Automation for LC/MS Sample Preparation: High Throughput In-Solution Digestion and Peptide Cleanup Enabled by the Agilent AssayMAP Bravo Platform

Application Note

Clinical Research

Authors

Jason D. Russell,
Zachary Van Den Heuvel,
Michael Bovee, and Steve Murphy
Agilent Technologies, Inc.
Madison, WI USA

Abstract

Protein and peptide sample preparation for LC/MS remains entrenched in workflows reliant upon serial manual manipulations resulting in protocols lacking in throughput, scalability, and transferability. These workflows are often dependent on highly skilled practitioners to achieve acceptable reproducibility, or, in many cases, large variability is simply accepted as intrinsic to the workflow. To address these issues, routine sample handling tasks common to LC/MS proteomic workflows have been automated using the Agilent AssayMAP Bravo platform and its suite of proteomic tools engineered to bring reproducibility, scalability, protocol portability, and ease-of-use to LC/MS sample preparation. The platform comprises a best-in-class liquid handler, disposable microchromatography cartridges, and simple user-customizable protocols to enable high-throughput protein digestion and peptide cleanup. We report analytical figures-of-merit for digestion of BSA followed by cleanup demonstrating day-to-day reproducibility yielding % CVs less than 5 % for the majority of the 25 BSA peptide targets. In addition, we offer a full characterization of AssayMAP RP-S and C18 reversed-phase cartridges for peptide cleanup.

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Introduction

Protein and peptide analysis has undergone a revolution, in part, due to advancements in tandem mass spectrometry, soft ionization techniques, bioinformatics, and chromatographic separations. This confluence of analytical innovation and computational power has enabled scientists to probe protein dynamics and make quantitative measurements with unprecedented sensitivity and precision empowered by workflows featuring liquid chromatography interfaced with mass spectrometry (LC/MS). As the field of quantitative proteomics by LC/MS matures, the demands for analytical precision will become greater as will the desire to increase sample throughput. However, despite the tremendous innovations in LC/MS for proteomics, advancements in sample preparation have failed to match pace with the other critical technologies. Nowhere has the sluggish progress of sample preparation for quantitative proteomics become more evident than in the field of biomarker discovery and research. Hundreds of putative protein biomarkers have been revealed by LC/MS assays and documented in the literature. Yet, the vast majority of these biomarker studies have not progressed beyond initial investigations involving a small set of biological samples. Attempts to scale up sample processing are often met with concomitant increases in assay variability due to compounded error resulting from individual or small batch processing of samples through multistep workflows. Such variability can cloak biologically significant changes in protein expression that pilot studies suggested were indicative of disease state or susceptibility.

Sample preparation for quantitative proteomics by LC/MS remains entrenched in workflows reliant upon serial manual manipulations resulting in protocols lacking in throughput, scalability, and transferability; all essential workflow attributes for the use of LC/MS and for high-throughput screening and process control. These workflows are often dependent on a relatively finite number of highly skilled practitioners to achieve acceptable reproducibility, severely limiting the ability to increase sample processing capacity while maintaining the desired assay quality. The success of protein analysis by LC/MS is contingent on reproducible sample preparation. The lack of intuitive, reliable, sample-scalable automation has further limited the adoption of LC/MS for assays requiring large sample campaigns or demanding high throughput. Sample preparation methods must be developed that can scale. Such scaling can be achieved if built upon a foundation of automation engineered specifically for protein sample preparation and, importantly, the needs of proteomicists.

This application note presents the Agilent AssayMAP Bravo automation platform and its suite of proteomic tools purposefully engineered to bring reproducibility, scalability, and ease-of-use to LC/MS sample preparation. The platform comprises a best-in-class liquid handler with 96 probe syringes that allow precise positive displacement flow control, disposable 5 µL packed-bed cartridges, and flexible, customizable protocols with simple user interfaces to enable automated, high-throughput protein digestion and peptide cleanup in a microtiter plate format. Here, we introduce the use of the AssayMAP Bravo for the digestion and cleanup of a single protein model system and report the analytical figures-of-merit of the combined workflow.

Experimental

In-solution digestion

The In-Solution Digestion protocol provides the user with the option of processing one to four 96-well plates with between 1–12 columns of samples (8–96 samples/plate) in parallel. For this experiment, 32 samples were processed per plate. The protocol sequentially draws reagent from the appropriate source plates, adds reagent to the samples, and mixes the solution in the sample plate following the inputs provided in the application interface (Figure 2). Flexibility is built into the system by turning steps on or off in the application interface and altering the composition of the various reagents to customize reaction conditions. Bovine serum albumin (BSA) was dissolved in water to a concentration of 15 µg/µL, and 15 µL (225 µg) was pipetted into each well of the first four columns of a U-bottom microtiter plate. This was repeated on three other plates to yield four sample plates and a total of 128 independent samples for digestion. The denaturation mixture plate was prepared such that a guanidine-based denaturant mixture was dispensed to Columns 1 and 2 and a urea-based denaturant mixture dispensed to Columns 3 and 4. Iodoacetamide was added to the first four columns of the U-bottom alkylant plate, *Tris* buffer was

Table 1. Samples, reagents, and analytical instrumentation.

On-deck samples and reagents	
In-solution digestion v1.0	
Sample	Bovine serum albumin (BSA), 15 µg/µL in water
Denaturation mixtures	8 M guanidine HCl with 25 mM TCEP and 150 mM *Tris* (pH 8) 9 M urea with 25 mM TCEP and 150 mM *Tris* (pH 8)
Alkylant	300 mM iodoacetamide
Diluent mixture	50 mM *Tris* (pH 8)
Protease	Trypsin (1 µg/µL in 50 mM acetic acid)
Wash station	Deionized water
Peptide cleanup v1.1	
Sample	TFA-acidified tryptic digests of BSA (pH ~ 2.6), 0.75 µg/µL
Priming buffer	50 % ACN/0.1 % TFA
Utility (equilibration) buffer	0.1 % TFA
Elution buffer	70 % ACN/0.1 % formic acid
Wash station	Deionized water
Analytical instrumentation	
Automation	
Core automation platform	Agilent AssayMAP Bravo (G5542A)
Agilent AssayMAP digest and cleanup starter kits	96 RP-S cartridges and labware for digestion and cleanup (G6596-60034) 96 C18 cartridges and labware for digestion and cleanup (G5496-60013)
Agilent AssayMAP Bravo accessories	Risers, 146 mm (G5498B#055) Peltier Thermal Station with STC controller (G5498B#035) Custom Plate Nest (G5498B#017) PCR Plate Insert (G5498B#013)
Additional accessories	Agilent PlateLoc Thermal Microplate Sealer (G5402A)
LC/MS	
Mass spectrometer	Agilent 6550 iFunnel QTOF LC/MS Dual Agilent Jet Stream ESI
LC system and columns	Agilent 1290 Infinity LC System Agilent AdvanceBio Pepide Mapping Columns (C18): Analytical: 2.1 × 250 mm, 2.7 µm (651750-902) Guard: 2.1 mm Fast Guard (851725-911)

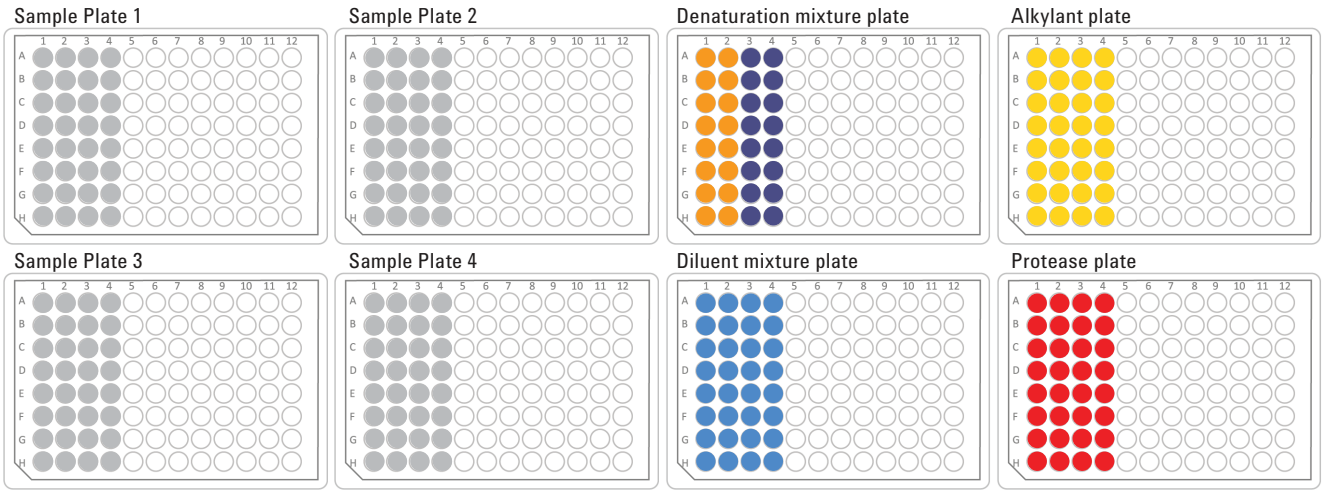


Figure 1. Microtiter plate layout for the multiplate digestion of BSA. Sample plates contained BSA (15 µg/µL), the Denaturation Mixture plate contained a guanidine-based denaturant mixture (orange) or urea-based denaturant mixture (purple). The Alkylant plate contained iodoacetamide while the Diluent Mixture plate contained *Tris* buffer and the Protease plate contained trypsin.

added to the first four columns of the 1-mL, deep-well diluent mixture plate, and trypsin was added to the first four columns of the PCR Protease plate. See Figure 1 plate layouts and Table 1 for a list of reagents.

The Plate Stacking utility in the In-Solution Digestion protocol was used to assemble a stack of five U-bottom plates at Location 2 on the AssayMAP Bravo deck which served as lids for each sample plate and the protease. These plates shield samples from light during the alkylation step and minimize the risk of airborne contamination. They also provide for a stringent syringe wash between each liquid handling step (stringent syringe washing was not used in this experiment). Reagent and sample plates were placed on the deck as shown in Figure 2. The Application Settings were entered on the In-Solution Digest user interface using the values indicated in Figure 2. The protocol was initiated, and the denaturant mixture was added and mixed with the sample. The protocol automatically paused to allow the sample plates to be removed from the deck. The sample plates were sealed with a removable seal using a PlateLoc, and placed in a 60 °C incubator for 1 hour. The plates were briefly centrifuged, seals were removed, and the samples were placed back on deck The protocol was continued with the addition of alkylant, alkylation incubation (45 minutes), diluent addition, and trypsin addition, which were carried out uninterrupted on deck. Upon completion of the protocol, the sample plates were removed from the deck, sealed using the PlateLoc, and placed into a 37 °C incubator for overnight digestion. Samples were removed from the incubator, briefly centrifuged, and each sample plate was acidified by the addition of 30 µL of 10 % TFA using the Single Liquid Addition utility. The In-Solution Digest protocol was repeated the next day using identical conditions. Acidified BSA digests were then processed through the Peptide Cleanup Protocol.

Peptide cleanup

AssayMAP offers two cartridge options for performing reversed-phase cleanup; a cartridge packed with a traditional C18 (16 % carbon load, 150Å pore size) stationary phase and a cartridge packed with an underivitized polystyrene divinylbenzene stationary phase (RP-S, 100Å pore size). Each cartridge type may exhibit subtle differences in selectivity depending on the sample and choice of reagents. In addition, C18 cartridges offer stable performance from pH ~2–8; while RP-S cartridges have a broader operational pH range (~ pH 1–13).

The Peptide Cleanup protocol was opened from the Workflow Navigator. Buffers (Table 1) were dispensed into

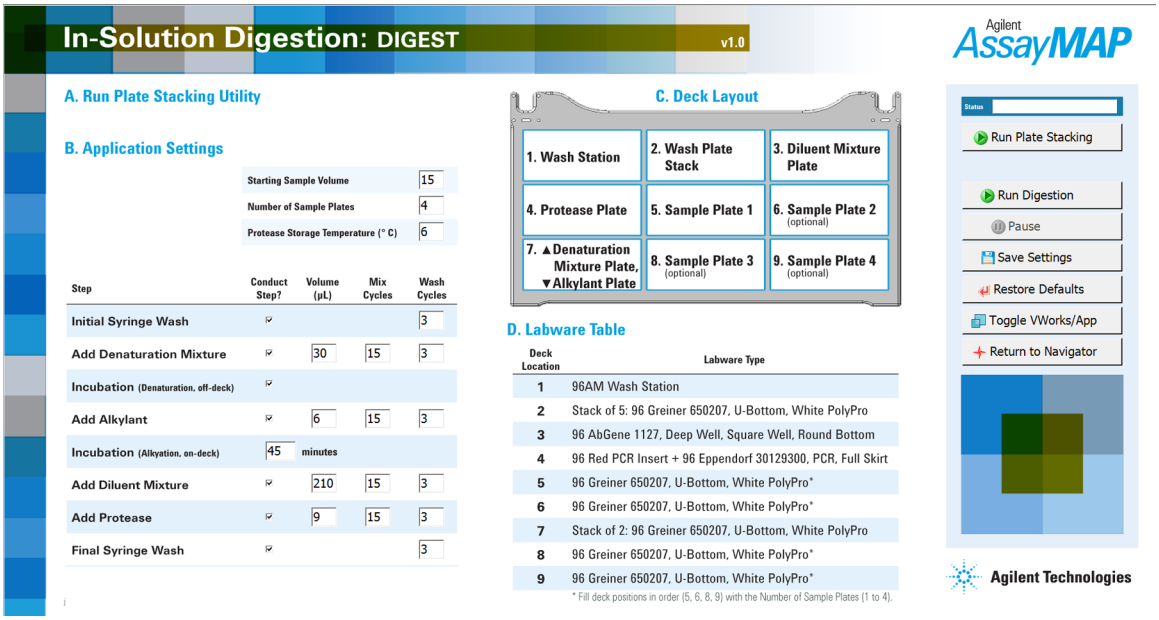


Figure 2. User interface for the Agilent AssayMAP In-Solution Digestion protocol with the parameters used for multiplate digestion of BSA.

the first four columns of 12-column plates corresponding to the reagent plates for Syringe Wash (priming) Buffer, Utility (equilibration/washing), or Elution Buffer. Empty U-bottom or PCR plates were used for the Flow Through Collection and Eluate Collection plates. The Cartridge Transfer utility was used to transfer four columns of AssayMAP C18 (Day 1) or RP-S (Day 2) cartridges from the cartridge rack to the first four columns of the Cartridge Seating Station placed at deck Location 2. The deck was configured as indicated in Figure 3 with a single U-bottom sample plate containing acidified BSA at Position 4. The Application Settings were entered on the Peptide Cleanup user interface and labware types were selected in the Labware Table as shown in Figure 3. The protocol was started and after the cartridges were primed and equilibrated, a volume of 100 µL of BSA digest (75 µg) was loaded onto each cartridge. The Peptide Cleanup protocol was repeated for the other three sample plates from the In-Solution Digest protocol. Eluates were lyophilized and stored at –80 °C. Eluates were reconstituted, and 1.25 µg was injected from each sample well. Signals from 25 peptides were monitored by LC/MS.

Peptide cleanup - recovery

For Peptide Cleanup recovery studies, acidified BSA digests (0.75 µg/µL) from the In-Solution Digest protocol were pooled and diluted to create this concentration series: 0.75, 0.50, 0.25, 0.125, 0.0625, and 0.03125 µg/µL. Samples were subjected to Peptide Cleanup using the settings in Figure 3 whereby 100 µL of each concentration was loaded onto AssayMAP C18 cartridges in triplicate and in parallel. Eluates were lyophilized and stored at –80 °C. Eluates were reconstituted, and 0.5 µg was injected from each load concentration. Signals from 25 peptides were monitored by LC/MS and directly compared to the peak areas of the same peptides with online LC cleanup of 0.5 µg of digest at each concentration. (0.67 µL to 16 µL injection volumes ranging from highest to lowest BSA concentration).

Peptide cleanup – differential mass loading

For Peptide Cleanup differential loading studies, an acidified BSA digest (4.75 µg/µL) from a bulk in-solution tryptic digest was diluted to create this concentration series: 4.0, 3.0, 2.0, 1.0, 0.75, 0.50, 0.25, and 0.10 µg/µL. Samples were subjected to Peptide Cleanup using the settings in Figure 3 whereby 100 µL of each concentration was loaded onto AssayMAP RP-S and C18 cartridges in duplicate with all experiments conducted in parallel. Eluates were lyophilized and stored at –80 °C. Eluates were reconstituted, and 1.0 µg was injected from each load concentration and peptide signals were monitored by LC/MS.

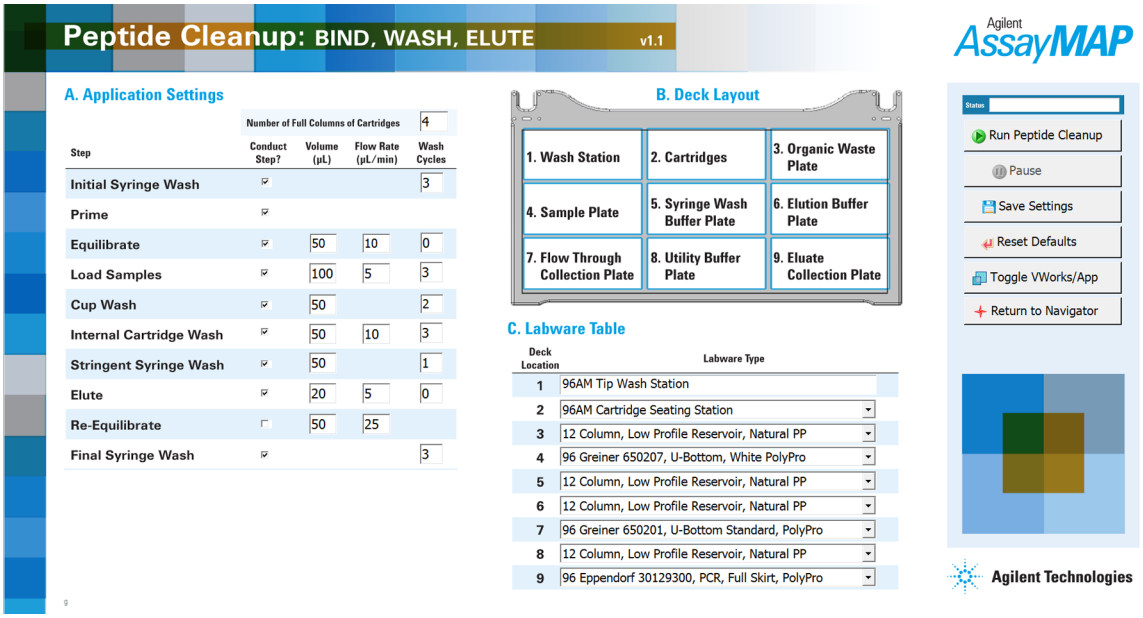


Figure 3. User interface for the Agilent AssayMAP Peptide Cleanup protocol with the parameters used for cleanup of BSA digests.

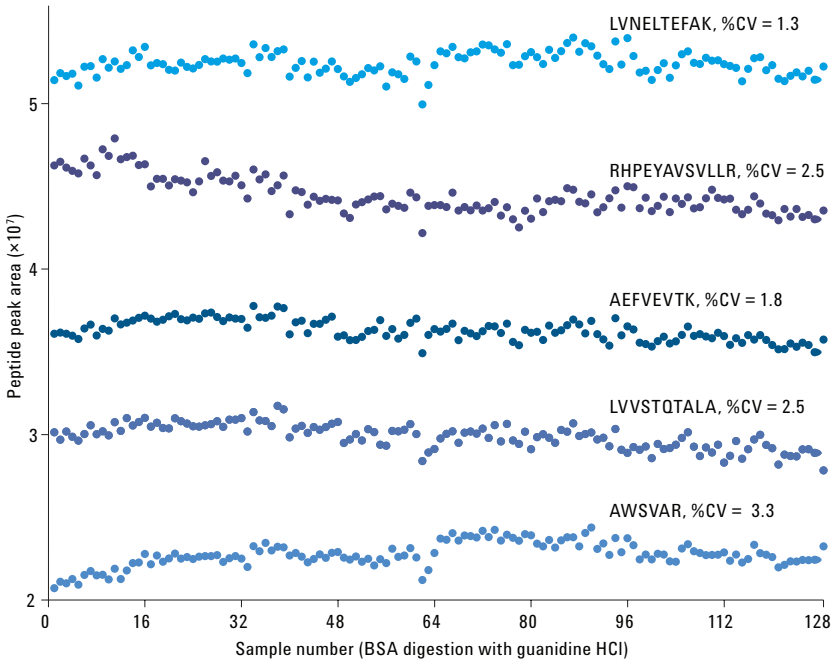
Results and Discussion

In-solution digestion with peptide cleanup

BSA digests from Day 1 (C18 cleanup) and Day 2 (RP-S cleanup) were analyzed by LC/MS. Extracted ion chromatograms (EICs) from 25 representative tryptic peptides of BSA were generated for each LC/MS run and the peak area for each peptide was plotted. Figure 4 (top) shows the peak areas of five tryptic BSA peptides (guanidine denaturation) tracked across both days representing 128 sample digestion and cleanup events. The % CV for these peptides range from 1.3 to 2.5 % across all replicates. The table in Figure 4 summarizes the % CV distributions for the 25 peptides sorted by day and by denaturant. The average % CV for the 25 BSA peptides was less than 3.7 % for both days with urea, and less than 2.6 % with guanidine. These results reveal remarkable signal consistency from these peptides for the entire workflow from digestion and cleanup through LC/MS analysis. This consistency, as evidenced by the multiplate data, permits high-throughput sample preparation extending the parallel processing capacity to 384 samples for In-Solution Digestion. Similarly, Peptide Cleanup can be performed on 96 samples simultaneously. In either case, throughput needs can be readily scaled to facilitate processing of tens, to hundreds, to thousands of samples per week.

Despite the low % CVs for the majority of the peptides that were monitored, not every peptide from BSA was well behaved under both digestion conditions. This, in some regard, is a reflection of the complexities of proteolysis where certain conditions favor or disfavor the liberation of certain peptides. For instance, the peptide FKDLGEEHFK contains a missed cleavage at a lysine residue flanked on its C-terminal end by an aspartic acid residue; a sequence motif known to slow the rate of trypsinization in some proteins. This is not an ideal peptide for targeted quantification by LC/MS, but sometimes a non-ideal peptide may be the only option for quantification. In this case, the peptide FKDLGEEHFK produced 4–5× more signal compared to the zero missed-cleavage peptide, DLGEEHFK, and produced more high *m/z* y-ions offering greater specificity for multiple reaction monitoring (MRM) experiments. However, with a urea-based denaturation the peak area % CV across both days was 39.2 %, but the intra-day % CVs were 5.5 % and 8.8 % suggesting poor conditions for denaturation. With a guanidine-based denaturation, the peak area % CV across both days was 5.8 % with intra-day % CVs of 2.5 % and 1.9 %.

Because of the high sample multiplexing ability of the AssayMAP Bravo platform, denaturation conditions were evaluated in parallel to find conditions to favor the consistent production of a non-ideal target peptide. It is possible to titrate different concentrations of multiple denaturants into the Denaturation Mixture plate to facilitate the rapid identification of denaturation conditions to favor the consistent liberation of target peptides from their respective proteins. In addition, because sample preparation can be done in parallel and



Urea	Day 1	Day 2
No. of samples	64	62
No. of peptides monitored	25	25
Avg. peak area % CV	3.3	3.7
No. peptides %CV <5	23	21
No. peptides 5 > % CV < 10	2	3
No. peptides % CV > 10	0	1

Guanidine HCl	Day 1	Day 2
No. of samples	64	64
No. of peptides monitored	25	25
Avg. peak area % CV	2.3	2.6
No. peptides % CV <5	25	23
No. peptides 5 > % CV < 10	0	1
No. peptides % CV > 10	0	1

Figure 4. Results of multiday, multiplate, in-solution digestion and cleanup of BSA. BSA was digested using the In-Solution Digestion protocol on multiple days using either urea- or guanidine-based denaturation. The Peptide Cleanup protocol was used with C18 (Day 1) or RP-S (Day 2) cartridges. Eluates were dried, reconstituted, and 1.25 µg was analyzed by LC/MS. Signal was monitored from 25 peptides and peak integration was performed on extracted ion chromatograms. Five peptides generated from denaturation with guanidine are shown with peak area % CVs less than 3.3 % across all 128 samples. The accompanying table shows the peptide peak area % CV distribution for each day and denaturant type.

on different plates, denaturing conditions can be assessed across each sample plate and independently incubated. Both denaturation time and temperature can be simultaneous optimized along with the composition of the denaturing solution in a massive multiplexed manner to accelerate method development.

Peptide cleanup – recovery versus load mass

A dilution series of BSA digests generated from the In-Solution Digestion protocol was used to assess peptide recovery from C18 cartridges relative to online LC cleanup of the same samples. Peak areas from 25 peptides were monitored and binned into three groups based upon calculated relative hydrophobicity:

- RH < 22.5, low hydrophobicity, 10 peptides
- 22.5 < RH < 35, moderate hydrophobicity 8 peptides
- RH > 35, high hydrophobicity, 7 peptides

As shown in Figure 5 (top), peptides representative of low hydrophobicity, LKECCDKPLLEK (RH = 14.5) and AEFVEVTK (RH = 22.2), had quantitative recovery across the mass load range. The other 8 peptides in this group displayed comparable recoveries. Recovery of the moderately hydrophobic peptide LVNELTEFAK (RH = 32.0) was equal to, or greater than, online LC cleanup from 3 to 75 µg.

However, the % recovery of the moderately hydrophobic peptide LVVSTQTALA (RH = 25.5) decreased from > 94.5 % at mass load amounts greater than 50 µg to 21.8 % at the lowest load amount (Figure 5, middle). The other six peptides in this relative hydrophobicity group had high recoveries across all mass loads. In general, peptide recovery strongly correlated with relative hydrophobicity calculations. However, as this example revealed, not all peptides followed this trend. Two peptides from the high-to-extreme hydrophobicity range, and TVMENFVAFVDK (RH = 45.1) and GLVLIAFSQYLQQCPFDEHVK (RH = 52.4), exhibited greater than 70 % recovery at load amounts exceeding 12.5 µg (Figure 5, bottom) as did the other five peptides in this hydrophobicity group.

The recovery was further reduced as the load mass decreased. As expected, very hydrophobic peptides were a challenge to recover at low mass loads, but recovery, even when low, was reproducible. Taken as a whole, these data reveal strong, consistent peptide recovery performance across the mass load range for peptides of low-to-moderate relative hydrophobicity. The average % recovery and % CV (in parentheses) for all 25 peptides was 87.5 % (2.6 %), 92.7 % (1.1 %), 93.0 % (2.1 %), 96.8 % (1.3 %), 100.0 % (1.1 %), and 99.7 % (1.4 %) for 3.125 µg, 6.25 µg, 12.5 µg, 25 µg, 50 µg, and 75 µg cartridge loads, respectively. In total, the average % recovery and % CV across all mass loads was 94.9 % and 1.6%, respectively.

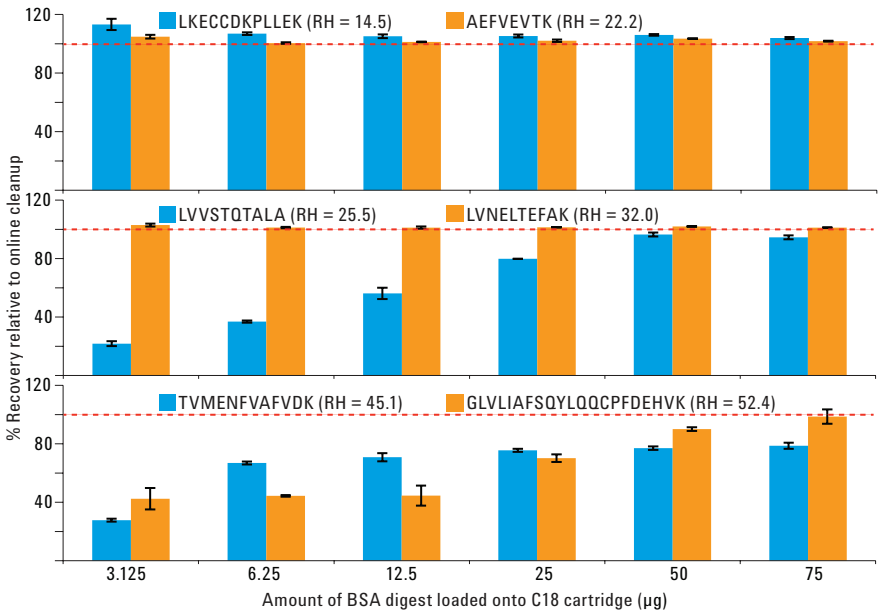


Figure 5. Peptide recovery as a function of BSA load mass. BSA digests were diluted to establish a concentration series from 0.03125 to 0.75 µg/µL. Sample volumes of 100 µL were processed using the Peptide Cleanup protocol with C18 cartridges (n = 3) and an estimated 0.5 µg of each eluate was analyzed. Peptide recoveries were normalized to online LC cleanup of 0.5 µg of digest at each concentration. Twenty-five peptides were monitored and binned into three groups based upon relative hydrophobicity: low hydrophobicity (Top panel, ten peptides), moderate hydrophobicity (Middle panel, eight peptides), and high hydrophobicity (Bottom panel, seven peptides). In general, high % recovery is achieved with peptides of low-to-moderate relative hydrophobicity (RH). However, % recovery did not always correlate with RH as demonstrated by the two peptides LVVSTQTALA and LVNELTEFAK (Middle panel). For all 25 peptides analyzed, the average % recovery and % CV (in parentheses) was 87.5 % (2.6 %), 92.7 % (1.1 %), 93.0 % (2.1 %), 96.8 % (1.3 %), 100.0 % (1.1 %), and 99.7 % (1.4 %) for 3.125 µg, 6.25 µg, 12.5 µg, 25 µg, 50 µg, and 75 µg cartridge loads, respectively. In total, the average % recovery and % CV across all mass loads was 94.9 % and 1.6 %, respectively.

Peptide cleanup – analyte bias versus load capacity

The BSA digest binding capacity of AssayMAP C18 and RP-S cartridges was investigated by loading 10–400 µg of digest on cartridges followed by an analysis of 1-µg injections of the eluates. In Figure 6, overlaid total ion chromatograms (TICs) of RP-S and C18 eluates are divided into regions of calculated relative hydrophobicity (RH, calculated using SSRCalc)[1], and arranged according to increasing mass load. As the load increased from 10–200 µg, the TIC signal of peptides of low-to-moderate relative hydrophobicity (RH = ~8-48) remained largely unchanged. The TIC signal of peptides with RH values between 14.5 and 48 were stable up to 300 µg. At 400 µg, the binding capacity for these peptides was exceeded. Very hydrophilic peptides (RH < ~8) became competitively displaced as the load mass increased above 100 µg and there were losses due to nonspecific binding of very hydrophobic peptides (RH > 48) at the 10 µg load amount. The capacity and binding characteristics of C18 and RP-S are nearly identical except that C18 exhibits slightly better retention of hydrophilic peptides as the load amounts were increased above 100 µg. The sample binding characteristics and capacities of AssayMAP reversed-phase cartridges are dependent upon several experimental variables including sample mass, sample complexity, and flow rate during sample binding. Importantly, the AssayMAP Bravo Peptide Cleanup protocol permits very quick assessment of cartridge capacities and binding characteristics to facilitate optimization of the conditions for sample cleanup.

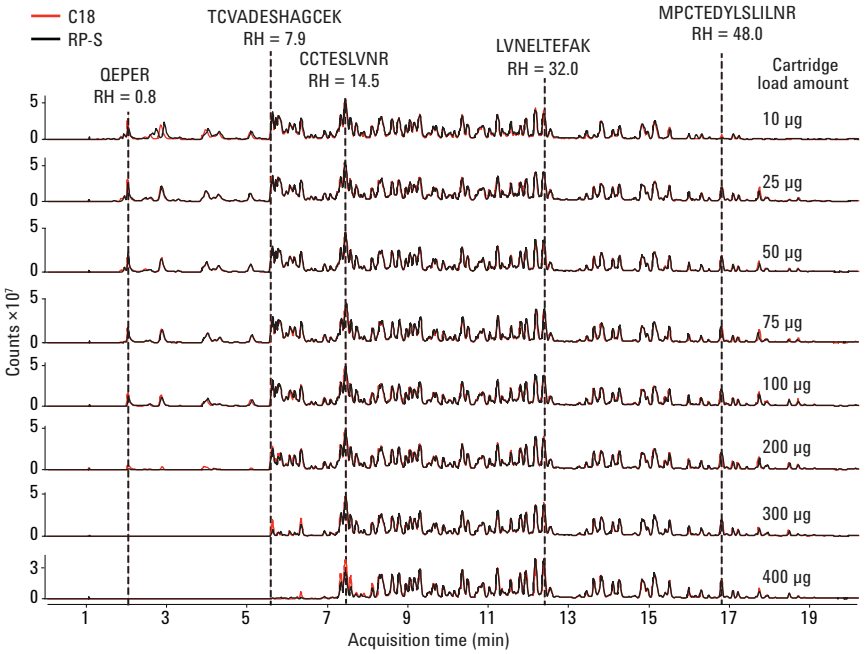


Figure 6. Peptide Cleanup load capacity. RP-S and C18 cartridges were loaded with increasing amounts of BSA digest ranging from 10-400 µg using the Peptide Cleanup protocol. Eluates were dried, reconstituted, and 1.0 µg was analyzed by LC/MS. There is little to no bias for recovery of peptides with load amounts of 25 to 100 µg regardless of relative hydrophobicity (RH). As the load amount surpasses 100 µg, hydrophilic peptides become competitively displaced by more hydrophobic peptides. With load amounts less than 25 µg, there is reduced recovery of very hydrophobic peptides.

Conclusions

The Agilent AssayMAP Bravo and its suite of tools for LC/MS sample preparation have brought a degree of reproducibility, scalability, flexibility, and ease-of-use to automation not available on any other platform. This technology minimizes hand-on time, maximizes throughput, and brings portable precision to common, yet critical, sample preparation tasks for quantitative proteomics. The In-Solution Digestion protocol allows parallel processing of 8 to 384 samples while Peptide Cleanup permits concurrent processing of 8 to 96 samples. These two protocols can be easily scaled to process tens, to hundreds, to thousands of samples per week with a single AssayMAP Bravo. Coupling together, the AssayMAP protocols for In-Solution Digestion and Peptide Cleanup yielded average peak area % CVs from 2.3–3.7 % for 25 peptides from BSA monitored across multiplate, multiday, experiments using two different denaturants. A full characterization of AssayMAP RP-S and C18 reversed-phase cartridges described important figures-of-merit including load capacity, binding characteristics, and peptide recovery for a tryptic digest of BSA. Excellent recovery of low-to-moderately hydrophobic peptides is achieved with load amounts as low as 3 µg while more than 100 µg of BSA digest can be loaded before extremely hydrophilic peptides are competitively displaced.

Reference

1. V. Spicer, *et al.*, "Sequence-specific retention calculator. A family of peptide retention time prediction algorithms in reversed-phase HPLC: Applicability to various chromatographic conditions and columns", *Anal. Chem*., 79 (22), 8762-8768, **2007**.

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