Full characterization and confirmation of diverse oligonucleotides by ion-pairing chromatography coupled with the Q Exactive™ HF-X HRMS

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ABSTRACT

Purpose: Characterize oligonucleotides varying in length between 17 to 120 nucleotides, by high resolution accurate mass spectrometry using the Thermo Scientific™ Q Exactive™ HF-X mass spectrometer with available decreased pressure mode for optimum resolution and sensitivity.

Methods: Oligonucleotides were analyzed using the Thermo Scientific™ UltiMate 3000 HPLC system by ion-pairing chromatography using the Thermo Scientific™ DNAPac™ RP analytical column. Chromatographic separation was monitored by analysis on the Q Exactive HF-X instrument with BioPharma option operated in negative electrospray ionization mode at varying resolutions, and using standard or decreased trapping gas pressures available with the Protein Mode option.

Results: DNA oligonucleotides ranging from 17 to 120 bases in length were analyzed by LC-MS. Short DNA oligonucleotide primers ranging from 17 to 23 bases were chromatographically separated in triplicate analyses, by a 13 minute gradient and deconvoluted with Thermo Scientific™ BioPharma Finder™ 3.0 software. Large oligonucleotides of 90 and 120 bases in length were accurately confirmed with full resolution and mass accuracy of < 3 ppm. Results show added improvement in signal intensity, resolution, and mass accuracy when reduced trapping gas pressure available with Protein Mode, are used.

INTRODUCTION

Oligonucleotides are used in many molecular biology applications including gene therapy by antisense and small interfering RNA (siRNA), primers for sequencing and amplification in polymerase chain reactions (PCR), probes to detect complementary DNA and RNA strands via hybridization reactions, and as aptamers engineered to bind specific molecular targets. Although these are seemingly simple entities comprised of combinations of only four different bases, the potential for numerous structural modifications and adductions can make characterization of these diverse biomolecules complex. However, continuing advancements in LC-MS/MS technology provide increasingly selective and sensitive methods that have facilitated improved qualitative and quantitative analysis. We present here the ability to accurately characterize a diverse group of oligonucleotides by high resolution accurate mass spectrometry for research purposes.

MATERIALS AND METHODS

Sample Preparation: Oligonucleotide standards 17 to 120 nucleotides in length were purchased from Integrated DNA Technologies and resuspended in 100 µM EDTA supplemented with 1 % methanol, to a final concentration of either 1 µg/mL (10 primer mix) or 5 µM (90 and 120mer).

Liquid Chromatography Method

System:	UltiMate 3000 HPLC
Column:	DNAPac RP 2.1 x 100 mm, 4 µm
Mobile Phases:	A: 400 mM HFIP, 16.3 mM TEA in Water
	B: Methanol
Column Temp.:	75 ºC
Auto Sampler Temp.:	4 ºC
Injection Volume:	2 µL, unless noted
Gradient:	Figure 1

Figure 1. LC gradient # 1: used for analysis of the Oligo Primers.

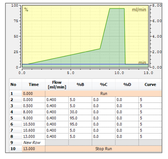


Figure 2. LC gradient # 2: used for analysis of 90mer and 120mer.

Table 1. HESI Source Parameters. Source conditions held constant for both small oligo primers and large intact oligonucleotides.

HESI Source Parameters			
Polarity	Negative	Sheath Gas	60
Spray Voltage	3.0 kV	Auxiliary Gas	15
Capillary Temp	400 ºC	Sweep Gas	1
S-Lens RF	80	Auxiliary Gas Temp	325 ºC

Table 2. Full MS Parameters

	Oligo Primer (17-23mer)	Large Oligo (90 and 120mer)		Oligo Primer (17-23mer)	Large Oligo (90 and 120mer)
Scan Range	*m/z* 650 – 2100	*m/z* 850 – 2300	AGC	1e6	
Resolution	120,000 or 240,000		Max Inject Time	200 ms	
Microscans	1		Protein Mode	OFF	ON

RESULTS

Experiment # 1: Full MS of Oligonucleotide Primer Mix Separated

Figure 3. Chromatographic separation of a mixture of oligonucleotide primers analyzed in triplicate with mass spectra zoomed in on a specified mass ranges.

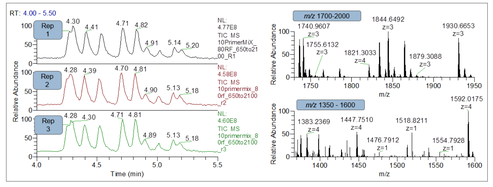


Figure 4. Full MS deconvoluted with Biopharma Finder 3.0. Full MS spectra were deconvoluted using sliding windows and the Xtract deconvolution feature. All oligos were detected with excellent mass accuracy and reproducibility.

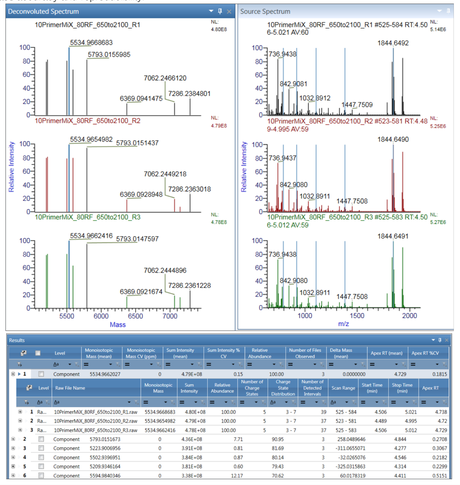


Table 4. Deconvolution of 10 oligonucleotide primer mix using Biopharma Finder 3.0 software

Oligo Identity	Sequence	RT (min)	Theoretical Monoisotopic Mass	Deconvoluted Monoisotopic Mass	Monoisotopic Mass CV (ppm)	Mass Accuracy Mean (ppm)
SP6 upstream	ATTTAGGTGACACTATAG	4.73	5534.962	5534.9662	0.1223	0.76
pET 5' (T7)	ACGGCTACCTTGTTATCGACTT	4.84	5793.014	5793.0152	0.0730	0.20
Bluescript KS	TCGAGGTCGACGGTATC	4.28	5223.895	5223.9007	0.0143	1.09
pET 3'	CTAGTTATTGCTCAGCGG	4.55	5502.935	5502.9397	0.0508	0.85
M13 Reverse (-27)	CAGGAAACAGCTATGAC	4.31	5209.929	5209.9346	0.0476	1.08
BGH Reverse	TAGAAGGCACAGTCGAGG	4.41	5594.982	5594.9840	0.1685	0.36
M13 Forward (-41)	GTAAAACGACGGCCAGT	5.16	7286.228	7286.2370	0.1806	1.23
M13 Reverse (-48)	CAGGAAACAGCTATGAC	5.21	7062.233	7062.2453	0.1577	1.75
16S rRNA Rev	ACGGCTACCTTGTTACGACTT	5.03	6369.077	6369.0931	0.1575	2.52
pGEX 3'	CCGGGAGCTGCATGTGTCAGAGG	4.89	7142.208	7142.2158	0.0837	1.09

Experiment # 2: Intact Analysis of 90mer Oligonucleotide

Table 5. 90mer DNA Oligonucleotide Sample Description

Oligo Identity	Sequence	RT (min)	Theoretical Monoisotopic Mass
90mer DNA Oligo	ATGCAAATTTTCGTCAAGACTTTAACCGGTAAGA CTATTACCCTGGAAGTTGAATCTTCTGACACTAT TGACAATGTCAAGTCCAAGATC	2.26	27653.596

Figure 5. 90mer DNA Oligonucleotide 240,000 Resolution with Protein Mode: Total Ion Chromatogram of duplicate injections of 20 µM of 90mer oligonucleotide. Duplicate injections showed strong reproducible signal.

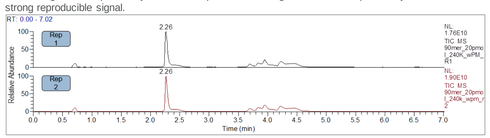


Figure 6. 90mer DNA Oligonucleotide 240,000 Resolution with Protein Mode: Charge state distribution of 20 pmol of the 90mer oligonucleotide and close inspection of charge state z = -20. 90mer oligonucleotide is fully resolved at 240,000 resolution with protein mode.

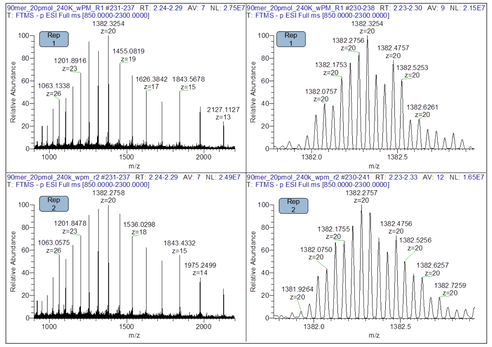


Figure 7. 90mer DNA Oligonucleotide analyzed at 240,000 Resolution with Protein and Standard Modes: Charge state distribution of the 90mer oligonucleotide with close inspection of charge state z=-20. The 90mer oligonucleotide is fully resolved at 240,000 resolution with protein mode with three times improvement in signal intensity.

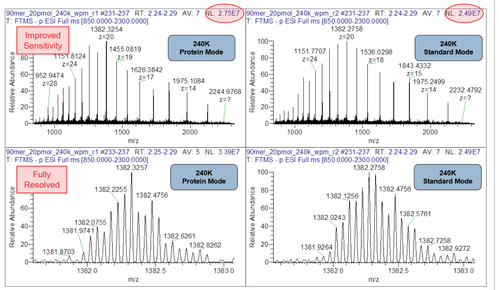


Figure 8. 90mer DNA Oligonucleotide analyzed at 120,000 Resolution with Protein Mode and Standard Mode: Close inspection of *z* = - 20 demonstrates improved sensitivity when using reduced trapping gas pressures available with Protein Mode.

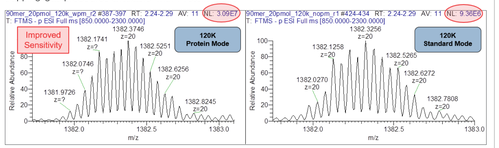


Table 6. Mass Accuracy Observed in duplicate analysis of 90mer Oligonucleotide for Different Methods

Method	Theoretical Monoisotopic Mass	Experimental Monoisotopic Mass (Average)	Mass Accuracy (ppm)
240,000 Protein Mode	27653.596	27653.6322	1.309
240,000 Standard Mode	27653.596	27653.6026	0.239
120,000 Protein Mode	27653.596	27653.6148	0.680
120,000 Standard Mode	27653596	27653.6702	2.683

Experiment # 3: Intact Analysis of 120mer Oligonucleotide

Table 7. Characterization of 90mer DNA Oligonucleotide at high resolution.

Oligo Identity	Sequence	RT (min)	Theoretical Monoisotopic Mass
120mer DNA Oligo	ATGCAAATTTTCGTCAAGACTTTAACCGGTAAGA CTATTACCCTGGAAGTTGAATCTTCTGACACTAT TGACAATGTCAAGTCCAAGATCCAAGACAAGGA AGGTATTCCACCTGACCAA	2.29	36913.148

Figure 9. 120mer DNA Oligo at 240,000 Resolution with Protein Mode: Total ion chromatogram of two replicate 20 pmol injections.

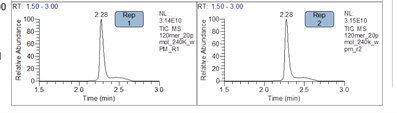
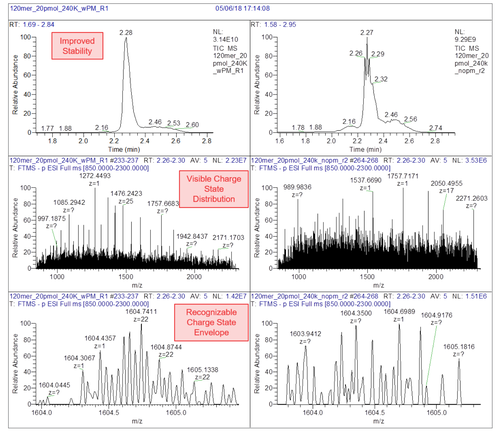


Figure 10. 120mer DNA Oligonucleotide 240,000 Resolution with Protein and Standard Mode: Charge state distribution of 20 pmol on column of the 120mer oligonucleotide and close inspection of charge state z = - 22.



CONCLUSIONS

Oligonucleotides as a class of biomolecules have traditionally not been widely characterized by mass spectrometry due to the difficulty in acquiring high resolution accurate mass data. In this study, we demonstrate the ease of acquiring high resolution accurate mass spectrometry data using the Q Exactive HF-X mass spectrometer in decreased trapping gas pressure mode, available with the Protein Mode option. We were able to fully resolve oligonucleotides ranging in size from 17 to 90 nucleotides with a mass accuracy of < 3 ppm. Additionally, mixtures of oligonucleotides can be easily fractionated using ion-pairing chromatography in conjunction with the DNAPac RP analytical column. Deconvolution of oligonucleotide data can be easily and reliably achieved using BioPharma Finder 3.0 software.

- DNAPac RP analytical column with an ion pairing gradient was effective in separating oligonucleotides ranging from 17 to 190 nucleotides in length.
- BioPharma Finder 3.0 with the sliding windows Xtract algorithm detected all 10 primers included in the mix.
- Intact analysis of a 90mer oligonucleotide was successfully detected with high resolution mass accuracy at 120,000 and 240,000 resolution.
- Signal intensity of the 90mer was improved by using the decreased trapping gas pressure available in Protein Mode. Analysis of the 120mer at 240,000 resolution in Protein Mode was greatly improved with a recognizable charge state distribution when using Protein Mode lending to the conclusion that large intact oligonucleotide analysis is greatly improved with decreased trapping gas pressure.

TRADEMARKS/LICENSING

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