RNA sequencing
VEuPathDB Workshop 2021

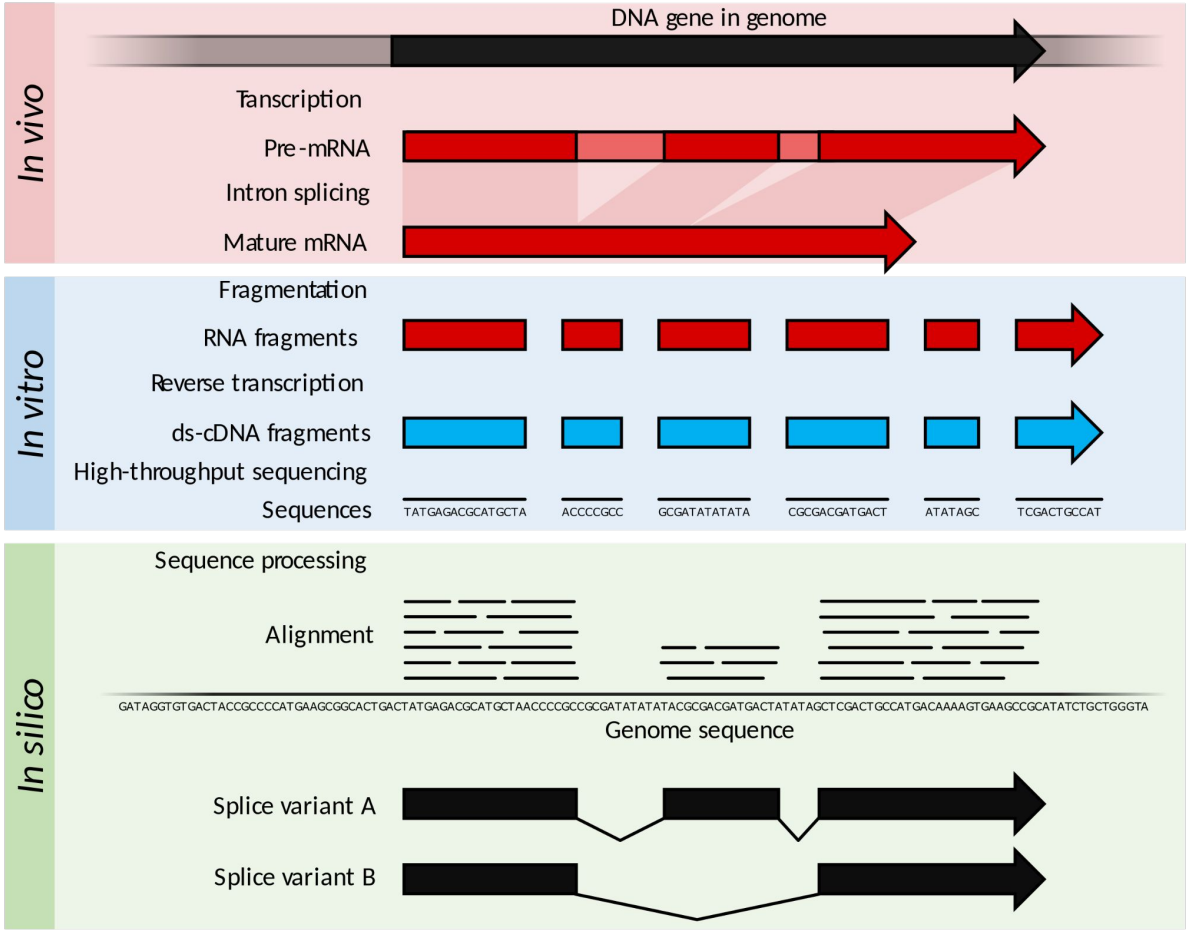
Kathryn Crouch
kathryn.crouch@glasgow.ac.uk

Why do we want to sequence the transcriptome?

Why do we want to sequence the transcriptome?

- Capture full unbiased view of full repertoire of transcripts for gene model prediction
- Functional studies for conditions such as stress and drug resistance
- Explore alternative splicing and complex patterns of expression and regulation

Transcriptome sequencing



Experimental Considerations

- Do you have enough biological replicates?
- Do you have enough RNA?
- Is the RNA what you want?
- Are you using an appropriate selection method?
- Are you interested in strand differentiation?
- Do you have appropriate controls?

Experimental Considerations

- Do you have enough biological replicates?
  - At least three are recommended

- Do you have enough RNA?
- Is the RNA what you want?
- Are you using an appropriate selection method?
- Are you interested in strand differentiation?
- Do you have appropriate controls?

Experimental Considerations

- Do you have enough biological replicates?
- Do you have enough RNA?
  - If you want to capture rare transcripts, you will need to sequence more deeply

- Is the RNA what you want?
- Are you using an appropriate selection method?
- Are you interested in strand differentiation?
- Do you have appropriate controls?

Experimental Considerations

- Do you have enough biological replicates?
- Do you have enough RNA?
- Is the RNA what you want?
  - If you are sequencing clinical or environmental samples, consider the abundance of the target organism

- Are you using an appropriate selection method?
- Are you interested in strand differentiation?
- Do you have appropriate controls

Experimental Considerations

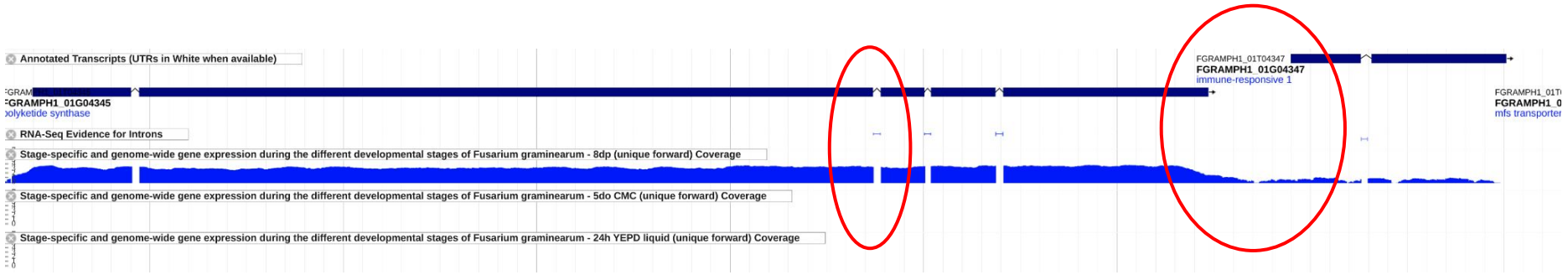
- Do you have enough biological replicates?
- Do you have enough RNA?
- Is the RNA what you want?
- Are you using an appropriate selection method?
  - PolyA selection is common - but not all RNA species are poly-adenylated
- Are you interested in strand differentiation?
- Do you have appropriate controls

Experimental Considerations

- Do you have enough biological replicates?
- Do you have enough RNA?
- Is the RNA what you want?
- Are you using an appropriate selection method?
- Are you interested in strand differentiation?
  - Stranded library kits allow you to distinguish the strand from which the sequencing read originated.

- Do you have appropriate controls

What Can We Learn from RNA-seq?



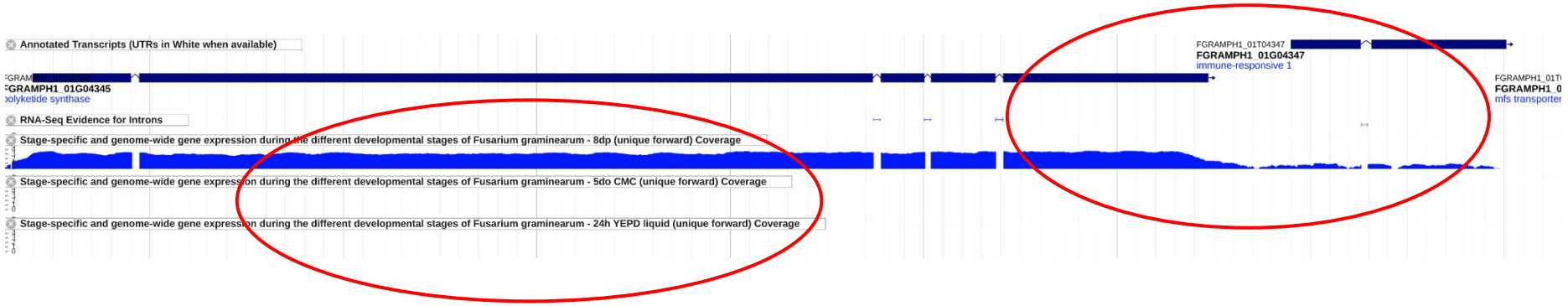
Gene Model Prediction

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/mven959f

What Can We Learn from RNA-seq?



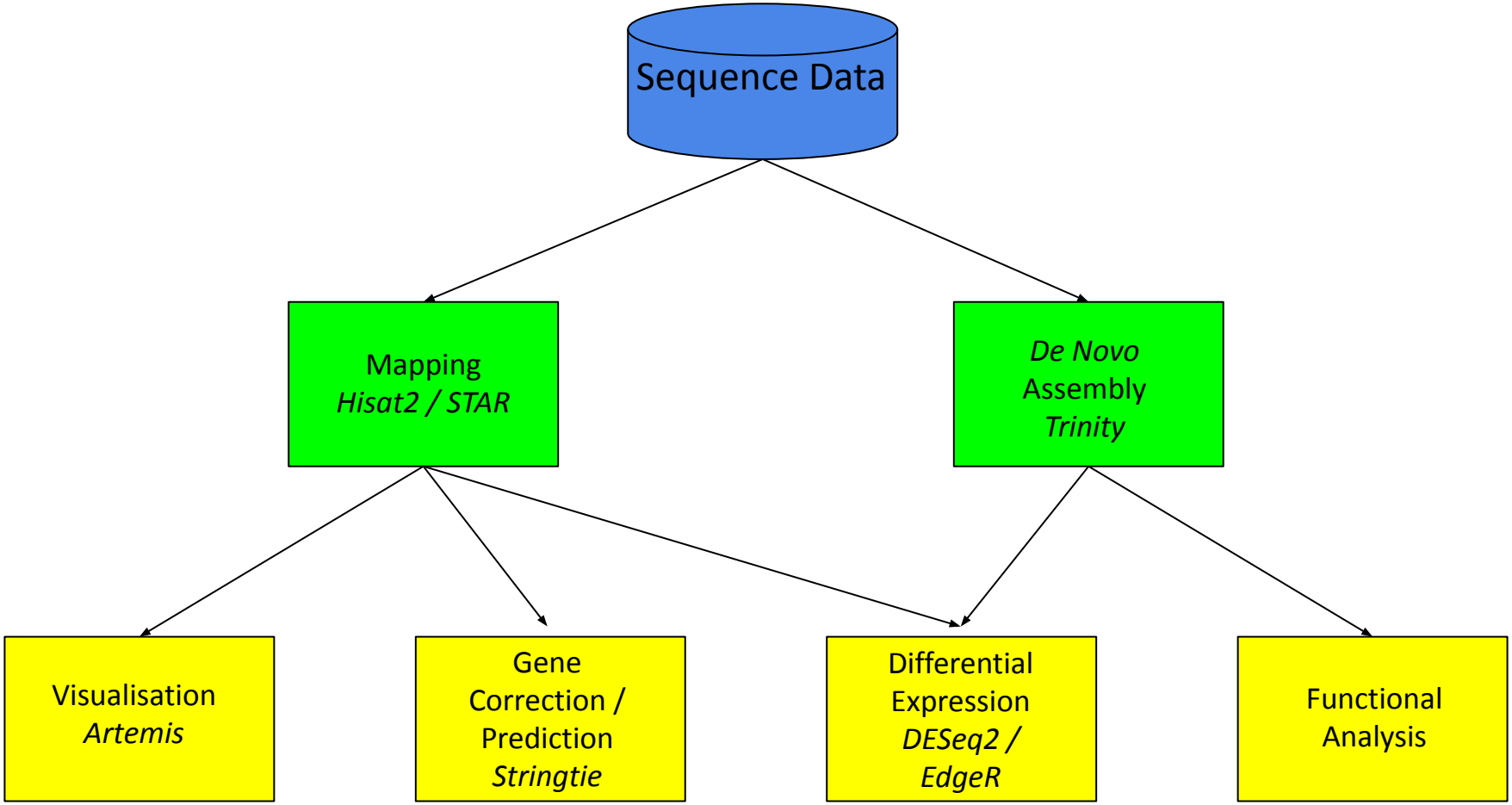
Differential Expression

Depth of coverage can help us learn about transcript abundance

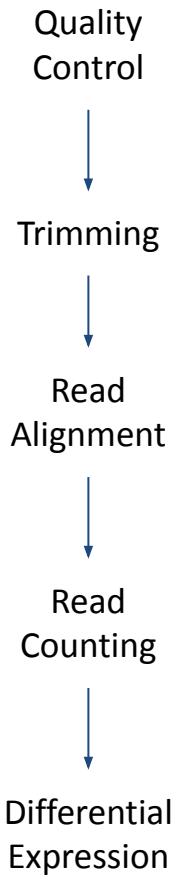
- Differential transcript abundance can be observed both within and between samples

https://tinyurl.com/mven959f

How do we get to that point?



Differential Expression Analysis



File Formats: FASTQ

```
@NS500205:127:HW272BGXX:1:11101:7788:1040 1:N:0:TCTCGCGC+AGGCTATA
CCTTGNTCCGTCATATTTTTTAGCATTGCAATGACGCTAAGTCCCGATTGACGCGCACGTGCTCACCCGGTTTCC
+
AAAAA#EEEEEEEEEEEEEEEEEEEAEEEEEAEEEEEEEEEEEEEEEEEEEAEEEEEEEEEEAEEEEEEEEEA
```

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%

Differential Expression Analysis

Quality Control

↓

Trimming

↓

Read Alignment

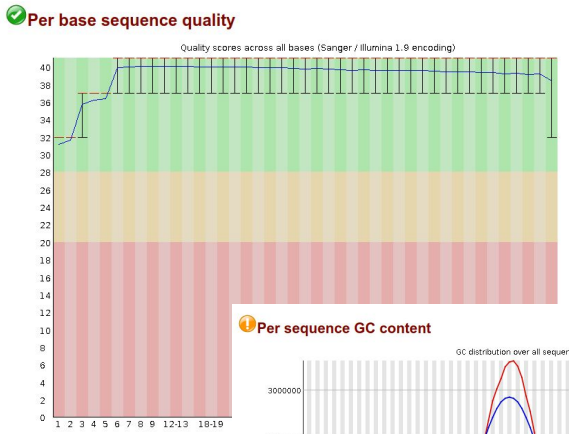
↓

Read Counting

↓

Differential Expression

- 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

Differential Expression Analysis

Quality Control

↓

Trimming

↓

Read Alignment

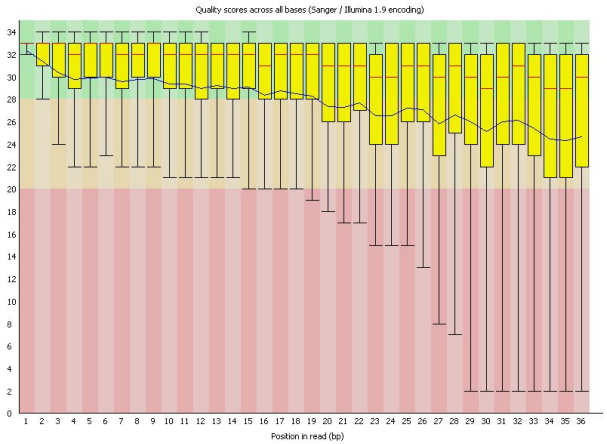
↓

Read Counting

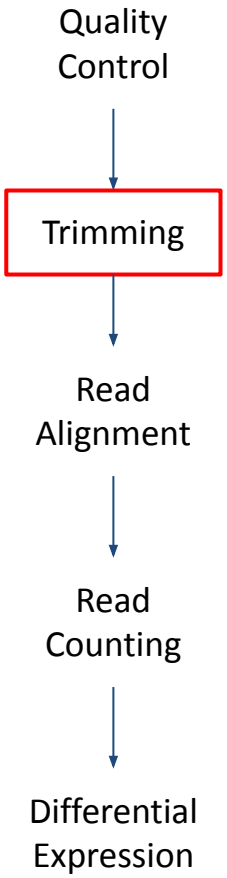
↓

Differential Expression

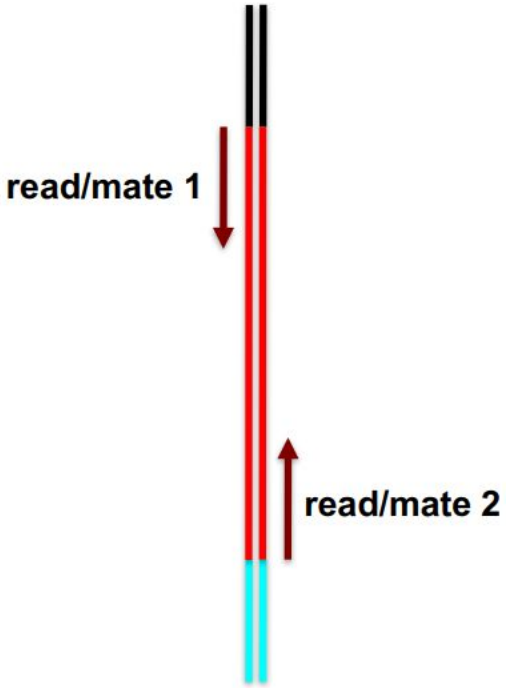
- Trimmomatic http://www.usadellab.org/cms/?page=trimmomatic
- Sequence quality tends to decrease towards the 3' end of reads - these can affect mapping
- Trimmomatic will:
  - Remove poor quality bases from the 3' end of each read
  - Check for reads that are too short and discard them
  - Check that all reads still have a pair and discard those that don't
- Some trimming tools can also remove adaptors



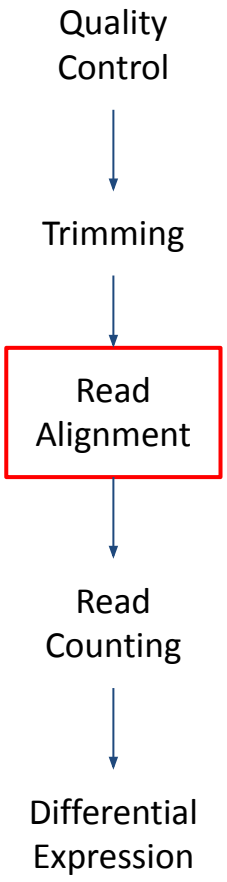
Differential Expression Analysis



- Sickle https://github.com/najoshi/sickle
- 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



Differential Expression Analysis



What is an alignment?

Two sequences:

```
ATTGAAAGCTA
GAAATGAAAAGG
```

How would you align one to the other?

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

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

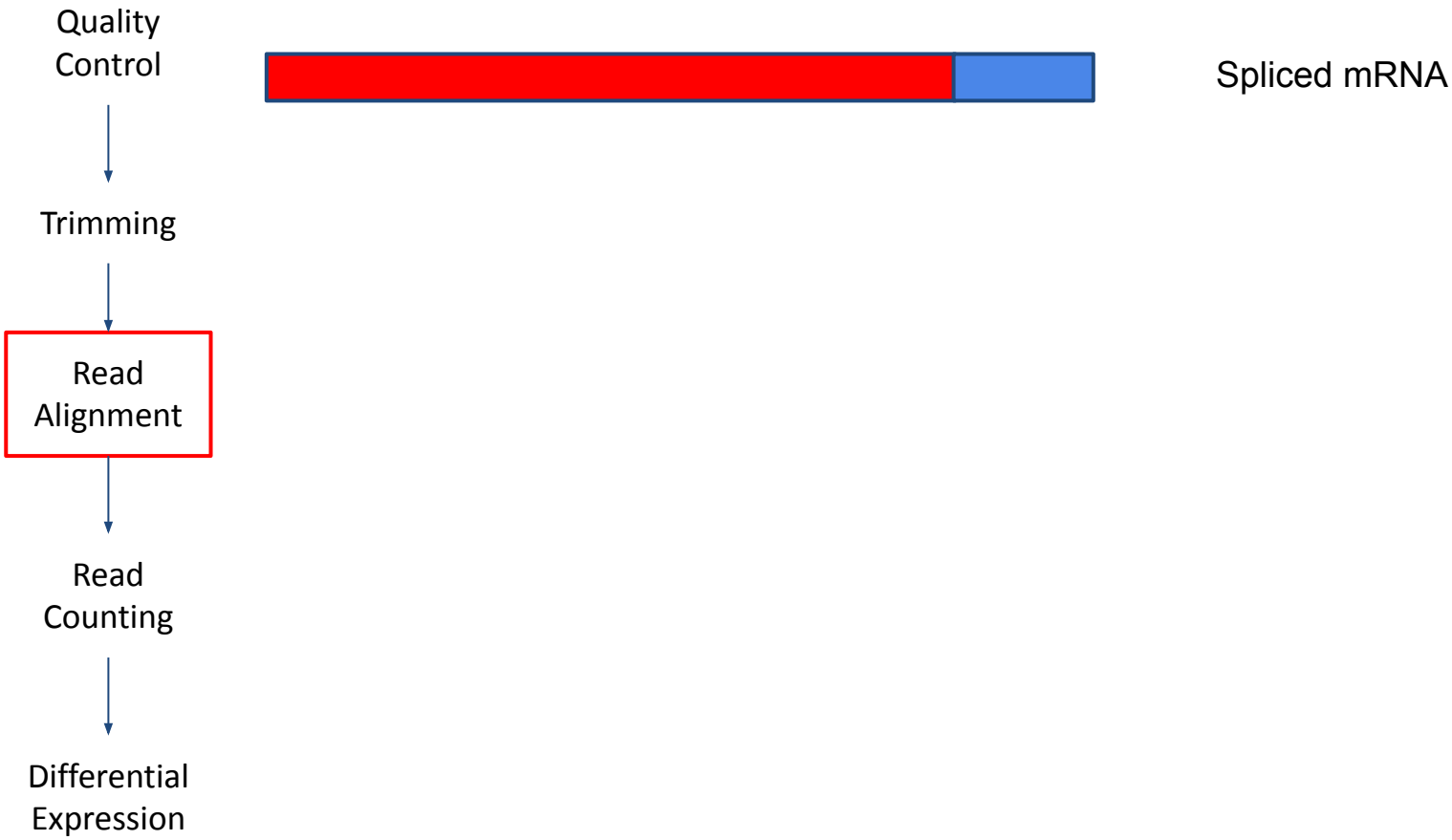
Which one is better??

Alignment scoring:

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

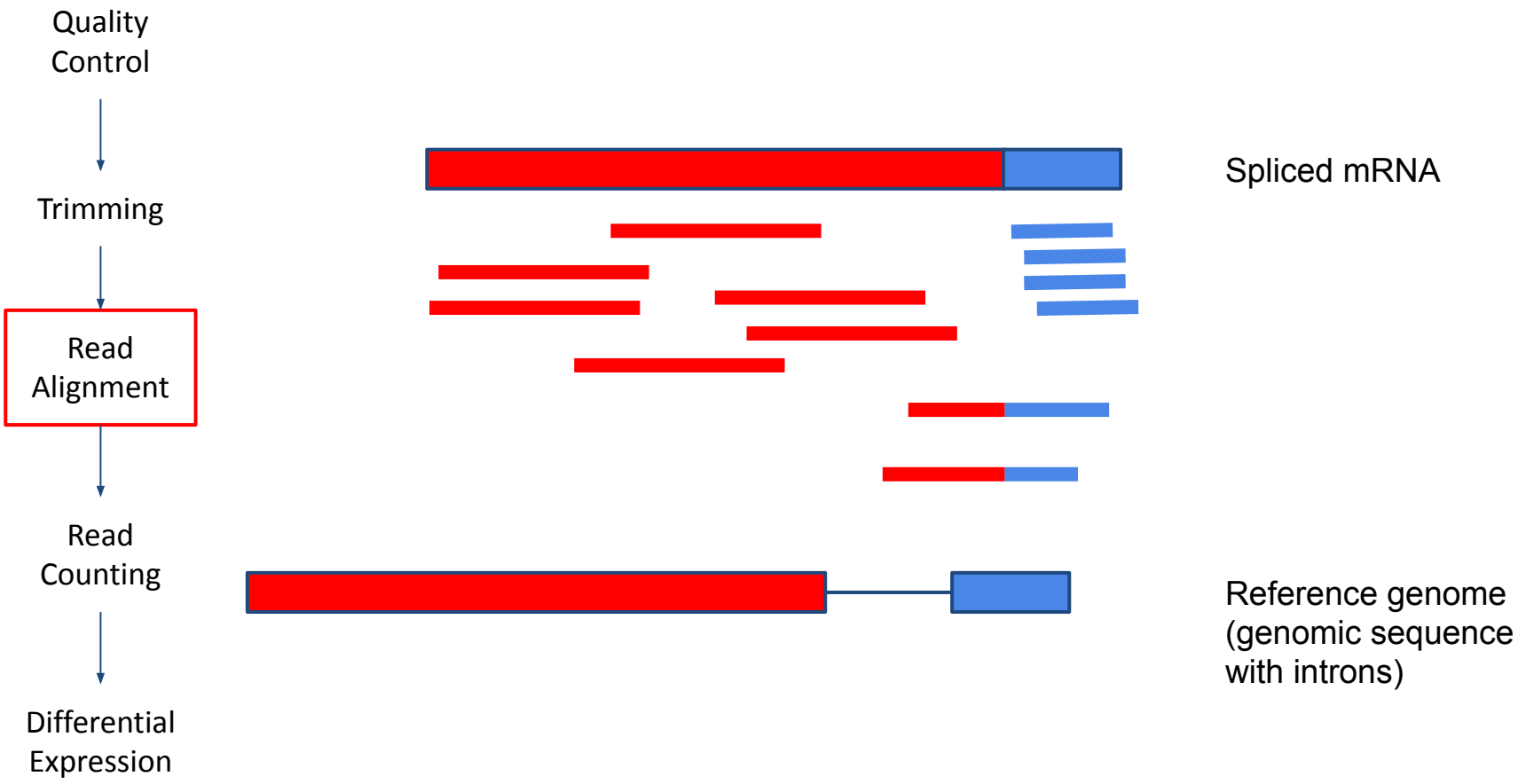
Now alignment 1 scores -4 and alignment 2 scores -10 so we would choose alignment 1

Differential Expression Analysis

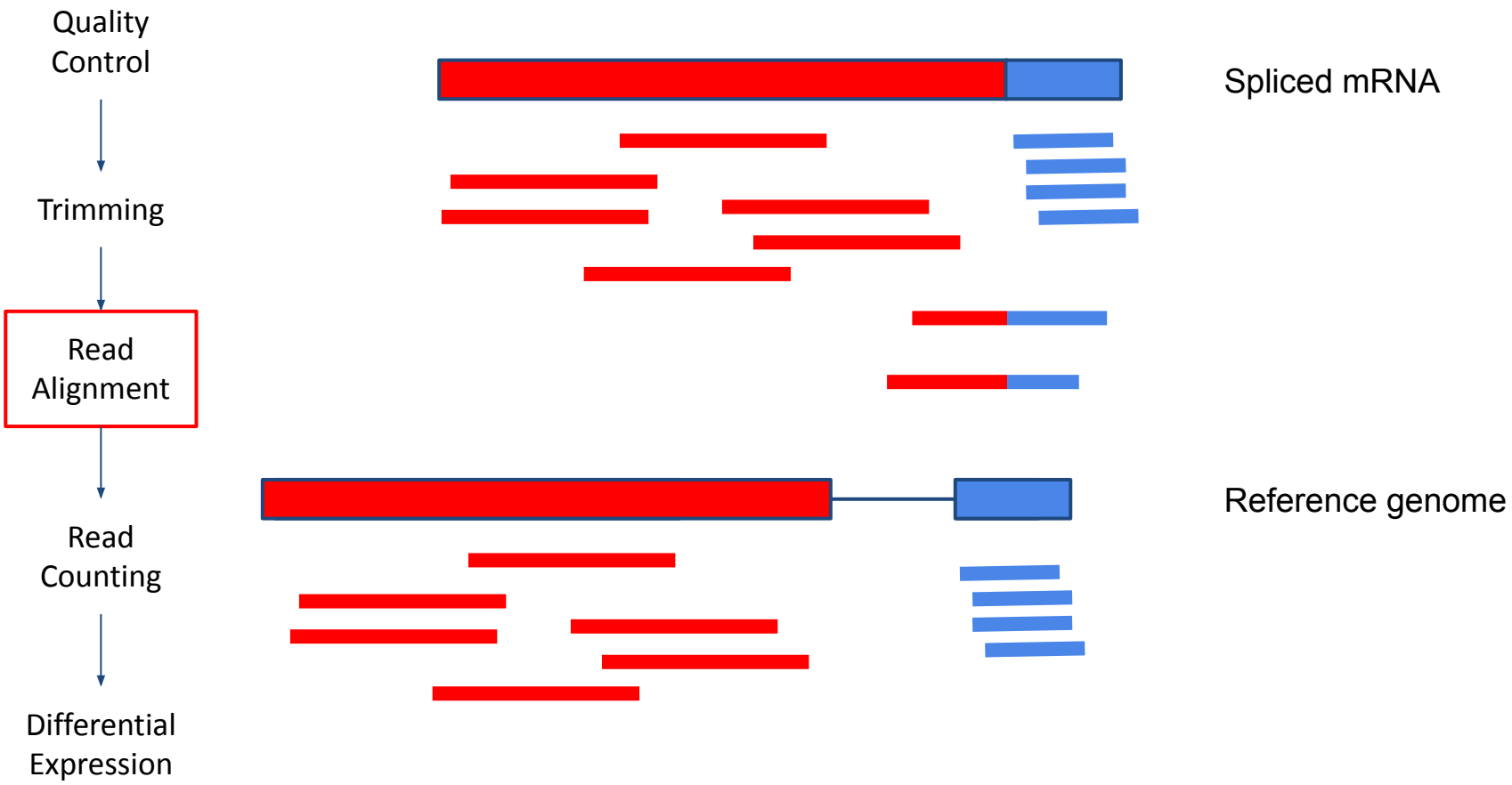


Differential Expression Analysis

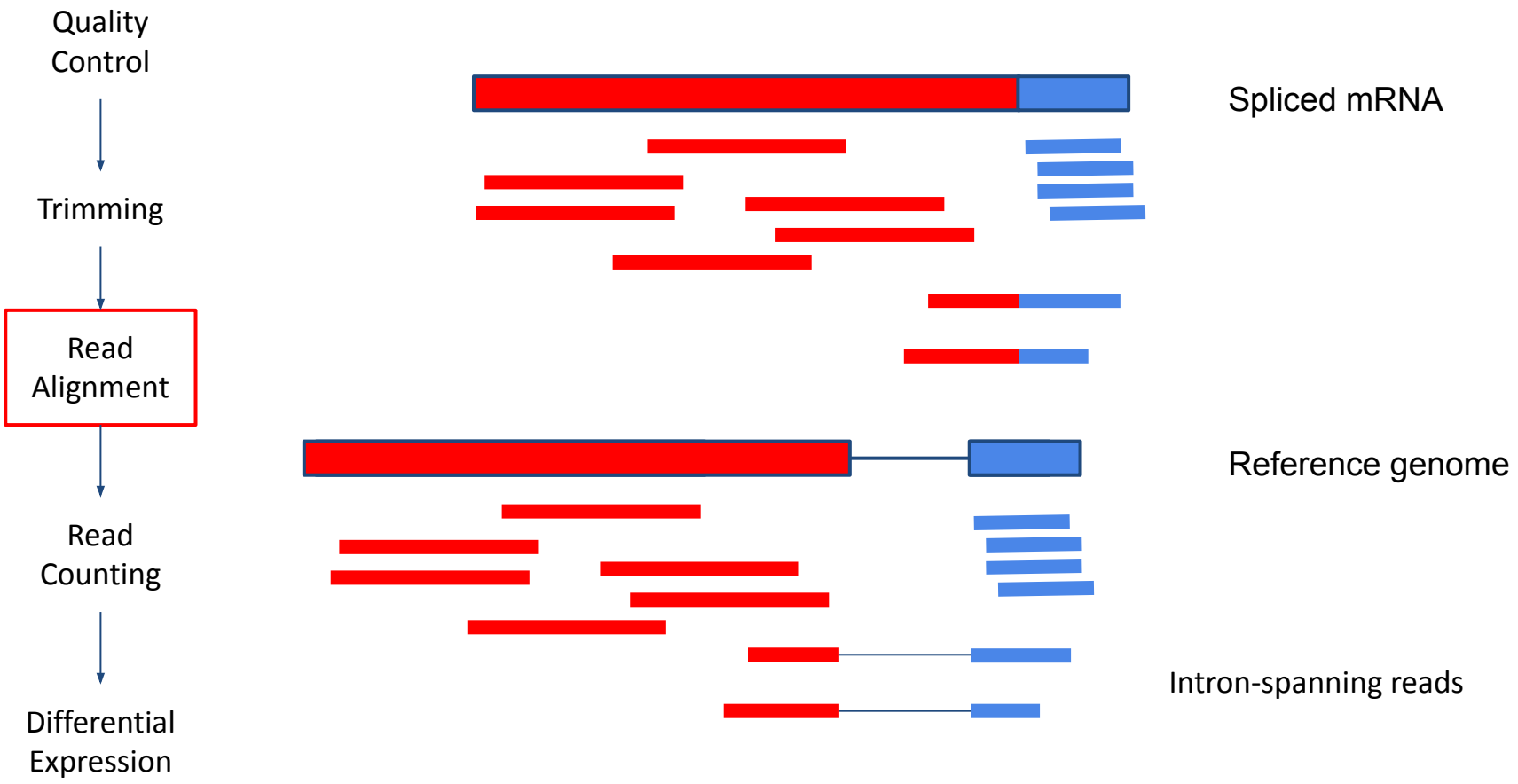
Differential Expression Analysis



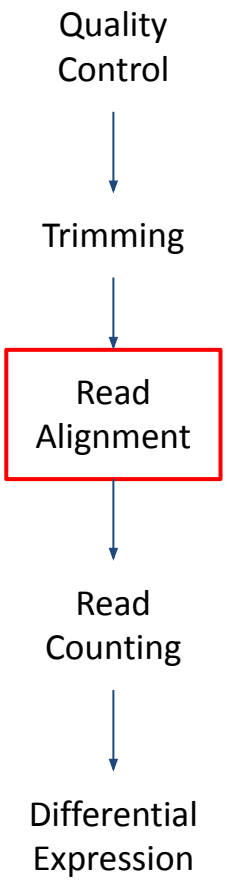
Differential Expression Analysis



Differential Expression Analysis



Differential Expression Analysis



Mapping Tools for RNA-seq data

- Capable of aligning millions of reads to a genome
  - In a reasonable amount of time
  - Using a reasonable amount of memory
- Use heuristic algorithms
  - "Good enough" not perfect
- 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

Differential Expression Analysis

Quality Control

↓

Trimming

↓

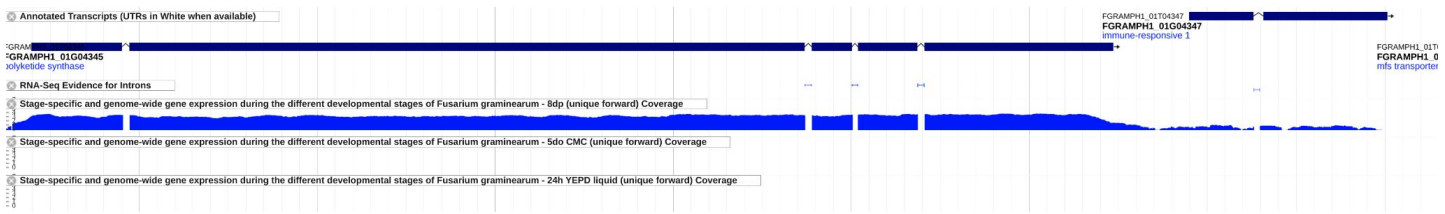
Read Alignment

↓

Read Counting

↓

Differential Expression



Quantifying Expression

At this point, we can load our alignment into a genome browser and look at expression

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.

Differential Expression Analysis

Quality Control

↓

Trimming

↓

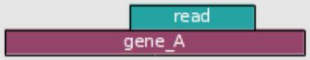
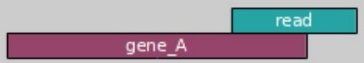
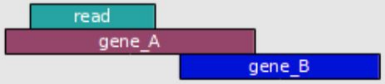
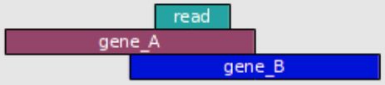
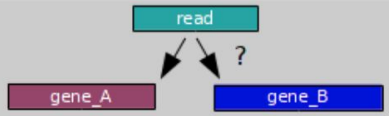
Read Alignment

↓

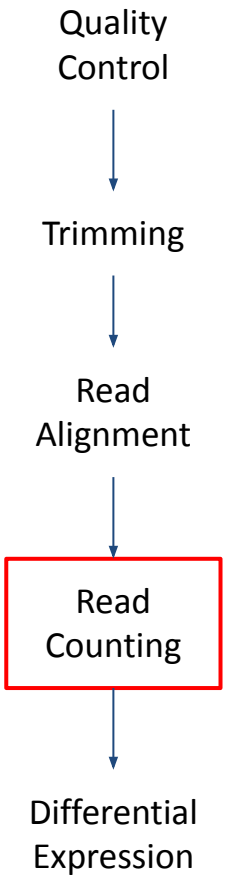
Read Counting

↓

Differential Expression

	union	intersection_strict	intersection_nonempty
	gene_A	gene_A	gene_A
	gene_A	no_feature	gene_A
	gene_A	no_feature	gene_A
	gene_A	gene_A	gene_A
	gene_A	gene_A	gene_A
	ambiguous (both genes with --nonunique all)	gene_A	gene_A
	ambiguous (both genes with --nonunique all)		
	alignment_not_unique (both genes with --nonunique all)		

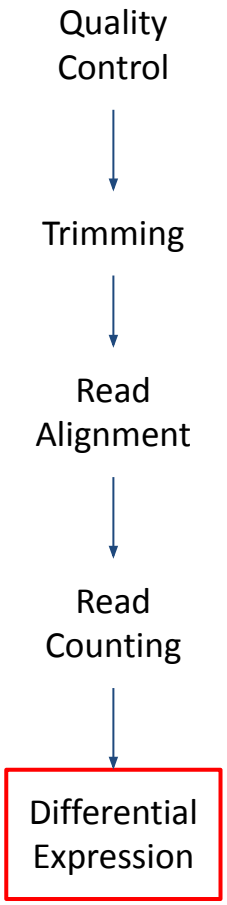
Differential Expression Analysis



Counting Reads Mapped to Genes

- Counting can be strand-specific with a stranded library
- Decide whether to count:
  - Only reads that align uniquely to one feature (stringent, most common)
  - Reads that can be aligned to multiple features (might be useful for multigene families)
- Decide at what level to count reads
  - Per gene - robust and easy to analyse but lacks information about differential isoform expression
  - Per transcript - may allow identification of differential expression of known isoforms. Harder to interpret.
  - Per exon - may allow identification of differential expression of novel isoforms. Harder to interpret
- Htseq-count https://htseq.readthedocs.io/en/release_0.11.1/count.html
- featureCounts http://subread.sourceforge.net/

Differential Expression Analysis



Differential Expression

- Compare conditions to see which transcripts differ in abundance
  - Knockout/knockdown/mutant vs wild-type
  - Different lifecycle or cell cycle stages
  - Different nutrient sources
  - Virulent vs avirulent strains
- Sequence coverage depth is used as a proxy for transcript abundance
  - We've already seen how we can visualise this in the alignment
  - Counting the reads mapping to each gene gives us a means to quantify this
- Normalisation for sequencing depth must be carried out
  - Need to account for the total number of reads sequenced
  - Sequencing more reads from one sample will increase the number of reads mapped to each gene even if relative transcript abundance has not changed

Differential Expression Analysis

Quality Control

↓

Trimming

↓

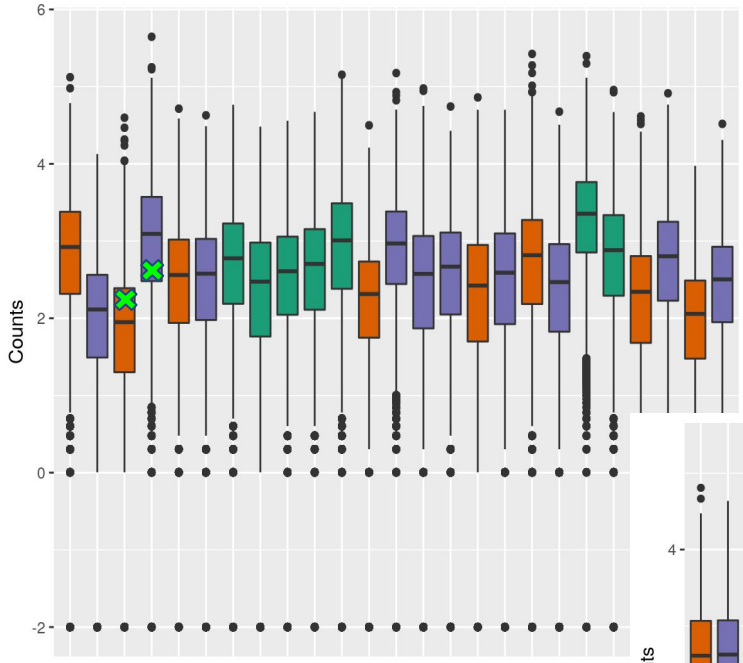
Read Alignment

↓

Read Counting

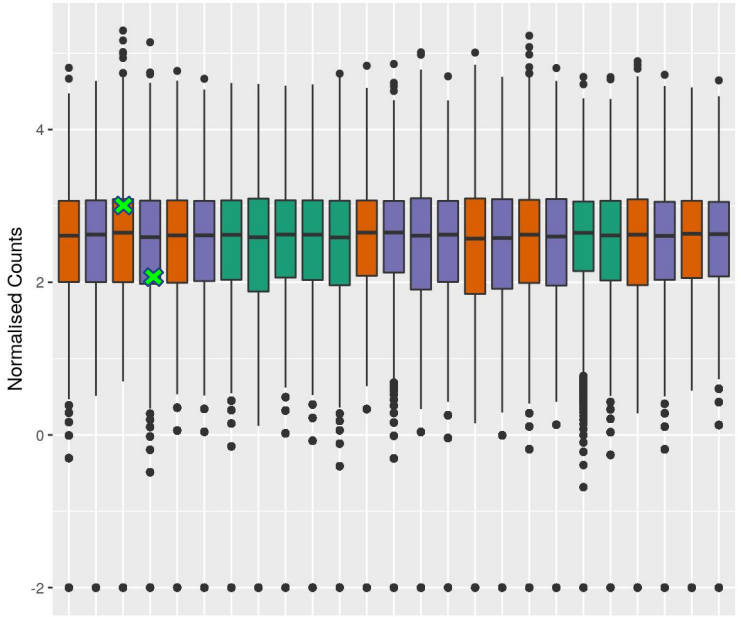
↓

Differential Expression



Raw count data

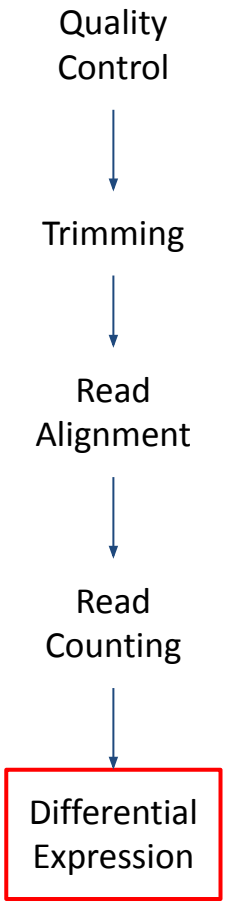
Each box represents is one sample and shows the distribution of read counts for each gene



Normalised count data

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

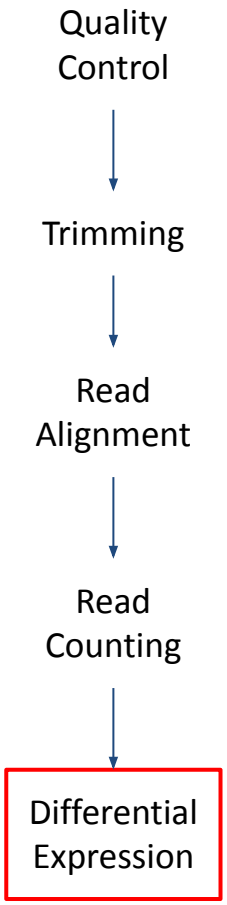
Differential Expression Analysis



Differential Expression - Statistical Analysis

- Robust statistical comparison of quantitative differences in transcript abundance
  - Single Factor experiments comparing two conditions
  - Multi Factor experiments investigating multiple experimental conditions
- RNA-seq data is not normally distributed.
  - Most packages assume a negative binomial distribution
  - Some packages may transform the data to fit it better to this model
  - Common tests used are Wald test (DESeq2) and F-test (EdgeR)
  - Log Ratio test can be useful for time courses
- Output is a table, which reports for each gene
  - Log2 fold-change - a measure of the magnitude and direction of differential expression
  - P-value
  - Q-value (FDR-adjusted P-value)

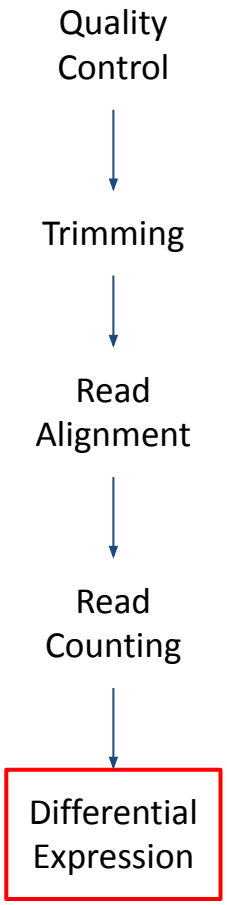
Differential Expression Analysis



Differential Expression - Error Types

- Type II error
  - "False negative", "acquitting a criminal"
  - Falsely accepting a null hypothesis
- Type I error
  - "False positive", "convicting an innocent person"
  - Falsely rejecting a null hypothesis
  - Potentially dangerous in science as it results in false discovery
  - Inherently linked to significance level - if you set a p-value cutoff at 0.01 you accept a positive result has a 1 in 100 chance of being a false positive
- If you test 10,000 genes using a p-value cutoff of 0.01, you expect to find 100 false positives
  - Q-value is an adjustment of the P-values from individual tests to reflect this
  - **ALWAYS USE THE Q-VALUE**

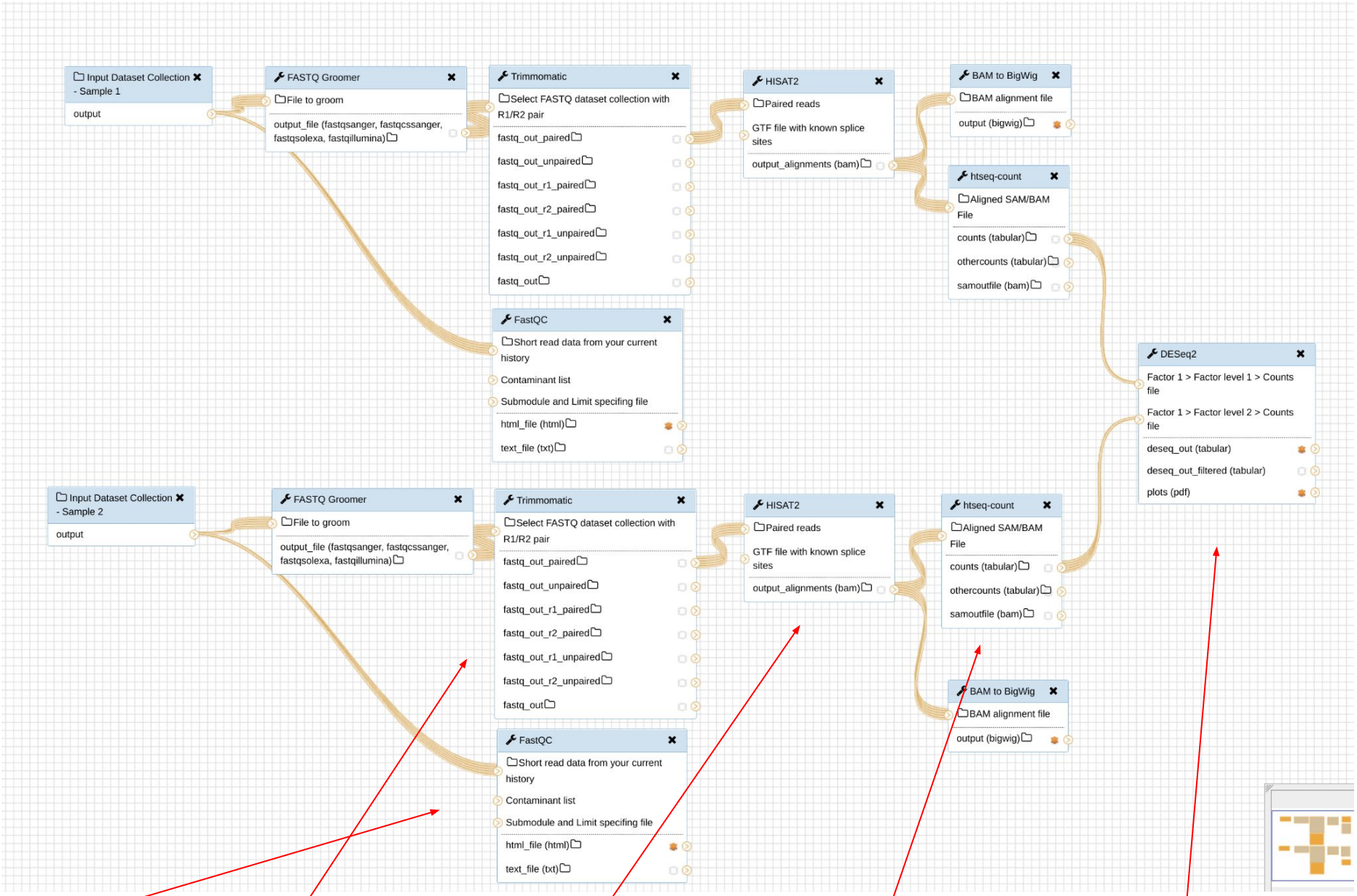
Differential Expression Analysis



Differential Expression - Statistical Analysis

- Tools for differential expression analysis usually combine data normalisation, data transformation and statistical testing into a single package
  - For this reason, it is best to use one package for the whole workflow if you aren't sure

- Some common tools include:
  - DESeq2: https://bioconductor.org/packages/release/bioc/html/DESeq2.html
  - EdgeR: https://bioconductor.org/packages/release/bioc/html/edgeR.html
  - Limma: https://bioconductor.org/packages/release/bioc/html/limma.html



QC → Trimming → Alignment → Read Counting → Differential Expression