Application note | 002270

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Pharma

Automated, compliance-ready, intact mass confirmation of modified oligonucleotides using an LC-MS platform

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Keywords

Oligonucleotide analysis, intact mass confirmation, average mass, ion-pairing reversed-phase liquid chromatography (IPRP-LC), Vanquish Flex Binary UHPLC, ISQ EM single quadrupole mass spectrometer, DNAPac reversed-phase column, Chromeleon Chromatography Data System (CDS)

Application benefits

- Fast and automated mass confirmation for oligonucleotides using LC-UV-MS based intact mass analysis with the compliance-ready Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS)
- Using the eWorkflow™ feature in Chromeleon CDS together with the Thermo Scientific™ Vanquish™ Flex UHPLC and Thermo Scientific™ ISQ™ EM single quadrupole mass spectrometer, the entire workflow is streamlined from sample, sequence, data generation, data processing, to reporting

Goal

Demonstrate the implementation of an eWorkflow procedure on a UHPLC-UV-MS system operated under compliance-ready Chromeleon CDS for automated mass confirmation of modified oligonucleotides from sample to report

Introduction

Therapeutic oligonucleotide characterization is an important component of drug development and manufacturing. Given the diversity of the types of oligonucleotides, a robust and accurate analytical method is needed to confirm the identity and determine purity for quality control needs. Herein, we describe an intact mass analysis workflow which uses LC-UV-MS analysis along with the eWorkflow feature in Chromeleon CDS for average mass confirmation of a set of five 20mer phosphorothioated oligonucleotides

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comprising the identical base sequence, containing a varying level from one to five phosphodiester modifications (Table 1). Samples were analyzed by ion-pairing reversed phase liquid chromatography using a trap-and elute technique followed by data acquisition on the ISQ EM single quadrupole mass spectrometer. The eWorkflow procedure in Chromeleon CDS provides a compilation of instrument methods, sequence setup, view settings, processing method, report template, and the injection sequence table that can be, but does not have to be, populated with sample-specific details, such as sample names, injection volume, target masses, and target mass tolerance, resulting in either a sample-specific or rather generic eWorkflow. After the eWorkflow procedure is created, it can be shared among users across sites and deployed for automatic data generation from sample to report.

Here, we demonstrate the implementation of a generic eWorkflow for the analysis of oligonucleotides only requiring input of sample-specific details (Figure 1).

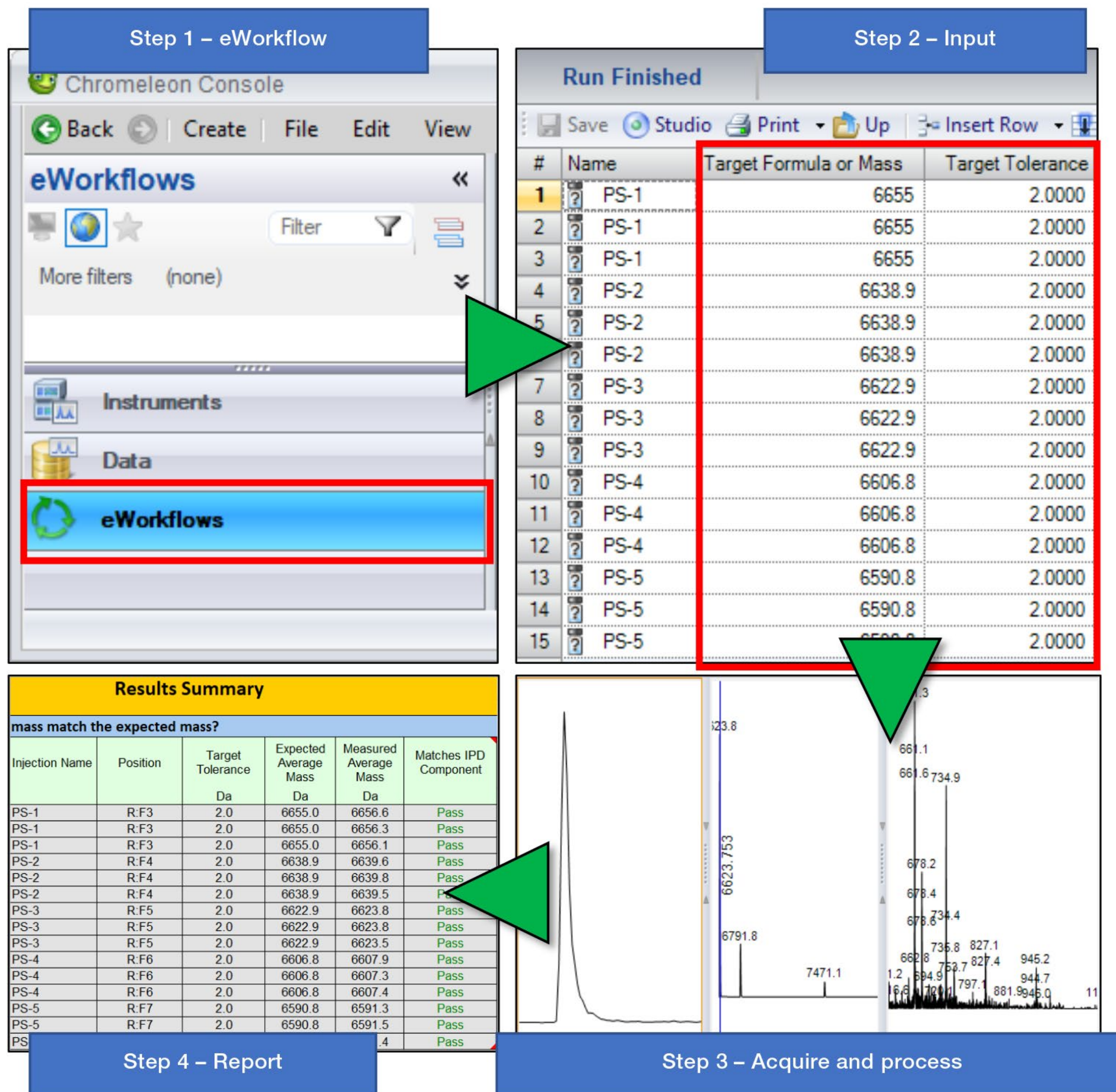

Figure 1. Represented is the step-by-step workflow for oligonucleotide mass confirmation. Step 1 is the loading of the eWorkflow procedure including all pertinent methods required for analysis automation. Step 2 is populating the injection sequence table with sample names, injection volumes, as well as target masses and tolerances imported from the oligonucleotide mass calculator in Thermo Scientific™ BioPharma Finder™ 5.2 software. Step 3 is raw data acquisition and automated data deconvolution and processing. Step 4 is the automatically generated and concise report.

Experimental

Reagents and consumables

- Oligonucleotide samples, HPLC purified, see Table 1 for sequence information (Integrated DNA Technologies)
- 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), 99.9% (Thermo Scientific Chemicals, P/N AC293410500)
- Triethylamine (TEA), 99% (Thermo Scientific Chemicals, P/N 157911000)
- Thermo Scientific™ DNAPac™ RP HPLC column, 2.1 × 50 mm, 4 µm (P/N 088924)
- Thermo Scientific™ Water, UHPLC-MS grade (P/N W8-1)
- Thermo Scientific™ Methanol, UHPLC-MS grade (P/N A458-1)
- Thermo Scientific™ 9 mm Screw Thread Vials, Polypropylene, 12 × 32 mm, 400 µL (P/N C4000-11)
- Thermo Scientific™ 9 mm Autosampler Vial Screw Thread Caps, Polypropylene (P/N C5000-50)

Sample preparation

The dry samples PS_1, PS_2, PS_3, PS_4, and PS_5 (Table 1) were reconstituted individually in UHPLC-MS grade water yielding a concentration of ~2.0 pmol/µL.

Table 1. Oligonucleotide sample sequences, chemical formula, and theoretical average mass. Both chemical formula and theoretical average mass were obtained from the oligonucleotide mass calculator in BioPharma Finder 5.2 software. ATCG: DNA bases; U: Uracil; r: ribose; p: phosphodiester bond; s: phosphorothioate bond

Oligonucleotide name	Sequence*	Average mass (Da)
PS_1	Ur-sAr-sCr-sAr-sGr-sCr-sAr-sUr-sCr-sGr-sGr-sCr-sCr-sUr-sGr-sGr-sAr-sCr-sAr-pUr	6655.0
PS_2	Ur-pAr-sCr-sAr-sGr-sCr-sAr-sUr-sCr-sGr-sGr-sCr-sCr-sUr-sGr-sGr-sAr-sCr-sAr-pUr	6638.9
PS_3	Ur-pAr-sCr-sAr-sGr-sCr-sAr-sUr-sCr-sGr-sGr-sCr-sCr-sUr-sGr-sGr-sAr-pCr-sAr-pUr	6622.9
PS_4	Ur-pAr-sCr-pAr-sGr-sCr-sAr-sUr-sCr-sGr-sGr-sCr-sCr-sUr-sGr-sGr-sAr-pCr-sAr-pUr	6606.8
PS_5	Ur-pAr-sCr-pAr-sGr-sCr-sAr-sUr-sCr-sGr-sGr-sCr-sCr-sUr-sGr-pGr-sAr-pCr-sAr-pUr	6590.8

Instrumentation

Thermo Scientific™ Vanquish™ Flex UHPLC system consisting of:

- System Base Vanquish Horizon/Flex (P/N VF-S01-A-01)
- Vanquish Binary Pump F (P/N VF-P10-A-01)
- 35 µL Mixer kit (P/N 6044.3870)
- Vanquish Split Sampler FT (P/N VF-A10-A-02)
- Vanquish Column Compartment H (P/N VH-C10-A-02)
- Active Pre-column heater (P/N 6732.0110)
- Variable Wavelength Detector F (P/N VF-D40-A)
- 2.5 µL SST flow cell (P/N 6077.0360)
- 2-Position/6-Port switching valve (1,500 bar) (P/N 6036.2520)
- ISQ EM single quadrupole mass detector (P/N ISQEM-ESI)

Chromatography

The Vanquish Flex UHPLC system was used for ion-pairing reversed-phase (IPRP) LC separation of the oligonucleotide samples. The used high-throughput trap-and-elute chromatographic method was previously published.[1] For all sample analyses, 2 µL of sample was injected onto the DNAPac RP column. Instrument method settings are provided in Table 2.

Table 2. LC-UV-MS chromatographic conditions for the trap-and-elute technique used to analyze the set of oligonucleotides

Parameter	Value		
Column	DNAPac RP 2.1 x 50 mm, 4 µm (P/N 088924)		
Mobile phase	A: HFIP 0.1%, 0.1% TEA, in water B: HFIP 0.1%, 0.1% TEA, in MeOH		
Gradient	Time (min)	Solvent A (%)	Solvent B (%)
	0	99	1
	0.4	99	1
	0.4	75	25
	1.0	75	25
	1.0	0	100
	1.6	0	100
	1.6	99	1
	4	99	1
Flow rate	0.7 mL/min		
Column temperature	70 °C, forced air mode 70 °C, active pre-heater		
Autosampler temperature	4 °C		
Autosampler wash solvent	10% MeOH in water		
Injection volume	2 µL		
UV detector settings	λ = 260 nm, 100 Hz		

Mass spectrometry

A full scan method was applied using the ISQ EM single quadrupole mass spectrometer and the optimized conditions using 0.1% HFIP and 0.1% TEA as the ion pairing additive concentrations. Additionally, the previously developed MS settings are provided in Table 3.[1]

Table 3. ISQ EM single quadrupole mass spectrometer source and scan settings used to analyze the array of oligonucleotides

HESI source settings	
Vaporizer temperature	350 °C
Ion transfer tube temperature	350 °C
Source voltage	-3,000 V
Sheath gas pressure	75 psig
Aux gas pressure	7.5 psig
Sweep gas pressure	0 psig
Scan settings	
Mass range	600–2,000 *m/z*
Dwell/scan time	0.5 s
Polarity	Negative
Spectrum type	Profile
Source CID voltage	0 V

Target mass and tolerance in injection sequence table

To be able to automate the comparison of the theoretical target mass with a specified tolerance to the measured average mass, one needs to enter the target mass and tolerance in the injection sequence table. To do this, the two optional table columns *Target Formula or Mass* and *Target Tolerance* are made visible in the injection table. It is important to note that for unit resolution data using the ReSpect™ algorithm for the deconvolution step, the target average mass has to be entered in the injection table as seen in Figure 2.

Intact mass analysis using Chromeleon CDS

Full scan data were processed using the ReSpect deconvolution algorithm available in the Intact Protein Deconvolution (IPD) feature in Chromeleon CDS version 7.3.2. The source spectra were generated by automatic averaging across a retention time range from 0.70 to 0.90 minutes. The deconvolution settings are listed in Table 4, with most parameters representing default settings.

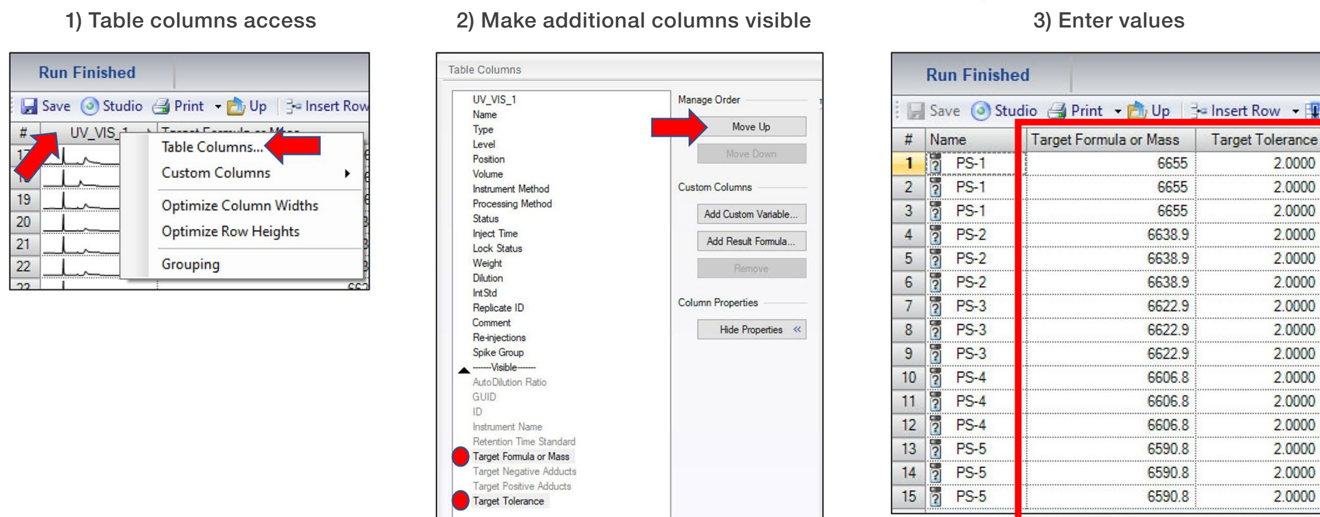

Figure 2. Adding the necessary additional table columns in the injection sequence table. 1) Right-click on a table heading and select the menu option *Table Columns.* 2) Select the two optional table columns *Target Formula or Mass* and *Target Tolerance* in the *Visible* section. Click the Move Up button until the two column options are placed above the *Visible* section and in a position that is desired. 3) The two additional columns are now present in the injection sequence table. Enter here the expected average masses and the tolerance(s).

Table 4. Parameter settings applied for the spectral deconvolution step

Parameter	Value
Peak retention window	0.7–0.8 min
Algorithm	ReSpect
Output mass range	2,000–20,000
Deconvoluted spectra display mode	Isotopic profile
Model mass range	2,000–160,000 Da
Deconvoluted Mass Tolerance	20 ppm
Peak model	Nucleotide
Resolution	Raw file specific
Charge carrier	H^+
Charge high	30
Charge low	2
High number adjacent charges	1
Low number adjacent charges	2
Intensity threshold scale	0.01
Min peak significance	2
Negative charge	True
Noise compensation	True
Noise rejection	No noise
Number of peak models	1
Peak model width scale	1
Quality score threshold	0
Relative abundance threshold	5
Target peak mass	10,000
Target peak shape left	2
Target peak shape right	2

Results and discussion

All samples were analyzed by LC-UV-MS using a generic eWorkflow that required input of sample-specific details in the injection sequence table for deployment. The chromatographic step utilizes a simple trap-and-elute technique that first loads the oligonucleotide onto the column; for the first 24 seconds without MS acquisition, a valve diverts this portion of the chromatography to waste which washes the non-retaining impurities from the column without contaminating the mass spectrometer. Next, the oligonucleotides are eluted using a step gradient. All oligonucleotides eluted at almost the same retention time without separation of impurities, which would require a longer gradient to separate from the target oligonucleotide as shown in Figure 3. The data processing comprising the steps from the chromatographic peak to the mass spectrum followed by the deconvolution step is showcased in Figure 4 based on the data obtained for PS-2. The source spectrum was automatically generated by averaging full scan data across the peak in the total ion chromatogram (TIC) with a retention time ranging from 0.7 to 0.9 minutes.

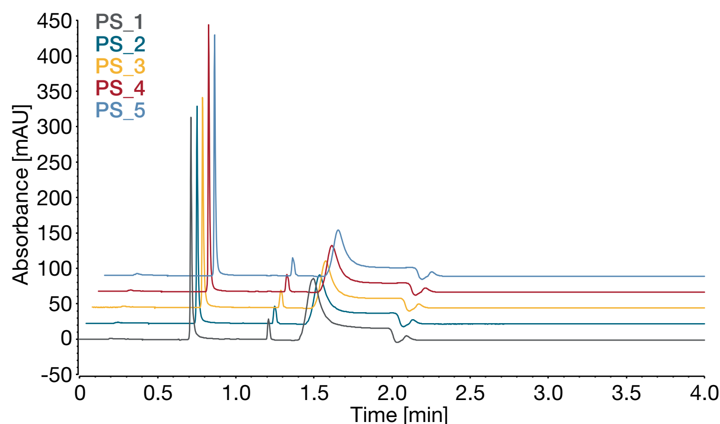

Figure 3. UV chromatograms of each modified oligonucleotide highlighting the high-throughput trap-and-elute technique

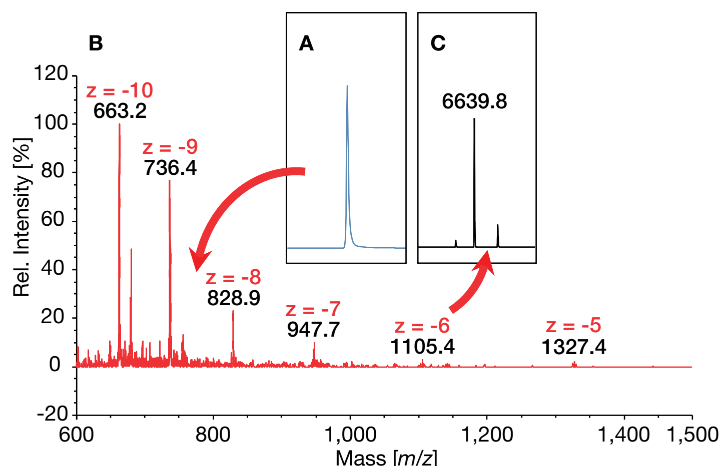

Figure 4. Averaging the full scan data in the retention time ranging from 0.7 to 0.9 minutes across the chromatographic peak (A) resulting in the the source spectrum (B). From the source spectrum, the deconvoluted average mass is generated (C), here represented by the PS_2 sample

As shown in Figure 5, an intact mass analysis summary report was generated by applying small modifications to the default oligonucleotide report template provided with the Chromeleon CDS version 7.3.2 software. In this report, both expected and measured average mass as well as the target mass tolerance for all tested samples are shown. Intact mass analysis of partially phosphorothiolated RNA with 20 nucleotide bases was evaluated using this automated average mass confirmation workflow. This study highlights the simple, high-throughput, compliance ready, intact mass confirmation from sample to a concise report based on a generic eWorkflow procedure, allowing for a quick mass confirmation assessment by using the pass/fail result provided for each individual sample analyzed.

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Results Summary					
mass match the expected mass?					
Injection Name	Position	Target Tolerance Da	Expected Average Mass Da	Measured Average Mass Da	Matches IPD Component
PS-1	R:F3	2.0	6655.0	6656.6	Pass
PS-1	R:F3	2.0	6655.0	6656.3	Pass
PS-1	R:F3	2.0	6655.0	6656.1	Pass
PS-2	R:F4	2.0	6638.9	6639.6	Pass
PS-2	R:F4	2.0	6638.9	6639.8	Pass
PS-2	R:F4	2.0	6638.9	6639.5	Pass
PS-3	R:F5	2.0	6622.9	6623.8	Pass
PS-3	R:F5	2.0	6622.9	6623.8	Pass
PS-3	R:F5	2.0	6622.9	6623.5	Pass
PS-4	R:F6	2.0	6606.8	6607.9	Pass
PS-4	R:F6	2.0	6606.8	6607.3	Pass
PS-4	R:F6	2.0	6606.8	6607.4	Pass
PS-5	R:F7	2.0	6590.8	6591.3	Pass
PS-5	R:F7	2.0	6590.8	6591.5	Pass
PS-5	R:F7	2.0	6590.8	6591.4	Pass

Figure 5. Intact mass analysis summary report generated from data obtained from a set of five 20mer phosphorothioated oligonucleotides (PS-1 to PS-5) acquired on the ISQ EM single quadrupole mass spectrometer

Conclusions

Here, we demonstrate the deployment of a generic eWorkflow procedure for the compliance-ready intact mass confirmation of a set of five phosphorothioated oligonucleotide samples.

- The fully automated workflow from sample to report was used to quickly evaluate the achieved mass accuracy based on the expected mass and defined mass tolerance provided in the injection sequence table prior to deploying the generic eWorkflow.
- Obtained masses upon deconvolution of the Full MS spectra (using the ReSpect algorithm implemented in Chromeleon CDS) resulted in mass accuracies ranging from 0.4 to 1.6 Da with an average of 0.77 Da, which was in all cases well below the set mass tolerance of 2 Da, resulting in a "Pass" result for all samples analyzed as reflected in the summary report.
- The eWorkflow can be downloaded in the Thermo Scientific™ AppsLab library. It can be readily deployed on a platform consisting of the LC-MS components described in this study, only requiring the population of the injection sequence table with the user´s sample-specific details regarding sample name, injection volume, target mass, and target mass tolerance.

Reference

1. Thermo Fisher Scientific, Application Note 000457: High-throughput analysis of oligonucleotides using a single quadrupole mass spectrometer for quality control. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-000457-hplc-vanquish-flex-isq-oligonucleotide-an000457-na-en.pdf

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