

# High Sensitivity SISCAPA-based Peptide Quantitation Using UHPLC and the 6490 QQQ with iFunnel Technology

## Application Note

Clinical Research

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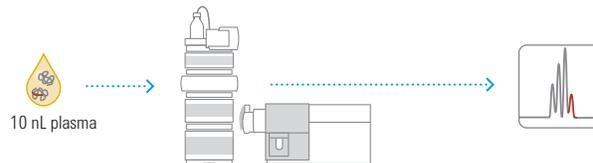
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### Introduction

Assays that are both specific and quantitative for target proteins and peptides are critical for biomarker research, particularly those at low concentrations in plasma, where absolute instrument sensitivity has been a key limitation.

Quantitation of proteotypic peptides by multiple reaction monitoring (MRM) MS allows specific, internally-standardized measurement of protein biomarkers in digests of plasma, and can achieve sub-ng/mL detection levels when specific anti-peptide antibodies are used to enrich target peptides from the digest (SISCAPA® technology). The Stable Isotope-Labeled Standards with Capture on Anti-Peptide Antibodies (SISCAPA) procedure enhances sensitivity by enriching a target peptide from an arbitrarily large amount of sample digest, as well as drastically reducing the complexity of the resulting sample and thus potentially enabling faster LC/MS analysis (Figure 1).

#### DIRECT INJECTION OF UNFRACTIONATED PLASMA DIGEST



#### SISCAPA ENRICHMENT OF TARGETED PEPTIDES

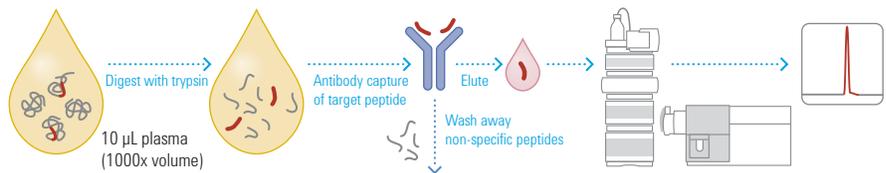


Figure 1. Overview of the SISCAPA protocol for the specific capture of target peptides by purpose-designed anti-peptide antibodies that yield a >100,000-fold enrichment.

Up to this point, the need to measure candidate biomarkers at low absolute abundance (often sub-fmol) in complex matrices such as plasma has focused attention on nanoflow chromatography with nanospray electrospray ionization (ESI) as the preferred analytical platform, despite its limited robustness in high-throughput environments such as clinical research laboratories. Here we evaluate the sensitivity and throughput that can be achieved using more robust standard flow chromatography interfaced with the Agilent 6490 Triple Quadrupole MS with iFunnel technology to analyze peptides captured by a multiplex SISCAPA panel incorporating 11 rabbit monoclonal antibodies to specific peptides from 10 proteins spanning a wide concentration range in plasma.

In this work, LC/MS analysis was performed using an Agilent 1290 Infinity LC system with a more conventional and robust standard-flow (2.1 mm column at 600  $\mu$ L/min) LC method. The enhanced sensitivity of the Agilent 6490 Triple Quadrupole with iFunnel technology yielded sensitivity at this standard flow rate that is comparable to nanoflow LC-MRM for SISCAPA assays. The improved sensitivity of the Agilent 6490 is achieved by enhancing the sampling and transmission of ions in the mass spectrometer. This improved ion transfer is based on an Agilent Jet Stream (AJS) source (ESI with thermal gradient focusing) coupled to ion optics that include a hexabore ion sampling capillary and a dual stage ion funnel for handling the increased gas load. Use of normal flow chromatography also allowed reduction of the analytical run time from ~30 min (nanoflow) to 5 min (normal flow), providing 6-fold higher throughput.

## Experimental

### SISCAPA

Proteotypic tryptic peptides (initially five peptides per protein) were selected representing a series of known protein biomarkers: protein C inhibitor, LPS binding protein, transferrin receptor, osteopontin, ferritin light chain, mesothelin, alpha-fetoprotein, HER2/neu, CA-125, and thyroglobulin. Proteotypic peptides were also selected for thyroglobulin and included those reported by Hoofnagle<sup>1</sup>. Each peptide was synthesized with an added N-terminal cysteine and coupled to the carrier protein KLH. Pools of five such immunogens were injected into two rabbits and titers were followed with a peptide ELISA. Affinity-purified polyclonal antibodies against the two peptides for each protein showing highest titers were prepared and characterized in SISCAPA assays, after which rabbit monoclonal antibodies (RabMAbs) were prepared (Epitomics, Inc.) against the best performing peptide for each target,

except for thyroglobulin, for which RabMAbs were made against two peptides. RabMAbs were selected for high-affinity binding to peptides in solution by surface plasmon resonance analysis, cloned, and expressed. Each RabMAb was independently covalently immobilized on magnetic beads and a pool of all 11 reagents was prepared.

### Automation of SISCAPA

An automated SISCAPA protocol was carried out on 96-well plates of plasma digest samples using an Agilent Bravo robot<sup>2</sup>. The pool of 11 RabMAbs on magnetic beads (1  $\mu$ g of each RabMAb per capture reaction) was used to capture the 11 corresponding stable, isotope-labeled internal standards (with <sup>13</sup>C on Arg or Lys) and cognate endogenous peptides from 10  $\mu$ L samples of plasma digest matrix spiked with varying amounts of labeled or unlabeled target peptide. After extensive washing of the antibody-coated beads, bound peptides were eluted in 20  $\mu$ L of 5 % acetic acid for LC-MRM analysis.

Abbreviation	Protein Name	Protein Accession Number	Target Peptide
PCI	PAI3	Q9UG30	EDQYHYLLDR
OPN	Osteopontin	Q15683	YPDAVATWLNPDPSQK
TfR	Transferrin receptor	P02786	GFVEPDHYVVVGAQR
FLC	Ferritin light chain	P02792	LGPEAGLGEYLFER
AFP	Alpha-fetoprotein	P02771	GYQELLEK
Her-2	Her-2	P04626	AVTSANIQEFAGCK
CA125	Mucin-16 (Ovarian carcinoma antigen)	Q8WXI7	ELGPYTLDR
Tg1	Thyroglobulin	P01266	FSPDDSAGASALLR
Tg2	Thyroglobulin	P01266	VIFDANAPVAVR
LPSBP	LPS binding protein	P18428	LAEGFPLPLLK
Meso	Mesothelin	Q13421	LLGPHVEGLK

Table 1. Proteins and targeted proteotypic peptides for the 11-plex SISCAPA assay.

## LC/MS Analysis

Parameters for each of the 11 target peptides and cognate labeled standards were optimized, permitting use of retention-time scheduled MRM data collection. All the peptides yielded narrow, well-shaped chromatographic peaks at distinct elution times, and displayed a wide range of ionization efficiencies (Figure 2).

Using twelve-point dilution curves of both labeled internal standard peptides (reverse curves) and of unlabeled synthetic peptide (standard addition curves), the response was characterized for these 11 assays in pooled human plasma digest matrix.

Three separate peptide fragments (transitions) were measured for each peptide and its labeled cognate (66 MRM channels).

### LC Conditions

<b>Column</b>	Eclipse Plus EC-C18, 1.8 $\mu$ m, 2.1 x 50 mm
<b>Column temperature</b>	35 $^{\circ}$ C
<b>Flow rate</b>	0.6 mL/min
<b>Mobile phase A</b>	0.1 % formic acid in water
<b>Mobile phase B</b>	0.1 % formic acid in 90 % acetonitrile in water
<b>Gradient</b>	10 to 14 % B in 0.01 min, then 16 % B at 2 min, 22 % B at 3 min, 40 % B at 3.7 min, 70 % B at 3.8 min, and 10 % B and 0.8 mL/min at 3.9 min.
<b>Stop time</b>	5 min
<b>Injection volume</b>	20 $\mu$ L

### MS Conditions

<b>Drying gas</b>	15 L/min, 150 $^{\circ}$ C
<b>Sheath gas</b>	11 L/min, 200 $^{\circ}$ C
<b>Nebulizer</b>	30 psig
<b>Nozzle</b>	300 V
<b>Cell Acc</b>	4 V
<b>Collision energy</b>	Optimized for each transition
<b>Cycle time</b>	500 ms
<b>Delta EMV</b>	200 V

## Results and Discussion

The data clearly demonstrate that a majority of the analytes can be measured in the digest of 10  $\mu$ L plasma using the optimized standard-flow approach, providing lower limits of quantitation comparable to those observed previously using nanoflow approaches. At the same time, the use of a 5 min analytical cycle time provided approximately 6-fold higher sample throughput than nanoflow methods (Figure 2).

With the increased sensitivity from thermal gradient ion focusing electrospray ionization (AJS source) and increased ion sampling with the hexabore capillary and dual ion funnel gain, standard flow LC becomes a workable, sensitive alternative to nanoflow LC-MRM for SISCAPA assays. This method uses a 2.1 x 50 mm column which is sufficient for the separation of the 11 peptides in this multiplexed assay. The 2.1 mm column also provides superior loading and peak capacity compared to nanoflow chromatography, resulting in excellent separation and retention time reproducibility.

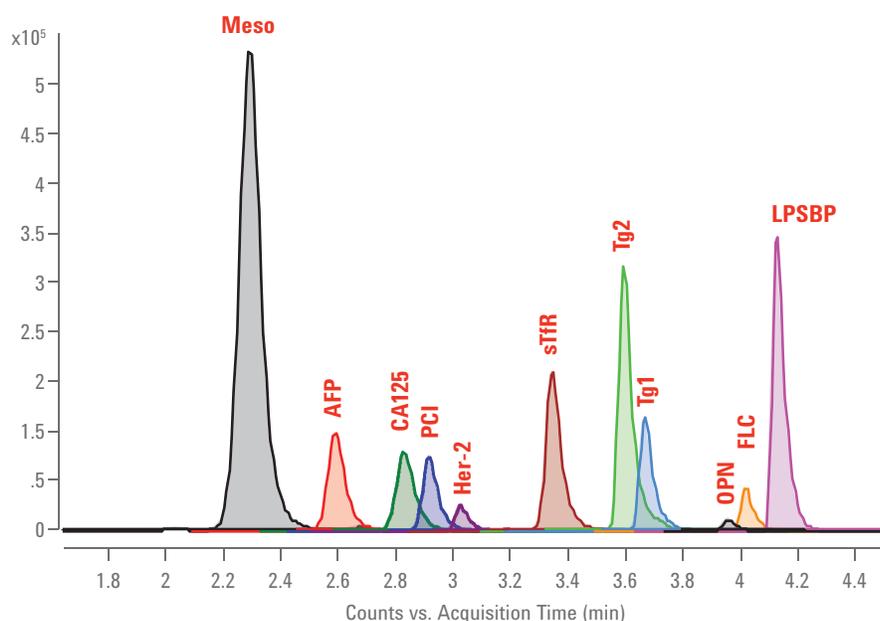


Figure 2. Separation of the 11 target peptides using a rapid LC/MS method.

Figure 3 shows the standard curves for mesothelin obtained by adding varying amounts of either the heavy internal standard peptide or a synthetic version of the endogenous (unlabeled, light) peptide.

In the forward curve (red), varying amounts of light synthetic target peptide have been added to the digest, along with a constant amount of heavy peptide. In this case, the level of the endogenous peptide present (5 fmol per 10  $\mu$ L digest, equivalent to 18 ng/mL protein) was measured with a replicate injection average CV of 2 % (within-run). In the reverse curve (blue), varying amounts of heavy internal standard peptide (11 three-fold dilutions) have been added to the digest, yielding a curve which decreases downward to a plateau at the lower limit of detection. This is in the neighborhood of 50 amol/10  $\mu$ L digest (equivalent to 200 pg/mL protein) and was measured with an average CV of 8 %.

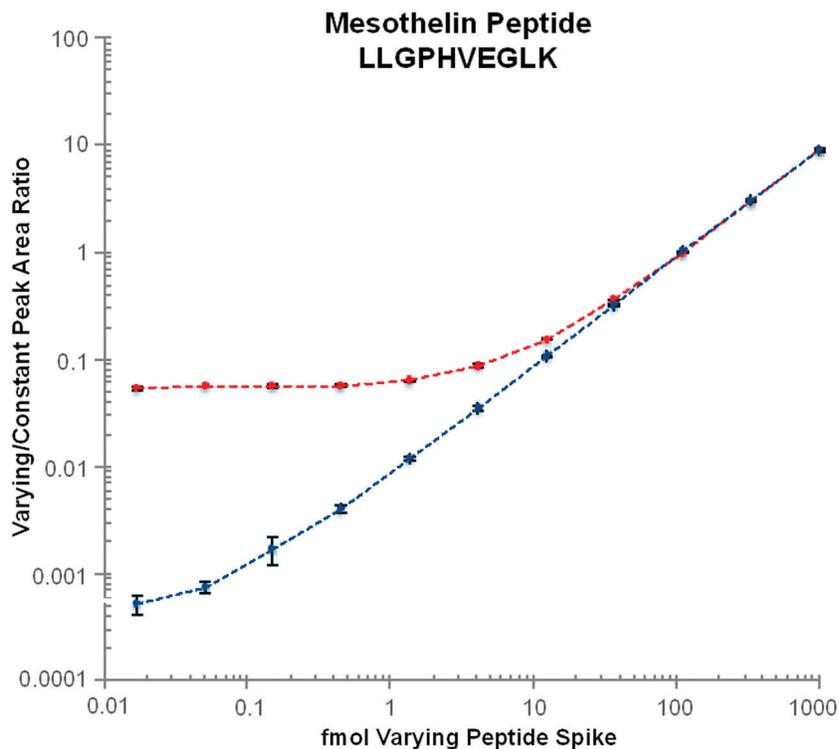


Figure 3. Standard curves showing quantitative MS results (ratio of light and heavy peptide MRM peak areas) for a mesothelin peptide captured by a RabMab from a tryptic digest of 10  $\mu$ L of human plasma.

## Conclusions

This work demonstrates the use of an integrated, automated, high-throughput MS-based protein assay platform. The results attained with standard flow LC/MS using iFunnel technology on an Agilent 6490 Triple Quadrupole MS were equivalent to those previously achieved with nanoflow LC/MS, but with greater robustness and much higher sample throughput.

## References

1. Hoofnagle *et al.* Quantification of Thyroglobulin, a Low-Abundance Serum Protein, by Immunoaffinity Peptide Enrichment and Tandem Mass Spectrometry. *Clinical Chemistry*, **2008**, 54(11):1796-1804.
2. Anderson, L. *et al.* Automation of a SISCAPA Magnetic Bead Workflow for Protein Biomarker Quantitation by Mass Spectrometry Using the Agilent Bravo Automated Liquid Handling Platform. *Agilent Application Note*, **2011**, publication number 5990-7360EN.

[www.agilent.com/chem/QQQ](http://www.agilent.com/chem/QQQ)

SISCAPA technology (U.S. patent 7,632,686) is developed and commercialized by SISCAPA Assay Technologies, Inc. ([www.SISCAPA.com](http://www.SISCAPA.com)). SISCAPA kits and antibodies are commercialized by Epitomics, Inc. SISCAPA® is a registered trademark of Anderson Forschung Group.

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