Peptide Mapping

Biologics Explorer Software 5.0 Guidelines

Powered by Genedata Expressionist®

Genedata solutions in silico

SCIEX The Power of Precision

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Peptide Mapping Guidelines

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Part A
General Guidelines for Peptide Mapping Workflows

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Overview of Applications for Peptide Mapping Workflows

- Use the Peptide Mapping workflows for the analysis of enzymatically digested biotherapeutic molecules, for:
  - Sequence coverage and confirmation
  - Glycopeptide analysis
  - Post-translational modification (PTM) analysis
  - Target PTM profiling
  - Disulfide-bond (DSB) analysis
  - Conjugate analysis
  - Sequence variant analysis (SVA)
- Use Pepmap workflows _Simple, _Extended, _Comparative or _SVA for replicate analyses with common peak boundaries across all samples.
- Use Pepmap workflow _BatchProcessing for analysis of the same or different molecules, with each sample treated as an individual replicate with no shared peak boundaries.
- Use Pepmap workflow _ReviewSnapshots to open saved results from other Pepmap workflows.

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Peptide Mapping Workflows

- **Pepmap_Simple**:
  - A Peptide Mapping workflow for routine characterization, with identification and quantification of the most common modifications and glycosylations.
- **Pepmap_Extended**:
  - A Peptide Mapping workflow that has more search nodes, for identification of less common PTMs to maximize sequence coverage.
- **Pepmap_BatchProcessing**:
  - A version of the Pepmap_Extended workflow that analyzes each data file on a sample-by-sample basis.
- **Pepmap_Comparative**:
  - A version of the Pepmap_Extended workflow that has activity nodes to complete a differential analysis between sample sets.
- **Pepmap_SVA**:
  - A version of the Pepmap_Extended workflow that has more search nodes for identification of potential sequence variants.
- **Pepmap_ReviewSnapshots**:
  - A workflow to open or review saved results.

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2. Common Activity Nodes for Peptide Mapping Workflows

GENERAL GUIDELINES FOR PEPTIDE MAPPING WORKFLOWS

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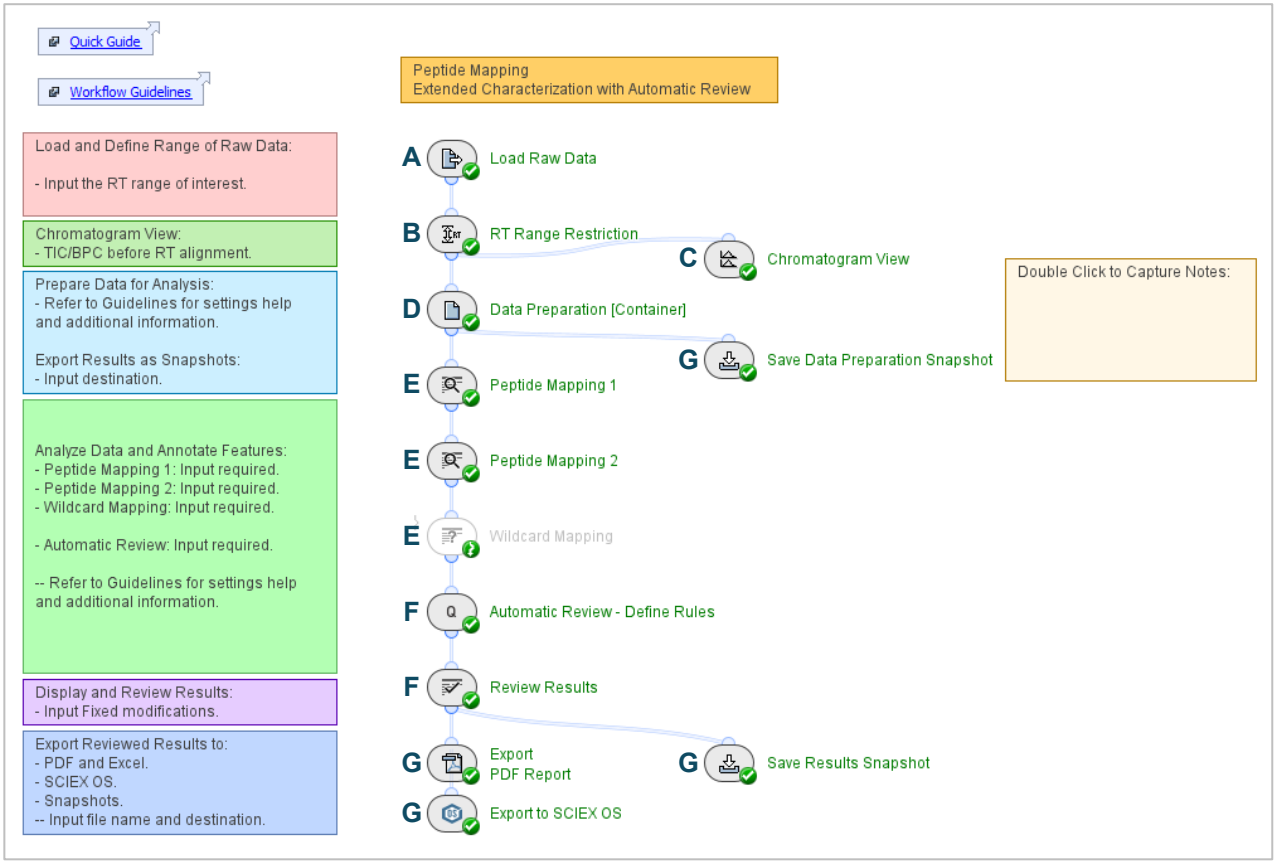
Example of a Typical Peptide Mapping Workflow



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Common Activity Nodes in Peptide Mapping Workflows

A. *Load Raw Data* → Input required*

B. *RT Range Restriction* → Input required*

C. *Chromatogram View* (before RT alignment)

D. *Data Preparation [Container]*

i. *Chromatogram Chemical Noise Subtraction*
ii. *Chromatogram RT Alignment*
iii. *Chromatogram View* after Alignment
iv. *Chromatogram Peak Detection*
v. *Chromatogram Isotope Clustering*
vi. *Singleton Filter*
vi. *Charge Grouping*
vii. *Adduct Grouping*
viii. *MS/MS Consolidation*
ix. *MS/MS Peak Detection*
x. *MS/MS Deisotoping*

E. Peptide Identification → Input required*

F. *Review Results* → Input required*

G. Report and Export Results

*Optimize settings for new samples

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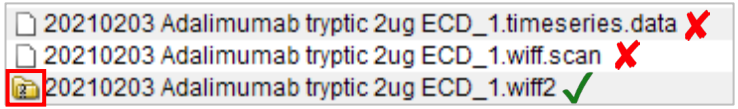
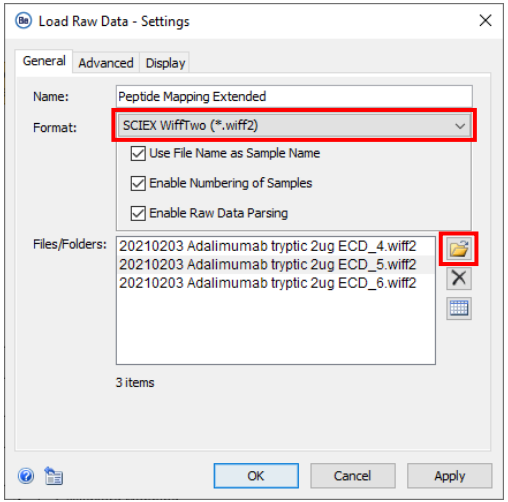
Load Raw Data: Data Files

- To upload raw data files, click the folder icon .
  - Select container files with the format wiff or wiff2.
    - If data was acquired with the ZenoTOF 7600 system, then select only the wiff2 format.
    - Do not select the auxiliary files with the same name.



  - To select samples in a wiff or wiff2 container file:
    - Double-click the wiff or wiff2 container to see the sample files.
    - Select the samples to upload.



Note: For information about Batch Processing, refer to **B:** 5.Guidelines for Peptide Mapping Batch Processing Workflows.

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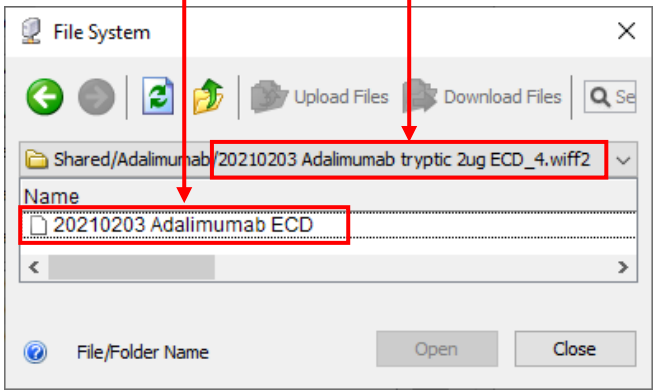
Load Raw Data: Use File Name as Sample Name

- The **File Name** of the wiff or wiff2 container file might not be the same as **Sample Name** in the wiff or wiff2 container file.
  - The name of the **Data File** in the SCIEX OS software becomes the name of the wiff or wiff2 container file (**File Name**) in Biologics Explorer software.



- Individual acquisitions with different wiff or wiff2 **File Names** might have the same **Sample Name**.

Note: If entries in the **Experiment Table** have the same **Sample Name**, there can be an effect on the quantitative information reported.

- Select **Use File Name as Sample Name** in *Load Raw Data* to use the **File Name** in the **Experiment Table**.
  - If **Format: Auto Detect** is selected, then the **Sample Name** is used in the **Experiment Table**.

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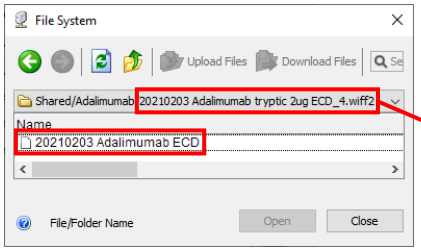
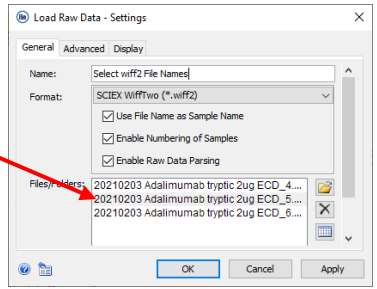
Load Raw Data: Use File Name as Sample Name

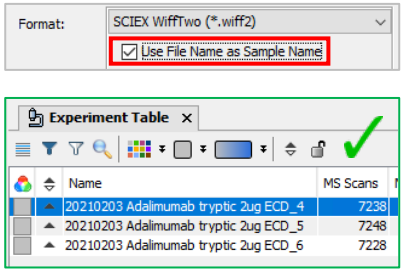
1. To load replicates that have one sample in each wiff or wiff2 container, select **Use File Name as Sample Name**:

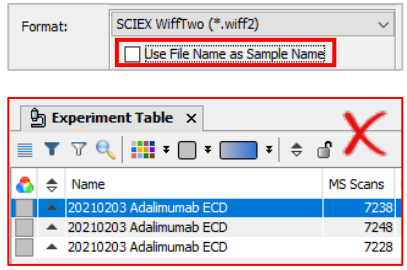


Single sample in a wiff2

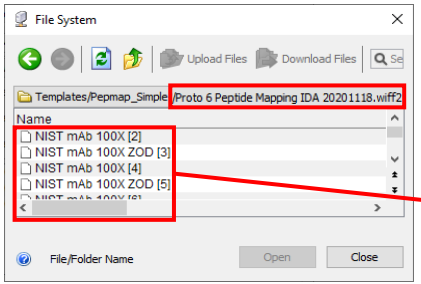


wiff2 container file selected

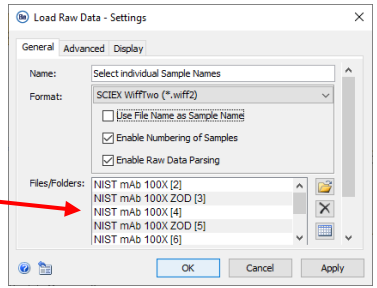




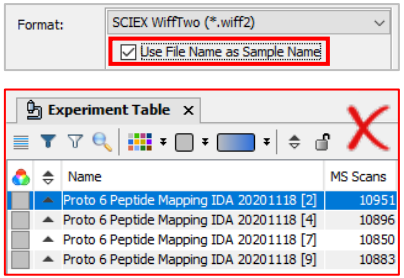
2. To load multiple samples from in the same wiff or wiff2 container, do not select **Use File Name as Sample Name**:

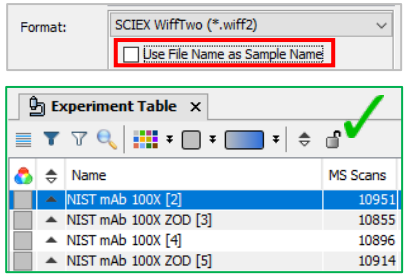


Multiple samples in a wiff2



Individual samples selected





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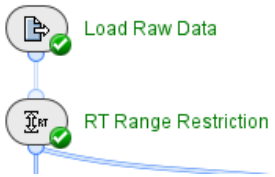
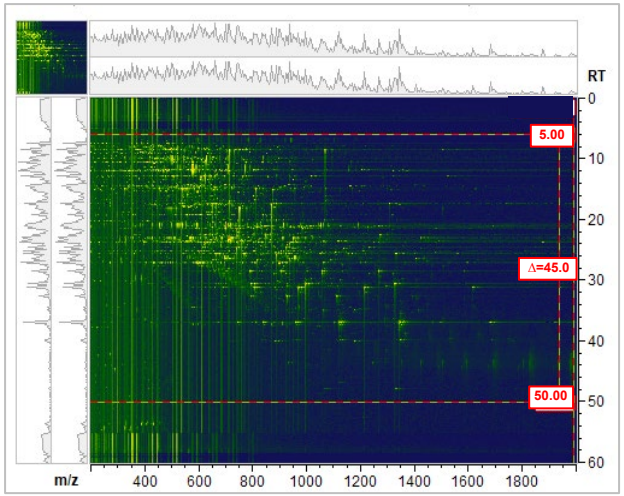
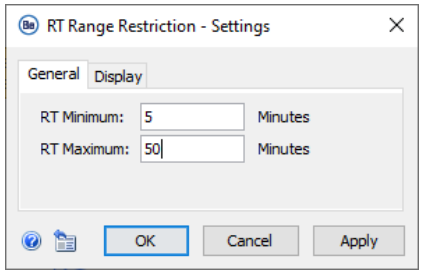
Restrict the RT Range





- To identify the retention time (RT) ranges that contain meaningful data, open (double-click) the results of *Load Raw Data*.
  - Exclude stray signals caused by valve switching or column wash.
  - Focus on the separation range.



Note: If the fields are blank, or if *RT Range Restriction* has the **Bypass** icon activated, then the full RT range is used.

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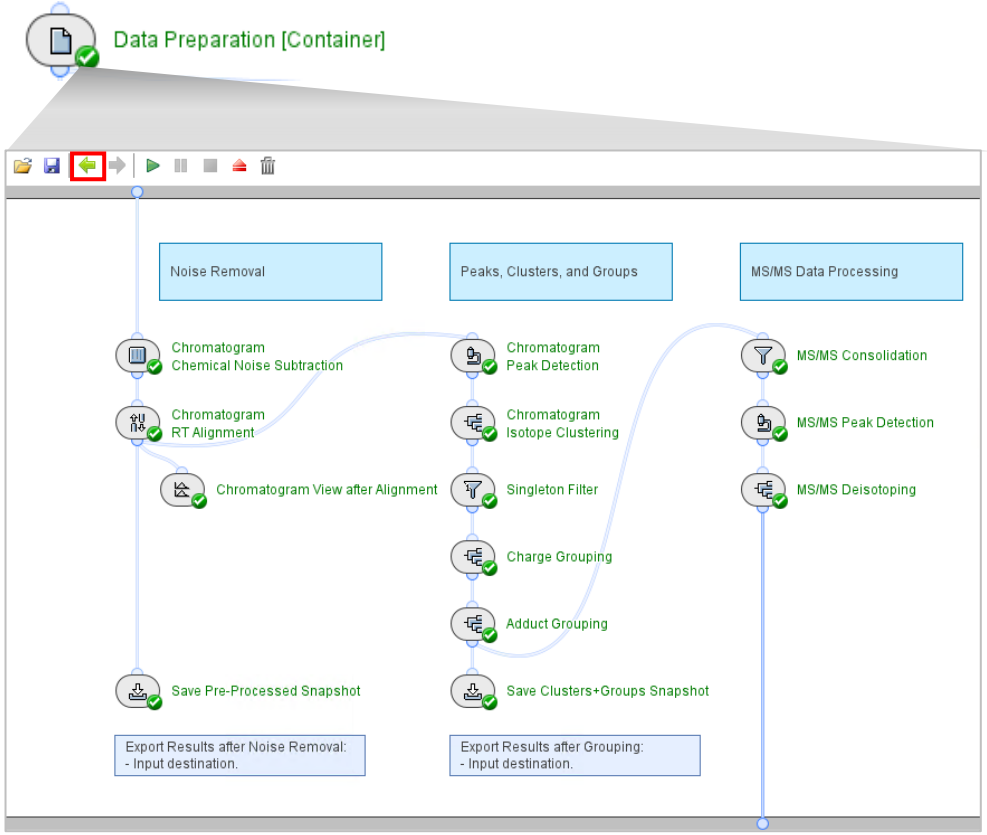
Data Preparation [Container]



- Activity nodes in the *Data Preparation [Container]* process the raw data for analysis.
  - Only optimize the settings of these activity nodes under specific conditions, for example, when troubleshooting a low sequence coverage.
- To return to the main workflow, click the icon.

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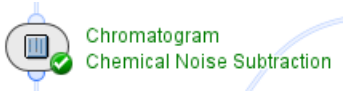
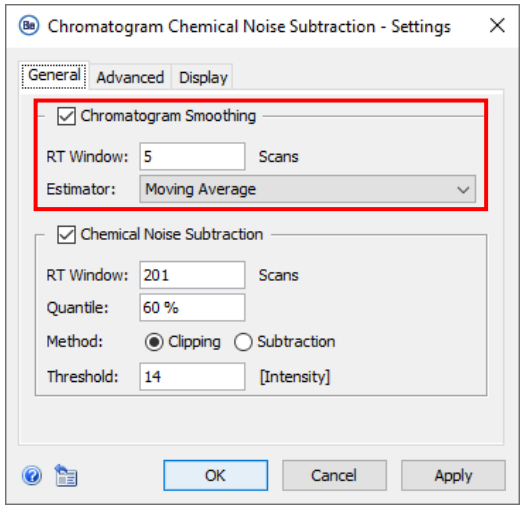
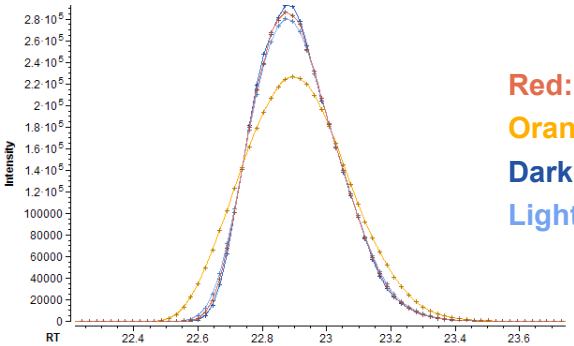
Chromatogram Chemical Noise Subtraction: Smoothing





Chromatogram Smoothing is used to improve the RT profile of peaks for peak detection.

- **Estimator:**
  - **Moving Average** replaces the intensity of each data point with the mean average intensity of the data points in the **RT Window**. High values cause peak widths to increase, but peak volume is not changed.
  - **Binomial** is an iterative form of **Moving Average** that has less effect on peak widths at high scan values.



Red: Moving Average (5 scans)
Orange: Moving Average (15 scans)
Dark Blue: Binomial (5 scans)
Light Blue: Binomial (15 scans)

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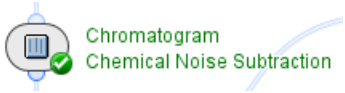
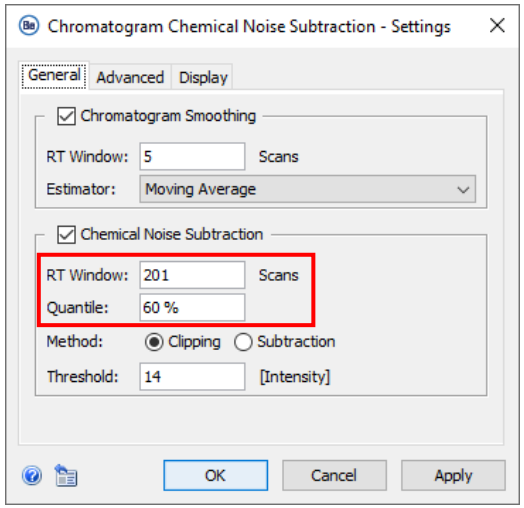
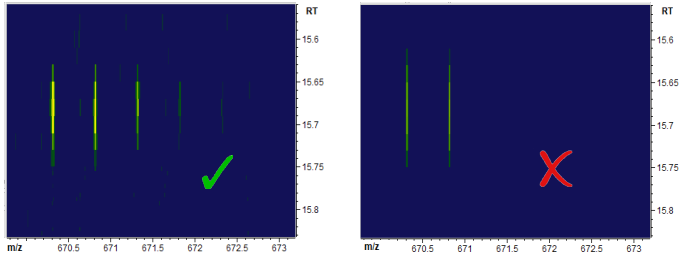
Chromatogram Chemical Noise Subtraction





- **Chemical Noise Subtraction** decreases the length of long-tailing peaks.
- Change this setting if the default values remove too much signal.
- If too much signal if removed, it can be identified by:
  - Excessive cutoff of the tails of very wide (extended RT) peaks.
  - Loss of low-intensity isotope peaks from singly (+1) or doubly (+2) charged clusters, or from low-intensity clusters of interest:



- To decrease the amount of noise removal (keep more signal):
  - Decrease the **Quantile**.
  - Increase the **RT Window**.

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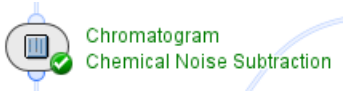
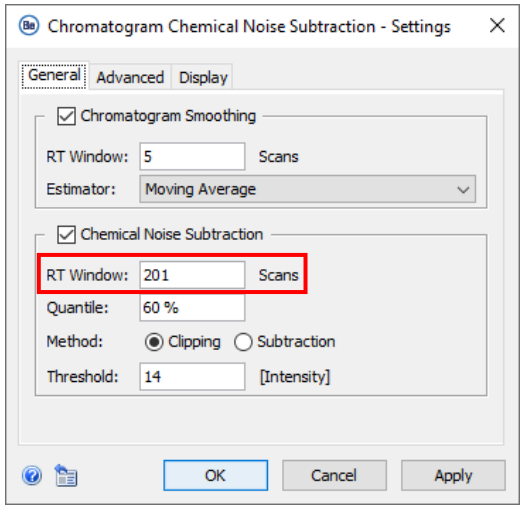
Chromatogram Chemical Noise Subtraction: RT Window

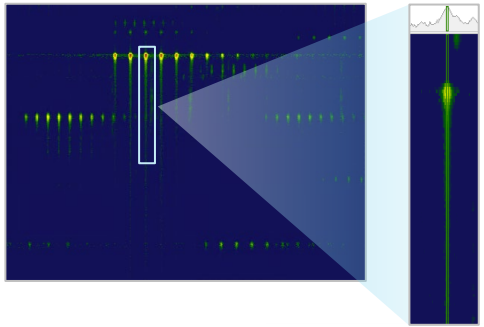




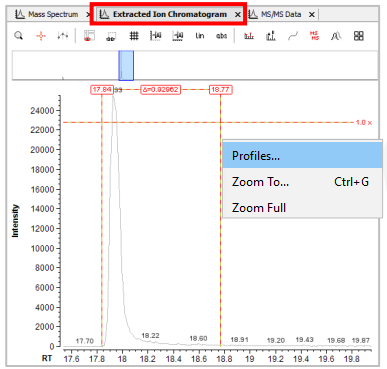
- Generally, the **RT Window** should be at least double the number of scans across the largest peak in the dataset.

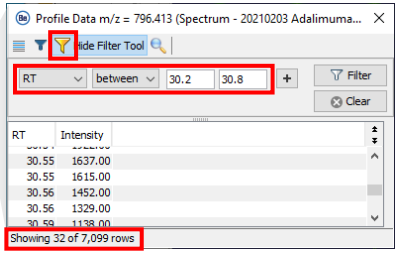
To calculate the number of scans:

1. Find a feature that extends over a longer RT than other features in the ion map.
2. Take a vertical slice to create an **Extracted Ion Chromatogram**.
3. Right-click in the **Extracted Ion Chromatogram** window, and then select **Profiles**.
4. Use the **Advanced Filter Tool** to select the RT range for the peak.
5. Record the number of scans shown, and then enter at least double this value for the **RT Window** in the settings.



Take a vertical slice





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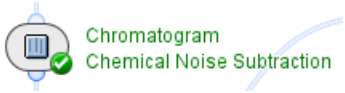
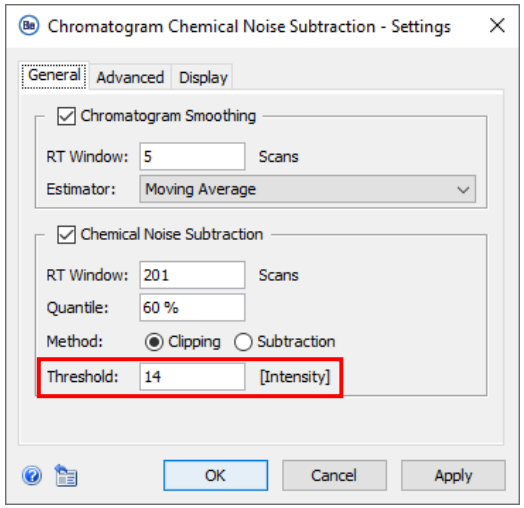
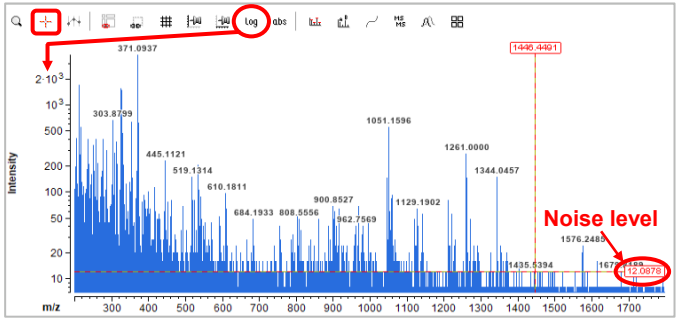
Chromatogram Chemical Noise Subtraction: Threshold





- If the noise level is significantly different from the **Threshold** value pre-set in the *Chromatogram Chemical Noise Subtraction* activity node, then change this setting.
- To measure the noise level and identify an applicable **Threshold** intensity value:
  1. Drag the intensity axis of the mass spectrum until the noise level can be seen, or use the icon in the tool bar to change the axis from the linear to the logarithmic scale.
  2. Use the crosshair tool to measure the intensity of the noise level.



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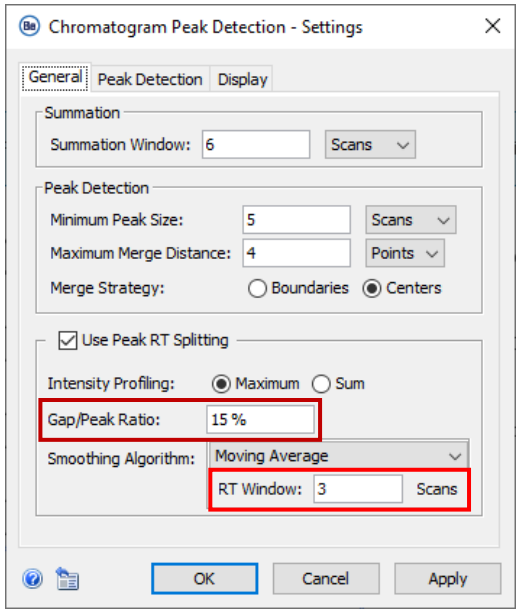
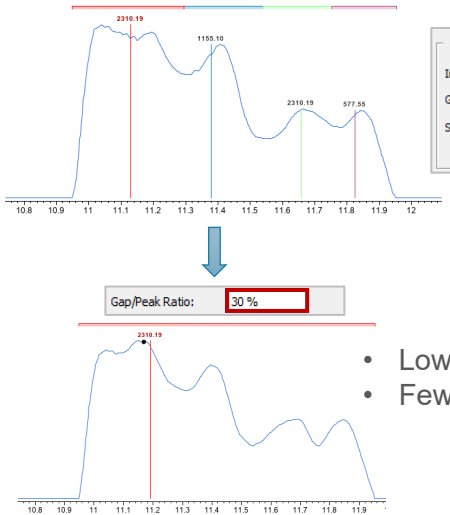
Chromatogram Peak Detection: Use Peak RT Splitting

- Shoulder peaks, in the RT direction, can be detected as a single peak, or as multiple separate peaks.
  - To increase the number of peaks detected (increase split sensitivity):
    - Decrease the **Gap/Peak Ratio**.
    - Decrease or remove **Smoothing**.





- Lower split sensitivity
- Fewer peaks

- Higher split sensitivity
- More peaks

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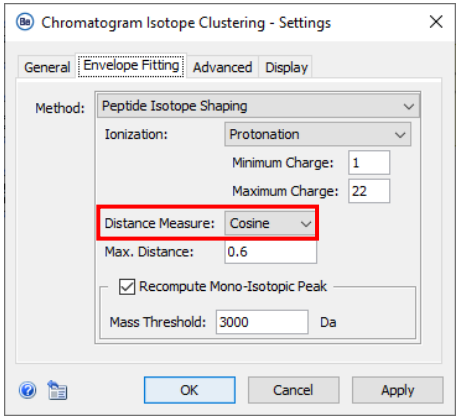
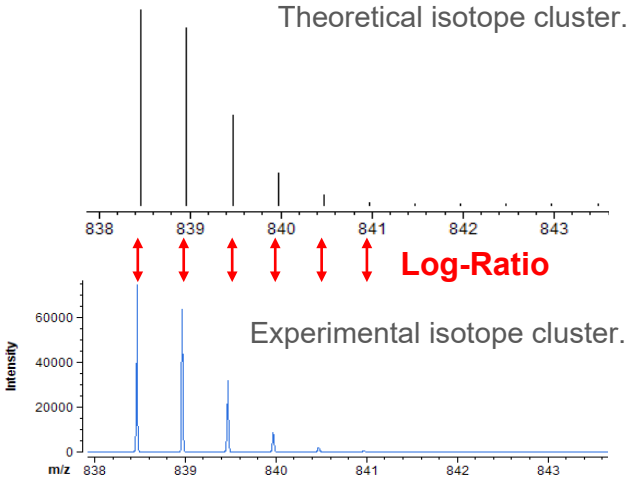
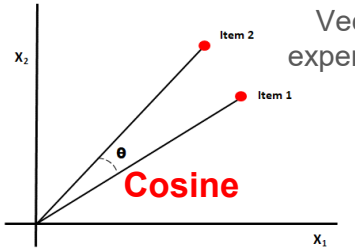
Chromatogram Isotope Clustering: Distance Measure

- The **Distance Measure** compares each experimental peak to the theoretical isotope profile for that peptide.



- ***Log Ratio*** treats all peaks in a cluster equally.
- ***Cosine*** converts each cluster into a vector, with the contribution of each peak relative to the abundance. Therefore, smaller peaks have less impact.





Vectors of theoretical and experimental isotope clusters.

- If a peptide of interest has not been clustered as required, then compare the results of **Log Ratio** and **Cosine**.

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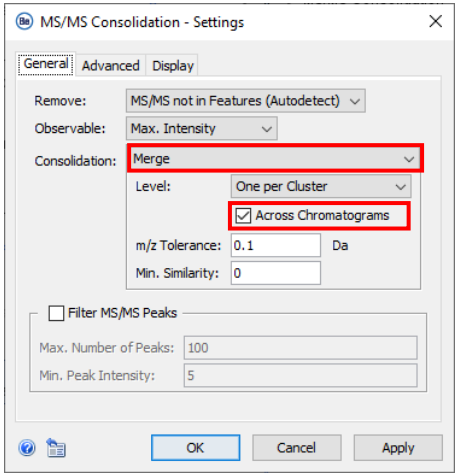
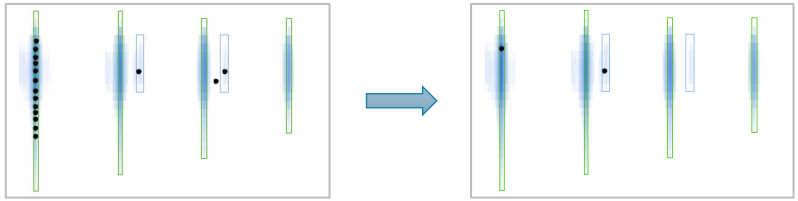
MS/MS Consolidation: Merge Across Chromatograms

- This activity node merges MS/MS data across equivalent peaks and clusters.
  - Consolidation can improve MS/MS spectra, and increase identifications.
  - Consolidation can decrease false positives if MS/MS spectra are too ambiguous.





- To use MS/MS data from technical replicates to increase confidence in identifications, select to **Merge** MS/MS data **Across Chromatograms**.
  - Merged MS/MS data are assigned to the sample with the highest intensity for that spectrum.
- To measure individual sample sequence coverage, do not select this option.

Coverage

Tra_HC

	Total	Mass Only	MS/MS Only	Mass and MS/MS
Overall	94.0%	0.0%	0.0%	94.0%
Trastuzumab [1]	94.0%	0.4%	0.0%	93.6%
Trastuzumab [2]	94.0%	0.4%	0.0%	93.6%
Trastuzumab [3]	94.0%	0.4%	0.0%	93.6%
Trastuzumab [4]	94.0%	0.0%	0.0%	94.0%

☐ Across Chromatograms

Coverage

Tra_HC

	Total	Mass Only	MS/MS Only	Mass and MS/MS
Overall	94.0%	0.0%	0.0%	94.0%
Trastuzumab [1]	94.0%	34.7%	0.0%	59.3%
Trastuzumab [2]	94.0%	7.1%	0.0%	86.9%
Trastuzumab [3]	94.0%	21.3%	0.0%	72.7%
Trastuzumab [4]	94.0%	10.0%	0.0%	84.0%

☑ Across Chromatograms

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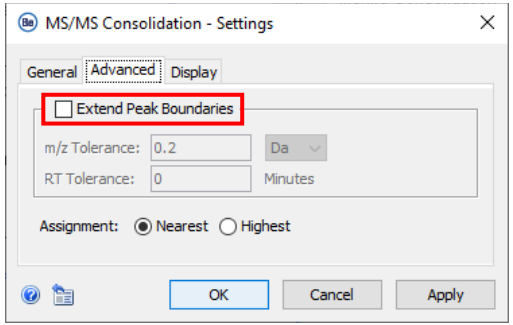
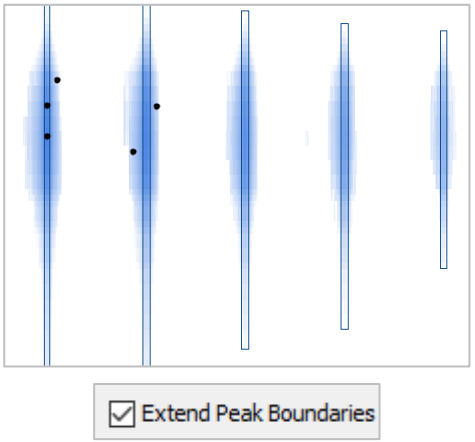
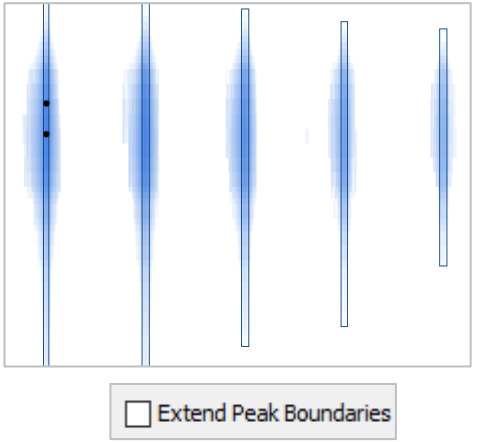
MS/MS Consolidation: Extend Peak Boundaries



- To analyze data acquired from a TripleTOF 5600/5600+, TripleTOF 6600/6600+, or X500B QTOF mass spectrometer, activate the **Extend Peak Boundaries** option.
  - This setting includes MS/MS data located outside of the peak boundaries in the consolidation.



MS/MS data outside of the peak boundaries is kept.



MS/MS data outside of the peak boundaries is removed.

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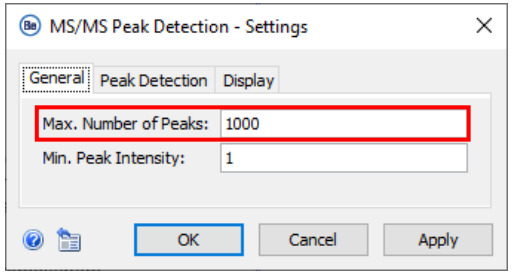
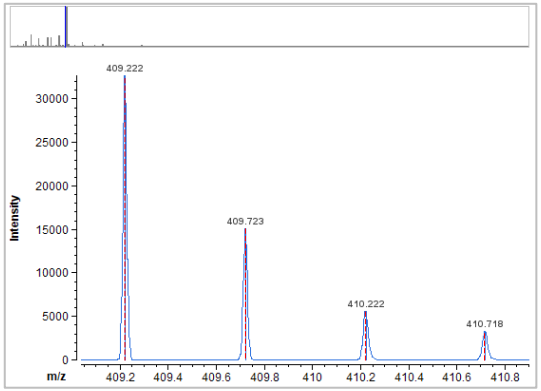
MS/MS Peak Detection



- This activity node detects peak centroids in MS/MS profile data.
  - Peak centroids are required for Peptide Mapping.



- To increase the likelihood of identifying long (high mass) peptides with EAD data, increase the **Max. Number of Peaks** to 5000 to keep more of the low-intensity MS/MS ions.
  - The number of MS/MS peaks detected, compared to the number of MS/MS peaks used for identification, has an effect on the score.
  - To compensate for the higher number of peaks, decrease the **Min. Score** in *Peptide Mapping.*
    - Note: A lower score increases the risk of false positive identifications.

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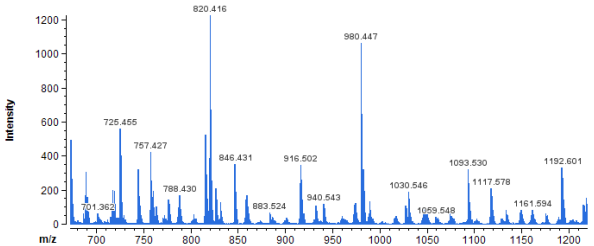
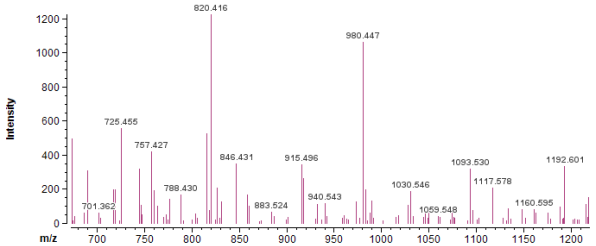
MS/MS Deisotoping

- This activity node removes the isotopic peaks in high-resolution MS/MS data to give singly-charged (deisotoped) monoisotopic peaks.
  - Deisotoping decreases the number of MS/MS peaks in the fragment spectra.
  - The number of MS/MS peaks detected, compared to the number of MS/MS peaks used for identification, has an effect on the score.

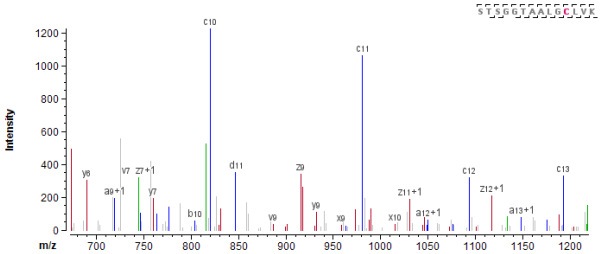
Before *MS/MS Deisotoping*



After *MS/MS Deisotoping*



After *Peptide Mapping*



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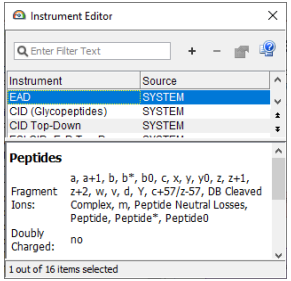
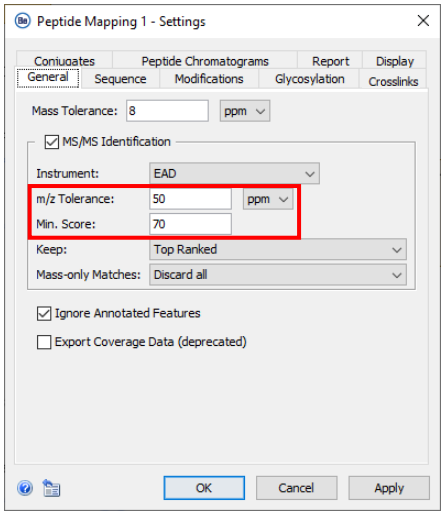
Peptide Mapping: General

- **Instrument**: Select the fragmentation type used for data acquisition.
  - To review or change the types of fragment ions used for identification:
    - Browse to **File** > **Tools** > **Instrument Editor**.





- **m/z Tolerance**:
  - The **m/z Tolerance** value is not an indicator of the instrument mass accuracy. It adjusts for the possible impact on the *m/z* of MS/MS pre-processing.
    - Decrease the **m/z Tolerance** to decrease the number of false positive or ambiguous annotations.
- **Min. Score**:
  - The number of MS/MS peaks has an effect on the optimal score threshold.
    - The MS/MS score is related to the number of identified and unidentified peaks in the spectrum.
    - Information-rich MS/MS spectra with many peaks can be confidently identified with a lower score.
  - Increase the **Min. Score** to decrease the number of false positives or ambiguous annotations.

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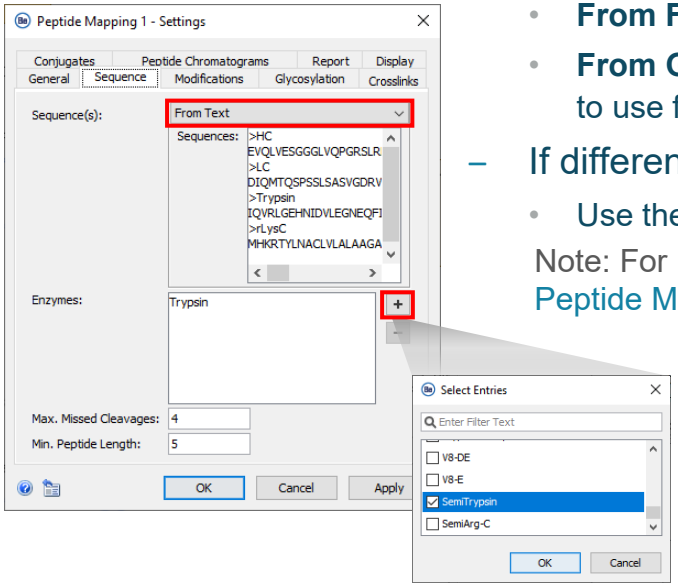
Peptide Mapping: Sequence

- **Sequence(s)**:
  - If all samples have the same sequence, enter it in one of these formats:
    - **From Text**: Enter the protein sequence in the **Sequences** box.
    - **From Fasta File**: Select a FASTA file that contains the sequence of interest.
    - **From Global File**: Select a FASTA file with multiple entries, and then select the sequences to use from the pop-up window.
  - If different samples require different sequences:
    - Use the Batch Processing workflow.

Note: For more information about Batch Processing, refer to the section **B:** 5.Guidelines for Peptide Mapping Batch Processing Workflows.



- **Enzymes**:
  - To see the list of system-configured and user-defined enzymes, use the **+** icon to open the **Select Entries** dialog.
  - Adjust enzyme specificity, maximum number of missed cleavages, and minimum peptide length as required.

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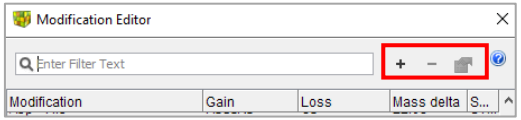
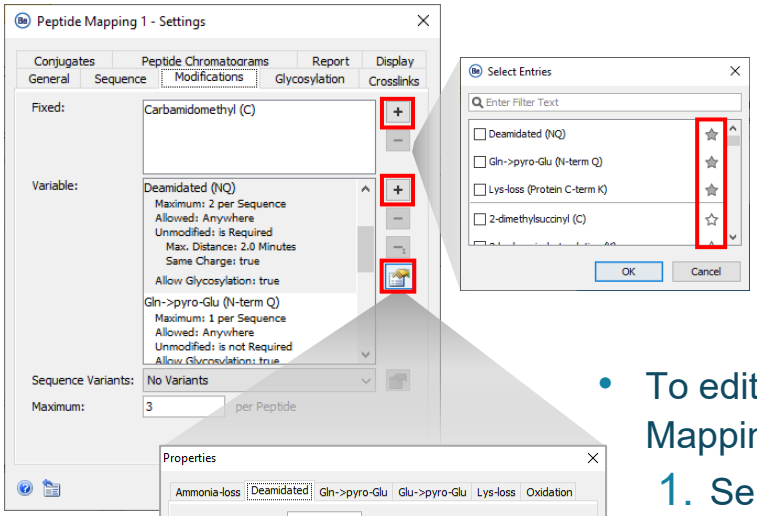
Peptide Mapping: Modifications

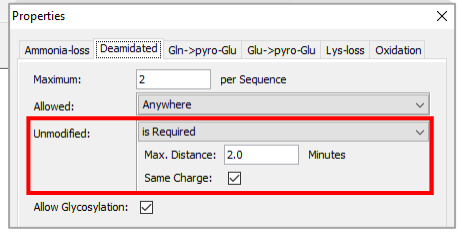
- To review existing modifications or to add custom modifications:
  - Browse to **File** > **Tools** > **Modification Editor**.





Modifications tab:

- To see the list of available **Fixed** or **Variable** modifications, use the **+** icon to open the **Select Entries** dialog.
  - To add commonly used modifications as favorites, select the star ☆ icon.



- To edit the properties of modifications selected for a particular Peptide Mapping search:
  1. Select all **Variable** or **Fixed** modifications to edit.
  2. Use the  icon to open the **Properties** dialog.
     - Select if the **Unmodified** peptide is required for the modification to be identified, and if so, how closely the two forms must elute.

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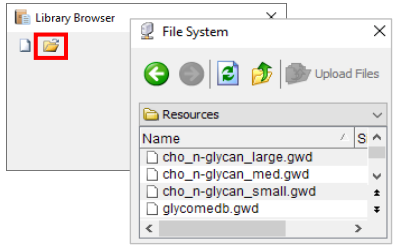
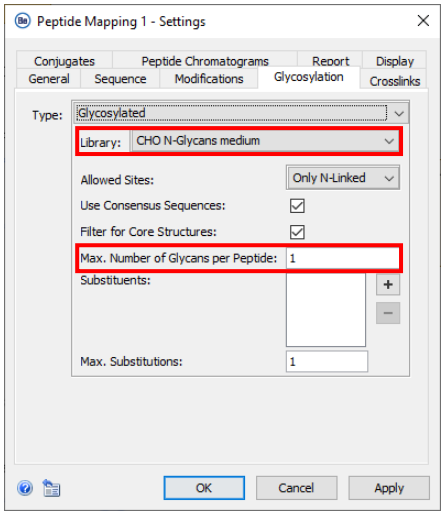
Peptide Mapping: Glycosylation (1)

Glycosylation tab:





- **Library**: Select a system-configured or user-defined library.
  - To review or change a glycan library: Browse to **File** > **Tools** > **Library Browser** > **Resources**.
- A search for multiple glycosylation sites can produce a high number of results and take a long time to complete. General guidance to control the search space:
  - **Max. Number of Glycans per Peptide**: For the search to proceed, the predefined threshold number of Estimated Glycopeptide Candidates cannot be exceeded.

    Note: For more information, refer to the next page: *Peptide Mapping*: Glycosylation Tab (2).
  - **Allowed Sites: Only *N*-linked**:
    - **Max. Number of Glycans per Peptide:** The maximum allowed value is 4.
    - Fewer missed cleavages and variable modifications decreases the search time.
  - **Allowed Sites: Only *O*-linked**: The search criteria must be controlled because every serine (S) and threonine (T) residue is a potential *O*-glycosylation site.
    - Long peptides that contain many potential glycosylation sites have a large effect on the number of Estimated Glycopeptide Candidates, and the subsequent processing time.
    - To decrease the total number of candidates and the search time, use enzymes that create shorter peptides. For example, select Trypsin/P so that cleavage is not restricted at RP/KP.

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Peptide Mapping: Glycosylation (2)

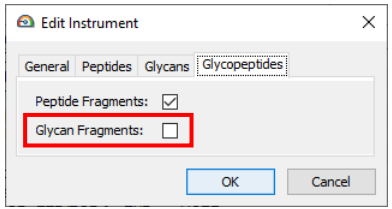
Example of permitted search combinations for *O*-glycans in Etanercept digested with trypsin:

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	✓	✓	✓	×	×



Note: Disable **Glycan Fragments** for glycopeptides in the **Edit Instrument** settings to decrease the time required for complex glycan searches. For glycopeptide analysis of CID data, enable **Glycan Fragments** for better MS/MS coverage.

Glycosylation tab:

- A combination of factors is used to calculate the number of Estimated Glycopeptide Candidates, which controls if the search will proceed:
  1. The number of glycans in the glycan library (including substituents).
     - Use the smallest library size that contains the applicable glycans of interest.
     - Select **Filter for Core Structures** to decrease the number of candidates.
  2. The **Max. Number of Glycans per Peptide**.
     - Enter the estimated number of glycans for the molecule. Larger libraries can be used with fewer glycans per peptide, and *vice versa*.
  3. The theoretical sites of glycosylation on a peptide.
     - The number of missed cleavages and the enzyme specificity have an effect on the total number of theoretical sites of glycosylation.
- Other search parameters have an effect on the overall search time.
  - To decrease 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.
    - Decrease the number of glycans per peptide.
    - Decrease the size of the glycan library.

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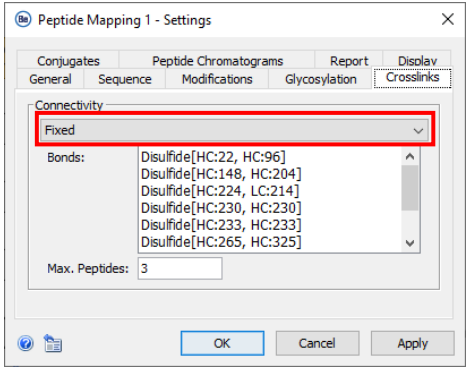
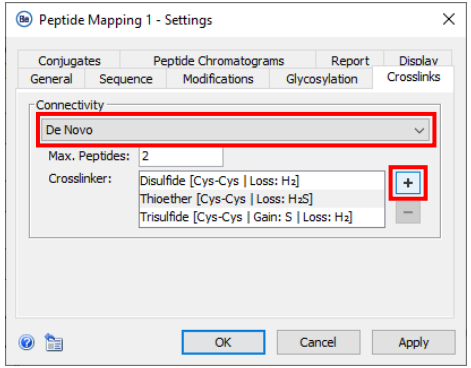
Peptide Mapping: Crosslinks (1)





Crosslinks tab:

- For reduced samples: Set **Connectivity** to **None**.
- For non-reduced samples, do one of the following:
  1. Set **Connectivity** to **Fixed**.
     - Use the correct syntax to enter the known disulfide bonds: enter the type of crosslink, and then the protein and amino acid position for each bond.
       - For example: **Disulfide[HC:22, HC:96]** or **Trisulfide[HC:371, LC:194]**
       - The chain names must be identical to those specified in the **Sequence** tab.
       - Different crosslinks cannot contain the same location.
     - Enter the number of crosslinked peptides to include in the search.
       - A **Max. Peptides** value of 3 or more increases the search time.
  2. Set **Connectivity** to **De Novo**.
     - Use the **+** icon to select the crosslinkers of interest.
     - Enter the number of crosslinked peptides to include in the search.
       - A **Max. Peptides** value of 3 or more increases the search time.
     - Enter the expected bonds in the **Crosslinks** tab of *Automatic Review*.

Note: For more information, refer to the page: *Automatic Review*: Crosslinks in this section (**A:** 2. General Guidelines for Peptide Mapping Workflows).

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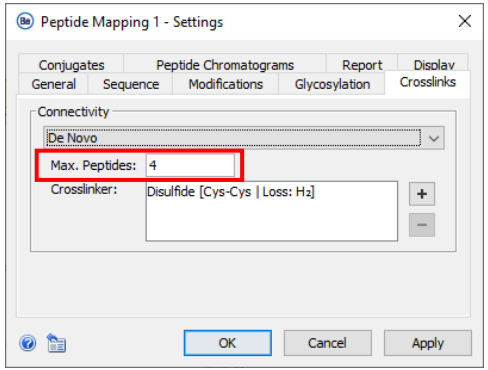
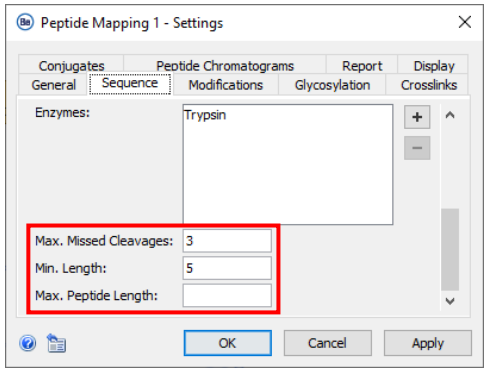
Peptide Mapping: Crosslinks (2)





The **De Novo** search can take a long time to complete and create a large number of possible crosslinked peptides. For the search to start, the number of possible crosslinked candidates cannot be more than the threshold of 2.5 million.

- The following *Peptide Mapping* parameters have an effect on the number of Crosslink Candidates:
  - **Crosslinks** tab:
    - **Max. Peptides**: Enter the maximum number of peptides in a complex.
      - A **Max. Peptides** value of 3 or more increases the search time.
  - **Sequence** tab:
    - **Max. Missed Cleavages**: Enter the maximum number of missed cleavages in a peptide.
      - In a crosslinked complex, each individual peptide can have up to this number of missed cleavages.
    - **Min. Length:** Enter the minimum length of a feature.
      - In a crosslinked complex, the minimum length is the total length of all peptide chains. For example, the crosslinked complex XX=XXXXX has a length of 7.
    - **Max. Peptide Length:** Enter the maximum length of a peptide in a complex.
      - In a crosslinked complex, the maximum length is the length of each individual peptide.

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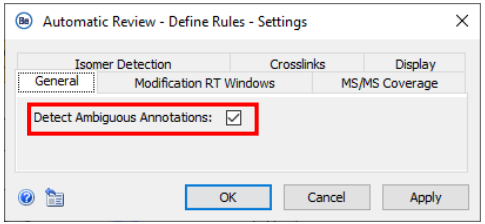
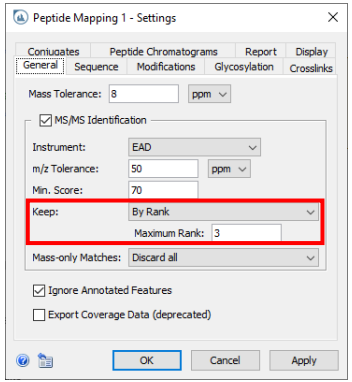
Automatic Review: Ambiguous Annotations

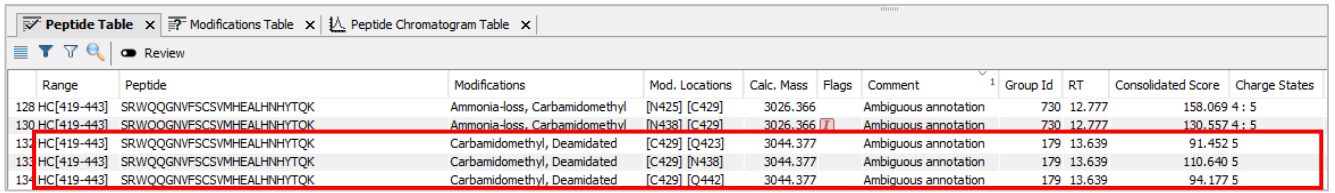
- Use *Automatic Review* to define the criteria to highlight *Peptide Mapping* results that require manual inspection.
  - Information is added to the **Flag** or **Comment** columns for the applicable entries in the **Peptide Table** in *Review Results*.



General tab:

- To add Ambiguous annotation to the **Comment** column in the **Peptide Table** for features that have multiple annotations, select **Detect Ambiguous Annotations**.





	Range	Peptide	Modifications	Mod. Locations	Calc. Mass	Flags	Comment	Group Id	RT	Consolidated Score	Charge States
128	HC[419-443]	SRWQQGNVFSCSVMHEALHNHYTQK	Ammonia-loss, Carbamidomethyl	[N425] [C429]	3026.366		Ambiguous annotation	730	12.777	158.069	4 : 5
130	HC[419-443]	SRWQQGNVFSCSVMHEALHNHYTQK	Ammonia-loss, Carbamidomethyl	[N438] [C429]	3026.366		Ambiguous annotation	730	12.777	130.557	4 : 5
132	HC[419-443]	SRWQQGNVFSCSVMHEALHNHYTQK	Carbamidomethyl, Deamidated	[C429] [Q423]	3044.377		Ambiguous annotation	179	13.639	91.452	5
133	HC[419-443]	SRWQQGNVFSCSVMHEALHNHYTQK	Carbamidomethyl, Deamidated	[C429] [N438]	3044.377		Ambiguous annotation	179	13.639	110.640	5
134	HC[419-443]	SRWQQGNVFSCSVMHEALHNHYTQK	Carbamidomethyl, Deamidated	[C429] [Q442]	3044.377		Ambiguous annotation	179	13.639	94.177	5

Note: For more information on this example, refer to the page: *Review Results*: Isomer Differentiation in this section (**A:** 2. General Guidelines for Peptide Mapping Workflows).

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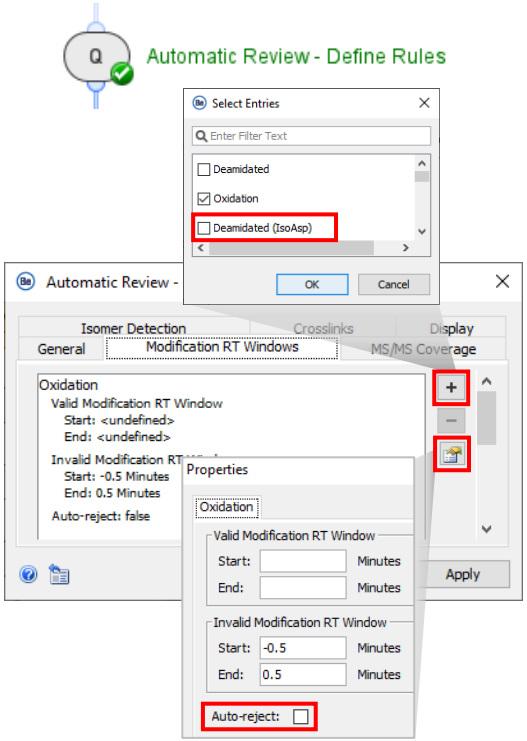
Automatic Review: Modification RT Windows



Modification RT Windows tab:

- To see the list of available modifications, use the **+** icon to open the **Select Entries** dialog.
  1. Select the modifications to monitor with *Automatic Review*.
     - Note: **Deamidated** and **Deamidated (IsoAsp)** are two separate modification entries in the dialog.
  2. Enter a time window when a modified peak must be detected in relation to the unmodified peak.
     - **Valid Modification RT Window**: Modified peaks with a RT outside of this window have a Q (Questionable) in the **Flag** column and information added to the **Comment** column.
     - **Invalid Modification RT Window**: Modified peaks with a RT inside this window have a Q (Questionable) in the **Flag** column and information added to the **Comment** column.

Modifications	Flags	Comment	RT
Carbamidomethyl, Oxidation	Q	Oxidation RT window rule violated (unmodified molecule at RT 12.7770)	12.490
Oxidation	Q	Oxidation RT window rule violated (unmodified molecule at RT 7.7863)	7.652

- To automatically reject peptides that do not meet the specified criteria, select **Auto-reject**.

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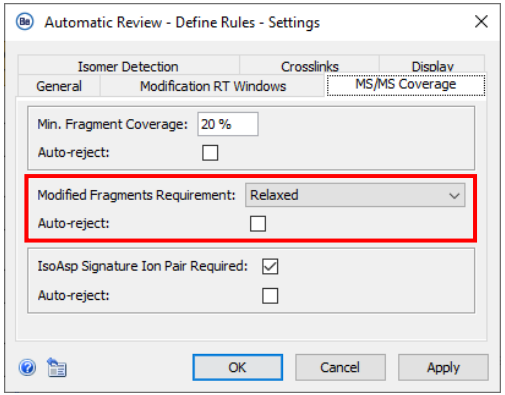
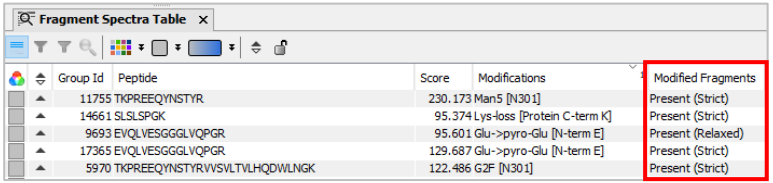
Automatic Review: MS/MS Coverage

MS/MS Coverage tab:

- **Min. Fragment Coverage**: Enter a minimum peptide MS/MS coverage.
  - Peptides with MS/MS coverage below the threshold have a **Q** flag and the comment MS/MS coverage below XX% in the **Peptide Table** of *Review Results*.
- **Modified Fragments Requirement**: Select the level of MS/MS fragment ion evidence that is required to validate peptides with **Variable** modifications.
  - **Strict**: Requires evidence of MS/MS ions on both sides of a modified amino acid.
    - Variable modifications that do not meet this criterion have a **Q** flag and the comment Modified fragments (strict) not found in the **Peptide Table** of *Review Results*.
  - **Relaxed**: Requires evidence of MS/MS ions on one side of a modified amino acid.
    - Variable modifications that do not meet this criterion have a **Q** flag and the comment Modified fragments (relaxed) not found in the **Peptide Table** of *Review Results*.
  - **None**: Requires no evidence of MS/MS fragment ions on either side of a modified amino acid. Select this option to accept all modified peptides.



- To automatically reject peptides that do not meet the specified criteria, select **Auto-reject**.



Modified peptides have either **Present (Strict)** or **Present (Relaxed)** in the **Modified Fragments** column in the **Fragment Spectra Table**.

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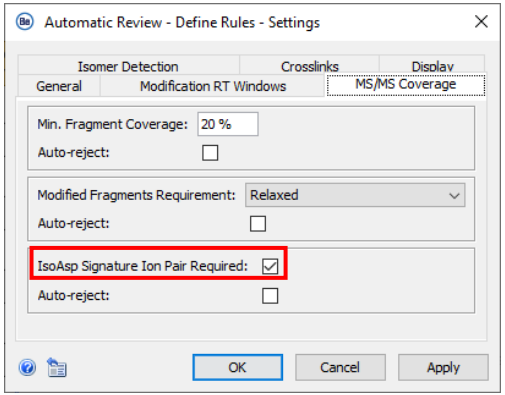
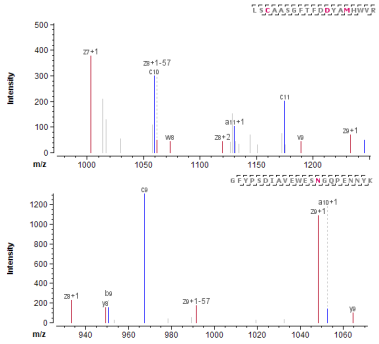
Automatic Review: MS/MS Coverage

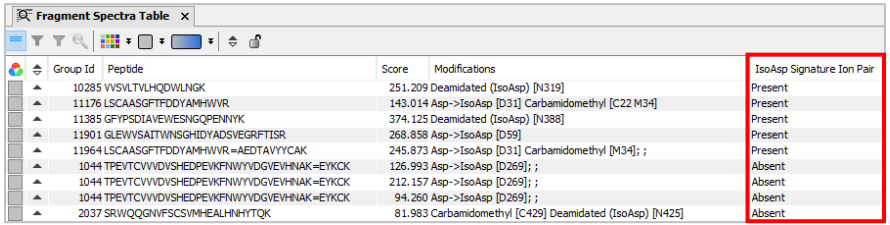


- To automatically reject peptides that do not meet the specified criteria, select **Auto-reject**.

MS/MS Coverage tab:

- **IsoAsp Signature Ion Pair Required**: Select this option if peptides that contain **Asp→IsoAsp** or **Deamidated (IsoAsp)** modifications must have at least one of the signature fragment ion pairs (c and c+57, or, z and z-57) identified.
  - Signature ion pairs include all relevant c or z class ions, for example, z+1, z+1-57, z+1, z-57.
  - Peptides that do not meet this criterion have a **Q** flag and the comment IsoAsp signature ion pair not found in the **Peptide Table** of *Review Results*.
  - Relevant peptides have either **Present** or **Absent** in the **IsoAsp Signature Ion Pair** column in the **Fragment Spectra Table**.





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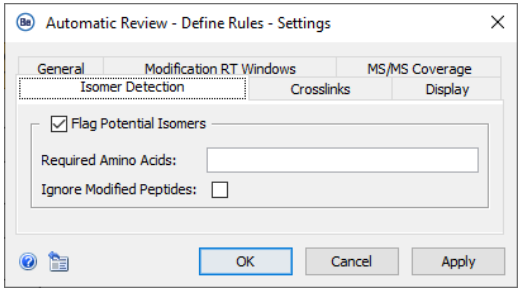
Automatic Review: Isomer Detection



Isomer Detection tab:

- Select this option to add an **I** (Potential Isomer) flag to peptides that have identical annotations but different Group IDs. This indicates the presence of potential isomers for manual review.
  - **Required Amino Acids:** Enter the single letter code for amino acids that must be in a peptide for it to be identified as an isomer candidate.
  - **Ignore Modified Peptides:** Select this option to ignore isomer candidates that contain modifications.

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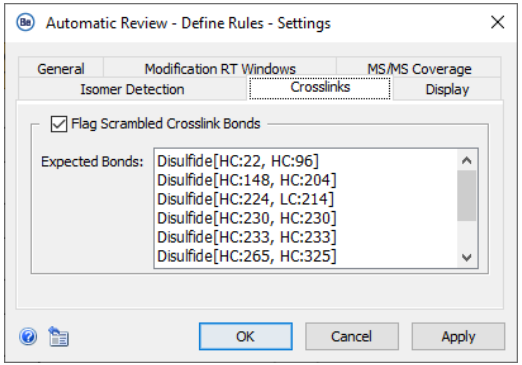
Automatic Review: Crosslinks



Crosslinks tab:

- Select **Flag Scrambled Crosslink Bonds** to add a ≠ flag and the comment Contains unexpected crosslink bonds to the **Peptide Table** in *Review Results*.
  - **Expected Bonds:** Use the correct syntax for the known disulfide bonds. Enter the type of crosslink, and then the protein and amino acid position for each bond.
    - For example: Disulfide[HC:22, HC:96] or Trisulfide[HC:371, LC:194]
    - The protein names must be identical to those in the **Sequence** tab.
    - Different crosslinks cannot contain the same location.

Note: In Biologics Explorer Software 5.0, peptide mapping results of crosslinked peptides with **Modification RT Windows** flags should be manually assessed. **Auto-Reject** should not be selected.

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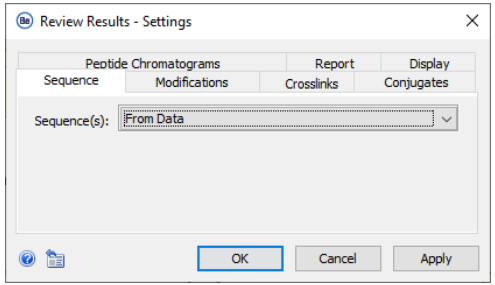
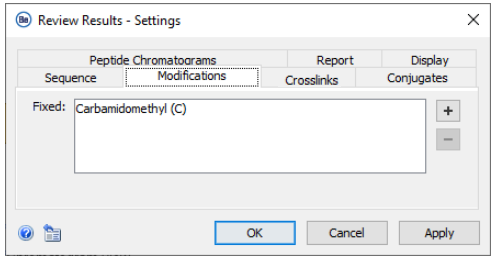
Review Results: Configure Settings



Sequence tab:

- **Sequence(s)**:
  - To use the sequence information specified in the previous *Peptide Mapping* activity nodes, select **From Data**.



Modifications tab:

- Select the **Fixed** modifications specified in the previous *Peptide Mapping* activity nodes.

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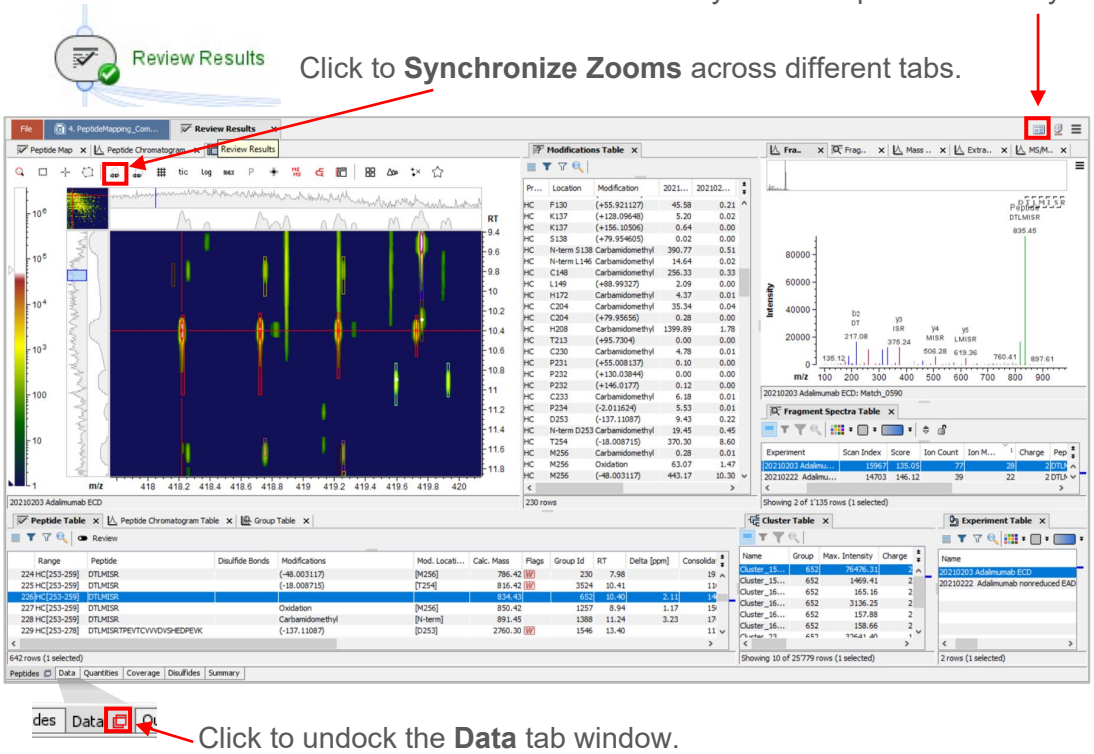
Review Results: Create Custom Layouts

Click to save the active layout or to open a saved layout.



- Each pane can be undocked and then docked at a new location.
- The location where the pane will be docked is highlighted by a blue box.
- To move useful tables and visualizers to the **Peptides** tab:
  1. To undock the **Data** tab, click the icon.
  2. To undock any pane from the **Data** tab window, drag it to a new location on the **Peptides** tab.
- Favorite layouts can be saved and opened with the **Layout** icon.

Note: For more information, refer to the document: Biologics Explorer Software Quick Guide.

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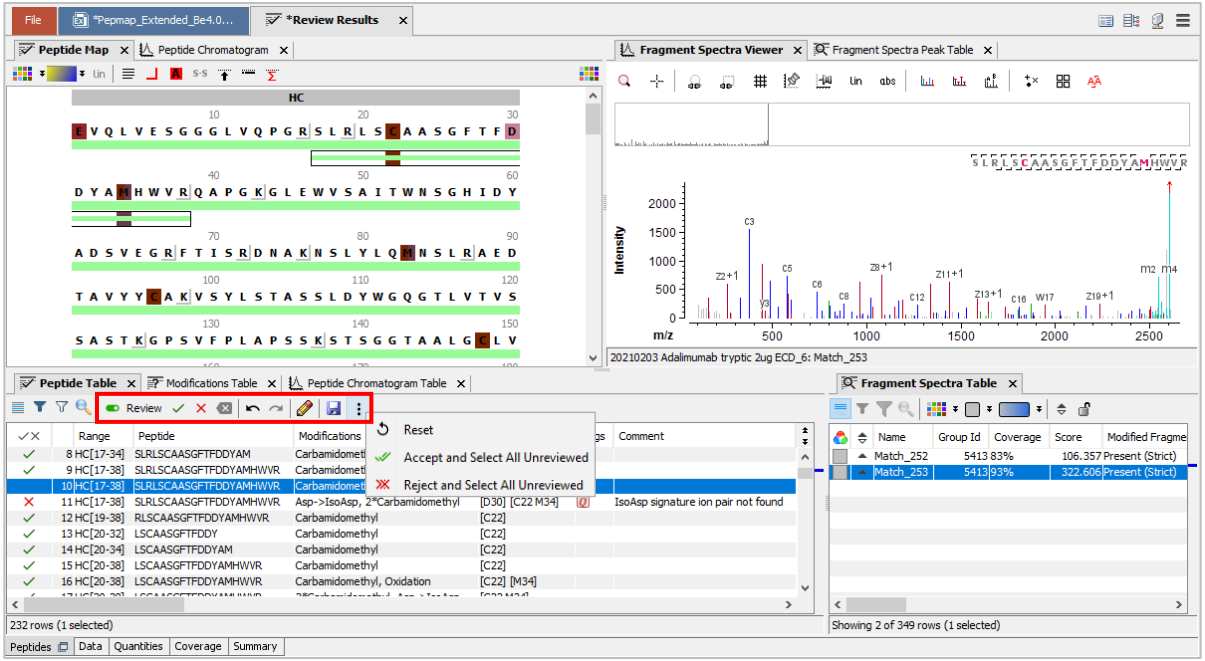
Review Results: Review Peptide Mapping Results

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



1. Activate the **Review** mode, and then **Accept** or **Reject** annotations.
   - To add a comment, either type in the applicable row in the **Comment** column, or use the icon to add the same comment to multiple rows.
2. To apply the changes, click the **Save** icon, and then select **Save and Reload**.

The activity node then automatically updates the peptide **Quantities** table.

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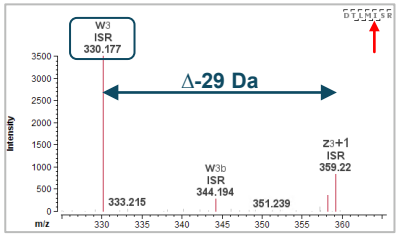
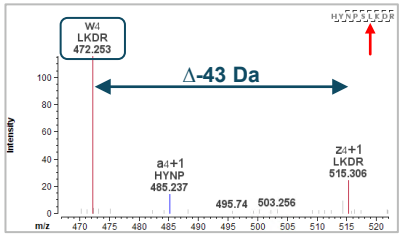
Review Results: Isomer Differentiation

- MS/MS analysis with EAD creates diagnostic internal fragment ions that help to identify different isomeric amino acid residues.

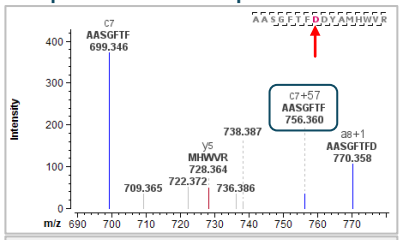
Isoleucine



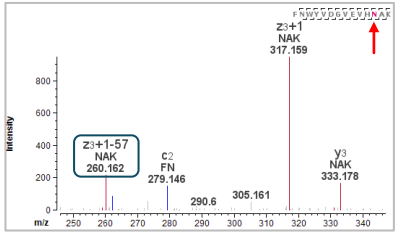
Leucine

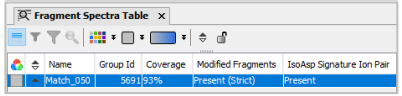


Aspartic acid → IsoAsp



Deamidated asparagine (IsoAsp)





- Leucine (Leu) or isoleucine (Ile):
  - Ions are annotated as: $\mathbf{w}_n$ or $\mathbf{w}_{nb}$.
    - Leucine: $\mathbf{w}_n$ ion at a 43 Da mass shift from the corresponding z (or z+1) ion.
    - Isoleucine: $\mathbf{w}_n$ ion at a 29 Da mass shift from the corresponding z (or z+1) ion.
- Isoaspartic acid (IsoAsp):
  - Ions are annotated as: $\mathbf{c}_n$**+57** or $\mathbf{z}_m$**-57** (or $\mathbf{z}_m$+1**-57**).
    - $\mathbf{c}_n$**+57** or $\mathbf{z}_m$**-57** ions in the MS/MS spectra identify isoaspartic acid.
    - Aspartic acid does not have these diagnostic internal fragment ions because the peptide backbone does not have a methylene group.

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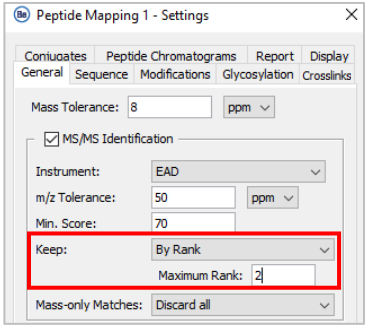
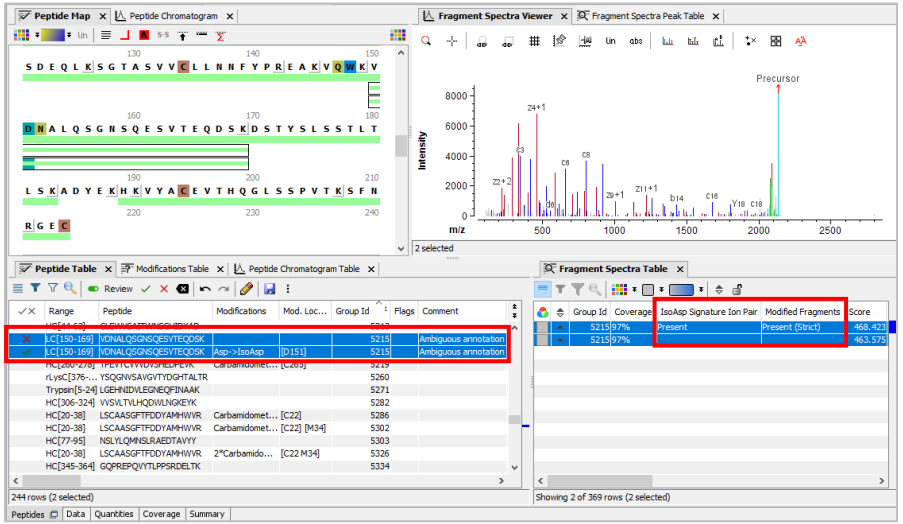
Review Results: Isomer Differentiation

- To see alternative identifications for the same peptide, use **Keep: By Rank** and **Maximum Rank: 2** in the settings for *Peptide Mapping*.



- For data review in the **Peptide Table** in *Review Results*:
  1. Click the **Group Id** column header to order entries numerically. Ambiguous annotations have the same **Group Id**.
     - Select **Detect Ambiguous Annotations** in *Automatic Review*, to add information to the **Comment** column.
  2. Use the diagnostic fragment ions in the MS/MS spectra to validate identifications.
     - To add information about diagnostic fragment ions to the **Comment** column in the **Peptide Table**, select **IsoAsp Signature Ion Pair Required** in *Automatic Review*.
  3. Select **Accept** or **Reject** for each result as required.
  4. To apply the changes, click the **Save** icon, and then select **Save and Reload**.



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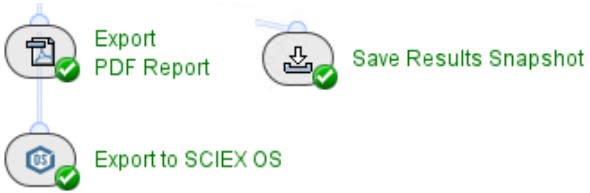
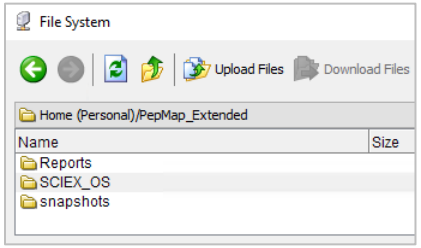
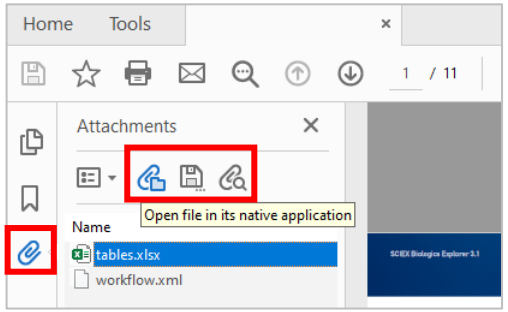
Export and Report Results





- There are three types of activity nodes to report or export results at the end of a workflow:
  - ***Save Results Snapshot***: Reviewed results are saved as sbf files that can be opened in the Pepmap_ReviewSnapshots workflow.
  - ***Export PDF Report***: A summary of results is saved as a PDF.
  - ***Export to SCIEX OS***: Results are saved as txt files that can be used for applications that use the SCIEX OS software.
- Select or add the folders where the results will be stored.



- The exported PDF Report includes:
  - A PDF document.
  - An embedded Excel file.
  - An embedded workflow file (xml) that includes all of the settings.
    - To open the xml file, drag the saved PDF Report into the workflow home page in the Biologics Explorer software.

Note: For more information, refer to the document: Biologics Explorer Software Quick Guide.

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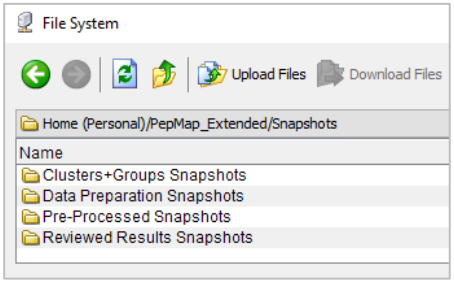
Export Intermediate Results for Further Analysis

- The *Save Snapshot* activity nodes save intermediate results at different stages of a workflow.
  - An individual sbf file is saved for each sample processed in the workflow.
  - The sbf file contains the properties of the processed data that are required to continue analysis from that point in the workflow.
- To use a *Save Snapshot* activity node to save intermediate results:
  - Deactivate the **Block** icon.
  - Select or add the folders where the sbf files will be stored.



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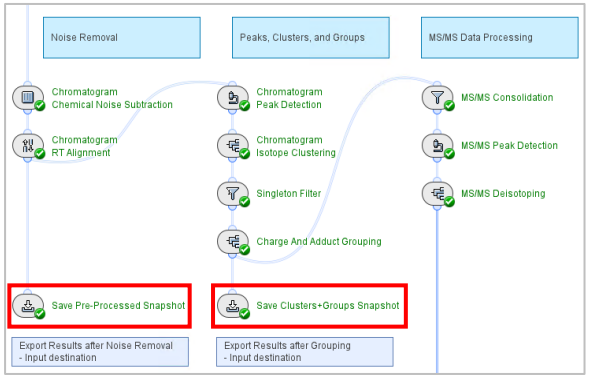
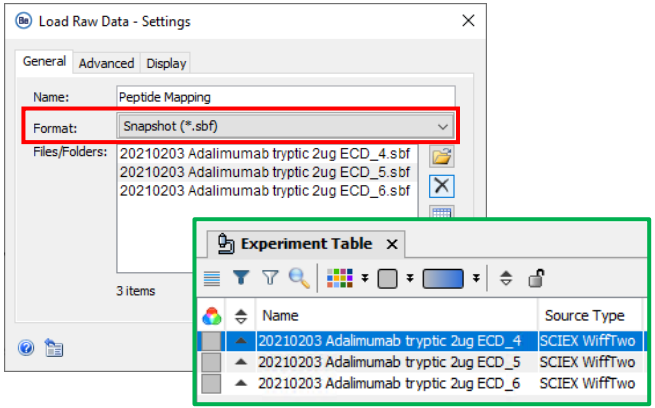
Use Intermediate Results for Further Analysis





- To continue data analysis with saved Snapshots, such as those from in the *Data Preparation [Container]*:
  1. Open a suitable Peptide Mapping workflow.
  2. Select the sbf files to import into *Load Raw Data*.
  3. Change the **Format** to **Snapshot (*.sbf)** or **Auto Detect**.
  4. Activate the **Bypass** icon on the activity nodes in the workflow that are before the Snapshots were saved.
     - For example: To load sbf files from *Save Clusters+Groups Snapshot*, activate the **Bypass** icon for all activity nodes between *Load Raw Data* and *MS/MS Consolidation*.

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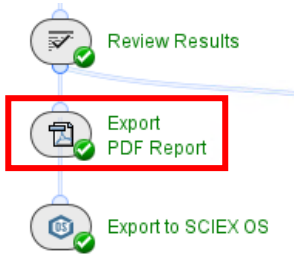
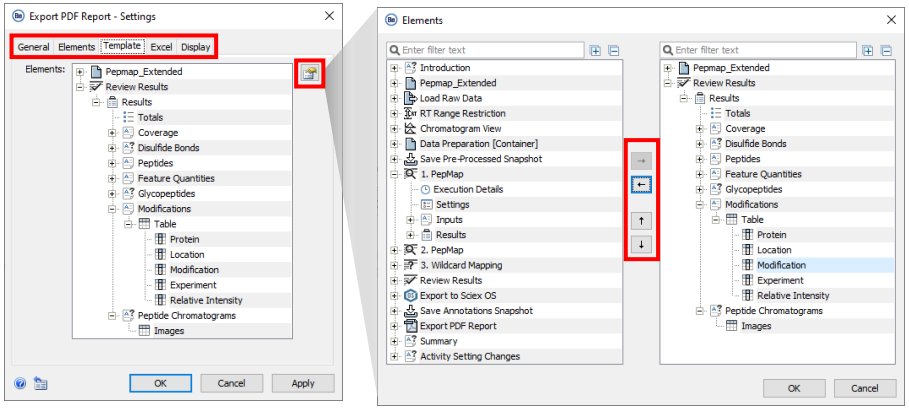
Export PDF Report



- The output of *Export PDF Report* includes:
  - A PDF document.
  - An optional Excel file.
  - An embedded workflow file (xml) that includes all of the settings.
    - To open the xml file, drag the saved PDF Report into the workflow home page in the Biologics Explorer software.
    - Note: For more information, refer to the document: Biologics Explorer Software Quick Guide.

- **General** tab: Enter the name and saved location of the exported report.
- **Template** tab: Use the **Edit Selection** icon to select the **Elements** to be included in the report.
  - Select only columns of interest in reported tables. The layout of the tables is controlled by the number of columns.
- **Excel** tab: Use the **Edit Selection** icon to select the **Tables** to be included in the report.
  - All columns in a selected table are reported.



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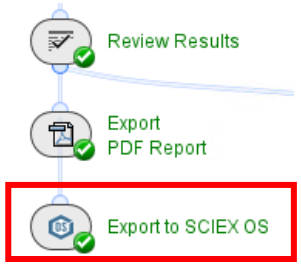
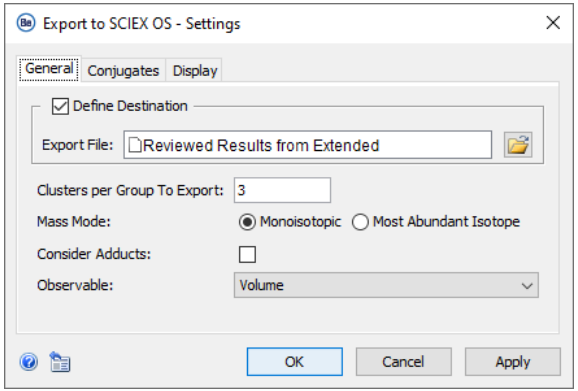
Export to SCIEX OS



Export to SCIEX OS creates a txt file of peptide mapping data that can be imported into the SCIEX OS software for further data processing.

- **General** tab:
  - Enter the name and saved location of the exported report.
  - Select the requirements of the export, for example, the number of clusters for each group, and if adducts are to be included.
  - Use **Observable: Volume** for data acquired using a QTOF mass spectrometer.

  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.



- The *Export to SCIEX OS* activity node should not be used in combination with the *Wildcard Mapping* activity node.
  - Activate the **Bypass** icon on the *Wildcard Mapping* activity node when required.

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Part B
Guidelines for Specific Peptide Mapping Workflows

SCIEX
The Power of Precision

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1. Simple Peptide Mapping

WORKFLOW SPECIFIC INFORMATION AND GUIDELINES

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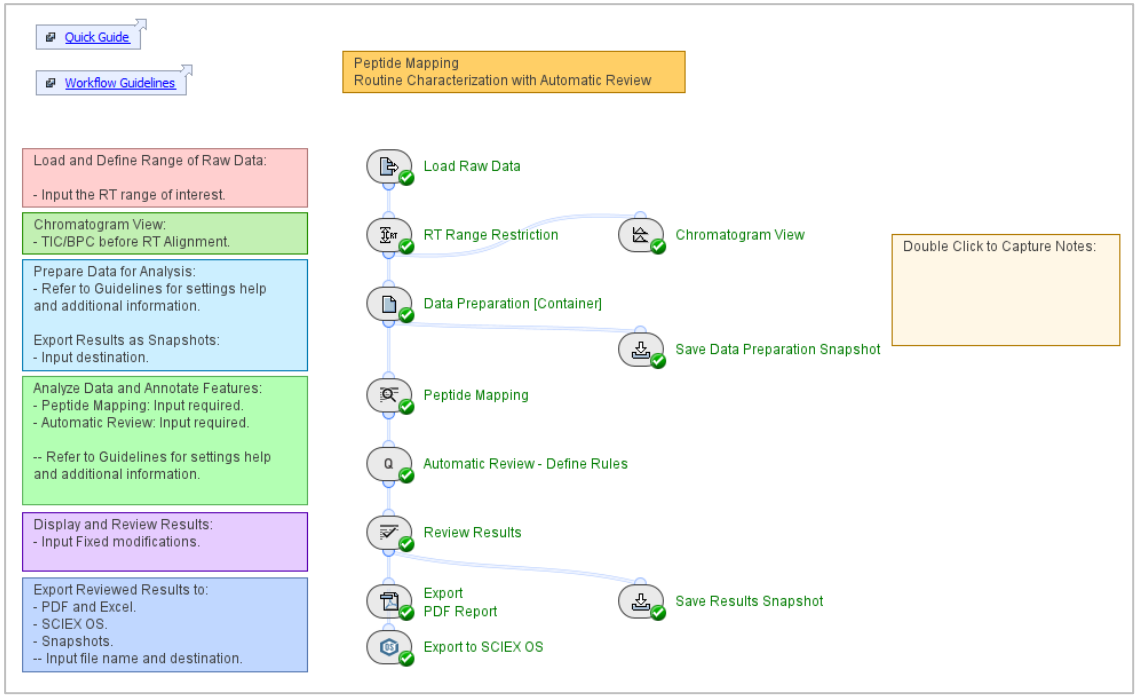
Simple Peptide Mapping Workflow



Pepmap_Simple_Be5.0

- Use this workflow for routine analysis of non-complex biotherapeutic molecules.
- Edit the combination of search parameters in the *Peptide Mapping* activity node to identify peptides and modifications, including glycosylation.

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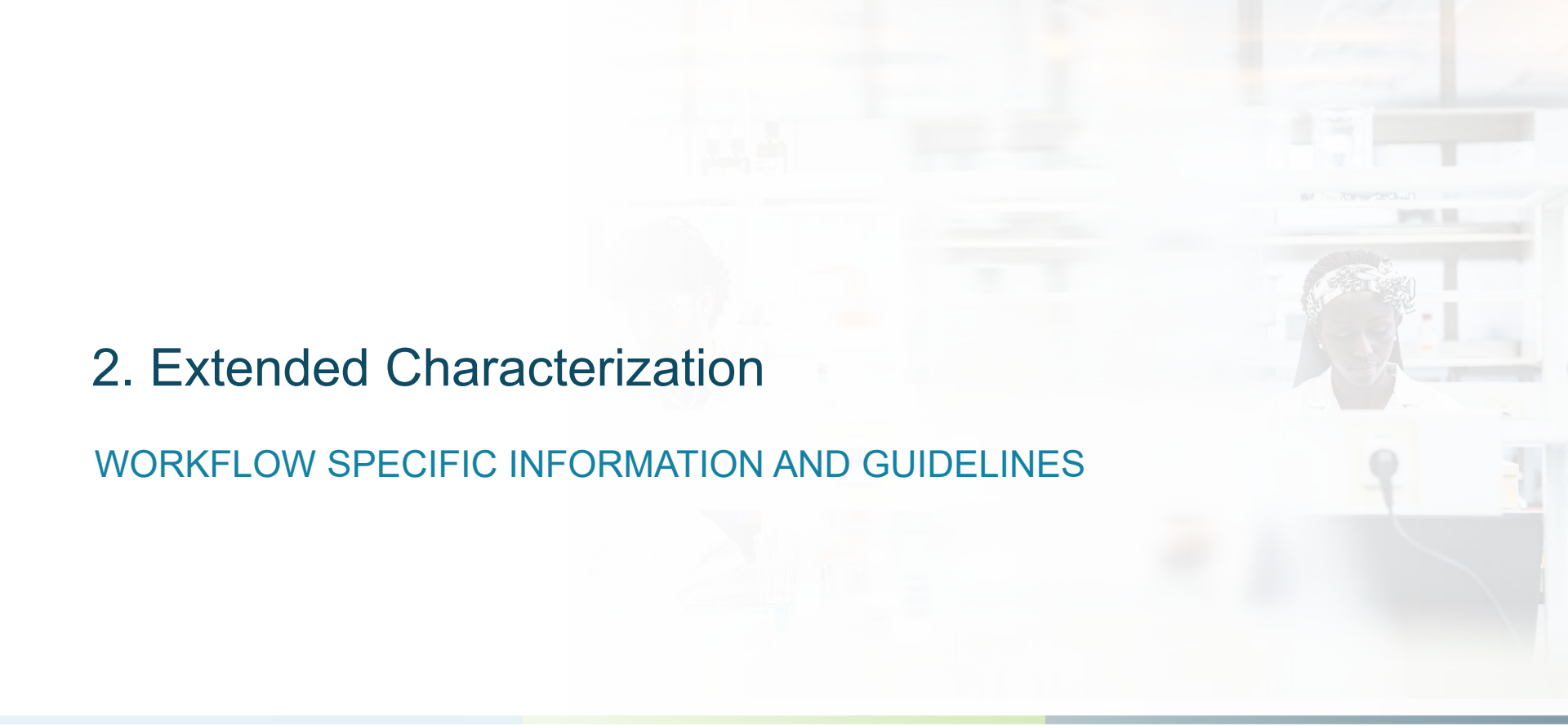
2. Extended Characterization

WORKFLOW SPECIFIC INFORMATION AND GUIDELINES



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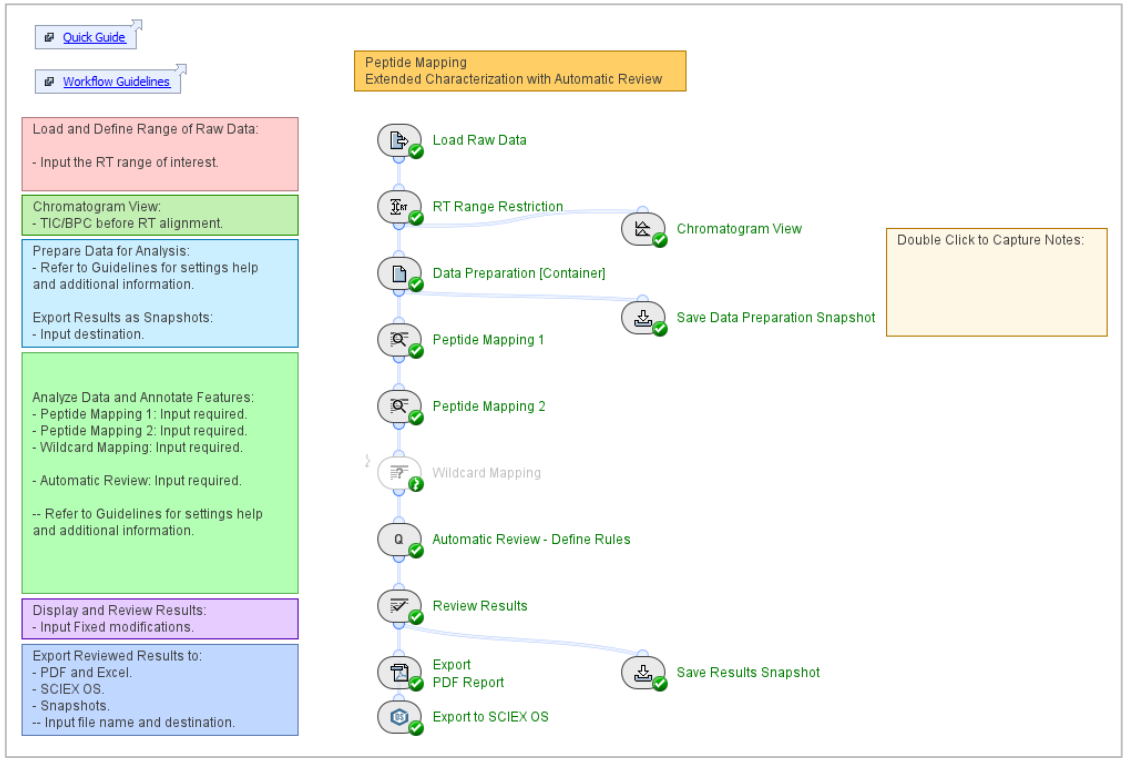
Extended Peptide Mapping Workflow



Pepmap_Extended_Be5.0

- Use this workflow for a more comprehensive Peptide Mapping analysis of biotherapeutic molecules.
- Combine results from up to three consecutive *Mapping* activity nodes to extend the search space and increase identifications, but keep false positives to a minimum.

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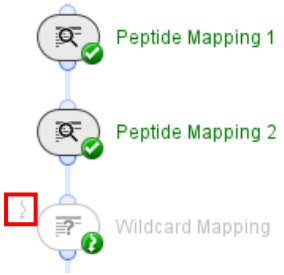
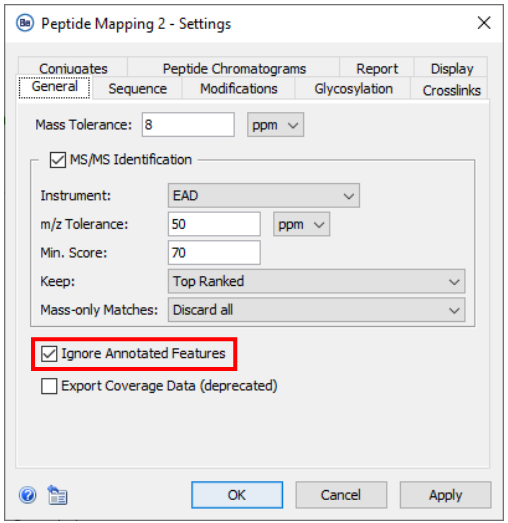
Extended Characterization





- This workflow uses up to three consecutive search nodes to extend the search space but minimize false positives:

Peptide Mapping 1

– To identify the most likely peptides and modifications.

Peptide Mapping 2

– To search for less common modifications.

– **Ignore Annotated Features**: Makes sure that only unannotated features from the previous search are considered.

- **Example Use Case**: For biotherapeutics with *N*- and *O*-glycosylation, false positives are decreased when *Peptide Mapping 1* is used to identify *N*-glycans, and *Peptide Mapping 2* is used to identify *O*-glycans.

Wildcard Mapping

– To search for unexpected modifications.

- Add identified modifications to a *Peptide Mapping* activity node.
- Activate the **Bypass** icon when *Wildcard Mapping* is not required.

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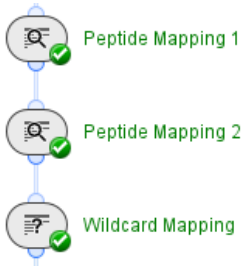
Step-Wise Peptide Mapping: Application Example



- Comprehensive post translational modification (PTM) analysis.
  - Suggested settings for this type of analysis:

Peptide Mapping 1

- **Sequence** tab. **Enzymes:** Fully specific (**Trypsin**).
- **Modifications** tab: Abundant and most likely modifications.

Peptide Mapping 2

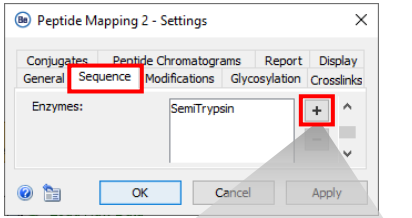
- **Sequence** tab. **Enzymes:** Semi-specific (**SemiTrypsin**).
- **Modifications** tab: Shorter list of most likely modifications.

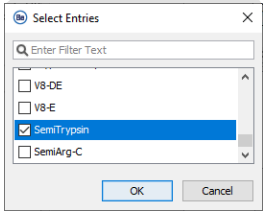
Or:

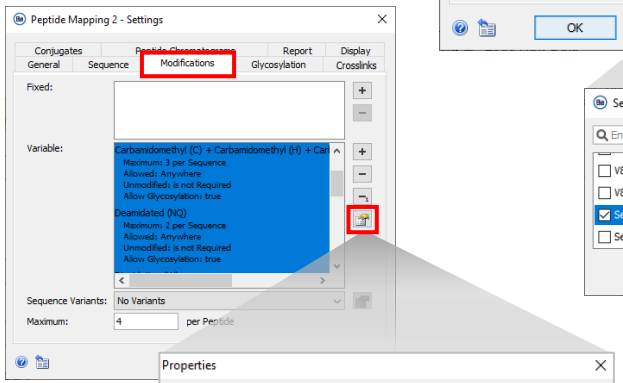
- **Sequence** tab: **Enzymes** - Fully specific (**Trypsin**).
- **Modifications** tab. Alternative set of less common modifications that might be present at low abundance.

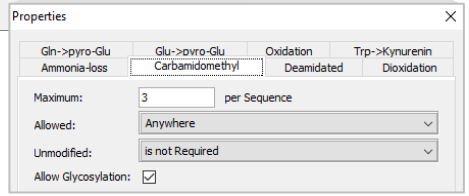
Wildcard Mapping

- Select **All Peptide Candidates** for annotations related to unknown modifications.









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3. Sequence Variant Search

WORKFLOW SPECIFIC INFORMATION AND GUIDELINES

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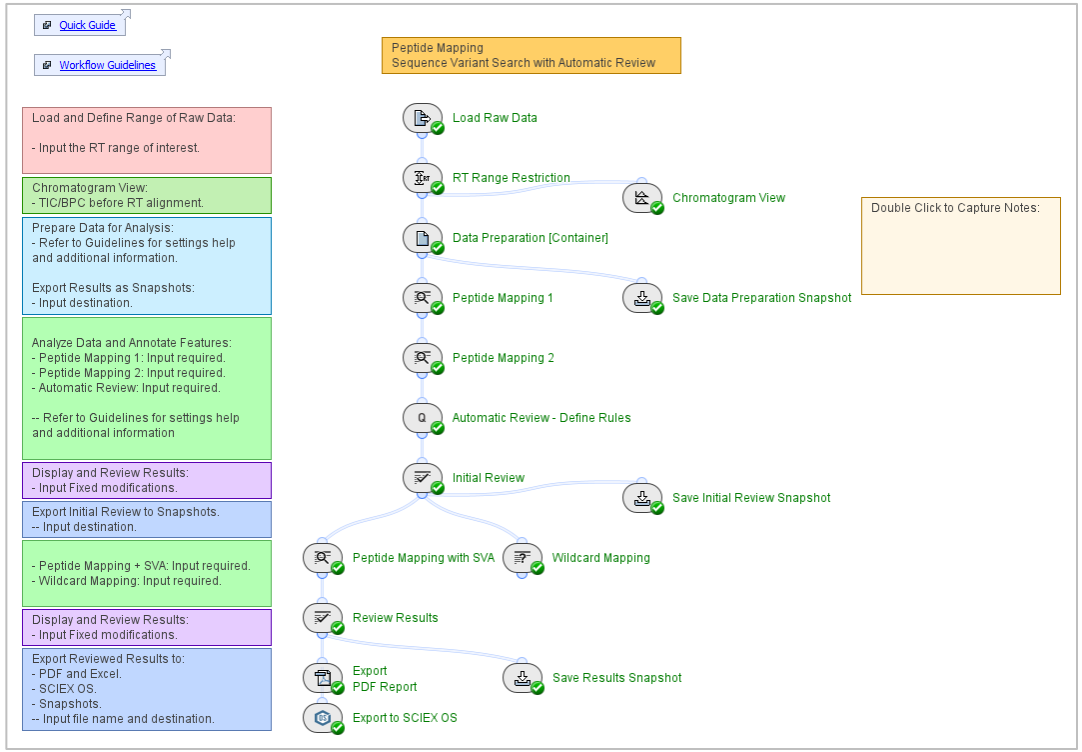
SVA Peptide Mapping Workflow



Pepmap_SVA_Be5.0

- Use this workflow to detect potential sequence variants.
  - Note: High quality MS and MS/MS data is required for confident identification of sequence variants. Instrument acquisition should be optimized for SVA.
- Two consecutive *Peptide Mapping* activity nodes identify the non-variant peptides and remove them from the search space.
  - Note: For more information about step-wise Peptide Mapping, refer to the section: **B:** 2.Guidelines for Extended Peptide Mapping Workflows.
- A further *Peptide Mapping with SVA* activity node searches for sequence variants.
- Possible SVA identifications can be compared with the results from the *Wildcard Mapping* activity node for verification.

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SVA Peptide Mapping Workflow

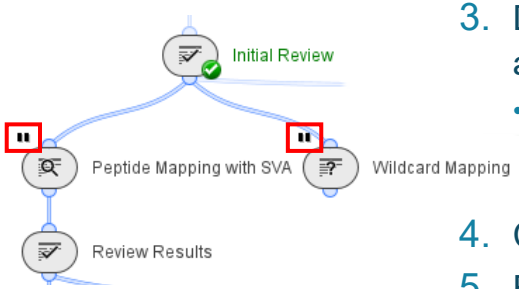
To detect potential sequence variants:

1. Use *Peptide Mapping 1* and *Peptide Mapping 2* to complete a typical analysis for non-variant peptides.
   - Refine the *Peptide Mapping* settings for the molecule under investigation.

   Note: For more information about step-wise Peptide Mapping, refer to the section: **B:** 2.Guidelines for Extended Peptide Mapping Workflows.

   ⟶ Identified peptides are removed from the search space, which decreases false positives in the next stage.
2. Complete an initial review of the data.
   - Click the **Save** icon, and then click **Save and Reload**.

   ⟶ Features from the rejected annotations are considered again in the *Peptide Mapping with SVA* search.



3. Deactivate the **Pause** icons on the *Peptide Mapping with SVA* and *Wildcard Mapping* activity nodes, and then click the **Play** icon to run them.
   - To increase the number of possible identifications, decrease the **Min. Score** in *Peptide Mapping with SVA* and *Wildcard Mapping*. A lower **Min. Score** increases the false positives for review. Manually review identifications that are close to the **Min. Score** threshold to reject incorrect annotations
4. Compare identifications in *Peptide Mapping with SVA* and *Wildcard Mapping*.
5. Review, and then **Accept** and **Reject** entries in the **Peptide Table** as required.

Note: For settings information for identification of low abundance peptides, refer to the pages: *MS/MS Peak Detection* and *MS/MS Deisotoping* in **A:** 2.General Guidelines for Peptide Mapping Workflows.

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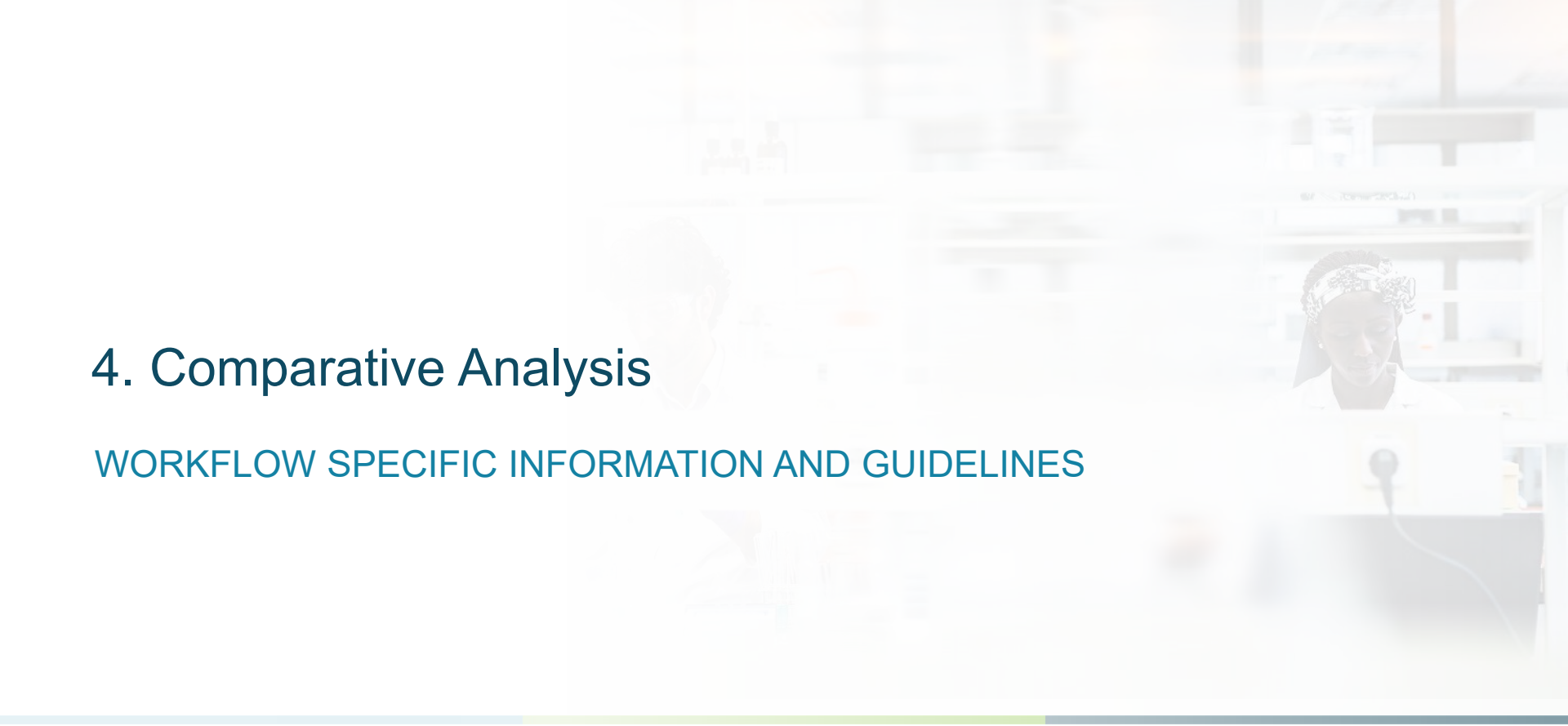

4. Comparative Analysis

WORKFLOW SPECIFIC INFORMATION AND GUIDELINES

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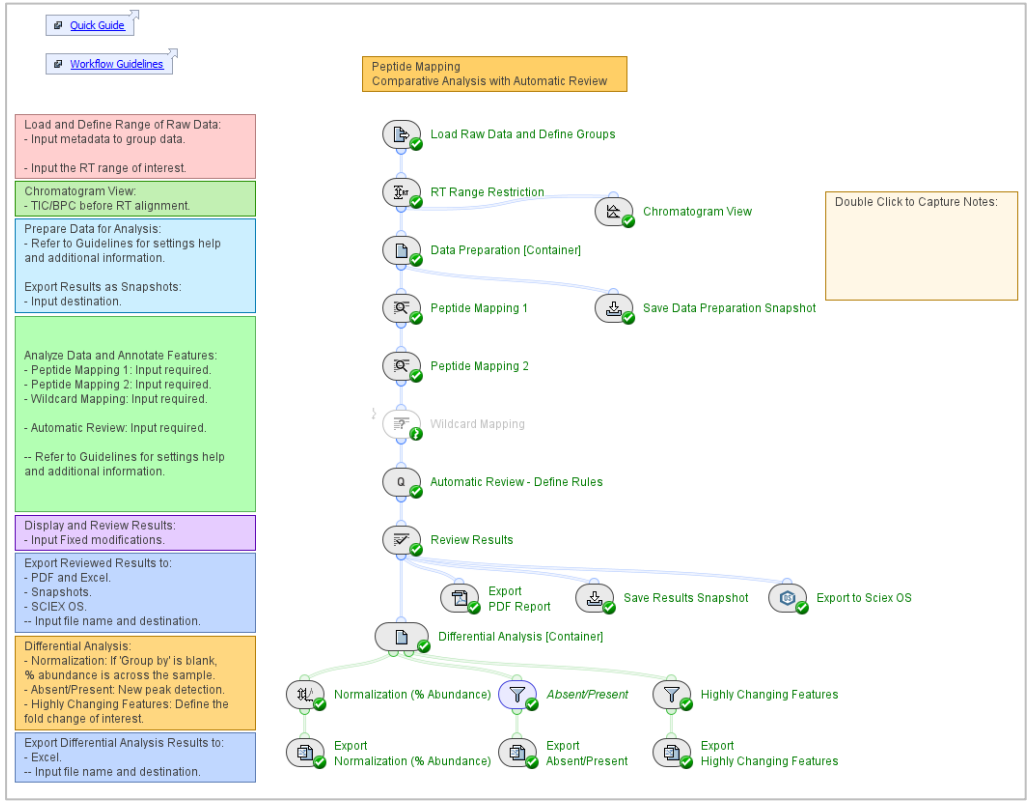
Comparative Peptide Mapping Workflow



Pepmap_Comparative_Be5.0

- Use this workflow to compare two sets of samples, for example:
  - Reduced and non-reduced samples.
  - Stressed and unstressed samples.
  - A reference sample with samples from a new batch.
- Combine results from up to three consecutive *Mapping* activity nodes.
  - Note: For more information about step-wise Peptide Mapping, refer to the section: **B:** 2.Guidelines for Extended Peptide Mapping Workflows.
- The *Differential Analysis [Container]* prepares data for comparisons to be made.

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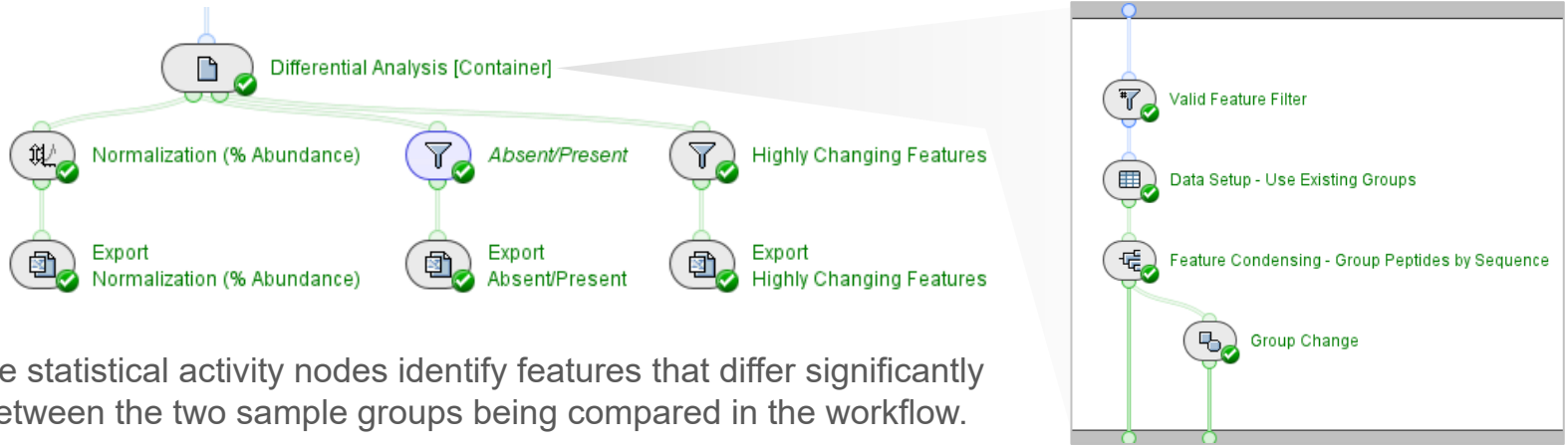
Comparative Peptide Mapping



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

- The activity nodes connected with green lines contain statistical tools that can be used to compare two datasets.
- The workflow reports:
  - The relative (%) abundance of peptides in each dataset.
  - The peptides that are absent in one sample set, but present in the other.
  - The peptides that have a specified fold-change difference between sample sets.

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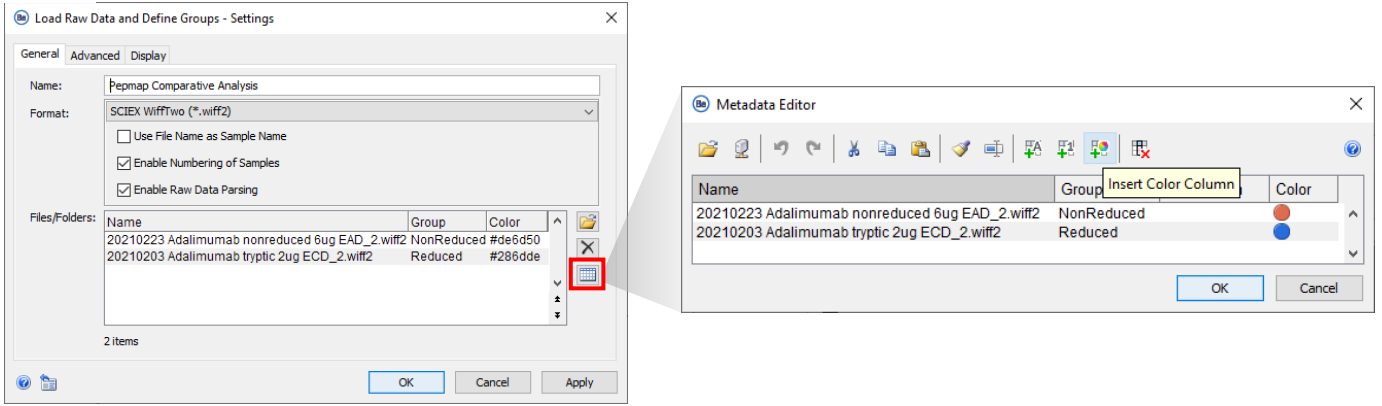
Load Raw Data and Define Groups

Load Raw Data and Define Groups



- Click the table icon to open the **Metadata Editor**.
  - Enter the **Group** names for the files to be compared.
  - Optionally, add a **Color** column and enter a color for each group.

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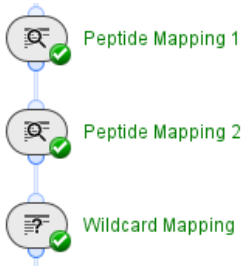
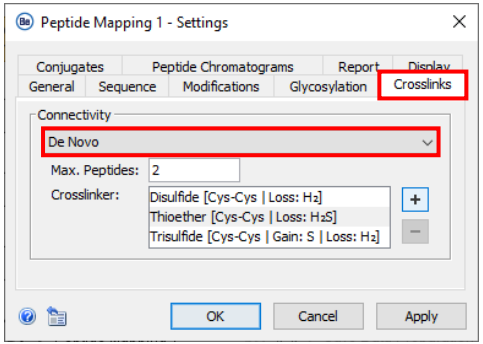
Application Example 1: Disulfide Bond Analysis



- Comparative analysis of reduced and non-reduced samples.



- Recommended settings for this type of analysis:

Peptide Mapping 1

- **Sequence** tab. **Enzymes:** Fully specific (**Trypsin**).
- **Disulfide** tab. **Connectivity: De Novo**
- **Modifications** tab. Variable alkylation of cysteine.

Peptide Mapping 2

- **Sequence** tab. **Enzymes:** Semi specific (**SemiTrypsin**).
- **Disulfide** tab. **Connectivity: None**.

Wildcard Mapping

- Select **All Peptide Candidates** for more annotations related to unknown modifications.

Note: For more information about step-wise Peptide Mapping, refer to the section: **B:** 2.Guidelines for Extended Peptide Mapping Workflows.

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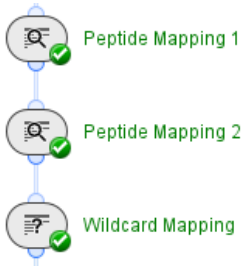
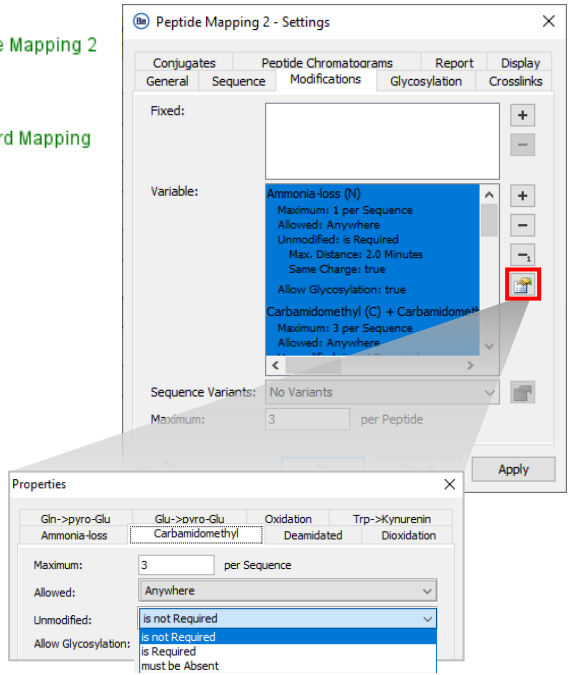
Application Example 2: Stress Tests and Variability



- Comparative analysis for stress testing and lot-to-lot variability.



Recommended settings for this type of analysis:

Peptide Mapping 1

- **Sequence** tab. **Enzymes:** Fully specific (**Trypsin**).
- **Modifications** tab. Abundant and most likely modifications.

Peptide Mapping 2

- **Sequence** tab. **Enzymes:** Semi specific (**SemiTrypsin**).
- **Modifications** tab. Shorter list of most likely modifications.

Or:

- **Sequence** tab. **Enzymes:** Fully specific (**Trypsin**).
- **Modifications** tab. Alternative set of less common modifications that might be present at low abundance.

Wildcard Mapping

- Select **All Peptide Candidates** for annotations related to unknown modifications.

Note: For more information about step-wise Peptide Mapping, refer to the section: **B:** 2.Guidelines for Extended Peptide Mapping Workflows.

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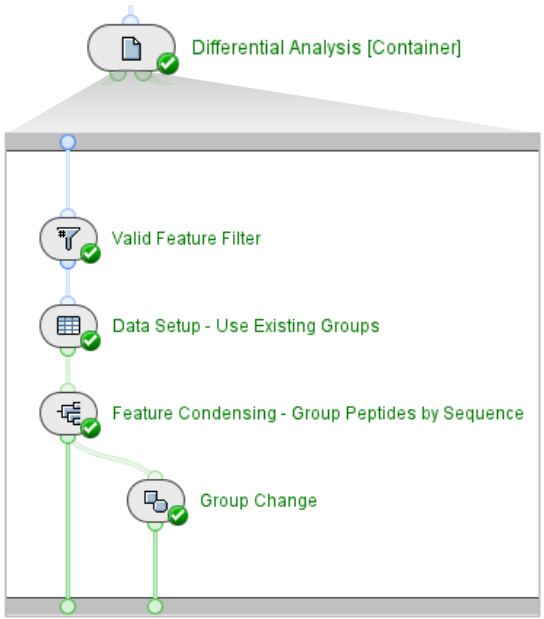
Differential Analysis [Container]



Note: Available settings are related to the previous activity nodes. To see the list of possible **Group by** options, run *Data Setup*, and then edit the *Feature Condensing* settings.

- *Valid Feature Filter*
  - Removes any features below a set threshold, and any found in less than a set % or number of experiments.
  - Removes insignificant differences or signal caused by noise or artifacts. If predicted peptides are not found, then optimize this setting.
- *Data Setup*
  - Prepares data in a matrix form for the next activity nodes.
  - If groups were not defined in *Load Raw Data*, use **Group: Manually** and assign each sample to a group.
- *Feature Condensing*
  - Uses annotations to group features.
  - Calculates a single intensity value for each of the created groups.
- *Group Change*
  - Calculates relative and fold-change differences between experiments.
  - If there are multiple experiments, then the reported change is the maximum difference between any two experiments.

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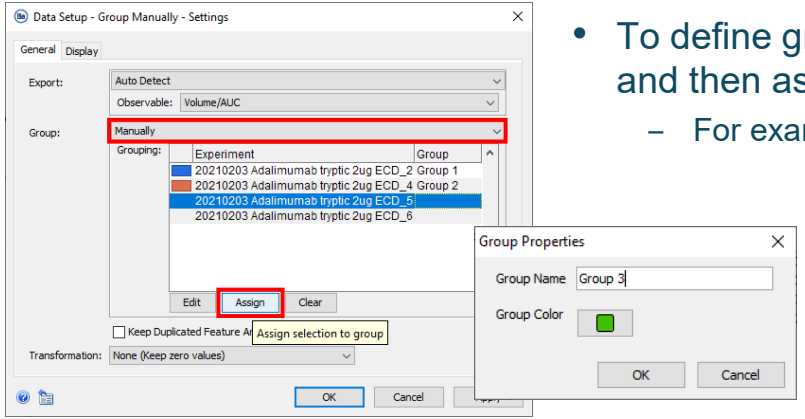
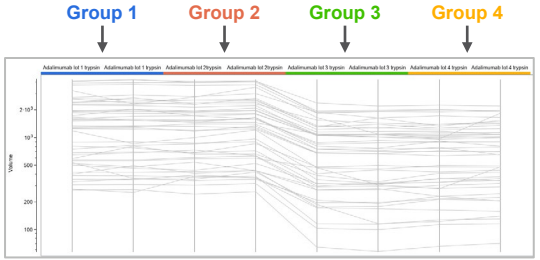
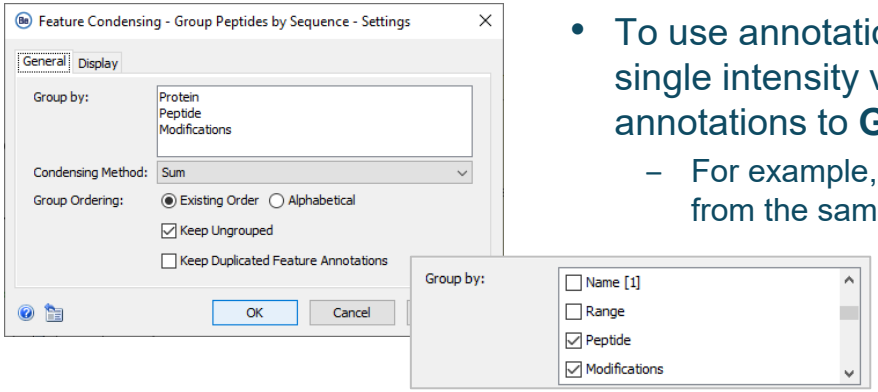
Differential Analysis [Container]: Data Preparation



- To define groups in the *Data Setup* activity node, select **Group: Manually**, and then assign each sample to a group.
  - For example, Group 1 to Group 4 for lot-to-lot comparability:





- To use annotations to combine the features in each group, and calculate a single intensity value for the combined feature, select the types of annotations to **Group by** in *Feature Condensing*.
  - For example, to combine peptides with the same sequence and modifications, from the same protein, select: **Protein**, **Peptide**, and **Modification**.

Note: To see the list of possible **Group by** options, run *Data Setup*, and then edit the *Feature Condensing* settings.

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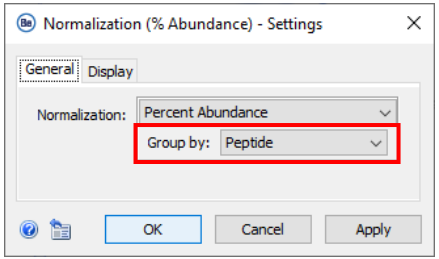
Normalization (% Abundance)



- *Normalization (% Abundance)*
  - **Percent Abundance**: For each experiment, the values of all members of each group are added. Then, the value of each member of a group is divided by the corresponding total and multiplied by 100.
    - If **Group by** is set to Peptide, then the **Percent Abundance** is calculated for each peptide.
    - If **Group by** is blank or set to **Undefined**, then the **Percent Abundance** is calculated for the whole sample.

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
	100%	100%

***Feature Condensing*: Group by**
Protein Peptide Modification

***Normalization*: Group by Peptide**

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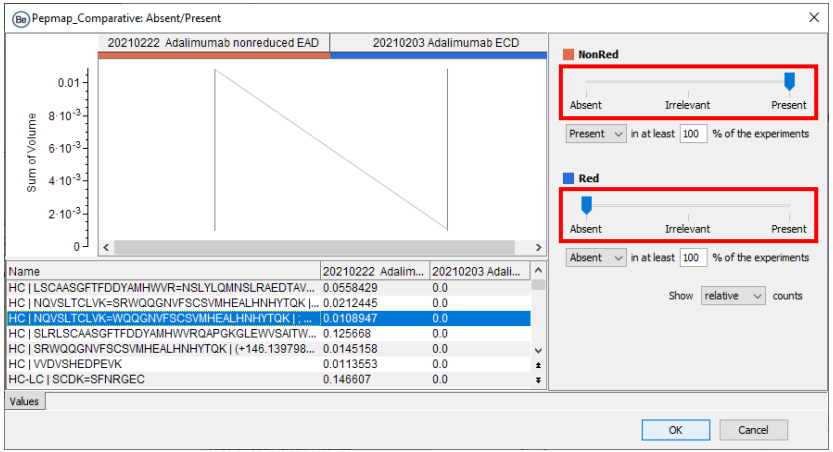
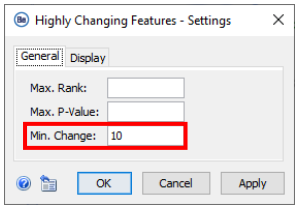
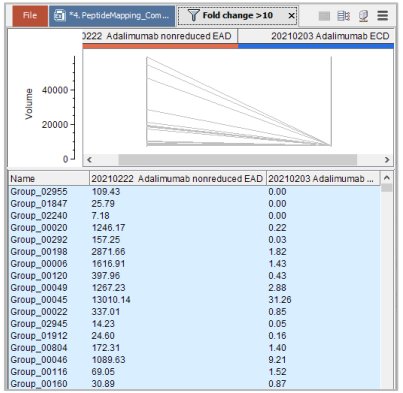
Absent/Present and *Highly Changing Features*

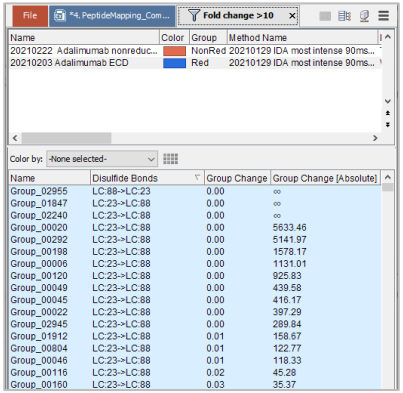
- *Absent/Present*
  - Compare features between two sample groups.
  - To filter the results, move the sliders in the input window that opens when the activity node is run.
    - For example, for disulfide bond analysis, the features of interest are likely to be **Absent** in the reduced sample and **Present** in the non-reduced sample.



- *Highly Changing Features*
  - Set the required minimum fold change.







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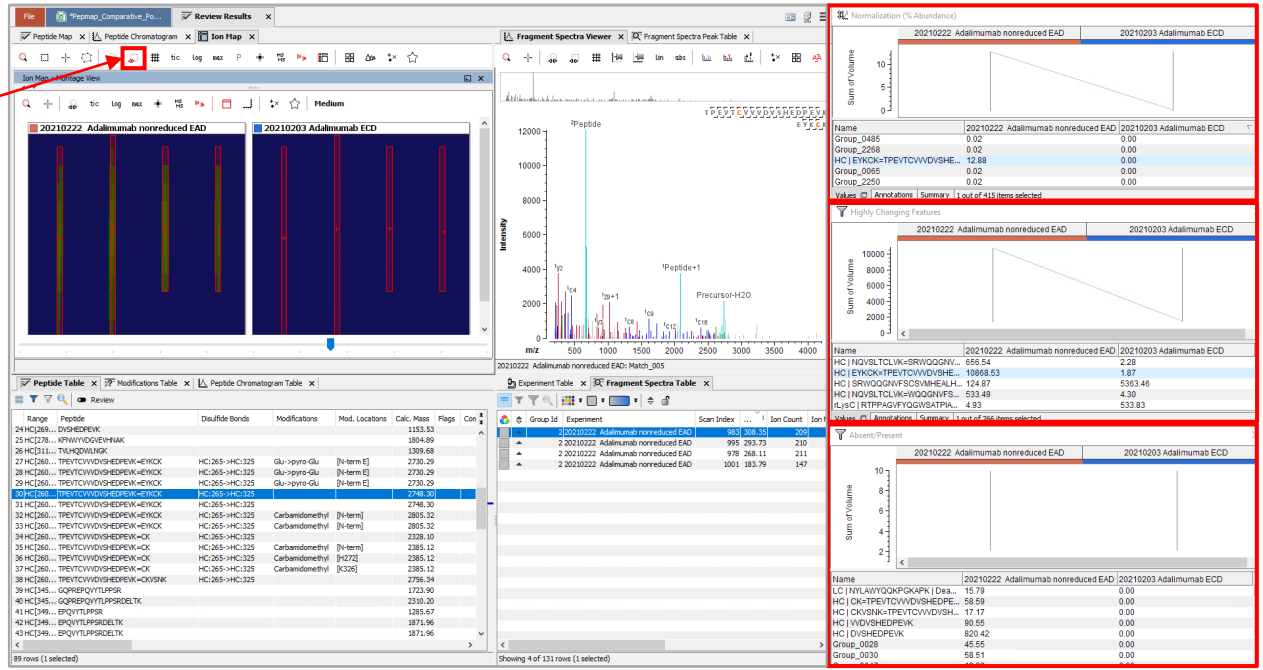
Synchronize Selections for Simplified Data Review

- To automatically update (dynamically link) the selection in all results windows:
  1. Open the results windows for *Absent/Present*, *Highly Changing Features* and *Review Results*.
  2. Click the **Synchronize selection** icon in **Ion Map** tab of *Review Results*.

Synchronize selection



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5. Peptide Mapping Batch Processing

WORKFLOW SPECIFIC INFORMATION AND GUIDELINES

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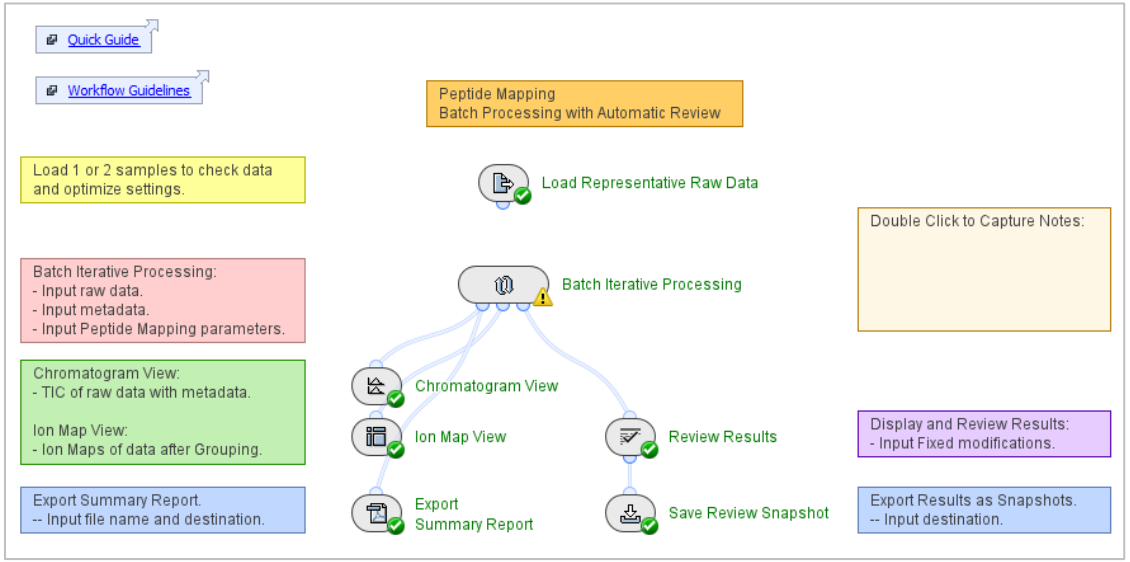
Peptide Mapping Batch Processing Workflow



Pepmap_BatchProcessing_Be5.0

- Use this workflow to analyze multiple samples independently, in an iterative manner.
- Use the Pepmap_Extended workflow to optimize the workflow settings for any new samples before running a Batch Processing workflow.
  - Note: For more information about step-wise Peptide Mapping, refer to the section: **B:** 2.Guidelines for Extended Peptide Mapping Workflows.
- Use the Pepmap_ReviewSnapshots workflow to review data and create reports for samples analyzed with the Batch Processing workflow.

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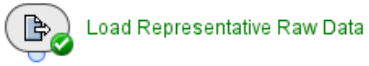
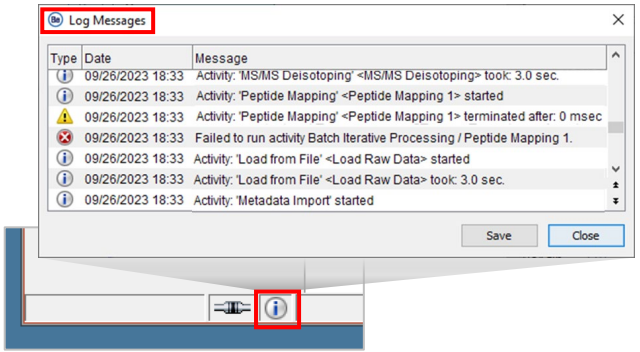
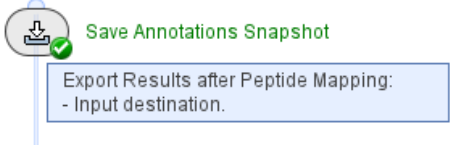
Recommendations for the Batch Processing Workflow







- To use the Batch Processing workflow:

1. Use the *Load Representative Raw Data* activity node to first review a small number of representative samples.
   - Examine the RT range and noise level.
   - Use the Pepmap_Extended workflow to optimize the workflow settings for any new samples.
2. Use a small *RT Range Restriction*, for example 10 min to 15 min, and a smaller sample set, so that the Batch Processing workflow contains all applicable metadata and completes successfully.
   - To monitor progress, double-click the icon to open the **Log Messages** dialog.
3. Use the *Save Annotations Snapshot or Save Review Snapshot* activity node to save Peptide Mapping results. Load each saved sbf file separately in the Pepmap_Review Snapshots workflow to review the results and create individual PDF reports.

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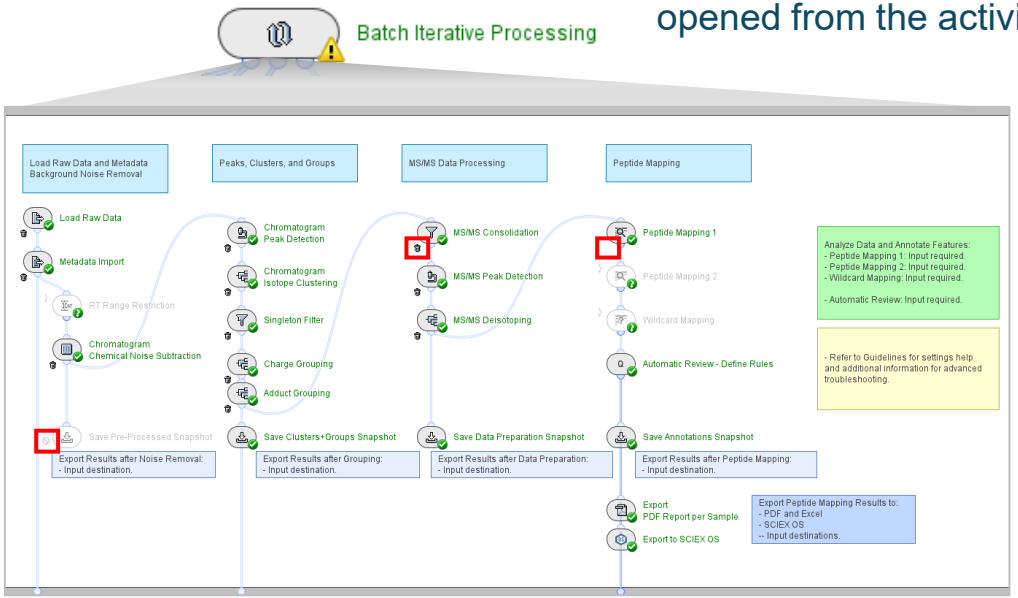
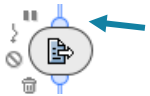
Batch Iterative Processing Container

- The *Batch Iterative Processing* container is not the same as other Biologics Explorer software containers.
  - Only intermediate results from the last sample to be processed can be opened from the activity nodes in the *Batch Iterative Processing* container.



Note: If activity nodes in the container have the **Bypass** icon activated, then the container shows a yellow warning symbol.

- To open the intermediate results of an activity node, deactivate the **Trash** icon before the workflow is started.
- Do not activate the **Trash** icon for *Peptide Mapping* activities that are used in the PDF Report.



Activity nodes in the *Batch Iterative Processing* container do not have a **Run** or **Reset** icon.

- Individual activity nodes in the *Batch Iterative Processing* container cannot be run alone.
  - To use a *Save Snapshot* or *Export* activity node in the *Batch Iterative Processing* container, deactivate the **Block** icon before the workflow is started.

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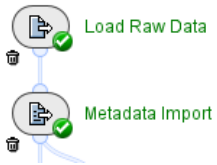
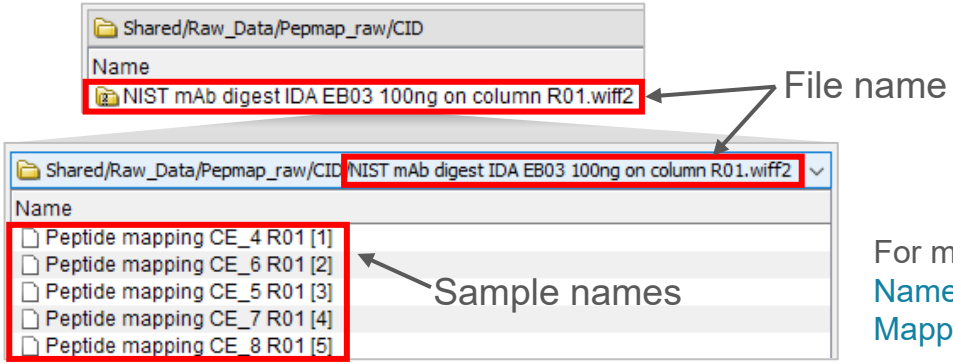
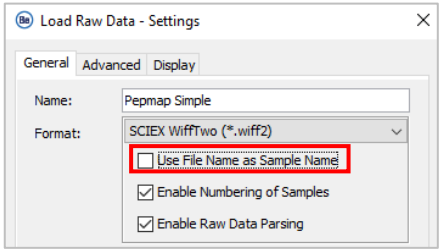
Load Raw Data: Experiment Names and Metadata



- To analyze replicate samples from different acquisition files:
  1. Select **Use File Name as Sample Name** in *Load Raw Data*.
  2. Use the **File Name** (name of the wiff or wiff2 container file) in the **Experiment** column of the txt file for *Metadata Import*.
- To analyze multiple samples from a single acquisition file:
  1. Do not select **Use File Name as Sample Name** in *Load Raw Data*.
  2. Use the **Sample Name** in the **Experiment** column of the txt file for *Metadata Import*.





For more information, refer to the page: *Load Raw Data*: Use File Name as Sample Name in **A:** 2.General Guidelines for Peptide Mapping Workflows.

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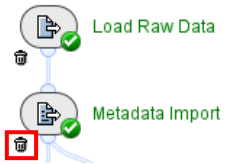
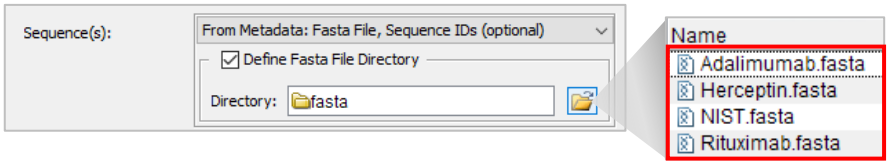
Metadata Import



- To analyze multiple samples with the same sequence:
  - Deactivate the **Trash** icon, and then activate the **Bypass** icon for *Metadata Import*.
  - On the **Sequences** tab in *Peptide Mapping*, select **From Text** or **From Fasta File**.
- To analyze multiple samples with different sequences:
  - Use *Metadata Import* to select the FASTA file (protein sequence) that will be used for identification in the *Peptide Mapping* activity nodes.

- Upload a txt file with *Metadata Import* that links each sample to the correct FASTA file.
  - The name in the **Experiment** column must be the same as in the **Experiment** table in *Load Raw Data*.
  - The name in the **Fasta File** column must be the same as the name of the FASTA file that is in the specified **Fasta File Directory**, including the file extension (fasta or txt).

	A	B
1	Experiment	Fasta File
2	20210203 Adalimumab tryptic 2ug ECD_1	Adalimumab.fasta
3	20210130 Herceptin IDA ECD Most intense _1	Herceptin.fasta
4	20210130 Rituximab IDA ECD Most intense _1	Rituximab.fasta
5	20210901_NISTmAb_TimeCourse_Control_4ul_EAD_1	NIST.fasta



Note: For more information, refer to the page: *Review Results*: Protein Name in FASTA Files in **B:** 5.Guidelines for Peptide Mapping Batch Processing Workflows.

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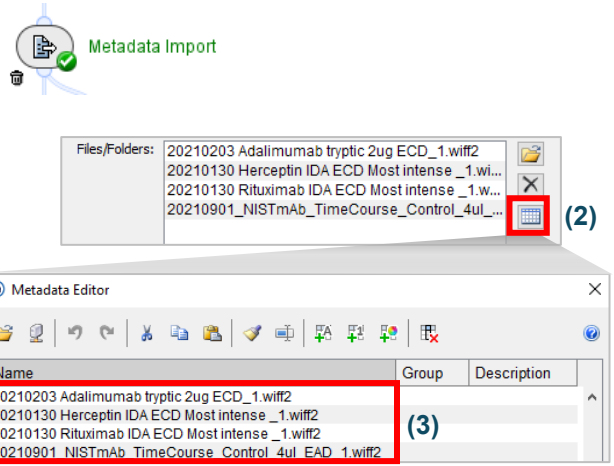
Metadata Import: How to Create the Metadata File



Note: Any metadata added in the *Load Raw Data* **Metadata Editor** table must be completed for all rows (all samples).

Experiment	Fasta File
20210203 Adalimumab tryptic 2ug ECD_1	Adalimumab.fasta
20210130 Herceptin IDA ECD Most intense _1	Herceptin.fasta
20210130 Rituximab IDA ECD Most intense _1	Rituximab.fasta
20210901_NISTmAb_TimeCourse_Control_4ul_EAD_1	NIST.fasta

(4)

- To create the metadata file in Excel or Notepad:
  1. Select the samples for batch processing in *Load Raw Data*.
  2. Open the **Metadata Editor** table.
  3. Select all of the entries in the **Metadata Editor** table, and then select copy.
  4. Paste the entries into the **Experiment** column of the metadata txt file.
     - Delete ".wiff" or ".wiff2" from the end of each name. (Tip: Use the Replace command in Excel or Notepad.)
  5. Type the applicable FASTA file name in each row in the **Fasta File** column.
  6. Save the file in txt format, and then upload the file in the *Metadata Import* activity node.

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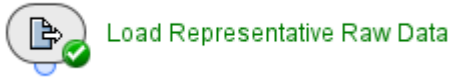
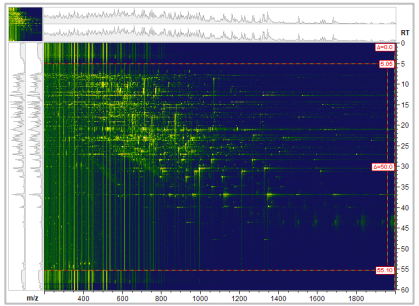
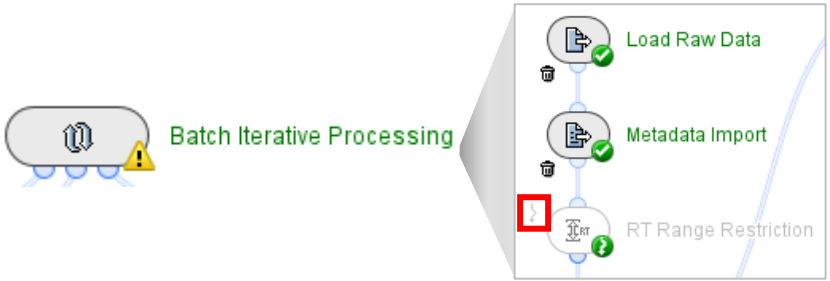
Restrict the RT Range

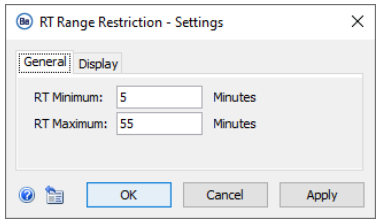


- Use the *Load Representative Raw Data* activity node to review a small number of representative samples outside of the *Batch Iterative Processing* container.

- To identify the RT ranges where there is meaningful data, open (double-click) *Load Representative Raw Data* after the data is loaded.
- If the RT ranges are consistent across all samples, then deactivate the **Bypass** icon and enter **RT Minimum** and **RT Maximum** values in the *RT Range Restriction* activity node in the *Batch Iterative Processing* container.







Note: If the fields are blank, or if *RT Range Restriction* has the **Bypass** icon activated, then the full RT range is used.

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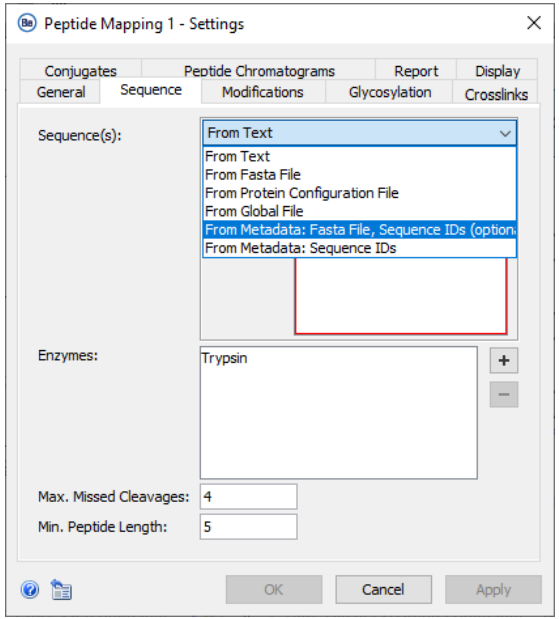
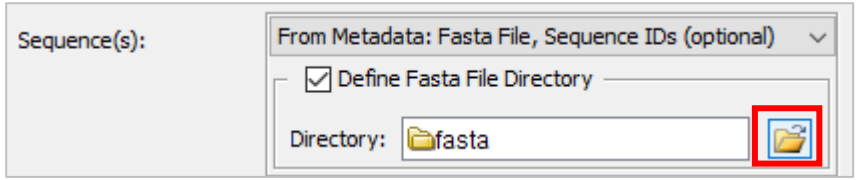
Peptide Mapping: Sequence tab



Sequence tab:

- **Sequence(s)**:
  - If all samples have the same sequence, then select **From Text** and type the sequence, or **From Fasta File** and select the applicable file.
  - If different samples require different sequences, then select **From Metadata: Fasta File, Sequence IDs (optional)**, and then browse to the location of the folder that contains all of the applicable FASTA files.



- **Enzymes**:
  - Adjust enzyme specificity, maximum number of missed cleavages, and minimum peptide length as required.

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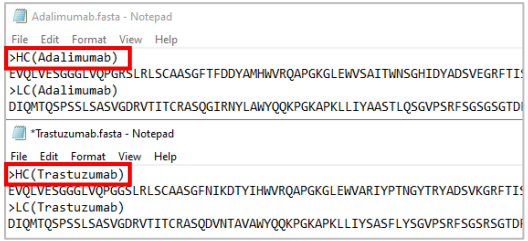
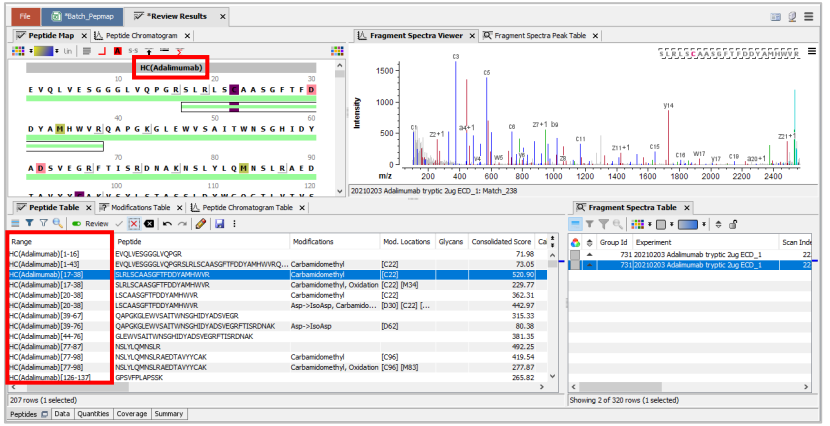
Review Results: Protein Name in FASTA Files

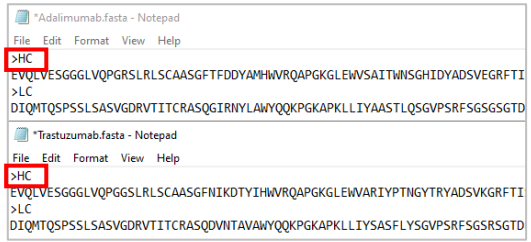
- If the protein sequence names <u>are unique</u> across the FASTA files used for identification:



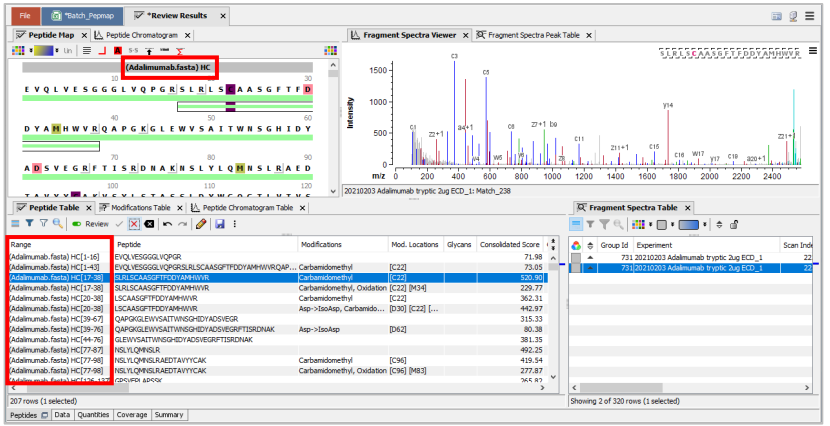
  - The protein sequence name in *Review Results* is the same as the name in the FASTA file.



- If the protein sequence names <u>are not unique</u> across the FASTA files used for identification:



  - The protein sequence name in *Review Results* includes the FASTA file name.



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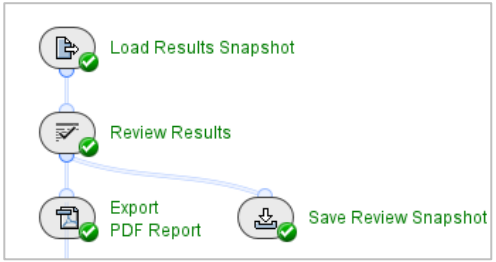
Export PDF Report

- There are two locations to save a PDF Report in the Batch Processing workflow:

1. *Export PDF Report per Sample*, in the *Batch Iterative Processing* container.
   - Creates a report for each sample, that contains the results from the *Peptide Mapping* activity nodes, before review.

2. *Export Summary Report*, out of the *Batch Iterative Processing* container.
   - Creates a report that contains the sequence coverage results for all samples from the *Review Results* activity node and any warnings from the workflow.



- To create individual reports of reviewed results:
  1. Use either *Save Annotations Snapshot* in the *Batch Iterative Processing* container, or *Save Review Snapshot* out of the *Batch Iterative Processing* container to save Peptide Mapping results.
  2. Review the sbf files separately in the Pepmap_ReviewSnapshots workflow.
  3. Use *Export PDF Report* in the Pepmap_ReviewSnapshots workflow to create one report per sample.

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6. Review Snapshots

WORKFLOW SPECIFIC INFORMATION AND GUIDELINES

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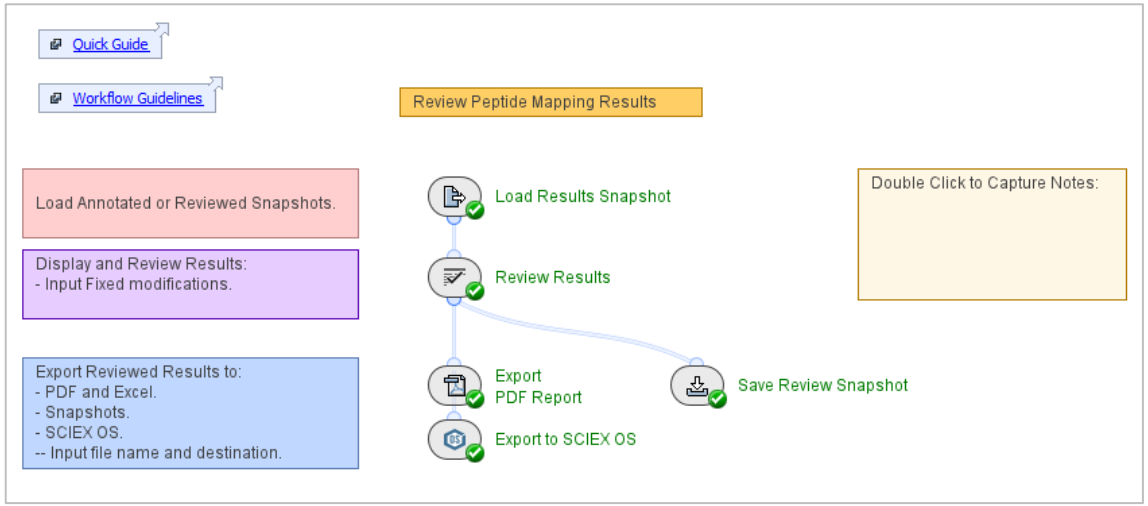
Review Snapshots Workflow



Pepmap_ReviewSnapshots_Be5.0

- Use this workflow to review saved Snapshot results that have peptide annotations.
  - For example, to review individual results Snapshots from the Batch Processing workflow and create a report.

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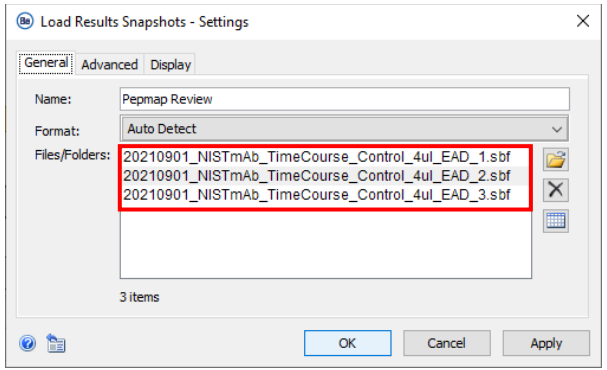
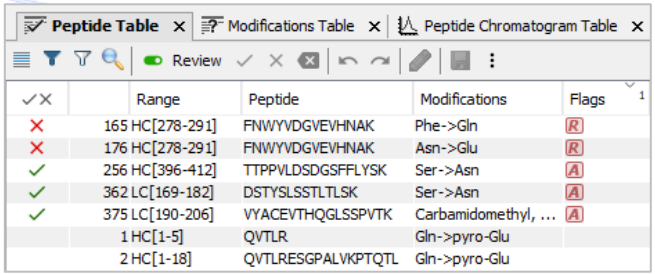
Review Saved Results

Load Results Snapshots



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

Review Results



- The *Review Results* activity node opens a copy of the previous analysis.
  - Any previously accepted or rejected peptides have the applicable entry in the **Flags** column.
  - A further review is then possible.
  - The reviewed sbf files and a new report can be saved.

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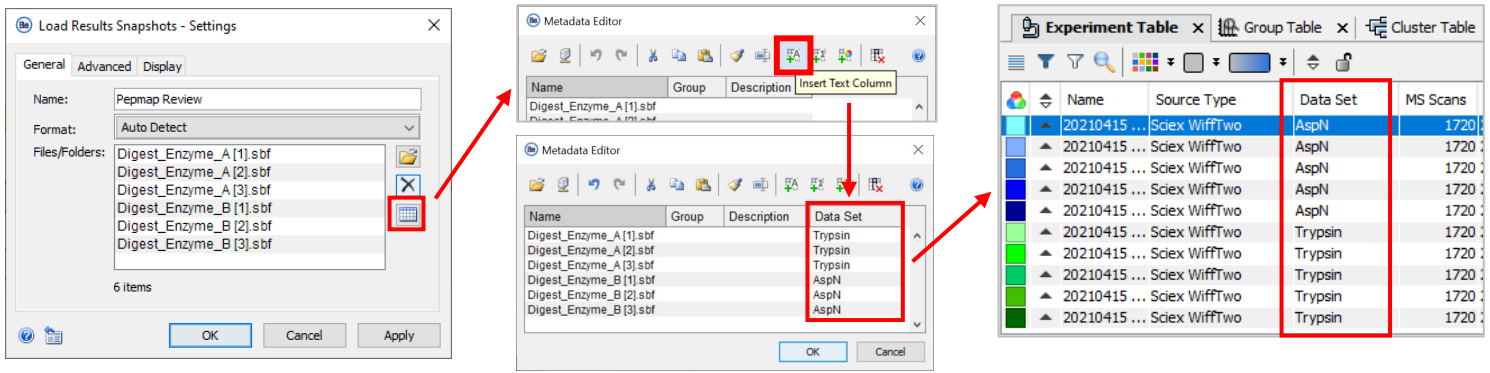
Load Snapshots: Metadata

- Special Use Case: To combine samples with different sample preparation methods that have been processed in different Peptide Mapping workflows.
  - For example: Samples of the same molecule that have been digested with different enzymes, and then analyzed separately using a Peptide Mapping workflow.

1. On the **General** tab, click the icon to open the **Metadata Editor**.
2. Add a column called **Data Set**.
   - Note: Spaces and capitalization are critical. For example, **Data set** will not work.

- The overall **Sequence Coverage** takes all of the samples into account.



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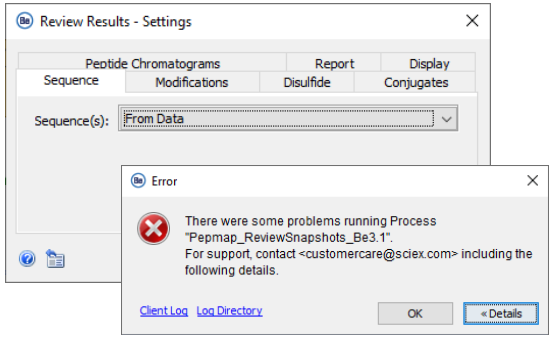
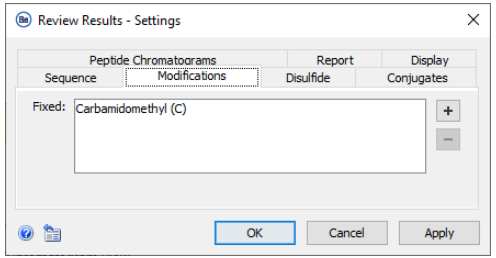
Review Results: Configure Settings





Sequence tab:

- **Sequence(s)**:
  - If sbf files were saved from data analyzed with Biologics Explorer software 4.0, then select **From Data**.
  - If sbf files were saved from data analyzed with Biologics Explorer software 3.1 (or earlier versions), then:
    - If all samples have the same sequence, then select **From Text** and type the sequence, or **From Fasta File** and add the applicable file.
    - If different samples require different sequences, then select **From Metadata: Fasta File, Sequence IDs (optional).**
    - Note: For more information about Batch Processing, refer to the section: **B:** 5.Guidelines for Peptide Mapping Batch Processing Workflows.

Modifications tab:

- Select the **Fixed** modifications specified in the previous *Peptide Mapping* activity nodes.

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