

# High Throughput Quantitative Proteomics Using High Resolution Multiple Reaction Monitoring Assays on a Triple Quadrupole Mass Spectrometer

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## Overview

**Purpose:** Develop a fast, robust method for accurate, quantitative analysis of targeted proteins in complex mixtures.  
**Method:** Whole human serum (Sigma) was analyzed by nano flow LC/MS/MS with a 75  $\mu$ m diameter C18 PicoFit<sup>®</sup> column on a TSO Quantum Ultra<sup>™</sup> instrument. 103 MRM transitions were monitored for the targeted quantitative analysis of 53 major proteins in human serum (1). Data were acquired at different Q1 quadrupole resolution settings (0.7 FWHM, 0.4 FWHM & 0.2 FWHM) and different scan times (20ms & 2ms). MRM-triggered MS/MS scans (1+10EA signal threshold) were also acquired for peptide verification. In addition, a known amount of interleukin 6 (IL-6) was spiked into the whole human serum to test the ability to predict MRM transitions in silico with an predictive software, P3 Predictor<sup>™</sup> (2), and successfully detect and quantitate the predicted peptides with the TSO Quantum triple quadrupole mass spectrometer.

**Results:** All MRM transitions were unambiguously detected from the whole human serum, using high resolution (0.2 FWHM) in the Q1 quadrupole. Most of the peptide sequences were also confirmed by MRM-triggered MS/MS spectra. Among 53 targeted proteins, 51 proteins produced suitable quantitative data (CVs <20%). The CVs (n=6) went from 5-26% (50% of MIDs had CV's <10%). Proteins present at concentrations as low as  $\mu$ g/ml levels, such as L-selectin and fibronectin (1), were reliably detected, yielding a dynamic range of greater than 4 orders of magnitude (from lowest peak areas of 6E+04 for fibronectin beta chain to highest peak areas of 6E+08 from albumin peptides) in a single experiment. More than 20 scans were acquired for each MRM transition when using a 2ms scan time. The predicted MRM transitions for IL-6 were clearly detected. The linear dynamic range for spiked IL-6 was over four orders of magnitude (0.02 – 400 fmol on column) and the limit of detection was 20 amol on column with a S/N of 22.

## Introduction

A common endpoint for a biomarker discovery experiment is a list of putative marker proteins. A reasonable next step is to then perform targeted quantitative measurements of these proteins in an expanded patient population to assess their validity as markers. Analytical accuracy and precision are required for unambiguous quantitative analysis of targeted proteins to very complex mixtures. Wide dynamic range and high sensitivity are critical for detecting low abundance proteins. One approach for this application is the use of tandem mass spectrometry to monitor a unique peptide (or peptides) from a protein of interest by a selected reaction monitoring (SRM) assay, or by simultaneous analysis of many peptides by a multiple reaction monitoring (MRM) assay. This approach can further provide absolute quantitation of targeted proteins by incorporation of appropriate stable isotope labeled peptides as internal standards. Although this approach gives very specific and sensitive responses for targeted peptides, it is still difficult to differentiate between the targeted peptide signal and chemical background, particularly when detecting very low abundance proteins in highly complex samples. The unique high resolution SRM (H-SRM) capability of the TSO Quantum Ultra can overcome this problem by increasing MRM assay specificity.

In this presentation, we demonstrate the TSO Quantum Ultra mass spectrometer's unparalleled capability for highly sensitive and accurate multiple protein quantitation from human serum by using high resolution multiple reaction assays. An MRM assay was developed for detecting 53 targeted proteins from human serum by using both wide mass resolution and high resolution on the Q1 quadrupole. The sensitivity, reproducibility, dynamic range and overall performance advantages of high resolution MRM assays were evaluated. Additionally, a specific MRM assay was developed for detecting a known biomarker (IL-6) from human serum.

## Methods

### Sample Preparation

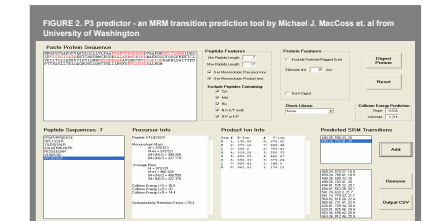
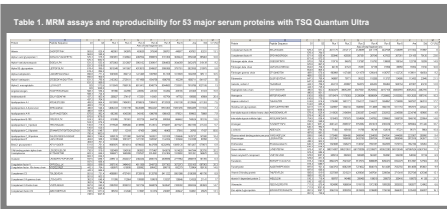
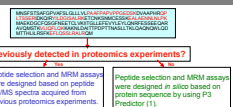
Whole Human Serum and interleukin 6 (Sigma) were used. The serum was diluted 40 times with 6M Guanidine. 1 ml of the diluted human serum sample and 10 $\mu$ g of IL-6 were reduced and S-carboxymethylated, exchanged into 100mM ammonium bicarbonate buffer and enzymatically digested. The digested mixtures were dried with a SpeedVac (Thermo Electron) device and reconstituted with 200 $\mu$ l water containing 0.1% TFA.

### Peptide Selection and MRM Transition Design

Figure 1 shows two basic approaches for peptide selection and MRM transition design. If the targeted protein was detected in previous LC/MS/MS experiments, the peptides that had been detected repeatedly from these experiments, but were unique for one single protein, or contained 3 or more common Cys, Met and/or His residues, were selected for MRM assays. Usually, multiple fragment ions for each selected peptide will be used to maximize specificity.

If no HPLC/MS/MS data was available for the targeted protein, an MRM predictor (P3 Predictor) was used to predict candidate peptides and multiple fragment ions for MRM assay design (Figure 2). P3 Predictor takes amino acid sequences of targeted proteins of interest and also contains a list of known peptides. A user simply selects one or multiple candidate peptides from the list and P3 Predictor automatically generates MRM transitions with proper collision energies and adds them to an output csv method which Quantum can accept directly. In our experiments, for the 53 major serum proteins, 103 MRM transitions (Table 1) were used based on previous work by Anderson et al. (1). Six MRM transitions (560,627,616,38, 560,627,31,41, 560,628,619,42, 663,369,612,46, 663,369,610,54) were also included and were based on the P3 Predictor tool. The collision energies were assigned by a generic formula of CE=0.034 x m/z + 3.314.

FIGURE 1. Basic approaches for peptide selection and MRM assay design

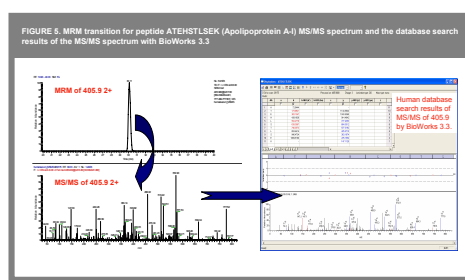
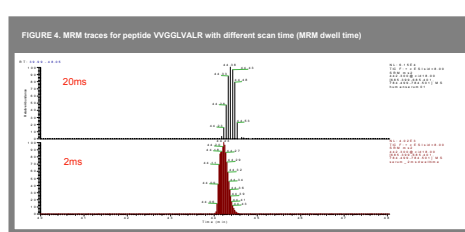
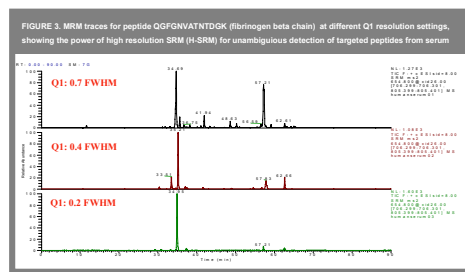


**HPLC**  
A PicoFit C18 column, 75 $\mu$ m x 100mm was used for peptide separation. A Surveyor<sup>™</sup> MS pump was used to produce and deliver a solvent gradient (A:0.1%FA/2%ACN/98% $H_2O$ , B:0.1%FA/100%ACN) to the column by means of a flow splitter. The flow rate post splitter was 300nL/min. The linear ramp was from 2% B to 50% B in 85 min. Samples were loaded directly onto the column by a Micro AS autosampler after the flow splitter. The sample loading rate was 5 $\mu$ L/min and loading time was 15 min.

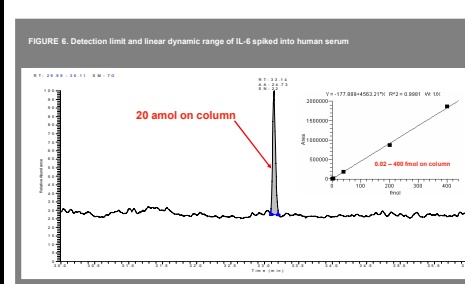
**MS**  
TSO Quantum Ultra with Ion Max<sup>™</sup> source equipped with a column adapter for nanoflow (New Objectives) was used. SRM: SRM set up 1: Q1: 0.7 FWHM, Q2: 0.7 FWHM Scan Event 1: Q1 & Q2, 0.7 FWHM, Q2, 1.5mTorr Scan width, 0.002 m/z; Scan time, 20ms SRM set up 2: Q1: 0.4 FWHM, Q2: 0.7 FWHM Scan Event 2: DO precursor mass from Scan Event 1. Q1: 0.7 FWHM, signal threshold 30,000 counts, 1.5 mTorr, CE, 0.034 x parent m/z + 3.314; Scan width, 0.002 m/z; Scan time, 20ms & 2ms

## Results

Quantitative results for 53 targeted major serum proteins by using 103 MRM assays with the same human serum sample. Five nano flow HPLC/MS/MS experiments were performed in each of which the same 103 MRMs were monitored with different resolutions and scan times mentioned above. The high resolution MRM assays (Q1: 0.2 FWHM) gave the best results and clearly resolved targeted analyte transitions from interference peaks that were seen at lower Q1 resolutions. Fig. 3 showed one example where high resolution helped to unambiguously detect peptide QGFGVNTVTDGK (representing fibrinogen beta chain) from complex serum. The 103 MRMs were detected with enough scans for reliable quantitation by using both 20ms and 2ms scan times, although the 2ms scan time gave twice as many scans with some decrease in signal intensity (Fig. 4). For testing the method's reproducibility, the same MRM experiment at Q1: 0.7 FWHM and 20 ms scan time was repeated six times and the results were summarized in Table 1. Among the 53 targeted proteins, 51 proteins produced acceptable quantitative data, while only two were not reliably observed. For the whole serum digest, CVs (n=6) were from 5-26% (50% of MRMs had CV's <10%). Proteins present at concentrations down to  $\mu$ g/ml levels, such as L-selectin and fibronectin (1), were reliably detected, yielding a dynamic range of greater than 4 orders of magnitude (from lowest peak areas of 6E+04 from fibrinogen beta chain to the highest peak areas of 6E+08 from albumin peptide) in a single experiment. The MS/MS spectra acquired once the MRM intensity exceeded 30,000 counts showed rich y series and some b series fragment ions and were of good quality, permitting database searching with SEQUEST<sup>™</sup> in BioTools V3.3 (Fig. 5).



Detection limit and linear dynamic range of IL-6, using MRM assays developed with P3 Predictor. An IL-6 digest mixture was spiked into human serum to create a dilution series, and was run sequentially to evaluate the detection limit and linearity of the developed MRM assay. Good analytical sensitivity and linearity were seen (Figure 6). The linear dynamic range for spiked IL-6 was over four orders of magnitude (0.02 – 400 fmol on column) and the limit of detection was 20 amol on column with a S/N of 22.



## Conclusions

An H-SRM based specific, sensitive, robust assay for targeted proteins, developed on a TSO Quantum Ultra triple quadrupole mass spectrometer, offers significant benefits for biomarker verification studies. 103 MRM transitions were monitored for the quantitation of 53 serum proteins.

- Q1 resolution of 0.2 FWHM dramatically reduces non-specific interference from serum background, improving assay specificity. At Q1 resolution of 0.4 or 0.7 FWHM, significant interference was seen for many targeted transitions.
- Analytical assay performance was excellent.
  - %CV varied from 5-26%, with 50% of protein CV's < 10%
  - Peptide response was linear over 4 orders of magnitude
  - Sensitivity was excellent, with the ability to detect proteins present at  $\mu$ g/ml levels and to detect IL-6 at levels as low as 20 amol on column
  - MRM-triggered MS/MS spectra were of good quality and in most cases sufficient to permit confirmation of peptide ID by database searching with SEQUEST

## References

- Leigh Anderson and Christie L. Hunter (2006) Quantitative Mass Spectrometric Multiple Reaction Monitoring Assays for Major Plasma Proteins. Mol. Cell. Proteomics 5, 4, 573-588.
- Michael J. MacCoss et al. (2008) Private communication.

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