SEQUENCE CONFIRMATION OF OLIGONUCLEOTIDES VIA AUTOMATED TOP-DOWN SPECTRAL ANNOTATION

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OVERVIEW

Sequence analysis / confirmation of full length modified and non-modified oligonucleotides using Spectrum tools. Top-down MS/MS fragment ions are automatically annotated and a report is generated that shows percent sequence coverage, observed mass and ppm mass error calculations.

INTRODUCTION

- Resurgent growth in the development of oligonucleotide-based therapeutics and the regulatory requirements for characterizing these molecules have fueled demand for analytical workflows and software tools that can streamline this work, especially for sequence analysis/confirmation. Top-down characterization workflows allow for sequence confirmation of both non-modified and modified oligonucleotides, however, annotation of oligonucleotide fragments is often done manually, which is both challenging and highly time consuming.
- Here we present three analytical workflows to obtain top-down spectra from modified and non-modified oligonucleotides, and a new software tool called Spectrum that automates the annotation of MS/MS spectra to determine the location of modifications and for overall sequence confirmation.
- We explored the capabilities of this approach with a mixture of oligonucleotides including a standard ssRNA, a backbone and nucleobase modified ssRNA and a backbone modified ssDNA. The four most abundant charge states were analyzed for each oligonucleotide and a collision energy range between 15-65 V was used to generate fragment ions. Higher charge states provided the highest sequence coverage ranging from 80-90 %. By using our Spectrum data analysis tool, fast and automatic annotation of top-down spectra resulted in fast method development and quick turn around of results.

Xevo G2 XS QTof MS system

METHODS

Materials

Oligonucleotides were purchased Integrated DNA Technologies, Coralville Iowa (IDT) including a 20mer RNA with 2'-O-methyl modifications, a 20mer RNA with a phosphothioate backbone and 2'-O-methyl modifications and a 20mer DNA with phosphothioate backbone. Oligonucleotide were reconstituted in RNAse free MiliQ water and diluted to a equimolar RNA_DNA oligonucleotide mixture (RDMix) ,10 µM final concentration for LCMS analysis.

Instrument Conditions

An ACQUITY™ UPLC® I-class system equipped with a Oligonucleotide BEH (ethylene Bridge Hybrid) C18 column (2.1 x 50 mm, 1.7 µm particles) was used to separate the three oligos in the RDMix at a flow rate of 200 µL/min and a column temperature of 60 °C. The mobile phase composition was: Solvent A: 10mM Triethylamine (TEA) and 50mM Hexafluoro-2-propanol (HFIP) in Milli-Q water and Solvent B: 5mM TEA, 25mM HFIP in 50% methanol.

TIME (minutes)	FLOW (ml/min)	%A	%B
0	0.2	90	10
10.0	0.2	60	40
11.0	0.2	40	60
12.0	0.2	40	60
12.1	0.2	90	10
15.0	0.2	90	10

LC Gradient

Experimental Design

LC-MS data was acquired using three different workflows. The first workflow (full MS) acquired all mass spectral information across a m/z range from 500 - 3000 using negative ESI-MS mode. The data was processed to identify the charge states in the range of -3 to –9, (Table III). which were targeted selectively by MS/MS in a second LC-MS analysis using the same LC conditions. The second workflow (automated data dependent MS/MS) was configured to select the four most abundant precursors for MS/MS and subsequent fragmentation analysis. Fixed collision energies were used based upon precursor m/z, with values ranging between 15-55V. The third workflow (MS^E) used data independent acquisition with no precursor ion selection. Alternating low and high energy MS scans result in the simultaneous capture of both precursor ions (low energy) and their corresponding fragment ions (high energy—ramped from 10-55V)(see Table I for detailed parameters).

Workflow	Mass Range m/z	Collision Energy / V
1. Full MS	500 - 3000	6
2. MS/MS	300 - 3000	15 - 55 (Fixed)
3. MS^E	300 - 3000	10 - 55 (Ramped)

Table I. MS range and collision energies applied in the three workflows

Informatics

Masslynx and our Spectrum data analysis tool were used for data acquisition and processing.

RESULTS

Xevo® G2-XS QTof

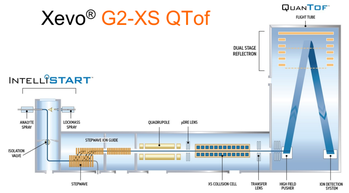

Figure 1. Diagram of the Xevo G2XS QTof mass spectrometer. Data-independent acquisition of alternative low and high collision energy MS scans resulting in MS and MS/MS data collected simultaneously providing top-down data without having to select specific precursors. During data-dependent acquisition, a precursor is isolated by the quadrupole and fragmented with a fixed collision energy in XS collision cell.

Oligo ID	Oligo Sequence
RNA RNA* DNA*	A-G-C-G-C-C-A-G-A-mC-U-mG-A-A-G-A-C-U-G-G A*-G*-C*-G*-C*-C*-A*-G*-A*-mC*-U*-mG*-A*-A*-G*-A*-C*-U*-G*-G A*-G*-C*-G*-C*-C*-A*-G*-A*-C*-T*-G*-A*-A*-G*-A*-C*-T*-G*-G

** Phosphothio backbone*

Table II. Oligonucletide standards contained in analysis mixture. mC and mG represents 2'-O-methyl modifications.

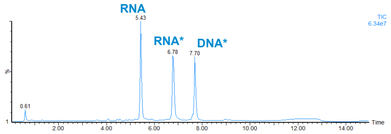

Figure 2. Example chromatogram of RNA-DNA Oligo Mix.

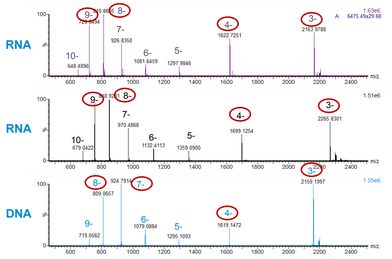

Figure 3. MS Spectra and charge state distribution (selected precursors circled) of each oligo in the RNA-DNA Oligo Mix. RNA with phosphodiester backbone (RNA); Modified RNA with phosphothioate backbone (RNA*); DNA with phosphothioate backbone (DNA*).

Top-Down Spectral Annotation Workflow

Raw High Energy Spectra → MaxEnt3 Deconvolution Monoisotopic Output → Spectrum Data Analysis Tool Automatic Spectral Annotation

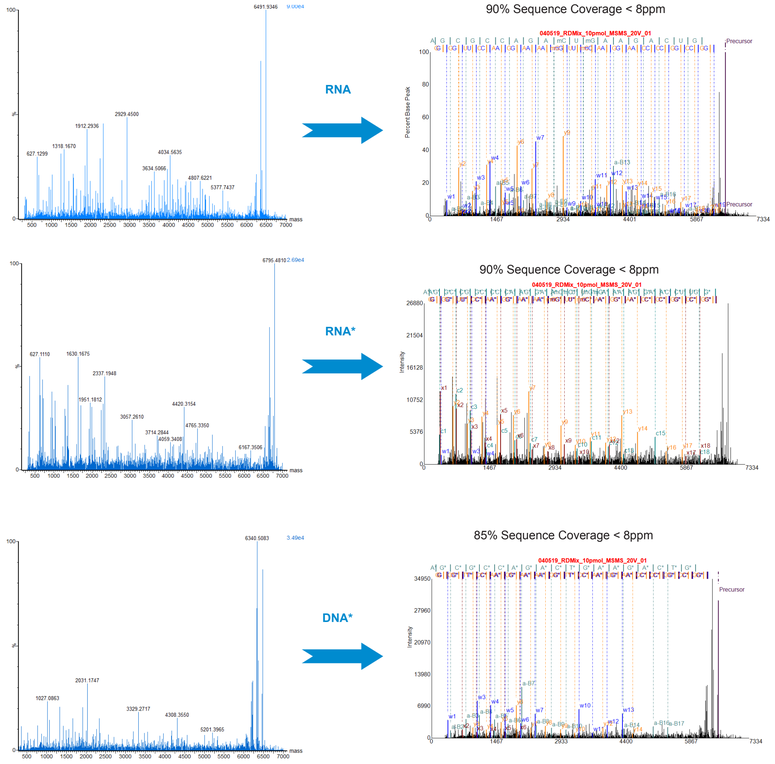

Figure 4. Deconvoluted MS/MS Spectra from selected precursors and their subsequent annotated fragment ions resultant from spectrum data analysis tools processing for RNA, RNA* and DNA*.

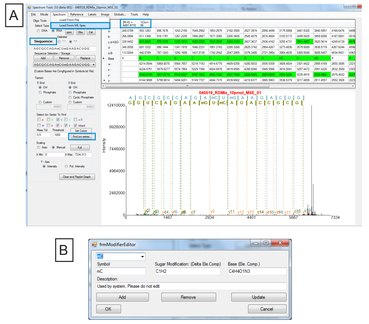

Figure 5. Spectrum data analysis tool (A). Based on the entered oligonucleotide sequence, the spectrum tool generates a comprehensive list of expected fragment ions with both 5' and 3' termini. The deconvoluted MS^E spectra can be automatically imported into Spectrum Tools. Standard and custom modified nucleobases are fully supported (B), Spectrum analysis tool will automatically search for the predicted fragment ions and annotate them when found.

Workflow	m/z	Charge state	%Sequence Coverage (CE/V)		
			RNA	RNA*	DNA*
MS/MS	2163.9788	-3	45 (55)		
	1622.7521	-4	90 (45)		
	810.8605	-8	85 (20)		
	720.6494	-9	**90** (20)		
	2265.8301	-3		30 (55)	
	1699.1254	-4		55 (55)	
	848.9281	-8		**90** (20)	
	745.6031	-9		40 (15)	
	2159.1997	-3			40 (55)
	1619.1472	-4			65 (35)
	924.7914	-7			**85** (20)
	809.0657	-8			70 (20)
MSE	500-3000	-3:-10	85 (10-55)	55 (10-55)	75 (10-55)

Table III. Summary results of the MS/MS and MS^E experiments. The charge state and %sequence coverage in this study are indicated along with the corresponding optimized collision energy.

The charge state and collision energy that provided highest sequence coverage are highlighted in bold.

CONCLUSIONS

- Using our spectrum data analysis tool, we demonstrated a fast, automated process for sequence verification of both non-modified and modified oligonucleotides.
- Extent of fragmentation across charge states was evaluated. Higher charge states (low m/z) showed overall richer fragmentation spectra with lower collision energy applied as compared to lower charge states (high m/z) where higher collision energy was required.
- Data dependent and data independent acquisition showed similar % sequence coverage on the unmodified RNA and modified DNA samples. For the modified RNA sample, data dependent MS/MS resulted in greater sequence coverage.

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