Application Note

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Assessing the Purity of an Antisense Oligonucleotide Sample by LC/MS

Using a high-sensitivity unit mass detector

Authors

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Abstract

This application note outlines a method for assessing the purity of antisense oligonucleotides (ASOs) using the Agilent InfinityLab Pro iQ Plus LC/MS system. While high-resolution mass spectrometry is typically employed for analyzing large biomolecules, such as oligonucleotides, the LC/MS approach described here enables the use of a unit mass detector in a more robust and routine fashion. The Agilent Oligo Analysis Accelerator (OAA) enhances peak integration and spectral interpretation, streamlining the quality control workflow. This method supports rapid analysis of oligonucleotide purity, assay performance, and basic impurity profiling.

Introduction

Antisense oligonucleotides (ASOs) are an established therapeutic nucleic acid modality, manufactured by solid-phase synthesis. Because of their complexity, a common way to assess the purity of ASOs is liquid chromatography/mass spectrometry (LC/MS). Although high-resolution MS might be appropriate for workflows such as characterization and sequence confirmation, unit mass detection provides a more robust and practical method for routine testing labs.

Single quadrupole LC/MS has previously been shown to effectively support purity, assay, and impurity profiling for routine testing of single-stranded therapeutic oligos, such as ASOs.[1] This method relies on MS full scan to quantitate closely related impurities that elute under the main peak. The LC/MS conditions ensure that analytes are predominantly in the 4– charge state when entering the gas phase. For example, an oligo that is ~ 8 kDa would have a predominant *m/z* value of ~ 2,000. This would either be at the upper limit or possibly exceed the scan range for some unit mass detectors. Furthermore, this method has a limit of quantitation (LOQ) of ≥ 0.2%, requiring a sensitive mass detector with efficient high mass transmission.

In this application note, the Agilent InfinityLab Pro iQ Plus LC/MS system is used to determine the purity of an ASO. The full scan spectrum from this unit mass detector enables the identification and quantification of low-level impurities at method specifications. This study establishes proof of concept for the use of this system in the routine testing of oligonucleotides for QC lot release.

Experimental

Instrument configuration

This experiment was conducted using the following instrument configuration:

- Agilent InfinityLab Pro iQ Plus LC/MS system (G6170A)
- Agilent 1290 Infinity II bio binary pump (G7120A)
- Agilent 1290 Infinity II bio multisampler (G7167B)
- Agilent 1290 Infinity II bio column compartment (G7116B)
- Agilent 1260 Infinity II diode array detector HS (G7117C)

Although this analysis used an Agilent Infinity II LC configuration, comparable results can be achieved on the Agilent Infinity III LC system with no changes to method parameters.

Sample preparation

A 20-mer, non-HPLC purified antisense oligo (ASO-1) was obtained from Integrated DNA Technologies (Coralville, IA, USA). Samples were reconstituted in de-ionized water to 1 mg/mL, then further diluted to 0.1 mg/mL in de-ionized water.

Software

Data acquisition was performed in Agilent OpenLab CDS, version 2.8, using the LC/MS parameters shown in Tables 1 and 2. Data analysis was performed in Oligo Analysis Accelerator (OAA) for OpenLab CDS, version 1.0.

LC/MS settings

Table 1. Source parameters for the Agilent InfinityLab Pro iQ Plus (6170A).

Mass Spectrometry Parameters	
MS	6170A
Source	Electrospray ionization (ESI)
Scan Mode	Negative polarity
Drying Gas Flow	12.0 L/min (standard) 13.0 L/min (harsh)
Gas Temperature	260 °C (standard) 350 °C (harsh)
Nebulizer Pressure	25 psi
Capillary Voltage	4,000 V
Scan	*m/z* 1,450 to 2,175
Scan Time	1,000 ms
Fragmentor	100 V
Gain Factor	1

Table 2. HPLC parameters.

Parameter	Value
Column	Agilent AdvanceBio Oligonucleotide, 2.1 × 150 mm, 2.7 µm (p/n 659750-702)
Sampler Temperature	8 °C
UV Detection	260/4 nm (Ref 400/80 nm) Peak width > 0.05 min (5 Hz)
Mobile Phase A	10% ACN, 5 mM tributylammonium acetate, 1 µM EDTA
Mobile Phase B	80% ACN, 5 mM tributylammonium acetate, 1 µM EDTA
Flow Rate	0.25 mL/min
Multi Wash	20:80 Water:methanol, flush port, 5 seconds 90:10 Water:methanol, flush port, 3 seconds
Column Temperature	50 °C
Post Time	1.0 min
Gradient Program	Time (min) %B 0 45 22 80 25 80 26 45

Results and discussion

This method demands exceptional sensitivity, precision, and a broad mass range, making the Agilent InfinityLab Pro iQ Plus an ideal solution for the task. To determine the purity of an ASO, a full scan spectrum is extracted from the main peak, which tends to be broad (2 to 3 minutes) due to the shallow gradient program. Any *m/z* values in the average spectrum that exceed an established threshold can then be used to generate an extracted ion chromatogram (EIC). The limit of quantification (LOQ) is established at 0.2%, and because many impurities are near isobaric, any spectral overlap may interfere with the data. Thus, a sensitive and selective method is required for this analytical workflow.

The Pro iQ Plus has a mass range of *m/z* 2 to 3,000. This method typically requires a full scan of *m/z* ± 150 around the 4– charge state of the full-length product (FLP). For example, an oligo with a molecular weight of 8,000 Da would require a scan range from *m/z* 1,750 to 2,150. Additionally, sufficient ion transmission at higher *m/z* values is important, as EICs are used for the relative quantitation of each impurity. Figure 1 shows the ion transmission stability for mass axis assignment and peak width at *m/z* 2,234, monitored over 24 hours. These data demonstrate the robustness and stability of large molecule transmission on the Pro iQ Plus.

Prior to selecting the ions that exceed the threshold for EIC integration, the method requires a comparison of the sample under different MS conditions. Figure 2 shows the overlay of so-called "standard" and "harsh" spectra from the ASO-1 sample. This overlay of spectra on a relative scale is used to determine if any ions exceeding the threshold are adducts. Ions that exceed the threshold under standard conditions, but not under harsh conditions, are regarded as adducts. This is because the higher temperature conditions used for harsh conditions minimize adduct formation during electrospray desorption of the ASO. The spectra obtained under both conditions demonstrate excellent selectivity and sensitivity for the ions in the 4– charge state.

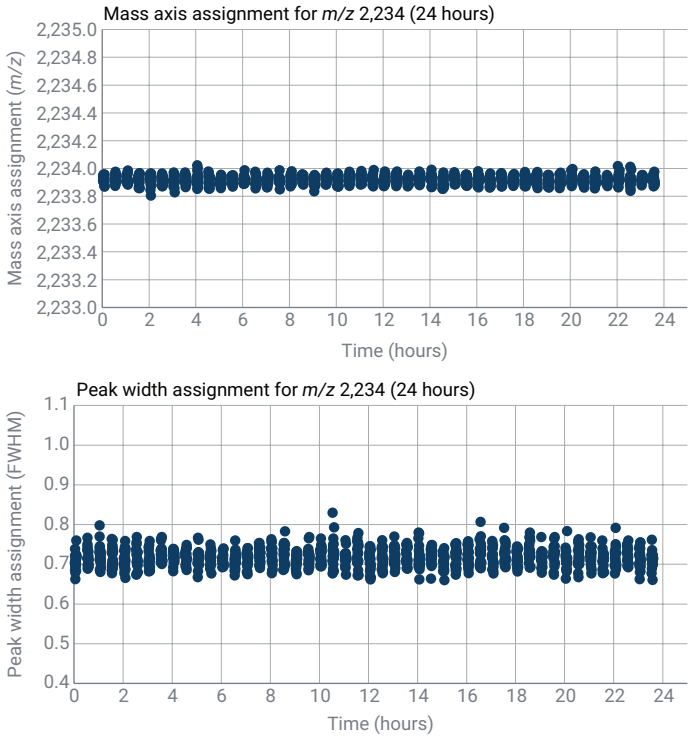

Figure 1. Mass axis assignment (*m/z*) and peak width (FWHM) of the calibrant ion (*m/z* 2,234) monitored over a continuous, 24-hour time period to demonstrate the stability of large molecule transmission on the Agilent Pro iQ Plus.

Table 3 shows the ions identified using the OAA algorithm. These ions exceed thresholds under both standard and harsh conditions, with any "known" ions being within *m/z* ± 0.5 of values in the imported ion list. Interestingly, several unknown ions with *m/z* values greater than the FLP are observed. These may be longmers (for example, n + 1) or other process-related impurities. These again are not chromatographically resolved, and thus it is critically important that they are measured by the MS detector.

The ions in Table 3 were used to generate EICs, which were then integrated into the next step. This information is required for two purposes. First, the relative peak areas are used to calculate the MS purity of the peak. Second, the earliest- and latest-eluting impurities are used to determine the UV integration of the main peak (Figure 3). Thus, both MS and UV purities can be used to determine the overall purity of the ASO sample.

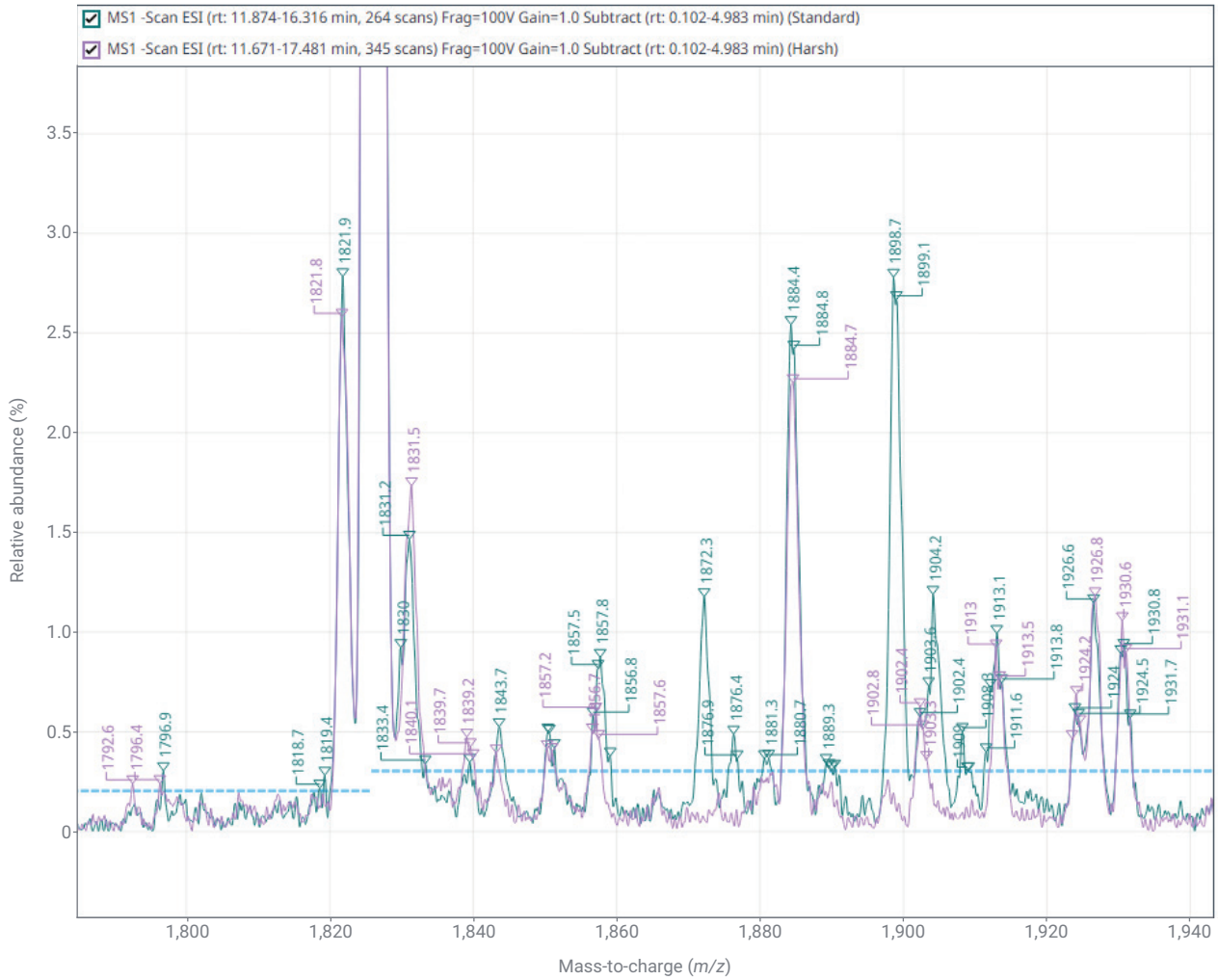

Figure 2. Overlay of ASO-1 full scan spectra under standard (teal) and harsh (violet) conditions. The Oligo Analysis Accelerator software user interface enables direct inspection of the overlaid spectra. Dashed lines indicate the thresholds: 0.2% "prepeak" (*m/z* values less than the 4– charge state of the full-length product) and 0.3% "postpeak" (*m/z* values greater than the 4– charge state of the full-length product). The software then automatically classifies ions accordingly based on whether the harsh-condition ions are still above the threshold.

Table 3. Classified ions from ASO-1 that exceed method thresholds.

Name	*m/z*
Full-Length n	1,826.3
Full-Length n (P=O)1*/Loss of Methylene	1,822.3
N – p(MOE A)	1,725.5
N – p(MOE MeU)/n – p(MOE MeC)	1,727.9
N – p(dA)	1,744
Abasic Depurination Species (Loss of Ade + H_20)	1,797
n + p(dA)	1,908.5
n + p(dG)	1,912.5
Dithioate/Thioate	1,830.2

Name	*m/z*
N3-(2-cyanoethyl)thymine (CNET)	1,839.5
Unknown	1,884.4
Unknown	2,087.1
Unknown	1,857.8
Unknown	1,651.1
Unknown	1,902.4
Unknown	1,774.1
Unknown	1,843.7
Unknown	1,850.7

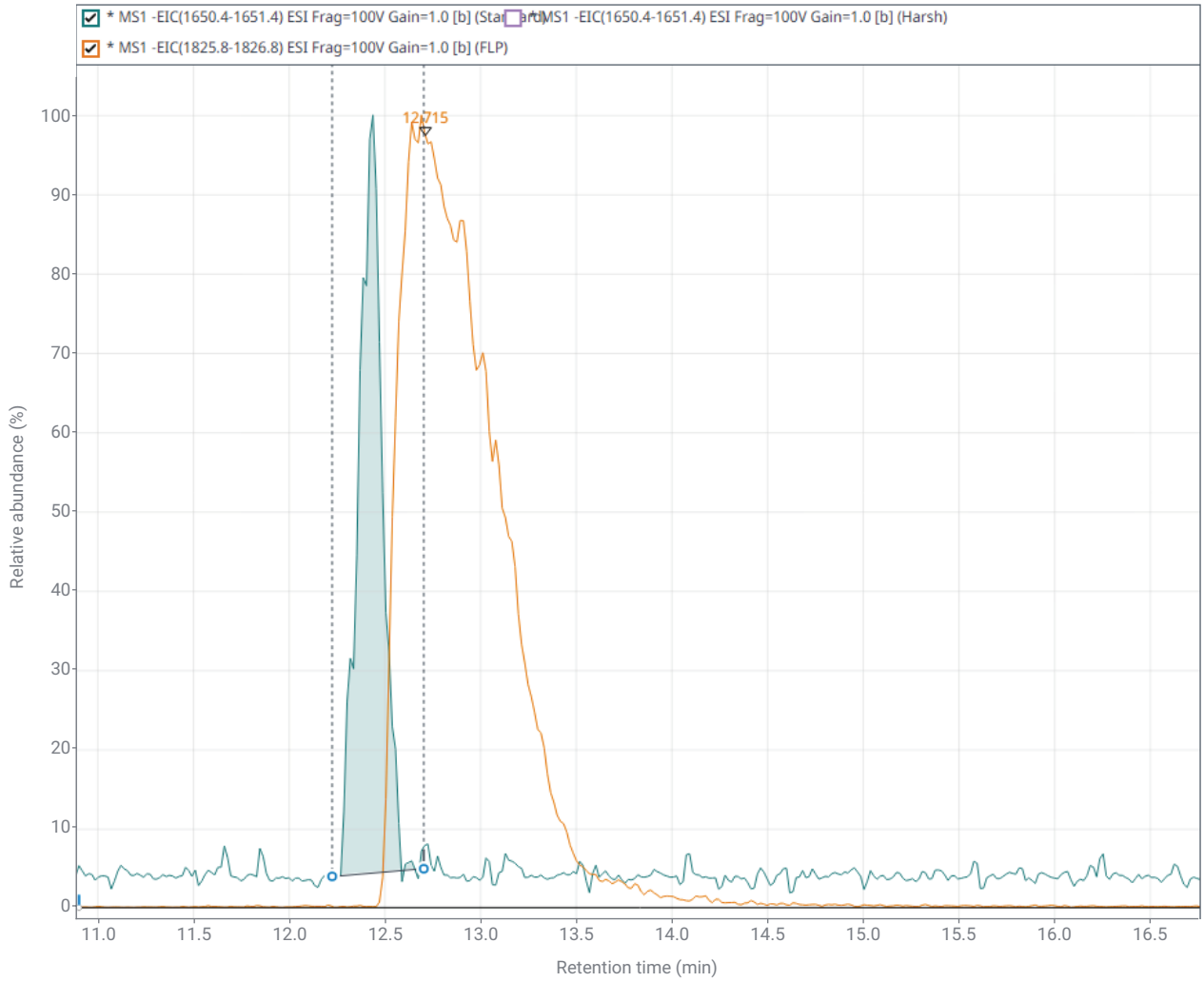

Figure 3. Extracted ion chromatogram integration within the Oligo Analysis Accelerator software, which allows for direct integration within the application. The *m/z* 1,650 ion (unknown) is used to determine the integration of the UV peak. The start of integration for the earliest-eluting impurity is applied as the start of integration for the UV peak, with UV and MS retention time offsets also considered in this automated calculation.

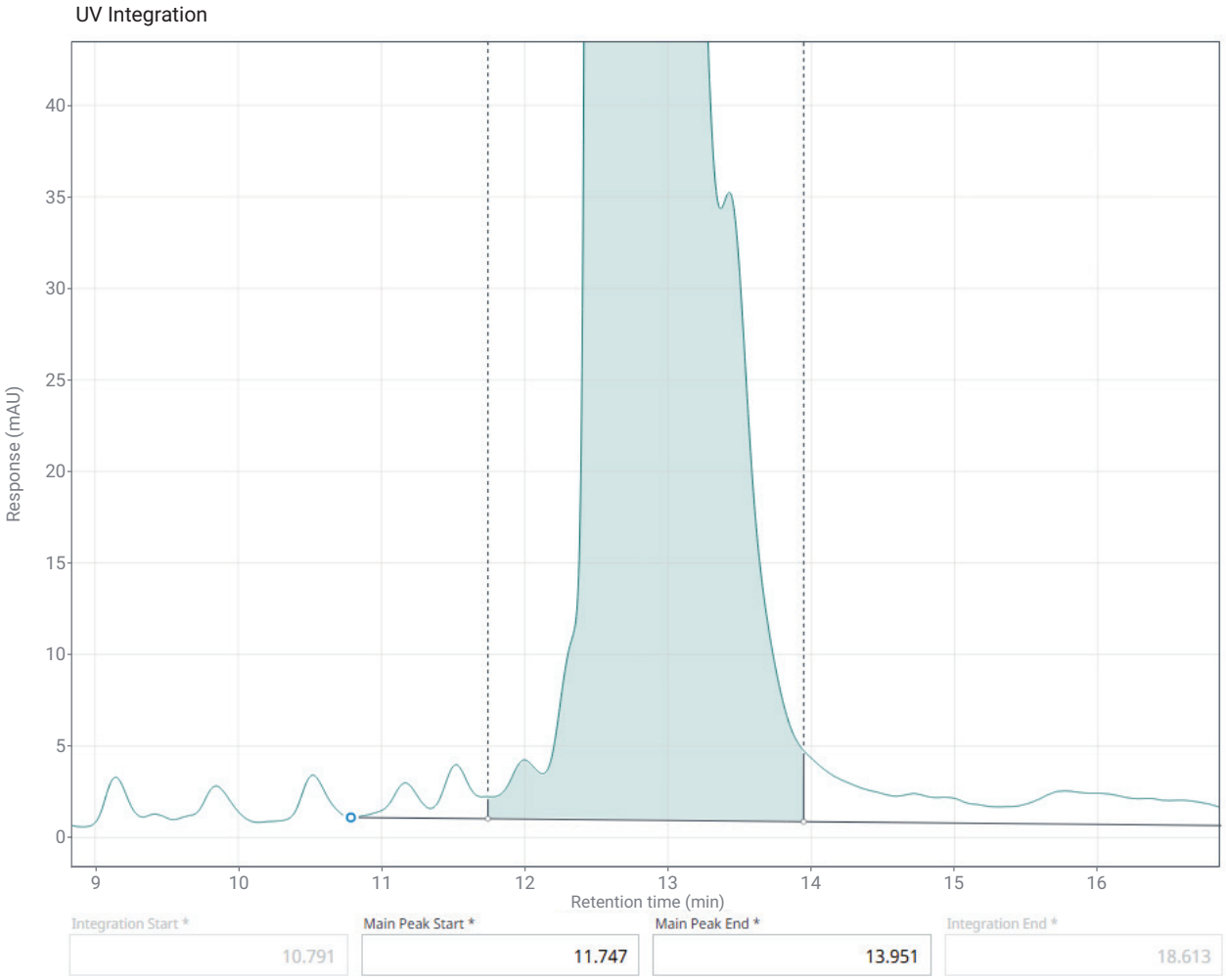

Figure 4. UV integration of the main peak, based on EIC integration for early- and late-eluting impurities.

The relative peak areas for each EIC are shown in Table 4. The resulting MS purity of 86.9% contrasts with the calculated UV purity of 98.0% (Table 5). Although this method was developed to allow for the coelution of closely related impurities, chromatographic method development may help ensure more accurate quantitation. This is especially important considering that many of the unknown impurities are likely n – 1 or alkylated impurities, which elute later than the FLP. In either case, a sensitive and selective MS detector is crucial for determining the purity of an oligonucleotide.

Table 4. Relative peak area results for all ions.

Name	*m/z*	Category	EIC Peak Area (%)
Full-Length n	1,826.3	Full-length n	85.126
Full-Length n (P=O)1*/Loss of Methylene	1,822.3	Full-length (P=O)	2.184
n – p(MOE A)	1,725.5	n – 1	0.197
n – p(MOE MeU)/n – p(MOE MeC)	1,727.9	n – 1	0.489
Abasic Depurination Species (Loss of Ade + H_2O)	1,797	Abasic	0.271
n + p(dA)	1,908.5	n + 1	0.271
n + p(dG)	1,912.5	n + 1	0.578
n + p(MOE MeU)/n + p(MOE MeC)	1,924.6	n + 1	0.338
n + p(MOE A)	1,927	n + 1	0.663
n + p(MOE + G)	1,931	n + 1	0.624
Dithioate/Thioate	1,830.2	Others	0.688
N3-(2-cyanoethyl)thymine (CNET)	1,839.5	Others	0.21
Unknown	1,884.4	–	1.831
Unknown	2,087.1	–	0.59
Unknown	1,857.8	–	0.545
Unknown	1,913.8	–	0.614
Unknown	1,651.1	–	0.334
Unknown	1,902.4	–	0.47
Unknown	1,774.1	–	0.453
Unknown	1,843.7	–	0.356
Unknown	1,850.7	–	0.25
Unknown	1,749.7	–	0.256
Unknown	1,833.4	–	0.216
Unknown	1,478.4	–	0.242
Unknown	1,457	–	0.162
Unknown	1,507.4	–	0.113

Table 5. UV and MS purity results.

Criteria	Result	Expected Result
UV Purity	98.0%	98.0%
MS Purity	86.9%	–
Most Abundant Mass (Da)	7,307.6	7,307.4

Conclusion

This method, as cited in previous literature[1], has become the gold standard for LC/MS-based purity, assay, and impurity profiling of therapeutic, single-stranded oligonucleotides. The method requires a selective and sensitive detector capable of acquiring full scan spectra from the main peak to ensure accurate quantitation of impurities. Additionally, depending on sample, the method may require scans above *m/z* 2,000, which is a limiting factor for many unit mass detectors. Due to its excellent ion transmission at high mass ranges, the Agilent InfinityLab Pro iQ Plus can meet these method requirements for both sensitivity and selectivity, thereby allowing QC analysis of oligonucleotide impurities.

Reference

1. Rentel, C.; Gaus, H.; Bradley, K.; Luu, N.; Kolkey, K.; Mai, B.; Madsen, M.; Pearce, M.; Bock, B.; Capaldi, D. Assay, Purity, and Impurity Profile of Phosphorothioate Oligonucleotide Therapeutics by Ion Pair–HPLC–MS. *Nucleic Acid Ther.* **2022**, *32(3)*, 206–220. DOI: 10.1089/nat.2021.0056

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