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List of Abbreviations

AML Acute Myeloid Leukaemia 2
ATRA all-*trans* retinoic acid 8
TMM Trimmed Mean of *M*-values 26
RIN RNA Integrity Number 12
PCR Polymerase Chain Reaction 13
FAB French-American-British 3
WHO World Health Organisation 3
LLE Liquid-Liquid Extraction 11
NGS Next-Generation Sequencing 9
SNP Single Nucleotide Polymorphism 6
DEG Differentially Expressed Genes 11
DGE Differential Gene Expression 9
BCV Biological Coefficient of Variance 29
FDR False Discovery Rate 29
GO Gene Ontology 30
KEGG Kyoto Encyclopedia of Genes and Genomes 30
logFC log_2 fold change 27
GSEA Gene Set Enrichment Analysis 31

1

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 project 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. Using this technology, we can detect the presence or absence of certain transcriptomic hallmarks of cancer.

1.1 | Acute Myeloid Leukaemia

Acute Myeloid Leukaemia (AML) is an aggressive form of cancer of the haematopoietic system (Figure 1.1) 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 (Juliusson et al., 2009; Khwaja et al., 2016). Acute myeloid leukaemia is synonymous with acute myelogenous leukemia, acute myelocytic leukemia, or acute nonlymphocytic leukemia.

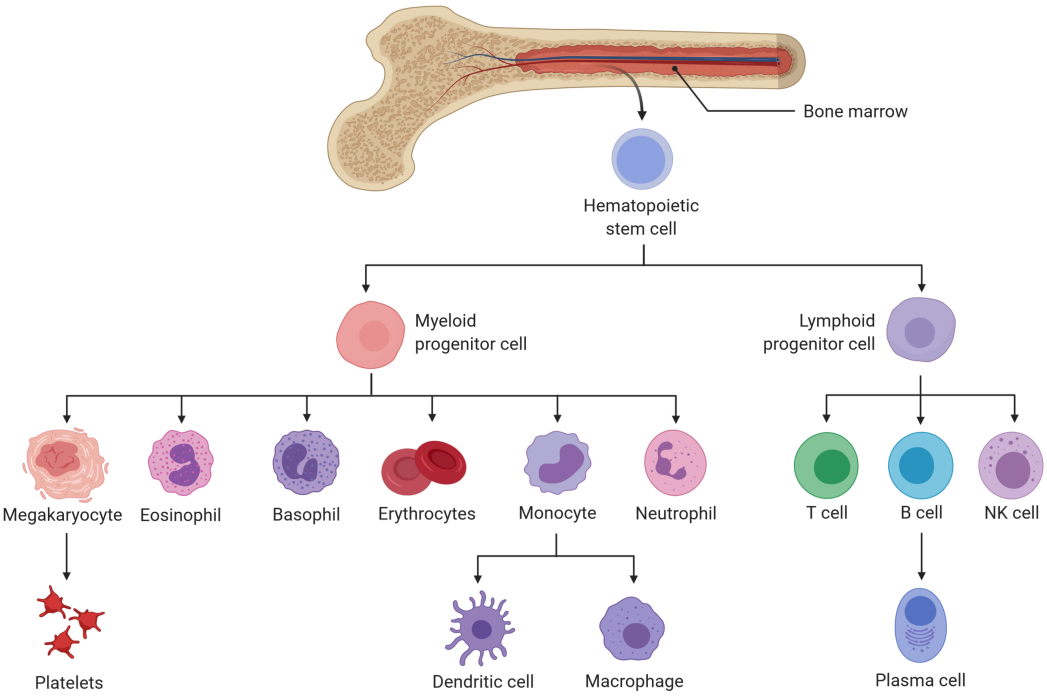

Figure 1.1: An overview of the main branches of haematopoietic stem cell differentiation pathways showing the myeloid and lymphoid lineages. Created using BioRender.com.

1.1.1 | 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).

1.1.1.1 | 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

1.1.1.2 | 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 their their abnormality (similar to Table 1.2, although not all are officially recognised as 'recurring abnormalities').

1.1.2 | 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).

1.1.2.1 | 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 1.2).

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, MHY11	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)

1.1.2.2 | Genetic Abnormalities

If we reduce our frame of reference to the genetic level, we find that the aforementioned structural variants (Table 1.2) can trigger the activation of an *oncogene*, or their fusion products (Table 1.2) 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 DiNardo and Cortes (2016) and Lindsley et al. (2015).

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

1.1.3 | 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).

1.1.4 | 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, Gallagher et al. (1979) 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 1.1.1). Subsequent analysis of the cells' karyotype, performed by Dalton Jr et al. (1988), 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 Jacobson et al. (2020) 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 1.1.3, 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.

1.2 | 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). Since its first publications in 2008 (Cloonan et al., 2008; Lister et al., 2008; Nagalakshmi et al., 2008), 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).

The following section is an overview of the techniques used to transform the nucleotide sequences residing inside living cells into letters on a screen. While this project deals with the data analysis part of RNA-seq, some background on the origins of said data is essential.

1.2.1 | Experimental Design

An RNA-seq experiment is customised according to research goals and often limited by the budget. Since many of the following options are an accuracy/expense tradeoff, the researcher should be knowledgeable on the options to effectively allocate funds, particularly if the sequencing will be outsourced to another company. This section was placed here to retain the chronological order in which an RNA-seq experiment would take place, although some of the below descriptions include some technical detail which will be explained in future sections.

Read length Before sequencing, it is possible to specify the number of base-pairs of the DNA fragments each read would contain. A distinction is made between reads which emerge from second-generation sequencing machines (short-reads) and third-generation sequencing machines (long-reads). Smaller reads lead to greater ambiguity during alignment as they have a greater probability of being multi-mapped, and partly determine the optimal alignment algorithm (Albert, 2020). This is especially true in regions of low-complexity or in the presence of structural variants (Rhoads and Au, 2015). The exception to this rule is in the study of small RNAs, where read lengths drop to <30bp (Albert, 2020).

Depth of coverage Coverage is the average number of reads that will cover a given sequence, meaning that it is determined by the read length and number of reads. It is commonly denoted with an x, e.g. 30x coverage means that a nucleotide is covered by an average of 30 reads. Low coverage is susceptible to ambiguity and sequencing errors.

Paired-end reads Fragments of cDNA are typically longer than the read length, so some sequencing information may be lost. Paired-end sequencing, as opposed to single-end sequencing, allows both ends of the cDNA fragment to be sequenced. The distance between each paired-end read is known, which is fed into alignment algorithms that use this information to improve alignment. This especially improves regions of low complexity (Albert, 2020).

Sample replicates Technical replicates originate from the same biological source to produce multiple samples which are all processed in the same manner. This gives isolates the non-biological variation, allowing for the evaluation of the instruments and methodology used. By contrast, biological replicates originate from different biological sources and are meant to test the biological variance of the samples. A mixture of the two types may be used, however given a limited budget, biological replicates are preferred in RNA-seq because technical variation is minimal (Bullard et al., 2010), by far outweighed by biological variation (Liu et al., 2014). Schurch et al. (2016) found that using three biological replicates gave 20% to 40% of the Differentially Expressed Genes (DEG)s (varies according to the tool) compared to a full set of 42 replicates (representing the 'true' population). This rises to >85% when considering genes with a $\log_2$ fold change of >2.

1.2.2 | 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 electrophorisis, 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.

1.2.3 | 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).

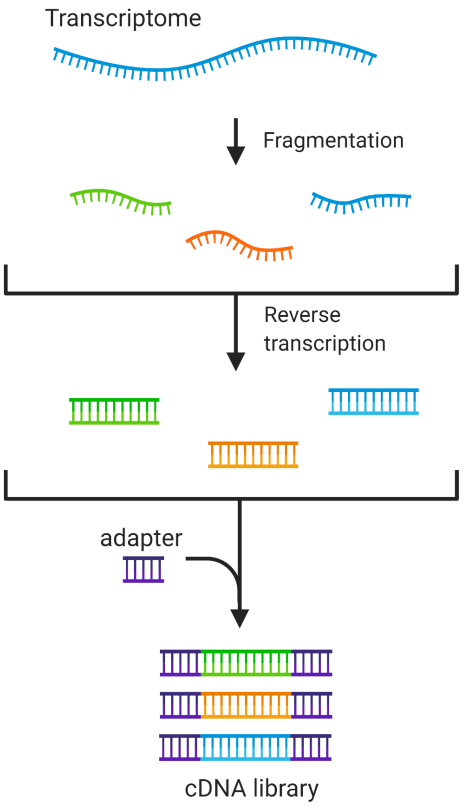

Figure 1.2: Illumina library preparation. Created using BioRender.com.

1.2.4 | 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) complimentary 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).

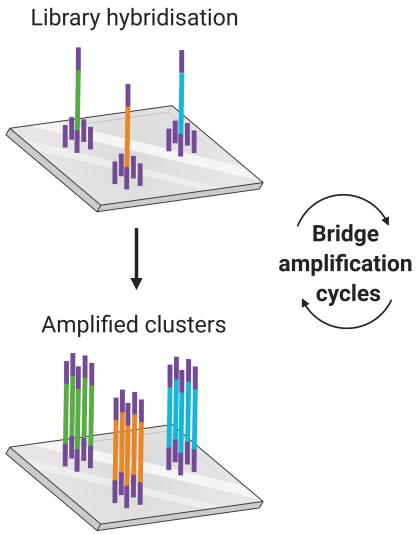

Figure 1.3: Illumina clonal amplification. Created using BioRender.com.

1.2.5 | 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.

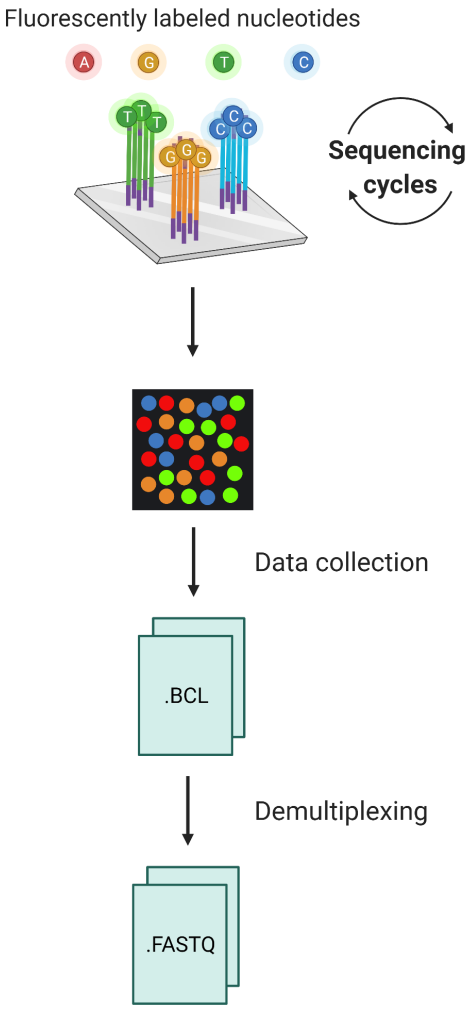

Figure 1.4: Illumina sequencing by synthesis. Created using BioRender.com.

1.3 | 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. They were inspired by a multitude of online tutorials and resources, accessed between 29/05/2022 and 10/06/2022:

- https://chagall.med.cornell.edu/RNA-seqcourse/
- https://training.galaxyproject.org/training-material/topics/transcriptomics/tutorials/rb-RNA-seq/tutorial.html
- https://btep.ccr.cancer.gov/wp-content/uploads/RNA-seq_BETP_2019rev.pdf
- https://www.bioconductor.org/packages/devel/bioc/vignettes/DEGreport/inst/doc/DEGreport.html
- https://training.galaxyproject.org/training-material/topics/sequence-analysis/tutorials/quality-control/tutorial.html
- https://chagall.med.cornell.edu/RNA-seqcourse/Intro2RNA-seq.pdf

1.3.1 | Quality Control

The first part of any sequencing 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:

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

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

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[1] 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.

[1]The complete Q-score encoding table: https://support.illumina.com/help/BaseSpace_OLH_009008/Content/Source/Informatics/BS/QualityScoreEncoding_swBS.htm

1.3.1.1 | FastQC

Citation: Andrews et al. (2010)
Documentation: `https://www.bioinformatics.babraham.ac.uk/projects/fastqc/Help/3%20Analysis%20Modules/1`
Dependencies: Java, Picard BAM/SAM Libraries (included in download)

In the rapidly changing field of nucleotide sequencing, FastQC 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 (Li et al., 2009) 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 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.
- **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.

1.3.1.2 | FastQScreen

Citation: Wingett and Andrews (2018)

Documentation: `https://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/_build/html/index.html`

Dependencies: Linux-based OS, Bowtie/Bowtie2/BWA

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 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.

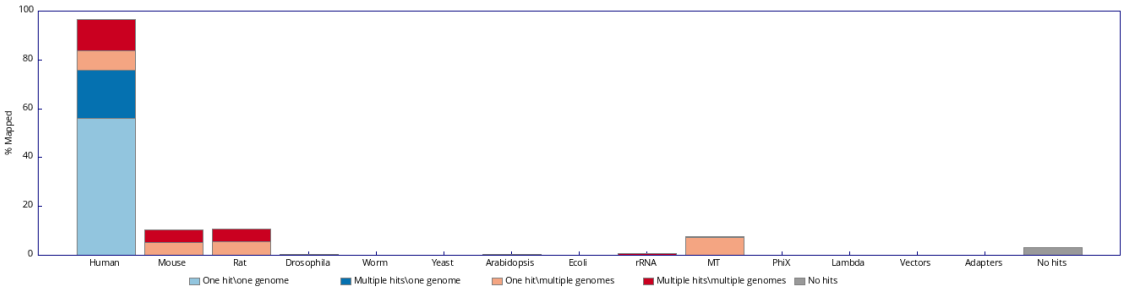

Figure 1.5: An example of a good FastQScreen output result, with human mapping close to 100% and some multi-mapping to mouse and rat genomes.

1.3.1.3 | MultiQC

Citation: Ewels et al. (2016)
Documentation: `https://multiqc.info/docs/`
Dependencies: Python 3

MultiQC 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.

1.3.2 | 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). Some (Liao and Shi, 2020) doubt the necessity of trimming at all.

1.3.2.1 | Cutadapt: Short reads and Adapter sequences

Cutadapt citation: Martin (2011)
Cutadapt documentation: `https://cutadapt.readthedocs.io/en/v4.0/guide.html`
Cutadapt dependencies: Python 3.7 or newer
Trim Galore! citation: Krueger (2019)
Trim Galore! documentation: `https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/`
Trim Galore! dependencies: cutadapt, FastQC

One of the primary functions of Cutadapt (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! 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
- Requires a three nucleotide overlap between read and adapter for an adapter to be found

1.3.2.2 | Prinseq++: Low complexity and No Basecalls

Short for: PReprocessing and INformation of SEQuence data
Citation: Cantu et al. (2019)
Documentation: `https://github.com/Adrian-Cantu/PRINSEQ-plus-plus`
Dependencies: C++

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 1.3.2.1. The data should be filtered to some degree based on these metrics, which is facilitated by ready-made tools such as Prinseq++. 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++ present 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 (Morgulis et al., 2006). 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

1.3.3 | 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 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).

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,

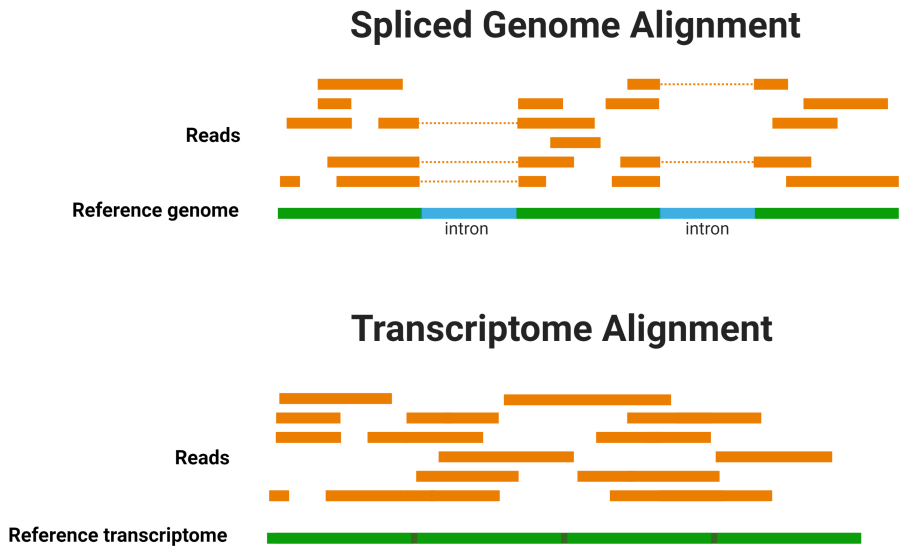

Figure 1.6: Alignment against a reference genome or a reference transcriptome. Created using BioRender.com.

although this distinction is arbitrary. Many conventional short-read mappers are not suitable for reads under 30bp, necessary for the study of small RNAs (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 (Andrews, 2016).

1.3.3.1 | STAR

Short for: Spliced Transcripts Alignment to a Reference
Citation: Dobin et al. (2013)
Documentation: `https://physiology.med.cornell.edu/faculty/skrabanek/lab/angsd/lecture_notes/STARmanual.pdf`
Dependencies: 64 bit Linux or Mac OS X

STAR 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 RNA-seq reads to a genome.

The algorithm first searches for the Maximum Mappable Prefix (MMP) which acts 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 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 RNA-seq 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 [2].

1.3.4 | Quantification

The following step associates the aligned reads with the respective genes or transcripts found at their locus. The counts of the mapped reads are proportional to the cell's expression of that particular gene/transcript. Quantifying at the transcript-level is more detailed than the gene-level, but not all research questions require this level of detail. The final output of the combined samples should be a table resembling Table 1.4.

Pachter (2011) provides a detailed (albeit slightly outdated) review of the mathematical models behind transcript quantification, such as the Expectation–Maximization (EM) algorithm, and how they affect downstream analyses. EM estimates the maximum likelihood of proper alignment in the presence of latent variables (Brownlee, 2019; Pachter, 2011).

Table 1.4: An example of a read count table, values representing the number of reads aligned to that gene. In bulk RNA-seq, each sample represents the pooled RNA of a large number of cells, most likely of different cell types.

Genes	**Sample**$_1$	**Sample**$_2$	**Sample**$_3$	...
A2BG	10	30	0	...
AML	30	3	3	...
AMT2	0	0	10	...
ARST5	5300	1900	3250	...
...	...	...	...	...

1.3.4.1 | RSEM

Short for: RNA-Seq by Expectation Maximization
Citation: Li and Dewey (2011)
Documentation: `http://deweylab.github.io/RSEM/README.html`
Dependencies: 64 bit Linux/Mac OS, C++, Perl, R, STAR/HISAT2/Bowtie2

RSEM uses a statistical model based on Li et al. (2010), an implementation of the EM algorithm to address the issue of ambiguous read mapping, and assign reads to

[2] `https://samtools.github.io/hts-specs/SAMv1.pdf`

their appropriate gene or transcript. RSEM gives the user the option to produce both, and normalises the counts in the process. For each sample, RSEM produces two tab-delimited text files: one quantified at the gene-level and another at the transcript-level. Each row of these file represents the respective gene or transcript (the transcript file is larger due to alternative splicing), with the columns including the IDs, expected counts and normalised counts (TPM and FPKM)

An aligner (STAR, Bowtie2 or HISAT2) may be called directly through RSEM, to combine alignment and quantification (and potentially normalisation) into a single step. The genome index to be used by the aligner may be generated through `rsem-prepare-reference` and alignment + quantification may be performed with `rsem-calculate-expression`.

RSEM's default options:

- Accepts FASTQ files as input for alignment.
- Outputs a gene-centric file, with the following columns: `gene_id, transcript_id(s), length, effective_length, expected_count, TPM and FPKM`
- Outputs a transcript-centric file, with the following columns: `transcript_id, gene_id, length, effective_length, expected_count, TPM, FPKM, IsoPct`

1.3.5 | Normalisation

To adjust for confounding variables which are not biologically relevant, the read counts must first be normalised. The main factors to account for are sequencing depth (Robinson and Oshlack, 2010), gene length (Oshlack and Wakefield, 2009) and GC content (Risso et al., 2011). Effective gene expression analysis should calculate the abundance of the transcripts as a fraction of the entire RNA repertoire for that particular sample. A number of methods have evolved over the years to tackle these issues. Dillies et al. (2013) and Bullard et al. (2010) extensively explore the different approaches one may take. Despite their frequent misuse in published studies, within-sample comparison methods (FPKM (Trapnell et al., 2010), RPKM (Mortazavi et al., 2008), TPM (Li and Dewey, 2011), Total Counts (Dillies et al., 2013)) should be avoided in DGE analysis as they only account for differences within the same sample, and not between samples (Dündar et al., 2015; Zhao et al., 2020).

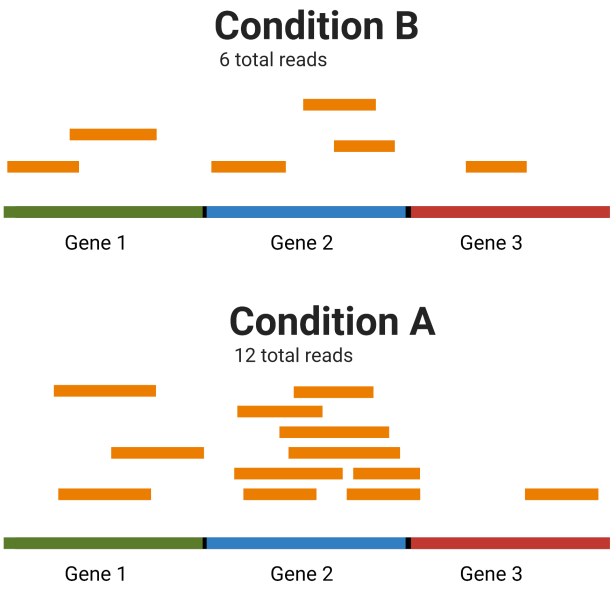

Figure 1.7: Potential differences between samples in library composition. Condition A has more reads aligned to Gene 1 than Condition B, but it is considered more highly expressed in Condition B since the *proportion* of the reads is higher. After accounting for library differences, Gene 2 is more highly expressed in Condition A, and Gene 3 is more highly expressed in Condition B. Created using BioRender.com.

1.3.5.1 | Trimmed Mean of *M*-values (TMM)

The Trimmed Mean of *M*-values (TMM) is implemented in edgeR (Robinson et al., 2010) through the `calcNormFactors` function. It is recommend by the edgeR vignette[3] if one wishes to continue performing DGE analysis using that library. It was first introduced in Robinson and Oshlack (2010), who explain the underlying mathematics in detail. TMM assumes that the majority of genes, in both samples, are not differentially expressed, although the model is robust against deviations to this assumption (Robinson and Oshlack, 2010).

TMM performs better for between-samples comparisons, as opposed to within-sample comparisons (Dündar et al., 2015). Robinson and Oshlack (2010) recognise that it makes intuitive sense that differences in library size should be normalised (i.e. depth or coverage, as seen in Figure 1.7), but they consider this scaling too simplistic for many biological applications.

[3] `https://www.bioconductor.org/packages/release/bioc/vignettes/edgeR/inst/doc/edgeRUsersGuide.pdf`

The observed counts for gene g in library k, calculated from the read quantification step (subsection 1.3.4), are represented as Y_{gk}. The total reads in library k are represented as N_k. The M-value for gene g and libraries k and k' may be calculated as:

$$M_g = log_2 \frac{Y_{gk}/N_k}{Y_{gk'}/N_{k'}}$$

The absolute expression level, A, for gene g is calculated as:

$$A_g = \frac{log_2(Y_{gk}/N_k) * Y_{gk'}/N_{k'}}{2}$$

The next step is to trim the means of the M-values and A-values. A mean is trimmed when a percentage of the data is truncated at the upper and lower ends. By default this is 30% for the M-values and 5% for the A-values, but these settings may be changed (Robinson and Oshlack, 2010).

The final step is the calculation of the normalisation factor and weighted mean of the trimmed M_g using precision (inverse of the variance) weights. The calculations used in this step are too complex for the scope of this project (see Robinson and Oshlack (2010) for further details).

1.3.6 | Differential Expression Analysis

The crux of the RNA-seq pipeline is to decide through statistical testing whether a given gene's expression varies significantly between samples, and if this variation can be explained by the difference in the cells' biology. Genes with very low read counts cannot be reliably represented across all samples, and are indistinguishable from background noise (McIntyre et al., 2011). These lowly expressed genes should be filtered before differential expression analysis as they are more likely to be incorrectly identified as DEGs. All tools which measure gene expression aim to estimate two metrics based on normalised read counts from replicated samples:

1. The *magnitude* of the differential expression, represented as the log_2 fold change (logFC).
2. The *significance* of the difference, represented as a p-value adjusted for multiple testing.

The three most commonly used tools for DGE judging by citation counts at the time of writing are DESeq2 (Love et al., 2014), edgeR (Robinson et al., 2010) and limma (Ritchie et al., 2015), which all take the same basic approach. Regression-based models

are used to estimate the difference in normalised read counts for each gene or transcript of interest, which are tested for a significant difference (Dündar et al., 2015).

In differential expression analysis we are testing whether each gene in our list is significantly up- or down-regulated when compared to the reference sample. This test is performed for thousands of genes, which is where we run into the multiple testing problem. These large numbers of comparisons suddenly make small Type I error rates relevant, running the risk of falsely identifying certain genes as differentially expressed. To account for this risk, *p*-values are adjusted based on how many tests are to be considered (Feise, 2002). The Bonferroni correction (Dunn, 1961) is one such method, although considered by some (Feise, 2002) to be too conservative, potentially tipping the scale to the other end and inducing Type II errors (declaring a result not statistically significant when it is). Other, less conservative solutions have been proposed, such as the Bonferroni-Holm (Holm, 1979) or Hochberg (Hochberg and Tamhane, 1987) techniques.

1.3.6.1 | EdgeR

The Bioconductor package edgeR allows the implementation of a wide array of statistical methods applicable to DGE analysis. EdgeR accepts a matrix of reads normalised by TMM as input (see Section 1.3.5.1 for details). Prior to differential expression analysis, this matrix is filtered according to read counts using the function `filterByExpr` which removes genes with <10 read counts. Two primary routes may be taken using Figure 1.8, the classic route which involves exact tests (Robinson and Smyth, 2007, 2008), or the Generalized Linear Model (GLM) route, although certain features of the two may be combined. The GLM tests for DGE are likelihood ratio tests (LRTs) (McCarthy et al., 2012) and quasi-likelihood F-tests (QLFs) (Lun et al., 2016; Lund et al., 2012).

The `exactTest` function is based on quantile-adjusted conditional maximum likelihood (qCML) method. It produces a matrix of pseudo-counts[4] which are designed to speed up computational analysis and not to be interpreted as regular normalised counts. The qCML method is only applicable on datasets with a single factor.

GLMs are an adaptation of classical linear models to cater for non-normally distributed data (Dunn et al., 2018). The QLF dispersion estimate and test can be performed with the functions *glmQLFit* and *glmQLFTest*. This fits a negative binomial GLM to the TMM normalised read counts. The LRT compares the goodness of fit of two competing models through the functions *glmFit* and *glmLRT*.

[4]Note that the meaning of the term *pseudo-counts* may change according to the context, and may be used by other studies to refer to something different.

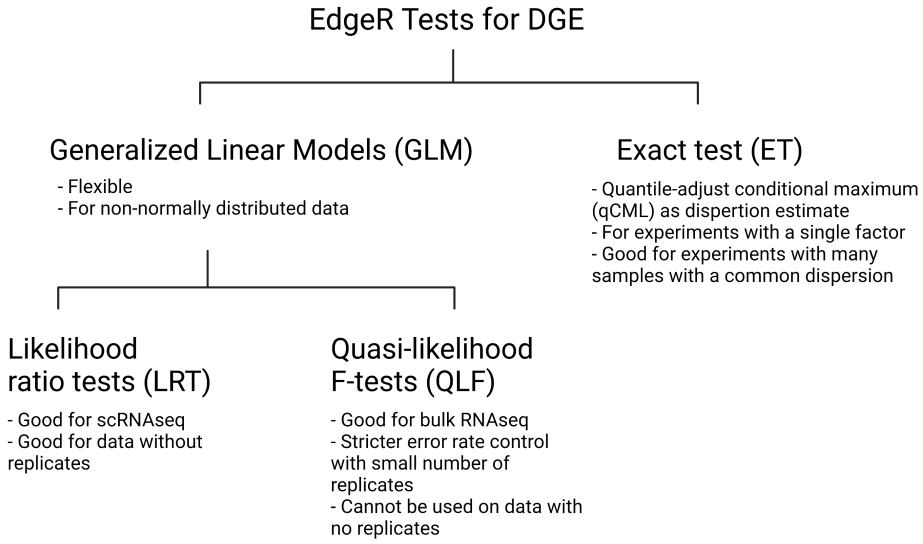

Figure 1.8: Summary of the three options provided by edgeR for DGE identification, based on comments and recommendations by its vignette.

EdgeR and DESeq2 are quite similar in that they both make use of a negative binomial distribution to model read counts, and estimate dispersion based on the approximate conditional inference, first proposed by Cox and Reid (1987). EdgeR is recommended for experiments with fewer than 12 replicates (Schurch et al., 2015), and unlike DESeq2, allows for the analysis of data with no replicates, although highly discouraged by the vignette.

The biggest challenge when working with data without replicates is the estimation of dispersion, which is mathematically impossible given a single sample. In cases when there is no other alternative, the vignette suggests giving a nominal value to the Biological Coefficient of Variance (BCV), from which we may derive the dispersion. The vignette suggests a few estimates for the BCV which are based on previous experiments as the dispersion, such as *0.1 for data on genetically identical model organisms*. The dispersion is equal to this value squared, which is little more than an educated guess, but is a better alternative to assuming no variance.

To account for the previously described multiple testing problem, the `topTags` function adjusts p-values using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) to control the False Discovery Rate (FDR). This produced value is the proportion of false positives one might expect to get from a test.

To gain further biological insight, the `goana` and `kegga` functions may be used to annotate genes according to their associated Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways respectively. This may be particularly useful in downstream tests for gene set analyses.

The end result should be an edgeR object containing a matrix of each the logFC, adjusted p-values and (optionally) annotations for each differentially expressed gene. The final step is filtering on the matrix by setting a (largely arbitrary) logFC and/or *p*-value cutoff, and sorting the data by one of these metrics for easier biological analysis.

1.3.7 | Downstream Analysis

The steps of the pipeline up until this point are quite standard, although there are various approaches one may take, the aim of each step is clear and consistent across all RNA-seq studies. Further exploration of the data is highly specific to the experimental design and research question. Putting the data into its biological context is a type of sanity check. Deviation from biological expectations is often an indication of issues in the upstream analysis.

1.3.7.1 | Graphical Representation

Graphical representation of the gathered information about the samples thus far conveys the information in a more human-readable format and allows for easier pinpointing of differences and potential batch effects. In plots which cluster data according to similarity, replicates should form distinct clusters. Two replicates which apparently switched clusters is a good indication of a potential sample swap.

Multidimensional Scaling (MDS) RNA-seq data deals with thousands of dimensions, making it difficult to interpret and impossible to use conventional plotting methods. MDS is type of non-linear dimensionality reduction used to mitigate this issue (Yin, 2007). It is a between-sample measure of similarity which plots pairwise distances using Cartesian coordinates (Mead, 1992).

Principal Component Analysis (PCA) Similar to MDS in scope except that it is a *linear* dimensionality reduction technique. PCA transforms the data to find the combination of variables which explains the maximum variation in the data.

Heatmaps and Clustering Often used in combination with each other, with a colour gradient in the heatmap signifying the logFC for each DEG in the list, which are clustered according to similar expression patterns.

Mean-Difference (MD) In other fields, it is used to determine if two methods of measurement are in agreement (Fry, 2008). In RNA-seq it is used to compare the logFC of each gene of a given sample against the mean logFC of that respective gene.

Volcano Plot Plots the results of differential expression that plots the significance (adjusted *p*-values) against the logFC. Thresholds of each are often indicated by a change in colour of the points.

1.3.7.2 | Annotation and Enrichment Analyses

The differentially expressed gene matrix may be annotated using the AnnotationDbi (Carlson, 2015) R package which may access annotation libraries such as the human org.Hs.eg.db (M, 2019) which is based on Entrez gene identifiers (Maglott et al., 2005). The systems biology of the data is of particular interest to this project, which may be investigated with enrichment analyses. GO terms and KEGG (Kanehisa et al., 2017) pathway enrichment allow for comparisons between genes according to their functional role in a biological system. Further enrichment analyses may fork into three approaches, as described by (Khatri et al., 2012) and Alhamdoosh et al. (2017):

Over-Representation Analysis (ORA) The gene list emerging from differential expression is compared to a list of genes associated with a specific pathway. The genes which overlap between the input list and the pathway list are tested for over- or under-representation (usually based on hypergeometric, chi-square, or binomial distribution) (Khatri et al., 2012). While this information is useful, ORA is limited in that it only tests for the presence of a gene, and ignores any additional information (logFCs, *p*-values, the effect of its products on other genes, etc).

Gene Set Enrichment Analysis (GSEA) The limitations to the ORA approach led to the development of an alternative method, GSEA. The philosophy behind GSEA is that although large fold-changes in individual genes can have a significant biological effect, so can weaker changes in genes with a disproportionate effect on the pathway. Luo et al. (2009) describe the term *gene set* as a pre-defined group of functionally related genes, which may share a common biological pathway or ontology term. GSEA is generally performed in three steps: (i) generation of gene-level statistics (e.g. ANOVA) which may be transformed (e.g. absolute values), (ii) statistical results are combined into a single value per gene set (e.g. Wilcoxon rank sum (Barry et al., 2005)) and (iii) the statistical significance of the gene-set-level statistic are assessed. Although an improvement over the previous

method, GSEA treats gene sets separately and does not consider that a gene may be involved in multiple sets.

Pathway Topology (PT) Building upon the previous two technologies, PT approaches take into account interactions between gene products, and the nature of their interaction (e.g. activation or inhibition). KEGG and STRING (Szklarczyk et al., 2019) are examples of knowledge-bases which have sufficient information to perform PT. There are several potential approaches which are difficult to generalise but are reviewed extensively in Ihnatova et al. (2018) and Ma et al. (2019).

1.3.7.3 | GAGE and Pathview

GAGE (Luo et al., 2009) performs GSEA, where gene-set-level statistics are generated to check which gene sets are differentially expressed. GAGE performs pair-wise comparison between samples by default to test for significantly differentiated KEGG pathways.

Pathview (Luo and Brouwer, 2013), like GAGE, is a Bioconductor (Gentleman et al., 2004) R package which visualises GAGE results as an image of the chosen KEGG pathway highlighting the differentially expressed genes according to their logFCs. Its sister library SBGNview (Dong et al., 2022) offers a wider array of gene set knowledge-bases such as PANTHER (Mi et al., 2005), Reactome (Croft et al., 2010) and SMPDB (Frolkis et al., 2010). Pathview supports two methods for pathway visualisation: the native KEGG view and the third party Graphviz (Ellson et al., 2001). Luo and Brouwer (2013) states that Graphiz provides better control over the graphical nodes and edges, at the expense of certain pathway metadata, namely cell types and temporal information.

GO term analysis is supported by GAGE, although frequently neglected due to the popularity of *pathway* analysis as opposed to the more generalised *gene set* analysis (Luo et al., 2009). Since GO terms do not contain information on molecular interactions, pathways similar to those constructed by Pathview are not an option. The data may be represented by heatmaps or scatterplots as arguments in the `geneData` function.

1.4 | Evaluation Criteria

Every step in the pipeline is followed by some sort of QC and potentially filtering of bad data, from trimming adapter sequences to filtering multimapped reads to removing very lowly expressed genes. It is not unlikely that errors of any form slip through, and for these reason the final results will be evaluated.

The list of DEGs will be checked against a list of housekeeping genes which will act similar to negative controls. Housekeeping genes are required for basic cellular func-

tion and must be expressed in both normal and pathological cells. While they may be differentially expressed in some cases (Greer et al., 2010), they are generally uniformly expressed with low variance. Repositories such as the Housekeeping and Reference Transcript Atlas (HRT Atlas) (Hounkpe et al., 2021) were used as sources for the gene lists. The genes and pathways affected by cancer, even those specifically affected by AML are well studied, annotated and aggregated in repositories such as GeneCards (Stelzer et al., 2016) or OMIM (Hamosh et al., 2005). Thus the biological relevance of these genes in relation to these pathways will be checked.

In future work, if more funds are acquired, similar work could be conducted with more technical replicates to confirm the findings of this project with greater statistical power. To decrease the number of sequencing errors, Sanger Sequencing could potentially be used, given its 99.999% accuracy rate and long read lengths of up to ~1000bp (Shendure and Ji, 2008).

1.5 | Related Work

This multidisciplinary project is based on multiple rapidly developing fields, thus it is important for the researcher to keep updated with the latest literature. This section will describe the methods and findings of literature which this project will build upon, namely those which cover: (i) the biochemical components of extra virgin olive oil and their potential application to clinical practice, (ii) compounds with chemotherapeutic potential in AML and the resultant DEGs and (iii) tools to construct an effective RNA-seq pipeline given our specific data.

1.5.1 | Biochemistry of Olive Oil

The Mediterranean diet is rich in fruits, vegetables, fish, and olive oil, and is linked to lower rates of atherosclerosis, cancer and cardiovascular disease (Cicerale et al., 2012; Fabiani et al., 2002; Owen et al., 2000; Tripoli et al., 2005). This is attributed in part to the higher intake of extra virgin olive oil, and more specifically, its phenolic compounds. These have been the subject of extensive investigation as a result of their antimicrobial, antioxidant and anti-inflammatory properties (Bendini et al., 2007; Cicerale et al., 2012; Serreli and Deiana, 2018; Tripoli et al., 2005). Tripoli et al. (2005) attributes olive oil's slightly bitter and pungent taste to hydroxytyrosol and oleuropein, which both exhibited antioxidant activity.

Owen et al. (2000) split the biochemical profile of olive oils into three classes: (i) simple phenols (e.g. hydroxytyrosol, tyrosol), (ii) secoiridoids (e.g. oleuropein) and (iii)

the lignans (e.g. pinoresinol). All three classes have shown antioxidant properties.

Gatt et al. (2021a) was the first to characterise the phenolic profiles of Maltese extra virgin olive oils. Through liquid-liquid extraction and liquid chromatography mass-spectrometry, they found that the major constituents are tyrosol, hydroxytyrosol (Figure 1.9) and their derivatives. This is in accordance to olive oils from other sources ((Angerosa et al., 1995)), although the compound concentrations may vary. These two classes of simple phenols have been the main focus of clinical research related to olive oil extracts.

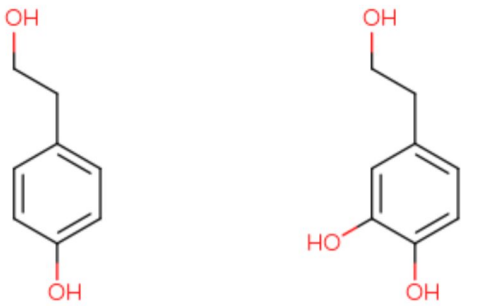

Figure 1.9: Chemical structures of tyrosol and hydroxytyrosol. The rest of the phenolic profile of extra virgin olive oils consists of derivatives of these two compounds.

There is abundant literature on the role of natural phenolic compounds in general in the prevention and treatment of cancer. This is achieved by regulating the cell cycle (Jafari et al., 2014) and the epigenome (Pan et al., 2015). These compounds may complement traditional chemotherapeutic treatments, potentially lessening the use of chemicals with severe side-effects on the patients. Gatt and Schembri Wismayer (2021) provide a comprehensive review on phenolic effects on leukaemia *in vitro*, *in vivo* and in clinical practice. Literature on the effects of specifically olive oil-derived phenolic compounds on cancer shall be covered in Section 1.5.2.

1.5.2 | Differentiation of HL60 cells by Phenolic Compounds Derived from Olive Oil

There is a great scientific initiative to identify compounds which induce apoptosis, necrosis or differentiation in cancerous cells, and to analyse the effects these compounds have on the biochemistry of the cell. Even the seemingly specific topic of the differentiation of HL60 cells by phenolic compounds derived from olive oil, yields myriad

peer-reviewed studies, earning it a distinct section in this literature review.

Differentiation therapy has changed the prognosis of AML, and has shown a surge in academic interest after the success of ATRA (see Section 1.1.3). In successful differentiation therapy, the undifferentiated myeloid cells will be induced to take an epigenetic path, culminating in apoptosis (Mark Welch et al., 2017; Santos-Beneit and Mollinedo, 2000).

The similarity in experimental design and goals makes Gatt et al. (2021b) a particularly important study to build upon. The study was successful in inducing differentiation and significant apoptosis in an HL60 cell line using tyrosol derived from Maltese extra virgin olive oil as a differentiation agent. The differentiated cells exhibited visual characteristics of neutrophils and monocytes, which were subjected to a bulk RNA-seq analysis. They defined a total of 199 DEGs. The *Myeloid differentiation* GO term (GO:0030099) genes were significantly upregulated (OSCAR, RELB, VEGFA, GAB2, JUNB, DNASE2, ICAM, CCL3), particularly monocyte genes. Transcription factors IRF1, IRF7, STAT2, RelB, NFKB2, ATF3, and BCL3 and chemokines CCL3 and CCL4 were all found to be upregulated. At a pathway-level, Neutrophil Degranulation (CEACAM6, CLEC5A, FPR1, SERPINA1, ELANE, AZU1, PRG2) and Cholesterol biosynthesis (LSS, SQLE, ACAT2, DHCR7 HMGCS1, FDFT1) were found to be downregulated.

Fabiani et al. (2008) found hydroxytyrosol treatment over a period of 25 hours to bring about an upregulation in cyclin-dependent protein kinase inhibitors CDKN1A and CDKN1B, which induced apoptosis. Cell proliferation inhibition was directly proportional to the increase in time and phenoli extract concentration.

In multidrug resistant HL60 cells, Crescimanno et al. (2009) induced the expression of the granulocytic CD11 or monocytic CD14 cell surface antigens using a crude phenolic extract.

1.5.3 | Determining the Optimal Tools

As a result of the decentralised and rapidly changing nature of the field of bioinformatics, there is a lack of standardisation. There is currently no one-size-fits-all tool or library, so the bioinformatician must evaluate the numerous trade-offs of the available tools, for every step of their sequencing pipeline, in accordance to their individual dataset and their desired result. To find the most appropriate tools for our data, an extensive literature search was conducted. The studies were split into three categories, each harbouring its own tabular summary which includes the tools used at each step:

1. Review papers, meta-analyses, studies which benchmark tools (Table 1.5)

2. RNA-seq experiments with somewhat similar conditions and goals (Table 1.6)
3. Ready-made, packaged pipelines (Table 1.7)

NOTE Studies which presented a new tool were excluded due to their inherent bias.

The first category will summarise the findings of studies which compare similar tools. The second and third sections aim to garner information on which tools are being used in practice and integrated into pipelines.

Table 1.5: Studies which compare RNA-seq tools or work-flows, including their conclusion summarised to one or two sentences.

Reference	Preprocessing	Alignment	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 betwen 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

Table 1.6: Studies which compare RNA-seq tools or work-flows, including their conclusion summarised to one or two sentences.

Reference	**Preprocessing**	**Mapping**	**Quantification**	**Normalisation**	**Differential expression**
Cardoso-Moreira et al. (2019)	FactoMineR	GSNAP	HTSeq	TMM	EdgeR
Mostafavi et al. (2014)	Ridge regression of log-transformed read counts	Tophat	HTSeq	/	LRT
Shiozawa et al. (2017)	/	RUM version 2.0.4	Genomon-fucion (fusion trancripts only)	TMM	edgeR, limma, ConsensusClusterPlus
Schubert et al. (2018)	FastQC, cutadapt	STAR v2.5.0c, GENCODE v19 for annotation	subread feature-Counts	Deseq	/
Schmiedel et al. (2018)	/	TopHat	HTSeq	Deseq	DESeq2
Lee et al. (2020)	Trimmomatic, Htseq	STAR	RSEM v.1.3.0	CCA, TPM	/
Wang et al. (2013)	/	/	/	/	IDEG6
Gatt et al. (2021b)	/	TopHat	SeqMonk v1.39.0	DEseq2	DEseq2

Table 1.7: Publications which introduce a packaged RNA-seq pipeline and their used tools.

Reference	**Preprocessing**	**Mapping**	**Quantification**	**Normalisation**	**Differential expression**
Cornwell et al. (2018)	RseQC	STAR	Cufflinks	DEseq	DEseq2
Ewels et al. (2020)	FastqQC, Trim Galore!, SortMeRNA, RSeQC, dupRadar, Qualimap, Preseq, DESeq2, MultiQC	STAR, HiSAT2	Salmon, RSEM	RSEM (TPM)	/
Salazar (2021)	Cutadapt, MultiQC	STAR	/	DEseq	DEseq2
Zhang and Jonassen (2020)	FastQC, Trim Galore, Qualimap2, MultiQC	HISAT2, Salmon	/	TMM, DEseq	edgeR, DESeq2
Kalari et al. (2014)	RSeQC	Tophat (Bowtie), MAP-RSeq	HTSeq, featureCounts, BEDTools Suite	MAP-Rseq (RPKM)	edgeR
Torres-García et al. (2014)	RNA-SeQC	*Custom*	/	RNA-SeQC (RPKM)	/

1.5.3.1 | Adapting DGE Analysis to a Lack of Replicates

The accuracy of any statistical analysis is dependent on the number of replicates. Technical replicates allow the isolation of the non-biological variation to evaluate the quality of the instruments and methodology used. Biological replicates originate from different biological sources and are meant to test the biological variance of the samples. Liu et al. (2014) find that in RNAseq biological variation is by far more important, and given a choice between the two, the researcher should invest in biological replicates. Bullard et al. (2010) confirm this claim, finding that technical variation in RNAseq experiments is minimal. Schurch et al. (2016) found that using three biological replicates gave 20% to 40% of the DEGs (varies according to the tool) compared to a full set of 42 replicates (representing the 'true' population). This rises to >85% when considering genes with a $\log_2$ fold change of >2. Schurch et al. (2016) state that ideally an RNAseq experiment for DGE should have a minimum of six replicates per condition for all experiments and 12 replicates for experiments where the identification of *all* the DEGs, even the lowly expressed ones, is important.

However, in practice, performing an experiment with large numbers of replicates is not always possible. Budget constraints and the still-high cost of sequencing are a common issue in RNAseq experiments. To make the most of such datasets, several DGE tools advertise their ability to work with just a single reading per experimental condition (Al Seesi et al., 2014; Anders and Huber, 2010; Feng et al., 2012; Gim et al., 2016; Wang et al., 2010). Notably, DESeq2 does not support datasets without replicates. There is an unfortunate lack of review papers and independent studies which benchmark these tools, except for brief comments in Schurch et al. (2016) who recommend edgeR (`exactTest`) or DESeq2 for experiments with <12 replicates per condition. For this reason this section will be reviewing the available tools adapted to performing DGE without replicates.

The first tool in this review will be **GFOLD** (Feng et al., 2012), developed specifically for datasets lacking in replicates. The developers acknowledge the dependence of p-values on variance estimation, which is impossible without replicates. A unique GFOLD value replaces the standard metrics of significance (p-values) and expression change ($\log_2$ fold changes). Feng et al. (2012) describe the value as a relative change of the expression level. GFOLD is unique in that it is the only tool in this comparison that is called through the Linux command line, instead of being an R library.

LPESeq (Gim et al., 2016) introduces the Local-Pooled-Error (LPE) method for few or single-replicate DGE analysis. This method attempts to estimate transcript-specific variance using the raw values of each transcript per condition. Hypothesis testing for

significant difference is then performed to identify the differentially expressed genes.

IsoDE (Al Seesi et al., 2014) is a non-parametric method (i.e. it assumes no statistical distribution) and is based on bootstrapping. The algorithm generates FPKM estimates from read counts of each condition which undergo pairwise comparison.

An MA-plot-based method, the R library **DEGseq** (Wang et al., 2010) (not to be confused with DESeq) accepts input .bed or .eland input files and outputs an XHTML page with p-values, gene expression values and expression differences in the form of Q-values.

The final tool in this review is the Bioconductor R library **edgeR** (Robinson et al., 2010). It may be more accurately described as a collection of methods, neither of which are specifically adapted to data without replicates, but the documentation provides recommendations to adapt the analysis to with the lack of replicates. EdgeR and its normalisation technique TMM have already been covered extensively in Section 1.3.6.1 and Section 1.3.5.1 respectively.

1.5.3.2 | Conclusion on Tool Choice

The following is the reasoning behind the final decisions made on the tool choices of each step of the pipeline, based on the literature reviewed. Extensive descriptions of the final tools implemented in the pipeline can be found throughout Section 1.3.

Tools which measure quality metrics without altering the data require little justification for their use, their mention occasionally being omitted from RNA-seq studies Table 1.6. **FastQC** is a staple of sequencing quality control, providing a detailed analysis of the contents of FASTQ files and drawing attention to signs of low quality reads. While FastQC results are detailed, they lack the detection of external nucleotide contaminants in the data. This information was supplemented with the results from **FastQScreen** which aligns the experimental sequences to common contaminant sequences (e.g. mouse, *Drosophila*, rRNA) and provides a graph marking any successful alignments.

To trim the data, we have chosen **Cutadapt**, which pipes its output back to FastQC through the wrapper script **Trim Galore!**. Additional filtering was performed by **Prinseq++** which may detect and remove regions of low-complexity. The functionality of these tools were found to complement each other. Filtering and trimming the data at this stage was relatively unimportant, as suggested by the findings of He et al. (2020) and because the raw data of this project was of good quality to begin with. Liao and Shi (2020) doubt the necessity of trimming at all.

Despite **STAR** needing a lot of RAM (Dobin et al., 2013) and being slower than more

light-weight aligners (Srivastava et al., 2020), these were not limiting factors due to our access to a High Performance Computer (HPC) and small number of samples. Srivastava et al. (2020) found that the choice between quasi-mappers (e.g. Salmon) and traditional aligners (e.g. STAR) is a trade-off between speed and accuracy. Similarly Zhang et al. (2017) found that pseudo-aligners Salmon and Kallisto require less runtime while maintaining similar accuracy to STAR. Schaarschmidt et al. (2020) find that the effect aligners have on the final list of DEGs is negligible, and that all tested aligners can be used equally for RNA-seq. Even if STAR provides a marginal increase in accuracy over quasi-aligners, this is preferred over the improvements in speed or computational resources provided by other aligners.

The most difficult and time-consuming choice to make was the tool to perform differential gene expression. The invested effort was justified by the findings of Williams et al. (2017), which state that the method of DGE exhibits the strongest impact on the results. Our data consists of three time points (1hr, 6hr, 12hr) and a control, each lacking replicates. Since we cannot accurately estimate the variance or dispersion with a single reading, many DGE tools simply fail to function. Although one may argue that low replicates is not a major issue due to the negligible technical variance of RNAseq (Bullard et al., 2010) and the minimal biological variance in our data which is derived from the same ATRA-resistant HL-60 cell line.

Due to the lack of review papers and benchmarks comparing the tools which claim to function well without replicates, they were manually tested and investigated (see Section 1.5.3.1). The lesser-known tools (GFOLD, LPESeq, DEGseq, IsoDE), while developed specifically to function without replicates, were found to be limited in functionality when compared to **edgeR**. Additionally, due to their rather unconventional approach to DGE, their output was found to be incompatible with other downstream libraries, limiting the potential for data exploration.

1.6 | Summary

This project goes into technical detail of AML, sequencing technologies, statistical analyses and the computational tools required to transform RNA-seq data into biologically meaningful results. In this section we provided adequate background to understand these aspects of the dissertation, assuming some basic prior knowledge of bioinformatics. The section *RNA-seq: in silico* covers the crux of this project, first describing each step of a generic RNA-seq pipeline, the potential approaches for that step, and a description of the specific tools used in this project.

In the *Related Work* section, we gave an overview of the work done to date related to the biochemistry of olive oil, in particular the phenolic candidates for differentiation therapy, and the effect these compounds had on the transcriptome of HL-60 cells. We concluded with a literature overview of available tools for RNA-seq analysis, how they compare with one another and which are the ones being used in practice. This was used to justify the tool choices made for our pipeline given our goals and set of data, achieving the first objective of this project. In particular, we discussed the best approach to tackle performing DGE analysis without replicate samples.

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