

## An Oligonucleotide Impurity Analysis Workflow Using LabSolutions Insight™ Biologics Software

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### User Benefits

- ◆ LabSolutions Insight Biologics software offers a simple workflow for the characterization of oligonucleotides and oligonucleotide impurities.
- ◆ Allows comprehensive characterization of oligonucleotide impurities.
- ◆ Fragment coverage is shown in a simple graphical format that highlights missing and modified nucleotides.

### Introduction

Oligonucleotide therapeutics have undergone rapid development in recent years and are attracting interest as a new modality in drug discovery. Such development has created a demand for the comprehensive detection and identification of impurities in oligonucleotide therapeutics to ensure their safety and efficacy. This Application News describes an impurity analysis workflow for oligonucleotides based on the LCMS-9050 quadrupole time-of-flight mass spectrometer system and LabSolutions Insight Biologics software.

### Samples

An unrefined, phosphorothioate-modified 20-mer oligonucleotide with the nucleotide sequence CTG CTA GCC TCT GGA TTT GA (unrefined PS 20-mer) was analyzed.

### Analytical Conditions

Analysis was performed with the Nexera™ XS inert system and LCMS-9050 system in data dependent acquisition (DDA) mode. The LC conditions used are shown in Table 1 and the MS conditions used are shown in Table 2.

Table 1 LC Conditions

[HPLC Conditions] (Nexera XS inert)

Column: Shim-pack Scepter™ Claris C18-120, 50 mm x 2.1 mm I.D., 1.9 μm\*1  
 Mobile Phases: A) Aqueous solution of 100 mM HFIP and 10 mM TEA  
 B) 50 % Methanol solution of 50 mM HFIP and 5 mM TEA  
 Gradient Program:

Time (min)	Flowrate (mL/min)	A. Conc	B. Conc
0.00	0.3	95	5
1.00	0.3	95	5
26.00	0.3	60	40
26.10	0.3	10	90
30.00	0.3	10	90
30.10	0.3	95	5
34.00	0.3	95	5

Column Temp.: 60 °C  
 Injection Volume: 2 μL

\*1: P/N: 227-31210-02

Table 2 MS Conditions

[MS Conditions] (LCMS-9050)

Ionization: ESI (Negative mode)  
 Mode: MS scan (m/z 550 to 2500), DDA  
 Interface Voltage: -3.0 kV  
 Nebulizing Gas Flow: 3.0 L/min  
 Drying Gas Flow: 10.0 L/min  
 Heating Gas Flow: 10.0 L/min  
 DL Temp.: 250 °C  
 Block Heater Temp.: 400 °C  
 Interface Temp.: 350 °C

### Configuring the Analysis Parameters

LabSolutions Insight Biologics is data analysis software that characterizes oligonucleotides and oligonucleotide impurities. First, the user creates an oligonucleotide sequence in the parameter configuration window using the nucleobases, linkers, ribose and modifications provided by the software. Nucleobases, linkers, ribose, and base modifications can be added and removed in each tab as required. Once an oligonucleotide sequence is entered, the software displays the molecular structure, monoisotopic mass, and structural formula (right side) of that oligonucleotide (Fig. 1). The structural formula is also updated in real time based on the entered sequence, allowing for easy visual identification of entry errors.

As shown in Fig. 2, nucleobases can be displayed with various colors for easy identification, and fragmentation sites can be also shown.

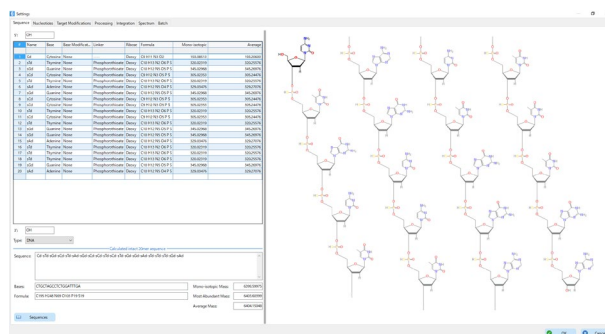


Fig. 1 Parameter Configuration Window

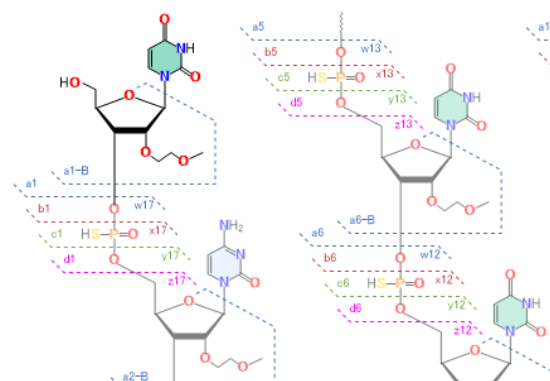


Fig. 2 Colored Display of Fragmentation Sites

A "Target Modifications" tab is also used to select the anticipated impurities. In addition to impurities such as different strand lengths, missing nucleobases, depurination/depyrimidination, deamination, and protecting groups, as well as additional ions, unknown modifying groups, the software can also search for molecular changes added by the user.

## ■ Results

The unrefined PS 20-mer was detected by photodiode array and mass spectrometry. The chromatograms obtained by each method are shown in Fig. 3. The LC chromatogram (top) and MS chromatogram (bottom) are aligned to allow a direct comparison between the peaks.

The mass spectrum has been displayed as a component chromatogram, which is based on MS1 spectra and combines signals from different valences and isotopes. Generation of a component chromatogram is a unique feature of this software,

whereby all individual signals contributing to the component have been summed into a single XIC chromatogram. Fig. 4 shows the multivalent ion mass spectrum and deconvoluted mass spectrum of the PS 20-mer. The spectra of oligonucleotide impurities were also deconvoluted and used to search for impurities.

An exhaustive search for impurities based on MS1 data identified over 30 impurities with different strand lengths, missing bases, and additional ions.

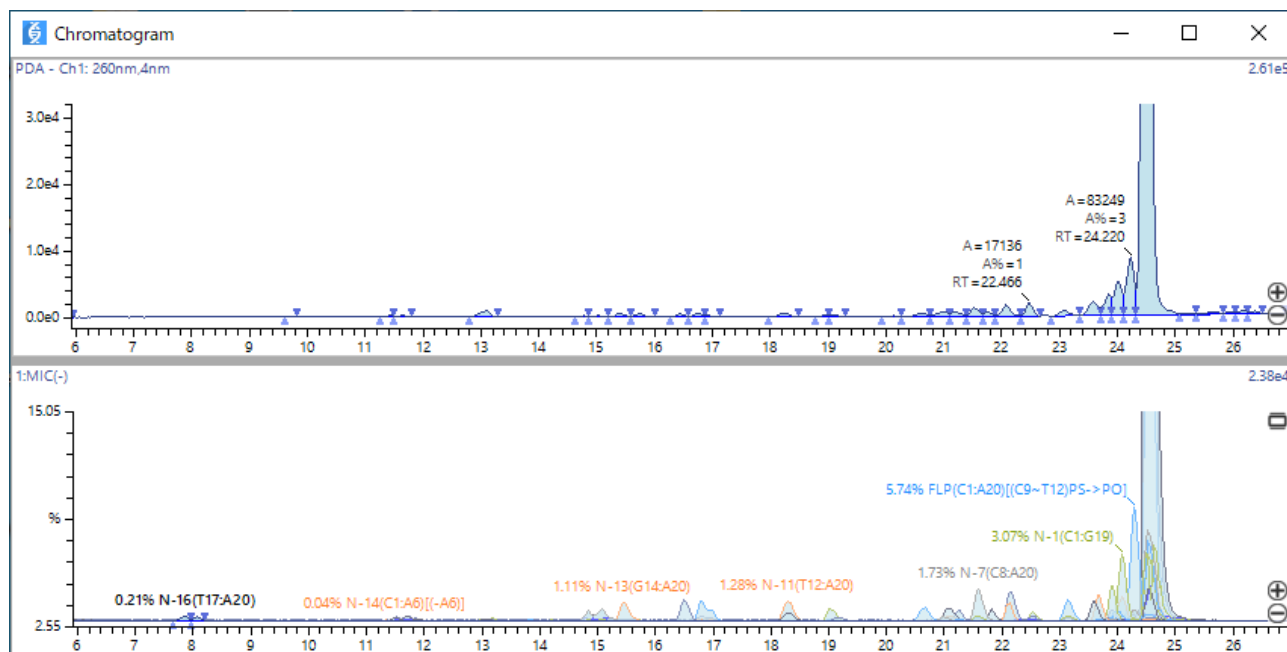


Fig. 3 Unrefined PS 20-mer UV Chromatogram (Top) and Component Chromatogram (Bottom)

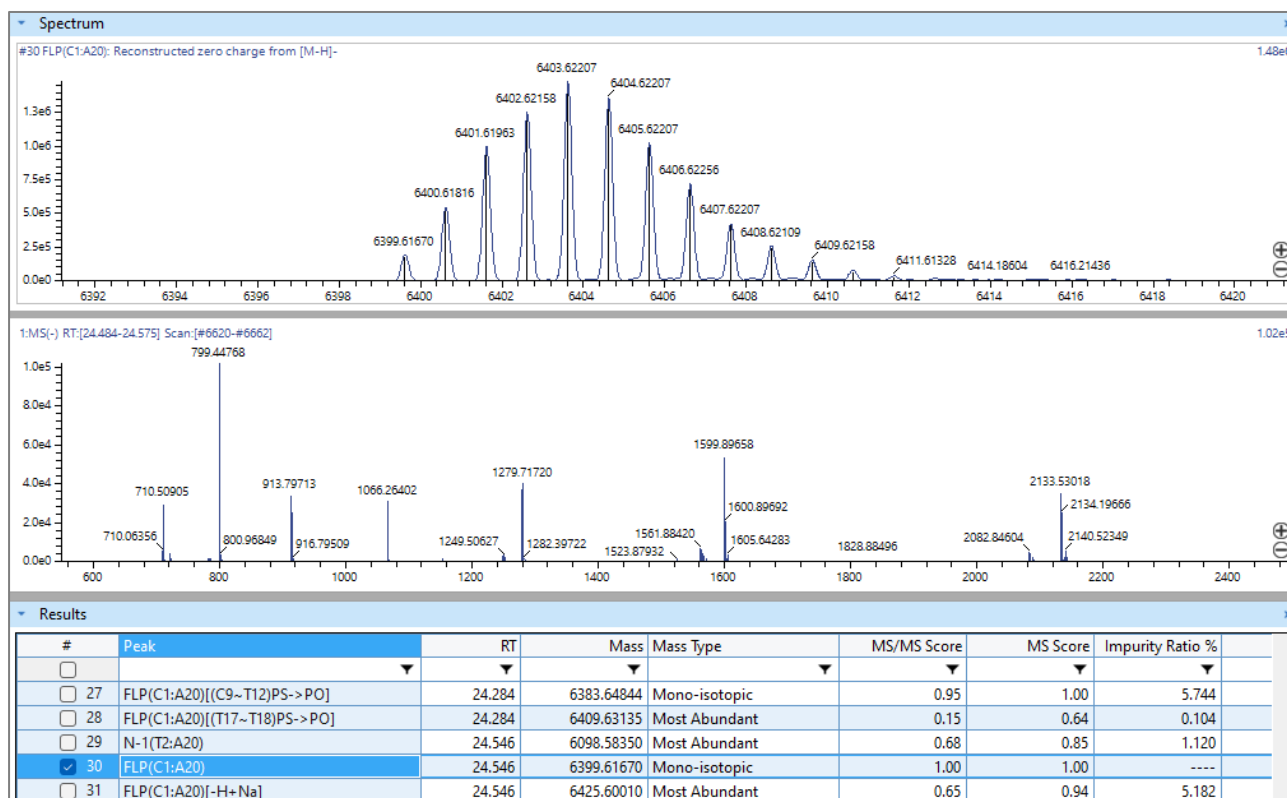


Fig. 4 PS 20-mer Mass Spectrum (Top) and Deconvoluted Mass Spectrum (Bottom)

The software also displays sequence coverage based on the MS2 fragmentation spectra.

The results obtained from aligning the fragments against the PS 20-mer sequence are shown in Figs. 5 and 6. The fragment ions generated by the analysis are cleaved at every nucleobase on the PS 20-mer and have sequences that match the PS 20-mer.

The software displays sequence coverage in two modes, and users can switch between these modes depending on the information of interest: a fill mode that shows signal information and the completeness of the aligned fragment ions, and a branch mode that shows the fragment sequence.

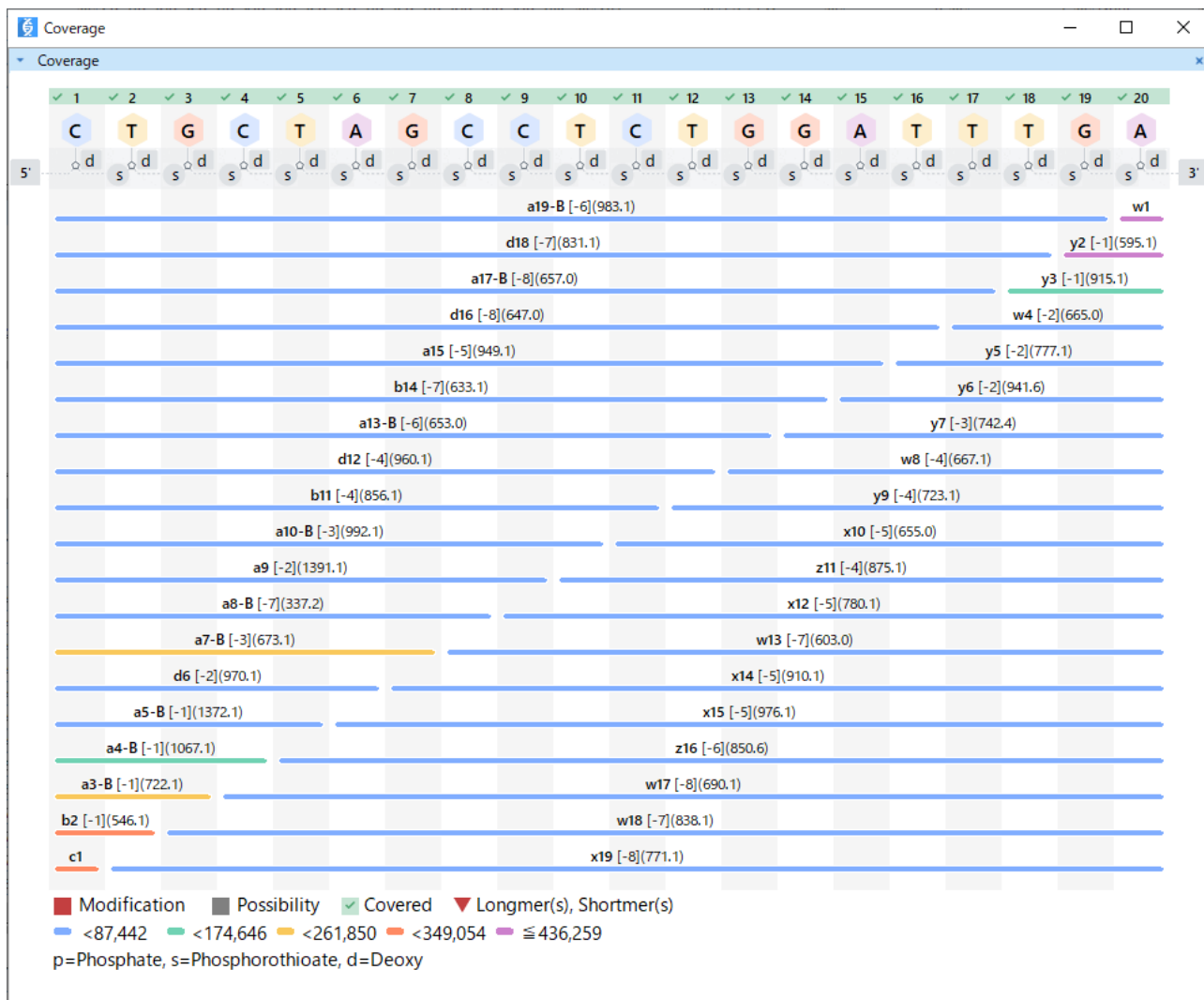


Fig. 5 PS 20-mer Sequence Coverage (Fill Mode)

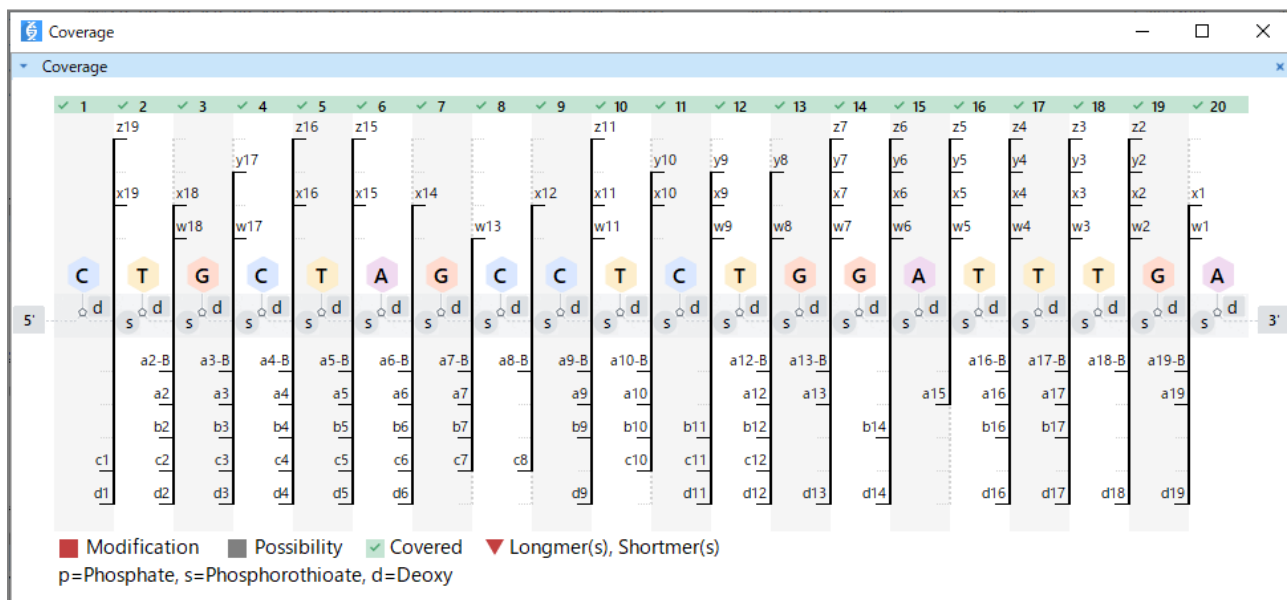


Fig. 6 PS 20-mer Sequence Coverage (Branch Mode)

## ■ Examining the Sequence of an Impurity

The sequence of an impurity missing 14 nucleotides from the 5' end (hereinafter N-14) and present as 0.5 % of the main sample component was examined. The validity of the data is shown by the high percentage sequence coverage, as indicated by the green tick marks above the verified nucleobases in Fig. 7.

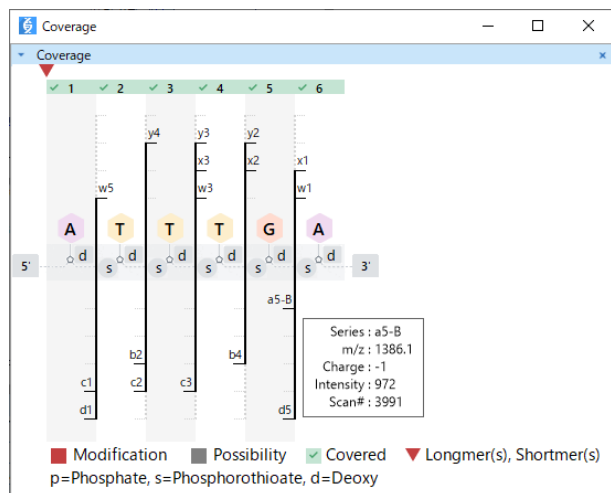
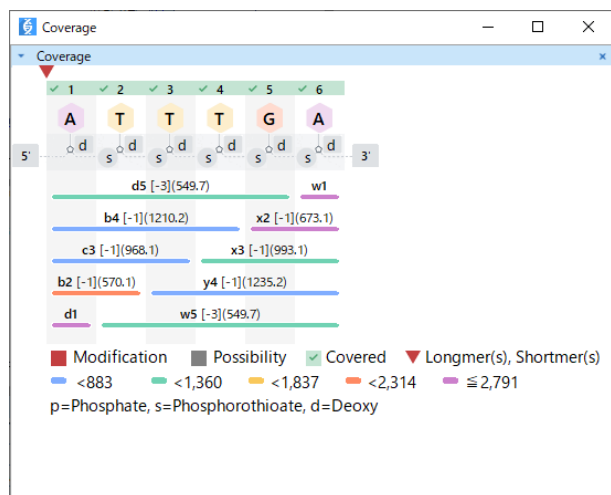


Fig. 7 N-14 Sequence Coverage

## ■ Conclusion

LabSolutions Insight Biologics software can comprehensively characterize and identify the sequences of oligonucleotide impurities.

The analysis workflow described in this article achieved complete sequence coverage not only for the main sample component, but also for an impurity with a relative abundance of 0.5 % compared with the main component.

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