Targeted Quantitative Protein Analysis in Human Serum Using High Resolution Multiple Selected 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.

Methods: Whole human serum (Sigma), in which the most abundant 12 proteins had been depleted, was analyzed by nanoflow LC-MS/MS with a 75 µm diameter C18 PicoFrit® column on a TSQ Quantum Ultra™ instrument. 320 SRM transitions were monitored for the targeted quantitative analysis of 97 major proteins in human serum. Data were acquired at both unit mass resolution (0.7FWHM) and high mass resolution (0.2FWHM) for Q1 quadrupole resolution setting with 5 ms scan time. SRM-triggered MS/MS scans were also acquired for peptide verification.

Results: Among 97 targeted proteins, 95 proteins were unambiguously detected from the depleted human serum, using high resolution (0.2 FWMH) in the Q1 quadrupole and multiple fragment ions from one peptide. Most of the peptide sequences were also confirmed by SRM-triggered MS/MS spectra. Proteins present at concentrations as low as µg/ml levels, such as L-selectin and fibronectin (1), were reliably detected, yielding a dynamic range of greater than 4 orders of magnitude. With the fast 5 ms duty cycle, each SRM transition was acquired with more than 20 scan points, providing reliable relative quantitative results for serum samples having different concentrations.

Introduction

Due to the increase of protein biomarker candidates from biomarker discovery experiments, it becomes more and more important to verify and eventually validate these putative biomarkers for clinical use. Unlike in the discovery phase, hundreds of samples are needed to be run for the verification of the putative marker proteins. A high throughput, robust, selective and sensitive method is required for this verification stage, and the selected reaction monitoring (SRM) method with tandem mass spectrometry has been shown to be quite suitable for this application (1,2,3). Although this approach gives very specific and sensitive responses for targeted peptides, it is often 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 TSQ Quantum Ultra can overcome this problem by increasing SRM assay specificity. In addition, the fast scan speed (short duty cycle) of the TSQ Quantum Ultra enables the monitoring of over 300 SRM transitions in one second.

In this presentation, we demonstrate the TSQ Quantum Ultra mass spectrometer's capability for highly sensitive and selective multiple protein quantitation from depleted human serum by using high resolution selected reaction monitoring assays. A 320-SRM assay was developed for detecting 97 targeted proteins from depleted human serum by using both unit mass resolution and high resolution on the Q1 quadrupole. The sensitivity, reliability, dynamic range and overall performance advantages of the high resolution SRM assays were evaluated.

Methods

Sample Preparation

400 µl of human serum (Sigma) was used. The 12 abundant proteins were removed from the serum by using ProteomeLab™ IgY-12 High Capacity LC2 column (6.4x63 mm, Beckman Coulter) following the standard operating protocol provided by the vendor. The depleted serum sample was reduced and S-carboxymethylated, exchanged into 100mM ammonium bicarbonate buffer and enzymatically digested. The digested mixtures were dried with a SpeedVac (Thermo Fisher Scientific) device and reconstituted with 100µl water containing 0.1% formic acid.

Peptide Selection and SRM Transition Design

There are two basic approaches for peptide selection and SRM transition design (3). If the targeted protein was detected in previous LC/MS/MS experiments, the peptides which had been detected repeatedly from these experiments and were unique for one single protein were selected for SRM assays. Usually, multiple fragment ions for each selected peptide were used to maximize specificity.

If no LC/MS/MS data was available for the targeted protein, P3 Predictor (4) was used to predict candidate peptides and multiple fragment ions for SRM assay design. In our experiments, 103 SRM transitions were chosen for the 53 major serum proteins based on previous work by Anderson et al. (2). 217 SRM transitions were used for targeting the other 44 proteins based on protein identification results from the LTQ™ mass spectrometer (Thermo Fisher Scientific) of the depleted serum sample (data is not shown here). The collision energies were assigned by a generic formula of CE=0.034 x *m/z* +3.314.

HPLC

A PicoFrit C18 column, 75µm x 100mm (New Objective) was used for peptide separation. A Surveyor™ MS pump was used to produce and deliver a solvent gradient (A:0.1%FA/2%ACN/98%H2O, 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 (A:2µl & B:1µl) were loaded directly onto the column by a Micro AS autosampler after the flow splitter.

MS

TSQ Quantum Ultra with Ion Max™ source equipped with a column adapter for nanoflow (New Objectives) was used.
SRM (total 320 transitions): Q1, 0.2 & 0.7 FWHM; Q3, 0.7 FWHM
Q2, 1.5 mTorr (Ar), Scan time, 5 ms

Results

Among the 97 targeted proteins, 95 proteins were detected with the above methods (Table 1). The high resolution SRM assays (Q1: 0.2 FWHM) gave the best results for clearly resolving targeted analyte transitions from interference peaks that were seen at unit mass resolution. Figure 1 shows just one example where high resolution helped to unambiguously detect peptide FVYHLSDLCK (representing fibrinogen beta chain) from the still complex depleted serum.

FIGURE 1. Selected SRM traces for peptide VGAHAGEYGAEALER (Hemoglobin alpha chain) at different Q1 resolution settings, showing the power of high resolution SRM (H-SRM) for unambiguous detection of targeted peptides from serum

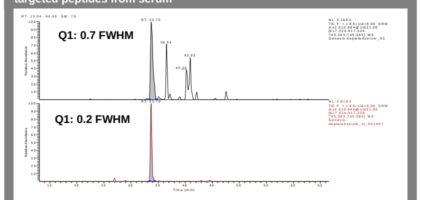


FIGURE 2. Selected SRM traces for peptide SLTSCLDSK (Apoliprotein M). 22 scans were acquired within a 5 ms duty cycle in this 320-SRM assay.

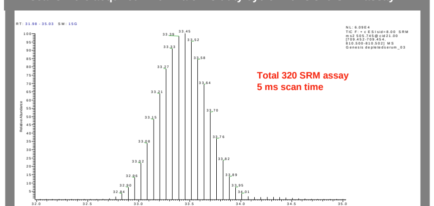
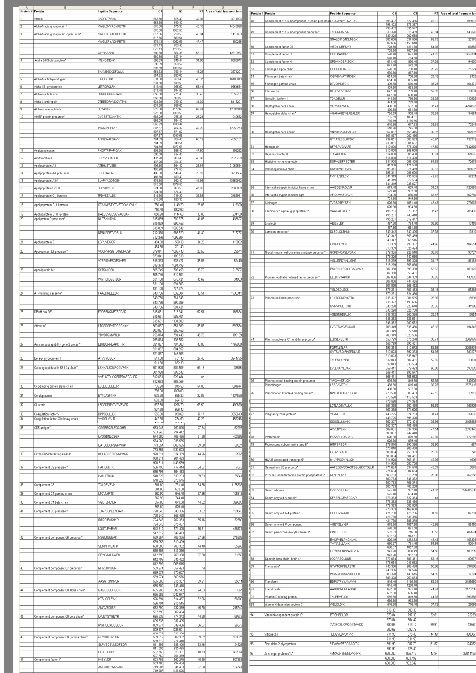


Table 1. The Q1/Q3 settings and retention time of 320 SRM assays for 97 serum proteins with TSQ Quantum Ultra . The total area of multiple fragments of each peptide is also shown.



*** Proteins identified from the depleted human serum sample with LTQ.**

By using the fast 5ms scan time, each SRM trace was acquired with more than 20 scans (Figure 2) which allowed reliable peak integration for the 320 SRM assay. Table 2 shows a portion of the relative quantitation results from sample A and B. Most resulted in ratios close to the expected ratio of 2.0. Considering that 320 SRM transitions were monitored simultaneously, these results were quite acceptable. Proteins present at low concentrations, such as Apolipoprotein M, Attractin, and Citron Rho-interacting kinase were reliably detected, yielding a dynamic range of greater than 4 orders of magnitude (1E+04 from Citron Rho-interacting kinase to 3E+08 from zinc finger protein 510 peptide) in a single experiment.

Table 2. Partial relative quantitative results between serum sample A and sample B

Protein	Peptide Sequence	A (2ul injection) total area of fragment ions	B (1ul injection) total area of fragment ions	Ratio A/B
Afamin				2.40
	DADPDTFFAK	3011923	1256884.00	2.40
Alpha 2-HS-glycoprotein				1.99
	HTLNQIDEVK	9553977	3932738.00	2.43
	EHAVEGDCDFQLLK	3971201	2560308.00	1.55
Appliprotein D precursor				2.26
	NILTSNNIDVK	4356279	1765819	2.47
	NPNLPPETVDSLK	7177779	3508807	2.05
Appoliprotein L1 precursor				2.54
	VQQNVPSGTDTGDPQSK	295710	100288	2.95
	VTEPISAESGEQVER	524409	247104	2.12
Attractin				2.52
	LTGSSGFVTDGPGNYK	6925345	3175224	2.18
	YDVDTQMWTILK	1091396	382774	2.85
CD5 antigen				2.69
	CSGEEQSLEQCQHR	622937	215220	2.89
	LVGGDNLCSGR	4033868	1552702	2.60
	EATLQDCPSGPWGK	503277	195360	2.58
Complement C5 precursor				2.13
	TDAPDLPEENQAR	1990450	1218536	1.63
	IDTQDIEASHYR	323805	141187	2.29
	LQGTLPVEAR	4988711	2027939	2.46
Plasminogen				2.19
	LSSPAVITDK	23751169	10843131.00	2.19
Plasminogen kringle-4 binding protein				2.36
	NWETEITAQPDGGK	109123	57817	1.89
	LDTLAQEVALLK	11479063	4058265	2.83
Serum amyloid A-4 protein				2.38
	GPGGVWAAK	8077815	3395853	2.38
Serum amyloid P-component				2.11
	VGEYSLYIGR	7171651	3395853.00	2.11

Conclusions

Reliable quantitation of proteins in a complex matrix (human serum) was demonstrated. An H-SRM based specific, sensitive, robust assay for targeted proteins, developed on a TSQ Quantum Ultra triple quadrupole mass spectrometer, offers significant benefits for biomarker verification studies. 320 SRM transitions were monitored for the quantitation of 97 serum proteins.

1) **H-SRM** (Q1 resolution of 0.2 FWHM) dramatically reduces non-specific interferences from the complex serum background, improving assay specificity. Alternatively, at Q1 unit mass resolution of 0.7 FWHM, significant interference was seen for many targeted transitions.

2) **Fast SRM cycle time**: only 5 ms were required for each peptide transition. The total cycle time for monitoring 320 peptide transitions was 2-3 s. Because of this rapid scan time, all transitions were monitored during the entire LC/MS separation – there was no need to "schedule" the SRM experiments. Approximately 20 data points were taken across a typical chromatographic peak width of 1 minute.

3) **SRM-triggered MS/MS**: Using Data Dependent MS/MS, when a targeted peptide transition was seen, a full scan MS/MS spectrum was automatically acquired. Peptide structure was confirmed by MS/MS. The MS/MS data could also be used to confirm the intensity of a predicted MS/MS fragment ion chosen for the transition and to allow potential selection of a more intense fragment to improve peptide signal. (Data not shown)

4) Analytical assay performance was good and reliable. Acceptable quantitative results were acquired.

References

(1) Nader Rifai,Michael A Gillette & Steven A Carr (2006) Protein biomarker discovery and validation: the long and uncertain path to clinical utility. Nature Biotechnology, Vol 24, 28, 971-983.

(2) 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.

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(4) Michael J. MacCoss et. al. (2006) Private communication.

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