Improved immunoaffinity enrichment methods for tyrosine phosphorylated, lysine acetylated, and lysine succinylated peptides with high sensitivity and specificity.

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INTRODUCTION

Posttranslational modifications (PTMs) regulate and are associated with diverse biological processes, for instance tyrosine phosphorylation (pY) with cell signaling, lysine acetylation (AcK) with epigenetic regulation, and lysine succinylation (SuccK) with metabolic flux. Immunoaffinity purification (IAP) is an established technique for the study of PTMs (Rush et al. 2005). Recently we introduced an antibody-based magnetic bead conjugate IAP method that enables identification and quantification of sites of lysine ubiquitination with improved sensitivity and specificity over preexisting agarose bead-based methods. Here we extend the repertoire of PTM types by presenting three novel magnetic bead-based IAP methods that enable the identification and quantification of pY, AcK, and SuccK sites, all with similar improvements over their respective agarose bead-based methods.

METHODS

Tryptic mouse and human peptides were used as input material for pY, AcK, and SuccK magnetic IAP beads. Peptides were incubated with beads using PTMScan® HS IAP Bind Buffer #1 for 2 hours at 4°C. Beads were washed with cold PTMScan® HS Wash Buffer then water, and the bound peptides were eluted with trifluoroacetic acid. Eluted peptides were desalted and analyzed on ThermoFisher™ Q Exactive or Fusion Lumos mass spectrometers using data-dependent acquisition. Peptides were resolved using a 90min reversed phase gradient from 7.5 to 32% acetonitrile on a 25cm or 50cm C18 column. Peptides were identified by Comet at a 2% false discovery rate. Label free quantitation was performed with Skyline. A BCA assay was used to assess intact antibody elution.

CONCLUSIONS

We developed a set of optimized IAP magnetic bead-based methods that provide a 2-fold improvement in identification of pY, AcK, and SuccK sites over the respective preexisting agarose bead-based methods when using the same input amount. We also observed a 98-99% reduction of antibody released from the magnetic beads during elution, which eliminates the need for multiple desalting rounds and increases stability of the LC system. In addition, the optimized method uses magnetic beads instead of agarose beads, which simplifies the benchwork and facilitates automation.

REFERENCES

-Rush J, Moritz A, Lee KA et al. 2005. Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. *Nature Biotechnology*. 23, 94-101.

PTMScan HS Workflow

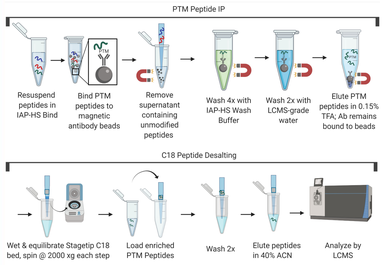

Figure 1. PTMScan® HS workflow starting at the IAP step, which uses different buffers and involves less desalting rounds compared to the preexisting PTMScan® Classic workflow.

Elution free from Ab Contamination

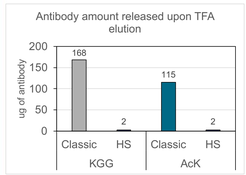

Figure 2. HS IAP beads release less antibody (Ab) during TFA elution compared to respective Classic IAP beads.

Performance of PTMScan HS Ubiquitin

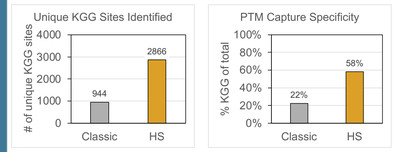

Figure 3. Comparison of IAP performance between PTMScan® Classic and HS Ubiquitin beads using mouse embryo peptides, analyzed on Q-Exactive.

Performance of PTMScan HS Phospho-Tyrosine

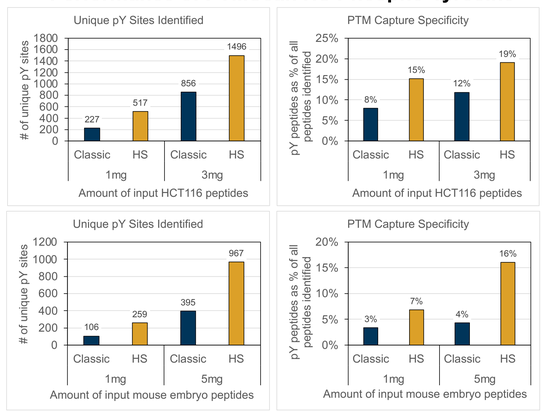

Figure 4. Comparison of IAP performance between PTMScan® Classic and HS Phospho-Tyrosine beads using various amounts of human and mouse input peptides, analyzed on Fusion Lumos, with approximately 2-fold improvement in unique pY sites identified and PTM capture specificity across conditions.

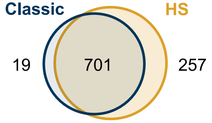

Figure 5. Overlap of pY peptides detected at the MS1 level from PTMScan® Classic and HS pulldowns using HCT116 input. Majority of peptides recovered using HS IAP beads are also recovered using Classic IAP beads.

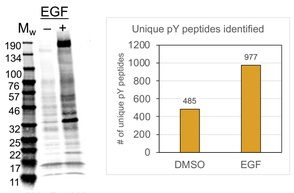

Figure 6. Detection of phospho-tyrosine peptides with PTMScan® HS beads using peptides derived from A431 cells treated with 100ng/mL EGF or DMSO for 5 minutes. The increased number of unique pY peptides is consistent with Western Blot results showing induction of pY protein levels.

Performance of PTMScan HS Acetyl-Lysine

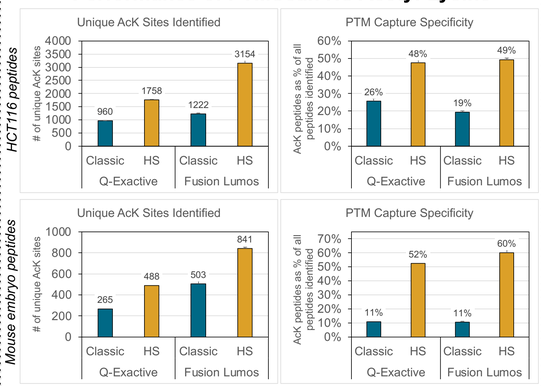

Figure 7. Comparison of IAP performance between PTMScan® Classic and HS Acetyl-Lysine beads using 1mg of human and mouse input peptides, analyzed on different instruments, with approximately 2-fold improvement in unique AcK sites identified and PTM capture specificity across conditions.

Figure 8. Overlap of AcK peptides detected at the MS1 level with Fusion Lumos from PTMScan® Classic and HS pulldowns using HCT116 input. Majority of AcK peptides recovered with HS IAP beads are also recovered with Classic IAP beads.

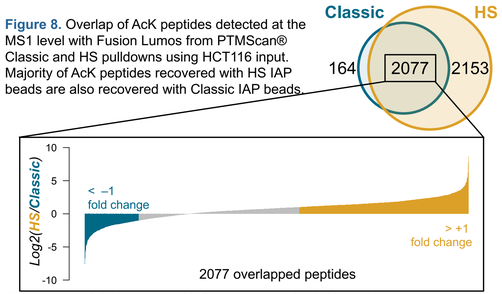

Figure 9. Log2 fold change of MS1 peak areas for AcK peptides that were detected at MS1 level for both PTMScan® Classic and HS pulldowns. There is a trend of more AcK peptides being recovered at greater abundance in HS relative to Classic.

Performance of PTMScan HS Succinyl-Lysine

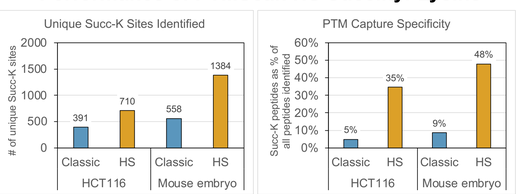

Figure 10. Comparison of IAP performance between PTMScan Classic and HS Succinyl-Lysine beads using 1mg of human and mouse input peptides, with approximately 2-fold improvement in unique succinyl sites identified and over 4-fold improvement in PTM capture specificity.

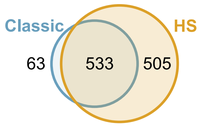

Figure 11. Overlap of Succ-K peptides detected at the MS1 level from PTMScan Classic and HS pulldowns using HCT116 input. Majority of Succ-K peptides recovered with HS IAP beads are also recovered with Classic IAP beads.

Summary of PTMScan HS Targets and Kits

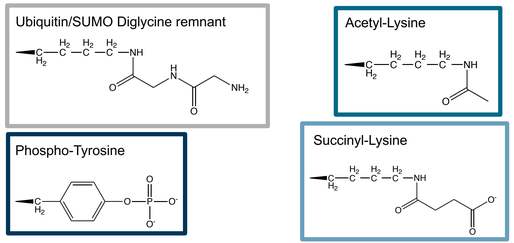

Figure 12. Structures of PTMs that can now be enriched with HS IAP beads using optimized buffers at improved sensitivity and specificity over preexisting approaches, along with improved ease of handling and automation.

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