Automated Immunoaffinity-based Proteomic Methods for the Study of Post-translational Modification

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Introduction

Post-translational modification (PTM) of proteins, including phosphorylation, acetylation, methylation, and ubiquitination, are critical events in all aspects of cellular signaling. Antibody-based enrichments of post-translationally modified peptides combined with LC-MS/MS have proven to be powerful methods for the study of PTMs in a wide variety of cells and tissues, and in profiling various disease states (1-4). These antibody-based methods involve complex protocols that necessitate great care to achieve optimal results and reproducibility. Here, manual (batch mode) versus automated protocols have been compared with respect to the number of post-translationally modified peptides identified and the corresponding relative abundance of those peptides between the two sample preparation procedures. The AssayMAP Bravo Platform (Agilent Technologies, Inc.) allows automation of antibody-based peptide enrichments, simplifying the enrichment protocol and providing results that can be superior to manual methods.

Methods

Human cell lines or mouse tissues were lysed, digested with trypsin, and desalted over C18 columns. Peptides from 2 mg of samples were processed using the standard batch-mode PTMScan® protocol (**Figure 1**) or the AssayMAP Bravo system (**Figure 2**) using Protein A cartridges and the antibody purification application. Motif antibodies (100 µg) were loaded onto the cartridges and washed with PBS. Peptides resuspended in immunoaffinity purification (IAP) buffer were loaded onto Protein A/Antibody cartridges, washed using IAP buffer and water, and eluted in 0.15% TFA. Enriched peptides were purified on StageTips and analyzed by LC-MS/MS on an Orbitrap Velos™ mass spectrometer using a top 20 data-dependent analysis method. MS/MS spectra were assigned to peptide sequences using SORCERER™ (5), and label-free quantification was performed using Progenesis® (Nonlinear Dynamics). Two independent immunoprecipitation reactions were performed for all antibodies using the AssayMAP Bravo Platform (Bravo 1 and Bravo 2). Replicate injections were run for each sample. Bars represent average number of identifications across replicate injections, and error bars are –/+ 1 standard deviation.

References

1. Rush, J. et. al. (2005) *Nat Biotechnol*. 23, 94–101.
2. Lee, K.A. et. al. (2011) *J Biol Chem*. 286, 41530–41538.
3. Stokes, M.P. et. al. (2012) *Mol Cell Proteomics*. 11, 187–201.
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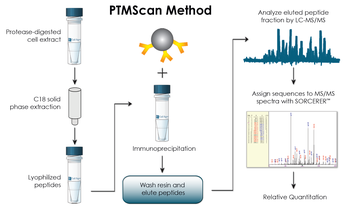


Figure 1: The Batch Mode PTMScan® Method. Samples are digested to peptides, run over reverse phase columns, immunoaffinity purified with the appropriate motif antibody, C18 purified, and subjected to LC-MS/MS.

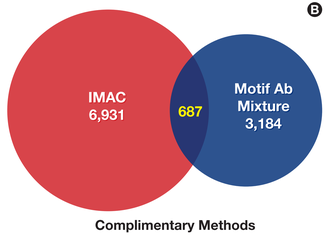
Figure 2: The AssayMAP Bravo System. Antibodies were bound to Protein A cartridges and washed. Peptides were then bound to antibody/Protein A beads, washed, eluted, C18 purified, and analyzed using LC-MS/MS.

Phospho-Serine/Threonine

A

Antibody Description	Motif
Akt Substrate	RXX(s/t)
Akt Substrate	RXRXX(s/t)
AMPK Substrate	LXRXX(s/t)
ATM/ATR Substrate	(s/t)Q
ATM/ATR Substrate	(s/t)QG
Cdk Substrate	(K/R)(s/t)PX(K/R)
CK Substrate	(s/t)(D/E)X(D/E)
MAPK Substrate	PX(s/t)P
PKA Substrate	(K/R)(K/R)X(s/t)
PKC Substrate	(K/R)X(s/t)X(K/R)
PKD Substrate	LXRXX(s/t)
PLK Binding Motif	S(s/t)P
tP Motif	(s/t)P
tPE Motif	(s/t)PE
tXR Motif	(s/t)XR
14-3-3 Binding Motif	(R/K)XX(s/t)XP

B



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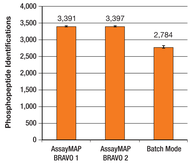

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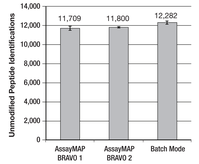
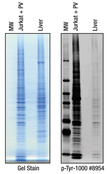


Figure 3: Ser/Thr Motif Antibody Mixture. A. List of antibodies included in the Ser/Thr motif antibody mixture with consensus phosphorylation motifs. **B.** Venn diagram of peptide identifications using the motif antibody mixture and IMAC. Number of phosphorylated (Orange, **C**) and unmodified (Grey, **D**) peptide identifications from mouse embryo for the Ser/Thr Motif Antibody mixture using the AssayMAP Bravo Platform or the standard batch mode method.

Phospho-tyrosine

A



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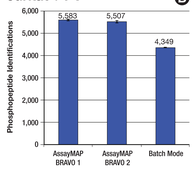

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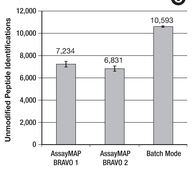

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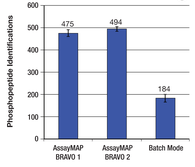

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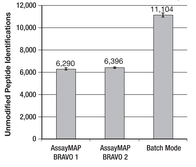

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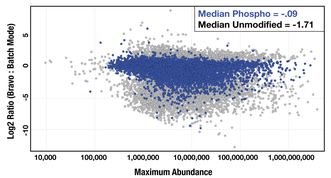
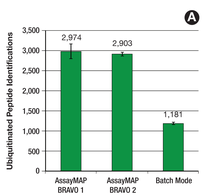


Figure 4: Phospho-tyrosine (p-Tyr-1000) Antibody. A. Gel Stain and western blot analysis on extract from Jurkat cells treated with pervanadate (PV: high p-Tyr signal) and mouse liver extract (low p-Tyr signal). Western blot performed using phospho-tyrosine (P-Tyr-1000) Rabbit mAb #8954. Number of tyrosine phosphorylated (Blue) and unmodified (Grey) peptides identified with the AssayMAP Bravo Platform or the standard batch mode method in Jurkat cells treated with pervanadate (**B** & **C**) or mouse liver (**D** & **E**). **F.** Log2 ratio versus intensity plot comparing Bravo method to batch mode method. Blue = phosphopeptides, Grey = unmodified peptides. The median log2 ratio for each is indicated.

Ubiquitin Branch

A



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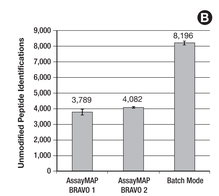

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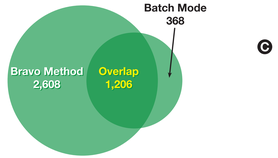

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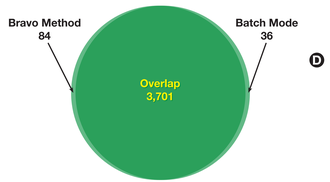

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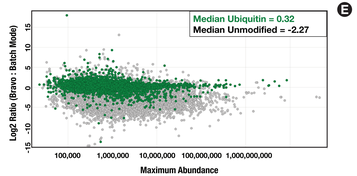
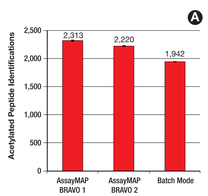


Figure 5: Ubiquitin Branch Motif Antibody. Number of ubiquitinated (Green, **A**) and unmodified (Grey, **B**) peptides identified in extract from mouse embryo using the Bravo method and the standard batch mode method. Venn diagrams of overlap between Bravo method and standard method at the MS2 (identification, **C**) and MS1 (feature, **D**) levels. **E.** Log2 ratio versus intensity plot comparing Bravo method to batch mode method. Green = ubiquitinated peptides, Grey = unmodified peptides.

Acetyl-lysine

A



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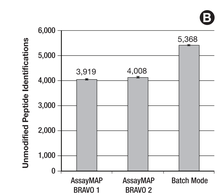

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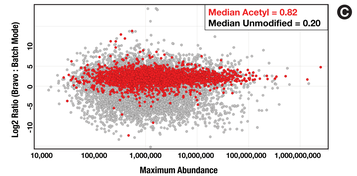

Figure 6: Acetyl-lysine Antibody. Number of acetylated (Red, **A**) and unmodified (Grey, **B**) peptides identified using the Bravo method or the standard batch mode method. **C.** Log2 ratio versus intensity plot comparing Bravo method to batch mode method. Red = acetylated peptides, Grey = unmodified peptides.

Conclusions

The AssayMAP Bravo Platform outperformed a traditional batch mode method for immunoaffinity purification of post-translationally modified peptides with all antibodies tested. In each case, the Bravo method resulted in a higher number of modified peptides identified along with a lower number of non-specific, unmodified peptides. The improved performance was likely due to the decrease in unmodified peptides in the Bravo samples, as relative abundance of the modified, target peptides changed little between methods (**Figures 4F, 5E, 6C**), and nearly all modified peptides were present in both methods at the MS1 feature level (**Figure 5D**).

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