Intact Mass Analysis

Biologics Explorer 2.0 Quick Guide

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Intact Mass Analysis: Biologics Explorer Quick Guide

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- Denatured Intact Protein
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- Antibody Drug Conjugates
- Subunit Analysis
- Fragment Analysis
- Comparability Test or Dilution Series
- Stress Test

Part A

Software and Workflows

1. OVERVIEW OF APPLICATIONS

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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:
  - Screening of multiple samples (process development, instrument method development)
  - Lot-to-lot comparability studies
  - Innovator *vs.* biosimilars 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

Part A

Software and Workflows

2. USING BIOLOGICS EXPLORER

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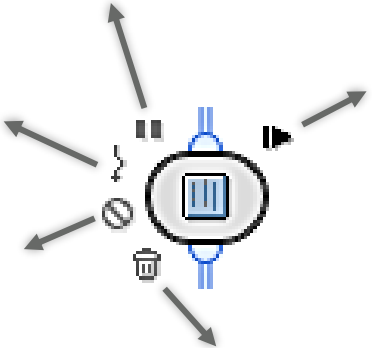
Using Biologics Explorer

ACTIVITY NODE ICONS

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

Bypass: Skip this task
when running the workflow.



Run
or **Reset**.

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

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

Using Biologics Explorer

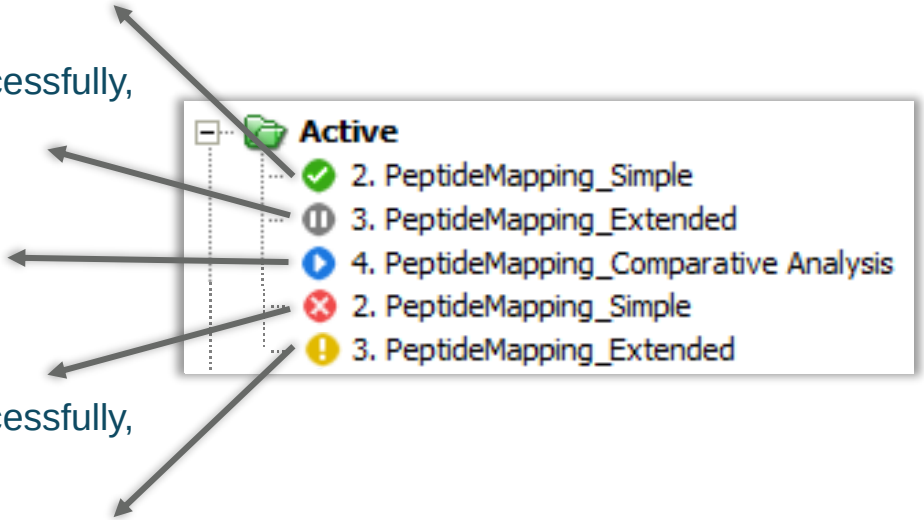
WORKFLOW ICONS

Workflow Completed
All activity nodes have completed successfully.

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

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

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



Workflow Warning
Some activity nodes are incomplete.

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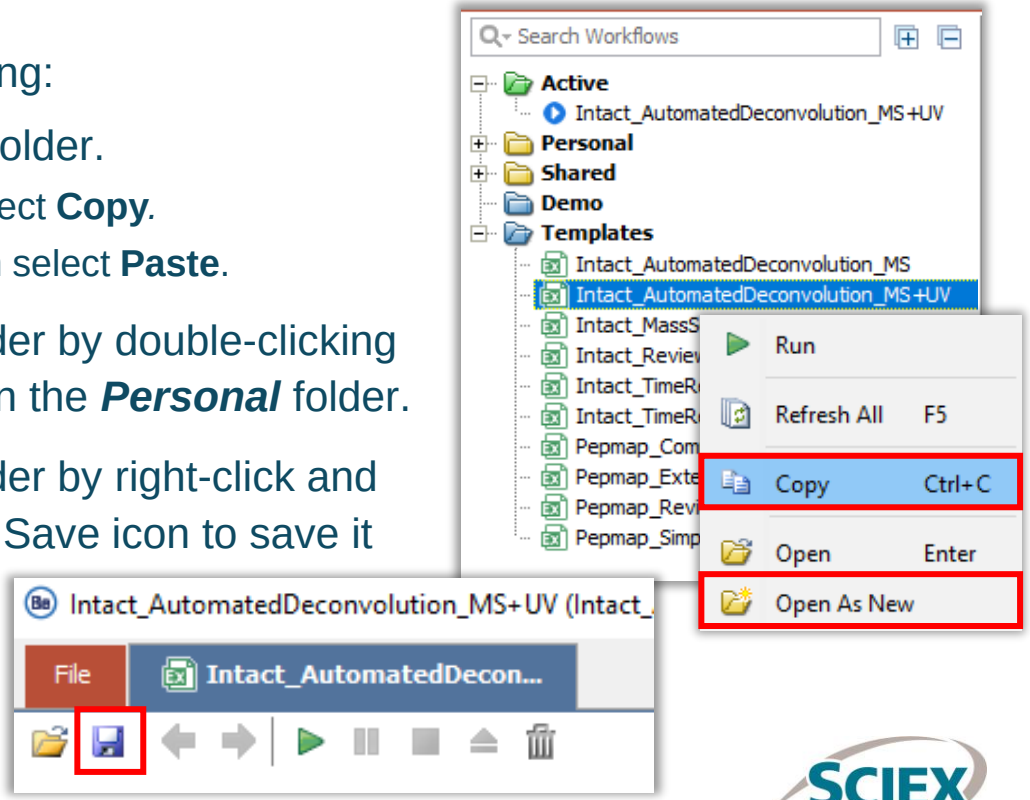
Using Biologics Explorer

START AND SAVE WORKFLOWS

To open a workflow, do one of the following:

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



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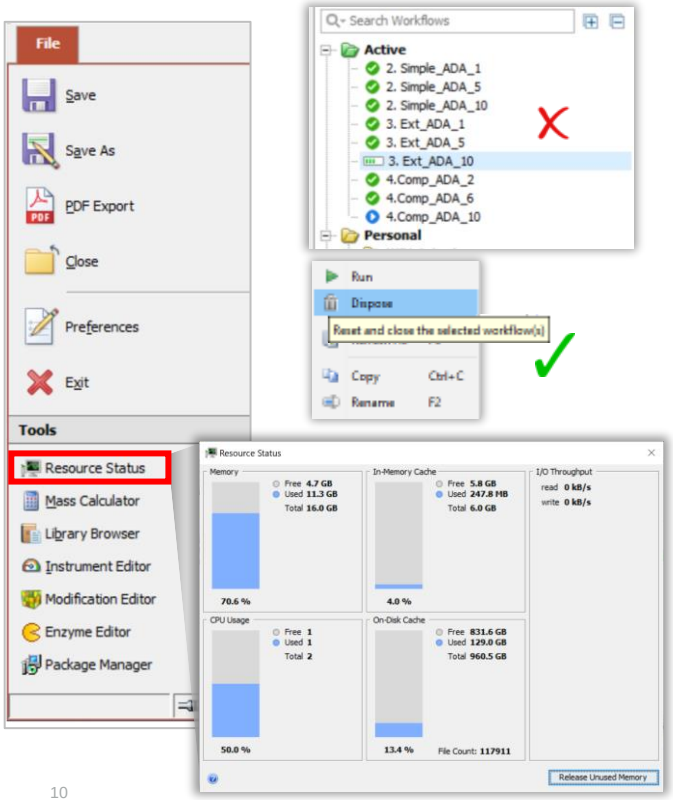
RECOMMENDATIONS FOR CORRECT USE OF THE RESOURCES



- Follow best practices to make sure that Biologics Explorer has sufficient memory and computing power:
  - Only run one workflow at a time: Some activity nodes are very resource intensive. Co-processing might use up all available resources.
  - To conserve memory, activate the Trash icon whenever possible in optimized workflows.
  - After reviewing data and saving results, reset or dispose workflows before starting a new analysis.
  - Use *Save Snapshot* activity nodes to enable completed results to be saved or reviewed in the Intact_ReviewSnapshots workflow.
- The processing computer should have at least 250 GB of free disk space and 6 GB of In-Memory Cache.
  - Files being processed for intact protein workflows should not add up to more than 12 GB.

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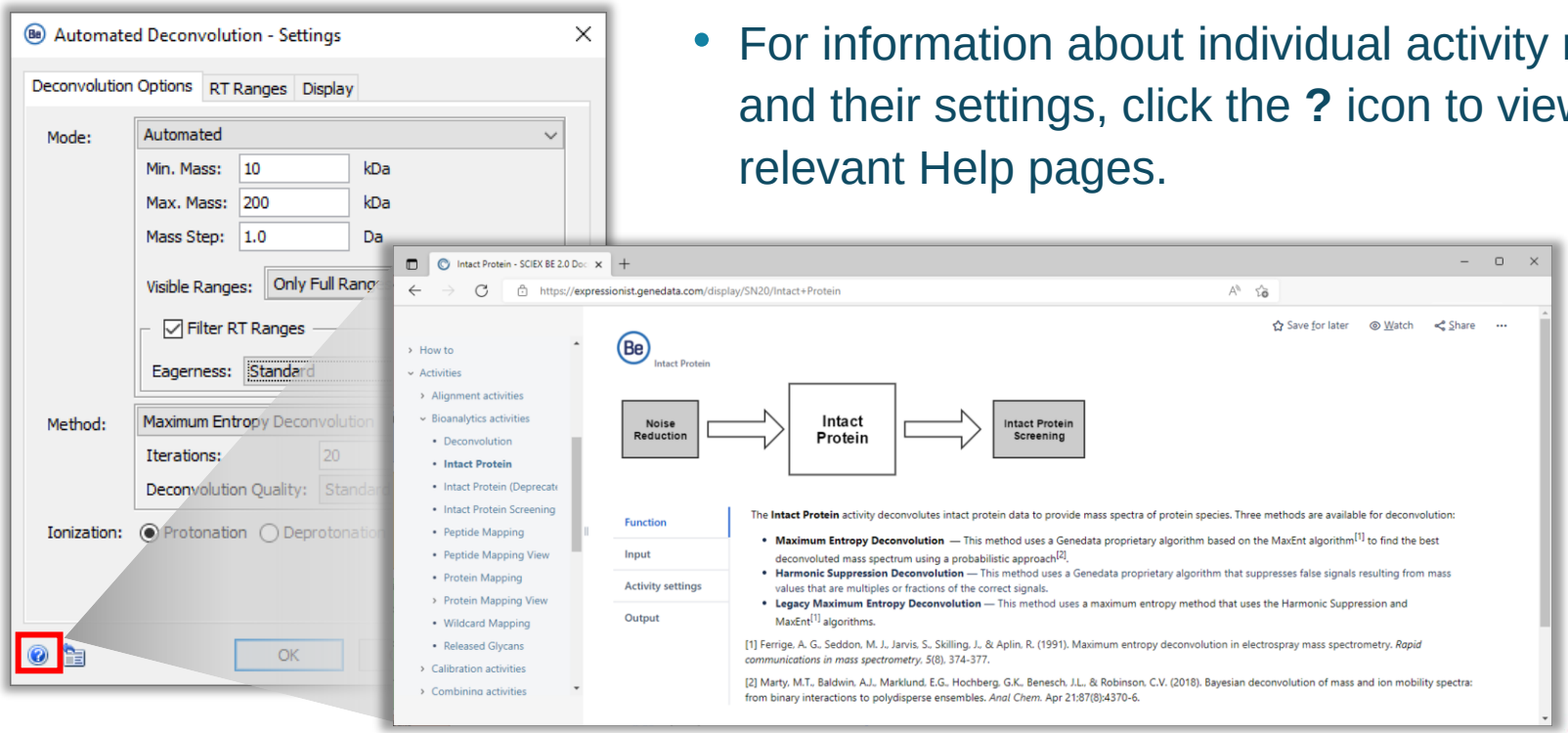
Using Biologics Explorer

ACCESS THE ONLINE HELP



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

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Part A

Software and Workflows

3. GENERAL GUIDELINES FOR INTACT WORKFLOWS

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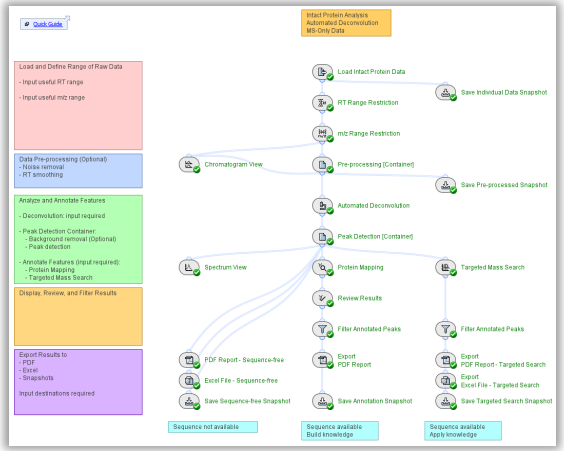
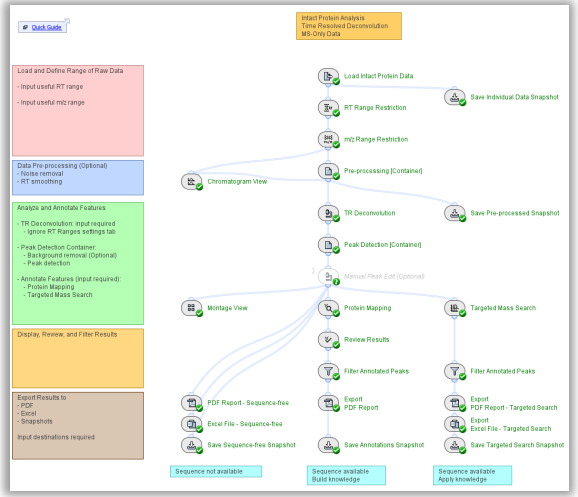
General Intact Workflow Guidelines

WORKFLOW TYPES



Intact_Automated Deconvolution

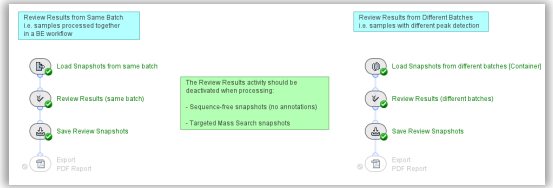
With and without UV processing



Intact_TimeResolved Deconvolution

With and without UV processing

Intact_MassScreening



Intact_ReviewSnapshots

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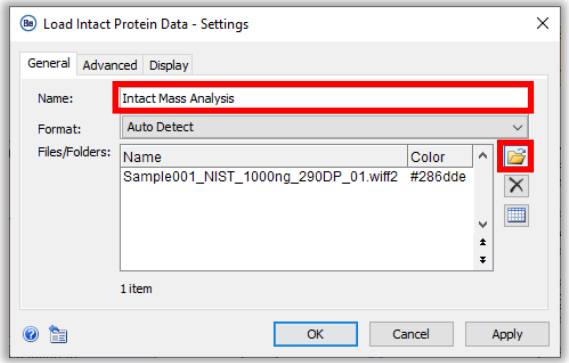
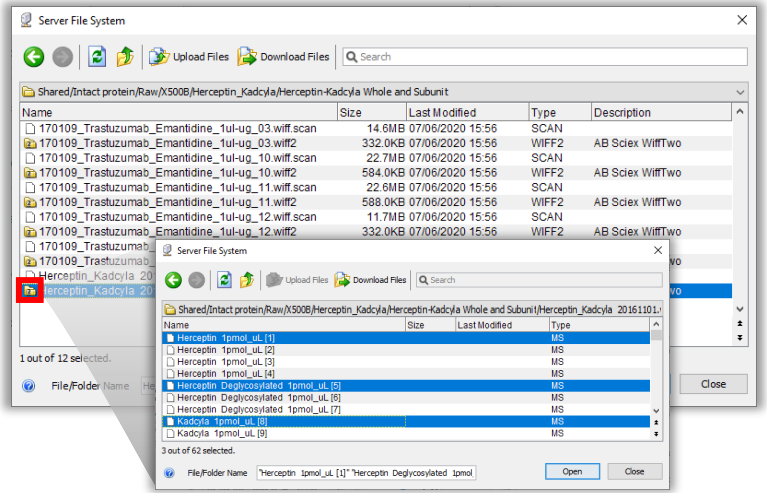
General Intact Workflow Guidelines

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

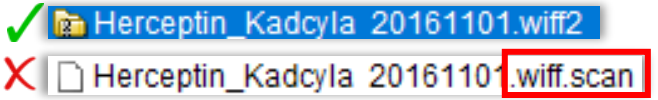
Load Intact Protein Data: Format (Auto Detect)





General tab.

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



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

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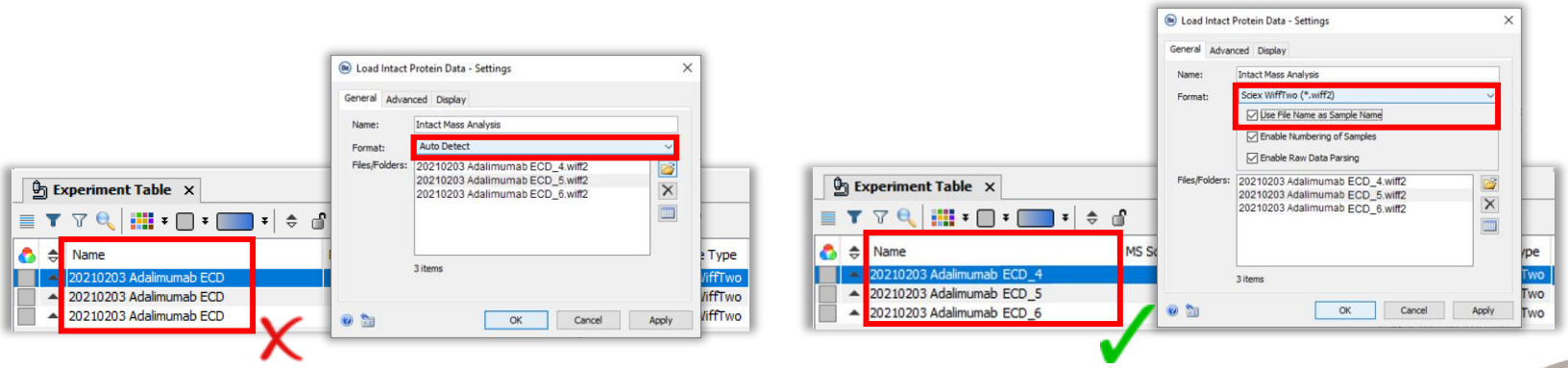
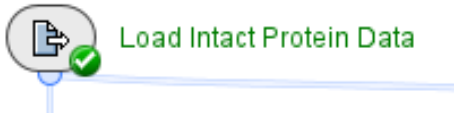
Load Intact Protein Data: Format

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

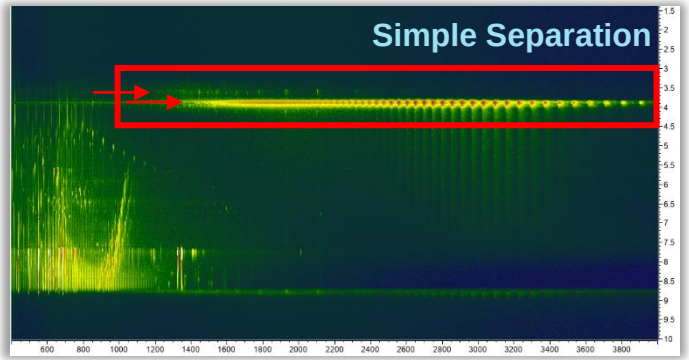


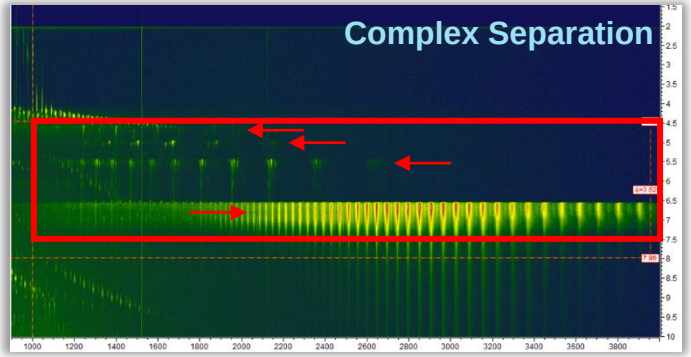
Restriction of RT and *m/z* Ranges

- Run the *Load Intact Protein Data* activity node and then open (double-click) it when data loading is complete.



- Identify the retention time (RT) and mass-to-charge ratio *(m/z)* ranges where there is meaningful data present.
  - Exclude stray signals caused by valve switching or column wash.
  - Focus on the separation range.
  - Unless minor components or contaminants at lower masses are of interest, limit the analysis to the target protein elution range.





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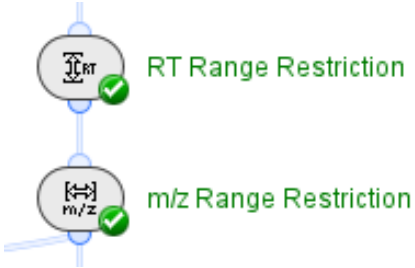
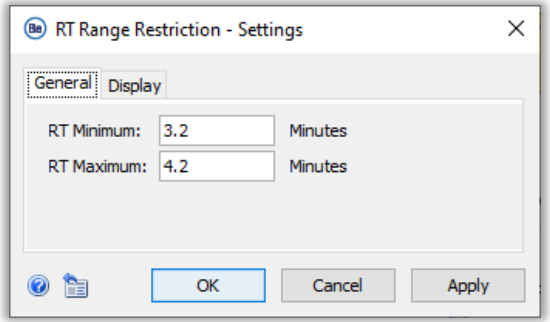
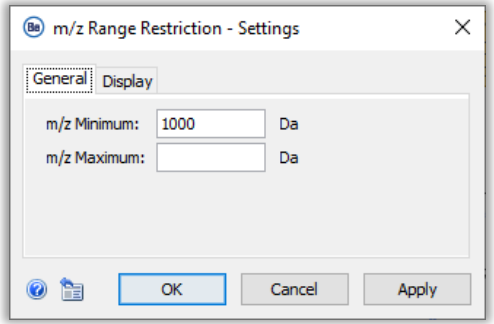
Restriction of RT and *m/z* Ranges



- Type the identified useful RT and *m/z* ranges.
  - When input boxes are left empty, the full RT or *m/z* range is used.



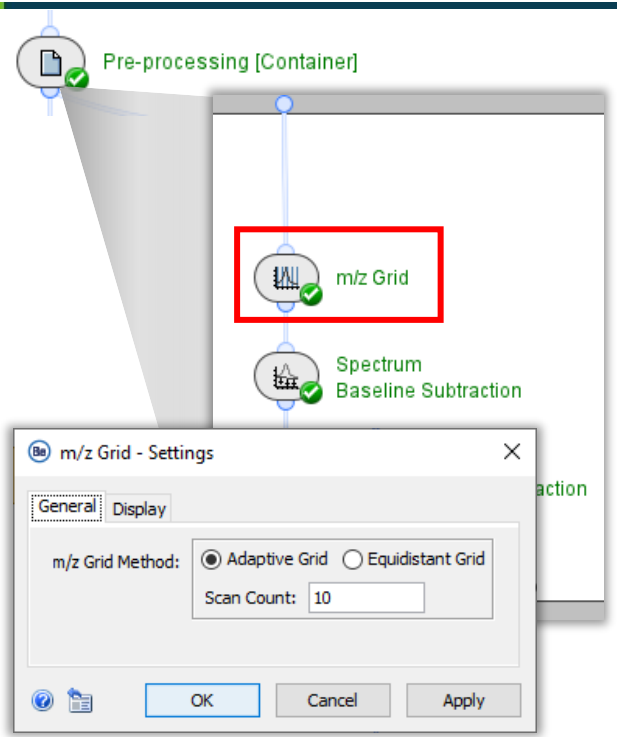


m/z Grid

- Use the *m/z Grid* activity node when working with multiple data files to make sure 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.



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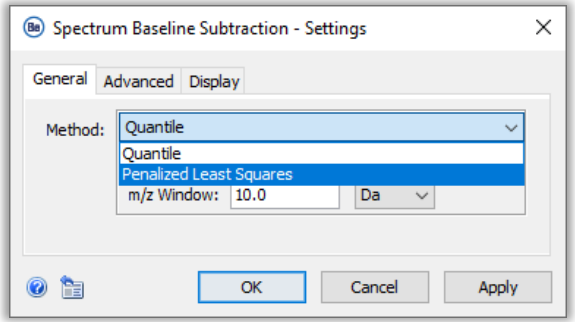
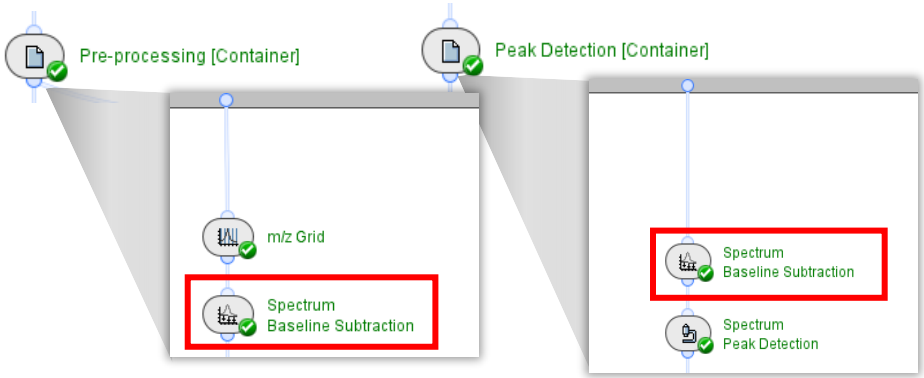
Spectrum Baseline Subtraction

- *Spectrum Baseline Subtraction* improves deconvolution by removing background noise and limiting the production of satellite peaks in the deconvoluted data.
- This activity node can also be used after deconvolution to improve peak detection.

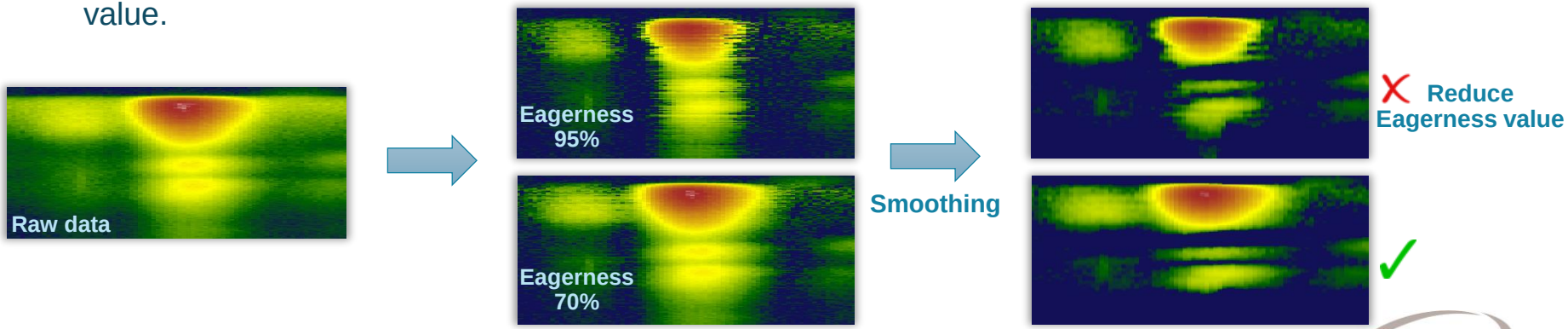




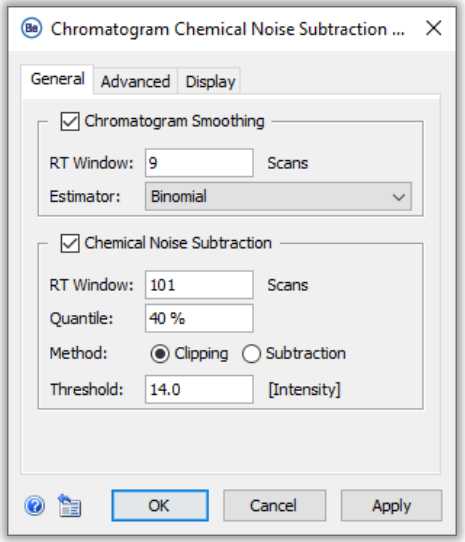
- **Quantile** subtraction affects all signals:
  - It requires little or no smoothing afterwards.
  - It is much faster than **Penalized Least Squares** when used with high resolution data.
  - It should be used with care during the analysis of intact whole proteins, to avoid removing meaningful signals.
- **Penalized Least Squares** subtraction affects low intensity signals only.

Spectrum Baseline Subtraction

- **Penalized Least Squares** reduces valley height between large peaks, and so reduces satellite peaks in deconvoluted spectra.
  - It can be time intensive, particularly when used with **Time Resolved Deconvolution** and with higher resolution data (such as subunits and fragments).
- High **Eagerness** values (greater than 90%) require extensive **Smoothing** in the *Chromatogram Chemical Noise Subtraction* activity node.
  - If features in the heat map continue to display irregular borders after smoothing, reduce the **Eagerness** value.



Chromatogram Chemical Noise Subtraction

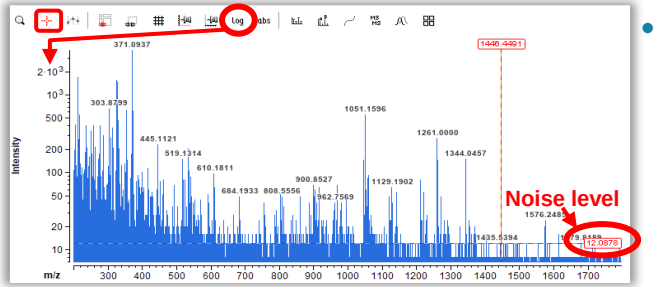


Chromatogram Smoothing is used to improve the RT profile of peaks.

- Smoothing is necessary after **Penalized Least Squares** (in *Spectrum Baseline Subtraction*) particularly if a high **Eagerness** value was used.
- **Estimator:**
  - **Moving Average** generates additional data points using the mean intensity of data points in the **RT Window**. High values will therefore cause peak widths to increase, but peak volume is conserved.
  - **Binomial** is an iterative form of **Moving Average** that has less impact on peak widths.

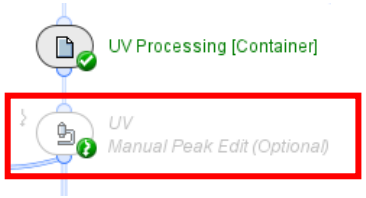
Chemical Noise Subtraction can help to:

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

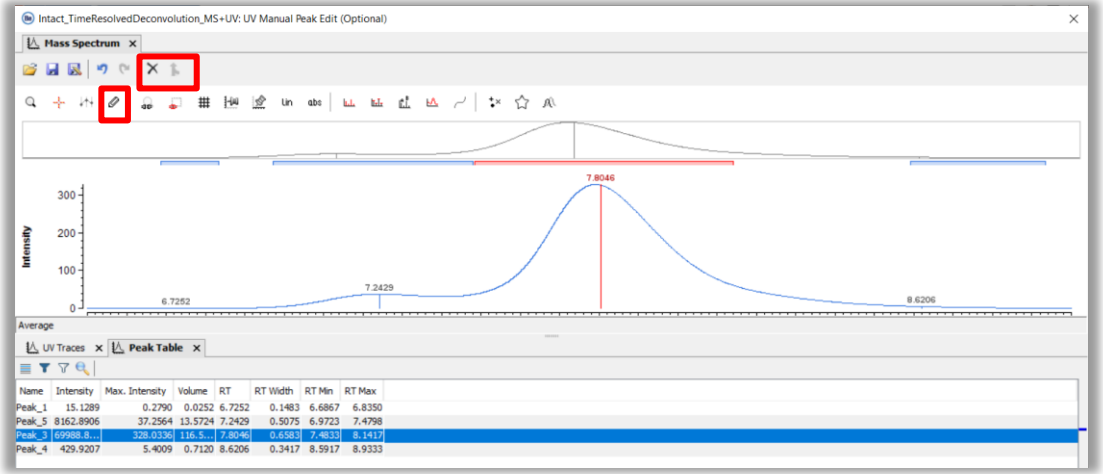


- Settings:
  - **RT Window:** Increase the value to subtract less data.
  - **Quantile:** Decrease the value to subtract less data.
  - **Threshold:** Inspect the noise level to determine an appropriate intensity value.
    1. Expand the mass spectrum intensity axis by dragging it until the noise level is readable, or change the axis from linear to the log scale using the icon in the tool bar.
    2. Use the crosshair tool to measure the intensity of the noise level.

UV Processing: *UV Manual Peak Edit*



- This activity is bypassed by default.
- Use *UV Manual Peak Edit* to manually refine the peak detection within the UV chromatogram by:
  - Changing the boundaries of peaks of interest.
  - Splitting overlapping peaks.
  - Deleting undesired peaks.
  - Drawing new peaks.



- *UV Manual Peak Edit* can be a convenient alternative to optimizing *UV Peak Detection* parameters for difficult peaks.

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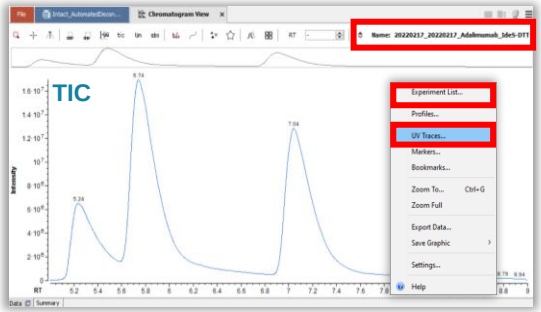
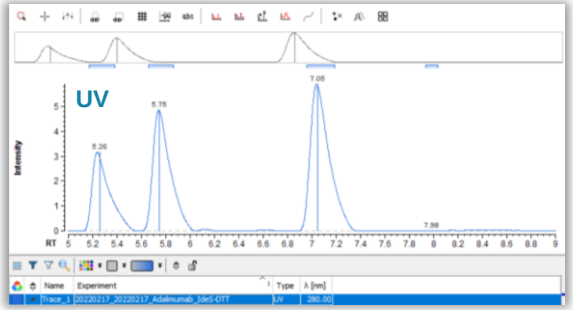
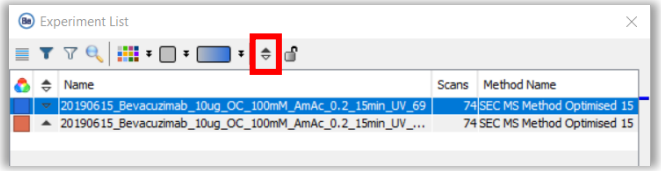
Chromatogram View

- *Chromatogram View* visualizes the TIC chromatograms of each sample, before and after pre-processing.
- The **Experiment List** and **UV Chromatograms** from before and after *UV Processing* can be accessed by right-clicking on the plot.





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



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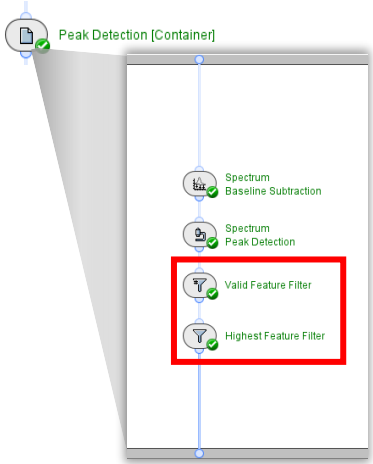
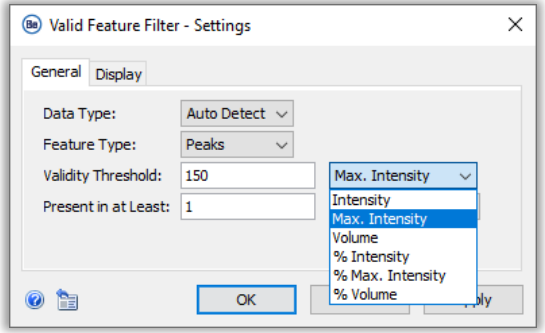
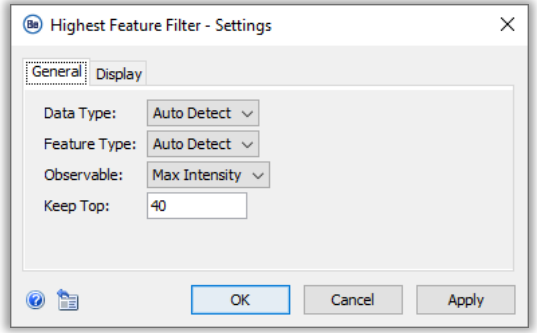
Feature Filters

- The *Feature Filter* activity nodes can be used to limit how many peaks are included, so that only the most relevant peaks are kept and those originating from noise are excluded.
  - Use the **Bypass** icon to restrict use if filtering peaks is not required.







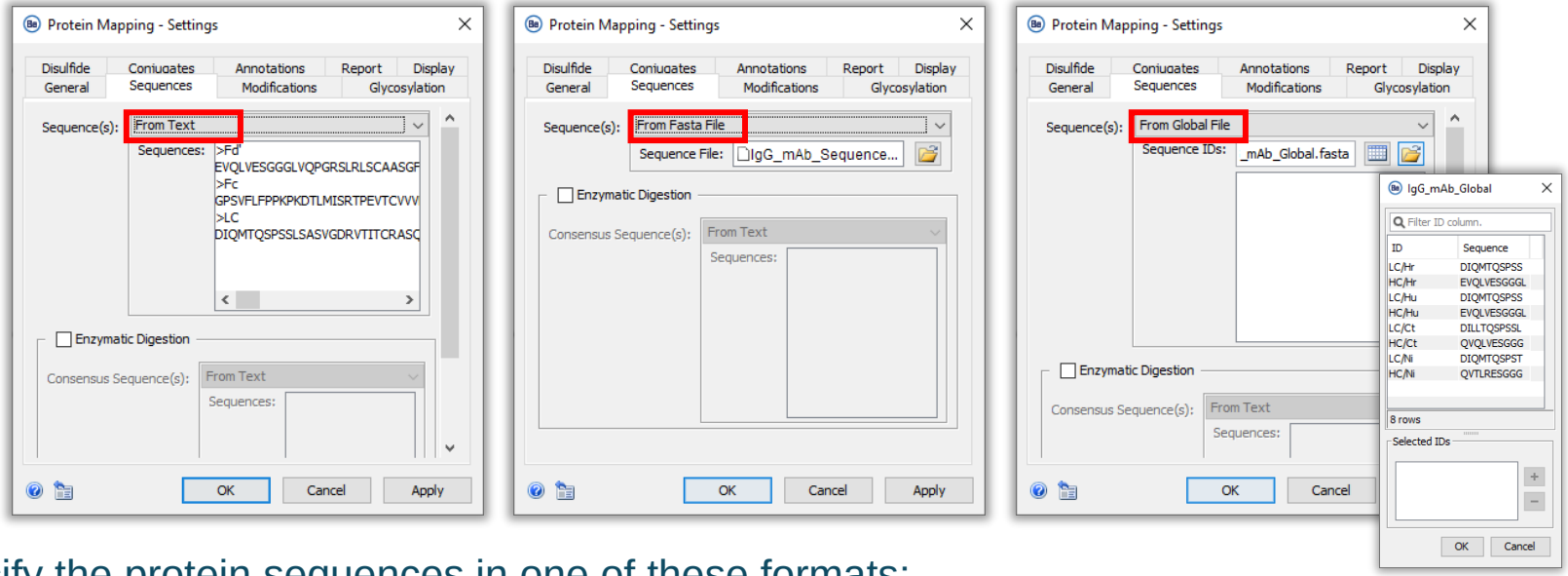
Valid Feature Filter

- Defines the minimum peak intensity and percentage of experiments that each peak must be present in to be included.

Highest Feature Filter

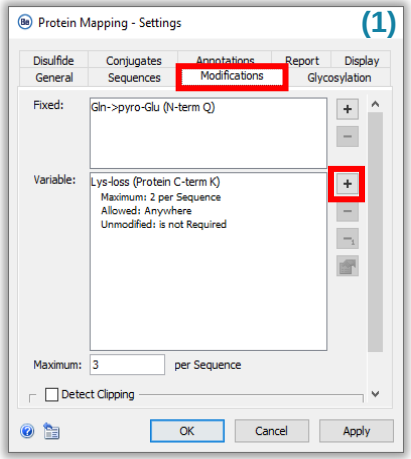
- Defines how many of the most abundant peaks will be reported in each identified RT range.

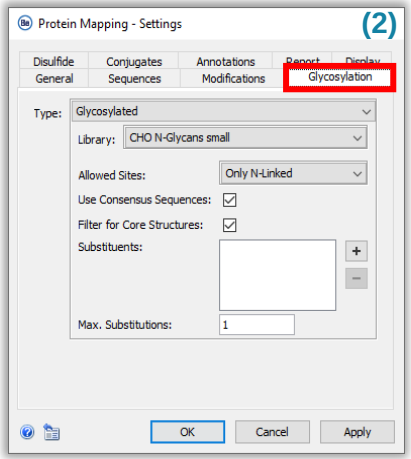
Protein Mapping: Sequences

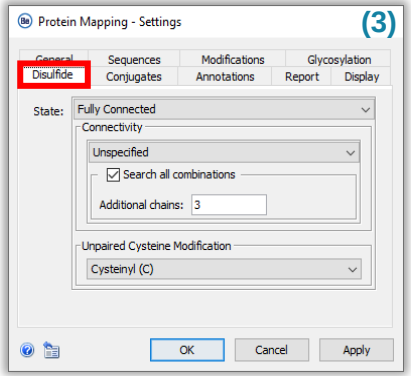


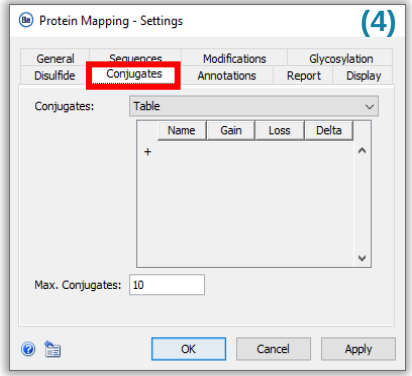
- Specify the protein sequences in one of these formats:
  - **From Text**: Specify the protein sequence in the **Sequences** box.
  - **From Fasta File**: Upload a fasta file containing sequence of interest.
  - **From Global File**: Upload a fasta file with multiple entries, from which the sequences to use can be selected from the additional pop-up window.

Protein Mapping: Modifications



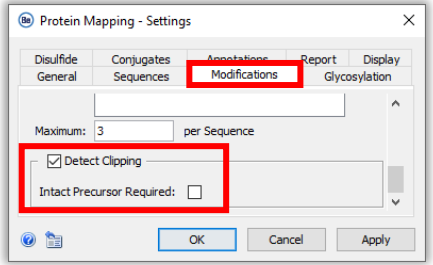


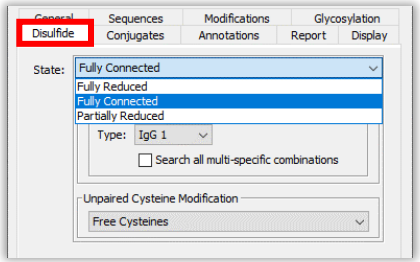


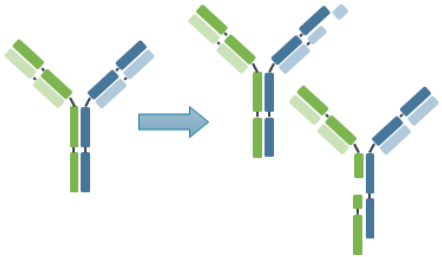


1. Specify **Fixed** and **Variable** PTMs.
   - With protein sequences carrying N-term E, it is recommended to set Glu-pyroGlu (N-term E) as a variable modification, because the modified form is not expected to be predominant.
2. Specify glycosylation parameters.
   - Set the **Type** to **Glycosylated**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

Protein Mapping: Detect Clipping







- The **Detect Clipping** functionality matches masses for potential (*in silico*) clipping events, with peaks detected in the data.
- Select **Detect Clipping** in the **Modifications** tab to identify MS peaks originating from a single clipping event at any sequence position.
  - This setting requires disulfide bonds to be **Fully Connected** or **Fully Reduced**. (Analysis of partially reduced samples is not supported).
  - The **Detect Clipping** functionality cannot be used in conjunction with the **Enzymatic Digestion** functionality on the **Sequences** tab.
- The number of combinatorial possibilities within the defined search space should be constrained. Too many variables will result in:
  - ⚠ Extended workflow processing times or workflows that are unable to run to completion.
  - ⚠ High levels of false positive identifications, requiring excessive data review.

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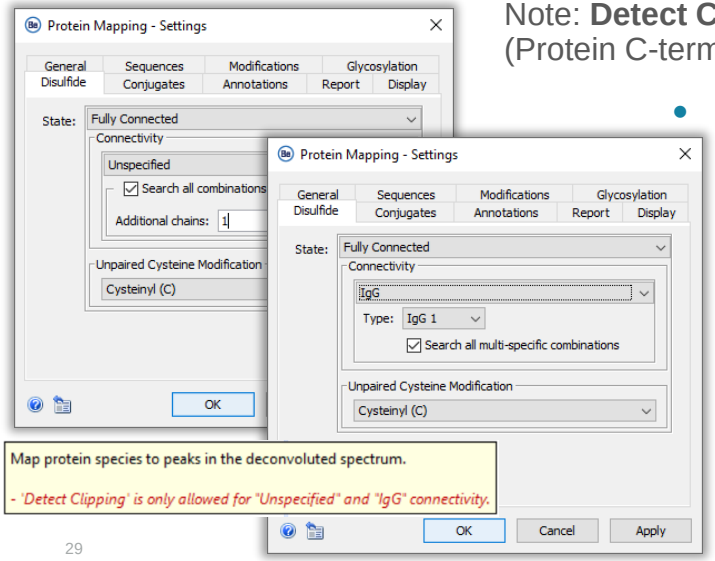
Protein Mapping: Detect Clipping Recommendations

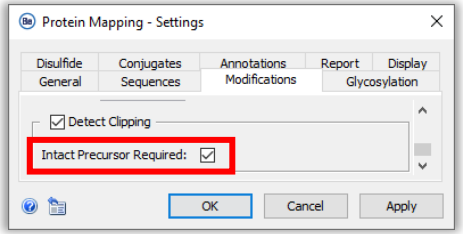
- General guidance to constrain the search space by focusing on specific peaks of interest:
  - Use *Feature Filter* activity nodes to remove peaks that are unlikely to be derived from a clipping event.
  - Use *Manual Peak Edit* in TRD workflows to delete peaks that are unlikely to be from clipping events.
  - Only search for modifications that are known to be present and 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.

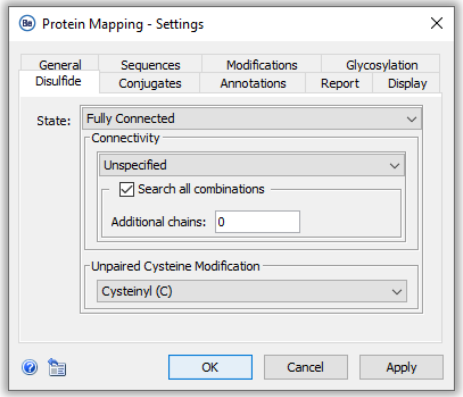


Note: **Detect Clipping** will report C-terminal lysine loss as a duplicate annotation if Lys-Loss (Protein C-term K) is also included as a modification in the *Protein Mapping* search.

- Combining **Detect Clipping** with **Disulfide** tab settings:
  - To detect clipped species originating from disulfide-linked proteins, select **Fully Connected** with **Unspecified** or **IgG** connectivity.
    - If **Intact Precursor Required** is selected, **Additional chains** should be set to 0 when **Search all combinations** is selected.
    - If **Intact Precursor Required** is <u>not</u> selected, **Additional chains** can be set to 0 or 1 when **Search all combinations** is selected.
  - To identify clips of fragments from a reducing IdeS digest, it is recommended to investigate each fragment individually.

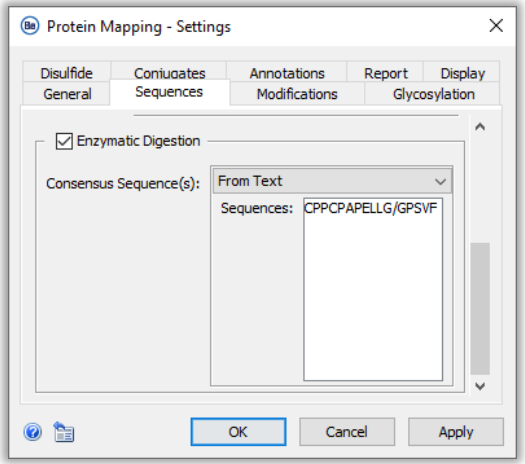
Protein Mapping: Detect Clipping - Intact Precursor Required

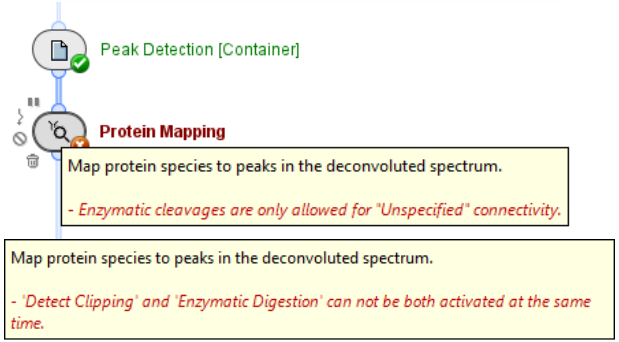




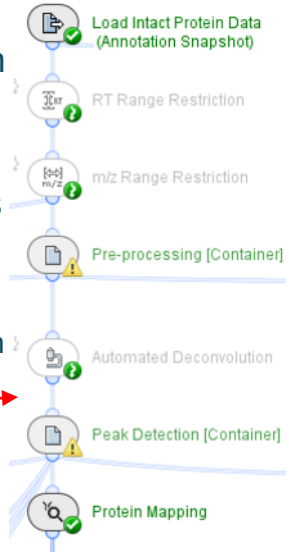
- If **Intact Precursor Required** is selected, clipped proteins containing modifications are only identified if an unclipped form carrying the same modifications is also identified in the data.
- When **Intact Precursor Required** is selected, the search time increases but false positives are reduced.
  - The algorithm confirms that all identified clipped forms are matched to a full-length unclipped form.
  - Any clipped forms without an unclipped match are rejected.
- Combining **Intact Precursor Required** with **Disulfide** tab settings:
  - Only use **Intact Precursor Required** with one of the following:
    - **Connectivity: IgG. Search all multi-specific combinations** can be selected if required.
    - **Connectivity: Unspecified. Search all combinations** can be selected, with **0 Additional chains**.

Protein Mapping: Enzymatic Cleavage





- Enzymatic cleavage is a sample preparation strategy that generates protein subunits for MS analysis.
- Select **Enzymatic Digestion** and provide a **Consensus Sequence** to define the cleavage point for protein mapping.
  - 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.
    - To achieve this, run the workflow sequentially. The output from *Save Annotations Snapshot* can be re-analyzed with any unrequired activity nodes bypassed.
- When using **Enzymatic Digestion**:
  - Select **Unspecified** connectivity on the **Disulfide** tab.
  - Do not select **Detect Clipping** on the **Modifications** tab.



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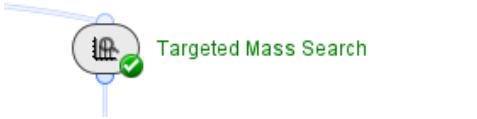
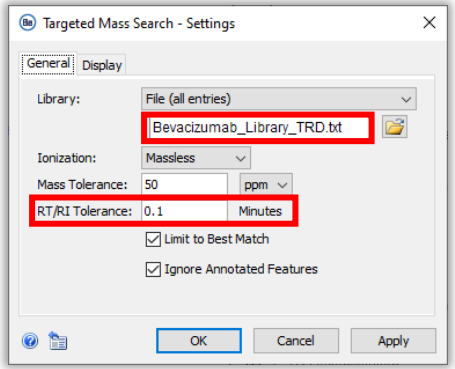
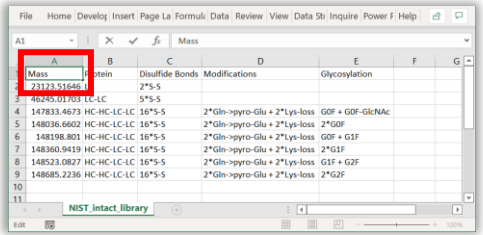
Targeted Mass Search

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

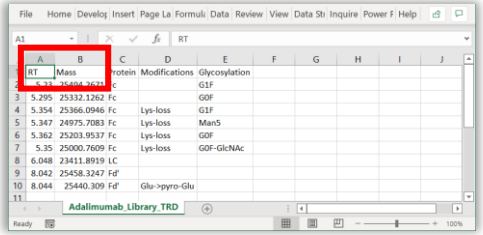




- Format for **Automated Deconvolution** workflows:
  - The first column (Mass) is mandatory.



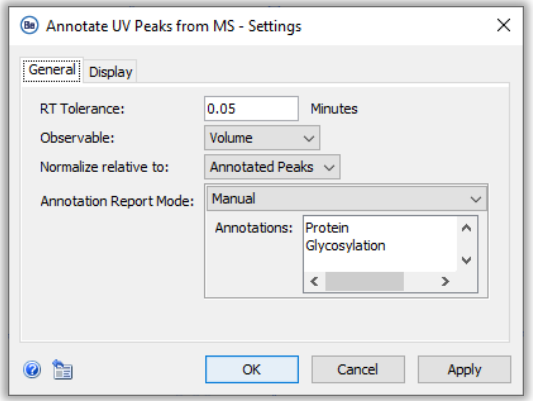
- Format for **Time Resolved Deconvolution** workflows:
  - The first two columns (RT and Mass) are mandatory.

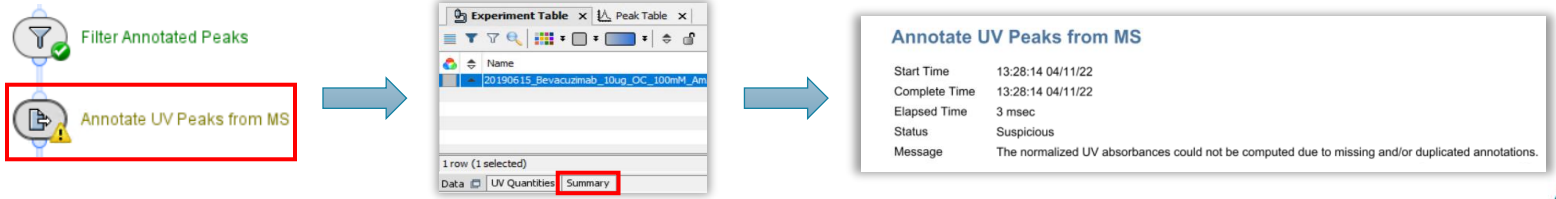


- Remove unrequired columns, or insert columns to provide additional information, as required.
- Adjust **RT/RI Tolerance** to account for chromatographic shifts.

Annotate UV Peaks from MS

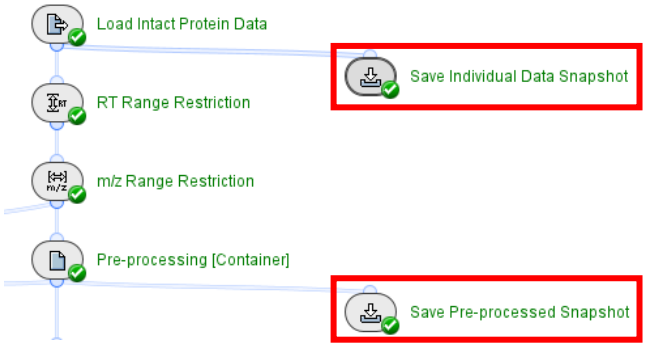
- This activity uses MS peak information to annotate the corresponding peaks in the UV trace.
  - A corresponding peak must elute within the specified **RT Tolerance**.
- The relative ratio of the UV peaks is also calculated, based on UV absorbance.
  - A protein sequence is required to normalize UV absorbance values.
  - If the UV normalization factor cannot be computed, 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.

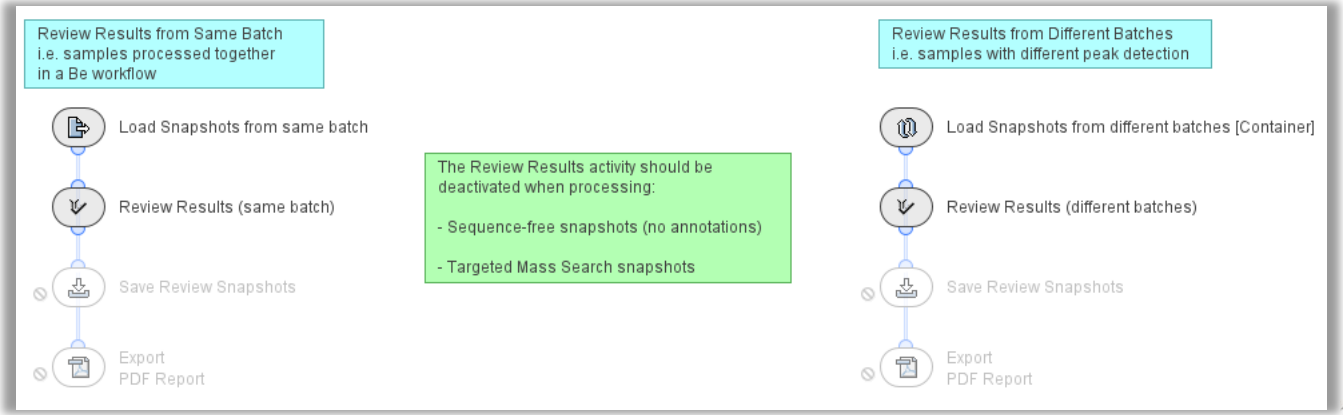




Reporting: *Save Snapshots*

- Store intermediate results after different stages throughout the workflows.
  - *Save Snapshot* activity nodes create a file (sbf) that contains the required properties of the processed data.
- To review the stored data, open the saved sbf files in the *Load Snapshots* activity nodes in the Intact_ReviewSnapshots workflows.





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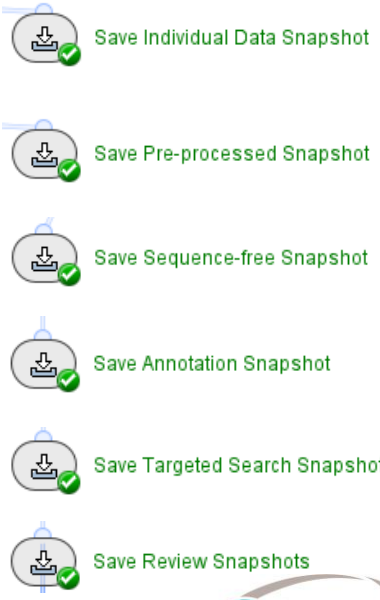
Reporting: *Save Snapshots*

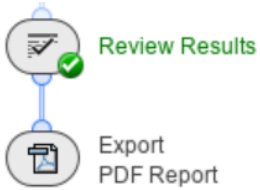
- Select or create the folders where results will be stored.
  - The default location is: C:\ProgramData\Sciex\BiologicsExplorer\data\data\user

Options for saving 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*: Saved after *Peak Detection*, stores peak information after deconvolution, without requiring a protein sequence.
4. *Save Annotation Snapshot*: Saved after the review process, stores all intermediate information, including feature annotations, with filtering and results from manual review.
5. *Save Targeted Search Snapshot*: Saved after *Filter Annotated Peaks*, stores peak information after annotation using a *Targeted Mass Search.*
6. *Save Review Snapshots*: Saved after the review process in Intact_ReviewSnapshots workflow. Stores all information, like the *Save Annotation Snapshot*.

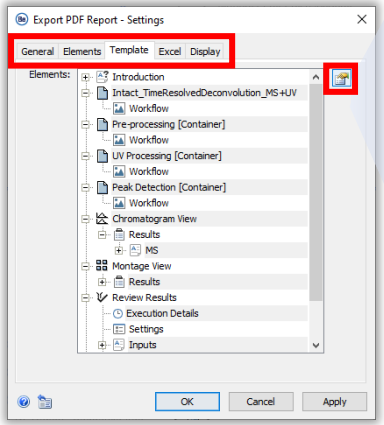


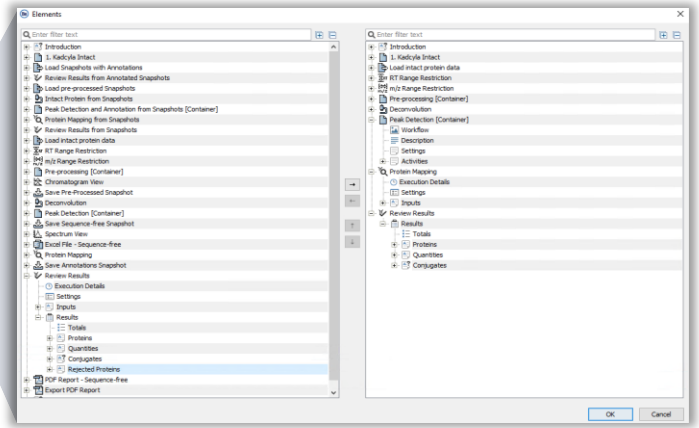
Reporting: *Export PDF Report*



- The *Export PDF Report* activity node produces:
  - A PDF document.
  - An Excel file with spectral information from deconvolution.
  - An Executed workflow (xml), that includes the settings used to generate results.
    - To open the xml file, drag and drop it into the Biologics Explorer workflow home page.

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





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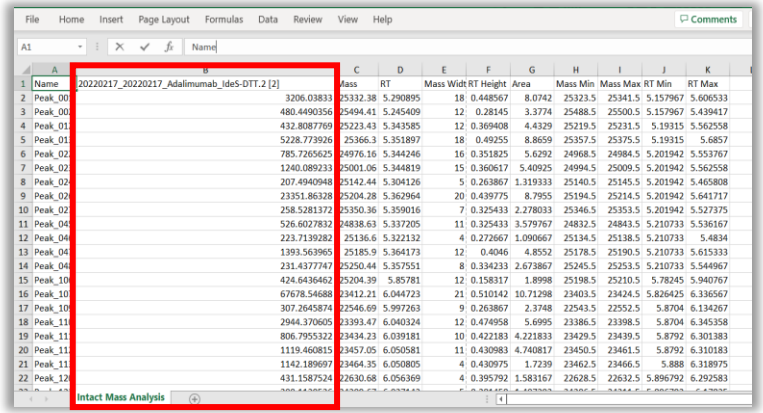
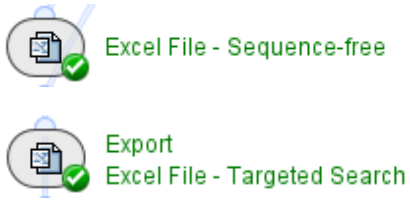
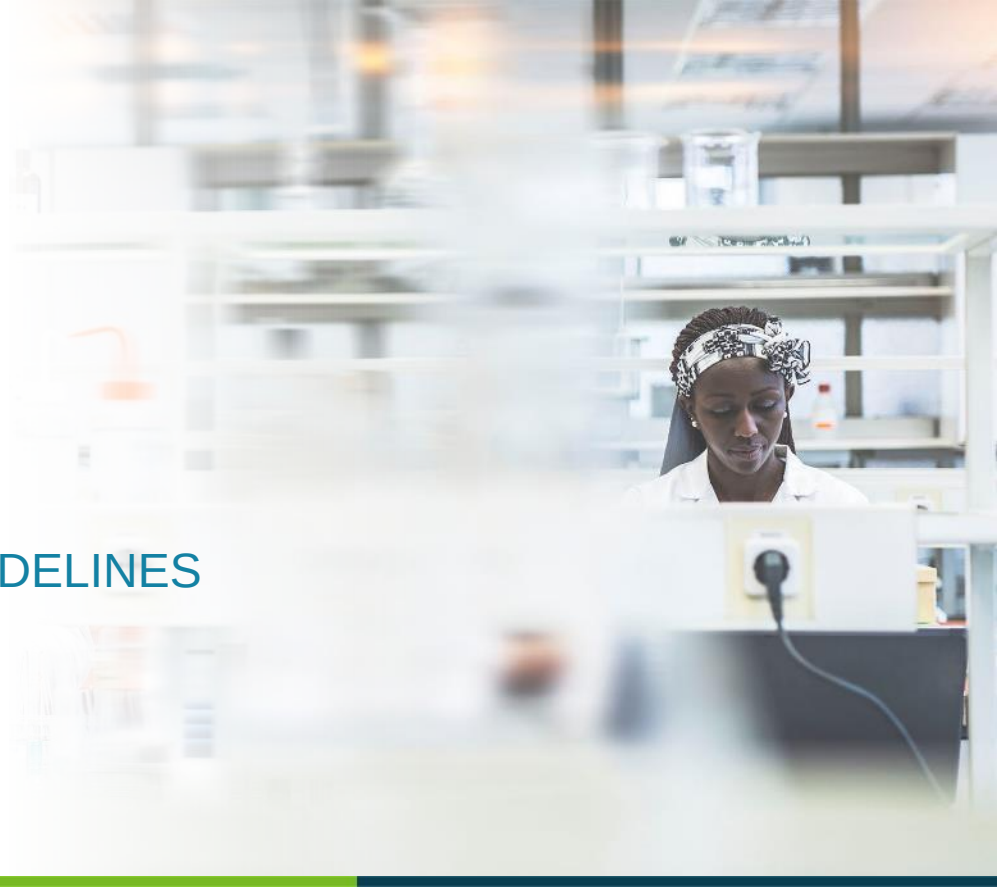
Reporting: Save or Export an Excel File

- The generated 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.**





- Select or create the folders where the results will be stored.
  - The default location is: C:\ProgramData\Sciex\BiologicsExplorer\data\data\user



Part A

Software and Workflows

SPECIFIC INTACT WORKFLOW GUIDELINES

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Automated Deconvolution with MS or MS and UV Data

WORKFLOW GUIDELINES

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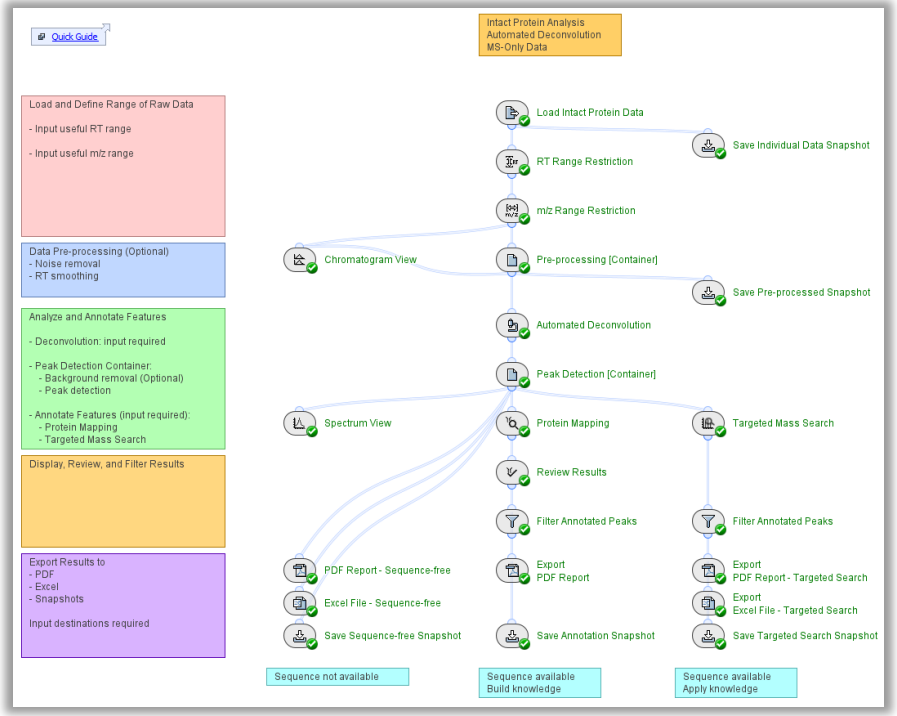
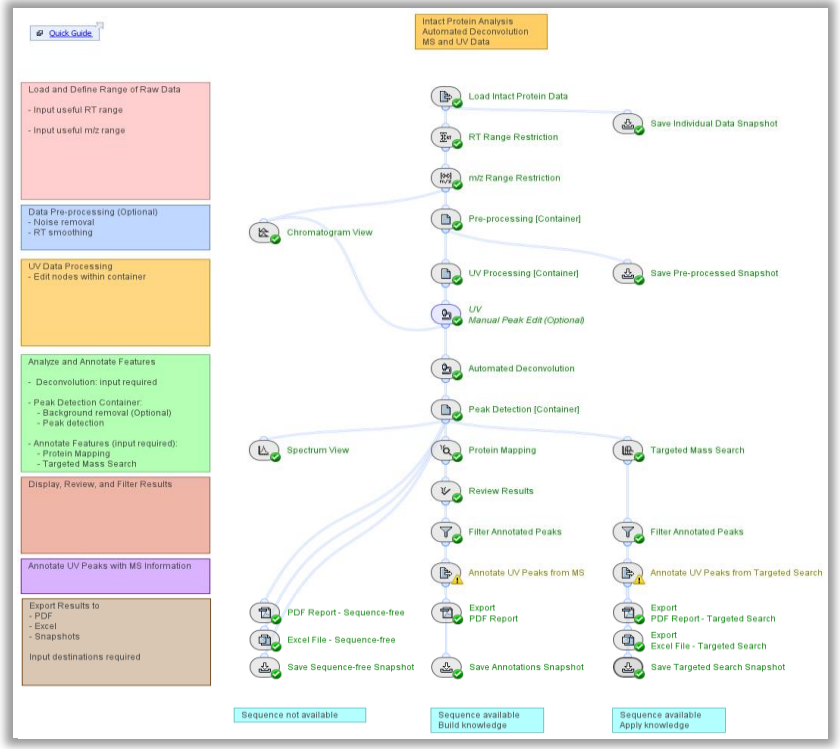
Automated Deconvolution Workflows: Design



Intact_AutomatedDeconvolution_ MS Workflow



Intact_AutomatedDeconvolution_ UV+MS Workflow

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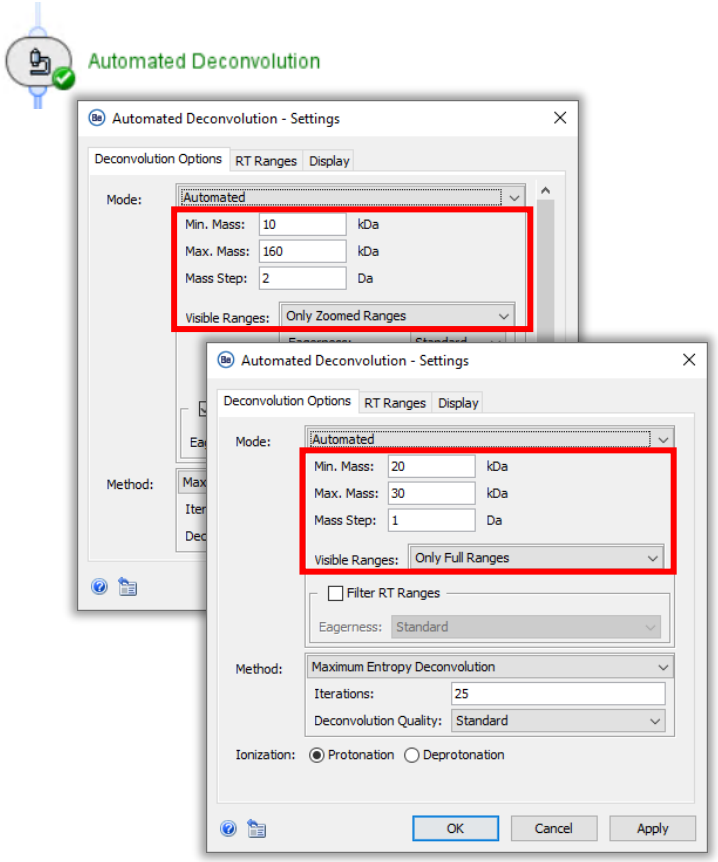
Automated Deconvolution Workflows: Overview

- **Automated Deconvolution** is optimal when species of interest are chromatographically well resolved.
- Biologics Explorer software applies a common peak detection approach when analyzing multiple samples together (batch analysis).
  - To make sure that annotations and quantification are consistent, use similar data. For example, analyze only all intact protein or all subunit datasets together.
  - If multiple samples with highly variable peak intensities are being analyzed, refer to the slide '*Automated Deconvolution*: Manual RT Ranges' for instructions on optimizing the workflow settings.

Automated Deconvolution: Deconvolution Options



Configure settings on the **Deconvolution Options** tab to define the relevant mass range and visualization options for deconvoluted spectra.

- **Mass range**:
  - Use a wide mass range to prevent prominent harmonics peaks when analyzing multiple species.
  - Use a narrow mass range to focus on a single entity.
- **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 produces 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.

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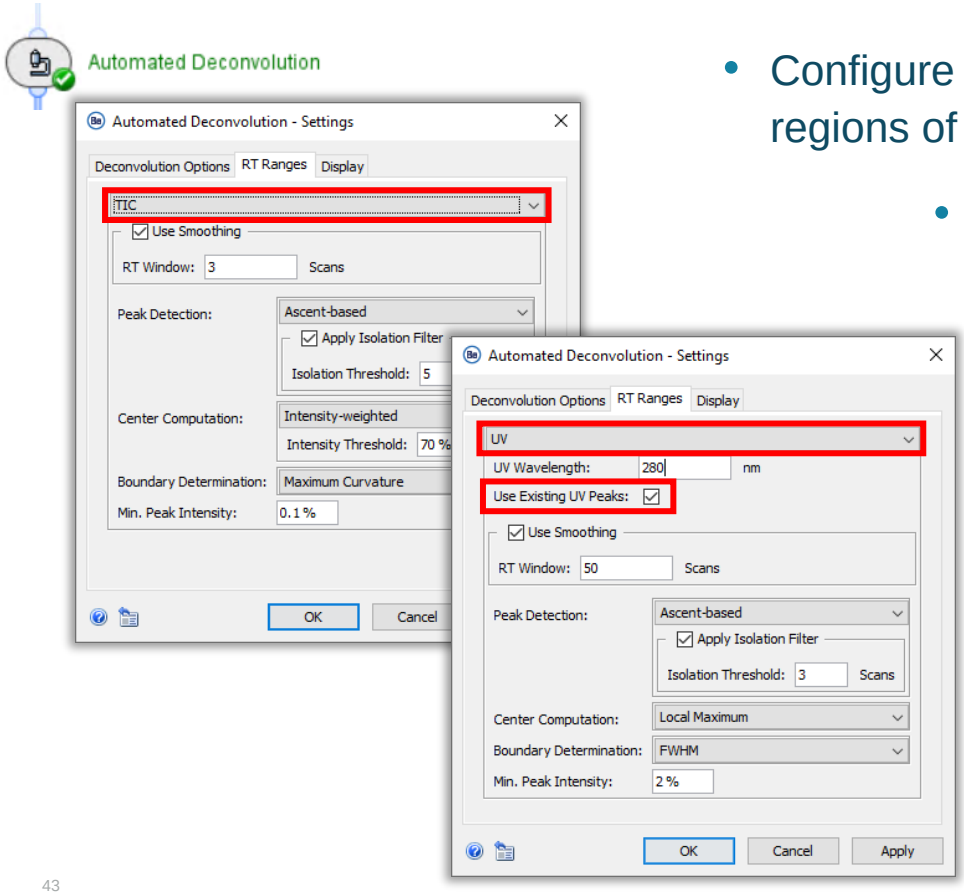
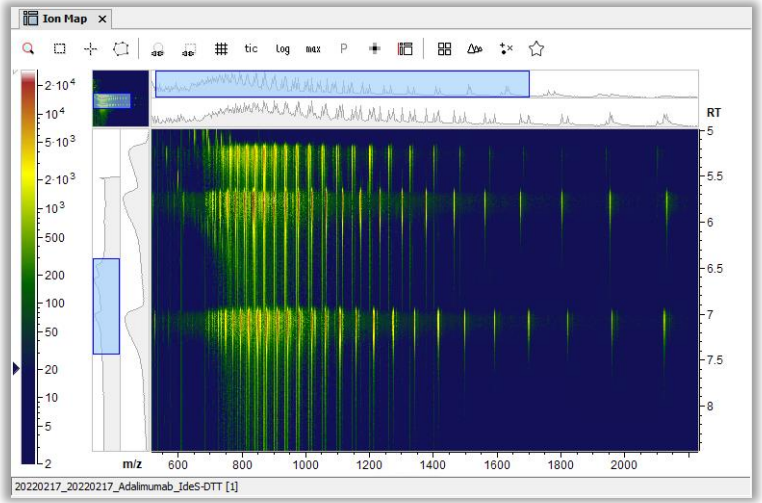
Automated Deconvolution: RT Ranges

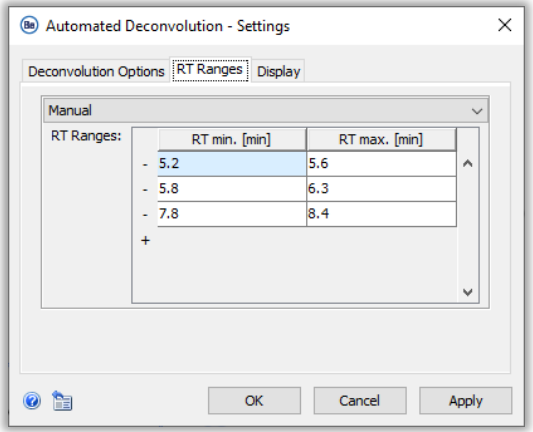


- Configure settings in the **RT Ranges** tab to identify RT regions of interest.
  - Select one of these options from the list:
    - **TIC**: Identify RT ranges using the total ion chromatogram.
    - **Manual**: Define RT ranges manually.
    - **UV**: Define RT ranges based on peaks in the UV data.
      - 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.





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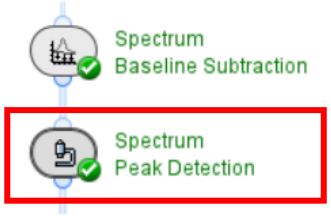
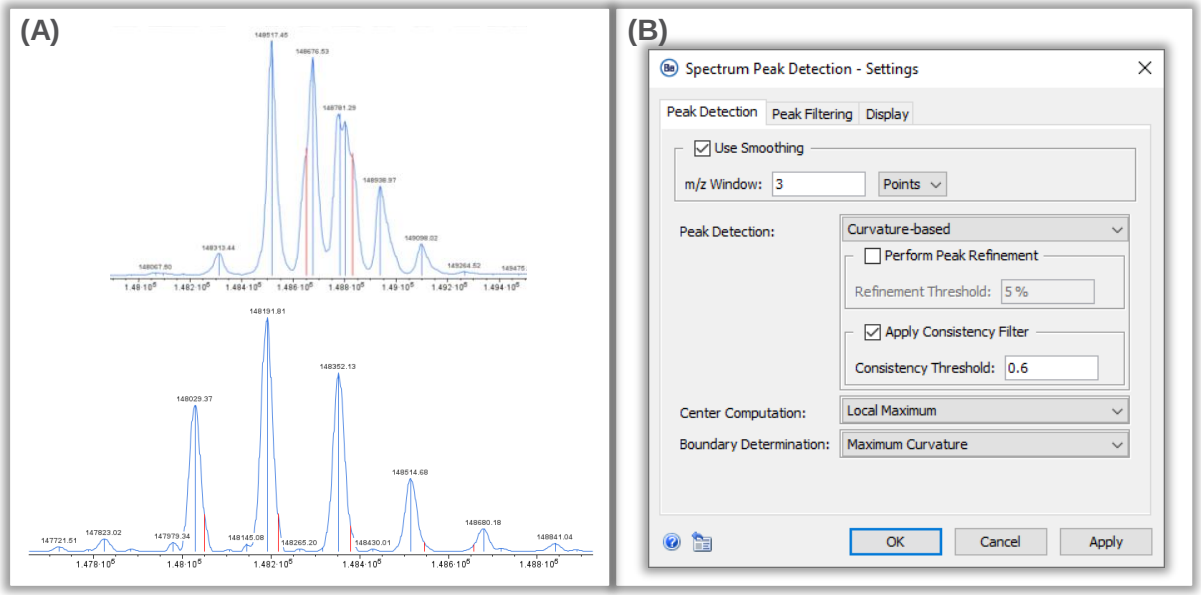
Spectrum Peak Detection

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





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.

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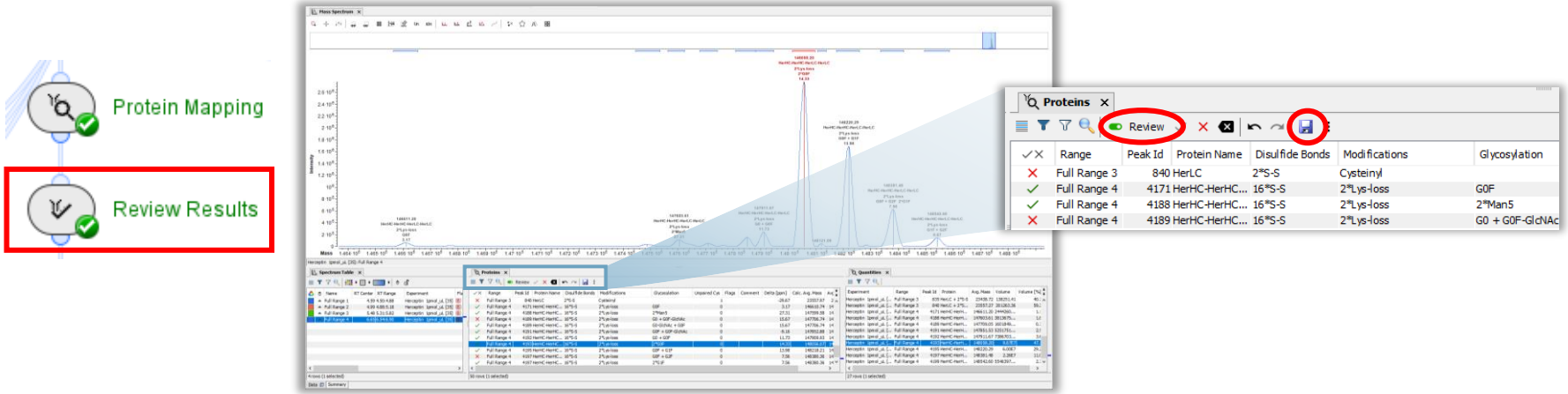
Review Results: Accept and Reject Annotations

- Open the *Review Results* activity node:
  1. Activate the **Review** mode and accept one annotation for all relevant peaks.
  2. Reject all other redundant annotations.
  3. Click **Save** to apply the changes. The activity node runs again, automatically recalculating quantities.

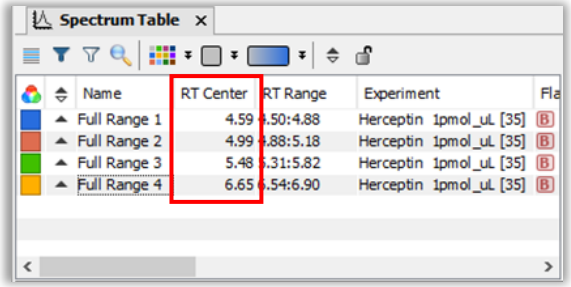


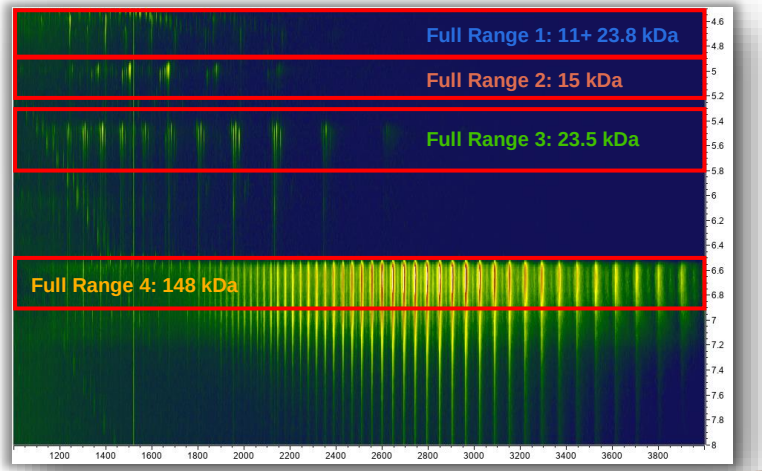
- *Review Results* is a critical step for the analysis of proteins with complex glycosylation patterns and for ADCs to correctly calculate DAR values.

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 corresponding spectrum.





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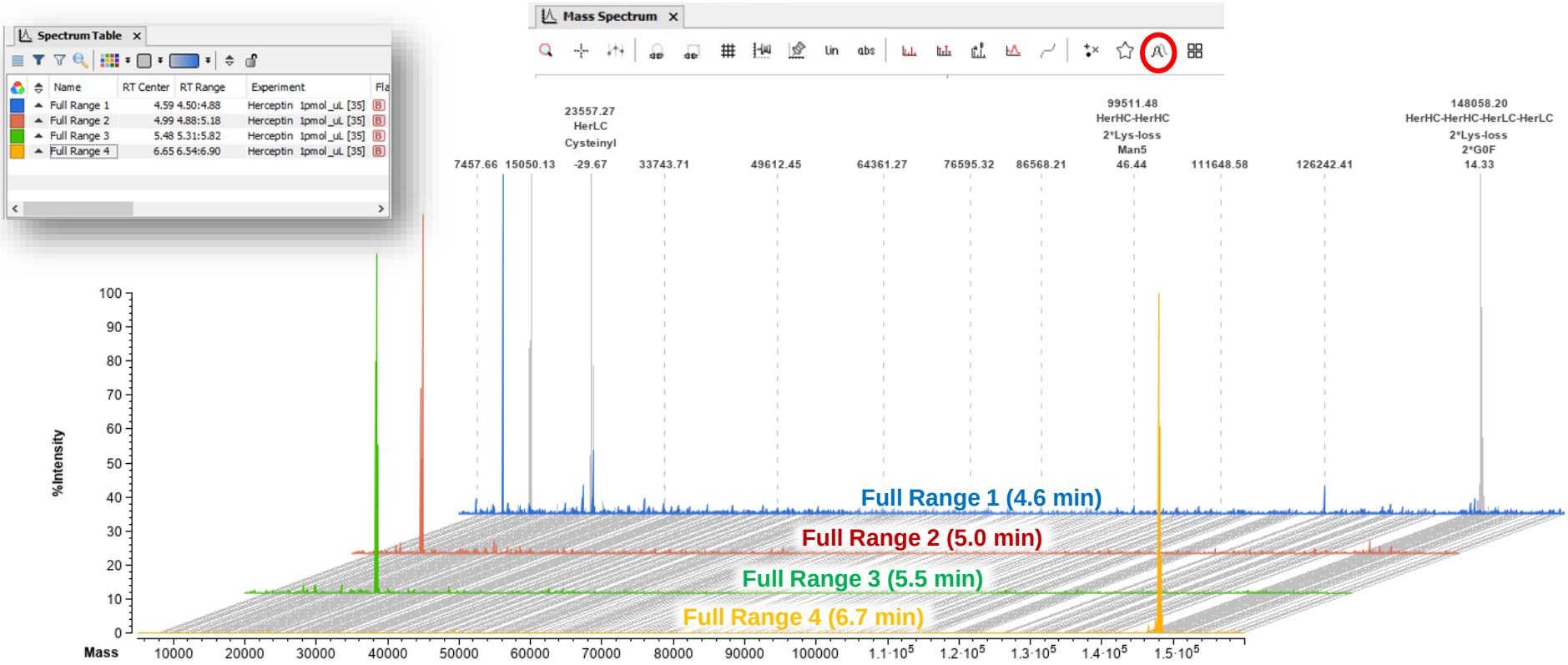
Review Results: Spectrum Offset View



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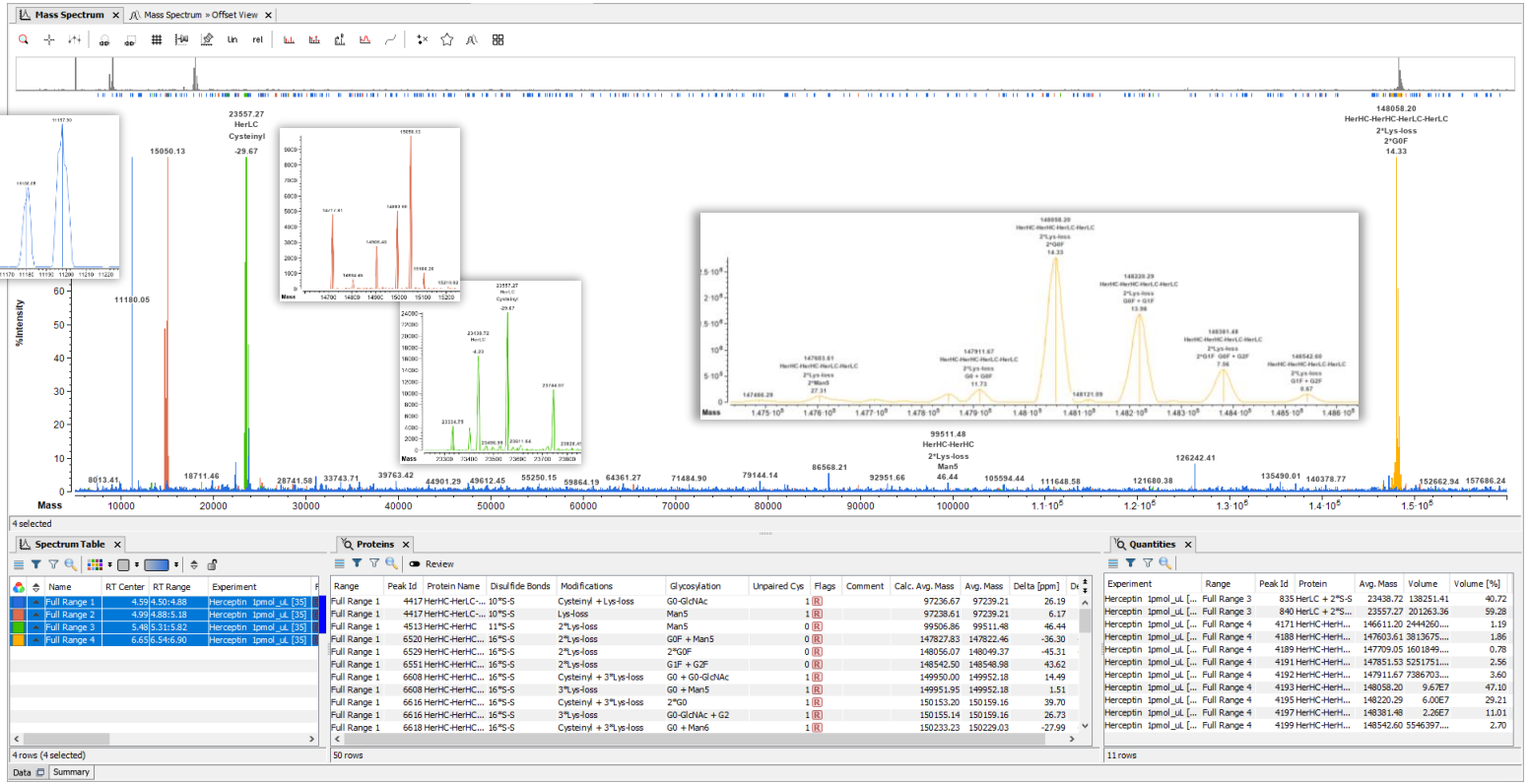
Review Results: Spectrum Overlapped View



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Time Resolved Deconvolution with MS or MS and UV Data

WORKFLOW SPECIFIC GUIDELINES

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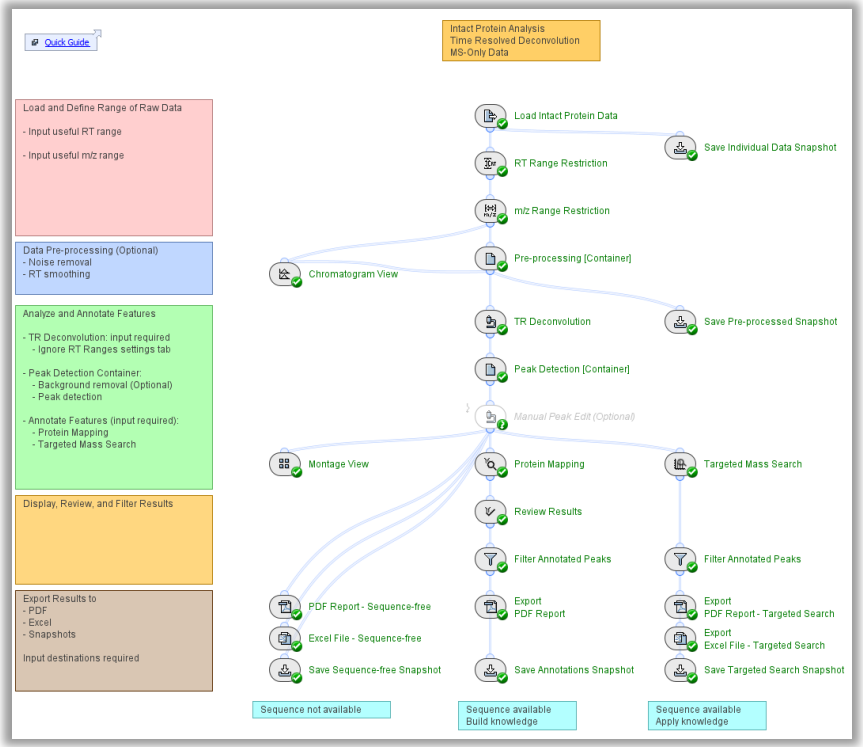
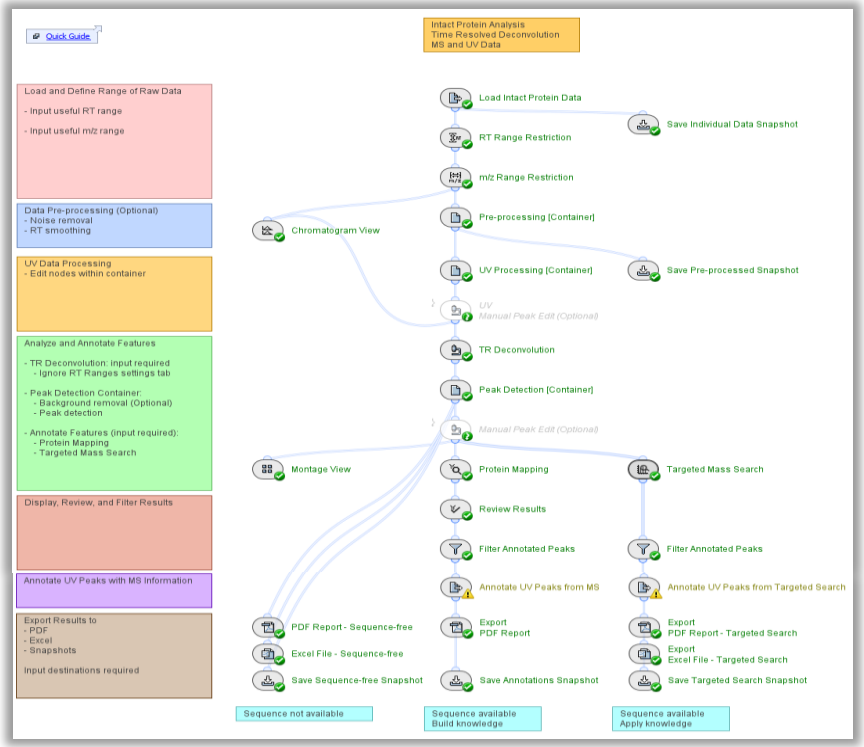
TRD Workflows: Design



Intact_TimeResolvedDeconvolution_MS Workflow



Intact_TimeResolvedDeconvolution_UV+MS Workflow

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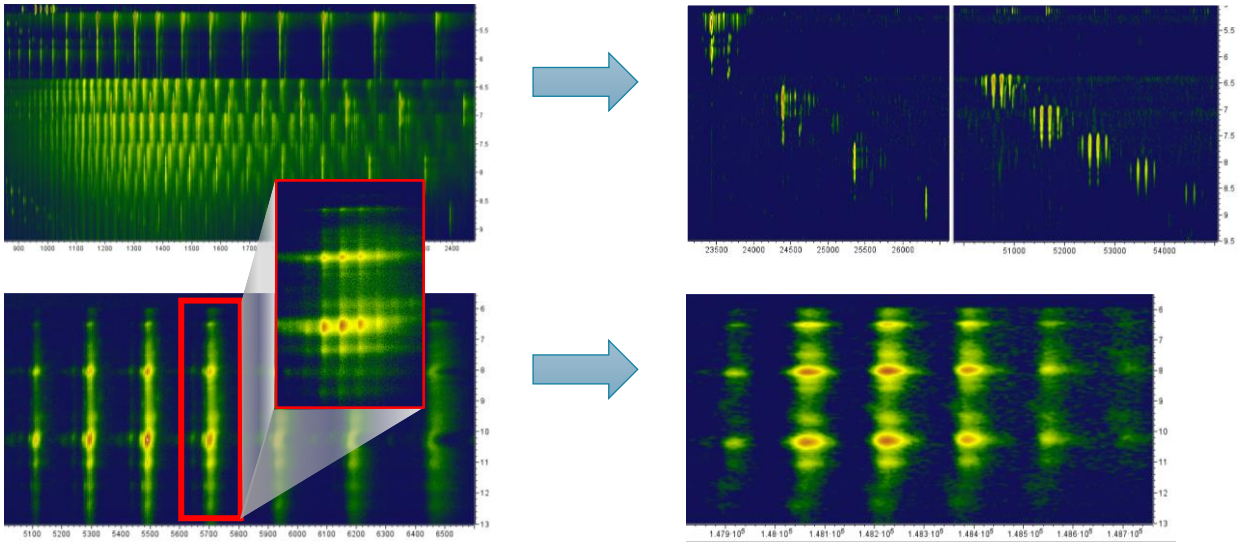
TRD Workflows: Overview

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

Before TRD (*m/z*)



After TRD (mass)

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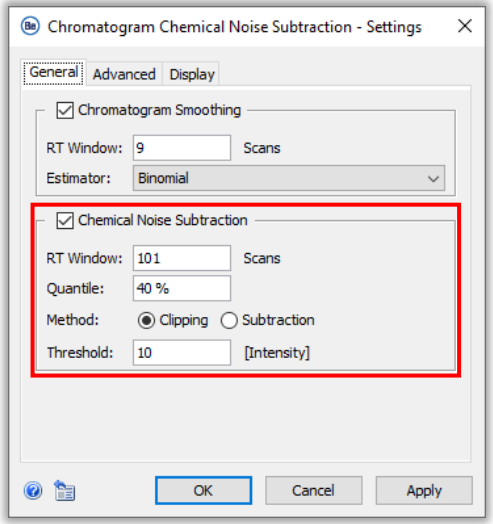
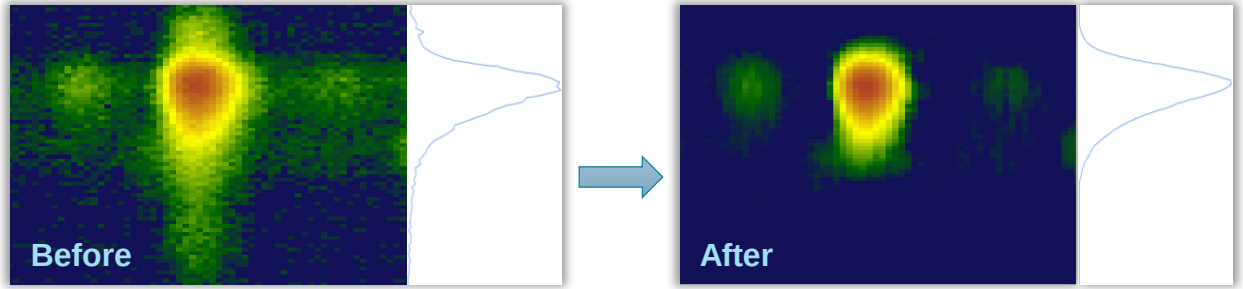
Chemical Noise Subtraction: Optimization



- **Chemical Noise Subtraction** is used with **TRD** data to help supress satellite peaks and improve peak detection by:
  - Removing extensive tailing on wide peaks.
  - Reducing overall background noise.

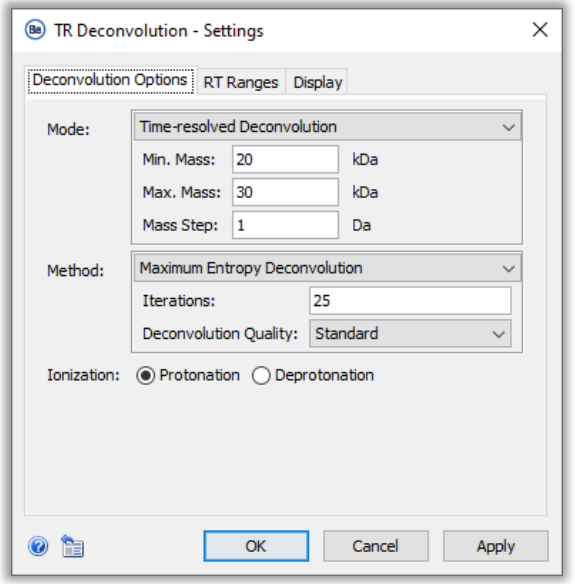


- Typical range for settings:
  - **RT Window***:* Approx. 1.3x the number of scans across the largest peaks.
  - **Quantile**: 40-50%.
- Summary of impact of settings:
  - Larger **RT Window** = Less data subtracted.
  - Higher **Quantile** = More data subtracted.

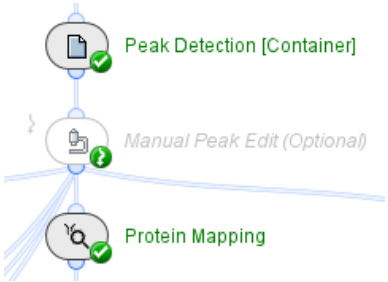
TR Deconvolution: Impact on Speed of Data Analysis

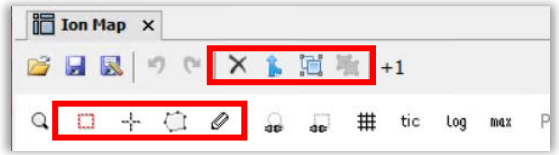
TRD is a more computer-resource intensive algorithm than Automated Deconvolution.

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

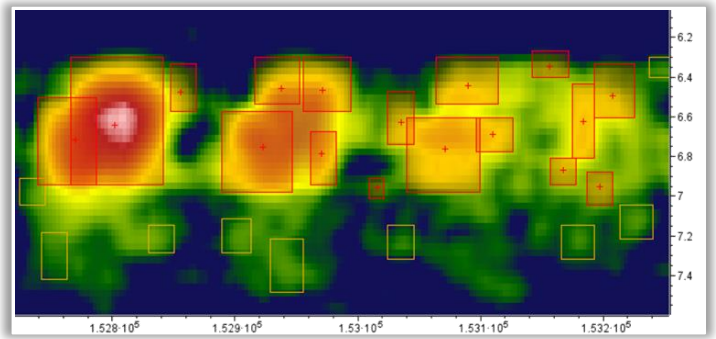


Manual Peak Edit (Optional)



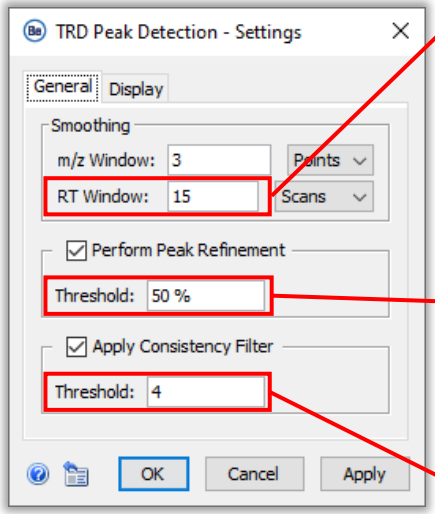


- This activity is bypassed as default.
- The peaks detected automatically in the deconvoluted ion map can be refined using *Manual Peak Edit* by:
  - Changing the boundaries of peaks.
  - Splitting overlapping peaks.
  - Deleting peaks.
  - Drawing new peaks.



- Use *Manual Peak Edit* to accurately re-assign intensity distributions of overlapping components to individual peaks.
  - This enables very fine details of complicated analyses to be captured.

TRD Peak Detection: Optimization



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

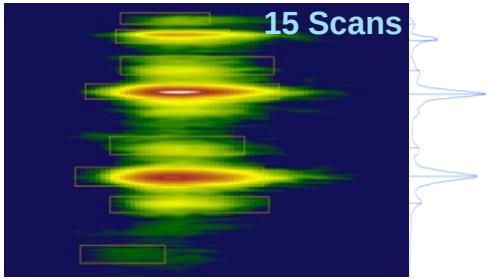
Lower value = Increase in splitting in RT direction.

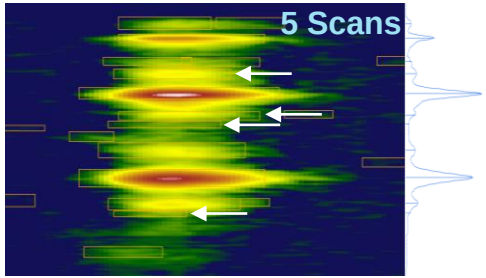
Affects the number and width of peaks along the mass axis (↔).

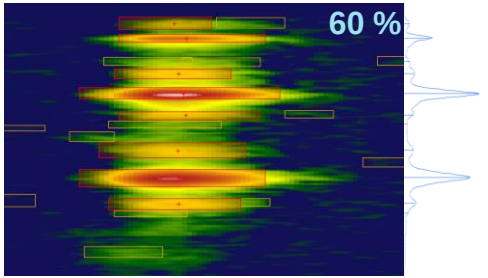
Lower value = Higher probability that peaks will be split in *m/z* direction.

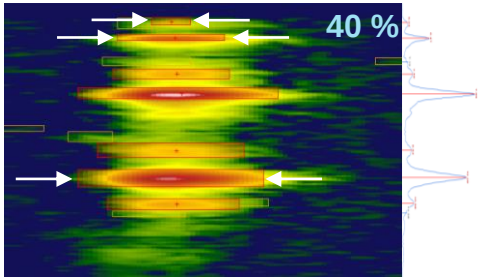
Affects sensitivity and therefore the number of background peaks.

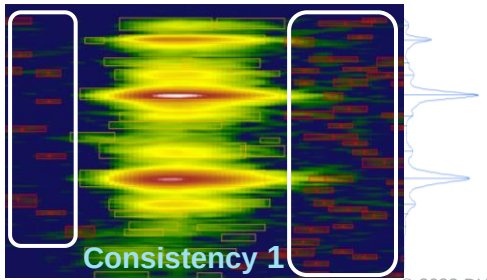
Lower value = Increase in *m/z* splitting.











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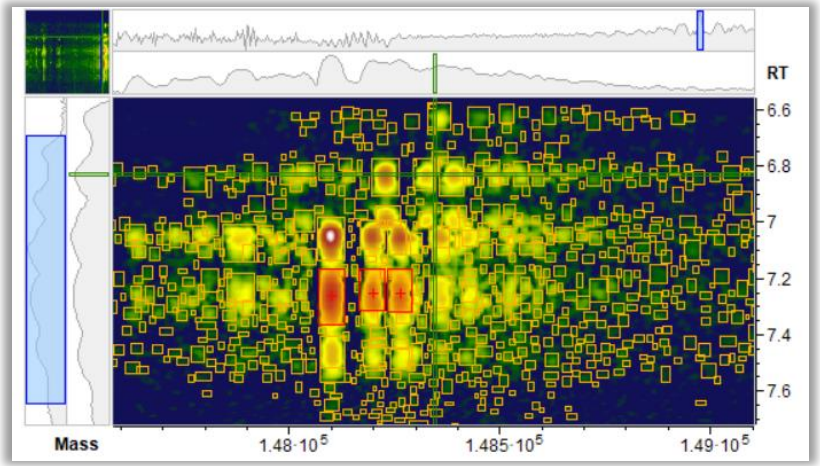
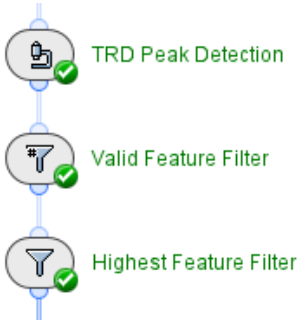
TRD Peak Detection: Optimization

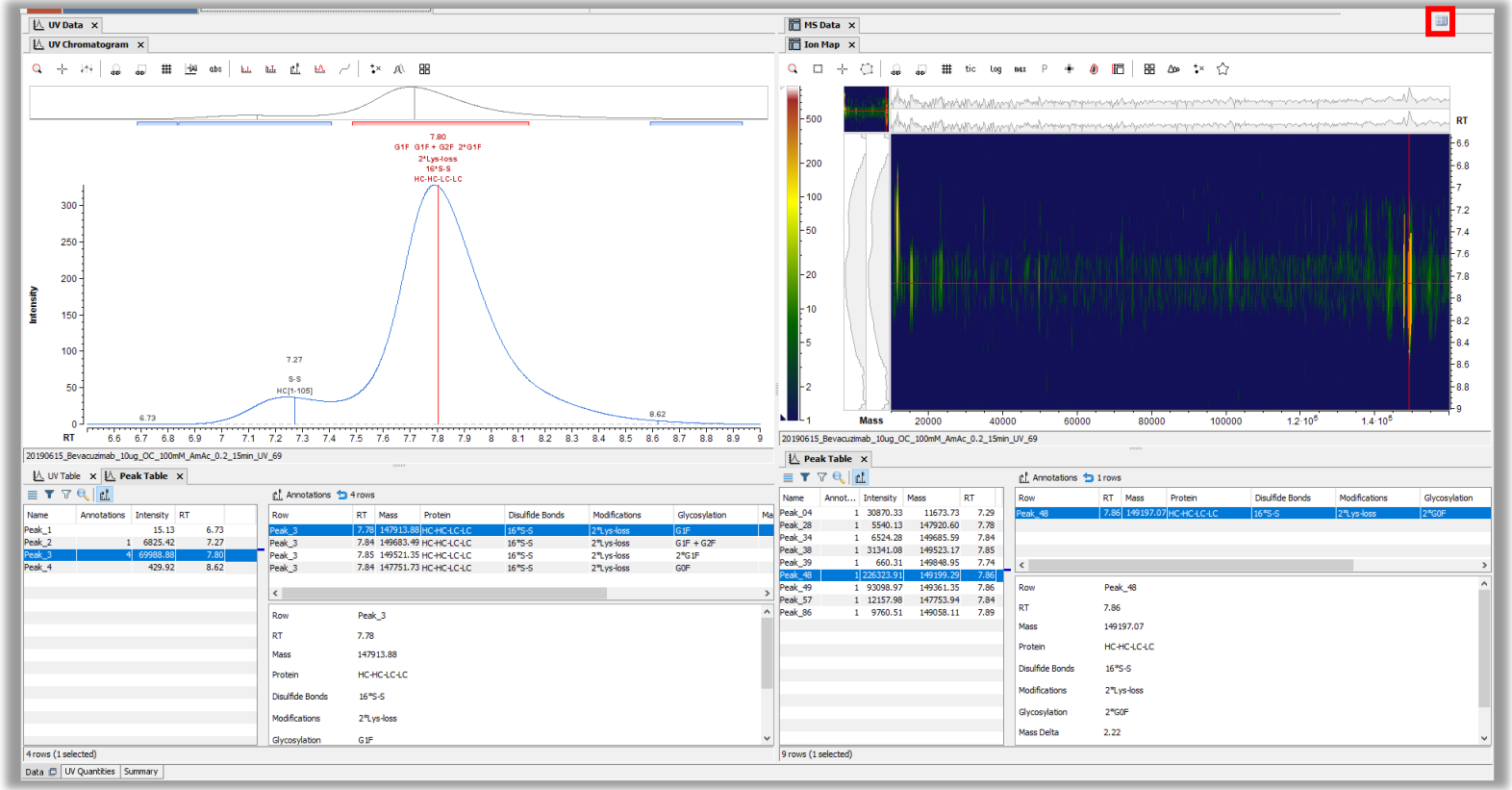


- If multiple small features are detected in a complex deconvoluted ion map:
  - The sensitivity can be lowered by increasing the **Consistency Filter Threshold** (to 2 or 3).



- The *Valid Feature Filter* and *Highest Feature Filter* activity nodes can also be used to decrease the number of selected features.

Recommended Layout for Reviewing TRD Results with UV Data



- Favorite layouts can be saved and accessed with the Layout icon.

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Intact Mass Screening

WORKFLOW SPECIFIC GUIDELINES

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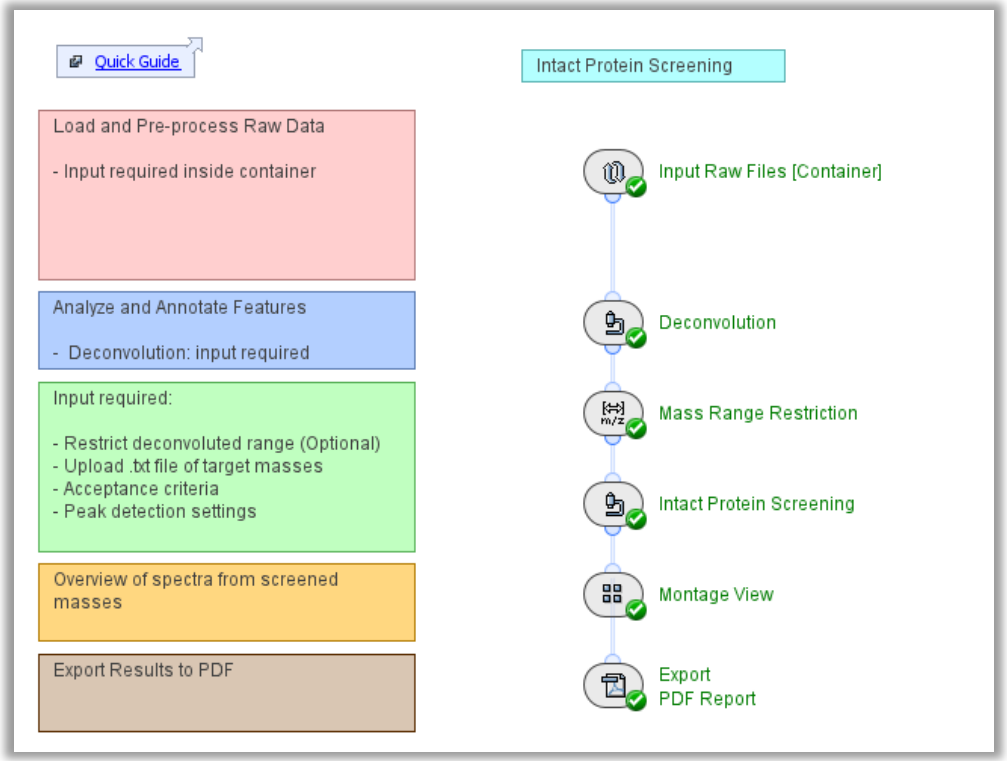
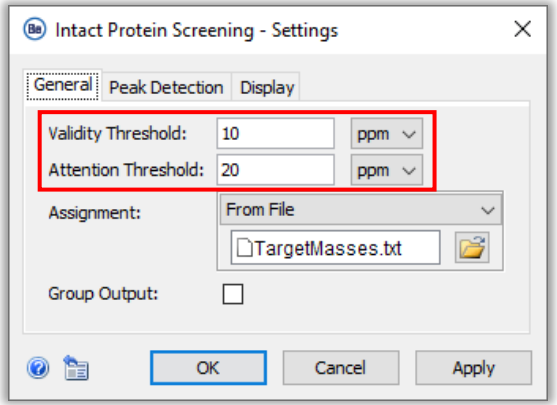
Intact Mass Screening Workflow: Design



Intact_MassScreening

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.

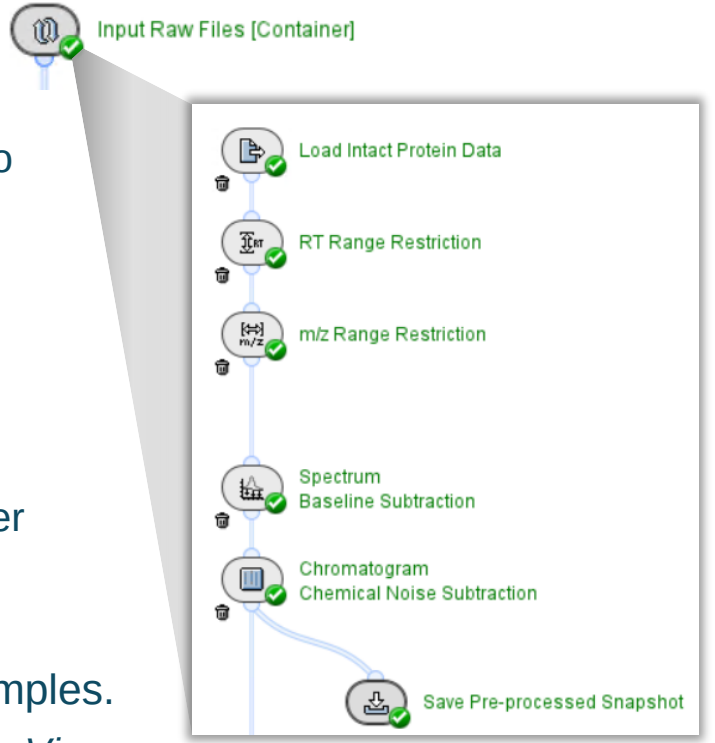


Zoomed Range 3

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) 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 being screened the *Montage View* activity node can be bypassed.

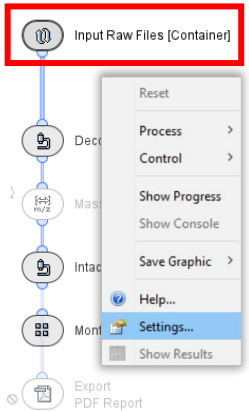


Intact Mass Screening Workflow: Conditions and Behavior

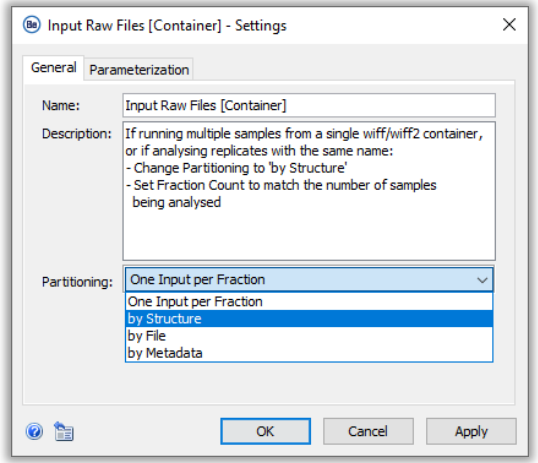
- For each sample, more than one mass can be searched, however, certain conditions should be met in the submitted samples:
  - Samples must belong to the same molecule type, for example, intact proteins, subunits or fragments.
  - Samples should have consistent chromatography, the same number of components, and similar expected deconvolution ranges.
  - Samples must have the same number of either **Full** or **Zoomed RT Ranges**.
- The workflow is designed to have the following behavior:
  - 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: Analyzing Replicates with the Same Name

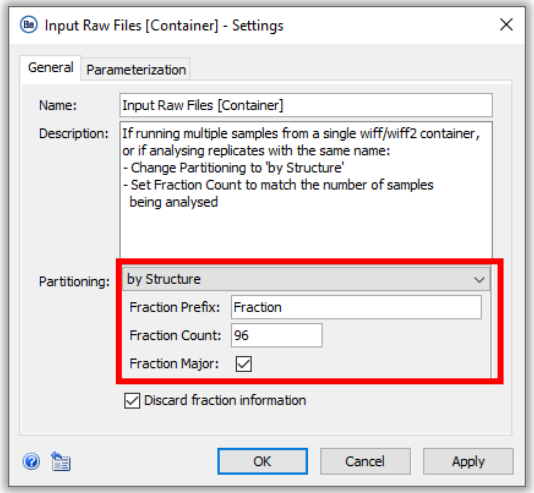
- The settings of the *Input Raw Files* container must be changed for:
  - Analysis of replicate samples that have the same file name.
  - Loading of multiple different samples from within a single wiff or wiff2 container.



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



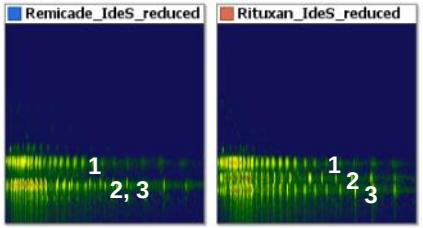
(2) Change **Partitioning** to **by Structure**.

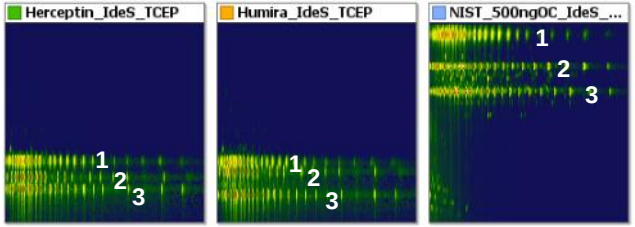


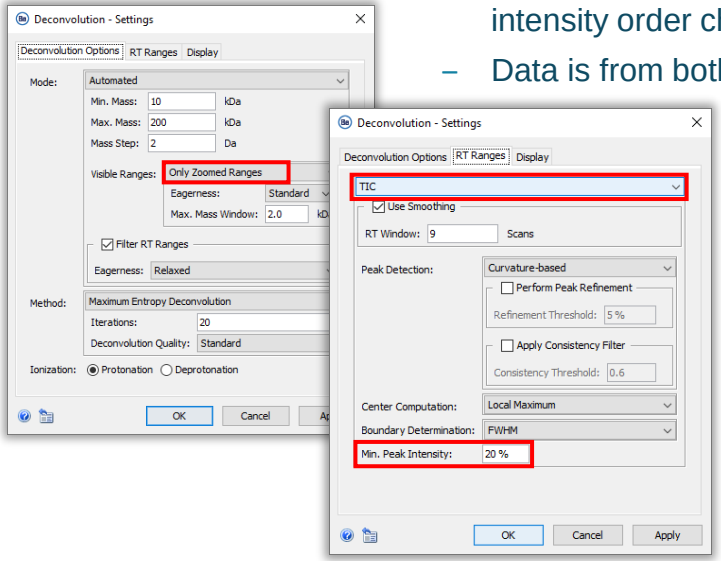
(3) Specify the number of samples loaded as the **Fraction Count**.

Deconvolution: Optimization for Complex Datasets

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

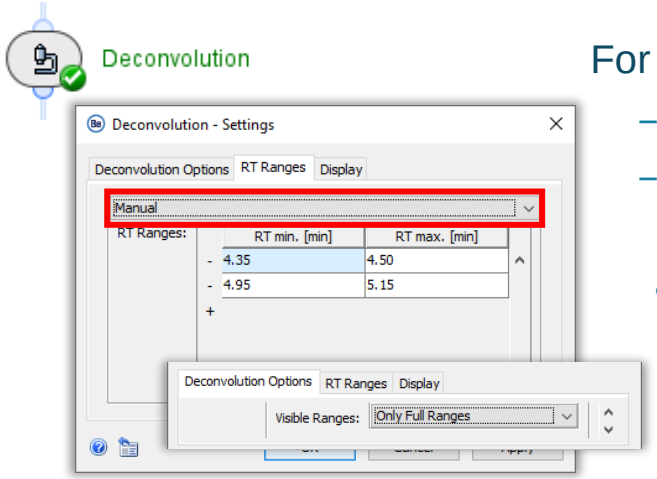


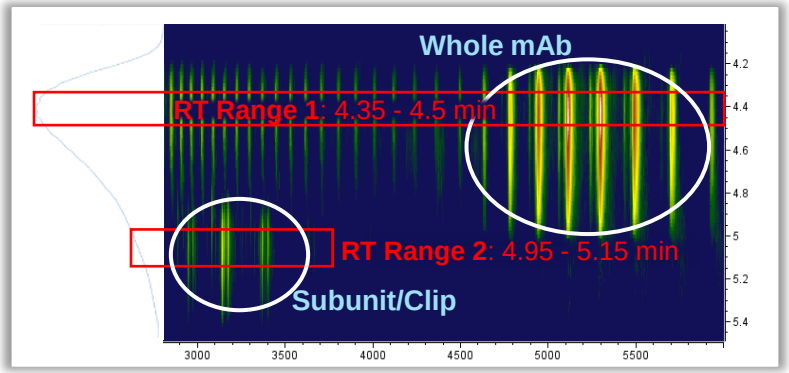




- To overcome the potential challenges with this type of dataset:
  - Use **TIC** to automatically identify the specific RT ranges for each sample.
  - Use a **Min. Peak Intensity** of 20% (or higher) to limit the number of deconvoluted RT ranges to those related to the target fragments.
  - Use **Only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

Deconvolution: Optimization for Present/Absent 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.

- Using **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.

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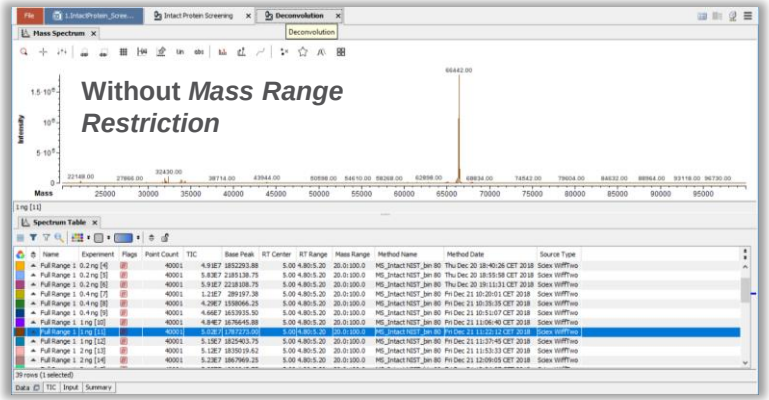
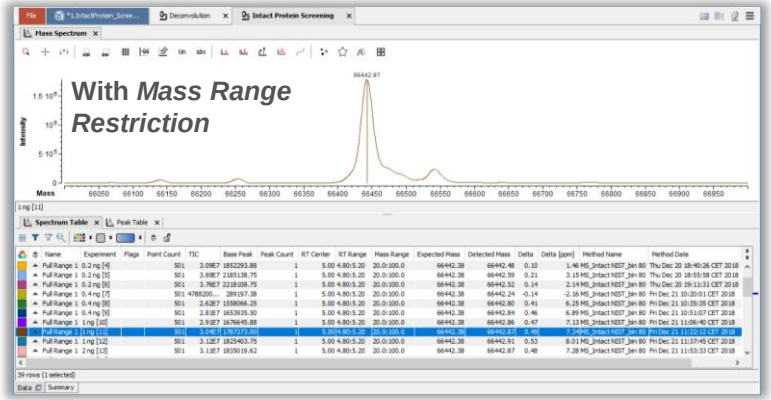
Mass Range Restriction

- This activity is bypassed by default.
- *Mass Range Restriction* can be used to restrict the visualization of deconvoluted spectra to a specific target mass.
  - 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**.

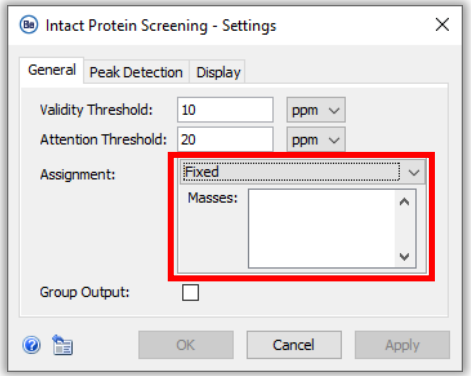




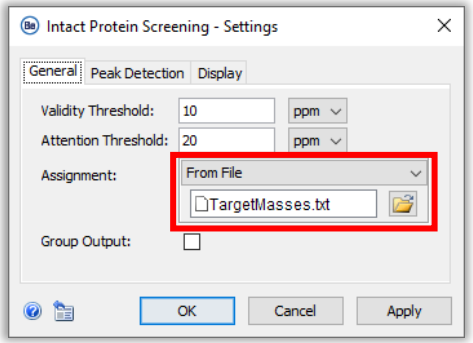
Defining Target Masses for *Intact Protein Screening*

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.

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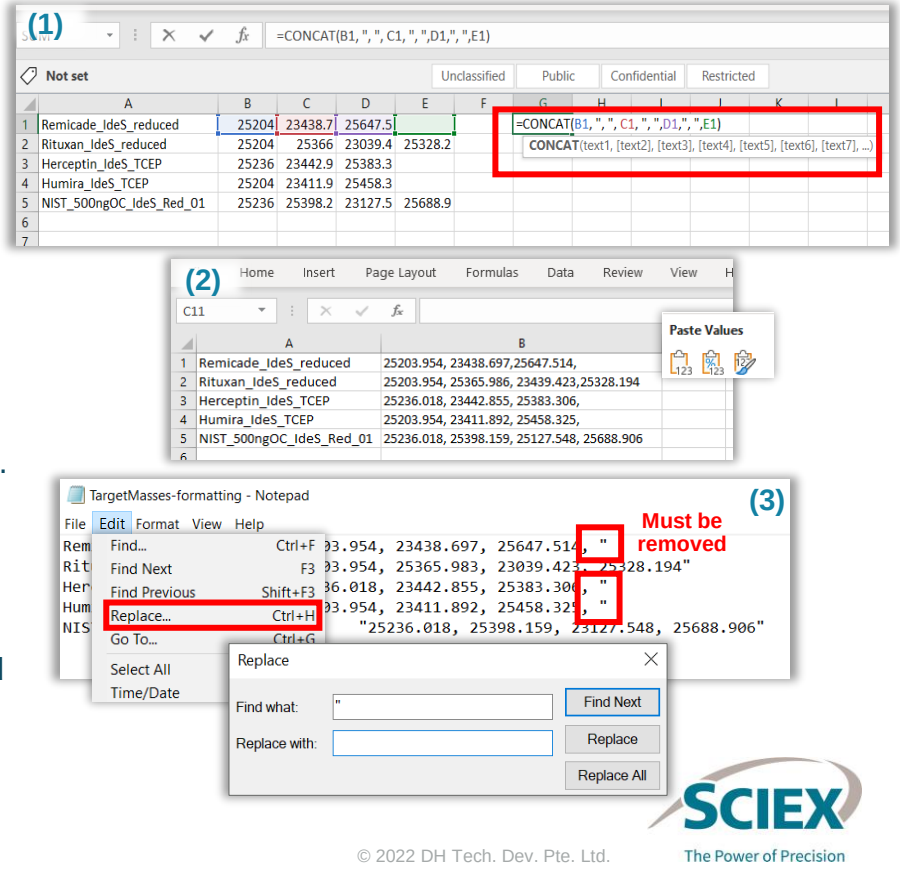
Intact Protein Screening: Target Masses File Format

To create a txt file for screening:

1. Create a table containing sample names and target masses. Do not include table headers.
   - Use the CONCATENATE function in Excel to combine columns containing 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.



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Review Stored Results

WORKFLOW SPECIFIC GUIDELINES

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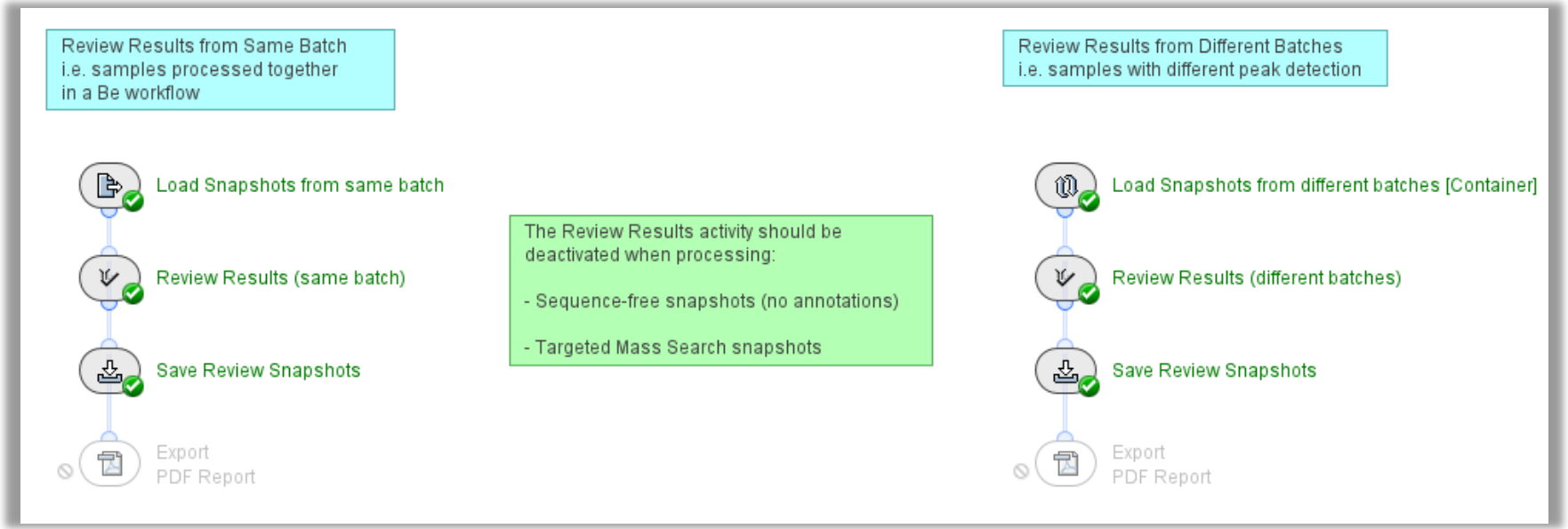
Review Stored Results Workflow: Design



Intact_ReviewSnapshots

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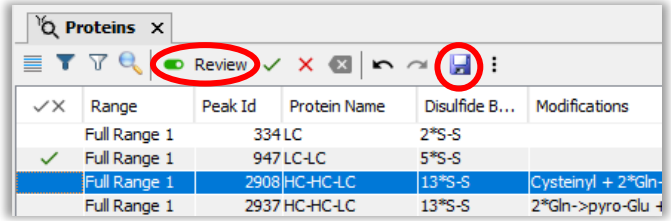
Review Stored Results Workflow: Overview

- These workflows can open all saved Snapshots (sbf) saved from any Intact Mass workflow in Biologics Explorer software.
  - View results from previous analyses using 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).



- When using *Sequence-free, Targeted Mass Search, Individual Data,* and *Pre-processed Snapshots,* bypass the *Review Results* activity node.
  - These snapshots do not include the sequence information required by *Review Results*.
- After further review, save the modified *Annotations Snapshots* as *Review Snapshots*, or export the results as a PDF report.

Part B

Workflow Applications

REFINED SETTINGS FOR SPECIFIC APPLICATIONS

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Selecting Time-Resolved or Automated Deconvolution

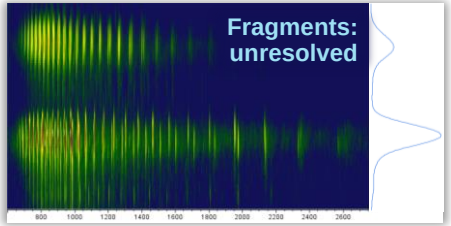
- **Automated:** Ranges for deconvolution are automatically determined.
  - Recommended for chromatographically well-resolved species.

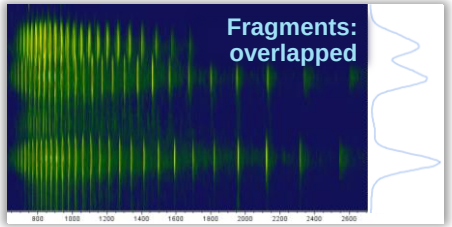
- **Time-resolved:** Deconvolution is completed on each scan in RT.
  - Recommended for data containing complex mixtures that are poorly resolved.
    - Creates a 2D ion map view of deconvoluted data, and so provides better visibility of overlapping or non-resolved peaks.
    - Simplifies quantitation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

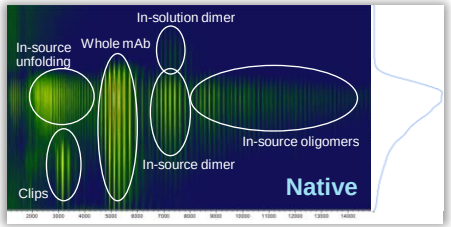
Use Cases for Time-Resolved and Automated Deconvolution

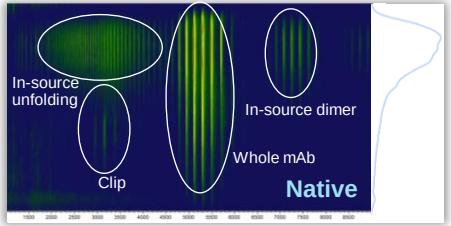
Key use cases for Time-Resolved Deconvolution:

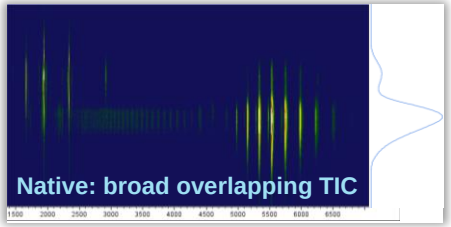
Complex Chromatography

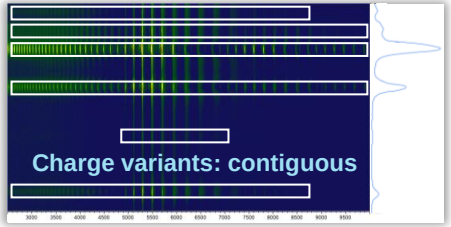






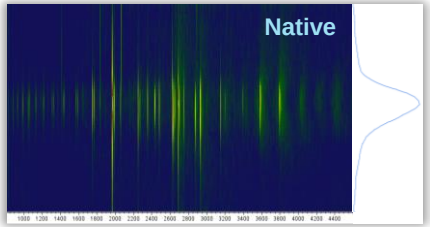


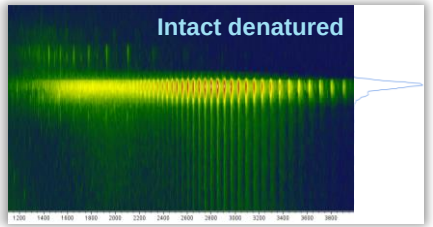


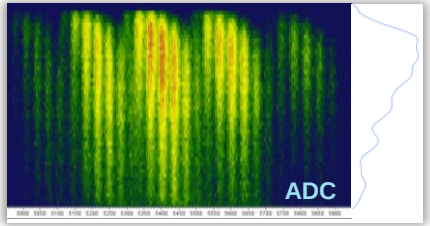


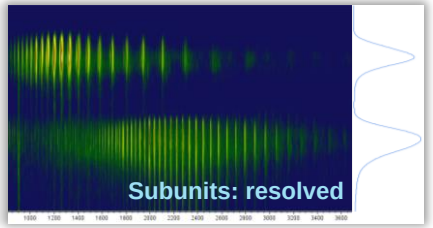
Key use cases for Automated Deconvolution:

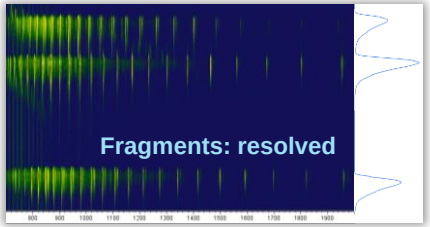
Simple Chromatography











SCIEX
The Power of Precision

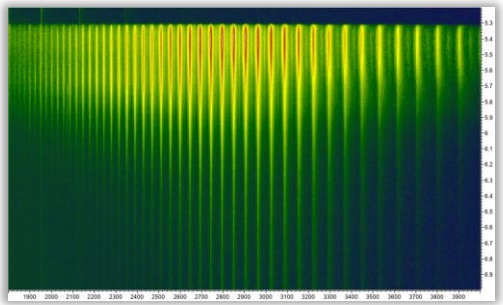
Denatured Intact Protein

Denatured Protein: Single Entity

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

Suggested initial settings:

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



Denatured intact protein

[1]Deconvolution, [2]Protein Mapping

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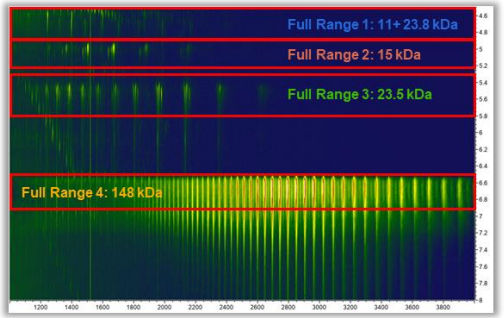
Denatured Protein: Complex Sample

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
Suggested 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
RT Ranges [1] (Ignore with TRD)	**RT Window**: 3 - 5 **Isolation Threshold**: 3 - 7 **Min. Peak Intensity**: <1% Or manually input all ranges for higher sensitivity and better efficiency.
Mass Tolerance (ppm) [2]	20 - 50 (depending 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.



Denatured intact protein with impurities

[1]Deconvolution, [2]Protein Mapping

SCIEX
The Power of Precision

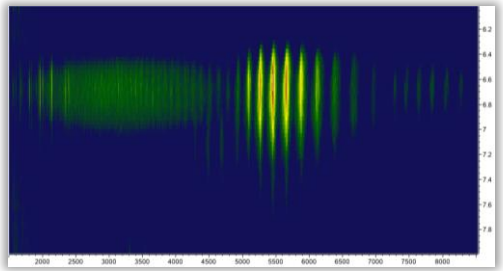
Native Intact Protein

Native Protein: Single Entity

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

Suggested initial settings:

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



Native intact protein

[1]Deconvolution, [2]Protein Mapping

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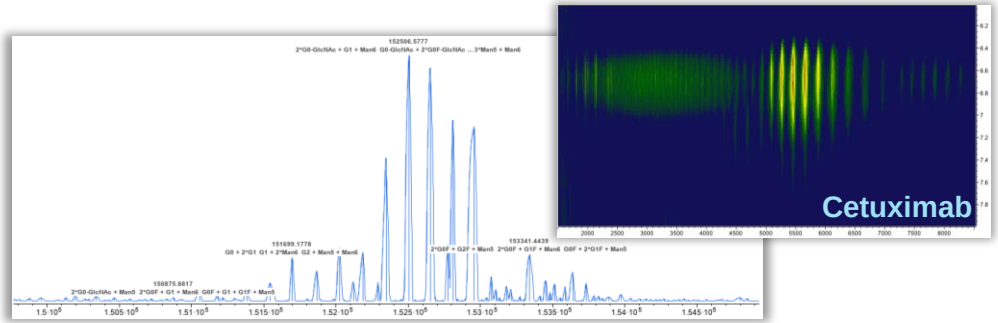
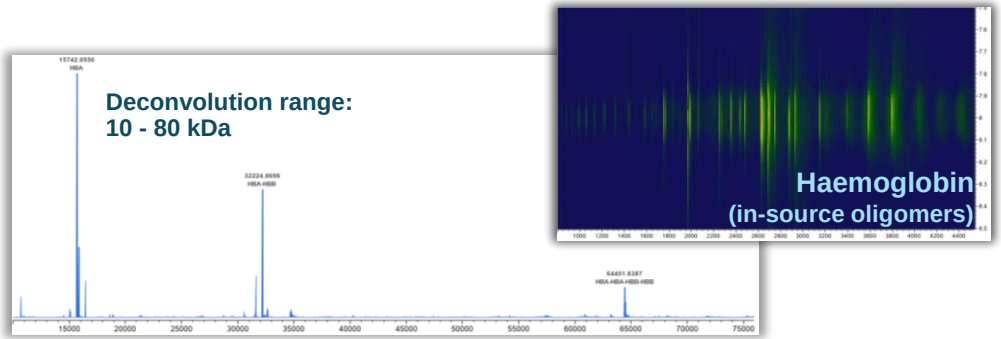
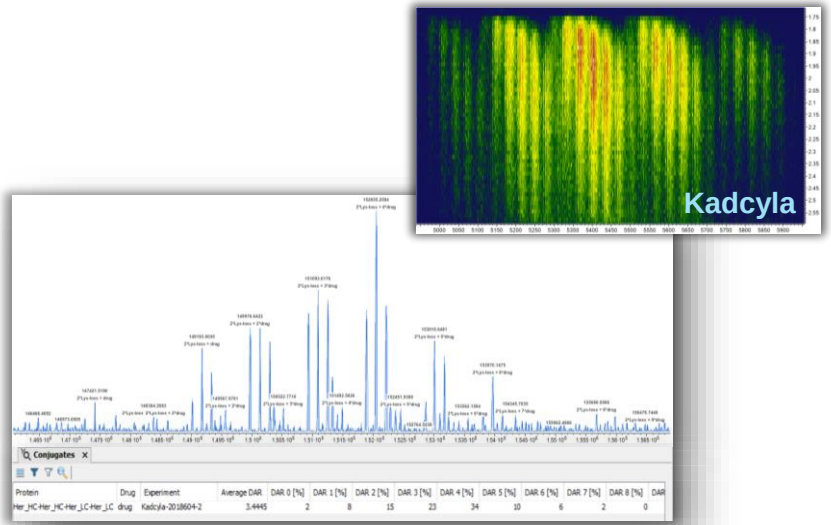
The Power of Precision

Native Protein: Single Entity

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







SCIEX
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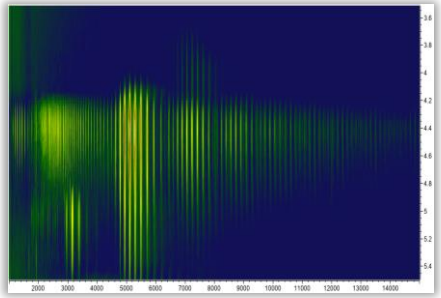
Native Protein: Complex Sample

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
Suggested 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 (Da) [1]	2
RT Ranges [1] (Ignore with TRD)	**RT Window**: 3 - 5 **Isolation Threshold**: 5 - 15 (depending on separation between components). **Min. Peak Intensity**: <1% Or manually input all ranges for higher sensitivity and better efficiency.
Mass Tolerance (ppm) [2]	20 - 50 (depending 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.



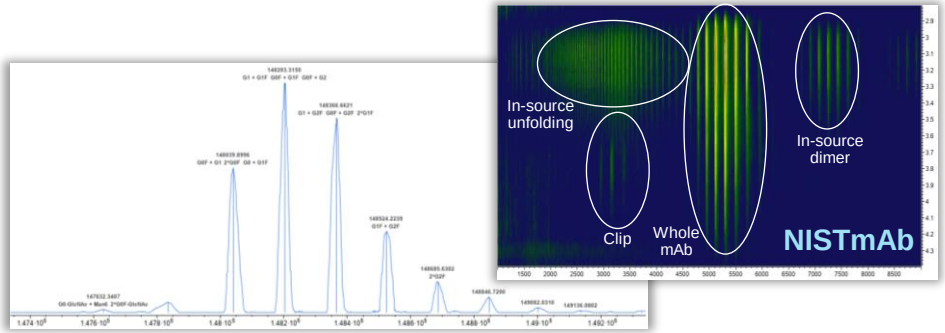
Native complex sample

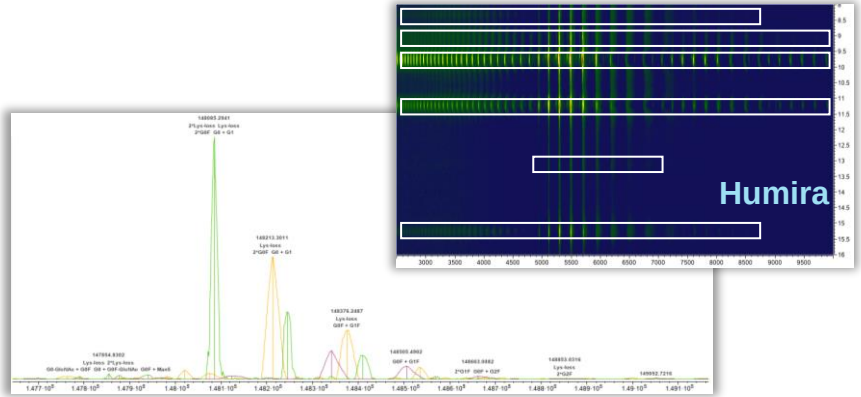
[1]Deconvolution, [2]Protein Mapping

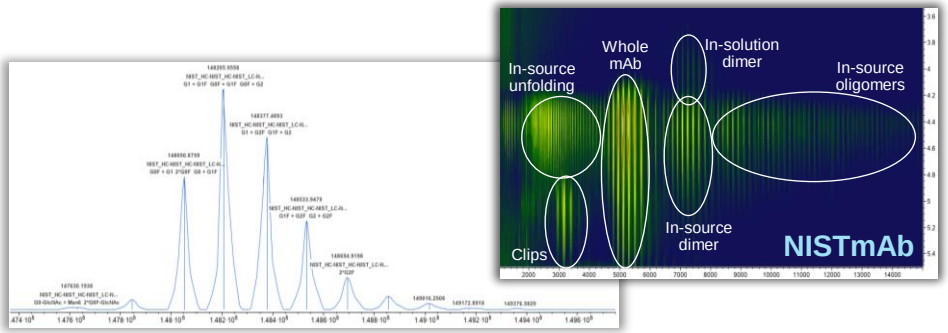
SCIEX
The Power of Precision

Native Protein: Complex Sample

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







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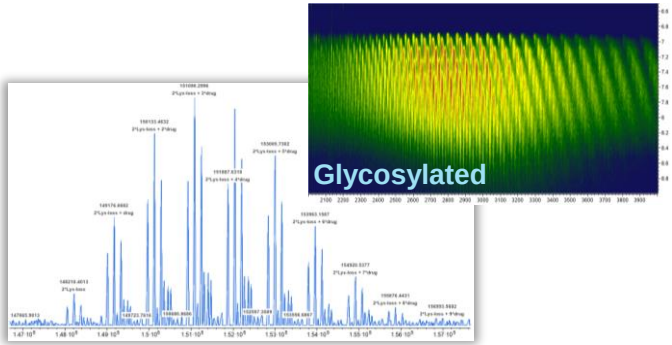
Antibody Drug Conjugates

ADC: Whole Protein

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

Suggested initial settings:

Sample type:	ADC: Denatured
Suggested workflow	Intact_AutomatedDeconvolution
Deconvolution range[1]	140 kDa - 160 kDa **Visible Ranges: Only Full Ranges** for visualization and reporting.
Mass step (Da) [1]	2
RT Ranges [1]	Manually input a single RT range that contains all ADC signals.
Mass Tolerance (ppm) [2]	20 - 50 (depending 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*: Remove redundant annotations to enable correct computation of DAR.



[1]Deconvolution, [2]Protein Mapping

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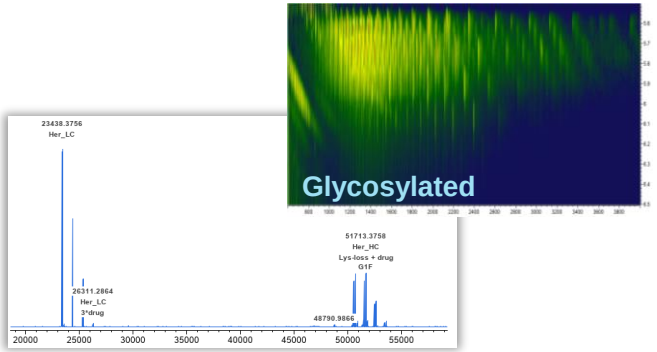
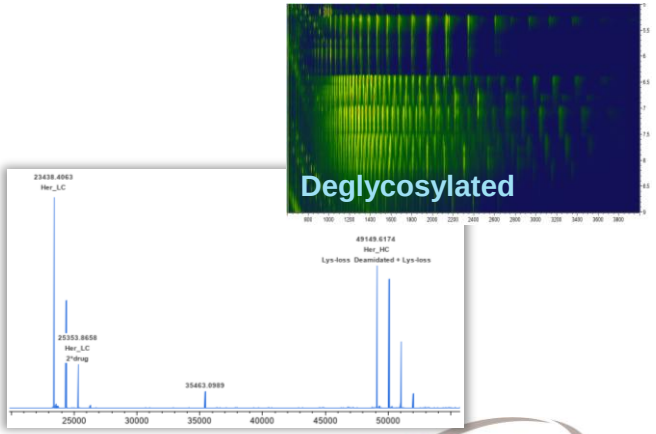
ADC: Subunits

FOCUS: Reduced ADC.

Suggested initial settings:

Sample type:	ADC: Denatured and reduced
Suggested workflow	Intact_AutomatedDeconvolution
Deconvolution range [1]	10 kDa - 60 kDa **Visible Ranges: Only Full Ranges** for visualization and reporting.
Mass step (Da) [1]	1 - 2
RT Ranges [1]	Manually input a single RT range that contains all ADC signals.
Mass Tolerance (ppm) [2]	10 - 20 (depending 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*: Remove redundant annotations to enable correct computation of DAR





[1]Deconvolution, [2]Protein Mapping

SCIEX
The Power of Precision

Subunit Analysis

Subunit Analysis

FOCUS: Heavy chain and light chain.

Suggested initial settings:

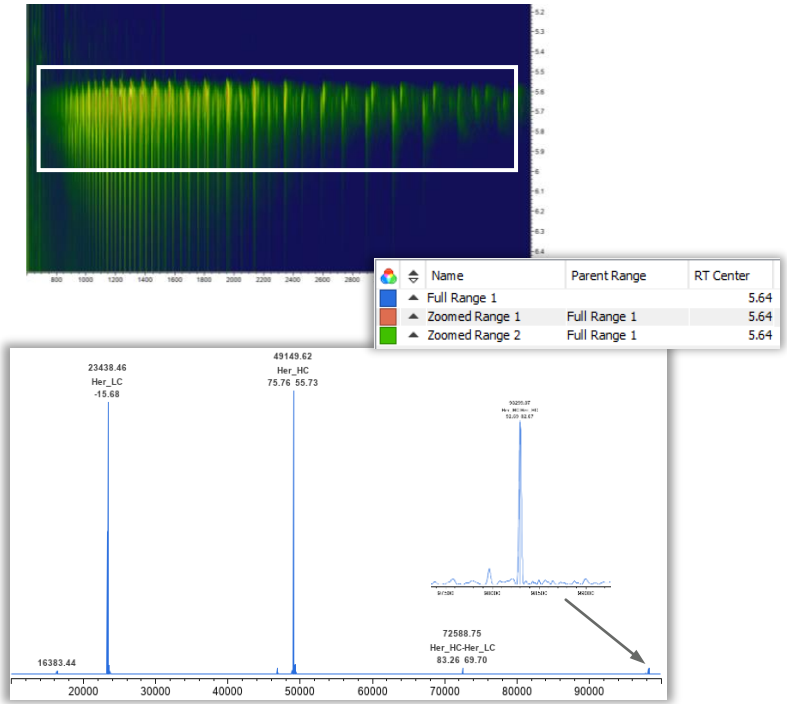
Sample type:	Protein reduced to HC and LC
Suggested workflow	Intact_AutomatedDeconvolution Intact_TimeResolvedDeconvolution (for fragments with complex modifications).
Deconvolution range [1]	20 kDa - 60 kDa or 20 kDa - 110 kDa to identify partially connected subunits. **Visible Ranges: All Ranges** particularly when subunits are unseparated.
Mass step (Da) [1]	1 - 2
RT Ranges [1] (Ignore with TRD)	**RT Window**: 3 - 9 **Isolation Threshold**: 3 - 10 (depending on the separation between components). Or manually input all ranges for many highly contiguous peaks.
Mass Tolerance (ppm) [2]	10 - 20 (depending 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

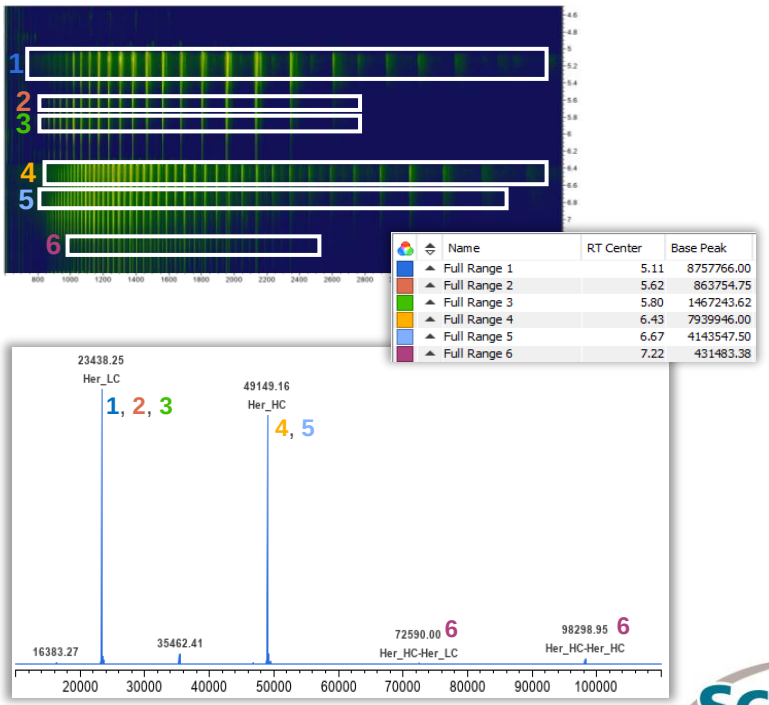
Subunit Analysis

FOCUS: Heavy chain and light chain.

Fully reduced



Partially connected



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Fragment Analysis

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
Suggested 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 (Da) [1]	1 - 2
RT Ranges [1] (Ignore with TRD)	**RT Window**: 3 - 9 **Isolation Threshold**: 3 - 10
Mass Tolerance (ppm) [2]	10 - 20 (depending 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

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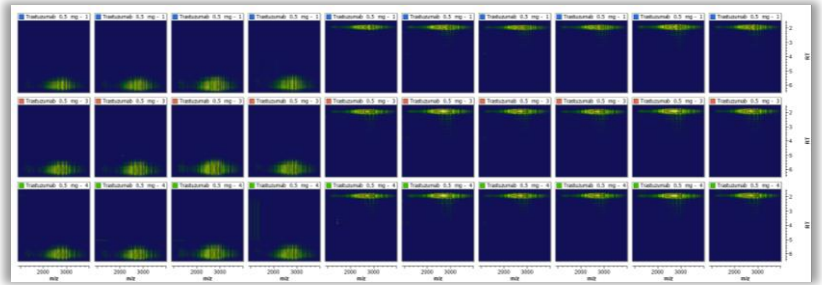
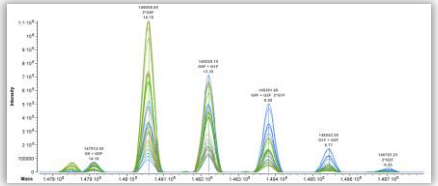
Comparability Test
or
Dilution Series

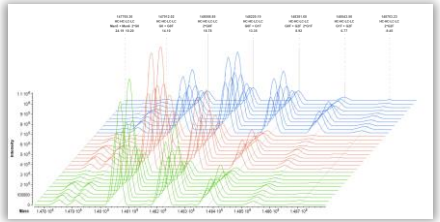
Comparability Test

Suggested initial settings:

Sample type:	Whole protein
Suggested workflow	Intact_AutomatedDeconvolution
Deconvolution range [1]	140 kDa - 160 kDa **Visible Ranges: Only Full Ranges** for visualization and reporting.
Mass step (Da) [1]	2
RT Ranges [1]	Manually input a single RT range that contains all of the target protein signals.
Mass Tolerance (ppm) [2]	20 - 50 (depending 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.*







- Reference mAb
- Biosimilar 1
- Biosimilar 2

[1]Deconvolution, [2]Protein Mapping

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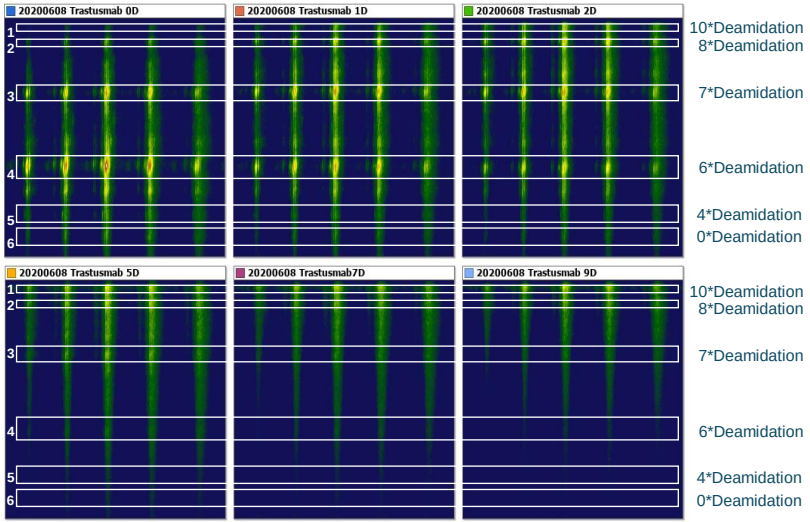
Stress Test

Stress Test

Suggested initial settings:

Sample type:	Whole protein
Suggested workflow	Intact_AutomatedDeconvolution
Deconvolution range [1]	140 kDa - 160 kDa **Visible Ranges: Only Full Ranges** for visualization and reporting.
Mass step (Da) [1]	2
RT Ranges [1]	Manually input all RT ranges for common peak detection across all samples and for meaningful relative quantitation.
Mass Tolerance (ppm) [2]	20 - 50 (depending 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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