Application Note

Pharma & Biopharma
Oligonucleotides

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Oligonucleotide Characterization by Agilent 1290 Infinity II Bio LC and 6545XT AdvanceBio LC/Q-TOF

Oligonucleotide identity, impurities analysis and sequence determination using BioConfirm 12.0 target plus impurities (TPI) and sequence confirmation workflows

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Introduction

Synthetic oligonucleotide therapeutics show high potential in the treatment of many challenging diseases. They are present in various sizes and forms and are often chemically modified to enhance product effect, stability, specificity and delivery efficiency. They are usually classified into categories like small interfering RNA (siRNA), antisense oligonucleotides (ASO), microRNA (miRNA), aptamers, and CRISPR gene editing guide RNA (gRNA). The most common quality attributes of synthetic oligonucleotides are their mass, purity, contaminating impurities, and sequence.

Characterization of oligonucleotides requires robust analytical instrumentation and methods as well as ease-of-use data analysis tools. Agilent 1290 Infinity II Bio LC System is made of biocompatible material for use with challenging mobile phase conditions in biopharma applications. Biocompatibility mitigates non-specific sample binding to flow path and it ensures the integrity of biomolecules and robustness of the system. The Target Plus Impurities (TPI) and Sequence Confirmation workflows in Agilent MassHunter BioConfirm software have already been introduced in two separate application notes.[1,2] In this study, both workflows were carried out to characterize two oligonucleotide samples using Agilent 1290 Infinity II Bio LC System coupled to Agilent 6545XT AdvanceBio LC/Q-TOF (Figure 1).

Experimental

Materials and methods

Triethylamine (TEA) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RNase-free water and 1xTE buffer pH 7.5 was purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). Methanol (InfinityLab Ultrapure LC/MS grade, part number 5191-4497) was obtained from Agilent Technologies.

Oligonucleotide (RNA) Resolution Standard (part number 5190-9028) was obtained from Agilent. An 18-mer antisense oligonucleotide (ASO) and 28-mer aptamer were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA) with standard desalting purification. Sample details are listed in Table 1. Codes for building blocks and modifications are annotated individually.

Sample preparation

Oligonucleotide RNA Resolution Standard was dissolved with 1 mL RNase-free water. The final concentration was 2 pmol/µL.

The other oligonucleotide samples were dissolved in respective volumes of 1xTE buffer pH 7.5 to achieve the final concentration of 100 pmol/µL.

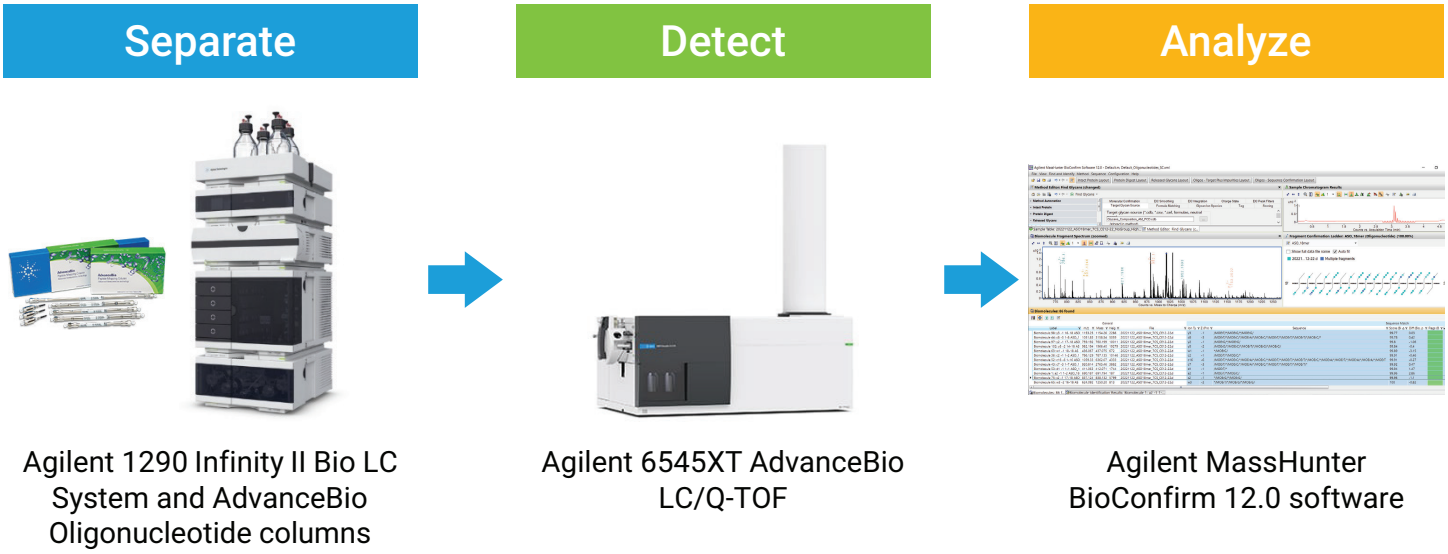

Figure 1. Analytical components of the oligonucleotide characterization for both TPI and SC workflows.

Table 1. Oligonucleotide sample details and sequence code notations. All sequences are in the 5' to 3' direction.

Name	Length	Sequence
ASO	18-mer	/52MOErT/*/i2MOErC/*/i2MOErA/*/i2MOErC/*/i2MOErT/*/i2MOErT/*/i2MOErT/*/i2MOErC/*/i2MOErA/*/i2MOErT/*/i2MOErA/*/i2MOErA/*/i2MOErT/*/i2MOErG/*/i2MOErC/*/i2MOErT/*/i2MOErG/*/32MOErG/
Aptamer	28-mer	/52FC/mGmGrArA/i2FU//i2FC/mAmG/i2FU/mGmAmA/i2FU/mG/i2FC//i2FU//i2FU/mA/i2FU/mA/i2FC/mA/i2FU//i2FC//i2FC/mG/3InvdT/

Code	Description
*	Phosphorothioate bond
A	2'-deoxyribose adenine
C	2'-deoxyribose cytosine
G	2'-deoxyribose guanine
mA	2'-O-methyl A
mG	2'-O-methyl G

Code	Description
rA	Ribose adenine
rG	Ribose guanosine
/52MOErT/	5' 2-methoxyethoxy T
/i2MOErA/	Internal 2-methoxyethoxy A
/i2MOErC/	Internal 2-methoxyethoxy C
/i2MOErT/	Internal 2-methoxyethoxy T

Code	Description
/i2MOErG/	Internal 2-methoxyethoxy G
/32MOErG/	3' 2-methoxyethoxy G
/52FC/	5' Fluoro C
/i2FU/	Internal Fluoro U
/i2FC/	Internal Fluoro C
/3InvdT/	3' inverted T

Instrumentation

- Agilent 1290 Infinity II Bio LC including:
  - Agilent 1290 Infinity II Bio High-Speed Pumps (G7132A)
  - Agilent 1290 Infinity II Bio Multisampler (G7137A) with Agilent Infinity II Sample Cooler (Option #100)
  - Agilent 1290 Infinity II Multicolumn Thermostat with Bio Heat Exchanger (G7116B)
  - Agilent 1290 Infinity II Diode Array Detector (G7117B)
- Agilent 6545XT AdvanceBio LC/Q-TOF

LC/MS analysis

LC/MS analyses were conducted on a 1290 Infinity II Bio LC coupled with a 6545XT AdvanceBio LC/Q-TOF system equipped with a Dual Agilent Jet Stream source. Agilent MassHunter Acquisition Workstation software (version 11.0) was used, with compliance supporting features enabled. LC separation was obtained with an Agilent AdvanceBio Oligonucleotide column, 2.1 × 50 mm, 2.7 µm (part number 659750-702).

LC/MS method parameters were listed in Tables 2 and 3.

Table 2. Liquid chromatography parameters.

Agilent 1290 Infinity II Bio LC	
Column	AdvanceBio Oligonucleotide, 2.1 × 50 mm, 2.7 µm (p/n 659750-702)
Thermostat	4 °C
Solvent A	15 mM TEA and 400 mM HFIP in water
Solvent B	Methanol
Gradient for TPI workflow	0 to 1 min, 10% B 1 to 10 min, 10 to 40% B 10 to 11 min, 40 to 95% B
Gradient for SC workflow	0 to 0.5 min, 15% B 0.5 to 4 min, 15 to 40% B 4.1 to 5 min, 95% B
Post Time	6 min
Column Temperature	65 °C
Flow Rate	0.5 mL/min

Table 3. MS data acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF System	
General source parameters shared by two workflows	
Source	Dual AJS
Polarity	Negative
Gas Temperature	275 °C
Gas Flow	12 L/min
Nebulizer	35 psi
Sheath Gas Temperature	350 °C
VCap	3,500 V
Nozzle Voltage	2,000 V
Fragmentor	175 V
Skimmer	65 V
Acquisition Mode	HiRes (4 GHz)
Reference Mass	1,033.988109

Agilent 6545XT AdvanceBio LC/Q-TOF System	
Parameters for TPI workflow	
Acquisition Mode	MS only
Mass Range	300 to 3,200 *m/z*
Acquisition Rate	4 spectra/s
Parameters for SC workflow	
Acquisition Mode	Targeted MS/MS
MS Acquisition Range	400 to 3,000 *m/z*
Acquisition Rate/Time	1 spectra/s
MS/MS Acquisition Range	100 to 3,000 *m/z*
Acquisition Rate/Time	2 spectra/s
Isolation Width	Medium (~4 *m/z*)
Collision Energy (CE)(eV)	Optimized individually

Results and discussion

Characterization of synthetic oligonucleotides encompasses oligonucleotide identity confirmation, relative quantification of related impurities, and oligonucleotide sequence confirmation. This formerly challenging task was made easy recently with the introduction of two integrated workflows within MassHunter BioConfirm 12.0 software.

Separation of the oligonucleotide standard

Agilent supplies oligonucleotide standards for both DNA and RNA samples. It is a good practice to run the standards prior to analyzing the actual samples for system suitability testing of system performance such as column resolution and detector sensitivity. In this work, Agilent RNA resolution standard was used to check the performance. With the ion-pairing reversed-phase chromatography, excellent resolution was achieved among four different lengths of standards as shown in Figure 2.

Identity and impurities analysis of ASO 18-mer with Target Plus Impurities (TPI) workflow

The Target Plus Impurities (TPI) workflow has been developed in MassHunter BioConfirm 12.0 for oligonucleotide identity confirmation and relative quantification of impurities. Either Find-by-Formula or Maximum Entropy algorithm can be applied in this workflow.

In this section, Max Entropy Deconvolution algorithm, an untargeted approach, was used to confirm the identity of an ASO 18-mer sample (average mass 7,127.2001 Da). The ASO sample was chromatographically separated from a series of related impurities. Approximately 3.5 µg of sample was injected onto the column with a flow rate of 0.5 mL/min. The 10-minute gradient enabled good separation of the full-length ASO from a series of impurities as shown in the inset of Figure 3A. The charge state distribution of the ASO was in the mass range of *m/z* 700 to 2,500 Da (charge −3 to −9) as depicted in Figure 3B. The zoomed view on the inset of Figure 3B demonstrates excellent MS isotopic resolution for the −7 charge state of the oligonucleotide. The optimized Q-TOF conditions warranted excellent quality deconvoluted MS spectra of the ASO peak with mass accuracy as low as −0.73 ppm by Max Entropy Deconvolution. In addition, low-abundance truncation species (~5,700 and 6,700 Da) and the extension species (~7,600 Da) were also detected, as shown in Figure 3C.

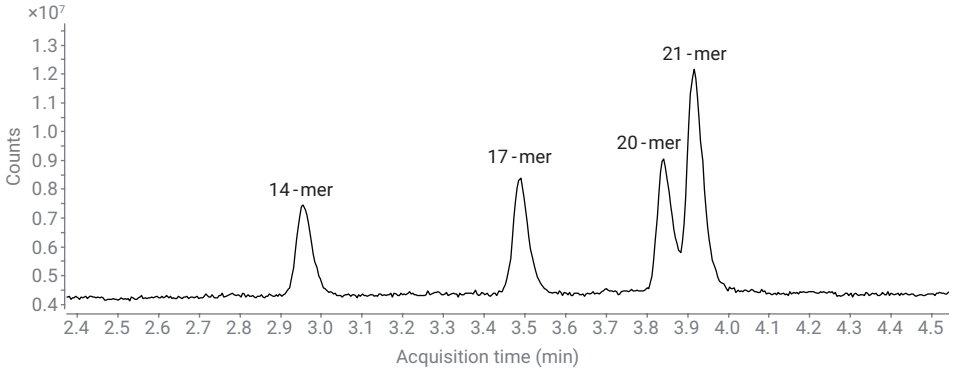

Figure 2. LC/MS analysis of Agilent RNA resolution standard.

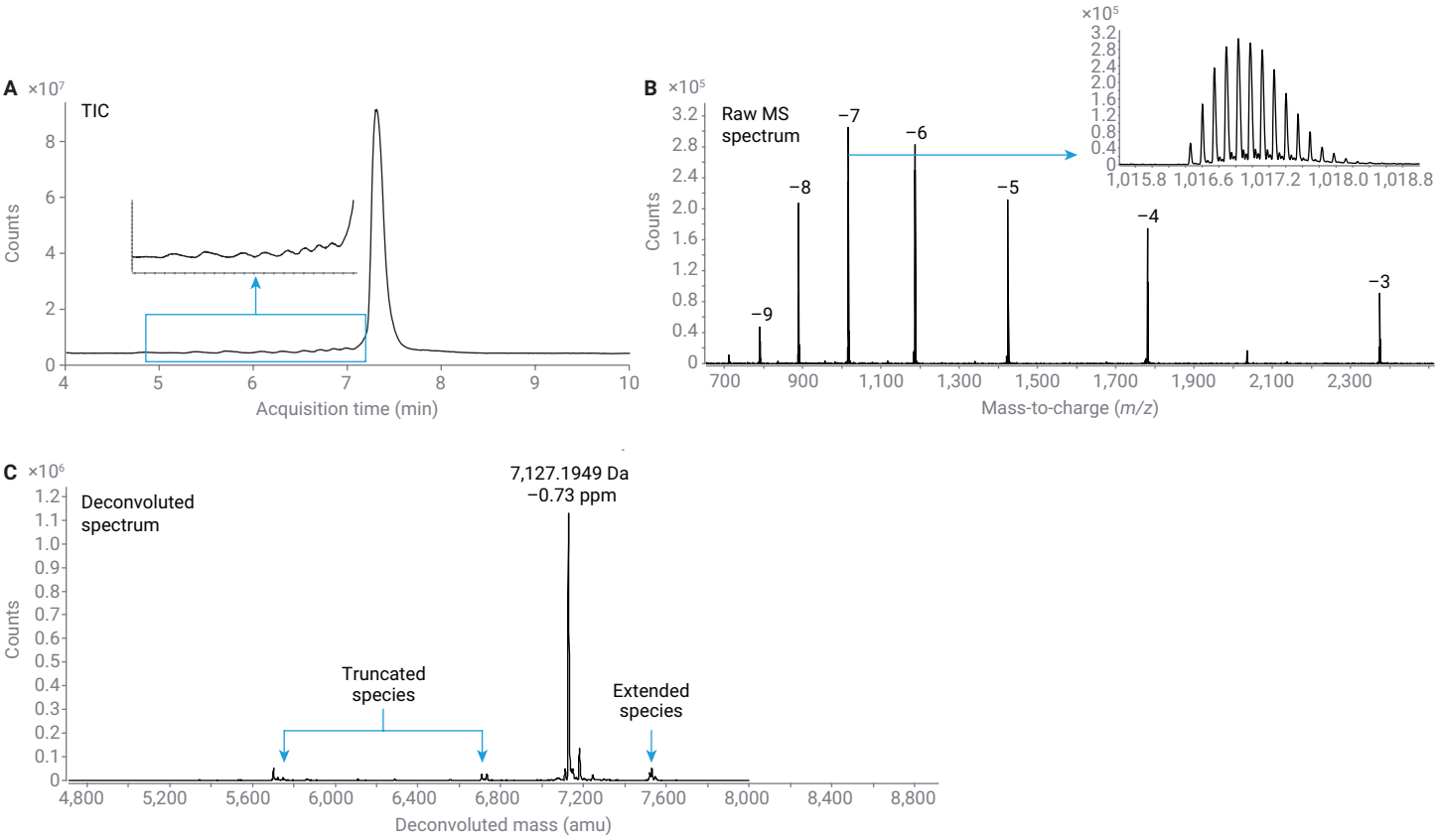

Figure 3. LC/MS analysis of ASO 18-mer. (A) Total ion chromatogram (TIC); (B) raw MS spectrum; (C) deconvoluted MS spectra of the ASO peak eluting from 7.09 to 7.37 minutes.

The oligonucleotide impurities could exist in different formats such as truncations, additions, and abasic oligonucleotides.[3] Impurities may derive from manufacturing process, shipment, and storage. Their presence could impact negatively on drug efficacy and safety. As a result, impurity analysis is an indispensable part in the characterization of synthetic oligonucleotides.

In this part, Find-by-Formula algorithm, a targeted approach, was used in the relative quantification analysis of ASO 5'-truncates with linker. Figure 4A demonstrates the extracted ion chromatograms (EICs) profiles of the full-length ASO and the series of the 5' truncates distributed across the entire separation. Accurate monoisotopic masses and reproducible relative quantitation results for all targeted impurities were demonstrated in Figure 4B. Relative quantification as low as 0.4% was achieved with high confidence.

Table 4 summarizes the total 17 oligonucleotides including targeted full-length ASO and its 5'-truncated impurities. Other types of impurities such as 3'-truncates with or without linker could also be detected at even lower level (data not shown).

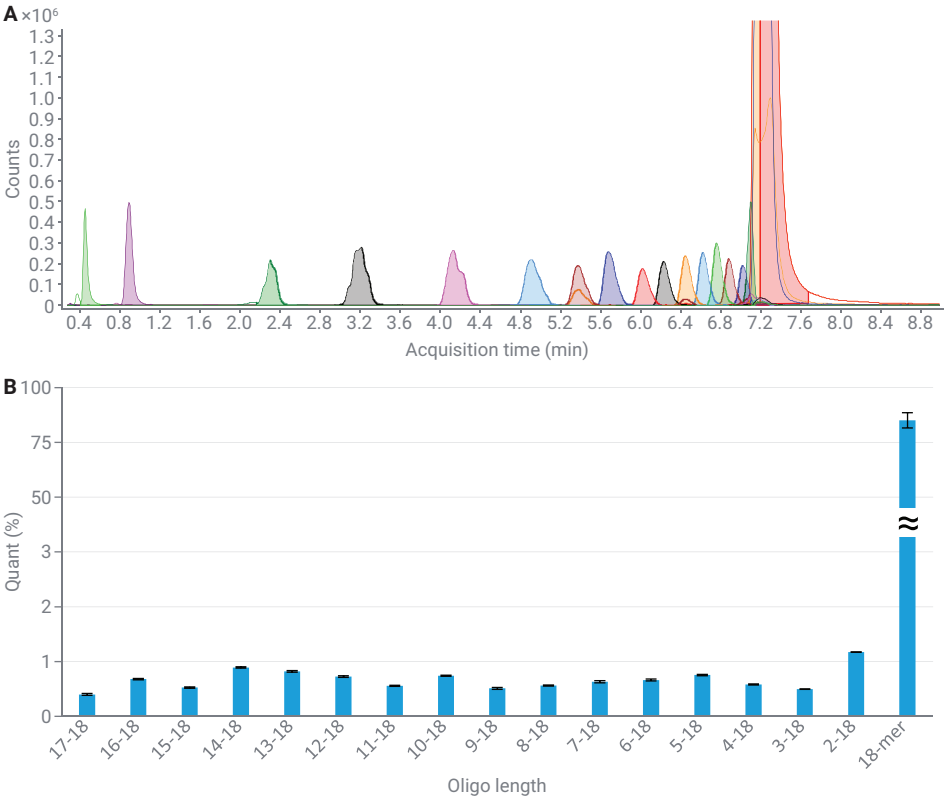

Figure 4. Relative quantification analysis of ASO18-mer and targeted impurities by the Find-by-Formula algorithm of Agilent BioConfirm 12.0. (A) Extracted ion chromatograms (EICs) of the full-length ASO and its impurities. (B) Relative quantitation analysis results. Excellent reproducibility with low RSD (<5%) was achieved over a total of six sample injections.

Table 4. Impurity analysis summary on ASO 18-mer (n = 6).

Peak	Oligo Length	Sequence	RT (min)	Calculated Mono Mass	Measured Mass	Avg Mass Accuracy (ppm) (n = 6)"	Avg % Quant (n = 6)"	Std Dev	RSD (%)
1	17-18	ASO_18mer {5-Trunc-L(x16)}	0.45	760.2000	760.2003	0.40	0.40	0.02	4.29
2	16-18	ASO_18mer {5-Trunc-L(x15)}	0.90	1,154.2600	1,154.2605	0.46	0.68	0.01	1.71
3	15-18	ASO_18mer {5-Trunc-L(x14)}	2.31	1,547.3359	1,547.3354	-0.33	0.53	0.01	2.59
4	14-18	ASO_18mer {5-Trunc-L(x13)}	3.20	1,966.4024	1,966.4013	−0.56	0.89	0.01	1.46
5	13-18	ASO_18mer {5-Trunc-L(x12)}	4.12	2,360.4623	2,360.4611	−0.51	0.82	0.01	1.79
6	12-18	ASO_18mer {5-Trunc-L(x11)}	4.90	2,763.5339	2,763.5316	−0.82	0.73	0.02	2.07
7	11-18	ASO_18mer {5-Trunc-L(x10)}	5.36	3,166.6054	3,166.6028	−0.85	0.56	0.01	1.86
8	10-18	ASO_18mer {5-Trunc-L(x9)}	5.67	3,560.6654	3,560.6628	−0.73	0.74	0.01	1.58
9	9-18	ASO_18mer {5-Trunc-L(x8)}	6.01	3,963.7369	3,963.7339	−0.77	0.51	0.02	3.37
10	8-18	ASO_18mer {5-Trunc-L(x7)}	6.21	4,356.8129	4,356.8084	−1.04	0.56	0.01	2.15
11	7-18	ASO_18mer {5-Trunc-L(x6)}	6.44	4,750.8729	4,750.8683	−0.96	0.63	0.02	2.94
12	6-18	ASO_18mer {5-Trunc-L(x5)}	6.61	5,144.9328	5,144.9274	−1.07	0.67	0.02	2.47
13	5-18	ASO_18mer {5-Trunc-L(x4)}	6.75	5,538.9928	5,538.9875	−0.96	0.76	0.01	1.83
14	4-18	ASO_18mer {5-Trunc-L(x3)}	6.87	5,932.0688	5,932.0620	−1.14	0.58	0.01	1.69
15	3-18	ASO_18mer {5-Trunc-L(x2)}	7.01	6,335.1403	6,335.1337	−1.04	0.50	0.01	1.04
16	2-18	ASO_18mer {5-Trunc-L}	7.09	6,728.2163	6,728.2055	−1.60	1.18	0.01	0.47
Target	18-mer	ASO_18mer	7.19	7,122.2762	7,122.2699	−0.89	89.18	0.14	0.16

Sequence confirmation of oligonucleotides with Sequence Confirmation workflow

The newly developed sequence confirmation workflow in BioConfirm 12.0 software streamlined the previously challenging and time-consuming data analysis process in determination of oligonucleotides sequence. The algorithm followed the standard nomenclature of oligonucleotide fragmentation. A unique fragment ions annotation for the Fragment Confirmation Ladder was applied and shown in Figure 5.

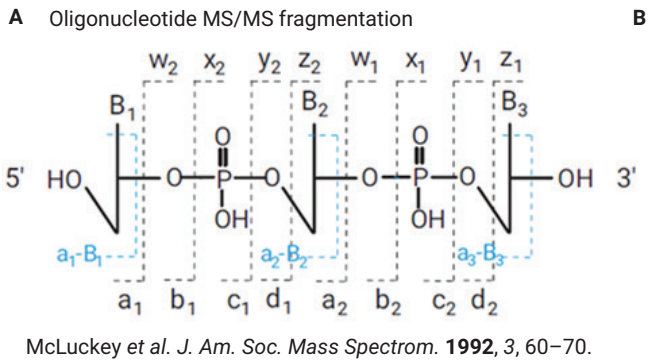

Figure 5. Nomenclature of oligonucleotide fragmentation (A) and annotation for oligonucleotide Fragment Confirmation Ladder (B). The colors of letters represent the different types of fragment ions identified and labeled in the MS/MS spectra.

Two synthetic oligonucleotides (ASO 18-mer, aptamer 28-mer) with varied sequence, length, modifications were investigated in this study using the sequence confirmation workflow. The LC/MS/MS method was optimized for individual oligonucleotides in the aspects of optimal charge state selection, collision energy (CE) and MS/MS acquisition time to obtain the best fragmentation results and highest sequence coverage. Grouping MS/MS scans by charge state or collision energy or both in the sequence confirmation algorithm further enhances sequence coverage.

A rapid 5-minute LC gradient method was developed for sequence confirmation workflow. Good MS/MS spectra were obtained for both samples.

For the 2-methoxyethoxylated ASO sample (average mass 7,127.2001 Da), most intensive charge state −7 was selected as precursor (*m/z* 1,017.0337) and collision energy 15 eV was applied for MS/MS fragmentation. Adequate fragments were generated for sequence confirmation with 100% sequence coverage from just one single injection (Figure 6). Similarly, the 100% sequence coverage of 28-mer aptamer (average mass 9,116.5566 Da), which contains fluoro U/T and methylated A/G, was successfully achieved with one injection whereby charge states −10 precursor ion (*m/z* 910.5288) was fragmented at collision energy 18 eV (Figure 7).

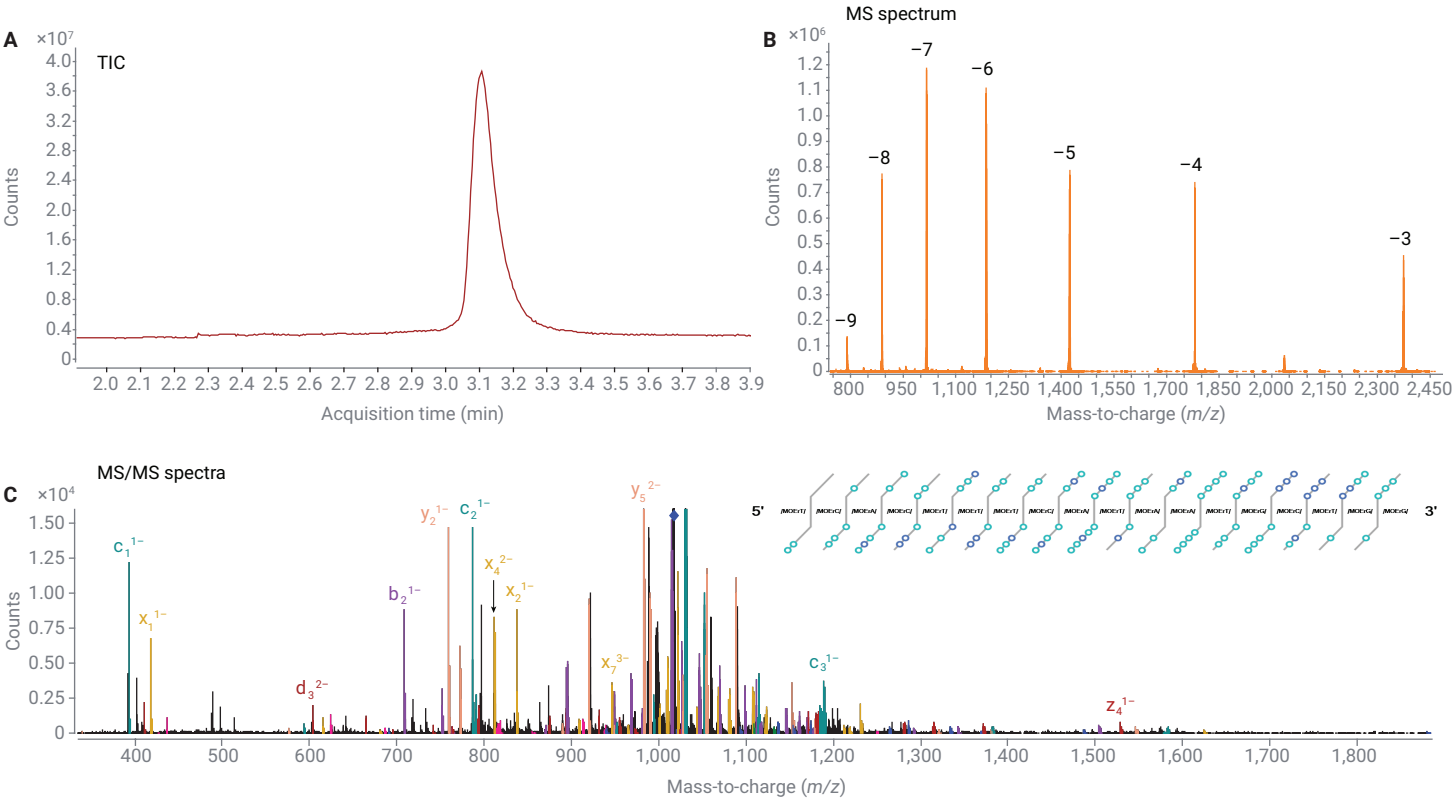

Figure 6. LC-MS/MS analysis of ASO sequence confirmation. (A) total ion chromatogram (TIC); (B) raw MS spectrum; (C) MS/MS spectra with fragment confirmation ladder.

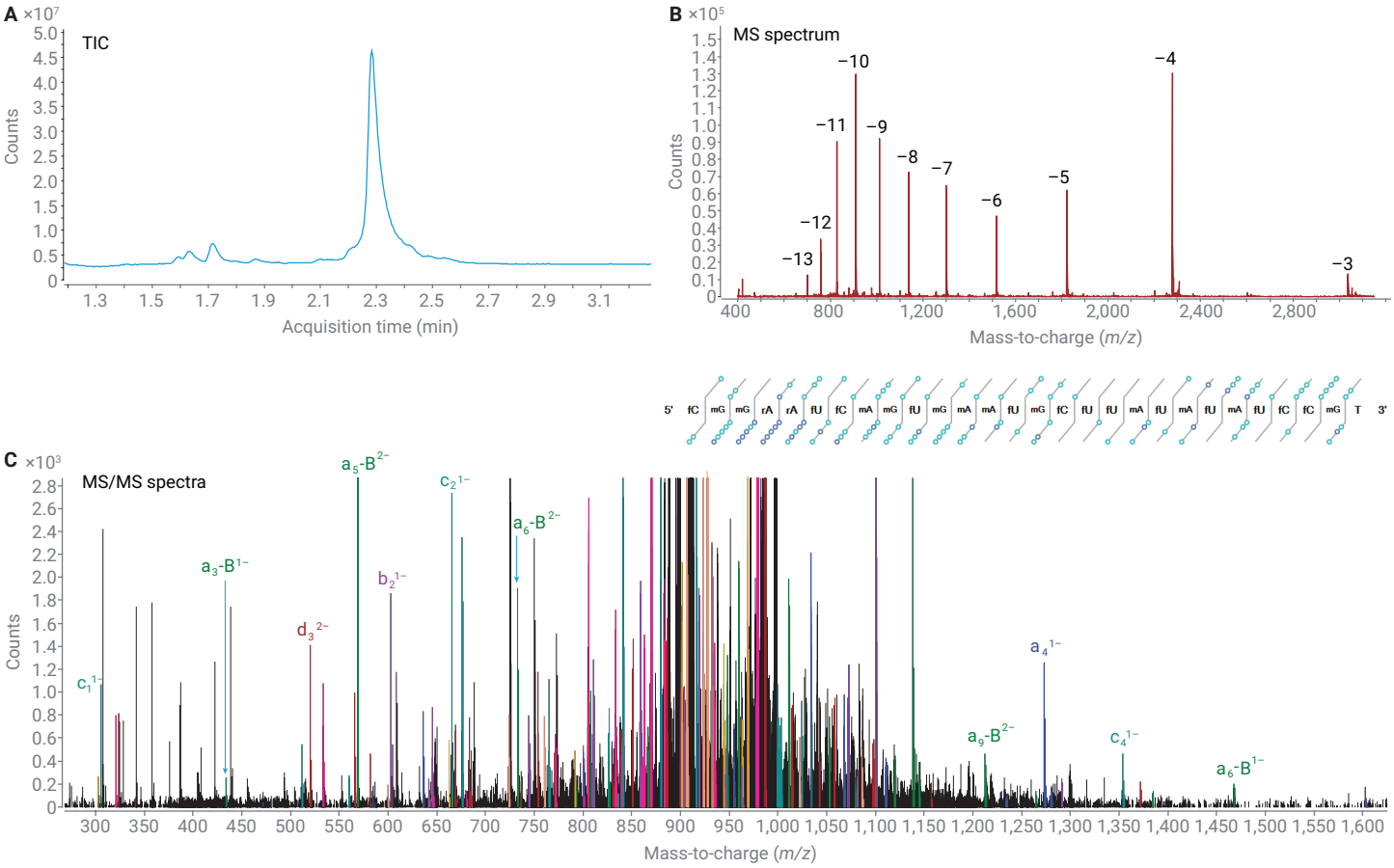

Figure 7. LC/MS/MS analysis of aptamer sequence confirmation. (A) total ion chromatogram (TIC); (B) raw MS spectrum; (C) MS/MS spectra with fragment confirmation ladder.

Conclusion

The Agilent 1290 Infinity II Bio LC coupled with an Agilent 6545XT AdvanceBio LC/Q-TOF demonstrated excellent chromatographic separation, reproducible quantification results, and superior LC/MS and HRAM MS/MS data. The two novel Targeted Plus Impurities (TPI) and Sequence Confirmation workflows in the Agilent MassHunter BioConfirm software version 12.0 greatly improve the data processing efficiency of oligonucleotide identity, impurities analysis, and sequence confirmation.

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