AUTOMATED WORKFLOWS FOR INTACT MASS, PURITY AND SEQUENCE CONFIRMATION OF OLIGONUCLEOTIDES

Waters™

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OVERVIEW

An automated workflow, suitable for regulated and non-regulated laboratories, has been developed and implemented for analysis of oligonucleotide impurities. The workflow provides purity calculations and intact mass measurements for all oligonucleotide impurities, followed by sequence verification using their MS/MS fragmentation spectra.

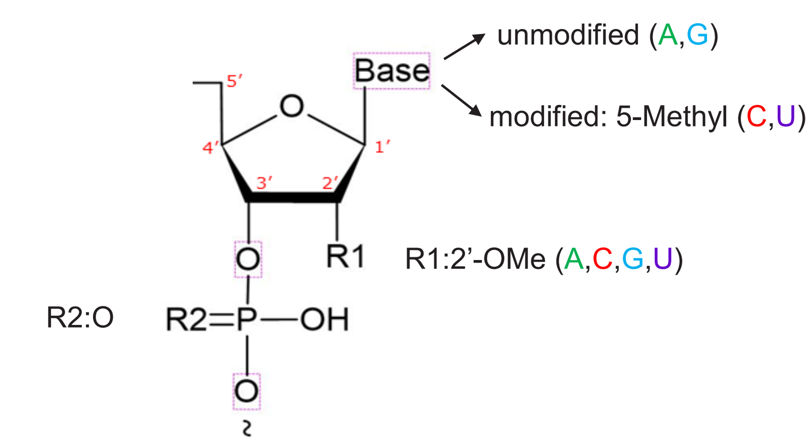
INTRODUCTION

- Synthetic oligonucleotides have emerged in recent years as a powerful alternative to small molecule and protein therapeutics [1].
- Manufacturing and quality control of oligonucleotide therapeutics requires highly selective and sensitive LC-MS methods for impurity detection and quantification.
- A critical step for identification of oligonucleotide impurities is mass spectrometry based sequencing [2] and data interpretation.
- The most often used mass spectrometry-based method for oligonucleotide analysis has been reversed-phase chromatography employing a variety of ion-pairing reagents and modifiers in negative ESI-MS mode (IP-RP LC-MS). Automated workflows for intact level analysis of oligonucleotides on the BioAccord™ LC-MS platform have been recently described [3-7].
- Here we are introducing an automated workflow for oligonucleotide impurity analysis that combines two *waters_connect*™ applications: INTACT Mass and CONFIRM Sequence. This workflow is supported on TOF and QTOF instruments operated under *waters_connect*.
- The INTACT Mass Application performs automated, fast deconvolution of oligonucleotide spectra across the entire chromatographic space, providing fast impurity assignments as well as the required metrics (mass accuracy and abundance) to support impurity analysis
- The CONFIRM Sequence application is used for fast processing of both MS/MS and MS^E (no specific precursor selection) fragmentation spectra for verification of sequence coverage. The software displays the relevant matching information (graphically and in table format) and provides statistical analysis on each matched fragment ion. The sequence coverage can be viewed in a "dot-map" form to easily assess the coverage of a predicted sequence, or to locate an impurity modification, and is capable of high-throughput data analysis of pre-acquired data.

METHODS

Materials

A 21-mer heavily modified oligonucleotide, containing a 2'-OMe modification on 19 of its nucleotides, having the sequence GUA ACC AAG AGU AUU CCA UTT and the elemental composition C229H306N76O143P20 was purchased from ATDBio (Southhampton, UK). Stock solutions were prepared in DI water at a concentration of 1 µM (or 2.34 µg/mL), from which a 10 µL volume was injected, which corresponds to loading 10 picomoles of the 21-mer oligonucleotide on-column.



Modifications of the 21-mer oligonucleotide

LC Conditions

Oligonucleotide separations were performed on an ACQUITY™ H-Class Bio UPLC system equipped with a 2.1 x 100 mm ACQUITY Premier OST column (P/N 186009485). The column flow rate was 300 µL/min and the column temperature was 60°C. The mobile phase composition was: Solvent A: 7 mM triethylamine (TEA) and 40 mM hexafluoro-2-propanol (HFIP) in Milli-Q water (pH 8.6) and Solvent B: 3.5 mM TEA, 20 mM HFIP in 50% methanol. Separations were performed using a 25-min gradient from 25-35% B and the total runtime was 40 min. UV chromatograms were recorded at a wavelength of 260 nm using a TUV detector.

MS conditions

ESI-MS spectra of oligonucleotides were acquired on a Xevo™ G2-XS QTOF instrument in negative ion mode over the m/z range of 500-5,000 with a full scan rate of 1Hz. The optimized ESI source parameters include: capillary voltage 2.5 kV, cone voltage 45V, source temperature 120°C and desolvation temperature 450°C. Doubly, triply and quadruply charged precursors of the 21-mer and its impurities were fragmented by CID in the collision cell using voltages in the range of 10-70 V. In addition, MS^E (DIA—data independent) datasets were acquired for the same oligonucleotides on the Xevo G2-XS instrument as well as on a BioAccord Tof System with the ACQUITY Premier UPLC system.

Informatics

Data acquisition and processing was performed using *waters_connect*™ software. ESI-MS spectra of oligonucleotides were processed automatically using the INTACT Mass app. The oligonucleotide purity assessments were calculated based on the UV response from the UV chromatogram using the same software. Individual MS/MS spectra and MS^E datasets were processed by the CONFIRM Sequence app to establish the optimum collision energy for fragmentation of each oligonucleotide precursor.

RESULTS

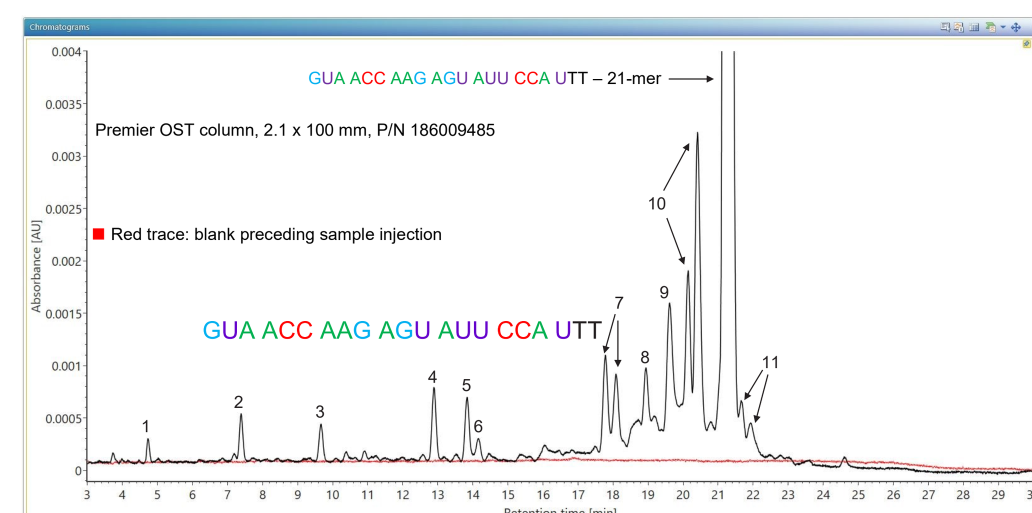


Figure 1. LC-UV chromatogram recorded on an ACQUITY Premier OST column.

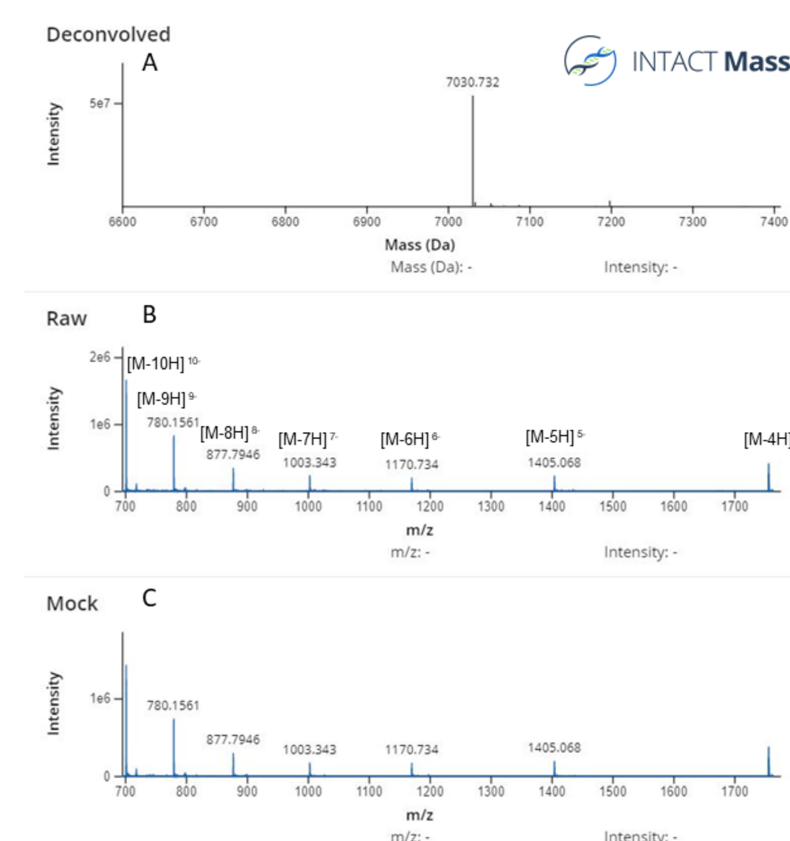


Figure 2. INTACT Mass ESI-MS spectra of the 21-mer heavily modified oligonucleotide: (A): deconvolved spectrum obtained using the BayesSpray deconvolution algorithm; (B) raw MS spectrum; (C) mock spectrum used for assessing the fidelity of the deconvolution process.

Figure 3. Screenshot with the processing results generated by the INTACT Mass app for the 21-mer oligonucleotide and its impurities. The dataset was deconvolved using the BayesSpray charge deconvolution algorithm and 11 oligonucleotide impurities were identified with mass accuracies of under 15 ppm. The first impurity displayed in the table, an 11-mer oligonucleotide, has the lowest detected abundance, at 0.2% according to the UV measurement.

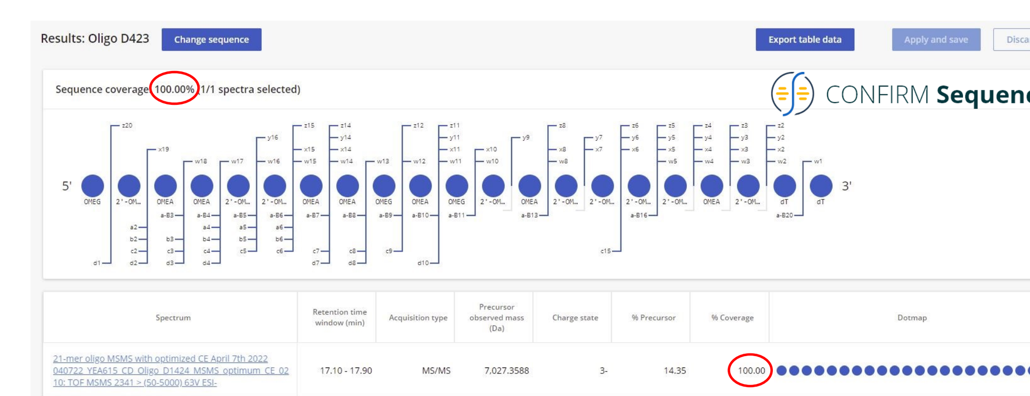


Figure 4. CONFIRM Sequence screenshot showing excellent MS/MS fragmentation coverage (100%) in a dot-map format. The $[M\text{-}3H]^{3-}$ precursor of the 21-mer heavily modified oligonucleotide (m/z = 2342.0) was fragmented using an *optimized fixed* collision energy (set at 63 V) in the collision cell of a Xevo G2-XS QTof instrument.

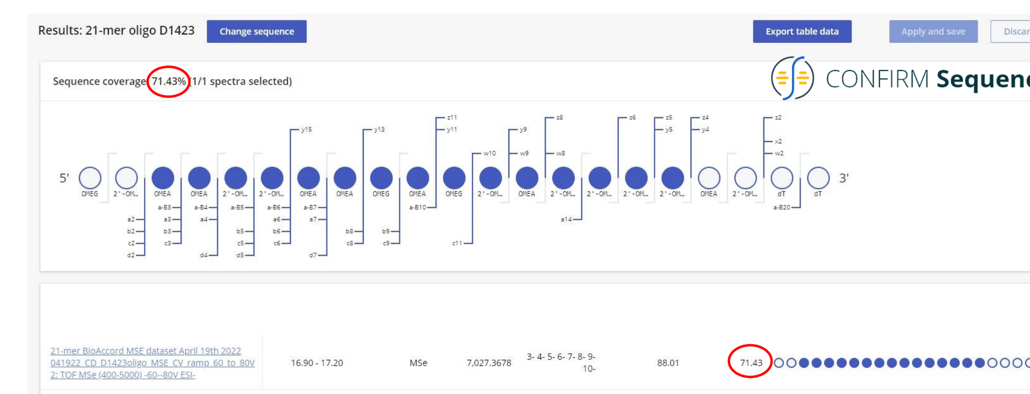


Figure 5. Dot-map sequence coverage (~ 70%) obtained from high energy MS^E untargeted fragmentation. All precursors of the 21-mer heavily modified oligonucleotide (see Figure 2B) were fragmented using an *optimized cone voltage ramp* (from 60 to 80 V) applied to the Step Wave of a BioAccord TOF instrument.

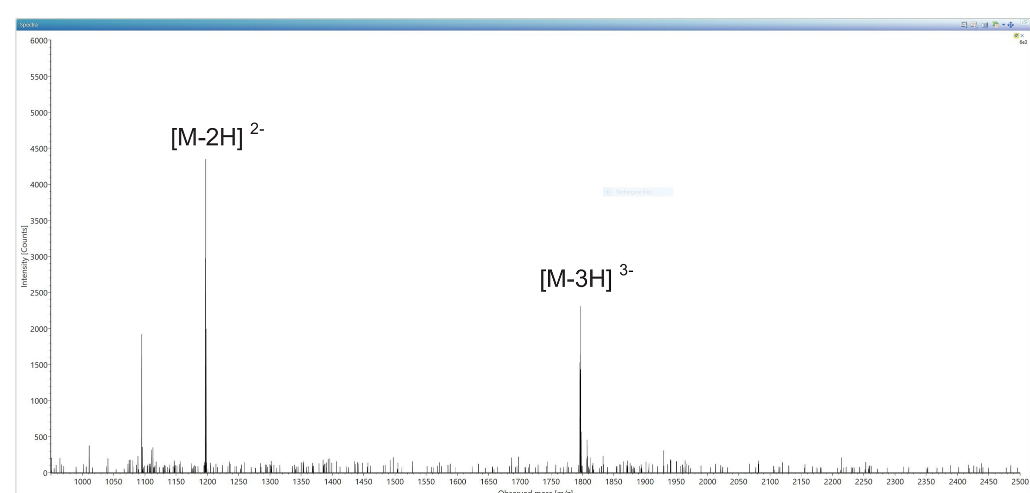


Figure 6. Ion pairing reversed phase ESI-MS spectrum of an 11-mer oligonucleotide impurity which is the least abundant impurity present in the 21-mer oligonucleotide sample (0.2% relative abundance, labeled as peak 1 in the chromatogram shown in Figure 1).

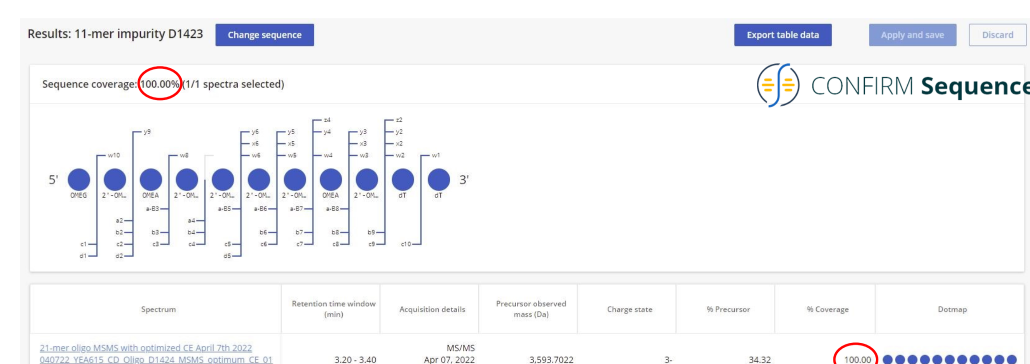


Figure 7. Maximum sequence coverage (100%) obtained from the MS/MS fragmentation of the $[M\text{-}3H]^{3-}$ precursor of the 11-mer oligonucleotide impurity. The precursor was fragmented with an *optimized fixed* collision energy (36 V) in the collision cell of a Xevo G2-XS instrument.

Figure 8. CONFIRM Sequence screenshot showing the MS/MS spectrum recorded for the 21-mer oligonucleotide. The $[M\text{-}3H]^{3-}$ precursor of this oligonucleotide (m/z = 2342.0) was fragmented using an *optimized fixed* collision energy (set at 63 V) in the collision cell of a Xevo G2-XS QTof instrument. The fragment ions labeled in green were matched to the oligonucleotide sequence according to the dot-map diagram shown in Figure 4.

GUA A*C AAG AGU AUU CCA UTT - C missing in position 5

Figure 9. Confirm Sequence screenshot showing the sequence coverage of a 20-mer oligonucleotide impurity belonging to the peak doublet 10 shown in Figure 1. The most abundant isomer of this impurity, eluting just before the FLP, was identified as an oligonucleotide missing a 2'-OMe 5 Me cytidine residue in position 5. This oligonucleotide sequence displayed a significantly higher sequence coverage (80%) than an alternative sequence which is missing the same residue at position 16. There are 4 modified cytidines in the 21-mer sequence, but because they are located as pairs, there are only two possible 20-mer impurities missing a single 2'-OMe 5 Me cytidine. The software looked for a missed 2'-OMe 5 Me cytidine from the sequence of the 21-mer and found strong evidence (80% sequence coverage) for assigning this impurity to the sequence: GUA A*C AAG AGU AUU CCA UTT.

Oligo length	Peak label	Retention time (min)	Oligonucleotide sequence	Elemental composition	Most abundant precursor monoisotopic mass / charge state	Second precursor/ charge state	Optimum Collision Energies (V)	MSMS Sequence Coverage (%)	COMBINED Seq Coverage (%)	TUV Area Percentage (%)
11-mer	1	4.73	GU AUU CCA UTT	C119 H161 N33 O77 P10	1196.8952 (-3)	1795.8464 (-2)	36 / 60	100.0	100.0	0.18
12-mer	2	7.39	AGU AUU CCA UTT	C130 H175 N38 O83 P11	1311.2512 (-3)	1967.3805 (-2)	40 / 59	100.0	100.0	0.51
14-mer	3	9.67	AG AGU AUU CCA UTT	C152 H203 N48 O96 P13	1545.295 (-3)	2318.4461 (-2)	46 / 60	100.0	100.0	0.46
15-mer	4	12.89	AAG AGU AUU CCA UTT	C163 H217 N53 O102 P14	1659.651 (-3)	2489.9802 (-2)	49 / 60	81.3	86.7	0.89
16-mer	5	13.83	C AAG AGU AUU CCA UTT	C174 H233 N56 O109 P15	1770.6752 (-3)	1327.7546 (-4)	51 / 40	75.0	83.2	0.83
17-mer	6	14.15	CC AAG AGU AUU CCA UTT	C184 H261 N59 O116 P16	1881.7307 (-3)	1411.0622 (-4)	-	-	-	1.34
18-mer	7	17.8/18.1	CC AAG AGU AUU CCA UTT	C184 H261 N59 O116 P16	1996.0555 (-3)	1496.7898 (-4)	-	-	-	2.38
21-mer	8	18.94	AUA ACC AAG AGU AUU CCA UTT	C229 H306 N76 O142 P20	2336.1198 (-3)	1751.8380 (-4)	-	-	-	2.32
20-mer	9	19.62	GU* ACC AAG AGU AUU CCA UTT	C218 H292 N71 O137 P19	2227.0954 (-3)	1670.0697 (-4)	59 / 50	76.2	76.2	2.32
20-mer	10	20.1/20.4	AUA A*C AAG AGU AUU CCA UTT	C218 H290 N73 O136 P19	2230.4272 (-3)	1672.5686 (-4)	59 / 50	80.0	80.0	6.29
21-mer	FLP	21.28	GUA ACC AAG AGU AUU CCA UTT	C229 H306 N76 O143 P20	2341.4514 (-3)	1755.8367 (-4)	63 / 50	100.0	100.0	82.03
21-mer	11	21.6/21.9	GUA ACC AAG AGU AUU CCA UTT	C229 H307 N76 O143 P20	2341.7874 (-3)	1756.0887 (-4)	-	-	-	0.45

Table I. Eleven oligonucleotide impurities were identified in a 21-mer extensively modified oligonucleotide. Eight impurities and the full length product (FLP) were sequenced using a Xevo G2-XS instrument and the individual MS/MS spectra fragmented with optimum collision energies were processed using the CONFIRM Sequence app. The MS/MS sequence coverage for the FLP and its impurities were above 75%. The lowest abundance impurity, an 11-mer oligonucleotide was sequenced with 95% sequence coverage, while the sequence of the FLP (21-mer oligonucleotide) was confirmed with 100% coverage. The total sequence coverage corresponds to the combined sequence obtained from the MS/MS fragmentation of two precursors of each oligonucleotide.

CONCLUSIONS

- An automated workflow relying on two recently introduced *waters_connect*™ applications (INTACT Mass and CONFIRM Sequence) was applied for the impurity analysis of a 21-mer heavily modified oligonucleotide and its impurities
- The INTACT Mass app provided purity calculations based on the UV data, as well as accurate mass measurements for all 11 oligonucleotide impurities, with mass accuracies under 10 ppm
- The MS/MS spectra acquired for eight oligonucleotide impurities along with the MS/MS spectra acquired for the 21-mer extensively modified FLP were processed using the CONFIRM Sequence app to confirm their expected sequences
- The workflow described here demonstrates the capability of the CONFIRM Sequence app to achieve maximum sequence coverage (100%) even for low abundance oligonucleotide impurities, down to ~ 0.2% abundance levels.
- The CONFIRM Sequence app is capable of finding sequence omissions, insertions or sequence scrambling

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