Streamlining workflow from characterization to monitoring of therapeutic oligonucleotides impurities across IPRP-LC-HRAM-MS platforms

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ABSTRACT

Advances in the synthesis of chemically modified oligonucleotides have permitted the development of novel therapeutics. In order to support fast growing therapeutic oligonucleotides programs, sensitive and robust analytical strategies are desired to efficiently characterize and monitor these novel modalities and their impurities during development, manufacturing and quality control. Herein, we developed a system performance evaluation test (SPET) that uses a 6-oligonucleotide mixture to monitor relevant metrics of LC-MS system based on a comprehensive set of acceptance criteria. Upon passing the SPET, the LC-MS system was used to separate a modified synthetic RNA sample and its impurities using a Thermo Scientific™ DNAPac™ RP column on the Thermo Scientific™ Vanquish™ Horizon UHPLC system. The utilization of a high-resolution accurate mass (HRAM) Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer coupled with ion pairing reverse phase LC (IPRP-LC), and Thermo Scientific™ BioPharma Finder™ software 5.0 enabled confident identification and characterization of full-length product (FLP) and impurities. Relative quantification of both FLP and impurities are performed using a full MS based method, which was seamlessly transferred to two fit-for-purpose full MS only Thermo Scientific™ Orbitrap Exploris™ MX mass detectors using Chromeleon eWorkflow procedure.

INTRODUCTION

IPRP-LC-MS2 is required to provide base-by-base sequence confirmation and localization of modifications for complete characterization, whereas full MS is often chosen for identity and impurity test to be deployed in the quality control lab. In this study, HRAM data dependent MS2 (ddMS2) data were acquired on an Orbitrap hybrid system coupled with IPRP-LC for characterizing synthetic oligonucleotide full length products (FLPs) and their impurities. The full MS based monitoring assay was subsequently created and transferred to two HRAM full MS systems. Seamless method transfer was demonstrated by obtaining comparable results across 3 systems.

MATERIALS AND METHODS

Sample Preparation

For SPET, oligonucleotide mixture ranging from 10mer to 55mer was obtained from Life Technologies, Paisley, United Kingdom. For modified RNA characterization and monitoring experiments, desalted and HPLC purified modified single stranded RNA was obtained from Integrated DNA Technologies.

HPLC Conditions

For SPET, 25pmol of the oligonucleotide mixture was injected onto a Thermo Scientific DNAPac RP columns and separated chromatographically using a Thermo Scientific Vanquish Horizon UHPLC system. UV data was also collected at 260nm at 20Hz. Similarly, 1µg of RNA sample was used for characterization and monitoring experiments. Gradient details are outlined in Table 1 below.

Table 1. LC methods for SPET and oligonucleotide characterization and monitoring

	System performance evaluation test (SPET)	Oligonucleotide characterization and monitoring
UHPLC column	Thermo Scientific™ DNAPac™ RP 2.1 x 50 mm, 4 µm (P/N 088924)	Thermo Scientific™ DNAPac™ RP 2.1 x 250 mm, 4 µm (P/N 303324)
Flow Rate	0.2 mL/min	0.3 mL/min
Solvent A	water with 15mM TEA and 50mM HFIP	water with 30mM TEA and 100mM HFIP
Solvent B	50% methanol	50% methanol with 30mM TEA and 100mM HFIP
Gradient	Time (min) %B 0 10 1 10 11 25 11.5 90 14 90 14.5 10 20 10	Time (min) %B 0.0 5 1 5 42 25 45 90 60 90 60.5 5 75 5
Injection Volume	1 µL	10 µL
Thermostatting mode	Still Air	
Column oven temperature	50°C	

Table 2. Source and MS setting for oligonucleotide characterization and monitoring

Instrument	Orbitrap Exploris 240 Mass Spectrometer	Orbitrap Exploris MX Mass Detector
MS source parameters		
Negative ion (V)	2500	Same as Orbitrap Exploris 240
Sheath Gas (Arb)	35	
Aux Gas (Arb)	10	
Sweep Gas (Arb)	0	
Ion transfer tube temperature (°C)	300	
Vaporizer temperature (°C)	150	
Full scan parameters		
Expected LC Peak Width (s)	6	Same as Orbitrap Exploris 240
Resolution	120,000	
Scan range (m/z)	420 – 1600	
Time range (min)	1 – 50 minutes	
AGC target	1E6	
Maximum injection time (ms)	50	
Data dependent MS² scan parameters		
MIPS filter		Not applicable
Monoisotopic peak determination	Peptide	
Intensity filter		
Intensity threshold (counts)	5.0e4	
Charge states filter		
Include charge state(s)	2-50	
Dynamic Exclusion filter		
Dynamic exclusion mode	Custom	
Exclusion duration (s)	8	
dd-MS² scan		
Isolation window (m/z)	2	
HCD collision energy (%)	17,20,23	
Resolution	30000	
AGC target	3e6	
Maximum injection time (ms)	100	
Microscans	3	

MS Conditions

For characterization experiments, data was collected using a Thermo Scientific Orbitrap Exploris 240 mass spectrometer. Sample analysis was performed using data dependent MS/MS acquisition. For monitoring experiments, data was collected using an Thermo Scientific Orbitrap Exploris MX mass detector. The MS source and scan settings for both systems are outlined in Table 2.

Data Processing

Data analyses was performed with Thermo Scientific BioPharma Finder 5.0, using the oligonucleotide sequencing workflow. Enterprise compliance ready Thermo Scientific™ Chromeleon™ CDS 7.3.1 software was used for all instrument control, data acquisition, processing, and reporting.

RESULTS

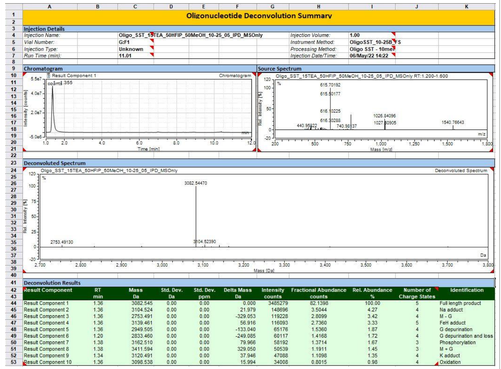
System performance evaluation test

The SPET evaluates LC-MS system performance by measuring system performance metrics relating to the LC and the MS systems across 10 replicate injections of the oligonucleotide standard mixture. Table 3 outlines the system performance metrics and their predetermined acceptance criteria. The SPET only passes when all measured system performance metrics for all monitored oligonucleotides pass. In addition to system performance metrics evaluation, SPET performs an intact mass deconvolution on all oligonucleotides. The source spectrum was generated by averaging the full MS scans across a retention time window for each oligonucleotide. The resultant components are identified based on the measured delta mass, and relative abundance of those components are measured using the deconvoluted spectrum for each oligonucleotide. Figure 1 shows an example of oligonucleotide deconvolution report summary for the 10mer.

Table 3. SPET details including monitored oligonucleotide sequences, related LC and MS system performance metrics, and associated acceptance criteria for each metric

Performance check	Oligonucleotides Sequence	System performance metrics	Acceptance criteria
LC-MS test	1. GAG CGG CTG T **(10mer)** 2. GAG CGG CTG TGA GCG GCT GT **(20mer)** 3. GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT **(30mer)** 4. GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT GAG CGG CTG T **(40mer)** 5. GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT GAG CGG CTG TGA GCG GCT GT **(50mer)** 6. GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT GAG CGG CTG TGA GCG GCT GTG AGC G **(55mer)**	• Retention time reproducibility • Peak area reproducibility • Peak height reproducibility • Peak height range • Peak width at 10% height reproducibility • Peak width at 10% height	• RT %RSD ≤ 2% • Peak area %RSD ≤ 10% • Peak height %RSD ≤ 10% • Peak height range between 2E7 to 2E8 counts • Peak width at 10% height %RSD ≤ 10% • Peak width at 10% height ≤ 0.5 min
Intact mass deconvolution		• Mass accuracy of deconvoluted FLP mass • Relative abundance of Na and K adducts	• Mass accuracy of deconvoluted FLP mass ≤ 5 ppm • Relative abundance of Na and K adducts ≤ 20%

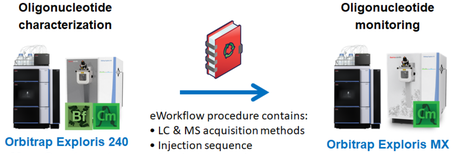
Figure 1. Example of oligonucleotide deconvolution summary report for 10mer. The report includes injection details, an extracted ion chromatogram of full-length product, averaged source spectrum over a retention time window, overall deconvoluted spectrum, and deconvolution results showing top 10 most abundant components.



Oligonucleotide Workflow

The oligonucleotide workflow consists of 2 steps as illustrated in Figure 2. In the first step, Thermo Scientific Orbitrap Exploris 240 mass spectrometer coupled with IPRP-LC was used for separating the synesthetic RNA and its impurities. Thermo Scientific Biopharma Finder software 5.0 was used to process the data dependent MS2 data for confident identification and characterization of the FLP and impurities. Next, routine monitoring of the relative abundance of the FLP and impurities were performed using a full MS scan method. Due to harmonization of the hardware and instrument control software across the Orbitrap Exploris platform, Chromeleon eWorkflow procedure was deployed to facilitate direct method transfer from Thermo Scientific Orbitrap Exploris 240 mass spectrometer setup to two fit-for-purpose full MS only Thermo Scientific Orbitrap Exploris MX mass detectors for routine monitoring of oligonucleotides.

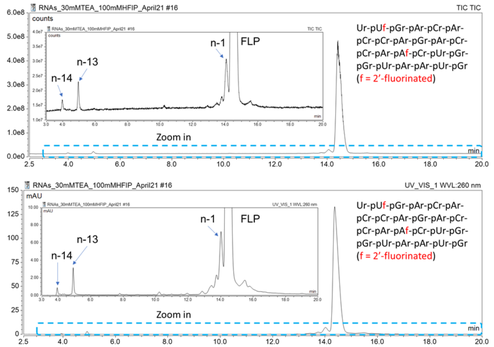
Figure 2. Schematic overview of the oligonucleotide workflow from oligonucleotide characterization to monitoring



Chromatographic Separation

Chromatographic separation of RNA sample was achieved using a 42-minute gradient ranging from 5% to 25% solvent B. Using this gradient, we could separate the full-length product (FLP) from the impurities resulting from 5' truncation (n-x). Both UV trace (260nm) and total ion chromatogram (TIC) from full MS data were plotted, resulting in identical impurity profiles as shown in Figure 3 for 2'-fluorinated 24mer RNA (2'F-24mer).

Figure 2. Impurity profiles for 2'F-24mer. TIC based impurity profile is shown on the top and UV based (260nm) impurity profile is shown on the bottom. Low level impurities were identified using BPF 5.0 with a confidence score of 80 or higher and mass accuracy of the measured monoisotopic mass within 3ppm of the theoretical.



Oligonucleotide Characterization

Thermo Scientific BioPharma Finder 5.0 provides interactive report and automated tools for identification and mapping of the oligonucleotide sequences. The monoisotopic mass and the MS2 fragmentation pattern of the identified components are compared to the predicted oligonucleotide components. A confidence score is provided based on the evaluation of mass accuracy, isotopic distribution, charge state determination, and correlation between the predicted and measured fragmentation pattern. The software also calculates an average structural resolution (ASR) value, which in an ideal case, all bonds between each individual nucleotide residue has been broken and resulting fragment ions matched the predicted MS/MS spectra. The combination of high confidence score with low delta mass ppm deviation and a low ASR value (e.g., 1.0) gives strong confidence in the sequence being correctly matched. Figure 3 shows the confident identification of 2'F-24mer FLP as multiple charge states (from -5 to -11) yielded an ASR value of 1.0 with mass deviation of less than 3ppm between the measured and the theoretical monoisotopic mass of the FLP. Figure 4 illustrates the identification of FLP and N-1 truncated impurity based on intact mass deconvolution and matching of experimental against predicted MS/MS spectra for identified charge states.

Figure 3. Data processing results of 2'F-24mer using Thermo Scientific BioPharma Finder 5.0. Selected ion chromatogram (top) and TIC (bottom) of 2'F-24mer sample are shown in top left panel. Fragment coverage map of FLP based on MS/MS spectra of -6 charge state is shown in top right panel. Component result table listing the identified oligonucleotide components is shown on the bottom panel.

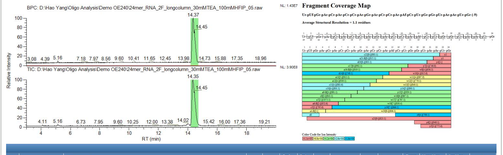
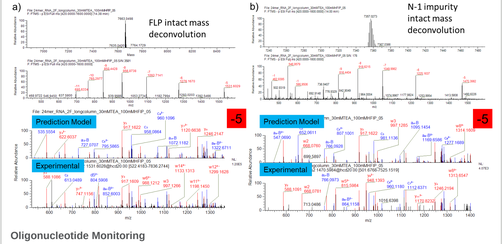


Figure 4. Identification of 2'F-24mer a) FLP and b) N-1 truncated impurity using Thermo Scientific BioPharma Finder 5.0. Identification is based on mass deviation of the measured monoisotopic mass of deconvoluted spectrum resulting from all identified charge states, as well as an overall correlation of the experimental and predicted MS/MS spectra for the identified charge states.



Oligonucleotide Monitoring

Traditionally, oligonucleotide impurity profiling is performed using LC-UV data. This method requires baseline separation of all components with optimized LC condition for consistent quantitation. However, with the LC-HRMS method, we can quantify the relative abundance by taking the peak area sum of observed isotopes for identified charge states of a given component and divide by the total peak area sum of all components. For consistency, the top 5 most abundant isotopes for each identified charge states were monitored in this study. The resulting relative abundance levels were compared with the relative abundance levels measured based on LC-UV data (i.e., peak area of the component over the peak area sum of all identified components). As shown in Table 4, not only LC-HRMS-MS method gave comparable % relative abundance for all identified components against the results obtained from LC-UV, it exhibits excellent consistency within replicate injections (N=3). To demonstrate ease of use, Chromeleon eWorkflow procedure was deployed in parallel on two fit-for-purpose full MS only Thermo Scientific Orbitrap Exploris MX mass detectors for quantitative evaluation of the relative abundance of the FLP and impurities. As shown in Table 5, not only the results obtained on two Thermo Scientific Orbitrap Exploris MX mass detectors are comparable to the results obtained on Thermo Scientific Orbitrap Exploris 240 mass spectrometer, but they are also reproducible with minimal instrument to instrument variation.

Table 4. Evaluation of % relative abundance for 2'F-24mer FLP and impurities using LC-UV and LC-HRMS-MS method. Only full MS scan data collected on Thermo Scientific Orbitrap Exploris 240 mass spectrometer is shown here.

Components	LC-UV (N=3)		LC-HRMS-MS (N=3)	
	% Relative abundance	%RSD	% Relative abundance	%RSD
FLP	94.4	0.2	97.4	0.1
N-1	4.5	2.0	1.7	2.0
N-13	0.83	4.4	0.62	8.1
N-14	0.26	26.8	0.25	9.1

Table 5. Evaluation of % relative abundance for 2'F-24mer FLP and impurities across 3 Thermo Scientific Orbitrap Exploris MS instruments. Only full MS scan data was used and the reported % relative abundance is the average of 3 replicate injections.

Components	% Relative abundance		
	Orbitrap Exploris 240 Mass Spectrometer	Orbitrap Exploris MX Mass Detector #1	Orbitrap Exploris MX Mass Detector #2
FLP	97.4	97.6	97.7
N-1	1.7	1.64	1.65
N-13	0.62	0.54	0.48
N-14	0.25	0.19	0.16

CONCLUSIONS

- A SPET was developed to evaluate system performance metrics related to LC and MS systems
- A LC-HRMS-ddMS2 method was developed to mass resolve oligonucleotide impurities using the Orbitrap Exploris 240 mass spectrometer
- Complete characterization and identification of oligonucleotide impurities were enabled through the use of BioPharma Finder 5.0 software
- Combination of low delta mass deviation of the measured monoisotopic mass and good correlation of the experimental MS/MS fragmentation against the predicted spectra gave confident identification to the impurities and FLP
- A LC-HRMS-MS method was developed to monitor the % relative abundance of identified impurities using the Orbitrap Exploris 240 mass spectrometer and Orbitrap Exploris MX mass detector
- Chromeleon eWorkflow procedure facilitated direct method transfer between Orbitrap Exploris platforms for reproducible quantitation results

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