Development of a fully automated magnetic workflow for phosphoproteome profiling

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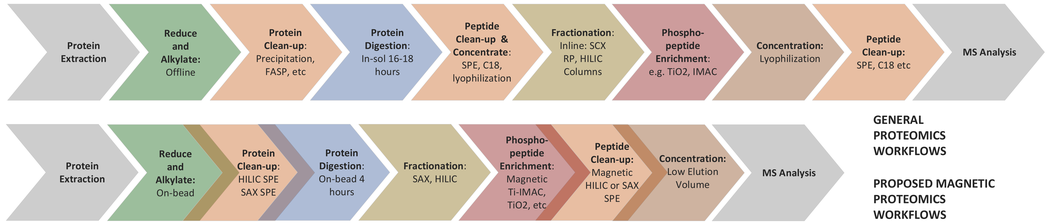
GRAPHICAL ABSTRACT



INTRODUCTION

As clinical proteomics applications start to reach maturity, this necessitates the requirement for robust and routine high throughput sample preparation workflows that allow processing of large sample cohorts. However, efficient sample preparation remains the *Achilles Heel* for mass spectrometry analysis, with current methods lacking the throughput, transferability and reproducibility required to deal with these large clinical sample numbers in a routinized laboratory setting. To address these we focus on the implementation of versatile and automatable magnetic bead based sample preparation workflows. Magnetic beads are considered desirable since these are easy to handle, linearly scalable, and high throughput compatible with the relatively simple integration of a magnetic stand in a variety of liquid handling stations making it independent of the liquid handling or magnetic bead handling station that may be present in a sample preparation laboratory. We have previously demonstrated protein and peptide clean-up workflows using magnetic HILIC for Solid Phase Extraction (HILIC SPE) from a broad range of common contaminants. Further, the use of magnetic Ti-IMAC for highly efficient phosphopeptide enrichment has previously been illustrated by Tape *et al.* 2014 and Baath *et al.* 2019. In this study we demonstrate the coupling of the clean-up to phosphopeptide enrichment. We further evaluate the option to combine a range of phosphopeptide enrichment chemistries (Ti-IMAC, Zr-IMAC, TiO_2, ZrO_2, Fe-NTA, Fe-IMAC) for possible deeper phosphoproteome coverage, by identifying buffers suitable for enrichment using various combinations of bead chemistries.

AUTOMATION

Although our aim is to fully automate mass spectrometry workflows, the protocols are also suitable for manual preparation with the ability to perform parallel sample processing using a laboratory magnetic stand. The protocols can be transferred to a variety of liquid or bead handling systems. All current experiments were automated on a KingFisher™ Duo (ThermoFisher) magnetic bead handling station (protocols available on request).

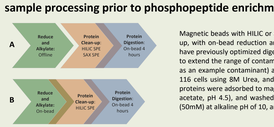
Automation of magnetic clean-up workflows can be achieved with KingFisher magnetic bead handlings stations. Examples of the conditions used for HILIC SPE of proteins using ACN (**left**) with SAX clean-up using alkaline conditions (**middle**), and phosphopeptide enrichment (**right**). For the clean-up workflows, the on-bead digestion takes place at position 8 since this is the position of the heating element. Beads may be transferred to position 2 or 3 if an extra elution step is required, or on-bead reduction and alkylation are included. Similarly, an extra elution step may be included for phosphopeptide elution if required.

LC MS/MS & BIOINFORMATICS

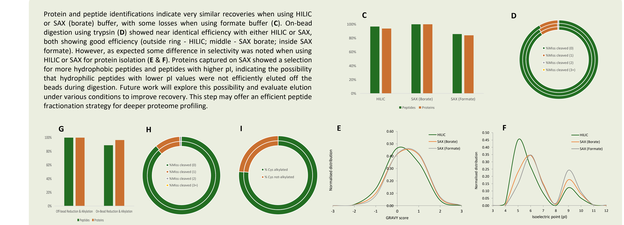
Samples were analysed using an AB SCIEX TripleTOF 6600 coupled to a Dionex nanoRSLC via a nanoSpray III interface with 60 minute gradient. Spectral data was searched using PEAKS Studio 6 (Ma *et al.*, 2003, Bioinformatics Solutions Inc). In the case of HCT 116 samples a Swiss-Prot mammalian database, supplemented with sequences of common contaminant proteins. A 0.1% and 1% FDR cut-off was applied at the PSM and peptide/protein levels respectively.

OPTIMIZATION OF SAMPLE CLEAN-UP

AIM: To evaluate particle chemistry, and the effects of on-bead reduction and alkylation on automated sample processing prior to phosphopeptide enrichment.



Magnetic beads with HILIC or Strong Anion Exchange (SAX) chemistry were evaluated for the automation of protein clean-up, with on-bead reduction and alkylation (**B**) (off-bead run as a control (**A**)), followed by on-bead trypsin digestion. We have previously optimized digestion to 4 hrs using Trypsin (1:10 in 20 mM AmmBic pH 8). Due to the potential compatibility to extend the range of contaminants that can be removed by automated clean-up, we evaluated the use of SAX (with Urea as an example contaminant) as an alternative for clean-up of proteins using HILIC-SPE. Proteins were extracted from HCT 116 cells using 8M Urea, and 10 µg aliquots were isolated using magnetic HILIC SPE or SAX SPE. Briefly; for HILIC SPE, proteins were adsorbed to magnetic HILIC microparticles using acetonitrile (ACN - 15%) under acidic conditions (ammonium acetate, pH 4.5), and washed with 95% ACN. For SAX SPE, proteins were adsorbed to SAX in Formate or Borate buffer (50mM) at alkaline pH of 10, and washed twice with the same buffer. All experiments were performed in duplicate.



The efficiency for off- and on-bead reduction and alkylation (**A** vs **B**) was evaluated using HILIC SPE with 50 µg protein extracted in 1% SDS. Protein and peptide recoveries (**G**) for on-bead digestion (right) showed slightly decreased peptide recovery (~10%), resulting in a reduction of ~3% identified proteins. The peptide ID's are relative to 10211, and proteins ID's to 1598, and experiments were performed in duplicate. To evaluate whether this was due to possible missed cleavages or inefficient reduction and alkylation we compared missed cleavages (**H**) and alkylation of cysteine residues (**I**). The results were similar for both parameters indicating efficient alkylation and trypsin digestion.

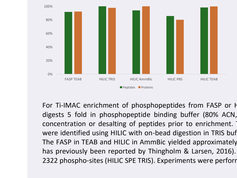
SUMMARY

- **HILIC and SAX are suitable for automated protein isolation and on-bead digestion**
- **Fully automated on-bead reduction and alkylation can be performed, but some optimization of this step is still required to improve sample coverage**
- **SAX shows promise for sample clean-up, with the possibility to extend the range of contaminants that can be removed in the fully automated workflow.**

INTEGRATION OF HILIC CLEAN-UP WITH PHOSPHO-ENRICHMENT: Ti-IMAC

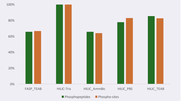
AIM: Evaluate compatibility of clean-up with the phosphoenrichment prior to MS analysis

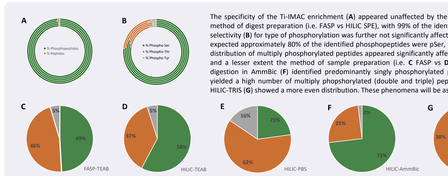
In order to evaluate the coupling of HILIC clean-up of proteins to phosphopeptide enrichment we used Ti-IMAC as the preferred candidate for phosphoproteome coverage (Tape *et al.*, 2014), with particular emphasis on enrichment efficiency and recovery. All phospho-enrichment protocols were used as per manufacturer's instructions (ReSyn Biosciences, South Africa).



Samples of 200 µg protein extracts in 1% SDS were processed either using FASP (standard, with TEAB as digest buffer) or automated HILIC SPE (with a variety of digest buffers, TRIS, AmmBic, PBS or TEAB). Post digestion samples of 5µl were kept for LC-MSMS analysis while the remainder was used for Ti-IMAC phosphopeptide enrichment. In comparison to the standard sample processing (FASP), HILIC showed approximately 10% improved coverage. Relative ID's showed that Tris, AmmBic and TEAB are the preferred buffers for on-bead trypsin digestion. The peptide recoveries are relative to 10977 and proteins ID's to 1947 (Peptides: HILIC SPE in TRIS; Proteins HILIC SPE in AmmBic). Experiments were performed in duplicate.

For Ti-IMAC enrichment of phosphopeptides from FASP or HILIC SPE (above) the protocol was initiated by diluting the digests 5 fold in phosphopeptide binding buffer (80% ACN, 1M Glycolic acid, 5% TFA), negating the requirement for concentration or desalting of peptides prior to enrichment. The highest number of phosphopeptides and phospho-sites were identified using HILIC with on-bead digestion in TRIS buffer, followed by PBS and TEAB, which offered similar results. The FASP in TEAB and HILIC in AmmBic yielded approximately 40% lower phosphopeptides/phospho-sites (similar to what has previously been reported by Thingholm & Larsen, 2016). The percentages are relative to 1719 phosphopeptides and 2322 phospho-sites (HILIC SPE TRIS). Experiments were performed in duplicate.





The specificity of the Ti-IMAC enrichment (**A**) appeared unaffected by the selection of tryptic digest buffer nor the method of digest preparation (i.e. FASP vs HILIC SPE), with 99% of the identified peptides being phosphorylated. The selectivity (**B**) for type of phosphorylation was further not significantly affected by buffer or method of preparation. As expected approximately 80% of the identified phosphopeptides were pSer, followed by 20% pThr and 2-4% pTyr. The distribution of multiply phosphorylated peptides appeared significantly affected by the digestion buffer (**D**, **E**, **F** & **G**), and a lesser extent the method of sample preparation (i.e. **C** FASP vs **D** HILIC SPE). Interestingly HILIC SPE with digestion in AmmBic (**F**) identified predominantly singly phosphorylated peptides while on-bead digestion in PBS yielded a high number of multiply phosphorylated (double and triple) peptides. FASP-TEAB (**C**), HILIC-TEAB (**D**) and HILIC-TRIS (**G**) showed a more even distribution. These phenomena will be assessed in future experiments.

SUMMARY

- **The automated clean-up and digestion of proteins was successfully coupled to phosphopeptide enrichment without the requirement for additional desalting and/or concentration steps**
- **Automated HILIC SPE with on-bead trypsin digestion performed favourably in comparison to FASP with in in-solution protein digestion**
- **The selection of digestion buffer significantly affects the identification of singly vs multiply phosphorylated peptides, this warrants further evaluation to potentially improve phosphoproteome coverage**

OPTIMIZATION OF PHOSPHOPEPTIDE ENRICHMENT BUFFERS

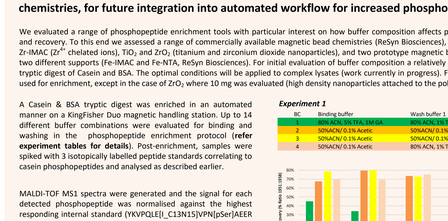
AIM: Identify the ideal buffer conditions for phosphopeptide enrichment for a range of enrichment chemistries, for future integration into automated workflow for increased phosphoproteome coverage.

We evaluated a range of phosphopeptide enrichment tools with particular interest on how buffer composition affects phosphopeptide enrichment efficiency and recovery. To this end we assessed a range of commercially available magnetic bead chemistries (ReSyn Biosciences), including Ti-IMAC (chelated Ti^{4+} ions), Zr-IMAC (Zr^{4+} chelated ions), TiO_2 and ZrO_2 (titanium and zirconium dioxide nanoparticles), and two prototype magnetic beads containing Fe^{3+} ions chelated to two different supports (Fe-IMAC and Fe-NTA, ReSyn Biosciences). For initial evaluation of buffer composition a relatively simple mixture was generated from a tryptic digest of Casein and BSA. The optimal conditions will be applied to complex lysates (work currently in progress). For all experiments 1 mg of beads was used for enrichment, except in the case of ZrO_2 where 10 mg was evaluated (high density nanoparticles attached to the polymer beads).

A Casein & BSA tryptic digest was enriched in an automated manner on a KingFisher Duo magnetic handling station. Up to 14 different buffer combinations were evaluated for binding and washing in the phosphopeptide enrichment protocol (**refer experiment tables for details**). Post-enrichment, samples were spiked with 3 isotopically labelled peptide standards correlating to casein phosphopeptides and analysed as described earlier.

Experiment 1

BC	Binding buffer	Wash buffer 1	Wash buffer 2
1	80% ACN, 5% TFA, 1M GA	80% ACN, 1% TFA	10% ACN, 0.2% TFA
2	50%ACN/ 0.1% Acetic	50%ACN/ 0.1% Acetic	50%ACN/ 0.1% Acetic
3	50%ACN/ 0.1% Acetic	50%ACN/ 0.1% Acetic	10% ACN, 0.2% TFA
4	50%ACN/ 0.1% Acetic	80% ACN, 1% TFA	10% ACN, 0.2% TFA



MALDI-TOF MS1 spectra were generated and the signal for each detected phosphopeptide was normalised against the highest responding internal standard (YKVPQLE[_C13N15]VPN[pSer]AEER 1958.4m/z) in order to determine enrichment efficiency under each buffer composition (BC). The data for the highest responding peptide (1951m/z) was plotted to illustrate the general trends (**right**), while the recovery of all phosphopeptides is illustrated for each enrichment chemistry (**below**). Specificity is calculated based on the total number of non-phosphorylated peptides detected in each sample. BC 1 is standard buffer system recommended by the bead manufacturer, ReSyn Biosciences.

With all commercially available phospho-enrichment tools (Ti-IMAC, Zr-IMAC, TiO_2, ZrO_2) buffer BC1 resulted in the highest specificity, but the lowest recovery. Removing glycolic acid (GA, refer BC2) and replacing TFA with acetic acid (Acetic) improved phosphopeptide recovery but concomitantly reduced specificity. For iron (Fe) based supports BC2 appeared most suitable for enrichment, with a balance between recovery and specificity. BC4 improved specificity, particularly for Zr-IMAC and TiO_2, while retaining high phosphopeptide recovery and was selected for further evaluation with Ti-IMAC, Zr-IMAC and TiO_2, while BC3 was selected for further evaluation with ZrO_2 magnetic microparticles.

Experiment 2

BC	Binding buffer	Wash buffer 1	Wash buffer 2
3	50%ACN/ 0.1% Acetic	10% ACN, 0.2% TFA	10% ACN, 0.2% TFA
4	50%ACN/ 0.1% Acetic	80% ACN, 1% TFA	10% ACN, 0.2% TFA
5	50%ACN/ 0.1% Acetic, 0.1M GA	80% ACN, 1% TFA	10% ACN, 0.2% TFA
6	50%ACN/ 0.1% Acetic, 0.5M GA	80% ACN, 1% TFA	10% ACN, 0.2% TFA
7	80%ACN/ 0.1% Acetic	80% ACN, 1% TFA	10% ACN, 0.2% TFA
8	50%ACN/ 0.1% Acetic, 0.1M GA	50%ACN/ 0.1% Acetic	10% ACN, 0.2% TFA
9	50%ACN/ 0.1% Acetic, 0.5M GA	50%ACN/ 0.1% Acetic	10% ACN, 0.2% TFA
10	80%ACN/ 0.1% Acetic	50%ACN/ 0.1% Acetic	10% ACN, 0.2% TFA

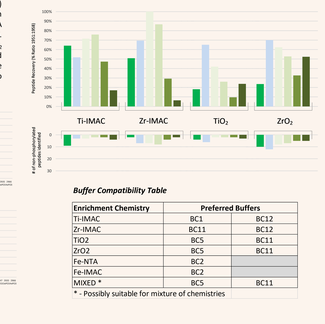
Aliphatic hydroxy acids have been noted to affect phospho-enrichment (Sugiyama *et al.*, 2007), and were thus investigated for optimization of buffers in this study. BC3 and BC4, chosen from Exp 1, were further modified in attempt to improve the specificity while retaining phosphopeptide recovery. In the case of Ti-IMAC the addition of 0.1M GA in the bead buffer (BC5) significantly improved the specificity whilst maintaining recovery. An increase in the ACN concentration (BC7) had a similar effect, i.e. high recovery with improved specificity. For Zr-IMAC the inclusion of GA improved specificity at 0.5M (BC6), but reduced recovery, while BC4 and BC5 (the only difference being 0.1M GA) showed similar recoveries and specificity.

Due to possible increased competition in complex samples, these conditions will be investigated further. Unlike Ti-IMAC, for Zr-IMAC high ACN reduced specificity (BC7). For TiO_2 a low concentration of GA (0.1M, BC5) improved recovery with no loss in specificity, while an increased concentration (0.5M, BC6) reduced recovery. The increase in ACN concentration (BC7) improved recovery but reduced specificity. For ZrO_2, the addition of 0.1M GA improved both specificity and recovery (BC8). Similar to TiO_2, increased ACN (BC10) improved recovery but reduced specificity.

The aim of experiment 3 was to further evaluate the effects of hydroxy acids including glycolic (GA), lactic (LA) and tartaric (TA) acids on phosphopeptide enrichment with Ti-IMAC, Zr-IMAC, TiO_2 and ZrO_2 magnetic microparticles (Sugiyama *et al.*, 2007). In all cases (except ZrO_2) the addition of TA (BC13 and BC14) resulted in significant decrease in phosphopeptide recovery. The decrease of GA from 1 to 0.1M GA improved phosphopeptide recovery without major effect on specificity (refer BC1 vs BC11 for each chemistry) while 0.1M LA performed similarly to 0.1M GA (BC12 vs BC11). In the case of Ti-IMAC and Zr-IMAC the lower pH buffer BC11 (TFA with 0.1M GA) performed better than BC5 (Acetic with 0.1M GA). This was opposite when comparing TiO_2 and ZrO_2, with TiO_2 showing an increase in recovery (BC5 vs BC11). The combined results from three experiments were used to derive a buffer table to guide future work where combinations will be applied to complex lysates.

Experiment 3

BC	Binding buffer	Wash buffer 1	Wash buffer 2
5	50%ACN/ 0.1% Acetic, 0.1M GA	80% ACN, 1% TFA	10% ACN, 0.2% TFA
11	80% ACN, 5% TFA, 0.1M GA	80% ACN, 1% TFA	10% ACN, 0.2% TFA
12	80% ACN, 5% TFA, 0.1M LA	80% ACN, 1% TFA	10% ACN, 0.2% TFA
13	80% ACN, 5% TFA, 0.1M TA	80% ACN, 1% TFA	10% ACN, 0.2% TFA
14	50%ACN/ 0.1% Acetic, 0.1M TA	80% ACN, 1% TFA	10% ACN, 0.2% TFA



Buffer Compatibility Table

Enrichment Chemistry	Preferred Buffers	
Ti-IMAC	BC1	BC12
Zr-IMAC	BC11	BC12
TiO2	BC5	BC11
ZrO2	BC5	BC11
Fe-NTA	BC2	
Fe-IMAC	BC2	
MIXED *	BC5	BC11

* - Possibly suitable for mixture of chemistries

SUMMARY

- **Combinations of buffers were evaluated for their compatibility with a variety of phosphopeptide enrichment chemistries**
- **The effects of buffer components were evaluated and used to derive a compatibility table**
- **The buffer compatibility table enables the mixing of enrichment chemistries and will be evaluated to improve global phosphoproteome coverage**

CONCLUSIONS & FUTURE WORK

- This work aims to develop a fully automated magnetic bead based workflow for deep phosphoproteome profiling that allows for processing of up to 96 samples in parallel in less than 8hrs
- The pipeline consists of modular and interchangeable blocks including:
  - i. Efficient protein isolation from detergents or denaturants (including SDS or UREA) with integrated reduction and alkylation, and on-bead tryptic digestion of proteins on magnetic HILIC or SAX microparticles
  - ii. Phosphopeptide enrichment using magnetic Ti-IMAC which does nor require sample desalting or lyophilization post digestion, enabling seamless and automatable protocol integration
- To improve global phosphoproteome coverage we have identified buffer conditions suitable for combining phosphopeptide enrichment chemistries. We are in process of applying these combinations to complex samples for identifying ideal conditions.
- The features of this enrichment include:
  - i. All chemistries are from the same supplier negating the possible effect of the support material.
  - ii. Simple integration into automated protocols owing to their magnetic format.
- Future work will focus on:
  - i. Improving the on-bead reduction and alkylation protocol
  - ii. Optimization and evaluation of alternate methods of clean-up such as SAX SPE and PAC (Baath *et al.*, 2019)
  - iii. Evaluate a range of elution buffers for HILIC SPE for coupling to phosphopeptide enrichment.
  - iv. Application of combinations of phosphopeptide enrichment chemistries for improved phosphoproteome coverage
  - v. Optimization of off-bead peptide fractionation (prior to enrichment) for deeper phosphoproteome coverage

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