# INTACT MASS - A VERSATILE SOFTWARE FOR RAPID MASS CONFIRMATION OF OLIGONUCLEOTIDES AND THEIR IMPURITIES

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## OVERVIEW

Here we are demonstrating an automated, compliance-ready LC-MS workflow for intact mass confirmation and purity analysis of synthetic oligonucleotides including small interfering RNA (siRNA) and large single guide (sg) RNA oligonucleotides and their impurities.

## INTRODUCTION

- Synthetic oligonucleotides have emerged in recent years as a powerful alternative to small molecule and protein therapeutics [1].
- Manufacturing and quality control of oligonucleotide therapeutics requires highly selective and sensitive LC-MS methods for impurity detection and quantification.
- The most often used mass spectrometry-based method for oligonucleotide analysis has been reversed-phase chromatography employing a variety of ion-pairing reagents and modifiers in negative ESI-MS mode (IP-RP LC-MS).
- An automated workflow for analysis of synthetic oligonucleotides employing the BioAccord LC-MS system was recently described [2-5].
- Here we introduce an automated, compliance-ready workflow embedded in the waters_connect INTACT Mass application which is shown to provide better than 10 ppm mass accuracy for intact mass confirmation of siRNA oligos and their impurities and better than 20 ppm mass accuracy for larger oligonucleotides - single guide (sgRNA) 100-mers and the corresponding impurities.

*BioAccord System with ACQUITY™ Premier System*

## METHODS

**Experimental Conditions**
A BioAccord system with an ACQUITY™ Premier UPLC® system equipped with a 2.1 x 100 mm Premier OST column (P/N 186009485) was used for all oligonucleotide separations. For separation of the 21-mer siRNA oligonucleotide and its impurities, a IP-RP mobile phase containing 7 mM TEA, 40 mM HFIP pH 8.6 was used as Solvent A, while the composition of Solvent B was 3.5 mM TEA, 20 mM HFIP in 50% methanol. Gradient separations were performed from 25% B to 35% B over 25 min. For separation of the 100-mer sgRNA oligonucleotide and its impurities, the IP-RP mobile phase contained 8 mM DIPEA (diisopropylethylamine), 40 mM HFIP (Solvent A) and 4 mM DIPEA, 4 mM HFIP in 75% acetonitrile (Solvent B). Gradient separations were performed from 12% B to 17% B over 25 min. For both oligonucleotides investigated here the column flow rate was 0.3 mL/min and the column temperature was 60°C.

**Sample Preparation**
A 21-mer heavily modified oligonucleotide, containing a 2'-OMe modification on 19 of its nucleosides, having the sequence GUA ACC AAG AGU AUU CCA UTT and the elemental composition C229H306N76O143P20 was purchased from ATDBio (Southhampton, UK). Stock solutions were prepared in DI water at a concentration of 1 µM (or 2.34 µg/mL), from which a 10 µL volume was injected, which corresponds to loading 10 picomoles of the 21-mer oligonucleotide on-column.
Ten nanomoles of a 100-mer sgRNA oligonucleotide encoding for the HPRT1 enzyme (hypoxanthine phosphoribosyltransferase1) were purchased from Integrated DNA Technologies (Coralville, IA). The oligonucleotide sequence 5'-G*A*U* GAU CUC UCA ACU UUA ACG UUU UAG AGC UAG AAA UAG CAA GUU AAA AUA AGG CUA GUC CGU UAU CAA CUU GAA AAA GUG GCA CCG AGU CGG UGC U*U*U* U-3' has the elemental composition of C959 H1183 N376 O691 P99 S6 and an average molecular weight of 32,292.5513 Da. The RNA oligonucleotide contains a 2'-OMe modification on its first three 5' nucleotides (G*A*U*) as well as on its last three 3' nucleotides (U*U*U*) and the asterisk indicates that all these six nucleotides are phosphorothioated. Stock solutions of the sgRNA oligonucleotide were prepared in DI water at a concentration of 5 µM, from which a 10 µL volume was injected onto a RP column, which corresponds to loading 50 picomoles of the 100-mer oligonucleotide on-column.

## RESULTS

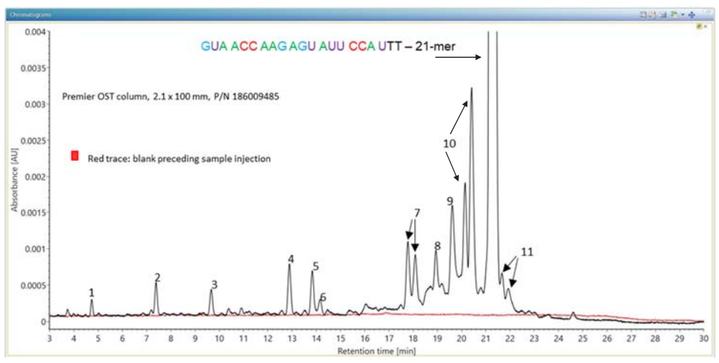


Figure 1. TUV chromatograms showing the separation of oligonucleotide impurities from a 21-mer heavily modified oligonucleotide on an ACQUITY Premier OST column.

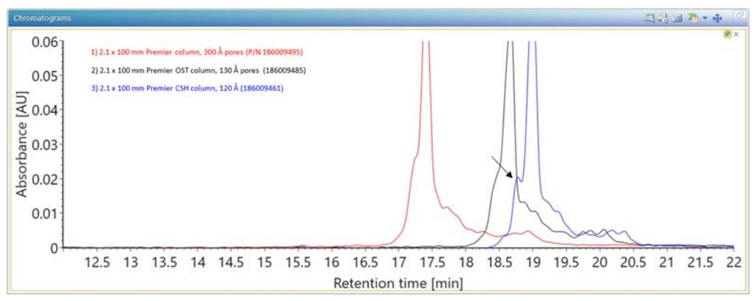


Figure 3. TUV TUV chromatograms showing the separation of the 100-merr sgRNA and its impurities on three Premier columns with the same dimensions (2.1 x 100 mm) and packed with three different types of C18 1.7 µm particles: 130 Å pores for the OST Premier column (black trace), 300 Å pores for the Premier PST column (red trace) and 130 Å pores for the CSH (charged surface hybrid) column (blue trace). An oligonucleotide impurity identified as the 100-mer oligo containing only a single modification (PS to PO conversion, ~ 16 Da modification) is clearly resolved much better by the CSH column (see the black arrow sign) compared to the other two columns. The separations were performed under identical experimental conditions.

INTACT Mass

Peak no:

| | Component | Observed mass (Da) | Expected mass (Da) | Mass error (ppm) | Identity result | Observed TIC RT (mins) | Observed UV RT (mins) | LC area | LC amount (%) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | D1423 n-OMeA[5] & n-OMe5MeC[2] & n-OMeG[2] & n-OMe5MeU | 3,593.702 | 3,593.707 | -1.5 | Pass | 4.74 | 4.69 | 1,222 | 0.2 |
| 2 | D1423 n-OMeA[4] & n-OMe5MeC[2] & n-OMeG[2] & n-OMe5MeU | 3,936.762 | 3,936.775 | -3.4 | Pass | 7.37 | 7.34 | 2,737 | 0.5 |
| 3 | D1423 n-OMeA[3] & n-OMe5MeC[2] & n-OMeG & n-OMe5MeU | 4,638.916 | 4,638.907 | 1.9 | Pass | 9.66 | 9.61 | 2,625 | 0.5 |
| 4 | D1423 n-OMeA[2] & n-OMe5MeC[2] & n-OMeG & n-OMe5MeU | 4,981.988 | 4,981.975 | 2.7 | Pass | 12.88 | 12.83 | 5,276 | 0.9 |
| 5 | D1423 n-OMeA[2] & n-OMe5MeC & n-OMeG & n-OMe5MeU | 5,315.046 | 5,315.048 | -0.3 | Pass | 13.80 | 13.77 | 4,704 | 0.8 |
| 6 | D1423 n-OMeA & n-OMeG & n-OMe5MeU | 5,991.181 | 5,991.188 | -1.2 | Pass | 17.76 | 17.72 | 7,942 | 1.4 |
| 7 | D1423 n-OMeA | 6,684.310 | 6,684.308 | 0.3 | Pass | 19.59 | 19.55 | 13,473 | 2.4 |
| 9 | D1423 n-OMe5MeU | 6,693.332 | 6,693.320 | 1.9 | Pass | 20.13 | 20.08 | 13,283 | 2.3 |
| 10 | D1423 n-OMe5MeC | 6,694.315 | 6,694.304 | 1.8 | Pass | 20.39 | 20.34 | 26,001 | 4.5 |
| 8 | D1423 unknown[-NH5] | 7,008.354 | 7,008.334 | 2.9 | Pass | 20.13 | 20.08 | 13,283 | 2.3 |
| | D1423 MAIN PEAK | 7,027.390 | 7,027.376 | 2 | Pass | 21.22 | 21.19 | 469,430 | 82.0 |
| 11 | D1423 Deamination | 7,028.315 | 7,028.360 | -6.5 | Pass | 20.39 | 20.34 | 26,001 | 4.5 |

Figure 2. Screenshot with the processing results generated by the INTACT Mass software for the analysis of a 21-mer oligonucleotide and its impurities. The dataset was deconvolved using the BayesS-pray charge deconvolution algorithm and eleven oligonucleotide impurities were identified with mass accuracies of under 10 ppm. The first impurity displayed in this table, an 11-mer oligonucleotide, has the lowest detected abundance at 0.2% according to the UV measurement.

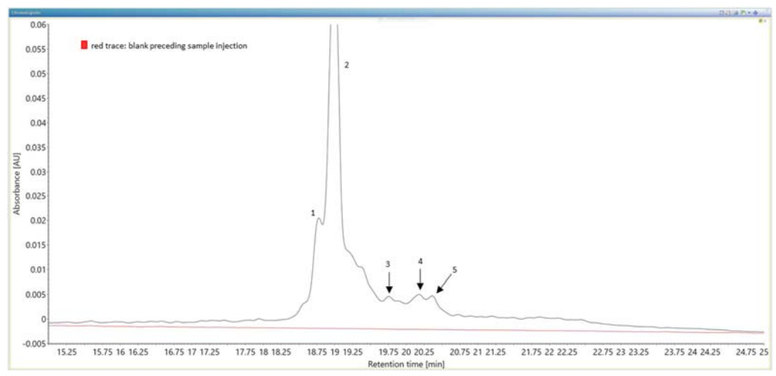


Figure 4. TUV chromatogram showing the separation of oligonucleotide impurities from a 100-mer sgRNA oligonucleotide. The sample was analyzed on a 2.1 x 100 Premier CSH column using a 25-min gradient. The red trace corresponds to the blank preceding sample injection.

INTACT Mass

Peak no:

| | Type | Molecule ID | Component | Observed mass (Da) | Expected mass (Da) | Mass error (ppm) | Identity result | Observed TIC RT (mins) | Observed UV RT (mins) | LC area | LC amount (%) | MS response | MS amount (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Summary | | | | | | Pass | | | | 73.8 | | |
| | Product | 32,291.5513 | 32291.5513 All Forms | | | | Pass | | | | 73.8 | 55,435,131 | 42.5 |
| 1 | Impurity | 32,291.5513 | 32291.5513 PS to PO | 32,275.46 | 32,275.49 | -0.9 | Pass | 16.50 | | 493,486 | 26.2 | 11,251,127 | 28.8 |
| 2 | Product | 32,291.5513 | 32,291.5513 MAIN PEAK | 32,291.47 | 32,291.55 | -2.4 | Pass | 16.71 | | 1,391,591 | 73.8 | 45,964,392 | 39.1 |
| Na adduct | Product | 32,291.5513 | 32291.5513 +Na | 32,312.99 | 32,313.53 | -16.9 | Pass | 16.71 | | 1,391,591 | 73.8 | 9,470,739 | 8.0 |
| 3 | Impurity | 32,291.5513 | 32291.5513 PS to PO,CNET | 32,328.38 | 32,328.55 | -5.2 | Pass | 16.71 | | 1,391,591 | 73.8 | 8,068,217 | 6.9 |
| 3 | Impurity | 32,291.5513 | 32291.5513 PS to PO,Isobutyryl | 32,345.57 | 32,345.58 | -0.2 | Pass | 16.71 | | 1,391,591 | 73.8 | 7,341,735 | 6.2 |
| 4 | Impurity | 32,291.5513 | 32291.5513 Isobutyryl | 32,361.10 | 32,361.64 | -16.8 | Pass | 16.71 | | 1,391,591 | 73.8 | 4,972,482 | 4.2 |
| 5 | Impurity | 32,291.5513 | 32291.5513 n+Phosphate | 32,372.20 | 32,371.53 | 20.8 | Warning | 16.71 | | 1,391,591 | 73.8 | 1,348,086 | 1.1 |

Figure 5. Section of the INTACT Mass report displaying the processing results obtained for the analysis of the 100-mer sgRNA oligonucleotide. Four closely related impurities, all 100-mers, were putatively identified with mass accuracies under 20 ppm. Impurity abundances were calculated based on their ESI-MS response and the lowest detected impurity was an extra phosphorylated 100-mer oligonucleotide (peak no 5) present at ~ 1% abundance level.

## CONCLUSIONS

- The compliance-ready waters_connect INTACT Mass Application performs automated, fast deconvolution of oligonucleotide spectra across the entire chromatographic space, providing fast impurity assignments as well as the required metrics (mass accuracy and abundance) to support impurity analysis.
- The INTACT Mass Application is shown to provide better than 10 ppm mass accuracy for intact mass confirmation of siRNA oligonucleotides and their impurities and better than 20 ppm mass accuracy for longer sgRNA oligonucleotides analyzed with an IP-RP LC-MS assay.
- The workflow described here provided purity information for sample components, down to ~ 0.2% abundance levels for siRNA oligonucleotides and down to ~ 1% abundance levels for sgRNA oligonucleotides.
- The BioAccord System with ACQUITY™ Premier and the Premier CSH Column are ideally suited for detailed intact mass confirmation and purity of siRNA and single guide RNA oligonucleotides (sgRNA)

### REFERENCES:

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