

Appendix 1: Bons, J., Rose, J., Zhang, R.; Burton, J. B., Carrico, C., Verdin, E., Schilling, B. In-depth Analysis of the Sirtuin 5-regulated Mouse Brain Malonylome and Succinylome using Library-free Data-Independent Acquisitions, *Proteomics*

Generation of a LC-MS/MS Variable Window Data-independent Acquisition Method for a Thermo Orbitrap Eclipse Platform, and Instructions for Data Processing with directDIA (Spectronaut)

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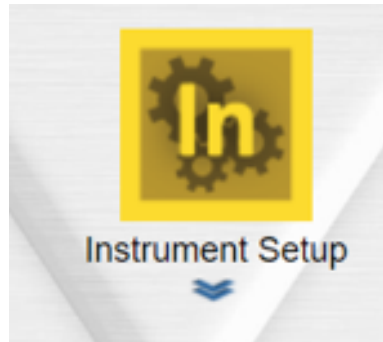
Note: use hyperlinks to go to the individual chapters by clicking on the page numbers above.

Chapter 1. Generate a LC-MS/MS method file for variable window DIA

1. Open **XCalibur** and navigate to the **XApps Page**.

Note: Here we use XCalibur version 4.3.73.11.

2. Click **Instrument Setup** to start a new method.



3. Click **Orbitrap Eclipse** to modify the MS method.



4. Set the **Method Duration** to the length of the chromatographic gradient.

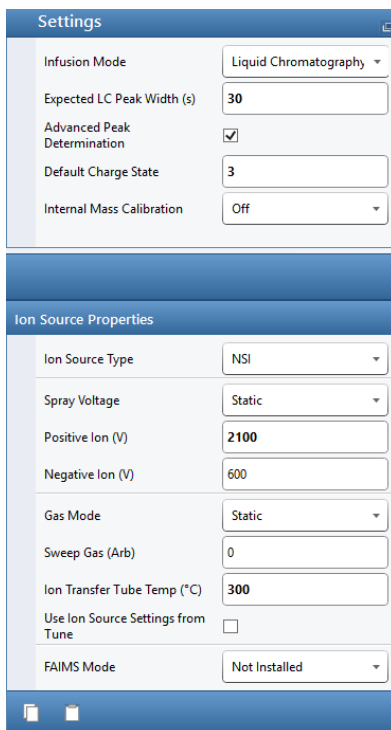
Note: In this study, the length of the chromatographic gradient is 210 min.

5. On the **Settings** menu, change the following parameters:

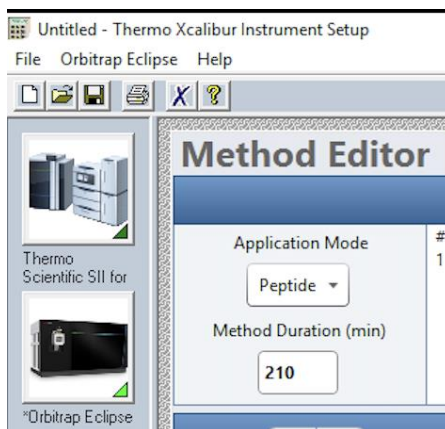
- set the **Expected LC Peak Width** to 30 s
- check **Advanced Peak Determination**
- set the **Default Charge State** to 3
- set the **Internal Mass Calibration** to Off.

6. On the **Ion Source Properties** menu, change the source conditions to conditions that were optimized for the system, which should be in the following ranges:

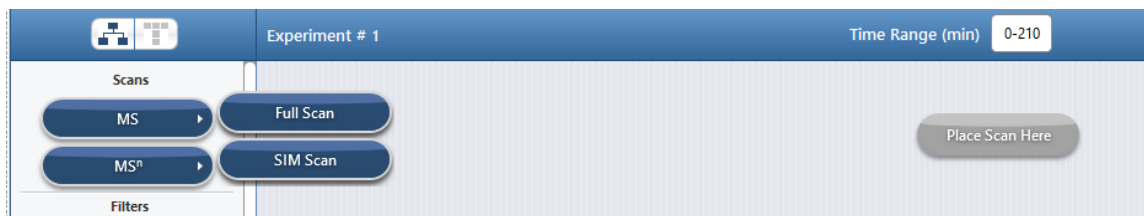
- set the **Positive Ion (V)** to 1900 – 2300 V
- set the **Sweep Gas (Arb)** to 0
- set the **Ion Transfer Tube Temp (°C)** to 290 – 310.



7. Click on **Scan Parameters**, and set to **Standard View**.



8. On the **Scans** menu, click **MS** and drag **Full Scan** into the **Experiment #1** window where it says **Place Scan Here** to set **Experiment #1** to **MS OT**.

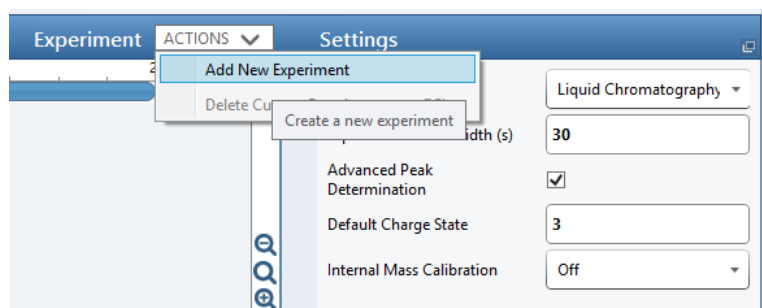


9. On the **MS Scan Properties** menu, change the following parameters:

- set the **Orbitrap Resolution** to 120000
- set the **Scan Range (m/z)** to 350 – 1650
- set the **AGC Target** to Custom
- set the **Normalized AGC Target (%)** to 750
- set the **Maximum Injection Time Mode** to Custom
- set the **Maximum Injection Time (ms)** to 60.

MS Scan Properties	
Detector Type	Orbitrap
Orbitrap Resolution	120000
Mass Range	Normal
Use Quadrupole Isolation	<input checked="" type="checkbox"/>
Scan Range (m/z)	350-1650
RF Lens (%)	30
AGC Target	Custom
Normalized AGC Target (%)	750
Maximum Injection Time Mode	Custom
Maximum Injection Time (ms)	60
Microscans	1
Data Type	Profile
Polarity	Positive
Source Fragmentation	<input type="checkbox"/>
Scan Description	

10. Under the **Experiment Actions** dropdown menu, click **Add New Experiment**.



11. On the **Scans** menu, click **MSⁿ** and drag **MSⁿ** into the **Experiment #2** window where it says **Place Scan Here** to set **Experiment #2** to **tMS² OT CID**.

12. Under the **Targeted MSⁿ Scan Properties** menu, change the following parameters:

- set the **MSⁿ Level (n)** to 2
- set the **Isolation Mode** to Quadrupole
- set the **Isolation Window (m/z)** to 1.6
- set the **Activation Type** to HCD
- set the **Collision Energy Mode** to Fixed
- set the **HCD Collision Energy (%)** to 27
- set the **Detector** to Orbitrap
- set the **Orbitrap Resolution** to 30000
- set **TurboTMT** to Off
- set the **Mass Range** to Normal
- set the **Scan Range Mode** to Define First Mass
- set the **First Mass (m/z)** to 200
- set the **RF Lens (%)** to 30
- set the **AGC Target** to Custom
- set the **Normalized AGC Target (%)** to defined in table by clicking the icon



- set the **Maximum Injection Time Mode** to Auto
- set the number of **Microscans** to 1
- set the **Data Type** to Profile
- set the **Polarity** to Positive
- set the **Loop Control** to N
- set the **N (Number of Spectra)** to 26
- set the **Dynamic Retention Time** to Off
- set the **Time Mode** to Start/End Time.

Targeted MSⁿ Scan Properties

MSⁿ Level (n)

Multiplex Ions

Isolation Mode

Isolation Window (m/z)

Activation Type

Collision Energy Mode

HCD Collision Energy (%)

Detector Type

Orbitrap Resolution

TurboTMT

Mass Range

Scan Range Mode

First Mass (m/z)

RF Lens (%)

AGC Target

Normalized AGC Target (%)

Maximum Injection Time Mode

Targeted MSⁿ Scan Properties

First Mass (m/z)

RF Lens (%)

AGC Target

Normalized AGC Target (%)

Maximum Injection Time Mode

Microscans

Data Type

Polarity

Source Fragmentation

Use EASY-IC™

Loop Control

N (Number of Spectra)

Dynamic Retention Time

Scan Description

Time Mode

▲ Select table icon to add property to mass list table.

Mass List Table ADD DELETE IMPORT EXPORT

	Compound	Formula	Adduct	m/z	z	t start
1				524.265	1	0

13. Import the isolation window strategy to the **Mass List Table** in the **Targeted MSⁿ Scan Properties** menu:

- prepare the **DIA Variable Window text file** from the Supplementary Table S1 that defines the variable width isolation window strategy
- in the **Mass List Table** click **Import**
- select the **DIA Variable Window text file**
- ensure that **t start (min)** and **t stop (min)** in the **Mass List Table** corresponds to the length of the gradient.

Mass List Table									
Compound	Formula	Adduct	m/z	z	t start (min)	t stop (min)	Isolation Window (m/z)	Normalized AGC Target (%)	
1			366.5	3	0	210	33	6000	
2			395	3	0	210	26	6000	
3			418	3	0	210	22	6000	
4			438	3	0	210	20	6000	
5			457	3	0	210	20	6000	
6			475	3	0	210	18	6000	
7			493	3	0	210	20	6000	
8			511.5	3	0	210	19	6000	
9			529.5	3	0	210	19	6000	
10			547.5	3	0	210	19	6000	
11			565.5	3	0	210	19	6000	
12			584	3	0	210	20	6000	
13			603.5	3	0	210	21	6000	
14			623.5	3	0	210	21	6000	
15			644.5	3	0	210	23	6000	
16			666.5	3	0	210	23	6000	
17			689	3	0	210	24	6000	
18			713	3	0	210	26	6000	
19			740.5	3	0	210	31	6000	
20			771	3	0	210	32	6000	
21			804.5	3	0	210	37	6000	
22			842	3	0	210	40	6000	
23			887.5	3	0	210	53	6000	
24			946	3	0	210	66	6000	
25			1027.5	3	0	210	99	6000	
26			1363	3	0	210	574	6000	

14. Click **File** and **Save** the instrument method file.

Chapter 2. Data Pre-Processing (Optional Step)

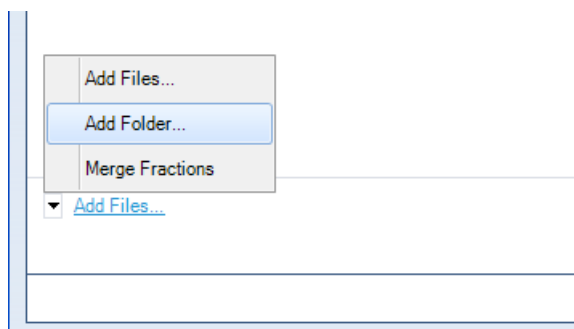
Once the DIA data is acquired, there are several data processing pipelines available to process the data. As an example, a DIA data set was acquired using the above method, then pre-processed using the HTRMS Converter program before directDIA analysis using Spectronaut (Biognosys, Schlieren, Switzerland). The HTRMS file conversion is optional and decreases the time to search the data using Spectronaut by converting vendor specific file formats into a **High Time Resolution Mass Spectrometry (HTRMS)** file format. The optional file conversion step is optimal for large sample sizes.

1. Install the HTRMS Converter program from Biognosys onto the MS computer.

Note: This requires a restart.

2. Open the **HTRMS Converter program** to set the program up to convert acquired data-independent acquisition files to a *.htrms peaks list in a monitored folder.

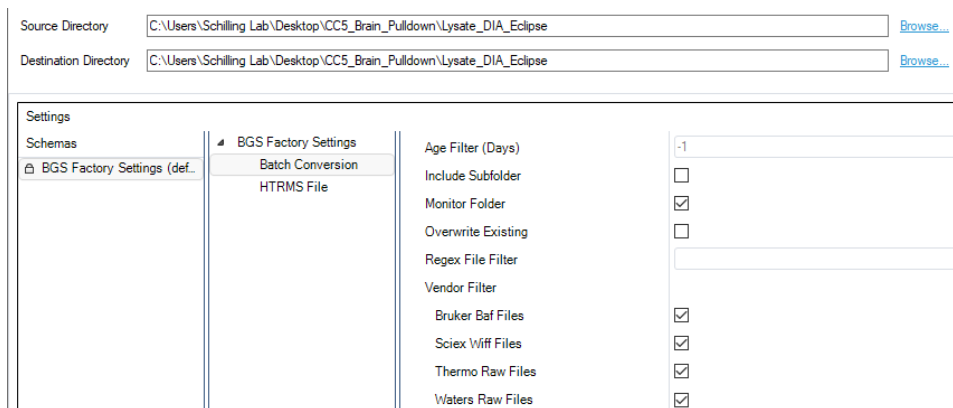
3. Select **Add Folder**.



4. Select the **Source** and **Destination Directories**.

5. Ensure that **Monitor Folder** is enabled.

6. Click **Ok**.



Chapter 3. Data Processing

Spectronaut data processing using directDIA analysis searches each sample acquisition to form a spectral library, then searches each sample acquisition against the library to identify precursors, peptides, proteins, and protein groups.

1. Open **Spectronaut**.

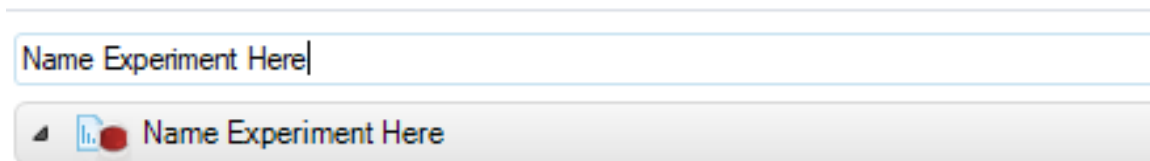
Note: This tutorial is based on Spectronaut version 15.7.220308.50606.

2. Select the **Pipeline** tab:

- click **Set up a directDIA analysis from File**
- navigate to the experimental vendor specific files or *.htrms files and select all files pertinent to the experiment
- Click **Open**.

Name	Date modified	Type	Size
210408_068_CC5_15_Lysate_5KOB1_DIA	4/15/2021 1:29 PM	RAW File	5,424,359 KB
210408_070_CC5_11_Lysate_WTB1_DIA	4/15/2021 6:20 PM	RAW File	5,412,406 KB
210408_072_CC5_16_Lysate_5KOB2_DIA	4/15/2021 11:12 PM	RAW File	4,279,200 KB
210408_074_CC5_12_Lysate_WTB2_DIA	4/16/2021 4:03 AM	RAW File	4,842,153 KB
210408_076_CC5_17_Lysate_5KOB3_DIA	4/16/2021 8:54 AM	RAW File	4,904,507 KB
210408_079_CC5_13_Lysate_WTB3_DIA	4/16/2021 1:46 PM	RAW File	4,307,309 KB
210408_080_CC5_18_Lysate_5KOB4_DIA	4/16/2021 6:37 PM	RAW File	4,912,960 KB
210408_082_CC5_14_Lysate_WTB4_DIA	4/16/2021 11:28 PM	RAW File	4,413,818 KB

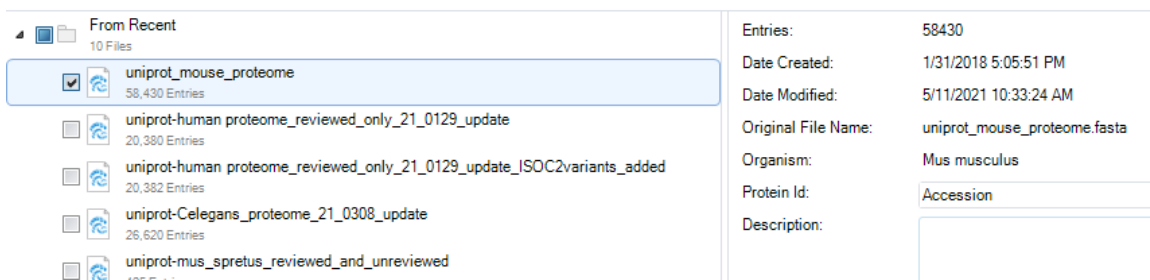
3. Name the experiment with a unique identifier.



4. Click **Next**.

5. Select the appropriate organism **protein database**.

Note: In this study, the FASTA file “uniprot_mouse_proteome” is used.



Property	Value
Entries:	58430
Date Created:	1/31/2018 5:05:51 PM
Date Modified:	5/11/2021 10:33:24 AM
Original File Name:	uniprot_mouse_proteome.fasta
Organism:	Mus musculus
Protein Id:	Accession
Description:	

6. Click **Next**.

Chapter 3.1. Protein Lysate: directDIA Search Settings

Create a **new search and extraction settings schema** for directDIA analysis of a digested protein lysate without specific post-translational modifications. Defaults should be preserved unless specifically mentioned in the text below.

1. Navigate to the **Pulsar Search Peptides** tab:

- the *Enzymes/Cleavage Rules* should be set to “Trypsin/P”
- set the *Digest Type* to “Specific”
- set the *Missed Cleavages* to “2”
- enable *Toggle N-terminal M*.

The screenshot shows the 'Pulsar Search Peptides' settings interface. On the left, a navigation tree is visible with 'Peptides' selected. The main panel is titled 'Enzymes / Cleavage Rules' and contains the following settings:

- Enzymes / Cleavage Rules:** A list of checkboxes with 'Trypsin/P' checked and 'Trypsin', 'LysC', 'LysC/P', and 'GluC' unchecked.
- Digest Type:** A dropdown menu set to 'Specific'.
- Max Peptide Length:** A text input field containing '52'.
- Min Peptide Length:** A text input field containing '7'.
- Missed Cleavages:** A text input field containing '2'.
- Toggle N-terminal M:** A checked checkbox.

2. Select the **Pulsar Search Modifications** tab:

- set the *Max Variable Modifications* to “5”
- set the *Fixed Modifications* to “Carbamidomethyl (C)”
- set the *Variable Modifications* to “Acetyl (Protein N-Term)” and “Oxidation (M)”.

The screenshot shows the 'Pulsar Search Modifications' settings interface. On the left, a navigation tree is visible with 'Modifications' selected. The main panel is titled 'Select Modifications' and contains the following settings:

- Max Variable Modifications:** A text input field containing '5'.
- Fixed Modifications:** A list of modification tags with 'Carbamidomethyl (C)' selected.
- Variable Modifications:** A list of modification tags with 'Acetyl (Protein N-term)' and 'Oxidation (M)' selected.

3. Navigate to the **DIA Analysis Identification** tab:

- set the *Precursor PEP Cutoff* to “1”
- set the *Precursor Qvalue Cutoff* to “0.01”
- set the *Protein Qvalue Cutoff (Experiment)* to “0.01”
- set the *Protein Qvalue Cutoff (Run)* to “0.05”.

The screenshot displays the BGS Factory Settings interface. On the left, a tree view shows the navigation structure with 'DIA Analysis Identification' selected. The main panel is divided into two columns. The left column lists various settings, and the right column shows their values. The 'DIA Analysis Identification' settings are as follows:

Setting	Value
Exclude Duplicate Assays	<input checked="" type="checkbox"/>
Generate Decoys	<input checked="" type="checkbox"/>
Decoy Method	Mutated
Preferred Fragment Source	NN Predicted Fragments
Decoy Limit Strategy	Dynamic
Library Size Fraction	0.1
Machine Learning	Per Run
Precursor PEP Cutoff	1
Precursor Qvalue Cutoff	0.01
Protein Qvalue Cutoff (Experiment)	0.01
Protein Qvalue Cutoff (Run)	0.05
Single Hit Definition	By Stripped Sequence
Exclude Single Hit Proteins	<input type="checkbox"/>
Pvalue Estimator	Kernel Density Estimator

4. Click on the **DIA Analysis Quantification** tab:

- set the *Minor (Peptide) Grouping* to “by Stripped Sequence”
- set the *Major Group Quantity* to “Sum peptide quantity”
- set the *Major Group Max* to “7”
- set the *Major Group Min* to “1”
- set *Minor Group Quantity* to “Sum precursor quantity”
- set the *Minor Group Max* to “10”
- set the *Minor Group Min* to “1”
- set the *Data Filtering* to “Qvalue sparse”
- set the *Imputing Strategy* to “No Imputing”
- set the *Normalization Strategy* to “Local Normalization”
- set the *Row Selection* to “Q-value sparse”.

<ul style="list-style-type: none"> ▲ BGS Factory Settings <ul style="list-style-type: none"> ▲ Pulsar Search <ul style="list-style-type: none"> Peptides Labeling Modifications Identification Tolerances Workflow Result Filters ▲ DIA Analysis <ul style="list-style-type: none"> Data Extraction XIC Extraction Calibration Identification Quantification PTM Workflow Workflow Protein Inference Post Analysis Pipeline Mode 	<ul style="list-style-type: none"> Interference Correction <input checked="" type="checkbox"/> Only Identified Peptides <input checked="" type="checkbox"/> Exclude All Multi-Channel Interferences <input checked="" type="checkbox"/> MS1 Min <input type="text" value="2"/> MS2 Min <input type="text" value="3"/> Protein LFQ Method <input type="text" value="Automatic"/> Proteotypicity Filter <input type="text" value="None"/> Major (Protein) Grouping <input type="text" value="by Protein Group Id"/> Minor (Peptide) Grouping <input type="text" value="by Stripped Sequence"/> Major Group Quantity <input type="text" value="Sum peptide quantity"/> Major Group Top N <input checked="" type="checkbox"/> Max <input type="text" value="7"/> Min <input type="text" value="1"/> Minor Group Quantity <input type="text" value="Sum precursor quantity"/> Minor Group Top N <input checked="" type="checkbox"/> Max <input type="text" value="10"/> Min <input type="text" value="1"/> Quantity MS-Level <input type="text" value="MS2"/> Quantity Type <input type="text" value="Area"/> Data Filtering <input type="text" value="Qvalue sparse"/> Imputing Strategy <input type="text" value="No Imputing"/> Cross Run Normalization <input checked="" type="checkbox"/> Normalization Filter Type <input type="text" value="None"/> Normalization Strategy <input type="text" value="Local Normalization"/> Row Selection <input type="text" value="Qvalue sparse"/>
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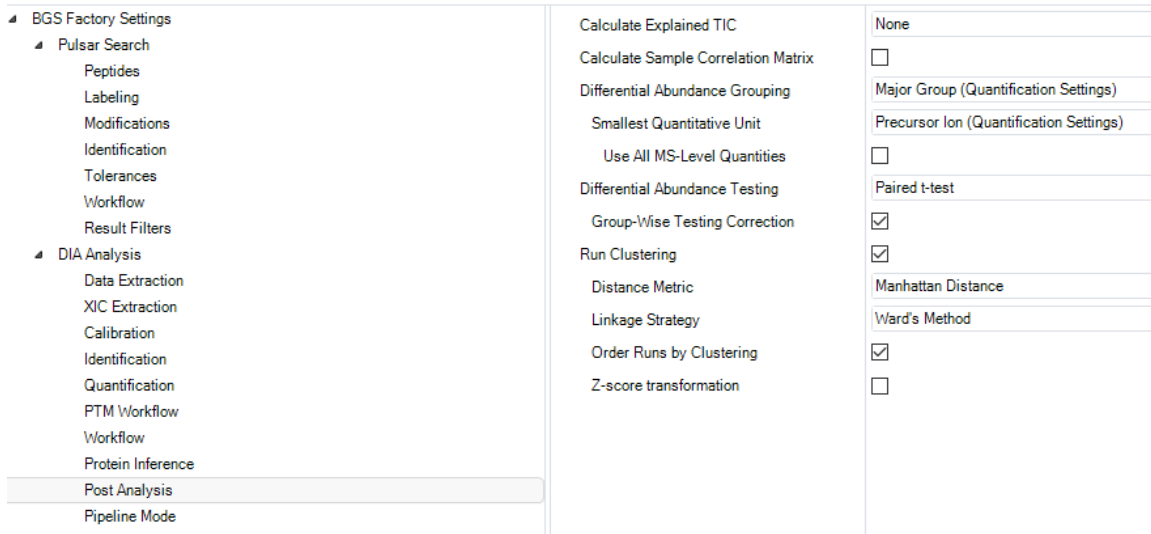
5. Select the **DIA Analysis Workflow** tab”

- set the *Profiling Strategy* to “iRT Profiling”.

<ul style="list-style-type: none"> ▲ BGS Factory Settings <ul style="list-style-type: none"> ▲ Pulsar Search <ul style="list-style-type: none"> Peptides Labeling Modifications Identification Tolerances Workflow Result Filters ▲ DIA Analysis <ul style="list-style-type: none"> Data Extraction XIC Extraction Calibration Identification Quantification PTM Workflow Workflow Protein Inference Post Analysis Pipeline Mode 	<ul style="list-style-type: none"> Method Evaluation <input type="checkbox"/> MS2 DeMultiplexing <input type="text" value="Automatic"/> Profiling Strategy <input type="text" value="iRT Profiling"/> Carry-over exact Peak Boundaries <input type="checkbox"/> Profiling Row Selection <input type="text" value="Minimum Qvalue Row Selection"/> Qvalue Threshold <input type="text" value="0.01"/> Profiling Target Selection <input type="text" value="Automatic Selection"/> Run Limit for directDIA Library <input type="text" value="-1"/> Unify Peptide Peaks Strategy <input type="text" value="None"/>
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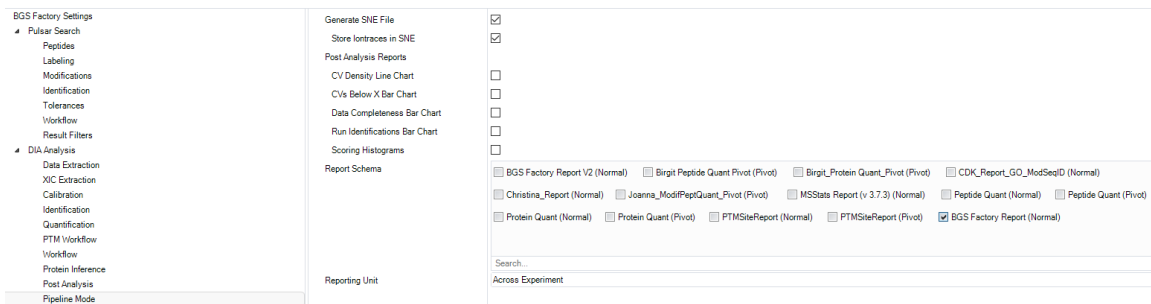
6. Navigate to the **DIA Analysis Post Analysis** tab:

- set the *Smallest Quantitative Unit* to “Precursor Ion (Quantification Settings)”
- set the *Differential Abundance Testing* to “Paired t-test”
- enable *Group-Wise Testing Correction*.



7. Click on the **DIA Analysis Pipeline Mode** tab:

- enable *Generate SNE File*
- select the appropriate *Report Schema* as described in **Chapter 4. Data Reporting:** “Protein_Quant_Pivot” and “Peptide_Quant_Pivot”.



8. Click **Next**.

9. Go to **Chapter 3.3** to complete modifying search settings.

Chapter 3.2. Post-translational Modifications: directDIA Search Settings

Create a **new search and extractions settings schema** for directDIA of a digested protein lysate enriched for specific post-translational modified peptides.

Note: Below is an example of a schema for directDIA analysis of a digested protein lysate enriched for succinylated peptides.

1. Navigate to the **Pulsar Search Peptides** tab

- set the *Enzymes/Cleavage Rules* to “Trypsin/P”
- set the *Digest Type* to “Specific”
- set *Missed Cleavages* to “2”
- enable *Toggle N-terminal M*.

The screenshot shows the BGS Factory Settings interface. On the left, a tree view shows the navigation structure: BGS Factory Settings > Pulsar Search > Peptides. The main panel is titled 'Enzymes / Cleavage Rules' and contains the following settings:

- Enzymes / Cleavage Rules:** A list of checkboxes with 'Trypsin/P' selected. Other options include 'Trypsin', 'LysC', 'LysC/P', and 'GluC'.
- Digest Type:** A dropdown menu set to 'Specific'.
- Max Peptide Length:** A text input field containing '52'.
- Min Peptide Length:** A text input field containing '7'.
- Missed Cleavages:** A text input field containing '2'.
- Toggle N-terminal M:** A checkbox that is checked.

2. Select the **Pulsar Search Modifications** tab:

- set the *Max Variable Modifications* to “8”
- set the *Fixed Modifications* to “Carbamidomethyl (C)”
- set the *Variable Modifications* to “Acetyl (Protein N-Term)”, “Oxidation (M)”, and “Succinyl”.

Note: Update “Succinyl” with the post-translational modification(s) of interest.

The screenshot shows the BGS Factory Settings interface with the 'Modifications' tab selected. The main panel is titled 'Max Variable Modifications' and contains the following settings:

- Max Variable Modifications:** A text input field containing '5'.
- Select Modifications:** A section with two sub-sections:
 - Fixed Modifications:** A list box containing 'Carbamidomethyl (C)'.
 - Variable Modifications:** A list box containing 'Acetyl (Protein N-term)', 'Oxidation (M)', and 'Succinyl'.

3. Navigate to the **DIA Analysis Identification** tab:

- set the *Precursor PEP Cutoff* to “1”
- set the *Precursor Qvalue Cutoff* to “0.01”
- set the *Protein Qvalue Cutoff (Experiment)* to “0.01”
- set the *Protein Qvalue Cutoff (Run)* to “1”.

<ul style="list-style-type: none"> ▲ BGS Factory Settings <ul style="list-style-type: none"> ▲ Pulsar Search <ul style="list-style-type: none"> Peptides Labeling Modifications Identification Tolerances Workflow Result Filters ▲ DIA Analysis <ul style="list-style-type: none"> Data Extraction XIC Extraction Calibration <li style="background-color: #e0e0e0;">Identification Quantification PTM Workflow Workflow Protein Inference Post Analysis Pipeline Mode 	<ul style="list-style-type: none"> Exclude Duplicate Assays <input checked="" type="checkbox"/> Generate Decoys <input checked="" type="checkbox"/> <ul style="list-style-type: none"> Decoy Method <input type="text" value="Mutated"/> Preferred Fragment Source <input type="text" value="NN Predicted Fragments"/> Decoy Limit Strategy <input type="text" value="Dynamic"/> Library Size Fraction <input type="text" value="0.1"/> Machine Learning <input type="text" value="Per Run"/> Precursor PEP Cutoff <input type="text" value="1"/> Precursor Qvalue Cutoff <input type="text" value="0.01"/> Protein Qvalue Cutoff (Experiment) <input type="text" value="0.01"/> Protein Qvalue Cutoff (Run) <input type="text" value="1"/> Single Hit Definition <input type="text" value="By Stripped Sequence"/> Exclude Single Hit Proteins <input type="checkbox"/> Pvalue Estimator <input type="text" value="Kernel Density Estimator"/>
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4. Select the **DIA Analysis Quantification** tab:

- set the *Minor (Peptide) Grouping* to “Modified sequence”
- set the *Major Group Quantity* to “Mean peptide quantity”
- set the *Major Group Max* to “3” and Min to “1”
- set *Minor Group Quantity* to “Mean precursor quantity”
- set the *Minor Group Max* to “3” and Min to “1”
- set the *Data Filtering* to “Qvalue sparse”
- set the *Imputing Strategy* to “No Imputing”
- ensure that the *Normalization Strategy* is unchecked.

<ul style="list-style-type: none"> BGS Factory Settings <ul style="list-style-type: none"> ▲ Pulsar Search <ul style="list-style-type: none"> Peptides Labeling Modifications Identification Tolerances Workflow Result Filters ▲ DIA Analysis <ul style="list-style-type: none"> Data Extraction XIC Extraction Calibration Identification <li style="background-color: #e0e0e0;">Quantification PTM Workflow Workflow Protein Inference Post Analysis Pipeline Mode 	<ul style="list-style-type: none"> Interference Correction <input checked="" type="checkbox"/> <ul style="list-style-type: none"> Only Identified Peptides <input checked="" type="checkbox"/> Exclude All Multi-Channel Interferences <input checked="" type="checkbox"/> MS1 Min <input type="text" value="2"/> MS2 Min <input type="text" value="3"/> Protein LFQ Method <input type="text" value="Automatic"/> Proteotypicity Filter <input type="text" value="None"/> Major (Protein) Grouping <input type="text" value="by Protein Group Id"/> Minor (Peptide) Grouping <input type="text" value="by Modified Sequence"/> Major Group Quantity <input type="text" value="Mean peptide quantity"/> Major Group Top N <input checked="" type="checkbox"/> <ul style="list-style-type: none"> Max <input type="text" value="3"/> Min <input type="text" value="1"/> Minor Group Quantity <input type="text" value="Mean precursor quantity"/> Minor Group Top N <input checked="" type="checkbox"/> <ul style="list-style-type: none"> Max <input type="text" value="3"/> Min <input type="text" value="1"/> Quantity MS-Level <input type="text" value="MS2"/> Quantity Type <input type="text" value="Area"/> Data Filtering <input type="text" value="Qvalue sparse"/> <ul style="list-style-type: none"> Imputing Strategy <input type="text" value="No Imputing"/> Cross Run Normalization <input type="checkbox"/>
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5. Click on the **DIA Analysis PTM Workflow** tab:

- enable *PTM Localization*
- set the *Probability Cutoff* to “0.75”.

BGS Factory Settings <ul style="list-style-type: none">▲ Pulsar Search<ul style="list-style-type: none">PeptidesLabelingModificationsIdentificationTolerancesWorkflowResult Filters▲ DIA Analysis<ul style="list-style-type: none">Data ExtractionXIC ExtractionCalibrationIdentificationQuantificationPTM WorkflowWorkflowProtein InferencePost AnalysisPipeline Mode	PTM Localization <ul style="list-style-type: none"><input checked="" type="checkbox"/>Probability Cutoff <input type="text" value="0.75"/>PTM Analysis <input checked="" type="checkbox"/>Flanking Region <input type="text" value="7"/>Multiplicity <input checked="" type="checkbox"/>PTM Consolidation <input type="text" value="Sum"/>Run Clustering <input type="checkbox"/>	
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6. Select the **DIA Analysis Workflow** tab

- set the *Profiling Strategy* to “None”.

BGS Factory Settings <ul style="list-style-type: none">▲ Pulsar Search<ul style="list-style-type: none">PeptidesLabelingModificationsIdentificationTolerancesWorkflowResult Filters▲ DIA Analysis<ul style="list-style-type: none">Data ExtractionXIC ExtractionCalibrationIdentificationQuantificationPTM WorkflowWorkflowProtein InferencePost AnalysisPipeline Mode	Method Evaluation <ul style="list-style-type: none"><input type="checkbox"/>MS2 DeMultiplexing <input type="text" value="Automatic"/>Profiling Strategy <input type="text" value="None"/>Run Limit for directDIA Library <input type="text" value="-1"/>Unify Peptide Peaks Strategy <input type="text" value="None"/>	
--	---	--

7. Navigate to the **DIA Analysis Post Analysis** tab:

- set the *Differential Abundance* Grouping to “Minor Group (Quantification Settings)”
- the *Smallest Quantitative Unit* should be set to “Precursor Ion (Quantification Settings)”
- set *Differential Abundance Testing* to “Paired t-test”
- ensure that *Group-Wise Testing Correction* is disabled.

The screenshot shows the BGS Factory Settings interface. On the left, a tree view shows the navigation menu with 'DIA Analysis' expanded and 'Post Analysis' selected. The main panel is divided into two columns. The left column contains the following settings: Calculate Explained TIC, Calculate Sample Correlation Matrix, Differential Abundance Grouping (set to 'Minor Group (Quantification Settings)'), Smallest Quantitative Unit (set to 'Precursor Ion (Quantification Settings)'), Use All MS-Level Quantities (unchecked), Differential Abundance Testing (set to 'Paired t-test'), Group-Wise Testing Correction (unchecked), Run Clustering (checked), Distance Metric (set to 'Manhattan Distance'), Linkage Strategy (set to 'Ward's Method'), Order Runs by Clustering (checked), and Z-score transformation (unchecked). The right column contains a dropdown menu with 'None' selected.

8. Click on the **DIA Analysis Pipeline Mode** tab:

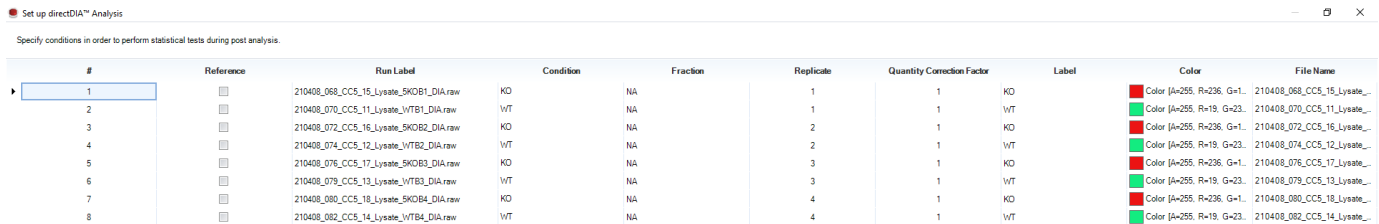
- enable *Generate SNE File*
- select the appropriate *Report Schema* as described in **Chapter 4. Data Reporting:** “Peptide_Quant_Pivot” and “PTM_Localization”.

The screenshot shows the BGS Factory Settings interface with the 'DIA Analysis Pipeline Mode' tab selected. The left navigation menu is the same as in the previous screenshot. The main panel is divided into two columns. The left column contains the following settings: Generate SNE File (checked), Store Iontraces in SNE (checked), Post Analysis Reports (CV Density Line Chart, CVs Below X Bar Chart, Data Completeness Bar Chart, Run Identifications Bar Chart, Scoring Histograms), Report Schema (with a list of schemas including 'BGS Factory Report V2 (Normal)', 'Birgit_Peptide Quant_Pivot (Pivot)', 'Birgit_Protein Quant_Pivot (Pivot)', 'CDK_Report_GQ_ModSeqID (Normal)', 'Christina_Report (Normal)', 'Joanna_ModifPeptQuant_Pivot (Pivot)', 'MSSStats Report (v 3.7.3) (Normal)', 'Peptide Quant (Normal)', 'Peptide Quant (Pivot)', 'Protein Quant (Normal)', 'Protein Quant (Pivot)', 'PTMSiteReport (Normal)', 'PTMSiteReport (Pivot)', and 'BGS Factory Report (Normal)' where 'BGS Factory Report (Normal)' is checked), and Reporting Unit (set to 'Across Experiment').

9. Go to **Chapter 3.3** to complete modifying search settings.

Chapter 3.3. directDIA Search Settings for Protein Lysate and Post Translational Modifications

1. Set the conditions for each experimental vendor specific file or *.htms file included in the experiment.



Set up directDIA™ Analysis

Specify conditions in order to perform statistical tests during post analysis.

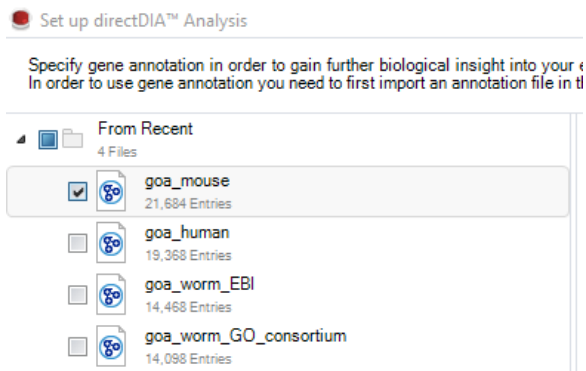
#	Reference	Run Label	Condition	Fraction	Replicate	Quantity Correction Factor	Label	Color	File Name
1	<input type="checkbox"/>	210408_068_CCS_15_Lysate_SKDB1_DIA.raw	KD	NA	1	1	KD	Color [A=255, R=236, G=1...	210408_068_CCS_15_Lysate...
2	<input type="checkbox"/>	210408_070_CCS_11_Lysate_VTB1_DIA.raw	WT	NA	1	1	WT	Color [A=255, R=19, G=23...	210408_070_CCS_11_Lysate...
3	<input type="checkbox"/>	210408_072_CCS_16_Lysate_SKDB2_DIA.raw	KD	NA	2	1	KD	Color [A=255, R=236, G=1...	210408_072_CCS_16_Lysate...
4	<input type="checkbox"/>	210408_074_CCS_12_Lysate_VTB2_DIA.raw	WT	NA	2	1	WT	Color [A=255, R=19, G=23...	210408_074_CCS_12_Lysate...
5	<input type="checkbox"/>	210408_076_CCS_17_Lysate_SKDB3_DIA.raw	KD	NA	3	1	KD	Color [A=255, R=236, G=1...	210408_076_CCS_17_Lysate...
6	<input type="checkbox"/>	210408_078_CCS_13_Lysate_VTB3_DIA.raw	WT	NA	3	1	WT	Color [A=255, R=19, G=23...	210408_078_CCS_13_Lysate...
7	<input type="checkbox"/>	210408_080_CCS_18_Lysate_SKDB4_DIA.raw	KD	NA	4	1	KD	Color [A=255, R=236, G=1...	210408_080_CCS_18_Lysate...
8	<input type="checkbox"/>	210408_082_CCS_14_Lysate_VTB4_DIA.raw	WT	NA	4	1	WT	Color [A=255, R=19, G=23...	210408_082_CCS_14_Lysate...

2. Click *Export Condition Setup*, name the file, and click **Save**.

3. Click **Next**.

4. Select the appropriate **Gene Ontology Annotations** for the experiment.

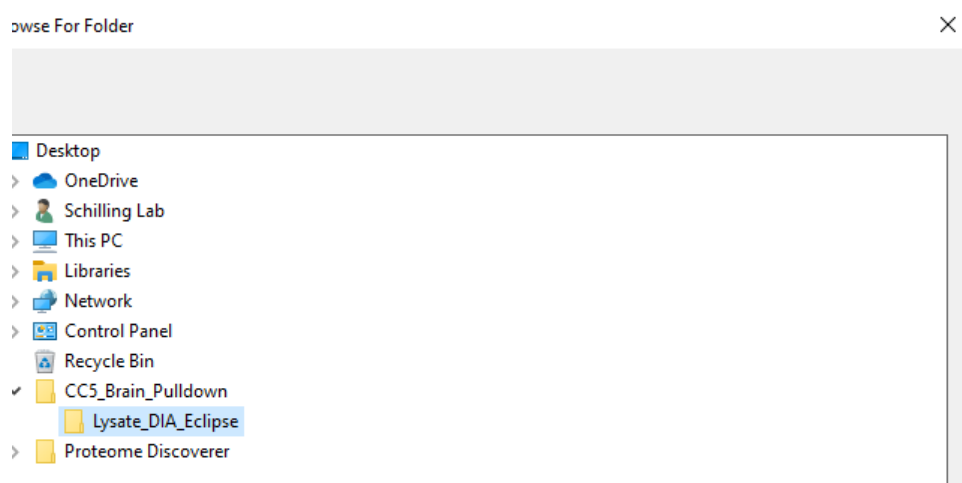
Note: In this study, the file “Mus musculus (GO Annotations Uniprot)” is used.



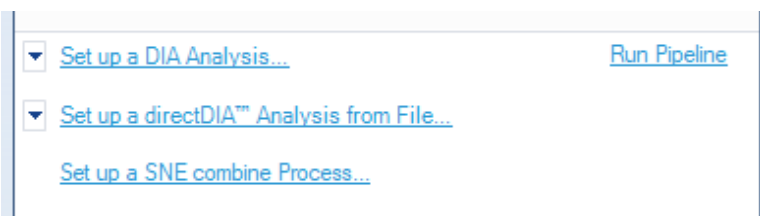
5. Click **Next**.

6. Click **Browse**:

- select the appropriate **Output Directory** to save the sne. file and export the reports, plots, and candidate protein files.



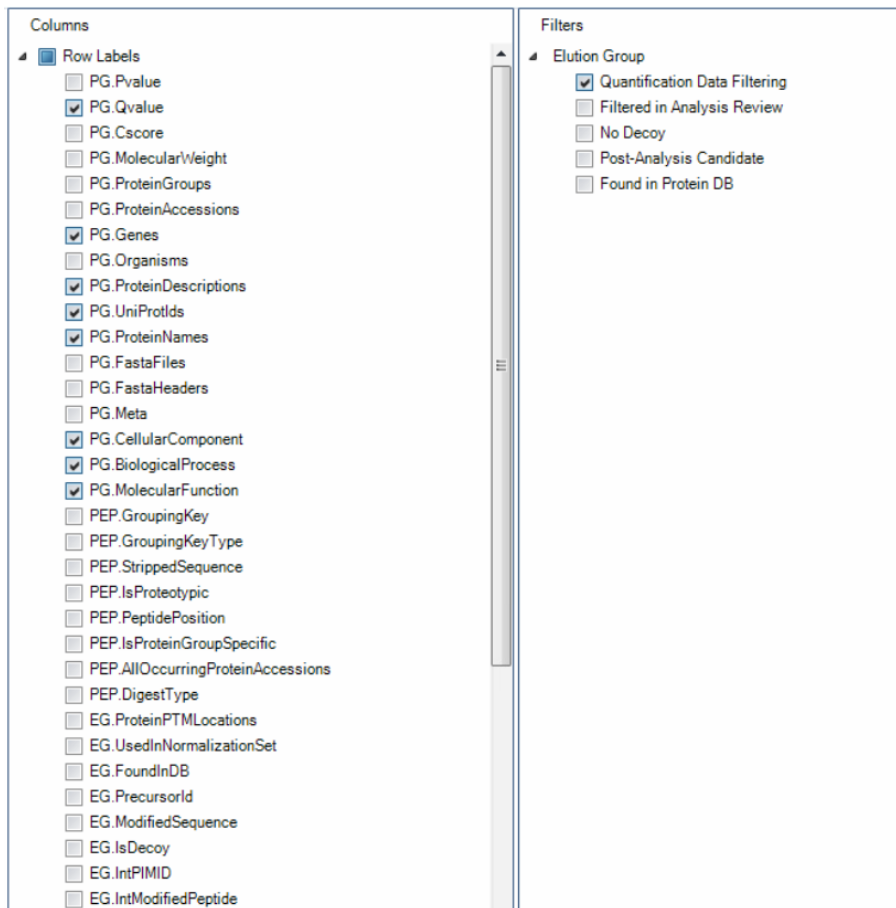
7. Click **Ok**.
8. Click **Finish** to queue the analysis.
9. Click **Run Pipeline** to start the analysis queue.



Chapter 4. Data Reporting

Chapter 4.1. Protein Quantification Report

1. Navigate to the **Report** tab to set up a custom report.
2. Select the **BGS Factory Report** under the **Run Pivot Report** dropdown to create a custom Protein Quantification Pivot Report, "Protein_Quant_Pivot".
3. Select the following **Row Labels**:
 - PG.Qvalue
 - PG.Genes
 - PG.ProteinDescriptions
 - PG.UniProtIDs
 - PG.ProteinNames
 - PG.CellularComponent
 - PG.BiologicalProcess
 - PG.MolecularFunction.



4. Select the following **Cell Values**:

- PG.NrOFFprecursorsIdentified
- PG.NrOFFprecursorsUsedForQuantification
- PG.Quantity.

The screenshot displays a software interface with two main panels: 'Columns' on the left and 'Filters' on the right. The 'Columns' panel is expanded to show a list of options under the 'Cell Values' category. The following items are checked with blue checkmarks: 'PG.NrOFFprecursorsIdentified', 'PG.NrOFFprecursorsUsedForQuantification', and 'PG.Quantity'. The 'Filters' panel is expanded to show the 'Elution Group' category, where 'Quantification Data Filtering' is checked with a blue checkmark. Other filter options like 'Filtered in Analysis Review', 'No Decoy', 'Post-Analysis Candidate', and 'Found in Protein DB' are unchecked.

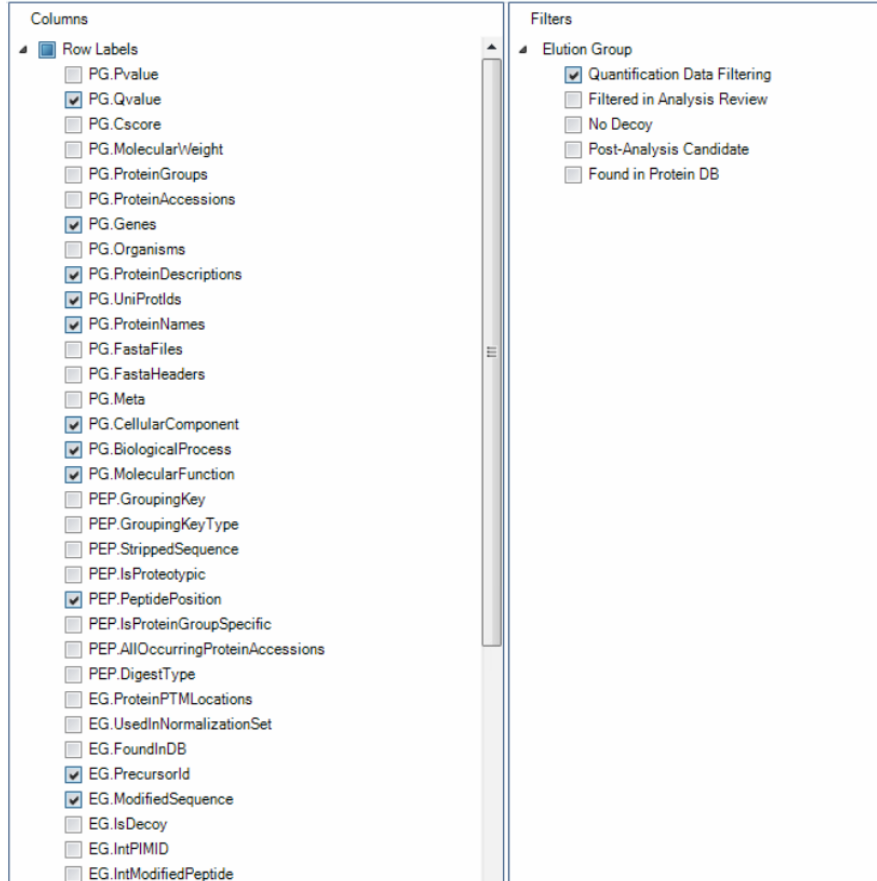
5. Select the following **Filters**:

- Quantification Data Filtering.

6. Select **Save As** and save the report scheme as "Protein_Quant_Pivot".

Chapter 4.2. Peptide Quantification Report

1. Navigate to the **Report** tab to set up a custom report.
2. Select the **BGS Factory Report** under the **Run Pivot Report** dropdown to create a custom Peptide Quantification Pivot Report, "Peptide_Quant_Pivot".
3. Select the following **Row Labels**:
 - PG.Qvalue
 - PG.Genes
 - PG.ProteinDescriptions
 - PG.UniProtIDs
 - PG.ProteinNames
 - PG.CellularComponent
 - PG.BiologicalProcess
 - PG.MolecularFunction
 - PEP.PeptidePosition
 - EG.PrecursorId
 - EG.ModifiedSequence.



4. Select the following **Cell Values**:

- PEP.MS2Quantity
- EG.PTMProbabilities
- EG.PTMSites
- EG.TotalQuantity (Settings).

The screenshot displays a software interface with two main panels: 'Columns' on the left and 'Filters' on the right. The 'Columns' panel lists various data fields, with several checked. The 'Filters' panel shows the 'Elution Group' filter with 'Quantification Data Filtering' checked.

Columns:

- EG.IntModifiedPeptide
- Cell Values
- PG.ManuallyAccepted
- PG.Cscore (Run-Wise)
- PG.QValue (Run-Wise)
- PG.PValue (Run-Wise)
- PG.IsIdentified
- PG.RunEvidenceCount
- PG.NrOfStrippedSequencesMeasured
- PG.NrOfModifiedSequencesMeasured
- PG.NrOfPrecursorsMeasured
- PG.NrOfStrippedSequencesIdentified
- PG.NrOfModifiedSequencesIdentified
- PG.NrOfPrecursorsIdentified
- PG.IsSingleHit
- PG.NrOfStrippedSequencesUsedForQuantification
- PG.NrOfModifiedSequencesUsedForQuantification
- PG.NrOfPrecursorsUsedForQuantification
- PG.Quantity
- PG.MS1Quantity
- PG.MS2Quantity
- PG.MS1ChannelQuantities
- PG.MS2ChannelQuantities
- PG.IBAQ
- PEP.RunEvidenceCount
- PEP.Quantity
- PEP.MS1Quantity
- PEP.MS2Quantity
- PEP.MS1ChannelQuantities
- PEP.MS2ChannelQuantities
- EG.MeanApexRT
- EG.ApexRT
- EG.PTMLocalizationProbabilities
- EG.PTMAssayProbability
- EG.PTMAssayCandidateScore
- EG.PTMPositions
- EG.PTMProbabilities
- EG.PTMSites
- EG.Qvalue
- EG.SignalToNoise
- EG.NormalizationFactor
- EG.IsImputed
- EG.TargetReferenceRatio (Settings)
- EG.TargetQuantity (Settings)
- EG.ReferenceQuantity (Settings)
- EG.TotalQuantity (Settings)
- FG.ReporterIons

Filters:

- Elution Group
 - Quantification Data Filtering
 - Filtered in Analysis Review
 - No Decoy
 - Post-Analysis Candidate
 - Found in Protein DB

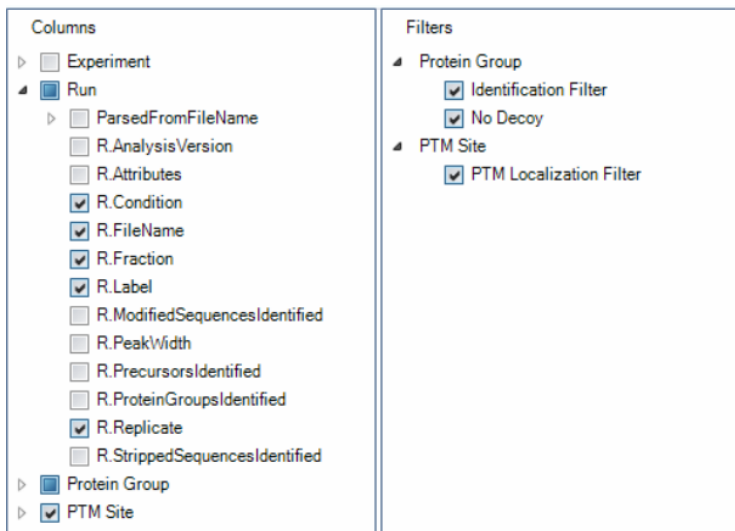
5. Select the following **Filters**:

- Quantification Data Filtering

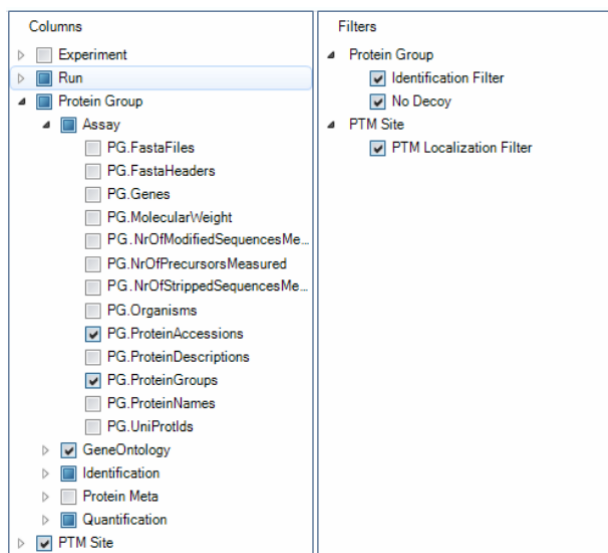
6. Select **Save As** and save the report scheme as "Peptide_Quant_Pivot".

Chapter 4.3. PTM Site Localization Report

1. Navigate to the **Report** tab to set up a custom report.
2. Select the **BGS Factory Report (default)** under the **PTM Site Report** tab and **Normal Report** dropdown to create a custom PTM Site Localization Report, "PTM_Localization".
3. Select the following **Run** settings:
 - R.Condition
 - R.FileName
 - R.Fraction
 - R.Label
 - R.Replicate.

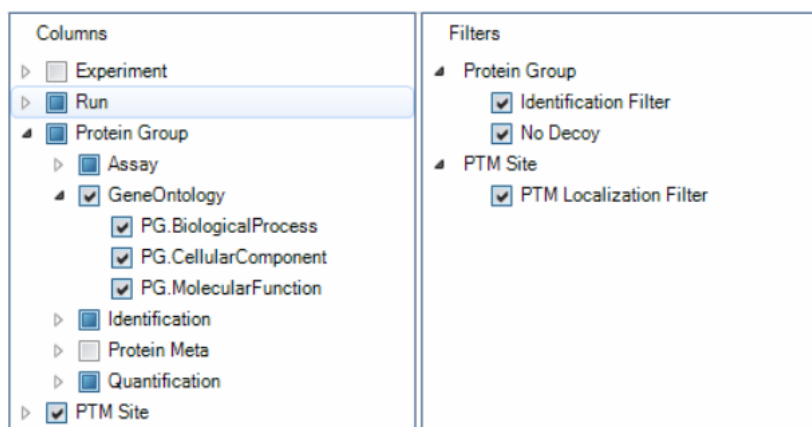


4. Select the following **Protein Group Assay** settings:
 - PG.ProteinAccessions
 - PG.ProteinGroups.



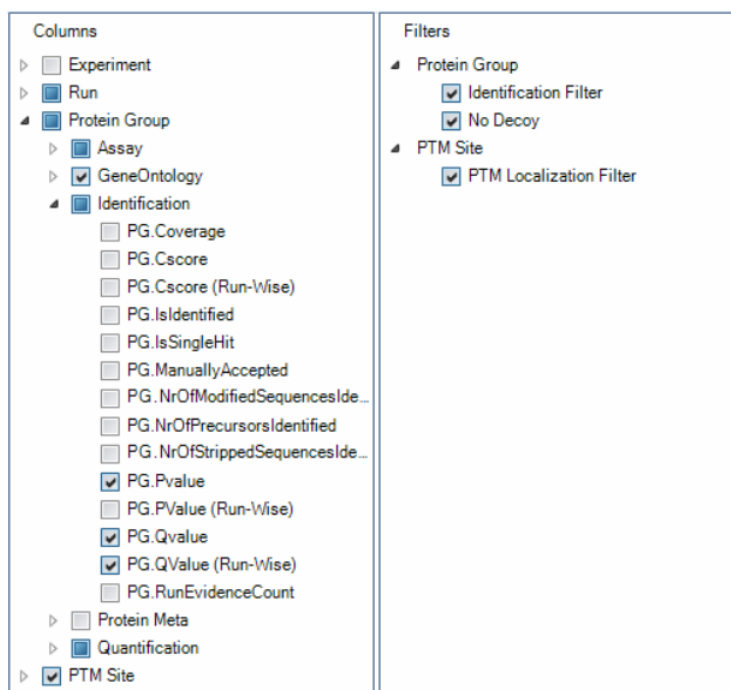
5. Select the following **Protein Group Gene Ontology** settings:

- PG.BiologicalProcess
- PG.CellularComponent
- PG.MolecularFunction.



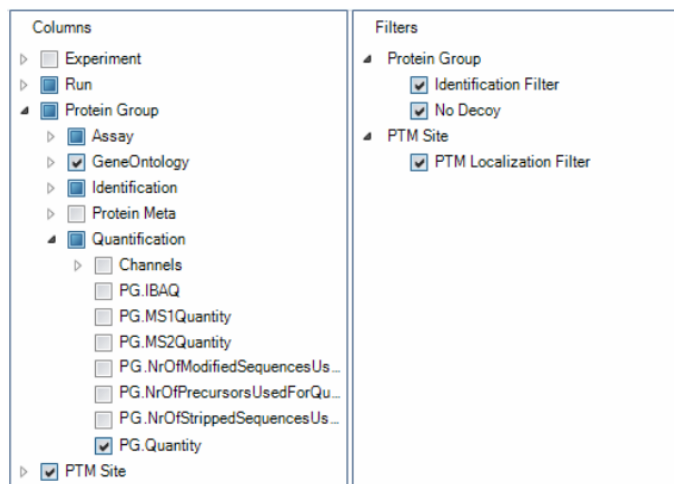
6. Select the following under **Protein Group Identification** settings:

- PG.Pvalue
- PG.Qvalue
- PG.Qvalue (Run-Wise).



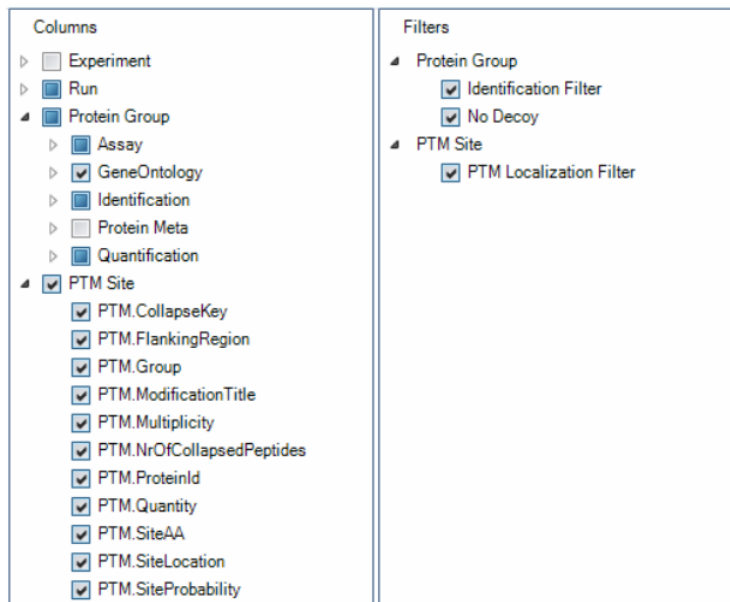
7. Select the following under **Protein Group Quantification** settings:

- PG.Quantity.



8. Select the following under **PTM Site** settings:

- PTM.CollapseKey
- PTM.FlankingRegion
- PTM.Group
- PTM.ModificationTitle
- PT.Multiplicity
- PTM.NrOFCollapsedPeptides
- PTM.ProteinId
- PTM.Quantity
- PTM.SiteAA
- PTM.SiteLocalization
- PTM.SiteProbability



9. Select the following **Filters** settings:

- Identification Filter
- No Decoys
- PTM Localization Filter.

10. Select **Save As** and save the report scheme as “PTM_Localization”.