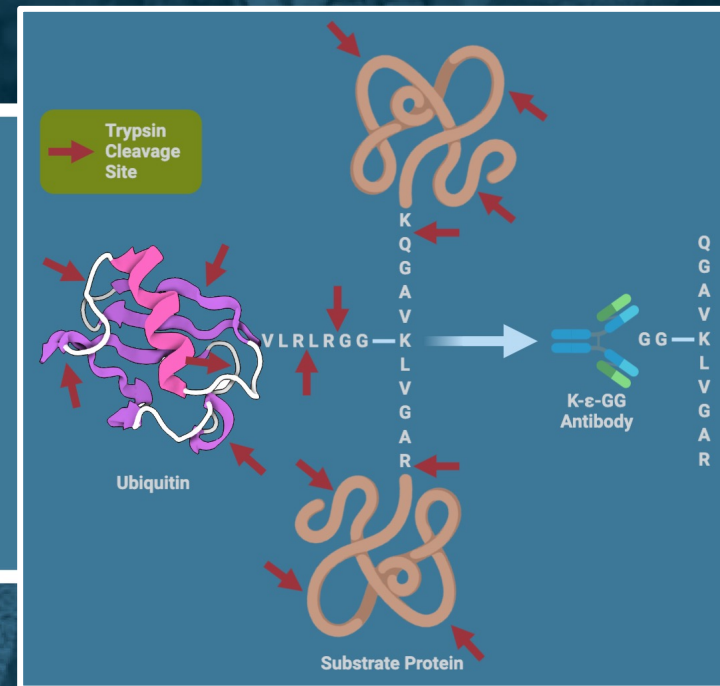


# Improved immuno-affinity enrichment method for ubiquitinated peptides with high sensitivity, specificity and robustness

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# Abstract

## Introduction

Ubiquitination is a critical post-translational modification in eukaryotic cells, triggering protein degradation or cell signaling events. A widely used method for ubiquitination site detection involves tryptic cleavage of ubiquitinated proteins generating a di-glycine remnant (K-GG) at sites of ubiquitination followed by immuno-affinity enrichment using an anti-K-GG antibody and LC-MS/MS analysis. This method allows identification and quantification of thousands of sites of ubiquitination from cells and tissues but requires large starting sample amounts and results in co-isolation of many non-specifically binding unmodified peptides. In this study, we introduce an improved immuno-affinity enrichment method for K-GG peptides that overcomes these issues.

## Methods

Tryptic mouse liver peptides were used to compare different types of beads, binding and washing buffers, and antibody amounts. Enriched K-GG peptides were analyzed on Thermo Q Exactive or Fusion Lumos mass spectrometers and identified by SEQUEST. A BCA assay was used to assess intact antibody elution.

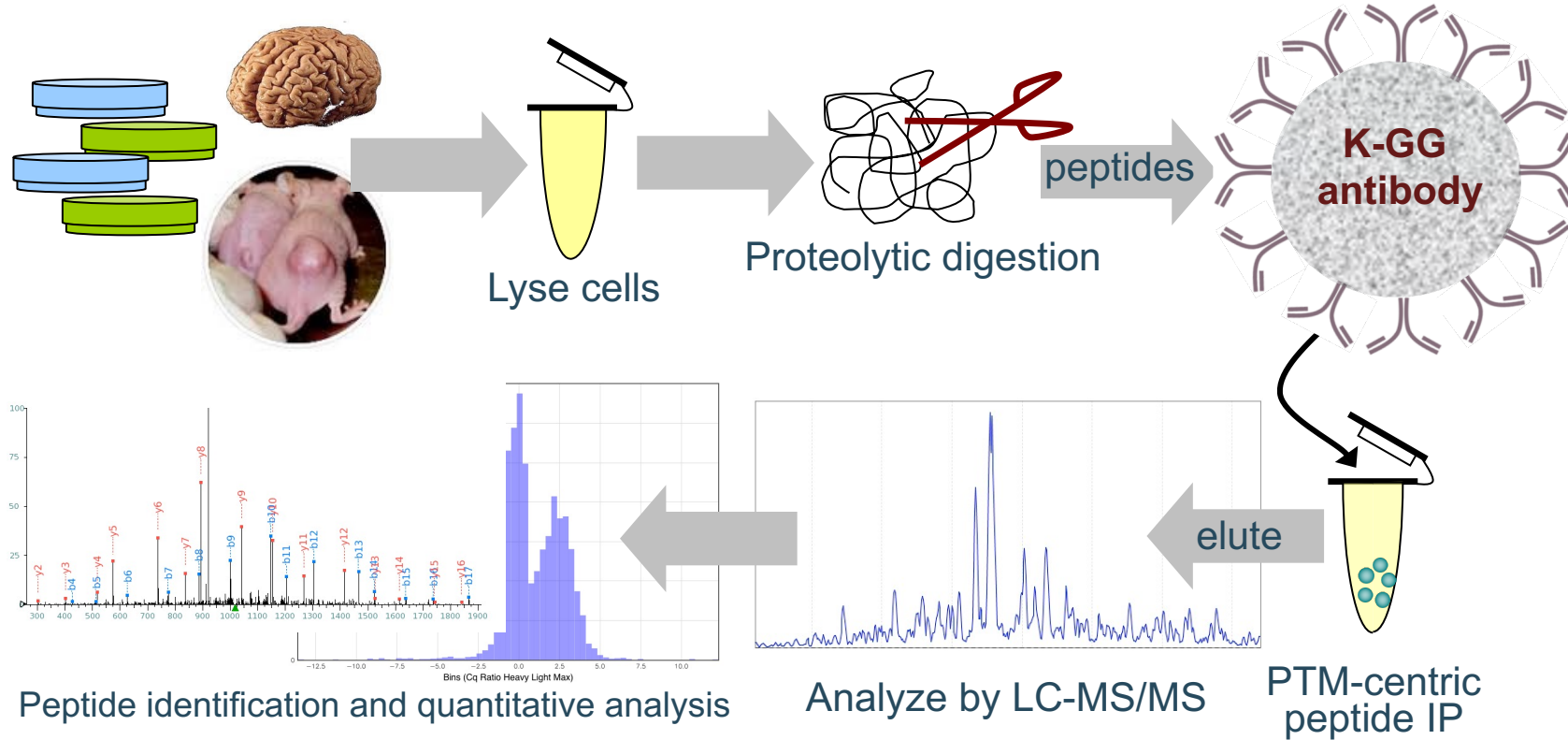
## Results

We have developed an optimized immuno-affinity enrichment method that provides 2-fold improved sensitivity, 2-3 fold improved specificity, and ~99% reduction in antibody released from beads upon acid elution. One milligram of mouse liver protein yields nearly 6000 ubiquitinated peptides identified, numbers typically generated with 10+ mg of starting material using the original method. The decrease in antibody eluted from the beads with K-GG peptides reduces the demand for C18 stage-tip cleaning and increases stability of the LC system. In addition, the optimized method uses magnetic beads instead of agarose beads, which greatly speeds and simplifies the procedure.

## Conclusions

Optimized K-GG peptide immuno-affinity enrichment method significantly improves sensitivity, specificity, and robustness of ubiquitinated peptide identification and quantification.

# PTMScan<sup>®</sup> method



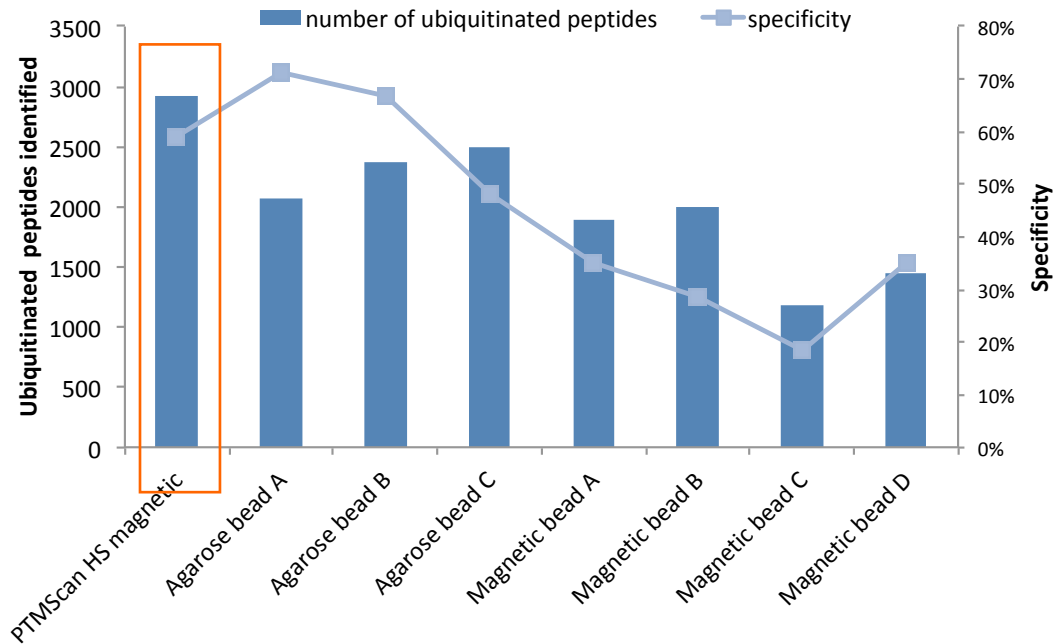
## Desired method improvements:

- Increase sensitivity
- Reduce required sample input amount
- Faster sample prep
- Eliminate carbamylation
- Eliminate antibody elution from beads
- Simplified enrichment using magnetic beads
- Eliminate peptide lyophilization requirement
- Increase specificity

PTMScan is a proven method for enriching post-translationally modified peptides from complex biological mixtures. First published for enriching phosphotyrosine-containing peptides, it has since been applied to dozens of PTMs and phosphorylation motifs. The method is limited, however, in several technical aspects (see list at right), preventing its application in many studies, particularly those with low available sample amounts. Using the K-e-GG antibody specific for the ubiquitin remnant produced after tryptic digestion (see figure on slide 1), we have improved this method through thorough testing and evaluation of each step. Here we present those improvements related to peptide immunoenrichment and their impact on sensitivity, specificity, and sample cleanliness.



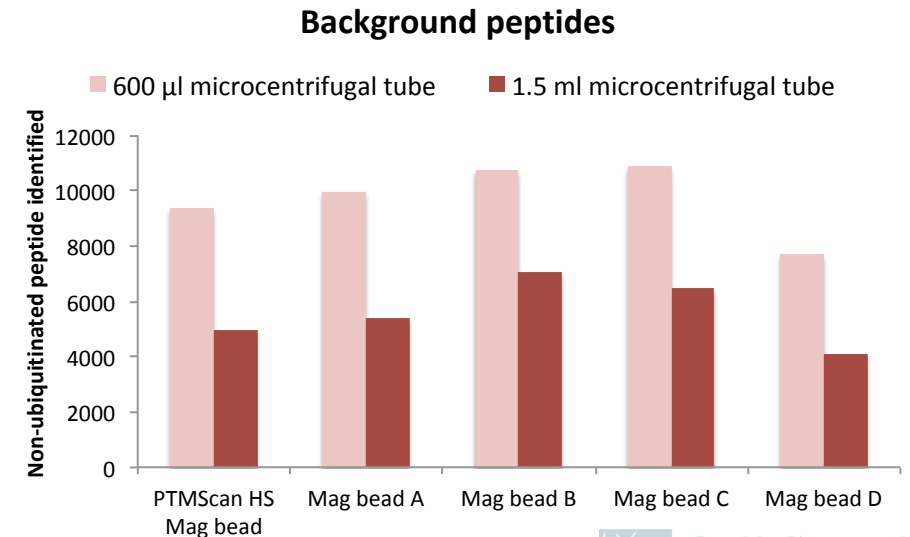
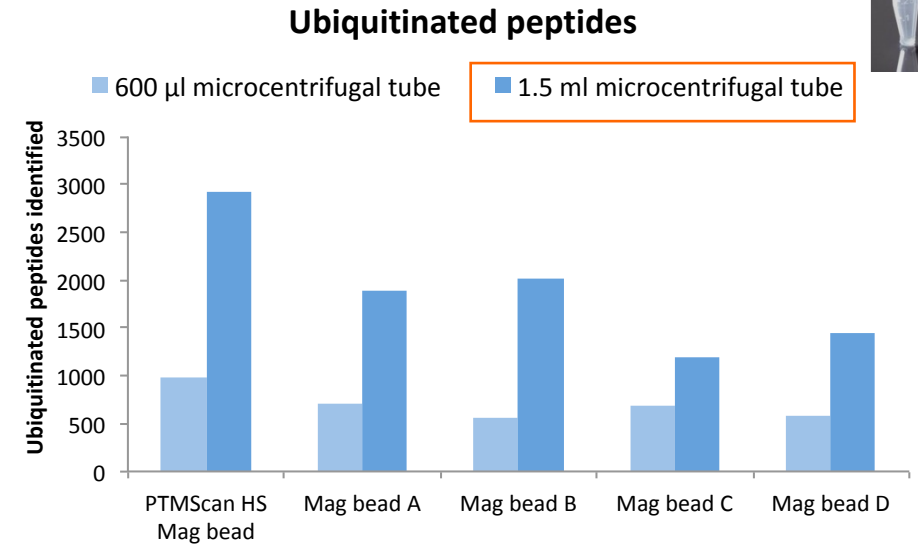
# Bead selection for immunoaffinity enrichment



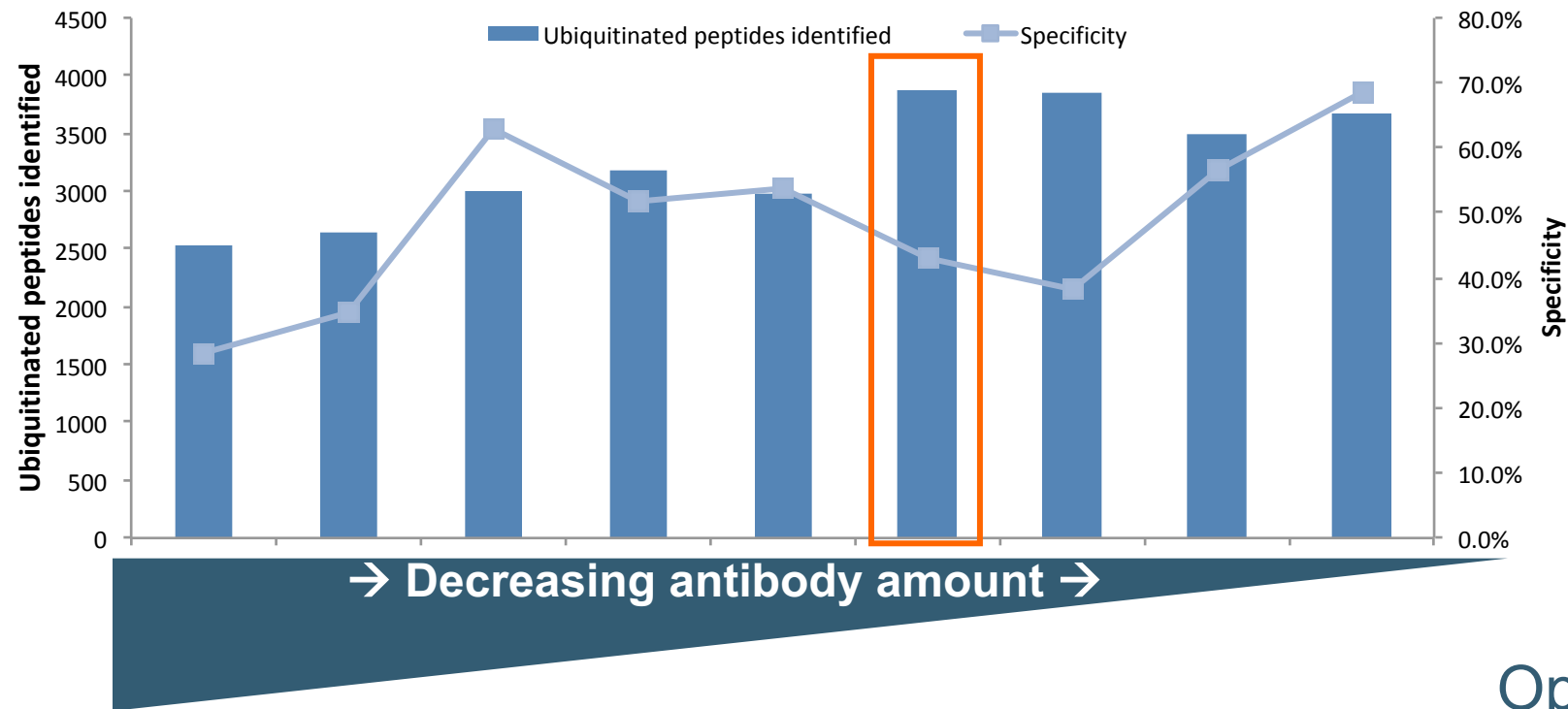
**Top panel:** Multiple agarose and magnetic beads were evaluated for binding capacity (data not shown) and performance in the PTMScan assay, both in number of K-GG peptides identified upon enrichment from 1 mg mouse liver tryptic peptides (blue bars above) and specificity of enrichment (fraction of identified peptides that contain the K-GG modification, blue line above). The magnetic beads selected had the highest antibody binding capacity of all tested, highest recovery of K-GG peptides, and were preferred based on ease of use to other beads.

**Right panel:** Regardless of magnetic beads selected, 1.5 mL microcentrifuge tubes enable more efficient washing, resulting in increased yield of K-GG peptides (top panel) and fewer unmodified peptides (bottom panel) than smaller 600uL tubes.

# Evaluating tube size



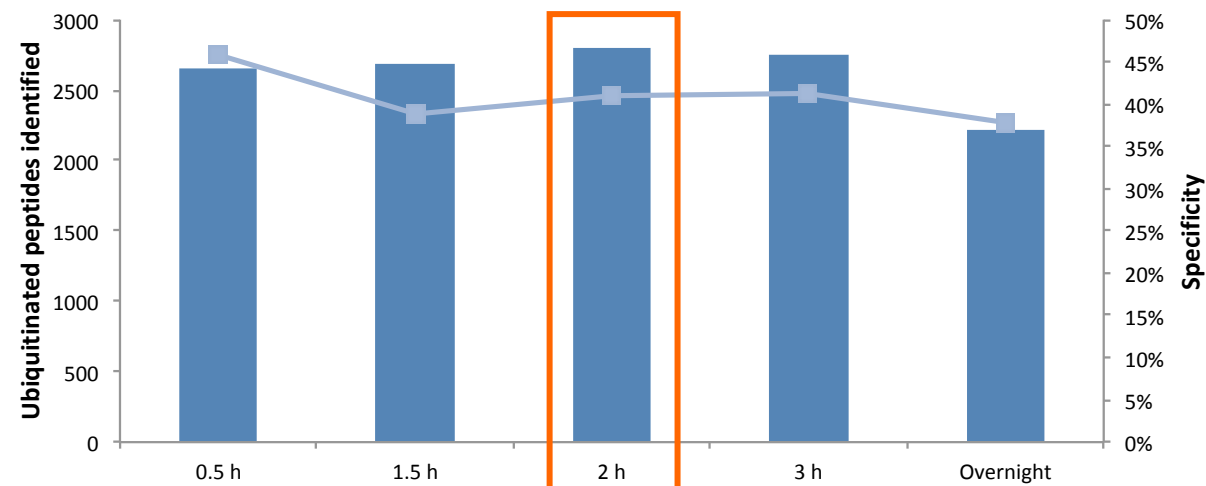
# Optimizing amount of antibody per immunoenrichment



**Left panel:** Trend observed that lowering antibody input amount results in higher K-GG peptide recovery from 1 mg input mouse liver tryptic peptide. The antibody amount indicated in red box was selected to enable efficient enrichment of a broad range of samples that may be richer in ubiquitination than the moderately ubiquitinated mouse tissue peptides used in this study.

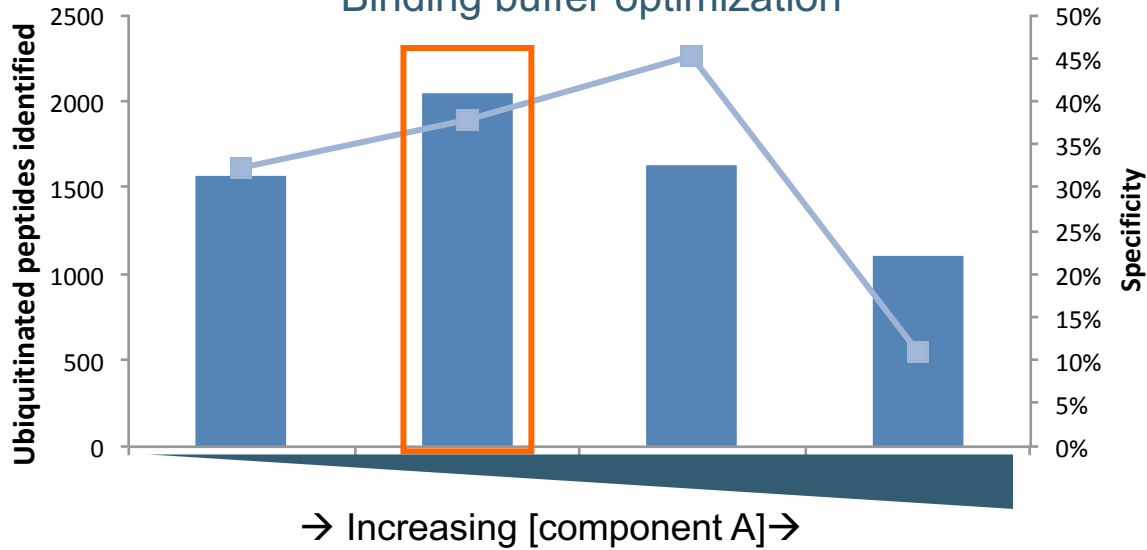
**Right panel:** The amount of time peptides are incubated with antibody-conjugated beads was evaluated, with a two-hour incubation time selected for final protocol. Overnight incubation results in reduced sensitivity and specificity, but all time points between 30 min and 3 hours had similar performance. Unique K-GG peptides are represented by dark blue bars while specificity of enrichment (fraction of identified peptides that contain the ubiquitin remnant) is plotted with the light blue line.

# Optimizing incubation time



# Optimizing binding and washing buffers

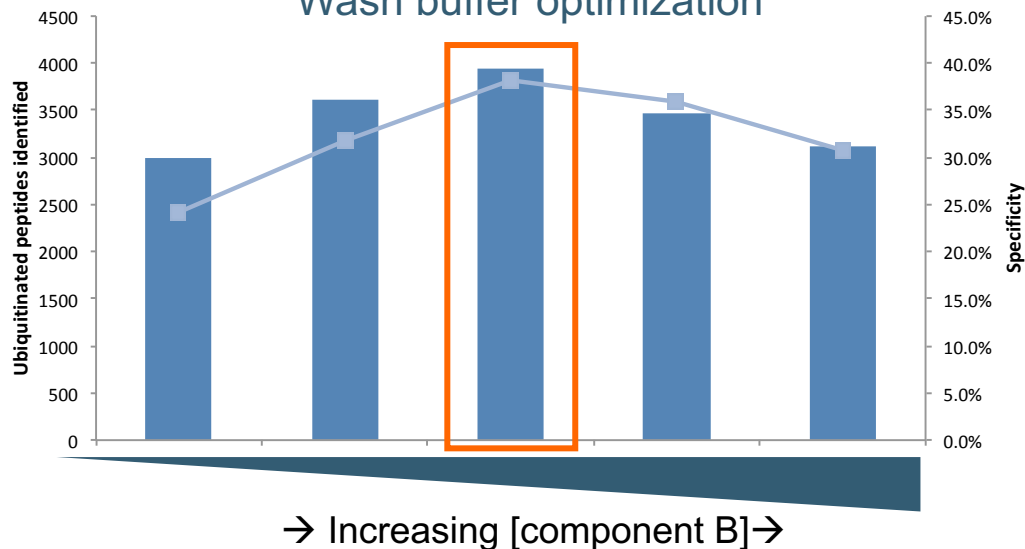
## Binding buffer optimization



→ Increasing [component A]→

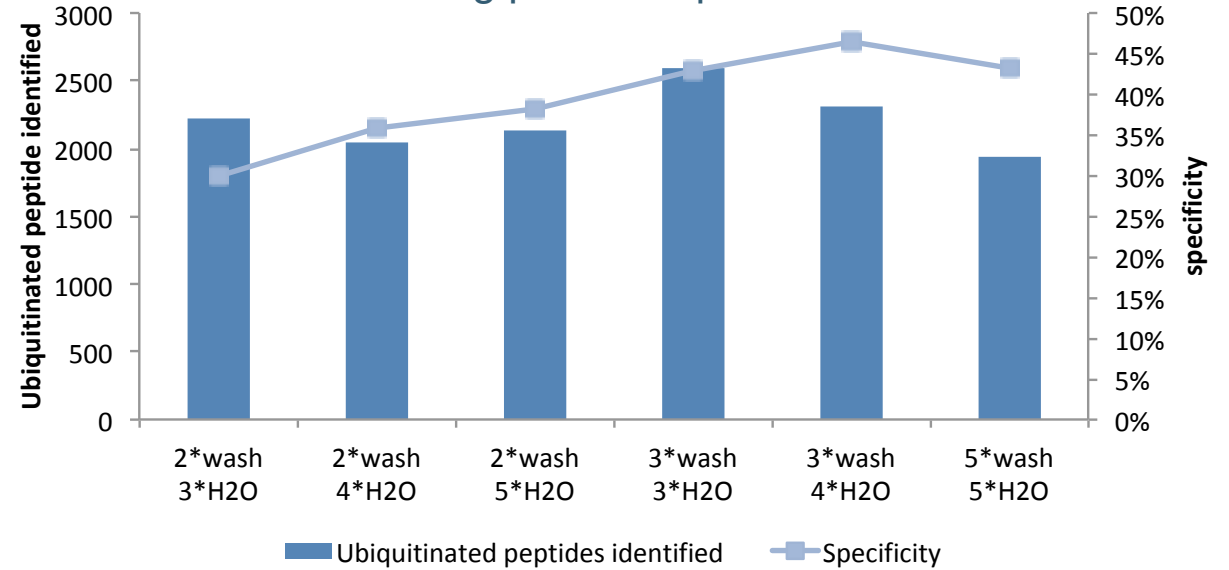
Ubiquitinated peptides identified Specificity

## Wash buffer optimization



→ Increasing [component B]→

## Washing protocol optimization



Ubiquitinated peptides identified Specificity

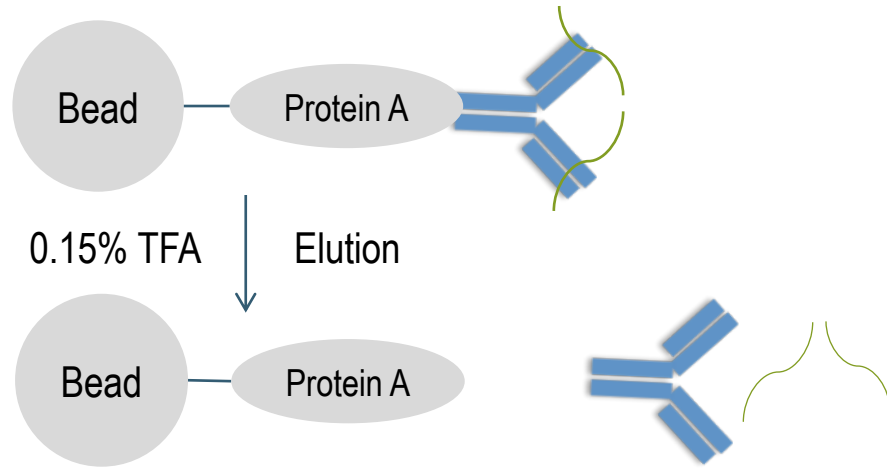
**Left panels:** Example data shown for binding and wash buffer optimization, with increasing concentration of one binding buffer component in upper panel and increasing concentration of one wash buffer component in lower panel. Selected value outlined in red.

**Upper right panel:** Optimizing number of washes with both wash buffer and water. Three washes with each resulted in highest recovery of K-GG peptides in this study but in a second study four washes with wash buffer and two water washes was found to be superior and was selected for the final method.

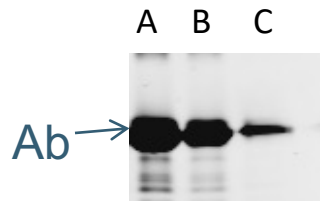
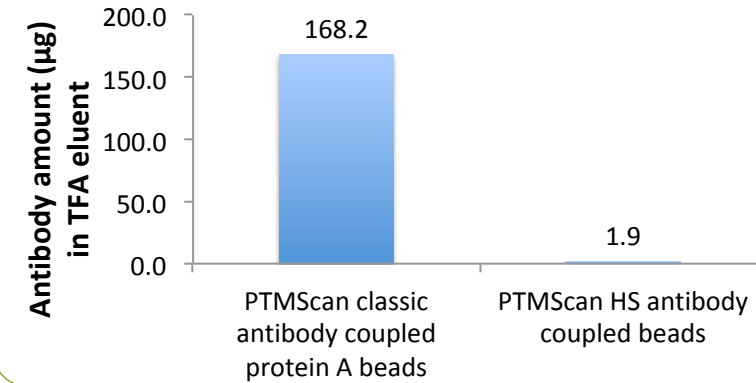
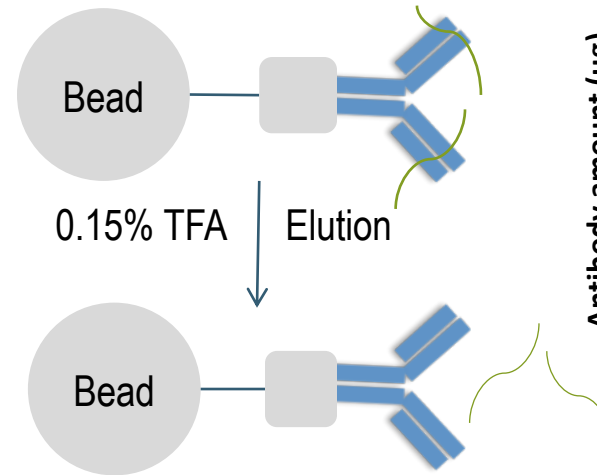
All studies were performed with 1 mg mouse liver tryptic peptides and assessed for both number of unique K-GG containing peptides identified and specificity of enrichment (fraction of identified peptides with K-GG).

# Eliminating antibody elution from beads

## Protein A bead-antibody coupling



## Magnetic bead-antibody coupling



AKT substrate antibodies coupled to protein A agarose bead

A. 1<sup>st</sup> TFA eluent

B. 2<sup>nd</sup> TFA eluent

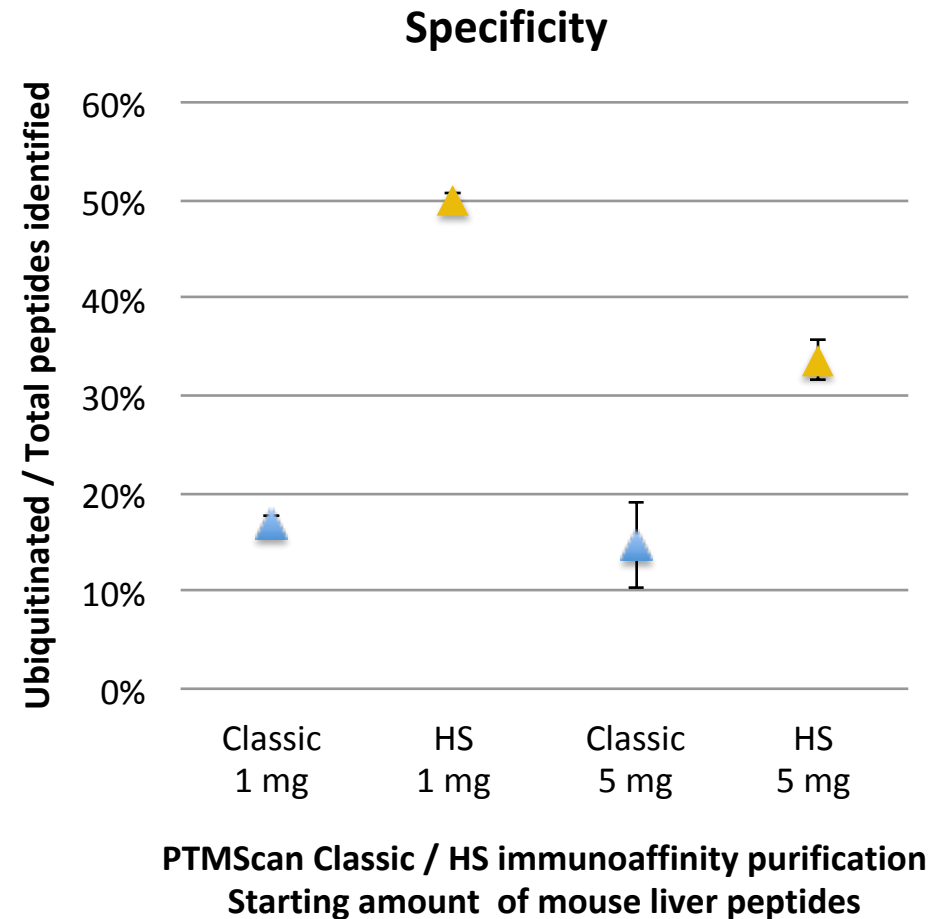
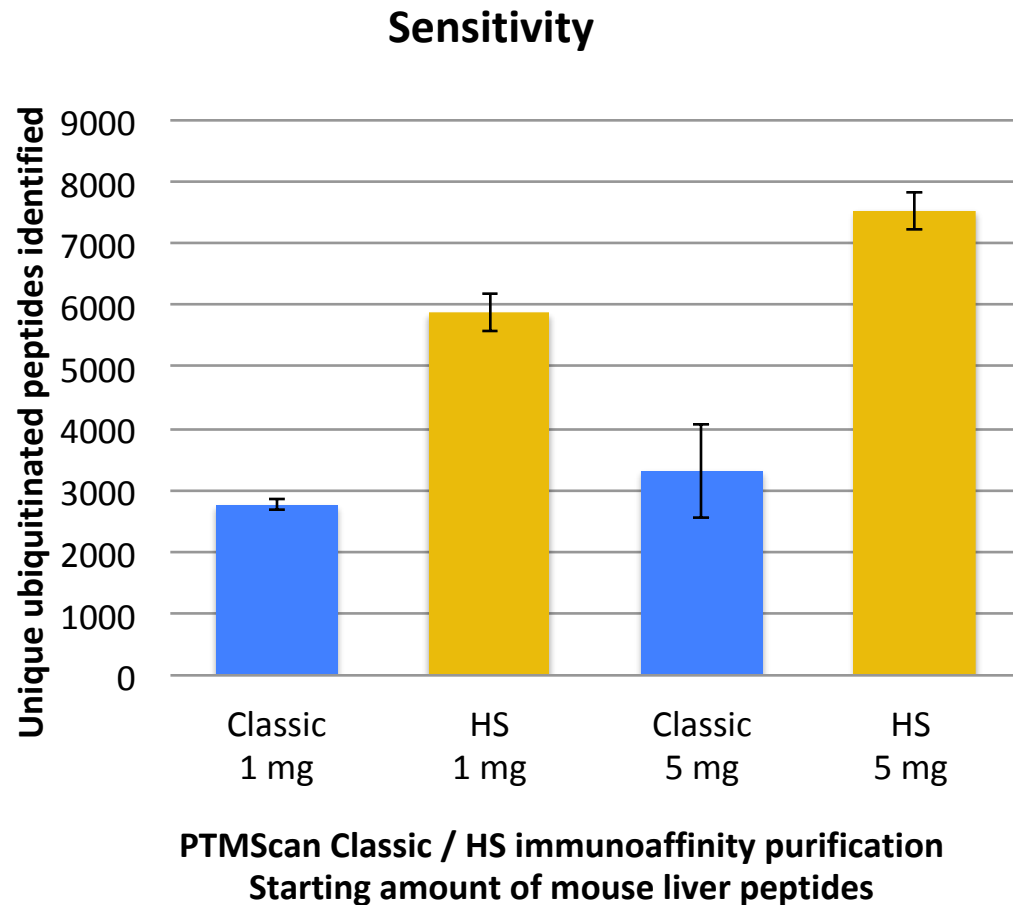
C. eluent from C18 STAGE tip

- Majority of antibody elutes from beads under acidic conditions
- Thorough C18 Tip cleaning required for antibody removal
- Intact antibody may cause LC-MS system contamination

Upgraded antibody coupling to magnetic beads ensures antibody stays bound upon peptide elution, preventing negative impacts to LCMS system. Western blot at left shows amount of antibody eluted during standard agarose bead/protein A coupled immunoenrichment. Multiple rounds of STAGE tip may be required to thoroughly remove antibody from peptide sample.

Graph at upper right shows amount of antibody eluted (determined using BCA assay) with new magnetic bead coupling chemistry is vastly reduced or eliminated.

# Comparing PTMScan Classic and improved PTMScan HS immunoenrichment



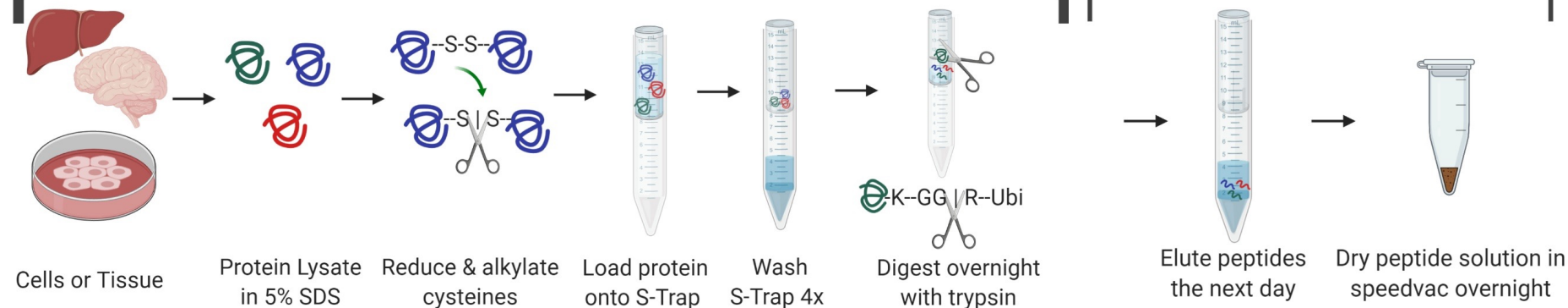
Combining all improvements to the PTMScan immunoenrichment step in the new PTMScan HS method results in more than double the recovery of K-GG containing peptides and significantly improved enrichment specificity. Pre-enrichment peptides for the PTMScan Classic enrichment (blue) and the PTMScan HS enrichment (yellow) are identical. All improvements are resulting from modifications to the immunoenrichment and washing portions of the method.



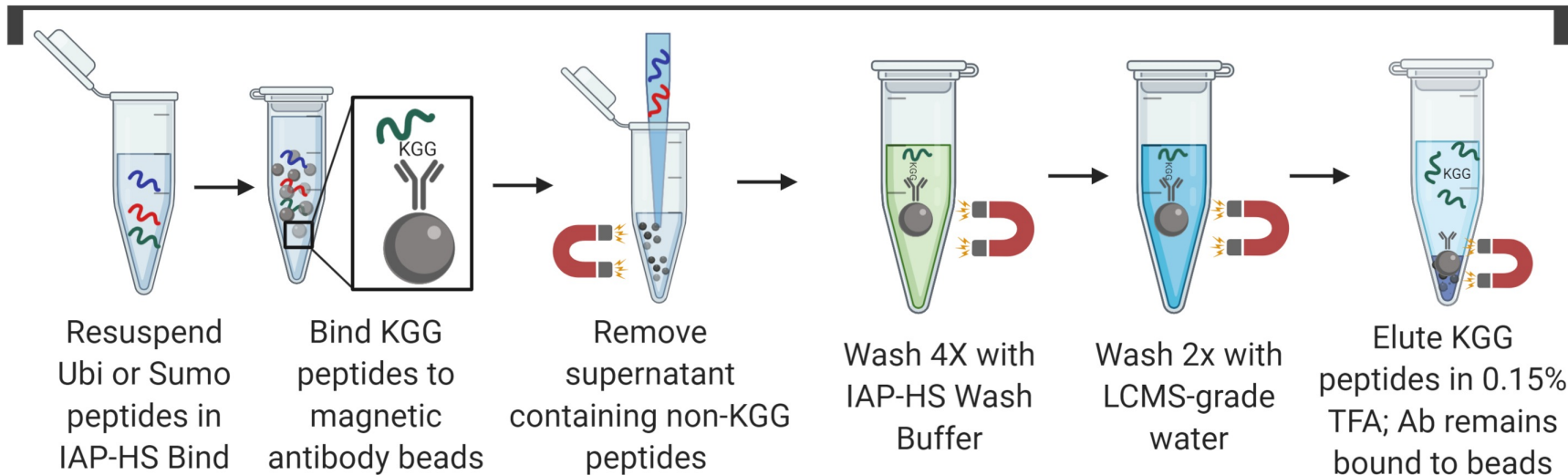
# Complete workflow for PTMScan HS

Day 1: Digest Ubiquitinated Proteins with Trypsin

Day 2: Elute Peptides & Dry

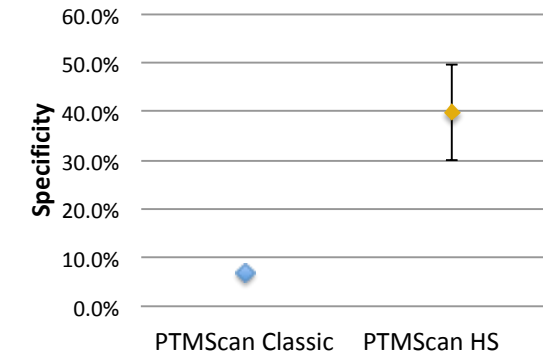
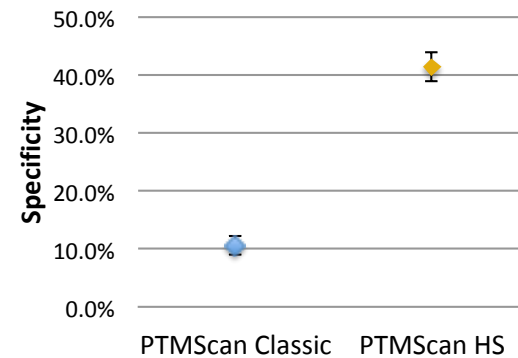
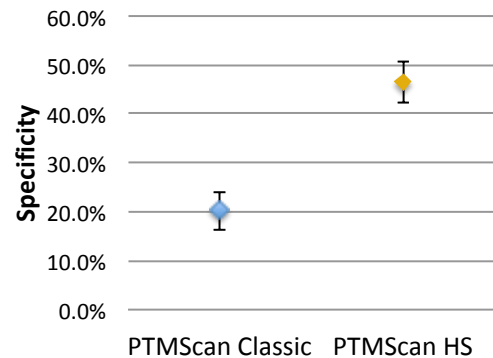
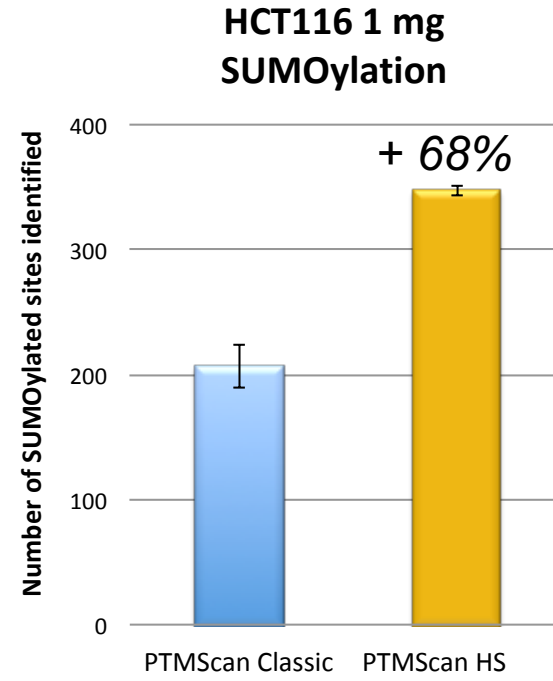
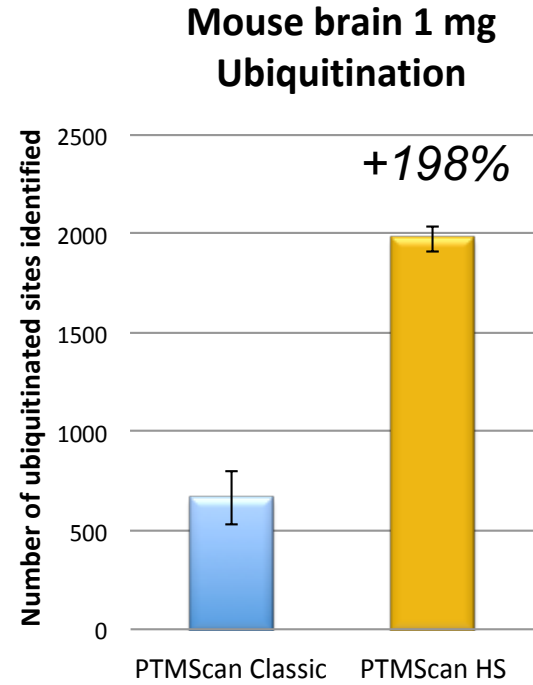
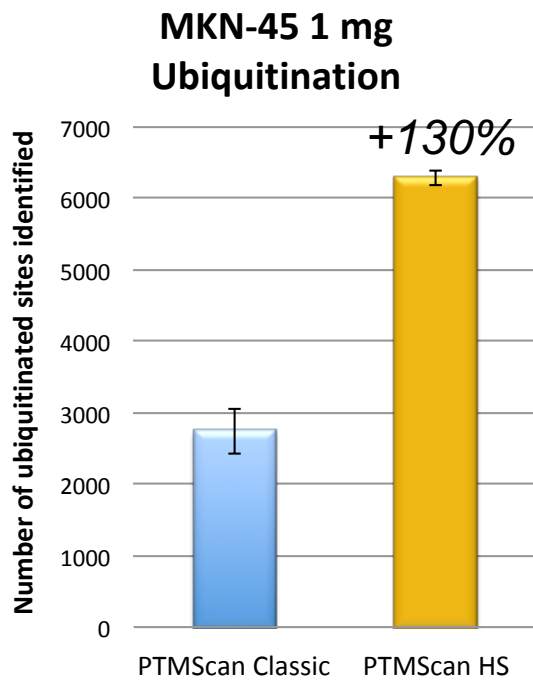


Day 3: Peptide Enrichment and LC-MS Analysis



- ✓ Increase sensitivity
- ✓ Reduce required sample input amount
- ✓ Faster sample prep
- ✓ Eliminate carbamylation
- ✓ Eliminate antibody elution from beads
- ✓ Simplified enrichment using magnetic beads
- ✓ Eliminate peptide lyophilization requirement
- ✓ Increase specificity

# Incorporating improvements to both sample preparation and immunoenrichment



Incorporating complete workflow improvements to sample lysis, digestion, peptide purification, and immunoenrichment results in significant improvements in sensitivity and specificity of enrichment. For MKN-45 and HCT116 cell lines, equal starting cell numbers were used for PTMScan Classic and PTMScan HS protocols. For mouse brain, equal amounts of wet weight tissue were used. Trypsin digestion was used for ubiquitin peptide generation (two left panels) and wild-type aliphatic protease (WALP) digestion was used for SUMO peptide generation.

PTMScan HS uses a simple, robust magnetic bead protocol to provide high sensitivity and high specificity K-GG peptide enrichment.