Overview of RNA-Seq

A Beginner's Guide

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Contents

1 Learning Objectives:

- Understand applications of RNA sequencing
- Introduce the overall differential expression workflow
- Understand experimental design concepts such as replicates and batch effects
- Understand different types of library preps, their requirements and uses.

2 Overview of RNA-seq

RNA-seq is an exciting experimental technique that is utilized to explore and/or quantify gene expression within or between conditions.

As we know, genes provide instructions to make proteins, which perform some function within the cell. Although **all cells contain the same DNA sequence**, muscle cells are different from nerve cells and other types of cells because of the **different genes that are turned on in these cells and the different RNAs and proteins produced**.

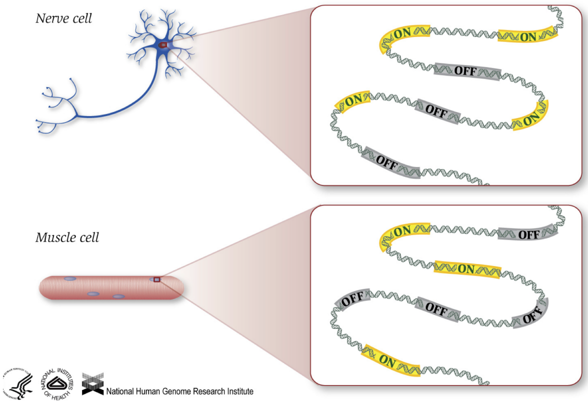
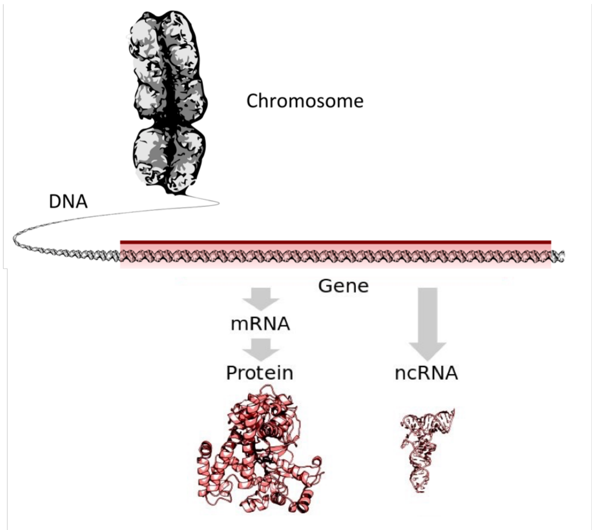


Figure 1: Gene Expression in Cells

Different biological processes, as well as mutations, can affect which genes are turned on and which are turned off, in addition to, *how much* specific genes are turned on/off.

To make proteins, the DNA is transcribed into messenger RNA, or mRNA, which is translated by the ribosome into protein. However, some genes encode RNA that does not get translated into protein; these RNAs are called non-coding RNAs, or ncRNAs. Often these RNAs have a function in and of themselves and include rRNAs, tRNAs, and siRNAs, among others. All RNAs transcribed from genes are called transcripts.

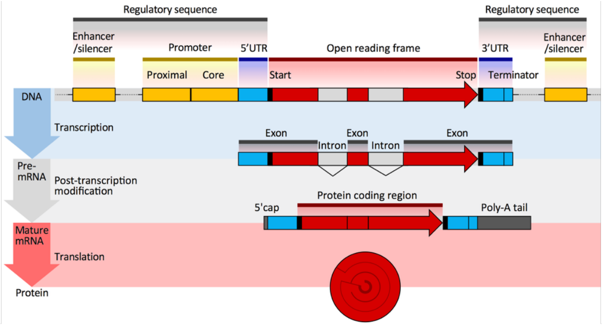


Wikimedia Commons Chromosome DNA Gene.svg and DNA to protein or ncRNA.svg" by Thomas Shafee, used under Creative Commons Attribution 4.0 International / Combined originals and added highlights

Figure 2: Gene Products

To be translated into proteins, the RNA must undergo processing to generate the mRNA. In the figure below, the top strand in the image represents a gene in the DNA, comprised of the untranslated regions (UTRs) and the open read frame. Genes are transcribed into pre-mRNA, which still contains the intronic sequences. After post-transciptional processing, a 5' cap and polyA tail are added and the introns are spliced out to yield mature mRNA transcripts, which can be translated into proteins.

While mRNA transcripts have a polyA tail, many of the non-coding RNA transcripts do not.



Wikimedia Commons Gene structure eukaryote 2 annotated.svg by Thomas Shafee, used under Creative Commons Attribution 4.0 International

Figure 3: Gene Structure

3 Transcriptomics

The transcriptome is defined as a collection of all the transcript readouts present in a cell. RNA-seq data can be used to explore and/or quantify the transcriptome of an organism, which can be utilized for the following types of experiments:

- **Differential Gene Expression**: *quantitative* evaluation and comparison of transcript levels between conditions
- **Transcriptome assembly**: building the profile of transcribed regions of the genome, a *qualitative* evaluation
- **Refinement of gene models**: building better gene models and verifying them using transcriptome assembly
- **Metatranscriptomics**: community transcriptome analysis

4 Illumina Sequencing

4.1 Illumina Library preparation

The general workflow for library preparation is detailed in the step-by-step images below.

Briefly, the RNA is isolated from the sample and contaminating DNA is removed with DNase.

The RNA sample then undergoes either selection of the mRNA (polyA selection) or depletion of the rRNA. The resulting RNA is fragmented.

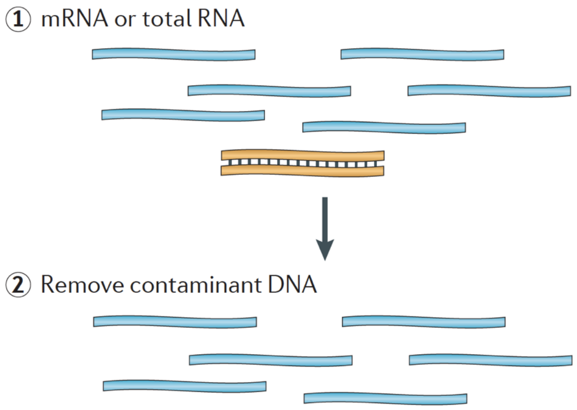


Figure 4: Library Prep

Generally, ribosomal RNA represents the majority of the RNAs present in a cell, while messenger RNAs represent a small percentage of total RNA, ~2% in humans. Therefore, if we want to study the protein-coding genes, we need to enrich for mRNA or deplete the rRNA.

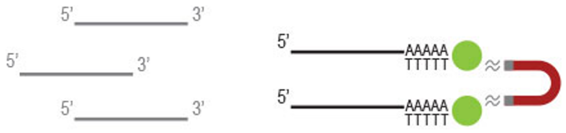


Figure 5: PolyA Tail

The size of the target fragments in the final library is a key parameter for library construction. DNA fragmentation is typically done by physical methods (i.e., acoustic shearing and sonication) or enzymatic methods (i.e., non-specific endonuclease cocktails and transposase tagmentation reactions.

The RNA is then reverse transcribed into double-stranded cDNA and sequence adapters are then added to the ends of the fragments.

The cDNA libraries can be generated in a way to retain information about which strand of DNA the RNA was transcribed from. Libraries that retain this information are called stranded libraries, which are now

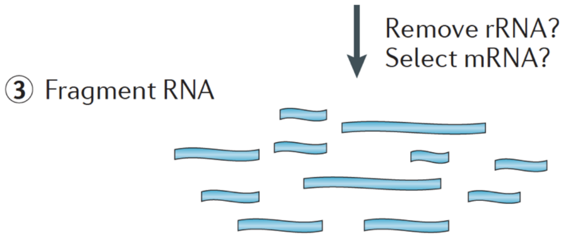


Figure 6: Library Prep

standard with Illumina's TruSeq stranded RNA-Seq kits. Stranded libraries should not be any more expensive than unstranded, so there is not really any reason not to acquire this additional information.

There are 3 types of cDNA libraries available:

- Forward (secondstrand) – reads resemble the gene sequence or the secondstrand cDNA sequence
- Reverse (firststrand) – reads resemble the complement of the gene sequence or firststrand cDNA sequence (TruSeq)
- Unstranded

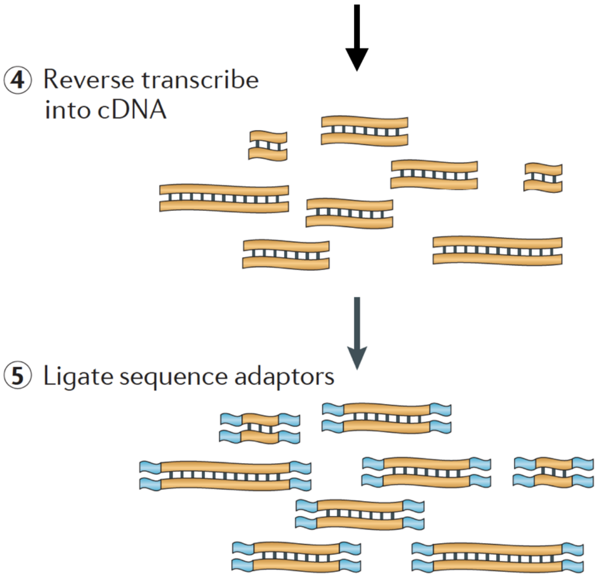


Figure 7: Library Prep Continued

Finally, the fragments are PCR amplified if needed, and the fragments are size selected (usually ~300-500bp) to finish the library.

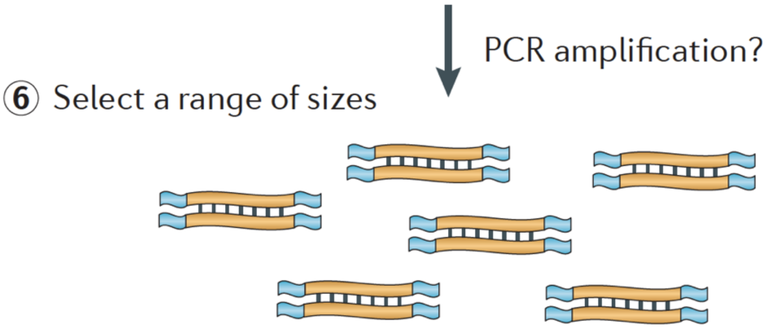


Figure 8: Library Prep Final Step

Image credit: Martin J.A. and Wang Z., Nat. Rev. Genet. (2011) 12:671–682

4.2 Strandedness

The implication of **stranded** libraries is that one could distinguish whether the reads are derived from the forward or reverse-encoded transcripts.

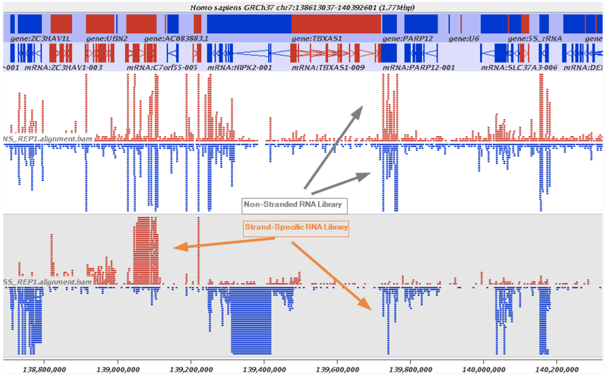


Figure 9: Strandedness

- Red = positive strand
- Blue = negative strand

4.3 Single-end versus Paired-end

After preparation of the libraries, sequencing can be performed to generate the nucleotide sequences of the ends of the fragments, which are called **reads**. You will have the choice of sequencing a single end of the cDNA fragments (single-end reads) or both ends of the fragments (paired-end reads).

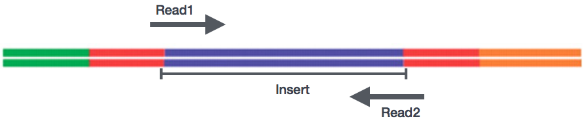


Figure 10: Paired End Reads

- SE - Single end dataset => Only Read1
- PE - Paired-end dataset => Read1 + Read2
  - often are 2 separate FastQ files!

Generally single-end sequencing is sufficient unless it is expected that the reads will match multiple locations on the genome (e.g. organisms with many paralogous genes), assemblies are being performed, or for splice isoform differentiation.

4.4 Different sequencing platforms

There are a variety of Illumina platforms to choose from to sequence the cDNA libraries.

Image credit: Adapted from Illumina

Differences in platform can alter the length of reads generated, the quality of reads, as well as the total number of reads sequenced per run and the amount of time required to sequence the libraries. The different platforms each use a different flow cell, which is a glass surface coated with an arrangement of paired oligos that are complementary to the adapters added to your template molecules. **The flow cell is where the sequencing reactions take place**.

Image credit: Adapted from Illumina

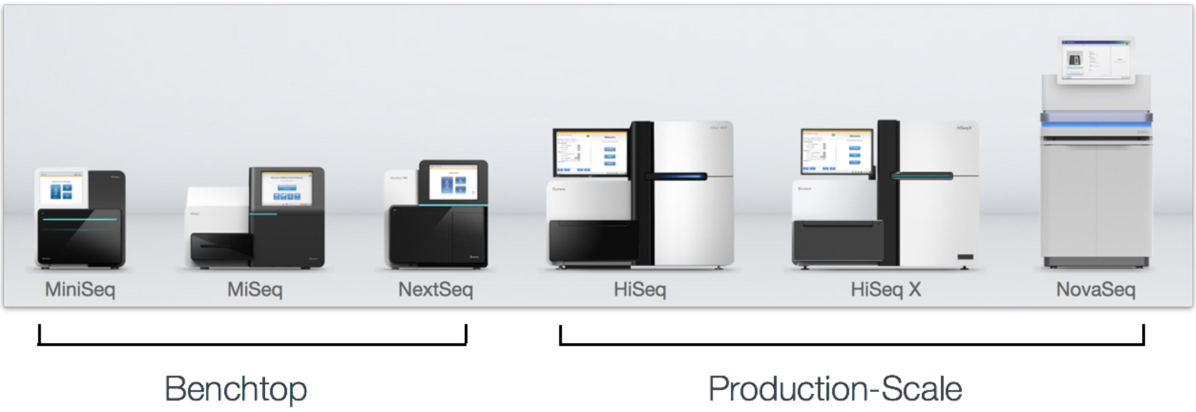


Figure 11: Illumina Platforms

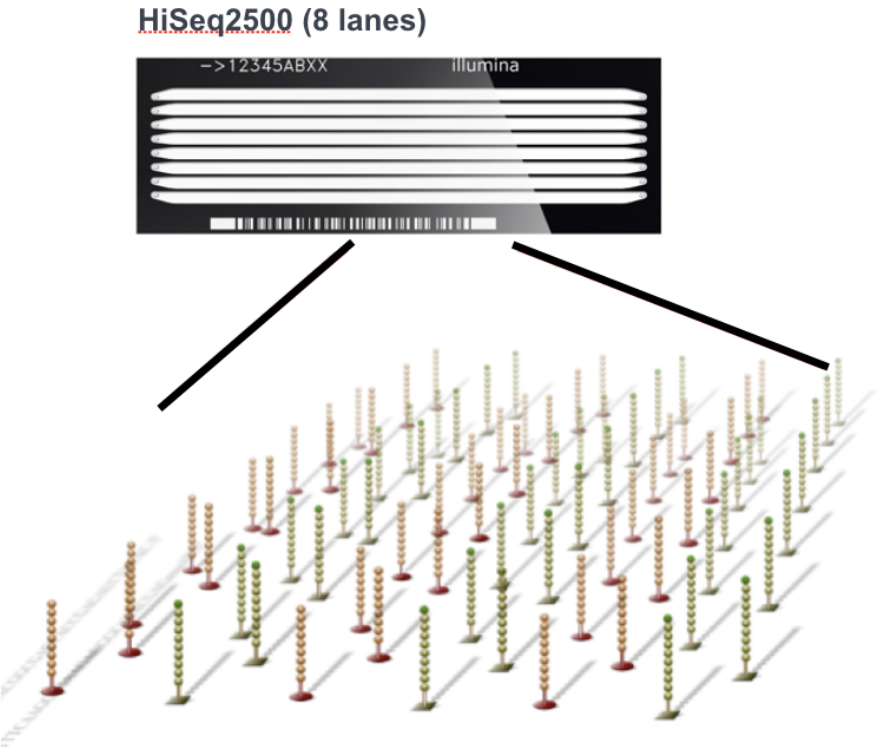


Figure 12: Flow Cell

4.5 Multiplexing

Depending on the Illumina platform (MiSeq, HiSeq, NextSeq), the number of lanes per flow cell, and the number of reads that can be obtained per lane varies widely. **The researcher will need to decide on how many reads they would like per sample** (i.e. the sequencning depth) and then based on the platform you choose calculate how many total lanes you will require for your set of samples.

Typically, charges for sequencing are per lane of the flow cell and you will be able to run multiple samples per lane. Illumina has therefore devised a nice multiplexing method which allows libraries from several samples to be pooled and sequenced simultaneously in the same lane of a flow cell. This method requires **the addition of indices** (within the Illumina adapter) or special barcodes (outside the Illumina adapter) as described in the schematic below.

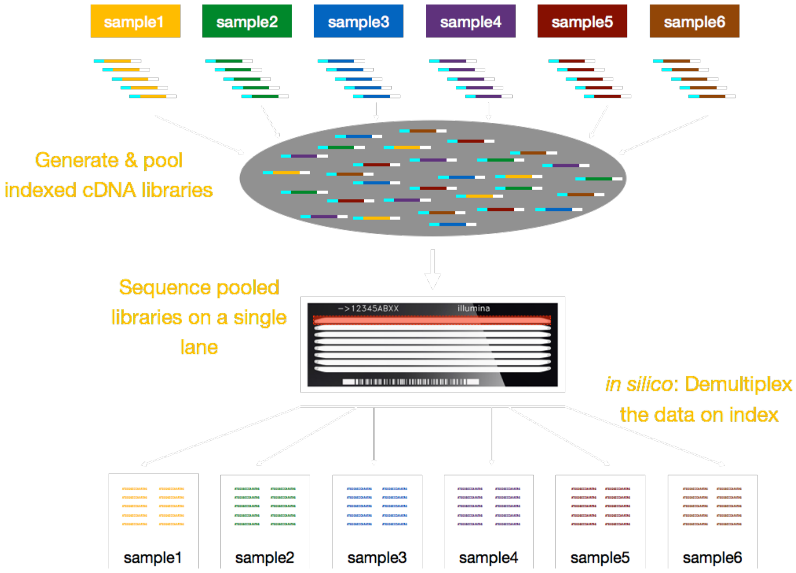

sample1
sample2
sample3
sample4
sample5
sample6
Generate & pool indexed cDNA libraries
Sequence pooled libraries on a single lane
—>12345ABXX
illumina
in silico: Demultiplex the data on index
sample1
sample2
sample3
sample4
sample5
sample6

Figure 13: Demultiplexing

- **General gene-level differential expression:**
  - ENCODE guidelines suggest 30 million SE reads per sample (stranded).
  - 15 million reads per sample is often sufficient, if there are a good number of replicates (>3).
  - Use of an HiSeq or NextSeq, or NovaSeq for sequencing

5 Differential gene expression

Differential gene expression analysis allows us to explore the gene expression changes that occur in disease or between different conditions, by measuring the quantity of RNA expressed by all genes in each of the different conditions.

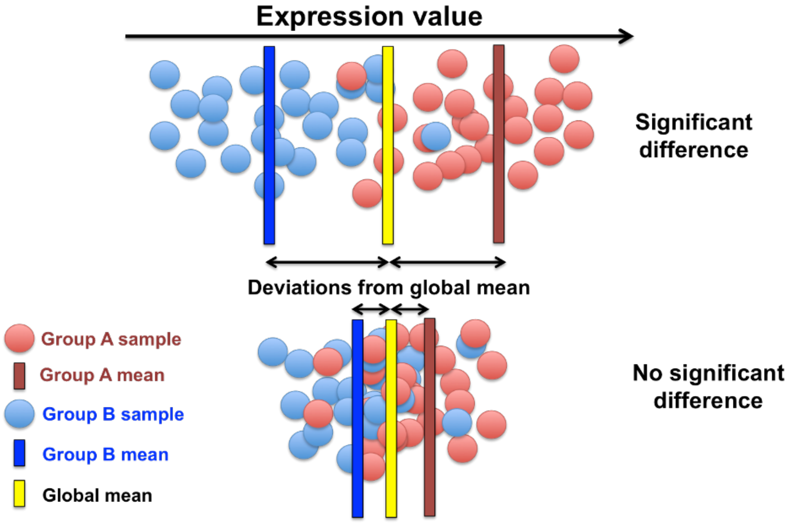


Figure 14: Differential Gene Expression

Using this analysis we can answer questions such as:

- What genes are differentially expressed between conditions?
- Are there any trends in gene expression over time or across conditions?

Citation: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0138236

- Which groups of genes change similarly over time or across conditions?
- What processes or pathways are important for my condition of interest?

Citation: https://elifesciences.org/articles/63003

To perform differential gene expression analysis, we perform the following steps:

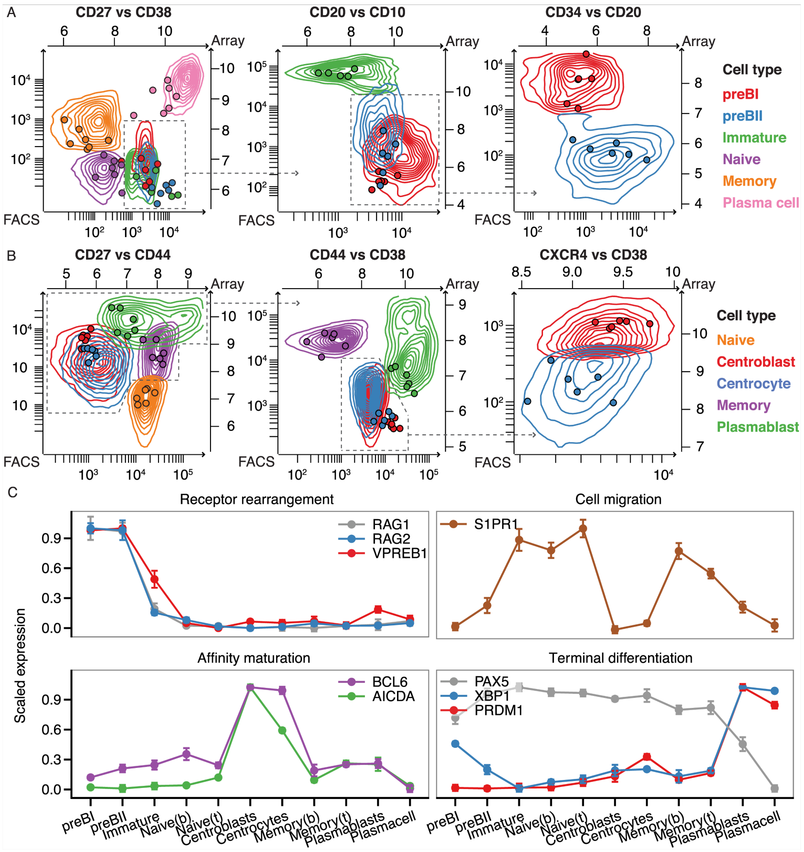


Figure 15: Trends DE

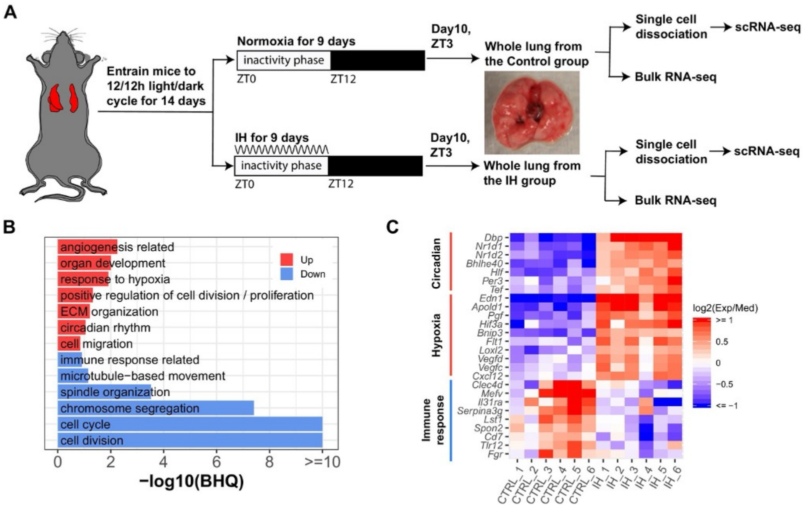


Figure 16: Pathways DE

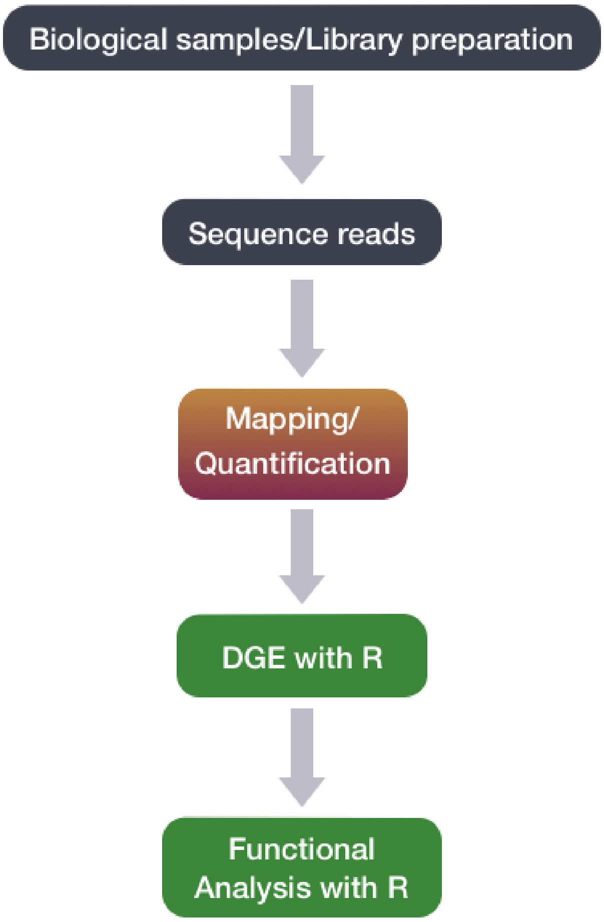


Figure 17: DE Workflow

6 Experimental Design

Understanding the steps in the experimental process of RNA extraction and preparation of RNA-Seq libraries is helpful for designing an RNA-Seq experiment and important to consider when selecting a dataset to analyze. There are special considerations that should be highlighted which can greatly affect the quality of a differential expression analysis.

These important considerations include:

1. Number and type of **replicates**
2. Avoiding **confounding** variables
3. Addressing **batch effects**

6.1 Replicates

Experimental replicates can be performed as **technical replicates** or **biological replicates**.

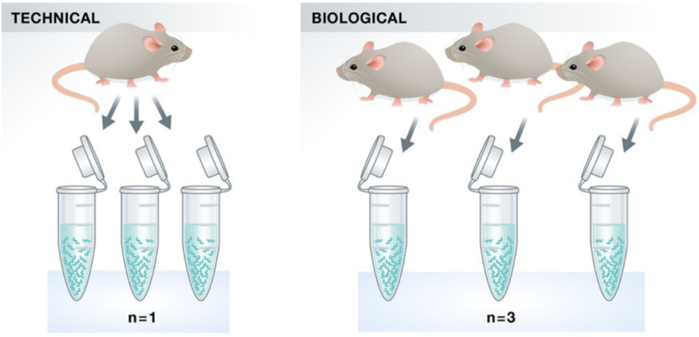


Figure 18: Biological Replicates

Image credit: Klaus B., EMBO J (2015) ***34****: 2727-2730*

- **Technical replicates:** use the same biological sample to repeat the technical or experimental steps in order to accurately measure technical variation and remove it during analysis.

- **Biological replicates** use different biological samples of the same condition to measure the biological variation between samples.

Biological replicates are absolutely essential for differential expression analysis. In fact, the more biological replicates, the better for estimates of biological variation and the more precise our estimates of the mean expression levels. This leads to more accurate modeling of our data and identification of more differentially expressed genes.

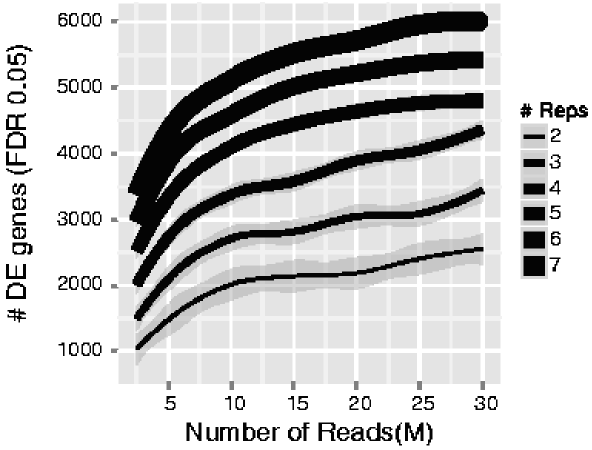


Figure 19: DE Replicates

Image credit: Liu, Y., et al., Bioinformatics (2014) ***30****(3): 301–304*

As the figure above illustrates, **biological replicates are of greater importance than sequencing depth**. The figure shows the relationship between sequencing depth and number of replicates on the number of differentially expressed genes identified [1]. Note that an **increase in the number of replicates tends to return more DE genes than increasing the sequencing depth**. Therefore, generally more replicates are better than higher sequencing depth, with the caveat that higher depth is required for detection of lowly expressed DE genes and for performing isoform-level differential expression.

Replicates are almost always preferred to greater sequencing depth for bulk RNA-Seq.

7 Confounding

A confounded RNA-Seq experiment is one where you **cannot distinguish the separate effects of two different sources of variation** in the data.

For example, we know that sex has large effects on gene expression, and if all of our *control* mice were female and all of the *treatment* mice were male, then our treatment effect would be confounded by sex. **We could not differentiate the effect of treatment from the effect of sex.**

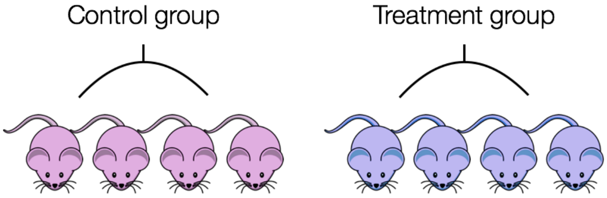


Figure 20: Confounding Variables

To AVOID confounding:

- Ensure animals in each condition are all the **same sex, age, litter, and batch**, if possible.
- If not possible, then ensure to split the animals equally between conditions

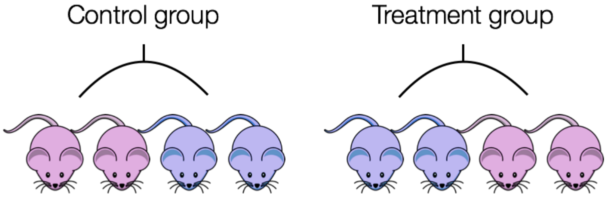


Figure 21: Non Confounded Design

8 Batch effects

Batch effects are a significant issue for RNA-Seq analyses, since you can see significant differences in expression due solely to the batch effect.

Image credit: Hicks SC, et al., bioRxiv (2015)

8.1 How to know whether you have batches?

- Were all RNA isolations performed on the same day and with the same kit?
- Were all library preparations performed on the same day?

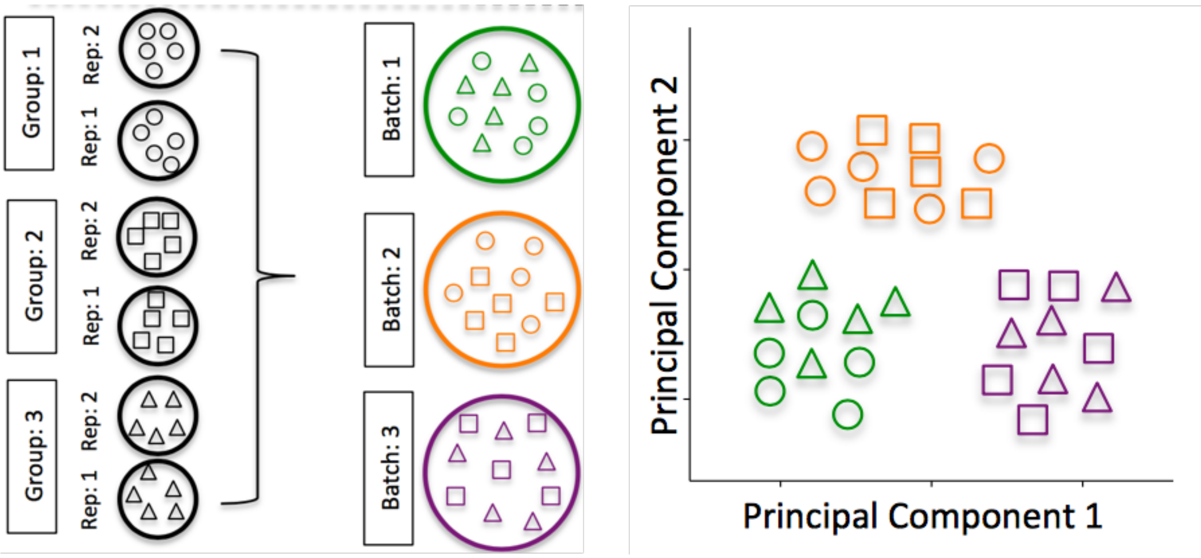


Figure 22: Non Confounded Design

- Did the same person perform the RNA isolation/library preparation for all samples?
- Did you use the same reagents for all samples?
- Did you perform the RNA isolation/library preparation in the same location?

If *any* of the answers is **'No'**, then you have batches.

8.2 Best practices regarding batches:

- Design the experiment in a way to **avoid batches**, if possible.
- If unable to avoid batches:
  - **Do NOT confound** your experiment by batch:
    Image credit: Hicks SC, et al., bioRxiv (2015)
  - **DO** split replicates of the different sample groups across batches. The more replicates the better!
    Image credit: Hicks SC, et al., bioRxiv (2015)
  - **DO** include batch information in your **experimental metadata**. During the analysis, we can regress out the variation due to batch so it doesn't affect our results if we have that information.

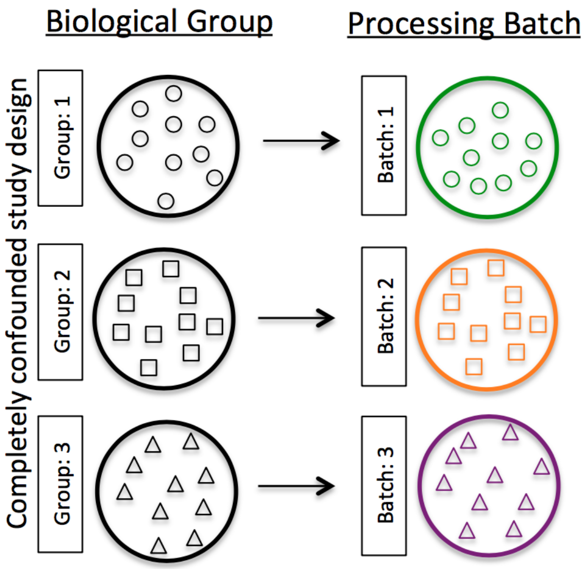


Figure 23: Confounded Batch

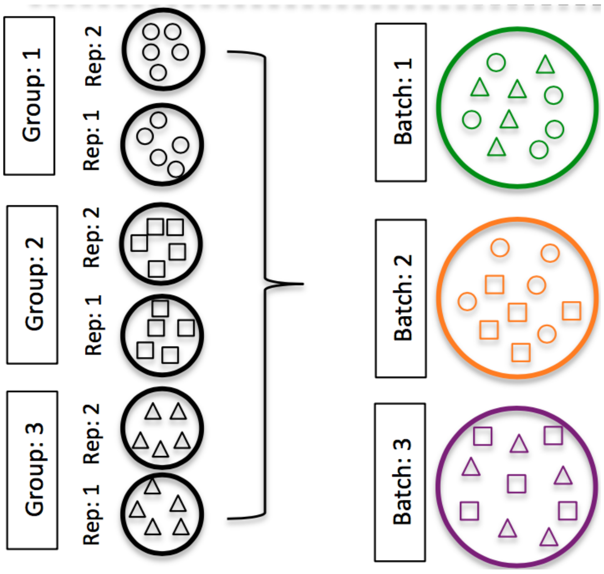
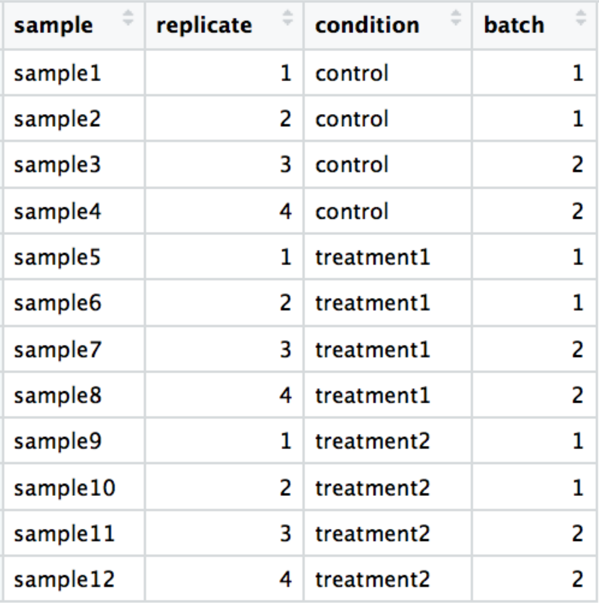


Figure 24: Batch Effect



sample	replicate	condition	batch
sample1	1	control	1
sample2	2	control	1
sample3	3	control	2
sample4	4	control	2
sample5	1	treatment1	1
sample6	2	treatment1	1
sample7	3	treatment1	2
sample8	4	treatment1	2
sample9	1	treatment2	1
sample10	2	treatment2	1
sample11	3	treatment2	2
sample12	4	treatment2	2

Figure 25: Metadata

8.3 Citation

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Authors: Mary Piper, Meeta Mistry, Radhika Khetani
Other sources - https://umich-brcf-bioinf.github.io/rnaseq_demystified_workshop/site/Module3a_Design_Prep_Seq#2_Experimental_Design_and_Practicalities