Manual for

Phospho-Analyst

Note

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

Quick Start

- Open a web browser and navigate to https://phosphoanalyst.erc.monash.edu/
- Open the "**Analysis**" sidebar tab
- Upload **Phospho (STY)Sites.txt** file generated by MaxQuant
- Upload your **phosphosite experimental design** table or modify the auto generated **"Template"** by clicking on it
- Upload your **Protein Group.txt** generated by MaxQuant (optional)
- Upload your **protein experimental design** table or modify the auto generated **"Template"** by clicking on it (optional)
- *Optional*: Adjust the various parameters such as the p-value cut-off, the log2 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 / 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. Note that this might take a while to complete.

Input Files

Phospho-Analyst contains four input files, the first two files are must have and the rest files 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.
Positions within proteins	The proteins in which the modification is found
Intensity (for each sample and each multiplicity)	Intensities calculated by MaxQuant
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. Or after uploading a **Phospho (STY)Sites.txt** file clicking on the “Template” button. Here is an example:

label	**condition**	**Replicate**
G1	Day0	1
G2	Day0	2
G3	Day0	3
G4	Day0	4

G5	Day0	5
G6	Day0	6
G7	Day2	1
G8	Day2	2
G9	Day2	3
G10	Day2	4
G11	Day2	5
G12	Day2	6
G13	Day7	1
G14	Day7	2
G15	Day7	3
G16	Day7	4
G17	Day7	5
G18	Day7	6

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 "S01" if a “Intensity S01___1” column is present in your Phospho (STY)Sites.txt file. Or after uploading a Phospho (STY)Sites.txt file clicking on the “**Template**” button.

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 (Similar to the example shows in phosphosite experimental design table). Or after uploading a Protein Group.txt file clicking on the "**Template**" button.

Note: Number of groups can be the same or different from the **phosphosite experimental design table**, but the condition names must be exactly the same with that in the **phosphosite experimental design table.**

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
- localization probability of <0.75 are removed
- Phosphosite intensity columns are expanded from wide to long format and separate the multiplicity information
- peptide sequences 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. Then, a variance stabilizing transformation type normalization is 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 pair-wise comparisons. Moreover, if the conditions are more than two groups, an ANOVA test is also be implemented.

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

Missing value imputation options

- **Perseus-type (default)**: 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 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

Proteins identified by single peptides

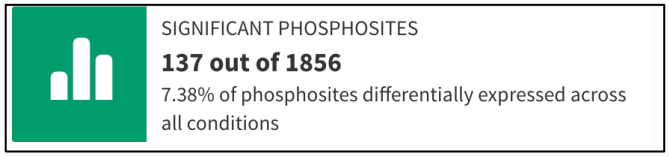
A checkbox is provided to include proteins with single peptide observations; the default is set to exclude them

Heatmap Clusters

The number of clusters used to group all identified differentially expressed proteins can be modified here (default = 6 clusters; see “heatmap” section on page 10).

Output

Experimental summary



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

Results table

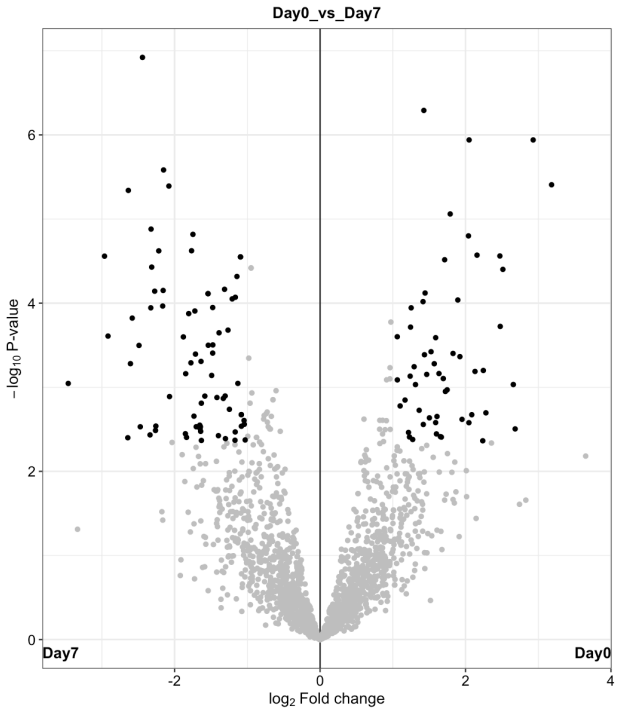
The Phosphosite names, phosphosite IDs, gene names and protein names of the quantified phosphosites are listed in this table. In addition, the following columns are shown:

- **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
- **Adjusted ANOVA p-value**: ANOVA_p.adj (Only appears in > 2 groups)
- **ANOVA p-value**: ANOVA_p.val (Only appears in > 2 groups)
- **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

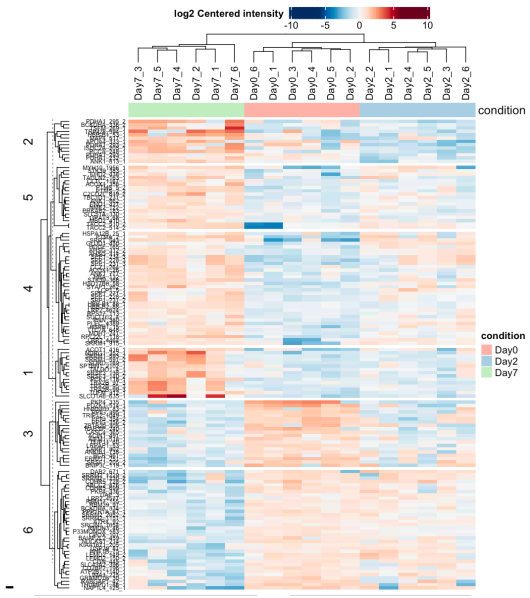
Result Plots

- **Volcano plot** (for each pairwise comparison): A volcano plot is a graphical 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 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.

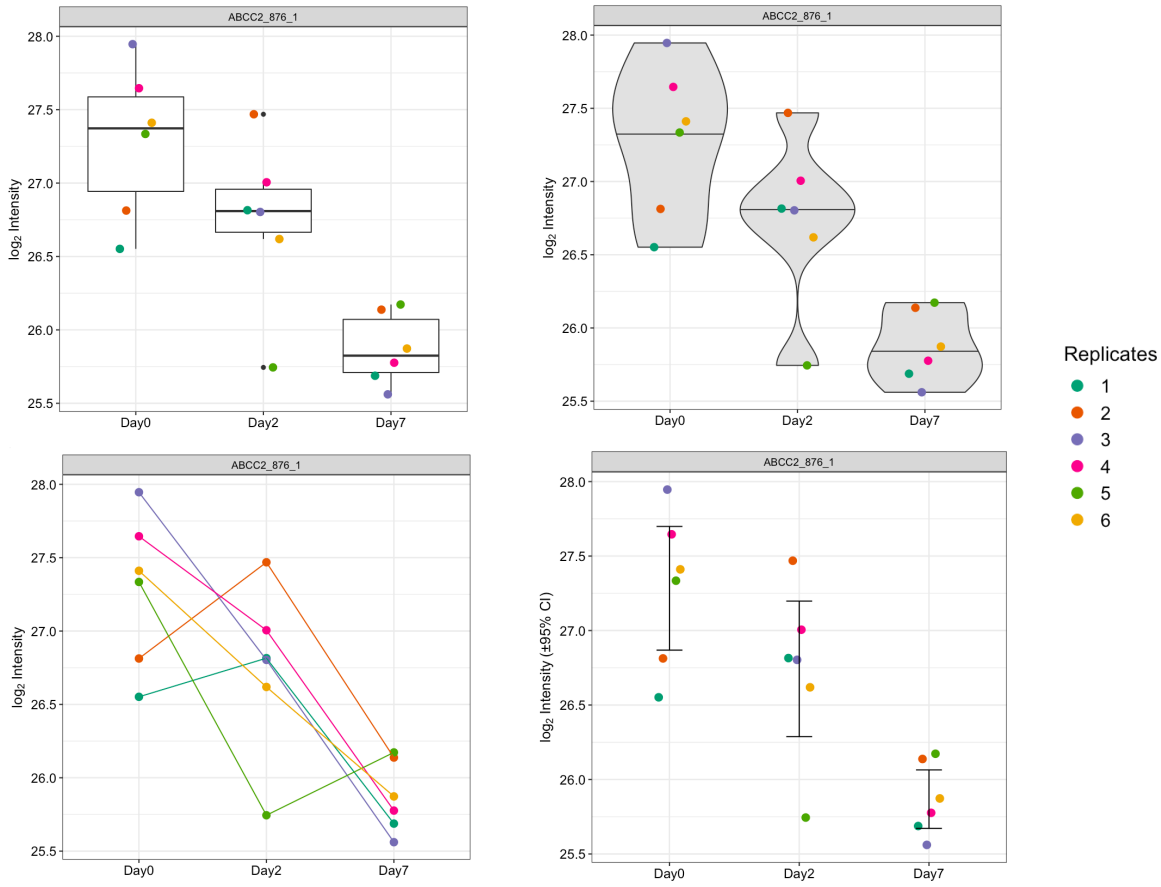
Likewise, phosphosites/proteins selected in the volcano plot are shown in the “Results Table”. Displayed volcano plots can be downloaded using “*Save Highlighted Plot*” button.



- **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 "*Advance option*” parameter.

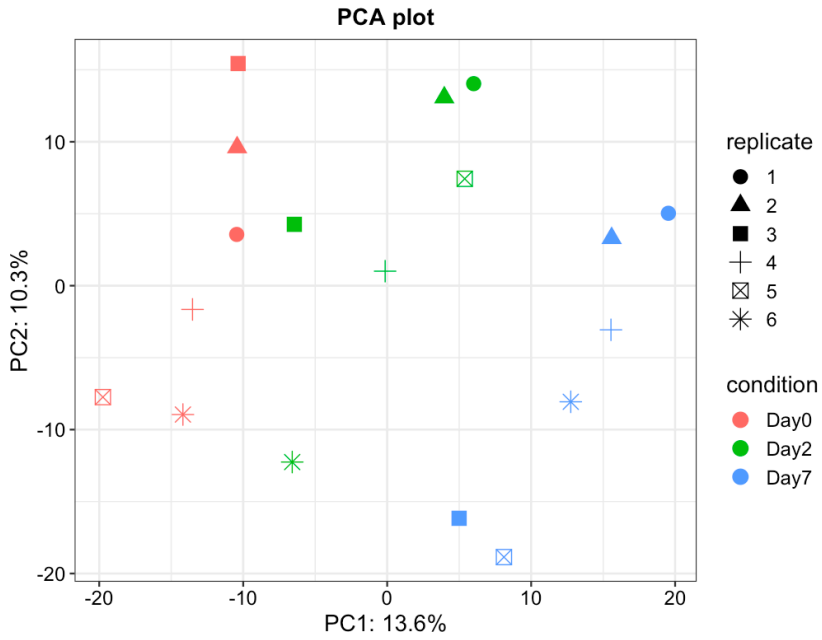


- **Individual Plot**: By selecting single or multiple rows/proteins from the "Results Table", individual intensities of a given phosphosite/protein are plotted across all replicates of a condition either as box plot, violin plot, interaction plot or intensity plot.
    1) A boxplot is a "box and whisker" 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.
    4) An intensity plot displays a line representing the 95% confidence interval.

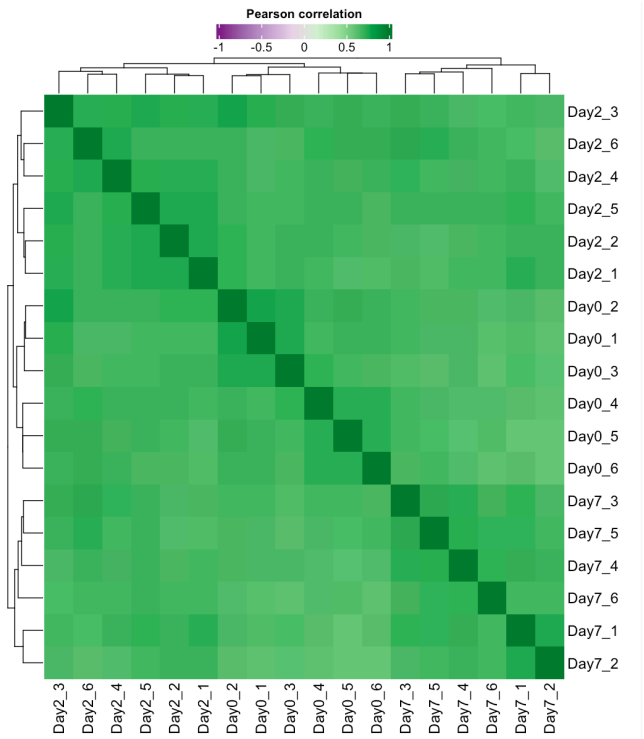


Quality Control (QC) Plots

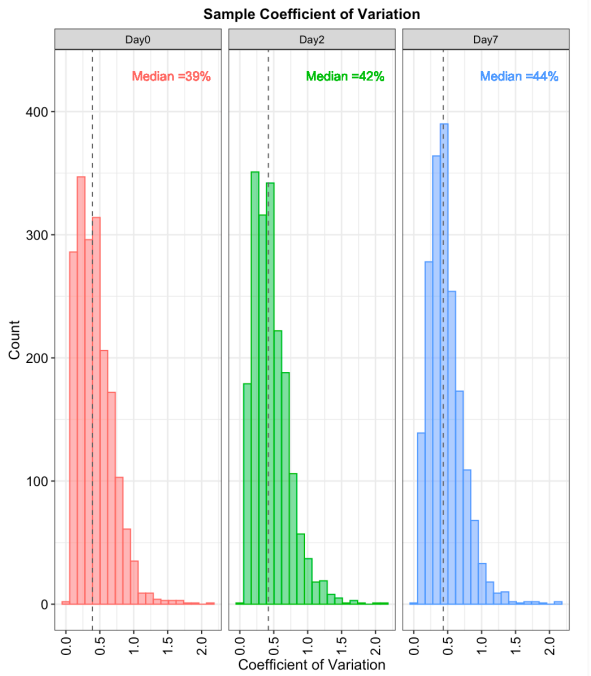
- **PCA plot**: A Principal Component Analysis (PCA) is a technique used to emphasize variation and bring out strong patterns in a dataset. In brief, the more similar 2 samples are, the closer they cluster together.



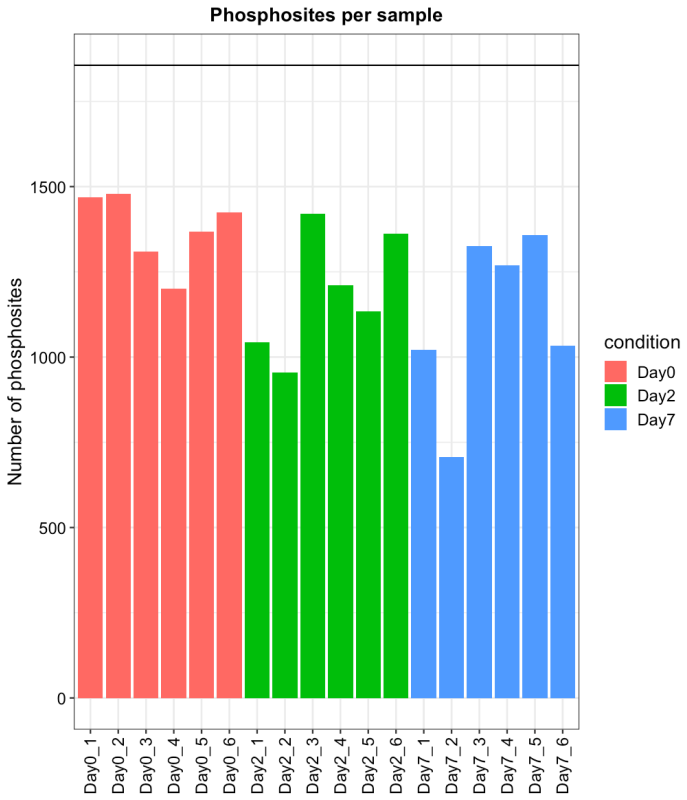
- **Sample Correlation**: A correlation matrix is plotted as a heatmap to visualize the Pearson correlation coefficient between the various samples.



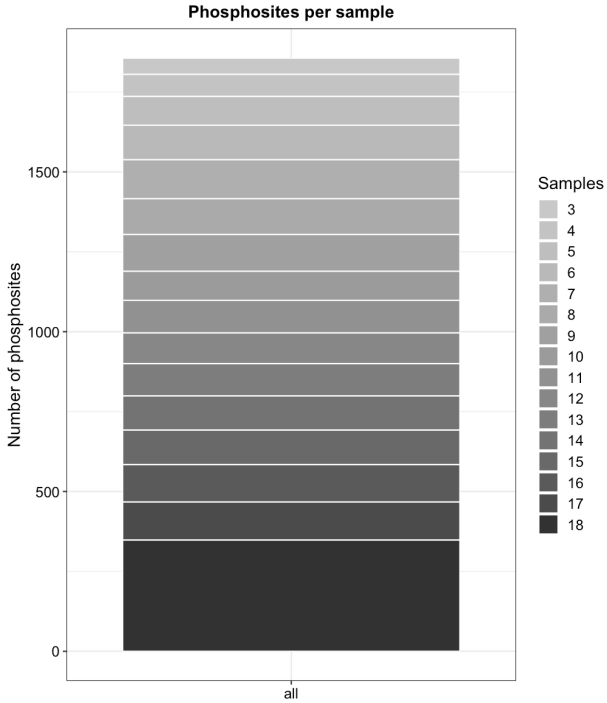
- **Sample CVs**: A histogram plot showing the distribution of protein level coefficient of variation (CV) for each condition. Each plot also contains a vertical line, which indicates the median CV percentage for that condition.



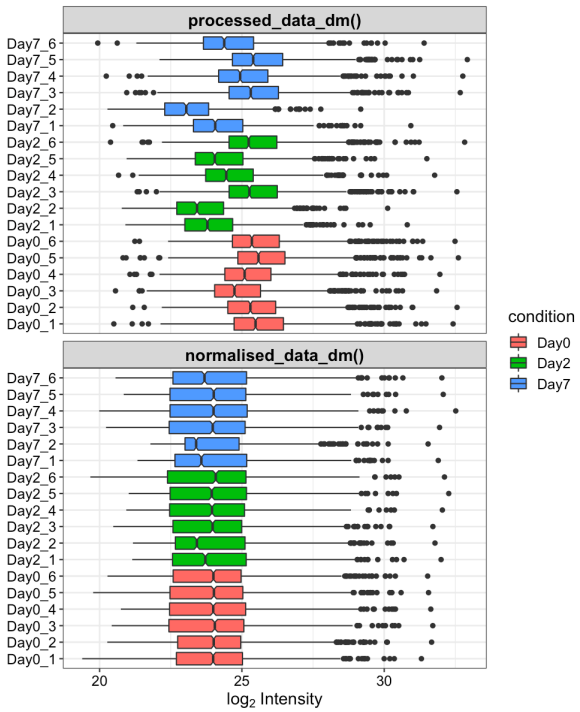
- **Phosphosite/Protein Numbers**: Bar plots representing the number of identified and quantified phosphosites/proteins in each sample after the data pre-filtering process described before.



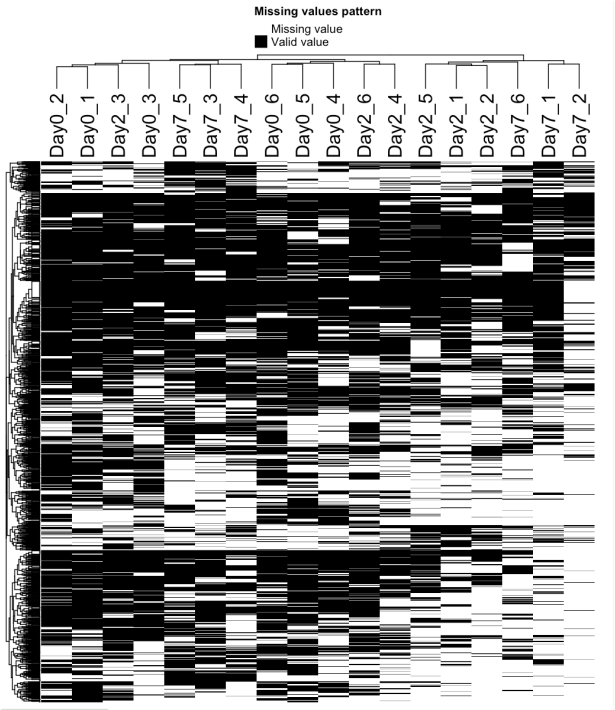
- **Sample coverage**: This plot provides a summary of how many phosphosites/proteins have been quantified consistently in how many samples after the data pre-filtering process described before. In the example shown below, approx. 500 phosphosites have been identified in all 12 samples, approx. 1000 proteins in 9 samples (i.e. three value had to be imputed) etc.



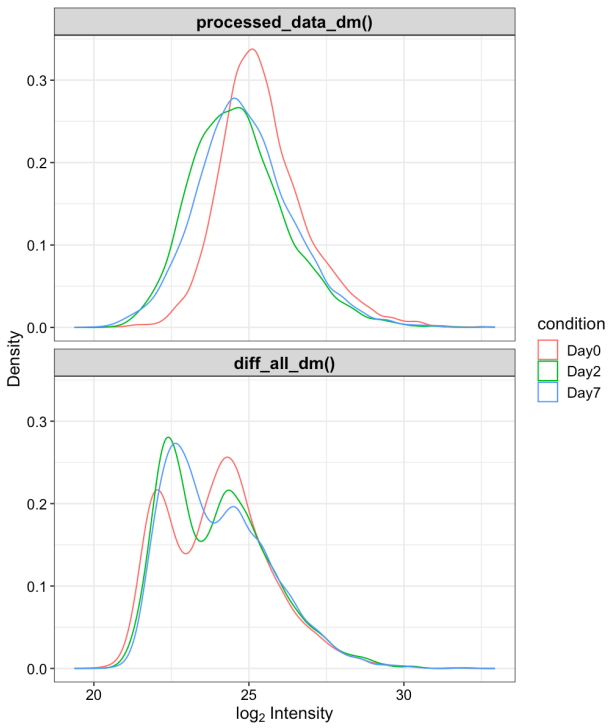
- **Normalization**: These two plots represent the effect of the variant stabilizing normalization (vsn) method on the phosphosite/protein intensity distribution in each sample. This step removes any non-biological related variations and makes the analysis of the results more reliable.



- **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.

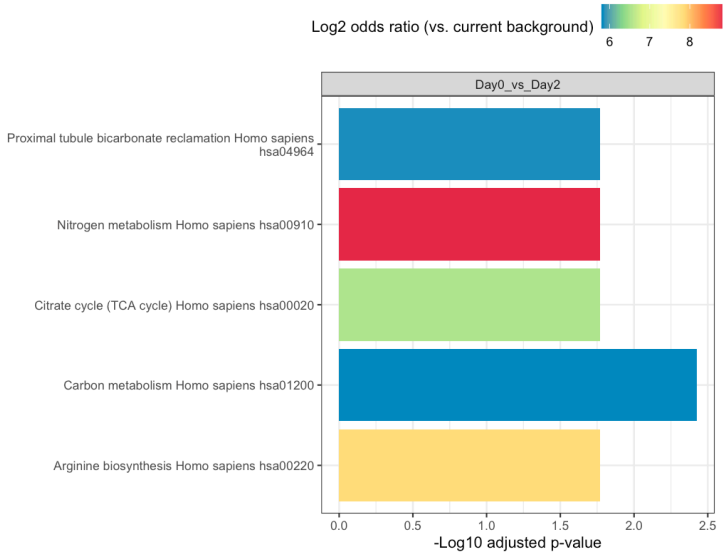


- **Imputation**: A density plot of protein intensity (log_2) distribution for each condition after and before missing value imputation being performed

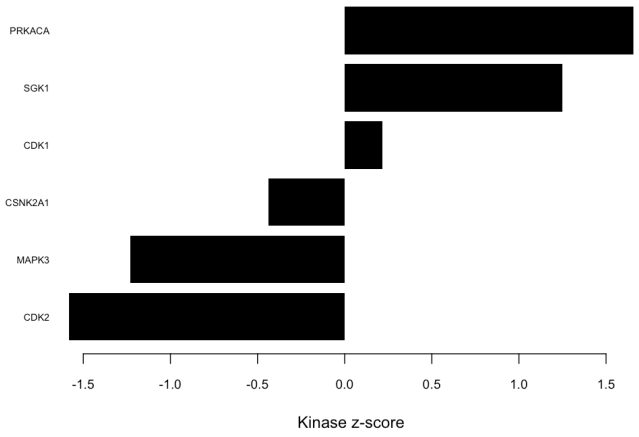


Enrichment Analysis

- **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.



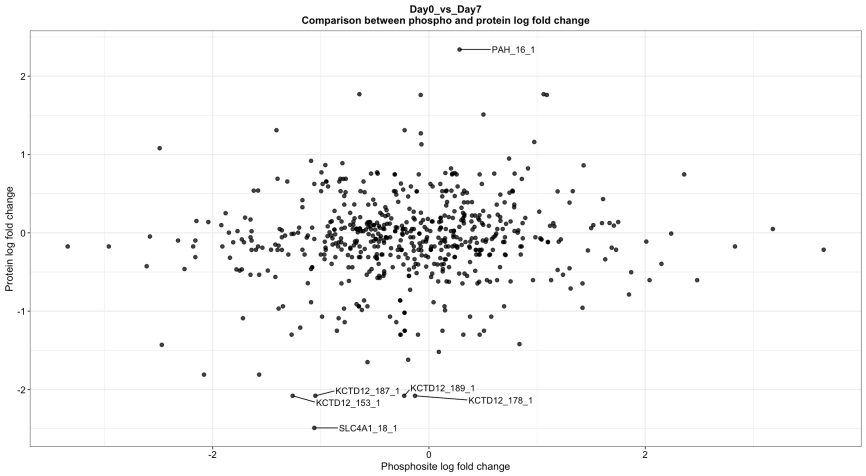
- **Kinase-Substrate enrichment analysis:** On both Phophosite and Phosphosite(corrected) page, Kinase-Substrate enrichment is performed using KSEAApp. The result is displayed as a bar chart and can be downloaded in tabular format.



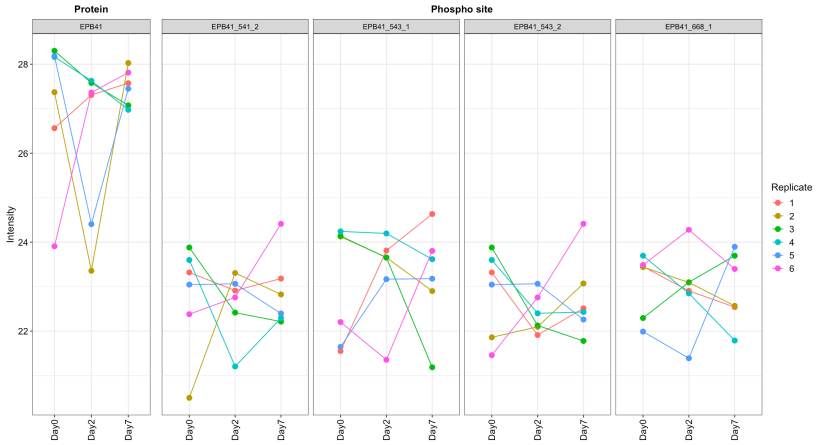
Comparison plots

- **Log fold change scatter plot**: For straightforward visualisation, the scatter plot is using phosphosite level $\log_2$ fold changes as x-axis and protein group $\log_2$ fold changes as y-axis. It

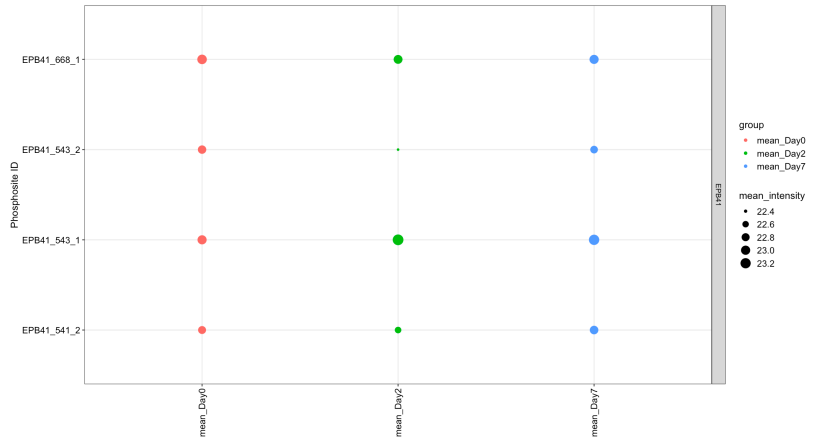
could be seen, proteomics data might not have any changes at protein level, but has significant changes at phosphosite level.



- **Interaction plot**: This plot using combined data to show the interaction between different replicates. It is clearly distinct 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 colored 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.



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.; 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.