Pellicular Anion-Exchange Oligonucleotide Chromatography Coupled with High-Resolution Accurate Mass (HRAM) Mass Spectrometry

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Application Note 1021

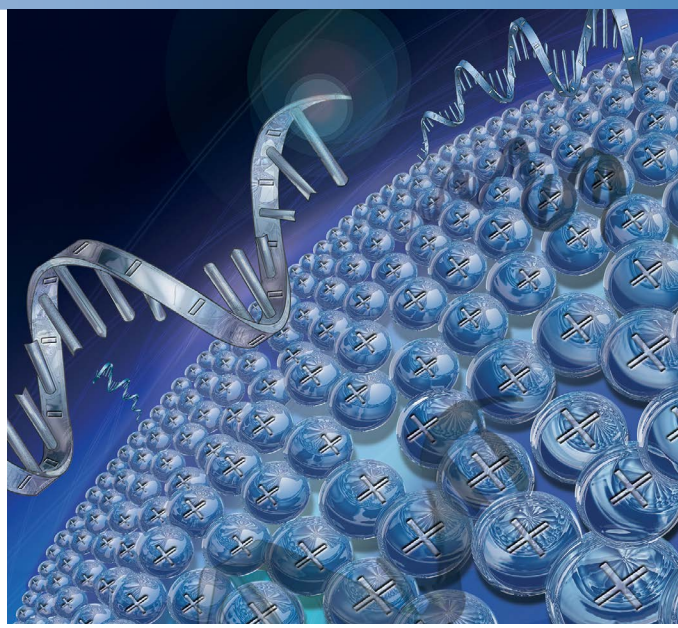
Key Words
Liquid Chromatography/Mass Spectrometry (LC/MS), Q Exactive Mass Spectrometer, Orbitrap Technology, Linkage Isomers, DNAPac Column, Acclaim PolarAdvantage II Column

Goal
To couple high-resolution pellicular anion-exchange liquid chromatography (pAXLC) of oligonucleotides with HRAM mass spectrometry (MS) and automated desalting to identify impurities and isomeric variants in synthetic oligonucleotides

Introduction
Recent advances in nucleic acid chemistry reveal possible therapeutic models targeting previously untreatable conditions. These models employ relatively short nucleic acid sequences decorated with protecting groups to preclude rapid metabolic elimination. The presence of these protecting groups—or modifications introduced during synthesis, processing, or administration—can produce isomeric variants. These variants must be identified, quantified, and filed in accordance with regulatory requirements. Reversed-phase liquid chromatography (RPLC)/MS is a generally accepted method for identification; however, some variants are linkage- or diastereoisomers that are not readily resolved by RPLC/MS.

One example of an isomeric variant is the 2',5'-linkage isomer. In ribonucleic acid interference (RNAi) duplexes, unprotected normal (3',5'-) linkages undergo phosphoryl migration during annealing of the guide and passenger RNAi strands to generate 2',5'-linkages.[1] High-performance pAXLC resolves these RNA linkage isomers.[2,3]

Another variant, the phosphorothioate linkage, introduces diastereoisomers that also can be resolved by pAXLC[4]—just as many other backbone and base modifications can be resolved by pAXLC[5]—but not by RPLC/MS. Two-dimensional chromatographic approaches for automated oligonucleotide separation and desalting prior to MS help identify and quantify these isomers.[2-5] This study demonstrates the coupling of pAXLC to HRAM mass spectrometry to complete the analysis process.



Equipment and Software
- Thermo Scientific™ Dionex™ UltiMate™ 3000 Biocompatible LC system,* including:
  - LGP-3400BM Quaternary Rapid Separation Pump
  - WPS-3000TBFC Thermostatted Biocompatible Pulled-Loop Well Plate Autosampler with Integrated Fraction Collection (P/N 5825.0020)
  - TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
  - UVD 340U UV-vis Detector
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software version 6.8
- Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap™ mass spectrometer controlled and interrogated using Thermo Scientific™ Xcalibur™ software version 2.0

* Equivalent results can also be obtained using the UltiMate 3000 BioRS system.

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Reagents and Standards

- Sodium Chloride (Sigma-Aldrich® P/N S7653)
- Trizma® Base (Sigma-Aldrich P/N T1503)
- 2-Amino-2-Methyl-1-Propanol (Sigma-Aldrich P/N 08580)
- *N,N*-Diisopropylethylamine (DIPEA, Sigma-Aldrich P/N D125806)
- Ethylenediaminetetraacetic Acid (EDTA) (Sigma-Aldrich)
- Diisopropylamine (Sigma-Aldrich P/N 471224)
- Methanesulfonic Acid (Fluka/Sigma-Aldrich P/N 64280)
- Sodium Hydroxide Solution (Fisher Scientific P/N SS254)
- 1,1,1,3,3,3-Hexafluoro-2-Propanol (HFIP, TCI America H0424l)
- Methanol (Honeywell P/N 230-4)
- Deionized (DI) water, Type I reagent grade, prepared using a water purification system
- Tris buffer as concentrate prepared by titration of Trizma base with HCl to pH 8. Buffer (40 mM) prepared by dilution of the concentrate.

Oligonucleotides

- Dio-3
- Dio-7

The sequence 5'-AUG AAC UUC AGG GUC AGC UUG -3' was the same for both Dio-3 and Dio-7. Dio-3 harbors 2',5'-linkages at positions 1 and 2, whereas Dio-7 harbors these linkages at positions 10 and 12. These products were obtained from Integrated DNA Technologies, Inc.

Chromatographic Conditions

First Dimension

Column:	Thermo Scientific™ DNAPac™ PA200, 8 µm, Analytical, 4 × 250 mm (P/N 063000)				
Eluents:	A) 40 mM Tris, pH 8.0 B) 1.25 M NaCl in A C) 5 mM Ammonium Formate, pH 6, 1.5% CH_3OH D) 40% CH_3OH in C				
Gradient:	Time (min)	% A	% B	% C	% D
	0.0	74	26	0	0
	13.3	58	42	0	0
	13.8	20	80	0	0
	14.8	20	80	0	0
	15.3	74	26	0	0
	18.0	74	26	0	0
Flow Rate:	850 µL/min				
Temp:	30 °C				
Detection:	Absorbance at 260 nm				

Note: Fraction collection was programmed between 3.0 and 15.3 min.

Collect target oligonucleotide peaks in the autosampler during anion-exchange chromatography as described above, then desalt in a second dimension step by switching the column selection valve in the TCC-3000RS.

Second Dimension

Column:	Thermo Scientific™ Acclaim™ PolarAdvantage II (PA2), 3 µm, Analytical, 4.6 × 50 mm (P/N 063189)				
Gradient:	Time (min)	% A	% B	% C	% D
	0.0	0.0	0.0	100	0.0
	3.0	0.0	0.0	100	0.0
	3.2	0.0	0.0	0.0	100
	4.2	0.0	0.0	0.0	100
	4.9	0.0	0.0	100	0.0
	8.0	0.0	0.0	100	0.0
Flow Rate:	500 µL/min				
Temp:	30 °C				
Detection:	Absorbance at 260 nm				

Note: Fraction collection was programmed between 4.9 and 5.4 min.

Sample Preparation

Dissolve oligonucleotides in DI water to 1.5 mg/mL and dilute as needed for purification and analysis.

Liquid Chromatography

Separate the 20 µL oligonucleotide samples by pAXLC as described above, collecting major peaks into 96 deep (2 mL) wellplates. When oligonucleotide pAXLC separation and collection is finished, switch the column selection valve from the DNAPac PA200 column position to the Acclaim PA2 column position. Then inject 166 µL aliquots of the collected fractions onto the Acclaim PA2 desalting column, as described above. Recollect the desalted fractions eluting at 4.9–5.4 min into a standard (400 µL) 96 wellplate for later analysis by MS.

Mass Spectrometry

Prepare a Q Exactive mass spectrometer for direct infusion of the desalted oligonucleotides (flow rate at 50 µL/min). Prior to MS analysis, dilute the purified desalted oligonucleotides in the carrier solution of Hail et al. (i.e., 0.75% HFIP, 0.375% DIPEA, 40% methanol, and 10 µM EDTA in DI water).[6] Set the spray voltage to (-) 2.5 kV, the sheath gas flow to 10, the auxiliary gas flow to 2, and the capillary temperature to 300 °C. Set the S-lens level to 50, the heater temperature to 0, the resolving power (Resolution) to 70,000, and the AGC target to 1E6 for full-scan mode. Set the maximum IT to 80 ms and full-scan mass range from 600 to 1150 m/z.

Data Analysis

Process the chromatographic data using Chromeleon CDS software to determine the purified oligonucleotide concentrations. Process the desalting data using the purified oligonucleotide concentration values to determine the desalted oligonucleotide fraction concentrations in the standard well plates. Control the Q Exactive mass spectrometer with the Xcalibur software and process the resulting mass spectra. Obtain the theoretical isotope distributions by entering the elemental composition derived from the oligonucleotide sequence (C200 H247 N79 O146 P20) in the Xcalibur software Isotope Simulation utility. Compare the observed and calculated values using Microsoft® Excel® software.

Results and Discussion

In this study, the desalting system was coupled with a Q Exactive mass spectrometer operating at a resolving power of 70,000. Two identical sequence oligonucleotides—each harboring a pair of 2',5'-linkages at different positions in the sequence—were separated and collected using pAXLC, then desalted using a 4 × 50 mm polar-embedded reversed-phase column prior to analysis on the Q Exactive mass spectrometer. Charge states from -7 to -11 were identified, and the mass accuracy of each isomer at each charge state was examined. The measured monoisotopic intact mass of each of the desalted oligonucleotide samples was 6709.9084 Da. The differences between the observed and calculated mass values were within 2.9–4.7 ppm for the seven measured isotopic variants of these oligonucleotide isomers.

Oligonucleotide Separation and Purification by pAXLC

Table 1 provides the oligonucleotide sequence for two isomers of the antisense strand of an enhanced green fluorescent protein (eGFP) small interfering RNA (siRNA) that were modified by introduction of aberrant 2',5'-linkages at specific but different positions in the sequence. The resulting identical-sequence RNA isomers (Dio-3 and Dio-7) were employed for this study.

Table 1. Oligonucleotides analyzed.

eGFP siRNA Sequences	
Antisense	5'-AUG AAC UUC AGG GUC AGC UUG-3'
Sense	3'-UCUAC UUG AAG UCC CAG UCG A-3'
Underlined residues have 2'-5' linkages at * positions.	
Dio3	5'-A*U*G AAC UUC AGG GUC AGC UUG -3'
Dio7	5'-AUG AAC UUC A*GG* GUC AGC UUG -3'

Note: These identical-sequence RNA samples were purified by pAXLC using the system depicted in Figure 1.

Purify the identical-sequence isobaric RNA samples using pAXLC, then desalt the samples on a polar-embedded reversed-phase column using the system depicted in Figure 1. With the Column Selection (CS) valve in the 1_10 position, and using eluents A and B, collect the DNAPac PA200 column-resolved components into the autosampler collection vials. After purification, switch the CS valve to the 1-2 position (switch flow from the DNAPac column to the Acclaim PA2 column), and use eluents C and D for the desalting step.

As shown in Table 1, these oligonucleotides differ only in the positions at which they harbor two 2',5'-linkages. These two isobaric oligonucleotide isomers were separated using identical conditions on the same day with the same eluents. Dio-3 eluted 26 s after Dio-7 and the two isomers were baseline resolved (Figure 2).

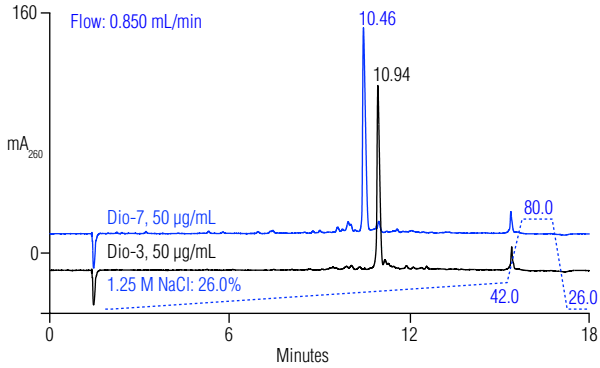

Figure 2. Resolution of Dio-3 and Dio-7 by pAXLC.

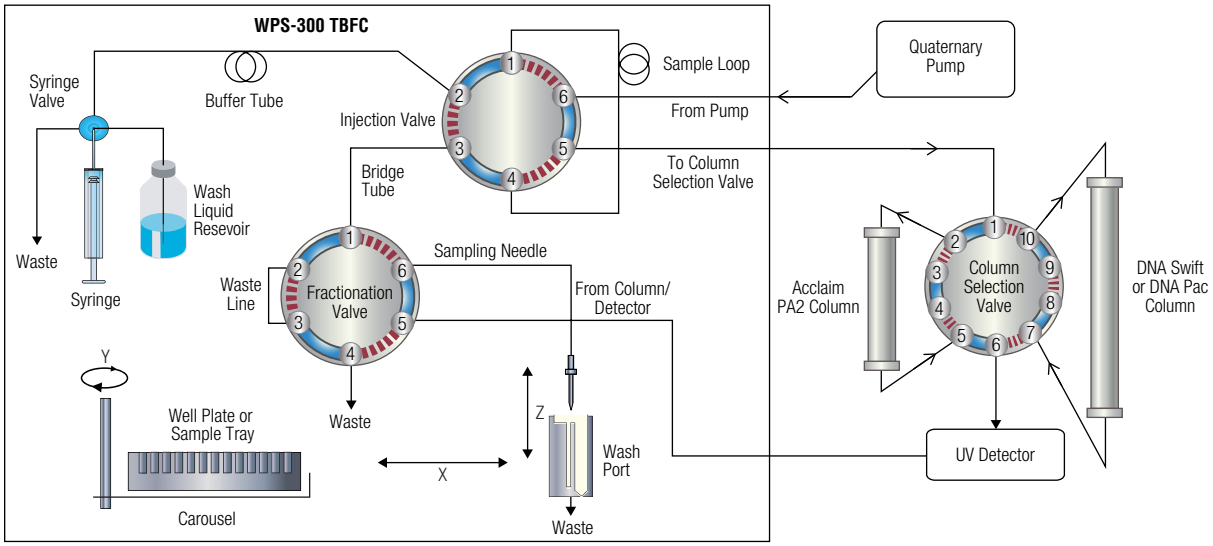

Figure 1. Automated oligonucleotide purification and desalting using a 10-port column selection valve.

In Figure 3, the left panel depicts the purification of the Dio-3 isomer by pAXLC on the DNAPac PA200 column and indicates the fractions collected (Fraction 28). That fraction was subsequently desalted using the Acclaim PA2 column as shown in the right panel of Figure 3. The baseline upset at the column void (0.8–1.8 min) indicates elution of the bolus of salt cocollected with the oligonucleotide during pAXLC. The oligonucleotide was eluted through a short elution step at 40% CH_3OH in which the desalted isomer was collected between 4.9 and 5.4 min. Column equilibration followed when eluent D returned to 0% (100% eluent C), thus preparing the system to desalt the next oligonucleotide.

After collection and desalting, the oligonucleotides were diluted into the MS carrier solution of Hail et al.[6] and infused into the Q Exactive mass spectrometer. In Figure 4, the top panel shows a representative spectrum of Dio-7 and the bottom panel presents a spectrum of Dio-3. These panels show the presence of charge states -7 through -9, as labeled in the Dio-3 spectrum.

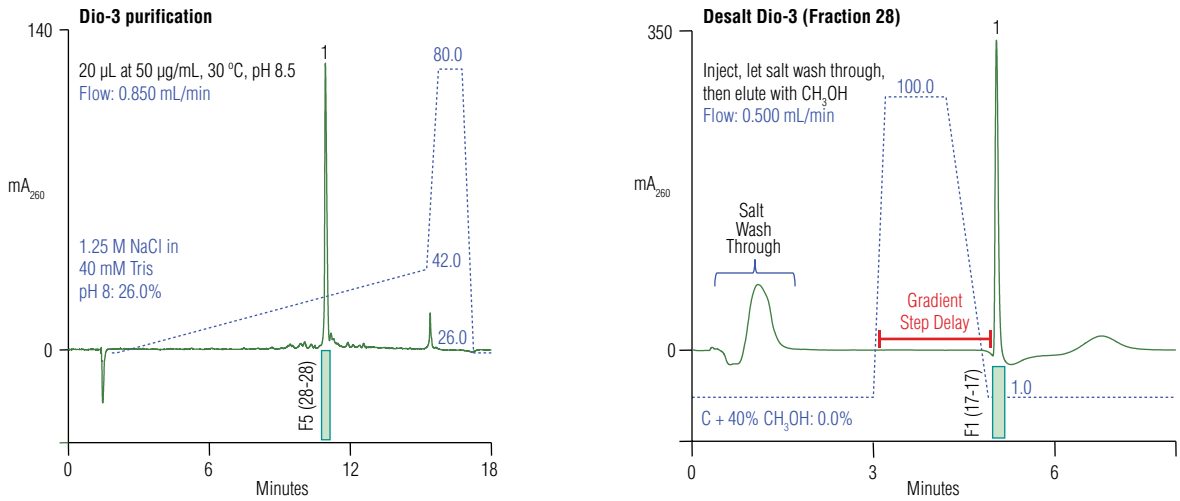

Figure 3. Purification and desalting of Dio-3 for electrospray ionization (ESI)-MS.

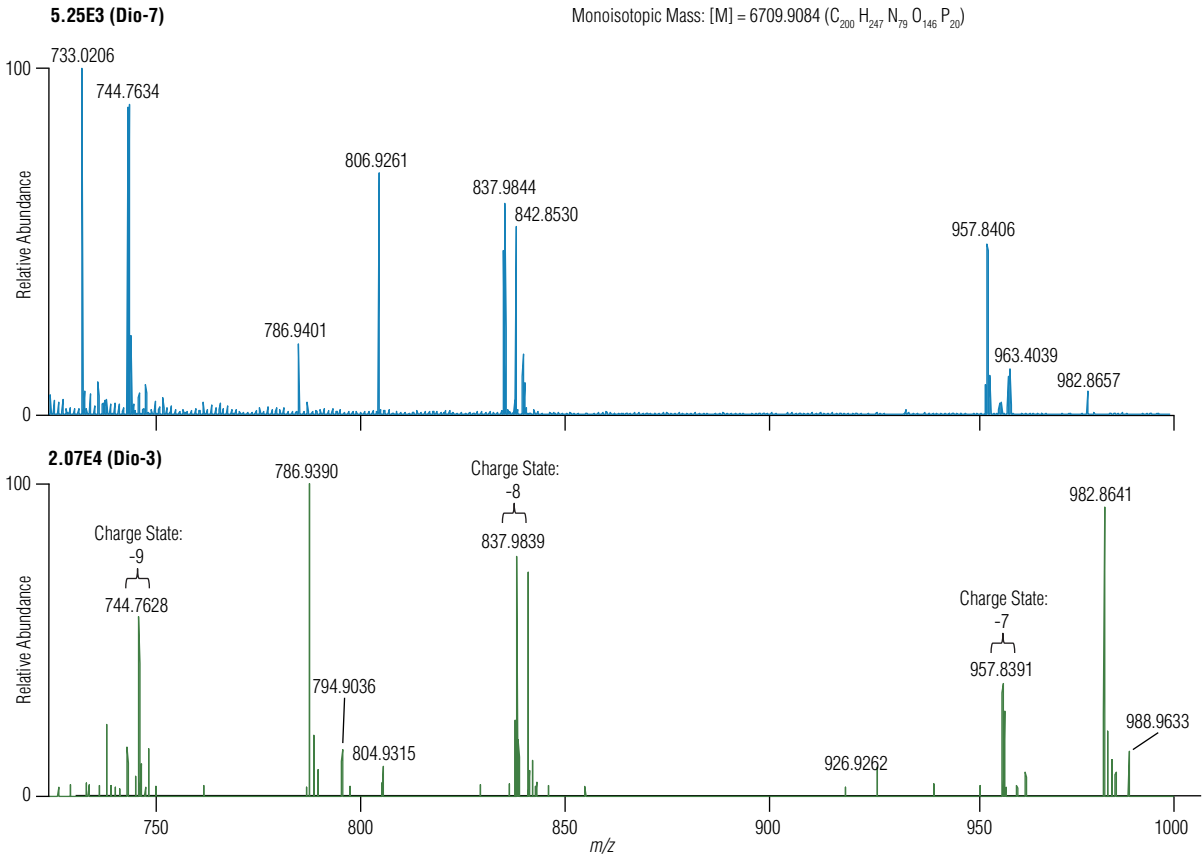

Figure 4. High-resolution ESI mass spectra of purified, desalted oligonucleotides.

Figure 5 displays the zoom-in mass spectra of both Dio-7 and Dio-3 at charge -7. The isotopic mass and distribution for both oligonucleotides are essentially identical.

Figure 6 shows the zoom-in mass spectra of both Dio-7 and Dio-3 at charge -8. The envelopes for both oligonucleotide isomers are also essentially identical.

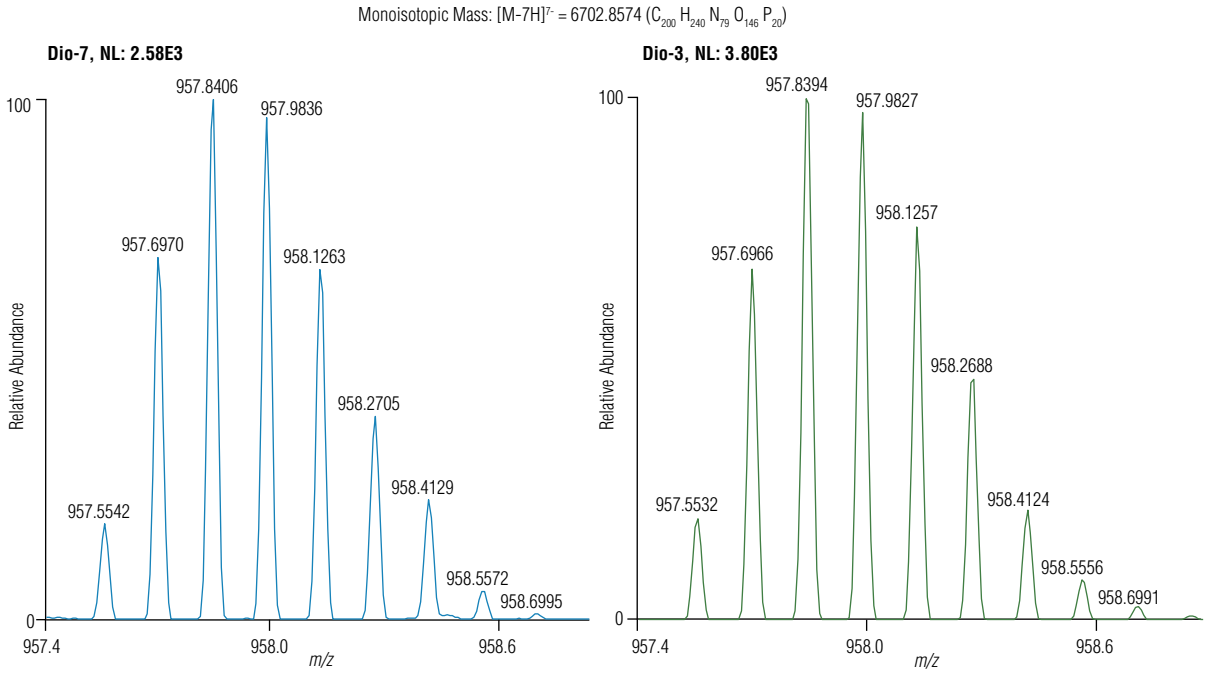

Figure 5. Q Exactive mass spectrometer nucleic acid MS data: mass envelope for charge state 7.

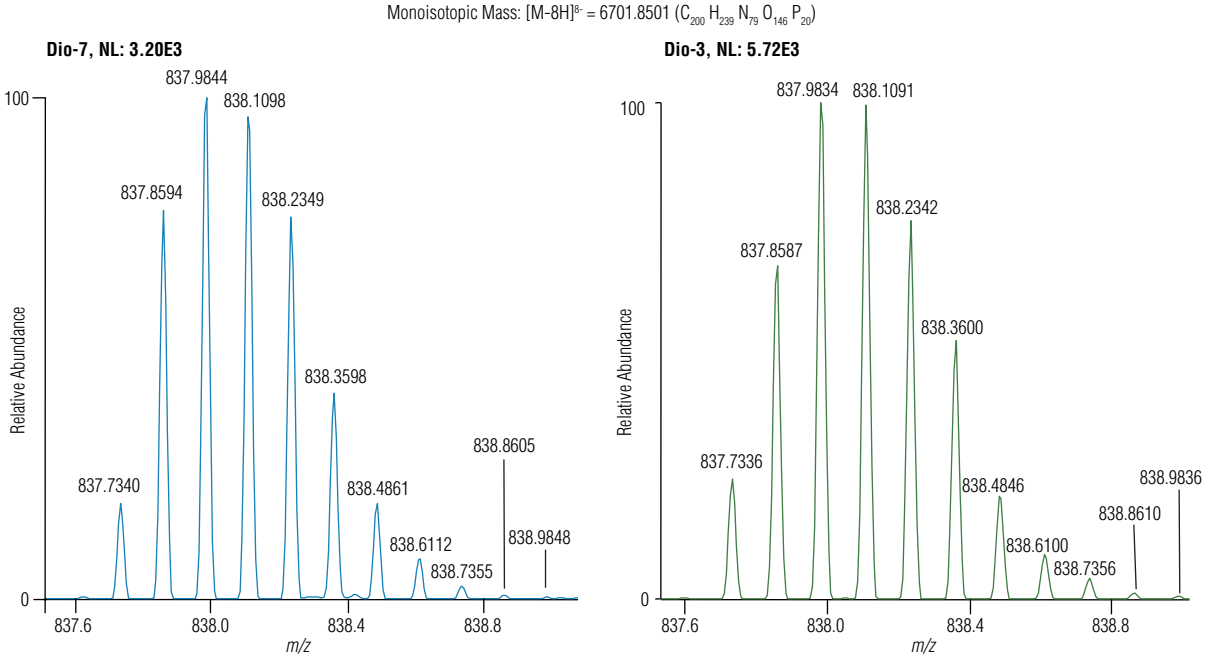

Figure 6. Q Exactive mass spectrometer nucleic acid MS data: mass envelope for charge state 8.

Figure 7 shows the zoom-in mass spectra of Dio-7 and Dio-3 at charge -9. As in Figures 5 and 6, the isotopic distribution and mass matched well for Dio-7 and Dio-3.

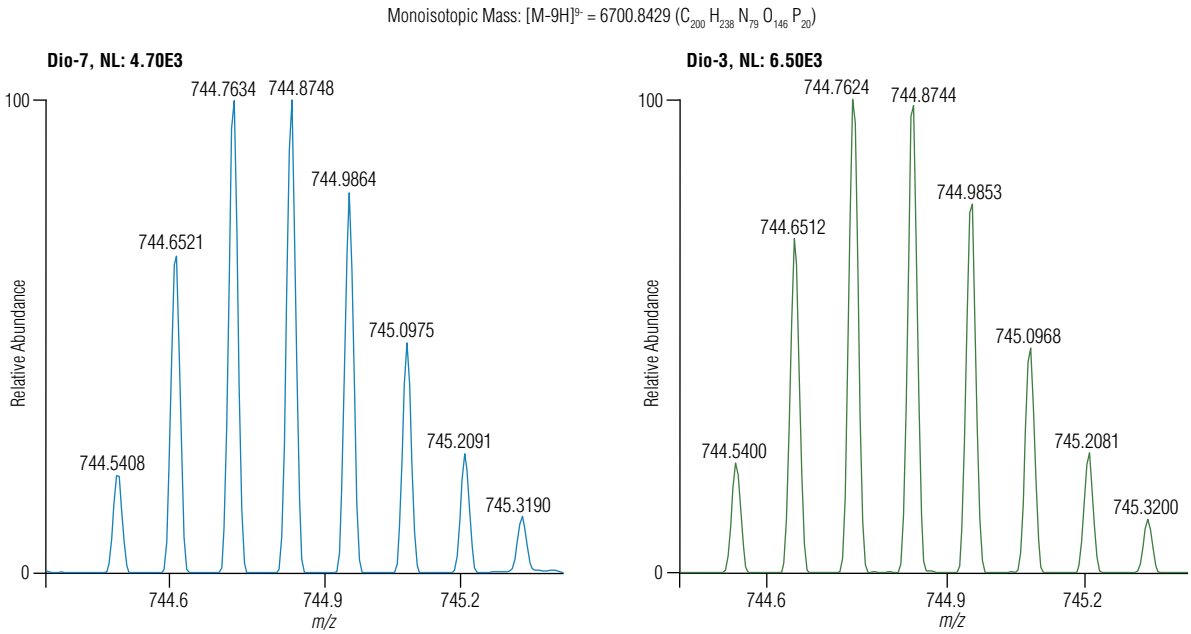

Figure 7. Q Exactive mass spectrometer nucleic acid MS data: mass envelope for charge state 9.

Table 2 details the calculated vs observed mass values for the -7 to -11 charge state isotope envelopes of Dio-3. This table also shows the calculated mean and standard deviation of the differences (in ppm) for the isotopes at each charge state. The average differences range from 2.94 to 4.07 ppm for external mass calibration. These data were generated with external calibration; no lock mass was needed to show good mass accuracy without the frequent external calibration required by other instruments.

Table 2. Analysis of mass error for Dio-3.

Dio-3 Charge State	5'-A*U*G AAC UUC AGG GUC AGC UUG -3'							Mass Accuracy (ppm)				
		Isotope #	-7	-8	-9	-10	-11	-7	-8	-9	-10	-11
Mass Values	Calculated	1	957.5500	837.7302	744.5370	669.9825	608.9833	3.342	4.059	4.029	4.179	3.613
	Observed		957.5532	837.7336	744.5400	669.9853	608.9855					
	Calculated	2	957.6933	837.8556	744.6485	670.0828	609.0745	3.446	3.700	3.626	4.179	3.612
	Observed		957.6966	837.8587	744.6512	670.0856	609.0767					
	Calculated	3	957.8366	837.9810	744.7600	670.1831	609.1657	2.923	2.864	3.223	4.029	4.104
	Observed		957.8394	837.9834	744.7624	670.1858	609.1682					
	Calculated	4	957.9800	838.1064	744.8715	670.2835	609.2569	2.818	3.222	3.893	4.028	3.611
	Observed		957.9827	838.1091	744.8744	670.2862	609.2591					
	Calculated	5	958.1233	838.2319	744.9829	670.3838	609.3482	2.505	2.744	3.222	3.878	2.298
	Observed		958.1257	838.2342	744.9853	670.3864	609.3496					
	Calculated	6	958.2663	838.3570	745.0941	670.4839	609.4391	2.609	3.578	3.624	4.027	3.610
	Observed		958.2688	838.3600	745.0968	670.4866	609.4413					
	Calculated	7	958.4096	838.4824	745.2056	670.5842	609.5304	2.921	2.624	3.355	4.175	2.953
	Observed		958.4124	838.4846	745.2081	670.5870	609.5322					
							Mean	**2.94**	**3.26**	**3.57**	**4.07**	**3.40**
							Std Dev	**0.35**	**0.54**	**0.32**	**0.11**	**0.59**

*Note: Underlined residues have 2'-5' linkages at * positions.*

Table 3 details the calculated vs observed mass values for the -7 to -11 charge state isotope envelopes of Dio-7. This table also shows the calculated mean and standard deviation of the differences (in ppm) for each charge state. The differences range from 3.00 to 4.70 ppm with external mass calibration.

Table 3. Analysis of mass error for Dio-7.

Dio-7 Charge State	5'-AUG AAC UUC A*GG* GUC AGC UUG -3'							Mass Accuracy (ppm)				
		Isotope #	-7	-8	-9	-10	-11	-7	-8	-9	-10	-11
Mass Values	Calculated	1	957.5500	837.7302	744.5370	669.9825	608.9833	4.386	4.536	5.104	2.239	3.120
	Observed		957.5542	837.7340	744.5408	669.9840	608.9852					
	Calculated	2	957.6933	837.8556	744.6485	670.0828	609.0745	3.863	4.535	4.834	2.239	1.970
	Observed		957.6970	837.8594	744.6521	670.0843	609.0757					
	Calculated	3	957.8366	837.9810	744.7600	670.1831	609.1657	4.176	4.057	4.565	4.775	3.776
	Observed		957.8406	837.9844	744.7634	670.1863	609.1680					
	Calculated	4	957.9800	838.1064	744.8715	670.2835	609.2569	3.758	4.057	4.430	2.536	0.821
	Observed		957.9836	838.1098	744.8748	670.2852	609.2574					
	Calculated	5	958.1233	838.2319	744.9829	670.3838	609.3482	3.131	3.579	4.698	3.431	2.626
	Observed		958.1263	838.2349	744.9864	670.3861	609.3498					
	Calculated	6	958.2663	838.3570	745.0941	670.4839	609.4391	4.383	3.340	4.563	4.624	4.102
	Observed		958.2705	838.3598	745.0975	670.4870	609.4416					
	Calculated	7	958.4096	838.4824	745.2056	670.5842	609.5304	3.443	4.413	4.697	4.325	4.594
	Observed		958.4129	838.4861	745.2091	670.5871	609.5332					
							Mean	3.88	4.07	4.70	3.45	3.00
							Std Dev	0.48	0.47	0.22	1.13	1.31

*Note: Underlined residues have 2'-5' linkages at * positions.*

Conclusion

- pAXLC resolves important therapeutic nucleic acid variants, including 2', 5'-linkage isomers, as shown in Figure 2.
- Automated coupling of oligonucleotide purification on the DNAPac PA200 column and desalting on the Acclaim PA2 column was demonstrated in Figure 3.
- Purification of oligonucleotides by pAXLC, followed by automated desalting, can be directly coupled to HRAM ESI-MS—or passively collected for later delivery to the mass spectrometer—using the approach depicted in Figure 1 with the results in Figure 3.
- The Q Exactive mass spectrometer provides highly accurate mass values for each oligonucleotide isotope at each of five charge states after purification by pAXLC, as demonstrated in Figures 5–7 and Tables 2–3.

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