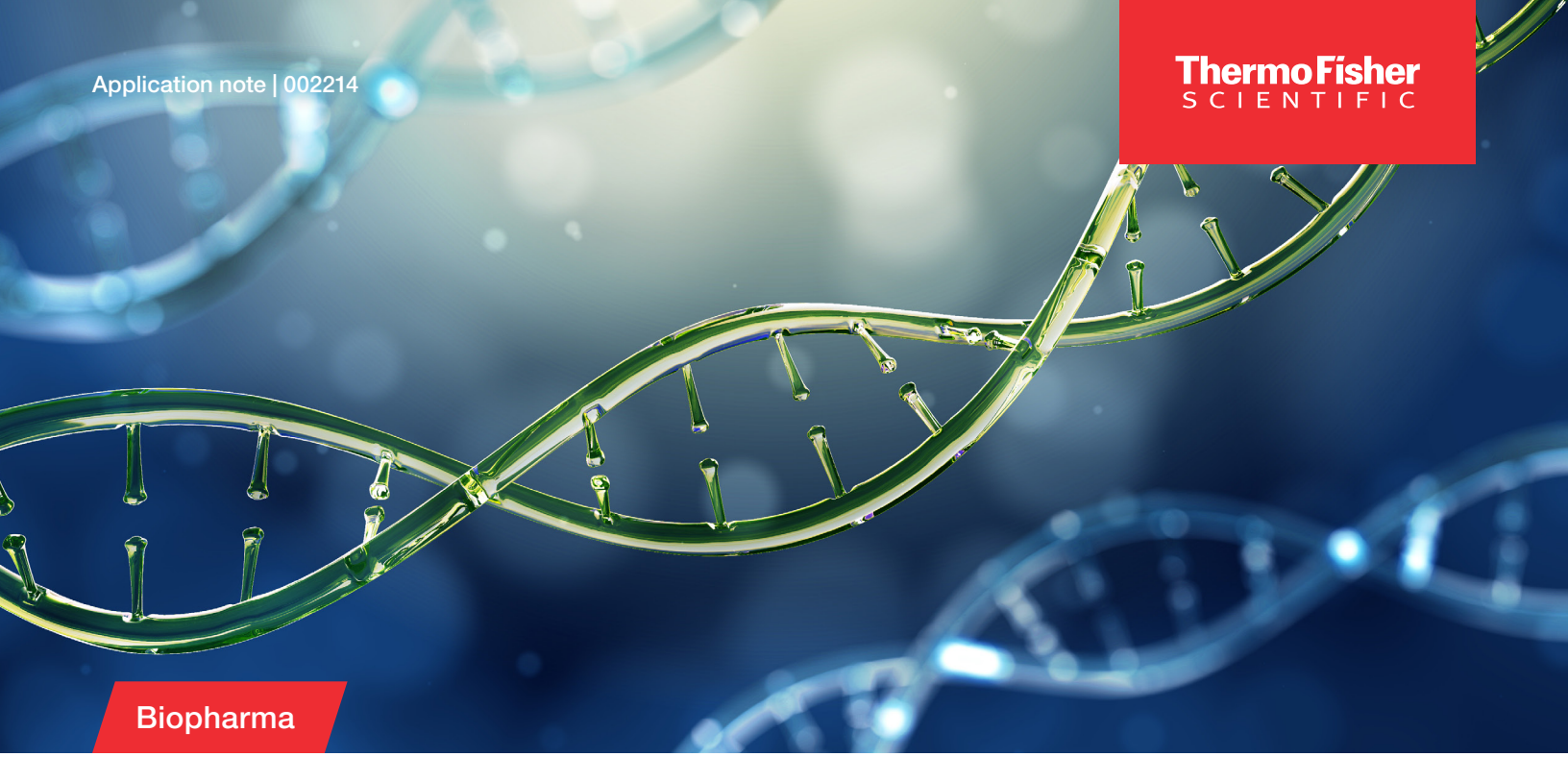
Application note | 002214



Biopharma

Monoisotopic mass confirmation of modified and unmodified oligonucleotides by LC-HRAM MS

Authors

Hao Yang[1], Keeley Murphy[2], Kristi Akervik[3], Min Du[2]

[1]Thermo Fisher Scientific, San Jose, CA, US
[2]Thermo Fisher Scientific, Boston, MA, US
[3]Thermo Fisher Scientific, Austin, TX, US

Keywords

Oligonucleotide analysis, intact mass determination, monoisotopic mass, liquid chromatography high-resolution accurate-mass mass spectrometry (LC-HRAM-MS), ion-pairing reversed phase liquid chromatography (IPRP-LC), Vanquish Horizon UHPLC, Orbitrap Exploris MX mass detector, DNAPac reversed phase column, Chromeloen Chromatography Data System (CDS), BioPharma Finder oligonucleotide mass calculator

Application benefits

Quick and confident monoisotopic mass confirmation of modified and unmodified oligonucleotides up to 100mer with less than 3 parts per million (ppm) mass accuracy using an LC-HRAM MS method that was developed on a Thermo Scientific™ Orbitrap Exploris™ MX mass detector

Goal

- Demonstrate the use of UHPLC-HRAM MS with the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) for accurate monoisotopic mass confirmation of unmodified and modified oligonucleotides
- Demonstrate the use of the Thermo Scientific™ BioPharma Finder™ software oligonucleotide mass calculator for determining the chemical formula, monoisotopic and average mass based on input oligonucleotide sequences

Therapeutic oligonucleotide characterization and analysis is an important component of drug development and quality control. Given the diversity of the types of oligonucleotides, a robust and accurate analytical method is needed to confirm the identity and determine the purity for quality control needs. While conventional intact mass analysis of oligonucleotide products is performed using nominal mass detectors to provide average mass confirmation, high-resolution accurate-mass mass spectrometric techniques, which allow monoisotopic mass confirmation with a high degree of mass accuracy, have gained increasing attention.

thermo scientific

Herein, we describe an intact mass analysis that uses the Xtract algorithm in Chromeleon CDS for monoisotopic mass confirmation of modified and unmodified oligonucleotides with lengths up to 100 nucleotides. Intact mass analysis was executed in four quick steps as shown in Figure 1. First, we obtained the chemical formula by entering the oligonucleotide sequence in the oligonucleotide mass calculator in BioPharma Finder software, version 5.2. Next, the chemical formula and set mass accuracy were entered in the target formula and target tolerance columns, respectively, in the injection sequence prior to data acquisition. After data were acquired, mass deconvolution was performed using the Xtract algorithm with a sequence specific isotope table. Lastly, a report was generated showing the evaluation of measured monoisotopic masses against the theoretical monoisotopic masses that were calculated based on the chemical formula of the input sequences. Using this method, accurate monoisotopic mass confirmation with less than 3 ppm mass accuracy was achieved for all tested oligonucleotides.

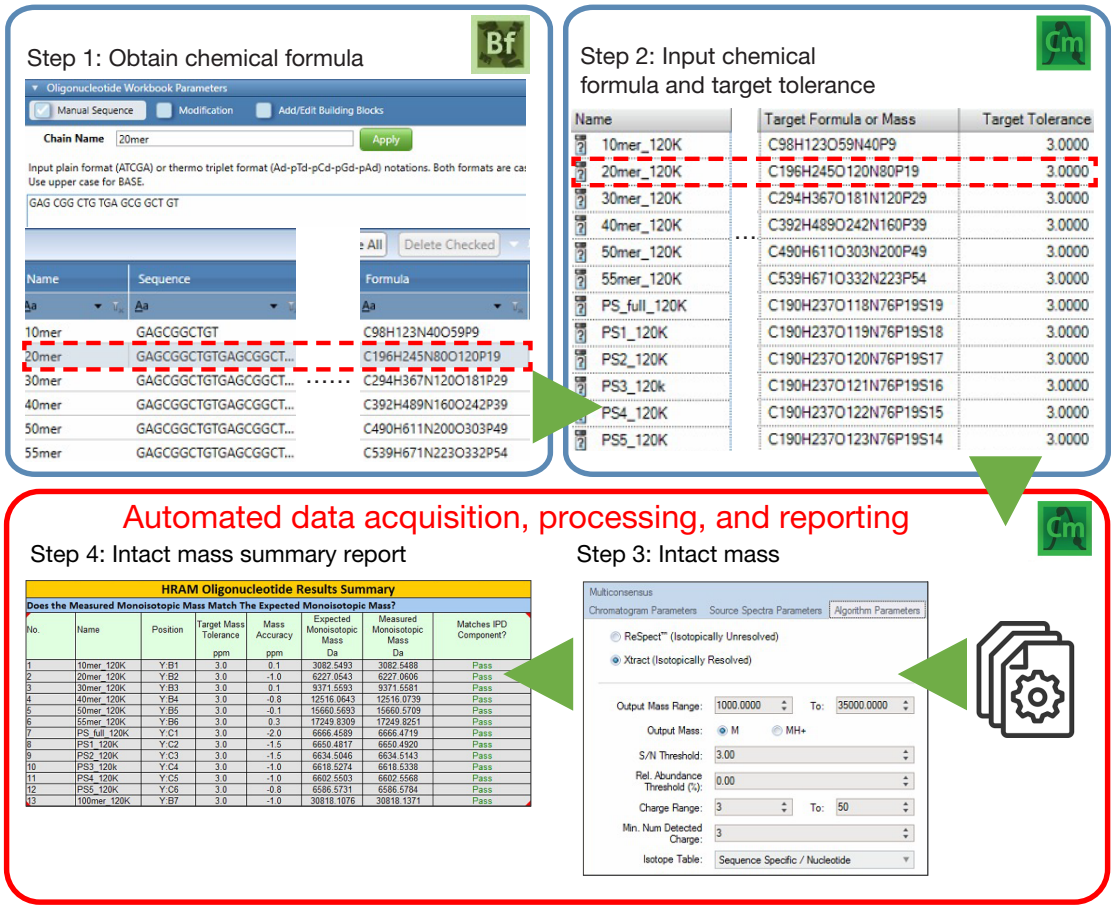


Figure 1. Schematics for intact mass analysis of modified and unmodified oligonucleotides. Step 1: input oligonucleotide sequences into the oligonucleotide mass calculator in BioPharma Finder software to generate the chemical formula. This step is optional if the user already has the chemical formula. Step 2: import the chemical formula list to the target formula column and enter the set mass accuracy in the target tolerance column in the injection sequence. Step 3: acquire and process raw data using the Xtract algorithm with a sequence specific isotope table. Step 4: evaluate the measured monoisotopic mass and mass accuracy for all tested samples in the summary report. Both Steps 3 and 4 are automated with auto reporting enabled.

Experimental

Reagents and consumables

- Oligonucleotide samples, HPLC purified, see Table 1 for details
- 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), 99.9% (Thermo Scientific™ Chemicals, P/N 293410500)
- Triethylamine (TEA), 99% (Thermo Scientific™ Chemicals, P/N 157911000)
- Dibutylamine (DBA), 99.5% (Sigma-Aldrich, 471232-100ML)
- 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™ Acetonitrile, UHPLC-MS grade (P/N A956-1)
- 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)

Oligonucleotide sample preparation

Oligonucleotide samples with lengths ranging from 10 to 55 were obtained from Life Technologies and were prepared at a concentration of 2.5 pmol/µL. The 100 oligomer and phosphorothiolated (PS) oligonucleotides were purchased from Integrated DNA Technologies and were reconstituted in UHPLC grade water at a concentration of 1 pmol/µL. Detailed oligonucleotide sequences, chemical formula, and theoretical monoisotopic mass for each of the samples are listed in Table 1.

Table 1. Oligonucleotide sequences, information, chemical formula, and theoretical monoisotopic mass. Both chemical formula and theoretical monoisotopic mass were obtained from the oligonucleotide mass calculator in BioPharma Finder software, version 5.2.

Oligonucleotide name	Sequence*	Chemical formula	Monoisotopic mass (Da)
10mer	GAG CGG CTG T	C98H123O59N40P9	3082.5493
20mer	GAG CGG CTG TGA GCG GCT GT	C196H245O120N80P19	6227.0543
30mer	GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT	C294H367O181N120P29	9371.5593
40mer	GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT GAG CGG CTG T	C392H489O242N160P39	12516.0643
50mer	GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT GAG CGG CTG TGA GCG GCT GT	C490H611O303N200P49	15660.5693
55mer	GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT GAG CGG CTG TGA GCG GCT GTG AGC G	C539H671O332N223P54	17249.8309
PS_Full	Ur-sAr-sCr-sAr-sGr-sCr-sAr-sUr-sCr-sGr-sGr-sCr-sCr-sUr-sGr-sGr-sAr-sCr-sAr-sUr	C190H237O118N76P19S19	6666.4589
PS_1	Ur-sAr-sCr-sAr-sGr-sCr-sAr-sUr-sCr-sGr-sGr-sCr-sCr-sUr-sGr-sGr-sAr-sCr-sAr-**p**Ur	C190H237O119N76P19S18	6650.4817
PS_2	Ur-**p**Ar-sCr-sAr-sGr-sCr-sAr-sUr-sCr-sGr-sGr-sCr-sCr-sUr-sGr-sGr-sAr-sCr-sAr-**p**Ur	C190H237O120N76P19S17	6634.5046
PS_3	Ur-**p**Ar-sCr-sAr-sGr-sCr-sAr-sUr-sCr-sGr-sGr-sCr-sCr-sUr-sGr-sGr-sAr-**p**Cr-sAr-**p**Ur	C190H237O121N76P19S16	6618.5274
PS_4	Ur-**p**Ar-sCr-**p**Ar-sGr-sCr-sAr-sUr-sCr-sGr-sGr-sCr-sCr-sUr-sGr-sGr-sAr-**p**Cr-sAr-**p**Ur	C190H237O122N76P19S15	6602.5503
PS_5	Ur-**p**Ar-sCr-**p**Ar-sGr-sCr-sAr-sUr-sCr-sGr-sGr-sCr-sCr-sUr-sGr-**p**Gr-sAr-**p**Cr-sAr-**p**Ur	C190H237O123N76P19S14	6586.5731
100mer	CAG TCA GTC AGT CAG TCA GTC AGT CAG TCA GTC AGT CAG TCA GTC AGT CAG TCA GTC AGT CAG TCA GTC AGT CAG TCA GTC AGT CAG T	C975H1226O598N375P99	30818.1076

*Sequence annotations
ATCG: DNA bases; U: Uracil; r: ribose; **p**: phosphodiester bond; s: phosphorothioate bond

Chromatography

The Thermo Scientific™ Vanquish™ Horizon UHPLC system was used for IPRP-LC separation of the oligonucleotide standards. For the 100mer oligo, a gradient of 1–25 %B at a flow rate 0.25 mL/min over 11 minutes with a mobile phase consisting of 15 mM DBA and 25 mM HFIP in water (solvent A) and acetonitrile (solvent B) was used to separate its impurities from the full-length product. For the other oligonucleotides, a flow rate of 0.7 mL/min with a previously reported[1] step gradient consisting of 1 %B for 0.4 minutes, 25 %B for 0.6 minutes, 100 %B for another 0.6 minutes, followed by 1 %B for 2.4 minutes was applied. The column temperature was set to 70 °C in forced air mode. For all sample analyses, 10 µL of sample was injected onto the DNAPac column.

Mass spectrometry

A full scan method with a resolution setting of 120,000 at *m/z* 200, mass range from *m/z* 600 to 1600, and 3 microscans was applied using the Orbitrap Exploris MX mass detector. For the 100mer, data were acquired in intact protein mode with a low pressure setting, whereas for the other oligonucleotides, data were acquired in peptide mode with the standard pressure setting. For experiments performed at 0.25 mL/min, the negative ion spray voltage was set at 2,500 V, sheath/aux/sweep gases were set at 50/10/1, respectively, and ion transfer tube and vaporizer temperatures were set at 325 and 350 °C, respectively. For experiments performed at 0.7 mL/min, the negative ion spray voltage was set at 3,000 V, sheath/aux/sweep gases were set at 60/15/2, respectively, and ion transfer tube and vaporizer temperatures were set at 350 °C.

Oligonucleotide mass calculator

The chemical formula is required for mass accuracy calculation and the use of a sequence specific isotope table for mass deconvolution of sulfur-containing oligonucleotides. As shown in Figure 1, the chemical formula for each tested sample was obtained from the input sequence using the oligonucleotide mass calculator in BioPharma Finder software, version 5.2.

Intact mass analysis using compliant-ready Chromeleon CDS 7.3.2

The data processing step for the intact mass analysis was executed automatically using pre-defined processing methods. The methods are detailed here. Full scan data were processed using the Xtract deconvolution algorithm under the integrated intact protein deconvolution feature within Chromeleon CDS version 7.3.2. This algorithm is specifically designed for processing of isotopically resolved accurate mass data. For the 100mer oligo, a single peak was detected within the retention time window between 11.8 to 12.1 minutes; whereas for the other oligonucleotides, a single peak was detected within the retention time window between 0.75 to 0.95 minutes. For each peak, the source spectrum was automatically generated from averaging the data points across the peak. Unless specified, default settings in “average over selected retention time with Xtract” method were used for mass deconvolution. The oligonucleotide output mass range was set between 1,000 and 35,000 Da, and the charge range was set between 3 and 50.

Intact mass analysis summary report

As shown in Figure 2, an intact mass analysis summary report template was built based on the default oligonucleotide report for optimal presentation of the obtained results. In this report, both expected and measured monoisotopic mass, mass accuracy, and target mass tolerance for all tested samples are shown.

HRAM Oligonucleotide Results Summary

Does the Measured Monoisotopic Mass Match The Expected Monoisotopic Mass?

No.	Name	Position	Target Mass Tolerance ppm	Mass Accuracy ppm	Expected Monoisotopic Mass Da	Measured Monoisotopic Mass Da	Matches IPD Component?
1	10mer_120K	Y:B1	3.0	0.1	3082.5493	3082.5488	Pass
2	20mer_120K	Y:B2	3.0	-1.0	6227.0543	6227.0606	Pass
3	30mer_120K	Y:B3	3.0	0.1	9371.5593	9371.5581	Pass
4	40mer_120K	Y:B4	3.0	-0.8	12516.0643	12516.0739	Pass
5	50mer_120K	Y:B5	3.0	-0.1	15660.5693	15660.5709	Pass
6	55mer_120K	Y:B6	3.0	0.3	17249.8309	17249.8251	Pass
7	PS_full_120K	Y:C1	3.0	-2.0	6666.4589	6666.4719	Pass
8	PS1_120K	Y:C2	3.0	-1.5	6650.4817	6650.4920	Pass
9	PS2_120K	Y:C3	3.0	-1.5	6634.5046	6634.5143	Pass
10	PS3_120k	Y:C4	3.0	-1.0	6618.5274	6618.5338	Pass
11	PS4_120K	Y:C5	3.0	-1.0	6602.5503	6602.5568	Pass
12	PS5_120K	Y:C6	3.0	-0.8	6586.5731	6586.5784	Pass
13	100mer_120K	Y:B7	3.0	-1.0	30818.1076	30818.1371	Pass

Figure 2. Example intact mass analysis summary report. The report reflects the assessment of the mass accuracy for each analyzed sample based on the provided target chemical formula, and reflects a “pass” result when the achieved mass accuracy is below the target mass tolerance set to 3 ppm.

Results and discussion

Intact mass analysis of unmodified, single-stranded DNA with length ranging from 10 to 100mer and partial and fully phosphorothiolated RNA (PS_Full) with 20 nucleotide bases was performed using a high-resolution accurate-mass method with a scan resolution of 120,000 at *m/z* 200. As illustrated in Figure 3 for the analysis of PS_Full, the source spectrum was generated by averaging full scan data across the highlighted peak in the total ion chromatogram (TIC) with a retention time ranging from 0.75 to 0.95 minutes. A total of seven charge states ranging from -5 to -11 were observed, which resulted in a deconvoluted monoisotopic mass of 6666.4719. Similarly, a charge state profile ranging from -11 to -20 was observed for the analysis of 55mer, which resulted in a deconvoluted monoisotopic mass of 17249.8251 as shown in Figure 4; a charge state profile ranging from -20 to -42 was observed for the analysis of 100mer, which resulted in a deconvoluted monoisotopic mass of 30818.1371 as shown in Figure 5. With this method, isotopic resolution of all peaks was achieved, and the measured monoisotopic masses were all within 3 ppm mass accuracy with respect to the expected monoisotopic masses. When a resolution setting of 60,000 was used for the analysis of the 100mer, peaks could not be isotopically resolved, and the deconvolution provided an average mass instead of monoisotopic mass (data not shown). The measured monoisotopic masses are compared to the expected monoisotopic masses calculated from the input chemical formula in the sequence, and the results are shown in a summary report illustrated in Figure 2. A green "pass" is awarded if the absolute value of the achieved mass accuracy is below the target mass tolerance, here set to 3 ppm.

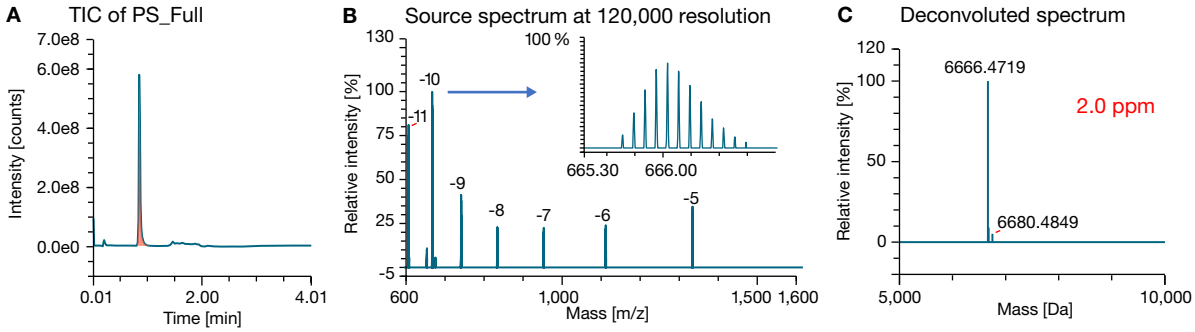


Figure 3. Intact mass analysis of PS_Full. (A) TIC; (B) source spectrum generated at 120,000 resolution by averaging the full scan data with retention time ranging from 0.75 to 0.95 minutes; (C) deconvoluted spectrum of PS_Full using the Xtract algorithm, with theoretical monoisotopic mass and measured mass accuracy labeled in red.

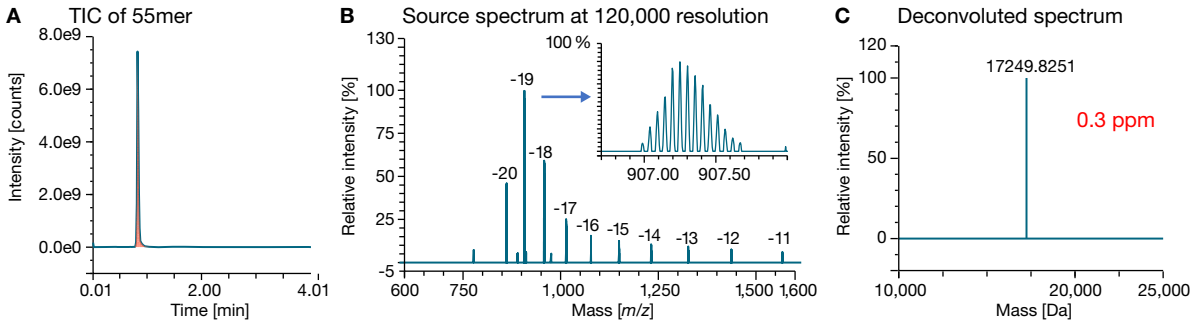


Figure 4. Intact mass analysis of 55mer. (A) TIC; (B) source spectrum generated at 120,000 resolution by averaging the full scan data with retention time ranging from 0.75 to 0.95 minutes; (C) deconvoluted spectrum of 55mer using the Xtract algorithm, with theoretical monoisotopic mass and measured mass accuracy labeled in red.

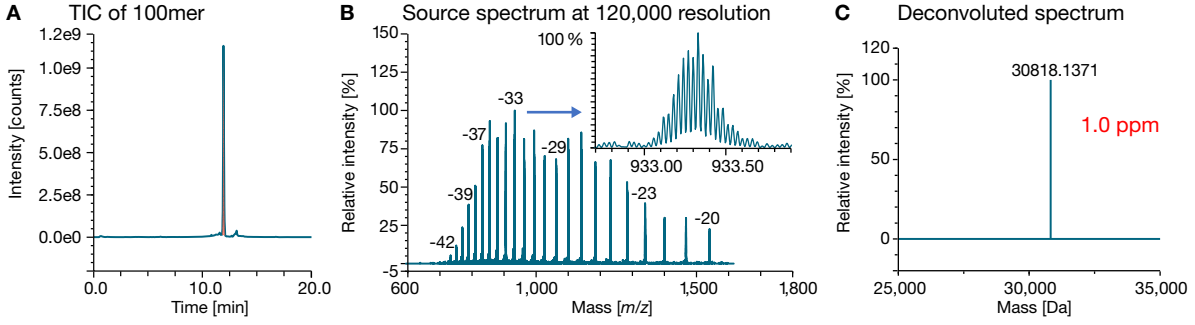


Figure 5. Intact mass analysis of 100mer. (A) TIC; (B) source spectrum generated at 120,000 resolution by averaging the full scan data with retention time ranging from 11.8 to 12.1 minutes; (C) deconvoluted spectrum of 100mer using the Xtract algorithm, with theoretical monoisotopic mass and measured mass accuracy labeled in red.

Conclusion

We developed a simple full scan method with mass resolution of 120,000 at *m/z* 200 on the Orbitrap Exploris MX mass detector for intact MS analysis of unmodified and modified oligonucleotides. This method provides the following:

- A quick way to obtain chemical formula, monoisotopic mass, and average mass for any given oligonucleotide sequences using the oligonucleotide mass calculator within BioPharma Finder software, version 5.2
- Confident monoisotopic mass confirmation with less than 3 ppm mass accuracy for all tested oligonucleotides
- A summary report for quick evaluation of the measured mass accuracy against set target mass accuracy representing pass/fail results for each tested oligonucleotide

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

Learn more at **thermofisher.com/oligonucleotides**