Integrating Complex Multi-Method Workflows on a Single Hamilton® Microlab STAR™ Workstation using INtip™ Chemistries

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integrated micro-chromatography systems

INTRODUCTION

Automating complex, multi-step methods requires personnel with laboratory science, robotics programming skills, and design of experiment expertise. They must invest a significant amount of time outlining the workflow and transferring methods for robotic automation and obtaining such resources can be tasking. Here, we automated the complex phosphopeptide enrichment methods starting from crude cell lysate to liquid chromatography-mass spectrometry (LC-MS) ready processed samples as an example.

- Automated cell lysate processing
- Sequential sample processing for small number of samples (8+ samples) to larger number of samples
- Multi-step solid phase extraction processes using INtip chemistries
- Options and variability functions displayed on graphical user interface
- Modular pre-templated programs for protein digestion, peptide desalting and phosphopeptide enrichment run in single run or in three separate stages

METHODS

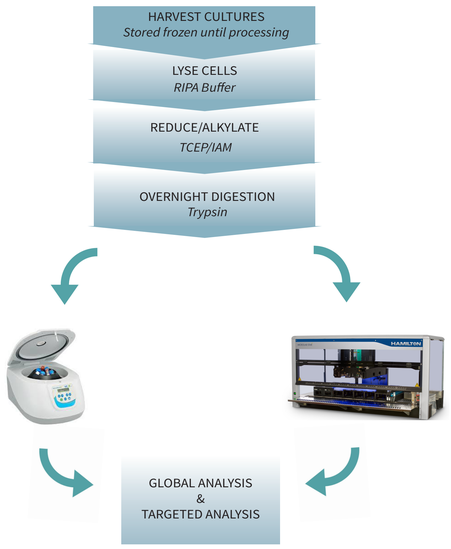

Figure 1. *General workflow for desalting and phosphopeptide enrichment.*

- HCT116 cells cultured in Gibco™ DMEM/F-12, containing 15 mM of HEPES and supplemented with 10% FBS.
- Cells were passaged by trypsin/EDTA treatment at 80-90% confluence.
- The cells were passaged at least 5 times before treating with 10 mM H_2O_2 or H_2O_2/Na_3VO_4 for 30 min.
- The cells lysed with RIPA buffer containing protease and phosphatase inhibitor cocktail.
- 10 mM TCEP was added to reduce proteins at 56 °C for 30 min, then 25 mM iodoacetamide alkylation for 30 min in dark followed by overnight trypsin digestion (1:50 enzyme to protein ratio) at 37 °C.
- Phosphopeptide enrichments using TiO_2-based spin tip methods were performed using the vendors' recommended protocol.
- Automated sample processing performed on Hamilton Microlab STAR using IMCStips packed with polystyrene divinylbenzene polymer for desalting followed by 300 µL IMCStips packed with two different phosphopeptide enrichment resins, ZrO_2 and PolyTi and 1:1 PolyTi/ZrO_2 mix (Mixed Mode).

Table 1. *Mass spectrometric analysis of phosphopeptides*

	Global Analysis	Targeted Analysis
Mass spectrometer	Orbitrap Velos Pro, Thermo Fisher	TSQ Endura
Liquid chromatography	Ultimate 3000 nano-UHPLC	Vanquish UHPLC
Mobile phase A	0.1% formic acid in water	0.1% formic acid in water
Mobile phase B	0.1% formic acid in 80% acetonitrile	0.1% formic acid in acetonitrile
LC gradient	2% - 30% B for 60 min	2%-35% for 10 min
Analytical column	50-cm in-house packed column [360 µm OD x 75 µm inner diameter (ID)] with C18 resin (2.2 µm, 100 A; Michrom Bioresources)	Waters BEH C18 (100 x 2.1 mm, 1.7 µm)
Column oven temperature	50°C	40°C

Global Analysis

MS spectra were acquired by data dependent scans consisting of MS/MS scans of the fifteen most intense ions from the full MS scan with dynamic exclusion option at 10 seconds. Spectra were searched using Sequest HT algorithm within the Proteome Discoverer v2.2 (Thermo Scientific) in combination with the human UniProt protein FASTA database (2017 March, 20,119 entries).
Search parameters were as follows:

- FT-trap instrument, parent mass error tolerance of 10 ppm,
- Fragment mass error tolerance of 0.02 Da (monoisotopic), variable modifications of 15.995 Da (oxidation) on methionine and 79.966 Da (phosphorylation) on serine, threonine and tyrosine, fixed modification of 57.021 Da (carbamidomethylation) on cysteine.

Targeted Analysis

- Phosphopeptide enrichment was performed on three phosphopeptides spiked in trypsin digested BSA
- 8 replicates were analyzed each week for 3 weeks, each week running a new enrichment process.

RESULTS

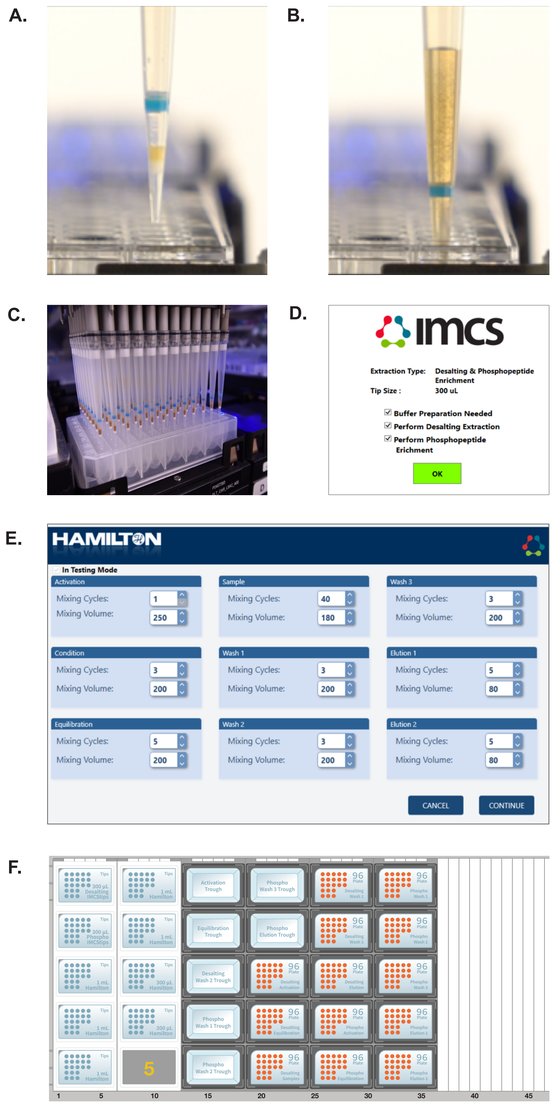

Figure 2. *Dispersive pipette extraction is a solid phase extraction tool that leverages turbulent mixing inside a pipette tip for efficient solid phase extractions. The loose resin contained within the tip* **(A)** *is dispersed as the liquid sample is aspirated* **(B)**. *Larger throughput of IMCStips on the Hamilton Microlab STAR can be used to perform solid phase extractions* **(C)**. *The script is provided with intuitive graphical user interfaces (GUIs) for the automated method. The first GUI provides options for buffer aliquoting, peptide desalting, and phosphopeptide enrichment* **(D).** *Extraction parameters page enables the scientist to adjust solution volumes and mixing cycles for each step in the phosphopeptide enrichment process* **(E).** *Buffer aliquoting transfers bulk reagents to 96-well plates, eliminating the need for reagent pipetting. Desalting of peptides is typically performed prior to phosphopeptide capture, and this method can be uncoupled and run independently. The deck layout for the automated method for desalting and phosphopeptide enrichment indicates where each of the consumables are placed prior to running the script* **(F)**.

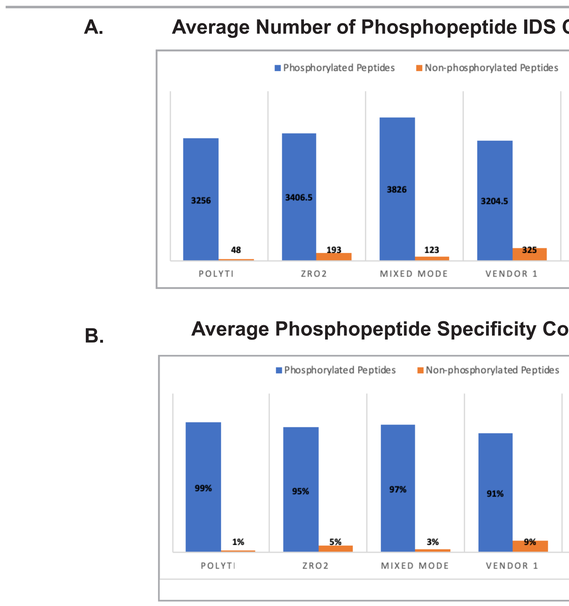

Figure 3. *The total number of phosphopeptides identified after enrichment* (***A***) *and their respective specificities for the different resin type, and automated / manual workflows* (***B***). *P, Z, and H were processed using the IMCStips using automated phosphopeptide enrichment method. SC1 and 2 were processed follow the vendors'protocol. All samples were processed in duplicates. The numbers shown are averages from duplicate runs.*

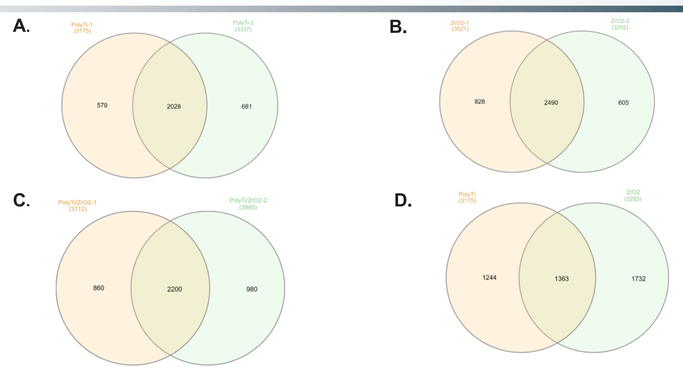

Figure 4. *Venn diagrams of phosphopeptide overlap from duplicate sample enrichments. The duplicate sample preparations for PolyTi* (***A***), *ZrO_2* (***B***) *and the combination of both resins in a single pipette tip* (***C***) *show an average of 76%, 78%, and 71% overlap, respectively. In comparison, the two different resins PolyTi and ZrO_2 show 48% percent of overlap* (***D***). *This reduction in overlap of phosphopeptide coverage is expected for these two resin types, as the distribution of the singly- and doubly-phosphorylated peptides were also varied for the two resin types.*

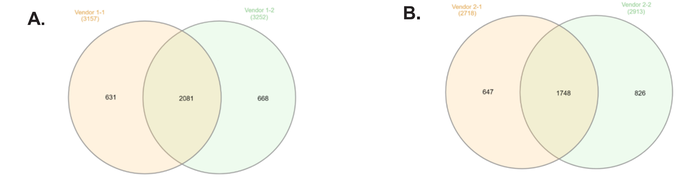

Figure 5. *Venn diagrams of phosphopeptide overlap from duplicate sample enrichments using spin columns. The duplicate sample preparations for SC1* (***A***) *and SC2* (***B***) *show an average of 76% and 70% overlap, respectively.*

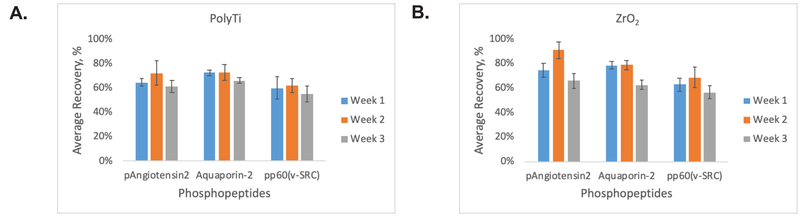

Figure 6. *The average recovery for each phosphopeptide from three different phosphopeptide enrichments and MRM assays performed once a week over three weeks using* (***A***) *PolyTi resin and* (***B***) *ZrO_2 resin (n=8 per week).*

CONCLUSION

We established a completely automated phosphopeptide enrichment method using two different resins in the IMCStips that allow for reproducible phosphopeptide sample preparation from cell lysates. This method is easily integrated onto the Hamilton Microlab STAR workstation without any need of additional hardware.

- Identified over 3000 phosphopeptides
- > 90% phosphopeptide specificity
- > 70% sample to sample overlap
- Reproducible quantitative results using an MRM assay.

This automation program demonstrates a standardized workflow for complex, multi-step methods that provides consistency and ease of use, allowing researchers to focus on data analysis and experimental design rather than slogging through monotonous technical protocols.

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