Manual for

Phospho-Analyst

Note

Phospho-Analyst has been developed to automate downstream statistical analysis of label-free, quantitative phosphoproteomic datasets preprocessed with MaxQuant.

Quick Start

- Open a web browser and navigate to https://analyst-suites.org/apps/phospho-analyst/
- Open the "**Analysis**" sidebar tab
- Upload **Phospho (STY)Sites.txt** file generated by MaxQuant [1].
- Upload your **phosphosite experimental design** table or modify the auto generated **"Template"** by clicking on it.

  *For the latter, it is required that the **Phospho (STY)Sites.txt** file is already uploaded.*
- Optional: Upload your **Protein Group.txt file** generated by MaxQuant.

  Please note that this does not refer to the proteinGroups.txt file that can be found in the same folder as the 'Phospho (STY) Sites.txt' file. It rather refers to a separate experiment analysing accompanied protein level changes. See also figure 2 of the manuscript.
- Optional: Upload your **protein experimental design** table or modify the auto generated **"Template"** by clicking on it.

  *For the latter, it is required that the **Protein Group.txt** file is already uploaded.*
- Optional: Adjust the various parameters such as the p-value cut-off, the log2 fold change cut-off, the normalisation type, imputation type and/or the type of the FDR correction in the "**Advanced Options**" sidebars
- Press "**Start Analysis**" to perform differential expression analysis and wait for the results to appear in the background.

  Please note if the number of experimental groups is >2, it will take a few minutes to perform the ANOVA calculation.
- To perform a Gene Ontology / Pathway Enrichment analysis and/or Kinase-Substrate Enrichment on the significantly regulated proteins, press "**Run Enrichment**" in the bottom right section of the results after selecting the desired Database (molecular function, biological process, cellular component, KEGG, Reactome) and/or Kinase-Substrate.

  Please note that this might take a while to complete.

Input Files

Phospho-Analyst contains four input files, two of which are mandatory and two of which are optional.

1) The MaxQuant **Phospho (STY)Sites.txt** file

It is also possible to upload a custom-made text file, but it is essential for the integrity of the R code that this file contains the following columns:

Columns	Description
id	Identifiler(s) of phosphosite(s) contained in all phosphosites
Phospho (STY) Probabilities	peptide sequences with localization probabilities of phosphosites
Gene names	Name(s) of the gene(s) associated to the phosphosite(s) contained within the group.
Protein names	Name(s) of protein(s) this phosphosite is associated with
Protein	Identifier of the protein this phosphosite is associated with
Intensity (for each sample and each multiplicity)	Intensities calculated by MaxQuant
Amino acid	Specified amino acid residue
Positions within proteins	The proteins in which the modification is found
Localization prob	When the value is less than 0.75, the particular phosphosite was regarded as low confident observation
Reverse	When marked with '+', this particular phosphosite contains no protein, made up of at least 50% of the peptides of the leading protein, with a peptide derived from the reversed part of the decoy database.
Potential contaminant	When marked with '+', this particular phosphosite was found to be a commonly occurring contaminant.

2) A **phosphosite experimental design table**

A tab separated file containing only three columns: "label", "condition", "replicate". The column headers including all entries are case sensitive.

Alternatively, after uploading the **Phospho (STY)Sites.txt** file, the wizard function can be used by clicking on the "Template" button. Here is an example of an experimental design table:

label	condition	replicate
EasyPhos_Pharmacological_CTRL_1	Pharmacological_CTRL	1
EasyPhos_Pharmacological_CTRL_2	Pharmacological_CTRL	2
EasyPhos_Pharmacological_CTRL_3	Pharmacological_CTRL	3
EasyPhos_Pharmacological_CTRL_4	Pharmacological_CTRL	4
EasyPhos_Pharmacological_L41-10um_1	Pharmacological_L41-10um	1
EasyPhos_Pharmacological_L41-10um_2	Pharmacological_L41-10um	2
EasyPhos_Pharmacological_L41-10um_3	Pharmacological_L41-10um	3
EasyPhos_Pharmacological_L41-10um_4	Pharmacological_L41-10um	4
EasyPhos_Pharmacological_L41-1um_1	Pharmacological_L41-1um	1
EasyPhos_Pharmacological_L41-1um_2	Pharmacological_L41-1um	2
EasyPhos_Pharmacological_L41-1um_3	Pharmacological_L41-1um	3
EasyPhos_Pharmacological_L41-1um_4	Pharmacological_L41-1um	4
EasyPhos_Pharmacological_ALG-10um_1	Pharmacological_ALG-10um	1
EasyPhos_Pharmacological_ALG-10um_2	Pharmacological_ALG-10um	2
EasyPhos_Pharmacological_ALG-10um_3	Pharmacological_ALG-10um	3
EasyPhos_Pharmacological_ALG-10um_4	Pharmacological_ALG-10um	4
EasyPhos_Pharmacological_ALG-1um_1	Pharmacological_ALG-1um	1
EasyPhos_Pharmacological_ALG-1um_2	Pharmacological_ALG-1um	2
EasyPhos_Pharmacological_ALG-1um_3	Pharmacological_ALG-1um	3
EasyPhos_Pharmacological_ALG-1um_4	Pharmacological_ALG-1um	4

Note: The entries in the "label" column must match the labels present in the Intensity columns of the **Phospho (STY)Sites.txt** file. For example, write "CTRL_1" if a "Intensity CTRL_1___1" column is present in your Phospho (STY)Sites.txt file.

3) The MaxQuant **Protein Group.txt** file (Optional)

It is also possible to upload a custom-made text file, but it is essential for the integrity of the R code that this file contains the following columns:

Columns	Description
Gene names	Name(s) of the gene(s) associated to the protein(s) contained within the group
Protein IDs	Identifier(s) of protein(s) contained in the protein group
Protein names	Name(s) of protein(s) contained within the group
LFQ intensity (for each sample)	LFQ intensities calculated by MaxLFQ algorithm
Razor + unique peptides	Number of distinct peptide sequences associated with each protein in protein group
Only identified by site	When marked with '+', this particular protein group was identified only by a modification site
Reverse	When marked with '+', this particular protein group contains no protein, made up of at least 50% of the peptides of the leading protein, with a peptide derived from the reversed part of the decoy database.
Potential contaminant	When marked with '+', this particular protein group was found to be a commonly occurring contaminant.

4) A **protein experimental design table** (Optional)

A tab separated file containing only three columns: "label", "condition", "replicate". The column headers including all entries are case sensitive.

Alternatively, after uploading the **ProteinGroups.txt** file, the wizard function can be used by clicking on the "Template" button.

Important Note: Whilst the number of replicates per experimental condition can differ between the proteomics (**ProteinGroups.txt** file) and phosphoproteomics (**Phospho (STY)Sites.txt** file) data, the number and names of the experimental conditions must be identical between the datasets.

Phospho-Analyst's processing pipeline

Data pre-filtering

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

- Reverse sequences are removed

- Potential contaminant sequences are removed

- Sequences with a localization probability of <0.75 are removed

- Phosphosite intensity columns are expanded from a wide to a long format to separate the multiplicity information

- Peptide sequence information is extracted

- Phosphosites with a high proportion of missing values are removed

  In detail, a dynamic exclusion strategy is applied (see table below). It is important to note that the number of valid values is assessed per group/condition and a phosphosite is kept in the analysis if this requirement is met at least once in any group/condition. For example, in an experiment with 3 groups/conditions and with 3 replicates in each group/condition, a given phosphosite will be kept even if it is completely absent in 2 groups, but present in the third group with (at least) 2 valid values (see table below). Or in other words, although the total number of missing values is 78% (7 out of 9), the phosphosite would be retained in the analysis as it met the "valid value requirement" in at least one group/condition.

Number of replicates	Minimum number of valid values required (in at least one condition)
Two or Three	2
Four or Five	3
Six or Seven	4
More than Seven	X / 2 + 1 rounded down, where X is the number of replicates*

E.g. If there are 8 replicates, then 5 valid values in at least one condition are necessary to keep a protein or peptide-level data in the analysis.

Differential expression analysis

After pre-filtering, all intensities are converted to a $\log_2$ scale and replicates are grouped by conditions based on the information provided in the experimental design table. Missing values are imputed using the 'Missing not At Random' (MNAR) method, which uses random draws from a Gaussian distribution left-shifted by 1.8 StDev Ad (standard deviation) with a width of 0.3. A variance stabilising transformation type normalisation is further applied. Finally, protein-wise linear models combined with empirical Bayes statistics are used for the differential expression analyses. 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 pairwise comparisons. However, if there are more than two experimental conditions, an ANOVA test is also performed.

Advanced Options

Significant protein filtering criteria
- Adjusted p-value cutoff: default is **0.05**
- $\log_2$ fold change cutoff: default is **1**

Test to use for differential expression analysis
- For paired datasets, a checkbox is provided to perform paired test for differential expression analysis; the default test is unpaired

Normalisation type
- **vsn (default)**: Performs variance stabilizing transformation using the "normalize_vsn" function inside the DEP package.
- **median**: Method "scale" of normalizeBetweenArrays function from limma package is applied to scale the expression values to have the same median.
- median subtraction: Median center normalisation.

Missing value imputation options
- **Perseus-type (default)**: This method is based on the popular missing value imputation procedure implemented in the *Perseus software* [2]. 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 leftcensored 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

Peptides localization prob cut-off (only in Phosphosite analysis)
Filter to keep only rows with a localization probability more than a specific value: default is **0.75**

Proteins identified by single peptides (only in total proteome analysis)
A checkbox is provided to include proteins with single peptide observations; the default is set to exclude them

<u>Output</u>

Experimental summary

SIGNIFICANT PHOSPHOSITES
2111 out of 21523
9.81% of phosphosites differentially expressed across all conditions
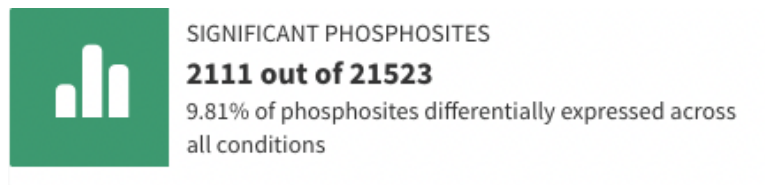
The number and proportion of all differentially expressed phosphosites across all pairwise comparisons is shown (considering the user defined thresholds for FDR and $\log_2$ fold change).

Results table

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

- **Peptide Sequence** Amino acid sequence
- **Amino Acid** Phosphorylated amino acid
- **Localization.prob** Decimal values up to 1; the probability of phosphosite localization
- **Adjusted ANOVA p-value**: ANOVA_p.adj (Only appears in > 2 groups)
- **ANOVA p-value**: ANOVA_p.val (Only appears in > 2 groups)
- **$\log_2$ 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 phosphosite has been observed to be significantly regulated in any pairwise comparison
- **Significant** (for each pairwise comparison): Boolean values (true or false) if a given phosphosite 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 phosphosite
- **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 visualisation by plotting the "**log₂ fold changes**" on the x-axis versus the $-\log_{10}$ "p-values" on the y-axis. Potentially interesting candidate phosphosites/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). Additionally, if there are more than two groups, use "**Apply ANOVA**" can change to display the plot based on ANOVA p-values. The volcano plots are fully interactive and proteins/rows selected in the "Results Table" are highlighted in maroon on the volcano plot. Likewise, phosphosites/proteins selected in the volcano plot are shown in the "Results Table". Displayed volcano plots can be downloaded using "*Save Highlighted Plot*" button.
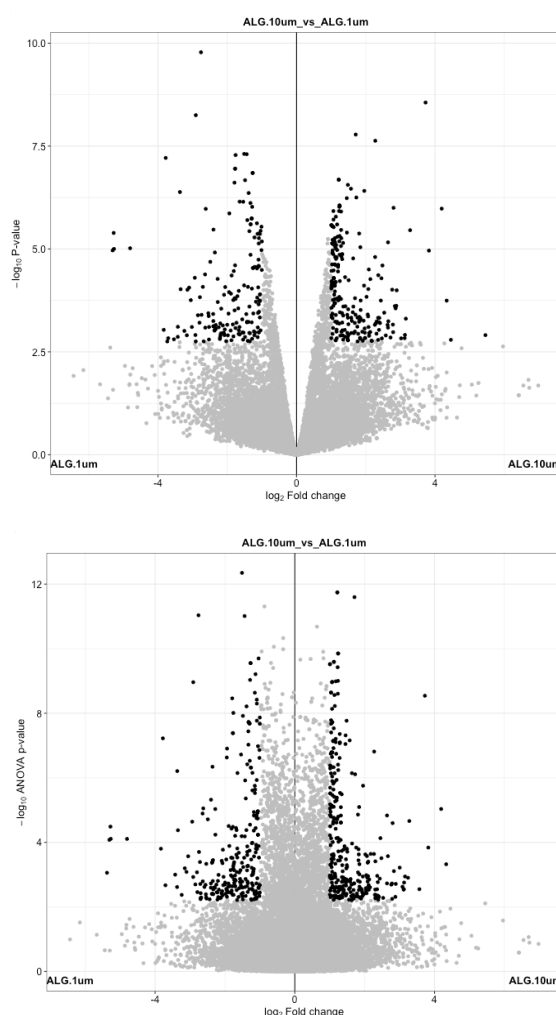
- **Heatmap**: The heatmap representation provides an overview of all differentially expressed proteins (rows) across all samples (columns). The results of hierarchical clustering on both phosphosites/proteins (rows) and sample (columns) level are indicated on the left and top side of the heatmap, respectively. By default, all differentially expressed phosphosites/proteins have been grouped into 6 clusters, which can be downloaded to obtain phosphosites/proteins information from each individual cluster. Alternatively, the user can change the number of clusters in the range of 1 to 20 by modifying the "*Number of clusters in heatmap*" parameter.
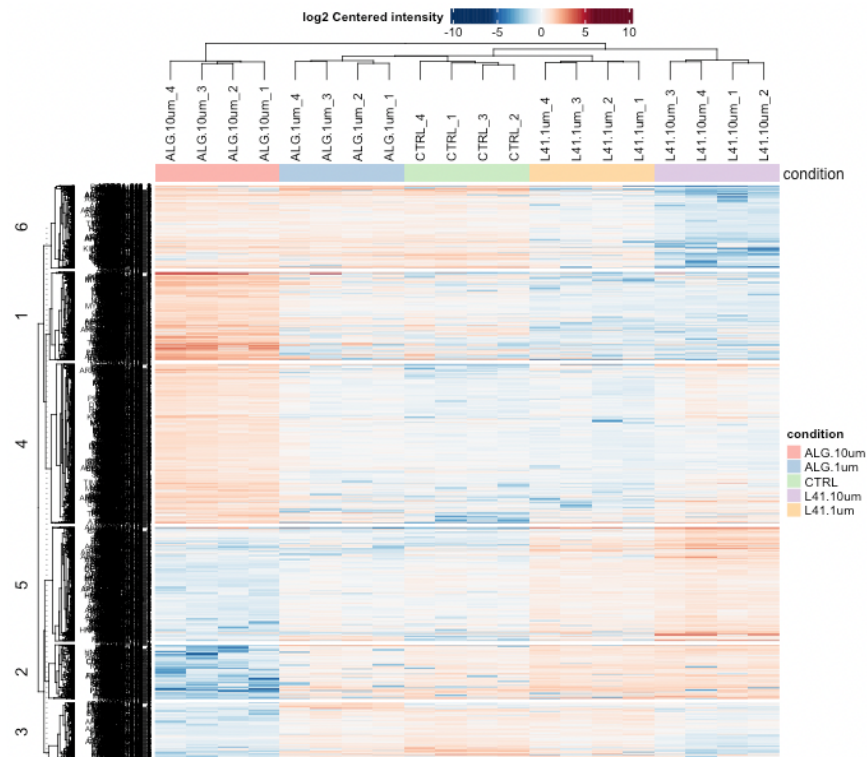
- **Individual Plot**: By selecting single or multiple rows/proteins from the "Results Table", individual intensities of a given phosphosite/protein are plotted across all replicates of a condition either as box plot, violin plot, interaction plot or intensity plot.
  1) A boxplot is a "box and whisker" representation of the phosphosite/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) $\log_2$ phosphosite/protein intensity.
  2) A violin plot is identical to a boxplot except that the box is replaced by a density area.
  3) 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.

10

4) An intensity plot displays a line representing the 95% confidence interval.
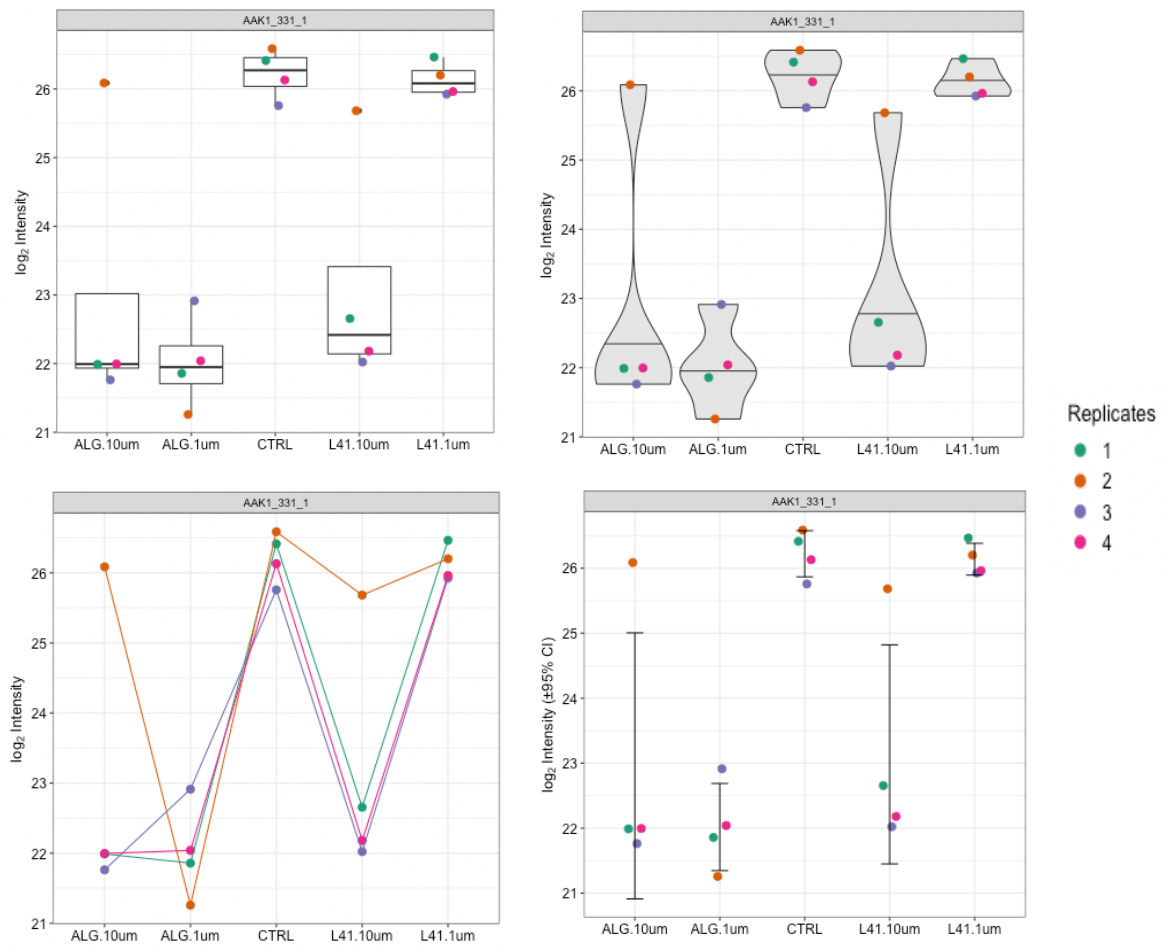
- **Abundance Plot**:

  1) **Abundance rank**: A rank/abundance plot illustrates a dynamic distribution of phosphosites by plotting the mean abundance values against corresponding rank.
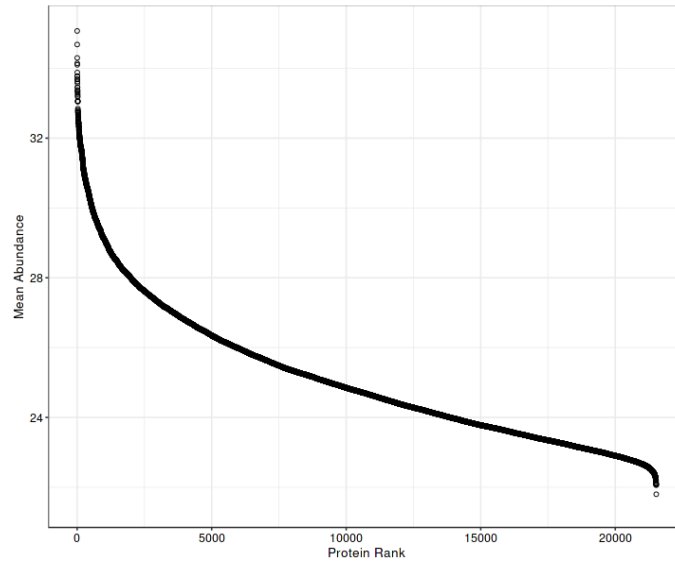

  2) **Abundance comparison (for each pairwise comparison)**: A scatter plot to represent the comparison of mean phosphosite abundance between each pairwise across all phosphosites with a 1:1 dashed line as reference.
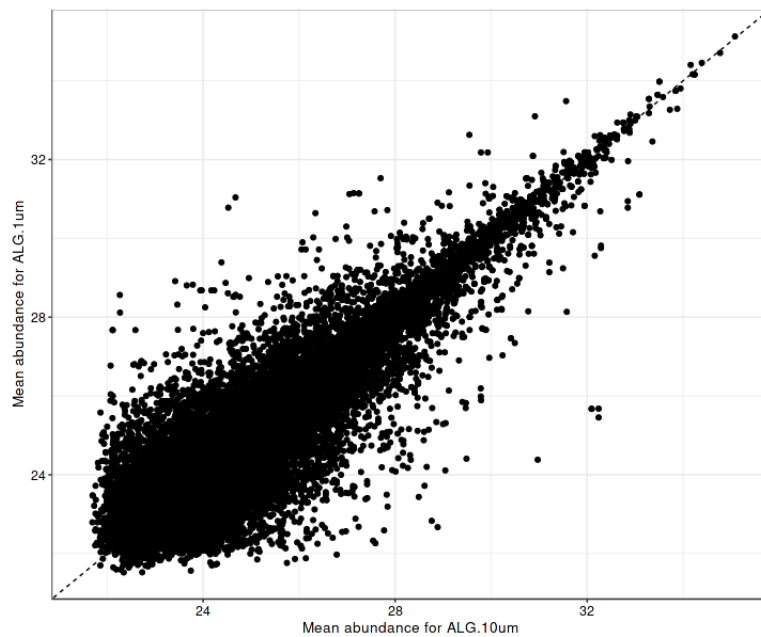
Quality Control (QC) Plots

- **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.
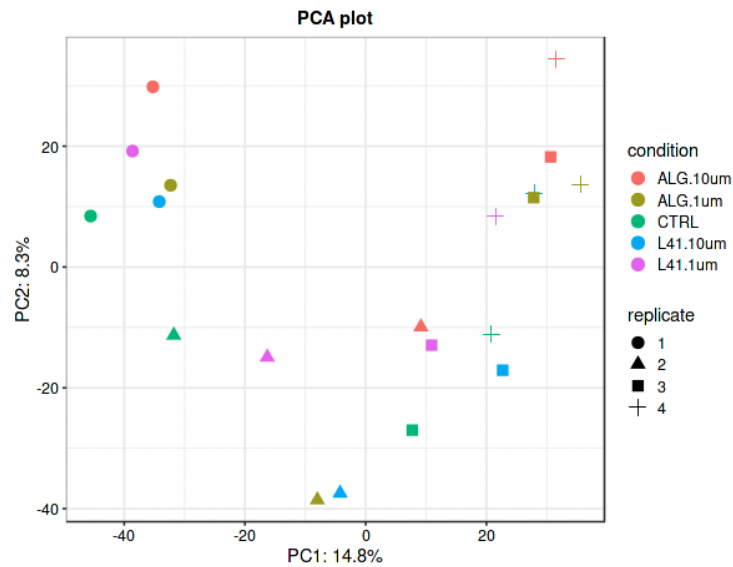
- **Sample Correlation**: A correlation matrix is plotted as a heatmap to visualize the Pearson correlation coefficient between the various samples.
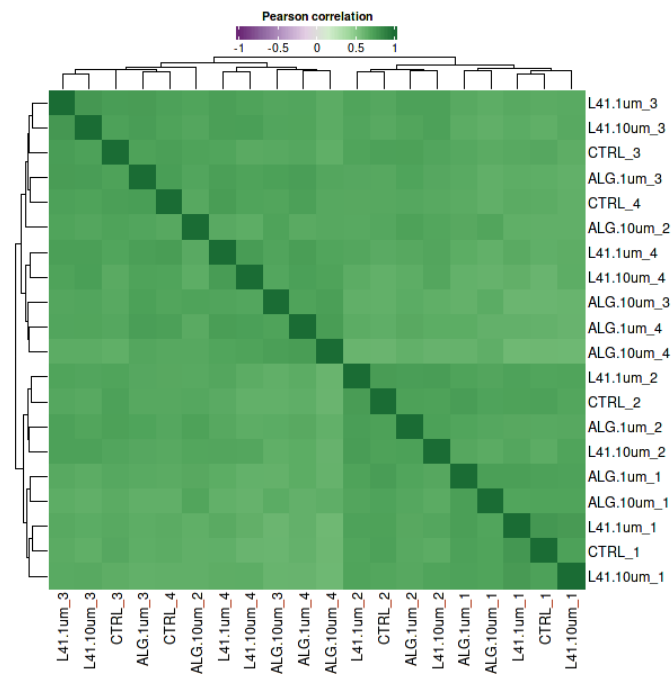
- **Sample CVs**: A histogram plot showing the distribution of protein level coefficient of variation (CV) for each condition. Each plot also contains a vertical line, which indicates the median CV percentage for that condition.
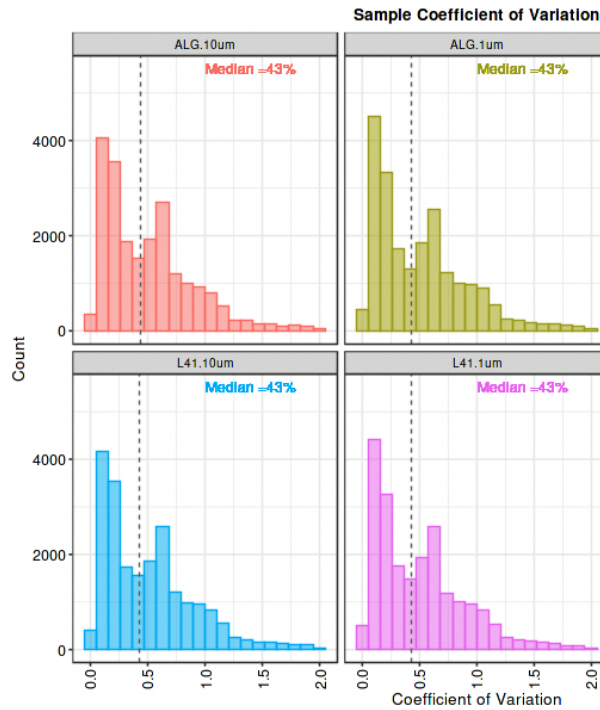
- **Phosphosite/Protein Numbers**: Bar plots representing the number of identified and quantified phosphosites/proteins in each sample after the data pre-filtering process described before.
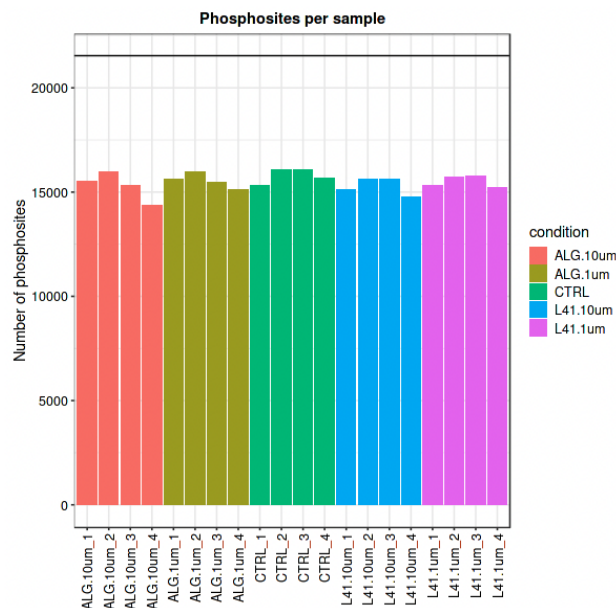
- **Sample coverage**: This plot provides a summary of how many phosphosites/proteins have been quantified consistently in how many samples after the data pre-filtering process described before. In the example shown below, approx. 500 phosphosites have been identified in all 12 samples, approx. 1000 proteins in 9 samples (i.e. three value had to be imputed) etc.
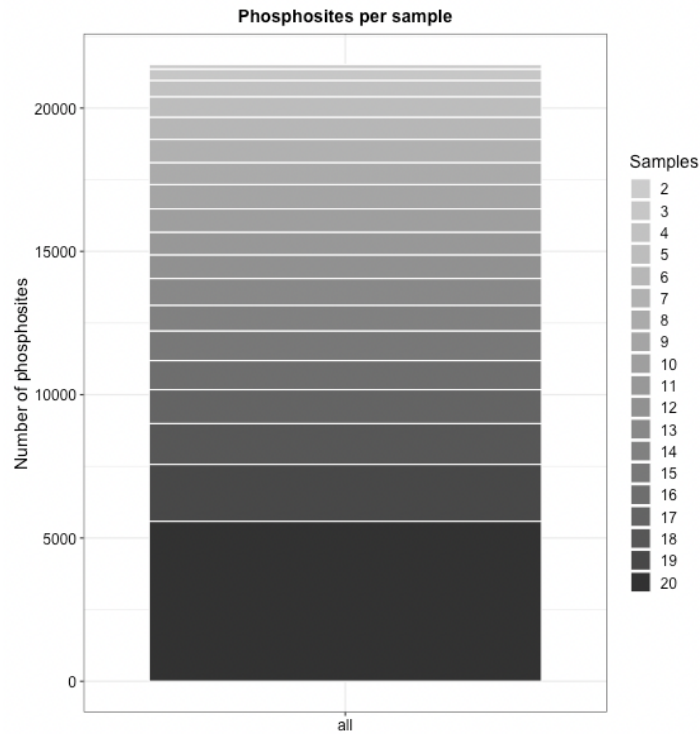

- **Normalization**: These two plots represent the effect of the variant stabilizing normalization (vsn) method on the phosphosite/protein intensity distribution in each sample. This step removes any non-biological related variations and makes the analysis of the results more reliable.
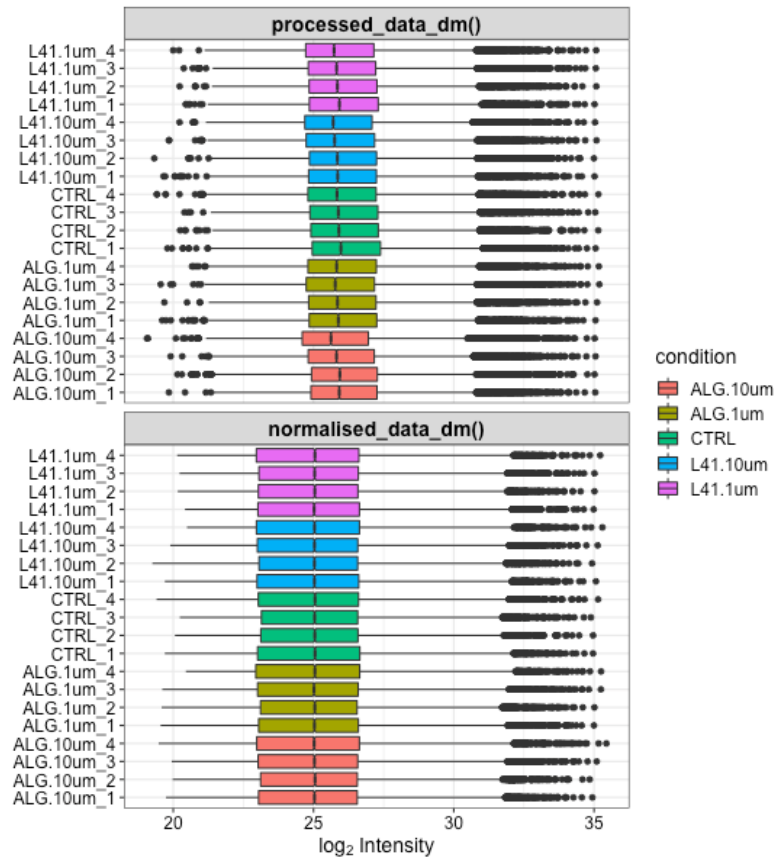
- **Missing values- Heatmap**: To explore the number and pattern of missing values in the data, this heatmap indicates whether a value of a given protein (rows) in a given sample (columns) is missing (0; white) or not (1; black). Only phosphosites/proteins with at least one missing value are visualized.

Missing values pattern

Missing value
■ Valid value
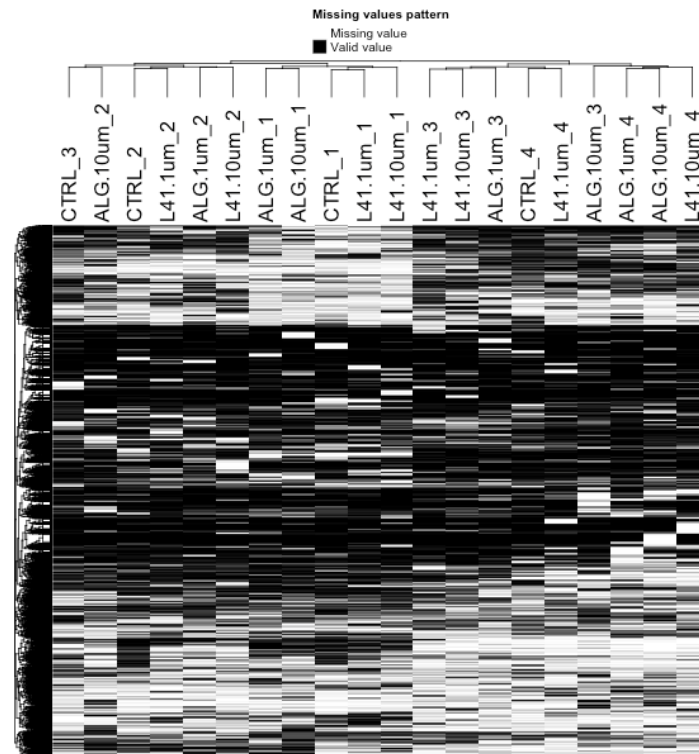
- **Imputation**: A density plot of protein intensity ($\log_2$) distribution for each condition after and before missing value imputation being performed
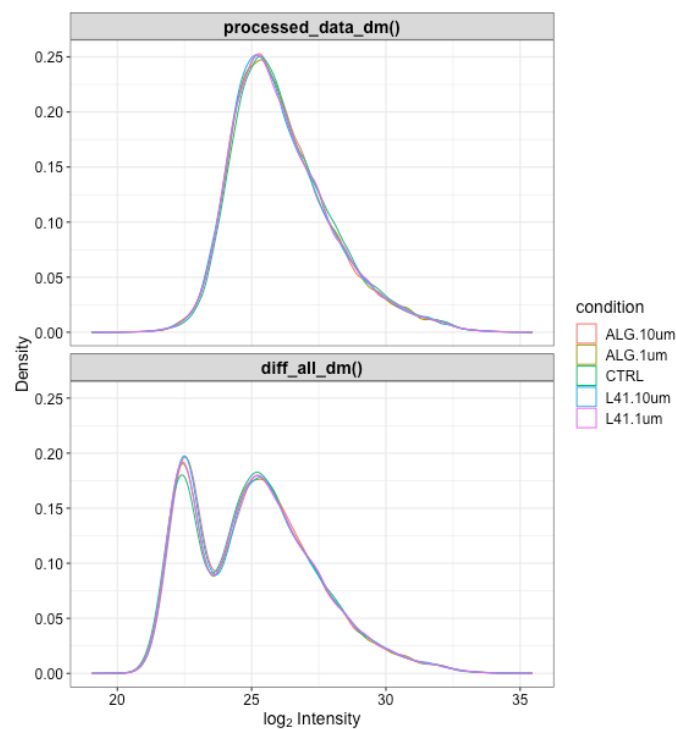

Enrichment Analysis

- **Gene Ontology (GO) / Pathway enrichment:** These analyses can be performed in Phospho_Analyst on all significantly regulated phosphosites/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.
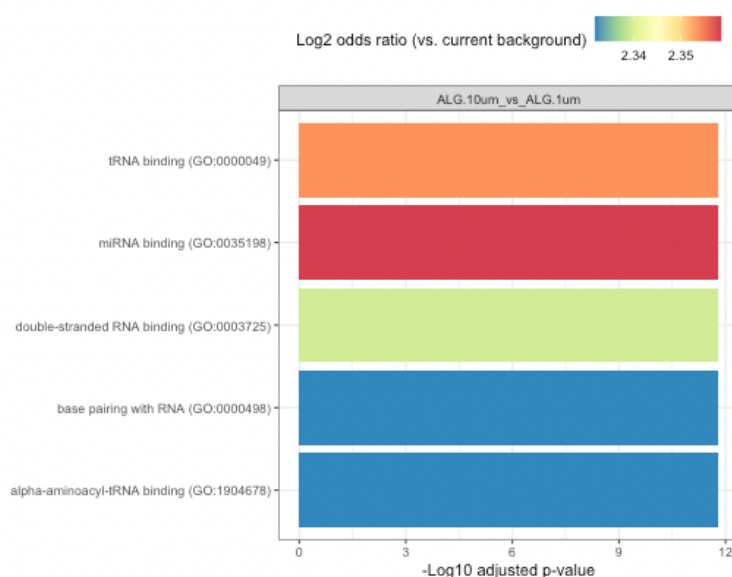

- **Kinase-Substrate enrichment analysis:** On both Phosphosite and Phosphosite(corrected) page, Kinase-Substrate enrichment is performed using KSEAApp. The result is displayed as a bar chart and can be downloaded in tabular format.
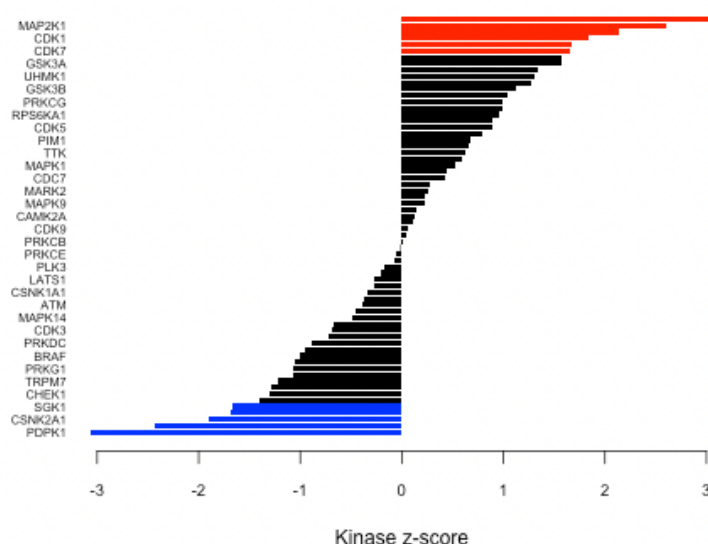
Comparison plots

- **Log fold change scatter plot**: This scatter plot is using phosphosite level log$_2$ fold changes as x-axis and protein group log$_2$ fold changes as y-axis to allow a quick assessment of whether the underlying protein has been observed to be significantly regulated as well
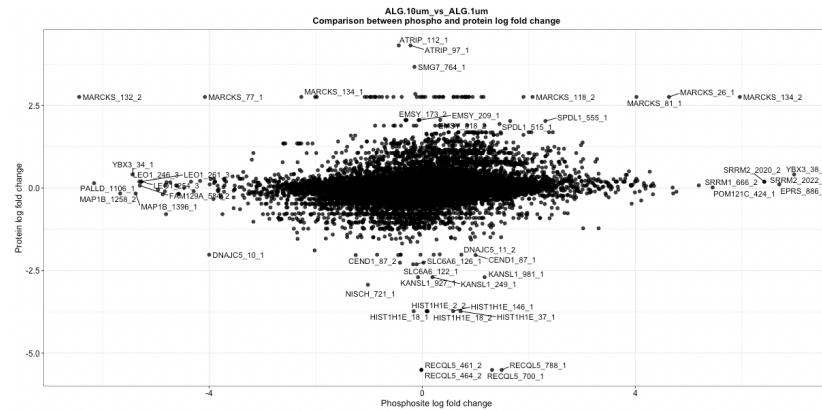
- **Interaction plot**: This plot uses combined data to show the interaction between different replicates. Enabling distinction of abundance at the protein and phosphosite level.
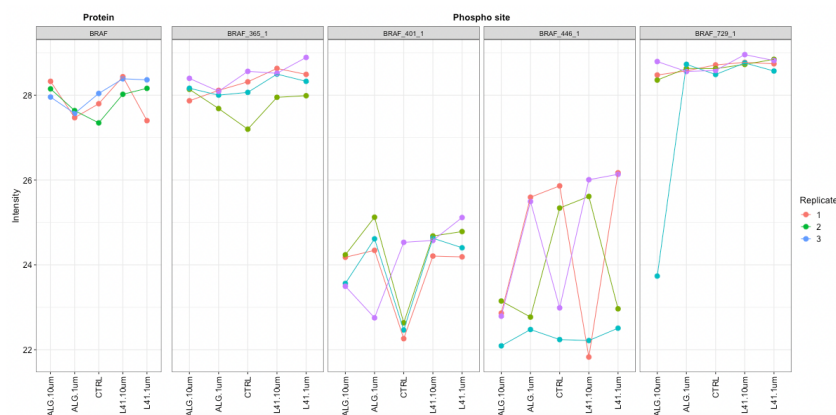
- **Bubble plot**: The plot displays the phosphosite intensity values of a selected protein group. The experiment groups of different conditions are coloured differently on the x-axis. The intensity values of distinct phosphosites are represented on the y-axis, and the size of the points represents the mean intensity values of the same phosphosite.
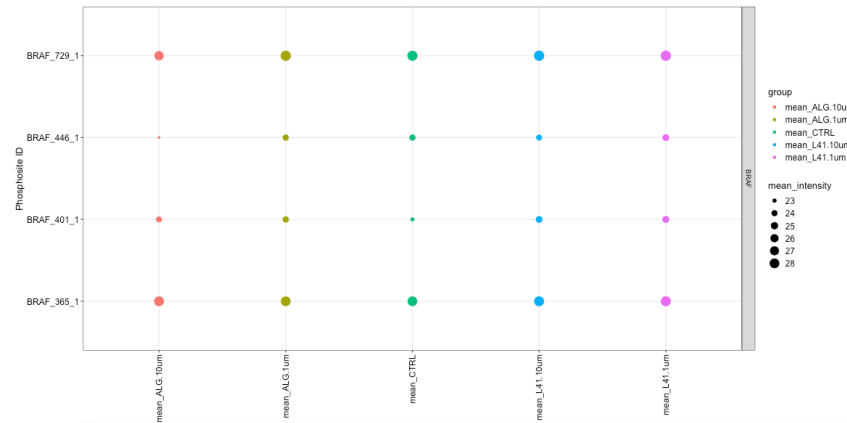
Venn plot (On Phosphosite Absence/Presence page)

Draw after selecting occurrence of each sample, and/or filtering by conditions. The overlap is coloured by number of phosphosites.
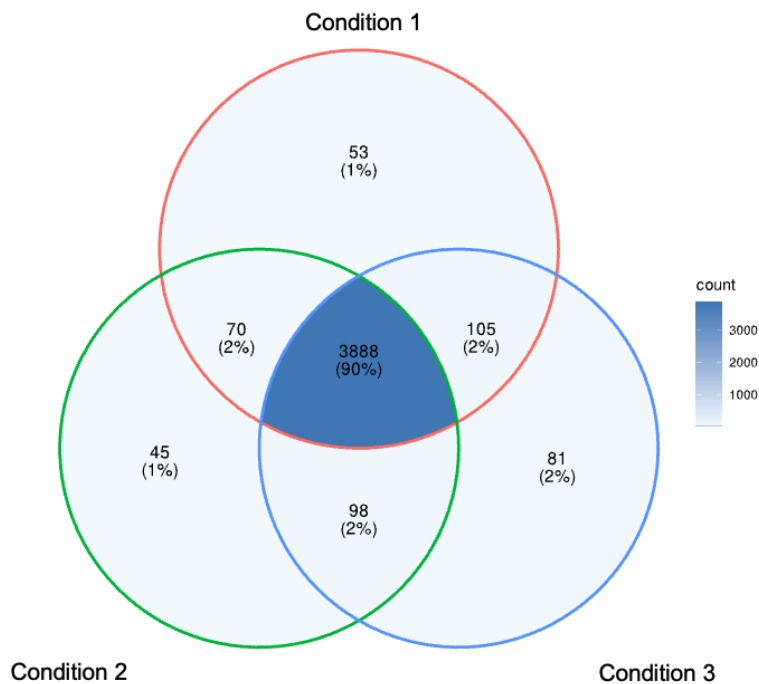
Download options

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 "*Results Table*"
  2) **Original data matrix**: A condensed data matrix showing phosphosite/protein intensities and missing values in each sample before imputation
  3) **Imputed data matrix**: A condensed data matrix showing phosphosite/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)
  5) Phosphomatics input: A dataset can be used
- **Download Report** (pdf format): A summary report document including summary statistics and data exploration and QC plots.

References

1. Tyanova, S.; Temu, T.; Cox, J. The MaxQuant Computational Platform for Mass Spectrometry-Based Shotgun Proteomics. *Nature Protocols* **2016**, *11* (12), 2301–2319.

2. 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.