Magnetic HILIC: An enabling & versatile tool for robust automated MS sample preparation workflows

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Introduction

In proteomics research an increased sample cohort is required to detect small, yet significant, modifications in the proteome related to e.g. onset and/or progression of a disease. This concomitantly necessitates increased throughput in analysis of clinical samples. 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 large sample numbers. To address these limitations, we have developed sample preparation workflows consisting of modular, robust, automatable, versatile steps in routine Mass Spectrometry sample preparation. This research outlines several workflows for robust clean-up of proteins and peptides using magnetic HILIC. The workflows are linearly scalable, fully automatable, and compatible for clean up from a range of common MS contaminants.

Methods

MagReSyn® HILIC used in this study was gifted by ReSyn Biosciences (Pty) Ltd. An outline of the sample preparation workflows tested in this study are illustrated in ***Figure 1***. For evaluation of HILIC for protein clean-up HCT 116 (colon carcinoma) cell extract was solubilized using a range of common extraction conditions, while samples extracted with 1% SDS were further spiked with a range of common contaminants frequently used in cell lysis preparations.

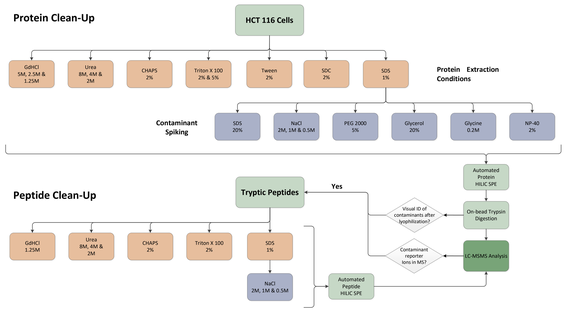
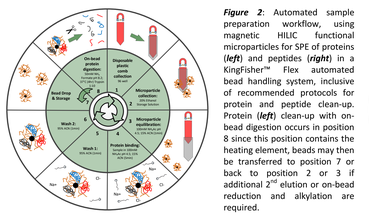


Figure 1: Flow diagram illustrating the experimental setup for evaluation of HILIC compatibility with a range of possible contaminants.

Automated sample clean-up and digestion was performed on KingFisher™ magnetic bead handling stations (Thermo Scientific, USA), using 96 deep-well microtiter plates, suitable for processing of up to 12 (Duo) and 96 (Flex) samples in parallel. The respective systems have a 12- or 96-pin robotic magnet head with disposable plastic comb preventing sample cross-contamination when handling and mixing of magnetic particles. ***Figure 2*** illustrates KingFisher™ Flex plate layout for sample clean-up with on-bead digestion of proteins, and workflow for clean-up of peptides. The automated protocols have been optimized for efficient pick-up, mixing and transfer of HILIC microparticles, and for reduced loss of protein and peptide during handling. KingFisher™ protocols (as well as several other systems) are available upon request (contact author). The protocol takes less then 45 min to process up to 96 samples in parallel with an additional 4 hr optimized tryptic digestion in the case of the protein clean-up workflow.



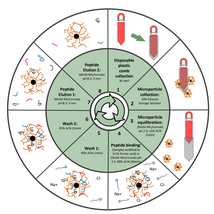


Figure 2: Automated sample preparation workflow, using magnetic HILIC functional microparticles for SPE of proteins (***left***) and peptides (***right***) in a KingFisher™ Flex automated bead handling system, inclusive of recommended protocols for protein and peptide clean-up. Protein (***left***) clean-up with on-bead digestion occurs in position 8 since this position contains the heating element, beads may then be transferred to position 7 or back to position 2 or 3 if additional 2[nd] elution or on-bead reduction and alkylation are required.

Samples were analysed using an AB Sciex TripleTOF 6600 coupled to a Dionex nanoRSLC. Peptide samples were first de-salted on an Acclaim PepMap C18 trap (75 µm × 2 cm) for 5 min at 5 µl.min^{-1} using 2% acetonitrile:0.2% formic acid, then separated on Acclaim PepMap C18 RSLC column (75 µm × 15 cm, 2 µm particle size). Peptide elution was achieved using a flow-rate of 0.5 µl.min^{-1} with a 60 min gradient, 4-50% B (A: 0.1% formic acid; B: 80% acetonitrile/0.1% formic acid). The samples were analyzed using a Sciex 6600 TripleTOF® mass spectrometer operated in Data Dependant Acquisition mode. Precursor scans were acquired from *m/z* 360-1500 using an accumulation time of 250 ms followed by 80 MS2 scans, acquired from *m/z* 100-1800 at 25 msec each, for a total scan time of 2.3 sec.

Methods cont...

Spectral data was searched using PEAKS Studio 6 (Bioinformatics Solutions Inc; Ma *et al.*, 2003) using a Swiss-Prot mammalian database supplemented with sequences of common contaminating proteins. A 0.1% and 1% FDR cut-off was applied at the PSM and protein levels respectively. Data was analyzed using MaxQuant (Cox & Mann, 2008) and Perseus (Tyanova *et al.*, 2016) software packages. Data was further inspected for reporter ions of detergents and other contaminants using Skyline (MacClean *et al.*, 2010). Samples exhibiting reporter ions were further cleaned-up using the peptide level HILIC workflow (HILIC-HILIC). Contaminants with no reporter ions were lyophilized and visually inspected for residual contamination presenting as crystals or other artefacts in the sample tubes.

Results & Discussion

The binding conditions for protein and peptide clean-up were previously extensively (but not exhaustively) optimized for increased recovery and identification of proteins and peptides using HILIC for SPE. Parameters evaluated included pH, ionic strength and solvent concentration (acetonitrile) of the binding buffer, as well as the bead:protein ratio. These optimized conditions (***Figure 2***) were applied to the clean-up of samples containing a range of contaminants (***Figure 1***). The results of the clean-up are illustrated in ***Figure 3***. In the cases where residual contamination was noted by visual inspection (after lyophilization) or presence of reporter ions in MS this data is illustrated in ***Figure 4***. To avoid confusion, the method of protein HILIC SPE followed by peptide HILIC SPE was termed HILIC HILIC.

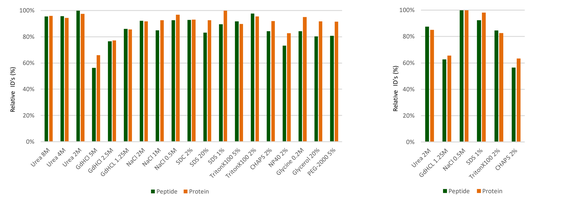


Figure 3: Results from clean-up and on-bead tryptic digestion of protein extracted from HCT 116 with a range of detergents and denaturants, and spiked with a range of common contaminants. High concentrations of salts appear to interfere with protein binding to the HILIC microparticles, but can largely be overcome by dilution.

Figure 4: Peptide Samples exhibiting contamination through reporter ions (LC-MS), or observed presence of contaminants (lyophilization), were subjected to further clean-up using peptide level HILIC.

Magnetic HILIC showed a wide compatibilty with a range of possible extraction conditions using the recommended protocols for protein clean-up with on-bead tryptic digestion. Salts did however still appear to impact binding, but to a large extent this could be overcome by dilution of the sample to reduce molarity. Protein HILIC followed by peptide HILIC further reduced contamination in the samples where MS reporter ions or visible contamination were observed (***Figure 5***). Peptide level clean-up did however result in reduced recovery in several instances including removal of residual Guanadinium hydrochloride and CHAPS. Further optimization is currently underway to improve recovery.

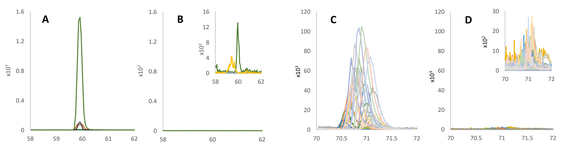
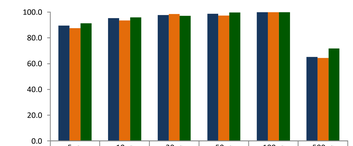


Figure 5: Samples containing residual contaminants (MS reporter ions) from protein clean-up were further subjected to peptide clean-up using magnetic HILIC SPE. Examples of the results from the HILIC HILIC workflow are illustrated above. **A**: CHAPS after protein HILIC SPE, and **B** HILIC HILIC. **C**: indicates HILIC SPE of TritonX100 samples (2%) with subsequent HILIC HILIC (**D**) to remove residual contaminant. Inserts show level of residual contamination (Y -axis adjusted).

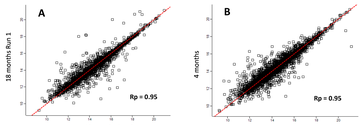
Figure 6: Scalability of HILIC protein clean-up workflow was evaluated from 5 to 500 µg of protein (HCT 116 cells extracted with 1% SDS). At a protein content of 500 µg protein precipitation was observed (due to high concentration of protein required for automated processing), and a maximum of 100 µg is currently recommended for automated sample preparation (higher protein content can be processed by increasing solubility of proteins using SDS, and/or the binding volume to effectively dilute the protein concentration and possibility to aggregate), while at low protein content of 5 µg manual clean-up is recommended due to the low effective concentration and volume of the sample. The best sample coverage was obtained at 100 µg of protein with PSM data of 23491, peptide count of 11851, correlating to 1927 unique identified proteins.

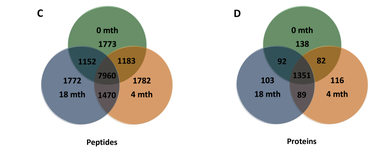


	5ug	10ug	20ug	50ug	100ug	500ug
PSM	89.5	95.4	97.6	98.8	100.0	65.3
Peptides	87.6	93.5	98.6	97.3	100.0	64.3
Proteins	91.4	95.9	97.1	99.7	100.0	71.8

Results & Discussion cont...

Considering MagReSyn® HILIC is a relatively new product, several batches were requested from ReSyn Biosciences for side-by-side comparison to evaluate reproducibility of the product. The specific instruction was to provide batches from a variety of manufacturing dates, but within the shelf-life of the product. Three batches of 18 months, 4 months and 0 months old were gifted for this study. The batches were evaluated for protein and peptide recoveries, and examples of the Pearson correlations for intra and inter batch reproducibility are illustrated in ***Figure 7***. There appeared to be little batch to batch variability for the batches evaluated in this study.





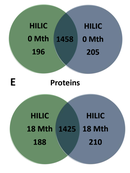


Figure 7: Batch to batch variability of MagReSyn® HILIC. **A**: Pearson correlation for replicate HILIC sample protein clean-ups from the same batch (18 months); **B**: Pearson correlation of HILIC protein clean-up performed using two batches of varying ages; **C**: Venn diagram of peptide; **D**: protein coverage for inter-batch evaluation; **E**: Reproducibility of protein clean-up using new and 18 month old HILIC.

The effect of sequential elutions was evaluated for the HILIC peptide clean-up workflow to assess possibility for increased sample coverage since this is a relatively new workflow. Two sequential elutions resulted in greater than 95% coverage, which can considered suitable for routine clean-up, but three elutions may be considered for deeper proteome coverage (***Figure 8***)

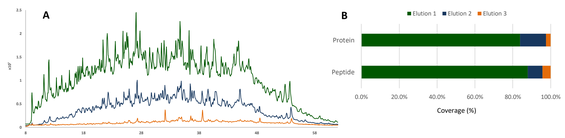


Figure 8: Total ion chromatographs (**A**) of sequential elutions of peptides from magnetic HILIC microparticles, and the effect of multiple elutions on sample coverage is illustrated in **B**.

Although the beads are engineered for automation with the aim of improving reproducibility, the HILIC SPE workflows are further suitable for manual processing. ***Figure 9*** illustrates good correlation for HILIC peptide clean up (1% SDS), but for protein clean-up with on bead digestion, automation does provide improved sensitivity, likely due to the pick-up and transfer of magnetic beads during each step thereby removing potential contamination from peptides adsorbed to the plastics. Sample coverage is consistently higher using automated sample processing as illustrated in ***Figure 10***.

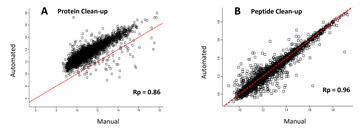


Figure 9 (Left): Pearson correlation for automated vs manual processing of protein samples (with on bead trypsin digestion) showing a preference for automated processing of samples (**A**), while peptide clean-up appears to perform similarly during automated and manual processing of samples (**B**). Automation data was generated using KingFisher™ Duo magnetic bead handling station with adapted protocols from ***Figure 2***.

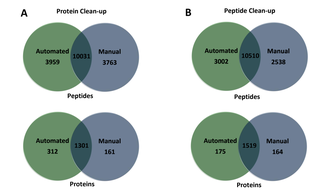


Figure 10 (Right): Overlap in samples prepared using HILIC clean-up of proteins with on-bead tryptic digestion (**A**), and HILIC clean-up of peptides (**B**) using manual and automated sample processing to show comparative sample coverage of HCT 116 samples prepared with 1% SDS. Automated sample processing provided improved coverage in both protein and peptide level clean-up. Samples were processed using a KingFisher™ Duo magnetic bead handling station with 50 µg of starting material. Evaluation using alternate automation sample preparation robots and systems is currently in progress.

Results & Discussion cont...

The most common method for peptide clean-up is considered C18 based SPE using pipette tips (e.g. STAGE or ZipTip®) or C18 filter plates (96 well) for higher throughput sample processing. Unfortunately this technique is currently not suitable for clean-up of detergents, and primarily used for removal of salts. We therefore ran a comparative assessment of HILIC and C18 STAGE tips to assess the application for clean-up from 1M urea, since C18 is not suitable for clean-up of peptides from detergents. Parameters for evaluation included coverage, reproducibility, and possible bias introduced for the two techniques. Results of this comparison are presented in ***Figure 11*** below.

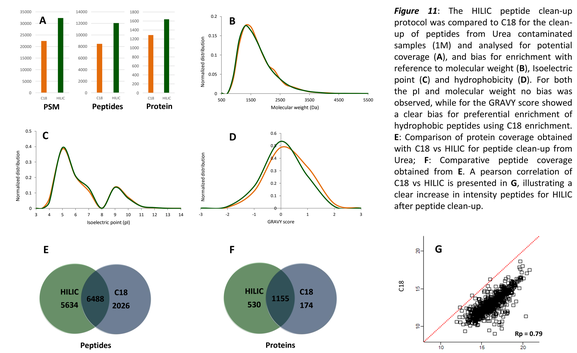


Figure 11: The HILIC peptide clean-up protocol was compared to C18 for the clean-up of peptides from Urea contaminated samples (1M) and analysed for potential coverage (**A**), and bias for enrichment with reference to molecular weight (**B**), Isoelectric point (**C**) and hydrophobicity (**D**). For both the pI and molecular weight no bias was observed, while for the GRAVY score showed a clear bias for preferential enrichment of hydrophobic peptides using C18 enrichment. **E**: Comparison of protein coverage obtained with C18 vs HILIC for peptide clean-up from Urea; **F**: Comparative peptide coverage obtained from **E**. A pearson correlation of C18 vs HILIC is presented in **G**, illustrating a clear increase in intensity peptides for HILIC after peptide clean-up.

Manually processed replicates of tryptic peptide digests (HCT116), contaminated with 1M Urea, were further analysed for intra-sample reproducibility when using C18 and HILIC for clean-up (***Figure 12***). The use of HILIC showed increased reproducibility for clean-up even though salts such as urea can interfere with HILIC binding of peptides.

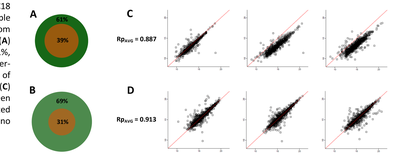


Figure 12: Replicate samples of HILIC and C18 were compared to assess the intra-sample variability for the extraction of peptides from 1M urea. Samples prepared using C18 (**A**) indicated an overlap of approximately 61%, while HILIC (**B**) indicated an increased inter-sample overlap of 70% (**B**). Examples of Pearson correlations of C18 vs C18 (**C**) replicate and HILIC vs HILIC (D) have been included. HILIC consistently demonstrated increased signal intensity, and showed no deviation from the median.

Conclusions

This work demonstrates the robust clean-up of proteins and peptides from a broad range of common sample contaminants using MagReSyn® HILIC for SPE. The workflows are suitable for automated and manual processing of samples, although automation may be preferred for improved reproducibility. HILIC SPE for peptide clean-up compares favourably to C18 clean-up showing improved coverage and reproducibility with no bias towards hydrophobic peptides. All workflows are automatable using magnetic bead handling stations such as the KingFisher™, allowing for processing of up to 96 samples in parallel for high throughput applications. The suitability for clean-up from a broad range of contaminants ensures researchers are less restricted by the limitations associated with MS compatibility of reagents, and rather by the biological question they intend answering.

References

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