Rapid Antibody Digestion Enabled by Automated Reversed-Phase Desalting on the Agilent AssayMAP Bravo Platform

Application Note

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Introduction

Monoclonal antibodies are a rapidly growing class of biotherapeutics whose rigorous characterization by mass spectrometry requires reproducible denaturation and digestion. One of the major bottlenecks in this workflow is the trypsin digestion step, which typically requires an overnight incubation. This long incubation significantly delays decisions required to move projects forward. The slow digestion is mainly due to the presence of denaturant in the digestion reaction. The time required for digestion can be significantly decreased by removing, rather than simply diluting, the denaturant with size exclusion chromatography (SEC)[1]. Although effective, SEC is labor-intensive and difficult to automate. An alternative approach for removing denaturant is reversed-phase chromatography, which is more amenable to automation. This Application Note presents the development of an automated reversed-phase whole protein desalting solution on the AssayMAP Bravo that decreases the time required for trypsin digestion of denatured antibodies to as little as 1 hour. When this application is combined with the Affinity Purification and In Solution Digestion applications on the AssayMAP Bravo, one can automate the purification, denaturation, reduction, alkylation, cleanup, and digestion of antibodies. The AssayMAP platform can thereby reduce the time required for a workflow that begins with affinity purification of antibodies from complex matrices and ends with digested peptides ready for LC/MS analysis to less than 8 hours.

Agilent Technologies

AssayMAP Bravo Platform

The AssayMAP Bravo platform consists of easy-to-use yet flexible software, the Bravo liquid handler with an AssayMAP head, and 5-µL packed-bed microchromatography cartridges. The AssayMAP software contains pre-optimized protocols for LC/MS sample preparation workflows designed to give bench scientists access to the power of automation without requiring them to become automation experts. The AssayMAP head contains 96 probe syringes, which allows eight to 96 samples to be processed in parallel. Each syringe probe forms a liquid/liquid contact with the top of the cartridge resin bed, allowing positive displacement control of fluid movement across the resin bed which, combined with the precise liquid handling capabilities of the Bravo, enables chromatographic performance, such as quantitative binding, efficient washing, and low elution volumes, in a highly parallel format. The final component of the AssayMAP platform is the cartridge, which can be packed with a variety of resins to automate sample preparation for a wide range of LC/MS workflows. The cartridges are packed to exacting specifications that ensure packed-bed consistency and high reproducibility.

The AssayMAP platform supports protein quantification and characterization workflows including affinity purification, enzymatic digestion, protein and peptide cleanup, peptide mapping, peptide fractionation, N-Glycan analysis, and phosphopeptide enrichment. The software for running each of these workflows comes with default settings to allow the workflow to be rapidly implemented, with flexibility that accommodates optimization if required. This Application Note demonstrates how to optimize the critical parameters for denatured antibody desalting using AssayMAP RP-W cartridges (reversed-phase, wide pore).

Experimental Methods

The Protein Cleanup application, like most AssayMAP applications, has five major steps: cartridge priming, equilibration, sample loading, cartridge washing, and elution of the target analyte. Parameters for each of these steps were optimized using RP-W cartridges and human IgG1 (hIgG1) as a model antibody. This Application Note may be used as a guide for the optimization of conditions for other proteins of interest in case the conditions shown here do not give satisfactory results.

Materials

Antibodies were purchased from Sigma-Aldrich (SILuLite) and Athens Research and Technology (hIgG1 and hIgG polyclonal). Guanidine, TCEP, iodoacetamide, trifluoroacetic acid, and ammonium bicarbonate were purchased from Sigma-Aldrich. Tris base and Tris HCl were purchased from EMD Millipore. Zeba Spin desalting plates were purchased from Thermo Scientific, and used according to the manufacturer's instructions. Cell-free conditioned medium from a Chinese Hamster Ovary (CHO) cell culture was purchased from Aldevron and stored in frozen aliquots at –20 °C. Sequencing grade trypsin was purchased from Agilent Technologies.

Table 1. Instrumentation and consumables.

Automation	
Platform	Agilent AssayMAP Bravo (G5542BA)
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) Orbital Shaking Station w/control Unit (G5498B#033)
Additional accessories	Agilent PlateLoc Thermal Microplate sealer (G5402A)
Cartridges	PG-W (G5496-60008) RP-W (G5496-60086)
Analytical instrumentation	
Mass spectrometer	Agilent 6550 iFunnel Q-TOF LC/MS, Dual Agilent Jet Stream ESI
LC systems and columns	Agilent 1290 Infinity LC System
	Agilent 1260 Infinity Bio-Inert LC System
	Agilent Poroshell 300SB-C18, 2.1 × 75 mm, 5 µm (660750-902)
	Agilent AdvanceBio Peptide Mapping, 2.1 × 250 mm, 2.7 µm (651750-902)
	Agilent AdvanceBio Peptide Mapping Fast Guard, 2.1 × 5 mm (851725-911)

Antibody denaturation, reduction, alkylation, and dilution

Denaturation and reduction was performed by adding three volumes of 8 M guanidine, 13.3 mM TECP, and 500 mM Tris pH 8.0 to the antibody sample to give a final concentration of 6 M guanidine, 10 mM TCEP, and 375 mM Tris pH 8.0. The samples were incubated for 60 minutes at 60 °C. Following denaturation and reduction, samples were alkylated by adding the appropriate amount of iodoacetamide to the denatured and reduced antibody sample to give a final concentration of 20 mM. This solution was incubated for 30 minutes at ambient temperature protected from light. The sample was then acidified and diluted to 2.5 M guanidine with 1.75 % TFA (1 % TFA final), unless otherwise noted, in preparation for binding to RP-W cartridges. Reactions were prepared in bulk for Figures 2–13, which focus on cartridge performance and comparison to other methods for reducing denaturant concentration.

Individual reactions were prepared for Figure 14, which focuses on a complete workflow using three AssayMAP applications: Affinity Purification, Protein Cleanup, and In-Solution Digestion: Single Plate.

Running the Protein Cleanup application

Unless otherwise indicated, the Protein Cleanup application was run in two sequential runs to minimize the time that the eluent was on the deck and susceptible to evaporation, as changes in the organic concentration can negatively impact recovery (Figure 8). The majority of the total run time for the Protein Cleanup takes place during the first of these two sequential runs. During the first run, the default settings (Figure 1) were used for Initial Syringe Wash, Prime (100 µL of 60 % acetonitrile, 0.1 % TFA at 300 µL/min), Equilibrate (50 µL of 0.1% TFA at 10 µL/minute), Load Sample (variable volume depending on the experiment at 5 µL/min), Cup Wash (50 µL of 0.1 % TFA), and Internal Cartridge Wash (50 µL of 0.1 % TFA at 10 µL/min), but the Stringent Syringe Wash, Elution, and Final Syringe Wash steps were turned off. At the end of this run, the deck was not altered except to add the eluent plate. The second run of the Protein Cleanup protocol, which only takes approximately 5 minutes and immediately follows the first run, had the Initial Syringe Wash, Prime, Equilibrate, Load Sample, Cup Wash, and Internal Cartridge Wash steps turned off. The second run used default settings for the Stringent Syringe Wash (50 µL of 60 % acetonitrile, 0.1% TFA unless indicated otherwise) and Final Syringe Wash. The Elution settings were 15 µL (60 % acetonitrile, 0.1 % TFA at 5 µL/min unless indicated otherwise) for Volume, and 2 µL (dispensed to waste) for Eluate Discard, which resulted in a total eluent volume of 13 µL. The Existing Volume was set to 143 µL (0.1 % TFA for Figures 2–11, or 50 mM ammonium bicarbonate for Figures 12–14).

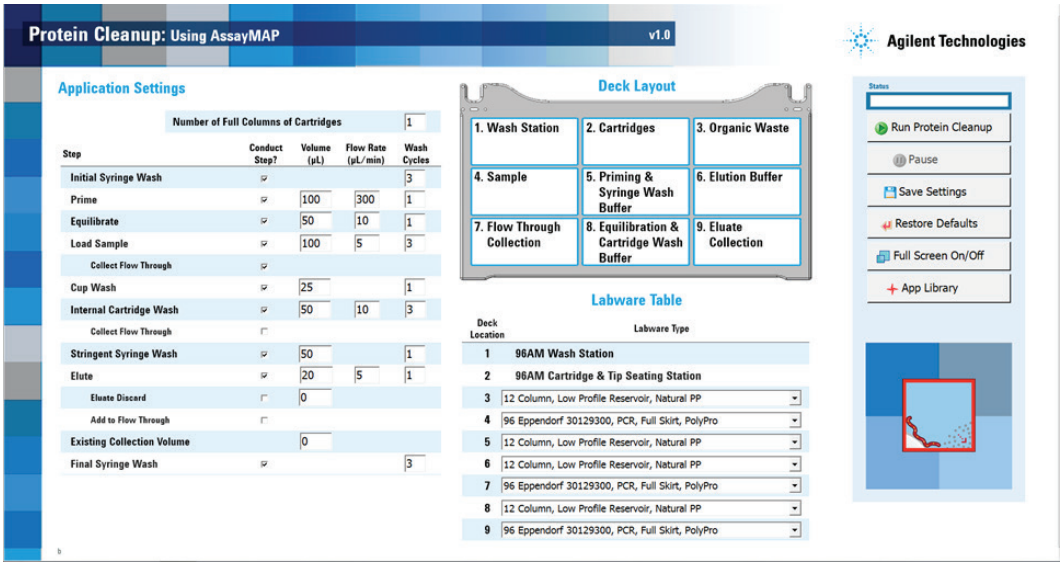


Figure 1. Protein Cleanup application user interface with default settings.

Binding capacity under optimized conditions

Dynamic binding capacity was determined using hIgG1 that was serially diluted in water, then denatured, reduced, alkylated, and diluted. The RP-W cartridges were primed, equilibrated, loaded with a serial dilution of sample (N = 3 per hIgG1 mass load), washed, and eluted using the Protein Cleanup application. Antibody recovered from RP-W cartridges was quantified using an Agilent 1260 Bio-Inert HPLC fitted with an Agilent Poroshell 300 SB-C18 column, on the basis of absorbance at 214 nm relative to a standard curve. Figure 2 shows the results.

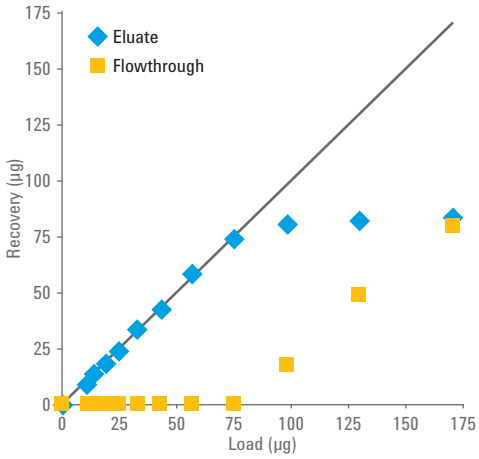


Figure 2. Quantitative recovery of denatured antibodies using RP-W cartridges under optimized conditions. An hIgG1 antibody was serially diluted, denatured, reduced, alkylated, and adjusted to 2.5 M guanidine and 1 % TFA. The samples were loaded onto RP-W cartridges (n = 3 per data point). The cartridges were washed, and the bound antibody was eluted. Flowthroughs and eluates were collected and quantified. Error bars represent ±1 S.D. The identity line is shown in gray for reference.

Prime optimization

The concentration of acetonitrile required to prime the RP-W cartridges was determined. The Protein Cleanup application was used as described above except that RP-W cartridges were primed with 0–100 % acetonitrile in triplicate. A 30 µg amount of denatured, reduced, alkylated, and diluted hIgG1 was cleaned up per cartridge. Antibody in the eluate and flowthrough was quantified as described above. Figure 3 shows the results.

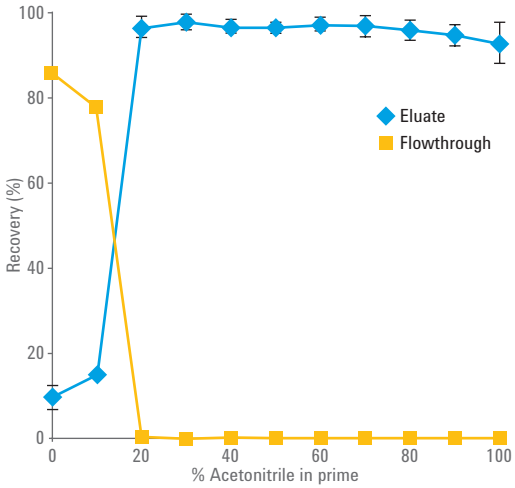


Figure 3. Optimization of the priming solution. RP-W cartridges were primed with increasing concentrations of acetonitrile, and equilibrated in 0.1 % TFA. Denatured, reduced, alkylated, and diluted hIgG1 was loaded onto RP-W cartridges (n = 3 per data point). The cartridges were washed, and the bound antibody was eluted. Flowthroughs and eluates were collected and quantified. Error bars represent ±1 S.D.

Load sample optimization

The effects of guanidine concentration, alkylation, sample volume, eluent evaporation, and flow rate were examined.

The effect of guanidine concentration in the sample

Human IgG1 was serially diluted in water, then denatured, reduced, alkylated, and diluted. The Protein Cleanup application was run three times (n = 3 per sample per run), once for each guanidine concentration of 5.0, 2.5, or 1.25 M with respective load volumes of 155, 310, or 620 µL. Antibody in the eluate and flowthrough was quantified as described above. Figure 4 shows the results.

The effect of sample alkylation

SILuLite antibody was denatured, reduced, then incubated in the presence or absence of iodoacetamide as described above. A 40 µg amount of these samples was diluted as described and loaded onto RP-W cartridges. The cartridges were washed, and the bound antibody was eluted using 50, 60, or 70 % acetonitrile in 0.1 % TFA. The eluate was quantified by HPLC. Figure 5 shows the results.

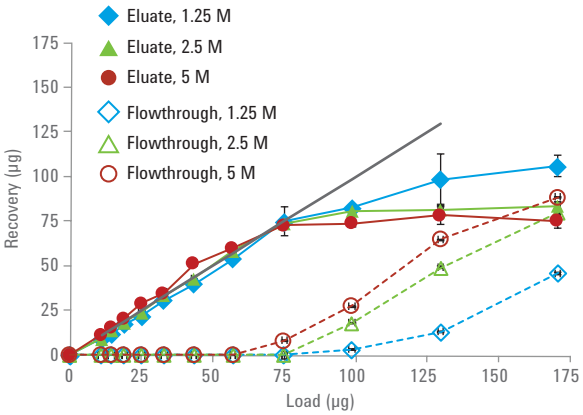


Figure 4. Effect of guanidine concentration on recovery. A serial dilution of hIgG1 was denatured, reduced, alkylated, and diluted to 1.25, 2.5, or 5.0 M guanidine. The samples were loaded onto RP-W cartridges (n = 3 per mass load). The cartridges were washed, and bound antibody was eluted. Eluates (closed symbols, solid lines) and flowthroughs (open symbols, dashed lines) were collected and quantified. Error bars represent ±1 S.D. The identity line is shown in gray for reference.

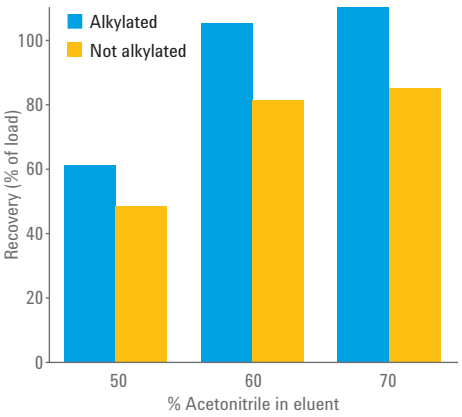


Figure 5. Effect of alkylation on recovery. SILuLite antibody was denatured, reduced, and either alkylated or not alkylated before loading 40 µg onto duplicate RP-W cartridges. Bound antibody was eluted with varying acetonitrile concentrations in 0.1 % TFA. Eluates were collected and quantified.

The impact of sample load volume and eluent evaporation

Human IgG1 was denatured, reduced, alkylated, and diluted as described above. The samples were further diluted with 2.5 M guanidine to volumes that would allow 25, 50, and 75 µg to be loaded in total sample volumes of 200, 300, or 1,000 µL. In this way, the 25, 50, and 75 µg mass loads could be applied to the RP-W cartridges in different total sample volumes while maintaining the guanidine concentration at 2.5 M in all samples. These volumes correspond to the maximum capacities of the labware for AssayMAP applications. The Protein Cleanup application was run once for each load volume as described above except that two elution conditions and two sample load flow rates were tested. The eluent was placed on the AssayMAP deck immediately before the elution step (Figure 6A) or at the start of the run (Figures 6B and 6C). The sample load flow rate was 5 µL/min for Figures 6A and B, whereas it was 20 µL/min in Figure 6C. Antibody in the eluate and flowthrough was quantified as described above.

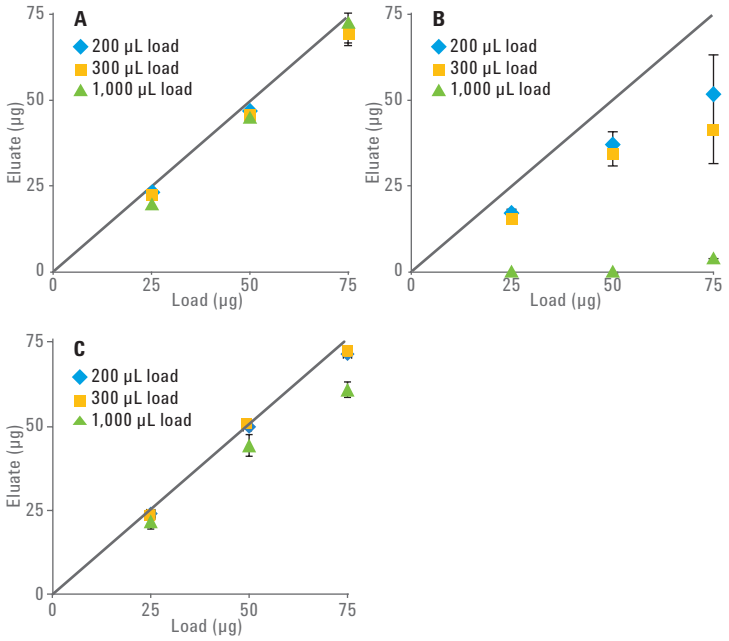


Figure 6. Effect of sample volume and eluent evaporation on recovery. Human IgG1 was denatured, reduced, alkylated, and diluted to 200, 300, or 1,000 µL. The samples were loaded onto RP-W cartridges (n = 3 per data point) at 5 µL/min (Panels A and B) or 20 µL/min (Panel C). The cartridges were washed, and the bound antibody was eluted. The eluates were collected and quantified. The panels compare recoveries when the eluent was placed on the deck immediately before the elution step (A), or placed on the deck for the entire run (Prime, Equilibrate, Load, and Wash steps) (B and C), and was therefore susceptible to evaporation. Error bars represent ±1 S.D.

The effect of sample loading flow rate

Human IgG1 was serially diluted, denatured, reduced, alkylated, and diluted. The samples were loaded onto RP-W cartridges at 2, 5, 10, or 20 µL/min in separate runs using the Protein Cleanup application. For each run, the cartridges were washed and eluted as decribed. Antibody in the eluate and flowthrough was quantified. Figure 7 shows the results.

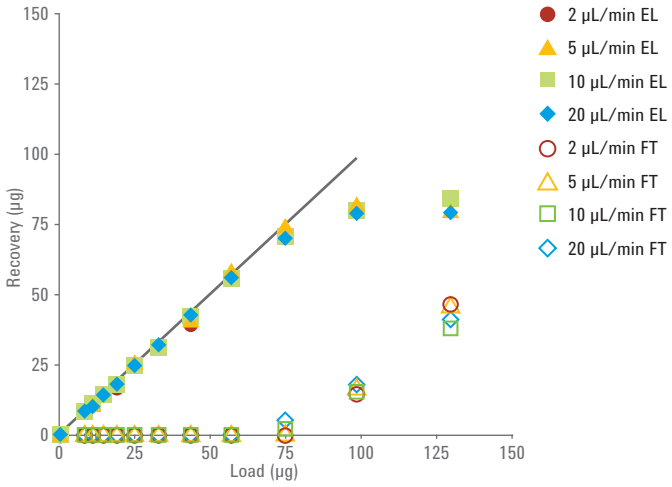


Figure 7. Effect of the sample loading flow rate on recovery. A serial dilution of hIgG1 was denatured, reduced, and alkylated. The samples were loaded onto RP-W cartridges (n = 3 per condition) at sample load flow rates of 2 µL/min (red symbols), 5 µL/min (yellow), 10 µL/min (green), and 20 µL/min (blue). Cartridges were washed, and the bound antibody was eluted. The identity line is shown in gray for reference. Eluates (closed symbols, EL) and flowthroughs (open symbols, FT) were collected and quantified.

Elution optimization

The effect of eluent pH and acetonitrile concentration was evaluated. Human IgG1 was denatured, reduced, and alkylated. A 50 µg amount of this sample was loaded onto RP-W cartridges using the Protein Cleanup application as described above except that the cartridges (n = 3 per elution condition) were eluted with 40, 50, 60, or 70 % acetonitrile in either 0.1 % TFA (pH 3) or 50 mM ammonium bicarbonate (pH 8). Eluted antibody was quantified as described above. Figure 8 shows the results.

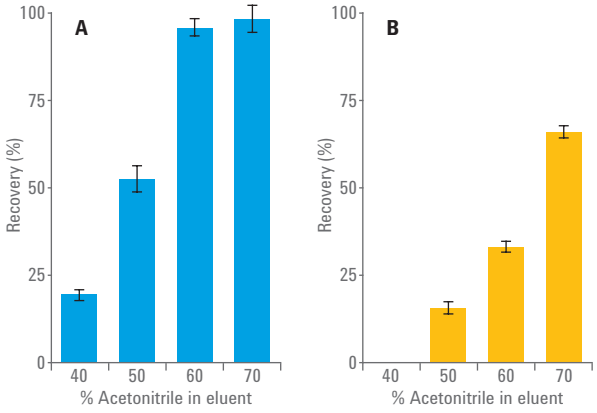


Figure 8. Effect of eluent composition on recovery. Human IgG1 was denatured, reduced, and alkylated. The samples were loaded onto RPW cartridges (n = 3 per condition). The cartridges were washed, and the bound antibody was eluted with various acetonitrile concentrations in (A) 0.1 % TFA, pH 3, or (B) ammonium bicarbonate, pH 8. Error bars represent ±1 S.D.

The minimum volume required for elution from RP-W cartridges was examined. Human IgG1 was denatured, reduced, alkylated, and diluted as described. A 50 µg amount of this sample was loaded onto eight RP-W cartridges using the Protein Cleanup application. The cartridges were washed, and elution was performed at 5 µL/min with 60 % acetonitrile in 0.1 % TFA, using a special protocol that dispensed eluent through cartridges in 12 sequential 2-µL fractions into 48 µL of 0.1 % TFA in the elution plate wells. Eluted antibody was quantified as described above. Figure 9 shows the results.

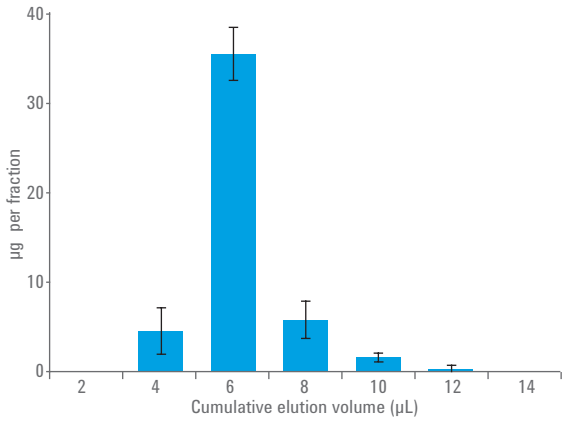


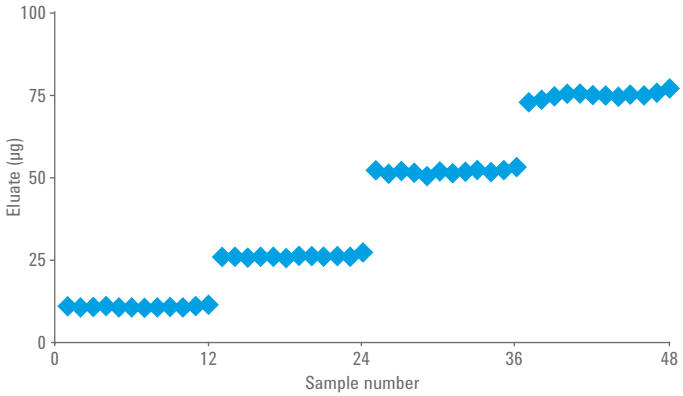
Figure 9. Minimum required elution volume. Human IgG1 was denatured, reduced, and alkylated. Samples were loaded onto RP-W cartridges (n = 8 per data point). The cartridges were washed, and bound antibody was eluted in 2-µL fractions using a custom elution protocol with 48 µL of diluent added in advance to each well of the elution plate. Eluted antibody in the 50-µL fractions was quantified. Error bars represent ±1 S.D.

Protein cleanup reproducibility and recovery

Various concentrations of hIgG1 were denatured, reduced, alkylated, and diluted. The Protein Cleanup application was used in combination with RP-W cartridges to clean up 10, 25, 50, and 75 µg of these samples (n = 12 for each loaded mass). Eluted mass was quantified and plotted with respect to loaded mass. CVs and recoveries for each set of 12 replicates are reported in the table insert (Figure 10).

Protein cleanup recovery of various hIgG antibodies

Various concentrations of three commercially available hIgG antibodies were denatured, reduced, alkylated, and diluted. Amounts of 25, 50, or 75 µg of these samples were desalted on RP-W cartridges (n = 3) using the Protein Cleanup application. Eluted antibodies were quantified as described. Figure 11 shows the results.



Samples	Load (µg)	% Recovery	% CV
1–12	10	104	2.6
13–24	25	103	1.8
25–36	50	103	1.3
37–48	75	99	1.4

Figure 10. Reproducibility and recovery. Various concentrations of hIgG1 were denatured, reduced, and alkylated. Twelve samples at each concentration were loaded onto RP-W cartridges, the cartridges were washed, and the bound antibodies were eluted and quantified. CVs and recovery were determined for each set of 12 replicates (summarized in the inset table).

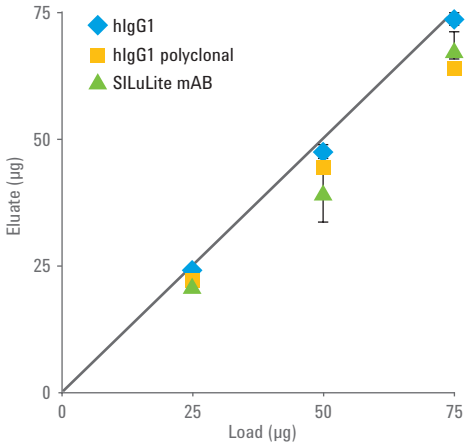


Figure 11. Recovery of three different hIgG antibodies. Various concentrations of the hIgG1, hIgG polyclonal, and SILuLite antibodies were denatured, reduced, and alkylated. Samples containing 25, 50, or 75 µg were loaded onto RP-W cartridges (n = 3 per condition). Eluted antibody was quantified. Error bars represent ±1 S.D. The identity line is shown in gray for reference.

Trypsin digestion time course

Human IgG1 was denatured, reduced, alkylated, then either diluted to 0.9 M guanidine, desalted using RP-W cartridges, or desalted using an SEC 96-well spin plate. SEC samples were processed according to the instructions provided by the manufacturer, without further optimization. A 25 µg amount of these diluted or desalted samples were digested with trypsin in quadruplicate for 0.5, 1, 2, 4, or 18 hours at 37 °C. The reactions were stopped at each time point by the addition of 10 % TFA to a final concentration of 1 %, then refrigerated. Peptides in digestion reactions that contained 0.9 M guanidine were desalted using the AssayMAP Peptide Cleanup application and C18 cartridges. All samples were adjusted to the same protein concentration of approximately 150 µg/mL, and 0.5 µg injections were analyzed on an Agilent 1290 HPLC and an Agilent 6550 Q-TOF mass spectrometer as described below. Figure 12 shows Total Ion Chromatograms (TICs) for each desalting method. Twelve peptides were quantified at each digestion time point based on MS1 peak area for each desalting method. Figure 13 shows the results.

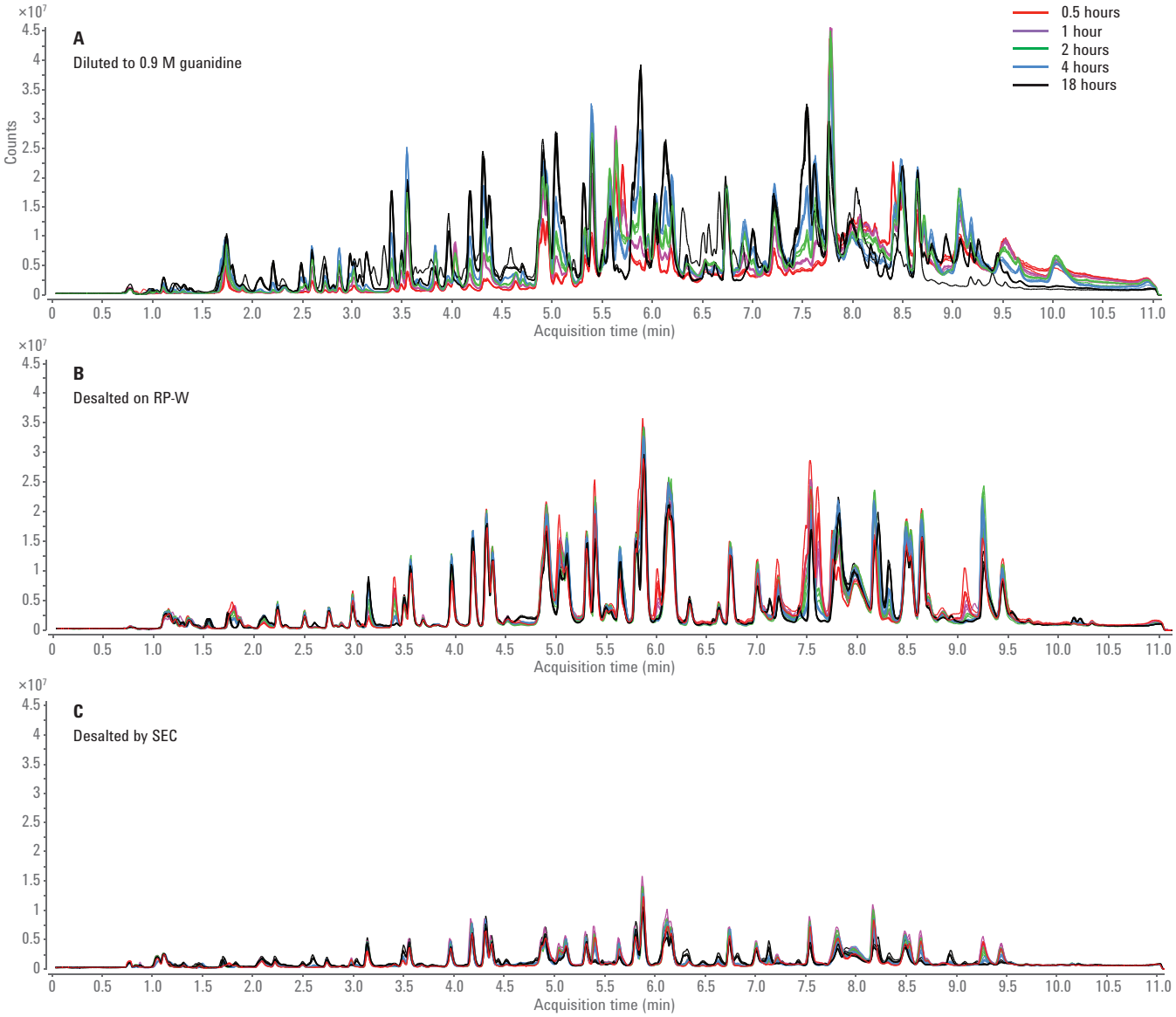


Figure 12. Qualitative analysis of an antibody digest time course. Human IgG1 was denatured, reduced, and alkylated, and samples were: A) diluted to 0.9 M guanidine; B) desalted using RP-W cartridges and eluted into 50 mM ammonium bicarbonate (ABC) and 5 % acetonitrile final; C) desalted into 50 mM ABC using size exclusion chromatography. Following these various treatments, the samples were trypsin digested for 0.5 hours (red trace), 1 hour (purple), 2 hours (green), 4 hours (blue), or 18 hours (black). TICs of replicates (n = 4) at each time point were overlaid using Agilent MassHunter, to illustrate the digest progression for each guanidine concentration reduction method.

Workflow reproducibility

A workflow beginning with antibody purification and ending with LC/MS quantification of digested peptides was executed using AssayMAP automated sample preparation. Thirty two samples of serum-free cell culture supernatant from a nonantibody-expressing CHO cell culture were spiked with hIgG1 (n = 16 samples) or Sigma SILuLite (n = 16). The antibodies were purified from the matrix using the AssayMAP Bravo Affinity Purification application and protein G (PG-W) cartridges using optimal binding conditions previously described (Bovee, *et al.*)[2]. Briefly, the PG-W cartridges were loaded with the samples and washed with PBS, then the purified antibodies were eluted with 10 µL of 5 % acetic acid directly into a 96-well PCR plate containing 30 µL of denaturation solution (8 M guanidine, 13.3 mM TCEP, 0.5 M Tris pH 8) in each well for a final concentration of 6 M guanidine, 10 mM TCEP, and 0.375 M Tris pH 8. The eluate was automatically mixed by the AssayMAP Bravo at the end of the run. The eluate plate was removed from the deck, sealed, and incubated for 60 minutes at 60 °C to denature and reduce the purified antibodies. The plate was allowed to cool at ambient temperature for 5 minutes before centrifugation for 1 minute at 500xg to collect condensation. The sample plate and a plate containing iodoacetamide were placed on the AssayMAP Bravo deck with opaque lids along with a nonlidded plate containing post-alkylation diluent. The In-Solution Digestion: Single Plate application was used to alkylate then dilute the denatured antibodies by removing the lids from the sample plate and iodacetamide plate with the gripper arm, aspirating 5 µL of 180 mM iodoacetamide per syringe from the iodacetamide plate, adding the iodoacetamide to the samples (20 mM final concentration), mixing, relidding both plates, and incubating for 30 minutes at ambient temperature. After alkylation was complete, the gripper arm removed the lid from the sample plate, 51 µL of diluent (1.9 % TFA) was aspirated from the diluent plate and added to the samples to give a final concentration of 2.5 M

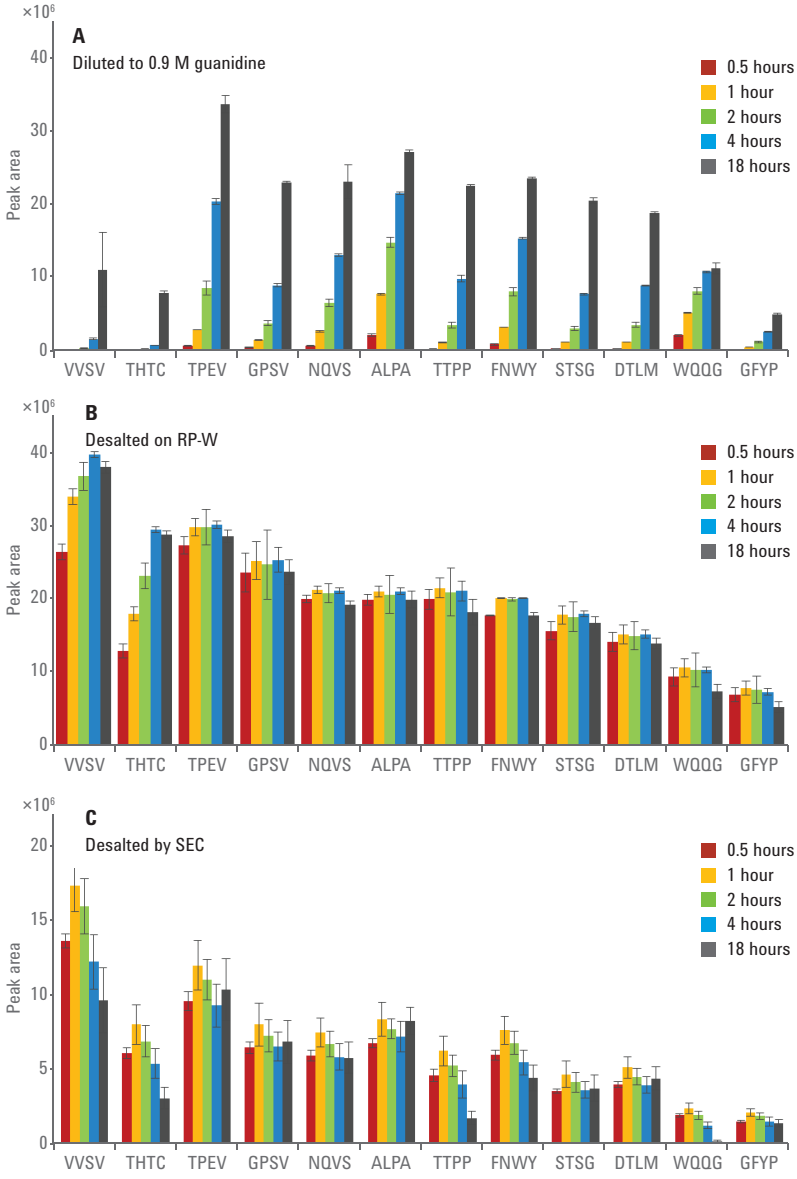


Figure 13. Quantitative analysis of an antibody digest time course. Human IgG1 was denatured, reduced, alkylated, then treated in one of three ways: A) The sample was diluted to 0.9 M guanidine; B) the sample adjusted to 2.5 M guanidine and 1 % TFA, desalted using RP-W cartridges, and diluted to 5 % acetonitrile; C) the sample was desalted using size exclusion chromatography. (The Y-axis for C was scaled for easier visual comparison with A and B.) Following these various treatments, the samples were trypsin digested for 0.5 hours (red trace), 1 hour (yellow), 2 hours (green), 4 hours (blue), or 18 hours (black). All 12 of the conserved peptides were quantified based on MS1 peak area. Error bars represent ±1 S.D. Peptides ranged from 7–26 amino acids in length, but are abbreviated to the first four amino acids for simplicity. Complete peptide sequences are shown in Figure 14D.

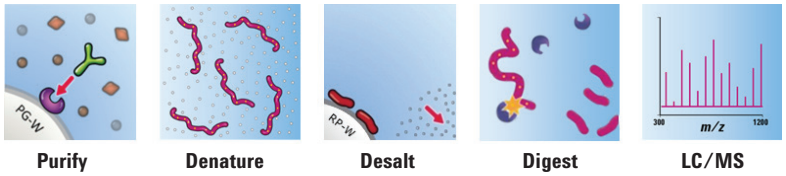
guanidine and 1 % TFA, the samples were mixed, and the lid was placed back on the sample. This sample plate became the load plate for the Protein Desalting application, which was run as described above. Finally, the In-Solution Digestion application was used to initiate the digestion step through the addition of trypsin (1:25 final by mass) to each sample. The protocol was automatically paused to allow the plate to be sealed, a 1-hour on-deck incubation at 37 °C was performed, the seal was removed, and the protocol was completed by adding 20 µL of 10 % TFA to the samples to inactivate the trypsin (1 % TFA final). Peptides were analyzed as described below. Figure 14 shows the results.

LC/MS data acquisition and analysis

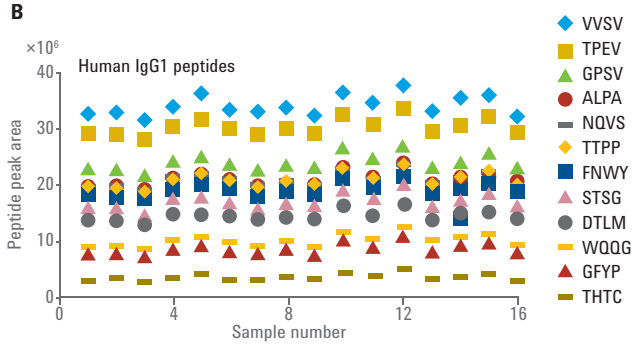
Peptides were separated with an AdvanceBio Peptide Mapping C18 column (2.1 × 150 mm, 2.7 µm) at a flow rate of 0.4 µL/min with a 20-minute method (from 5 to 40 %B over 10 minutes; A = 0.1 % formic acid in water, B = 0.1 % formic acid in ACN). MS scans were acquired over the mass range 100–1,700 *m/z* at a scan rate of 8 spectra/sec. MS/MS scans were acquired over the mass range of 100–1,700 *m/z* at a scan rate of 3.5 spectra/sec. The three most intense precursor peaks were selected for auto MS/MS fragmentation. Dynamic exclusion was set for 12 seconds.

For the hIgG1 and SILuLite antibodies analyzed by LC/MS in this study, the amino acid sequence was only available for the SILuLite antibody. Since hIgG1 antibodies have highly conserved sequences, MS/MS spectra from both antibodies were searched against the SILuLite sequence using SpectrumMill (Agilent Technologies). For peptide identification, a mass tolerance of 10 ppm was permitted for intact peptide masses, and 50 ppm for CID-fragmented ions, with allowance for two missed cleavages in the trypsin digests, oxidized methionine, and deamidated asparagine as potential variable modifications. Carboxyamidomethylation (C) was set as a fixed modification.

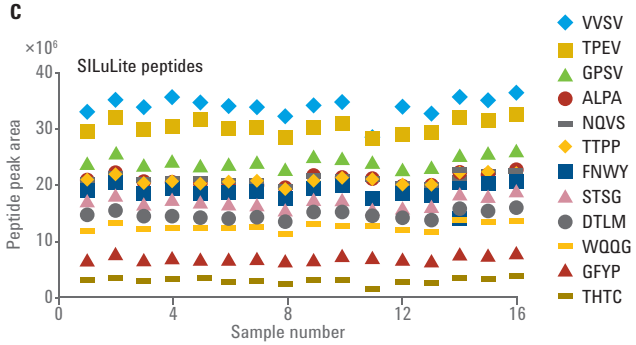
A



B



C



D

Peptide	% CV hIgG1	% CV SILuLite
VVSVLTVLHQDWLNGK	5.5	5.4
TPEVTCVVVDVSHEDPEVK	5.2	4.1
GPSVFPLAPSSK	6.3	4.2
ALPAPIEK	6.3	4.6
NQVSLTCLVK	6.4	4.3
TTPPVLD	7.0	4.3
FNWYVDGVEVHNAK	6.3	5.0
STSGGTAALGCLVK	8.4	6.1
DTLMISR	7.2	5.0
WQQGNVFSCSVMHEALHNHYTQK	11.2	5.7
GFYPSDIAVEWESNGQPENNYK	12.5	6.5
THTCPPCPAPELLGGPSVFLFPPKPK	18.2	17.1

Figure 14. Reproducibility of an antibody sample prep workflow using the Agilent AssayMAP Bravo. A) Icons illustrate the main steps of the biotherapeutic antibody sample prep workflow that was run to generate the data in this figure using the AssayMAP Bravo. Human IgG1 or SILuLite (n = 16 for each antibody) were processed in parallel. Each antibody type was purified from a CHO cell-free matrix using PG-W cartridges, denatured, reduced, alkylated, desalted on RP-W cartridges, and digested for 60 minutes using the AssayMAP Bravo. The resulting peptides were analyzed by LC/MS. Peak areas for the 12 fully tryptic peptides are shown in graphs B and C. CVs for individual tryptic peptides are summarized in the table (D). Peptide abbreviations are underlined in the table.

Twelve peptides were quantified using MS1 peak integration. M, M+H, and M+2H precursor mass for each peptide were extracted and integrated using skyline.

Results and Discussion

Trypsin digestion is a key component of the sample preparation for many protein quantification and characterization workflows, yet it remains a major bottleneck, often requiring overnight incubation for complete digestion. It has been reported that even small amounts of guanidine hydrochloride greatly inhibit trypsin activity, and removal of guanidine results in nearly complete digestion in as little as 30 minutes (Ren, *et al.*, Analytical Biochemistry 392:12, 2009[1]). Desalting proteins to hasten trypsin digestion typically involves SEC, which is difficult to perform in an automated fashion, especially for large numbers of samples or large volumes. Desalting by reversed-phase can address these limitations because analytes are retained on the solid phase while salts and matrix components flow through. This approach uses much smaller resin bed volumes, simplifying automation and sample volume control.

Optimized RP-W cartridge performance

Reversed-phase desalting of denatured, reduced, alkylated, and diluted antibodies using the Protein Cleanup application, RP-W cartridges, and a hIgG1 under optimal conditions is shown in Figure 2. Briefly, a serial dilution of human IgG1 antibody was denatured and reduced with guanidine and TCEP, respectively, followed by alkylation with iodoacetamide. Samples were then diluted and acidified before loading onto AssayMAP RP-W cartridges. The cartridges were washed, and bound antibody was eluted and diluted to reduce the acetonitrile concentration prior to quantification by HPLC. Figure 1 shows the Protein Cleanup application with default settings. These settings can be used as shown for protein cleanup, but the highest yield and reproducibility for large volume samples will be obtained if the protocol is run using a load flow rate up to 20 µL/min or by placing the eluate on the deck immediately before the elution step (see Figure 6) to minimize evaporation altering the concentration of the eluent. Figure 2 shows that RP-W cartridges can quantitatively bind and elute up to 75 µg of denatured, reduced, and alkylated antibody per RP-W cartridge with less than 10 % breakthrough.

Prime optimization

AssayMAP cartridges are shipped with dry resin, and require a priming step to prepare them for use. The priming step uses a rapid flow rate to purge entrained air from the resin bed. RP-W cartridges require priming with a concentration of organic solvent sufficient to remove all air, and condition the surface of the resin. This step is essential for maximum capacity and reproducibility. To determine the minimum effective acetonitrile concentration for priming, RP-W cartridges were primed with a range of acetonitrile concentrations, equilibrated in 0.1 % TFA, loaded with denatured, reduced, and alkylated hIgG1, washed, and eluted directly into 11 volumes of diluent. The final dilution step was included to reduce the organic concentration to 5 % prior to quantification by HPLC. As shown in Figure 3, RP-W cartridges must be primed with at least 20 % acetonitrile to quantitatively recover antibody during the elution step. Since elution also requires greater than 20 % acetonitrile (see below), reagent preparation is simplified when the same solution is used for priming and elution.

Load sample optimization

Sample loading parameters that were investigated include guanidine concentration, alkylation, sample load volume, and flow rate. The effect of sample dilution on recovery from RP-W cartridges was tested using a serial dilution of denatured, reduced, alkylated hIgG1 diluted to 5.0, 2.5, or 1.25 M guanidine. The RP-W cartridges were loaded with sample, washed, and the bound antibody was eluted. Sample dilution had little effect on recovery up to approximately 75 µg. Dilution is necessary for maximum binding capacity, but this requires a larger sample volume and a correspondingly longer time to load the samples (Figure 4).

The effect of alkylation on the binding of denatured and reduced antibodies was examined by denaturing, reducing, and either alkylating with iodoacetamide, or not alkylating a serial dilution of hIgG1. The samples were loaded onto RP-W cartridges. The cartridges were washed and eluted with various concentrations of acetonitrile. The results in Figure 5 show that alkylation is required for the best recovery under the standard conditions used in this study. The magnitude of this effect is antibody-dependent (data not shown). Alkylation has the effect of reducing the hydrophobicity of modified cysteines[3]. Nonalkylated proteins may require optimized conditions different from those described here for alkylated antibodies.

The effect of sample volume and eluent evaporation on recovery was tested with 25, 50, or 75 µg hIgG1 that had been denatured, reduced, alkylated, and diluted to 200, 300, or 1,000 µL. These volumes reflect the capacity limits of the three 96-well labware options: PCR plate, round-bottom plate, and 1 mL deepwell plate, respectively. The samples were desalted using a two-step Protein Cleanup protocol where the eluent was added immediately before elution, or they were desalted using the Protein Cleanup protocol where the eluent was put on the deck at the beginning of run, then the protocol was conducted in one continuous run. If a two-step approach is used, the recovery is high regardless of sample volume (Figure 6A). If the elution is on the deck for the entire length of the run, there may be some decrease in recovery with large sample volumes (Figures 6B and 6C).

The time the sample is exposed to the resin in the cartridge (residence time) is a critical factor for efficient binding. Residence time is inversely related to flow rate. The relationship between flow rates and recovery was examined by loading serial dilutions of hIgG1 that had been denatured, reduced, alkylated, diluted then loaded onto RP-W cartridges at flow rates of 2, 5, 10, or 20 µL/min. The cartridges were then washed, and bound antibody was eluted. Antibody recovered in both the flowthrough and the eluate was quantified. For RP-W cartridges, the efficient capture of denatured hIgG1 was similar for load flow rates up to 20 µL/min (Figure 7). Higher flow rates were not examined since 20 µL/min is sufficient for loading the maximum sample volume (1 mL) in less than 1 hour. The default load flow rate was set to 5 µL/min to be consistent with the default flow rate of other AssayMAP cartridges, which show diminishing recovery as flow rate increases from 2 to 20 µL/min.

The effect of sample acidification on recovery was also tested using denatured, reduced, and alkylated hIgG1. Sample pH did not significantly affect sample recovery for sample loads up to 75 µg. It was, however, observed that sample acidification tended to improve reproducibility of recovery, and it was never observed to negatively impact recovery (data not shown).

Internal cartridge wash optimization

The Protein Cleanup application wash step uses 50 µL (10 column volumes) at 10 µL/min by default. No benefit was observed by increasing the wash volume or decreasing the wash flow rate (data not shown). Both of these parameters can be adjusted if desired.

Elute optimization

AssayMAP Bravo applications provide elution options that give the user control over the volume and buffer composition of the eluted sample. The effect of pH, acetonitrile concentration, and eluent volume were examined.

To determine the optimal eluent composition, hIgG1 was denatured, reduced, alkylated, diluted, then loaded onto RP-W cartridges. The cartridges were washed, then eight eluent conditions (four acetonitrile concentrations at pH 8 or pH 3) were tested. Elution with greater than 70 % acetonitrile was not tested due to the risk of protein precipitation. Figure 8 shows acidification of the eluent with 0.1 % TFA resulted in maximal recovery at 60–70 % acetonitrile, but at pH 8 recovery was poor at all acetonitrile concentrations. This illustrates the importance of optimization, since even at the lower pH recovery drops off significantly below 60 % acetonitrile.

Having established the optimal eluent formulation, the minimum volume required for recovery of the entire sample was examined. A 50 µg load of denatured, reduced, and alkylated hIgG1 was applied to eight replicate RP-W cartridges, and the cartridges were washed. The antibody was eluted from the cartridges using a specialized protocol that sequentially dispenses 2 µL of eluate into each well of a collection plate. Eluted antibody was quantified to generate the elution profile shown in Figure 9. The first fraction represents the column void volume, which contained no detectable antibody. The next four to five fractions (8 to 10 µL total) contained the antibody. The total recovery was greater than 90 % of the load.

RP-W Reproducibility

Human IgG1 was denatured, reduced, alkylated, and diluted to give mass loads of 10, 25, 50, and 75 µg. The samples were loaded onto RP-W cartridges (n = 12 per mass load), washed, and eulted using the Protein Cleanup application. The CV and recovery for each mass load were determined. The CV was found to be less than 5 %, and the recovery greater than 90 %, as shown in Figure 10. CVs below 5 % are consistent with reproducibilities that have been determined for other AssayMAP cartridges, highlighting the precision that can be achived using this platform.

Performance for multiple antibodies

To determine how well the standard conditions used in this study translate to other antibodies, two additional hIgG antibodies were examined. The first was a polyclonal hIgG preparation from normal serum. The second was the monoclonal standard antibody SILuLite from Sigma. All three antibodies were serially diluted, denatured, reduced, alkylated, and 25, 50, or 75 µg was loaded onto RP-W cartridges. As shown in Figure 11, the recovery for the hIgG1 was greater than 90 %, and CVs were below 5 %, as expected. The recovery and variability for the other two antibodies were not quite as good as hIgG1 but still worked well without further optimization. These findings suggest that the standard conditions presented here can be used with a variety of antibodies, and that optimal conditions may vary for different antibodies.

Rapid trypsin digestion

Trypsin digestion of denatured proteins is inefficient and incomplete in the presence of denaturant. Trypsin digestion following desalting on RP-W cartridges or SEC was compared with simply diluting the denaturant. Human IgG1 was denatured, reduced, alkylated, and either diluted to 0.9 M guanidine, desalted using RP-W cartridges, or desalted using a 96-well SEC spin plate. Quadruplicate samples were digested with trypsin for 0.5, 1, 2, 4, or 18 hours at 37 °C in parallel. Figure 12 shows the TICs of all reactions (20 overlaid traces), for each guanidine reduction method. The colors red, purple, green, blue, and black indicate the respective digest time points of 0.5, 1, 2, 4, and 18 hours. In the case of dilution-only reactions, which were digested in the presence of 0.9 M guanidine, the five colors form visually distinct groups of quadruplicates, indicating a gradual progression toward complete digestion that required up to 18 hours for most of the peaks. By contrast, both desalting methods showed much less variation over the digestion time course. This is in agreement with previously published results showing that removal of guanidine decreases digestion

time and increases the reproducibility of trypsin digestion[1]. It was also observed that SEC desalting resulted in smaller TIC areas than RP-W desalting or simple dilution, indicating lower recovery. SEC is a well-accepted method for desalting proteins, and it is likely that this is an antibody-specific observation, and that further optimization of this method would result in better recovery.

Twelve fully tryptic peptides were identified that are conserved between the hIgG1 antibodies used in this study. Extracted ion chromatograms of all 12 peptides were generated from MS1 data, and peak areas were integrated and quantified for each of the time points (Figure 13). When guanidine was simply diluted to 0.9 M (panel A), complete liberation of nearly all the peptides required more than 4 hours, as indicated by the continuous increase in bar height across the time course. Only the peptide beginning with WQQG reached a plateau by 4 hours (compare blue and black bars). Samples that were desalted prior to digestion using either RP-W cartridges (panel B) or an SEC spin plate (panel C) show the majority of peptides reaching maximum levels by 1 hour.

Workflow reproducibility

A common sample preparation workflow for biotherapeutic antibody analysis is the purification of a target antibody from a complex matrix, denaturation and digestion of the purified antibody, and quantification of selected peptides. The reproducibility of this workflow was examined using the AssayMAP Bravo for the sample preparation steps. Human IgG1 or SILuLite antibody was spiked into cell-free medium from a nonantibody-expressing CHO cell culture (n = 16 per antibody). The antibodies were purified from this matrix using the AssayMAP Affinity Purification application and protein G (PG-W) cartridges[2]. The antibodies were eluted directly into a solution containing guanidine and TCEP, and incubated for 60 minutes at 60 °C in a sealed plate. The AssayMAP Bravo In-Solution Digestion application was used to alkylate and dilute the samples. The Protein Cleanup application and RP-W cartridges were used to desalt the samples, and the In-Solution Digestion application was used to digest the antibodies prior to LC/MS analysis. Twelve conserved peptides were quantified for each sample. Figure 13 shows that CVs for the entire workflow were less than 10 % for at least nine out of 12 peptides examined. The peptides with CVs higher than 10 % were those with the smallest peak areas. The average CVs for all 12 peptides were 8.4 % for hIgG1 peptides, and 6.0 % for the SILuLite peptides.

Conclusions

The Agilent AssayMAP platform is a scalable and easy-to-use automation platform that can simultaneously improve the reproducibility of LC/MS workflows and reduce labor. The AssayMAP Affinity Purification, In-Solution Digestion, and Protein Cleanup applications can be used to purify antibodies from complex matrices, denature, reduce, alkylate, clean up, and digest the antibodies, all in less than 8 hours, with CVs less than 10 %. This represents a significant improvement in time-to-results compared to overnight digestion, so you can move projects forward faster.

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