

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 iTRAQ¹ and TMT² (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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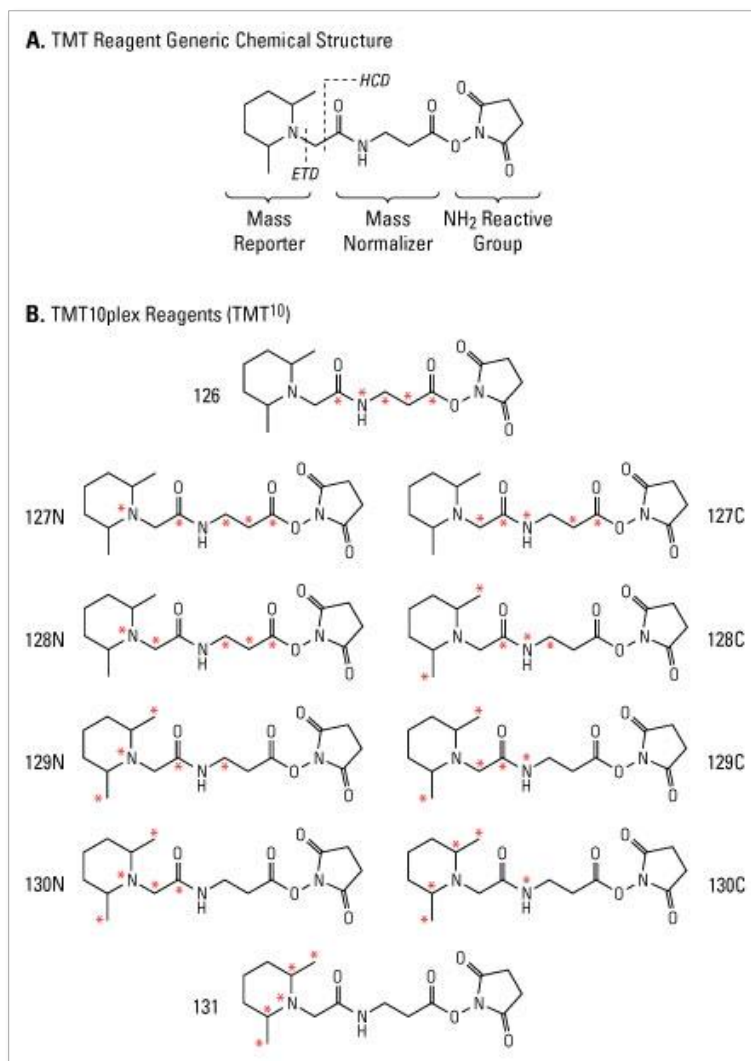
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In this product data sheet, you see listed the ten different labeling reagents, *e.g.* [TMT10-128N](#) where 128 corresponds to the mass in Dalton of the released reporter ion, and N or C whether the mass difference is encoded at a Nitrogen or Carbon 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? [\[4.2a\]](#)



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:

Search Settings - TMT 10-plex

Database

Database (FASTA)

Modifications

Fixed Modifications (3)

Name	Mass
Carbamidomethylation of C	57.02
TMT 10-plex of K	229.16
TMT 10-plex of peptide N-term	229.16

Variable Modifications (1)

Name	Mass
Oxidation of M	15.99

Most Used Modifications

Name	Mass
Acetylation of K	42.01
Acetylation of protein N-term	42.01
Deamidation of N	0.98
Deamidation of Q	0.98
Phosphorylation of S	79.97
Phosphorylation of T	79.97
Phosphorylation of Y	79.97
Pyroglutamine from E	-18.01
Pyroglutamine from Q	-17.03
Pyroglutamine from carbamidomethylated C	-17.03
TMT 6-plex of K	229.16
TMT 6-plex of peptide N-term	229.16
ITRAQ 4-plex of K	144.10
ITRAQ 4-plex of Y	144.10

Protease & Fragmentation

Digestion

Enzyme

Specificity

Max Missed Cleavages

Fragment Ion Types

Precursor m/z Tolerance

Fragment m/z Tolerance

Precursor Charge

Isotopes

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

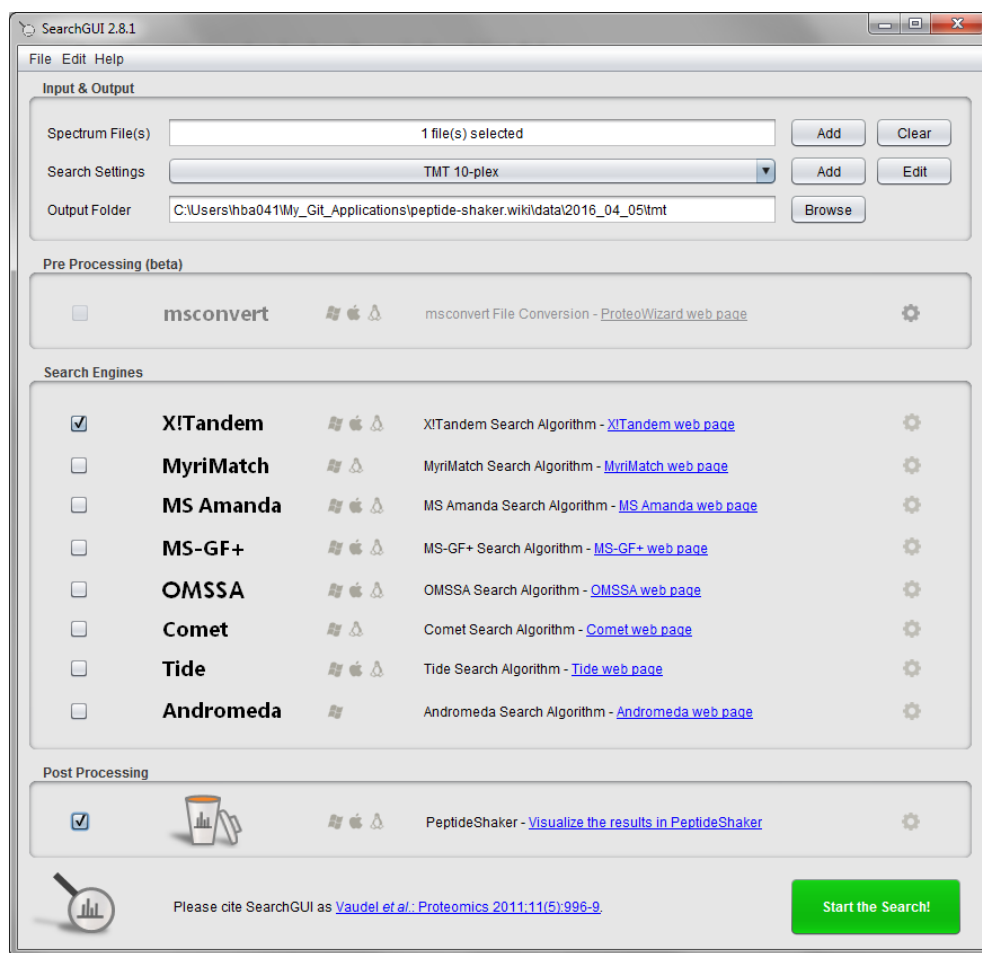


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Save the settings and go back to the main SearchGUI dialog.

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 an 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.



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



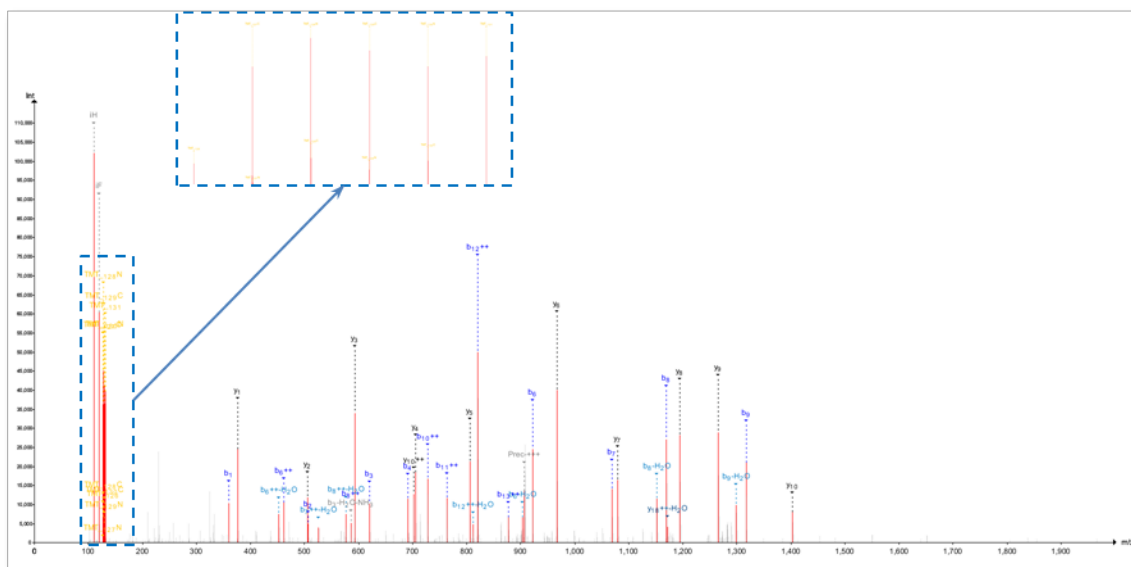
Your **PeptideShaker** project should already have been saved, if not save it now *via* the **File -> Save As...** menu.

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

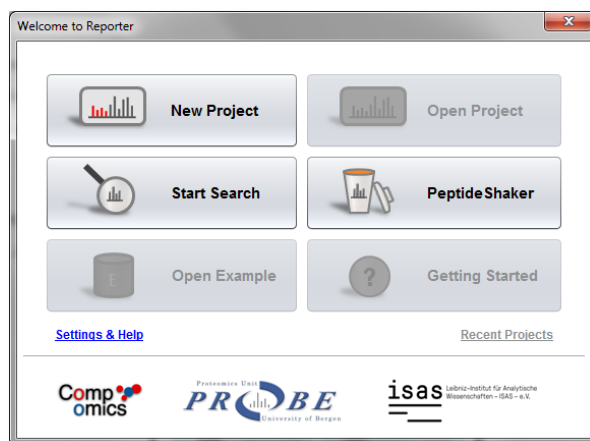
Peptides (135/154 - 130 confident, 5 doubtful)

PI	Sequence	Start	#Spectra	Confidence
1	TMT-EFNAETTFTHADICTLSEK-COC	525	86	100
2	TMT-DVFLGMFLYEYAR-COOH	348	71	100
3	TMT-RHPDYSVLLLR-COOH	361	40	100
4	TMT-LVAASQAALL-COOH	599	28	100
5	TMT-PCAEYLSVNLQCVLHEK-	470	25	100
6	TMT-ARRHDPDYSVLLLR-COOH	359	24	100
7	TMT-AVDDFAFVEK-COOH	570	22	100
8	TMT-RHPYFAPPELLFFAK-COOH	169	20	100
9	TMT-EFNAETTFTHADICTLSEK-C	525	18	100
10	TMT-RMPCAEDYLSVNLQCVLHEI	469	14	100
11	TMT-AVMDFAFVEK-COOH	570	13	100
12	TMT-ALVIAFAQYLCQPFEDHVK-C	45	12	100
13	TMT-HPYFAPPELLFFAK-COOH	170	12	100
14	TMT-MPCAEDYLSVNLQCVLHEK-	470	12	100
15	TMT-RPCAEYLSVNLQCVLHEI	469	12	100
16	TMT-RPCSALEVDITYPK-COOH	509	12	100
17	TMT-LVNEVTEFAK-COOH	66	11	100

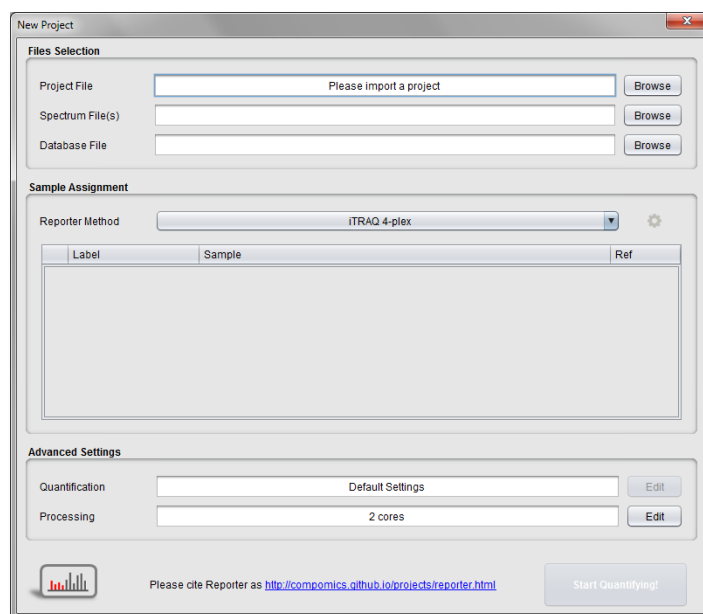
When zooming in the low mass range of the spectrum, you can see the Reporter ions annotated:



At startup you should see this dialog:

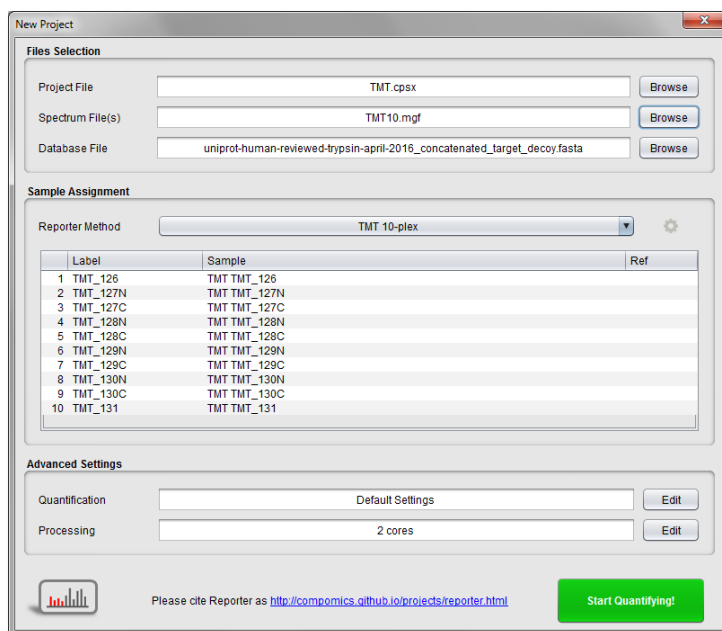


Click on New Project, you will see the following dialog:



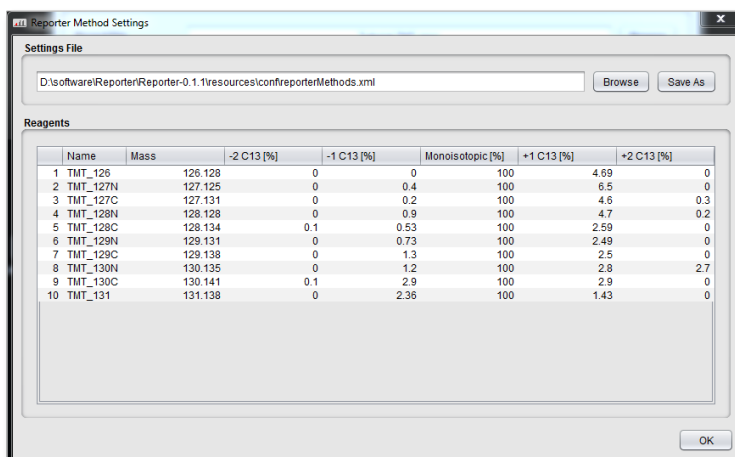
Click the 'Browse' button next to 'Project File' and select the just saved [PeptideShaker](#) project (.cpsx file).

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 in⁴. *Why is isotope correction required? Is it important?*

[4.2d]



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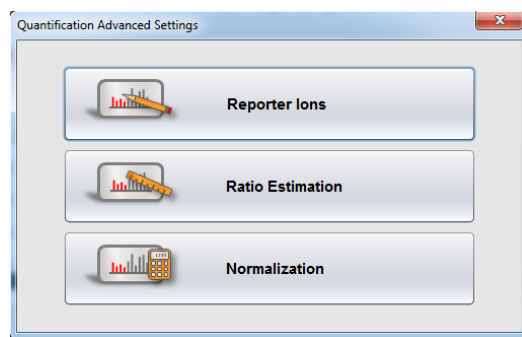
Close the *Reporter Methods Settings* dialog and go back to project creation.

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:

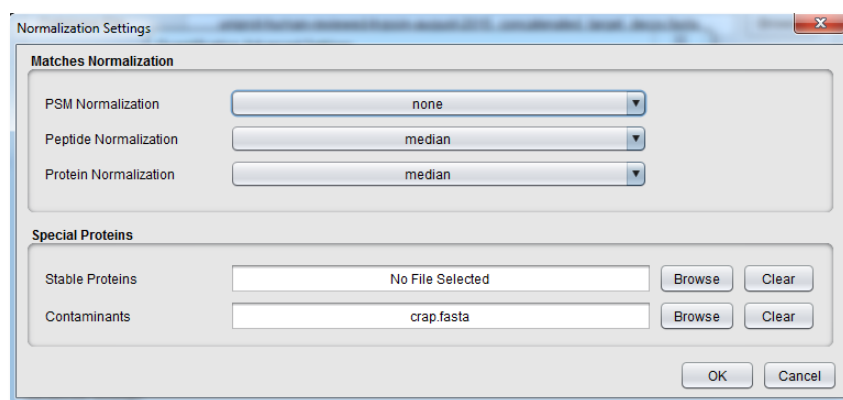
Label	Sample	Ref
1 TMT_126	TMT TMT_126	✓
2 TMT_127N	TMT TMT_127N	
3 TMT_127C	TMT TMT_127C	✓
4 TMT_128N	TMT TMT_128N	
5 TMT_128C	TMT TMT_128C	✓
6 TMT_129N	TMT TMT_129N	
7 TMT_129C	TMT TMT_129C	✓
8 TMT_130N	TMT TMT_130N	
9 TMT_130C	TMT TMT_130C	✓
10 TMT_131	TMT TMT_131	

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

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.



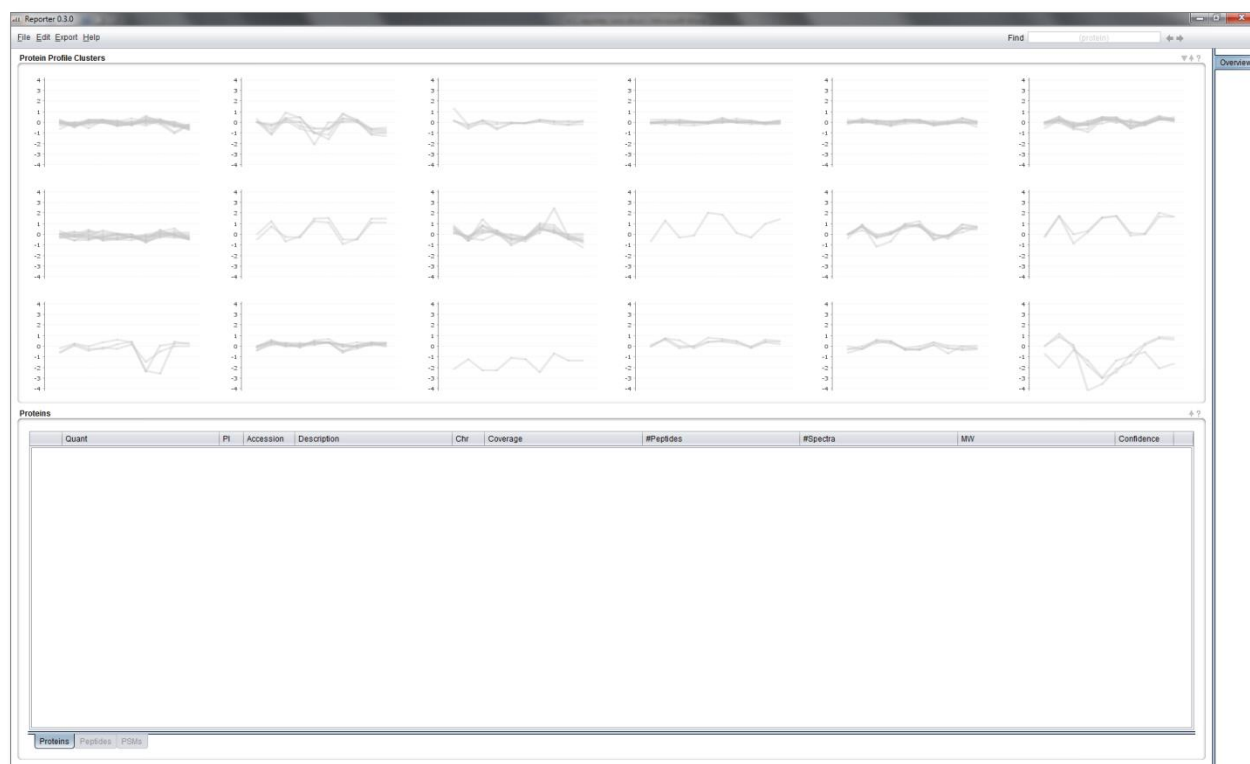
Select **Normalization**:



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]

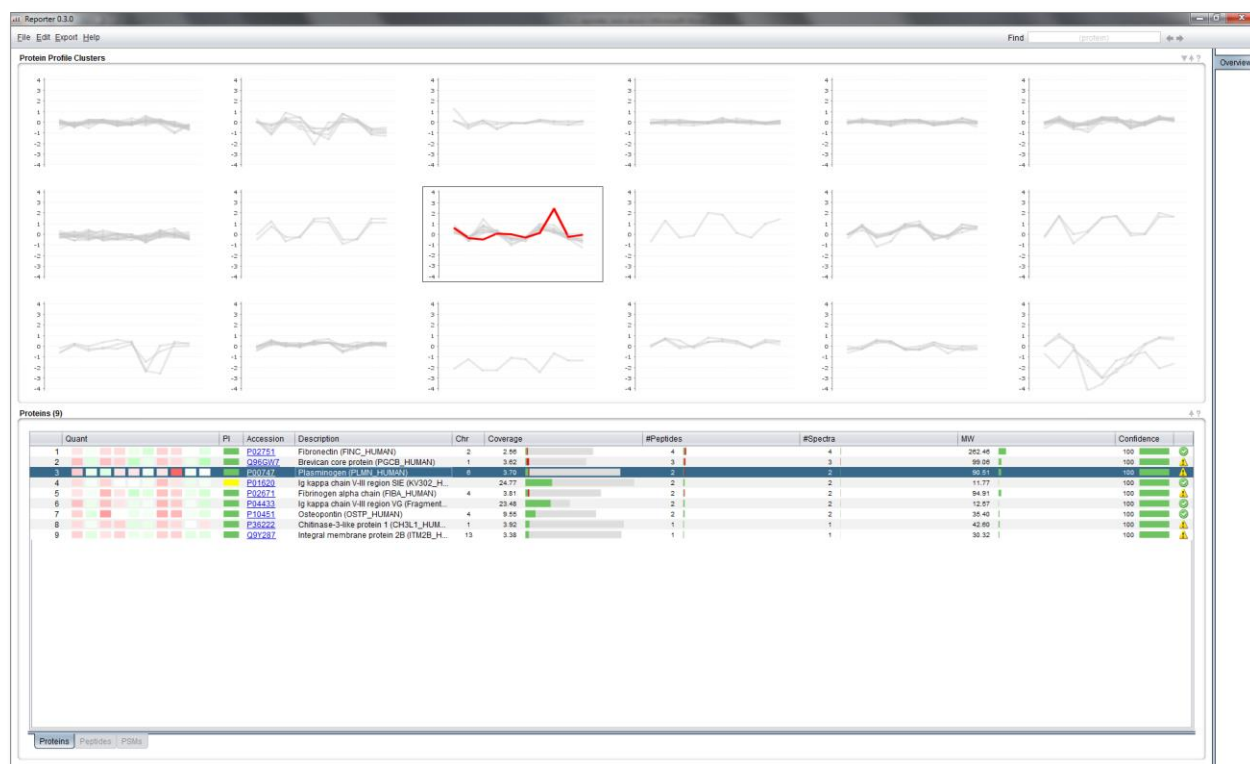
Leave the settings to default and go back to the project creation dialog. Click on 'Start Quantifying!', upon calculation, you should see clusters similar to this:

**Tip:**

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



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>).

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).

