RNASeqGUI

User Manual*

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Contents

1 Introduction **5**
- 1.1 Overview of RNASeqGUI R package 5
- 1.2 Other GUIs for RNASeq data analysis 6
- 1.3 Scope and availability 6

2 RGTK2 installation guide **8**
- 2.1 For Linux users 8
- 2.2 For MacOS users 8
- 2.3 For Windows users 9

3 Installation of R and the required R-packages **10**

4 Quick start **15**

5 What's new **16**

6 Structure of RNASeqGUI_1.0.0 main interface **19**

7 How to create a new project or select an existing one **23**

8 BAM EXPLORATION SECTION **25**
- 8.1 Bam Exploration Interface 25

9 COUNT SECTION **29**
- 9.1 Read Count Interface 29

10 PRE-ANALYSIS SECTION **32**
- 10.1 Data Exploration Interface 32
- 10.2 Normalization Interface 35
- 10.3 Filtering Interface 36

11 DATA ANALYSIS SECTION **38**
- 11.1 Data Analysis Interface 38
- 11.2 EdgeR Exact Test 40
- 11.3 EdgeR GLM for Multi-Factor Design 41
- 11.4 DESeq 42
- 11.5 DESeq Complex Design 44
- 11.6 DESeq2 45
- 11.7 DESeq2 Complex Design 47
- 11.8 NoiSeq 49
- 11.9 BaySeq 49

12 POST ANALYSIS SECTION **52**
12.1 Result Inspection Interface 52
12.2 Result Comparison Interface 54

13 GO/PATHWAY SECTION **55**
13.1 Graphite Interface 55
13.2 David Interface 55
13.3 Gage Interface 57

14 REPORT AND UTILITY SECTION **59**
14.1 Reproducible Research: the *Report* 59
14.2 Utility Interface 64

15 Usage Example **65**
15.1 Data Preparation 65
15.2 Usage of RNASeqGUI 67

16 How to customize RNASeqGUI **81**
16.1 Adding a new button in just three steps 81

17 Technical Details **83**

18 Errors/Warnings/Bugs **84**
18.1 Read Count Interface Errors 84
18.1.1 Warning messages: In .deduceExonRankings(exs... . . . 84

Acknowledgement **85**

RNASeqGUI

1 Introduction

1.1 Overview of RNASeqGUI R package

This manual describes *RNASeqGUI R package* [Russo Angelini 2014] that is a graphical user interface for the identification of differentially expressed genes from RNA-Seq experiments.
R (`http://cran.r-project.org/`) is an open source object oriented language for statistical computing and graphics. RNASeqGUI package includes several well known RNA-Seq tools, available as command line in `www.bioconductor.org`.

RNASeqGUI main interface is divided into seven sections. Each section is dedicated to a particular step of the data analysis process. The first section covers the exploration of the `bam` files. The second concerns the counting process of the mapped reads against a gene annotation file (GTF). The third focuses on the exploration of count-data and on data preprocessing, including the normalization procedures. The fourth is about the identification of the differentially expressed genes that can be performed by several methods, such as: **EdgeR Exact Test, Edge GLM for Multi-Factor Design, DESeq, DESeq for Complex Design, DESeq2, DESeq2 for Complex Design, NoiSeq, BaySeq**.
The fifth section regards the inspection of the results produced by these methods and the quantitative comparison among them.
The six section regards the Gene-Set and Pathway analysis.
Finally, in the spirit of **Reproducible Research** in the seventh section we find the **Report** button that the user can click to generate the report (in html format) that stores of all steps performed during the analysis. The report includes the documentation of the methods used along with the plots generated and all the chunks of codes that have been executed during the RNASeqGUI usage. Moreover, this section also contains the Utility Interface that allows different types of modifications of the input count files.
Cached Computation [Peng 2006] is used to speed up repetitive and computational expensive function calls by using results stored in pre-computed data-bases.
Moreover, results can be viewed and explored on a web browser thanks to *ReportingTools* [Huntley *et al.*, 2013] library that allows the user to navigate

through them.

1.2 Other GUIs for RNASeq data analysis

This package was implemented following and expanding the idea presented in [Villa-Vialaneix *et al.*, 2013] and in `http://tuxette.nathalievilla.org/?p=866&lang=en`.
The idea of RNASeqGUI is similar to that one presented in [Wettenhall *et al.*, 2004, Sanges *et al.*, 2007, Lohse *et al.*, 2012, Pramana *et al.*, 2013, Wettenhall *et al.*, 2006, Angelini *et al.*, 2008] with specific attention on RNA-Seq data analysis. Moreover, RNASeqGUI is designed to facilitate RNA-seq work-flow analysis (via its organization in several different sections and interfaces and via the inclusions of numerous concise and clear vignettes) and also to facilitate the extensibility of the GUI (via its software development organization that facilitate the task of expanding and redesign its interfaces). In fact, it is extremely easy to add new buttons that calls new functionalities. Therefore, a user can customize RNASeqGUI interfaces for his own purposes and benefits by adding the methods he needs mostly (for more details see **Section 16 How to customize RNASeqGUI: Adding a new button in just three steps**). Hence, we think that RNASeqGUI represents a useful and valid alternative to other existing GUIs.

1.3 Scope and availability

RNASeqGUI is an R package designed for the identification of differentially expressed genes across multiple biological conditions. This software is not just a collection of some known methods and functions, but it is designed to guide the user during the entire analysis process. Moreover, the GUI is also helpful for those who are expert R-users since it speeds up the usage of the included RNA-Seq methods drastically. Current implementation allows to handle the simple experimental design where the interest is on the experimental condition, future work will cover complex designs.

RNASeqGUI is freely available at (see Figure 1) :

`http://bioinfo.na.iac.cnr.it/RNASeqGUI/Download`

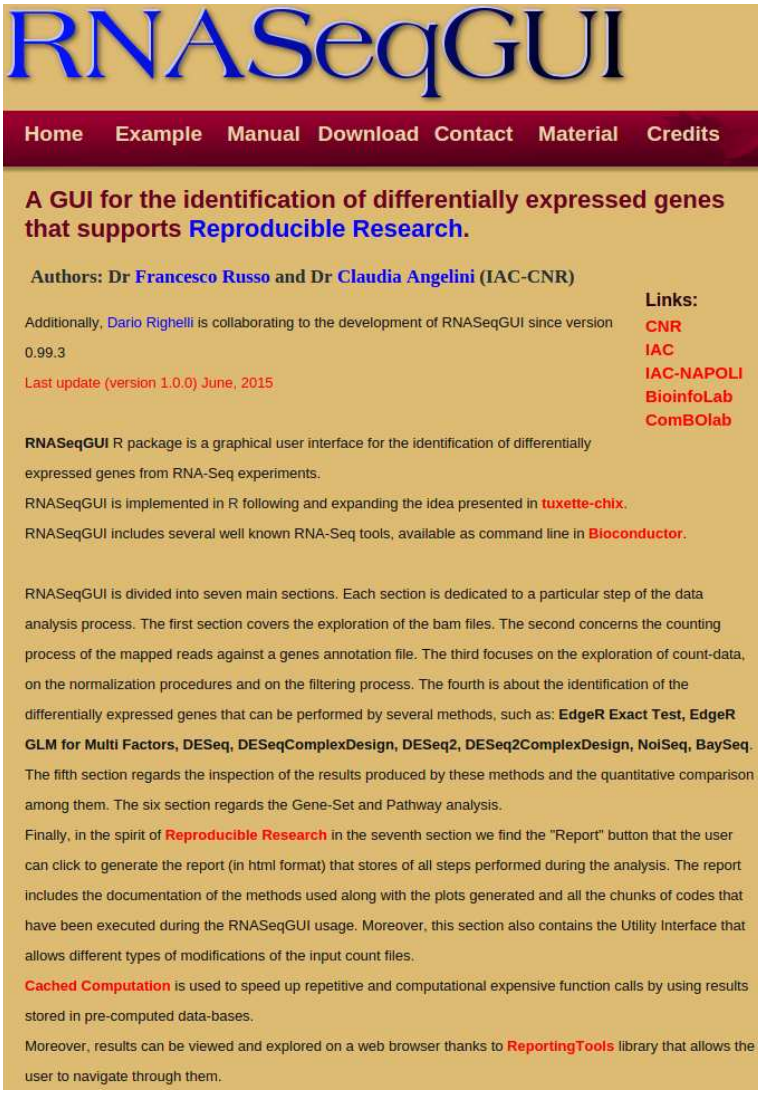


Figure 1: The `http://bioinfo.na.iac.cnr.it/RNASeqGUI` web page

2 RGTK2 installation guide

RNASeqGUI package requires the RGTK2 graphical library [Lawrence *et al.*, 2010] to run. The installation process consists in two steps. The first depends on the operating system (devoted to installation the GTK+ 2.0, an open-source GUI tool written in C). The second regards the required R packages.

2.1 For Linux users

We tested RNASeqGUI on Ubuntu 12.04 (precise) 64-bit, Kernel Linux 3.2.0-37-generic, GNOME 3.4.2.

1 - Open a terminal and type:

```
sudo apt-get update

sudo apt-get install libgtk2.0-dev
```

2 - Type:

```
sudo apt-get install libcurl4-gnutls-dev
```

3 - Type:

```
sudo apt-get install libxml2-dev
```

4 - Then, go to Section **3**.

2.2 For MacOS users

1 - Install `Xcode` developer tools (at least version 5.0.1) from Apple Store (it is free).

2 - Install `XQuartz-2.7.5.dmg` from `http://xquartz.macosforge.org/landing/`

3 - Install `GTK_2.24.17-X11.pkg` from `http://r.research.att.com`

WARNING: Please, install the binary version `GTK_2.24.17_X11.pkg` for Mac OS 10.6 Snow Leopard even though you have Mac OS 10.9 Mavericks.

4 - Then, go to Section **3**.

2.3 For Windows users

1 - download `gtk+-bundle_2.22.1-20101229_win64.zip` from `http://ftp.gnome.org/pub/gnome/binaries/win64/gtk+/2.22/` .

2 - This is a bundle containing the GTK+ stack and its dependencies for Windows. To use it, create some empty folder like $C:\backslash opt\backslash gtk$.

3 - Unzip this bundle.

4 - Now, you have to add the bin folder to your `PATH` variable. Make sure you have no other versions of `GTK+` in `PATH` variable. To do this, execute the following instructions: Open `Control Panel`, click on `System and Security`, click on `System`, click on `Advanced System Settings`, click on `Environment Variables`. In the `Environment Variables` window you will notice two columns `User variables` for a user name and `System variables`. Change the `PATH` variable in the `System variables` to be $C:\backslash opt\backslash gtk\backslash bin$.

5 - Then, go to Section **3**.

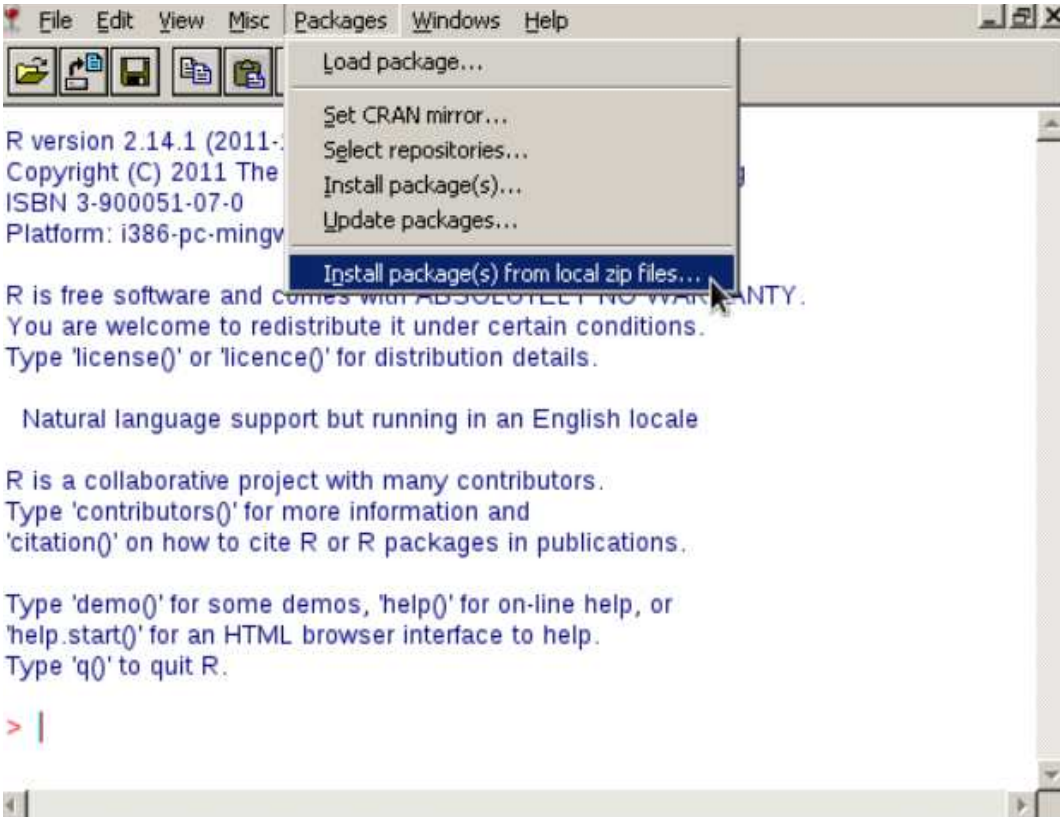


Figure 2: Select "Install packages(s) from local zip files", under the "Packages" pull-down menu.
From `http://outmodedbonsai.sourceforge.net/InstallingLocalRPackages.html`

3 Installation of R and the required R-packages

1 - Install **R version 3.1.2 (31/10/2015)** from `http://cran.r-project.org/` according to your operating system.

2 - Download RNASeqGUI package from `http://bioinfo.na.iac.cnr.it/RNASeqGUI/Download`. For Windows operating system, download the *zip* binary file. For MacOS and Linux download the *tar.gz* file.

- For Windows users: select "Install packages(s) from local zip files", under the "Packages" pull-down menu, as in the Figure 2.

- For MacOS users: under "Package and Data" pull-down menu, select "Package Installer", see Figure 3.

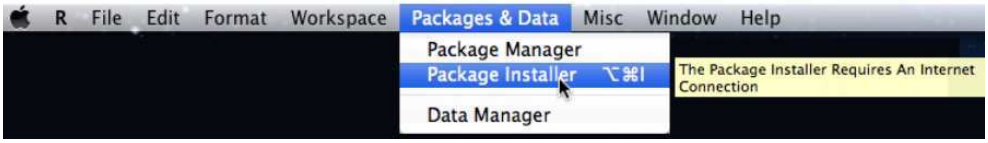


Figure 3: Under "Package and Data" pull-down menu, select "Package Installer". From `http://outmodedbonsai.sourceforge.net/InstallingLocalRPackages.html`

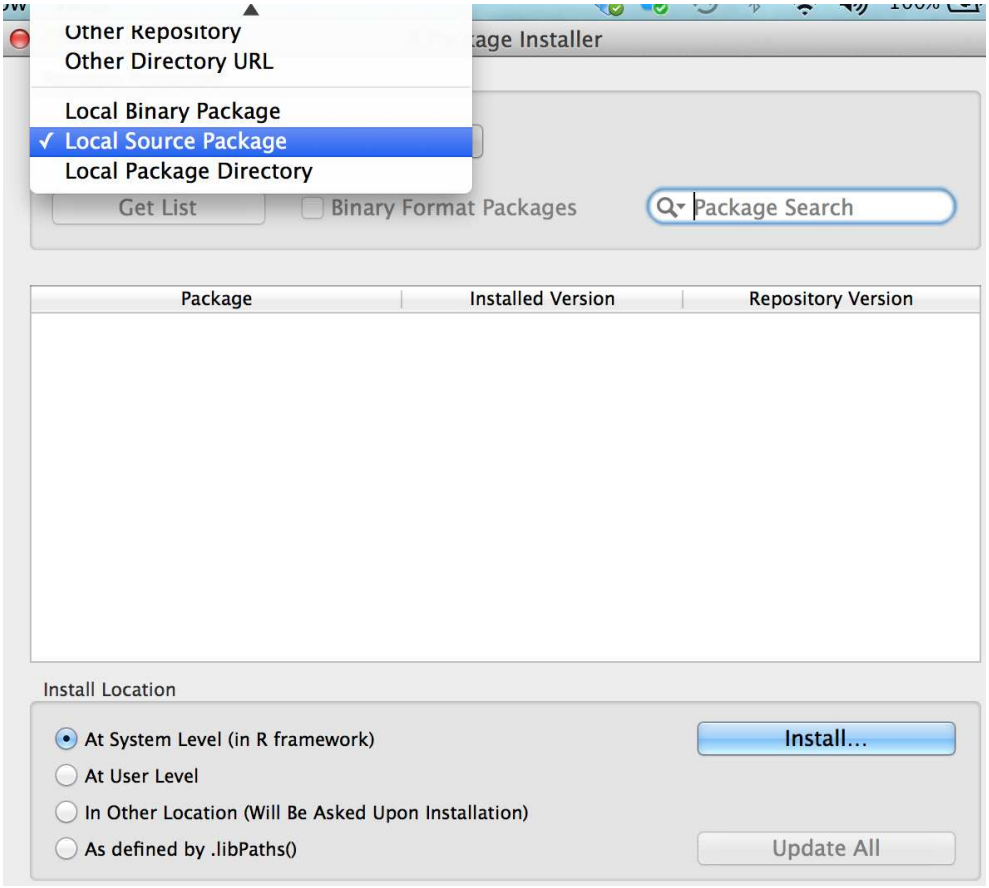


Figure 4: In the "Package Installer", pull down the top-left menu, select "Local Source Package" and navigate to where you have downloaded the source package.

In the "Package Installer", pull down the top-left menu, select "Local Source Package" and navigate to where you have downloaded the source package, see Figure 4.

- For Linux users: open a shell and go to the directory containing the package tree and type the command

```
sudo R CMD INSTALL -l /path/to/library RNASeqGUI
```

3 - Finally, if the libraries required by RNASeqGUI are not automatically downloaded and installed, we suggest the user to install all the packages that are needed to run RNASeqGUI package before loading it. Open R and type (the order of the list below is important):

For **MacOS***: go to* `http://cran.r-project.org/web/packages/RGtk2/index.html` *and choose the binary version for* `OS X Snow Leopard binaries: r-release: RGtk2_2.20.29.tgz`*. Then, in the "Package Installer", pull down the top-left menu and select "Local Binary Package".*

```
install.packages("e1071")
install.packages("ineq")
install.packages("RGtk2")
install.packages("RCurl")
install.packages("digest")
install.packages("ggplot2")
install.packages("RColorBrewer")
install.packages("VennDiagram")
install.packages("XML")
install.packages("tcltk")
install.packages("knitr")
install.packages("filehash")
install.packages("latticeExtra")
```

3 - Type (the order of the list below is important):

```
source("http://bioconductor.org/biocLite.R")
biocLite("biomaRt")
biocLite("DEXSeq")
biocLite("pasilla")
```

```
biocLite("GenomicRanges")
biocLite("GenomicFeatures")
biocLite("Rsamtools")
biocLite("edgeR")
biocLite("baySeq")
biocLite("NOISeq")
biocLite("DESeq")
biocLite("DESeq2")
biocLite("gplots")
biocLite("EDASeq")
biocLite("leeBamViews")
biocLite("preprocessCore")
biocLite("scatterplot3d")
biocLite("BiocParallel")
biocLite("digest")
biocLite("Rsubread")
biocLite("gage")
biocLite("pathview")
biocLite("biomaRt")
biocLite("ReportingTools")
biocLite("graphite")
```

4 - Once the installation is complete, please, check that all the packages listed above have been installed correctly. To see this, copy and paste the following list into `R` to see whether there are errors coming out.

```
library(e1071)
library(ineq)
library(RGtk2)
library(RCurl)
library(digest)
library(ggplot2)
library(RColorBrewer)
library(VennDiagram)
library(XML)
library(tcltk)
library(knitr)
library(filehash)
library(latticeExtra)
library(biomaRt)
library(DEXSeq)
```

```
library(pasilla)
library(GenomicRanges)
library(GenomicFeatures)
library(Rsamtools)
library(edgeR)
library(baySeq)
library(NOISeq)
library(DESeq)
library(DESeq2)
library(gplots)
library(EDASeq)
library(leeBamViews)
library(preprocessCore)
library(scatterplot3d)
library(BiocParallel)
library(digest)
library(Rsubread)
library(gage)
library(pathview)
library(biomaRt)
library(ReportingTools)
library(graphite)
```

In case an error message is displayed, repeat step 3 for the missing packages, otherwise go to Section **4**.

4 Quick start

If you have successfully gone through the installation you are ready to use RNASeqGUI, as follows.

1 - Open `R`.

2 - Type

```
library(RNASeqGUI)
```

in the R environment. Wait for the package to be loaded.

3 - Finally, type

```
RNASeqGUI()
```

After that, a dialog window, as that one shown in Figure 5, will appear and you can start interacting with the program.

5 What's new

- June, 2015 RNASeqGUI_1.0.0 was released

  In the version RNASeqGUI_1.0.0, we present some new features, such as:

  1 - New *Gage Interface* with the possibility to have three different types of conversion (from ENSEMBL ids or gene names or gene codes to ENTREZ genes).

  2 - A **Heatmap** function in the *Gage Interface*,

  3 - A **Pathway Barplot** function in the *Gage Interface*,

  4 - New Utility Interface with **Keep Columns** function,

  5 - New Utility Interface with **Round** function,

  6 - New *David Interface* for the pathway and Go analysis,

  7 - New **Heatmap** function in the *Data Exploration Interface* that can be applied to a provided list of genes,

  8 - Several minor bugs fixed.

- March 11, 2015 RNASeqGUI_0.99.4 was released

  In the version RNASeqGUI_0.99.4, we present some new features, such as:

  1 - Cached Computation was added,

  2 - Several minor bugs fixed.

- December 22, 2014 RNASeqGUI_0.99.3 was released

  In the version RNASeqGUI_0.99.3, we present some new features, such as:

  1 - Filtering Interface inside the PRE-ANALYSIS SECTION,

  2 - GENE/SET PATHWAY SECTION that contains *Graphite Interface* and *Gage Interface*,

  3 - **Convert** button inside the *Utility Interface*,

  4 - `NoiSeqBio` function automatically called when the user clicks on the **Run NoiSeq** button with biological replicates,

  5 - New function for **Plot all Counts** button inside the *Data Exploration Interface*,

  6 - Several bugs fixed.

- July 16, 2014 RNASeqGUI_0.99.2 was released

  In the version RNASeqGUI_0.99.2, we present some new features, such as:

  1 - Reactive Data Exploration via a web browser thanks to *ReportingTools* package (**Show Results** button for all the methods),

  2 - Reproducible Research thanks to *knitr* package (**Log file** button),

  3 - Complex Design Analysis for EdgeR, DESeq and DESeq2,

4 - Utility Interface,

5 - FeatureCounts (a new alternative method included in the *Read Count Interface*),

6 - Venn Diagrams DE 4 sets in the *Result Inspection Interface*,

7 - `bplapply` function of *BiocParallel* package was introduced again to speed up the *Count Section*.

- May 15, 2014 RNASeqGUI_0.99.1 was released

  In the version RNASeqGUI_0.99.1

  1 - We fix a bug present in DESeq and in DESeq2, since up and down regulated genes where swapped,

  2 - Minor point. In this version, we replaced "bplapply" function of BiocParallel with "lapply" function since with BiocParallel_0.4.1 RNASeqGUI worked fine, but with the latest version (BiocParallel_0.7.0) we found some problems. We are now trying to find out why things have changed.

- March 26, 2014 RNASeqGUI_0.99.0 was released

  First release of RNASeqGUI

6 Structure of RNASeqGUI_1.0.0 main interface

The RNASeqGUI main interface is divided into seven *Sections*, as shown in Figure 5, such as:

- **BAM EXPLORATION SECTION**,
- **COUNT SECTION**,
- **PRE-ANALYSIS SECTION**,
- **DATA ANALYSIS SECTION**,
- **POST ANALYSIS SECTION**,
- **GO/PATHWAY SECTION**,
- **REPORT AND UTILITY SECTION**.

Each section corresponds to a particular step of the RNA-Seq data analysis work-flow and contains one or more *Graphical Interfaces* that can be called by clinking the corresponding button.
Inside each interface, there is a `How to use this interface` button that displays a vignette to help the user to use the interface (see Figure 11) and there are several available *functionalities* (also called *functions* or *methods* in the rest of the manual). Each function takes specific inputs that can be numeric ones, strings or both and generate an output that can be a plot, a text file or both.
The sections of RNASeqGUI will be described one by one in the next sections of this manual.

Figure 5: Sections of RNASeqGUI_0.99.5 main interface

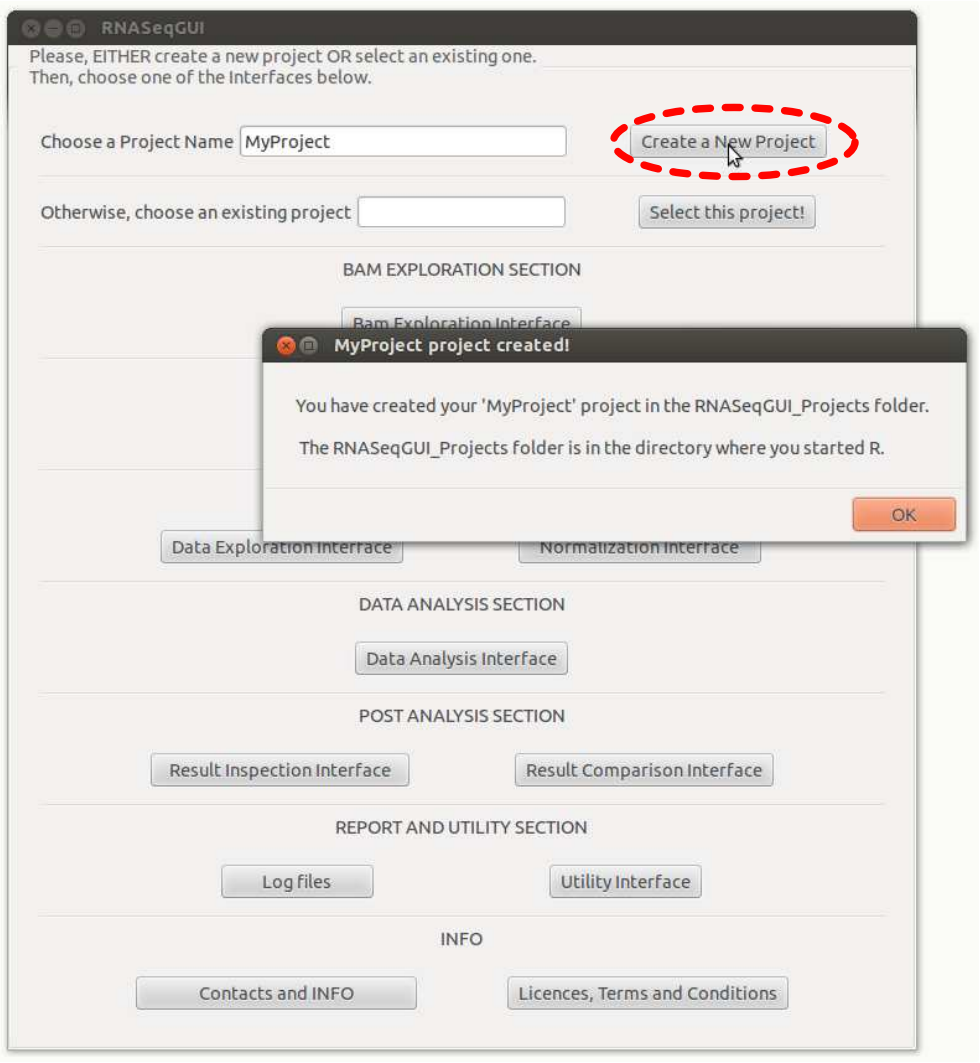


Figure 6: Creation of a new project

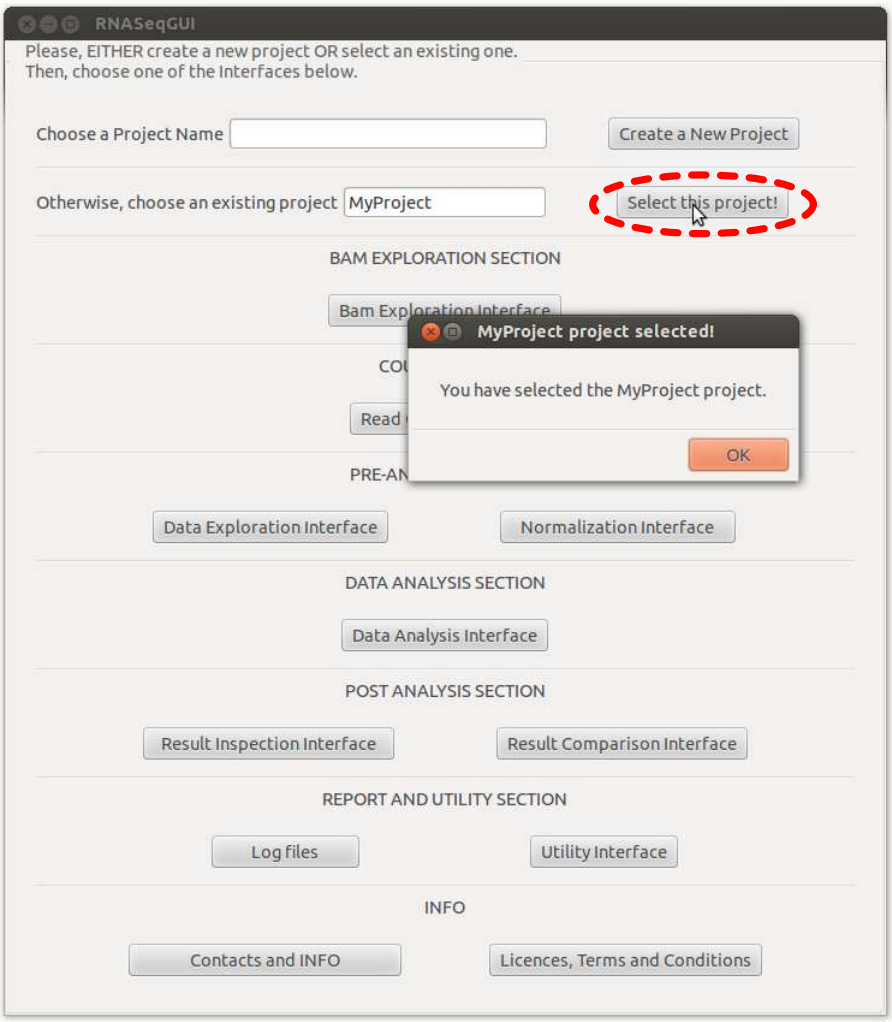


Figure 7: Selection of an existing project

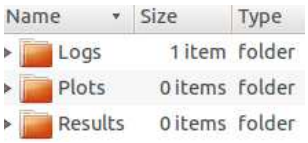


Figure 8: Structure of the **MyProject** directory

7 How to create a new project or select an existing one

To start using RNASeqGUI, you must either create a new project by choosing a name for it (suppose you choose as name `MyProject`) and then clicking on the `Create a New Project` button (see Figure 6) or select an existing project by typing the name and then clicking on the `Select this Project!` button (see Figure 7). The two cases are explained below.

1. In the first case, if you are using RNASeqGUI for the first time a directory called **RNASeqGUI_Projects** is created in your current working directory (type `getwd()` in the R environment to know where you are). Inside **RNASeqGUI_Projects** directory, a project folder is created with the name chosen by you (in this case with the name `MyProject`).

   At any moment, you can see or change your working directory with the following R commands, respectively.

   ```
   getwd()
   ```

   ```
   setwd("path/you/want/to/set")
   ```

   The creation of **RNASeqGUI_Projects** directory will only occur the first time you start using RNASeqGUI. Subsequently, when you click the `Create a New Project` button, RNASeqGUI checks whether the **RNASeqGUI_Projects** folder already exists in your working directory. If this folder, was already created then RNASeqGUI does not create a copy of it and all the projects you will create will be stored in it.

   Now, inside **RNASeqGUI_Projects**, you find **MyProjects** directory. Inside this directory, three folders are automatically created (see Figure 8), such as: **Logs**, **Results**, **Plots**.
   In the **Logs** folder, a `report.Rmd` file is created to report all the actions you perform and which parameters you use by performing those actions. A session information that summaries all the versions of the used packages is automatically written in the `report.Rmd` file (see Figure 40) at the creation of the project and each time you star this project

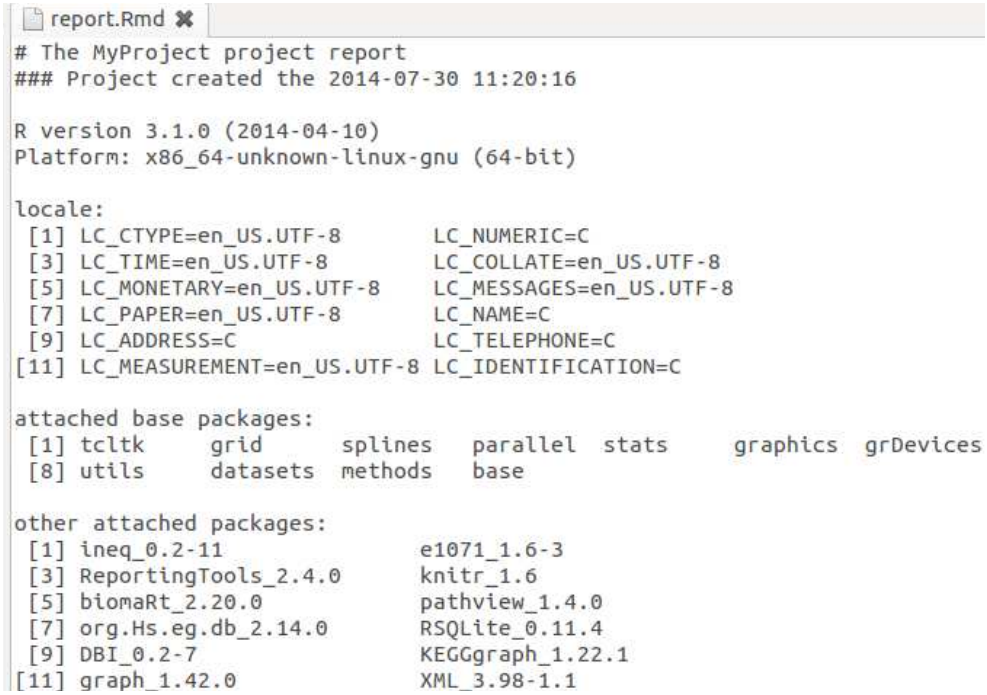


Figure 9: An example of the file `report.Rmd` automatically created in **Logs** directory at the creation of MyProject project. Note that the session information is included.

again.

2. In the second case, an existing project is selected, see Figure 7. RNASeqGUI checks whether the selected name already exists in the **RNASeqGUI _Projects** folder. If no project with the chosen name is found, a message warns the user that the selected project does not exist.
   When an existing project is restarted, RNASeqGUI continues to write in the same `report.Rmd` file created previously.

8 BAM EXPLORATION SECTION

8.1 Bam Exploration Interface

In the first section of the GUI, we find the *Bam Exploration Interface* (see Figure 10) that can be easily called by clicking the corresponding button. In this interface we find five different methods to explore the bam files: **Read Counts, Mean Quality of the Reads, Per Base Quality of Reads, Reads Per Chromosome, Nucleotide Frequencies**. Each of these functions takes a folder name as input. This input folder must contain all the bam files that the user wants to explore. To select the entire bam folder, select just one bam file inside the bam folder you want to use. The entire folder will be loaded. To use this interface you can also click on `How to use this Interface` button and a vignette window will appear on the screen describing the interface usage briefly, as shown in Figure 11.

- The **Read Counts** makes use of `barplot` function of the `graphics` package. This function returns an histogram (as the one shown in Figure 49) showing the number of mapped reads in each bam file (stored in the input folder) and a txt (tab-delimited) file summarizing the counts.
- The **Mean Quality of the Reads** makes use of `plotQuality` function of the `EDASeq` package [Risso *et al.*, 2011]. This function returns a plot showing the quality of each base of the reads averaged across all bam files.
- The **Per Base Quality of Reads** makes use of `plotQuality` function of the `EDASeq` package [Risso *et al.*, 2011]. This function returns as many box-plots as the number of bam files stored in the provided input folder. Each box-plot shows the quality of the reads per each base. This function makes use of `bplapply` function of the `BiocParallel` package [Morgan *et al.*, 2014] to parallelize the code in order to reduce the execution time.
- The **Reads Per Chromosome** makes use of `barplot` function of the `graphics` package. This function returns as many histograms as the number of bam files stored in the provided input folder. Each histogram shows the number of reads are present in each chromosome. This function makes use of `bplapply` function of the `BiocParallel` package [Morgan *et al.*, 2014] to parallelize the code in order to reduce the execution time.

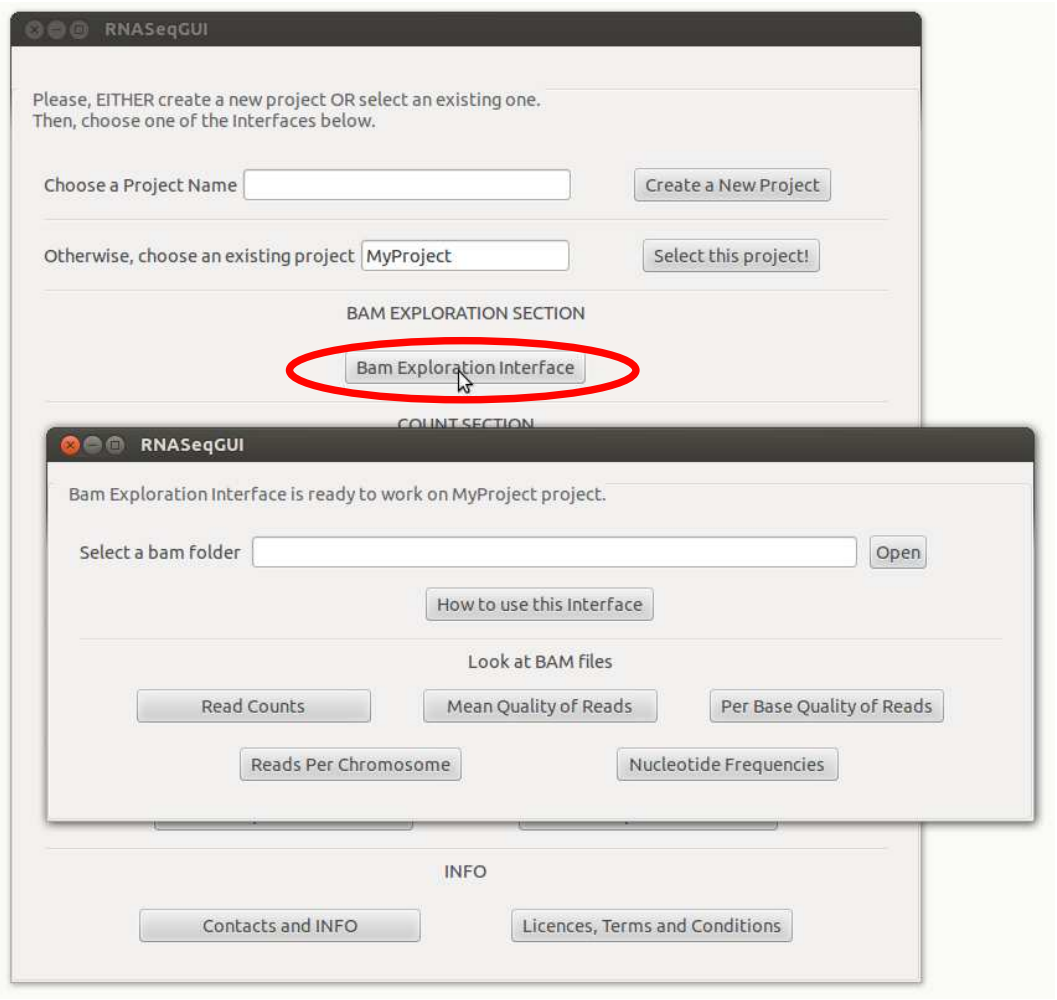


Figure 10: By clicking the **Bam Exploration Interface** button (in the red cycle), the interface to explore bam files will be displayed.

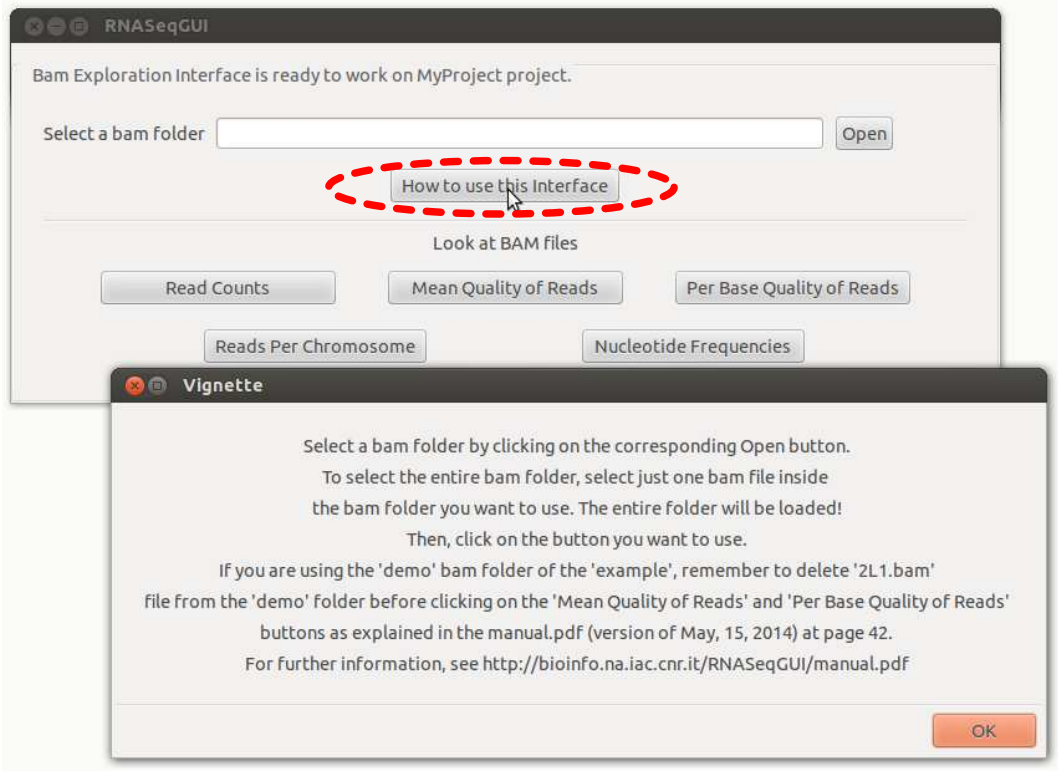


Figure 11: By clicking `How to use this Interface` button, a vignette window will appear on the screen.

- The **Nucleotide Frequencies** makes use of `plotNtFrequency` function of `EDASeq` package [Risso *et al.*, 2011]. This function returns a plot showing the percentage of each nucleotide at each position of the reads.

Figures will be stored in folder **Plots**, tables in folder **Results**.

9 COUNT SECTION

9.1 Read Count Interface

In the second section of the GUI, you find two functions for counting reads: **SummarizeOverlaps** [Lawrence *et al.*, 2013] and **FeatureCounts** [Liao *et al.*, 2013].

- **SummarizeOverlaps** takes four inputs (see Figure 12). The first input must be the name of the folder containing the bam files we want to process. The second input must be an annotation file in *GTF* format (General Transfer Format). The third input specifies the count mode that can be one of the following: `Union`, `IntersectionStrict` and `IntersectionNotEmpty`. The fourth input is `Ignore Strand?` check-box that allows to perform a strand specific counting task or not.

  The **SummarizeOverlaps** button calls `summarizeOverlaps` function of the the *GenomicRanges* package [Lawrence *et al.*, 2013] to obtain gene counts and returns a data-frame, as the one shown in Figure 13. The first column of this data-frame represents the `Gene Id`, while the other columns correspond to the names of the loaded bam files. The other entries report the number of reads that have hit a particular gene for each sample (see `www.bioconductor.org/packages/release/bioc/vignettes/GenomicRanges/inst/doc/summarizeOverlaps.pdf` for more information about the counting modes).

- The second one is **FeatureCounts** of the *Rsubread* package [Liao *et al.*, 2013]. This method takes four inputs (see Figure 12). The first input must be the name of the folder containing the bam files we want to process. The second input must be an annotation file in *GTF* format (General Transfer Format). The third input is the `Strand Number` field that can be one of the following: 0 (unstranded), 1 (stranded), 2 (reversely stranded). The fourth input is `Number of threads` field that specifies the number of the threads to use for the counting process. The fifth input is `Paired End?` check-box that allows the counting mode either for paired-end reads or for single-end ones.

  The **FeatureCounts** button calls `FeatureCounts` function of the Rsubread package to obtain gene counts and returns a data-frame, as the one shown in Figure 13. The first column of this data-frame represents

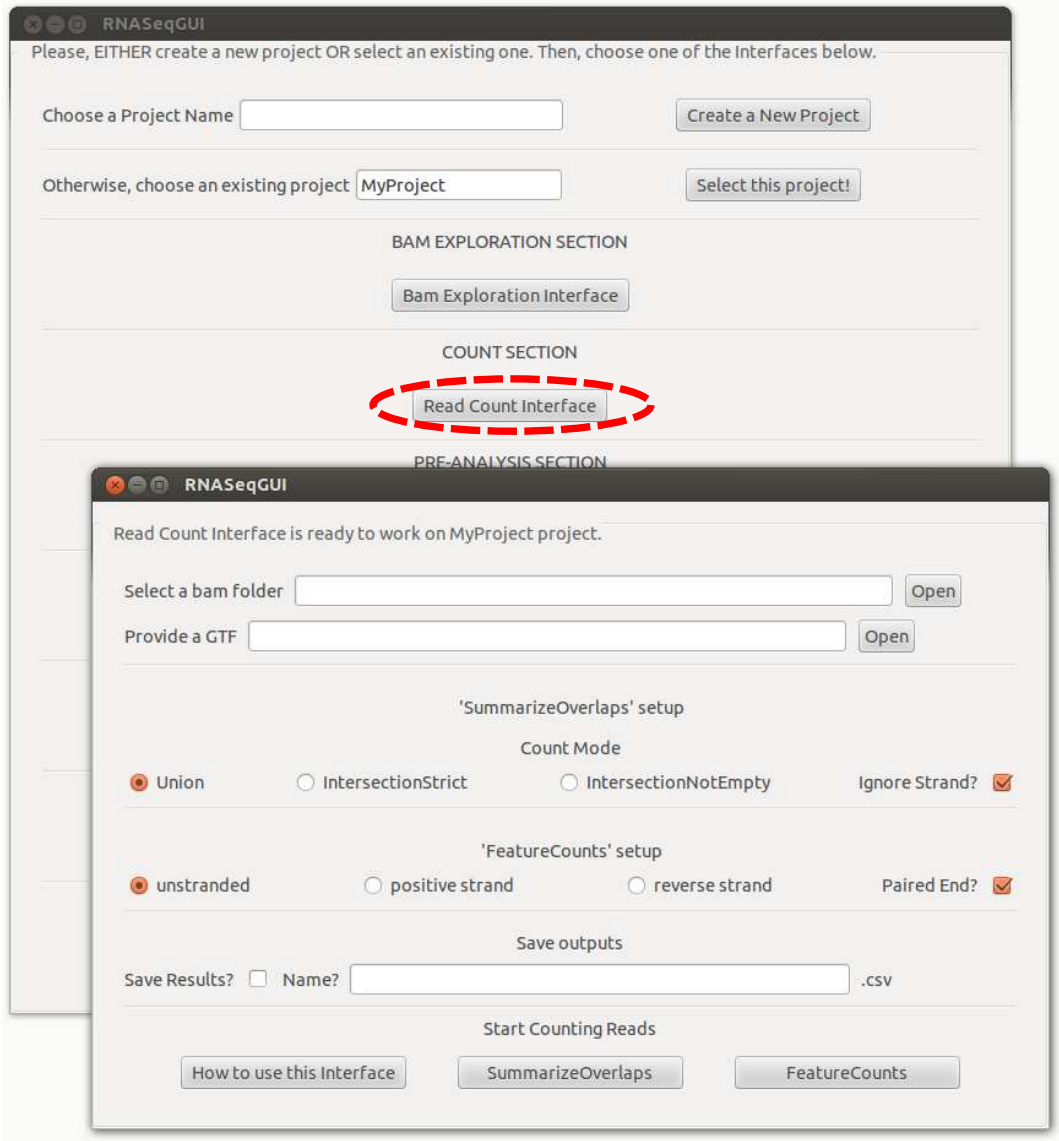


Figure 12: Read Count Interface

Gene Id	control_1	control_2	treated_1	treated_2
ENSG00000000003	455	463	583	598
ENSG00000000005	0	0	0	1
ENSG00000000419	1174	1210	1545	1533
ENSG00000000457	260	256	305	349
ENSG00000000460	550	607	709	741
.....	.....	.....	.....	.....
.....	.....	.....	.....	.....

Figure 13: An example of a count file with 20062 genes. The row names are given by the Gene Id in the annotation file (gtf), the column names are given by the alignment file names (the bam files)

the `Gene Id`, while the other columns correspond to the names of the loaded bam files. The other entries report the number of reads that have hit a particular gene for each sample (see `http://bioinformatics.oxfordjournals.org/content/30/7/923.full.pdf` for more information about the counting modes).

Read counting process can be a very computational demanding task, especially for large experiments with several samples and big alignment files. The R environment is not optimized from this point of view. Therefore, the counting task can be problematic on standard PC with limited clock speed and memory space. In this case, it could be beneficial either to process samples independently or to import count tables (in the format specified in Figure 13) in RNASeqGUI obtained from other tools, such as HTSeq-count (`www-huber.embl.de/users/anders/HTSeq/`). Therefore, this function makes use of `bplapply` function of the `BiocParallel` package [Morgan *et al.*, 2014] to parallelize the code in order to reduce the execution time.

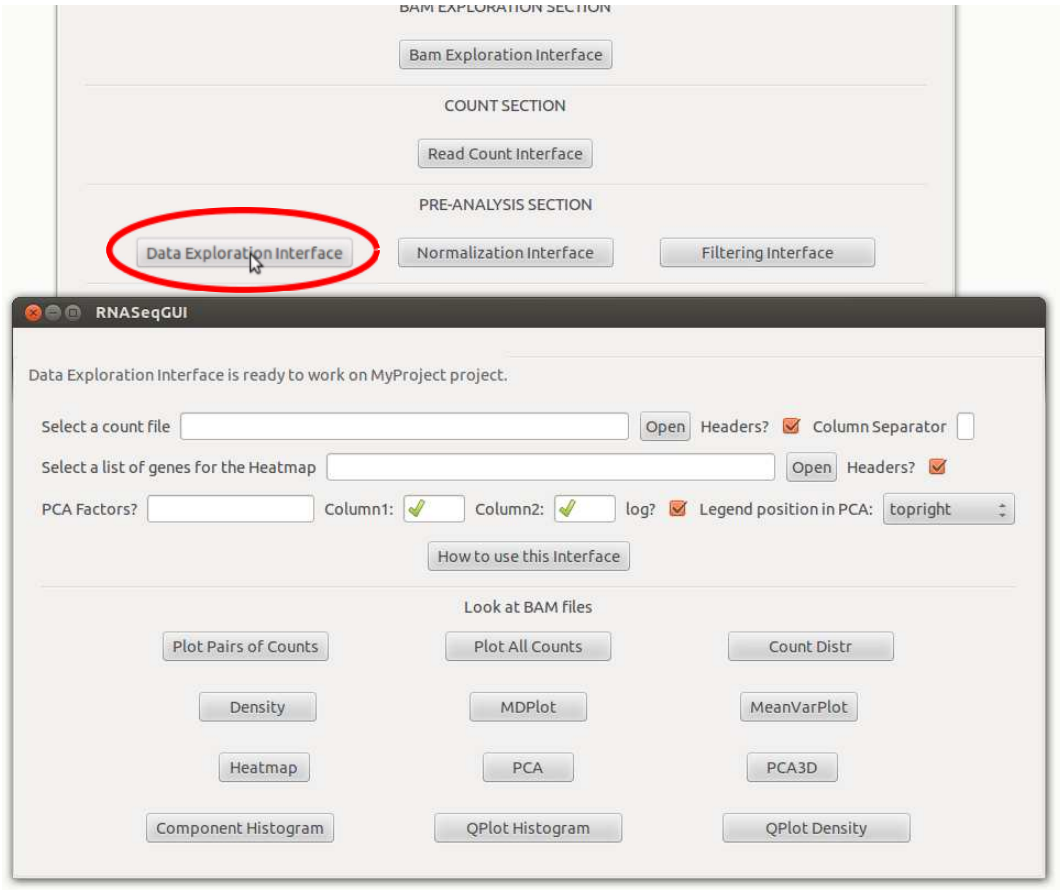


Figure 14: Data Exploration Interface

10 PRE-ANALYSIS SECTION

The third section of the GUI contains two interfaces: *Data Exploration Interface* (see Figure 14), *Normalization Interface* (see Figure 15) and *Filtering Interface* (see Figure 16). Both interfaces take an input count file that must be tab-delimited and must have the structure shown in Figure 13. The rows represent genes ids and the columns represent the samples.

10.1 Data Exploration Interface

In *Data Exploration Interface* there are twelve methods, such as: **Plot Pairs of Counts, Plot all Counts, Count Distr, Density, MDPlot, MeanVarPlot, Heatmap, PCA, PCA3D, Component Histogram, Qplot Histogram, Qplot Density**.

- The **Plot Pairs of Counts** makes use of `plot` function of the `graphics` package. This function takes a count file as input (in *txt* or *cvs* format) where the rows correspond to the gene ids and the columns correspond to the samples. This function also takes two integers, one specifying `Column1` and the other specifying `Column2` of the count file (see Figure 14) and plots the counts of sample in `Column1` against the counts of sample in `Column2`. Moreover, for this function it is possible to plot either the raw counts or the log of the counts (we add 1 to each number in the count file to avoid the problem of $log(0)$).

- The **Plot all Counts** makes use of `spm` function of the `car` package. This function takes a count file as input and produces all possible plots that can be generated by each column in the file against all the other columns. If the input text file has n columns then $n(n-1)$ plots will be produced. An example of this plot is shown in Figure 56. For this function, the `log` check box does not change anything.

- The **Count Distr** makes use of `boxplot` function of the `graphics` package. This function takes a count file as input and generates a box plot showing the distribution of the counts for each column in the file. An example of this plot is shown in Figure 54. Moreover, for this function it is possible to generate the box plot either of the raw counts or the log of the counts (we add 1 to each number in the count file to avoid the problem of $log(0)$).

- The **Density** makes use of `density` function of the `stats` package. This function takes a count file, and a sample specified by an integer in `Column1` as input and produces a curve representing the density function of the counts for the selected sample. The method is available in two modes. By default the log of the counts (we add 1 to each number in the count file to avoid the problem of $log(0)$) will be used to generate the density function. It is possible to uncheck this mode by clicking in the `log?` check-box (see Figure 14).

- The **MDPlot** makes use of `MDplot` function of the `EDASeq` package [Risso *et al.*, 2011]. This function takes a count file and two integers `Column1` and `Column2` and returns a plot showing the mean of the two selected columns against their difference gene by gene. For this function, the `log` check box does not change anything.

- The **MeanVarPlot** makes use of `meanVarPlot` function of the `EDASeq` package [Risso *et al.*, 2011]. This function takes a count file and returns

a plot showing the mean of all columns found in the file against the variance gene by gene. For this function, the `log` check box does not change anything.

- The **Heatmap** makes use of `heatmap` function of the `stats` package. This function takes a count file and an integer `N` in the `How many genes in the Heatmap?` field. The function returns an heat-map of the $\texttt{N}^{th}$ most expressed genes (on average). The columns of the heatmap are the samples, while the rows in the heat-map represent the gene ids of the most expressed ones. An example of heat-map is shown in Figure 58. Moreover, for this function it is possible to generate the heatmap either of the raw counts or the log of the counts (we add 1 to each number in the count file to avoid the problem of $log(0)$).

- The **PCA** makes use of `prcomp` function of the `stats` package. This function takes a count file, a comma separated sequence of strings (e.g.: `a,b,c,d`) indicating what are the labels for the legend, to be specified in the field `Factors` (see Figure 14) and `Legend position in PCA` that can be: topright, bottomright, topleft, bottomleft. The **PCA** function returns the principal component analysis plot between the first two components. An example of PCA plot is shown in Figure 57. For this function, the `log` check box does not change anything.

- The **PCA3D** makes use of `scatterplot3d` function of the `scatterplot3d` package. This function takes the same inputs of the **PCA** function and returns the 3D PCA plot between the first, the second and the third principal component. For this function, the `log` check box does not change anything.

- The **Component Histogram** makes use of `screeplot` function of the `stats` package. This function takes a count file and returns an histogram showing the variance level of each component. For this function, the `log` check box does not change anything.

- The **Qplot Histogram** makes use of `qplot` function of the `ggplot2` package. This function takes a count file and and returns an histogram showing the count level of each column in the count file. Moreover, for this function it is possible to generate the histogram either of the raw counts or the log of the counts (we add 1 to each number in the count file to avoid the problem of $log(0)$).

- The **Qplot Density** makes use of `qplot` function of the `ggplot2` package. This function takes a count file and and returns a plot showing

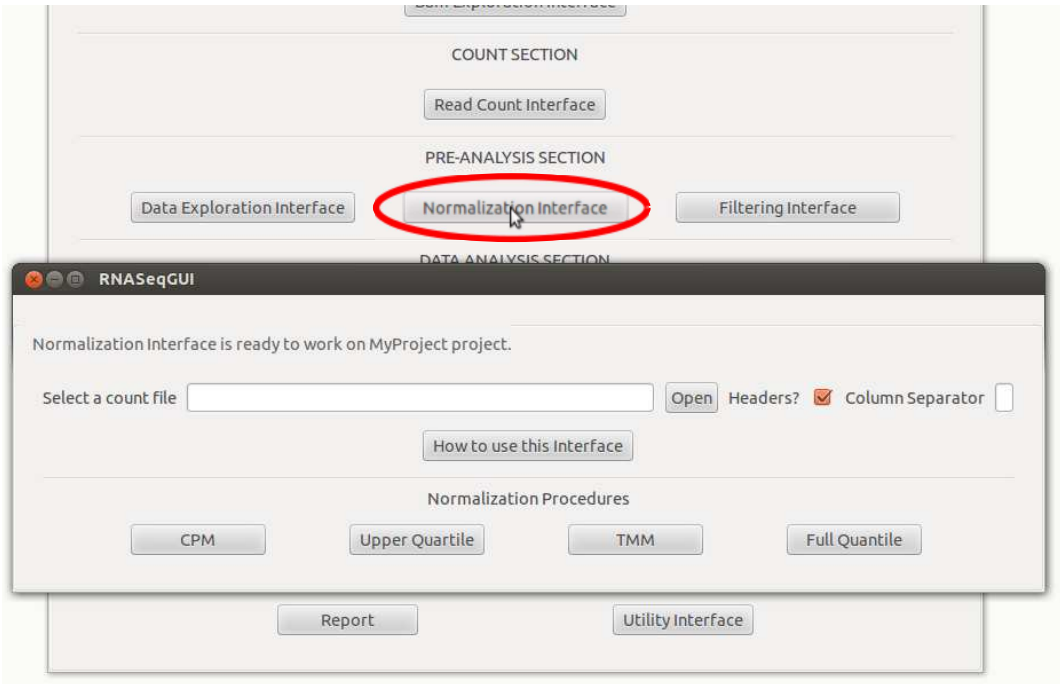


Figure 15: Normalization Interface

the density function of each column in the count file. Moreover, for this function it is possible to generate the density either of the raw counts or the log of the counts (we add 1 to each number in the count file to avoid the problem of $log(0)$).

10.2 Normalization Interface

The *Normalization Interface* (see Figure 15) includes four normalization procedures: **CPM, Upper Quartile, TMM, Full Quantile**.

- **CPM** makes use of `rpkm` function of the `NOISeq` package [Tarazona *et al.*, 2011]. This function takes a count file as specified in Figure 13 and returns a count file with normalized numbers. This function performs the RPKM [Mortazavi *et al.*, 2008] normalization.

- **Upper Quartile** makes use of `calcNormFactors` function of the `edgeR` package [Robinson *et al.*, 2010]. This function takes a count file as specified in Figure 13 and returns a count file with normalized numbers. This function performs the Upper Quartile [Bullard *et al.*, 2010] normalization.

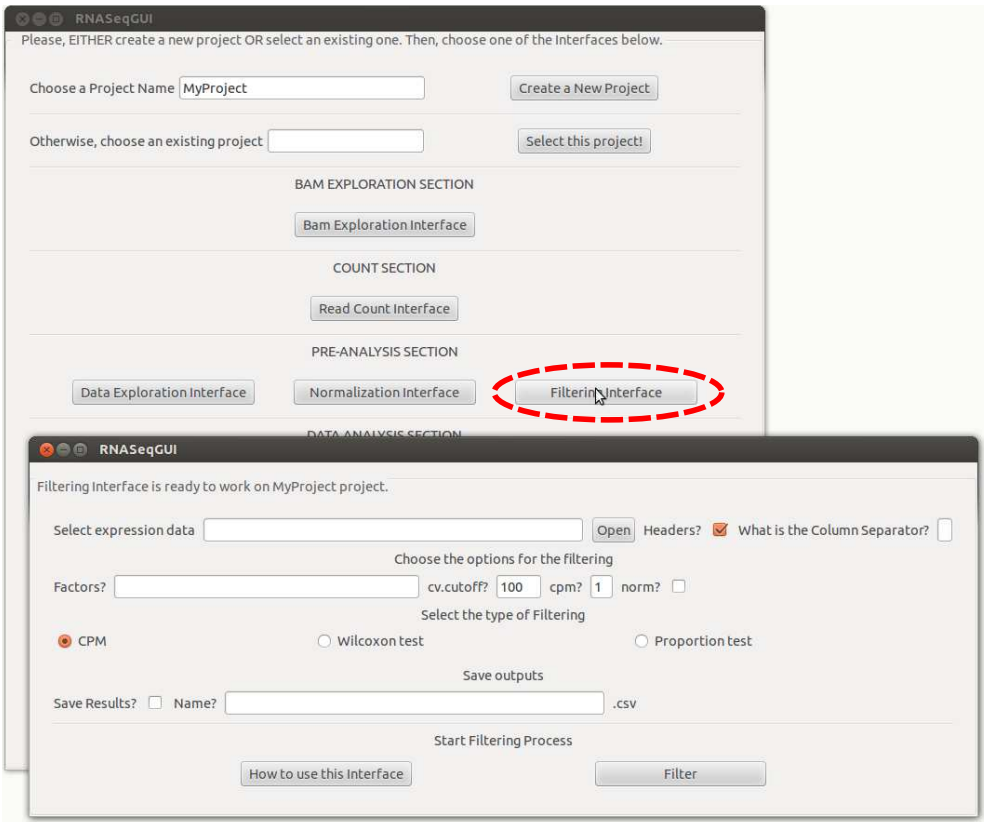


Figure 16: Filtering Interface

- **TMM** makes use of `calcNormFactors` function of the `edgeR` package [Robinson *et al.*, 2010]. This function takes a count file as specified in Figure 13 and returns a count file with normalized numbers. This function performs the TMM [Robinson *et al.*, 2010] normalization.

- **Full Quantile** makes use of `normalize.quantiles` function of the `preprocessCore` package. This function takes a count file as specified in Figure 13 and returns a count file with normalized numbers. This function performs the Full Quantile [Bolstad *et al.*, 2003, Smyth *et al.*, 2005] normalization.

10.3 Filtering Interface

In the *Filtering Interface* there is the **Filter** button as shown in Figure 16.

Select expression data by clicking on the corresponding `Open` button.
In the `Factors?` field, specify the factors of the expression data separated

by columns.
Choose a `cv.cutoff` that is a cutoff for the coefficient of variation per condition to eliminate features with inconsistent expression values. To be used only for CPM method.
Choose a `cpm` (counts per million) under which a feature is considered to have low counts in a sample.
The `cpm` for a condition with s samples is cpm x s. To be used only for CPM method and the Proportion test method.
Check the `norm` check-box to specify if the data have been normalized or not.
Select with the radio button the method to be used: `CPM`, `Wilcoxon test` or `Proportional test`.
Finally, click on **Filter** button.
For further information, see
`www.bioconductor.org/packages/release/bioc/vignettes/NOISeq/inst/doc/NOISeq.pdf`
at page 14.

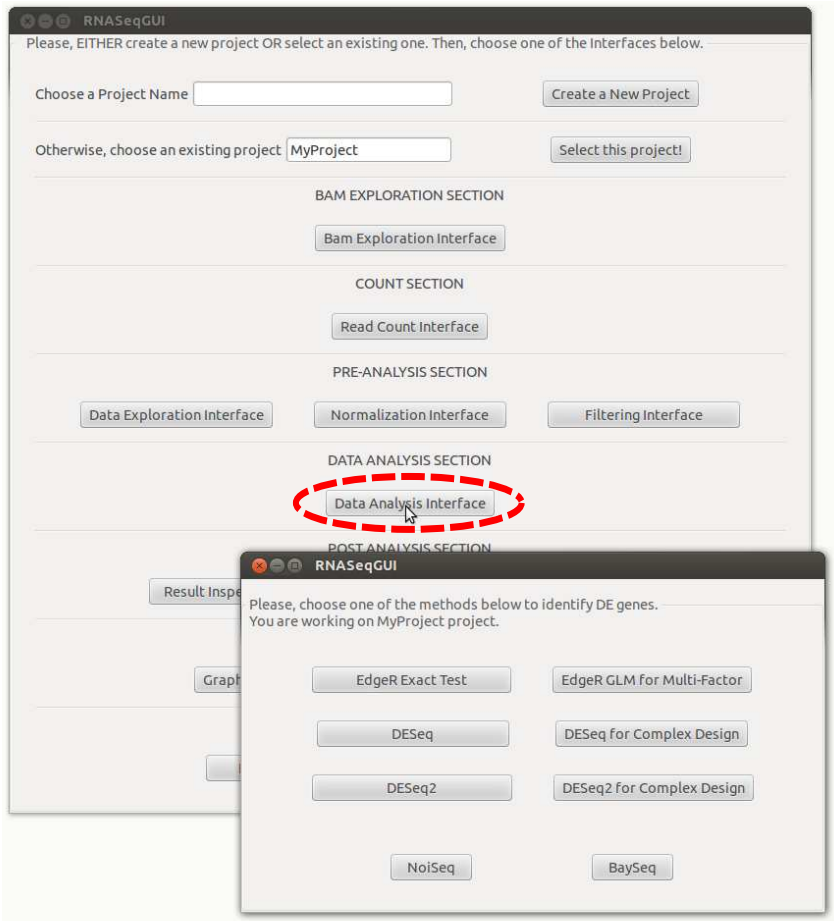


Figure 17: Data Analysis Interface

11 DATA ANALYSIS SECTION

11.1 Data Analysis Interface

This section contains the Data Analysis Interface shown in Figure 17 and represents the core of RNASeqGUI. This interface includes eight different statistical methods to detect differentially gene expression, such as: **EdgeR, EdgeRComplexDesign, DESeq, DESeqEdgeRComplexDesign, DESeq2, DESeq2EdgeRComplexDesign, NoiSeq, BaySeq**.

Results of all methods can be viewed and explored on a web browser thanks to *ReportingTools* [Huntley *et al.*, 2013] library that allows the user to navigate through them (see figure Figure 59).

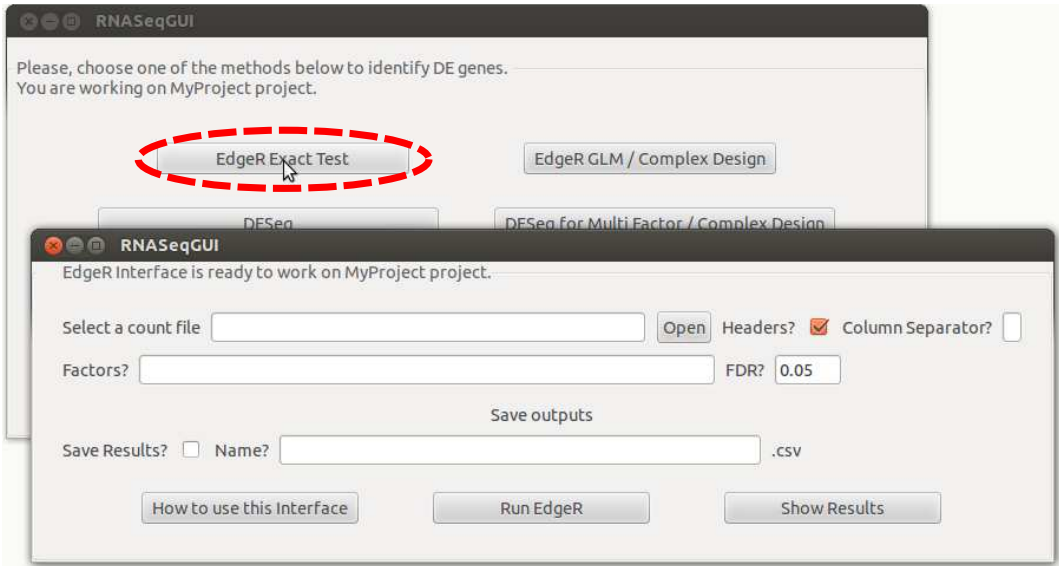


Figure 18: EdgeR interface

id	logFC	logCPM	PValue	FDR
ENSG..003	0.023	9.181	0.736	1
ENSG..005	2.357	1.058	1	1
ENSG..419	0.072	10.003	0.178	0.571
ENSG..457	-0.043	8.418	0.612	0.966
ENSG..460	-0.0006	9.164	1	1
ENSG..938	2.5e-15	0.888	1	1
ENSG..971	0.078	1.472	1	1
.....	.....	.....	.....	.....
.....	.....	.....	.....	.....

Figure 19: The first text file produced by the EdgeR method. The first column reports the gene ids, `logFC` reports the log of the fold-changes, `logCPM` reports the the log of the counts per million, `PValue` reports the p-values and `FDR` reports the false discovery rates calculated by the Benjamini and Hochberg's algorithm.

id	logFC	logCPM	PValue	FDR
ENSG..3756	-0.151	10.652	0.001	0.035
ENSG..4777	-0.523	8.455	2.6e-10	4.3e-08
ENSG..5961	-0.506	6.340	0.002	0.049
ENSG..6025	-0.577	8.699	2.8e-14	7.1e-12
ENSG..6047	-0.627	6.027	0.001	0.027
ENSG..6118	-0.152	10.456	0.001	0.039
ENSG..6282	-0.418	9.966	1.0e-14	3.3e-12
.....	.....	.....	.....	.....
.....	.....	.....	.....	.....

Figure 20: The EdgeR second text file showing the differentially expressed genes only. Columns are the same as in Figure 19.

11.2 EdgeR Exact Test

- The **EdgeR** method [Robinson *et al.*, 2007, Robinson *et al.*, 2008] [Robinson *et al.*, 2010, McCarthy *et al.*, 2012] (see Figure 18) takes an input count file (as the one shown in Figure 13) via the `Open` button.
  In the `Factors?` field the user can specify each condition of the count file loaded.
  In the `FDR?` field the user can specify the False Discovery Rate corrected by the Benjamini and Hochberg's algorithm to infer which are the differentially expressed genes.
  Finally, click on the **Run EdgeR** button.

  Run EdgeR returns two text files and two plots.

  The first text file shows the overall result obtained by edgeR (see Figure 19), while the second text file extracts the subset of differentially expressed genes only (see Figure 20).

  The output count file is saved with the name specified by the user in the `Name?` field (see Figure 18).
  If no name is specified by the user, then the first output count file is named with the name of the input file plus "`_results_EdgeR.txt`" suffix. The second file is named with the name of the input file plus "`_fdr=0.05_DE_genes_EdgeR.txt`" suffix, where 0.05 is the chosen FDR. Both text files are saved in the **Results** folder.

  The first plot shows the Biological Coefficient of Variation for a given CPM (Count Per Million) and is named with the name of the input file plus "`_Dispersion_EdgeR.pdf`" suffix. The second plot shows the

relative similarities of the samples and is named with the name of the input file plus "_MDS_EdgeR.pdf" suffix. Both plots are saved in the **Plots** folder.

11.3 EdgeR GLM for Multi-Factor Design

If you want to perform a multiple test or you have a more complex design you can use the *EdgeR GLM for Multi Factor* interface (see Figure 21).
Suppose you have two treatments (T1, T2) and one control (U). For instance, `Factors?: U, U, T1, T1, T2, T2`.
In the `LibTypes?` field the user can specify an extra feature regarding the factors.
Suppose that `LibTypes` specifies the type of reads used in your experiment for each factor.
For instance, `LibTypes?:single-end,paired-end,single-end,paired-end, paired-end,single-end`.
Finally, you need to specify the `Coefficient?` field.
Set `Coefficient?: 2` , to compare T1 vs U
Set `Coefficient?: 3` , to compare T2 vs U
`Coefficient?: 1` , should not be used.
Finally, click on the **Run EdgeRComplexDesign** button.
For further information, see `www.bioconductor.org/packages/release/bioc/vignettes/edgeR/inst/doc/edgeR.pdf` .

Run EdgeRComplexDesign returns two text files and two plots.

The first text file shows the overall result obtained by **Run EdgeRComplexDesign**, while the second text file extracts the subset of differentially expressed genes only.

The output count file is saved with the name specified by the user in the `Name?` field (see Figure 21).
If no name is specified by the user, then the first output count file is named with the name of the input file plus "_results_EdgeRComplexDesign.txt" suffix. The second file is named with the name of the input file plus "_fdr=0.05_DE_genes_EdgeREdgeRComplexDesign.txt" suffix, where 0.05 is the chosen FDR. Both text files are saved in the **Results** folder.

The first plot shows the Biological Coefficient of Variation for a given CPM (Count Per Million) and is named with the name of the input file plus "_Dispersion_EdgeREdgeRComplexDesign.pdf" suffix. The

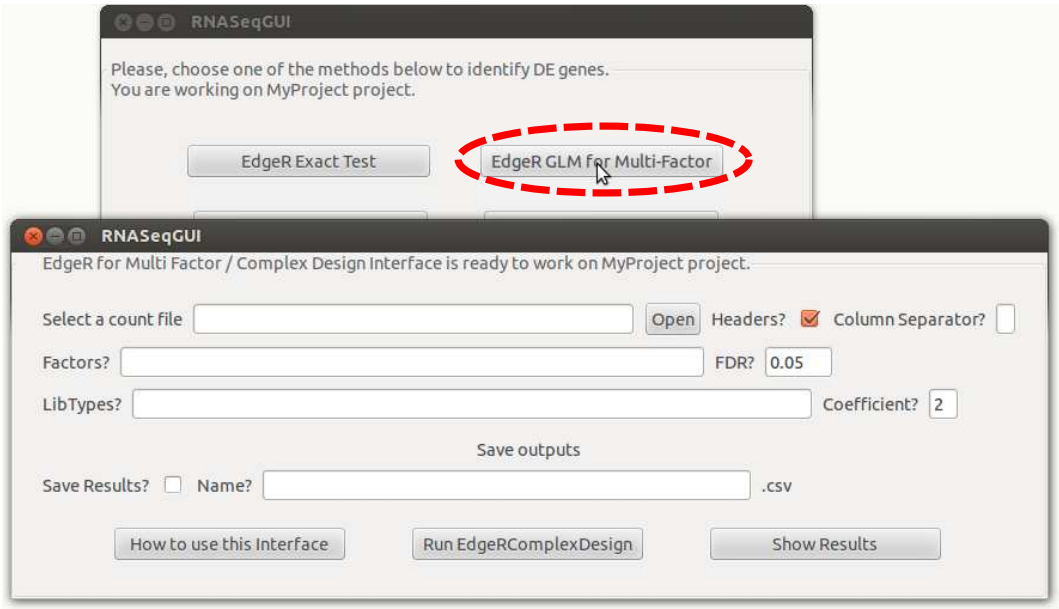


Figure 21: Run EdgeR GLM for Multi Factor

second plot shows the relative similarities of the samples and is named with the name of the input file plus "`_MDS_EdgeRComplexDesign.pdf`" suffix. Both plots are saved in the **Plots** folder.

11.4 DESeq

- The **DESeq** method [Anders *et al.*, 2010] (see Figure 22) takes an input count file (as the one shown in Figure 13) via the `Open` button.
  In the `Factors?` field the user can specify each condition of the count file loaded.
  In the `Padj?` field the user can specify the P-value adjusted corrected by the Benjamini and Hochberg's algorithm to infer which are the differentially expressed genes.
  In the `LibTypes?` field the user can specify an extra feature regarding the factors.
  For the count example in the Figure 13, `LibTypes?` is set to be: `paired-end,paired-end,paired-end,single-end`.
  In the `Treated` field the user can specify which factor is the treated one.
  In the `Control` field the user can specify which factor is the control one.

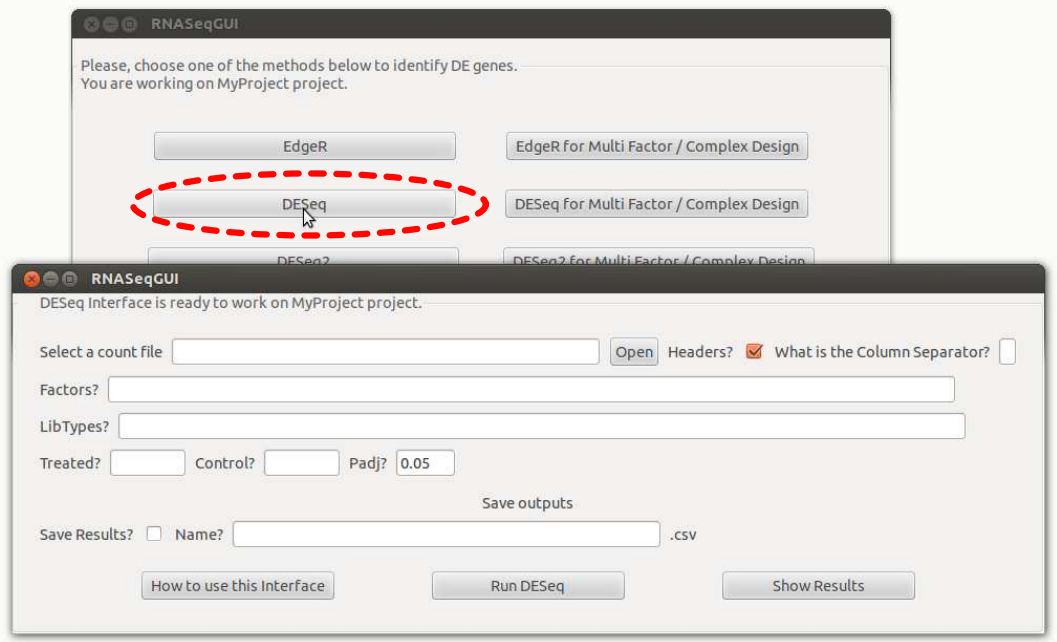


Figure 22: DESeq interface

Finally, click on the **Run DESeq** button.

Run DESeq returns two text files and two plots.

The first text file shows the results of this method (see Figure 24), while the second text file shows the differentially expressed genes only.

The output count file is saved with the name specified by the user in the `Name?` field (see Figure 22).

If no name is specified by the user, then the first output count file is named with the name of the input file plus "`_results_DESeq.txt`" suffix.

The second file is named with the name of the input file plus "`_padj=0.05_DE_genes_DESeq.txt`" suffix, where 0.05 is the chosen p-value adjusted.

Both text files are saved in the **Results** folder. The generated plot shows the dispersion value for a given mean of normalized counts.

This plot is named with the name of the input file plus "`_Dispersion_DESeq.pdf`" suffix and it is saved in the **Plots** folder.

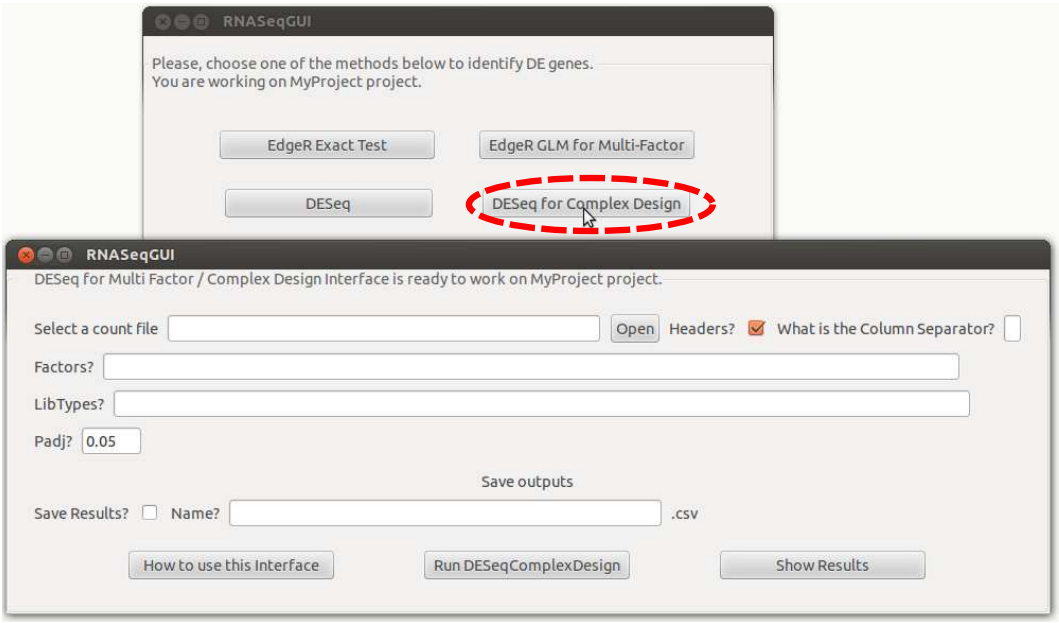


Figure 23: DESeq Multi Factor / Complex Design

11.5 DESeq Complex Design

If you want to perform a multiple test or you have a more complex design you can use the *DESeq Complex Design* interface (see Figure 23).
Suppose you have two treatments (T1, T2) and one control (U). For instance, `Factors?: U, U, T1, T1, T2, T2`.
In the `LibTypes?` field the user can specify an extra feature regarding the factors.
Suppose that `LibTypes` specifies the type of reads used in your experiment for each factor.
For instance, `LibTypes?:single-end,paired-end,single-end,paired-end, paired-end,single-end`.
Finally, click on the **Run DESeqComplexDesign** button.
A file with For further information, see `www.bioconductor.org/packages /release/bioc/vignettes/DESeq/inst/doc/DESeq.pdf` .

Run DESeqComplexDesign returns two text files and two plots.

The first text file shows the results of this method, while the second text file shows the differentially expressed genes only.

id	baseMean	baseMeanA	baseMeanB	foldChange	log2FoldChange	pval	padj
ENSG...0003	625.025	630.902	619.147	0.981	-0.027	0.774	1
ENSG...0005	0.264	0.528	0	0	-Inf	0.985	1
ENSG...0419	1106.882	1136.118	1077.646	0.948	-0.076	0.297	0.935
ENSG...0457	367.367	362.361	372.374	1.027	0.039	0.744	1
ENSG...0460	617.493	618.055	616.931	0.998	-0.002	0.982	1
....	.....	.....	.....	.....	.....	.....	...
....	.....	.....	.....	.....	.....	.....	...

Figure 24: DESeq output. The first column reports the gene ids, `baseMean` reports the mean normalised counts, averaged over all samples from both conditions, `baseMeanA` reports the mean normalised counts from condition A, `baseMeanB` mean normalised counts from condition B, `foldChange` reports the fold changes from condition A to B, `log2FoldChange` reports the logarithm (to basis 2) of the fold changes, `pval` reports the p values for the statistical significance and `padj` reports the p values adjusted for multiple testing calculated by the Benjamini-Hochberg algorithm.

The output count file is saved with the name specified by the user in the `Name?` field.

If no name is specified by the user, then the first output count file is named with the name of the input file plus "`_results_DESeqComplexDesign.txt`" suffix.

The second file is named with the name of the input file plus "`_padj=0.05_DE_genes_DESeqDESeqComplexDesign.txt`" suffix, where 0.05 is the chosen p-value adjusted.

Both text files are saved in the **Results** folder. The generated plot shows the dispersion value for a given mean of normalized counts.

This plot is named with the name of the input file plus "`_Dispersion_DESeqComplexDesign.pdf`" suffix and it is saved in the **Plots** folder.

11.6 DESeq2

- The **DESeq2** method [Anders *et al.*, 2010] (see Figure 25) takes an input count file (as the one shown in Figure 13) via the `Open` button and returns two text files and three plots.

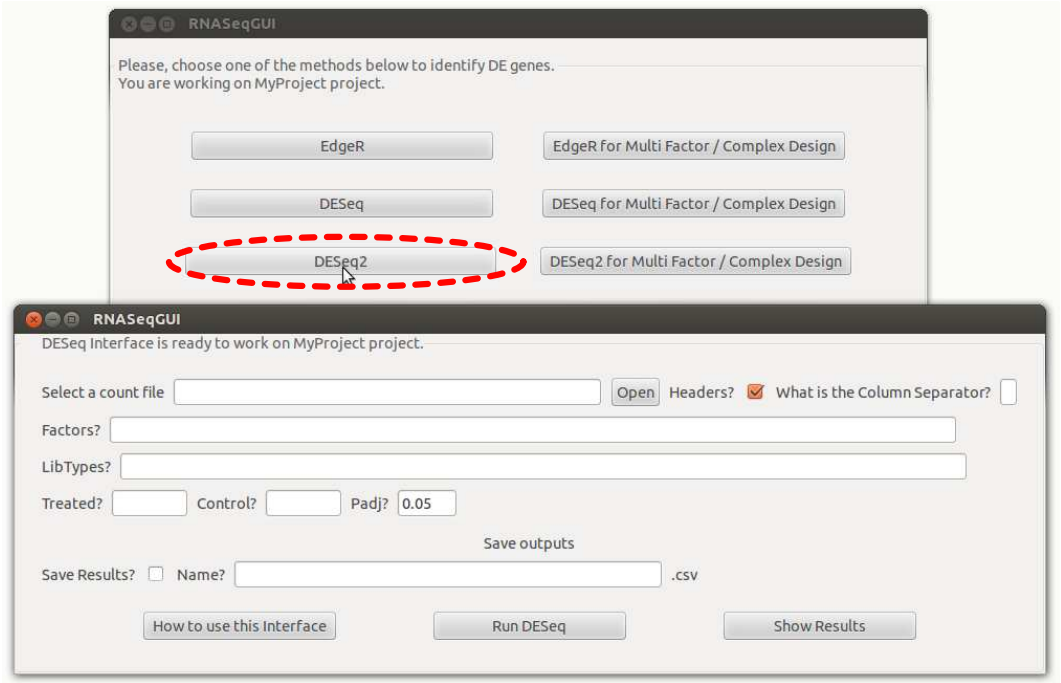


Figure 25: DESeq2 interface

The first text file shows the results of this method (see Figure 24), while the second text file shows the differentially expressed genes only.

The output count file is saved with the name specified by the user in the `Name?` field (see Figure 25).

If no name is specified by the user, then the first file is named with the name of the input file plus "`_results_DESeq2.txt`" suffix. Both text files are saved in the **Results** folder.

The second file is named with the name of the input file plus "`_padj=0.05_DE_genes_DESeq2.txt`" suffix, where 0.05 is the chosen adjusted p-value for rejection.

The first plot shows the dispersion value for a given mean of normalized counts and it is named with the name of the input file plus the "`_Dispersion_DESeq2.pdf`" suffix.

The second plot shows the dispersion mean value for a given mean of normalized counts and it is named with the name of the input file plus the "`_Dispersion_Mean_DESeq2.pdf`" suffix.

The third plot shows the dispersion local value for a given mean of

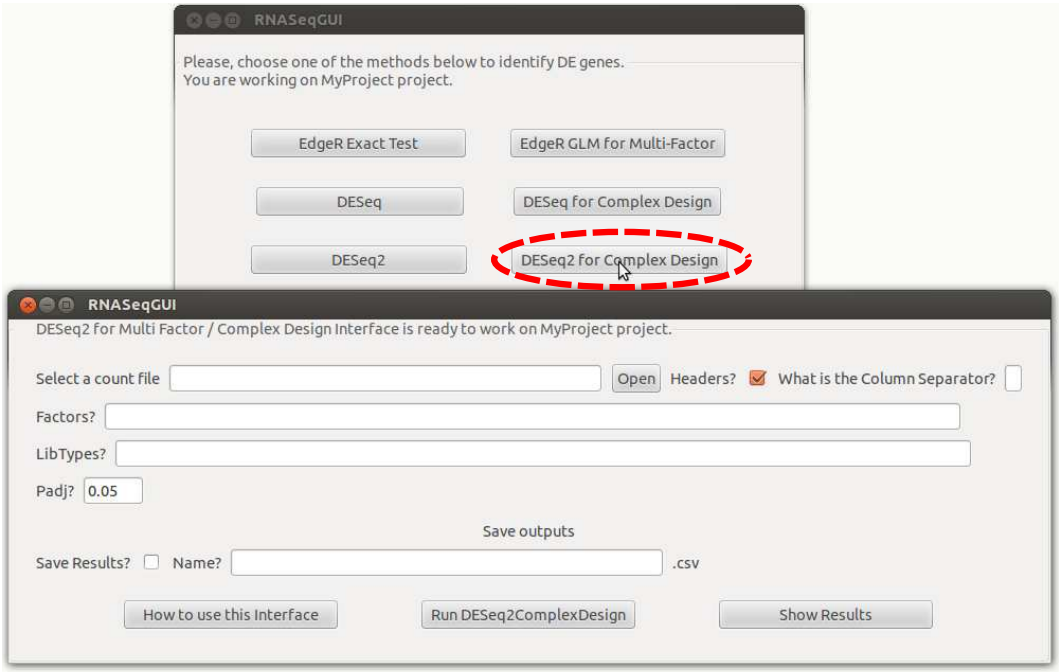


Figure 26: DESeq2 Multi Factor / Complex Design

normalized counts and it is named with the name of the input file plus the `_Dispersion_Local_DESeq2.pdf` suffix.

All plots are saved in the **Plots** folder.

11.7 DESeq2 Complex Design

If you want to perform a multiple test or you have a more complex design you can use the *DESeq2 Complex Design* interface (see Figure 26).
Suppose you have two treatments (T1, T2) and one control (U). For instance, `Factors?: U, U, T1, T1, T2, T2`.
In the `LibTypes?` field the user can specify an extra feature regarding the factors.
Suppose that `LibTypes` specifies the type of reads used in your experiment for each factor.
For instance, `LibTypes?:single-end,paired-end,single-end,paired-end, paired-end,single-end`.
Finally, click on the **Run DESeq2ComplexDesign** button.
A file with For further information, see `www.bioconductor.org/packages`

id	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
ENSG00000000003	625.025	-0.025	0.079	-0.318	0.750	0.954
ENSG00000000005	0.264	-0.014	0.020	-0.675	0.499	0.911
ENSG00000000419	1106.882	-0.072	0.062	-1.174	0.240	0.768
ENSG00000000457	367.367	0.035	0.095	0.365	0.714	0.937
ENSG00000000460	617.493	-0.002	0.079	-0.033	0.973	0.994
.....	.....	.....	.....	.....	.....	....
.....	.....	.....	.....	.....	.....	....

Figure 27: DESeq2 output. The first column reports the gene ids, `baseMean` reports the base mean over all rows, `log2FoldChange` reports the logarithm (to basis 2) of the fold changes, `lfcSE` reports the standard errors, `stat` reports the Wald statistic, `pval` reports the p values for the statistical significance and `padj` reports the p values adjusted for multiple testing calculated by the Benjamini-Hochberg algorithm.

`/release/bioc/vignettes/DESeq/inst/doc/DESeq2.pdf` .

Run DESeq2ComplexDesign returns two text files and two plots.

The first text file shows the results of this method, while the second text file shows the differentially expressed genes only.

The output count file is saved with the name specified by the user in the `Name?` field.

If no name is specified by the user, then the first output count file is named with the name of the input file plus "`_results_DESeq2.txt`" suffix.

The second file is named with the name of the input file plus "`_padj=0.05_DE_genes_DESeq2.txt`" suffix, where 0.05 is the chosen p-value adjusted.

Both text files are saved in the **Results** folder. The generated plot shows the dispersion value for a given mean of normalized counts.

This plot is named with the name of the input file plus "`_Dispersion_DESeq2.pdf`" suffix and it is saved in the **Plots** folder.

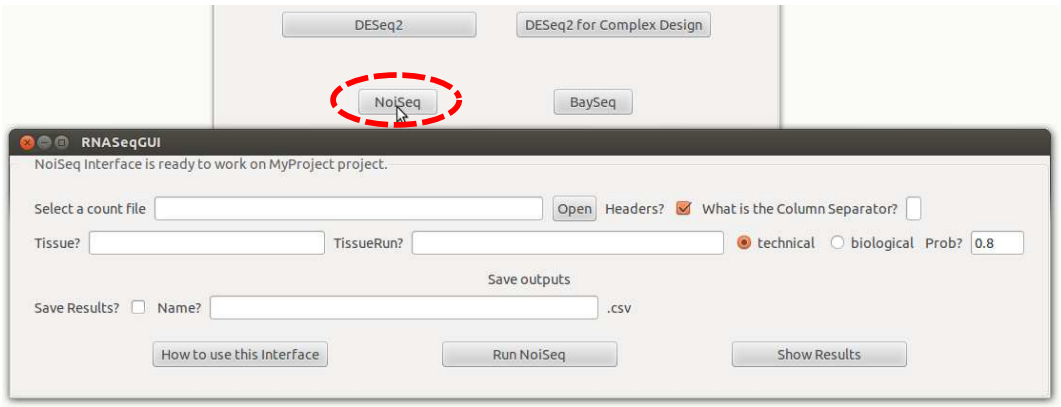


Figure 28: NoiSeq Interface

11.8 NoiSeq

- The **NoiSeq** [Tarazona *et al.*, 2011] method (see Figure 28) takes an input count file (as the one shown in Figure 13) via the `Open` button and returns two text files.

  The first text file shows the results of this method (see Figure 29), where M is the log2 ratio of the two conditions. The second text file shows the differentially expressed genes only.

  The first file is named with the name of the input file plus "`_results_Noiseq.txt`" suffix.

  The output count file is saved with the name specified by the user in the `Name?` field (see Figure 28).

  If no name is specified by the user, then the second file is named with the name of the input file plus "`_prob=0.8_DE_genes_Noiseq.txt`" suffix, where 0.8 is the chosen posterior probability for rejection.

  Both text files are saved in the **Results** folder.

  Both plots are saved in the **Plots** folder.

11.9 BaySeq

- The **BaySeq** [Hardcastle *et al.*, 2010] method (see Figure 30) takes an input count file (as the one shown in Figure 13) via the `Open` button, a list of factors (e.g. `treated,treated`, `control,control`) in the

id	control_mean	treated_mean	M	D	prob	ranking
ENSG00000000003	575.05	582.71	-0.019	7.659	0.104	-7.659
ENSG00000000005	0.22	0.47	-1.083	0.251	0.037	-1.112
ENSG00000000419	1000.84	1049.17	-0.068	48.333	0.405	-48.333
ENSG00000000457	345.75	334.47	0.047	11.275	0.164	11.275
ENSG00000000460	572.81	570.80	0.005	2.004	0.028	2.004
.....	.....	.....	.....	.....	....	....
.....	.....	.....	.....	.....	....	....

Figure 29: NoiSeq result file. The first column reports the gene ids, `control_mean` is the mean across the control samples, `treated_mean` is the mean across the treated samples, `M` is the log2-ratio of the means of the two conditions) and `D` is the difference between the two conditions means, `prob` is the probability of differential expression, the `ranking` is a summary statistic of M and D values (equal to $-sign(M) \times \sqrt{M^2 + D^2}$).

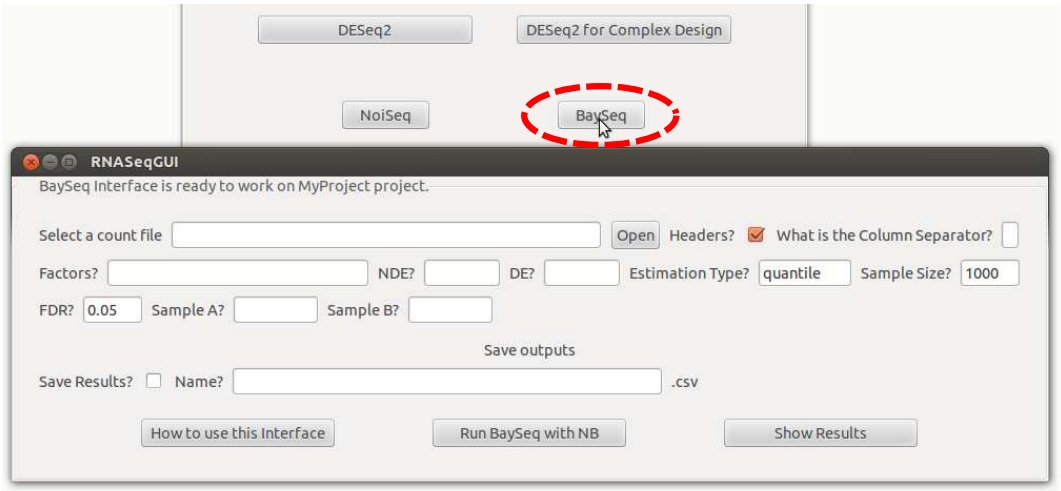


Figure 30: BaySeq Interface

id	rowID	control_1	control_2	treated_1	treated_2	Likelihood	FDR.DE
ENSG..971	row_7	1	1	1	1	0.261	0.738
ENSG..419	row_3	1132	1070	1088	1138	0.217	0.760
ENSG..457	row_4	354	348	392	377	0.111	0.803
ENSG..003	row_1	633	590	618	661	0.074	0.833
ENSG..460	row_5	618	580	653	621	0.067	0.853
ENSG..005	row_2	0	1	0	0	0.051	0.869
.....	...	...	...	...	...	....	....
.....	...	...	...	...	...	...	....

Figure 31: BaySeq result file. Bayseq reports the input counts and the number of the row (`rowID`) in the first columns and the `Likelihood` and the false discovery rate (`FDR.DE`) in the remaining columns.

`Factors?` field, a `NDE` list (e.g. `1,1,1,1`), a `DE` list (e.g. `1,1,2,2`), an Estimation Type? (e.g. `quantile`), the `SampleSize` (e.g. `1000`), an `FDR` level, `SampleA` (e.g. `treated`) and `SampleB` (e.g. `control`).

The **BaySeq** function returns two text files and two plots.

The first text file shows the results of this method (see Figure 31), while the second text file shows the differentially expressed genes only.

The output count file is saved with the name specified by the user in the `Name?` field (see Figure 30).

If no name is specified by the user, then the first file is named with the name of the input file plus "`_results_BaySeq.txt`" suffix. Both text files are saved in the **Results** folder.

The second file is named with the name of the input file plus "`_fdr=0.05_DE_genes_BaySeq.txt`" suffix, where 0.05 is the chosen FDR for rejection..

The first plot shows the log ratios of the counts against the mean average of the counts and it is named with the name of the input file plus the `_PlotMA_BaySeqNB.pdf` suffix.

The second plot shows the posterior likelihood. This plot is named with the name of the input file plus the `_Posteriors_BaySeqNB.pdf` suffix.

This method is very time consuming.

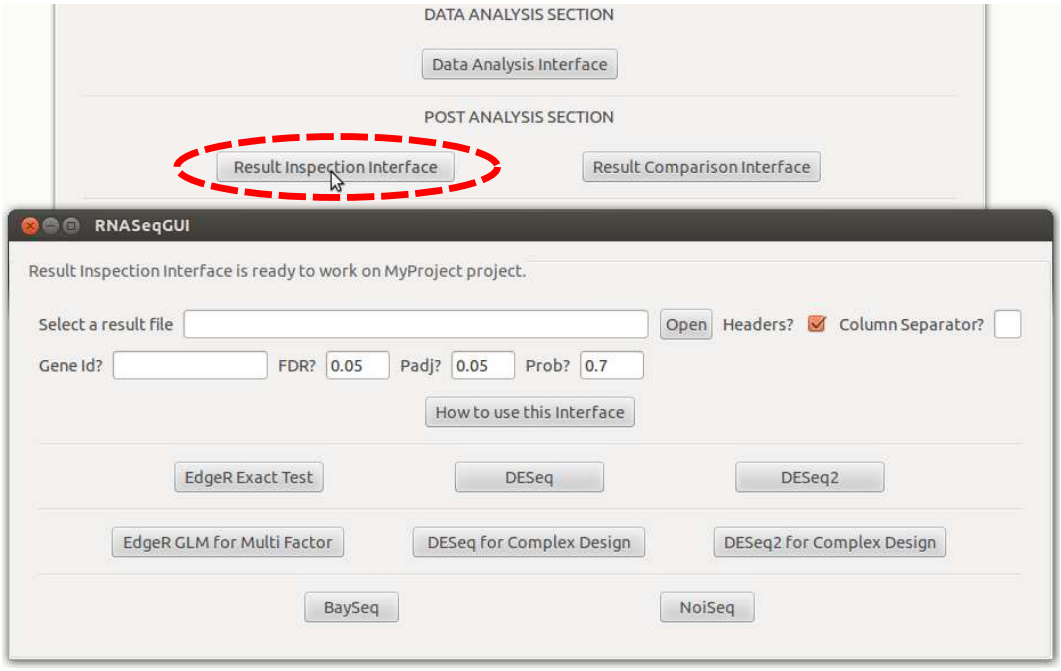


Figure 32: Result Inspection Interface

12 POST ANALYSIS SECTION

In the fifth section of the GUI, called Post Analysis Interface, there are two interfaces: **Result Inspection Interface** (see Figure 32) and **Result Comparison Interface** (see Figure 34). The first interface includes the possibility to generate several plots for each methods. The second allows to compare the outcomes obtained from several methods.

12.1 Result Inspection Interface

To explore the results of a specific method, we have to click on the used method in **Data Analysis Section** (say EdgeR) and the interface in Figure 32 will display the functions available for the selected method (for EdgeR **Plot FC**, **FDR Hist**, **P-value Hist** functions are available). If we click all buttons in Figure 32, the interface will grow and we get the interface shown in Figure 33.

Therefore, for each method, we have **Plot FC**, **FDR Hist** (or **P-value Hist**) and **Volcano Plot** functions, except for the BaySeq method since this method already provides an *MAplot* and a *PosteriorPlot* during the

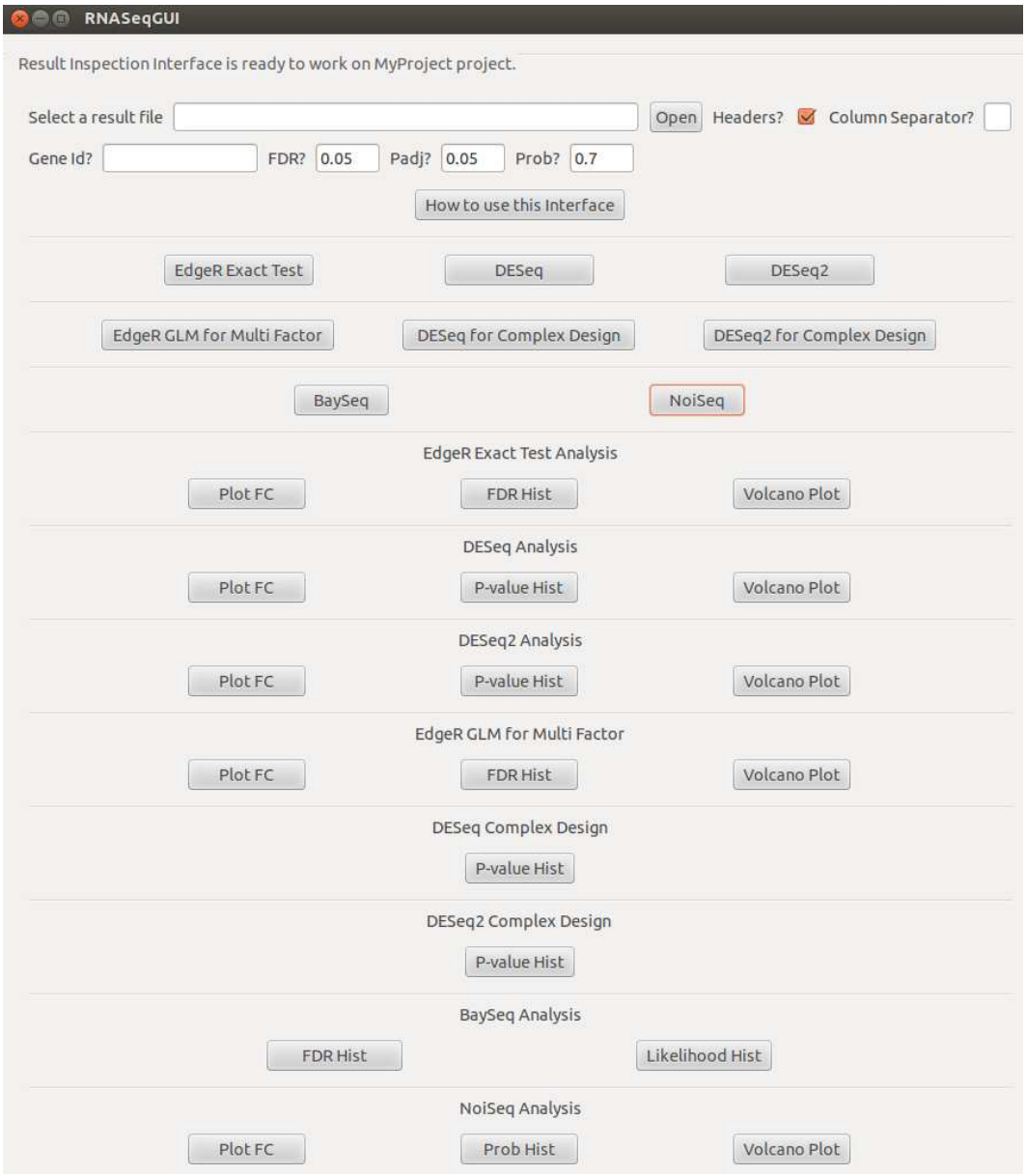


Figure 33: Result Inspection Interface after clicking all the eight buttons at the top.

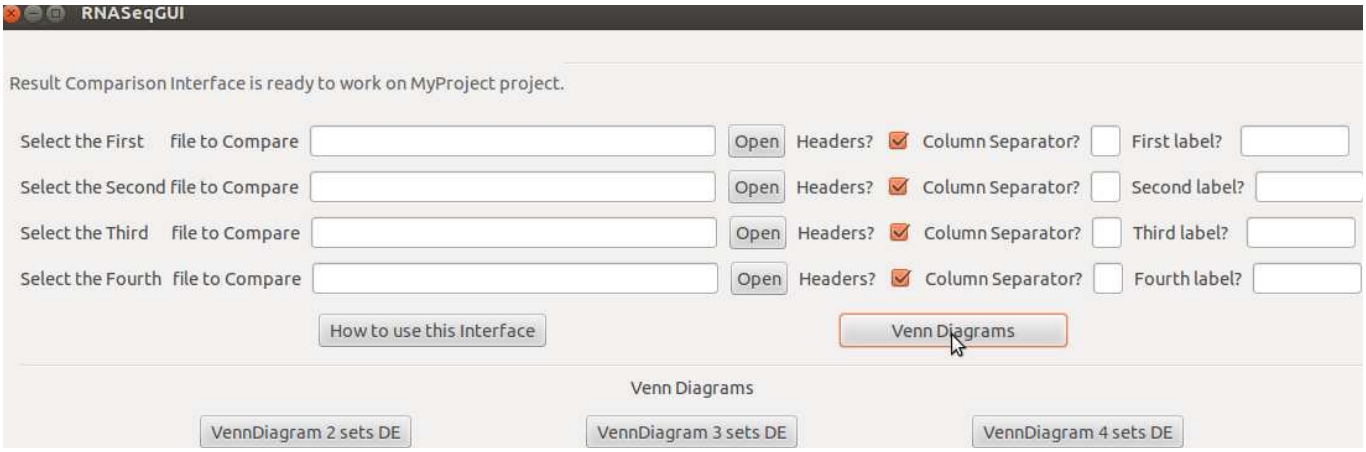


Figure 34: Result Comparison Interface

analysis process that can be run in the **BaySeq Analysis Interface**.
For each function (e.g.: **FDR Hist**, **P-value Hist**, **Likelihood Hist**) of each method, we just need to provide a "full result" file placed in the **Results** folder. For **Volcano Plot** and **Plot FC** functions, we must provide a path to a "full result" file (as the one shown in Figure 19) and a `FDR`, `P-value` or `Prob` value (it depends on the chosen method) to point out the differentially expressed genes (shown in red). In this case, it is also possible to provide a gene id, provided into the `Gene Id` field, to point out that particular gene in the Volcano or FC plot (that gene will be displayed in green).
All generated plots are saved in pdf format in the **Plots** folder.

12.2 Result Comparison Interface

The second interface includes the possibility to generate Venn diagrams of two, three or four result text files (See Figure 34).
The user must provide two, three or four text files reporting the results of the used methods and the corresponding labels to recognize these files in the generated diagrams.
A Venn diagram is generated and saved in the **Plots** folder. Moreover, a text file (showing the gene ids belonging to the intersection of the selected methods) is created and saved in the **Results** folder.

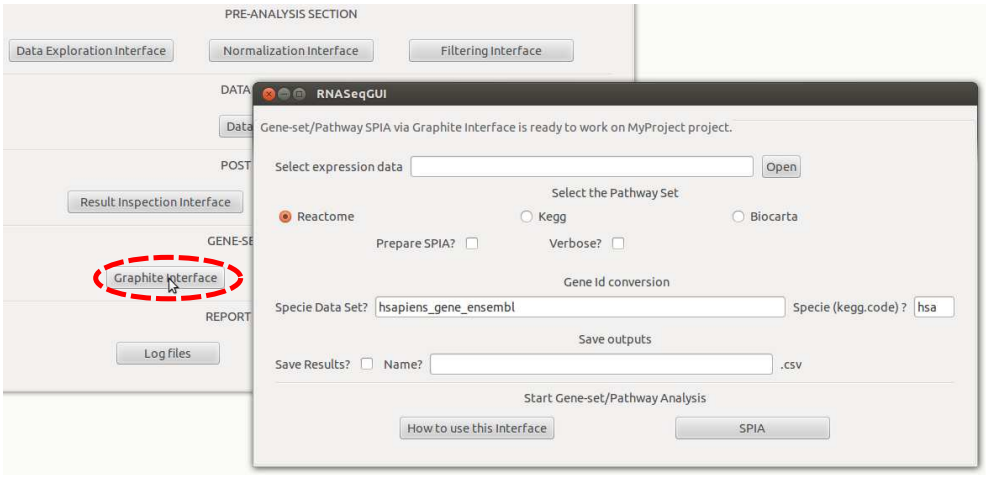


Figure 35: Graphite Interface

13 GO/PATHWAY SECTION

13.1 Graphite Interface

In the *Graphite Interface* there is the **SPIA** button as shown in Figure 35.

Select expression data by clicking on the corresponding `Open` button.
Select the Gene Sets by choosing either `Reactome`, `Kegg` or `Biocarta`.
Check the `Prepare SPIA` check-box if you don't have a SPIA database file prepared.
Check the `Verbose` check-box if you want additional output in the R shell.
The `Species Data Set` and the relative `Kegg code` is uneditable because the tool is actually performed only for human species.
Finally click on **SPIA** button.
For further information, see
`http://www.bioconductor.org/packages/release/bioc/html/graphite.html`

13.2 David Interface

In the *David Interface* there is the **DAVID** button as shown in Figure 36.
Select differential expression data file by clicking on the corresponding 'Open' button.
It can also be a single column file within a list of differential expressed genes.
In the column field, insert the number of the column containing the gene list.
In the Gene Identifier field, select the identifier of your genes.
Important: If your genes are genesymbols, you have to select the specie of

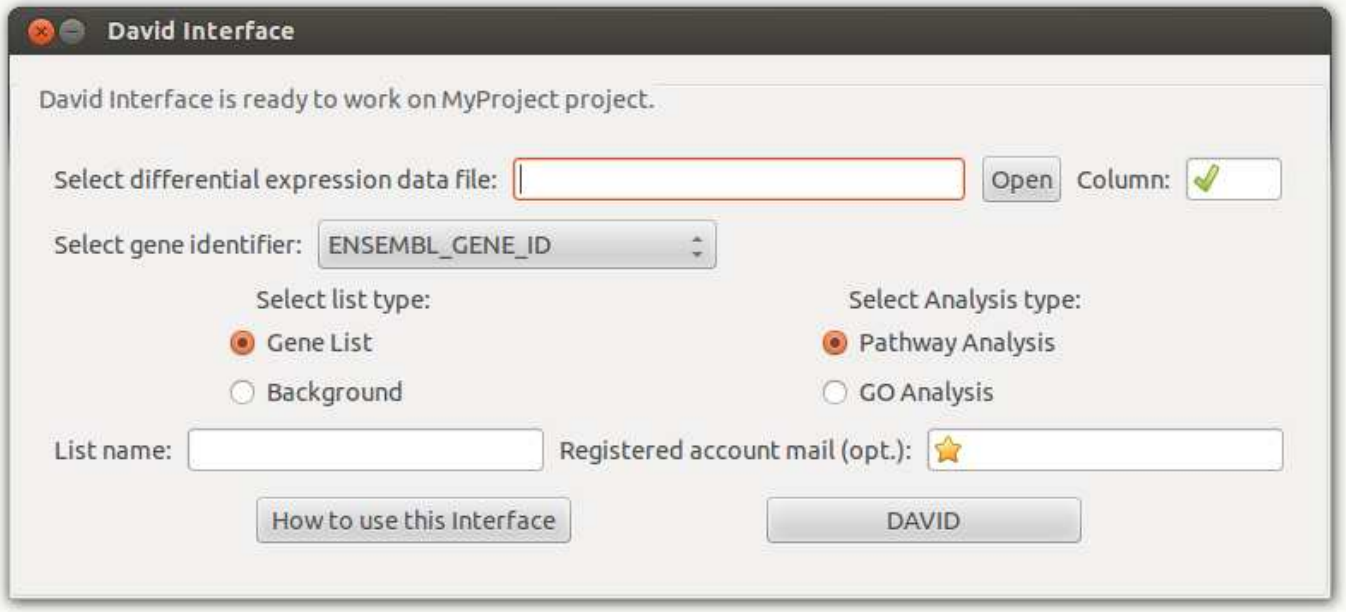


Figure 36: David Interface

the genes, because they will be converted to ensembl gene id.
This is due to DAVID constraints.
The list type indicate if you are using a gene list or a background list.
Further information here:
`http://david.abcc.ncifcrf.gov/helps/list$_$manager.html#upload` .
You can choose two types of analysis: Pathways or Gene Ontology.
In the next step a popup window will be open.
For the Pathway Analysis: you have to choose one of the pathway database to use.
For the Gene Ontology: there are two categories you can choose: FAT and ALL.
In each category all the choosen terms type will be analysed together.
In the list name field you have to enter a name for your gene list.
Notice: DAVID connects this name to your gene list for a certain amount of time.
So, if you change the gene list, you have to change the list name until DAVID forgot the initially associated gene list.

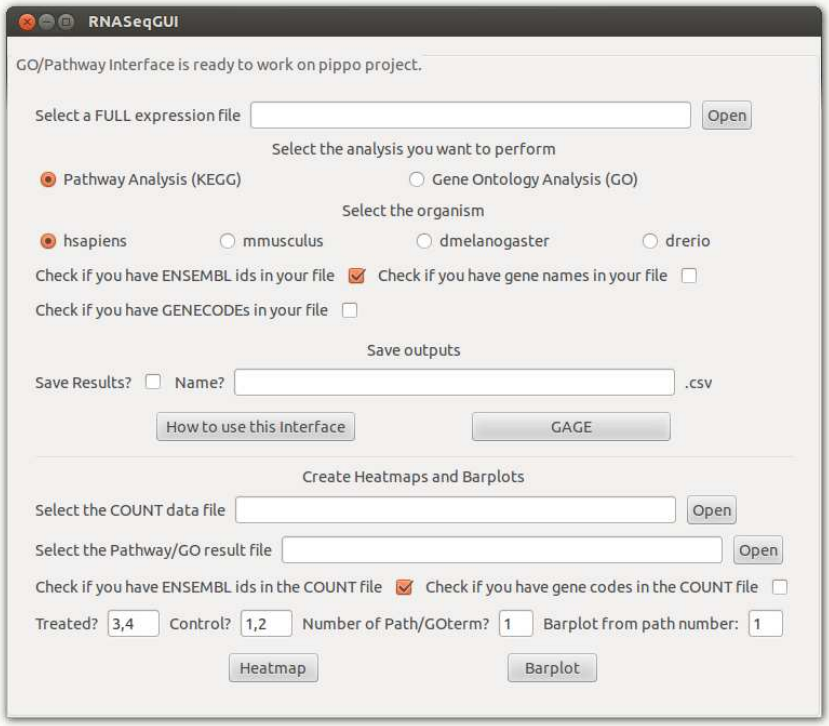


Figure 37: Gage Interface

13.3 Gage Interface

In the *Gage Interface* there are three buttons, as shown in Figure 37.

- The first one is the **GAGE** button. Select full expression data by clicking on the corresponding `Open` button. Select either `Pathway Analysis` or `GO Analysis`. Select the organism you are investigating on. Specify the gene id type you have in the expression file. Finally, click on **GAGE** button. For further information, see `http://bioconductor.org/packages/release/bioc/html/gage.html` .

- The second button is the **Heatmap** one. If you want to produce an Heatmap, select the starting count file and the PATHWAY/GO result file. Specify the gene id type you have in the count file. Specify which columns are the treated and which are the control ones in the count file. Specify the `number of the Path/GOterm` for which you want to

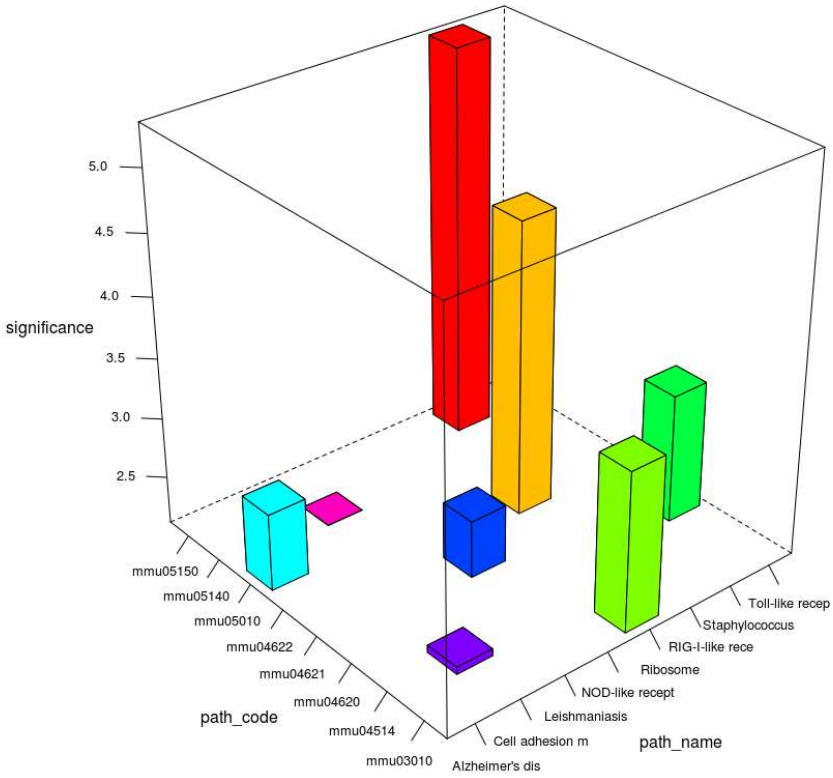


Figure 38: Example of the output of **Barplot** function.

create an heatmap. Finally, click on the **Heatmap** button.

- The third button is the **Barplot** one. If you want to produce a Barplot, select a PATHWAY/GO result file. Specify from which Path/GOterm you want to create a Barplot by choosing a starting point in the `Barplot from path number` field. Finally, click on the **Barplot** button. A plot such as the one shown in (Figure 38) will be produced.

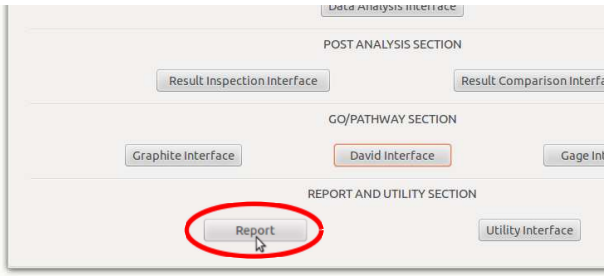


Figure 39: By clicking the **Report** button the file report.html is generated.

14 REPORT AND UTILITY SECTION

14.1 Reproducible Research: the *Report*

In the spirit of **Reproducible Research**, RNASeqGUI is able to automatically generate a report, in *html* format, of all steps performed during the analysis of a specific project (see figure 39). Reports are produced in R markdown format via *knitr* library and they include the documentation of the methods used and the R code that has been executed during the RNASeqGUI usage.
Hence, all the functionalities used by the user are automatically saved in a report file (as the one shown in Figure 40) inside the **Logs** directory of the user project. This report reports the session information that describes all used package versions by RNASeqGUI at the time of the project creation, along side with the name of the project, time, date and the parameters (fdr, padj, etc.) the user selected during the usage of the GUI.

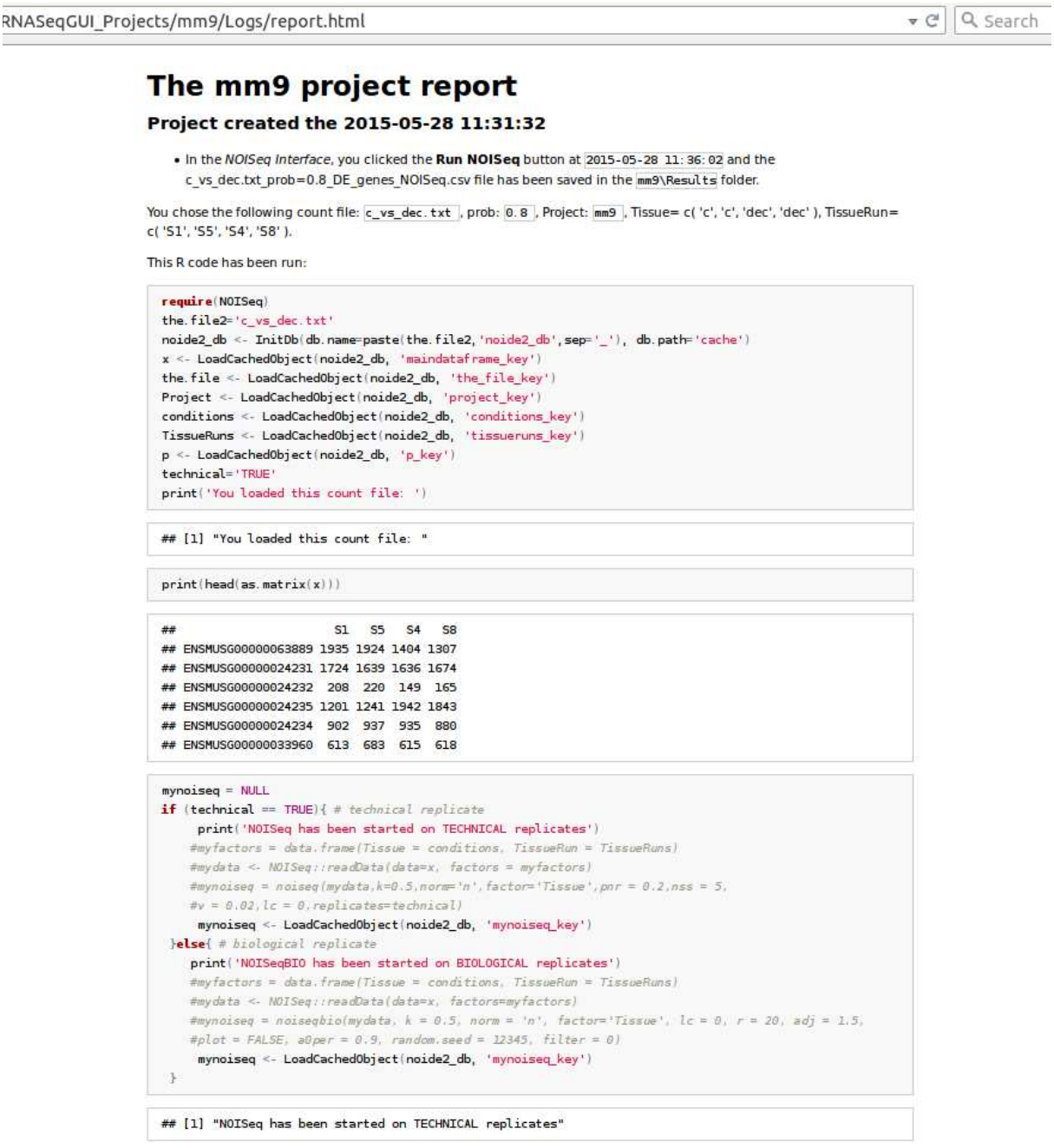


Figure 40: An example of the html report file generated by the **Report** button from the log file report.Rmd.

```
plot(log10(results_NoiSeq[,2] * results_NoiSeq[,1]),log10(results_NoiSeq[,2]/results_NoiSeq[,1]),col='bla
main=paste('PlotFC ',the.file2,sep=''), xlab=paste('log10( ', colnames(results_NoiSeq)[2],' * ',
colnames(results_NoiSeq)[1],')',sep=''),ylab=paste('log10( ',colnames(results_NoiSeq)[2],
' / ',colnames(results_NoiSeq)[1],' )',sep=''),pch=19,cex=0.3)
DE_genes_NoiSeq = subset(results_NoiSeq, prob>p)
points(log10(DE_genes_NoiSeq[,2] * DE_genes_NoiSeq[,1]), log10(DE_genes_NoiSeq[,2]/DE_genes_NoiSeq[,1]),
pch=19, col='red', cex=0.5)
```

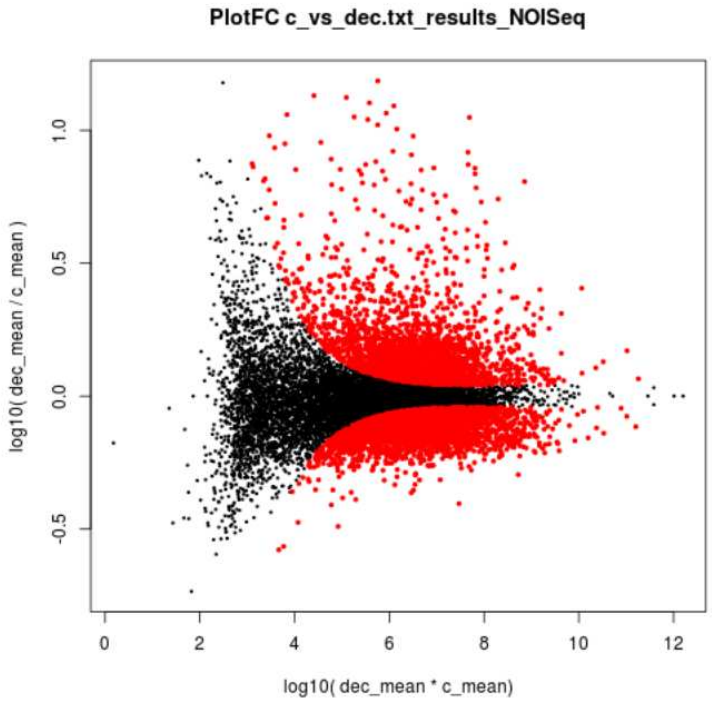


Figure 41: An example of the html report file generated by the **Report** button from the log file report.Rmd. We can notice the chunk of code that produced the NOISeq Fold Change plot. This code can be copy and pasted in an R console to be run independently and it will generate the same plot shown in this report.

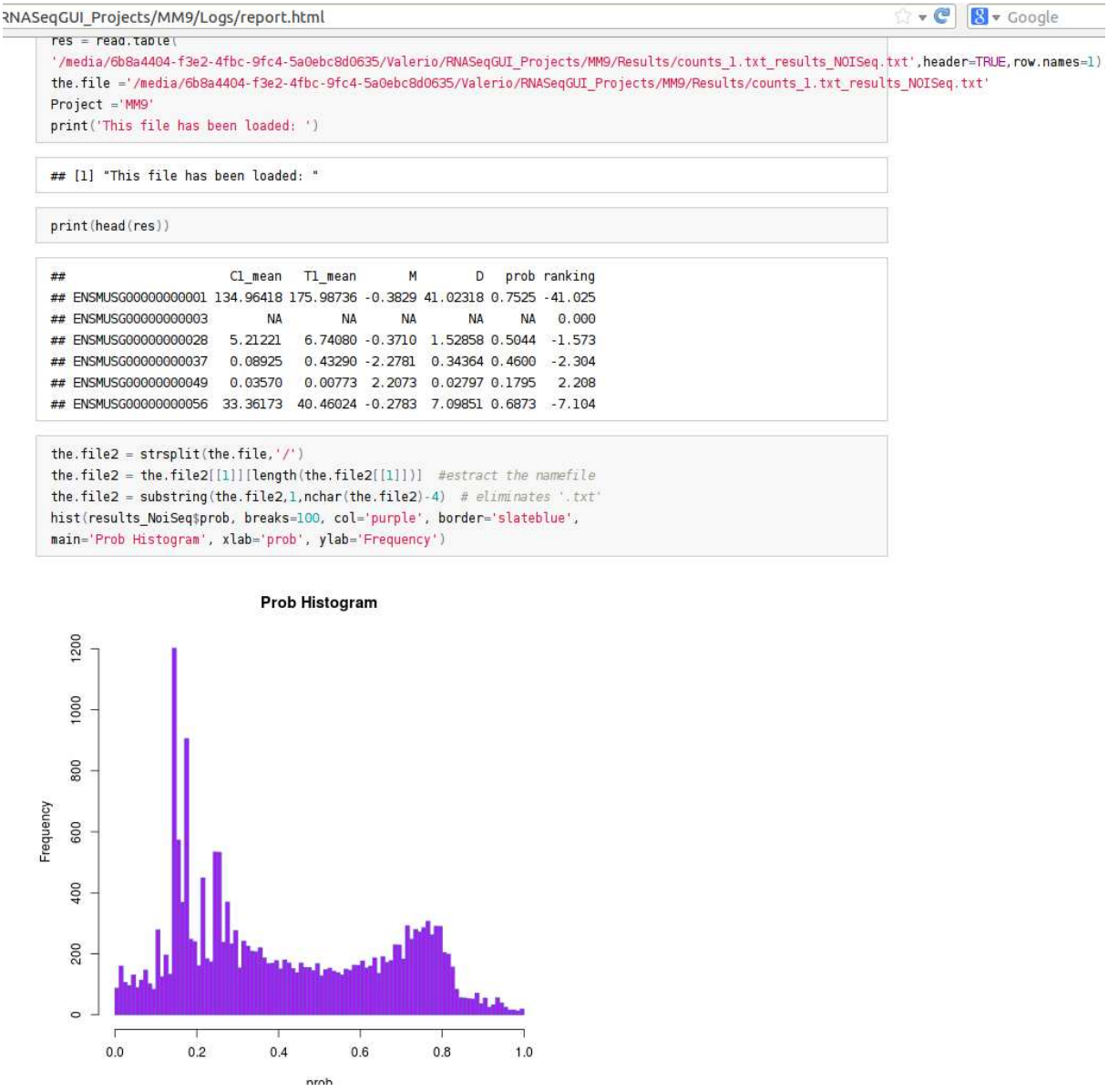
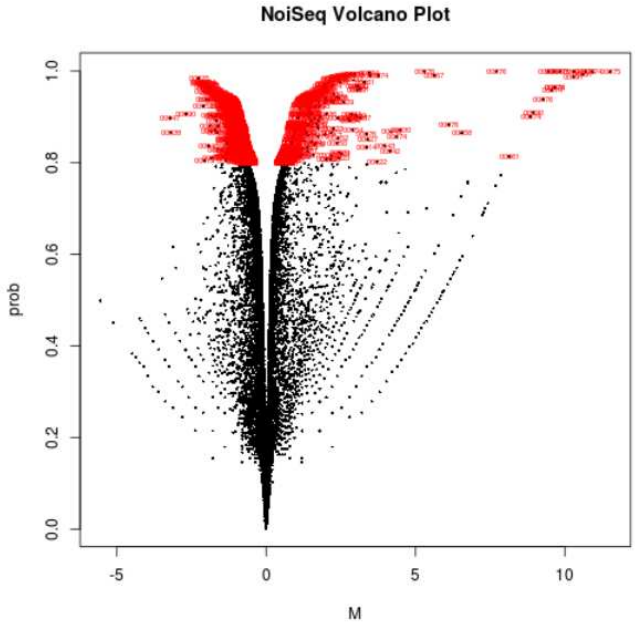


Figure 42: An example of the html report file generated by the **Report** button from the log file report.Rmd.

```
the.file2 = strsplit(the.file,'/')
the.file2 = the.file2[[1]][length(the.file2[[1]])]  #estract the namefile
the.file2 = substring(the.file2,1,nchar(the.file2)-4)  # eliminates '.txt'
plot(results_noi$M, results_noi$prob, col = 'black', main='NoiSeq Volcano Plot', xlab='M', ylab='prob', pch=16,cex=0.4)
DE_genes_NoiSeq = subset(results_noi, prob>p)
text(DE_genes_NoiSeq$M, DE_genes_NoiSeq$prob, label=substring(row.names(DE_genes_NoiSeq),11,15), col='red', cex=0.5)
```




```
if (name!=''){    OneGene = subset(results_noi, row.names(results_noi)==name)
    text(OneGene$M, OneGene$prob, label=name, col='green', cex=0.6) }
a=paste(getwd(),'/RNASeqGUI_Projects/',Project,'/Plots/',sep='')
outputName=paste(the.file2,'_Volcano_NOISeq.pdf', sep='')
b=paste(a,outputName,sep='/')
# dev.print(device = pdf, file=b)
```

Figure 43: An example of the html report file generated by the **Report** button from the log file report.Rmd.

14.2 Utility Interface

In the Utility Interface we find three buttons, as shown in Figure 44.

- The first one is the **Bind Count Files** button that binds several counting files of the same length together.

  Select a count folder by clicking on the corresponding `Open` button. To select the entire folder, select just one file inside the folder you want to use. The entire folder will be loaded. Please, be sure that the folder only contains the files you want to bind. Finally, click on **Bind Count Files** button.

- The second button is the **Convert** one that replaces the gene ids with gene names in a file.
  Select a count file by clicking on the corresponding `Open` button.
  Finally, click on **Convert** button. For further information, see
  `http://bioinfo.na.iac.cnr.it/RNASeqGUI/Manual.html`

- The third button is the **Modify Count** one that creates a new count file containing the columns of interest only. Select a count file by clicking on the corresponding `Open` button. Select the number of columns to keep. Finally, click on **Modify Count** button. For further information, see
  `http://bioinfo.na.iac.cnr.it/RNASeqGUI/Manual.html`

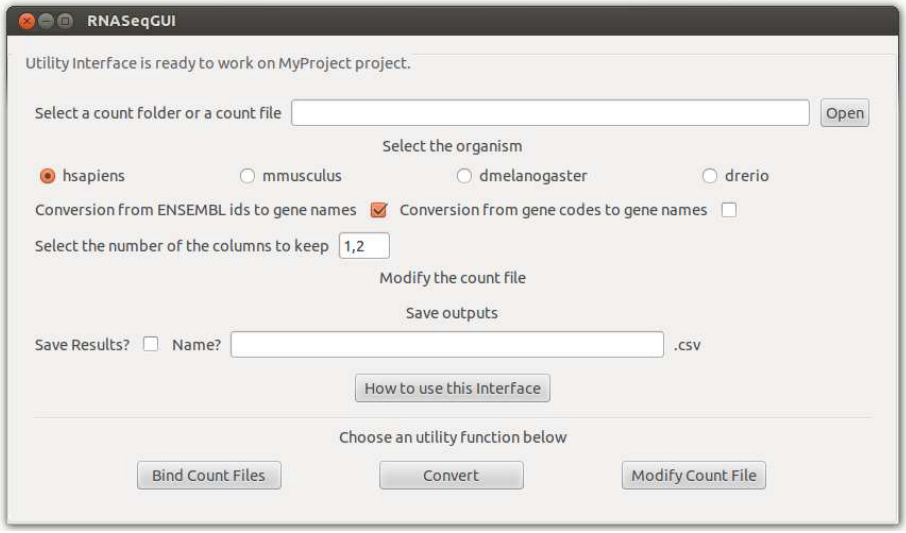


Figure 44: The Utility Interface.

15 Usage Example

We can start using RNASeqGUI by downloading the example data at `http://bioinfo.na.iac.cnr.it/RNASeqGUI/Example`, as shown in Figure 45.

We download the folder called **example_RNASeqGUI.tar.gz**, we extract this bundle and open it. Inside this, we find a folder called **demo**, a gtf file called `2L_Drosophila_ melanogaster.BDGP5.70.gtf` and a text file called `README.txt` file.

15.1 Data Preparation

In this usage example, we start the analysis of the RNA-Seq data from bam files and we compare the results of EdgeR, DESeq and NOISeq against each other.
We downloaded the dataset published by [Brooks *et al.*, 2011]. This dataset has already been used in [Anders *et al.*, 2013] as a real data working example.
We downloaded the data from `http://www.ncbi.nlm.nih.gov/sra?term=SRP001537` by following the instructions described in [Anders *et al.*, 2013] at the page 1771. The entire experiment is available at `http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18508`.

The dataset consists of seven samples. Three samples represent the response

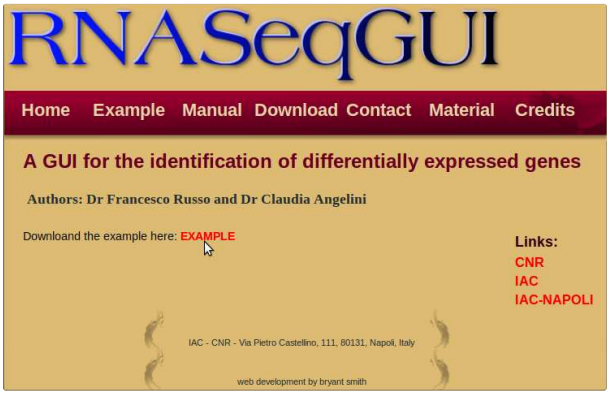


Figure 45: At `http://bioinfo.na.iac.cnr.it/RNASeqGUI/Example` we can download the example.

BamFileName	NameOfTheReducedBam	LibraryType	LibraryLayout
CG8144_RNA-1	2L_1	treated	single
CG8144_RNA-3	2L_3	treated	paired
CG8144_RNA-4	2L_4	treated	paired
Untreated-1	2L_U1	untreated	single
Untreated-3	2L_U3	untreated	paired
Untreated-4	2L_U4	untreated	paired
Untreated-6	2L_U6	untreated	single

Figure 46: Experimental design

to a treatment and four samples are controls. Each sample is a cell culture of *Drosophila melanogaster* (For more details about this experiment see [Brooks *et al.*, 2011]).

We downloaded and aligned the *fastq* files by running `tophat2` [Kim *et al.*, 2013] as described in [Anders *et al.*, 2013] at page 1774. Once the bam files were obtained (we called them CG8144_RNA-1, CG8144_RNA-3, CG8144_RNA-4, Untreated-1, Untreated-3, Untreated-4, Untreated-6 as in in [Anders *et al.*, 2013]), it is possible to perform the analysis with RNASeqGUI.

For illustrative purpose and for keeping the computational cost of the demonstrative example under control, we limit our attention to chromosome `2L`. Alignment data (bam files) are contained in the folder called `demo` inside the **Bam** folder, with the following names: `2L_1.bam`, `2L_3.bam`, `2L_4.bam`, `2L_U1.bam`, `2L_U3.bam`, `2L_U4.bam`, `2L_U6.bam` (see Figure 46).

15.2 Usage of RNASeqGUI

We open R, then we type

```
library(RNASeqGUI)
```

and we type

```
RNASeqGUI()
```

Once the main RNASeqGUI interface (see Figure 5) has appeared on the screen, we create a new project (for instance, we can call it `demoProject`) and then we click on `Bam Exploration Interface` button. We select the **demo** folder with the `Open` button. After that, we start the analysis by using the `Read Counts` button in the *Bam Exploration Interface*. This action creates the plot shown in Figure 49. The bam files in the **demo** folder are loaded in alphabetically order and their name are displayed at x axis in Figure 49 alphabetically. This plot is automatically saved in pdf format in the **Plots** folder of the project you selected.

A text file is also generated and saved in the **Results** folder with the `demo_Read Count.txt` name, as shown in Figure 50. This file shows the number of reads for each bam file.

Critical: We cannot use the **Mean Quality of Reads** or **Per Base Quality of Reads** function for this dataset, since the `2L_1.bam` file was generated by pulling *fastq* files containing reads of different length (This file correspond to `CG8144_RNAi-1` at page 1774 of [Anders *et al.*, 2013]). To use these functions, we need bam files containing reads of the same length. Otherwise, we get the following error:

```
Error in as.vector(x, "character"):  cannot coerce type 'environment' to vector of type 'character'.
```

If the user wants to use these functions, in this case the `2L_1.bam` file must be temporary removed from the **demo** folder before using them. In this case, if we use those functions without the `2L_1.bam` file, we get the plots in Figure 47 and in Figure 48, respectively.

Subsequently, we click on *Read Count Interface* and select the bam folder **demo** and the `2L_Drosophila_melanogaster.BDGP5.70.gtf` annotation file. We select `Union` as `Counting Mode` and check the `Ignore Strand` box, as shown in Figure 51. Hence, we click on `Count Reads` button. As result of this action, a text file named `2L_counts.csv` (see Figure 52) is generated and saved in the **Results** folder. A file named `counts.txt` is also generated in case the user forgets to use the `Save Results?` check-box at the bottom of the interface. The column names in Figure 51 follow the alphabetical order

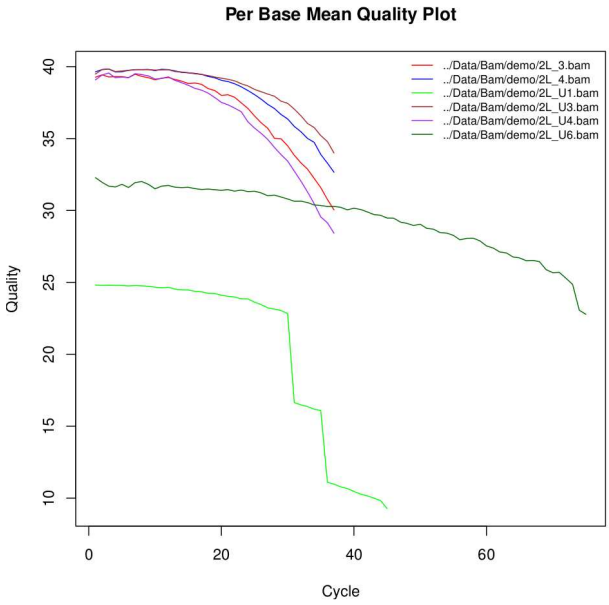


Figure 47: **Mean Quality of Reads** of the bam files stored in the folder **demo** without the `2L_1.bam` file.

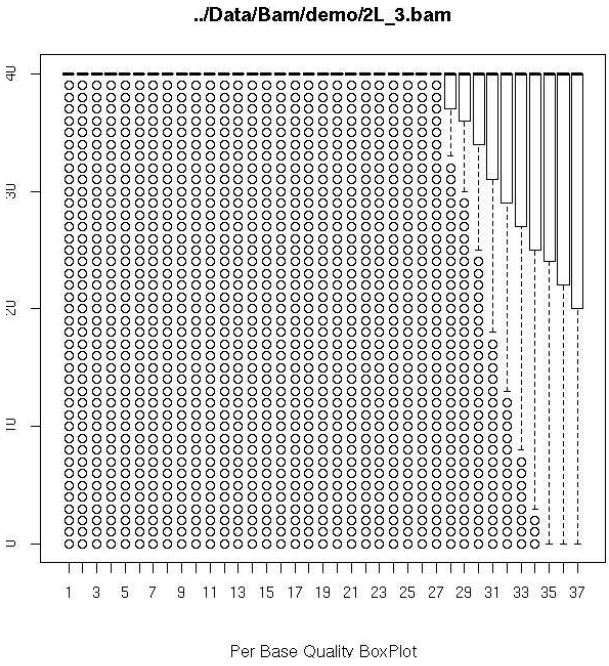


Figure 48: **Per Base Quality of Reads** of the bam files stored in the folder **demo** without the `2L_1.bam` file.

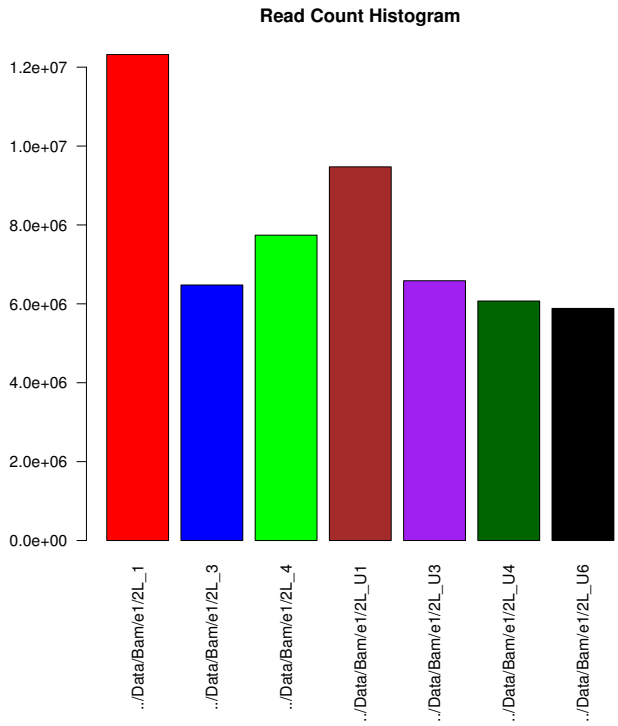


Figure 49: Read Count Histogram of the bam files stored in the folder **demo**.

fileName	NumberOfReads
../Data/Bam/demo/2L_1	12320205
../Data/Bam/demo/2L_3	6477978
../Data/Bam/demo/2L_4	7741241
../Data/Bam/demo/2L_U1	9473462
../Data/Bam/demo/2L_U3	6586330
../Data/Bam/demo/2L_U4	6071744
../Data/Bam/demo/2L_U6	5883666

Figure 50: The `demo_ReadCount.txt` file saved in the **Results** folder.

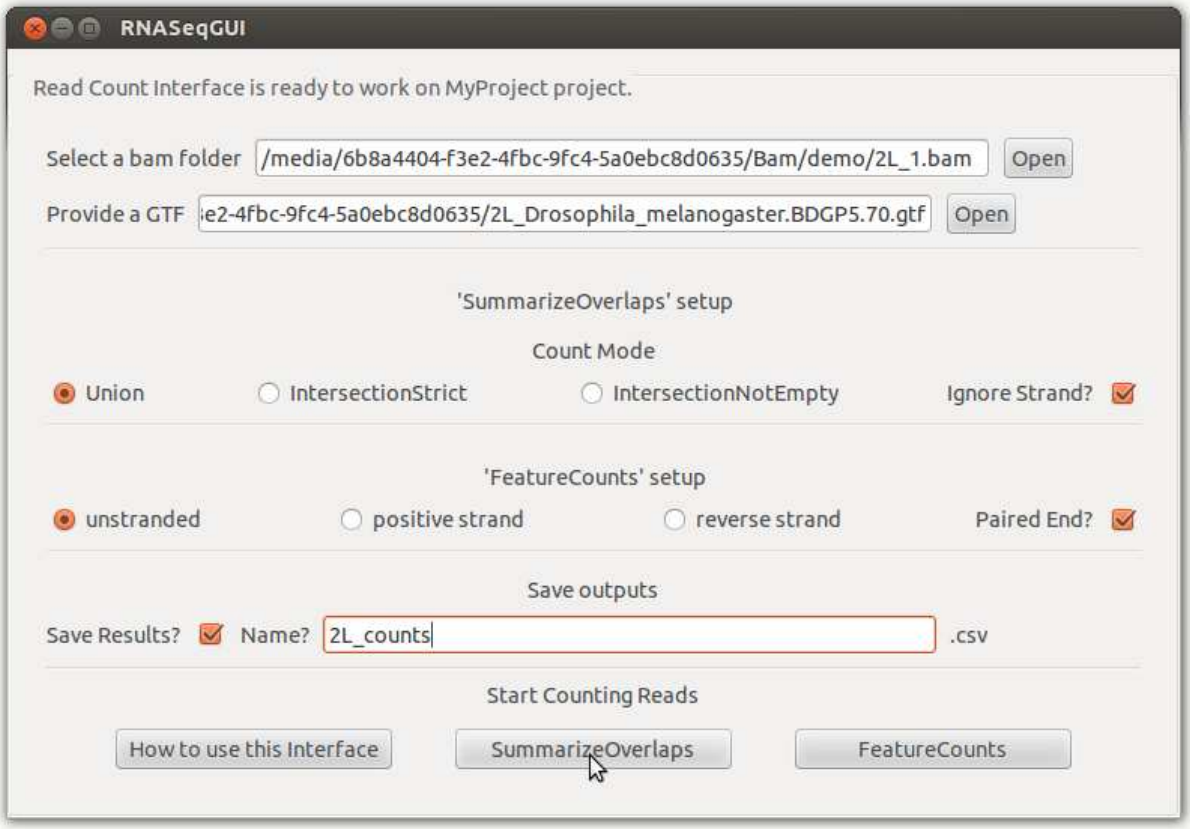


Figure 51: We select the `demo` bam folder and the `2L_Drosophila_melanogaster.BDGP5.70.gtf` file. We check `Union` in the *Count Mode* setup and we check `Ignore Strand?`. We save the result with name `2L_counts`. Finally, we click on `SummarizeOverlaps` button.

id	2L_1	2L_3	2L_4	2L_U1	2L_U3	2L_U4	2L_U6
FBgn0000018	528	485	546	613	441	501	485
FBgn0000052	2300	2968	3555	2921	3097	3244	2626
FBgn0000053	2361	2982	3790	2307	2352	2542	1856
FBgn0000055	1	0	0	0	0	0	0
FBgn0000056	0	0	0	0	0	0	0
FBgn0000061	4	2	2	1	1	5	0
FBgn0000075	2	2	1	4	4	3	1
FBgn0000097	3849	3727	4546	4656	4227	3448	2569
....	....	....	....	....	....	....	....
....	....	....	....	....	....	....	....

Figure 52: The `2L_counts.csv` file created by **Count Reads** function and saved in the **Results** folder.

of the bam files placed in the **demo** folder.

Now, we can explore the obtained count file, shown in Figure 52.

We click on *Data Exploration Interface* button. Once this interface has appeared on the screen (see Figure 53), we select the `2L_counts.csv` file.

First, we use the **Count Distr** and the **Plot All Counts** functions by clicking the corresponding buttons (see Figure 53). The generated plots are shown in Figure 54 and Figure 56, respectively. From Figure 54, we can see that all the count means (the black lines in the box plot) and all the count distributions are almost aligned. Therefore, we decide not to normalize the counts since a normalization procedure does not seem to be necessary.
To better understand whether a normalization procedure is needed, we can also use the **MDPlot** by plotting each sample counts (by selecting `Column1` and `Column2` fields) against all the other sample counts.
Anyway, if we use the full quantile normalization procedure by clicking the **Full Quantile** button in the *Normalization Interface*, we get the plot show in Figure 55 and a text file of normalized counts saved in **Results** folder.

Subsequently, we use the **PCA** function by typing the `1,3,4,U1,U3,U4,U6` sequence in the `PCA Factors?` field (see Figure 53) to specify the labels that will be displayed in the legend at the top-right of the plot generated by this function (shown in Figure 57).

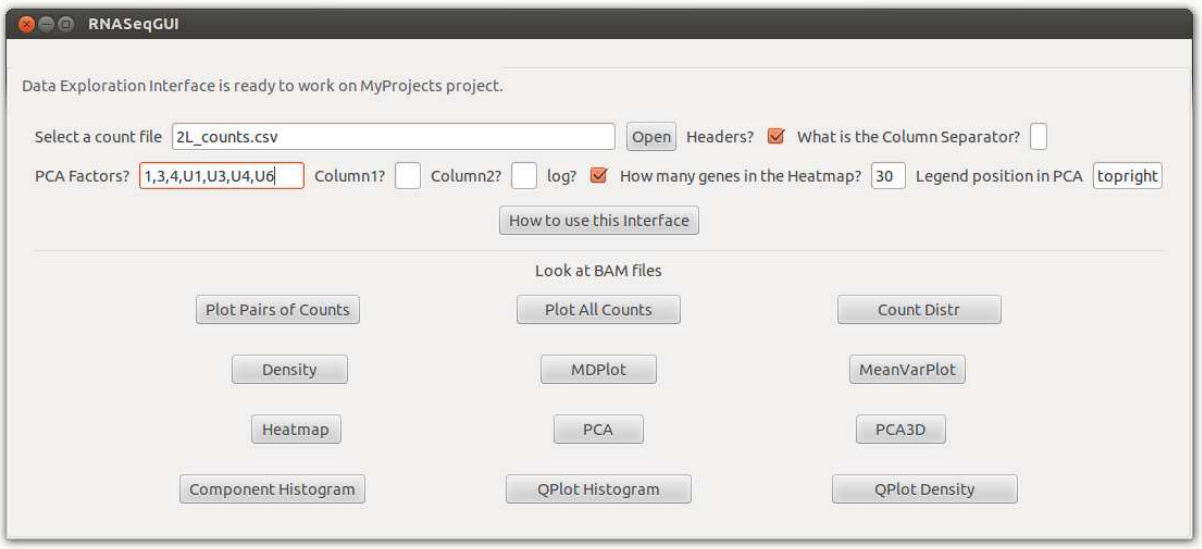


Figure 53: Data Exploration Interface

Finally, we can use the **HeatMap** function to see what are the first (say thirty) most expressed genes. Therefore, we typed the number 30 in the `How many genes in the Heatmap?` field (see Figure 58). From the heatmap, we can notice that the the most expressed gene is the one called FBgn0000559 (look at the bottom of the Figure 58).

Now, we can start with the analysis. We decide to use EdgeR, DESeq and NOISeq and compare the results among them.

We click on *Data Analysis Interface* button.

We start the EdgeR analysis by clicking on the **EdgeR** button. In the *EdgeR Analysis Interface*, we select the `2L_counts.csv` count file.
We type the `T,T,T,U,U,U,U` sequence in the `Factors?` field to specify which are the treated samples (called `T`) and which are the untreated ones (called `U`) as reported in Figure 46. We choose a 0.05 value as the `FDR`. Finally, we click on **Run EdgeR** button. The EdgeR analysis is performed and two result text files are created and saved in the **Results** folder.

We click on **DESeq** button. In the *DESeq Analysis Interface*, we select the `2L_counts.csv` count file. We type the `T,T,T,U,U,U,U` sequence in the `Factors?` field to specify the treated and untreated samples as in EdgeR analysis. We type `single-end,paired-end,paired-end,single-end,pair`

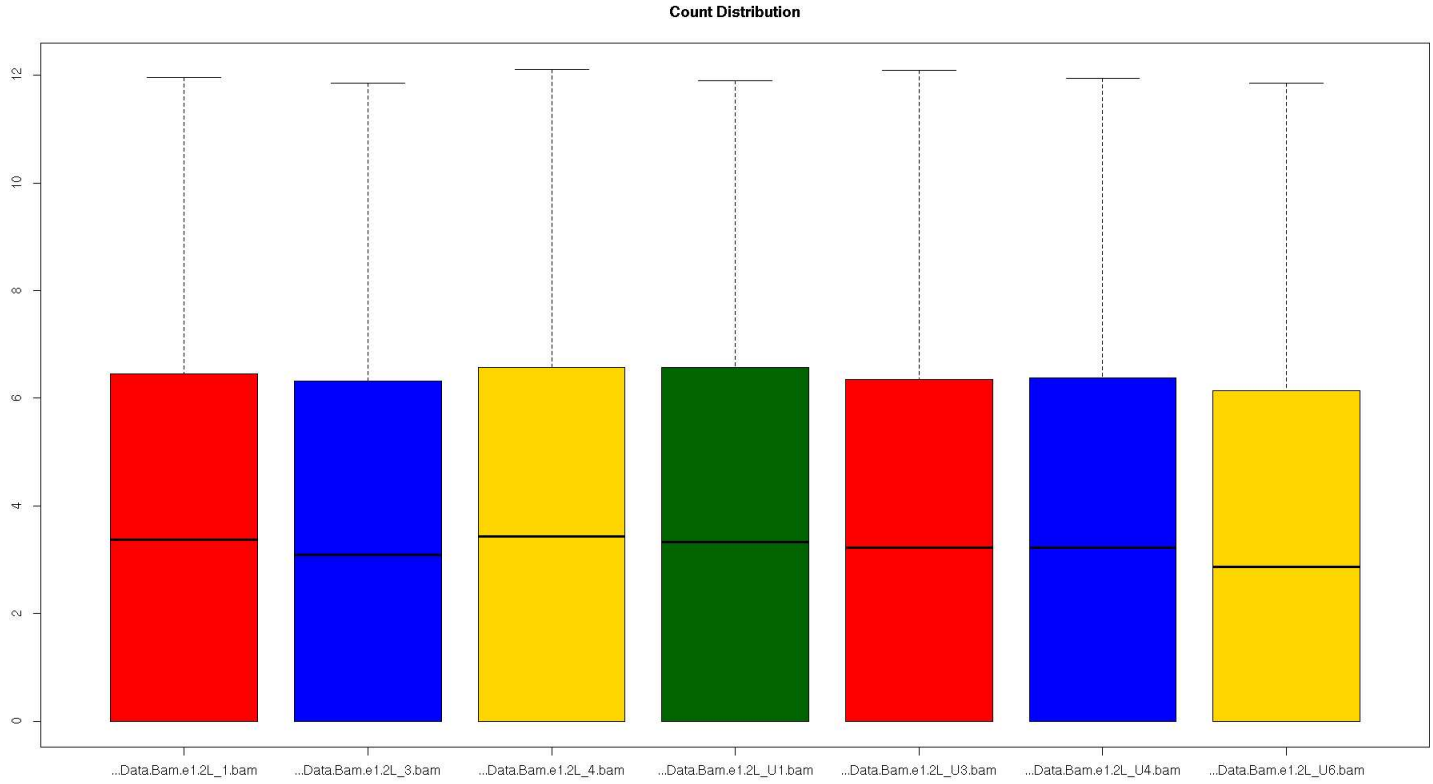


Figure 54: Box plot generated by the **Count Distr** function.

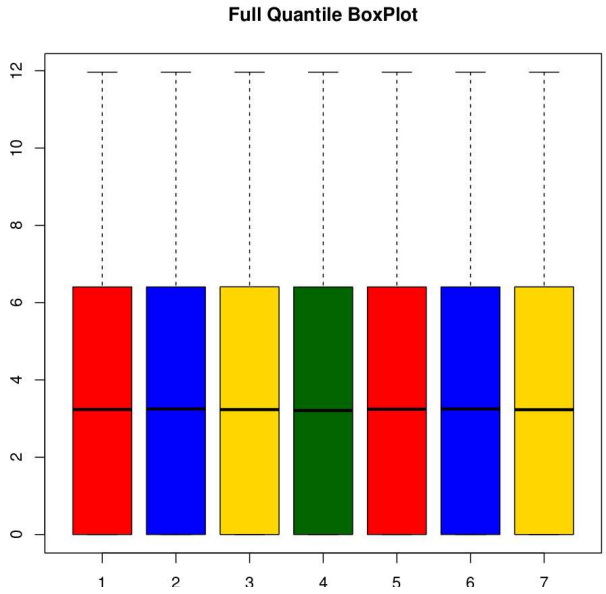


Figure 55: Boxplot of the counts shown in Figure 54 after the full quantile normalization.

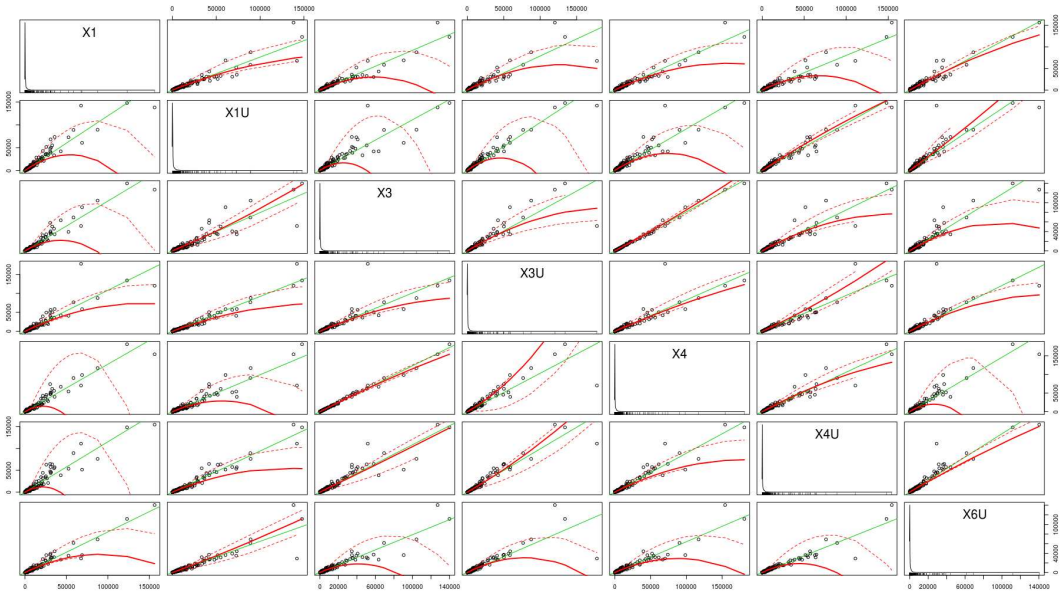


Figure 56: Count plots generated by the **Plot All Counts** function.

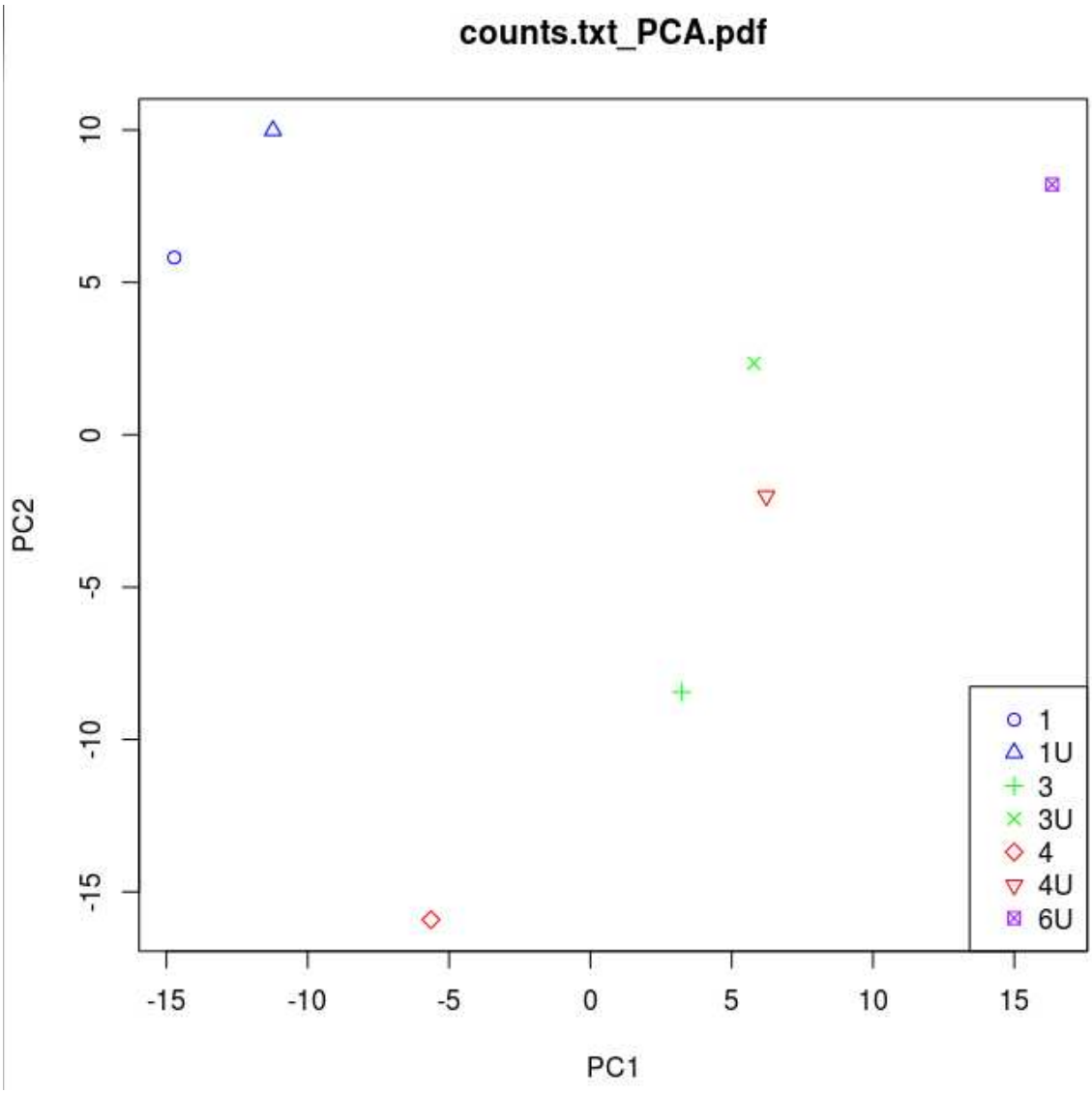


Figure 57: PCA plot generated by the **PCA** function.

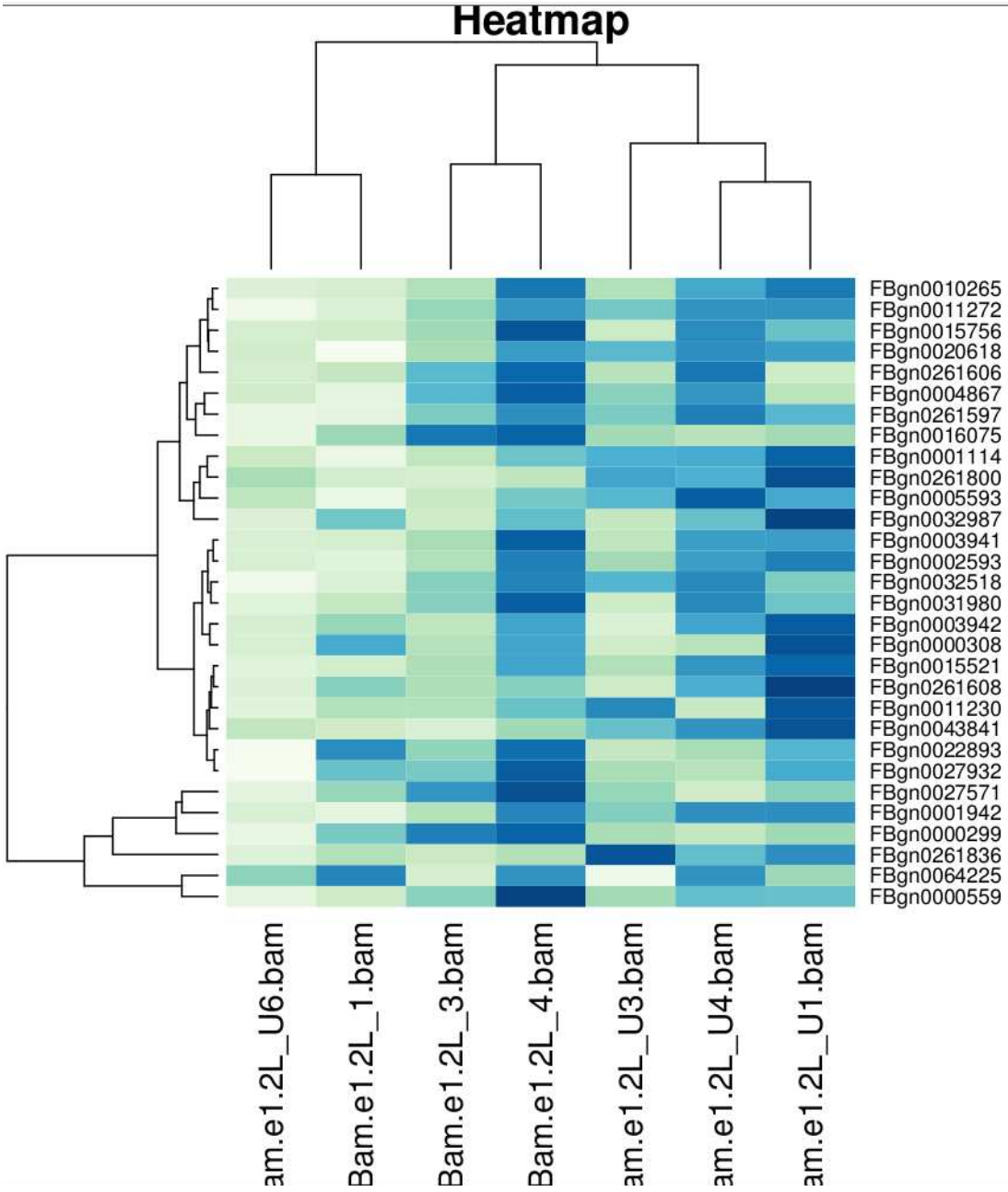


Figure 58: Heatmap

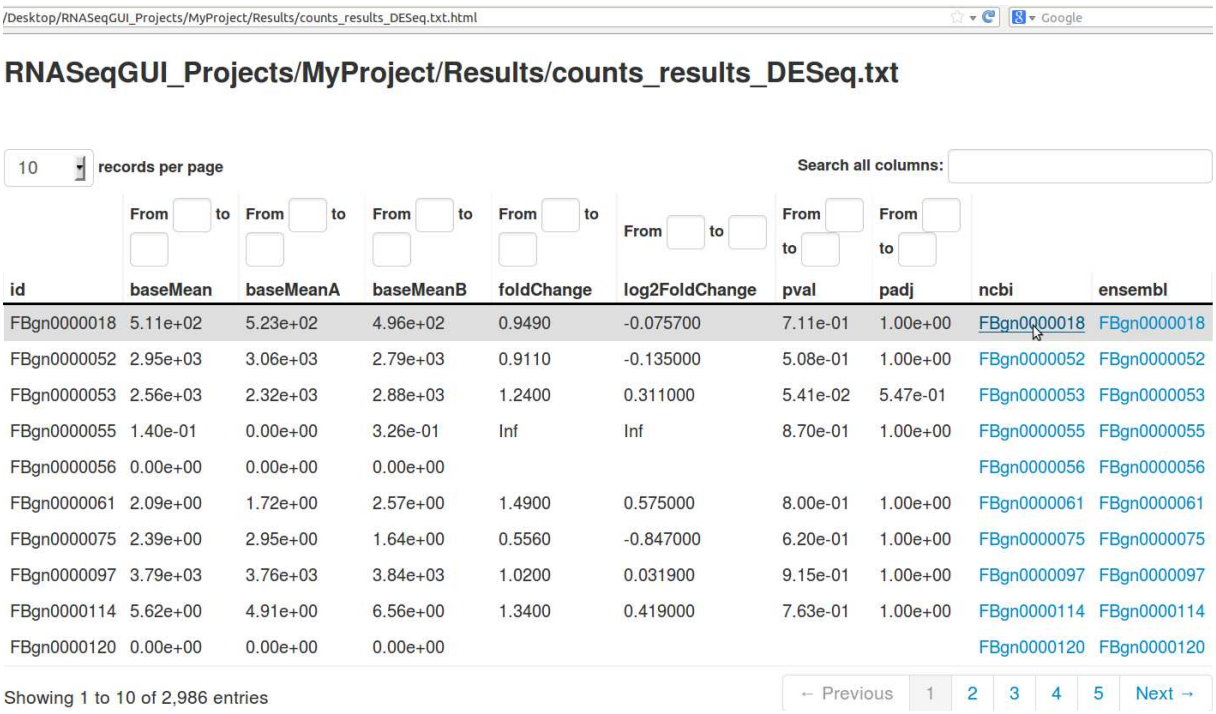


Figure 59: Result file shown via a web browser after clicking on the **Show Result** button of the DESeq Interface. The gene names in blue color are clickable and address the user to either NCBI or Ensembl databases.

`ed-end,paired-end,single-end` in the `LibTypes` field to specify the library layout as reported in Figure 46. We choose a 0.05 value as the `Padj`. Finally, we click on **Run DESeq** button. The DESeq analysis is performed and two result text files are created and saved in the **Results** folder. We can look at results by clicking on the **Show Result** Figure 59.

We click on **NOISeq** button. In the *NOISeq Analysis Interface*, we select the `2L_counts.csv` count file. We type the `T,T,T,U,U,U,U` sequence in the `Factors?` field. We type `T1,T3,T4,U1,U3,U4,U6` in the `TissueRun` field to specify the library layout as specified in Figure 46. We select `biological` in the `Replicate?` field. We choose a 0.6 value as the `prob`. Finally, we click on **Run NOISeq** button. The NOISeq analysis is performed and two result text files are created and saved in the **Results** folder.

Once all the results have been obtained, we can start inspecting them by clicking on *Result Inspection Interface*. We click on **EdgeR**, **DESEq** and **NOISeq** buttons at the same time. At each click we can see the *Result*

Inspection Interface growing (see the top-right of the Figure 60).

For each method, we select the corresponding result file (by giving the all path to the file in the `Select File` field) and we click on **Plot FC** on **FDR Hist** and on **Volcano Plot** of each method. We also provide a gene id to display a specific gene (in this case we type `FBgn0000559` in the `Gene Id field`, as shown in Figure 60, that is the most expressed gene found in the heatmap in Figure 58).

Finally, we compare the results by clicking on *Result Comparison Interface*.

We fill all the fields as shown in Figure 61. We click on **VennDiagrams3setsDE** button. This action creates two files. The first file is the pdf shown in Figure 62 and saved in **Plots** folder. The second file is a text file, called `NOISEQ_DESEQ_EDGER_genes_in_intersection.txt` and saved in the **Results** folder. This text file reports the 86 gene-ids that fall in the intersection of all the three methods (see in Figure 62).

All the functionalities we have used are automatically saved in a report file inside the **Logs** directory.

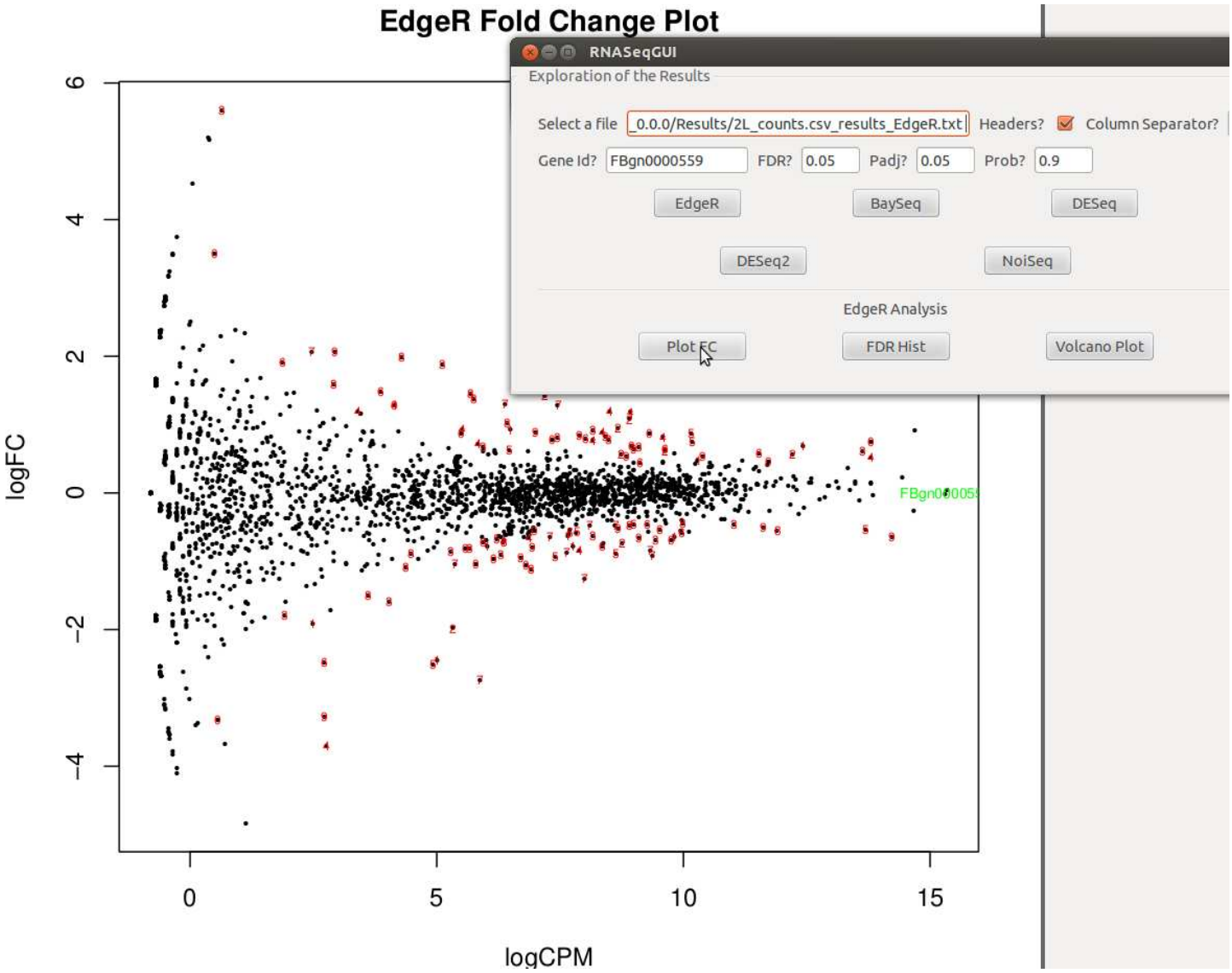


Figure 60: Fold Change Plot generated by using the function PlotFC of EdgeR

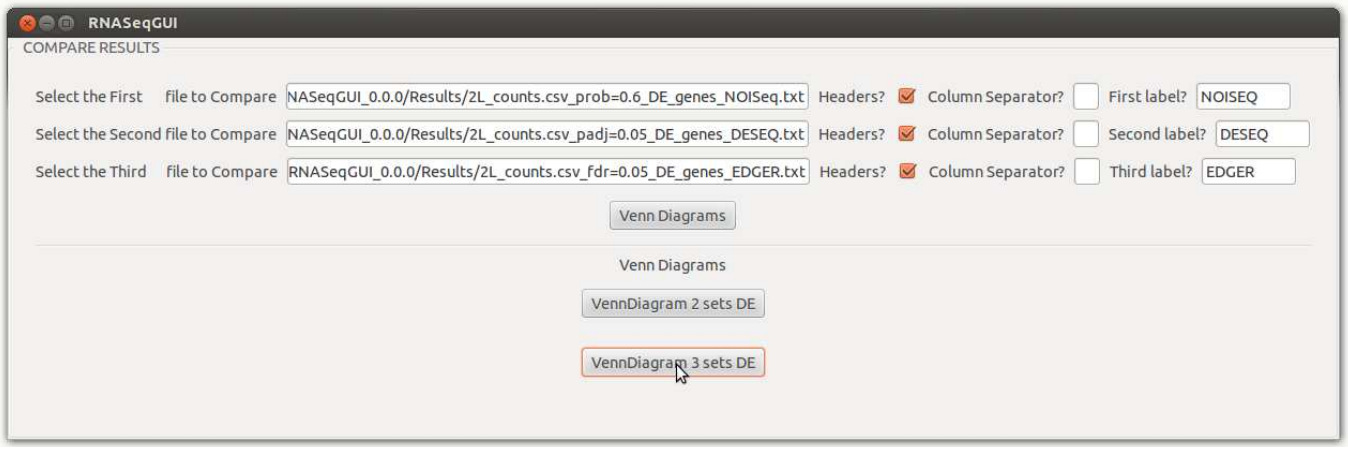


Figure 61: Result Comparison Interface

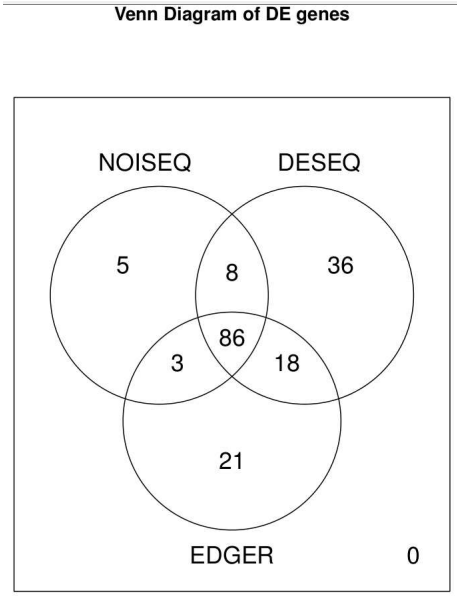


Figure 62: Venn Diagram

16 How to customize RNASeqGUI

It is extremely easy to add new buttons that calls new functions. Hence, a user can customize RNASeqGUI interfaces for his purposes and benefits by adding the methods he needs mostly.

16.1 Adding a new button in just three steps

For the sake of example, suppose you have written a function that generates a heat-map as the one written below.

```
MyHeatmap <- function(x,geneNum){
  require(RColorBrewer)
  n <- as.numeric(geneNum)
  x <- as.matrix(x)
  means=rowMeans(x)
  select = order(means, decreasing=TRUE)[1:n]   # show first n genes
  hmcol = colorRampPalette(brewer.pal(7,"Greens"))(100)
  heatmap(x[select,],col=hmcol,margins=c(5,8),main="MyHeatMap")
}
```

If you want to add `MyHeatmap` function to RNASeqGUI, follow these tree simple steps.

1 - Place `MyHeatmap` function in a file (for instance, called MyHeatmap.R) in the **R** folder inside the **RNASeqGUI** directory.

2 - Open calculateGUI1.R file (This is the file that generates the *Data Exploration Interface*) and copy the following 3 lines and paste them at the bottom of this file before "}" parenthesis.

```
#Here you create the button, called "MY OWN FUNCTION"
MYOWNBUTTON <- gtkButtonNewWithMnemonic("MY OWN FUNCTION", show = TRUE)
#Associate the button to MyHeatmapConn that calls MyHeatmap function
gSignalConnect(MYOWNBUTTON , "clicked", MyHeatmapConn)
the.buttons$packStart(MYOWNBUTTON,fill=F)
```

3 - Finally, Copy the following code

```
MyHeatmapConn<- function(button, user.data) {
  res <- NULL
  # Get the information about data and the file
  the.file <- filename$getText()
  the.sep <- sepEntry$getText()
  the.headers <- headersEntry$active
  the.geneNum <- geneNum$getText()
  d <- read.table(the.file,sep=the.sep,header=the.headers,row.names=1)
  # Select numerical variables
  numVar <- sapply(1:ncol(d),function(x){is.numeric(d[,x])})
  if (sum(numVar)==0) { error <- "ERROR: No numerical variables in the data!"
  }else{res=MyHeatmap(d,the.geneNum)} #HERE YOU CALL THE FUNCTION YOU DEFINED!
}
```

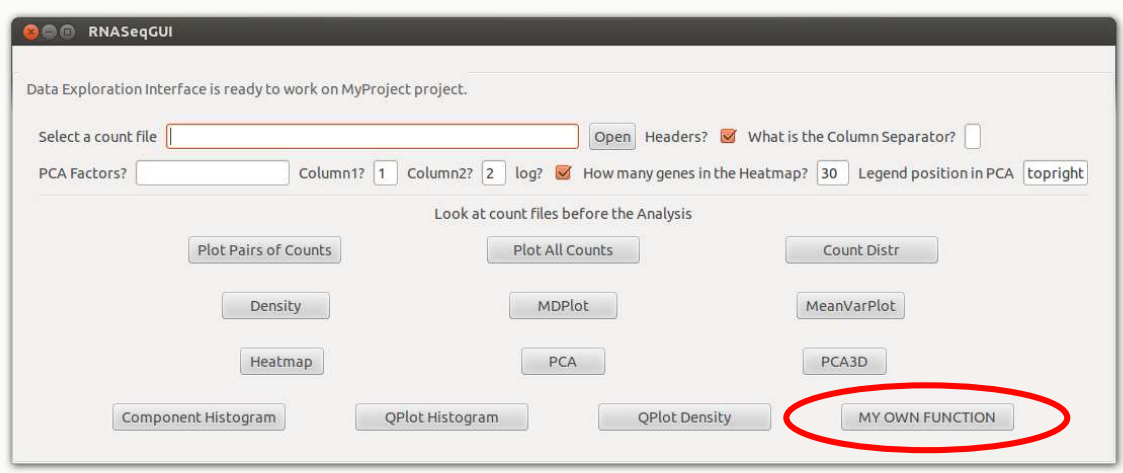


Figure 63: A new button called `MY OWN FUNCTION` is created

and paste it before the two following lines below that are written inside the calculateGUI1.R file.

```
# Create window
window <- gtkWindow()
```

At this point, `MY OWN FUNCTION` button is created and the result is the one shown in Figure 63. By clicking this button, we call `MyHeatmapConn` function that calls `MyHeatmap` function defined before.

```
 [1] filehash_2.2-2           ineq_0.2-13
 [3] e1071_1.6-4              ReportingTools_2.6.0
 [5] RSQLite_1.0.0            DBI_0.3.1
 [7] knitr_1.9                Rsubread_1.16.1
 [9] digest_0.6.8             scatterplot3d_0.3-35
11] preprocessCore_1.28.0     leeBamViews_1.1.1
13] BSgenome_1.34.1           rtracklayer_1.26.2
15] EDASeq_2.0.0              ShortRead_1.24.0
17] GenomicAlignments_1.2.2   RColorBrewer_1.1-2
19] gplots_2.16.0             pasilla_0.5.1
21] DEXSeq_1.12.2             BiocParallel_1.0.3
23] DESeq2_1.6.3              RcppArmadillo_0.4.650.1.1
25] Rcpp_0.11.5               Rsamtools_1.18.3
27] Biostrings_2.34.1         XVector_0.6.0
29] GenomicFeatures_1.18.3    AnnotationDbi_1.28.1
31] Biobase_2.26.0            GenomicRanges_1.18.4
33] GenomeInfoDb_1.2.4        IRanges_2.0.1
35] S4Vectors_0.4.0           BiocGenerics_0.12.1
37] RGtk2_2.20.31             RNASeqGUI_0.99.5

oaded via a namespace (and not attached):
 [1] acepack_1.3-3.3          annotate_1.44.0          AnnotationForge_1.8.2
 [4] aroma.light_2.2.1        base64enc_0.1-2          BatchJobs_1.5
 [7] BBmisc_1.9               biomaRt_2.22.0           biovizBase_1.14.1
10] bitops_1.0-6              brew_1.0-6               Category_2.32.0
13] caTools_1.17.1            checkmate_1.5.1          class_7.3-10
16] cluster_1.15.2            codetools_0.2-8          colorspace_1.2-6
19] DESeq_1.18.0              dichromat_2.0-0          edgeR_3.8.6
22] evaluate_0.5.5            fail_1.2                 foreach_1.4.2
25] foreign_0.8-61            formatR_1.0              Formula_1.2-0
28] gdata_2.13.3              genefilter_1.48.1        geneplotter_1.44.0
31] GGally_0.5.0              ggbio_1.14.0             ggplot2_1.0.0
34] GO.db_3.0.0               GOstats_2.32.0           graph_1.44.1
37] grid_3.1.0                gridExtra_0.9.1          GSEABase_1.28.0
40] gtable_0.1.2              gtools_3.4.1             Hmisc_3.15-0
43] hwriter_1.3.2             iterators_1.0.7          KernSmooth_2.23-12
46] lattice_0.20-29           latticeExtra_0.6-26      limma_3.22.7
49] locfit_1.5-9.1            MASS_7.3-39              Matrix_1.1-5
52] matrixStats_0.14.0        munsell_0.4.2            nnet_7.3-8
55] OrganismDbi_1.8.1         PFAM.db_3.0.0            plyr_1.8.1
58] proto_0.3-10              RBGL_1.42.0              RCurl_1.95-4.5
61] reshape_0.8.5             reshape2_1.4.1           R.methodsS3_1.7.0
64] R.oo_1.19.0               rpart_4.1-8              R.utils_2.0.0
67] scales_0.2.4              sendmailR_1.2-1          splines_3.1.0
70] statmod_1.4.20            stringr_0.6.2            survival_2.37-7
73] tcltk_3.1.0               tools_3.1.0              VariantAnnotation_1.12.9
76] XML_3.98-1.1              xtable_1.7-4             zlibbioc_1.12.0
```

Figure 64: Session info

17 Technical Details

To see the versions of the used methods, we type

```
sessionInfo()
```

and we get the list shown in Figure 64.

18 Errors/Warnings/Bugs

18.1 Read Count Interface Errors

18.1.1 Warning messages: In .deduceExonRankings(exs...

```
> Warning messages:
> In .deduceExonRankings(exs, format = "gtf") :
> Infering Exon Rankings.  If this is not what you expected, then
> please be sure that you have provided a valid attribute for
> exonRankAttributeName
```

This happens when in the provided GTF file there is no exon ranking information. Therefore, the only way to get exon rank information is by deducing it based on the provided coordinate positions. This inference task can be performed by the parser, but it takes time to be completed. Moreover, the parser makes assumptions on your data. Hence, it is better to avoid it when possible. That's s why the `deduceExonRankings` function is throwing a warning about the exon ranking inference process.

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