Characterization of a synthetic double stranded siRNA using high-resolution mass spectrometry

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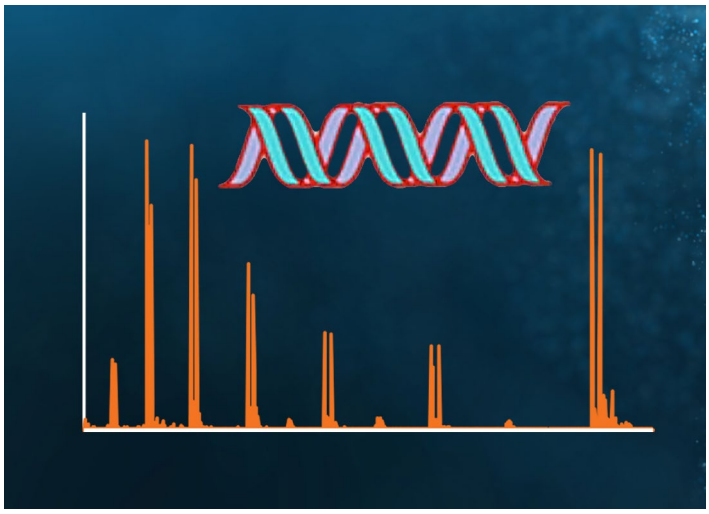

Key benefits

- Separation and detection of a double-stranded siRNA and its diastereomers
- Confident identification and mapping of the sense and antisense oligonucleotides
- High resolution accurate mass (HRAM)-based data-dependent tandem mass spectrometry (ddMS^2) for characterization of oligonucleotides

Goal

Demonstrate the capability of separating and characterizing a double-stranded siRNA and its diastereomers using a Thermo Scientific™ Vanquish™ Horizon UHPLC system coupled with a Thermo Scientific™ DNAPac™ RP column, a Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer, and Thermo Scientific™ BioPharma Finder™ software

Introduction

Therapeutic oligonucleotides, such as antisense oligonucleotides (ASOs) and small interference RNA (siRNA), have attracted increasing interest from the industry due to the successes of applying these new modalities for the treatment of rare diseases and their potential for some common diseases.[1]

The modern analytical tools are indispensable for the characterization of oligonucleotides with increasing

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complexity in structure and impurity content. Mass spectrometry coupled to ion-pairing reversed-phase liquid chromatography has proven to be a powerful tool for oligonucleotide analysis.[2,3] We have recently described an HRAM-based ddMS^2 method for confident identification, mapping, and quantitation of oligonucleotides and their impurities.[4,5] In addition, the capabilities of ddMS^2 for differentiation of isomeric sequences and structural characterization of unexpected impurities were highlighted.[4,5]

In this application note, we extend the application of the ddMS^2 method to characterization of a synthetic double-stranded siRNA consisting of complementary sense and antisense oligonucleotides. The separation, detection, and identification of siRNA diastereomers will be highlighted.

Experimental

Equipment

- Thermo Scientific Orbitrap Exploris 120 Mass Spectrometer (P/N BRE725531)
- Thermo Scientific Vanquish Horizon UHPLC System consisting of
  - Thermo Scientific™ System Base Vanquish™ Horizon/Flex (P/N VF-S01-A-02)
  - Thermo Scientific™ Vanquish™ Binary Pump H (P/N VH-P10-A-02)
  - Thermo Scientific™ Vanquish™ Split Sampler HT (P/N VH-A10-A-02),
  - Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A-02)
  - MS Connection Kit Vanquish (P/N 6720.0405)

Software

- Thermo Scientific™ BioPharma Finder™ 4.1 software (P/N OPTON-30988)

Columns

- Thermo Scientific™ DNAPac™ RP column (4 µm, 2.1 × 50 mm, P/N 088924)

Solvents

- Thermo Scientific™ UHPLC-MS Water (P/N W81)
- Fisher Chemical™ Optima™ LC/MS Grade Methanol (P/N A456-1)
- 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), >99.0%, TCI Chemicals (P/N H0424)
- N,N-Diisopropylethylamine (DIPEA), >99.0%, TCI Chemicals (P/N D1599)
- Triethylamine (TEA), >99.0%, TCI Chemicals (P/N T0424)

Oligonucleotide samples

A synthetic double-stranded siRNA consisting of a sense and an antisense oligonucleotide 20mer was purchased from Integrated DNA Technologies (IDT) (Coralville, IA, USA). Below is the sequence of this siRNA.

- AGC UGA CCC UGA AG*U UCA UC (sense)
- UCG ACU GGG ACU UCA AGU AG (antisense)

The sense oligonucleotide contains a phosophorothioate group at G^{14} (annotated as G* in the sequence), creating a chiral center that results in the presence of diastereomers. A stock solution of 1 mg/mL was prepared for siRNA. and subsequently diluted to 100 µg/mL prior to LC-MS analysis.

Ion-pairing reversed-phase liquid chromatography

Two solvent compositions consisting of HFIP and DIPEA or TEA were prepared for separation of siRNA. Figure 1 displays the LC gradients used with these two solvents.

- DIPEA solvents:

  A: 2% (~190 mM) HFIP and 0.1% (~5.7 mM) DIPEA in water (pH 7.8)

  B: 0.075% (~7.1 mM) HFIP and 0.0375% (~2.1 mM) DIPEA in methanol

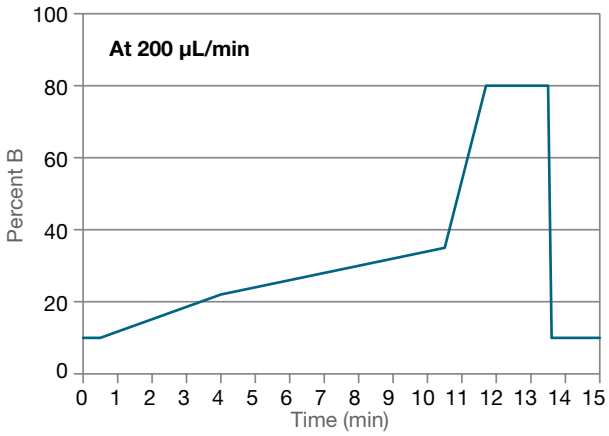
- TEA solvents:

  A: 0.5% TEA (~35 mM), 1.25% HFIP (~120 mM) HFIP in water (pH 8.9)

  B: 0.5% TEA (~35 mM), 1.25% HFIP (~120 mM) HFIP in 75:25 water:methanol

(a)

Time (min)	%B
0	10
0.5	10
4	22
10.5	35
11.7	80
13.5	80
13.6	10
15	10



(b)

Time (min)	%B
0	20
10	70
10.1	90
12	90
12.1	20
15	10

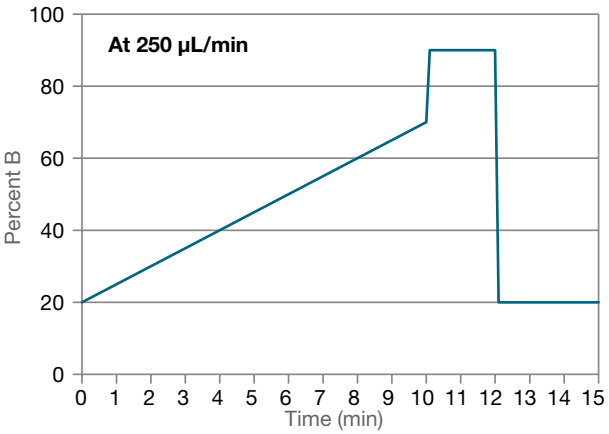


Figure 1. LC gradients for siRNA separation at a flow rate of (a) 200 µL/min using DIPEA solvents and (b) 250 µL/min using TEA solvents

MS acquisition

Table 1 lists the key MS parameters of the Full Scan/ddMS^2 (Top4) method employed in this study.

Table 1. Full Scan/ddMS^2 (Top4) method settings

Full Scan	Value
Orbitrap resolution	120,000
Scan range	570–2,000
RF lens (%)	70
AGC target	Auto
Polarity	Negative
ddMS^2 (Top4)	**Value**
Isolation window *(m/z)*	2
Collison energy mode	Stepped
Collision energy type	Normalized
HCD collision energies (%)	15-17-19 or 17-19-21
Orbitrap resolution	30,000
Time (ms)	200
AGC target	Standard

Data analysis

The processing of ddMS^2 data using BioPharma Finder software has been described in detail in two recent application notes.[4,5] Briefly, the sequences of sense and antisense oligonucleotides were created in Sequence Manager. The oligonucleotide mapping was performed using the Oligonucleotide Analysis workflow in BioPharma Finder software.

Results and discussion

Separation and identification of siRNA using DIPEA solvents (pH ≈ 8)

It is known that the composition and pH of ion paring solvents as well as column temperature significantly affect the state (denaturing vs. non-denaturing) of an siRNA duplex in solution and its chromatographic separation. Two LC-MS experiments were performed to gain insight into the effects of these factors on separation of the double stranded siRNA and its diastereomers. The separation of the double-stranded siRNA using DIPEA solvents (pH ≈ 8) at a column temperature of 70 °C, a condition previously used for separating single stranded oligonucleotides,[4,5] resulted in two distinct chromatographic peaks (Figure 2) that correspond to the sense and antisense oligonucleotides, respectively, as evidenced by the presence of single peak series in the mass spectra. The two sequences were confidently confirmed by HRAM (<2 ppm), high quality MS^2 spectra, and complete sequence coverages (Figure 2). No diastereomers were detected under this LC-MS condition. These results indicate that the sense and antisense oligonucleotides were present as denaturing single strands in the DIPEA solvents (pH ≈ 8).

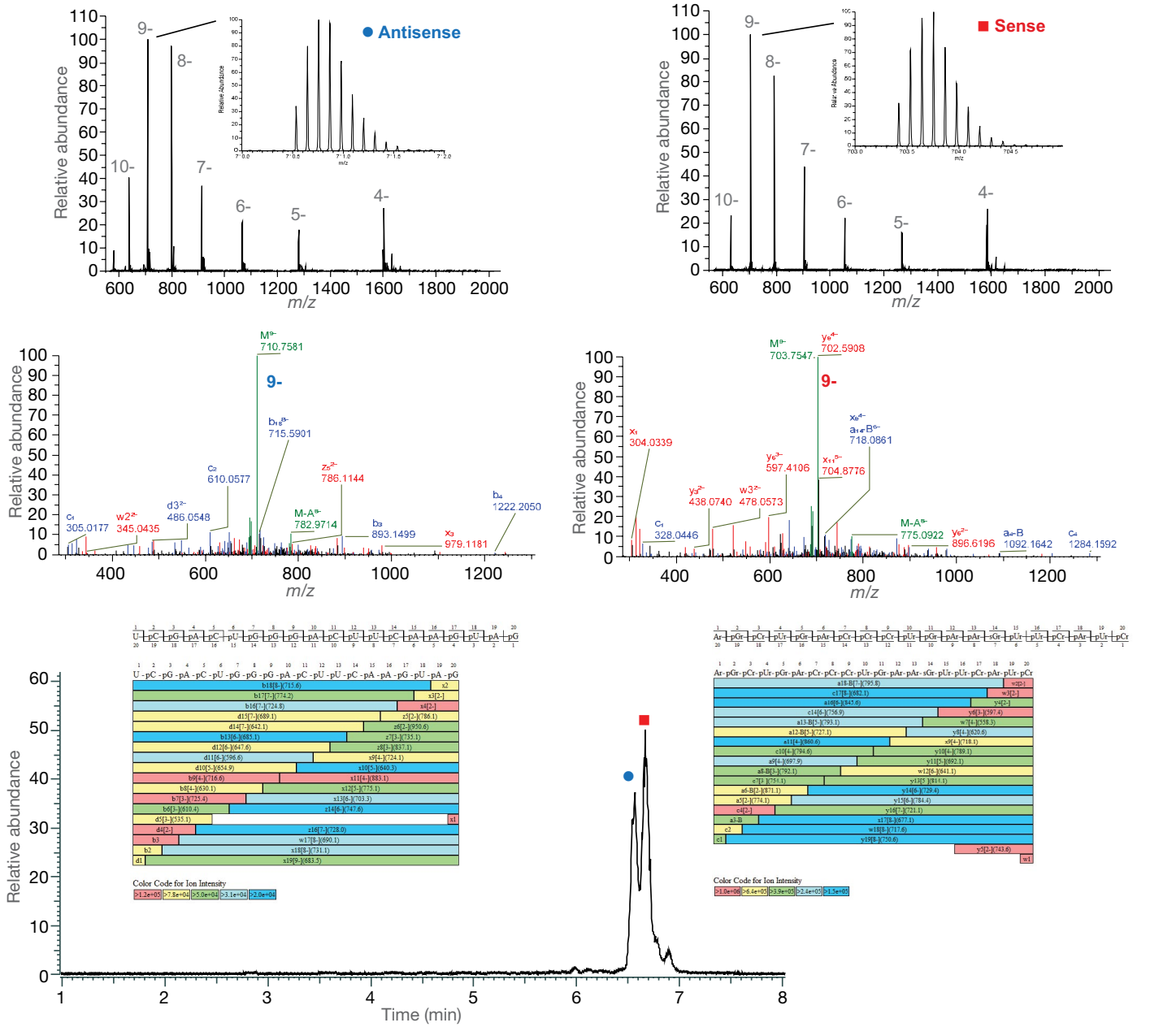


Figure 2. The MS^1 spectra, $ddMS^2$ spectra, and fragment coverage maps of sense and antisense oligonucleotides in siRNA separated using DIPEA solvents at a column temperature of 70 °C. Red square: sense oligonucleotide; blue circle: antisense oligonucleotide. Insets in the MS^1 spectra (top) display the isotopically resolved 9-charge states of two oligonucleotides. The $ddMS^2$ data (middle) was acquired using an NCE of 15-17-19. Sequence coverage maps (bottom)

Separation and detection of siRNA using TEA solvents (pH ≈ 9)

The use of TEA solvents at higher pH (≈9) and a lower column temperature (30–50 °C) led to separation and detection of chromatographic peaks 2 and 3 (Figure 3a), both of which correspond to the non-denaturing siRNA duplex, as confirmed by the co-presence of the sense and antisense peak series in their respective mass spectra (Figure 3c and 3d). The single stranded sense oligonucleotide in excessive amount was also detected at early retention time (see peak 1 in Figure 3a) and its accurate mass was confirmed by the mass spectrum (Figure 3b).

The chromatographic peaks 2 and 3 in Figure 3a can be assigned as two distinct diastereomers of the non-denaturing siRNA duplex. The two diastereomers were formed due to the presence of a chiral center created by a phosphorothioate group in the sense oligonucleotide. The column temperature affected the separation of these two diastereomers (Figure 3a). While two species were readily separated at a column temperature of 30–50 °C, their separation was diminished at ≥60 °C.

Identification of sense and antisense oligonucleotides in diastereomers

The ddMS^2 (Top4) results (Figure 4) confidently confirmed the identities of sense and antisense oligonucleotides detected in the experiment described above (Figure 3). The high quality MS^2 spectra led to complete sequence coverage of these two oligonucleotides (Figure 4). Taken together, these results highlight the power of LC-ddMS^2 for separation, detection, and identification of a double-stranded siRNA in single experiment.

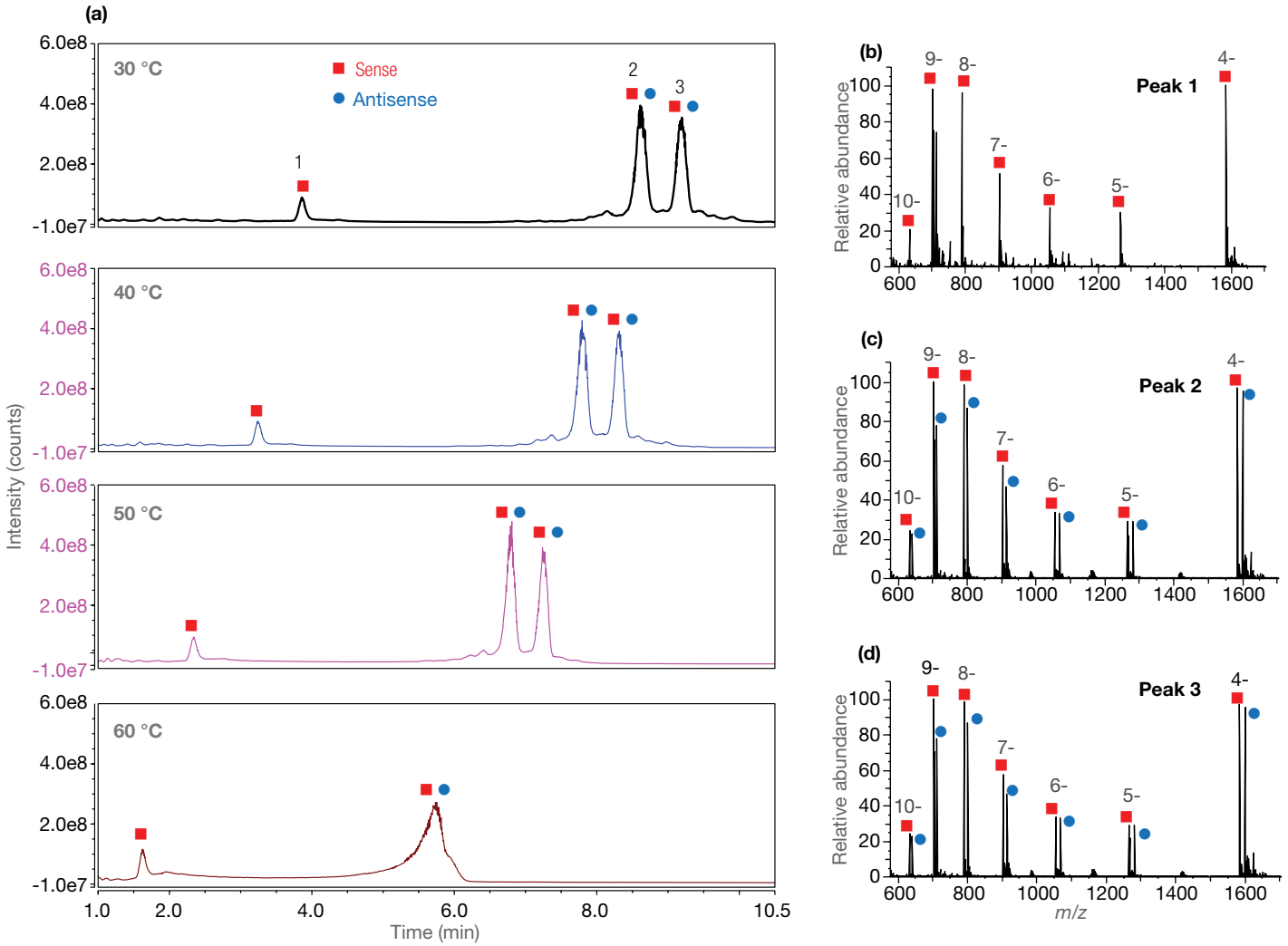


Figure 3. (a) Total ion chromatograms (TICs) of siRNA separated using TEA solvents at the column temperatures of 30–60 °C; (b-d) representative mass spectra for chromatographic peaks 1-3 in (a) at 30 °C

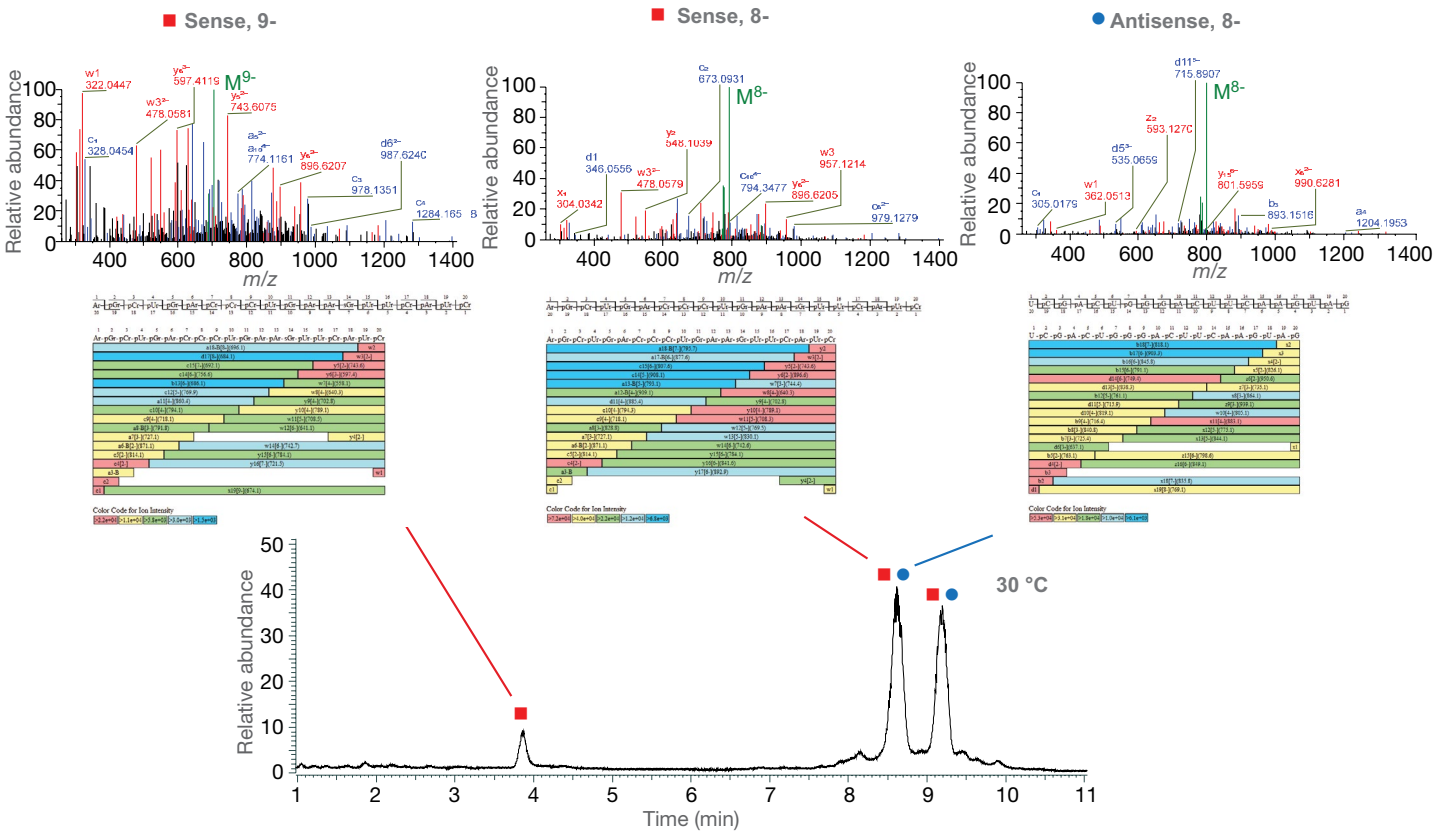


Figure 4. MS² spectra and fragment coverage maps of sense and antisense oligonucleotides separated using TEA solvents (pH ≈ 9) at a column temperature of 30 °C. The ddMS² data was acquired using an NCE of 17-19-21.

Conclusion

In summary, the Orbitrap Exploris 120 mass spectrometer, coupled with the Vanquish Horizon UHPLC system, the DNAPac RP column, and BioPharma Finder software, is a powerful tool for characterizing double-stranded siRNA and its diastereomers. This hardware and software combination can be employed for in-depth characterization of oligonucleotides.

References

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