

## An Automated Compliance-Ready LC-MS Workflow for Intact Mass Confirmation and Purity Analysis of Oligonucleotides

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### APPLICATION BENEFITS

- An automated compliance-ready LC-MS workflow for mass confirmation of both modified and unmodified oligonucleotides
- An LC-UV assay for purity and relative quantification of oligonucleotide impurities

### WATERS SOLUTIONS

[BioAccord™ System](#)

[ACQUITY™ UPLC™ I-Class PLUS System](#)

[ACQUITY UPLC Tunable UV \(TUV\) Detector](#)

[ACQUITY UPLC Oligonucleotide C<sub>18</sub> Column](#)

[waters\\_connect™ HUB](#)

### KEYWORDS

BioAccord, ESI-ToF, Oligonucleotide analysis, Intact mass analysis, DNA, RNA, OST, MassPREP, waters\_connect

### INTRODUCTION

Oligonucleotide therapeutics have emerged in recent years as a powerful alternative to small molecule and protein therapeutics.<sup>1</sup> Manufacturing and quality control of oligonucleotide therapeutics requires highly selective and sensitive LC-MS methods. The method most often used for mass spectrometry-based oligonucleotide analysis has been reversed-phase chromatography employing a variety of ion-pairing reagents and modifiers in negative ESI-MS mode. Integrated LC-MS workflows for oligonucleotide analysis have been recently implemented on quadrupole and QToF LC-MS platforms.<sup>2-4</sup>

The Waters™ BioAccord System featured in Figure 1 was introduced in 2019 as a compact, robust, and easy-to-use platform for routine biopharmaceutical analysis. Here, we describe a streamlined, compliance-ready workflow for oligonucleotide analysis employing the BioAccord System operating under waters\_connect HUB data acquisition and processing software. The fully integrated BioAccord LC-MS System is comprised of an ACQUITY UPLC I-Class PLUS System, an ACQUITY UPLC Tunable Ultraviolet (TUV) Detector and the ESI-ToF-based ACQUITY RDa™ Mass Detector. All the LC-MS data presented in this application note was acquired in full scan MS mode and was processed in waters\_connect, which provided for automated spectral deconvolution and reporting of intact oligonucleotide mass measurement, as well as UV-based purity analysis. Both unmodified and modified oligonucleotides were analyzed using the workflow introduced here.



Figure 1. Waters BioAccord LC-MS and informatics platform.

## EXPERIMENTAL

## REAGENTS AND SAMPLE PREPARATION

Triethylamine (TEA, 99.5% purity, catalog number 65897-50ML) and methanol (LC-MS-grade, catalog number 34966-1L) were obtained from Honeywell (Charlotte, NC), while 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, 99% purity, catalog number 105228-100G) was purchased from Sigma Aldrich (St Louis, MO). HPLC-grade deionized (DI) water was purified using a MilliQ system (Millipore, Bedford, MA). Mobile phases were prepared fresh and used on the same day. The MassPREP™ OST (Oligonucleotide Separation Technology) standard (p/n: [186004135](#)) was dissolved in DI water to prepare a dilution series containing 1 µM, 100 nM, and 10 nM concentrations. The 25-mer, fully phosphorothioated (PPT) oligonucleotide (5'-C\*T\*C\*T\*C\* G\*C\*A\*C\*C\* C\*A\*T\*C\*T\* C\*T\*C\*T\*C\* C\*T\*T\*C\*T\*-3') was purchased from Integrated DNA Technologies (Coralville, IA) and diluted into DI water to prepare a 1-µM solution. A 100-mer oligonucleotide (5'- TGCCA GTTGC AGTTG TTTCC GAGCA GAAAC TCATC TCTGA AGAGG ATCTG GAGCA GAAAC TCATC TCTGA AGAGG ATCTG CACAC GCTGG AGCTG CCGCG-3') was purchased from Integrated DNA Technologies and diluted with DI water to prepare a 1-µM solution. The injection volume was 10 µL for all samples.

LC-MS system: BioAccord incorporating the ACQUITY RDa Mass Detector, ACQUITY UPLC I-Class PLUS and ACQUITY UPLC TUV Detector

## LC conditions

OST column: 2.1 × 50 mm, packed with 1.7 µm C<sub>18</sub> particles (p/n: [186003949](#))

Column temp.: 60 °C

Flow rate: 300 µL/min

Mobile phase A: 80 mM HFIP (hexafluoroisopropanol), 7 mM TEA (triethylamine) in DI water

Mobile phase B: 40 mM HFIP, 3.5 mM TEA in 50% methanol

## Gradient table:

Time (min)	Flow rate (µL/min)	Solvent A composition (%)	Solvent B composition (%)	Curve profile
0.00	300	78	22	Initial
10.00	300	60	40	6
11.00	300	15	85	6
12.00	300	15	85	6
12.10	300	78	22	6
15.00	300	78	22	6
60.00	300	78	22	6
61.00	20	50	50	6

Sample temp.: 6 °C

Injection volumes: 10 µL

Purge solvent: 50% MeOH

Sample Manager wash solvent: 50% MeOH

Seal wash: 20% acetonitrile in DI water

## MS conditions

Ionization mode: ESI-

Capillary voltage: 0.8 kV

Cone voltage: 40 V

Source temp.: 120 °C

Desolvation temp.: 400 °C

Desolvation gas (N<sub>2</sub>) pressure: 6.5 bar

TOF mass range: 400–5000

Acquisition rate: 2 Hz

Lock-mass: waters\_connect Lockmass Solution (p/n: 186009298)

Data acquisition software: waters\_connect

Data processing software: waters\_connect

## RESULTS AND DISCUSSION

As shown in Figure 2, excellent separation of the polyT oligonucleotides and their truncated impurities was achieved on the ACQUITY UPLC OST Column in under eight minutes using the ion-pairing RP conditions listed in the experimental section. Several low-level oligonucleotide impurities were detected in both the UV and the TIC chromatograms, facilitating the assignment of all the truncated oligo species based on their corresponding ESI-MS spectra recorded in negative ion mode. Overall, 26 oligonucleotide components were confirmed in the OST MassPREP standard. The ESI-MS spectra of the five major components present in this sample (dT15, dT20, dT25, dT30, and dT35) are presented in Figure 3A. All spectra display a bimodal distribution for the observed charge states, with the lower charge states (from -3 to -5) and the higher charge states (between -7 and -15) reaching two distinct maxima for each oligo. This spectral feature is typical for ion-pair reversed-phase separations of oligonucleotides and provides a wide mass range ( $m/z = 600\text{--}3000$ ) and a relatively large number of charge states (6–12) for deconvolution. The resolution of the ACQUITY RDa Mass Detector ( $>10,000$ ) is adequate for resolving the isotopes of the highly charged oligonucleotide ions, as demonstrated in Figure 3B, which shows that the monoisotopic peak of the  $[M-12H]^{-12}$  charge state of the dT35 oligo can be clearly distinguished. The isotopic resolution of individual charge states helps the BayesSpray deconvolution algorithm<sup>5</sup> to produce more accurate mass measurements for the oligonucleotide deconvoluted average masses.

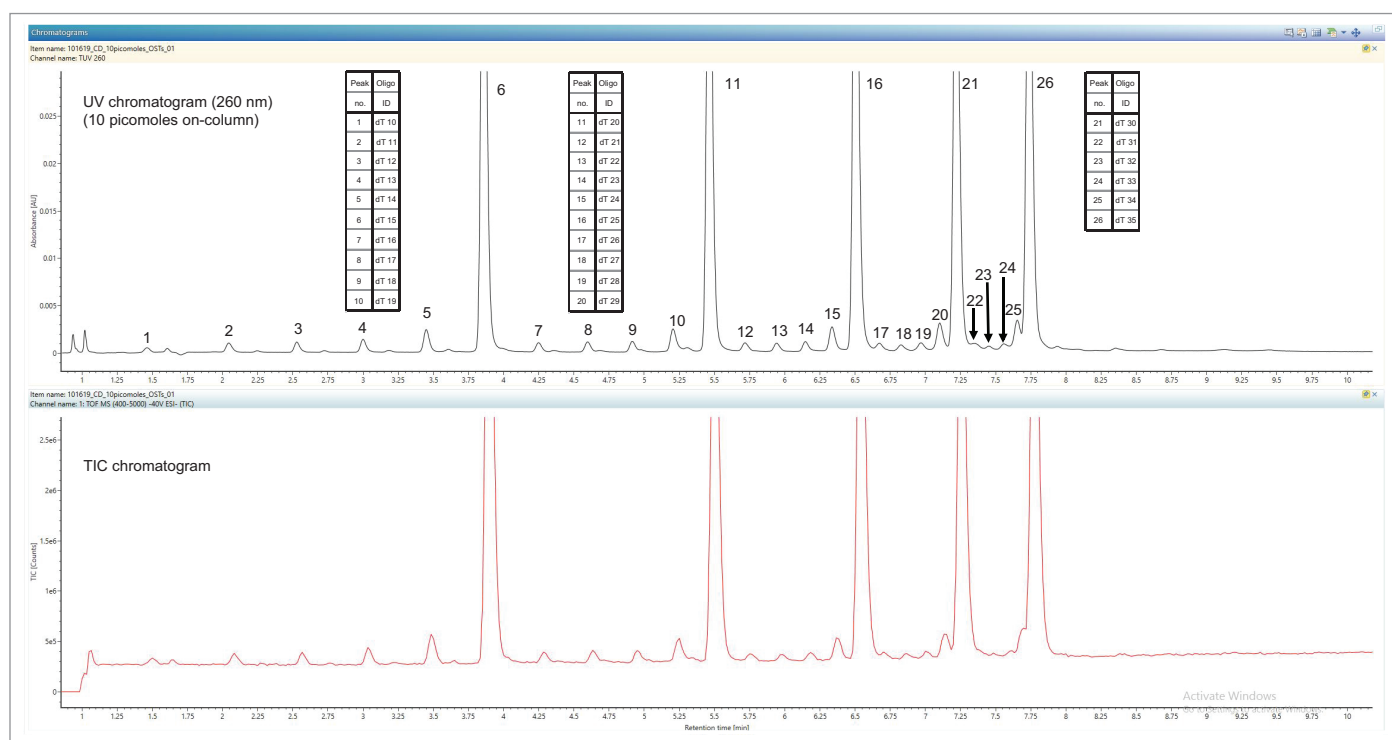


Figure 2. TUV and TIC chromatograms showing the separation of the major and minor oligonucleotide components of the OST MassPREP standard. The amount of sample loaded on-column was 10 picomoles for each of the five major oligonucleotides (dT15, dT20, dT25, dT30, and dT35). Peak identification was performed based on the deconvoluted average mass of each oligonucleotide peak from the TIC chromatogram and is listed in the figure inset.

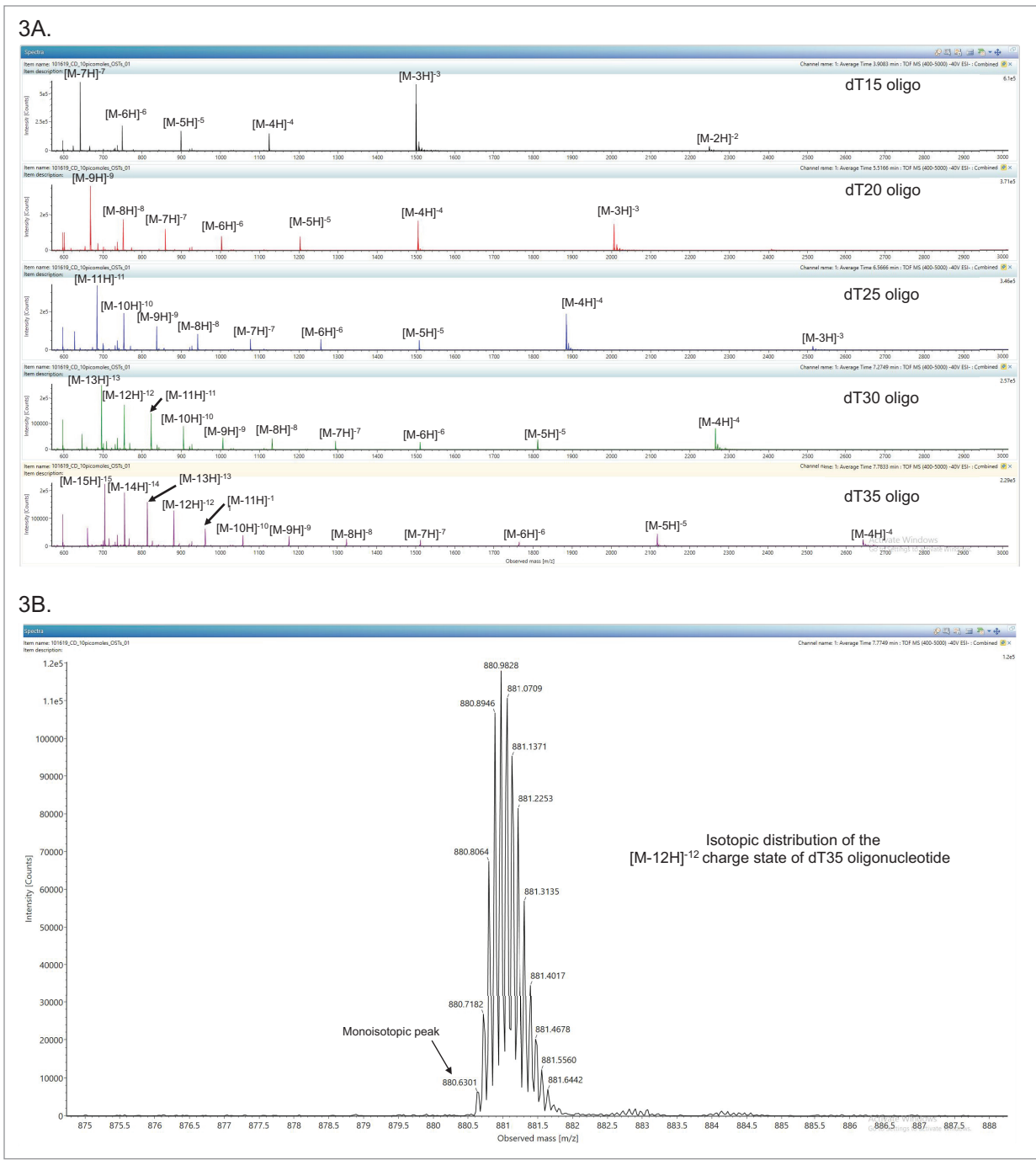


Figure 3. ESI-MS spectra recorded for the five major components of the OST MassPREP standard (A). All spectra contained a bimodal distribution for the detected charge states, typical for the ion-pair RP conditions used for separation of the oligonucleotide mixture. Detailed view of the [M-12H]<sup>-12</sup> charge state of the dT35 oligonucleotide (10,585 Da MW) reveals the monoisotopic peak signal at m/z=880.6301 (B). With mass resolution >10,000, the BioAccord System resolves the higher charge states of medium size oligonucleotides.

Based on the information collected from the UV chromatogram shown in Figure 2 (using the individual retention times of each oligo component), an automated data processing method was created in waters\_connect to perform peak integration of all 26 oligonucleotide components present in the UV chromatogram, in order to calculate the abundance of each oligo species. In addition, the same processing method applied the BayesSpray deconvolution algorithm to the raw ESI-MS spectra of the same components (collected in the TIC trace) to calculate the corresponding accurate average deconvoluted masses. A screenshot from the processed data is shown in Figure 5, which lists the predicted accurate average oligonucleotides masses, the experimentally measured masses, and the corresponding mass accuracy errors. For all analyzed components, regardless of their abundance, the mass accuracy error was less than 15 ppm and the pooled mass accuracy error was 5.3 ppm for all 26 measurements.

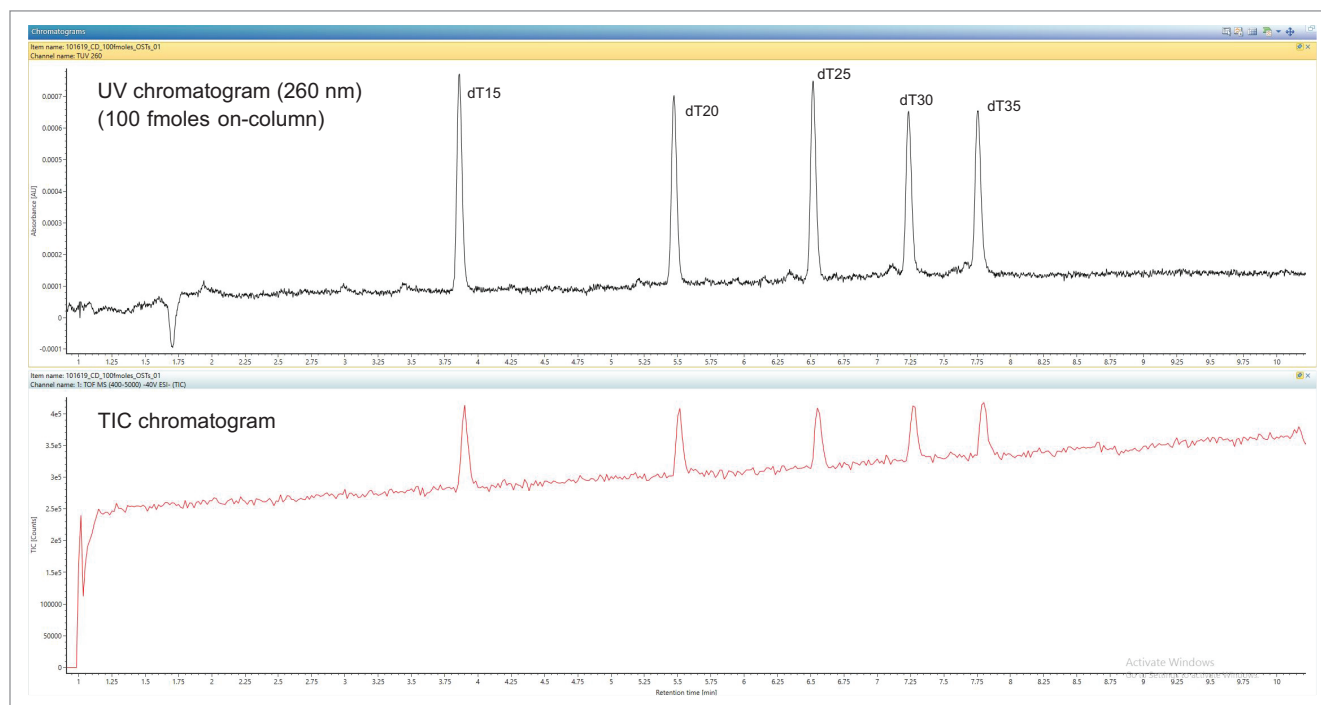


Figure 4. TUV and TIC chromatograms showing the performance of the BioAccord System when the 1- $\mu$ M OST standard was diluted 100 times (100 fmoles sample loading). All five major oligos are clearly detected in both the UV and TIC chromatograms, indicating that the LC-MS system and method has adequate sensitivity for detecting low-level oligo impurities.

A more detailed table summarizing the elemental composition of each oligonucleotide component, its RT, and abundance (%), along with the calculated accurate average deconvoluted mass and the mass error, is presented in Table 1. The results presented in Figure 5 and Table 1 clearly illustrate how the waters\_connect workflow can provide fast and accurate results for purity analysis and intact mass confirmation of major oligonucleotides and their impurities.

Peak number	Oligo ID	Elemental composition					Retention time (min)	Abundance (%)	Average mass (Da)	Mass accuracy (ppm)
		Carbon	Hydrogen	Nitrogen	Oxygen	Phosphorus				
1	dT10	100	131	20	68	9	1.50	0.29	2979.9672	3.5
2	dT11	110	144	22	75	10	2.08	0.28	3284.1604	-2.8
3	dT12	120	157	24	82	11	2.57	0.34	3588.3536	6.2
4	dT13	130	170	26	89	12	3.03	0.45	3892.5467	5.6
5	dT14	140	183	28	96	13	3.48	0.77	4196.7399	-11.1
<b>6</b>	<b>dT15</b>	<b>150</b>	<b>196</b>	<b>30</b>	<b>103</b>	<b>14</b>	<b>3.90</b>	<b>19.76</b>	<b>4500.9331</b>	<b>-11.8</b>
7	dT16	160	209	32	110	15	4.28	0.31	4805.1263	-8.4
8	dT17	170	222	34	117	16	4.63	0.34	5109.3195	-7.2
9	dT18	180	235	36	124	17	4.95	0.36	5413.5126	-5.9
10	dT19	190	248	38	131	18	5.25	0.84	5717.7058	-7.6
<b>11</b>	<b>dT20</b>	<b>200</b>	<b>261</b>	<b>40</b>	<b>138</b>	<b>19</b>	<b>5.50</b>	<b>17.62</b>	<b>6021.8990</b>	<b>-9.4</b>
12	dT21	210	274	42	145	20	5.75	0.29	6326.0922	-5.2
13	dT22	220	287	44	152	21	5.98	0.30	6630.2854	0.0
14	dT23	230	300	46	159	22	6.18	0.34	6934.4785	-0.5
15	dT24	240	313	48	166	23	6.37	0.79	7238.6717	-11.6
<b>16</b>	<b>dT25</b>	<b>250</b>	<b>326</b>	<b>50</b>	<b>173</b>	<b>24</b>	<b>6.53</b>	<b>18.94</b>	<b>7542.8649</b>	<b>-3.0</b>
17	dT26	260	339	52	180	25	6.70	0.19	7847.0581	0.9
18	dT27	270	352	54	187	26	6.87	0.20	8151.2513	-1.1
19	dT28	280	365	56	194	27	7.00	0.27	8455.4444	0.4
20	dT29	290	378	58	201	28	7.13	0.99	8759.6376	-0.9
<b>21</b>	<b>dT30</b>	<b>300</b>	<b>391</b>	<b>60</b>	<b>208</b>	<b>29</b>	<b>7.25</b>	<b>16.69</b>	<b>9063.8308</b>	<b>0.8</b>
22	dT31	310	404	62	215	30	7.35	0.29	9368.0240	9.5
23	dT32	320	417	64	222	31	7.45	0.19	9672.2172	-6.5
24	dT33	330	430	66	229	32	7.56	0.19	9976.4104	5.4
25	dT34	340	443	68	236	33	7.65	0.96	10280.6036	6.6
<b>26</b>	<b>dT35</b>	<b>350</b>	<b>456</b>	<b>70</b>	<b>243</b>	<b>34</b>	<b>7.77</b>	<b>18.01</b>	<b>10584.7967</b>	<b>9.2</b>
									RMS ERROR:	5.3 ppm

Table 1. Twenty-six oligonucleotide components identified in the MassPREP OST standard. The mass accuracy error for the accurate average masses measured on the BioAccord LC-MS System was better than 15 ppm for each oligo.

To illustrate the sensitivity of the oligonucleotide assays performed on the BioAccord LC-MS System, a 100-fold more diluted OST sample (10 nM) was analyzed on the same instrument. The UV and TIC chromatograms for this sample (100 fmoles of five major oligos loaded on-column) are displayed in Figure 5. All five major components are clearly visible in both traces, indicating the ability of the BioAccord System to detect low-level oligonucleotide amounts with both optical and mass spectrometric detection.

Component Summary ▾								
▲	Protein name	Modifiers	Response	Observed mass (Da)	Expected mass (Da)	Mass error (mDa)	Mass error (ppm)	Observed RT (min)
6	dT15		7606760	4500.8799	4500.93310	-53.2	-11.8	3.90
7	dT16		121148	4805.0860	4805.12630	-40.3	-8.4	4.28
8	dT17		131039	5109.2835	5109.31940	-35.9	-7.0	4.63
9	dT18		124778	5413.4809	5413.51260	-31.7	-5.9	4.95
10	dT19		256082	5717.6622	5717.70580	-43.6	-7.6	5.25
11	dT20		7561908	6021.8424	6021.89900	-56.6	-9.4	5.50
12	dT21		78630	6326.0595	6326.09220	-32.7	-5.2	5.75
13	dT23		67818	6934.4747	6934.47850	-3.8	-0.5	6.18
14	dT24		260808	7238.5879	7238.67170	-83.8	-11.6	6.37
15	dT25		9023372	7542.8419	7542.86490	-23.0	-3.0	6.53
16	dT26		60509	7847.0650	7847.05810	6.9	0.9	6.70
17	dT27		43903	8151.2426	8151.25130	-8.7	-1.1	6.87
18	dT14		319470	4196.6933	4196.73990	-46.6	-11.1	3.48
19	dT28		70344	8455.4479	8455.44440	3.5	0.4	7.00
20	dT29		252132	8759.6296	8759.63760	-8.0	-0.9	7.13
21	dT30		5854481	9063.8378	9063.83080	7.0	0.8	7.25
22	dT31		25097	9368.1131	9368.02400	89.1	9.5	7.35
23	dT32		20615	9672.1540	9672.21720	-63.2	-6.5	7.45
24	dT33		36178	9976.4639	9976.41030	53.6	5.4	7.57
25	dT34		242194	10280.6710	10280.60360	67.4	6.6	7.65
26	dT35		4224434	10584.8940	10584.79670	97.3	9.2	7.77

Figure 5. Screenshot showing the results of the waters\_connect automatic processing method. All 26 oligonucleotide components from the OST sample were detected and measured with mass accuracy of better than 15 ppm, regardless of their abundance.

Therapeutic oligonucleotides contain a natural DNA/RNA sequence that is often chemically modified in order to resist degradation by naturally occurring nucleases. There are three major types of chemical modifications, including backbone, sugar, and nucleobase modifications. While therapeutic oligos are mostly modified on the backbone and sugar moieties, the nucleobase modification is the preferred choice for oligonucleotides used in molecular diagnostics. One of the first introduced backbone modifications involves the replacement of an oxygen atom with sulfur in the phosphate backbone to produce a phosphorothioated (PPT) oligonucleotide. This type of molecule requires a unique isotopic model for charge deconvolution in order to obtain accurate average mass. The PPT oligo isotopic model takes into account the natural abundances of the sulfur isotopes. To demonstrate the intact mass analysis of PPT oligos, a 25-mer fully phosphorothioated oligo (with the sequence 5'-C\*T\*C\*T\*C\* G\*C\*A\*C\*C\* C\*A\*T\*C\*T\* C\*T\*C\*T\*C\* C\*T\*T\*C\*T\*-3') was analyzed on the BioAccord System using the same experimental conditions as the analysis of the OST oligonucleotides. The UV and TIC traces recorded for this oligo are displayed in Figure 6A. The accurate average mass of this oligo is 7,776.3314 Da, as calculated based on its elemental composition of C237 H310 N72 O131 P24 S24. The ESI-MS spectrum of the 25-mer PPT oligo (shown in Figure 6B) was charge deconvoluted using BayesSpray and the PPT oligonucleotides isotopic model from waters\_connect, and the deconvoluted mass was compared with the predicted accurate average mass.

As indicated in the screenshot captured from the waters\_connect report, presented as an inset in Figure 6B, the mass error for this measurement was 1.5 ppm. Clearly, accurate mass measurements can be achieved on the BioAccord System when using the PPT oligo isotopic model for deconvolution of phosphorothioates.

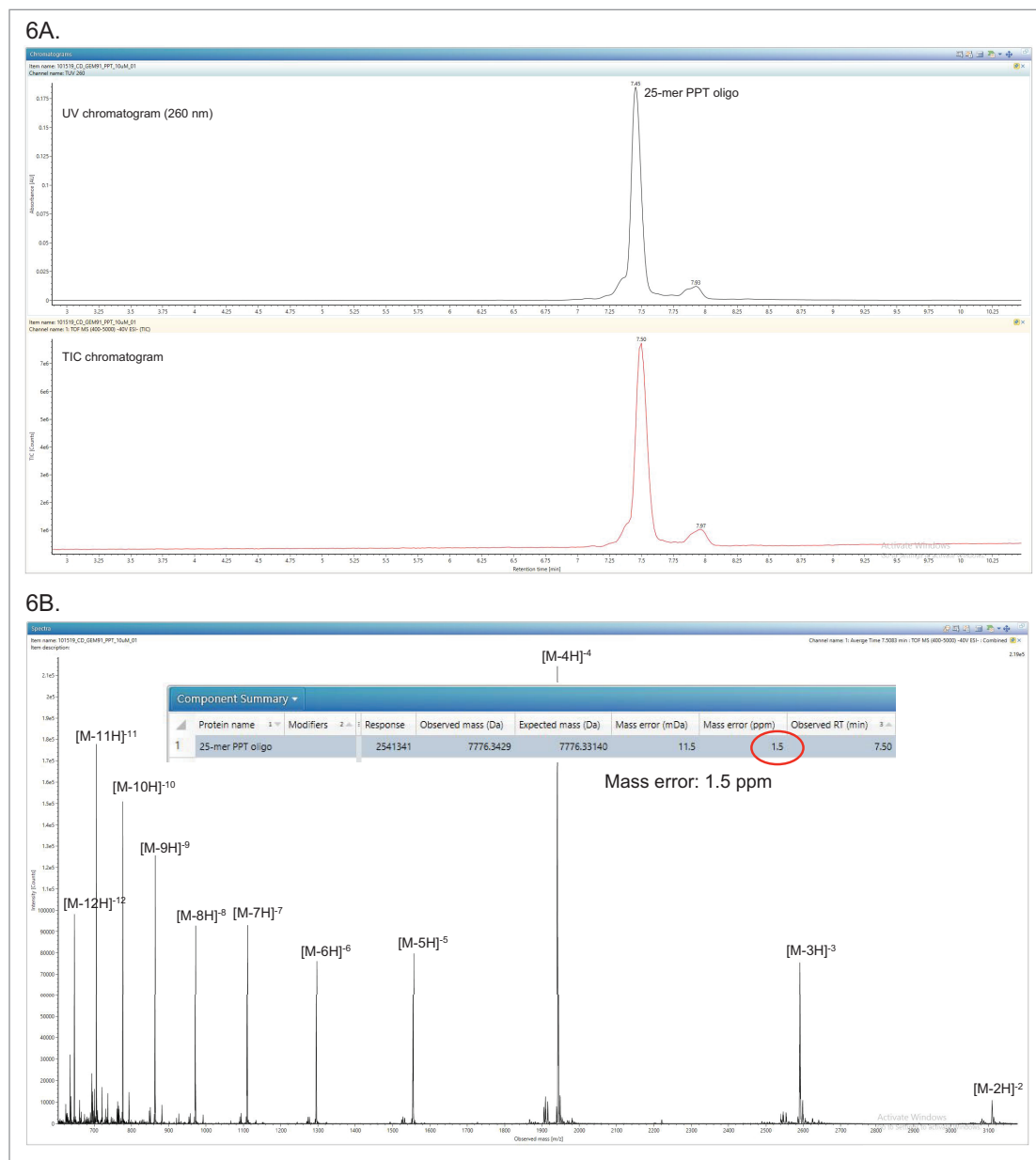


Figure 6. TUV and TIC chromatograms of the 25-mer fully phosphorothioated (PPT) oligonucleotide (A) and ESI-MS spectrum of the 25-mer PPT oligonucleotide (B). The inset of this figure contains a screenshot from the waters\_connect processing method indicating that the measured deconvoluted mass was only 1.5 ppm off from the calculated MW.



The analysis of larger oligonucleotides, typically used in CRISPR applications, is described in the next experiment. A 100-mer oligonucleotide with the sequence 5'- TGCCA GTTGC AGTTG TTTCC GAGCA GAAAC TCATC TCTGA AGAGG ATCTG GAGCA GAAAC TCATC TCTGA AGAGG ATCTG CACAC GCTGG AGCTG CCGCG-3' was analyzed on the BioAccord System using a modified 10-min gradient starting at 35% and ending at 45% Eluent B (3.5 mM TEA, 40 mM HFIP in 50% methanol). The corresponding UV and TIC chromatograms from Figure 7A indicate the presence of a major oligonucleotide eluting at 6.9 min.

According to the elemental composition of this 100-mer (C975 H1223 N384 O595 P99), the calculated accurate average mass is 30,907.7613 Da. The ESI-MS spectrum of this oligo (displayed in Figure 7B) was automatically deconvoluted using the BayesSpray charge deconvolution algorithm from waters\_connect and the experimentally measured deconvoluted mass was 26.9 ppm off from the expected mass, as indicated in the screenshot from Figure 7B. This result demonstrates the ability of the BioAccord System to provide highly accurate mass confirmation results even for large oligonucleotides (>100 oligomers).

Using the workflow described here, the BioAccord LC-MS System enables users to perform rapid and accurate intact mass confirmation as well as purity analysis for a variety of modified and unmodified oligonucleotides.

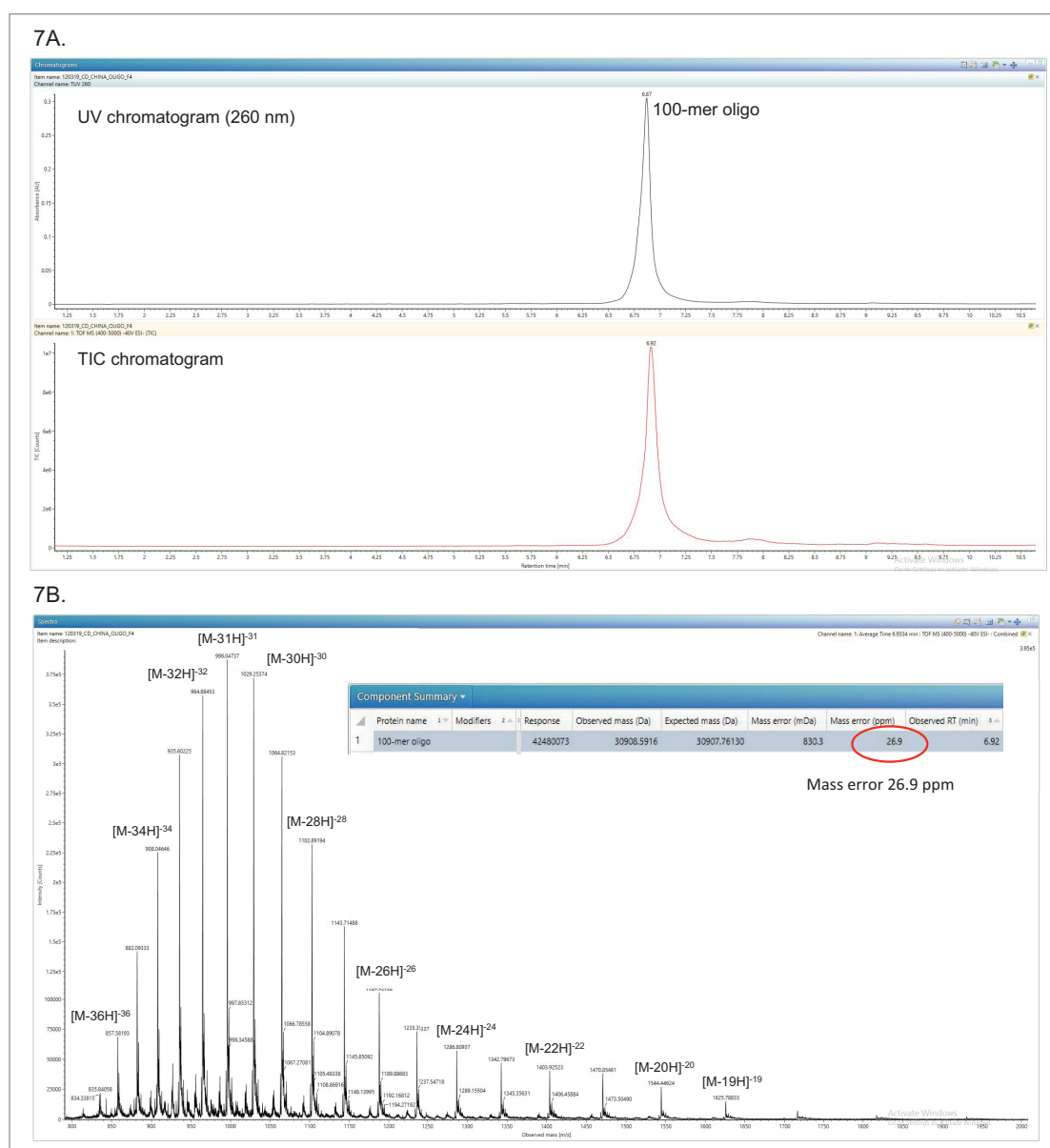


Figure 7. TUV and TIC chromatograms of the 100-mer oligonucleotide (A) and ESI-MS spectrum of the 100-mer oligonucleotide (B). The inset of this figure contains a screenshot from the waters\_connect processing method indicating that the measured deconvoluted mass for this oligo deviated 26.9 ppm from its calculated accurate average mass.

## CONCLUSIONS

- A waters\_connect workflow is shown to provide good mass accuracy (5–15 ppm) for intact mass confirmation of oligonucleotides of various lengths (10–35 mers), as well as phosphorothioated oligos.
- In addition, the automated processing method that was developed provides purity information for all the oligo sample components that are separated by ion-pair reversed-phase chromatography.
- This automated, compliance-ready LC-MS workflow provides for a robust, easy-to-use solution for fast mass confirmation and purity analysis of oligonucleotides.

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