Improve Sensitivity for Antisense Oligonucleotide Quantification in Plasma Using MicroLC-MRM Methodology

***Featuring the* SCIEX QTRAP® 6500+ LC-MS/MS System with the M5 MicroLC System**

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Oligonucleotide therapeutics are rapidly growing as a class of therapeutic compounds. While oligonucleotide therapeutics have been investigated for the treatment of a number of diseases over the past 30 years, the number of regulatory approvals has been limited due to challenges in delivery of the therapeutic molecule to the target. Significant research has focused on improvement in the delivery of oligonucleotides as well as improvement of their therapeutic half-life. Therefore, an increase in the number of candidates entering clinical trials and in approvals has been observed, which triggers higher demands on bioanalytical assay development.

Traditional approaches include hybridization enzyme linked immunosorbent assays (ELISA), liquid chromatographic fluorescence (LC-FLR), or polymerase chain reaction (PCR) approaches. While these approaches demonstrate good quantification capabilities, including high throughput and high sensitivity, they usually struggle with providing desired selectivity to distinguish between the target oligonucleotide and its impurities or metabolites.

As the orthogonal technology, mass spectrometry has been routinely used for bioanalytical studies of oligonucleotides, but the extent of its use to date has been limited because its sensitivity does not allow it to quantify the ultra-low analyte levels in matrices. Improvements in mass spectrometry sensitivity as well as the implementation of microflow chromatography have enabled sensitivity in LC-MS assays to approach what was previously only achievable with hybridization ELISA. Presented in this technical note is the use of the QTRAP 6500+ LC-MS/MS System coupled with the M5 MicroLC System with the OptiFlow® Source for improved sensitivity to quantify oligonucleotides in matrix.

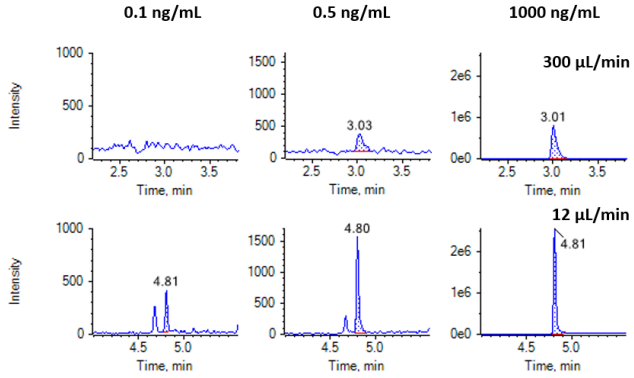

Figure 1. Comparison of High-flow (top) and Microflow (bottom) XICs of Fomivirsen at Selected Spiked-In Levels.

Key Features of the MicroLC-MRM Workflow for Oligonucleotide Quantification

- The QTRAP 6500+ LC-MS/MS System provides the sensitivity and quantification power to analyze oligonucleotides in matrix
- The QTRAP 6500+ LC-MS/MS System is compatible with both high-flow and microflow HPLC analysis with minimum optimization required
- The OptiFlow Source offers flexibility in column selection for challenging oligonucleotide separations, while requiring no probe or electrode position optimization
- The M5 MicroLC System provides accurate control of flow rates down to 1 µL/min with direct-inject and trap-elute capabilities for fast and large sample volume loading

Materials and Methods

Samples and Reagents: The ion paring reagents HFIP and DIEA were purchased from Sigma Aldrich. The customized oligonucleotide standards were synthesized by Sigma, including an oligonucleotide that is designed to be structurally equivalent to fomivirsen and an internal standard (IS). Fomivirsen is a synthetic antisense oligonucleotide composed of 21 bases (5'-G*C*G*T*T*T*G*C*T*C*T*T*C*T*T*C*T*T*G*C*G-3') linked by phosphorothioate bonds. The IS is an oligonucleotide composed of 23 bases (5'- ACGGCTACCTTGTTACGACTTCA-3'). The OTX Clarity SPE kit was purchased from Phenomenex.

Sample Preparation: 100 µL of rat plasma was extracted through the OTX Clarity SPE plate using the recommended protocol. The SPE plate was activated with 1 mL methanol and equilibrated with 1 mL equilibration buffer. After equilibration, rat plasma was loaded to the plate and washed with 1 mL washing buffer for 3 times and subsequently eluted with 1 mL elution buffer. The eluted samples were dried down with N_2 gas and reconstituted in 100 µL mobile phase A (100 mM HFIP and 15 mM DIEA in water) containing 100 µM EDTA. The calibration curve sample set with 0.1 ng/mL to 1000 ng/mL of fomivirsen was prepared by spiking fomivirsen into extracted rat plasma.

LC-MS Conditions for Comparative Analysis: On-column MRM method development was performed because the MRM parameters are highly dependent on the LC-MS conditions. In order to compare the sensitivity difference between analytical flow LC-MS and microflow LC-MS approaches, each sample was injected and analyzed in triplicate on the same QTRAP 6500+ System with the same MS dependent parameters (Table 1), coupled with either an ExionLC™ system and Ion Drive™ Turbo V Ion Source, or an M5 MicroLC System and an OptiFlow Turbo V Ion Source. Table 2-5 summarize the gas/source and liquid chromatography conditions that were optimized for each specific hardware configuration and flow rate.

Data Processing: The MRM data were processed by using SCIEX OS-Q Software 1.5 with a targeted quantitative workflow. The MQ4 algorithm was used for peak integration.

Table 1: MRM Method and MS Parameters.

ID	Q1	Q3	DP	CE	CXP	EP
Fomivirsen [M-9H]	741.0	319.0	-74	-43	-12	-10
Fomivirsen [M-8H]	834.0	319.0	-55	-46	-12	-10
IS-1	730.0	303.2	-72	-49	-12	-10
IS-2	657.1	328.1	-83	-37	-12	-10

Table 2: Chromatographic Conditions for Analytical Flow.

Time	Flow Rate (mL/min)	%A	%B
0	0.3	84	*16*
0.5	0.3	*84*	*16*
3.5	*0.3*	*50*	*50*
3.6	*0.3*	*0*	*100*
4.0	*0.3*	*0*	*100*
4.1	*0.3*	*84*	*16*
5.0	*0.3*	*984*	*16*

Parameter	Value
Stationary phase	*C18 column (2.1 mm X 50 mm, 1.7 µm, 130 Å)*
Mobile phase A	*15mM DIEA 100mM HFIP in water*
Mobile phase B	*15mM DIEA 100mM HFIP in 90% methanol*
Flow rate	*0.3 mL/min*
Column temperature	*60 ºC*
Injection volume	*10 µL*

Table 3: Mass Spectrometer Conditions for Analytical Flow.

Parameter	Value	Parameter	Value
Curtain gas:	35	CAD GAS:	*High*
Ion source gas 1:	70	*Ion Spray Voltage:*	*-4500*
Ion source gas 2:	*70*	*Source Temperature:*	*400*

Table 4: Chromatographic Conditions for Microflow.

Time	Flow Rate (µL/min)	%A	%B
0	12	85	*15*
3.0	12	*85*	*15*
7.0	12	*50*	*50*
7.1	12	*5*	*95*
8.5	12	*5*	*95*
8.6	12	*85*	*15*
10.0	12	*85*	*15*

Table 4: Chromatographic Conditions for Microflow Continued.

Parameter	Value
Stationary phase	*Phenomenex Gemini 3 um C18 110 A LC Column 50 x 0.3 mm, Capillary*
Mobile phase A	*15mM DIEA 100mM HFIP in water*
Mobile phase B	*15mM DIEA 100mM HFIP in 90% methanol*
Flow rate	*12 µL/min*
Column temperature	*60 ºC*
Injection volume	*10 µL*

Table 5: Mass Spectrometer Conditions for Microflow.

Parameter	Value	Parameter	Value
Curtain gas:	25	CAD GAS:	*High*
Ion source gas 1:	30	*Ion Spray Voltage:*	*-4500*
Ion source gas 2:	*30*	*Source Temperature:*	*300*

Results and Discussion

Two synthetic oligonucleotides were analyzed in this assay. One corresponded to the sequence of fomivirsen and the other was used as the internal standard. The plasma was extracted using the OTX Clarity SPE sample preparation solution and the fomivirsen and internal standard were spiked into the extracted plasma for analysis.

As shown in Figure 2, analysis of the spiked samples using high-flow chromatography provides high quality quantification of fomivirsen, with an LOD of 0.5 ng/mL and an LLOQ of 1 ng/mL. The blank sample shows no interference from matrix species which enables low limits of detection. A calibration curve is obtained for quantification of fomivirsen from 1 ng/mL to 1000 ng/mL. Each sample is analyzed in triplicate to demonstrate the statistical relevance. All data show %CV below 8% and accuracies from 85% to 110% (Figure 5).

Following the results with high-flow chromatography, the impact of moving to the microflow regime was investigated. In this case the injection volume was identical to that used in the high-flow work. Using the trap and elute functionality of the microflow LC overall analysis times are similar to those achieved using high flow which is advantageous when sample throughput is required along with high sensitivity.

As shown in Figure 3 and 6, the assay LLOQ using microflow LC is improved. The linearity is obtained over the range of 0.1 ng/mL to 1000 ng/mL. The accuracy and repeatability of the microflow assay was similar to that achieved using high flow which is important as improvements in sensitivity must be reliable to ensure actionable results. When directly comparing the data from the high-flow and microflow experiments, the microflow LC data show significant improvement of sensitivity, at an average of 5-fold for S/N (Figure 5).

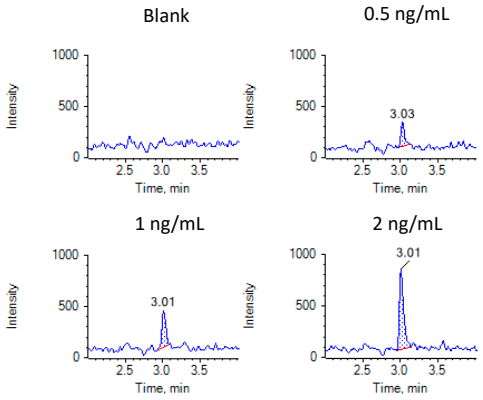

Figure 2. XICs of Oligonucleotide Quantification Using High-Flow HPLC. Shown are blank, 0.5, 1 and 2 ng/mL.

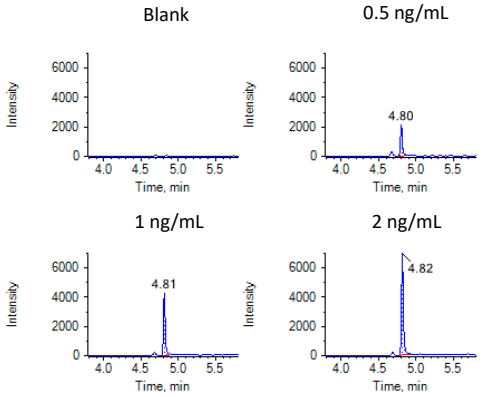

Figure 3. XICs of Oligonucleotide Quantification Using Microflow HPLC. Shown are blank, 0.5, 1 and 2 ng/mL.

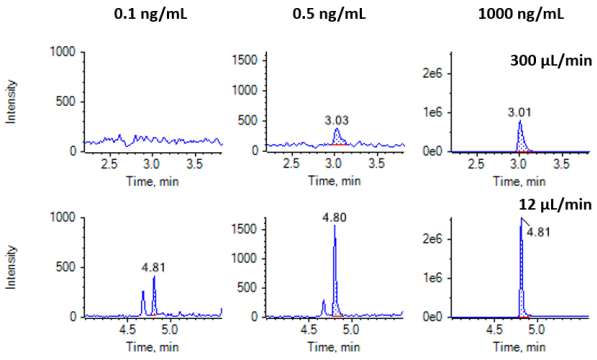

Figure 4. Comparison of High-Flow (top) and Microflow (bottom) XICs of Fomivirsen at Selected Spiked-In Levels.

Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Devia...	Percent CV	Accuracy	Value #1	Value #2	Value #3
1	F-8-1	1.00	3 of 3	1.087e0	7.666e-2	7.05	108.69	1.169e0	1.017e0	1.075e0
2	F-8-1	2.00	3 of 3	1.748e0	1.348e-1	7.71	87.42	1.891e0	1.731e0	1.623e0
3	F-8-1	5.00	3 of 3	4.653e0	7.019e-2	1.51	93.05	4.640e0	4.728e0	4.589e0
4	F-8-1	10.00	3 of 3	8.845e0	6.562e-1	7.42	88.45	9.473e0	8.899e0	8.164e0
5	F-8-1	50.00	3 of 3	5.143e1	5.078e-1	0.99	102.86	5.089e1	5.149e1	5.190e1
6	F-8-1	100.00	3 of 3	1.075e2	3.804e-1	0.35	107.48	1.079e2	1.074e2	1.071e2
7	F-8-1	500.00	3 of 3	5.334e2	6.841e0	1.28	106.68	5.393e2	5.351e2	5.259e2
8	F-8-1	1000.00	3 of 3	1.054e3	4.000e0	0.38	105.37	1.057e3	1.054e3	1.049e3

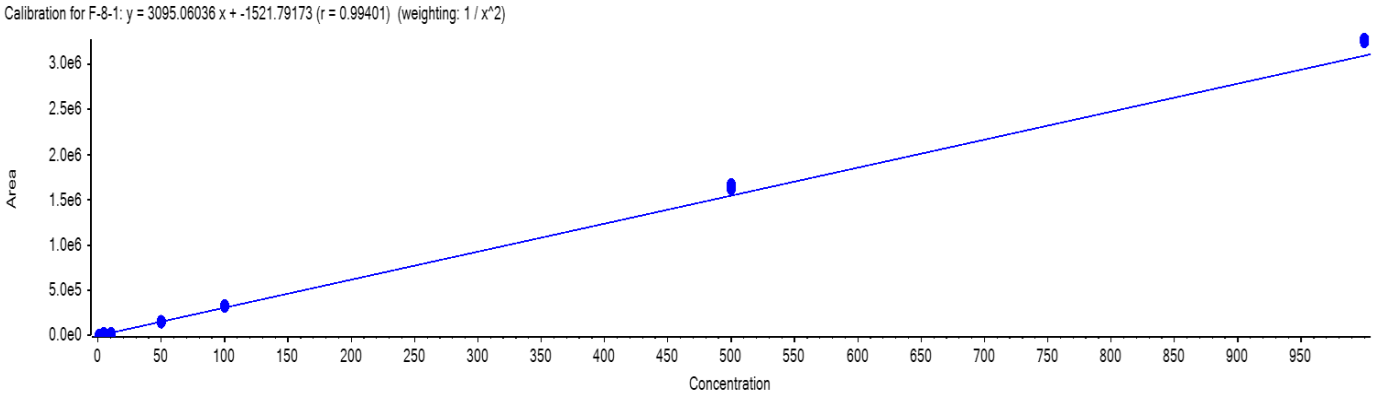

Figure 5. Statistics and Calibration Curve for Quantification of Fomivirsen Using High-flow Chromatography.

Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Devia...	Percent CV	Accuracy	Value #1	Value #2	Value #3
1	F-8-1	0.10	3 of 3	9.801e-2	1.601e-2	16.33	98.01	8.519e-2	1.160e-1	9.289e-2
2	F-8-1	0.20	3 of 3	2.035e-1	2.329e-2	11.45	101.75	2.248e-1	2.071e-1	1.786e-1
3	F-8-1	0.50	3 of 3	5.179e-1	2.306e-2	4.45	103.58	5.433e-1	4.982e-1	5.122e-1
4	F-8-1	1.00	3 of 3	1.039e0	3.125e-2	3.01	103.93	1.061e0	1.054e0	1.003e0
5	F-8-1	2.00	3 of 3	1.976e0	1.688e-1	8.54	98.81	1.983e0	2.142e0	1.804e0
6	F-8-1	5.00	3 of 3	5.139e0	1.399e-1	2.72	102.77	5.295e0	5.094e0	5.026e0
7	F-8-1	10.00	3 of 3	1.011e1	9.487e-2	0.94	101.11	1.022e1	1.008e1	1.004e1
8	F-8-1	50.00	3 of 3	4.992e1	6.323e-1	1.27	99.83	4.995e1	5.053e1	4.927e1
9	F-8-1	100.00	3 of 3	9.874e1	2.432e0	2.46	98.74	9.729e1	9.738e1	1.015e2
10	F-8-1	500.00	3 of 3	4.850e2	1.234e1	2.54	97.01	4.957e2	4.878e2	4.715e2
11	F-8-1	1000.00	3 of 3	9.446e2	2.837e1	3.00	94.46	9.726e2	9.454e2	9.159e2

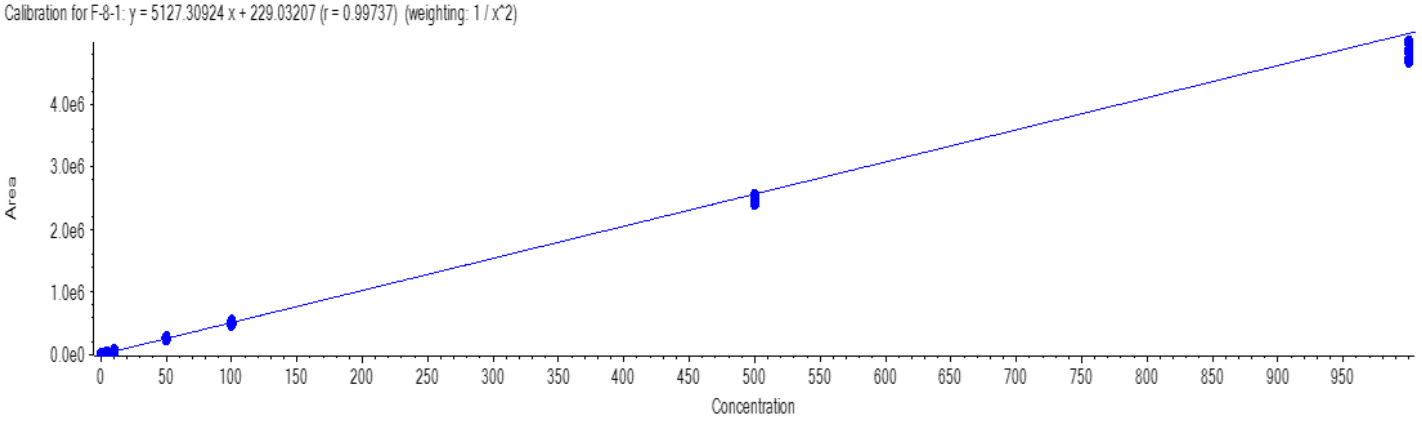

Figure 6. Statistics and Calibration Curve for Quantification of Fomivirsen Using Micro Flow Chromatography.

Conclusions

An ultra-sensitive microflow LC-MS/MS workflow for quantifying antisense oligonucleotides in matrix with high robustness, high throughput and wide dynamic range is reported. A 5-fold S/N improvement is achieved compared to the high-flow LC method.

In addition, it is also notable that the reduced flow rate of the microflow assay significantly enhances the instrument robustness by introducing less contamination from ion pairing reagents as the required mobile phase additives.

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