Intact Mass Analysis

Biologics Explorer 2.0 Quick Guide

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Intact Mass Analysis: Biologics Explorer Quick Guide

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Intact Mass Analysis: Biologics Explorer Quick Guide

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- Comparability Test or Dilution Series
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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

- Post-translational modification (PTM) characterization
- Glycosylation pattern analysis
- 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 mixtures

- Protein subunits/fragments

- Drug conjugates

Multimeric proteins



Part A Software and Workflows

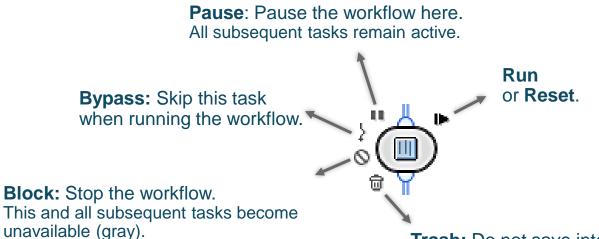
2. USING BIOLOGICS EXPLORER





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ACTIVITY NODE ICONS



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.



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. Active 2. PeptideMapping_Simple **Workflow Ready** PeptideMapping_Extended No activity nodes have been completed. The PeptideMapping_Comparative Analysis workflow is ready to start. 2. PeptideMapping Simple 3. PeptideMapping_Extended Workflow Error Some activity nodes have been completed successfully, but at least one activity node cannot run.

Workflow Warning Some activity nodes are incomplete.



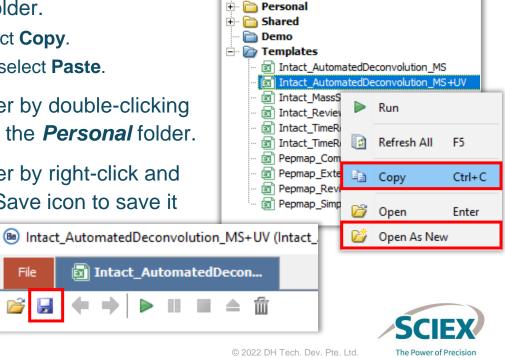
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START AND SAVE WORKFLOWS

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**.
 - Right-click the **Personal** folder and then select **Paste**. 2.
- 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.

File



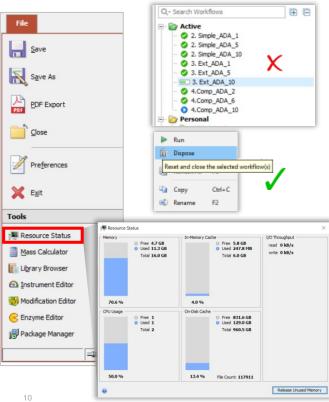
Q - Search Workflows

Intact AutomatedDeconvolution MS+UV

🖃 📄 Active

F -

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.



ACCESS THE ONLINE HELP

	d Deconvolution - Settings Options RT Ranges Display	×		formation about individual act eir settings, click the ? icon to
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	Visible Ranges: Only Full Rang	 □ Solitate Protein - SCIEX BE 2.0 Doc × ← → C □ https://expres 	+ sionist.genedata.com/display/SN20/Intact+Protein	A* 16
Method:	Filter RT Ranges Eagerness: Standard Maximum Entropy Deconvolution Iterations: 20	 How to Activities Alignment activities Bioanalytics activities Deconvolution 	Be Intact Protein	
Ionization:	Deconvolution Quality: Standar Protonation Operotonation	Intact Protein Intact Protein (Deprecati Intact Protein Screening Peptide Mapping Peptide Mapping View Protein Mapping	Function Maximum Entropy deconvoluted mass	ity deconvolutes intact protein data to provide mass spectra of protein species. Three methods are available for deconvolution: y Deconvolution — This method uses a Genedata proprietary algorithm based on the MaxEnt algorithm ^[1] to find the best spectrum using a probabilistic approach^[2] . sion Deconvolution — This method uses a Genedata proprietary algorithm that suppresses false signals resulting from mass
		Protein Mapping Protein Mapping View Wildcard Mapping	Activity settings values that are mul • Legacy Maximum Output MaxEnt ^[1] algorithm	tiples or fractions of the correct signals. Entropy Deconvolution — This method uses a maximum entropy method that uses the Harmonic Suppression and ns.
	ОК	Released Glycans Calibration activities Combining activities	communications in mass s [2] Marty, M.T., Baldwin, A	M. J. Jarvis, S. Skilling, J., & Aplin, R. (1991). Maximum entropy deconvolution in electrospray mass spectrometry. <i>Rapid pectrometry</i> , <i>5</i> (8), 374-377. J., Marklund, E.G., Hochberg, G.K., Benesch, J.L., & Robinson, C.V. (2018). Bayesian deconvolution of mass and ion mobility spectration polydisperse ensembles. <i>Anal Chem.</i> Apr 21:87(8):4370-6.

individual activity nodes k the ? icon to view the



- 0

Part A Software and Workflows

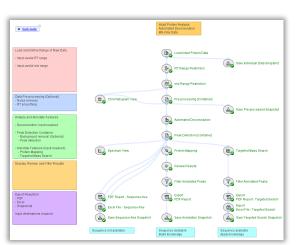
3. GENERAL GUIDELINES FOR INTACT WORKFLOWS



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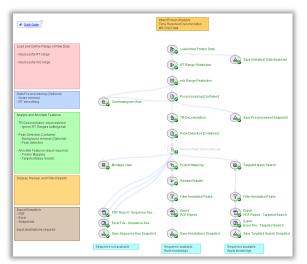
General Intact Workflow Guidelines

WORKFLOW TYPES



Intact_Automated Deconvolution

With and without UV processing



Intact_TimeResolved Deconvolution

With and without UV processing



Intact_MassScreening



Intact_ReviewSnapshots



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
- Protein Mapping
- J. Targeted Mass Search
- K. Annotate UV Peaks from MS
- L. Reporting and Exporting

Load Intact Protein Data: Format (Auto Detect)

General Advan	Protein Data - Settings X					
Name:	Intact Mass Analysis					
Format:	Auto Detect 🗸					
Files/Folders:	Name Color Sample001_NIST_1000ng_290DP_01.wiff2 #286dde					
	1 rem					
0	OK Cancel Apply					

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Herceptin Deglycosylated 1pmol_uL	[7]			MS		~	
Kadcyla 1pmol_ul. [8]]		MS		*	
C Kadcyla 1pmol_uL [9]				MS		Ŧ	
3 out of 62 selected.	L [1]" "Herceptin De	glycosylated	1pmol	Oper	Close		

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.

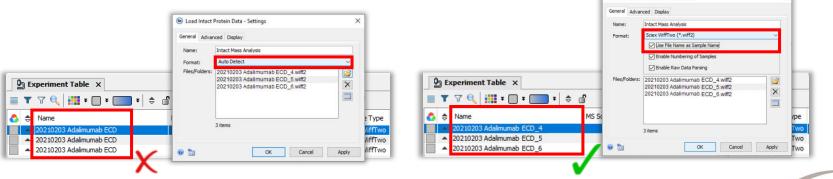
Herceptin_Kadcyla 20161101.wiff2
 Herceptin_Kadcyla 20161101.wiff.scan

- 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.



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.





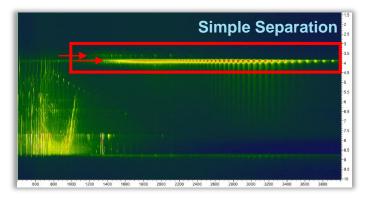
Load Intact Protein Data - Settings

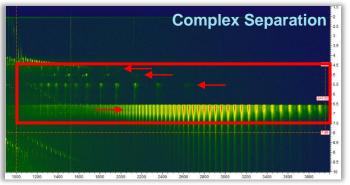
Restriction of RT and *m/z* Ranges

• 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.







GENERAL GUIDELINES FOR INTACT WORKFLOWS

Restriction of RT and *m*/*z* Ranges

🕼 👌 RT Range Restriction

m/z Range Restriction

[;;;;] m/z ____ • Type the identified useful RT and *m/z* ranges.

 When input boxes are left empty, the full RT or m/z range is used.

🐵 RT Range Re	striction - Setti	ngs	×
General Display	Y		
RT Minimum: RT Maximum:	3.2 4.2	Minutes Minutes	
0 🗎 [ОК	Cancel	Apply

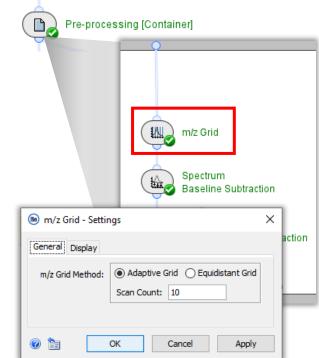
🐵 m/z Range Re	estriction - Set	tings	×
General Display			
m/z Minimum:	1000	Da	
m/z Maximum:		Da	
	ОК	Cancel	Apply



GENERAL GUIDELINES FOR INTACT WORKFLOWS

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.





Spectrum Baseline Subtraction

• Spectrum Baseline Subtraction improves deconvolution by removing background noise and limiting the production of satellite peaks in the deconvoluted data.

Pre-processing [Container]

W)

m/z Grid

Spectrum Baseline Subtractior

• This activity node can also be used after deconvolution to improve peak detection.

Be Spectru	m Baseline Subtraction - Settings	X
General A	dvanced Display	
Method:	Quantile ~ Quantile Penalized Least Squares m/z Window: 10.0 Da ~	
۵ 🛅	OK Cancel Apply	

- 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

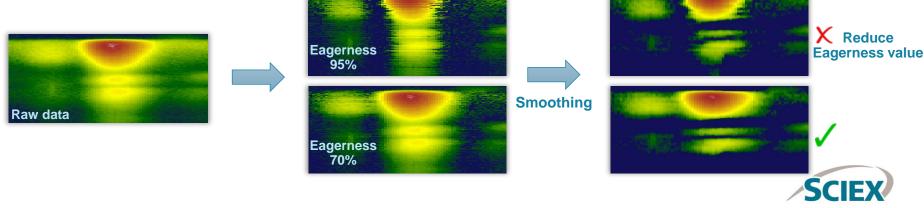
Spectrum Peak Detection

Peak Detection [Container]

21

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.

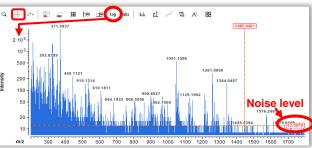


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The Power of Precision

Chromatogram Chemical Noise Subtraction

Chromatogi	ram Chemical N	Voise Subtrac	tion >	<
General Advar	nced Display			
Chromat	togram Smoothin	g		
RT Window:	9	Scans		
Estimator:	Binomial		\sim	
Chemica	l Noise Subtractio	on ———		
RT Window:	101	Scans		
Quantile:	40 %			
Method:	Clipping) Subtraction		
Threshold:	14.0	[Intensity]		
-				
@ 🛅 📃	ОК	Cancel	Apply	



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.

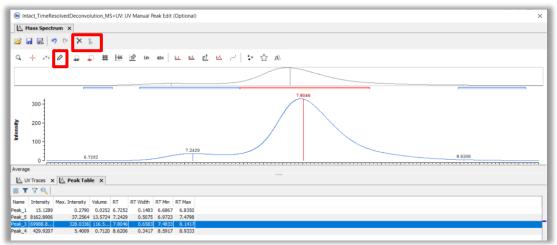
Chemical Noise Subtraction can help to:

- 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.

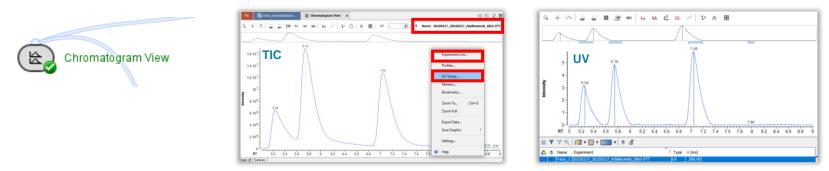


UV Processing [Container

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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.



The Power of Precision

Feature Filters

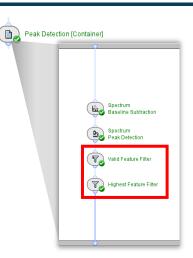
- The *Feature Filter* activity nodes can be used to limit how many peaks are included, so that only the most relevant peaks are kept and those originating from noise are excluded.
 - Use the **Bypass** icon to restrict use if filtering peaks is not required.

🐵 Valid Feature Filter	- Settings		×
General Display			
Data Type:	Auto Detect V		
Feature Type:			
Validity Threshold:	150	Max. Intensity 🗸 🗸	
Present in at Least:	1	Intensity Max. Intensity Volume % Intensity	
0	ОК	% Max. Intensity % Volume	

Valid Feature Filter

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

ure Filter - Settings	×
Auto Detect \lor	
Auto Detect \lor	
Max Intensity $$	
40	
OK Cancel Apply	
	Auto Detect V Max Intensity V 40



Highest Feature Filter

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



GENERAL GUIDELINES FOR INTACT WORKFLOWS

Protein Mapping: Sequences

Protein Mapping - Settings ×	Protein Mapping - Settings X Order Mapping - Settings X	ing - Settings ×
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Consensus Sequence(s); From Text	- DEnzymatic	LC/Ct DILTQSPSSL HC/Ct QVQLVESGGG LC/N DIOMTOSPST
Sequences:	Consensus Seq	uence(s): From Text Sequences: Sequences: Selected IDs
OK Cancel Apply Apply OK Cancel Apply C	🙆 🛅 OK Cancel Apply	OK Cancel

- 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

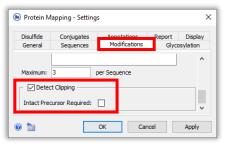
			(1)		n Mapping - Settir	-	
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					Substituents:		+
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Protein					I Sequences	ngs Modifications	(
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. Specify Fixed and Variable PTMs.

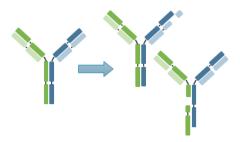
- With protein sequences carrying N-term E, it is recommended to set Glu-pyroGlu (N-term E) as a variable modification, because the modified form is not expected to be predominant.
- 2. Specify glycosylation parameters.
 - Set the **Type** to **Glycosylated** or **Deglycosylated**:
 - **Deglycosylated** 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.
- Specify the names and masses of conjugates if the target protein is an ADC.



Protein Mapping: Detect Clipping



Ceneral		Jences	Modifications	Glyco	sylation			
Disulfide	Conj	ugates	Annotations	Report	Displa			
State:	Fully Conr	nected			~			
	Fully Redu	ced						
	Fully Conn	ected						
	Partially R	educed						
	Type:	IgG 1	~					
	Search all multi-specific combinations							
	Unpaired	Cysteine	Modification					
		/steines						



- 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:
 - A Extended workflow processing times or workflows that are unable to run to completion.
 - A High levels of false positive identifications, requiring excessive data review.



Protein Mapping: Detect Clipping Recommendations

Display

- 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.

Be Protein Genera Disulfide			Glycosylation	\sim		e: De otein	
State:	Fully Connected Connectivity Unspecified	Be Protein	∽ Mapping - Setti	ings	_		_
	Additional chains: 1	General Disulfide	Sequences Conjugates		ications ations	Glyce Report	osylation Displa
	Unpaired Cysteine Modification	State:	Fully Connected Connectivity IIgG Type: IgG 1 V Se	L V	specific co	ombinations	~
0	ОК		Unpaired Cysteir Cysteinyl (C)	ne Modification	۱		~
	n species to peaks in the dec ipping' is only allowed for "L			nectivity.			
		0		ОК	Can	cel	Apply

tect Clipping will report C-terminal lysine loss as a duplicate annotation if Lys-Loss 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 not 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.



The Power of Precision

Protein Mapping: Detect Clipping - Intact Precursor Required

🐵 Protein M	apping - Setting	5		×
Disulfide General	Conjugates Sequences	Annotations Modifications	Report Glyco	Display osylation
Detec	t Clipping			^
Intact Pred	cursor Required:			*
0		OK Can	cel	Apply

🐵 Proteir	n Mapping - Settings	×
General Disulfide		
State:	Fully Connected Connectivity Unspecified Search all combinations Additional chains: 0 Unpaired Cysteine Modification Cysteinyl (C)	
0	OK Cancel Apply	

- 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 fulllength 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

Protein M	apping - Setting	s			
Disulfide	Coniudates	Annotat		Report	Display
General	Sequences	Modific	ations	Glyco	osylation
- 🗹 Enzym	natic Digestion —				^
Consensus	Sequence(s): F	rom Text			\sim
	5	Sequences:	СРРСРА	PELLG/GPS	VF
					<u> </u>
1		ОК	Can	el	Apply

Peak Detection (Container) Protein Mapping Map protein species to peaks in the deconvoluted spectrum. Enzymatic cleavages are only allowed for "Unspecified" connectivity. Map protein species to peaks in the deconvoluted spectrum. 'Detect Clipping' and 'Enzymatic Digestion' can not be both activated at the same

- 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. **b**. Load Intact Protein Data
 - 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.

B.0

1'a

(Annotation Snaps

Pre-processing [Container]

Peak Detection [Container]

Protein Mapping

time.

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.

🕀 🐊 Targeted Mass Search

Targeted Mass S	earch - Setting	gs	×
General Display			
Library:	File (all entries) ~	
	Bevacizuma	ab_Library_TRD.txt 🛛 📓	
Ionization:	Massless	~	
Mass Tolerance:	50	ppm 🗸	
RT/RI Tolerance:	0.1	Minutes	
	Limit to Bes	st Match	
	Ignore Ann	otated Features	
0 🛅	OK	Cancel Apply	

- 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.

Fi	le Home D	evelor Insert	Page La Formu	la Data Review View Data	Sti Inquire Power F	Help 🖪	2
A1		: × - 🗸	fx Mass				×
I	A	в	с	D	E	F	G 🔺
	Mass	Potein	Disulfide Bonds	Modifications	Glycosylation		
2	23123.51646	1	2*S-S				
3	46245.01703	LC-LC	5*S-S				
4	147833.4673	HC-HC-LC-LC	16*S-S	2*Gln->pyro-Glu + 2*Lys-loss	GOF + GOF-GlcNAc		
5	148036.6602	HC-HC-LC-LC	16*S-S	2*Gln->pyro-Glu + 2*Lys-loss			
6	148198.801	HC-HC-LC-LC	16*S-S	2*Gin->pyro-Glu + 2*Lys-loss	GOF + G1F		
7	148360.9419	HC-HC-LC-LC	16*S-S	2*Gln->pyro-Glu + 2*Lys-loss	2*G1F		
8	148523.0827	HC-HC-LC-LC	16*S-S	2*Gln->pyro-Glu + 2*Lys-loss	G1F + G2F		
9	148685.2236	HC-HC-LC-LC	16*S-S	2*Gln->pyro-Glu + 2*Lys-loss	2*G2F		
10							_
11		T late at Ille					-
	NIS	T_intact_libra	ary 🛞	: •			
Edit	10				Ш — —	+	100%

A1			× v	fx RT							
I	А	В	с	D	E	F	G	н	1	J	[
ı	RT	Mass	rotein	Modifications	Glycosylation						
2	5.22	25404 2671	c		G1F						
3	5.295	25332.1262	Fc		GOF						
4	5.354	25366.0946	Fc	Lys-loss	G1F						
5	5.347	24975.7083	Fc	Lys-loss	Man5						
6	5.362	25203.9537	Fc	Lys-loss	GOF						
7	5.35	25000.7609	Fc	Lys-loss	G0F-GlcNAc						
8	6.048	23411.8919	LC								
9	8.042	25458.3247	Fd'								
10	8.044	25440.309	Fd'	Glu->pyro-Glu							
11				orary_TRD							

- 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.

Filter Annotated Peaks	Seperiment Table × ▶ Peak Table × ▼ ∇ ♀ ▼ ▼ ▼ ↓ ⊕ ⊕ ▼ ∇ ♀ ↓ ▼ ▼ ↓ ↓ ⊕ ⊕ ● ∇ ∇ ♀ ↓ ↓ ▼ ↓ ↓ ↓ ⊕ ● Name ■ 201900615 Betvacumab. 10ug OC. 100mM AR ■ ■ ■ ■	. [Annotate L Start Time	JV Peaks from MS 13:28:14 04/11/22	1
<u> </u>			Complete Time	13:28:14 04/11/22	
(🕒) Annotate UV Peaks from MS			Elapsed Time	3 msec	
			Status	Suspicious	
Y-			Message	The normalized UV absorbances could not be computed due to missing and/or duplicated annotations.	
	1 row (1 selected)				
	Data 🗇 UV Quantities Summary				
					SCIE)

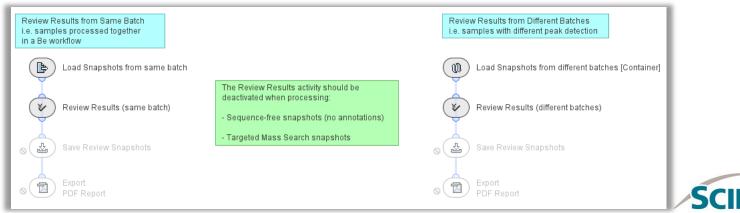
RT Tolerance:	0.05	Minutes	
Observable:	Volume	\sim	
Normalize relative to:	Annotated Pea	iks ∨	
Annotation Report Mode:	Manual		~
	Annotations:	Protein Glycosylation	^
		<	>

The Power of Precision

GENERAL GUIDELINES FOR INTACT WORKFLOWS

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.





The Power of Precision

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.



- 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.











Save Sequence-free Snapshot



Save Annotation Snapshot



Save Targeted Search Snapshot



Save Review Snapshots



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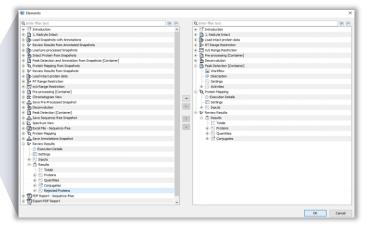
Reporting: Export PDF Report



- 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.

Export PDF Report - Settings	×
General Elements Template Excel Display	
Elements:	 ▲ ▲
CK Cancel	Apply





GENERAL GUIDELINES FOR INTACT WORKFLOWS

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.

A1		- i × ✓ fx Name										
	A	B		с	D	E	F	G	н	1	J	к
1	Name	20220217_20220217_Adalimumab_IdeS-DT	T.2 [2]	Aass	RT	Mass Widt	RT Height	Area	Mass Min	Mass Max	RT Min	RT Max
2	Peak_00		3206.03833	25332.38	5.290895	18	0.448567	8.0742	25323.5	25341.5	5.157967	5.606533
3	Peak_00		480.4490356	25494.41	5.245409	12	0.28145	3.3774	25488.5	25500.5	5.157967	5.439417
4	Peak_01		432.8087769	25223.43	5.343585	12	0.369408	4.4329	25219.5	25231.5	5.19315	5.562558
5	Peak_01		5228.773926	25366.3	5.351897	18	0.49255	8.8659	25357.5	25375.5	5.19315	5.6857
6	Peak_02		785.7265625	24976.16	5.344246	16	0.351825	5.6292	24968.5	24984.5	5.201942	5.553767
7	Peak_02		1240.089233	25001.06	5.344819	15	0.360617	5.40925	24994.5	25009.5	5.201942	5.562558
8	Peak_02		207.4940948	25142.44	5.304126	5	0.263867	1.319333	25140.5	25145.5	5.201942	5.465808
9	Peak_02		23351.86328	25204.28	5.362964	20	0.439775	8.7955	25194.5	25214.5	5.201942	5.641717
10	Peak_02		258.5281372	25350.36	5.359016	7	0.325433	2.278033	25346.5	25353.5	5.201942	5.527375
11	Peak_04		526.6027832	24838.63	5.337205	11	0.325433	3.579767	24832.5	24843.5	5.210733	5.536167
12	Peak_04		223.7139282	25136.6	5.322132	4	0.272667	1.090667	25134.5	25138.5	5.210733	5.4834
13	Peak_04		1393.563965	25185.9	5.364173	12	0.4046	4.8552	25178.5	25190.5	5.210733	5.615333
14	Peak_04		231.4377747	25250.44	5.357551	8	0.334233	2.673867	25245.5	25253.5	5.210733	5.544967
15	Peak_10		424.6436462	25204.39	5.85781	12	0.158317	1.8998	25198.5	25210.5	5.78245	5.940767
16	Peak_10		67678.54688	23412.21	6.044723	21	0.510142	10.71298	23403.5	23424.5	5.826425	6.336567
17	Peak_10		307.2645874	22546.69	5.997263	9	0.263867	2.3748	22543.5	22552.5	5.8704	6.134267
18	Peak_11		2944.370605	23393.47	6.040324	12	0.474958	5.6995	23386.5	23398.5	5.8704	6.345358
19	Peak_11		806.7955322	23434.23	6.039181	10	0.422183	4.221833	23429.5	23439.5	5.8792	6.301383
20	Peak_11		1119.460815	23457.05	6.050581	11	0.430983	4.740817	23450.5	23461.5	5.8792	6.310183
21	Peak_11		1142.189697	23464.35	6.050805	4	0.430975	1.7239	23462.5	23466.5	5.888	6.318975
22	Peak_12		431.1587524	22630.68	6.056369	4	0.395792	1.583167	22628.5	22632.5	5.896792	6.292583



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



Part A Software and Workflows

SPECIFIC INTACT WORKFLOW GUIDELINES



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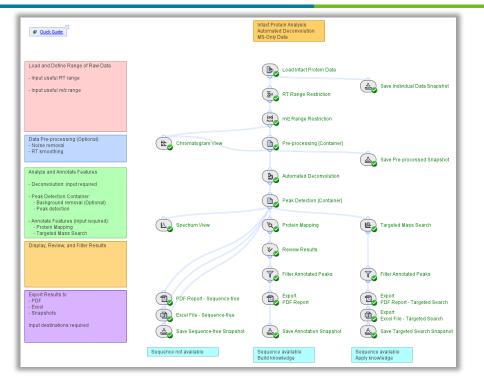
Automated Deconvolution with MS or MS and UV Data

WORKFLOW GUIDELINES

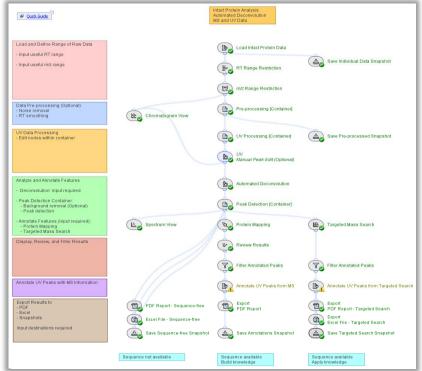


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Automated Deconvolution Workflows: Design



Intact_AutomatedDeconvolution_ MS Workflow



Intact_AutomatedDeconvolution_ UV+MS Workflow



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

	Automate	d De	convolut	ion						
	B Automate	d Deco	onvolution - S	Settings					×	
	Deconvolution	Option	IS RT Ranges	Displa	/					
	Mode:	Autor	nated					~	^	
		Min. I	Mass: 10		kDa					
			Mass: 160		kDa					
		Mass	Step: 2		Da					
		Visible		nly Zoom		-		\sim		
			Automat			tion - Settin				
							-			
		_ ₽	Deconvolutio	n Option	s RT	Ranges Di	isplay			
		Ea	Mode:	Auton	nated]
	Method:	Max			lass:	20	_	kDa		
		Iter			Mass:		_	kDa		
		Dec		Mass	Step:	1		Da		
				Visible	Range	es: Only F	Full Ra	nges		~
	0				Filter R	T Ranges				
1				Eage	rness:	Standard				
			Method:	Maxim	um Ent	ropy Decon	voluti	on		
				Iterat	ions:		25			
				Decor	nvolutio	on Quality:	Stan	dard		~
			Ionization:	Pro	tonatio	on O Depr	otona	ition		
			0			(ОК		Cancel	Арр
				_	-	_	-	-	_	

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.



Automated Deconvolution: RT Ranges

Automated Deconvol Automated Deconvolution Automated Deconvolution	_	×		•	C re	onf gio	igu ns	
Deconvolution Options RT Ra	anges Display		~				9.0	
Peak Detection:	Ascent-based Apply Isolation Filter Isolation Threshold: 5		Automated Deconvol			~		
Center Computation: Boundary Determination: Min. Peak Intensity:	Intensity-weighted Intensity Threshold: 70 % Maximum Curvature 0.1 %		UV UV Wavelength: Use Existing UV Peaks: Use Smoothing - RT Window: 50	280	Scans	nm		~
0 🖻 🚺	OK Cancel		Peak Detection: Center Computation: Boundary Determination Min. Peak Intensity:	I	cal Maximu	solation Fil		Scans
		0	1		ОК	Canc	el	Apply

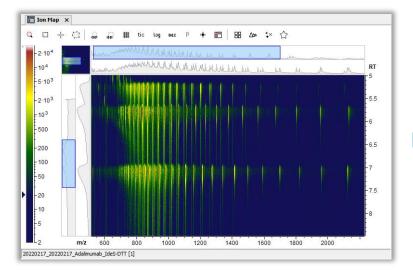
- Configure settings in the RT Ranges tab to identify RT regions of interest.
 - Select one of these options from the list:
 - TIC: Identify RT ranges using the total ion chromatogram.
 - **Manual**: Define RT ranges manually.
 - UV: Define RT ranges based on peaks in the UV data.
 - To use UV data to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

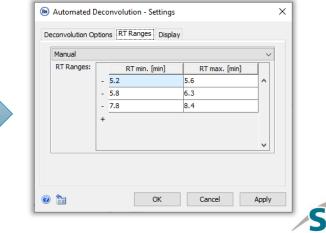


Automated Deconvolution: Manual RT Ranges

Automated Deconvolution

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

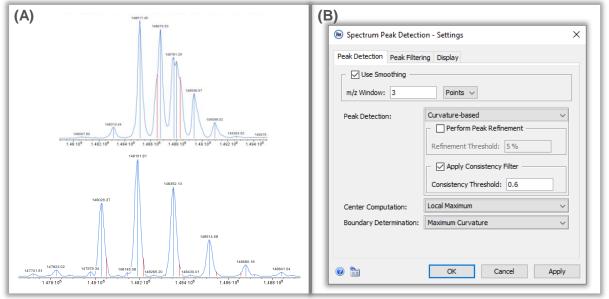


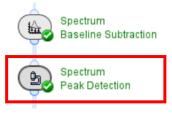




Spectrum Peak Detection

- The default 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.



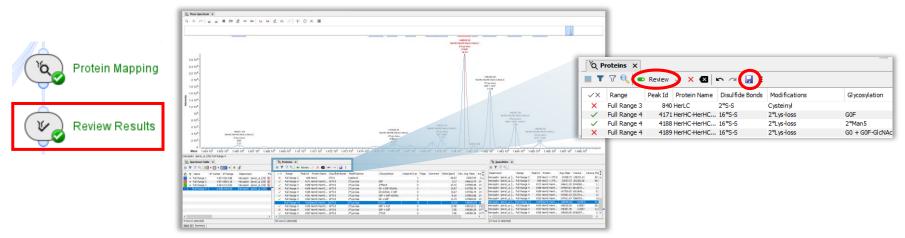


- A. Deconvoluted spectra produced when **Curvaturebased** peak detection is used to resolve shoulder peaks.
- B. Example settings used to resolve the shoulder peaks in the spectra shown.



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.

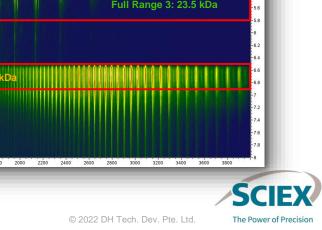


• *Review Results* is a critical step for the analysis of proteins with complex glycosylation patterns and for ADCs to correctly calculate DAR values.



Review Results: Spectrum Table

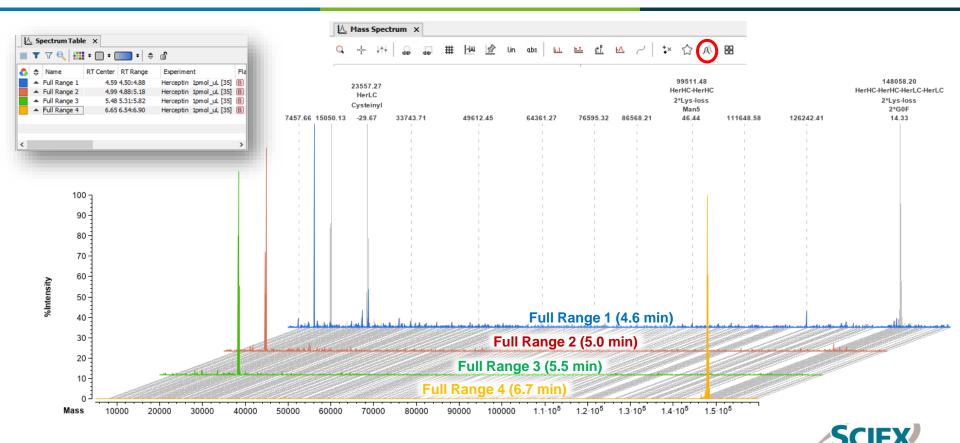
- The Spectrum Table lists the detected RT ranges, with the **RT Center** of the protein signals that were detected.
- ↓ Spectrum Table × 🖓 🔍 👬 Ŧ 🔲 Ŧ 🥅 Ŧ 🔶 🔐 Name RT Center RT Range Experiment ۰ Herceptin 1pmol_uL [35] B Full Range 1 4.59 4.50:4.88 Herceptin 1pmol_uL [35] B Full Range 2 4.99 4.88:5.18 Full Range 3 5.48 5.31:5.82 Herceptin 1pmol_uL [35] B Full Range 4 6.65 54:6.90 Herceptin 1pmol_uL [35] B < Full Range 1: 11+ 23.8 kDa Full Range 2: 15 kDa Full Range 3: 23.5 kDa Full Range 4: 148 kDa 1400 1600 1800 2000 2200 2400 2600 3000 3200 3400 3600 3800



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

GUIDELINES FOR AUTOMATED DECONVOLUTION WORKFLOWS

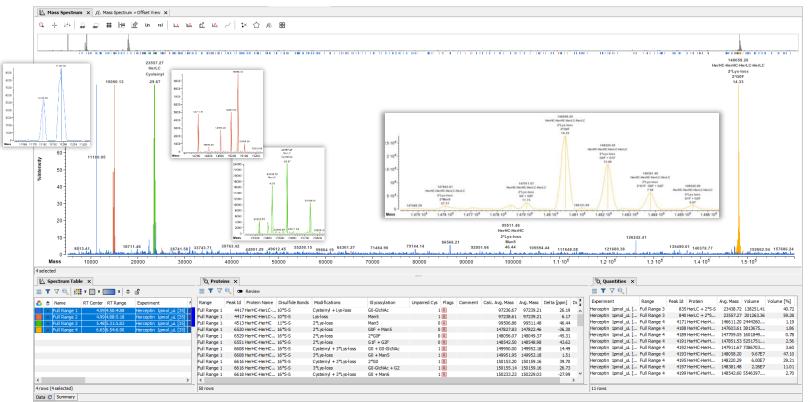
Review Results: Spectrum Offset View



The Power of Precision

GUIDELINES FOR AUTOMATED DECONVOLUTION WORKFLOWS

Review Results: Spectrum Overlapped View





Time Resolved Deconvolution with MS or MS and UV Data

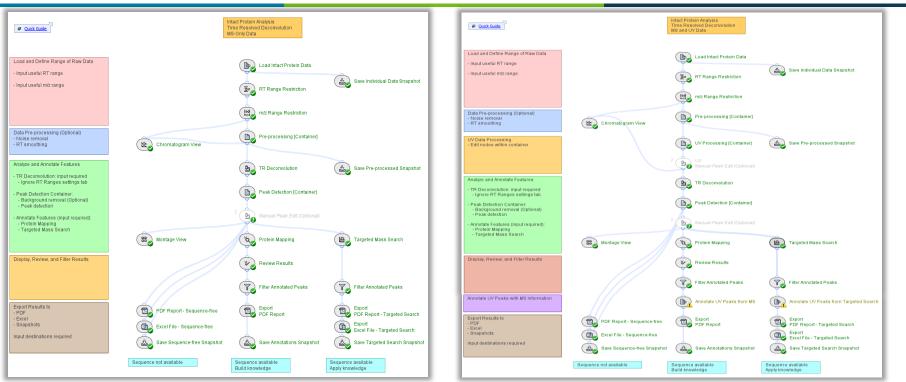
WORKFLOW SPECIFIC GUIDELINES



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Intact TimeResolvedDeconvolution

TRD Workflows: Design

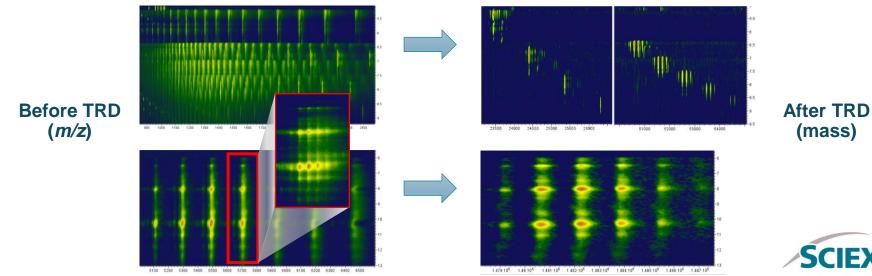


Intact_TimeResolvedDeconvolution _UV+MS Workflow



MS Workflow

- **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.

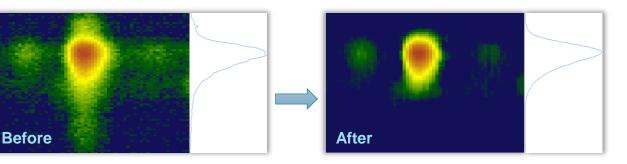


The Power of Precision

Chemical Noise Subtraction: Optimization

🐵 Chromatogram Chemical Noise Subtraction - Settings 🛛 🗙							
General Advanced Display							
Chromatogram Smoothing							
RT Window: 9 Scans							
Estimator: Binomial ~							
Chemical Noise Subtraction							
RT Window: 101 Scans							
Quantile: 40 %							
Method: Clipping O Subtraction 							
Threshold: 10 [Intensity]							
OK Cancel Apply							

- 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.

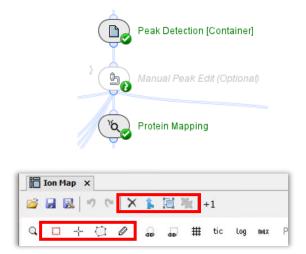
- The deconvoluted mass range and data density have a significant impact on processing time.
 - Fragment/subunit datasets often have higher data density than whole mAb datasets.
- The Min./Max. Mass and Mass Step should be optimized for each data-set.
 - A smaller mass range and larger mass step can 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.

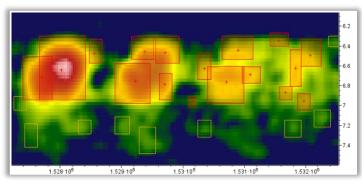
🛞 TR Decon	volution - Set	tings				Х
Deconvolution	Options RT	Ranges Di	splav			
		-				
Mode:	Time-resolve	d Deconvolu	ution		~	
	Min. Mass:	20		kDa		
	Max. Mass:	30		kDa		
	Mass Step:	1		Da		
Method:	Maximum Ent	tropy Decon	volut	ion	~	
	Iterations:		25			
	Deconvolutio	on Quality:	Star	ndard	\sim	
Ionization:	Protonatio	n O Depr	oton	ation		
	0	0-4				
		ОК		Cancel	Apply	



GUIDELINES FOR TIME RESOLVED DECONVOLUTION WORKFLOWS

Manual Peak Edit (Optional)

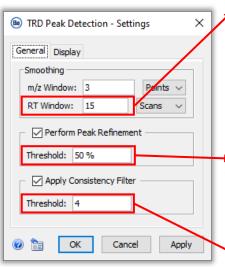




- This activity is bypassed as default.
- The peaks detected automatically in the deconvoluted ion map can be refined using *Manual Peak Edit* by:
 - Changing the boundaries of peaks.
 - Splitting overlapping peaks.
 - Deleting peaks.
 - Drawing new peaks.
 - Use *Manual Peak Edit* to accurately re-assign 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 (\$).

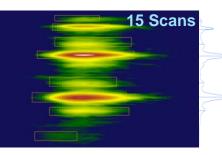
Lower value = Increase in splitting in RT direction.

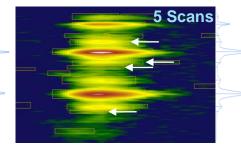
Affects the number and width of peaks along the mass axis (↔).

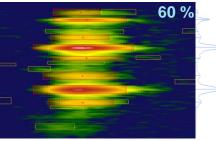
Lower value = Higher probability that peaks will be split in m/z direction.

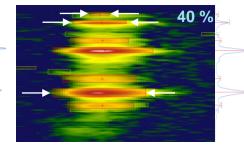
Affects sensitivity and therefore the number of background peaks.

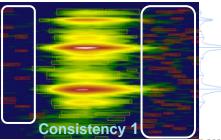
Lower value = Increase in m/z splitting.







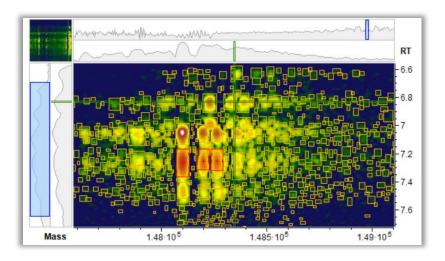






GUIDELINES FOR TIME RESOLVED DECONVOLUTION WORKFLOWS

TRD Peak Detection: Optimization



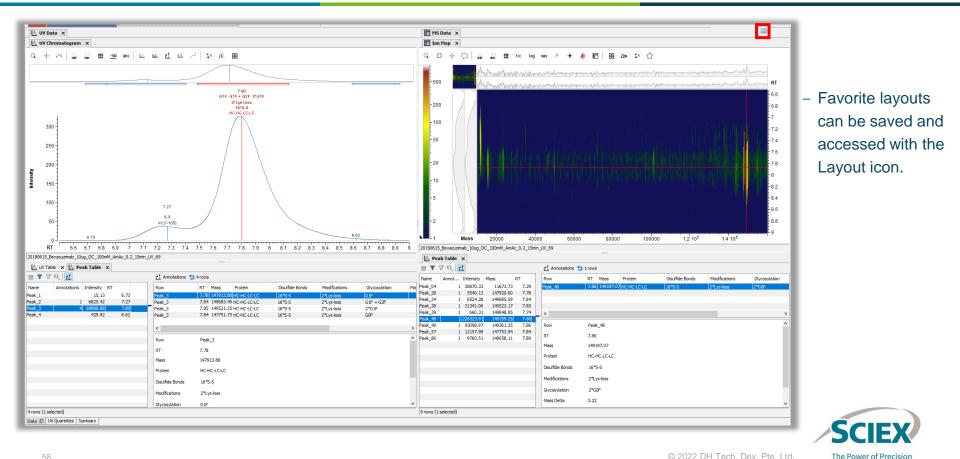
- If multiple small features are detected in a complex deconvoluted ion map:
 - The sensitivity can be lowered by increasing the Consistency Filter Threshold (to 2 or 3).



• 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



Intact Mass Screening

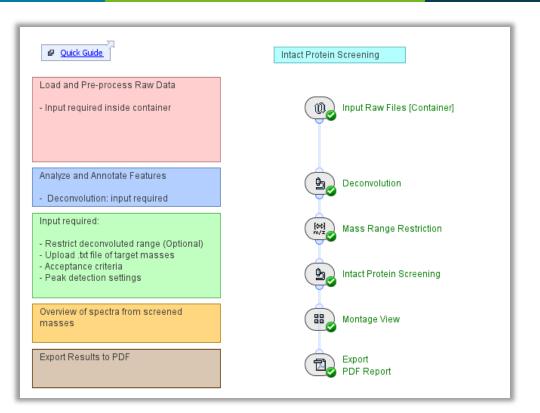
WORKFLOW SPECIFIC GUIDELINES





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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 (\checkmark): The calculated mass is below the Validity Threshold.
 - Critical (!!): The calculated mass is between the Validity and Attention Threshold.
 - Invalid (X): The calculated mass is above the Attention Threshold.

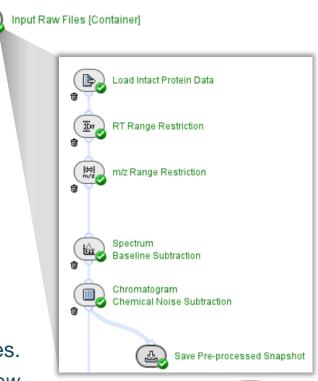
Name	Expected Mass	Detected Mass	Delta [Da]	Delta [ppm]	Valid
Remicade_IdeS_reduced	25647.51	25647.35	-0.17	-6.0	v
Rituxan_IdeS_reduced	25328.19	25327.79	-0.41	-16.0	X
Herceptin_IdeS_TCEP	25383.31	25383.14	-0.16	-6.0	1
Humira_IdeS_TCEP	25458.33	25457.95	-0.37	-15.0	!!
NIST 500ngOC IdeS Red 01	25688.91	25688.72	-0.19	-7.0	1

(B) Intact Protein Screening - Settings	×				
General Peak Detection Display					
Validity Threshold: 10 ppm ~ Attention Threshold: 20 ppm ~					
Assignment: From File	~				
Group Output:					
🔞 🛅 OK Cancel	Apply				



Intact Mass Screening Workflow: Overview

- To reduce the computational memory used for highthroughput 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.

۲	Input Raw	Files (Containe	r]
		Reset	
(h)	Deco	Process	>
	Deet	Control	>
	Mas	Show Progress	8
Ť		Show Console	
_	Intac	Save Graphic	>
Ť	0	Help	
88	Mont 🗳	Settings	
		Show Results	
0	Export PDF Repo	ort	

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

General Para	ameterization
Name:	Input Raw Files [Container]
Description:	If running multiple samples from a single wiff/wiff2 container, or if analysing replicates with the same name: - Change Partitioning to 'by Structure' - Set Fraction Count to match the number of samples being analysed
Partitioning:	One Input per Fraction
Partitioning:	One Input per Fraction V One Input per Fraction
Partitioning:	One Input per Fraction by Structure
Partitioning:	One Input per Fraction

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

	🐵 Input Raw F	iles [Container] - Settings	×		
	General Parar	neterization			
	Name:	Input Raw Files [Container]			
⇒	Description:	If running multiple samples from a single wifffwiff2 container, or if analysing replicates with the same name: - Change Partitioning to 'by Structure' - Set Fraction Count to match the number of samples being analysed			
	Partitioning:	by Structure 🗸 🗸			
		Fraction Prefix: Fraction			
		Fraction Count: 96			
		Fraction Major: 🔽			
		Discard fraction information			
	0 🛅	OK Cancel Apply			

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



options RT Ranges Display

w: 9

ction:

mputation: Determination:

0 🎦

Scans

Curvature-based

Local Maximum

FWHN

20 %

OK

Refinement Threshold: 5 %

Consistency Threshold: 0.6

Cancel

Apply

Deconvolution: Optimization for Complex Datasets

Deconvolution

Deconvoli	ution - Setting	js				×	1
Deconvolution	Options RT	Ranges Di	splay				
Mode:	Automated Min. Mass:	10	kDa			~	
	Max. Mass:	200	kDa			80 D	econvol
	Mass Step: 2			_		Deco	nvolutior
	Visible Range	Eager		Standard	~	Т	IC
	- Filter F		Mass Window:	2.0	kD.		RT Windo
	Eagerness:					P	eak Dete
Method:	Maximum Ent	ropy Decon	volution				
	Iterations:		20				
Ionization:	Deconvolution Quality: Standard						
0 🛅		OK	Cano	el	Aŗ		Center Co Ioundary
						Ν	ʻlin. Peak

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.

Herceptin IdeS TCEP

22

- 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.



Remicade IdeS reduced

2.3

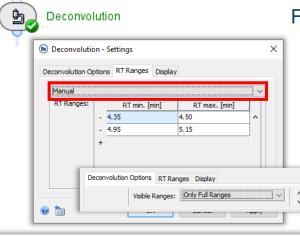
Humira IdeS TCEP

Rituxan IdeS reduced

NIST 500naOC IdeS

MONDARY CONTRACTOR

Deconvolution: Optimization for Present/Absent RT Ranges



Whole mAb 4.2 **RT Range 1: 4.35 - 4.5 min RT Range 2: 4.95 - 5.15 min Subunit/Clip** 3000 3500 4000 4500 5000 5500

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.



Mass Range Restriction

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**.

Interchangement Interchang	No. Control Stream Spacework (or x) Spacework (or x) <t< th=""></t<>
Q + 35 章 型 團 图 図 ■ ■ 図 2 2 1 2 合 A BB	(4) (4) (4) (4) (4) (4) (4) (4) (4) (4)
Without Mass Range	With Mass Range
2 5 10 ⁴ 0 10 ¹⁴ 10 ¹⁴ 1	ž 5 10 ³ 0 Mass ¹ 6650 6100 6150 6250 6250 6250 6250 6250 6250 6250 6550 6650 66
[reg[1]	1 ng (11)
L spectrum Table x	L Spectrum Table x L Peak Table x
0 0	O Imme Depresent High Non-Na Name April (Name April (
Sala (2) ThC [Input [Summay]	Data (D) Semary

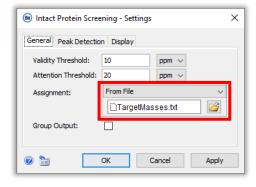


Defining Target Masses for Intact Protein Screening

(B) Intact Protein Screening - Settings								
General Peak Detection Display								
Validity Threshold:	10	ppm 🗸						
Attention Threshold:	ppm \checkmark							
Assignment:	Fixed Masses:	J						
		~	,					
Group Output:								
0 1	OK (Cancel App	bly					

Intact Protein Screening

- Specify the masses of interest for screening:
 - If the <u>same masses</u> are expected across all samples, then change
 Assignment to Fixed and type the values in the Masses section.



If <u>different masses</u> 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.



Ь.

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.

lot set					U	nclassified	Public	Confide	ential Restri	cted
А		В	С	D	E	F	G	н	1	К
emicade_IdeS_reduce	d 🚺	25204	23438.7	25647.	5		=CONCAT(B	1, ", ", C1, ",	",D1,", ",E 1)	
tuxan_IdeS_reduced		25204			4 25328.2		CONCAT	text1, [text2]	, [text3], [text4]	, [text5], [text6], [
erceptin_IdeS_TCEP			23442.9			L				
umira_IdeS_TCEP IST 500ngOC IdeS Re	od 01		23411.9		3 5 25688.9					
21_2001BOC_I062_V	u_01	23230	23390.2	23127.	5 23066.9					
-	(2)	Home	Insert	Pag	e Layout	Formula	is Data	Review	View H	1
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			_reduced eS_TCEP				, 23439.423, , 25383.306,	25328.194		
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Hum Replace	0.00		Ctrl+H 03	8.954,	23411.	892, 29	5458.325	, "		
NIS Go To	-		Ctrl+G	"25	236.018	, 25398	3.159, <mark>2</mark>	3127.548	3, 25688.9	906"
	-	Replac						>		
Select All		- Cplac								
Time/Dat	e	Find w	aat.					Find Next		
		rinu wi	iat.							
		Replac	e with:					Replace		
		L .						Replace All		

Review Stored Results

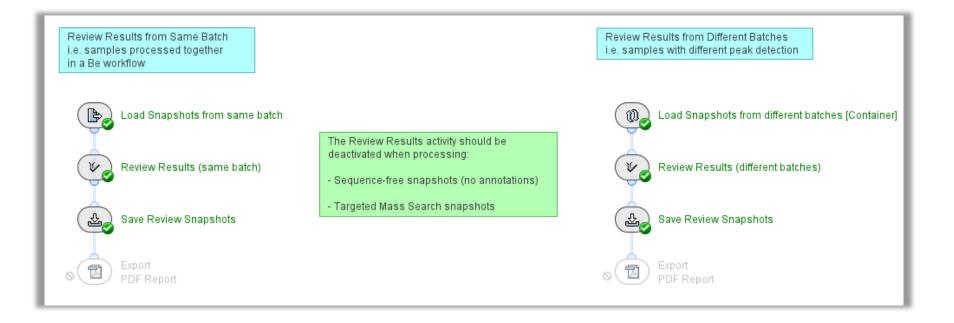
WORKFLOW SPECIFIC GUIDELINES





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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).

¹ Q Proteins X							
√×	Range	Peak Id	Protein Name	Disulfide B	Modifications		
	Full Range 1	334	LC	2*S-S			
~	Full Range 1	947	LC-LC	5*S-S			
	Full Range 1	2908	HC-HC-LC	13*S-S	Cysteinyl + 2*Gln-		
	Full Range 1	2937	HC-HC-LC	13*S-S	2*Gln->pyro-Glu +		

- 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



(Distant) (S)

Selecting Time-Resolved or Automated Deconvolution

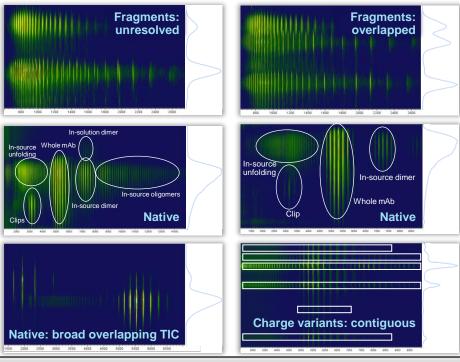
- Automated: Ranges for deconvolution are automatically determined.
 - Recommended for chromatographically well-resolved species.
- **Time-resolved:** Deconvolution is completed on each scan in RT.
 - Recommended for data containing complex mixtures that are poorly resolved.
 - Creates a 2D ion map view 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

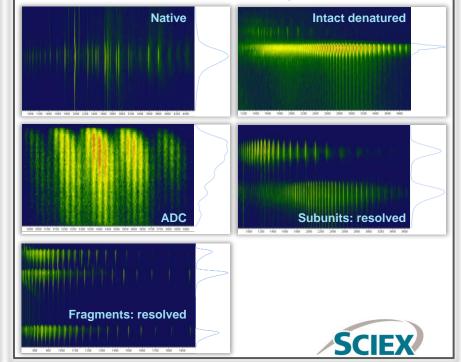
Key use cases for Time-Resolved Deconvolution:

Complex Chromatography



Key use cases for Automated Deconvolution:

Simple Chromatography



Denatured Intact Protein

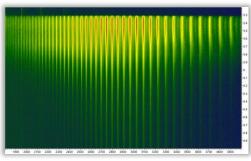


Denatured Protein: Single Entity

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



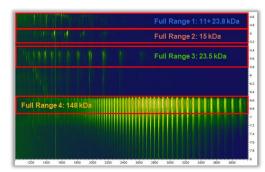
Denatured Protein: Complex Sample

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



¹Deconvolution, ²Protein Mapping

Native Intact Protein

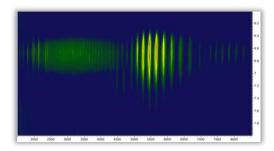


Native Protein: Single Entity

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).



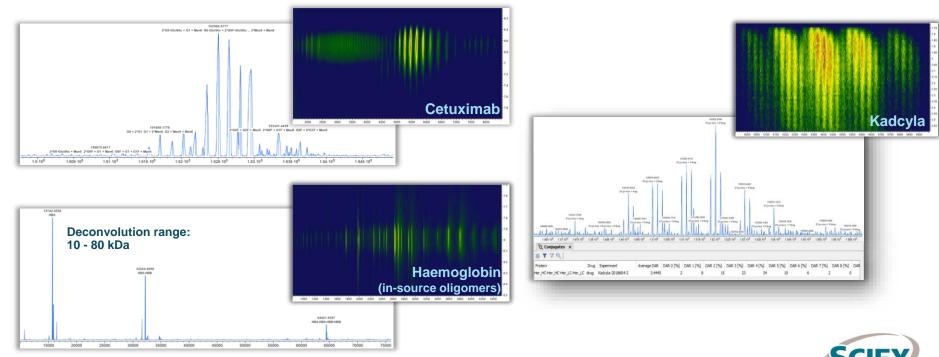
Native intact protein



¹Deconvolution, ²Protein Mapping

Native Protein: Single Entity

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

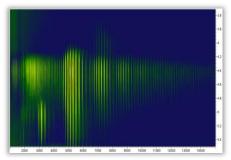


Native Protein: Complex Sample

FOCUS: All visible RT ranges.

Including low-mass degradation products (unconnected subunits, clips).

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: <i>Fully Connected.</i> Connectivity: Unspecified + 3 Additional chains. Cysteinyl for unpaired cysteines.



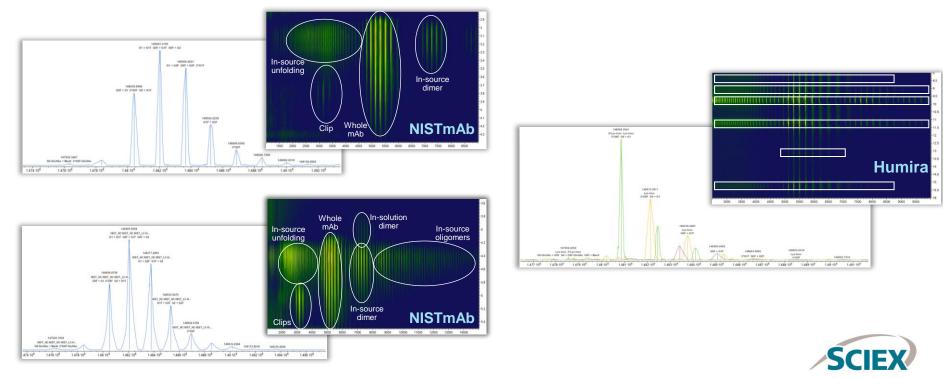
Native complex sample



Native Protein: Complex Sample

FOCUS: All visible RT ranges.

Including low-mass degradation products (unconnected subunits, clips).



Antibody Drug Conjugates

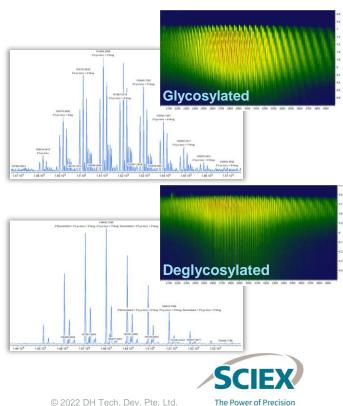


ADC: Whole Protein

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	<i>Review Results</i> : Remove redundant annotations to enable correct computation of DAR.

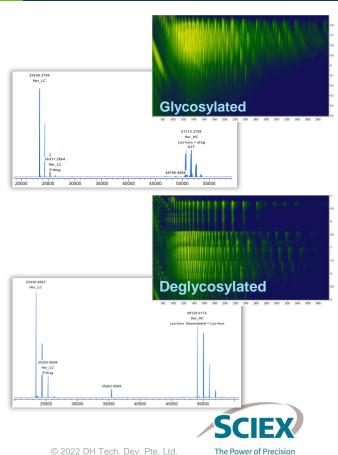


¹Deconvolution, ²Protein Mapping 85

ADC: Subunits

FOCUS: Reduced ADC.

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	<i>Review Results</i> : Remove redundant annotations to enable correct computation of DAR



Subunit Analysis



FOCUS: Heavy chain and light chain.

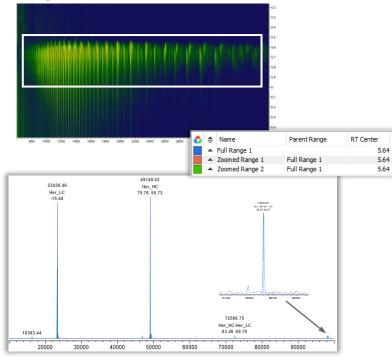
Sample type:	Protein reduced to HC and LC
Suggested workflow	Intact_AutomatedDeconvolution Intact_TimeResolvedDeconvolution (for fragments with complex modifications).
Deconvolution range ¹	20 kDa - 60 kDa or 20 kDa - 110 kDa to identify partially connected subunits. Visible Ranges: All Ranges particularly 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.



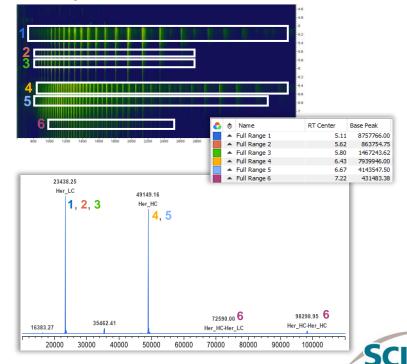
Subunit Analysis

FOCUS: Heavy chain and light chain.

Fully reduced



Partially connected



Fragment Analysis



Fragment Analysis

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

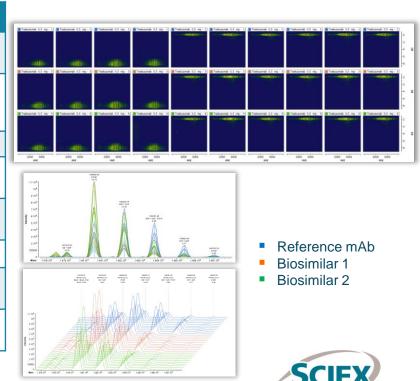
Sample type:	F(ab')2 and ScFc, or, LC, Fd' and ScFc
Suggested workflow	Intact_AutomatedDeconvolution Intact_TimeResolvedDeconvolution (for fragments with complex modifications).
Deconvolution range ¹	20 kDa - 110 kDa or 20 kDa - 30 kDa with reduced fragments Visible Ranges: Only Zoomed Ranges or All 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.



Comparability Test or Dilution Series



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 <i>Targeted Mass Search</i> .

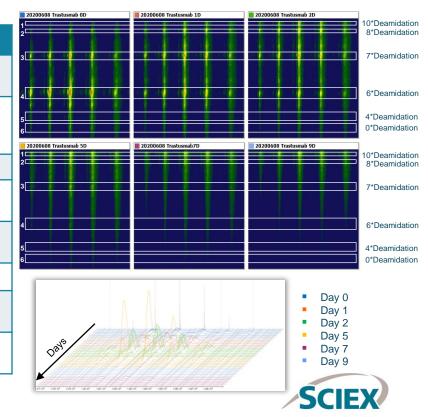


Stress Test



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 <i>Targeted Mass Search</i> .





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