

## Ion-Pair Reversed-Phase (IP-RP) LCMS-9030 (Q-TOF) Mass Spectrometer for Separation and Identification of Oligonucleotides

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### User Benefits

- ◆ High-resolution separation of 10 to 60 base oligonucleotides using an ion-pairing buffer system
- ◆ High-accuracy mass analysis of oligonucleotides using LCMS-9030 (Q-TOF) mass spectrometer

### Introduction

Oligonucleotide therapeutics are short synthetic DNA or RNA polymers with the potential to treat a wide range of diseases. Currently, 13 oligonucleotide therapeutics have been granted new drug approval (NDA) by the U.S. Food and Drug Administration (FDA). On December 11, 2020, the U.S. FDA issued the first emergency use authorization (EUA) for the Pfizer-BioNTech COVID-19 mRNA Vaccine to be used in the US, which has further spurred interest in the oligonucleotide therapeutics.

Typical oligonucleotide therapeutics are approximately 15-30 bases, except aptamer oligonucleotides, which are commonly 40-60 bases. While shorter oligonucleotides (less than 20 bases) can be easily resolved by HPLC, the separation of longer sequences becomes progressively more challenging.

This article introduces an ion-pair reversed-phase (IP-RP) LC-MS system for high-resolution separation and high-accuracy mass analysis of oligonucleotides. Shimadzu LCMS-9030 (Q-TOF) mass spectrometer is used for LC-MS analysis.

### Experimental

#### Oligonucleotide Sample:

A DNA oligonucleotide ladder standard were purchased from the Integrated DNA Technologies (IDT), which contains eight oligonucleotides with 10, 15, 20, 25, 30, 40, 50, and 60 bases (Table 1).

#### Sample Preparation:

The oligonucleotide ladder standard was dissolved with Milli-Q® water to make it a 5 µg/mL solution for LC-MS analysis.

### Analytical Conditions:

HPLC and MS analytical conditions are shown in Table 2. In this analysis, the ion-pairing reagents of triethylamine (TEA) and hexafluoroisopropanol (HFIP) were used as the mobile phase in order to achieve the best reversed-phase separation of oligonucleotides.

Table 2 Analytical conditions

HPLC conditions (Shimadzu Nexera™ UHPLC)	
Column:	Shim-pack™ Scepter HD-C18-80 (100 mm x 2.1 mm I.D.; 1.9 µm)
Column Temp.:	60 °C
Flow rate:	0.2 mL/min
Mobile phase A:	15 mM TEA and 400 mM HFIP in 5% methanol
Mobile phase B:	50% mobile phase A + 50% methanol
Gradient program:	B. Conc. 31% (0 min)→47% (20 min)→75% (21-22 min)→31% (23 min)
Injection volume:	5 µL
MS conditions (Shimadzu LCMS-9030)	
Ionization:	ESI (Negative mode)
Mode:	MS (m/z 500 - 1400)
Probe voltage:	-3 kV
Gas flows (L/min):	Nebulizing, 3; Drying, 12; Heating, 12
Temperatures (°C):	Heat block, 400; DL, 250; Interface, 350

Table 1 DNA oligonucleotide ladder standard

Standards	Sequences	Formula	M.W. (Da)
10-mer	ATCGC GGATT	C98H124N37O59P9	3043
15-mer	GCTGC GACGA GGCTG	C146H183N61O88P14	4634
20-mer	ATCGC GGATT AGCAC TACGT	C195H246N75O118P19	6117
25-mer	ATCTC GGATT AGCAC TACGC ATCGG	C243H307N93O148P24	7642
30-mer	ATCGC GGATT AGCAC TACGC ATCGG TTACA	C292H368N113O177P29	9191
40-mer	ATCGC GGATT AGCAC TACGC ATCGG TTACA AACGA GTACC	C389H489N154O234P39	12274
50-mer	ATCGC GGATT AGCAC TACGC ATCGG TTACA AACGA GGACC TGATG CACTT	C487H612N191O295P49	15379
60-mer	ATCGC GGATT AGCAC TACGC ATCGG TTACA AACGA GGACC TGATG CACTT TGACA GCATG	C585H734N231O354P59	18493

## Data Processing:

MS data were processed by using Shimadzu LabSolutions version 5.99 SP2.

## Results and Discussion

### Separation of Oligonucleotides:

Separation of oligonucleotides was achieved on a Shim-pack Scepter HD-C18-80 column (100 mm x 2.1 mm I.D.; 1.9  $\mu$ m), using an ion-pairing buffer system containing TEA and HFIP. The resulting chromatogram shows an efficient separation of 10, 15, 20, 25, 30, 40, 50, and 60-mer oligonucleotides in 20 min gradient elution (Figure 1).

### Mass Accuracy:

LCMS-9030 (Q-TOF) mass spectrometer was operated in negative mode for high-accuracy mass analysis of oligonucleotides. All the eight targets were successfully identified with the most abundant mass accuracy of less than 2.5 ppm (Table 3). For example, Figure 2 shows mass spectrum of 20-mer oligonucleotide (most abundant m/z was 763.5020 [M-8H]<sup>8-</sup>), having a mass accuracy of 1.96 ppm.

## Conclusion

An analytical method was developed on the LCMS-9030 (Q-TOF) mass spectrometer using an ion-pairing buffer system of TEA and HFIP, with the aim to separate and identify the 10 to 60 base oligonucleotides. Overall, the method enables high-resolution separation and high-accuracy mass analysis of oligonucleotides, in which a mass error of less than 2.5 ppm was guaranteed.

Table 3 The most abundant mass accuracy of oligonucleotides on LCMS-9030

Standards	M.W. (Da)	Cal. m/z	Meas. m/z	ppm	Adduct ion
10-mer	3043	1013.1763	1013.1773	0.99	[M-3H] <sup>3-</sup>
15-mer	4634	925.7547	925.7557	1.08	[M-5H] <sup>5-</sup>
20-mer	6117	763.5005	763.5020	1.96	[M-8H] <sup>8-</sup>
25-mer	7642	954.1569	954.1585	1.68	[M-8H] <sup>8-</sup>
30-mer	9191	834.4997	834.5014	2.04	[M-11H] <sup>11-</sup>
40-mer	12274	817.1994	817.2013	2.33	[M-15H] <sup>15-</sup>
50-mer	15379	853.3599	853.3615	1.87	[M-18H] <sup>18-</sup>
60-mer	18493	1026.3331	1026.3349	1.75	[M-18H] <sup>18-</sup>

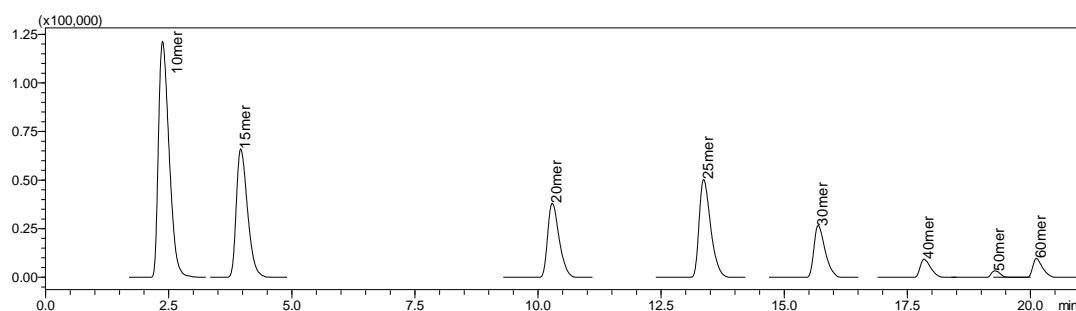


Figure 1 Separation of 10 to 60 base oligonucleotides

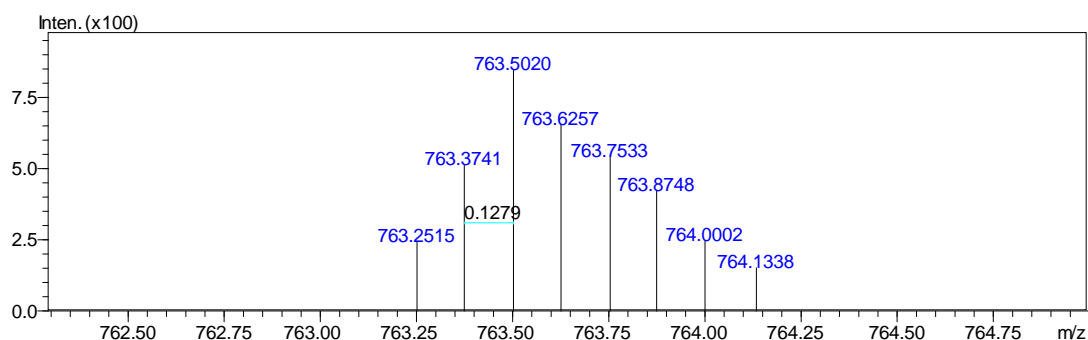


Figure 2 High-resolution mass spectrum of 20-base oligonucleotide.

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