Using transcriptomics to identify routes to survival under stress in bacteria

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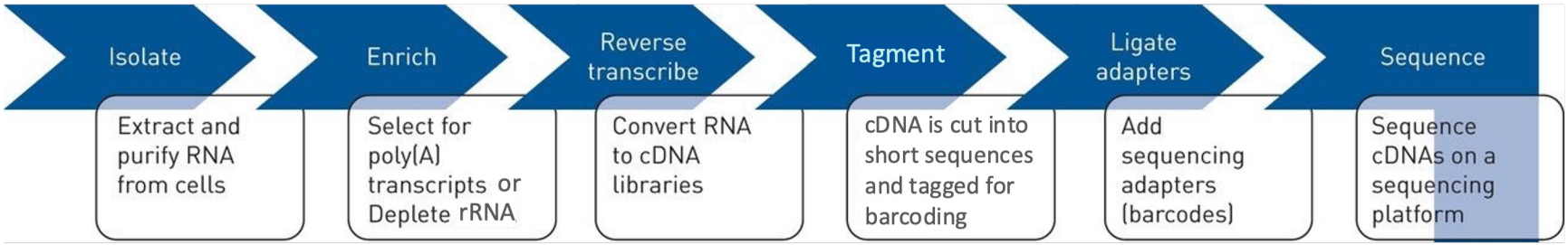
Transcriptomics

- Transcriptomics is set of techniques which use NGS of mRNA transcripts to study global gene expression
- By quantifying which genes are being upregulated or downregulated relative to a control, the molecular basis underpinning physiological responses to specific conditions can be interrogated
- RNA-seq relies on purification of total RNA from cells, depletion of ribosomal RNA (or enrichment of mRNA) and conversion of mRNA to complementary DNA with reverse transcriptase
- When cDNA is obtained, sequencing libraries can be generated in the same manner as genomic DNA
- Sequencing by synthesis (Illumina sequencing) is the most common platform for transcriptomics
- Unlike genomic sequencing which expresses sequencing volume as genome coverage or read depth, transcriptomic sequencing just uses total read count (5 – 20 million reads is the standard read count for bacterial RNA-seq)



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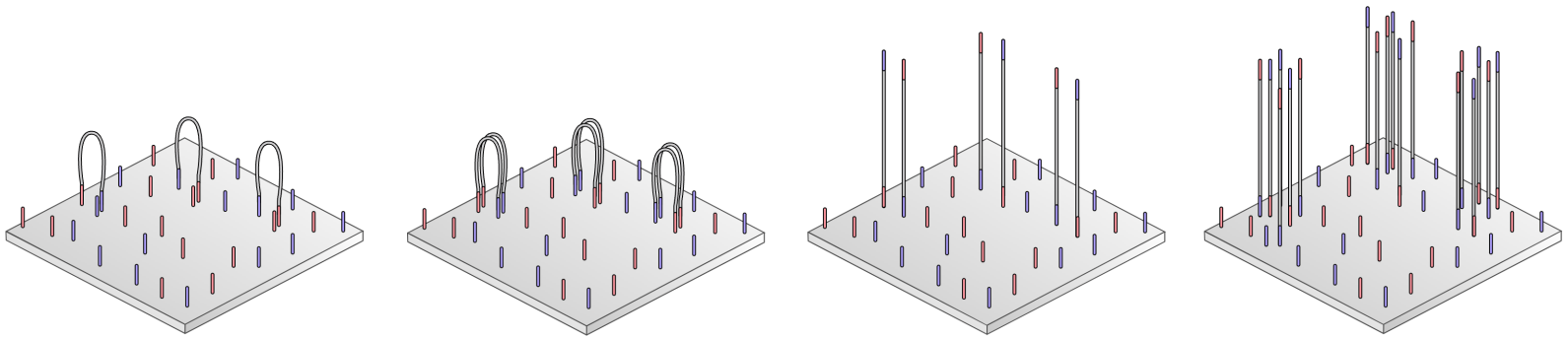
Illumina Sequencing

- DNA is fragmented into ~350 bp sequences and tagged with primers which allow sequencing adapters to be ligated and fragments are amplified by PCR to generate a ‘sequencing library’
- The libraries are normalized to a standard concentration and pooled together, with the unique combination of index adapters allowing for the pool to be demultiplexed back into their respective libraries after sequencing
- The pooled libraries are transferred to a flow cell where they anneal to random oligonucleotides complementary to adapters at either end forming bridges
- Each fragments is amplified through successive cycles of PCR known as solid-phase bridge amplification forming clusters of clonal fragments which are subsequently linearized and the reverse strands are cleaved away
- The forward strands are then sequenced by detecting the emission of light from each cluster as complementary fluorescently-tagged nucleotides compete to anneal



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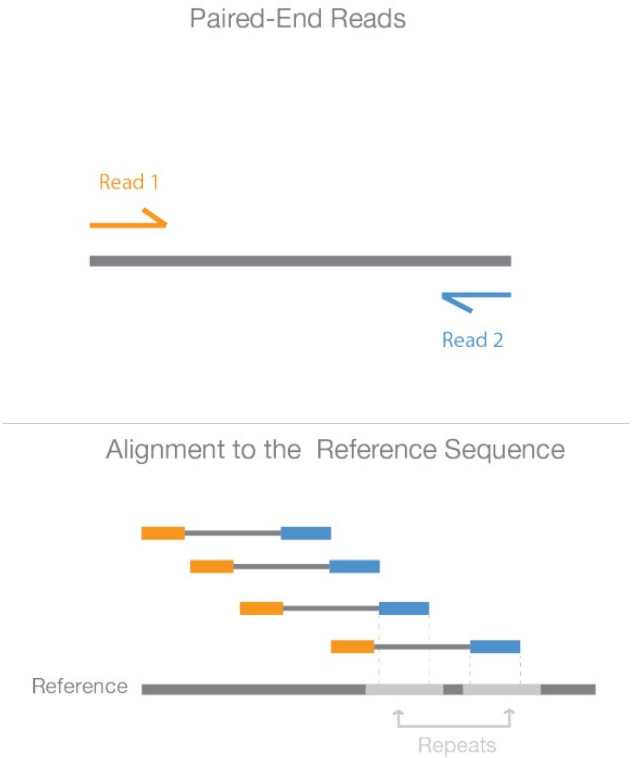
Paired-end Sequencing

- Illumina sequencing is now generally performed in paired-end format to generate 2 reads per sample known as R1 and R2
- After the first sequencing pass, oligos on the flow cell are unblocked and the sequencing product denatured from the forward strand, bridges form again and a reverse strand is transcribed
- The forward strand is cleaved and the sequencing reaction is then performed again to generate a second read
- Reads typically have a length of typically 50 - 150 bp however fragment inserts are usually longer than the sum of the two read lengths, leaving an unsequenced part in the middle of the fragment.
- When the distance between read pairmates is known (i.e. average fragment size), paired-end sequencing can help overcome a major limitation in alignment algorithms associated with repeat sequences.



File Types

FASTA format

- Contains sequence identifier prefixed by '>'
- Contains consensus sequence
- The format for contigs, assemblies and annotated genomes

```
>Mus_musculus_tRNA-Ala-AGC-1-1 (chr13.trna34-AlaAGC)
GGGGGTGTAGCTCAGTGGTAGAGCGCGTGCTTAGCATGCACGAGGcCCTGGGTTCGATCC
CCAGCACCTCCA
```

FASTQ format

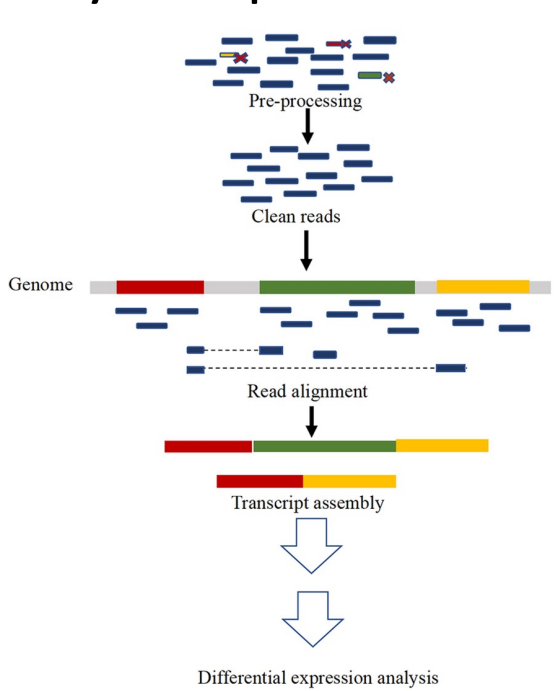
- Contains sequence identifier prefixed by '@'
- Contains read sequence
- Contains phred quality score corresponding to each base
- Format for raw reads
- Generally compressed in .gz files

```
@071112_SLXA-EAS1_s_7:5:1:817:345
GGGTGATGGCCGCTGCCGATGGCGTC
AAATCCCACC
+
IIIIIIIIIIIIIIIIIIIIIIIII
IIII9IG9IC
```

Phred scores (Q)

- Measures probability of errors in basecalling
- Phred scores range from Q2 – 40, with Q30 being the usual quality cutoff representing a 1:1000 chance of error
- The phred score is an important metric for quality control and trimming reads
- Two main schemes for phred scoring is phred+33 and phred+64 which encode different ASCII characters for each value

RNA-seq Analysis Pipeline



Steps	Tools
Quality control	FASTQ NGSQC/RNA-SeQC
Pre-processing	Trimmomatic/PRINSEQ/Soapnuke
Read alignment	Tophat2/HISAT2/STAR/BWA/Bowtie
Transcript assembly	Cufflinks/StringTie/Trinity/ SOAPdenovoTrans/Trans-ABySS
Expression quantification	FeatureCount/HTSeq-count /Cufflinks/eXpress/RSEM/ DEXSeq/Kallisto/Sailfish/Salmon
Differential expression analysis	DESeq2/edgeR

- First-generation transcript quantifiers take alignment files as input (Cufflinks, featureCounts)
- Second-generation quantifiers are alignment free so just take reads (Kallisto, Salmon, Sailfish)
- Code-free ecosystems are now also available for performing bulk RNA-seq (iDEP, RNAlysis)

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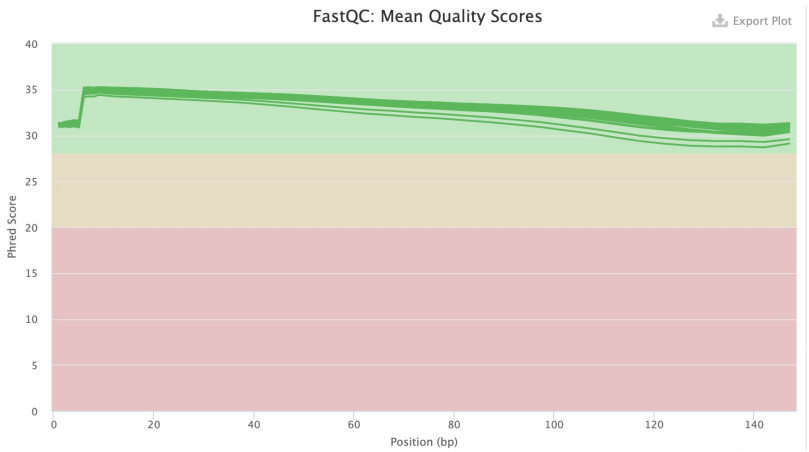
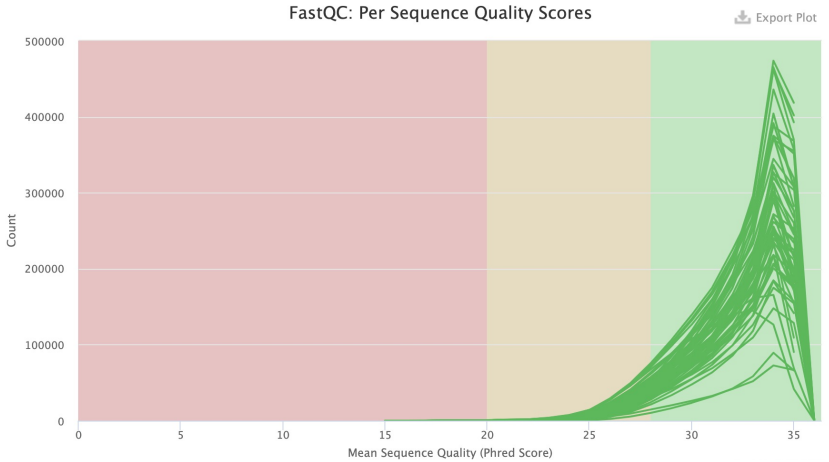
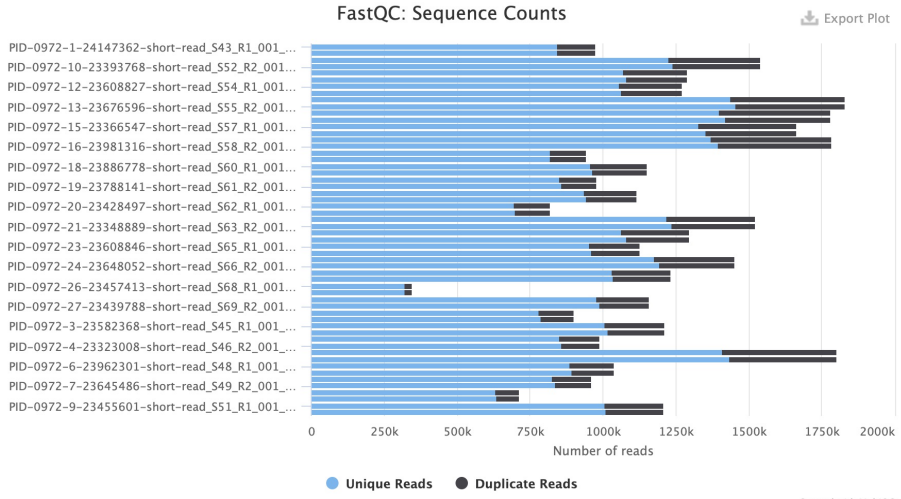
Quality Control

- The gold standard for quality checking short read sequencing data is FastQC (LongQC and Nanoplot are good alternatives for long-read data)
- FastQC will construct summary statistics from FASTQ files for base position quality, quality per read, GC content, overrepresented sequences and sequencing adaptor contamination
- Multiple FastQC reports can be aggregated and visualised with MultiQC
- Confirming sequencing read quality should always be the first thing done when you receive sequencing data







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Read Trimming

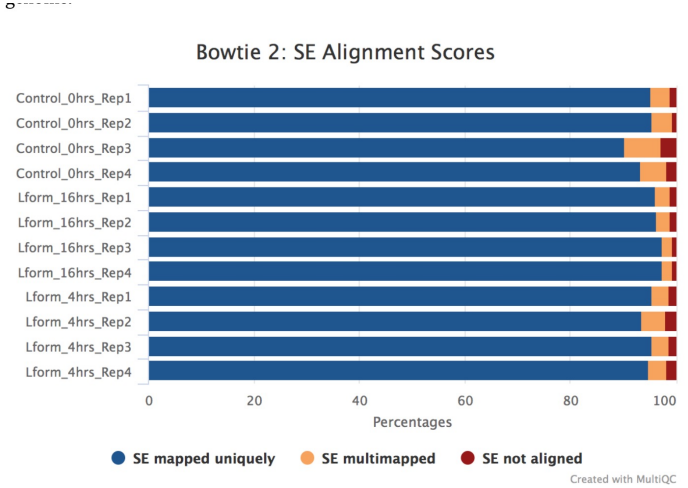
- Low quality reads should be removed from sequencing data using filtering software such as Trimmomatic
- Trimmomatic uses several user-defined parameters to determine which sequences to trim/drop based on length and phred score

Parameter	Function
ILLUMINACLIP	Cut adapter and other illumina-specific sequences from the read
LEADING/TRAILING	Cut bases off the start/end of a read, if below a threshold quality
SLIDINGWINDOW	Scans from the 5' end and clips the read once the average quality within the window falls below a threshold
MINLEN	Drop the read if it is below a specified length
AVGQUAL	Drop the read if the average quality is below the theshold

- The order in which these parameters are invoked affects which reads are trimmed, the recommended order is shown above
- In paired-end mode, Trimmomatic requires both pairmates and will output trimmed reads in both paired and unpaired formats
- For trimmed reads in paired format will, if a read is dropped, then the pairmate will be dropped irrespective of its own quality
- It is good practice to run quality control again after read trimming, however when the raw read quality is high and read filtering is low (<1% trimmed reads) it's probably overkill

Read alignment

- The percentage of mapped reads is a global indicator of the overall experimental quality
- When performing alignment and assembly, it is imperative to use the paired reads as these tools rely on read concordance to operate correctly (i.e. read #705,531 in R1 should be the mate of read #705,531 in R2)
- Bowtie2 and HISAT2 are two of the most popular bwa aligners for microbial RNA-seq




```
Coor     12345678901234  5678901234567890123456789012345
ref      AGCATGTTAGATAA**GATAGCTGTGCTAGTAGGCAGTCAGCGCCAT

+r001/1        TTAGATAAAGGATA*CTG
+r002         aaaAGATAA*GGATA
+r003       gcctaAGCTAA
+r004                  ATAGCT..............TCAGC
-r003                        ttagctTAGGC
-r001/2                                   CAGCGGCAT
```

```
@HD VN:1.6 SO:coordinate
@SQ SN:ref LN:45
r001   99 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002    0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA    *
r003    0 ref  9 30 5S6M       *  0   0 GCCTAAGCTAA       * SA:Z:ref,29,-,6H5M,17,0;
r004    0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003 2064 ref 29 17 6H5M       *  0   0 TAGGC             * SA:Z:ref,9,+,5S6M,30,1;
r001  147 ref 37 30 9M         =  7 -39 CAGCGGCAT         * NM:i:1
```

- Alignment tools typically output data in SAM/BAM format
- Sequence alignment map (SAM) files store alignment information of short reads mapped against reference sequences.
- BAM files are binary-translated SAM files which offers smaller file sizes reducing computation times

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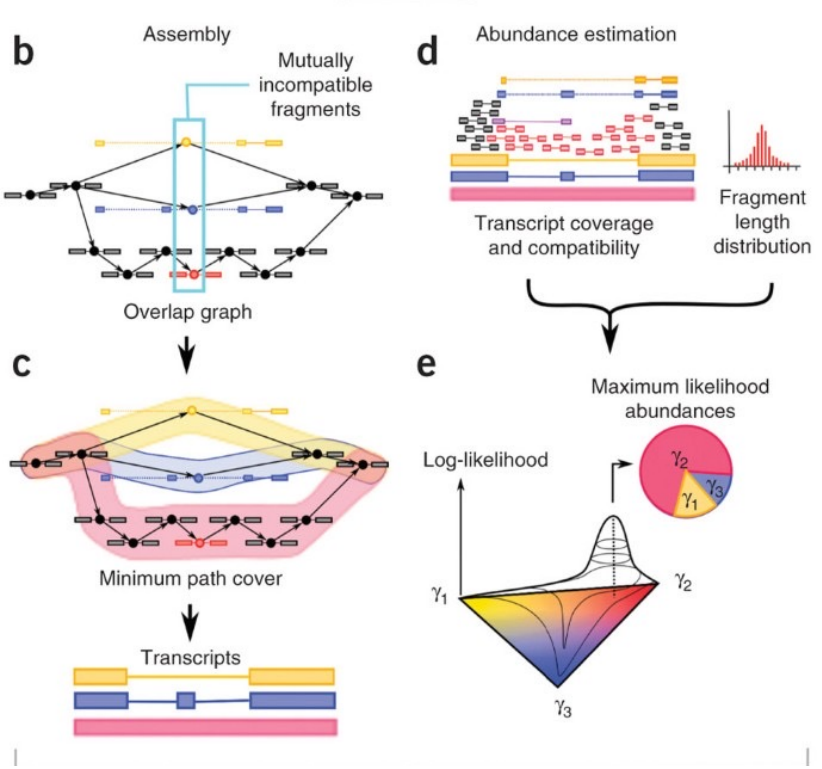
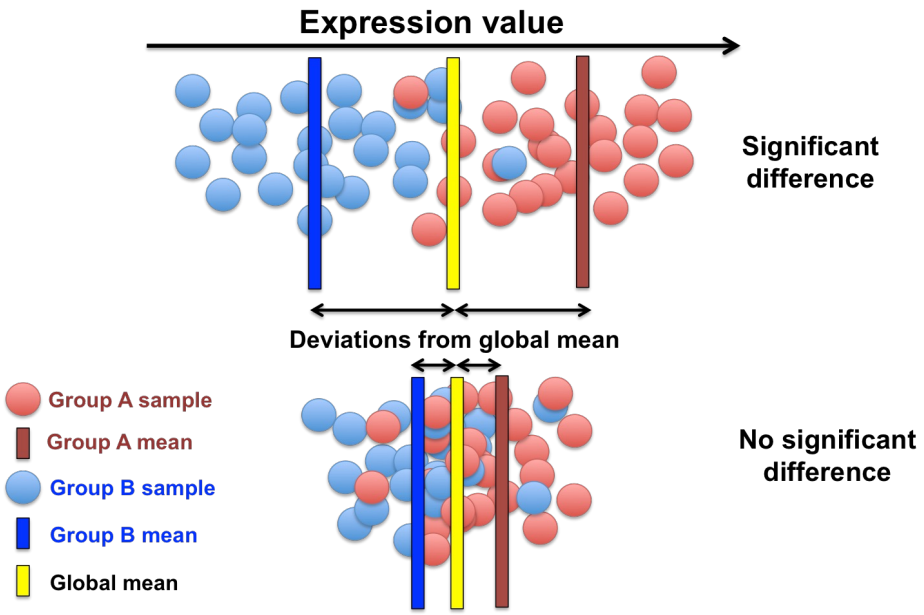
Transcript Quantification

- Assembly of aligned reads into mRNA transcripts is largely deprecated for bacterial RNA-seq, modern counting tools perform this step automatically during transcript quantification
- Manual transcript assembly against a transcriptome reference may still be necessary in eukaryotic RNA-seq due to gene splicing
- Baseline gene expression is generally measured as RPKM (reads per kilobase per million reads) or FPKM (fragments per kilobase per million reads)
- These metrics often undergo further normalization in order to correct for transcript length, GC content and read size distributions



Differential Expression Analysis

- Differential expression analysis takes count data and fits models to discover changes in expression between experimental groups.
    - Differential expression analysis is specifically designed for comparing expression **between** samples
    - For comparisons of genes **within** a sample, RPKM/FPKM are more reliable
- edgeR and DEseq2 are the two most common DE tools. DEseq2 is more conservatives and its estimations and considered to have lower rate of type I errors although can miss smaller changes in expression.
- Differential expression tools allow for the modelling of complex experimental designs, such as multiple groups with multiple replicates.
- Differential expression is measured in log2 fold change between the test and control groups, and an adjusted p-value is calculated based on the Wald test
- A differentially expressed gene (DEG) is called when the log2FC is > 2 and the adj p-value (also referred to as false discovery rate (FDR)) is < 0.05



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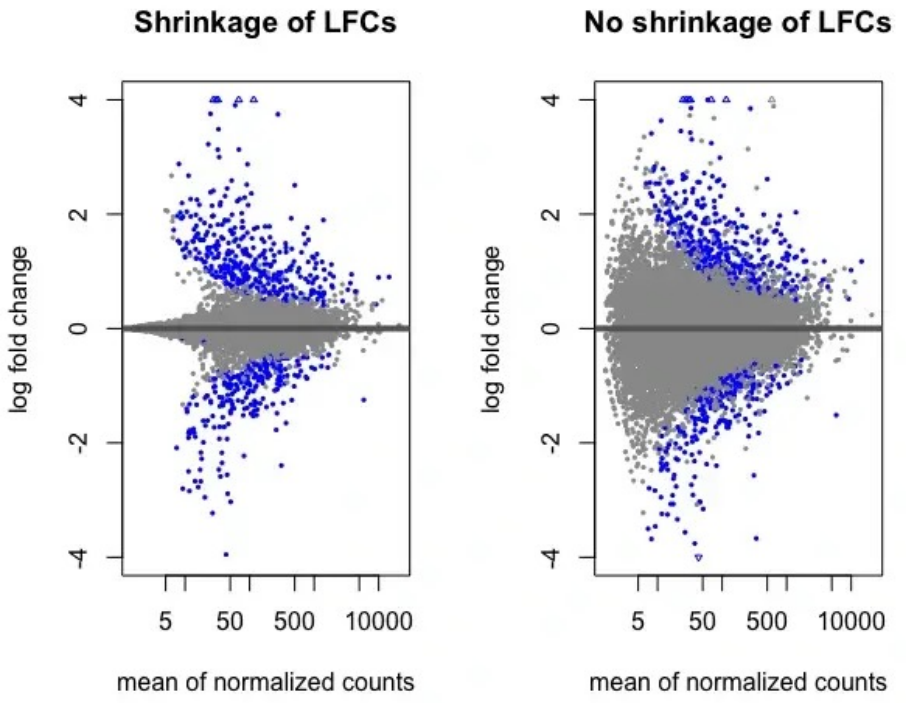
Modelling Differential Expression

- DESeq2 generates a negative binomial model from the ratios of normalized counts against a ‘size factor’ – a statistic calculated to correct for library size, transcript length and composition bias.
- A shrinkage estimator is used stabilize parameter estimates by ”shrinking” each point closer to to the mean, with larger variances shrinking more.
- Typically, the number of replicates for an RNA-seq experiment is small (2 – 4) and the number of dimensions within the data (i.e. genes) is large, therefore it is difficult to reliably estimate variance.
- As genes of similar expression can be assumed to possess similar variance, clustering is performed to increase the effective sample size which increases statistical power of significance calculations.



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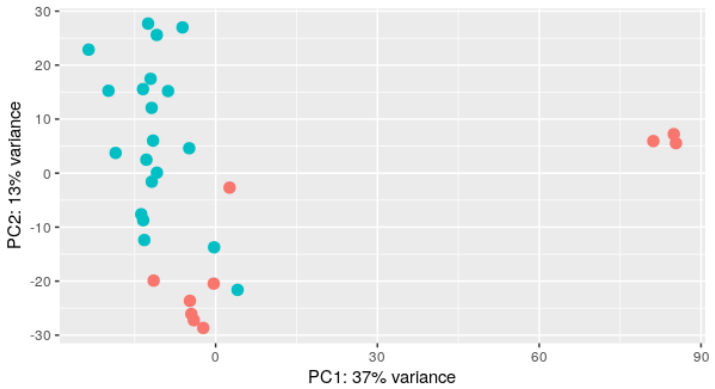
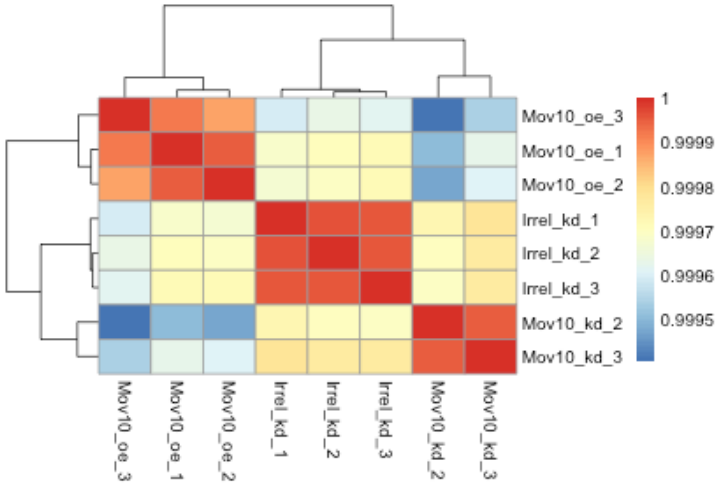
Validating Differential Expression Analysis

- Principal component analysis (PCA) is a dimensionality reduction technique used to determine inter-group variation
  - PCA calculates the maximum variance across each dimension
  - A principal component is generated from each dimension
  - The top 2 or 3 principal components (which reflect the greatest amount of variance able to be visualized on a 2 or 3 dimensional axis) are used to address how well groups cluster
  - The greater variance accounted for in a PCA plot, the more robust the conclusions on inter-group variation are
  - Homogenous variance across principal components is often due to non-linear relationships, in which case non-linear dimensionality reduction methods should be used such as t-SNE or UMAP.
  - Hierarchical clustering can also be used to determine this





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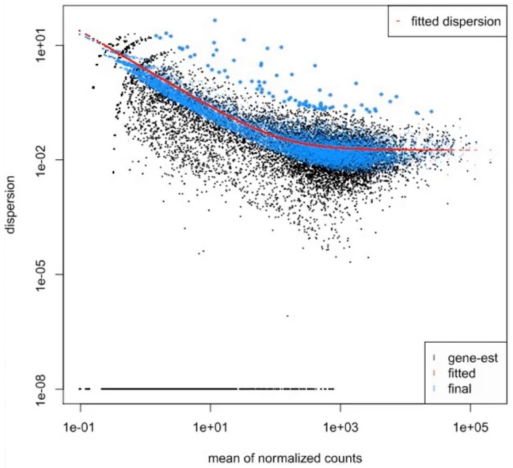
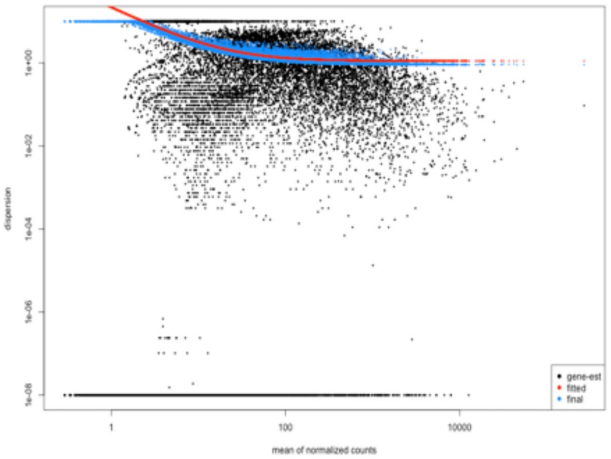
Validating Differential Expression Analysis

- Dispersion plots are used to estimate within-group variation, which shows the data pre- and post-shrinkage
  - Good models should cluster dispersion estimations around the fitted maximum likelihood estimate
  - Dispersion should decrease with increasing mean count

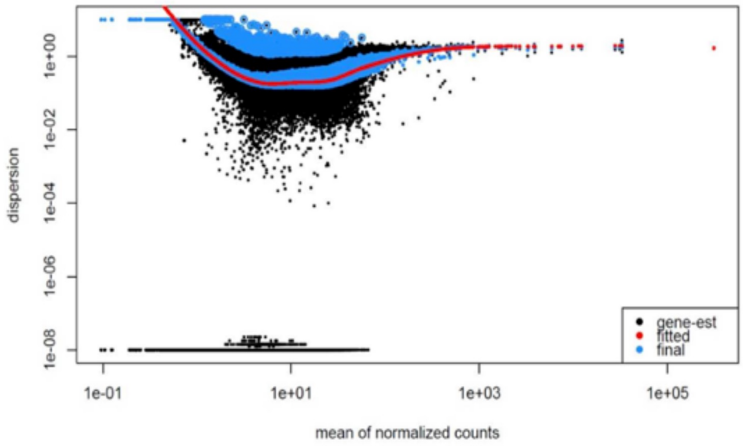
Good estimate



Bad estimate – Dispersions do not follow fit



Bad estimate – Dispersions do not monotonically decrease



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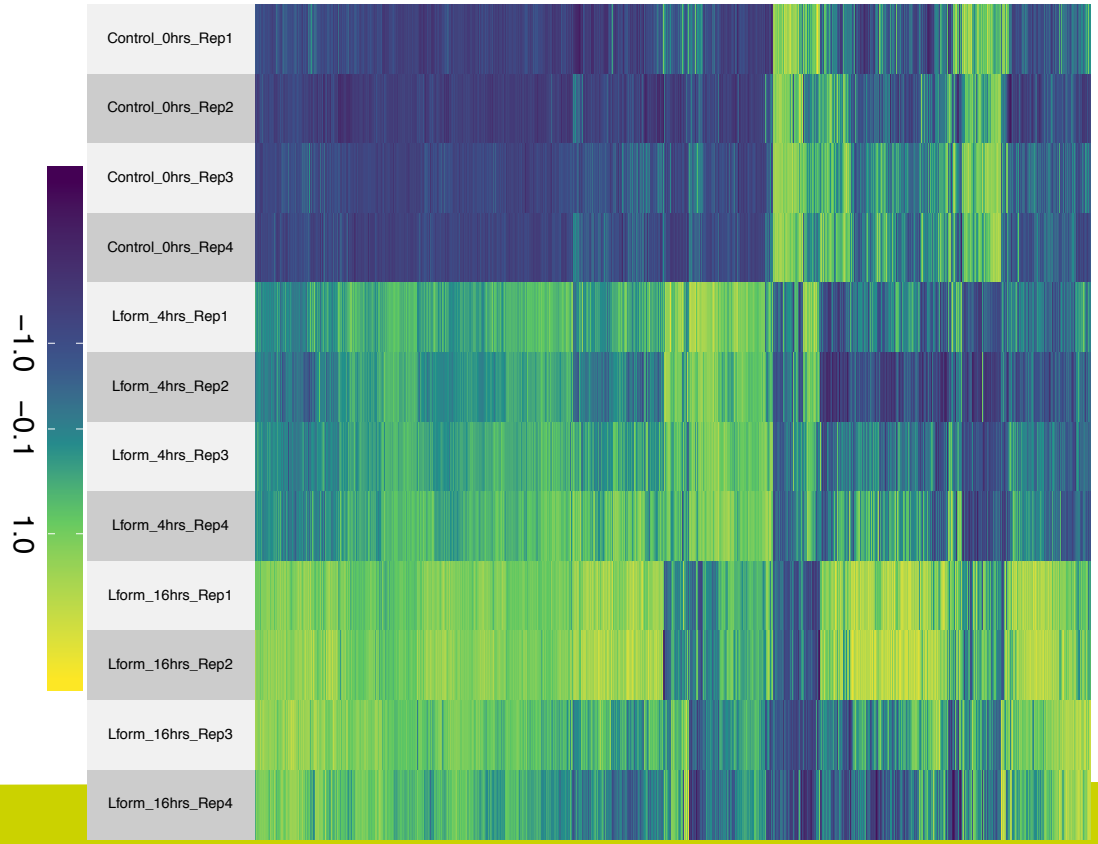
The heatmap is the highest level for visualization of global gene expression changes



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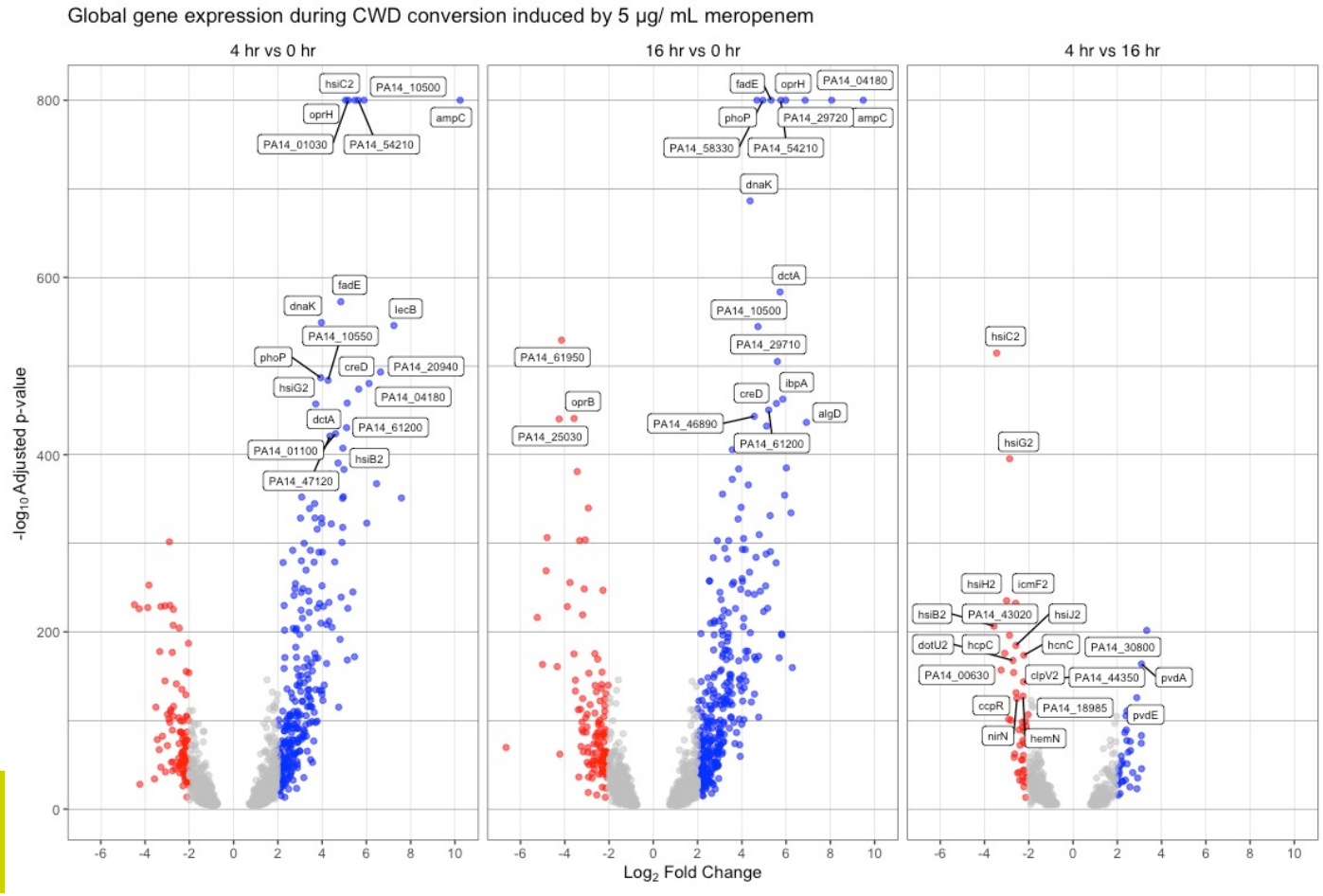
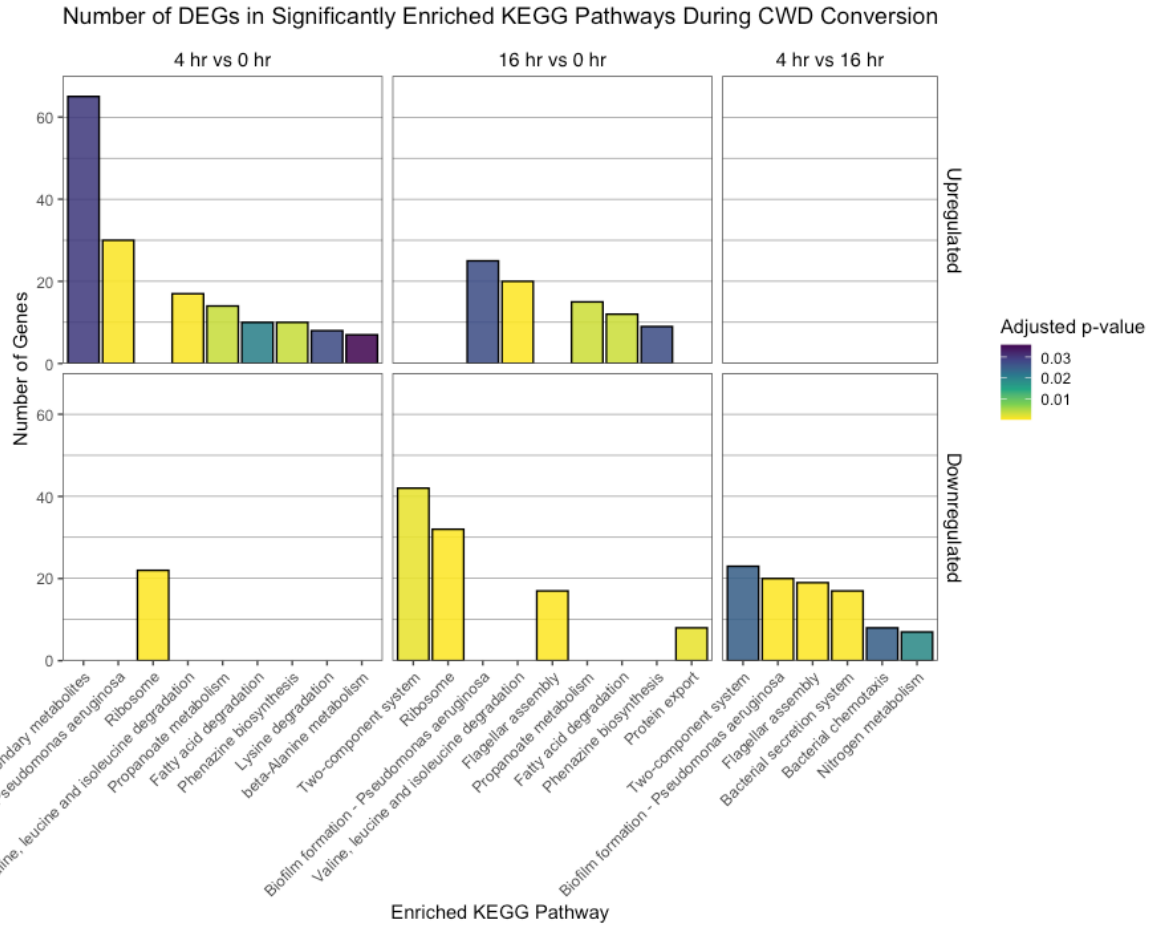
The volcano plot allows for the most significantly differentially-expressed genes to be visualised



Shared functions can also be classified in differentially expressed genes through enrichment analysis (KEGG, GO, COG)



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