Oligonucleotide mapping using BioPharma Finder software

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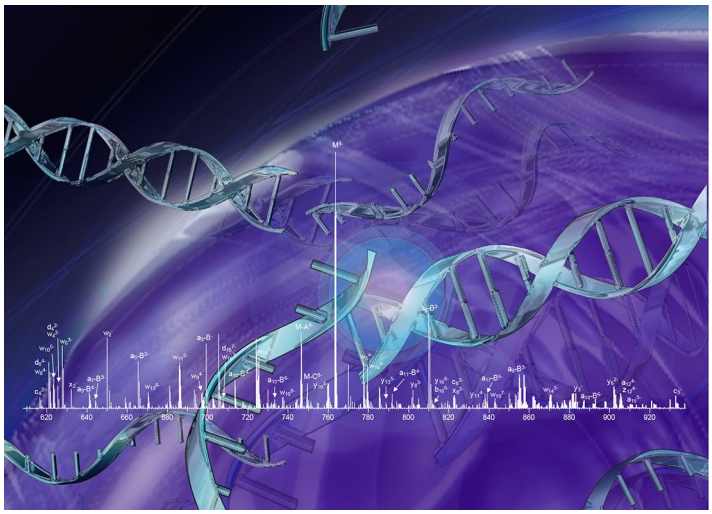
Application benefits

- High resolution accurate mass mass spectrometry (HRAM MS) coupled with data dependent tandem MS ($ddMS^2$) allows accurate mass measurement and confident identification and mapping of oligonucleotides.
- Thermo Scientific™ BioPharma Finder™ 4.0 software offers a complete toolset for characterizing oligonucleotides using $ddMS^2$ data.
- Comparative analysis of $ddMS^2$ data leads to fast method optimization for analyzing oligonucleotides in different sizes.

Goal

This work aims to develop and optimize a liquid chromatography (LC) $ddMS^2$ method for the untargeted analysis of oligonucleotides using Orbitrap-based HRAM MS. We will describe oligonucleotide identification and mapping using the new Oligonucleotide Analysis workflow



available in BioPharma Finder software. The key features that enable comparative analysis of multiple raw files and facilitate optimization of $ddMS^2$ methods will be highlighted.

Introduction

Since the first antisense oligonucleotide (ASO) drug was approved by the U.S. Food and Drug Administration (FDA) in 1998, therapeutic oligonucleotides, such as ASO and siRNA, have gained substantial interest in both academia and pharmaceutical industry.[1-3] Currently, eleven oligonucleotide drugs have received approval from the FDA, including nine since 2013, with over 150 drugs in the clinical pipeline (Phase I to Pre-registration). Oligonucleotide therapeutics have been successfully developed for the treatment of rare diseases such as neurological and

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hepatic diseases. The applications of oligonucleotide drugs to treat muscle and cardiovascular diseases are being pursued.[1-3] The potential of oligonucleotide therapies for alleviating the coronavirus disease-19 (COVID-19) pandemic was also recently discussed.[4]

The advancement of oligonucleotide therapeutics drives the need to develop robust analytical approaches for characterizing these novel drugs. While the LC-MS method using MS^1 only data has proven to be a viable tool for mass measurement and confirmation of therapeutic oligonucleotides,[5,6] it does not provide information about their sequences base-by-base or exact location of the modifications. By comparison, the $ddMS^2$ method allows confident identification and mapping of oligonucleotides, thereby enabling umambiguous sequence determination and localization of the modifications. Additionally, $ddMS^2$ does not require construction of a target mass list, as in the case of a parallel reaction monitoring (PRM) experiment, and hence is easy to execute. Currently, the application of $ddMS^2$ to oligonucleotide analysis is challenging due to the lack of a complete software package for automatic and confident MS/MS interpretation. To address this need, new tools are added in BioPharma Finder software that can process $ddMS^2$ data for fast identification and mapping of oligonucleotides. It offers the following key features for oligonucleotide analysis:

1. Sequence Manager supports RNA and DNA sequences and provides ease-of-use and flexibility in sequence building with user-defined custom building blocks;
2. Oligonucleotide Analysis workflow offers a powerful comparative analysis of multiple raw files, automatic MS^2 annotation, confident sequence identification, and site localization of modifications;
3. Intact Mass Analysis workflow supports modified oligonucleotides and provides automatic annotation using sequence or formula.

The first two features will be highlighted in this application note.

Experimental

Equipment

- Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer
- Thermo Scientific™ Vanquish™ Horizon UHPLC System

Data analysis software

- BioPharma Finder 4.0 software

Oligonucleotide samples

The lyophilized oligonucleotides were purchased from Integrated DNA Technologies (IDT) (Coralville, IA, U.S.A.). Table 1 lists the six DNA and RNA oligonucleotides analyzed in this study. A stock solution of 1 mg/mL was prepared for each oligonucleotide per quantity specified by IDT. This solution was diluted to 10 µg/mL before $ddMS^2$ analysis. A mixture of 15mer, 20merAC, and 25merMod at 10 µg/mL each were also analyzed by $ddMS^2$.

Table 1. List of synthetic oligonucleotides from IDT. The sequences follow a 5′-1′-2′ notation defined in BioPharma Finder software, as illustrated in Figure 1. The sequence difference of two isomeric 20mers (20merAC and 20merCA) is highlighted in bold. The mass values in the table correspond to the monoisotopic peaks.

Name	Sequence	MW (Da)
15mer	Cd-pAd-pGd-pTd-pCd-pAd-pGd-pTd-pCd-pAd-pGd-pTd-pCd-pAd-pGd	4,574.808
20merAC	Cd-pAd-pGd-pTd-pCd-pAd-pGd-pTd-pCd-pAd-pGd-pTd-**pAd-pCd**-pGd-pTd-pCd-pAd-pGd-pTd	6,114.057
20merCA	Cd-pAd-pGd-pTd-pCd-pAd-pGd-pTd-pCd-pAd-pGd-pTd-**pCd-pAd**-pGd-pTd-pCd-pAd-pGd-pTd	6,114.057
25merMod	Cd-pAd-pGd-pTd-pMe-pAe-pGd-pId-pTd-pId-pCd-pAf-pGd-pCd-pId-pAd-pGd-pId-pTd-pCd-pId-pAd-pGd-pTd	7,864.345
45mer	Cd-pAd-pGd-pTd-pCd-pAd-pGd-pTd-pCd-pAd-pGd-pTd-pCd-pAd-pGd-pTd-pCd-pAd-pGd-pTd-pCd-pAd-pGd-pTd-pAd-pAd-pAd-pAd-pAd-pAd-pCd-pAd-pGd-pTd-pCd-pAd-pGd-pTd-pCd-pAd-pGd-pTd-pCd-pAd-pGd-pTd-pCd-pAd-pGd	13,864.369
RNA20mer	Cr-pAr-pGr-pUr-pCr-pAr-pGr-pUr-pCr-pAr-pGr-pUr-pCr-pAr-pGr-pUr-pCr-pAr-pGr-pUr	6,363.877

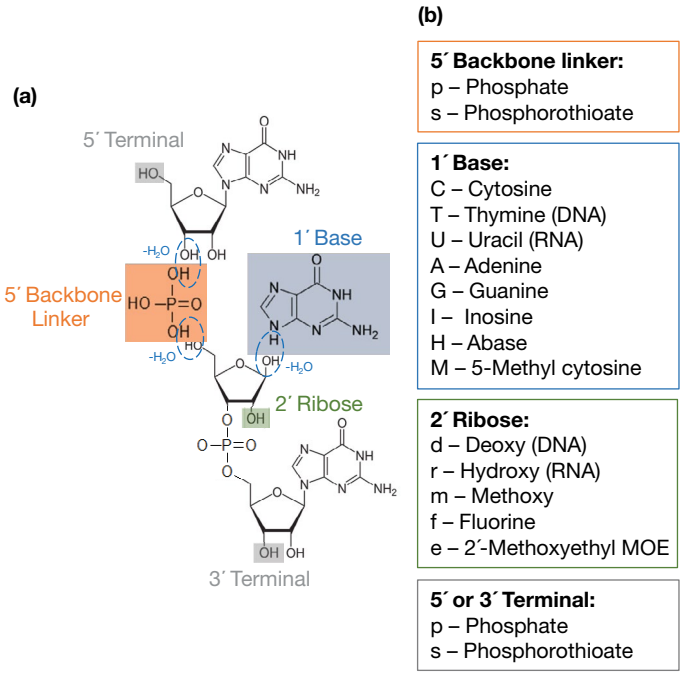


Figure 1. Oligonucleotide sequence notation in BioPharma Finder software. (a) An oligonucleotide sequence is annotated using a 5′-1′-2′ notation. (b) List of common annotations of 5′ backbone linker, 1′ base, and 2′ ribose building blocks as well as 5′ and 3′ terminus. For example, an unmodified CAG is annotated as Cd-pAd-pGd for DNA and Cr-pAr-pGr for RNA in BioPharma Finder software. The CAG with phosphorothioate at 5′ backbone linkers is annotated as Cd-sAd-sGd for DNA.

Solvents

- Water, UHPLC-MS Grade, Thermo Scientific (P/N W81)
- Methanol (MeOH), UHPLC-MS Grade, Thermo Scientific (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)
- Solvent A: 2% (~190 mM) HFIP and 0.1% (~5.7 mM) DIPEA in water (pH 7.8)

For a 500 mL solution, add 489.5 mL H_2O, 10 mL HFIP, and 0.5 mL DIPEA.

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

For a 500 mL solution, add 499.4 mL MeOH, 375 µL HFIP, and 187.5 µL DIPEA.

Ion-pairing reversed phase liquid chromatography

Oligonucleotide separations were performed with a Thermo Scientific™ DNAPac™ RP column, (4 µm, 2.1 × 50 mm, P/N 088924) using a Vanquish Horizon UHPLC system. The autosampler was held at 5 °C while the column was maintained at 60 °C with the column oven Thermostatting Mode set to Still Air. The solvents were prepared in the original UHPLC solvent bottles to minimize salt contamination. The LC gradient used in this study is shown in Figure 2.

Time (min)	%B
0	20
0.5	20
5	40
6.5	60
6.7	80
6.9	80
7	20
9	20

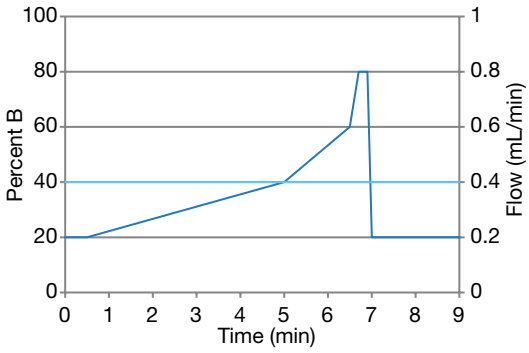


Figure 2. LC gradient for oligonucleotide separation at a flow rate of 0.4 mL/min

Mass spectrometry

The Q Exactive Plus mass spectrometer was operated in negative ion mode with calibration performed using the negative ion CalMix within the Exactive Series Tune software. The source and MS method settings are provided in Table 2. The data was acquired in the standard pressure mode using Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS). (Note: Data can also be acquired with Thermo Scientific™ Xcalibur™ software.) Data analysis was performed using BioPharma Finder software.

Table 2. Source and Full MS/ddMS² method settings. [i]S-lens RF level was varied from 40 to 100 when optimized for 20merAC but was kept at 60 for the rest of experiments. [ii]A resolution setting of 70,000 (at *m/z* 200) was used for the Full MS portion of Full MS-ddMS² experiments. However, a resolution setting of 140,000 was utilized for Full MS only experiments. [iii](N)CE or stepped (N)CE was extensively optimized for 20merAC and 25merMod, as listed in Table 3.

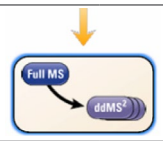
MS source setting	Value
Sheath gas flow rate	40
Aux gas flow rate	20
Sweep gas flow rate	2
Spray voltage (kV)	2.8
S-Lens RF Level	60[i]
Aux gas heater temp. (°C)	320
Capillary temp. (°C)	300
Sweep water flow	100 mL/min
Properties of the method	**Value**
Global settings	
Use lock masses	Off
Chrom. peak width (FWHM)	6 s
Time	
Method duration	9.0 min
Properties of Full MS/ dd-MS² (Top5)	**Value**
General	
Runtime	0 to 6 min
Polarity	Negative
Default charge state	2
Full MS	
Resolution	70,000[ii]
AGC target	1e6
Maximum IT	100 ms
Scan range	*m/z* 550 to 2000
	
dd-MS² / dd-SIM	
Resolution	35,000
AGC target	1e5
Maximum IT	150 ms
Loop count	5
TopN	5
Isolation window	*m/z* 2.0
Fixed first mass	*m/z* 300.0
(N)CE / stepped (N)CE	nce: 13, 15, 17[iii]
dd settings	
Minimum AGC target	5,000
Intensity threshold	3.3e4
Charge exclusion	1
Peptide match	Preferred
Exclude isotopes	On
Dynamic exclusion	3.0 s

Table 3. List of stepped (N)CEs tested for 20merAC, 25merMod, RNA20mer, and 45mer

Oligonucleotides	Stepped (N)CE	Parameters
20merCA	NCE	10-12-14, 11-13-15, 12-13-14, 12-14-16, 12-15-18, 13-14-15, 13-15-17, 14-15-16, 14-16-18, 15-17-19, 16-18-20, 17-19-21, 18-20-22, 22-24-26, 24-26-28, 26-28-30 (16 in total)
25merMod	NCE	10-11-12, 10-12-14, 11-13-15, 12-14-16, 12-16-20, 13-15-17, 14-16-18, 15-17-19, 16-18-20, 17-19-21, 18-20-22 (11 in total)
	CE	16-17-18, 17-18-19, 18-20-22, 19-20-21, 24-26-28, 26-28-30, 28-30-32, 31-32-33, 32-33-34, 33-34-35, 34-35-36, 35-36-37, 37-38-39, 39-40-41 (14 in total)
RNA20mer	NCE	10-12-14, 12-14-16, 13-15-17, 14-16-18, 15-17-19 (5 in total)
45mer	NCE	10-12-14, 11-13-15, 12-14-16, 13-15-17, 14-16-18, 15-17-19, 16-18-20 (7 in total)

Oligonucleotide analysis in BioPharma Finder software

A new oligonucleotide sequence can be created in Sequence Manager by importing a FASTA file or entering it manually. In this study, all the sequences were added manually in the Manual Input Sequence pane (Figure 3a) as plain format (e.g., CAG TCA GTC AGT ACG TCA GT for 20merAC), which was automatically converted into the Thermo triplet format (Table 1 and Figure 1) once entered. For 25merMod, the sequence of the unmodified 25mer was entered as described above, which was then followed by choosing the modified base and 2′ ribose in the Edit Sequence pane (Figure 3b) using drop-down menus. The default variable modifications, including dephosphorylation at 5′ terminal, phosphorylation at 3′ terminal (for DNA oligonucleotides), and backbone linker and base-specific modifications (e.g., base loss), were used for searching ddMS² data (Figure 3c). BioPharma Finder software also allows users to edit the existing building blocks or add new variable modifications (Figure 3d).

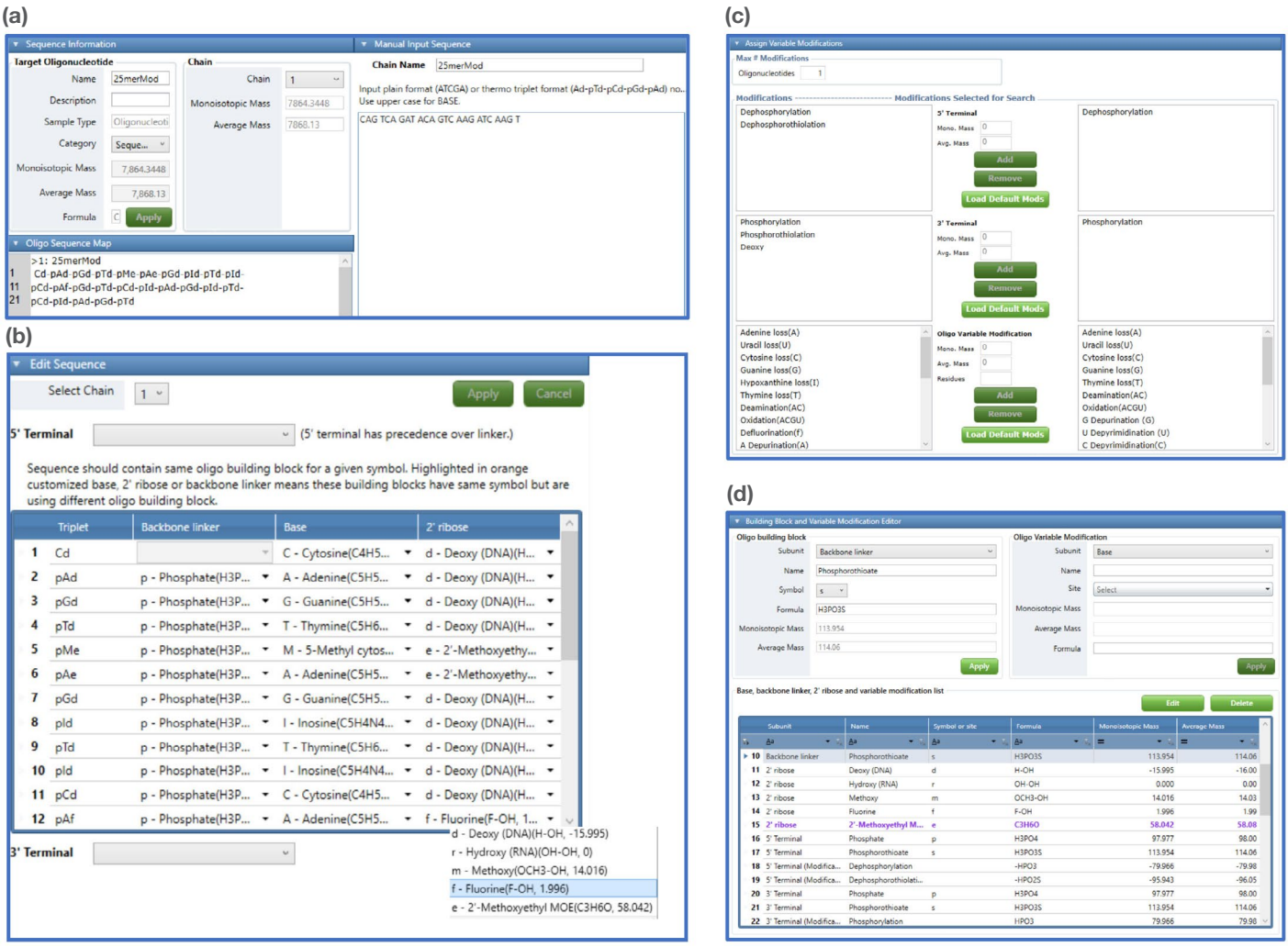


Figure 3. BioPharma Finder software provides flexibility in creating and modifying oligonucleotide sequences. The sequence can be added as a plain format in (a) Manual Input Sequence and modified in (b) Edit Sequence using drop-down menus, with variable modifications defined in (c) Assign Variable Modifications. The Building Block and Variable Modification Editor (d) allows the user to modify the building blocks or add new variable modifications.

The identification and mapping of oligonucleotides were performed in the Oligonucleotide Analysis workflow within BioPharma Finder software. The processing method for searching ddMS^2 data was created (as a new method) from the Basic Default Method by making two minor changes to the identification parameters: 1) increase maximum oligonucleotide mass from 11,000 to 15,000 under Oligonucleotide Identification, and 2) uncheck "Enable Mass Search for Unspecified Modifications" under Advanced Search. Other identification parameters include: Use MS/MS = Use All MS/MS, Mass Accuracy = 5 ppm for all oligonucleotides except 45mer and 10 ppm for 45mer, and minimum confidence = 0.80. For component detection, the task of "Final All Ions in the Run" was chosen. All other component detection parameters such as MS noise level and S/N threshold were automatically determined by BioPharma Finder software.

During method optimization, multiple raw files were processed in the multi-consensus format, which allows a comparative analysis of all data in one result file. The results were reviewed in the Process and Review and Mapping pages, where the comparative analysis was facilitated by a rich amount of information about the oligonucleotides identified, including extracted ion chromatogram (XIC), sequence annotations, trend ratio or MS area, fragment coverage map, full scan *vs*. deconvolution spectra, and experimental vs. predicted (or another experimental) MS^2 spectra. The comparisons of trend MS area, fragment coverage sequence, and MS^2 spectra helped determine the best method settings for a specific oligonucleotide.

Results and discussion

HRAM data of oligonucleotides

The oligonucleotides were separated based on ion-paring chemistry using a Vanquish Horizon UHPLC system coupled with a DNAPac RP column. The experimental condition employed in this study offers highly reproducible retention and separation of oligonucleotides in different sizes. Figure 4 displays typical mass spectra of 20merAC and 45mer. The inset in Figure 4b showcases excellent baseline separation for the isotopes of the 15- of 45mer at a resolution setting of 140,000 (at *m/z* 200), leading to the accurate mass measurement of the 45mer (0.5 ppm for the monoisotopic peak of the 15-).

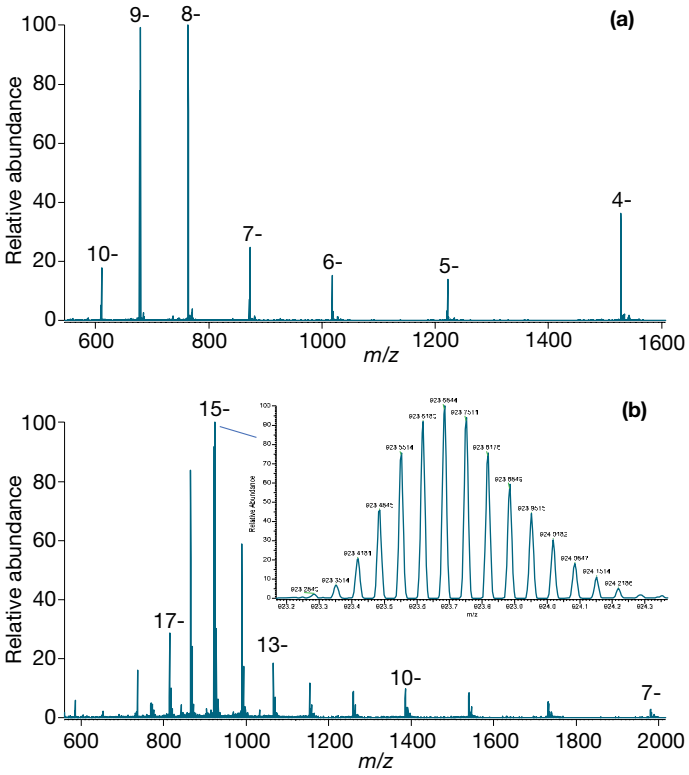


Figure 4. Mass spectra of (a) 20merAC and (b) 45mer with the inset displaying the isotopic distribution of the 15-. Both spectra were acquired at a resolution setting of 140,000 (at *m/z* 200).

Optimization of S-lens RF level

BioPharma Finder software provides an array of plots and graphs for comparative analysis of multiple raw files acquired with different method settings. This feature can be utilized to determine the optimal S-lens RF level for analyzing oligonucleotides. In this study, eight ddMS^2 raw files of 20merAC were acquired by varying S-lens RF levels from 40 to 100 and were then searched in a multi-consensus format in BioPharma Finder software. Figure 5 displays Trend MS Area plots of three different charge states (4-, 8-, and 10-) of 20merAC at eight different S-lens RF levels (Figure 5a-5c) and their selected ion chromatograms (SICs) at two extreme RF settings (RF = 40 and 100) (Figure 5d-5f). The plots show that while the S-lens RF level had little effect on the most abundant charge state (8-) of 20merAC (Figure 5b), it greatly affected the intensities of the 4- and 10- (Figure 5a and 5c), albeit in an opposite manner. An S-lens RF level of 60-65 provided a good signal for all the charge states of 20merAC.

Optimization of HCD collision energy (CE) for oligonucleotides

A key method parameter for obtaining high-quality ddMS^2 data of oligonucleotides is the HCD CE. The strategy described above for optimization of S-lens RF level was employed to determine the optimal HCD CE (absolute) or normalized CE (NCE) for 20merAC, 25merMod, and 45mer (Table 3). To assess the quality of MS^2 data, the fragment coverage map and annotated MS^2 spectra were compared across multiple raw files acquired using different stepped (N)CE settings (Table 3). Figure 6 shows that a stepped NCE of 10-12-14 was insufficient for fragmenting the 8- of 20merAC (Figures 6a and 6b). By comparison, a complete fragment coverage was obtained for 20merAC using stepped NCE15-17-19 (Figure 6c and 6d).

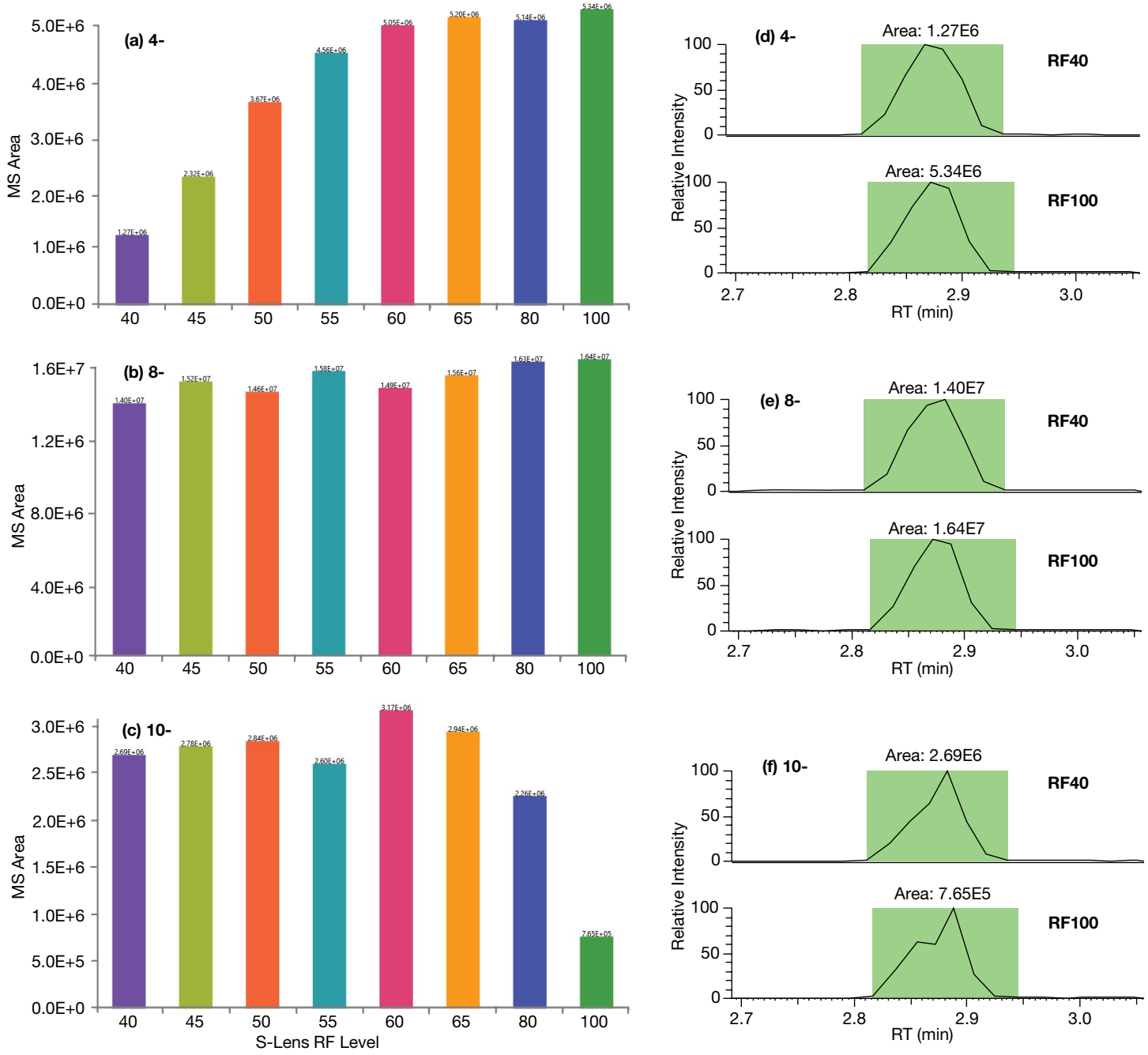


Figure 5. Trend MS Area plots of the (a) 4-, (b) 8-, and (c) 10- of 20merAC at S-lens RF levels of 40–100 and SICs of three charge states (d-f) at RF settings of 40 and 100. The SICs confirmed the same peaks used for comparison

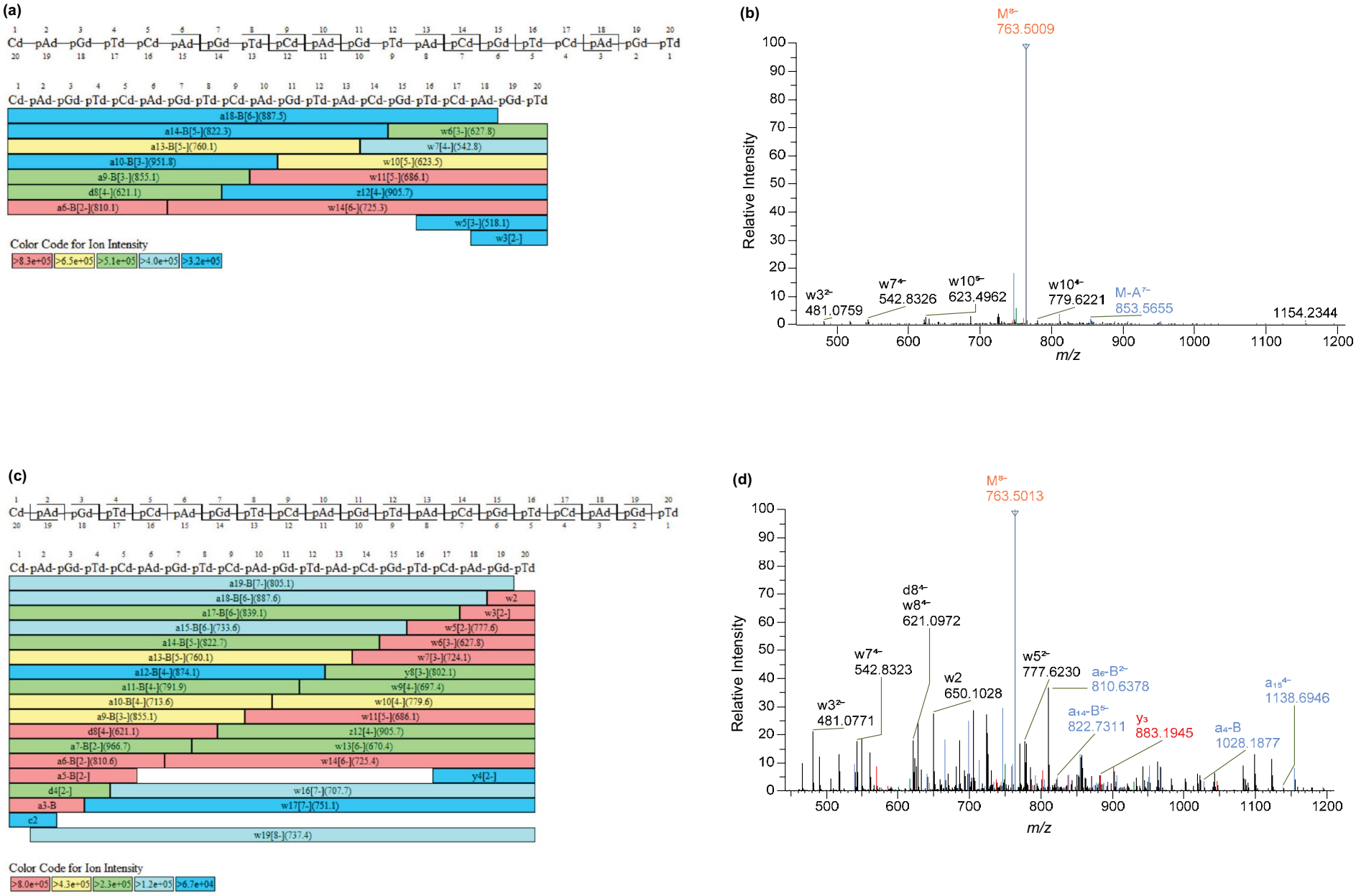


Figure 6. Fragment coverage map and annotated MS² spectra of 20merAC (8-) acquired at stepped NCEs of 10-12-14 (a and b) and 15-17-19 (c and d). The two annotated MS² spectra (b and d) were compared in a stacked format in BioPharma Finder software.

The average structural resolution (ASR) is a score calculated from the total number of base residues and the number of oligonucleotide fragments detected. An ASR of 1.0 indicates the detection of fragments that cover all bond cleavages between oligonucleotide residues. The further the ASR deviates from 1.0, the less complete the fragment coverage map for an oligonucleotide is. The ASR can be used as a guide to determine the optimal range of stepped NCEs. Table 4 lists ASRs measured for the charge states 4- to 10- of 20merAC at eleven stepped NCEs. This table reveals that a broad range of stepped NCEs can provide complete or nearly complete fragment coverages for different charge states of 20merAC (ASR = 1.0-1.2, in the green background), with the best results obtained from stepped NCEs of 15-17-19, 16-18-20, and 17-19-21.

Similarly, a range of stepped (N)CEs were determined for high charge states (8- to 12-) of 25merMod based on the ASRs obtained (Table 5). Figure 7 displays fragment coverage maps and annotated MS^2 spectra of the most abundant charge state (10-) of 25merMod at two-stepped (N)CEs that produced complete sequence coverage (ASR = 1.0). By contrast, none of the stepped (N)CEs tested in this study gave satisfactory fragment coverage for the 4- of 25merMod (Table 5), indicating the importance of using the right charge state(s) and (N)CE(s) for achieving complete fragment coverage. However, the MS^2 of the 4- generated abundant large fragments (e.g. b_{24} and y_{24}) that may provide complementary information to that obtained from higher charge states.

Table 4. ASRs measured for the charge states 4- to 10- of 20merAC at eleven stepped NCEs (out of sixteen tested). Green: ASR = 1.0-1.2, Yellow: ASR = 1.3-1.5, Red: ASR > 1.5+.

NCEs	101214	111315	121416	131517	141618	151719	161820	171921	182022	222426	262830
4-	1.3	1.3	1.1	1.2	1.3	1.3	1.3	1.3	1.4	1.7	1.7
5-	1.3	1.3	1.3	1.3	1.2	1.1	1.1	1.1	1.3	1.3	1.3
6-	5.0	2.2	1.5	1.3	1.3	1.1	1.2	1.1	1.1	1.1	1.3
7-	5.0	2.0	1.3	1.3	1.3	1.1	1.0	1.1	1.0	1.1	1.3
8-	2.0	1.4	1.3	1.2	1.2	1.0	1.0	1.1	1.1	1.0	1.5
9-	1.8	1.2	1.1	1.1	1.0	1.1	1.0	1.0	1.2	1.3	1.7
10-	1.1	1.1	1.1	1.1	1.1	1.0	1.1	1.1	1.1	1.4	1.5

Table 5. ASRs measured for selected charge states (4- , 8-, 10-, and 12-) of 25merMod at twelve stepped (N)CEs (out of twenty-five tested). Green: ASR = 1.0-1.2, Yellow: ASR = 1.3-1.5, Red: ASR > 1.5+.

	Stepped CEs						Stepped NCEs					
	161718	192021	242628	283032	343536	383940	121416	141618	151719	161820	171921	182022
4-	25.0	25.0	6.3	3.1	2.5	1.9	1.9	1.6	1.8	1.9	1.9	2.1
8-	8.3	1.9	1.1	1.0	1.0	1.5	1.7	1.3	1.1	1.1	1.1	1.1
10-	1.1	1.0	1.0	1.3	1.6	2.1	1.3	1.1	1.0	1.0	1.0	1.0
12-	1.0	1.0	1.6	1.8	2.1	2.3	1.0	1.0	1.0	1.0	1.1	1.0

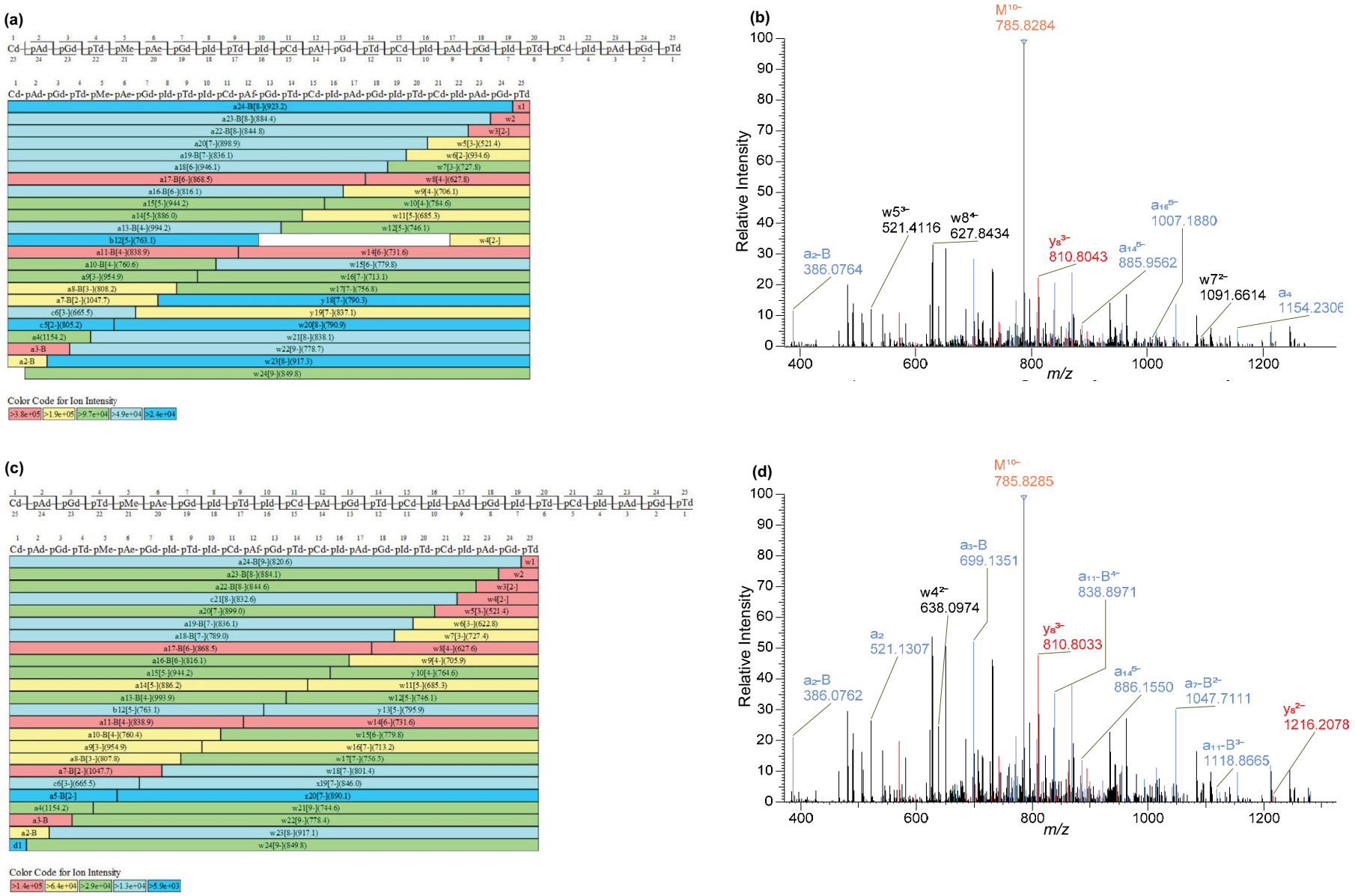
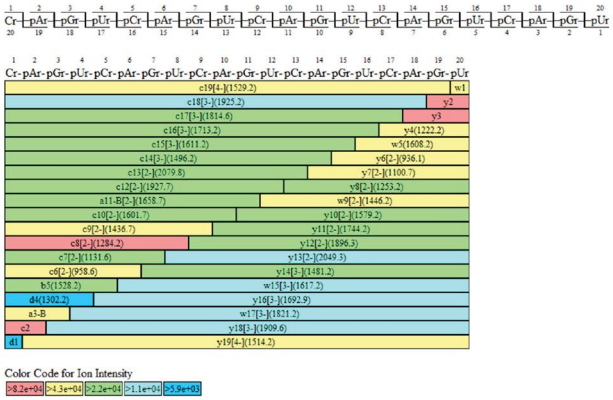


Figure 7. Fragment coverage map and annotated MS² spectra of 25merMod (10-) at stepped NCE15-17-19 (a and b) and CE19-20-21 (c and d).

The optimal range of stepped NCEs determined for 20merAC was further applied to RNA20mer and 45mer. Complete or nearly complete sequence coverage was obtained for these two oligonucleotides (Figure 8).

(a) RNA20mer, 9-



(b) 45mer, 16-

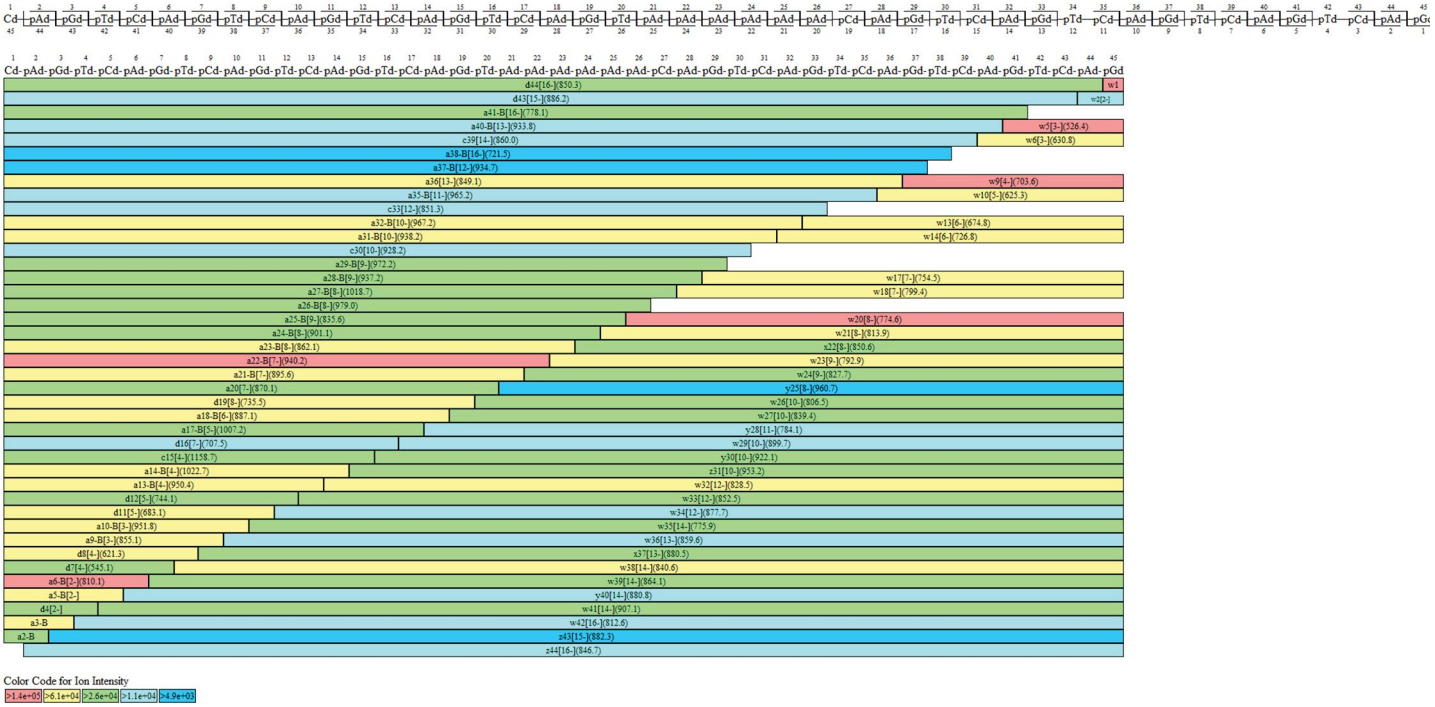


Figure 8. Fragment coverage maps of (a) 9- of RNA20mer and (b) 16- of 45mer acquired at stepped NCEs of 15-17-19, and 13-15-17, respectively

Identification of oligonucleotides by ddMS² in a mixture

From the result described above, it is possible to use a single stepped NCE setting to achieve complete coverages for oligonucleotides in similar sizes. In our further test, a stepped NCE of 15-17-19 was found to provide complete fragment coverages (ASR = 1.0) for three oligonucleotides (15merGT, 20merAC, and 25merMod) in a mixture (Figure 9). This result further demonstrates the power of employing BioPharma Finder software to facilitate the optimization of (N)CEs for oligonucleotide mapping.

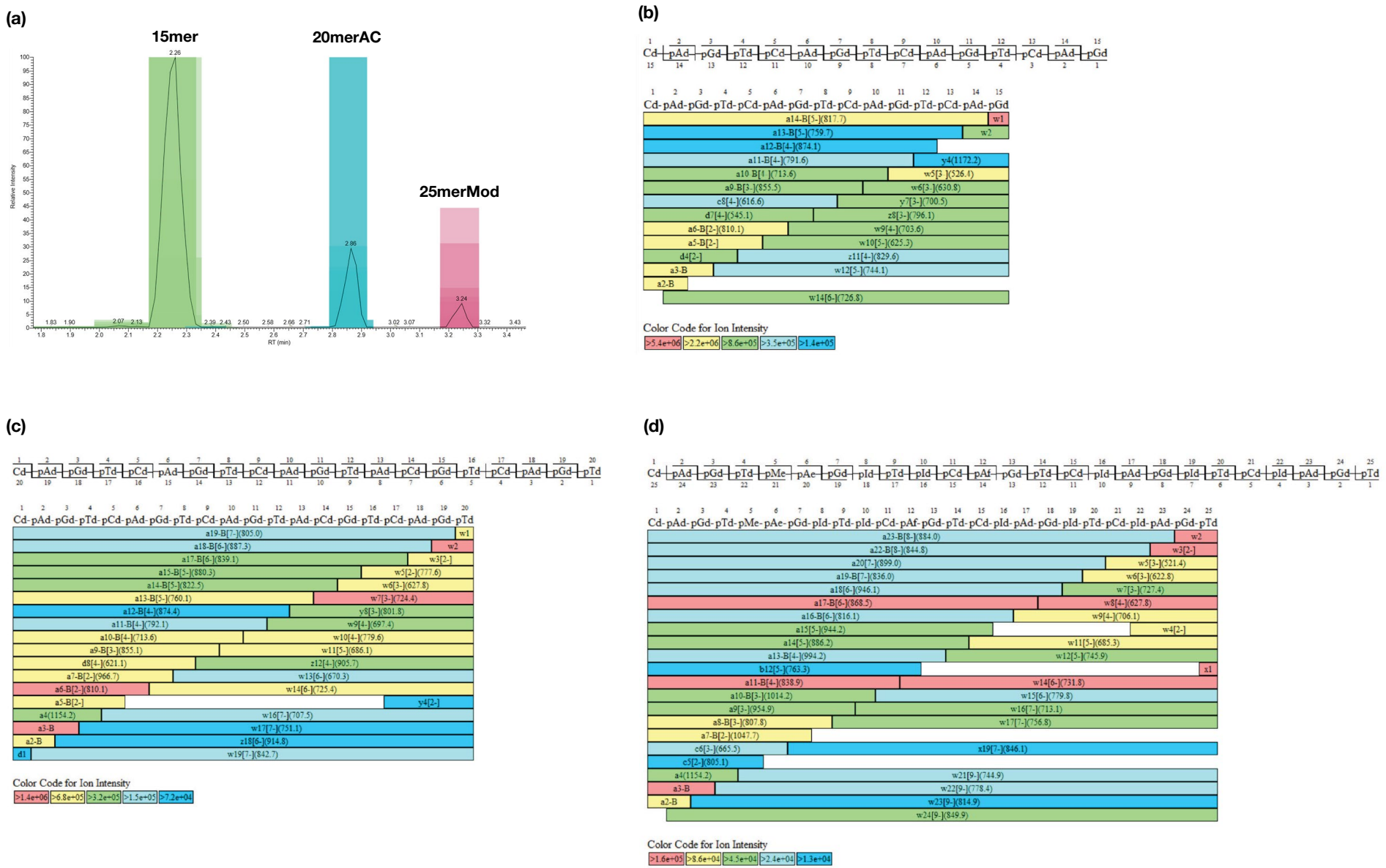


Figure 9. (a) Shaded chromatographic representation showing identification of three oligonucleotides in a mix; (b-d) Fragment coverage maps of 15mer 7- (b), 20merAC 8- (c), and 25merMod 10- (d), at a stepped NCE of 15-17-19

Fragmentation of isomeric 20merAC and 20merCA

BioPharma Finder software also offers tools to facilitate the characterization of isomeric oligonucleotides. Figure 10 displays the zoomed MS^2 spectra of the 6- of isomeric 20merAC and 20merCA as viewed in the stacked format in BioPharma Finder software. A mass difference of 8 Da observed for the w_7^{3-} peak aided unambiguous identification of these two isomers. The mass differences seen for the w_7 fragments (2- to 4-) from two isomers were also displayed in their respective fragment coverage maps (data not shown).

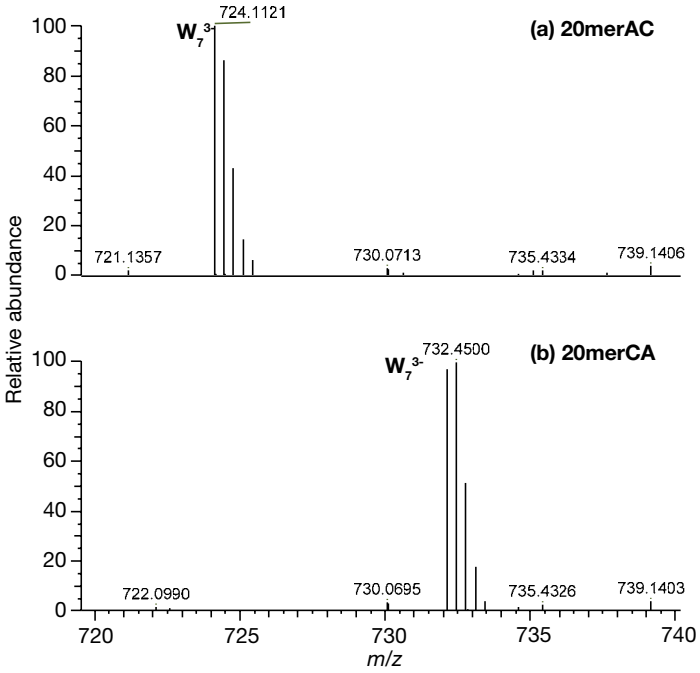


Figure 10. Zoomed MS^2 spectra of the 6- of (a) 20merAC and (b) 20merCA at NCE15-17-19 showing the diagnostic w_7^{3-} peak. These two spectra were viewed in the stacked format in BioPharma Finder software.

Conclusion

This application note describes a robust HRAM based $ddMS^2$ approach for accurate mass measurement and high confidence identification and mapping of oligonucleotides. The HRAM capability offered by the Orbitrap provides baseline resolving power and high mass accuracy for analyzing oligonucleotides. The ability of BioPharma Finder software to automatically interpret the $ddMS^2$ data of oligonucleotides opens up a new avenue for in-depth analysis of this novel modality. The powerful tools available in BioPharma Finder 4.0 software for comparative data analysis enables easy and fast method optimization, for which this application note can be used as a guide.

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