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# Contents

<b>1</b>	<b>Background &amp; Literature Overview</b>	<b>1</b>
1.1	Differentiation Pathways in the Haematopoietic System . . . . .	2
1.2	Acute Myeloid Leukaemia . . . . .	2
1.2.1	Classification and Subtypes . . . . .	3
1.2.2	Pathogenesis . . . . .	4
1.2.3	Transcriptomic abnormalities . . . . .	8
1.2.4	Treatment Methods . . . . .	8
1.2.5	The Model Cell Line: HL-60 . . . . .	9
1.3	RNA-seq: <i>in vitro</i> . . . . .	10
1.3.1	RNA extraction . . . . .	11
1.3.2	Library Preparation . . . . .	12
1.3.3	Clonal amplification . . . . .	14
1.3.4	Sequencing and Nucleobase Detection . . . . .	15
1.4	RNA-seq: <i>in silico</i> . . . . .	17
1.4.1	Quality Control . . . . .	17
1.4.2	Preprocessing . . . . .	20
1.4.3	Alignment . . . . .	21
1.4.4	Read Quantification . . . . .	25
1.4.5	Read Normalisation . . . . .	26
1.4.6	Differential Expression Analysis . . . . .	26
1.4.7	Downstream Analysis . . . . .	26
1.5	Evaluation Criteria . . . . .	26
1.6	Related Work . . . . .	26
1.7	Summary . . . . .	28

<b>2</b>	<b>Materials &amp; Methods</b>	<b>29</b>
2.1	Preliminary Study . . . . .	29
2.1.1	Phenolic extraction . . . . .	29
2.1.2	Cell Culturing and RNA extraction . . . . .	30
2.1.3	Determination of RNA Quality and Sequencing . . . . .	30
2.2	RNA-seq Pipeline . . . . .	31
2.2.1	Quality Control . . . . .	33
2.2.2	Preprocessing . . . . .	34
2.2.3	Read alignment and Quantification . . . . .	35
2.2.4	Reassessing the Quality . . . . .	36
2.2.5	Normalisation and Differential Gene Expression . . . . .	36
2.2.6	Functional analysis . . . . .	39
2.3	Summary . . . . .	39
	<b>References</b>	<b>41</b>

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## List of Figures

1.1	Stem cell differentiation . . . . .	2
1.2	Library preparation . . . . .	13
1.3	Illumina clonal amplification . . . . .	14
1.4	Sequencing by synthesis . . . . .	16
1.5	FastQScreen plot example . . . . .	19
1.6	Alignment against a reference genome or a reference transcriptome . . . . .	23
2.1	General overview of the RNA-seq pipeline. . . . .	32



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## List of Abbreviations

<b>AML</b> Acute Myeloid Leukaemia . . . . .	2
<b>ATRA</b> all- <i>trans</i> retinoic acid . . . . .	9
<b>TMM</b> Trimmed Mean of <i>M</i> -values . . . . .	38
<b>RIN</b> RNA Integrity Number . . . . .	11
<b>PCR</b> Polymerase Chain Reaction . . . . .	14
<b>FAB</b> French-American-British . . . . .	3
<b>WHO</b> World Health Organisation . . . . .	4
<b>EMBL</b> European Molecular Biology Laboratory . . . . .	30
<b>HPLC</b> High-Performance Liquid Chromatography . . . . .	29
<b>HSC</b> Hematopoietic Stem Cells . . . . .	2
<b>LLE</b> Liquid-Liquid Extraction . . . . .	11
<b>STAR</b> Spliced Transcripts Alignment to a Reference . . . . .	24
<b>NGS</b> Next-Generation Sequencing . . . . .	10
<b>SNP</b> Single Nucleotide Polymorphism . . . . .	7
<b>DEG</b> Differentially Expressed Genes . . . . .	39
<b>DGE</b> Differential Gene Expression . . . . .	10
<b>BCV</b> Biological Coefficient of Variance . . . . .	38
<b>FDR</b> False Discovery Rate . . . . .	39



## Background & Literature Overview

The typical multicellular organism stores its genetic code as deoxyribonucleic acid (DNA), found identically in all its somatic cells (unless *de novo* mutations occur). DNA is a biological polymer, consisting of a double-stranded polynucleotide chain. Each nucleotide monomer consists of a phosphate group, deoxyribose (a five-carbon sugar), and one of four nucleobases: adenine (A), cytosine (C), guanine (G), or thymine (T). These two strands are held together with a series of hydrogen bonds between the nucleobases, forming Watson-Crick base pairs.

DNA is just the general starting point in a series of information transfers described by the *Central Dogma of Molecular Biology*, which the cell uses to ultimately produce its molecular products (Cobb, 2017). Through the process of *transcription*, the code from one strand of DNA is transferred onto a primary ribonucleic acid (RNA) transcript. RNA is similar to DNA except that it is single-stranded, has ribose as its five-carbon sugar and uses the nucleobase *uracil* instead of *thymine*. This primary transcript is modified into ribosomal RNA (rRNA), transfer RNA (tRNA) or messenger RNA (mRNA). All three are involved in *protein synthesis*, although mRNA is especially relevant to this dissertation since the protein sequence can be deduced from the mRNA sequence. These molecular products shape the cell's appearance, define how it interacts with external or internal stimuli, and allows it to perform its intended functions. They give each cell type a characteristic RNA profile which can be measured through RNA-seq (Described in Section ???).

## 1.1 | Differentiation Pathways in the Haematopoietic System

Acute leukaemia occurs earlier in the differentiation pathway, allowing the blasts to divide more rapidly. Figure 1.1 shows the mature cell types that result from myeloid and lymphoid cell lineages, which both share Hematopoietic Stem Cells (HSC) as the common progenitor cell type.

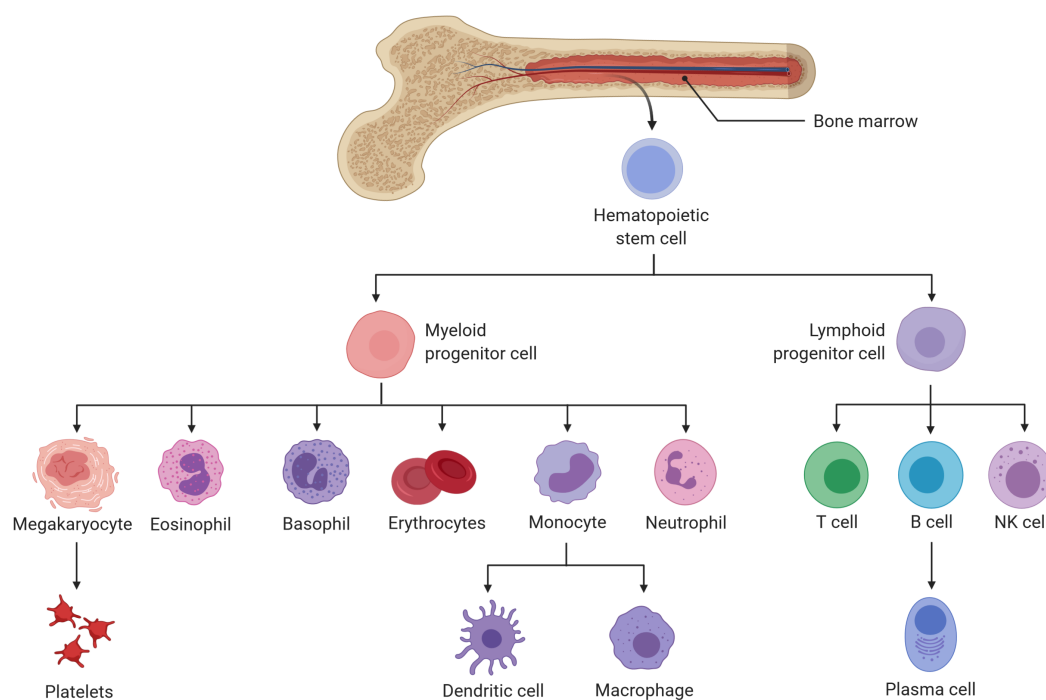


Figure 1.1: An overview of the main branches of haematopoietic stem cell differentiation pathways showing the myeloid and lymphoid lineages. Created using BioRender.com.

## 1.2 | Acute Myeloid Leukaemia

Acute Myeloid Leukaemia (AML) is an aggressive form of cancer which is characterised by its rapid proliferation of myeloblasts. This occurs when undifferentiated myeloid cells acquire mutations which hinder further differentiation but allows for their clonal proliferation (Khwaja et al., 2016). This comes at the expense of the production of their healthy, differentiated counterparts: erythrocytes, platelets and granulocytes (Khwaja



et al., 2016). It is an exception to cancers in that it does not form a tumour, which is usually analysed to determine the severity. Instead AML is staged according to its subtype and other variables (The American Cancer Society, 2018). It is the most common form of acute leukaemia, with an incidence rate of 4.3 per 100,000 in the United States (Kouchkovsky and Abdul-Hay, 2016). One of the main risk factors is age, with a median age of diagnosis of 70 years, and with a slight male predominance (Juliussen et al., 2009; Khwaja et al., 2016). Acute myeloid leukaemia is synonymous with acute myelogenous leukemia, acute myelocytic leukemia, or acute nonlymphocytic leukemia.

### 1.2.1 | Classification and Subtypes

AML is one of four main branches of leukaemia classification, the others being Acute Lymphoblastic Leukaemia (ALL), Chronic Myeloid Leukaemia (CML) and Chronic Lymphoblastic Leukaemia (CLL) (Shimanovsky, 2021). Despite their cytogenic differences, there have been multiple reports of chronic leukaemia types transitioning into the more aggressive, acute form over time (Frenkel et al., 1981; Jacobs et al., 1984; Kaur et al., 2016). Treatment may vary depending on the subtype of the disease, which is why a rigid classification system and correct identification is important (Shimanovsky, 2021).

Each of these four leukaemia subtypes is subdivided into more specific classifications. AML in particular is genetically and morphologically heterogeneous and can involve any single or a combination of myeloid lineages (Kouchkovsky and Abdul-Hay, 2016; Swerdlow et al., 2017).

#### 1.2.1.1 | The FAB classification system

The French-American-British (FAB) classification, first produced in 1976, was an early attempt to distinguish subtypes of AML (Bennett et al., 1976). The divisions were based on cell morphology and the relative quantities of myeloblasts and erythroblasts (acs).

Table 1.1: The FAB classification of AML.

M0	Undifferentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyelocytic leukemia (APL)
M4	Acute myelomonocytic leukemia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
M7	Acute megakaryoblastic leukemia

### 1.2.1.2 | The WHO classification system

A more modern, and now more widely used system, is that devised in the *World Health Organisation (WHO) Classification of Tumors of Hematopoietic and Lymphoid Tissues*, now in its revised 4th edition (Swerdlow et al., 2017). AML is here defined as having >20% of the cells in the bone marrow being myeloblasts. The WHO based their classification on a mixture of genetic, morphological and cytochemical criteria and based on the presence of other conditions. They define seven subcategories:

1. AML with recurrent genetic abnormalities
2. AML with myelodysplasia-related changes (MRC)
3. Therapy-related myeloid neoplasms (t-MN)
4. AML related to previous chemotherapy or radiation
5. Myeloid sarcoma (also known as granulocytic sarcoma or chloroma)
6. Myeloid proliferations related to down syndrome (DS)
7. AML with chromosomal translocations and inversions

These may be further classified according to their specific genetic or karyotypic abnormalities (Swerdlow et al., 2017). Cases which do not fall into any of the above groups, are labelled as 'AML, not otherwise specified (NOS)' and are subject to a form of classification similar to the FAB (The American Cancer Society, 2018). Cases classified as having 'recurrent genetic abnormalities' are often sub-categorised and described according to their abnormality (similar to table ??, although not all are officially recognised as 'recurring abnormalities').

### 1.2.2 | Pathogenesis

The genetic abnormalities leading to AML are heterogeneous and complex, meaning that there are many different combinations of causative genetic or cytogenetic abnormalities which may lead to the AML phenotype (Lindsley et al., 2015; Swerdlow et al., 2017). The genetic and karyotypic profile can have profound prognostic impact, affecting both therapeutic strategy and survival rate (Mrózek et al., 2000; Swerdlow et al., 2017).

#### 1.2.2.1 | Cytogenic Abnormalities

Approximately 55% of AML patients have at least one cytogenic abnormality (Meyer and Levine, 2014). Stölzel et al. (2016) note that patients with 3 unrelated cytogenic

abnormalities, have a worse overall survival rate than AML patients with a normal karyotype, and that the patients at most risk had  $\geq 4$  unrelated cytogenic abnormalities. There are some exceptions, where the presence of certain abnormalities actually *increases* survival rate with good response to treatment (Table ??).

Table 1.2: Recurrent abnormalities in AML and their effects. This table makes use of the International System for Human Cytogenomic Nomenclature (ISCN) to describe chromosomal abnormalities (Jean McGowan-Jordan, 2020). The information given prior to the parentheses denotes the type of chromosomal abnormality (for example *t* for translocation, and *inv* for inversion). The contents of the first pair of parenthesis refer to the affected chromosome(s). The second pair of parentheses, if present, refers to the specific part of the respective chromosome(s) affected (the short arm *p* or the long arm *q*, and which region or band of these arms).

Aberration	Prognosis	Fusion Genes	Note	Reference
t(8;21)(q22;q22)	Favourable	RUNX1, RUNX1T1	Common (~5% of all AML)	Reikvam et al. (2011) Peterson and Zhang (2004)
inv(16)(p13;q22) t(16;16)(p13;q22)	Favourable	CBFB, MYH11	Common	Plantier et al. (1994) Shigesada et al. (2004)
t(15;17)(q24;q21)	Favourable	PML, RARA	Common (~10% of adult AML)	De Braekeleer et al. (2014)
t(9;11)(p22;q23)	Poor	KMT2A, MLLT3	Frequency decreases with age	Chandra et al. (2010) Metzler et al. (2004)
t(6;9)(p23;q34)	Poor	DEK, CAN/NUP214	Rare, associated with an internal tandem duplication (ITD) mutation on FLT3	Chi et al. (2008)
inv(3)(q21.3;q26.2) t(3;3)(q21.3;q26.2)	Poor	RPN1, MECOM	Rare, low response to standard chemotherapy	Sitges et al. (2020)
t(1;22)(p13;q13)	Poor	RBM15, MKL1	Rare, almost exclusively found in infants with acute megakaryocytic leukaemia	Carroll et al. (1991) Bernstein et al. (2000)
Monosomy	Very poor	/	Loss of chromosome, frequency increases with age	Breems et al. (2008)

### 1.2.2.2 | Genetic Abnormalities

If we reduce our frame of reference to the genetic level, we find that the aforementioned structural variants (Table ??) can trigger the activation of an *oncogene*, or their fusion products (Table ??) can become an oncogene themselves. Some genes have the potential to cause cancer under abnormal conditions and are called *proto-oncogenes*, and if said conditions are met, become the carcinogenic oncogenes. This carcinogenicity can be triggered by either a structural variant, a Single Nucleotide Polymorphism (SNP) or gene amplification (Tabin et al., 1982). This can cause up-regulation, over-activity or a change in function of the respective protein (Tabin et al., 1982). These proteins are often the targets of cancer drugs (Liu et al., 2004).

Cells have evolved mechanisms to prevent carcinogenesis, through *tumour suppressor genes*. These genes are typically involved in the regulation of cell division, DNA repair or induction of apoptosis. While proto-oncogenes require their up-regulation to induce cancer, tumour suppressor genes require down-regulation or complete deactivation. Knudson (1971) suggested a 'two-hit hypothesis', that most tumour suppressor genes require the deactivation of both alleles for carcinogenesis to occur. Knudson theorised that early onset retinoblastoma (cancer of the retina) was caused by an inherited mutation (the first 'hit') and a second acquired mutation (the second 'hit'). Knudson explained late-onset of the disease as being non-inherited, with both 'hits' being acquired.

Table 1.3: Recurring genetic abnormalities in AML. Compiled and adapted from Di-Nardo and Cortes (2016) Table 1 and Lindsley et al. (2015) Figure 2.

Role	Role description	Mutated genes
Signalling pathways	Internal or external chemical communication	NRAS, KRAS, PTPN11, NF1, CBL, KIT, FLT3
DNA methylation	Epigenetic modifier, adds methyl groups to DNA	DNMT3A, TET2, IDH1, IDH2
Chromatin modifiers	Epigenetic modifier, remodels chromatin	ASXL1, EZH2, BCOR
Transcription factors	Involved in transcribing DNA into RNA	CEBPA, RUNX1, GATA2
Tumour suppressors	DNA repair, initiation of apoptosis, halting cell growth	TP53
Spliceosome complex	Ribonucleoprotein complex involved in splicing RNA	SRSF2, U2AF1, SF3B1, ZRSR2
Cohesin complex	Protein complex involved in chromatid cohesion	STAG2, SMC3, SMC1A, RAD21
Others	Other proto-oncogenes	WT1, PHF6, TP53, NPM1

### 1.2.3 | Transcriptomic abnormalities

Pathways

### 1.2.4 | Treatment Methods

Surgery, chemotherapy, radiotherapy, immunotherapy and hormone therapy are common treatments used to kill cancer cells. While the specifics are partly dependent on the particular AML subtype and the patient's condition, some variation of chemotherapy is standard practice. Treatment is typically split into four phases spread over a period of 2-3 years (Malard and Mohty, 2020):

1. **Induction** - Uses chemotherapeutic drugs with the intention of achieving complete remission (no symptoms or signs of cancer) and restore normal cellular activity. Cytarabine (AraC) is one of the most commonly used chemotherapeutic drugs for AML, often used in conjunction with others such as daunorubicin (Robak and Wierzbowska, 2009).
2. **Consolidation** - Consists of several short sequential courses of chemotherapy every two weeks, usually using stronger doses.

3. **Intensification** - Also called reinduction therapy, includes drugs similar to those used during the induction phase.
4. **Long-term maintenance** - Chemotherapy is performed for 2-3 years after complete remission to prevent, or slow down, the growth of any cancer remnants. At times, a bone marrow or stem cell transplant is sometimes necessary to replenish the supply of healthy hematopoietic cells

In recent decades, advances in our knowledge of cancer biology and the development of more efficient high-throughput sequencing techniques, have lead to the identification of novel treatments which specifically target cancer cells, such as differentiation therapy. A key characteristic of cancer cells is remaining in a stem-cell like state, which allows for their rapid proliferation. Differentiation therapy is a relatively modern approach which attempts to induce the process of differentiation, where the malignant cells mature and lose their ability to proliferate, rendering them virtually harmless. The first successful differentiation agent was all-*trans* retinoic acid (ATRA), also known as tretinoin, used to treat acute promyelocytic leukaemia (APL) (Chomienne et al., 1990). This revolutionary drug managed to achieve a 90% survival rate in APL patients, without the severe cytotoxic side-effects of traditional non-targeted chemotherapy (Kim et al., 2015). There have been many attempts to emulate this with other compounds, with mixed results (Nowak et al., 2009).

### 1.2.5 | The Model Cell Line: HL-60

A 36-year-old Caucasian woman was being treated for AML at the MD Anderson Cancer Center in Texas, 1977, when she consented to being part of a study on her disease. Researchers took a blood sample, from which they extracted blasts for their analysis. Three years later, ? would describe for the first time the HL-60 cell line, now one of the most widely used AML cell lines. The cells were described as having primarily neutrophilic and promyelocytic morphology, and thus initially placed into the FAB-M3 'acute promyelocytic leukemia' category (see Section 1.2.1). Subsequent analysis of the cells' karyotype, performed by ?, revealed that they lacked the t(15;17) translocation characteristic of FAB-M3, and were categorised as FAB-M2, but development in nomenclature led the cell-line to finally being placed in the 'AML with maturation' category, using the WHO system.

Early karyotypic studies had identified the t(5;17) (Von Hoff et al., 1990) and t(9;14) translocations, together with a complex structural variant between chromosomes 5, 7, and 16 (Liang et al., 1999). A more recent study by ? used genome wide chromatin

conformation capture (Hi-C) and RNA-seq to study structural variants in HL-60 genetic branches. They have shown the heterogeneity in HL-60 cell lines, but identified novel structural variants thought to be found in the original HL-60 sample: t(5;7)(q31.2;q32.3), t(5;16)(q33.3;q23.2-q23.3), t(7;16)(q32.3;q24.1), t(9;14)(q31.1;q23.2), and t(5;17)(q11.2;p11.2).

As mentioned in Section 1.2.4, ATRA has been a success story in AML differentiation therapy, and since its discovery, has been extensively used on HL-60 cells. This has led to the evolution of an ATRA-resistant branch of the HL-60 cell line, which was used during the study (Gatt, 2016) that laid the foundation for this dissertation. Fu et al. (2005) were successful in reverting this resistance through gene knockdown of MCL-1, which seems to produce the protein responsible for ATRA resistance.

## 1.3 | RNA-seq: *in vitro*

RNA sequencing (RNA-seq) is the application of a Next-Generation Sequencing (NGS) technique to measure the quantity of RNA sequences in a biological sample, in a given moment (Wang Zhong, 2009). While this dissertation deals with the data analysis part of RNA-seq, some background on the origins of said data is essential. RNA-seq has gradually been replacing microarrays as the standard technology in molecular biology to analyse Differential Gene Expression (DGE). Its main advantage is that it allows for the sequencing of the entire transcriptome, while microarrays only allow for predefined regions to be sequenced (Rao et al., 2019).

Sanger sequencing is considered as the first generation in a series of changes in sequencing technology, developed in 1977 and dominated the nucleic acid sequencing industry for over 30 years (Behjati and Tarpey, 2013). Next-generation sequencing (or second generation sequencing) revolutionised the industry, its massively parallel capabilities allowing for greatly increased throughput, sequencing millions of fragments at a time instead of Sanger sequencing's just one. At the time of writing, we are currently in the process of transitioning into the third generation of nucleic acid sequencing, which allows for longer reads (>1000 bp as opposed to 35-600 bp). Longer reads translate to greater overlap between the reads, and thus greater certainty during assembly or alignment, particularly when considering regions of low-complexity or structural variants (Rhoads and Au, 2015).

We should make a distinction between two popular types of RNA-seq: the classic bulk RNA-seq, and single-cell RNA-seq (scRNA-seq). Bulk RNA-seq, which this project has made use of, takes the average gene expression of a sample, which may be composed of many cell types, while scRNA-seq investigates the transcriptome of each



individual cell. RNA-seq is traditionally used to profile transcriptomes, but it may be used in the identification of expressed SNPs, identification of novel transcripts, the detection of fused genes and alternative splicing (Han et al., 2015; Zhao et al., 2014).

### 1.3.1 | RNA extraction

The first step in any RNA-seq workflow is the extraction of RNA from the biological sample. This is complicated by the chemical instability of RNA due to its hydroxyl groups at the 2' and 3' positions, facilitating RNase activity (RNA-degrading enzymes) (Green and Sambrook, 2019). This issue is compounded by the ubiquity and chemical resilience of RNases, meaning that special care must be taken to avoid contamination of glassware and instruments that interact with the RNA (Green and Sambrook, 2019). One method uses liquid nitrogen to deactivate any RNase enzymes and freeze the samples, which are pulverised to extrude the cell contents (Wang and Vodkin, 1994).

The data serving as the basis for this dissertation was provided by Gatt (2016), who followed the RNeasy® Mini kit (QIAGEN, 2014) which makes use of an extraction technique called *acid guanidinium thiocyanate-phenol-chloroform* (AGPC) extraction (Chomczynski and Sacchi, 1987). This is based on Liquid-Liquid Extraction (LLE) (Mazzola et al., 2008), where under acidic conditions the cell's RNA partitions into the aqueous phase while the DNA, proteins and lipids partition into the organic phase, aided by centrifugation. The organic phase is composed of phenol (which dissolves the protein) and chloroform (which dissolves the lipids). Guanidinium thiocyanate is part of the kit's buffer solution, and acts as a chaotropic agent, meaning it disrupts water's hydrogen bonds. This is added to the organic phase to disrupt the hydrophobic properties of protein (including RNases), aiding in their denaturation. Ethanol is added to precipitate the RNA and any residual DNA. A spin-column is used to bind nucleic acids to a silica membrane, and wash away any proteins, carbohydrates, fatty acids and any traces of salts, aided by centrifugation (Matson, 2009). The end-result is a purified aqueous nucleic acid solution.

RNA concentration is commonly checked through quantitation using a spectrophotometer, which measures the ability of the sample to absorb UV light at wavelengths of 260nm and 280nm. A score is assigned to the sample's ability to absorb each of the two wavelengths, and the purity of the sample is often quantified using the ratio between the two scores (A260/280 ratio). A pure RNA sample should yield an A260/280 ratio of 2.0 (Scientific, 2013).

An additional quality metric commonly checked before sequencing, is the integrity of the RNA. This can be quantified via the RNA Integrity Number (RIN) algorithm,

applied to the results of capillary electrophoresis, which separates the RNA fragments based on their length (Schroeder et al., 2006). In most labs, the electrophoresis and computation of the RIN is performed automatically in an electropherogram (Chamieh et al., 2015). A poor RIN may indicate RNase contamination during extraction, that could have degraded the RNA.

### 1.3.2 | Library Preparation

'RNA' is a generic term, which includes both coding and non-coding RNA. Ribosomal RNA (rRNA) is a form of non-coding RNA which comprises 80% to 95% of the total RNA (Kukurba and Montgomery, 2015; O'Neil et al., 2013) and must be removed before sequencing.

There are two main competing methods available, each with their unique advantages and restraints: poly-A enrichment and rRNA depletion. The 3' end of messenger RNA (mRNA) undergoes polyadenylation prior to transcription, meaning that a long chain of adenine nucleotides called the *poly-A tail* is added. With poly-A enrichment, RNA fragments with a poly-A tail are enriched with oligo (dT) primers, thus selecting for the mRNA (Zhao et al., 2014). The alternative approach is an active removal of the rRNA using commercially available kits, such as the Illumina *Ribo-Zero Plus rRNA Depletion Kit*. These kits use oligonucleotides complementary to the rRNA sequences to reduce their abundance (Griffith et al., 2015; Peano et al., 2013).

The next two steps are fragmentation and conversion to complimentary DNA (cDNA), the order of which may vary. In the Illumina workflow used to generate the data for this dissertation, the RNA strands were first fragmented, and reverse transcribed to their cDNA counterparts (Pease and Sooknanan, 2012). A short, artificially synthesised oligonucleotide called an *adapter* sequence is ligated to each of the cDNA fragments, using the ligase enzyme, together with sequence motifs such as barcode sequences (Pease and Sooknanan, 2012) (Figure 1.3).

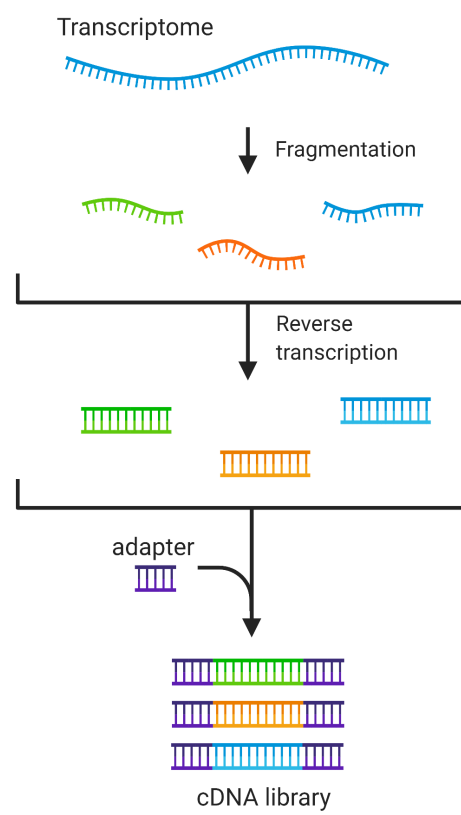


Figure 1.2: Illumina library preparation. Created using BioRender.com.

### 1.3.3 | Clonal amplification

The following step amplifies the fragments of the cDNA library to a level detectable by the sequencing machine, using a form of Polymerase Chain Reaction (PCR). The Illumina Sequencing by Synthesis technology makes use of the flow-cell-based method of *bridge amplification* (Illumina, 2010), as opposed to emulsion PCR, a similar technology used in Ion Torrent Semiconductor Sequencing which makes use of bead surfaces (Williams et al., 2006).

In bridge amplification (Illumina, 2010), the previously prepared adapter-ligated cDNA library is attached to a flow cell, which is a hollow glass slide with multiple channels, coated with a lawn of oligonucleotides (called oligos in short) complementary to the sequences which form part of the adapters. Strands of cDNA bind to these oligos, and polymerase creates the complement of the hybridised strand. Each double-stranded cDNA molecule is then denatured and enters a number of bridge-amplification cycles. Each molecule is amplified, forming clusters of identical cDNA sequences adjacent to each other (Figure 1.3).

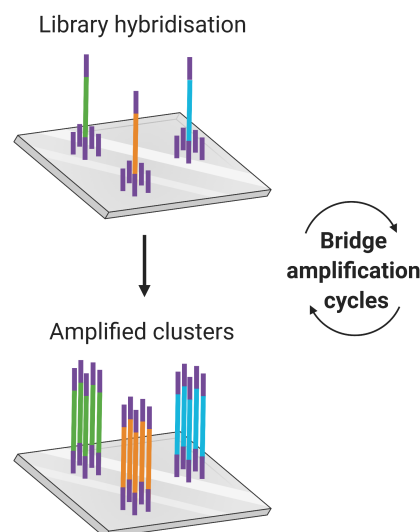


Figure 1.3: Illumina clonal amplification. Created using BioRender.com.

### 1.3.4 | Sequencing and Nucleobase Detection

Sequencing by Synthesis (Illumina, 2010) makes use of fluorescently-labelled deoxynucleoside triphosphate (dNTP). Each sequencing cycle binds a dNTP molecule to the millions of clusters in parallel, with each of the four nucleotides emitting a different coloured light upon binding and laser excitation. The sequencing machine captures the light being emitted from the flow cell as an image and identifies the first base of each fragment. The cycle repeats itself for the second base, third base, and so on, until the end of the sequence (Figure 1.4). The raw sequencing data is stored as Binary Base Call (BCL) files.

Multiple samples may be sequenced simultaneously during a single run, where they are multiplexed by the machine, meaning they are pooled into a single data stream. Unique identifiers called barcode sequences (added to the cDNA fragments during library preparation) allow for the recognition of the different samples, and demultiplexing of the BCL files into text-based FASTQ files (Cock et al., 2010). These are immediately compressed to reduce costs associated with data storage and data transfer. While the *de facto* data compression format used is gzip (Deutsch et al., 1996), and bzip is used on occasion (Seward, 1996), the underlying compression algorithms used are unspecialised and inefficient for genomic data.

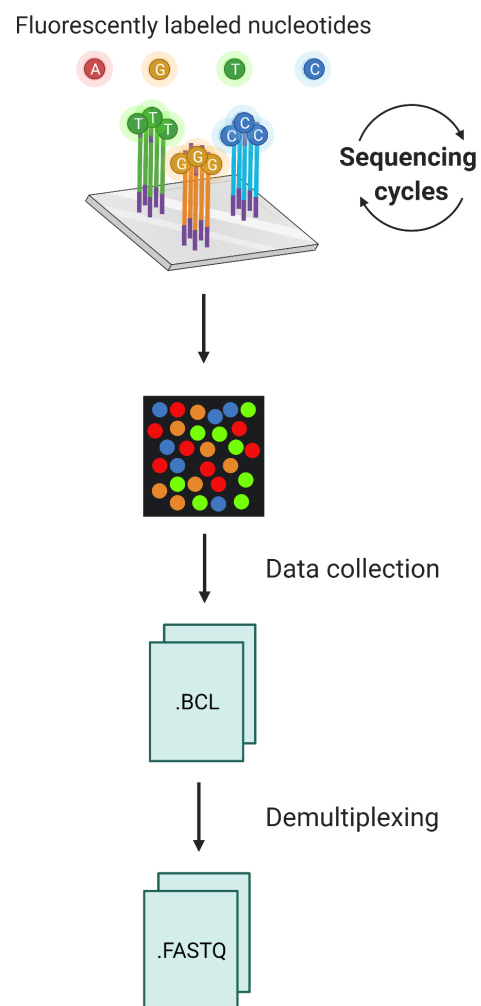


Figure 1.4: Illumina sequencing by synthesis. Created using BioRender.com.

## 1.4 | RNA-seq: *in silico*

Once the FASTQ files emerge from the sequencing machines, we may move into the dry lab and feed the data into an RNA-seq data analysis pipeline. While the specific tools which make up the pipeline will vary according to the type of data and goals of the researcher, all RNA-seq pipelines share a common skeleton. The following subsections will first provide a general overview of the respective step in the pipeline, and then delve into the specific tools used in this project.

### 1.4.1 | Quality Control

The first part of any pipeline should be to analyse the quality of the data received from the sequencing machine. If poor quality sequencing information is identified, it is truncated to mitigate inaccuracies in the downstream pipeline. Some imperfections and uncertainties in sequencing are unavoidable, thus the reading of each base call by the sequencer is assigned a Phred quality score. These are numerical scores generally ranging from 10 to 60, logarithmically related to the probability of an erroneous base-call, represented as a single ASCII character (Ewing et al., 1998). They are calculated as follows:

$$Q = -10\log_{10}P$$

$$P = 10^{-\frac{Q}{10}}$$

where:

$Q$  = Phred-scale quality score

$P$  = Probability of an erroneous base call

A common convention is to write the value of the Phred score after the letter  $Q$ , so we may say that a base call with quality of  $Q30$  has a 0.1% chance of being erroneous. The FASTQ files used for this project are Sanger/Illumina 1.9 encoded, meaning that the assigned character to the score is equal to its value as an ASCII code + 33. So  $Q30$  would correspond to the ASCII character with an ASCII code<sup>1</sup> of 53, which is the question mark character ? (Ewing et al., 1998). The lack of a base call is represented as an  $N$  in place of the nucleotide.

<sup>1</sup>The complete Q-score encoding table: [https://support.illumina.com/help/BaseSpace\\_OLH\\_009008/Content/Source/Informatics/BS/QualityScoreEncoding\\_swBS.htm](https://support.illumina.com/help/BaseSpace_OLH_009008/Content/Source/Informatics/BS/QualityScoreEncoding_swBS.htm)

### 1.4.1.1 | FastQC

In the rapidly changing field of nucleotide sequencing, FastQC (Andrews et al., 2010) has been one of the few constants. It has become a staple quality control tool for high throughput sequencing data, accepting BAM (Li, 2009), SAM (?) or FASTQ files as input, from which it produces an HTML-based report using a number of modules measuring various quality metrics. The software rates each of these modules using a green check-mark signifying that it 'passed' QC, a yellow exclamation mark 'warning', or a red cross 'failed'. However these flags are set to DNA sequencing standards, and have limited applicability with other types of sequencing, such as RNA-seq, where a number are expected to fail. These modules are thoroughly described in its documentation<sup>2</sup> and summarised below. Care should be taken as the X-axis is non-uniform for a number of the produced graphs.

#### Modules used in FastQC:

- **Basic Statistics** - Some basic information on the file: its name, type of quality score, total read count, read length and GC content.
- **Per Base Sequence Quality** - The aggregated Q-scores at each position of the reads, represented by a box-plot.
- **Per Sequence Quality Scores** - The number of reads on the y-axis and the average Q-score on the x-axis.
- **Per Base Sequence Content** - A relative abundance line graph showing the percentage abundance of each of the four nucleotides across all the reads.
- **Per sequence GC content** - The percentage abundance of each of the four nucleotides across all the reads, overlaid on the expected distribution.
- **Per base N content** - Percentage of bases at each position of the sequence with no base call, represented as an N.
- **Sequence Length Distribution** - Shows the distribution of sequence lengths, measured in number of base-pairs (bp). The module will raise a warning if all sequences are not the same length and an error if any of the sequences have zero length.
- **Sequence Duplication Levels** - Percentage of reads in the library which come from sequences with duplication. Two lines indicate the percentages of the raw and the deduplicated libraries.

---

<sup>2</sup><https://www.bioinformatics.babraham.ac.uk/projects/fastqc/Help/3%20Analysis%20Modules/>



- **Overrepresented Sequences** - A list of sequences which account for  $\geq 0.1\%$  of the total reads. These are compared to common contaminants to try identify them.
- **Adapter Content** - A cumulative line graph where a sequence library adapter sequence is identified at that base position.

### 1.4.1.2 | FastQScreen

While FastQC is certainly a useful and well-maintained tool, it is not exhaustive of the possible QC metrics for FASTQ files. For this reason, other tools such as FastQScreen<sup>3</sup> (Wingett and Andrews, 2018) may be used to supplement the results.

FastQScreen maps the sample reads against the genomes of common contaminants and against that of a human for comparison using a third party alignment tool such as Bowtie (Langmead et al., 2009), Bowtie2 (Langmead and Salzberg, 2012) or BWA (Li and Durbin, 2009). A bar chart (Figure 1.5) and its respective data table are produced which show the percentage reads mapped for each genome, and what percentage did not map at all. With human samples, one should expect some multi-mapping to the mouse and rat genomes, given their genetic similarities.

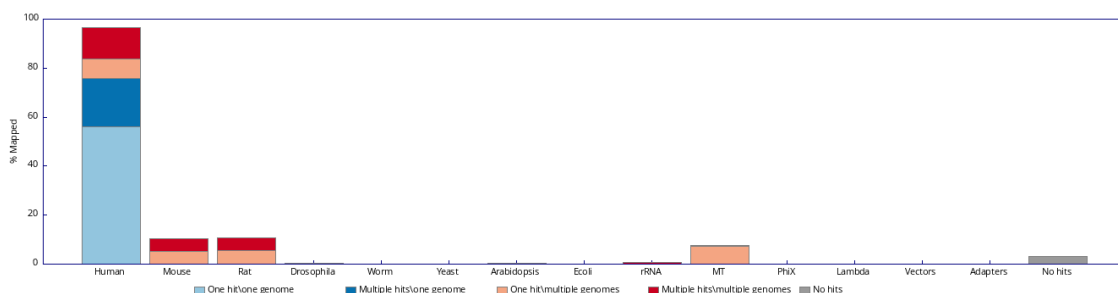


Figure 1.5: An example of a good FastQScreen output result, with human mapping close to 100% and some multi-mapping to mouse and rat genomes.

<sup>3</sup>[https://www.bioinformatics.babraham.ac.uk/projects/fastq\\_screen/\\_build/html/index.html](https://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/_build/html/index.html)

### 1.4.1.3 | MultiQC

MultiQC<sup>4</sup> (Ewels et al., 2016) provides a convenient way of collating multiple QC reports across multiple samples into a single interactive HTML report. It supports the input of 114 tools as of version 1.11, including the reports from tools found further downstream, in the preprocessing, alignment or quantification parts of the pipeline.

## 1.4.2 | Preprocessing

If poor quality data is identified, it should be cleaned to avoid negative effects in the downstream analysis. Quality trimming which is too aggressive may similarly negatively impact downstream analysis, thus care must be taken to select appropriate quality thresholds (Davis, 2019).

### 1.4.2.1 | Cutadapt: Short reads and Adapter sequences

One of the primary functions of Cutadapt<sup>5</sup> (Martin, 2011) (as indicated by its name) is to trim adapter sequences, which may be given as a string following the `-a` parameter. Additionally, Cutadapt may be given a read length threshold (`-length`) to remove short reads which are susceptible to multimapping and ambiguity during alignment (Deschamps-Francoeur et al., 2020).

Trim Galore!<sup>6</sup> (Krueger, 2019) is a wrapper script that may be used to instantly redirect the trimmed reads from Cutadapt back to FastQC to reassess the data quality. It accepts the same arguments as Cutadapt, with an additional `-fastqc_args` which accepts additional arguments to be passed on to FastQC as a string. This combines both Cutadapt and FastQC parameters into a single command.

#### **Cutadapt's default options:**

- Outputs the trimmed FASTQ file and simultaneously generates its FastQC report.
- Assumes Sanger/Illumina 1.9 quality encoding (ASCII code +33 = Phred score)
- Trims adapter and up- or downstream sequence
- Allows a maximum error rate of 10 % (Error rate = number of errors divided by length of matching region)
- Removes up to one adapter per read

---

<sup>4</sup><https://multiqc.info/docs/>

<sup>5</sup><https://cutadapt.readthedocs.io/en/v4.0/guide.html>

<sup>6</sup>[https://www.bioinformatics.babraham.ac.uk/projects/trim\\_galore/](https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/)

- Requires a three nucleotide overlap between read and adapter for an adapter to be found

### 1.4.2.2 | Prinseq++: Low complexity and No Basecalls

Ambiguity in reads may manifest itself in the form of low complexity regions, and reads with a high number of *N*'s, in addition to those discussed in Section 1.4.2.1. The data should be filtered to some degree based on these metrics, which is facilitated by ready-made tools such as Prinseq++<sup>7</sup> (Cantu et al., 2019). Prinseq++ is a C++ multi-threaded implementation of the perl-coded Prinseq-lite software (Schmieder and Edwards, 2011).

Regions of low-complexity (also called compositionally biased regions) are a natural part of biological sequences, playing an important role in protein translation (Frugier et al., 2010), and have a functional role in some proteins (Ntountoumi et al., 2019). Nevertheless, due to their repetitive nature, they tend to result in multimapping and low alignment confidence scores, especially when exacerbated with short read lengths. To quantify low-complexity regions, Prinseq++ presents the DUST (Tatusov and Lipman, unpublished) and Entropy approaches. Both are different algorithms which employ a scoring function based on nucleotide frequencies which ultimately generate a score between 0 and 1 as a measure for sequence complexity (?). The DUST module is incorporated in BLAST (Altschul et al., 1997) for the same purpose, to mask low-complexity regions. Prinseq++ filters reads which exceed the stipulated DUST score (`-lc_dust`) or Entropy (`-lc_entropy`) thresholds.

The ambiguous base *N* represents no basecall, and a threshold for the maximum number of *N*'s in a sequence may be set using `-ns_max_n`.

#### Prinseq++'s default options:

- Outputs the filtered FASTQ, and the filtered reads as separate files.
- Removes sequences with a DUST score < 0.5
- Removes sequences with an Entropy score < 0.5
- Trims recursively from both ends of the sequence chunks of length 2 if the mean quality of the first 5 bases is < 20

### 1.4.3 | Alignment

Quick and computationally efficient pairwise comparison and alignment of two sequences consisting of billions of reads is a classic problem in bioinformatics. We have

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<sup>7</sup><https://github.com/Adrian-Cantu/PRINSEQ-plus-plus>

amassed large volumes of literature describing potential strategies to tackle the problem, occupying different niches.

Aligners may take one of two approaches: global alignment or local alignment. Global alignment algorithms, such as Needleman and Wunsch (1970), aligns both sequences from their first amino acid residue through to their last and is more suitable for sequences of approximately equal lengths. By contrast, local alignment algorithms, such as Smith et al. (1981) and BLAST, are more suited for sequences that are suspected to overlap only partially.

There are some semantics associated with this particular step which should be clarified before proceeding further. *Alignment* and *mapping* are often used interchangeably, but there are subtle differences. According to the BioStar Handbook (Albert, 2020) and a presentation by Heng Li, *alignment* is the optimal placement of a read against a genome, while *mapping* suggests less certainty, and that the optimal placement is not always possible. Which term to use is dependent on the data and goals of the study, although modern tools often combine the two approaches, which continues to blur the line separating the terms.

In RNA-seq, the reference sequence one aligns against may be either a genome or a transcriptome. Since reads from our FASTQ file originate from processed mRNA, the reads may span across multiple exons. This cannot be simply mapped onto a reference genome because of the presence of intronic and non-coding regions (Nekrutenko). To map transcript-derived reads which against a genome, a splice-aware aligner must be used (Figure 1.6).

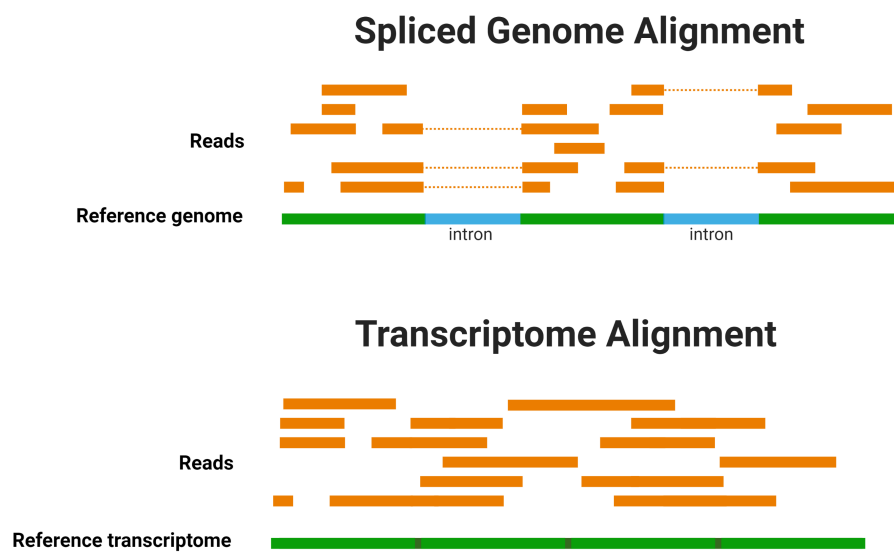


Figure 1.6: Alignment against a reference genome or a reference transcriptome. Created using BioRender.com.

Alignment algorithm efficiency is at least semi-dependent on read length, with each having ideal range of lengths, although this is rarely stated in the documentation (Albert, 2020). A distinction is often made between short-read and long-read mappers, although this distinction is arbitrary. Many conventional short-read mappers are not suitable for reads under 30bp, necessary for the study of microRNAs (Albert, 2020; Ziemann et al., 2016).

Quasi-mappers and pseudo-aligners (most notably Salmon (Patro et al., 2017) and Kallisto (Bray et al., 2016) respectively) differ from classical alignment. They utilise *k*-mer matching to match reads and corresponding transcripts (Nekrutenko). They require less runtime than other existing alignment tools (Zhang et al., 2017), while their accuracy is disputed, with Srivastava et al. (2020) finding that they are less accurate and Schaarschmidt et al. (2020); Zhang et al. (2017) argue that their accuracy is comparable to conventional aligners.

Some implementation of the mapping quality (MAPQ) value is used by all conventional aligners and it is a standard field in the SAM/BAM file formats. It is analogous to the Phred score in a FASTQ file, and allows for easy filtering of bad quality reads. Unlike the Phred score, there is no single standardised definition or formula, with slight variations existing across various sources (?).

### 1.4.3.1 | STAR

Spliced Transcripts Alignment to a Reference (STAR)<sup>8</sup> (Dobin et al., 2013) is an open-source software package that performs local, splice-aware alignment in two major steps: (1) seed search and (2) clustering, stitching and scoring. It was coded in C++ for the specific purpose of mapping RNAseq reads to a genome.

The algorithm first searches for the Maximum Mappable Prefix (MMP) which acts as a seed from which to extend its alignment. This must be an exact identical match with the reference genome. These MMPs are clustered according to proximity to identify a set of *anchor* seeds. STAR stitches together the seeds identified in the first step, and if alignment within one window does not cover the entire read, it will try to find multiple windows to cover the read, resulting in a chimeric alignment. This means that different parts of the same read may map to distant genomic loci, possibly to different strands or chromosomes, which is especially useful when dealing with cancer-derived transcriptomes given the frequency of structural variants. A local alignment scoring

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<sup>8</sup>[https://physiology.med.cornell.edu/faculty/skrabaneck/lab/angsd/lecture\\_notes/STARmanual.pdf](https://physiology.med.cornell.edu/faculty/skrabaneck/lab/angsd/lecture_notes/STARmanual.pdf)

system guides the stitching, with matches, mismatches, indels and splice junction gaps translating to different scores.

An index must be generated prior to alignment, which is generated from a reference genome and its respective annotation file in the GTF format. This hastens the algorithm in a way similar to how one might use the index in a book, which points to the specific locations of certain headers (Trapnell and Salzberg, 2009).

STAR provides the user with great flexibility, with many parameters, such as the scoring system weighting and the size of search windows, being user-defined. Dobin and Gingeras (2015) provide excellent descriptions of nine different datatype- and output-dependent strategies that one may take when mapping RNAseq reads with STAR.

#### STAR's default options:

- Generates a genome index using a reference file and its respective annotation (GTF) file.
- Aligns an experimental transcriptome using the genome index and outputs an alignment file (SAM, unsorted BAM or BAM sorted by coordinates) and various log files.
- Uses a mapping quality metric MAPQ, calculated as  $10 * \log_{10}(1 - \frac{1}{N_{map}})$ , where  $N_{map}$  is the number of places the read maps to. A value of 255 is given to uniquely mapped reads.
- Passes on NH HI AS nM as SAM attributes as defined in the SAM format specifications<sup>9</sup>.

### 1.4.4 | Read Quantification

Table 1.4: An example of a read count table. Each sample represents the pooled RNA of a large number of cells, often of different cell types.

Genes	Sample <sub>1</sub>	Sample <sub>2</sub>	Sample <sub>3</sub>	...
A2BG	10	30	0	...
AML	30	3	3	...
AMT2	0	0	10	...
ARST5	5300	1900	3250	...
...	...	...	...	...

<sup>9</sup><https://samtools.github.io/hts-specs/SAMv1.pdf>

#### 1.4.4.1 | RSEM

li2011rsem RSEM has built-in support for Bowtie2/STAR/HISAT2 alignment programs, which enables alignment and gene-level abundance estimation to be performed in a single step.

#### 1.4.5 | Read Normalisation

##### 1.4.5.1 | TMM

(Robinson et al., 2010)

#### 1.4.6 | Differential Expression Analysis

feng2012gfold, gim2016lpeseq, (Robinson et al., 2010)

##### 1.4.6.1 | EdgeR

statistical models used by edger uwekk

#### 1.4.7 | Downstream Analysis

luo2013pathview

### 1.5 | Evaluation Criteria

### 1.6 | Related Work

**In this section you need to explain similar work in literature.** Make sure to give a systematic overview of studies with related/similar work and highlight similarities/differences to your work (perhaps in the form of a table)



Table 1.5: Studies which compare RNA-seq tools or work-flows, including the tools used at each step and their conclusion summarised to one or two sentences. Those which propose a novel tool were not considered due to their inherit bias.

Reference	Preprocessing	Mapping	Quantification	Normalisation	Differential expression	Summarised conclusion
Williams et al. (2017)	/	Bowtie2, HISAT2, Kallisto, Salmon, Sailfish, SeqMap, STAR, TopHat2	Sailfish, Kallisto, Salmon	/	Ballgown, baySeq, BitSeq, cuffdiff, DESeq2, EBseq, NOISeqBIO, SAMseq, Sleuth, edgeR, limma, NBPseq	Different workflows exhibit a precision/recall tradeoff, the method of differential gene expression exhibited the strongest impact on performance
Zhang et al. (2017)	/	Cufflinks, RSEM, TIGAR2, eXpress, Sailfish, Kallisto, Salmon	Sailfish, Kallisto, Salmon	/	/	Pseudo-aligners require less runtime and achieve similar accuracy. Salmon and RSEM (BAM input) performed the best considering computational resources and accuracy
Schaarschmidt et al. (2020)	/	BWA, CLC, HISAT2, RSEM, Kalliso, Salmon, STAR	RSEM, Kallisto, Salmon, idxstat, featureCounts	DEseq	DESeq2, CLC	All mappers can be equally used for RNA-Seq, with an outlier being the CLC software combined with it's own differential gene expression module
MacManes (2014)	Trimmomatic, FastX, BioPieces, BLAT, Jellyfish	Bowtie2	/	FPKM	/	Suggests a Phred score cutoff of 2 or 5 for transcriptome assembly
He et al. (2020)	Cutadapt, FastP, Trimmomatic	BWA, Novoalign	/	/	/	Differences between preprocessing techniques are marginal
Lin et al. (2016)	/	/	/	Total Count, Median, upper quartile, Quantile, RPKM, ...	edgeR, DESeq, SAS	Best normalisation approach is to use DESeq and model the data using edgeR or DESeq
Everaert et al. (2017)	/	Tophat, STAR, Kallisto, Salmon	HTSeq, Cufflinks, Kallisto, Salmon	/	/	Each method yielded a small set of lowly expressed genes specific to that method
Srivastava et al. (2020)	Trim galore!	Salmon, STAR, Bowtie2	tximport, RSEM	DEseq, TMM, limma	DESeq2, edgeR, limma	Quasi-mappers are faster but aligners more accurate
Teng et al. (2016)	/	Flux Capacitor, Cufflinks, eXpress, RSEM, Sailfish, kallisto, Salmon	HTSeq, Cufflinks, Kallisto, Salmon	/	/	RSEM slightly outperforming the rest with two methods clearly under-performing

Note that this section may be sectioned based on the different aspects of your dissertation.

Talk about papers that used RNA-seq for the treatment of cancers Despite STAR needing a lot of RAM, this was not a limiting factor due to our access to **HPC!** (**HPC!**) and small number of samples

## 1.7 | Summary

## Materials & Methods

### 2.1 | Preliminary Study

The transcriptomic data used as the basis of this dissertation originates from the doctoral study of Dr Vassallo Gatt (Gatt, 2016). Three samples of the ATRA-resistant HL-60 cell line were incubated and treated with a phenol mixture for varying lengths of time (1, 6, and 12 hours), while a fourth sample served as the negative control, using the growth medium RPMI 1640 as the 'treatment'. This section is a summary of the laboratory procedure utilised by Dr Vassallo Gatt, and is intended to give context to the data.

#### 2.1.1 | Phenolic extraction

Phenolic compounds were isolated from Maltese extra-virgin olive oil by LLE, followed by separation of fractions using preparative-scale High-Performance Liquid Chromatography (HPLC). LLE transferred the water-soluble compounds (including phenols) from their organic solvent to an aqueous one. The heavier aqueous solution was extracted using a separating funnel while the raffinate was discarded. HPLC was used to separate the components of the remaining solution based on their differing chemical interactions with an adsorbent column. As the solution was pumped through the column, phenols flowed at a different rate from the other compounds, leading to the isolation of the phenolic fraction. This is likely a mixture of phenols which requires further analysis to determine its chemical composition and to identify the active compound(s).

### 2.1.2 | Cell Culturing and RNA extraction

ATRA-resistant HL-60 cells were mixed with the phenolic fraction and the mixture was used to seed three wells out of a 6-well plate. The control was seeded similarly, but with growth medium instead of the phenols, which should theoretically not effect their growth. Samples were incubated for their stipulated time period, after which the treated cells began showing characteristic morphological signs of differentiation such as the presence of lobed nuclei, vacuoles, and a decreased nucleus:cytoplasm ratio.

The samples were frozen, then thawed on ice and subjected to RNA extraction according to the RNeasy® Mini kit (QIAGEN, 2014), which makes use of the acid guanidinium thiocyanate-phenol-chloroform (AGPC) extraction technique (Chomczynski and Sacchi, 1987). This involved the separation of the mixture into two partitions: an organic phase containing DNA and protein, and an aqueous phase containing the RNA, induced by the addition of chloroform. The aqueous phase was separated into a separate microcentrifuge tube to which ethanol was added for precipitation of nucleic acids. The mixture was subjected to multiple cycles of spin column-based nucleic acid purification. Between centrifugation cycles the flow-through was discarded and lysis buffer was added to remove silica-bound proteins, carbohydrates, fatty acids and any traces of salts (Matson, 2009). A final centrifugation was performed to elute the RNA in RNase-free water.

### 2.1.3 | Determination of RNA Quality and Sequencing

The RNA was analysed using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific) which confirmed that the concentrations and A260/280 values were of acceptable quality. The total RNA extracted was then shipped to the European Molecular Biology Laboratory (EMBL) in Heidelberg, Germany where the RNA integrity was analysed by gel analysis and then sequenced using an Illumina HiSeq 2000 Sequencing System. The steps followed were typical of the Illumina Stranded mRNA-seq workflow (Illumina, 2010), consisting of poly-A enrichment, RNA fragmentation, cDNA synthesis, ligation of TruSeq adapters, cluster generation, sequencing by synthesis, sequence identification, demultiplexing of the data and the assignment of Phred (Q) scores to each base call (Pease and Sooknanan, 2012; Wang et al., 2011; Zhong et al., 2011).

The transcriptomic data was received in the form of four FASTQ (Cock et al., 2010) files, one per experimental time point. They were composed of 51 base-pairs (bp) single-ended reads, with 30x coverage, and were Sanger/Illumina 1.9 encoded, which uses the ASCII character corresponding to the Phred score, and adds '33' to it (Ewing et al., 1998).

These served as the starting point for the bulk RNAseq pipeline.

## 2.2 | RNA-seq Pipeline

RNA-seq analysis is performed in a number of steps, each requiring one or more different tools. The data 'flows' through these tools, which are constituents of the pipeline. Choosing the correct tools is a non-trivial task, and the process is explained further in Section ???. Each step and tool used in this pipeline is covered in detail in Section 1.4. The pipeline for this analysis is represented as the flowchart in Figure 2.1.

Data was stored and processed until the Quantification stage on a high performance computer, managed by the University of Malta with the following specifications: 56-core Intel(R) Xeon(R) CPU E5-2660 v4 @ 2.00GHz with 128GB RAM, running the Ubuntu v18.04.5 operating system. Read count data was then transferred to a VirtualBox v6.1.32 (Oracle, 2010) virtual environment running Ubuntu v20.04.4, with the following partitioned resources: Intel(R) Core(TM) i5-7200U CPU @ 2.50GHz with 4GB RAM.

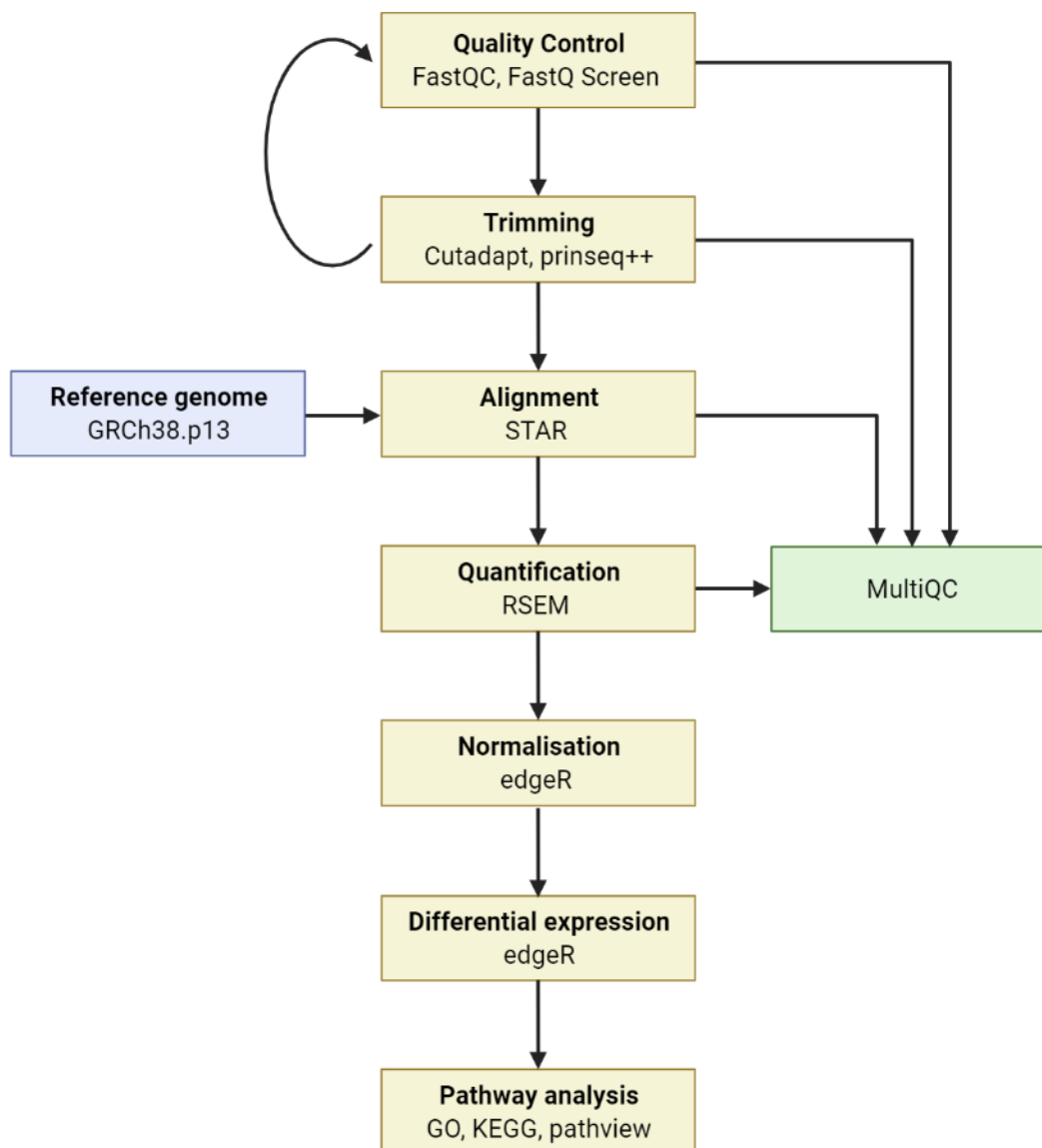


Figure 2.1: General overview of the RNA-seq pipeline. Created with BioRender.com.

### 2.2.1 | Quality Control

The first step of any sequencing pipeline should be the assessment of the quality of the raw data files. Sequencing information of poor quality is identified if present, and if necessary, truncated to mitigate inaccuracies in the downstream pipeline. To assess the quality of the FASTQ files, they were ported to FastQC v0.11.9 (Andrews et al., 2010) using eight threads (Listing 2.1). This extracted information on the sequences, including Phred scores, GC content, N content, sequence length distribution, sequence duplication levels, overrepresented sequences and adapter sequences. FastQC rates each of these modules using a green check-mark signifying that it 'passed' QC, a yellow exclamation mark 'warning', or a red cross 'failed'.

Two modules failed consistently throughout the four samples received: 'Sequence Duplication Levels' and 'Per base sequence content'. This is normal and expected for RNA data, and is explained in further detail in ???. Traces (<0.5%) of TruSeq adapters were detected in the FASTQ files, which is corroborated by the sequencer's manual (Illumina, 2010) stating that it makes use of the 'TruSeq family of reagents'.

```
1 # FastQC accepts multiple input files, so we can use wildcards
2 fastqc \
3 -t 8 \
4 -o ./raw_FastQC_out \
5 *.fq
```

Listing 2.1: FastQC command

Next, Fastq Screen v0.14.0 (Wingett and Andrews, 2018) was used to check for RNA contaminants from other common sources, by comparing the reads against a set of sequence databases (Listing 2.2). Perl, an aligner (Bowtie (Langmead et al., 2009), Bowtie2 (Langmead and Salzberg, 2012) or BWA (Li and Durbin, 2009)) and a Linux-based operating system are required. Bowtie2 was used to align the sample reads to the reads in the contaminant database.

```
1 # FastQScreen also accepts multiple file inputs
2 fastq_screen \
3 --aligner bowtie2 \
4 --conf ./fastq_screen.conf \
5 *.fq
```

Listing 2.2: FastqScreen command

### 2.2.2 | Preprocessing

Poor quality reads lead to poor downstream sequence analysis. Thus, it is common practice in RNA-seq to trim the undesired regions. Cutadapt (Martin, 2011) was used to trim the previously detected TruSeq adapter sequences and short reads, using a threshold of <45 bp. Between 1.3 and 1.6% of the base-pairs were trimmed across the four samples. Cutadapt was accessed through the wrapper script Trim Galore! v0.6.7 (Krueger, 2019) which instantly redirects the trimmed data back to FastQC to assess the improvement in quality, if any.

```

1 trim_galore \
2 --phred33 \
3 --fastqc \
4 -a "GATCGGAAGAGCACACGTCTGAACTCCAGTCAC" \
5 --length 45 \
6 -o trim_galore_output \
7 --fastqc_args "-o trimmed_FastQC_out -t 8" \
8 --cores 4 \
9 *.fq

```

Listing 2.3: Trim Galore! trimming

The trimmed files were further filtered using Prinseq++ (Cantu et al., 2019) which removed ambiguous reads containing >7 N's and sequences with a DUST score<sup>1</sup> of <0.1.

```

1 samples=(control, 1hour, 6hour, 12hour)
2 mkdir prinseq_out
3
4 for sample in ${samples[@]};
5 do
6     prinseq++ \
7     -fastq ./trim_galore_output/${sample}_trimmed.fq \
8     -out_name ./prinseq_out/${sample}_filtered.fq \
9     -out_bad ./prinseq_out/${sample}_bad.fq \
10    -ns_max_n 7 \
11    -lc_dust=0.1 > ./prinseq_out/control_prinseq_report.txt
12 done

```

Listing 2.4: Prinseq++ filtering

<sup>1</sup>A value between 0 and 1 generated by the DUST algorithm which is a measure of sequence complexity. Here, its purpose is to mask low-complexity regions.



### 2.2.3 | Read alignment and Quantification

The trimmed and filtered FASTQ files were aligned to the GRCh38.p13 reference genome (NCBI, 2019) using the STAR 2.7.9a aligner (Dobin et al., 2013). STAR was called through RSEM v1.3.3 (Li and Dewey, 2011), which after alignment estimates gene and isoform expression levels. RSEM must be accessed through a 64 bit Linux or Mac OS command-line, and must have C++, Perl and R installed as dependencies.

Reference transcripts from the reference genome were first generated through the `rsem-prepare-reference` program, with the help of its respective GTF file (Listing 2.5).

```

1 mkdir RSEM
2 mkdir RSEM/reference
3 rsem-prepare-reference --gtf mm9.gtf \
4                         --star \
5                         --star-sjdboverhang 50 \
6                         --num-threads 8 \
7                         --gtf Homo_sapiens.GRCh38.104.gtf \
8                         Homo_sapiens.GRCh38.dna.primary_assembly.fa \
9                         ./RSEM/reference/GRCh38

```

Listing 2.5: reference generation

The `-star-sjdboverhang` is an argument passed to STAR which sets the maximum possible overhang for the reads. It should be equal to the read length minus one. In our case, the read length is 51, thus a value of 50 is used. The final variable is a prefix to the output files. This may be used as the output path for the generated reference files.

To calculate expression values, the `rsem-calculate-expression` command is used. As before, the final argument is a prefix to the output files, and the argument before that is the path to the reference files created in the previous command. RSEM first uses STAR to align the filtered sample reads to the generated reference reads, creating a BAM file sorted by its coordinates. It outputs two quantification files per sample, with differing suffices: one ending in `'genes.results'` and another `'isoform.results'`. These are both tab-delimited text files with similar headers, with the former dedicating a row for each gene and the latter dedicating a row for each transcript. The isoform-centric file thus contains more data, which are unnecessary for our objectives. Of the columns in the gene-centric file, two will be used in the subsequent step: `'gene_id'` which holds the Ensembl gene ID, and `'TPM'` which holds the normalised read count for the respective gene.

```

1 mkdir RSEM/expression
2 samples=(control, 1hour, 6hour, 12hour)
3
4 for sample in ${samples[@]};
5 do

```

```

6      rsem-calculate-expression \
7      --num-threads 16 \
8      --star \
9      --sort-bam-by-coordinate \
10     ./prinseq_out/${sample}*good_out.fq \
11     ./RSEM/reference/GRCh38 \
12     ./RSEM/expression/${sample}
13 done

```

Listing 2.6: RSEM expression command

### 2.2.4 | Reassessing the Quality

The produced files from FastQC, FastQScreen, Trim Galore! and RSEM, were funnelled into MultiQC v1.11 (Ewels et al., 2016) which summarises their output in the form of an HTML report. At the time of writing, the latest version of MultiQC did not support Prinseq++ and its log files were inspected manually instead.

```

1 multiqc \
2 -o ./multiqc_out \
3 ./trim_galore_output/*txt \
4 ./fastqc/FastQC_out/. \
5 ./FastQ_Screen_out/raw/. \
6 ./RSEM/expression/.

```

Listing 2.7: MultiQC command

### 2.2.5 | Normalisation and Differential Gene Expression

We have chosen edgeR v3.14 (Robinson et al., 2010) as the package to help identify differentially expressed genes, which supports datasets that lack replicates such as our own. The instructions on the vignette<sup>2</sup> were followed, and are summarised as follows.

The four gene read count files generated through RSEM were imported into the R script v3.6.3 (R Core Team, 2020) and had their gene IDs (1st column) and expected read counts (5th column) compiled into a single DGEList object which consists of multiple data-frames.

```

1 files <- list("1hour.genes.results", "12hour.genes.results",
2 "6hour.genes.results", "control.genes.results")
3 path <- "/path/to/files/"

```

<sup>2</sup><https://www.bioconductor.org/packages/release/bioc/vignettes/edgeR/inst/doc/edgeRUsersGuide.pdf>

```

4 labels = c('1hr', '12hr', '6hr', '0hr')
5 group <- factor(labels)
6 y <- readDGE(files, path=path, columns=c(1,5), group=group, labels=labels)

```

Listing 2.8: Importing count files to R

### 2.2.5.1 | Filtering Low Count Genes

Genes with low counts (<10) are generally considered as noise, as they are not expressed at any biologically meaningful level and filtering them would reduce the amount of statistical tests required in the downstream analysis (Law et al., 2016). EdgeR provides the function `filterByExpr` which serves this purpose.

```

1 library(edgeR)
2 keep <- filterByExpr(y, group=group)
3 table(keep) # How many genes were removed and how many remain
4 y <- y[keep,keep.lib.sizes=FALSE]

```

Listing 2.9: Filtering low count genes

### 2.2.5.2 | Gene Annotation

The remaining genes were annotated using the `AnnotationDbi` library v3.14 (Carlson, 2015) which is dependent on the `org.Hs.eg.db` v3.10.0 (M, 2019) human annotation database. These were used to add the Entrez ID annotation to the `DGEList` object, which will be used for pathway analysis further downstream. `AnnotationDbi` provides other useful annotation options, but were excluded as combination of annotations was causing a one to many mapping error. This step was later repeated (after pathway analysis) to add gene symbol annotations. This proved useful for later research into their biology, since most papers refer to the gene by its gene symbol.

```

1 library(org.Hs.eg.db)
2 library(AnnotationDbi)
3
4 # This step was repeated at a later stage with "SYMBOL" as the
5 # column argument, which adds the gene symbol.
6 Symbol <- mapIds(org.Hs.eg.db, keys=rownames(y), keytype="ENSEMBL",
7                 column="ENTREZID")
8
9 y$genes <- data.frame(Symbol=Symbol)
10 head(y$genes)

```

Listing 2.10: Annotation step

### 2.2.5.3 | Normalisation

The read counts are then normalised by the `calcNormFactors` function which uses the Trimmed Mean of *M*-values (TMM) method. This is recommended by the edgeR vignette and is explained in detail in Robinson and Oshlack (2010). The aim of normalisation is to mitigate any technical bias on the data.

```
1 y <- calcNormFactors(y)
2 design <- model.matrix(~group)
```

Listing 2.11: TMM normalisation

EdgeR has multiple methods to determine which genes are differentially expressed, but the classic `exactTest` was deemed as most appropriate. This is a pairwise test which compares the means between two groups of counts with a negative-binomial distribution. The calculation of the dispersion of our data is required prior to its use, however this is not mathematically possible due to our lack of replicates. In such cases the edgeR vignette suggests that estimating the dispersion based on the experimental conditions is more scientifically sound than assuming no variation. It should be emphasised that this technique is inaccurate, but was deemed as the best possible option for statistical analysis without replicates (further detail in Section ??).

The Biological Coefficient of Variance (BCV) is equal to the square root of the dispersion, and the vignette suggests a few estimates for the BCV, such as '0.1 for data on genetically identical model organisms'. While this is human data, not a model organism, the data originate from the same genetically identical cell line, and this value was deemed appropriate. Thus the dispersion used for `exactTest` is equal to the  $BCV^2$ , or 0.01. The test was performed a total of three times, where each treated sample was compared with the control. This returns a `DGEEExact` object for each comparison, containing the  $\log_2$  fold change (`logFC`),  $\log_2$  Counts Per Million (`logCPM`), p-values and annotations for each gene.

```
1 bcv <- 0.1
2 et_12 <- exactTest(y, pair=c(1, 2), dispersion=bcv^2)
3 et_1 <- exactTest(y, pair=c(1, 3), dispersion=bcv^2)
4 et_6 <- exactTest(y, pair=c(1, 4), dispersion=bcv^2)
```

Listing 2.12: Exact test function

### 2.2.5.4 | Adjusting p-values and Cut-offs

Multiple testing is an inherent risk of RNA-seq due to its vast amounts of comparisons and adjusting p-values is a common technique implemented into the RNAseq pipeline

to help compensate for this. The False Discovery Rate (FDR) with the Benjamini-Hochberg controlling procedure was the chosen p-value adjustment method. This produced value is the proportion of false positives one might expect to get from a test. Each of the DGEExact objects was transformed in this way using the topTags function. Differential expression was sorted by the FDR and any genes with an FDR < 0.05 were filtered out, which means that we are willing to accept that 5% of all Differentially Expressed Genes (DEG) will be false positives. Filtering on the logFC was then performed manually, using a threshold of 1.5.

```

1 FDR_thresh <- 0.05 # removes rows with FDR less than this
2 et_1_topTags <- topTags(et_1, n=nrow(et_1$table), adjust.method="BH",
3 sort.by="PValue", p.value=FDR_thresh)$table
4 et_12_topTags <- topTags(et_12, n=nrow(et_12$table), adjust.method="BH",
5 sort.by="PValue", p.value=FDR_thresh)$table
6 et_6_topTags <- topTags(et_6, n=nrow(et_6$table), adjust.method="BH",
7 sort.by="PValue", p.value=FDR_thresh)$table
8
9 FC_thresh <- 1.5 # removes rows with a logFC less than this
10 et_1_topTags <- et_1_topTags[abs(et_1_topTags$logFC) > FC_thresh, ]
11 et_12_topTags <- et_12_topTags[abs(et_12_topTags$logFC) > FC_thresh, ]
12 et_6_topTags <- et_6_topTags[abs(et_6_topTags$logFC) > FC_thresh, ]

```

Listing 2.13: Adjusting p-values and filtering data based on the logFC and FDR

## 2.2.6 | Functional analysis

?

## 2.3 | Summary

Empty for now.



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