A streamlined LC-MS workflow for intact oligonucleotide analysis with enhanced MS sensitivity

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This technical note highlights a streamlined ion-pairing reverse-phase liquid chromatography-mass spectrometry (IP RPLC-MS) workflow for intact oligonucleotide analysis with enhanced MS sensitivity. This workflow combines the benefits of high MS sensitivity offered by the ZenoTOF 8600 system and easy-to-use features of Biologics Explorer software, leading to rapid quality and impurity assessment of oligonucleotides with increased confidence and user experience.

Oligonucleotide therapeutics are a diverse group of drugs targeting a variety of diseases through different mechanisms of action.[1] Synthetic oligonucleotides often carry various modifications and product-related impurities. Intact IP RPLC-MS is a common approach to verify the identity of a synthetic oligonucleotide and assess the level of impurities. A streamlined workflow–from data acquisition to result interpretation–is essential to enable rapid mass confirmation and impurity assessment, accelerating the development of therapeutic oligonucleotides.

Key features of the streamlined IP RPLC-MS workflow for intact oligonucleotide analysis

- **High sensitivity**: The improved hardware of the ZenoTOF 8600 system leads up to 10X gain in MS sensitivity compared to the previous platform, enabling oligonucleotide impurity analysis with reduced sample load
- **Streamlined**: Biologics Explorer software provides an easy-to-use, optimized data analysis workflow for oligonucleotide sequence creation, intact deconvolution and mass mapping
- **Flexibility**: Biologics Explorer software offers the flexibility for advanced users to fine-tune processing parameters or algorithms to achieve the optimal result
- **Excellent visualization**: The 2-dimensional ion map within Biologics Explorer software enables rapid data/result review and impurity assessment
- **Consistent recovery:** Biozen Oligo LC column combines core-shell particle technology and bioinert hardware, significantly increasing the chromatographic efficiency

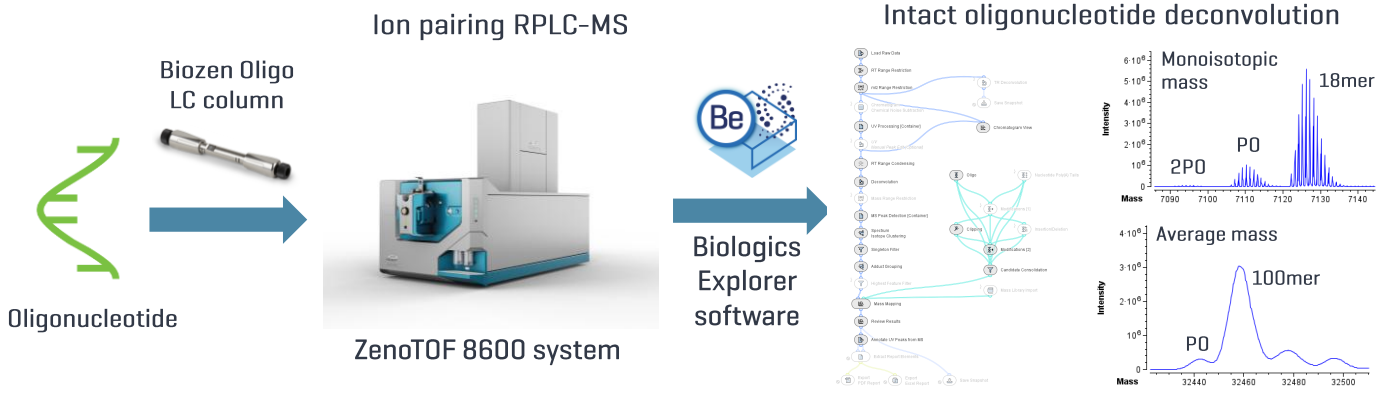

Figure 1. A streamlined workflow for intact oligonucleotide analysis. Oligonucleotides are separated based on IP RPLC using a Phenomenex Biozen Oligo LC column. Intact MS data of oligonucleotides are acquired using the ZenoTOF 8600 system with improved MS sensitivity. Intact oligonucleotide deconvolution is performed using an easy-to-use, optimized data analysis workflow within Biologics Explorer software. This powerful software provides ease and flexibility in defining oligonucleotide sequences, creating custom-modifications and performing intact devolution based on monoisotopic or average mass. In addition, Biologics Explorer software offers excellent tools for data/result visualization and advanced settings for fine optimization when necessary.

Methods

Sample preparation: A desalted oligonucleotide 18mer and an HPLC-purified sgRNA 100mer were purchased from Integrated DNA Technologies (IDT). A stock solution of 1 mg/mL was prepared for 2 oligonucleotides. This solution was diluted to 10 ng/µL, from which 5 µL (50 ng) was injected for IP RPLC-MS analysis.

IP RPLC separation: Oligonucleotides were separated based on IR RPLC using a Phenomenex Biozen Oligo LC column (2.6 µm, 50 x 2.1 mm) installed on a Nexera XS inert HPLC system (Shimadzu). The separation was performed using the gradient shown in Table 1 with a column temperature of 60°C and flow rate of 0.4 mL/min. Mobile phases A and B are water and methanol/water (1:1 v/v), respectively, with 50 mM 1,1,3,3,3-hexafluoroisopropanol (HFIP, Sigma-Aldrich) and 15 mM diisopropylethylamine (DIPEA, Sigma-Aldrich).

Table 1. Gradient for IP RPLC separation.

Time (Min)	Mobile phase A (%)	Mobile Phase B (%)
Initial	80	20
1	80	20
10	30	70
11	5	95
13	5	95
13.1	80	20
15	80	20

Mass spectrometry: TOF MS data were acquired in the negative mode using SCIEX OS software on a ZenoTOF 8600 system (SCIEX). The key instrument parameters are listed in Table 2.

Table 2. Source and TOF MS parameters of the ZenoTOF 8600 system.

Parameter	Value
Workflow	Intact proteins
Curtain gas	40
CAD gas	7
Ion source gas 1	50 psi
Ion source gas 2	50 psi
Temperature	400°C
Spray voltage	-3,000 V
Time bins to sum	8
Start mass	620 Da
Stop mass	3,000 or 4,000 Da
Accumulation time	0.5 s
QJet DP	-40 V
Collision energy	-10 V

Data analysis: MS data was automatically interpreted using an intact oligonucleotide deconvolution workflow (Figure 2) within Biologics Explorer software, Version 7.0.1 (SCIEX). The oligonucleotide sequences were defined based on the nomenclature of 3 building blocks, including sugar, linker and base, as will be described in the following sections. The conversion of a phosphorothioate (PS) to phosphodiester (PO) linkage was set as a modification. A mass step of 0.1 Da and 1 Da was used to deconvolute the MS data of the 18mer and 100mer, respectively. Mass mapping was performed for the 18mer and 100mer based on their monoisotopic and average masses, respectively.

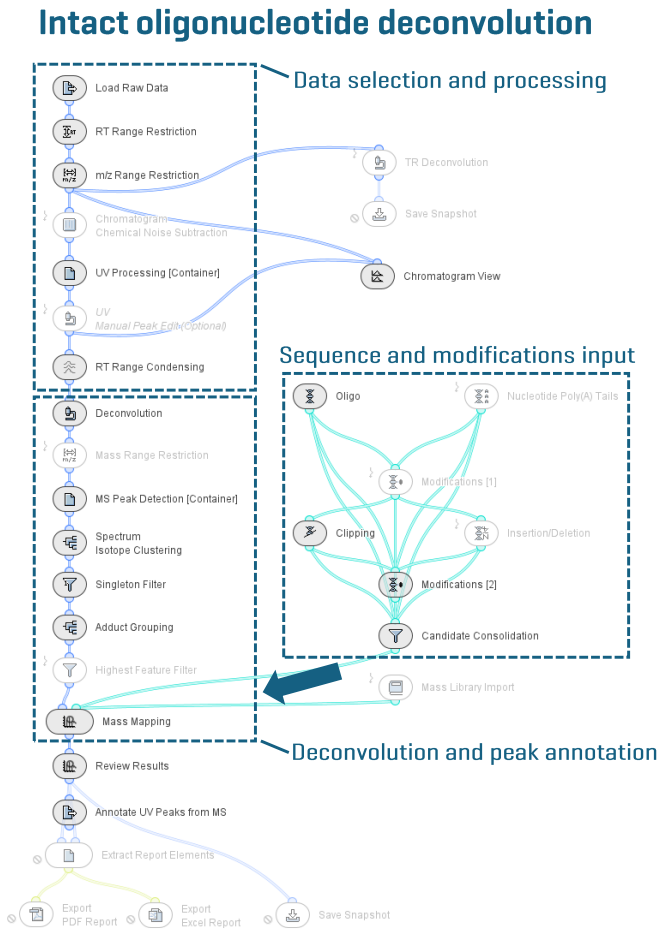

Figure 2. Intact oligonucleotide deconvolution workflow from Biologics Explorer software. Biologics Explorer software offers a prebuilt, optimized data analysis workflow for intact oligonucleotide deconvolution and mass mapping based on monoisotopic or average mass. This workflow is easy to use while offering flexibility and advanced settings for further optimization when necessary.

Intact oligonucleotide deconvolution workflow

Built upon powerful algorithms from Genedata Expressionist®, Biologics Explorer software offers optimized, easy-to-use data analysis workflows, such as intact MS, peptide mapping and top/middle-down MS, for rapid characterization of biopharma molecules. These streamlined workflows require minimal user's input and hence are quick to learn and easy to run. For advanced users, the workflows offer the flexibility to fine-tune various settings or algorithms.

Figure 2 shows an overview of the intact oligonucleotide deconvolution workflow introduced in the Version 7.0.1 of Biologics Explorer software. Compared to the intact protein deconvolution workflow, this data analysis workflow includes a cluster of activities for creating oligonucleotide sequences and their sequence variants (Figure 3). Any of these activities can be enabled or disabled depending on the need and goal. This allows the flexibility and versatility in creating candidate sequences of an oligonucleotide and its variants with or without modifications. A consolidated list of monoisotopic or average masses from the Candidate Consolidation activity serves as the input for mass mapping with the deconvolution results for peak assignment and annotation (Figures 2 and 3).

Oligonucleotide sequence generation

Biologics Explorer software offers user-friendly tools to help define and create oligonucleotide sequences with or without modifications. The nucleotide editor contains a list of sugars, linkers and bases commonly found in oligonucleotides and the custom entries created by the user (Figure 4A). Each of the building blocks is represented with a unique 1-letter abbreviation. For example, 5-methyl-cytosine and 2'-

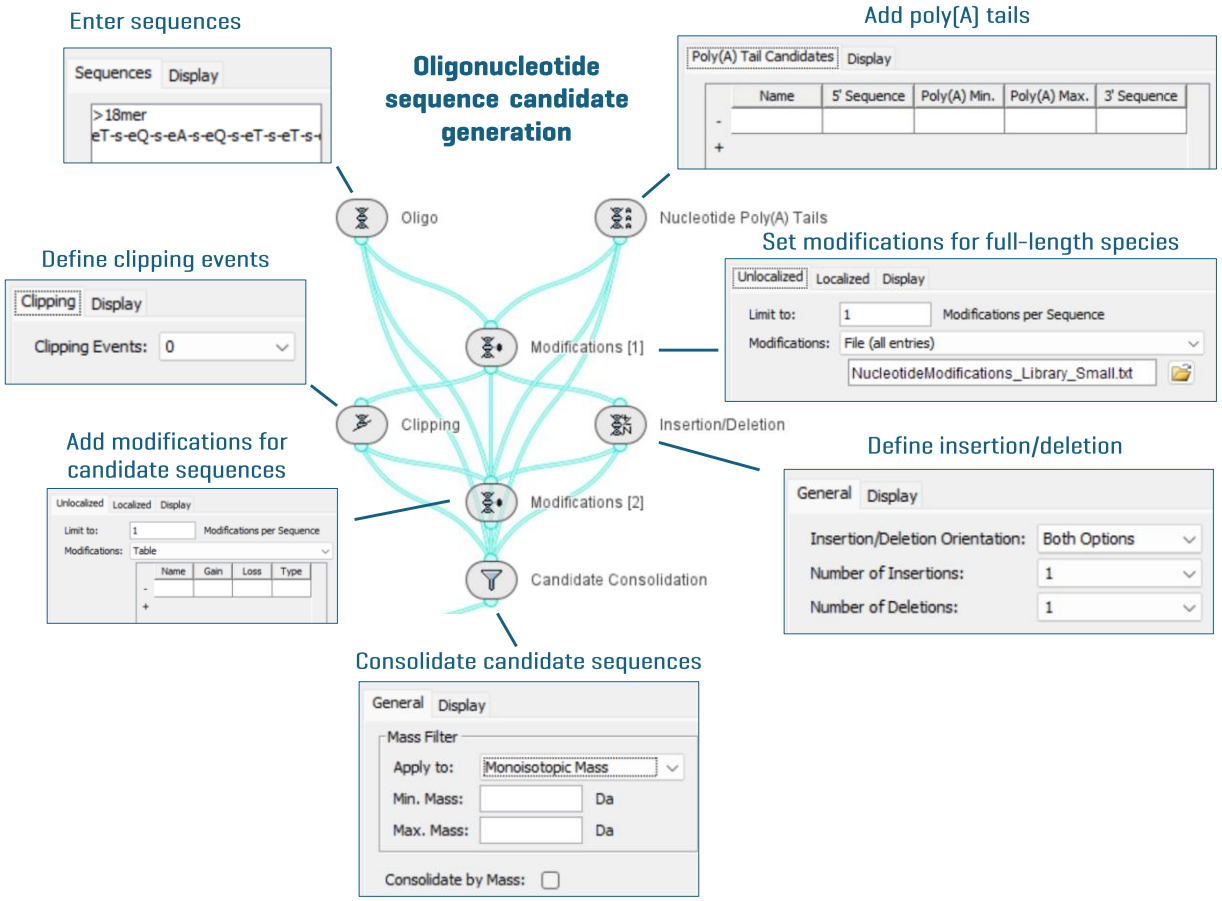

Figure 3. Oligonucleotide sequence candidate generation. Biologics Explorer software provides flexibility in creating oligonucleotide sequence candidates for mass mapping. The oligonucleotide sequence is defined based on the sugars, linkers or bases defined in the Nucleotide Editor (Figure 4), where users can also add custom entries. The poly(A) tails and insertion/deletion are specified in 2 separate activities, which may be disabled if they are not applicable. Up to 2 clipping events on the 3' and/or 5' ends can be set. The modifications can be defined for the sequence with or without the poly(A) tails (see "Modification [1]" activity) or for the variants resulting from clipping or insertion/deletion (see "Modification [2]" activity). The monoisotopic or average masses of all the candidate sequences are consolidated for mass mapping with the deconvolution results.

methoxyethylribose (2'-MOE) were defined as Q and e, respectively. With these building blocks defined, the sequence of the 18mer analyzed in this study was defined as the following:

eT-s-eQ-s-eA-s-eQ-s-eT-s-eT-s-eT-s-eQ-s-eA-s-eT-s-eA-s-eA-s-eT-s-eG-s-eQ-s-eT-s-eG-s-eG

where the dash ("-") symbols are optional. This sequence was entered into the Oligo node (Figures 2 and 3) for the generation of a candidate mass list for mass mapping. The oligonucleotide sequence can also be copied to the mass calculator for formula and mass calculation (Figure 4B).

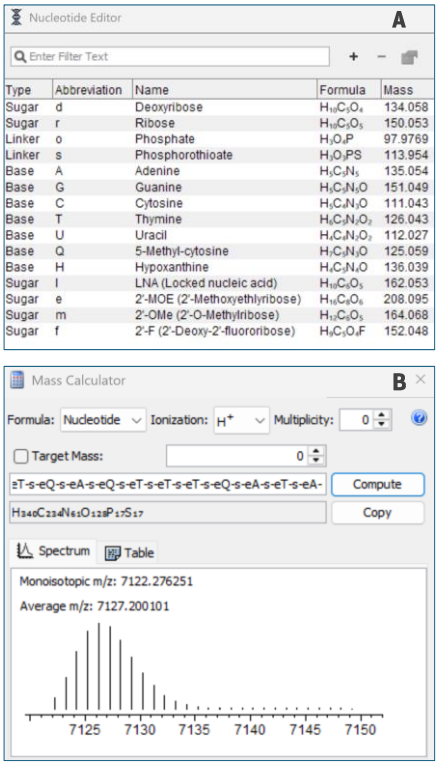

Figure 4. Nucleotide editor and mass calculator. Biologics Explorer software provides useful tools to facilitate sequence creation and mass calculation. The nucleotide editor contains a list of common sugars, linkers and bases. The user can add custom building blocks to the list (A). The mass calculator provides the molecular formula and monoisotopic and average masses for the oligonucleotide sequence entered (B).

Intact deconvolution and mass mapping

Biologics Explorer software provides excellent visualization tools for rapid assessment of data quality and impurity level. Figure 5 shows the ion maps and mass spectra from IP RPLC-MS analyses of 18mer and 100mer (50 ng) using the ZenoTOF 8600 system. The ion map offers a 2-dimensional view of the raw data of these 2 oligonucleotides (Figures 5A and 5C). The mass spectrum at specific RT (Figures 5B and 5D) or extracted ion chromatogram (XIC) of a specific m/z (not shown) can be

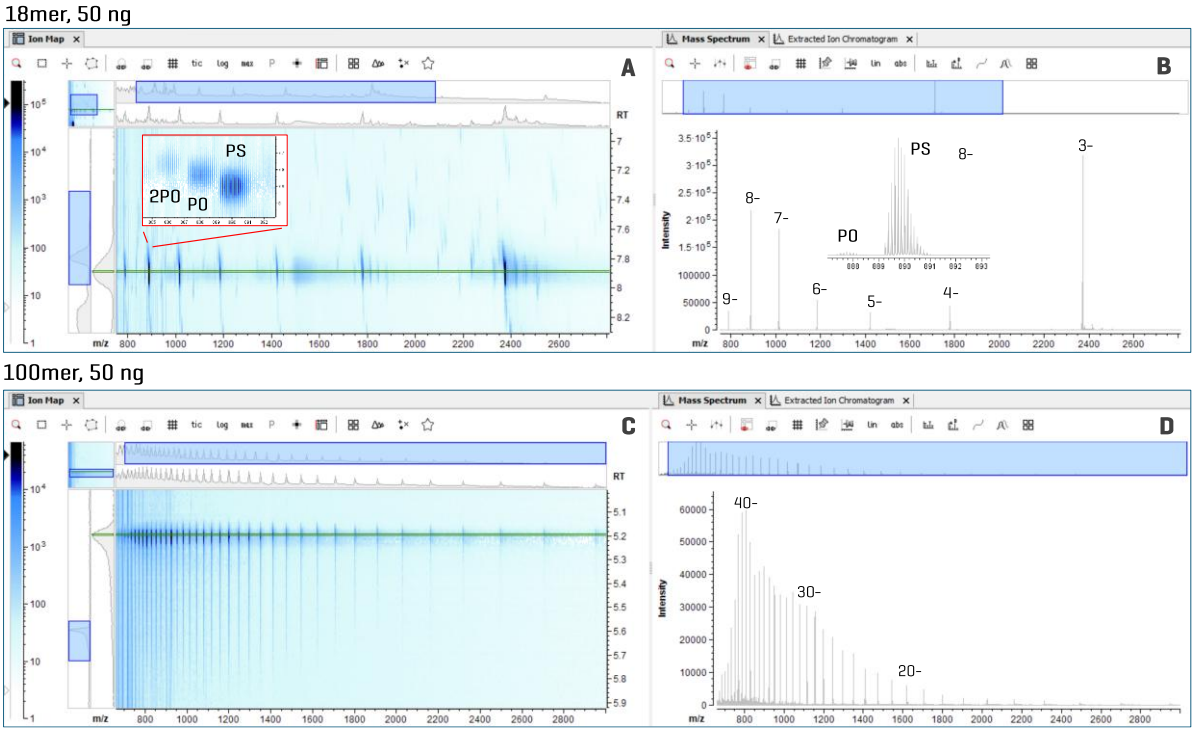

Figure 5. Visualization of 18mer and 100mer data from IP RPLC-MS analysis. Biologics Explorer software provides powerful tools for excellent visualization of the data and results. The ion maps (A and C) and mass spectra (B and D), which can be viewed side-by-side, allow rapid assessment of sample quality and impurity level in the 18mer and 100mer samples. For the 18mer, the expanded views of the ion map (A) and mass spectrum (B) show the detection of the PO species and minimal salt adducts.

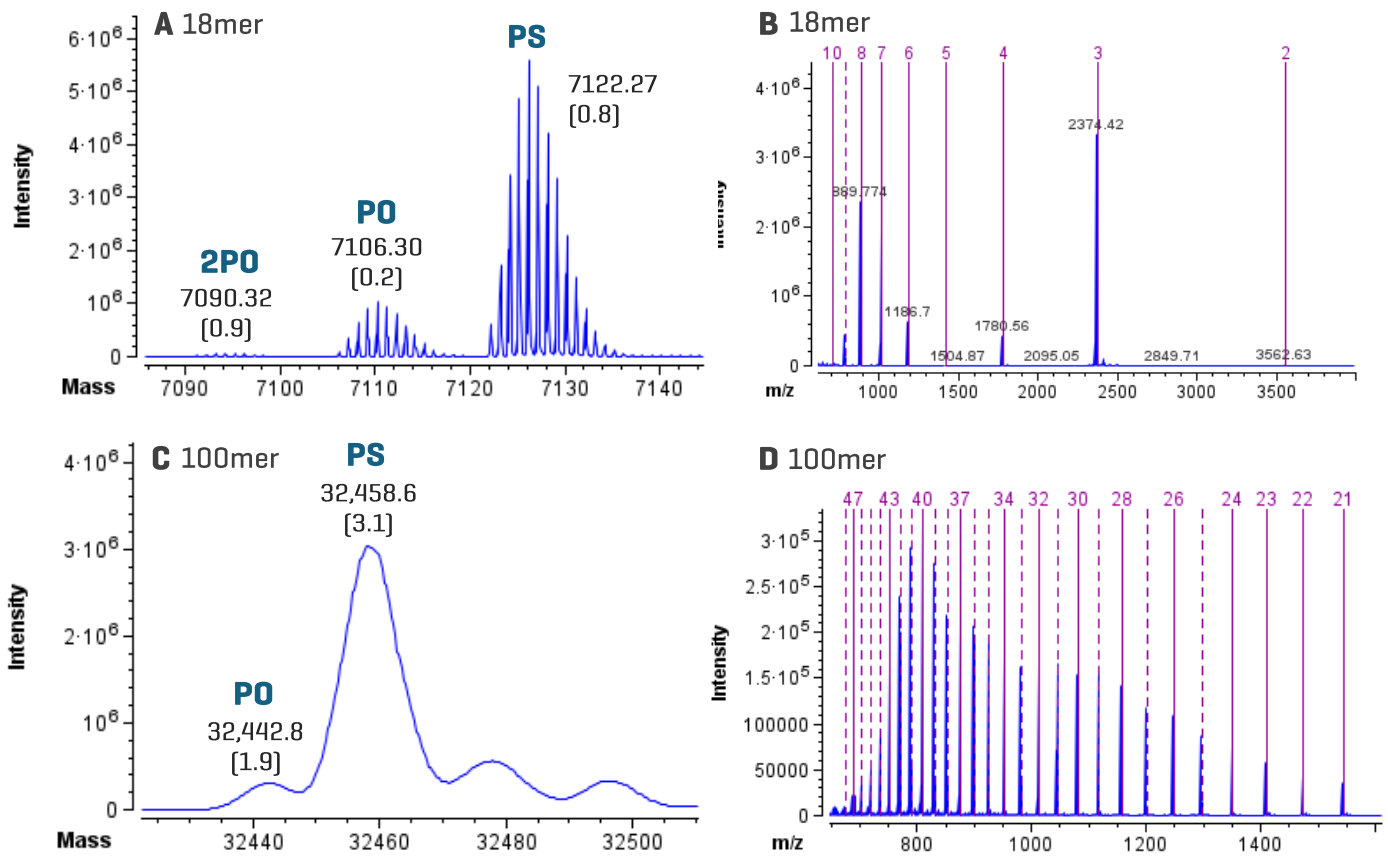

Figure 6. Deconvolution results of the 18mer and 100mer. The 18mer (A and B) and 100mer (C and D) data were deconvoluted and mapped against the candidate sequences based on the monoisotopic and average masses, respectively. The ZenoTOF 8600 system offered high MS sensitivity to allow the detection of low-abundant 2PO species in the 18mer with a 50 ng sample load (A). A High mass accuracy (<5 ppm) was obtained for all the PS and PO species detected. The input mass spectra of 18mer (B) and 100mer (D) used for intact deconvolution can be viewed side-by-side with the corresponding deconvolution spectra within Biologics Explorer software, improving user experience with data analysis.

conveniently viewed side-by-side with the ion map. A closer view of the 18mer data revealed the presence of the full-length product (PS) and its PO species, which were partially separated from the PS (insets in Figures 5A and 5B).

Intact oligonucleotide deconvolution and mass mapping were performed for the 18mer using the monoisotopic mass and for the 100mer using the average mass. Figure 6 shows the devolution results of 2 oligonucleotides. The ZenoTOF 8600 system offered high MS sensitivity for the detection of a low-abundant 2PO species with 2 desulfurization (PS→PO) in the 18mer (Figure 6A). A high mass accuracy (<5 ppm) was achieved for all the PS and PO species.

In summary, this technical note highlights a streamlined workflow leveraging high MS sensitivity of the ZenoTOF 8600 system and powerful while intuitive tools from Biologics Explorer software for enhanced intact oligonucleotide analysis.

Conclusions

- A streamlined IP RPLC-MS workflow was developed for enhanced intact oligonucleotide analysis, leveraging the high MS sensitivity offered by the ZenoTOF 8600 system and an easy-to-use, optimized data analysis workflow from Biologics Explorer software.
- Biologics Explorer software provides an array of powerful tools for oligonucleotide sequence creation, intact deconvolution and mass mapping.

- The ion map offers excellent visualization of oligonucleotide data for rapid sample quality and impurity assessment.
- Biozen Oligo LC column combines core-shell particle technology and bioinert hardware, significantly increasing the chromatographic efficiency.
- The streamlined workflow can be easily implemented for rapid oligonucleotide analysis by different levels of LC-MS scientists.

Acknowledgements

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References

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