Analysis of locked nucleic acid (LNA) oligonucleotides (ON) and related impurities using accurate mass spectrometry

Featuring SCIEX TripleTOF® 6600 LC-MS/MS System with IonDrive™ Turbo V Ion Source and ProMass Software

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Recent advances in the synthesis and purification of therapeutic oligonucleotides (ON) as well as the administration have paved the way to their viability as a therapeutic modality. The most popular approach for ON production is arguably phosphoramidite solid-state synthesis from 3' to 5'direction.[1] Although the approach has been significantly optimized over time, it is still a serial synthesis approach, adding the nucleotides step-by-step to achieve the desired product. As such, even very minute yield impurities at each step are multiplicative in the final product. Therefore, synthetic ONs can contain significant levels of impurities related to the misincorporation of nucleotides at the 5' end, which can be very difficult to be purified out or characterized. Enhancing the therapeutic function by modifying bases, sugars and the phospho-backbone, can pose additional challenges for the separation of closely related ON species during analytical studies. In addition, fragile ONs can show undesired fragmentation using LC-MS which needs to be avoided for accurate characterization.

Here, the separation and optimization for a phosphorothioated, locked nucleic acid (LNA) ON and related shortmers using high resolution TOF instrumentation are demonstrated.

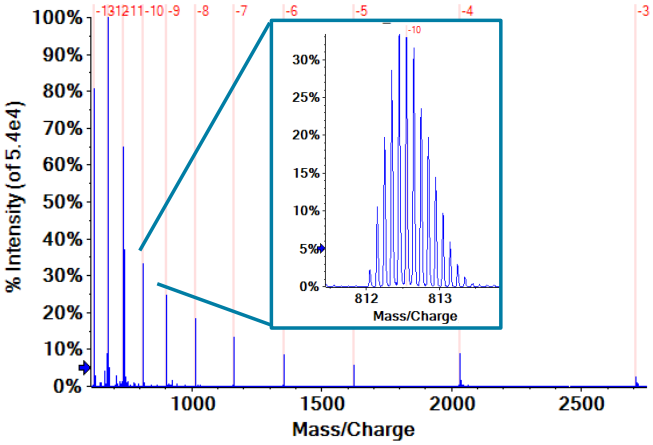


Figure 1. High-resolution TOF-MS spectra for 24-mer LNA ON.
Insert shows zoom-in to isotopically resolved charge state (z = -10).

Key Features of SCIEX QTOF technology for therapeutic ON characterization

- Efficient ionization and transmission of analytes in negative mode while reducing undesired fragmentation using the IonDrive™ TurboV Ion Source and QJet® Ion Guide
- Excellent TOF-MS raw data quality for therapeutic ON for confidence in confirmation
- High mass accuracy with exceptional negative ion performance for ON analysis
- Integration of data in ProMass Software for robust mass confirmation and purity assessment enabling high throughput analysis

Methods

Sample Preparation: Synthetic ONs were purchased from Integrated DNA Technologies as custom sequences mimicking a therapeutic product and related shortmers (Table 1). Phosphorothioated locked nucleic acid containing ONs (Figure 2) were chosen as these modifications are commonly used for therapeutic purposes.[2] The samples were reconstituted in 100 mM ethylenediaminetetraacetic acid (EDTA). All three ONs were mixed in similar amounts and further diluted in deionized water prior to use.

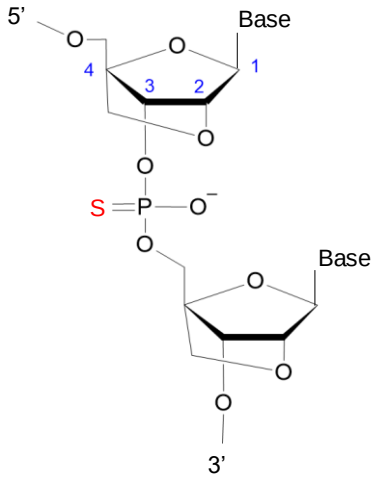


Figure 2. Schematic of phosphorothioated locked nucleic acid. Phophorotioate: a non-bridging oxygen is replaced by a sulfur (red). Locked nucleic acid: the 2' oxygen is linked to the 4' carbon.

Chromatography: The separation was accomplished using an Agilent 1290 Infinity UHPLC fitted with a Oligonucleotide BEH Column, 2.1 mm×100 mm with 1.7 µm particles and 130 Å. Mobile phase A consisted of 20 mM *N,N*-diisopropylethylamine (DIEA) with 100 mM 1,1,1,3,3,3-hexafluoro isopropanol (HFIP) in water while mobile phase B was 20 mM DIEA with 100 mM HFIP in methanol. A flow rate of 300 µl/min was used with the gradient shown in Table 2. The column temperature was held at 90 ºC. An injection volume of 5 µL was employed.

Table 2. Gradient for ON analysis.

Time [min]	Mobile Phase A [%]	Mobile Phase B [%]
Initial	*82*	*18*
7.0	*75*	*25*
7.1	*2*	*98*
8.5	*2*	*98*
8.6	*82*	*18*
11.5	*82*	*18*

Mass Spectrometry: A SCIEX TripleTOF® 6600 System equipped with an IonDrive™ Turbo V Ion Source was used for analysis. The MS parameters are listed in Table 3. The intact protein mode was turned on.

Data Processing: Data were visualized and processed using the BioToolKit within SCIEX OS Software as well as the SCIEX ProMassProcessor and ProMass Software (Novatia LLC).

Table 3. MS parameters.

Parameter	Setting
Scan Mode	*TOF-MS negative*
Gas 1	*60 psi*
Gas 2	*60 psi*
Curtain Gas	*40 psi*
Source Temperature	*350 °C*
Ion Spray Voltage	*-4500 V*
Time Bins to Sum	*4*
Accumulation Time	*1.0 sec*
Mass Range	*600 - 2,750 m/z*
Declustering Potential	*-50 V*
Collision Energy	*-10 V*
QJet RF Amplitude (XA1)	*200 V*

Table 1. Sequences of ON analytes.

Length	Sequence 5' to 3'
24-mer	*+T*+A*+G*rU*rC*rC*rA*rC*rA*rG*rA*rG*rC*rC*rG*rU*rG*rU*rU*rA*rG*+C*+A*+T*
23-mer	*+A*+G*rU*rC*rC*rA*rC*rA*rG*rA*rG*rC*rC*rG*rU*rG*rU*rU*rA*rG*+C*+A*+T*
22-mer	*+G*rU*rC*rC*rA*rC*rA*rG*rA*rG*rC*rC*rG*rU*rG*rU*rU*rA*rG*+C*+A*+T*

+: locked nucleic acid
*: phosphorothioated base
r: RNA

Optimization of LC and MS parameters for phosphorothioated LNA analysis

Phosphorothioate linkages are a dominating modification for ONs in therapeutic use, replacing a non-bridging oxygen by a phosphorus in order to enhance stability against nucleases and thus modulating the pharmacokinetics and pharmacodynamics of a drug (Figure 2).[2] With this enhancement of the therapeutic function a diastereomer is formed for each phosphorus center resulting in broad peaks for chromatographic methods.[1,3] In addition, target binding can be improved by connecting the 2'oxygen to the 4' carbon, resulting in a locked nucleic acid (Figure 2).[2] Such modifications pose additional analytical challenges[3], e. g. the ability to separate shortmers, *n*-1 failure sequences with *n* representing the number of nucleotides of the desired product.

Since the melting temperature (T_m) of ONs is affected by these modifications, too, the column oven temperature of the LC method was evaluated as a first step. Temperatures ranging from 60ºC to 90ºC were tested in 10ºC increments (data not shown). A temperature of 90ºC was chosen as being most beneficial for the peak shape of the LNA analytes. A temperature well above the T_m prevents the formation of secondary structures, thus improving the chromatographic behavior.

Due to the hydrophilic nature of ONs, their analyses via reversed-phase methods using C18 columns requires the use of ion-pairing agents to enhance their retention at the stationary phase. Aim for this study was to prevent the usage of highly toxic ion-pairing agents such as N,N-dimethylcyclohexylamine. With a slope of 1% acetonitrile per minute using 20 mM DIEA as an ion pairing reagent, a separation of the *n*-1 and *n*-2 failure sequences could be achieved within a total run time of 11.5 min (Figure 3).

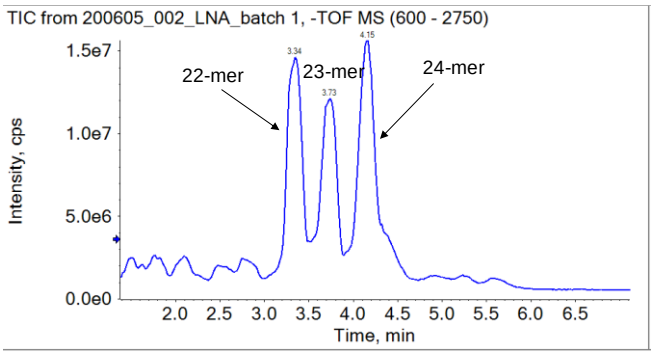


Figure 3. Total ion chromatogram for LNA impurity analysis. The 24-mer was separated from the spiked-in failure sequences.

Since the fragile analytes can show undesired fragmentation (Figure 4, bottom), a focus was put on tuning the front end appropriately. Two main factors contributing to undesired fragmentation were evaluated: the source temperature and the radio frequency (RF) of the QJet® Ion Guide (XA1).

For the mass range of 600 – 2,750 *m/z* used for this analysis, the default value of XA1 is set to 300 V. This default value is suitable for a wide range of applications from small molecules to proteins and ensures the transfer of the analytes into the Q0 with high efficiency. However, for oligo analysis it is recommended to verify the default setting. A variety of ON classes including deoxyribonucleic acid (DNA), ribonucleic acids (RNA) containing modifications such as 2'O-methoxyethyl RNA and LNA, with sizes suitable for therapeutic purposes (17 to 24 bases) were tested. For all ONs tested, lowering the XA1 to ~200 V minimized undesired fragmentation in the front end (Figure 3), while maintaining the overall intensities.

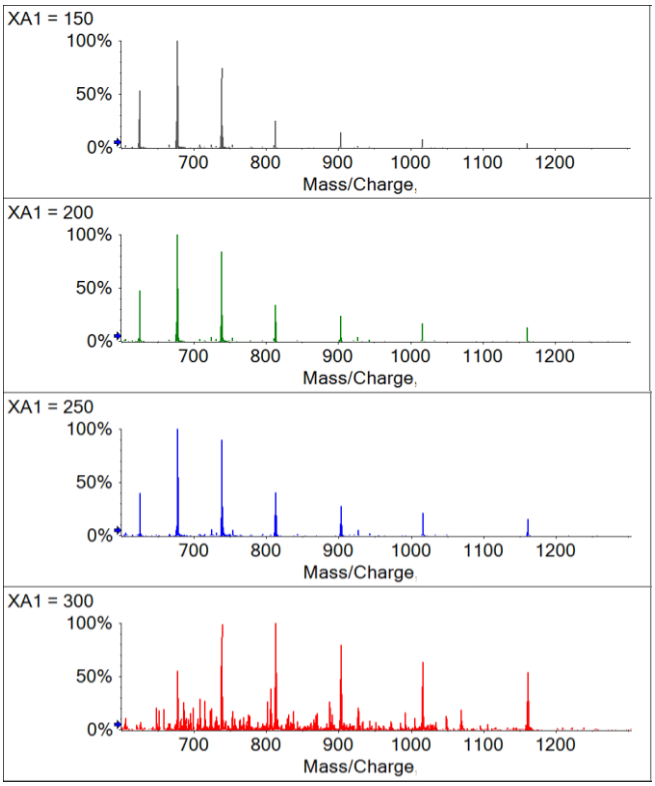


Figure 4. TOF-MS raw spectra of the analysis of the 24-mer LNA with different XA1 values. For XA1 values above 250 V fragmentation was observed.

Another concern during characterization of ONs is the loss of a purine base, guanidine or adenosine, respectively, in the source (Figure 5). Tuning the source temperature is key to prevent undesired in-source depurination. With the IonDrive™

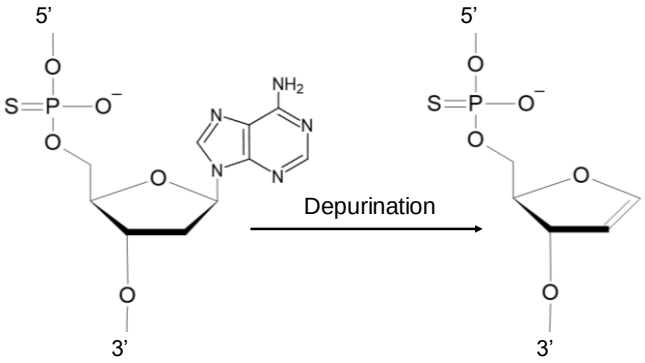


Figure 5. Process of depurination in the gas phase. Example shows loss of an adenine base in phosphorothioated DNA.

Turbo V Ion Source and its large heater diameter, an efficient desolvation, declustering and ionization can be achieved, without causing depurination of the fragile analytes. For the LNA sample, source temperature above 350 °C showed increasing depurination (Figure 6, arrow).

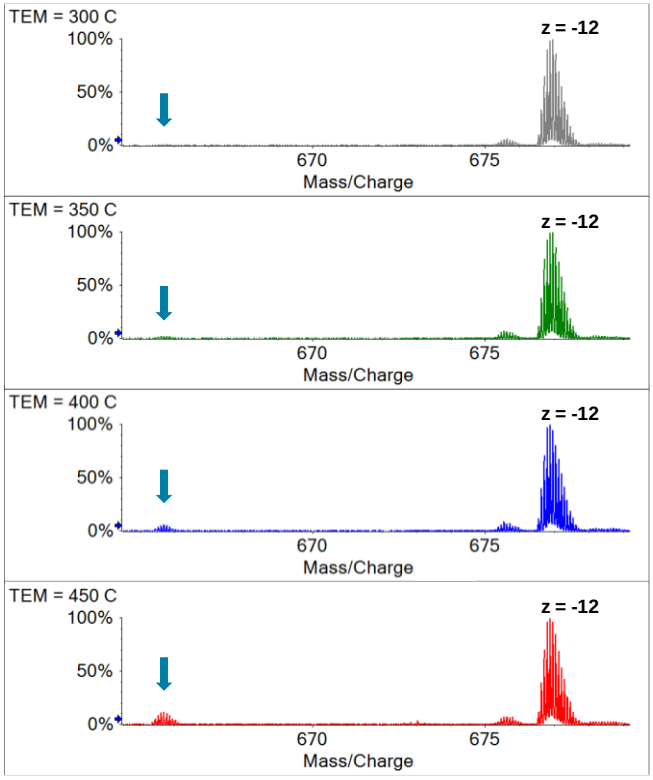


Figure 6: TOF-MS raw spectra of the analysis of the 24-mer LNA with different source temperatures. Zoom into highest abundant charge state z = -12. For temperature values above 350°C, depurination (indicated by arrow) was observed.

Characterization of LNA ON

Using the optimized method the spiked-in samples were evaluated. Great TOF-MS data quality was achieved for the main product and the related shortmers (Figure 7). With the BioToolKit in SCIEX OS Software an isotopically resolved reconstruction was performed and the masses obtained were compared against theoretically calculated masses for the monoisotopic peak (Table 4). Excellent mass accuracy (below 1.5 ppm error) was obtained for all analytes (Figure 7, Table 4), giving high confidence in the exact measurement of the ONs with high-resolution TOF-MS.

Table 4. Mass accuracies for reconstructed monoisotopic peaks of different LNAs.

LNA	$MW_{measured}$ [Da]	$MW_{theoretical}$ [Da]	Mass Error [ppm]
22-mer	*7425.526*	*7425.517*	*1.2*
23-mer	*7782.543*	*7782.547*	*-0.5*
24-mer	*8130.564*	*8130.565*	*-0.1*

With the final method, no depurination was observed (Figure 8). Furthermore, undesired salt adducts could be kept to a minimum (Figure 8). ON usually contain salts after synthesis and require a desalting step. However, they also tend to pick up salts, such as sodium and potassium, from the storage or dilution buffer and/or the LC system. Achieving low adducts (below 5 %) is key for a successful characterization and is a common requirement within the industry for lot release of therapeutic ON products. Mindful buffer preparation (usually on a daily basis) and a thoroughly cleaned, dedicated LC system for ON analysis play an important role, but also the a highly efficient declustering in the ion source while preventing fragmentation.

For all of the phosphorothioated LNA analytes used in this study, a mass shift of -16 Da was observed (B1-B3 in Figure 7, Figure 8). This mass shift can be linked to the presence of residual phosphate in the phosphorothioated ON.[1] Residual phosphate is considered a major process-related impurity for phosphorothioated ON products due to incomplete thiolation or PO exchange.[1] The separation of such an impurity is highly sequence specific and not necessarily possible in all cases using RP-LC.[1] For the targeted analyte a partial separation was observed being in alignment with the expectation of the phosphodiester-containing impurity being more hydrophilic compared to the fully phosphorothioated product (Figure 9). Charged-based separation might be the method of choice via anion exchange chromatography or capillary electrophoresis.

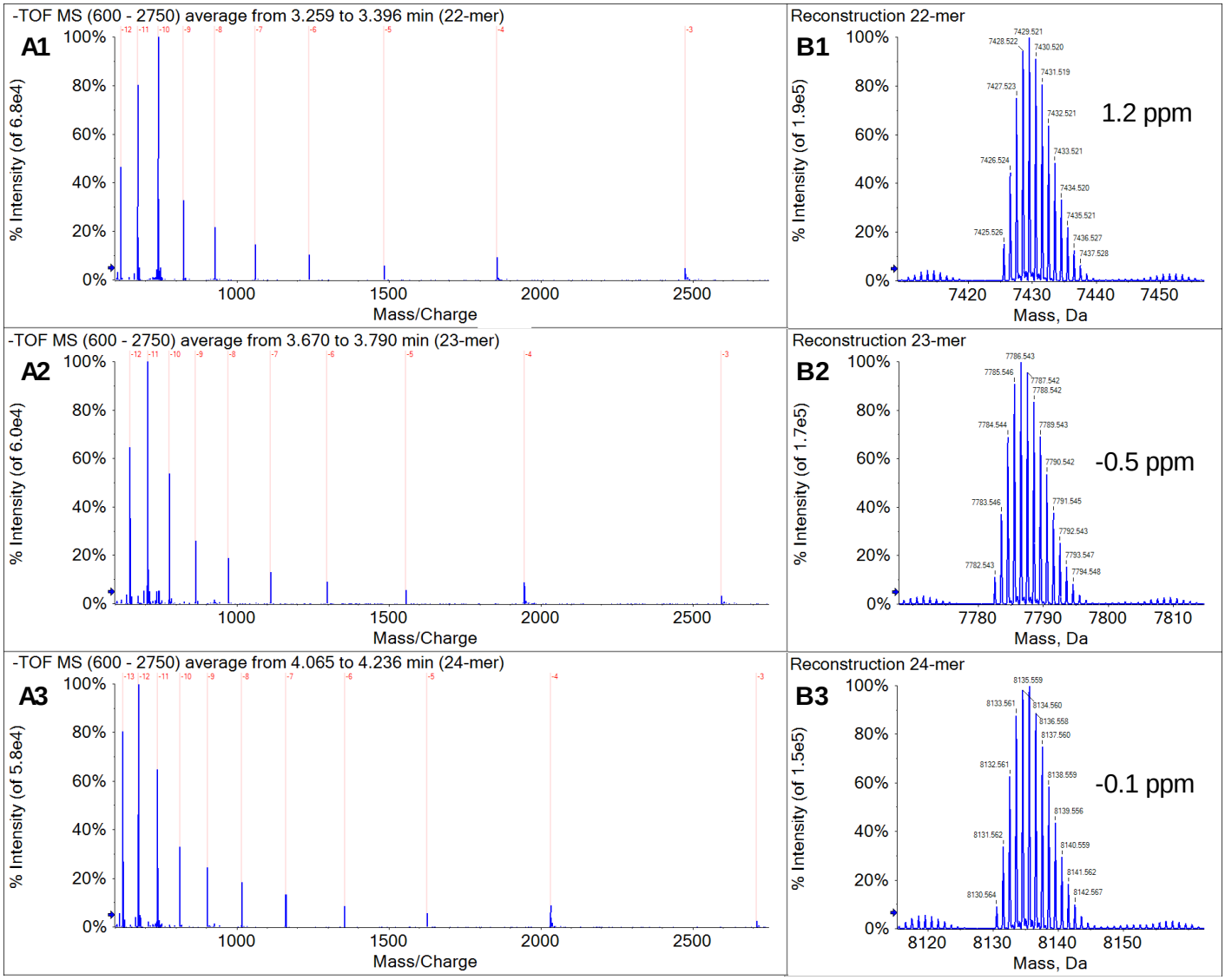


Figure 7. Data for characterization of LNA and related impurities. A: average TOF-MS spectra for 22-mer (A1), 23-mer (A2) and 24-mer (A3). C: zoom-in to isotopically resolved reconstructed data for 22-mer (B1), 23-mer (B2) and 24-mer (B3) including error calculation for monoisotopic peak in ppm.

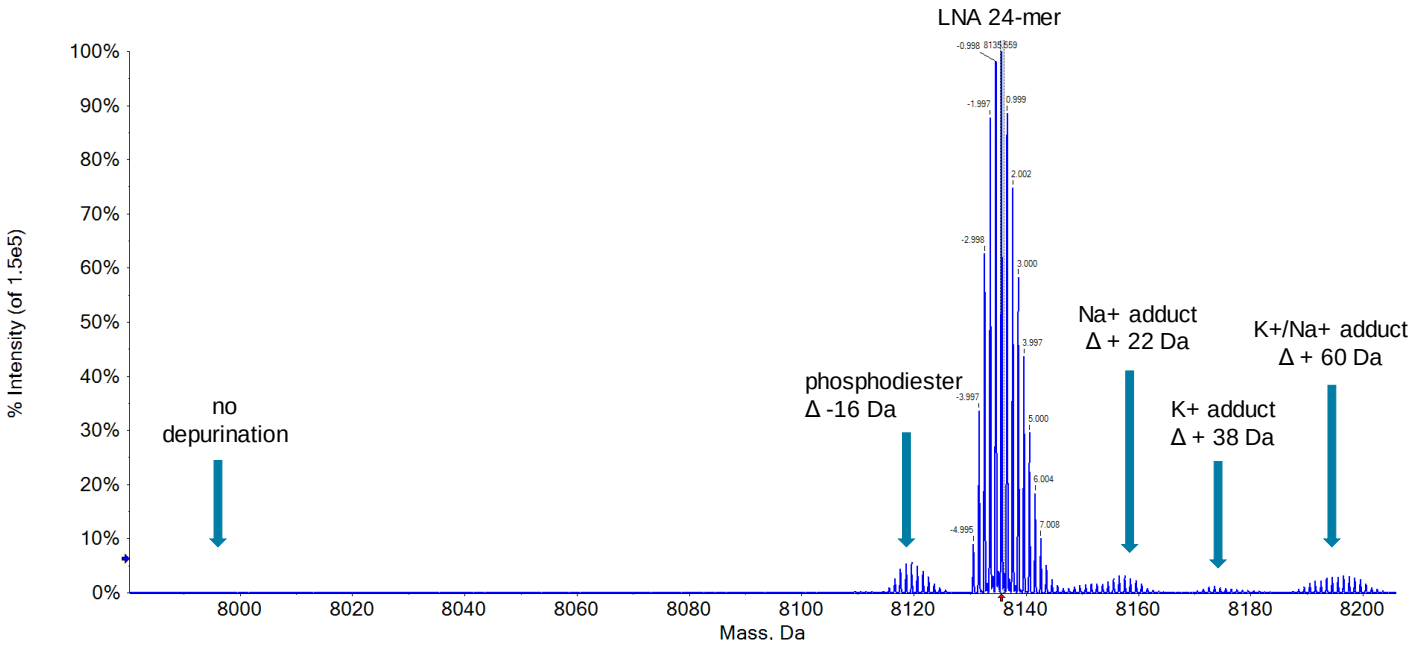


Figure 8. Isotopically resolved reconstructed data for 24-mer LNA product. Major species represents the product of interest (phosphorothioated 24-mer of LNA). Smaller peaks with positive mass shift compared to major species show sodium (Na+) and potassium (K+) salt adducts (all below 5 %). A small amount of the product with a phosphodiester (Δ-16 Da) was detected, whereas no depurination was observed.

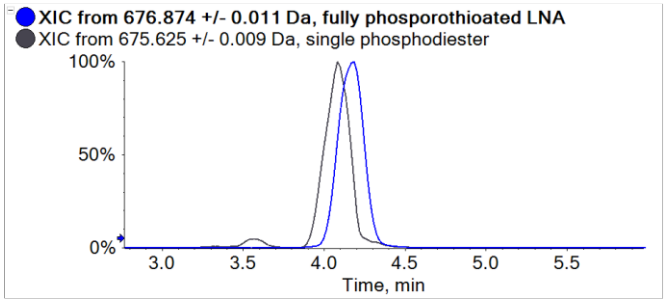


Figure 9. Partial separation of single phosphodiester linkage. Extracted ion chromatograms (XIC) of phosphorothioated 24-mer LNA (blue) and 24-mer product with one phosphodiester (grey).

ProMass Software for high throughput analysis

SCIEX raw data formats .wiff and .wiff2 can be exported using the SCIEX ProMassProcessor followed by processing with ProMass Software as described earlier.[4] Within the SCIEX ProMass Processor, impurities such as 5' end or 3' failure sequences can be chosen for processing (Figure 10), allowing for identification in an automatic fashion via batch processing of ON data in the ProMass software. With this approach, high throughput analysis is possible. Upon processing, an intuitive color coding scheme expedites the review of data, allowing a scientist to focus on the samples deviating from the desired state for instance (see sample browser with green, red and purple color coding in Figure 11 top). For each sample further information can be obtained, such as information on the matching of the target compound and automatic matching of impurities based on the processing criteria set by the user (Figure 11 bottom table) speeding up the evaluation of batches.

In this case, the target mass of the 24-mer LNA was predefined and the processing settings were set to sequence with 5' ladders and impurities (Figure 10), to showcase the identification of the target compound alongside with impurities related to the misincorporation of nucleotides at the 5' end. As a result, the spiked-in failure sequence were identified (sequence ladder summary in Figure 11). In addition, the process-related phosphodiester-containing product (~16 Da loss) was identified (last line of the sequence ladder summary in Figure 11).

As a useful tool e.g. for comparing batches, an automatic percentage calculation of the different impurities/ the purity of the desired product either based on UV-data or MS-data input can be obtained (Figure 11, bottom).

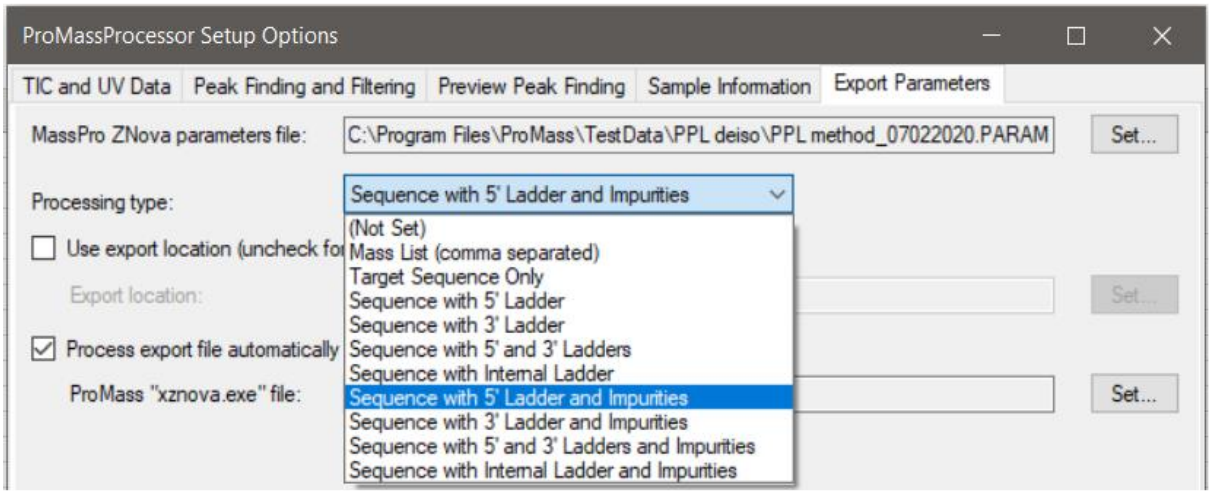
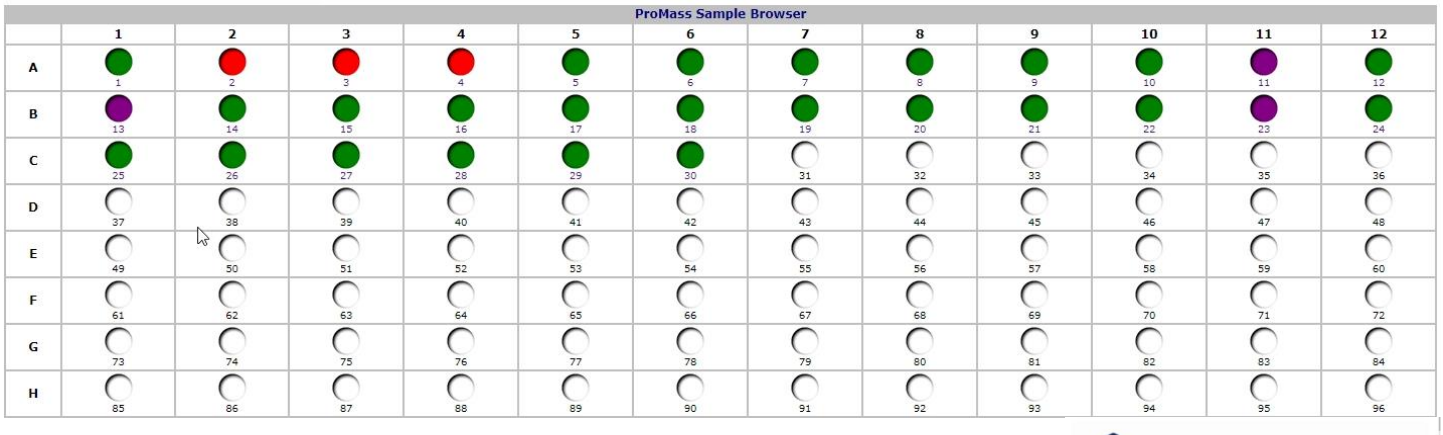


Figure 10. Processing parameters within the SCIEX ProMass Processor Software. The drop-down menu allows for an optional search of different impurities.



Target Mass Summary

RT (min)	Target Mass (Da)	Observed Mass (Da)	Mass Error	Intensity	% Abundance (in Spectrum)	%Purity (Estimate)	Identity	Result Code
4.150	8130.5614	8130.559	-0.0024 Da (-0.3 ppm)	1.79E+006	20.82	8.62	Target Mass	

Sequence Ladder Summary

RT (min)	Calculated Mass (Da)	Observed Mass (Da)	Mass Error	Intensity	Sequence
3.337	7425.5140	7425.537	0.0230 Da (3.1 ppm)	1.87E+006	+G*3-+T24
4.150	8130.5614	8130.559	-0.0024 Da (-0.3 ppm)	1.79E+006	+T*1-+T24
3.729	7782.5435	7782.554	0.0105 Da (1.3 ppm)	1.51E+006	+A*2-+T24, 8130.5614 (Minus +T*)
3.729	7783.5274	7783.560	0.0326 Da (4.2 ppm)	1.08E+006	8130.5614 (Minus +C*)
3.729	7785.5319	7785.536	0.0041 Da (0.5 ppm)	3.24E+005	8130.5614 (Minus rA*)
4.150	8114.5842	8114.566	-0.0182 Da (-2.2 ppm)	7.54E+004	8130.5614 (loss of thioate)

Chromatogram Summary

RT (min)	Base Peak Mass (Da)	Intensity	Spectral Quality	LC/MS Peak Area	LC/MS Area Percent
3.337	7426.525	2.31E+006	ok	1.61E+008	31.40
3.729	7782.554	1.51E+006	ok	1.39E+008	27.18
4.150	8130.559	1.79E+006	ok	2.12E+008	41.42

Figure 11. Results of processing LNA data in ProMass Software. Top: sample summary with colored wells indicating pass (green), fail (red) or marginal differences (purple) based on user-defined criteria. Bottom: tabular summary for one sample including target mass and impurity overview with retention time, calculated and observed monoisotopic mass and mass error.

Conclusions

- Method parameters were successfully optimized for the analysis of synthetic phosphorothioated LNA
- Undesired fragmentation and depurination were circumvented and adduct formation was kept to a minimum using soft still efficient ionization via the IonDrive™ TurboV Ion Source and transfer via the QJet® Ion Guide suitable for a range of ON
- Excellent TOF-MS raw data quality and high mass accuracy were achieved for all analytes
- High throughput data analysis with ProMass Software was showcased, allowing for quick turnaround times in assessing products and process-related impurities

References

1. Bonilla JV and Srivatsa GS: Handbook of Analysis of Oligonucleotides and Related Products, New York, CRC Press, 2011.
2. Smith E and Zain R (2018) Therapeutic Oligonucleotides: State of the Art. Annu Rev Pharmacol. 59:605-630.
3. Nikcevic I, et al. (2012) Detecting low-level synthesis impurities in modified phosphorothioate oligonucleotides using liquid chromatography-high resolution mass spectrometry. Int J Mass Spectrom. **304**(2-3):98-104.
4. High Resolution Analysis of Synthetic Oligonucleotides, SCIEX technical note RUO-MKT-02-10050-A

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