Automated Plasma Processing for Quantitative, Targeted LC/MS Analysis of Proteins

Application Note

Authors

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Introduction

Variability in the reproducibility of protein digestion is the major source of measurement variance in stable isotope dilution, multiple reaction monitoring mass spectrometry (SID-MRM-MS), and immuno-MRM-MS (SISCAPA) assays.[1] This application note describes how the Agilent Bravo Automated Liquid Handling Platform was used for automated digestion in order to improve the reproducibility between experimental runs and reduce the labor intensity of this step when large numbers of samples are evaluated. A Bravo Platform at the Broad Institute was configured for automated reduction, alkylation, and proteolytic digestion of plasma from human patients and animal samples to complete this assessment. This application note compares the automated trypsin digestion of plasma in 96-well plates using the Bravo Platform to manual trypsin digestion in microcentrifuge tubes, demonstrating comparable analytical results between manual and automated methods. Automation on the Bravo Platform allows us to process up to 96 samples per day (as compared to 24 processed manually), enables parallel reagent addition, and improves lab productivity. A future report will detail our use of the Agilent Bravo in targeted immuno-MRM enrichment.

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Experimental

Plasma samples of were spiked with 27 proteins fully labeled with ^{15}N ("heavy proteins", Argonne National laboratories) to a concentration of 0.33 pmol/µL. Half of the samples were added to a 96 deepwell plate and transferred to the Bravo Platform for digestion, and half were kept in microfuge tubes for manual processing (Figure 1).

Next, urea was added to a final concentration of 6 M and Tris (2-carboxyethyl) phosphine (TCEP) was added to a final concentration of 25 mM. Samples were heated and mixed at 800 rpm, for 30 minutes at 37 °C. Iodoacetamide (IAA) was then added to a final concentration of 80 mM, and samples were incubated for 30 minutes at ambient temperature, in the dark without shaking. The Bravo Platform deck was configured as shown in Figure 2, and the instrument was entirely enclosed to avoid light exposure throughout the reduction, alkylation, and digestion procedure.

Enzymes were prepared at 0.5 µg/µL in 50 mM acetic acid to prevent autolysis and were added to a plate on the Peltier thermal unit set to 4 °C prior to sample processing. Enzymes were added directly to the samples and pH 8.1 was maintained throughout. Plates were mixed and incubated on the temperature-controlled shaker unit on the Bravo Platform deck as shown in Figure 2. Bravo Platform deck positions were configured as follows:

- The sample plate was located at position 5;
- Pipette tips were located at positions 1, 2, 3, and 8;
- Denaturation, reduction, alkylation, and quenching reagents were in a 384-well plate in position 7;
- Trizma base was located at position 9.

Heating and mixing were done on an Agilent Heating Shaking Station (p/n G5498B#009) located at position 4, and enzyme preparations were in a 384-well plate at position 6 on an Agilent Peltier Thermal Station (p/n G5498B#021). Position 4 and 6 devices were controlled within the Agilent VWorks Automation Control software using the MTC Controller (p/n G5498B#015). The matching microfuge samples were mixed in an Eppendorf Thermomixer.

Next, mass spectrometry grade lysyl endoproteinase lys-C (Wako Pure Chemical Industries, Ltd. p/n 125-05061) was added to produce an enzyme to substrate ratio of 1:50. The samples were then incubated at 37 °C and mixed at 800 rpm. Urea concentration was reduced to ~1 M by adding 0.2 M Trizma (pH 8.1). Next, sequencing grade modified trypsin (Promega p/n V5111) was added to an enzyme:substrate ratio of 1:100, and the samples were incubated at 37 °C and mixed at 800 rpm. After 2 hours, a second aliquot of trypsin was added (E:S of 1:100), and incubation and mixing continued overnight (~15 hours). Enzymatic activity was quenched by adding 50 % formic acid to a final concentration of 1%.

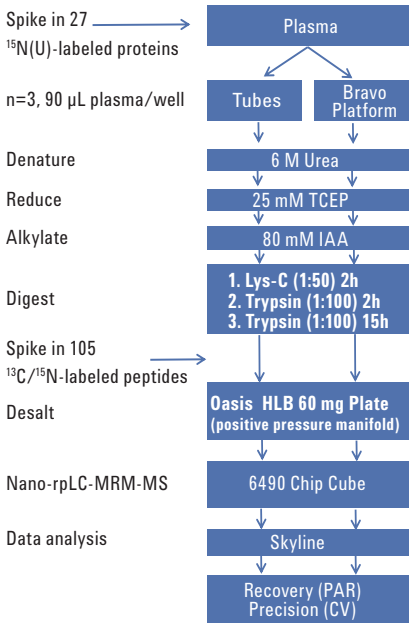

Figure 1. Digestion workflow performed on the Bravo Platform versus liquid handler with a light-tight enclosure on the workbench.

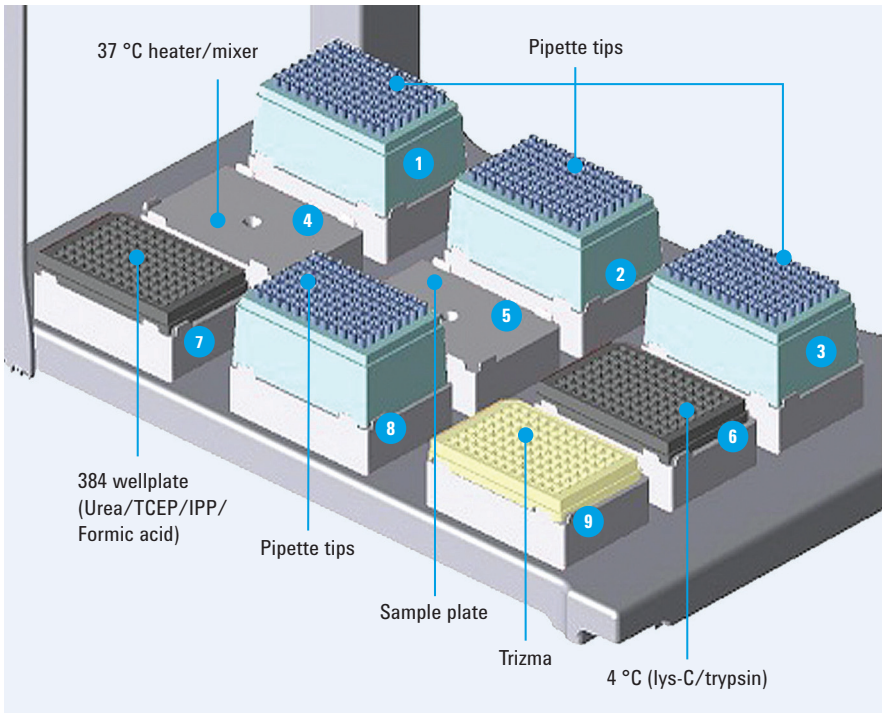

Figure 2. Deck locations of buffers and pipette tips for automated trypsin digestion using the Agilent Bravo Platform with gripper (p/n G5523A#003) and a 96LT Disposable-tip Pipette Head (p/n G5498B#042).

Following reduction, alkylation, and digestion, a mixture of the synthetic heavy-labeled peptides was added to each well at a molar concentration equal to the original heavy (^{15}N) protein spikes. $^{13}C/^{15}N$-labeled peptide standards were added to enable the monitoring of digestion efficiency and reproducibility as determined by SID-MRM-MS.

Next, the digested plasma samples were desalted using Oasis HLB60 plates on a positive pressure manifold. Desalted samples were reconstituted in a solution containing 3 % acetonitrile and 5 % acetic acid (3 % ACN, 5 % HOAc) and centrifuged for 5 minutes at 5,000 x g. The supernatant was diluted 1:10 in 3 % ACN, 5 % HOAc and transferred to an HPLC vial. One µL of the sample was injected onto the Agilent HPLC-Chip with dual trap/analytical columns and analyzed on an Agilent 6490 Triple Quadrupole LC/MS System.

Between one and five unique tryptic peptides were chosen to monitor by MRM-MS for each of the proteins using a combination of empirical digestion data, public database information, and ESP,[2] an algorithm that aids in the selection of high response peptides for electrospray mass spectrometry. Three transition ions specific for each labeled form of the peptide (one for the synthetically-labeled C-terminal arginine or lysine standard, and one for the ^{15}N (U)-labeled peptide derived from the protein), were selected and programmed into an MRM experiment that contained a LC gradient method optimized for dynamic MRM acquisition.

Results and Discussion

The mass spectrometry data were used to determine peptide recovery and digestion reproducibility by calculating the ratio of each analyte tryptic peptide (derived by trypsin digestion of the heavy protein) to its corresponding heavy synthetic peptide spike. For example, a peak area ratio (PAR) of 0.8 would represent a digestion recovery of 80 %. Process triplicate plasma digestions were injected once on the 6490 Triple Quadrupole LC/MS System with an HPLC-Chip and analyzed by dynamic LC-MRM-MS.

PAR and CV values were calculated for 102 of the 105 peptides in the study to determine recovery and precision, respectively (Table 1) and plotted as histograms (Figure 3). The median recovery of peptides from automated digestion and manual digestion were both approximately 50 %, and the histogram representing the range of recoveries for peptides was also very similar for automated versus manual digestion (Figure 3A). The median CV for the reproducibility of the process triplicate digestion was approximately 6 % for both automated and manual digestion, and the histograms were also very similar (Figure 3B).

Table 1. Median PAR and CV for 102 peptides (3 transitions/peptide) measured by LC/MS, with MRM, on an Agilent 6490 Triple Quadrupole LC/MS System.

Sample processing	Median recovery (PAR)	Median precision (CV)
Manual (tubes)	50 %	5.9 %
Bravo Platform (plates)	56 %	6.4 %

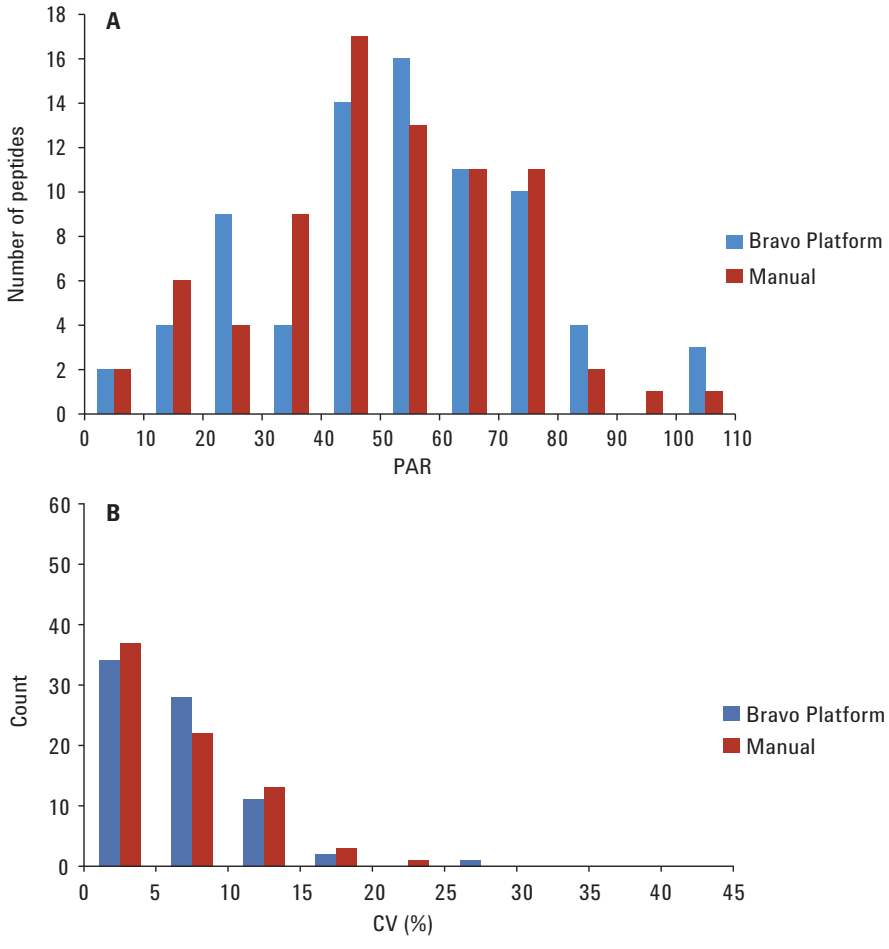

Figure 3. Histogram plots of (A) recovery as determined by heavy protein to heavy peptide peak area ratio (PAR) and (B) process reproducibility (CV).

A plot of the recovery of each peptide from manual versus automated digestion shows very strong correlation, indicating that manual and automated digestion are essentially identical with respect to peptide recovery (Figure 4).

Conclusions

The Bravo Automated Liquid Handling Platform was used for the automated digestion of plasma samples for SID-MRM LC/MS analysis to improve the reproducibility between experimental runs and to reduce the labor intensity of this step when large numbers of samples are evaluated. The analytical results achieved were comparable to those obtained by a highly trained technician using a manual method of trypsin digestion in microfuge tubes based on PAR and CV values.

In addition, automated preparation on the Bravo Platform enables processing of up to 96 samples at one time, 4 times the number that can be processed manually. This allows for more samples or replicates to be completed in a single experimental run, and, since all samples are processed in parallel, there is a reduced risk of variability between samples. The automated solution presented in this application note affords the possibility of increasing overall lab productivity and achieving more consistent results, while freeing scientists from routine, manual tasks.

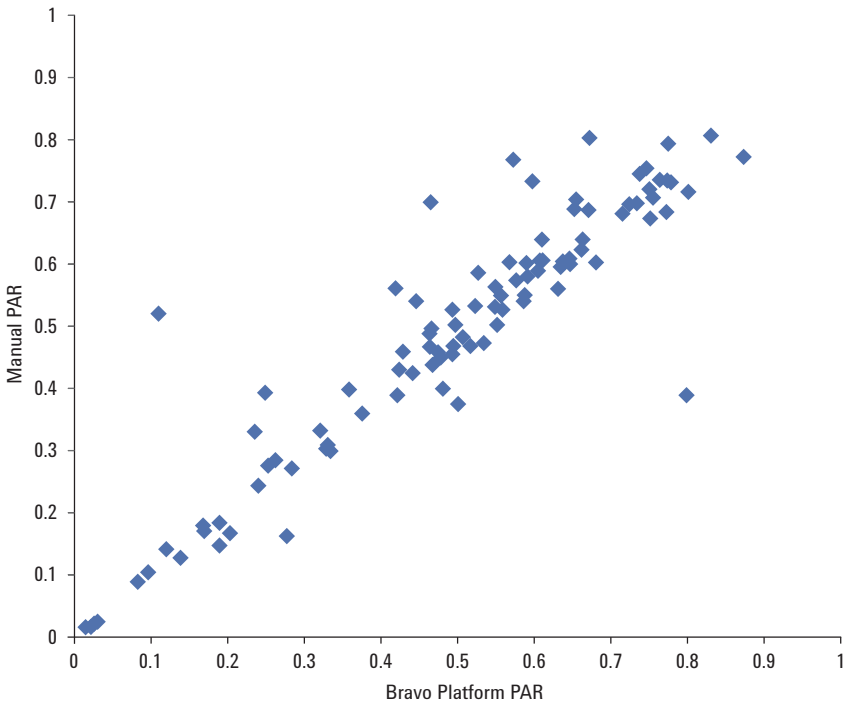

Figure 4. A high degree of correlation was demonstrated between plasma digestions performed manually in tubes and those completed on the Bravo Platform using multiwell plates.

References

1. Kuhn, E., *et al.* Inter-laboratory Evaluation of Automated, Multiplexed Peptide Immunoaffinity Enrichment Coupled to Multiple Reaction Monitoring Mass Spectrometry for Quantifying Proteins in Plasma. *Mol Cell Proteomics,* **2011**, *doi:10.1074/mcp.M111.013854.*
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