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Automation of a SISCAPA Magnetic Bead Workflow for Protein Biomarker Quantitation by Mass Spectrometry

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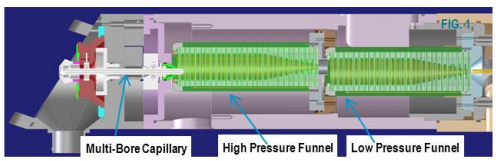
Introduction

Quantitation of proteotypic peptides by SRM-MS allows specific, internally-standardized measurement of protein biomarkers in digests of plasma and can achieve sub-nanogram/mL detection levels when specific anti-peptide antibodies are used to enrich target peptides from the digest (SISCAPA). We have developed an automated protocol for implementing this immunoaffinity enrichment of biomarker peptides. The effectiveness of the protocol was evaluated using a multiplex SISCAPA panel based on 11 rabbit monoclonal antibodies to specific peptides from 10 proteins spanning a wide plasma concentration range. Results will be presented for the reproducibility and sensitivity of rapid LC/MS analysis performed on a dual ion-funnel-equipped triple quadrupole instrument using both standard flow and nanoflow LC approaches.

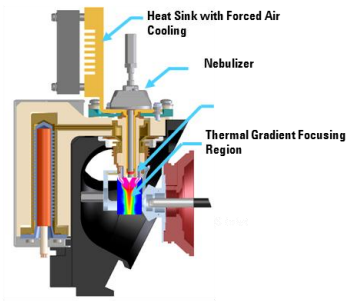
Ion Funnel Technology

The Agilent 6490 QQQ incorporates iFunnel technology which is a combination of three fundamental innovations:

- Agilent Jet Stream technology – ESI with thermal gradient ion focusing confinement
- Hexabore sampling capillary with 6 independent parallel bores to enable sampling a much larger fraction of the ions
- Dual-stage ion funnel for efficient removal of large gas volumes and ion transfer to Q1 optics



- Agilent JetStream is ESI with thermal gradient ion focusing confinement
- The standard heat sink was modified to provide additional shielding of the nebulizer to accommodate low flow rates

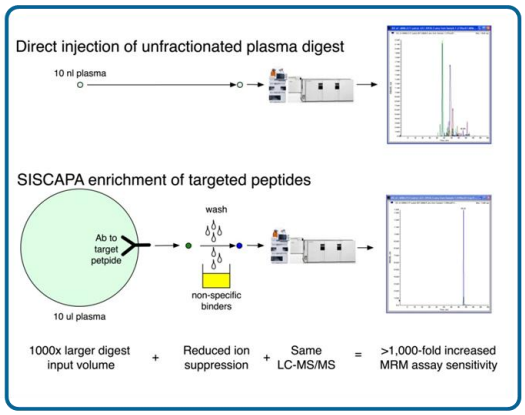


SISCAPA Methodology

Proteotypic tryptic peptides (initially 5 peptides per protein) were selected representing known protein biomarkers: PAI3 (protein C inhibitor), LPS binding protein, transferrin receptor, osteopontin, ferritin light chain, mesothelin, alpha-fetoprotein, HER2/neu, CA-125 and thyroglobulin. Proteotypic peptides for thyroglobulin included those reported by Hoofnagle.

Each peptide was synthesized with an added N-terminal cysteine and coupled to KLH. Pools of five such immunogens were injected into two rabbits, whose titers to each peptide were followed by a "peptide ELISA". Affinity-purified polyclonal antibodies against the two peptides for each protein showing highest titers were characterized in SISCAPA assays, after which rabbit monoclonal antibodies (RabMAbs) were prepared (Epitomics, Inc.) against the best performing peptide for each target, except for Tg, for which mAb's were made against two peptides. RAbMAbs were selected for high affinity binding to peptides in solution by surface plasmon resonance analysis.

Each RAbMAb was independently covalently immobilized on magnetic beads and a pool of all 11 RabMAb reagents was used in an automated procedure to capture the 11 corresponding stable isotope labeled internal standards and cognate endogenous peptides from plasma digest matrix, after which bound peptides were eluted for LC-SRM analysis.

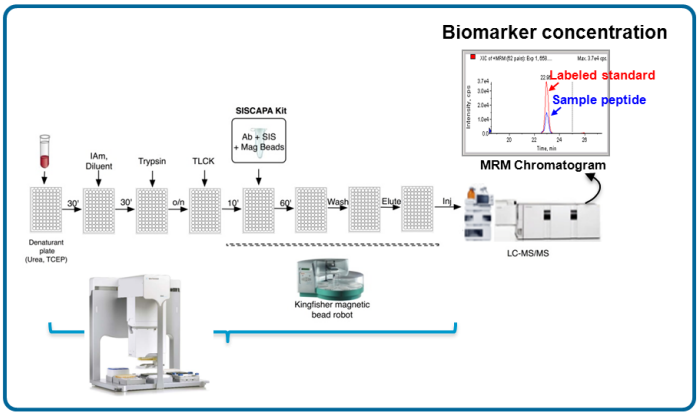


Enrich target peptides and decrease sample complexity

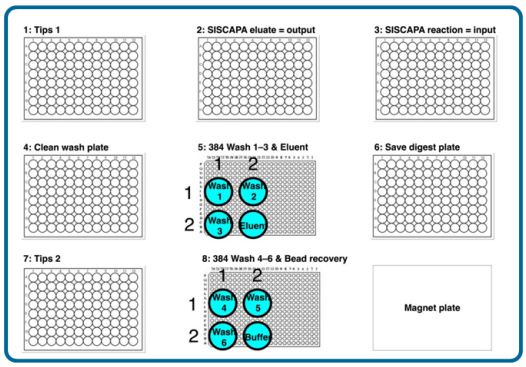
The near-purity of these analyte peptides, in contrast to the enormous matrix background of unfractionated plasma digests and their generally low absolute abundance (often sub-fmol) has to date focused attention on nanoflow chromatography and nanospray ESI as the preferred analytical platform despite its limited robustness for high-throughput applications in clinical laboratories.

Automation Workflow

Magnetic Bead Implementation of SISCAPA Assay Technology



SISCAPA Capture/Elution Implemented on Bravo



The multiplexed capture reaction setup, bead wash and analyte elution steps for a SISCAPA assay have been automated on a Bravo Automated Liquid Handling Platform (Agilent) to allow processing of 96 samples in less than 30 minutes. The eluted peptides were delivered in a volume (20 µl) and solvent (5% acetic acid) suitable for injection into a reversed-phase LC system.

LC/MS Method Development

With the increased sensitivity from thermal gradient ion focusing electrospray ionization and increased ion sampling with the hexabore capillary and dual ion funnel gain, standard flow LC becomes a workable, sensitive alternative to nanoflow LC-SRM for SISCAPA assays. For this work, optimized chromatography and MS conditions was developed for standard flow LC using 2.1 mm i.d. columns.

- Parameters for each of the 11 target peptides and cognate labeled standards were optimized, permitting use of retention-time scheduled SRM data collection.
- The 2.1 mm column provides superior loading and peak capacity compared to nanoflow which results in excellent separation and retention time reproducibility.
- The initial method had a 20 min runtime (figure A top right) and all peptides yielded narrow, well-shaped peaks at distinct elution times.
- In general, the retention time for all analytes showed less than 0.2% RSD across the concentration range for the standard curves (both forward and reverse curves).
- A faster chromatographic method has been developed using a 5 minute runtime (Figure B, lower right). This method uses a 2.1 x 50 mm column which is sufficient for the separation of this multiplexed assay.
- Column temperature was found to be an important parameter in the optimization of the shorter method.

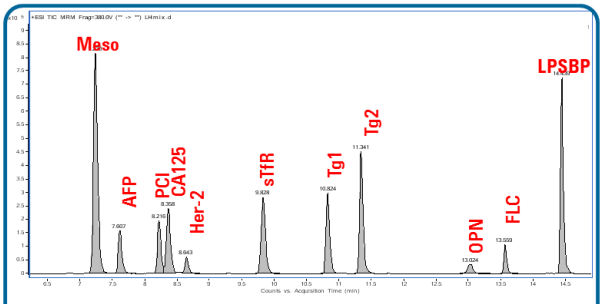


Figure A: Initial 20 min method.

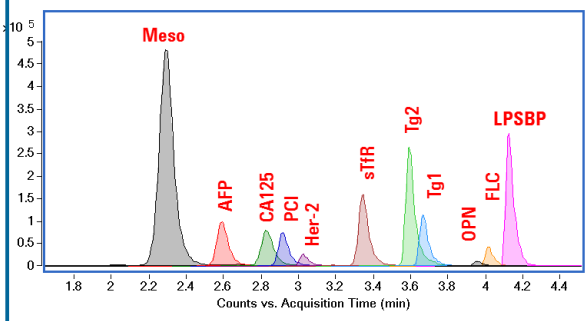
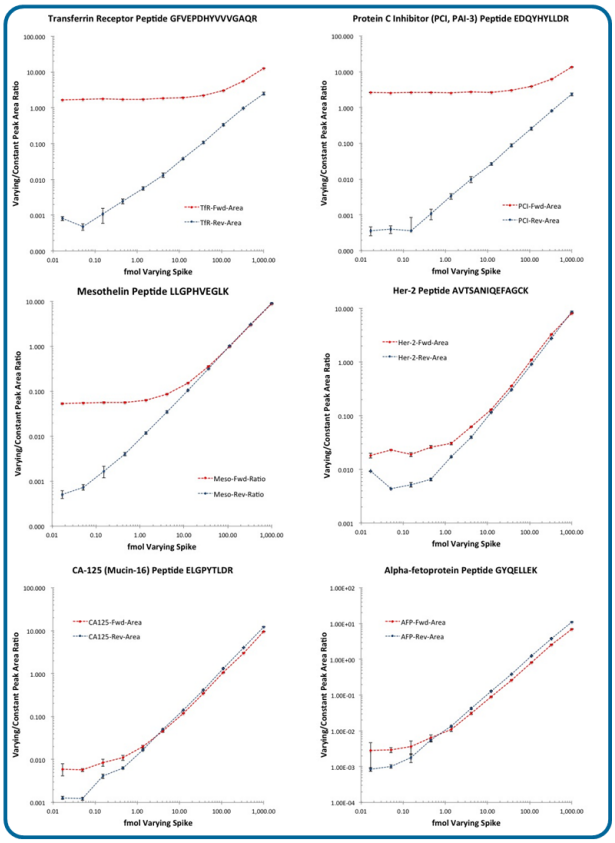


Figure B: Optimized 5 min method.

Results and Discussion



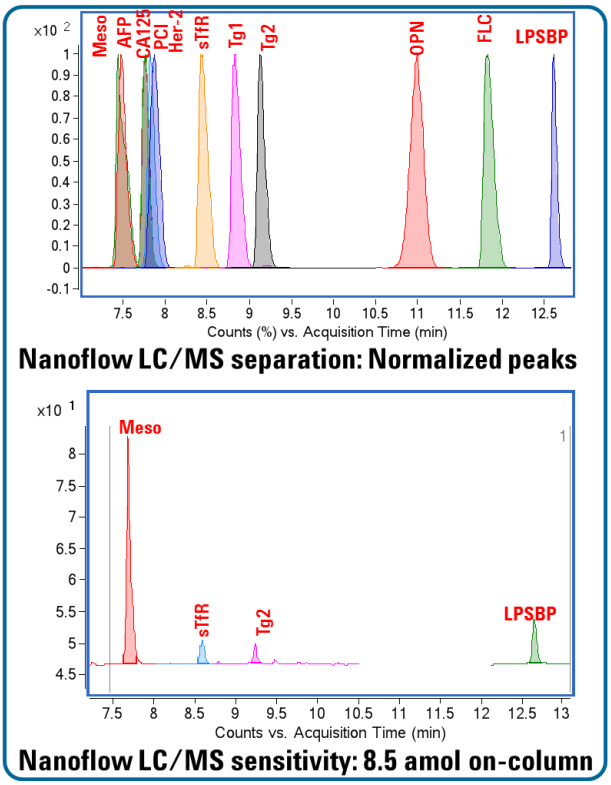
Forward and reverse curves for 6 of the peptides

Using twelve-point dilution curves of both labeled internal standard peptides (i.e., reverse curves) and of unlabeled synthetic peptide (i.e., standard addition curves), we have characterized the response of these 11 assays in pooled human plasma digest matrix. The data clearly demonstrate that a majority of the analytes can be measured in the digest of 10 µl plasma using the optimized standard chromatography-ion-funnel MS method.

The figures above show example standard curves obtained by adding varying amounts of either the heavy internal standard peptide ("Reverse" curve: 3-fold dilutions down from 400 fmol per peptide) or a synthetic version of the endogenous (unlabeled, light) peptide ("Forward" or standard addition curve). The difference between the curves is the presence of endogenous light peptide derived from the endogenous protein in the plasma sample digested to provide the analytical matrix.

Rapid nanoflow LC/MS of standard peptides

Preliminary experiments have been done with peptide standards (not SISCAPA) to evaluate the sensitivity of microfluidic-based nanoflow LC interfaced to the ion funnel QQQ MS. The results below show the separation and sensitivity achieved to date.



Nanoflow LC/MS separation: Normalized peaks

Nanoflow LC/MS sensitivity: 8.5 amol on-column

Conclusions

- An integrated, automated, high-throughput MS-based protein assay platform is now feasible using Agilent components
- Multiplexed SISCAPA sample preparation can be automated for high-throughput assays
- Standard flow LC/MS using ion funnel technology on a QQQ mass spectrometer was equivalent to that achieved previous using nanoflow LC/MS
- Future development will focus on further assessment of the robustness of the rapid LC/MS analysis