Dynamic MRM: A Clear Advantage for High-throughput Protein Quantitation

Application Note

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Abstract

An MS/MS approach based on Multiple Reaction Monitoring (MRM) is commonly used in the biomarker validation process and in other protein quantitation applications. The Dynamic MRM (DMRM) algorithm in the Agilent 6400 Series Triple Quadrupole LC/MS system automatically constructs DMRM timetables for multiple analytes throughout the LC/MS analysis based on the retention time window for each analyte. It allows the instrument to acquire MRM data only during a stated retention time window, thus reducing the number of concurrent ion transitions. In this study, 194 MRM transitions from a digest of complex protein standard was analyzed using DMRM and MRM modes in the Agilent 6410 Triple Quadrupole LC/MS system. Improved sensitivity, better peak symmetry, and up to ten-fold increase in the signal-to-noise (S/N) ratio are achieved using DMRM.

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Introduction

The most common MS/MS approach to validate biomarker candidates from a discovery experiment has been to generate a highly specific MRM-based assay. Hundreds of peptides may have to be quantified in a validation process of biomarker discovery. The DMRM algorithm in the Agilent 6400 Series Triple Quadrupole LC/MS system automatically constructs DMRM timetables for multiple analytes using retention times and detection windows (Delta RT). It allows the instrument to acquire MRM data only during the retention time window such that the MS duty cycle is not wasted by monitoring ions during periods of time when the compounds are not expected to elute. It also reduces the number of concurrent ion transitions, thus maximizing dwell time and should therefore improve sensitivity. DMRM utilizes a constant cycle time to ensure uniform distribution of data points. This will improve the sampling of chromatographic peaks resulting in better peak symmetry that enables reproducibility in retention time measurement, peak areas, and accuracy of quantitation. In this study, 194 MRM transitions from a commercially available complex protein standard were analyzed using DMRM and MRM modes in the Agilent 6410 Triple Quadrupole LC/MS system to show the benefits of DMRM in a high-throughput analysis.

Experimental Procedure

This study used the 'Complex Proteomics Standard' (Agilent Technologies, part no. 400510), which is composed of a complex mixture of proteins extracted from *Pyrococcus furiosus* (Pfu). The protein mixture was reduced, alkylated, and digested using trypsin (obtained from Agilent Technologies, part no. 204310) as described in the user manual. The digest was analyzed on an Agilent 6520 Accurate-Mass Q-TOF LC/MS system coupled to a 1200 Series HPLC-Chip/MS system for the initial identification of proteins/ peptides. An Agilent 6410 Triple Quadrupole LC/MS system coupled to an HPLC-Chip/MS system was used for MRM experiments. Insulin-like growth factor-1 (IGF-1) obtained from Sigma was reduced, alkylated, digested, and spiked in the Pfu digest before mass spectral analysis. 1 µg of Pfu digest spiked with 1 femtomole of IGF-1 was loaded on-column in each LC/MS analysis. IGF-1 was spiked in the sample in order to have six selected MRM transitions from peptides with known concentrations.

LC and MS conditions used for the identification of the proteins/peptides

The analysis was conducted using an HPLC-Chip/MS system with a 40 nL enrichment column and a 75 mm x 43 mm analytical column packed with ZORBAX 300SB-C18 5 µm (300Å). 0.1% formic acid in water (A) and 90% acetonitrile in water with 0.1% formic acid (B) were used as solvents for elution. Flow rates were as follows: 3 µL/min on the capillary pump for loading the sample on the enrichment column and 600 nL/min from the nano pump for the analytical column. Samples were loaded on the enrichment column using 3% B. The gradient used for the analysis was as follows: 3% B at 0 min, 12% B at 5 min, 30% B at 75 min, 60% B at 80 min, 95% B at 83 min, and 3% B at 85-90 min.

Spectra were recorded in positive ion mode with a capillary voltage of 1950 V, drying gas flow of 5 L/min at 325°C, and fragmentor voltage of 135 V. MS/MS spectra were acquired in auto MS/MS mode with the MS acquisition rate (*m/z* 300–3,200) of 8 spectra/sec and MS/MS acquisition rate (*m/z* 100–3,000) of 3 spectra/sec. The collision energy for each precursor was automatically determined using the following equation: [(*m/z*) / 100]*slope + intercept, where the slope was 3.6 and the intercept was -4.8.

Selection of MRM transitions

MS/MS data from the Q-TOF analysis was searched against the NCBInr database using Spectrum Mill software. Two or three MRM transitions were selected from the MS/MS spectra of 71 peptides from 32 proteins. Precursor ion masses, product ion masses, and the retention time information for a total of 194 MRM transitions were obtained from the search results and used for MRM experiments.

LC and MS conditions used for MRM experiments

The same mobile phases and the same HPLC-Chip used for identification of peptides on the 6520 Q-TOF were used for the MRM experiments on the 6410 Triple Quadrupole. Samples were loaded on the enrichment column using 3% B. The run time for the LC/MS analysis was reduced to 45 min using the following gradient: 3% B at 0 min, 12% B at 3 min, 30% B at 37 min, 60% B at 40 min, 95% B at 42 min, and 3% B at 45 min.

The MS source conditions were the same as those for the Q-TOF experiments. 194 MRM transitions were monitored in MRM and dynamic MRM modes. A 5 ms dwell time (time spent on each transition) was used for the MRM experiments resulting in a cycle time of 1650 ms, which provided 15-30 data points across the chromatographic peaks. The same cycle time of 1650 ms was used for the DMRM experiment so that DMRM and MRM can be compared under identical acquisition conditions. The dwell time is automatically adjusted in DMRM. The collision energy for each transition was calculated using the same equation used for the Q-TOF experiments. Because the collision cell and ion optics are similar in the 6520 Q-TOF and 6410 Triple Quadrupole, the same fragmentation was observed when identical collision energies were used.

Results and Discussion

The chromatography used in the Q-TOF experiment could be duplicated on the 6410 Triple Quadrupole owing to the excellent retention time reproducibility achieved using the HPLC-Chip[1]. Within each DMRM timetable, all ion transitions had the same dwell time; however, dwell times were varied for each timetable to ensure that all analytes are quantitatively sampled and a sufficient number of data points are acquired across all detected peaks.

Table 1 summarizes the peptide sequences, proteins from which these peptides are generated, peptide mass, precursor ions, and product ions of the MRM transitions as shown in **Figures 1** and **2**. From Figure 1, it is clear that there is a significant improvement in apparent sensitivity in the DMRM mode as compared to MRM. For example, the transition 719.5→387.0 from one of the spiked IGF-1 peptides **APQTGIVDECCFR** (at a concentration of 1 femtomole on-column) is clearly seen in DMRM while the analyte signal is within the noise level in MRM mode.

Peptide No.	Peptide sequence	Protein name	Peptide mass (Da)	Precursor ion (*m/z*)	Product ion mass (*m/z*)
1	APQTGIVDECCFR	Spiked IGF-1	1437.4	719.5	387.0
2	TYPIDATDVVFTFWR	Dipeptide-binding protein	1830.0	915.9	347.2
3	ALYILGNYYVPEVILGQNR	Dipeptide-binding protein	2194.2	732.4	700.4
4	AVTILIR	Thermosome	784.5	393.3	514.3
5	AFYDVYNIAK	Glutamate dehydrogenase	1202.6	602.3	608.3
6	TLSQSESGWDLIQQGVSYIVPIR	Alpha-amylase	2575.3	1288.7	385.2

Table 1. List of peptides from which MRM transitions are shown in Figures 1 and 2.

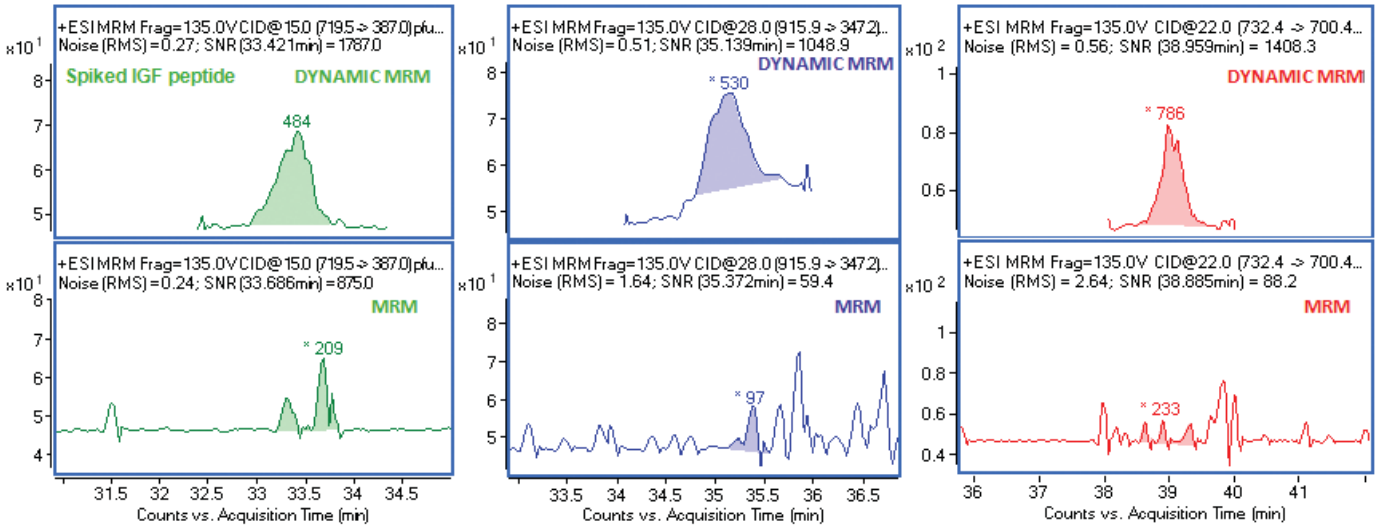

Figure 1. Examples of transitions demonstrating substantial sensitivity enhancement with DMRM.

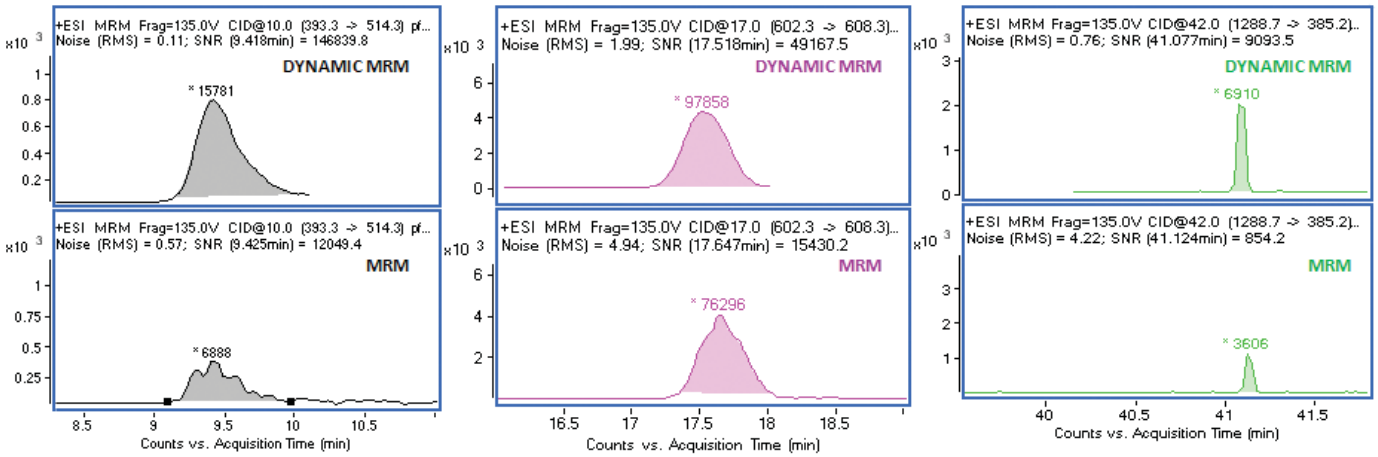

Figure 2. Comparison of peak symmetry obtained in MRM and DMRM.

Table 2 summarizes peak areas, S/N ratios of the MRM peaks, and the number of concurrent MRMs in the selected transitions. The number of concurrent transitions in DMRM is reduced to 15-25 as compared to 194 transitions in MRM. All transitions have a dwell time of 5 ms in MRM mode while the minimum dwell time reported in DMRM mode is 53.4 ms (10-fold difference). Hence, improved sensitivity and improved S/N ratios are observed in dynamic MRM, which are shown in Table 2.

Another advantage of using DMRM is the improved sampling across the chromatographic peak resulting in better peak symmetry. This enables reproducible retention time measurement (as the apexes of the peaks are sampled sufficiently) and more accurate quantitation. Figure 2 shows the comparison of peak symmetry obtained in MRM and DMRM experiments.

Peptide No.	MRM Transition	No. of concurrent MRMs in DMRM	Peak Area		S/N	
			MRM	DMRM	MRM	DMRM
1	719.5 → 387.0	19	No signal	**484**	No signal	**1787.0**
2	915.9 → 347.2	20	No signal	**530**	No signal	**1048.9**
3	732.4 → 700.4	20	No signal	**786**	No signal	**1408.3**
4	393.3 → 514.3	25	6888	**15781**	12049.4	**146839.8**
5	602.3 → 608.3	15	76296	**97858**	15430.2	**49167.5**
6	1288.7 → 385.2	16	3606	**6910**	854.2	**9093.5**

Table 2. Peak areas and S/N observed in selected transitions in MRM and DMRM modes.

Conclusions

- Improved sensitivity is achieved in DMRM due to longer dwell times.
- Improved sensitivity achieved in DMRM enables detection of some low abundant peptides in a complex mixture.
- Up to ten-fold improvement in the S/N ratio is observed in some transitions in DMRM.
- Better sampling across the chromatographic peak results in improved peak symmetry and enables reproducible retention time measurement.
- Dynamic MRM is an essential feature in triple-quadrupole LC/MS for high-throughput protein quantitation.

Reference

1. N. Tang, C. Miller, J. Roark, N. Kitagawa, and K. Waddell, "High-Throughput Protein Quantitation Using Multiple Reaction Monitoring," Agilent publication number 5990-4276EN, **2009**.

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