High Throughput, High Sensitivity and Selectivity Targeted Quantitative Protein Analysis Using a Triple Quadrupole Mass Spectrometer

Reiko Kiyonami, Amol Prakash & Vlad Zabrouskov

Thermo Fisher Scientific, San Jose, CA, USA

Overview

Purpose: Demonstrate the advantages of high resolution selective reaction monitoring using the TSQ Quantum Ultra (Thermo Scientific) for targeted quantitative proteomics in complex digests.

Methods: Whole human serum (SIGMA™), was filtered, enzymatically digested and analyzed by nanoflow LC-MS/MS with a 75 µm diameter C18 PicoFrit® column (New Objective) on a Thermo Scientific TSQ Quantum Ultra™ instrument. Thirteen serum proteins with variable concentration ranging from 1.25E09 pg/mL of haptoglobin to 3.40E05 pg/mL of vitronectin(1) were targeted. An assay containing 61 SRM transitions was designed targeting 20 selected peptides from 13 proteins of interest employing newly developed SRM prediction and data processing software (2). SRM triggered QED MS/MS experiment and SRM experiments at different resolutions for the Q1 quadrupole (0.2 FWHM & 0.7 FWHM) and at different dwell times (5 ms, 10 ms & 20 ms) was carried out. The assay precision and quantitative accuracy tests were evaluated.

Results: All thirteen targeted proteins were identified by SRM triggered QED MS/MS data. They were also detected by time aligning multiple fragment ions from each peptide isolated by H-SRM. Proteins present at concentrations as low as ng/mL, such as vitronectin, were reliably detected, yielding a dynamic range of quantitation greater than four orders of magnitude for abundant proteins like haptoglobin to low abundant proteins such as vitronectin in a single experiment. No significant signal loss was observed when using different dwell times. Although both H-SRM and SRM produced acceptable % CV variation range, the H-SRM assay gave much higher analytical assay precision. 90-95% of the H-SRM assay had CVs<15% at all three different dwell times (5 ms, 10 ms & 20 ms). Excellent quantitative accuracy was acquired by using H-SRM assay. On average, only ±4% of quantitation error was observed for our relative quantitation test.

Introduction

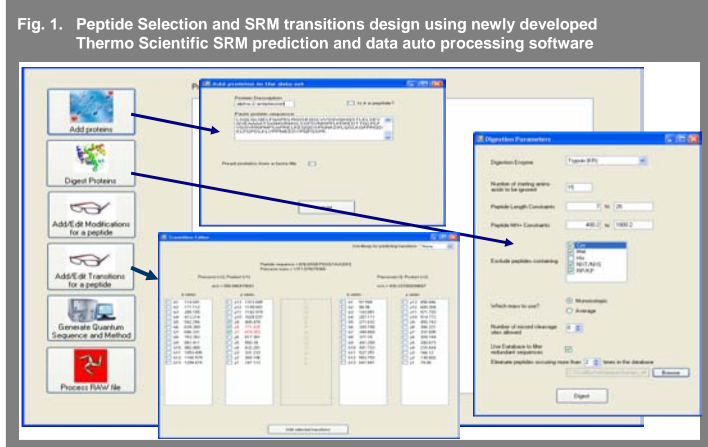
A selected reaction monitoring (SRM) workflow based on tandem mass spectrometry allows highly sensitive and selective quantitation of unique peptides from the protein of interest in complex biological matrices and is very useful for biomarker discovery and validation (3,4). Although unit mass resolution SRM approach gives selective and sensitive responses for targeted peptides in most cases, it is often difficult to differentiate between the targeted peptide signals and the chemical background, particularly when detecting low abundance peptides in highly complex matrixes. It is also difficult to maintain the SRM assay's accuracy and precision in the presence of protein isoforms and contaminant interferences in the complex matrix. The unique high resolution selectivity of the TSQ Quantum Ultra for both precursor ion (Q1) and fragment ion (Q3) can overcome these problems by greatly increasing SRM assay specificity.

Here, we demonstrate the advantages of the high resolution selected reaction monitoring assay on TSQ Quantum for sensitive, selective and accurate targeted protein quantitation in complex whole human serum digests. The sensitivity, reproducibility at different dwell times, quantitative accuracy and overall performance advantages of high resolution multiple selected reaction monitoring assay were evaluated.

Methods

Sample Preparation: An aliquot of 25 µL of human serum was used. The serum sample was diluted forty times with 975 uL of 100 mM ammonium bicarbonate buffer, ultrafiltrated and enzymatically digested. The digested mixtures were dried and reconstituted with 250 µL water containing 0.1% formic acid.

Fig. 1. Peptide Selection and SRM transitions design using newly developed Thermo Scientific SRM prediction and data auto processing software



Peptide Selection and SRM Transition Design

There are two basic approaches for peptide selection and SRM transition design (4). If the targeted protein was detected in previous LC/MS/MS experiments, the peptides which were detected repeatedly from these experiments and unique for that protein are selected for SRM assays. Alternatively, if no LC/MS/MS data was available for the targeted protein, the peptide selection and SRM assay are performed *in silico* based on the known protein sequences. For this purpose, Thermo Scientific has developed a new software for targeted protein quantitation (2). This software can be used for predicting candidate peptides and choosing multiple fragment ions for SRM assay design, building an instrument method and a sequence file, and also for automatic peptide peak confirmation and quantitative data processing (Fig.1). We have used this software to design all the SRM assays described in this work. A total of 61 SRM transitions designed from 20 unique peptides representing the thirteen targeted proteins was used.

HPLC: A PicoFrit C18 column, 75µm x 100mm was used for peptide separation. A Thermo Scientific 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 using a flow splitter. The post-split flow rate was 300 nL/min. The linear ramp was from 2% B to 50% B in 85 min. Samples were loaded directly onto the column by a Thermo Scientific MicroAS autosampler after the flow splitter. All the experiments used 2 uL injection except for the quantitative accuracy experiment in which both 2 uL and 1 uL injections were used.

MS: Thermo Scientific TSQ Quantum Ultra with Ion Max™ source equipped with a column adapter for nanoflow (New Objective) was used.

SRM (61 transitions):

SRM 1: Q1, 0.7 FWHM; Q3, 0.7 FWHM CE: 0.034 x *m/z* of precursor ion + 3.314

SRM 2 (H-SRM): Q1, 0.2 FWHM; Q3, 0.7 FWHM Scan width: 0.002 *m/z*

SRM1 & SRM2: Q2, 1.5 mTorr (Ar) Scan time: 5 ms, 10 ms, 20 ms

SRM triggered QED MS/MS:

DD precursor mass from Scan Event 1; Q1, 0.7 FWHM; signal threshold 5,000 counts; Q2: 1.5 mTorr, CE: 0.034 x precursor mass *m/z* + 3.134; Dynamic exclusion settings: repeat count 2; duration, 30 s; exclusion time, 60 s; exclusion list size, 50.

Results

Peptide peak detection: There are two approaches for peptide peak detection. The traditional approach is to acquire SRM triggered MS/MS data for database search to confirm the sequence of the peptide. TSQ Quantum can provide very sensitive QED MS/MS data by using unit mass resolution (0.7 FWHM) or higher for precursor ion isolation. Fig. 2 shows the SRM triggered QED MS/MS data and the SEQUEST® database search results for the low abundance protein of Vitronectin (340 ng/mL). The peptide FEDGVLDPDYPR was confirmed with the SEQUEST search result.

Fig. 2. Peptide FEDGVLDPDYPR confirmed by SEQUEST search results with QED MS/MS spectrum

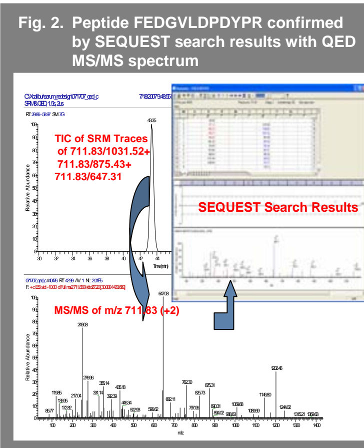


Fig. 3. Peptide AAISGENAGLVR confirmed by alignment of H-SRM multiple y ions

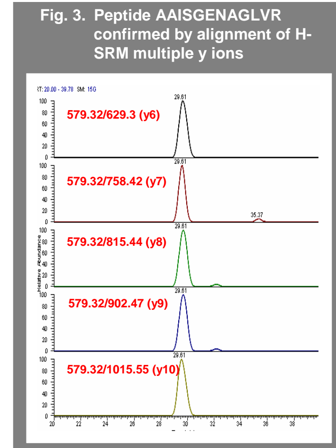


Table 1. The mean raw peak areas and CVs from the detection of 13 proteins in whole human serum using H-SRM assay with 3 different dwell times

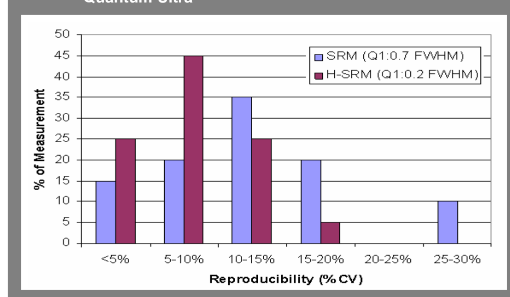
	B	C	D	E	F 5ms mean area	G 10ms mean area	H 20ms mean area	I 5ms %CV	J 10ms %CV	K 20ms %CV
1	AMBP	ETLLQDFR	511.27	565.27	5306979	5987942	7087222	14.4	2.9	14.2
		ETLLQDFR	511.27	678.36						
		ETLLQDFR	511.27	791.44						
2	Appoliprotein M	AFLLTPR	409.25	746.46	2343721	2667912	3021895	12.2	19.4	2.9
		AFLLTPR	409.25	599.39						
		AFLLTPR	409.25	486.30						
3	Complement C4-A	VDFTLSSER	527.26	839.43	4086351	4453567	4770800	6.4	4.2	12.8
		VDFTLSSER	527.26	692.36						
		VDFTLSSER	527.26	591.31						
4	Complement C4_B	VGDTLNLNLR	557.82	629.37	4538032	4656495	5500169	1.3	7.3	10.6
		VGDTLNLNLR	557.82	742.46						
		VGDTLNLNLR	557.82	843.51						
5	comple.component1 q	TINVPLR	406.75	598.37	6376783	7902119	7490320	7.0	11.7	9.4
		TINVPLR	406.75	484.32						
		TINVPLR	406.75	385.26						
6	complement factor B	YGLVTYATYPK	638.34	1112.60	5167312	5154807	6359996	10.6	9.1	17.3
		YGLVTYATYPK	638.34	942.49						
		YGLVTYATYPK	638.34	843.42						
		YGLVTYATYPK	638.34	742.38						
		EELLPAQDIK	578.32	897.54	14983067	16315794	17005929	4.6	11.1	4.7
		EELLPAQDIK	578.32	784.46						
		EELLPAQDIK	578.32	671.37						
7	gelsolin isoform b	HVVPNEVVVQR	638.36	1138.66	3546385	3770675	3734683	14.0	15.2	3.3
		HVVPNEVVVQR	638.36	1039.59						
		HVVPNEVVVQR	638.36	940.52						
8	haptoglobin	TEGDGVYTLNDK	656.31	1081.52	15685232	18022858	14891978	3.0	7.5	0.7
		TEGDGVYTLNDK	656.31	909.47						
		TEGDGVYTLNDK	656.31	753.38						
		VGYVSGWGR	490.75	881.43	109038887	129683137	117215733	15.5	11.4	6.2
		VGYVSGWGR	490.75	661.34						
		VGYVSGWGR	490.75	562.27						
		NFPSPVDAAFR	610.81	959.49	60599383	64702391	71769403	2.6	6.3	9.4
		NFPSPVDAAFR	610.81	862.44						
		NFPSPVDAAFR	610.81	775.41						
9	histidine-rich glycoprotein	DGYLFQLLR	562.81	789.50	3727491	4003966	4353635	7.1	3.0	10.0
		DGYLFQLLR	562.81	676.41						
		DGYLFQLLR	562.81	529.35						
10	inter-alpha-trypsin ingibitor	EVAFDLEIPK	580.81	932.51	7159915	8615872	9203943	8.0	5.9	1.4
		EVAFDLEIPK	580.81	861.47						
		EVAFDLEIPK	580.81	714.40						
		AAISGENAGLVR	579.32	902.47	3453932	4457886	4001541	10.9	13.3	8.8
		AAISGENAGLVR	579.32	815.44						
		AAISGENAGLVR	579.32	629.37						
11	kininogen 1	TVGSDTFYSFK	626.30	1051.47	5916403	5984698	6819773	9.2	1.9	16.4
		TVGSDTFYSFK	626.30	994.45						
		TVGSDTFYSFK	626.30	792.39						
		DFVQPPTK	466.25	669.39	118000371	11127580	12340433	2.3	12.7	6.5
		DFVQPPTK	466.25	570.32						
		DFVQPPTK	466.25	442.27						
12	Serum amyloid P-compon	VGEYSLYIGR	578.80	1057.53	214563	228545	300006	13.3	5.7	9.5
		VGEYSLYIGR	578.80	871.47						
		VGEYSLYIGR	578.80	708.40						
		IVLGQEQDSYGG	697.35	1181.54	1398887	1702529	1732344	7.3	13.6	9.6
		IVLGQEQDSYGG	697.35	1068.46						
		IVLGQEQDSYGG	697.35	883.38						
13	vitronectin	VDTVDPPYPR	579.79	944.48	27542274	29601332	29024927	17.5	7.6	7.8
		VDTVDPPYPR	579.79	744.37						
		VDTVDPPYPR	579.79	629.34						
		FEDGVLDPDYPR	711.83	1031.52	26678354	25506256	28376037	8.7	8.9	11.8
		FEDGVLDPDYPR	711.83	875.43						
		FEDGVLDPDYPR	711.83	647.31						

Another approach for peptide peak confirmation is to monitor multiple fragment ions from one peptide and use the time alignment of the multiple fragment traces to confirm the peptide identity. The Quantum Ultra can use its unique H-SRM capability to differentiate the targeted peak from interfering peaks, monitor over three hundred H-SRM transitions within one single HPLC-MS/MS run without any peptide peak retention time information (5) and process the alignment of multiple fragment traces automatically (2). This allows for simultaneous qualitative and quantitative analysis of multiple targeted peptides one HPLC-MS/MS run. All the targeted proteins were detected using both approaches. Proteins present at concentrations as low as ng/mL levels, (such as vitronectin), were reliably detected. This established a dynamic range of greater than four orders of magnitude for detecting abundant proteins such as haptoglobin and low abundant proteins such as vitronectin in a single experiment.

H-SRM and SRM assays precision (reproducibility test results)

The SRM and H-SRM assays were analyzed in triplicate at each dwell time. No significant signal loss was observed for faster dwell times. Among the 20 peptides, ETLLQDFR showed the largest variation and lost 25% signal intensity at 5 ms dwell time compared with 20 ms dwell time (Table 1). By dramatically reducing non-specific interference from serum background, the H-SRM assay had high assay precision across all dwell times. Table 1 shows the %CV variation of the H-SRM assay at different dwell times. The reproducibility of the H-SRM assay was excellent and approximately 95% of peptides had %CV values <15 % for all three dwell times. The SRM assay also gave acceptable quantitative precision, although the variation of %CVs was higher compared to H-SRM assay. Figure 4 shows the %CV comparison between H-SRM and SRM assays performed at 20 ms dwell time. With the H-SRM assay, 70% of peptides had CVs <10% and 95% of peptides had CVs <15%. Only one peptide had a %CV between 15 and 20%. Conversely, for the SRM assay, 35% of the peptides had %CV values <10%, 70% of peptides had CVs <15%, 20% of peptides had CVs between 15 - 20%. The remaining 10% of peptides had CVs between 25-30%.

Fig. 4. The %CV comparison of SRM and H-SRM using the Quantum Ultra



Quantitative accuracy

The excellent assay precision of the H-SRM assay made accurate quantitation possible using the Quantum Ultra. Table 2 shows the relative quantitative results of 13 targeted proteins (A: 1 uL injection and B: 2 uL injection) with the H-SRM assay. On average, an error of ±4% was observed.

Table 2. Relative Quantitative Results of Targeted Serum Proteins with H-SRM assay for sample A (1 uL of serum) and sample B (2 uL of serum)

A	B Protein	C Sample A (1ul) Mean Area (n=3)	D Sample B (2 ul) Mean Area (n=3)	E B/A Ratio Observed	F B/A Ratio Expected	G % Error of Ratio*
1	AMBP			1.94	2.00	-3.0
	ETLLQDFR	7640072	14663553	1.94		
2	Appoliprotein M			2.09	2.00	4.5
	AFLLTPR	3549729	7445575	2.09		
3	Complement C4-A			2.14	2.00	7.0
	VDFTLSSER	7356641	15764496	2.14		
4	Complement C4_B			2.12	2.00	6.0
	VGDTLNLNLR	6522740	13834291	2.12		
5	comple.component1 q			1.82	2.00	-9.0
	TINVPLR	11992826	21609996	1.82		
6	complement factor B			2.07	2.00	3.5
	YGLVTYATYPK	5585992	11391670	2.04		
	EELLPAQDIK	21306581	44757519	2.10		
7	gelsolin isoform b			1.93	2.00	-3.5
	HVVPNEVVVQR	2310372	4464778	1.93		
8	haptoglobin			1.99	2.00	-0.5
	TEGDGVYTLNDK	22164154	45309950	2.04		
	VGYVSGWGR	185320005	371465118	2.00		
	NFPSPVDAAFR	66257716	128839733	1.94		
9	histidine-rich glycoprotein			1.92	2.00	-4.0
	DGYLFQLLR	2768997	5323262	1.92		
10	inter-alpha-trypsin ingibitor heavy			2.12	2.00	6.0
	EVAFDLEIPK	6577817	14680692	2.23		
	AAISGENAGLVR	4909825	9834816	2.00		
11	kininogen 1			1.95	2.00	-2.5
	TVGSDTFYSFK	6089979	12656487	2.07		
	DFVQPPTK	18825551	34420368	1.83		
12	Serum amyloid P-componenet			2.00	2.00	0.0
	VGEYSLYIGR	453911	934968	2.06		
	IVLGQEQDSYGGK	2239569	4376917	1.95		
13	vitronectin			1.96	2.00	-2.0
	VDTVDPPYPR	36737187	71271250	1.94		
	FEDGVLDPDYPR	27485185	54140444	1.97		

* % of Error of Ratio = (Observed B/A ratio-Expected B/A ratio)/Expected B/A ratiox100

Conclusions

An H-SRM assay was developed using a TSQ Quantum Ultra triple quadrupole mass spectrometer. This gave excellent analytical assay precision and quantitative accuracy for targeted protein quantitation in whole human serum by dramatically reducing non-specific interference from complex serum background. The H-SRM assay improves assay specificity and offers significant advantages for biomarker verification and validation studies.

1) An H-SRM assay allowed confident peptide peak confirmation and quantitation from a single HPLC-MS/MS analysis.
2) Sensitivity was excellent, with the ability to detect proteins present at ng/mL levels.
3) The dynamic range for detecting proteins at different concentrations was over four orders of magnitude.
4) Excellent analytical H-SRM assay precision was obtained for all selected dwell times (5 ms, 10 ms & 20 ms). Approximately 95% of peptides gave %CVs <15%. No significant signal loss was observed when using shorter dwell time.
5) The quantitative accuracy of the H-SRM assay was excellent. The average relative quantitation error was ±4%.
6) The SRM triggered QED/MS/MS data were of high quality and allowed confirmation of peptide ID by database searching.

References

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