Robust Targeted Protein Quantification by LC/MS Enabled by a Versatile Sample Preparation Platform for Automated Protein Digestion and Cleanup

Jason Russell, Zachary Van Den Heuvel, Michael Bovee, Scott Fulton, and Steve Murphy
Agilent Technologies, Inc., Madison, WI
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Introduction

Protein quantification by mass spectrometry is dependent on laborious, manual sample preparation workflows required to liberate signature peptides from target proteins. Manual sample preparation can lead to variable, irreproducible, user-dependent results problematic for protocol transfers, cross-laboratory comparisons, and throughput scaling. The success of high-throughput protein quantification by MS is critically dependent on sample preparation, but the lack of robust automation has stalled its wider adoption in clinical settings and other high sample throughput applications. If high-throughput protein quantification by MS is going to succeed in areas traditionally dominated by non-MS techniques (such as ELISAs), sample preparation methods that scale to meet throughput demands must be developed that maintain the reproducibility and sensitivity achieved on smaller scales. Such scaling can be achieved if built upon a foundation of precision automation engineered specifically for protein sample preparation.

We present a robust, high throughput, automated sample preparation platform for protein quantification by LC/MS. We describe proof-of-principal experiments for in-solution protein digestion, peptide cleanup, and strong-cation exchange fractionation (SCX) of complex peptide mixtures using this automation platform.

AssayMAP Bravo Technology

The Agilent AssayMAP Bravo automated sample preparation platform consists of:

- 5-μL packed bed cartridges packed with a variety of resins
- AssayMAP Bravo with a 96-channel probe syringe head that mates with the microchromatography cartridges and provides highly precise positive displacement flow control.
- Easy-to-use software interfaces and protocols

AssayMAP Bravo Protocols

In-Solution Digestion: DIGEST

A. Application Settings:
- Enzyme: Trypsin
- Enzyme concentration: 50 μg/mL
- Enzyme: Chymotrypsin
- Enzyme concentration: 20 μg/mL
- Inhibitors: None
- Incubation time: 15 minutes
- Incubation temperature: 37 °C
- Autoclave temperature: 121 °C
- Autoclave pressure: 15 psi

B. Deck Layout:
- 4. Inhibitors Plate
- 5. Samples Plate 1
- 6. Samples Plate 2
- 7. Samples Plate 3
- 8. Samples Plate 4

C. Laminar Table:
- 1. 96-Well Sample Plate
- 2. 96-Well Digest Plate
- 3. 96-Well Reaction Plate
- 4. 96-Well Wash Plate
- 5. 96-Well Elution Plate
- 6. 96-Well Blank Plate

Three protocols were developed to enable high-throughput sample processing and rapid method development. Simple interfaces allow users to turn steps on/off and alter volumes and flow rates. Key features include:

- In-solution Digestion: Parallel processing of 4 microtiter plates (8-384 samples) including denaturation, reduction, alkylation, dilution, and addition of protease.
- Peptide Cleanup: Quantitative elution in as little as 10 μL from a reversed-phase cartridge.
- Fractionation: 2-6 fractions can be collected using ionic strength or pH elution with SCX cartridges, or using ACN elution with high-pH reversed-phase cartridges.

Experimental

Automated trypsin digestion, reversed-phased cleanup, and fractionation was performed using bovine serum albumin (BSA) and Escherichia coli protein lysate on an AssayMAP Bravo. Cartridges packed with C18 or polystyrene divinylbenzene (RP-S) resins were used for reversed-phase cleanup. Cartridges packed with SCX resin were used for peptide fractionation.

Analytical work was performed using Agilent’s:

- 1290 Infinity Binary LC System
- AdvanceBio Peptide Mapping Column (2.1 x 250 mm) with LC flow rates of 400 μL/min. Gradients generally consisted of increasing ACN with 0.1% formic acid.
- 6550 iFunnel Q-TOF MS with a Dual Agilent Jet Stream source. All data were collected in auto MS/MS mode.
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Figures of Merit for Automated In-solution Digestion and Peptide Cleanup

- LVNELTEFAK, %CV = 1.3
- RHPEYAVSVLLR, %CV = 2.5
- AEFVEVTK, %CV = 1.8
- LVVSTQTALAL, %CV = 2.5
- AWSVAR, %CV = 3.3

Multiday, Multiplate, Digestion and Cleanup of BSA

- BSA was denatured using urea and guanidine HCl. (16 samples for each denaturant on 4 plates) reduced, alkylated, and digested in parallel on two separate days (64 digests with each denaturant on each day).

- Digests (75 µg) were cleaned/desalted using C18 cartridges (day 1) or RP-S cartridges (day 2) and signals of 25 tryptic peptides were monitored.

- The scatter plot (left) shows combined day 1/day 2 data for 5 representative peptides from 128 independent BSA digestions.

- The table shows the %CVs for each day and denaturant. Automated digestion and peptide cleanup was very reproducible with average peak area %CVs less than 4%.

Peptide Recovery after Reversed-phase Cleanup

- AEFVEVTK (RH = 22.2) LKECCDQPLLEK (RH = 14.5)
- LVNELTEFAK (RH = 32.8) RHPEYAVSVLLR (RH = 25.7)
- GLVLIAFSGQYGGCPDENVK (RH = 52.4) LTFTHADICTLPDTEK (RH = 38.8)

BSA digests were loaded onto C18 cartridges (n = 3) at different concentrations to generate the indicated load mass. Signals normalized to online cleanup of 1.25 µg of the same digest at highest concentration tested (0.75 µg/µL).

- High recovery was achieved for peptides with low relative hydrophobicity (RH) values (top).

- Recovery of moderately hydrophobic peptides (middle) was high for most peptides but some did show some loss.

- High recovery of very hydrophobic peptides (bottom) was achieved at moderate load masses.

- Recovery at each load mass was reproducible with the average CV = 1.5%.

Various amounts of tryptic BSA digests were cleaned up with C18 and RPS cartridges. The TIC from these experiments show:

- Unbiased recovery for peptide load amounts of 25 to 100 µg.

- Competitive displacement from the cartridge of extremely hydrophilic peptides with load amounts exceeding 100 µg.

- Diminished recovery of very hydrophobic peptides with load amounts below 25 µg.
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Reproducible Digestion, Fractionation, and Cleanup of Complex Mixtures

### Complex Mixture Analysis

Protein lysate (~105 µg) from *E. coli* was digested on two different days (n = 4, each day) followed by this workflow:

1) Cleanup with C18 cartridges using a 20 µL elution volume (70% ACN) followed by a 10x dilution in SCX equilibration buffer containing 25% ACN.

2) Fractionation with SCX cartridges using a 20 µL elution volume for each of the 6 collected fractions

3) Fractions (24 fractions in total for each day) were diluted 10x in 0.1% TFA and again subjected to C18 cleanup

An estimated 4-12 µg of digested protein lysate was injected per fraction and separated over a 60-minute gradient. Peptide-spectrum matches (PSMs) and resulting unique peptide sequences determined from database searches were filtered to a 1% false discovery rate (FDR).

- On average, each replicate yielded over 38,000 PSMs and 15,000 unique peptide sequences with more than 10,000 peptides identified as exclusive to a single fraction.

- In total, more than 67% of the unique peptide sequences could be found exclusively in a single fraction.

### Conclusions

- Automated digestion and cleanup produced %CV values < 4% for a multiday, multiplate study of BSA.

- Peptide recovery is reproducible across a wide range of load masses.

- Complex workflows involving automated digestion, fractionation and multiple rounds of cleanup give %CV values < 8% without the use of internal standards.

- Small elution volumes minimize or eliminates the need for dry down in complex workflows.

- The ability to perform reproducible protein sample preparation on a versatile instrument platform which enables scaling of sample preparation makes robust, high-throughput, protein quantification attainable.

**TIICs from 8 *E. coli* replicates for each fraction demonstrate the high degree of reproducibility for the entire workflow.**

- 2.3 to 7.6% peak area CVs from EICs without the use of internal standards or using targeted MS/MS.

- Less than 4.4 ppm average mass error without the use of reference mass correction and an average 0.1% retention time drift across the replicates.