Manual for

LFQ-Analyst

LFQ-Analyst has been developed to automate downstream statistical analysis of label-free, quantitative proteomics datasets preprocessed with MaxQuant

Quick start

- Open a web browser and navigate to https://bioinformatics.erc.monash.edu/apps/LFQ-Analyst/
- Open the **“Analysis”** sidebar tab
- Upload your **proteinGroups.txt** file generated by MaxQuant
- Upload your **experimental design table**
- *Optional*: Adjust the p-value cut-off, the $\log_2$ fold change cut-off, the imputation type and/or the type of the FDR correction in the “Advanced Options” sidebar
- Press “Start Analysis” to perform differential expression analysis and wait for the results to appear in the background

- To perform a Gene Ontology and/or Pathway Enrichment analysis on the significantly regulated proteins, press “Run Enrichment” in the bottom right section of the results after selecting the desired GO database (molecular function, biological process or cellular component) and/or pathway database (KEGG or Reactome). Note that this might take a while to complete.

Input Files

LFQ-Analyst requires two input files:

1) The MaxQuant **proteinGroups.txt** file that **Must** contain a *Gene name* and *Protein IDs* column in addition to "*LFQ intensity*" columns.
2) An **experimental design table**: A tab separated file containing **only three** columns: "*label*", "*condition*", "*replicate*". The column headers including all entries are **case sensitive**. Here is an example

label	**condition**	**replicate**
Total_309B	Benign	1
Total_445B	Benign	2
Total_555B	Benign	3
Total_588B	Benign	4
Total_309M	Malignant	1
Total_445M	Malignant	2
Total_555M	Malignant	3
Total_588M	Malignant	4

Note: The entries in the "label" column must match the labels present in the **LFQ Intensity** columns of the **proteinGroups.txt** file. For example, write **"Total_309B"** if a **"LFQ Intensity Total_309B"** column is present in your proteinGroups.txt file.

***LFQ-Analyst's* processing pipeline**

Data pre-filtering

The following steps are applied to the data before differential expression analysis is performed:

- Potential contaminant sequences are removed
- Reverse sequences are removed
- Proteins that have been only "identified by site" are removed
- Proteins that were quantified by a single Razor or unique peptide are removed
- Proteins with a high proportion of missing values are removed. In detail: see table below

Number of replicates	**Allowed missing values (in at least one condition)**
Two	0
Three	1
Four or Five	2
Six or Seven	3
More than Seven	Number divided by 2, rounded down*

* For example, if the number of replicates is 9, then the number of allowed missing values is 4.

Differential expression analysis

Protein-wise linear models combined with empirical Bayes statistics are used for the differential expression analysis. We use the *Bioconductor* package *limma* to carry out the analysis using the information provided in the experimental design table. Of note, differential expression analyses are performed for all possible pair-wise comparisons.

Advanced Options

Significant protein filtering criteria

- Adjusted p-value cutoff: default is **0.05**
- Log_2 fold change cutoff: default is **1**

Missing value imputation options

- **Perseus-type:** This method is based on the popular missing value imputation procedure implemented in the *Perseus* software(1).The missing values are replaced by random numbers drawn from a normal distribution of 1.8 standard deviation down shift and with a width of 0.3 of each sample.
- **bpca:** Bayesian missing value imputation
- **knn:** Missing values replace by nearest neighbor averaging technique
- **QRILC:** A missing data imputation method that performs the imputation of left-censored missing data using random draws from a truncated distribution with parameters estimated using quantile regression.
- **MinDet:** Performs the imputation of left-censored missing data using a deterministic minimal value approach. Considering an expression data with n samples and p features, for each sample, the missing entries are replaced with a minimal value observed in that sample. The minimal value observed is estimated as being the q-th quantile (default q = 0.01) of the observed values in that sample.
- **MinProb:** Performs the imputation of left-censored missing data by random draws from a Gaussian distribution centered to a minimal value. Considering an expression data matrix with n samples and p features, for each sample, the mean value of the Gaussian distribution is set to a minimal observed value in that sample. The minimal value observed is estimated as being the q-th quantile (default q = 0.01) of the observed values in that sample. The standard deviation is estimated as the median of the feature standard deviations. Note that when estimating the standard deviation of the Gaussian distribution, only the peptides/proteins which present more than 50% recorded values are considered.

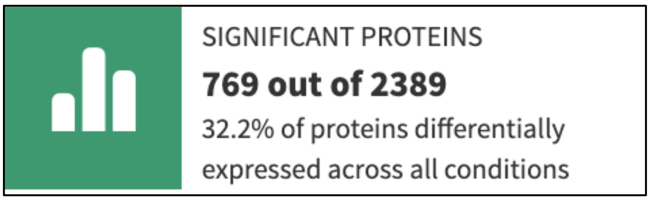
- **min:** Replaces the missing values by the smallest non-missing value in the data.
- **zero:** Replaces the missing values by **0**.

False Discovery Rate (FDR) correction option

- Benjamini Hochberg (BH) method
- t-statistics correction: Implemented in fdrtools

Output

Experimental summary



SIGNIFICANT PROTEINS

769 out of 2389

32.2% of proteins differentially expressed across all conditions

The number and proportion of all differentially expressed proteins across all pair-wise comparisons is shown (considering the user-defined thresholds for FDR and $\log_2$ fold change).

LFQ Results table

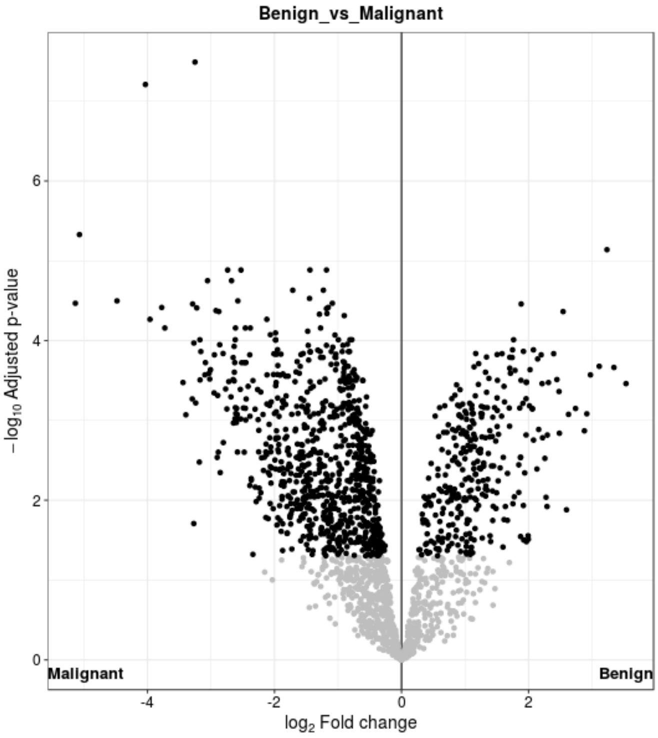
The gene names, Protein IDs and protein names of the quantified proteins are listed in this table. In addition, the following columns are shown:

- **Log$_2$ fold change** (for each pairwise comparison)
- **Adjusted p-value** (for each pairwise comparison): p.adj
- **P-value** (for each pairwise comparison): p.val
- **Significant**: Boolean values (true or false) if a given protein has been observed to be significantly regulated in **any** pairwise comparison
- **Significant** (for each pairwise comparison): Boolean values (true or false) if a given protein has been observed to be significantly regulated in **this particular** pairwise comparison
- **Imputed:** Boolean values (true or false) if at least one value had to be imputed for a given protein
- **Num_NAs**: Number of missing values across all samples that had to be imputed

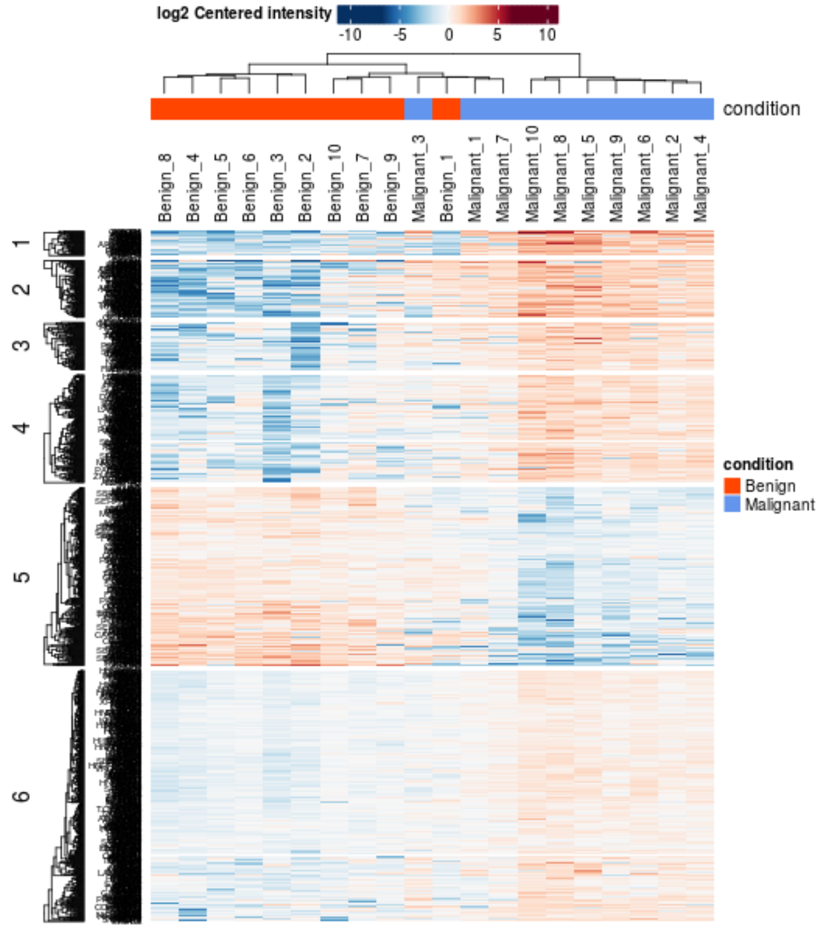
Result Plots

- **Volcano plot** (for each pairwise comparison): A volcano plot is a graphical visualization by plotting the "**log$_2$ fold changes**" on the x-axis versus the –log$_{10}$ "**p-values**" on the y-axis. Potentially interesting candidate proteins are located in the left and right upper quadrant. Checkboxes are available to use "**adjusted**

p-values” on the y-axis (instead of p-values) and to display the names of all significantly regulated proteins (which can be quite overwhelming). The volcano plots are fully interactive and proteins/rows selected in the “LFQ Results Table” are highlighted in maroon on the volcano plot. Likewise, proteins selected in the volcano plot are shown in the “LFQ Results Table”. Displayed volcano plots can be downloaded using "*Save Highlighted Plot*" button.



- **Heatmap**: The heatmap representation provides an overview of all differentially expressed proteins (rows) across all samples (columns). The results of hierarchical clustering on both protein (rows) and sample (columns) level are indicated on the left and top side of the heatmap, respectively. By default, all differentially expressed proteins have been grouped into 6 clusters, which can be downloaded to obtain protein information from each individual cluster. Alternatively, the user can change the number of clusters in the range of 1 to 20 by modifying the ‘*Advance option’* parameter.



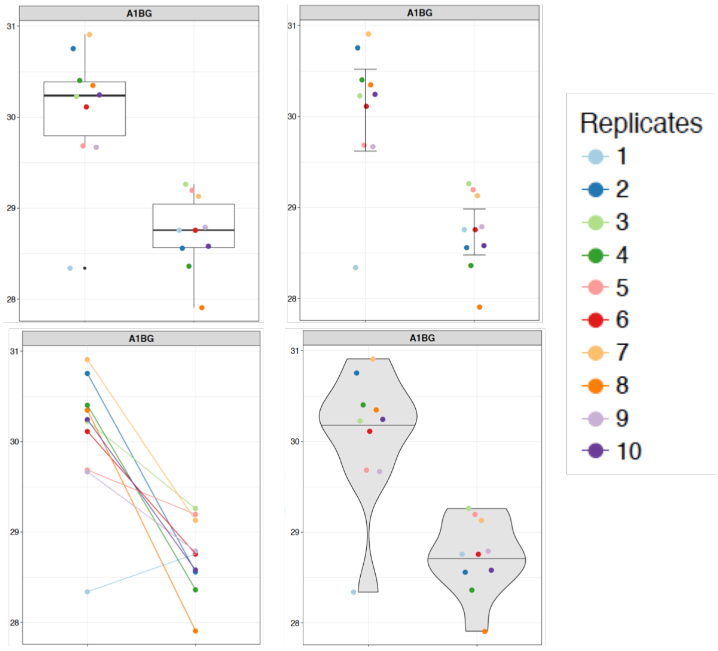
- **Protein Plot**: By selecting single or multiple rows/proteins from the "LFQ Results Table", individual LFQ-intensities of a given protein are plotted across all replicates of a condition either as box plot, violin plot, interaction plot or intensity plot.

  A boxplot is a "box and whisker" representation of the protein intensity distribution in each replicate grouped by condition. It visualizes five statistical values of the dataset: the minimum (lower vertical line), first quartile (Q1; lower box), median (horizontal line), third quartile (Q3; upper box) and maximum (upper vertical line) $\log_2$ protein intensity.

  A violin plot is identical to a boxplot except that the box is replaced by a density area.

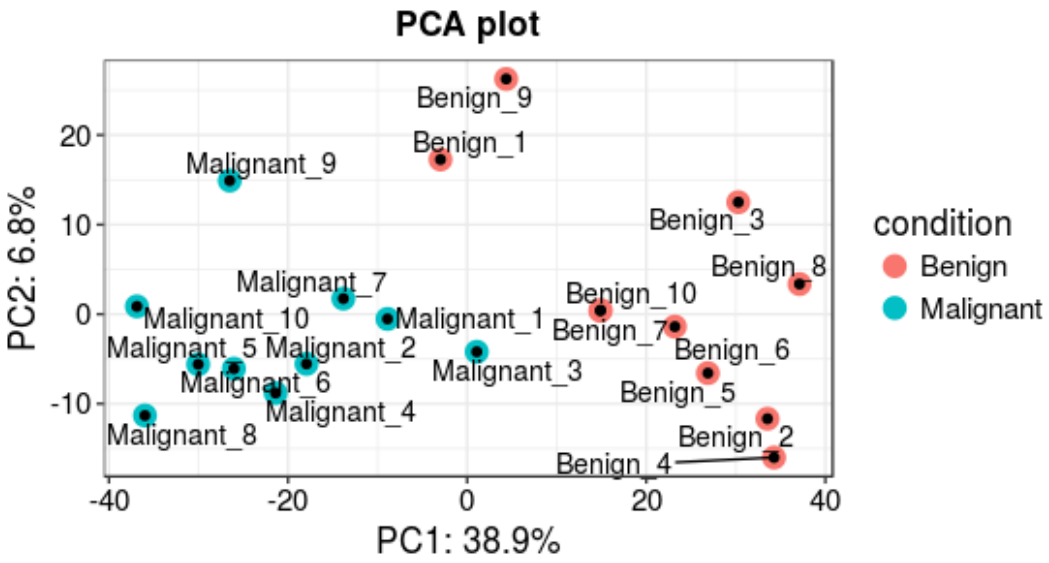
  An interaction plot shows the corresponding replicates of two groups connected by a line, i.e. replicate 1 of group 1 is connected to replicate 1 of group 2, replicate 2 of group 1 is connected to replicate 2 of group 2 and so on. An interaction plot is typically used for a paired dataset.

An intensity plot displays a line representing the 95% confidence interval.

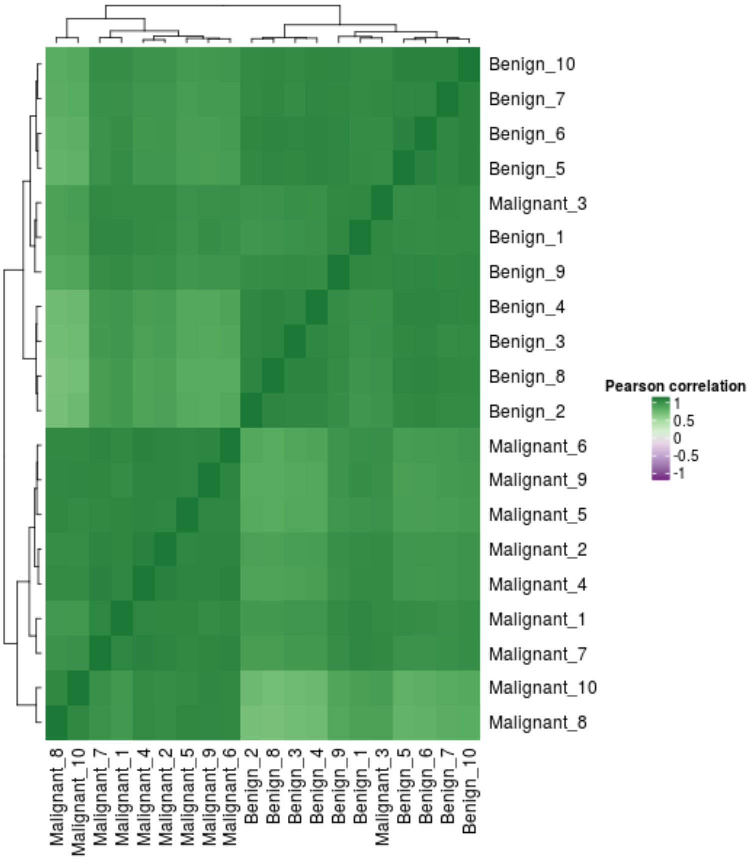


QC plots

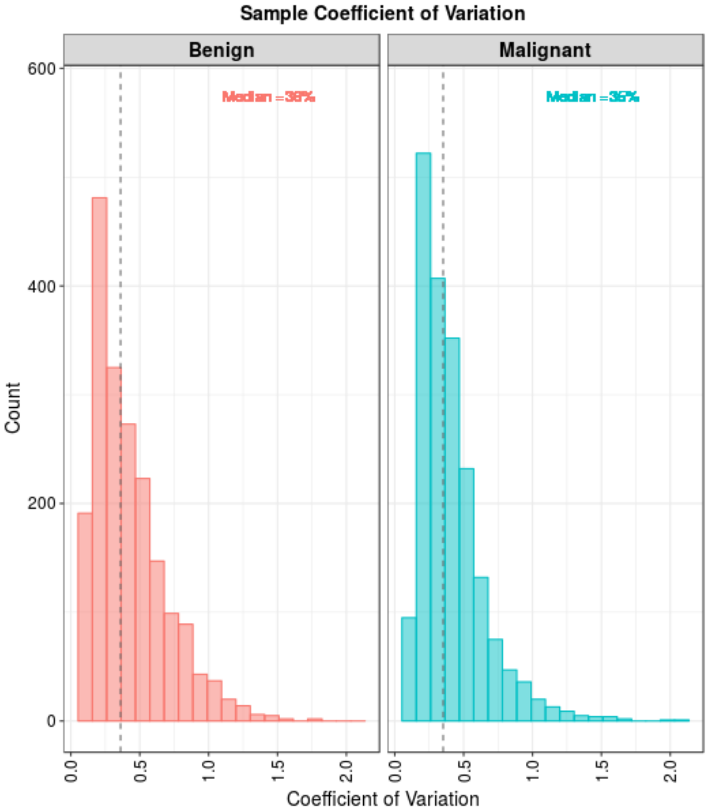
- **PCA plot**: A Principal Component Analysis (PCA) is a technique used to emphasize variation and bring out strong patterns in a dataset. In brief, the more similar 2 samples are, the closer they cluster together.



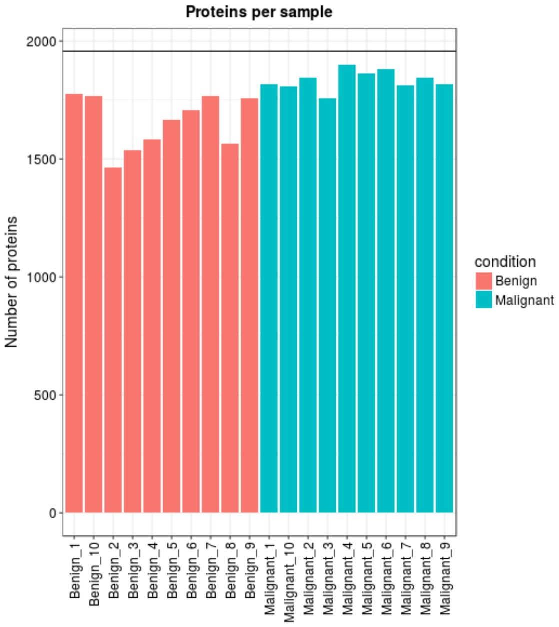
- **Sample Correlation**: A correlation matrix is plotted as a heatmap to visualize the Pearson correlation coefficient between the various samples.



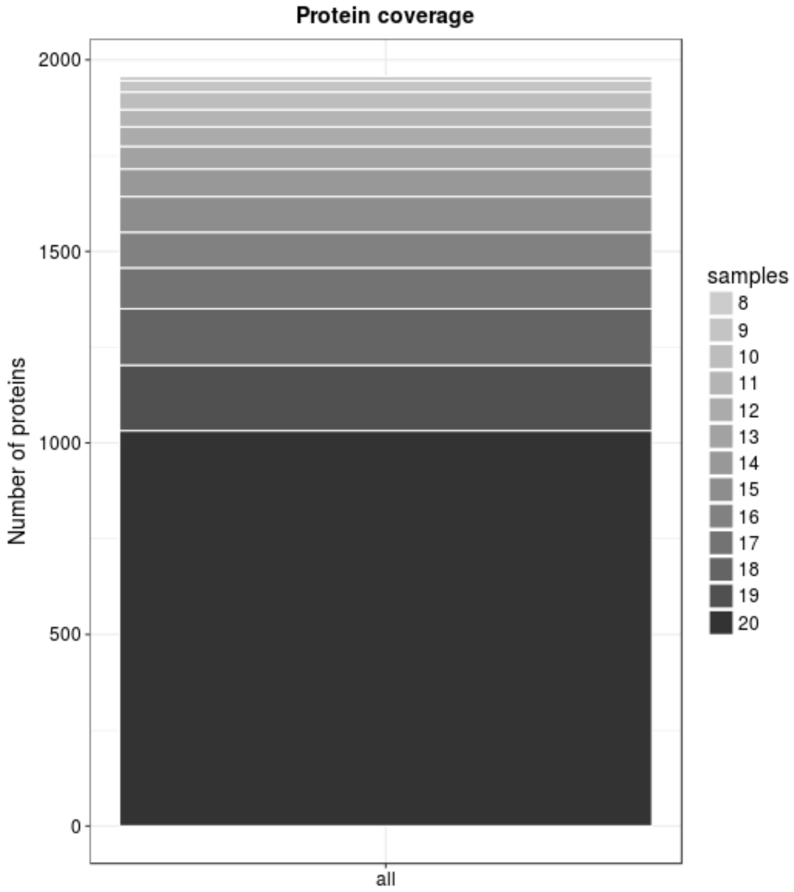
- **Sample CVs**: A histogram plot showing the distribution of protein level coefficient of variation (CV) for each condition. Each plot also contains a vertical line, which indicates the median CV percentage for that condition.



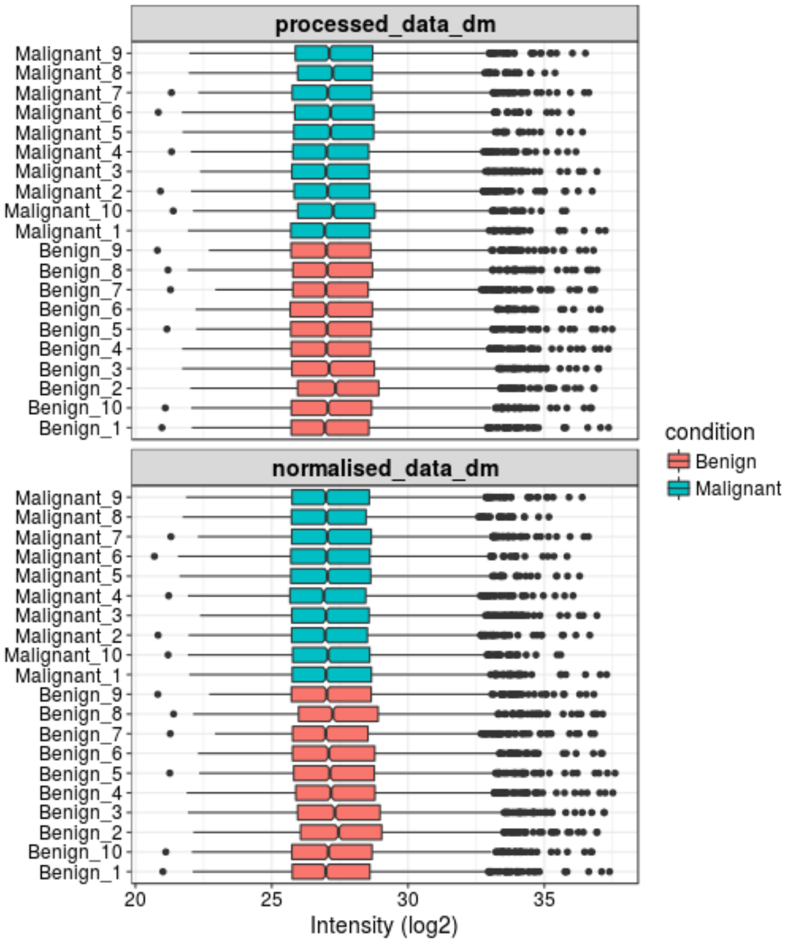
- **Protein Numbers**: Bar plots representing the number of identified and quantified proteins in each sample after the data pre-filtering process described on page 5.



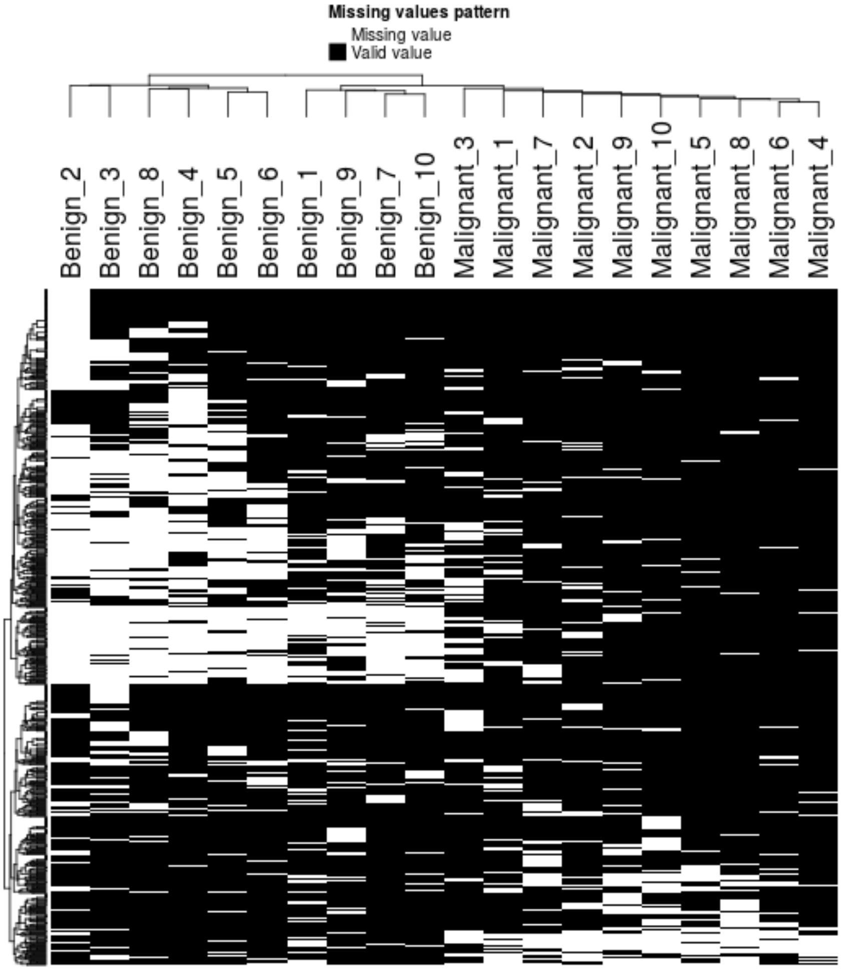
- **Sample coverage**: This plot provides a summary of how many proteins have been quantified consistently in how many samples after the data pre-filtering process described on page 5. In the example shown below, approx. 1000 proteins have been identified in all 20 samples, approx. 200 proteins in 19 samples (i.e. one value had to be imputed), approx. 150 proteins in 18 samples (i.e. two values had to be imputed) etc.



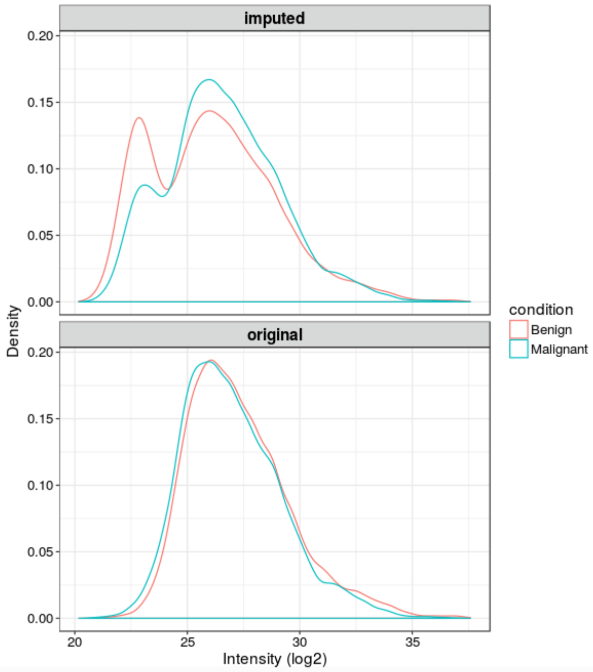
- **Normalization**: These two plots represent the effect of the variant stabilizing normalization (vsn) method on the protein intensity distribution in each sample. **Please note**: As MaxQuant is normalizing protein intensities using the MaxLFQ algorithm, *LFQ-Analyst* is **not** performing any further normalization. These plots are just drawn for visualization purposes.



- **Missing values- Heatmap**: To explore the number and pattern of missing values in the data, this heatmap indicates whether a value of a given protein (rows) in a given sample (columns) is missing (0; white) or not (1; black). Only proteins with at least one missing value are visualized.

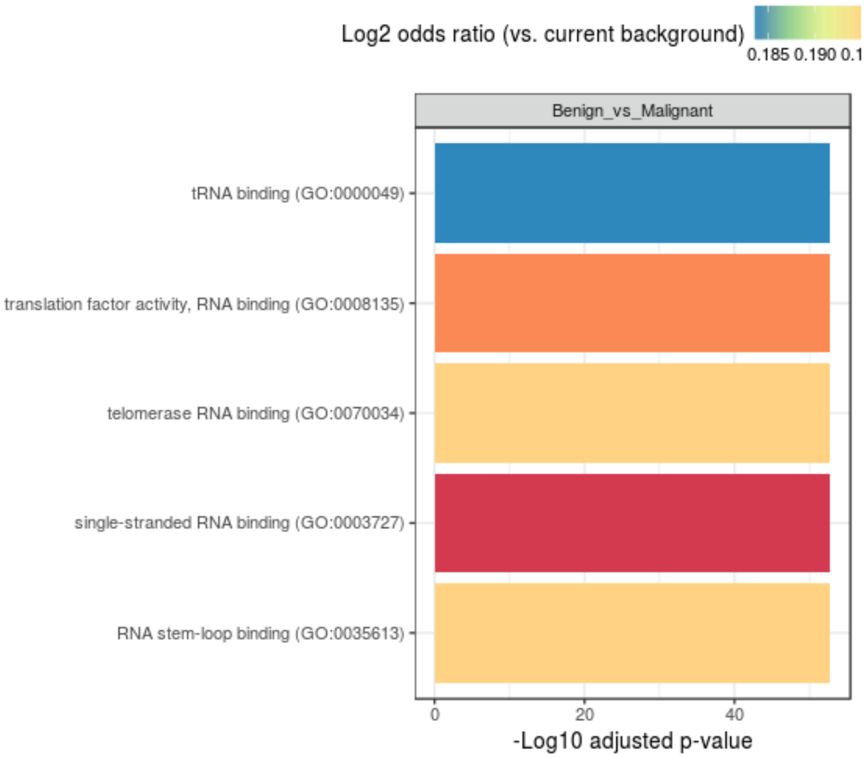


- **Imputation**: A density plot of protein intensity (log2) distribution for each condition after and before missing value imputation being performed.



Enrichment Analysis

Gene Ontology (GO) and/or Pathway enrichment analysis can be performed in *LFQ-Analyst* on all significantly regulated proteins. A selection of three GO terms (Molecular Function, Cellular Component and Biological Process) and two pathway databases (KEGG and Reactome) are available and the analysis is performed using application program interface (API) calls to EnrichR. The result is displayed as a bar chart and can be downloaded in tabular format.



Download options

Individual download options are available for all result plots and enrichment results. In addition, pre-defined data tables and a compilation of all plots can be downloaded using the button on the top the of results page:

- **Download data tables** (csv format):
  1) **Results**: Same as "*LFQ Results Table*"
  2) **Original data matrix**: A condensed data matrix showing protein intensities and missing values in each sample before imputation
  3) **Imputed data matrix**: A condensed data matrix showing protein intensities in each sample after missing value imputation
  4) **Full results**: An extensive table showing all results before and after imputation) including $\log_2$ fold changes and p-values.
- **Download Report** (pdf format): A summary report document including summary statistics and data exploration and QC plots.

Reference

1. Tyanova, S.; Temu, T.; Sinitcyn, P.; Carlson, A.; Hein, M. Y.; Geiger, T.; Mann, M.; Cox, J., The Perseus computational platform for comprehensive analysis of (prote) omics data. *Nature methods* **2016,** 13, (9), 731.