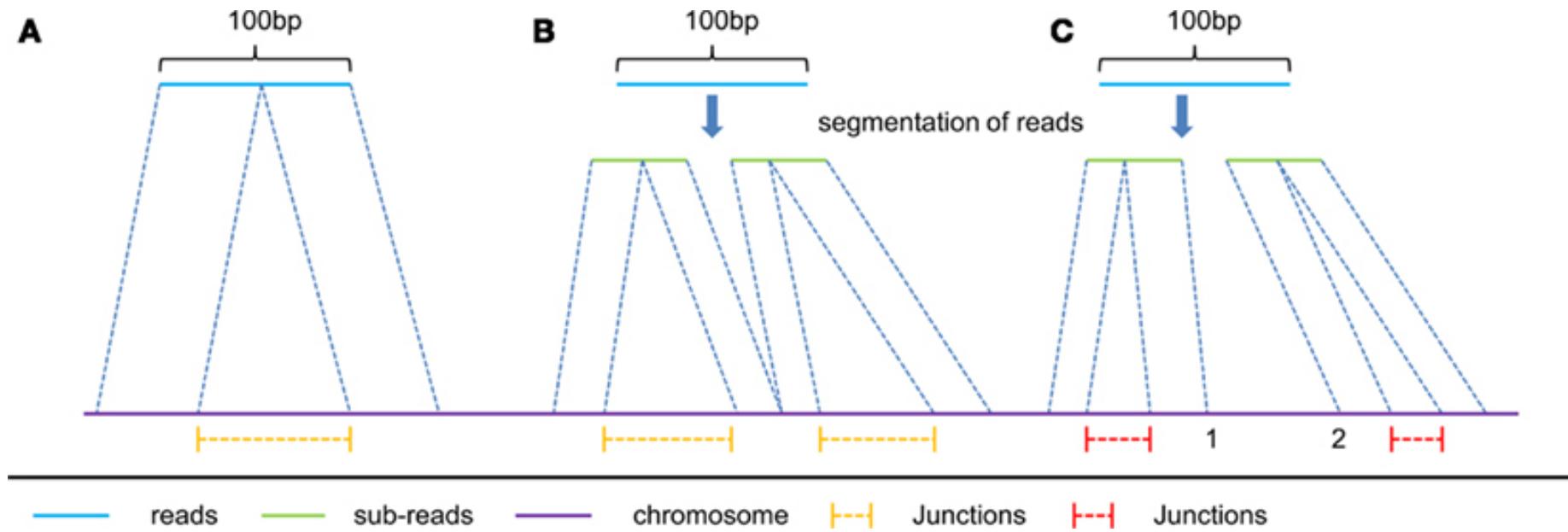
- Given sequence data,
 - Assembly seeks to put together the puzzle without knowing what the picture is
 - Mapping tries to put together the puzzle pieces directly onto an image of the picture
- In mapping the question is more, given a small chunk of sequence, where in the genome did this piece most likely come from.
- The goal then is to find the match(es) with either the “best” edit distance (smallest), or all matches with edit distance less than max edit dist. Main issues are:
 - Large search space
 - Regions of similarity (aka repeats)
 - Gaps (INDELS)
 - Complexity (RNA, transcripts)

Consideration

- Placing reads in regions that do not exist in the reference genome (reads extend off the end) [mitochondrial, plasmids, structural variants, etc.].
- Sequencing errors and variations: alignment between read and true source in genome may have more differences than alignment with some other copy of repeat.
- What if the closest fully sequenced genome is too divergent? (3% is a common alignment capability)
- Placing reads in repetitive regions: Some algorithms only return 1 mapping; If multiple: map quality = 0
- Algorithms that use paired-end information => might prefer correct distance over correct alignment.

Intron/exon junctions

- In RNA-seq data, you must also consider effect of splice junctions, reads may span an intron



Some Aligners

- Spliced Aligners
 - STAR
 - HiSAT2 (formerly Tophat [Bowtie2])
 - GMAP - GSNAp
 - SOAPsplice
 - MapSplice
- Aligners that can 'clip'
 - bwa-mem
 - Bowtie2 in local mode

https://en.wikipedia.org/wiki/List_of_sequence_alignment_software

Genome vs Transcriptome

- May seem intuitive to map RNAseq data to transcriptome, but it is not that simple
 - Transcriptomes are rarely complete
 - Which transcript of a gene, canonical transcripts? Shouldn't map to all splice variants as these would show up as 'multi-mappers'.
- More so, a mapper will to its damndest to map every read, somewhere, provided the result meets its minimum requirements.
 - Need to provide a mapper with all possible places the read could have arisen from, which is best represented by the genome. Otherwise you get mis-mapping because its close enough.

Genome and Annotation

Genome sequence fasta files and annotation (gff, gtf) files go together! These should be identified at the beginning of analysis.

- Genome fasta files should include all primary chromosomes, unplaced sequences and un-localized sequences, as well as any organelles. Should not contain any contigs that represent patches, or alternative haplotypes.
- If you expect contamination, or the presence of additional sequence/genome, add the sequence(s) to the genome fasta file.
- Annotation file should be GTF (preferred), and should be the most comprehensive you can find.
 - Chromosome names in the GTF must match those in the fasta file (they don't always do).
 - Star recommends the Genecode annotations for mouse/human

Pseudo-aligners (salmon and kalisto)

- Quasi-mapping
- Probabilistic
- Map to transcripts, not genome
- Does transcript quantifications (or gene)
- Blazing FAST and can run on most laptops
- Experience suggests differences between “traditional” mappers are in the low abundance genes.

Preparing a sam file for counting and stats

- Samtools is used to manipulate mapping files for counting, common steps include:
 - samtools view [to convert from sam to bam]
 - samtools sort [possibly by read and not by position]
 - samtools index
 - samtools idxstats
 - samtools flagstat
 - samtools stats
- Check with the counting application as to its input requirements.

QA/QC

- Mapper produce summary statistics, view the summary report (in a text editor) and compare across samples.
 - Other additional summary statistics can be produced with:
samtools flagstat
samtools idxstats
samtools stats
- Produce a multi-dimensional scaling (MDS) plots of the summary files, the purpose is to look for patterns in the plot that are non-random, and may be influenced by technical artifacts

Estimating Gene Abundance

So you want to do a:

RNASeq Experiment – Differential Expression Analysis

Counting as a measure of expression

- The more you can count (and HTS sequencing systems can count a lot) the better the measure of copy number for even rare transcripts in a population.
 - Most RNA-seq techniques deal with count data. Reads are mapped to a reference genome, transcripts are detected, and the number of reads that map to a transcript (or gene) are counted.
 - Read counts for a transcript are roughly proportional to the gene's length and transcript abundance.
- Technical artifacts should be considered during counting
 - Mapping quality
 - Map-ability (uniqueness), the read is not ambiguous
- Options are (HTSEQ STAR, featureCounts)

Read Counting with HTSEQ

Problem:

- Given a sam/bam file with aligned sequence reads and a list of genomic feature (genes locations), we wish to count the number of reads (fragments) than overlap each feature.
 - Features are defined by intervals, they have a start and stop position on a chromosome.
 - For this workshop and analysis, features are genes which are the union of all its exons. You could consider each exon as a feature, for alternative splicing.
- Htseq-count has three overlapping modes
 - union:
 - intersection-strict
 - intersection-nonempty

Htseq-count (and STAR)

	union	intersection _strict	intersection _nonempty
 A single red bar representing gene_A is overlaid by a single teal bar representing a read.	gene_A	gene_A	gene_A
 A single red bar representing gene_A is overlaid by a teal bar representing a read that starts within the gene.	gene_A	no_feature	gene_A
 Two red bars representing genes A and B are overlaid by a teal bar representing a read that spans both genes.	gene_A	no_feature	gene_A
 Two red bars representing genes A and B are overlaid by two teal bars representing two separate reads, one for each gene.	gene_A	gene_A	gene_A
 Two red bars representing genes A and B are overlaid by a single teal bar representing a read that spans both genes.	gene_A	gene_A	gene_A
 Two red bars representing genes A and B are overlaid by a single teal bar representing a read that spans both genes.	ambiguous	gene_A	gene_A
 Two red bars representing genes A and B are overlaid by a single teal bar representing a read that spans both genes.	ambiguous	ambiguous	ambiguous

Counting genes – STAR

- Counts coincide with HTSeq-counts under default parameters (union and test all orientations). Need to specify GTF file at genome generation step or during mapping.
- Output, 4 columns
 - GenID
 - Counts for unstranded
 - Counts for first read strand
 - Counts for second read strand
- Choose the column that makes sense and generate a matrix table, columns are samples, rows are genes.

QA/QC

- View summary report (in a text editor)
- Produce a multi-dimensional scaling (MDS) plots of the summary files, the purpose is to look for patterns in the plot that are non-random, and may be influenced by technical means.
- Statistics such as:
 - % multi-mapped reads
 - % Uniquely mapped reads
 - Splice sites
 - Unmapped
 - Chimeric
 - Etc.

Differential Expression Analysis

So you want to do a:

RNASeq Experiment – Differential Expression Analysis

Differential Expression Analysis

- Differential Expression between conditions is determined from count data, which is modeled by a distribution (ie. Negative Binomial Distribution, Poisson, etc.)
- Generally speaking differential expression analysis is performed in a very similar manner to DNA microarrays, once and normalization have been performed.
- A lot of RNA-seq analysis has been done in R and so there are many packages available to analyze and view this data. Two of the best are:
 - DESeq, developed by Simon Anders (also created htseq) in Wolfgang Huber's group at EMBL
 - edgeR (extension to Limma [microarrays] for RNA-seq), developed out of Gordon Smyth's group from the Walter and Eliza Hall Institute of Medical Research in Australia
 - http://bioconductor.org/packages/release/BiocViews.html#_RNASeq

Basic steps procedure – EdgeR/limma voom

- Read count data into R
- Filter genes (uninteresting genes, e.g. unexpressed)
- Calculate normalizing factors (sample-specific adjustments)
- Calculate dispersion (gene-gene variance-stabilizing transformation)
- Fit a statistical model to your experiment
- Perform likelihood ratio tests on comparisons of interest (using contrasts)
- Adjust for multiple testing, Benjamini-Hochberg (BH) or q-value common
- Check results for confidence
- Attach annotation if available and write tables

Filtering genes

- Most common filter is to *remove* genes that are less than X normalized read counts (cpm) across a certain number of samples. Ex: `rowSums(cpms <=1) < 3`, require at least 1 cpm in at least 3 samples to keep.
- A second less used filter is for genes with minimum variance across all samples, so if a gene isn't changing (constant expression) its inherently not interesting therefor no need to test

NORMALIZATION

- In differential expression analysis, only sample-specific effects need to be normalized, NOT concerned with comparisons and quantification of absolute expression.
 - Sequence depth – is a sample specific effect and needs to be adjusted for.
 - RNA composition - finding a set of scaling factors for the library sizes that minimize the log-fold changes between the samples for most genes (uses a trimmed mean of M-values between each pair of sample)
 - GC content – is NOT sample-specific (except when it is)
 - Gene Length – is NOT sample-specific (except when it is)
- Normalization in edgeR/voom is model-based, you calculate normalization factors using the function calcNormFactors which by default uses TMM (trimmed means of M values). **Assumes most genes are not DE.**

RPKM vs FPKM vs model based

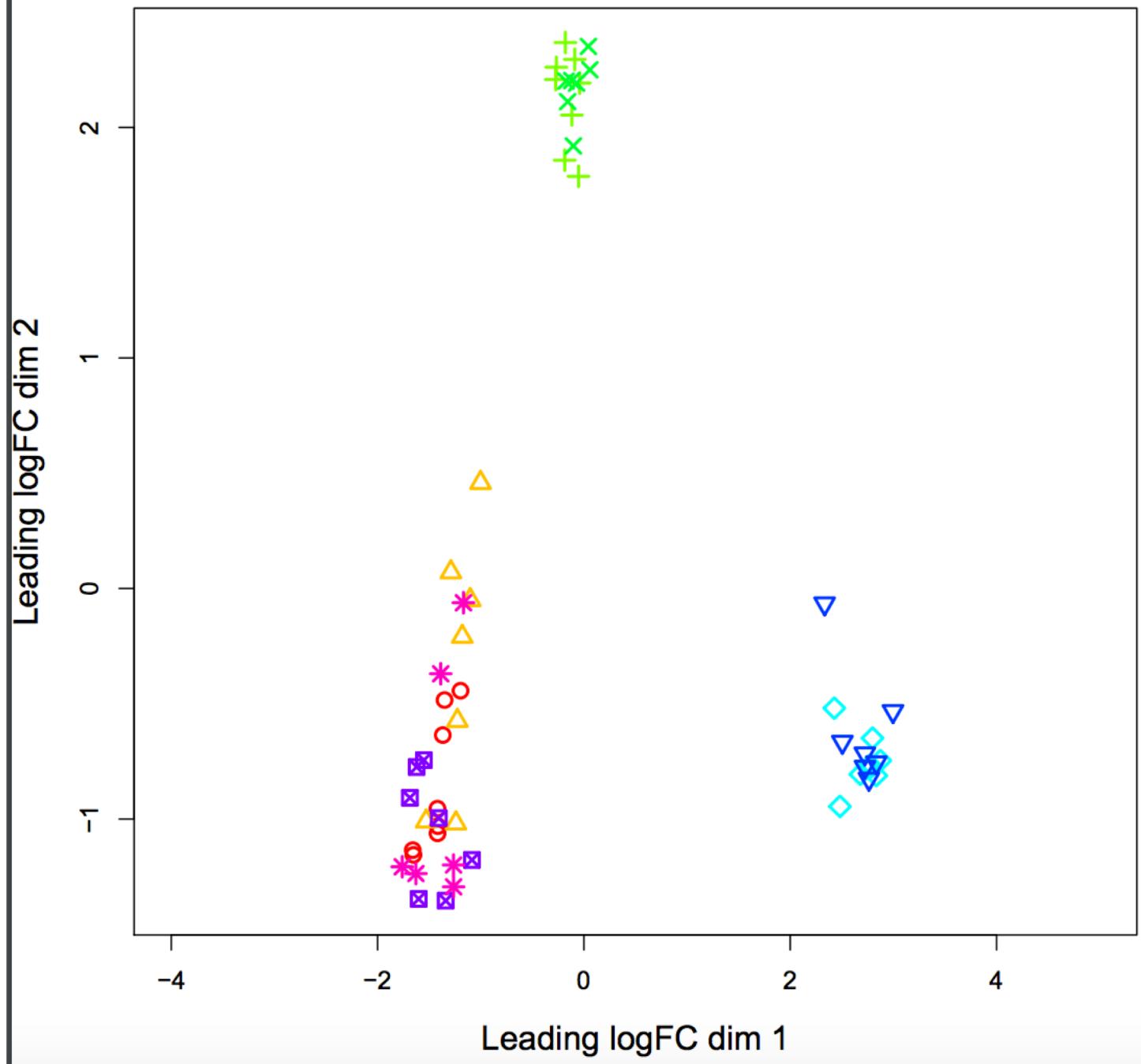
- RPKM - Reads per kilobase per million mapped reads
- FPKM - Fragments per kilobase per million mapped reads
- logCPM – log Counts per million [good for producing MDS plots, estimate of normalized values in model based]
- Model based - original read counts are not themselves transformed, but rather correction factors are used in the DE model itself.

Transformation

- Transformation turn the gene count data into a distribution more suitable for statistical analysis (more “normal” like).
- Most common:
 - Limma- trended transformation, logCPM, best when total counts per sample are relatively close to each other (~3-fold).
 - Voom is best used when the library sizes are quite variable across samples.

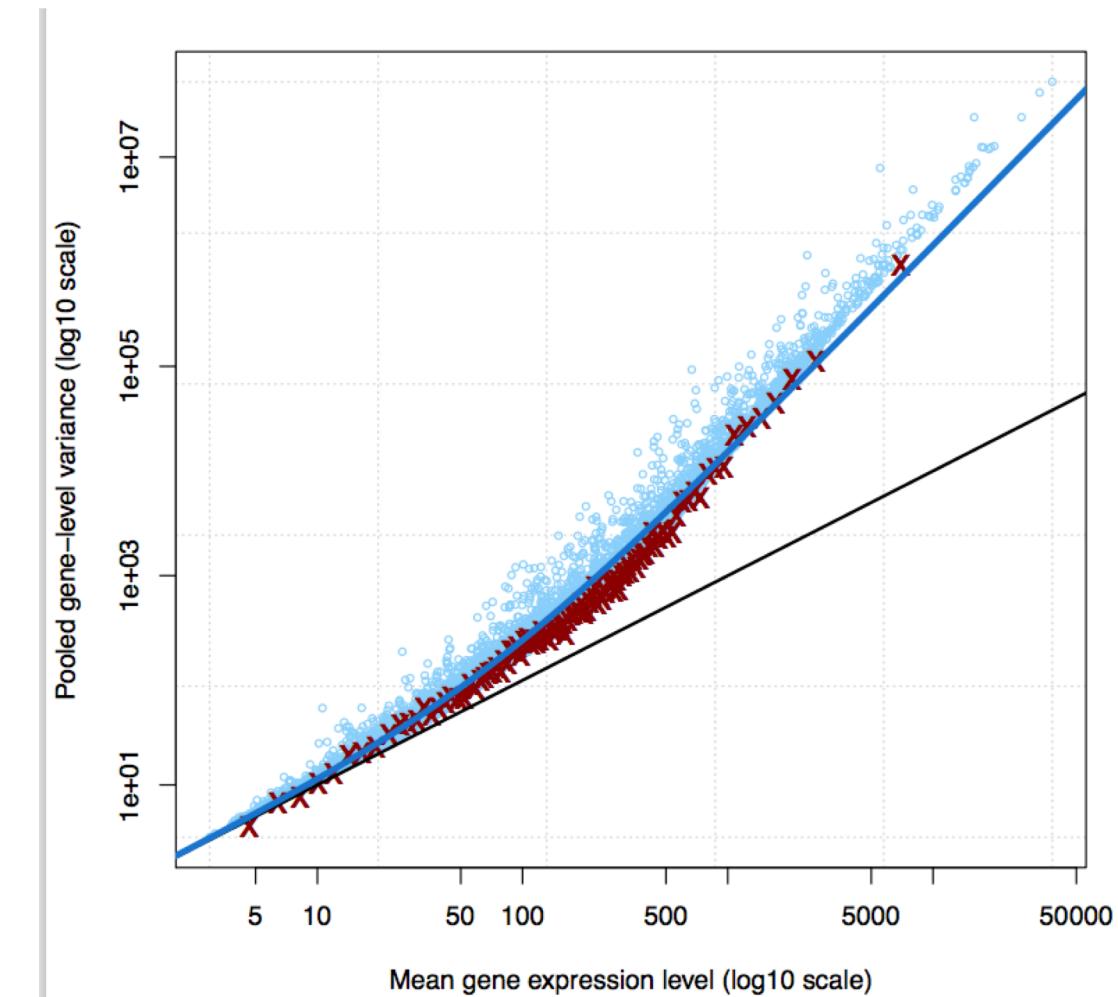
QA/QC

- MDS plots of logCPM



Variance Stabilization - eBayes

- The variance characteristics of low expressed genes are different from high expressed genes, if treated the same, the effect is to over represent low expressed genes in the DE list.



Multiple testing correction

- Simply a must! Best choices are:
 - FDR (false discovery rate), such as BH.
 - Qvalue
- The FDR (or qvalue) is a statement about the list and is no longer about the gene (pvalue). So a FDR of 0.05, says you expect 5% false positives in the list of genes with an FDR of 0.05 or less.
- The statement “Statistically significant” **means** FDR of 0.05 or less.
 - My opinion is these genes **do not** require further validation (e.g. with qrtPCR)
 - You can dip below the FDR of 0.05, but in my opinion you then need to validate those genes (e.g. with qrtPCR)

Manual

- Both edgeR and limma voom have VERY comprehensive user manuals
 - Limma voom
<https://bioconductor.org/packages/release/bioc/vignettes/limma/inst/doc/usersguide.pdf>
 - edgeR
<http://bioconductor.org/packages/release/bioc/vignettes/edgeR/inst/doc/edgeRUsersGuide.pdf>

Summarization and Visualization

So you want to do a:

RNASeq Experiment – Differential Expression Analysis

The Top Table

- The basic table
 - Gene_ID: The Gene Id from the GTF file
 - logFC: log fold change, positive values indicate up-regulation, negative numbers indicate down-regulation
 - logCPM: log counts per million, average ‘expression’ value of the gene
 - LR: likelihood ratio of the test (ignore)
 - Pvalue: raw p-value for that gene (best to sort on)
 - FDR: false discover rate for that gene
- Annotation is added in additional columns.

Visualization and Next step tools

Visualization

1. Integrated Genome Viewer (<https://www.broadinstitute.org/igv/>)

Further Annotation of Genes

1. DAVID (<http://david.abcc.ncifcrf.gov/tools.jsp>)
2. ConsensusPathdb (<http://cpdb.molgen.mpg.de/>)
3. NetGestalt (<http://www.netgestalt.org/>)
4. Molecular Signatures Database (<http://www.netgestalt.org/>)
5. PANTHER (<http://www.pantherdb.org/>)
6. Cognoscente (<http://vanburenlab.medicine.tamhsc.edu/cognoscente.shtml>)
7. Pathway Commons (<http://www.pathwaycommons.org/>)
8. Reactome (<http://www.reactome.org/>)
9. PathVisio (<http://www.pathvisio.org/>)
10. Moksiskaan (<http://csbi.ltdk.helsinki.fi/moksiskaan/>)
11. Weighted Gene Co-Expression Network Analysis (WGCNA)s
12. More tools in R Bioconductor

Gene Set enrichment analysis (GSEA) And GO/Pathway Enrichment

Gene set enrichment analysis

- A computational method that determines whether an a priori set of genes (e.g. gene ontology group, or pathway) shows statistically significant, concordant differences between two biological states (e.g. phenotypes)

Gene Ontology/Pathways enrichment analysis

- Given a set of genes that are up-regulated, which gene ontologies or pathways are over-represented (or under-represented) using annotations for that gene set.

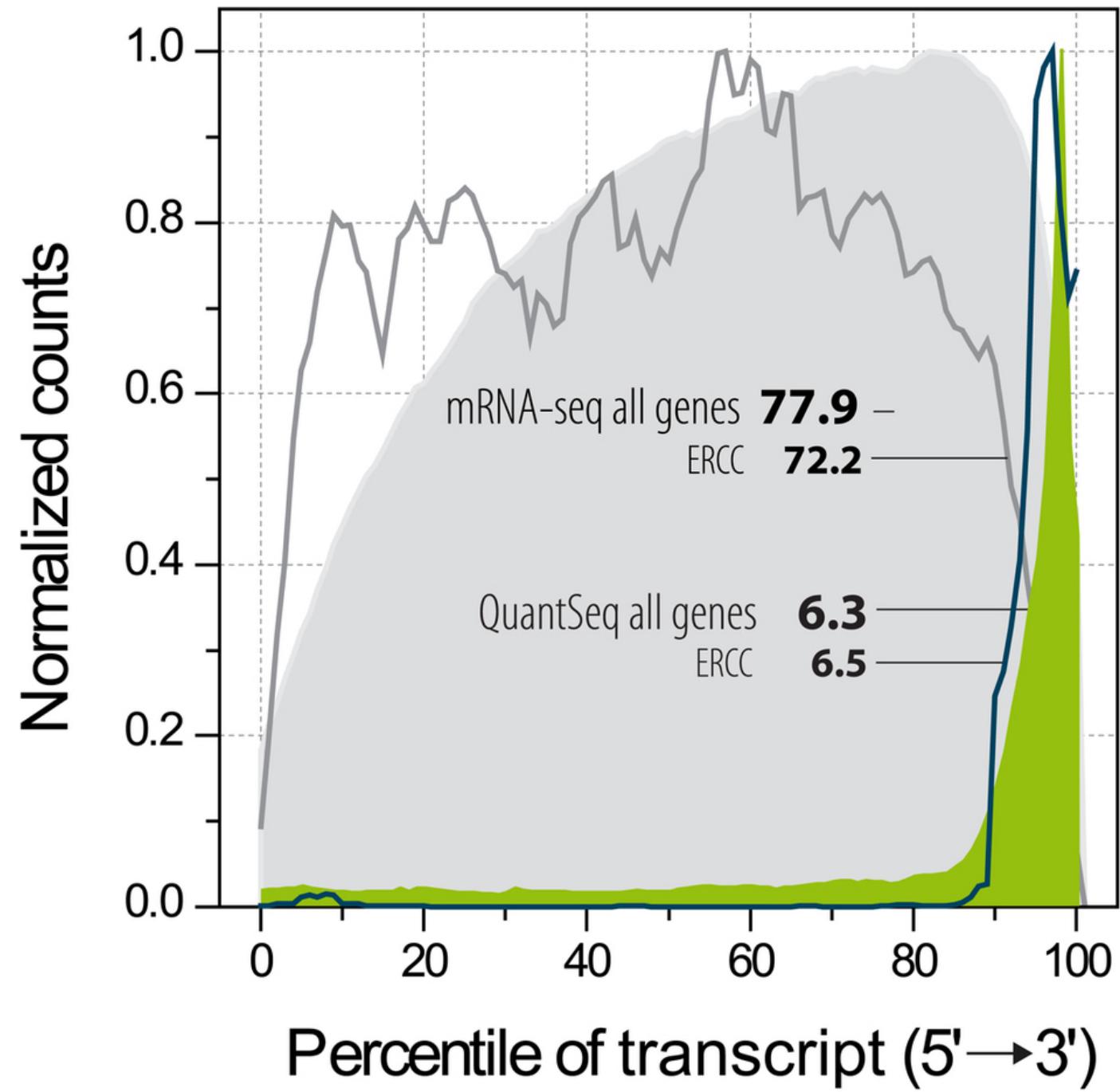
Tag-seq

So you want to do a:

RNASeq Experiment – Differential Expression Analysis

What is TagSeq

- Generic name for 3' biased transcriptome sequencing
- Standard RNAseq has a bias associated with transcript length
- In TagSeq methods one transcript -> one read
- Interested in Differential Gene Expression AND not splice variation, transcript sequence, etc.



Protocol

Reverse transcription
(oligo-dT priming)



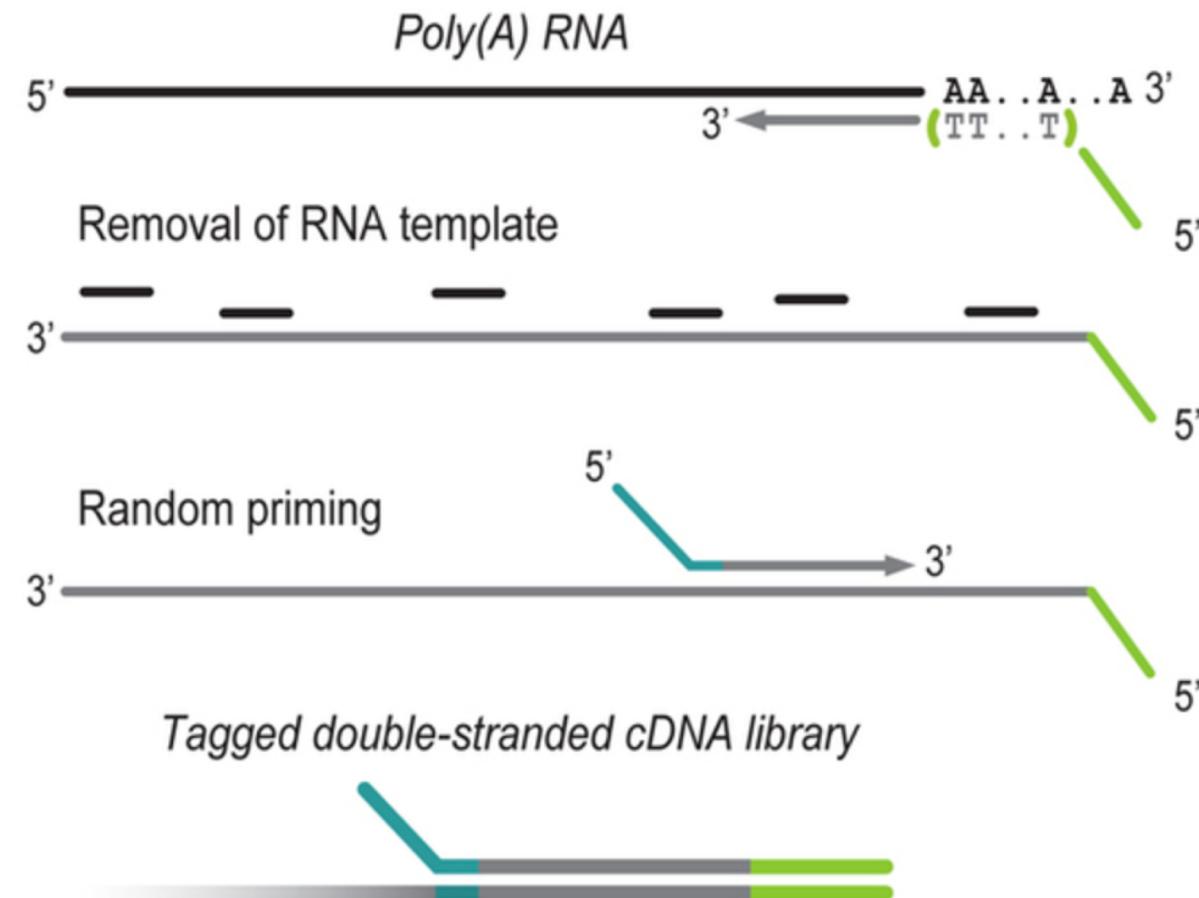
RNA removal



Second-strand
synthesis



Purification



Benefits

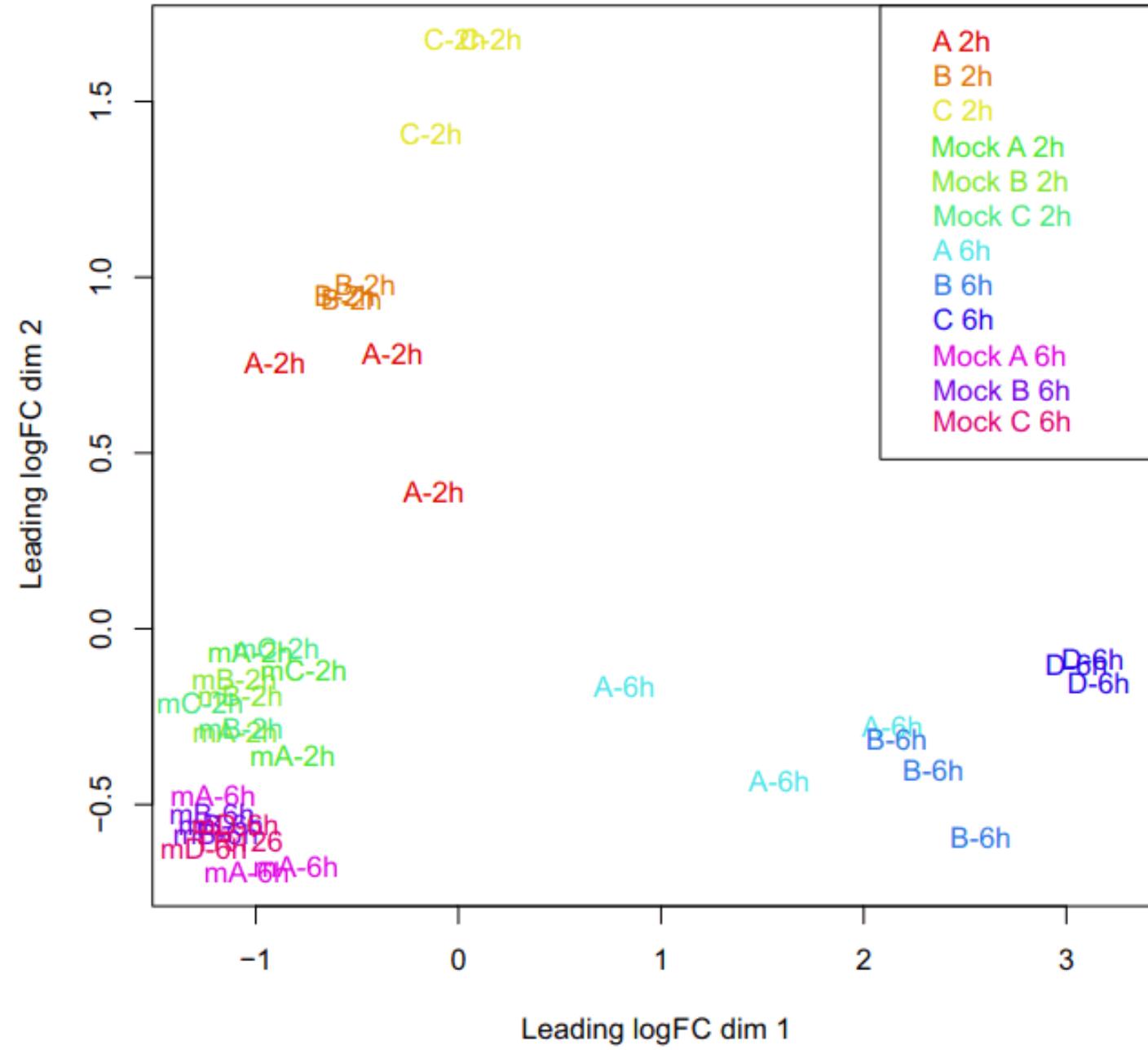
- Low input and low quality samples (say as low as 100pg)
- Faster library preparation protocol, means cheaper libraries
- Strand specific
- Can pinpoint 3' to polyA junctions and obtain accurate information about the 3' UTR (REV kit)
- Best suited for gene counting
- Significant cost saving through multiplexing, need less sequencing per sample, comparable to microarrays
- Single read sequencing is sufficient

Disadvantages

- Data does not contain any transcript splicing information
- Requires (sort of) a reference genome with good annotation (plus known UTRs)
- Only applicable to Eukaryotic samples (requires polyA)
- protocol is a (a bit) more sensitive to chemical contaminants (spin column cleaned RNA samples are recommended)

Bioinformatics

- When sequencing only the interior (non-polyA) read
 - Need to trim off the first 11bp that represents the random primer
 - Align with STAR, which will soft clip off any potential adapter and span any intron-exon gap you may land in
- When sequencing pairs, can follow the same protocol as 'normal' RNAseq paired end, but need to also trim that same 11bp, now possibly on read2
- Rest is the same as standard RNAseq



Software

So you want to do a:

RNASeq Experiment – Differential Expression Analysis

Software

Preprocessing:

- Python 2.7
 - Modules: argparse, optparse, distutils
- bowtie2 - contaminant screening
 - <http://bowtie-bio.sourceforge.net/bowtie2/index.shtml>
- Super-Deduper – Identify and remove PCR duplicates
 - <https://github.com/dstreett/Super-Deduper>
- Sickle – Trim low quality regions
 - <https://github.com/dstreett/sickle>
- Scythe – Identify and remove adapters in SE reads
 - <https://github.com/ucdavis-bioinformatics/scythe>
- FLASH2 – Join overlapping reads, identify and remove adapter in PE reads
 - <https://github.com/dstreett/FLASH2>
- HTStream – Complete preprocessing pipeline, supersedes all above
 - <https://github.com/ibest/HTStream>

Software

Mapping:

- Bwa mem – map reads to a reference
 - <http://sourceforge.net/projects/bio-bwa/files/>
- samtools – processing of sam/bam file
 - <http://www.htslib.org/>

Read Counting:

- samtools – processing of sam/bam file
 - <http://www.htslib.org/>
- HTSeq-0.6.1 htseq_count – count reads occurrences within genes
 - <http://www-huber.embl.de/users/anders/HTSeq/>

OR simultaneous read mapping and counting:

- Star
 - <https://github.com/alexdobin/STAR> [performs both alignment and counting]

Software

Analysis of differential expression:

- R <http://www.r-project.org/>
 - R Packages: EdgeR, limma from bioconductor – differential expression analysis
 - <http://bioconductor.org/packages/release/bioc/html/edgeR.html>
 - <http://bioconductor.org/packages/release/bioc/html/limma.html>
 - <https://genomebiology.biomedcentral.com/articles/10.1186/gb-2014-15-2-r29>
 - RStudio
 - <https://www.rstudio.com/>

Files and File Types

So you want to do a:

RNASeq Experiment – Differential Expression Analysis

Sequencing Read files

fasta files

```
>sequence1  
ACCCATGATTGCGA
```

qual files

```
>sequence1  
40 40 39 39 40 39 40 40 40 40 40 20 20 36 39 39
```

fastq files

```
@sequence1  
ACCCATGATTGCGA  
+  
IIHHIHIIII55EHH
```

Quality Scores

$$Q = -10 \log_{10} P$$

Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10000	99.99%

Qscore Conversion

$Q_{sanger} = -10 \log_{10} P$ - based on probability (aka phred)

$Q_{solexa} = -10 \log_{10} \frac{P}{1-P}$ - based on odds

S - Sanger	Phred+33,	raw reads typically (0, 40)
X - Solexa	Solexa+64,	raw reads typically (-5, 40)
I - Illumina 1.3+	Phred+64,	raw reads typically (0, 40)
J - Illumina 1.5+	Phred+64,	raw reads typically (3, 40)
L - Illumina 1.8+	Phred+33,	raw reads typically (0, 41)

Illumina Read naming conventions

CASAVA 1.8 Read IDs

- @EAS139:136:FC706VJ:2:2104:15343:197393 1:Y:18:ATCACG
 - EAS139 the unique instrument name
 - 136 the run id
 - FC706VJ the flowcell id
 - 2 flowcell lane
 - 2104 tile number within the flowcell lane
 - 15343 'x'-coordinate of the cluster within the tile
 - 197393 'y'-coordinate of the cluster within the tile
 - 1 the member of a pair, 1 or 2 (paired-end or mate-pair reads only)
 - Y Y if the read fails filter (read is bad), N otherwise
 - 18 0 when none of the control bits are on, otherwise it is an even number
 - ATCACG index sequence

SAM/BAM Files

- SAM (Sequence Alignment/Map) format = unified format for storing read alignments to a reference sequence(Consistent since Sept. 2011).
 - <http://samtools.github.io/hts-specs/SAMv1.pdf>
 - <http://samtools.github.io/hts-specs/SAMtags.pdf>
- BAM = binary version of SAM for fast querying

SAM/BAM files

SAM files contain two regions

- The header section
 - Each header line begins with character '@' followed by a two-letter record type code
- The alignment section
 - Each alignment line has 11 mandatory fields. These fields always appear in the same order and must be present, but their values can be '0' or '*', if the corresponding information is unavailable, or not applicable.

Sam columns

```
7172283 163 chr9 139389330 60 90M = 139389482 242 TAGGAGG... EHHHHHH...
7705896 83 chr9 139389513 60 90M = 139389512 -91 GCTGGGG... EBCHHFC...
7705896 163 chr9 139389512 60 90M = 139389513 91 AGCTGGG... HHHHHHH...
```

1	QNAME	query template name
2	FLAG	bitwise flag
3	RNAME	reference sequence name
4	POS	1-based leftmost mapping position
5	MAPQ	mapping quality
6	CIGAR	CIGAR string
7	RNEXT	reference name of mate
8	PNEXT	position of mate
9	TLEN	observed template length
10	SEQ	sequence
11	QUAL	ASCII of Phred-scaled base quality

Sam flags

Bit	Description
0x1	template having multiple segments in sequencing
0x2	each segment properly aligned according to the aligner
0x4	segment unmapped
0x8	next segment in the template unmapped
0x10	SEQ being reverse complemented
0x20	SEQ of the next segment in the template being reversed
0x40	the first segment in the template
0x80	the last segment in the template
0x100	secondary alignment
0x200	not passing quality controls
0x400	PCR or optical duplicate
0x800	supplementary alignment

Mapq explained

- MAPQ, contains the "phred-scaled posterior probability that the mapping position" is wrong.
- In a probabilistic view, each read alignment is an estimate of the true alignment and is therefore also a random variable. It can be wrong. The error probability is scaled in the Phred. For example, given 1000 read alignments with mapping quality being 30, one of them will be incorrectly mapped to the wrong location on average.
- A value 255 indicates that the mapping quality is not available.

Mapq explained

- The calculation of mapping qualities is simple, but this simple calculation considers many of the factors below:
 - The repeat structure of the reference. Reads falling in repetitive regions usually get very low mapping quality.
 - The base quality of the read. Low quality means the observed read sequence is possibly wrong, and wrong sequence may lead to a wrong alignment.
 - The sensitivity of the alignment algorithm. The true hit is more likely to be missed by an algorithm with low sensitivity, which also causes mapping errors.
 - Paired end or not. Reads mapped in pairs are more likely to be correct.

Mapq explained

- When you see a read alignment with a mapping quality of 30 or greater, it usually implies:
 - The overall base quality of the read is good.
 - The best alignment has few mismatches.
 - The read has few or just one ‘good’ hit on the reference, which means the current alignment is still the best even if one or two bases are actually mutations, or sequencing errors.

In practice however, each mapper seems to compute the MAPQ in their own way.

Sam cigar

- Compact Idiosyncratic Gapped Alignment Report (CIGAR) SAM flag field:

Op	BAM	Description
M	0	alignment match (can be a sequence match or mismatch)
I	1	insertion to the reference
D	2	deletion from the reference
N	3	skipped region from the reference
S	4	soft clipping (clipped sequences present in SEQ)
H	5	hard clipping (clipped sequences NOT present in SEQ)
P	6	padding (silent deletion from padded reference)
=	7	sequence match
X	8	sequence mismatch

CIGAR Example

	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	2	
Ref Pos:	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0
Reference:	C	C	A	T	A	C	T		G	A	A	C	T	G	A	C	T	A	A	C
Read:					A	C	T	A	G	A	A	T	G	G		C	T			

POS: 5

CIGAR: 3M1I6M1D2M

** mismatches are not considered in standard CIGAR

GFF/GTF files

- The GFF (General Feature Format) format consists of one line per feature, each containing 9 columns of data (fields). The GTF (General Transfer Format) is identical to GFF version 2.
- Fields must be tab-separated and all fields must contain a value; “empty” fields should be denoted with a “.”.
- Columns:
 - Seqname: Name of the sequence chromosome
 - Source: the program, or database, that generated the feature
 - Feature: feature type name, (e.g. gene, exon, cds, etc.)
 - Start: start position of the feature, sequences begin at 1
 - End: stop position of the feature, sequences begin at 1
 - Score: a floating point value (e.g. 0.01)
 - Strand: Defined as ‘+’ (forward),or ‘-’ (reverse)
 - Frame: One of ‘0’, ‘1’, ‘2’, ‘0’ represents the first base of a codon.
 - Attribute: A semicolon-separated list of tag-value pairs, providing additional information about each feature.

GFF/GTF files

Sample GTF output from Ensembl data dump

```
1 transcribed_unprocessed_pseudogene    gene        11869 14409 . + . gene_id "ENSG00000223972"; gene_name "DDX11L1"; ge  
1 processed_transcript                 transcript  11869 14409 . + . gene_id "ENSG00000223972"; transcript_id "ENST000
```

Sample GFF output from Ensembl export:

```

X Ensembl Repeat 2419108 2419128 42 . .
X Ensembl Repeat 2419108 2419410 2502 - .
X Ensembl Repeat 2419108 2419128 0 . .
X Ensembl Pred.trans. 2416676 2418760 450.19 - 2 genscan=GENSCAN00000019335
X Ensembl Variation 2413425 2413425 . +
X Ensembl Variation 2413805 2413805 . +

```