Utilizing High Resolution Accurate Mass Spectrometry for Quantification, MRM^{HR} and SWATH® Acquisition Workflows

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INTRODUCTION

Within the bioanalytical laboratory triple quadrupole instruments have become the gold standard for high performance quantification. MRM acquisition is used to achieve the highest degrees of sensitivity, linear dynamic range, accuracy and precision.

High resolution accurate mass spectrometry (HRAMS) has gained rapid attention as an approach for quantitative bioanalysis [1]. There are several reasons for adopting this approach including the drive for higher selectivity especially in complex matrices and flexibility with fragment ion selection.

HRAMS delivers more information than a traditional tandem triple quadrupole mass spectrometer. When operating HRAMS the typical approach is to acquire both ToF MS and MRM^{HR} (ToF MS/MS) scans. This provides both MS and MS/MS level quantification capabilities with selectivity derived from higher mass resolving power and mass accuracy [2].

With MRM^{HR} quantification there are two ways to acquire the data; with a fixed collision energy or with collision energy spread. With a fixed collision energy we can acquire in an analogous form to MRM on a triple quadrupole. Here the most intense fragment ion is optimized and quantified. With the collision energy spread approach all fragment ions from low, medium and high energy are acquired with the user being able to pick and choose the most relevant fragments. This also opens the ability for fragment ion summation and using multiple fragment ions is highly beneficial when analysing peptides, oligonucleotides or any other compounds with a high number of lower intensity specific fragments

The drive for faster scanning HRAMS instrumentation has brought the ability to perform unbiased data independent acquisition (DIA) workflows into the forefront. Here acquisition strategies of MS/MS^{ALL} with SWATH® acquisition have pushed the speed, selectivity and sensitivity of DIA. The approach of SWATH® acquisition is to acquire MS/MS data on all components within a sample. This allows for true quant/qual workflows.

In this study we look at three case studies utilising either MRMHR or SWATH acquisition for quantification using typical bioanalytical assays.

MATERIALS AND METHODS

Sample Preparation: Thymidine and Plerixafor were spiked into human plasma, extracted and reconstituted in 100µL Water.

UHPLC Conditions: A Shimadzu Nexera UHPLC system was used. For the analysis of Thymidine a Waters Acquity T3 HSS, 50x2.1mm, 1.8µm column at 30˚ C with a gradient of mobile phase A of 0.1% formic acid in water and mobile phase B 0.1% formic acid in acetonitrile was used at a flow rate of 600µL/min. The injection volume was set to 5µL. A 2 minute gradient elution profile was utilized with a total runtime of 6 minutes.

For the analysis of Plerixafor a Phenomenex Kinetex Biphenyl, 100x2.1mm, 2.6µm column at 30˚ C with a gradient of mobile phase A of 0.1% formic acid in water and mobile phase B 0.1% formic acid in methanol was used at a flow rate of 400µL/min. The injection volume was set to 2.5µL. A 1 minute gradient elution profile was utilized with a total runtime of 2.2 minutes.

HRAMS Conditions: A SCIEX TripleTOF® 6600 high resolution accurate mass (HRAMS) LC-MS/MS system with the IonDrive™ Turbo V source and both Atmospheric Pressure Chemical Ionization (APCI) and Electrospray Ionization (ESI) probes were used.

Software: Analyst® TF 1.7 control software and MultiQuant™ 3.0 quantification software were used.

RESULTS

To test the quantitative performance of the MRM^{HR} and SWATH workflows key analytical criteria were examined. The assay selectivity, sensitivity, linear dynamic range, precision, accuracy and data point numbers were all measured by running small verification batches.

Case Study 1:

The analysis and quantification of Thymidine in extracted mouse plasma was the first case study chosen. This assay required the formation of a formic acid adduct in negative ion mode APCI (atmospheric pressure chemical ionisation). This assay is currently run on TripleQuad™ 6500 and the goal by transferring the assay onto a TripleTOF® 6600 would be to determine the underlying performance. The performance on the SCIEX TripleQuad™ 6500 was a LLOQ of 5ng/mL determined as 5 times the peak area of the analyte in a matrix zero.

The first analytical criteria to determine was the number of data points across the chromatographic peak due to the UHPLC conditions. The data point calculation is shown in Figure 1.

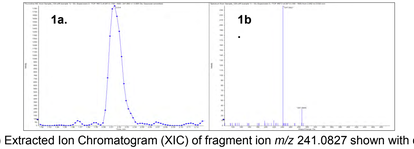
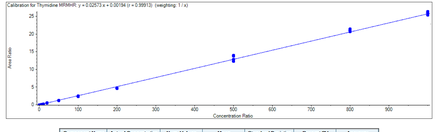


Figure 1. a) Extracted Ion Chromatogram (XIC) of fragment ion *m/z* 241.0827 shown with data points, extracted with a 10mDa (+/-5mDa) width. **b)** ToF MS/MS spectrum for peak shown in **a)**

As shown in figure 1a. the chromatography conditions used resulted in a peak width of 2.4 seconds with 15 data points across the peak, therefore meeting our standard requirement for quantification. In figure 1b. The ToF MS/MS spectrum is shown for Thymidine behind the 10mDa XIC shown in 1a. Here we have a good sensitivity MS/MS spectrum which has been acquired in MRM^{HR} high sensitivity mode. This results in a mass resolution of 23k and mass accuracy of the fragment less than 2ppm.



Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
Thymidine MRMHR	0.50	6 of 6	0.5626440	0.0257926	5.11	112.53
Thymidine MRMHR	1.00	6 of 6	1.1184424	0.0662775	5.93	111.84
Thymidine MRMHR	5.00	6 of 6	4.9703174	0.4184341	8.41	99.57
Thymidine MRMHR	10.00	6 of 6	10.1709752	0.7240678	7.14	101.71
Thymidine MRMHR	20.00	6 of 6	19.0139899	0.8225647	4.33	95.07
Thymidine MRMHR	50.00	6 of 6	45.2850917	2.4205887	5.35	90.57
Thymidine MRMHR	100.00	6 of 6	92.9621668	2.8293936	3.04	92.96
Thymidine MRMHR	200.00	6 of 6	183.5998388	2.6873649	1.46	92.00
Thymidine MRMHR	500.00	6 of 6	504.7731271	26.7871562	5.31	100.95
Thymidine MRMHR	800.00	6 of 6	817.2440109	10.8896266	1.33	102.16
Thymidine MRMHR	1000.00	6 of 6	1006.3915258	17.8824162	1.78	100.64

Figure 2 & Table 1. Calibration curve and associated statistics for accuracy and precision

The next analytical criteria to be tested included; sensitivity, linear range, accuracy and precision. This was tested by the acquisition of a small verification batch of standards and quality controls (QC's) at low, medium and high concentration. Figure 2 shows the calibration curve achieved across the desired range of 0.5ng/mL to 1000 ng/mL. As can be seen the full LC-MS/MS assay shows good linear range with a r value of 0.9991. Table 1 shows the accuracy and precision for 6 replicates at each of the standard concentrations. As can be seen the assay performs within recommended performance with accuracy 100% (+/-15%) and percentage co-efficient of variation (CV) less than 10%. Table 2 shows the bracketed quality controls at low, medium and high concentration (15, 80 & 800ng/mL). As can be seen good performance is observed with accuracy +/- 15% and precision less than 5% variation.

Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
Thymidine MRMHR	15.00	9 of 9	14.3711953	0.6724307	4.68	95.81
Thymidine MRMHR	80.00	9 of 9	75.0608573	2.8474235	3.79	93.83
Thymidine MRMHR	800.00	9 of 9	802.9062163	23.1052737	2.88	100.36

Table 2. Accuracy and precision statistics for Low, Medium and High QC

Finally in comparing the assay performance between the SCIEX TripleQuad™ 6500 and TripleTOF® 6600 the LLOQ (with a peak area 5 times the matrix blank) is at a concentration of 1ng/mL (compared to 5ng/mL). This shows the underlying performance of the TripleTOF® 6600 is excellent while meeting the criteria for a bioanalytical lab.

Case Study 2:

One of the key benefits of the TripleTOF® is the acquisition of all fragment ions. This allows for no up-front optimization and if one particular fragment ion is unselective another can be extracted without re-optimization. The other benefit to this is being about to sum multiple fragment ions to reduce the limits of quantification. This is a major benefit to multiply charged molecules where there is often a large choice of fragment ions. One particular case is for the compound Plerixafor. This is an unusual small molecule that forms a doubly charged precursor species and fragments into a large number of fragment masses.

The flexibility of acquiring full scan MS/MS for quantification is shown in figure 3. As can be seen in figure 3a for Plerixafor there are a minimum of 8 to 10 similar intensity fragment ions to choose from. In figure 3b and c it shows the two approaches to quantification on the TripleTOF®. Firstly in 3b seven of the fragment ions have been extracted with a 10mDa extraction width. Taking a measurement of signal to noise on the most intense fragment (peak to peak) a value of approximately 249:1 is achieved. In figure 3c the same fragment masses have been summed and achieves a greater peak intensity and signal to noise, with a value of 1230:1. Therefore by summing multiple fragment ions a five fold increase in both peak intensity and signal to noise has been achieved.

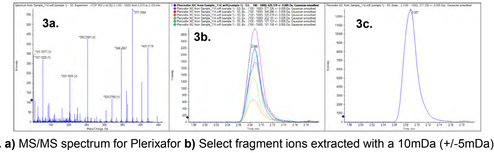
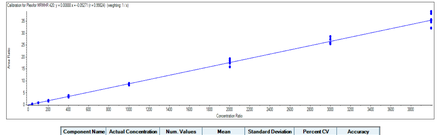


Figure 3. a) MS/MS spectrum for Plerixafor **b)** Select fragment ions extracted with a 10mDa (+/-5mDa) width. **c)** Summed fragment ions extracted with a 10mDa width.

As with case study 1 the same analytical criteria were tested with the acquisition of a small verification batch of standards and quality controls at low, medium and high concentration. Figure 4 shows the calibration curve achieved across the desired range of 40ng/mL to 4000ng/mL. As can be seen the assay shows good linear range with a r value of 0.9982.



Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
Plexifor MRMHR	40.00	6 of 6	4.08e4	5.029e-1	1.23	102.09
Plexifor MRMHR	100.00	6 of 6	1.033e2	3.692e0	3.57	103.34
Plexifor MRMHR	200.00	6 of 6	1.995e2	1.295e1	6.47	97.81
Plexifor MRMHR	400.00	6 of 6	3.969e2	2.334e1	5.88	99.22
Plexifor MRMHR	1000.00	6 of 6	9.665e2	2.679e1	2.99	96.65
Plexifor MRMHR	2000.00	6 of 6	2.002e3	1.271e2	6.08	100.09
Plexifor MRMHR	3000.00	6 of 6	2.991e3	1.339e2	4.65	99.70
Plexifor MRMHR	4000.00	6 of 6	4.044e3	2.939e2	7.01	101.10

Figure 4 & Table 3. Calibration curve and associated statistics for accuracy and precision

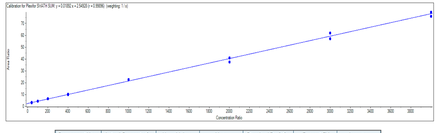
Table 3 shows the accuracy and precision for 6 replicates at each of the standard concentrations. As can be seen the assay performs within recommended performance with accuracy 100% +/- 15% and precision less than 10% variation. Table 4 shows the bracketed quality controls at low, medium and high concentration (120, 400 & 3000ng/mL). As can be seen good performance is observed with accuracy 100% +/- 15% and precision less than 8% variation.

Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
Plexifor MRMHR	120.00	9 of 9	1.153e2	8.904e0	7.72	96.11
Plexifor MRMHR	400.00	9 of 9	3.774e2	2.553e1	6.76	94.36
Plexifor MRMHR	3000.00	9 of 9	2.909e3	1.637e2	5.63	96.97

Table 4. Accuracy and precision statistics for Low, Medium and High QC

Case Study 2 – SWATH

The quantitative performance and applicability of data independent acquisition with SWATH® was tested using the Plerixafor assay. The assay conditions were kept exactly the same with only the mode of acquisition changing. Due to time limitations only a duplicate run of the standards and quality controls was achievable. In future work this will be expanded to test a more rigorous sized data set. Figure and table 5 show the initial performance achieved. It shows good linear range across the range of 40ng/mL to 4000ng/mL with a r value of 0.9989.



Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
Plexifor SWATH...	40.00	2 of 2	3.755e1	1.466e0	3.89	93.88
Plexifor SWATH...	100.00	2 of 2	9.605e1	9.810e-1	1.02	96.05
Plexifor SWATH...	200.00	2 of 2	2.119e2	4.972e0	2.35	105.93
Plexifor SWATH...	400.00	2 of 2	4.030e2	8.098e0	2.01	100.74
Plexifor SWATH...	1000.00	2 of 2	1.060e3	2.825e0	0.26	106.01
Plexifor SWATH...	2000.00	2 of 2	1.942e3	1.255e2	6.46	97.12
Plexifor SWATH...	3000.00	2 of 2	3.007e3	1.844e2	6.13	100.25
Plexifor SWATH...	4000.00	2 of 2	3.974e3	1.204e2	3.03	99.35

Figure 5 & Table 5. Calibration curve and associated statistics for accuracy and precision

Table 5 shows the accuracy and precision for duplicates at each of the standard concentrations. As can be seen the initial assay performs within recommended performance with accuracy 100% +/- 15% and precision less than 10% variation. Table 6 shows the bracketed quality controls at low, medium and high concentration (120, 400 & 3000ng/mL). As can be seen good performance is observed with accuracy 100% +/- 15% and precision less than 8% variation.

Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
Plexifor SWATH...	120.00	3 of 3	1.179e2	9.489e0	8.05	98.26
Plexifor SWATH...	400.00	3 of 3	4.059e2	1.789e1	4.41	101.46
Plexifor SWATH...	3000.00	3 of 3	3.050e3	1.485e2	4.87	101.67

Table 6. Accuracy and precision statistics for Low, Medium and High QC

CONCLUSIONS

In this work we have described how the TripleTOF® 6600 can routinely meet the sensitivity, stability, speed and reproducibility required for bioanalysis. The demands of a bioanalytical laboratory are now met with HRAMS delivering the added flexibility and complementarity to current technologies for all molecule types.

REFERENCES

1 Fung E, Jemal M, and Aubrey A. (2013) *Bioanalysis* 5(10). 1277-1284
2 Campbell L and Le Blanc Y. (2012) *Bioanalysis* 4(5). 487-500

TRADEMARKS/LICENSING

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