Peptide Mapping

Biologics Explorer 2.0 Quick Guide

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Peptide Mapping: Biologics Explorer Quick Guide

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Part A

Software and Workflows

1. OVERVIEW OF APPLICATIONS

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Overview of Applications for Peptide Mapping Workflows

- These workflows are primarily designed for peptide mapping analyses of enzymatically digested biotherapeutic molecules:
  - Sequence coverage and confirmation
  - Glycopeptide analysis
  - Post-translational modification (PTM) analysis
  - Target PTM profiling
  - Disulfide-bond (DSB) analysis
  - Conjugate analysis
  - Sequence variant analysis (SVA)
- Batch analysis of replicate samples of the same molecule is possible:
  - In-depth characterization
  - Comparison of multiple samples: Process development, instrument method development
  - Stress tests
  - Reduced *vs.* non-reduced sample comparisons

Part A

Software and Workflows

2. USING BIOLOGICS EXPLORER

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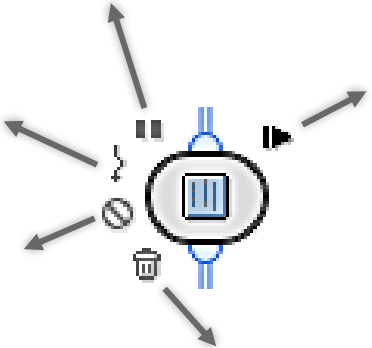
Using Biologics Explorer

ACTIVITY NODE ICONS

Pause: Pause the workflow here.
All subsequent tasks remain active.

Bypass: Skip this task
when running the workflow.



Run
or **Reset**.

Block: Stop the workflow.
This and all subsequent tasks become unavailable (gray).

Trash: Do not save intermediate data.
When this icon is activated, the results for this particular activity node cannot be viewed.
Using the Trash icon helps to save memory. Use this feature after workflow settings have been optimized.

Using Biologics Explorer

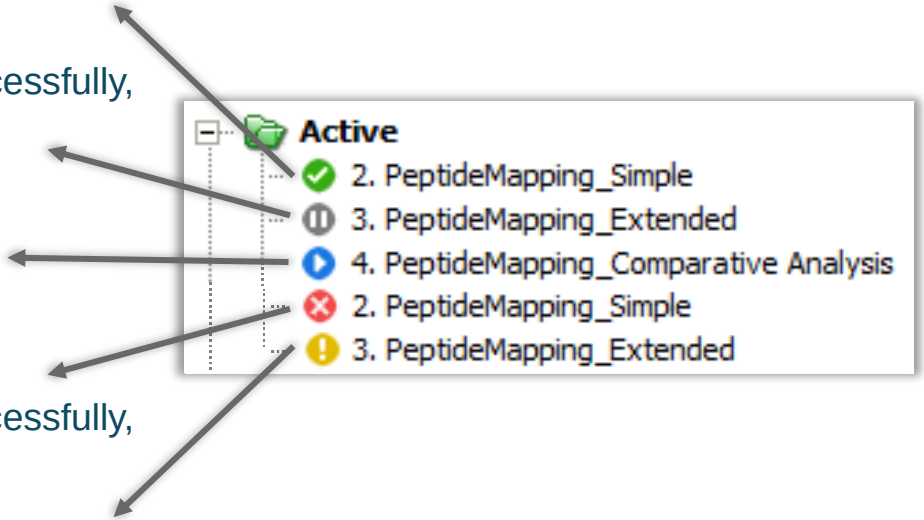
WORKFLOW ICONS

Workflow Completed
All activity nodes have completed successfully.

Workflow Paused
Some activity nodes have been completed successfully, but some have not yet started.

Workflow Ready
No activity nodes have been completed. The workflow is ready to start.

Workflow Error
Some activity nodes have been completed successfully, but at least one activity node cannot run.



Workflow Warning
Some activity nodes are incomplete.

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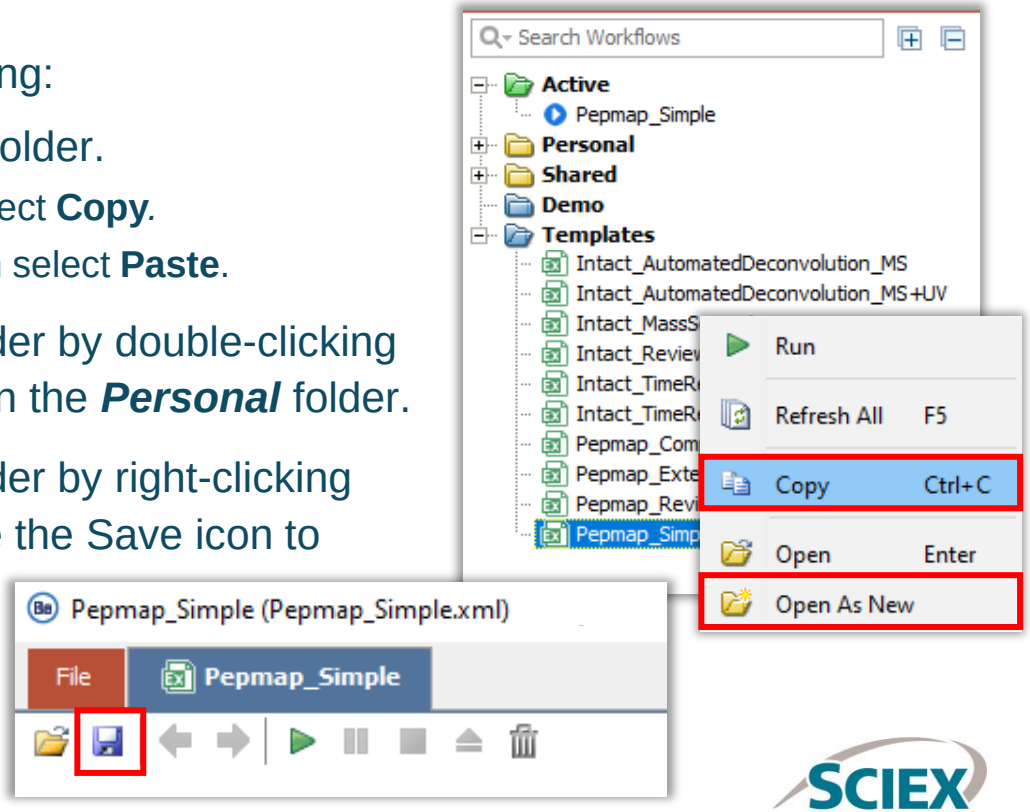
Using Biologics Explorer: General Overview

START AND SAVE WORKFLOWS

To open a workflow, do one of the following:

- Copy a workflow from the ***Templates*** folder.
  1. Right-click on the workflow and then select **Copy**.
  2. Right-click the ***Personal*** folder and then select **Paste**.
- Open a workflow in the ***Templates*** folder by double-clicking and then use the **Save** icon to save it in the ***Personal*** folder.
- Open a workflow in the ***Templates*** folder by right-clicking and then selecting **Open As New**. Use the Save icon to save it in the ***Personal*** folder.



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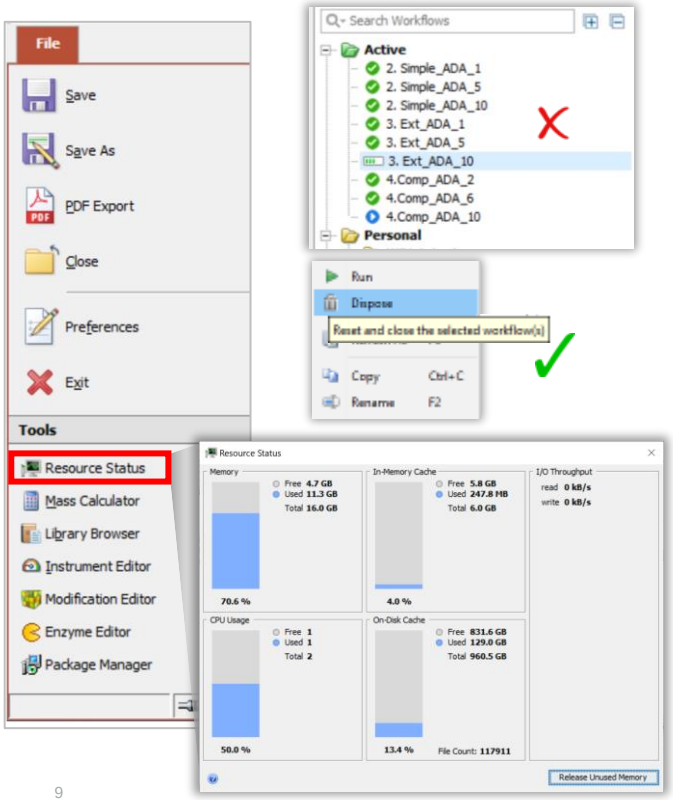
Using Biologics Explorer: General Overview

RECOMMENDATIONS FOR CORRECT USE OF THE RESOURCES



- Follow best practices to make sure that Biologics Explorer has sufficient memory and computing power:
  - Only run one workflow at a time: Some activity nodes are very resource intensive. Co-processing might use up all available resources.
  - To conserve memory, activate the Trash icon whenever possible in optimized workflows.
  - After reviewing data and saving results, reset or dispose workflows before starting a new analysis.
  - Use *Save Snapshot* activity nodes to enable completed results to be saved or reviewed in the Pepmap_ReviewSnapshots workflow.
- The processing computer should have at least 250 GB of free disk space and 6 GB of In-Memory Cache.
  - Files being processed for peptide mapping workflows should not add up to more than 4 GB.

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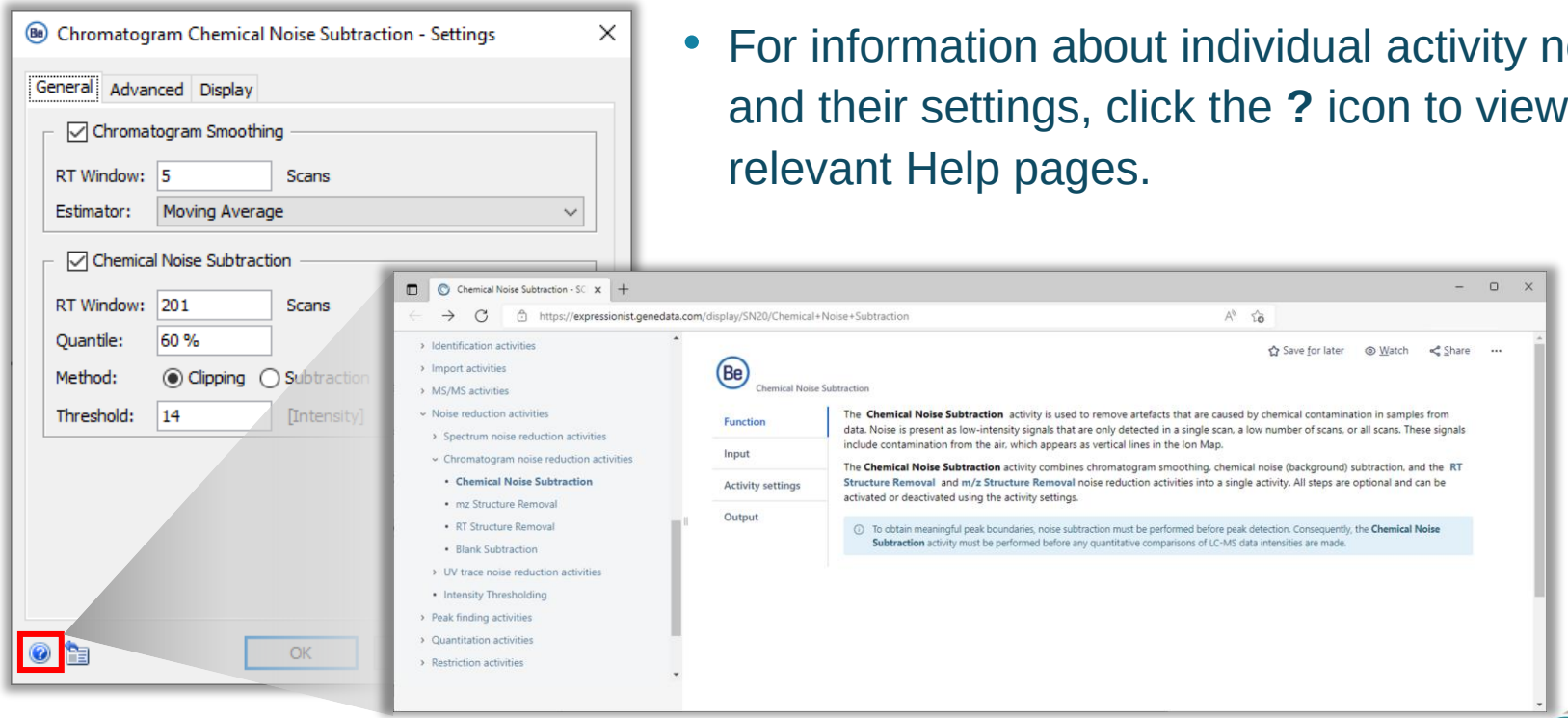
Using Biologics Explorer: General Overview

ACCESS THE ONLINE HELP



- For information about individual activity nodes and their settings, click the **?** icon to view the relevant Help pages.

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Part A

Software and Workflows

3. GENERAL GUIDELINES FOR PEPTIDE MAPPING WORKFLOWS

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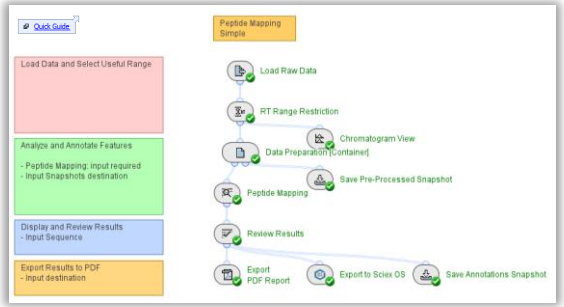
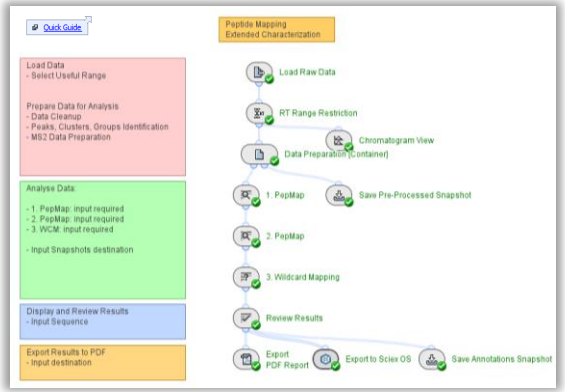
General Peptide Mapping Workflow Guidelines

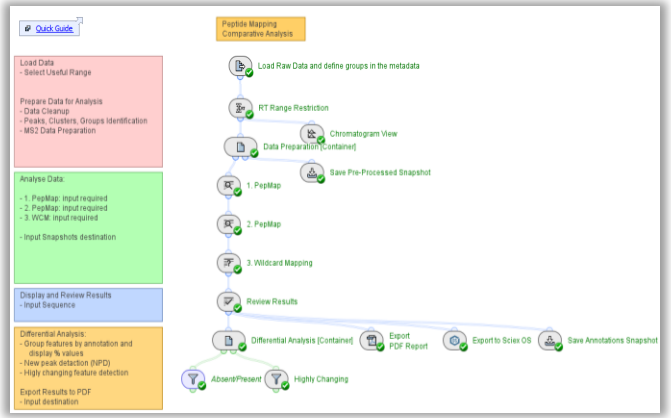
WORKFLOW TYPES



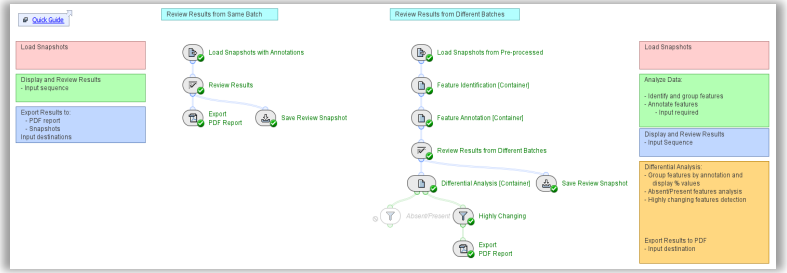
Pepmap_Simple



Pepmap_Extended



Pepmap_Comparative



Pepmap_ReviewSnapshots

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General Peptide Mapping Workflow Guidelines

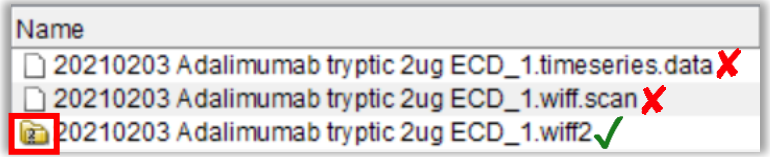
COMMON ACTIVITY NODES IN THE PEPTIDE MAPPING WORKFLOWS

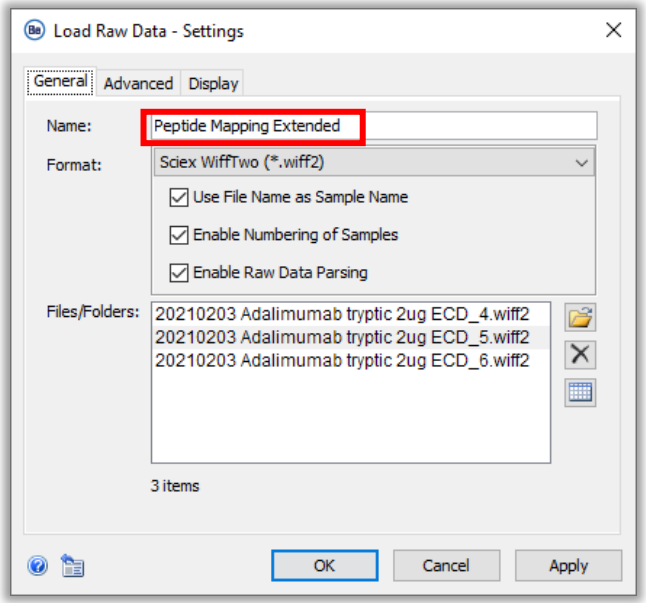
A. *Load Raw Data*
B. *RT Range Restriction*
C. *Chromatogram View*
D. *Data Preparation [Container]*
   i. *Chromatogram Chemical Noise Subtraction*
   ii. *Chromatogram RT Alignment*
   iii. *Chromatogram Peak Detection*
   iv. *Chromatogram Isotope Clustering*
   v. *Singleton Filter*
   vi. *Charge and Adduct Grouping*
   vii. *MS/MS Consolidation*
   viii. *MS/MS Peak Detection*
   ix. *MS/MS Deisotoping*
E. *Peptide Mapping*
F. *Review Results*
G. Reporting and Exporting

Load Raw Data: Add Analysis Name and Data Files

General tab.

- Type into the **Name** field to define the analysis.
- Upload raw data files:
  - Select only wiff or wiff2 container files.
    - When analyzing data from the ZenoTOF 7600 system, use only wiff2 files and not wiff files.
  - Do not select the auxiliary files with the same name.

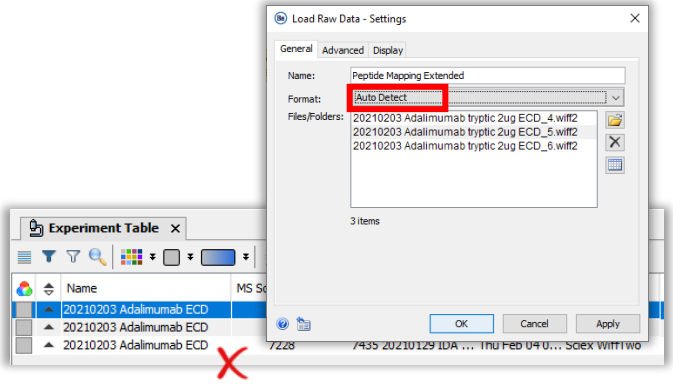


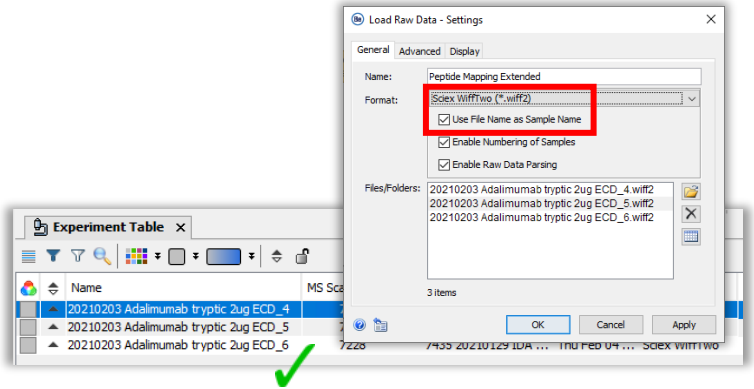


  - To view files within a wiff1 or wiff2 container, double-click the wiff or wiff2 container to open it.
    - Choose the files to upload from the list of embedded files.

Load Raw Data: Format

- If individual sample files within a wiff or wiff2 container have the same name, then do not use the **Auto Detect** option.
- To make sure that unique sample names are present in the *Experiment Table*, and that *Review Results* displays the correct quantitative information for each sample:
  1. From the **Format** dropdown list select either **Sciex Wiff** or **Sciex WiffTwo**.
     - Only use wiff2 for data acquired using the ZenoTOF 7600 system.
  2. Select the **Use File Name as Sample Name** check box.





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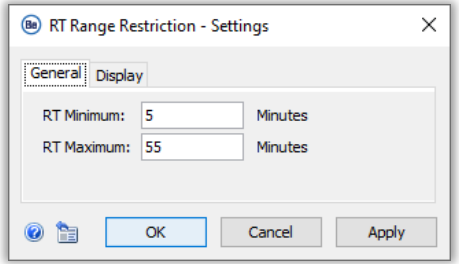
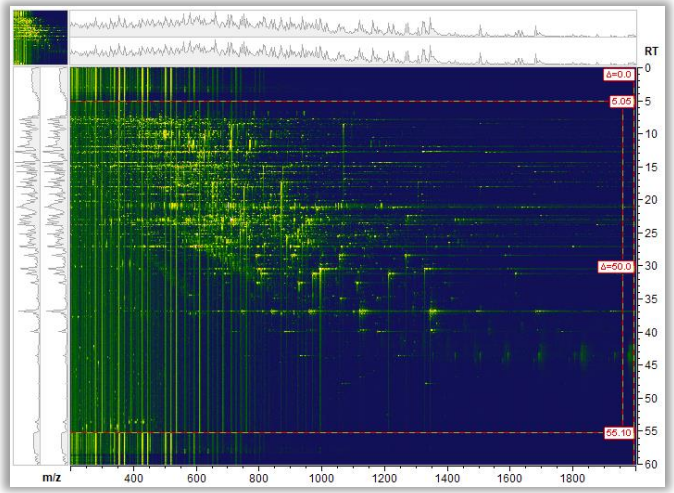
Restrict the RT Range

- Run the *Load Raw Data* activity node and then open (double-click) it when data loading is complete.
- Identify the retention time (RT) range where there is meaningful data present.
  - Exclude stray signals caused by valve switching or column wash.
  - Focus on the separation range.

Note: If the fields are blank, then the full RT range is used.





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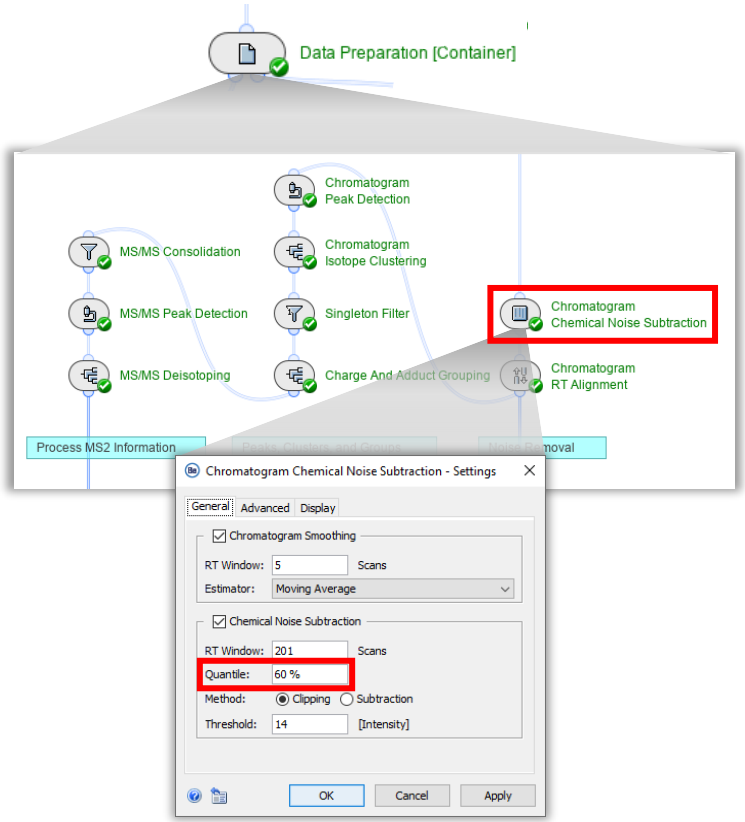
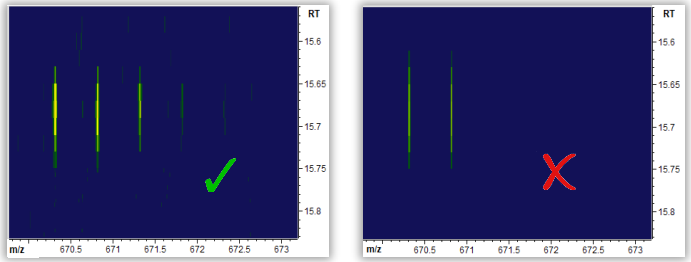
Data Preparation: Chemical Noise Subtraction - Quantile



- Change this setting only if the default noise removal is too harsh, which can be identified by:
  - Loss of low intensity isotope peaks from singly (+1) or doubly (+2) charged clusters, or from low intensity clusters of interest.
  - Excessive cutoff of the tails of very wide (extended RT) peaks.



- If clusters of interest are affected, or if the unwanted peak modifications that are described above appear too frequently, then set the **Quantile** to a lower value, such as 50%.

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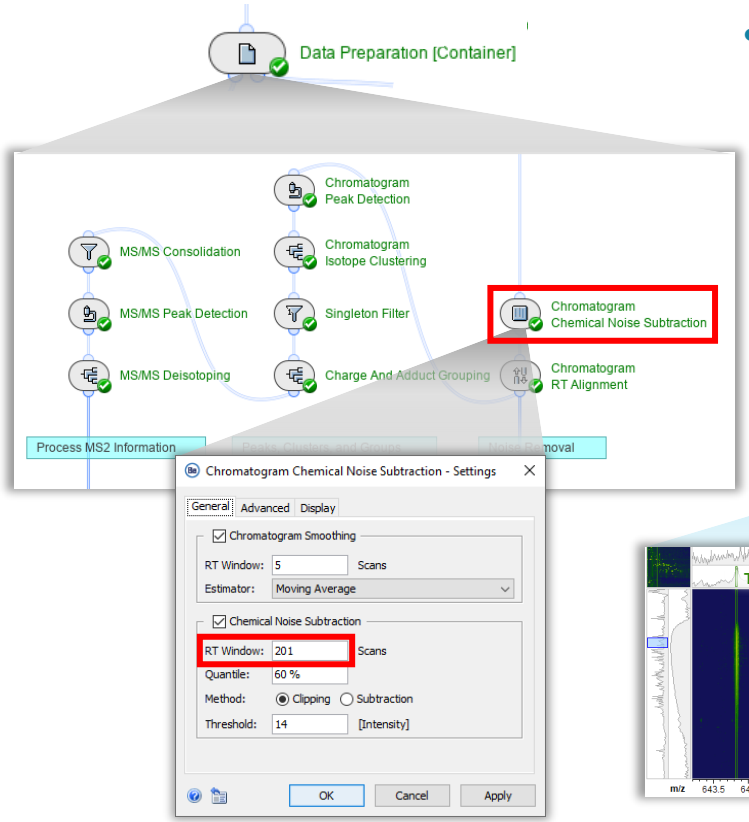
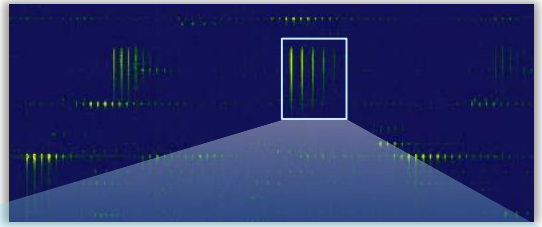
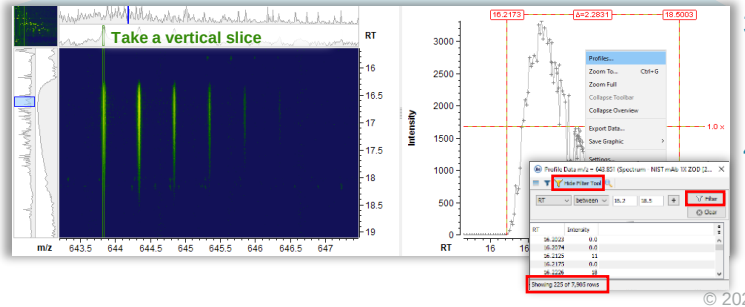
Data Preparation: Chemical Noise Subtraction - RT Window



- If the largest peak in the dataset contains less than 50 scans, then decrease the **RT Window** (to 101 or 151 scans, for example).
  - As a rule of thumb, the **RT Window** should be at least double the number of scans across the largest peak in the dataset.

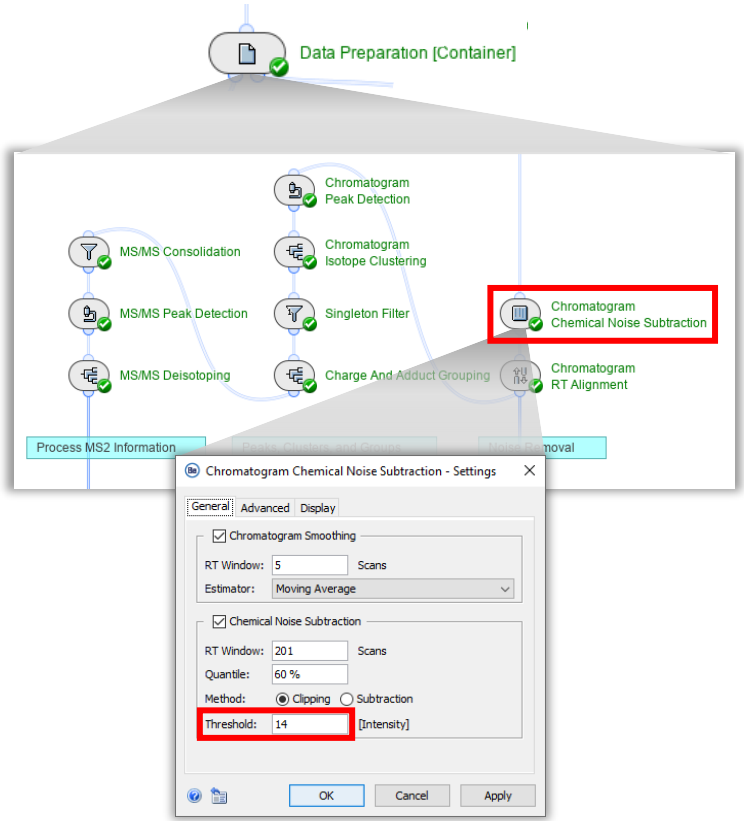




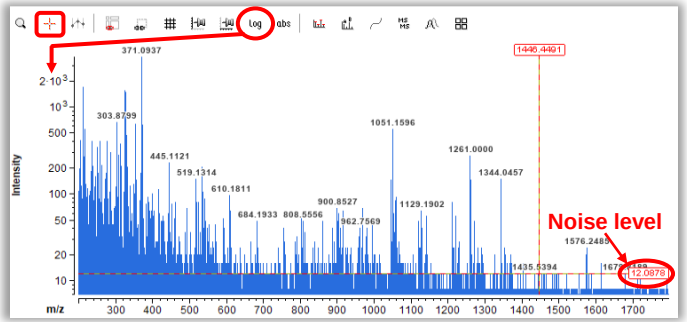
To determine the number of scans:

1. Locate a feature that extends over a longer RT than other features in the ion map.
2. Take a vertical slice to generate an Extracted Ion Chromatogram.
3. Right-click in the Extracted Ion Chromatogram window and select **Profiles**.
4. Use the **Advanced Filter Tool** to select the RT range for the peak.

Data Preparation: Chemical Noise Subtraction - Threshold



- If the noise level is significantly different from the **Threshold** value pre-set in the *Chromatogram Chemical Noise Subtraction* activity, then change this setting.
- To measure the noise level and determine an appropriate **Threshold** intensity value:
  1. Expand the mass spectrum intensity axis by dragging it until the noise level is readable, or change the axis from linear to the log scale using the icon in the tool bar.
  2. Use the crosshair tool to measure the intensity of the noise level.



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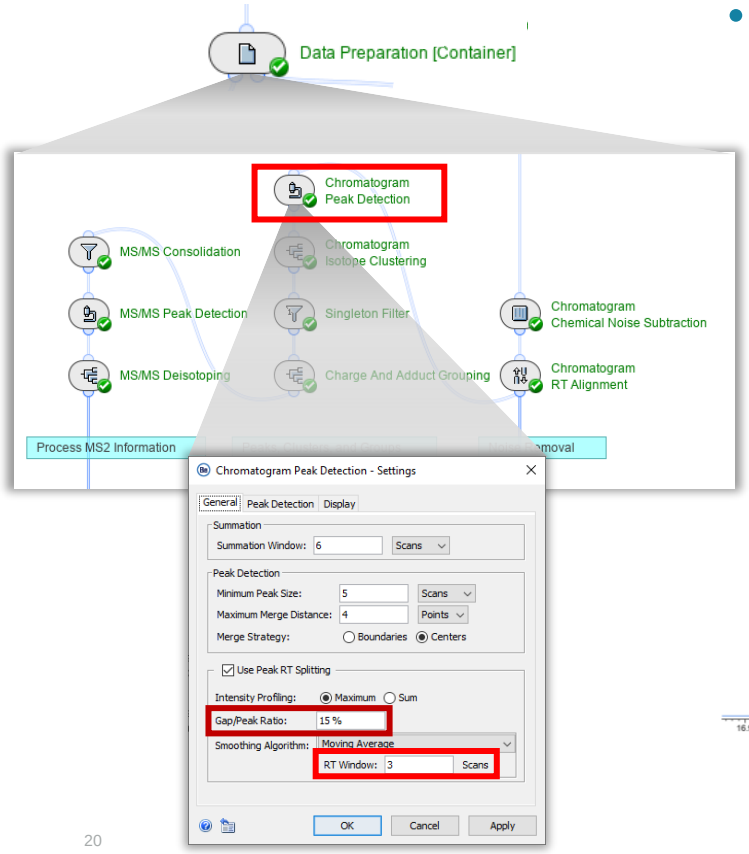
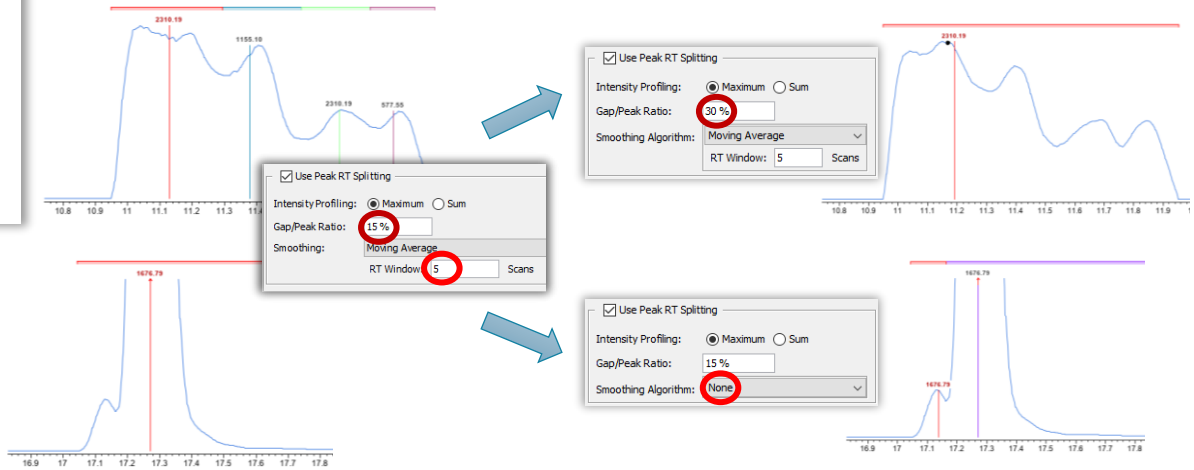
Data Preparation: *Chromatogram Peak Detection*



- The peak splitting of closely eluting components in the RT direction can be modified as required.
  - To decrease splitting: Increase the **Gap/Peak Ratio**.
  - To increase split sensitivity: Decrease or remove **Smoothing**.



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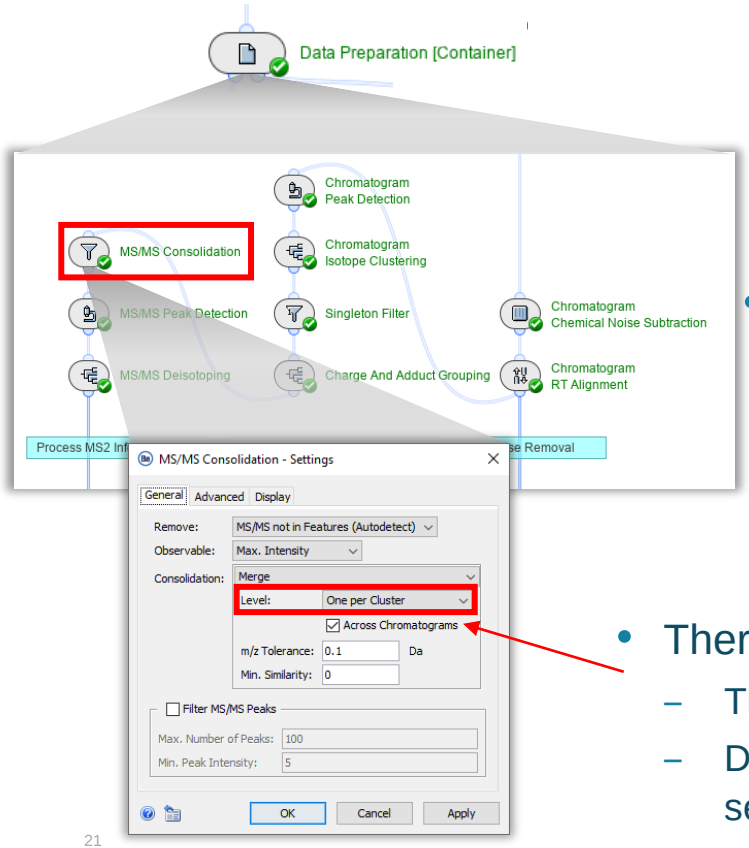
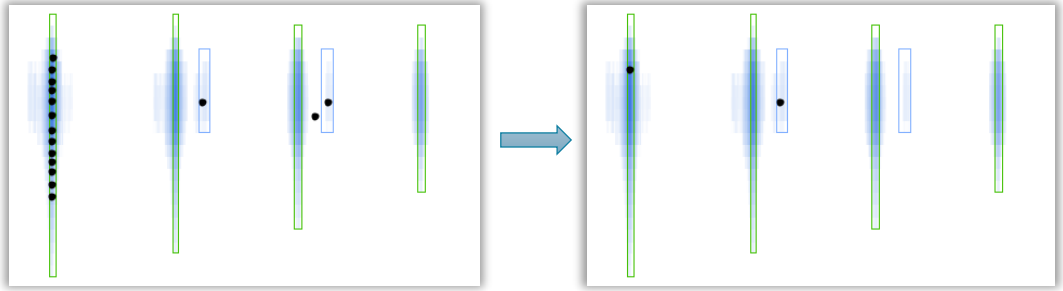
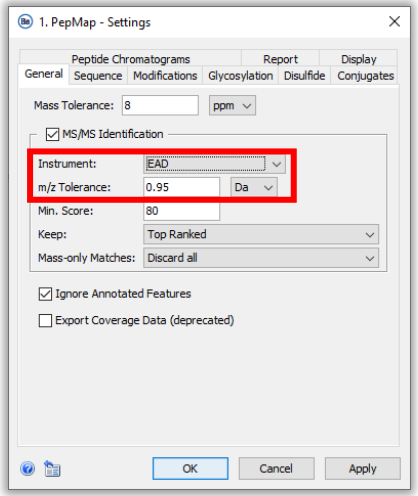
Data Preparation: *MS/MS Consolidation*





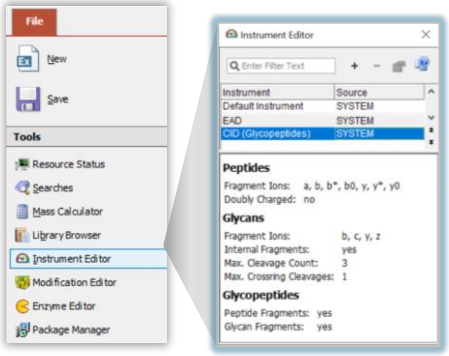
- This activity node merges MS/MS data across equivalent peaks and clusters.
  - Consolidation can improve MS/MS spectra, resulting in more identifications.
  - Consolidation can reduce false positives if MS/MS spectra are too ambiguous.
- There is an option to merge MS/MS **Across Chromatograms.**
  - This improves confidence in identifications across technical replicates.
  - Do not use this option when assessing individual sample sequence coverage.

Peptide Mapping: Configure Settings (1)



General tab:

- **Instrument**: Select according to specific experimental set-up.
  - To review or modify: Navigate to **File** > **Tools** > **Instrument Editor**.



- **m/z Tolerance**:
  - The default value (0.95 Da) is not a reflection of MS mass accuracy. It increases the possible options for identification as a compromise for the potential impact of MS/MS pre-processing on the *m/z*.
    - Reduce the **m/z Tolerance** to 20 ppm when data generated using the ZenoTOF 7600 system is being analysed. The **m/z Tolerance** can also be reduced to 20 ppm for analysis of data generated using other MS systems.
    - Reducing the **m/z Tolerance** limits the number of false positives or ambiguous annotations that are generated.
  - The default value can be increased if required, for example, if the data has wide error distributions.

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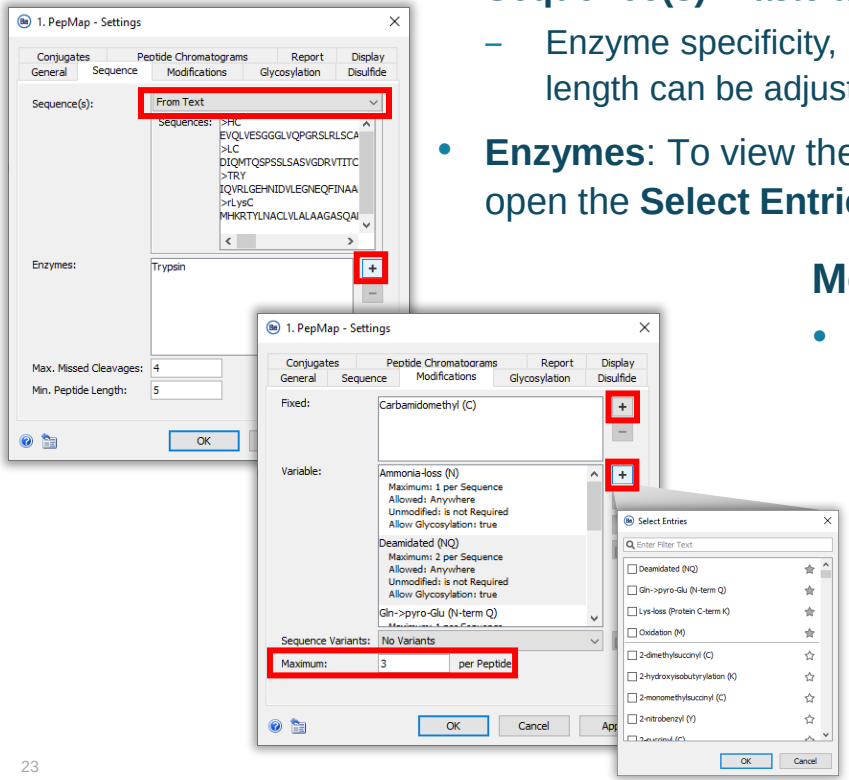
Peptide Mapping: Configure Settings (2)



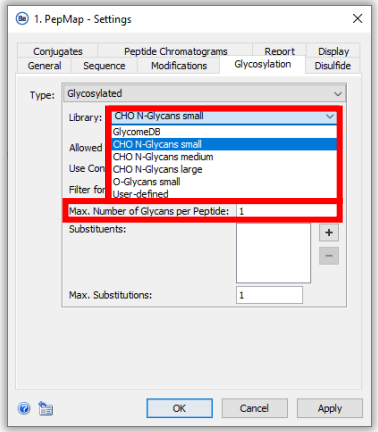
Sequence tab:

- **Sequence(s)**: Paste as text or upload as a FASTA file.
  - Enzyme specificity, maximum number of missed cleavages, and minimum peptide length can be adjusted as required.
- **Enzymes**: To view the list of system-configured and user-defined enzymes, open the **Select Entries** dialog using the + on the left.

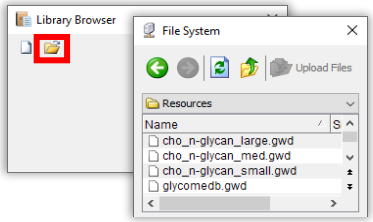
Modifications tab:

- View the list of possible **Fixed** or **Variable** modifications by opening the **Select Entries** dialog using the + on the left.
  - Commonly used modifications can be added as favorites by selecting the star icon.
  - To analyze over- or under-alkylation, set the alkylating reagent to **Variable** for cysteine and other target amino acids.

Peptide Mapping: Configure Settings (3)



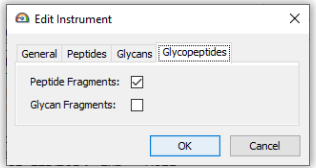
Glycosylation tab:



- **Library**: Select a system-configured or a user-defined library.
  - To review or modify a glycan library: Navigate to **File** > **Tools** > **Library Browser** > **Resources**.
- **Max. Number of Glycans per Peptide**: There is a threshold number of Estimated Glycopeptide Candidates allowed for the search to proceed (refer to next page for details).
  - **Allowed Sites: Only N-linked**: There is typically fewer potential consensus sequences per peptide, so searches for *N*-glycosylation are generally more tolerant of search criteria:
    - Up to 4 *N*-glycans per peptide is the maximum allowed value.
    - The number of missed cleavages and variable modifications impacts the search time.
  - **Allowed Sites: Only O-linked:** Every serine and threonine (S and T) residue is a potential site for *O*-glycosylation.
    - Long peptides containing many potential glycosylation sites dramatically impact the number of Estimated Glycopeptide Candidates, and the subsequent processing time.
    - Using enzymes that result in shorter peptides, for example using Trypsin/P in the settings so that cleavage is not restricted at RP/KP, can help to reduce the search time and limit the total number of candidates.

Note: Disabling **Glycan Fragments** for glycopeptides in the **Edit Instrument** settings reduces the time required for complex glycan searches.

It is recommended to have **Glycan Fragments** enabled for data acquired in CID mode.



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Peptide Mapping: Configure Settings (4)

Example of permitted search combinations for *O*-glycans in Etanercept digested with trypsin:

Enzyme: Trypsin
Missed cleavages: 1

Glycans/ peptide	Size of glycan library				
	3	4	5	6	7
3	✓	✓	✓	✓	✓
4	✓	✓	✓	✓	✗
5	✓	✓	✗	✗	✗
6	✗	✗	✗	✗	✗
7	✗	✗	✗	✗	✗

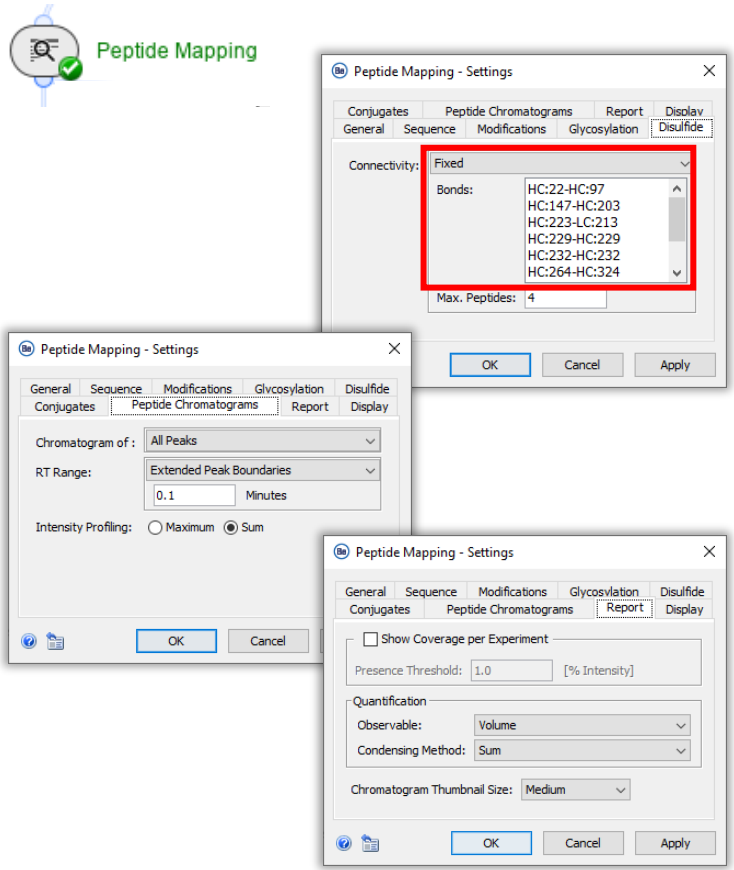
Enzyme: Trypsin/P
Missed cleavages: 0

Glycans/ peptide	Size of glycan library				
	3	4	5	6	7
3	✓	✓	✓	✓	✓
4	✓	✓	✓	✓	✓
5	✓	✓	✓	✓	✗
6	✓	✓	✓	✗	✗
7	✓	✓	✓	✗	✗

Glycosylation tab:

- A combination of factors is used to calculate the number of Estimated Glycopeptide Candidates to determine if the search will proceed:
  - The number of glycans in the glycan library (including Substituents).
    - Use the smallest library size that contains the relevant glycans of interest,
    - Selecting **Filter for Core Structures** can help to limit the candidates.
  - The maximum allowed number of glycans per peptide.
    - Do not exceed what is expected for the molecule. Lower values allow larger libraries to be used, and *vice versa*.
  - The theoretical sites of glycosylation on a peptide.
    - The number of missed cleavages and the enzyme specificity contribute to this.
- Other search parameters contribute to the overall search time.
  - To reduce the time to completion:
    - Disable **Glycan fragments** in the **Edit Instrument** settings.
    - Minimize the variable modifications and their number per peptide.
    - Maximize the minimum peptide length.
    - Reduce the number of glycans per peptide.
    - Reduce the size of the glycan library.

Peptide Mapping: Configure Settings (5)



Disulfide tab:

- For non-reduced samples: Set **Connectivity** to **Fixed.** Specify the expected disulfide bridges using the correct syntax, for example; HC:22-HC:97.
  - The chain names must match those specified in the **Sequence** tab.
- For reduced samples: Set **Connectivity** to **None**.

Peptide Chromatograms tab:

- These settings control the layout of peptide chromatograms when viewing the results of this activity, and do not need to be changed.

Report tab:

- We recommend setting **Volume** as the **Observable** for data acquired using a QTOF system.

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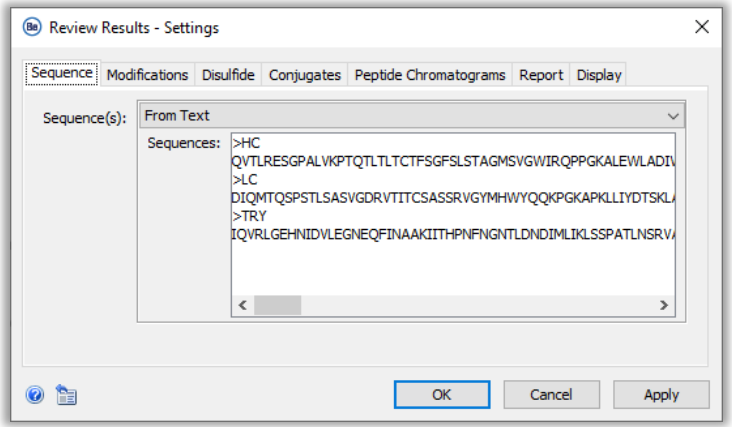
Review Results: Configure Settings

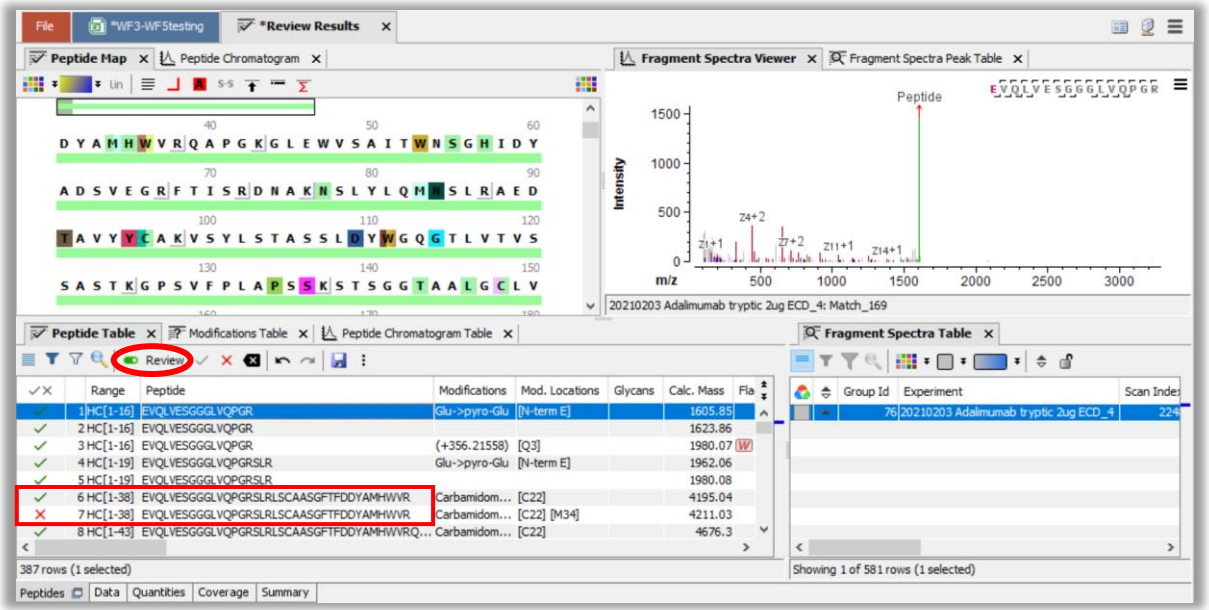


Sequence tab:

- **Sequence(s)**: Paste as text or upload as a FASTA file.
  - Use the same FASTA protein sequences in the *Peptide Mapping* activity node and the *Review Results* activity node.

Review Results: Review Peptide Mapping Results

- Open the *Review Results* activity node to review the combined results of any preceding *Peptide Mapping* activity nodes.



1. Activate the **Review** mode and accept one annotation for all relevant peaks.
2. Reject all other redundant annotations.
3. Click **Save** to apply the changes.

The activity node runs again to automatically recalculate peptide quantities.

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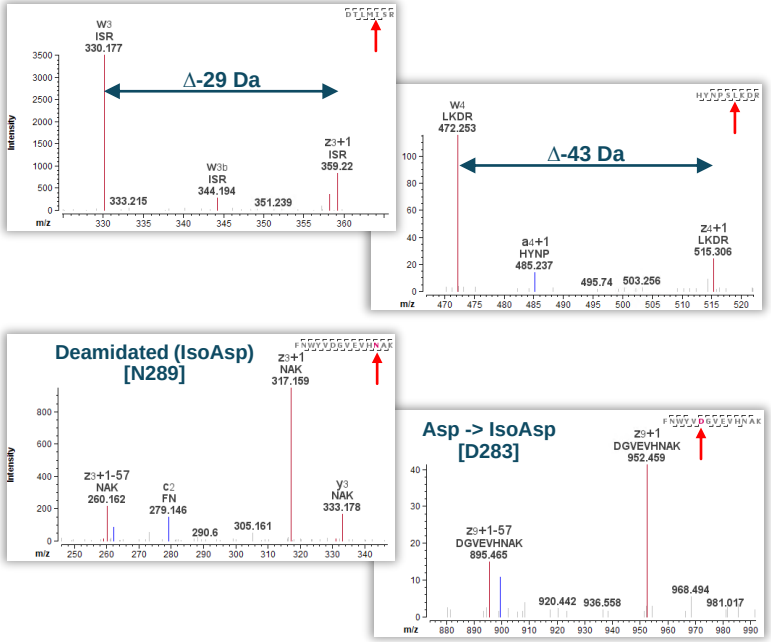
Review Results: Isomer Differentiation



Note: Use an **m/z Tolerance** of <20 ppm in the *Peptide Mapping* activity node for optimal results.

- During MS/MS analysis using EAD, diagnostic internal fragment ions are produced that enable differentiation between isomeric amino acid residues.
  - To confirm the presence of leucine (Leu) or isoleucine (Ile):
    - Ions are annotated in the MS/MS spectra as $\mathbf{w_n}$ or $\mathbf{w_{nb}}$.
      - Leucine: A $\mathbf{w_n}$ ion at a 43 Da mass shift from the corresponding z+1 ion.
      - Isoleucine: A $\mathbf{w_n}$ ion at a 29 Da mass shift from the corresponding z+1 ion.
  - To confirm the presence of aspartic acid (Asp) or isoaspartic acid (IsoAsp):
    - Ions are annotated in the MS/MS spectra as $\mathbf{c_n+57}$ or $\mathbf{z_m+1-57}$.
      - c_n+57 or z_m-+157 ions annotated in the MSMS spectra signify the presence of isoaspartic acid.
      - Aspartic acid does not generate these diagnostic internal fragment ions because there is no methylene group in the peptide backbone.

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Review Results: Isomer Differentiation (Cyclization and Deamidations)

Peptide Table | Modifications Table | Peptide Chromatogram Table

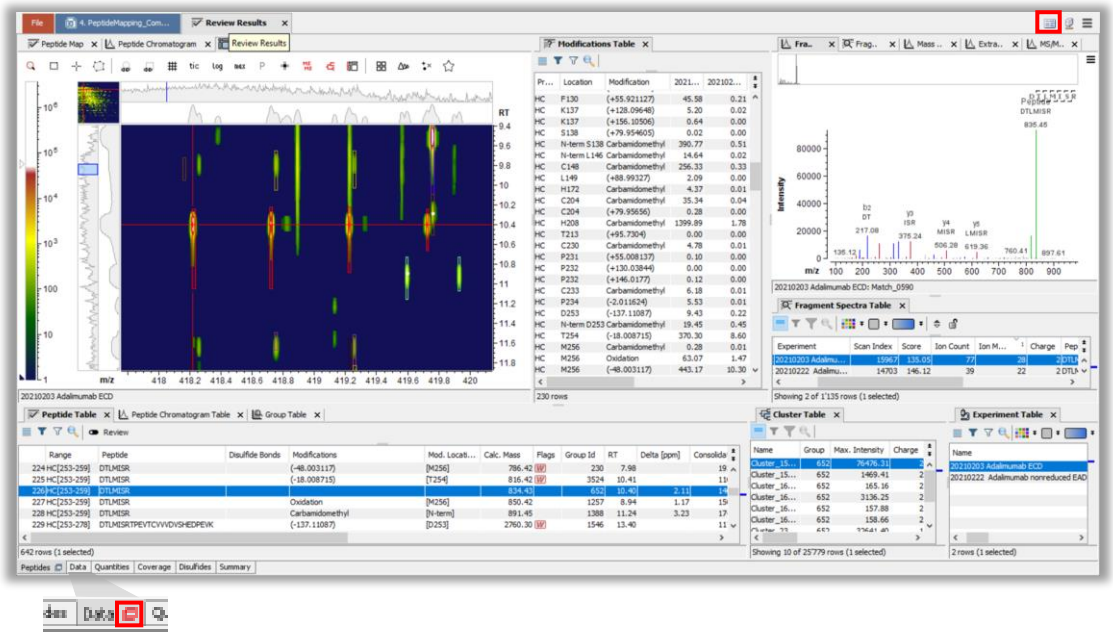
Review

Range	Peptide	Disulfide Bonds	Modifications	Mod. Locat...
39 TRY[45-54]	LSSPATLNSR			
40 TRY[55-62]	VATVSLPR			
10 HC[278-291]	FNWYVDGVEVHNAK		Asp->IsoAsp	[D283]
29 HC[413-419]	LTVDKSR		Asp->IsoAsp	[D416]
32 LC[45-52]	LLIYDTSK		Asp->IsoAsp	[D49]
8 HC[259-277...	TPEVTCVVVDVSHEDPEVK=VVSVLTVLHQDWLNGKEYKCK	HC:264->HC:324	Deamidated	[N318]
12 HC[278-291]	FNWYVDGVEVHNAK		Deamidated	[N289]
11 HC[278-291]	FNWYVDGVEVHNAK		Deamidated (IsoAsp)	[N279]
13 HC[278-291]	FNWYVDGVEVHNAK		Deamidated (IsoAsp)	[N289]
17 HC[305-320]	VVSVLTVLHQDWLNGK		Deamidated (IsoAsp)	[N318]
18 HC[305-320]	VVSVLTVLHQDWLNGK		Deamidated (IsoAsp)	[N318]
20 HC[305-323]	VVSVLTVLHQDWLNGKEYK		Deamidated (IsoAsp)	[N318]
21 HC[305-323]	VVSVLTVLHQDWLNGKEYK		Deamidated (IsoAsp)	[N318]
22 HC[305-323]	VVSVLTVLHQDWLNGKEYK		Deamidated (IsoAsp)	[N318]
14 HC[292-304]	TKPREEQYNSTYR		G0F	[N300]
15 HC[292-304]	TKPREEQYNSTYR		G1F	[N300]
7 HC[252-258]	DTLMISR		Oxidation	[M255]

- To simplify data review, it is possible to filter for relevant modifications. For example:
  - Asp → IsoAsp
  - Deamidated
  - Deamidated (IsoAsp)
- The diagnostic internal fragment ions present in the MS/MS spectra can then be used to validate identifications, and results Accepted or Rejected as required.

Review Results: Create Custom Layouts

Click to save or load saved Layouts.



Click to disconnect the **Data** tab window.

- Each pane can be disconnected and docked at a new location.
- The location where the disconnected pane will be docked is highlighted by a blue box.
- To move the Ion Map into the **Peptides** tab:
  1. Select the **Data** tab and click the icon to disconnect the **Data** tab window.
  2. The ion map, or any pane from the **Data** tab window, can then be undocked and dragged to a new location on the **Peptides** tab.
- Favorite layouts can be saved and accessed with the Layout icon.

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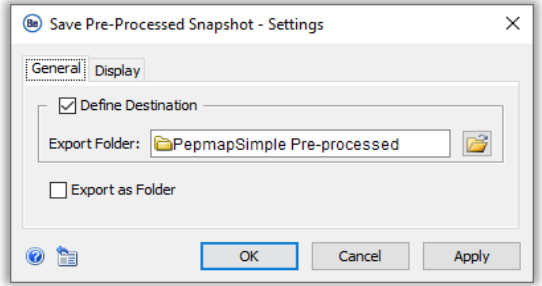
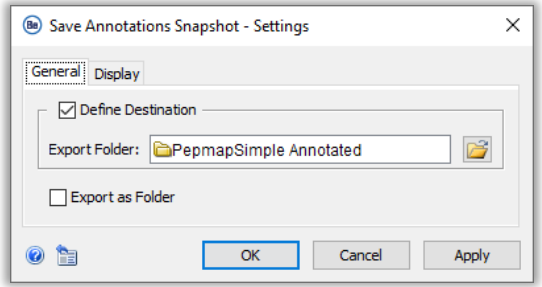
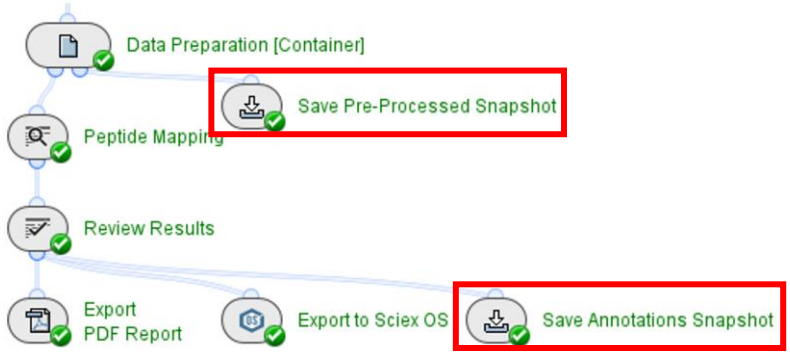
Reporting: Define Destination for Snapshots and Reports

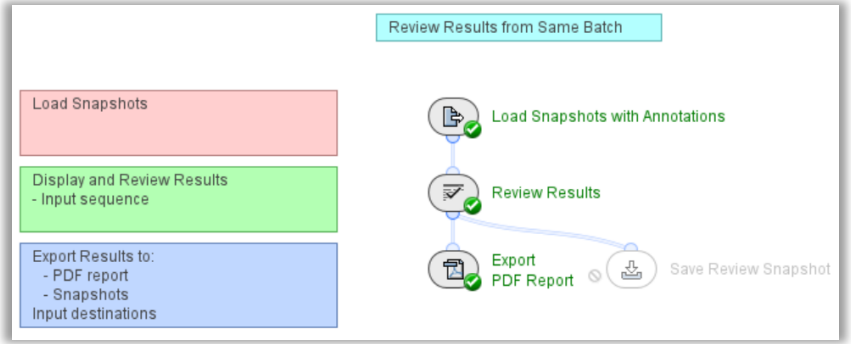
- Select the folders in which results will be stored.
- Two types of results are stored:
  - **Snapshots:**
    - Snapshots are intermediate results saved permanently as sbf files.
      - Snapshots saved from pre-processed data store the intermediate results generated *after Chromatogram Chemical Noise Subtraction* and *Chromatogram RT Alignment*.
      - Snapshots saved after *Review Results* store all of the intermediate information, including annotations of features.
  - **PDF Reports**, which embed:
    - A PDF document.
    - An Excel file with spectral information from deconvolution.
    - An Executed workflow (xml file), that includes the settings used to generate results.
      - To open the xml file, drag and drop it into the Biologics Explorer workflow home page.





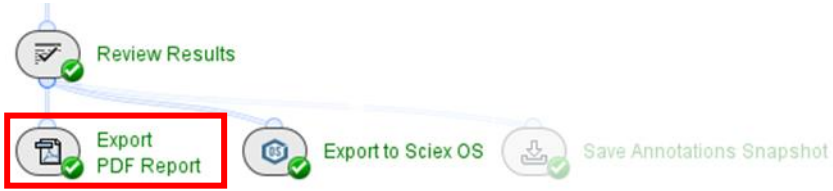
Reporting: Save Intermediate Results

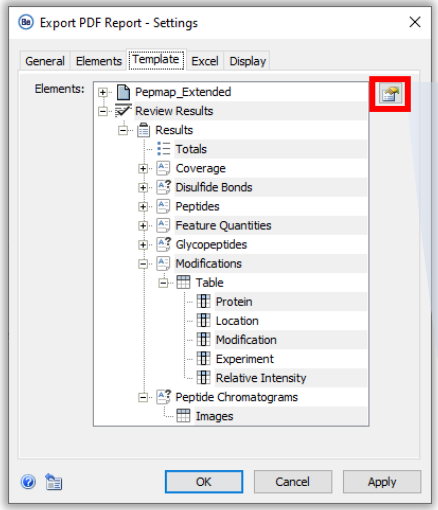


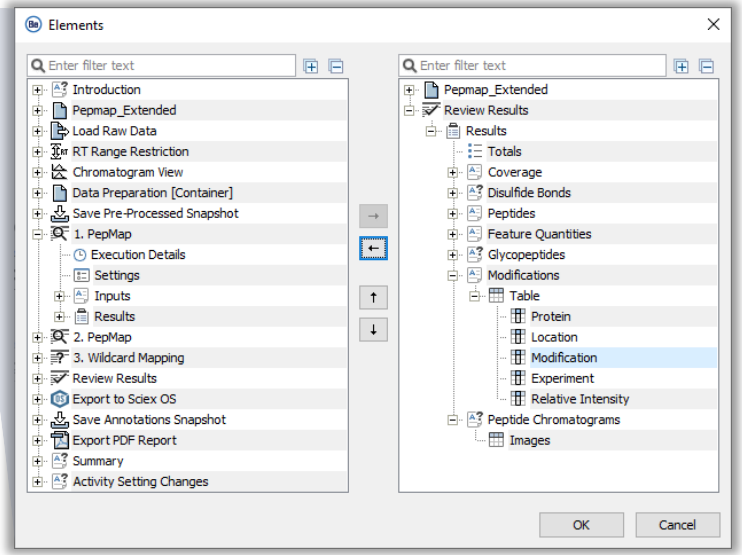


- Store intermediate results after different activity nodes through-out the workflows.
  - *Save Snapshot* activity nodes create a file (sbf) that contains the required properties of the processed data.
- To review stored data, open sbf files in the *Load Snapshots* activity nodes in the Pepmap_ReviewSnapshots workflows.
  - Load snapshots from *Save Annotations Snapshot* to review results from the same dataset.
  - Load snapshots from *Save Pre-processed Snapshot* to analyze results from different batches.
- Saved sbf files can also be loaded into the *Load Raw Data* activity node in any workflow.
  - Load snapshots from *Save Annotations Snapshot* and bypass all activity nodes except for those required for peptide mapping to add more searches to an analysis.

Reporting: *Export PDF Report*

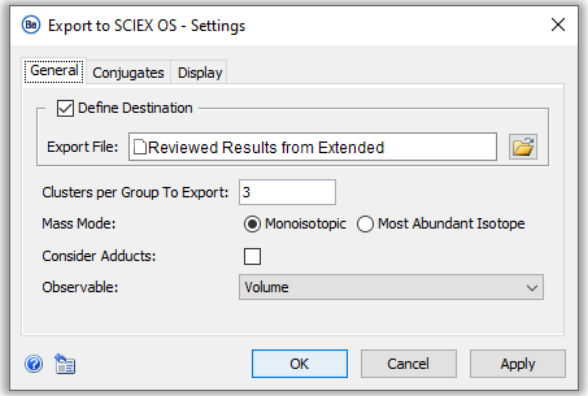






- **General** tab: Define the destination of the exported report.
- **Template** tab: Customize the contents of the report.
- **Excel** tab: Customize the tables that will be exported with the report.

Reporting: *Export to SCIEX OS*



- **General** tab:
  - Define the destination of the reviewed results.
  - Define the requirements of the export, for example, the required number of clusters for each group.
- The *Export to SCIEX OS* activity node should not be used in combination with the *Wildcard Mapping* activity node.
  - The *Wildcard Mapping* activity node can be bypassed in relevant workflows.

Note: The modification position exported with the *Export to SCIEX OS* activity node is relative to the peptide, not the protein. For example, DTL[M]ISR would be M4 not M255.

Part B

Workflows and Applications

GUIDELINES FOR SPECIFIC PEPTIDE MAPPING WORKFLOWS

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Simple Peptide Mapping

WORKFLOW SPECIFIC GUIDELINES

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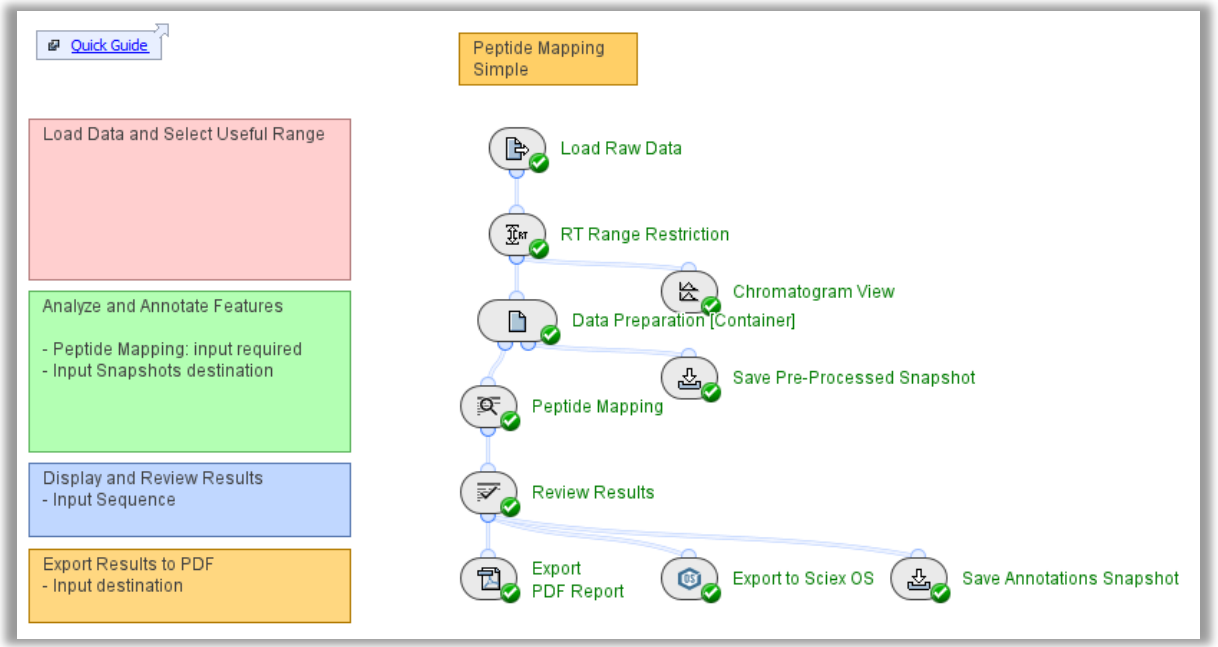
Simple Peptide Mapping Workflow: Design



Pepmap_Simple

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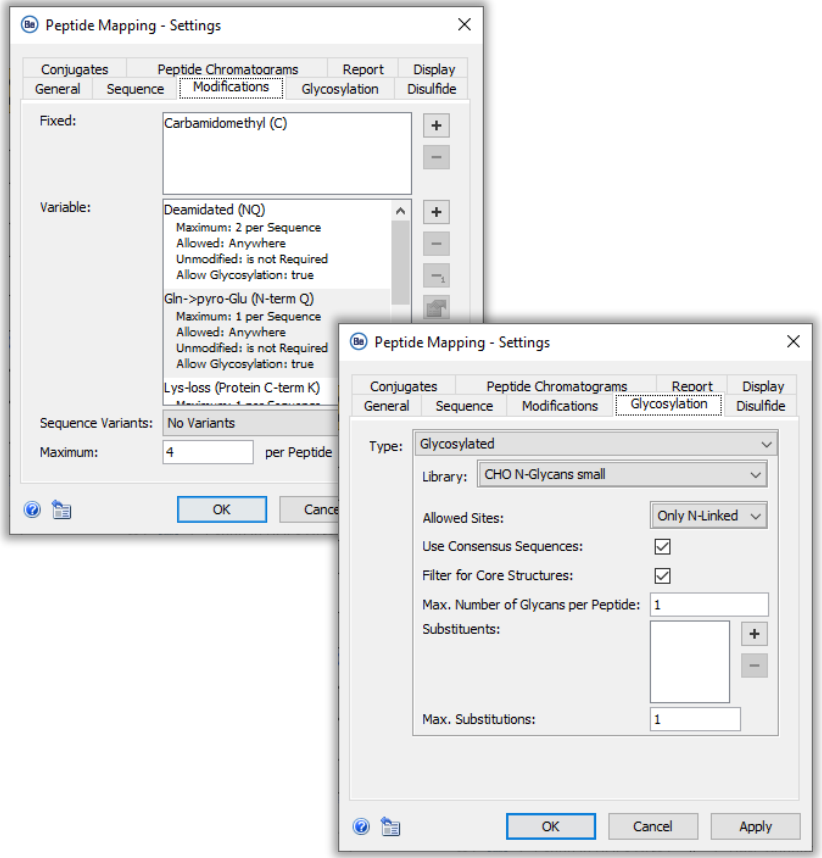
Simple Peptide Mapping Workflow: Overview



- Use this workflow when completing routine analyses with non-complex biotherapeutic molecules.
- The combination of search parameters in the *Peptide Mapping* activity node identifies commonly expected peptides and modifications, including glycosylation.

Extended Peptide Mapping

WORKFLOW SPECIFIC GUIDELINES

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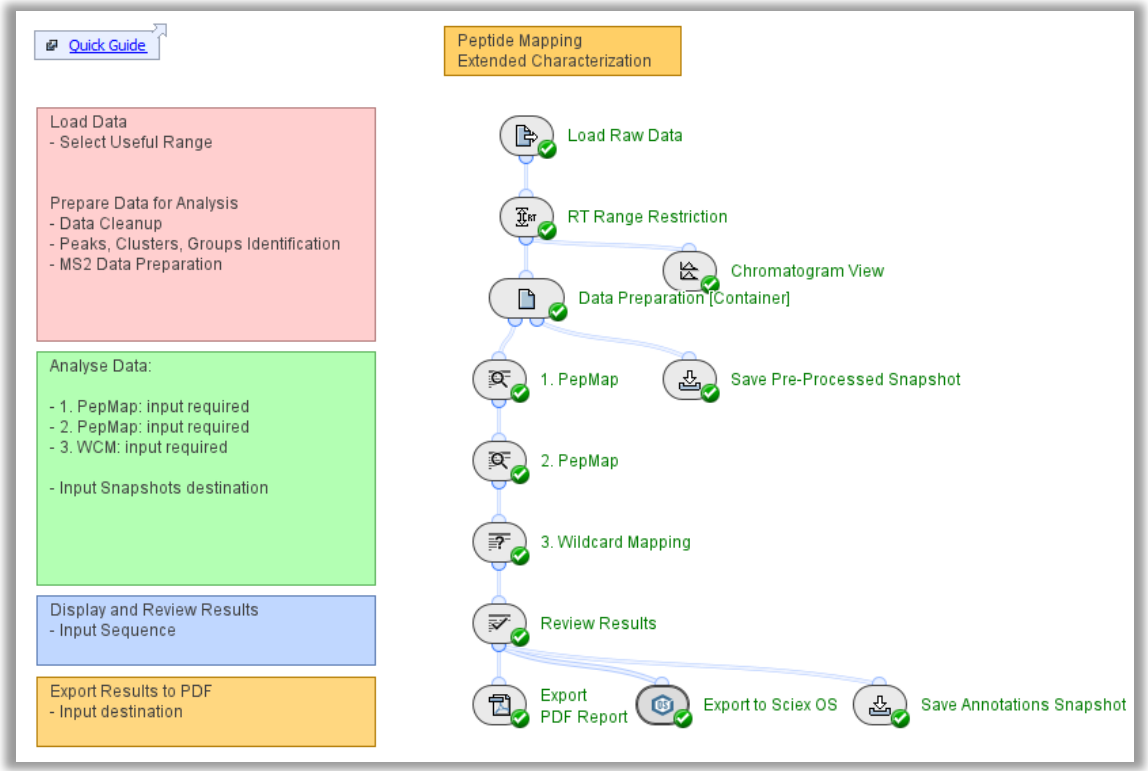
Extended Peptide Mapping Workflow: Design



Pepmap_Extended

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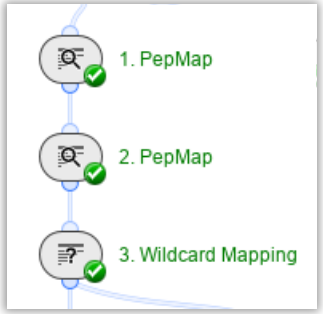
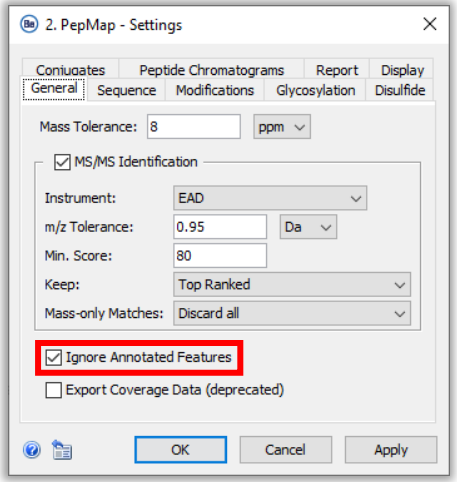
Extended Peptide Mapping





- For a more comprehensive peptide mapping analysis, results from up to three consecutive activity nodes can be combined to extend the search space while minimizing false positives:

1. PepMap

- Identifies the most expected peptides and modifications.

2. PepMap

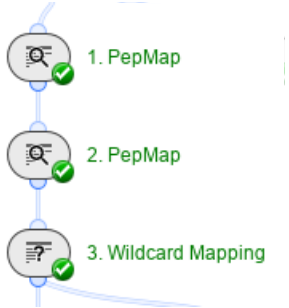
- Digs deeper into the sample.
- **Ignore Annotated Features**: Makes sure that only unannotated features from the previous search are considered.

3. Wildcard Mapping

- Searches for unexpected modifications, which can be subsequently added to *1.PepMap* or *2.PepMap* activity nodes.

Note: For biotherapeutics with expected *N*- and *O*-glycosylation, false positives are reduced when *1.PepMap* is used to identify expected *N*-glycans, and *2.PepMap* focuses on the typically less well characterised *O*-glycans.

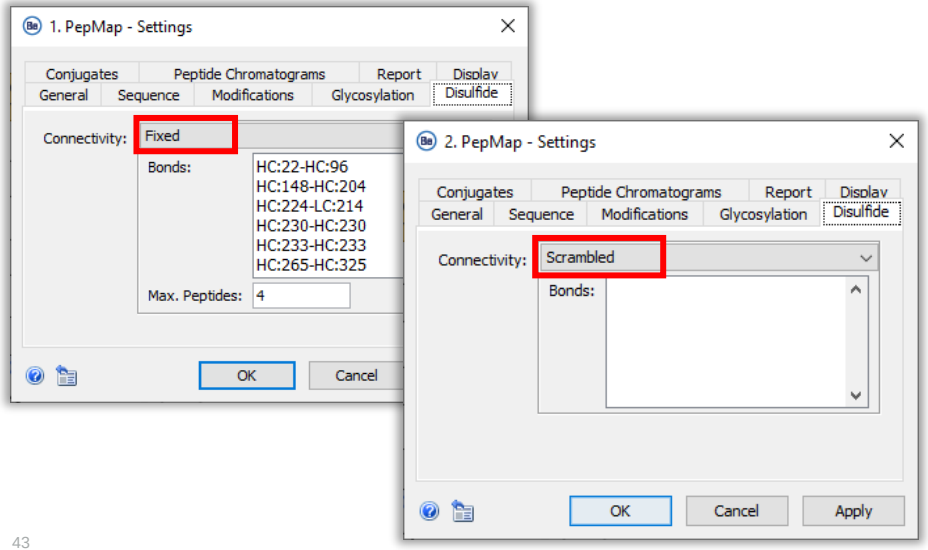
Step-Wise Peptide Mapping: Application Examples



- Three consecutive peptide mapping steps can be combined, depending on the type of analysis required.

Example 1: Disulfide bond (DSB) analysis for non-reduced samples.

- Key settings specific to this type of analysis:



1. PepMap

- **Sequence** tab: **Enzyme** - Fully specific.
- **Disulfide** tab: **Fixed Connectivity**.
  - Define the expected disulfide bridges using the correct syntax (HC:22-HC:96).

2. PepMap

- **Sequence** tab: **Enzyme** - Fully specific.
- **Disulfide** tab: **Scrambled Connectivity**.

3. Wildcard Mapping

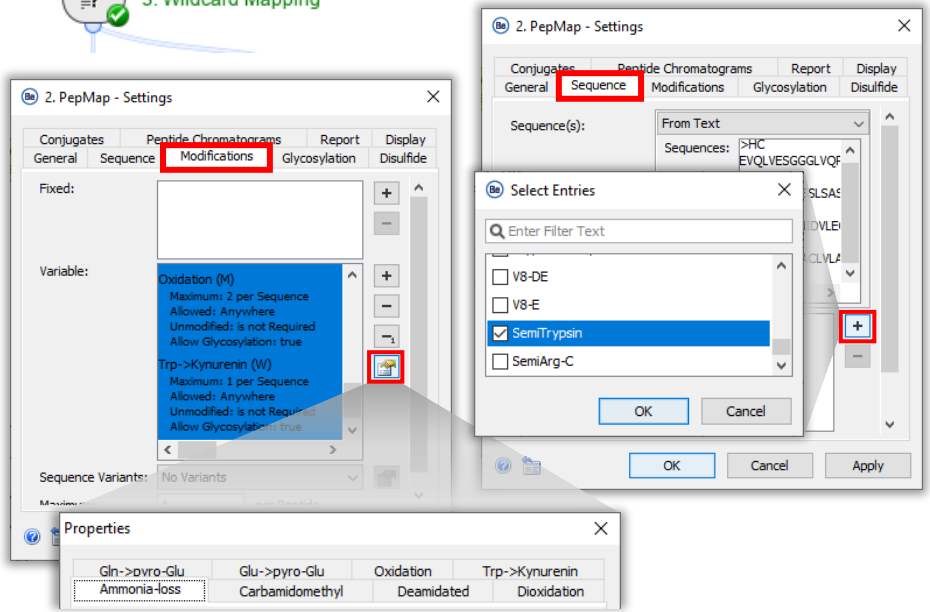
- On **All Peptide Candidates** for more annotations relating to unknown modifications.

Step-Wise Peptide Mapping: Application Examples

- Three consecutive peptide mapping steps can be combined, depending on the type of analysis required:

Example 2: Post translational modification (PTM) analysis.

- Key settings specific to this type of analysis:



1. PepMap

- **Sequence** tab: **Enzyme** - Fully specific.
- **Modifications** tab: Abundant and expected modifications.

2. PepMap

- **Sequence** tab: **Enzyme** - Semi-Specific.
- **Modifications** tab: Shorter list of expected modifications.

Or:

- **Sequence** tab: **Enzyme** - Fully specific.
- **Modifications** tab: Alternative set of less common modifications that might be expected at low abundance.

3. Wildcard Mapping

- On **All Peptide Candidates** for annotations relating to unknown modifications.

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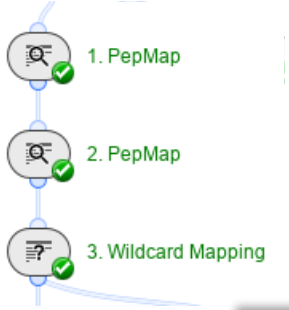
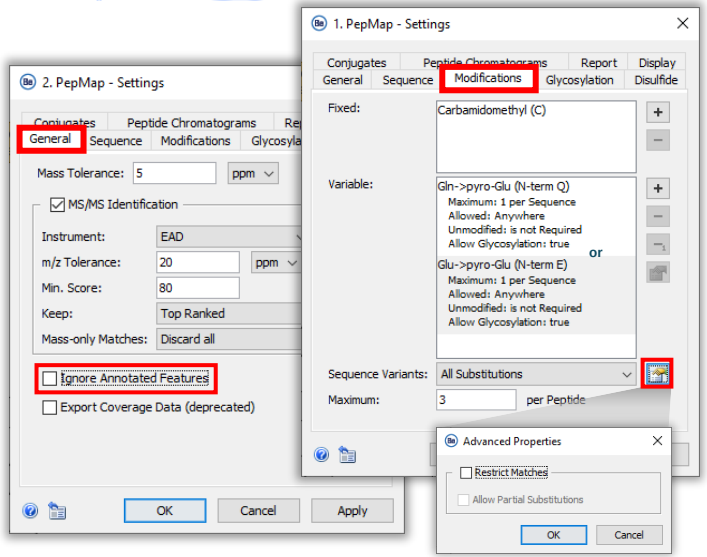
Step-Wise Peptide Mapping: Application Examples



- Three consecutive peptide mapping steps can be combined, depending on the type of analysis required:

Example 3: Sequence Variant Analysis (SVA).

- Key settings specific to this type of analysis:



1. PepMap

- **Sequence** tab: **Enzyme** - Fully specific. No missed cleavages.
- **Modifications** tab: Fixed alkylation (cys) with variable modifications limited to the predominant form (for example, pyro-glutamation). **Sequence Variants - All Substituents** (**Restrict Matches** cleared).

2. PepMap

- **General** tab: Low **Mass Tolerance**. Clear **Ignore Annotated Features**.
- **Sequence** tab: **Enzyme** - Semi-Specific. 1-2 missed cleavages.
- **Modifications** tab: Variable alkylation on commonly modified amino acids to detect overalkylation, with all other expected variable modifications.

3. Wildcard Mapping

- On **Only Annotated Peptides**.
  - Use *Review Results* to compare additional annotations on the same features to rule out false positives.

Comparative Peptide Mapping

WORKFLOW SPECIFIC GUIDELINES

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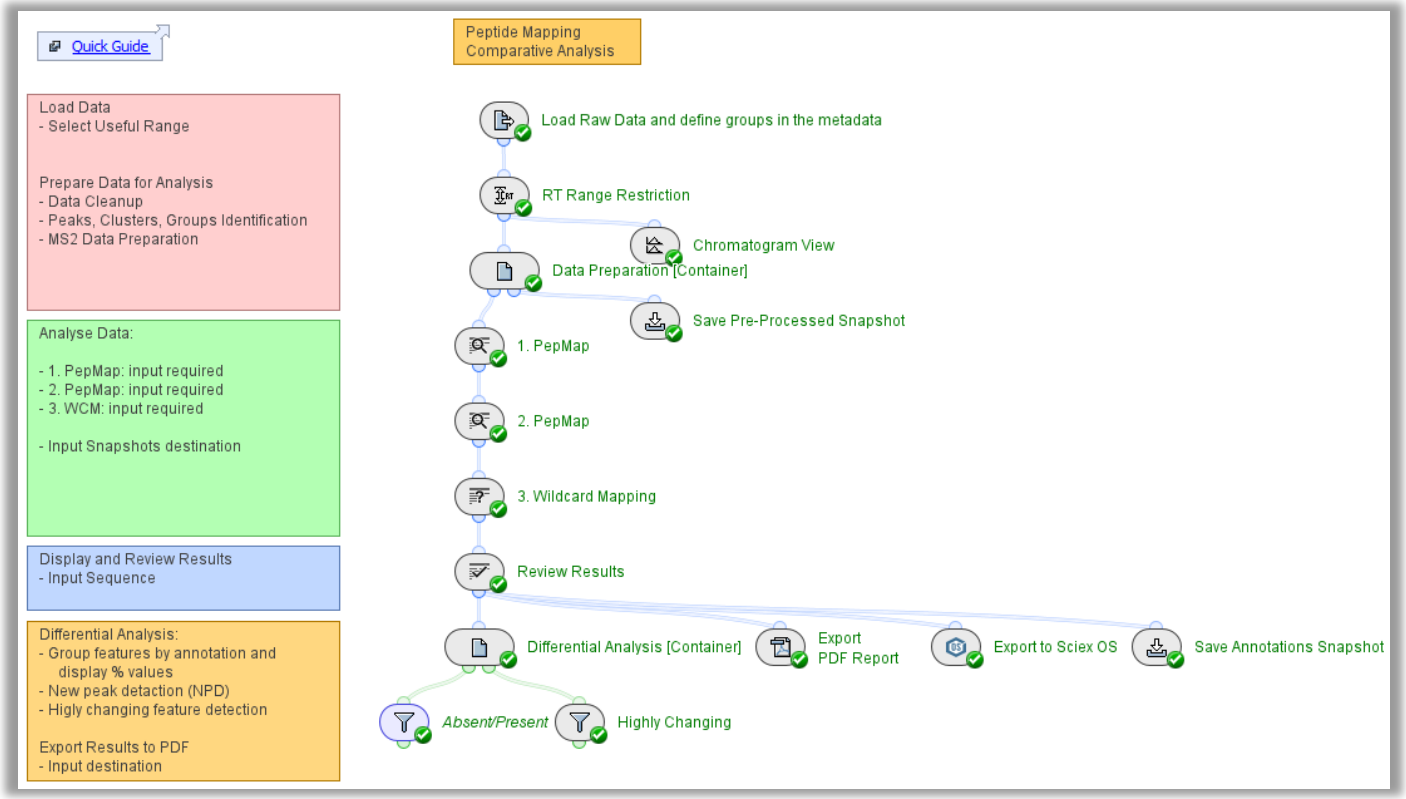
Comparative Peptide Mapping Workflow: Design



Pepmap_Comparative

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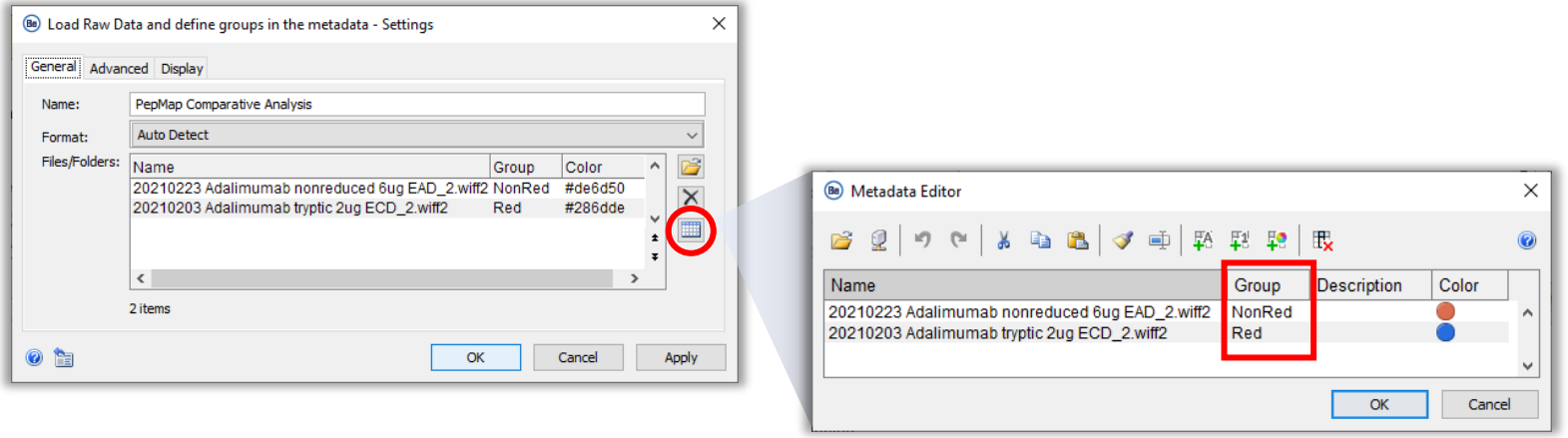
Comparative Peptide Mapping

These statistical activity nodes identify the features that differ significantly between the two sample groups being compared in the workflow.

- The activity nodes connected with green lines contain statistical tools.
- These statistics activity nodes can be used to compare two datasets, and report peptides that are either:
  - Absent in one sample set but present in the other.
  - Have a specified fold-change difference between sample sets.

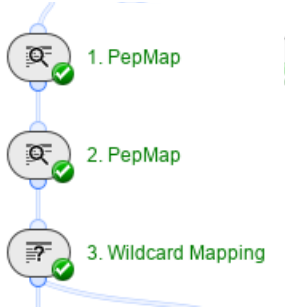
- Example use cases:
  - To compare reduced and non-reduced samples.
  - To compare stressed and unstressed samples.
  - To compare a reference sample with samples from a new batch.

Load Raw Data and Define Groups



- On the **General** tab, click the table icon to open the **Metadata Editor.**
  - Specify the **Group** names for the files to be compared.
  - Optionally, add a **Color** column and define colors for each group.

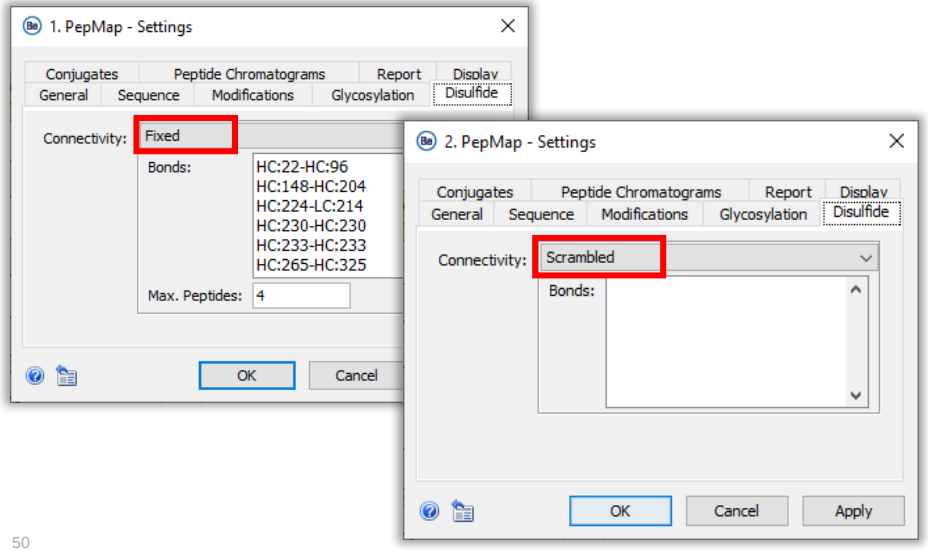
Step-Wise Peptide Mapping: Application Examples



- Three consecutive peptide mapping steps can be combined, depending on the type of analysis required:

Example 1: DSB analysis for comparing reduced and non-reduced samples.

- Key settings specific to this type of analysis:



1. PepMap

- **Sequence** tab: Fully specific.
- **Disulfide** tab: **Fixed Connectivity**.
  - Define the expected disulfide bridges using the correct syntax (HC:22-HC:96).
- **Modifications** tab: Variable alkylation (cys).

2. PepMap

- **Sequence** tab: Fully specific.
- **Disulfide** tab: **Scrambled Connectivity**.

3. Wildcard Mapping

- On **All Peptide Candidates** for more annotations relating to unknown modifications.

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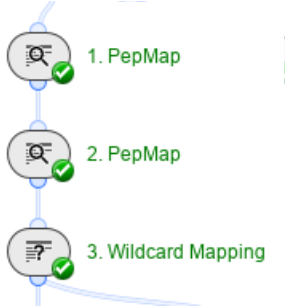
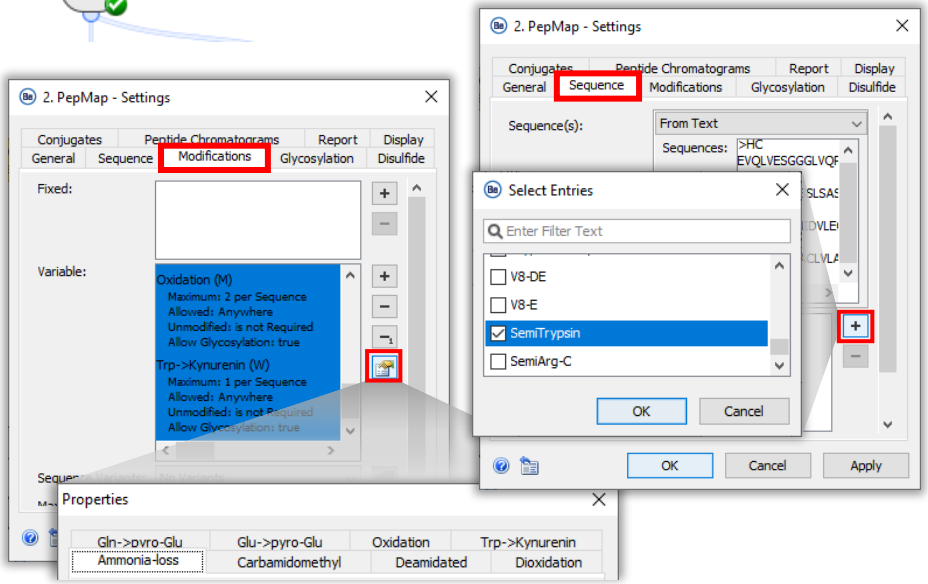
Step-Wise Peptide Mapping: Application Examples



- Three consecutive peptide mapping steps can be combined, depending on the type of analysis required:

Example 2: Comparative analysis for stress testing and lot-to-lot variability.

- Key settings specific to this to this type of analysis:



1. PepMap

- **Sequence** tab: Fully specific.
- **Modifications** tab: Abundant and expected modifications.

2. PepMap

- **Sequence** tab: Unspecific enzyme.
- **Modifications** tab: Shorter list of expected modifications.

Or:

- **Sequence** tab: Fully specific.
- **Modifications** tab: Alternative set of less common modifications that might be expected at low abundance.

3. Wildcard Mapping

- On **All Peptide Candidates** for more annotations.

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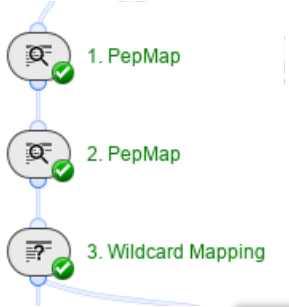
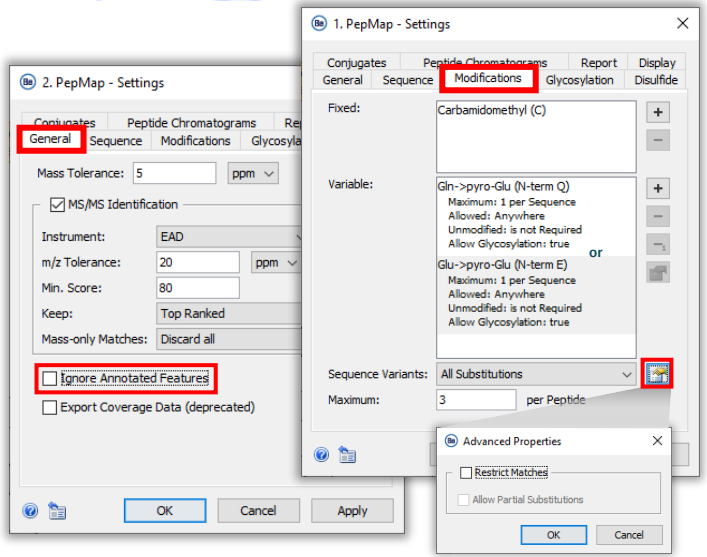
Step-Wise Peptide Mapping: Application Examples



- Three consecutive peptide mapping steps can be combined, depending on the type of analysis required:

Example 3: SVA for comparing wild type (*WT*) and mutant samples.

- Key settings specific to this type of analysis:



1. PepMap

- **Sequence** tab: **Enzyme** - Fully specific. No missed cleavages.
- **Modifications** tab: Fixed alkylation (cys) with variable modifications limited to the predominant form (for example, pyro-glutamation). **Sequence Variants - All Substituents** (**Restrict Matches** cleared).

2. PepMap

- **General** tab: Low **Mass Tolerance**. Clear **Ignore Annotated Features**.
- **Sequence** tab: **Enzyme** - Semi-Specific. 1-2 missed cleavages.
- **Modifications** tab: Variable alkylation on commonly modified amino acids to detect overalkylation, with all other expected variable modifications.

3. Wildcard Mapping

- On **Only Annotated Peptides**.
  - Use *Review Results* to compare additional annotations on the same feature to rule out false positives.

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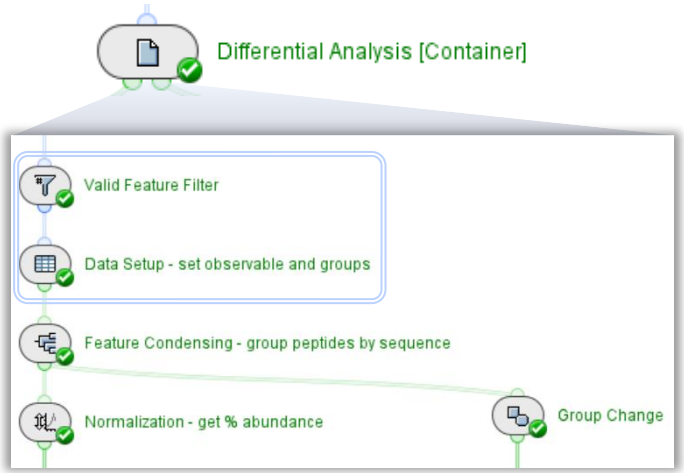
Differential Analysis Activity Node Settings



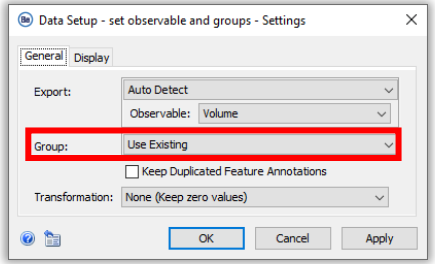
- *Valid Feature Filter*: Removes any features below a set threshold, and those present in less than a set % or number of experiments.
  - This filtering removes insignificant differences or any signal due to noise or artifacts. If expected peptides are not present, optimize this setting.
- *Data Setup*: Prepares data in a matrix form that can be used as input for filtering and statistics tasks.
  - If Groups were not set in *Load Raw Data*, they can be defined here.

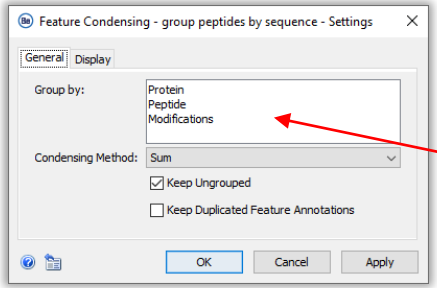
- *Group Change*: Calculates relative and fold-change differences between experiment groups.
  - If multiple experiment groups are present, then the reported change is the maximum difference between any two groups.

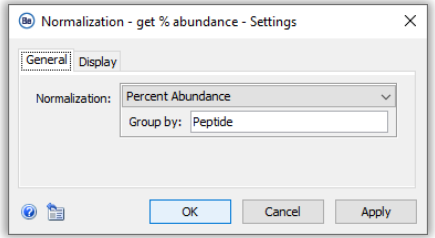
- *Feature Condensing*: Groups features based on their annotations.
  - Computes a single intensity value for each of the created groups.
- *Normalization*: Provides a comparable basis for further statistical analysis.
  - **Percent Abundance**: Values are summed across all members of each group for each experiment. Each value is divided by the sum from the corresponding group and multiplied by 100.

Note: Settings are linked to previous activity nodes: Run *Data Setup* before editing *Feature Condensing.*

Differential Analysis: Data Preparation





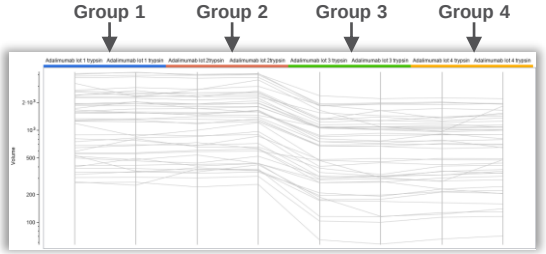


- Define Groups in the *Load Raw Data* activity node by editing the Metadata table (preferred option).
- If required, define Groups in the *Data Setup* activity node, using **Group: Manually** and assigning each sample to a group.

- *Feature Condensing*: Combines features based on existing annotations and calculates a single intensity value (sum, average, median, or max) for each group. For example, the same peptide, from the same protein, with the same modification will be summed using these settings.
  - Run the *Data Setup* activity node <u>before</u> editing the *Feature Condensing* settings.

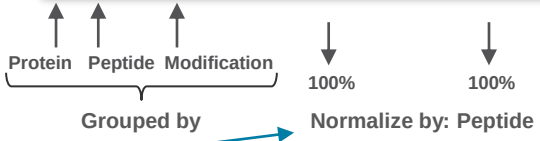
- *Normalization*: This will report **Percent Abundance** of condensed features relative to the selected type of annotation. For example, if **Group by** is set to **Peptide**, then the % abundance is calculated per peptide. If **Group by** is left blank, then the % abundance is calculated across the whole sample.

Lot-to-lot comparability



DSB analysis

Name	20210222 Adalimumab non...	20210203 Adalimuma...
HC | DTLMISR	78.46	70.35
HC | DTLMISR | (-18.01052015889502) [T2]	8.62	10.19
HC | DTLMISR | (-48.00280214761824) [M4]	10.89	4.93
HC | DTLMISR | Carbamidomethyl [ ]	0.53	1.33
HC | DTLMISR | Oxidation [M4]	1.51	13.20



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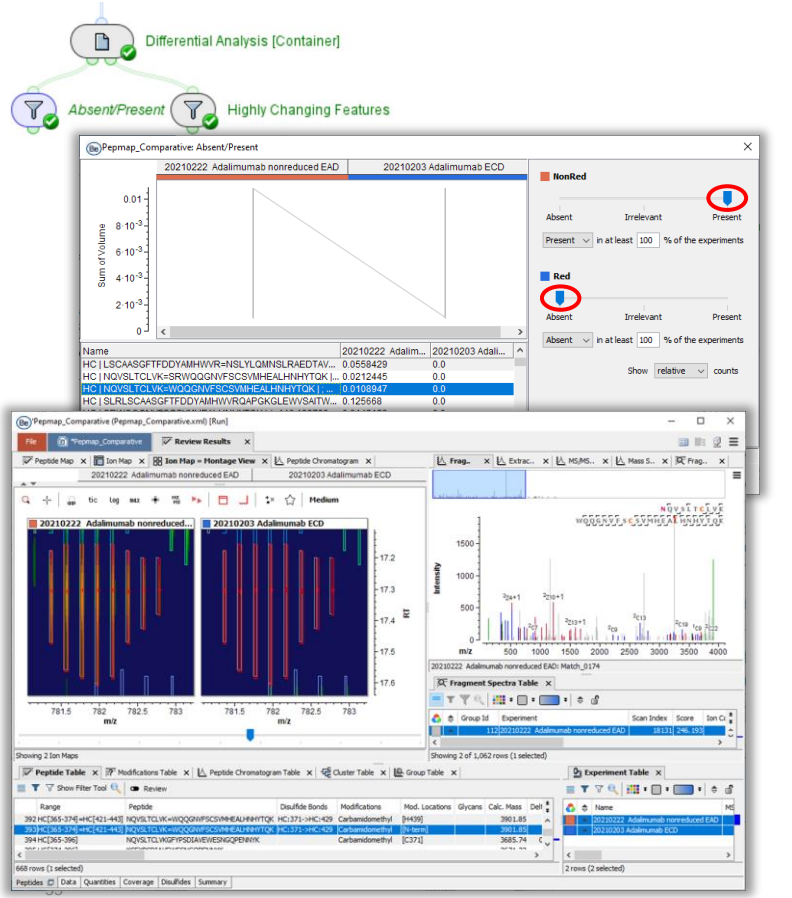
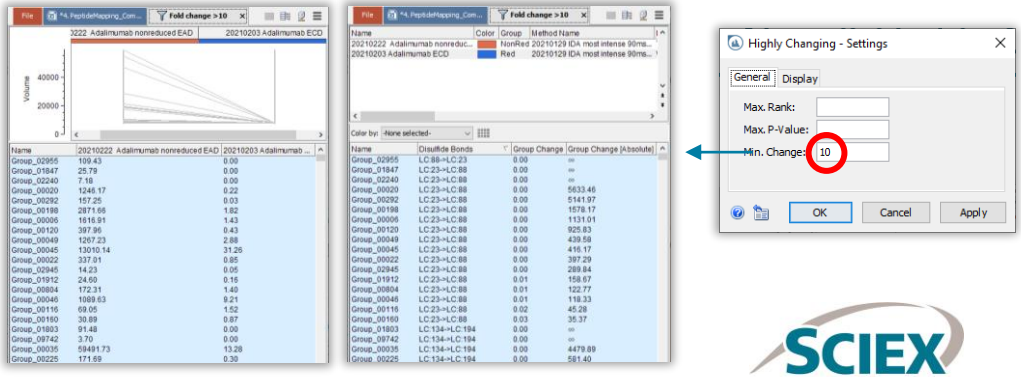
New and Highly Changing Feature Detection



- The *Absent/Present* activity node can facilitate New Peak Detection (NPD).
  - The sliders are applied in the input window that opens when the activity node is run.
  - Moving the sliders filters the results. For example, for DSB analysis, the desired features are expected to be **Absent** in the reduced sample and **Present** in the non-reduced sample.
- For *Highly Changing Features*, the desired minimum fold change must be set in the activity node settings.



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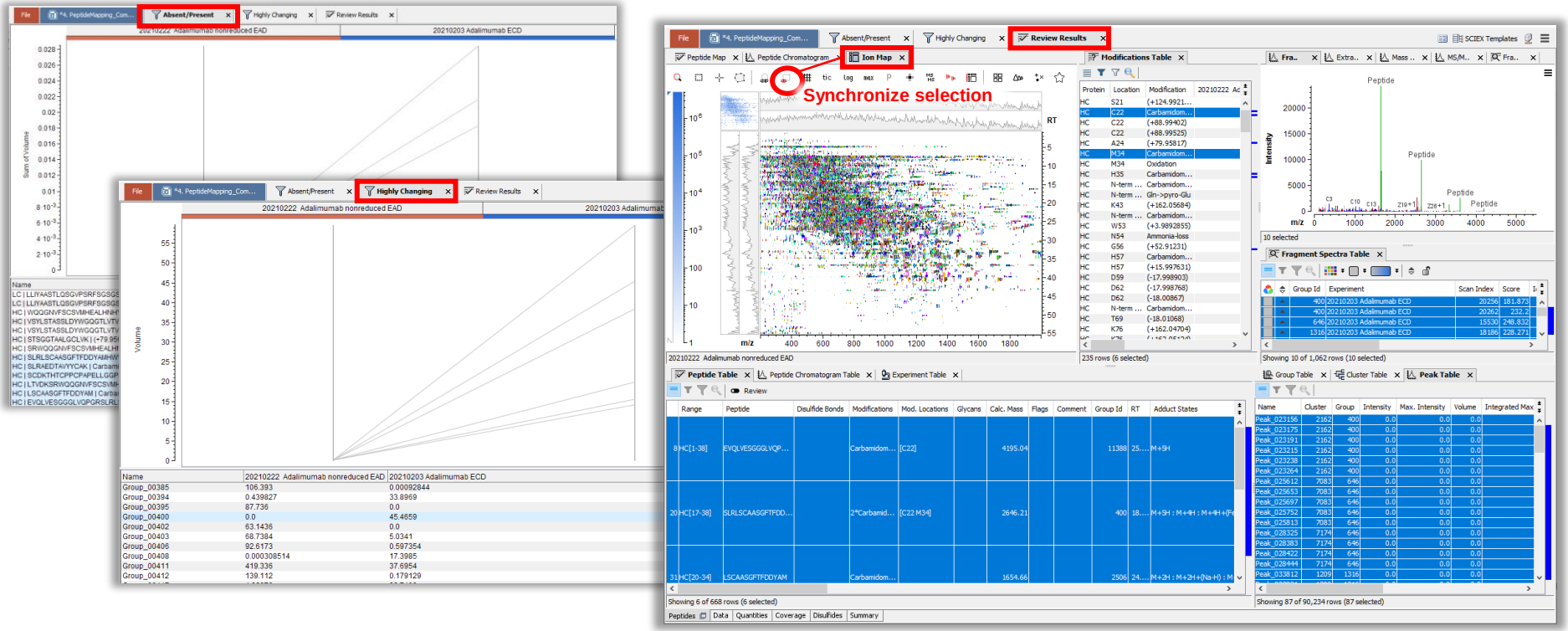
Synchronized Selections for Simplified Data Review



Open all three windows and activate **Synchronize selection** in *Review Results* for dynamic linking.

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Review Stored Results

WORKFLOW SPECIFIC GUIDELINES

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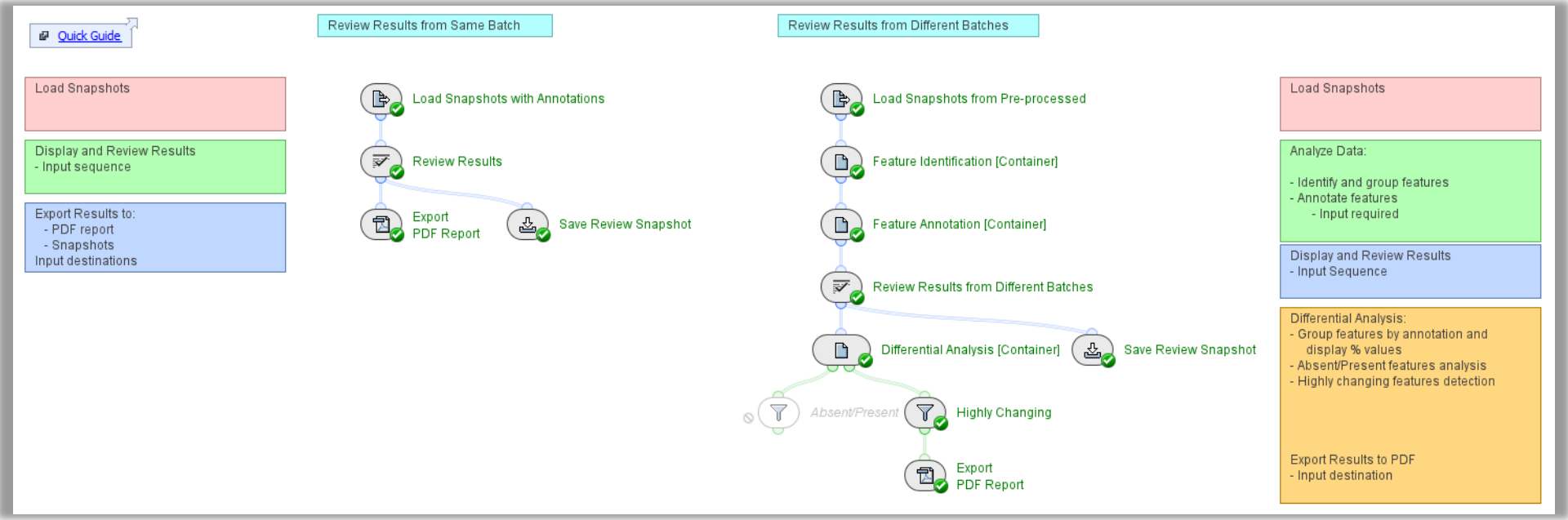
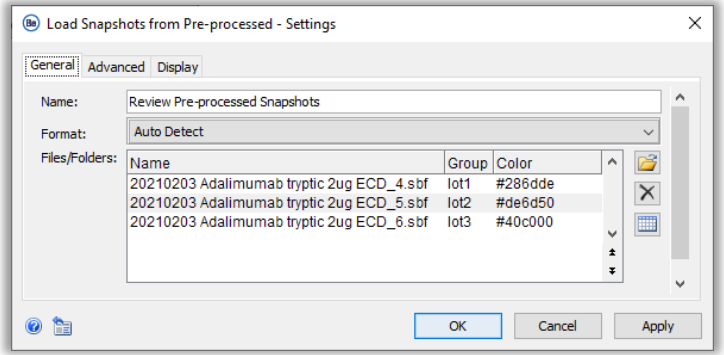
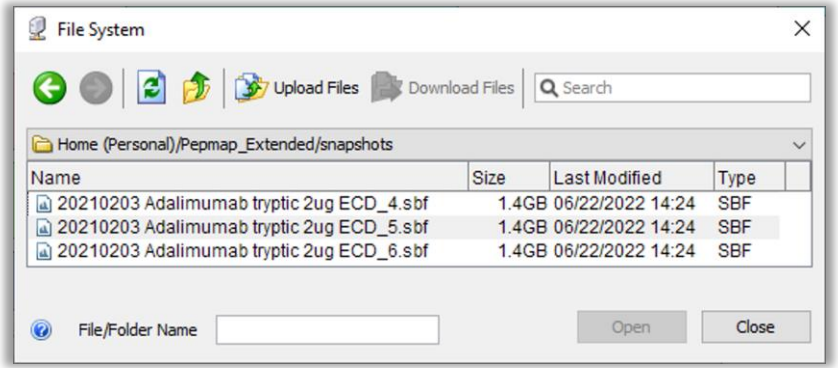
Review Peptide Mapping Snapshots Workflow: Design



Pepmap_ReviewSnapshots

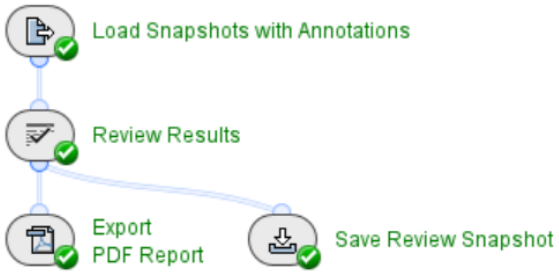
Review Results from Same Batch



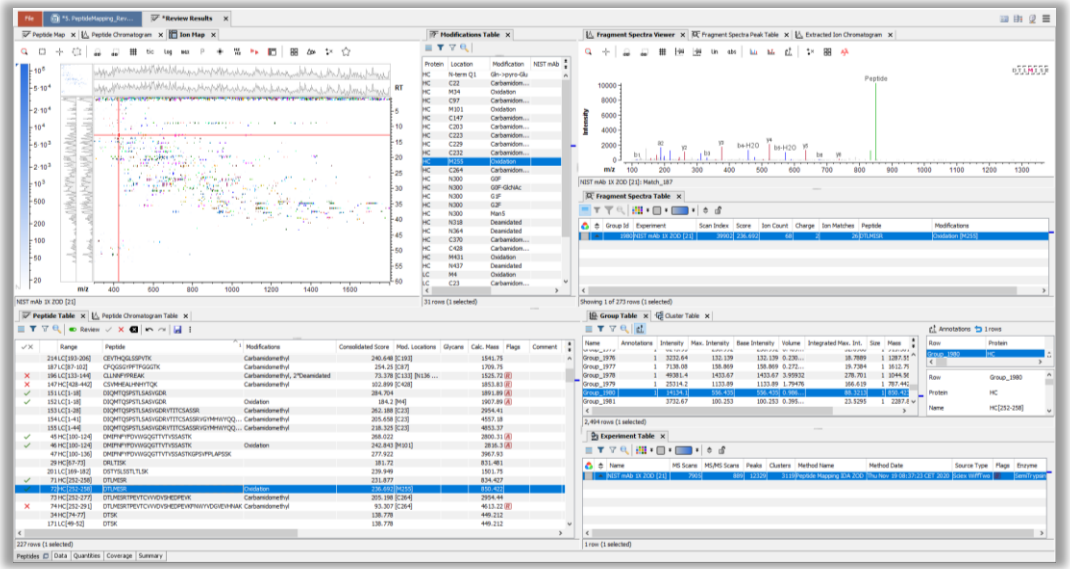


- When multiple samples are analyzed in other Peptide Mapping workflows, each sample generates its own sbf file.
- When loading saved Snapshots into the Pepmap_ReviewSnapshots workflow, select all individual sbf files within the parent folder.
  - Data will not load if parent folder is selected.

Review Results from Same Batch

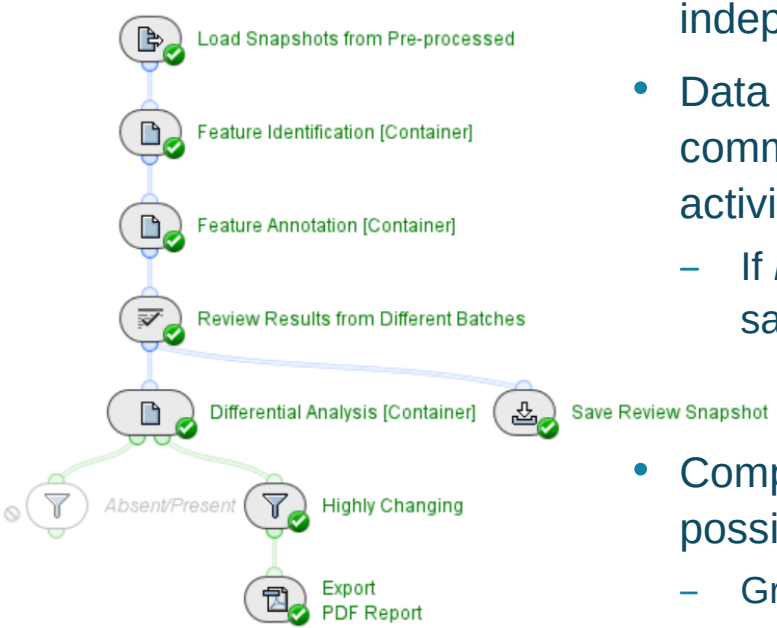


- Use this workflow to review previous results from samples that have been analyzed together previously and have peptide annotations.



- The *Review Results* activity node opens a copy of the previous analysis, including any previously accepted and rejected peptides that have the relevant entry in the **Flags** column.
- A further review is then possible, and the reviewed snapshots can be saved, if required.

Review Results from Different Batches



- Data from different analyses can be pre-processed independently and then the Snapshots combined.
- Data requires feature detection including *RT Alignment* and common *Peak Detection*, followed by the *Peptide Mapping* activity nodes for feature annotation.
  - If *Pre-processed Snapshots* are used to process data from the same batch, then bypass the *RT Alignment* activity node.
- Comparisons between different groups of samples are also possible.
  - Groups defined in the original workflow are maintained.
  - If not previously defined, groups can be defined within the *Load Snapshots* or the *Data Setup* activity nodes.

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