Development of a fully automated high throughput magnetic workflow for phosphoproteome profiling

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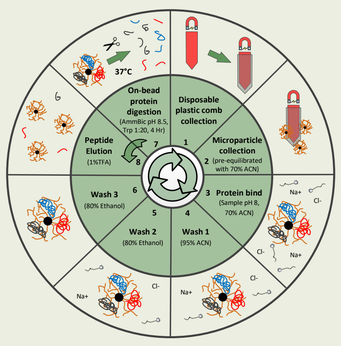
INTRODUCTION

The requirement for robust and routine high throughput sample preparation workflows has become a necessity as clinical proteomics reaches maturity. The workflows will enable processing of large sample cohorts with the throughput, robustness and reproducibility required for a routinized laboratory setting. In this study we illustrate a fully automated phosphoproteomics workflow, with further elaboration on options for clean-up from dilute and high content samples, using SAX and HILIC respectively. We further demonstrate improved phosphoproteome coverage using a range of bead enrichment chemistries. Magnetic beads are considered desirable since these are easy to handle, simple to automate, linearly scalable, and high throughput compatible on a range of magnetic bead handling stations.

AUTOMATED CLEAN-UP TO PHOSPHOPEPTIDE ENRICHMENT

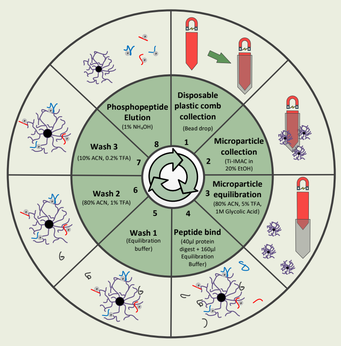
We recently demonstrated the rapid and automated enrichment of phosphopeptides using MagReSyn® Ti-IMAC HP (prototype supplied by ReSyn Biosciences) with clean-up using PAC of proteins extracted with GdHCL using MagReSyn® Amine as the precipitation nucleus.

Protein Aggregation Capture Protocol



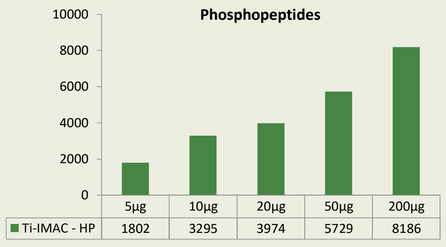
3 to 5 Hrs per 96 samples

Phosphopeptide Enrichment Protocol



45 min per 96 samples

The benefits of coupling of PAC to phosphopeptide enrichment will only require **30 to 40 minutes labour**. LC was performed using an EvoSep 1 with 21 minute gradient, coupled to a ThermoFisher Orbitrap HF-X 15000 resolution.



	5µg	10µg	20µg	50µg	200µg
Ti-IMAC - HP	1802	3295	3974	5729	8186

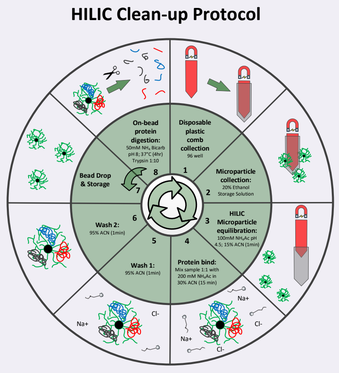
AUTOMATED PROTEIN AND PEPTIDE CLEAN-UP: SAMPLE DEPENDENT ALTERNATIVES

Although the above developed protocol is suitable for phosphopeptide enrichment from a range of biological samples, we have started investigating the use of alternate clean-up and enrichment tools to further extend the workflow to a diverse range of biological samples, and to try and increase possible coverage offered by this workflow.

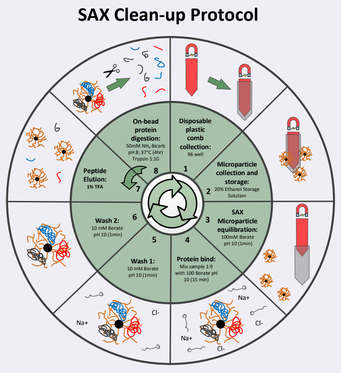
For example, the use of SAX (Strong Anion Exchange) bead chemistry can be used for capture of proteins and peptides from dilute samples such as urine and ability to potentially apply pH based fractionation, but is not suitable for sample preparation using SDS (accumulation on beads).

HILIC requires lower dilution to capture proteins, and is therefore potentially more suitable for high-content samples, and can be extended to the clean-up of peptides and glycans.

HILIC Clean-up Protocol

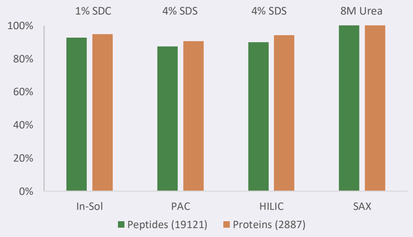


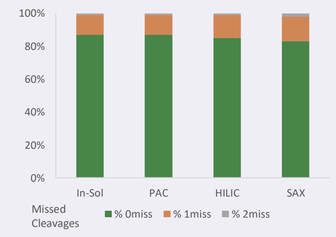
SAX Clean-up Protocol



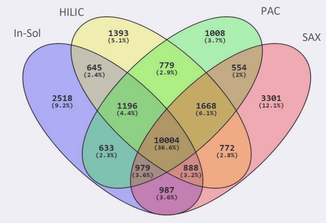
Conditions	Amine (PAC)	HILIC	SAX
Bind	50-80% ACN (*or* EtOH) pH 8	15% ACN, 100mM NH4Ac pH 4.5	100mM Borate pH 10
Wash	80% EtOH & 95% ACN	95% ACN	10mM Borate pH 10
Digest	LysC/Trp (1-4hr)	LysC/Trp (1-4hr)	LysC/Trp (1-4hr)
Elute	H_2O *or* 0.5% TFA *or* DMSO	1% TFA	1% TFA (frac pH 10→2)
Range (automated)	sub 1µg - 200µg	5µg - 0.5mg	20 µg - 5mg
Extraction	SDS, NP40, Tween, SDC	SDS, NP40, Tween, SDC	Urea
Clean-up of	Proteins	Proteins, Peptides & Glycans	Proteins & Peptides
Fractionation	NA	NA	Salt or pH Gradient
Top-down	NA	NA	Salt or pH Gradient

Sample preparation was automated on a KingFisher® Duo magnetic bead handling station and analysed using an SCIEX TripleTOF 6600 coupled to a Dionex nanoRSLC using 60 minute nano-flow gradients. Data was searched using Sciex Protein Pilot (Shilov *et al*., 2007) against a Uniprot Swiss Prot *H. sapiens* database supplemented with sequences of common contaminant proteins. A 1% FDR cut-off was applied at the PSM, peptide and protein levels.

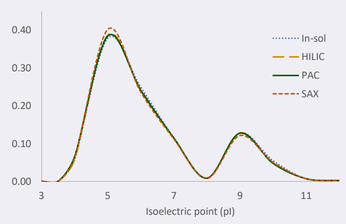


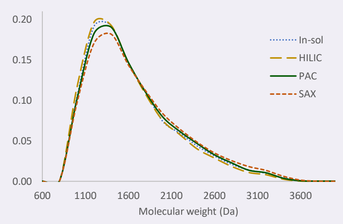


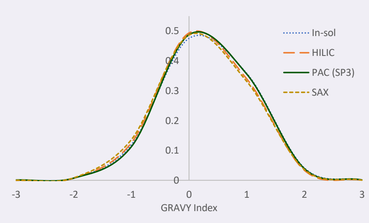
Peptide overlap for sample preparation techniques



When comparing the range of workflows for automated protein clean-up we noted little difference in the coverage of samples using the new protocols developed in this study (**above left**). SAX did show a slightly increased recovery, but we believe similar recovery can be achieved using TFA to aid elution with PAC and HILIC (experiments in progress). On-bead HILIC and SAX digestion showed similar digest efficiency as in solution digestion (**above centre**), and the further optimisation of this step is currently underway. Venn overlaps (**above right**) of the data indicated the highest number of unique peptides was identified by SAX, followed by in-solution digestion. The sample preparation techniques do not appear to show any bias with respect to peptide properties (**figures below**). We intend to investigate the coupling of the optimized protocols to automated phosphopeptide enrichment.







AUTOMATED PHOSPHOPEPTIDE ENRICHMENT: INCREASING SAMPLE COVERAGE

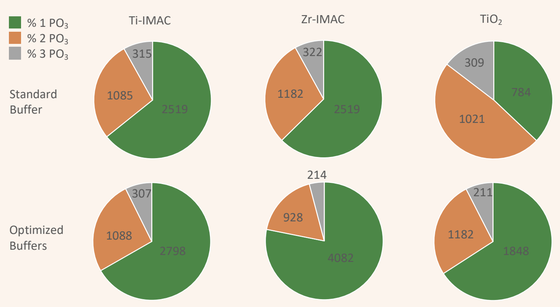
Magnetic beads from ReSyn Biosciences have been extensively used phosphopeptide enrichment, with Ti-IMAC being preferred by the majority of research groups (Baath *et al*., 2019; Niemi *et al*., 2019., Yángüez *et al*., 2018). Although the product has been shown to provide a high number of phosphopeptide identifications, specificity can vary based on sample preparation. Recently, the Olsen group has reported >99% specificity using Ti-IMAC by decreasing the bead to protein ratio, thereby improving competition of phosphopeptides for the support material. However, the impact of this approach on total coverage is not yet known. We therefore use the recommended ratio provided by the supplier for the optimization of phosphopeptide enrichment. ReSyn Biosciences offers a range of products offering complimentary sample coverage, the challenges and benefits of which are outlined below.

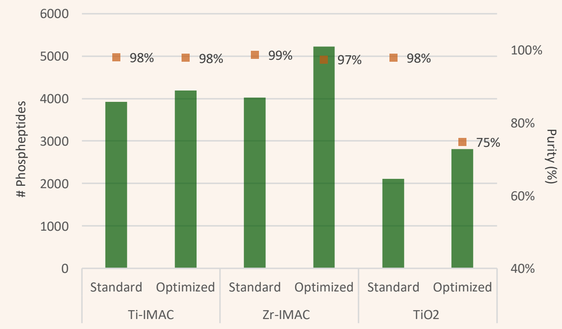
Product	Active Group	Advantages	Disadvantages	Notes
Ti-IMAC	Titanium ions	High Coverage	Sensitive to contaminants	Selective towards mono-phosphorylated peptides
Zr-IMAC	Zirconium ions	High Coverage (New Buffer)	Sensitive to chelating agents	Selective towards mono-phosphorylated peptides
Ti-Dioxide	Titanium dioxide nanoparticles	Resistant to Contaminants	Lower capacity	Bias towards multiply phosphorylated peptides
Zr-Dioxide	Zirconium dioxide nanoparticles	Resistant to Contaminants	Low capacity	Bias towards multiply phosphorylated peptides

We recently reported (ASMS 2019) on extensive optimization of binding buffer for phosphopeptide enrichment, in particular the effect of hydroxy acids on the capacity and specificity. Initially performed on a simple protein mixture, we confirm the effect of the new binding buffers using a complex lysate. Proteins from HEPG2 cells were extracted by sonication with pre-chilled OPB (50mM TEAB, 1% SDC, 10mM TCEP, 40mM CAA). Proteins were precipitated overnight with acetone, and resuspended in 50mM AmmBic containing 1% SDC. Protein (10mg) was digested overnight and peptides desalted using Oasis HLB cartridges.

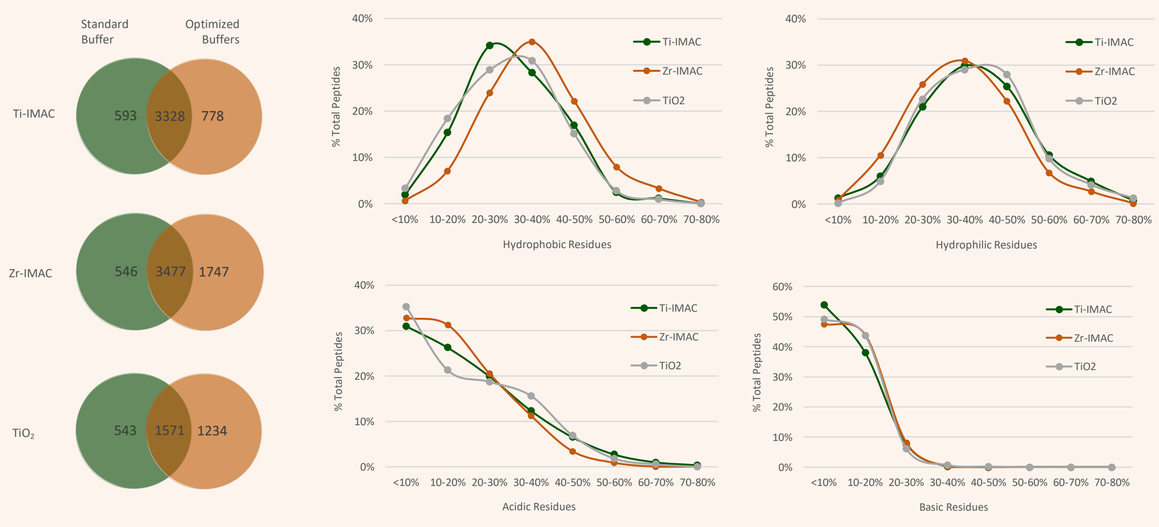
Buffer	Binding Buffer
Standard	80% ACN, 5% TFA, 1M Glycolic Acid
Ti-IMAC	80% ACN, 5% TFA, 0.1M Lactic Acid
Zr-IMAC	80% ACN, 5% TFA, 0.1M Glycolic Acid
TiO_2	50% ACN, 0.1% Acetic Acid, 0.1M Glycolic Acid

Phosphopeptide enrichment was performed from 200 µg of lyophilized protein digest on a KingFisher Duo, and samples analysed using a Dionex nanoRSLC using 60 minute nano-flow gradients, coupled to a SCIEX 6600 TripleTOF. Data was searched using Proteome Discoverer (MASCOT) with 50 ppm peptide tolerance, 0.2 Da tolerance, and 2 miss cleavages. Peptide properties were extracted using a custom R-script.

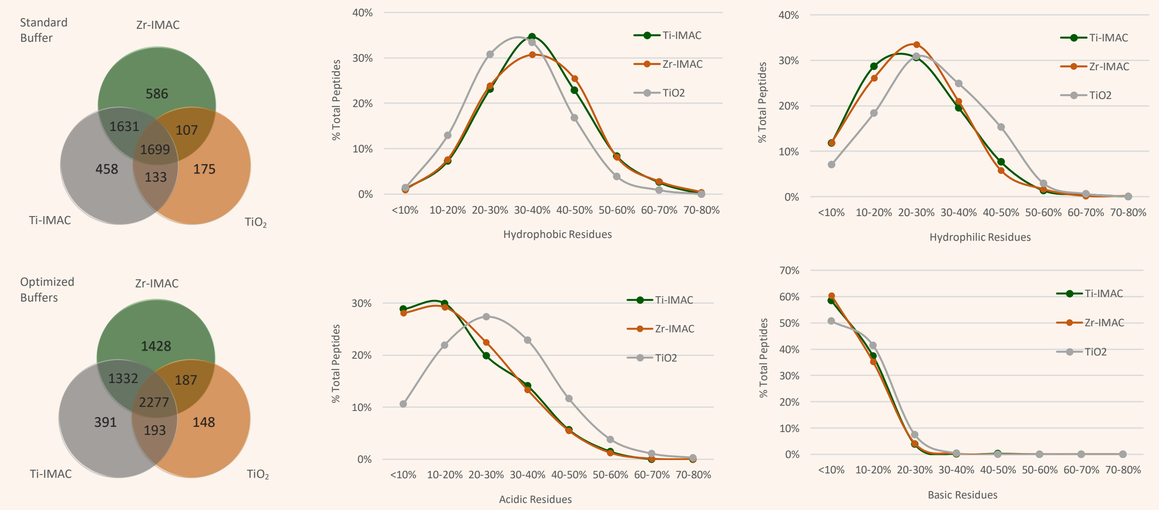




The use of optimized buffers for Ti-IMAC and Zr-IMAC increase sample coverage, with Zr-IMAC increasing by 20%, while retaining a high specificity of over 95% (**above**). The increased coverage offered by TiO2 also resulted in a decrease in the specificity to around 75% (**above**). Standard enrichment with TiO_2 captures multi-phosphorylated peptides (**left**), while the optimized buffer system normalizes to the standard IMAC profile. It appears that the increased coverage for Zr-IMAC results from improved capture of mono-phosphorylated peptides at the expense of multi-phosphorylated.



When comparing the phosphopeptide overlaps for the standard and optimized buffers, we can see that the Zr-IMAC and TiO2 show significant improvement in coverage with the new buffer systems (**above left**). Analysis of peptide properties (**above right for standard buffer, below right optimized buffers**) further elucidates selectivity of the three phosphopeptide enrichment chemistries. With standard buffers Zr-IMAC shows higher selectivity for hydrophobic residues, while the use of optimized buffer for Ti-IMAC increases hydrophobic residues (a possible reason for improved coverage using this new buffer system). Optimized buffers for Ti-IMAC and Zr-IMAC normalized their selectivity for the peptides (highly overlapping profiles, **below right**). The new buffer system for TiO2 provides a vastly different peptide property profile, increasing selectivity for acidic peptides and shifting towards hydrophilic peptides.



CONCLUSIONS & FUTURE WORK

- We demonstrate a fully automated workflow for automated phosphoproteome profiling suitable for a range of samples
- SAX magnetic beads provide a suitable alternative for high volume but low concentration samples such as urine, with the option of fractionation (pH) for deep proteome profiling
- HILIC chemistry is potentially suitable for high content samples or where precipitation is not desirable
- We have optimized protocols and buffers for automated enrichment using three magnetic bead variants
- Reduction of glycolic acid in the binding buffer improved the performance of Zr-IMAC, and for the conditions described in this study it outperformed Ti-IMAC, likely due to increased capture of mono-phosphorylated peptides
- Analysis of the properties of enriched peptides showed that dioxide enriched samples showed a vastly different profile than that of IMAC
- We are evaluating buffer conditions with the aim of combining dioxide and IMAC chemistry in an attempt to improve coverage for single-shot phosphoproteomics by enriching single- and multi-phosphorylated peptides
- The fully automated platforms will be applied to a range of samples to identify the best conditions for each sample type

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