CANCER RESEARCH UK | CAMBRIDGE INSTITUTE

Introduction to RNAseq Methods

March 2023

HTS Applications - Overview

DNA Sequencing

- Genome Assembly
- SNPs/SVs/CNVs
- DNA methylation
- DNA-protein interactions (ChIPseq)
- Chromatin Modification (ATAC-seq/ChIPseq)

RNA Sequencing

- Transcriptome Assembly
- **Differential Gene Expression**
- Fusion Genes
- Splice variants

Single-Cell

- RNA/DNA
- Low-level RNA/DNA detection
- Cell-type classification
- Dissection of heterogenous cell populations

RNAseq Workflow

Experimental Design

Library Preparation

Sequencing

Bioinformatics Analysis

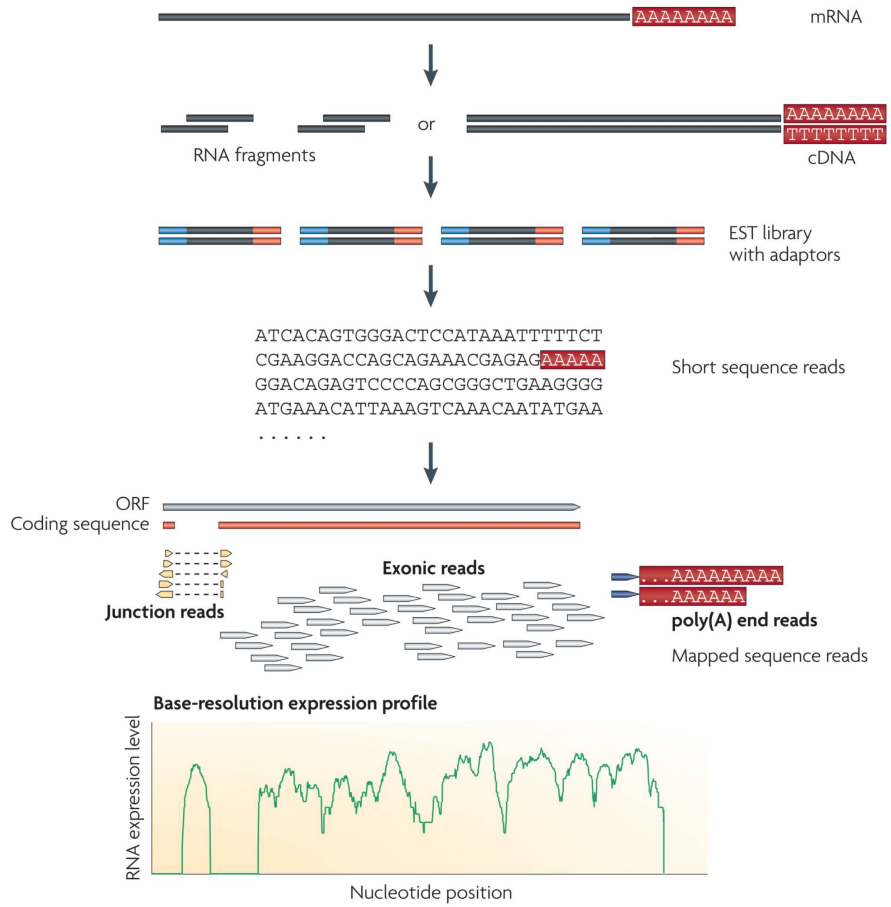


Image adapted from: Wang, Z., et al. (2009), Nature Reviews Genetics, 10, 57–63.

Designing the right experiment

A good experiment should:

- Have clear objectives
- Have sufficient power
- Be amenable to statisical analysis
- Be reproducible
- More on experimental design later

Designing the right experiment

Practical considerations for RNAseq

- Coverage: how many reads?
- Read length & structure: Long or short reads? Paired or Single end?
- Controlling for batch effects
- Library preparation method: Poly-A, Ribominus, other?

Designing the right experiment - How many reads do we need?

The coverage is defined as:

$$\frac{Read\ Length \times Number\ of\ Reads}{Length\ of\ Target\ Sequence}$$

The amount of sequencing needed for a given sample is determined by the goals of the experiment and the nature of the RNA sample.

- For a general view of differential expression: 5–25 million reads per sample
- For alternative splicing and lowly expressed genes: 30–60 million reads per sample.
- In-depth view of the transcriptome/assemble new transcripts: 100–200 million reads
- Targeted RNA expression requires fewer reads.
- miRNA-Seq or Small RNA Analysis require even fewer reads.

Designing the right experiment - Read length

Long or short reads? Paired or Single end?

The answer depends on the experiment:

- Gene expression – typically just a short read e.g. 50/75 bp; SE or PE.
- kmer-based quantification of Gene Expression (Salmon etc.) - benefits from PE.
- Transcriptome Analysis – longer paired-end reads (such as 2 x 75 bp).
- Small RNA Analysis – short single read, e.f. SE50 - will need trimming.

Designing the right experiment - Replication

Biological Replication

- Measures the biological variations between individuals
- Accounts for sampling bias

Technical Replication

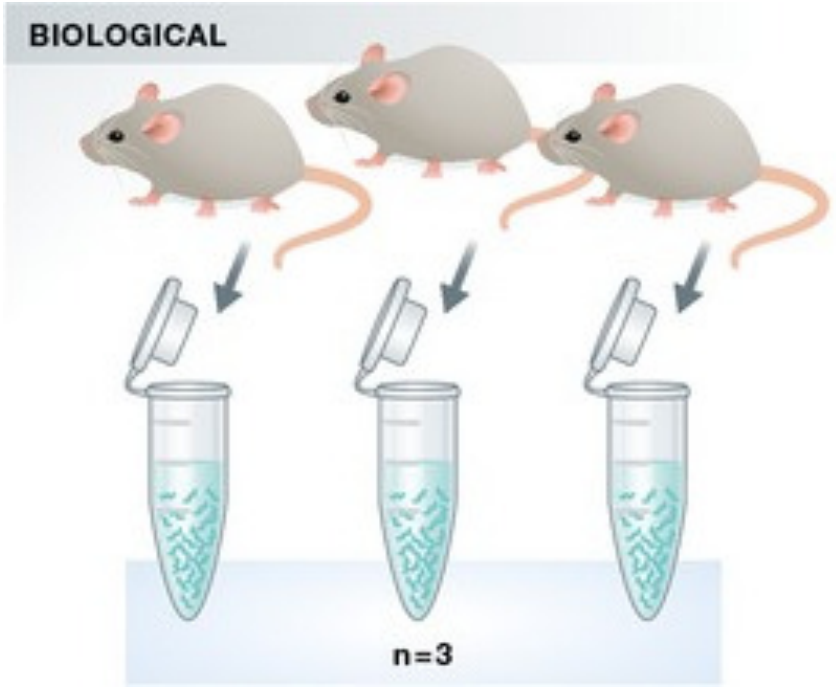
- Measures the variation in response quantification due to imprecision in the technique
- Accounts for technical noise

Designing the right experiment - Replication

Biological Replication

Each replicate is from an indepent biological individual

- *In Vivo*:
  - Patients
  - Mice
- *In Vitro*:
  - Different cell lines
  - Different passages



Designing the right experiment - Replication

Technical Replication

RNA Sequencing

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Single-Cell

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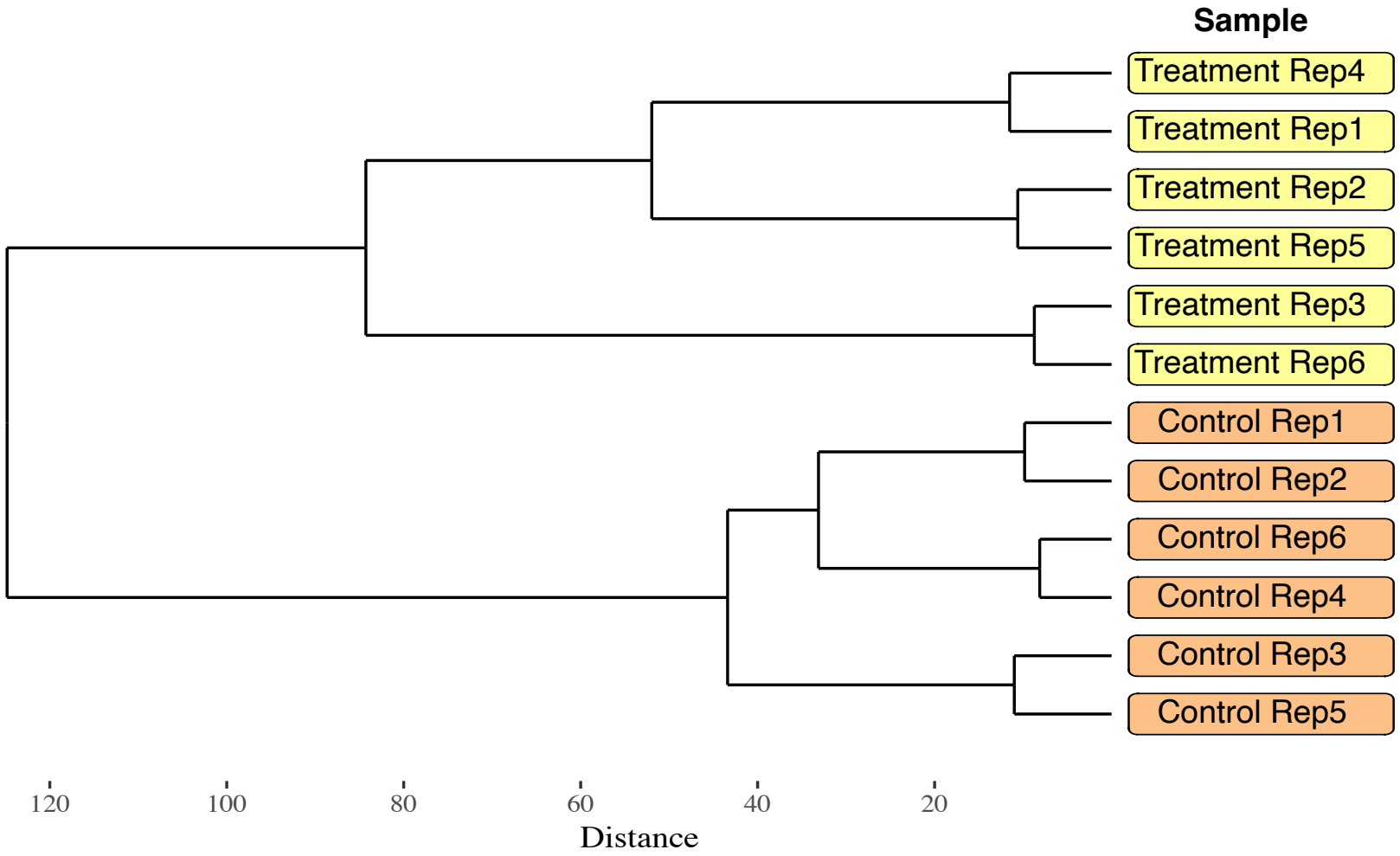
Replicates are from the same individual but processed separately

- Experimental protocol
- Measurement platform

Designing the right experiment - Batch effects

- Batch effects are sub-groups of measurements that have qualitatively different behavior across conditions and are unrelated to the biological or scientific variables in a study.
- Batch effects are problematic if they are confounded with the experimental variable.

Designing the right experiment - Batch effects



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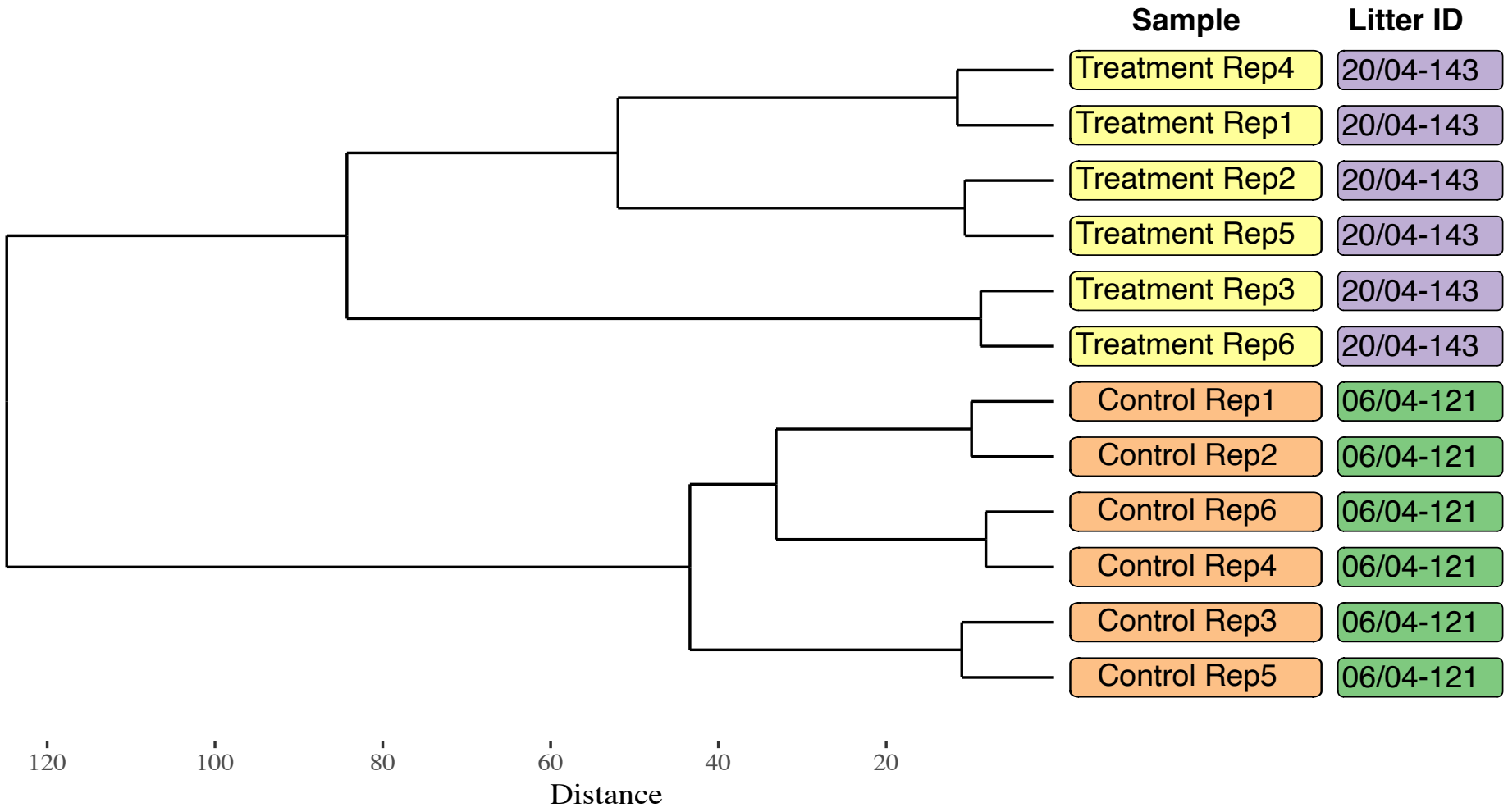

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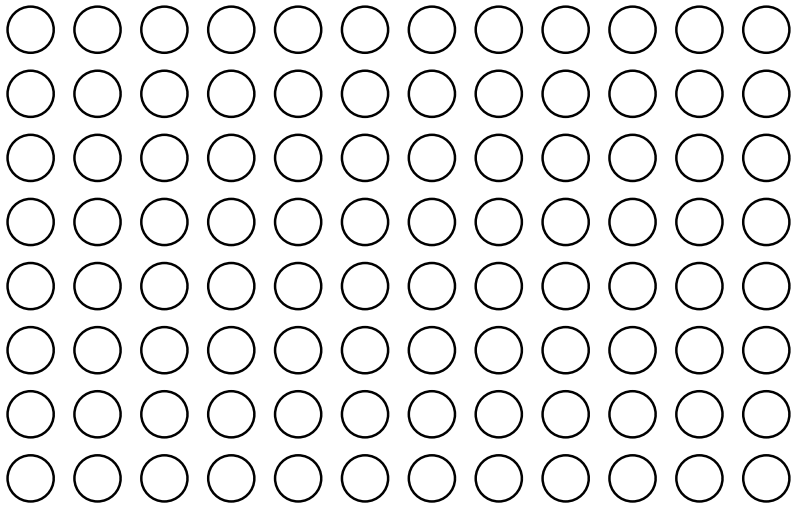

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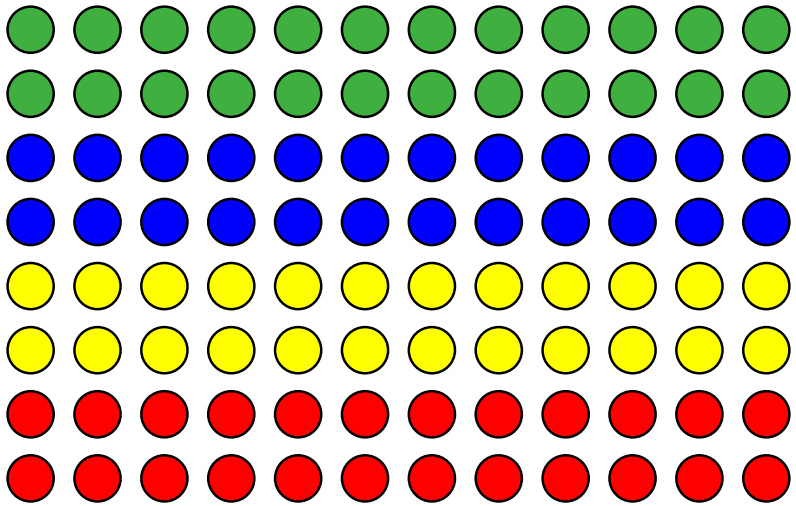

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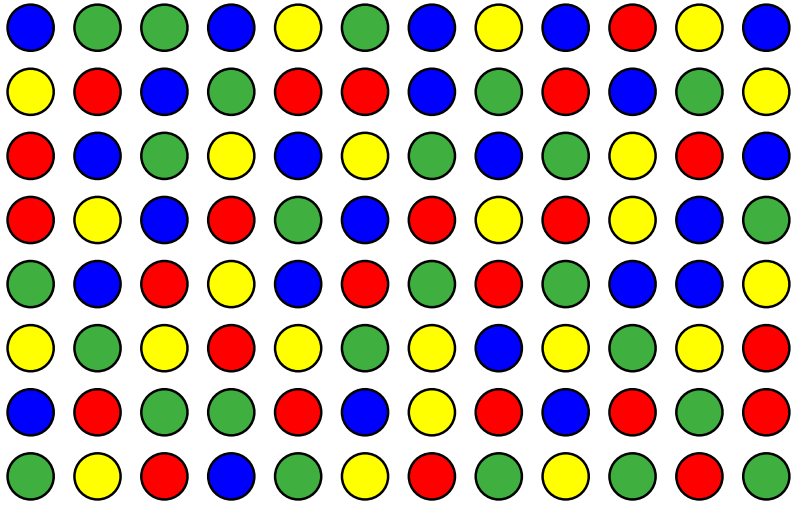

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- Randomise all technical steps in data generation in order to avoid batch effects
- **Record everything**: Age, sex, litter, cell passage ..

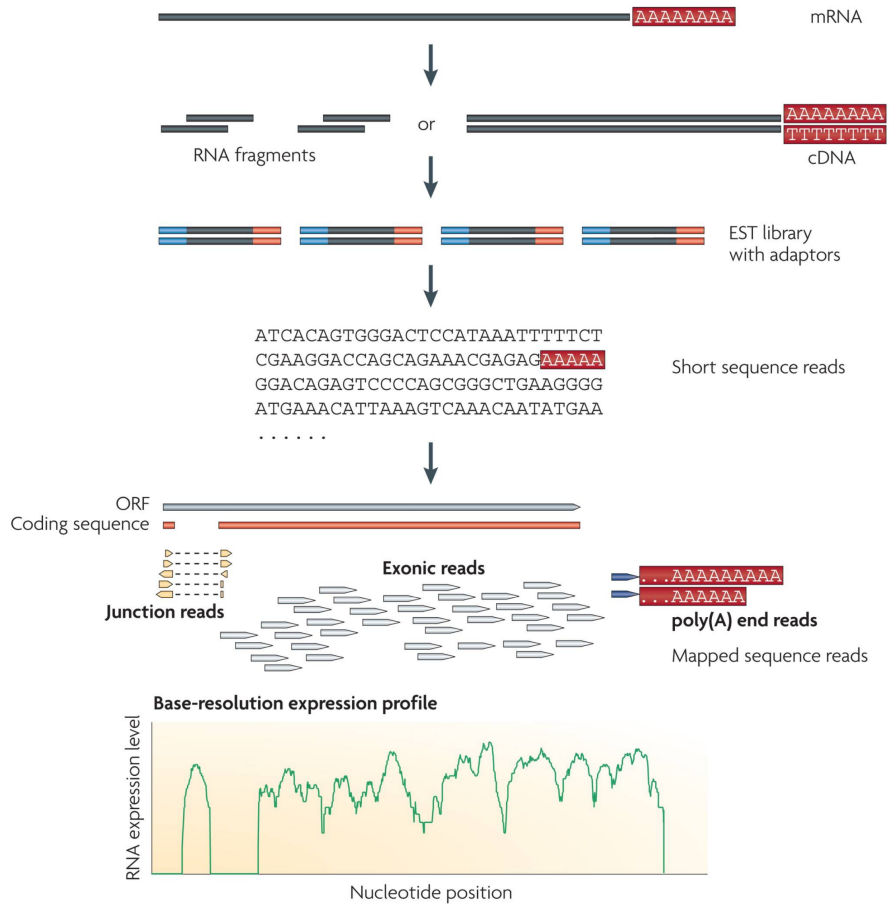
RNAseq Workflow

Experimental Design

Library Preparation

Sequencing

Bioinformatics Analysis



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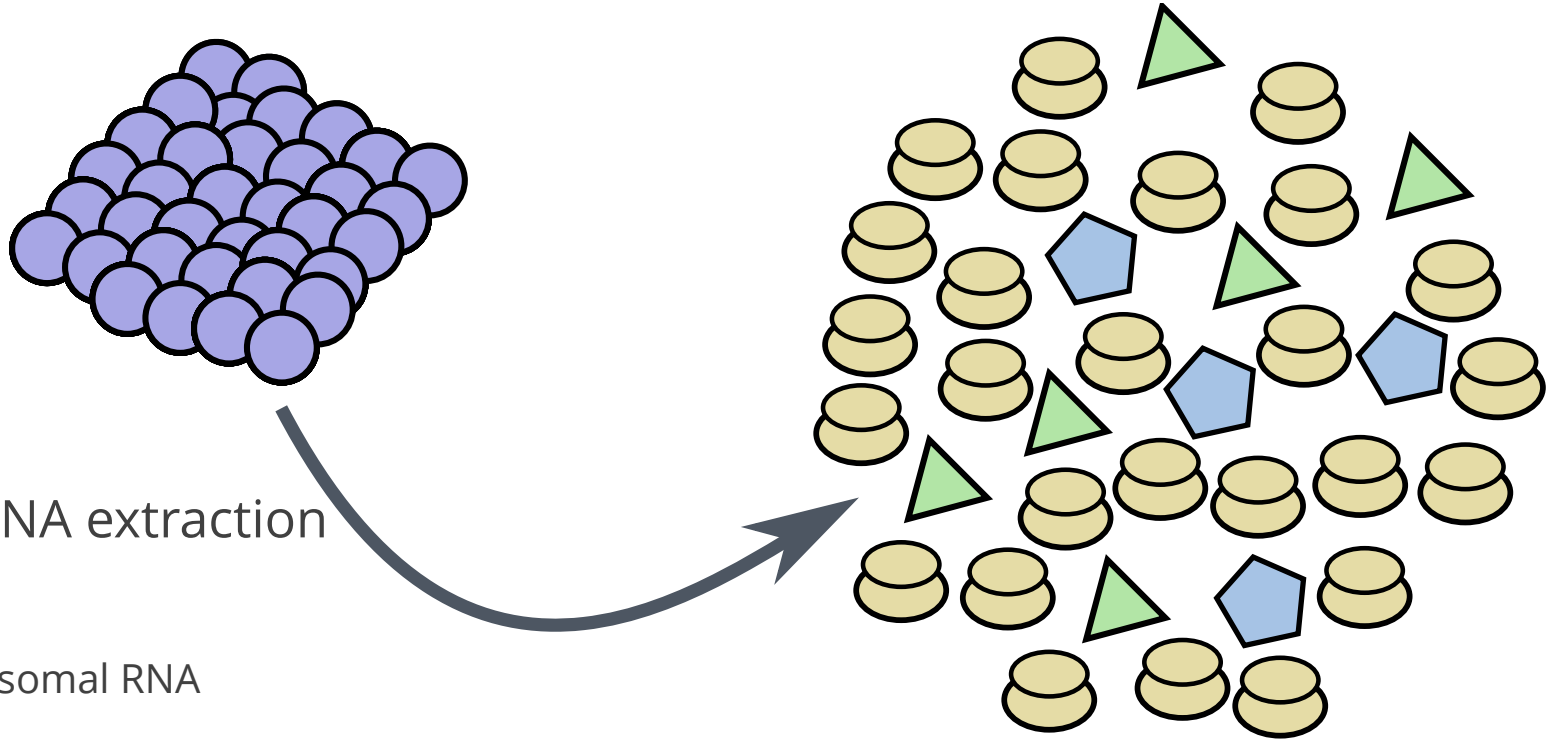


Total RNA extraction

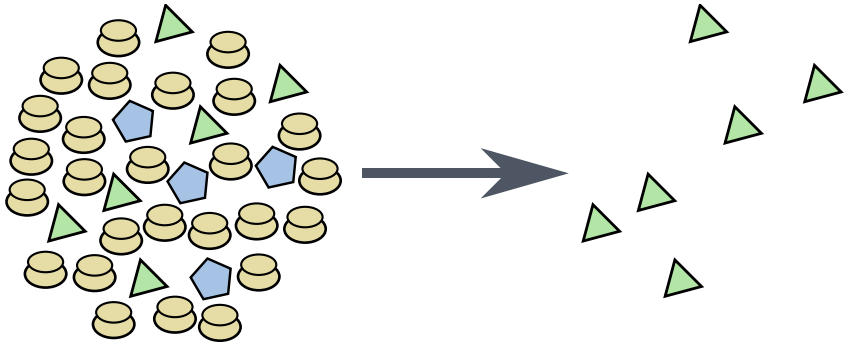
- Ribosomal RNA

- Poly-A transcripts

- Other RNAs e.g. tRNA, miRNA etc.

Library preparation

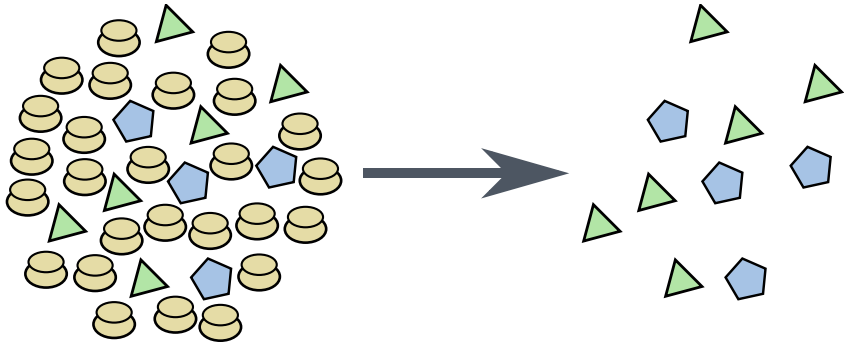
Poly-A Selection



Poly-A transcripts e.g.:

- mRNAs
- immature miRNAs
- snoRNA

Ribominus selection



Poly-A transcripts + Other mRNAs e.g.:

- tRNAs
- mature miRNAs
- piRNAs

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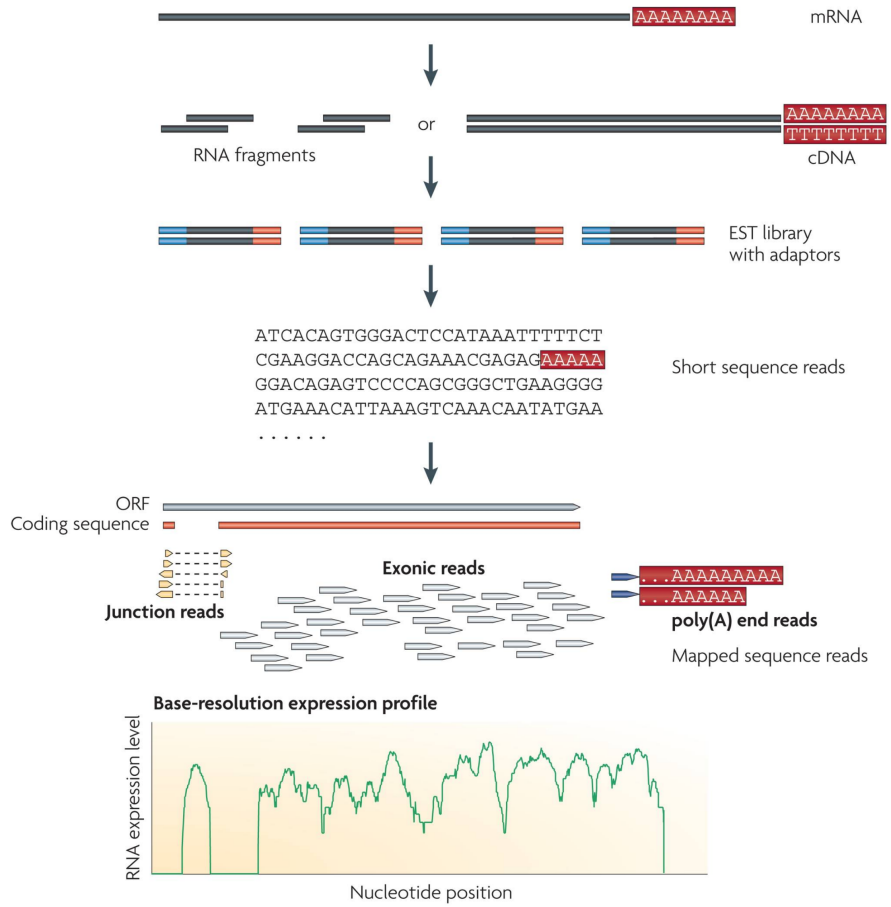

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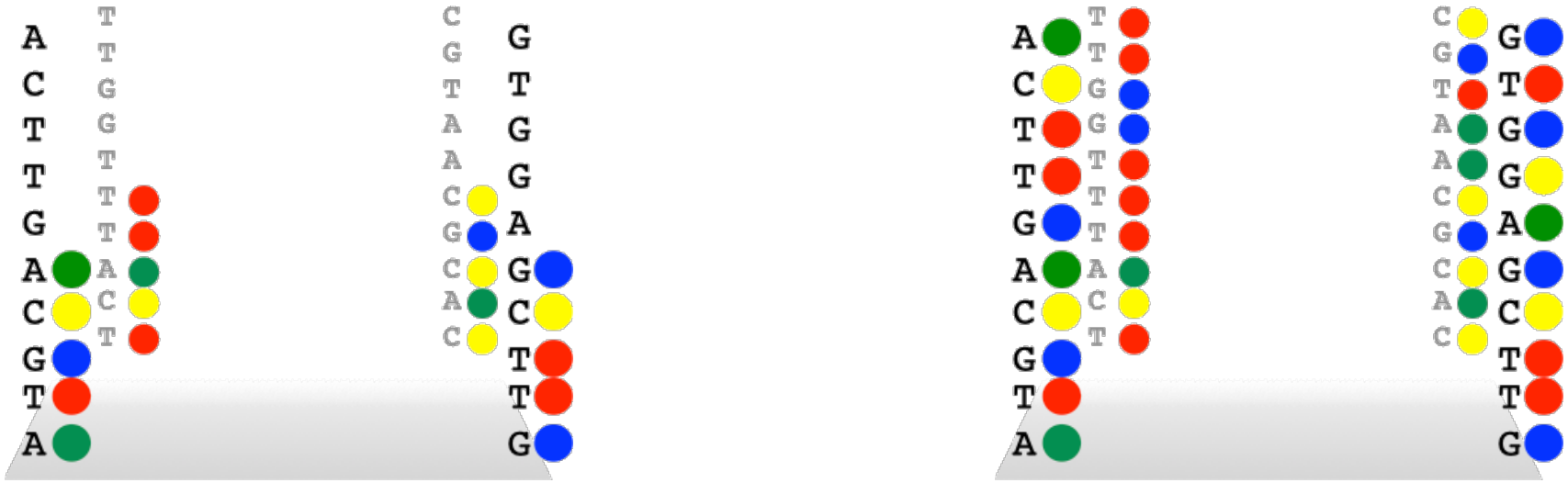
Sequencing by synthesis

A complimentary strand is synthesized using the cDNA fragment as template.

Each nucleotide includes a fluorescent tag and as the new strand is synthesized, the colour of the fluorescence indicates which base is being added.

The sequencer records the order of these flashes of light and translates them to a base sequence.

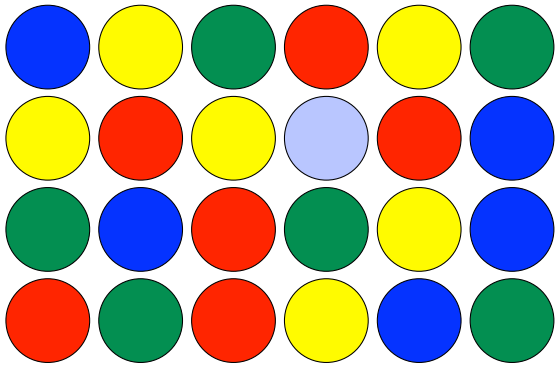
see this animation



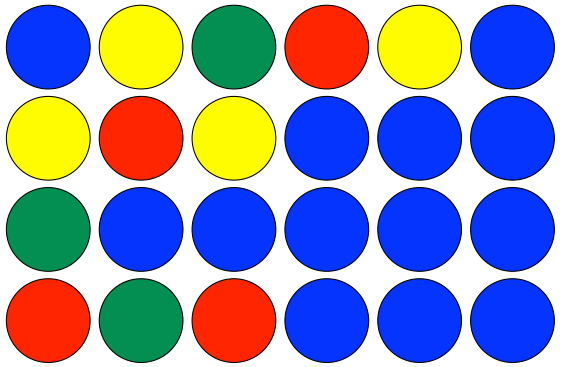
Sequencing by synthesis - sequencing errors

Sequencing errors cause uncertainty in calling the nucleotide at a given location. These reductions in confidence would be reflected int he quality scores in your fastq output.

If a probe doesn't shine as bright as it should, the sequencer is less confident in calling that base.



If there are lots of probes the same colour in the same region the sequencer finds it harder to identify the individual reads.



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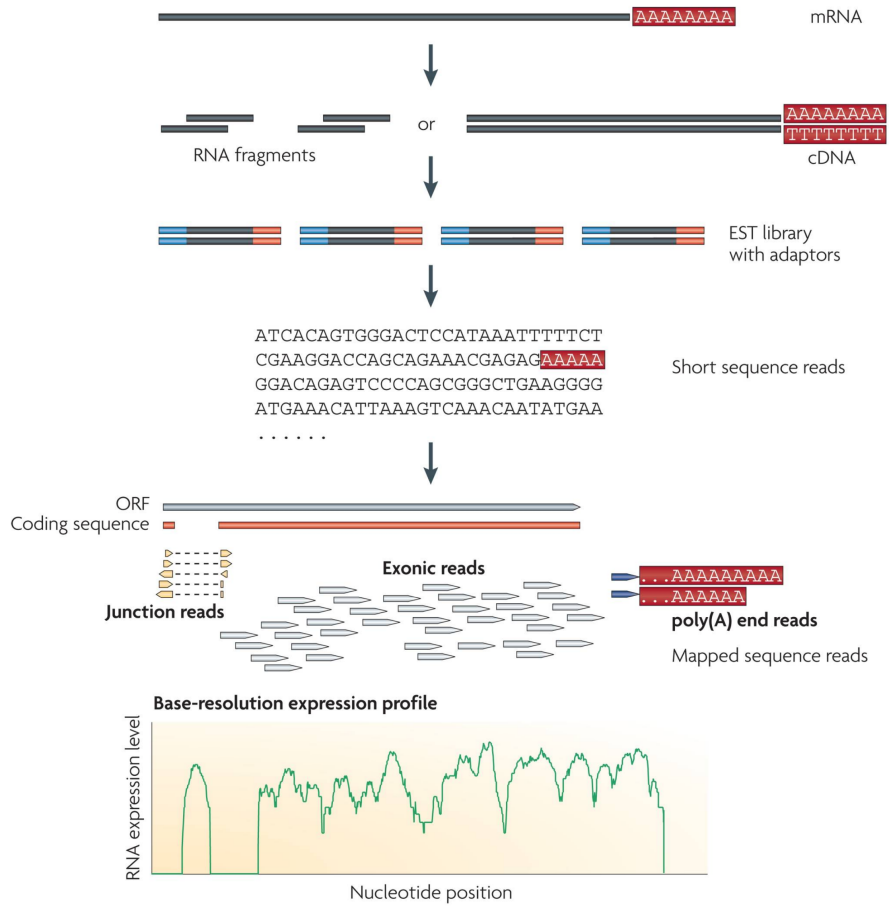

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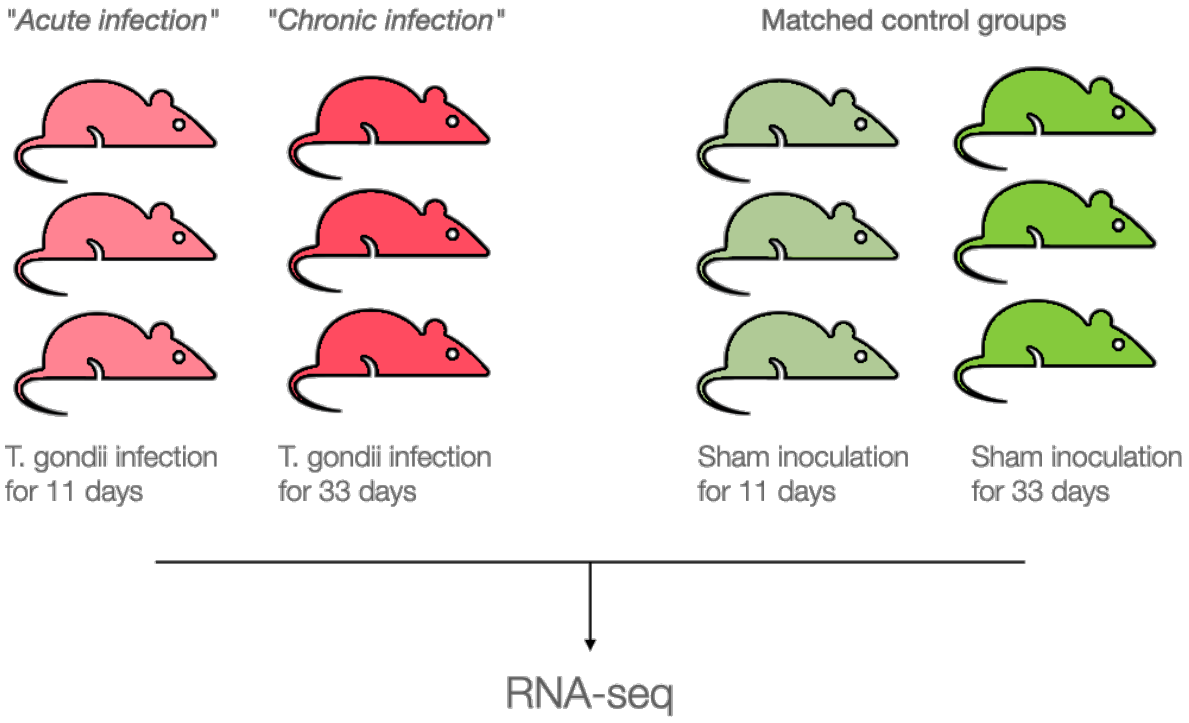
Case Study

Transcriptomic Profiling of Mouse Brain During Acute and Chronic Infections by *Toxoplasma gondii* Oocysts

Rui-Si Hu[1,2], Jun-Jun He[1], Hany M. Elsheikha[3], Yang Zou[1], Muhammad Ehsan[1], Qiao-Ni Ma[1], Xing-Quan Zhu[1,4] and Wei Cong[5]**



Differential Gene Expression Analysis Workflow