High-Throughput Protein Quantitation Using Multiple Reaction Monitoring

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Introduction

Peptide quantitation using multiple reaction monitoring (MRM) has been established as an important methodology for biomarker validation. Quantitative proteomics can require high throughput as often hundreds of target peptides need to be monitored in each sample and thousands of biological samples may need to be analyzed. Dynamic MRM algorithm allows the system to acquire transition ion data only during the retention window when each peptide is eluting. This reduces the number of concurrent MRM and therefore improves quantitation and sensitivity. In this study, we spiked Peroxidase at different concentrations into human plasma and demonstrated the entire workflow from biomarker discovery to validation. Reproducibility of MRM abundances and retention time at nanoflow range were studied with 443, 2000 and 3293 MRM transitions using Dynamic MRM methods.

Experimental

Sample Preparation

Human plasma sample was purchased from Sigma (St. Louis, MO). The sample was depleted of 14 highly abundant proteins using a HU-14 immunoaffinity column (Agilent) following the standard protocol. After depletion, the sample was buffer-exchanged into an ammonium bicarbonate solution, reduced, alkylated (IAA) and digested with trypsin under denaturing conditions. Horseradish peroxidase was purchased from Sigma (St. Louis, MO) and digested with trypsin. Peroxidase digest was spiked at 500 amole (A) or 5 fmol (B) per 0.5 µg human plasma digest.

Experimental

LC/MS Analysis

Agilent 6410 Triple Quadrupole (QQQ) and 6520 Accurate Mass Quadrupole Time-of-Flight (Q-TOF) mass spectrometers were connected to the HPLC-Chip interface.

HPLC-Chip: Protein ID chip with 150 x 0.075 mm analytical column and 40 nL enrichment column. Sample load: 0.5 µg of human serum digest with different amount of peroxidase digest spiked in. Injection volume: 1µL. Flow: 300 nL/min analytical pump, 3 µL/min loading pump. Mobile phases A: 0.1% FA, B: 90% acetonitrile, 0.1% FA. Gradients: 3% B at 0 min.,10%B at 3 min, 12%B at 8 min., 30%B at 42 min., 45%B at 45 min., 70%B at 50 min. 90%B at 90 min., then 3%B at 55.1 min. Stop time: 60 min. Post time: 10 min.

MS Conditions: Drying gas: 5 L/min, 325°C; Collision energy: slope 3.6, offset -4.8; Capillary voltage: 1800V.

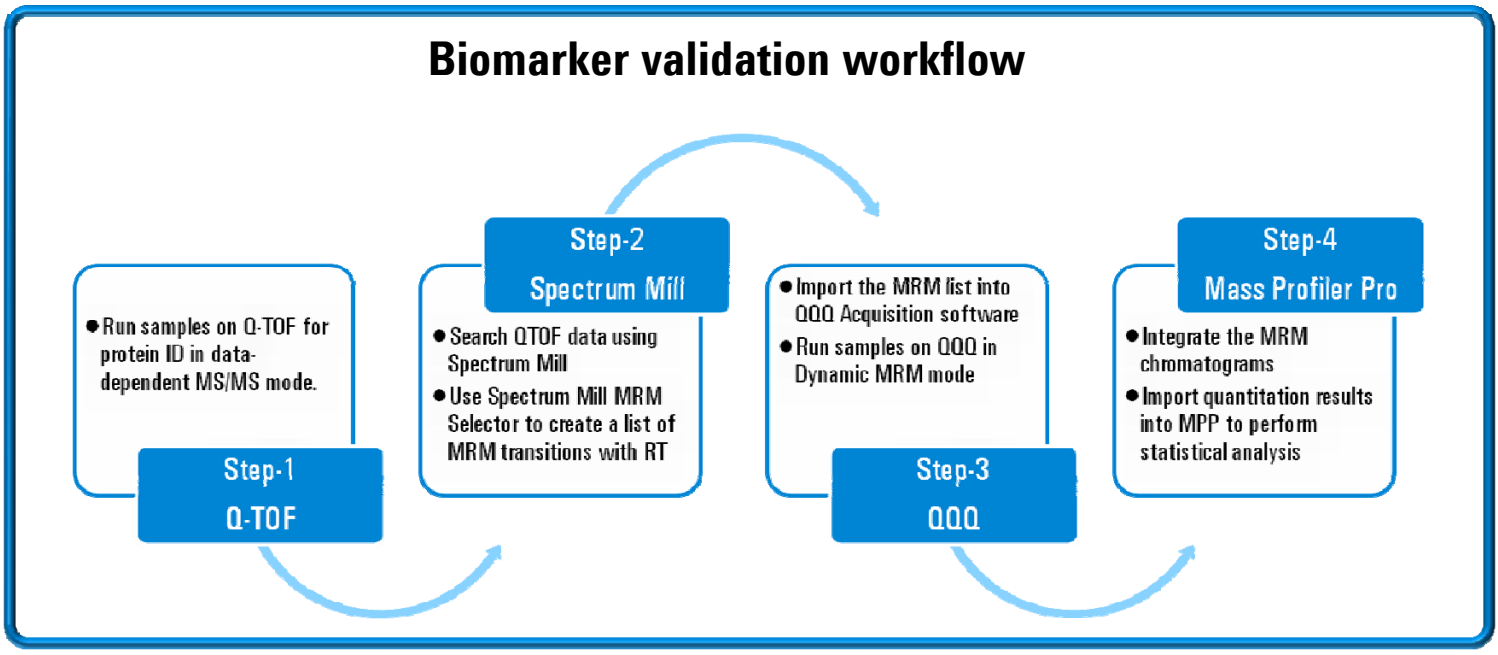
Software

Spectrum Mill

Q-TOF data was searched against the SwissProt database using Agilent Spectrum Mill MS Proteomics Workbench. The dynamic MRM methods were generated using MRM Selector in Spectrum Mill.

Mass Profiler Professional (MPP)

Quant batch report XML files were imported into Mass Profiler Professional. Spiked-in peptide features were analyzed in context of human serum peptides via principal components analysis (PCA). Additionally, a naïve hierarchical clustering analysis was performed.



Step 1: Q-TOF

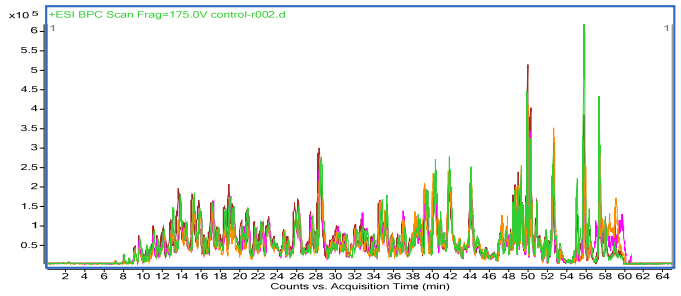


Figure 1: Overlaid 5 replicate runs of depleted human plasma tryptic digest on HPLC-Chip/Q-TOF. The samples were analyzed in data-dependent MS/MS mode for protein identification.

Step 2: Spectrum Mill

Q-TOF data was searched using Spectrum Mill. A Dynamic MRM list were generated using MRM Selector based on the validated peptide hits, which contains protein accession number and peptide sequence, MRM transition values, retention time (RT), peak width, collision energy and fragmentor value.

MRM Selector parameters:

•number of peptides per protein,

•number of product ions per peptide, choice of above precursor and y-ions

•Peptide score and %SPI

•Required AA and disallowed AA

•Peptide pI

•Protein accession number

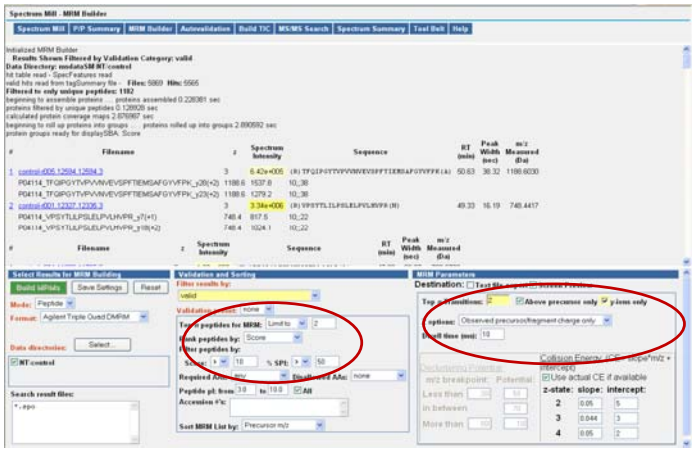


Figure 2: MRM Selector generates dynamic MRM methods from discovery Q-TOF data.

Step 4: QQQ

The dynamic MRM lists containing hundreds to thousands of MRMs were imported into a QQQ acquisition method. A cycle time was set to obtain at least 15 data points across the peaks. The dwell time for each MRM was calculated by the software.

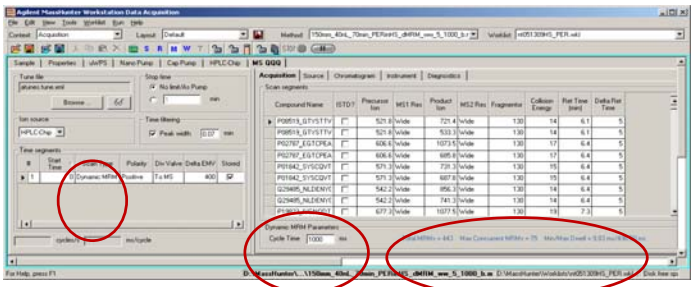


Figure 3. Example of Dynamic MRM method.

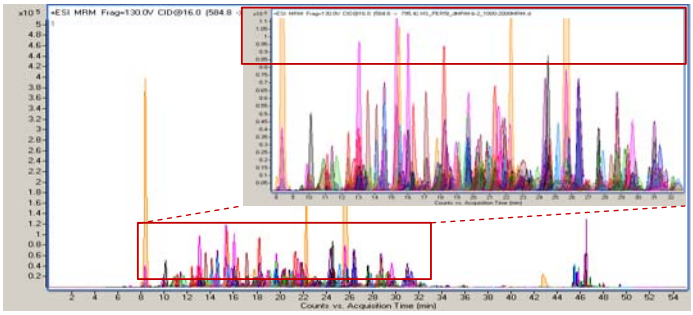


Figure 4. Overlaid 2000 MRM chromatograms acquired in a single run using Dynamic MRM.

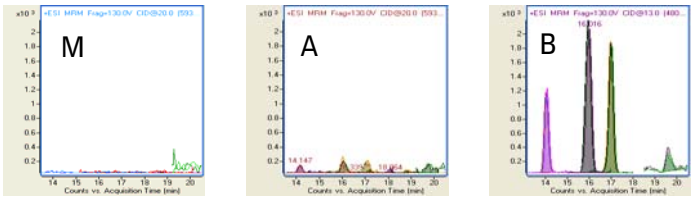


Figure 4. Overlaid MRM chromatograms of peroxidase peptides showed excellent reproducibility of retention time and MS response. M: human plasma matrix; A: 500 amole peroxidase spiked into human plasma; B: 5 fmol peroxidase spiked into human plasma.

# MRM	RT window (min)	Cycle time (ms)	Min. dwell (ms)	Max. # concurrent MRM	%RSD Area	RSD RT (min)
443	2	1000	16.5	50	2.5	0.038
443	1	1000	29.83	30	3.2	0.016
2000	2	1000	2.75	160	4.5	0.030
3293	1	1050	2.18	185	4.7	0.025

Table 1. Reproducibility of MS response and RT.

Step 4: Mass Hunter Mass Profiler Pro

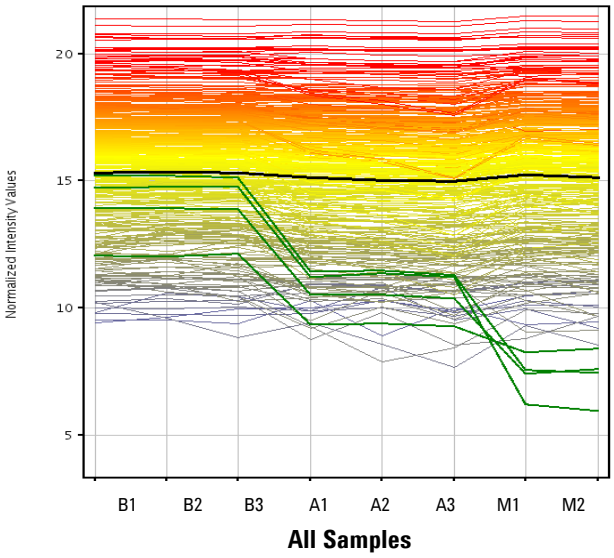


Figure 6. Profile of 443 MRM abundances across all samples. Four peptides from peroxidase were highlighted in green. The mean of 443 MRM abundances is displayed (black) to show the peptides from plasma did not vary from sample to sample.

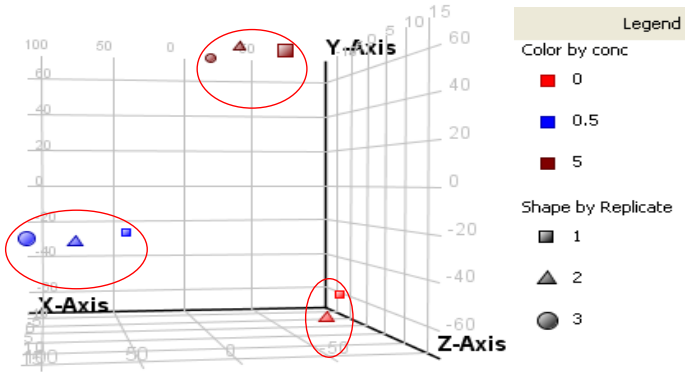


Figure 7. PCA analysis of different samples. Samples at different peroxidase concentrations were correctly grouped together.

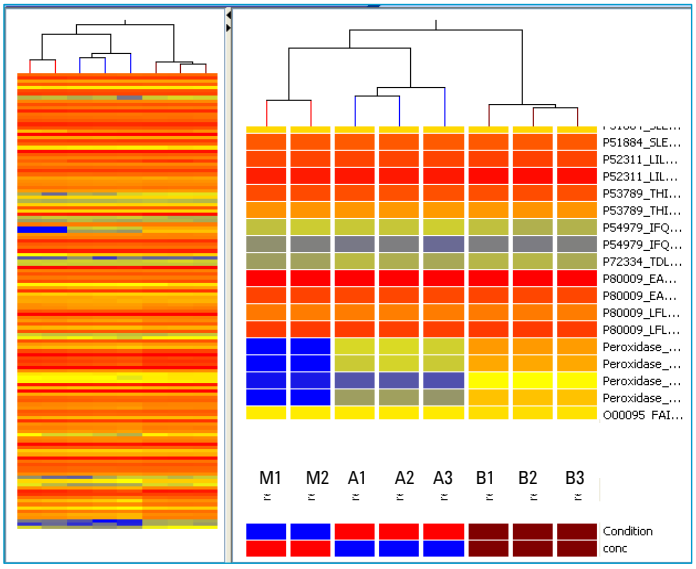


Figure 8. Hierarchical clustering successfully clustered samples at different peroxidase concentration. A condition was generated with peroxidase concentration color-coded on the tree branches as in Figure 7, along with the peptide features labeled on each row. The heat map is colored from blue to red, where blue is low abundance and red is high abundance. The full view of all the features is on the left. The zoom view is on the right.

Compound	Corrected p-val...	p-value	Retention Time
Peroxidase_DTI	0	0	15.952
Peroxidase_GFP	0	0	16.999
Peroxidase_YYV	0	0	14.034
Peroxidase_SSD	0	0	19.595

Table 2. Analysis of Variance. A one-way ANOVA on concentration was performed on the peptide abundances. Benjamini-Hochberg multiple testing correction was applied. Additionally, a filter for fold change >= 5.0 was applied to the list. The four peroxidase peptides each had a corrected p-value of 0.0.

Conclusions

- A complete biomarker workflow from Q-TOF discovery to QQQ validation has been demonstrated.
- MRM Selector allows the user to create dynamic MRM methods based on experimental discovery data.
- Hundreds to thousands of peptides were monitored in dynamic MRM mode in a single LC/MS run, producing excellent RSDs on MS abundance and retention time.
- Excellent reproducibility of the HPLC-Chip/MS system is the key element for high-throughput and sensitive analysis of biomolecules.