Reporter Ion Based Quantification

In reporter ion based quantification, peptides obtained from the digestion of different samples are labeled using isobaric tags, and pooled. The different samples will remain undistinguishable until the data interpretation step, where so-called reporter ions, released upon fragmentation of the tags will be found in the low mass range of the spectrum. Upon identification, the characteristic mass of a reporter ion allows identifying the tag used to label a given sample, and the intensity of the ions in a spectrum can be used to relatively quantify the abundance of the corresponding peptide between the different samples. The two most common reporter based techniques are iTRAQ[1] and TMT[2] (Tandem Mass Tag), where iTRAQ supports 4 or 8 samples, while TMT 2, 6 or 10 samples. Overall, the same data interpretation procedure applies to all reagents.

In the resources folder you will find an mgf file named TMT10.mgf. It corresponds to the acquisition of a TMT experiment, where ten human cerebrospinal fluid samples were labeled and then pooled together, so-called TMT 10-plex. When purchasing a labeling kit, you will be given a product data sheet similar as the one on the following page. It is extremely important to keep this certificate of analysis and not discard it with the box as it contains important information required for the interpretation of the data. We recommend attaching the certificate of analysis for the kit(s) used for an experiment to any publication and to the dataset when submitting data to public repositories.

Tip:

*Systematically scan and save the **product data sheet** of the labelling kit(s) used for an experiment!*

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PRODUCT DATA SHEET

TMT10plex™ Label Reagent Set

Product Number: 90110B **Lot Number:** OJ190396B

Form: The TMT10plex™ Label Reagents are supplied dried, 0.8 mg/tube. Make a stock solution by reconstituting each tube with 41 µl acetonitrile.

Note: Please refer to Table 3 of the instruction booklet for cross-referenced TMTsixplex products, reporter ion masses or mass tolerance window in your data analysis software.

**Reporter ion isotopic distributions are for informational use only and are not required isotope correction factors for Proteome Discoverer software TMT10plex quantitation method. Reporter ion isotopic distributions (-2, -1, +1, +2) are primarily for carbon isotopes with reporter ion interference for each mass tag shown in parentheses.

****Reporter Ion Isotopic Distributions:**

Mass Tag	Reporter Ion	-2	-1	Monoisotopic	+1	+2
TMT^{10}-126	126.127726	0	0	100%	4.69 (127C)	0 (128N)
TMT^{10}-127N	127.124761	0	0.4	100%	6.5 (128N)	0 (128C)
TMT^{10}-127C	127.131081	0	0.2 (126)	100%	4.6 (128C)	0.3 (129N)
TMT^{10}-128N	128.128116	0	0.9 (127N)	100%	4.7 (129N)	0.2 (129C)
TMT^{10}-128C	128.134436	0.10 (126)	0.53 (127C)	100%	2.59 (129C)	0 (130N)
TMT^{10}-129N	129.131471	0 (127N)	0.73 (128N)	100%	2.49 (130N)	0 (130C)
TMT^{10}-129C	129.137790	0 (127C)	1.3 (128C)	100%	2.5 (130C)	0 (131)
TMT^{10}-130N	130.134825	0 (128N)	1.2 (129N)	100%	2.8 (131)	2.7
TMT^{10}-130C	130.141145	0.1 (128C)	2.9 (129C)	100%	2.9	0
TMT^{10}-131	131.138180	0 (129N)	2.36 (130N)	100%	1.43	0

Stability: One year from date of product receipt.

Storage: Store at -20°C.

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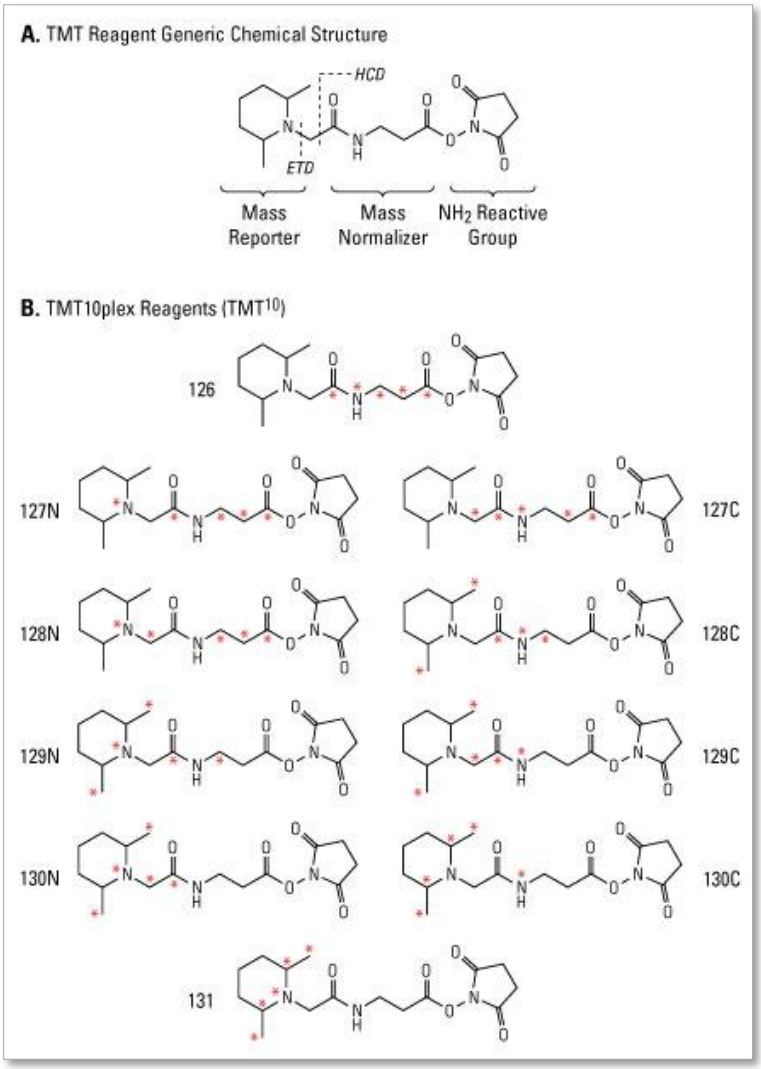
In this product data sheet, you see listed the ten different labeling reagents, *e.g.* TMT10-128N where 128 corresponds to the mass in Dalton of the released reporter ion, and N or C whether the mass difference is encoded at a Nitrogen or Carbon atom. For every reagent, the mass of the reporter ion is given along with purity information. It is these masses which we will look for in spectra.



What is the smallest mass difference between two reporter ions? What does it imply for the acquisition and interpretation of such data? [4.2a]

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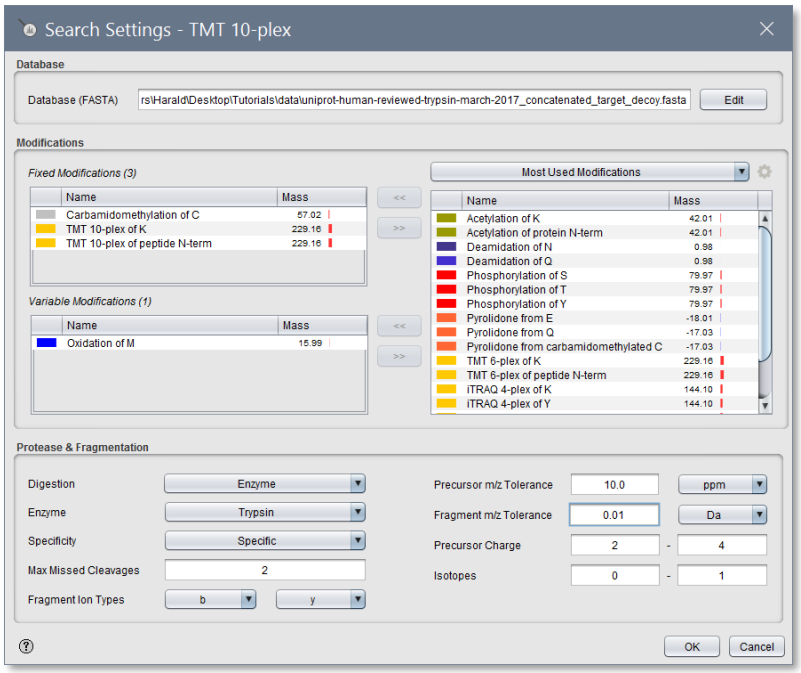
The interpretation strategy consists in three steps: (1) peptide to spectrum matching, (2) PTM localization, protein inference and validation, and (3) quantification of the peptides and proteins. For these, we will use SearchGUI, PeptideShaker, and Reporter, respectively. If you are not already familiar with SearchGUI and PeptideShaker, please refer to the Identification chapters of this tutorial. Reporter is provided in the software folder, and can be downloaded from http://compomics.github.io/projects/reporter.html. Please note that Reporter is still in active development, available in beta version only – you might therefore encounter issues. If you find any, or have any suggestions for improvements, please let us know!

Open SearchGUI and select the file TMT10.mgf as the spectrum file. Click 'Add' behind the 'Settings File' to open the Identification Settings dialog and give the settings a name, for example 'TMT 10-plex'. Next, click the Spectrum Matching settings and for the database choose the human database created in the "Database Generation" chapter (also available in the resources folder). As modifications select, as fixed: Carbamidomethylation of C, TMT 10-Plex of K and TMT 10-Plex of peptide N-term; and, as variable: Oxidation of M. Select 'Trypsin' as the enzyme and change the Fragment Ion Tolerance to 0.01 Da. You should now have the following settings:



How would you adapt these settings when working with other reagents? [4.2b]

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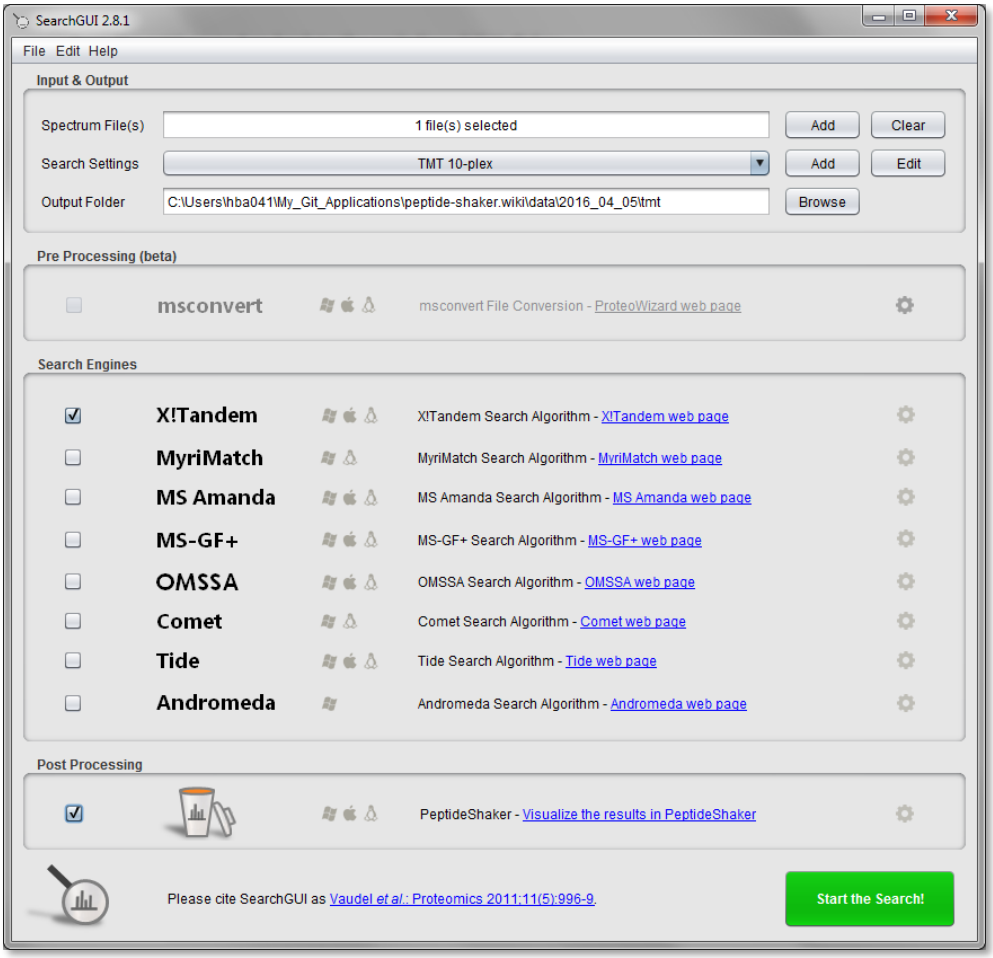
Save the settings and go back to the main SearchGUI dialog.

To save some time, we will here only use the X! Tandem search engine, so make sure that it is the only search engine selected. Next, select and output folder, and set up post-processing in PeptideShaker choosing a Project Name and Sample Name.

You should have the following:



Click the 'Start the Search!' button.

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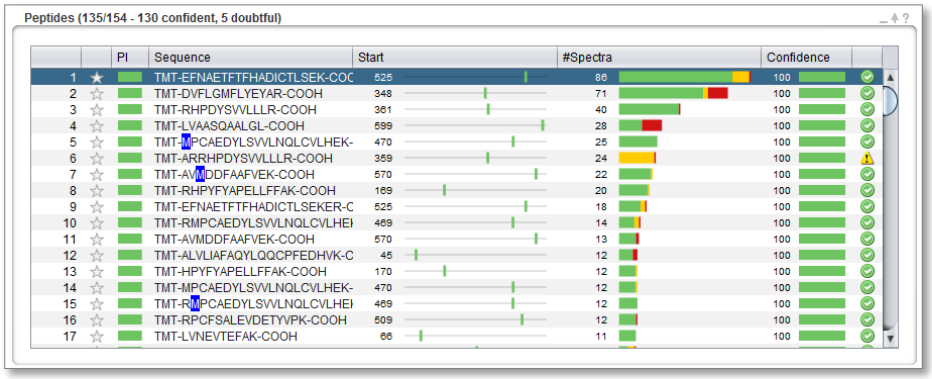
When the processing completes, you should have the search results displayed in PeptideShaker:

Your PeptideShaker project should already have been saved, if not save it now *via* the *File -> Save As...* menu.

Note how all the peptides have TMT annotated at the n-terminal:



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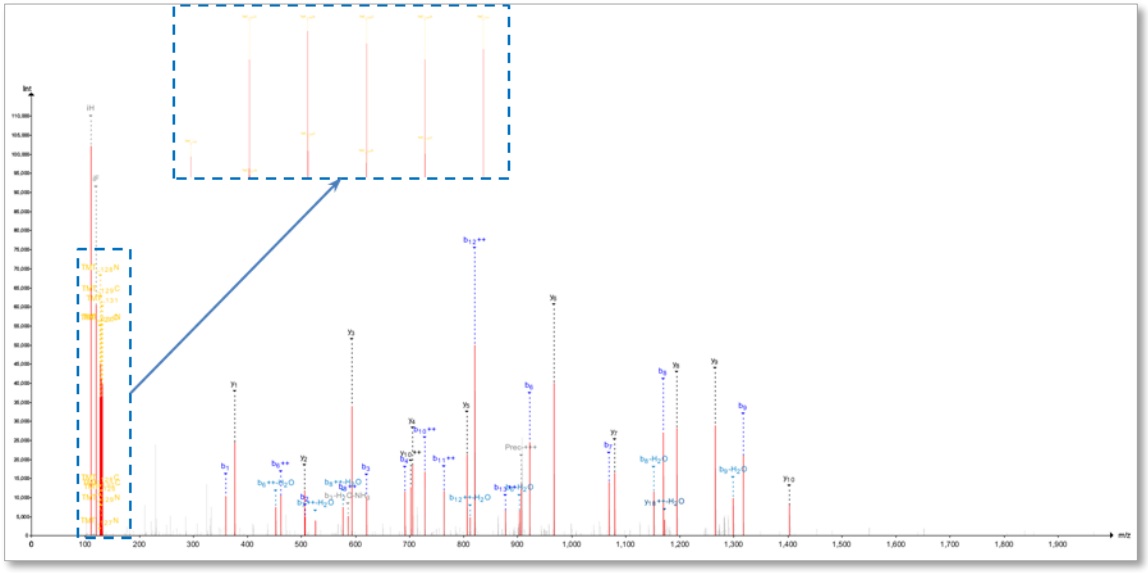
When zooming in the low mass range of the spectrum, you can see the Reporter ions annotated:



Can you see any differences between the height (intensity) of the peaks? *Do you see the same pattern when looking at other PSMs from the same peptide? How similar should the pattern be across the PSMs? What about across different peptides from the same protein? What could affect the results?* *[4.2c]*

In order to convert these intensities into peptide and protein relative abundances, we are going to use Reporter. Note that PeptideShaker and Reporter cannot be used at the same time. Close PeptideShaker and double-click on the Reporter jar file.

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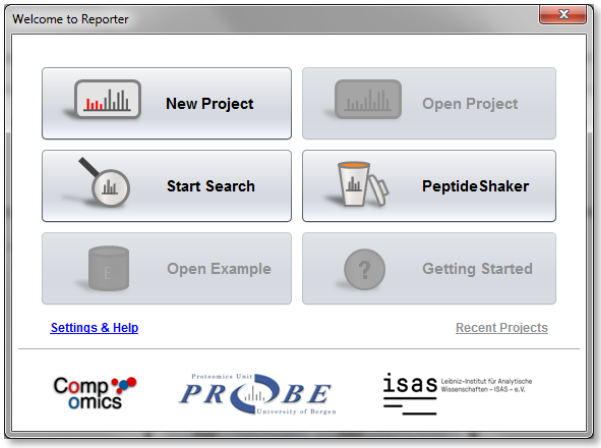
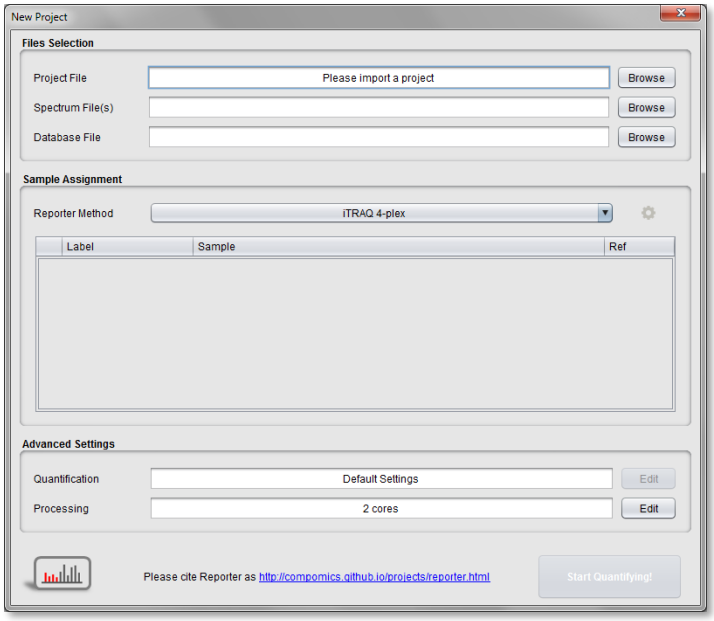
At startup you should see this dialog:



Click on New Project, you will see the following dialog:



Click the 'Browse' button next to 'Project File' and select the just saved PeptideShaker project (.cpsx file).

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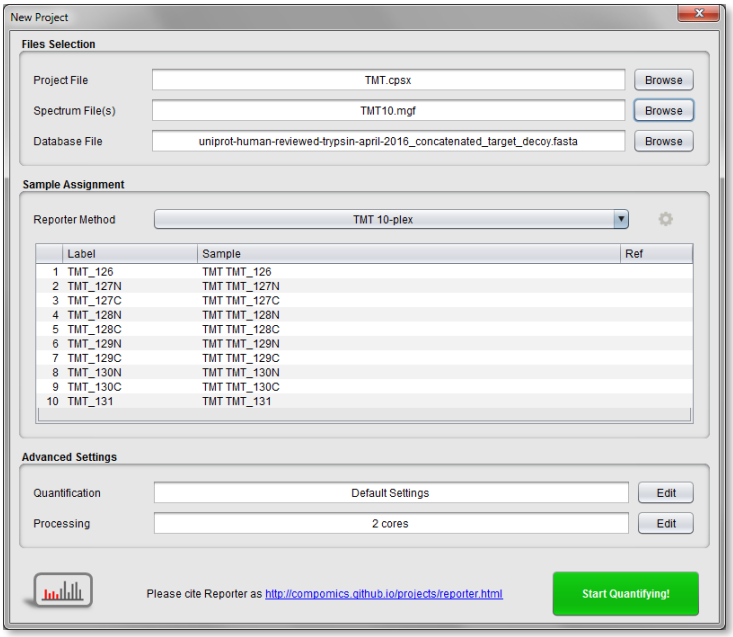
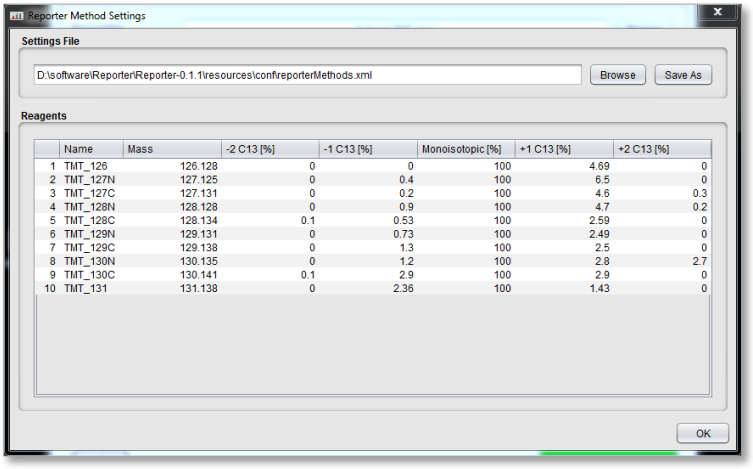
Upon import, you should see the following:



Note that Reporter automatically inferred from your search settings that TMT 10-Plex labels were used. Alternatively, you can select the *Reporter Method* in the *Sample Assignment* section.

Click on the cogwheel next to the selected *Reporter Method*. The following dialog should appear:



This dialog allows you to input the purity coefficients from the product data sheet. Isotope correction will be automatically applied by Reporter as reviewed in[4]. *Why is isotope correction required? Is it important? [4.2d]*

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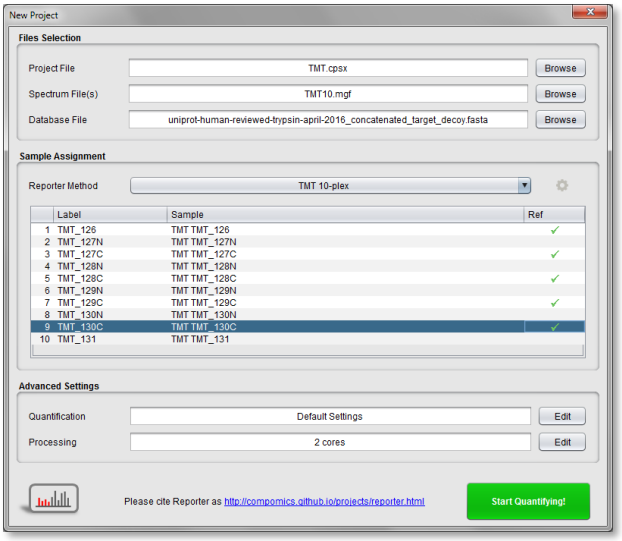
Close the *Reporter Methods Settings* dialog and go back to project creation.

In the sample assignment table, note that you can edit the label of the sample assigned to every label. Note also that you can select reference samples for the estimation of ratios. Here select 126, 127C, 128C, 129C, and 130C as reference. You should have the following:



Which samples should be used as a reference? What if we do not have a reference? [4.2e]

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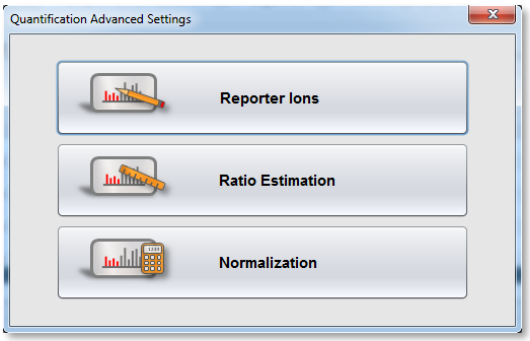
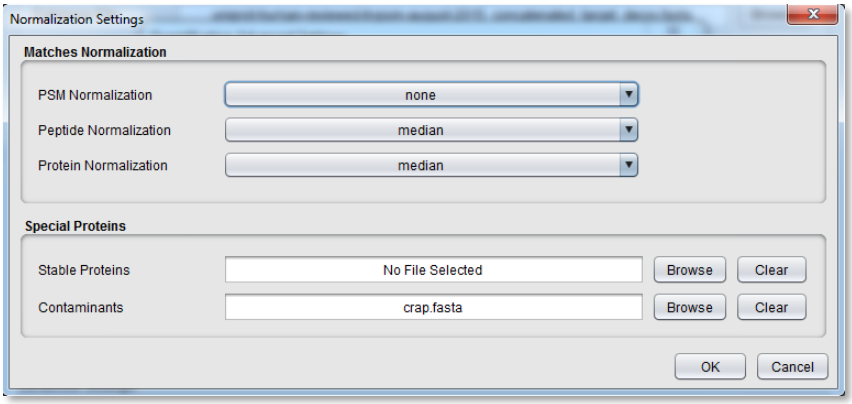
In the Quantification settings, you are able to fine tune the details of the quantification strategy. Click on the 'Edit' button, you will see the following.



Select Normalization:



In this dialog, you can select different statistical estimators to use for the normalization of ratios. You can also provide proteins to consider stable in priority, and contaminants to exclude from the normalization strategy.

What is the best normalization strategy? Why are contaminants excluded? [4.2f]

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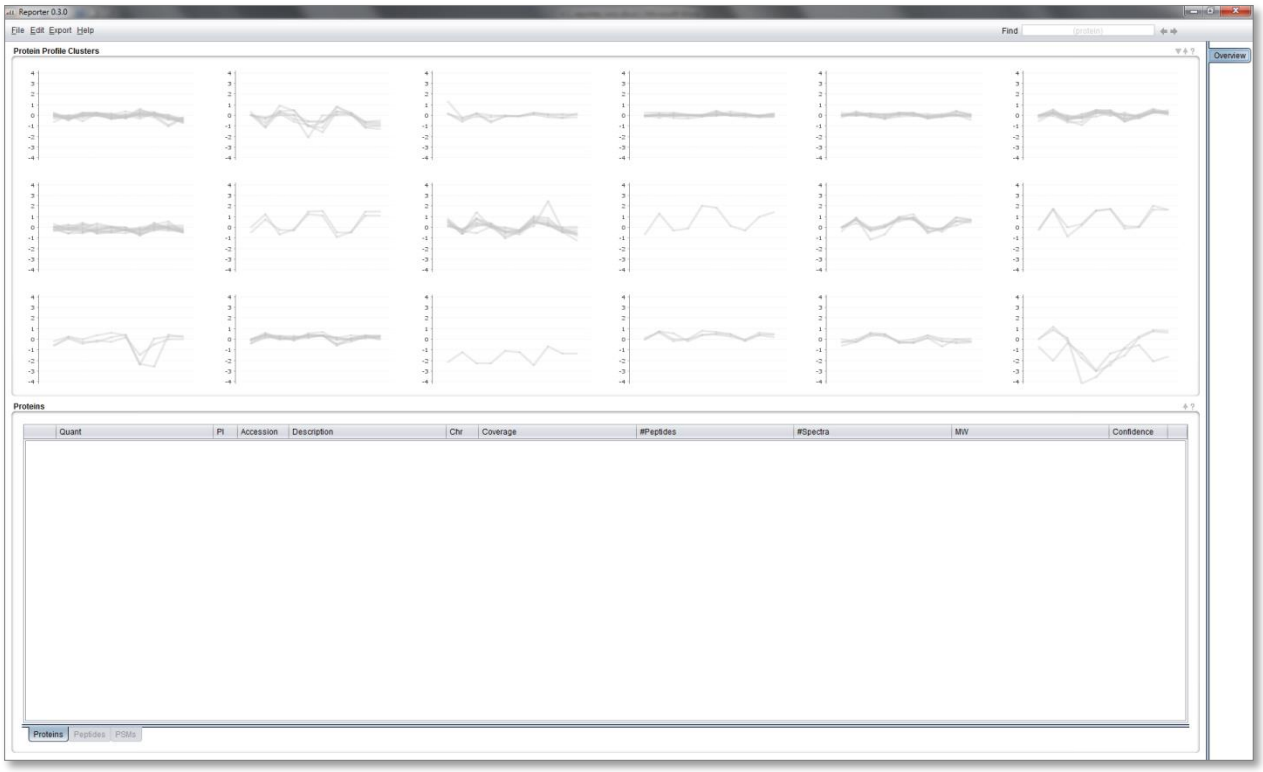
Leave the settings to default and go back to the project creation dialog. Click on 'Start Quantifying!', upon calculation, you should see clusters similar to this:



Tip:

You can search a protein or change the number of clusters in the upper right corner!

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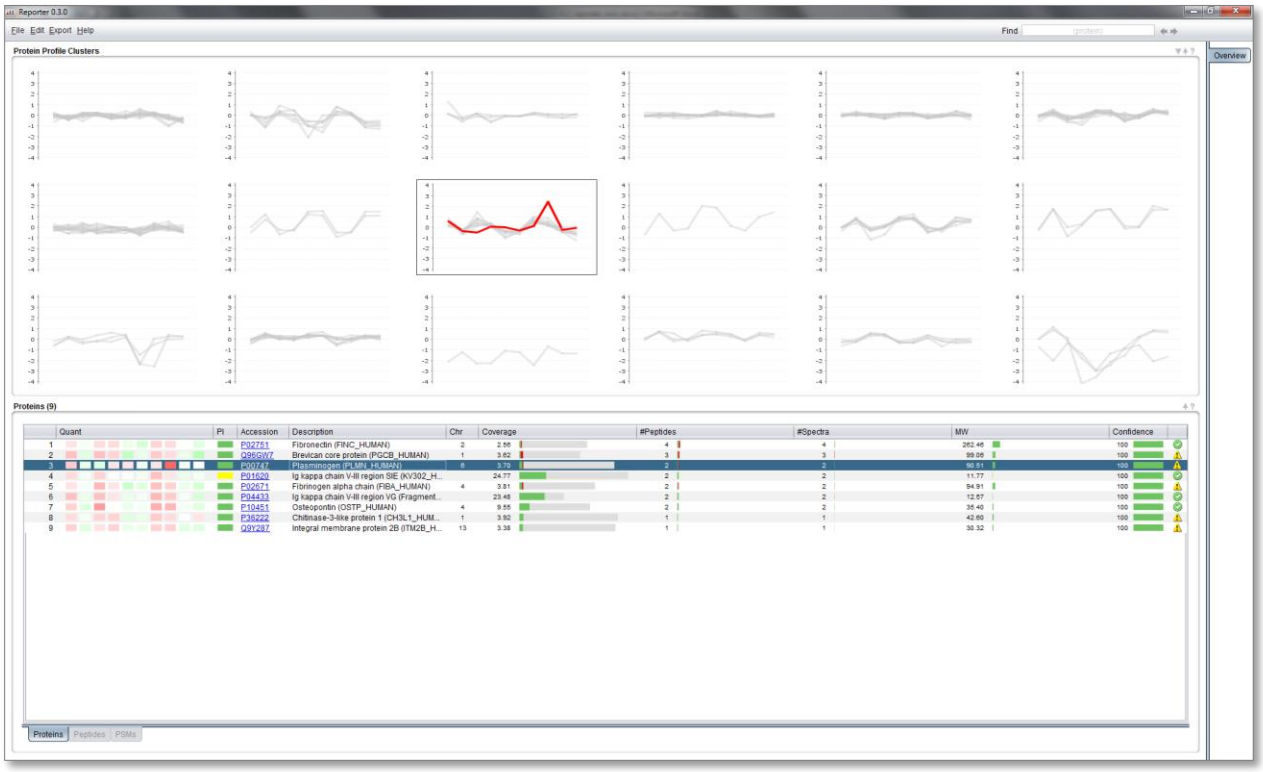
As you can see, Reporter clustered the proteins according to their expression profiles. If you select a cluster, the containing proteins will be listed and their ratios displayed to the left in the table.



For follow-up processing of the quantification values, it is possible to export the values with annotation *via* the *Export -> Identification Features*, either as Microsoft Excel spreadsheet or as standard text files - notably compatible with Perseus (http://maxquant.org).

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References

1. Ross, P.L. et al. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Molecular & cellular proteomics : MCP* **3**, 1154-1169 (2004).
2. Thompson, A. et al. Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Analytical chemistry* **75**, 1895-1904 (2003).
3. Kocher, T. et al. High precision quantitative proteomics using iTRAQ on an LTQ Orbitrap: a new mass spectrometric method combining the benefits of all. *Journal of proteome research* **8**, 4743-4752 (2009).
4. Vaudel, M., Sickmann, A. & Martens, L. Peptide and protein quantification: A map of the minefield. *Proteomics* **10**, 650-670 (2010).

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