Gene Counts and Data Quality

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WHG

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Gene Counts and Data Quality

- Goals:
  - Learn how the process used to get from raw reads to count tables excludes certain reads from consideration.
  - Show what can drive differences between samples.
  - Recognise common Quality Control (QC) issues in data.

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Gene Counts and Data Quality

- Presentation:
  - Focus on differential expression (DE) projects.
  - A recap of a typical RNA Sequencing pipeline for DE.
  - Roundup of common QC issues.
  - Examples of tools used in the default pipelines in WHG
- Practical:
  - Work with toy datasets to get experience recognising QC problems.

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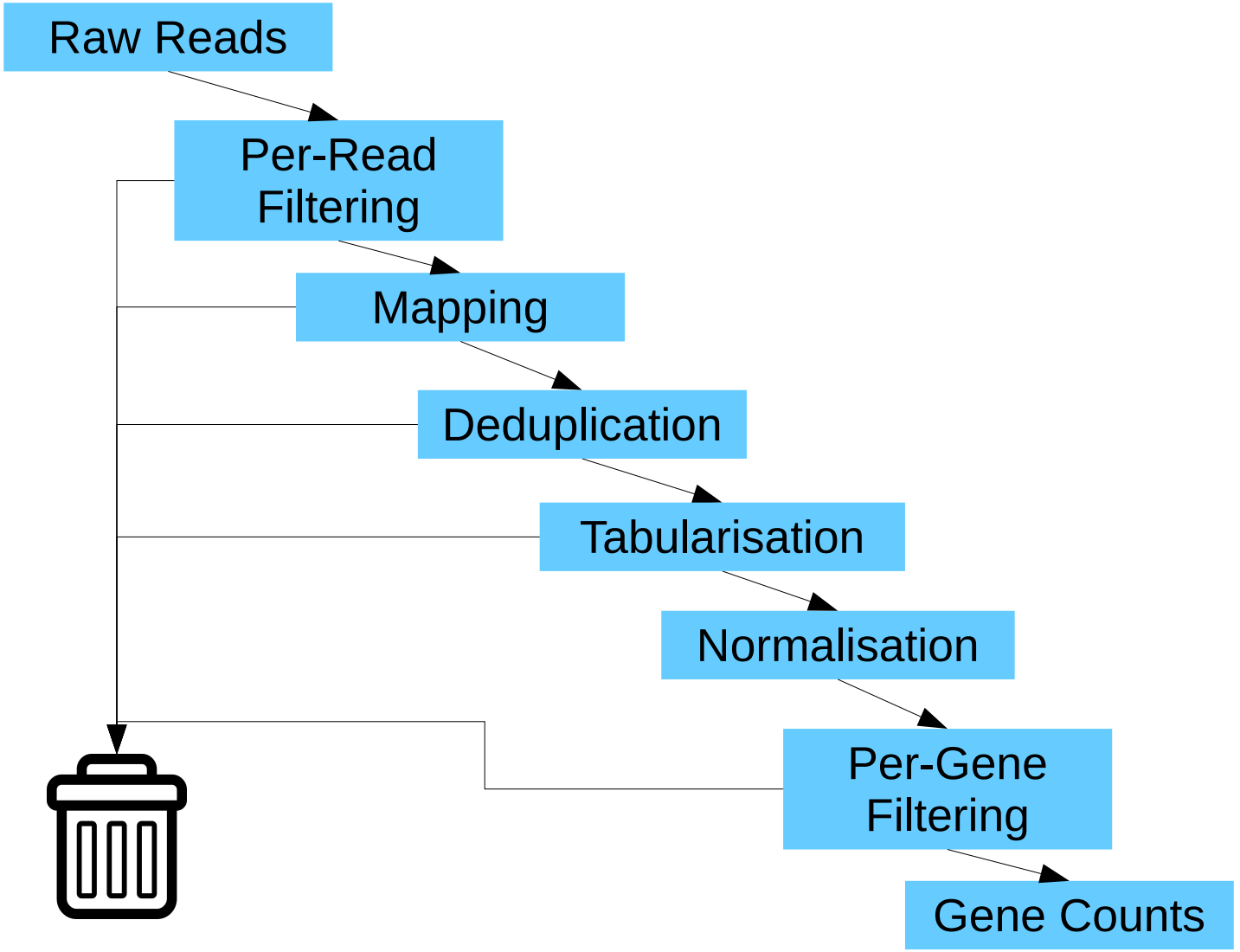
Reads to Leads



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Reads to Leads

- Per-read filtering
  - Use metrics from the sequencing pipeline to exclude reads of low overall quality.
  - Usually an option during the mapping step.
- Mapping
  - Map to transcriptome for the organism.
  - HiSat2

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Reads to Leads

- Deduplication
  - A tool identifies duplicate reads so they can be excluded from further analysis.
  - Picard MarkDuplicates
- Tabularisation
  - Match mapped reads to features (i.e. genes).
  - Produce count table.
  - featureCounts

Reads to Leads

- Normalisation
  - Adjusts count table to take into account any variation in counts not due to gene expression.
  - Many methods, including:
    - Counts per million (cpm) – adjusts for library size.
    - Transcripts per million (tpm) – adjusts for library size and gene length.
    - Variance stabilisation (vsn) – adjusts so that the variance is not dependent on the mean.
  - DESeq2 (R package)

Reads to Leads

- Per-gene filtering
  - Where the overall level of expression is very low, reliable differential expression analysis cannot be performed.
  - Identify genes with low expression across all experimental groups and filter them out.
  - If a gene of interest is filtered out in this way, no work-around other than a repeat of sequencing at greater depth.

What Reads are Removed?

- Poor quality reads.
- Unmapped reads.
  - Do not map to the transcriptome.
- Duplicate reads.
- Reads that do not map to a unique gene.
  - Map to intron or intergenic region.
  - Do not map uniquely.
- Reads for marginally-expressed genes.

Assignment Summaries

- Tabularisation attempts to match reads to features.
  - Sometimes this cannot be done.
- Most tools report how many reads are unable to be tabularised, grouped by reason.
  - The details vary depending on the tool.
- Many tools will exclude reads based on earlier criteria.
  - Either way is fine, as long as you know what filtering rules are being followed.

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What Drives Differences?

- Quality issues.
- Technical aspects.
- Differential expression.

Quality Issues

- Quality issues can arise at every stage of the process:
  - Sample gathering
    - Batch effects.
    - Sample labelling issues.
    - Poor experimental design.
  - Lab work
    - Contamination.
    - Low input material.
    - Batch effects.
  - Sequencing technicalities
    - Sequencing machine problems.

Dealing with Quality Issues

- Many of these quality problems manifest as a sample or set of samples having a very different profile to the rest of the data.
  - Treated as outliers.
  - Outliers generally have to be discarded before analysis.
  - If the problem is limited to a particular middle step, the sample can be sequenced again.
- Some problems have a systematic effect on the samples and can be adjusted for in the analysis.

Technical Aspects

- Technical aspects:
  - Tissue type.
  - Kit type.
  - Other experimental variables that should have been held constant for the entire project.

Differential Expression

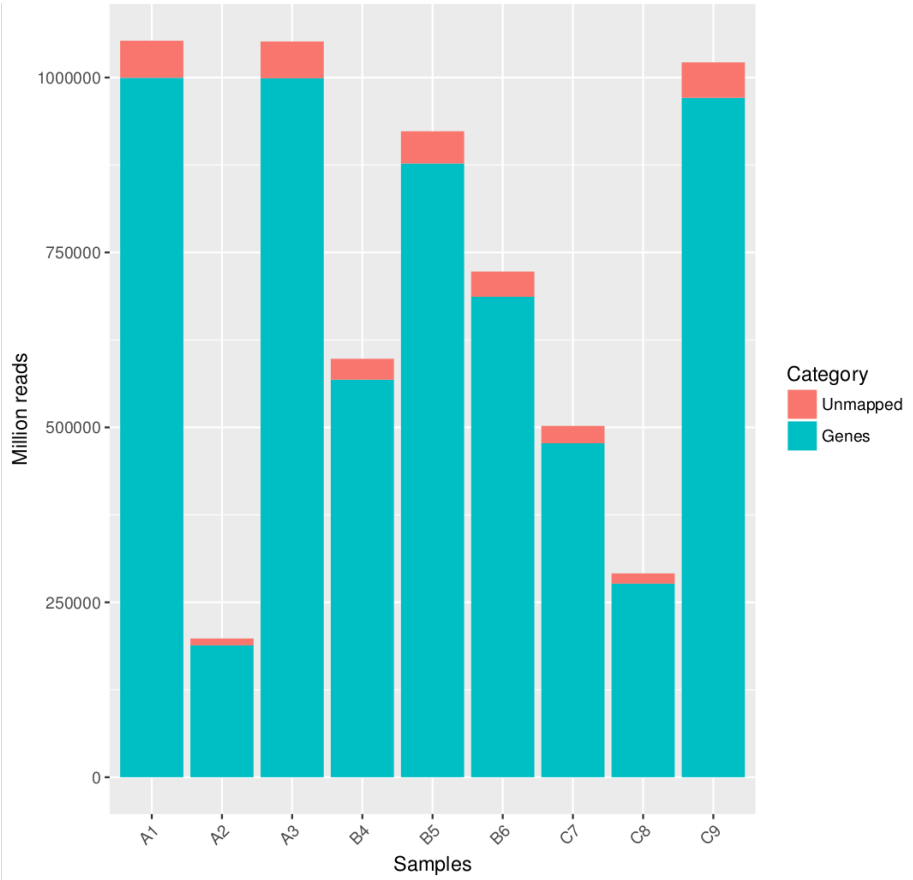
- Differential expression:
  - Treatment levels.
  - Disease condition.
  - Knockdown models.
  - Time factors.

Visualising QC

- Visual inspection can identify quality issues.
  - Outlier samples.
  - Potential batch effects.
  - Possible sample swaps.
- Often requires confirmation of the issue from outside the data before it can be adjusted for.
- Usually a problem can be seen in multiple visualisations.
- Being able to recognise common issues from visualisations saves time.

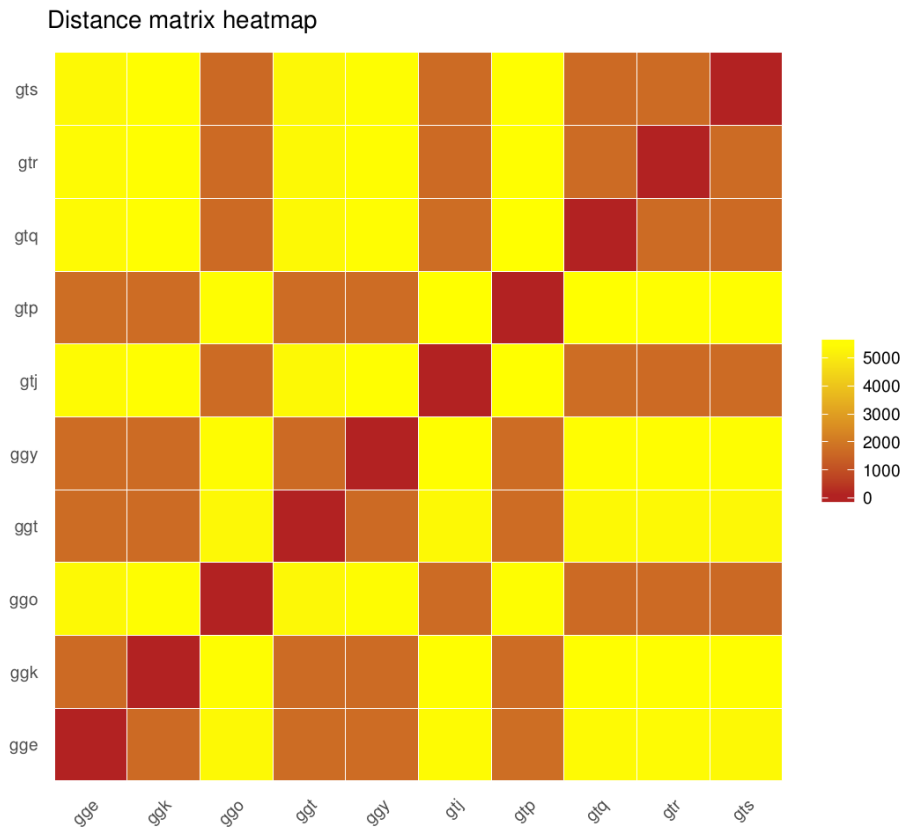
Visualising QC – Read Counts

- Shows total reads and proportion of reads assigned to different categories.
- Identifies outliers on the basis of total reads or read assignment.
- Quick way to spot failed samples or uneven depth.



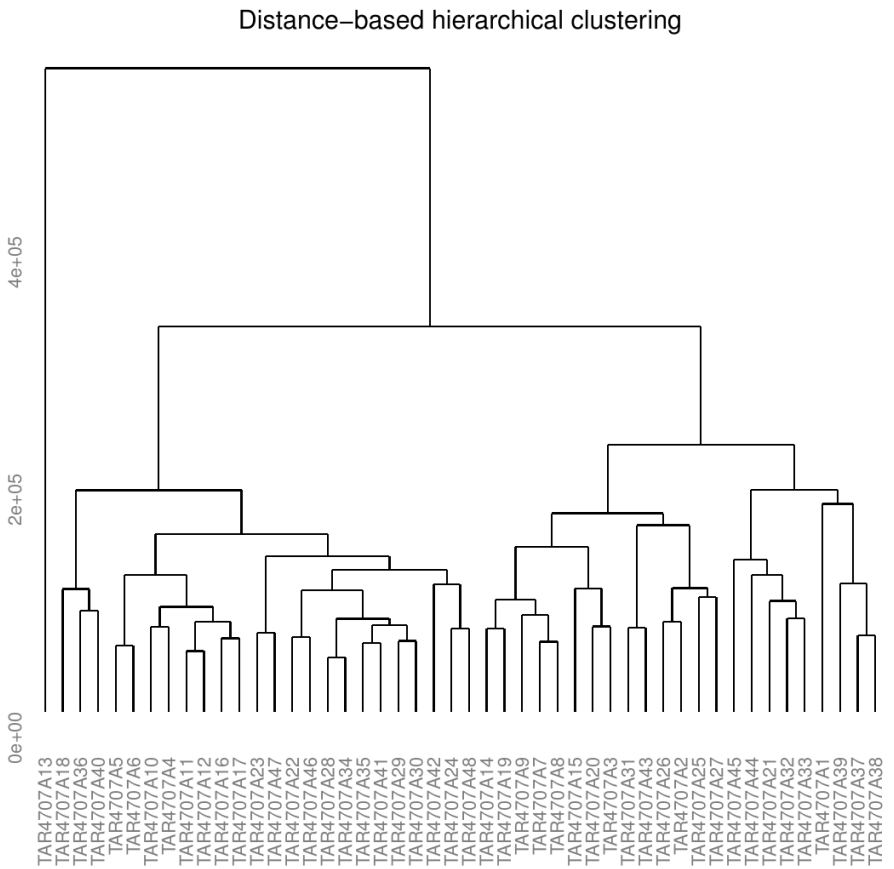
Visualising QC - Heatmaps

- Show similarity between samples.
- Many different ways of measuring that similarity.
- Can identify sample groups.
- Do not indicate underlying structure.



Visualising QC - Dendrograms

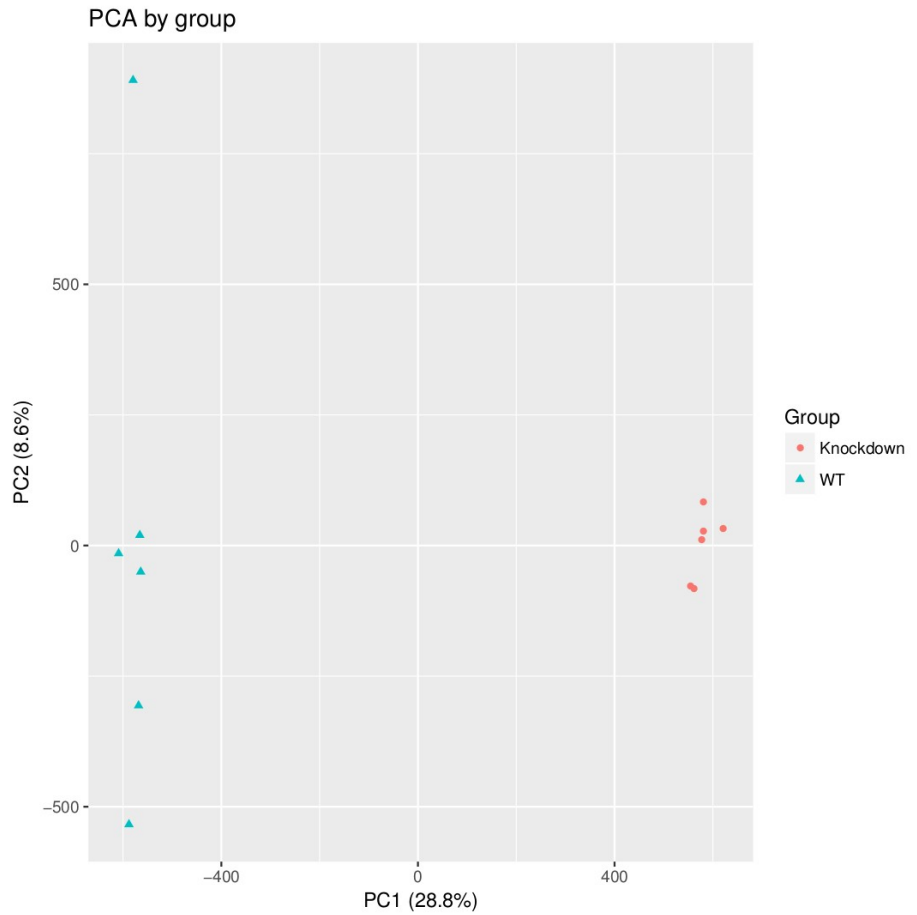
- Show similarity between samples.
- Many different ways of measuring that similarity.
- Can identify sample groups.
- Can infer hierarchical clustering from the tree.
- Often added to heatmaps.

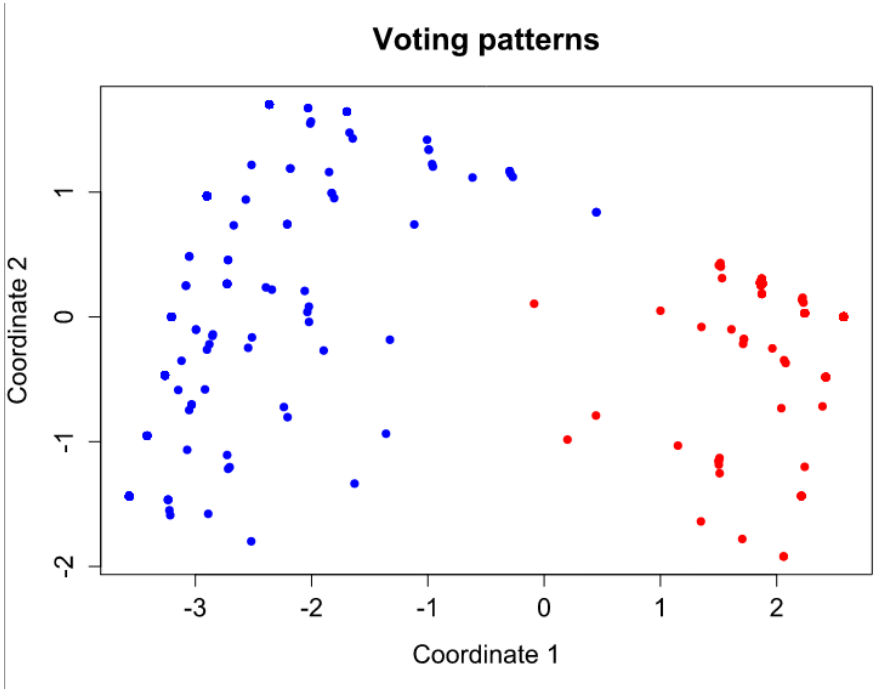


Visualising QC – PCA and MDS

- PCA
  - 'Principal Components Analysis'.
  - Multidimensional technique for revealing underlying clustering.
  - Identify multiple separate groups.
  - Provides indication of how much variability lies in each dimension.
- MDS
  - 'Multidimensional Scaling' plots.
  - Multidimensional technique for revealing underlying clustering.
  - Identify multiple separate groups.

Visualising QC – PCA and MDS





Visualising QC – PCA and MDS

- The most common plots show only the first 2 dimensions of these techniques.
- Higher dimensions can show more layers of information regarding the structure of the data.
- Ideally, each dimension will correspond to one source of variation.
    - Dimension 1 might be treated/untreated.
    - Dimension 2 might be time since treatment.
    - Dimension 3 might be a batch effect.
    - Etc.

Visualising QC - Limitations

- Good QC plots do not guarantee a successful project with useful analysis.
- If gene expression differs only slightly between experimental groups, the underlying pattern can be very difficult to spot visually if the visualisation is based on the full data.
- When there are multiple factors in the experiment, some may have a much larger effect on gene expression and make the differences of others harder to spot.

QC Issues

- QC problems of a given type typically affect the visualisations in a recognisable and consistent way.
- Visual inspection is therefore a powerful tool for identifying what type of problem has been encountered.
- This does not replace formal statistical techniques for finding clusters or determining significant differences in expression between groups.

QC Issues – Failed Sample

- Characteristics:
  - Significantly lower total read count.
  - Proportion of read categories different from the rest of the data.
  - Isolated in PCA and MSD plots, sometimes to the point that the rest of the plot is unreadable.
- Solution:
  - Exclude that sample and re-examine QC for further issues.

QC Issues – Batch Effect

- Characteristics:
  - Experimental groups expected to be a single cluster are split into separate clusters.
  - These splits are in a consistent direction across different experimental groups.
- Solution:
  - Verify that a potential lab-based batch effect corresponds to the pattern in QC.
  - Introduce a variable for that batch effect in the analysis.

QC Issues – Sample Mix Ups

- Characteristics:
  - Clusters exist in the data, but do not correspond to the experimental groups.
  - The number of samples in each cluster make sense for the experiment.
- Solution:
  - Double check sample naming. If an error is found, correct the names and continue checking.
  - Never try to use the data to infer the correct naming.

QC Issues – Failed Project

- Characteristics:
  - No visible clustering.
  - Or variation very small.
- Solution:
  - Conduct analysis and see if there is truly nothing to be found.
  - Work with the lab to discover what went wrong.
  - Repeat the experiment.

Conclusions

- Visual inspection of QC metrics can identify patterns and problems with RNA sequencing.
  - This is mostly an exercise in lateral thinking.
- Experience reviewing these projects permits educated guesses as to what causes these patterns.
- Often any problems can be worked around at the analysis stage.
  - If not, it is useful to know that before trying.