Quantification and Qualification of Oligonucleotides by High Resolution/Accurate Mass Orbitrap MS

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Overview

Purpose: Identification and quantitation of oligonucleotides by UHPLC-high resolution Orbitrap MS.

Methods: Oligonucleotides were first qualified in full scan MS mode at 70,000 resolution, then quantified with both selected ion monitoring (SIM) at 70,000 resolution and targeted MS/MS methods at 17,500 resolution with a 8-min gradient on Thermo Hypersil Gold column.

Results: Samples prepared in a human plasma based solution to reduce non-specific binding indicates good linearity with a calibration range of 1-5nM to10µM. Performing SIM (70,000 resolution) shows 5 fold more sensitivity than targeted MS/MS method (17,500 resolution). With faster scan speed, there are at least 20 scans across a 9-sec UHPLC peak around LOQ at 70,000 resolution.

Introduction

Qualitative and quantitative analysis of oligonucleotides in biological matrices are important aspects of the drug development process. There is a trend towards the use of high resolution mass spectrometry (HRMS) as an alternative to overcome the limitations of nominal resolution provided by triple quadrupole MS. [1-2] A high throughput generic UHPLC-HR-MS assay is demonstrated for oligonucleotide analysis using Q-Exactive Orbitrap MS. The qualitative information provided during quantitation of analytes within one injection is a crucial advantage to accelerate drug development process by reducing instrument analysis time and sample consumption.

Methods

Sample Preparation

Oligonucleotides (ODNs)(15mer- and 20mer-),bought from TriLink BioTechnologies, were dissolved in water. The concentration of stock solutions were quantified by Thermo NanoDrop UV spectrometer. A 20-mer Phosphodiester ODN (5'-ATT CAG TTC ACT TAT CGT AT-3') was diluted in 3 different carriers (1)Solvent A (2%HFIP+0.4%TEA in Water) (2) 0.1% Human Plasma in Solvent A (3) 0.1% Human Plasma + 0.1%TFA+ 10%MeOH. The synthetic ODN was quantified with 15-mer phosphorothioate oligonucleotide (5'-ATT CAG TTC ACT TAT-3') as internal standard. Samples were analyzed on a Thermo Q-Exactive Orbitrap equipped with XRS™ UHPLC pump and Open Accela autosampler.

Liquid Chromatography

Column: Hypersil Gold C18 column (2.1 x 50 mm, 1.9 µm)

Injection Volume: 10 µL

LC: XRS™ UHPLC pump

Solvent A: 2%HFIP+0.4%TEA in Water

Solvent B: 2%HFIP+0.4%TEA in Methanol

Flow Rate: 300 µL/min

Gradient:

Time	A%	B%
0.0	90	10
5.0	60	40
6.0	10	90
8.0	10	90
8.1	95	10

Mass Spectrometry

Spray Voltage (-)	3800 kV
Capillary Temperature (-)	320 ºC
Sheath Gas (-)	45
Aux Gas (-)	15
Sweep Gas (-)	0
Heater Temperature (-)	400 ºC
S-lens	50
Negative MS Scan	1 microscan
Full Scan	R = 70,000; AGC = 1e6; IT = 250 ms; Lock Mass = off
SIM	R = 70,000; AGC=2e5; IT= 350ms; Isolation= 4 amu
MS/MS	R = 17,500; AGC = 2e5; IT= 500 ms; Isolation= 4 amu; HCD = 20

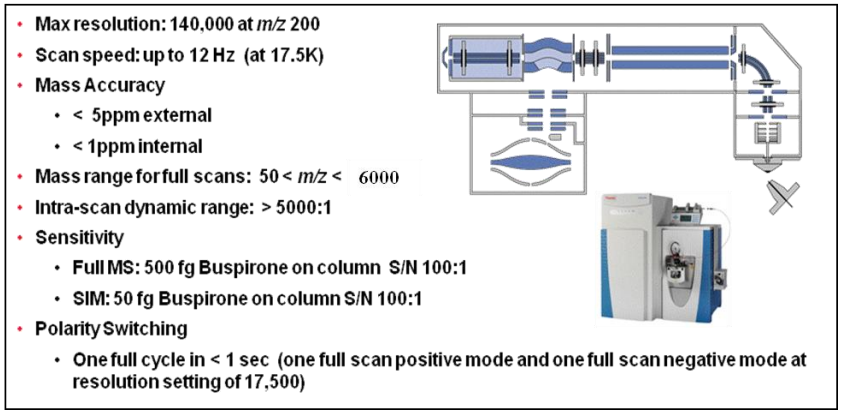


FIGURE 1. Q Exactive Benchtop Orbitrap Mass Spectrometer

Data Analysis

Oligonucleotides were initially analyzed by full scan mode at 70,000 resolution to obtain the charge state distribution envelope. The most abundant ions, $[M-9H]^{9-}$ for 20-mer and $[M-3H]^{3-}$ for 15-mer, were then employed for quantitation. A comparison of ODN quantitation using both selected ion monitoring (SIM) and targeted MS/MS was performed with a 8-min gradient to separate analyte with internal standard. The calibration curves of ODNs were generated by LCquan™ software. For SIM method, the four most abundant isotopes of deprotonated ODNs were summed for the quantitation. For targeted MS/MS, four relative abundant and unique fragmentation ions were chosen to do quantification.

Results

Oligonucleotides Charge State Distribution and SIM Quantitation

Analyte and internal standard ODNs were analyzed by LC-MS at full scan mode from *m/z* 400 to 2000 at 70,000 resolution. The charge state distributions of two ODNs are shown in Figure 2 and 4, respectively. For 20-mer ODN, the most abundant ion is $[M-9H]^{9-}$. Four isotopes, *m/z* 671.7792, 671.8902, 672.0001 and 672.1119 were selected for quantitation for SIM method(Figure 3). For 15-mer ODN, $[M-3H]^{3-}$ is the most abundant ion, three isotopes, *m/z* 1579.1551, 1579.4875 and 1579.8208 were used for quantitation(Figure 5).

FIGURE 2. Full Scan MS Spectrum of 20-mer Synthetic Oligonucleotide (5'-ATT CAG TTC ACT TAT CGT AT-3')

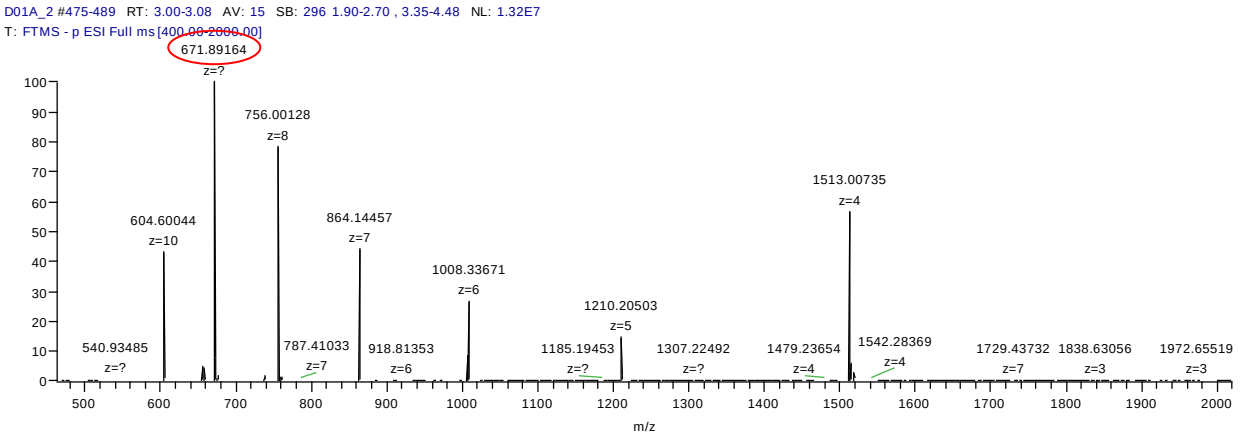


FIGURE 3. Zoom-in MS Spectrum of $[M-9H]^{9-}$ 20-mer Oligonucleotide 5'-ATT CAG TTC ACT TAT CGT AT-3')

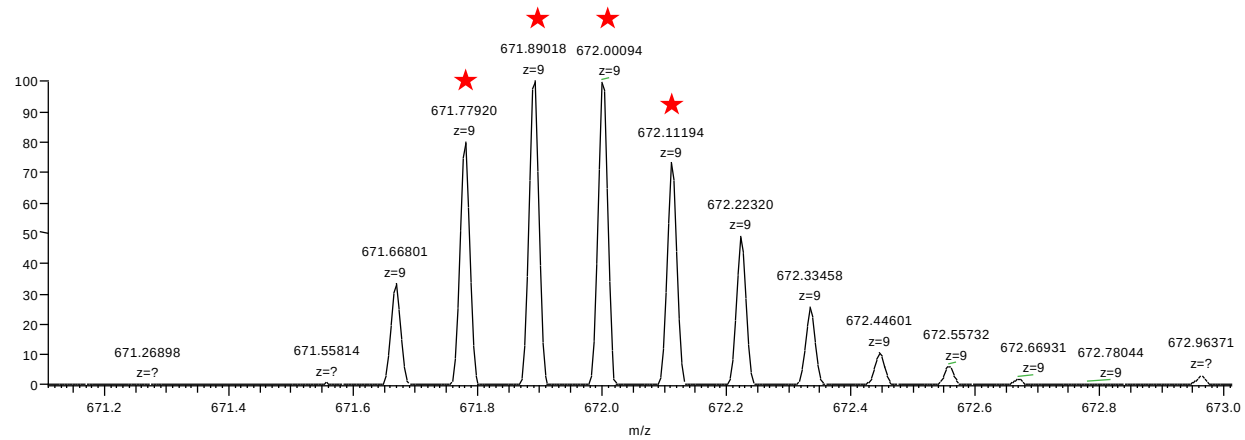


FIGURE 4. Full Scan MS Spectrum of 15-mer synthetic Oligonucleotide (5'-ATT CAG TTC ACT TAT-3')

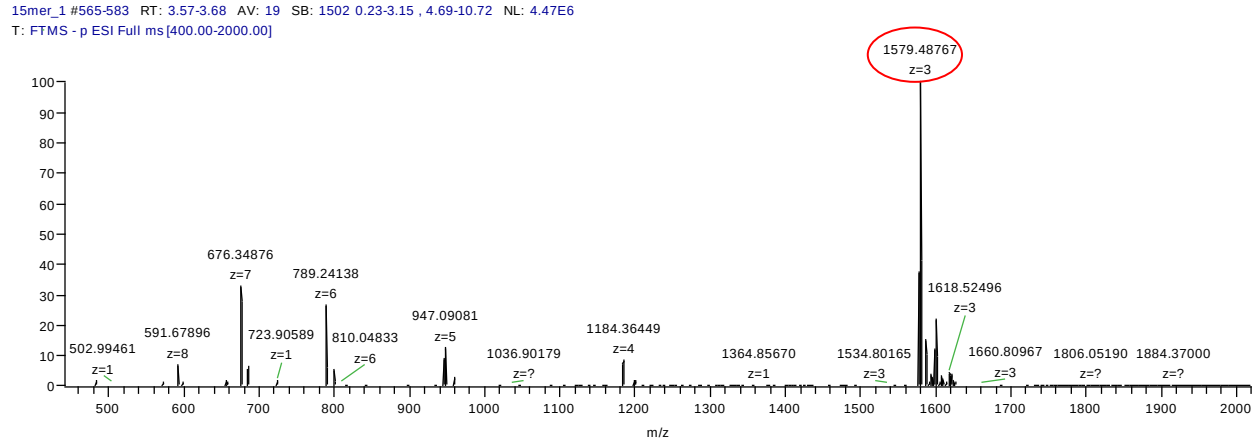
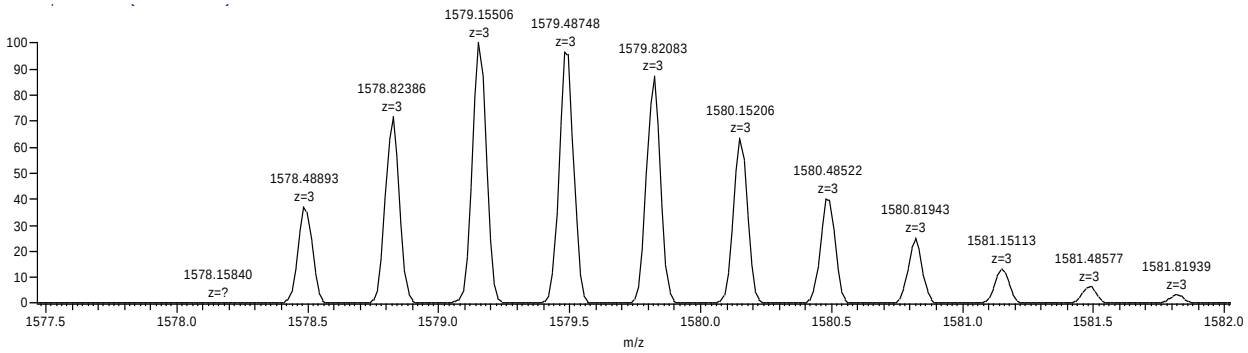


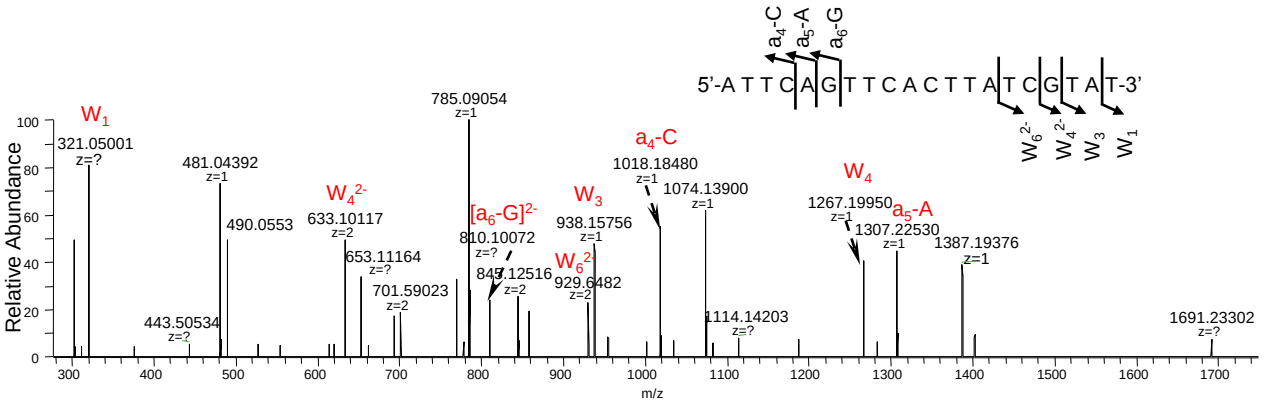
FIGURE 5. Zoom-in MS Spectrum for $[M-3H]^{3-}$ of 15-mer Oligonucleotide (5'-ATT CAG TTC ACT TAT-3')



Targeted MS/MS Quantitation

Four fragmentation ions, in Figure 6, *m/z* 938.1536, 1018.1786, 1267.2055 and 1387.1949 from $[M-4H]^{4-}$ (*m/z* 1513.0074) of 20-mer ODN were used for targeted MS/MS quantitation. While for 15-mer ODN, fragmentation ions *m/z* 968.0706, 833.0189, 1138.0447 and 1306.1107 from $[M-3H]^{3-}$ (*m/z* 1579.4876) precursor ion were chosen (spectrum not shown).

FIGURE 6. HCD MS/MS spectrum of $[M-4H]^{4-}$ (*m/z* 1512.0073) from 20-mer ODN (CE=20)



Calibration Curve

To avoid analyte absorption, three different carriers(see Method) and two sample vials (polypropylene and silanized glass) were evaluated. Silanized glass vials and human plasma in10%MeOH were found to give the least absorption. ODN (20-mer) was then quantified by both SIM and MS/MS methods. SIM method (70,000 resolution) shows 5 fold more sensitivity than the targeted MS/MS method (17,500 resolution) (Figure 7).

FIGURE 7. SIM and MS/MS Chromatograms near LOD

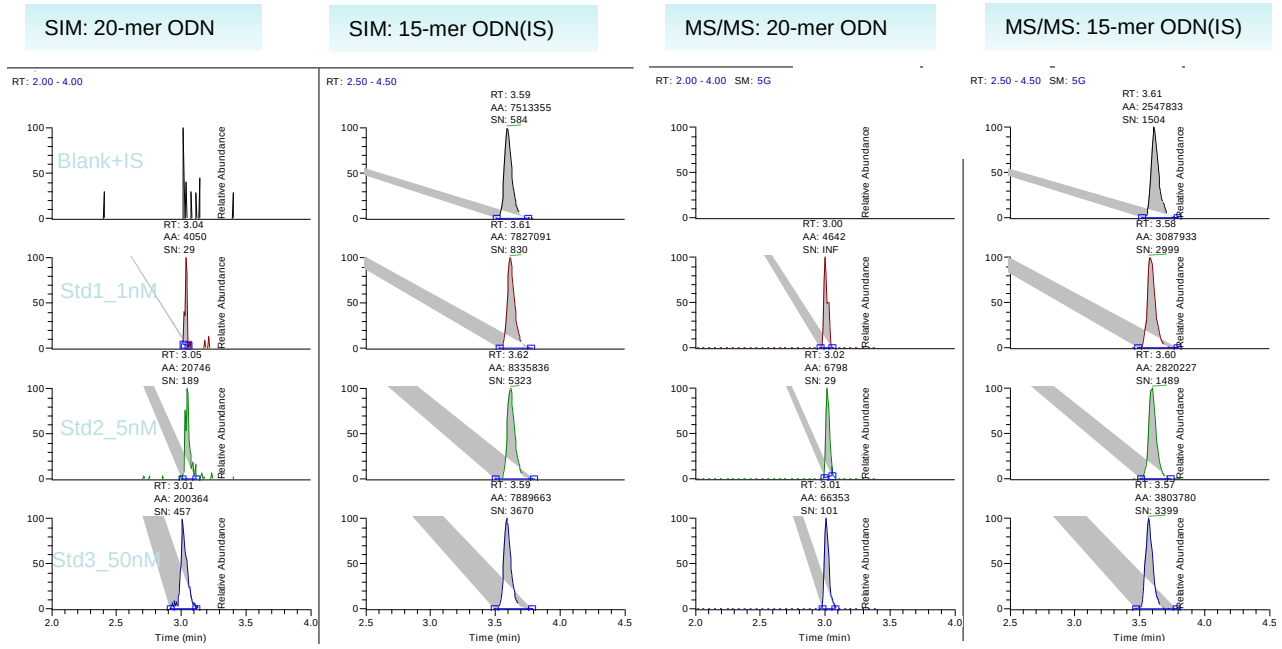


FIGURE 8. Calibration Curve of 20-mer ODN by SIM Method

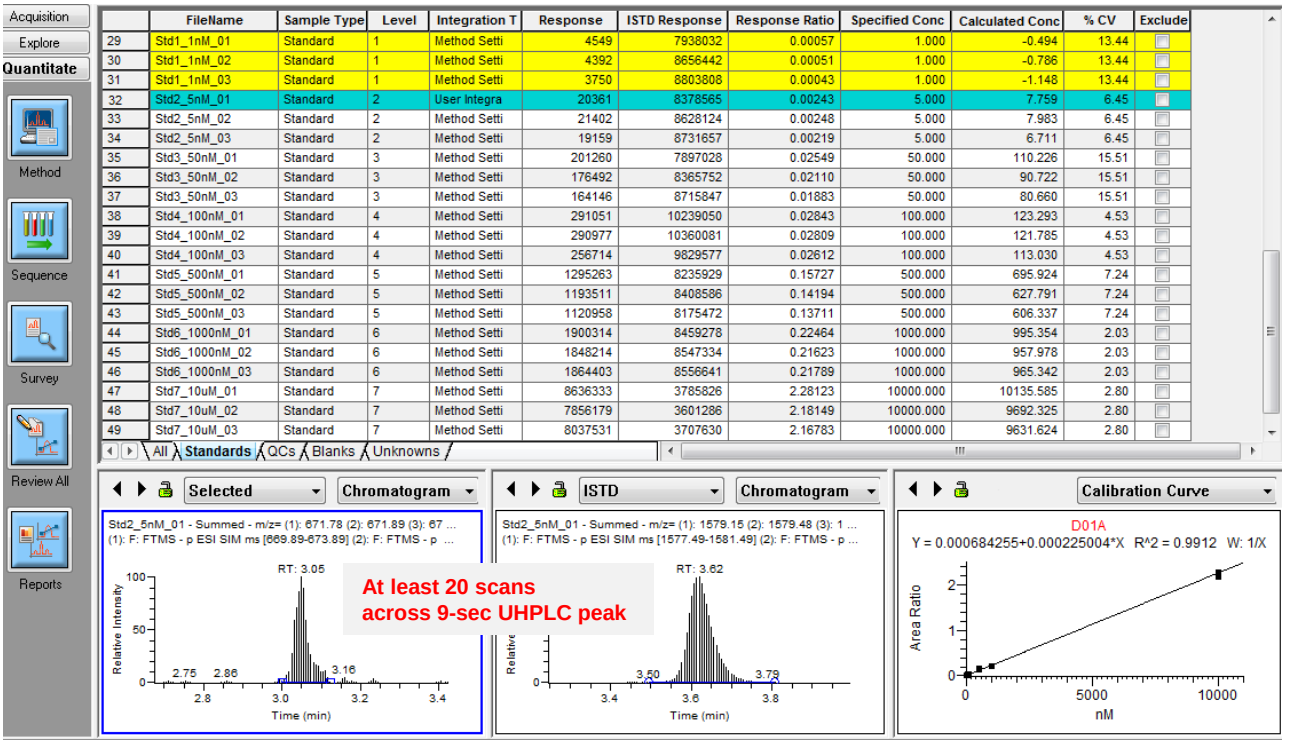
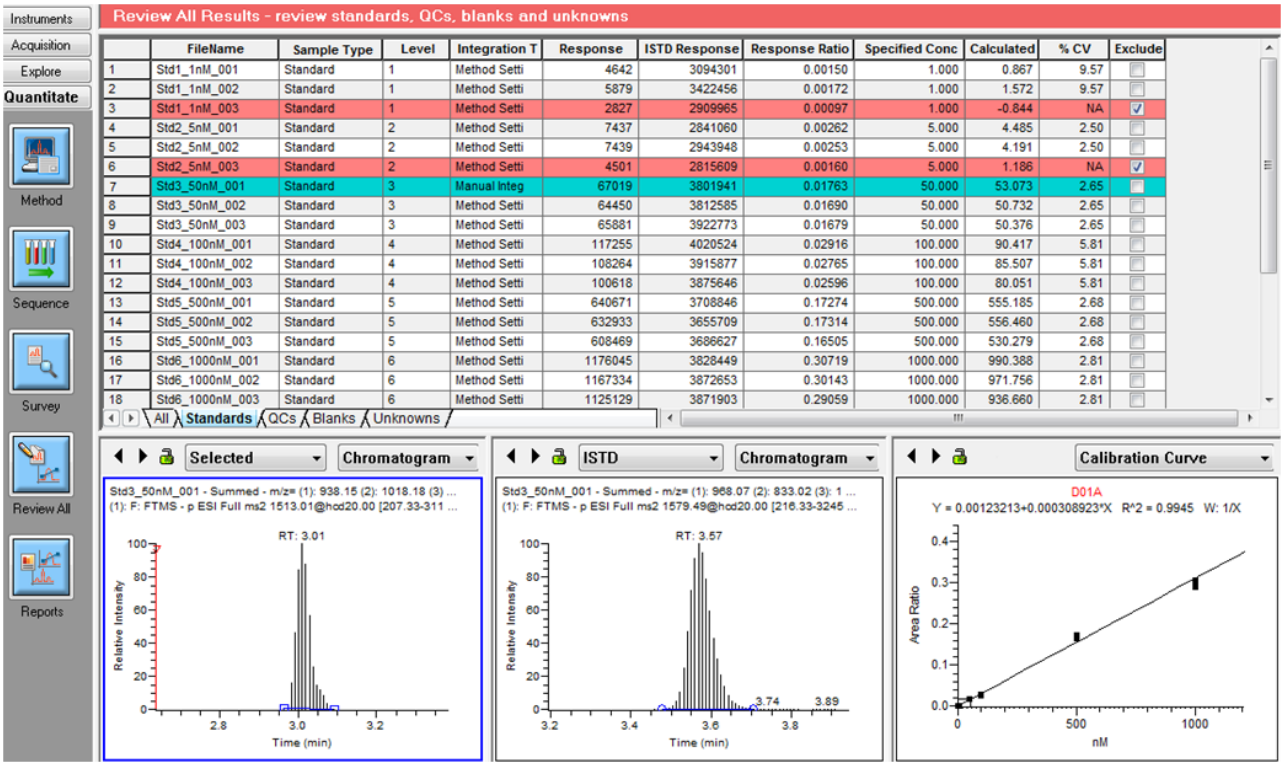


FIGURE 9. Calibration Curve of 20-mer ODN by MS/MS Method



	FileName	Sample Type	Level	Integration T	Response	ISTD Response	Response Ratio	Specified Conc	Calculated	% CV	Exclude
1	Std1_1nM_001	Standard	1	Method Setti	4642	3094301	0.00150	1.000	0.867	9.57	
2	Std1_1nM_002	Standard	1	Method Setti	5879	3422456	0.00172	1.000	1.572	9.57	
3	Std1_1nM_003	Standard	1	Method Setti	2827	2909965	0.00097	1.000	-0.844	NA	✓
4	Std2_5nM_001	Standard	2	Method Setti	7437	2841060	0.00262	5.000	4.485	2.50	
5	Std2_5nM_002	Standard	2	Method Setti	7439	2943948	0.00253	5.000	4.191	2.50	
6	Std2_5nM_003	Standard	2	Method Setti	4501	2815609	0.00160	5.000	1.186	NA	✓
7	Std3_50nM_001	Standard	3	Manual Integ	67019	3801941	0.01763	50.000	53.073	2.65	
8	Std3_50nM_002	Standard	3	Method Setti	64450	3812585	0.01690	50.000	50.732	2.65	
9	Std3_50nM_003	Standard	3	Method Setti	65881	3922773	0.01679	50.000	50.376	2.65	
10	Std4_100nM_001	Standard	4	Method Setti	117255	4020524	0.02916	100.000	90.417	5.81	
11	Std4_100nM_002	Standard	4	Method Setti	108264	3915877	0.02765	100.000	85.507	5.81	
12	Std4_100nM_003	Standard	4	Method Setti	100618	3875646	0.02596	100.000	80.051	5.81	
13	Std5_500nM_001	Standard	5	Method Setti	640671	3708846	0.17274	500.000	555.185	2.68	
14	Std5_500nM_002	Standard	5	Method Setti	632933	3655709	0.17314	500.000	556.460	2.68	
15	Std5_500nM_003	Standard	5	Method Setti	608469	3686627	0.16505	500.000	530.279	2.68	
16	Std6_1000nM_001	Standard	6	Method Setti	1176045	3828449	0.30719	1000.000	990.388	2.81	
17	Std6_1000nM_002	Standard	6	Method Setti	1167334	3872653	0.30143	1000.000	971.756	2.81	
18	Std6_1000nM_003	Standard	6	Method Setti	1125129	3871903	0.29059	1000.000	936.660	2.81	

Conclusion

1. Oligonucleotides were identified by high resolution accurate mass data with charge distribution. Their sequences were confirmed by MS/MS accurate mass spectrum. A 20-mer synthetic ODN was used as a model compound and was quantified by both SIM and targeted MS/MS methods.
2. Samples with human plasma as a carrier indicates good linearity with a calibration range of 1-5nM to10µM for SIM method (R^2=0.9912, Linear 1/X with CV<16%), while 5nM-1µM for MS/MS method (R^2=0.9945, Linear 1/X with CV<10%).
3. SIM method (70,000 resolution) shows 5 fold more sensitive than targeted MS/MS method (17,500 resolution) for the synthetic ODN.
4. There are at least 20 scans across a 9-sec UHPLC peak around LOQ at 70,000 resolution.
5. SIM at 140,000 resolution and targeted MS/MS provide better selectivity for the assay with complex matrices.

Reference

1. Wang LX. Oligonucleotide Bioanalysis: Sensitivity versus Specificity. *Bioanalysis*, 2011,3,1299-1303.
2. Yuan WW, Wang LX, Enriquez C, Meng M, Wang J, Cook K and Bennett P. Quantitation of Oligonucleotides in Human Plasma Using Q-Exactive Orbitrap High Resolution MS. *Poster presentation at 60th ASMS Conference, 2012.*

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