NGS Analysis and Galaxy Part 1

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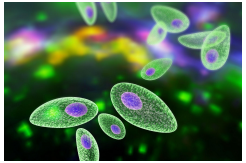
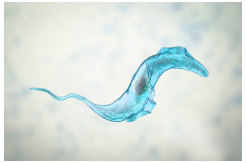
Introduction

University of Glasgow

Who am I?




- PhD in comparative immunology 2005
- Worked in industry (big pharma and small biotech)
- MSc bioinformatics 2013
- Core bioinformatician WCIP 2013 - 2021
- Create online resources for public use

VEuPathDB
Eukaryotic Pathogen, Vector & Host Informatics Resources

MicrobiomeDB
A Microbiome Resource

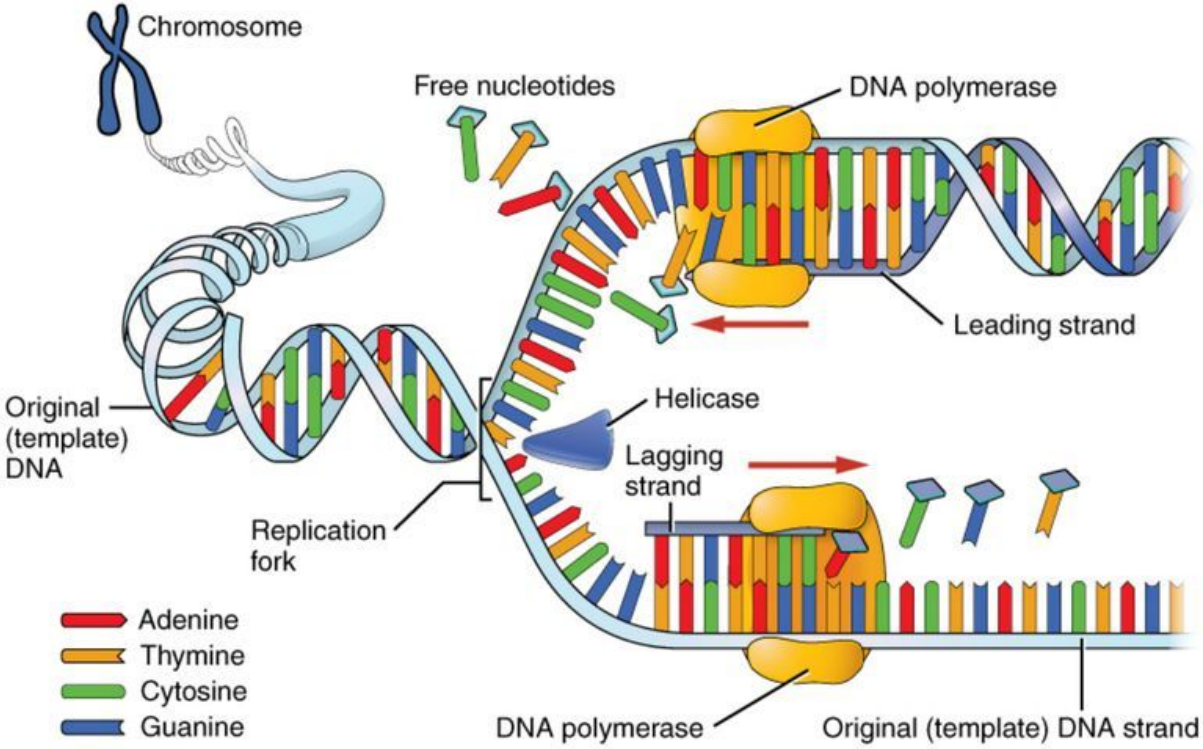
COMPANION

Outline

- Background
  - How does sequencing work?
  - What does the data look like?
- Pre-processing for Analysis
  - QC and trimming
  - Aligning to a reference genome
- RNA-seq and differential expression
- Using Galaxy

How Does Sequencing Work?

- Carry out replication under controlled conditions
- Artificially slow down the reaction to see the order in which bases are incorporated



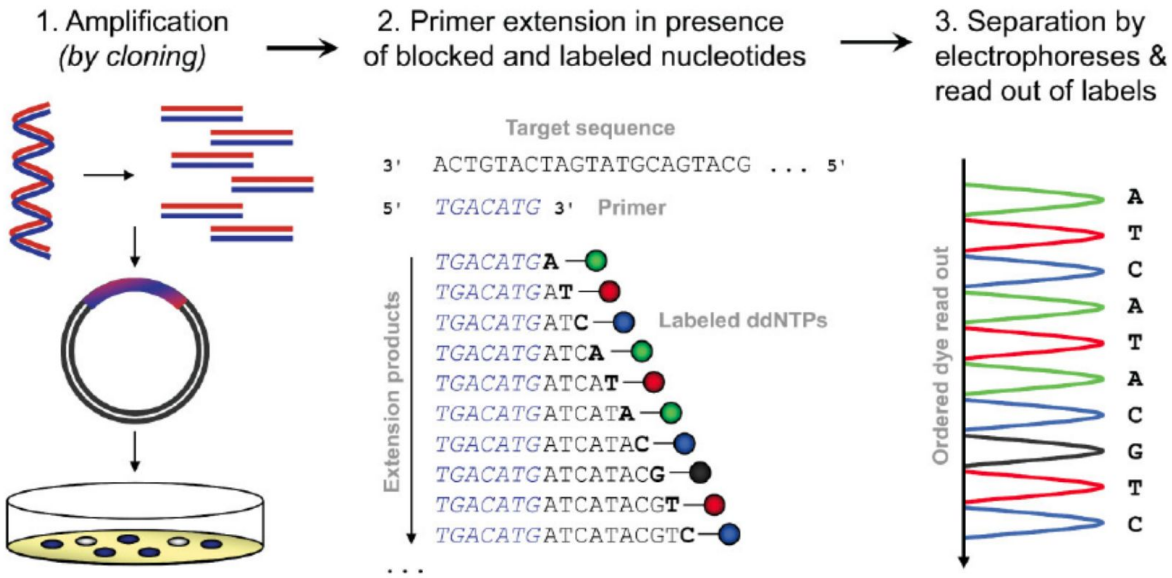
How Does Sequencing Work?

Sanger Sequencing (1975)

Uses modified nucleotides (ddNTPs) that cannot be extended

Each ddNTP is labelled with a different dye so you can see the order in which they are incorporated

Long reads and low error rate, but low throughput



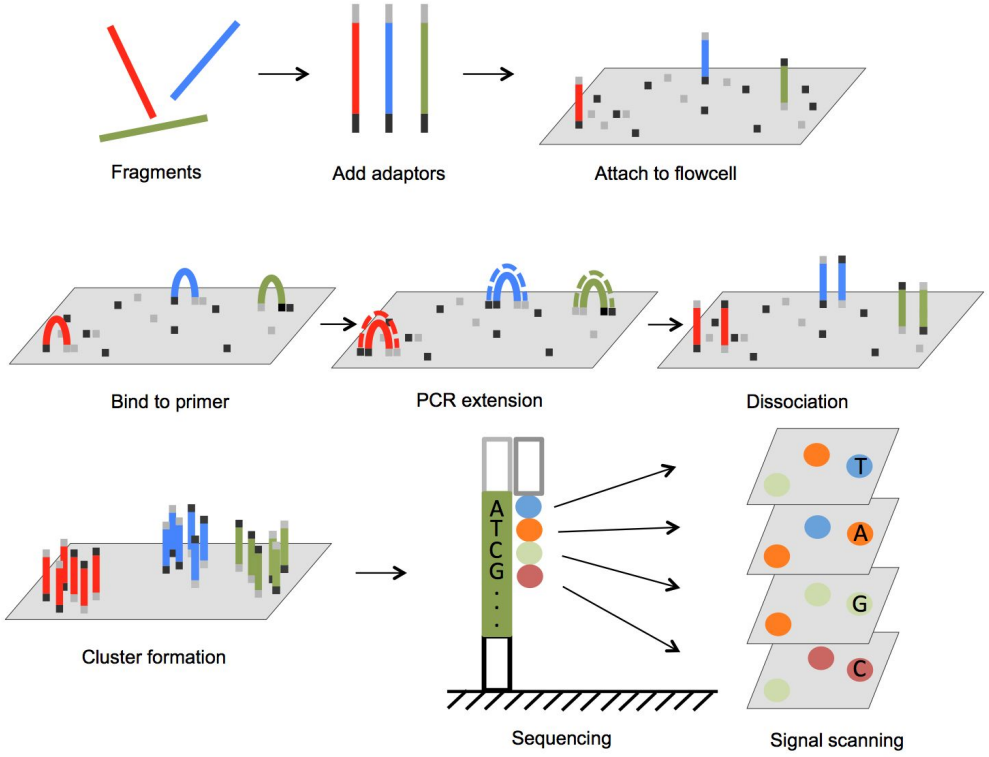
How Does Sequencing Work?

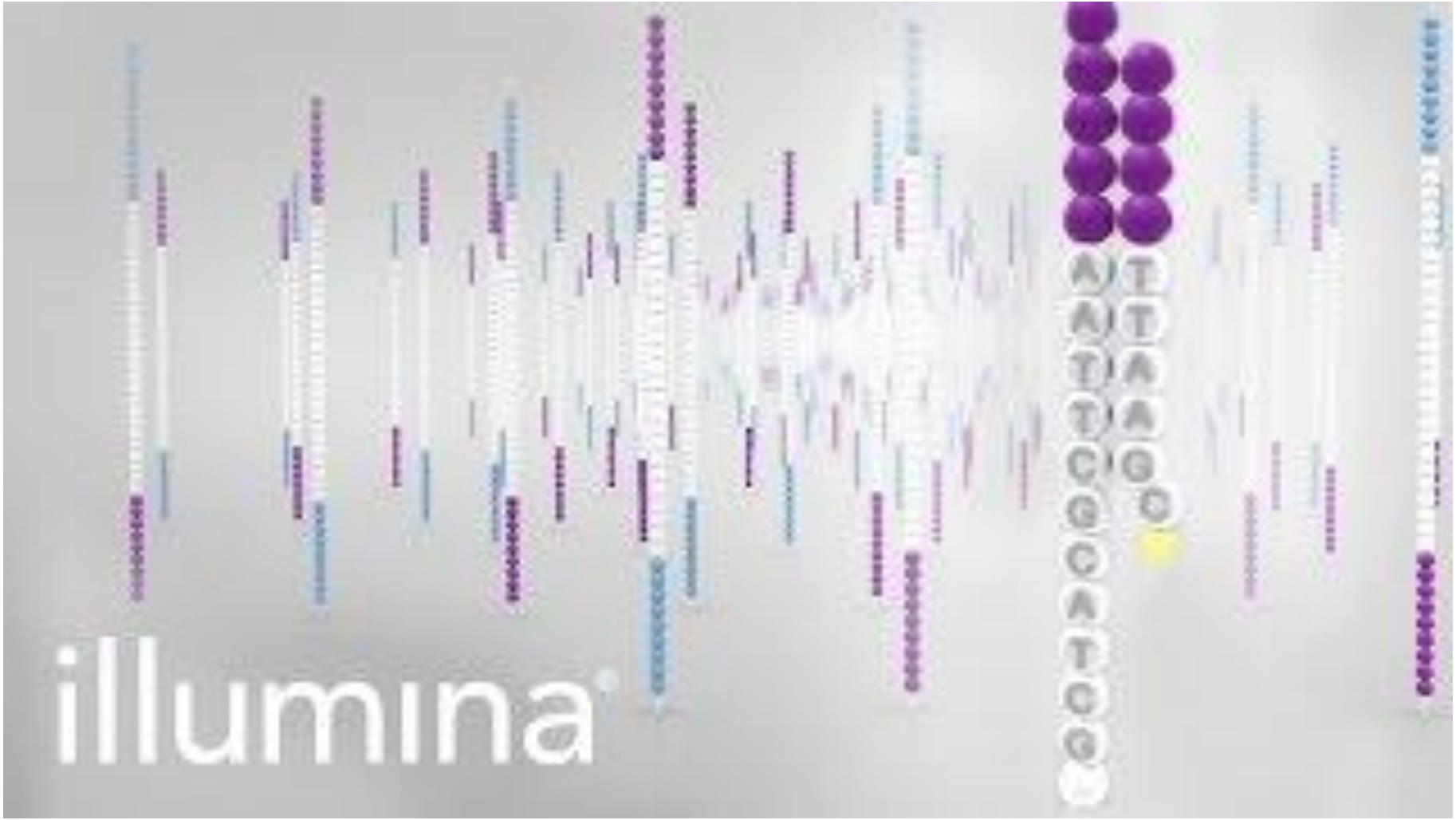
Illumina Sequencing (2005)

DNA fragments are adaptor-ligated and attached to a flow cell

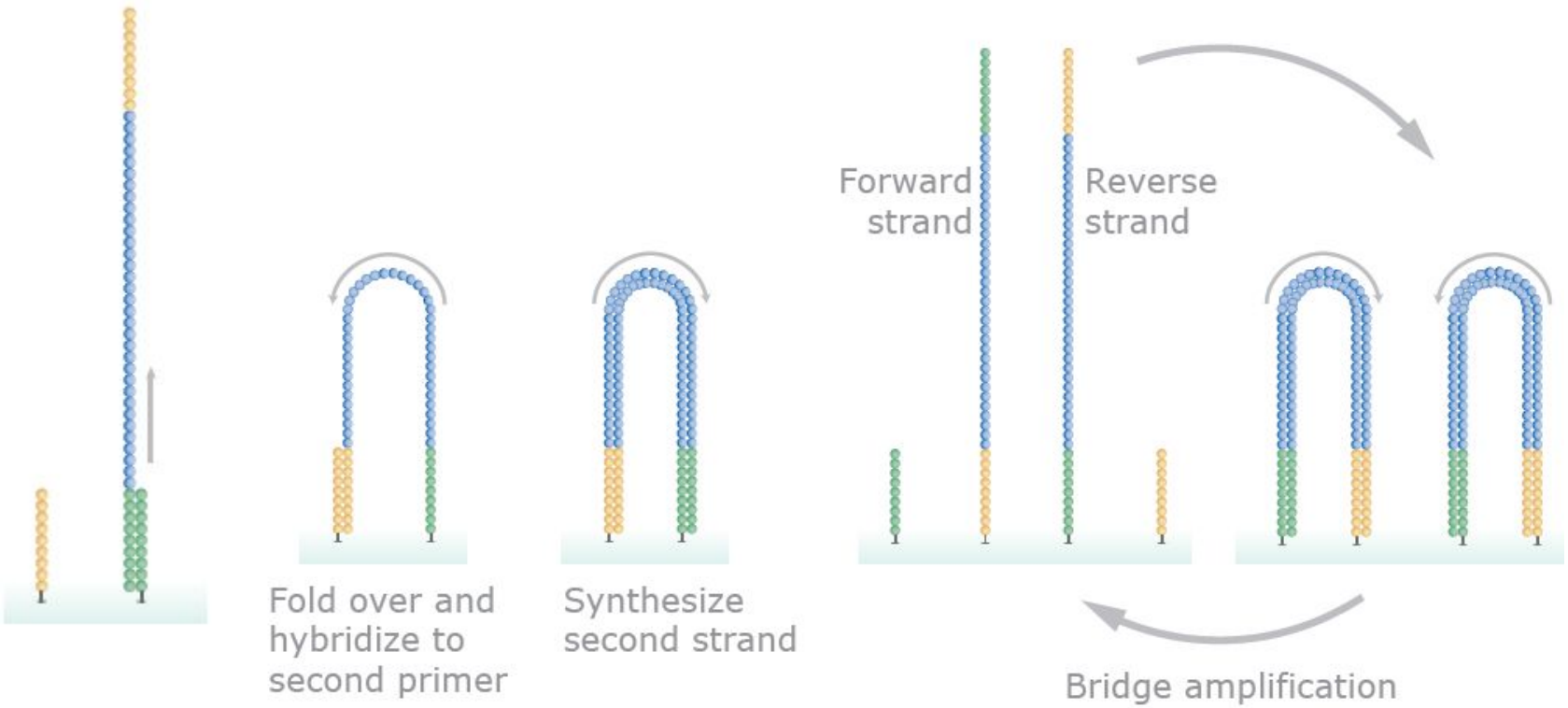
PCR is carried out in situ to form clusters

Sequencing can be carried out on millions of clusters simultaneously

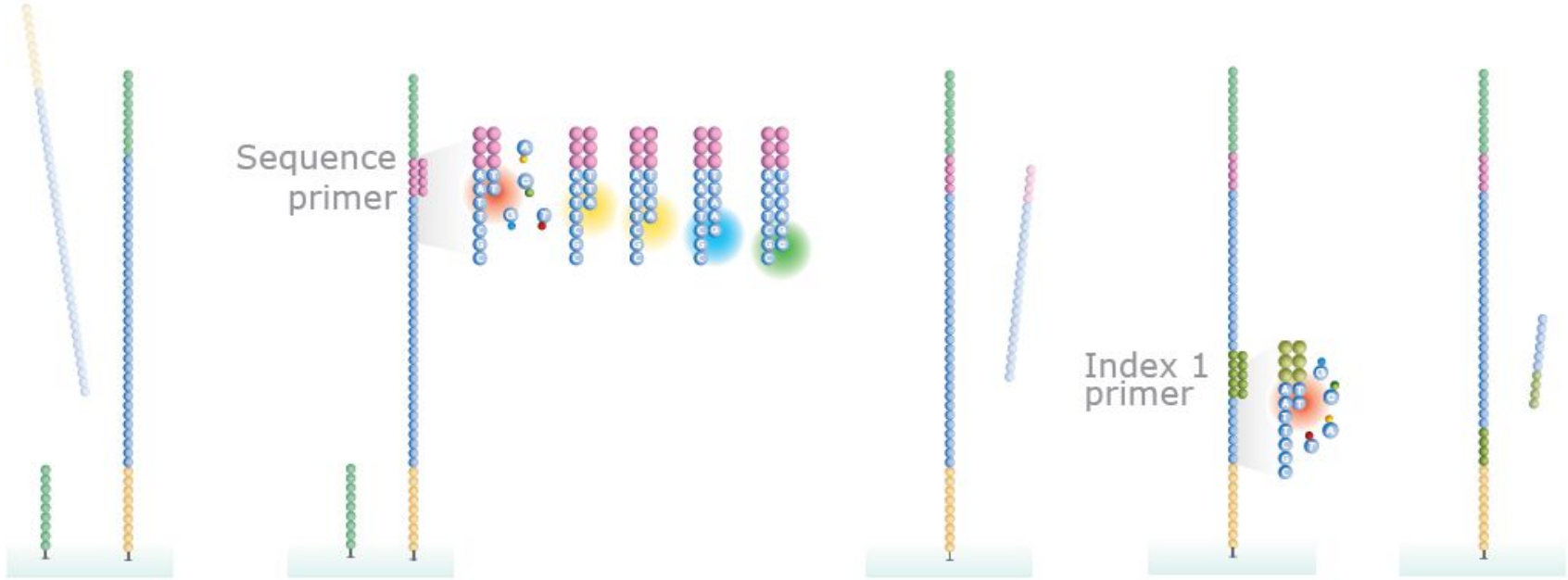



A
A
T
T
C
G
C
A
T
C
G
T
T
A
A
G
C
illumina

How Does Sequencing Work?



How Does Sequencing Work?



The reverse strand is cleaved and washed away

With each cycle, four fluorescently tagged nucleotides compete for addition to the growing chain. Only one is incorporated based on the sequence of the template.

The read product is washed away

Sequence Index1

The read product is washed away

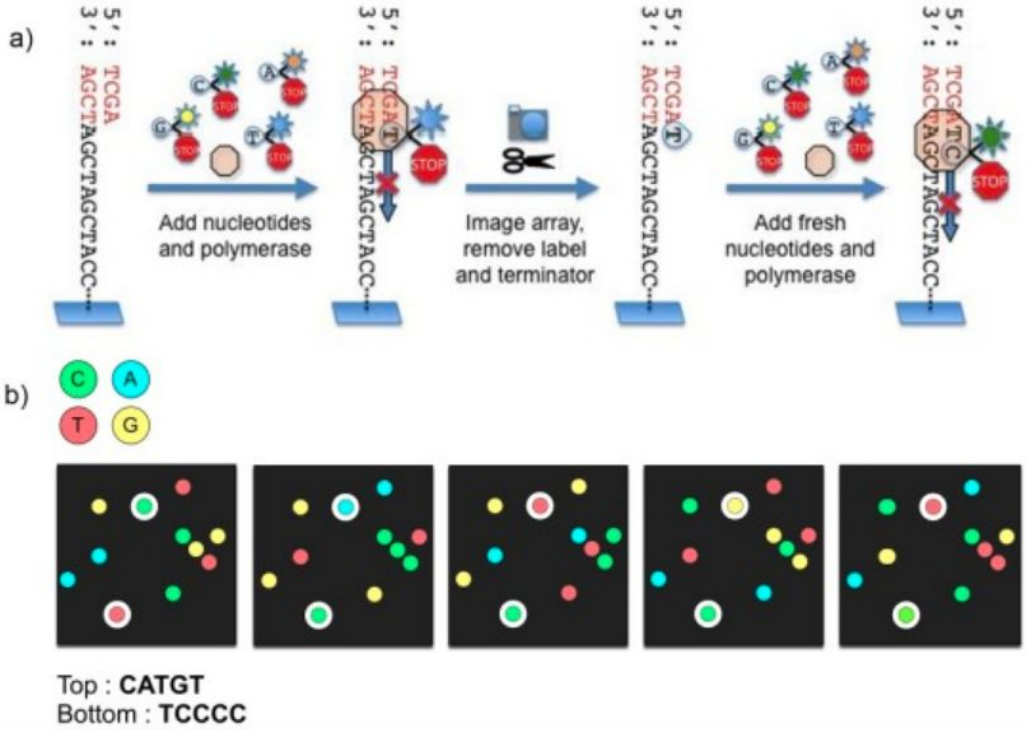
How Does Sequencing Work?

Illumina Sequencing

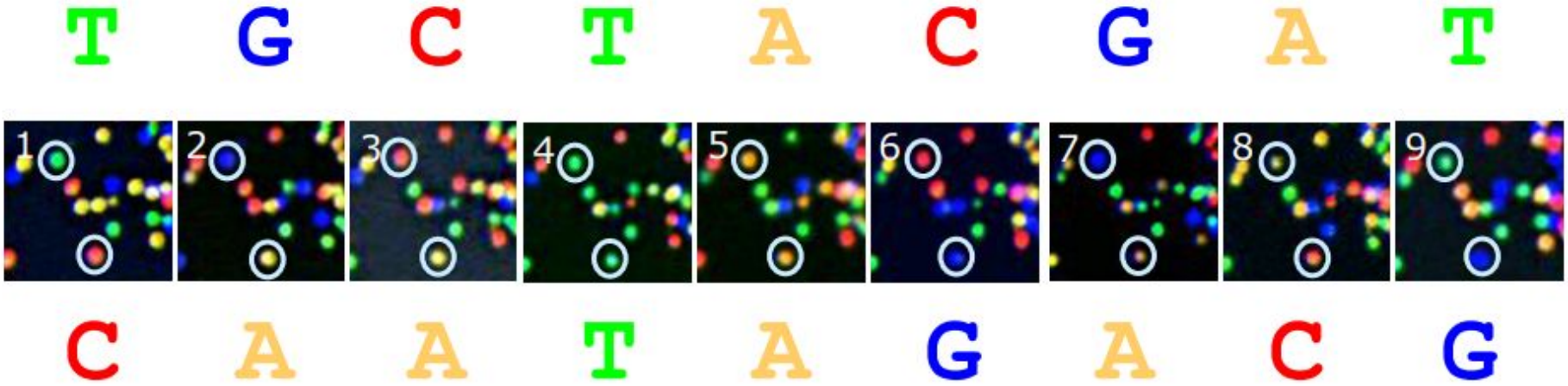
Blocked and labelled nucleotides are added

1 nucleotide is incorporated and an image is taken of the array

Label and block are removed and cycle repeats



How Does Sequencing Work?

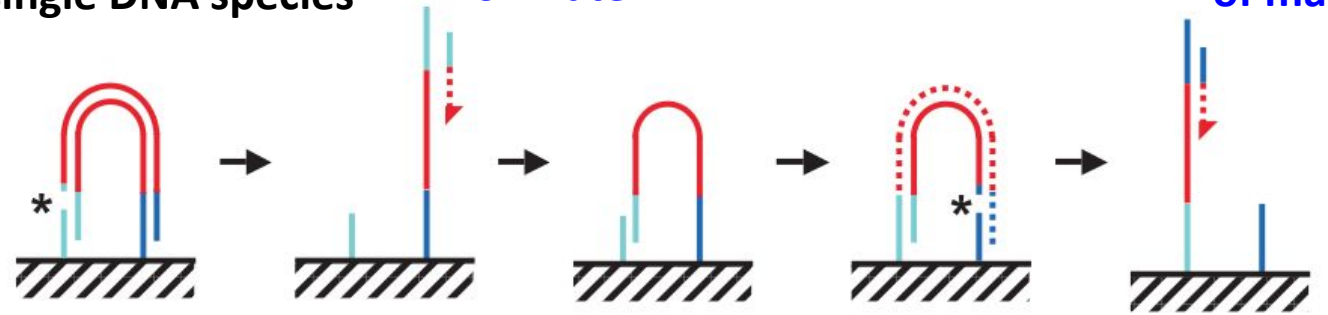


How Does Sequencing Work?

Denaturing
& washing
➔ single DNA species

Sequencing of mate 1

Sequencing of mate 2



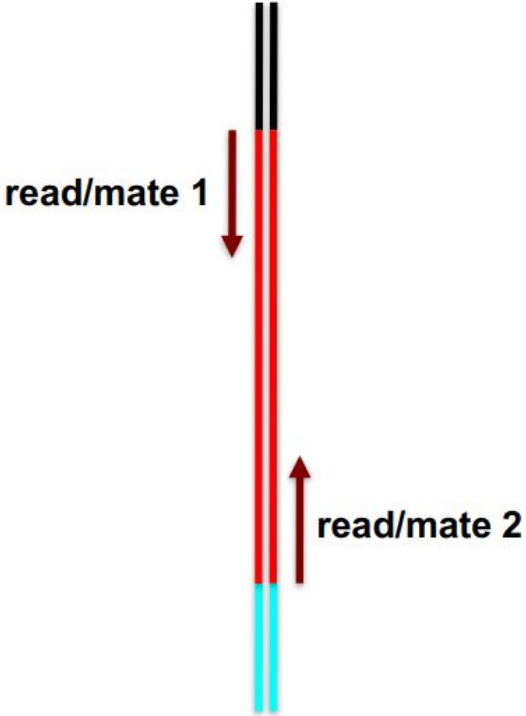
Adaptor-specific DNA-cleavage

Re-synthesis of complementary DNA strand & adaptor-specific DNA-cleavage

Paired End Illumina Sequencing

A short read is sequenced from each end of each fragment

Blue and black are adaptors



Paired End Sequencing

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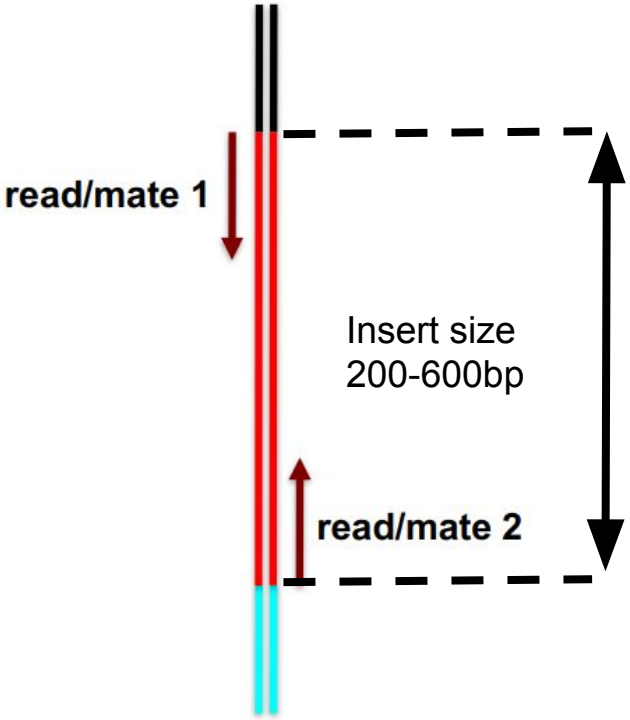
Insert or fragment size is the distance between adaptors



Paired End Sequencing

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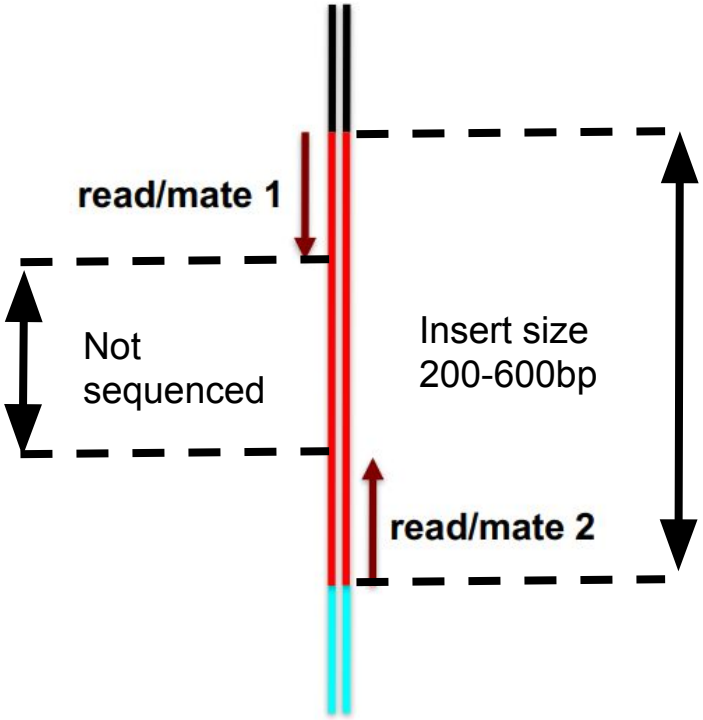
The region between the reads is not sequenced - it is covered by other fragments



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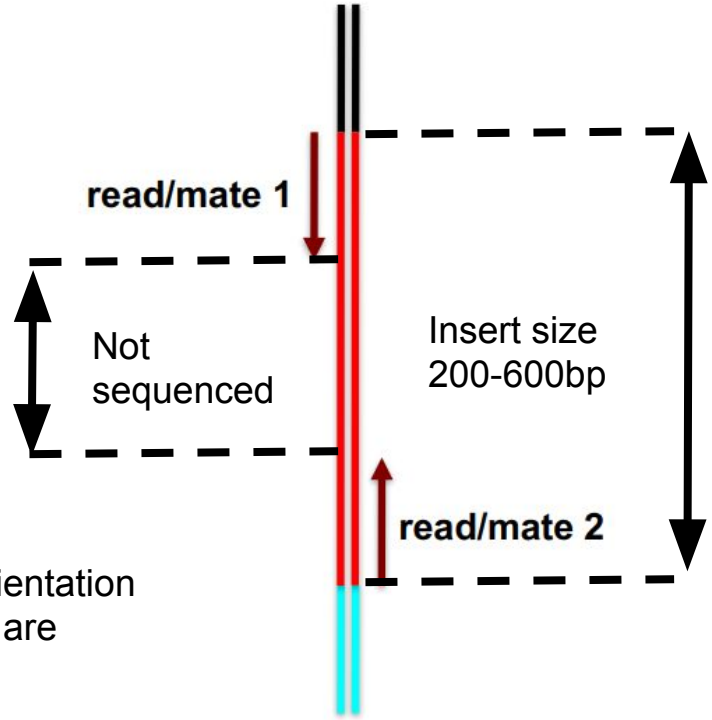

Reads that map in the correct orientation and the expected distance apart are “concordant” or “proper pairs”

Concordant alignments are prioritised

File Formats: FASTQ

```
@NS500205:127:HW272BGXX:1:11101:7788:1040 1:N:0:TCTCGCGC+AGGCTATA
```

For paired-end reads you will have two files

4 lines per read

- Line 1 is a unique header (this will be shared between the pairs)

File Formats: FASTQ

```
@NS500205:127:HW272BGXX:1:11101:7788:1040 1:N:0:TCTCGCGC+AGGCTATA
CCTTGNTCCGTCATATTTTTTAGCATTGCAATGACGCTAAGTCCCGATTGACGCGCACGTGCTCACCCGGTTTCC
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For paired-end reads you will have two files

4 lines per read

- Line 1 is a unique header (this will be shared between the pairs)
- Line 2 is the sequence of the read

File Formats: FASTQ

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CCTTGNTCCGTCATATTTTTTAGCATTGCAATGACGCTAAGTCCCGATTGACGCGCACGTGCTCACCCGGTTTCC
+
AAAAA#EEEEEEEEEEEEEEEEEEEEAEEEEEAEEEEEEEEEEEEEEEEEEEEAEEEEEEEEEEAEEEEEEEEEA
```

For paired-end reads you will have two files

4 lines per read

- Line 1 is a unique header (this will be shared between the pairs)
- Line 2 is the sequence of the read
- Line 4 is the quality for each base
  - Quality is encoded using ASCII
  - http://www.asciitable.com/

Phred quality scores are logarithmically linked to error probabilities

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 10,000	99.99%
50	1 in 100,000	99.999%
60	1 in 1,000,000	99.9999%

Alignment-based Analysis

Quality Control

↓

Read Trimming

↓

Alignment

Analysis of NGS Sequencing

Quality Control

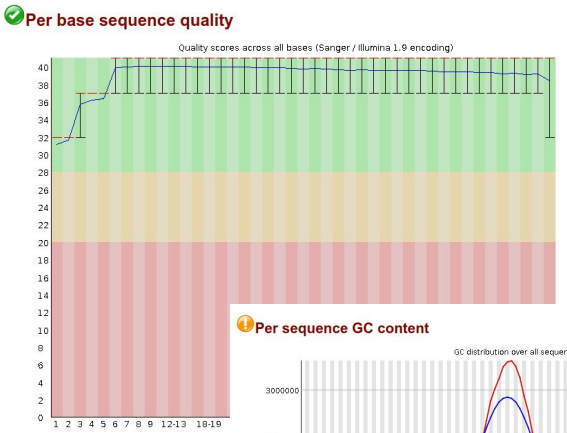
↓

Read Trimming

↓

Alignment

- FASTQC https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
  - Overall sequencing quality
  - GC content
  - N content
  - Read length distribution
  - Over-represented sequences
  - Adaptor content
- Output is an html file that can be opened in a web browser



Overrepresented sequences

Sequence	Count	Percentage	Possible Source
ACAAGTGTGTAACATTAATTTGCAAGTTTGCAACGCTGTTCTTTAGTGTT	70896	0.12562741276052788	No Hit

Analysis of NGS Sequencing

Quality Control

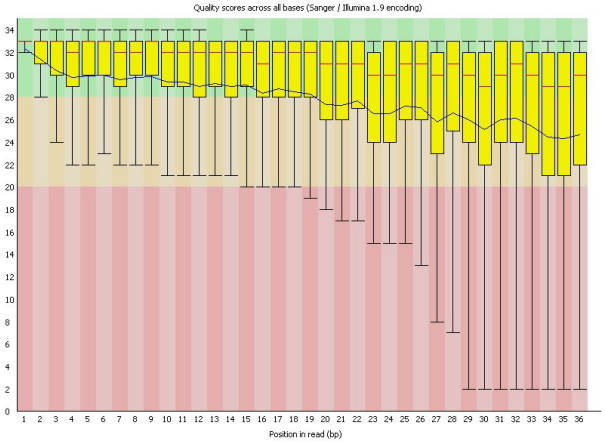
↓

Read Trimming

↓

Alignment

- Trimmomatic https://github.com/usadellab/Trimmomatic
- Sequence quality tends to decrease towards the 3’ end of reads - these can affect mapping
- Sickle will:
  - Remove poor quality reads from the 3’ end of each read
  - Check for reads that are too short and discard them
  - Check that all reads still have a pair and discard those that don’t
- Some trimming tools can also remove adaptors



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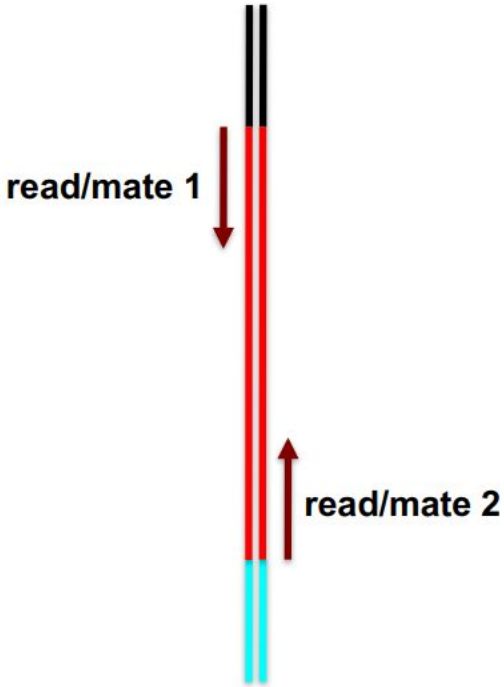

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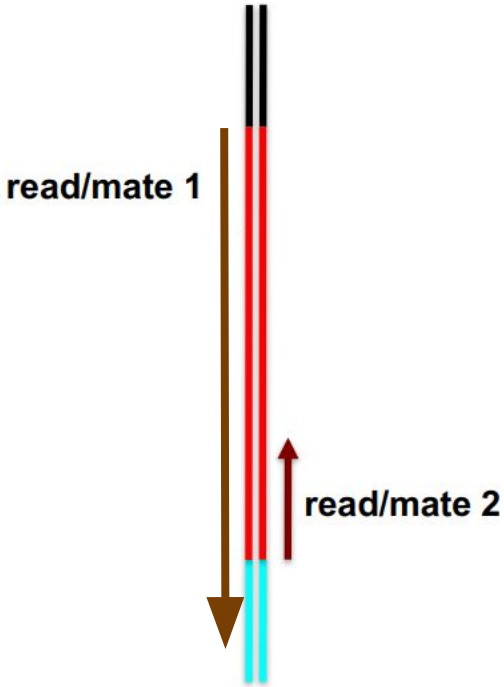

Analysis of NGS Sequencing

Quality Control

↓

Read Trimming

↓

Alignment

What is an alignment?

Two sequences:

```
ATTGAAAGCTA
GAAATGAAAAGG
```

How would you align one to the other?

Analysis of NGS Sequencing

Quality Control

↓

Read Trimming

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Alignment

What is an alignment?

Two sequences:

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How would you align one to the other?

```
--ATTGAAA-GCTA
  | |||||  |
GAAATGAAAAGG

ATTGAAA-GCTA---
   |||| |   |
---GAAATGAAAAGG
```

Which one is better??

Analysis of NGS Sequencing

Quality Control

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Read Trimming

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Alignment

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Two sequences:

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ATTGAAAGCTA
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How would you align one to the other?

```
--ATTGAAA-GCTA
  | |||||  |
GAAATGAAAAGG--

ATTGAAA-GCTA---
   |||| |   |
---GAAATGAAAAGG
```

Which one is better??

Alignment scoring:

- 1 for a match
- -1 for a mismatch
- -2 for a gap

Now alignment 1 scores -4 and alignment 2 scores -10 so we would choose alignment 1

Alignment algorithms are designed to align data in a reasonable time on a standard computer

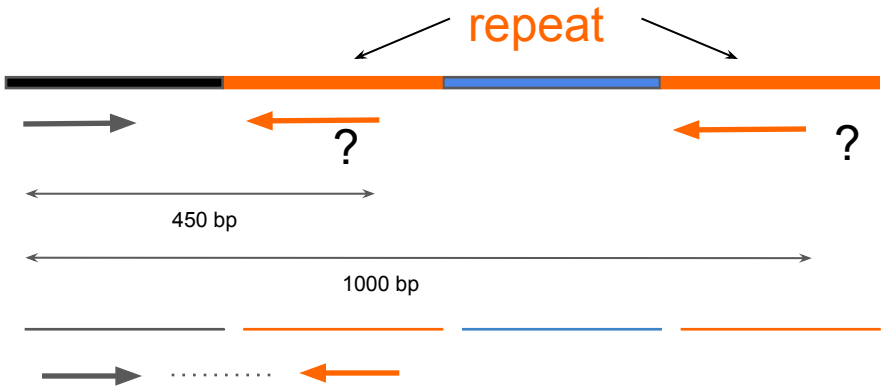
Heuristic - not exhaustive, but “good enough”

Improving Alignments with Paired End Reads

Paired end reads can resolve alignments in repetitive regions

A read that could map in multiple locations due to repetitions in the genome can be located accurately by inference from the position of its pair

In this case, we know that the insert size is ~400 bp, so we can infer that the first alignment is more likely to be correct.



Aligning RNA-seq Data

Quality Control

↓

Read Trimming

↓

Alignment

Spliced mRNA

Aligning RNA-seq Data

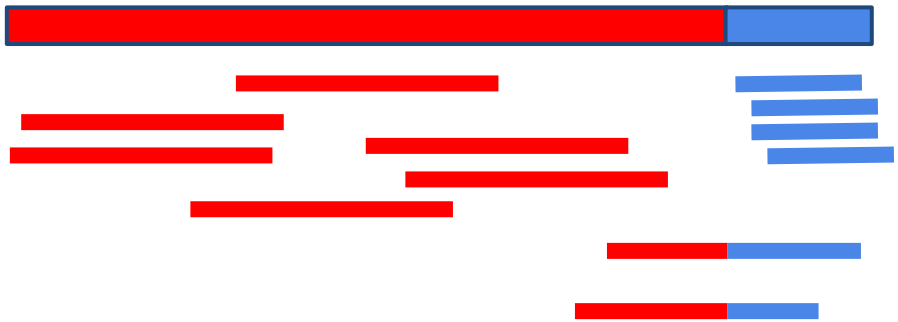
Quality Control

↓

Read Trimming

↓

Alignment



Spliced mRNA

Aligning RNA-seq Data

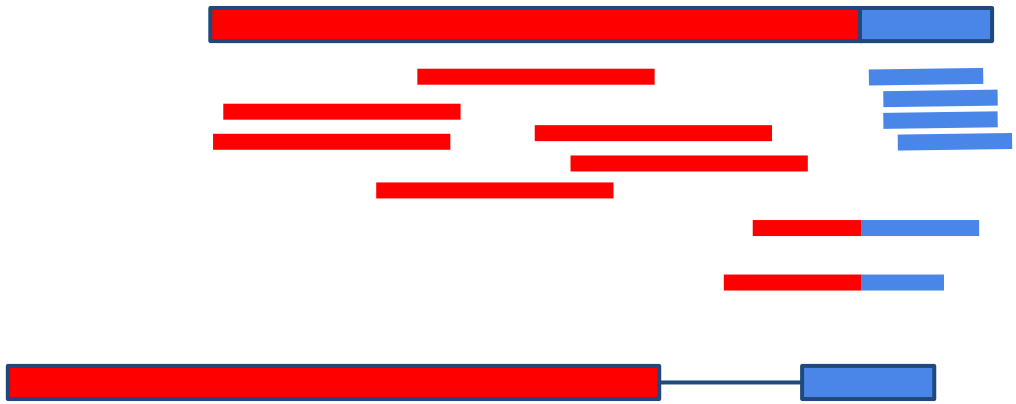
Quality Control

↓

Read Trimming

↓

Alignment



Spliced mRNA

Reference genome (genomic sequence with introns)

Aligning RNA-seq Data

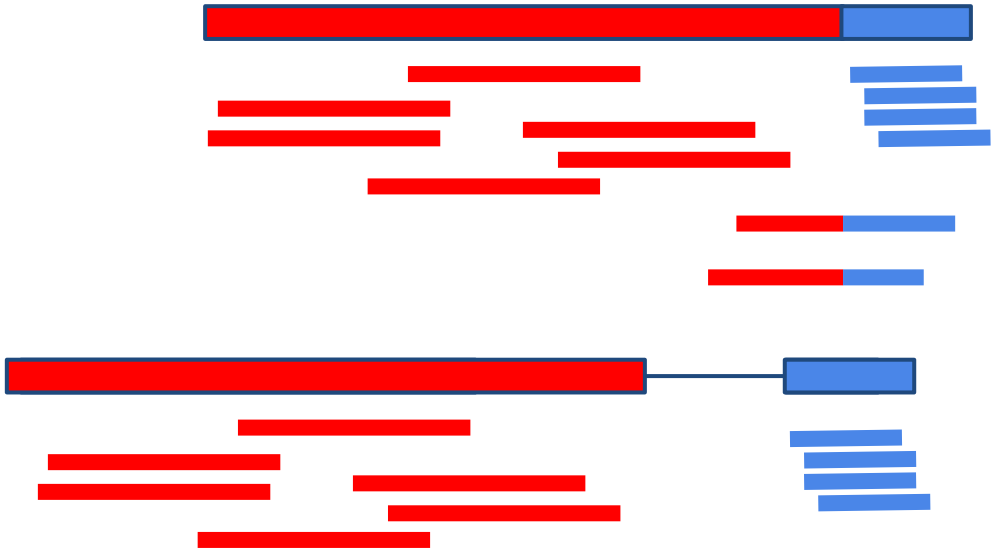
Quality Control

↓

Read Trimming

↓

Alignment



Spliced mRNA

Reference genome

Aligning RNA-seq Data

Quality Control

↓

Read Trimming

↓

Alignment

Alignment Tools

Quality Control

↓

Read Trimming

↓

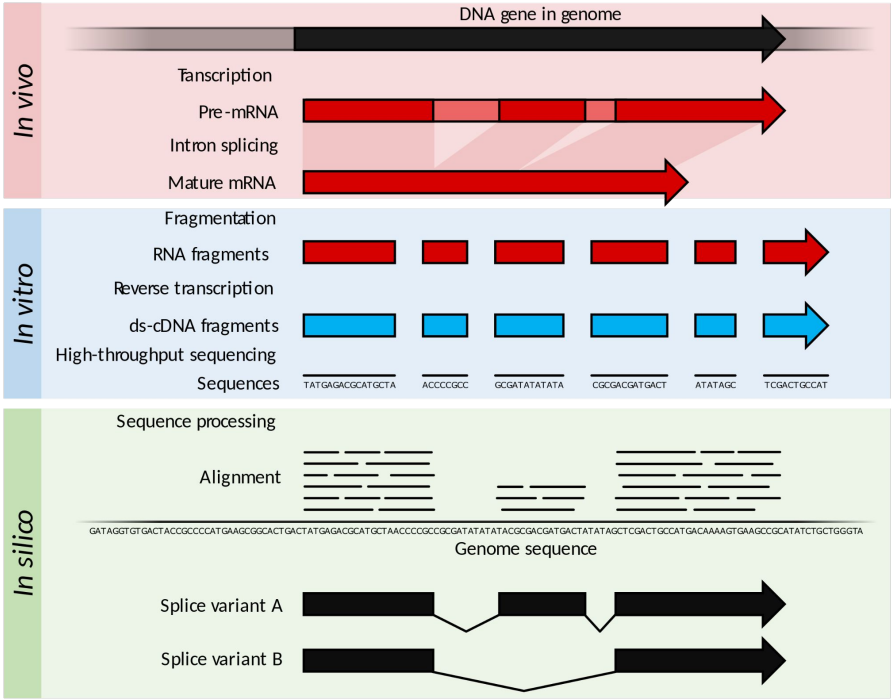
Alignment

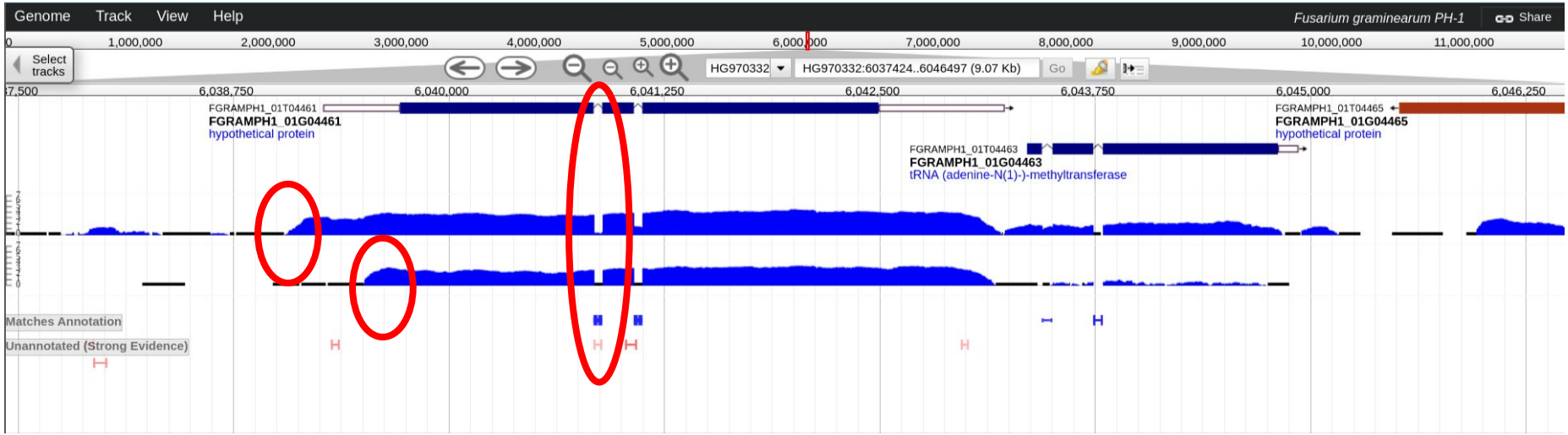
Mapping Tools for RNA-seq data

- Must be capable of aligning intron-spanning reads
- Hisat2
  - Fast, sacrifices sensitivity
  - http://daehwankimlab.github.io/hisat2/
- STAR
  - Very sensitive, but slow
  - https://github.com/alexdobin/STAR

Transcript Sequencing (RNA-seq)

Transcriptome sequencing



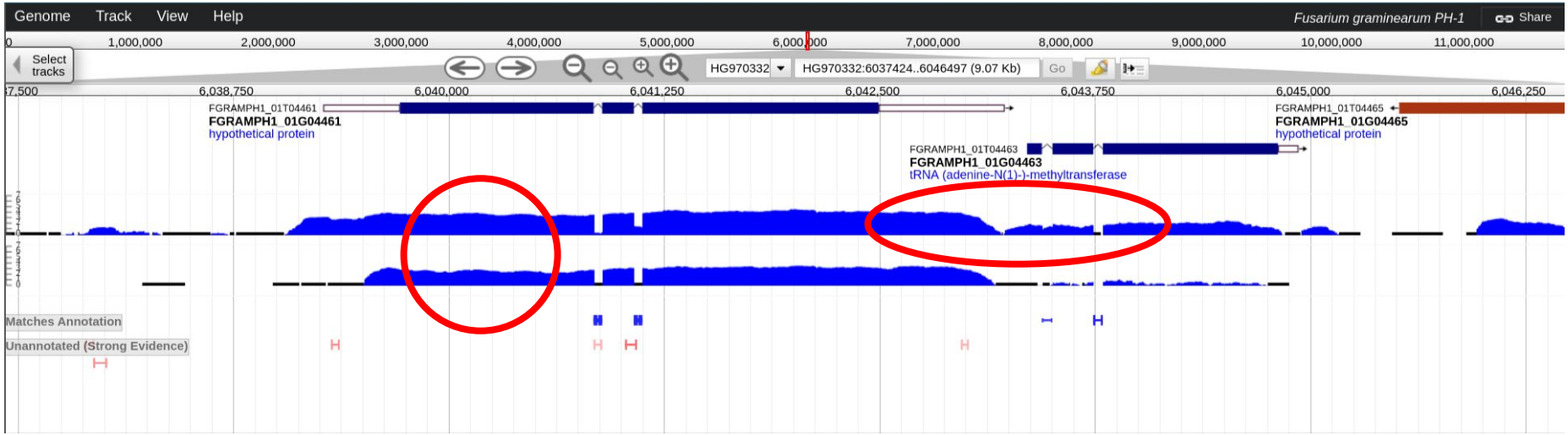


Gene Model Prediction

Alignment of RNA-seq reads to a genomic reference can help us to predict and confirm gene model structure

- Introns can be predicted based on coverage and on individual reads that cross splice junctions
- UTRs can be predicted based on coverage
- Differential splicing can also be predicted from coverage

https://tinyurl.com/mrryern4



Differential Expression

Depth of coverage can help us learn about transcript abundance

- Differential transcript abundance can be observed both within and between samples

https://tinyurl.com/mrryern4

Quantifying Expression

Quality Control

↓

Trimming

↓

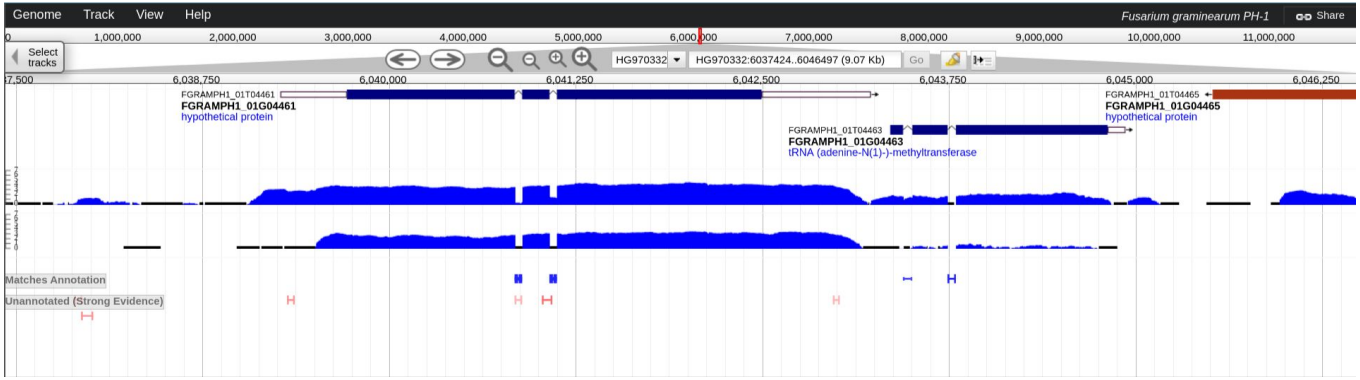
Read Alignment

↓

Read Counting

↓

Differential Expression



Quantifying Expression

We've seen that we can see expression differences in a genome browser

Looking at plots like this is great for one gene, but it is too much to look at every gene individually and is not statistically robust

To examine transcript expression globally and perform robust statistics, we must count how many reads map to each gene.

Quantifying Expression

Quality Control

↓

Trimming

↓

Read Alignment

↓

Read Counting

↓

Differential Expression

	union	intersection _strict	intersection _nonempty
read, gene_A	gene_A	gene_A	gene_A
read, gene_A	gene_A	no_feature	gene_A
read, gene_A, gene_A	gene_A	no_feature	gene_A
read, read, gene_A, gene_A	gene_A	gene_A	gene_A
read, gene_A, gene_B	gene_A	gene_A	gene_A
read, gene_A, gene_B	ambiguous (both genes with --nonunique all)	gene_A	gene_A
read, gene_A, gene_B	ambiguous (both genes with --nonunique all)		
read, ?, gene_A, gene_B	alignment_not_unique (both genes with --nonunique all)		

Read Counting Tools:

htseq-count: https://htseq.readthedocs.io/en/release_0.11.1/count.html

FeatureCounts: https://subread.sourceforge.net/featureCounts.html

Kallisto: https://pachterlab.github.io/kallisto/

Normalisation

Quality Control

↓

Trimming

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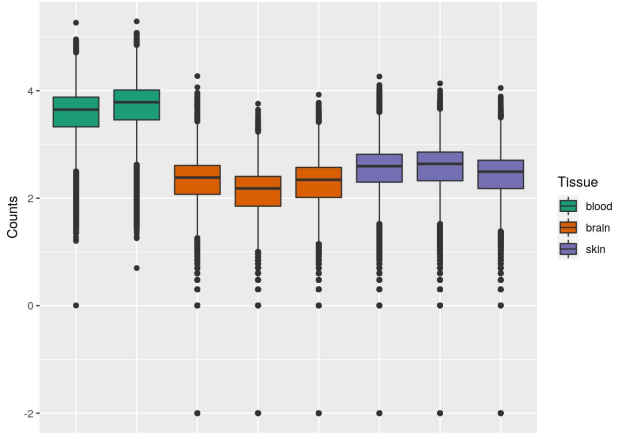
Read Alignment

↓

Read Counting

↓

Differential Expression

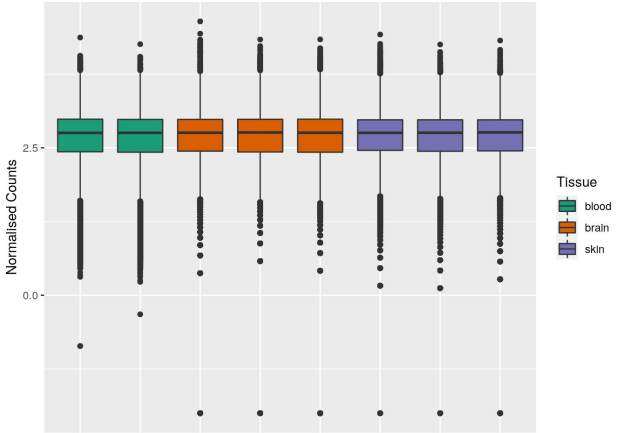


Raw count data

Each box represents is one sample and shows the distribution of read counts for each gene

Normalised count data

After normalising, count distributions are aligned so individual genes can be compared



With Quantitative Data We Can…

Quality Control

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Trimming

↓

Read Alignment

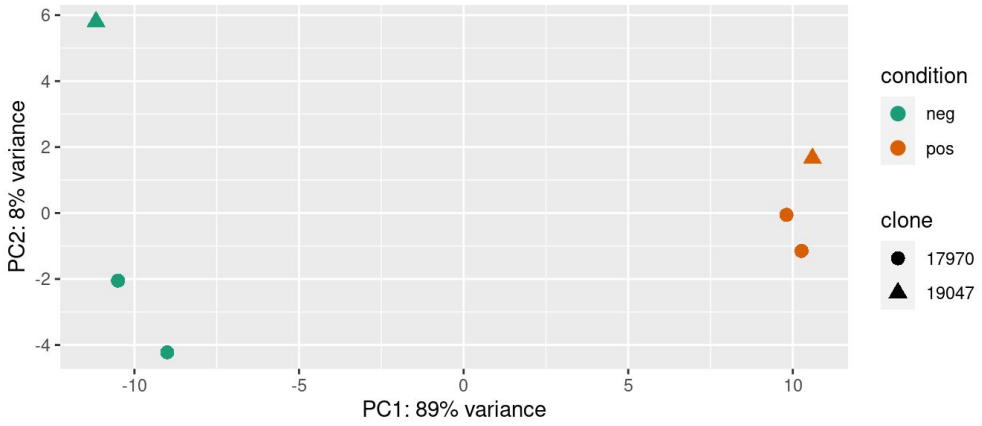
↓

Read Counting

↓

Differential Expression

- Explore our data



With Quantitative Data We Can…

Quality Control

↓

Trimming

↓

Read Alignment

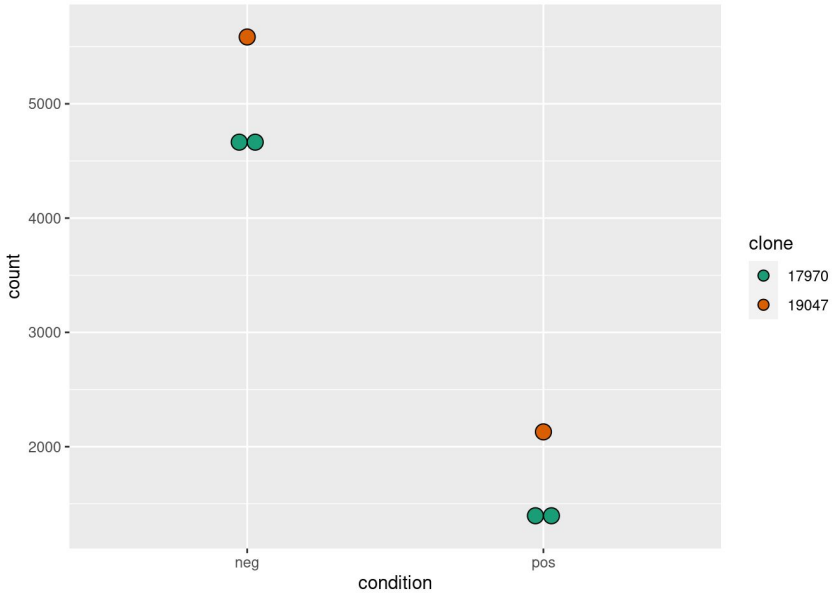
↓

Read Counting

↓

Differential Expression

- Explore our dataset
- Look at expression for individual genes



With Quantitative Data We Can…

Quality Control

↓

Trimming

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Read Alignment

↓

Read Counting

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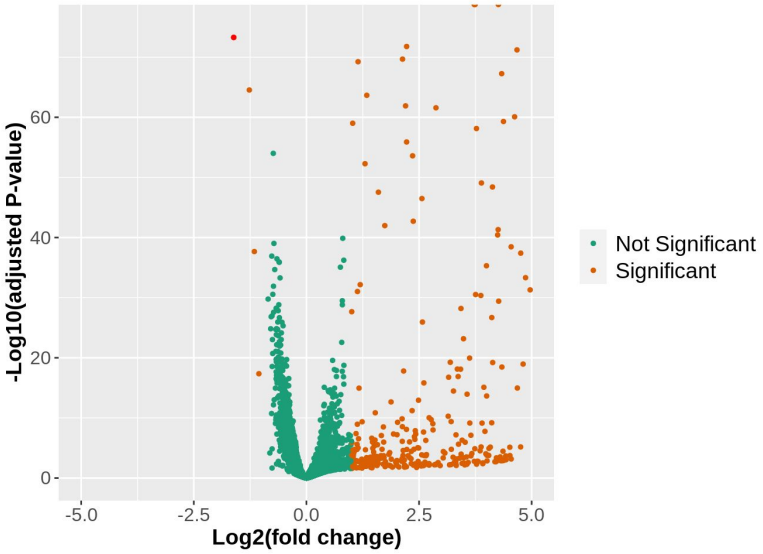
Differential Expression

- Explore our dataset
- Look at expression for individual genes
- Do pairwise statistical tests (differential expression)



With Quantitative Data We Can…

Quality Control

↓

Trimming

↓

Read Alignment

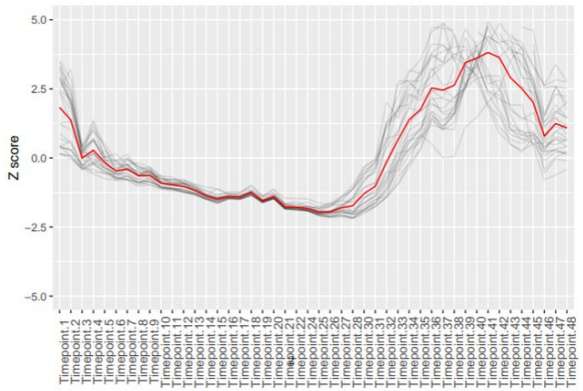
↓

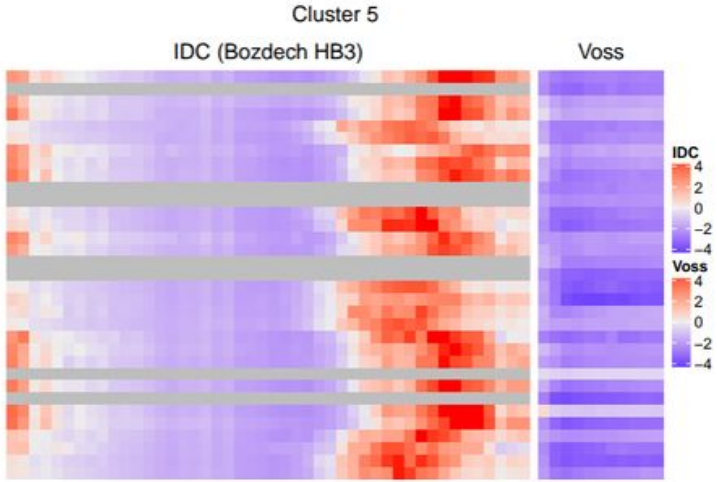
Read Counting

↓

Differential Expression

- Explore our dataset
- Look at expression for individual genes
- Do pairwise statistical tests (differential expression)
- Do advanced analysis (clustering, coexpression, etc.)

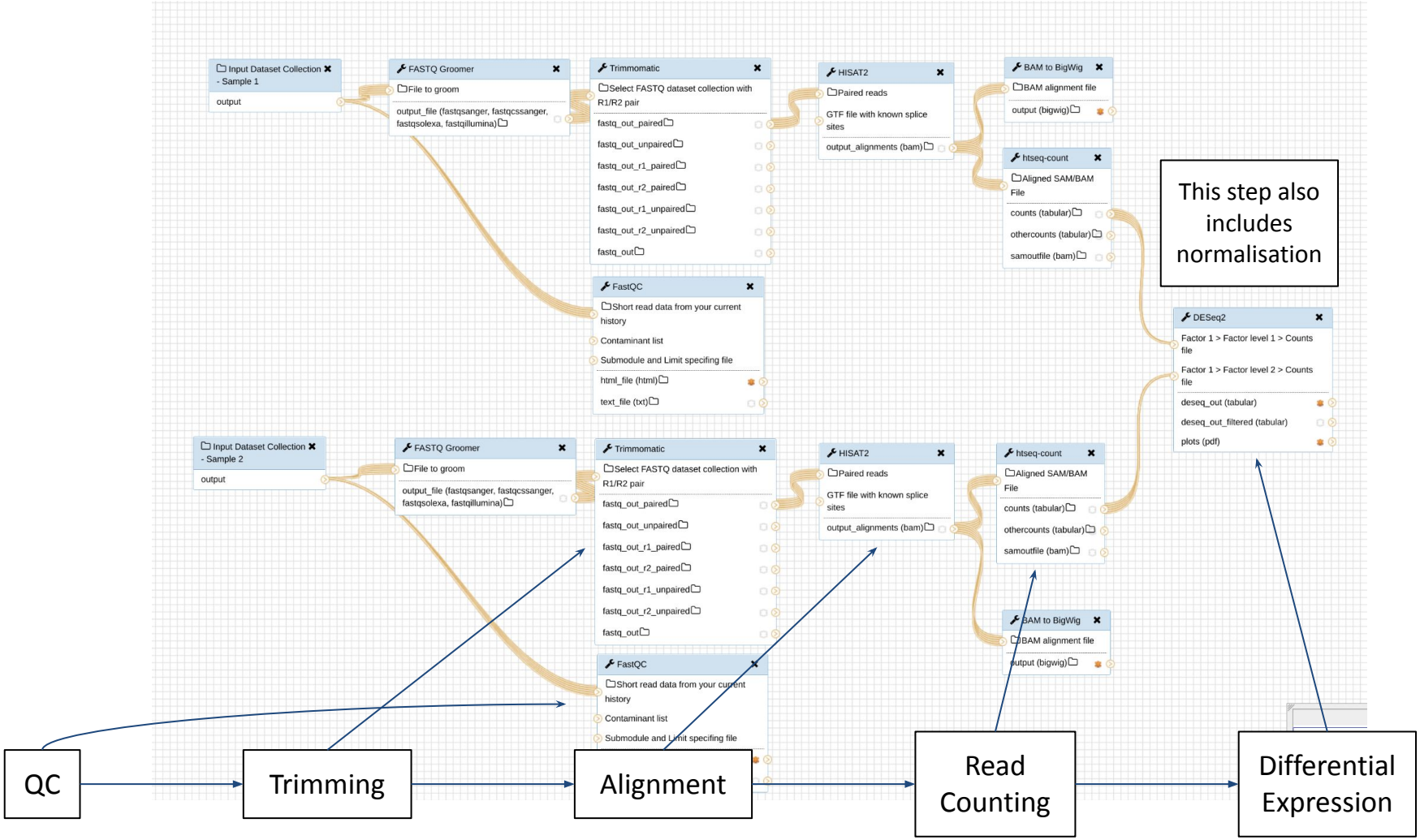




This session

1. Set up workflow for RNA-seq
2. Set up workflow for SNP calling (we will talk about this later)
3. Let them run overnight - we’ll look at the output tomorrow
4. Once they are running you can log off

Galaxy


Input Dataset Collection - Sample 1
output
FASTQ Groomer
File to groom
output_file (fastqsanger, fastqcssanger, fastqsolexa, fastqillumina)
Trimmomatic
Select FASTQ dataset collection with R1/R2 pair
fastq_out_paired
fastq_out_unpaired
fastq_out_r1_paired
fastq_out_r2_paired
fastq_out_r1_unpaired
fastq_out_r2_unpaired
fastq_out
HISAT2
Paired reads
GTF file with known splice sites
output_alignments (bam)
BAM to BigWig
BAM alignment file
output (bigwig)
htseq-count
Aligned SAM/BAM File
counts (tabular)
othercounts (tabular)
samoutfile (bam)
This step also includes normalisation
FastQC
Short read data from your current history
Contaminant list
Submodule and Limit specifing file
html_file (html)
text_file (txt)
DESeq2
Factor 1 > Factor level 1 > Counts file
Factor 1 > Factor level 2 > Counts file
deseq_out (tabular)
deseq_out_filtered (tabular)
plots (pdf)
Input Dataset Collection - Sample 2
output
FASTQ Groomer
File to groom
output_file (fastqsanger, fastqcssanger, fastqsolexa, fastqillumina)
Trimmomatic
Select FASTQ dataset collection with R1/R2 pair
fastq_out_paired
fastq_out_unpaired
fastq_out_r1_paired
fastq_out_r2_paired
fastq_out_r1_unpaired
fastq_out_r2_unpaired
fastq_out
HISAT2
Paired reads
GTF file with known splice sites
output_alignments (bam)
htseq-count
Aligned SAM/BAM File
counts (tabular)
othercounts (tabular)
samoutfile (bam)
BAM to BigWig
BAM alignment file
output (bigwig)
FastQC
Short read data from your current history
Contaminant list
Submodule and Limit specifing file
QC
Trimming
Alignment
Read Counting
Differential Expression