Dealing with the Dirty Dozen: Universal Unbiased pre-MS Clean-Up Using Magnetic HILIC SPE

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Introduction

Although mass spectrometry (MS) is a powerful technique for analysing complex protein samples, reproducible sample preparation remains elusive for robust MS analysis. Furthermore, current methods lack throughput and/or automation creating a bottleneck in sample analysis. To alleviate some of these limitations ReSyn Biosciences introduced MagReSyn® HILIC, for routine, automatable and robust sample preparation workflows that integrate sample clean-up and digestion using multi-mode magnetic HILIC for solid phase extraction (SPE). In a routine workflow this can be followed by on-bead tryptic digestion, and direct LC-MSMS analysis. This workflow was introduced at ASMS 2017, with initial evaluation for unbiased SPE (download from www.resynbio.com). The technique was automated on KingFisher™ magnetic bead handling stations, providing processing capability of 12 to 96 samples (inclusive of digestion) without time consuming offline steps such as centrifugation. Here we evaluate the versatility of HILIC for clean-up by processing protein samples extracted, or spiked, with various agents including detergents and chaotropes. The poster further describes the first application of HILIC SPE for peptide clean-up, alluding to the potential use sample preparation of peptides.

Methods

An outline of the sample preparation workflows evaluated in this study is illustrated in ***Figure 1***. Briefly, HCT 116 (colon carcinoma) cells were lyzed and protein solubilized using a range of common detergents or chaotropes. Samples extracted with SDS were further spiked with common MS contaminants. Proteins were bound to MagReSyn® HILIC (ReSyn Biosciences) microparticles using acetonitrile (15%), followed by washing with 95% ACN to remove detergents, chaotropes and salts, prior to on-bead trypsin digestion. We further evaluated the use of HILIC SPE technique for clean-up of peptides. This was tested using a tryptic digest of Bovine Serum Albumin (BSA) and Casein spiked with 1% SDS. All protein-HILIC, digestion, and peptide-HILIC steps were automated using a magnetic bead handling station (KingFisher™ Duo; protocols available on demand). Samples were analyzed using an AB SCIEX TripleTOF 6600 coupled to a Dionex nanoRSLC. 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 was used for analysis, while for the BSA-Casein mix a custom database was compiled with the sequences for both proteins. Both SwissProt and custom databases were supplemented with sequences of common contaminating proteins. A 0.1% and 1% FDR cut-off was applied at the PSM and protein levels respectively.

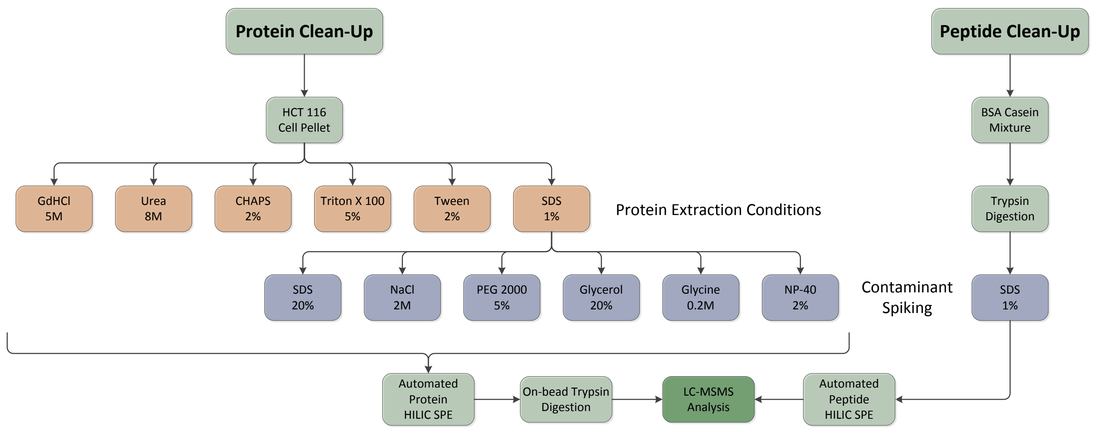


Figure 1: Protein and peptide clean-up workflows using HILIC SPE for a range of common extraction conditions and sample contaminants. For protein clean-up, SDS extracted samples were spiked with common additives to assess removal of these from SDS extracted protein samples. We further conducted a preliminary evaluation of HILIC SPE for clean-up of peptides from a standard peptide mix.

Table 1: List of common contaminants and their application in Cell/Molecular Biology, with recommended clean-up procedures (extracted from Pierce Protein Handbook, online resource made available by ThermoFisher).

Compound	Application in Cell/Molecular Biology	Recommended Clean-Up Technique/Compatibility	MagReSyn® HILIC SPE
SDS	Anionic deterget for protein solubilization (disrupts protein-protein interactions)	Filtration; Spin columns (0.5-1% max)	20%
CHAPS	Zwitterionic detergent used for protein extraction (preserves protein-protein interactions)	Filtration; Spin columns (0.5-1% max)	2%
Triton X-100	Nonionic detergent used for protein extraction (preserves protein-protein interactions)	TCA/Acetone Precipitation; Spin columns (0.5-1% max)	5%
NP-40	Nonionic detergent used for protein extraction (preserves protein-protein interactions)	Acetone Precipitation; Spin columns (0.5-1% max)	2%
Tween-20	Nonionic detergent used for protein extraction (preserves protein-protein interactions)	Acetone Precipitation	2%
Urea	Chaotrope used for protein solubilzation (stabilize unfolded protein)	Filtration; Reverse phase	8M
GdHCl	Chaotrope used for protein solubilzation (stabilize unfolded protein)	Filtration; Reverse phase	5M
NaCl	Increase ionic strength of buffers and improve protein solubility	Filtration; Reverse phase	2M
PEG 2000	Polymer used for fractional precipitation of proteins and peptides	Filtration; Dialysis; Activated Carbon	5%
Glycerol	Stabilization of proteins in solution	Filtration; Dialysis	20%
Glycine	Elution of proteins during immunoprecipitation	Filtration; Reverse phase	0.2M

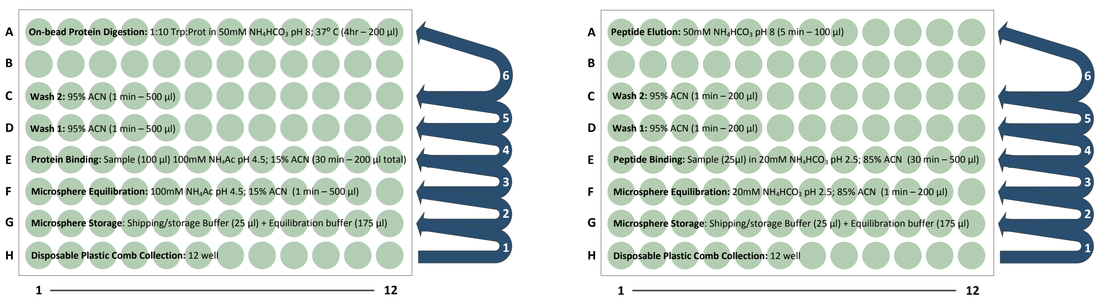


Figure 2: Automated HILIC SPE clean-up workflows on KingFisher™ Duo magnetic handling station for protein (left) and peptide (right) clean up (available on request; e-mail the author).

Results & Discussion

The number of PSM, peptide and protein matches indicated adequate coverage for all samples except Sodium Chloride and Guanadine Hydrochloride, and to a lesser extent Urea, which resulted in significant loss of the protein sample. The extent of the loss due to concentration and/or the potential change in pH of the binding buffer is as yet uncertain. In any event, the solution to improve recovery is likely dilution of the starting material with a suitable benign buffer. Analysis of the PSM, peptides and proteins with respect to peak area, CV, and retention times was used to confirm the removal of the contaminants from the protein samples. Protein P10809 was selected for the subsequent comparative analysis since it was present in all samples analysed.

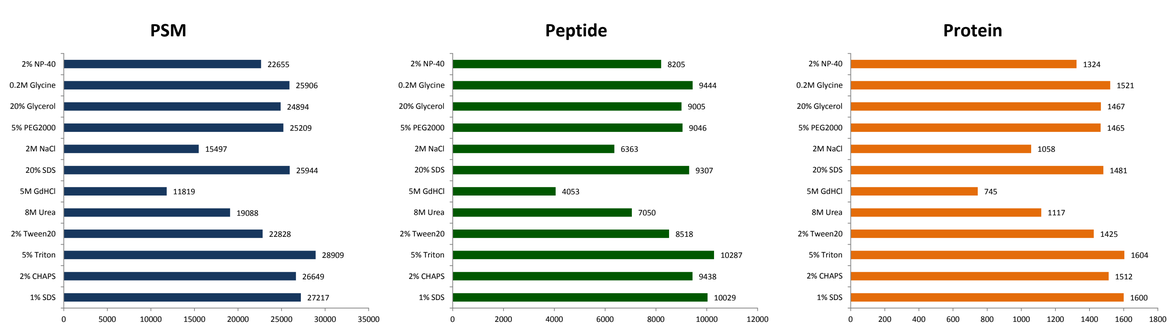


Figure 3: PSM, Peptide, and Protein coverage of the range extracted and spiked samples analysed in this study.

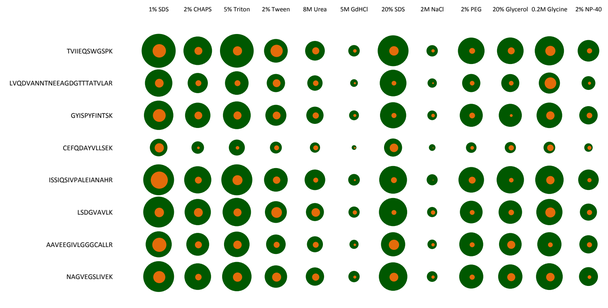


Figure 4 (left): Peak areas for peptides (y-axis) from protein P10809 for each extraction condition (x-axis). Green bubbles represent comparative peak area (largest bubble 1.2E+07), and orange bubbles represent CV% for triplicate analysis.

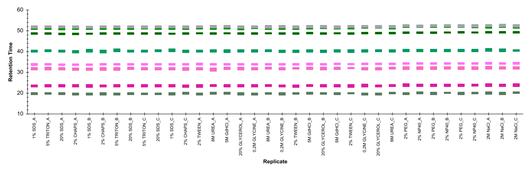


Figure 5 (above): Retention time (RT) for each of the peptides from protein P10809 (shown in the order of analysis). No significant shifts in RT and peak widths were observed, an indication that samples were essentially contaminant free.

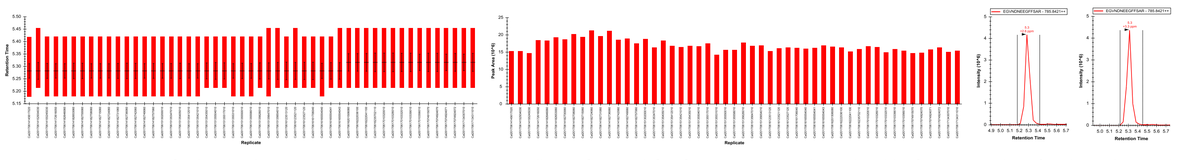


Figure 6: Contamination of either the chromatography column or ion source generally manifests as shifts of retention time, wider peak widths, fronting or tailing of peaks, or decreased signal due to ion suppression. Therefore, to further evaluate possible instrument contamination during a 3 day analysis, LCMS performance was monitored by injecting a peptide standard (Glu-1Fibrinopeptide B) between each sample run. As illustrated by the data, no significant shifts in RT, peak width, or changes in peak area (signal) were observed during the experimental setups. The two XIC's represent the first and last injections for the experiment.

Results & Discussion cont...

In order to further probe for the presence of residual contamination post HILIC SPE clean-up, an MS1 transition library was compiled and analyzed using Skyline. The library contained MS1 reporter ions for all detergents used in the experimental sets including SDS, CHAPS, Triton X-100, Tween-20 and NP-40 as well the polyether PEG. Using this approach we detected some reporter ions for CHAPS and Triton X-100 in the respective samples (refer ***Figure 7***), indicating residual detergent contamination. However, as illustrated in ***Figures 4, 5 and 6*** their presence did not appear to affect the number of protein identifications or LCMS performance, indicating that the bulk of the contamination was removed by HILIC. Interestingly NP-40, a non-ionic detergent with very similar structure to Triton X-100, was not detected in the samples and thus was efficiently removed by HILIC SPE. This was likely due to inadequate bead:detergent ratio since a concentration of 2% was used for NP-40, while 5% was evaluated for Triton X-100. We therefore propose that reducing the starting concentration of Triton X-100 to 2%, or increasing the bead quantity, will likely result in complete removal of the detergent by HILIC SPE (experimentation underway).

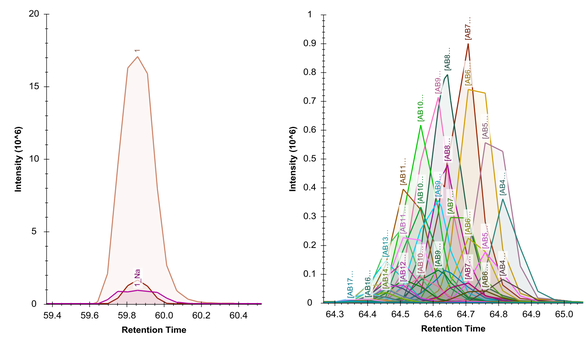
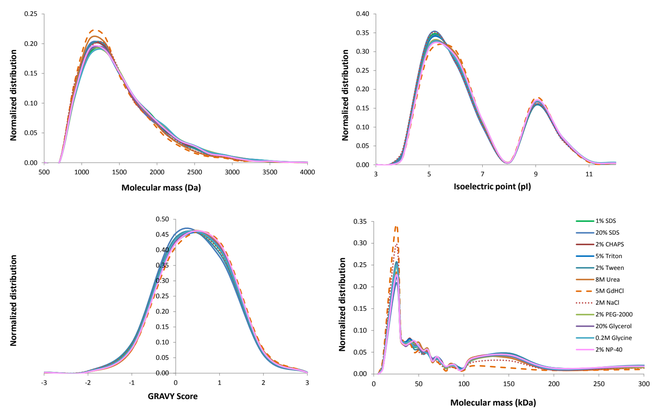


Figure 7: Residual detergent species in samples extracted using CHAPS (left) and Triton-X100 (right).



For protein clean-up, the peptide distribution after trypsin digestion was analyzed in terms of molecular mass, Isoelectric point (pI) and hydropathicity (GRAVY score). The data suggests no major bias for samples cleaned up using protein HILIC SPE (refer ***Figure 8***). However, the protein mass profile of samples extracted with GdHCl and NaCl (refer ***Figure 8***) indicate some bias for selection of smaller proteins. In particular large proteins in the range of 100 to 200 kDa were lost during HILIC SPE, with proteins around 20-30 kDa being preferentially enriched. We propose that the high ionic strength of buffers containing GdHCl and NaCl may likely result in lower protein affinity toward the hydrophilic microparticle. We are currently evaluating the limits of this selectivity to potentially remove this MW bias.

Figure 8 (left): Peptide distribution of protein samples cleaned up with HILIC SPE. No significant bias was observed for peptide molecular mass (top left), Isoelectric point (pI – top right), or hydropathicity (GRAVY score) of peptides. Protein level analysis identified bias towards smaller proteins in the case of high ionic strength buffers.

In this study we have focused on clean-up of protein samples prior to MS analysis for a range of common MS contaminants. We demonstrated that the workflow provides a suitable mechanism for clean-up of 12 major components. However, due to the sheer range of contaminants, and their possible mixtures, some optimization may be required for complete removal from a sample prior to MS analysis. The preliminary evaluation of HILIC SPE for clean-up of peptide samples containing SDS indicated reproducible sample preparation and good coverage (***Figure 10***). This initial evaluation was conducted on a standard peptide mixture, and we will be moving to analysis of complex lysates. We propose this may be suitable for removing residual contamination from digested peptide samples, or used as a 'polishing' step prior to MS analysis. Typically this is currently performed using reverse phase (RP) SPE media packed in tips, e.g. StageTip or ZipTips®, prior to MS analysis. We further propose there are several advantages of using HILIC over RP, namely: unlike RP SPE, HILIC SPE is compatible for the clean-up of detergents and polymer based contaminants; in the case of RP SPE an offline drying step is required prior to LCMS analysis to remove organic content from the RP SPE, while in the case of HILIC SPE peptide elution is achieved using aqueous conditions enabling direct analysis; the magnetic nature of the HILIC microparticles allow for routine automation using magnetic handling stations, in turn enabling increased throughput and lower technical variability.

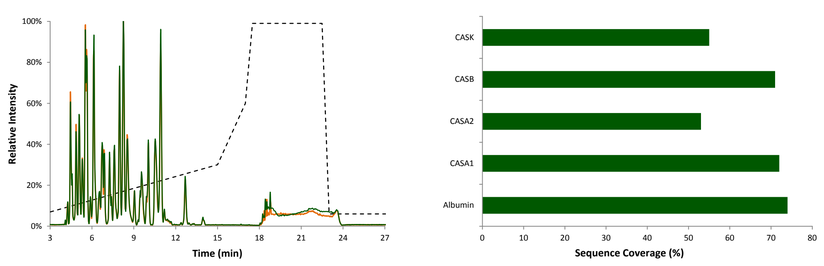


Figure 10 (left): Preliminary evaluation of magnetic HILIC SPE for clean-up of peptide samples spiked with SDS to a final concentration of 1%. Results indicate efficient depletion of the detergent as represented by the highly reproducible base peak chromotagrams (BPC's – far left; dotted line represents ACN gradient elution) as well as the comprehensive sequence coverage obtained for each of the proteins in the test sample. A subsequent study on the clean-up of peptides from a range of biological samples, and spiked with a range of contaminants is currently underway.

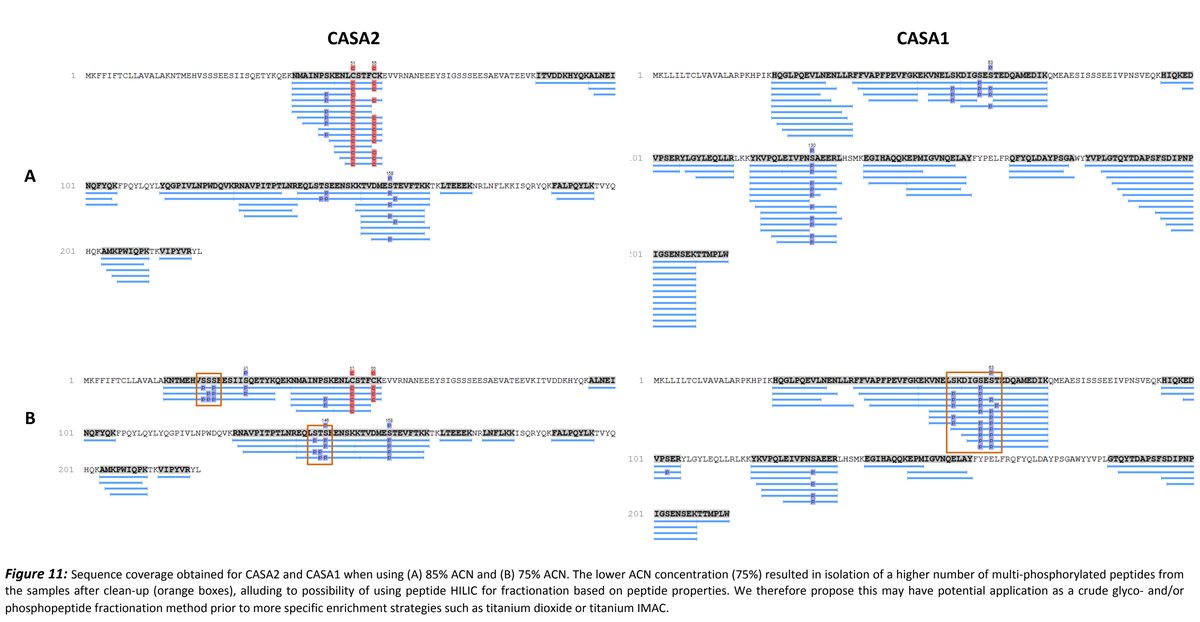


Figure 11: Sequence coverage obtained for CASA2 and CASA1 when using (A) 85% ACN and (B) 75% ACN. The lower ACN concentration (75%) resulted in isolation of a higher number of multi-phosphorylated peptides from the samples after clean-up (orange boxes), alluding to possibility of using peptide HILIC for fractionation based on peptide properties. We therefore propose this may have potential application as a crude glyco- and/or phosphopeptide fractionation method prior to more specific enrichment strategies such as titanium dioxide or titanium IMAC.

Conclusions

Research requiring MS analysis have to date been limited by lack of robust and automatable sample preparation technologies. There are currently few universal solutions for the removal of contaminants in protein and peptide samples. The sheer variety of possible sample contaminants and variability in sample preparation conditions has made it near impossible to provide a universal solution for sample clean-up. The concomitant result is that research is guided by the compatibility of sample preparation and extraction limitations, as opposed to the research question at hand. We propose that the broad applicability of HILIC SPE for clean-up provides a suitable solution to expedite research, and provide the reproducibility required for routine sample analysis by MS. We have previously shown that this technique provides an unbiased workflow for protein clean-up, and now demonstrate application to a wide variety of possible sample contaminants and extraction conditions. We believe that this will enable researchers to focus primarily on answering the biological question, without the possible concomitant problems of instrument contamination and poor signal due to ion suppression.

References

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