Automation of a SISCAPA Magnetic Bead Workflow for Protein Biomarker Quantitation by Mass Spectrometry Using the Agilent Bravo Automated Liquid Handling Platform

Application Note

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Abstract

An automated protocol has been developed implementing SISCAPA immunoaffinity enrichment of biomarker peptides prior to quantitation by mass spectrometry. In this protocol, magnetic beads coated with anti-peptide antibodies bind specific target peptides from tryptic digests of samples such as plasma and serum, after which the beads are washed to remove unbound peptides, and the bound, purified peptides are eluted in small volumes for injection into an LC/MS/MS system. Internal standards (stable isotope-labeled versions of the same peptides) allow accurate quantitation. The Agilent Bravo implementation allows SISCAPA processing of 96 samples in less than 30 minutes.

Introduction

Mass spectrometry (MS) has become the benchmark technology for analysis of peptides based on three important advantages: 1) precise quantitation (particularly when used with internal standards); 2) near-absolute structural specificity (based on unique molecular fragmentation patterns); and 3) facile multiplexing of hundreds of measurements in a single analytical run. These advantages can be exploited in the analysis of “proteotypic” peptides, which occur uniquely in a single protein sequence, to reveal the concentrations of the parent proteins in proteolytic digests of complex samples such as patient plasma or serum. The principal limitation of the technology in research on protein biomarkers has been sensitivity for low-abundance proteins, which may be present at levels 10 orders of magnitude below the major plasma proteins such as albumin. This problem has been solved through specific capture of target peptides by purpose-designed anti-peptide antibodies (the SISCAPA technology), yielding >100,000-fold enrichment of target vs albumin peptides, and thus extending the sensitivity of quantitative mass spectrometry to match that of immunoassays. When implemented using established affinity carriers such as magnetic beads, large numbers of samples can be processed with efficiency and precision. As a result, the inherent quality advantages of MS measurement can be obtained over the full range of existing and candidate proteins biomarkers.

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Materials

- Agilent Bravo Automated Liquid Handling Platform (G5409A)
- 96-channel LT Disposable Tip Head (04730-202)
- VarioMag Teleshake Plate Shaker (on Bravo deck)
- 24-pin magnet array (e.g., Novagen Cat. No. 71101-3)
- 96-well, polypropylene conical bottom (V-bottom) plate, having 450 µL capacity per well (e.g., Nalge/Nunc 249944) for processing capture reactions
- 96-well, polypropylene skirted PCR plate for SISCAPA-enriched peptides (e.g., Axygen Cat. No. PCR-96-FS-C)
- 96-deepwell, polypropylene conical bottom (V-bottom) plate, having 2 mL capacity (e.g., Geiner 780280)
- 384-deepwell, polypropylene conical bottom (V-bottom) plate, having 200 µL capacity (e.g., Greiner 781271)
- Agilent 96LT 200 µL sterile, filtered pipette tips (08585-102)
- Agilent VWorks Automation Control software
- SISCAPA antibodies covalently coupled to Protein G magnetic beads (e.g., Dynal Dynabeads G)
- Stable isotope-labeled versions of target peptides at 100 fmol/µL
- Wash Solution: PBS with 0.03% CHAPS detergent and 0.02% NaN_3, pH 7.4
- Elution Solution: 5% acetic acid

Protocol Workflow

The SISCAPA protocol described here begins with tryptic digests of plasma (or serum) samples to which the two SISCAPA reagents (antibody-coated magnetic beads and stable isotope-labeled peptides) have been added and incubated for 3-15 hours. In typical practice, the tryptic digestion has been carried out in advance, the resulting tryptic peptides cleansed by solid phase extraction (SPE), e.g., on an Oasis plate, lyophilized, and reconstituted in Wash Solution (PBS + 0.03% CHAPS + 0.02% NaN_3, pH 7.4).

In the following Bravo protocol, the bead wash/elution process is implemented as two sequential scripts (A and B) written using the VWorks software. In script A, stock solutions provided in rows of a 96-deepwell plate are distributed into wells of two 384-deepwell plates in order to provide five separately-accessible sets of 96-wells in two deck positions for use in protocol B (**Table 1, Figure 1**). After script A is complete, the tips and stock solution plate are removed, leaving the two 384-deepwell plates in deck positions 5 and 8, and additional labware is mounted (**Table 1, Figure 2**). The SISCAPA reaction plate (in which each well contains a sample digest, antibody-coated magnetic beads and added heavy standard peptides) is placed on a plate shaker mounted at deck position 3. Script B then carries out the following general process:

1. Separation of beads from digest using magnetic methods
2. Three sequential washes of beads in 150 µL fresh Wash Buffer with minimal carryover volume
3. Elution of bound peptides in 20 µL low-pH Elution Buffer
4. Collection ~~the~~ beads at neutral pH for potential re-use

Table 1: Bravo Deck Setups

	Plate Prep (Script A)	Wash/Elute (Script B)
1	*(empty)*	Tips 1 (New)
2	*(empty)*	SISCAPA Eluate (=Output)
3	*(empty)*	SISCAPA Reaction (=Input)
4	96-Deepwell Stock Solutions	Clean 96-well Washplate
5	384-Deepwell Wash 1-3 & Eluent	384-Deepwell Wash 1-3 & Eluent
6	Tips (Used)	Save Digest Plate
7	Tips (New)	Tips 2 (New)
8	384-Deepwell Bead Recovery	384-Deepwell Bead Recovery
9	(magnet plate)	(magnet plate)

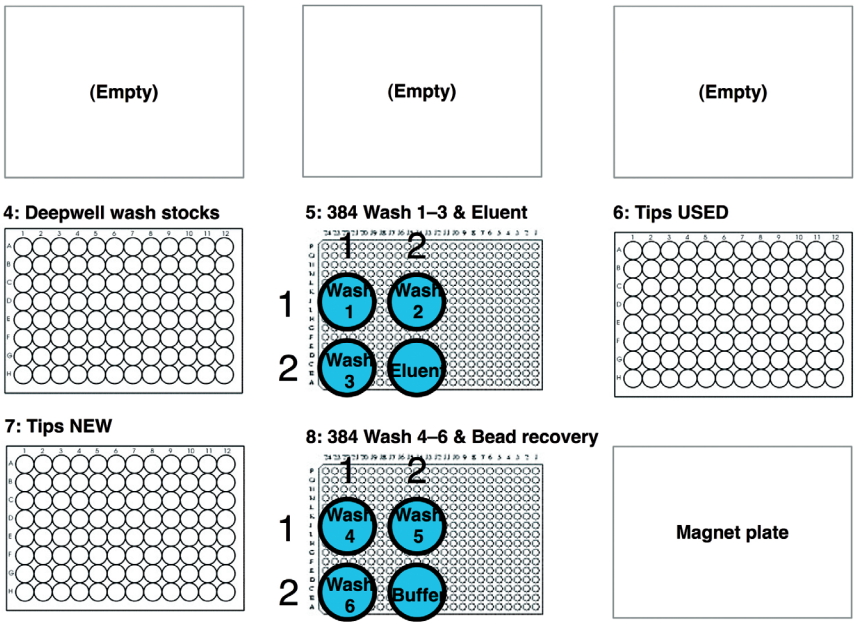

Figure 1. Bravo Deck Layout for Script A

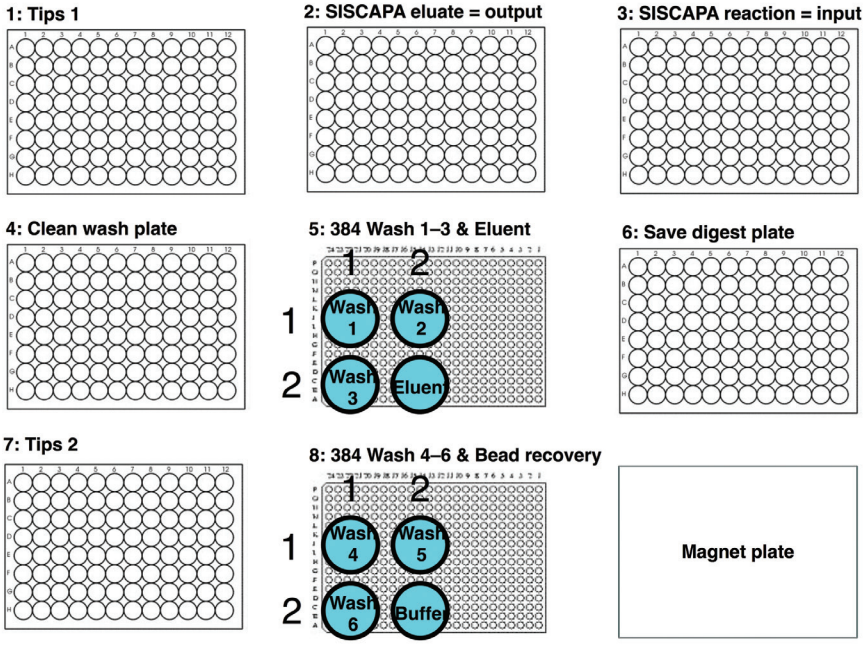

Figure 2. Bravo Deck Layout for Script B

In order to minimize carryover of non-specifically bound peptides on surfaces of plate wells or pipette tips, two sets of tips are used: one "clean" for introducing fresh solutions and collecting the final eluate, and one "dirty" used to remove digest and used wash solutions. In addition, the bead suspension is moved during the second wash cycle from the original SISCAPA Reaction plate to a Clean Washplate prior to the final wash and elution. During each cycle of magnetic separation, the plate containing the beads is moved by the Bravo arm onto a magnetic plate causing the beads to clump to one side of the well, after which all the free liquid can be removed by aspirating from the bottom, center of the well. Subsequently fresh liquid is dispensed into the well, and the plate moved back to the shaker, which resuspends the beads in the fresh solution (1,000 rpm for 60 sec). Script B requires 20 min, in which the time between removal of beads from sample digest and final peptide elution is less than 13 min.

The eluted peptides are delivered in a volume (20 µL) and solvent (5% acetic acid) suitable for subsequent injection into a reversed-phase LC system feeding a triple quadrupole MS, in which multiple reaction monitoring (MRM) methods are used to quantitate the stable isotope-labeled ("heavy" internal standard) and sample-derived ("light" analyte) version of each peptide. Analyte concentration is calculated as the peak area ratio of light/heavy MRM's multiplied by the known concentration of the added heavy peptide (so-called stable isotope dilution method).

Results and Conclusion

A multiplex SISCAPA experiment was carried out using 1 µg of each of 11 rabbit monoclonal antibodies covalently linked to magnetic beads to capture 11 respective target peptides and cognate stable isotope-labeled standards from digests of 10 µL samples of human plasma. The captured and eluted peptides were analyzed using reversed-phase (C18) chromatography at 400 µL/min and delivered to an Agilent 6490 Triple Quadrupole LC/MS with iFunnel technology. Three separate peptide fragments (transitions) were measured for each peptide and its labeled cognate (66 MRM channels). The LC peak shape and elution times of the peptides are shown in **Figure 3**.

Figure 4 shows 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 reaction) 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 soluble transferrin receptor (sTfR) protein in the plasma sample digested to provide the analytical matrix.

The results demonstrate that the Agilent Bravo Automated Liquid Handling Platform can implement the SISCAPA peptide enrichment method, generating samples suitable for LC/MS/MS analysis.

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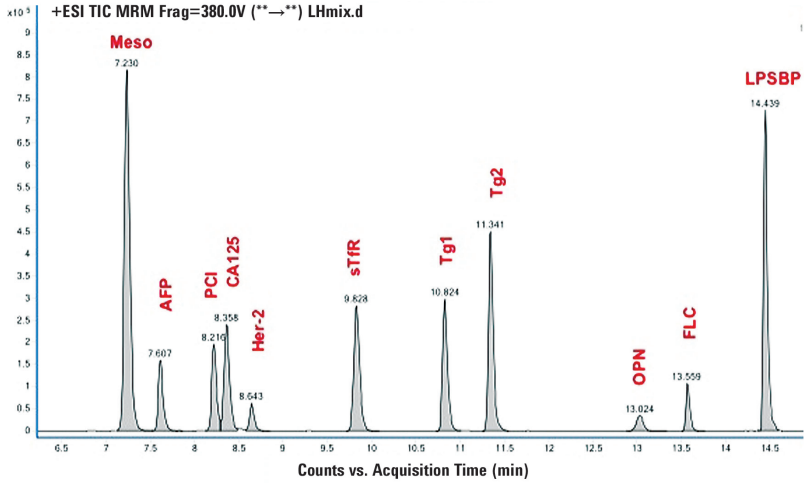

Figure 3. Peaks in the LC chromatogram produced by the best transition for each of the 11 peptides monitored.

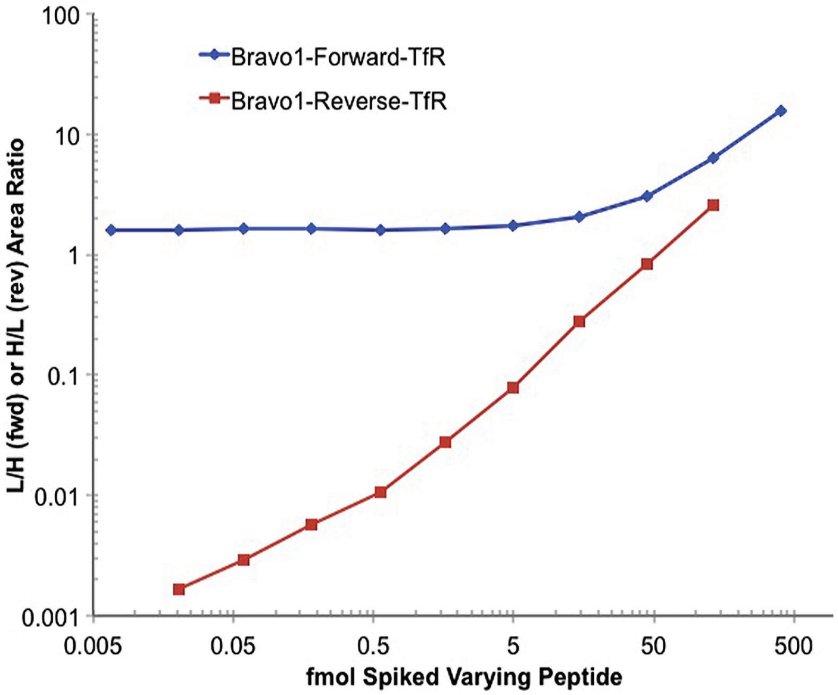

Figure 4. Standard curves showing quantitative MS results (ratio of light and heavy peptide MRM peak areas) for a peptide (GFVEPDHYVVVGAQR - unique to the circulating fragment of human transferrin receptor sTfR) captured by a rabbit monoclonal antibody from a tryptic digest of 10 µL human plasma. In the Reverse curve (red), varying amounts of Heavy internal standard peptide (eleven 3-fold dilutions down from 400 fmol) have been added to the digest, yielding a curve decreasing downward to a plateau at the lower limit of detection. In the Forward curve (blue), varying amounts of Light synthetic target peptide (eleven 3-fold dilutions down from 400 fmol) have been added to the digest, along with a constant amount (50 fmol) of heavy peptide. In this case, the level of the endogenous peptide (250 fmol) present in the sample digest is evident.

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