Intact Mass Analysis

Biologics Explorer Software 4.0 Guidelines

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Intact Mass Analysis

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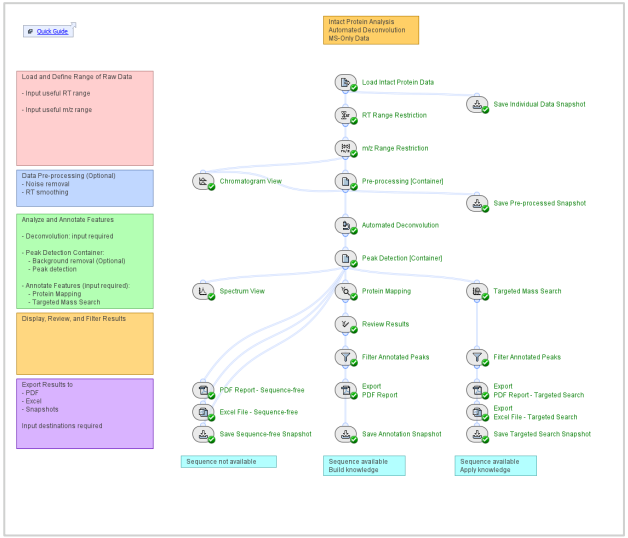
Part A
General Guidelines for Intact Mass Workflows

Overview of Applications for Intact Mass Workflows

- These workflows are mainly designed for single sample analysis:
  - Intact mass confirmation
  - Glycosylation pattern analysis
  - Post-translational modification (PTM) characterization
  - Drug-Antibody Ratio (DAR) calculations
- Batch analysis is also possible, if chromatography is consistent across samples:
  - To screen multiple samples (process development, instrument method development)
  - Lot-to-lot comparability studies
  - Innovator to biosimilar comparability studies
  - Stress tests
- These types of molecules can be analyzed:
  - Whole proteins (native or denatured)
  - Protein subunits/fragments
  - Multimeric proteins
  - Protein mixtures
  - Drug conjugates

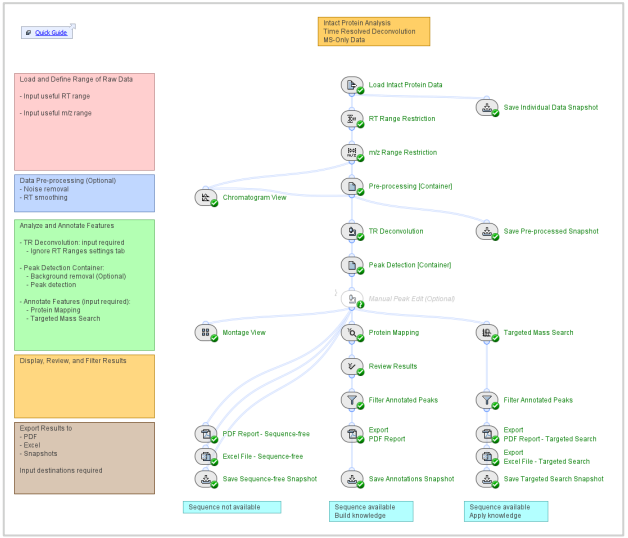
General Intact Mass Workflow Guidelines

WORKFLOW TYPES



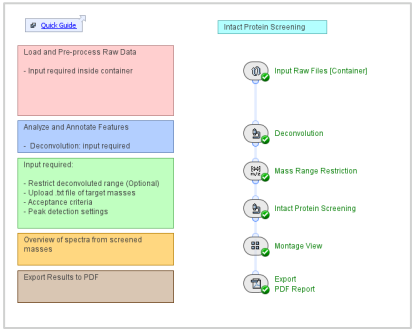
Intact_AutomatedDeconvolution

- with and without UV processing

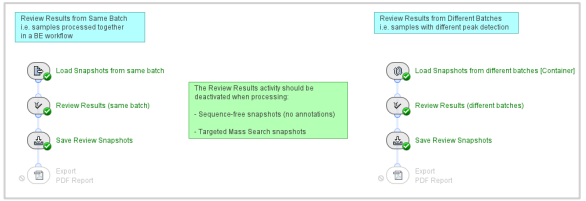


Intact_TimeResolvedDeconvolution

- with and without UV processing



Intact_MassScreening

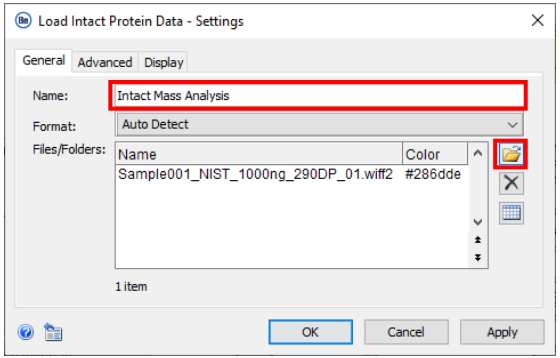


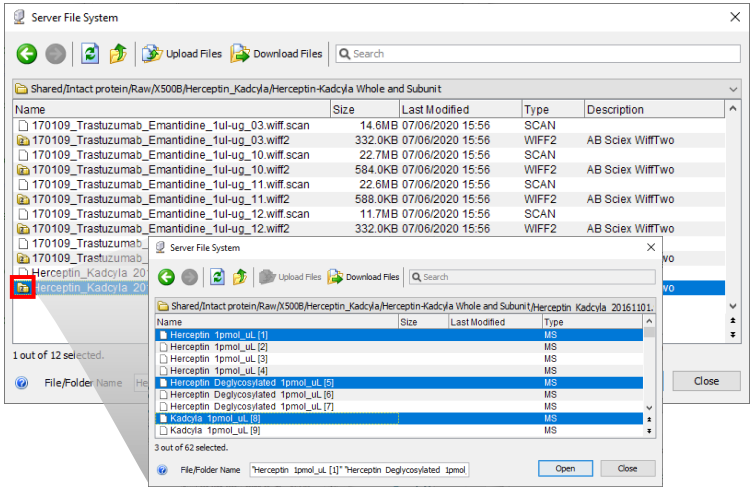
Intact_ReviewSnapshots

Common Activity Nodes in the Intact Mass Workflows

A. *Load Intact Protein Data*
B. *RT Range Restriction* and *m/z Range Restriction*
C. *m/z Grid*
D. *Spectrum Baseline Subtraction*
E. *Chromatogram Chemical Noise Subtraction*
F. *UV Processing [Container]*
G. *Chromatogram View*
H. Feature Filters
I. *Protein Mapping*
J. *Targeted Mass Search*
K. *Annotate UV Peaks from MS*
L. Reporting and Exporting

Load Intact Protein Data: Data Files



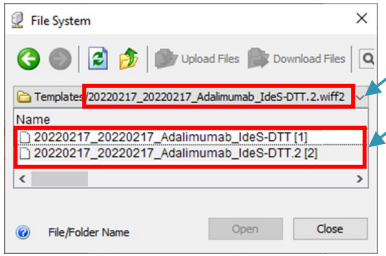


- To upload raw data files, click the folder icon .
  - Select container files with the format wiff or wiff2.
    - If data was acquired with the ZenoTOF 7600 system, select only the wiff2 format.
    - Do not select the auxiliary files with the same name.

  - 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.
      - Note: For more information about Batch Processing, refer to the section: Guidelines for Intact Mass Batch Processing.

Load Intact Protein Data: Format

- The wiff or wiff2 **File Name** and the associated **Sample Name** might not be the same.



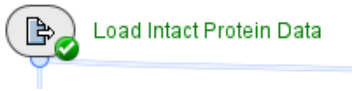
  - The **File Name** is the name of the wiff or wiff2 container file.
  - The **Sample Name** is the name of the data file in the wiff or wiff2 container file.
  - Individual acquisitions with different **File Names** might have the same **Sample Name**.
    - If entries in the **Experiment Table** have the same **Sample Name**, it can affect the quantitative information reported.

- To load multiple experiments that have <u>one acquisition</u> in the wiff or wiff2 container:
  1. From the **Format** list select either **SCIEX Wiff (*.wiff)** or **SCIEX WiffTwo (*.wiff2)**.
  2. Select **Use File Name as Sample Name** to use the **File Name** in the **Experiment Table**.
     - If **Format: Auto Detect** is selected, then the **Sample Name** will be used in the **Experiment Table**.

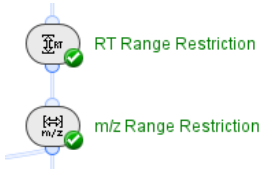
- To load experiments that have <u>multiple acquisitions</u> in the wiff or wiff2 container:
  1. From the **Format** list select either **SCIEX Wiff (*.wiff)** or **SCIEX WiffTwo (*.wiff2)**.
  2. <u>Do not</u> select **Use File Name as Sample Name**.
     - If **Format: Auto Detect** is selected, then the **Sample Name** will be used in the **Experiment Table**.
  - Note: For more information about Batch Processing, refer to the section: Guidelines for Intact Mass Batch Processing.

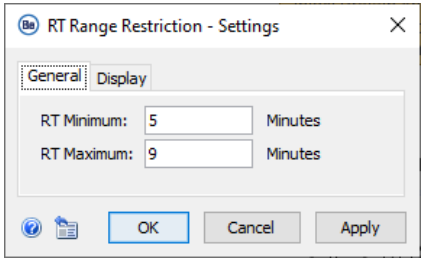
Restriction of RT and *m/z* Ranges

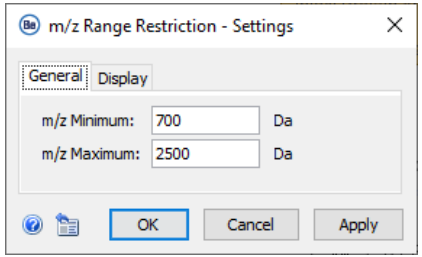
- To identify the retention time (RT) and mass-to-charge ratio *(m/z)* ranges that contain meaningful data, open (double-click) the results of *Load Intact Protein Data*.

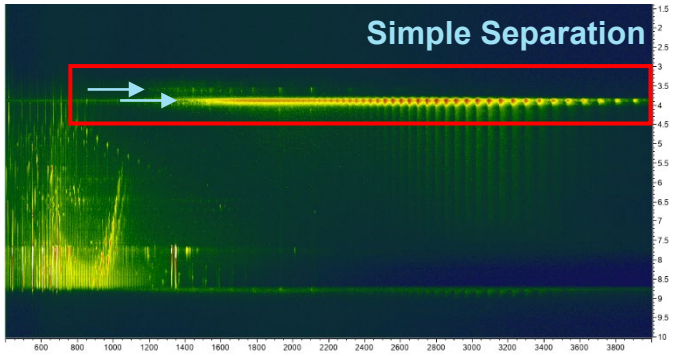


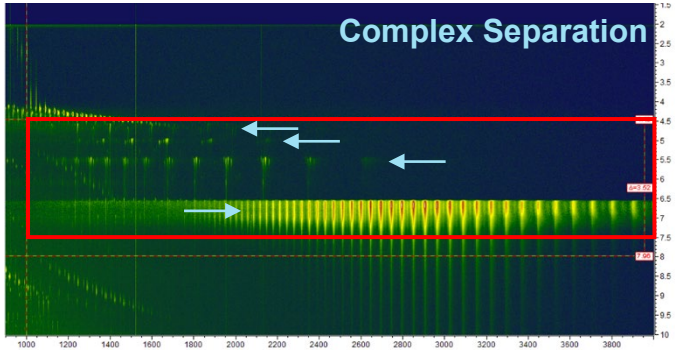
  - Unless minor components or contaminants at lower masses are of interest, restrict the RT and m/z ranges to the target protein.











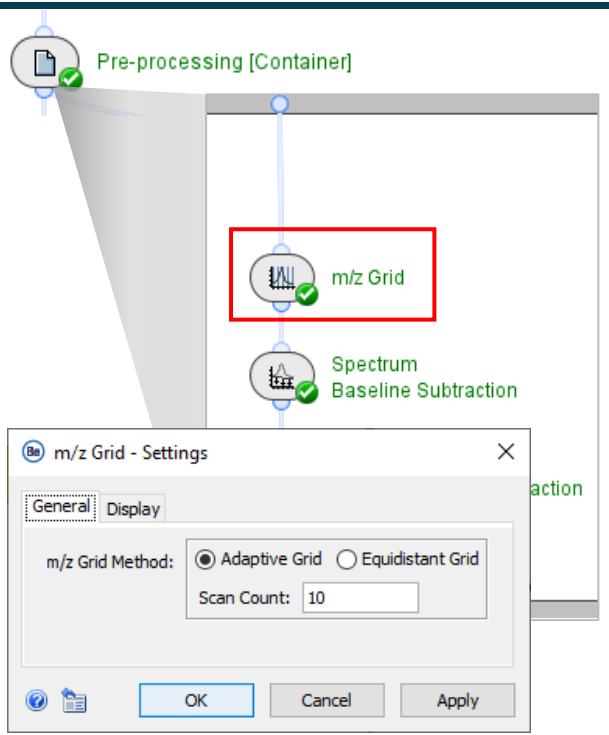
Note: To use the full RT or *m/z* ranges, leave the fields blank, or activate the **Bypass** icon.

m/z Grid

- Use the *m/z Grid* activity node to analyze multiple data files so that they are all are sampled at the same *m/z* positions for peak detection.

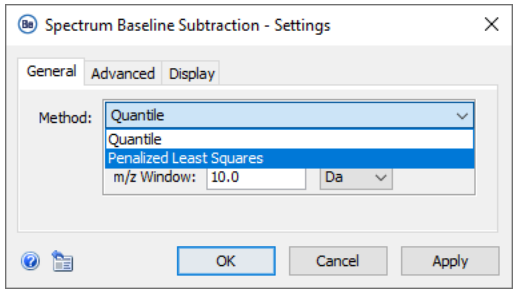
- The default setting is **Adaptive Grid**.
  - This grid adapts to the data density.
  - Use this setting to analyze samples with a large mass range.

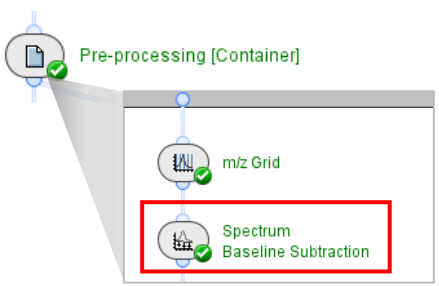
- Use the **Equidistant Grid** setting to analyze replicate samples, and data with under-sampled or noisy peaks.
  - An optimal value for **Equidistant Grid** spacing provides sufficient data points for low mass peaks of interest, without oversampling high mass peaks.

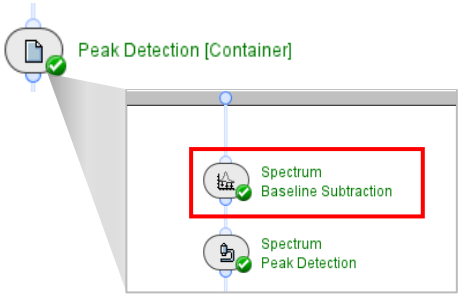


Spectrum Baseline Subtraction

- *Spectrum Baseline Subtraction* removes background noise and decreases the intensity of satellite peaks in the deconvoluted data.
- This activity node is also used after deconvolution to optimize peak detection.



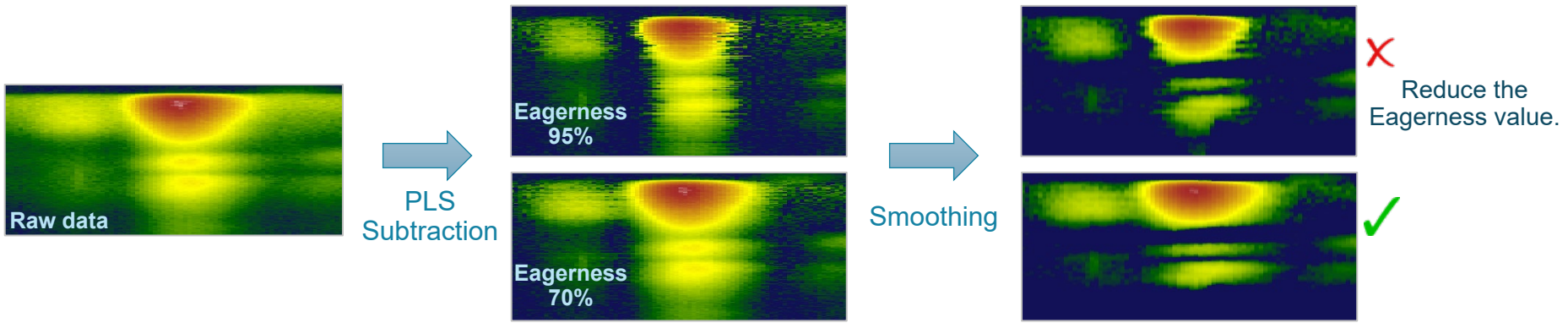




- **Quantile** subtraction affects all signals:
  - It requires little or no smoothing afterwards.
  - It is much faster than **Penalized Least Squares** when used with high resolution data.
  - It should be used with care for the analysis of intact proteins to prevent removal of meaningful signals.
- **Penalized Least Squares** subtraction has an effect on low intensity signals only.

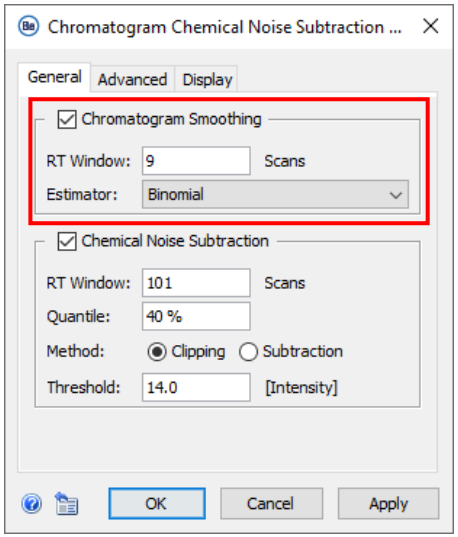
Spectrum Baseline Subtraction

- **Penalized Least Squares** decreases the valley height between large peaks, which decreases the intensity of satellite peaks in deconvoluted spectra.
  - High **Eagerness** values (greater than 90%) require extensive **Smoothing** in the *Chromatogram Chemical Noise Subtraction* activity node.
  - If features in the ion map have irregular borders after smoothing, then decrease the **Eagerness** value.



- Note: Penalized Least Squares can be time intensive, particularly when used with **Time Resolved Deconvolution** and with higher resolution data (such as subunits and fragments).

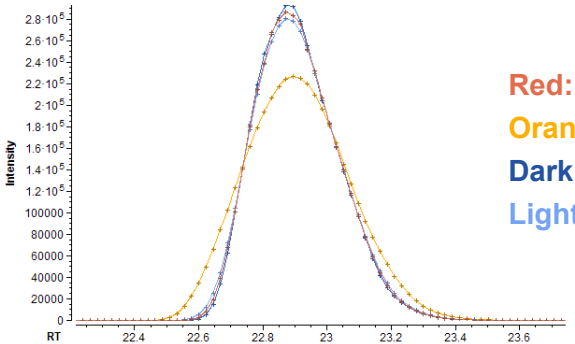
Chromatogram Chemical Noise Subtraction: Smoothing



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

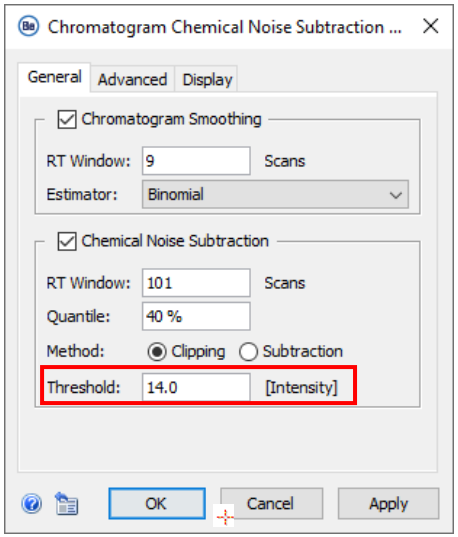
- Use **Chromatogram Smoothing** after **Penalized Least Squares** (in *Spectrum Baseline Subtraction*), especially if a high **Eagerness** value was used.

- **Estimator:**
  - **Moving Average** uses the mean intensity of data points in the **RT Window** to add more data points. High values cause peak widths to increase, but peak volume is conserved.
  - **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)

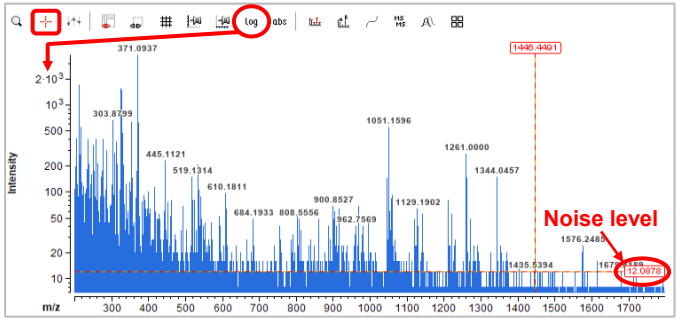
Chromatogram Chemical Noise Subtraction: Threshold



Chemical Noise Subtraction can help to:

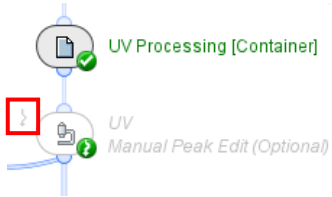
- Reduce the broad or long-tailing peaks often observed with native data.
- Suppress satellite peaks and improve peak detection with TRD.

- To decrease the amount of noise removal (keep more signal):
  - Decrease the **Quantile**.
  - Increase the **RT Window**.
- If the noise level is significantly different from the **Threshold** value pre-set in *Chromatogram Chemical Noise Subtraction*, then change this setting.



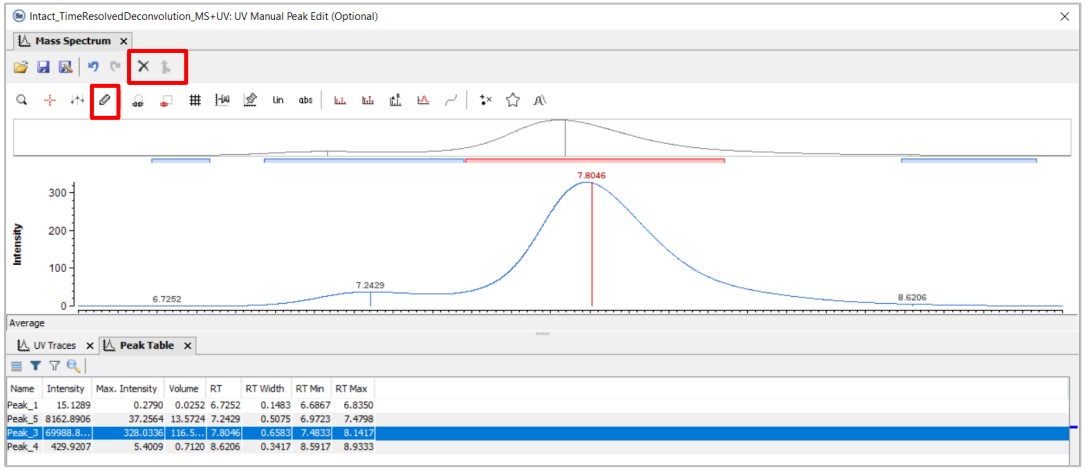
- To measure the noise level and specify an appropriate **Threshold** intensity value:
  1. Drag the mass spectrum intensity axis until the noise level can be seen, or use the icon in the tool bar to change the axis from the linear to the logarithmic scale.
  2. Use the crosshair tool to measure the intensity of the noise level.

UV Manual Peak Edit

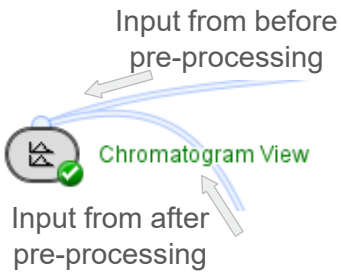


- This activity node is optional. To use *UV Manual Peak Edit*, deactivate the **Bypass** icon.
- Use *UV Manual Peak Edit* to manually change the peaks that were detected in the UV chromatogram.
  - For difficult peaks, it is recommended to use *UV Manual Peak Edit* to refine peak detection, and not to optimize *UV Peak Detection* parameters.

- Select the **Edit Mode** icon to:
  - Move the peak boundaries.
  - Split peaks that overlap.
  - Delete peaks.
  - Draw new peaks.

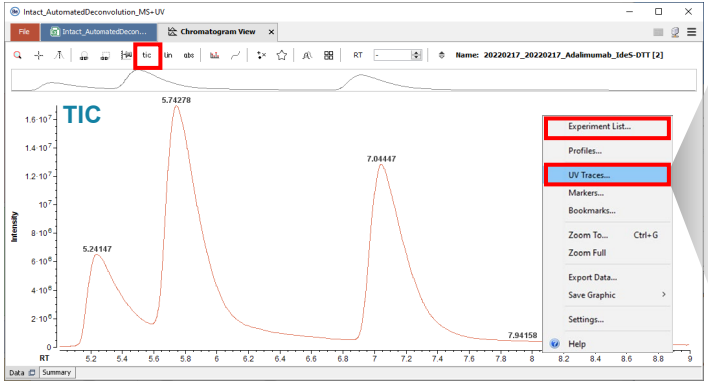


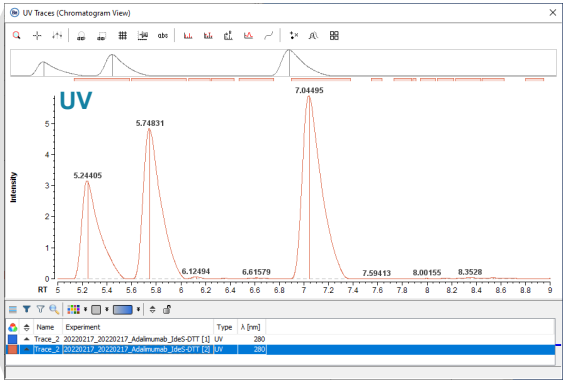
Chromatogram View

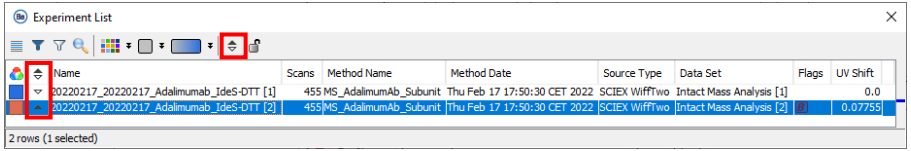


- *Chromatogram View* shows the Total Ion Chromatogram (TIC) or Base Peak Chromatogram (BPC) of the data before and after pre-processing.
- To see the **Experiment List** and **UV Chromatograms** from before and after *UV Processing*, right-click on the plot.

To change between TIC and BPC, use the icon in the Tool bar.



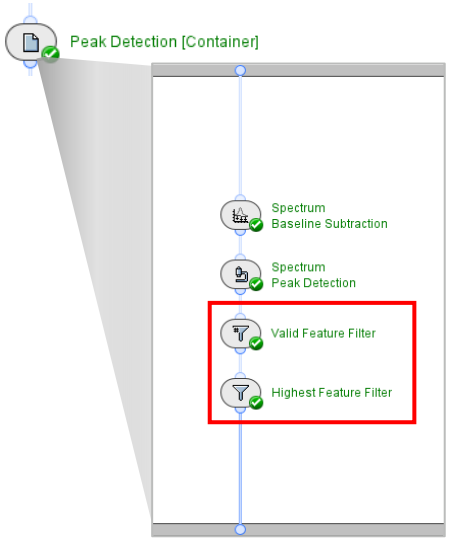


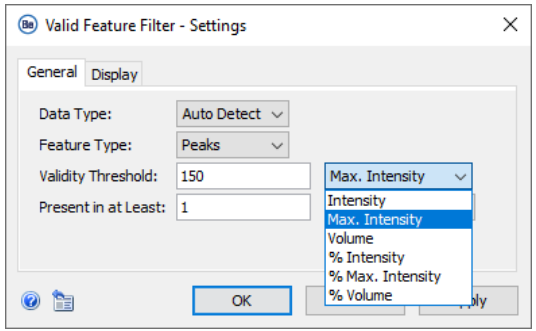


Multiple chromatograms can be selected from the **Experiment List**, and then overlayed or flipped to show mirror views.

Feature Filters

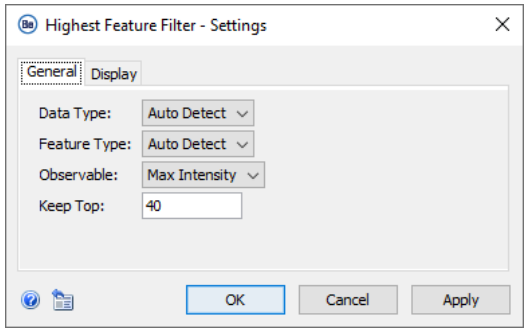
- Use the *Feature Filter* activity nodes to limit how many peaks are included in the results.
  - Keep only the most relevant peaks and exclude those that originate from noise.
  - Activate the **Bypass** icons to keep all possible peaks.





Valid Feature Filter

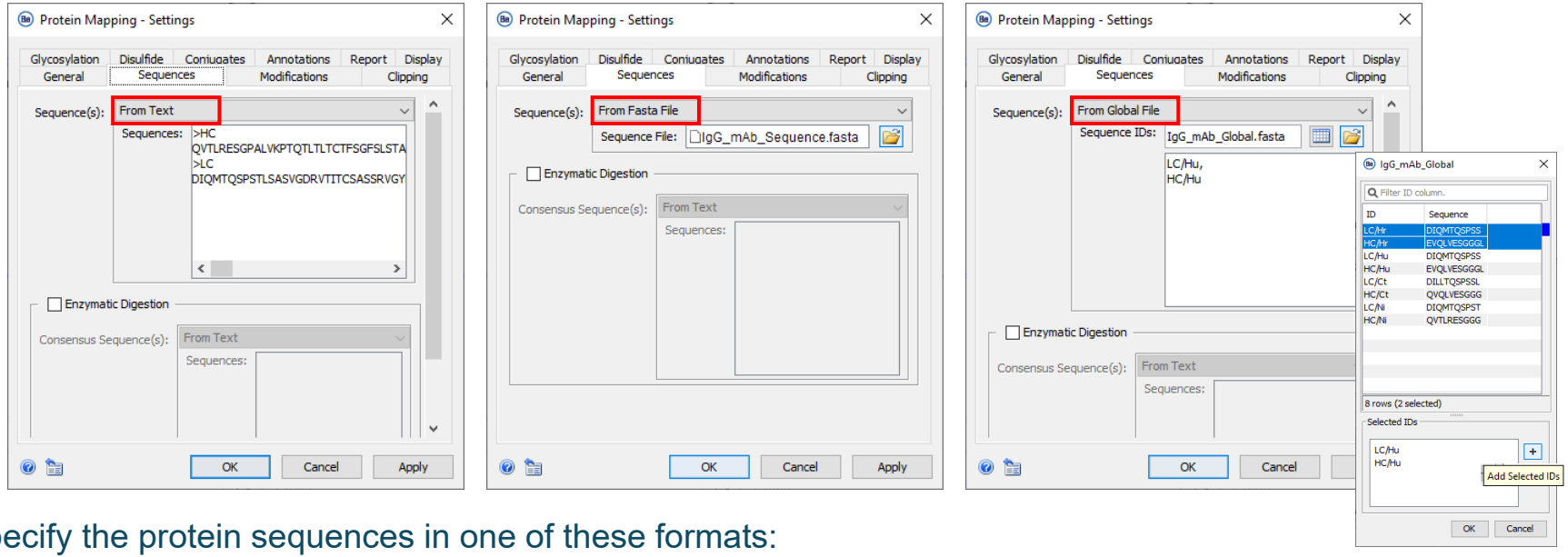
- Specify the minimum peak intensity that a peak must have to be kept.



Highest Feature Filter

- Specify the number of abundant peaks that will be reported in each identified RT range.

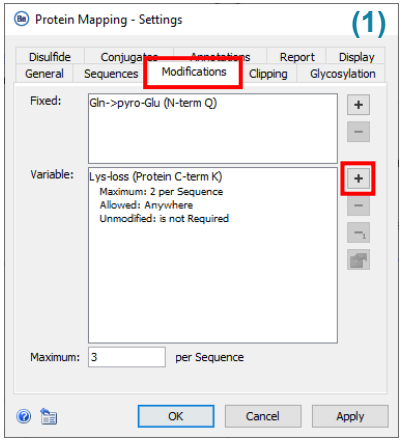
Protein Mapping: Sequences

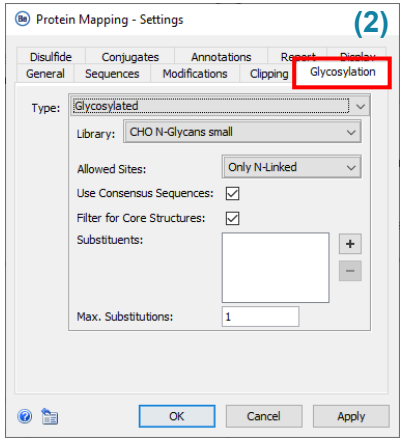


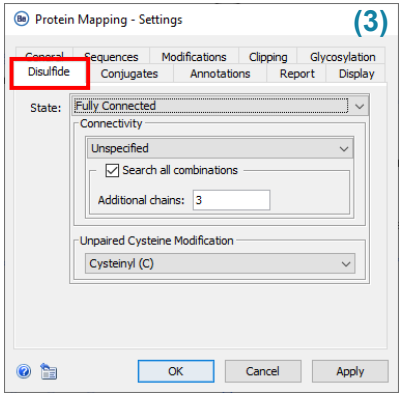
- Specify the protein sequences in one of these formats:
  - **From Text**: Specify the protein sequence in the **Sequences** box.
  - **From Fasta File**: Upload a FASTA file that contains the sequence of interest.
  - **From Global File**: Upload a FASTA file with multiple entries, and then select which sequences to use from the pop-up window.

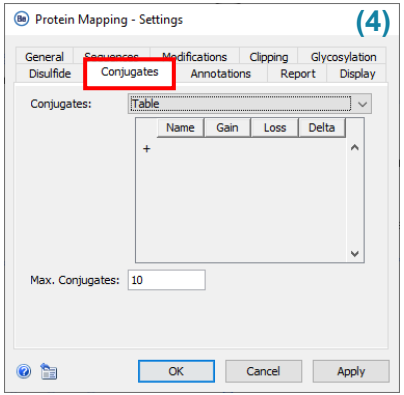
Note: For information about Batch Processing, refer to the section: Guidelines for Intact Mass Batch Processing.

Protein Mapping: Modifications



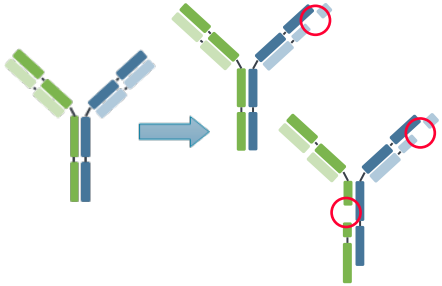


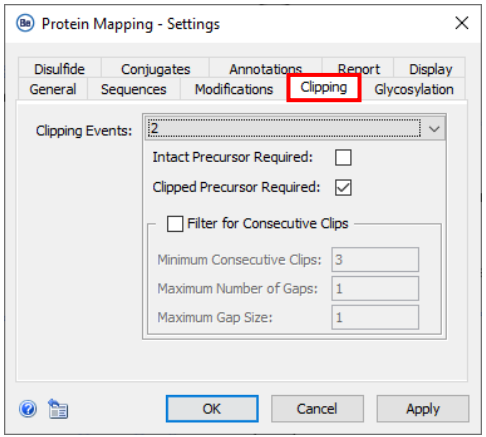




1. Specify **Fixed** and **Variable** PTMs.
   - With protein sequences that have a glutamic acid at the N-terminal, if the modified form is not expected to be the main form, set **Glu-pyroGlu (N-term E)** as a **Variable** modification.
2. Specify glycosylation parameters.
   - Set the **Type** to **Glycosylated** or **Deglycosylated**:
     - **Deglycosylated** assumes a deamidation event at every *N*-glycosylation consensus site, so there is no need to specify deamidation as variable modification.
     - **Glycosylated**: Select the glycan library to use.
3. Specify the type of cysteine connectivity.
   - Both mAbs and unconnected subunits can be annotated together. A variable PTM can be set for free cysteines.
4. If the target protein is an ADC, then specify the names and masses of **Conjugates**.

Protein Mapping: Clipping





- The **Clipping** functionality matches masses for potential (*in silico*) clipping events with peaks detected in the data.
- In the **Clipping** tab, select the number of **Clipping Events** that are expected on either the same chain or a different connected chain.
  - For **1 Clipping Event** chain recombination and losses are considered.
    - For example, for a mAb of structure `LC1-LC2-HC1-HC2`, configurations such as `LC1-LC1-LC1-LC1`, `LC1-LC1-HC1-HC1` and losses derived from them, are considered.
  - For **2 Clipping Events** only clips on the expected protein configuration, and chain-losses of this configuration, are considered.
- **Intact Precursor Required**: Clipped forms will be reported only if the unclipped form with the same modifications is also detected.
- **Clipped Precursor Required**: Doubly-clipped forms will be reported only if a singly-clipped form with the same modifications is also detected.

Protein Mapping: Clipping Filters

- Filters control the number of combinatorial possibilities in the defined search space. Too many variables will result in:
  - ⚠ Extended processing times or workflows that do not run to completion.
  - ⚠ High levels of false positive identifications, that require excessive data review.
- **Filter for Consecutive Clips**: Only protein forms that relate to consecutive clipping events, which must contain identical modifications, will be included in the results

Detected matches	Protein_Xx [1-25] Protein_Xx [2-25] Protein_Xx [3-25] Protein_Xx [4-25] Protein_Xx [5-25] Protein_Xx [6-25] Protein_Xx [7-25]	Protein_Xx [1-25] Protein_Xx [2-25] Protein_Xx [4-25] Protein_Xx [5-25] Protein_Xx [6-25] Protein_Xx [7-25]	Protein_Xx [1-25] Protein_Xx [3-25] Protein_Xx [4-25] Protein_Xx [7-25]	Protein_Xx [1-25] Protein_Xx [3-25] Protein_Xx [4-25] Protein_Xx [7-25]
Settings	Min. Consecutive Clips: **5** Max. Number of Gaps: **1** Max. Gap Size: **1**	Min. Consecutive Clips: **5** Max. Number of Gaps: **1** Max. Gap Size: **1**	Min. Consecutive Clips: **5** Max. Number of Gaps: **1** Max. Gap Size: **1**	Min. Consecutive Clips: **4** Max. Number of Gaps: **2** Max. Gap Size: **2**
Results	Detected matches as output.	Detected matches as output.	No results listed.	Detected matches as output.

Protein Mapping: Clipping Recommendations

Setting (Tab)	Feature	Sub Feature	1 Clip	2 Clips
General	Consolidate Matches		×	×
Sequences	Enzymatic Digestion		×	×
Clipping	Intact Precursor Required		✓	✓
	Clipped Precursor Required		×	✓
	Consecutive Clip Events Filter		✓	✓
Modifications	Fixed/Variable		✓	✓
Glycosylation	Type		✓	✓
Disulfide	Fully Reduced		✓	✓
	Fully Connected	Unspecified	✓	✓
		IgG	✓	✓
		Complex	×	×
	Partially Reduced	IgG	✓	✓
		Complex	×	×
Conjugates			✓	✓

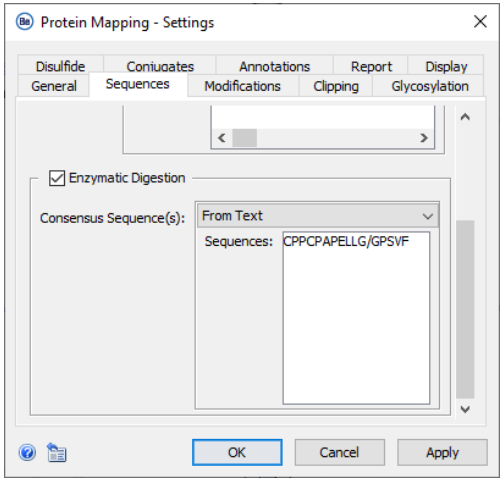
- To identify clips of mAb fragments from a reduced IdeS digest, we recommend that each fragment be investigated individually.
- C-terminal lysine loss will be reported as a duplicate annotation if Lys-Loss (Protein C-term K) is also included as a modification in the *Protein Mapping* search.

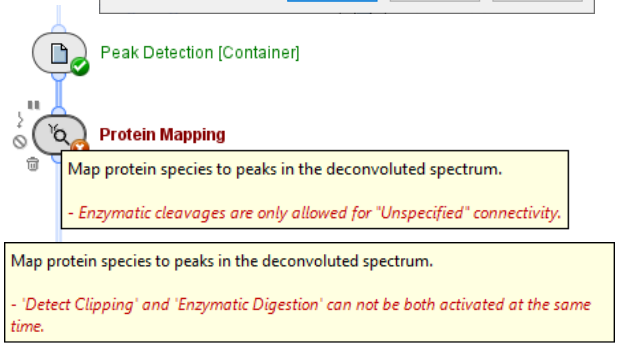
- A search for protein clips can produce a high number of results and take a long time to complete.

General guidance to control the search space:

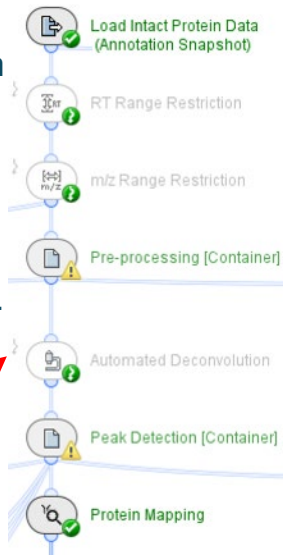
- When **Intact Precursor Required** is selected with **Unspecified Connectivity**, then **Additional chains** should be set to 0.
- Remove peaks that are unlikely to be from a clipping event:
  - Use the *Feature Filter* activity nodes or the *Manual Peak Edit* in TRD workflows.
- Only search for modifications that are known to be present in reasonable abundance:
  - Keep the number of variable modifications and the number of glycans in the selected library to a minimum. For example, only include the glycoforms that make up the majority (95%) of the glycan profile.

Protein Mapping: Enzymatic Cleavage



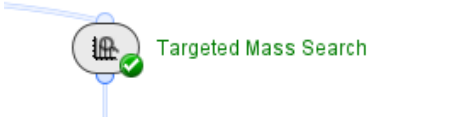


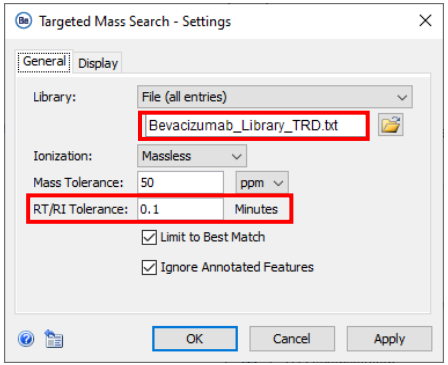
- Enzymatic cleavage is a sample preparation strategy that results in protein subunits for MS analysis.
- To define the cleavage point for Protein Mapping, select **Enzymatic Digestion** and provide a **Consensus Sequence**.
  - When multiple consensus sites are added, the algorithm assumes there are cleavages at all positions, not that they are alternatives.
  - To find unspecific cleavages around a single consensus sequence, for example `CPPCPAPELLG/GPSFV` and `CPPCPAPELLGG/PSFV`, search for each one independently.
    - Run the workflow sequentially with each variation.
    - To re-analyze the output from *Save Annotations Snapshot*, activate the **Bypass** icon for any unrequired activity nodes.
- To use **Enzymatic Digestion**:
  - Select **Unspecified** connectivity on the **Disulfide** tab.
  - Select **0 Clipping Events** on the **Clipping** tab.



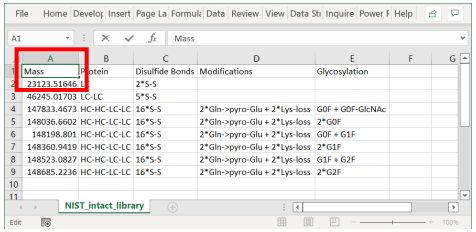
Targeted Mass Search

- The input library for *Targeted Mass Search* must be in the form of a tab-separated text file that can be edited in Excel.

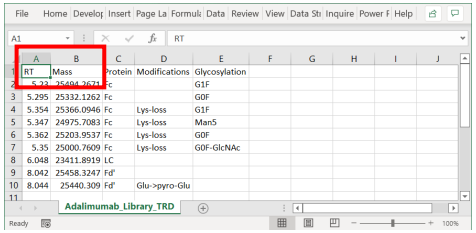




- Format for **Automated Deconvolution** workflows:
  - The first column (**Mass**) is mandatory.



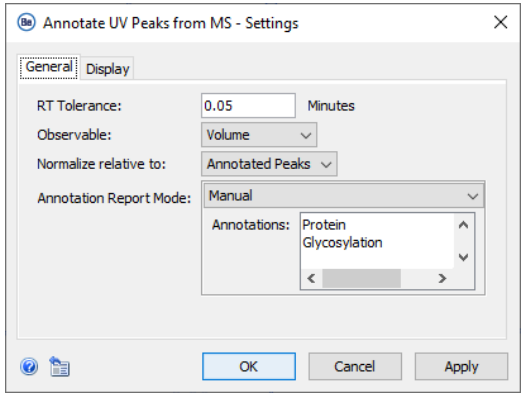
- Format for **Time Resolved Deconvolution** workflows:
  - The first two columns (**RT** and **Mass**) are mandatory.

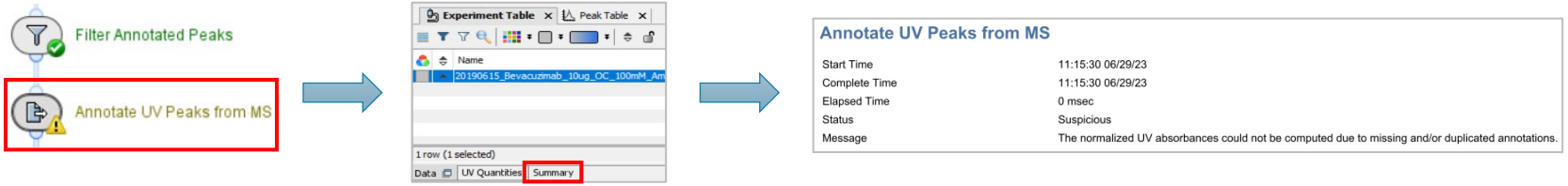


- Remove unrequired columns, or insert columns to provide additional information, as required.
- Adjust **RT/RI Tolerance** to account for chromatographic shifts.

Annotate UV Peaks from MS

- This activity node uses MS peak information to annotate the related peaks in the **UV Chromatogram**.
  - A related peak must elute within the specified **RT Tolerance**.
- The relative ratio of the UV peaks is also calculated, based on UV absorbance.
  - A protein sequence is required to normalize UV absorbance values.
  - If the UV normalization factor cannot be calculated, then the activity node displays a **yellow warning**.
    - UV normalization is not available with **Time Resolved Deconvolution**.
    - UV normalization is not applicable for *Targeted Mass Search* because this activity node does not contain the protein sequence.



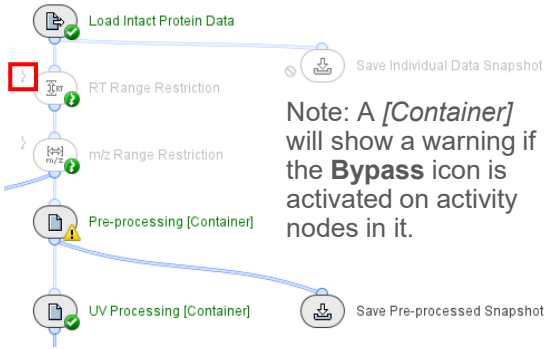


Export Intermediate Results for Further Analysis

- The *Save Snapshot* activity nodes store 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:
  - Deactivate the **Block** icon.
  - Select or add the folders where the sbf files will be stored.

- To use intermediate results from *Save Pre-processed Snapshot*:
  1. Select the sbf file to import into *Load Intact Protein Data*.
  2. Change the **Format** to **Auto Detect** or **Snapshot (*.sbf)**.
  3. Activate the **Bypass** icon on the activity nodes that are before the Snapshot was saved.
     - For example: To load sbf files from *Save Pre-processed Snapshot*, activate the **Bypass** icon for all activity nodes between *Load Intact Protein Data* and *UV Processing [Container]*.



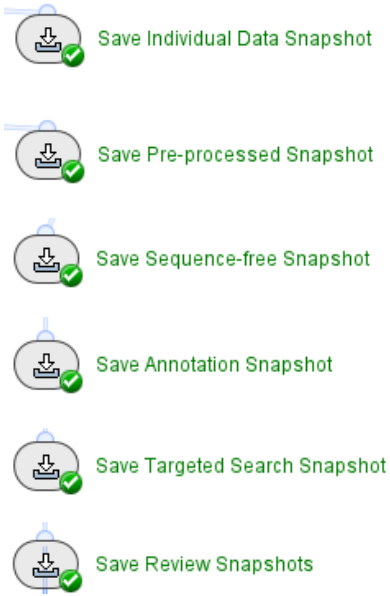
Note: A *[Container]* will show a warning if the **Bypass** icon is activated on activity nodes in it.

Reporting: *Save Snapshots*

- Select or create the folders where results will be stored.

Options to save Snapshots:

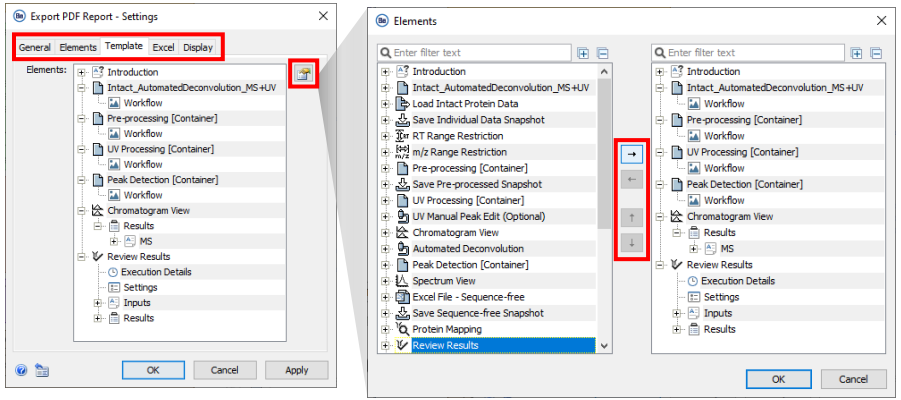
1. *Save Individual Data Snapshot*: Converts raw data into sbf format. Multiple samples in the same wiff or wiff2 container, as well as multiple experiments within the same run, are saved as individual snapshots.
2. *Save Pre-processed Snapshot*: Stores intermediate results from data cleanup and RT alignment before deconvolution.
3. *Save Sequence-free Snapshot*: Stores peak information after peak detection and deconvolution. Do not require a protein sequence.
4. *Save Annotation Snapshot*: Stores all intermediate information and feature annotations after the review process.
5. *Save Targeted Search Snapshot*: Stores peak annotation information from a *Targeted Mass Search.*
6. *Save Review Snapshots*: Stores all information after the review process in the Intact_ReviewSnapshots workflow.



Export PDF Report

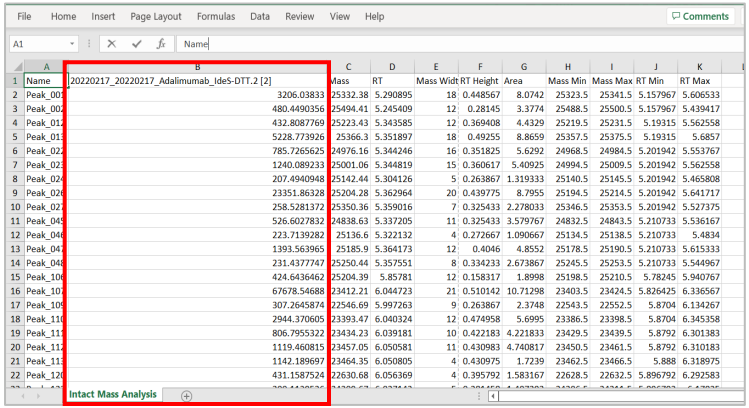
- The exported PDF Report includes:
  - A PDF document.
  - An Excel file with spectral information from deconvolution.
  - An embedded workflow (xml file) that includes all of the settings.
    - To open the xml file, drag the saved PDF Report into the workflow home page in the Biologics Explorer software.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

- **General** tab: Specify the name and saved location of the exported report.
- **Template** tab: Use the **Edit Selection** icon to specify 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 specify the **Tables** to be included in the report.
  - All columns in a selected table are reported.

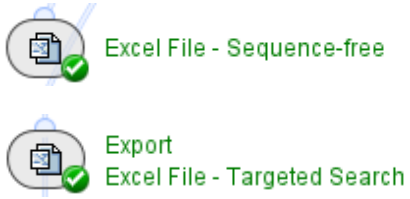


Save or Export an Excel File

- Exported Excel files contain peak-associated quantitative information:
  - **Automated Deconvolution**: Quantitative information is exported as **Maximum Intensity**.
  - **Time Resolved Deconvolution**: Quantitative information is exported as **Volume.**



	A	B	C	D	E	F	G	H	I	J	K
1	Name	20220217_20220217_Adalimumab_IdeS-DTT.2 [2]	Mass	RT	Mass Widt	RT Height	Area	Mass Min	Mass Max	RT Min	RT Max
2	Peak_00	3206.03833	25332.38	5.290895	18	0.448567	8.0742	25323.5	25341.5	5.157967	5.606533
3	Peak_00	480.4490356	25494.41	5.245409	12	0.28145	3.3774	25488.5	25500.5	5.157967	5.439417
4	Peak_01	432.8087769	25223.43	5.343585	12	0.369408	4.4329	25219.5	25231.5	5.19315	5.562558
5	Peak_01	5228.773926	25366.3	5.351897	18	0.49255	8.8659	25357.5	25375.5	5.19315	5.6857
6	Peak_02	785.7265625	24976.16	5.344246	16	0.351825	5.6292	24968.5	24984.5	5.201942	5.553767
7	Peak_02	1240.089233	25001.06	5.344819	15	0.360617	5.40925	24994.5	25009.5	5.201942	5.562558
8	Peak_02	207.4940948	25142.44	5.304126	5	0.263867	1.319333	25140.5	25145.5	5.201942	5.465808
9	Peak_02	23351.86328	25204.28	5.362964	20	0.439775	8.7955	25194.5	25214.5	5.201942	5.641717
10	Peak_02	258.5281372	25350.36	5.359016	7	0.325433	2.278033	25346.5	25353.5	5.201942	5.527375
11	Peak_04	526.6027832	24838.63	5.337205	11	0.325433	3.579767	24832.5	24843.5	5.210733	5.536167
12	Peak_04	223.7139282	25136.6	5.322132	4	0.272667	1.090667	25134.5	25138.5	5.210733	5.4834
13	Peak_04	1393.563965	25185.9	5.364173	12	0.4046	4.8552	25178.5	25190.5	5.210733	5.615333
14	Peak_04	231.4377747	25250.44	5.357551	8	0.334233	2.673867	25245.5	25253.5	5.210733	5.544967
15	Peak_10	424.6436462	25204.39	5.85781	12	0.158317	1.8998	25198.5	25210.5	5.78245	5.940767
16	Peak_10	67678.54688	23412.21	6.044723	21	0.510142	10.71298	23403.5	23424.5	5.826425	6.336567
17	Peak_10	307.2645874	22546.69	5.997263	9	0.263867	2.3748	22543.5	22552.5	5.8704	6.134267
18	Peak_11	2944.370605	23393.47	6.040324	12	0.474958	5.6995	23386.5	23398.5	5.8704	6.345358
19	Peak_11	806.7955322	23434.23	6.039181	10	0.422183	4.221833	23429.5	23439.5	5.8792	6.301383
20	Peak_11	1119.460815	23457.05	6.050581	11	0.430983	4.740817	23450.5	23461.5	5.8792	6.310183
21	Peak_11	1142.189697	23464.35	6.050805	4	0.430975	1.7239	23462.5	23466.5	5.888	6.318975
22	Peak_12	431.1587524	22630.68	6.056369	4	0.395792	1.583167	22628.5	22632.5	5.896792	6.292583



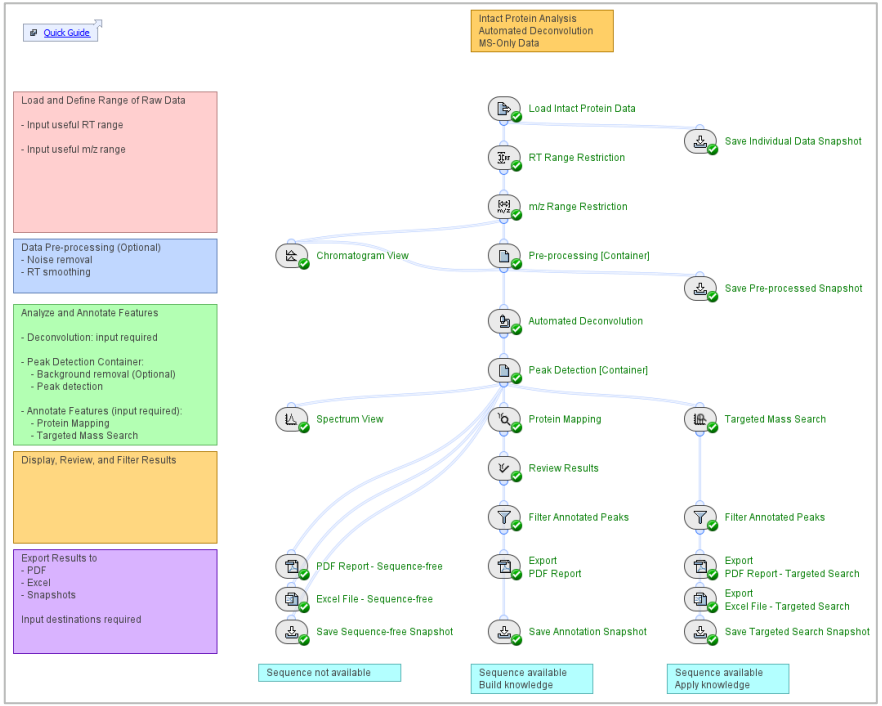
- Select or create the folders where the results will be stored.

Part B
Guidelines for Specific Intact Mass Workflows

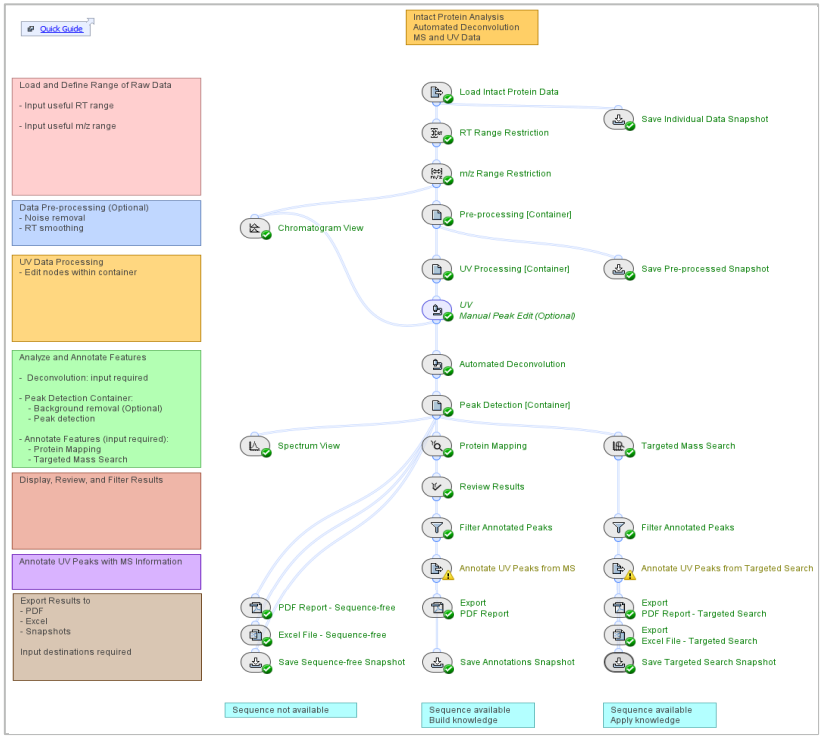
Automated Deconvolution with MS or MS and UV Data

WORKFLOW SPECIFIC GUIDELINES

Overview of the Automated Deconvolution Workflows



Intact_AutomatedDeconvolution_MS_Be4.0

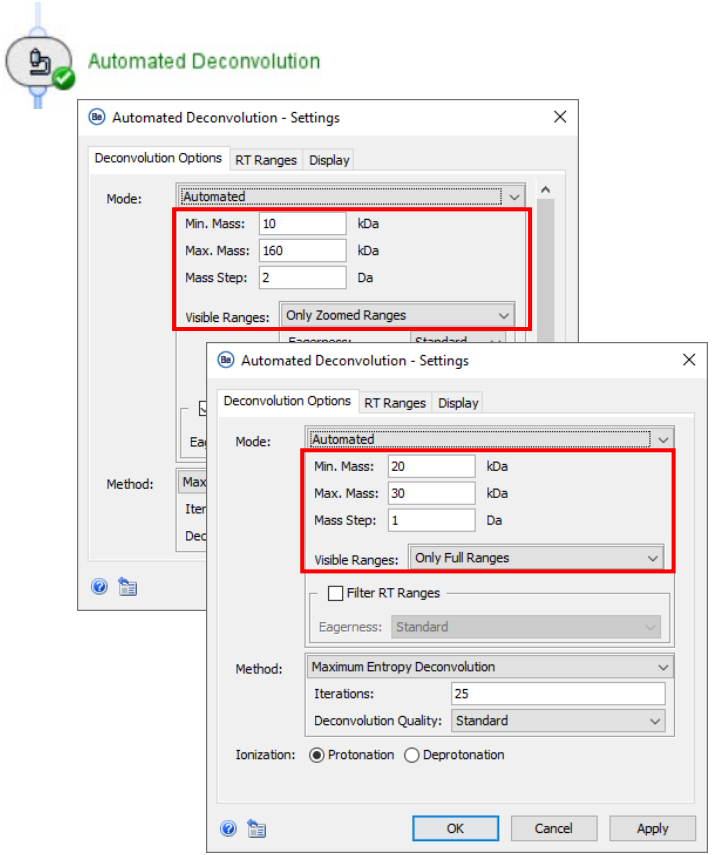


Intact_AutomatedDeconvolution_UV+MS_Be4.0

Automated Deconvolution Workflows: Overview

- Use **Automated Deconvolution** when species of interest are chromatographically well resolved.
- If multiple samples are analyzed together with an Intact_AutomatedDeconvolution workflow, a common peak detection is applied across all samples.
  - To make sure that annotations and quantification are consistent, use similar data. For example, analyze all intact protein, or all subunit datasets, together.
  - To optimize the workflow settings for samples with highly variable peak intensities, refer to the page: *Automated Deconvolution*: Manual RT Ranges.
  - To analyze multiple samples with individual peak detection, refer to the section: *Intact Mass Batch Processing*.

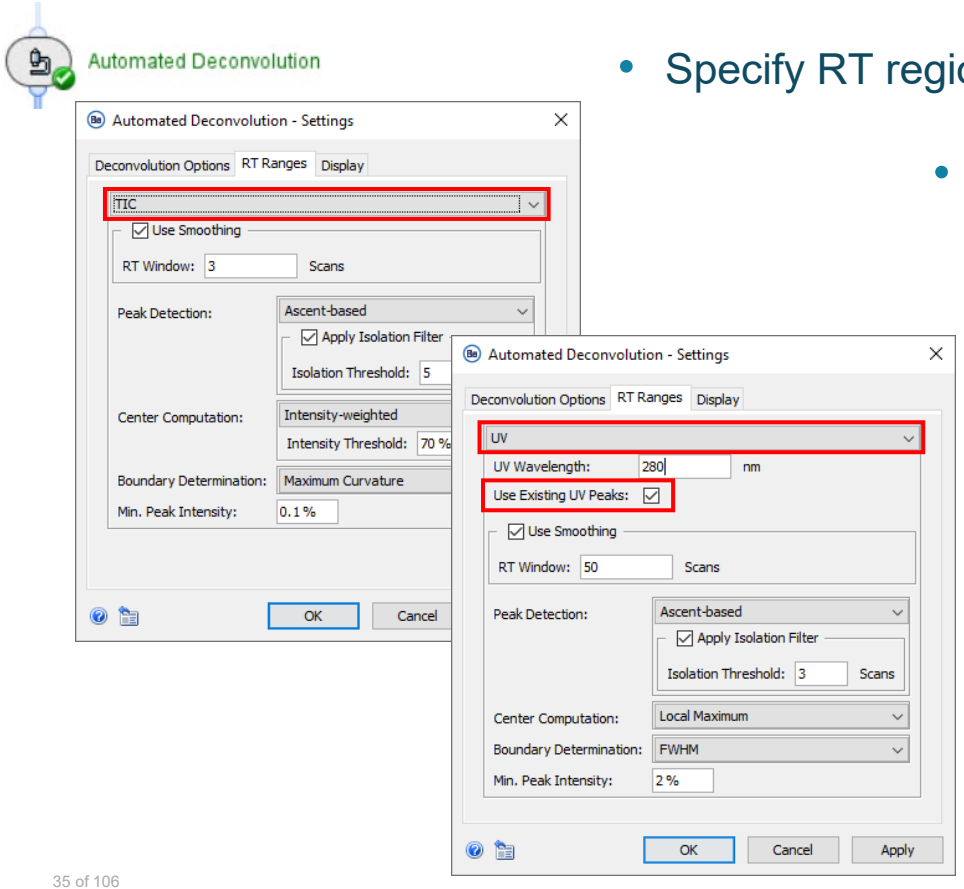
Automated Deconvolution: Deconvolution Options



Specify the relevant mass range and visualization options for the deconvoluted spectra.

- **Mass range**:
  - To analyze multiple species in the same data file, use a wide mass range to reduce prominent harmonics peaks.
  - To focus on a single species, use a narrow mass range.
- **Visible Ranges** controls how the results are displayed.
  - Use **Only Zoomed Ranges** if multiple components are detected in the same RT range.
- Set a **Mass Step** value that results in the same number of data points across peaks before and after deconvolution.
  - 0.1 Da - 0.2 Da for isotopically resolved data.
  - 1 Da for subunits (lower-resolution data).
  - 2 Da for intact proteins (lower-resolution data).
  - 3 Da if fewer datapoints are required.

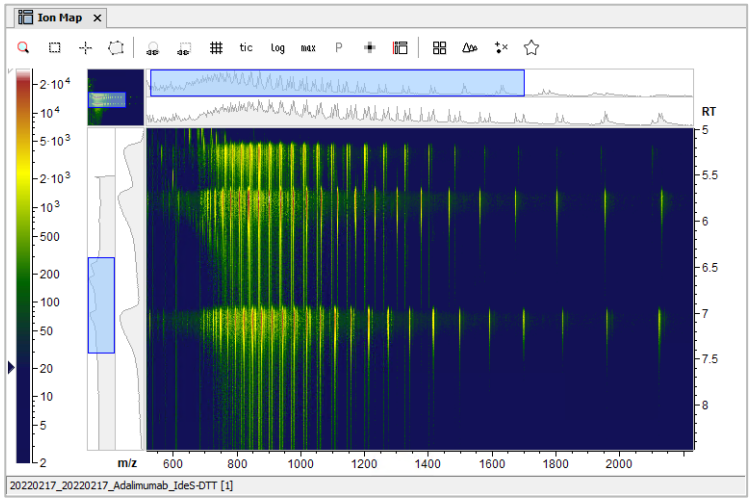
Automated Deconvolution: RT Ranges

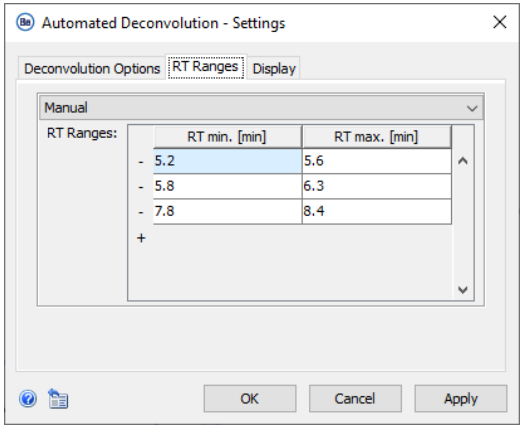


- Specify RT regions of interest.
  - Select one of these options from the list:
    - **TIC**: Use the the total ion chromatogram to identify RT ranges.
    - **Manual**: Specify RT ranges manually.
    - **UV**: Specify RT ranges based on peaks in the UV data.
      - To use UV data to define RT ranges:
        - Use the Intact_AutomatedDeconvolution_UV+MS workflow.
        - Define the **UV Wavelength**.
        - Select **Use Existing UV Peaks**. The subsequent peak detection settings on this tab are ignored.

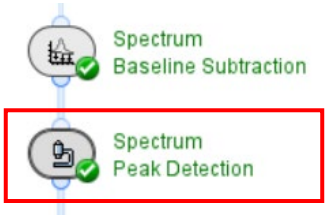
Automated Deconvolution: Manual RT Ranges

- Define the RT ranges manually if signal intensities differ significantly across samples in a batch analysis.
  - For example, in a dilution series or a time-course experiment.
- Select **Manual** mode in the **RT Ranges** tab.

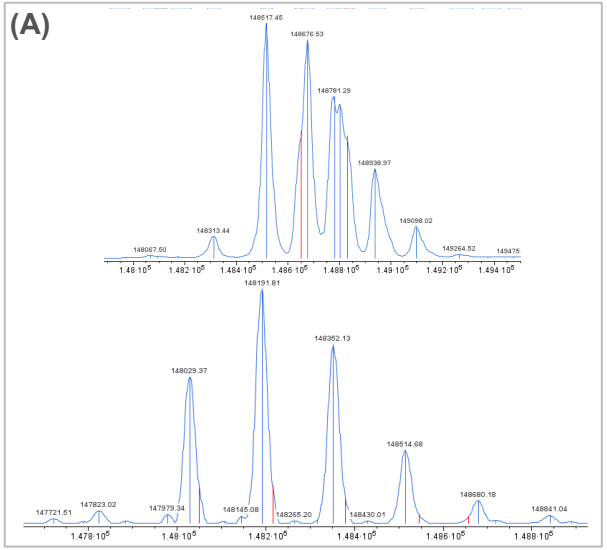


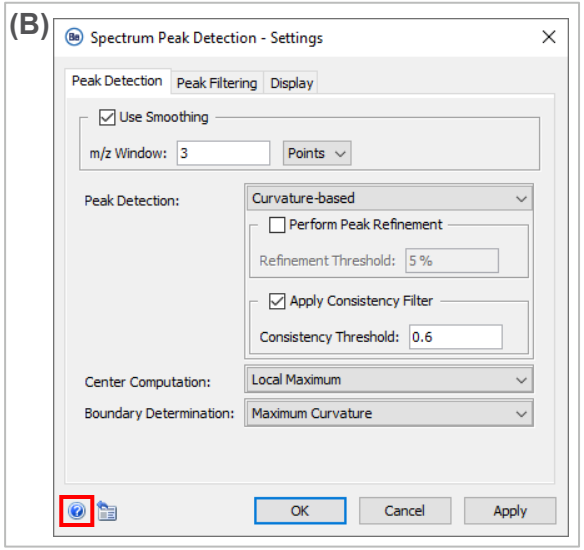


Spectrum Peak Detection



- The default peak detection settings (**Ascent-based**) are optimal for most use cases.
- Use **Curvature-based** peak detection to resolve shoulder peaks.
  - For a detailed description of the parameters, click the **?** icon to view the relevant Help pages.





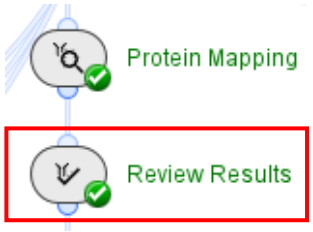
A. Deconvoluted spectra produced when **Curvature-based** peak detection is used to resolve shoulder peaks.

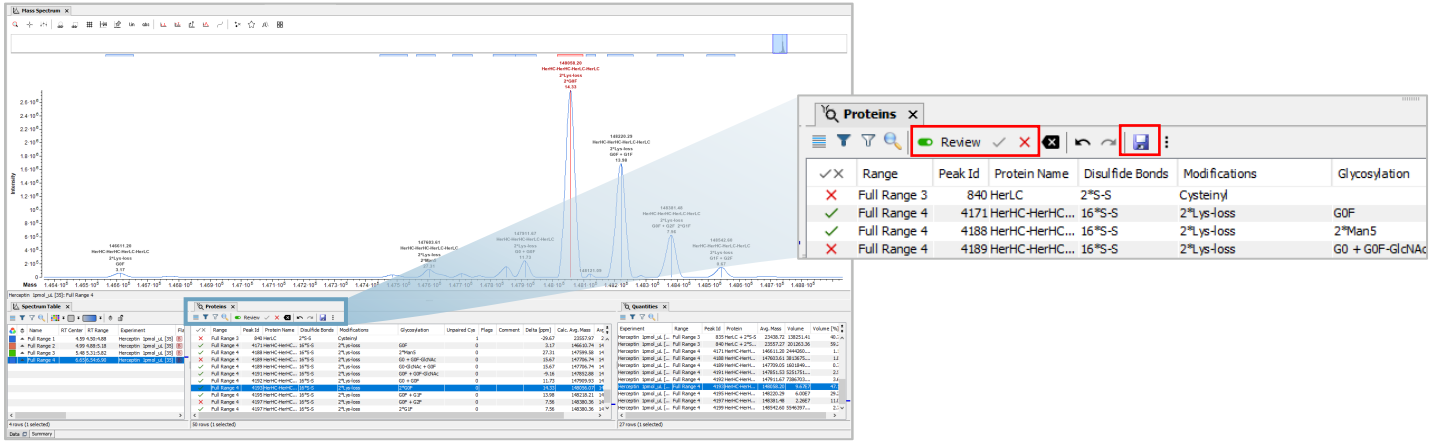
B. Example settings used to resolve the shoulder peaks in the spectra shown in (A).

Review Results: Accept and Reject Annotations

- In the completed *Review Results* activity node:
  1. Activate the **Review** mode and accept one annotation for all relevant peaks.
  2. Reject all other redundant annotations.
  3. To apply the changes, click the **Save** icon, and then select **Save and Reload**.

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

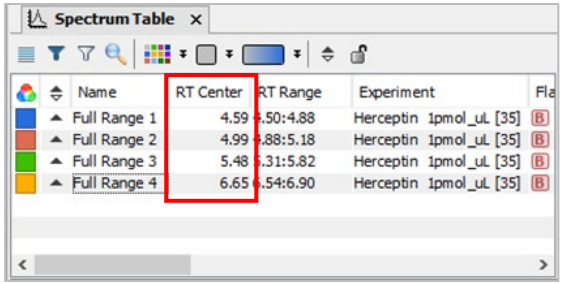




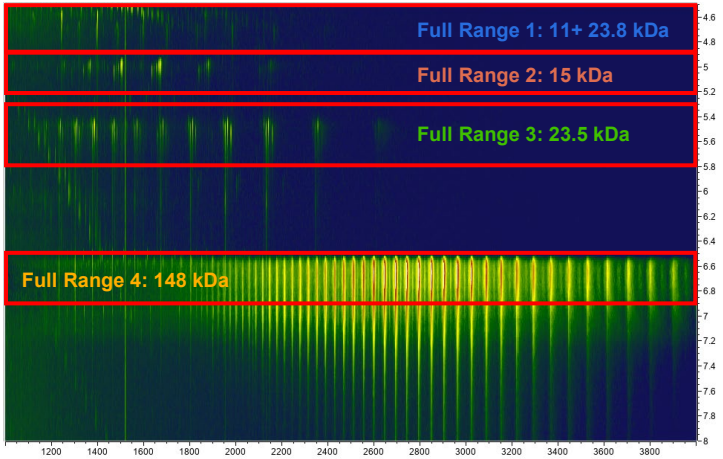
- *Review Results* is a critical step for the analysis of proteins with complex glycosylation patterns and for the correct calculation of DAR values for analysis of ADCs.

Review Results: Spectrum Table

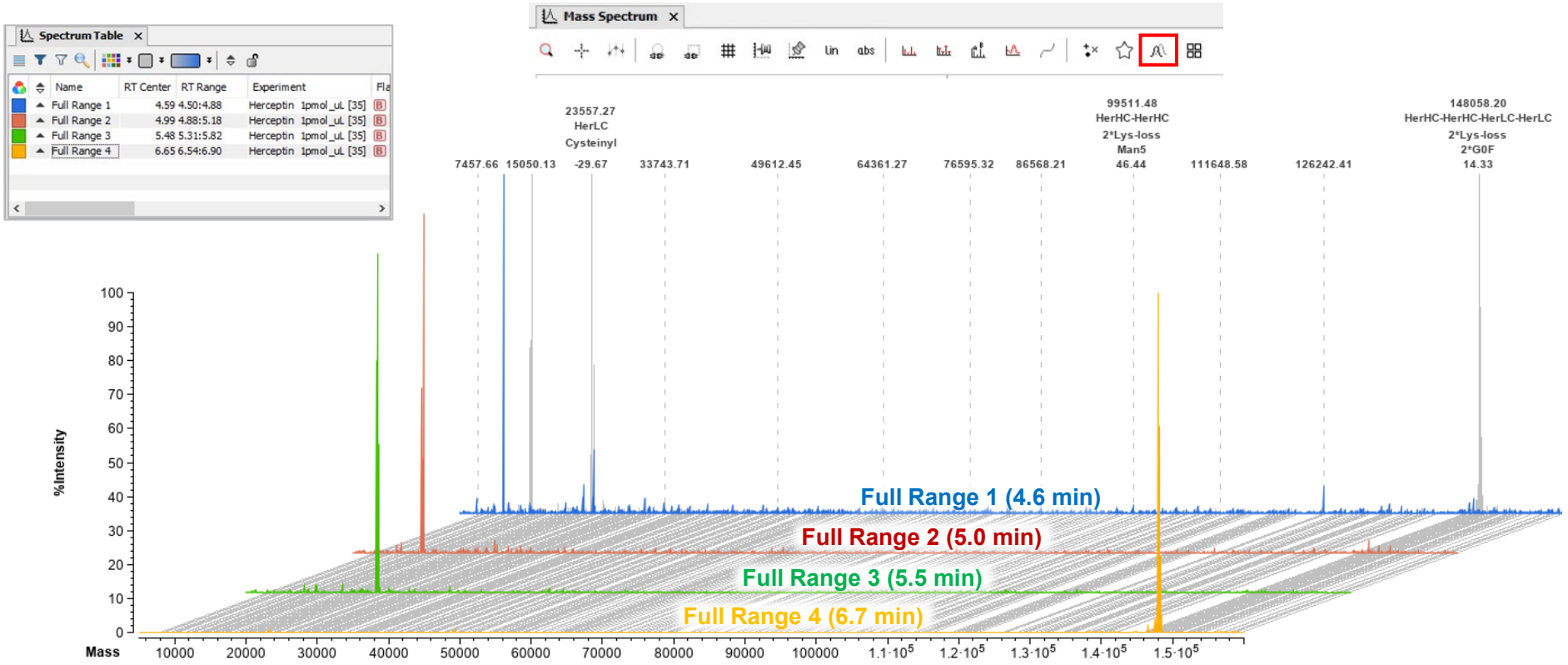
- The **Spectrum Table** lists the detected RT ranges, with the **RT Center** of the protein signals that were detected.



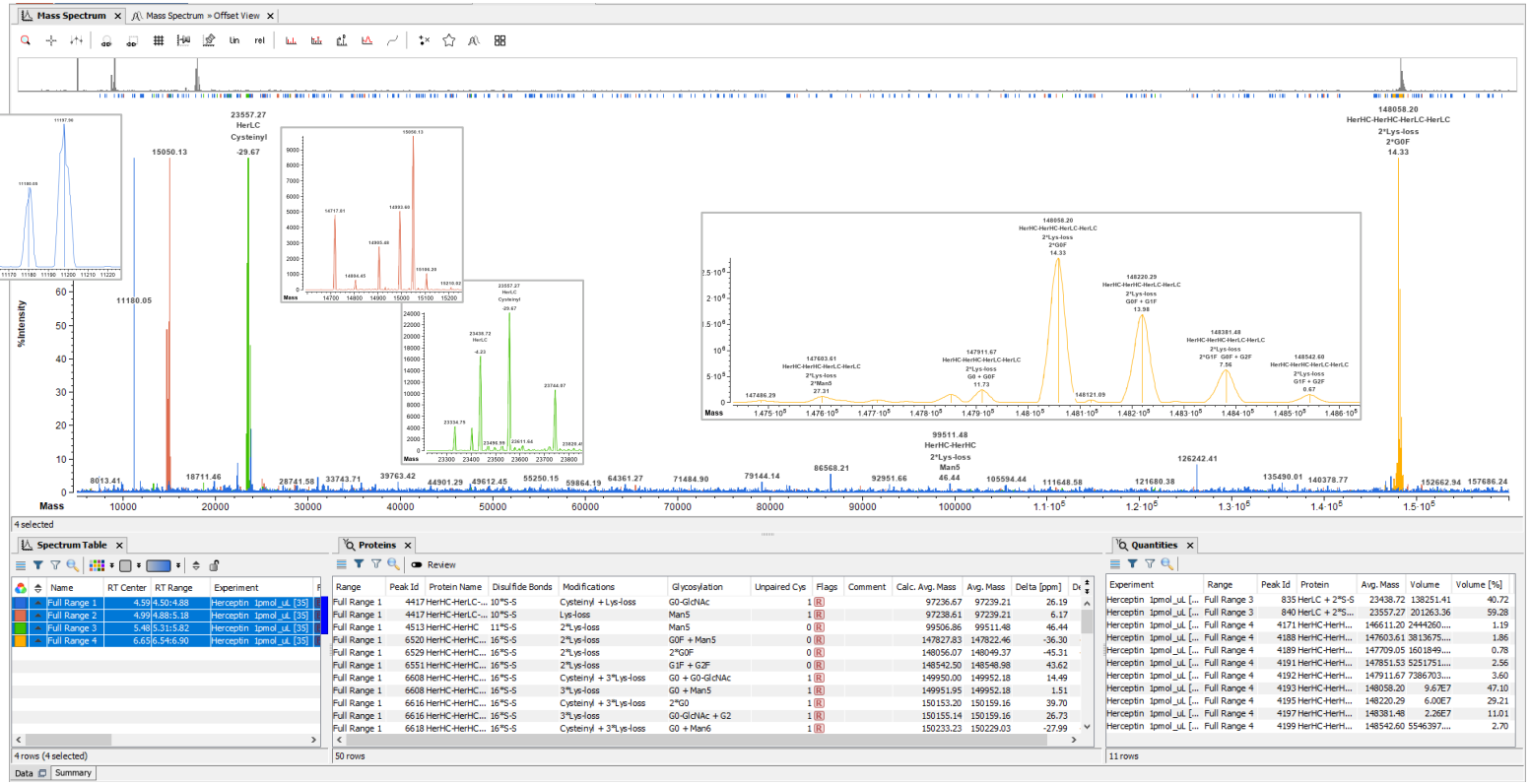
- All scans within each time range are summed and deconvoluted to produce the related spectrum.



Review Results: Spectrum Offset View

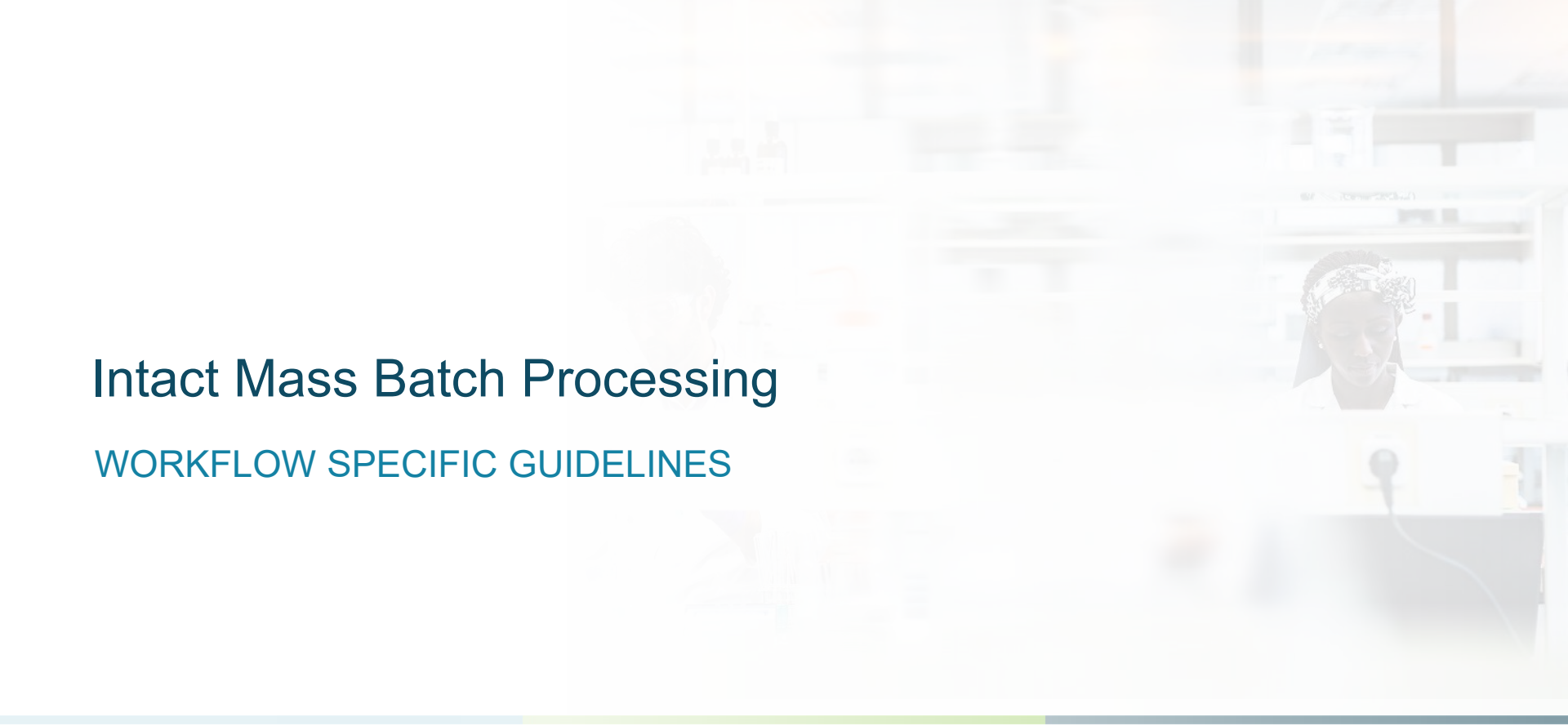


Review Results: Spectrum Overlapped View



Intact Mass Batch Processing

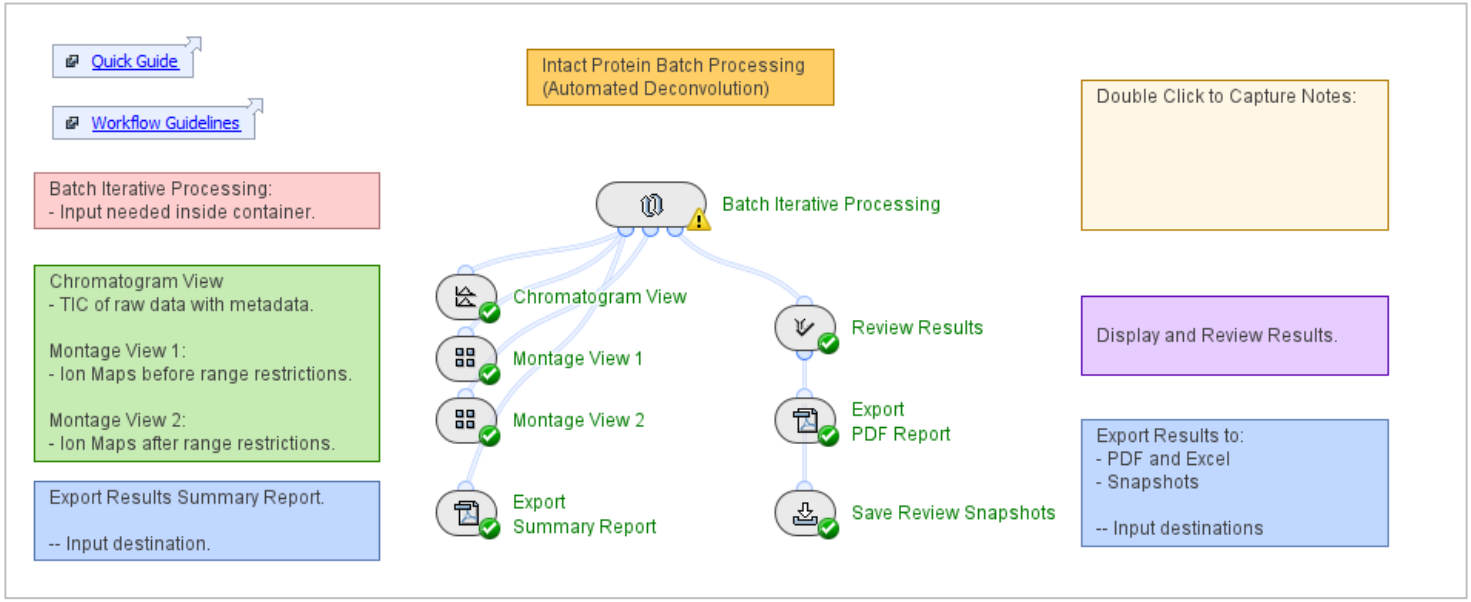
WORKFLOW SPECIFIC GUIDELINES



How to Use the Intact Mass Batch Processing Workflow

- The Intact Mass Batch Processing workflow is designed for sequential analysis of multiple samples of intact biotherapeutic molecules.
- Each sample is loaded and analyzed independently.
  - The samples do not need to have consistent chromatography.
  - The samples do not need to have the same protein sequence.
- All samples and their associated metadata are analyzed in the *Batch Iterative Processing* container.
  - Intermediate results for each sample are not saved when the *Batch Iterative Processing* container is used for data analysis.
  - Optimize workflow parameters on a representative sample before analysis of large numbers of samples.
  - To save memory so that large numbers of samples can be loaded and processed together, activate the **Trash** icon for activity nodes in the *Batch Iterative Processing* container.
  - Close and then open Biologics Explorer software between extensive Intact Mass Batch Processing analyses.

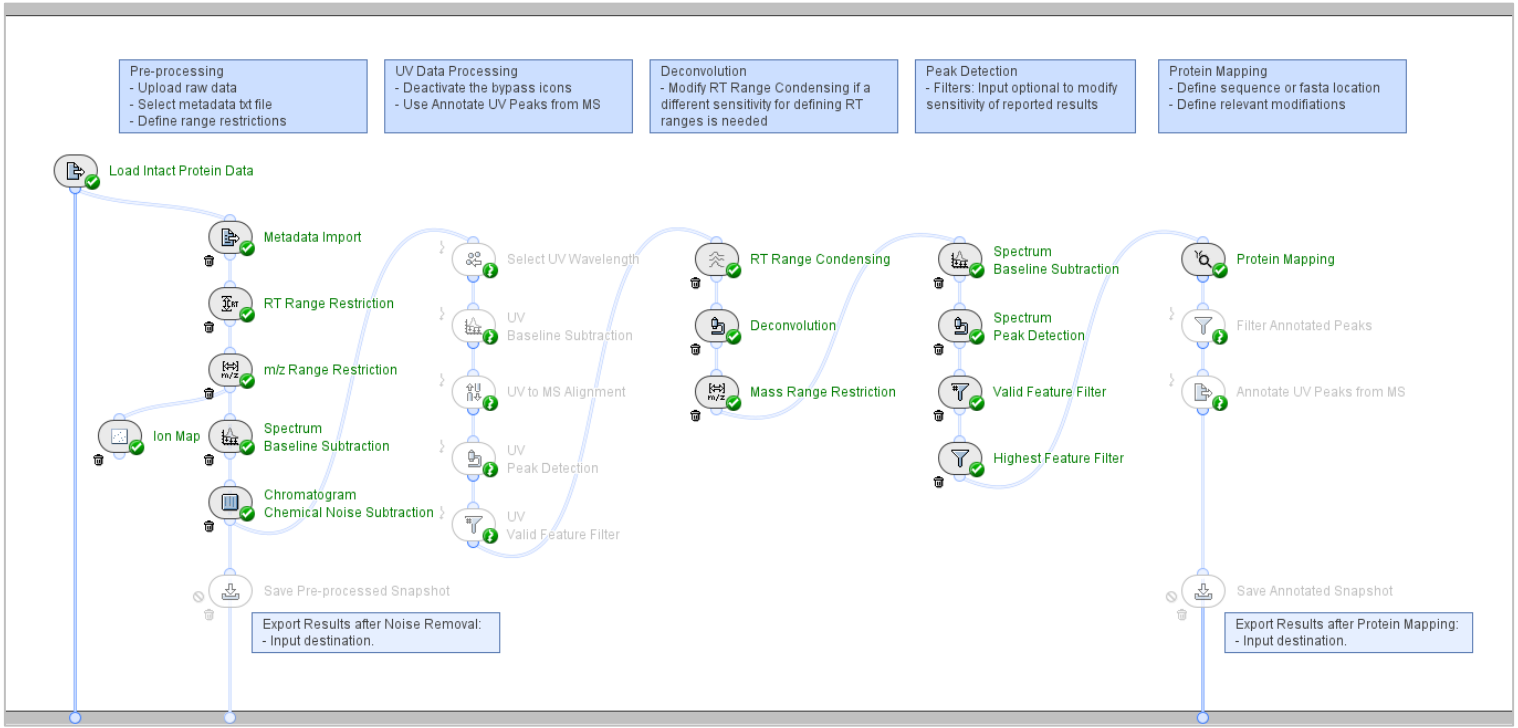
Overview of the Intact Mass Batch Processing Workflow



Intact_BatchProcessing_Be4.0

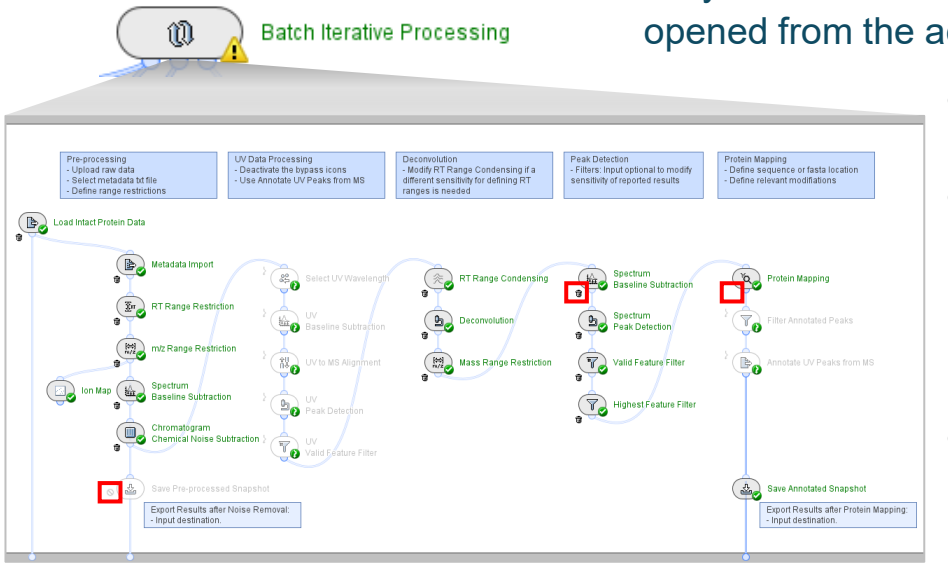
Overview of the Intact Mass Batch Processing Workflow

WORKFLOW STRUCTURE - INSIDE THE *BATCH ITERATIVE PROCESSING* CONTAINER



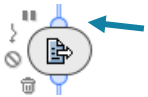
Batch Iterative Processing Container

- 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: When there are activity nodes in the container that have the **Bypass** icon activated, 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 activities that will be used in the PDF Report.

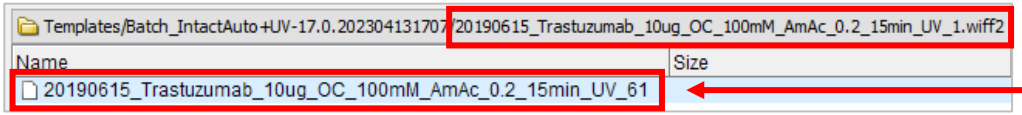


Activity nodes in the *Batch Iterative Processing* container do not have a **Run** or **Reset** icon.

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

- To optimize the workflow settings, use a smaller representative sample set in either the Batch Processing workflow with all **Trash** icons deactivated, or in an Intact_AutomatedDeconvolution workflow.

Load Intact Protein Data: Format



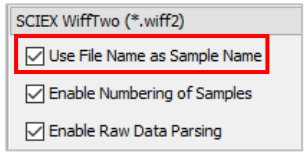
The **File Name** is the name of the wiff or wiff2 container file.

The **Sample Name** is the name of the data file in the wiff or wiff2 container file.

Note: The **File Name** and **Sample Name** might not be the same.

There are two options to load data for analysis with the Batch Processing workflow:

1. To analyze data from a wiff or wiff2 that contains a single sample:
   a. From the **Format** list select either **SCIEX Wiff (*.wiff)** or **SCIEX WiffTwo (*.wiff2)**.
      - If data was acquired with the ZenoTOF 7600 mass spectrometer, select only the wiff2 format.
   b. Select **Use File Name as Sample Name**.
      - Use the **File Name** (container file name) in the Experiment column of the metadata txt file.



2. To analyze data from a wiff or wiff2 that contains multiple samples:
   a. From the **Format** list select either **SCIEX Wiff (*.wiff)** or **SCIEX WiffTwo (*.wiff2)**.
      - If data was acquired with the ZenoTOF 7600 mass spectrometer, select only the wiff2 format.
   b. Do not select **Use File Name as Sample Name**.
      - Use **Sample Names** in the **Experiment** column of the metadata txt file.

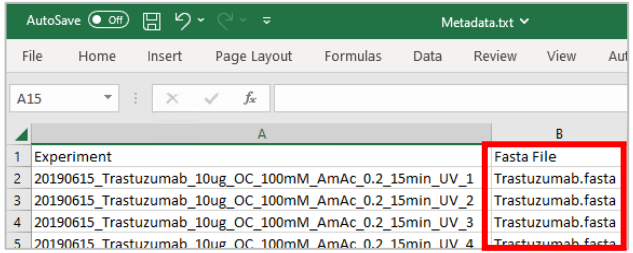
Note: For more information, refer to the page: *Metadata Import*.

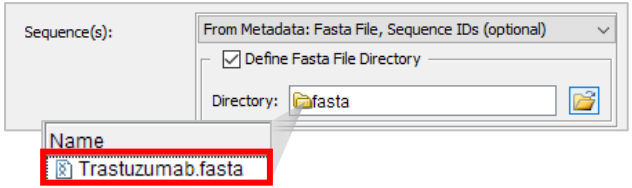
Metadata Import

- 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 specify which FASTA file (protein sequence) will be used for identification in the *Protein Mapping* activity nodes.

– Use *Metadata Import* to upload a txt file that links the **Sample Name** or **File Name** to a FASTA file.

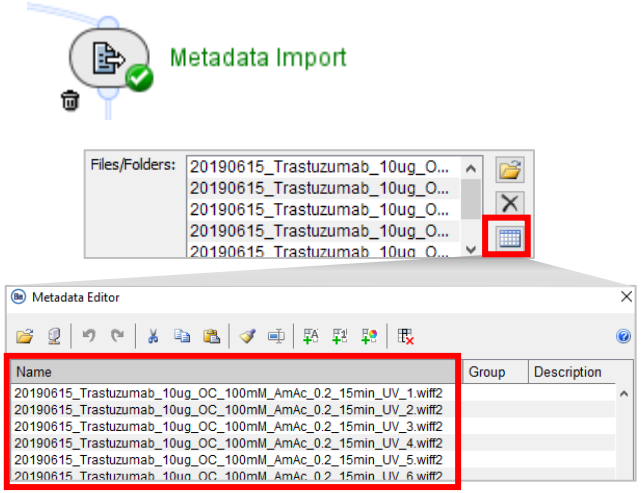
- The name in the **Experiment** column must be the same as the **Sample Name** or **File Name** in *Load Intact Protein Data*.
- The name in the **Fasta File** column must be the same as the name of the FASTA file that is in the **Fasta File Directory**, including the file extension (fasta or txt).



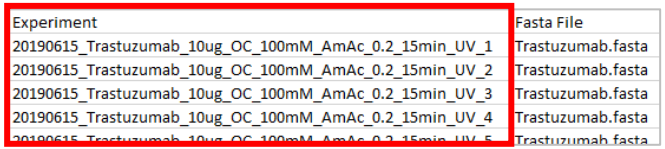


Note: For more information about recommended protein sequence names, refer to the page: *Review Results*: Protein Name in FASTA Files.

Metadata Import: Create the Metadata File



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

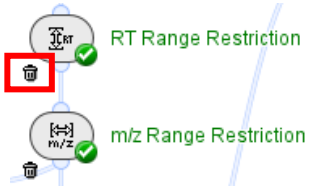


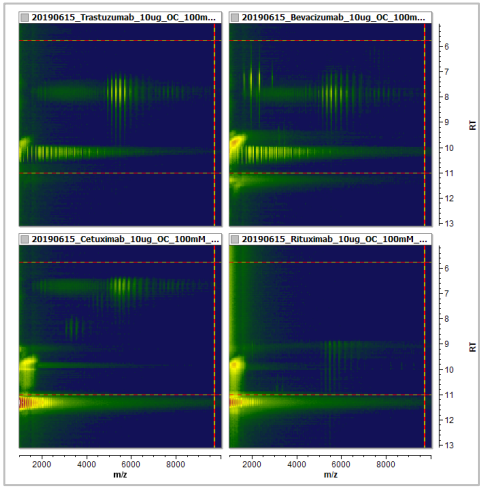
Experiment	Fasta File
20190615_Trastuzumab_10ug_OC_100mM_AmAc_0.2_15min_UV_1	Trastuzumab.fasta
20190615_Trastuzumab_10ug_OC_100mM_AmAc_0.2_15min_UV_2	Trastuzumab.fasta
20190615_Trastuzumab_10ug_OC_100mM_AmAc_0.2_15min_UV_3	Trastuzumab.fasta
20190615_Trastuzumab_10ug_OC_100mM_AmAc_0.2_15min_UV_4	Trastuzumab.fasta

- To create the metadata file in Excel or Notepad:
  1. Select the samples for batch processing in *Load Intact Protein Data*.
     - If **Use File Name as Sample Name** is selected, then select only wiff or wiff2 container files.
  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 in 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.

Restriction of RT and *m/z* Ranges

- To analyze multiple samples of the same molecule, select a single representative sample in the *Load Intact Protein Data* activity node in the *Batch Iterative Processing* container.
  - To optimize settings, deactivate the **Trash** icon for all activity nodes.
- To analyze multiple samples of different molecules, optimize workflow settings with an Intact_AutomatedDeconvolution workflow.
  - Make sure that the selected range is wide enough to include all samples.
  - If the molecules require very different ranges, then activate the **Bypass** icon on the *Range Restriction* activity nodes.

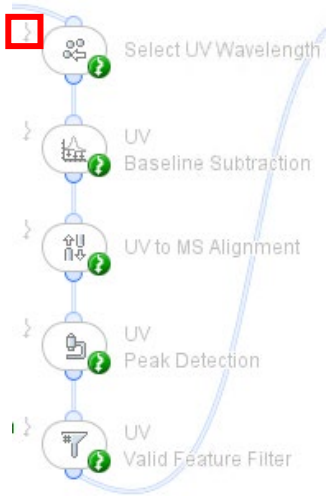




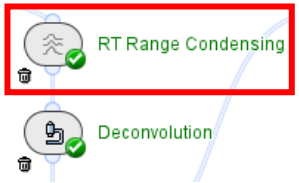
- To identify the ranges where there is meaningful data, open (double-click) *Load Intact Protein Data* after the data is loaded.
  - Remove ranges that contain minor components or contaminants at lower masses, unless they are of specific interest. Any signals from noise or contaminants will also be deconvoluted. This might cause inconsistent numbers of spectra (**Ranges**) per sample.

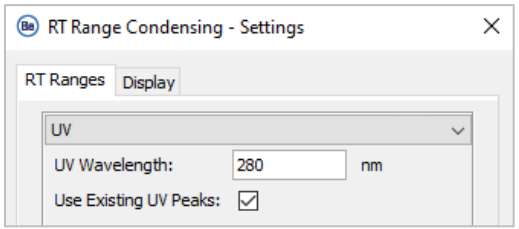
Note: If the fields are blank, or if *RT Range Restriction* has the **Bypass** icon activated, then the full RT range is used.

UV Data Processing



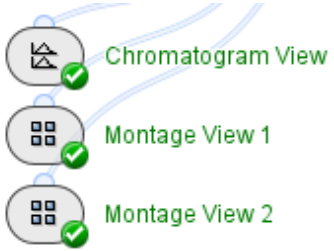
- All activity nodes for UV data have the **Bypass** icon activated in the template workflow.
- To use UV peaks in the data to identify the RT ranges for deconvolution:
  1. Deactivate the **Bypass** icon and then activate the **Trash** icon (to save memory) for *Annotate UV Peaks from MS* and all activity nodes with the prefix UV.
  2. Make sure that *Select UV Wavelength* contains the correct value.
  3. Select **UV** from the list in *RT Range Condensing*, specify the **UV Wavelength**, and then select **Use Existing UV Peaks**.
     - All other peak detection settings on this tab are ignored.



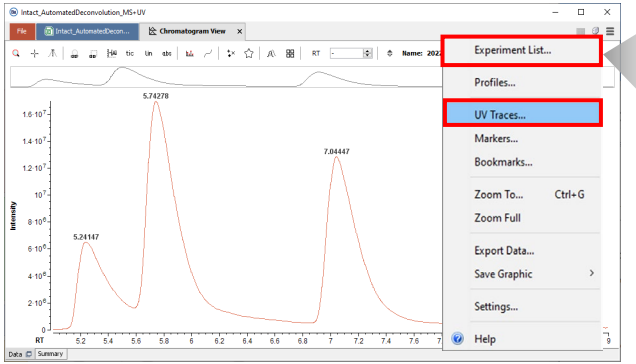


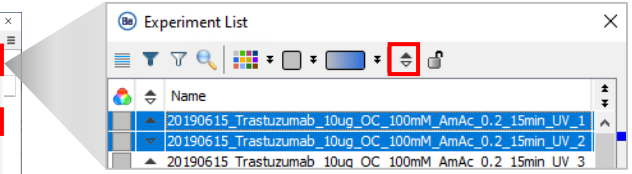
- *Annotate UV Peaks from MS* uses MS peak identifications to annotate the peaks in the UV chromatogram.
  - The peak must elute in the specified **RT Tolerance**.

Chromatogram View and *Montage View*

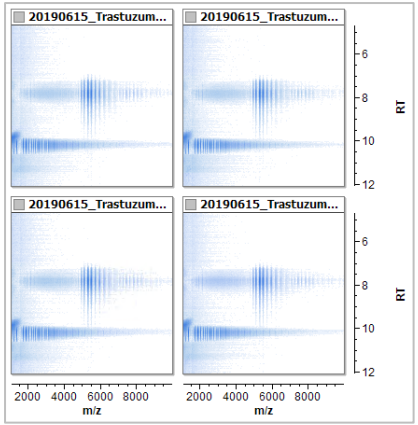


- *Chromatogram View* shows the TIC chromatograms and UV traces for each sample after the *Load Intact Protein Data* activity node.
  - Right-click in the plot to open the **Experiment List**.



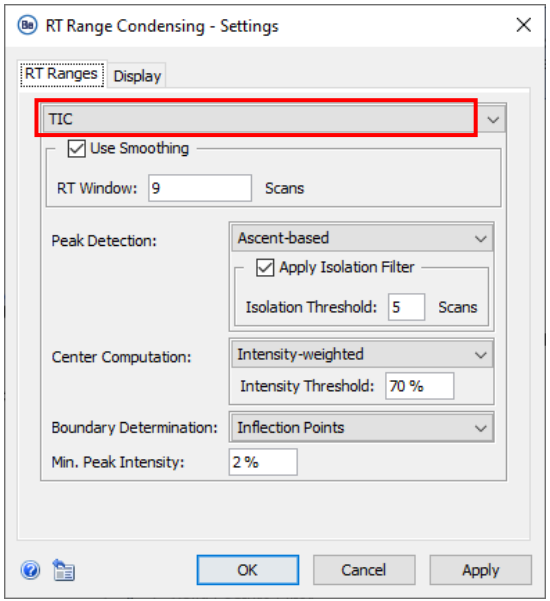


  - Optionally, select multiple chromatograms from the **Experiment List**, and then overlay or flip them to show mirror views.

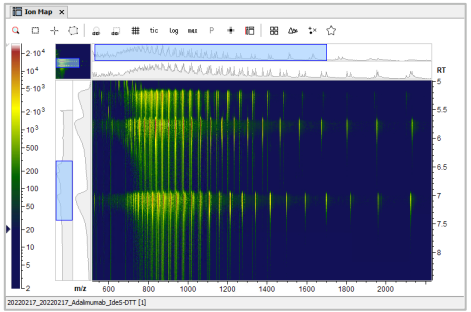


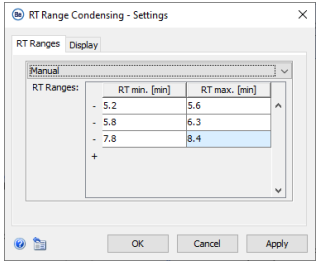
- The *Montage View* activity nodes show the ion maps of all samples analyzed in the *Batch Iterative Processing* container.
  - *Montage View 1* shows the ion maps of each sample before range restrictions.
  - *Montage View 2* shows the ion maps of each sample after range restrictions.

RT Range Condensing



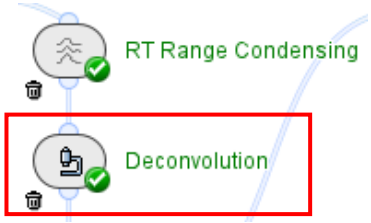
- Controls how RT regions of interest are identified.
  - **TIC**: Uses the total ion chromatogram to identify RT ranges.
  - **Manual**: Uses manually specified RT ranges.
    - Specify the RT ranges manually if signal intensities are significantly different across samples. For example, in a dilution series or a time-course experiment.



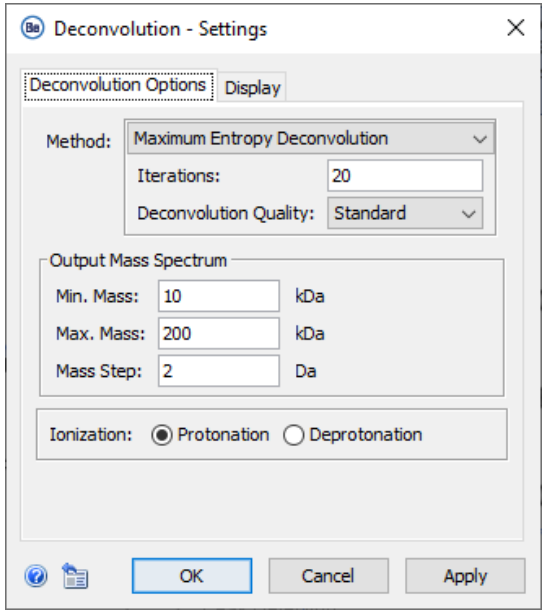


  - **UV**: Uses peaks in the UV data to identify RT ranges.
    - To use UV data to define RT ranges, refer to the page: UV Data Processing.

Deconvolution

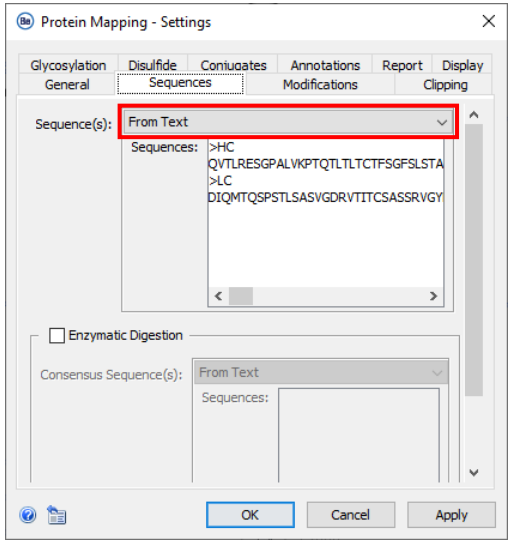


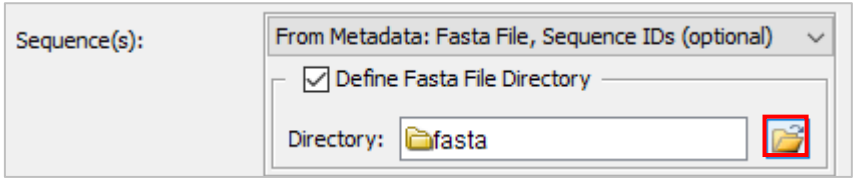
- The RT ranges detected in the previous activity node (*RT Range Condensing*) are deconvoluted with the **Maximum Entropy Method** (spectral deconvolution).



- **Output Mass Spectrum**:
  - To analyze multiple species, use a wide mass range to prevent prominent harmonics peaks.
  - Use a narrow mass range to focus on a single entity.
  - Set a **Mass Step** value that gives the same number of data points across peaks before and after deconvolution.
    - 0.1 Da - 0.2 Da for isotopically resolved data.
    - 1 Da for subunits (lower-resolution data).
    - 2 Da for intact proteins (lower-resolution data).
    - 3 Da if fewer datapoints are required.

Protein Mapping: Sequences

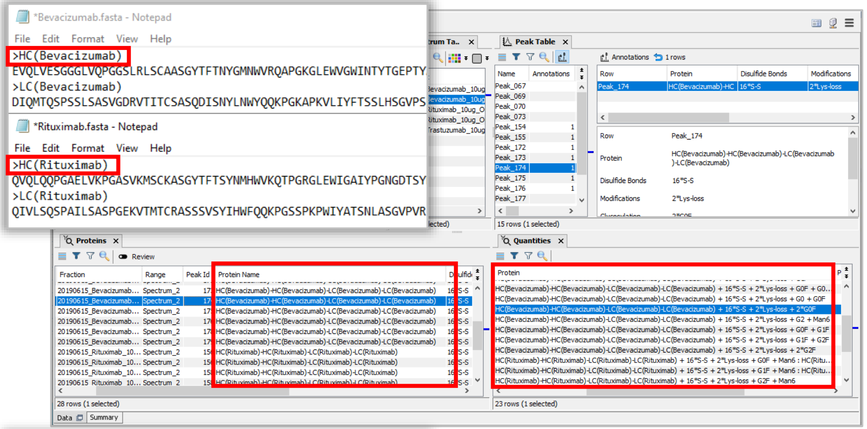


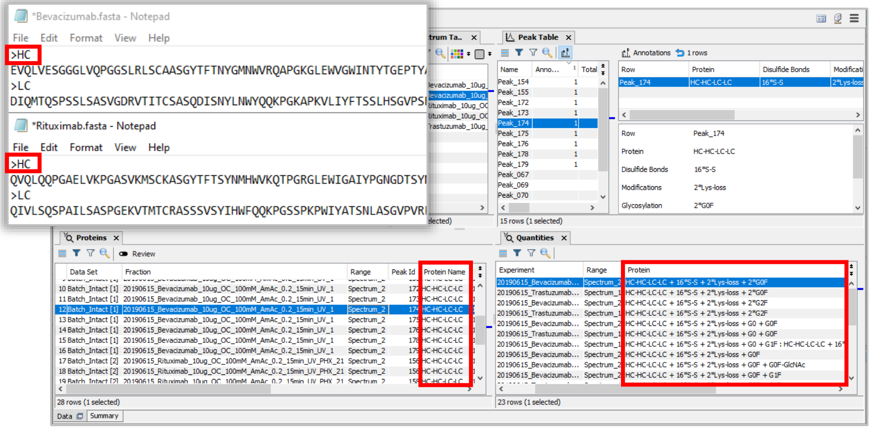


Sequences tab:

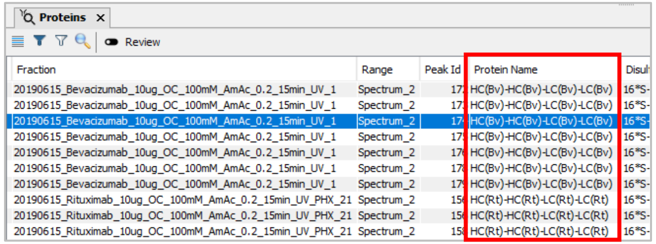
- **Sequence(s)**:
  - If all samples have the same sequence, then select **From Text** and type the sequence, or **From Fasta File** and select the applicable file.
  - If different samples require different sequences, then select **From Metadata: Fasta File, Sequence IDs (optional)**, and then browse to the location of the folder that contains all of the applicable FASTA files.
    - For more information, refer to the page: *Review Results*: Protein Name in FASTA Files.

Review Results: Protein Name in FASTA Files



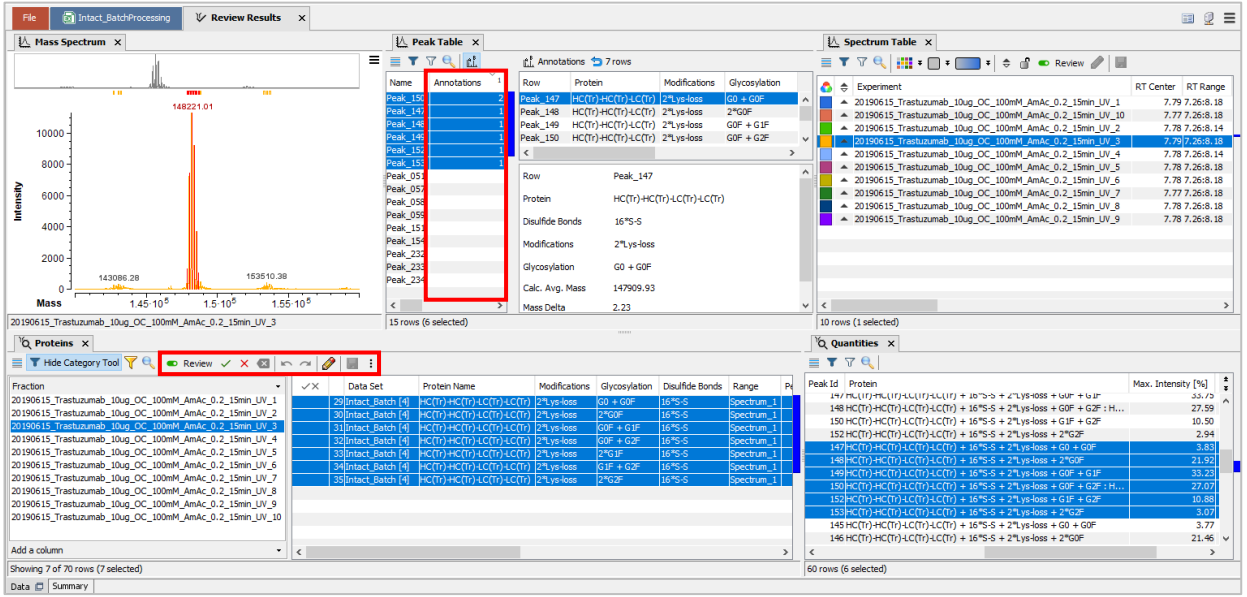


- The protein sequence names used in the FASTA files control the name given to the protein in *Review Results* and in the exported PDF report.
- If the protein sequence names are too long, then columns in tables can become too wide, and some columns might be absent from the exported PDF report.
  - For Peptide Mapping Batch Processing, we recommend that the protein sequence names are unique across the FASTA files.
  - If the same FASTA files will be used for both Intact Mass and Peptide Mapping Batch Processing, use shortened molecule names, rather than just chain names, to maintain uniqueness:

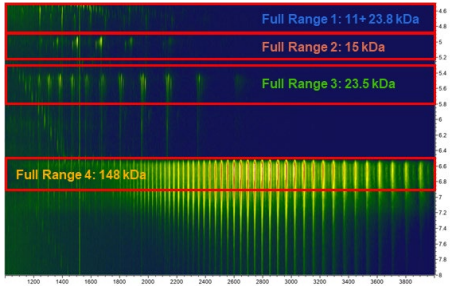


Review Results

- Use the **Categorical Filter** to review entries in the **Proteins** table by specific information, such as the experiment name or identified glycan.
  - To identify annotated peaks, sort the **Annotations** column in the **Peak Table**.
  - If an entry in the Peak Table that is not related to the selected **Categorical Filter** is clicked, it will make the visualizations blank. Click elsewhere to restore the visualizations.



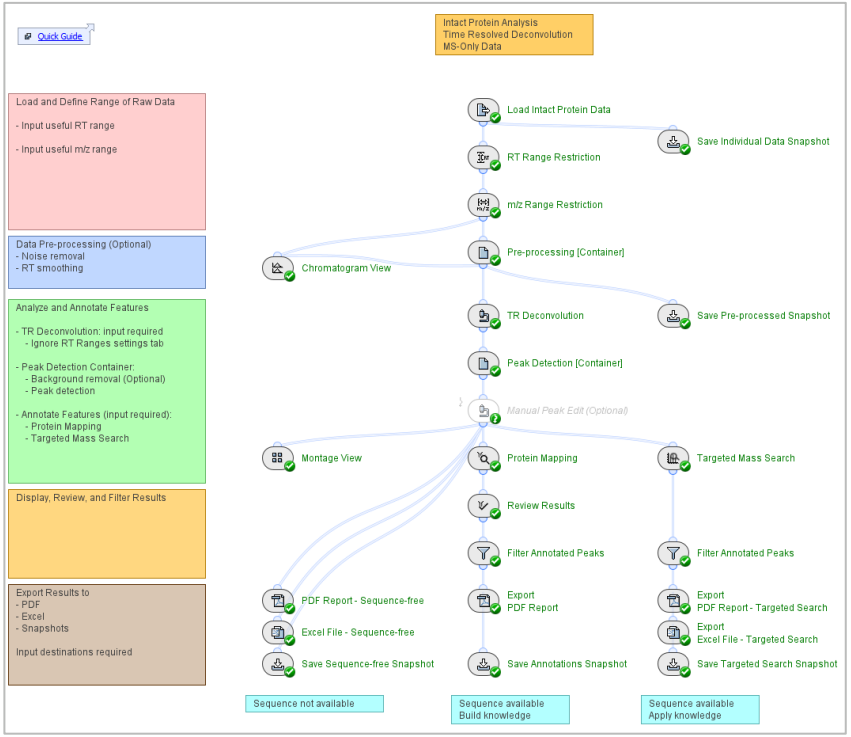
- The **Spectrum Table** lists the detected RT ranges, with the **RT Center** of the protein signals that were detected.
  - All of the scans in each time range are summed and deconvoluted to make the related spectrum.



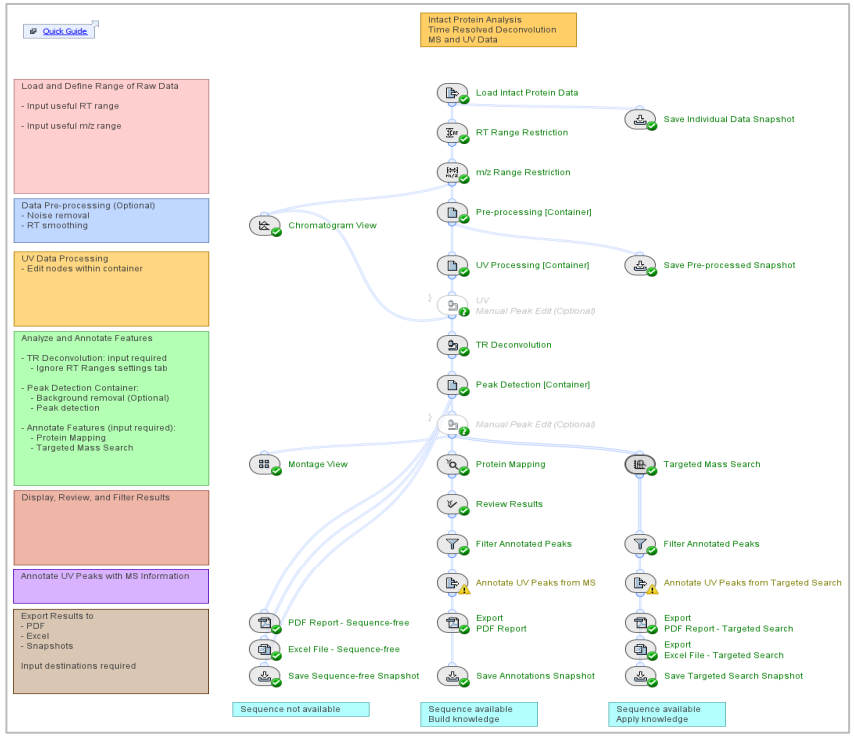
Time Resolved Deconvolution with MS or MS and UV Data

WORKFLOW SPECIFIC GUIDELINES

Overview of the Time Resolved Deconvolution Workflows



Intact_TimeResolvedDeconvolution_MS_Be4.0

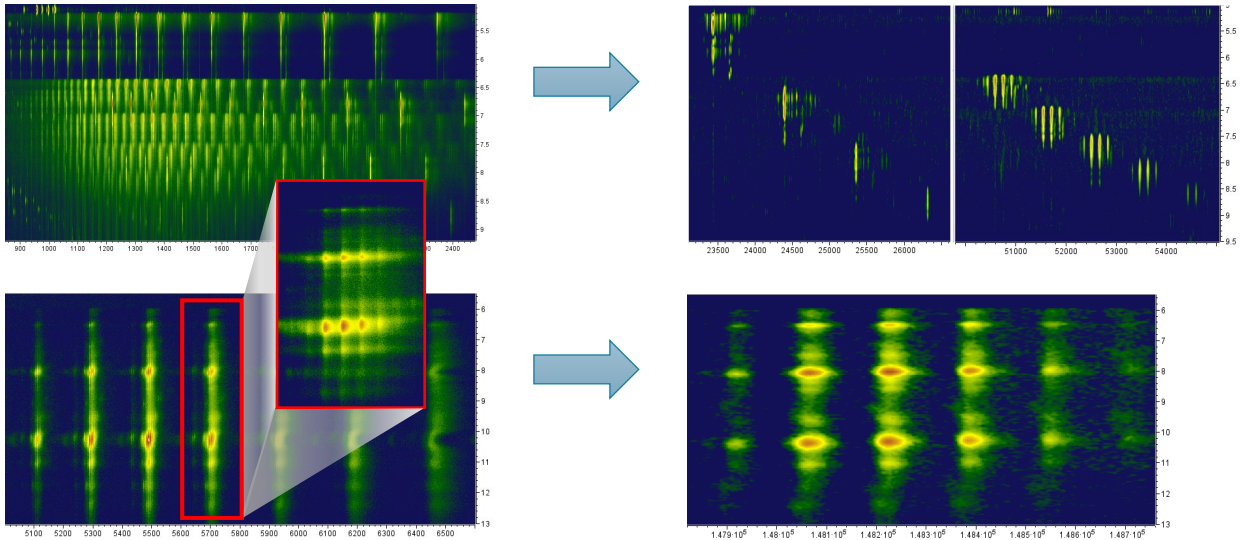


Intact_TimeResolvedDeconvolution_UV+MS_Be4.0

TRD Workflows: Overview

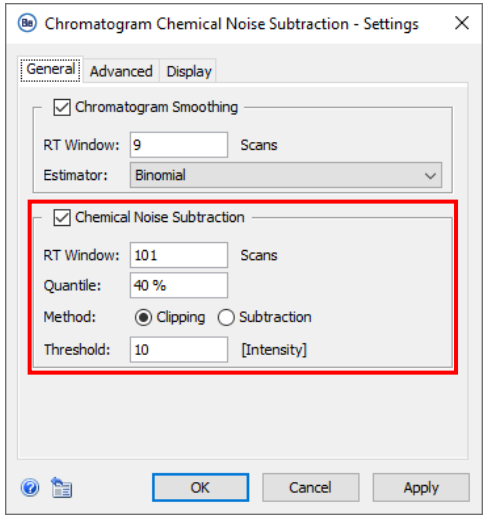
- **TRD** is essential for robust quantification for analysis of multiple components that have similar masses and overlapping chromatographic profiles.
- **TRD** also provides deep insight for characterization of modified proteoforms, and shows details for peaks associated with oxidations, partial reductions and adducts, for example.

Before TRD (*m/z*)

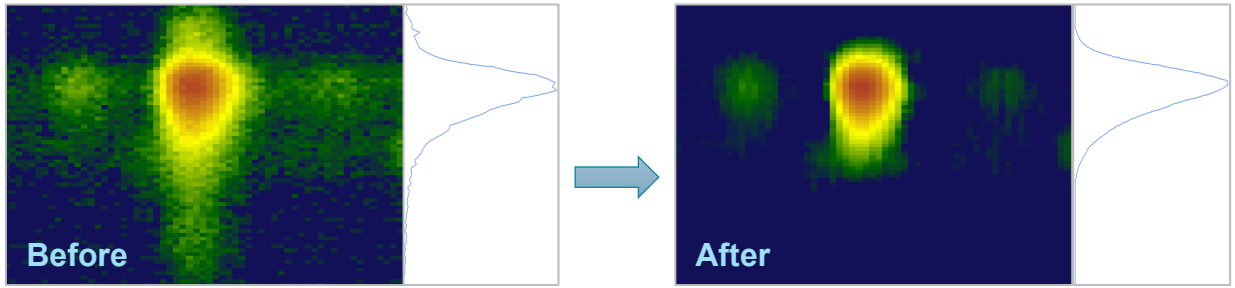


After TRD (mass)

Chemical Noise Subtraction: Optimization



- **Chemical Noise Subtraction** is used with **TRD** data analysis to suppress satellite peaks and improve peak detection by:
  - Removal of extensive tailing on wide peaks.
  - Reduction of overall background noise.

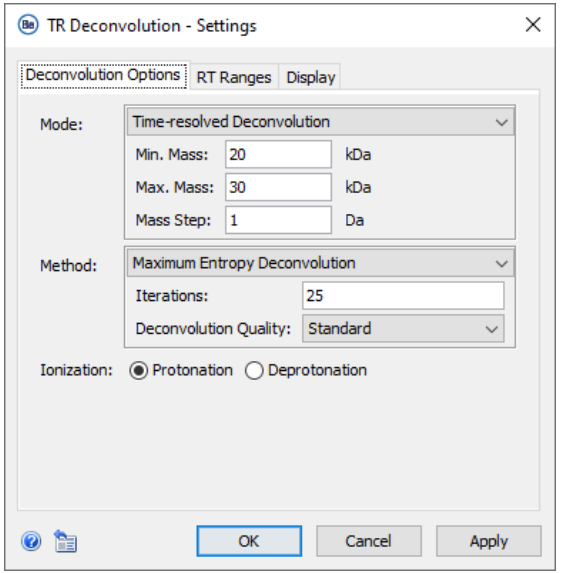


- Typical range for settings:
  - **RT Window***:* Approximately 1.3 times the number of scans across the largest peaks.
  - **Quantile**: 40% or 50%.
- Summary of the impact of these settings:
  - Larger **RT Window** = Less data subtracted.
  - Higher **Quantile** = More data subtracted.

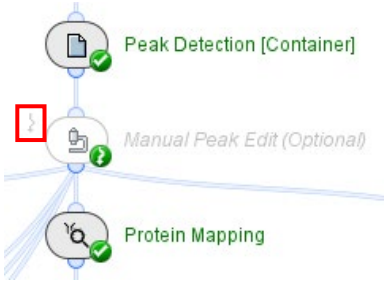
TR Deconvolution: Impact on Speed of Data Analysis

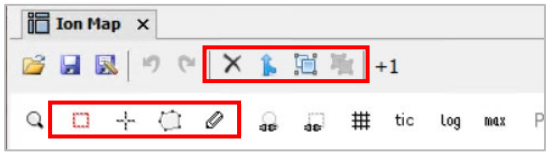
Time-resolved Deconvolution is a more computer-resource intensive algorithm than **Automated Deconvolution**.

- The deconvoluted mass range and data density have a significant impact on processing time.
  - Fragment and subunit datasets often have higher data density than whole mAb datasets.
- The **Min. Mass**, **Max. Mass** and **Mass Step** should be optimized for each dataset.
  - A smaller mass range and larger **Mass Step** can reduce the required processing time, if applicable to the specific dataset.
- When **Time-resolved Deconvolution** is selected, the settings on the **RT Ranges** tab are ignored.
  - Deconvolution occurs over the entire RT range to give a deconvoluted ion map.

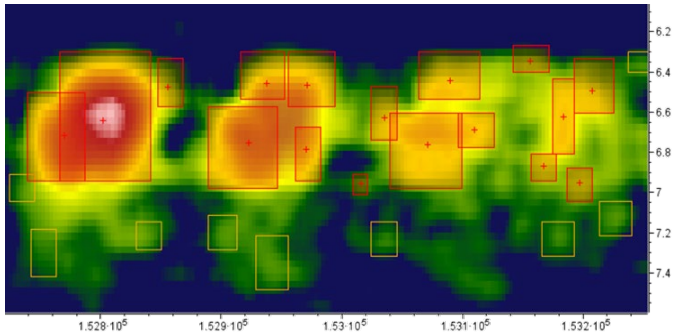


Manual Peak Edit (Optional)



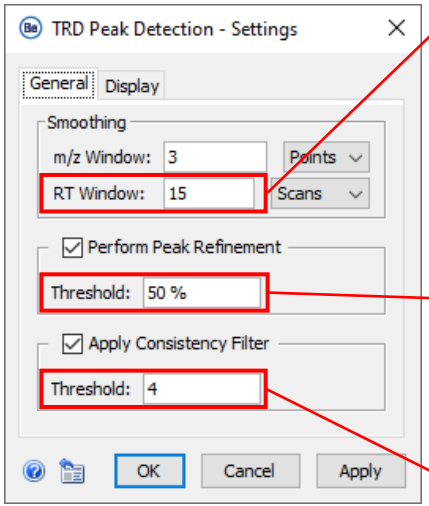


- This activity node is optional. To use *Manual Peak Edit*, deactivate the **Bypass** icon.
- Use *Manual Peak Edit* to manually change the peaks that were detected in the time resolved ion map.
  - It is recommended to use *Manual Peak Edit* to accurately re-assign intensity distributions of overlapping components to individual peaks, and not to optimize *TRD Peak Detection* parameters.



- Select the **Edit Mode** icon to:
  - Move the peak boundaries.
  - Split peaks that overlap.
  - Delete peaks.
  - Draw new peaks.

TRD Peak Detection: Optimization



Affects the number of peaks split along RT (↕).

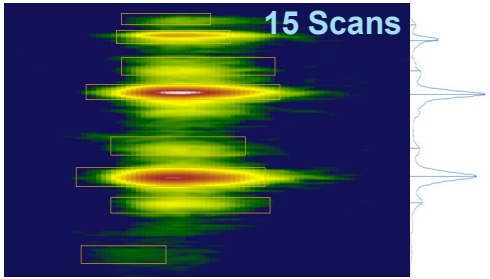
Lower value = Increase in splitting in the RT direction.

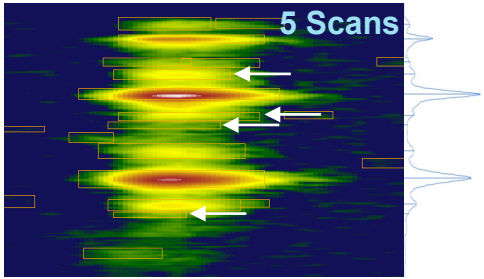
Affects the number and width of peaks along the mass axis (↔).

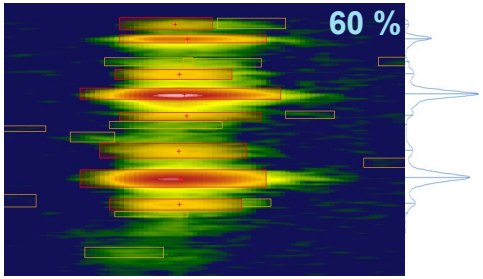
Lower value = Higher probability that peaks will be split in the *m/z* direction.

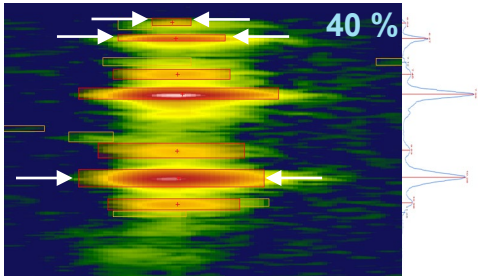
Affects sensitivity and therefore the number of background peaks.

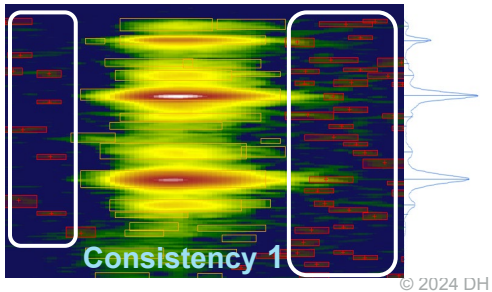
Lower value = Increase in splitting in the *m/z* direction.



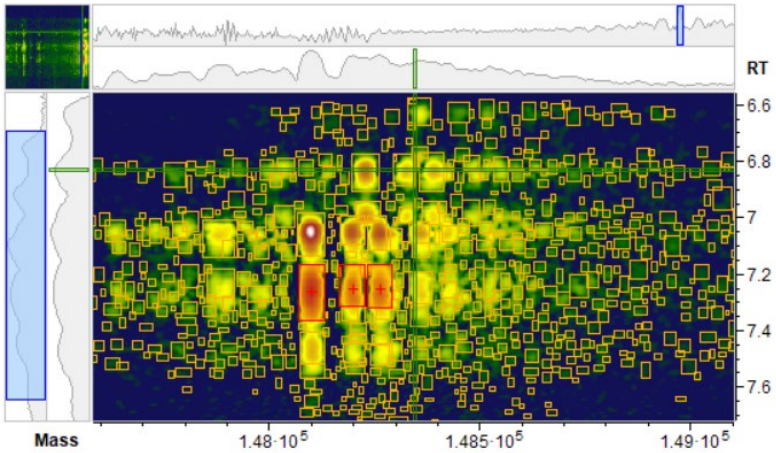




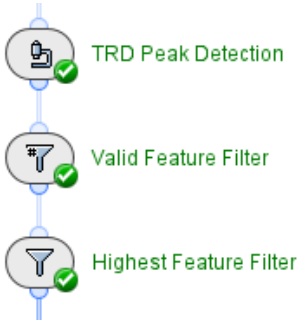




TRD Peak Detection: Optimization

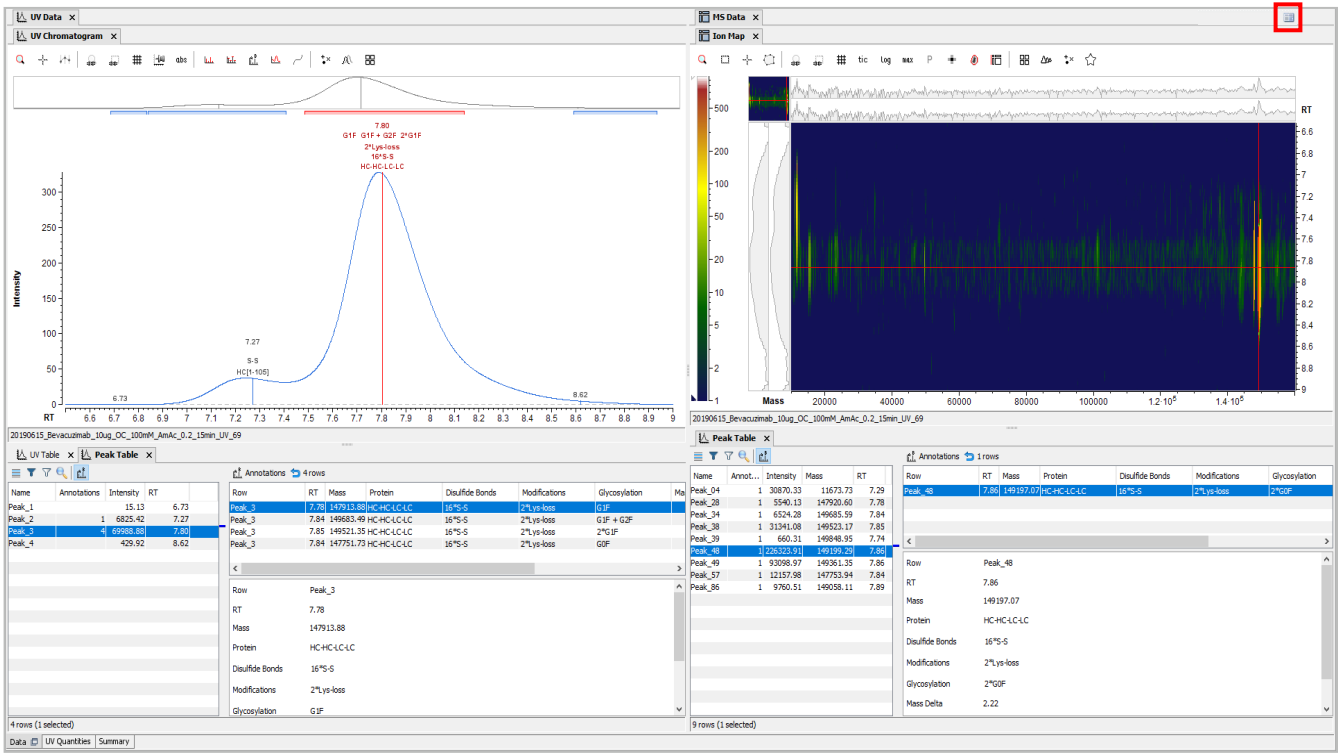


- If multiple small features are detected in a complex deconvoluted ion map, the **Consistency Filter Threshold** is set too low:
  - Increase the **Consistency Filter Threshold** to reduce the sensitivity and detect fewer peaks.



- Use the *Valid Feature Filter* and *Highest Feature Filter* activity nodes to decrease the number of selected features.

Recommended Layout to Review TRD with UV Results

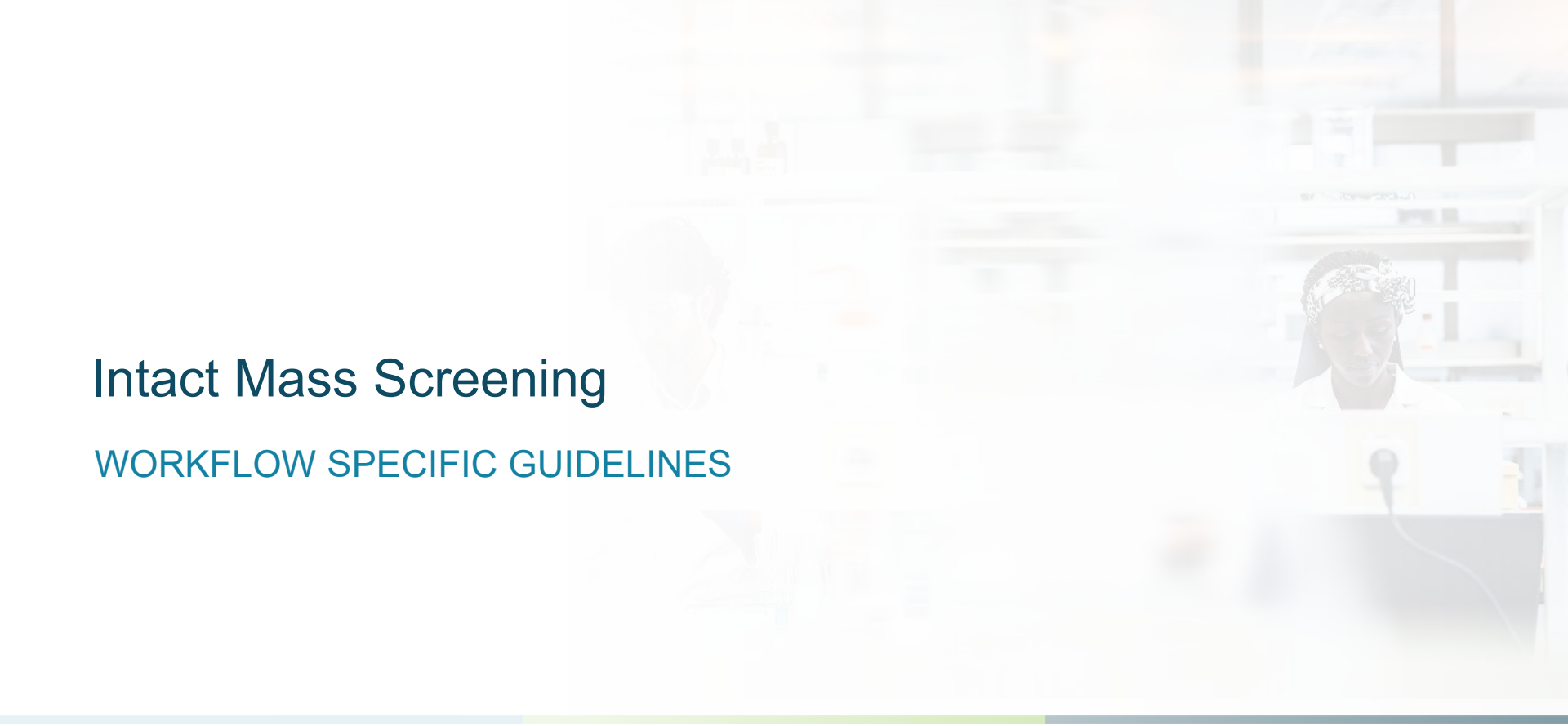


- Use the **Save Layouts** icon to save a preferred location for each window

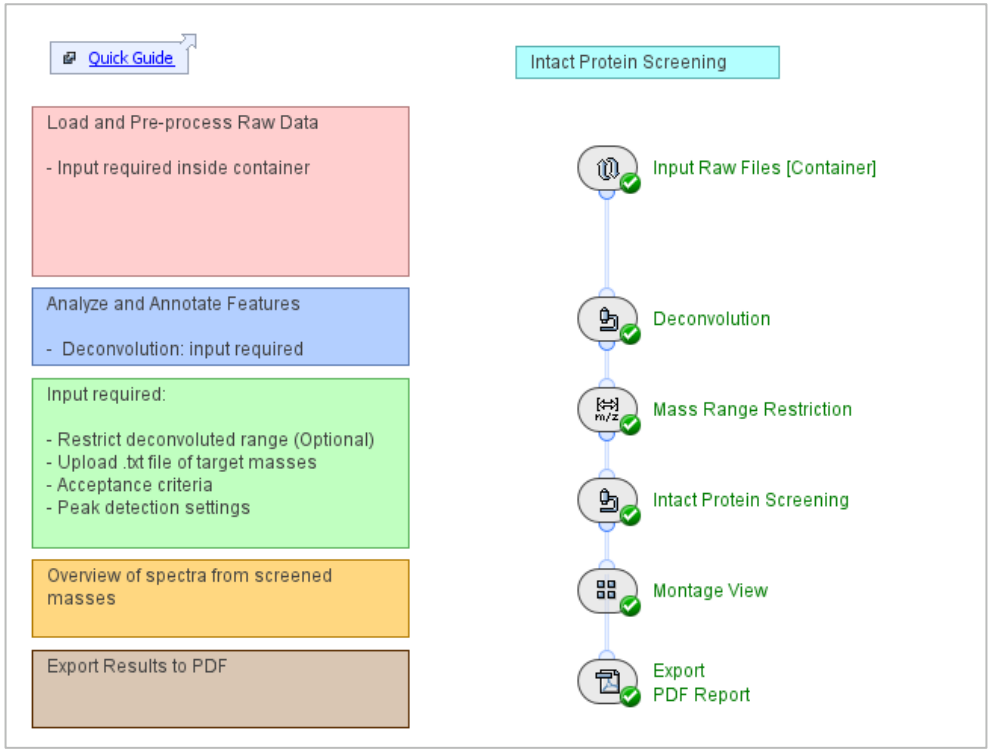
  Note: For more information, refer to the document: Biologics Explorer Quick Guide.

Intact Mass Screening

WORKFLOW SPECIFIC GUIDELINES



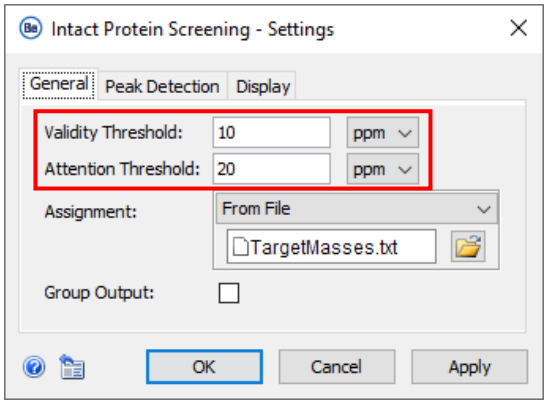
Overview of the Intact Mass Screening Workflow



Intact_MassScreening_Be4.0

Intact Mass Screening Workflow: Overview

- This workflow provides quick deconvolution for high-throughput screening of large batches of samples.
- It can verify the presence, or absence, of target masses within specified limits of mass confidence (ppm or Da).
- The visual summary table identifies each sample as:
  - Valid (✓): The calculated mass is below the Validity Threshold.
  - Critical (!!): The calculated mass is between the Validity and Attention Threshold.
  - Invalid (✗): The calculated mass is above the Attention Threshold.

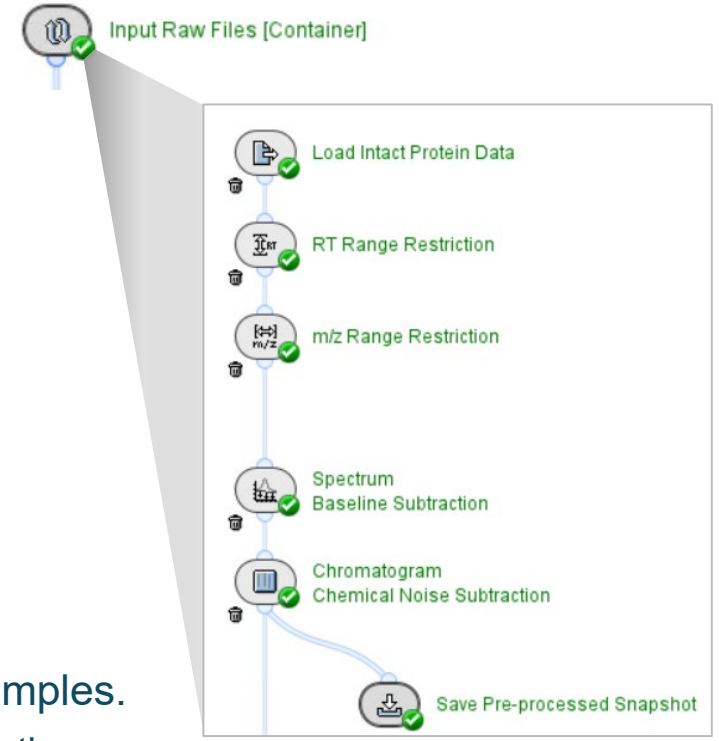


Zoomed Range 3

Name	Expected Mass	Detected Mass	Delta [Da]	Delta [ppm]	Valid
Remicade_IdeS_reduced	25647.51	25647.35	-0.17	-6.0	✓
Rituxan_IdeS_reduced	25328.19	25327.79	-0.41	-16.0	✗
Herceptin_IdeS_TCEP	25383.31	25383.14	-0.16	-6.0	✓
Humira_IdeS_TCEP	25458.33	25457.95	-0.37	-15.0	!!
NIST_500ngOC_IdeS_Red_01	25688.91	25688.72	-0.19	-7.0	✓

Intact Mass Screening Workflow: Overview

- To reduce the computational memory used for high-throughput screening:
  - The *Input Raw Files* container uses an iterative process to pre-process each sample in the batch independently.
  - **Trash** is activated by default in the container so that intermediate results are deleted as soon as they are passed to the subsequent activity node.
  - Pre-processing results from each pre-processed sample can be saved as a snapshot (sbf) file that can be used in other intact mass analysis workflows for further investigation, if required.



- The *Montage View* activity node can display up to 200 samples.
  - If more than 200 samples are analyzed together, then activate the **Bypass** icon on the *Montage View* activity node.

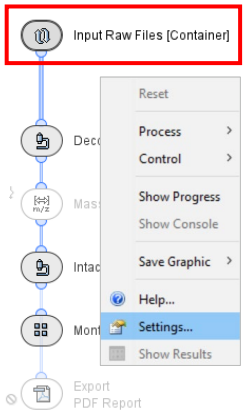
Intact Mass Screening Workflow: Conditions and Behavior

- For each sample, more than one mass can be searched. However, certain conditions should be met in the submitted samples:
  - Samples must belong to the same molecule type. For example, all samples should be either intact proteins, subunits, or fragments.
  - Samples should have consistent chromatography, the same number of components, and similar expected deconvolution ranges.
  - Samples must have the same number of either **Full** or **Zoomed RT Ranges**.

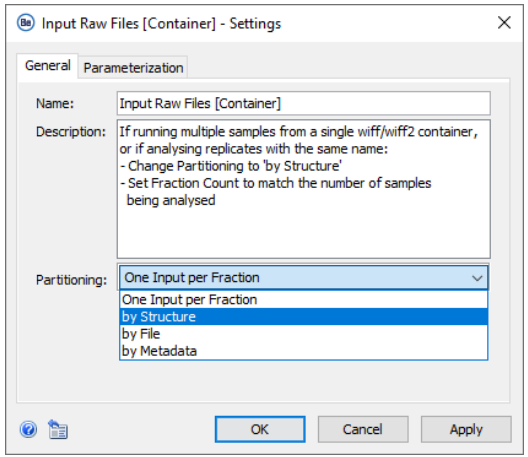
- The workflow is designed to analyze data as follows:
  - The highest peak in each deconvoluted RT range (**Full** or **Zoomed RT Range**) of each sample is detected independently and assigned to a single match based on the list of masses.
  - Results do not include annotation.
  - The values of the detected masses are reported.

Input Raw Files: Replicates with the Same Name

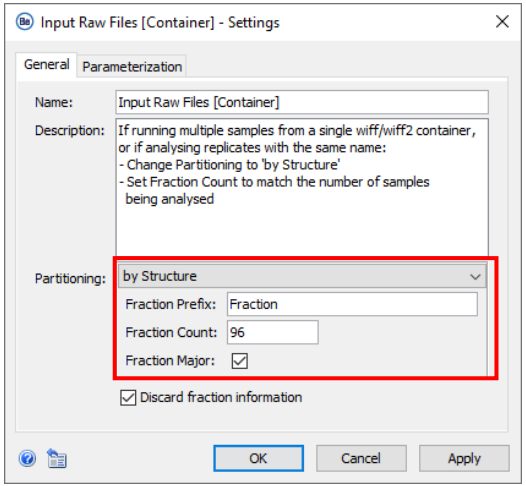
- The settings of the *Input Raw Files* container must be changed to:
  - Analyze replicate samples that have the same file name.
  - Load multiple different samples from in a single wiff or wiff2 container.



(1) Right-click on *Input Raw Files [Container]* to access Settings.



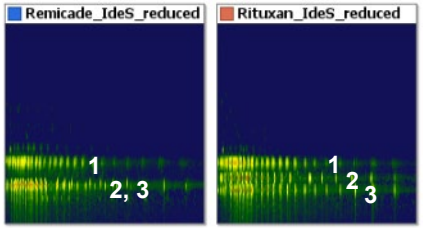
(2) Change **Partitioning** to **by Structure**.

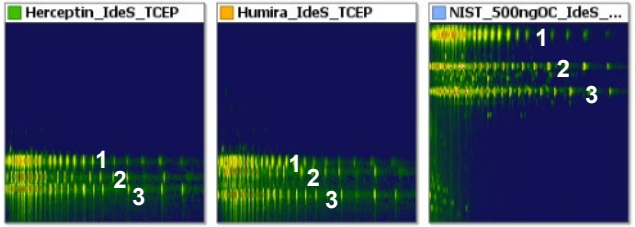


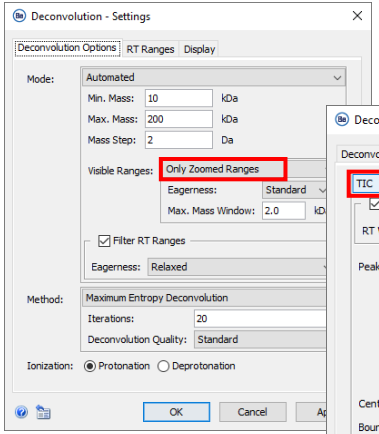
(3) Specify the number of samples loaded as the **Fraction Count**.

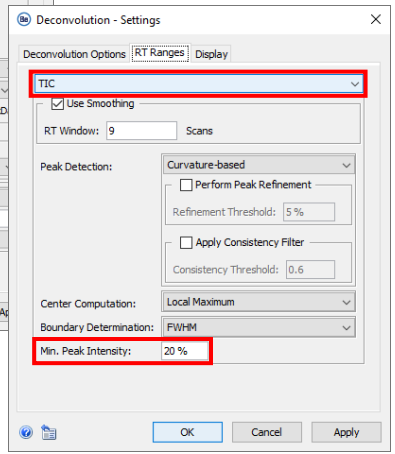
Deconvolution: Complex Datasets

- The template workflow represents a highly complex screening application:
  - There are three target components (mAb fragments), from different mAbs.
  - Chromatography is inconsistent.
  - The Fc/2 glycoforms have similar intensity, and the intensity order changes across the samples.
  - Data is from both wiff and wiff2 files.



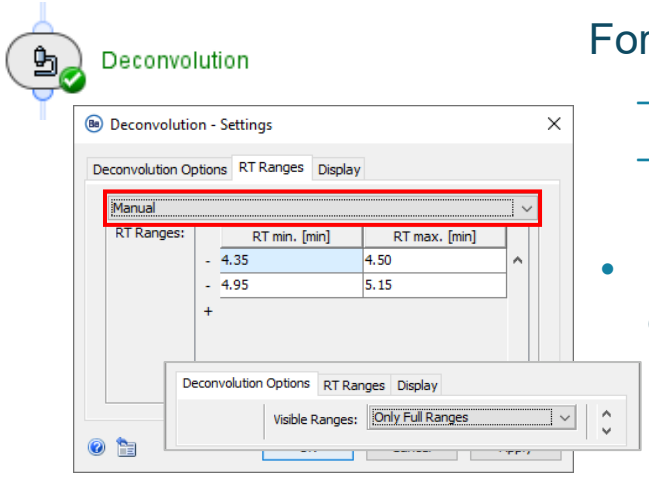


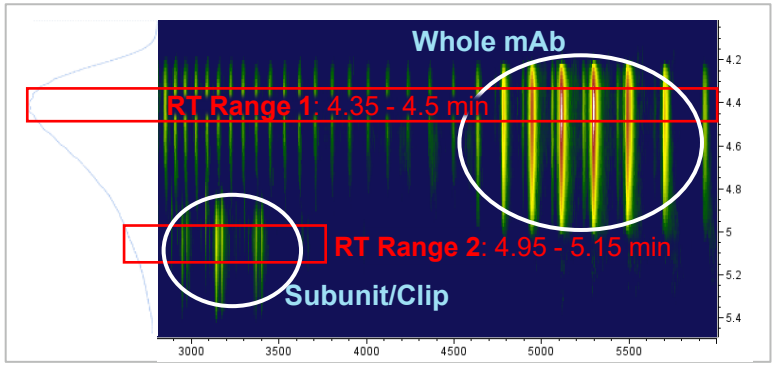




- To overcome the potential challenges with this type of dataset:
  - Use **TIC** to automatically identify the specific RT ranges for each sample.
  - Use a **Min. Peak Intensity** of 20% (or higher) to limit the number of deconvoluted RT ranges to those related to the target fragments.
  - Use **Only Zoomed Ranges** as these are expected to be the same for all screened samples in the same batch, if they are the same type of molecule.

Deconvolution: RT Ranges





For the use case where a screening workflow is used to analyze:

- Main target component: Whole mAb (always expected).
- Known side product: Misconnected subunit that may be present or absent.

- Use of the **TIC** to identify the RT ranges would result in variable numbers of RT ranges per sample. This inhibits the screening workflow.

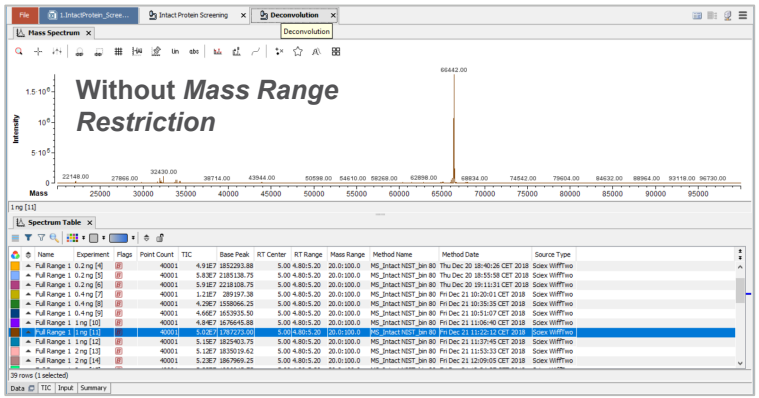
The optimal settings for **RT Ranges** when components are not always present are:

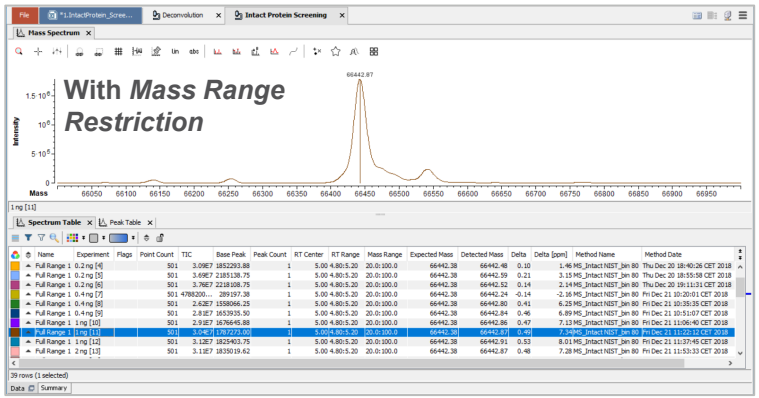
- Use **Manual** to specify the RT ranges for each component (small RT windows focused on the apex of the elution profiles).
- Set **Visible Ranges** to **Only Full Ranges**.
- The workflow will complete, if:
  - The chromatography is consistent over the sample batch.
  - There is some separation between the components.

Mass Range Restriction

Mass Range Restriction

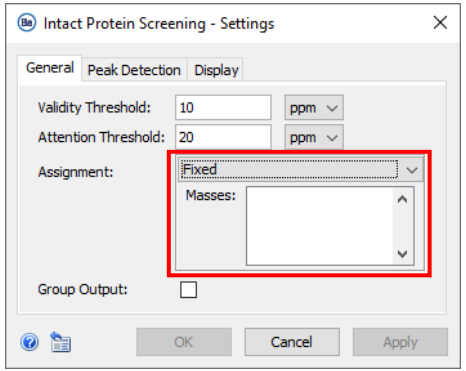
- This activity node is optional. To use *Mass Range Restriction*, deactivate the **Bypass** icon.
- *Mass Range Restriction* can be used to restrict how the deconvoluted spectra of specific target mass is shown.
  - The restriction continues to be applied when **Only Full Ranges** is used.
  - This activity node can be used as an alternative to **Only Zoomed Ranges**.



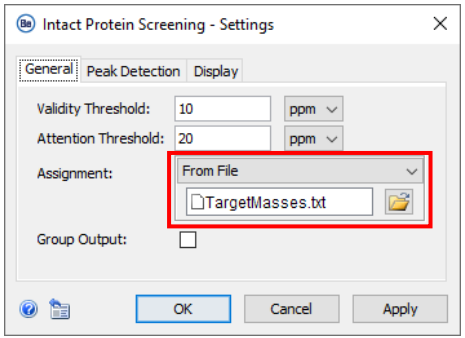


To Define Target Masses for *Intact Protein Screening*

- Specify the masses of interest for screening:



- If the same masses are expected across all samples, then change **Assignment** to **Fixed** and type the values in the **Masses** section.



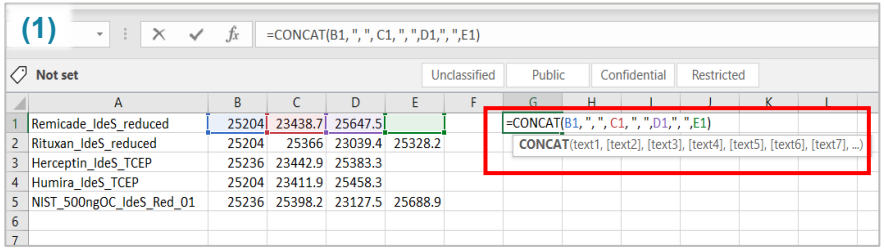
- If different masses are expected across the samples, then change **Assignment** to **From File** and upload a txt file that contains the target masses for each sample.

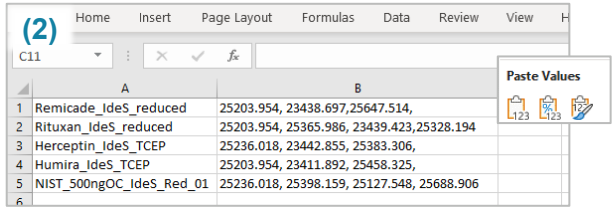
- There is no need to change the default **Peak Detection** settings.

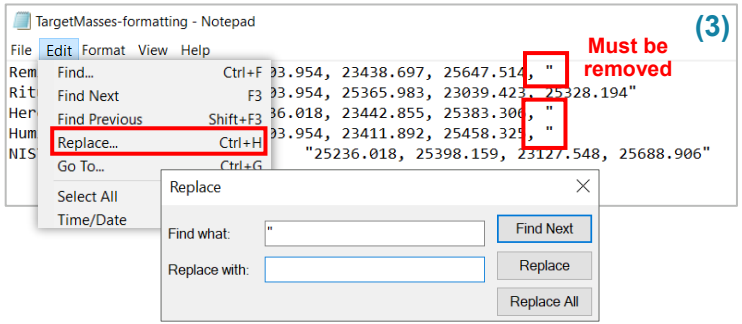
Intact Protein Screening: Target Masses File Format

To create a txt file for screening:

1. Create a table that contains sample names and target masses. Do not include table headers.
   - Use the CONCATENATE function in Excel to combine columns that contain the targeted masses into one column.
   - Notice the format **“, “**.
2. Remove formulas from the table.
   - Copy the concatenated column and select **Paste Values**.
   - Delete the original columns to produce a two-column table.
   - Save the spreadsheet as txt file.
3. Open the txt file and remove any additional, unrequired characters.
   - Manually remove duplicate commas or commas at the end of a sequence of masses.
   - If **“** is present: Use the Replace tool to replace “ with a space. Then save the txt file.

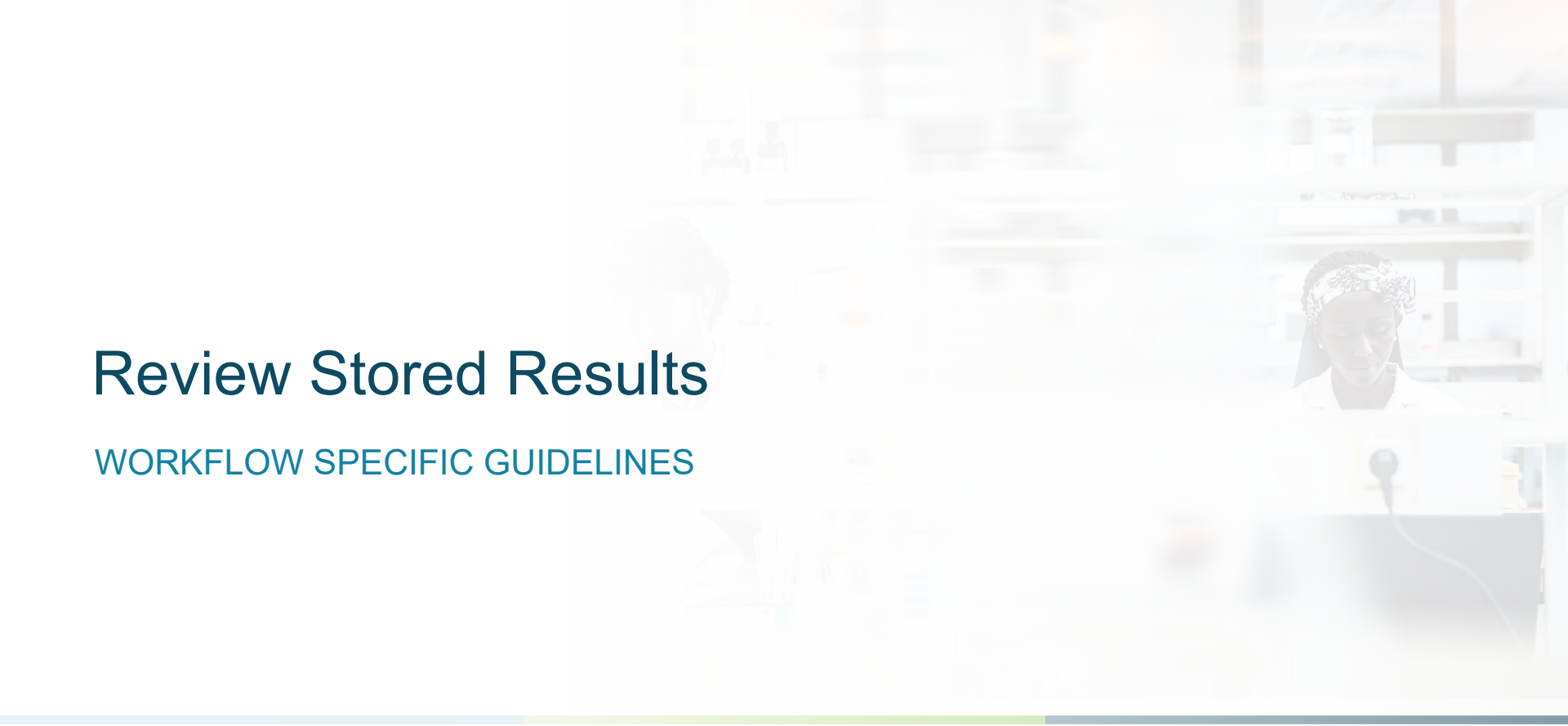




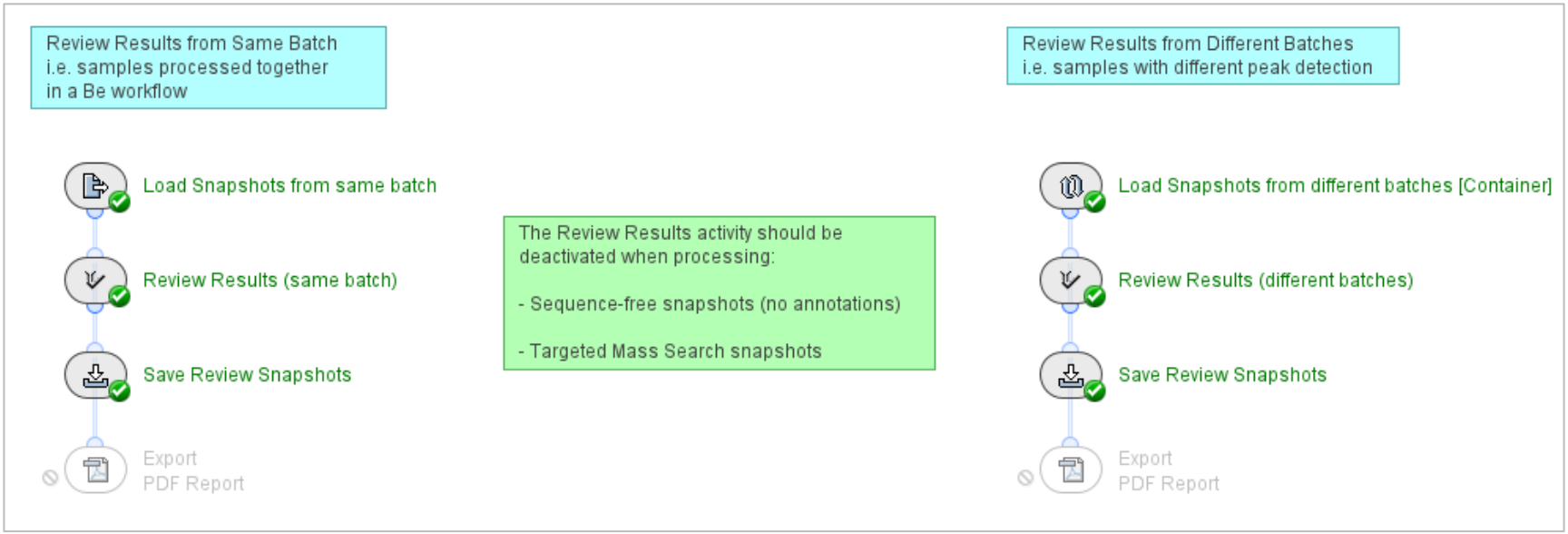


Review Stored Results

WORKFLOW SPECIFIC GUIDELINES



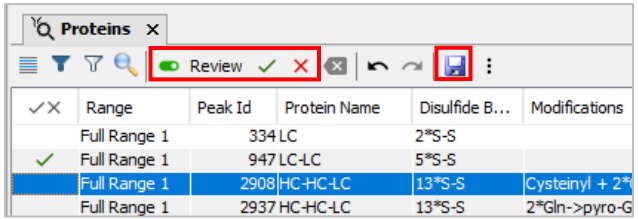
Overview of the Review Snapshots Workflow

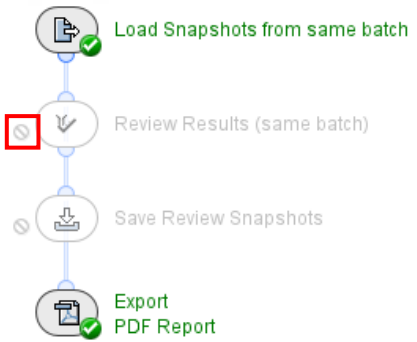


Intact_ReviewSnapshots_Be4.0

Review Snapshots Workflow: Overview

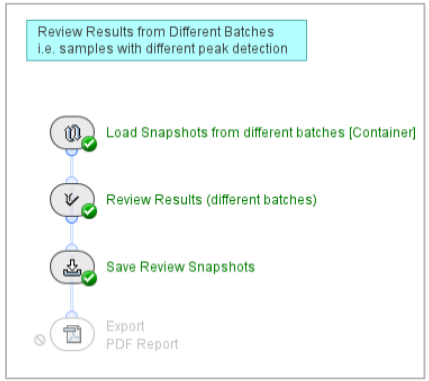
- These workflows can be used to:
  - Open all saved snapshot (sbf) files created from any Intact Mass workflow in Biologics Explorer software.
  - View results from previous analyses with data analyzed in the same or different batches.
  - Complete further review and, if required, change results that were accepted or rejected in the initial review process (*Annotations Snapshots* only).



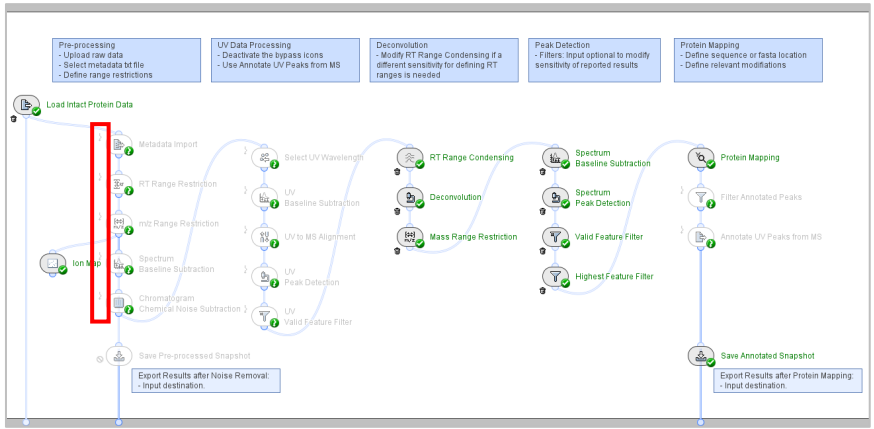


- After further review, the results can be saved as *Review Snapshots* and exported as a PDF report.
- To export a PDF report from *Sequence-free, Targeted Mass Search, Individual Data,* and *Pre-processed Snapshots,* activate the **Block** icon on the *Review Results* activity node.
  - These snapshots do not include the sequence information required by *Review Results*.

Review Snapshots Workflow: Batch Processing



- To open sbf files with protein annotations that were saved with the Batch Processing workflow:
  - Load Snapshots from *Save Annotated Snapshot* or *Save Review Snapshots* into the Review Results from Different Batches workflow in Intact_ReviewSnapshots.
    - The *Review Results* activity node opens a copy of the previous analysis, with any previously accepted or rejected proteins.
    - To save new results from any additional review, use the *Save Review Snapshots* activity node.



- To continue analysis of sbf files from *Save Pre-processed Snapshot* in the Batch Processing workflow:
  - Use the Batch Processing workflow.
  - Select the applicable sbf files in the *Load Intact Protein Data* activity node in the Batch Processing workflow.
  - Change the **Format** to **Snapshot (*.sbf)**.
  - Deactivate the **Trash** icon, and then activate the **Bypass** icon for all other activity nodes before *Save Pre-processed Snapshot*.

Part C
Refined Settings for Specific Applications

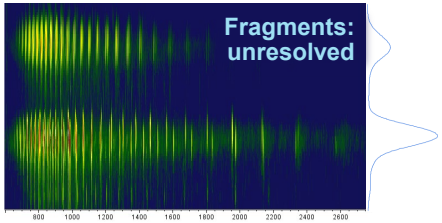
Select Time-Resolved or Automated Deconvolution

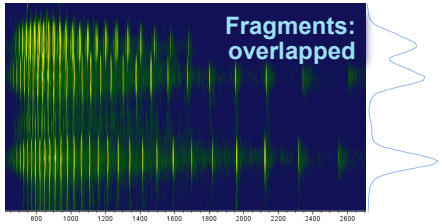
- **Automated:** Ranges for deconvolution are automatically determined.
  - Recommended for chromatographically well-resolved species.

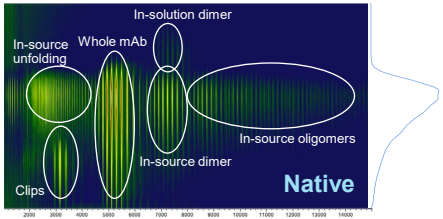
- **Time-resolved:** Deconvolution is completed on each scan in RT.
  - Recommended for data that contains complex mixtures that are poorly resolved.
    - Creates a 2D ion map view of deconvoluted data that provides better visibility of overlapping or non-resolved peaks.
    - Simplifies quantitation of overlapping or unresolved peaks.

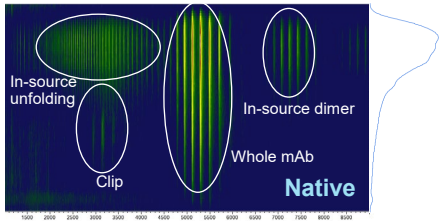
Use Cases for Deconvolution Types

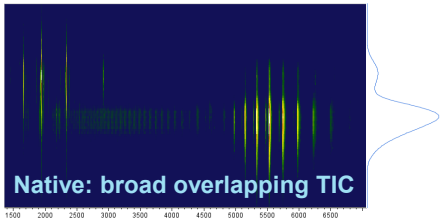
Key use cases for **Time-Resolved Deconvolution:** Complex Chromatography

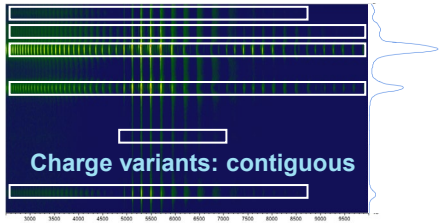




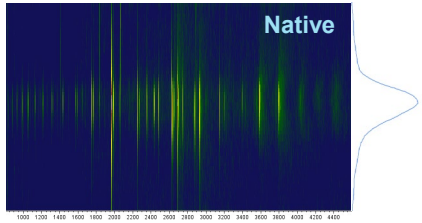


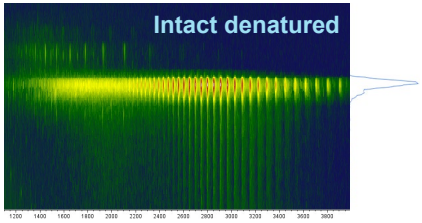


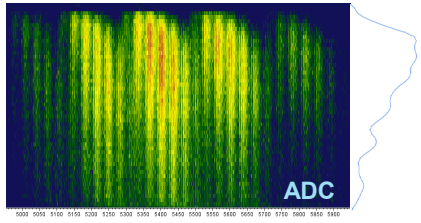


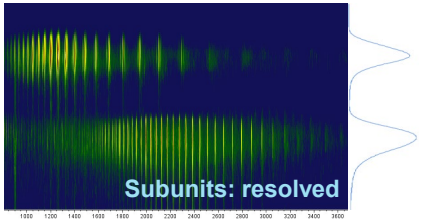


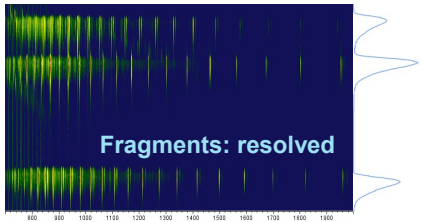
Key use cases **for Automated Deconvolution:** Simple Chromatography











Denatured Intact Protein

REFINED SETTINGS FOR SPECIFIC APPLICATIONS

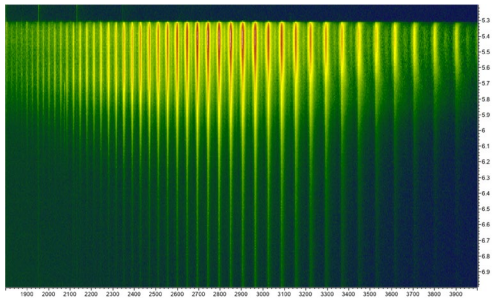
Denatured Protein: Single Entity

FOCUS: Single target protein.
No side products of interest.

Suggested initial settings:

Sample type:	Single denatured protein
Recommended workflow	Intact_AutomatedDeconvolution
Deconvolution range [1]	140 kDa - 160 kDa **Visible Ranges: Only Full Ranges** for visualization and reporting.
Mass step [1]	2 Da
RT Ranges [1]	**RT Window**: 5 - 9 (The goal is to identify a single RT range). **Isolation Threshold**: 5 - 15 (depends on scan frequency).
Mass Tolerance [2]	20 ppm - 50 ppm (depends on resolving power used).
Glycosylation [2]	**Deglycosylated** or **Glycosylated** with library selection.
Disulfide [2]	**State: Fully Connected**. **Connectivity: IgG** (if applicable, otherwise specify).

[1]Deconvolution, [2]Protein Mapping



Denatured intact protein

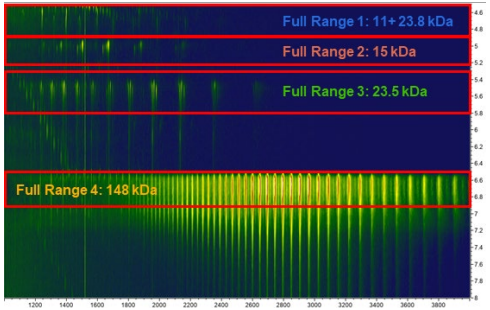
Denatured Protein: Complex Sample

FOCUS: All visible RT ranges.
Including low-mass degradation products (unconnected subunits, clips).

Suggested initial settings:

Sample type:	Denatured protein and its impurities/degradation products
Recommended workflow	Intact_AutomatedDeconvolution Intact_TimeResolvedDeconvolution (for multiple, overlapping RT ranges)
Deconvolution range [1]	10 kDa - 160 kDa **Visible Ranges: Only Zoomed Ranges** for visualization and reporting.
Mass step (Da) [1]	2 Da
RT Ranges [1] (Ignore with TRD)	**RT Window**: 3 - 5 **Isolation Threshold**: 3 - 7 **Min. Peak Intensity**: <1% For higher sensitivity and better efficiency manually specify all ranges.
Mass Tolerance [2]	20 ppm - 50 ppm (depends on resolving power used).
Glycosylation [2]	Deglycosylated or Glycosylated with library selection.
Disulfide [2]	**State: Fully Connected**. **Connectivity: Unspecified + 3 Additional chains**. **Cysteinyl** for unpaired cysteines.

[1]Deconvolution, [2]Protein Mapping



Denatured intact protein with impurities

Native Intact Protein

REFINED SETTINGS FOR SPECIFIC APPLICATIONS

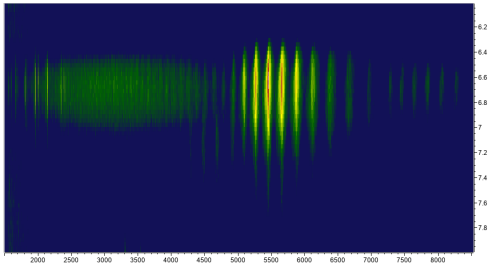
Native Protein: Single Entity

FOCUS: Single target protein.
No side products of interest.

Suggested initial settings:

Sample type:	Single native protein
Recommended workflow	Intact_AutomatedDeconvolution
Deconvolution range [1]	140 kDa - 160 kDa **Visible Ranges: Only Full Ranges** for visualization and reporting.
Mass step [1]	2 Da
RT Ranges [1]	**RT Window**: 5 - 9 (The goal is to identify a single RT range). **Isolation Threshold**: 5 - 15 (depends on scan frequency).
Mass Tolerance [2]	20 ppm - 50 ppm (depends on the resolving power used).
Glycosylation [2]	**Deglycosylated** or **Glycosylated** with library selection.
Disulfide [2]	**State: Fully Connected.** **Connectivity: IgG** (if applicable, otherwise specify).

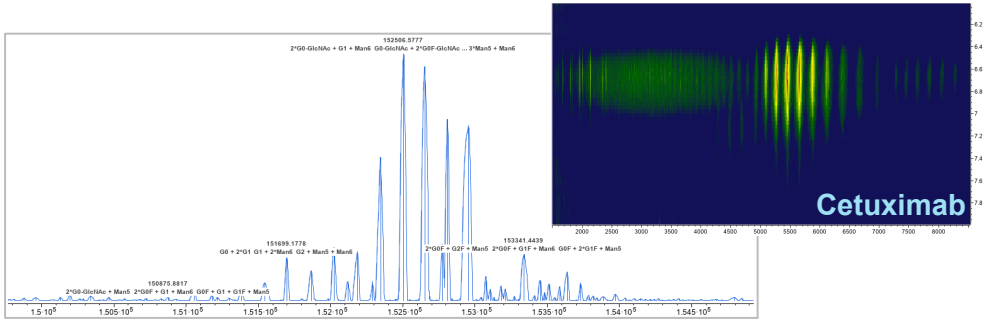
[1]Deconvolution, [2]Protein Mapping

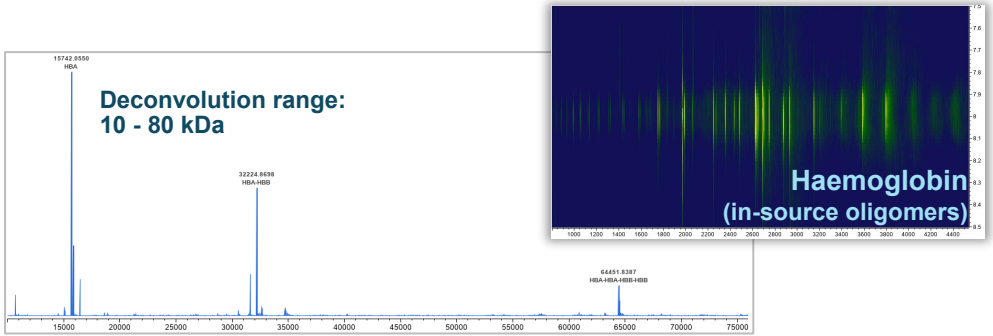


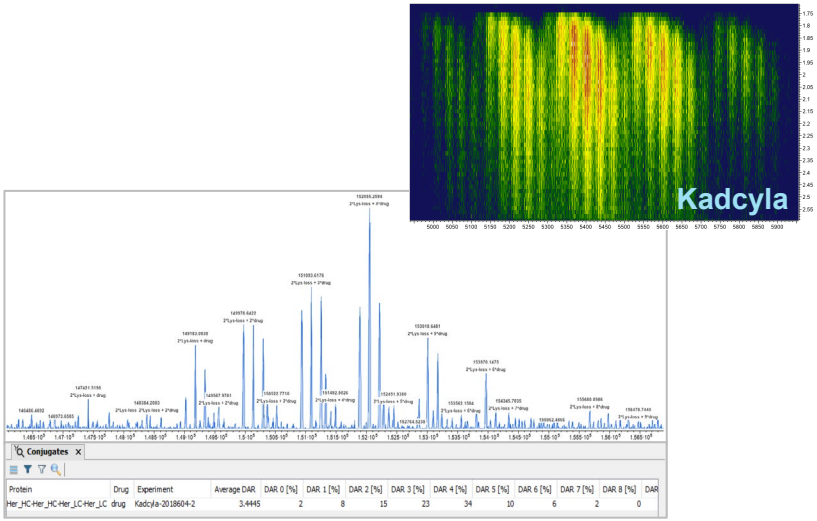
Native intact protein

Native Protein: Single Entity

FOCUS: Single target protein.
No side products of interest.







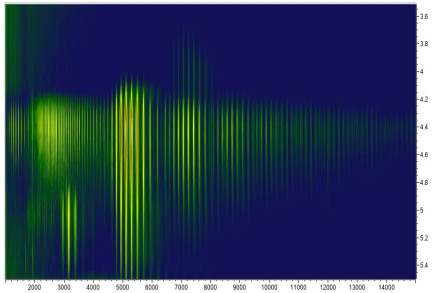
Native Protein: Complex Sample

FOCUS: All visible RT ranges.
Including low-mass degradation products (unconnected subunits, clips).

Suggested initial settings:

Sample type:	Native protein and its impurities/degradation products
Recommended workflow	Intact_TimeResolvedDeconvolution (for multiple, overlapping RT ranges). Intact_AutomatedDeconvolution (if peaks are resolved).
Deconvolution range [1]	10 kDa - 160 kDa **Visible Ranges: Only Zoomed Ranges** for visualization and reporting.
Mass step [1]	2 Da
RT Ranges [1] (Ignore with TRD)	**RT Window**: 3 - 5 **Isolation Threshold**: 5 - 15 (depends on separation between components). **Min. Peak Intensity**: <1% For higher sensitivity and better efficiency manually specify all ranges.
Mass Tolerance [2]	20 ppm - 50 ppm (depends on the resolving power used).
Glycosylation [2]	**Deglycosylated** or **Glycosylated** with library selection
Disulfide [2]	**State: *Fully Connected*.** **Connectivity: Unspecified + 3 Additional chains**. **Cysteinyl** for unpaired cysteines.

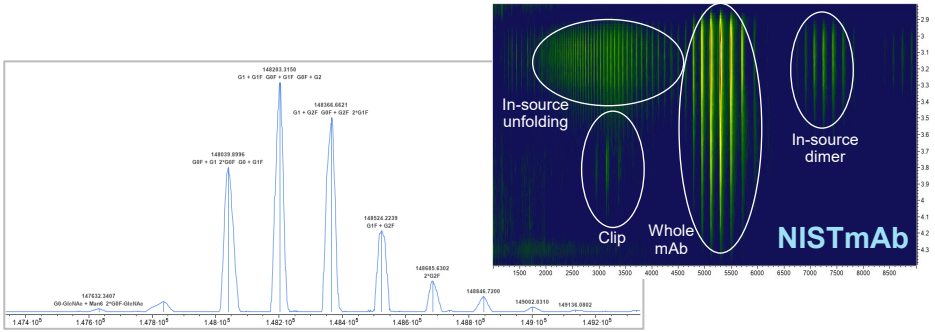
[1]Deconvolution, [2]Protein Mapping

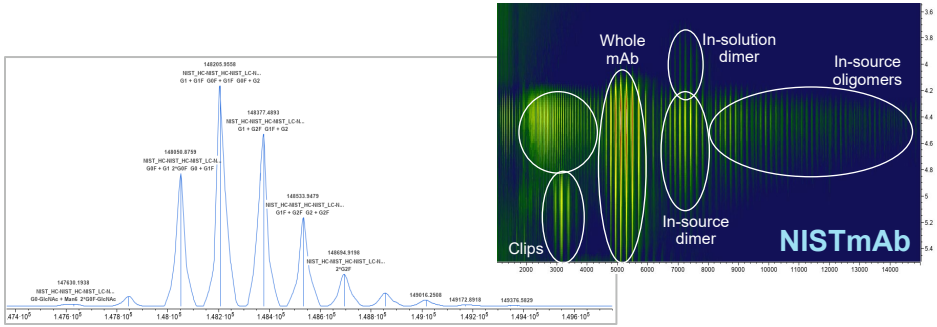


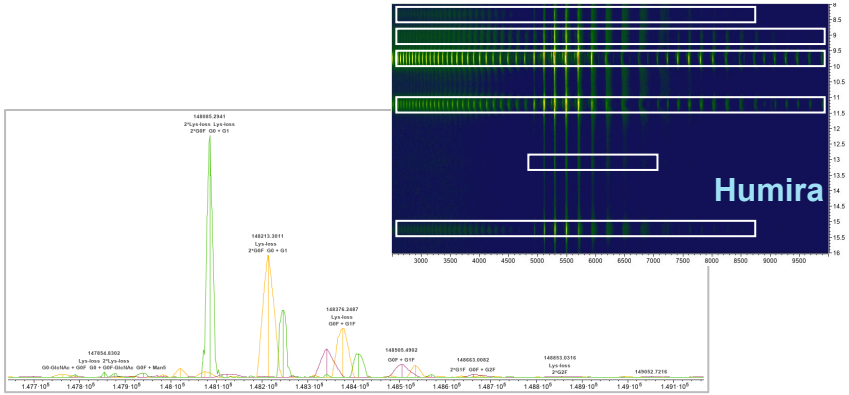
Native complex sample

Native Protein: Complex Sample

FOCUS: All visible RT ranges.
Including low-mass degradation products (unconnected subunits, clips).







Antibody Drug Conjugates

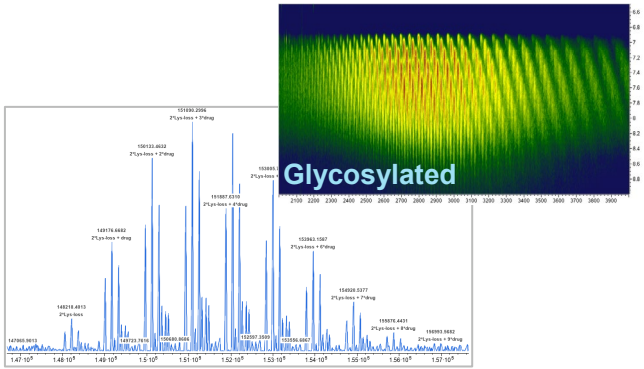
REFINED SETTINGS FOR SPECIFIC APPLICATIONS

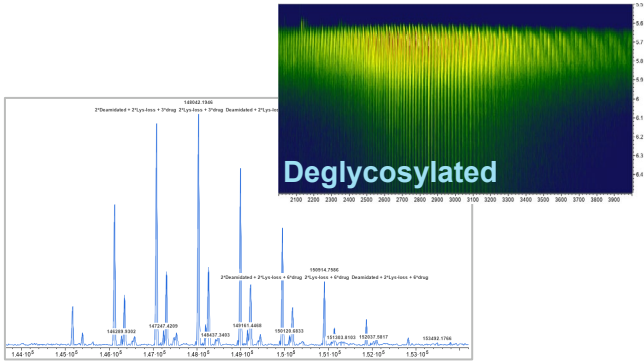
ADC: Whole Protein

FOCUS: Single target protein.
No side products of interest.

Suggested initial settings:

Sample type:	ADC: Denatured
Recommended workflow	Intact_AutomatedDeconvolution
Deconvolution range [1]	140 kDa - 160 kDa **Visible Ranges: Only Full Ranges** for visualization and reporting.
Mass step [1]	2 Da
RT Ranges [1]	Manually specify a single RT range that contains all of the ADC signals.
Mass Tolerance [2]	20 ppm - 50 ppm (depends on the resolving power used).
Glycosylation [2]	**Deglycosylated** or **Glycosylated** with library selection.
Disulfide [2]	**State: Fully Connected**. **Connectivity: IgG** (if applicable, otherwise specify).
Conjugates [2]	Specify the names and masses of conjugates.
Other notes	*Review Results*: Reject redundant annotations to make sure that the DAR is calculated correctly.





[1]Deconvolution, [2]Protein Mapping

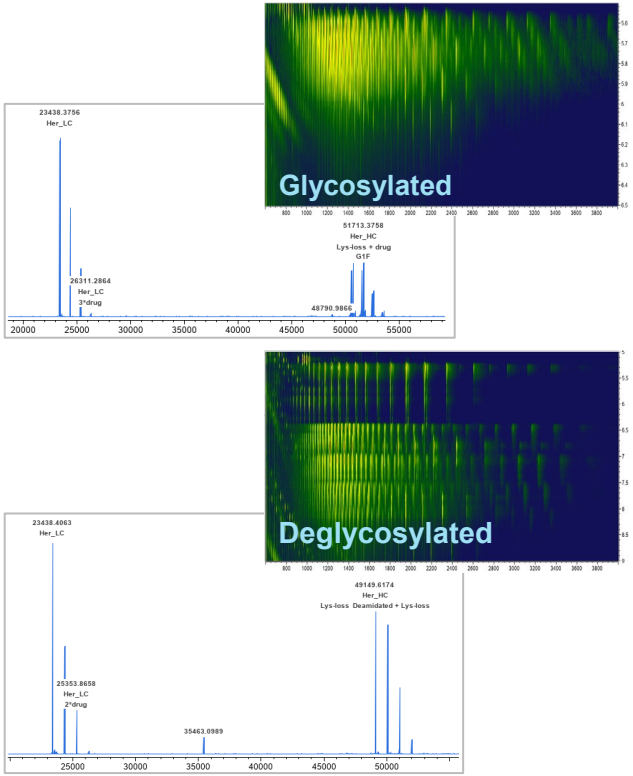
ADC: Subunits

FOCUS: Reduced ADC.

Suggested initial settings:

Sample type:	ADC: Denatured and reduced
Recommended workflow	Intact_AutomatedDeconvolution
Deconvolution range [1]	10 kDa - 60 kDa **Visible Ranges: Only Full Ranges** for visualization and reporting.
Mass step [1]	1 Da - 2 Da
RT Ranges [1]	Manually specify a single RT range that contains all of the ADC signals.
Mass Tolerance [2]	10 ppm - 20 ppm (depends on the resolving power used).
Glycosylation [2]	**Deglycosylated** or **Glycosylated** with library selection.
Disulfide [2]	**State: Fully Reduced**
Conjugates [2]	Specify the names and masses of conjugates.
Other notes	*Review Results*: Reject redundant annotations to make sure that the DAR is calculated correctly.

[1]Deconvolution, [2]Protein Mapping



Subunit Analysis

REFINED SETTINGS FOR SPECIFIC APPLICATIONS

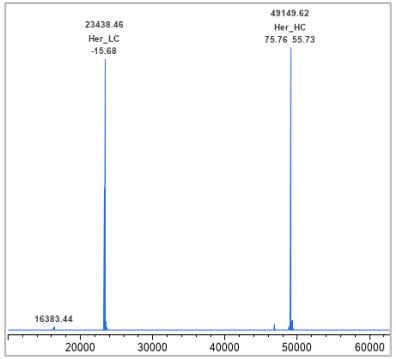
Subunit Analysis

FOCUS: Heavy chain and light chain.

Suggested initial settings:

Sample type:	Protein reduced to HC and LC
Recommended workflow	Intact_AutomatedDeconvolution Intact_TimeResolvedDeconvolution (for fragments with complex modifications).
Deconvolution range [1]	20 kDa - 60 kDa or 20 kDa - 110 kDa to identify partially connected subunits. **Visible Ranges: All Ranges** particularly when subunits are unseparated.
Mass step [1]	1 Da - 2 Da
RT Ranges [1] (Ignore with TRD)	**RT Window**: 3 - 9 **Isolation Threshold**: 3 - 10 (depends on the separation between components). For higher sensitivity and better efficiency manually specify all ranges.
Mass Tolerance [2]	10 ppm - 20 ppm (depends on calibration accuracy).
Glycosylation [2]	**Deglycosylated** or **Glycosylated** with library selection.
Disulfide [2]	Separate subunits - **State: Fully Reduced**. Partially connected subunits - **State: Partially Reduced, Connectivity: IgG**. Optional: Search for reduced intrachain bonds.

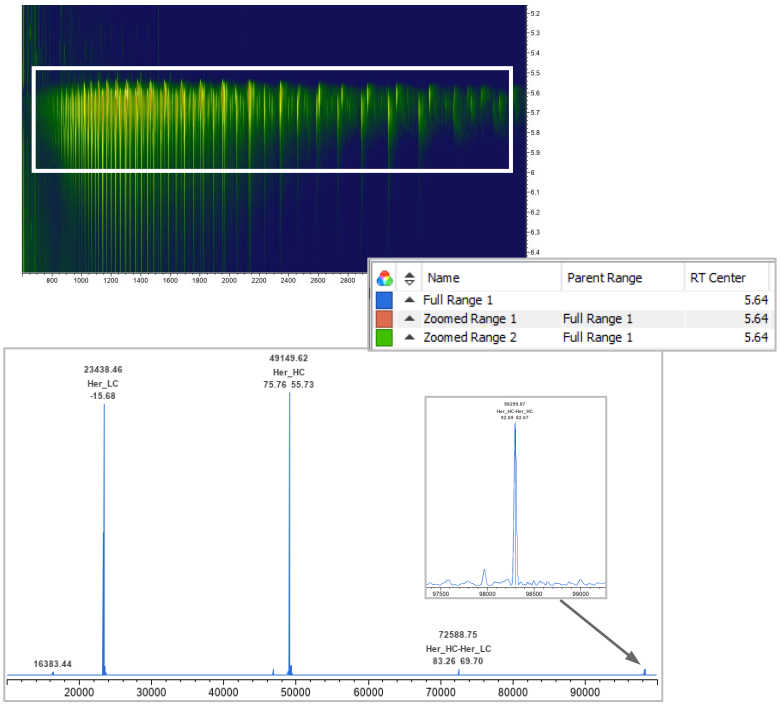
[1]Deconvolution, [2]Protein Mapping



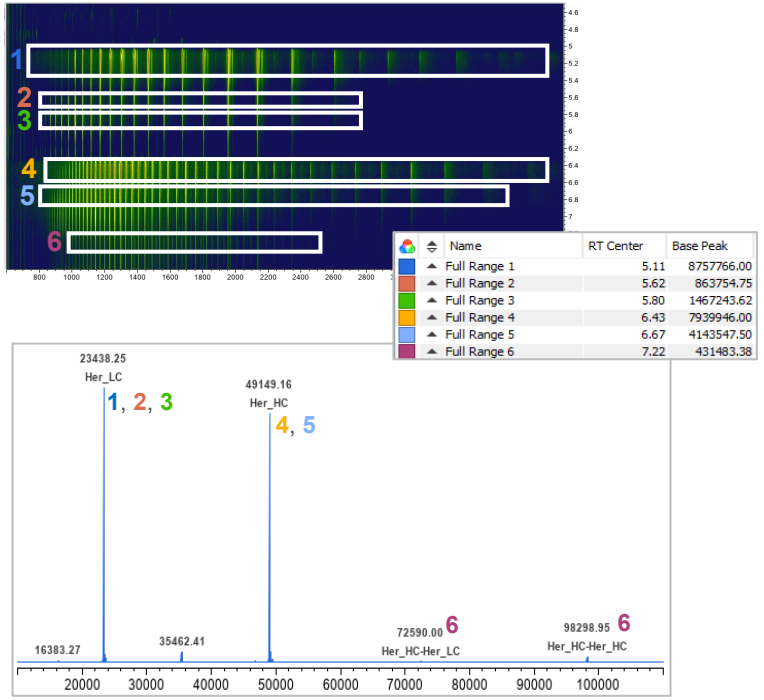
Subunit Analysis

FOCUS: Heavy chain and light chain.

Fully reduced



Partially connected



Fragment Analysis

REFINED SETTINGS FOR SPECIFIC APPLICATIONS

Fragment Analysis

FOCUS: IdeS digested protein, with or without further reduction.

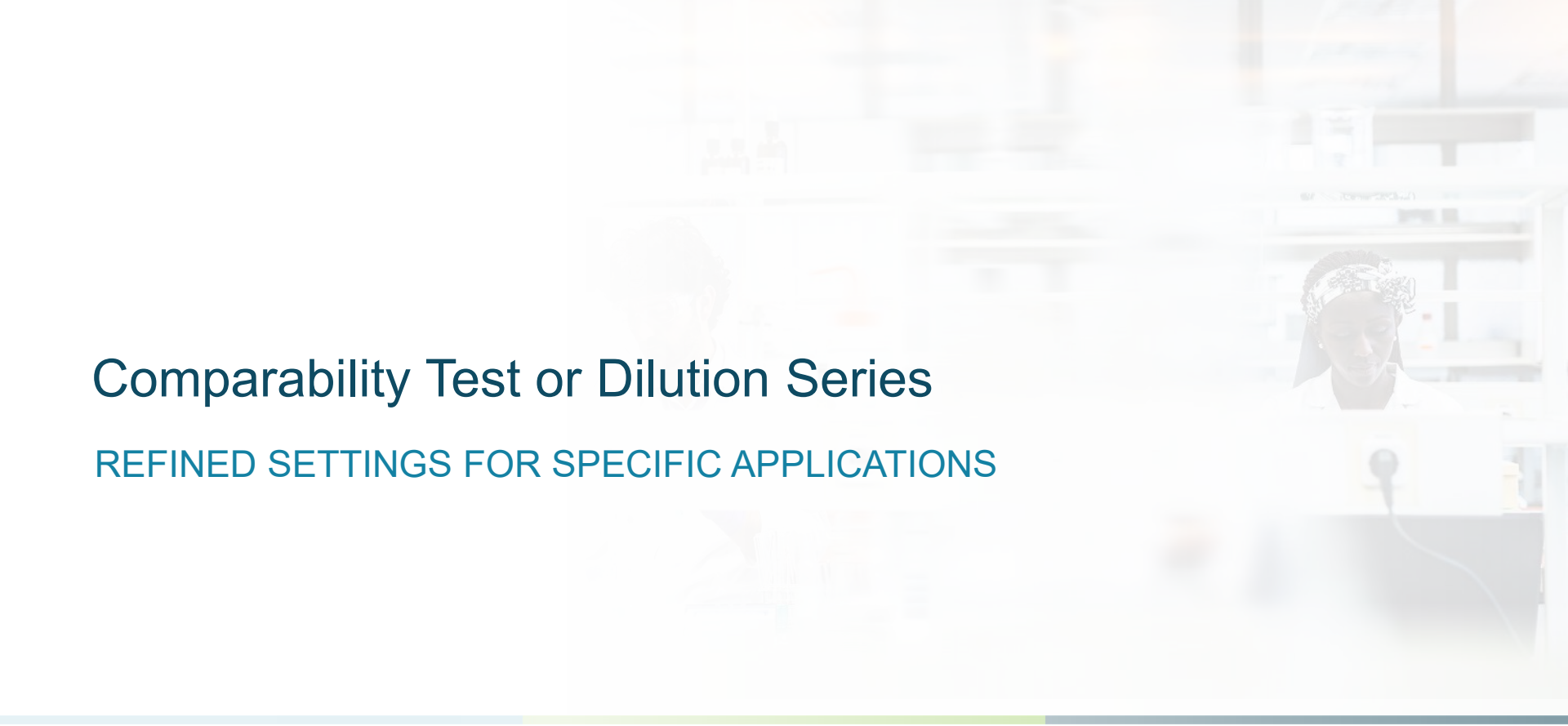
Suggested initial settings:

Sample type:	F(ab')2 and ScFc, or, LC, Fd' and ScFc
Recommended workflow	Intact_AutomatedDeconvolution Intact_TimeResolvedDeconvolution (for fragments with complex modifications).
Deconvolution range [1]	20 kDa - 110 kDa or 20 kDa - 30 kDa with reduced fragments **Visible Ranges: Only Zoomed Ranges** or **All Ranges** for better visualization (even if fragments are coeluting).
Mass step [1]	1 Da - 2 Da
RT Ranges [1] (Ignore with TRD)	**RT Window**: 3 - 9 **Isolation Threshold**: 3 - 10
Mass Tolerance [2]	10 ppm - 20 ppm (depends on the calibration accuracy).
Glycosylation [2]	**Deglycosylated** or **Glycosylated** with library selection.
Disulfide [2]	**State: Fully Connected**. **Connectivity: Unspecified + 3 Additional chains**. **Free Cysteines** for unpaired cysteines. **State: Fully Reduced** for reduced fragments.
Sequence	Each fragment sequence (Fc/2, LC, and Fd') must be listed separately.

[1]Deconvolution, [2]Protein Mapping

Comparability Test or Dilution Series

REFINED SETTINGS FOR SPECIFIC APPLICATIONS

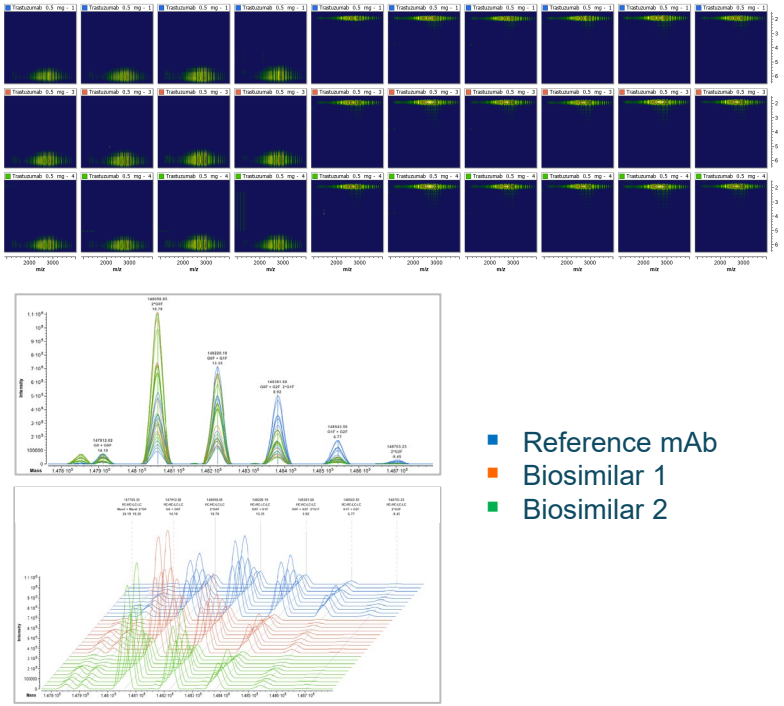


Comparability Test

Suggested initial settings:

Sample type:	Whole protein
Recommended workflow	Intact_AutomatedDeconvolution
Deconvolution range [1]	140 kDa - 160 kDa **Visible Ranges: Only Full Ranges** for visualization and reporting.
Mass step [1]	2 Da
RT Ranges [1]	Manually specify a single RT range that contains all of the target protein signals.
Mass Tolerance [2]	20 ppm - 50 ppm (depends on the resolving power used).
Glycosylation [2]	**Deglycosylated** or **Glycosylated** with library selection.
Disulfide [2]	**State: Fully Connected**. **Connectivity: IgG** (if applicable, otherwise specify).
Notes	For consistent peak annotations and quantification, create a library from reviewed results to use in a *Targeted Mass Search*.

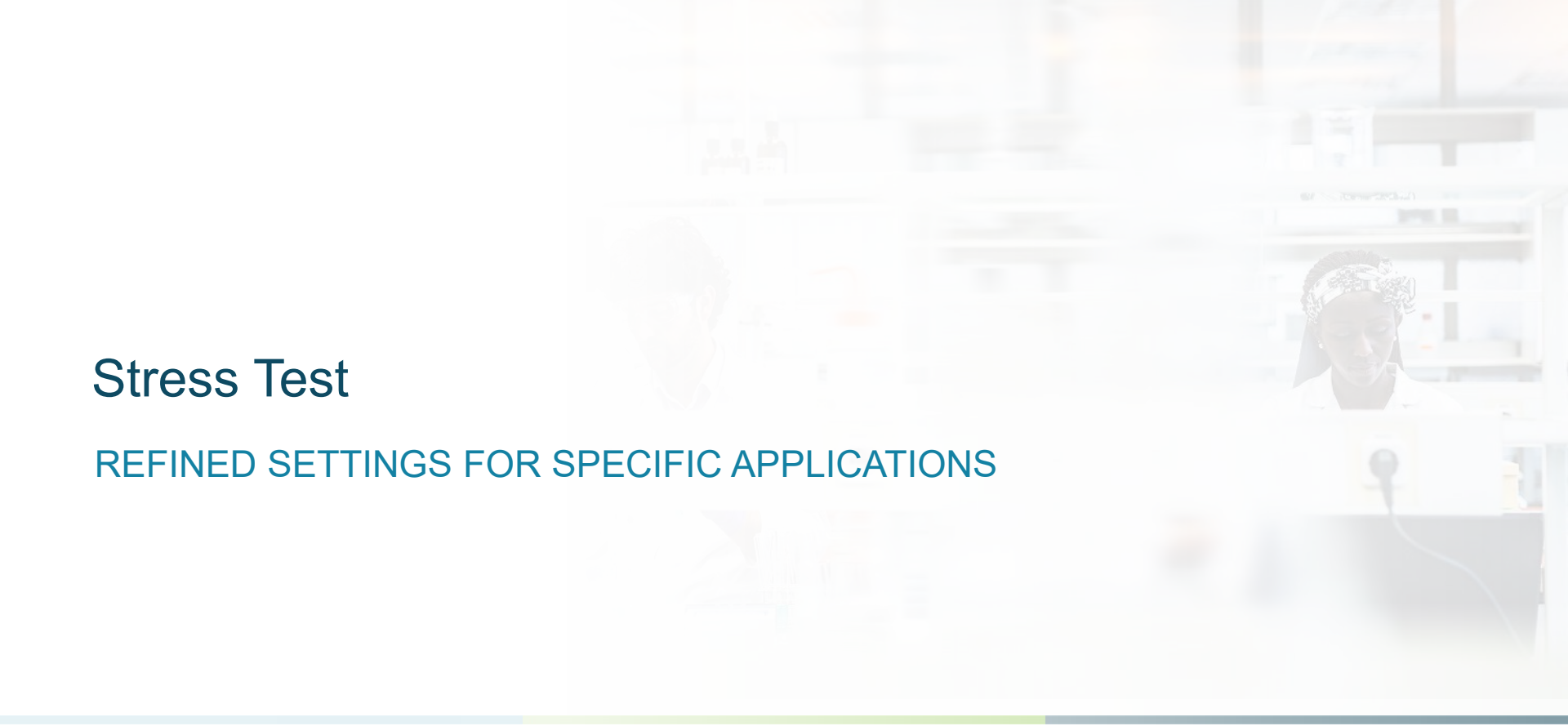
[1]Deconvolution, [2]Protein Mapping



- Reference mAb
- Biosimilar 1
- Biosimilar 2

Stress Test

REFINED SETTINGS FOR SPECIFIC APPLICATIONS

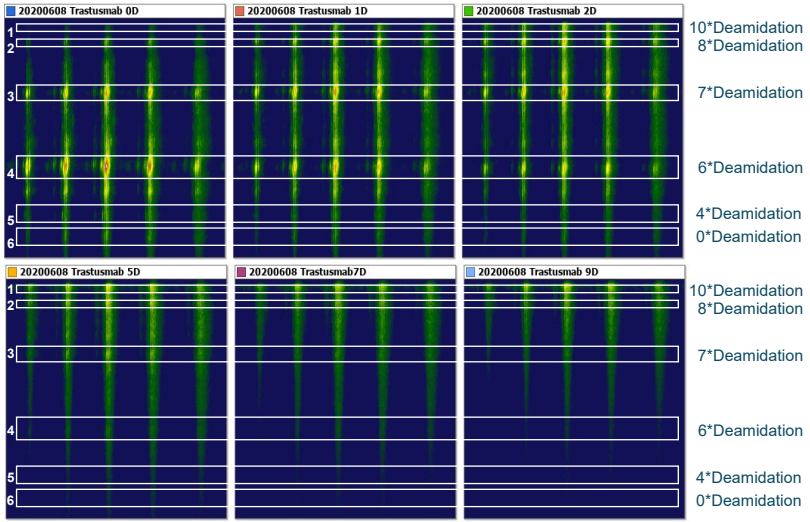


Stress Test

Suggested initial settings:

Sample type:	Whole protein
Recommended workflow	Intact_AutomatedDeconvolution
Deconvolution range [1]	140 kDa - 160 kDa **Visible Ranges: Only Full Ranges** for visualization and reporting.
Mass step [1]	2 Da
RT Ranges [1]	Manually input all RT ranges for common peak detection across all samples and for meaningful relative quantitation.
Mass Tolerance [2]	20 ppm - 50 ppm (depends on the resolving power used).
Glycosylation [2]	**Deglycosylated** or **Glycosylated** with library selection.
Disulfide [2]	**State: Fully Connected**. **Connectivity: IgG** (if applicable, otherwise specify).
Notes	For consistent peak annotations and quantification, create a library from reviewed results to use in a *Targeted Mass Search*.

[1]Deconvolution, [2]Protein Mapping



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