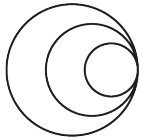


wellcome
connecting
science

Differential Expression using RNA-Seq

Vivek Iyer
Based extensively on slides from Victoria Offord

Wellcome Sanger Institute
7 June 2024

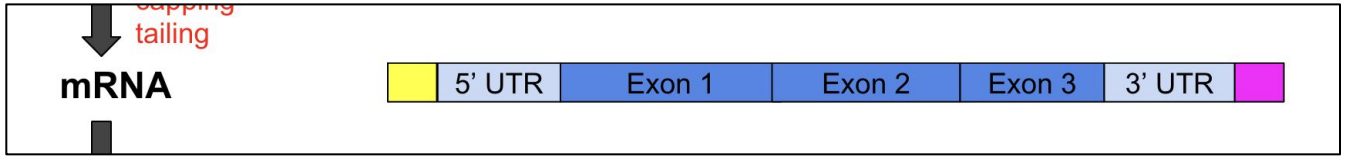
Learning outcomes

By the end of this module and tutorial you can expect to be able to:

- Appreciate the important **aspects of RNASeq experiment design**
- Understand the various technical **steps in RNASeq pipelines**
- **Align RNA-Seq reads** to a reference genome and a transcriptome
- **Visualise transcription data** using standard tools
- **Quantify the expression values** of your transcripts using standard tools
- **Perform QC** of NGS transcriptomic data
- Interpret **differential gene expression** data

This module in context

- A change of modality - sequence mRNA ! (indirectly)



- QC - basic thresholds and PCA
- See some actual stats -
  - Experiments are looking for associations between
    - gene expression (quantitative readout)
    - experimental conditions
  - Some sort of modelling needed
    - We'll see p-values and q-values flying around.
- Once you see these techniques, CRISPR screens make sense too

About me

Background

- PhD Theoretical physics 1996
- Software engineering (consultancy) 1996-2002
- Sanger 2002 - now
  - Java developer (Apollo genome browser)
  - Ensembl gene builder
  - High-throughput Mouse ESCell KO's (EuCOMM/KOMP)
  - Cancer bioinformatics / analysis (WGS, WES, CNV, CRISPR)
  - Human genetics programme informatics team (services to humgen)
    - WES/WGS variant calling and QC
    - RNA and scRNA calling and QC
    - TRE / "Data safe havens"
    - Software and disk space management

Scale + engineering

Lecture outline

- RNA-seq background
- Pipelines
  - Mapping to the genome (HISAT2 and IGV)
  - Mapping to the transcriptome and counting reads (Kallisto)
  - Read count normalisation
  - Differential expression and QC (Sleuth)
- What to do with a gene list
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What is the transcriptome?

"The complete set of transcripts in a cell

and their quantity

for a specific developmental stage or condition"

Wang *et al*. (2009)
Nature Reviews Genetics
(PubMed: 19015660)

What is the transcriptome?

"The complete set of transcripts in a cell

and their quantity

for a specific developmental stage or condition"

It's a snapshot
- Fixed point in time
- Fixed set of conditions

RNASeq - uses NGS technology to measure this

Wang *et al*. (2009)
Nature Reviews Genetics
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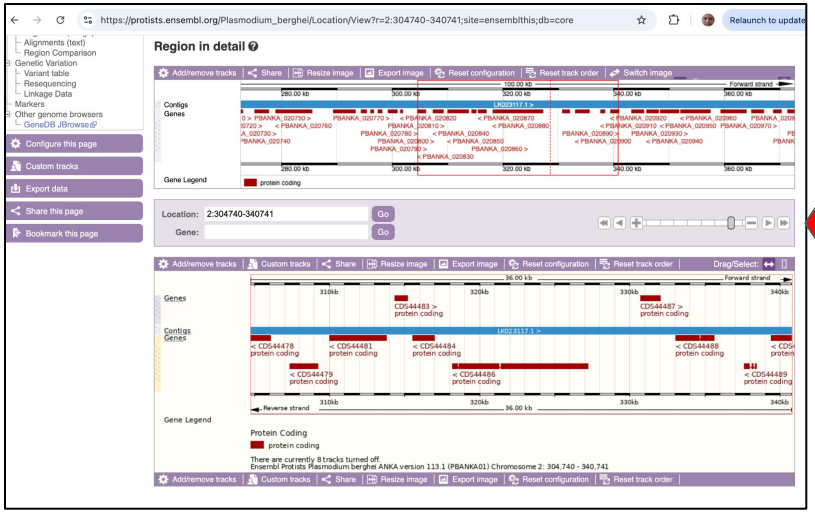
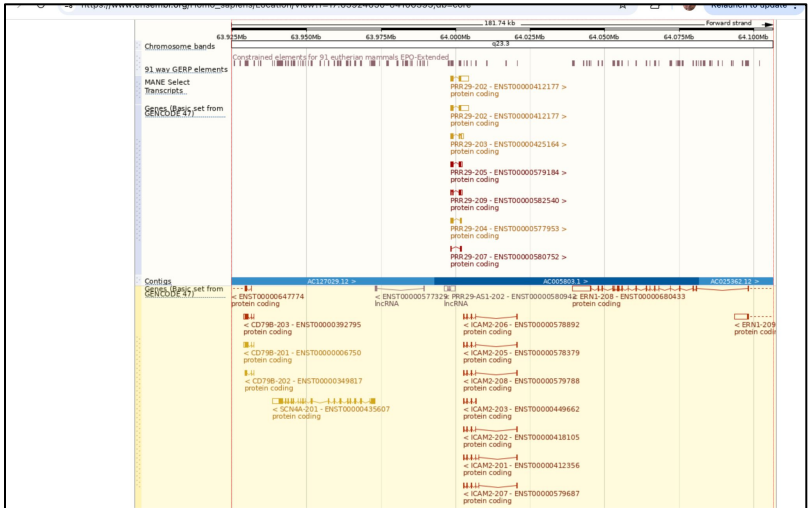


Plasmodium berghei

```
vvi   19254170 20 Oct 19:31 PccAS_v3_genome.fa
vvi   10554131 20 Oct 19:31 PccAS_v3_transcripts.fa
```

Transcriptome (10M) ~ 50% of genome (20M)
Exons are big cf introns / intergenic



Homo Sapiens

3.0 G GRCh38_15/Homo_sapiens.GRCh38_15.fa

434 M Homo_sapiens.GRCh38.cdna.all.fa

Transcriptome ~ 10% of genome
Exons are *small* compared to introns / intergenic

Central dogma

DNA

transcription

Pre-mRNA

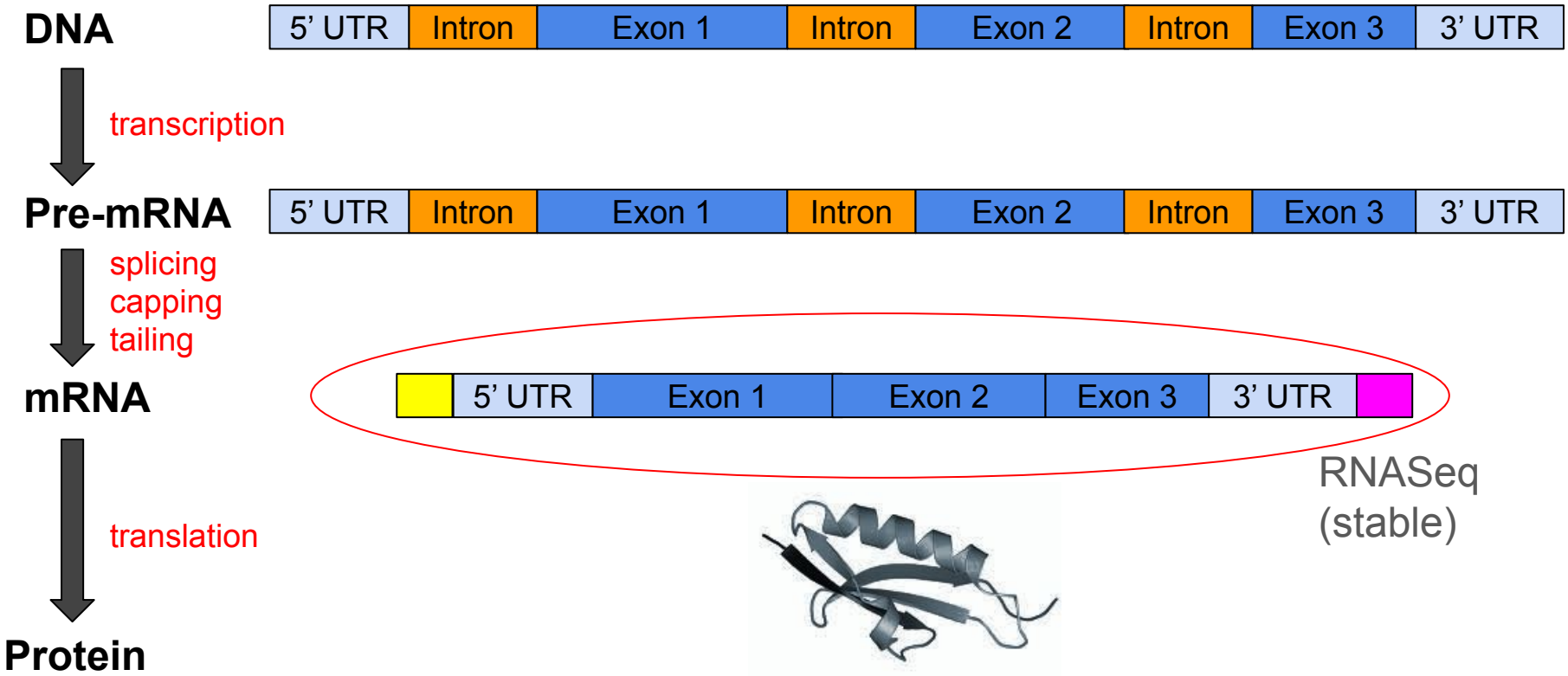
splicing
capping
tailing

mRNA

translation

Protein

Central dogma



Central dogma

DNA

transcription

Pre-mRNA

splicing
capping
tailing

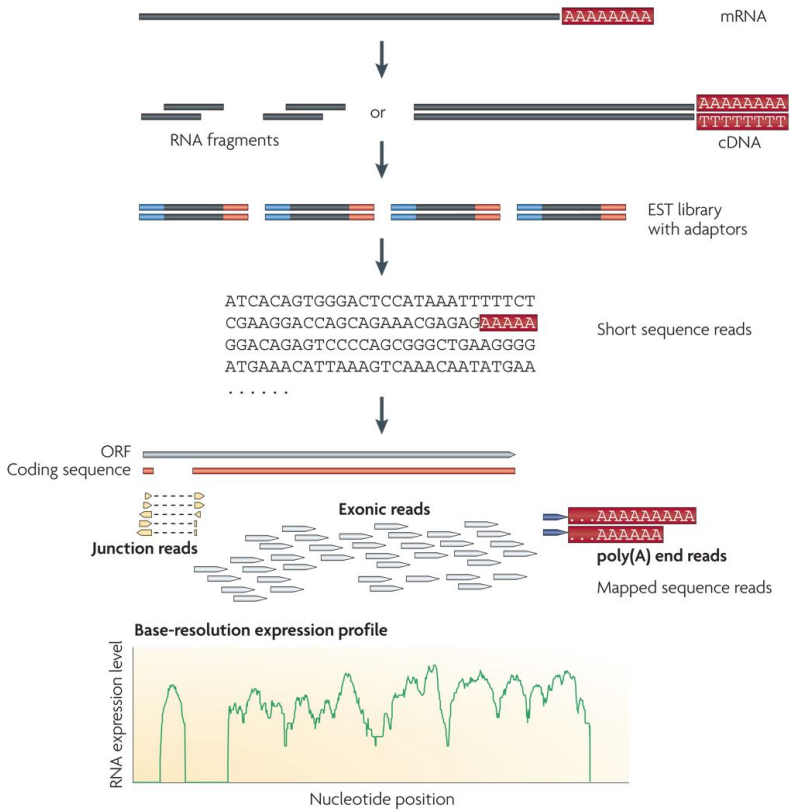
mRNA

translation

Protein

Harder to measure

RNA Sequencing



- Convert to cDNA
- Fragmented
- Adapters
- sequenced

- Direct sequencing of mRNA via long-read

Wang *et al*. (2009)
Nature Reviews Genetics
(PubMed: 19015660)

Experimental design

- Successful RNA-Seq studies start with a good study design
- Considerations for generating data to answer your biological question include:
    - library prep and type
    - sequencing depth
    - number of replicates

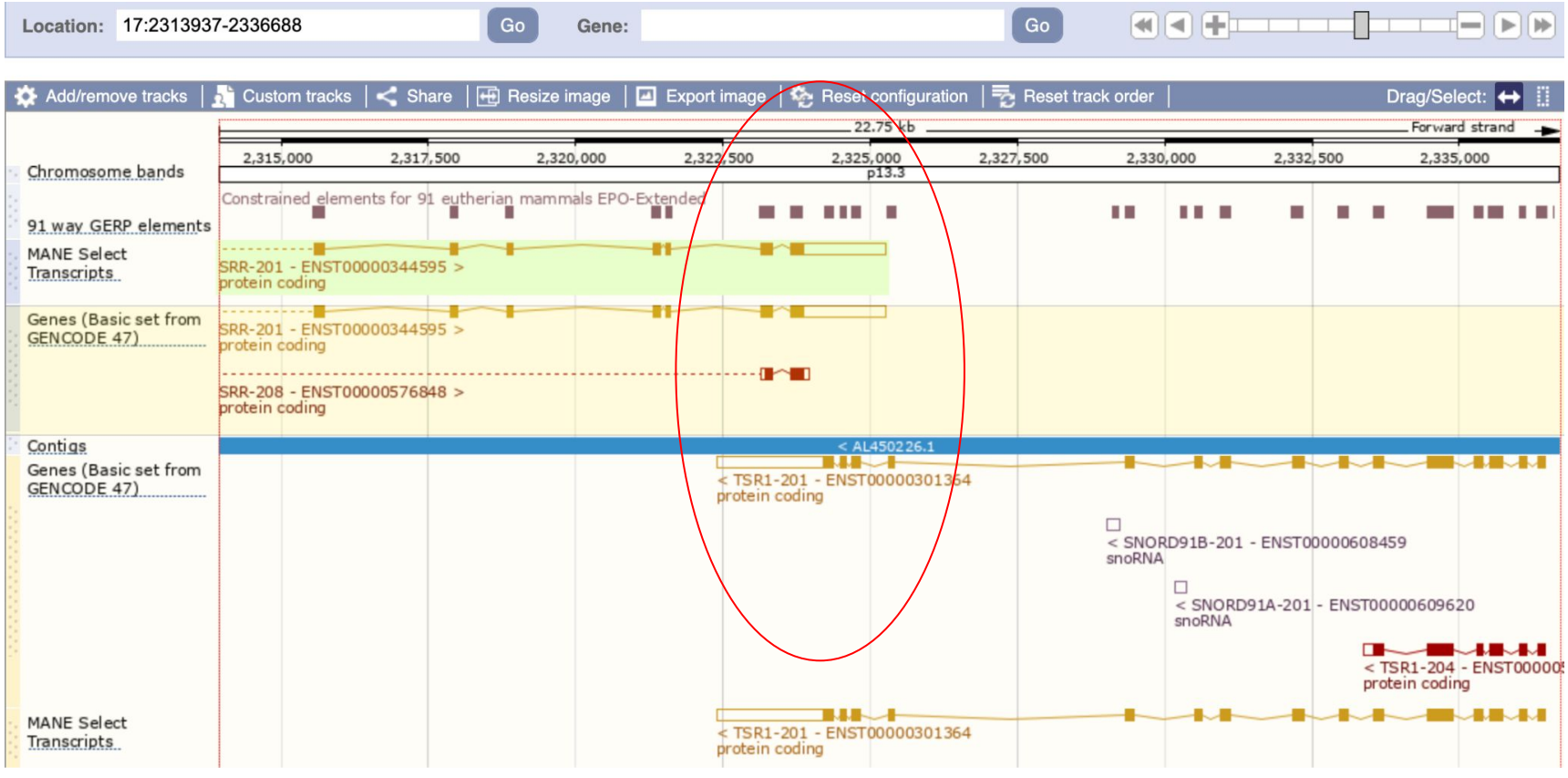
Experimental design - library preparation

- Total RNA = mRNA + **rRNA** + tRNA + regulatory RNAs...
- Ribosomal RNA can represent > 90% total RNA
- We need to enrich for the 1-2% mRNA *OR* deplete rRNA
  - enrichment typically needs good RIN and high RNA proportion
  - some samples (e.g. tissue biopsies) may not be suitable
  - bacterial mRNA not polyadenylated -> ribosomal depletion
- Be aware of protocol being used (e.g. some will remove small RNAs)

Experimental design - library type

- **Stranded vs unstranded**
  - strand-specific protocols better for detangling antisense or overlapping transcripts
- **Single or paired end**
  - paired end better for *de novo* transcript discovery or isoform expression analysis
  - < 55% reads will span 2 or more exons

SRR 3’UTR overlaps TSR1 3’UTR + coding



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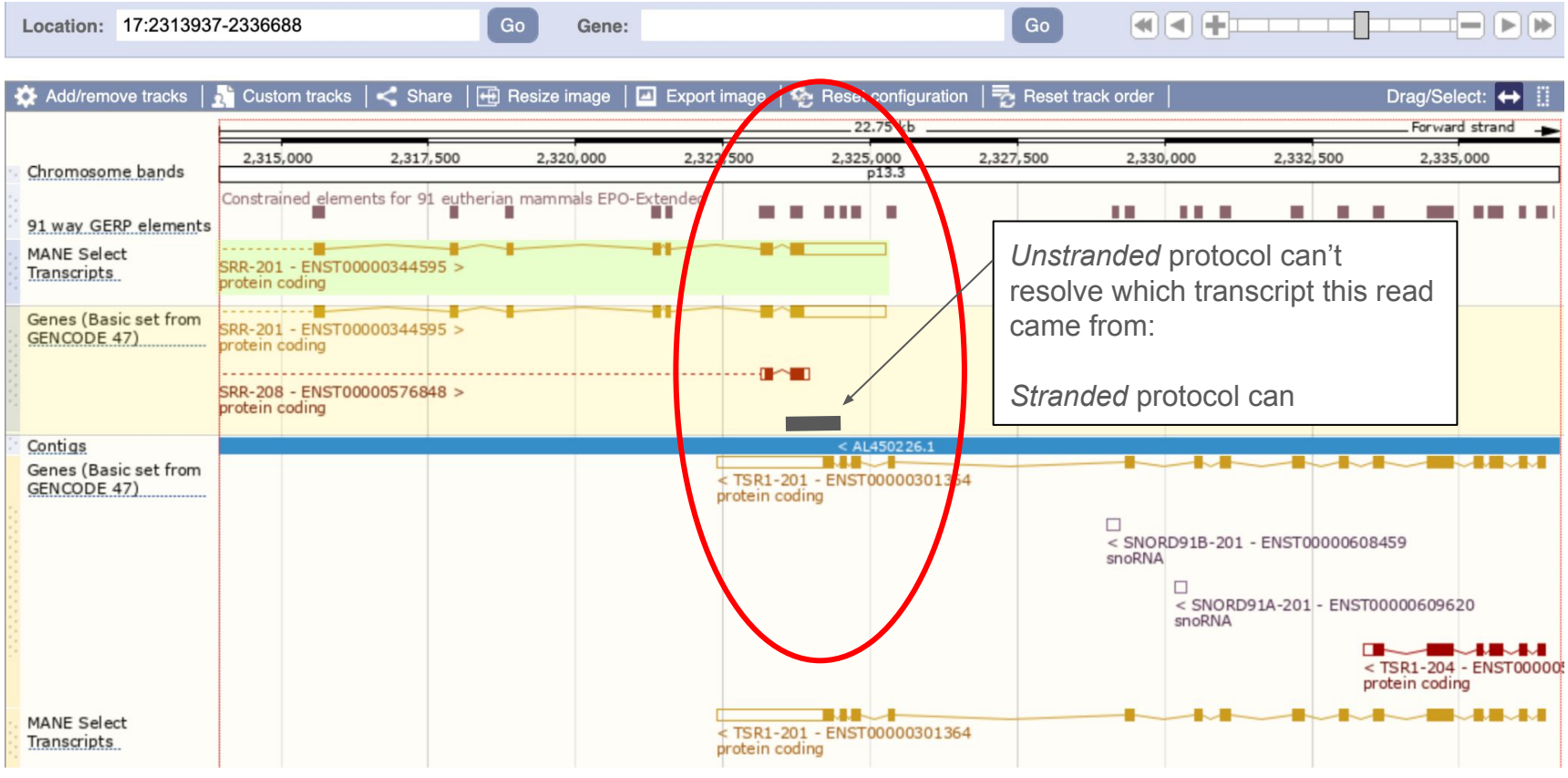

Experimental design - replicates

Biological replicates

- biologically distinct samples
- same type of organism treated or grown in the same condition
- understand biological variation (e.g. variation between individuals)
- relevant biological replicates are required

Technical replicates

- repeated measurements of the same sample
- understand the variation in equipment or protocols
- technical replicates are not generally required, but try to arrange samples on plates to minimise potential problems (***some packages adjust based on these "spike-ins"***)

Experimental design - sequencing depth

Sequencing depth: **encodeproject.org**

- 100bp Paired End, Human transcriptome: 30 million reads
- Novel transcripts, rare isoforms: 50-100 million reads

Next: this is how I think sequencing depth …

Experimental design - sequencing depth

This is how I think about it

- 30 million x 100bp PE ~ 3x10^7 x 100 ~ 3 x 10^9 bases

- Transcriptome ~ 150 Mbp ~ 1.5 x 10^8 bases => **“Coverage” ~ 20x** (=30/1.5)

Experimental design - sequencing depth

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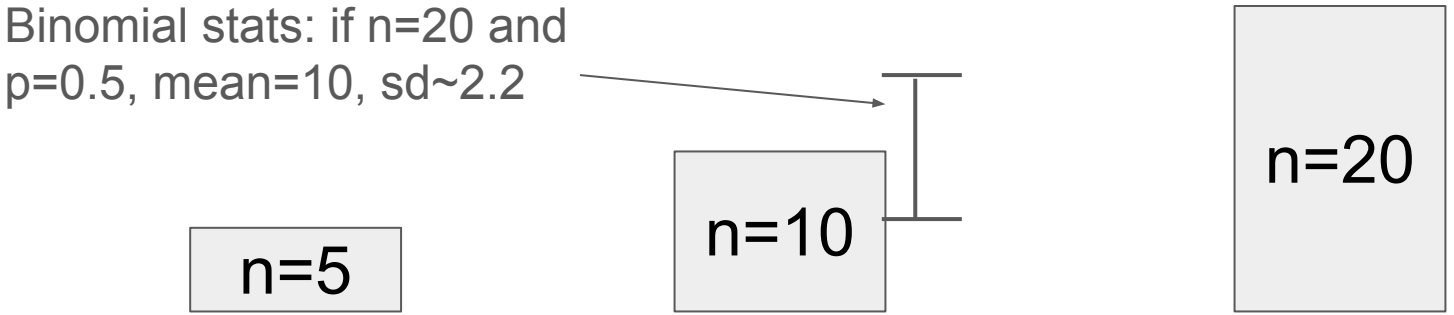
- **This means you can "reasonably" tell apart : full / half / quarter**



Experimental design - replicates

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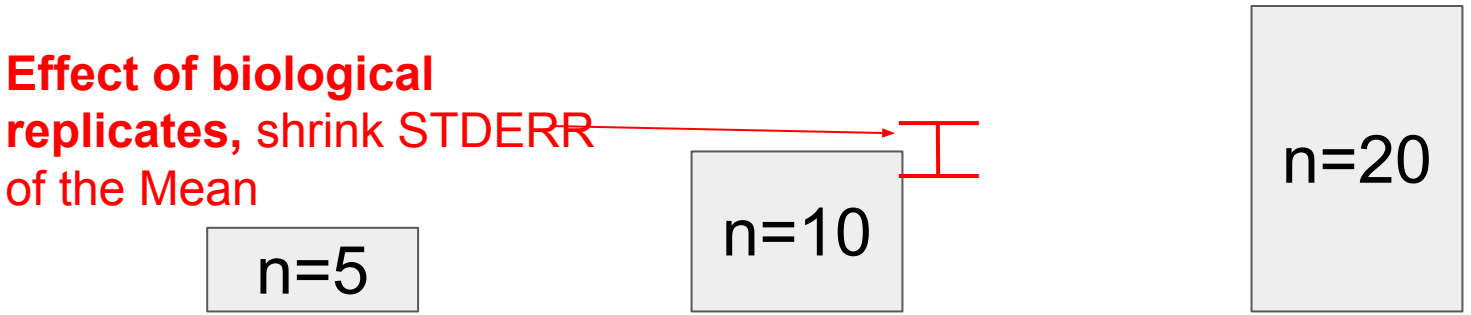


Experimental design - sequencing depth *vs* replicates

- Increasing sequencing depth can increase the ability to detect low expression transcripts (i.e. increases ability to detect DE genes)
    - Returns diminish beyond a certain sequencing depth
- Increasing biological replicates increases the **accuracy of logFC** and absolute expression levels (particularly in low expression transcripts)

Experimental design - sequencing depth / replicates

BIOINFORMATICS **DISCOVERY NOTE**

Vol. 30 no. 3 2014, pages 301–304
doi:10.1093/bioinformatics/btt688

Gene expression

Advance Access publication December 6, 2013

RNA-seq differential expression studies: more sequence or more replication?

Yuwen Liu[1,2], Jie Zhou[1,3] and Kevin P. White[1,2,3,*]

[1]Institute of Genomics and Systems Biology, [2]Committee on Development, Regeneration, and Stem Cell Biology and [3]Department of Human Genetics, University of Chicago, Chicago, IL 60637, USA

Associate Editor: Janet Kelso

Experimental design - sequencing depth / replicates

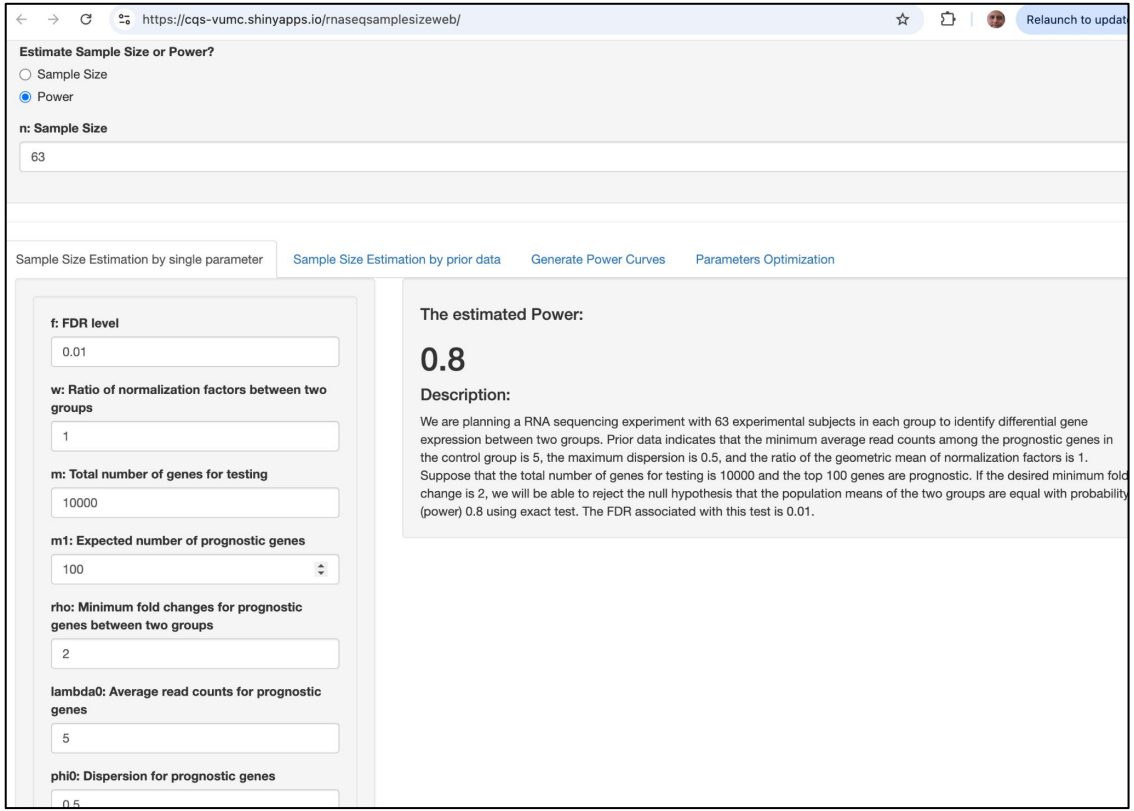
Use a power calculator to estimate sample size!

https://cqs-vumc.shinyapps.io/rnaseqsamplesizeweb/

Experimental design - sequencing depth / replicates

Plug in

- **Sample Size**
- FDR level
- Total number of genes
- *Expected variable genes*
- *Minimum FC between groups*
- **Average read counts**
- Dispersion for prognostic genes



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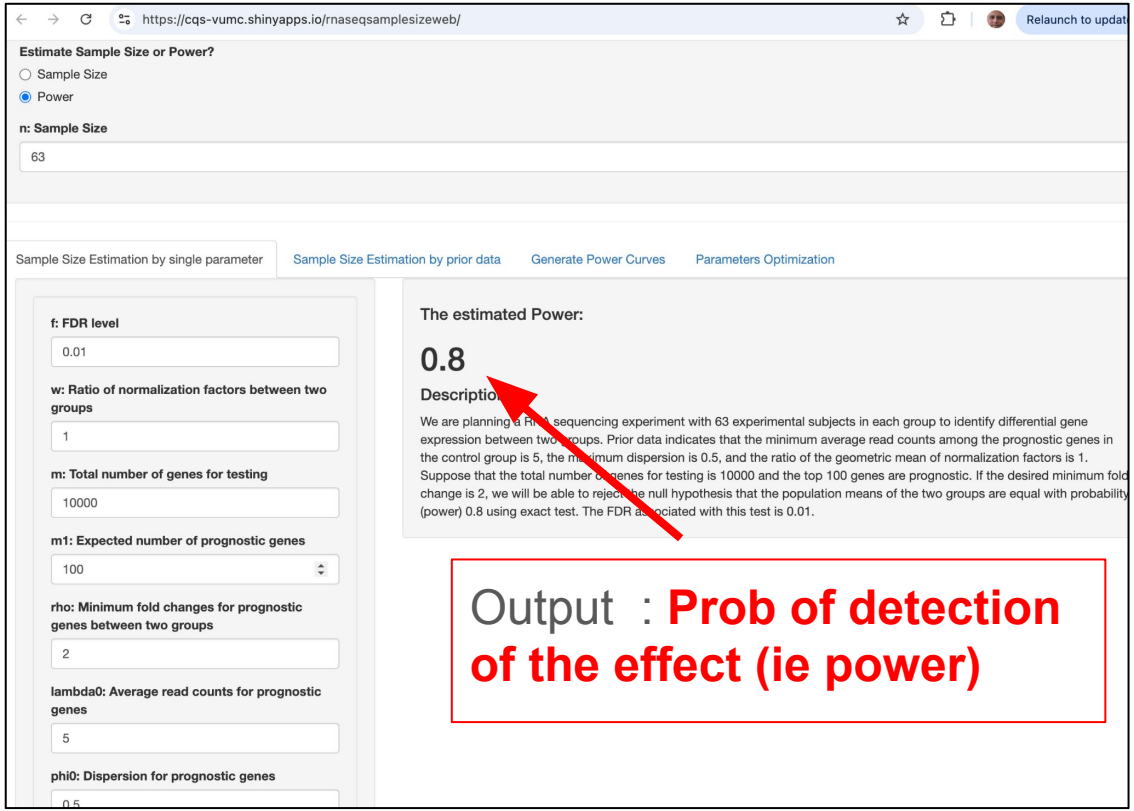

Output : **Prob of detection of the effect (ie power)**

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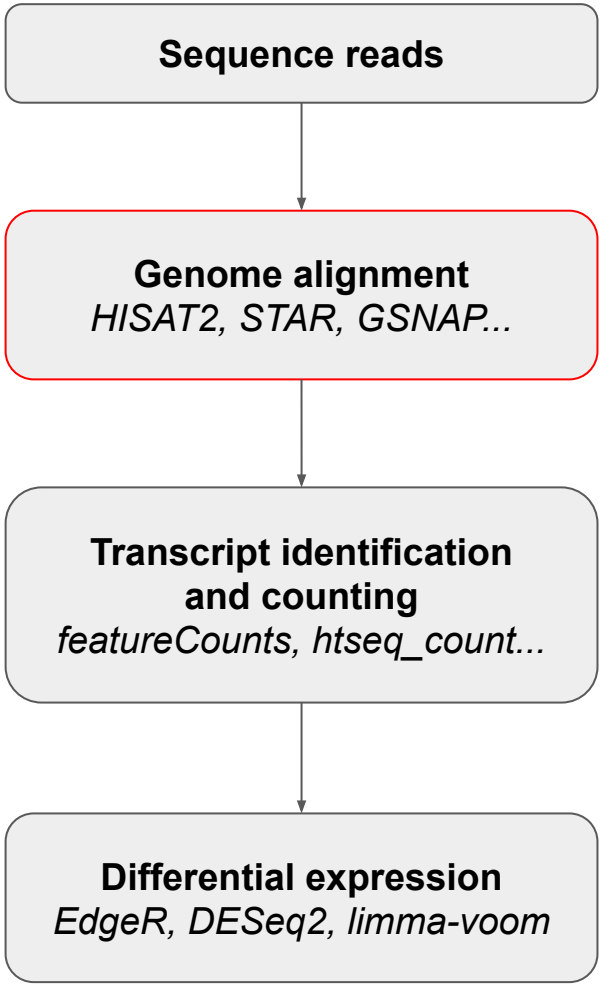
Key steps in any pipeline

1. Which genes/transcripts do our reads belong to? **mapping / assembly**
2. How many reads align to a specific gene/transcript? **quantification**
3. Do different sample groups express genes/transcripts differently? **DGE analysis**

No universal pipeline to cover every analysis!!!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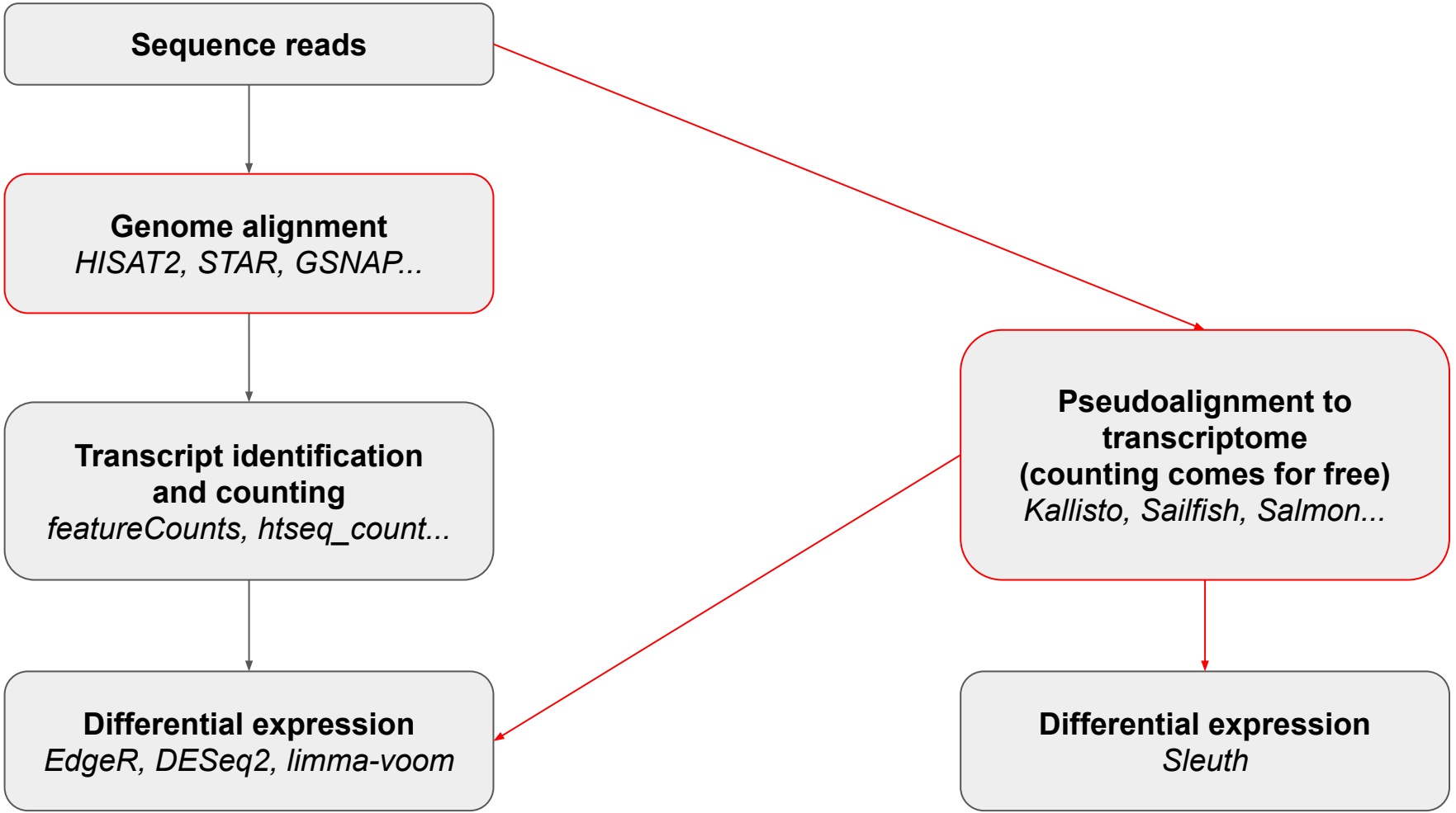
Genome alignment
HISAT2, STAR, GSNAP...
Transcript identification and counting
featureCounts, htseq_count...
Differential expression
EdgeR, DESeq2, limma-voom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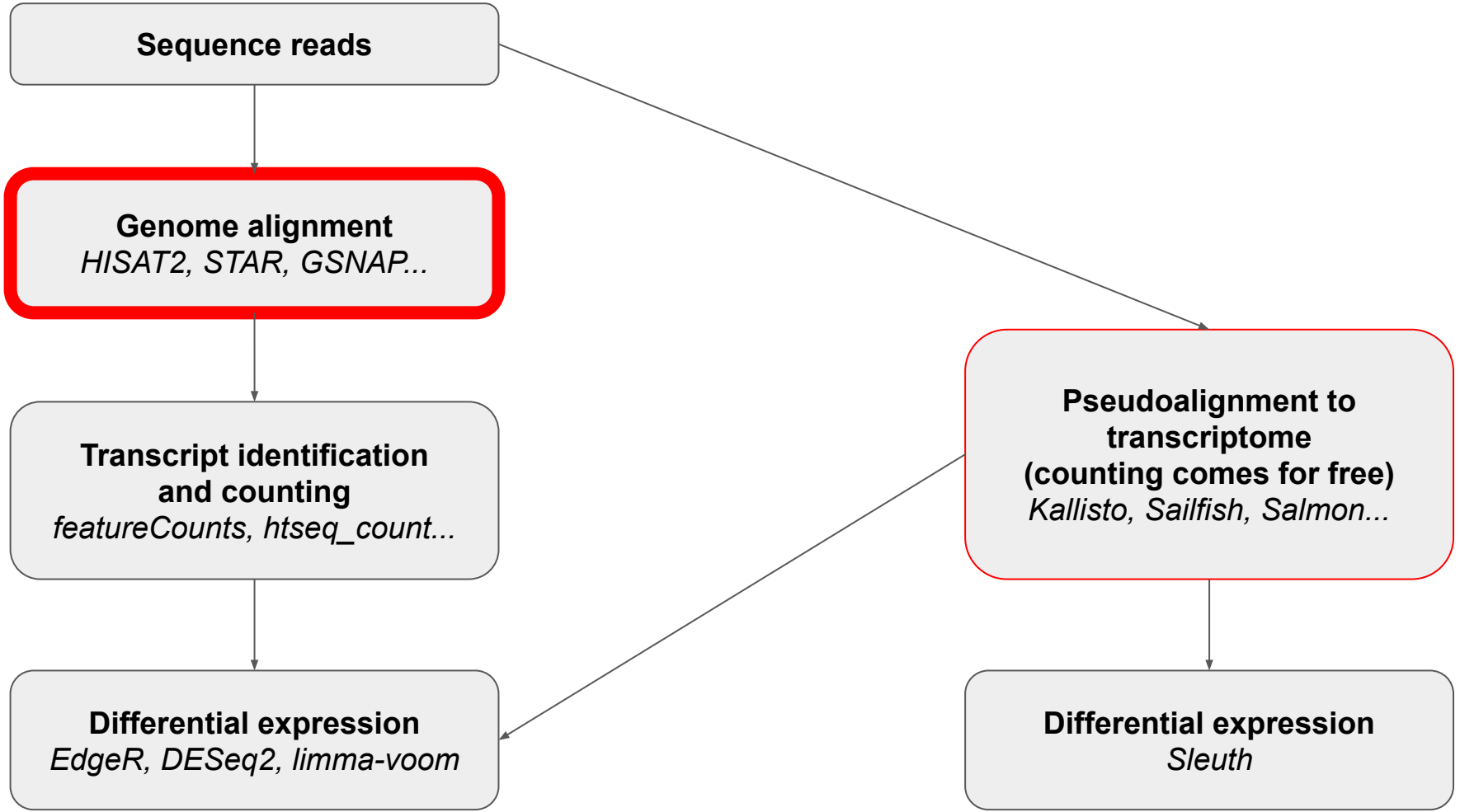
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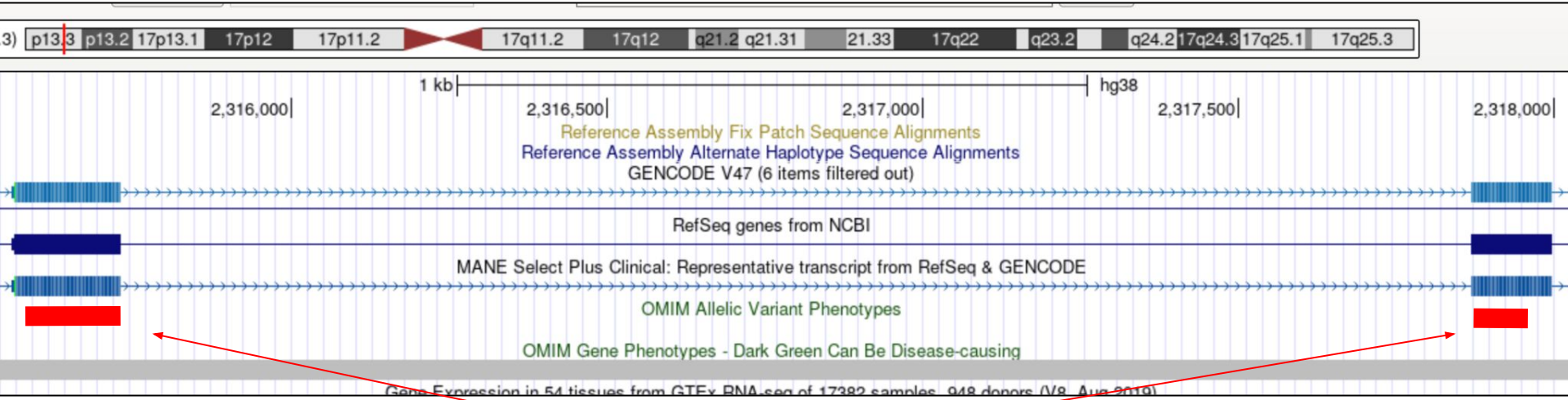
Mapping RNA-seq reads to the genome (**HISAT2**)

- Mapping to the genome is great for determining whether your RNA-seq data is of high quality and exploring the structure of genes of interest
- Eukaryotic genes have introns, which are not present in mature mRNA so special mapping algorithms are required (splice-aware)



mRNA read spanning intron *may not fully map back to genome* (bwa)

Need a splice-aware aligner

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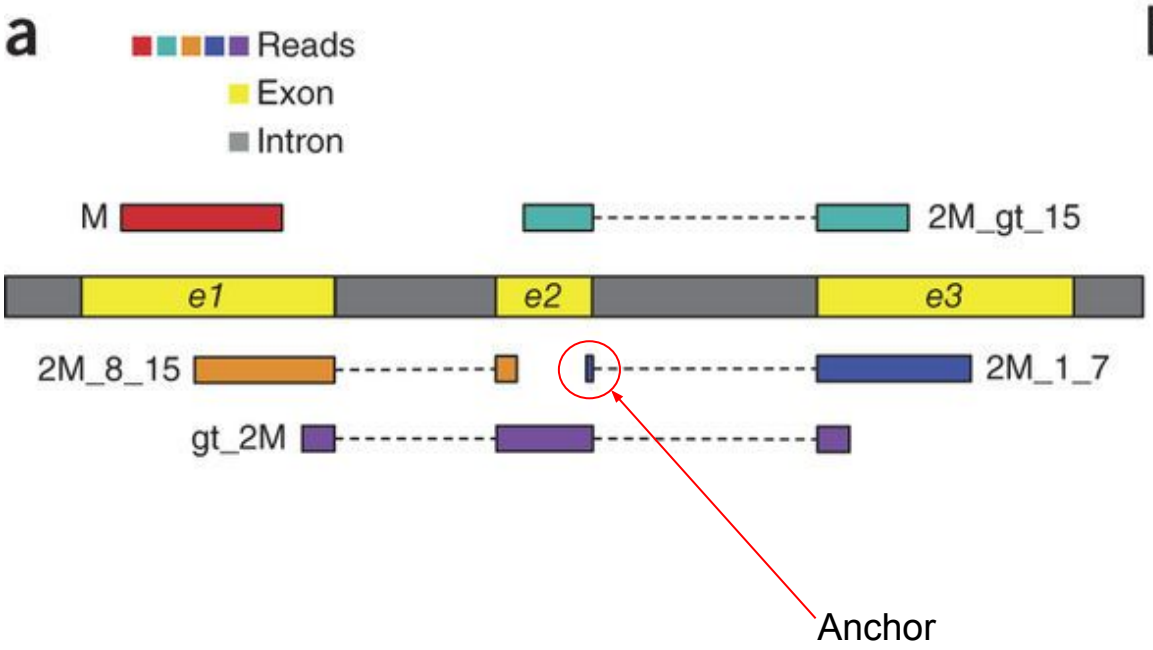
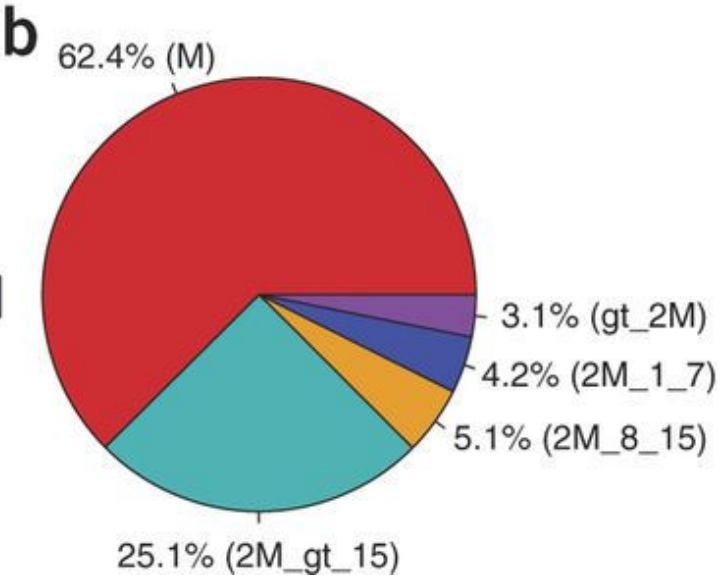
- **HISAT2** is only one such algorithm, but is accurate, fast and easy to use. (others include STAR, and bowtie2 (v old))

Splice aware alignment





Kim *et al*. (2015)
Nature Methods
(PubMed: 25751142)

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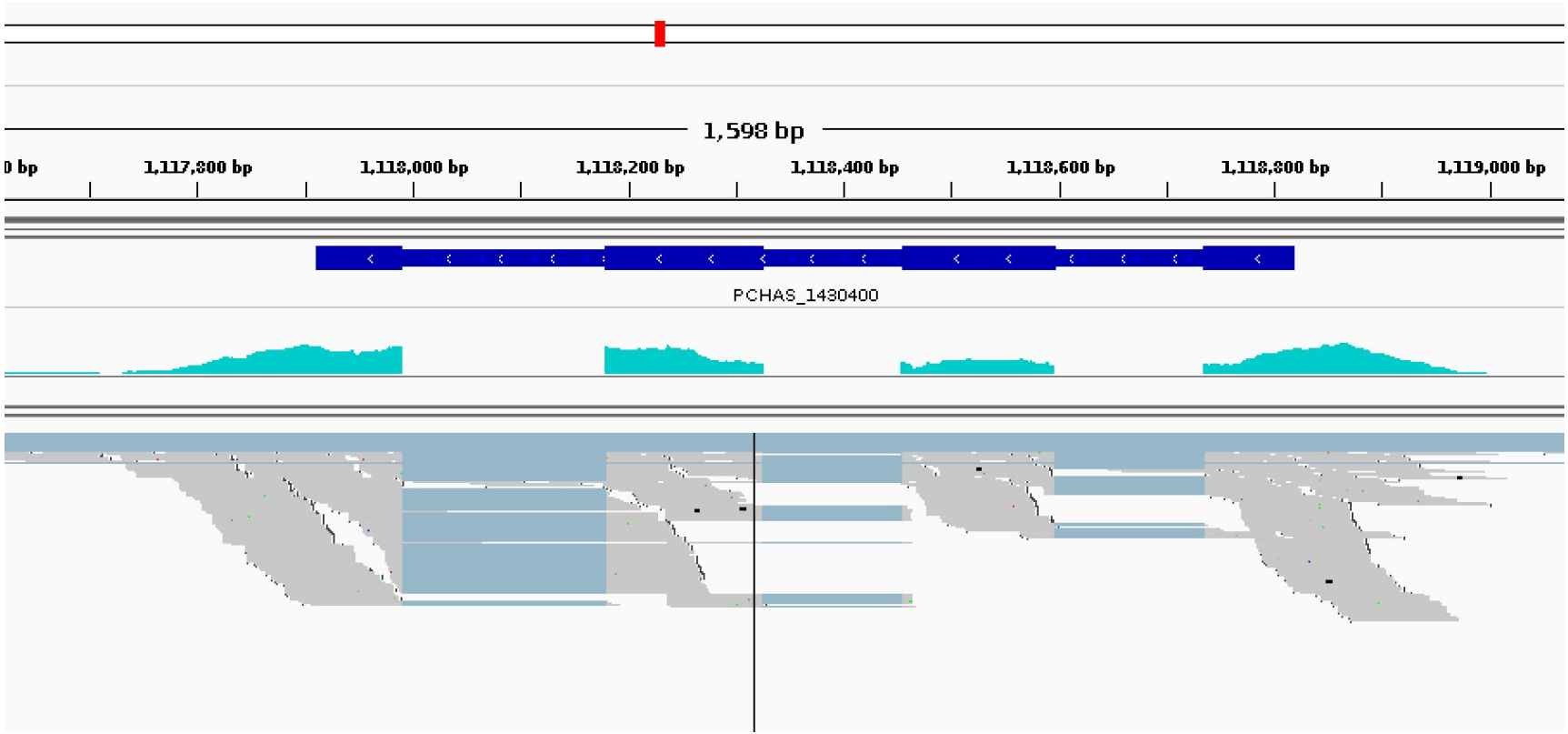
- **HISAT2** is only one such algorithm, but is accurate, fast and easy to use. (others include STAR, and bowtie2 (v old)
- HISAT2: memory footprint smaller, novel splice discovery faster

Visualisation: Integrative Genomics Viewer (IGV)



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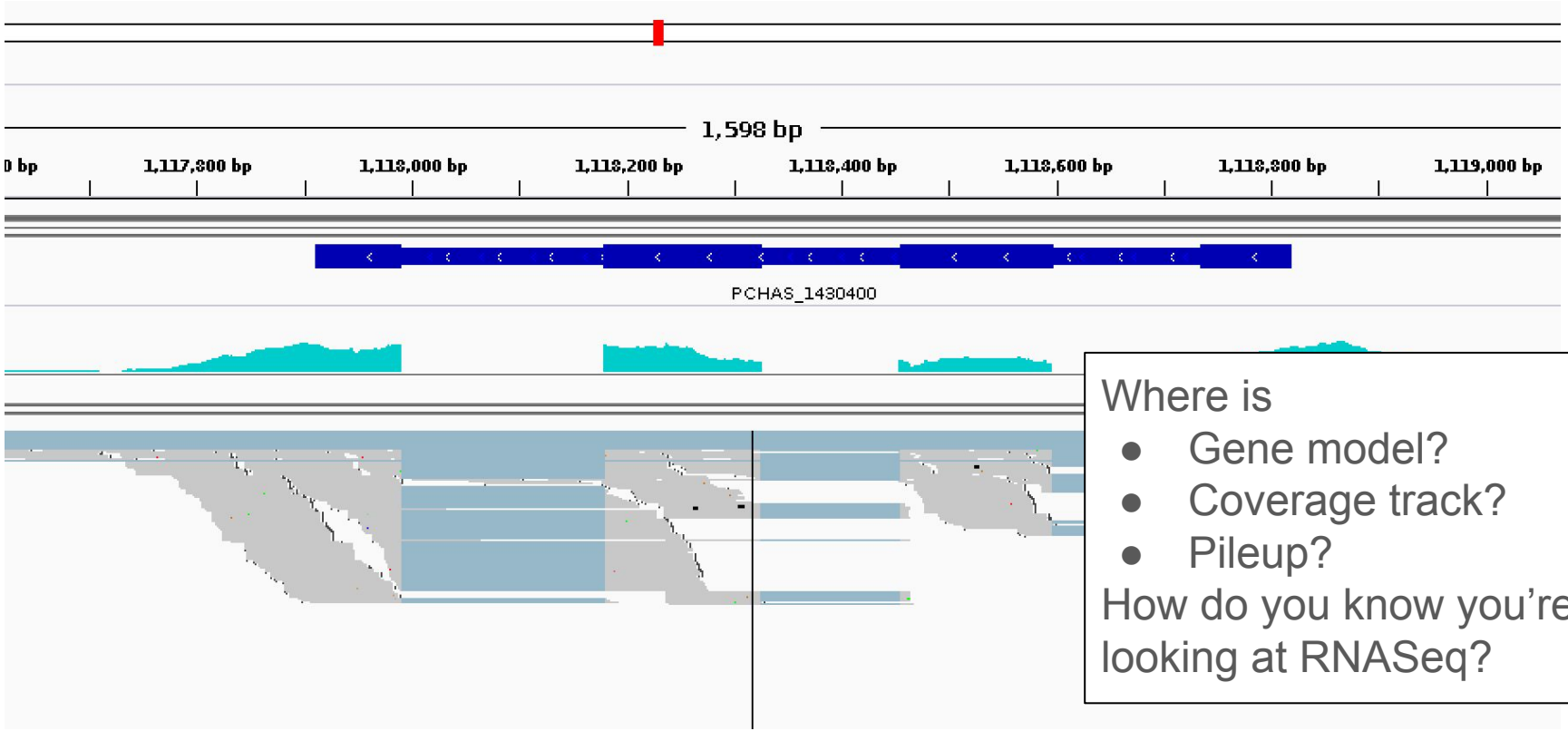

Where is

- Gene model?
- Coverage track?
- Pileup?

How do you know you're looking at RNASeq?

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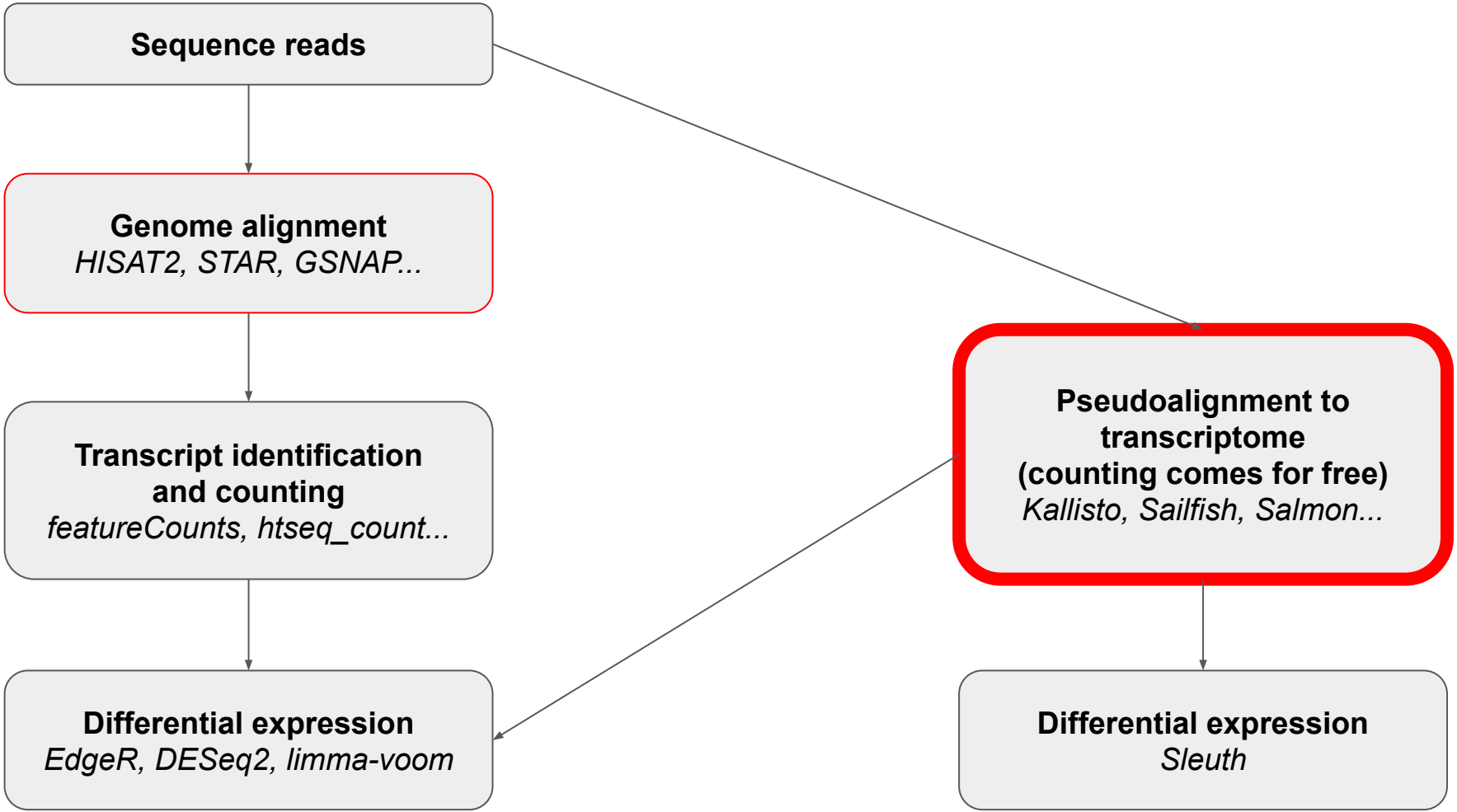
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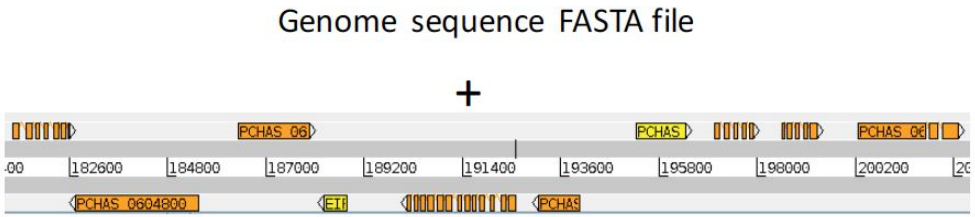
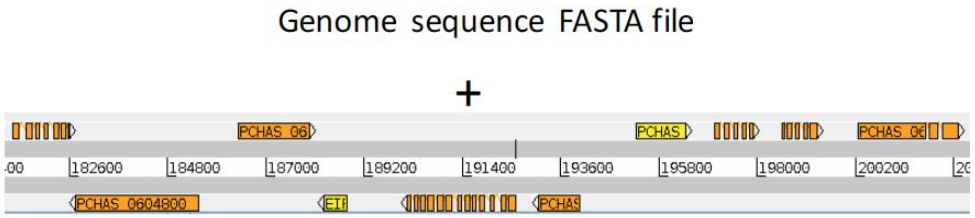


Kallisto has two steps:

1. Building an index from the spliced transcript sequences
2. Quantify reads against the index

WHAT IS THE DISADVANTAGE ??

Mapping to the transcriptome and counting reads (Kallisto)



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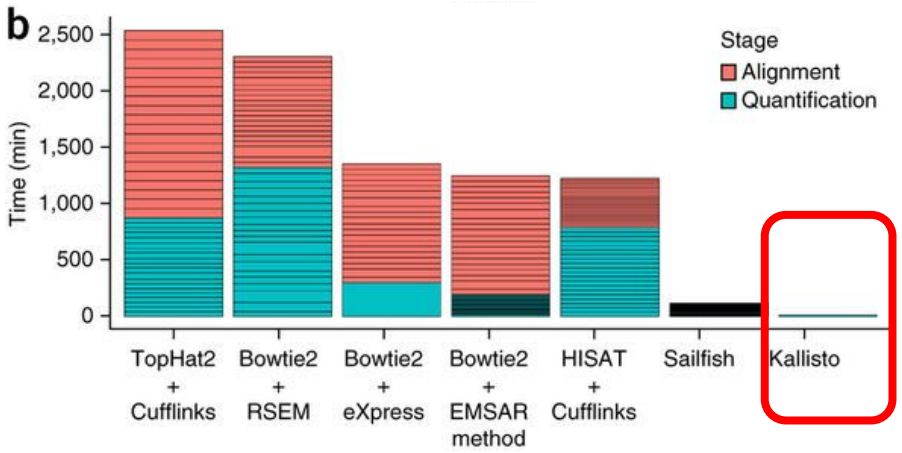
1. Building an index from the spliced transcript sequences
2. Quantify reads against the index

Kallisto cannot be used to identify novel transcripts

Mapping to the transcriptome and counting reads (Kallisto)

- It is faster because there is less target sequence
- ***pseudoalignment*** make this even faster
    - doesn't care where in each transcript reads map to, just which of the transcripts they map to
- Counting comes for free
- Multiple splice forms per gene introduce ambiguity into the mapping
    - **Mapping to the spliced transcript sequences** allows this ambiguity to be taken into account and allows transcript-specific read counts

Mapping to the transcriptome and counting reads (Kallisto)



Bray *et al*. (2016)
Nature Biotechnology
(PubMed: 27043002)

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- Good quality control method
- Novel splice junctions discovery
- Have to quantify afterwards
- If you have the compute, do it

- Very very fast
- Quantification comes for free
- Must have transcriptome defined
- Cant discover novel transcripts

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The result of quantification will look like this

Gene	Replicate 1 Counts	Replicate 2 Counts	Replicate 3 Counts
A (2,000 bases)	10	12	30
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C (1,000 bases)	5	8	15
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Can you directly compare these two gene counts?
Why ? / Why not ?

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Why ? / Why not ?
Overall sequencing depths differ

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Can you directly compare these two gene counts?
Why ? / Why not ?
Gene lengths differ

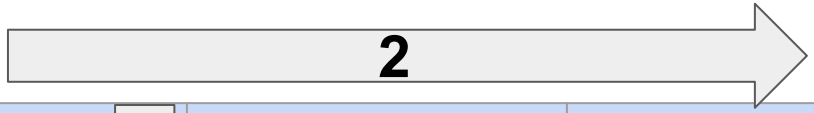
Normalisation

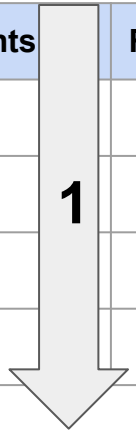
- Runs with more depth will have more reads mapping to each gene (**sequencing depth bias**)
- Longer genes will have more reads mapping to them (**gene length bias**)
- Most methods will normalise for **sequencing depth** *AND* **gene length**

Normalisation methods

- **RPKM** - reads per kilobase per million
- **FPKM** - fragments per kilobase per million
- **TPM** - transcripts per million

RPKM - adjust for sequencing depth, *then* gene size





Gene	Replicate 1 Counts	Replicate 2 Counts	Replicate 3 Counts
A (2,000 bases)	10	12	30
B (4,000 bases)	20	25	60
C (1,000 bases)	5	8	15
D (10,000 bases)	0	0	1

TPM - adjust for gene size, *then* sequencing depth

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A (2,000 bases)	10	12	30
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C (1,000 bases)	5	8	15
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Normalisation methods

- **RPKM** - reads per kilobase per million : GENE LENGTH FIRST
- **FPKM** - fragments per kilobase per million : GENE LENGTH FIRST
- **TPM** - transcripts per million : TOTAL READ COUNT FIRST

Normalisation methods

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- **TPM** - transcripts per million : TOTAL READ COUNT FIRST

Some of these methods have problems with highly expressed genes, so it's better to use more complicated normalisation procedures (**DESeq2 rlog**, **Sleuth**)

RPKM

BEFORE

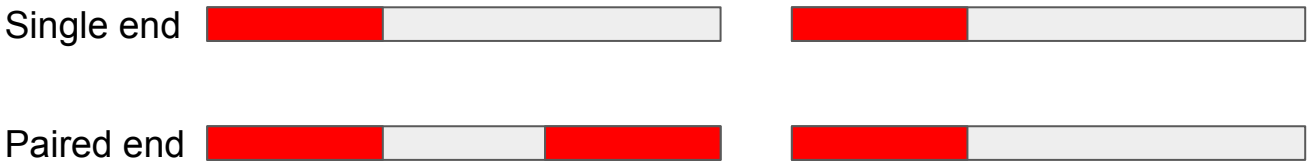
Gene	Replicate 1 Counts	Replicate 2 Counts	Replicate 3 Counts
A (2,000 bases)	10	12	30
B (4,000 bases)	20	25	60
C (1,000 bases)	5	8	15
D (10,000 bases)	0	0	1

AFTER

Gene (bases)	Replicate 1 RPKM	Replicate 2 RPKM	Replicate 3 RPKM
A (2,000 bases)	1.43	1.33	1.42
B (4,000 bases)	1.43	1.39	1.42
C (1,000 bases)	1.43	1.78	1.42
D (10,000 bases)	0	0	0.009

FPKM (fragments per kilobase million)

- RPKM for paired reads
- takes into account that two reads can map to one fragment (and so it doesn’t count this fragment twice)



RPKM vs TPM

RPKM

Gene	R1	R2	R3
A	1.43	1.33	1.42
B	1.43	1.39	1.42
C	1.43	1.78	1.42
D	0	0	0.009
Total	**4.29**	**4.5**	**4.25**

TPM

Gene	R1	R2	R3
A	3.33	2.96	3.326
B	3.33	3.09	3.326
C	3.33	3.95	3.326
D	0	0	0.02
Total	**10**	**10**	**10**

Easier to see the proportion of each gene within a sample as sum of TPMs same across samples

Adapted from StatQuest (http://statquest.org)

Lecture outline

- RNA-seq background
- **Pipelines**
  - Mapping to the genome (HISAT2 and IGV)
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- What to do with a gene list
- The exercise

Why QC our data?

- We gather samples and sequence
- We are trying to: detect associations between
  - genetics/expression/assay readout and
  - “condition”
- But things can go wrong
  - Samples can be tainted , e.g.
    - Mis-handled during collection (DNA or RNA degrades)
    - Sequencing of a lane can have problems (too little, too much, problem reagents)
  - Specific genomic loci can be problematic - e.g. sequencing “hotspots”, local capture problems
- Result
  - **Association is muddied** - false positive, false negative

Why QC our data?

	S1	S2	S3	S4	S5	…
L1						
L2						
L3						
L4						
L5						
…						
L						

Why QC our data?

	S1	S2	S3	S4	S5	…
L1						
L2						
L3						
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L						

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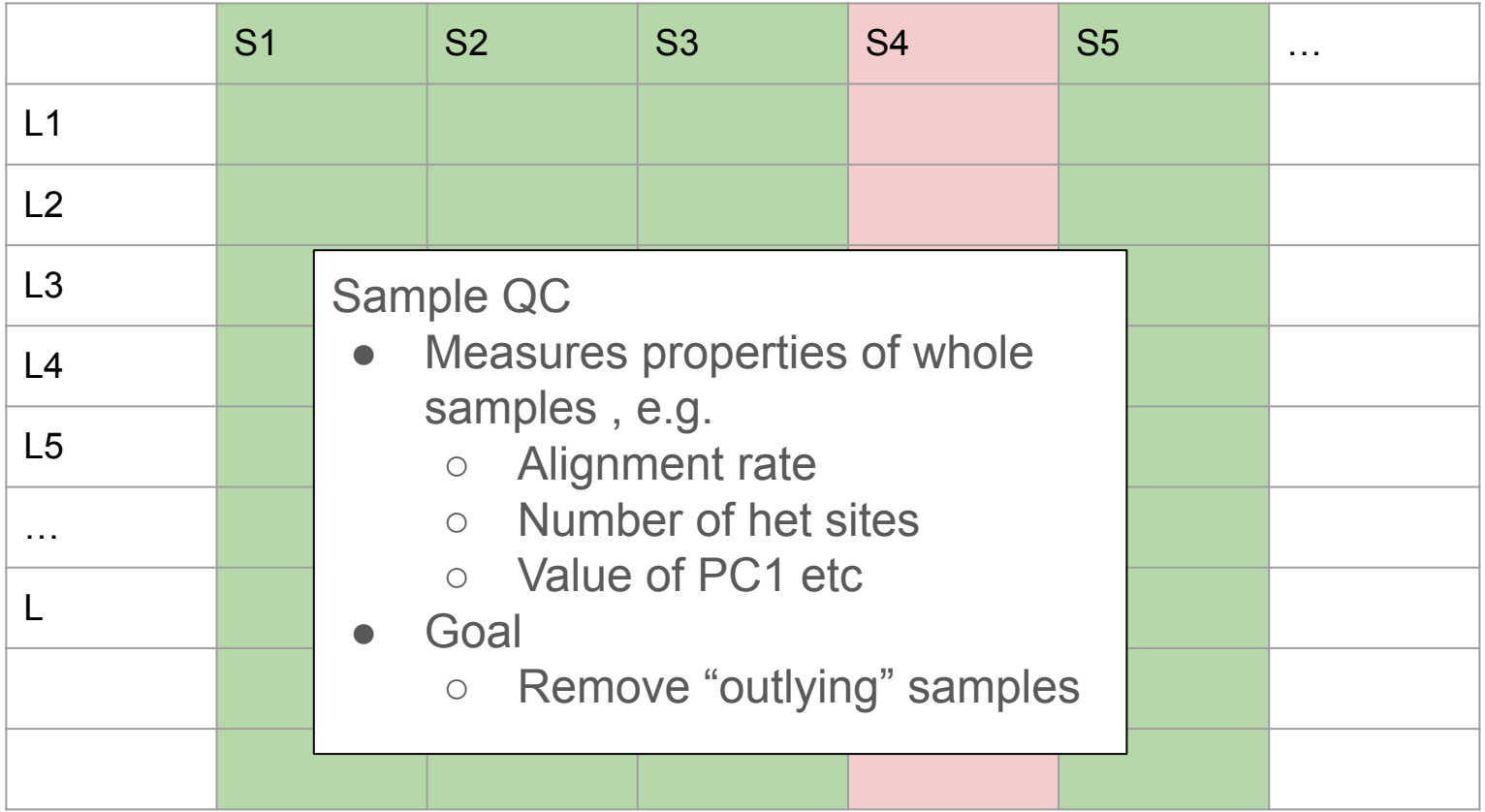
Why QC our data?



Why QC our data?

	S1	S2	S3	S4	S5	…
L1						
L2						
L3						
L4						
L5						
…						
L2000						
…						

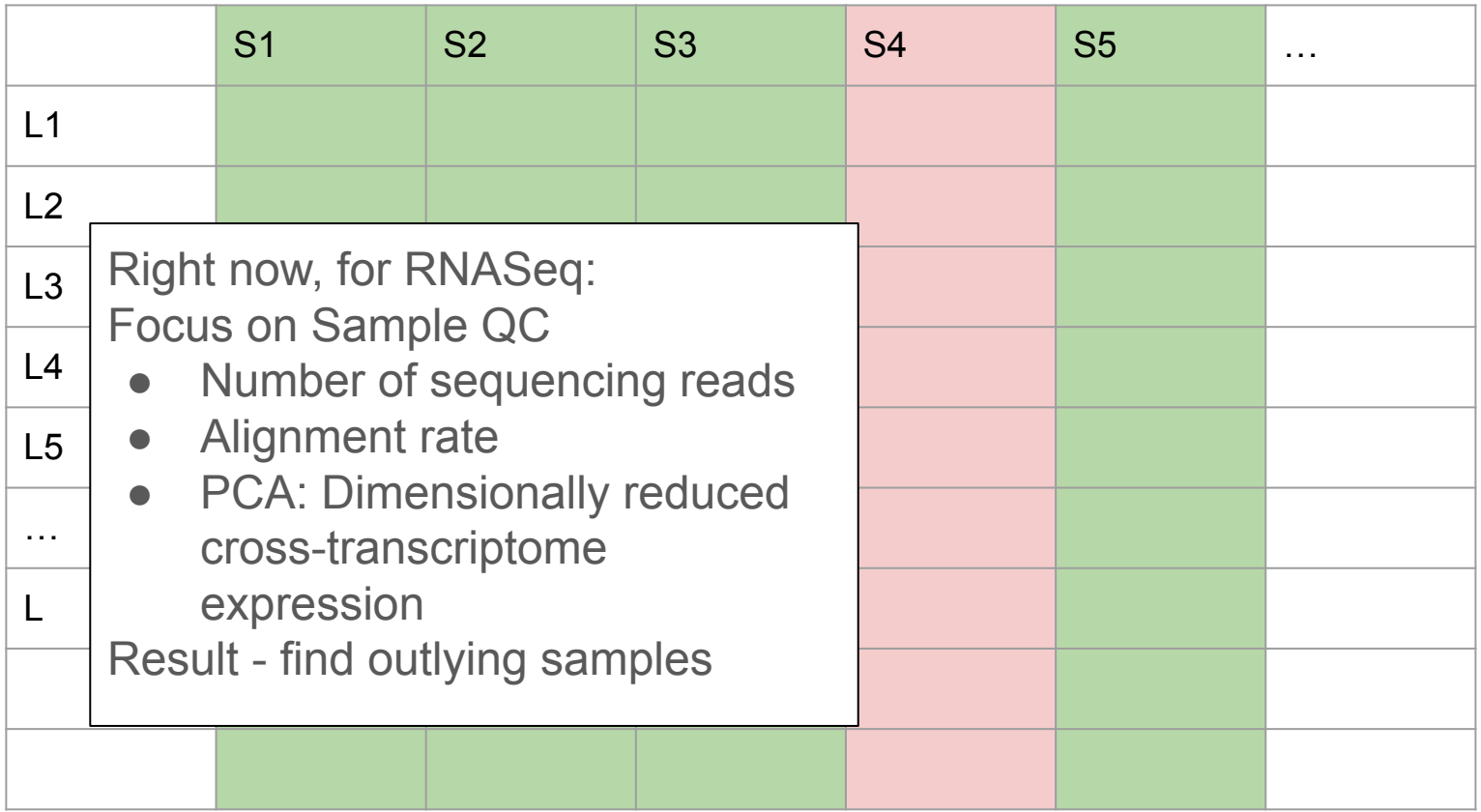
Why QC our data?

	S1	S2	S3
L1			
L2			
L3			
L4			
L5			
…			
L2000			
…			

Locus QC

- DNASeq:
  - Measures properties of single locus (all samples) , e.g.
  - Allele Depth
  - Strand bias
  - …
  - Train model on known false and true loci
  - Goal
    - Remove problematic loci
- RNASeq: remove low-expressed / low-variability genes

Why QC our data?



Why QC our data?

Flagging outlying samples based on read count / align rate

Sample	Number of reads	Alignment Rate	QC Pass?
S1	60M	85%	
S2	10M	80%	
S3	50M	85%	
S4	65M	50%	
S5	55M	55%	
S6	70M	75%	

Why QC our data?

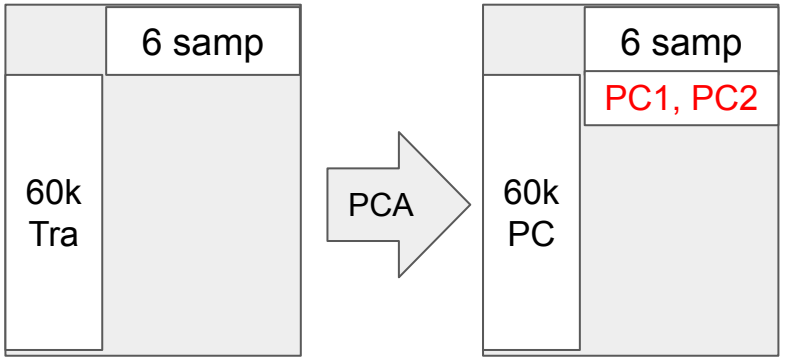
Flagging outlying samples based on read count / align rate

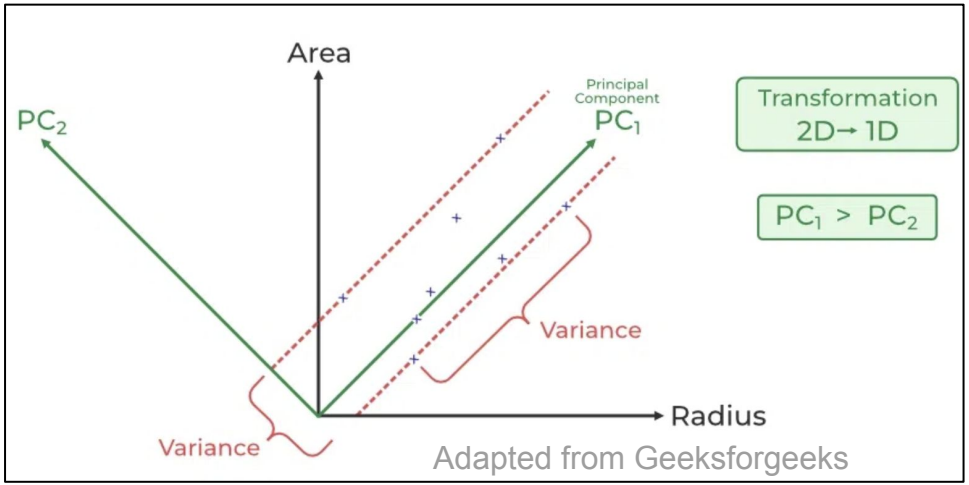
Sample	Number of reads	Alignment Rate	QC Pass?
S1	60M	85%	
S2	**10M**	80%	?
S3	50M	85%	
S4	65M	**50%**	?
S5	55M	**55%**	?
S6	70M	75%	

Why QC our data?

Flagging outlying samples based on PCA

- Start with N samples in D-dimensional space (RNASeq D~60k)
- Transform (rotate) into a *new* D-dim space (PC space)
- Dimensional reduction: Focus on first 2-4 PCs





Why QC our data?

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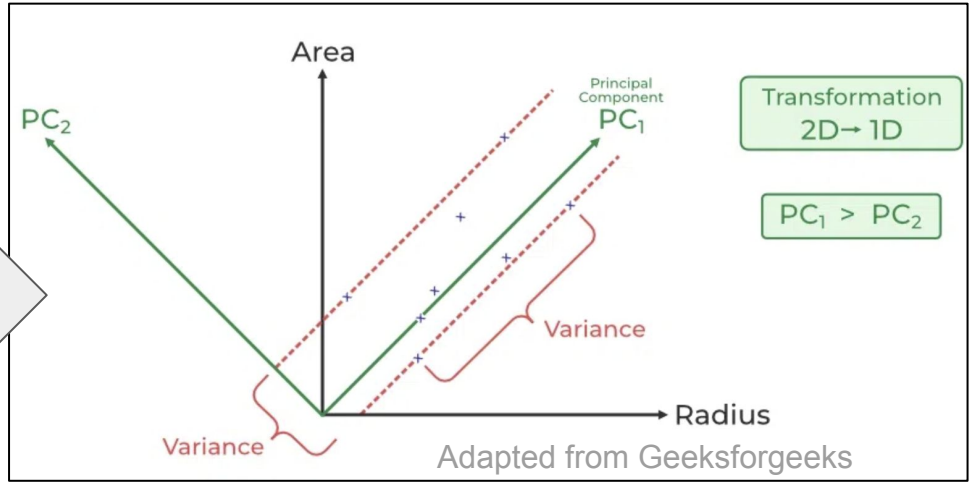
- Dimensional reduction: Focus on first 2-4 PCs

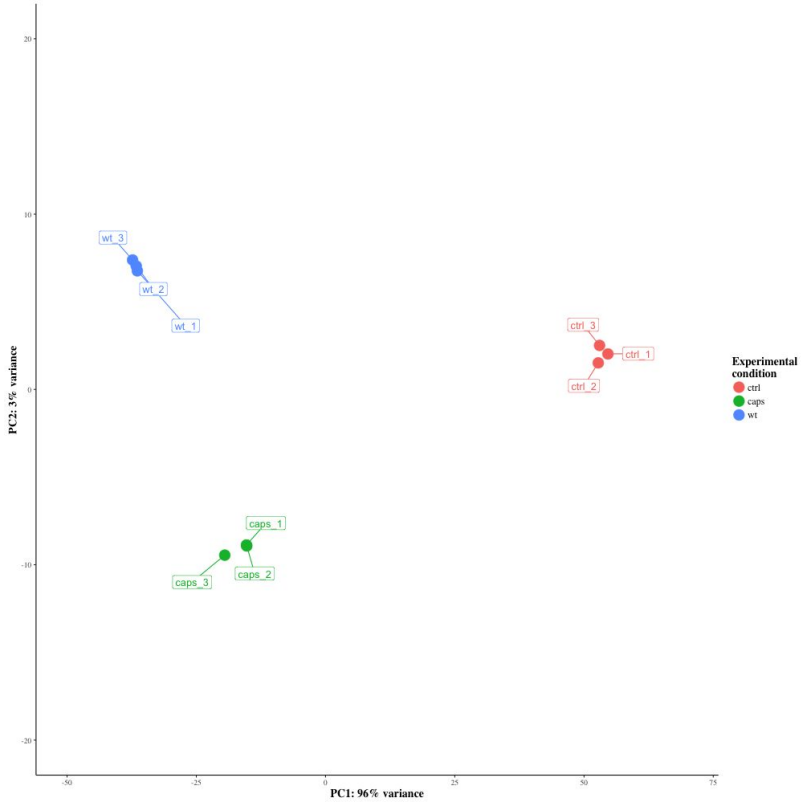
Visualise N samples in new dimensions

- Order new dimensions in order of sample variability
- The variability in each dimension is maximally independent (no covariance between dimensions)



Principal component analysis (PCA)

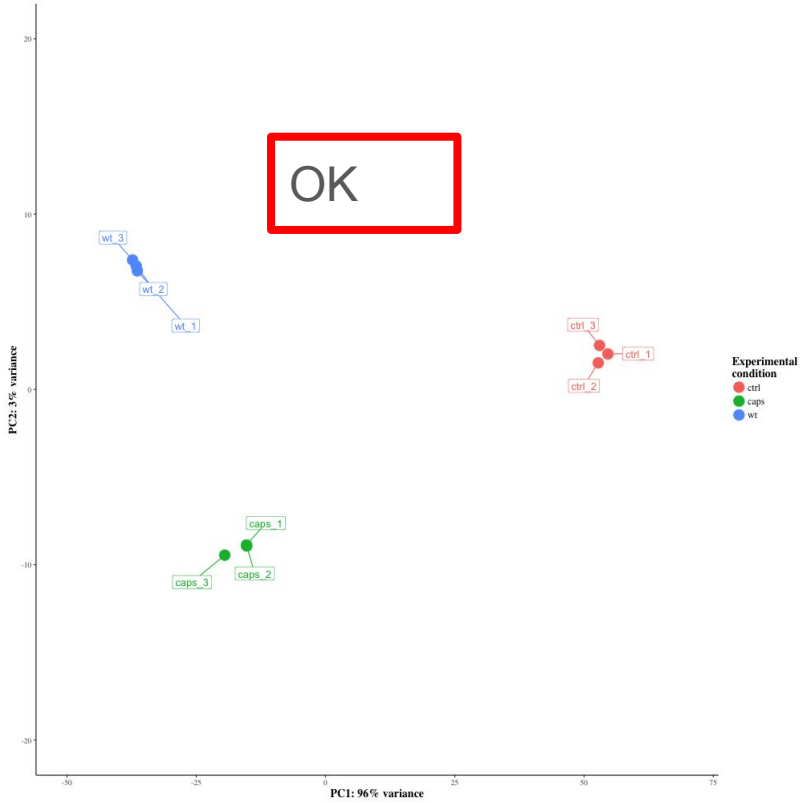
- Use to look at variation and strong patterns within data



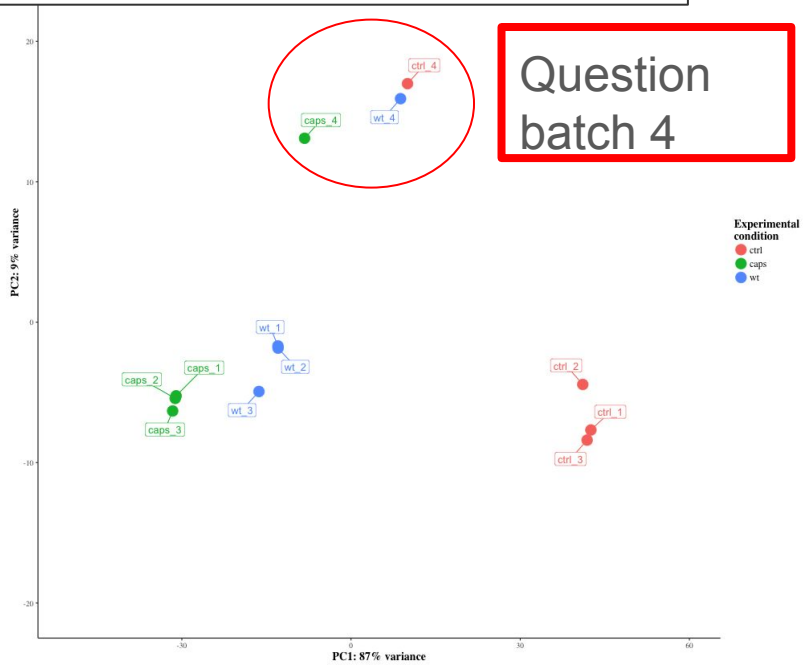
Why QC our data?

In practice, PCA picks up batch effects
Plot N samples in PC1 and PC2
You *don't want* samples clustering by an experimental artefact (eg processing batch)

OK



Question batch 4



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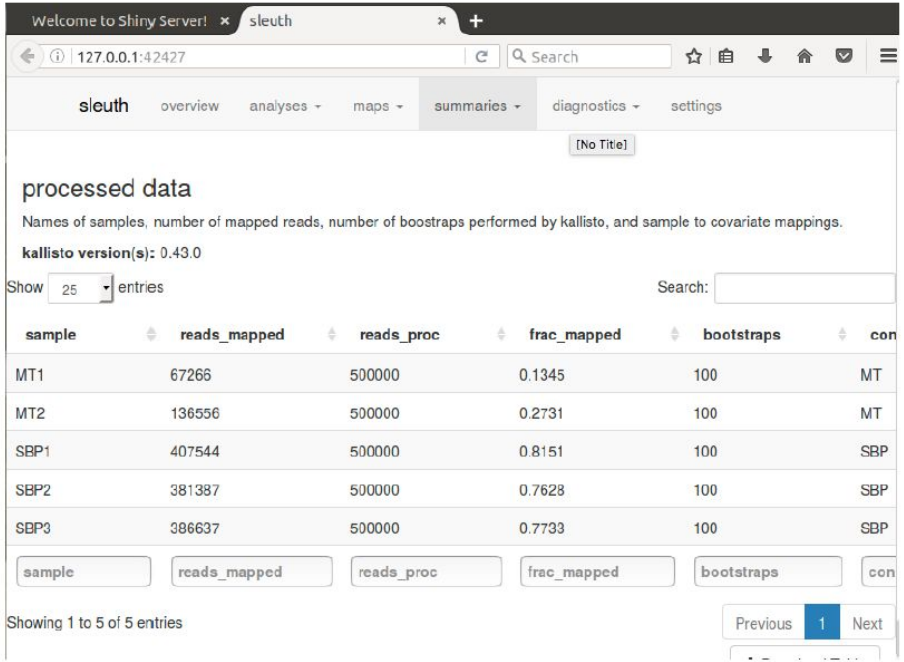
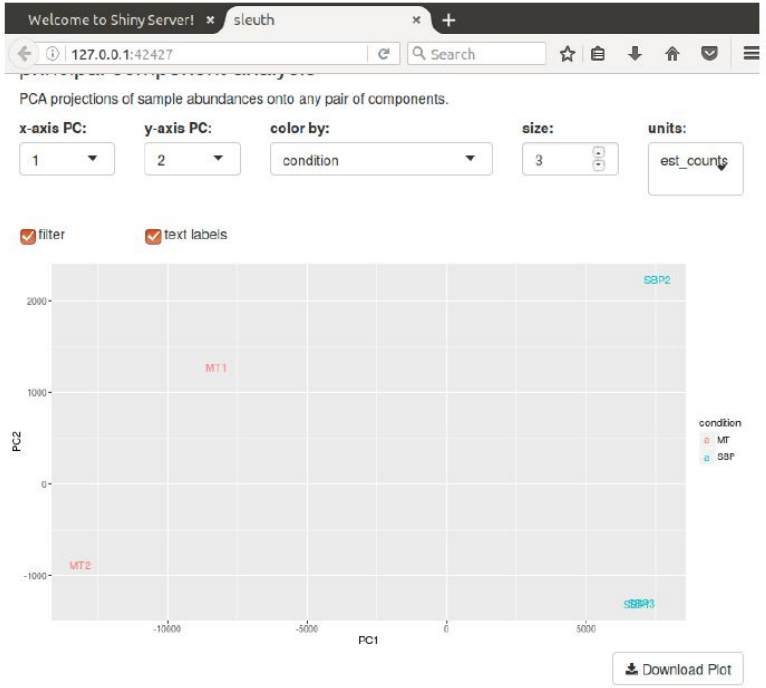
Determining differential expression (Sleuth)

- Many packages to do Differential Gene Expression
  - DESeq2, EdgeR, Limma/Voom
  - Sleuth - companion to Kallisto
- Why you can't (really) use a gene by gene t-test
  - "Size factors" - we can do better than the basic method in FPKM or TPM
  - **We don't normally have enough replicates to do traditional tests of significance for RNA-seq data (methods do gene-variance modelling in some way)**
  - **You may want to account for many different input conditions (e.g. experimental + genetic)**
    - Methods compare two linear models - with and without experimental condition
  - Need to account for multiple-testing effect (q-value vs p-value)

QC with Sleuth





What to do next with your gene list

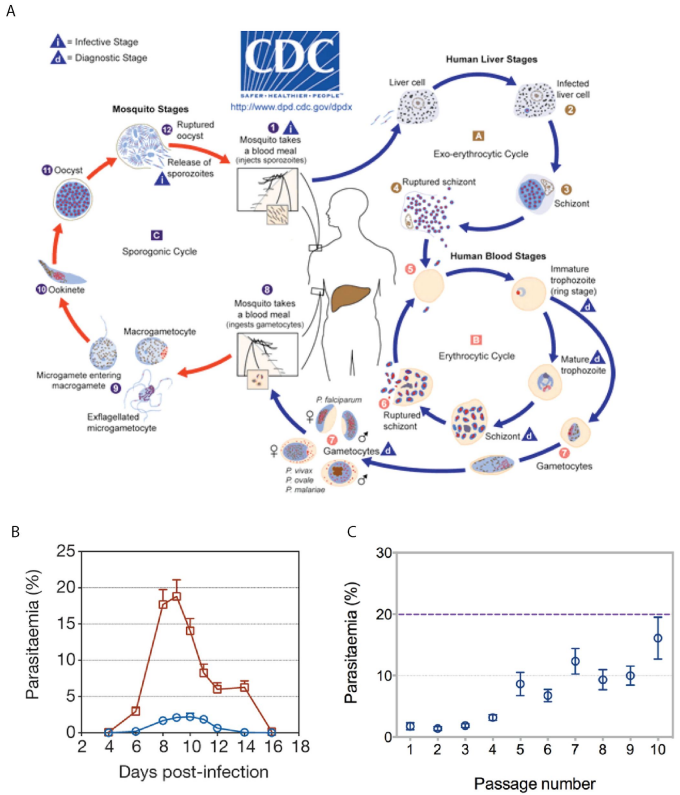
When you have a list of differentially expressed genes, things start to get difficult.

What to do:

1. Have a hypothesis already? Test it.
2. **GO term/pathway/gene-set enrichment analysis (GSEA, TopGO, InnateDB, Ingenuity Pathway Analysis etc.)**
3. Work through list, Google, read papers
4. Stare at a volcano plot of effect size x p-value, draw cutoffs in effect size and cherry pick genes ;-)

Then make a hypothesis about what genes are interesting and why. Can you test/explore this further bioinformatically? Design the next wet lab experiment

The exercise



- *Plasmodium chabaudi*
- rodent malaria parasite
  - exhibits many characteristics associated with the pathogenesis of human infection
- serial blood passage (SBP)
  - direct injection from mouse to mouse
  - results in severe disease
- infection with parasites via mosquitoes (MT)
  - develop lower parasitaemia (presence of parasites in the blood)
  - mild, chronic disease

IS THE TRANSCRIPTOME OF
- MOSQUITO TRANSMITTED PARASITE (MT) *DIFFERENT FROM*
- ONE WHICH HAS NOT PASSED THROUGH A MOSQUITO (SBP)?