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A Computational Platform for Gene Expression Analysis

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For Jury Evaluation

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Abstract

The advent of next generation sequencing methods has revolutionized the field of molecular biology in the past few years. Nowadays, we are able to produce enormous amounts of biological information, both quickly and at low cost. As such, tools have to evolve accordingly, in order to cope with such large volumes of information. In this report we discuss the usage of computer tools capable of conducting gene expression profiling based on information obtained through RNA Sequencing techniques, applied to a specific set of biological problems. In particular, we present the idealization process and implementation details of a web platform capable of addressing these problems, as well as the actual platform prototype. The prototype's functionality is showcased with a real case study, produced in collaboration with biology researchers. This report also includes a literature review, covering both the biological and technical aspects of the work, with special emphasis in machine learning techniques applied to data mining tasks. Lastly, we review the work done and results obtained so far and outline the possible future of the web platform.

Resumo

O advento das técnicas de sequenciação de nova geração revolucionou o campo da biologia molecular nos últimos anos. Hoje em dia somos capazes de produzir enormes quantidades de informação biológica rapidamente e a baixo custo. Assim sendo, as ferramentas devem também evoluir, a fim de lidarem com estas extensas quantidades de informação. Neste relatório discutimos o uso de ferramentas informáticas capazes de analisar perfis de expressão génica com base em informação obtida através de técnicas de *RNA Sequencing*, aplicadas a um conjunto específico de problemas biológicos. Em particular, apresentamos o processo de idealização e os detalhes de implementação de uma plataforma *web* capaz de resolver estes problemas, assim como o protótipo funcional dessa plataforma. As funcionalidades deste protótipo são demonstradas através de um caso de estudo real, produzido em colaboração com investigadores da área da biologia. Este relatório inclui também uma revisão da literatura, cobrindo os aspetos biológicos e técnicos deste trabalho, com um ênfase especial em técnicas de aprendizagem máquina aplicadas a tarefas de *data mining*. Por fim, revemos todo o trabalho efetuado e os resultados obtidos até ao momento e delineamos as possibilidades futuras para a plataforma *web*.

Acknowledgements

First of all, I would like to direct my gratitude to my supervisor, Rui Camacho (FEUP). His concern about my work and the helpfulness he has always shown were key factors for the success of this project. I extend the same gratitude to my co-supervisor, Nuno Fonseca (EMBL-EBI), for his endless patience in answering my questions.

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A very special thank you to my all my friends at FEUP, that were with me in what is surely one of the best periods in my life. In particular I would like to acknowledge Pedro. Our constant competition, along with our shared antics, made working on this project that much more fun and fulfilling.

To my parents, Joaquim and Maria, I leave a truly heartfelt thank you; for all the nights they spent awake worrying about me; for all their guidance and wisdom; for being with me in every good and bad moment of my life. I would not be the person I am today without them.

Lastly, but definitely not least, I thank my girlfriend, Raquel. Of everything I have and owe to her, nothing is more important than the smile she puts on my face every day, as she gives me the strength to face the world.

Diogo Teixeira

“ Often people, especially computer engineers, focus on the machines. They think, "By doing this, the machine will run faster. By doing this, the machine will run more effectively. By doing this, the machine will something something something." They are focusing on machines. But in fact we need to focus on humans, on how humans care about doing programming or operating the application of the machines. We are the masters. They are the slaves. ”

Yukihiro Matsumoto

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Abbreviations

***k*-NN** *k*-Nearest-Neighbors.

API Application Programming Interface.

BLAST Basic Local Alignment Search Tool.

cDNA Complementary DNA.

CPU Central Processing Unit.

CSS Cascading Style Sheets.

DBMS DataBase Management System.

DNA DeoxyriboNucleic Acid.

GUI Graphical User Interface.

HTML HyperText Markup Language.

IBMC *Instituto de Biologia Molecular e Celular*.

ILP Inductive Logic Programming.

mRNA Messenger RNA.

NCBI National Center for Biotechnology Information.

NGS Next Generation Sequencing.

PAM Partition Around Medoids.

RBP RNA Binding Protein.

RNA Ribonucleic Acid.

rRNA Ribosomal RNA.

SAM Sequence Alignment/Map.

SQL Structured Query Language.

tRNA Transfer RNA.

WTSS Whole Transcriptome Shotgun Sequencing.

Chapter 1

Introduction

Molecular biology is a branch of biology that studies biological activities of living beings, at a molecular level. The grounds for this field of study were set in the early 1930s, although it only emerged in its modern form in the 1960s, with the discovery of the structure of DNA. Among the processes studied by this branch of biology is gene expression. Gene expression (further explained in Chapter 2) is the process by which DNA molecules are transformed into useful genetic products, typically proteins, which are essential for living organisms. This knowledge is not only important in fields like evolutionary or molecular biology, but has crucial applications in fields such as medicine. One example of such an application is the usage of gene expression analysis in the diagnosis and treatment of cancer patients [PASH03].

With the advent of NGS (*Next Generation Sequencing*) techniques, researchers have at their disposal huge amounts of sequencing data, that is not only cheaper and faster to produce, but also more commonly available. This data can then be used to obtain relevant information about organisms' gene expression. But, as the cost of sequencing genomes was reduced, the cost of processing such information was increased. NGS techniques tend to produce much smaller reads[1] than previously used techniques, presenting a more challenging problem, from a computational standpoint [Wol13].

1.1 Domain Problem

Despite its great advancements in the past decades, molecular biology is still a relatively new subject and, as such, there are still some unknowns and partial knowledge in this area. In respect to gene expression, some mechanisms of this intricate process are yet to be fully understood. One such mechanism is the one that regulates the transcription speed into RNA. One of the objectives of this thesis is to understand how the final sequences of a gene's exons are responsible for the speed at which the exons themselves are transcribed. The other objective is to understand how RNA-binding protein (RBP) manipulation can be used to better understand an organism's gene expression. These are, however, complex tasks that can be further decomposed in the three main problems that will be addressed in the thesis, namely:

---

[1] A *read* is a single fragment of a genome/transcriptome, obtained through sequencing techniques.

- Sequencing read alignment against a reference genome and differential expression analysis between samples of different individuals (of the same species). This is effectively one of the most complex problems addressed in the thesis. We will use data obtained through a sequencing method called RNA Sequencing[2]. Further insight about this method will be given in Chapter 2, with particular emphasis for tools used to align and analyze this data (Section 2.2.2).
- Gene enrichment and RBP analysis. This part of the work aims to collect as much relevant information as possible about the particular genes being studied at the time, to help biologists to better understand their function. RBP knowledge is particularly important for gene manipulation and a very useful tool for better understanding gene expression, as will be further described in Chapter 2.
- Further analysis of the produced data, using machine learning techniques for data mining, specifically for clustering analysis. These techniques will be employed in an effort to give biologists more relevant information about gene expression, uncovering possible relationships in the retrieved information. This topic will be developed in Section 2.3.

Solving these problems requires the use of computational tools. As such, the development of a computer system (or multiple systems) to tackle these problems emerges as a secondary objective of the thesis. The details of the design of this system will be presented in Chapter 3, while its concrete implementation will be discussed in Chapter 4.

1.2 Motivation and Objectives

Gene expression analysis is essential for modern day molecular biology. Among many of the possible applications of this information, we can highlight: better classification and diagnosis of diseases, assessing how cells react to a specific treatment, and others.

While nowadays powerful computational tools exist to target almost any biology problem, many of those tools require a very specific set of technical skills and have a steep learning curve. Possibly the most important motivation behind this thesis, and ultimately its main objective, is to provide researchers with powerful yet simple and user friendly tools. This means developing a system simple enough that any user can learn to operate it in a short period of time with minimal effort, but sufficiently advanced to suit the user's research needs.

Another typical problem that biology researchers face nowadays is information dispersion and the repetitive and lengthy task of compiling that information. Researchers frequently have to manually join information originating from a multitude of different platforms, which use inconsistent formats and notations. Our second objective is therefore to provide a system that is able to take this burden off the user, making the process faster and simpler.

---

[2] RNA Sequencing is also referred to as *Whole Transcriptome Shotgun Sequencing*, or WTSS.

1.3 Project

The project itself revolves around the development of a prototype computer system, capable of solving the aforementioned problems. Due to the complexity of the complete system, its development followed a modular organization (further described in Chapter 3). The envisioned system architecture is divided into three major components:

Differential expression analysis pipeline is responsible for aligning reads against a reference genome and compare contrasts between different samples. The pipeline is based on the preexisting iRAP pipeline[3]. The pipeline's capabilities are further enhanced with both job configuration automation and differential expression results consolidation (combining results from multiple differential expression tools).

RNA-binding protein analysis workflow aggregates information about RBPs from multiple biologic web databases (Ensembl, NCBI, UniProt, etc.) and organizes it in ways that are useful to biology researchers. Moreover, this information is clustered using data mining techniques, in order to reveal groups of genes and RBPs that may hold biologic relevance.

Web platform is responsible for storing and managing genetic data, coordinating interaction between the other components of the system and providing a web interface for user interaction. This component is based mainly on typical web technologies, that is, a document based database for data storage (MongoDB), a web framework for business logic implementation (Padrino) and web markup and styling languages for interface implementation (HTML, CSS).

1.4 Structure of the Report

Besides the introduction chapter, this document is composed by five additional chapters. Chapter 2 introduces some basic biology and RNA-Seq concepts, that are essential to understand the problems with which this document deals. Furthermore, we describe the main techniques used for genome/transcriptome sequencing and assembly, their differences, applications and the tools and data formats typically used in those areas. Lastly, we give some insight about data mining algorithms and how they will be applied in the context of the project. Chapter 3 presents the design of the software solution. The basic system architecture is outline in this chapter, as well as relevant design decisions. Chapter 4 establish relevant implementation details for the developed solution, giving a more in depth knowledge about its inner workings. Used technologies are also reviewed, and their selection is justified. In Chapter 5 we present the case study that was used to assess the quality of the produced solutions. We review the test data set, test conditions and obtained results. Lastly, Chapter 6 sums up the what has been accomplished during the project. We review objective fulfilment and present some possibilities for future work.

---

[3] `https://code.google.com/p/irap/`

Chapter 2

Problem Domain and Technological Base Concepts

In this chapter we begin by making a more in-depth presentation of the process of gene expression. This will be followed by a literature and state-of-the-art review in the fields of read alignment, differential expression analysis and data mining. We will present the tools used in the development of the analysis pipelines and the web platform. Lastly, we review some results evaluation techniques and relevant data representation formats for genetic information.

2.1 Biological Base Concepts

Before dwelling in the details of the state of the art that are on the foundation of the thesis, it is important to explain some concepts of the domain of molecular biology.

2.1.1 Gene Expression

As explained in Chapter 1, gene expression is the mechanism by which an organism's DNA can be expressed into functional genetic products, like proteins. This process starts with the genetic code, or nucleotide sequence, of each gene. Different genes in an organism's DNA are responsible for the creation of different genetic products. The process of gene expression itself is composed by two main stages, transcription and translation [GEN].

Transcription is the stage at which genetic data in the form of DNA is used to synthesize RNA, being this the process that concerns the thesis' main question. Several different types of RNA are produced by this process, including *mRNA* (which specifies the sequences of amino acids that form a protein), *rRNA* and *tRNA*, both later used in the translation stage. Simplifying a gene's structure, it can be seen as composed by two types of sequences, introns and exons, as seen in Figure 2.1.

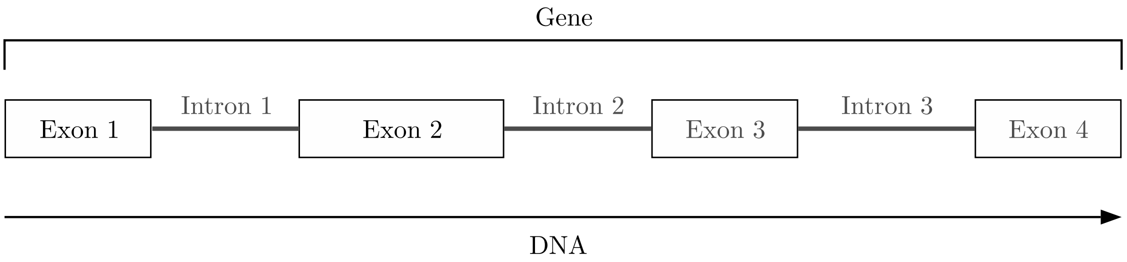
Gene
Exon 1
Intron 1
Exon 2
Intron 2
Exon 3
Intron 3
Exon 4
DNA

Figure 2.1: Overall structure of a gene, with its different areas (simplified).

The exons are useful in the gene expression process, being also known as coding regions. Introns, on the other hand, are not used in the process. They are present in an early stage mRNA molecule, the precursor mRNA, but are later removed (or spliced) in the final molecule before the translation stage [GEN]. Figure 2.2 illustrates the removal of introns from the mRNA molecule, during the splicing process.

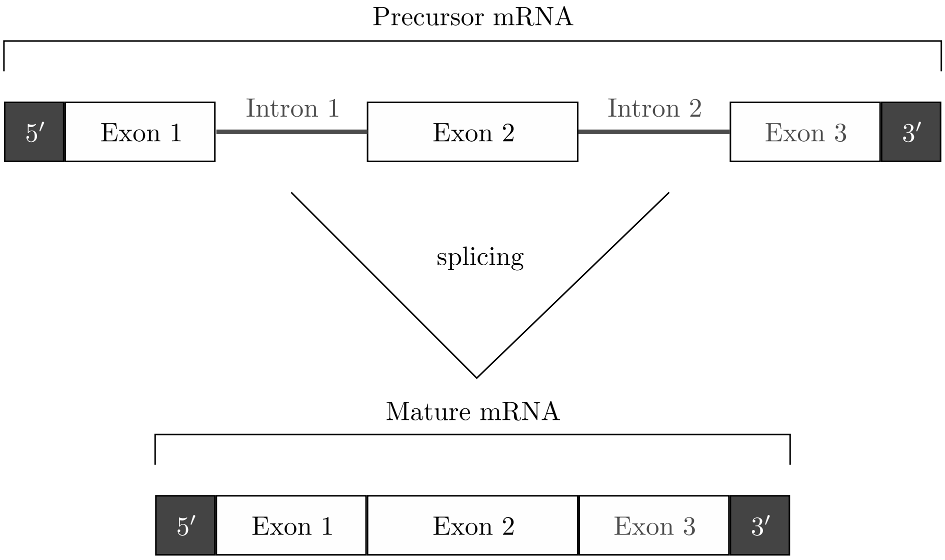
Precursor mRNA
5′
Exon 1
Intron 1
Exon 2
Intron 2
Exon 3
3′
splicing
Mature mRNA
5′
Exon 1
Exon 2
Exon 3
3′

Figure 2.2: The removal (splicing) of introns from the precursor mRNA, during the transcription process.

After the conclusion of the transcription process comes the translation process. In this process, the synthesized mRNA is used to specify the sequence of amino acids that constitute the particular protein being produced. The other types of RNA molecules (rRNA and tRNA) are also used in this stage of the gene expression process.

2.1.2 RNA-Binding Proteins

RNA-binding proteins, also referred to as RBP, regulate every aspect of the RNA metabolism, including pre-mRNA splicing, mRNA transport, location, stability and translation control

[CWD09, MMNMMN13, SH07, SH09], as shown in Figure 2.3.

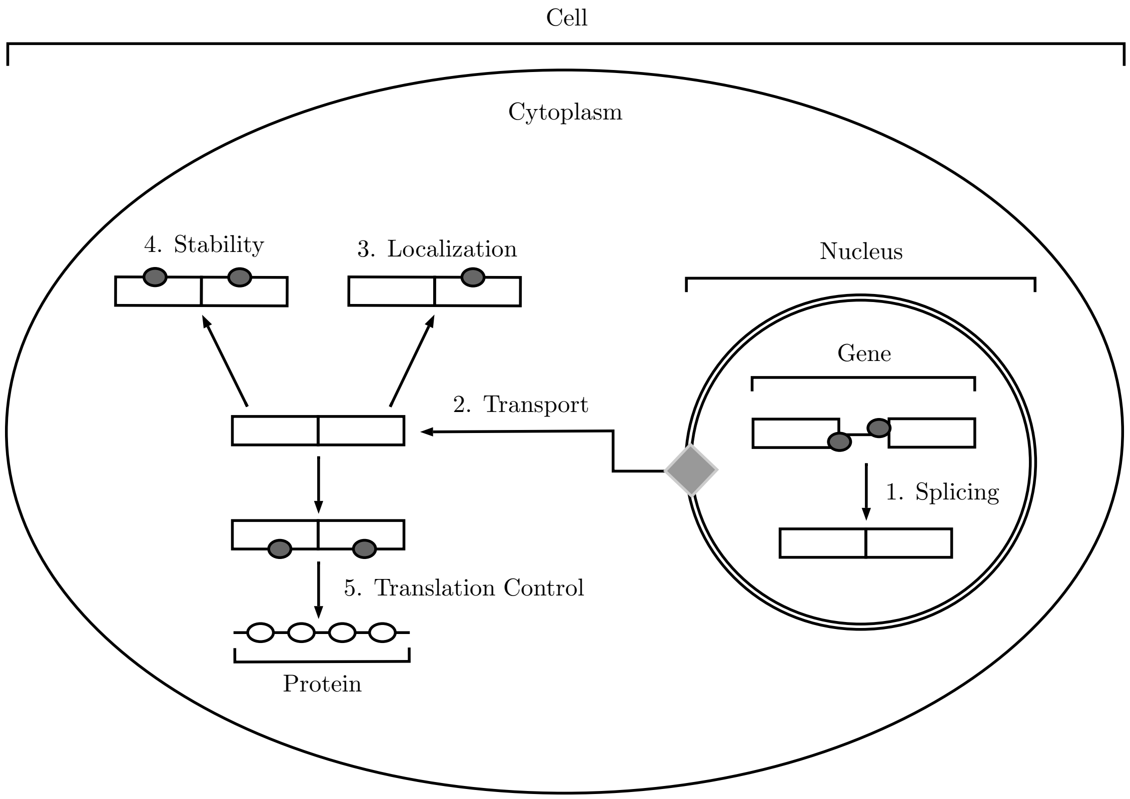

Figure 2.3: Diagram of a typical cell showing the multiple roles of RBPs in post transcriptional processes [JM11]. The grey ellipses represent RBPs. The numbered text represents the different processes in which RBPs take part. Multiple RBPs can bind with a single RNA at one or more locations, creating an abundance of different combinations and possibilities in every step of the RNA metabolism.

The binding of RBPs to RNA depends on different RNA-sequence specificities and affinities. This aspect, coupled with the existence of hundreds of RBPs in an organism, gives rise to a plethora of different combinations and outcomes to the RNA metabolism.

RBPs regulate gene expression in health and disease, and mutations affecting the function of RBPs may cause several diseases [CWD09]. Therefore, understanding the binding patterns of RBPs during a particular biological process is crucial to get insight into that process, both during health and disease conditions.

2.1.3 Sequencing

Obtaining genetic information is done experimentally, by employing a sequencing technique. For quite some time this process was carried out using the Sanger's and other similar sequencing methods [RF09]. Though effective, such methods were notably slow and costly, with large projects like the Human Genome Project (HGP) consuming roughly thirteen years and US$ 3 billion. These limitations were so severe that, other than the realm of human genetics, this kind of study was restricted to model organisms, such as the fruit fly and mouse genomes [Wol13]. The past few years have seen the appearance and rise in popularity of the NGS techniques. These techniques differ from the more classical ones by producing larger amounts of information, at lower cost. They are also typically more cost effective than previous techniques and can be easily employed by single laboratories, which has greatly contributed to their popularity.

The rise in popularity and availability of NGS techniques, coupled with the importance of RNA knowledge in understanding gene expression, led to the appearance of RNA-Seq. RNA-Seq makes use of these newly available deep-sequencing techniques to profile complete transcriptomes. This is, however, a difficult task to accomplish. NGS techniques produce shorter reads than their older counterparts, being that "*(...) transcriptome assembly from billions of RNA-Seq reads (...) poses a significant informatics challenge*" [MW11, p. 671].

Although this thesis does not deal with the problems of sequencing techniques, it is important to indicate that the read data sets that were used resulted from NGS techniques, in particular RNA-Seq. As such, suitable tools for this particular type of data were used.

2.1.4 Transcriptome Assembly

Transcriptome assembly is the process by which experimentally obtained RNA data reads can be organized and merged together in a partial or complete transcriptome. As stated above, the advent of next generation sequencing techniques, with their reduced costs, greatly increased the availability of transcript sequencing data.

For years, microarrays were the standard tool available for examining features of the transcriptome and global patterns of gene expression [Wol13]. However, microarrays are typically more oriented towards assembly against existing reference data, hence limiting its application to species with well known reference genomes. This is a severe constraint, as NGS techniques allow to cheaply obtain genetic information of previously non-studied species. This is one of the reasons that led to the inception of RNA-Seq. Contrary to microarrays, RNA-Seq techniques are able to wield results that are suitable for both reference guided assembly and *de novo* assembly approaches [WL09]. *De novo* or exploratory assembly has captured the interest of researchers in the past few years, leading to the appearance of multiple RNA-Seq tools that are capable of making this type of assembly without a reference genome [FEB+11]. Transcriptome assembly was not performed during this thesis, as its main focus in terms of the RNA-Seq process is read alignment and differential expression analysis.

2.1.5 Relevant Standard File Formats

As expected, the great diversity of RNA-Seq tools brings with it a wealth of file formats. Some of these formats are developed from the ground up to satisfy a specific need, while others are mere contextual adaptations or specializations of already established formats. Below we will present a few of the most popular and widely spread file formats, talking about their basic structure, the types of data they represent and their applications. Some examples of this file formats can be consulted in Appendix C.

FASTA

FASTA is the standard line and character sequence format used by NCBI [NCB], using this last organization's character code conventions (see Appendix C.4). It is a simple format, that can be used to easily store data represented by character sequences, like nucleotide (DNA, RNA) or amino acid (protein) sequences. This file format is widely use to store sequencing reads, DNA/RNA sequences and other character sequences in database systems. Its simplicity makes it extremely easy to manipulate and parse, presenting itself as an attractive solution for data transfer between different tools.

FASTQ

FASTQ is used to store character sequences, typically nucleotide sequences [CFG+10] (see Appendix C.3). It is quite similar to the standard FASTA format, in respect to the manner in which character sequences are represented. However, for every sequence, there is a second sequence of equal length, representing the quality scores of the original sequence. These quality scores are also represented as single characters, taking values between and including ASCII-33 to ASCII-126. It is typically used in the same situations as the FASTA format, when quality scores are available/relevant.

SAM and BAM

The SAM format is a text format for storing sequence alignment data [Lab] (see Appendix C.1). It is widely used to store mapping information between sequencing reads and a given reference genome. This sort of information is typically the product of sequencing alignment tools, that consume sequencing reads from FASTQ files and align them with a reference genome.

The BAM format contains exactly the same information as the SAM format and the same rules apply for both formats. The difference between both formats lies in their encoding. While SAM is a text based format, BAM is a binary format. This means that BAM sacrifices human readability for increased machine processing performance, as it is more efficient to work with compressed and indexed binary data.

VCF

VCF is a text file format used to store gene sequence variants [Smi13] (see Appendix C.2). In the past few years, as larger and larger genome sequencing projects became more common (like the 1000 Genomes Project[1]), storing such large amounts of information became a serious concern. To address these concerns the VCF format was created. Instead of storing the complete genome, VCF stores only the variations (and their respective positions) of newly sequenced genomes relatively to a known reference genome, typically in a compressed text file. As such, it is a format often used when building genome databases.

GFF and GTF

GFF is a text based file format to store gene features [San11] (see Appendix C.5). Many genome assembly tools execute this process in two separate steps: feature detection for identification of specific regions (exons, introns, etc.) and genome assembly, using those features as reference. However, it is beneficial to decouple these two steps, using different and more efficient tools for each. As such, the GFF format emerged as a protocol for feature information transfer between tools.

The GTF format is similar to the GFF format, in which it is based. It is also used in similar situations. However, GTF builds on top of GFF, defining additional conventions, specific to the domain of genetic information. Despite their initial relation, both formats continue to be developed individually.

2.2 RNA-Seq Analysis

2.2.1 RNA-Seq Pipeline

The analysis of RNA-Seq data is a complex process, with multiple stages. As such, in order to produce relevant results is usually used a pipeline. An analysis pipeline uses a set of tools, chained together in such a way that the output of one tool becomes the input to the succeeding tool.

A typical RNA-Seq analysis pipeline is composed by six essential stages [Fas12] (see Figure 2.4):

Read quality control and improvement is a pre-processing stage. It comprises the usage of quality control tools, whose function is to trim bad quality data, in order to improve the overall quality of the data set. Other than direct data manipulation, this stage might produce some statistical data about the reads, that can later be used to better drive the succeeding stages.

---

[1] The 1000 Genomes Project, started back in 2008, is an international effort to establish the most comprehensive catalogue to date of human genetic variations.

Sample contamination checking is also a pre-processing stage. As read data is obtained experimentally, it is not uncommon for contamination of the samples to occur. Bacterial contaminations, such as *E. coli*, are fairly common and can sometimes skew the analysis results. In some cases it is possible to detect these contaminations and remove the affected data, hopefully improving the final results.

Read alignment is the stage in which reads are positioned against a reference sequence. This sequence can be either a known and annotated reference genome (typically a combination of a FASTA file and a GTF file) or an assembled transcriptome, either assembled *de novo* or against a reference genome (see Section 2.1.4). This alignment will allow to assess gene abundance in later stages of the pipeline.

Quantification is the stage where transcript abundance is determined/estimated (gene expression). This involves counting the number of occurrences of certain transcripts in the read data. Typically this stage produces transcript count tables, that can later be used for differential expression analysis.

Differential expression is the stage where transcript abundances between different samples are compared. As such, the produced count data is used to predict differences between transcript abundances between two or more samples, effectively demonstrating differences in gene expression. A common task for differential expression analysis is the comparison between a control and a mutated sample.

Result reporting is the final stage in most pipelines. In this stage the resulting data is organized and represented in a manner that is useful to the user. This usually involves producing plots, tables and reports. Some pipelines may perform additional task before or after this stage, like gene set enrichment.

Note that this is only a generic architecture of a RNA-Seq analysis pipeline. In practice new stages can be added and other can be removed, to better suit the experiment at hand and the available data. In the given example (Figure 2.4) there is an additional stage, *gene model parsing*, that is only applied when the read are aligned against an annotated genome.

iRAP

iRAP[2] is a RNA-Seq analysis pipeline [FPMB14]. It implements a workflow similar to the one described above, albeit with some differences (see Figure 2.5). iRAP also allows some stages of the analysis to be skipped. Differential expression analysis is one such stage. This particular analysis will only be performed at user request. The gene set enrichment stage (Figure 2.5, in dashed line) is also optional. This stage uses Piano [VNN13], an R package capable of conducting gene set analysis using various statistical methods, from different gene level statistics

---

[2] `https://code.google.com/p/irap/`

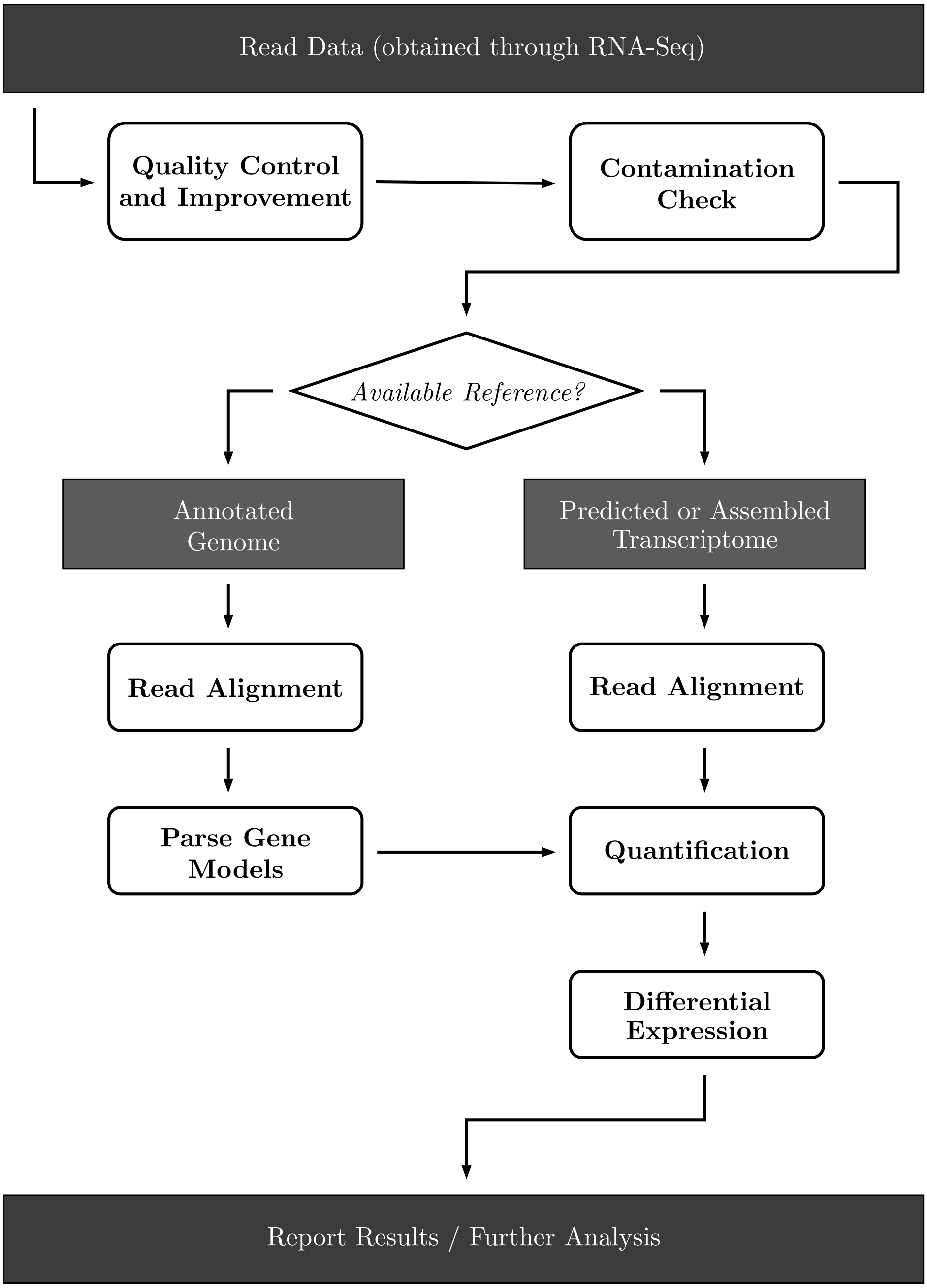
Read Data (obtained through RNA-Seq)
Quality Control and Improvement
Contamination Check
Available Reference?
Annotated Genome
Predicted or Assembled Transcriptome
Read Alignment
Read Alignment
Parse Gene Models
Quantification
Differential Expression
Report Results / Further Analysis

Figure 2.4: Representation of a standard RNA-Seq analysis pipeline [Fas12]. The analysis workflow has a slight variation depending on whether an annotated genome or an assembled transcriptome are used as reference for read alignment. Although this is a standard representation of the stages of RNA-Seq analysis, stages can be added or removed as needed to suit a particular assay.

and a wide range of gene-set collections. However, this stage will not be analysed in-depth, as gene set enrichment was not performed in this thesis.

One of the major strengths of iRAP is the ability to choose the tools that are used in each stage [FPMB14]. This allows for a vast array of pipeline customization possibilities, making it easy to adapt to a particular experiment. Below we will present a set of tools, integrated in iRAP, that were used during this thesis.

iRAP requires is a minimum set of user provided information to perform RNA-Seq analysis: the *annotated reference genome* and the *raw reads*, as previously mentioned. Note that the reads are organized in libraries. A library, in this case a cDNA library, is a collection of cDNA fragments that together constitute a portion of the transcriptome.

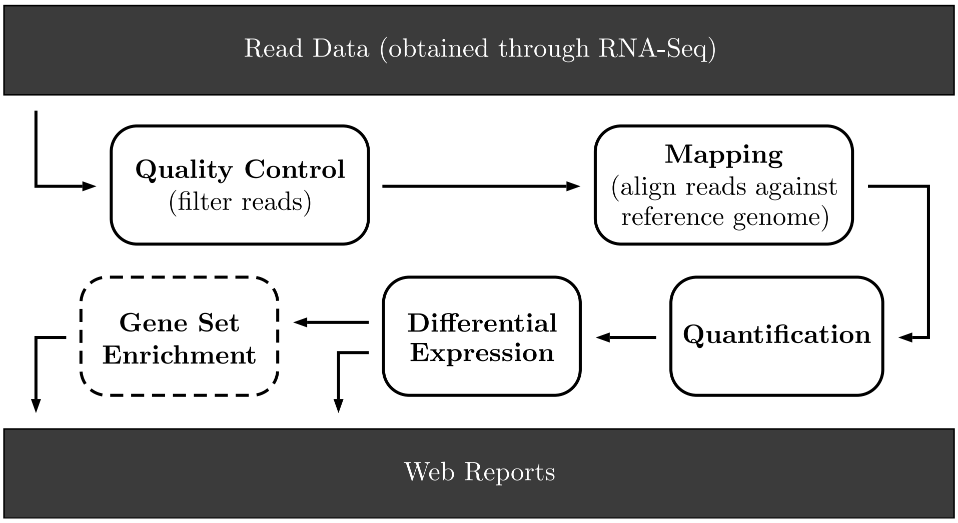
Read Data (obtained through RNA-Seq)
Quality Control
(filter reads)
Mapping
(align reads against reference genome)
Quantification
Differential Expression
Gene Set Enrichment
Web Reports

Figure 2.5: iRAP RNA-Seq data analysis pipeline. Note that the gene enrichment step (in dashed line) is optional and was not used.

2.2.2 RNA Sequencing, Read Alignment and Analysis Tools

We now present some bioinformatic tools, used to support the multiple steps of the RNA Sequencing, read alignment and data analysis process. It is important to note that none of these tools were used separately, but rather as parts of an analysis pipeline (also described below).

Tuxedo Suite

The Tuxedo suite is a free, open-source collection of applications that has been widely adopted as analysis toolset for fast alignment of short reads. It is composed by four separate tools (Bowtie, TopHat, Cufflinks and CummRbund) briefly reviewed below. These tools are extensively used for RNA Sequencing analysis. Although the applications are made for command line execution, there are several workflow managers, like Galaxy[3], that easily integrates with the suite, providing a web interface for its use. Note that not all components of the Tuxedo Suite were used.

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[3] http://galaxyproject.org/

Bowtie. Bowtie is an ultrafast, memory-efficient short read aligner [LTP+09]. Bowtie is typically used to build a reference index for the genome of the organism being studied, for posterior use by other tools, like TopHat. It can also output alignments in the standard SAM format, allowing Bowtie to interoperate with tools like SAM Tools. However, it should not be used as a general purpose alignment tool, as it was created and is more effective when aligning short read sequences against large reference genomes.

TopHat. TopHat is a fast splice junction mapper for RNA Sequencing reads [TPS09]. It uses Bowtie as the underlying alignment tool, using its results and a FASTA formated reference genome to identify splice junctions between exons.

Cufflinks. Cufflinks assembles transcripts, estimates their abundances, and tests for differential expression and regulation in RNA Sequencing samples [TWP+10]. It uses the SAM or BAM formatted files as input, typically the ones produced by TopHat, outputting GTF files as a result.

CummeRbund. Lastly, CummeRbund[4] is an R package (see Section 2.3.5) designed to help the visualization and analysis of Cufflinks' RNA Sequencing output. As such, it is not directly involved in the trascriptome alignment process. It takes the various output files from Cufflinks and uses them to build a SQLite database describing appropriate relationships between genes, transcripts, etc. This database is later used to convert that data to R objects which allows them to be used by plotting functions, as well as by other commonly used data visualization tools.

HTSeq

HTSeq is a programming framework used for processing data resulting from next generation sequencing methods [APH14], developed in Python. While many tools can efficiently align reads, sometimes data needs to be manipulated before being passed to those tools. This data can either be badly formated (or "dirty"), or simply in a format different from the one that is needed. The latter is a particularly common problem when trying to pass the results of one tool to the one that succeeds it in the pipeline. HTSeq is useful to easily create scripts that accomplish this task, acting as a "glue" between tools.

HTSeq provides parsers for many popular formats for representing genetic information (see Section 2.1.5). In addition, it ships with two standalone scripts, HTSeq-QA and HTSeq-Count. HTSeq-QA is used to provide an initial assessment of the quality of sequencing runs, producing plots with that information. HTSeq-Count takes a SAM/BAM file and GTF/GFF file containing gene models. It then counts, for each gene, how many aligned reads overlap that gene's exons.

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[4] `http://compbio.mit.edu/cummeRbund/`

2.2.3 Differential Expression Analysis Tools

Below we describe the tools that were used for differential expression analysis. These tools are integrated in the iRAP pipeline, are used in its fourth stage.

DESeq

DESeq [AH10] is available in an R package (see Section 2.3.5), included in the Bioconductor super package. DESeq takes count data generated from RNA-Seq analysis assays. As count data is discrete and skewed, it is not well approximated by a normal distribution. DESeq solves this problem by applying a test based on the negative binomial distribution, which can reflect these properties. This method has a much higher power to detect differential expression.

edgeR

edgeR [RMS10] is available in an R package (see Section 2.3.5), included in the Bioconductor super package. It provides methods for the statistical analysis of count data from comparative experiments on next generation sequencing platforms, among which is RNA-Seq, the most common source of data used with edgeR. It has many characteristics in common with the previously mentioned DESeq, as it also uses negative binomial models (among others) to distinguish biological from technical variation. Later we describe how both tools can be used together to produce better results.

2.2.4 File Manipulation and Pre-processing Tools

Sometimes data is badly formated or otherwise in a format that is not compatible with a specific tool. This is particularly frequent when passing data between two different tools in a pipeline. As such, we need some intermediate tools that are able to easily manipulate and transform data, making it useful again. Below we present some tools that can be used to accomplish this task.

SAM Tools

SAM Tools[5] is a library package designed for parsing and manipulating alignment files in the SAM/BAM format [LHW+09] (see Section 2.1.5). SAM Tools has two separate implementations, one in C and the other in Java, with slightly different functionality. Beyond manipulation of SAM and BAM files, this package is able to convert between other read alignment formats, sort and merge alignments and show them in a text-based viewer.

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[5] `http://samtools.sourceforge.net/`

FASTX

FASTX[6] (FASTX-Toolkit) is a collection of command line tools for pre-processing short read files. These short read files can be either in FASTA or FASTQ format. FASTX is used to manipulate these files before the aligning stage, in order to produce better results. It includes tools to convert files from FASTQ to FASTA format, assess statistics about the reads, filter and remove sequences based on their quality, among others. Although the toolkit contains only command line based tools, some of them are already integrated in the Galaxy web based workflow manager.

FastQC

FastQC[7] is a tool for quality control for NGS data, implemented in Java. Its main objective is to find errors and problematic areas in NGS read data. FastQC accepts FASTQ, SAM and BAM files, and is able to report results both inside the tool itself and by exporting HTML files. These reports contain, among other information, summary graphs and table that allow quick access to the data. FastQC can either be used as a standalone tool with its graphical interface, or as part of an analysis pipeline.

2.3 Data Mining

Data mining is the process of "*extracting or "mining" knowledge from large amounts of data*" [HKP06, p. 5]. As such, it consists of a set of techniques that can be used to find interesting patterns in large data sets, that translate in newfound knowledge. Data mining borrows techniques from multiple fields, such as artificial intelligence, machine learning, statistics, and database systems [CEF+12]. Its ultimate goal is to combine all those techniques and transform large and (apparently) meaningless sets of data into understandable and useful information. Thus, data mining was motivated by the perspective of harnessing the abundance of data, that characterizes today's information systems, to produce meaningful knowledge.

Because of their large quantities of input data, data mining tasks are usually totally, or at least partially, automated. As such, there are several algorithms for these tasks and tools that implement such algorithms, as presented in Section 2.3.2 and Section 2.3.5, respectively.

We can divide data mining into main types: *descriptive data mining* and *predictive data mining* [FPSS96]. Descriptive data mining is focused on finding the underlying structure of a given set of data. Instead of predicting future values, it concerns the intrinsic structure, relations and interconnectedness of the data being analyzed, presenting its interesting characteristics without having any predefined target. On the other hand, predictive data mining is used to predict explicit values, based on patterns determined from the data set. With predictive data mining we try to build models using known data and use those models as a base to predict future behavior.

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[6] http://hannonlab.cshl.edu/fastx_toolkit/

[7] http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

As we can see, data mining does not represent a single type problem. In fact there are several different types of problems that can be addressed by data mining techniques. Each of these problems may require a different data mining method. A brief review of the most common type of problems is given below.

Classification is a type of problem that tries to generalize the already known structure of a data set, so that it applies to new data sets. In other words, with classification we try to learn a function that is capable of mapping our data into predefined classes.

Regression tries to learn a function that models relationships between variables in the data set. That function can latter be used to find real value predictions of future behavior of data sets originated from the same population.

Clustering consists in identifying a finite set of categories or clusters of similar values, to describe the data set. As such, it is used without prior knowledge about the data structure.

Summarization provides a more compact representation of a subset of data, in a way that the summarized data retains the central points of the original data. This can be accomplished in several different ways, like using report generation or multivariate visualization techniques.

Dependency modeling finds a model which describes relationships between variables, revealing their dependencies.

Change and deviation detection tries to discover the most significant changes in the data, when compared with previously measured data. This method is useful to find interesting data variations or data errors.

Note that due to the nature of the work of this thesis we will focus on clustering analysis techniques and, as such, the following sections will contain a more in-depth review of these methods, along with descriptions of the used algorithms and tools.

2.3.1 Clustering Techniques

As explained above, clustering is the process of grouping data into *clusters*, in such a way that objects inside a cluster are very similar to each other, while being as different as possible from objects in other clusters [HKP06]. These similarities (and dissimilarities) are assessed based on the attributes of each object using a comparison method, often a distance function. Clustering is used in situations where the classes contained in the data set are unknown, either because they are difficult to determine or because such an assessment would be too costly or we known little about the domain and want to do a prospective study.

However, data clustering as a process is highly dependent on the data being analysed. For example, while some data sets can be easily clustered using "spherical" clusters, other can only be represented by "concave" clusters. In other words, there is no global technique to group similar objects, it needs to adapt to the problem at hand. This multitude of different interpretations of the cluster notion led to the appearance of many different clustering methods and algorithms. There are five major categories in which we can classify clustering methods: *partitioning methods*; *hierarchical methods*; *density-based methods*; *grid-based methods*; and *model-based methods* [HKP06].

Partitioning Methods

Partitioning methods are based around the construction of partitions of the whole data set. Each one of the constructed partitions represents a data cluster. Given a dataset with n elements, and a number k of clusters, the correct application of these methods must verify two conditions [HKP06]:

(1) each cluster must contain at least one object ($k \leq n$);
(2) a single object of the data set must only belong to one cluster.

Given a number k of clusters (typically chosen *a priori*), an iterative relocation is used in order to successively reclassify objects and hopefully improving their clustering [Mad12]. These methods try to maximize cohesion between objects in the same partition, while assuring that clusters are as distant as possible between themselves. In other words, the elements within a partition should be as similar or "close" as possible between them, while being as different as possible from elements in other groups.

Achieving the optimal partitioning would require the exhaustive enumeration and combination of all possible clusters. This is, of course, impractical and even unfeasible in some situations. As such, most partitioning methods adopt some sort of heuristic evaluation of their clusters' quality. For example, the *k-means* algorithm uses the mean value of the objects in a cluster to represent the same cluster; the *k-medoids* algorithm, which is centroid based, uses an object that is roughly at the center of the cluster to represent it. Typically these methods work well with "spherical" clusters, but may falter if clusters have more complex shapes ("concave" shaped clusters, for example).

Hierarchical Methods

These methods create an hierarchical division among objects in the data set. This hierarchy is represented as a tree structure. There are two strategies for hierarchical analysis, the *divisive* strategy and the *agglomerative* strategy [HKP06]. The *divisive* strategy consists of putting all objects in the same cluster and then divide them in multiple clusters, based on their distance. This process is repeated iteratively, until every object is in its own cluster. Inversely, the *agglomerative* strategy starts by putting each object in its own clustering, and then iteratively merges clusters until all objects are part of one cluster.

One notable weak point of hierarchical methods is that calculated results are irreversible; that is, once an objects is attributed to a cluster, that result will not be evaluated again. This may lead to incorrect labeling of some objects. These problems can be minimized by pre-processing the data set. Note that this single pass evaluation gives hierarchical methods good computational performance.

Density-based Methods

Unlike the previous methods that rely on the notion of *distance* between objects, density-based methods are based on the notion of *cluster density* [HKP06]. The basic idea of these methods is to keep growing a given cluster while the number of objects in its vicinity (or *density*) exceeds a certain user defined threshold. Density-based methods are particularly useful to discover clusters with irregular shapes. It should be noted that algorithms implementing these kind of analysis, like DBSCAN [EpKSX96], are usually very computationally heavy, both in terms of processing power and memory usage (although optimizations exist).

Grid-based Methods

Grid-based methods transform the data set object space into a finite number of cells, forming a grid structure [HKP06, Mad12]. All clustering operations are then conducted using the grid representation of the data set. Algorithms that implement this strategy are usually high-performance, as execution times are dependent on the number of cells in each dimension of the grid, rather than on the number of objects in the data set.

Model-based Methods

Model-based methods work by hypothesizing a mathematical model for each cluster and then find the objects that best fit those models [Mad12]. These methods typically follow the assumption that objects are distributed by clusters according to an underlying statistical probability. This also makes it possible for the number of clusters to be automatically determined, based on such statistics [HKP06].

2.3.2 Clustering Algorithms

Below we present the clustering algorithms that were used during the progress of this thesis. These methods were chosen based on their suitability for the tasks at hand, as will be described in Chapter 4.

k-Medoids

The k-medoids specification appeared as an evolution of the k-means algorithm. As k-means uses the mean value of the objects of a cluster to determine its center, it is sensitive to outliers (objects that deviate considerably from the data set average): an object with a large, disproportional value might skew the results. k-medoids mitigates this problem by using medoids, picking a specific object of the cluster as its "center" [HKP06]. Medoids are chosen randomly at the beginning. This means that the algorithm may produce different results depending on the starting objects (it does not reach an optimal solution). The remaining objects are then clustered with the medoid to which they are most similar.

The most common implementation of k-medoids is the Partitioning Around Medoids (PAM) algorithm (see Algorithm 2.1).

```
Input: $k$, the number of clusters; $D$, a data set containing $n$ objects.
Result: A set of $k$ clusters.
repeat
    assign each remaining object to the cluster with the nearest representative object;
    randomly select a non-representative object, $o_{random}$;
    compute the total cost, $S$, of swapping representative object, $o_j$, with $o_{random}$;
    if $S < 0$ then swap $o_j$ with $o_{random}$ to form the new set of $k$ representative objects;
until no change;
```

Algorithm 2.1: Partitioning Around Medoids (PAM) algorithm, a k-medoids implementation for partitioning based on medoid or central objects.

Average Linkage Hierarchical Clustering

Average linkage is a method for calculating distance between clusters in the standard hierarchical clustering analysis. In order to decide which clusters should be combined or divided, in agglomerative and divisive clustering respectively, we need a measure of dissimilarity between those clusters. In the average linkage method this dissimilarity is computed based on the average distance between all elements in both clusters. The average is calculated over all pairs of objects composed by one object from the first cluster and one object from the second. The linkage function is therefore defined as

$$D(X,Y) = \frac{1}{N_X \times N_Y} \sum_{i=1}^{N_X} \sum_{j=1}^{N_Y} d(x_i, y_i), \quad x_i \in X, y_j \in Y \tag{2.1}$$

where X and Y are two clusters; N_X and N_Y and the number of elements in clusters X and Y, respectively; and $d(x_i, y_j)$ is the distance between objects $x \in X$ and $y \in Y$.

Inductive Logic Programming

ILP is a subfield of machine learning that uses first order logic to represent both data and models [LD98, Mug91]. ILP infers hypotheses (models) from examples and background knowledge. The examples may be of two types: instances of the concept to be “learned” (positive examples), and non-instances of the concept (negative examples). Background knowledge is a set of predicates encoding all information that the experts find useful to construct the models. ILP might be used to tackle several machine learning and data mining problems, like classification, regression, clustering and association rules discovery.

The first and most important motivation for ILP systems is that they overcome the representation limitations of attribute-value learning systems, such as the previously mentioned data mining algorithms. Attribute-value systems base their representations of data in table based representations. Although effective in many situations, this representation is not very expressive and might not even be feasible for certain problems [BM95]. The second motivation for ILP is that by using a logical representation, the hypotheses are understandable and interpretable by humans, being therefore useful to explain the phenomenons that produce the data. This representation also means that background knowledge can be represented and employed in the induction process, in contrast to attribute-value models, where this information is difficult to represent. The expressive power of first-order logic enable an easy representation of data with complex structure, and the encoding of complex models that may combine numerical computation and relational information.

Despite these advantages, ILP cannot be applied indiscriminately to any clustering or classification problems. ILP systems are typically very heavy when it comes to computational resource consumption and run for long periods of time [FCSC03]. To ameliorate this situation there has been significant progress in parallel execution of ILP systems.

2.3.3 Clustering Evaluation and Assessment

The goal of most clustering methods is to achieve high similarity between objects in the same cluster, while maintaining the dissimilarity between other clusters [MRS08]. This is called an *internal evaluation criterion*, as it is only dependent on the clustered data itself. However, high internal evaluation scores do not necessarily translate to good effectiveness in a real application.

To provide a better judgement of clustering results we may use *external criteria* [MRS08]. Such criteria act as a surrogate for the judgement of a human field expert. As such, they must use a set of data outside the clustering data set, that was pre-classified by an expert. This set of data is considered a *golden standard*, that is, the best possible outcome for the clustering analysis, under reasonable conditions.

Note that in the specific case of this thesis no pre-classified data set was available. As such, we focused our assessments purely on internal evaluation methods. Despite this lack of automated external evaluation, our results were evaluated by experts in this field, as explained in Chapter 5.

Silhouette Coefficient

The silhouette coefficient is a direct representation of the *intra-cluster similarity and inter-cluster dissimilarity* concept. In other words, the silhouette coefficient improves as the cohesion between elements in the same group and the farthest the distance from that particular group to all the other groups [Rou87]. The silhouette coefficient is computed using the formula

$$s(i) = \begin{cases} 1 - \frac{a(i)}{b(i)} & \text{if } a(i) < b(i), \\ 0 & \text{if } a(i) = b(i), \\ \frac{b(i)}{a(i)} - 1 & \text{if } a(i) > b(i), \end{cases} \tag{2.2}$$

where $a(i)$ is the average dissimilarity between i and all other objects in the same cluster; and $b(i)$ is the lowest average dissimilarity between i and any other cluster to which i does not belong. The higher the value of $s(i)$, the more appropriately clustered the data point is. Inversely, low silhouette values are the result of unfitting clustering. The average $s(i)$ of a single cluster can be used to measure how tightly all its data points are grouped. The average $s(i)$ over the entire data set can be used to measure how appropriately the data has been clustered.

The silhouette coefficient can also be used to provide a visual representation of the clustering results, as dendograms do for hierarchical clustering analysis (Figure 2.6). This plot combines silhouettes widths for all objects in the data set, the average silhouette width for each cluster, and the silhouette of the complete data set.

Other than that, the average silhouette of the clustering results may be used to determine the best number of clusters. This is done by repeatedly executing the analysis with different k values, between a specified range. The chosen k value is the one that produces the best average silhouette.

2.3.4 Common Distance Measures

Many clustering algorithms rely on the notion of *distance* between objects in the data set. These distances are used as a measure of similarity/dissimilarity between those objects. Typically, objects that are close to each other are considered similar, as opposed to objects that are far away from each other and therefore considered different. Below we will present some common types of distance measures that are used in clustering methods.

Euclidean Distance

Euclidean distance is one the most common distance measures. It represents the geometric distance between two points, in a n-dimensional space [Mad12]. Euclidean distance can be

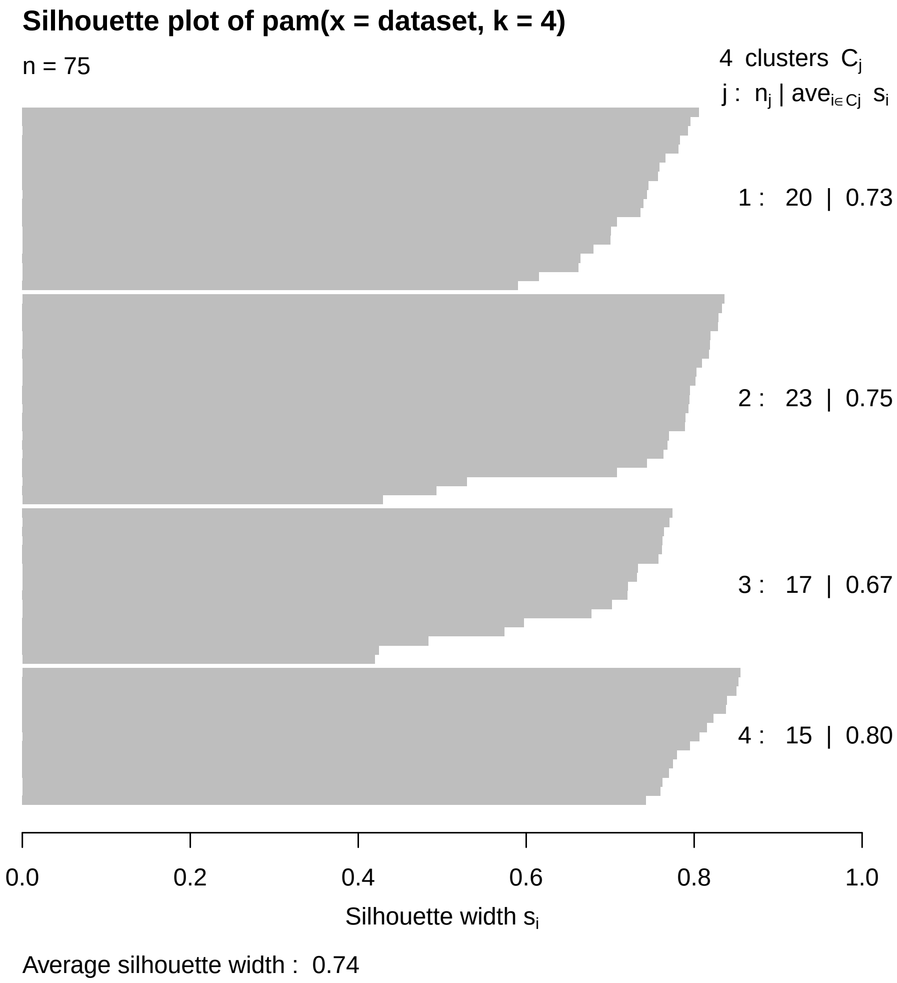

Figure 2.6: Example of a silhouette plot, using the PAM algorithm. It represents the silhouettes of every objects in the data set, as well as the average silhouette for each cluster ($k = 4$) and the average silhouette for the complete data set.

defined as shown in Equation 2.3 (for n-dimensions).

$$d(x,y) = \sqrt{\sum_{i=1}^{n} |x_i - y_i|^2} \tag{2.3}$$

Squared Euclidean Distance. The squared Euclidean distance is a simple variation of the stadard distance, obtained by squaring it (Equation 2.4). It is used when there is a need to attribute progressively greater weight to objects that are far apart from each other.

$$d(x,y) = \left(\sqrt{\sum_{i=1}^{n} |x_i - y_i|^2}\right)^2 = \sum_{i=1}^{n} |x_i - y_i|^2 \tag{2.4}$$

Manhattan Distance

The Manhattan distance between two objects is the sum of the differences between each of their components. In other words, it is equivalent to the distance between the two objects, if a n-dimensional grid-like path was followed [Mad12]. It is defined as shown in Equation 2.5.

$$d(x,y)=\sum_{i=1}^{n}\left|x_i-y_i\right| \tag{2.5}$$

Chebychev Distance

The Chebychev distance is less common than the previously mentioned distances. The Chebychev distance between two objects is defined as the maximum distance between components of the objects (Equation 2.6). Is is useful in situations where two object must necessarily be considered different if one of their components is different, as the use of the maximum value removes the dampening factors of other (closer) components.

$$d(x,y)=max\left\{\left|x_i-y_i\right|\right\} \tag{2.6}$$

Jaccard Distance

The Jaccard distance is a measure of dissimilarity between two objects. It is related to the Jaccard coefficient, a similarity measure. These measures are applicable to binary and non-binary data. In these cases, a simple geometric distance, like the Euclidean distance, might not accurately represent the distance similarity (or dissimilarity) between the two objects.

For binary attributes, the Jaccard coefficient (similarity) is computed as

$$sim(x,y)=\frac{q}{q+r+s} \tag{2.7}$$

where q is the number of attributes that are true for both objects; r is the number of attributes that are true for object x and false for object y; and s is the number of attributes that are false for object x and true for object y. Note that by definition the similarity coefficient is a value between 0 and 1, where 0 indicates that the objects are completely different, while 1 indicates that the objects are exactly equal. A distance measure can be obtained directly from this coefficient, as shown in Equation 2.8.

$$dist(x,y)=1-sim(x,y) \tag{2.8}$$

In this representation a value of 0 is given to two objects that are close, while the value 1 is attributed to distant objects.

Similarly, the Jaccard coefficient and distance can be computed for sets. This can be useful to determine distance between to objects whose attributes contain nominal values. The Jaccard coefficient for sets is calculated as shown in Equation 2.9, while the distance between sets is calculated in the same way than between binary attributes.

$$sim(x,y) = \frac{|x \cap y|}{|x \cup y|} \tag{2.9}$$

2.3.5 Clustering Tools

Except in rare cases of very specific problems, it typically makes no sense for someone to implement any data mining algorithm that they might need. In fact, today we have lots of data mining tools (many of which are free), that already implement many of those algorithms. These tools are usually customizable, making it easy to adapt them to most problems. Below we'll briefly review some of the most popular data mining tools, applicable to the specific needs of this thesis. Note that some of these tools, namely RapidMiner and Weka, were only used for testing purposes during the development of the project. As such, they are not part of the final prototype.

RapidMiner

RapidMiner [rap12] is a complete solution for data mining problems. it is available as a standalone GUI based application, as seen in Figure 2.7. It is a commercial application, although its core and earlier versions are distributed under an open source license and it offers a free version, beyond its multiple paid versions. Being one of the most popular data mining tools used today, its applications span several domains, including education, training, industrial and personal applications, among others. Its functionality can also be easily extended through the use of plugins[8], reflecting an increased value for this tool. One of such example in the area of bioinformatics is the integration plugin between RapidMiner and the Taverna[9] open source workflow management system [JEF11].

Weka

Weka [HNF+09] is an open source tool that implements several machine learning algorithms and allows its user to easily apply those algorithms to data mining tasks. Created at the University of Waikato, New Zeland in 1997[10], it is still in active development to date. Weka supports several common data mining tasks, like data preprocessing, classification, clustering, regression and data visualization. Its core libraries are written in Java and allow for an easy integration of its data mining algorithms in pre existing code and applications. Other than that, Weka can be used directly through a command line/terminal or through one of its

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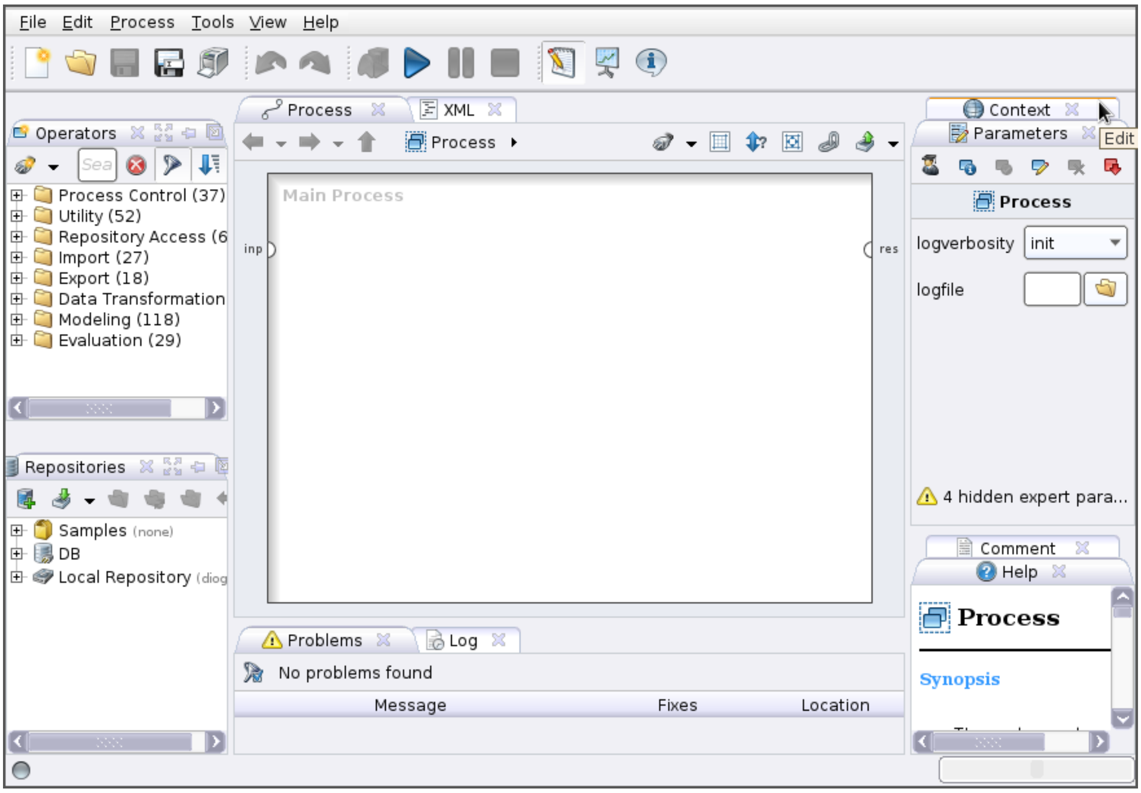
[8] Plugin is a software module that adds new functionality to an existing software application. Plugins are typically dependent on the platform they extend and can't be used as standalone tools.

[9] `http://www.taverna.org.uk/`

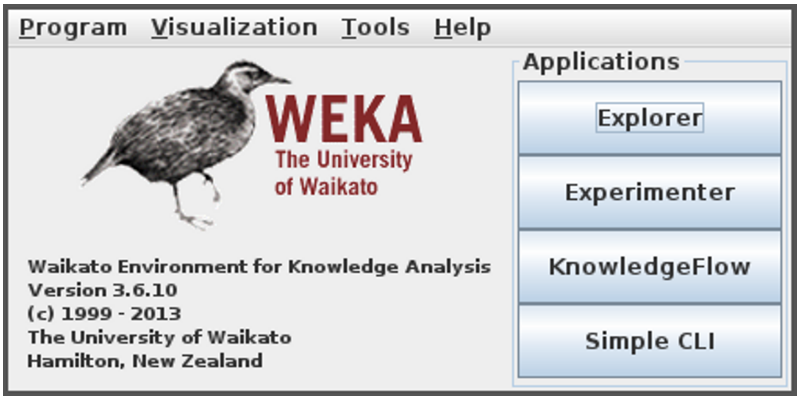
[10] The current version was completely rewritten in 1997, despite the first iteration of the tool being developed as early as 1993.


File Edit Process Tools View Help
Operators
Process Control (37)
Utility (52)
Repository Access (6
Import (27)
Export (18)
Data Transformation
Modeling (118)
Evaluation (29)
Repositories
Samples (none)
DB
Local Repository (diog
Process
XML
Main Process
inp
res
Problems
Log
Message
Fixes
Location
Context
Parameters
Edit
Process
logverbosity
init
logfile
4 hidden expert para...
Comment
Help
Process
Synopsis

No problems found

Figure 2.7: RapidMiner user interface.

multiple GUIs (Figure 2.8). Its simple API and well structure architecture allow it to be easily extended by users, should they need new functionalities.


Program Visualization Tools Help
WEKA
The University of Waikato
Applications
Explorer
Experimenter
KnowledgeFlow
Simple CLI

Waikato Environment for Knowledge Analysis
Version 3.6.10
(c) 1999 - 2013
The University of Waikato
Hamilton, New Zealand

Figure 2.8: Weka interface selection.

R Language

R [Iha98] is a free programming language and software environment for statistical computing and graphics generation. Originally developed by Ross Ihaka and Robert Gentleman at the University of Auckland, New Zealand in 1993, it is still under active development. R is typically used by statisticians and data miners, either for direct data analysis or for developing new statistical software [FA05].

R is an implementation of the S programming language[11], borrowing some characteristics from the Scheme programming language. Its core is written in a combination of C, Fortran and R itself. It is possible to directly manipulate R objects in languages like C, C++, Java and Prolog. R can be used directly through the command line or through several third party graphical user interfaces like Deducer[12]. There are also R wrappers for several scripting languages.

R provides several different statistical and graphical techniques, including linear and non-linear modeling, classical statistical tests, time-series analysis, classification, clustering, among others. It can also be used to produce publication-quality static graphics. Tools like Sweave [Lei02] allow users to embed R code in LaTeX documents, for complete data analysis.

Bioconductor Package. Bioconductor is a free and open source set of tools for genomic data analysis, in the context of molecular biology [Lei02]. It is primarily based on R. It is under active development, with two stable releases each year. Counting with more than seven hundred different packages, it is the most comprehensive set of genomic data analysis tools available for the R programming language. It contains many of the tools that are part of most open source biological analysis pipelines. It also provides a set of tools to read and manipulate several of the most common file formats used in molecular biology oriented applications, including FASTA, FASTQ, BAM and GFF.

2.4 Chapter Conclusions

In this chapter we gave a brief introduction of the molecular biology concepts that serve as base of the thesis. We have reviewed the concepts on RNA-Seq and data mining and presented short analyses of concrete tools and algorithms that were used during this thesis.

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[11] S is an object oriented statistical programming language, appearing in 1976 at Bell Laboratories.

[12] `http://www.deducer.org/pmwiki/index.php`

Chapter 3

Solution Description

In this chapter we present the designed solution. The main objectives of the solution's design are reviewed, along with the most important choices made. Both major components of the developed solution and their envisioned integration process are described in detail.

3.1 Overview

As discussed, from a purely biological standpoint, this thesis has two major objectives: to perform differential expression analysis of RNA-Seq data and to discover and characterize RBPs related to groups of genes. While both objectives are important for our particular domain problem, they present different problems and require different approaches. Furthermore, both parts of the problem are relevant by themselves.

Splitting the domain problem into two objectives led to the first important design decision. It was decided that the software solutions to both problems would be implemented independently and, at a later stage, integrated with each other, to form the complete system. This would also allow both tools to be used separately, leading to a wider array of possibilities in terms of future uses.

Designing software solutions with high performance, scalability and extensibility is also a major concern. A software solution should be able to quickly and efficiently fulfil a user's requests, while dealing with a large number of simultaneous users. It should allow new features to be easily added, in order to better respond to the users' needs. If these requirements are not met, the solution is at risk of being abandoned by its users, becoming therefore useless. The software solutions presented in this thesis were designed with these goals in mind, as is further discussed in Chapter 4.

3.2 Gene Expression Analysis Pipeline

The gene expression analysis pipeline uses iRAP [FPMB14] as its foundation. As discussed before, iRAP provides begin-to-end gene RNA-Seq data analysis. However, while iRAP is a tool aimed at a broad group of users, both beginners and advanced users, it is still a command line tool, with many configuration settings. This technological barrier may drive away some more inexperienced users. One of the objectives of this part of the work was to make it a simpler and more straightforward process, accessible to any user with basic computer literacy; another was to combine results of several different tools, with the aim of improving those results. Note that some of the features described below were not implemented, due to time constraints. As such, those features are presented as future work.

3.2.1 Analysis Configuration

iRAP is configured with an experience configuration file (see Appendix B). This file must define the name of the species being analysed, the reference and read data files, the tools to use for the analysis, read libraries, etc.. Although many of these configurations are difficult to infer and define automatically, it is possible to create a more user friendly way to configure the experiments. This is accomplished through a web form. This web form has two different views: the *normal view* allows users to configure only the essential settings of the experiment, those that cannot be inferred; the *advanced view* allows more experienced users to tweak every configuration.

3.2.2 Experimental Data Management

Gene expression analysis with iRAP requires two sets of files, *reference files* and *read data files*.

Reference files are both the reference genome (in FASTA format) and its annotations (in GTF or GFF formats). These files are automatically obtained from Ensembl's FTP endpoint. The current release contains information of a total of sixty six species. The tool automatically checks if a new release is available, and in that case downloads and substitutes the files. In the experiment configuration users need only to indicate which species they are analysing, and the correct files will be automatically passed to iRAP.

Read data files contain the reads obtained through sequencing, in FASTQ formats. The user needs to upload these files. One file must be provided for each library the user wants to analyse, and should be compressed. As some of these files can be quite large, users also have the possibility to provide a link to those files. If a link is provided, the files will be downloaded before the analysis starts.

3.2.3 Analysis Workflow

Once both the configuration file and the required data files are ready, the analysis process can begin. The analysis process starts by passing those files to iRAP (Figure 3.1). In this initial stage of the workflow the analysis will stop immediately after the quantification step (see Section 2.2.1).

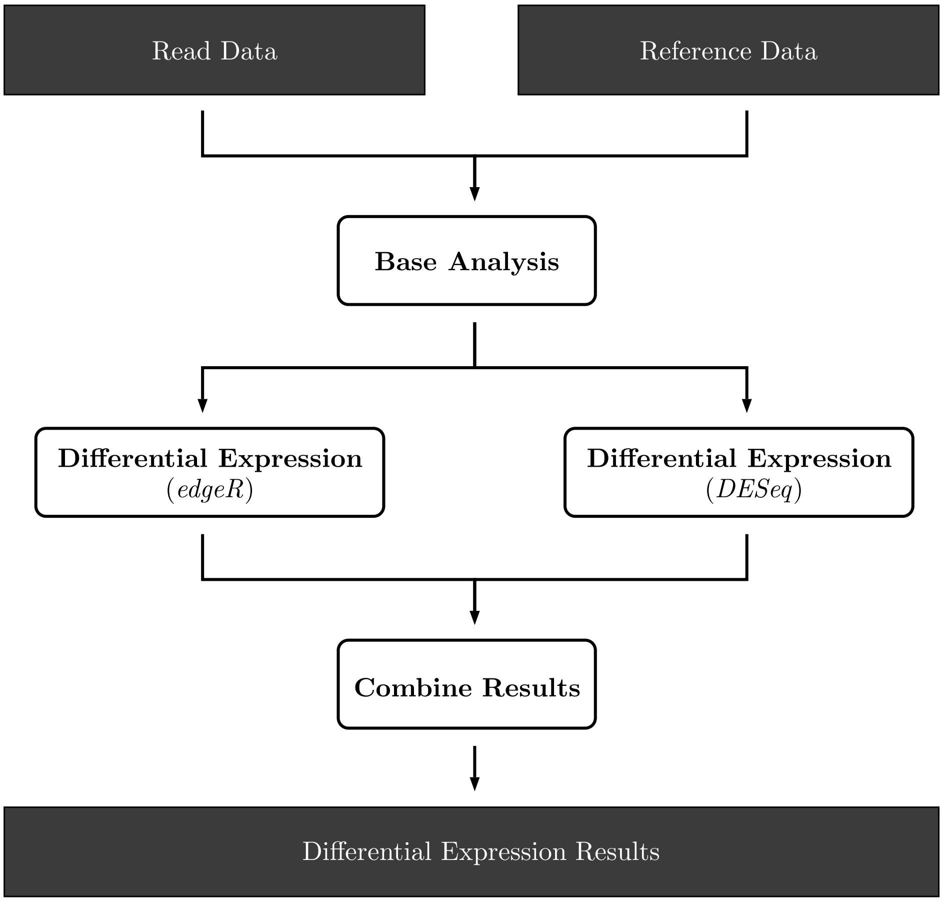
Read Data
Reference Data
Base Analysis
Differential Expression (edgeR)
Differential Expression (DESeq)
Combine Results
Differential Expression Results

Figure 3.1: Gene expression analysis pipeline workflow.

The next step is to sequentially execute differential expression analysis with the chosen tools, in this case DESeq [AH10] and edgeR [RMS10]. The resulting files are kept until all tools are executed. Note that there is no need for the analysis process to start from the beginning every time a new tool is used, as iRAP can (in most cases) reuse the results produced up until the point where a new tool is introduced. After all tools are executed, their results must be combined into a final list of gene identifiers. In order for a gene identifier to appear in the final combined results it must obey two constraints:

(1) the gene identifier must be present in all files;
(2) the average *p-value*[1] for that gene must be inferior to a maximum threshold in all files (0.05 by default).

---

[1] A *p-value* is used to assert the significance of statistical results. It represents the probability of obtaining the same as before in a new sample, given that the null hypothesis is true [Goo].

3.3 RNA Binding Protein Analysis Web Platform

The main objective of this system is to transform a complex, manually performed analysis, into an automated workflow. The platform should require as little information as possible from the user, inferring analysis parameters whenever possible. It should also be able to automatically group genes (and proteins), to reveal implied relationships between them that might be useful to experts. This platform was named Protein Binding Site Finder (PBS Finder) and shall henceforth be referred to by that name.

3.3.1 Experimental Data

The only data PBS Finder collects from the user is a list of gene (or transcript) identifiers that should be analysed. These identifiers can be in one of five different formats: *Ensembl Gene*, *Ensembl Transcript*, *Entrez*, *RefSeq* or *GenBank* (see Table 3.1). Users can submit jobs with any combination of the previous types and use identifiers from multiple species.

Ensembl Gene	ENSG00000224274	ENSRNOG00000013536
Ensembl Transcript	ENST00000003156	ENSRNOT00000117589
Entrez	11245	66850
RefSeq	NM_001107622.1	NM_031098.1
GenBank	U49845	C35522

Table 3.1: Two sets of examples of identifiers accepted by PBS Finder.

While PBS Finder can accept all these kinds of identifiers, internally the analysis can only be started with *Ensembl Gene* or *Entrez* identifiers, that map to Ensembl's and NCBI's pages, respectively. As such, the identifiers must be first separated into different groups and converted. This conversion is reviewed with more detail in Section 3.3.3.

3.3.2 User Information Management

In order to use PBS Finder a new user must create an account, providing their name and email, and choosing their authentication password. The account system was implemented so that jobs could be associated with their creator in a cross browser/system manner, in contrast with a typical cookie-based[2] data persistence. This allows each user to list their own jobs. Despite jobs being only directly accessible by their owner (creator), no effort was made to deny users access to each other's jobs through the job's URL. We believe that sharing results is an essential part of the investigation process.

To create a new job the user provides a job description and an identifier list. The identifier list is a newline-separated list (a single entry per line) of unique identifiers for genetic information databases. In the current version acceptable types of identifiers include Ensembl (both

---

[2] Modern websites sometimes need to save information about the user, for example credentials. This data can be saved in the client machine, through the use of a web browser cookie.

Gene and Transcript), Entrez, RefSeq and GenBank. Users can submit jobs with any combination of the previous types and use identifiers from multiple species. Upon job creation, users can choose to be notified by email when the job finishes.

3.3.3 Analysis Workflow

PBS Finder is based around a three stage analysis model (Figure 3.2): *base analysis*, *data set enrichment* and *clustering analysis*.

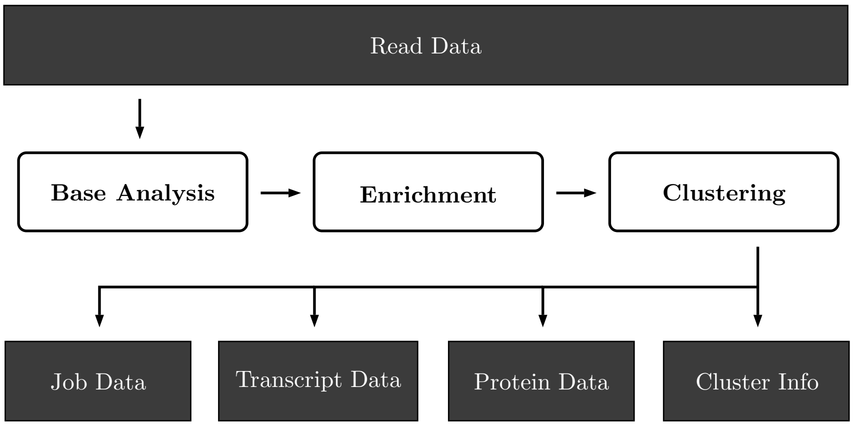

Figure 3.2: Simplified PBS Finder workflow.

Base analysis comprises data set filtering, retrieval of basic gene and transcript information and finding RBPs. The first released version of the platform implemented only this stage of the analysis. It contains all the information that is necessary to answer one of the base thesis problems. However, further stages build on top of this information, bringing a more valuable set of results to biologists.

In the enrichment stage additional information about every protein is retrieved. This information is obtained from UniProt[3], a web platform for curated protein information. This stage was implemented in the second release of the platform.

The clustering stage takes all the information collected in the previous stages and uses it to perform clustering analysis. There are two types of clustering analysis performed: *clustering using RBP presence* and *clustering using RBP attributes*. The objective of this analysis is to uncover implicit relationships in the collected data, that may be of use to biologists.

These stages are described in more detail in Chapter 4.

3.4 Tool Integration

In the designed solution both tools should be able to integrate, so that a user could perform the complete analysis creating a unique analysis job. This would be accomplished by using the interface of the RNA-Seq analysis pipeline as the master (or main) interface for job creation. When the analysis completes, the pipeline presents the preliminary results to the user and automatically launches a PBS Finder analysis for the result gene identifier list. At this point the RNA-Seq pipeline's interface should include a direct link to the created job in PBS Finder. That link can then be used by the user to view the results of the RBP localization analysis. Note that, while both tools are now integrated, the results of both analyses are independent. As such, they can be consulted/reviewed independently from each other, as if both had run separately.

---

[3] http://www.uniprot.org

3.5 Chapter Conclusions

In this chapter we reviewed the most important details about the design of the proposed software solution (implementation details are described in Chapter 4). We presented the modular, two subsystem model, that allows both tools to be used as standalone products, increasing the number of their possible uses. Lastly, we talked about the general details of the workflows of both tools, as well as the integration of those workflows.

Chapter 4

Implementation

In this chapter we will talk about some specific details of the implementation of the designed solution. We will also review some of the used technologies, and provide the reasoning behind their choice, where relevant.

4.1 Gene Expression Analysis Pipeline

Due to time constraints the gene expression analysis pipeline could not be fully finished. However, both the iRAP pipeline and result combining program were completed and functional. As these particular components were already described in the previous chapter, they will not be mentioned again.

4.2 RNA Binding Protein Analysis Web Platform

PBS Finder is implemented in two parts, following a client-server architecture: the *web interface* and the *analysis server*. In this case the web interface is the client and the analysis server is the server. This means that, whenever a user submits an analysis job, the web interface sends an analysis request to the server, including the data set given by the user. The server then processes the request and reports the results back to the web interface, that presents them to the user.

4.2.1 Web Interface

PBS Finder's web application interface is written in Ruby, using the Padrino[1] framework. Padrino is a web framework built on top of the Sinatra[2] web library. Padrino allows the creation of web applications that use Ruby to define their back-office logic.

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[1] http://www.padrinorb.com

[2] http://www.sinatrarb.com

User and job data is persisted in a database. In this case the MongoDB[3] database system was chosen. MongoDB is a document based, NoSQL database. While widely adopted relational database management system store information in large rely on large data banks and relational calculus, NoSQL databases store information in the form of files [SSK11]. NoSQL databases sacrifice the possibility to use some complex mechanisms of classical relational (structured queries, enforced information integrity, etc.), in order to achieve higher performance and scalability. NoSQL databases are most effective when the saved records are large but loosely structured information collections.

4.2.2 Analysis Server

The analysis server is responsible for managing analysis request sent by the web interface. The server is implemented on top of Distributed Ruby (dRuby), a distributed object system for Ruby. dRuby allows methods and behaviours to be invoked on an object that exists in a different process or even in a different computer, using a network connection. To the client it seems like the object is local, as it can be used as any other object. The server uses a master distributed object to receive analysis requests from the web interface.

The server launches each job concurrently. This behaviour allows the server to run multiple analyses at the same time. Note that the server is not bound to a single type of analysis, it can perform any type of analysis, as long as a valid implementation is available. The only two constraints to create a new type of analysis are that the analysis workflow must be encapsulated inside an instantiable class, and that class must implement a simple set of methods that allows the server to control it.

Algorithm 4.1 represents the behaviour of the server when a new request arrives. The first step is to determine if the requested analysis method is available. If it is, the respective class is instantiated, and the analysis data set (and other relevant information) are passed to it. Note that at the same time the server creates a file with a copy of all the parameters of that particular request. If for some reason the server stops working, analyses that were running at that time will be automatically restarted once the server is available again. After the object that will be responsible for the analysis is created, the server creates and starts a new thread for it. The server also saves an indication that that particular analysis is being executed.

Once the analysis is complete the server is notified, its results are communicated to the web interface. The server is also notified and eliminates both the indication from its internal list and the file with the analysis' parameters.

4.2.3 Analysis Workflow

A detailed overview of the RBP analysis workflow can be seen in Figure 4.1. This workflow is composed by three main stages: *base analysis*, *data set enrichment* and *clustering analysis*, all of which will be described in detail below.

---

[3] `http://www.mongodb.org`

```
receive request;
if request.analysis_method is available then
    instantiate request.analysis_method with request.parameters;
    save request.parameters to disk;
    run the analysis in a new thread;
    if success then
        report results;
    else
        report error;
    end
    delete request.parameters from disk;
else
    report error;
end
```

Algorithm 4.1: Processing a new analysis request from the web interface.

4.2.4 Base Analysis

The first step in the pipeline is to prepare the gene identifiers for further analysis. These identifiers can be have five different notations, as described in Section 3.2.3. As such, identifiers are separated according to their potential notation type in those five groups. However, some of those identifiers may be invalid, despite appearing to fit one of the possible notations. It is assumed that if an identifiers is valid, it can at least be converted to either *Ensembl Gene* or *Entrez* notation. This conversion is done using bioDBnet's [MCYS09] identifier conversion service. *Entrez* conversion is only attempted if *Ensembl Gene* conversion is not available. While none of those platforms are preferred above the other, converting as many identifiers as possible to one convention helps to maintain data set coherence.

Once only two lists remain, one with *Ensembl Gene* identifiers and the other with *Entrez* identifiers, information about those genes' transcripts must be collected. This information includes the transcript's name, its identifier, and its relevant genetic sequences. This information is collected using their respective platform (Ensembl or NCBI).

The transcript sequences are then used to retrieve the related RBPs using RBPDB, an online RNA binding protein database [CKZ+11]. For each sequence RBPDB finds every possible RBP, along with its name, location and binding sequence. This information is sufficient to answer one of this thesis' problems. However, it will be enriched and further analysed, bringing greater benefits to researchers.

4.2.5 Data Set Enrichment

Given the RBP names obtained in the base analysis stage, a query is made to UniProt in order to obtain their respective accession identifiers. These are used internally by UniProt to refer to specific proteins. With the accession identifiers, the pages for each RBPs can be retrieved

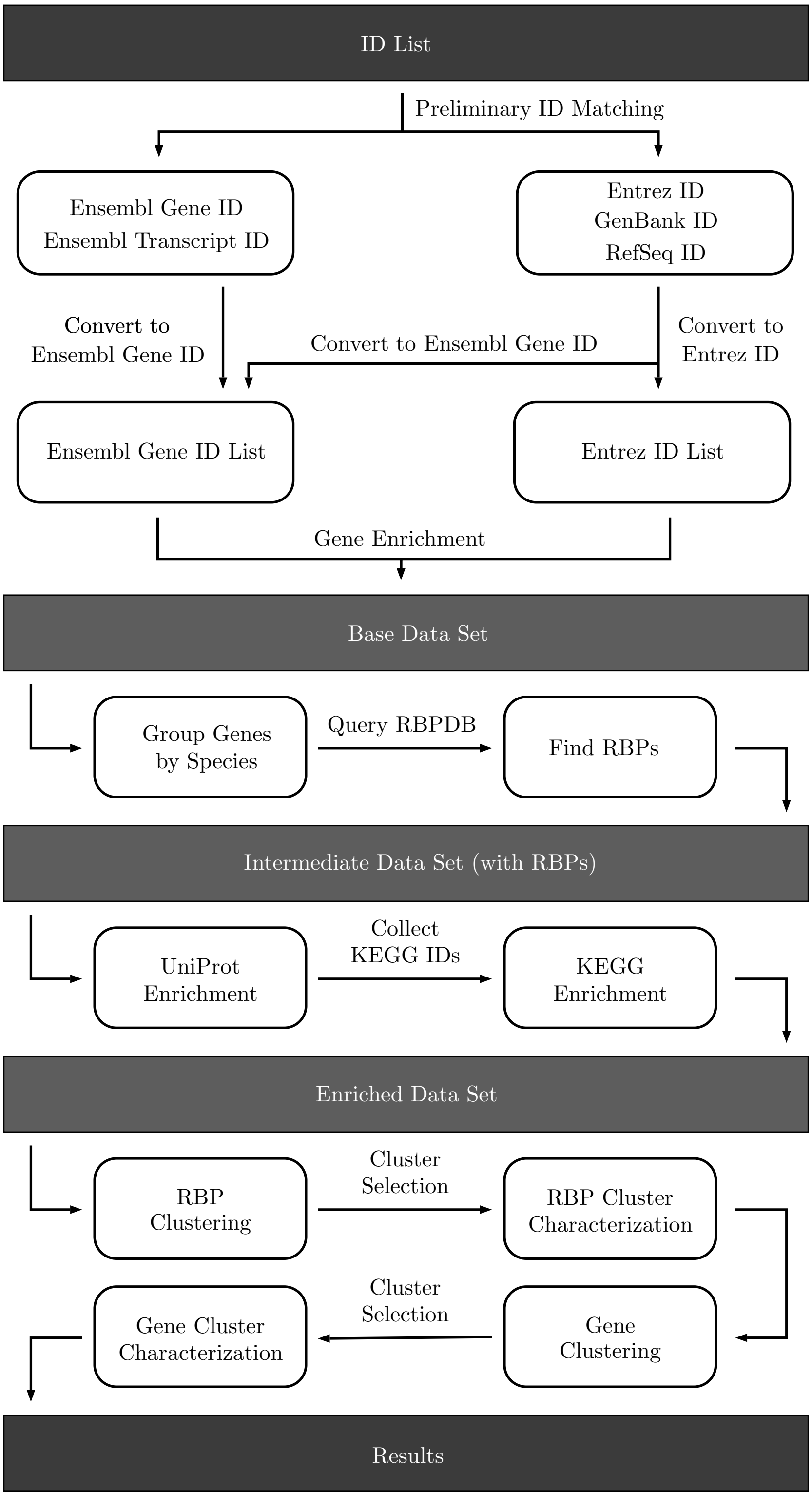
ID List
Preliminary ID Matching
Ensembl Gene ID
Ensembl Transcript ID
Entrez ID
GenBank ID
RefSeq ID
Convert to Ensembl Gene ID
Convert to Ensembl Gene ID
Convert to Entrez ID
Ensembl Gene ID List
Entrez ID List
Gene Enrichment
Base Data Set
Group Genes by Species
Query RBPDB
Find RBPs
Intermediate Data Set (with RBPs)
UniProt Enrichment
Collect KEGG IDs
KEGG Enrichment
Enriched Data Set
RBP Clustering
Cluster Selection
RBP Cluster Characterization
Gene Cluster Characterization
Cluster Selection
Gene Clustering
Results

Figure 4.1: PBS Finder workflow. Note that error paths were not represented for simplicity. However, every component implements health checks, that may stop the entire analysis if the minimum requirements for success are not met.

and analyzed individually. These pages contain information about their respective RBPs, including keywords, related tissues and information about the protein's ontologies (cellular components, molecular functions and biological processes).

The information collected from UniProt may also contains links to the KEGG web platform. Among other useful information, KEGG contains information about protein pathways. A pathway is a sequence of actions, related to specific molecules, that produce certain changes within a cell, when performed. This information is crucial to understand the function of RBPs. As such, it is collected for further analysis.

4.2.6 Clustering Analysis

The last stage of the workflow is clustering analysis. Two different types of clustering approaches were conceived: *clustering by RBP presence* and *clustering by RBP information*. Note that this analysis will not be performed if the quantity of available data is below certain thresholds (typically the tool will not analyse data sets that have less than ten genes and/or ten RBPs).

Clustering by RBP Presence

In the first case, the clustering analysis is only conducted at the gene level. Two genes are similar if they are associated with the same RBPs. Inversely, two genes that do not have any (or few) RBPs in common are considered “distant”. The tool makes an additional step in order to determine which RBPs are common in one particular group, while being absent in all the other groups. This information allows researchers to easily determine which RBPs can be used to interact with a particular group of genes, while not affecting the others.

Clustering by RBP Information

In the second case the clustering process has two phases. In the first phase RBPs are grouped based on their similarity. Similarity is computed using a set of attributes that include (depending on availability): pathways; tissues where the RBP is expressed; keywords describing their main functions; and other relevant information such as ontologies (biological processes, molecular function and cellular components). The tool compiles a list of defining features for each group (to later present to the user). However, in this case the RBP groups will be used to group similar genes. Unlike the previous method, similarity between genes is not only measured in how many RBPs they have in common. In this case any two genes may be considered similar if both are related to RBPs that were grouped together in the previous step, even if they do not share exactly the same RBPs between them.

Setup Variation

To produce the final results multiple clustering experiments are attempted. Due to the small amount of time that the chosen clustering algorithms need to run it is possible to execute a large number of attempts and then choose only the best results. Each attempt is a variation of the analysis parameters, being that each possible value for a parameter is combined with every value for the remaining parameters, giving rise to a large number of experiments. When clustering by RBP presence the variable parameters are: clustering algorithm (either hierarchical clustering or partitioning around medoids); number of clusters (between two and ten); and distance matrix representation (either using a strictly binary matrix or a Jaccard distance matrix). While clustering by RBP information the same variable attributes apply. However, the decision about what RBP information is used for their comparison is also variable, from a minimum of two information fields. For example, in a particular clustering attempt only information about the RBPs' pathways and related tissues may be used, while another attempt may use all available information.

Result Selection

With the large number of results obtained from all the clustering attempts, it is not reasonable to expect a user to manually chose the ones that are most interesting. As such, internal evaluation techniques for clustering are coupled with rules that define minimum acceptance criteria for results, narrowing the result space to only one for each type of clustering. Algorithm 4.2 represents the method used for result selection.

ILP Clustering

Clustering using ILP techniques was also attempted. The main objective of this approach was to uncover more information about about the implicit relationships present in the data sets. While conventional clustering methods present only the data set objects grouped in clusters, with ILP it is possible to understand exactly what rules were used to form those clusters.

The devised approach had two steps. First, the results for each new job would be represented in the form of Prolog clauses. Second, those clauses would be used as data set for the ERDA clustering system, running on top of YAP Prolog. However, due to problems with the available tools, this approach could not be included in the final version of PBS Finder.

4.2.7 Web Interface

Job View

Job view, also known as main view, is the default projection of a job's results (Figure A.1). It gives the user a matrix that matches every gene (and transcript) in the data set with their corresponding binding proteins, as well as an histogram of the genes (a bar chart of the counting of genes with which each RBP binds). This matrix also contains two columns that

```
sort clustering results by average silhouette (descending);
filter clustering results with clusters with less than 8% of data set objects;
abundance_frequency = 0.90;
while abundance_frequency ≥ 0.50 do
    foreach clustering result do
        classify as abundant any attribute with frequency ≥ abundance_frequency;
        remove abundant attributes that appear in multiple clusters;
        if at least one unique abundant attribute exists for each cluster then
            select cluster;
            break loop;
        else
            abundance_frequency = abundance_frequency - 0.05;
        end
    end
end
if a cluster was selected then
    return selected cluster;
else
    return cluster with highest average silhouette;
end
```

Algorithm 4.2: Selection of best clustering results.

give information about how the genes were grouped, based on the two distinct clustering techniques. In addition to this information, clicking any group of genes will give the user a list of all the characteristics that differentiate that particular group from all the others.

Transcript View

The transcript view (Figure A.2) offers more detailed information about the transcript's binding proteins, giving for each protein the base pairs that compose the binding site (and its start and stop positions) and a percentage score of the match's quality. A maximum of three different genetic sequences (5' UTR, 3' UTR and 3' UTR downstream) are also displayed, depending on their availability. Whenever possible, related links to other platforms are presented (Ensembl, NCBI and UniProt). Like the job view, it also contains clustering information, but in this case only one column, that shows how the RBPs related to that specific transcript were grouped. Also similarly to the job view, users can click any group to obtain relevant information about the defining characteristics of that particular group.

Protein View

The protein view (Figure A.3) displays relevant classification information for every RBP (when available). This information includes the protein's name, links to other platforms (UniProt, KEGG, Gene3D, etc.), tissues in which the protein might be expressed, its related ontologies and the pathways in which the protein participates. Each pathway is coupled with a link to the corresponding KEGG pathway browser page.

Results Exporting

Besides browsing, users can download their results as text files. Three different types of data projections of the results are provided: *RBP data*, *protein data* and the *complete data set*. RBP data can be downloaded in either comma-separated values (CSV) or tab-separated values (TSV) format. This file gives the user a match matrix of binding proteins, similar to the one in the job results view, as well as species, gene and transcript information. Protein data can also be downloaded in both CSV and TSV formats. It gives a more complete view of the results, including species, gene, transcript and protein identifiers and additional protein information (keywords, related tissues, ontologies, etc.). Finally, the complete data set consists of a Prolog predicate representation of the job results. This is the same representation of the results that was created with the usage of ILP clustering techniques in mind. While it was not possible to use those techniques, the produced data set representation might still be of use to researchers.

While in most cases these data representation conversion are apparently instantaneous, for large data sets they may take an additional amount of time. This would cause the web application to seemingly block if the user tried to download one of those files. To solve this problem all files are generated during the analysis process, then stored in the web application's MongoDB instance through GridFS[4]. This allows those files to be downloaded instantly, as they need only to be generated once.

4.3 Chapter Conclusions

In this chapter we presented a concrete approach to the development of the proposed solutions. We reviewed their most important features and implementation choices. Further examples of their usage is available through a case study in Chapter 5 (the case study applies only to PBS Finder).

---

[4] GridFS is a functionality integrated in MongoDB that allows a simple file system to be simulated inside a database. Files are divided into small chunks and those chunks are stored as regular MongoDB documents.

Chapter 5

Case Study

In this chapter we present a case study that was used as a proof of concept for the developed system. We characterize the experimental environment, the used data set and the obtained results. We also compare this method with the previous method, manual analysis.

5.1 Case Study Setup

This case study is aimed only at the RBP analysis tool (PBS Finder). The reason for this is that it was not possible to acquire a test data set in sufficient time. It was also not possible to have an expert validate those results in the available time. Moreover, the available case study data set was produced by IBMC experts. The same experts then validated the obtained results experimentally. The same experts then validated the obtained results experimentally.

5.2 Experimental Environment

The testing environment was reproduced in two different machines. This machines differ in their hardware and operative system, but every other variable in the environment (internet connection speeds, machine usage, etc.) holds for both machines. Performance results for the case study experiment for both machines are given in Section 5.5. Henceforth the machines will be referenced as *machine1* and *machine2*, as shown in Table 5.1.

	machine1	***machine2***
Operative system	OS X 10.9.3	Debian GNU/Linux Jessie/Sid
CPU	Intel Core i7 4850HQ (4 cores)	Intel Core 2 Quad Q9400 (4 cores)
CPU speed	2.30 GHz	2.66 GHz
Memory	16 GiB DDR3 (1600 MHz)	16 GiB DDR2 (800 MHz)
Internet connection	100 Mbps	100 Mbps

Table 5.1: Specifications of the test environments used for the case study experiments.

5.2.1 Analysis Data Set

The analysis data set is comprised by twenty three gene identifiers, from the *RhoGTPase* family. *RhoGTPases* comprise a family of molecular switches that control signal transduction pathways that link cell surface receptors to a variety of intracellular responses. In this particular case the genes are related to the species *Rattus norvegicus*, commonly known as *norway rat*. *Rattus norvegicus* is a model organism: a “simple”, non-human organism that is extensively studied, with the expectation that the discoveries made for that particular organism are useful to understand different organisms [FJ05]. Other examples of model organisms are the *E. coli* bacteria, *Drosophila melanogaster* (fruit fly) and *Mus musculus* (common mouse). The actual genes used for our tests can be found in Table 5.2. Note that the tool only needs to receive gene identifiers; gene names are not currently accepted as input data.

Gene name	***Gene ID***	***Transcript ID***
Rhoj	ENSRNOG00000021919	ENSRNOT00000031979
Rhog	ENSRNOG00000020393	ENSRNOT00000027641
Rac3	ENSRNOG00000048172	ENSRNOT00000073886
Rac2	ENSRNOG00000007350	ENSRNOT00000009994
Rhod	ENSRNOG00000019220	ENSRNOT00000026092
Rhof	ENSRNOG00000042607	ENSRNOT00000064390
Rhoh	ENSRNOG00000002540	ENSRNOT00000003425
Rnd	ENSRNOG00000020698	ENSRNOT00000028089
Rhoc	ENSRNOG00000012630	ENSRNOT00000017254
Cdc42	ENSRNOG00000013536	ENSRNOT00000029025
Cdc42	ENSRNOG00000013536	ENSRNOT00000018118
Rhoq	ENSRNOG00000015415	ENSRNOT00000020822
Rac1	ENSRNOG00000001068	ENSRNOT00000001417
Rhou	ENSMUSG00000039960	ENSMUST00000136615
Rhou	ENSMUSG00000039960	ENSMUST00000045487
Rhoa	ENSRNOG00000050519	ENSRNOT00000071664
Rnd1	ENSRNOG00000013621	ENSRNOT00000018276
Rnd3	ENSRNOG00000004624	ENSRNOT00000006111
Rhob	ENSRNOG00000021403	ENSRNOT00000008008
Rhobtb1	ENSRNOG00000000633	ENSRNOT00000000784
Rhobtb2	ENSRNOG00000017373	ENSRNOT00000023876
Rhobtb3	ENSRNOG00000012414	ENSRNOT00000016838
Rhov	ENSRNOG00000013380	ENSRNOT00000018277

Table 5.2: *RhoGTPase* family genes used as data set in the case study.

5.3 Analysis Results

Table 5.3 includes the relevant results for this case study. As expected, some RBPs are able to bind to almost all members of the *RhoGTPase* family (for example *Muscleblind-Like Splicing Regulator 1* (MBNL1)), where others were more specific (for example *Y Box Binding Protein*

1 (YBX1)). The latter kind is more interesting to analyse, as it might be used to manipulate a very specific set of genes, while leaving all others unaltered. In this case we chose the RBP YBX1 as the base of our validation, both because it only interacts with only five genes and because the genes it interacts with are representative of the data set, in terms of biological classification.

5.4 Result Validation

Result validation was conducted in two different fronts: *collected data completeness and correction*, and *biological results evaluation*.

Completeness and correction analysis were conducted both manually and automatically during the development of the tool. In some cases it was possible to automate these validations using *unit testing*[1] and *mocks*[2]. However, creating this test infrastructure is a lengthy process. As such, some results, as is the case with this case study, were manually validated. This validation involves manually visiting every website used to collect information and cross reference that information with the one collected by the tool.

The validation of biological results is more complex. It involves experimentally obtaining the same information that the tool produces, from biological samples. This validation was conducted at IBMC. However, it is a lengthy process, as obtaining this information may take several weeks. It has two main objectives: validating the number and type of RBPs that bind to each gene; and validate the adequacy of the gene clustering results.

Both validation processes were conducted successfully, thus proving that the developed platform produces adequate results.

5.5 Comparison with Previous Method

As with any newly developed automation tool, it is essential to assess if said tool is in fact an improvement when compared to the previously available methods. The results produced by the tool were already validated in Section 5.4. As such, the tool should now be evaluated from the user experience standpoint, namely, in terms of *task simplification* and *efficiency*.

5.5.1 Task Simplification

Figure 5.1 depicts how PBS Finder simplified the analysis process, from the user point of view. When conducting a manual analysis, the user must individually analyse every gene in the data set.

---

[1] Unit testing is a software testing method that focus on testing individual units of a developed software; these tests are used to assess which of those units are fit, and which do not work correctly.

[2] A mock, or mock object, is a simulated object that mimics the behavior of a complex component of a software system. Mock objects are particularly useful when the behavior of its real counterpart is hard/costly to predict and control

		SNRPA	*ELAVL2*	*HNRNPA1*	*NONO*	*PABPC1*	*ZRANB2*	*RBMY1A1*	*FUS*	*PUM2*	*SFRS9*	*MBNL1*	*EIF4B*
Cluster 1	***Rhoj***				×	×		×	×	×	×	×	×
	Rhog				×	×		×			×	×	×
	Rac3			×	×	×		×	×	×	×	×	×
	Rac2				×	×		×	×			×	×
	Rhod			×		×			×	×	×	×	×
	Rhof				×	×			×	×	×	×	×
	Rhoh							×		×	×	×	×
	Rnd2				×				×	×	×	×	×
	Rhoc	×		×	×				×	×	×	×	×
Cluster 2	***Cdc42***	×	×	×	×	×	×	×	×	×	×	×	×
	Cdc42		×		×			×	×	×	×	×	×
	Rhoq	×			×	×		×	×	×	×	×	×
	Rac1	×				×			×	×	×	×	×
	Rhou												
	Rhou	×	×	×	×	×		×	×	×	×	×	×
	Rhoa		×			×			×	×	×	×	×
	Rnd1				×	×			×		×	×	×
	Rnd3		×		×	×		×	×	×	×	×	×
	Rhob	×	×		×	×		×	×	×	×	×	×
	Rhobtb1	×	×	×	×	×	×	×	×	×	×	×	×
	Rhobtb2				×				×	×	×	×	×
	Rhobtb3	×	×	×	×	×	×	×	×	×	×	×	×
	Rhov				×	×		×	×	×	×	×	×

		EIF4B	*KHSRP*	*YTHDC1*	*VTS1*	*RBMX*	*KHDRBS3*	*SFRS1*	*ELAVL1*	*SFRS13A*	*SFRS7*	*A2BP1*	*QKI*
Cluster 1	***Rhoj***	×	×	×	×	×	×	×	×	×		×	×
	Rhog	×	×	×		×	×	×	×	×			×
	Rac3	×	×	×	×	×	×	×	×	×		×	×
	Rac2	×	×	×		×	×	×	×	×			
	Rhod	×	×	×	×	×	×	×	×	×		×	×
	Rhof	×	×	×	×	×	×	×	×	×			×
	Rhoh	×	×			×	×	×	×	×			
	Rnd2	×	×	×	×	×	×	×	×	×		×	
	Rhoc	×	×	×	×	×	×	×	×	×		×	
Cluster 2	***Cdc42***	×	×	×	×	×	×	×	×	×	×	×	×
	Cdc42	×	×	×		×	×	×	×	×		×	×
	Rhoq	×	×	×	×	×	×	×	×	×		×	×
	Rac1	×	×	×	×	×	×	×	×	×			×
	Rhou												
	Rhou	×	×	×	×	×	×	×	×	×		×	
	Rhoa	×	×	×	×	×	×	×	×	×			×
	Rnd1	×	×		×	×	×	×	×	×		×	
	Rnd3	×	×	×	×	×	×	×	×	×		×	
	Rhob	×	×	×	×	×	×	×	×	×	×	×	
	Rhobtb1	×	×	×	×	×	×	×	×	×		×	
	Rhobtb2	×	×		×	×		×	×	×			
	Rhobtb3	×	×	×	×	×	×	×	×	×		×	×
	Rhov	×	×	×	×	×	×	×	×	×			

		QKI	*SUS*	*YBX2-A*	*PSI*	*SAP-49*	*ACO1*	*YBX1*	*SFRS2*	*ZFP36*	*RBM4*	*PTBP1*
Cluster 1	***Rhoj***	×		×		×		×	×			
	Rhog	×					×					
	Rac3	×										
	Rac2			×								
	Rhod	×										
	Rhof	×					×		×		×	
	Rhoh											
	Rnd2						×		×			
	Rhoc											
Cluster 2	***Cdc42***	×										
	Cdc42	×	×	×	×	×	×	×				
	Rhoq	×		×		×	×			×	×	
	Rac1	×		×		×	×				×	
	Rhou											
	Rhou			×		×	×			×		
	Rhoa	×				×	×	×			×	
	Rnd1						×			×		
	Rnd3				×	×	×	×				×
	Rhob					×	×				×	
	Rhobtb1						×	×			×	
	Rhobtb2			×			×					
	Rhobtb3	×		×		×	×		×	×		
	Rhov					×	×					

Table 5.3: Case study results generated by PBS Finder. This table includes information about which RBPs bind with each gene, as well as about the gene clusters that were generated.

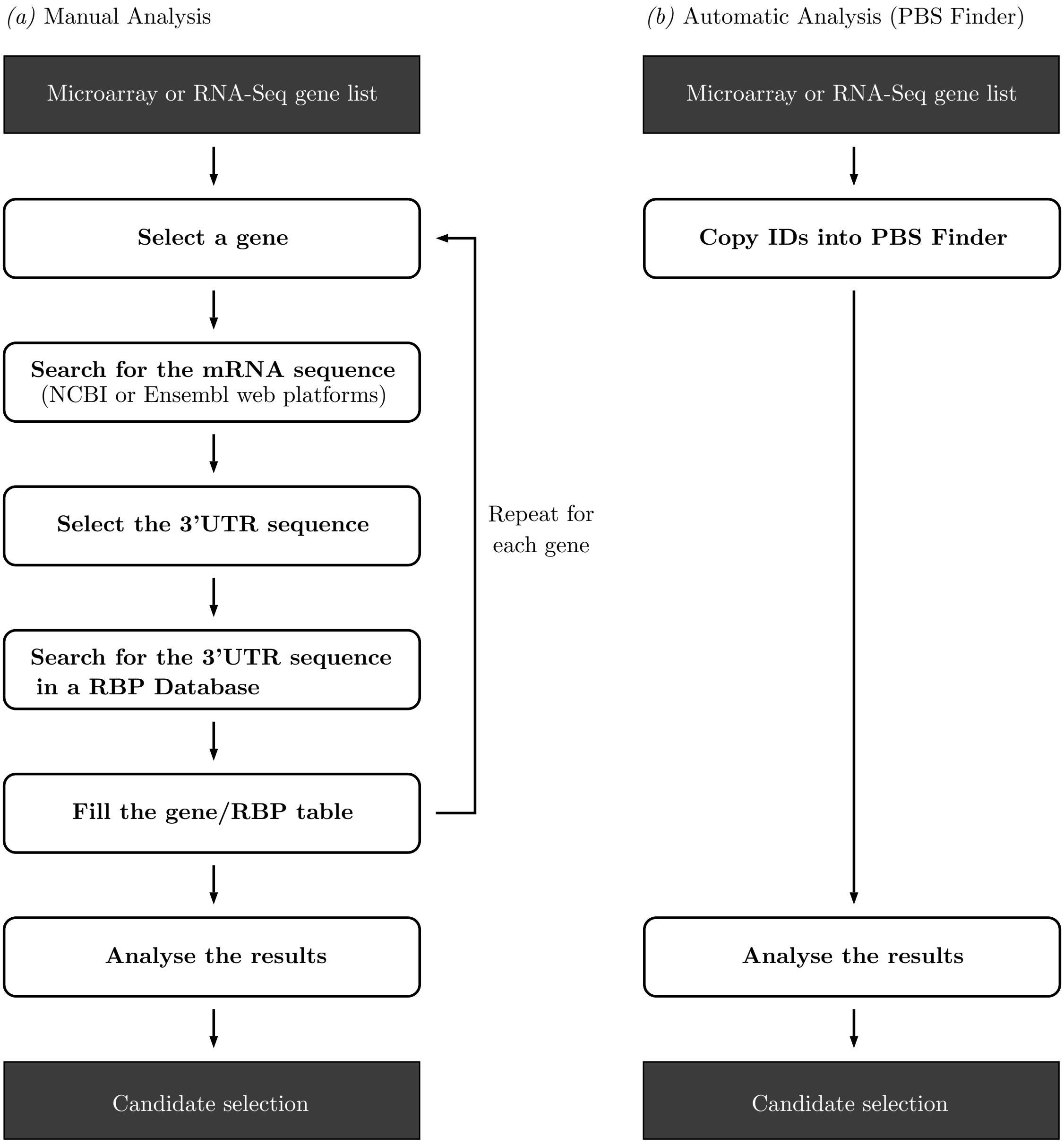
(a) Manual Analysis
(b) Automatic Analysis (PBS Finder)
Microarray or RNA-Seq gene list
Select a gene
Search for the mRNA sequence (NCBI or Ensembl web platforms)
Select the 3'UTR sequence
Search for the 3'UTR sequence in a RBP Database
Fill the gene/RBP table
Repeat for each gene
Analyse the results
Candidate selection
Microarray or RNA-Seq gene list
Copy IDs into PBS Finder
Analyse the results
Candidate selection

Figure 5.1: Comparison between manual RBP analysis and automatic RBP analysis (conducted with PBS Finder).

The first step is to select a gene for analysis. The gene identifier is then searched in either Ensembl or NCBI web platforms, depending on the type of the identifier. If the gene is found in those platforms, the next step involves finding the 3'UTR sequence in the results page and copying it. That sequence is then passed to an online RBP analysis platform, that should retrieve a list of RBPs that may bind to that particular sequence. Lastly, the RBP information must be manually combined in the results table. When this information is collected for every gene it is finally possible to conduct a useful analysis.

On the other hand, with PBS Finder the user needs only to provide the complete list of all gene identifiers in the data set. The tool will them automatically process those identifiers, find all relevant information and present it to the user, ready for further analysis. Note that these results contain additional information that is not available in the manual analysis: gene, transcript and protein information (names, additional sequences, pathways, etc.); links to external platforms; useful histograms; and gene and protein clustering results.

5.5.2 Efficiency

It is essential that the developed platform is able to conduct the analysis in a timely fashion, making tool performance a major concern. As such, we compared the average time that an expert would take to analyse the data set to the average time that the tool takes to analyse the same data set.

The consulted molecular biology expert estimated that, on average, it would take thirty minutes to analyse a single gene. This means that an expert would need an average of eleven and a half hours ($30\ minutes \times 23\ genes$) to process the entire case study data set.

On the other hand, the automated tool takes a shorter amount of time to conduct the analysis of the same data set. In order to assess the actual amount of time the tool took to analyse the entire data set we ran twenty sequential experiences, ten for each of the experimental environments. Table 5.4 depicts the duration of each one of those experiments, as well as their average duration. As expected, an automated tool widely outperforms a human expert in information collection and analysis.

	machine1	***machine2***
Experience 1	$1m\ 47s$	$6m\ 34s$
Experience 2	$1m\ 58s$	$6m\ 25s$
Experience 3	$1m\ 46s$	$6m\ 30s$
Experience 4	$1m\ 45s$	$6m\ 15s$
Experience 5	$2m\ 23s$	$6m\ 42s$
Experience 6	$1m\ 35s$	$6m\ 30s$
Experience 7	$1m\ 40s$	$6m\ 31s$
Experience 8	$1m\ 39s$	$6m\ 11s$
Experience 9	$1m\ 42s$	$6m\ 42s$
Experience 10	$1m\ 42s$	$6m\ 52s$
Average time	$1m\ 42s$	$6m\ 31s$

Table 5.4: Execution times of the case study data set in two different environments (sequential experiments). Note that while *machine2* has a significant loss in performance (due to its outdated hardware) it still achieves satisfactory execution times. This test also shows that it is possible to efficiently run PBS Finder in a home computer.

Table 5.5 contains the stress test results of both machines, compared to the estimated work time needed by an expert. In this case stress tests were executed with one hundred, five hundred and nine hundred gene identifiers. Even the slowest test can be completed by the machine with less performance (*machine2*) in under three hours. That same effort would take an expert approximately four hundred and fifty hours, which amounts to two and a half weeks on a twenty four hour working day, and to just under two months in normal working conditions (eight hours per day).

	machine1	***machine2***	***Manual method***
100 IDs	$9m\ 56s$	$11m\ 1s$	$\approx 50h$
500 IDs	$41m\ 47s$	$55m\ 51s$	$\approx 250h$
900 IDs	$1h\ 33m\ 32s$	$2h\ 7m\ 4s$	$\approx 450h$

Table 5.5: Result comparison between manual analysis and both test machines.

5.6 Chapter Conclusions

Through this case study we have shown that PBS Finder produces relevant and correct results for large-scale analysis of RBPs using data from NGS techniques. We have also shown that our tool significantly facilitates the work of the biologist, by making analysis of this data easier and quicker. As such, we consider PBS Finder a tool of great value for studying RNA biology.

Chapter 6

Conclusions

In this thesis we proposed the development of an integrated platform for RNA-Seq data analysis. This platform is able to perform the entirety of the analysis process, from the sequencing reads, to grouping genes by their RBPs. This means being able to perform read alignment, quantification and differential expression analysis tasks, as well as data set enrichment, RBP identification and clustering analysis of genes and proteins. Through a case study we showed the developed prototype in action, and assessed its correctness and efficiency. Below we will share our thoughts on the fulfilment of the objectives of this thesis, and present some possibilities for any future development in our solution.

6.1 Objective Fulfilment

Our objectives, in terms of studying the problem at hand and developing a solution to it, were completely fulfilled. The proposed solution corresponds to all of our expectations. However, as previously discussed, the implementation of the RNA-Seq data analysis pipeline system was not completed, due to time constraints. As such, our objective of prototyping and testing the complete system could not be completely achieved.

6.2 Future Work

The obvious continuation of the proposed work would be to finish the implementation and integration of the RNA-Seq data analysis pipeline. This would allow our solution to work as designed, integrating the complete analysis pipeline, from sequencing data to gene clustering and result visualization. Furthermore, it would be interesting to study the developed tools in terms of performance, under large volumes of information and requests. Whilst the tools were developed taking in consideration their performance, making them available in a large scale would take another kind of infrastructure.

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Glossary

Alternative splicing

Alternative splicing that causes a specific gene to be transcribed to several different RNA sequences, by means of different combinations of exons. This process is a major source of protein diversity in an organism [Bla03].

cDNA

DNA which has been reverse transcribed using RNA as a template [Lyo98].

Cytoplasm

The protoplasm of a cell contained within the cell membrane, but excluding the nucleus. It helps to move materials around the cell and is also responsible for dissolving cellular waste [Lyo98].

Exon

The portions of a genomic DNA sequence which will be represented in the final, mature mRNA. Exons may include coding sequences, the *5'* untranslated region or the *3'* untranslated region [Lyo98].

Expression

To “express” a gene is to cause it to function. A gene which encodes a protein will, when expressed, be transcribed and translated to produce that protein. A gene which encodes an RNA rather than a protein (for example, a rRNA gene) will produce that RNA when expressed [Lyo98].

Gene

A unit of DNA which performs one function. Usually, this is equated with the production of one RNA or one protein. A gene contains coding regions, introns, untranslated regions and control regions [Lyo98].

Genome

The total DNA contained in each cell of an organism. There are somewhere in the order of a hundred thousand genes, including coding regions, *5'* and *3'* untranslated regions, introns, *5'* and *3'* flanking DNA [Lyo98].

Intron

Introns are portions of genomic DNA which are transcribed (and thus present in the primary transcript) but which are later spliced out. Thus, they are not present in the mature mRNA [Lyo98].

mRNA

"Messenger RNA" contains sequences coding for a protein. The term mRNA is used only for a mature transcript (with all introns removed), rather than the primary transcript in the nucleus [Lyo98].

Nucleus

The nucleus is a membrane enclosed part of the cell [Lyo98]. It contains the cell's genetic information, in the form of DNA and RNA molecules [Lyo98].

rRNA

"Ribosomal RNA" describes any of several RNAs which become part of the ribosome, and thus are involved in translating mRNA and synthesizing proteins [Lyo98].

Shotgun cloning

The process of randomly shearing an organism's genomic DNA and cloning it into a suitable vector, resulting in a genomic library [Lyo98].

Shotgun sequencing

Sequencing the DNA library created by shotgun cloning [Lyo98].

Signal transduction

Process in which an extracellular signaling molecule activates a specific receptor located in the border (or inside) a cell; in turn this receptor triggers a chain of events inside the cell that leads to its response [LBZ00].

Transcription

The process of copying DNA to produce an RNA transcript. This is the first step in the expression of any gene. The resulting RNA will produce the desired protein molecule by the process of translation [Lyo98].

Translation

The process of decoding a strand of mRNA, thereby producing a protein based on the code [Lyo98].

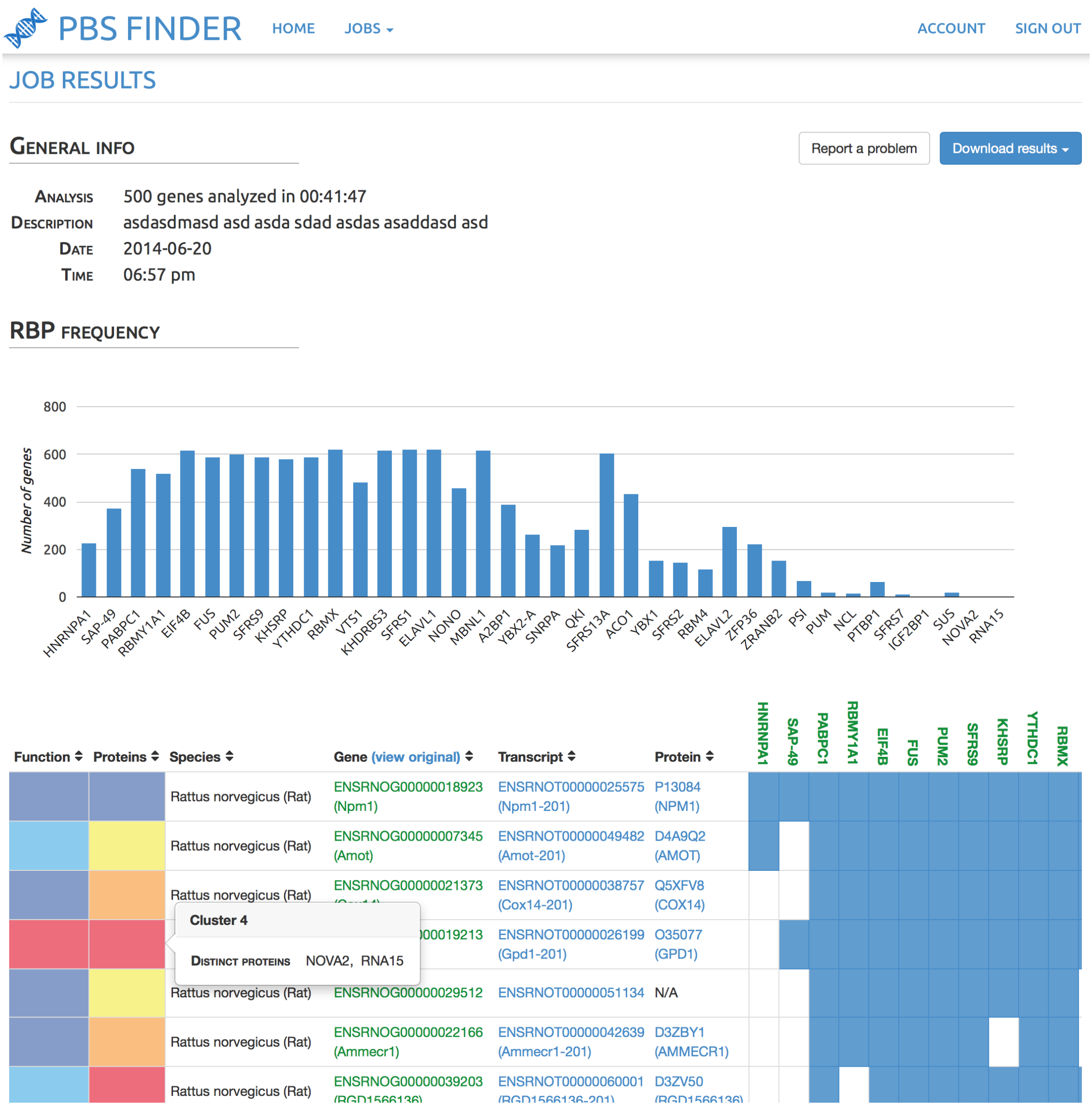
tRNA

“Transfer RNA” represents one of a class of rather small RNAs used by the cell to carry amino acids to the enzyme complex (the ribosome) which builds proteins, using an mRNA as a guide [Lyo98].

Appendix A

PBS Finder Screen Shots

2


PBS FINDER
HOME
JOBS
ACCOUNT
SIGN OUT
JOB RESULTS
Report a problem
Download results
RBP FREQUENCY
Number of genes
800
600
400
200
0
HNRNPA1
SAP-49
PABPC1
RBMY1A1
EIF4B
FUS
PUM2
SFRS9
KHSRP
YTHDC1
RBMX
VTS1
KHDRBS3
SFRS1
ELAVL1
NONO
MBNL1
A2BP1
YBX2-A
SNRPA
QKI
SFRS13A
ACO1
YBX1
SFRS2
RBM4
ELAVL2
ZFP36
ZRANB2
PSI
PUM
NCL
PTBP1
SFRS7
IGF2BP1
SUS
NOVA2
RNA15

General info

Analysis 500 genes analyzed in 00:41:47

Description asdasdmasd asd asda sdad asdas asaddasd asd

Date 2014-06-20

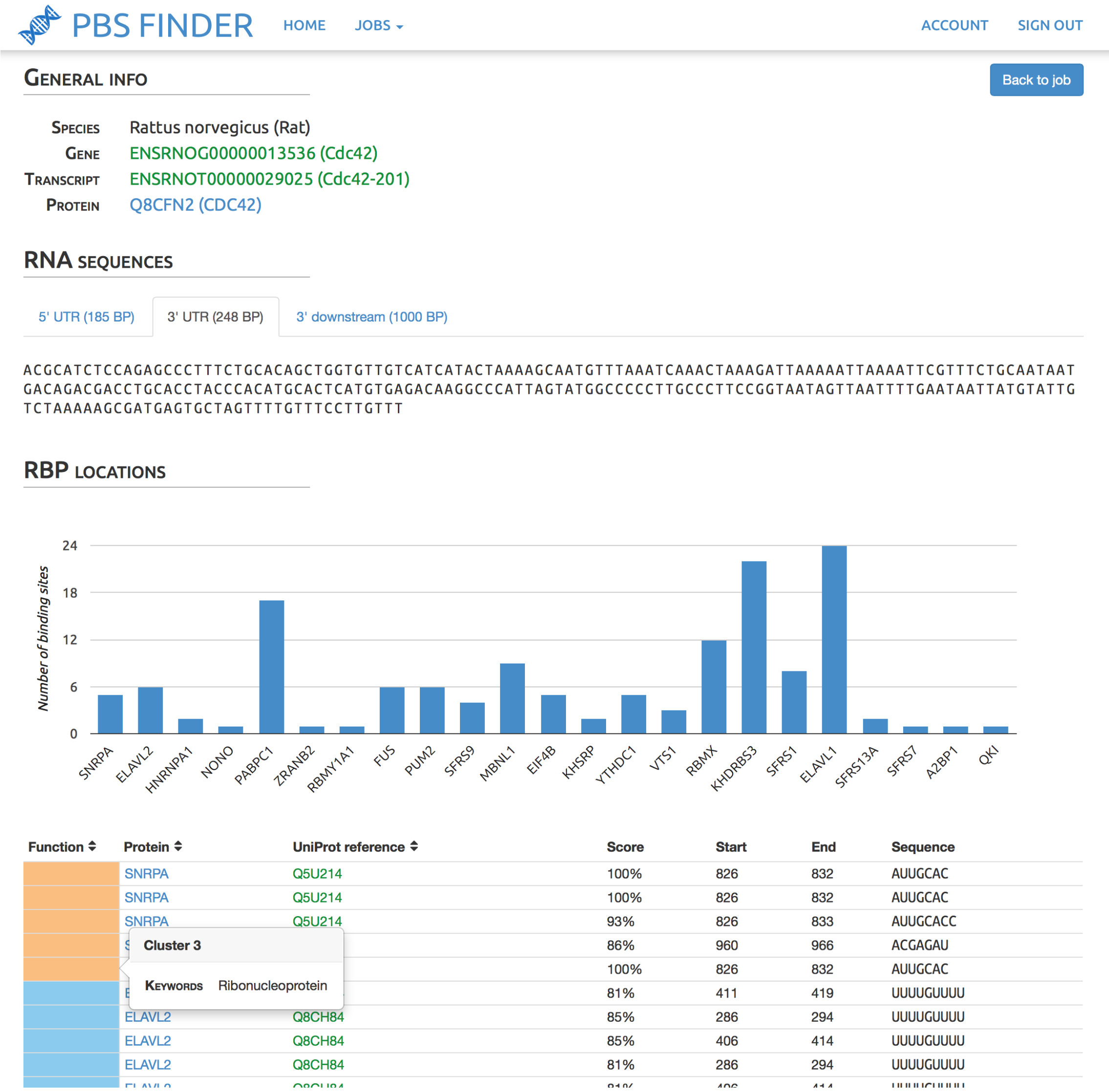
Time 06:57 pm

Function	Proteins	Species	Gene (view original)	Transcript	Protein	HNRNPA1	SAP-49	PABPC1	RBMY1A1	EIF4B	FUS	PUM2	SFRS9	KHSRP	YTHDC1	RBMX
🟪	🟪	Rattus norvegicus (Rat)	ENSRNOG00000018923 (Npm1)	ENSRNOT00000025575 (Npm1-201)	P13084 (NPM1)	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦
🟦	🟨	Rattus norvegicus (Rat)	ENSRNOG00000007345 (Amot)	ENSRNOT00000049482 (Amot-201)	D4A9Q2 (AMOT)	🟦		🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦
🟪	🟧	Rattus norvegicus (Rat)	ENSRNOG00000021373	ENSRNOT00000038757 (Cox14-201)	Q5XFV8 (COX14)			🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦
🟥	🟥		00019213	ENSRNOT00000026199 (Gpd1-201)	O35077 (GPD1)		🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦
🟪	🟨	Rattus norvegicus (Rat)	ENSRNOG00000029512	ENSRNOT00000051134	N/A			🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦
🟪	🟧	Rattus norvegicus (Rat)	ENSRNOG00000022166 (Ammecr1)	ENSRNOT00000042639 (Ammecr1-201)	D3ZBY1 (AMMECR1)			🟦	🟦	🟦	🟦	🟦	🟦		🟦	🟦
🟦	🟥	Rattus norvegicus (Rat)	ENSRNOG00000039203 (RGD1566136)	ENSRNOT00000060001 (RGD1566136-201)	D3ZV50 (RGD1566136)			🟦		🟦	🟦	🟦	🟦	🟦	🟦	🟦

Cluster 4

Distinct proteins NOVA2, RNA15

Figure A.1: Job view example, also known as main view. It is comprised by three main components: general information (which includes data download); RBP frequency histogram; and RNA binding protein matching table.


PBS FINDER
HOME
JOBS
ACCOUNT
SIGN OUT
Back to job
Number of binding sites
24
18
12
6
0
SNRPA
ELAVL2
HNRNPA1
NONO
PABPC1
ZRANB2
RBMY1A1
FUS
PUM2
SFRS9
MBNL1
EIF4B
KHSRP
YTHDC1
VTS1
RBMX
KHDRBS3
SFRS1
ELAVL1
SFRS13A
SFRS7
A2BP1
QKI

GENERAL INFO

SPECIES	Rattus norvegicus (Rat)
GENE	ENSRNOG00000013536 (Cdc42)
TRANSCRIPT	ENSRNOT00000029025 (Cdc42-201)
PROTEIN	Q8CFN2 (CDC42)

RNA SEQUENCES

5' UTR (185 BP) | 3' UTR (248 BP) | 3' downstream (1000 BP)

```
ACGCATCTCCAGAGCCCTTTCTGCACAGCTGGTGTTGTCATCATACTAAAAGCAATGTTTAAATCAAACTAAAGATTAAAAATTAAAATTCGTTTCTGCAATAAT
GACAGACGACCTGCACCTACCCACATGCACTCATGTGAGACAAGGCCCATTAGTATGGCCCCCTTGCCCTTCCGGTAATAGTTAATTTTGAATAATTATGTATTG
TCTAAAAAGCGATGAGTGCTAGTTTTGTTTCCTTGTTT
```

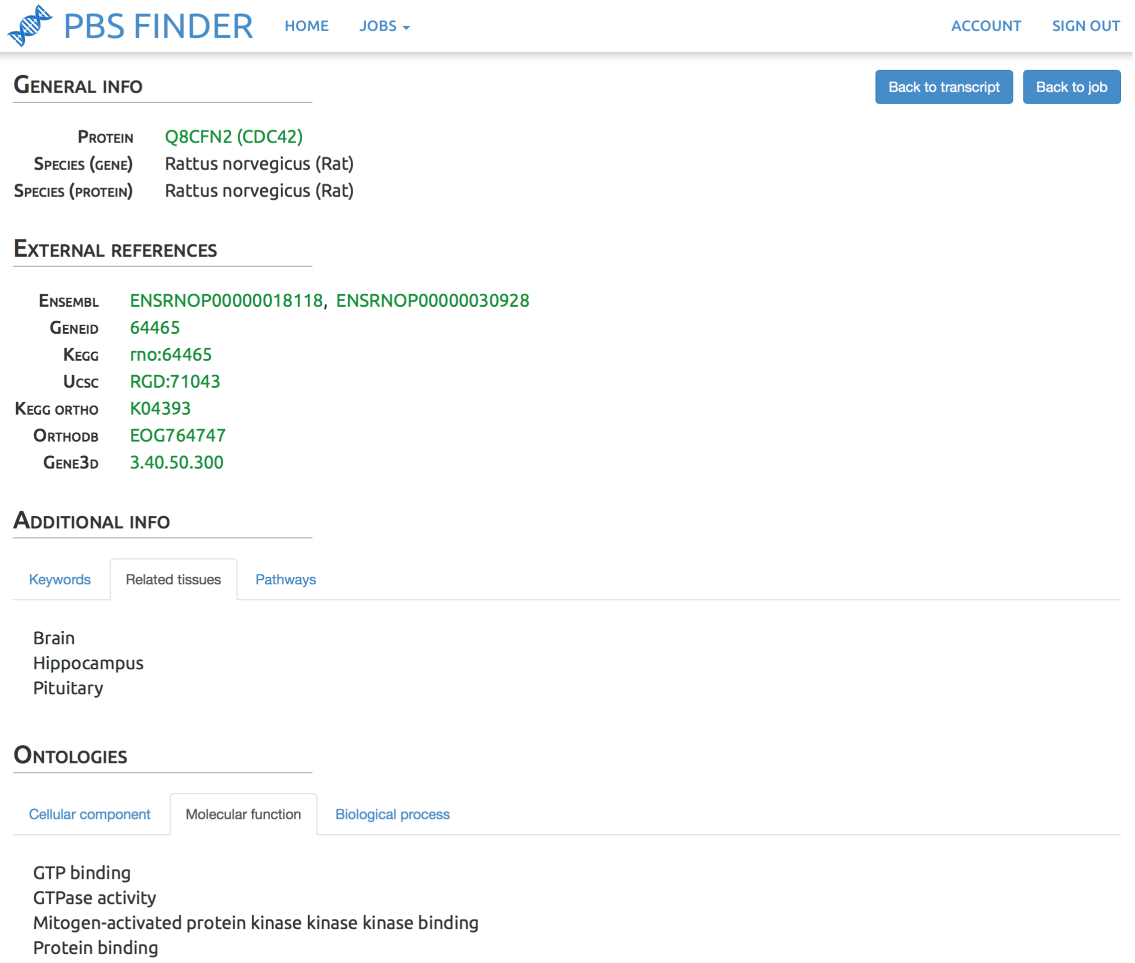
RBP LOCATIONS

Function	Protein	UniProt reference	Score	Start	End	Sequence
🟧	SNRPA	Q5U214	100%	826	832	AUUGCAC
🟧	SNRPA	Q5U214	100%	826	832	AUUGCAC
🟧	SNRPA	Q5U214	93%	826	833	AUUGCACC
🟧			86%	960	966	ACGAGAU
🟧			100%	826	832	AUUGCAC
🟦			81%	411	419	UUUUGUUUU
🟦	ELAVL2	Q8CH84	85%	286	294	UUUUGUUUU
🟦	ELAVL2	Q8CH84	85%	406	414	UUUUGUUUU
🟦	ELAVL2	Q8CH84	81%	286	294	UUUUGUUUU

Cluster 3

KEYWORDS Ribonucleoprotein

Figure A.2: Transcript view example. It contains four main areas: general information about the transcript; RNA sequences (5'UTR, 3'UTR and 3'UTR downstream are shown depending on availability); RBP locations histogram; and RBP location table (including clustering results and match sequences).


PBS FINDER
HOME
JOBS
ACCOUNT
SIGN OUT
Back to transcript
Back to job
Keywords
Related tissues
Pathways
Cellular component
Molecular function
Biological process

GENERAL INFO

PROTEIN	Q8CFN2 (CDC42)
SPECIES (GENE)	Rattus norvegicus (Rat)
SPECIES (PROTEIN)	Rattus norvegicus (Rat)

EXTERNAL REFERENCES

ENSEMBL	ENSRNOP00000018118, ENSRNOP00000030928
GENEID	64465
KEGG	rno:64465
UCSC	RGD:71043
KEGG ORTHO	K04393
ORTHODB	EOG764747
GENE3D	3.40.50.300

ADDITIONAL INFO

Brain
Hippocampus
Pituitary

ONTOLOGIES

GTP binding
GTPase activity
Mitogen-activated protein kinase kinase kinase binding
Protein binding

Figure A.3: Protein view example, containing four relevant areas: general information about the protein; links to other web platforms with relevant information about the protein (note that some of those platforms might not contain information about a particular protein, and therefore no links can be shown); additional info, including keywords, related tissues and pathways in which the protein is expressed; and lastly, information about the protein's ontologies, including cellular components, molecular functions and biological processes.

Appendix B

iRAP Example Configuration

```
# ==============================================================================
# Name of the experiment. (no spaces)
# All files produced by irap will be placed in a folder with the given name.
name=myexp

# ==============================================================================
# Name of the species.
species=homo_sapiens

# ==============================================================================
# FASTA file with the reference genome.
reference=Homo_sapiens.GRCh37.66.dna.fa

# ==============================================================================
# GTF file with the annotations.
gtf_file=Homo_sapiens.GRCh37.66.gtf

# ==============================================================================
# iRAP options (may be provided in the command line).

# Mapper
# mapper=<pick one supported by iRAP>

# Quantification method
# quant_method=<pick one supported by iRAP>

# Differential expression method
# de_method=<pick one supported by iRAP>

# Gene set enrichment (GSE) analysis
# gse_tool=piano

# Check data (reads) quality (on|off)
# qual_filtering=on

# Trim all reads to the minimum read size after quality trimming (y|n)
# (only applicable if qual_filtering is on)
# trim_reads=y

# Minimum base quality accepted (default is 10)
# min_read_quality=10

# Contamination check (cont_index parameter).  Reads that likely originate from
# organisms other than the one under study can be discarded during
# pre-processment of the reads. This is done by aligning the reads to the
# genomes of organisms that might be a source of contamination and discard
# those that map with a high degree of fidelity. By default iRAP will check if
# the data is contaminated by e-coli. An example to create a contamination
# "database" is provided in the examples/ex_add2contaminationDB.sh script. The
# value of the parameter should be the file name prefix of the bowtie index
# files.

# Disable contamination check
# cont_index=no

# Default value
# cont_index=$(data_dir)/contamination/e_coli

###########################################
# Miscellanious options

# Number of threads that may be used by iRAP
# max_threads=1

# Exon level quantification (y|n)
# exon_quant=y

# Transcript level quantification (y|n)
# transcript_quant=y

# =============================================================================
# Full or relative path to the directory where all the data can be found.
data_dir=data

# =============================================================================
# Only necessary if you intend to perform Differential Expression analysis.

# Contrasts
contrasts=purpleVsPink purpleVsGrey

# Definition of each constrast
purlpleVsPink=Purple Pink
```

```
purlpleVsGrey=Purple Grey

# Groups definition
Purlple=myLib1 myLib2
Pink=myLib3
Grey=myLib4

# Technical replicates
technical.replicates="myLib1,myLib2;myLib3;mylib4"

# ================================================================================
# Data
# *_rs      => read size
# *_qual    => quality encoding (33|64)
# *_sd      => standard deviation
# *_ins     => insert size

myLib1=f1.fastq
myLib1_rs=75
myLib1_qual=33

myLib2=f2.fastq
myLib2_rs=75
myLib2_qual=33

myLib3=f3_1.fastq f3_2.fastq
myLib3_rs=50
myLib3_qual=33
myLib3_ins=350
myLib3_sd=60

myLib4=f4_1.fastq f4_2.fastq
myLib4_rs=50
myLib4_qual=33
myLib4_ins=350
myLib4_sd=60

# List the names of your single-end (se) and paired (pe) libraries
se=myLib1 myLib2
pe=myLib3 myLib4
```

Appendix C

Examples of Biological Information Files

C.1 SAM Example

```
HD  VN:1.0 SO:coordinate
@SQ SN:seq1 LN:5000
@SQ SN:seq2 LN:5000
@CO Example of SAM/BAM file format.
B7_591:4:96:693:509 73  seq1  1 99  36M * 0 0
    CACTAGTGGCTCATTGTAAATGTGTGGTTTAACTCG  <<<<<<<<<<<<<<<;<<<<<<<<<5<<<<<;:<;7
    MF:i:18 Aq:i:73 NM:i:0  UQ:i:0  H0:i:1  H1:i:0
EAS54_65:7:152:368:113  73  seq1  3 99  35M * 0 0
    CTAGTGGCTCATTGTAAATGTGTGGTTTAACTCGT <<<<<<<<<<0<<<<655<<7<<<:9<<3/:<6): MF:i
    :18 Aq:i:66 NM:i:0  UQ:i:0  H0:i:1  H1:i:0
EAS51_64:8:5:734:57 137 seq1  5 99  35M * 0 0 AGTGGCTCATTGTAAATGTGTGGTTTAACTCGTCC
     <<<<<<<<<<<7;71<<;<;;<7;<<3;);3*8/5 MF:i:18 Aq:i:66 NM:i:0  UQ:i:0  H0:i:1
    H1:i:0
B7_591:1:289:587:906  137 seq1  6 63  36M * 0 0
    GTGGCTCATTGTAATTTTTTGTTTTAACTCTTCTCT  (-&----,----)-)-),'--)---',+-,),''*,
    MF:i:130  Aq:i:63 NM:i:5  UQ:i:38 H0:i:0  H1:i:0
EAS56_59:8:38:671:758 137 seq1  9 99  35M * 0 0
    GCTCATTGTAAATGTGTGGTTTAACTCGTCCATGG <<<<<<<<<<<<<<<;<;7<<<<<<<<7<<;:<5% MF:i
    :18 Aq:i:72 NM:i:0  UQ:i:0  H0:i:1  H1:i:0
EAS56_61:6:18:467:281 73  seq1  13  99  35M * 0 0
    ATTGTAAATGTGTGGTTTAACTCGTCCCTGGCCCA <<<<<<<<;<<<8<<<<<;8:;6/686&;(16666 MF:i
    :18 Aq:i:39 NM:i:1  UQ:i:5  H0:i:0  H1:i:1
EAS114_28:5:296:340:699 137 seq1  13  99  36M * 0 0
    ATTGTAAATGTGTGGTTTAACTCGTCCATGGCCCAG  <<<<<;<<<;<;<<<<<<<<<<<8<8<3<8;<;<0;
    MF:i:18 Aq:i:73 NM:i:0  UQ:i:0  H0:i:1  H1:i:0
```

C.2 VCF Example

```
##fileformat=VCFv4.0
##fileDate=20090805
##source=myImputationProgramV3.1
##reference=1000GenomesPilot-NCBI36
##phasing=partial
##INFO=<ID=NS,Number=1,Type=Integer,Description="Number of Samples With Data">
##INFO=<ID=DP,Number=1,Type=Integer,Description="Total Depth">
##INFO=<ID=AF,Number=.,Type=Float,Description="Allele Frequency">
##INFO=<ID=AA,Number=1,Type=String,Description="Ancestral Allele">
##INFO=<ID=DB,Number=0,Type=Flag,Description="dbSNP membership, build 129">
##INFO=<ID=H2,Number=0,Type=Flag,Description="HapMap2 membership">
##FILTER=<ID=q10,Description="Quality below 10">
##FILTER=<ID=s50,Description="Less than 50% of samples have data">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=GQ,Number=1,Type=Integer,Description="Genotype Quality">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Read Depth">
##FORMAT=<ID=HQ,Number=2,Type=Integer,Description="Haplotype Quality">
#CHROM POS     ID        REF ALT    QUAL FILTER INFO
                                 FORMAT      NA00001        NA00002
    NA00003
20     14370   rs6054257 G      A       29   PASS   NS=3;DP=14;AF=0.5;DB;H2
              GT:GQ:DP:HQ 0|0:48:1:51,51 1|0:48:8:51,51 1/1:43:5:.,.
20     17330   .         T      A       3    q10    NS=3;DP=11;AF=0.017
                  GT:GQ:DP:HQ 0|0:49:3:58,50 0|1:3:5:65,3   0/0:41:3
20     1110696 rs6040355 A      G,T     67   PASS   NS=2;DP=10;AF=0.333,0.667;AA=
    T;DB GT:GQ:DP:HQ 1|2:21:6:23,27 2|1:2:0:18,2   2/2:35:4
20     1230237 .         T      .       47   PASS   NS=3;DP=13;AA=T
                      GT:GQ:DP:HQ 0|0:54:7:56,60 0|0:48:4:51,51 0/0:61:2
20     1234567 microsat1 GTCT   G,GTACT 50   PASS   NS=3;DP=9;AA=G
                       GT:GQ:DP    0/1:35:4
```

C.3 FASTQ Example

```
@SRR014849.1 EIXKN4201CFU84 length=93
GGGGGGGGGGGGGGGGCTTTTTTTGTTTGGAACCGAAAGG
GTTTTGAATTTCAAACCCTTTTCGGTTTCCAACCTTCCAA
AGCAATGCCAATA
+SRR014849.1 EIXKN4201CFU84 length=93
3+&$#"""""""""""7F@71,’";C?,B;?6B;:EA1EA
1EA5’9B:?:#9EA0D@2EA5’:>5?:%A;A8A;?9B;D@
/=<?7=9<2A8==
```

C.4 FASTA Example

```
>gi|5524211|gb|AAD44166.1| cytochrome b [Elephas maximus maximus]
LCLYTHIGRNIYYGSYLYSETWNTGIMLLLITMATAFMGYVLPWGQMSFWGATVITNLFSAIPYIGTNLV
EWIWGGFSVDKATLNRFFAFHFILPFTMVALAGVHLTFLHETGSNNPLGLTSDSDKIPFHPYYTIKDFLG
LLILILLLLLLALLSPDMLGDPDNHMPADPLNTPLHIKPEWYFLFAYAILRSVPNKLGGVLALFLSIVIL
GLMPFLHTSKHRSMMLRPLSQALFWTLTMDLLTLTWIGSQPVEYPYTIIGQMASILYFSIILAFLPIAGX
IENY
```

C.5 GTF/GFF Example

```
## GFF
##gff-version 3
##sequence-region   ctg123 1 1497228
ctg123 . gene            1000  9000  .  +  .  ID=gene00001;Name=EDEN
ctg123 . TF_binding_site 1000  1012  .  +  .  ID=tfbs00001;Parent=gene00001
ctg123 . mRNA            1050  9000  .  +  .  ID=mRNA00001;Parent=gene00001;Name=
    EDEN.1
ctg123 . mRNA            1050  9000  .  +  .  ID=mRNA00002;Parent=gene00001;Name=
    EDEN.2
ctg123 . mRNA            1300  9000  .  +  .  ID=mRNA00003;Parent=gene00001;Name=
    EDEN.3
ctg123 . exon            1300  1500  .  +  .  ID=exon00001;Parent=mRNA00003
ctg123 . exon            1050  1500  .  +  .  ID=exon00002;Parent=mRNA00001,
    mRNA00002
ctg123 . exon            3000  3902  .  +  .  ID=exon00003;Parent=mRNA00001,
    mRNA00003

---------------------------------------------------

## GTF
140    Twinscan    inter         5141     8522     .    -    .    gene_id "";
    transcript_id "";
140    Twinscan    inter_CNS     8523     9711     .    -    .    gene_id "";
    transcript_id "";
140    Twinscan    inter         9712     13182    .    -    .    gene_id "";
    transcript_id "";
140    Twinscan    3UTR          65149    65487    .    -    .    gene_id "
    140.000"; transcript_id "140.000.1";
140    Twinscan    3UTR          66823    66992    .    -    .    gene_id "
    140.000"; transcript_id "140.000.1";
140    Twinscan    stop_codon    66993    66995    .    -    0    gene_id "
    140.000"; transcript_id "140.000.1";
```