Development of fully automated pipeline for phosphoproteome profiling

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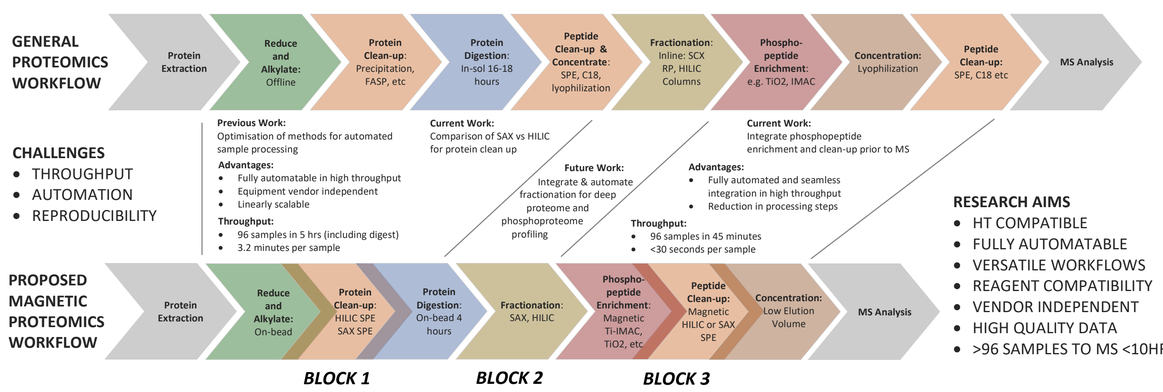
GRAPHICAL ABSTRACT



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. 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 fairly agnostic for the liquid handling or magnetic bead handling station that may be present in the 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, while the use of magnetic beads for highly efficient phosphopeptide enrichment has previously been demonstrated (Tape *et al*. 2014, Baath *et al.* 2018). In the current work we demonstrate the coupling of these steps into an automated workflow for phosphoproteome profiling requiring minimal liquid handling.

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 magnetic stand. The protocols can be transferred to a variety of liquid or bead handling systems. All experiments were performed on a KingFisher™ Duo (ThermoFisher) magnetic bead handling station (protocols available on request).

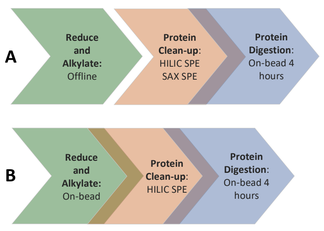
LC MS/MS & BIOINFORMATICS

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.

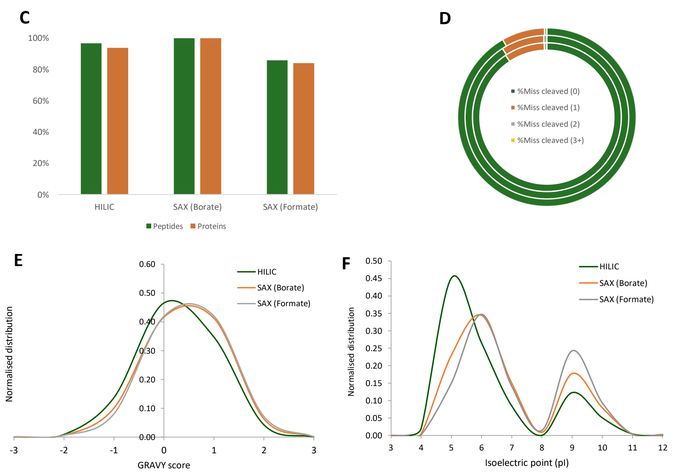
OPTIMIZATION BLOCK 1: METHODS, RESULTS & DISCUSSION

PROTEIN CLEAN UP, REDUCTION, ALKYLATION AND DIGESTION

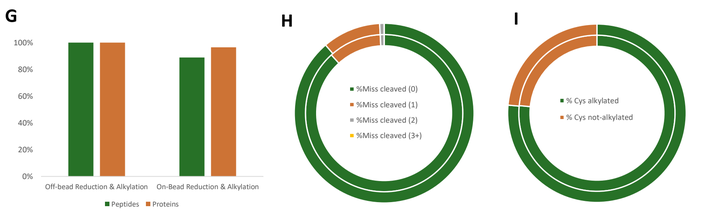
AIM: To evaluate particle chemistry, and the effect of on-bead reduction and alkylation on automated sample processing.



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 digestion. We have previously optimized digestion to 4 hrs using Trypsin (1:10 in 20 mM AmmBic pH 8). Due to the recalcitrance for removal of some detergents using HILIC SPE, we further evaluated the use of SAX (with Urea as an example contaminant) as an alternative for protein clean-up. Proteins were extracted from HCT 116 using 8M Urea, and 10 ug aliquots were isolated using magnetic HILIC or SAX for 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.



Protein and peptide identifications indicate very similar recoveries when using HILIC or SAX (borate) buffer, with some losses when using formate buffer (**C**). On-bead digestion using trypsin (**D**) showed near identical with either HILIC or SAX indicating efficient digestion when using either chemistry for protein isolation (outside ring - HILIC; middle - SAX borate; inside SAX formate). However, as expected some difference in selectivity was noted when using HILIC or SAX for protein isolation (**E** & **F**). Proteins captured on SAX showed a shift toward more hydrophobic peptides with higher pI values, indicating the possibility that hydrophilic peptides with lower pI values were not efficiently eluted off the beads during digestion. Future work will explore this possibility and evaluate elution under various conditions. This step may offer an efficient peptide fractionation strategy for deeper proteome profiling.



The efficiency of 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% in 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.

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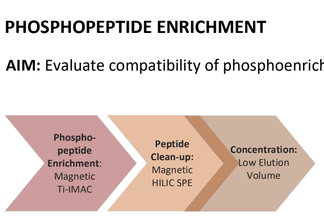
OPTIMIZATION BLOCK 3 & INTEGRATION WITH BLOCK 1: METHODS, RESULTS & DISCUSSION

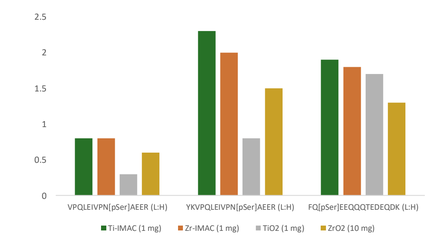
PHOSPHOPEPTIDE ENRICHMENT

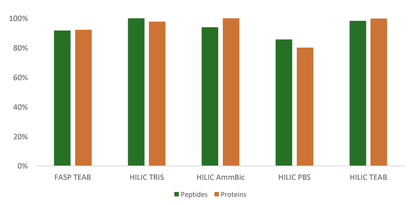
AIM: Evaluate compatibility of phosphoenrichment strategies with clean-up of sample prior to MS analysis



In order to optimize Block 3 we evaluated a range of phosphopeptide enrichment tools with particular interest in enrichment efficiency, capacity and recovery. To this end a range of functionalities were assessed including magnetic beads having immobilized titanium ions (Ti-IMAC), zirconium ions (Zr-IMAC), and titanium and zirconium oxides (TiO_2 and ZrO_2), using a standard protein mix. All enrichment protocols were used as per manufacturers instructions (ReSyn Biosciences).

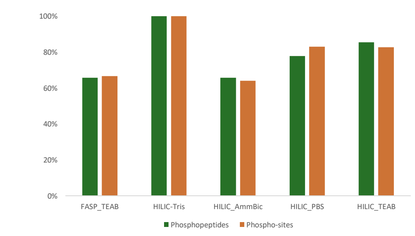
A Casein & BSA tryptic digest was processed (automated on a KingFisher™ Duo), using the range of magnetic micro-particles. Post phosphopeptide enrichment samples were spiked with 3x isotopically labelled peptide standards correlating to the phosphopeptides of casein, VPQLEIVPN[pSer]AEER, YKVPQLEIVPN[pSer]AEER and FQ[pSer]EEQQQTEDEQDK. MALDI-TOF MS1 spectra were generated and the ratio of light to heavy (L:H) phosphopeptide was calculated for each sample to determine enrichment efficiency. Experiments were performed in triplicate with each experiment further analysed in triplicate. The Ti-IMAC and Zr-IMAC enriched samples showed similar L:H ratios followed by TiO_2 and last ZrO_2. In the case of ZrO_2, 10x more beads had to be applied as compared to the other 3 enrichment chemistries. It is apparent that the TiO_2 may further require a higher quantity for improved enrichment. Considering the potential complementarity of the chemistries by Tape *et al* in 2014. Future work will include mixing the range of functionalities with the aim of increasing phosphoproteome coverage.

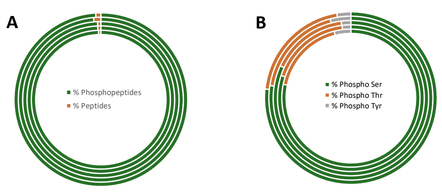




Samples of 200 µg protein extracts in 1% SDS were processed using FASP (TEAB as digest buffer) or HILIC (TRIS, AmmBic, PBS or TEAB as digest buffers) using a magnetic handling station. Post digestion 5µl from each sample was kept for LC-MSMS analysis while remainder was used for phosphopeptide enrichment on Ti-IMAC magnetic micro-particles. There was a 10% drop of in peptide and protein identifications in the case of the FASP processed samples and 15% drop-off when on-bead digestion was performed in PBS with the HILIC-based protocol. 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.

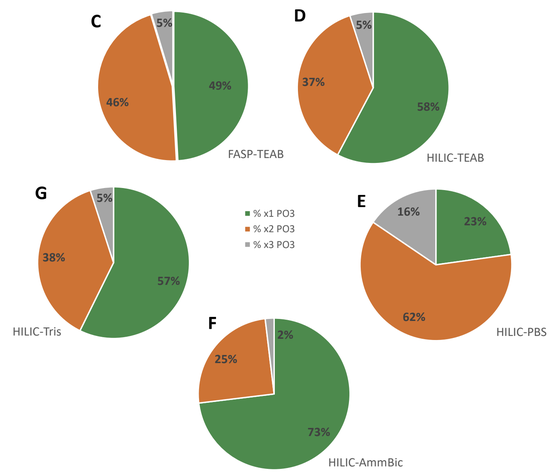
Phosphopeptide enrichment using digests generated from FASP or HILIC SPE (above) was performed with Ti-IMAC magnetic microparticles (selected from optimization experiment). The protocol was initiated by diluting the digests with 5 fold in phosphopeptide binding buffer (80% ACN, 1M Glycolic acid, 5% TFA), negating the requirement for concentration or desalting prior to enrichment. The highest number of phosphopeptides and phospho-sites were identified using the HILIC with 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 in literature, 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.

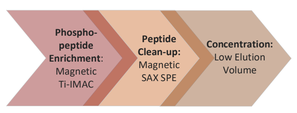
The distribution of phosphorylation levels i.e. single vs double vs triple phosphorylation, was significantly affected by the buffer used for digestion (**D**, **E**, **F** & **G**), and to a lesser extent the method of digest preparation (i.e. **C** vs **D** FASP vs HILIC SPE). The combination of HILIC SPE with digestion in AmmBic (**F**) resulted in predominantly singly phosphorylated peptides being identified. On-bead digestion performed in the presence of PBS yielded a significantly higher amount or multiply phopshorylated (double and triple) peptides, perhaps stabilised by the addition of native phosphate buffer, while the FASP-TEAB (**C**), HILIC-TEAB (**D**) and HILIC-TRIS (**G**) showed a more even distribution between singly and doubly phosphorylated peptides. This phenomenon will be assessed in future experiments where the HILIC based workflow is expanded for applications using multiple or complementary buffers e.g. on-bead digestion in TRIS followed by PBS incubation of HILIC micro-particles prior to phosphopeptide enrichment.



ALTERNATE BLOCK 3: METHODS, RESULTS & DISCUSSION

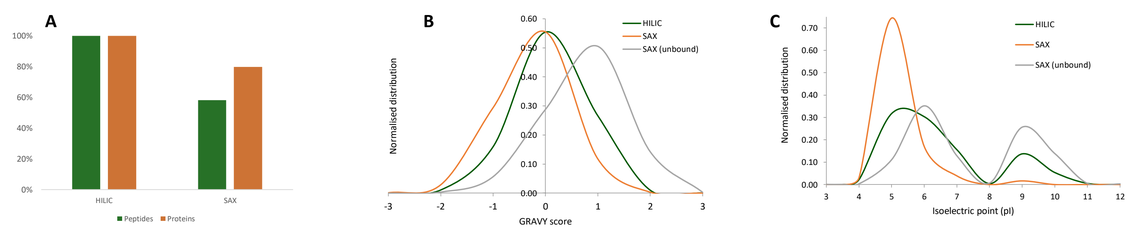
SAX CLEAN-UP PRE-MS ANALYSIS

AIM: Evaluate the application of SAX for clean-up of peptides for direct MS analysis of peptides



With the aim of fully automating the sample preparation workflow, we evaluated the option of using strong anion exchange (SAX) as a clean-up step prior to MS analysis. The use of this chemistry has several advantages for clean-up, primarily that binding occurs at alkaline pH (i.e. compatible with elution after Ti-IMAC), and elutes at acidic pH thereby removing the necessity for drying of samples (required with HILIC SPE of peptides).

A tryptic peptide digest of 10 µg was processed using magnetic HILIC SPE or SAX SPE using automated protocols. SAX SPE was performed with binding in 50 mM ammonium formate, and elution in 0.2% formic acid, HILIC SPE for peptides was performed as described previously. Experiments were performed in duplicate. Initial evaluation revealed that peptide and protein identifications were significantly reduced in the case of SAX processed samples (**A**) indicating loss of sample under the binding and elution conditions tested. The peptide recoveries are relative to 10393 peptide and 2177 protein ID's (HILIC SPE). The peptide GRAVY (**B**) and pI distributions (**C**) indicated that hydrophobic peptides and peptides with alkaline pI values did not bind well during SAX SPE, providing an explanation for the reduced binding efficiency. Due to the potential to go directly to MS without offline drying, and the fractionation potential for deep proteome profiling, we intend to elaborate on the current SAX workflow, and evaluate a range of binding and elution conditions.



CONCLUSIONS & FUTURE WORK

- This work describes a pipeline for phosphoproteome profiling that allows for automated processing of up to 96 samples in parallel in less than 8hrs with all methods automated on a magnetic bead handling station, KingFisher™ (protocols available on request)

- The pipeline consists of modular and interchangeable BLOCKS including:
  i. Efficient protein isolation in detergents or denaturants such as SDS or UREA with integrated reduction and alkylation, removal of contaminants, and on-bead tryptic digestion of proteins on magnetic HILIC or SAX microparticles
  ii. Phosphopeptide enrichment using magnetic Ti-IMAC, Zr-IMAC, TiO_2 or ZrO_2 that does nor require sample desalting or lyophilization post digestion allowing for seamless and automatable protocol integration
  iii. Optional desalting of peptides using HILIC or SAX magnetic micro-particles (with Ti-IMAC or Zr-IMAC this may be omitted)

- Future work will focus on:
  i. Improving the on-bead reduction and alkylation protocol
  ii. Optimization of SAX SPE for protein recovery and clean-up with coupling to on-bead digestion and peptide fractionation for deeper proteome and phospho-proteome profiling
  iii. Evaluate multiple buffer elution from HILIC SPE with coupling to the phosphopeptide enrichment protocol
  iv. Combination of various phosphopeptide enrichment products to improve phosphoproteome coverage (eg. Ti-IMAC and Zr-IMAC)
  v. Optimise SAX SPE for peptide binding, desalting, and concentration of phosphopeptides directly from alkaline elution, allowing for direct MS analysis of samples