

# Accurate Mass Analysis of Oligonucleotides Using a High Resolution Orbitrap Mass Spectrometer



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## Overview

**Purpose:** To demonstrate the advantage of high resolution and mass accuracy for determining the composition of short single stranded oligonucleotides using an Orbitrap mass spectrometer

**Methods:** DNA and RNA oligonucleotides were analyzed by LC/MS. The acquired accurate masses of the oligonucleotides were then used to determine base composition.

**Results:** The base composition for short single stranded DNA and RNA can be narrowed down and in some cases unequivocally determined from high resolution, accurate mass data. Examples of resolution, deconvolution of accurate mass data and determination of base composition are presented here.

## Introduction

The analysis of oligonucleotides has become important in the area of DNA and RNA therapeutics. With an LTQ Orbitrap XL mass spectrometer it is possible to acquire mass spectra with resolving powers on the order of 100,000 FWHM and mass accuracies with less than 3 ppm RMS error using external mass calibration. Several papers have been written that highlight the use of mass spectrometry to analyze DNA and RNA<sup>1</sup>, including siRNA<sup>2</sup> and PCR products.<sup>3</sup> The data presented here are for two DNA and two RNA simply to show the resolving power and mass accuracy capabilities of an Orbitrap™ mass spectrometry for oligonucleotides and show how high resolution, high mass accuracy data can help to narrow down the possible base composition of an oligonucleotide. The solvent system required to chromatographically separate oligonucleotides leads to data having high chemical noise. The high dynamic range and high resolving power of the Orbitrap allow for lower levels of analyte to be detected compared to lower resolution mass spectrometers.

## Methods

Two DNA and two RNA sequences were randomly chosen to use for these experiments and are listed in **Table 1**. Oligonucleotides were separated by liquid chromatography using the solvent system A: H<sub>2</sub>O with 0.75% HFIPA, 0.375% TEA, 10uM EDTA, B: 75/25 MeOH/H<sub>2</sub>O with 0.75% HFIPA, 0.375% TEA, 10uM EDTA on a standard C18 column. The effluent was electrosprayed in negative ion mode into an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Data were collected for the oligonucleotides at 7500, 15000, 30000, 60000 and 100000 resolving powers. The data from the observed charge states were then deconvoluted to obtain an accurate mass of the target oligonucleotide. These masses were then entered into a web-based oligonucleotide calculator<sup>4</sup> to determine possible compositions of the oligonucleotides.

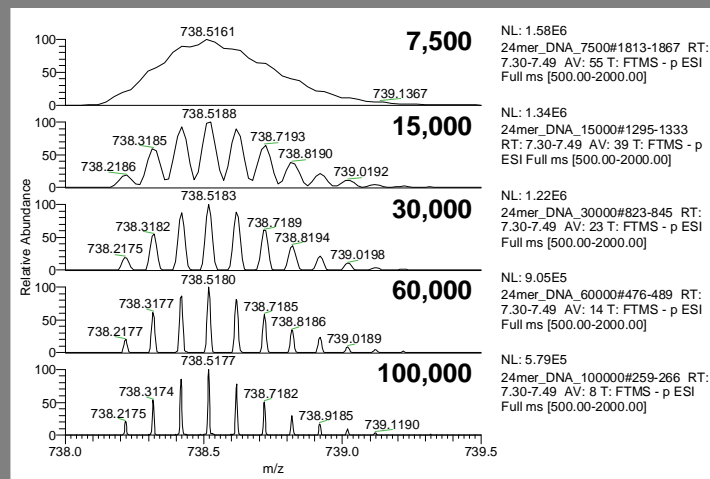
## Results

The sequences, base composition (T for DNA molecules and U for RNA molecules), chemical formulas and calculated monoisotopic masses are reported for each of the oligonucleotides are presented in **Table 1**. The two RNA molecules are 21 residues in length beginning with AA to mimic siRNA like molecules. **Figure 1** demonstrates how resolving power plays a part in determining accurate mass of the monoisotopic peak of the -10 charge state. By 30,000 resolving power the isotopes are fully resolved and an accurate mass can be determined for the -10 charge state. As the number of charges increase the resolving power need to resolve the isotopes increases.

TABLE 1. DNA and RNA sequences, compositions, chemical formulas and monoisotopic masses

Name	Sequence	A	G	C	T/U	Chemical formula	Monoisotopic mass
DNA	17mer	3	3	6	5	C <sub>164</sub> H <sub>210</sub> N <sub>58</sub> O <sub>162</sub> P <sub>16</sub>	5118.8830
	24mer	3	10	8	3	C <sub>232</sub> H <sub>292</sub> N <sub>95</sub> O <sub>142</sub> P <sub>23</sub>	7392.2513
RNA	RNA1	6	3	7	5	C <sub>198</sub> H <sub>248</sub> N <sub>76</sub> O <sub>144</sub> P <sub>20</sub>	6612.9172
	RNA2	8	6	4	3	C <sub>203</sub> H <sub>250</sub> N <sub>88</sub> O <sub>140</sub> P <sub>20</sub>	6778.9900

FIGURE 1. Comparison of resolving power for the -10 charge state of the 24mer oligonucleotide



The isotopic distributions for the most intense charge state observed for each of the oligonucleotides acquired at 60,000 resolving power are shown in **Figure 2**. Below each experimental spectrum is a calculated spectrum (calculated with a resolving power of 60,000). Note the accurate intensity distribution of the isotope pattern as well as the mass accuracy of each of the spectra. Part per million errors are reported for the monoisotopic peak of the charge state shown.

FIGURE 2. Comparison of experimental most intense charge state isotope distribution to calculated isotope distribution and accurate mass

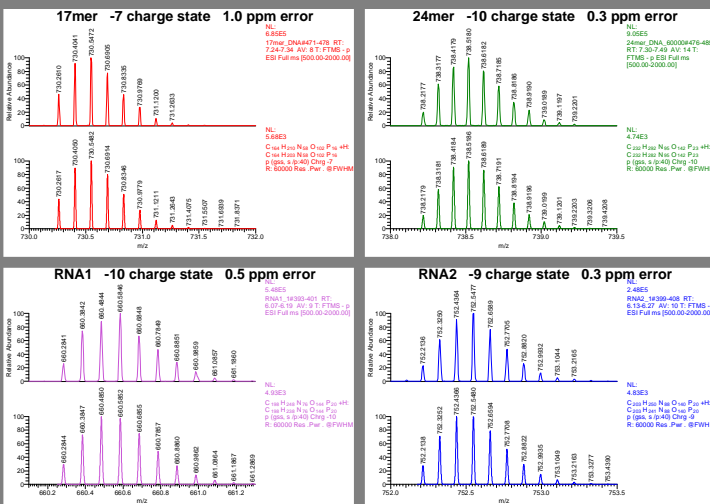


TABLE 2. Monoisotopic masses measured for the five most abundant charge states for each oligonucleotide with calculated monoisotopic values, the associated ppm error and mass differences

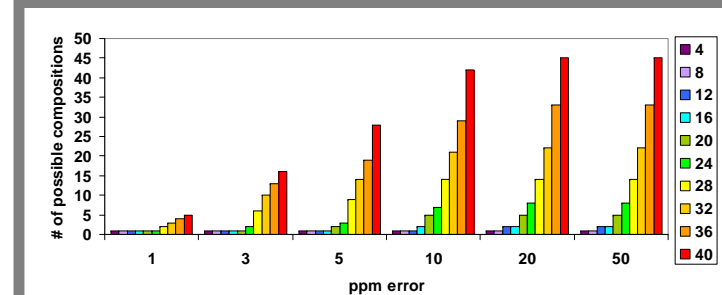
Name	charge	monoisotopic m/z of charge state			
		experimental	calculated	ppm error	mass difference
17mer	-4	1278.7121	1278.7135	1.0949	0.0014
	-5	1022.7693	1022.7693	0.0000	0.0000
	-6	852.1391	852.1399	0.9388	0.0008
	-7	730.2610	730.2617	0.9586	0.0007
	-8	638.8525	638.8531	0.9392	0.0006
24mer	-7	1055.0286	1055.0286	0.0000	0.0000
	-8	923.0244	923.0241	0.3250	0.0003
	-9	820.3539	820.3540	0.1219	0.0001
	-10	738.2177	738.2179	0.2709	0.0002
	-11	671.0154	671.0156	0.2981	0.0002
RNA1	-7	943.6945	943.6952	0.7418	0.0007
	-8	825.6068	825.6074	0.7267	0.0006
	-9	733.7610	733.7613	0.4089	0.0003
	-10	660.2841	660.2844	0.4543	0.0003
	-11	600.1667	600.1670	0.4999	0.0003
RNA2	-6	1128.8250	1128.8244	0.5315	0.0006
	-7	967.4198	967.4199	0.1034	0.0001
	-8	846.3667	846.3665	0.2363	0.0002
	-9	752.2136	752.2138	0.2659	0.0002
	-10	676.8918	676.8917	0.1477	0.0001

**Table 2** shows the monoisotopic masses measured for the five most intense charge states for each of the four oligonucleotides. The calculated monoisotopic mass is given as well as the ppm error and mass difference between the measured and calculated mass. Note that the data were collected using external mass calibration and all ppm errors are at or below 1.1 ppm and the highest mass difference reported is 0.0014 Da. The accurate masses from the observed charge states were then deconvoluted to determine the accurate mass of the oligonucleotide molecules. The deconvoluted masses for the oligonucleotides are presented in **Table 3** along with the calculated monoisotopic mass for the molecule and the ppm error and mass difference for each of the molecules. To demonstrate the power of high mass accuracy in the analysis of oligonucleotides, the web-base calculator Simple Oligonucleotide Sequencer<sup>4</sup> was used to calculate composition and number of possible compositions of bases for a given oligonucleotide mass with a given error. The constraints given were a minimum of 0 and a maximum of 50 A, G, C, T (DNA) or U (RNA) and no terminal phosphates. The calculated monoisotopic masses were input with  $\pm 5$ ppm,  $\pm 3$ ppm and  $\pm 1$ ppm errors and the number of possible compositions are reported in **Table 3**. As expected the correct compositions were reported from the calculator for the  $\pm 1$  ppm error calculations: 17mer A3 G3 C6 T5; 24mer A3 G10 C8 T3; RNA1 A6 G3 C7 U5; RNA2 A8 G6 C4 U3.

TABLE 3. Deconvoluted masses of oligonucleotides, error associated with these masses and calculated number of possible compositions at 5ppm, 3ppm and 1ppm error for each oligonucleotide

Name	molecular mass of oligonucleotide				possible compositions		
	deconvoluted	calculated	ppm error	mass difference	5ppm error	3ppm error	1ppm error
17mer	5118.8790	5118.8830	0.7814	0.0040	2	1	1
24mer	7392.2520	7392.2513	0.0947	0.0007	7	5	1
RNA1	6612.9123	6612.9172	0.7410	0.0049	1	1	1
RNA2	6778.9907	6778.9900	0.1033	0.0007	1	1	1

FIGURE 3. Theoretical calculation of number of possible base compositions with respect to ppm error for model oligonucleotides ranging from 4 to 40 bases



**Figure 3** is a theoretical representation of how increasing mass error increases the possible bases composition for model oligonucleotides from 4 to 40 bases. The model oligonucleotides are DNA molecules with equal numbers of A, G, C and T. So for example the composition of the 24mer is A6 G6 C6 T6. The accurate mass for this composition, 7349.2594 Da, was placed in the calculator and the mass error was varied to determine the number of possible compositions within the error;  $\pm 1$ ppm 1,  $\pm 3$ ppm 2,  $\pm 5$ ppm 3,  $\pm 10$ ppm 7,  $\pm 20$ ppm 8,  $\pm 50$ ppm 8. This demonstrates that higher mass accuracy can help to reduce the possible base compositions and/or unequivocally identify the composition of the measured oligonucleotide.

## Conclusions

- The Orbitrap mass spectrometer provides high mass accuracy and high resolution that are beneficial in the analysis of oligonucleotides.
- The resolving powers provided by the instrument were demonstrated and the need for higher resolving power to determine accurate mass was illustrated.
- Isotopic distributions for the most intense charge states for each of the oligonucleotides were shown and compared well to the calculated isotopic distributions.
- Monoisotopic masses for the five most intense charge states for each of the four oligonucleotides were reported with less than 1.1 ppm error
- Deconvolution of the data revealed monoisotopic masses for each of the oligonucleotide molecules with less than 0.8 ppm error
- Using the web-base calculator Simple Oligonucleotide Sequencer<sup>4</sup> the base composition of an oligonucleotide can be determined from a measured accurate mass

## References

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