

# Drug Discovery and Development

## High Resolution Analysis of Synthetic Oligonucleotides

### Reverse Phase Ion Pairing LC-MS Analysis Using the X500B QTOF System

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Oligonucleotide therapeutics and gene therapies are rapidly gaining attention as novel therapeutics. The potential disease areas for which oligonucleotide based therapies are being developed is rapidly expanding. In particular, these therapies hold high promise due to their specificity and the high potential for treating previously undruggable targets.

A defining characteristic of oligonucleotides, when compared to more commonly used protein based biotherapeutics, is that they are produced synthetically. Synthetic processes have been highly refined to enable production of high quality synthetic products with limited failure sequences or other associated impurities. Even when considering the high efficiency of modern synthetic processes there is still a need to verify oligonucleotide mass, sequence, and purity.

For the purpose of high throughput oligonucleotide analysis to verify purity and mass confirmation a common data processing solution is ProMass from Novatia LLC. This software enables rapid verification of molecular mass and assessment of purity. In addition, ProMass can annotate failure sequences and synthetic impurities based on the input of the target sequence and selection of search parameters.



Demonstrated in this technical note is the use of the SCIEX X500B QTOF System for the analysis of oligonucleotides using ion-pairing reverse phase chromatography. Highly efficient chromatographic separation with high performance negative ion mode performance produce results for unambiguous confirmation of sequences with minimal adduct formation or analysis induced modifications. Also discussed is the processing of this data using ProMass HR for automated verification of molecular mass and purity assessment.

### Key Feature of X500B QTOF System

- High mass accuracy with exceptional negative ion performance for oligonucleotide analysis
- TurboV™ source and QJet® ion guide provide efficient ionization and transmission of analytes while reducing contamination from use of ion-pairing reagents
- Instrument control using SCIEX OS enables accessibility and ease of use regardless of expertise level
- High resolution platform for qualitative and quantitative oligonucleotide workflows
- Integration of data with ProMass for high throughput and robust mass confirmation and purity assessment

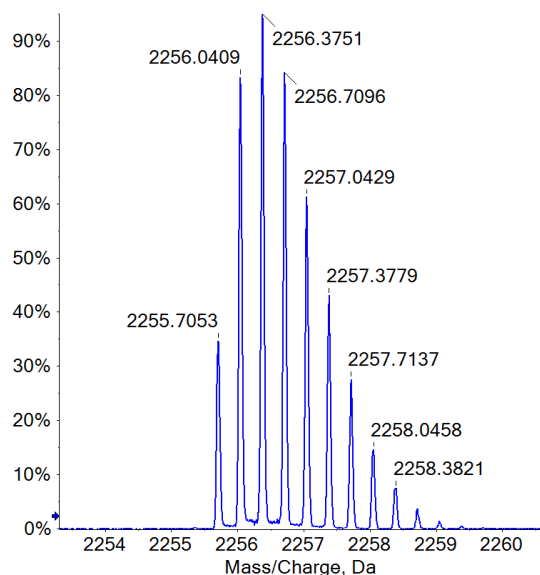


Figure 1. Isotopically resolved charge state for oligonucleotide CATGGTCCTGCTGGAGGTCGTG

## Methods

**Sample Preparation:** Synthetic oligonucleotides were purchased from IDT as custom sequences. The samples were reconstituted in water prior to use.

**Chromatography:** The separation was accomplished using an Agilent 1290 Infinity UHPLC fitted with a BEH Column, 2.1 mm x 100 mm with 1.7  $\mu\text{m}$  particles. Mobile phase A was 15 mM diisopropylethylamine (DIEA) with 100 mM 1,1,1,3,3,3-hexafluoro isopropanol (HFIP) in water while mobile phase B was 15 mM DIEA with 100 mM HFIP in 50:50 v/v % methanol:water. The gradient used is shown in Table 1. The column temperature was held at 60°C. An injection volume of 1  $\mu\text{L}$  was employed, resulting in 50 ng on column.

**Mass Spec:** A SCIEX X500B QTOF with a Turbo V™ source and TwinSpray probe was used for analysis. The MS parameters are listed in Table 1. The data was acquired using SCIEX OS Software 1.5

**Data processing:** All data were processed with either SCIEX OS or the ProMassProcessor and ProMass for SCIEX software.

## Results and Discussion

The analysis of oligonucleotides can be challenging for a wide variety of reasons. A specific challenge centers around the use of ion-pairing reagents which enable the separation of oligonucleotide species based on order of their charge which is generally indicative of their length. Typically, ion-pairing systems are present at low to mid millimolar concentrations and efficient removal of these components during the electrospray process is critical. In addition, the presence of alkali metal ions, namely sodium and potassium, can complicate analysis. Proper maintenance and regular mobile phase preparation with high quality reagents is required.

As shown in Figure 2, a high quality chromatographic separation is accomplished using ion-pairing reverse phase chromatography. In the work presented here a generic gradient was used which enables analysis of a wide range of oligonucleotide sequences with little requirement for the adjustment of the method.

Averaging the spectra under the main peak results in a typical raw spectrum for oligonucleotides. In this case, charge states spanning from -3 to -10 are shown in Figure 3. As evidenced in the spectrum, adduct formation is quite low and adduct formation is most pronounced in the lowest charge state species. As expected, each charge state demonstrates isotopic resolution. The inset of Figure 3 shows the zoomed view of the -3 charge state for the oligonucleotide

**Table 1. LC Gradient**

Time (min)	%A	%B	Flow Rate ( $\mu\text{L min}^{-1}$ )
Initial	70	30	300
1.0	70	30	300
6.0	10	90	300
7.0	10	90	300
8.0	70	30	300
10.0	70	30	300

**Table 2. MS Parameters**

	Experiment 1
Polarity	Negative
Intact Protein Mode	On
GS1 (psi)	70
GS2 (psi)	70
Curtain Gas	35
Temperature ( $^{\circ}\text{C}$ )	350
TOF Start Mass (Da)	600
TOF Stop Mass (Da)	2900
Accumulation Time (s)	0.25
Spray Voltage (V)	-4500
Declustering Potential (V)	-80
Collision Energy (V)	-10
Time bins to sum	4

Frequently the objective of intact mass measurement is confirmation of the target mass for a specific oligonucleotide sequence. In addition, there may be a need to identify failure sequences or impurities which are present in a sample. A common tool for this processing is ProMass which enables batch processing of oligonucleotide data. To enable the migration of data collected from SCIEX mass spectrometers the ProMassProcessor was used to define the peak selection criteria and sample information for export to ProMass.

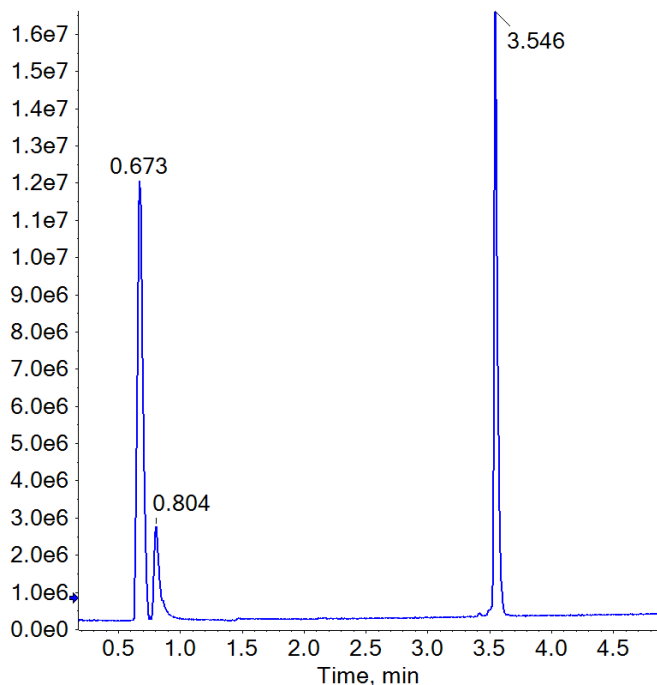


Figure 2. Total Ion Chromatogram for the analysis of a 22-mer oligonucleotide CAT GGT CCT GCT GGA GTT CGT G.

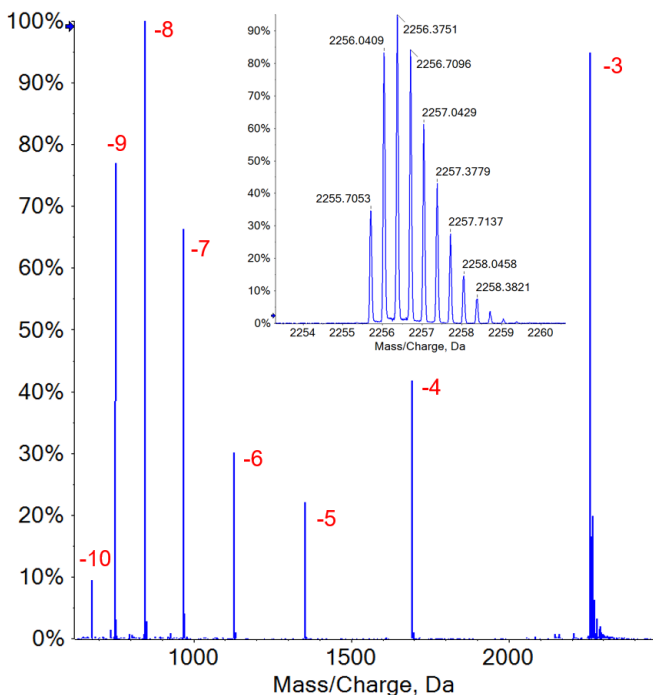


Figure 3. Raw mass spectrum of a 22-mer oligonucleotide CAT GGT CCT GCT GGA GGT CGT G

Within the ProMassProcessor, data files are selected for processing. Files may be move individually or in groups. There is no limit on the number of files that may be moved, however downstream processing in ProMass will increase as more files are selected.

Following file selection, peak selection criteria can be defined for both MS and, if collected simultaneously, the UV data for each experiment as shown in Figure 5A. In addition, peak finding criteria may be defined and additional peak filtering criteria such as minimum and maximum retention time, relative peak area and the maximum number of peaks to find are defined, Figure 5B.

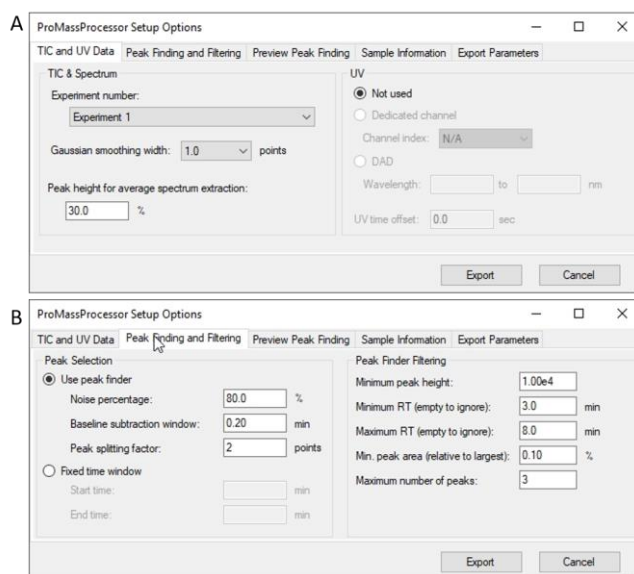


Figure 5. A. Definition of peak selection and experimental criteria in ProMassProcessor. B. Definition of peak finding in ProMassProcessor.

Prior to export it is possible to review the integration of each selected file using the defined criteria, Figure 6. If the integration is not suitable the parameters may be adjusted and will apply to each file. Alternatively, if there is a desire to include a specific peak from a file that falls outside of the defined criteria, or alternatively if a peak should be excluded it is possible to manually add or remove these peaks as shown in Figure 6.

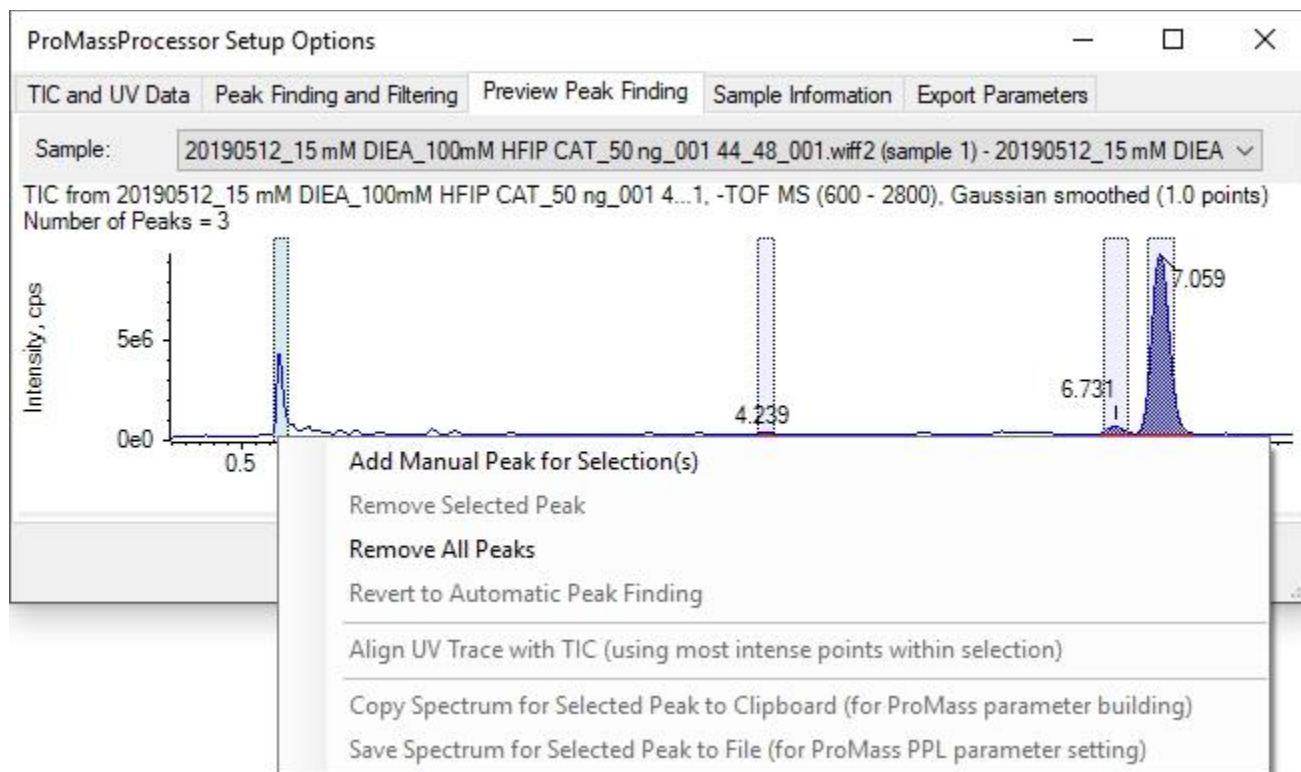
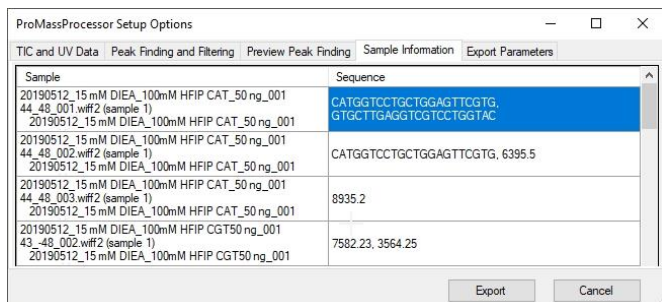


Figure 6. Preview of peak finding results in ProMassProcessor. Additional peaks may be defined manually if desired or already selected peaks may be removed.

For each sample the target information may be defined separately. The target information may be entered as a sequence which is defined using the standard nomenclature supported by ProMass, as a target mass or a combination of both. If more than one target is present in a sample, each species is separated using a comma. To expedite entry, values may be pasted from other sources such as Excel. The software is able to use defined oligonucleotide sequence information, calculate the theoretical mass sequence and, if selected, the associated failure sequences from both the 5' and 3' ends during ProMass processing.



Sample	Sequence
20190512_15 mM DIEA_100mM HFIP CAT_50 ng_001 44_48_001.wiff2 (sample 1)	CATGGTCCTGGAGTTCGTG, GTGCTTGAGGTGCTCTGGTAC
20190512_15 mM DIEA_100mM HFIP CAT_50 ng_001 44_48_002.wiff2 (sample 1)	CATGGTCCTGGAGTTCGTG, 6395.5
20190512_15 mM DIEA_100mM HFIP CAT_50 ng_001 44_48_003.wiff2 (sample 1)	8935.2
20190512_15 mM DIEA_100mM HFIP CGT50 ng_001 43_48_002.wiff2 (sample 1)	7582.23, 3564.25

Export Cancel

Figure 7. Definition of Sequence and/or mass information in ProMassProcessor.

The final stage in processing data is to export the data from the ProMassProcessor to ProMass. In this case the user selects the parameter file defined within ProMass that they would like to apply and the processing begin immediately. To initiate processing a licensed version of ProMass for SCIEX is required.

Following processing the results are displayed accessed through ProMass Browser from the main screen of ProMass, Figure 8. Users can either open the last processed results using the browser button or previously processed results by opening them directly. The results displayed in ProMass provide an easy to understand indication of which samples have passed or failed. The underlying data for each sample is easily investigated by selecting each well and reviewing the total ion chromatogram, raw MS data, and reconstructed MS data. In addition, a table of all identified components with their corresponding abundance, mass error and the sequence the mass corresponds to is also provided.

## Conclusions

- The X500B QTOF system generates high quality oligonucleotide data in the presence of ion-pairing mobile phase components
- High mass accuracy and resolving capabilities enable unambiguous confirmation of oligonucleotide mass
- Low adduct formation is obtainable with suitable cleaning protocols for system components and use of high quality reagents
- Data processing using ProMassProcessor and ProMass provides a semi-automated solution for oligonucleotide characterization and QC

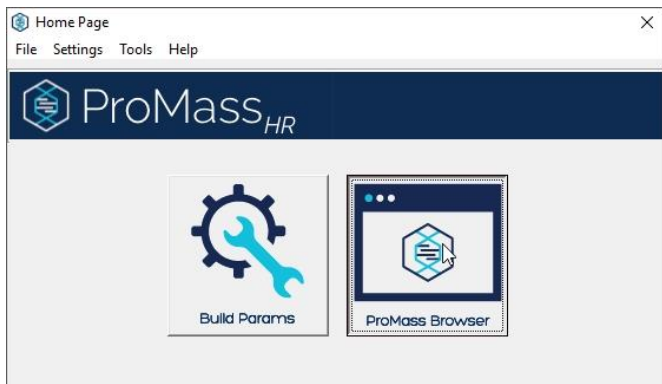


Figure 8. Home page of ProMass software



ProMass Sample Browser												
	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	25	26	27	28	29	30	31	32	33	34	35	36
D	37	38	39	40	41	42	43	44	45	46	47	48
E	49	50	51	52	53	54	55	56	57	58	59	60
F	61	62	63	64	65	66	67	68	69	70	71	72
G	73	74	75	76	77	78	79	80	81	82	83	84
H	85	86	87	88	89	90	91	92	93	94	95	96

Figure 9. Sample summary from ProMass Sample Browser. Colored wells indicate passing, failing, or marginal results based on criteria defined in ProMass parameters.

RT (min)	Calculated Mass (Da)	Observed Mass (Da)	Mass Error	Intensity	Sequence
9.864	6148.0	6146.6	-1.4 Da (-0.023 %)	1.84E+004	A1-T20 +3'Phos
9.864	6221.1	6219.6	-1.5 Da (-0.024 %)	1.92E+004	6372.2 (G depurination)
9.864	6237.1	6235.1	-2.0 Da (-0.032 %)	4.03E+004	6372.2 (A depurination)
9.864	6372.2	6371.1	-1.1 Da (-0.017 %)	1.23E+006	A1-T21
9.496	6043.0	6041.8	-1.2 Da (-0.020 %)	1.29E+003	6372.2 (Minus G)
9.496	6139.0	6138.0	-1.0 Da (-0.016 %)	3.23E+003	C2-T21 +5'Phos
9.327	6083.0	6082.1	-0.9 Da (-0.015 %)	8.11E+003	6372.2 (Minus C)
9.048	5849.8	5849.1	-0.7 Da (-0.012 %)	3.69E+003	G3-T21 +5'Phos
9.048	6068.0	6067.2	-0.8 Da (-0.013 %)	1.77E+004	A1-T20, 6372.2 (Minus T)
8.571	5520.6	5519.5	-1.1 Da (-0.020 %)	2.05E+003	G4-T21 +5'Phos
8.571	6059.0	6058.6	-0.4 Da (-0.007 %)	1.70E+004	C2-T21, 6372.2 (Minus A)
8.291	5769.8	5768.9	-0.9 Da (-0.016 %)	2.02E+004	G3-T21

Figure 10. Individual sample summary table displaying retention time, calculated and observed mass, mass error, intensity, and sequence match based on mass.

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