

ProStaR Tutorial

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Abstract

This document is a tutorial to *ProStaR*. It uses the data stored in the 'Exp1_R25_pept.txt' file, from the *DAPARdata* package, to illustrate on a walkthrough example the use of *ProStaR* to conduct a proteomics quantitative analysis.

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1 Preamble

This document is only a step by step illustration of the use of *ProStaR*. For a comprehensive description of *ProStaR* functionalities, we refer the readers to the "*DAPAR* and *ProStaR* user manual", available on the Bioconductor webpage of *ProStaR*.

2 Loading data

The text-format file of 'Exp1_R25_pept.txt' is part of the *DAPARdata* package. For a precise understanding of the dataset, please refer to the document "Description of the UPS{pep/prot}25 datasets" available on the bioconductor webpage of *DAPARdata*.

In a nutshell, it gathers the peptide intensities of 6 samples, containing each the same yeast lysate. In addition, several human proteins have been spiked in, with different concentrations: three samples theoretically contains 2.5 more human proteins than the three others. At the end of the quantitative analysis, all and only these proteins should be selected as significantly differentially abundant.

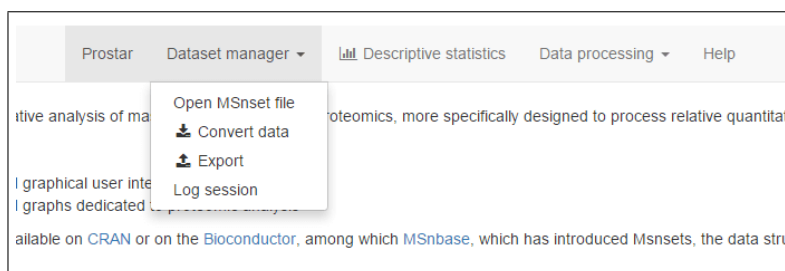


Figure 1: Dataset Manager Menu

The text file containing the data must be of tabular form, as those produced by MaxQuant software, for instance. It is to be uploaded by clicking on the "Convert data" of the "Dataset manager" submenu (see Fig. 1). This opens the page captured on Fig. 2 and composed of five panels: Select File, Data Id, Exp and Feat data, Samples and Convert.

In the panel **Select File**: Indicate where to find the text file containing the data. As in this example, the file contains peptide intensities that are not depicted on a logarithm scale, the corresponding options must be manually selected (see Fig. 2).

Prostar Dataset manager Descriptive statistics Data processing Help

These steps allow to create a MSnSet file from a tabulated-text file.

1 - Select file 2 - Data Id 3 - Exp. and feat. data 4 - Samples metadata 5 - Convert

Data file

Choisissez un fichier Aucun fichier choisi

Hint : before importing quantification file data, check the syntax of your text file.

Is it a peptide or protein dataset ?

☒ peptide dataset
☐ protein dataset

Check whether the data you want to analyze are already logged or not. If not, they will be automatically logged

☐ yes
☒ no

☒ Replace all 0 and NaN by NA

Figure 2: Conversion Text Data Page

In the panel **Data Id**: Indicate the column that contains the IDs of the peptides. These IDs have to be unique across all the rows. In the example dataset, it is simply the column referred to as "id". This is why "id" is selected in the drop down menu (see Fig. 3).

Prostar Dataset manager Descriptive statistics Data processing Help

These steps allow to create a MSnSet file from a tabulated-text file.

1 - Select file 2 - Data Id 3 - Exp. and feat. data 4 - Samples metadata 5 - Convert

Please select among the columns of your data the one that corresponds to a unique ID of the peptides .

If you choose the automatic ID, Prostar will build an index.

☐ Auto ID
☒ user ID

id

Figure 3: Using user-column 'id' as an ID in ProStaR

In the panel **Exp. and Feat. Data**: indicate the title of the columns that contains quantitative data of the peptides (See Fig. 4).

Prostar Dataset manager Descriptive statistics Data processing Help

These steps allow to create a MSnSet file from a tabulated-text file.

1 - Select file 2 - Data Id 3 - Exp. and feat. data 4 - Samples metadata 5 - Convert

Select the columns that are quantitation values by clicking in the fields below.

Quantitative Data

Intensity.C.R1 Intensity.C.R2 Intensity.C.R3 Intensity.D.R1
 Intensity.D.R2 Intensity.D.R3

Figure 4: Exp and Feat. Data panel

In the panel **Samples metadata**: Fill in the table according to the properties of the experimental design (see Fig. 5). In the example case, the first 3 samples correspond to 25 fmol of human proteins, while the others to 10fmol; the concentration is used to refer to the conditions. What matters here is to have different name for the two conditions, and to have a similar name within each condition. Because of the simple experimental design, the biological, technical and analytical replicates do not need to be filled.

ProStaR Dataset manager Descriptive statistics Data processing Help

These steps allow to create a MSnSet file from a tabulated-text file.

1 - Select file 2 - Data id 3 - Exp. and feat. data 4 - Samples metadata 5 - Convert

Warning : it is mandatory that the column "Label" is filled.

	Experiment	Label	Bio.Rep	Tech.Rep	Analyt.Rep
1	Intensity.C.R1	25fmol	R1		
2	Intensity.C.R2	25fmol	R2		
3	Intensity.C.R3	25fmol	R3		
4	Intensity.D.R1	10fmol	R1		
5	Intensity.D.R2	10fmol	R2		
6	Intensity.D.R3	10fmol	R3		

Figure 5: Metadata table to fill in (Samples metadata panel)

Finally, in the panel **Convert**, enter the name of the project and click on "Convert data" to build an MSnSet object containing the data. A message indicates when the conversion is over (see fig. 6).

ProStaR Dataset manager Descriptive statistics Data processing Help

Dataset versions
Original - peptide

These steps allow to create a MSnSet file from a tabulated-text file.

1 - Select file 2 - Data id 3 - Exp. and feat. data 4 - Samples metadata 5 - Convert

Enter the name of the study

UPSep25

Convert data

The conversion is done. Your dataset has been automatically loaded in memory. Now, you can switch to the Descriptive statistics panel to visualize your data.

Figure 6: The conversion of text file to MSnSet format is done

Please also notice in the screenshot that the value in the top-right box has changed from 'None' to 'Original - peptide'. All along the ProStaR pipeline, this box indicates the current state of the dataset. Here "original" means that no processing has been performed so far, and "peptide" means that the dataset contains peptide intensities.

3 Descriptive statistics

Once the data are loaded, their exploration/visualisation is the natural following step. By clicking on "Descriptive statistics" in the menu, one finds a series of 9 panels, that are helpful to understand the data:

The first one, referred to as **Overview**, shows very basics and general informations about your dataset. For our example we can see we have: 6 samples, 13919 peptides, 19.19% of missing values and 733 lines with only NA values (missing values).

The second one, referred to as **Missing values**, summarizes the missing values distributions with 3 plots (see fig. 7 from left to right), that are also use to monitor the imputation of missing values (see Section 6):

- **'# NA per columns'** i.e. the number of missing values per sample.
- **'# of lines by # of NA'**: how many lines (peptides in our example) have 1, 2, ..., up to 6 NA. The red bar bar of the barplot (6 here) corresponds to peptides that have only missing values, i.e. that have been identified but not quantified. Notice that this corresponds to the 733 lines already pointed on the previous paragraph.
- **'# of lines by # of NA per condition'**: It is the same plot as the second one, yet condition-wise, rather than entire dataset-wise. Notice that in this third plot, the number of lines with 0 missing values is presented, contrarily to the second one (where it would shrink too much the Y-axis).

The third one, referred to as **Data explorer**, allows the practitioner to navigate through the MSnSet containing the data. Several tables are available: quantitative data, peptides metadata, replicate metadata.

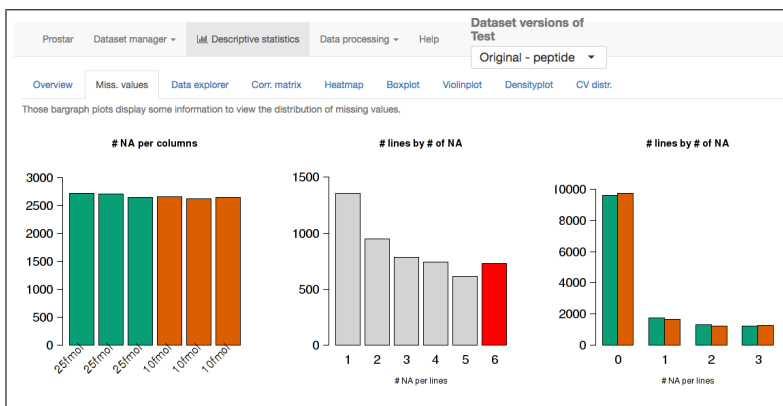


Figure 7: Missing Values repartition in the dataset

The other panels produce graphical representations of the dataset: **Boxplot**, **Densityplot**, **Correlation matrix**, **Heatmap**, **Violinplot** and **CV distribution** (see some of them on Fig. 8 to 10).

In particular:

- The correlation matrix (Fig. 8) shows well the replicates correlate better within conditions (25fmol together or 10fmol together). This confirms the quality of the dataset.
- On the density plots (Fig. 10) the distributions are Gaussian-like. It is a pre-requisite for most next coming statistical processing.

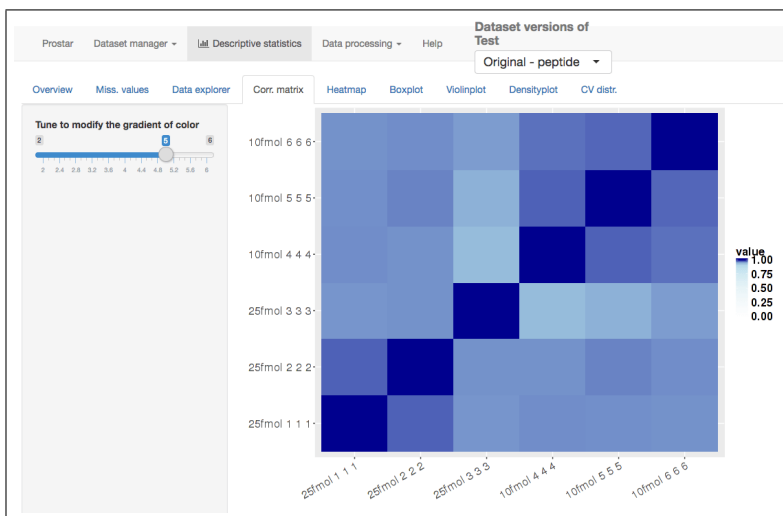


Figure 8: Correlation Matrix (for the 6 samples)

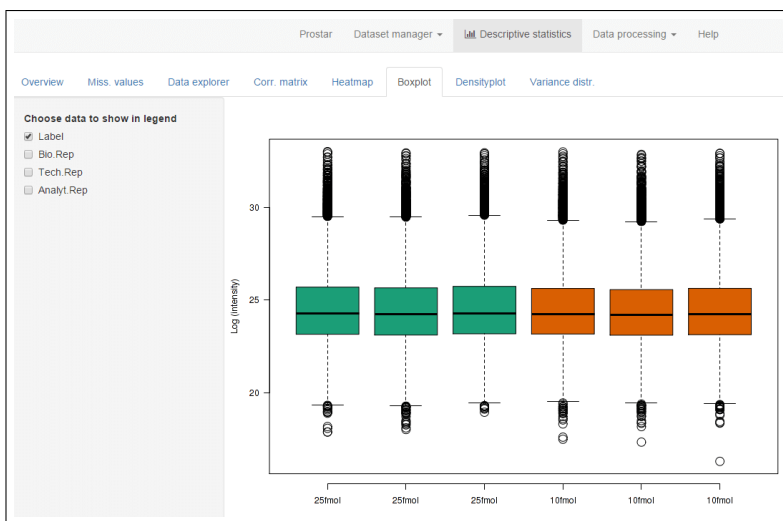


Figure 9: Boxplot (raw data)

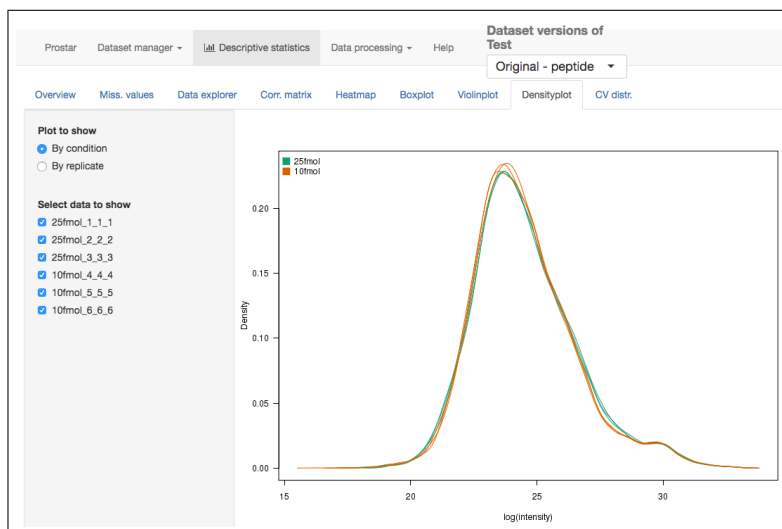


Figure 10: Density plots of the log2 raw data

4 Filtering

Clicking on 'Filter data' in the drop down submenu 'Data processing' allows the practitioner to filter out some lines of the dataset according to various criteria.

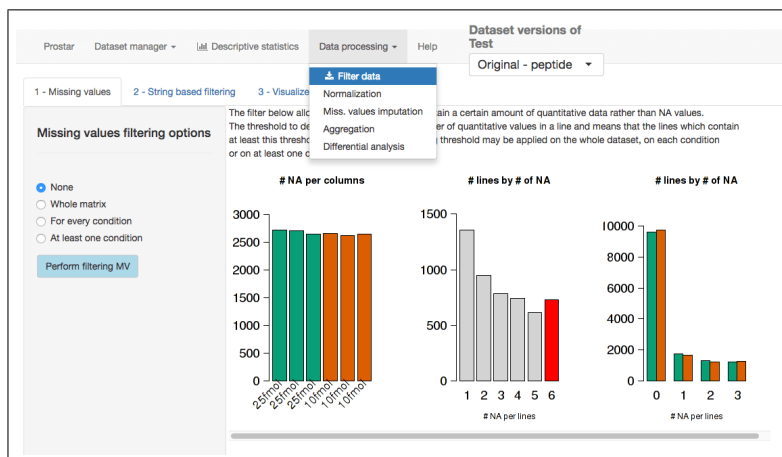


Figure 11: Screenshot of the missing value filtering panel

Graphics are interactively updated.

In the first panel, one filters out peptides with too many missing values. The same plots as those from the 'Descriptive statistics' panel are displayed (see Fig. 11). For this dataset, we propose to filter out peptides that have at maximum 1 missing value among the 3 peptide intensities of each condition (but other filtering option would also make sense, depending on the dataset,

and on the next coming processing). This results in the options selection that is illustrated on Fig. 12, where one sees that the 3 plots have been automatically updated, as soon as the 'Perform filtering MV' button has been clicked on.

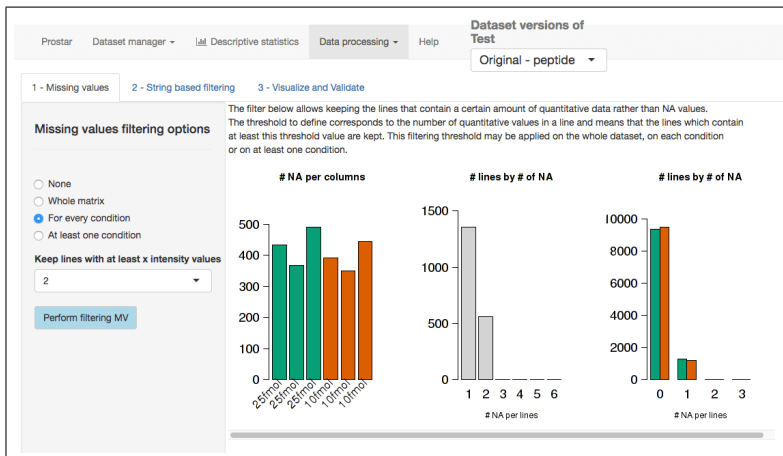


Figure 12: Screenshot of the missing value filtering panel

Graphics are interactively updated.

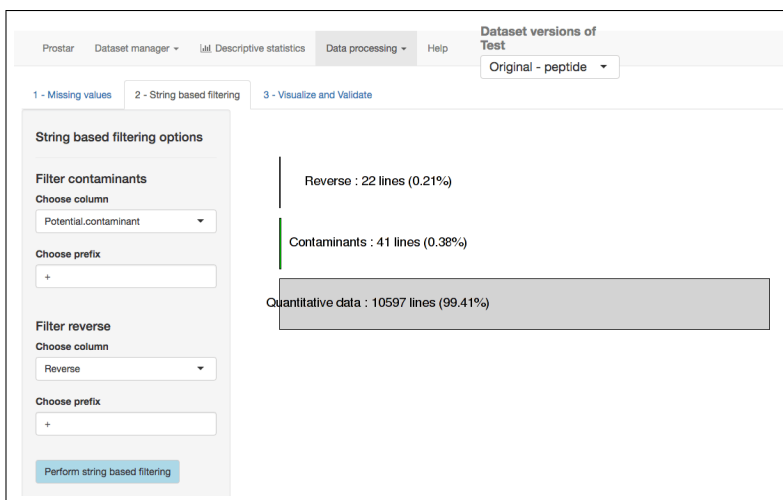


Figure 13: Screenshot of the string based filtering panel

Graphics are interactively updated.

Once this filtering has been performed, it is possible to move to the string-based filtering panel, which proposes to remove lines on the basis of some meta-data (i.e. peptide sequences that are known to be contaminants ou reverse sequences). This panels works as the previous one and is shown on Fig. 13.

Finally, one goes on the third panel, named 'Visualise and validate', that aims at performing the final validation of the filtering steps. To do so, one simply clicks on "Save filtered dataset" (Fig. 14). From that point on, the field in the top-right box is updated to "**Filtered** - peptide", indicating that ProStaR will work

on this filtered version of the dataset. As a result, all the panels are interactively updated through *ProStaR*. Hence, if one goes back to the 'Descriptive statistics' panels, all the plots will display this "**Filtered** - peptide" dataset. For example in the 'Overview' panel, it now appears that the filtered data consist of 10660 peptides (instead of 13919), with 3.88% of missing values.

ID	Intensity.C.R1	Intensity.C.R2	Intensity.C.R3	Intensity.D.R1	Intensity.D.R2	Intensity
3	3	19.976				
13	13	22.127				
22	22					
24	24	23.158			22.372	
25	25					
29	29	23.188	22.742	22.946		
42	42					
44	44			25.83	25.988	26.031
45	45	22.276				
46	46	22.354			22.109	

Figure 14: Panel for filter data validation

5 Normalization

Once the data are filtered, the next step is to normalize the data (in the "Data processing" dropdown menu). It works as the previous processing steps, yet the navigation is even simpler. First, chose the adequate normalization method (here we have chosen "Quantile Centering - within conditions with quantile=0.5"); Second perform the normalization; and finally, save it (see Fig. 16). As usual, notice that once the data are normalized, the top-right box indicates "Normalized - peptide".

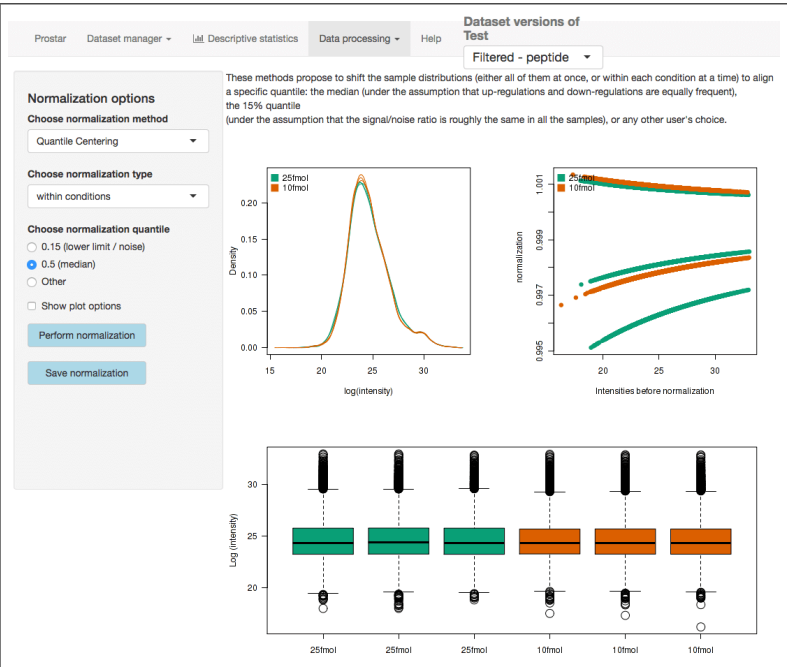


Figure 15: The normalization panel - before performing normalization

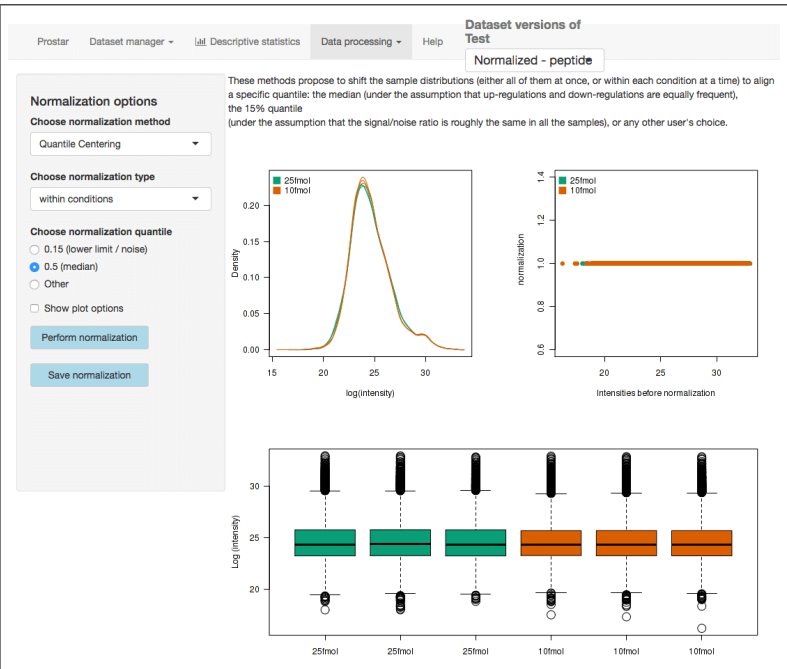


Figure 16: The normalization panel - after the normalization has been performed

6 Missing value imputation

The next step is to impute the missing values. On this panel (Fig. 17) two plots are of help to visualize the distribution of the missing values in the dataset, and to choose the adequate imputation method.



Figure 17: Panel Missing Values Imputation

On the left hand side (Fig. 17) is displayed the number of missing values as a function of the intensity of the peptides. The missing values heatmap (on the right hand side) allows to see once-at-a-glance the possible difference of distribution of the missing values between the conditions.

On this example, we impute the missing values with the imp4p method (with one iteration, imputation of LAPALA, the upper distribution bound is set to 2.5 and the distribution type used is beta). Fig. 18 represents the automatically updated plots. Once again, it is necessary to save the imputation before going on.

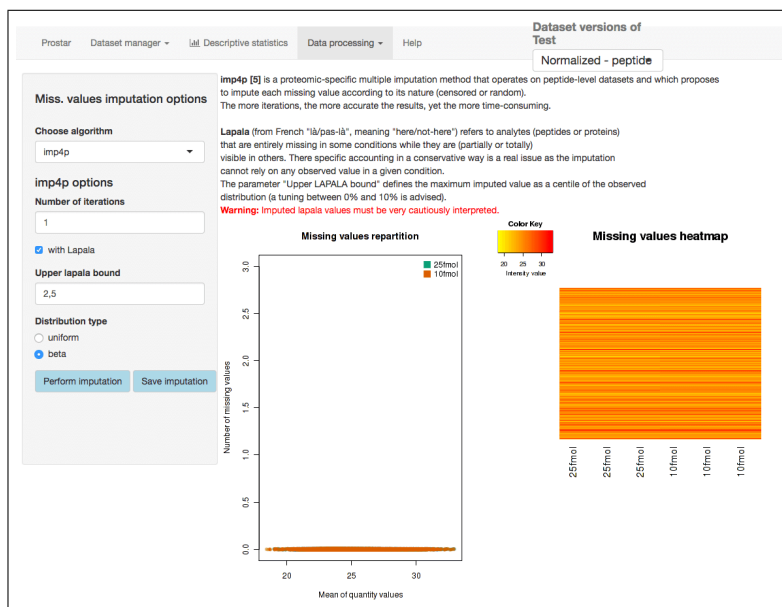


Figure 18: Panel Missing Values Imputation

7 Protein-wise aggregation

The dataset we work on is quantified at the peptide-level. Nevertheless, the aim of a proteomics quantitative analysis is to conclude at protein-level. Thus, we aggregate the peptide intensities into protein abundance values (the available aggregation methods are described in the 'DAPAR and ProStaR user manual').

On the panel "Aggregation" of the "Data processing" drop-down menu (Fig. 19), one has first to indicate in the "Aggregate peptides" tab, the protein ID to consider (here 'Protein.group.IDs'). As soon as the protein ID is indicated, some plots and statistics provide evidences on the number of (specific or shared) peptides per proteins (on the right hand side of the panel), so as to help with the tuning of the two other parameters (Fig. 19).

After the tuning of the other parameters, namely, the aggregation method (here 'mean'), and whether shared peptides are considered or not (here 'yes'), the aggregation can be performed. A message shows up indicating the aggregation was successfully conducted (see Fig. 20):

As usual with *ProStaR*, it is necessary to save the results. This is done on the next panel "Configure protein dataset". Here, one indicates among the peptide metadata of the dataset which one will be kept and aggregated to define the protein metadata (here, we consider the column "Leading.razor.protein" - Fig. 21). Once the aggregation is saved, please check that the top right box indicates now "Aggregated - proteins". From that point on, it is possible to go

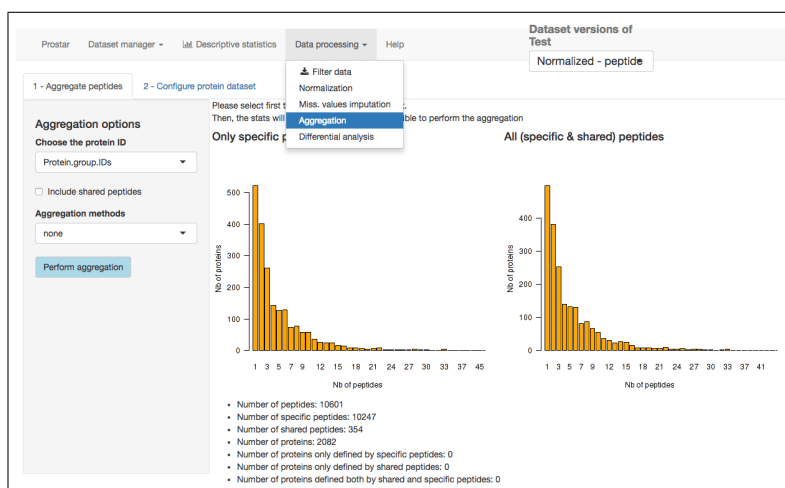


Figure 19: Aggregation step panel before aggregation

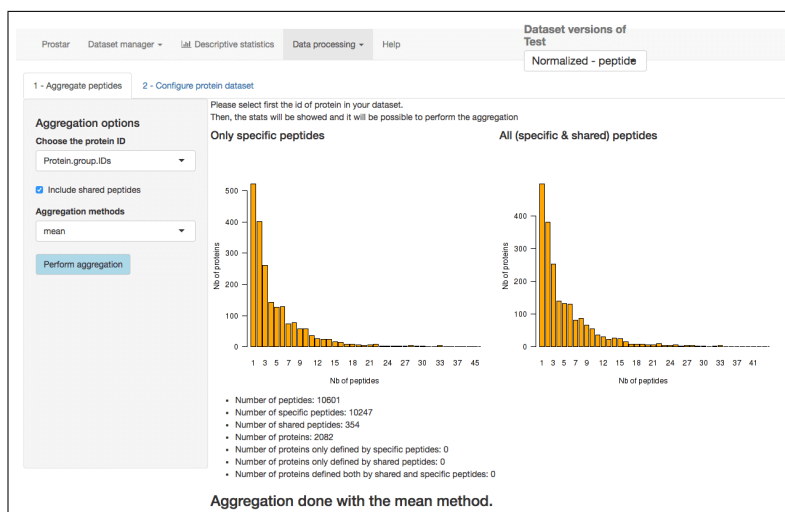


Figure 20: Aggregation step panel after aggregation

back to the "Descriptive statistics" tabs, and to investigate the new protein-wise dataset. For instance, in the "Overview" tab, it appears that one has the following information:

There is 6 samples in your data.

There is 2082 lines in your data.

Percentage of missing values: 0 %

The screenshot shows the 'Data processing' tab in the ProStaR interface. It features two tabs: '1 - Aggregate peptides' (active) and '2 - Configure protein dataset'. A checkbox for 'Filtering : remove the proteins that are defined by less than n peptides.' is present. Below it, a text box contains 'Leading,razor,protein'. A 'Save aggregation' button is at the bottom left. On the right, a dropdown menu for 'Dataset versions of Test' is set to 'Normalized - peptide'.

Figure 21: Aggregation step panel after aggregation

8 Differential analysis

Differential analysis is the final step to identify differentially abundant proteins. This is performed on the corresponding panel of the "Data processing" drop-down menu. have been performed if the original dataset had MV).

The first step of the differential analysis is to build a volcano plot. This requires conducting a statistical test on each protein, so as to fit them with a p -value each (which will be used as a complement to the fold-change to construct the plot). To do so, we advise to use Limma moderated t -test. Concretely, one has to define which are the two conditions to compare (select "condition 1" and "condition 2" - in the example case, it is obvious as there are only 2 different conditions) and to select "Limma" on the drop down menu, on the left hand side of the panel. Then, the volcano plot is displayed (Fig. 22).

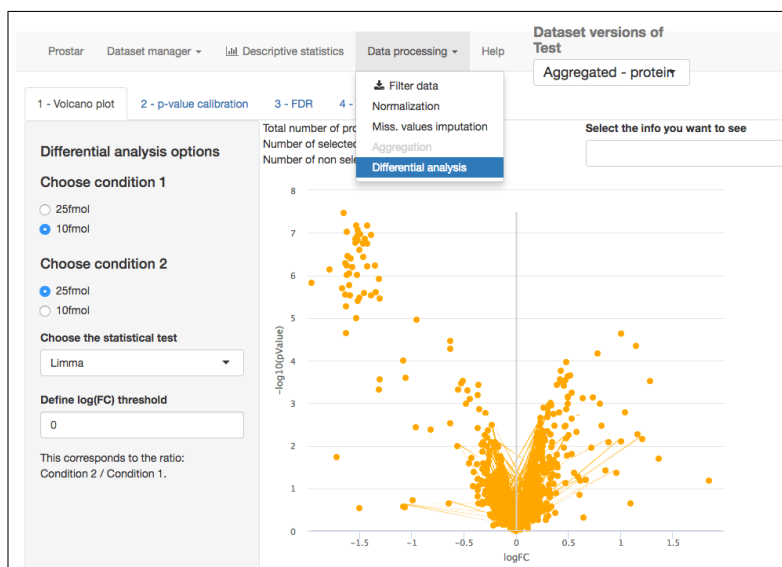


Figure 22: Volcano plot panel (Differential analysis)

Then, the practitioner is allowed to define a threshold on the fold-change, so as to filter out proteins which do not vary enough between the conditions (Fig. 23).

When the user puts the pointer of his mouse over a point of the plot, a tooltip window appears and shows some informations about that point. He can select the items to show in the Select widget where the different choices correspond to the columns of the feature meta-data table. The tooltip window is automatically updated. When the user clicks on a point, a table is displayed above the volcano plot. It shows the values of intensities for all the samples related to the selected point. The cells colored in blue indicate that the corresponding value was a missing value in the original dataset and has been imputed. The user can click and draw a rectangle on the plot to zoom in. By clicking on the button named "Reset zoom", the user can return to the entire plot.



Figure 23: Volcano plot panel (Differential analysis)

The **Calibrate Ana Diff** panel (fig. 24) helps the practitioner to check if the distribution of the p -values is well-calibrated. This calls the `cp4p` R package functions, and we refer to the corresponding tutorial: <https://sites.google.com/site/thomasburgerswebpage/download/tutorial-CP4P-4.pdf>. Here, we simply use the default setting with "pounds" method.

On the **FDR** panel (fig. 25), one adjusts the p -value threshold (here to 0.0001), and apply the adaptive Benjamini-Hochberg procedure to adjust the p -values (corrected by pounds methods, see previous tab), which leads to an estimator of an upper bound of the proportion of false discoveries (referred to as False Discovery Rate, or FDR - in the example, it is estimated to 0%).

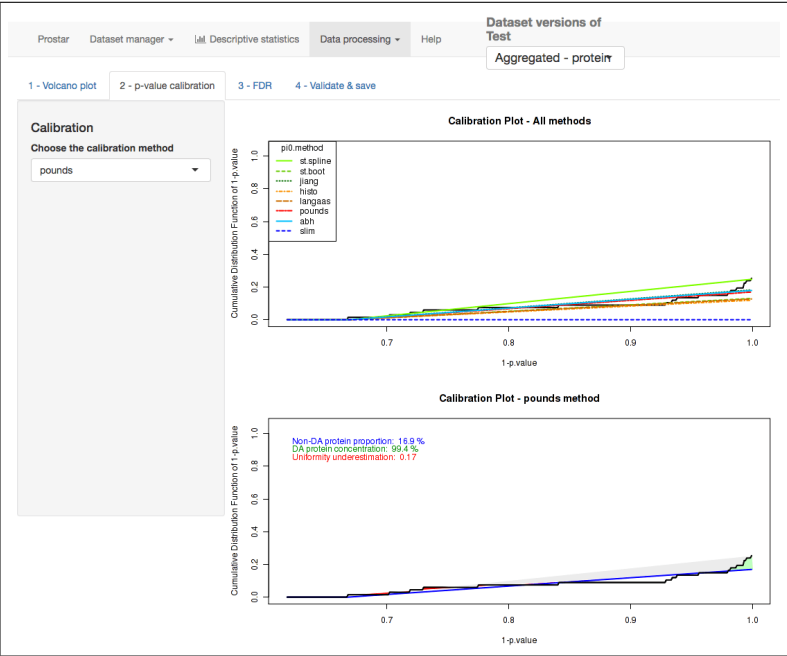


Figure 24: Calibration panel for the differential analysis)

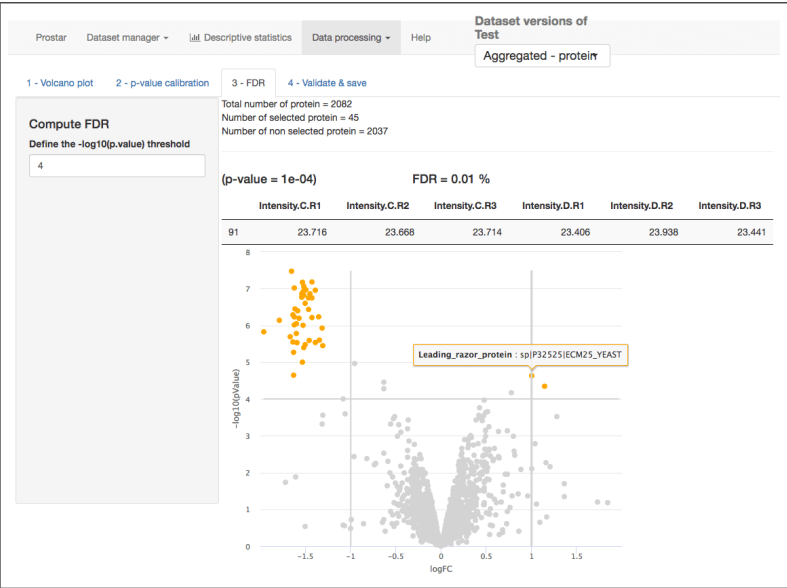


Figure 25: FDR visualization (Differential Analysis)

Finally, on the **Validate and Save** panel, one visualizes the selected 45 proteins and save a new dataset with only the latter ones.

9 Export

At the end of the quantitative analysis, or whenever an intermediate step is achieved along the processing pipeline, it is possible export the dataset. To do so, an "Export" panel is available in the "Dataset Manager" drop-down menu (see Fig. 26).

Let us note that the dataset which will be exported is the one that is indicated in the "Dataset versions" box. On the left of the panel ones specifies: the export format (MsnSet or Excel), and of course the name of the file exported. In the case of an Excel export, it is necessary to specify the protein or peptide ID that is used to map the data to the meta data (the file contains 3 sheets for the quantitative data, peptide/protein metadata and the replicate metadata).

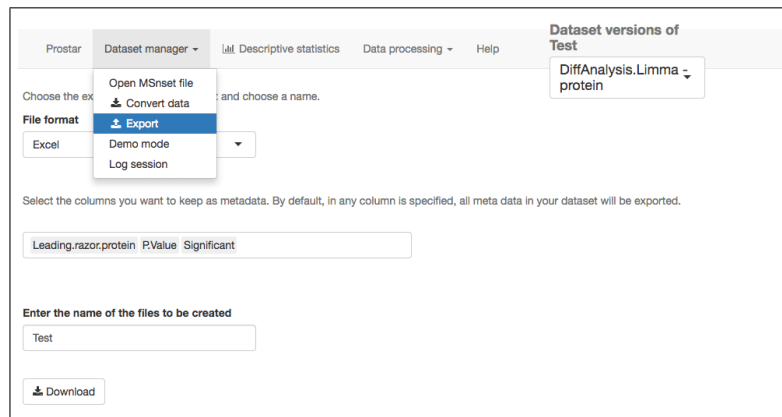


Figure 26: Export page of ProStaR