Application Kits for Standardizing MRM-based Quantitative Plasma Proteomic Workflows on the Agilent 6490 LC/MS System

Application Note

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Abstract

Quality control in the MS and proteomics community is necessary to enhance method reproducibility and transferability, but is currently limited for quantitative proteomics. To this end, two quality control (QC) application kits have been developed by MRM Proteomics Inc., Vancouver, B.C. for standardizing the sample preparation workflow or the LC/MS platform through the MRM-based quantitation of 40 plasma proteins. This Application Note describes the utility and significance of analyzing these kits on an Agilent standard-flow UHPLC-MRM/MS platform, which consists of an Agilent 1290 Infinity UHPLC system and Agilent 6490 Triple Quadrupole LC/MS System. Use of these kits helps assess the efficiency and reproducibility of the bottom-up proteomic workflow, and can also reveal any errors or performance deficits related to the sample preparation process or the LC/MS platform.

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Introduction

Mass spectrometry has emerged as one of the core analytical techniques for quantitating proteins in biological fluids, such as blood plasma – the most complex, yet most frequently investigated, human-derived proteome sample known. The determination of plasma protein concentrations is commonly accomplished through an absolute quantitative approach that combines targeted multiple reaction monitoring (MRM)-based detection with stable isotope-labeled standard (SIS) peptides implemented within a bottom-up proteomic method. This approach, a form of tandem mass spectrometry (Figure 1), has been gaining in popularity since its initial use in metabolite analyses in 1990[2] and in proteomic analyses in 1996[3].

MRM involves the mass selection of a precursor ion and a product ion, and has demonstrated exceptional robustness, sensitivity, and specificity in the multiplexed, high-throughput quantitation of plasma proteins[4]. Lack of method reproducibility and inter-laboratory transferability are major limitations that must be addressed if MRM-based assays are to be used in large-scale biomedical research of candidate disease biomarkers.

Addressing the aforementioned issues, Percy *et al*.[5] have developed two QC application kits for standardizing system performance on the most common LC/MS platforms used in the quantitative plasma proteomics field. The PeptiQuant™ LC/MS Platform Performance Kit (either daily or monthly version) is to be used in the performance assessment of the the LC/MS platform, while the PeptiQuant™ MRM-MS Workflow Performance Kit is intended for quality control of the entire bottom-up workflow (from denaturation through to detection). The quantitative technique applied in the standardization is the MRM with SIS peptide approach, and involves the multiplexed monitoring of 40 peptides corresponding to 40 plasma proteins. Since the analytes are high-to-moderate in abundance (from albumin at 41 mg/mL to L-selectin at 1.6 µg/mL), no sample prefractionation is required. This enables rapid and routine system standardization.

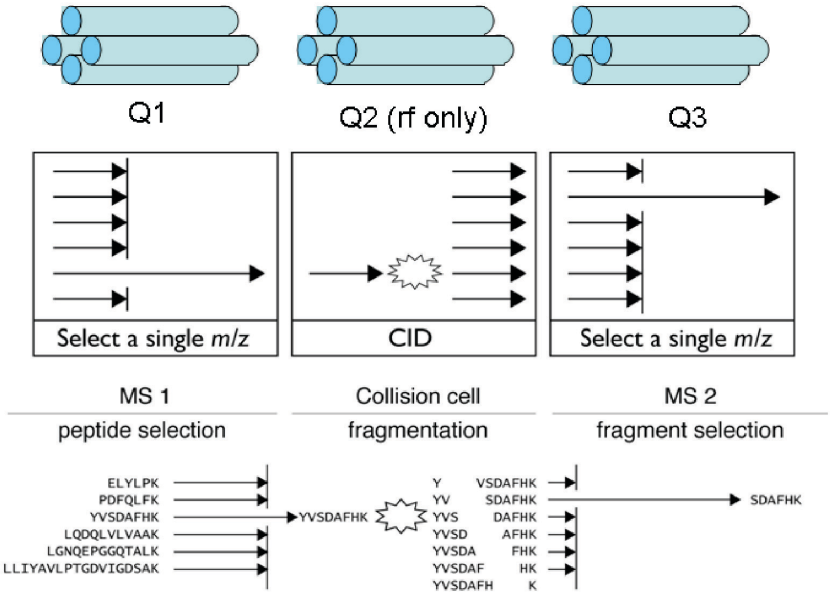

Figure 1. Schematic of the ion selection and fragmentation process performed in a triple quadrupole mass spectrometer, along with the principle behind MRM analysis. Reprinted from[1], with permission.

This Application Note describes the significance of the aforementioned kits in standardizing a commonly employed, absolute quantitative proteomic technique on an Agilent standard-flow UHPLC/MRM/MS platform. Central to the platform is a 6490 Triple Quadrupole LC/MS System with iFunnel technology, which is ideal for proteomic applications of complex samples due to its high sensitivity. Using these kits should help ensure that high quality data can be accurately reproduced by laboratories across the world using this LC/MS platform.

Experimental

Materials

The Platform Performance kit contains seven tryptic-digested plasma samples spiked with SIS peptides, while Workflow Performance kit contains the following three starting materials: human plasma, trypsin, and a SIS peptide mixture. All other chemicals, for example ammonium bicarbonate or dithiothreitol) and solvents (for example, acetonitrile or methanol) are of analytical reagent or LC/MS grade and can be obtained from a commercial vendor, such as Sigma-Aldrich (St. Louis, MO, USA).

Solution and sample preparation

The complete protocol for preparing the kits for quantitative proteomic analyses is detailed in the Percy *et al*. manuscript[5]. Briefly, the seven plasma digest lyophilates supplied in the PeptiQuant™ LC/MS Platform Performance Kit are to be rehydrated for direct LC/MRM/MS analyses, while the materials provided with the PeptiQuant™ MRM-MS Workflow Performance Kit are to be used in the preparation of seven standard samples for bottom-up LC/MRM/MS analyses. The latter preparation involves sequential reduction (with *tris*-(2-carboxyethyl) phosphine, final concentration 5 mM), alkylation (with iodoacetamide, final concentration 10 mM), and quenching (with dithiothreitol, final concentration 10 mM) of the 10× diluted plasma prior to overnight, tryptic digestion at a 10:1 substrate:enzyme ratio. Digestion is terminated with the sequential addition of a chilled SIS peptide mixture (from 250 to 0.025 fmol/µL for standard samples G to

A, respectively) and a chilled formic acid (FA) solution (2.1 %) into seven separate Eppendorf tubes. Following centrifugation to remove the acid-insoluble deoxycholate, the peptide supernatants are desalted by solid phase extraction (with 10 mg Waters Oasis HLB cartridges (Milford, MA, USA)), lyophilized, and rehydrated in 0.1% FA (final concentration 1 µg/µL) for LC-MRM/MS analyses.

LC/MS platform

The instrument platform was comprised of an Agilent 1290 Infinity ultra-high performance liquid chromatography (UHPLC) system interfaced to an Agilent 6490 Triple Quadrupole LC/MS System, using a standard-flow ESI (Jet Stream) source. The LC system specifically consists of a binary pump, a thermostatted column compartment, and a thermostatted micro well-plate autosampler. Pertinent LC/MS conditions for the acquisition method are listed below and are entered into the Agilent MassHunter Workstation Software (version B.05.01), as specified in the "Data Acquisition for 6400 Series Triple Quadrupole LC/MS Familiarization Guide".

Data analysis

All data are processed with the Agilent MassHunter Quantitative Analysis software (version B.05.00) using conventional batch and method creation user guides from Agilent. After ensuring correct peak selection and complete peak integration, seven-point peptide calibration curves (five replicates/level), with $1/x^2$ weighted linear regression, are generated. Only the concentration levels that are precise (average CV < 20 %) and accurate (80–120 % on average) remain on the curve. Performance characteristics can then be extracted and compared to the known reference values for accuracy assessment. Such measures of comparison include the precision of the LC/MRM/MS assay (defined as the CV of the natural (NAT):SIS response ratio for Standard E). The lower limit of quantitation (corresponds to the SIS concentration at the lowest level on the curve that satisfies the precision and

LC conditions

Parameter	Value			
Column	Agilent ZORBAX RRHD Eclipse Plus C18 UHPLC, 150 mm × 2.1 mm id, 1.8 µm (p/n 959759-902)			
Column temperature	50 °C			
Mobile phases	A: 0.1 % FA B: 0.1 % FA in 90 % ACN			
Autosampler temperature	4 °C			
Flow rate	0.4 mL/min			
Gradient program	**Time**	**A**	**B**	**Flow rate**
	Initial	97 %	3 %	0.4 mL/min
	2 minutes	89 %	11 %	0.4 mL/min
	15 minutes	81 %	19 %	0.4 mL/min
	22 minutes	71 %	29 %	0.4 mL/min
	25 minutes	55 %	45 %	0.4 mL/min
	27 minutes	10 %	90 %	0.4 mL/min
	29 minutes	10 %	90 %	0.4 mL/min
	30 minutes	89 %	3 %	0.4 mL/min
Injection volume	10 µL (corresponds to 10 µg digest and 0.1 to 1,000 fmol SIS on-column for standards A to G)			
Needle wash	2 seconds			
Draw speed	20 µL/min			
Eject speed	40 µL/min			
Analysis time	30 minutes			
Post-run equilibration	4 minutes			

Agilent 6490 Triple Quadrupole MS conditions

Parameter	Value
Ionization mode	positive ESI
Sheath gas temperature and flow	250 °C, 11 L/min
Nozzle voltage	300 V
Drying gas temperature and flow	150 °C, 15 L/min
Nebulizer gas pressure	30 psi
Capillary voltage	3,500 V
Fragmentor voltage	380 V
Cell acceleration potential	5 V
Acquisition mode	dynamic MRM
Q1/Q3 resolution	unit
Cycle time	260 ms
Collision energy	see peptide specific product sheets

All other instrument parameters are set by Agilent autotune and subsequent mass calibration functionality.

accuracy requirements), and the plasma protein concentration (calculated from the quotient of the NAT:SIS relative response ratio and the SIS concentration at each qualified level together with the protein molecular weight and a conversion factor to convert fmol/µL to ng/mL).

Results and Discussion

Performance standards are essential for improving the inter-laboratory transferability and platform reproducibility, but are currently lacking in the quantitative proteomics field. To address this shortcoming, two kits have been developed by Percy *et al*. for standardizing Agilent's standard-flow UHPLC/MRM/MS platform through a MRM with SIS peptide quantitative approach[5]. Reference values for LC/MS platform assessment include 4.1 % CV for the average signal stability and 0.06 % CV for the average retention time variability (both obtained from Standard E replicates). Assay attributes to evaluate the entire analytical workflow include the correlation of determination (R^2), the lower limit of quantitation (LLOQ), the dynamic range, and the plasma protein concentration. Peptide-specific reference values for these attributes are listed in the individual product sheets and correspond to an average R^2 of 0.99 and an average dynamic range of 103, with LLOQ's below the experimentally derived plasma protein concentrations for all 40 proteins in the multiplexed MRM assay. If the user adheres to the recommended LC/MS settings and strictly follows the kit protocols[5], an extracted ion chromatogram (EIC) trace and a linear regression curve similar to those illustrated in Figures 2 and 3 are achievable.

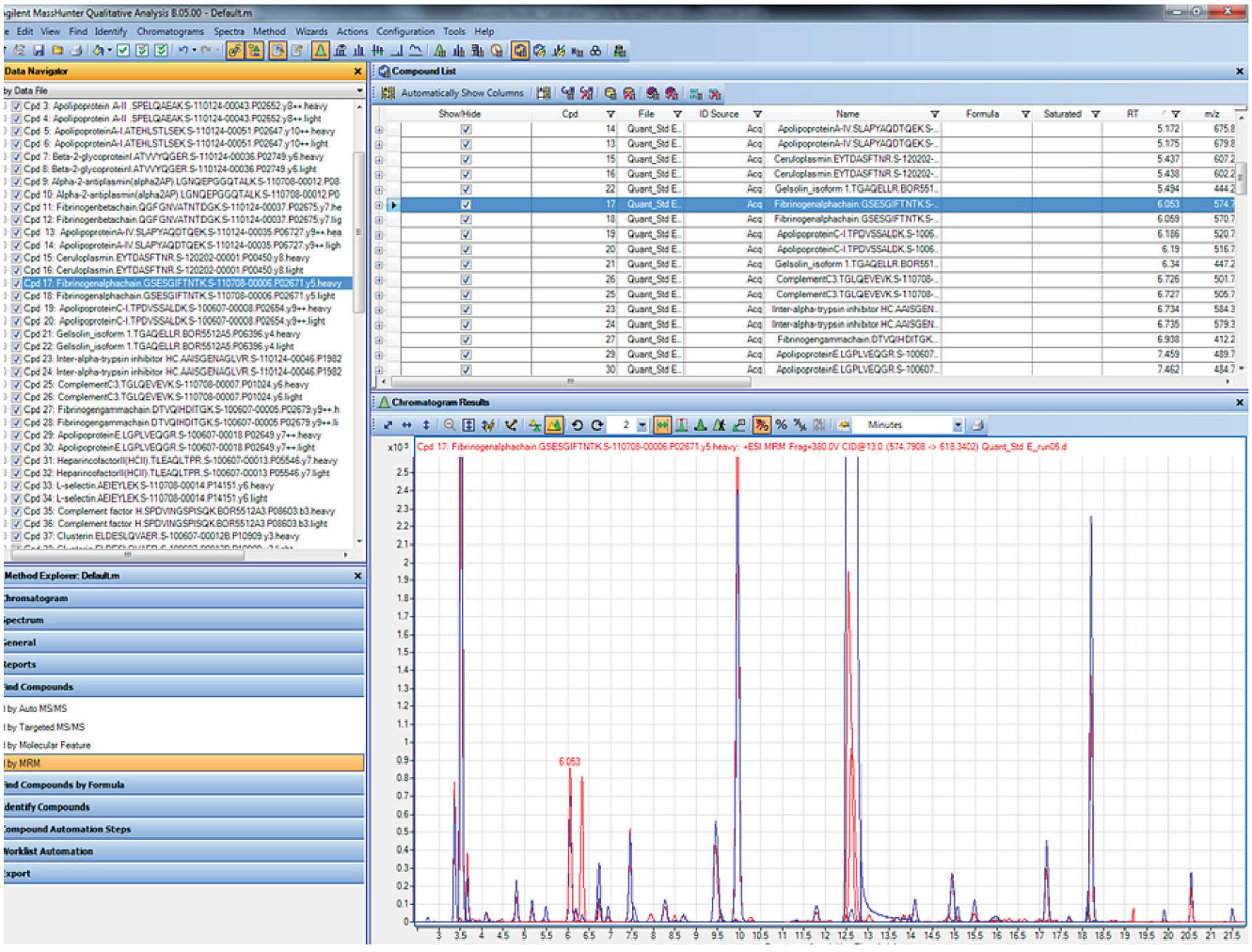

Figure 2. A representative EIC trace of 40 target peptides from the standard kits obtained on Agilent's standard-flow UHPLC/MRM/MS platform. The NAT peptides are displayed in blue, while the SIS peptides are in red.

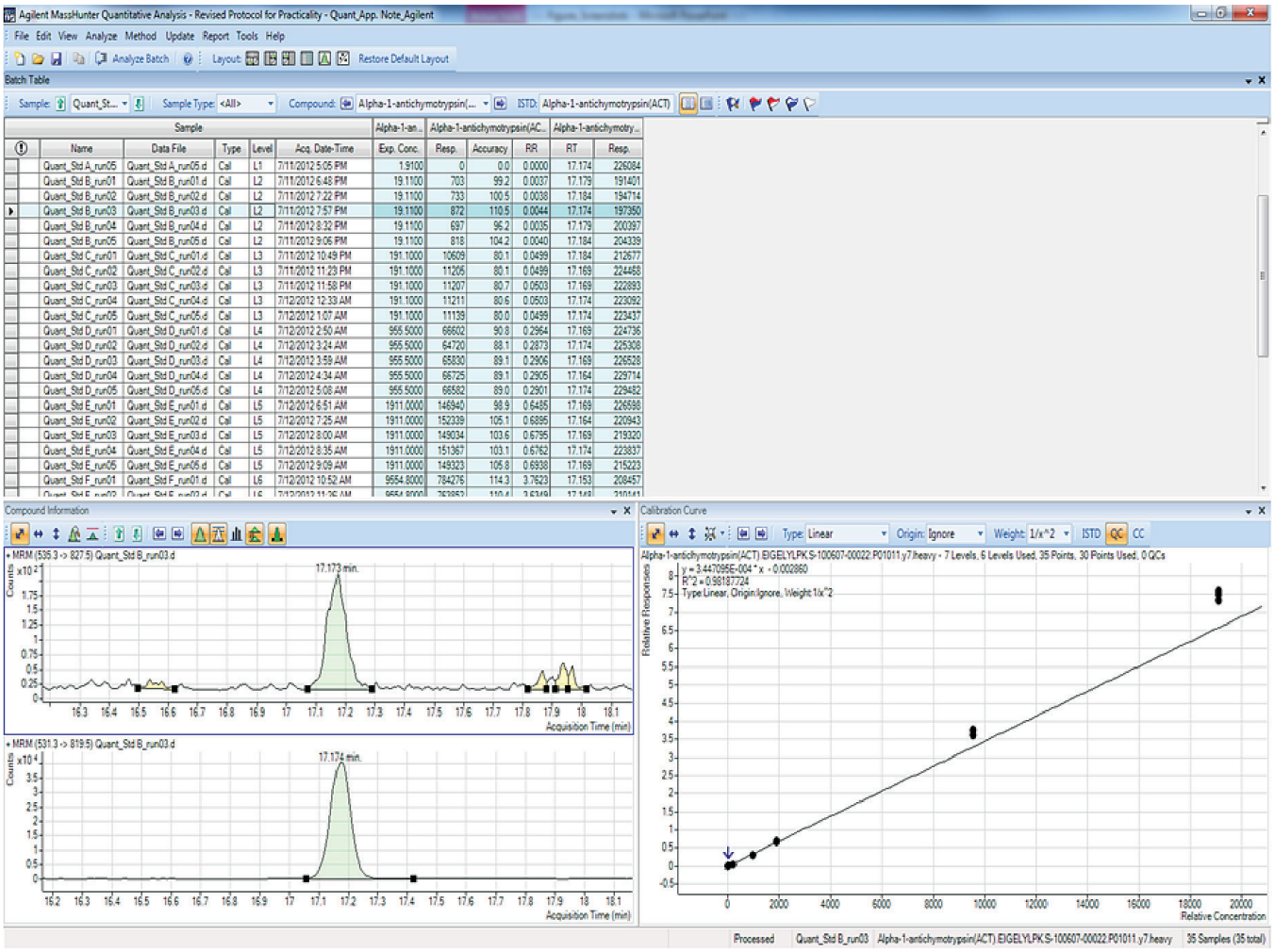

Figure 3. Quantitative performance of the kit on Agilent's standard-flow UHPLC-MRM/MS platform. Shown is a representative calibration curve for peptide EIGELYLPK from α_1-antichymotrypsin (103 dynamic range), with the EICs for the synthetic (SIS) and natural (NAT) peptides at the LLOQ also illustrated.

Deviations from the reference values reflect system or preparation errors that must be corrected before performing the quantitative plasma proteomics experiments of interest. Failure to do so will lead to inaccurate and imprecise quantitative results, which will have serious consequences on the analysis of real samples. Erroneous values can manifest in the absence of a NAT or SIS peptide signal or high retention time variability, and is indicative of a sample preparation or an LC-related issue, respectively. This was observed previously in the intra-laboratory testing of the Workflow Performance kit (Figure 4, adapted from[5]).

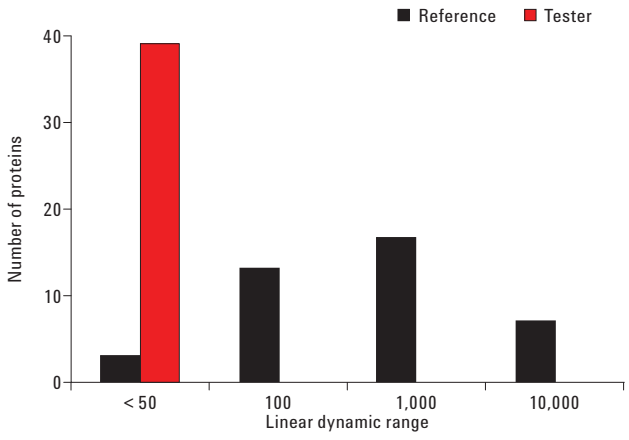

Figure 4. Dynamic range obtained from an intra-laboratory test of the standard kits. The distribution of values for the reference and test are in black and red, respectively.

The narrower dynamic range and higher LLOQ obtained was directly attributed to an error made in preparing the SIS peptide dilutions at the three lowest concentration levels. This was apparent from the absence of the SIS peptide signal at these lower levels and the presence of NAT signal (the normalizer) throughout.

Narrow dynamic ranges and dissimilar LLOQs or plasma protein concentrations reflect a sample preparation or MS-related issue. This was revealed in a separate intra-laboratory study of the Workflow Performance kit[5], where the LLOQ and dynamic range deviated by 3 fold from the reference, while all other metrics correlated appreciably. Further examination revealed the mass spectrometer to be the culprit of the diminished sensitivity. This was corroborated by a Checktune of the signal intensity of the calibrant ions. Alternatively, the Platform Performance kit can be used together with the Workflow Performance kit to verify instrument performance.

Conclusions

This Application Note demonstrates the utility and significance of two standard kits for method and platform standardization in quantitative plasma proteomics on an Agilent standard-flow UHPLC-MRM/MS platform. The quantitative approach uses MRM-based detection with isotopically labeled peptides as internal standards for the targeted quantitation of 40 plasma proteins. Together, the kits help pinpoint mistakes made by the technician and performance deficits related to the equipment that must be corrected prior to performing the quantitative experiments of interest.

References

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4. T.A. Addona, *et al*. "Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma" *Nat. Biotech.* 27, 633-641 (**2009**).

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