Approaches for quantification of oligonucleotides in extracted plasma using high resolution mass spectrometry

Featuring the SCIEX TripleTOF® 6600+ LC-MS/MS System and SCIEX OS-Q Software 1.7

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Oligonucleotide therapeutics and gene therapies are rapidly gaining attention as their potency improves and delivery challenges are addressed, this is particularly true of the antisense oligonucleotide (ASO) and the small interfering RNA (siRNA) therapeutics. Furthermore, the potential disease areas in which oligonucleotide therapies are being developed is rapidly expanding due to their potential for treating previously undruggable targets.

The increased interest in these modalities necessitates the development of flexible analytical approaches that can not only be used for traditional bioanalysis to understand the pharmacokinetic profiles but can also be employed to monitor the presence of degradation and metabolism products as well as confirm the target sequence. Routine approaches for oligonucleotide bioanalysis have involved hybridization-based ELISA (HELISA) primarily due to the low detection limits that can be achieved. However, hybridization assays lack the specificity to distinguish between the oligonucleotide product and related impurities/metabolites and as such they cannot be identified and subsequently quantified. Liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS) is an attractive technique for the analysis of oligonucleotide therapeutics.

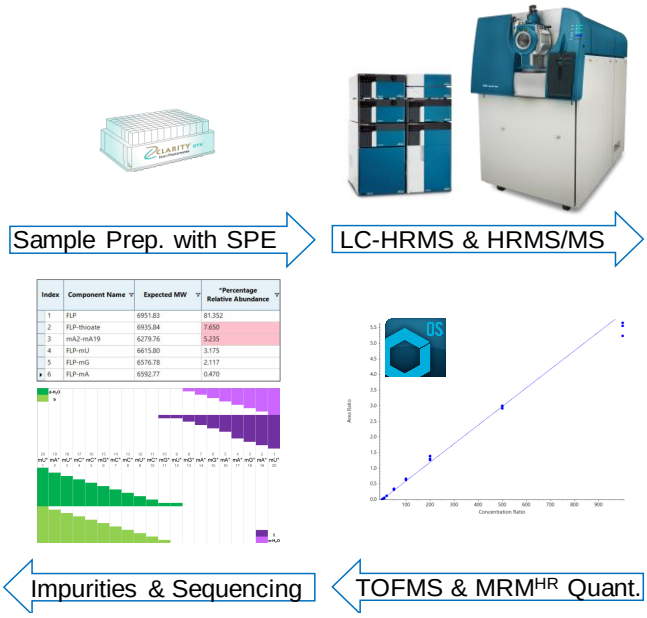

Figure 1. A schematic representation of the HRMS workflow.

LC-HRMS can effectively discriminate and quantify not only the target analyte but also impurities/metabolites in the sample. The SCIEX TripleTOF 6600+ LC-MS/MS System combines excellent quantitative performance with the selectivity of HRMS. In this work, the quantitative capabilities of both TOF MS and MRM^{HR} scans were investigated. The unique quantitative mass reconstruction capabilities within SCIEX OS-Q Software 1.7 are presented in addition to the highly selective MRM-based workflows. Finally, the HRMS benefits of the SCIEX TripleTOF 6600+ LC-MS/MS System are demonstrated for the detection and relative quantification of impurities using TOF MS and the determination of the target sequence with MRM^{HR}.

Key features of the SCIEX solution for oligonucleotides

- Exceptional negative ion performance with optimal sensitivity
- Selectivity benefits of HRMS to detect, identify and quantify not only the analyte but also impurities in the sample
- Flexibility and unique quantitative capabilities of SCIEX OS-Q Software 1.7
- High-quality MS/MS spectra for oligonucleotide sequencing

Methods

Samples and reagents: All reagents were purchased from Sigma Aldrich. This included the 1,1,1,3,3,3-hexafluoro isopropanol ≥ 99.8% (HFIP), diisopropylethylamine 99.5% (DIEA), methanol and ethylenediaminetriacetic acid (EDTA). The oligonucleotide standards were synthesized by Integrated DNA Technologies (IDT). The analyte was a fully phosphorothioated 20mer and each base was 2'-O-methylated. The sequence was mU*mA*mU*mC*mC*mG*mC*mC*mU*mC*mG*mU*mG*mA*mG*mA*mA*mG*mA*m'. The internal standard (IS) was a fully phosphorothioated 21mer with a sequence of. G*C*G*T*T*T*G*C*T*C*T*T*C*T*T*C*T*T*G*C*G. A carrier oligonucleotide was also employed to help prevent non-specific binding, the sequence was CATGGTCCTGCTGGAAGTTCGTG. The Clarity OTX SPE kit was purchased from Phenomenex. Human plasma from BioIVT was employed as the matrix.

Sample preparation: Aliquots of 200 µL neat plasma were combined with 200 µL of lysis-loading buffer. The lysed plasma samples were then extracted through the Clarity OTX SPE plate using the recommended protocol. Briefly, the sorbent was conditioned with 1 mL methanol and equilibrated with 1mL equilibration buffer. Following equilibration, the lysed plasma was loaded and then washed with 3 x 1 mL washing buffer. Finally, the sample was eluted with 1 mL of elution buffer. The eluted samples were dried down with N_2 gas and reconstituted in 200 µL water containing 100 µM EDTA, 2 µg/mL carrier oligonucleotide and 1 µg/mL IS. The calibration curve and quality control samples were then spiked between 0.5 ng/mL to 1000 ng/mL with the analyte, assuming an 80% recovery.

Chromatography: The separation was accomplished using an Agilent 1290 Infinity UHPLC fitted with a Waters Oligonucleotide BEH Column, 2.1 mm x 50 mm with 1.7 µm particles. Mobile phase A and B were 15 mM DIEA with 100 mM HFIP in water and methanol respectively. The gradient used is shown in Table 1. The column temperature was held at 70ºC. An injection volume of 20 µL was employed.

Table 1. LC gradient.

Time (min)	%A	%B	Flow Rate (µL/min)
Initial	*85*	*15*	*300*
3.0	*65*	*35*	*300*
3.1	*2*	*98*	*300*
4.5	*2*	*98*	*300*
4.6	*85*	*15*	*300*
6.0	*85*	*15*	*300*

Mass spectrometry: A SCIEX TripleTOF 6600+ LC-MS/MS System fitted with an IonDrive™ Turbo V Ion Source was utilized for all analyses. TOF MS and MRM^{HR} were investigated for quantification. The details of each scan type can be seen in Table 2. The source conditions employed are listed in Table 3. The instrument was calibrated using the carrier oligonucleotide. The calibration table is listed in Table 4. The data was acquired using Analyst® TF Software 1.8.0.

Table 2. Acquisition parameters.

	Value
TOF MS scan	
Mass Range (m/z)	*600 - 2800*
Delustering Potential (V)	*-50*
Collision Energy (V)	*-10*
Accumulation Time (ms)	*200*
MRM^{HR} scan	
Product Ion -10 Charge State Analyte (m/z)	*694.2*
Product Ion -9 Charge State- IS (m/z)	*741.4*
Q1 Resolution	*Low*
Mass Range (m/z)	*300-1000*
Delustering Potential (V)	*-50*
Collision Energy – Analyte (V)	*-35*
Collision Energy – IS (V)	*-25*
Accumulation Time (ms)	*50*

Table 3. Source parameters.

	Value
Polarity	*Negative*
GS1 (psi)	*70*
GS2 (psi)	*70*
Curtain Gas (psi)	*40*
Temperature (°C)	*400*
Spray Voltage (V)	*-4500*

Data processing: Explorer and Bio Tool Kit within the SCIEX OS-Q Software 1.7 were employed to visualize data. The quantification was performed using Analytics within SCIEX OS-Q Software 1.7, this included quantification of selected charge

states of the mass reconstructed spectra and MRM^{HR} quantification. ProMass was used to identify the impurities.

Table 4. Calibration table. The most abundant isotope of each charge state was employed for MS calibration while $[M-H]^{-9}$ was used for MS/MS.

MS	Precursor m/z	CE for MS/MS	DP for MS/MS	RT (min)	Fragment MS/MS	Fragment m/z
-9	751.4526	-40	-50	2.1	PO_3H	78.9591
-8	845.5112	-10	-50	2.1	$PO_5C_5H_7$	176.9958
-7	966.4412	-10	-50	2.1	$*w_2\text{-}H_2O \mid b_{21}$	303.0388
-6	1127.8497	-10	-50	2.1	$*w_3 \mid a_{21}\text{-}B$	506.0484
-5	1353.6211	-10	-50	2.1	w_2	650.1018
-4	1692.0275	-10	-50	2.1	$*w_2 \mid a_{22}\text{-}B$	810.0944
-3	2256.3799	-10	-50	2.1		

**Note: Multiple internal fragment possibilities with this mass*

Results

As mentioned above, HRMS is an attractive platform for the analysis of oligonucleotides as it is well suited towards sensitive bioanalysis and enables the ability to detect and quantify impurities/metabolites within the sample. Additionally the full scan MRM^{HR} data can be assessed to gain an understanding of the oligonucleotide and impurity sequences.

In this work, the fast scan speeds of the TOF instrumentation were leveraged to undertake a rapid comparison of the quantitative performance of the scan techniques simultaneously. Both TOF MS and MRM^{HR} were investigated. The acceptance criteria of the assay was +/- 15% deviation and +/- 20% deviation at the LLOQ for both the calibration curve and the QCs and this was used to assess the linear range of quantification for the assay.

TOF MS workflows

Mass reconstruction quantification: The acquired TOF MS data was first evaluated. Using this scan type all ions within the mass range of the experiment are detected. The key advantage of this approach is the simplicity of MS method set up and the ability to detect the presence of both the analyte and any potential impurities/metabolites. Figure 2 shows the charge state envelope and the reconstructed mass of the analyte. The left pane reveals the charge state envelope which distributes the signal from -11 to the -3 charge state. In the right pane, these charge states are reconstructed to the intact mass of the analyte (zero charge state) using the mass reconstruction algorithm.

The SCIEX OS-Q Software 1.7 offers two approaches for quantification for enhanced flexibility. The first approach discussed is the mass reconstruction approach, here quantification is undertaken based on the reconstructed spectral peak of the oligonucleotide, a unique feature of the SCIEX OS-Q Software 1.7. The raw charge states are reconstructed and the reconstructed MS spectral peaks are integrated for

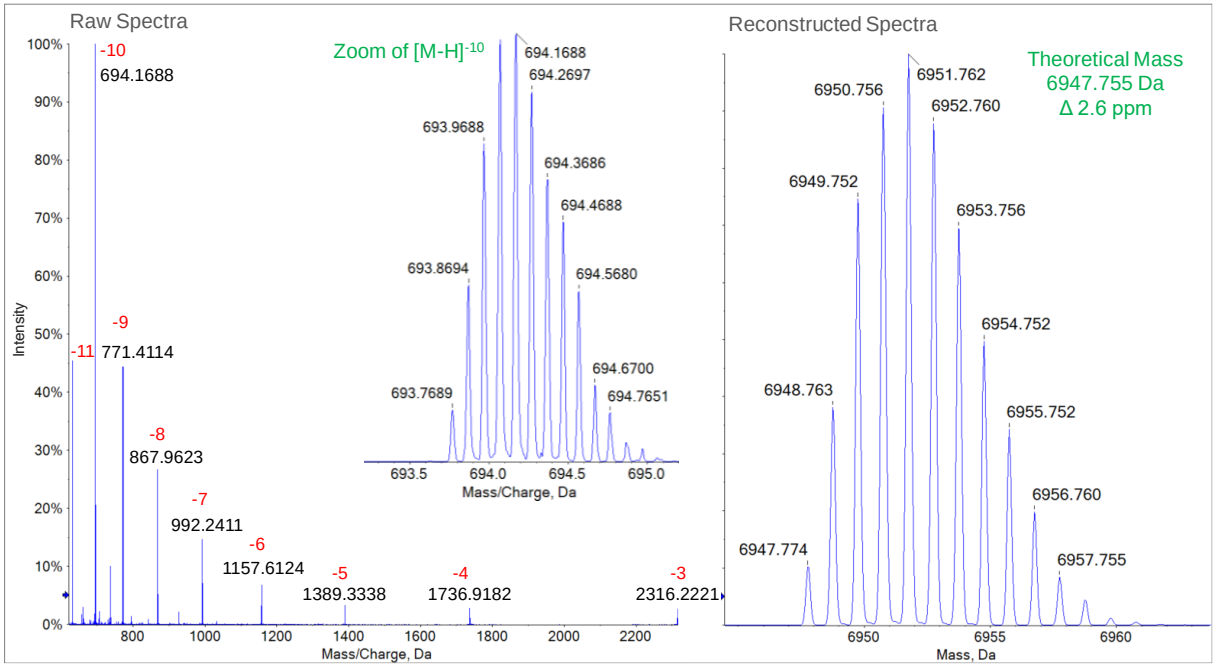

Figure 2. The TOF MS raw and reconstructed spectra of mU*mA*mU*mC*mC*mG*mC*mC*mU*mC*mG*mU*mG*mA*mG*mA*mA*mG*mA*mU.

quantification. This approach has the potential to simplify data processing and analysis as the appropriate charge states and isotopes for quantification no longer need to be determined.

The reconstruction parameters employed for quantification can be seen in Table 5.

Table 5. Source parameters.

	Value
Input Mass Range (m/z)	*600-2800*
Output Mass Range (Da)	*2000-7000*
Resolution	*30000*
Step Mass	*0.05*

The most abundant isotope of the reconstructed mass spectra was selected for quantification (Figure 3). The blue shading shows the integrated area of this spectra peak which was employed for quantification.

A linear regression with $1/x^2$ weighting was applied to the data with r = 0.99199. Figure 4 demonstrates the linear calibration curve ranging from 1 – 1000 ng/mL. Table 6 and Table 7 highlight the figures of merit of the assay for both the calibration curve and the QCs respectively. While Figure 5 displays the signal of the blank and 1 ng/mL calibrant.

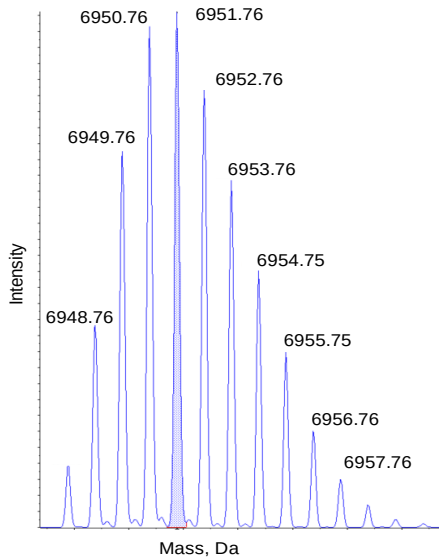

Figure 3. The most abundant isotope of the reconstructed spectra was selected for quantification. The shaded blue integration of the spectral peak is seen above.

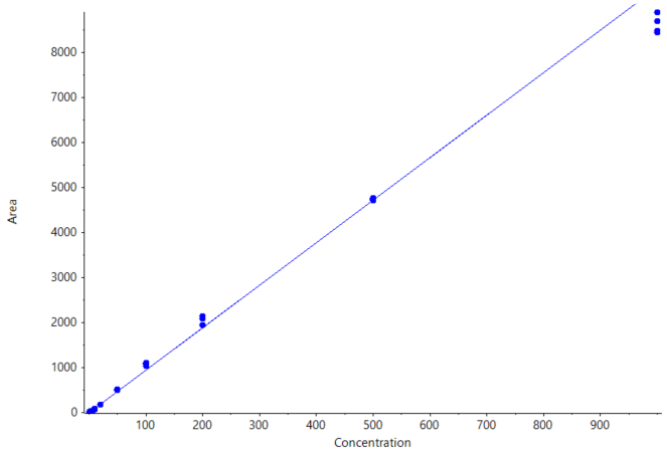

Figure 4. The calibration curve for the most abundant isotope of the reconstructed spectra, 6951.8 Da. A linear regression with $1/x^2$ weighting was applied to the data yielding r = 0.99199.

Table 6. The figures of merit for the calibration curve of the most abundant isotope of the analyte, 6951.8 Da.

Actual Conc. (ng/mL)	Calculated Conc. (ng/mL)	Accuracy (%)	CV (%)
1	*1.1*	*107.2*	*17.3*
2	*1.7*	*85.6*	*5.0*
5	*4.7*	*94.4*	*2.3*
10	*8.8*	*88.5*	*9.4*
20	*19.4*	*96.8*	*2.1*
50	*54.5*	*108.9*	*1.2*
100	*114.1*	*114.1*	*3.0*
200	*217.3*	*107.6*	*5.1*
500	*225.4*	*100.5*	*0.5*
1000	*913.8*	*91.4*	*2.4*

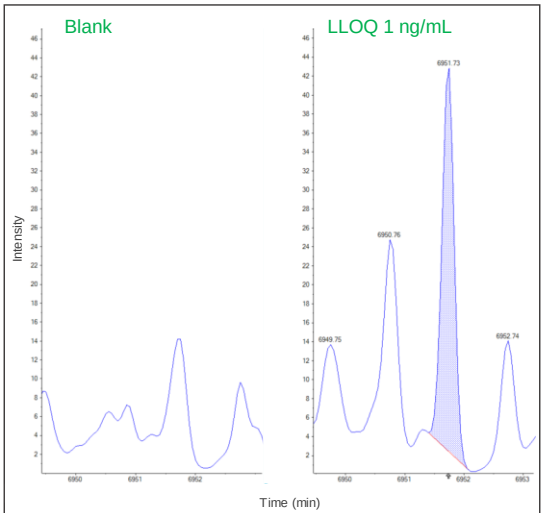

Figure 5. The blank sample and the LLOQ of 1 ng/mL for the selected isotope, 6951.8 Da, used for mass reconstruction quantification.

The generated results demonstrate that the SCIEX TripleTOF 6600+ LC-MS/MS System, in conjunction with the unique mass reconstruction feature of SCIEX OS-Q Software 1.7, was able to quantify the oligonucleotides extracted from plasma down to the ng/mL level.

As discussed, TOF MS is an attractive and flexible approach to detect and quantify not only the FLP but also impurities/metabolites in the sample as they can yield key information. The highest calibrant sample was analyzed with ProMass Software to identify and characterize the presence of related impurities within the sample. For further details on this workflow please refer to the SCIEX technical note “High resolution analysis of synthetic oligonucleotides”.

The masses of the identified impurities were imported into SCIEX OS-Q Software 1.7 and the relative abundances of the impurities was determined. A flagging threshold of >5% of the FLP was assigned to the percent relative abundances. The results are listed in Figure 6.

Index	Component Name	Expected MW	*Percentage Relative Abundance
1	FLP	6951.83	81.352
2	FLP-thioate	6935.84	7.650
3	mA2-mA19	6279.76	5.235
4	FLP-mU	6615.80	3.175
5	FLP-mG	6576.78	2.117
6	FLP-mA	6592.77	0.470

Figure 6. The relative abundances of impurities in the sample.

Table 7. The figures of merit for the QCs for the most abundant isotope of the analyte, 6951.8 Da.

Actual Conc. (ng/mL)	Calculated Conc. (ng/mL)	Accuracy (%)	CV (%)
Low	4.49	89.8	2.3
Medium	14.7	97.7	6.5
High	694.0	92.5	2.7

Selected charge state quantification: The traditional approach for TOF MS quantification uses extracted ion chromatogram (XIC) of chosen isotopic peaks of the selected charge states. This method can facilitate the quantification of using a discrete selected XIC peak or several XICs can be summed to improve S/N. In this example, the most abundant charge state, $[M-H]^{-10}$, was selected for quantification and from the $[M-H]^{-10}$ the two highest intensity isotopes were summed (Figure 7). As above a linear regression of $1/x^2$ was applied to the data yielding r = 0.99149 over the calibration range of 1 – 1000 ng/mL (Figure 8).

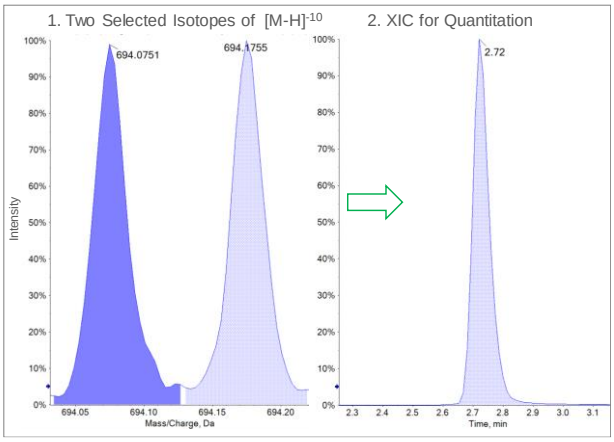

Figure 7. The most abundant isotopes of the $[M-H]^{-10}$ selected for XIC generation and subsequent quantification.

Table 8 and Table 9 highlight the figures of merit of the assay for both the calibration curve and the QCs, respectively.

Table 8. The figures of merit for the calibration curve for the summed isotopes of the selected $[M-H]^{-10}$ charge state.

Actual Conc. (ng/mL)	Calculated Conc. (ng/mL)	Accuracy (%)	CV (%)
1	*1.1*	*109.9*	*3.4*
2	*1.7*	*87.6*	*9.5*
5	*4.3*	*85.2*	*7.2*
10	*8.8*	*88.5*	*5.8*
20	*20.4*	*102.1*	*3.2*
50	*55.9*	*111.8*	*4.6*
100	*109.4*	*109.4*	*2.9*
200	*224.0*	*112.0*	*4.7*
500	*499.4*	*99.9*	*1.3*
1000	*919.9*	*91.9*	*4.0*

Table 9. The figures of merit for the QCs for the summed isotopes of the selected $[M-H]^{-10}$ charge state.

Actual Conc. (ng/mL)	Calculated Conc. (ng/mL)	Accuracy (%)	CV (%)
Low	4.3	86.4	3.9
Medium	15.7	104.5	4.9
High	718.7	95.8	4.3

The $[M-H]^{-10}$ charge state of each impurity can also be employed to assess the relative abundance of each impurity, this yielded similar results (data not shown).

MRM^{HR} workflows

MRM^{HR} is a powerful targeted quantitative approach offering additional selectivity than the TOF MS workflow. When an MRM^{HR} scan type is employed, the Q1 selects only the defined precursor ion, this ion is then fragmented within Q2 and all fragment ions are detected. The advantage of MRM^{HR} is that all product ions are detected, which enables the post-acquisition selection of the most suitable product ion(s). Another key benefit of this scan type is the ability to obtain sequence information about the analyte. The most abundant charge state $[M-10]^{-10}$ was selected for fragmentation. To ensure assay specificity only

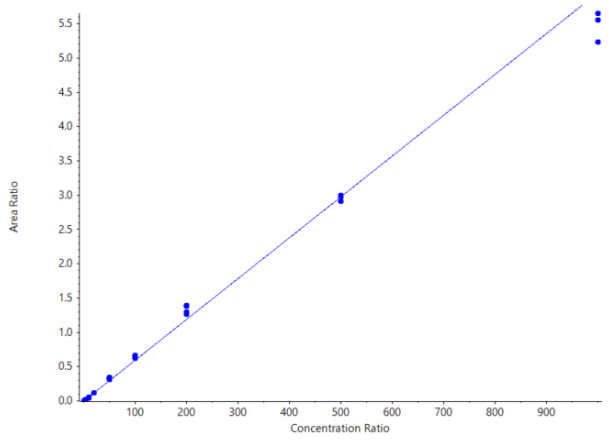

Figure 8. The calibration curve for the summed isotopes of the $[M-H]^{-10}$ charge state. A linear regression with $1/x^2$ weighting yielded r = 0.99149.

product ions with a m/z greater than 300 m/z were chosen for quantification. Figure 10 shows the product ion spectra of $[M-10]^{-10}$ with a collision energy of -35V.

In this analysis the product ion 374.03 Da (mG*-) was the most intense and as such the transition 694.16 → 374.03 *m/z* was utilized for quantification. Again a linear regression of $1/x^2$ provided the best fit for the data yielding r = 0.99116 over the calibration range of 1 – 1000 ng/mL (Figure 11). Table 10 and 11 display the figures of merit for both the calibrants and the QCs.

Finally, Figure 12 displays the signal of the blank and the LLOQ, the 1 ng/mL calibrant. In this example, the enhanced selectivity of the MRM^{HR} assay almost entirely removes any interferences from the extracted plasma matrix as seen in the blank spectra. This advantage is clear when comparing between Figure 9 and Figure 12.

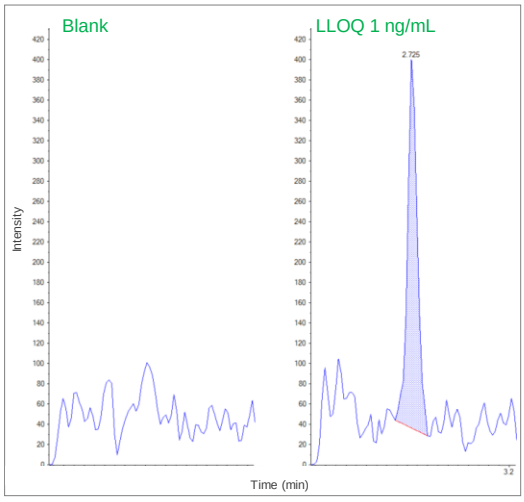

Figure 9. The blank and the LLOQ of 1 ng/mL for summed isotopes of the selected $[M-H]^{-10}$ charge state.

Table 10. The figures of merit for the calibration curve for the MRM^{HR} transition 694.16 → 374.03 *m/z*.

Actual Conc. (ng/mL)	Calculated Conc. (ng/mL)	Accuracy (%)	CV (%)
1	*1.1*	*106.2*	*9.5*
2	*1.8*	*91.7*	*9.7*
5	*4.8*	*95.4*	*2.5*
10	*8.6*	*86.5*	*4.9*
20	*17.6*	*87.9*	*4.7*
50	*56.5*	*113.0*	*9.4*
100	*94.1*	*94.1*	*10.4*
200	*223.1*	*111.5*	*4.2*
500	*526.4*	*105.3*	*3.2*
1000	*1070.0*	*107.0*	*5.1*

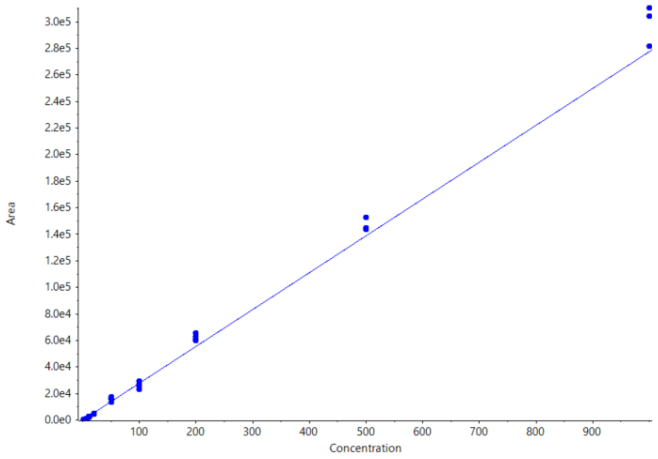

Figure 11. The calibration curve for the transition 694.16 → 374.03 *m/z*. A linear regression with 1/x^2 weighting was applied to the data yielding r = 0.99116.

Table 11. The figures of merit for the QCs for the MRM^{HR} transition 694.16 → 374.03 *m/z*.

Actual Conc. (ng/mL)	Calculated Conc. (ng/mL)	Accuracy (%)	CV (%)
Low	4.4	88.0	9.4
Medium	12.8	85.6	10.4
High	800.4	106.7	9.4

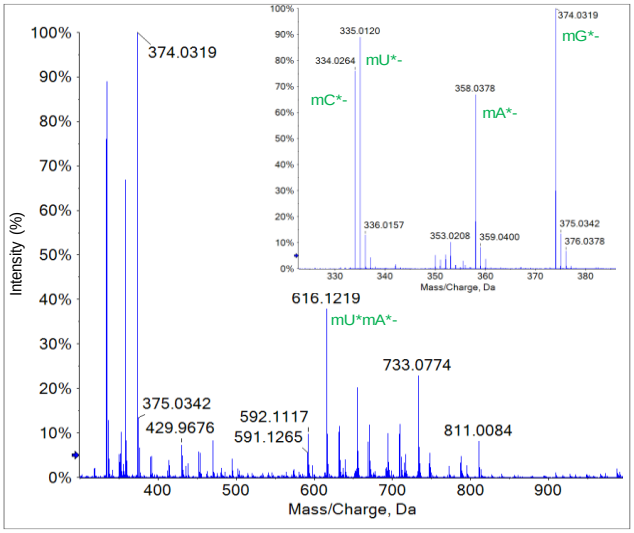

Figure 10. The MRM^{HR} product ion spectra of the [M-H]$^{-10}$ ion.

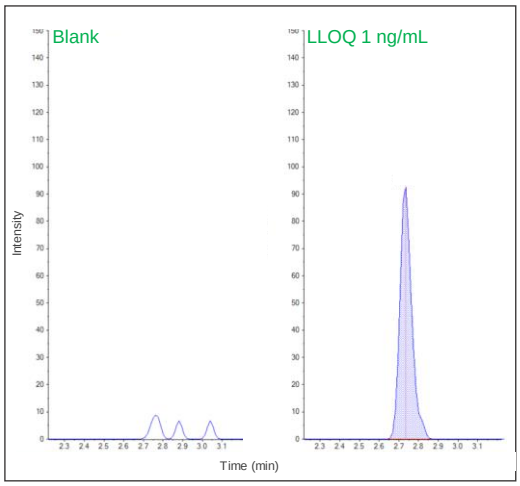

Figure 12. The blank and the LLOQ of 1 ng/mL for the transition 694.16 → 374.03 *m/z*.

While a CE of -35V was optimal for quantification as it provided the most intense product ions a lower CE of -20V for fragmenting $[M-H]^{-10}$ was more suitable for sequencing as it yielded larger and more specific product ions. Given the fast scan speeds of the TOF an additional transition could be included in the experiment with a CE selected for sequencing. However, in this investigatory example we elected only to consider the terminal fragments and, therefore, a neat standard was injected (100 ng on column) so low abundant fragments could be detected. The fragment ion assessed were b and d-H_2O from the 5' end and y and w-H_2O from the 3' end. A survey of the data for these ion series revealed the instrument was able to provide 100% sequence coverage of the analyte.

Figure 10 shows the MS/MS scan while Figure 14 shows six selected fragments ions, three from the 5' end and three from the 3' end. The demonstrated fragment ions were selected across a wide intensity range to showcase the superior instrument performance of the SCIEX TripleTOF 6600+ LC-MS/MS System in negative ion mode and the ability to generate product ion spectra of exceptional quality.

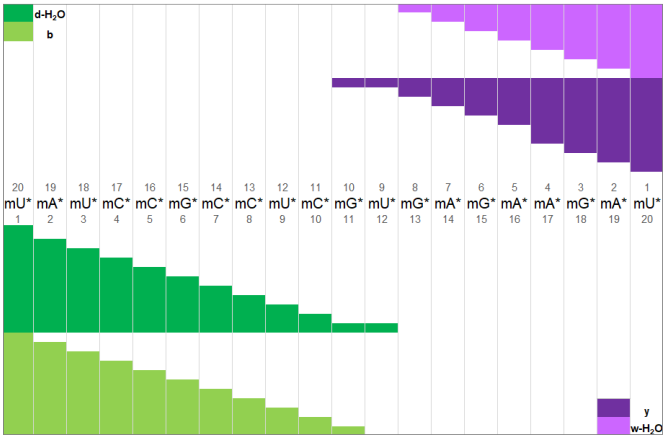

Figure 13. The identified fragments of the analyte, b and d-H_2O from the 5' end and y and w-H_2O from the 3' end.

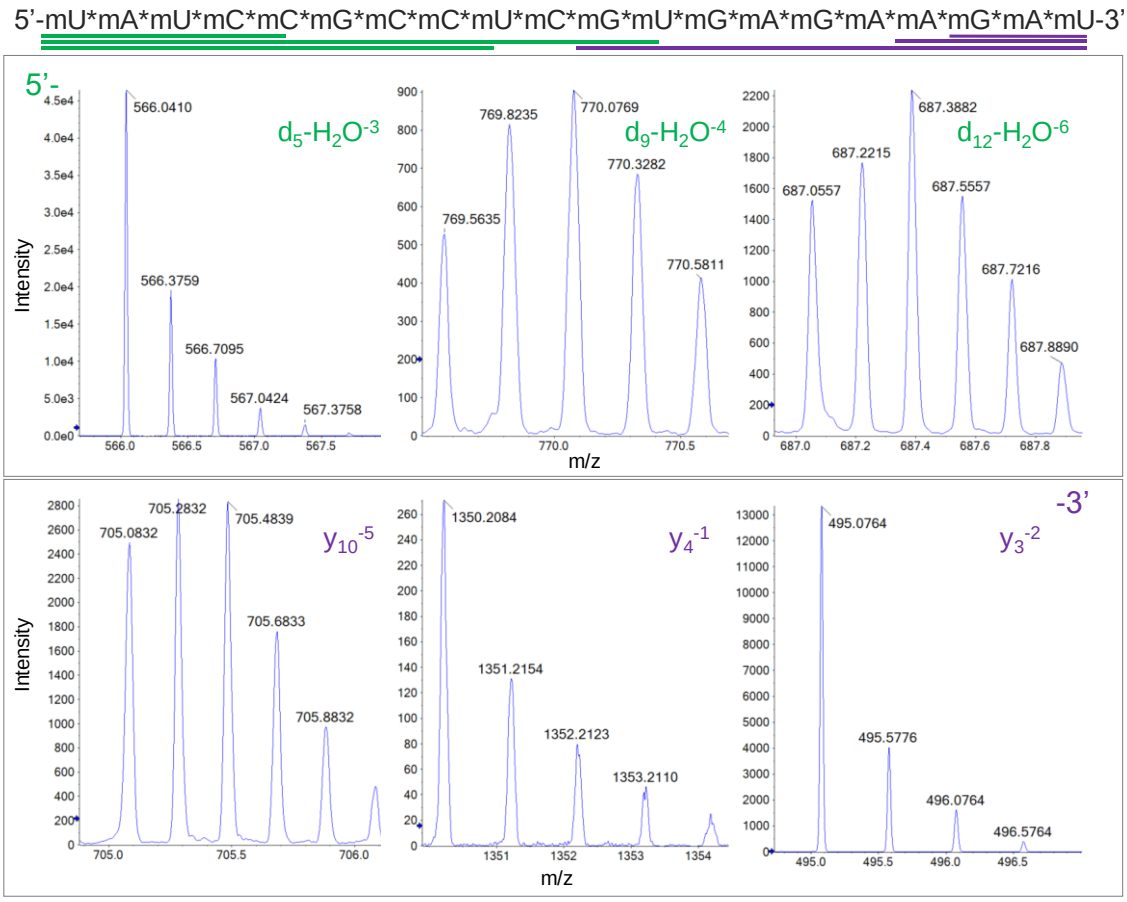

Figure 14. Selected fragments ions, three from the 5' end and three from the 3' end over a wide range of intensities.

Conclusions

- The three demonstrated workflows have provided similar quantitative performance with LLOQs of 1 ng/mL for mU*mA*mU*mC*mC*mG*mC*mC*mU*mC*mG*mU*mG*mA*mG*mA*mA*mG*mA*mU in extracted plasmams/ms
- The SCIEX TripleTOF 6600+ LC-MS/MS and SCIEX OS-Q Software 1.7 offer the flexibility to select a quantitative workflow works best for your analyses
- Utilizing this HRMS platform the identity of the impurities was determined and quantified with TOF MS
- The generated high-quality MS/MS spectra was used to confirm the target sequence of the analyte

References

1. Candish E, et al. 2019. High resolution analysis of synthetic oligonucleotides. SCIEX technical note RUO-MKT-02-10050-A.

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