# Intact Nucleotide Template Workflows

#### **Biologics Explorer Software Guidelines**

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# Intact Nucleotide Template Workflow

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#### **Part A** Overview of the Intact Nucleotide Template Workflow





### Overview of Applications for Intact Nucleotide Workflows

- Use the Intact Nucleotide template workflow with deconvolution to analyze these types of molecules:
  - Large synthetic oligonucleotides with their related impurities and modifications.
  - Large nucleotides with their related impurities and modifications, including Poly(A) Tails and 5' Caps.
- Use the Intact Nucleotide template workflow with no deconvolution to analyze these types of molecules:
  - Smaller oligonucleotides (less than 10 kDa) with their related impurities and modifications.
- The Nucleotide Candidate Generation activity nodes create a list of possible nucleotide forms and impurities to annotate MS features in *Mass Mapping*.
- The RT ranges for deconvolution of MS signals can be identified manually, or by the UV or TIC peaks.



#### Intact Nucleotide Template Workflows

#### IntactNucleotide\_Deconvolution:

• An intact nucleotide analysis workflow with spectral deconvolution for the identification of nucleotides and their impurities from MS1-only data (data without MS/MS fragmentation).

#### IntactNucleotide\_NoDeconvolution:

• An intact nucleotide analysis workflow with no deconvolution for the identification of nucleotides and their impurities from MS1-only data (data without MS/MS fragmentation).



#### Part B

#### Activity Nodes in the Intact Nucleotide Workflows

B



#### Intact Nucleotide Template Workflows



Note: For information about activity nodes that are used in all workflows, for example *Load Raw Data, Review Results,* or *Export PDF Report,* refer to the document: *Biologics Explorer Quick Guide*.



IntactNucleotide Deconvolution

#### Nucleotide Candidate Generation



IntactNucleotide\_NoDeconvolution

Use the Nucleotide Candidate Generation activity nodes to create a list of theoretical candidates of the full-length sequence with the applicable impurities and modifications.



#### **B:** ACTIVITY NODES IN THE INTACT NUCLEOTIDE WORKFLOW

### Nucleotide Candidate Generation: Nucleotide Editor

- To review the Nucleotide Building Blocks or to add custom (USER) Building Blocks, browse to File > Tools > Nucleotide Editor.
  - The most frequently used nucleotide bases, sugars, and linkers are included as SYSTEM building blocks that cannot be edited.

Some less frequently used bases and sugars are included as USER building blocks that can be edited.

<b>Q</b> Enter	Filter Text		+ - 😭			Ø
Туре	Abbreviation	Name	Formula	Mass	Source	
Sugar	d	Deoxyribose	H <sub>10</sub> C <sub>5</sub> O <sub>4</sub>	134.058	SYSTEM	
Sugar	r	Ribose	H <sub>10</sub> C <sub>5</sub> O <sub>5</sub>	150.053	SYSTEM	
Linker	0	Phosphate	H₃O₄P	97.977	SYSTEM	
Linker	S	Phosphorothioate	H <sub>3</sub> O <sub>3</sub> PS	113.954	SYSTEM	
Base	Α	Adenine	H <sub>5</sub> C <sub>5</sub> N <sub>5</sub>	135.054	SYSTEM	
Base	G	Guanine	H <sub>5</sub> C <sub>5</sub> N <sub>5</sub> O	151.049	SYSTEM	
Base	С	Cytosine	H <sub>5</sub> C <sub>4</sub> N <sub>3</sub> O	111.043	SYSTEM	
Base	Т	Thymine	H <sub>6</sub> C <sub>5</sub> N <sub>2</sub> O <sub>2</sub>	126.043	SYSTEM	
Base	U	Uracil	H <sub>4</sub> C <sub>4</sub> N <sub>2</sub> O <sub>2</sub>	112.027	SYSTEM	
Base	Q	5-Methyl-cytosine	H <sub>7</sub> C <sub>5</sub> N <sub>3</sub> O	125.059	USER	
Base	н	Hypoxanthine	H <sub>4</sub> C <sub>5</sub> N <sub>4</sub> O	136.039	USER	
Sugar	1	LNA (Locked nucleic acid)	H <sub>10</sub> C <sub>6</sub> O <sub>5</sub>	162.053	USER	
Sugar	m	2'-OMe (2'-O-Methylribose)	H <sub>12</sub> C <sub>6</sub> O <sub>5</sub>	164.068	USER	
Sugar	f	2'-F (2'-Deoxy-2'-fluororibose)	H₀C₅O₄F	152.048	USER	
Sugar	е	2'-MOE (2'-Methoxyethlyribose)	H <sub>16</sub> C <sub>8</sub> O <sub>6</sub>	208.095	USER	

🐵 Edit Buildir	ng Block	×
Type:	Sugar	~
Abbreviation:	m	
Name:	2'-OMe (2'-O-Methylribose)	
Formula:	H12C6O5	

- To edit a **USER** building block, select the entry, and then click the roon.
- To create a new **USER** building block, click the **+** icon.
  - The abbreviation for a base must be a single, uppercase letter.
  - The abbreviation for a sugar or linker must be a single, lowercase letter.





#### **B:** ACTIVITY NODES IN THE INTACT NUCLEOTIDE WORKFLOW

#### Nucleotide Candidate Generation: Mass Calculator

- Use the Mass Calculator to give the theoretical mass and isotopic distribution of a DNA, RNA, or nucleotide sequence.
  - To open the Mass Calculator, browse to File > Tools > Mass Calculator.
  - For a sequence that contains bases with standard (natural) phosphate linkers and ribose (RNA) or deoxyribose (DNA) as the sugar, select Format: RNA or DNA, and then use singlebase notation.
  - For a sequence that contains non-standard (synthetic) USER sugars or linkers, select Format: Nucleotide, and then use triplet notation:
    - Nucleotides triplets can be given in any order, with or without dashes. For
      - example: fA-o-dA-o-dC-o-dT-o-dA-o-dG-o
        - fAodAodCodTodAodGo
        - lU-oAr-oCr-oUr-oAr-oGr-o
        - lUoAroCroUroAroGro
  - To use the chemical formula in *Spectrum Isotope Clustering*, click the **Copy** button.

Note: For information, refer to the section: C: Guidelines for the Intact Nucleotide Workflows.





### Nucleotide Candidate Generation: Nucleotide Sequences

💩 Nuc	leotide Sequer	nces - Settings		×
Sequen	ces Display			
>DNA dGGT	(phosphate lin AAAGGGAG	ker)	 	
>RNA rGGU	(phosphate lin AAAGGGAG	ker)		
>RNA	with Custom B	uilding Blocks		

- Sequences: Use FASTA format:
  - For the input name, use the prefix >.
  - For DNA sequences, use the prefix d.
  - For RNA sequences, use no prefix, or the prefix **r**.
  - For sequences with custom (**USER**) Building Blocks, use triplet notation.
- The Input Sequence column in the Nucleotides Result Table is a reference for the Backbone Index.
  - The **Base Index** counts the bases. The **Backbone Index** also counts the linkers:

Input Sequence:	rG	-0-	rG	-0-	rU	-0-	rA	-0-	rA
Backbone Index:	1	2	3	4	5	6	7	8	9
Base Index:	1		2		3		4		5

Nucleotides X												
🔳 🔻 🖓 🔍												
Identifier	Sequence	Bases	Base Delta	Туре	From (Base)	To (Base)	From (Backbone)	To (Backbone)	Calc. Mass	Calc. Avg. Mass	Formula	Input Sequence
DNA (phosphate linker)	dGGTAAAGGGAG	11	N	Full-Length	1	. 11	1 1	21	3468.636	3470.295	H <sub>134</sub> C <sub>110</sub> N <sub>52</sub> O <sub>61</sub> P <sub>10</sub>	dG-o-dG-o-dT-o-dA-o-dA-o-dG-o-dG-o-dG-o-dA-o-dG
RNA (phosphate linker)	rGGUAAAGGGAG	11	N	Full-Length	1	. 11	L 1	. 21	3630.564	3632.262	H <sub>132</sub> C <sub>109</sub> N <sub>52</sub> O <sub>72</sub> P <sub>10</sub>	rG-o-rG-o-rU-o-rA-o-rA-o-rG-o-rG-o-rG-o-rA-o-rG
RNA with Custom Building Blocks	rGGUA-s-rQ-s-rAGG	8	N	Full-Length	1	. 8	3 1	. 15	2633.376	2634.776	$H_{98}C_{79}N_{35}O_{51}P_7S_2$	rG-o-rG-o-rU-o-rA-s-rQ-s-rA-o-rG-o-rG



Note: For information about Poy(A) Tail candidate generation, refer to the section: *C1: Guidelines for the Intact Nucleotide with Deconvolution Template Workflow*. •

# Nucleotide Candidate Generation: Modifications Settings



Modifications [2] - Settings								
Unlocalized Loc	alized Display							
Limit to:	1	Modifications per Sequence						
Modifications:	Table	~						
	Table File (all entries) File (selected e	ntries)						
		~						
0	ОК	Cancel Apply						

- Select an option to add the definition of the Modifications:
  - **Table:** Type the modifications of interest.
  - File (all entries): Use all of the modifications in a pre-defined txt file.
  - File (selected entries): Use a selection of the modifications from a pre-defined txt file.
    - To see a list of example modifications, browse to Library Browser > Resources > NucleotideModifications\_Library\_Small.
      - Open this file in the **Library Browser** to customize it for use in *Modifications*.

<b>2</b>		
File System		×
🌏 🌑 🔀 🎓 🕼 Upload Files 🎥 Dov	wnload Files	
Resources		~
Name	Size 535B	±
<		>

🗋 🖻 *NucleotideModifications_Library_Small 🗙								
📃 🔜 🤊 (*) 🛝 🥒 🕂 — 🖽 🎛 🧛								
O Films N	C-i	Less en Tress este						
	ame or Gain or	Loss or Type colu	mn.					
Name	Gain	Loss	Type	± *				
A Loss		C5H5N5		^				
C Loss		C4H5N3O						
G Loss		C5H5N5O						
Q Loss		C5H7N3O						
-		CELICN 202		~				



Note: The number of nucleotide candidates created by the selected settings cannot be more than the threshold (100,000). 12 of 57 TPUB-CUST-16012-

### Nucleotide Candidate Generation: *Modifications* Settings



- Unlocalized tab: Create modifications on the nucleotide sequence with a Gain or Loss (by chemical formula or mass in Da).
- Localized tab: Create modifications on a specified Character or Location (backbone index) with a Gain or Loss (by chemical formula or mass in Da).
  - For analysis of mRNA with a 5' capping species, use **5'** as the **Location** for the 5' cap.

(Be) Modification	s [1]	- Settings	;					×
Unlocalized Loc	alized	Display						
Limit to:	1		Modifications per	Sequence				
Modifications:	Tabl	e						$\sim$
		Name	Gain	Loss	On Characters	On Locations	Type	
	-	pp	H4P2O7	H2O		5'	Uncapped	^
	-	ppp	H5P3O10	H2O		5'	Uncapped	
	-	G Cap	C10H16N5O14P3	H2O		5'	Partial	
	-	Cap 0	C11H18N5O14P3	H2O		5'	Capped	
	-	Cap 1	C12H20N5O14P3	H2O		5'	Capped	
	+							~
0					ОК	Cance	A	\pply

- **Type**: Optionally, create a customized nucleotide Type for each modification.
  - If left blank, then the **Type** will show in the **Nucleotides** Results Table as Modified.

§ Nucleotides ×							
💻 🕇 👗 🗟							
Identifier	Sequence	Bases	Base Delta	Туре	Mod. Location (Backbone)	Modifications	
mRNA Cap 0 [1]	rGGGAGACG	18	N	Capped	Cap 0 [1]	1	
mRNA Cap 1 [1]	rGGGAGACG	18	N	Capped	Cap 1 [1]	1	
mRNA OH	rGGGAGACG	18	N	Modified	OH	1	
mRNA G Cap [1]	rGGGAGACG	18	N	Partial	G Cap [1]	1	
mRNA pp [1]	rGGGAGACG	18	N	Uncapped	pp [1]	1	
mRNA ppp [1]	rGGGAGACG	18	N	Uncapped	ppp [1]	1	
mRNA G Cap [1], OH	rGGGAGACG	18	N	Modified, Partial	G Cap [1], OH	2	



#### **B:** ACTIVITY NODES IN THE INTACT NUCLEOTIDE WORKFLOW

# Nucleotide Candidate Generation: Modifications and Impurities

- To create both modified <u>and</u> unmodified versions of candidates with insertions or deletions, activate the **Bypass** icon on *Modifications [1]*.
  - If the Bypass icon is not activated on *Modifications [1]*, then only modified versions of candidates with insertions or deletions are created.





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Туре 👻	Identifier	Seque	Bases	Base Delta	Туре
3' Clip	100 mer -(dG-o[193])	dATCG	99	N-1	Deletion (N-1)
3' Clip+Linker	100 mer -(dC-o[195])	dATCG	99	N-1	Deletion (N-1)
3' Clip+Linker, Modified	100 mer -(dG-o[197])	dATCG	99	N-1	Deletion (N-1)
3' Clip, Modified	100 mer +(dA-o[1])	dAATC	101	N+1	Insertion (N+1)
5' Clip	100 mer +(dT-o[3])	dATTC	101	N+1	Insertion (N+1)
5' Clip+Linker	100 mer +(dC-o[5])	dATCC	101	N+1	Insertion (N+1)
5' Clip+Linker, Modified	100 mer -(dC-o[195]) G Loss	dATCG	99	N-1	Deletion (N-1), Modified
5' Clip, Modified	100 mer -(dC-o[195]) T Loss	dATCG	99	N-1	Deletion (N-1), Modified
Deletion (N-1)	100 mer -(dG-o[197]) A Loss	dATCG	99	N-1	Deletion (N-1), Modified
Deletion (N-1), Modified	100 mer -(dG-o[197]) C Loss	dATCG	99	N-1	Deletion (N-1), Modified
Full-Length	100 mer -(dG-o[197]) G Loss	dATCG	99	N-1	Deletion (N-1), Modified
Insertion (N+1)	100 mer -(dG-o[197]) T Loss	dATCG	99	N-1	Deletion (N-1), Modified
Insertion (N+1), Modified	100 mer +(dA-o[1]) A Loss	dAATC	101	N+1	Insertion (N+1), Modified
Modified	100 mer +(dA-o[1]) C Loss	dAATC	101	N+1	Insertion (N+1), Modified

🖉 Nucleotides 🗙								
I I I I I I I I I I I I I I I I I I I								
Туре 👻	Identifier	Seque	Bases Base Delta	Туре				
3' Clip	100 mer -(dC-o[195]) G Loss	dATCG	99 N-1	Deletion (N-1), Modified				
3' Clip +Linker	100 mer -(dC-o[195]) T Loss	dATCG	99 N-1	Deletion (N-1), Modified				
3' Clip +Linker, Modified	100 mer -(dG-o[197]) A Loss	dATCG	99 N-1	Deletion (N-1), Modified				
3' Clip, Modified	100 mer -(dG-o[197]) C Loss	dATCG	99 N-1	Deletion (N-1), Modified				
5' Clip	100 mer -(dG-o[197]) G Loss	dATCG	99 N-1	Deletion (N-1), Modified				
5' Clip +Linker	100 mer -(dG-o[197]) T Loss	dATCG	99 N-1	Deletion (N-1), Modified				
5' Clip+Linker, Modified	100 mer +(dA-o[1]) A Loss	dAATC	101 N+1	Insertion (N+1), Modified				
5' Clip, Modified	100 mer +(dA-o[1]) C Loss	dAATC	101 N+1	Insertion (N+1), Modified				
Deletion (N-1), Modified	100 mer +(dA-o[1]) G Loss	dAATC	101 N+1	Insertion (N+1), Modified				
Full-Length	100 mer +(dA-o[1]) T Loss	dAATC	101 N+1	Insertion (N+1), Modified				
Insertion (N+1), Modified	100 mer +(dT-o[3]) A Loss	dATTC	101 N+1	Insertion (N+1), Modified				
Modified	100 mer +(dT-o[3]) C Loss	dATTC	101 N+1	Insertion (N+1), Modified				



#### Nucleotide Candidate Generation: Modifications Results



Nucleotides Result Table:

- The candidate **Identifier** is updated to include the modification.
- The Nucleotides Results Table is updated to contain more columns:
  - **Type:** Modified or as defined in the *Modifications* settings.
  - **Modifications**: The number of modifications on the candidate.
  - Mod. Location (Backbone): The name and location of the modification.

Nucleotides	×														
🔳 🝸 🏹 🔍															
Identifier	Sequence	Bases	Base Delta	Туре	Modifications	Mod. Location (Backbone)	From (Base)	To (Base)	From (Backbone)	To (Backbone)	Calc. Mass	Calc. Avg. Mass	Formula	Input Sequence	
NA 2×A-loss	dggtaaagggag	11	N	Modified	2	2×A-loss	1	11	1	21	3234.548	3236.072	H <sub>128</sub> C <sub>100</sub> N <sub>42</sub> O <sub>63</sub> P <sub>10</sub>	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-dG-o-dG-o-dG-o-dA-o-dG	
NA 2×C-loss	dGGTAAAGGGAG	11	N	Modified	2	2×C-loss	1	. 11	1	. 21	3282.570	3284.121	$H_{128}C_{102}N_{46}O_{61}P_{10}$	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-dG-o-dG-o-dG-o-dA-o-dG	
NA 2×G-loss	dggtaaagggag	11	N	Modified	2	2×G-loss	1	11	1	21	3202.558	3204.073	$H_{128}C_{100}N_{42}O_{61}P_{10}$	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-dG-o-dG-o-dG-o-dA-o-dG	
NA 2×T-loss	dGGTAAAGGGAG	11	N	Modified	2	2×T-loss	1	. 11	1	. 21	3252.571	3254.098	H <sub>126</sub> C <sub>100</sub> N <sub>48</sub> O <sub>59</sub> P <sub>10</sub>	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-dG-o-dG-o-dG-o-dA-o-dG	
NA A-loss	dGGTAAAGGGAG	11	N	Modified	1	A-loss	1	. 11	1	21	3351.592	3353.183	$H_{131}C_{105}N_{47}O_{62}P_{10}$	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-dG-o-dG-o-dG-o-dA-o-dG	
NA A-loss, C-loss	dGGTAAAGGGAG	11	N	Modified	2	A-loss, C-loss	1	. 11	1	. 21	3258.559	3260.096	H <sub>128</sub> C <sub>101</sub> N <sub>44</sub> O <sub>62</sub> P <sub>10</sub>	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-dG-o-dG-o-dG-o-dA-o-dG	
NA A-loss, G-loss	dGGTAAAGGGAG	11	N	Modified	2	A-loss, G-loss	1	. 11	1	21	3218.553	3220.072	$H_{128}C_{100}N_{42}O_{62}P_{10}$	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-dG-o-dG-o-dG-o-dA-o-dG	
NA A-loss, T-loss	dGGTAAAGGGAG	11	N	Modified	2	A-loss, T-loss	1	. 11	1	. 21	3243.560	3245.085	H <sub>127</sub> C <sub>100</sub> N <sub>45</sub> O <sub>61</sub> P <sub>10</sub>	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-dG-o-dG-o-dG-o-dA-o-dG	
NA C-loss	dGGTAAAGGGAG	11	N	Modified	1	C-loss	1	11	1	21	3375.603	3377.208	$H_{131}C_{106}N_{49}O_{61}P_{10}$	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-dG-o-dG-o-dG-o-dA-o-dG	
NA C-loss, G-loss	dGGTAAAGGGAG	11	N	Modified	2	C-loss, G-loss	1	. 11	1	. 21	3242.564	3244.097	H <sub>128</sub> C <sub>101</sub> N <sub>44</sub> O <sub>61</sub> P <sub>10</sub>	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-dG-o-dG-o-dG-o-dA-o-dG	
NA C-loss, T-loss	dggtaaagggag	11	N	Modified	2	C-loss, T-loss	1	11	1	21	3267.571	3269.110	H <sub>127</sub> C <sub>101</sub> N <sub>47</sub> O <sub>60</sub> P <sub>10</sub>	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-dG-o-dG-o-dG-o-dA-o-dG	
NA G-loss	dGGTAAAGGGAG	11	N	Modified	1	. G-loss	1	. 11	1	. 21	3335.597	3337.184	H <sub>131</sub> C <sub>105</sub> N <sub>47</sub> O <sub>61</sub> P <sub>10</sub>	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-dG-o-dG-o-dG-o-dA-o-dG	
NA G-loss, T-loss	dggtaaagggag	11	N	Modified	2	G-loss, T-loss	1	11	1	21	3227.565	3229.086	H <sub>127</sub> C <sub>100</sub> N <sub>45</sub> O <sub>60</sub> P <sub>10</sub>	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-dG-o-dG-o-dG-o-dA-o-dG	
NA T-loss	dGGTAAAGGGAG	11	N	Modified	1	. T-loss	1	. 11	1	. 21	3360.603	3362.197	H <sub>130</sub> C <sub>105</sub> N <sub>50</sub> O <sub>60</sub> P <sub>10</sub>	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-dG-o-dG-o-dG-o-dA-o-dG	



#### Nucleotide Candidate Generation: Clipping

Elipping		
🐵 Clipping - Setti	ngs	×
Clipping Display		
Clipping Events:	1 All Clips With and Without Linkers Without Linkers With Linkers	All Clips 3' Clips 5' Clips
0 🛅	OK Cancel	Apply

Nucleotides 🗙					
= 🗕 👗 🖌 🖉					
Identifier	Sequence	Bases	Base Delta	Туре	Clips
100 mer [1-9]	dATCGC	5	N-95	3' Clip	1
100 mer [1-11]	dATCGCG	6	N-94	3' Clip	1
100 mer [1-10]	dATCGC-o	5	N-95	3' Clip +Linker	1
100 mer [191-199]	dAGCGA	5	N-95	5' Clip	1
100 mer [190-199]	o-dAGCGA	5	N-95	5' Clip +Linker	1
100 mer [3-11]	dTCGCG	5	N-95	3' Clip, 5' Clip	2
100 mer [3-12]	dTCGCG-o	5	N-95	3' Clip +Linker, 5' Clip	2
100 mer [2-11]	o-dTCGCG	5	N-95	3' Clip, 5' Clip+Linker	2



- Clipping Events: Select 0, 1, or 2 clips.
- **Keep**: Select the applicable options from the lists:
  - All Clips, 3' Clips, 5' Clips.
  - With and Without Linkers, Without Linkers, With Linkers.

Note: For more information, click the ? icon to open the *Online Help*.

#### Nucleotides Result Table:

- The candidate Identifier is updated to include the backbone index of the clipped candidate.
- The **Nucleotides** Result Table is updated to contain more columns:
  - **Type:** The position of the clip and if it contains a linker.
  - **Clips**: The number of clips to create the candidate.
  - Base-Delta: The number of bases removed from the full-length sequence (N).

Note: The number of nucleotide candidates created by the selected settings cannot be more than the threshold (100,000).

### Nucleotide Candidate Generation: Insertion/Deletion



- Number of Insertions: Select 0 or 1 repetitions of a nucleotide.
- Number of Deletions: Select 0, 1, or
   2 removals of a nucleotide.

#### Nucleotides Result Table:

- The **Name** of the candidate is updated to include the inserted or deleted nucleotide and the backbone index location with the following format:
  - Insertion: +(sugarBase-linker[backbone index]).
  - Deletion: -(sugarBase-linker[backbone index]).
- The **Nucleotides** Result Table is updated to contain more columns:
  - Type: The change to the full-length sequence (N).
  - **Deletion/Insertion (Base)**: The nucleotide with the base index location.
  - **Deletion/Insertions (Backbone)**: The nucleotide with the backbone index location.
  - **Bases**: The number of bases in the modified sequence.
  - **Base-Delta**: The change to the number of bases from the full-length sequence (N).

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🕈 Nucleotides 🗙	§ Nucleotides ×												
Identifier	Sequence	Bases	Base Delta	Туре	Deletion (Base)	Deletion (Backbone)	Insertion (Base)	Insertion (Backbone)					
100mer -(dG-o[11-12])	dATCGCGA	99	N-1	Deletion (N-1)	dG-o[6]	dG-o[11-12]							
100mer -(o-dG[10-11])	dATCGCGA	99	N-1	Deletion (N-1)	o-dG[6]	o-dG[10-11]							
100mer -(dT-o[3-4], dG-o[11-12])	dACGCGAT	98	N-2	Deletion (N-2)	dT-o[2], dG-o[6]	dT-o[3-4], dG-o[11-12]							
100mer -(o-dT[2-3], o-dG[10-11])	dACGCGAT	98	N-2	Deletion (N-2)	o-dT[2], o-dG[6]	o-dT[2-3], o-dG[10-11]							
100mer +(dG-o[11-12])	dATCGCGG	101	N+1	Insertion (N+1)			dG-o[6]	dG-o[11-12]					
100mer +(o-dG[10-11])	dATCGCGG	101	N+1	Insertion (N+1)			o-dG[6]	o-dG[10-11]					



### Nucleotide Candidate Generation: Candidate Consolidation



B Candidate Consolidation - Settings ×										
General Display	/									
Mass Filter		1								
Apply to:	Monoisotopic Mass 🗸 🗸 🗸									
Min. Mass:	Da									
Max. Mass:	Da									
Consolidate by	Consolidate by Mass:									
OK Cancel Apply										

- Select Consolidate by Mass to combine candidates from all Nucleotide Candidate Generation activity nodes, and to merge candidates that have the same input name and input sequence from *Nucleotide Sequences*, and the same mass and modifications from the Nucleotide Candidate Generation activity nodes.
  - Use the Mass Filter to use only the candidates in a specified mass range.

#### Nucleotides Result Table:

- The Identifier column is updated to include all candidate information.
- The **Type** column includes the prefix of **Consolidated**.
- For consolidated candidates, columns that would have different information are empty.

🕈 Nucleotides 🗙	Nucleotides ×															
<b>=                                    </b>																
Identifier	Sequence	Bases	Base Delta	Туре	Clips	Modifications	Mod. Location (	From (Base)	To (Base)	From (Backbone)	To (Backbone)	Calc. Mass	Calc. Avg. Mass	Formula	Input Name	Input Sequence
FLP	dATCGCGGA	100	) N	Full-Length	0	0 0	)	1	100	1	199	30876.14	30890.81	H <sub>1224</sub> C <sub>976</sub> N <sub>383</sub> O <sub>594</sub> P <sub>99</sub>	FLP	dA-o-dT-o-dC-o-dG-o-d
FLP [1-15]	dATCGCGGA	8	3 N-92	3' Clip	1	ι Ο	)	1	. 8	1	15	2433.46	2434.63	H <sub>98</sub> C <sub>78</sub> N <sub>33</sub> O <sub>45</sub> P <sub>7</sub>	FLP	dA-o-dT-o-dC-o-dG-o-d
FLP [1-10] A-loss [1], H2O Loss	dATCGC-o	5	5 N-95	3' Clip +Linker, Base-loss, Modified	1	ι 2	A-loss [1], H2O Loss	1	. 5	1	10	1389.19	1389.84	H <sub>56</sub> C <sub>43</sub> N <sub>13</sub> O <sub>30</sub> P <sub>5</sub>	FLP	dA-o-dT-o-dC-o-dG-o-d
FLP [Oxidation] -(dG-o),-(o-dG)		99	9 N-1	Consolidated (Deletion (N-1), Modified)	0	) 1	L	1	. 100	1	199	30563.08	30577.61	H <sub>1212</sub> C <sub>966</sub> N <sub>378</sub> O <sub>589</sub> P <sub>98</sub>	FLP	dA-o-dT-o-dC-o-dG-o-d
FLP [Oxidation] +(dT-o), +(o-dT)		101	LN+1	Consolidated (Insertion (N+1), Modified)	0	) 1	L	1	100	1	199	31196.18	31211.01	H <sub>1237</sub> C <sub>986</sub> N <sub>385</sub> O <sub>602</sub> P <sub>100</sub>	FLP	dA-o-dT-o-dC-o-dG-o-d
FLP [A-loss] [1-23]	dATCGCGGA	12	2 N-88	Consolidated (3' Clip, Base-loss)	1	L 1	L	1	. 12	1	23	3548.60	3550.30	H <sub>143</sub> C <sub>113</sub> N <sub>42</sub> O <sub>70</sub> P <sub>11</sub>	FLP	dA-o-dT-o-dC-o-dG-o-d
FLP [H2O Loss] [1-17, 183-199]		9	9 N-91	Consolidated (3' Clip, 5' Clip, Modified)	1	L 1	l					2719.49	2720.80	${\sf H}_{109}{\sf C}_{88}{\sf N}_{35}{\sf O}_{51}{\sf P}_8$	FLP	dA-o-dT-o-dC-o-dG-o-d



Note: Isomeric species with different input names or input sequences are not consolidated

#### Nucleotide Candidate Generation: Mass Library Import



Mass Library Import - Settings	×
General Display	
Library: File (all entries)	
Candidates_Library.txt 💕	
OK Cancel Apply	

- Use the *Mass Library Import* activity node to identify features from a list imported as a txt file.
  - To **Bypass** the Nucleotide Candidate Generation activity nodes:
    - 1. Select all of the Nucleotide Candidate Generation activity nodes.
    - 2. Right-click, and then select **Control** > **Bypass Selection**.
  - To use Mass Library Import:
    - 1. Deactivate the **Bypass** icon.
    - 2. Browse to a saved output from the other Nucleotide Candidate Generation activity nodes, or to a previously prepared list of candidates.
      - The library txt file must contain a **Calc. Mass** or **Calc. Avg. Mass** column.
        - Select the applicable **Mass Mode** in the *Mass Mapping* activity node.

Identifier	Sequence	Bases	Base Delta	Туре	Clips	Modifications	Mod. Location (Backbone)	Deletion (Base)	Deletion (Backbone)	Insertion (Base)	Insertion (Backbone)	From (Base)	To (Base)	From (Backbone)	To (Backbone)	Calc. Mass	Calc. Avg. Mass	Formula	Input Sequence
Oligo	dGGTAAAGGGAG	11	1 N	Library (Full-Length)		0 (	0					1	11	1	21	3468.636	3470.295	H <sub>134</sub> C <sub>110</sub> N <sub>52</sub> O <sub>61</sub> P <sub>10</sub>	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-d
Oligo [1-5]	dGGT	3	3 N-8	Library (3' Clip)		1 (	0					1	3	1	5	900.195	900.642	H <sub>38</sub> C <sub>30</sub> N <sub>12</sub> O <sub>17</sub> P <sub>2</sub>	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-d(
Oligo [1-7]	dGGTA	4	1N-7	Library (3' Clip)		1 (	0					1	4	1	7	1213.253	1213.849	H <sub>50</sub> C <sub>40</sub> N <sub>17</sub> O <sub>22</sub> P <sub>3</sub>	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-d
Oligo [1-9]	dggtaa	5	5 N-6	Library (3' Clip)		1 (	0					1	5	1	9	1526.311	1527.056	H <sub>62</sub> C <sub>50</sub> N <sub>22</sub> O <sub>27</sub> P <sub>4</sub>	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-d
Oligo [1-17]	dggtaaaggg	9	9 N-2	Library (3' Clip)		1 (	0					1	9	1	17	2826.526	2827.882	H <sub>110</sub> C <sub>90</sub> N <sub>42</sub> O <sub>50</sub> P <sub>8</sub>	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-d
Oligo [1-6]	dGGT-o	3	3 N-8	Library (3' Clip+Linker)		1 (	0					1	3	1	6	980.162	980.622	H <sub>39</sub> C <sub>30</sub> N <sub>12</sub> O <sub>20</sub> P <sub>3</sub>	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-d
Oliao [1-8]	dGGTA-o	4	1N-7	Library (3' Clip +Linker)		1 (	0					1	4	1	8	1293.219	1293.829	H <sub>e</sub> ,C <sub>ao</sub> N, <sub>7</sub> O <sub>3e</sub> P,	dG-o-dG-o-dT-o-dA-o-dA-o-dA-o-d



All columns in the library txt file are shown in the output table. The **Type** column includes the prefix of **Library**. 19 of 57

Pause Here

Bypass Selection

**Disable Selection** 

**Dispose Results** 

lation

Process

Control

Show Progress

Show Console

Save Graphic

Help.,

Settings.

Show Results

# UV Processing [Container]



- To use UV data, select the correct value in *Select UV Wavelength*.
- To remove low intensity peaks that are not of interest, set a threshold in UV Valid Feature Filter.
  - If the Validity Threshold is set to a percentage of an observable (% Intensity/Total Intensity, % Max. Intensity or % Volume/AUC), then the largest peak is used to calculate the percentage, not the sum of all peaks.

Note: For more information, click the **?** icon to open the *Online Help*.

(B) UV Valid Feature	Filter - Sett	tings	×
General Display			
Data Type: Feature Type:	UV Peaks	~	
Validity Threshold: Present in at Least	2 20	% Max	Intensity     V       % of Experiments     V
0 🛍	OK	(	Intensity/Total Intensity Ta Max. Intensity Volume/AUC % Intensity/Total Intensity
			% Max. Intensity % Volume/AUC

#### • If there is no UV data:

- 1. Select all of the activity nodes in the UV Processing [Container].
- Right-click, and then select Control > Bypass Selection.







#### UV Manual Peak Edit



To manually change the peaks that were detected in the UV chromatogram, use UV Manual Peak Edit.





- To use UV Manual Peak Edit, deactivate the **Bypass** icon.

- Select the **Edit Mode** icon *⊘* to:
  - Move the peak boundaries.
  - Merge selected peaks into a single peak.
  - Delete peaks.
  - Draw new peaks.



#### Adduct Grouping



(B) Adduct Grouping - Settings	× • F
General Display	• F
RT Tolerance:     0.1       mass Tolerance:     20.0       Allowed Adducts:     K+	Minutes S
Grouping Stringency:	Belect Entries
Gap Size: 0 Detect Multimers:	
Merge Charge and Adduct Groups:  Mass Mode:	
OK Cance	el Apply
L	ОК

- Use Adduct Grouping to group isotopic clusters together that have the same neutral mass and RT.
  - Use the + to select from the list of available adducts.
  - Select the applicable Mass Mode:

 $\times$ 

☆ ☆ ☆

☆

Cancel

- For data with isotopically resolved peaks, or to use **Mass**, select **Monoisotopic**.
- For data with peaks that are not isotopically resolved, or to use Avg. Mass, select Average.

Note: For information about analysis of data with peaks that are not isotopically resolved, refer to the section: *D1: Guidelines for Specific Applications > Recommended Settings for Isotopically Nonresolved Data*.



# Adduct Grouping: Merge Charge and Adduct Groups



- Select **Merge Charge and Adduct Groups** to group isotopic clusters together into a single group for *Mass Mapping*.
- Do not select **Merge Charge and Adduct Groups** to identify adducts as separate groups for *Mass Mapping*.



**SCIEX** 

### Mass Mapping



ß	🐵 Mass Mapping - Settings 🛛 🗙 🗙									
General Display										
	Mass Tolerance:	20	ppm $ \smallsetminus $							
	Mass Mode:	Monoisotopic      Average								
		✓ Limit to Bes	t Match							
		Ignore Ann	otated Features							
		ОК	Cancel	Apply						

- Use *Mass Mapping* to annotate MS features with candidates created in the Nucleotide Candidate Generation activity nodes, or with candidates from the *Mass Library Import*.
- Select the applicable **Mass Mode**:
  - For data with isotopically resolved peaks, or to use Mass, select
     Monoisotopic.
  - For data with peaks that are not isotopically resolved, or to use Avg.
     Mass, select Average.
- Limit to Best Match: Select to use the nucleotide candidate with the lowest mass delta to the detected mass for annotation.
  - To see all annotations within the specified Mass Tolerance, do not select Limit to Best Match.



#### Annotate UV Peaks from MS



Note: If a UV peak has more than one MS annotation, then the activity node shows a yellow warning.

Annotate UV Peaks from MS - Settings ×											
General Display											
RT Tolerance:	0.1	Minutes									
Normalize relative to:	All Peaks	$\sim$									
UV Observable:	AUC	$\sim$									
MS Observable:	MS Observable: Intensity/Total Intensity 🗸										
Annotation Report Mode:	Manual		$\sim$								
	Annotations:	🗹 Туре	^								
		🗹 Identifier									
		Input Name									
		< >	Ť								
0 1	ОК	Cancel A	Apply								

- This activity node uses MS peak information to annotate the related peaks in the **UV Chromatogram**, and then calculate the relative UV and MS abundances.
  - A related peak must elute in the specified **RT Tolerance**.

Peak	RT	Annotations	UV Abs	orbance [/	AUC] R	elative UV Absorband	ce					
Peak_1	19.15	1	2.07		2	2.61 %						
Peak_2	22.22	3	1.90		20	0.84 %						
Peak_3	23.28	1	1.83		20	0.0 %						
Peak_4	24.15	2	1.76		1	0.02.04		1				
Dook	04.77	4	4 50	UV Peak	RT	Identifier	Туре	UV AUC	UV Relative	Adjusted UV	MS Relative Intensity	UV Peak Annotated
Data U	/ Quantities 🧯	🗦 UV Annotatio	ns   Sumn	Peak_1	19.15	Impurity	Full-Length	2.07	22.61 %	22.61 %	100.0 %	100.0 %
				Peak_2	22.22	Target [1-46]	3' Clip+Linker	1.90	20.84 %	0.12 %	0.57 %	100.0 %
1				Peak_2	22.22	Target [1-59]	3' Clip	1.90	20.84 %	20.13 %	96.58 %	100.0 %
				Peak_2	22.22	Target [60-119]	5' Clip+Linker	1.90	20.84 %	0.59 %	2.85 %	100.0 %
				Peak_3	23.28	Unknown	Full-Length	1.83	20.0 %	20.0 %	100.0 %	100.0 %
				Peak_4	24.15	Unknown [5-79]	5' Clip	1.76	19.23 %	0.06 %	0.32 %	100.0 %
				Peak_4	24.15	Target [1-99]	3' Clip	1.76	19.23 %	19.17 %	99.68 %	100.0 %
				Dook E	04 77	Toroot (04, 140)	E! Olin+Linkor	1 50	17 21 0/	0.07.04	0.42.04	100.0.%
				Data UV	/ Quantit	ies UV Annotations	🗦 Summary 15 it	ems				

- Normalize relative to:
  - All Peaks: Relative UV absorbance is calculated across all detected peaks.
  - **Annotated Peaks**: Relative UV absorbance is calculated across annotated peaks.
    - MS Relative Intensity is calculated across annotated peaks.
  - **Annotation Report Mode: Manual:** 
    - Select the information about the annotated features that is included in the result table.

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### Extract Report Elements



- Use the *Extract Report Elements* to customize the tables that will be included in the PDF report.
  - To see the columns that are available for selection in *MS Join* or *MS Quantities*, run the activity nodes that are immediately before them.

0

OK

- *MS Join*: If **Bypass** is activated for Adduct Grouping, then select Cluster.
- *MS Quantities*: Select the columns of interest for the table in the report.

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

	Elapsed Time	4 msec
	Status	Suspicious
	Message	The following expected columns are absent or empty: Review Status
Summary		



Sequence

Bases Base Delta Apply

ancel

# Part C

Guidelines for the Intact Nucleotide Workflows



Intact Nucleotide with Deconvolution Template Workflow Guidelines C1



#### Intact Nucleotide with Deconvolution Template Workflow

- This section contains information about these activity nodes of interest:
  - Chromatogram Chemical Noise Subtraction
  - Nucleotide Poly(A) Tails
  - RT Range Condensing
  - Deconvolution
  - Spectrum Peak Detection
  - Spectrum Isotope Clustering

Note: For information about activity nodes that are used in all workflows, for example *Load Raw Data, Review Results,* or *Export PDF Report,* refer to the document: *Biologics Explorer Quick Guide.* 





# Chromatogram Chemical Noise Subtraction: Optional



- To use *Chromatogram Chemical Noise Subtraction*, deactivate the **Bypass** icon.
  - Chromatogram Smoothing is used to improve the RT profile of peaks for peak detection.
  - **Chemical Noise Subtraction** should be used with care, and only when very high background noise has an effect on the quality of the deconvolution.

Note: For more information, click the ? icon to open the *Online Help*.



### Nucleotide Candidate Generation: Poly(A) Tails



Be	B Nucleotide Poly(A) Tails - Settings							
Po	oly(A	A) Tail Candida	tes Display					
		Name	5' Sequence	Poly(A) Min.	Poly(A) Max.	3' Sequence		^
	-	Segment 1	AAGGAGA	1	50		~	
	-	Segment 2		1	30	GAGG		- 11
	+				^	^		~
0	1			OK	Car	ncel	Арр	ly



• Poly(A) Tail Candidates:

- Use the + icon to add the correct number of rows.
- Add a 5' Sequence or 3' Sequence, or leave blank if not required.
- Type the minimum and maximum expected number of adenosines in the Poly(A) Tail under investigation.
- Activate the **Bypass** icon on *Clipping* and *Insertion/Deletion*.
- The **Identifier** and the **Poly(A) Bases** columns in the **Nucleotides** Result Table contain the number of adenosines in that candidate.

8 Nucleotides	×							
<u> </u>								
Identifier	Sequence	Bases	Poly(A) Bases	Туре	Input Name			
Segment 1 [10*A]	rAAGGAGAAGAAGAAGAAGAAAAAAAAAAA	30	10	Poly(A) Tail	Segment 1			
Segment 1 [11*A]	rAAGGAGAAGAGAAGGAAGAGAAAAAAAAAAAA	31	11	Poly(A) Tail	Segment 1			
Segment 1 [12*A]	rAAGGAGAAGAAGAAGAAGAAAAAAAAAAAAAAAAAAAA	32	12	Poly(A) Tail	Segment 1			
Segment 1 [13*A]	raaggagaagaagaaggaagaaaaaaaaaaaaa	33	13	Poly(A) Tail	Segment 1			
Segment 1 [14*A]	rAAGGAGAAGAGAAGGAAGAGAAAAAAAAAAAAAAA	34	14	Poly(A) Tail	Segment 1			
Segment 1 [15*A]	rAAGGAGAAGAGAAGGAAGAGAAAAAAAAAAAAAAAAAA	35	15	Poly(A) Tail	Segment 1			
Segment 2 [30*A]	raaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa	34	30	Poly(A) Tail	Segment 2			
Segment 2 [29*A]	raaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa	33	29	Poly(A) Tail	Segment 2			
Segment 2 [28*A]	raaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa	32	28	Poly(A) Tail	Segment 2			
Segment 2 [27*A]	raaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa	31	27	Poly(A) Tail	Segment 2			
Segment 2 [26*A]	raaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa	30	26	Poly(A) Tail	Segment 2			
Segment 2 [25*A]	raaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa	29	25	Poly(A) Tail	Segment 2			

#### RT Range Condensing



• *RT Range Condensing* detects regions of the ion map that contain signal, and then creates a single spectrum across the RT in these regions.

VL	~
UV Wavelength: 2	60 nm
Use Existing UV Peaks:	2
Use Smoothing	
RT Window: 50	Scans
Peak Detection:	Ascent-based Apply Isolation Filter Isolation Threshold: 3 Scans
Center Computation:	Local Maximum $\sim$
Boundary Determination:	FWHM 🗸
Min. Peak Intensity:	1%

• Select an option from the list:

- TIC: Uses the peaks in the total ion chromatogram to identify the RT ranges to condense.
- **UV**: Uses peaks in the UV data to identify the RT ranges to condense.
- Manual: For complex separations, identify the RT ranges to condense manually.



### RT Range Condensing: UV and TIC RT Ranges



	RT Range Condensing - S	ettings X
RTI	Ranges Display	
·	TIC - 🔽 Use Smoothing	
	RT Window: 7	Scans
	Peak Detection:	Ascent-based  V Apply Isolation Filter
		Isolation Threshold: 10 Scans
	Center Computation:	Intensity-weighted V Intensity Threshold: 70 %
	Boundary Determination:	Inflection Points $\checkmark$
	Min. Peak Intensity:	1%

🐵 RT Range Condensin	ıg - Settings		×
RT Ranges Display			
UV			~ ^
UV Wavelength:	280	nm	
Use Existing UV Peaks	s: 🔽		~
0	OK	Cancel	Apply

#### **RT Ranges: TIC**

- Peak Detection:
  - To identify local maxima in the MS signal, select **Ascent-based**.
  - To identify changes in the curvature of the MS signal, for example to identify shoulder peaks, select **Curvature-based**.

Note: For more information, click the ? icon to open the *Online Help*.

#### **RT Ranges: UV**

- 1. Select the **UV Wavelength**.
- 2. Select Use Existing UV Peaks.
- If Use Existing UV Peaks is selected, then other peak detection settings on this tab are ignored.



### RT Range Condensing: Manual RT Ranges



#### **RT Ranges: Manual**

- Select RT ranges manually if the components of interest are not chromatographically resolved.
  - For example, if the peaks in the TIC or the UV chromatogram do not show all of the components of interest.

[	Manual				~
	RT Ranges:		RT min. [min]	RT max. [min]	
		-	0.8	0.8	
		-	0.8	1.2	1
		-	1.5	1.8	
		-	1.8	2.0	
		-	2.1	2.6	
		+			<b>v</b>

g

TR Deconvolution



- To use *TR Deconvolution* to help to identify RT ranges of interest:
  - 1. Deactivate the **Bypass** icon.
    - 2. Review the results of TR Deconvolution.
  - 3. Type the RT ranges of interest in *RT Range Condensing*.

#### Deconvolution



Beconvolution - Settings >								
Deconvolution Options Display								
Method: Maximum Entropy Deconvolution								
	Iterations: 25							
	Deconvolution Quality: High $\sim$							
Coutput M	ass Spectrum							
Min. Mas	s: 10	kDa	i					
Max. Ma	ss: 100	kDa	i i i i i i i i i i i i i i i i i i i					
Mass Ste	ep: 0.1	Da						
Mass Step:     0.1     Da       Ionization:     Orotonation     Deprotonation       Image: OK     Cancel     Apply								

• The RT ranges detected in *RT Range Condensing* are deconvoluted.

- Deconvolution Quality:
  - Select **High** for isotopically resolved data.
  - Select Standard for lower-resolution data.
- Min. Mass and Max. Mass:
  - Use a wide mass range to decrease the number and intensity of harmonic peaks.
  - It is not recommended to use a Min. Mass value that is lower than the maximum *m/z* value of the data that will be deconvoluted.
- Mass Step: Set a value that keeps the peak resolution of the data.
  - 0.05 Da to 0.2 Da for isotopically resolved data.
  - 1 Da to 2 Da for lower-resolution data.



# MS Peak Detection [Container]: Spectrum Peak Detection

(	<b>b</b> _	D	econvolut	ion		
{		M	ass Rang	je Restri	tion	
(	Ê,	M	6 Peak D	etection (	Conta	(iner)
		_				]
		2	Spectrur Baseline	n 9 Subtract	ion	
	Ŀ		Spectrur Peak De	n tection		

Valid Feature Filter

Spectrum Peak Detection	on - Settings
Peak Detection Peak Filteri	ng Display
Use Smoothing —	
m/z Window: 5	Points ~
Peak Detection:	Curvature-based ~
	Perform Peak Refinement
	Refinement Threshold: 5 %
	Apply Consistency Filter
	Consistency Threshold: 0.6
Center Computation:	Local Maximum V
Boundary Determination:	Inflection Points $\sim$

The default *Spectrum Peak Detection* settings are applicable for most data with isotopically resolved peaks.

Note: For information about analysis of data with peaks that are not isotopically resolved, refer to the section: *D1: Guidelines for Specific Applications > Large Intact Nucleotides (Nonresolved Data)*.

Peak Detection:

- To identify local maxima in the MS signal, select **Ascent-based**.
- Increase the **Isolation Threshold** to increase the minimum distance between local maxima for a peak to be detected.
- To identify changes in the curvature of the MS signal, for example to identify shoulder peaks in the *m/z* direction, select Curvature-based.
  - Decrease the **Refinement Threshold** to increase the split sensitivity.
  - Increase the **Consistency Threshold** to decrease the split sensitivity.

Note: For more information, click the ? icon to open the *Online Help*.



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Apply

Da

Cancel

#### Spectrum Isotope Clustering



Recompute Mono-Isotopic Peak

Mass Threshold: 2500.0

OK

- Use *Spectrum Isotope Clustering* to group peaks together in an isotopic envelope.
  - Average Formula: Type a representative molecular formula for the nucleotide under investigation.
    - The chemical formula from the **Mass Calculator** can be copied and pasted here.
  - Ionization: Select Massless for deconvoluted data.
- Spectrum Isotope Clustering is required for Adduct Grouping with isotopically resolved and nonisotopically resolved data.
- Use Singleton Filter with isotopically resolved data to remove peaks that are not clustered.

Note: For information about analysis of data with peaks that are not isotopically resolved, refer to the section: *D1: Guidelines for Specific Applications > Recommended Settings for Isotopically Nonresolved Data*.



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# Spectrum Isotope Clustering: Distance Measure



The Distance Measure compares each experimental peak to the theoretical isotope profile for the representative molecular formula in Average Formula.
If a peak of interest has not been clustered as required, then compare the results of Log Ratio and Cosine:



 Log Ratio treats all peaks in a cluster equally.



*Cosine* converts each cluster into a vector, with the contribution of each peak relative to the abundance. Therefore, smaller peaks have less impact.



Intact Nucleotide with no Deconvolution Template Workflow Guidelines C2



#### Intact Nucleotide with No Deconvolution Template Workflow

- This section contains information about these activity nodes of interest:
  - Chromatogram Chemical Noise Subtraction
  - MS Peak Detection
  - Isotope Clustering
  - Charge Grouping

Note: For information about activity nodes that are used in all workflows, for example *Load Raw Data, Review Results,* or *Export PDF Report,* refer to the document: *Biologics Explorer Quick Guide.* 





# Chromatogram Chemical Noise Subtraction: Smoothing



Chromatogram Chemical Noise Subtraction - Settings	$\times$							
General Advanced Display								
- 🗹 Chromatogram Smoothing								
RT Window: 9 Scans								
Estimator: Binomial ~								
Chemical Noise Subtraction								
RT Window: 301 Scans								
Quantile: 70 %								
Method:      Clipping      Subtraction								
Threshold: 7 [Intensity]								

**Chromatogram Smoothing** is used to improve the RT profile of peaks for peak detection.

- Estimator:
  - Moving Average replaces the intensity of each data point with the mean average intensity of the data points in the RT Window. High values cause peak widths to increase, but peak volume is not changed.
  - **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: RT Window & Quantile



Chromatog	ram Chemical Noise Subtraction - Settings	$\times$
General Advar	nced Display	
Chroma	togram Smoothing	
RT Window:	9 Scans	
Estimator:	Binomial ~	
– 🗹 Chemica	Noise Subtraction	1
RT Window:	301 Scans	
Quantile:	70 %	
Method:	Clipping O Subtraction	
Threshold:	7 [Intensity]	
a 🍬	OK Cancel Apply	
9 💷	Cancer Apply	

Chemical Noise Subtraction decreases the length of long-tailing peaks.

- Change this setting if the default values remove too much signal.
- If too much signal if removed, it can be identified by:
  - Excessive cutoff of the tails of very wide (extended RT) peaks.
  - Loss of low-intensity isotope peaks from singly (+1) or doubly (+2) charged clusters, or from low-intensity clusters of interest:



- To decrease the amount of noise removal (keep more signal):
  - Decrease the Quantile.
  - Increase the **RT Window**.



# Chromatogram Chemical Noise Subtraction: Threshold



Chromatogi General Advar	ram Chemical M nced Display	Noise Subtraction - Set	tings X
Chromat	ogram Smoothin	g	
RT Window:	9	Scans	
Estimator:	Binomial		$\sim$
Chemica	Noise Subtracti	on	
RT Window:	301	Scans	
Quantile:	70 %		
Method:	Clipping	) Subtraction	
Threshold:	7	[Intensity]	
a 🛌	OK	Cancel	Apply

- If the noise level is significantly different from the **Threshold** value in *Chromatogram Chemical Noise Subtraction*, then change this setting.
- To measure the noise level and identify an applicable **Threshold** intensity value:
  - 1. Drag the intensity axis of the mass spectrum 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.





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#### **MS** Peak Detection



Beak Detection - Settings X	Decree
General Peak Detection Display	
Summation Summation Window: 10 Scans V	MS Peak Detection - S
Peak Detection	General Peak Detection
Minimum Peak Size: 11 Scans ~	Use Smoothing —
Maximum Merge Distance: 9 Points ~	m/z Window: 5
Merge Strategy: O Boundaries O Centers	
- 🔽 Use Peak RT Splitting Intensity Profiling: ( ) Maximum () Sum	Peak Detection:
Gap/Peak Ratio: 30 %	
Smoothing Algorithm: None ~	
OK Cancel Apply	Center Computation: Boundary Determination:

Decrease or remove Smoothing. X eak Detection - Settings Peak Detection Display Use Smoothing Points 🗸 Vindow: 5 Curvature-based Detection: Ascent-based Curvature-based Resolution-based Apply Consistency Filter Consistency Threshold: 0.6 ٠ Local Maximum Computation:

Inflection Points

Cancel

OK

 $\sim$ 

Apply

shoulder peaks) in the RT direction:

Decrease the Gap/Peak Ratio.

not been deconvoluted.

#### Peak Detection:

The default *MS Peak Detection* settings are applicable for most data that has

**Use Peak RT Splitting**: To increase the number of peaks detected (increase split sensitivity of

- To identify local maxima in the MS signal, select **Ascent-based**.
  - Increase the **Isolation Threshold** to increase the minimum distance between local maxima for a peak to be detected.
- To identify changes in the curvature of the MS signal, for example to identify shoulder peaks in the *m/z* direction, select Curvature-based.
  - Decrease the **Refinement Threshold** to increase the split sensitivity.
- Increase the Consistency Threshold to decrease the split sensitivity.
   Note: For more information, click the ? icon to open the Online Help.
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### Isotope Clustering



🐵 lsotope	Clustering - Settings	5	×
General Er	velope Fitting Adva	nced Display	
Method:	Average Formula Iso	otope Shaping v	
	Average Formula:	H233C185N73	
	Ionization:	Deprotonation ~	
		Minimum Charge: 1	
		Maximum Charge: 20	
	Distance Measure:	Cosine $\lor$	
	Max. Distance:	0.6	
	- 🔽 Recompute Mo	ono-Isotopic Peak	
	Mass Threshold: 3	3500.0 Da	
		,	
0 🛅	ОК	Cancel Apply	

- Use *Spectrum Isotope Clustering* to group peaks together in an isotopic envelope.
- *Isotope Clustering* is required for *Charge Grouping* and *Adduct Grouping*.
  - Average Formula: Type a representative molecular formula for the nucleotide under investigation.
    - The chemical formula from the **Mass Calculator** can be copied and pasted here.
  - Ionization: Select Deprotonation for data that was acquired in negative ion mode.



# *Isotope Clustering*: Distance Measure



Isotope Clustering - Settings >			
General Er	nvelope Fitting Advar	nced Display	
Method:	Average Formula Iso	tope Shaping v	
	Average Formula:	H233C185N73	
	Ionization:	Deprotonation ~	
		Minimum Charge: 1	
		Maximum Charge: 20	
	Distance Measure:	Cosine 🗸	
	Max. Distance:	0.6	
	🕞 🔽 Recompute Mo	no-Isotopic Peak	
	Mass Threshold: 3	500.0 Da	
0	ОК	Cancel Apply	

The Distance Measure compares each experimental peak to the theoretical isotope profile for the representative molecular formula in Average Formula.
If a peak of interest has not been clustered as required, then compare the results of Log Ratio and Cosine:



• Log Ratio treats all peaks in a cluster equally.



*Cosine* converts each cluster into a vector, with the contribution of each peak relative to the abundance. Therefore, smaller peaks have less impact.



# Singleton Filter and Charge Grouping

Ŀ	MS Peak Detectio	on
TE S	Isotope Clusterin	g
T	Singleton Filter	
E.	Charge Grouping	1
E.	Adduct Grouping	
		📵 Charge Gro
		General Displ

• Use *Singleton Filter* to remove peaks that are not clustered.

🐵 Charge Groupin	Charge Grouping - Settings		
General Display			
RT Tolerance:	0.05 Minutes		
Mass Tolerance:	20.0 ppm ~		
Ionization by:	-H+ ~		
Mass Mode:	Monoisotopic O Average		
Allow Gaps:			
🞯 🛅 🛛 ОК	Cancel Apply		

- Use *Charge Grouping* to group related clusters with different charge states together.
  - Ionization by: Select -H<sup>+</sup> for data that was acquired acquired in negative ion mode.



#### **Part D** Guidelines for Specific Applications





#### Recommended Settings for Isotopically Nonresolved Data Application Specific Information

D1



#### Nucleotide Deconvolution Workflow for Isotopically Nonresolved Data

- This section contains additional information about these activity nodes:
  - Spectrum Peak Detection
  - Spectrum Isotope Clustering
  - Singleton Filter
  - Adduct Grouping
  - Mass Mapping
  - Export Report Elements





# MS Peak Detection [Container]: Spectrum Peak Detection



- Use **Peak Detection: Ascent-based** for data with peaks that are not isotopically resolved.
  - To remove unwanted detection of peak shoulders, increase the Smoothing or Isolation
     Threshold.







### Spectrum Isotope Clustering

мз	Peak Detection (C	Container]			
Ge Sp	ectrum	🐵 Spectru	m Isotope Clustering	- Settings	2
So Iso	tope Clustering	General E	nvelope Fitting Advar	nced Display	
Sin	aleton Filter	Method:	No Shape Restriction		~
	giotori i itali		Ionization: Massles	S	$\sim$
	duct Grouping		ОК	Cancel	Apply
	additorouping		UN	Contect	

- *Spectrum Isotope Clustering* is required for *Adduct Grouping* with data that is not isotopically resolved.
  - Method: No Shape Restriction
  - Ionization: Massless

- Make sure that the results of *Spectrum Isotope Clustering* do not contain clusters with a **Size** that is more than 1.
  - To remove unwanted detection of peaks, increase the Smoothing or Isolation
     Threshold in Spectrum Peak Detection.







# Singleton Filter and Adduct Grouping



Adduct Grouping - Settings		$\times$
General Display		
RT Tolerance:	0.1 Minutes	
Mass Tolerance:	20.0 ppm ~	
Allowed Adducts:	K+ +	
	Na+	
Grouping Stringency:	Relaxed O Strict	
Gap Size:	0	
Detect Multimers:		
Merge Charge and Adduct Groups:		
Mass Mode:	O Monoisotopic   Average	]
🙆 🛅 ОК	Cancel Apply	

- Singleton Filter:
  - Activate the **Bypass** icon.
- Adduct Grouping:
  - Mass Mode: Average.







Mass Mapping - Settings	×
General Display	
Mass Tolerance: 0.1 Da 🗸	
Mass Mode: O Monoisotopic O Average	
Limit to Best Match	
Ignore Annotated Features	
🔞 🛅 OK Cancel	Apply

• Select Mass Mode: Average.





### Extract Report Elements



SCIEX

- MS Quantities:
  - Select Average Mass and Calc. Avg. Mass from the list.
    - To see the columns that are available for selection, run *MS Join*, and then select from the list.

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

Annotate UV Peaks from	Annotate UV Peaks from MS - Settings			
General Display				
RT Tolerance:	0.1	Minutes		
Normalize relative to:	All Peaks	$\sim$		
UV Observable:	AUC	$\sim$		
MS Observable:	Intensity/Total	Intensity $\sim$		
Annotation Report Mode:	Manual	~		
	Annotations:	Type ^		
		☑ Identifier		
		🗹 Average Mass		
		🗹 Calc. Avg. Mass 🗸 🗸		
0	OK	Cancel Apply		

📵 MS Qua	B MS Quantities - Settings			
Selection	Output Display			
+ 0	Column	Rename as		
-+	Spectrum	~		
- + 1	Identifier	~		
- +	Туре	~		
-+	Sequence	~		
- + -	Bases	~		
-+	Base Delta	~		
-+	Average Mass	~		
- +	Calc. Avg. Mass	~		
-+	Mass Delta [ppm]	~		
-+	Total Intensity [%]	~		
- + -	Review Status	~		
0 🛅	ОК	Cancel	Apply	

 Average Mass and Calc. Avg. Mass can also be selected in Annotate UV Peaks from MS.

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