Robust Protein Quantification Enabled by a Versatile Platform for Automated Sample Preparation

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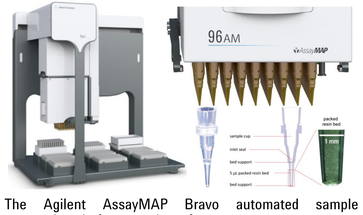
Introduction

The growing emphasis on biotherapeutics within the pharmaceutical industry has created an increasing demand for protein analysis techniques with high precision, sensitivity and throughput. In response, protein and peptide analysis has undergone a revolution due to advancements in tandem spectrometry, soft ionization techniques, bioinformatics and chromatographic separations. Analytical innovation and computational power has enabled scientists to make quantitative measurements with unprecedented sensitivity and precision. However, advancements in sample preparation have failed to match pace and continue to be highly manual. Manual sample preparation can lead to variable, user-dependent results problematic for protocol transfers, cross-laboratory comparisons, and throughput scaling. The success of protein analysis by LC/MS is critically dependent on reproducible sample preparation, but the lack of reliable, sample-scalable automation has limited the adoption of LC/MS for assays requiring large sample campaigns or increased throughput. Sample preparation methods must be developed that are robust and can scale. We present an automated sample preparation platform facilitating parallel processing of 8 to 96 samples that can be easily scaled. Coupling together, the AssayMAP protocols for In-Solution Digestion and Peptide Cleanup yielded average peak area %CVs <4% for a multiplate, multiday study of BSA.

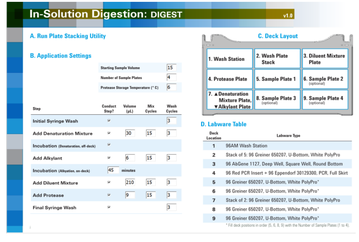
AssayMAP Bravo Technology



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 interface and protocols

AssayMAP Bravo Protocols



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:** 96 samples can be processed in parallel achieving quantitative elution in as little as 10 µL from a reversed-phase cartridge.
- **Fractionation**: 1-6 fractions can be collected in a step-wise fashion from 96 samples in parallel using ionic strength or pH elution with SCX cartridges. Reversed-phase fractionation can be accomplished at high or low pH with organic elution using reversed-phase cartridges.

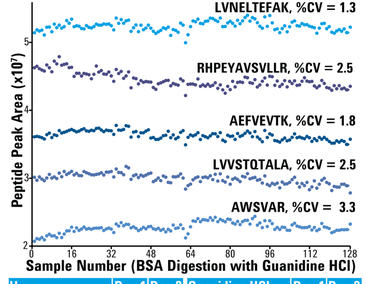
Experimental

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:

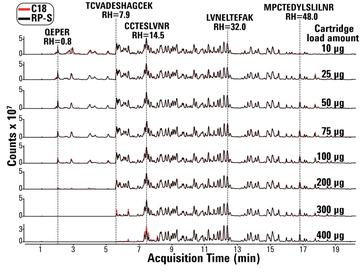
- 1290 Infinity Binary LC System
- AdvanceBio Peptide Mapping Column (2.1 x 250 mm) with LC flow rates of 400 µL/min. Gradients generally consisted of increasing ACN with 0.1% formic acid.
- 6550 iFunnel Q-TOF MS with a Dual Agilent Jet Stream source. All data were collected in auto MS/MS mode.

Figures of Merit for Automated In-solution Digestion and Peptide Cleanup



Urea	Day 1	Day 2	Guanidine HCl	Day 1	Day 2
#of samples	64	62	#of samples	64	64
#of peptides monitored	25	25	#of peptides monitored	25	25
Avg. peak area %CV	3.3	3.7	Avg. peak area %CV	2.3	2.6
# Peptides %CV <5	23	21	# Peptides %CV <5	25	23
# Peptides 5>%CV<10	2	3	# Peptides 5>%CV<10	0	1
# Peptides %CV >10	0	1	# Peptides %CV >10	0	1

Capacity of Reversed-phase Cartridges



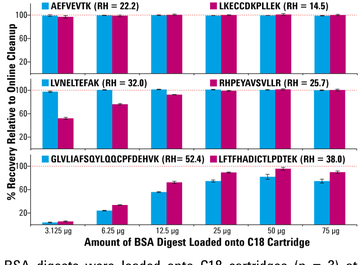
Various amounts of tryptic BSA digests were cleaned up with C18 and RPS cartridges. The TIC from these experiments show:

- 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.

Multiday, Multiplate, Digestion and Cleanup of BSA

- BSA was denatured using urea or 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



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 tested concentration (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 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%.

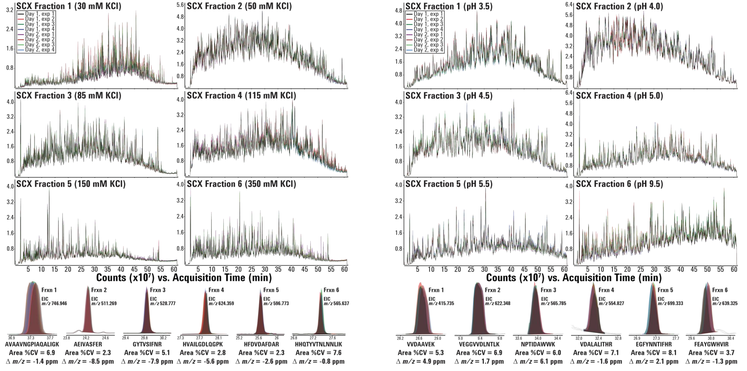
Reproducible Digestion, Fractionation, and Cleanup of Complex Mixtures

E. coli (n = 8) SCX frxn #	Avg. # of PSMs*	Avg. # of unique sequences*	Avg. # of unique sequences exclusive to frxn*	Avg. % of unique sequences exclusive to frxn
Flow thru/wash	117	83	39	47.0%
Frxn 1 (30 mM KCl)	2,853	1,544	471	30.5%
Frxn 2 (50 mM KCl)	9,035	6,424	3,471	54.0%
Frxn 3 (85 mM KCl)	7,692	5,245	1,774	33.8%
Frxn 4 (115 mM KCl)	9,011	5,424	2,221	40.9%
Frxn 5 (150 mM KCl)	5,858	3,538	1,055	29.8%
Frxn 6 (350 mM KCl)	5,711	3,195	1,200	37.6%
Summary	38,345	15,241	10,231	67.1%

*1% FDR

E. coli (n = 8) SCX frxn #	Avg. # of PSMs*	Avg. # of unique sequences*	Avg. # of unique sequences exclusive to frxn*	Avg. % of unique sequences exclusive to frxn
Flow thru/wash	178	109	44	40.4%
Frxn 1 (pH = 3.5)	7,157	3,678	1,686	45.8%
Frxn 2 (pH = 4.0)	11,001	6,544	2,933	44.8%
Frxn 3 (pH = 4.5)	8,020	5,083	1,502	29.5%
Frxn 4 (pH = 5.0)	8,977	4,997	1,371	27.4%
Frxn 5 (pH = 5.5)	7,503	4,214	730	17.3%
Frxn 6 (pH = 9.5)	9,023	4,709	1,580	33.6%
Summary	51,841	15,335	9,845	64.2%

*1% FDR



Complex Mixture Analysis. *E. coli* protein lysate (~105 µg) was digested on two different days followed by cleanup with C18 cartridges (20 µL elution) and a 10x dilution in SCX equilibration buffer. Fractions were eluted from SCX cartridges in 20 µL steps yielding 6 fractions plus the flow through. Fractions from ionic strength elution were diluted 10x, subjected to C18 cleanup to remove nonvolatile salts, and lyophilized. Fractions from pH elution contained volatile buffers and were lyophilized directly.

Fractions were separated over a 60-min gradient. On average, each replicate yielded over 38,000 peptide-spectrum matches (PSMs) and 15,000 unique peptide sequences for SCX fractionation by ionic strength (filtered to a 1% false discovery rate). Nearly 52,000 PSMs and more than 15,000 unique peptide sequences were identified using SCX fractionation by pH. In total, 64-67% of the unique peptide sequences, ~10,000 for each technique, 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 cleanup yield %CVs ≤ 8.1% without the use of internal standards.
- Small elution volumes minimize or eliminates the need for dry down after each step 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.