

# High-Throughput Protein Quantitation Using Multiple Reaction Monitoring

## Application Note

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### Abstract

Quantitative proteomics using multiple-reaction monitoring (MRM) has emerged as an important methodology for biomarker validation. Multiple-reaction monitoring on a triple quadrupole (QQQ) mass spectrometer provides superior sensitivity and selectivity for targeted peptides in a complex sample. MRM also offers high precision in quantitation and a fast scan speed, which makes it an ideal technology for validating biomarkers in a high-throughput fashion. This application note demonstrates the entire workflow for biomarker validation offered by Agilent Technologies using multiple reaction monitoring together with statistical analysis software. Thousands of peptide transitions were generated using Agilent Q-TOF and Spectrum Mill MRM selector. The transition list was imported into MassHunter acquisition software equipped with dynamic MRM functionality and monitored in a single LC/MS run. Finally, MRM quantitation results were analyzed using Mass Profiler Pro for principle component analysis (PCA), hierarchical clustering and ANOVA analysis.



**Agilent Technologies**

## 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. The 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 ion transitions and therefore improves quantitation and sensitivity. In this study, peroxidase was spiked at different concentrations into human plasma to demonstrate the entire workflow from biomarker discovery to validation. Reproducibility of peak abundances and retention time at nanoflow range were studied with 443, 2,000 and 3,293 ion transitions using dynamic MRM method on a nanoflow LC/MS system.

## 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), reduced, alkylated and digested with trypsin. Peroxidase digest was spiked at 500 amol (A) or 5 fmol (B) per 0.5  $\mu$ g human plasma digest.

### **LC/MS analysis**

With electrospray LC/MS, smaller chromatographic elution volumes result in enhanced peak height and thus greater sensitivity. A unique microfluidic chip, the HPLC-Chip, has been developed for nanoflow LC/MS that integrates sample enrichment and separation columns, microvalve connections and the nanospray tip on a biocompatible polyimide chip. Interfacing this device to a triple quadrupole mass spectrometer provides easy-to-use, highly reliable and highly sensitive LC/MS analysis. For this work, the HPLC-Chip was interfaced to an Agilent 6410 Triple Quadrupole (QQQ) LC/MS and an Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS.

### **HPLC-Chip Parameters:**

Chip and columns: Protein ID chip with 150 x 0.075 mm analytical column and 40 nL enrichment column.

Sample load: 0.5  $\mu$ g of human serum digest spiked with different amounts of peroxidase digest.

Injection volume: 1  $\mu$ L.

Flow: 300 nL/min analytical pump, 3  $\mu$ L/min loading pump.

Mobile phases: A. 0.1% formic acid (FA), B. 90% acetonitrile (ACN), 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 55 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: 1,800 V.

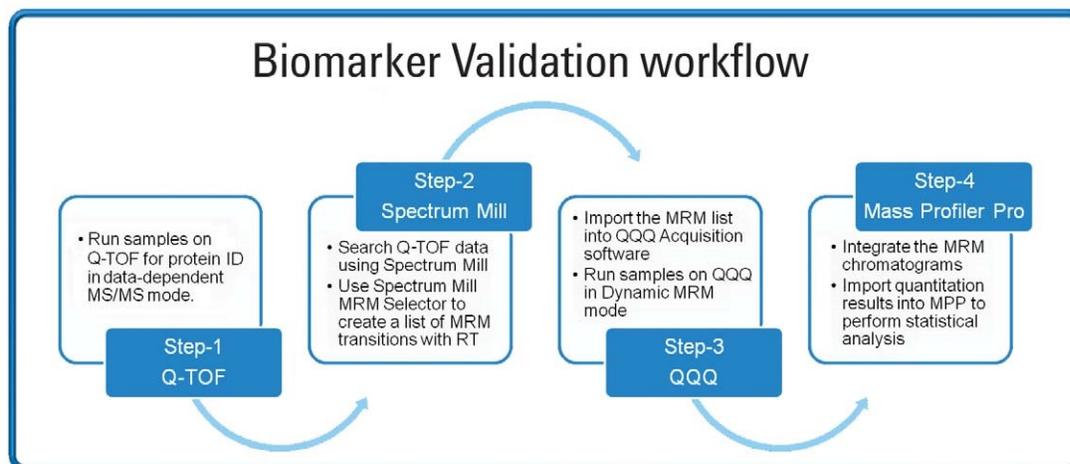
## Software

The Q-TOF data was searched against the SwissProt database using Agilent Spectrum Mill MS Proteomics Workbench. A new tool within Spectrum Mill, MRM Selector, was used to directly generate the dynamic MRM methods based on results from the Q-TOF database search results. After LC/MS analysis, the results were analyzed using MassHunter Quantitative

Analysis software. From this, quant batch report XML files were imported into Mass Profiler Professional software, a chemometrics software package designed specifically for mass spectrometry data. Spiked-in peptide features were analyzed in the context of human serum peptides via principal components analysis (PCA). Additionally, a naïve hierarchical clustering analysis was performed.

## Results and Discussion

The biomarker validation workflow is illustrated in **Figure 1**. In the first step, the samples were run on HPLC-Chip/Q-TOF in data-dependent MS/MS mode. The HPLC-Chip provided excellent reproducibility as shown by the overlaid base peak chromatograms (BPC) of 5 replicate injections (**Figure 2**).



**Figure 1. Biomarker validation workflow.**

## Step 1: Q-TOF

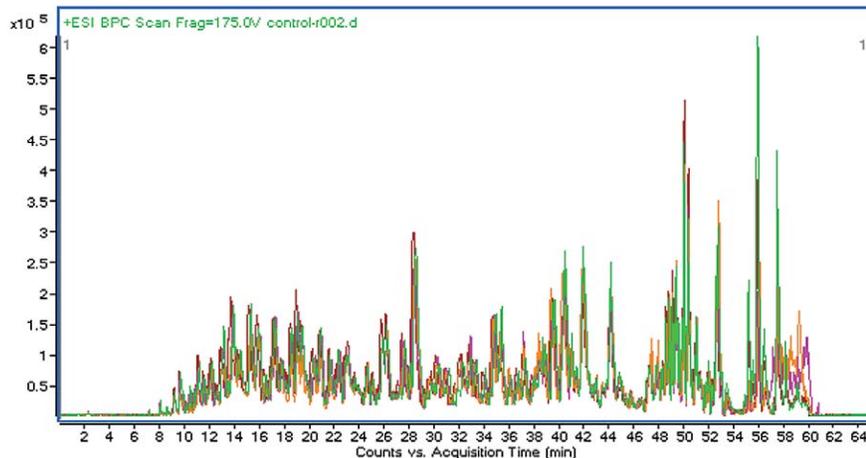


Figure 2. Overlaid BPCs from 5 replicate runs of depleted human plasma tryptic digest on HPLC-Chip/Q-TOF. The samples were analyzed in data-dependent MS/MS mode for protein identification.

## Step 2: Spectrum Mill

Q-TOF data was searched using Spectrum Mill. A dynamic MRM list was generated using MRM Selector based on the validated peptide hits. MRM Selector is a utility tool in Spectrum Mill workbench that allows the user to select the MRM transitions from the experimental MS/MS data. The user can input several parameters (listed below) to filter the ion transitions to be monitored on QQQ. The MRM Selector results contain protein accession number and peptide sequence, ion transition values, retention time (RT), peak width, collision energy and fragmentor values. The saved list can be pasted directly into the QQQ acquisition software.

Figure 3. MRM Selector generates dynamic MRM methods from discovery Q-TOF data.

### 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 pl
- Protein accession number

### Step 3: QQQ

The dynamic MRM lists containing hundreds to thousands of ion transitions were imported into a QQQ acquisition method. The cycle time was set to obtain at least 15 data points across the peaks.

In order to assess the reproducibility of the MS and RT with increasing number of ion transitions, four dynamic MRM experiments were set up using MRM Selector with 443, 2,000 and 3,293 ion transitions in each method. The retention time (RT) window for monitoring the ion transitions was also varied between 1-2 min resulting in different minimum dwell time and maximum number of concurrent MRMs. The RSD of MS response and RT from 12 peroxidase peptide transitions were calculated and are listed in **Table 1**. The %RSD of the MS responses was below 5% while the RSDs of RT were less than 0.04 min for the 60 min run.

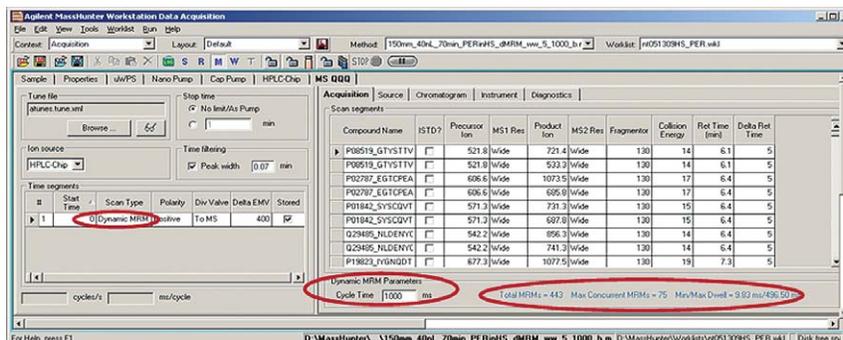


Figure 4. Example of dynamic MRM method.

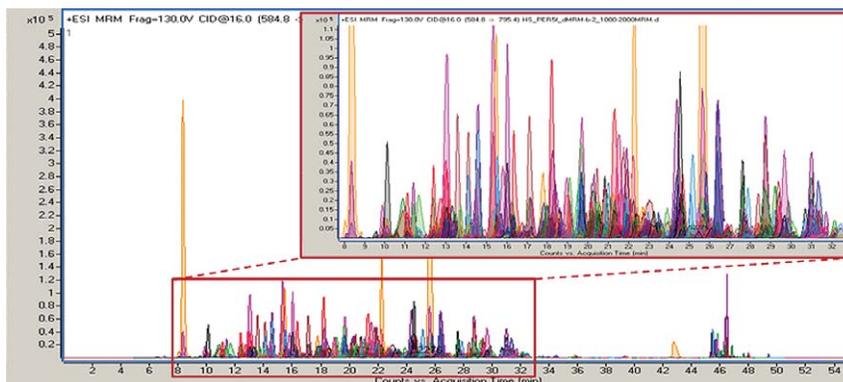


Figure 5. Overlaid 2,000 MRM chromatograms acquired in a single run using dynamic MRM.

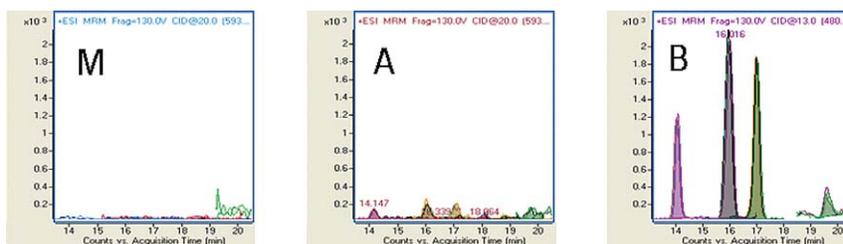


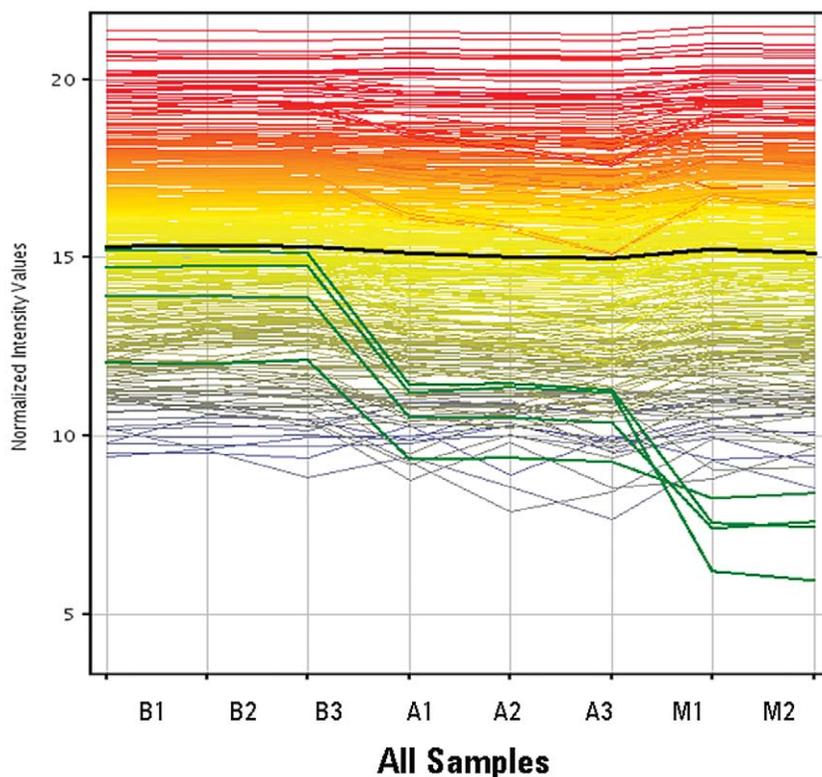
Figure 6. Overlaid MRM chromatograms of peroxidase peptides showed excellent reproducibility of retention time and MS response. M: human plasma matrix; A: 500 amol peroxidase spiked into human plasma; B: 5 fmol peroxidase spiked into human plasma.

# MRM	RT window (min)	Cycle time (ms)	Min. dwell (ms)	Max. # concurrent MRM	% RSD Area	RSD RT (min)
443	2	1,000	16.5	50	2.5	0.038
443	1	1,000	29.83	30	3.2	0.016
2,000	2	1,000	2.75	160	4.5	0.030
3,293	1	1,050	2.18	185	4.7	0.025

Table 1. Reproducibility of MS response and RT.

## Step 4: MassHunter Mass Profiler Pro

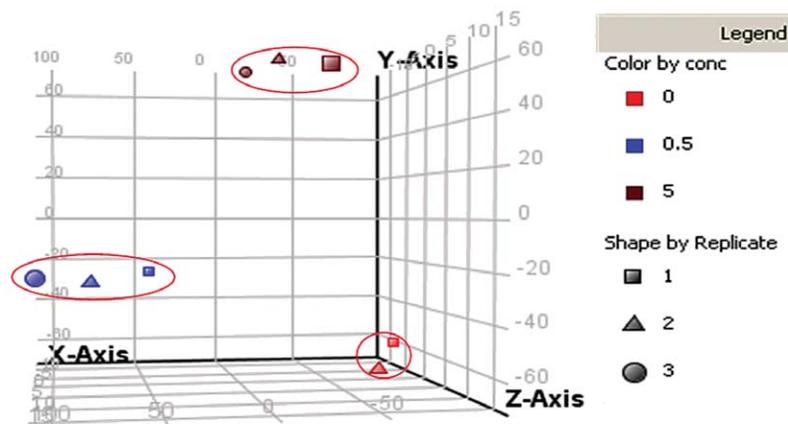
Mass Profiler Professional can easily classify, compare and analyze sample groups using a combination of powerful statistical and mathematical models to analyze complex MS data sets. **Figure 7** shows the abundance of 443 ion transitions across all samples (three B samples, three A samples and two controls). Each line represents one ion transition and the color represents the relative intensity of the ion transition (red being high, gray being low). The ion transitions for the four peptides from peroxidase were highlighted in green. The mean of the 443 abundances is displayed (black) to show that the peptides from plasma did not vary from sample to sample.



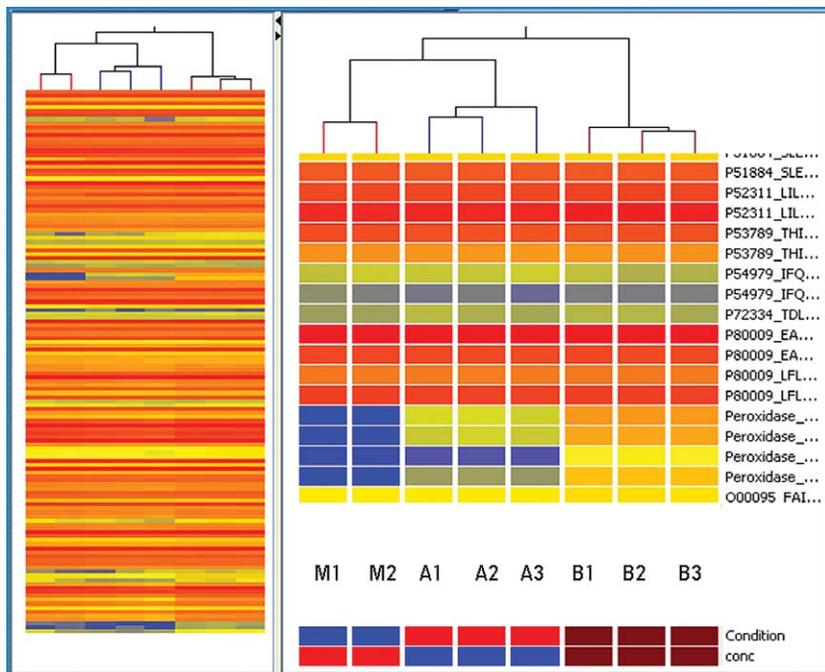
**Figure 7.** Profile of abundances across all samples for 443 ion transitions. Four peptides from peroxidase were highlighted in green. The mean of the 443 abundances is displayed (black) to show that the peptides from plasma did not vary from sample to sample.

Principal component analysis (PCA) can be used to find differences in groups in either an unsupervised fashion (classify without knowing the group assignments) or a supervised fashion (must classify groups before analysis). As shown in **Figure 8**, both levels of peroxidase-spiked plasma were clearly differentiated from the control plasma.

Clustering analysis by sample groups organizes the relationships based on the similarity of entities' abundance profiles. The tree diagram produced by hierarchical clustering in Mass Profiler Professional reveals the relationships between mass entities in one dimension and between samples in the other dimension. In **Figure 9**, the technical replicates for the different peroxidase levels were correctly grouped using hierarchical clustering.



**Figure 8.** PCA analysis of different samples. Samples at different peroxidase concentrations were correctly grouped together.



**Figure 9. Hierarchical clustering successfully clustered samples at different peroxidase concentrations.** A condition was generated with peroxidase concentration color-coded on the tree branches as in Figure 8, 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-value	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  $\geq 5.0$  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 using a spike-in study with human plasma. The protein database search results from Q-TOF discovery data was used to create dynamic MRM methods via MRM Selector, a new tool being developed in Spectrum Mill. These dynamic MRM methods allowed hundreds to thousands of peptides to be monitored in a single LC/MS run, while still producing excellent RSDs on MS abundance and retention time. Mass Profiler Professional software allowed chemometric analysis of the quantitative results for confirmation of the significant differences between samples. Excellent reproducibility of the HPLC-Chip/MS system is the key element for high-throughput and sensitive analysis of biomolecules.

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