There has been an exponential increase in the number of ‘potential’ protein biomarkers discovered; thus requiring the need for better quantification strategies to confirm or refute their ultimate utility for large scale validation studies. Also required is increased throughput which means reduced sample preparation and/or accelerated chromatography, both of which can increase the chance of interference that could confound robust quantification. The purpose of this study is to explore new MS methodologies on the QTRAP® 5500 System that enable higher selectivity for quantification. The different techniques rely on different properties of the molecule for specificity so their utility will depend to a large degree on the target molecules. In this study, we compare the quantification of Brain Natriuretic Peptide (BNP) using mass spectrometric methodologies including MRM, MRM³, and SIM with differential mobility separation.

High Selectivity Quantitation Strategies on the QTRAP® 5500 System

Three quantitative methodologies are available on the QTRAP® 5500 System to provide a solution to almost every quantitative problem:

- **Multiple Reaction Monitoring (MRM)** using the Scheduled MRM™ Algorithm will be the workhorse mode for many peptides in complex matrices. MRM is a very sensitive method which provides selectivity based on a specific precursor and MS/MS fragment masses. However, there will be cases where interferences or background can impact the ultimate sensitivity of the targeted assay and additional selectivity is required.

- **MRM³ Workflow** is possible because of the hybrid triple quadrupole linear ion trap configuration of the QTRAP® system. The MRM³ workflow adds selectivity to an assay through an additional fragmentation event and detection of secondary fragment ions.¹

- **SelexION™ Technology** is a planar differential mobility device (DMS) that separates peptides based on differences in their chemical properties, prior to entering Q1, providing an orthogonal level of separation and selectivity.²

Figure 1. High Selectivity Quantification Methods on the QTRAP® 5500 System. (Top) The SelexION™ Technology is an easy to install differential mobility device that can be used to provide additional selectivity to any quantitative experiment. (Bottom) MRM³ Workflow leverages the unique linear ion trap capability of the QTRAP system to provide additional specificity through additional fragmentation steps.
Methods

Sample Preparation: Human BNP peptide (0.5 mg, Sigma) was dissolved in 20% acetonitrile to make a stock of 5 µg/µL. For matrix preparation, human plasma was crashed using various concentrations of acetonitrile to test for endogenous peptide recovery. Plasma containing BNP was precipitated at 1:1, 1:2, and 1:3 ratios with plasma:acetonitrile and the resultant supernatant was diluted 1:1 with 0.1% formic acid (Figure 2). For determining sensitivity, the intact BNP was spiked into the crashed plasma matrix (0.25 equivalents of plasma on column) to create calibration curves.

HPLC Conditions: A Shimadzu HPLC system with a Thermo Aquasil C18, 2.1mm x 150mm column at 40 °C with a gradient of 2 to 70 % acetonitrile in 0.1% formic acid was used at a flow rate of 300 µL/min. The injection volume was set to 10 µL.

MS/MS Conditions: An AB SCIEX QTRAP® 5500 LC/MS/MS system equipped with Turbo V™ source was used. MRM transitions and MRM³ experiments for BNP were developed and used to measure the calibration curves. For the differential mobility separations, a QTRAP 5500 system equipped with SelexiON™ Technology was used and BNP was analyzed in Single Ion Monitoring (SIM) mode (using a parent to parent MRM with no collision energy).

Data Processing: Samples were analyzed in triplicate and data processed using MultiQuant™ Software to perform quantification and statistical analysis.
Comparing the Quantitative Workflows

The recovery from the various extraction procedure was determined by monitoring the intensity of the 6+ ion of BNP. It was determined that plasma precipitated with a 1:1 ratio of plasma:acetonitrile provided the best relative recovery of BNP, and was therefore used as a matrix for all of the studies. (Figure 2) An in-depth investigation of sample preparation was not done and the reported lower limit of quantitation (LLOQ) in plasma could likely be improved with further optimization. However, the goal of this study was to explore the technology, not to develop an optimized BNP sample preparation protocol.

Because of the hybrid nature of the QTRAP system technology, it has many powerful scan modes that can be leveraged to solve a biological problem (Figure 3). First, concentration curves using standard MRM and MRM<sup>3</sup> analyses were compared. Both techniques provided reasonably interference free traces at low BNP levels on column (Figure 4). The LLOQ for both the MRM and MRM<sup>3</sup> analyses were found to be 128 pg/mL on column. The LLOQ is expressed as peptide concentration per volume of matrix, and for these experiments matrix was 0.25 equivalents of plasma, therefore the effective detection limit of BNP in plasma is 512 pg/mL. (Figure 5)

To explore the utility of single ion monitoring (SIM) mode for analyzing large endogenous peptides that don’t fragment well, SIM analysis with and without the differential mobility was performed. Here, the high selectivity provided by the mobility separation showed a significant reduction in background (Figure 6). The use of DMS resulted in a 25 fold improvement in the LLOQ achieved on the 6+ ion (Figure 7).
Conclusions

Three quantitative methodologies can be used with the QTRAP® 5500 System:

- MRM provides selectivity based on the fragmentation of the peptide and monitoring of a specific product ion.
- When matrix interference is a problem with MRM, further selectivity can be achieved using MRM³, which provides a second level of selectivity based on monitoring a secondary product ion.
- Differential Mobility Separation (DMS) provides an orthogonal level of selectivity by separating components based on their chemical properties and mobility preceding the quadrupoles.

BNP peptide was chosen as a model analyte to investigate quantitation of large endogenous peptides. The BNP peptides provide good quality fragmentation for both MS/MS and MS³, thus good sensitivity was obtained using both the MRM and MRM³ workflow. However, many large peptides do not fragment well, therefore monitoring the MS signal using SIM mode in combination with DMS was also explored. Application of DMS provided a 25x improvement in LLOQ over SIM alone, through substantial reduction in background.

This study demonstrates the effectiveness of these high selectivity workflows and the versatility of the QTRAP® 5500 System for the selective detection and quantitation of peptide analytes using MRM, MRM³, SIM, and SIM with DMS.

References

1. MRM³ Quantitation for Highest Specificity in Complex Matrices. AB SCIEX Technical Note, Publication 0920210-03.

Figure 7. Low End of the Concentration Curves for the SIM Experiments. Shown are the lower points of the concentration curves for the SIM experiment on BNP 6+ charge state peptide, with DMS (blue trace) and without the DMS filtering (red trace). Due to the enhanced selectivity and near removal of a co-eluting interference, the LLOQ for the SIM with DMS on was substantially improved (~25 fold improvement) over the SIM only experiment.