

An RNA-seq Data Analysis Protocol

David Chisanga and Wei Shi

2021-02-22

Contents

Preface	5
1 Overview	7
2 Prerequisites	11
2.1 Data	11
2.2 SOFTWARE	11
3 Running Environment	13
3.1 RStudio	13
3.2 UNIX server + laptop	13
3.3 Laptop only	14
4 RNA-SEQ analysis protocol	15
5 More details on the protocol	23
5.1 Sequencing quality	23
5.2 Gene annotation	24
5.3 Index building	24
5.4 Read mapping	25
5.5 Read counting	26
5.6 Gene filtering	27
5.7 Sample clustering	27
5.8 Statistical testing of differential expression	27

6	Downstream analysis	29
6.1	Load previous results	29
6.2	Visualisations	29
6.3	Pathway Analysis	34

Preface

This tutorial describes a RNA-seq analysis protocol that can be used to analyse Bulk RNA-seq data on the Bioinformatics server. The guide provides a brief discussion and examples of the steps involved in the analysis of Bulk RNA-seq data, from raw data through to differential expression analysis.

Chapter 1

Overview

Discovering differentially expressed genes is one of the most important applications of RNA sequencing technologies. This RNA-seq analysis protocol provides step-by-step procedures for a successful analysis of an RNA-seq dataset. The protocol can be applied to RNA-seq data generated from any species that has a reference genome and an annotation of genes and it can be applied to both small and large RNA-seq experiments.

This protocol comprises three major steps including read mapping, read counting and statistical testing (Figure 1.1). Read mapping and counting are performed using Rsubread package (Liao et al., 2019) and statistical testing is carried out using limma package (Ritchie et al., 2015).

Read mapping.

Read mapping is the first step in an RNA-seq analysis. In this step, an index for the reference genome will be first built using *buildindex()* function and then reads will be aligned to the genome using the *align()* function (Liao et al., 2019, 2013b). Index building is a one-off operation. The built index can be reused in future RNA-seq analyses. Read mapping results generated from *align()* are saved to BAM or SAM format files. *align()* also returns a mapping summary including percentages of mapped reads, uniquely mapped reads and multi-mapping reads.

Read counting

After read mapping is completed, mapped reads can then be counted to genes using the *featureCounts()* function (Liao et al., 2013a). Other than read mapping data, a gene annotation must be provided to *featureCounts()* for read counting. Rsubread package includes inbuilt gene annotations for human and mouse genomes. These annotations were constructed based on the NCBI RefSeq gene annotation database (O’Leary et al., 2015). Gene annotations for other species can be downloaded from NCBI RefSeq or other databases. *featureCounts()* (Shi et al., 2020) returns a count table which includes read counts for each gene in each library. It also returns other information such as counting summary for

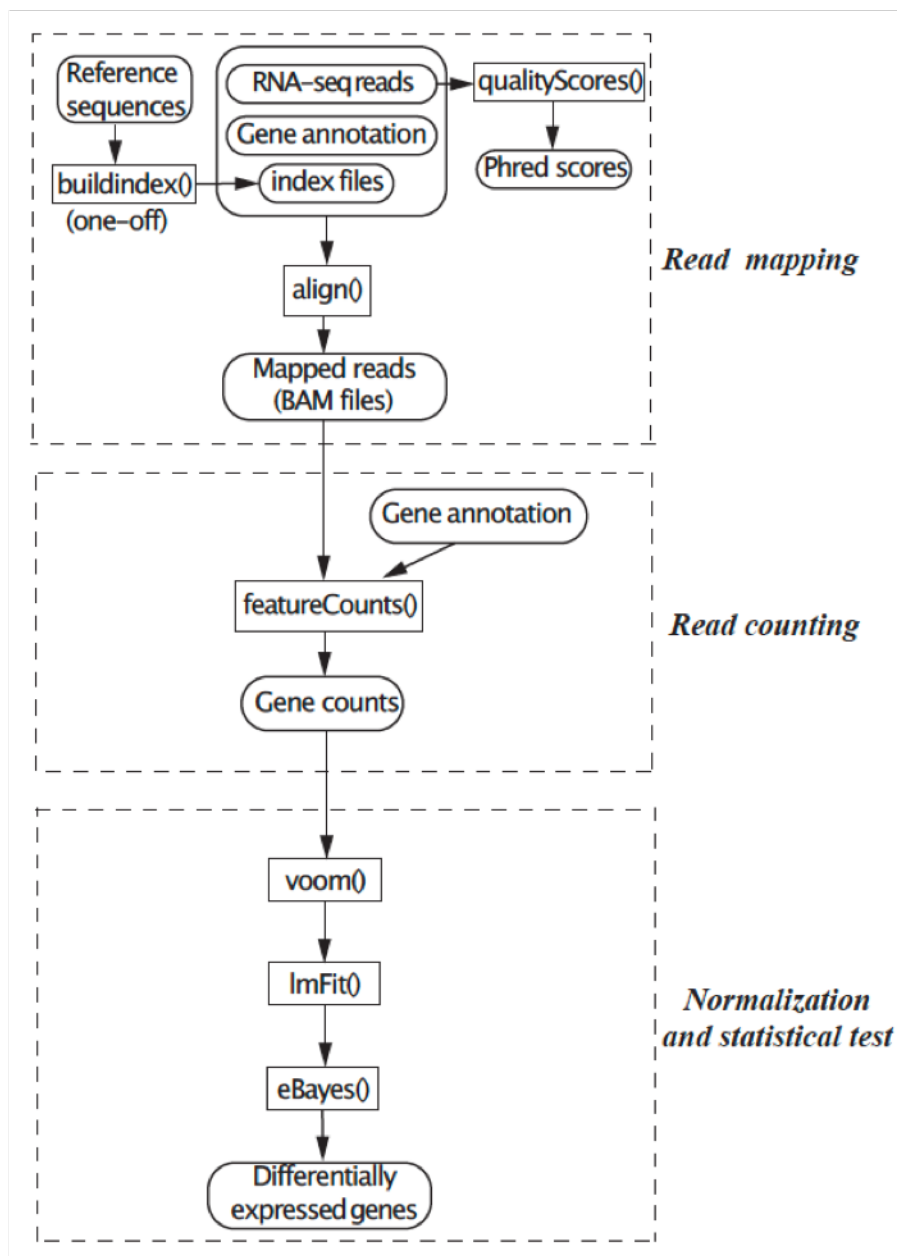


Figure 1.1: Overview of the RNA-seq analysis protocol. The three boxes enclosed by dashed lines represent the three major components of the protocol. Each rectangle represents an R function and each rounded rectangle denotes data that are input to or output from a function

each library and gene length.

Normalization

After read counts are generated, the *voom()* function in limma will be used to convert raw counts to counts per million (CPM), estimate the mean-variance relationship and compute observation levelweights (Law et al., 2014). The quantile normalization will also be applied to the data. Linear models are then fitted to the normalized data using *lmFit()* function. Empirical Bayes moderated t-statistic will be used to test the statistical significance of gene expression changes (Smyth, 2004). This test is performed using the *eBayes()* function in limma (Smyth et al., 2020). The output of statistical testing will include differentially expressed genes if any are found.

Chapter 2

Prerequisites

Before you get started with the rest of the analysis, it is important that you have the necessary data and software that will be used in this analysis.

2.1 Data

RNA-seq data

An RNA-seq dataset generated in a published study (Delconte et al., 2016) is used as an example dataset in this protocol.

Two samples (wild-type and Cish^{-/-} natural killer cells) are included in this dataset and each sample has two biological replicates. These data were already deposited in the Gene Expression Omnibus database (GSE79409). However for the convenience of this analysis, FASTQ files of the raw RNA-seq data were also saved in the ‘Workshop_RNAseq’ directory on Z drive.

A text file called “Targets.txt”, which includes relevant sample information, can also be found in this directory.

Reference genome data

A FASTA-format file including all chromosomal sequences of the GRCm38/mm10 genome was also saved in ‘Workshop_RNAseq’ directory on Z drive.

2.2 SOFTWARE

The following software tools should be installed on a UNIX server and on your laptop:

- R (<https://www.r-project.org>)
- Rsubread (<http://bioconductor.org/packages/release/bioc/html/Rsubread.html>)
- limma (<http://bioconductor.org/packages/release/bioc/html/limma.html>)
- edgeR (<http://bioconductor.org/packages/release/bioc/html/edgeR.html>)
- org.Mm.eg.db (<http://bioconductor.org/packages/release/data/annotation/html/org.Mm.eg.db.html>)
- statmod (<https://CRAN.R-project.org/package=statmod>)

Consult the R Project website for the installation of R (<https://www.r-project.org/>) (R Core Team, 2020). Make sure the latest release version of R is downloaded and installed. After R is installed, launch R and type the following commands to install *Rsubread*, *limma*, *edgeR*, *org.Mm.eg.db* and *statmod*:

```
if (!requireNamespace("BiocManager", quietly = TRUE))
  install.packages("BiocManager")

BiocManager::install(c("Rsubread", "limma", "edgeR", "org.Mm.eg.db"), update = T)

if (!requireNamespace("statmod", quietly = TRUE))
  install.packages("statmod")
```

Alternatively, you may use Rstudio to run this protocol.

Chapter 3

Running Environment

3.1 RStudio

This is the recommended approach for running this protocol. An RStudio application ('RStudio(2672)') has been created under the 'Analysis' tab in the Remote Access Facility. After you log into it (using your email account name and password), change to the directory '/data/RawPrimary/Public' (you can do this by choosing Session > Set Working Directory > Choose Directory and then using the '...' to specify the directory you want to change to) and then you will find a folder called 'Workshop_RNAseq' which includes all the materials included in this Workshop. You can make a copy of this folder and then start to run the protocol in your own folder.

3.2 UNIX server + laptop

Run read mapping and counting on a UNIX server and then perform the rest of the analysis on your laptop. Refer to the document "How to Access Linux Bioinformatics Analysis Platform.pdf" for how to access the Bioinformatics UNIX server. Once you logged in the server, you can issue the following commands to copy the Workshop data to a directory you create on the server and to launch R. You are then ready to run the protocol.

```
# connect AllStaffShare Drive to the server  
cifscreds add svr-fs95
```

```
# create your own directory, eg 'my_directory'  
cd /data/Processing/Public/  
mkdir my_directory
```

```
# copy the Workshop data to your directory  
cp -r /mnt/AllStaffShare/Workshop_RNAseq my_directory  
cd my_directory/Workshop_RNAseq
```

```
# load R  
module load R-bundle-Bioconductor  
R
```

3.3 Laptop only

Run your entire RNA-seq analysis on your laptop. If you choose this option, you should build a low-memory index (split index) for the reference genome before performing read mapping.

Chapter 4

RNA-SEQ analysis protocol

Please note that your analysis results might be slightly different from those shown in this protocol due to software version differences.

We first change the working directory to the Workshop folder

```
setwd("Workshop_RNAseq/")
```

Build index for a reference genome

Step 1

Start an R session and build an index for the reference genome *GRCm38/mm10*. The created index files will be saved to the current working directory. This index only needs to be built once and it can be reused in future RNA-seq data analyses.

TIMING ~40 mins

```
library(Rsubread)
```

```
buildindex("mm10_reference", "mm10.fa")
```

If you build an index on a laptop, it is recommended to build a split index. The amount of requested memory should be roughly half of the total memory available on your laptop. For example, if your laptop has 8GB memory, you may use the following command to build a split index with 4GB memory usage.

TIMING ~90 mins

```
buildindex("mm10_reference",  
           "mm10.fa",  
           indexSplit = TRUE,  
           memory = 4000)
```

Create sample-related information and evaluate quality of sequencing TIMING ~ 3 mins

Step 2

Create a tab-delimited text file that contains sample-related information such as FASTQ file names, sample names and cell types. For convenience, a file called “Targets.txt” has already been created for this dataset and we read in this file:

```
library(limma)
targets <- readTargets("Targets.txt")
targets
```

	Library	Sample	CellType
## 1	CIS_1_C6AG6ANXX_ATCACG_L008_R1.fastq.gz	CIS_rep1	CIS
## 2	CIS_2_C6AG6ANXX_CGATGT_L008_R1.fastq.gz	CIS_rep2	CIS
## 3	WT_3_C6AG6ANXX_TTAGGC_L008_R1.fastq.gz	WT_rep1	WT
## 4	WT_4_C6AG6ANXX_TGACCA_L008_R1.fastq.gz	WT_rep2	WT

Step 3

Choose one of the libraries and examine the Phred quality scores of read bases at each read position. Boxplots of Phred scores are generated for each base position.

```
qs <- qualityScores(targets$Library[1], offset = 33)
```

```
boxplot(qs,
        xlab = "Base position",
        ylab = "Phred score",
        cex = 0.1)
```

Align and count reads TIMING ~6 min per library

Step 4

Map sequence reads to mouse genome GRCm38/mm10. It is recommended to provide gene annotation to the read mapping. Mapped reads are saved to BAM files and a mapping summary is returned to R after read mapping is completed.

```
align.outfiles <- paste(targets$Sample, "bam", sep = ".")
align.summary <-
  align(
    "mm10_reference",
    targets$Library,
    output_file = align.outfiles,
```

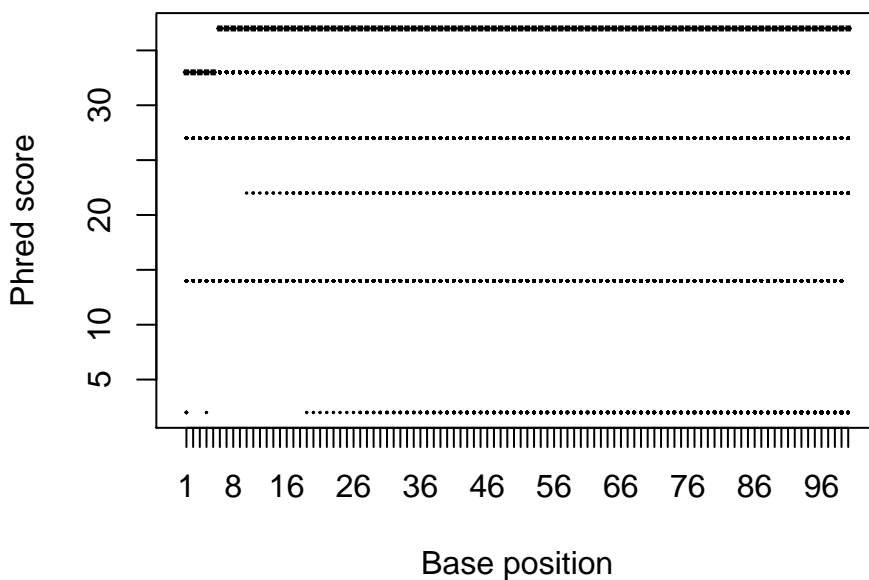



Figure 4.1: Distribution of Phred quality scores of read bases at each base position of the reads

```

    nthreads = 10,
    useAnnotation = TRUE,
    annot.inbuilt = "mm10"
  )
align.summary

```

Step 5

Assign reads to overlapping genes and generate an R object that contains a count table, gene annotation and counting summary. The count table contains number of reads assigned to each gene in each library. The gene annotation includes Entrez gene identifier, chromosomal coordinates of gene exons and gene length (total number of non-overlapping exonic bases each gene has). The counting summary gives the number of successfully assigned reads in each library and also numbers of reads that failed to be assigned due to filtering. The 'Status' column in this summary includes a 'Assigned' category and also multiple 'Unassigned' categories corresponding to different read filters used in counting.

```

counts.gene <- featureCounts(align.outfiles, "mm10", nthreads = 10)
counts.gene$stat

```

Save R data and switch from the UNIX server to a personal computer
TIMING < 1 min

(Steps 6 and 7 can be skipped if you run the protocol using ‘RStudio (2672)’ or laptop only)

Step 6 (optional)

Save all generated R objects to a file and then copy the file to your personal computer.

```
save.image("Counts.rdata")
```

Step 7 (optional)

Launch R on your personal computer and load R objects from the copied file.

```
load("Counts.rdata")
```

Perform differential expression analysis TIMING < 1 min

Step 8

Load required libraries.

```
library(limma)
library(edgeR)
library(statmod)
suppressMessages(library(org.Mm.eg.db))
```

Step 9

Create a design matrix.

```
ct <- factor(targets$CellType)
design <- model.matrix(~0 + ct)
colnames(design) <- levels(ct)
```

Step 10

Map Entrez gene identifiers to gene symbols and create an R object containing annotation data for each gene. The annotation data include Entrez gene identifier, gene symbol and gene length.

```

tmp <- org.Mm.egSYMBOL
entrez_symbol <- as.list(tmp[mappedkeys(tmp)])
entrez_symbol <- sapply(entrez_symbol, function(x)
  x[1])
genes <- counts.gene$annotation$GeneID
m <- match(genes, names(entrez_symbol))
genes <- data.frame(
  EntrezID = genes,
  Symbol = entrez_symbol[m],
  Length = counts.gene$annotation$Length,
  stringsAsFactors = FALSE
)

```

Step 11

Remove from analysis those genes that did not express, or expressed at a very low level, in all cell types. Here we require a gene to have a CPM value greater than 0.5 in at least two libraries to be included in the subsequent analysis. The reason we require at least two libraries is because there are two biological replicates generated for each sample in this dataset.

```

keep <- rowSums(cpm(counts.gene$counts) > 0.5) >= 2
sum(keep)

```

```
## [1] 11969
```

Step 12

Transform count data to log2-cpm, estimate the mean-variance relationship and compute observation-level weights. Log2-cpm expression values of genes are then normalized using the quantile method and converted to log2-rpkm (log2 reads per kilobases of exons per million reads) values.

```

y <-
  voom(counts.gene$counts[keep, ], design, normalize.method = "quantile")
y$genes <- genes[keep, ]
y$E <- y$E - log2(y$genes$Length / 1000)

```

Step 13

Cluster samples via multidimensional scaling.

```
plotMDS(y, labels=targets$CellType)
```

Step 14

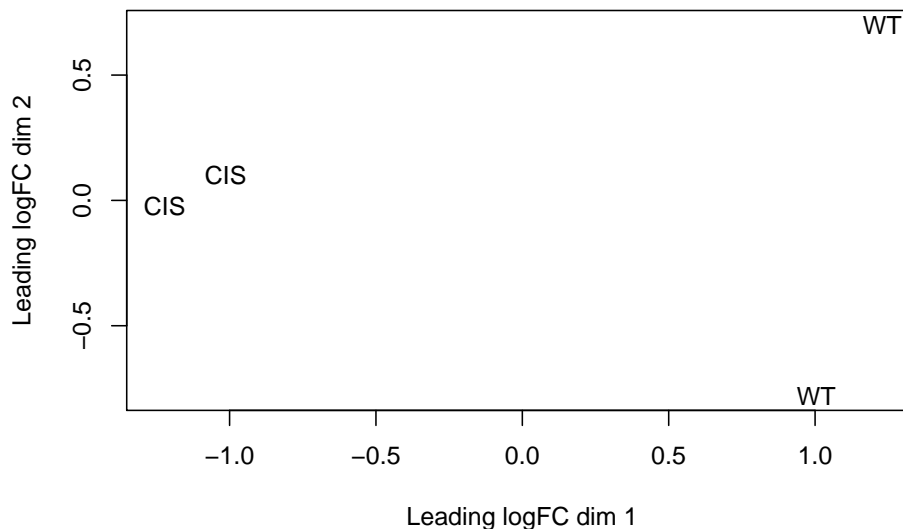


Figure 4.2: Sample clustering. Distances between samples represent leading log2-fold-changes.

Fit linear models to genes and perform statistical testing to discover differentially expressed genes. A contrast matrix is constructed to specify the comparisons between samples. The empirical Bayes moderated t-statistic is used to assess the differential expression of genes. A FDR (false discovery rate) cutoff is applied for calling differentially expressed genes. For this analysis, an FDR cutoff of 0.05 was applied (default cutoff value in `decideTests()`).

```
fit <- lmFit(y, design)
contr <- makeContrasts(CISvsWT = CIS - WT, levels = design)
fit.contr <- eBayes(contrasts.fit(fit, contr))
dt <- decideTests(fit.contr)
summary(dt)
```

```
##          CISvsWT
## Down      1662
## NotSig    8788
## Up        1519
```

Step 15

Display top 10 most differentially expressed genes:

```
options(digits=2)
topTable(fit.contr)
```

##	EntrezID	Symbol	Length	logFC	AveExpr	t	P.Value	adj.P.Val
##	12700	12700						
##	12700	12700						
##	69368	69368						
##	69368	69368						
##	21638	21638						
##	21638	21638						
##	436468	436468	Trav15d-1-dv6d-1					
##	436468	436468	Trav15d-1-dv6d-1					
##	22290	22290	Uty					
##	22290	22290	Uty					
##	26908	26908	Eif2s3y					
##	26908	26908	Eif2s3y					
##	26900	26900	Ddx3y					
##	26900	26900	Ddx3y					
##	20592	20592	Kdm5d					
##	20592	20592	Kdm5d					
##	59310	59310	Myl10					
##	59310	59310	Myl10					
##	13508	13508	Dscam					
##	13508	13508	Dscam					
##		B						
##	12700	10.1						
##	69368	8.2						
##	21638	6.6						
##	436468	6.3						
##	22290	6.1						
##	26908	6.0						
##	26900	6.0						
##	20592	5.8						
##	59310	5.7						
##	13508	5.6						

Step 16

Save all differentially expressed genes to a file and all other results for further downstream analysis.

```
de.genes <- topTable(fit.contr, coef="CISvsWT", p.value=0.05, n=Inf)
write.csv(de.genes, file="DE_genes.csv", row.names=FALSE)
```

Save all the results to be used later.

```
#save differential expression results
save.image("Counts2.RData")
```


Chapter 5

More details on the protocol

5.1 Sequencing quality

Sequencing output from a sequencer is stored in one or more FASTQ-format files. Each FASTQ file contains nucleotide sequences and sequencing quality strings for the reads generated from a library. The quality string for a read has the same length as the read sequence and each letter in the string encodes the sequencing quality score of the corresponding read base. The ASCII code of a letter is equal to the Phred quality score of the read base plus an integer offset. A Phred quality score denotes the sequencing quality of a read base. It is computed as $-10 \times \log_{10}P$, where P is the probability that a read base is incorrectly called

The `qualityScores()` function in **Rsubread** extracts quality strings from a FASTQ file and returns the Phred scores in a data matrix object in which rows are reads and columns are base positions. Distribution of Phred scores at each base position can be viewed by using the `boxplot()` function. For reads generated by the popular Illumina sequencers such as HiSeq and NextSeq, sequencing quality is usually lower at the two ends of the read (particular at the 3' end) compared to the middle part of the read. The vast majority of read bases in a FASTQ file are expected to have a Phred score greater than 13 (corresponding to P value of 0.05). Fig. 2 shows the boxplots of quality scores in a library that was sequenced in 2015 by an Illumina HiSeq 2000 sequencer. The boxplots show that this dataset has a high sequencing quality.

Box 1 | The Simplified Annotation Format (SAF)

The Simplified Annotation Format is defined in Rsubread and this format is used by

featureCounts to counts reads to genes or other genomic features. This format only includes five compulsory columns: 'GeneID', 'Chr', 'Start', 'End' and 'Strand'. Below is an example of SAF annotation for two RefSeq genes with Entrez gene identifiers 497097 and 100503874.

GeneID	Chr	Start	End	Strand**
497097	chr1	3204563	3207049	-
497097	chr1	3411783	3411982	-
497097	chr1	3660633	3661579	-
100503874	chr1	3637390	3640590	-
100503874	chr1	3648928	3648985	-

Gene identifiers included in the column 'GeneID' can be integer numbers (eg. Entrez g

5.2 Gene annotation

The Rsubread package contains inbuilt gene annotation for human and mouse, making it convenient to process RNA-seq data generated from human or mouse samples. This annotation was generated based on the NCBI RefSeq gene annotation. In this annotation, overlapping exons from the same gene were merged to form a single exon covering all overlapping exons. The inbuilt annotations can be used in both read counting and read mapping. The inbuilt annotations are in SAF format (Box 1). They can be retrieved using the `getInBuiltAnnotation()` function in Rsubread.

Alternatively, external gene annotations such as those generated in Ensembl database (9) or GENCODE database (10) can be used for counting and mapping. Formats of external gene annotation that are accepted by Rsubread functions include GTF and GFF3 (<https://genome.ucsc.edu/FAQ/FAQformat.html#format4>).

5.3 Index building

An index built for a reference genome contains a large hash table, which stores chromosomal locations of subreads (16bp mers) extracted from the reference genome. With the hash table, subreads extracted from a read can be quickly mapped to the genome via a quick search (hashing) in the table. Candidate mapping locations of the read will then be determined by the voting of mapped subreads and final mapping location will be determined by further evaluation of candidate mapping locations.

The index can be built as either a full index or a gapped index. A full index contains subreads extracted from every base location of the genome, whereas a gapped index contains subreads extracted from every three bases in the genome. The use of a full index will make read mapping faster, but it also causes more computer memory to be used. Full index and gapped index can be further split

into blocks. This can reduce memory usage but mapping time will increase on the other hand. See Box 2 for more information on gapped index and split index.

By default, *buildindex()* constructs a single-block full index for a reference genome. This allows maximum mapping speed to be achieved. To our knowledge, Rsubread is the only tool that allows users to tune the amount of memory used to achieve a desired balance between memory consumption and speed in read mapping. This flexibility allows Rsubread to be run on various computing platforms.

5.4 Read mapping

After an index is successfully built, sequencing reads can then be mapped to the reference genome via the *align()* function. Reads are mapped via a two-pass procedure. In the first pass, subreads (seeds) are extracted from each read and their locations in the genome are quickly determined by looking up the hash table included in the index. Mapped subreads then ‘vote’ for mapping locations of the read. This ‘*seed-and-vote*’ strategy has been demonstrated to be more efficient and accurate than the conventional ‘*seed-and-extend*’ strategy (Liao et al., 2019, 2013b). Indels will also be discovered in this step.

In the second pass, reads are re-aligned to the genome to determine their final mapping location by taking into account indels identified from the first pass. Note that *align()* does not perform full alignment for exon-spanning reads. It only aligns such reads to the exon they most overlap with.

Read bases overlapping other exons in such reads are soft-clipped. However this partial alignment is sufficient for RNA-seq expression quantification at the gene level because reads can be confidently assigned to one of the exons in the gene. To fully align exon-spanning reads, the *subjunc()* function in **Rsubread** can be used. *subjunc()* is slower than *align()*, but it is still computationally competitive. *subjunc()* is also a two-pass aligner. However it not only collects indels in its first pass, it also collects exon splicing sites. In its second pass, it will use detected exon splicing sites to further improve the mapping of exon-spanning reads.

Box 2 | Build an index with less memory usage

A gapped index can be built to reduce the index size.

Size of a gapped index is only one third of size of the full index.

Use of a gapped index could save more than 50% of memory used in read mapping.

During read mapping, the memory contains the index and also other data such as chromosomal sequences.

For human or mouse genome, size of the full index is ~15GB and size of the gapped index is 5GB.

During read mapping, ~18GB of memory is consumed when full index is used and ~8GB when gapped index is used.

Below is the command for building a gapped index:

```
> buildindex("mm10_reference", "mm10.fa", gappedIndex=TRUE)
```

The full index and gapped index can be split into blocks to further control the amount of memory to be used.

Users can specify the maximum memory allowed and `buildindex()` will automatically split the index into required number of blocks.

The index blocks will be loaded to memory sequentially during read mapping.

The more blocks an index is split into, the smaller the memory required for read mapping.

The following command builds a full index that is split into multiple blocks to achieve

```
> buildindex("mm10_reference", "mm10.fa", indexSplit=TRUE, memory=4000)
```

Rsubread aligners are the first to use the two-pass strategy to improve the mapping quality of RNA-seq reads. This strategy has later been adopted by other RNA-seq aligners. Mapping results from `align()` include both mapped and unmapped reads. The results are saved to files in BAM or SAM format. Indels identified in the mapping are saved to VCF-format files. `align()` also returns an **R** object that contains mapping statistics for each library.

Percentage of successfully mapped reads in a library is affected by multiple factors including sequencing quality, read length, paired-end or single-end, quality of reference sequences and aligner setting. For the single-end reads generated from the latest Illumina HiSeq/NextSeq/NovaSeq sequencers, `align()` typically reports around 80-90 percent of mapped reads. If paired-end reads are provided, the mapping percentage is expected to be slightly higher.

5.5 Read counting

After read mapping is completed, mapped reads can be counted to genes by `featureCounts()` function in **Rsubread**. `featureCounts()` compares chromosomal coordinates of mapped reads with chromosomal coordinates of exons in each gene. *CIGAR* (Concise Idiosyncratic Gapped Alignment Report) string of each mapped read is analyzed so that read assignment can be done precisely. `featureCounts()` provides a wide range of counting options. For example, overlap between a read and an exon can be determined based on the number of overlapping bases, fraction of overlapping bases and number of non-overlapping bases. Multi-mapping reads can be excluded from counting, or only have their primary alignment counted or have all their alignments counted.

Similarly, for multi-overlapping reads that overlap more than one gene they can be excluded from counting or be counted to all their overlapping genes.

`featureCounts()` accepts both name-sorted and location-sorted reads. It can output counting details for each individual read.

`featureCounts()` returns an **R** object that includes a count table, gene annotation and counting statistics. Percentage of successfully assigned reads is affected

by many factors including cell type, annotation quality and mRNA purity. The counting percentage could be highly variable between experiments, even for well annotated genomes such as human and mouse genomes. Typically around 50 – 90 percent of mapped reads are assigned to genes in a mouse experiment. For the RNA-seq data used in this protocol, ~70 percent of reads were successfully assigned.

5.6 Gene filtering

Genes that do not express or express at a very low level in all the samples are filtered out from analysis. Such genes are very hard to handle in the statistical testing. Typically ~50 percent of genes are excluded in the analysis of mouse RNA-seq data. For the RNA-seq data used in this protocol, 58 percent of genes were removed from analysis.

5.7 Sample clustering

Examining the clustering of samples is a useful way to check the quality of samples. Sample replicates should cluster together and different sample types should separate from each other.

5.8 Statistical testing of differential expression

A rigorous *false discovery rate (FDR)* threshold should be applied when calling differentially expressed genes. This threshold is usually *0.05*, but it might be relaxed to *0.1* or even *0.15* in some circumstances. The *treat()* function in **limma** might be considered for testing against a fold change of gene expression.

Chapter 6

Downstream analysis

In this chapter we will run through a series of example downstream analyses that can be performed on the identified differentially expressed genes.

6.1 Load previous results

The results from the differential analysis were saved as R-objects. These are imported below and used for the rest of the analysis

```
#load the required package  
library(limma)  
load("Counts2.RData") # loads the counts dataset
```

6.2 Visualisations

There are a number of ways in which you can visualise the results from the differential expression analysis, while in this chapter we attempt to cover the most common ways of visualising the data, they are by no means exhaustive.

6.2.1 Heatmaps

Heatmaps are a great way of demonstrating the differences in the expression patterns between different conditions in your dataset. Here we use the *coolmap* function within the **limma** package which is an extension of the *heatmap.2* function within the **gplots** package. The heatmap below shows the expression pattern of the top 100 differentially expressed genes between CIS vs WT.

```

heat.data <- y$E[head(rownames(de.genes), n = 100), ]
colnames(heat.data) <- targets$Sample
rownames(heat.data) <- head(de.genes$Symbol, n = 100)
coolmap(
  heat.data,
  margin = c(5, 4),
  cexCol = 0.8,
  lhei = c(0.8, 4),
  cexRow = 0.6
)

```

6.2.2 Mean-difference plot

A mean-difference plot or MD-plot is a plot that can be used to show the fold-change differences against the average expression values of all genes used in the analysis. This can be generated easily using the *plotMD* function within the **limma** package.

```
plotMD(fit.contr, column = 1, status = dt, cex=0.8)
```

Alternatively, an interactive mean-difference plot can be generated using *glMD-Plot* in **Glimma** which outputs an interactive html page with results displayed in the left panel and the expression values for the selected gene. A gene within the dataset can be searched through a search bar.

```

library(Glimma)
glMDPlot(fit.contr, coef=1, status=dt, main=colnames(fit.contr)[1],
  side.main="ENTREZID", counts=y$E, groups=colnames(contr),
  launch = F)

```

[Click here for interactive version](#)

Hover over points to see sample-wise expression

- Click on column names to sort by column
- Click rows on tables to highlight gene

6.2.3 Volcano plot

A volcano plot shows the relationship between the log fold-change on the x-axis and a measure of statistical significance on the y-axis. The measure of significance can be $-\log(\text{p-value})$ or the B-statistics. Here, we will use the *volcanoplot*

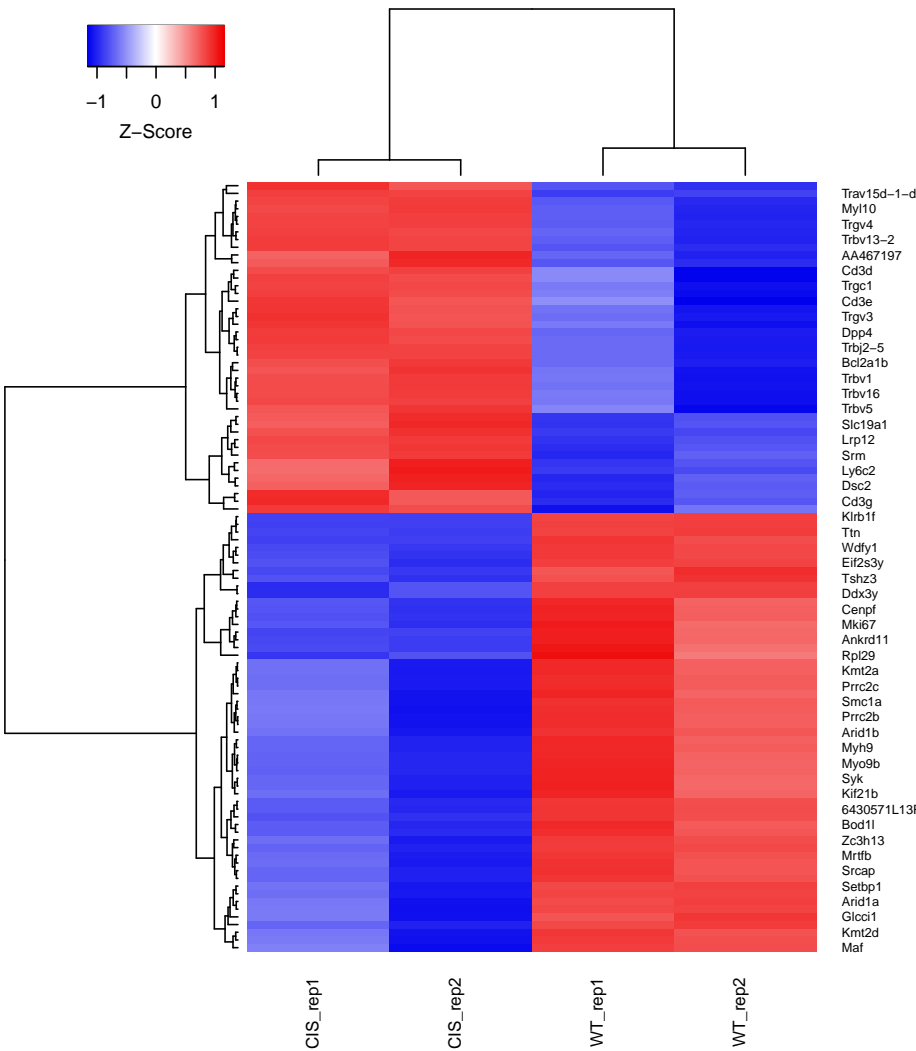


Figure 6.1: Heatmap plot of differentially expressed genes in CIS vs WT

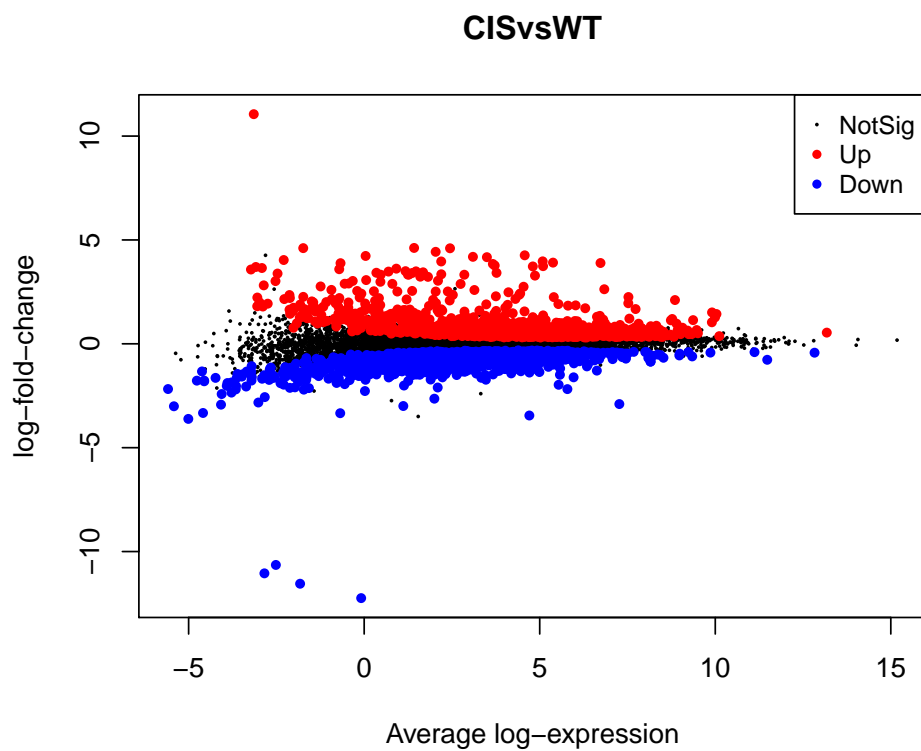


Figure 6.2: Mean-Difference plot. Represents relationship between mean expression and fold-changes

function within the **limma** package. In the volcano plot below, differentially expressed genes are highlighted with red for up-regulated and blue for down-regulated genes.

```
#highlight de genes  
col<-c("blue","black","red")[factor(dt[, "CISvsWT"])]  
volcanoplot(fit.contr,col=col)
```

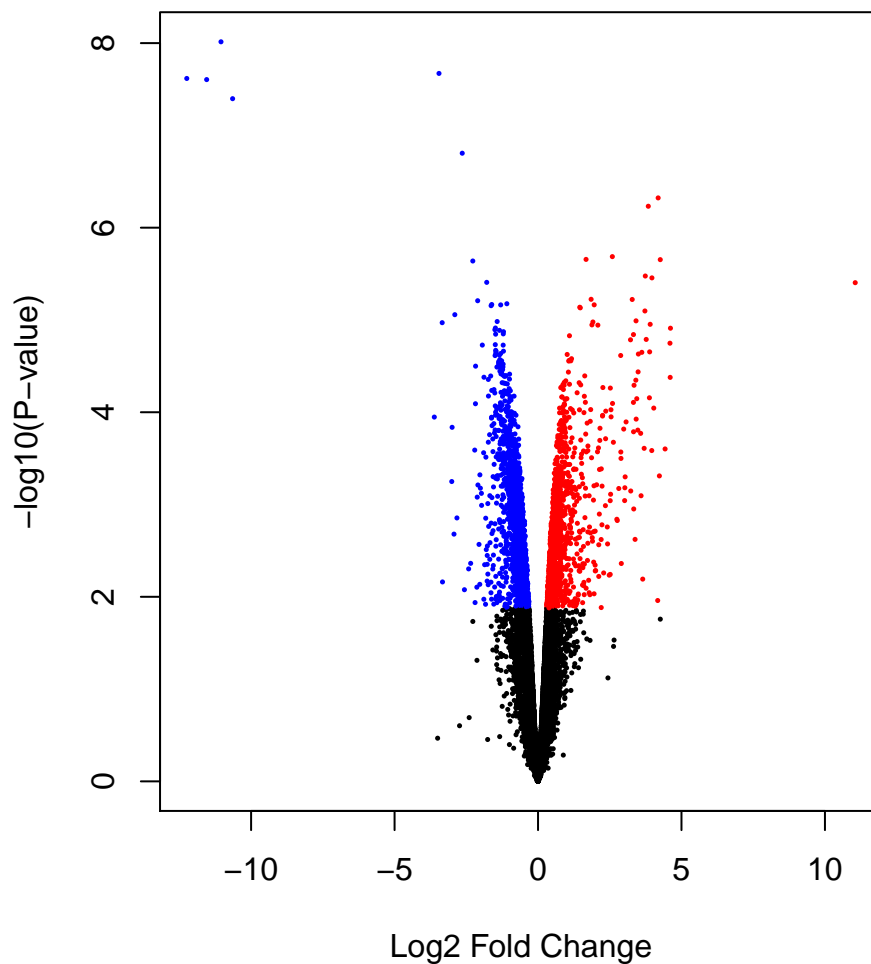


Figure 6.3: Volcano plot. Shows the relationship between fold-change and significance of change

6.3 Pathway Analysis

From the differential expression analysis there were some 3181 differentially expressed genes. This is quite a long list of genes to interpret and nearly impossible to go through a gene at a time to gain any meaningful biological understanding. A common downstream analysis step is to understand pathways/gene networks that the genes are implicated in.

6.3.1 Gene Ontology enrichment

Gene ontology (<http://www.geneontology.org/>) provides a controlled vocabulary for describing biological processes (BP ontology), molecular functions (MF ontology) and cellular components (CC ontology).

The GO ontologies themselves are organism-independent; terms are associated with genes for a specific organism through direct experimentation or through sequence homology with another organism and its GO annotation.

Terms are related to other terms through parent-child relationships in a directed acyclic graph.

You can use enrichment analysis as another way of drawing conclusions from your set of differentially expressed genes.

```
enrich.pvalue<-0.00001
```

Here, we use the *goana* function within the **limma** package to test for enrichment of differentially expressed genes between the CIS vs WT. Note that the *p-values* returned by *goana* are **unadjusted for multiple testing**. It is therefore, advisable that if the results are to be published, only terms with very small p-values should be included. For instance, in the example below, only terms with a p-value < 10^{-5} are retained.

We use the output from the differential expression analysis and test for over-representation for the Up and Down regulated genes separately.

```
if(!requireNamespace("GO.db"))
  BiocManager::install("GO.db")
library(GO.db)
go.rst<-goana(fit.contr,species="Mm")
#exclude terms not meeting our cut-off
go.rst<-go.rst[rowSums(go.rst[,c("P.Up","P.Down")]<enrich.pvalue)==1,]
topGO(go.rst)
```

```
##
## GO:0050794 regulation of cellular process BP 6084 593
```

##	G0:0050789	regulation of biological process	BP	6343	628
##	G0:0065007	biological regulation	BP	6647	675
##	G0:0048522	positive regulation of cellular process	BP	3439	335
##	G0:0005515	protein binding	MF	5634	584
##	G0:0048518	positive regulation of biological process	BP	3697	368
##	G0:0060255	regulation of macromolecule metabolic process	BP	3917	361
##	G0:0032501	multicellular organismal process	BP	3570	335
##	G0:0048856	anatomical structure development	BP	3061	287
##	G0:0019222	regulation of metabolic process	BP	4221	404
##	G0:0005488	binding	MF	7993	876
##	G0:0032502	developmental process	BP	3334	319
##	G0:0031323	regulation of cellular metabolic process	BP	3857	361
##	G0:0048731	system development	BP	2484	228
##	G0:0007275	multicellular organism development	BP	2804	261
##	G0:0016043	cellular component organization	BP	3824	409
##	G0:0006996	organelle organization	BP	2732	266
##	G0:0010604	positive regulation of macromolecule metabolic process	BP	2214	224
##	G0:0051171	regulation of nitrogen compound metabolic process	BP	3590	321
##	G0:0080090	regulation of primary metabolic process	BP	3689	332
##		Down P.Up P.Down			
##	G0:0050794	1177	1	5.2e-68	
##	G0:0050789	1206	1	6.1e-66	
##	G0:0065007	1233	1	6.5e-61	
##	G0:0048522	746	1	4.5e-50	
##	G0:0005515	1069	1	6.7e-50	
##	G0:0048518	779	1	1.6e-47	
##	G0:0060255	808	1	7.0e-46	
##	G0:0032501	754	1	1.0e-45	
##	G0:0048856	673	1	1.3e-45	
##	G0:0019222	853	1	1.4e-45	
##	G0:0005488	1360	1	1.5e-45	
##	G0:0032502	716	1	2.1e-45	
##	G0:0031323	796	1	6.2e-45	
##	G0:0048731	575	1	1.4e-44	
##	G0:0007275	628	1	1.7e-44	
##	G0:0016043	789	1	2.7e-44	
##	G0:0006996	607	1	2.5e-41	
##	G0:0010604	518	1	1.4e-40	
##	G0:0051171	738	1	1.8e-39	
##	G0:0080090	753	1	2.2e-39	

A simple way to visualise the enrichment results is through a bar plot as shown below which shows the top 10 enriched terms in each direction.

```

top.up <- head(go.rst[order(go.rst$P.Up, decreasing = F),], n = 10)
top.down <-
  head(go.rst[order(go.rst$P.Down, decreasing = F),], n = 10)
bar.data <-
  rbind(
    data.frame(
      Term = top.up$Term,
      P.value = top.up$P.Up,
      Dir = "up"
    ),
    data.frame(
      Term = top.down$Term,
      P.value = top.down$P.Down,
      Dir = "down"
    )
  )
bar.data <- bar.data[order(bar.data$P.value, decreasing = F),]
par(mai = c(0.8, 2, 0.5, 0.5))
bb <- barplot(
  -log10(bar.data$P.value),
  horiz = T,
  xlab = " -log10(p-value)",
  cex.names = 0.5,
  col = c("red", "blue")[factor(bar.data$Dir)]
)
axis(
  2,
  line = -0.8,
  at = bb,
  labels = bar.data$Term,
  tick = F,
  las = 2,
  cex.axis = 0.5
)
legend("topright", legend = c("Up", "Down"), pch = 22, pt.bg = c("red", "blue"))

```

Add gene information (optional)

Sometimes you may want to know what genes are enriched in each of the terms, this section allows you to add gene details.

First, we extract the genes that are associated with the catalytic activity GO terms. This results in a list mapping the GO term and the associated genes.

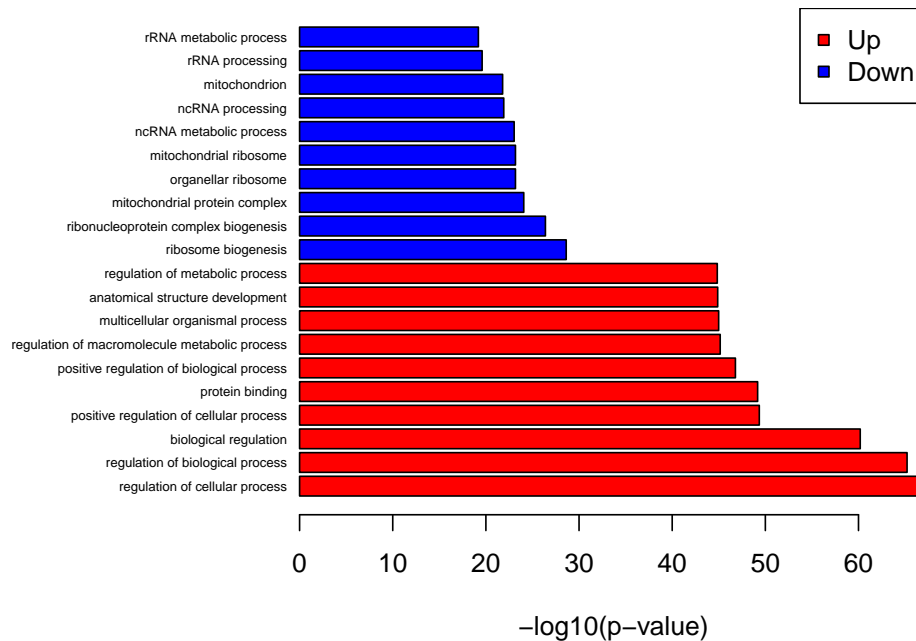


Figure 6.4: Enrichment plot. Top enrichments in either direction

```
library(GO.db)
library(org.Mm.eg.db)
go.EntrezID <- as.list(org.Mm.egGO2ALLEGS)
go.rst <- tibble::rownames_to_column(go.rst, var = "GO.ID")

#Add gene details
go.rst$Genes.Up <- unlist(lapply(go.rst$GO.ID, function(x) {
  xx <- go.EntrezID[[x]]
  if (is.null(xx) |
    with(go.rst[go.rst$GO.ID == x,], Up == 0 |
      P.Up > enrich.pvalue))
    return("-")
  x <- intersect(with(de.genes, EntrezID[logFC>0]), xx)
  x <-
    paste0(with(fit.contr$genes, Symbol[EntrezID %in% xx]), collapse = "|")
  return(x)
}))
go.rst$Genes.Down <- unlist(lapply(go.rst$GO.ID, function(x) {
  xx <- go.EntrezID[[x]]
  if (is.null(xx) |
    with(go.rst[go.rst$GO.ID == x,], Down == 0 |
```

```

        P.Down > enrich.pvalue))
    return("-")
  x <- intersect(with(de.genes,EntrezID[logFC<0]),xx)
  x <-
    paste0(with(fit.contr$genes, Symbol[EntrezID %in% xx]), collapse = "|")
  return(x)
})))

```

6.3.2 KEGG pathway enrichment

Pathway enrichment can also be performed against the Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.genome.jp/kegg/>) database. The KEGG database contains curated molecular pathways and disease signatures. To perform this analysis we use the *kegga* function within the **limma** which is similar to the *goana* function we used above.

```

kegg.rst<-kegga(fit.contr,species="Mm")
topKEGG(kegg.rst)

```

##	Pathway	N	Up	Down	P.Up
## path:mmu03008	Ribosome biogenesis in eukaryotes	75	37	4	1.2e-14
## path:mmu01100	Metabolic pathways	1014	200	75	2.2e-11
## path:mmu04810	Regulation of actin cytoskeleton	143	6	45	1.0e+00
## path:mmu05206	MicroRNAs in cancer	121	6	40	1.0e+00
## path:mmu04919	Thyroid hormone signaling pathway	93	3	32	1.0e+00
## path:mmu03010	Ribosome	137	39	1	6.1e-07
## path:mmu03020	RNA polymerase	29	14	1	3.2e-06
## path:mmu01240	Biosynthesis of cofactors	112	32	2	5.6e-06
## path:mmu04330	Notch signaling pathway	43	2	18	9.8e-01
## path:mmu00310	Lysine degradation	52	1	20	1.0e+00
## path:mmu04611	Platelet activation	76	0	25	1.0e+00
## path:mmu04062	Chemokine signaling pathway	119	5	34	1.0e+00
## path:mmu00190	Oxidative phosphorylation	117	31	0	4.0e-05
## path:mmu05200	Pathways in cancer	323	26	71	1.0e+00
## path:mmu05205	Proteoglycans in cancer	126	8	34	9.9e-01
## path:mmu04658	Th1 and Th2 cell differentiation	69	6	22	8.9e-01
## path:mmu05202	Transcriptional misregulation in cancer	120	16	32	4.6e-01
## path:mmu05224	Breast cancer	81	8	24	8.2e-01
## path:mmu03050	Proteasome	44	15	0	2.1e-04
## path:mmu05012	Parkinson disease	206	44	12	3.1e-04
##	P.Down				
## path:mmu03008	1.0e+00				
## path:mmu01100	1.0e+00				
## path:mmu04810	4.7e-08				

```
## path:mmu05206 5.7e-08
## path:mmu04919 4.3e-07
## path:mmu03010 1.0e+00
## path:mmu03020 9.9e-01
## path:mmu01240 1.0e+00
## path:mmu04330 6.4e-06
## path:mmu00310 9.3e-06
## path:mmu04611 1.9e-05
## path:mmu04062 2.1e-05
## path:mmu00190 1.0e+00
## path:mmu05200 4.1e-05
## path:mmu05205 7.5e-05
## path:mmu04658 1.0e-04
## path:mmu05202 1.5e-04
## path:mmu05224 1.8e-04
## path:mmu03050 1.0e+00
## path:mmu05012 1.0e+00
```

6.3.3 Gene Set Enrichment Analysis

GO and KEGG enrichment analysis uses the list of differentially expressed genes to test for over-representation of these genes. As such the results typically depend on the cut-offs used to get the differentially expressed genes.

Gene Set Enrichment Analysis is instead performed using *fry*, a gene set enrichment function within the **limma** package. Unlike above where we focused on a subset of genes(differentially genes), here all the genes in the set are used to test for gene expression signatures or pathways. A test is performed against the GO terms for demonstrative purposes, alternatively if you wish to test against a specific gene set, this can easily be done by importing that list.

We then use *fry* to test for enrichment between CIS and WT. We use the design matrix 'design',the expression values 'y' and the contrast matrix 'contr' from the imported results to perform the gene set test and the GO term and gene map that we created above.

```
go.all<-go.EntrezID[go.rst$GO.ID]
names(go.all)<-go.rst$Term
fry.GSEA<-fry(y,index = go.all,design = design,contrast = contr)
head(fry.GSEA)
```

##	NGenes	Direction	PValue
## meiotic nuclear division	108	Down	2.3e-07
## chromosomal region	274	Down	3.2e-06
## chromosome organization involved in meiotic cell cycle	42	Down	1.3e-04

## meiotic chromosome segregation	60	Down 1.6e-04
## chromosome, centromeric region	187	Down 3.3e-04
## 90S preribosome	28	Up 5.1e-04
##	FDR	PValue.Mixed
## meiotic nuclear division	0.00018	0.0131
## chromosomal region	0.00125	0.0162
## chromosome organization involved in meiotic cell cycle	0.03044	0.0094
## meiotic chromosome segregation	0.03044	0.0079
## chromosome, centromeric region	0.05207	0.0145
## 90S preribosome	0.05876	0.0039
##	FDR.Mixed	
## meiotic nuclear division	0.034	
## chromosomal region	0.034	
## chromosome organization involved in meiotic cell cycle	0.034	
## meiotic chromosome segregation	0.034	
## chromosome, centromeric region	0.034	
## 90S preribosome	0.034	

```
term.test<-rownames(fry.GSEA)[1]
```

Next, we can visualise the gene set enrichment results using a barcode plot. The barcode plot is used to plot the positions of genes within a set in ranked list of statistics. The statistics are ranked from left to right ie. from smallest to largest.

To do so, the gene identifies for the term should first be mapped to the index position of each gene in the expression matrix;

```
index<-rownames(fit.contr) %in% go.all[[rownames(fry.GSEA)[1]]]
barcodeplot(fit.contr$coefficients[, "CISvsWT"], index = index, labels = c("CS", "WT"),
            main=rownames(fry.GSEA)[1])
```

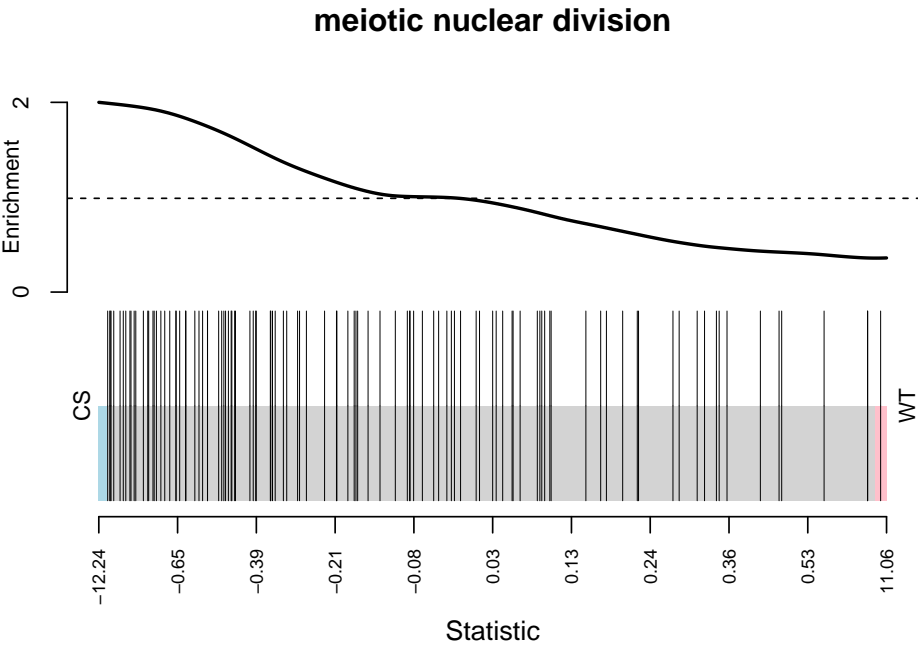



Figure 6.5: Barcode plot showing the enrichment of meiotic nuclear division in CS vs WT

Bibliography

- Delconte, R. B., Kolesnik, T. B., Dagley, L. F., Rautela, J., Shi, W., Putz, E. M., Stannard, K., Zhang, J.-G., Teh, C., Firth, M., et al. (2016). Cis is a potent checkpoint in nk cell-mediated tumor immunity. *Nature Immunology*, 17(7):816–824.
- Law, C. W., Chen, Y., Shi, W., and Smyth, G. K. (2014). voom: Precision weights unlock linear model analysis tools for rna-seq read counts. *Genome biology*, 15(2):R29.
- Liao, Y., Smyth, G. K., and Shi, W. (2013a). featurecounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*, 30(7):923–930.
- Liao, Y., Smyth, G. K., and Shi, W. (2013b). The subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic acids research*, 41(10):e108–e108.
- Liao, Y., Smyth, G. K., and Shi, W. (2019). The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. *Nucleic Acids Research*, 47:e47.
- O’Leary, N. A., Wright, M. W., Brister, J. R., Ciufu, S., Haddad, D., McVeigh, R., Rajput, B., Robbertse, B., Smith-White, B., Ako-Adjei, D., et al. (2015). Reference sequence (refseq) database at ncbi: current status, taxonomic expansion, and functional annotation. *Nucleic acids research*, 44(D1):D733–D745.
- R Core Team (2020). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
- Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., and Smyth, G. K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research*, 43(7):e47.
- Shi, W., Liao, Y., and with contributions from Jenny Dai, G. K. S. (2020). *Rsubread: Mapping, quantification and variant analysis of sequencing data*. R package version 2.4.2.

- Smyth, G., Hu, Y., Ritchie, M., Silver, J., Wettenhall, J., McCarthy, D., Wu, D., Shi, W., Phipson, B., Lun, A., Thorne, N., Oshlack, A., de Graaf, C., Chen, Y., Langaas, M., Ferkingstad, E., Davy, M., Pepin, F., and Choi, D. (2020). *limma: Linear Models for Microarray Data*. R package version 3.46.0.
- Smyth, G. K. (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Statistical applications in genetics and molecular biology*, 3(1).