Comparison of different resins for automated phosphopeptide enrichments for deep-profiling of Hct116 cell line.

Todd Mullis*[1], Sunil Hwang[2], Matthew Manter[2], Michael Walla[1], Jingyun Lee[3], Cristina Furdui[3], L. Andrew Lee[2], Qian Wang[1]

[1]University of South Carolina, Department of Chemistry and Biochemistry, Columbia, SC; [2]Integrated Micro-Chromatography Systems, Inc, Columbia, SC; [3]Proteomics and Metabolomics Shared Resource, Wake Forest School of Medicine, Winston-Salem

integrated micro-chromatography systems

ABSTRACT

Developed and optimized a completely automated phosphopeptide enrichment method using two different resins in the IMCStip that allows for high-throughput and reproducible sample preparation.

- Identified of over 3000 phosphopeptides per sample with an average sample to sample peptide overlap of 80%.
- Generated reproducible quantitative MS data using a SILAC approach to compare treated versus untreated phosphorylation profiles for the Hct116 cell line.

INTRODUCTION

Phosphorylation events of proteins are key signal transductions within eukaryotic cells and provide insightful molecular signatures of various human diseases. The global deep profilings of phosphopeptides have been reported in the selective phosphopeptide enrichment, mass spectrometry, and data analysis. However, phosphopeptide enrichment is a highly labor-intensive and lowthroughput process that leads to poor reproducibility. Here we provide detailed workflow using fully-automated dispersive pipette extraction for phosphopeptide enrichment method on a robotic liquid handling system. We use two different resin chemistries to compare the different phosphopeptide profiles by mass spectrometry. Specifically, the differences in number of identified phosphorylated peptides, enrichment specificities, and sample to sample reproducibilities based on specific peptides identified within the sample are shown for both automated and spin format methods. We also show a quantitative global phosphoproteomic approach using SILAC in order to determine sample to sample reproducibility using peptide abundance ratios.

METHODS

Hct116 cells were grown in Gibco™ DMEM/F-12, containing 15 mM of HEPES and supplemented with 10% FBS. Freshly thawed cells were passaged by trypsin/EDTA treatment at 80-90% confluence. The cells were passaged at least 5 times before oxidative stress treatment with 10 mM H_2O_2 or H_2O_2/Na_3VO_4. For Stable Isotope Labelling with Amino Acids in Cell Culture (SILAC), Hct116 cells were grown in Thermo Scientific™ DMEM:F-12 Media with 10% dialyzed FBS. 99% incorporation of heavy labelled amino acids ($^{13}C_6$ L-Lysine and $^{13}C_6{}^{15}N_4$ L-Arginine) was confirmed by mass spectrometry. The cells were lysed in RIPA buffer containing protease and phosphatase inhibitor cocktail. Cell lysates were reduced with 10 mM TCEP at 56 °C for 30 min, then alkylated with 25 mM iodoacetamide for 30 min in dark followed by overnight trypsin digestion (1:50 enzyme to protein ratio) at 37 °C. For automated sample processing, we used Microlab STAR from Hamilton Robotics. Phosphopeptide enrichments using conventional TiO_2-based spin tip methods from Vendor 1 and Vendor 2 were performed using the vendors' recommended protocol.

- Mass spectrometer .....Orbitrap Velos Pro, Thermo Fisher
- Liquid chromatography .....Ultimate 3000 nano-UHPLC
- Mobile phase A.....0.1% formic acid in water
- Mobile phase B.....0.1% formic acid in 80% acetonitrile
- LC gradient .....2% - 30% B for 60 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)
- Column oven temperature .....50 ºC

For phosphopeptide identification, we used an Orbitrap Velos Pro mass spectrometer coupled to Ultimate 3000 nano-UHPLC system. 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. For SILAC quantitation, the heavy labelled cells were used as the control.

RESULTS

The cells were incubated with H_2O_2 and sodium orthovanadate and harvested at various time points. The influence of orthovanadate in combination with peroxide on protein phosphorylation has been reported in the past (1-3). Hct116 phosphorylation events were monitored by western blot with anti-pMAPK (ERK 1/2, rabbit host, Cell Signaling Technologies) and 4G10 (anti-phosphotyrosine, mouse, Sigma Aldrich). ERK1/2 phosphorylation is immediately observed upon hydrogen peroxide and orthovanadate stimulation (**Figure 1 A**), whereas overall phosphotyrosine appears to increase over time (**Figure 1 B**), as suggested by the increase in overall intensity of the protein bands.

These results are consistent with reported studies by Zick and Sagi-Eisenberg, and Heffetz et al. (1-3). Heffetz et al reported that the combination of hydrogen peroxide and orthovanadate mimics insulin-like stimulatory effects with rapid onset of tyrosine phosphorylation (1,2). Zick and Sagi-Eisenberg demonstrated the increase in protein tyrosine phosphorylation in a dose dependent manner, along with a dramatic increase in inositol triphosphate (3). Based on such reports, similar stimulation was utilized for establishing the initial phosphopeptide enrichment workflows.

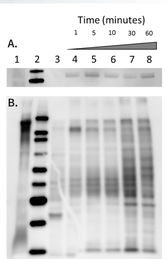

Figure 1. *Western blot images of HCT116 lysates. Lysates were separated on SDS-PAGE, blotted and probed with anti-phospho-MAPK (ERK 1/2) (A). The lysates were also probed with anti-pTyr antibody (4G10) (B). Lane 1: Gel Marker (Expedeon Two-Color SDS™ Marker), Lane 2: Western Blot Marker (Invitrogen™ Novex™ MagicMark™ XP Western Protein Standard), Lane 3: Unstimulated Hct116 cell lysate. Lanes 4-8: Hct116 cell lysate subjected to oxidative stress with increasing time. The control lane showed no phosphorylation of MAPK while peroxide and orthovanadate treated cells show MAPK phosphorylation consistent with previous report (5).*

Automation reduces the variations introduced during manual pipetting and sample processing. Especially for a workflow as complex as the phosphopeptide enrichment method, this workflow allows for automation, and a standardized phosphopeptide enrichment leading to reproducible sample to sample results. Prior reports have relied heavily on manual workflows, in particular using the spin column formats. In comparison to the centrifugation, the automated workflow relies far less on manual intervention, which is likely to lead to more consistent results.

Automated sample preparation using the Hamilton® STAR was optimized for 300 µL IMCStips packed with two different phosphopeptide enrichment resins, ZrO_2 and PolyTi and a 1:1 PolyTi/ZrO_2 mix (Hybrid). To increase enrichment efficiency, we optimized preenrichment desalting with IMCStips using a polymeric reverse phase resin. The overall workflow for the automated pre-desalting and phosphopeptide enrichment method is shown in **Table 1** and the associated deck layout for the method (**Figure 2**).

Step Number	Step name	Buffer Composition	Mixing Volume, µL	Number of Mix, #	Approx. Duration, Min.
1	Desalting Activation	100% ACN	200	3	2
2	Desalting Equilibration	1% TFA	200	3	2
3	Desalting Sample Bind	1% TFA	200	10	5
4	Desalting Wash 1	1% TFA	200	3	2
5	Desalting Wash 2	Water	200	3	2
6	Desalting Elution	100 mM glycolic acid, 1% TFA, 50%ACN	150	5	3
7	Phospho Activation	100% ACN	200	3	2
8	Phospho Equilibration	1% TFA	200	3	2
9	Phospho Sample Bind	100 mM glycolic acid, 1% TFA, 50% ACN	150	30	30
10	Phospho Wash 1	100 mM acetic acid, 1% TFA, 80% ACN	200	5	4
11	Phospho Wash 2	100 mM acetic acid, 1% TFA, 80% ACN	200	5	4
12	Phospho Wash 3	80% ACN	200	7	5
13	Phospho Elution 1	1.5% NH_4OH	100	5	4
14	Phospho Elution 2	1.5% NH_4OH	100	5	4

Table 1. *Automated workflow for desalting and phosphopeptide enrichment.*

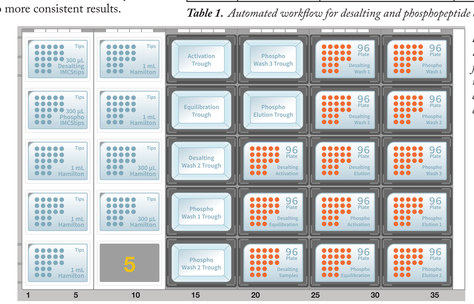

Figure 2. *Microlab STAR deck layout for the automated method for desalting and phosphopeptide enrichment.*

The different phosphopeptide enrichment resins and workflows were compared using a single source of material, Hct116 cells treated with hydrogen peroxide and sodium orthovanadate. The phosphopeptide enrichment for samples marked P, Z and H was preceded by reverse phase desalting procedure on the automated liquid handler. Over 3000 phosphopeptides were identified for four of the five different resins/methods, with SC2 having lowest number of phosphopeptides and specificity (**Figure 3 A**). The sample (H) processed in combination of the two resins provided the highest number of phosphopeptides, but less than the two samples (P, Z) processed separately. Using the PolyTi, ZrO_2, and hybrid, average phosphopeptide specificities of 99%, 95%, and 97% were obtained, respectively, whereas the spin formats resulted in lower specificities at 91% and 77% for SC1 and SC2, respectively (**Figure 3 B**).

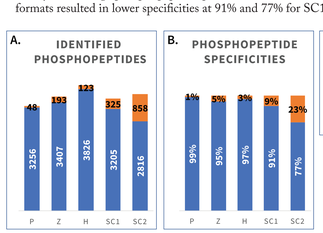

Figure 3. *The total number of phosphopeptides identified after enrichment **(A)** and their respective specificities for the different resin type, and automated / manual workflows **(B)**. P, Z, and H were processed using the IMCStip using automated phosphopeptide enrichment method. SC1 and 2 were processed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

The distribution of the singly-, doubly- and triply- phosphorylated peptides suggest higher selectivity for singly phosphorylated peptides when using ZrO_2, whereas PolyTi resin enriches both singly and doubly phosphorylated peptides (**Figure 4**). Approximately 90% of the identified phosphopeptides are singly phosphorylated peptides when using ZrO_2, whereas less than 50% of the identified phosphopeptides are singly- phosphorylated with PolyTi enriched samples. The highest number of doubly- and triply- phosphorylated peptides were identified using PolyTi resin. Previous studies have shown that the titania and zirconia have different affinities for the phosphorylated peptides, and the current results are consistent with these prior reports (5,6).

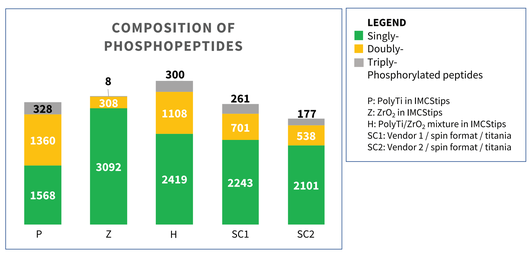

Figure 4. *The different distribution of singly-, doubly- and triply- phosphorylated peptides enriched using the different resins.*

Interestingly, the mixture of PolyTi/ZrO_2 leads to increased phosphopeptide identifications, but not a complete overlap compared to when each resin is used individually. This difference could be attributed to a variety of factors such as resin capacities, signal suppression or mass spectrometric limitations of the LTQ Orbitrap. This hybrid approach could be used when faster throughput is desired whereas, samples could be processed with both chemistries in two different tips for a more comprehensive phosphorylation profile.

Next, the phosphopeptide enrichment reproducibility was determined by comparing sample to sample overlap of peptides identified within the samples. The peptide overlap of 2 different samples after phosphopeptide enrichment with PolyTi, ZrO_2, and 1:1 PolyTi/ZrO_2 show averages greater than 75% (**Figure 5**).

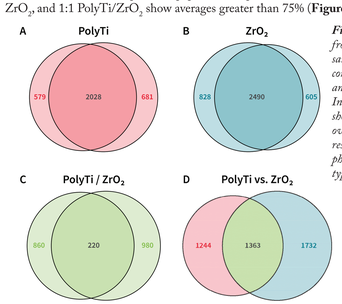

Figure 5. *Venn diagrams of phosphopeptide overlap from duplicate sample enrichments. The duplicate sample preparations for PolyTi (A), ZrO_2 (B) and the combination of both resins in a single pipette tip (C) show an average of 76%, 78%, and 71% overlap, respectively. In comparison, the two different 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.*

The manual methods using spin column formats show overlapping peptide identification of 76% for vendor 1 and 70% for vendor 2 (**Figure 6**).

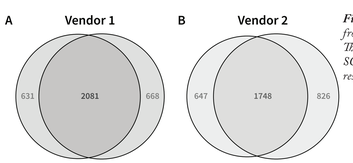

Figure 6. *Venn diagrams of phosphopeptide overlap from duplicate sample enrichments using spin columns. The duplicate sample preparations for SC1 (A) and SC2 (B) show an average of 76% and 70% overlap, respectively.*

Based on these results, the automated phosphopeptide enrichment method generates reproducible phosphorylation profiles similar or better than manual spin format methods using different resin chemistries with less hands-on time. Both reproducibility and automation are crucial for generating consistent profiles. These phosphorylation profiles are then compared to different disease states and healthy states to identify potential dysfunction in cellular signaling pathways (5). Prior studies have utilized various fractionation strategies to identify over 11,000 phosphopeptides, but this approach is utilizing non-fractionated identification of approximately 4,000 phosphopeptides with two different immobilized metal affinity resins (PolyTi and ZrO_2). Further studies coupling automated fractionation with SCX resins or use of higher end mass spectrometry may result in a greater number of identified phosphopeptides.

Implementing SILAC for the study of phosphorylation events upon different treatments has been useful in understanding how cells respond by using different signaling pathways (6). As previously stated, oxidative stress due to the accumulation of ROS is a common seen in different cancers. For researchers it is important to be able to not only detect these changes, but also quantify these changes in a reproducible manner (5,6). Using SILAC, we wanted to determine sample to sample reproducibility of calculated quantitative ratios between untreated and treated Hct116 cells after phosphopeptide enrichment using our automated method.

For each sample, light (oxidative stress treated) and SILAC heavy labelled cells were mixed 1:1 and automated phosphopeptide enrichment was done on each sample. The data was then processed in Proteome Discoverer in order to calculate fold-changes in the detected proteins. The calculated abundances were compared between biochemical replicate samples enriched using the same resin type to determine sample to sample reproducibility of the calculated quantitative ratios. These comparisons are shown in **Figures 7** for PolyTi and ZrO_2, respectively. By Pearson correlation the R-values for the comparison of calculated abundance ratios for two replicates from PolyTi and ZrO_2 were 0.77 and 0.81, respectively.

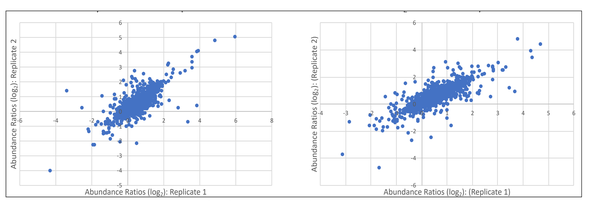

Figure 7. *A comparison of abundance ratios (log2) between two different 1:1 mixed SILAC samples that were subjected to phosphopeptide enrichment using PolyTi or ZrO_2. R-value equal to 0.77 and 0.81, respectively by Pearson correlation (P-Value < 0.00001).*

These results show that the implementation of our automated method with the IMCStip will allow researchers to standardize their sample preparation method yielding trustworthy quantitative MS data that will lead to insights of how different treatments or disease states effect phosphorylation effects (5,6).

CONCLUSIONS

Established a completely automated phosphopeptide enrichment method using two different resins using IMCStips that allow for reproducible phosphopeptide sample preparation from cell lysates.

Method is integrated onto the Hamilton® Microlab STAR platform without any need of additional hardware.

Identified over 3000 phosphopeptides in each sample for both PolyTi™ , ZrO_2, and 1:1 PolyzTi/ZrO_2 mix resins. Show >75% sample to sample overlap and reproducible quantitative results using a SILAC approach when comparing the calculated abundance ratios.

Automated workflow method can be used to standardize sample preparation leading to reproducible results to study phosphorylation events for biomedical research.

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*Contact: Todd Mullis, Graduate student. – Todd.Mullis@imcstips.com

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