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Application Note

Sensitive LC-MS/MS Bioanalytical Quantitation of Antisense Oligonucleotides

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Abstract

This application demonstrates the quantitative and qualitative capabilities of the Waters™ Xevo™ TQ Absolute Tandem Quadrupole Mass Spectrometer (MS/MS) and its suitability for oligonucleotide bioanalysis in human plasma.

Xevo TQ Absolute Tandem Quadrupole Mass Spectrometer.

Benefits

- Sensitive and routine quantitation of oligonucleotides in human plasma is demonstrated using the tandem quadrupole mass spectrometer, the Waters Xevo TQ Absolute

- The quantitation of a 25-mer fully phosphorothioated antisense oligonucleotide, Trecoversin (GEM91) and polyoligodeoxythymidine 15 to 30 mer oligonucleotide standards from 4.5 to 9 kDa molecular weight range extracted from human plasma is shown
- The Waters ACQUITY™ Premier UPLC System and Waters ACQUITY Premier Oligonucleotide C_{18} Column technologies enhanced performance for this application for several critical bioanalytical challenges including improved chromatographic recovery, improved LLOQs and increased linear dynamic range

Introduction

Oligonucleotides are challenging substrates to measure due to many factors including but not limited to: requiring ion pairing or non-reversed phased chromatography; requiring careful handling and sample prep; and often suffering from non-specific binding issues from consumables and metal surface interactions often present in chromatographic systems.

In this application note, we demonstrate the performance capabilities of the high-performance tandem Xevo TQ Absolute Tandem Quadrupole Mass Spectrometer coupled to the Waters ACQUITY Premier System for the analysis of oligonucleotides in biological matrices. Quantification of oligodeoxythymidines standards (Waters MassPREP™ Oligonucleotide Separation Technology (OST) Standard) and GEM91, a fully phosphorothioated antisense oligonucleotide [d(P-Thio)(C-T-C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T-T-C-T)-DNA] were investigated.

Experimental

Stock solutions of the Waters MassPREP Oligonucleotide Separation Technology (OST) Standard (p/n: 186004135 <https://www.waters.com/nextgen/global/shop/standards--reagents/186004135-massprep-oligonucleotide-standard.html>) and the oligodeoxynucleotide phosphorothioate, GEM91 (custom synthesized by Integrated DNA Technologies, Inc.) were prepared at 10 μM and 1 mg/mL respectively in TE buffer prepared

in-house (10 mM Tris + 1mM EDTA, adjusted to pH 8 with ammonium hydroxide). Plasma samples were prepared by serial dilution of stock solution in human plasma (purchased from BioIVT). Plasma samples were extracted using liquid-liquid extraction method with Phenol:Chloroform:Isoamyl Alcohol 25:24:1 followed by a second extraction with chloroform, +99%. Finally, all aqueous extracts were dried and reconstituted in 100 µM EDTA solution.

LC Conditions

LC system:	Waters ACQUITY Premier System (BSM)
Mobile phase A:	100 mM Hexafluoroisopropanol (HFIP) + 15 Mm N, N-Diisopropylethylamine (DIPEA) in Water
Mobile phase B:	100 mM Hexafluoroisopropanol (HFIP) + 15mM N, N-Diisopropylethylamine (DIPEA) in 80% acetonitrile
Vials/plate:	QuanRecovery™ with MaxPeak™ 700 µL Plate (p/n: 186009185) with Round Plug Pre-slit Silicone Cap-mat (p/n: 186006332)
Column(s):	Waters ACQUITY Premier Oligonucleotide C_{18} column, 1.7 µm, 2.1 x 50 mm (p/n: 186009484)
Column temp.:	50 °C
Sample temp.:	8 °C
Injection volume:	20 µL
Flow rate:	0.5 mL/min

Gradient

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.5	95.0	5.0	Initial
3.50	0.5	78.0	22.0	6
4.00	0.5	20.0	80.0	6
4.50	0.5	95.0	5.0	6
5.00	0.5	95.0	5.0	6

MS Conditions

Ionization mode:	ESI-
Acquisition type:	MRM
Capillary voltage:	2.5 kV
Cone voltage:	30 V
Source offset:	30
Source temp.:	150 °C
Desolvation temp.:	600 °C
Desolvation gas:	1000 L/Hr

Cone gas: 150 L/Hr

Collision gas: 0.15 mL/min

Name	Precursor *m/z*	Product *m/z*	Cone (V)	Collision (eV)
15T	561.8	125.1	30	35
20T	601.1	125.1	30	35
25T	627.3	303.1	30	20
30T	646.5	303.1	30	20
GEM91	597.2	319.1	30	20
GEM132 (IS)	732.7	319.0	30	40

Table 1. MRM transitions and conditions used for oligonucleotide analysis.

Data Management

Data was acquired using MassLynx™, v4.2 and processing was performed using TargetLynx™.

Results and Discussion

Chromatographic separation was performed on an ACQUITY Premier System, equipped with an ACQUITY Premier Oligonucleotide C_{18} Column, 130 Å, 1.7 µm, 2.1 x 50 mm (p/n: 186009484 < https://www.waters.com/nextgen/global/shop/columns/186009484-acquity-premier-oligonucleotide-c18-column-130a-17--m-21-x-50-mm.html>), using a 5-minute gradient (5–80% B) at flow rate of 0.5 mL/min. ACQUITY Premier Columns incorporate MaxPeak™ High Performance Surfaces (HPS) technology to column hardware which is critical for minimizing non-specific binding, thereby improving oligonucleotide recovery and

assay limits of detection. HPS technology was developed specifically to minimize metal interactions with analytes such as oligonucleotides and other analytes that have historically shown strong affinity towards metal surfaces.[1,2]

Typical spectra observed for these GEM91 are shown in Figure 1, showing both a general distribution of charge state clusters (MS Scan) and MS/MS spectra showing fragments generated for charge state selected as precursor of the MRM transition for GEM91. During method development, multiple ions may be monitored. If a preferred ion is interfered with, a different cluster and appropriate fragment ion may be easily chosen.

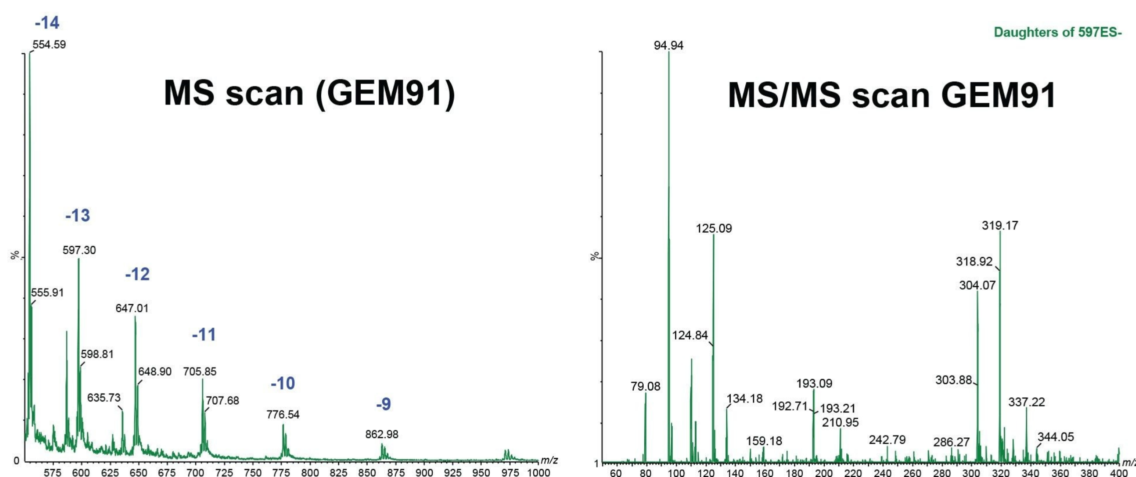

Figure 1. Representative MS (Left) and MS/MS (Right) spectrum of GEM91.

Figure 2A shows representative traces of GEM91 (*m/z* 597.2 > 319.1) LLOQ relative to the matrix background.

Figure 2B shows representative QC traces from 0.1 to 500 ng/mL.

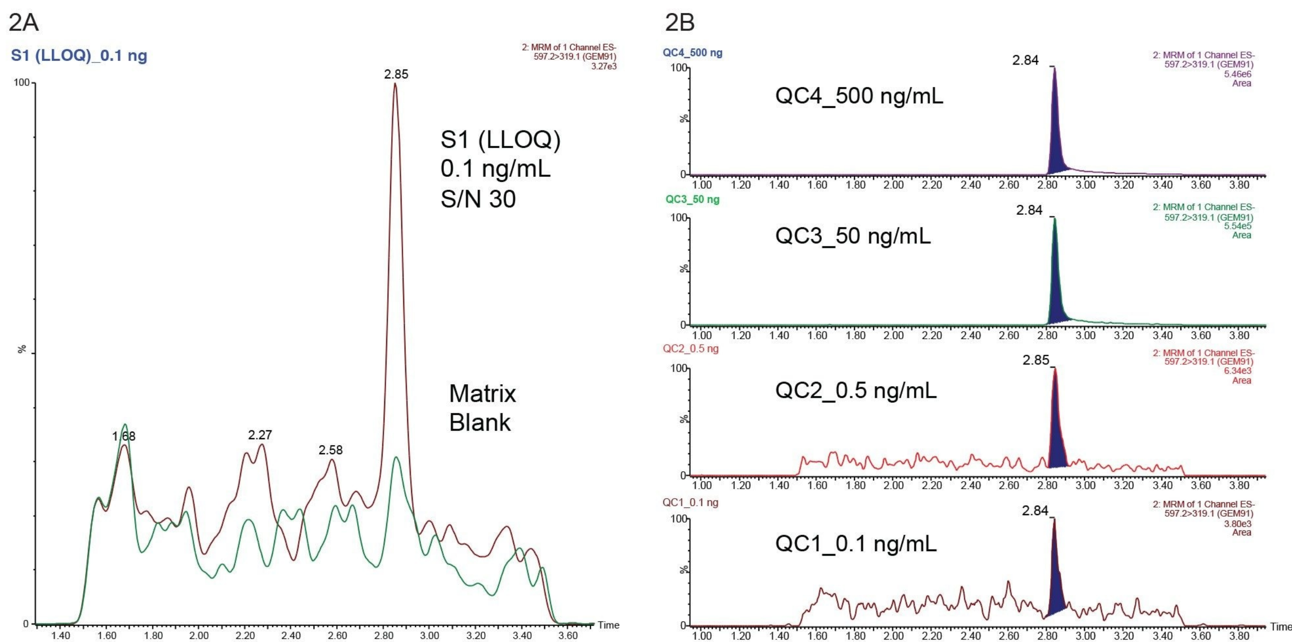

Figure 2A. GEM 91 (597.2>319.1) 0.1 ng/mL standard (LLOQ) overlaid with matrix blank (green trace).

Figure 2B. Representative QC traces for GEM91 (0.1–500 ng/mL).

A summary of standard curve and quality control (QC) performance for OST standards and GEM 91 on the Xevo Tandem Quadrupole Mass Spectrometer is highlighted in Table 2. The OST stock solution was prepared as µM stock and is reported in nM with corresponding LLOQs in ng/mL for reference shown on the right, GEM91 is reported both in nM and in ng/mL.

	OSTs (Accuracy (%))					GEM91		
Name	Std conc (nM)	15T	20T	25T	30T	Std conc (ng/mL)	Std conc (nM)	Accuracy (%)
Std-1	0.01	94.5	103.7	105.4	-	0.10	0.08	110.2
Std-2	0.02	107.3	94.8	92.9	101.6	0.20	0.16	82.1
Std-3	0.05	111.2	93.9	87.3	90.6	0.50	0.39	92.8
Std-4	0.10	96.0	95.6	111.9	111.6	1.00	0.78	98.4
Std-5	0.20	99.5	111.1	88.8	98.4	2.00	1.60	97.0
Std-6	1.00	101.1	104.8	104.8	100.9	10.0	7.80	97.8
Std-7	10	96.6	104.1	103.8	102.1	100	78.0	106.2
Std-8	100	99.3	99.8	105.2	111.1	1000	780	108.9
Std-9	500	98.6	100.5	101.2	93.9	5000	3890	107.4
Std-10	1000	95.9	91.9	98.6	89.9	10000	7780	99.1

Table 2. Calibration standards used in this experiment.

The lower limit of quantification (LLOQ) was 0.1 ng/mL with a dynamic range observed from 0.1–10,000 ng/ml. The calibration curves were linear with r^2 values >0.99 ($1/x^2$ weighting) with mean accuracy of all accepted calibration points between 87–112%. QC performance of Oligonucleotides OST and GEM 91 are reported in Table 3, with mean accuracies between 90–114% and CV's (not shown) between 1.1–10.5% for all Oligonucleotides. OST standards' results suggest that smaller oligonucleotides could have more sensitivity compared to longer ones. GEM91 which is of similar length to 25T standard has shown similar results, whereas 15T standard showed two times higher sensitivity at 0.05ng/mL as LLOQ.

	OSTs (Accuracy (%))					GEM91		
Name	Std conc (nM)	15T	20T	25T	30T	QC conc (ng/mL)	QC conc (nM)	Accuracy (%)
QC1	0.01	114.3	100.3	93.7	N/A	0.10	0.08	99.2
QC2	0.05	100.3	105.2	104.0	108.7	0.50	0.39	91.0
QC3	5.0	95.1	99.9	93.2	100.1	50	39.0	106.8
QC4	50	90.1	99.8	95.6	100.9	500	390	110.7
QC5	400	96.6	99.6	97.7	97.2	4000	3110	108.3
QC6	800	92.3	92.1	93.0	91.6	8000	6220	98.3

Table 3. QC samples used in this experiment.

Several transitions tested on the Xevo TQ Absolute were able to detect sub ng/ml of GEM91 oligonucleotide in biological matrix. Three transitions for the LLOQ of 0.1 ng/ml level of GEM91 are shown in Figure 3.

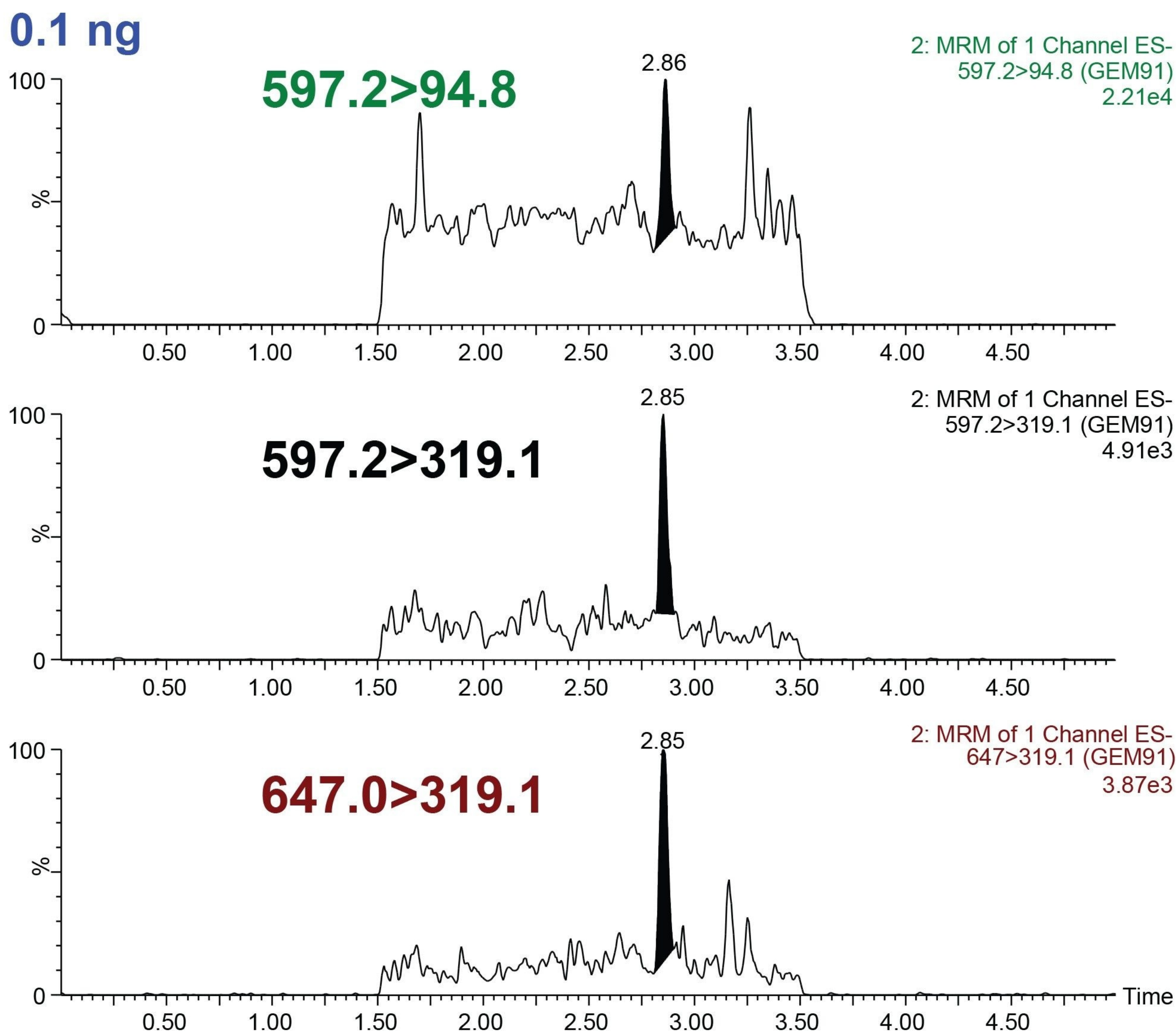

Figure 3. Demonstrates ability to detect 0.1 ng/mL with different transitions.

Lower mass transitions, corresponding to basic structural moieties (such as for example, the phosphorothioate fragment ion, measured by the 597.2>94.8 transition) are often the most intense fragment ion and attractive targets for quantitation, but may be challenging or less selective in complex plasma matrix background. In this study, both high counts and acceptable signal-to-noise was observed at both low and higher fragment-based mass transitions at the LLOQ levels. Traces for the -14 (597 Da) and -13 (647 Da) charge states to the 319.1 fragment ions show how the 597.2>94.8 MRM peak shape and performance compares to larger ions in this

assay. Ultimately, this flexibility facilitates the user to select a transition which exhibits the best sensitivity and selectivity across species/matrix changes in preclinical (and human) studies. QC, linearity, and bioanalytical figures of merit for this application note are shown for the -13 charge state (*m/z* 597.2) using the *m/z* 319.1 product ion, however, the Xevo TQ Absolute detected GEM91 at LLOQ of 0.1 ng/mL (>5x response compared to blank response) and showed similar bioanalytical performance across all transitions (shown in figure 3) in plasma samples.

Figure 4 shows the linear dynamic range obtained on the MS system for GEM91 (597.2>319.1) for the range 0.1 to 10,000 ng/mL and a close-up view of lower-level calibration standards below 10 ng/mL. Five orders linear dynamic range was observed.

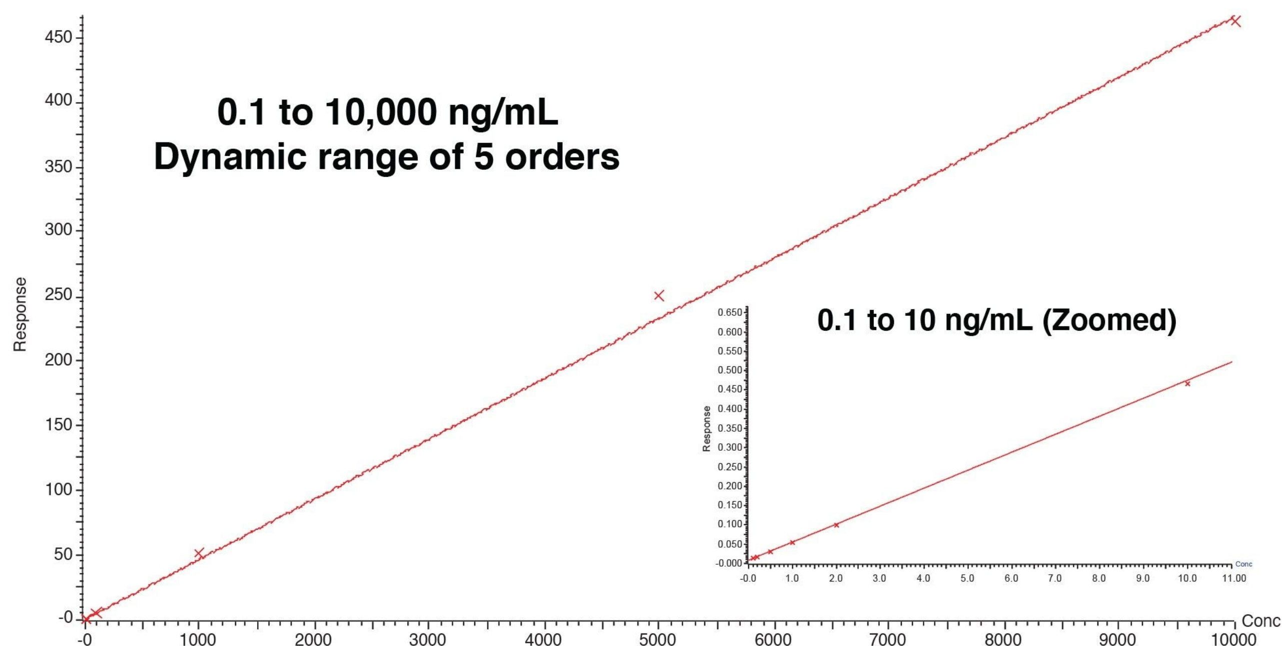

Figure 4. Standard curve for GEM 91 analyzed on the Xevo TQ Absolute showing 5x linear dynamic range, inset shows 0.1 to 10 ng/mL standards.

Conclusion

- With enhanced sensitivity for challenging negative ionization compounds, the Xevo TQ Absolute is capable of generating high quality data for routine liquid chromatography with tandem mass spectrometry (LC-MS/MS) based quantitation of oligonucleotides in biological matrices.
- Low ng/ml levels of sensitivity with a good dynamic range performance for oligonucleotides was observed in human plasma for both an antisense oligonucleotide as well as oligonucleotide performance standards.
- The use of ACQUITY Premier with MaxPeak High Performance Surfaces (HPS) technology in both the separations system and analytical column help to mitigate metal adsorption, ensuring robust and sensitive quantitation performance for quantitative bioanalytical assays.

References

1. Guilherme J. Guimaraes, J. Michael Sutton, Martin Gilar, Michael Donegan, Michael G. Bartlett, 'Impact of Nonspecific Adsorption to Metal Surfaces in Ion Pair-RP LC-MS Impurity Analysis of Oligonucleotides', *Journal of Pharmaceutical and Biomedical Analysis* Volume 208, 20 January 2022, 114439.
2. Jennifer M Nguyen , Martin Gilar, Brooke Koshel, Michael Donegan, Jason MacLean, Zhimin Li & Matthew A Lauber, 'Assessing the Impact of Nonspecific Binding on Oligonucleotide Bioanalysis', ***Future Science,*** BIOANALYSIS VOL*. 13, NO. 16.*
3. Kathryn Brennan, Mary Trudeau, Paul D. Rainville, Utilization of the ACQUITY Premier System and Column for Improved Oligonucleotide Bioanalytical Chromatographic Performance, Waters Application Note, 720007119, January 2021.

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ACQUITY Premier System <https://www.waters.com/waters/nav.htm?cid=135077739>

Xevo TQ Absolute

MassLynx MS Software <https://www.waters.com/513662>

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