#### Intact Mass Screening

**Biologics Explorer Software Guidelines** 

Powered by Genedata Expressionist®



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Zoomed Range 3

Remicade IdeS reduced

NIST 500ngOC IdeS Red 01

Rituxan\_IdeS\_reduced

Herceptin IdeS TCEP

Humira IdeS TCEP

Name

#### 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.

Detected Mass

25647.35

25327.79

25383.14

25457.95

25688.72

Delta [Da]

-0.17

-0.41

-0.16

-0.37

-0.19

Invalid (X): The calculated mass is above the Attention Threshold.

25647.51

25328.19

25383.31

25458.33

25688.91

Expected Mass

Delta [ppm]

-6.0

-16.0

-6.0

-15.0

-7.0

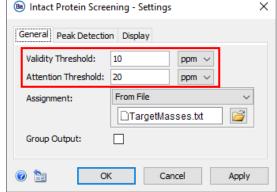
Valid

х

1

!!

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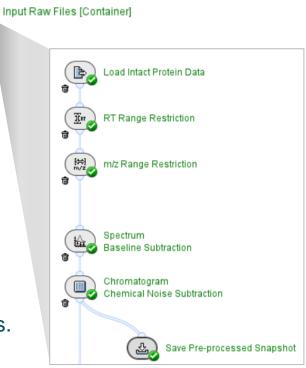




#### Intact Mass Screening Workflow: Overview

- 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 in the container so that intermediate results are deleted as soon as they are passed to the subsequent activity node to save memory.
  - Results from each pre-processed sample can be saved as a snapshot (sbf) file that can be used in other intact mass analysis workflows for further investigation, if required.
- The *Montage View* activity node can display up to 200 samples.
  - If more than 200 samples are analyzed together, then activate the Bypass icon on the *Montage View* activity node.



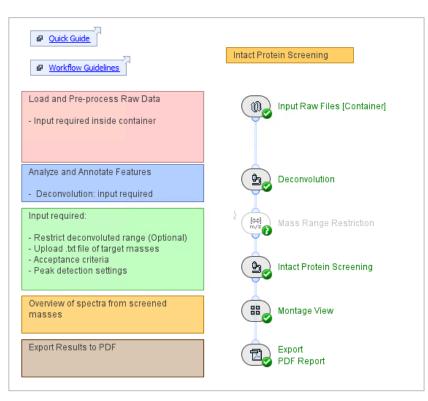




## Intact Mass Screening Workflow: Conditions and Behavior

- For each sample, more than one mass can be searched. However, certain conditions should be met in the submitted samples:
  - Samples must belong to the same molecule type. For example, all samples should be either intact proteins, subunits, or fragments.
  - Samples should have consistent chromatography, the same number of components, and similar expected deconvolution ranges.
  - Samples must have the same number of either **Full** or **Zoomed RT Ranges**.
- The workflow is designed to analyze data as follows:
  - The highest peak in each deconvoluted RT range (Full or Zoomed RT Range) of each sample is detected independently and assigned to a single match based on the list of masses.
  - Results do not include annotation.
  - The values of the detected masses are reported.

#### **Overview of the Intact Mass Screening Workflow**



#### Intact\_MassScreening\_Be4.0





#### Load Intact Protein Data



Name:	Intact Mass Screening
Format:	Auto Detect 🗸
Files/Folders:	Name 🔺 📴
	20171117_Remicade_IdeSreduce_1.wiff
	20171117_Rituxan_IdeSreduce_1.wiff
	20171120_Humira_IdeSTCEP_1.wiff
	Sample002 NIST 500ngOC Ides Red 011

🕖 Server File System							×
🚱 🌑 📓 🍺 🏂 Upload Files 🔓 Download Files	Q Search						
Shared/Intact protein/Raw/X500B/Herceptin_Kadcyla/Herceptin-K	adcyla Whole an	d Subunit					~
Name	Size	Last Mod	ified	Туре	Description		^
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2 170109 Trastuzumab Emantidine 1ul-ug 03.wiff2	332.0KB	07/06/20	20 15:56	WIFF2	AB Sciex Wiff	Гwo	
170109 Trastuzumab Emantidine 1ul-ug 10.wiff.scan		07/06/20		SCAN			
170109 Trastuzumab Emantidine 1ul-ug 10.wiff2		07/06/20		WIFF2	AB Sciex Wiff	Two	
170109 Trastuzumab Emantidine 1ul-ug 11.wiff.scan		07/06/20		SCAN			
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Name			Last Modified	Type	,000C/10 20101101.		*
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D Hercentin 1nmol ul [2]				MS			•
1 out of 12 selected. Herceptin 1pmol_uL [3]				MS			
Herceptin 1pmol_uL [4]				MS			
File/Folder Name He Herceptin Deglycosylated 1pmol_uL [				MS		C	lose
Herceptin Deglycosylated 1pmol_uL[				MS			
Herceptin Deglycosylated 1pmol_uL	7]			MS	~		
Kadcyla 1pmol_uL [8]				MS	*		
				MO	•		
3 out of 62 selected.							
Ple/Folder Name "Herceptin 1pmol uL	[1]" "Herceptin Ded	vcosvlated 1	pmol	Open	Close		
6 of 106							

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

Herceptin Kadcyla 20161101.wiff2

Herceptin Kadcyla 20161101.wiff.scan X

- To select samples in a wiff or wiff2 container file:
- Double-click the wiff or wiff2 container to see the sample files. .
- Select the samples to upload from the list of sample files. .
  - Note: For more information, refer to the page: Input Raw Files [Container]. \_



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

٢	Input Raw	Files [Containe	r]
		Reset	
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		Control	>
	Mas	Show Progress	
Ť		Show Console	
(b)	Intac	Save Graphic	>
Ţ	0	Help	
	Mont 😭	Settings	
		Show Results	
	Export PDF Rend		

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

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
	One Input per Fraction
	by Structure
	by File

# (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 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:	by Structure $\checkmark$	
	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**.

#### Spectrum Baseline Subtraction



• Spectrum Baseline Subtraction removes background noise and decreases the intensity of satellite peaks in the deconvoluted data.

B Spectrum Baseline Subtraction - Settings							
General A	dvanced Displa	зу					
Method:	Quantile		$\sim$				
	Quantile:	50 %					
	m/z Window:	10.0 Da 🗸					
0	ОК	Cancel A	pply				

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

## Chromatogram Chemical Noise Subtraction: Smoothing



General Advar	nced Display togram Smoothin	g
RT Window: Estimator:	5 Moving Averag	Scans Je V
Chemica	l Noise Subtracti	on
RT Window:	51	Scans
Quantile:	50 %	
Method:	Clipping	Subtraction
Threshold:	10	[Intensity]

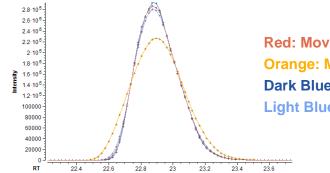
#### Chromatogram Smoothing is used to improve the RT profile of peaks.

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

#### Estimator:

.

- Moving Average uses the mean intensity of data points in the RT Window to add more data points. High values cause peak widths to increase, but peak volume is conserved.
- **Binomial** is an iterative form of **Moving Average** that has less effect on peak widths at high scan values.



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

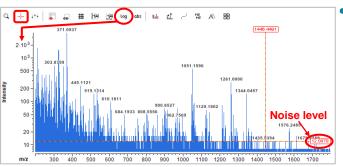
## Chromatogram Chemical Noise Subtraction: Threshold



RT Window:	5	Scans
Estimator:	Moving Average	ge v
Chemica	l Noise Subtract	ion ———
RT Window:	51	Scans
Quantile:	50 %	]
Method:	Clipping	Subtraction
Threshold:	10	[Intensity]

#### Chemical Noise Subtraction can help to:

- Reduce the broad or long-tailing peaks often observed with native data.
- Suppress satellite peaks and improve peak detection with TRD.
- To decrease the amount of noise removal (keep more signal):
  - Decrease the Quantile.
  - Increase the **RT Window**.
- If the noise level is significantly different from the **Threshold** value pre-set in *Chromatogram Chemical Noise Subtraction*, then change this setting.



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

#### **Deconvolution**



Deconvo	lution - Setting	gs				
econvolutio	on Options RT	Ran	ges Displa	у		
Mode:	Automated					
	Min. Mass:	10		kDa		
	Max. Mass:	20	0	kDa		
	Mass Step:	2		Da		
	Visible Range		Only Zoom	ned Ranges		
	fibilitie rearinge		Eagernes		Standard	~
			Max. Mas	s Window:	2.0	kD
	Filter R	IT R	anges —			
	Eagerness:	R	elaxed			
Method:	Maximum Ent	trop	y Deconvolu	ition		
	Iterations:		20			
	Deconvolutio	on O	uality: Sta	andard		

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

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

## *Deconvolution*: Complex Datasets

Scans Curvature-based

Local Maximum

OK

🕜 🛅

Perform Peak Refinement Refinement Threshold: 5 %

Apply Consistency Filter Consistency Threshold: 0.6

Cancel

Apply



Rituxan IdeS reduced

Deconvolution

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Deconvolu	ution - Setting	gs					×		inter
Deconvolution	Options RT	Range	es Displa	ay					D
Mode:	Automated					~		-	Data
	Min. Mass:	10		kDa					
	Max. Mass:	200		kDa		BB	) De	convolution - Settings	s
	Mass Step:	2		Da				volution Options RT R	20000 D: 1
	Visible Range	es:	Only Zoo	med Ranges			econ	Volution Options	Display
			Eagerne	ss:	Standard	~	TIC		
			Max. Ma	ss Window:	2.0	kD.	Г	Use Smoothing	
	- Filter R	RT Ran	nges				R	T Window: 9	Scans
	Eagerness:	Rela	axed				Pe	ak Detection:	Curvature-ba
									Perform
Method:	Maximum Ent	tropy (	Deconvol	lution		_			Refinement
	Iterations:		20	)		_			Kennenent
	Deconvolutio	on Qua	ality: St	tandard					Apply C
Ionization:	Protonation	on ()	) Deproto	onation					Consistency
0			OK	Cano	el	Ap	Ce	nter Computation:	Local Maximur
						-	Bo	undary Determination:	FWHM
							Mi	n. Peak Intensity:	20 %

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

Herceptin IdeS TCEP Humira IdeS TCEP NIST 500naOC IdeS M4949999999999999999

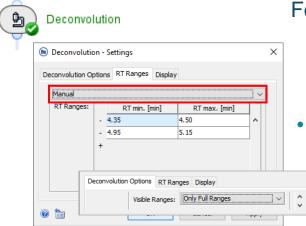
Remicade IdeS reduced

2.3

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

## Deconvolution: RT Ranges





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

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

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

#### Mass Range Restriction



Mass Range Restriction

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

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	ing[i]	[1ng[11]
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Althrup: 1.2 (p)     Alth		
Addamp 1 2-cp [0]     4001     5122 22880.7     504 455.2     20100     605.2     704 505     70     704		
Adjung 13 4 (2)     Adjung 14 (2)     Adjung 13 4 (2)     Adjung 14 (2)     Adj		
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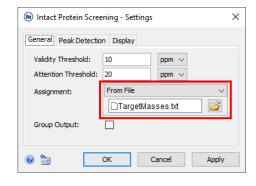
# To Define Target Masses for Intact Protein Screening



- lntact Protein Screening
- Specify the masses of interest for screening:

Intact Protein Scree	ening - Settings	1	×
General Peak Detection	n Display		
Validity Threshold:	10	ppm $\checkmark$	
Attention Threshold:	20	ppm $\checkmark$	
Assignment:	Fixed Masses:	· · · · · · · · · · · · · · · · · · ·	
Group Output:			
0	OK	Cancel Apply	r

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.

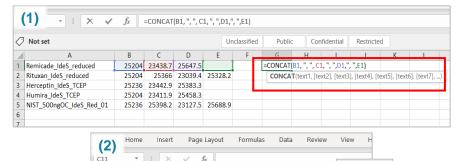
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# Intact Protein Screening: Target Masses File Format

#### To create a txt file for screening:

- Create a table that contains sample names and target masses. Do not include table headers.
  - Use the CONCATENATE function in Excel to combine columns that contain the targeted masses into one column.
  - Notice the format ", ".
- 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.



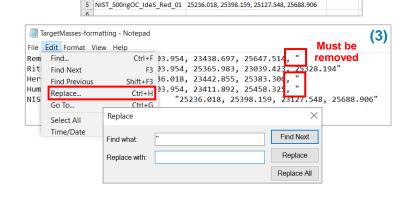


25203.954. 23438.697.25647.514.

25236.018, 23442.855, 25383.306

25203.954, 23411.892, 25458.325

25365.986, 23439.423, 25328.194



Remicade IdeS reduced

Rituxan IdeS reduced

Herceptin IdeS TCEP

4 Humira IdeS TCEP



Paste Values

123 223 12/

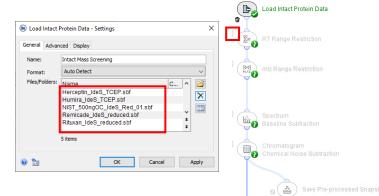
## Export Intermediate Results for Further Analysis



- The Save Snapshot activity node stores intermediate results after pre-processing.
  - An individual sbf file is saved for each sample processed in the workflow.
  - The sbf file contains the properties of the processed data that are required to continue analysis from that point in the workflow.



- To use a Save Snapshot activity node:
  - Deactivate the **Block** icon.
  - Select or add the folders where the sbf files will be stored.
- To use intermediate results from Save Preprocessed Snapshot.
  - 1. Select the sbf file to import into Load Intact Protein Data.
  - 2. Deactivate the **Trash** icon, and then activate the **Bypass** icon on the activity nodes that are before the Snapshot was saved.



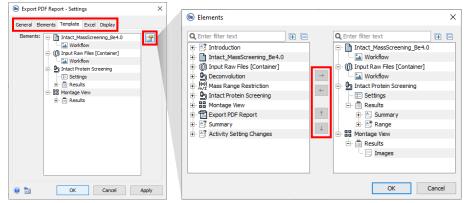
## Export PDF Report

Export

PDF Report



- The exported PDF Report includes:
  - A PDF document.
  - An Excel file with spectral information from deconvolution.
  - An embedded workflow (xml file) that includes all of the settings.
    - To open the xml file, drag the saved PDF Report into the workflow home page in the Biologics Explorer software.
    - Note: For more information, refer to the document: Biologics Explorer Quick Guide.
- **General** tab: Specify the name and saved location of the exported report.
- **Template** tab: Use the **Edit Selection** icon to specify the **Elements** to be included in the report.
  - Select only columns of interest in reported tables. The layout of the tables is controlled by the number of columns.
- Excel tab: Use the Edit Selection icon to specify the Tables to be included in the report.
  - All columns in a selected table are reported.





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