Automation of Phosphoenrichment using Magnetic Fe-NTA Beads and KingFisher™ Apex Magnetic Particle Processor

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ABSTRACT

Purpose: Phosphorylation is a critical post translational modification that modulates the function of numerous proteins and recent advances in the mass spectrometry (MS) instrumentation have enabled studying phosphorylation at proteomics scale in complex biological samples. Due to the low stoichiometry of phosphorylation in biological samples, IMAC has been widely used for enriching phosphorylated peptides. Here, we introduce an agarose-based Fe-NTA magnetic bead for manual and automated phosphopeptide enrichment workflows using Thermo Scientific™ Kingfisher™ Apex Magnetic Particle Processor for high throughput applications.

Methods: Nocodazole treated HeLa S3 cells were processed using Thermo Scientific™ EasyPep™ Maxi MS Sample preparation kit. Magnetic Fe-NTA beads were incubated with protein digests and magnetically separated from the supernatant manually or through automation using Kingfisher Apex Magnetic Particle Processor for the phosphopeptide enrichment. Peptides were quantified and normalized using the Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay prior to LC-MS analysis using a Thermo Scientific™ Orbitrap™ Q Exactive™ Plus mass spectrometer. Thermo Scientific™ Proteome Discoverer™ 2.4 software was used to localize the phosphorylation sites.

Results: Our optimized EasyPep chemistry combined with the large-scale format and subsequent phosphopeptide enrichment provides a complete workflow solution in less than 7 hours. We have identified ~8000-9000 phospshopeptides with ~95% phosphospecificity and CVs <5%. We have compared it to the existing resin workflows and observed identical performance in terms of phosphopeptide specificity and identification rates. We have also assessed the workflow on a Kingfisher Apex Magnetic Particle Processor which ensures reproducibility and eliminates the hands-on-challenges while handling a large number of samples.

INTRODUCTION

Phosphorylation is a post translational modification (PTM) that acts as a molecular switch in cell signaling pathways. Because phosphorylation misregulation is key factor in many human diseases, high throughput studies that identify changes in phosphorylation are important for understanding disease mechanisms. In these studies, reducing variability between samples is key. For studying the phosphoproteome, additional enrichment is required before mass spec analysis due to the lability and low stoichiometry of phosphorylation. This enrichment can be done using Immobilized Metal Affinity Chromatography (IMAC) with Fe-NTA which has selectivity for the phosphate group at the appropriate acidic pH. The purpose of this study was to use Fe-NTA magnetic agarose beads to optimize a Thermo Scientific™ KingFisher ™ Apex protocol for high-throughput phosphoenrichment.

MATERIALS AND METHODS

Sample Preparation

HeLa S3 cells were cultured in sMEM media supplemented with 10%FBS, 1X Glutamax, and 1% Pen/Strep. Cells were treated with Nocodazole at 0.1µg/ml for 18 hours. HeLa S3 harvested cells and CSF samples were processed into protein digests with Thermo Scientific™ EasyPep™ Maxi MS Sample preparation kit and Halt phosphatase inhibitor. Protein concentrations were determined using Pierce™ Rapid Gold BCA Assay kit. CSF samples were additionally labelled with Thermo Scientific™ TMTpro™ 16plex reagents .

Phosphoenrichment

KingFisher Apex Magnetic Particle Processor, KingFisher 96 Deep-Well plates, and Fe-NTA magnetic agarose beads were used for phosphopeptide enrichment. The digests were incubated with Fe-NTA magnetic beads, organic and aqueous wash steps were performed, and peptides were eluted in a basic buffer. Bead to digest ratios, elution times, wash volume, and plate rinses were tested to optimize the protocol. Peptide desalting using a mixed mode resin in a tip was done to reduce possible contaminants. For a positive control, manual phosphoenrichment was done using Fe-NTA magnetic beads on a magnetic stand.

LC-MS Analysis

Before LC-MS analysis, peptide concentration was determined using Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay kit. Samples were separated using a Thermo Scientific™ Dionex™ Ultimate™ 3000 Nano LC system using a 50 cm C18 Thermo Scientific™ EASY-Spray™ column with an acetonitrile gradient from 3% to 28% over 85 min, 28% to 45% over 30 min, at a flow rate of 300nL/min on a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer. CSF Samples were analyzed on an Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer. To identify plastic contaminants, Fe-NTA enrichment buffers were incubated on KingFisher 96 Deep-Well plates and directly injected into a Thermo Scientific™ QExactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer

RESULTS

Figure 1: Workflow for Phosphopeptide Enrichment

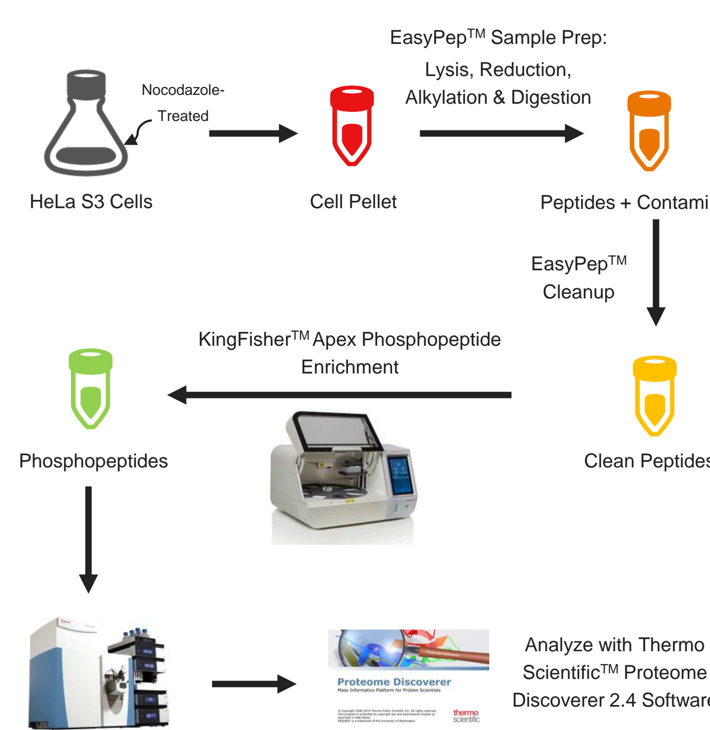
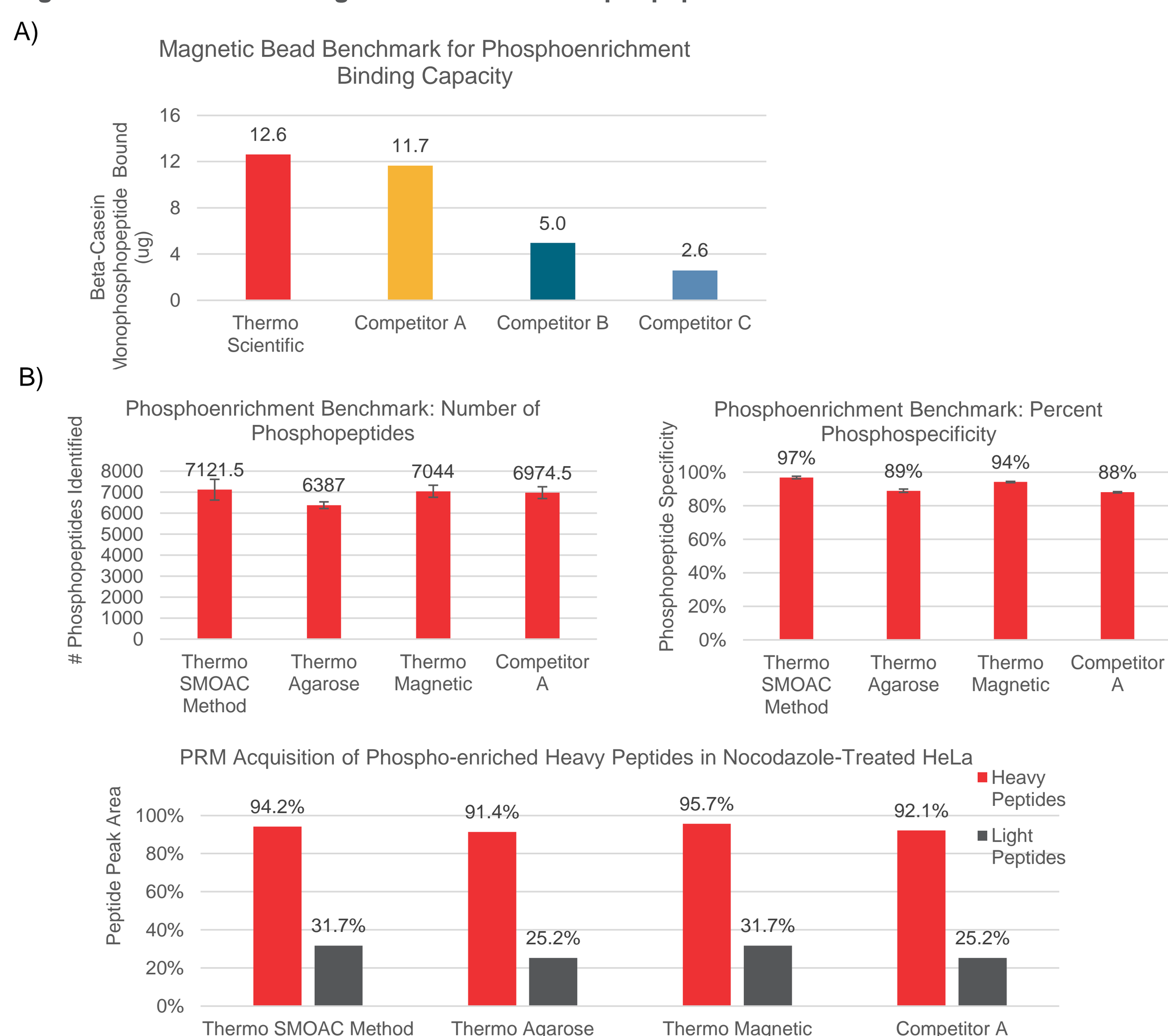
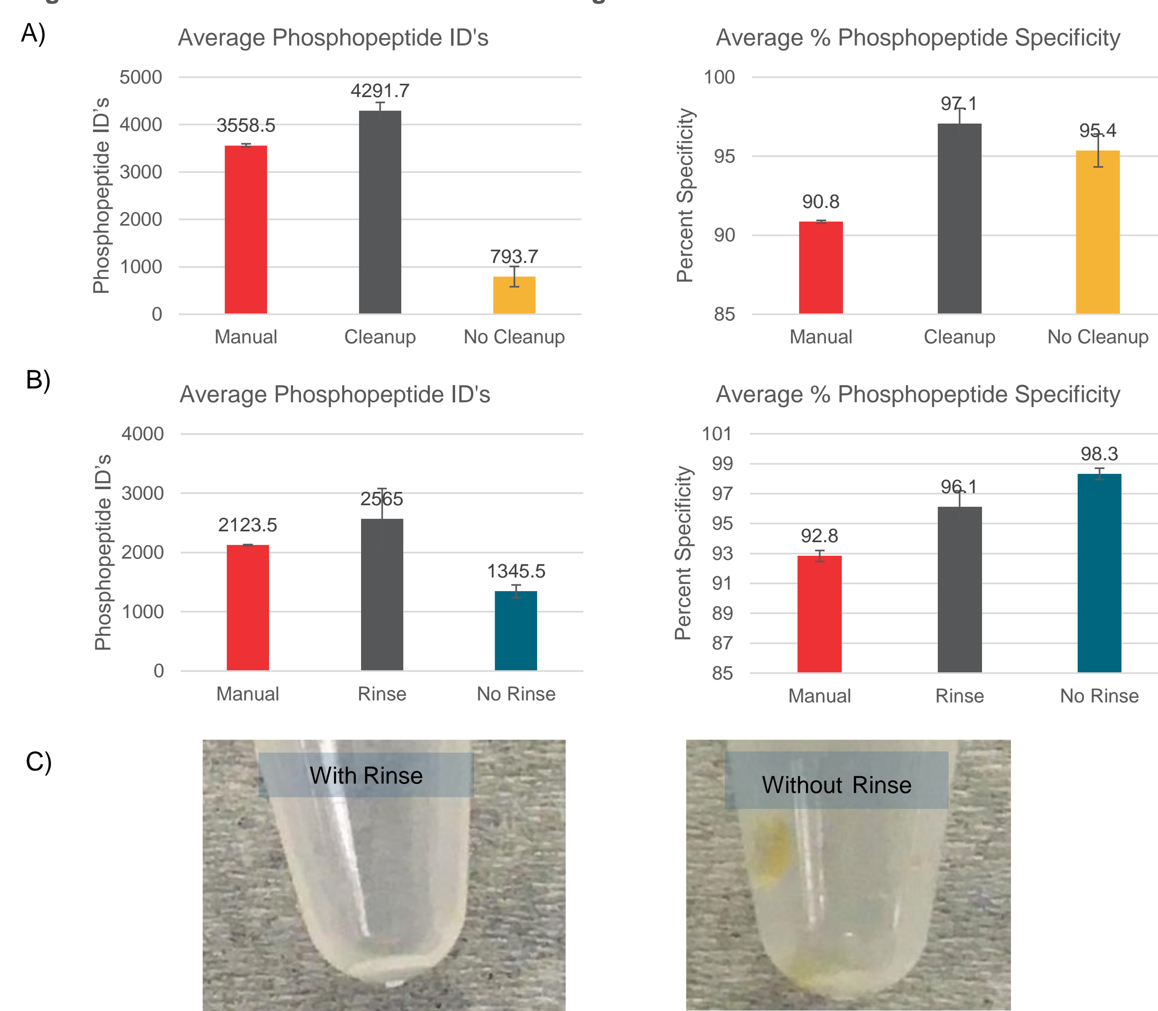


Figure 2: Evaluation of Magnetic Beads for Phosphopeptide Enrichment



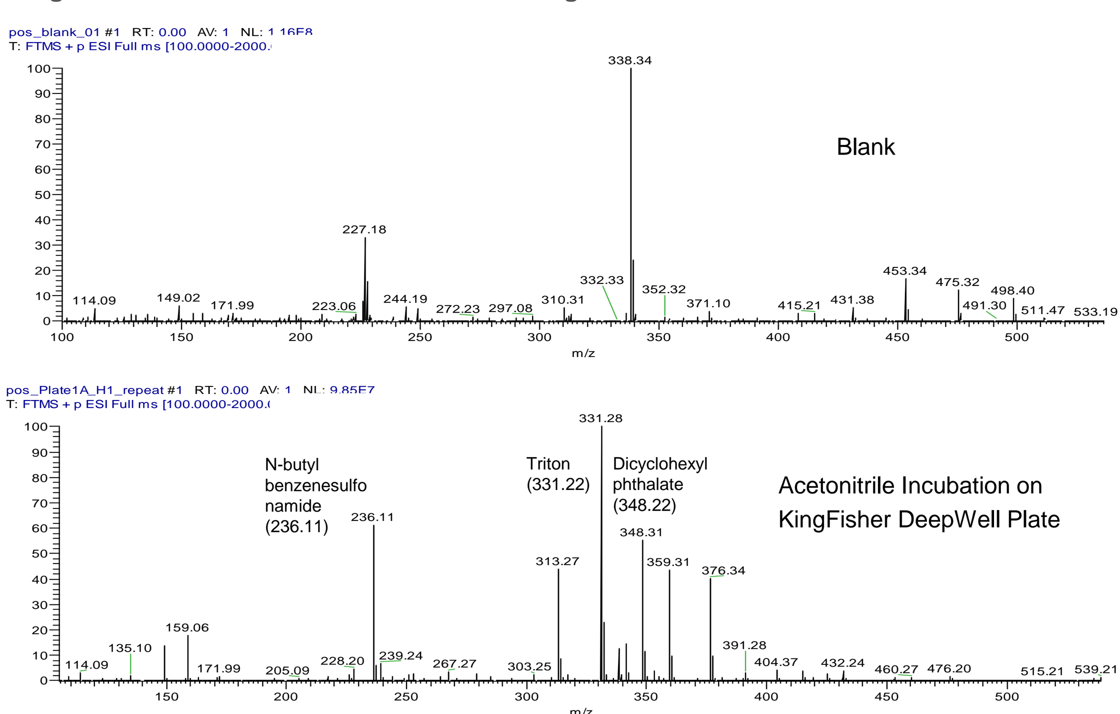
A). Magnetic beads were evaluated for total binding capacity versus the Thermo Scientific™ High-Select™ Fe-NTA Phosphopeptide Enrichment Kit and three competitor products used for phosphopeptide enrichment. 15ug of Beta Casein monophosphopeptide was allowed to bind to a fixed, equivalent amount of resin or bead for all samples. Protocols were followed according to manufacturer's instructions. Thermo Scientific™ Pierce™ Phosphoprotein Phosphate Estimation Assay Kit was used to quantitate the amount of protein remaining in the flow through after binding. Total amount of Beta Casein Monophosphopeptide bound was calculated by subtracting this value from the initial load. **B)** HeLa S3 cells treated with Nocodazole, were processed using EasyPep workflow, spiked with 131 Heavy AQUA phosphopeptides, and subjected to phosphoenrichment with either Thermo Scientific™ High-Select™ Fe-NTA Phosphopeptide Enrichment Kit (with or without SMOAC method), Magnetic Fe-NTA beads, or Competitor Beads A, which performed well in initial binding capacity assessments. Samples were analyzed by Mass spec for number of phosphopeptides versus total peptide identifications as well as targeted analysis to evaluate recovery of heavy and light peptides of interest in a complex matrix.

Figure 3: Current Solutions for Plastic Leaching



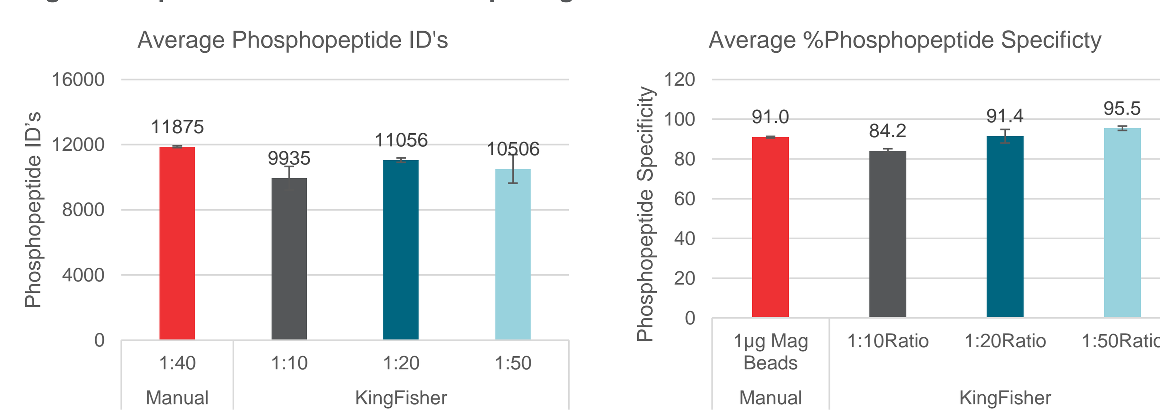
A) Evaluating post-enrichment cleanup: 500µg of EasyPep processed peptides (1µg/µl) were incubated with beads in a 1:50 ratio with a wash volume of 100µl and a one minute elution time. After enrichment, cleaned samples were desalted on a mixed mode resin. 500ng of enriched samples and 500ng of the Fe-NTA magnetic manual comparison sample were analyzed by LCMS as described in the methods. Including cleanup improved ID's by more than 4-fold and percent phosphospecificity was high (~97%). The disadvantages of cleanup include decreased yield and efficiency. **B)** Evaluating an elution plate rinse: 500µg of EasyPep processed peptides (1µg/µl) were incubated with beads in a 1:50 ratio with a wash volume of 100µl and a one minute elution time. Elution plate wells were either rinsed twice with acetonitrile or left empty before adding elution buffer and performing enrichment. 750ng of enriched samples and 500ng of the Fe-NTA magnetic manual comparison sample were analyzed by LCMS as described in the methods. Including a rinse improved phosphopeptide ID's by nearly two-fold and phosphopeptide specificity remained high (~96%). **C)** Images of the above rinse/no rinse samples that have undergone vacuum centrifugation for the same time period. Comparing with the sample on the right, decreased color and faster drying time indicates a decrease in plastic leachables with rinsing.

Figure 4: Evaluation of Leachables from KingFisher™ Plastic Consumables



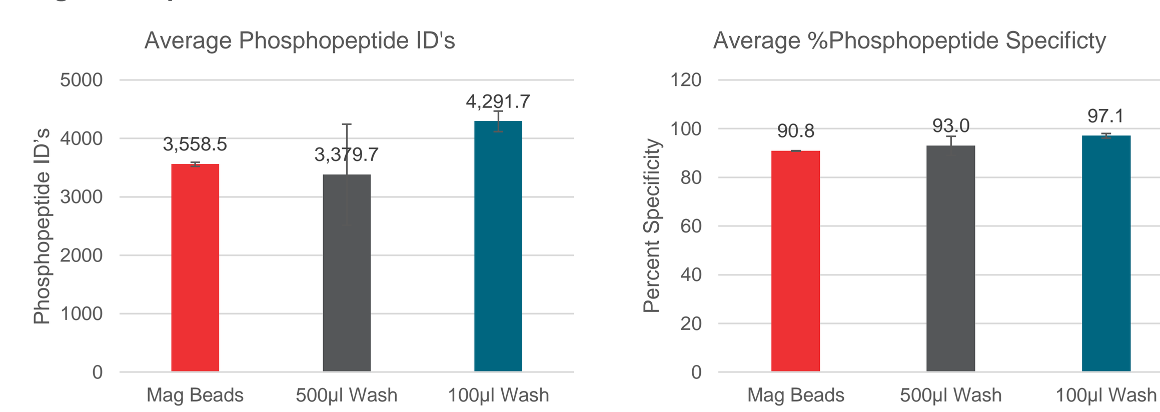
KingFisher plastics are designed for compatibility with DNA and RNA workflows and interaction with enrichment solvents leads to leaching of contaminants. Direct injection of a blank onto the Q Exactive™ HF mass spectrometer shows an acceptable number of contaminant peaks. Injection of acetonitrile and ammonium hydroxide incubated on KingFisher 96 Deep-Well plate at 4°C overnight shows an increased number of peaks consistent with plasticizers and detergents listed in MS contaminant databases. After several rounds of sample and blank injections, the contaminant peaks remaining in the final blank indicate the persistence of these contaminants and the need for extended flushing.

Figure 5: Optimization of Bead to Sample Digest Ratios



Using the Thermo Scientific™ KingFisher™ Apex, 500µg of EasyPep processed peptides (1µg/µl) were incubated with beads in a 1:10, 1:20 or 1:50 ratio. 500ng of enriched samples and 500ng of the Fe-NTA magnetic manual sample were analyzed by LC-MS as described in the methods. The results show that a 1:50 bead to sample ratio maximizes phosphopeptide specificity with only a minor loss in phosphopeptide ID's.

Figure 6: Optimization of Wash Volume



Using the Thermo Scientific™ KingFisher™ Apex, 500µg of EasyPep processed peptides (1µg/µl) were incubated with beads in a 1:50 bead to sample ratio. Organic wash volumes of 500µl or 100µl were compared. 500ng of enriched samples and 500ng of the Fe-NTA magnetic manual comparison sample were analyzed by LC-MS as described in the methods. 100µl wash volume slightly improved phosphopeptide ID's and percent phosphospecificity with greater reproducibility.

Figure 7: Cerebrospinal Fluid Sample Prep and LCMS Analysis Workflow

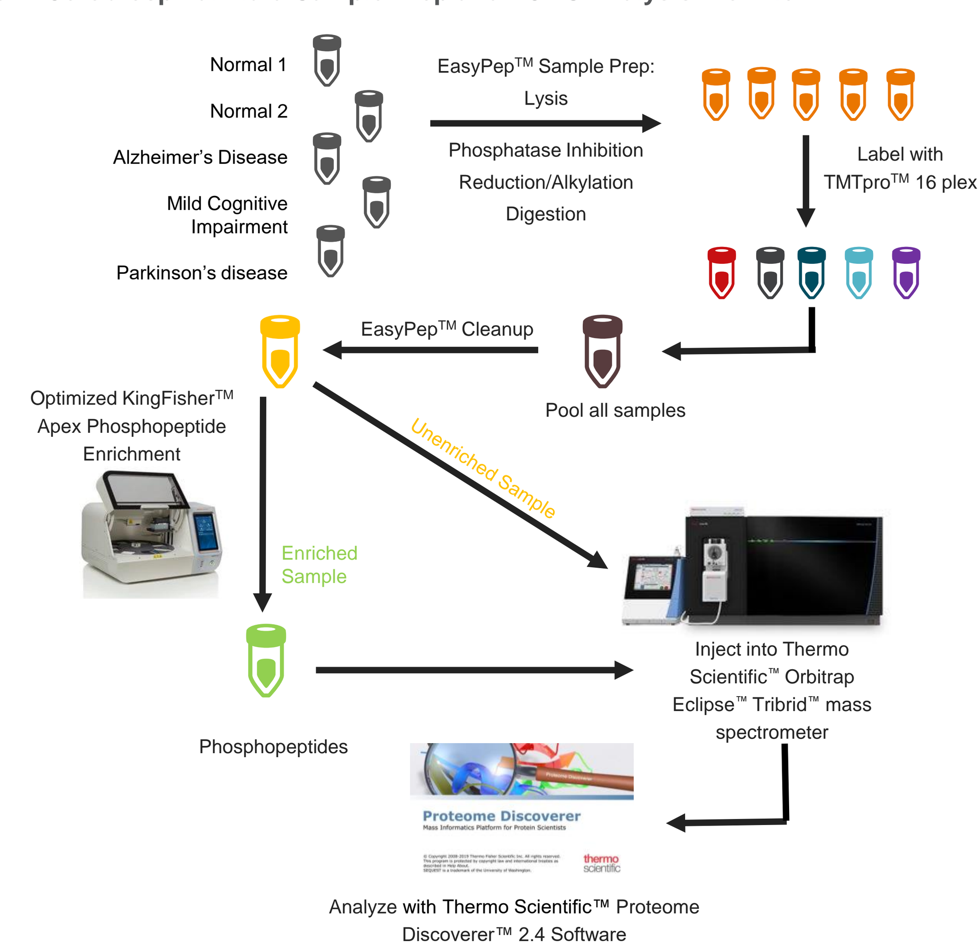
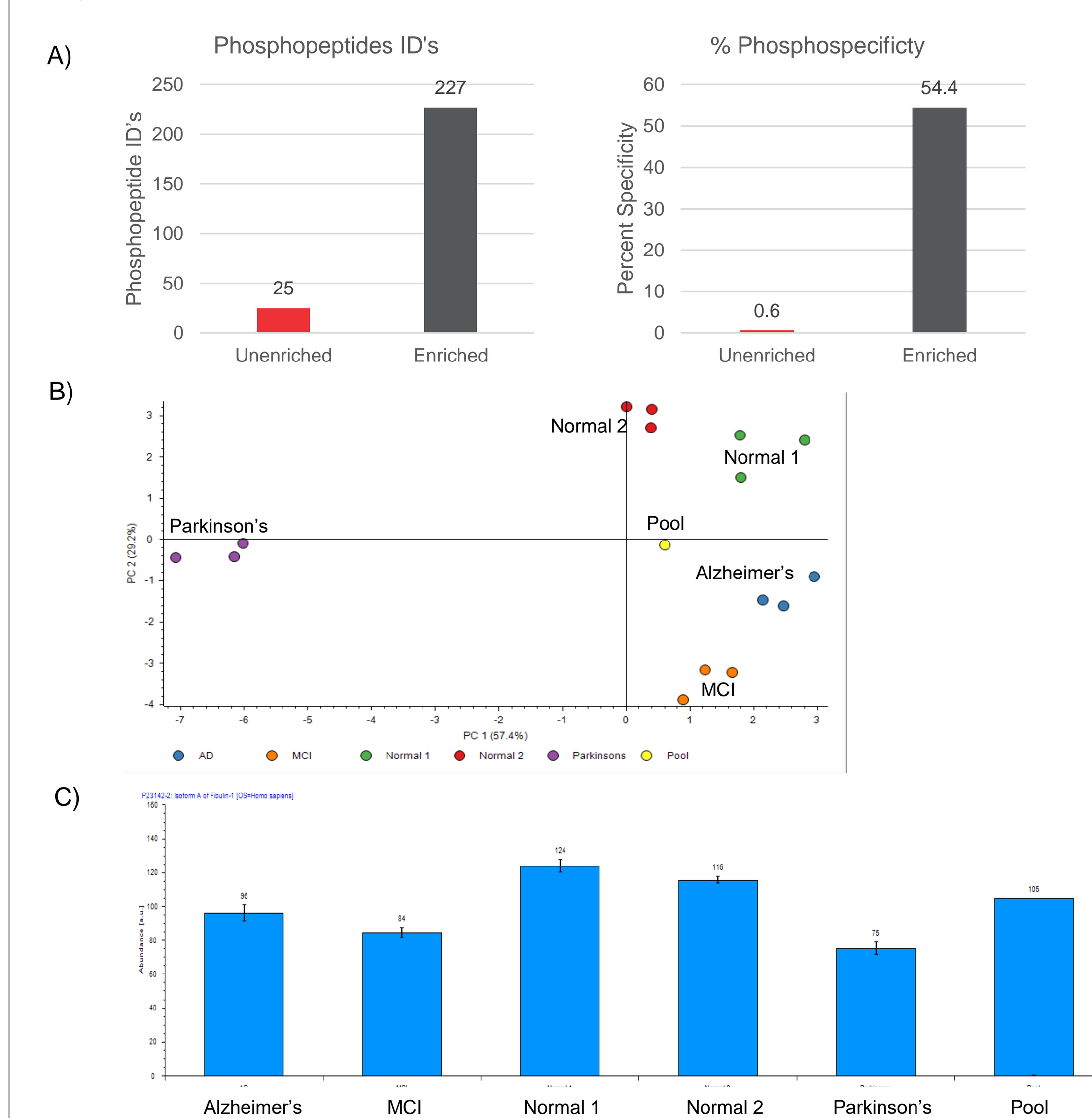


Figure 8: Application of Phosphoenrichment to Cerebrospinal Fluid Samples



CSF samples from two normal patients, one Alzheimer's Disease, one Mild Cognitive Impairment, and one Parkinson's patient were processed according to the unenriched and enriched workflows in Figure 6. A) Phosphopeptide ID's increased in the enriched sample from 25 to 227 with a specificity of 54%. B) PCA plot showing reproducibility with each samples appropriately showing as a distinct population. C) Abundance of Fibulin-1 Isoform A in phosphoenriched CSF samples. Fibulin-1 was found to be phosphorylated in the enriched samples but not in unenriched samples. Although not differentially phosphorylated, Fibulin-1 has lower expression in diseased samples which may correspond with Fibulin's ability to bind the N-terminus of APP – a protein implicated in AD[2].

CONCLUSIONS

- KingFisher automation of phosphoenrichment with Fe-NTA magnetic beads has low variability between samples with up to 10,000 phosphopeptide ID's and 95% phosphopeptide specificity and is comparable or improved to manual magnetic phosphoenrichment.
- KingFisher Apex automation was optimized with cleanup or an elution plate rinse, a 1:50 bead to sample digest ratio, a one minute elution time, and a 100µl wash volume.
- Application of this protocol to CSF samples resulted in 202 more phosphopeptide ID's and identification of phosphorylated proteins of interest in disease.

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TRADEMARKS/LICENSING

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