



icIEF-UV+MS Data Analysis for Intabio ZT System

Biologics Explorer Software 6.0 Guidelines

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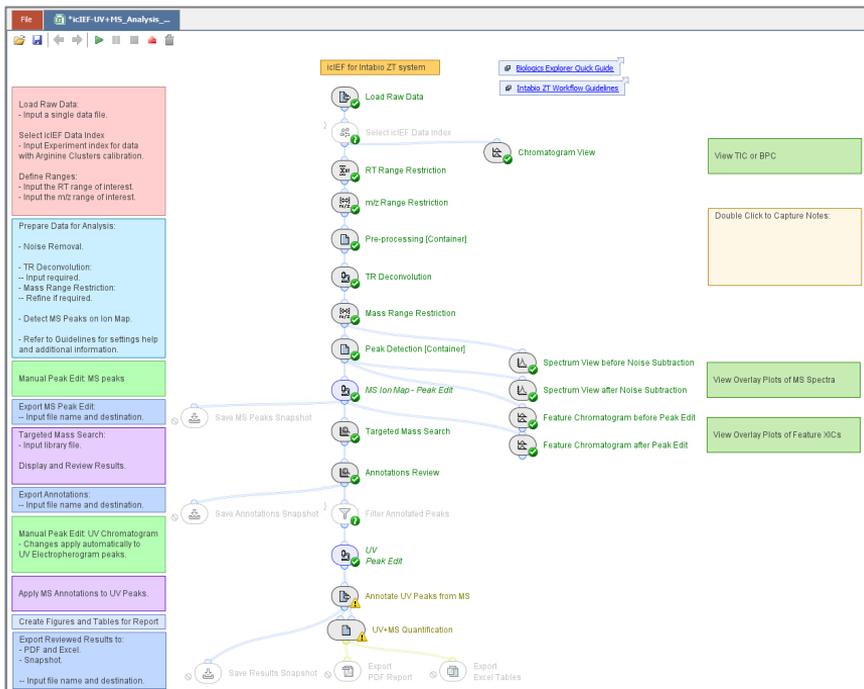
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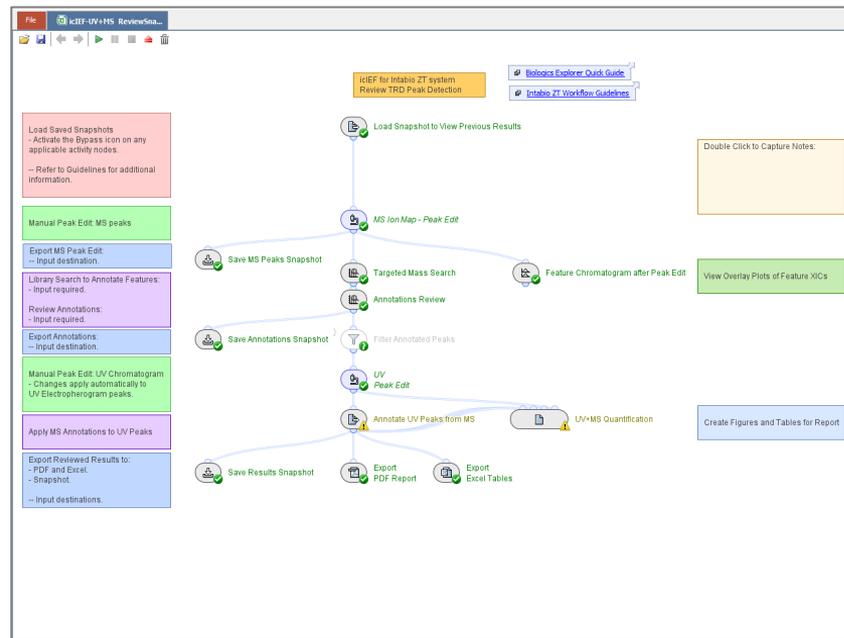
A: Overview of icIEF-UV+MS Data Analysis for the Intabio ZT System



Overview of the Workflows

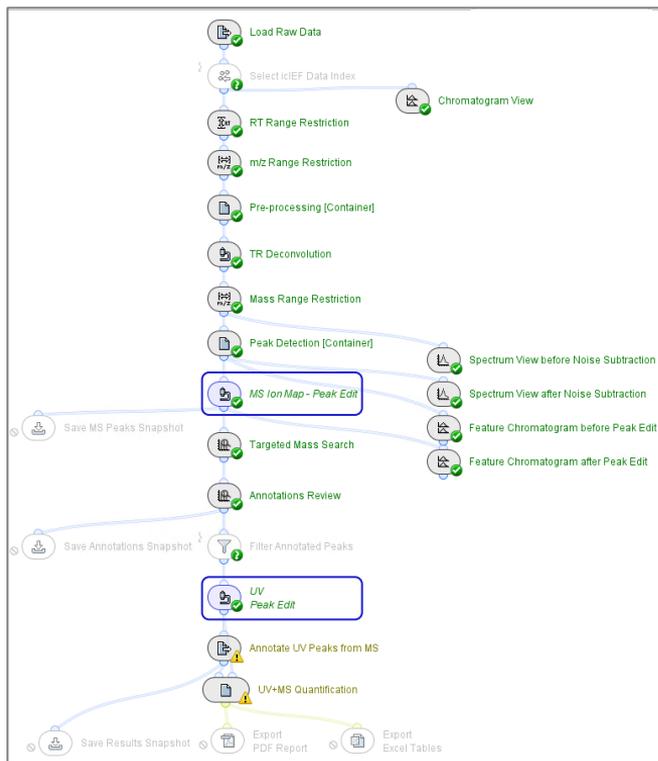


icIEF-UV+MS_Analysis_Be6.0



icIEF-UV+MS_ReviewSnapshots_Be6.0

How to Use the icIEF-UV+MS Workflows

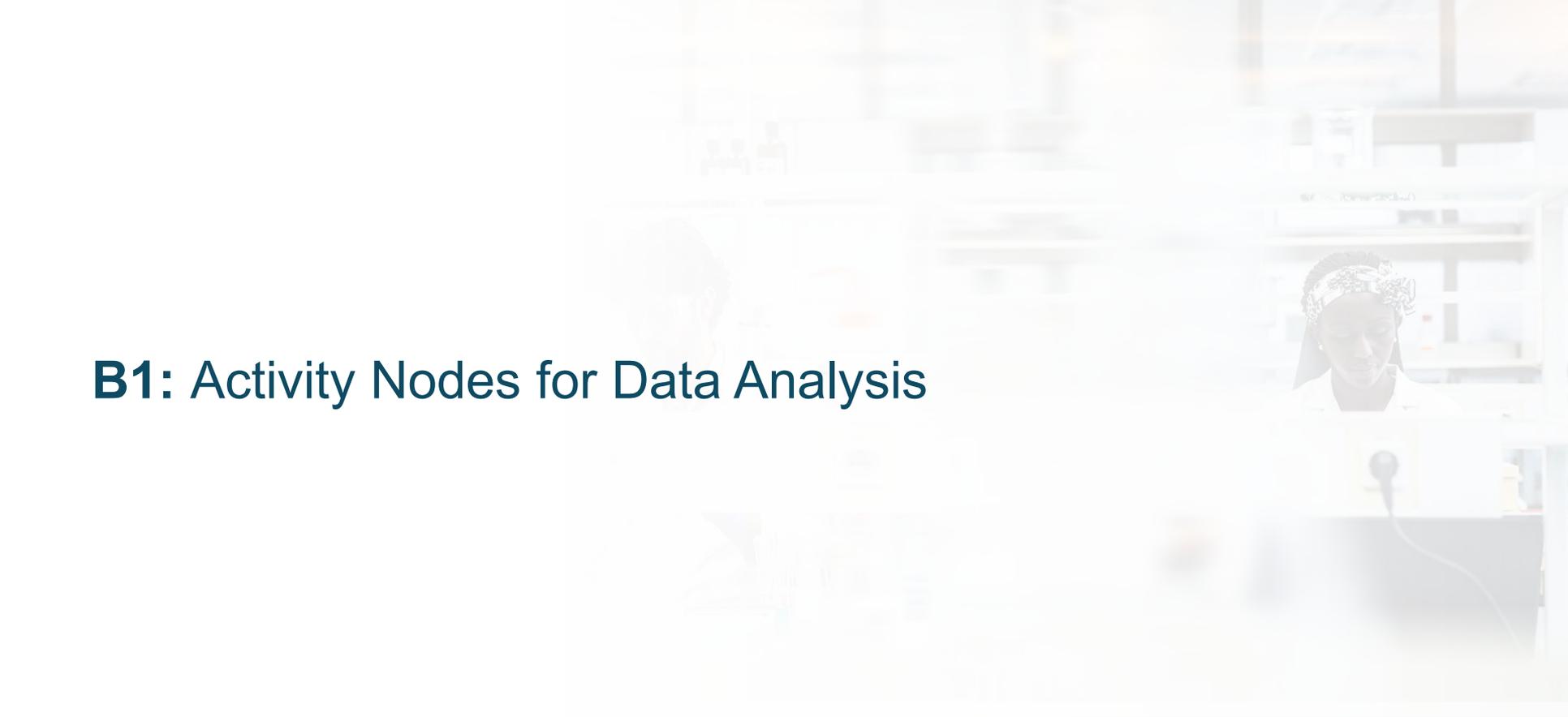


- Use the workflows to analyze a single sample from an icIEF-UV separation with the Intabio ZT system.
- The main path of the workflow processes the data.
- The branched activity nodes on either side contain tools to:
 - Visualize data before and after data processing.
 - Create tables and figures for the *Export Report* activity nodes.
 - Save intermediate data and final results as Snapshots (sbf files).
- The blue activity nodes require manual input to continue.
- Use the *Export Excel Report* for intermediate data analysis and quick access to results for optimization.
- Use the *Export PDF Report* for final data results.
 - Excel tables are also exported by the *Export PDF Report*.
- Use the icIEF-UV+MS_ReviewSnapshots workflow to continue analysis of saved sbf files.

B: Guidelines for icIEF-UV+MS Workflows



B1: Activity Nodes for Data Analysis

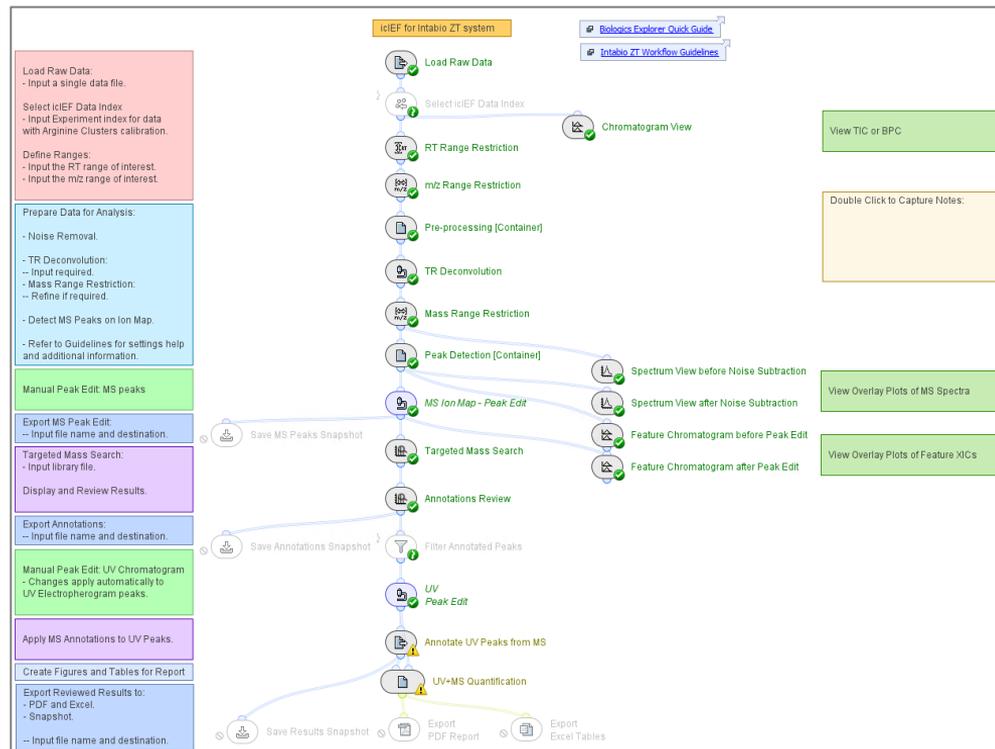


icIEF-UV+MS_Analysis Workflow

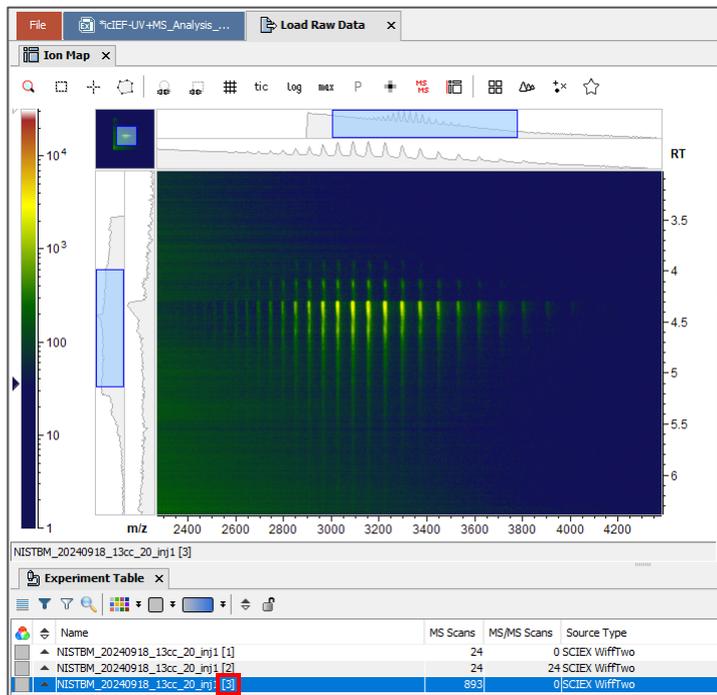
- This section contains information about these activity nodes of interest:

- *Select icIEF Data Index*
- *Spectrum Baseline Subtraction*
- *Chemical Noise Subtraction*
- *TR Deconvolution*
- *Peak Detection*
- *MS Ion Map - Peak Edit*
- *Targeted Mass Search*
- *Annotations Review*
- *Filter Annotated Peaks*
- *UV Peak Edit*
- *Annotate UV Peaks from MS*

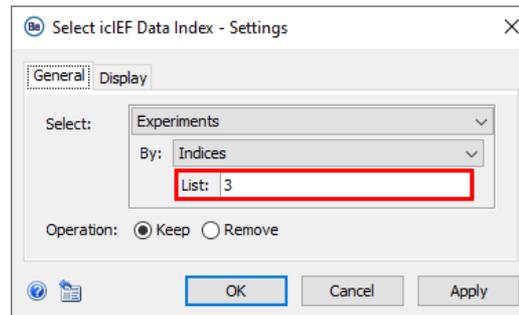
Note: For information about activity nodes that are used in all workflows, for example *Load Raw Data*, refer to the document: [Biologics Explorer Quick Guide](#).



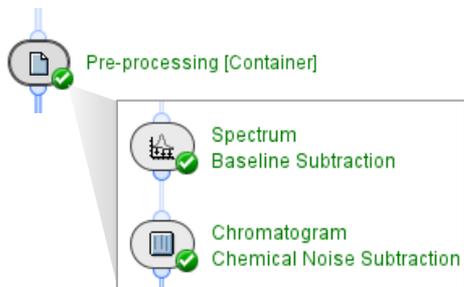
Select icIEF Data Index



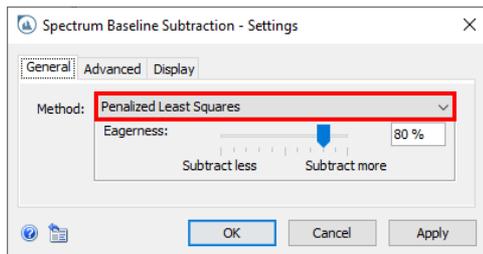
- To analyze data acquired with the Arginine Clusters calibration, deactivate the **Bypass** icon on *Select icIEF Data Index*.
 - Identify the applicable the **Experiments: Indices** value for the entry in the **Experiment Table** that contains the sample data.



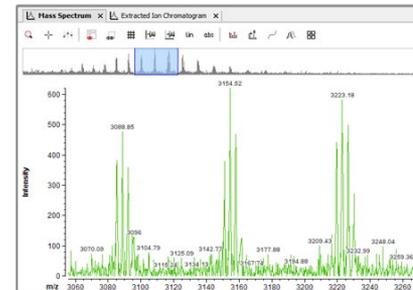
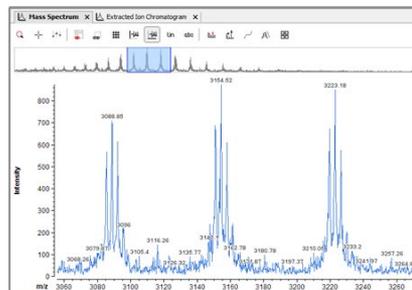
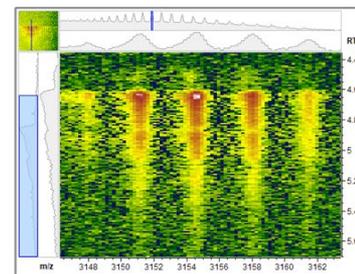
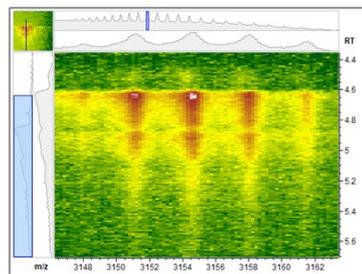
Spectrum Baseline Subtraction



- Use *Spectrum Baseline Subtraction* before deconvolution to remove background noise and decrease the intensity of satellite peaks in the deconvoluted data.
- Use *Spectrum Baseline Subtraction* after deconvolution to optimize peak detection.



- **Penalized Least Squares** subtraction has an effect on low intensity signals only.
- **Quantile** subtraction has an effect on all signals.
 - **Quantile** subtraction should be used with care for the analysis of intact proteins.

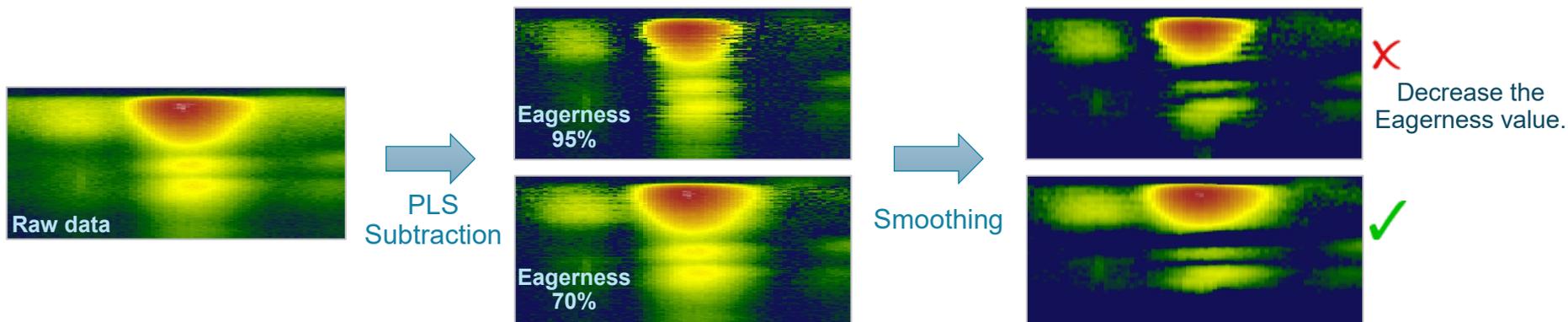


Before *Spectrum Baseline Subtraction*

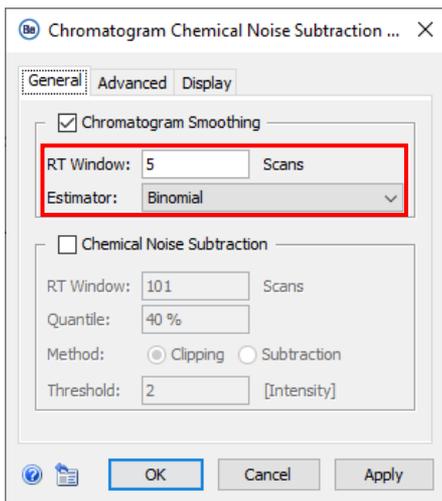
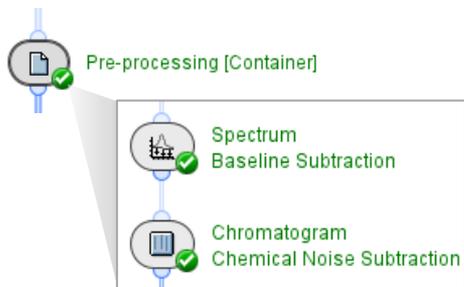
After *Spectrum Baseline Subtraction*

Spectrum Baseline Subtraction: Penalized Least Squares

- **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 *Chromatogram Chemical Noise Subtraction*.
 - If features in the ion map have irregular borders after smoothing, then decrease the **Eagerness** value.

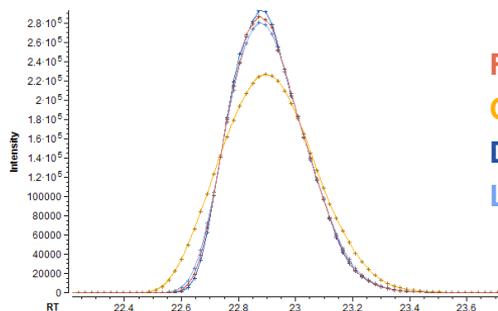


Chromatogram Chemical Noise Subtraction



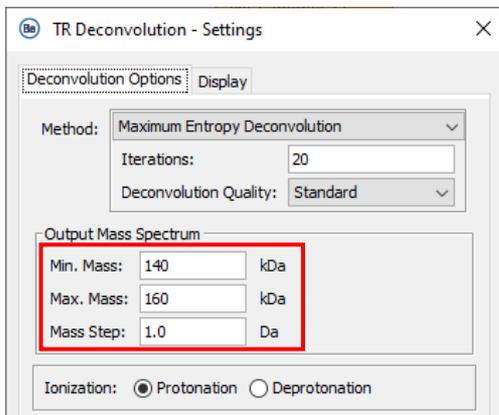
Chromatogram Smoothing is used to improve the RT profile of peaks.

- Use **Chromatogram Smoothing** after **Penalized Least Squares** (in *Spectrum Baseline Subtraction*), especially if a high **Eagerness** value was used.
- **Estimator:**
 - **Moving Average** replaces the intensity of each data point with the mean average intensity of the data points in the **RT Window**. High values cause peak widths to increase, but peak volume is conserved.
 - **Binomial** is an iterative form of **Moving Average** that has less effect on peak widths if a large **RT Window** (large number of scans) is used.



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

TR Deconvolution

TR Deconvolution - Settings

Deconvolution Options | Display

Method: Maximum Entropy Deconvolution

Iterations: 20

Deconvolution Quality: Standard

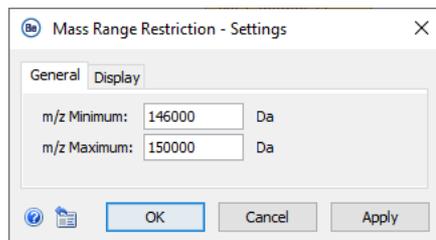
Output Mass Spectrum

Min. Mass: 140 kDa

Max. Mass: 160 kDa

Mass Step: 1.0 Da

Ionization: Protonation Deprotonation



Mass Range Restriction - Settings

General | Display

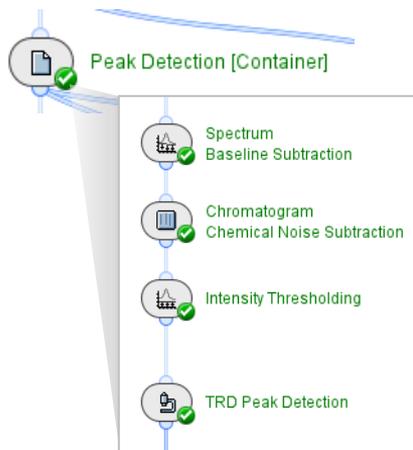
m/z Minimum: 146000 Da

m/z Maximum: 150000 Da

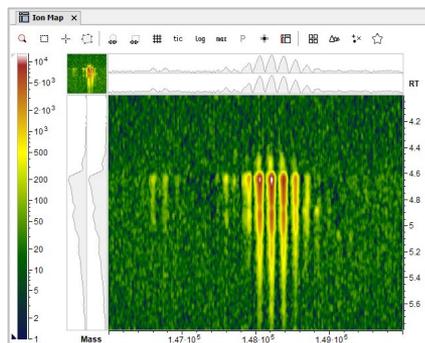
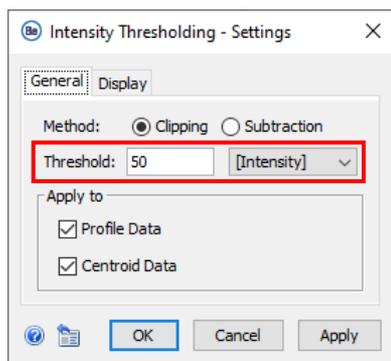
OK Cancel Apply

- **TR Deconvolution: Time-Resolved Deconvolution** occurs over the full RT range to create a deconvoluted ion map.
 - **Maximum Entropy Deconvolution:**
 - **Iterations:** Increase to 50 to increase peak definition.
 - **Deconvolution Quality:** The number of data points used for deconvolution.
 - Select **High** for isotopically-resolved data.
 - Select **Standard** for lower-resolution data.
 - **Min. Mass and Max. Mass:**
 - Use a wide mass range to reduce the number and intensity of harmonic peaks.
 - Optimize the **Mass Step:**
 - Set a value that keeps the peak resolution of the data.
 - 0.1 Da to 0.2 Da for isotopically-resolved data.
 - 1 to 2 Da for lower-resolution data.
- **Mass Range Restriction:** Enter the mass range that will be shown in the ion map.

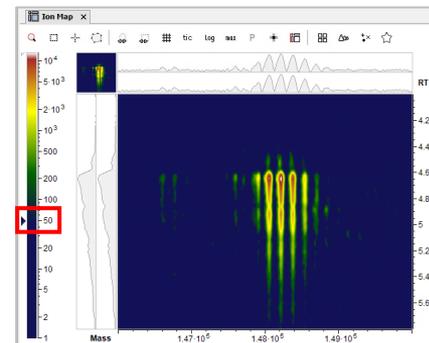
Peak Detection: Intensity Thresholding



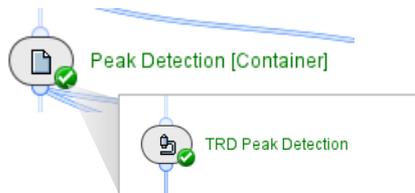
- To optimize peak detection after deconvolution, use *Spectrum Baseline Subtraction*.
 - Chromatogram Smoothing** is required after **Penalized Least Squares** subtraction.
- To control the level of background noise, use *Intensity Thresholding*.
 - Optimize the **Threshold** value for each dataset.
 - Make sure that sufficient noise is removed but the lowest-intensity proteoform of interested is detected.
 - Use the intensity slider on the ion map to find the correct value.



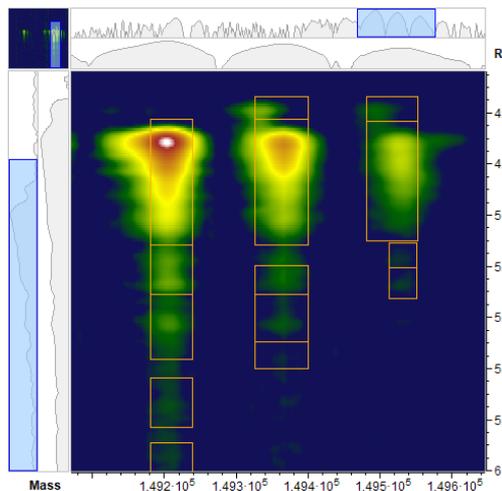
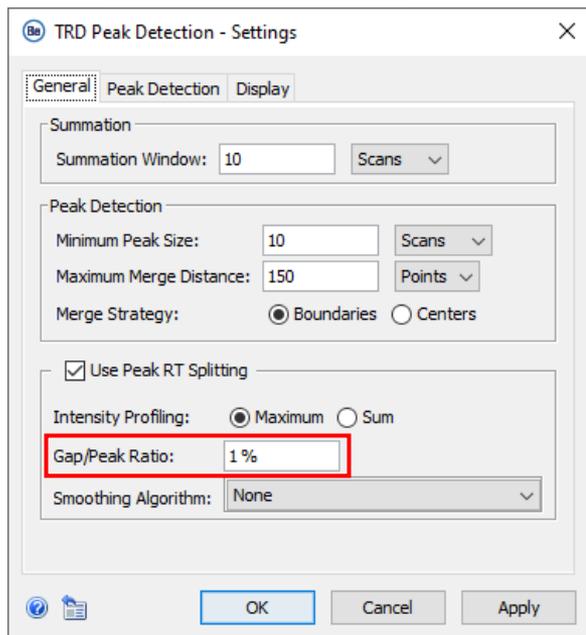
Visualize noise removal in the ion map with the intensity slider.



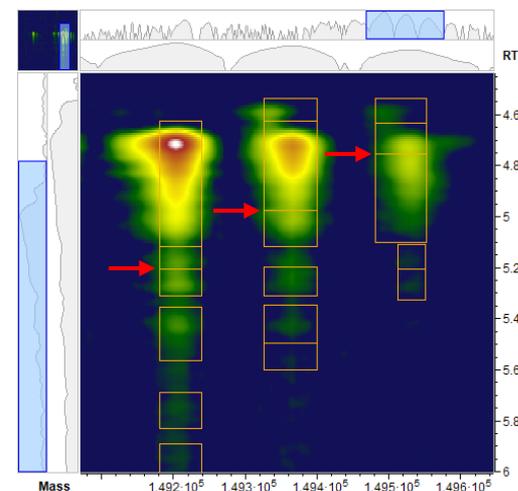
TRD Peak Detection: RT



- To control peak splitting in the RT direction, optimize the **Gap/ Peak Ratio**.
 - The percentage value specifies the minimum height difference between two closely eluting peaks that is required for them to be detected as separate peaks.

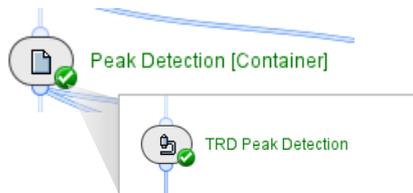


Gap/Peak Ratio: 10%

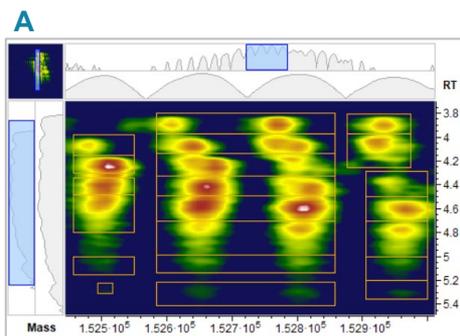
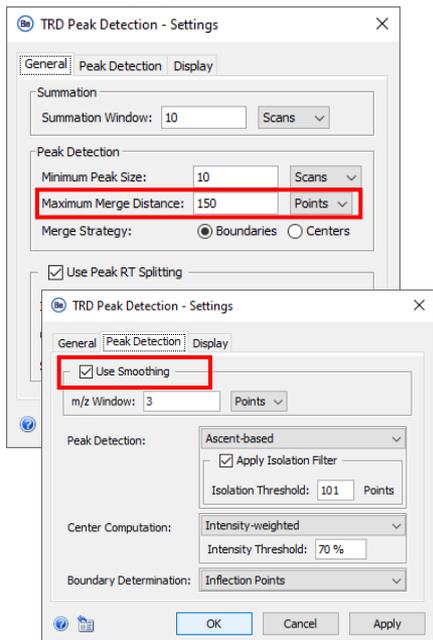


Gap/Peak Ratio: 1%

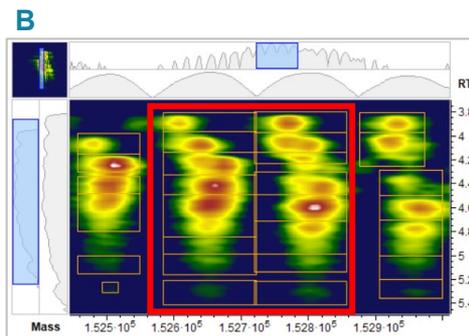
TRD Peak Detection: m/z



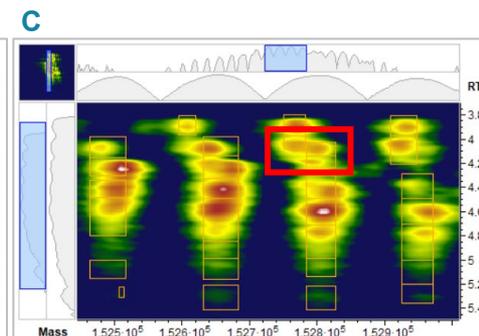
- To control peak splitting in the m/z direction, optimize **Use Smoothing** and **Maximum Merge Distance**.
- The following settings can improve m/z splitting for some samples:
 - Decrease the **Maximum Merge Distance** on the **General** tab (see **B** below).
 - Do not select **Use Smoothing** on the **Peak Detection** tab (see **C** below).



Maximum Merge Distance: 150 Points
Use Smoothing: Selected
 m/z Window: 3 Points

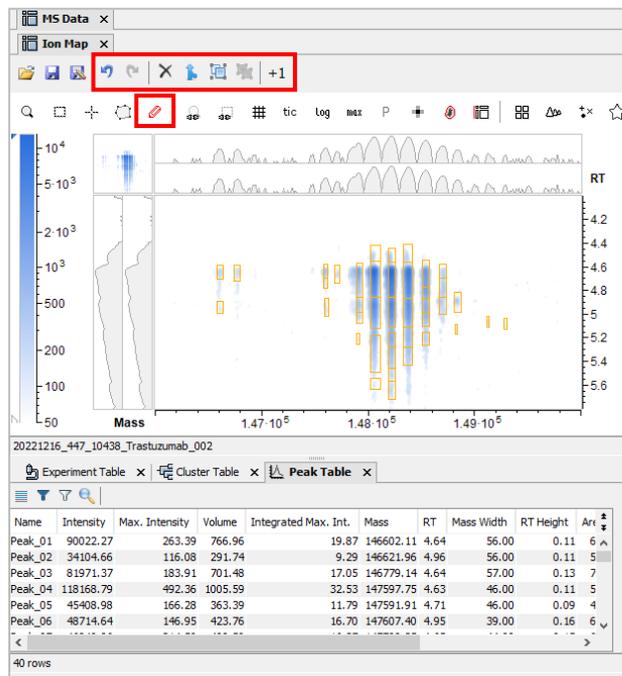


Maximum Merge Distance: 100 Points
Use Smoothing: Selected
 m/z Window: 3 Points

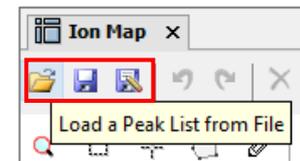


Maximum Merge Distance: 150 Points
Use Smoothing: Not selected

MS Ion Map - Peak Edit



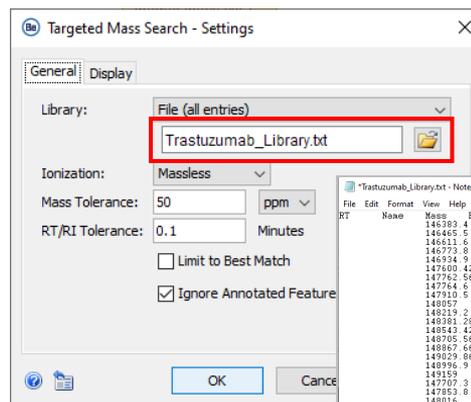
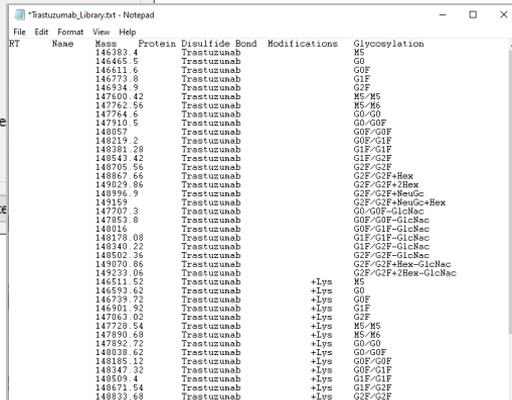
- To manually change the peaks in the ion map that were detected in *TRD Peak Detection* after deconvolution, use *MS Ion Map - Peak Edit*.
 - Select the **Edit Mode** icon  to:
 - Move the peak boundaries.
 - Merge or split selected peaks.
 - Delete peaks.
 - Draw new peaks.
 - Changes can be saved and the **Peak List** loaded again.
 - The saved **Peak List** keeps all characteristics of the original peaks.
 - Peak boundaries and peak centers (mass and RT coordinates) are not automatically updated.
 - If peak boundaries are changed, then the peak centers will be calculated again.



Targeted Mass Search

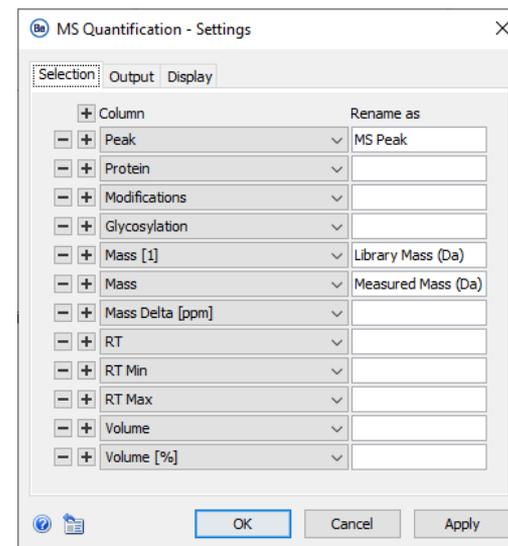


- The input library for *Targeted Mass Search* must be a tab-separated txt file.
 - The tab-separated txt file can be edited in Excel.
- To supply additional information, add columns to the library as required.
 - To control the columns that are shown in the final report, use the *MS Quantification* activity node, in the *UV+MS Quantification* container.

Trastuzumab_Library.bt - Notepad

RT	Name	Mass	Protein	Disulfide Bond	Modifications	Glycosylation
146283.4	Trastuzumab	MS				
146465.0	Trastuzumab	GO				
146611.6	Trastuzumab	G1F				
146773.8	Trastuzumab	G1F				
146934.9	Trastuzumab	G2F				
147000.42	Trastuzumab	MS+MS				
147762.55	Trastuzumab	MS+MS				
147754.6	Trastuzumab	GO-GO				
147910.5	Trastuzumab	GO-GOF				
148057	Trastuzumab	G0F-G0F				
148019.2	Trastuzumab	G0F-G1F				
148281.28	Trastuzumab	G1F-G1F				
148543.42	Trastuzumab	G1F-G2F				
148705.55	Trastuzumab	G2F-G2F				
148857.66	Trastuzumab	G2F-G2F+Hex				
149029.86	Trastuzumab	G2F-G2F+2Hex				
149156.9	Trastuzumab	G2F-G2F+HexGc				
149159	Trastuzumab	G2F-G2F+HexGc+Hex				
147707.9	Trastuzumab	GO-G0F-G1cNac				
147853.8	Trastuzumab	G0F-G0F-G1cNac				
148016	Trastuzumab	G1F-G1F-G1cNac				
148178.08	Trastuzumab	G1F-G1F-G1cNac				
148340.22	Trastuzumab	G1F-G2F-G1cNac				
148502.36	Trastuzumab	G2F-G2F-G1cNac				
149070.86	Trastuzumab	G2F-G2F+Hex-G1cNac				
149253.06	Trastuzumab	G2F-G2F+2Hex-G1cNac				
146511.52	Trastuzumab	+Iys MS				
146593.62	Trastuzumab	+Iys GO				
146739.72	Trastuzumab	+Iys G0F				
146901.92	Trastuzumab	+Iys G1F				
147063.02	Trastuzumab	+Iys G2F				
147728.54	Trastuzumab	+Iys MS+MS				
147890.68	Trastuzumab	+Iys MS+MS				
147892.72	Trastuzumab	+Iys GO-GO				
148038.62	Trastuzumab	+Iys GO-G0F				
148185.12	Trastuzumab	+Iys G0F-G0F				
148347.32	Trastuzumab	+Iys G1F-G1F				
148509.4	Trastuzumab	+Iys G1F-G1F				
148671.54	Trastuzumab	+Iys G1F-G2F				
148833.68	Trastuzumab	+Iys G2F-G2F				



Annotations Review



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

The screenshot displays the software interface with several panels:

- Annotations Review** window: Shows a table of annotations with columns for Name, RT Mn, RT, RT Max, and Max. Intensity. A red box highlights the 'Annotations' column header.
- Mass Spec.** window: Shows a mass spectrum plot with peaks labeled at 148217.518, 148607.601, 147598.338, and 148704.410.
- UV Chromatogram** window: Shows a chromatogram plot with peaks labeled at 4.470, 4.643 (Peak_5), 4.910 (Peak_4), 5.126, and 5.394 (Peak_1).
- UV Electropherogram** window: Shows an electropherogram plot with peaks labeled at 8.730 (Peak_1), 8.860 (Peak_3), 8.931 (Peak_4), 9.014 (Peak_5), and 9.097 (Peak_7).
- Chromatogram Table** window: Shows a table with columns for Name, RT Mn, RT, RT Max, and AUC [%].
- Electropherogram Table** window: Shows a table with columns for Name, pI Mn, pI Max, pI, AUC, and AUC [%].

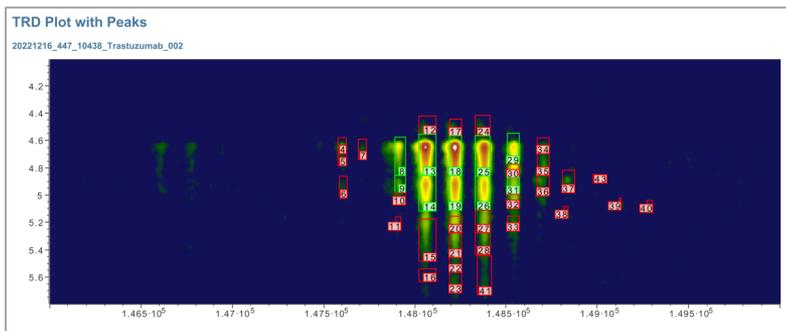
– Click on the **Annotations** column header to order by entry value.

Filter Annotated Peaks

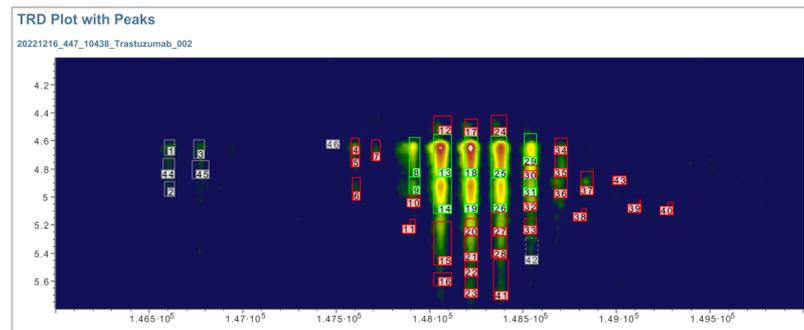


- Use *Filter Annotated Peaks* to remove peaks that are not annotated from the final results that are reported by *TRD Plot with Peaks*.
 - To use *Filter Annotated Peaks*, deactivate the **Bypass** icon.

With *Filter Annotated Peaks*:



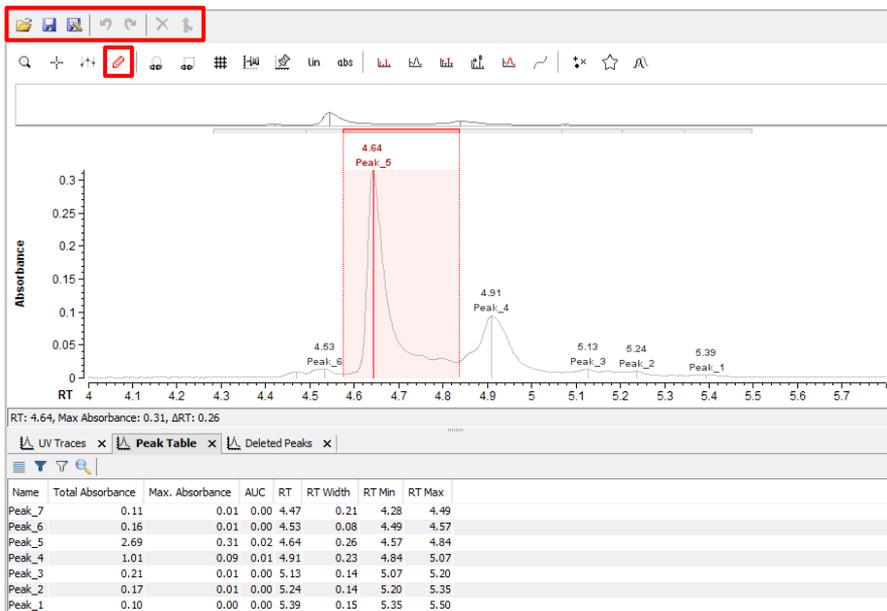
Without *Filter Annotated Peaks*:



- Solid **green** boundary + **peak number in green shading** = Peak was **accepted**.
- Solid **gray** boundary + **peak number in gray shading** = Peak was **rejected**.
- Solid **red** boundary + **peak number in red shading** = Peak is **annotated** but **unreviewed**.
- Dashed **gray** boundary + **peak number in gray shading** = Peak is **unannotated** and **unassigned**.

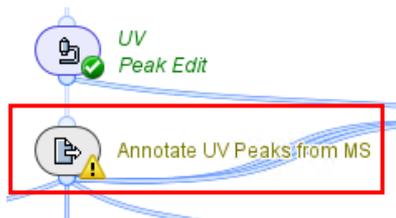


UV Peak Edit



- To manually change the UV peaks that were defined in the ibo file supplied by the Intabio ZT system, use *UV Peak Edit*.
- Select the **Edit Mode** icon  to:
 - Move the peak boundaries.
 - Merge selected peaks into a single peak
 - Delete peaks.
 - Draw new peaks.
- Changes made to the **UV Chromatogram** are also applied to the **UV Electropherogram**.
- Changes can be saved and applied to other data.

Annotate UV Peaks from MS



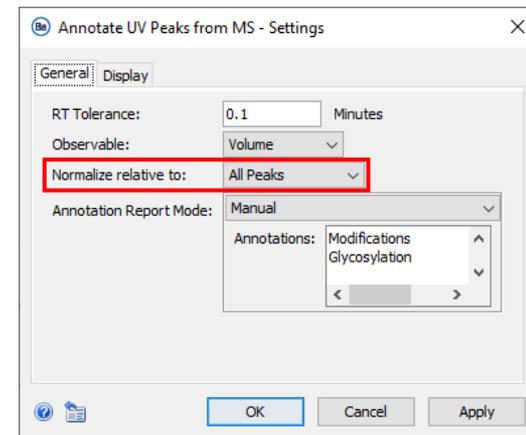
- This activity node uses MS peak information from *Annotations Review* to annotate the related peaks in the **UV Chromatogram** and **UV Electropherogram**.
 - A related peak must be detected in the specified **RT Tolerance**.

• Normalize relative to:

- All Peaks:** Relative UV absorbance is calculated across all detected peaks.
- Annotated Peaks:** Relative UV absorbance is calculated across all annotated peaks

• Annotation Report Mode: Manual:

- Select the information about the annotated features that is included in the result table.

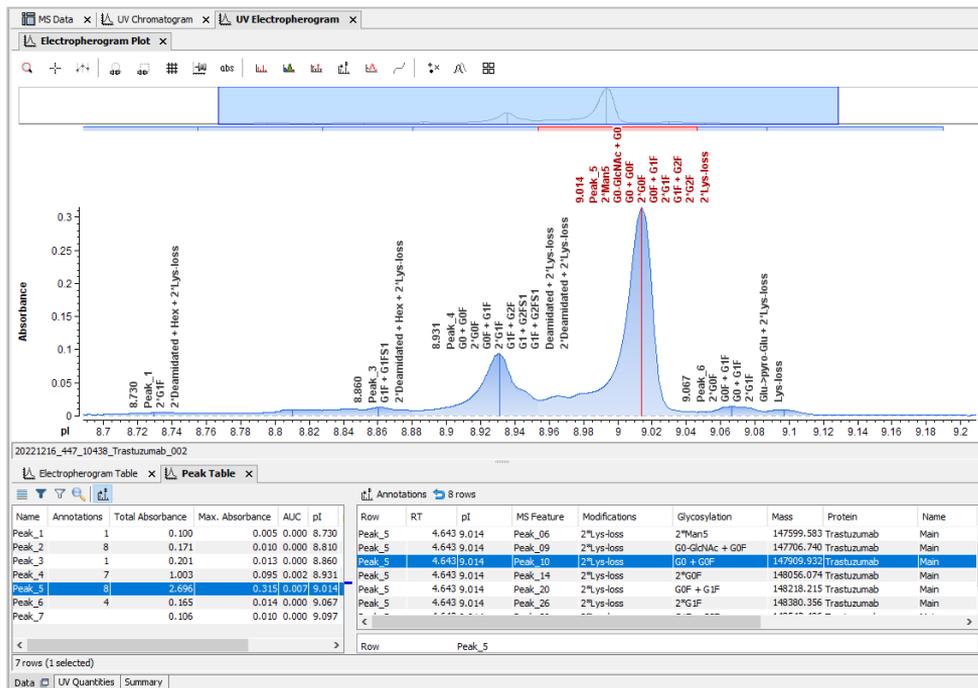


Note: If normalized UV abundances cannot be calculated, then the activity node shows a **yellow warning**.

Status	Suspicious
Message	The normalized UV absorbances could not be computed due to missing and/or duplicated annotations.

Annotate UV Peaks from MS

- To add labels to UV peaks, right-click the **Electropherogram Plot**, and then click **Settings**.
 - To see all MS identifications for each electropherogram peak, select **Orientation: Vertical** and increase the **Max. Visible Count**.



Settings

Labels | Grid

Color: █

Profiles: pi + -

Centroids: pi + -

Name + -

Glycosylation + -

Modifications + -

Orientation: Vertical + -

Magnification: 125% + -

Label Stacking: Horizontal + -

Value Stacking: Horizontal + -

Max. Text Width: 200

Max. Visible Count: 10

OK Cancel Apply

B2: Activity Nodes for Data Visualization



UV Electropherogram and UV Chromatogram Plots

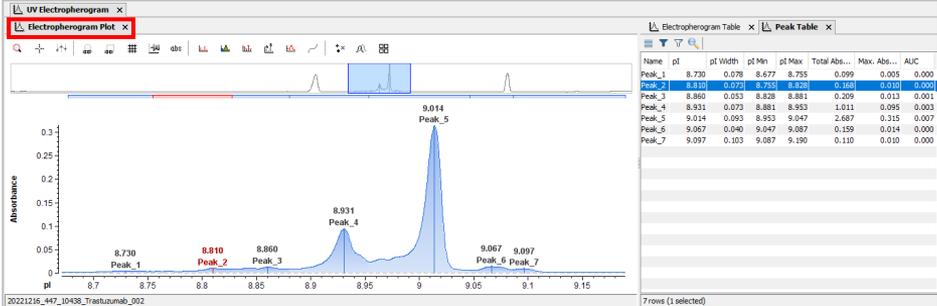
- The results of most of the activity nodes in the workflow contain the **UV Electropherogram Plot** and **UV Chromatogram Plot**.

- 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*.

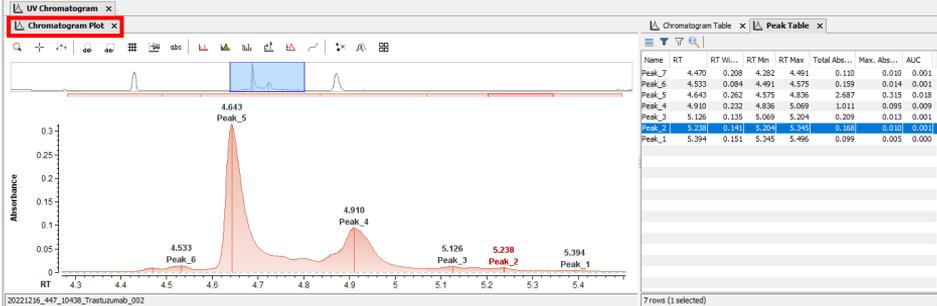
UV Electropherogram Plot

- Shows the icIEF image after separation, before mobilization.
- Data is in the pI scale.



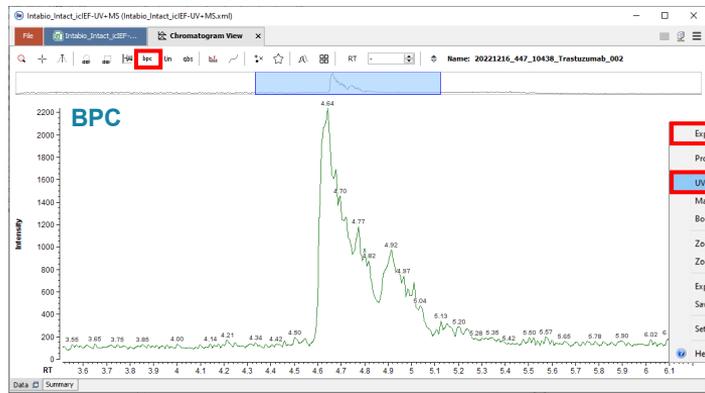
UV Chromatogram Plot

- Shows the icIEF image after manual alignment in the Intabio software.
- Data is in the RT scale.



Chromatogram View

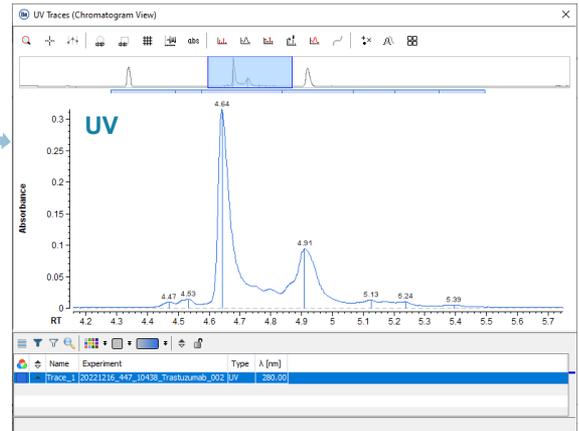
- Chromatogram View shows the total ion chromatogram (TIC), or base peak chromatogram (BPC), of the data before pre-processing.



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

Name	Scans	Method Name	Method Date	Source Type	UV Shift
20221215_447_10438_Trasuzumab_002	1134	20221216_NIST_nano_10mm	PM Dec 16 12:44:54 CET 2022	SCIEX WFTW	0.00

- Experiment List...
- Profiles...
- UV Traces...
- Markers...
- Bookmarks...
- Zoom To... Ctrl+G
- Zoom Full
- Export Data...
- Save Graphic >
- Settings...
- Help



To open the **Experiment List** and **UV Chromatogram**, right-click the plot.

Spectrum View

- Spectrum View before Noise Subtraction
- Spectrum View after Noise Subtraction

- To see a deconvoluted mass spectrum for each MS scan before and after noise subtraction, use the *Spectrum View* activity nodes.

The screenshot displays the Spectrum View software interface with three main panels: Mass Spectrum, UV Chromatogram, and UV Electropherogram. Each panel has a corresponding data table below it.

Mass Spectrum Table:

RT	Intensity
147267	~50
147654	~50
147723	~50
148002	~100
148052	~150
148383	~150
148782	~150
149300	~150
149446	~150
149775	~150
149775	~550

UV Chromatogram Table:

Name	RT	RT Width	RT Min	RT Max	Total Absorbance
Peak_7	4.4661	0.203772	4.26234	4.66988	0.109774
Peak_6	4.53253	0.0840570	4.48052	4.57453	0.159026
Peak_5	4.64289	0.291362	4.34959	4.83877	2.68741
Peak_4	4.90974	0.223401	4.83617	5.06857	1.01074
Peak_3	5.12642	0.13534	5.06857	5.20391	0.209178
Peak_2	5.23801	0.141271	5.20391	5.34518	0.168467
Peak_1	5.3939	0.151013	5.34518	5.49619	0.0994977

UV Electropherogram Table:

Name	RT	RT Width	RT Min	RT Max	Total Absorbance
Peak_1	8.72957	0.0779905	8.67674	8.75473	0.100456
Peak_2	8.81008	0.0729589	8.75473	8.82769	0.170988
Peak_3	8.8604	0.0528316	8.82769	8.88052	0.200919
Peak_4	8.92064	0.0729589	8.88052	8.95349	1.0031
Peak_5	9.01386	0.0838643	8.95349	9.04657	2.6962
Peak_6	9.06669	0.0402536	9.04657	9.08683	0.165204
Peak_7	9.08683	0.03348	9.08683	9.18997	0.106059

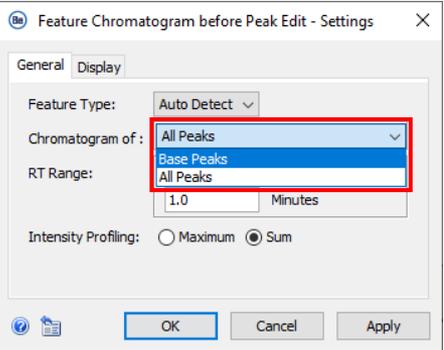
The interface also includes a 'Filter Tool' and a 'Peak Table' for each panel, allowing users to select time ranges and view detailed peak data.

- Use the **Filter Tool** in the **Spectrum Table** to select the time range of interest for each UV peak.
- The applicable scans are shown in the **Spectrum Table**.
- Use the **Peak Table** of the **UV Electropherogram** tab to view the **pI** information of each peak.

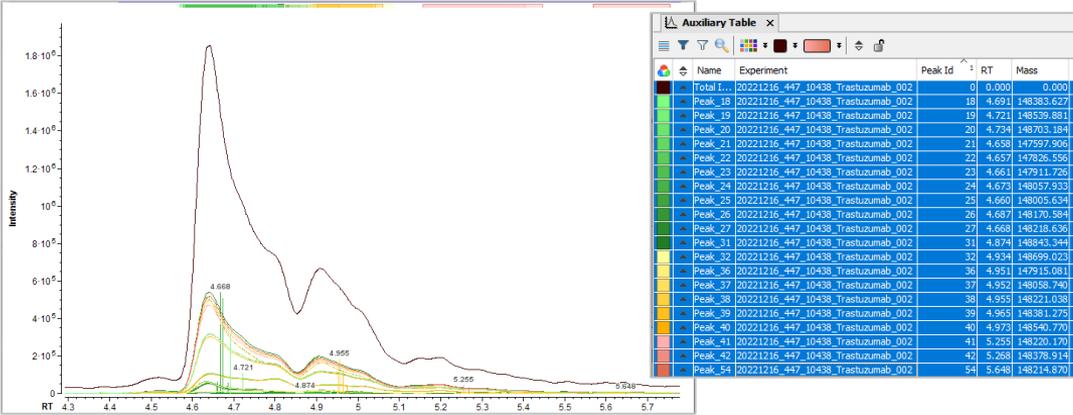
Feature Chromatogram

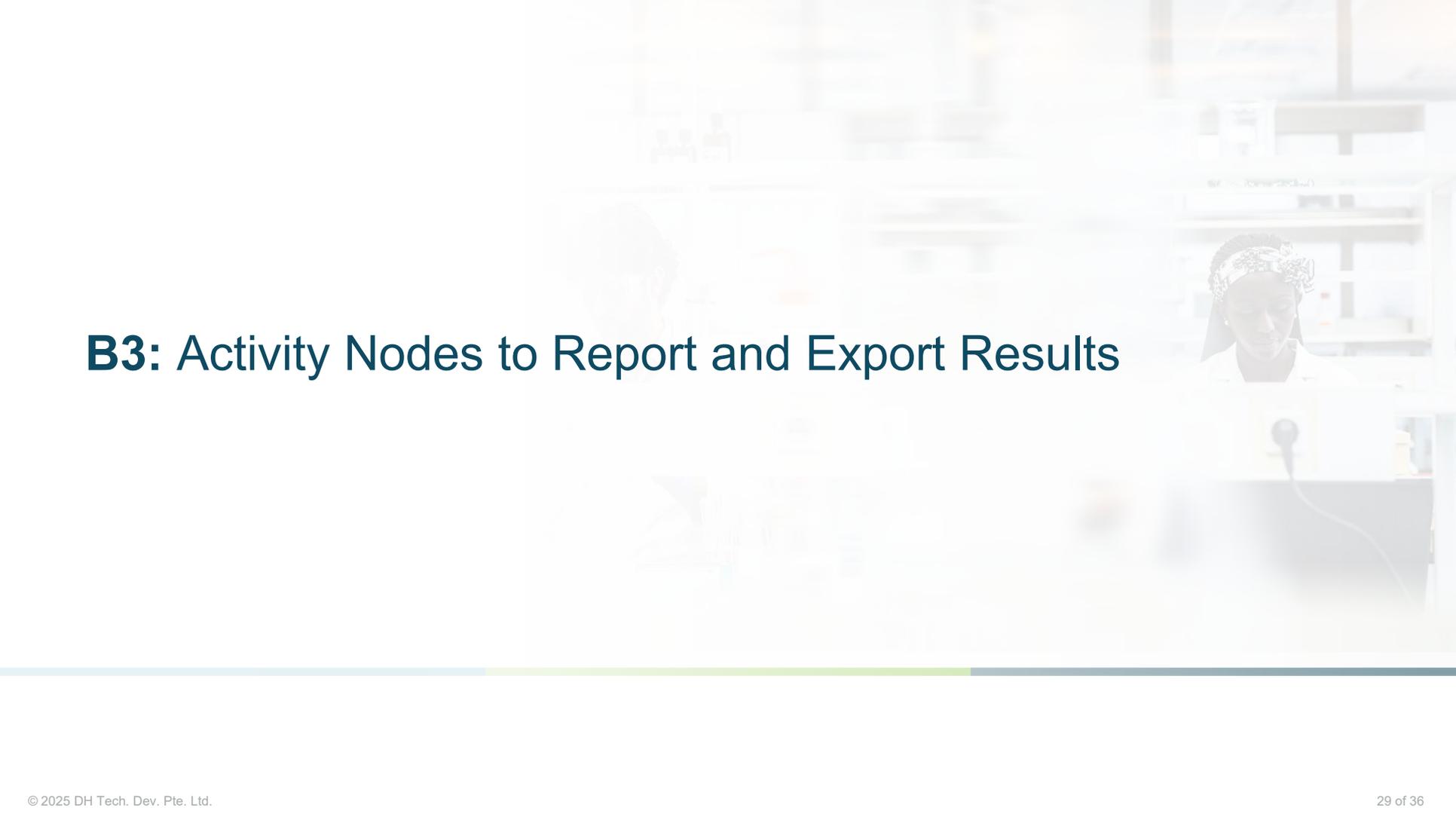


- To see the deconvoluted extracted mass spectrum for each feature before and after *MS Ion Map - Peak Edit*, use the *Feature Chromatogram* activity nodes.



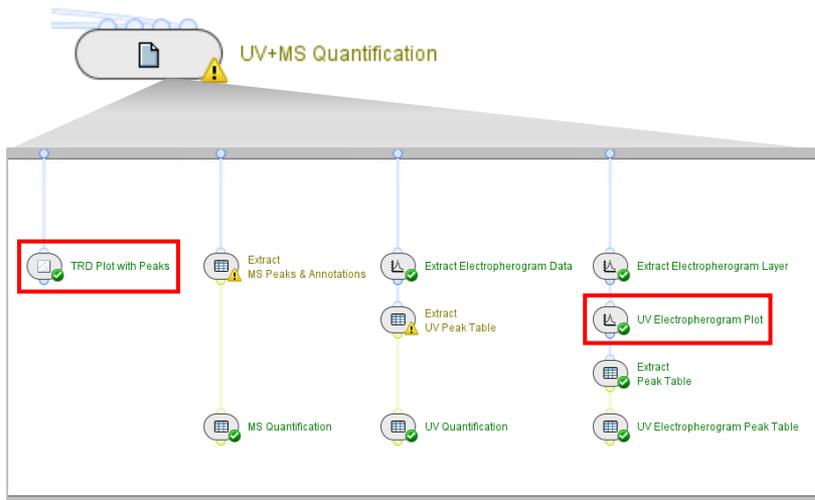
- To show the BPC, select **Base Peaks** in the **Chromatogram of** field in the settings.
- Visually align the results of this activity node with the UV Electropherogram to select the correct peak boundaries in the *MS Ion Map - Peak Edit* activity node.



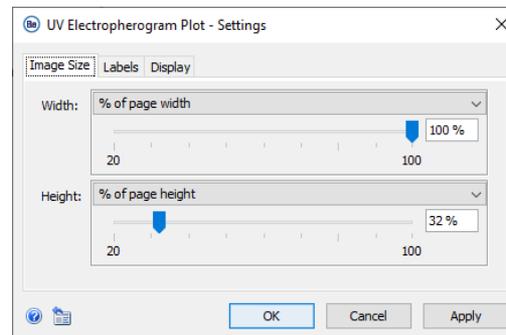
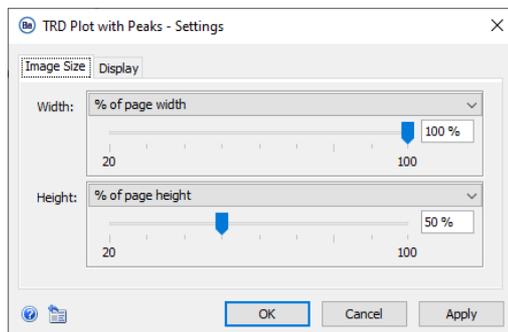


B3: Activity Nodes to Report and Export Results

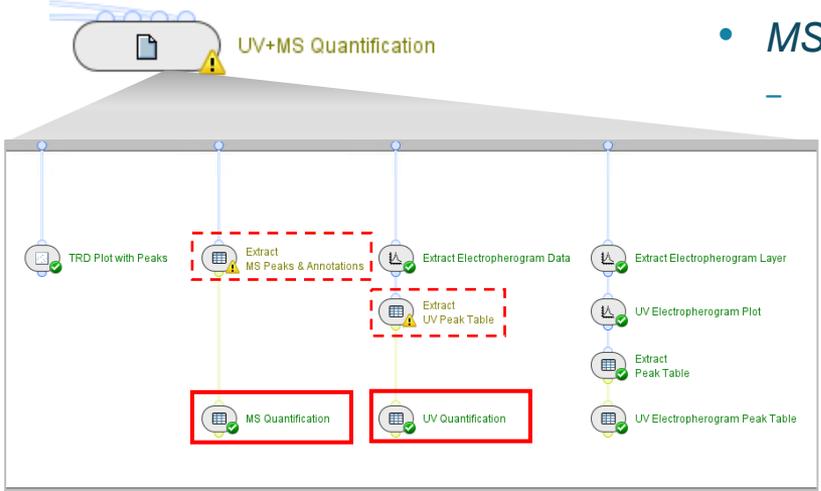
Extract Report Elements: *UV+MS Quantification*



- Use the activity nodes in *UV+MS Quantification* to specify what is included in the exported *PDF Report* or *Excel Tables*.
 - *TRD Plot with Peaks*
 - Select the size of the image in the PDF Report.
 - *UV Electropherogram Plot*
 - Select the size of the image in the PDF Report.
- Note: For more information, refer to the page: [Filter Annotated Peaks](#).



Extract Report Elements: *UV+MS Quantification*



- *MS Quantification and UV Quantification*
 - Use the +/- icons to select the columns to include in the PDF Report.

Results

MS Quantification

MS Peak	Protein	Modifications	Glycosylation	Library Mass (Da)	Measured Mass (Da)	Mass Delta (ppm)	RT	RT Min	RT Max	Volume	Volume [%]	Review Status	Comment
1					146607.601		4.642	4.590	4.705	766.960	0.216		
2					146614.485		4.948	4.889	4.994	291.738	0.082		
3					146774.883		4.643	4.590	4.722	701.485	0.197		
4	Trastuzumab			147600.420	147598.338	-14.106	4.634	4.582	4.698	1006.502	0.283		
5	Trastuzumab										0.102		
6	Trastuzumab										0.119		
7	Trastuzumab										0.115		
8	Trastuzumab										1.980		
9	Trastuzumab										0.488	accepted	Main
10	Trastuzumab										0.157		

Extract MS Peaks & Annotations - Settings

Output Display

Extracted Data: **Peaks and Annotations**

Include Peaks without Annotations:

Table Name: **Peaks**

MS Quantification - Settings

Selection Output Display

Column	Rename as	
+	Peak	MS Peak
+	Protein	
+	Modifications	
+	Glycosylation	
+	Mass [1]	Library Mass (Da)
+	Mass	Measured Mass (Da)
+	Mass Delta [ppm]	
+	RT	
+	RT Min	
+	RT Max	
+	Volume	
+	Volume [%]	
+	Protein	
+	Disulfide Bond	
+	Modifications	
+	Glycosylation	
+	Mass Delta	
+	Mass Delta [ppm]	
+	Review Status	
+	Comment	

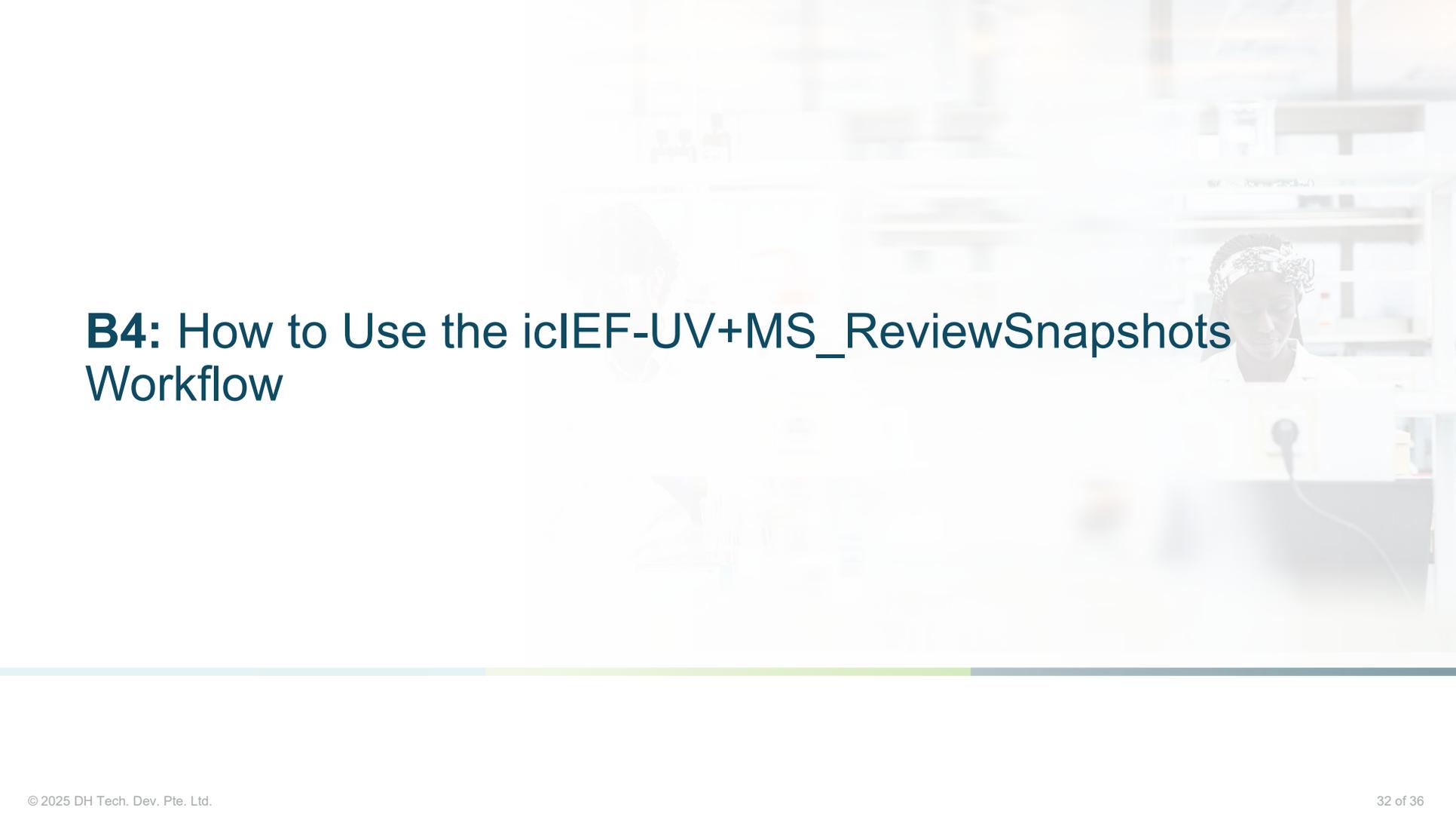
UV Quantification - Settings

Selection Output Display

Column	Rename as	
+	Peak	UV Peak
+	MS Feature	MS Peak
+	pi	
+	pi Min	
+	pi Max	
+	Mass	
+	Mass Delta [ppm]	
+	Modifications	
+	Glycosylation	
+	Total Absorbance	Total Absorbance (fo AUC)
+	Total Absorbance [%]	Total Absorbance %

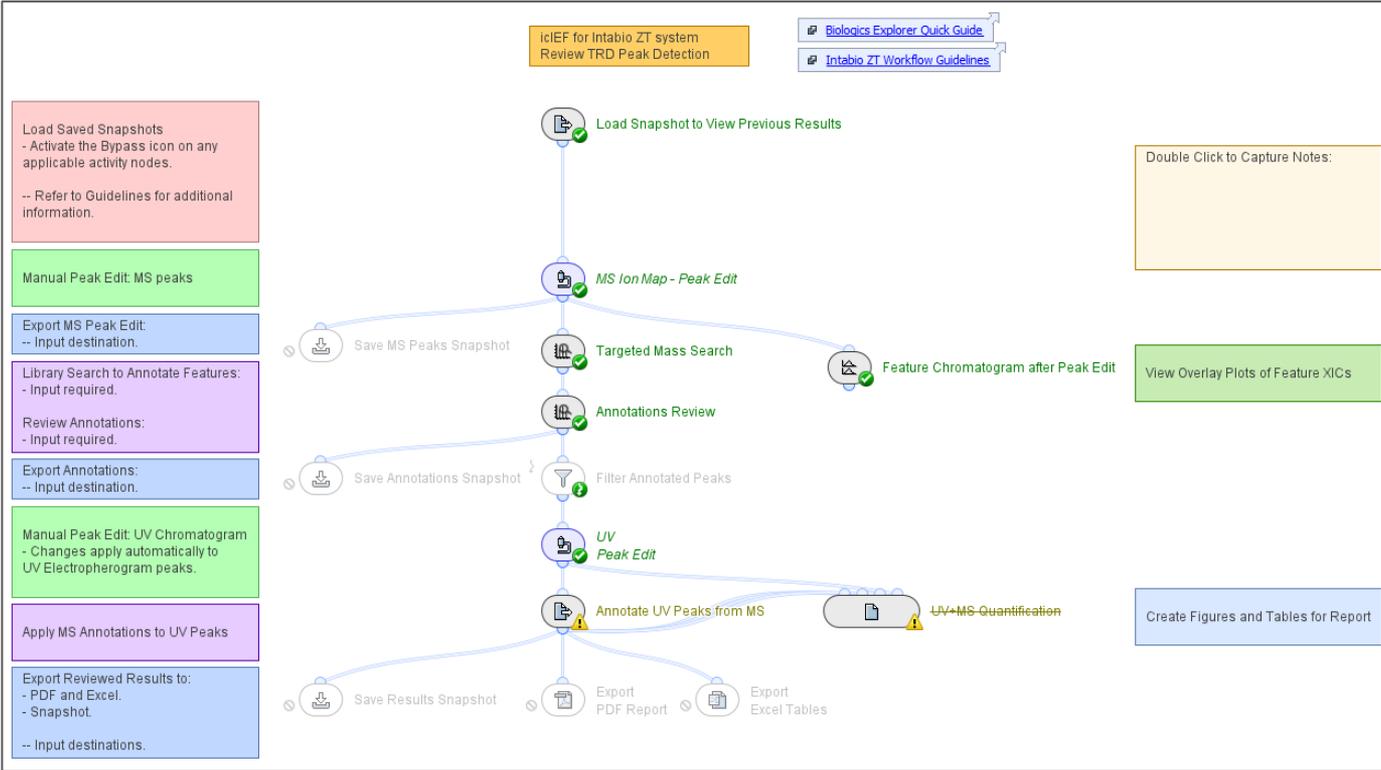
Note: If **Review Status** is selected, but there are no accepted identifications, then the activity node shows a **yellow warning**.

- **Review Status** and **Comment** do not apply to rejected peaks.
- To remove unannotated and rejected peaks from the reported tables, do not select **Include Peaks without Annotations** in the *Extract Peaks* activity nodes.



B4: How to Use the icIEF-UV+MS_ReviewSnapshots Workflow

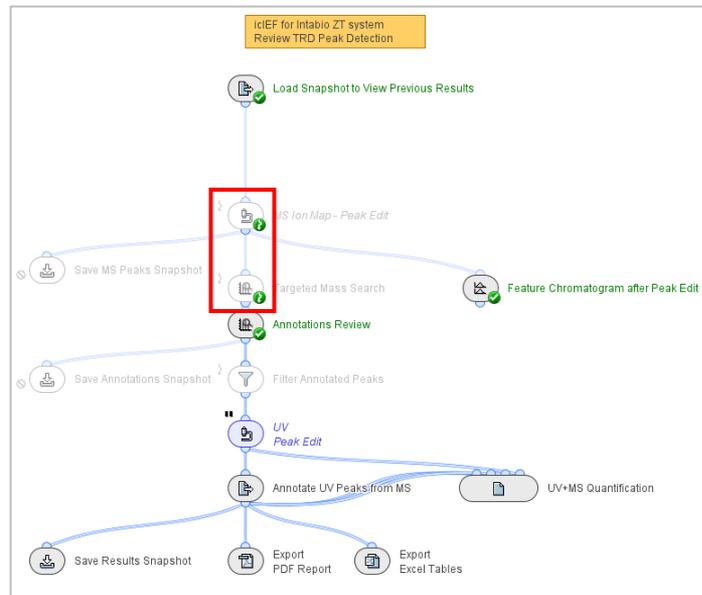
icIEF-UV+MS_ReviewSnapshots Workflow



How to Use the Review Snapshots Workflow

- Use the Review Snapshots workflow to:
 - Open a saved Snapshot (sbf) file created using an icIEF-UV+MS_Analysis Workflow.
 - If required, then continue to edit the peak detection on the ion map.

- To use intermediate results in a saved sbf file:
 1. Use the icIEF-UV+MS_ReviewSnapshots workflow.
 2. Select the sbf file in *Load Snapshots to View Previous Results*.
 3. Activate the **Bypass** icon on the activity nodes that are before the sbf file was saved.
 - For example: To load an sbf file from *Save Annotations Snapshot*, activate the **Bypass** icon for the activity nodes between *Load Snapshot to View Previous Results* and *Annotations Review*.
- The activity nodes in this workflow are used exactly as described for the icIEF-UV+MS_Analysis workflow.





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