



# Top/Middle-Down Protein Sequencing

Biologics Explorer Software 4.0 Guidelines

Powered by Genedata Expressionist®



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# Part A

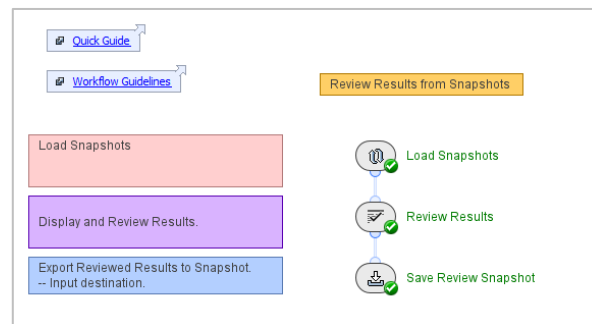
## Workflow Guidelines for Protein Sequencing



# Overview of Applications for Top/Middle-Down Workflows

- These workflows are designed for top-down or middle-down protein sequencing with MS/MS data of:
  - Antibody fragments: for example, Fd', Fc/2 and Lc after IdeS digestion and reduction of disulfide bonds.
  - Antibody chains: for example, Hc and Lc after reduction of disulfide bonds.
  - Proteins with masses up to approximately 50 kDa.
- Analyze each antibody fragment or chain separately, and then use the Top\_Middle-Down\_ReviewSnapshots workflow to show all of the results together.
- If the signal-to-noise ratio at high mass ranges is low, then data quality for protein sequencing can be increased if multiple consistent technical replicates are analyzed at the same time in the Top\_Middle-Down\_ProteinSequencing workflow.
- If the overall signal intensity is good, with a satisfactory signal-to-noise ratio, then a single sample can be sufficient for a successful analysis.

# Top/Middle-Down Protein Sequencing Workflows



## Top\_Middle-Down\_ReviewSnapshots\_Be4.0

## Top\_Middle-Down\_ProteinSequencing\_Be4.0


# Top/Middle-Down Protein Sequencing Workflow

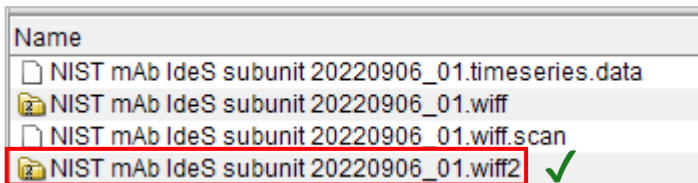


# Activity Nodes in the Protein Sequencing Workflow

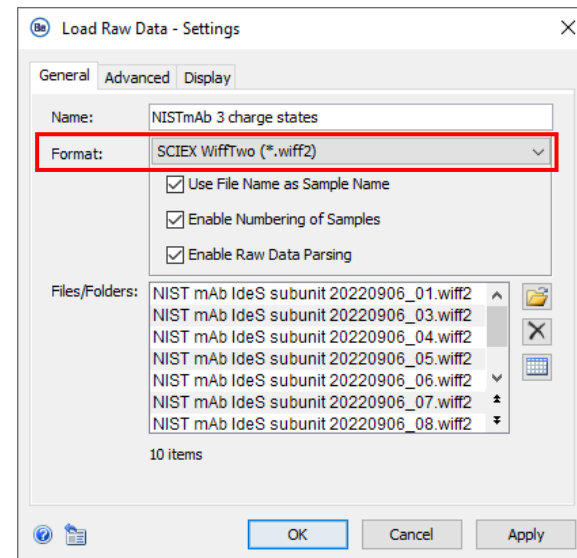
- A. *Load Raw Data*
- B. *RT Alignment*
- C. *Chromatogram View*
- D. *RT Range Restriction*
- E. *m/z Range Restriction*
- F. *Data Preparation [Container]*
  - i. *MS/MS Data Selection*
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  - iii. *Average Spectra Across Replicates*
  - iv. *Spectrum Smoothing*
  - v. *Baseline Flattening*
  - vi. *Noise Subtraction*
  - vii. *Intensity Thresholding*
  - viii. *MS/MS Range Restriction*
- G. *Filter Precursor Masses*
- H. *Spectrum Peak Detection*
- I. *Peak Clustering*
- J. *Singleton Filter*
- K. *Fragment Mapping*
- L. *Filters [Container]*
  - i. *Valid Feature Filter*
  - ii. *Highest Feature Filter*
  - iii. *Filter Annotated Peaks*
- M. *Define Experiment Name*
- N. *Review Results*
- O. *Save Results Snapshot*

# 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 mass spectrometer, then select only the wiff2 format.
    - Do not select the auxiliary files with the same name.



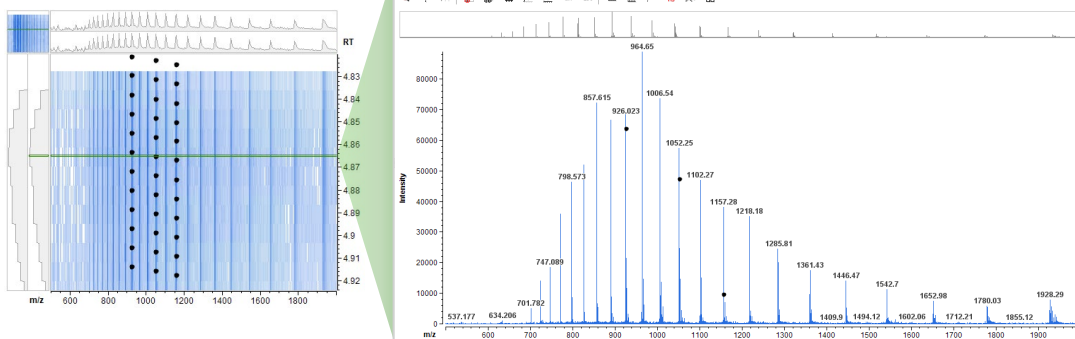
- To select samples in a wiff or wiff2 container file:
  - Double-click the wiff or wiff2 container to see the sample files.
  - Select the samples to upload from the list of sample files.



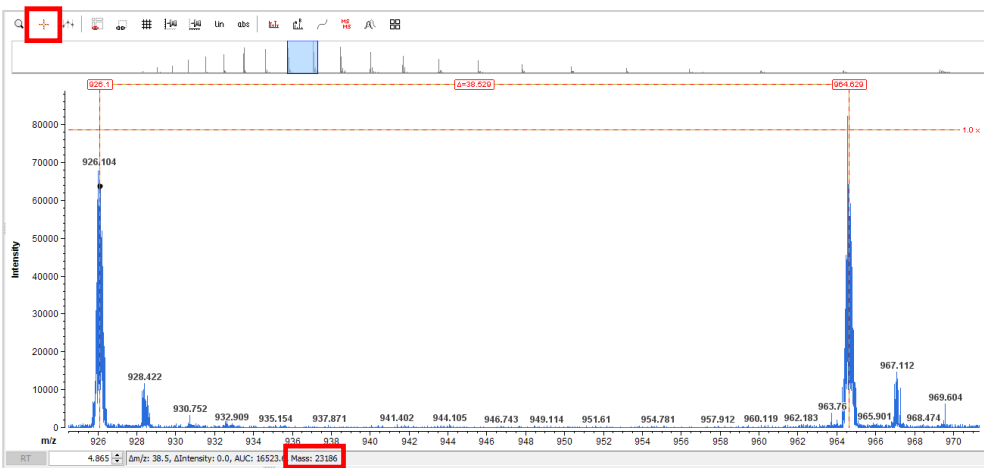


# Identify Charge States in Non-Deconvoluted Data

(1)



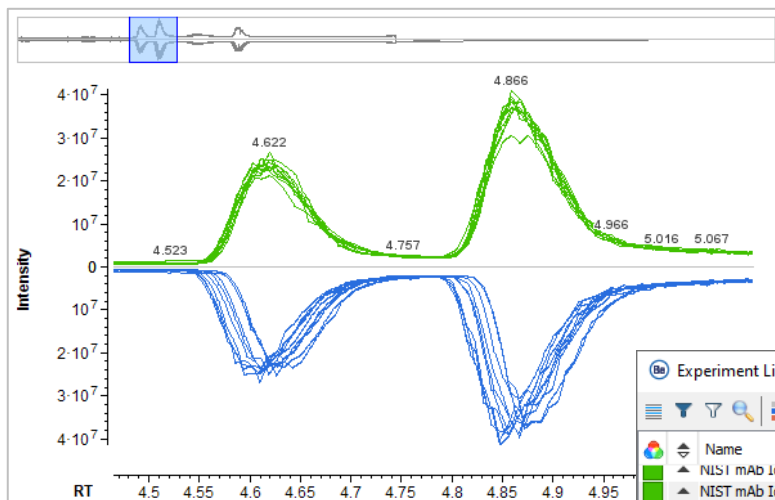
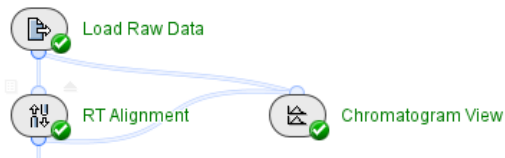
(2)



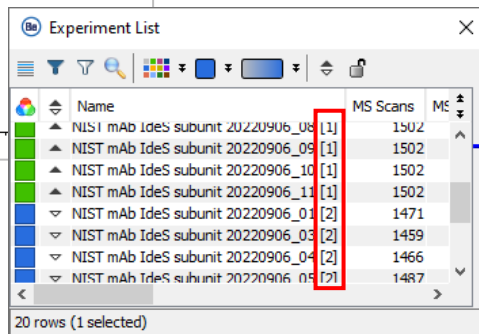
(3)

- To identify the charge state of a peak before deconvolution, for example, during optimization of the acquisition method and selection of the optimal charge states for MS/MS fragmentation:
  - Take a horizontal slice across the ion map to show the charge envelope in the **Mass Spectrum** window.
  - Use the **Measuring Tool** to measure the mass difference between two homologous peaks from consecutive charge states.
  - Divide the given **Mass** value by the  $m/z$  of the peak of interest to calculate the charge state of that peak.

# RT Alignment



- Use *RT Alignment* to remove minor deviations in peak elution times between multiple technical replicates.
- Use *Chromatogram View* to compare samples before and after alignment.
  - Right-click the chromatogram to open the **Experiment List**.
    - The samples with suffix [1] are **after alignment**.
    - The samples with suffix [2] are **before alignment**.



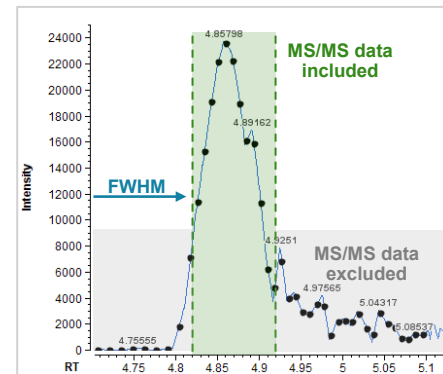
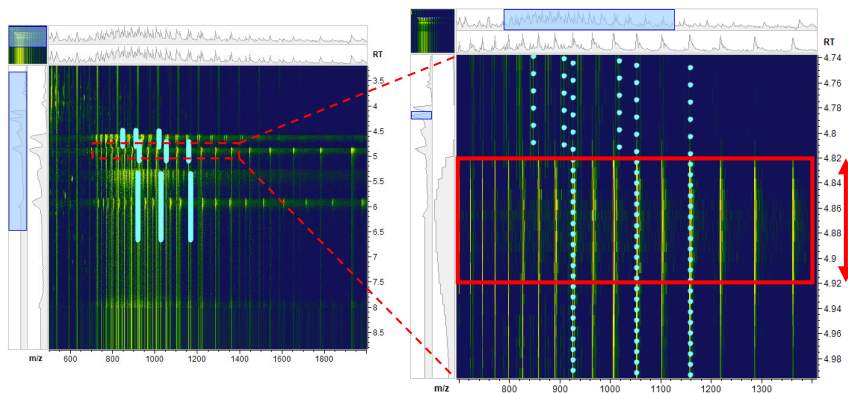
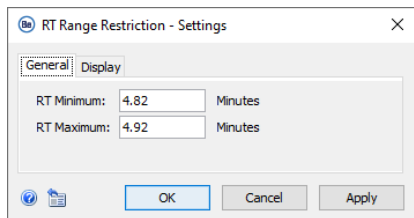
Name	MS Scans	MS
NIST mAb IdeS subunit 20220906_08 [1]	1502	
NIST mAb IdeS subunit 20220906_09 [1]	1502	
NIST mAb IdeS subunit 20220906_10 [1]	1502	
NIST mAb IdeS subunit 20220906_11 [1]	1502	
NIST mAb IdeS subunit 20220906_01 [2]	1471	
NIST mAb IdeS subunit 20220906_03 [2]	1459	
NIST mAb IdeS subunit 20220906_04 [2]	1466	
NIST mAb IdeS subunit 20220906_05 [2]	1487	

20 rows (1 selected)

# Restriction of RT and $m/z$ Ranges



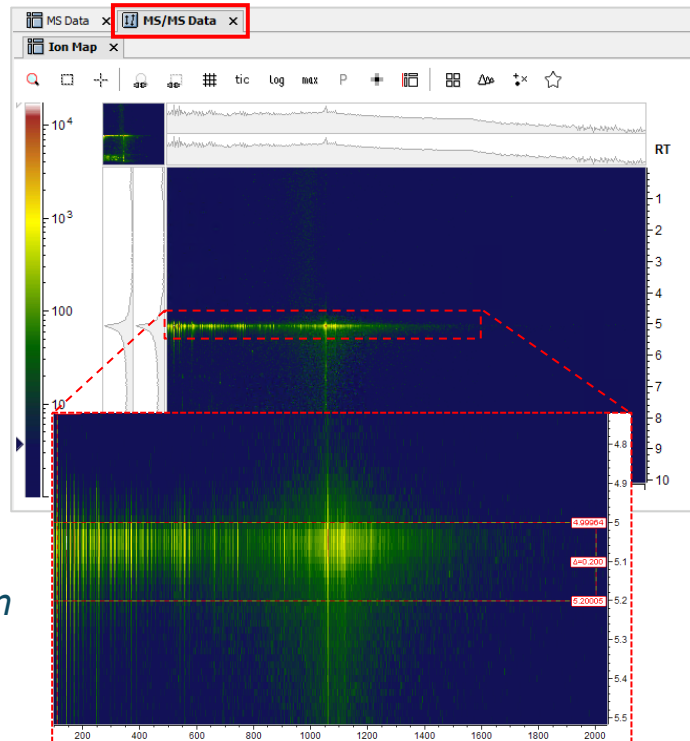
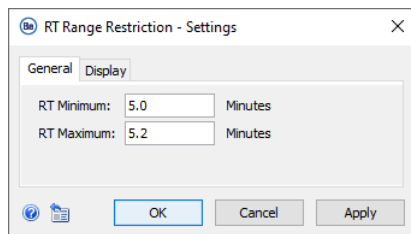
- To identify the RT ranges where there is meaningful data, open (double-click) *Load Raw Data* after the data is loaded.
- Identify a retention time (RT) range that includes MS/MS data from a single protein.
  - Select the RT range that contains MS/MS data acquired across the highest intensity region of the peak (above the full width at half maximum, FWHM) in most replicates.



- Change the  $m/z$  Range Restriction only if the specified RT range includes MS/MS data from other proteins or mAb fragments.

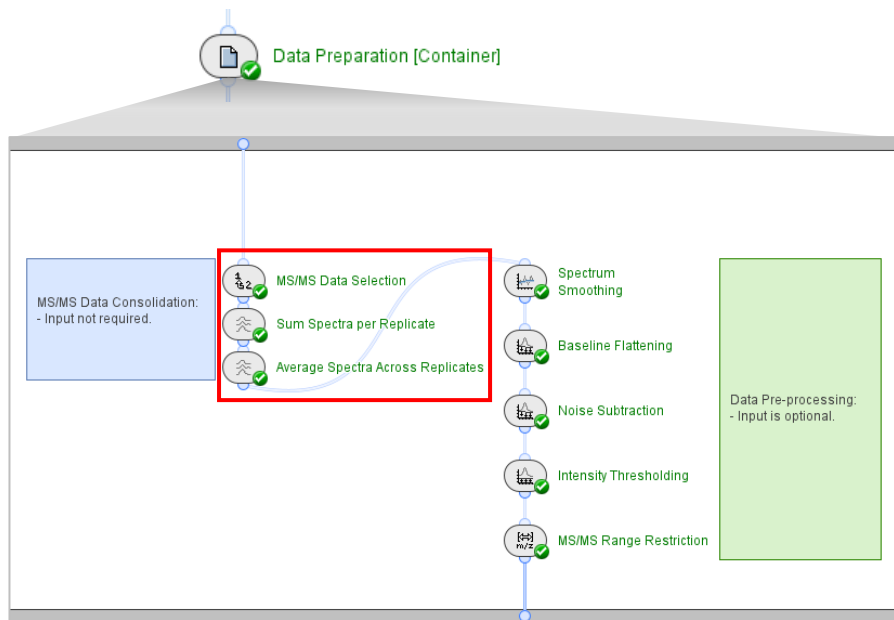
# Restriction of RT and $m/z$ Ranges: Special Case

- If protein sequencing data is acquired on only a single precursor, then the MS/MS data is found in the **MS/MS Data** tab as an ion map.
  - Select the RT range that includes the most intense region of the MS/MS.



- To include all MS/MS data, keep the  *$m/z$  Range Restriction* input fields empty or activate the **Bypass** icon.

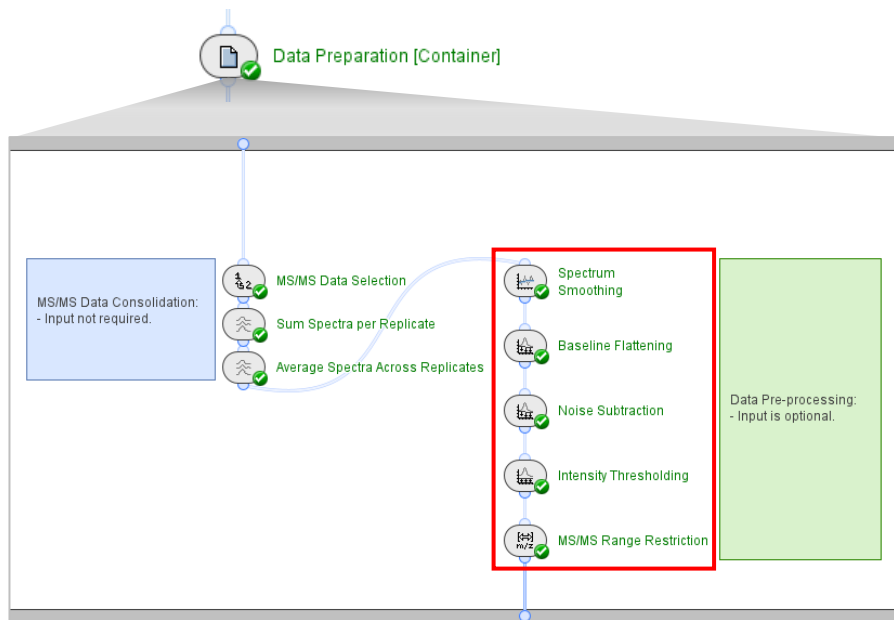
# Data Preparation: MS/MS Data Consolidation



**Note:** The activity nodes used for consolidation of MS/MS data do not require any input or optimization, even if only a single replicate is analyzed.

- *MS/MS Data Selection*
  - Selects and extracts MS/MS data to create a new ion map for further processing in the workflow.
- *Sum Spectra per Replicate*
  - Sums the MS/MS data from the selected RT range across all charge states for each replicate experiment.
- *Average Spectra Across Replicates*
  - Averages the summed spectra from each replicate across all replicates, to create a single high-quality MS/MS spectrum for *Fragment Mapping*.

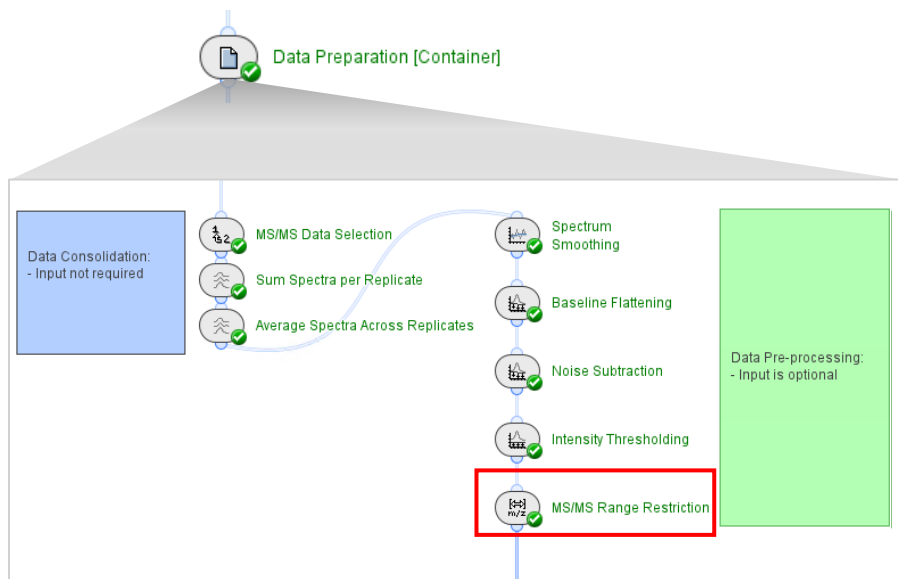
# Data Preparation: MS/MS Data Pre-Processing



**Note:** The activity nodes related to pre-processing of MS/MS data require input only under unusual circumstances. The settings have been optimized across a range of datasets to give reliable results for protein sequencing applications.

- **Spectrum Smoothing**
  - Removes small fluctuations in intensity to decrease the total number of detected peaks.
- **Baseline Flattening**
  - Removes low-intensity noise and decreases the baseline intensity.
- **Noise Subtraction**
  - Removes general noise and has an effect on all signals.
    - A decrease in the **Quantile** subtraction will increase sensitivity, but will also increase the number of false positive identifications significantly.
    - To increase sensitivity and thus increase bond coverage, it is recommended to decrease the thresholds in the *Filters* [Container].
- **Intensity Thresholding**
  - Removes any signal below the defined threshold.

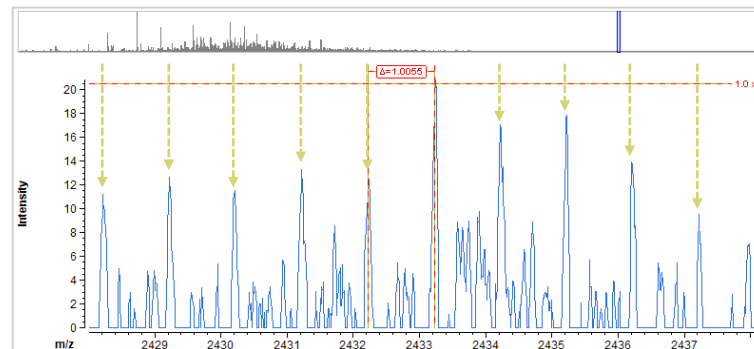
# Data Preparation: MS/MS Range Restriction



- To include all MS/MS data, keep the *MS/MS Range Restriction* input fields empty.

- *MS/MS Range Restriction*

- If the MS/MS data is acquired over a large  $m/z$  range, then data above and below defined  $m/z$  values can be removed to simplify data interrogation.
- For example, above 2000  $m/z$  the inherent 1 Da 'picket fence' ladder peaks that are typical of this data type have higher intensities than at lower  $m/z$ . The higher intensity of these peaks decreases the robustness of the isotopic clustering of fragment ions.



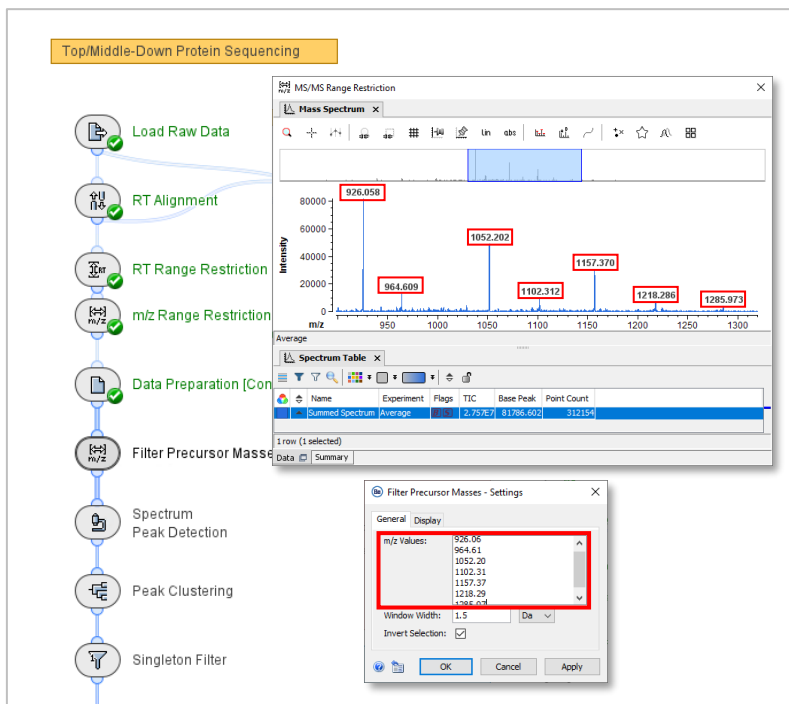
# Filter Precursor Masses



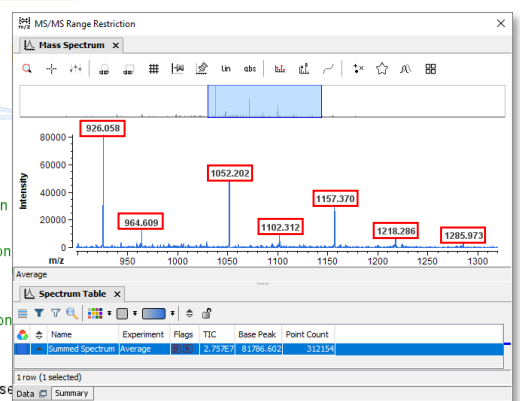
## • Filter Precursor Masses

- Removes data at defined  $m/z$  values.
  - To remove data related to the unfragmented precursors, type a list of masses that are applicable to the molecule of interest.
- Simplifies visualization of MS/MS clusters.

Top/Middle-Down Protein Sequencing



MS/MS Range Restriction



Average

Name	Experiment	Flags	TIC	Base Peak	Point Count
Summed Spectrum	Average		27.9727	31.766621	312154

1 row (1 selected)

Filter Precursor Masses - Settings

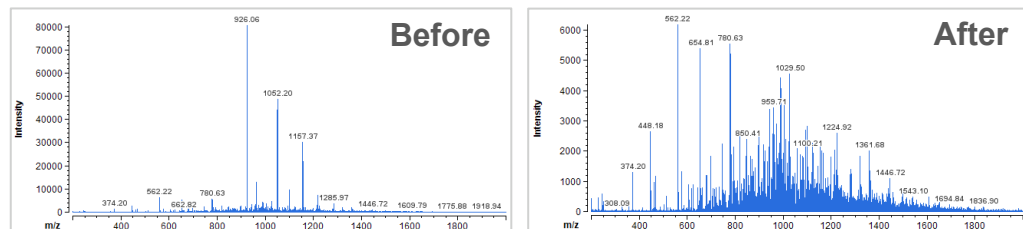
General Display

$m/z$  Values: 926.06, 964.61, 1052.20, 1102.31, 1157.37, 1218.29, 1285.97

Window Width: 1.5 Da

Invert Selection:

OK Cancel Apply

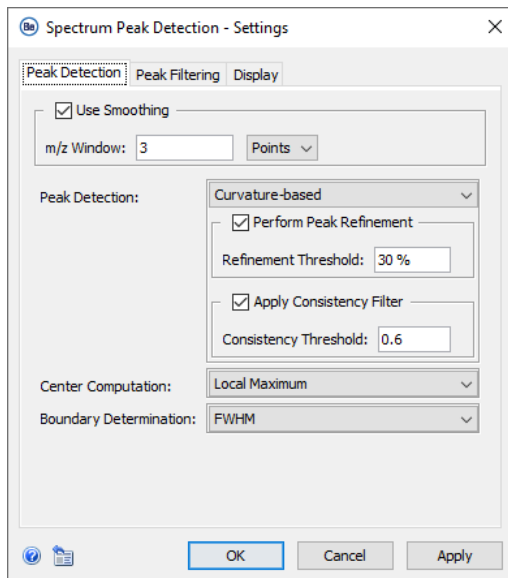


**Note:** Use the results of *MS/MS Range Restriction* to see the  $m/z$  values that should be removed:

1. Open the results of *MS/MS Range Restriction* in the *Data Preparation [Container]*.
2. Undock the *MS/MS Range Restriction* window.
3. Open *Filter Precursor Masses* from the workflow and specify values.



# Spectrum Peak Detection



- **Use Smoothing**

- Temporary smoothing is applied in the background for peak detection.

- **Peak Detection**

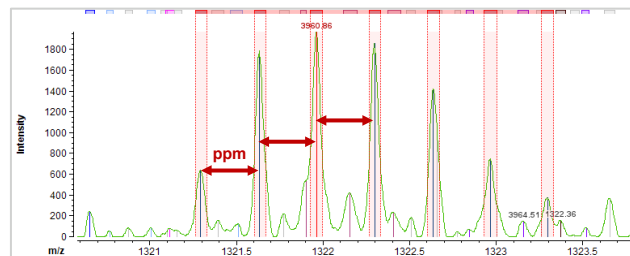
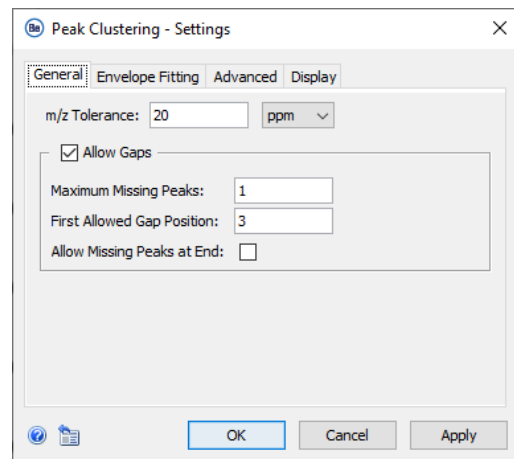
- **Curvature-based** peak detection is used to resolve shoulder peaks, which is recommended for analysis of data with complex and often overlapping isotopic cluster profiles.
- **Perform Peak Refinement:** For detection of subtle shoulder peaks.
  - **Refinement Threshold:** To increase peak splitting with subtle shoulder peaks, decrease the threshold percentage.
- **Apply Consistency Filter:** Considers peak width and curvature.
  - **Consistency Threshold:** Values between 0.4 and 0.6 are recommended.
  - To decrease peak splitting, increase the threshold, up to a maximum of 2.

# Peak Clustering: General



- **$m/z$  Tolerance**

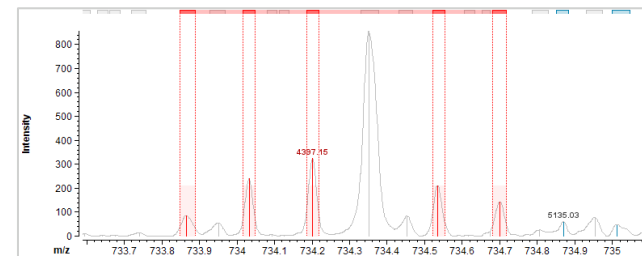
- Specify the maximum window of mass accuracy that each peak must have to be included in an isotopic cluster, related to either the detected or the recomputed monoisotopic mass.



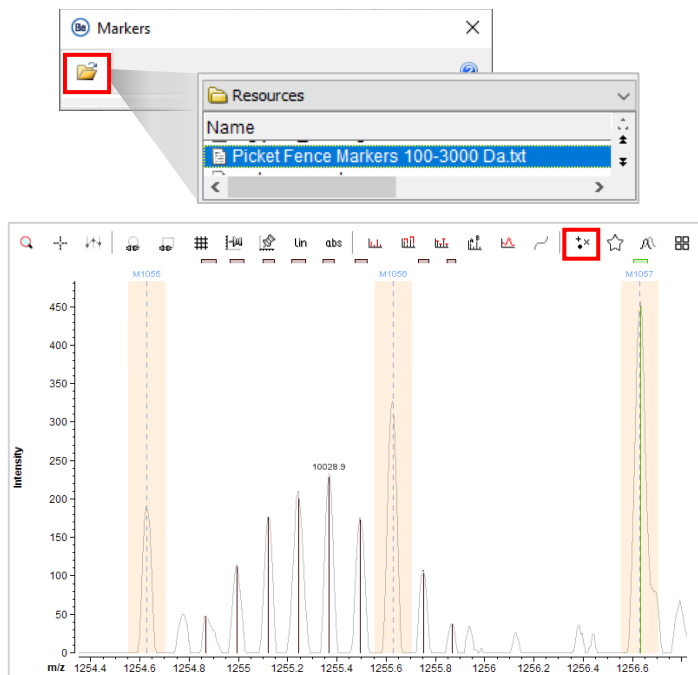
- Note: This setting is not a reflection of the mass accuracy of the mass spectrometer.

- **Allow Gaps**

- Isotopic envelopes with missing or overlapping peaks can be clustered.



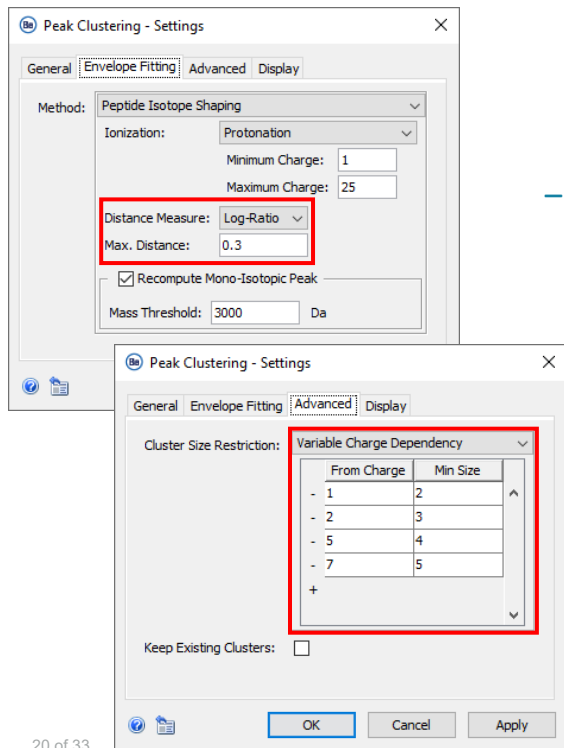
# Peak Clustering: 'Picket Fence' Peaks



**Note:** Use the **Picket Fence Markers** file in the **Resources** folder to show the position of the 'picket fence' peaks to help with manual interrogation of isotopic clusters during data review. To load the Marker File included in the template workflow, use the **Show Markers** icon.

- In MS/MS data acquired from large peptides and proteins there is an inherent 'picket fence' ladder-signal of peaks separated by approximately 1 Da.
  - To decrease the effect of 'picket fence' peaks in the MS/MS data, decrease the energy transmitted to the molecule during the MS data acquisitions.
- Use **Allow Gaps** to compensate for 'picket fence' peaks in the MS/MS data.
  - Some 'picket fence' peaks will overlap with true peaks and cause a change to the isotopic cluster shape.
  - Overlapping 'picket fence' peaks prevent clusters from being correctly annotated, unless the **Allowed Gaps** setting is used to ignore changed peaks.

# Peak Clustering: Envelope Fitting and Advanced Tab

The screenshot shows two overlapping instances of the 'Peak Clustering - Settings' dialog box. The top instance is on the 'Envelope Fitting' tab, and the bottom instance is on the 'Advanced' tab. Both instances have a red box highlighting specific settings.

**Top Instance (Envelope Fitting Tab):**

- Method: Peptide Isotope Shaping
- Ionization: Protonation
- Minimum Charge: 1
- Maximum Charge: 25
- Distance Measure: Log-Ratio
- Max. Distance: 0.3
- Recompute Mono-Isotopic Peak
- Mass Threshold: 3000 Da

**Bottom Instance (Advanced Tab):**

- Cluster Size Restriction: Variable Charge Dependency
- Keep Existing Clusters:

From Charge	Min Size
- 1	2
- 2	3
- 5	4
- 7	5
+	

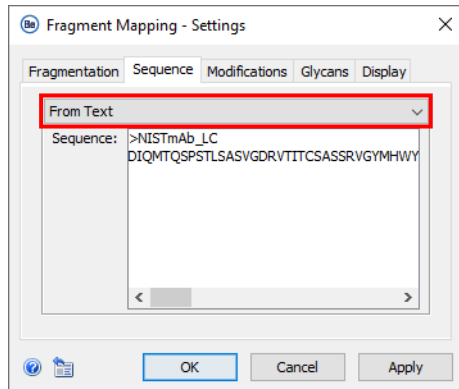
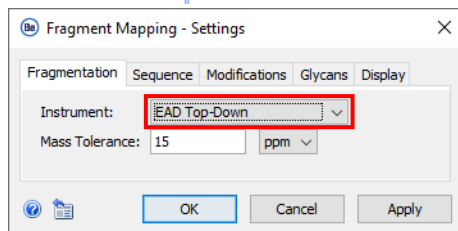
## • Distance Measure

- Compares the measured isotopic cluster profile to the theoretical isotopic profile shape (based on the Averagine model).
  - **Log Ratio:** Treats all peaks in a cluster equally in comparisons of the experimental profile and the theoretical profile.
- To decrease the required similarity between the measured and the theoretical isotopic clusters, increase the **Max. Distance**.
  - Note: When **Max. Distance** is increased to improve detection of one cluster, isotopic clustering in another area of the data might then fail.
  - When the **Max. Distance** is increased, the number of false positive identifications also increases.

## • Variable Charge Dependency

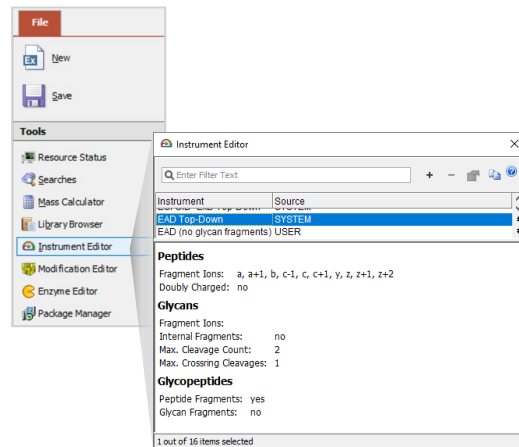
- Specify the minimum number of peaks required in an isotopic cluster, based on the charge of that cluster.

# Fragment Mapping: Configure Settings (1)



## Fragmentation tab:

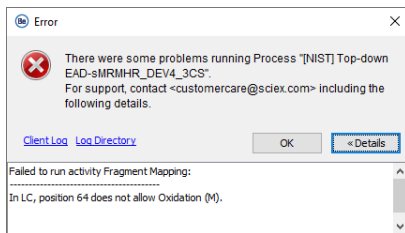
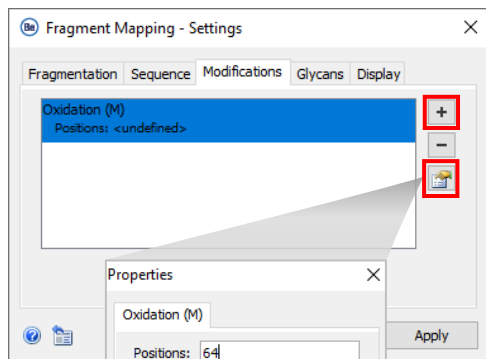
- **Instrument:** Select **EAD Top-Down**.
  - To review or change the types of fragment ions used for identification:
    - Browse to **File > Tools > Instrument Editor**.



## Sequence tab:

- **Sequence:** Select **From Text** and type the sequence, or select **From Fasta File** and add the applicable file.
- To analyze a mAb digested with IdeS:
  1. Run a separate *Fragment Mapping* search for each of Lc, Fd' and Fc/2.
  2. Use *Save Results Snapshot* to export the sbf file for each proteoform.
  3. Analyze the sbf files together with the Top\_Middle-Down\_ReviewSnapshots workflow.

# Fragment Mapping: Configure Settings (2)



## Modifications tab:

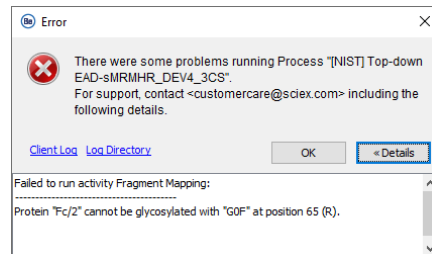
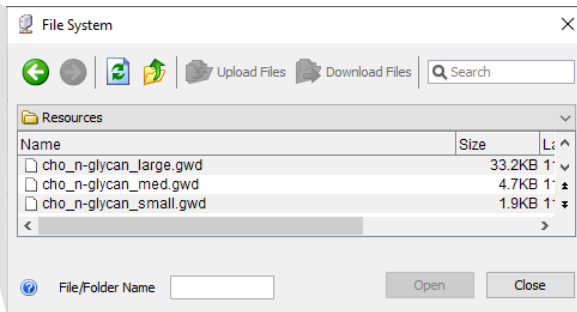
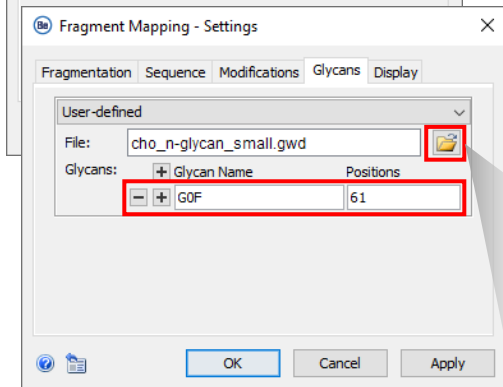
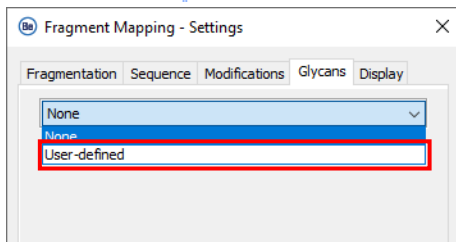
- All modifications are fixed.
- To see the list of available modifications, use the **+** icon to open the **Select Entries** dialog.
- To add information for the selected modification, click the **Configure selected entries** icon to open the **Properties** dialog.
  - Specify the expected amino acid position of the modification.
  - If the specified amino acid is not at that location in the protein sequence, then the activity node will show an error.
- To compare modifications specified at different amino acid positions:
  1. Run a separate *Fragment Mapping* search for each modification position.
  2. Use *Save Results Snapshot* to export the sbf file for each proteoform.
  3. Analyze the saved sbf files together with the *Top\_Middle-Down\_Review Snapshots* workflow.

# Fragment Mapping: Configure Settings (3)

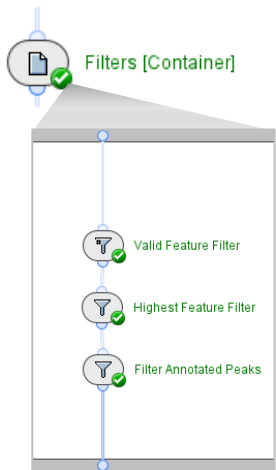


## Glycans tab:

- Select **User-defined** if the sequence contains a glycosylation.
  - **File:** Browse to **Resources** in the **File System**, and then select a glycan library that is applicable to the molecule being investigated.
    - To review or change a glycan library: Browse to **File > Tools > Library Browser**.
  - **Glycans:** Type the glycan name and its expected position in the sequence.
    - If multiple glycans are specified, then each will be searched for as a separate fixed modification.
    - If the specified amino acid is not at that location in the protein sequence, then the activity node will show an error.



# Filters: Remove Clusters



**Note:** To optimize the *Filters* and change the sensitivity, compare the results for both the bond coverage and total number of detected clusters in the *Fragment Mapping* activity node with the results of the *Review Results* activity node.

- **Valid Feature Filter**
  - To remove low-intensity clusters, specify the **Validity Threshold**.
    - If the most intense peak is below the defined threshold, then the cluster is removed.
- **Highest Feature Filter**
  - Specify the total number of clusters to keep.
- **Filter Annotated Peaks**
  - Removes any non-annotated peaks to simplify data visualization in *Review Results*.
    - To increase sensitivity, decrease the *Valid Feature Filter* threshold or increase the **Keep Top** number of clusters in *Highest Feature Filter*. An increase in sensitivity results in:
      - An increase in the number of detected clusters and, thus, in bond coverage.
      - An increased number of false positive identifications.
        - The increase in false positive identifications that results from a decrease in the *Valid Feature Filter* threshold is less than that caused by a decrease in the **Quantile** subtraction in the *Noise Subtraction* activity node.



# Define Experiment Name



Name	Experiment	Flags	Point Count	TIC	Base Peak
Summed Spectrum	Average		312154	2.01477E7	7312.77


Name	Experiment	Flags	Point Count	TIC	Base Peak
Summed Spectrum	NISTmAb: Fc with Oxi Met		312154	2.01477E7	7312.77

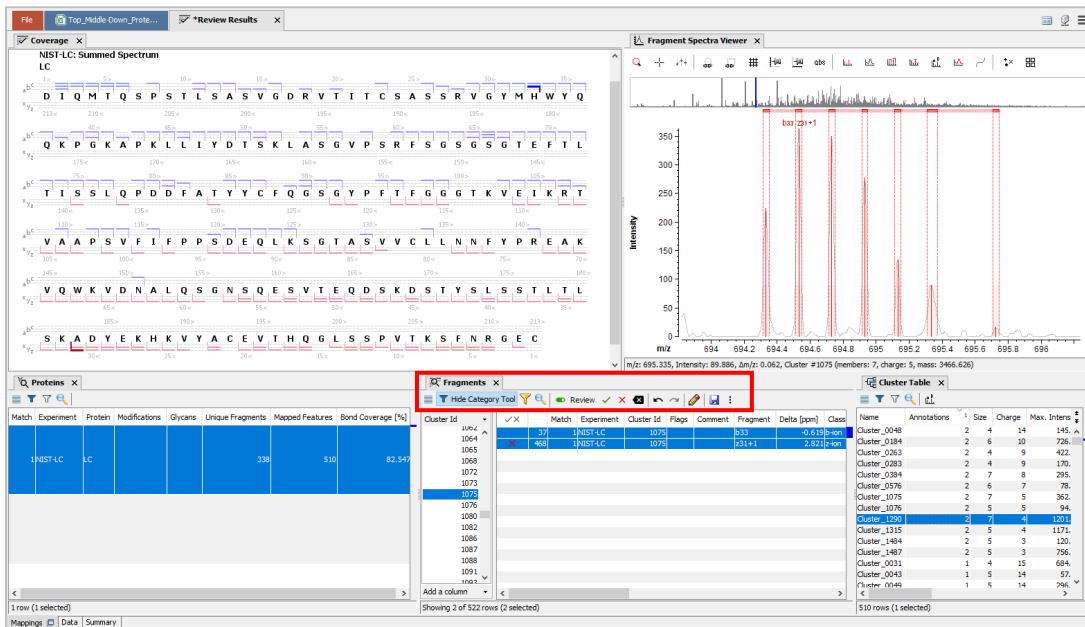
**Note:** To analyze results from different Protein Sequencing workflows that are saved as *Results Snapshots*, load them together in the Top\_Middle-Down\_ReviewSnapshots workflow.

- If multiple technical replicates are analyzed together, then the **Experiment** name is overwritten in the *Average Spectra Across Replicates* activity node.
- Use the *Define Experiment Name* activity node to specify a new **Experiment** name for the dataset.
- *Define Experiment Name* is also useful if the same data is analyzed with different search parameters in the *Fragment Mapping* activity node.
  - For example, for comparison of different results when a modification is specified at different amino acid locations.
  - It is a good practice to use *Define Experiment Name* to include unique information about the specific sample set and the workflow settings used during data analysis.

# Review Results: Review Protein Sequencing Results



- 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.
- To apply the changes, click the **Save** icon, and then select **Save and Reload**.



The screenshot shows the software interface in 'Review Results' mode. The main window displays a protein sequence with fragmentation points and a corresponding mass spectrum. A 'Review Results' window is open, showing a table of fragments with columns for Match, Experiment, Protein, Modifications, Glycans, Unique Fragments, Mapped Features, and Bond Coverage. The 'Review' tab is active, and a red box highlights the 'Review' button and the 'Comment' column. A 'Cluster Table' window is also visible, showing a list of clusters with columns for Name, Annotations, Size, Charge, Max. Intensity, and Bond Coverage.

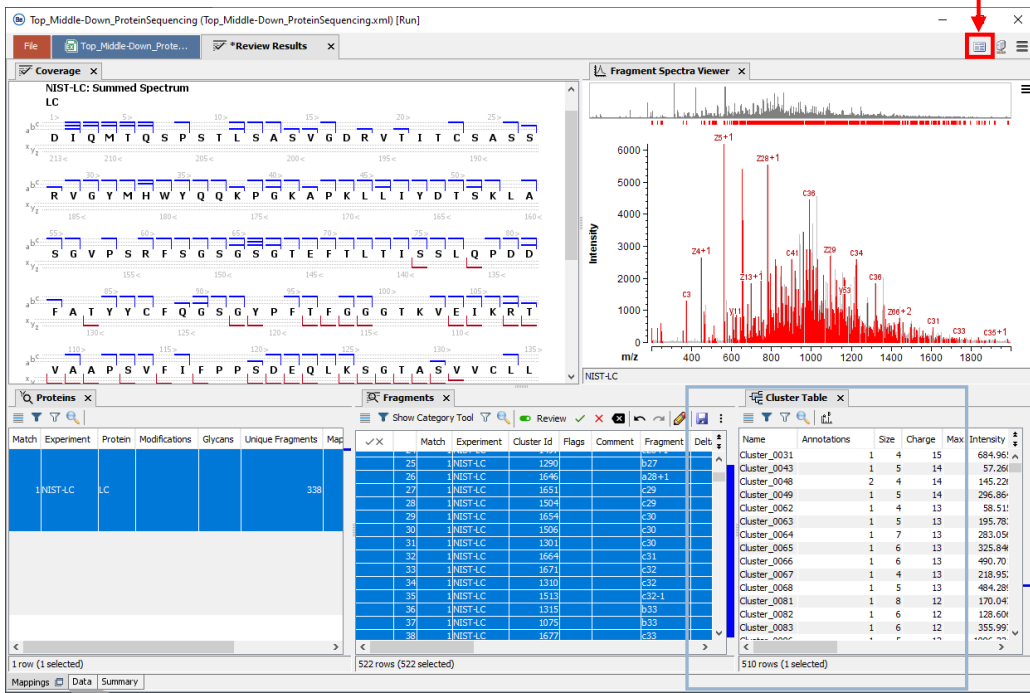
Match	Experiment	Protein	Modifications	Glycans	Unique Fragments	Mapped Features	Bond Coverage [%]
	1.NEST-LC	LC			330	510	82.54%

Cluster Id	Match	Experiment	Cluster Id	Flags	Comment	Fragment	Delta [ppm]	Class
1072	37	1.NEST-LC	1072			[33	-0.619	non
1073	460	1.NEST-LC	1073			[211+1	2.821	non

Name	Annotations	Size	Charge	Max. Intensity	Bond Coverage [%]
Cluster_0048		2	4	14	145
Cluster_0184		2	6	10	726
Cluster_0263		2	4	9	422
Cluster_0283		2	4	9	170
Cluster_0384		2	7	8	295
Cluster_0576		2	6	7	78
Cluster_1075		2	7	5	362
Cluster_1076		2	5	5	94
Cluster_1500		2	7	5	120
Cluster_1315		2	5	4	1171
Cluster_1484		2	5	3	120
Cluster_1487		2	5	3	786
Cluster_0031		1	4	15	684
Cluster_0043		1	5	14	57
Cluster_1044		1	6	14	794


# Review Results: Create Custom Layouts

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





The screenshot displays the 'Review Results' window with several panes:

- Coverage:** Shows protein sequences with b and y fragment ions labeled.
- Fragment Spectra Viewer:** Displays a mass spectrum with peaks labeled by m/z values (e.g., 25+1, 228+1, 24+1).
- Proteins:** A table listing identified proteins.
- Fragments:** A table listing identified fragments.
- Cluster Table:** A table listing identified clusters.

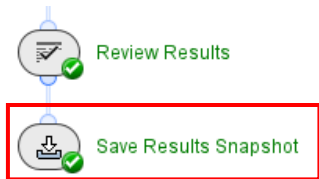
- Each pane can be undocked and then docked at a new location.
- The location where the undocked pane will be docked is highlighted by a blue box.
- To move useful tables and visualizers to the **Mappings** tab:
  1. To undock the **Data** tab, click the  icon.
  2. To undock any pane from the **Data** tab window, drag it to a new location on the **Mappings** 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](#).

des Data  

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

# Export Intermediate Results for Further Analysis



- Snapshots are intermediate results that are saved permanently as sbf files.
  - The *Save Result Snapshot* creates an sbf file that contain the results of the *Top\_Middle-Down\_ProteinSequencing* workflow.

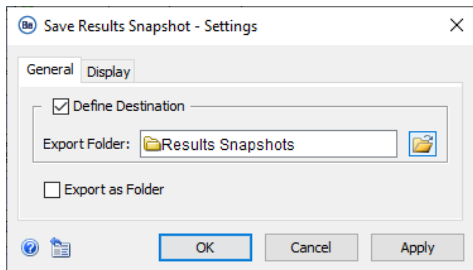
- To use a *Save Snapshot* activity node:

- Deactivate the **Block** icon.
- Select or add the folders where the sbf files will be saved.



- To review saved data:

1. Select the sbf files to import into the *Load Snapshots* activity node in the *Top\_Middle-Down\_ReviewSnapshots* workflow.
  - Note: Select individual sbf files from the parent folder. Data will not load if the parent folder is selected.

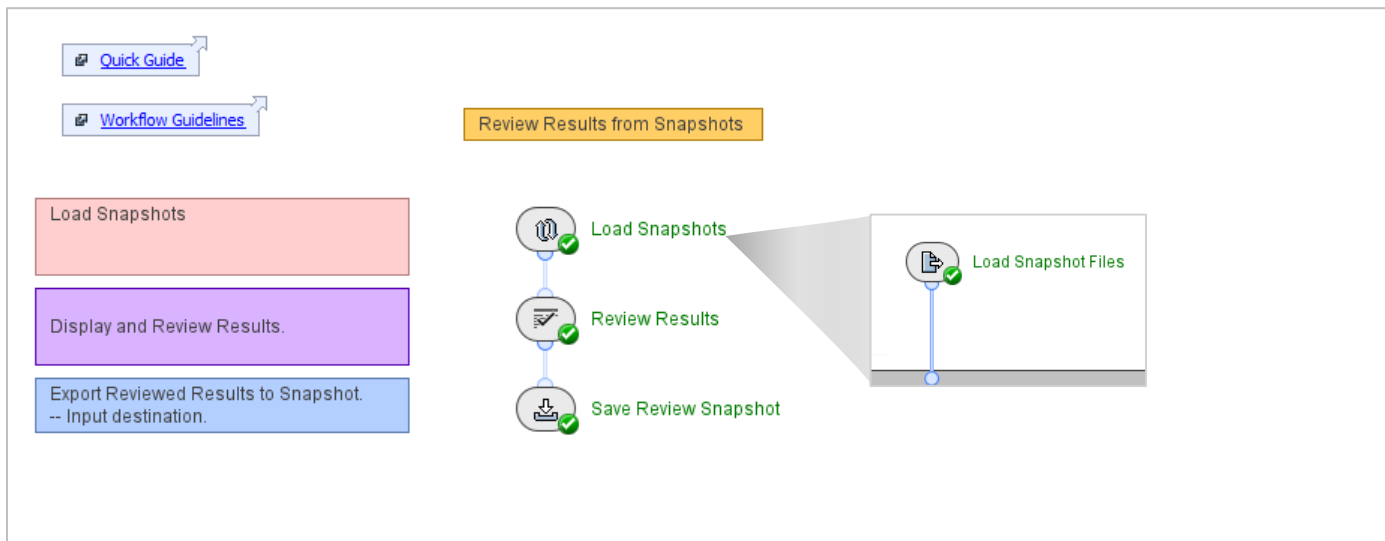


# Part B

## Workflow Guidelines to Review Snapshots

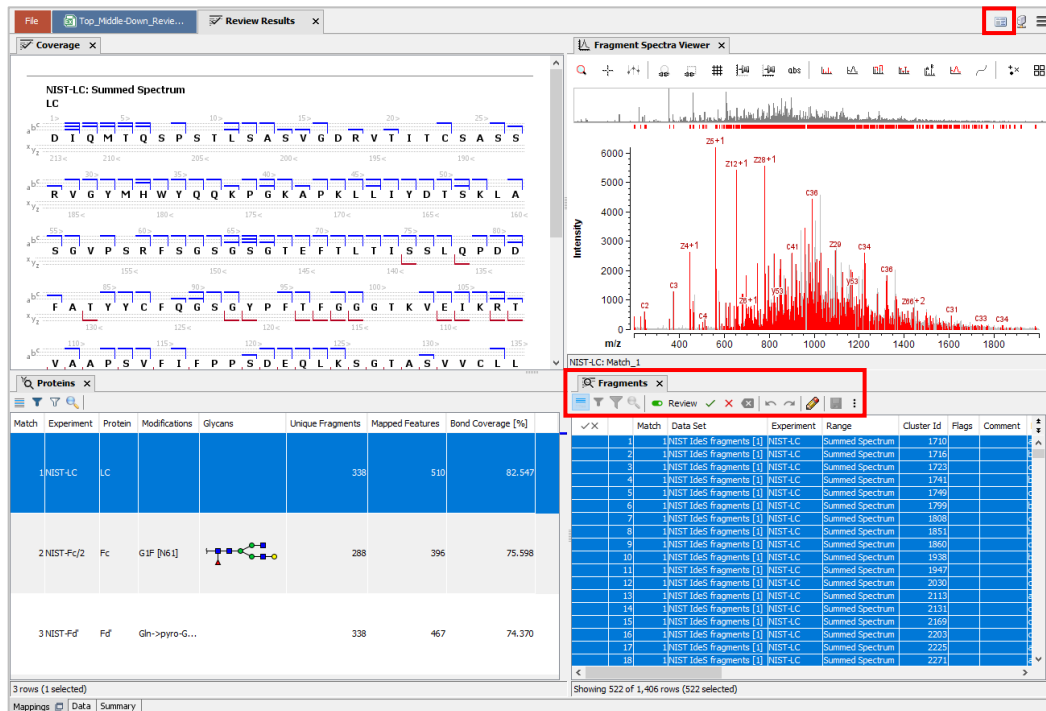


# Protein Sequencing Review Snapshots Workflow




Top\_Middle-Down\_ReviewSnapshots\_Be4.0

# Review Snapshots Workflow: Overview



The screenshot displays the Proteomics software interface with the following components:

- Protein Sequence:** NIST-LC: Summed Spectrum LC. Sequence: D I Q M T I Q S P S T L S A S V G D R V T I T C S A S S. Other sequences include R V G Y M H W Y Q Q K P G K A P K L L I Y D T S K L A, S G V P S R F S G S G T E F T L T I S S L Q P D D, F A T Y Y C F Q G S G Y P P F T F G G G T K V E I K R T, and V A A P S V F I F P P S D E Q L K S G T A S V V C L L.
- Mass Spectrum:** Fragment Spectra Viewer showing Intensity vs. m/z. Key peaks are labeled: C2, C3, C4, 24+1, 25+1, 212+1, 220+1, C36, C41, C29, C34, C38, 289+2, C33, C34.
- Protein Table:**

Match	Experiment	Protein	Modifications	Glycans	Unique Fragments	Mapped Features	Bond Coverage [%]
1	NIST-LC	LC			338	510	82.547
2	NIST-Fc/2	Fc	G1F [N61]		288	396	75.598
3	NIST-Fd	Fd	Gln->pyro-G...		338	467	74.370
- Fragment Table:**

Match	Data Set	Experiment	Range	Cluster Id	Flags	Comment
1	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	1710		
2	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	1716		
3	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	1723		
4	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	1741		
5	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	1749		
6	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	1799		
7	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	1808		
8	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	1851		
9	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	1860		
10	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	1938		
11	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	1947		
12	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	2030		
13	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	2113		
14	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	2131		
15	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	2169		
16	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	2201		
17	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	2222		
18	NIST IdS5 fragments [1]	NIST-LC	Summed Spectrum	2271		

- This workflow can be used to open saved snapshot (sbf) files created from any Protein Sequencing workflow in Biologics Explorer software.
- Use this workflow to analyze previous results from *Fragment Mapping* searches.
  - For example, to analyze all mAb fragments from an IdeS digest together, or to compare the sequence coverage with different modification locations.
- The same saved layout can be applied here as in the Protein Sequencing workflow.
- Use *Save Review Snapshot* to save a final reviewed sbf file for each sample.



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