

Introduction to RNAseq Methods

March 2021

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

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

Practical considerations for RNAseq

- ▶ Coverage
- ▶ Read length
- ▶ Library preparation method

Designing the right experiment

Coverage: How many reads do we need?

The coverage is defined as:

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

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.

Library preparation

- Ribosomal RNA
- Poly-A transcripts
- Other RNAs e.g. tRNA, miRNA etc.

Total RNA extraction

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

Capturing Variance - 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

Capturing Variance - Replication

Biological Replication

Each replicate is from an independent biological individual

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

Capturing Variance - Replication

Technical Replication

Replicates are from the same individual but processed separately

- ▶ Experimental protocol
- ▶ Measurement platform

Controlling 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.
- ▶ Batch effects that are randomly distributed across experimental variables can be controlled for.

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Controlling batch effects

Multiplexing

Hidden Confounding variables

- ▶ Think deeply about the samples you are collecting
- ▶ This will be covered in more detail tomorrow

Library preparation

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Digitising Oligonucleotides

Differential Gene Expression Analysis Workflow