Development of control peptides for immunoaffinity-based enrichment of posttranslationally modified peptides

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INTRODUCTION

Posttranslational modifications (PTMs) are important elements of many biological processes. A key aspect to experiments that aim to identify and quantify PTM sites is the usage of appropriate techniques such as immunoaffinity purification (IAP) or immobilized metal affinity chromatography (IMAC) to enrich for PTMs prior to liquid chromatography-mass spectrometry (LCMS) analysis. To facilitate IAP and IMAC workflows, we developed a series of synthetic peptides that contain a specific PTM and a stable heavy isotope. The goal of such synthetic peptides is to be added into samples during PTM enrichment and assayed as an internal control for workflow performance and reproducibility.

METHODS

We based the synthetic control peptide sequences on naturally occurring PTM sites consistently identified in our department's internal research using PTMScan® IAP and IMAC workflows.

Peptides were synthesized using FMOC protected amino acids (Gyros Protein Technologies) and Carboxyl Resins (EMD) on a CEM MultiPep 2 peptide synthesizer. Isotopically labeled FMOC protected amino acids ($^{13}C_6{}^{15}N_2$-Lysine or $^{13}C_6{}^{15}N_4$-Arginine) were sourced from Cambridge Isotope Labs. HBTU (Chem-Impex) and 4-Methylmorpholine (EMD) were used for activation of the amino acids. After synthesis, the peptides were cleaved with 87.5% Trifluoroacetic acid (TFA), 5% Dimethyl sulfide, 2.5% H_2O, 2.5% Triisopropylsilane, 2.5% Ethanedithiol. Cleaved peptides were precipitated twice with Diethyl ether. Crude peptides were purified using Reverse Phase C18 HPLC and an accurate peptidyl concentration was determined by amino acid analysis. Peptide purity and sequence were confirmed by LCMS. The final peptide stocks were reconstituted in 25% Acetonitrile (MeCN), 74.9% H_2O, and 0.1% TFA.

To assess the optimal concentration range for use, serial dilution of Control Peptides at 10 µL volume was added to sample peptides (e.g. mouse liver tryptic) at a final volume of 1.4-1.5 mL prior to PTM enrichment. Washes, elution, and desalting were performed as specified in the respective PTMScan® workflows.

CONCLUSIONS

We identified a set of synthetic Control Peptides that can be quantitatively enriched by the respective PTMScan® workflows without negative impact on the analysis of endogenous modified peptides. Such peptides can be used to assess the reproducibility of PTM enrichment steps independent of upstream sample peptide preparation steps.

ACKNOWLEDGMENTS

We thank Jason Reynolds and the rest of the CST Peptide Lab for purification and quantification of the control peptides. We thank members of the CST Proteomics Department and Production Team 11 for their advice and discussions during the development of the control peptides.

Control Peptide & PTMScan® Workflow

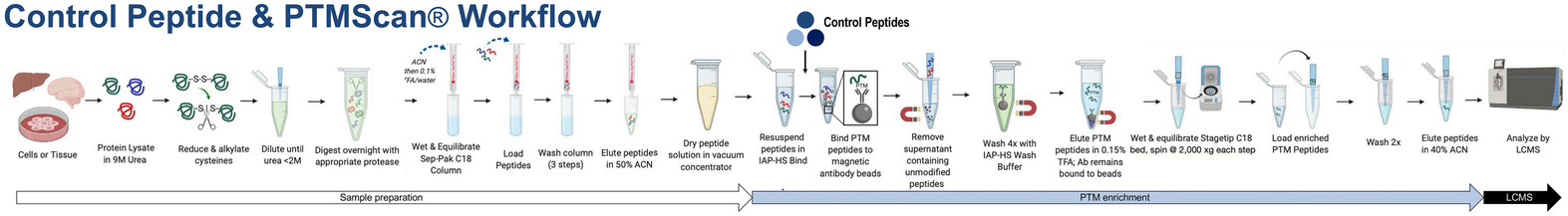

Figure 1: Users add an equal concentration of Control Peptides (blue circles) into each sample at the beginning of PTM enrichment in PTMScan® workflows, as an internal control for enrichment reproducibility. Control Peptides can be detected by LCMS.

Control Peptide Recovery from biological samples using PTMScan® reagents

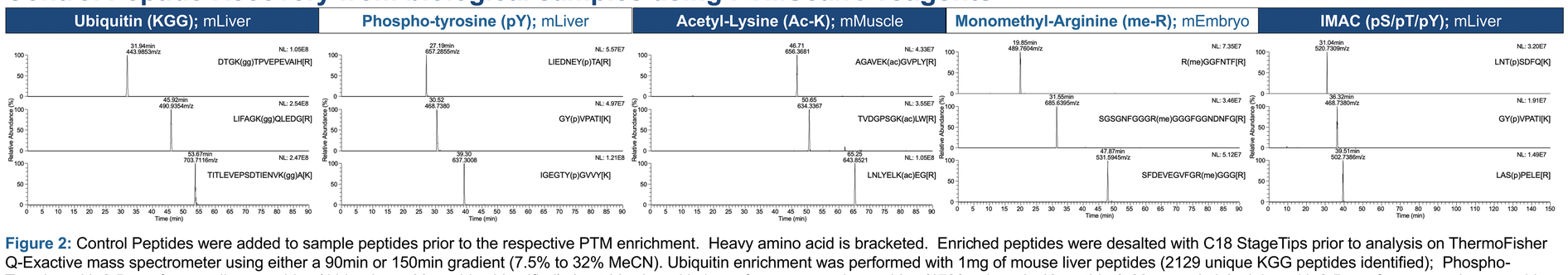

Figure 2: Control Peptides were added to sample peptides prior to the respective PTM enrichment. Heavy amino acid is bracketed. Enriched peptides were desalted with C18 StageTips prior to analysis on ThermoFisher Q-Exactive mass spectrometer using either a 90min or 150min gradient (7.5% to 32% MeCN). Ubiquitin enrichment was performed with 1mg of mouse liver peptides (2129 unique KGG peptides identified); Phospho-Tyrosine with 2.5mg of mouse liver peptides (414 unique pY peptides identified); Acetyl-Lysine with 1mg of mouse muscle peptides (1726 unique Ac-K peptides); Monomethyl-Arginine with 2.5mg of mouse embryo peptides (894 unique me-R peptides identified); IMAC with 0.5mg of mouse liver peptides (8398 unique pS/pT/pY peptides identified). Extracted ion chromatograms for each Control Peptide confirm recovery by PTMScan® beads.

Control Peptide Quantification Range

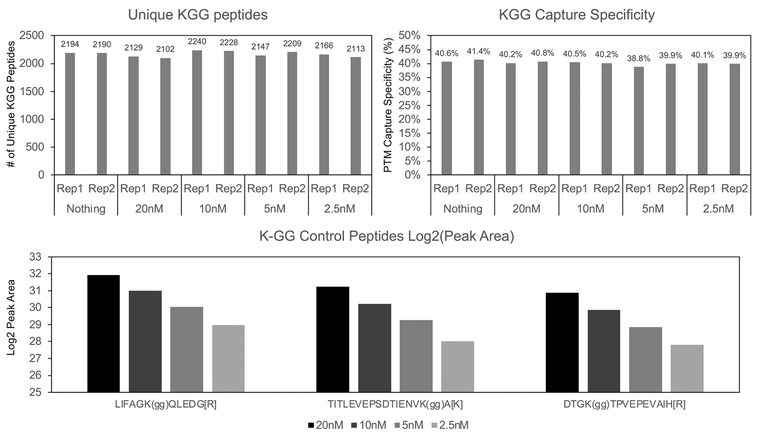

Figure 3: Addition of KGG Control Peptides across a dilution range does not impact the yield or specificity of recovering endogenous KGG modified peptides from 1mg of input mouse liver sample peptides using PTMScan® HS Ubiquitin beads. The recovery of the KGG Control Peptides was proportional to the amount added, and a 20nM stock concentration was determined optimal for all Control Peptides. In this Figure, "Rep" refers to duplicate injections of a single peptide sample. Data were acquired on ThermoFisher Q-Exactive instrument.

Control Peptide Reproducibility

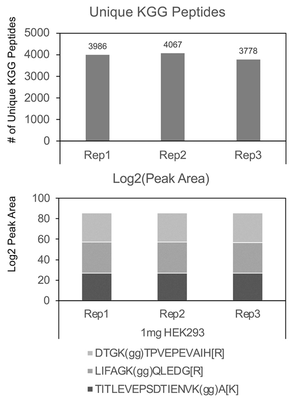

Figure 4: Recovery of KGG Control Peptides across three independent PTMScan® HS Ubiquitin experiments using 1mg of human HEK293 sample peptides. Data were acquired on ThermoFisher Fusion Lumos instrument.

Control Peptide & PTMScan® Compatibility

PTMScan Kit	PTMScan Control Peptide
PTMScan® Ubiquitin Remnant Motif (K-ε-GG) Kit	PTMScan® Control Peptides Ubiquitin/SUMO
PTMScan® HS Ubiquitin/SUMO Remnant Motif (K-ε-GG) Kit	
PTMScan® Acetyl-Lysine Motif [Ac-K] Kit	PTMScan® Control Peptides Acetyl-Lysine
PTMScan® HS Acetyl-Lysine Motif (Ac-K) Kit	
PTMScan® Phospho-Tyrosine Rabbit mAb (P-Tyr-1000) Kit	PTMScan® Control Peptides Phospho-Tyrosine
PTMScan® HS Phospho-Tyrosine (P-Tyr-1000) Kit	
PTMScan® Succinyl-Lysine Motif [Succ-K] Kit	PTMScan® Control Peptides Succinyl-Lysine
PTMScan® HS Succinyl-Lysine Motif (Succ-K) Kit	
PTMScan® Mono-Methyl Arginine Motif [mme-RG] Kit	PTMScan® Control Peptides Mono-Methyl Arginine
PTMScan® HS Mono-Methyl Arginine Motif (mme-RG) Kit	
PTMScan® Symmetric Di-Methyl Arginine Motif [sdme-RG] Kit	PTMScan® Control Peptides Symmetric Di-Methyl Arginine
PTMScan® HS Symmetric Di-Methyl Arginine Motif (sdme-RG) Kit	
PTMScan® Asymmetric Di-Methyl Arginine Motif [adme-R] Kit	PTMScan® Control Peptides Asymmetric Di-Methyl Arginine
PTMScan® HS Asymmetric Di-Methyl Arginine Motif (adme-R) Kit	
PTMScan® Pan-Methyl Lysine Kit	PTMScan® Control Peptides Pan-Methyl Lysine
PTMScan® O-GlcNAc [GlcNAc-S/T] Motif Kit	PTMScan® Control Peptides O-GlcNAc
PTMScan® Phospho-Akt Substrate Motif mAb 1 (RXXS*/T*) Kit	PTMScan® Control Peptides Phospho-Akt (RXXS*/T*)
PTMScan® Multi-Pathway Enrichment Kit	PTMScan® Control Peptides Multi-Pathway
PTMScan® Phospho-Enrichment IMAC Fe-NTA Magnetic Beads	PTMScan® Control Peptides Phospho-Enrichment IMAC

Table 1: List of all Control Peptides developed and demonstrated compatibility with existing respective PTM enrichment workflows, including both agarose and magnetic-bead based formats (i.e. PTMScan® HS and IMAC). Each set of Control Peptides for a particular PTM is comprised of at least 3 unique peptide components.

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