



Automated, High Precision Tryptic Digestion and SISCAPA-MS Quantification of Human Plasma Proteins Using the Agilent Bravo Automated Liquid Handling Platform

Application Note

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Abstract

An “addition only” tryptic protein-digestion protocol, followed by multiplexed SISCAPA peptide enrichment, was developed and automated in 96-well format on the Agilent Bravo Automated Liquid Handling Platform. These combined protocols, taking as little as 4 hours total, were coupled with high throughput mass spectrometry for quantitation of plasma proteins over a wide range of abundance. The complete workflow was demonstrated for suitability of routine quantitation of plasma protein biomarkers based upon sensitivity, multiplexing capability, scalability, reproducibility, and throughput.



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Introduction

Quantitation of proteotypic surrogate peptides in relation to internal standards allows quantitative, multiplexed measurement of proteins using methods derived from classical isotope-dilution mass spectrometry (MS) of small molecules¹. Triple quadrupole mass spectrometer (TQMS) instruments have measured light:heavy peptide ratios with CVs of less than 2 %². Since the precision of MS measurements depends on the variability of interfering ion signals (matrix and chromatography effects) as well as the intensity of peptide ion signals, achieving low CVs for low abundance peptides is not trivial and adds to the need for reproducible digestion. Quantitative

reproducibility of tryptic digestion is essential to support the evolution of mass spectrometric assays for identification and quantitation of peptides, and ultimate measurement of proteins as clinically relevant biomarkers.

This Application Note presents the development of a highly reproducible, automated "addition only" method for tryptic digestion of plasma, followed by the previously-described bead-based SISCAPA enrichment³ of target peptides and quantitation using TQMS. This method offers precision, simplicity of multiplexing, generality, and is used to improve sensitivity and increase the dynamic range and throughput of peptide MS assays. The combined,

automated workflow, together with an optimized liquid chromatography procedure, facilitates multiplexed peptide quantitation with a 3 minute LC/MS sample cycle time, significantly increasing the throughput of protein quantitation.

Materials and Methods

Hardware

An addition-only method was developed to enable facile, efficient, and highly reproducible tryptic digestion of plasma samples followed by SISCAPA enrichment of target peptides, using an Agilent Bravo Automated Liquid Handling Platform (Figure 1).

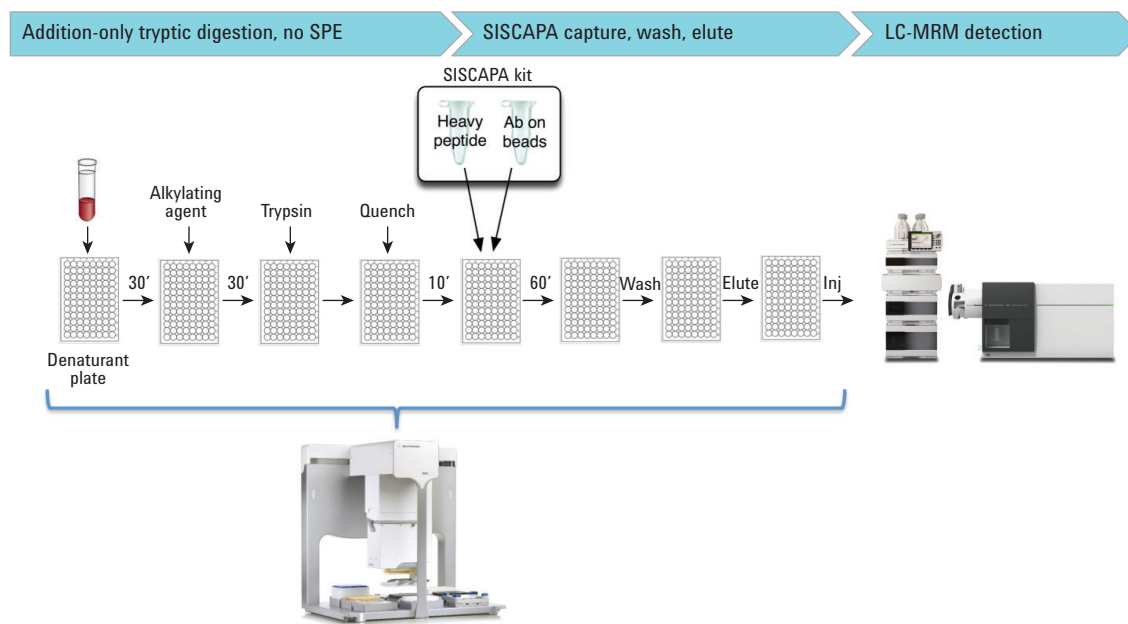


Figure 1. Protocol Overview: Trypsin digestion, SISCAPA enrichment, LC/MS quantitation.

All steps in the workflow protocol were carried out using the Bravo Automated Liquid Handling Platform with 96-channel LT disposable tip head and one VarioMag Teleshake plate shaker (Figure 2). The steps were programmed for execution using VWorks software.

The workflow requires use of three distinct Bravo Automated Liquid Handling Platform Layouts as shown in Figure 3.

Trypsin digestion (Figure 3A)

A 10 μL solution of pooled human plasma was added to each well of a 96-well plate containing a dried denaturation mix, followed by a 30-minute incubation at room temperature with vigorous mixing. The alkylation and diluting agents were then added to the sample plate, followed by the addition of trypsin at an enzyme:protein ratio of 1:10. The digest plate was sealed and manually moved to an incubator at 37 $^{\circ}\text{C}$ for tryptic digestion of 1 to 5 hours.

| Agilent Bravo Automated Liquid Handling Platform deck configuration | | |
|---|-------------------|-------------------------|
| 1 | 2 | 3 |
| Alignment station | Alignment station | Orbital shaking station |
| 4 | 5 | 6 |
| Alignment station | Alignment station | Alignment station |
| 7 | 8 | 9 |
| Alignment station | Alignment station | Alignment station |

Figure 2. Agilent Bravo Automated Liquid Handling Platform deck configuration for all methods.

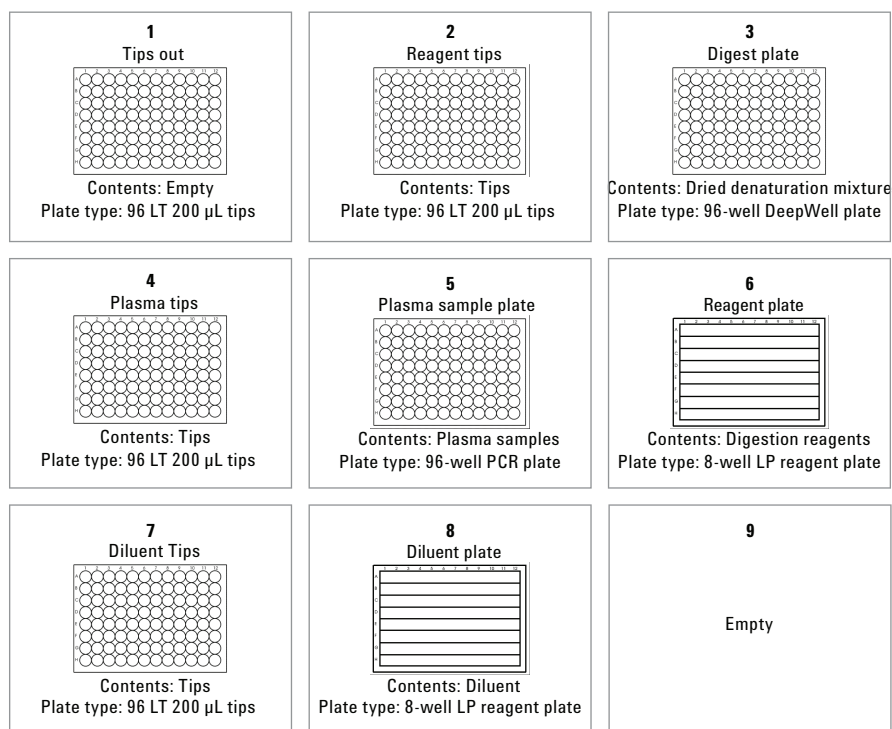


Figure 3A. Agilent Bravo Automated Liquid Handling Platform deck layout: trypsin digestion.

Stopping digestion, SIS addition, SISCAPA target peptide enrichment (Figure 3B)

During tryptic-digest incubation, the deck was rearranged as shown in Figure 3B. The tryptic cleavage reaction was stopped by adding a stopping agent. Each sample was spiked with 500 fmol each of SIS peptides and 0.5–1.0 µg each of corresponding antipeptide antibodies conjugated to magnetic beads. The sample plate containing the antibody-bead complex was vigorously shaken for 1 hour at 1,000 RPM to capture the peptides.

Bead wash and elution (Figure 3C)

The Bravo Automated Liquid Handling Platform deck was set up a third time, (Figure 3C). Three washes were performed to remove unbound matrix, with a custom magnet array designed by SISCAPA Assay Technologies (Figure 4) used to pull the magnetic beads to the sides of the wells for the unbound peptide digest to be removed. The enriched peptides were released from the beads using acid elution.

Liquid chromatography tandem mass spectrometry (LC/MS/MS)

Analysis was carried out using an Agilent 1290 Infinity ultra high performance liquid chromatograph and an Agilent 6490 TQMS (Agilent Technologies, CA). Agilent MassHunter Workstation Software was used for both data acquisition and analysis: MassHunter LC/MS Data Acquisition for Agilent 6400 Series Triple Quadrupole LC/MS v. B.06.00 was used for data acquisition while MassHunter Quantitative Analysis v. B.05.02 and MassHunter Qualitative Analysis v. B.06.00 were used for data analysis.

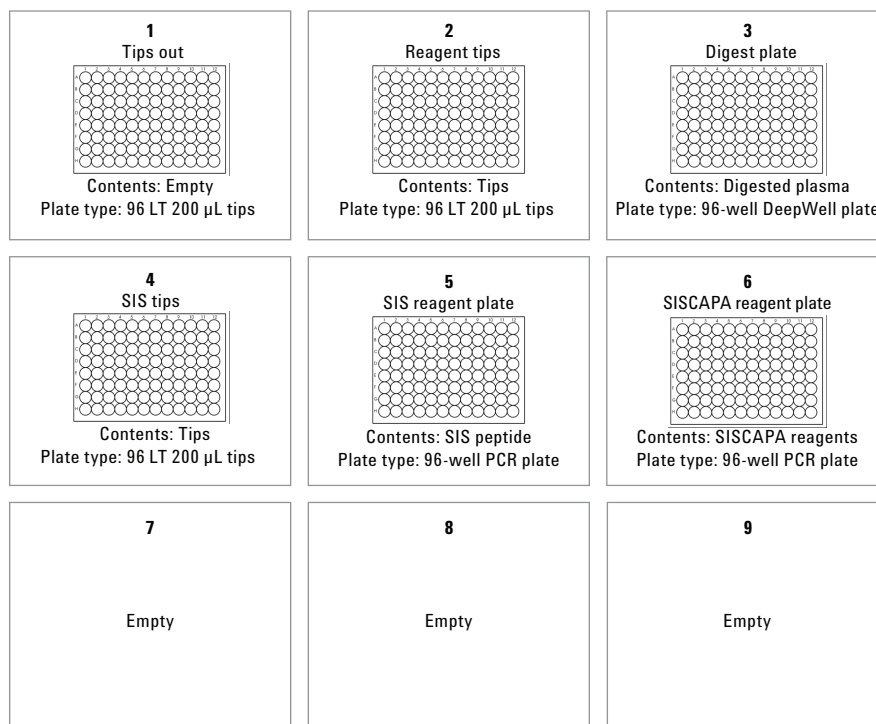


Figure 3B. Agilent Bravo Automated Liquid Handling Platform deck layout: stopping digestion, SIS addition, target peptide enrichment.

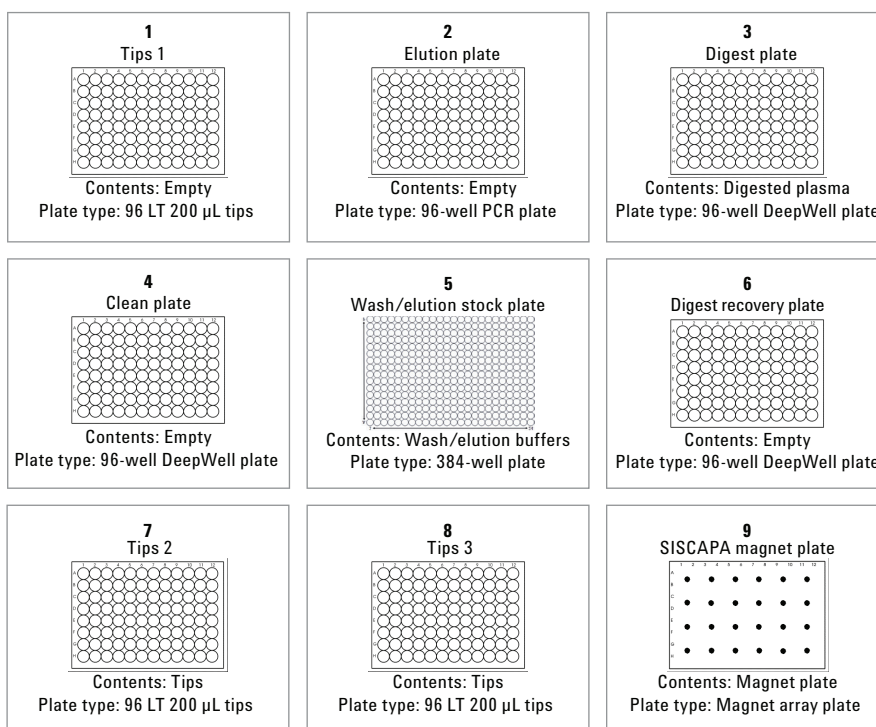


Figure 3C. Agilent Bravo Automated Liquid Handling Platform deck layout: bead wash and elution.

Results and Discussion

Sensitivity

The Limit of Detection (LOD) was calculated as the amount of the SIS peptide below which the CV was consistently greater than 50 % and Limit of Quantitation (LOQ) as the amount of the SIS peptide below which the CV was consistently greater than 20 %. The performance of four example peptide assays is summarized in Table 1.

Comparison to a reference method

A 5-level calibrator set for Soluble Transferrin Receptor (sTfR) was analyzed using the automated protocol and the results were compared to the results of the reference method provided with the calibrator set. As shown in Figure 5, a strong correlation ($R^2 = 0.998$) was observed between the SISCAPA measurement of sTfR and its measurement by this reference method. The linear regression model had a slope of 0.1963 ± 0.0003 and a y-intercept of 0.4039 ± 0.0082 at a 95 % confidence interval.



Figure 4. SISCAPA custom magnet array used for washing of magnetic beads.

Table 1. Agilent Bravo Automated Liquid Handling Platform SISCAPA assay performance data.

| | Mesothelin | Soluble transferrin receptor | Protein C inhibitor | LPS binding protein |
|--------------------------------------|-------------------|------------------------------|---------------------|---------------------|
| Measured in 10 μ L plasma (fmol) | 9.98 (\pm 0.7) | 97.8 (\pm 3.7) | 755 (\pm 105) | 392 (\pm 36) |
| LOD (fmol) | 0.99 (\pm 0.1) | 0.72 (\pm 0.07) | 1.5 (\pm 0.03) | 0.89 (\pm 0.15) |
| LOQ (fmol) | 5.11 (\pm 1.6) | 0.72 (\pm 0.07) | 1.5 (\pm 0.03) | 0.89 (\pm 0.15) |

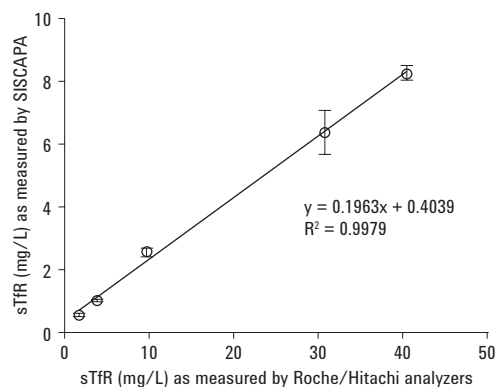


Figure 5. Comparison of sTfR measurements using robotic SISCAPA and a Roche/Hitachi analyzer.

Scalability of digestion and linearity of peptide responses

Linearity of peptide recovery is shown as a function of plasma volume for four targets (Figure 6) measured by mixing equal volumes of pooled human plasma (known to contain the four proteins under study) with chicken plasma (deficient in the target peptides). In all cases, linear responses ($R^2 > 0.999$) were observed

across four technical replicates. Assay performance is shown using forward and reverse curves (bottom panel).

Forward curves were generated by titrating synthetic light peptide from 10,000 fmol to 0 fmol while the corresponding SIS peptide was kept constant at 500 fmol. For SIS-varying reverse curves the SIS peptide was titrated from 10,000 fmol to 56 amol while

maintaining constant light peptide by spiking 500 fmol in all 12 samples. The curves were independently generated and processed using 10 μL /point of pooled human plasma in triplicate. Endogenous levels of the analytes (plateaued part of the forward curve) can be measured, along with limits of detection (LOD) and limits of quantitation (LOQ).

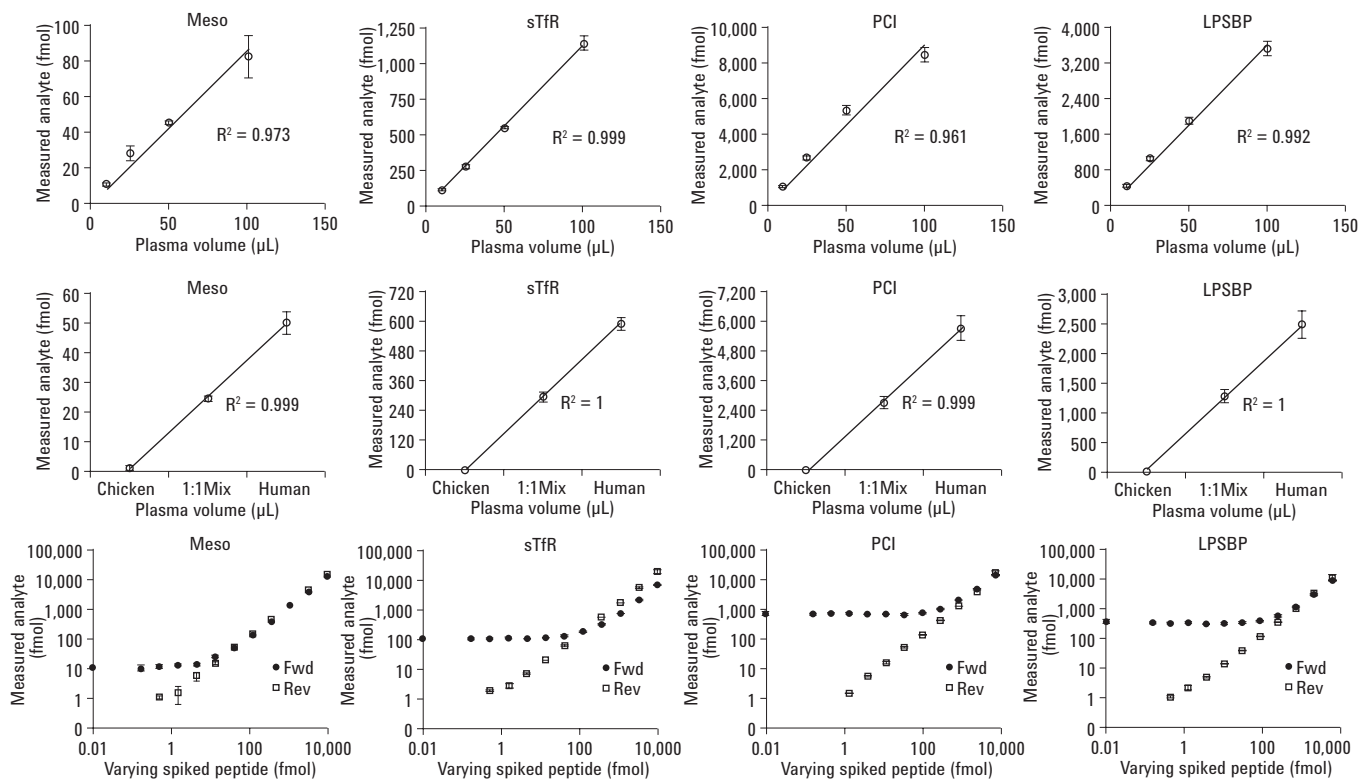


Figure 6. Scalability of digestion and linearity of peptide responses.

Reproducibility of trypsin digestion and SISCAPA enrichment

To evaluate the precision of the automated SISCAPA protocol, including the digestion and peptide enrichment steps, three sets of 12 samples (N = 36) were digested and processed independently on 3 days. Coefficients of variation for the total workflow (digestion + SISCAPA peptide enrichment + mass spectrometric analysis) were consistently less than 5 %, and as low as 2.8 % for proteotypic peptides (Table 2). The coefficients of variation attributable to the tryptic digestion step were estimated from 0 % to 2.2 % for given targets, with the limiting factor in SISCAPA assay precision being MS performance rather than sample preparation.

Throughput

This workflow, implemented on the Bravo Automated Liquid Handling Platform, facilitates digestion and enrichment of 96 samples in parallel in as little as 4 hours. The near-purity of analyte peptides enriched by SISCAPA antibody capture means the subsequent LC/MS quantitation is reduced to a total of 3 minutes per sample. Indeed, it has been demonstrated elsewhere that SISCAPA-enriched peptides can be quantitated using RapidFire MS for a cycle time of approximately 10 seconds per multiplexed sample. The combined throughput of this automated process is appropriate for industrial applications including biomarker verification and quantitative analysis.

Table 2. Precision of Agilent Bravo Automated Liquid Handling Platform digestion and SISCAPA workflow. Addition-only protocol on Agilent Bravo Automated Liquid Handling Platform – 10 µL plasma samples in 96-well plates

| | | %CV (n = 12) | | |
|-----------------------|-------|--------------|-----------------------|---------------------|
| | | Mesothelin | sTransferrin receptor | LPS binding protein |
| MS | Day 1 | 5.10 | 4.00 | 2.00 |
| MS | Day 2 | 5.40 | 2.80 | 2.00 |
| MS | Day 3 | 6.70 | 1.80 | 2.60 |
| SISCAPA + MS | Day 1 | 7.00 | 3.10 | 2.10 |
| SISCAPA + MS | Day 2 | 5.10 | 2.20 | 2.60 |
| SISCAPA + MS | Day 3 | 5.90 | 2.80 | 2.50 |
| Digest + SISCAPA + MS | Day 1 | 6.10 | 2.60 | 4.70 |
| Digest + SISCAPA + MS | Day 2 | 4.60 | 3.30 | 5.10 |
| Digest + SISCAPA + MS | Day 3 | 6.30 | 3.40 | 4.70 |

References

1. Rittenberg, D., Foster, G.L., A new procedure for quantitative analysis by isotope dilution with application to the determination of amino acids and fatty acids. *J. Biol. Chem.*, **1940**, 133, 737-774.
2. Anderson, *et al.* Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). *J. Proteome Res.*, **2004**, 3, 235-244.
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