Universal unbiased sample clean-up pre-mass spectrometry using MagReSyn® HILIC for SPE

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Abstract

The application note provides an adaptable guideline and protocol for routine, robust, automatable protein clean-up and digestion using multi-mode hydrophilic interaction chromatography magnetic microparticles for solid phase extraction (HILIC SPE). This is followed by on-bead tryptic digestion with direct LC-MSMS analysis of samples. The workflow is automatable (demonstrated using KingFisher™ magnetic bead handling stations) providing processing capability of up to 96 samples without the necessity of time consuming offline steps such as centrifugation. The performance of the magnetic HILIC SPE workflow is compared to other universal methods for pre-MS sample clean-up, including Filter Aided Sample Preparation (FASP) and Single-Pot Solid-Phase-enhanced Sample Preparation (SP3), with at least 30% improvement in identified PSM, peptides and proteins, without sample bias for proteins.

Introduction

Although mass spectrometry (MS) is a powerful technique for analysing complex protein samples, reproducible sample preparation remains an Achilles Heel for MS analysis, with current methods further lacking throughput and reproducibility. To address these limitations we have developed a routine and robust automatable sample preparation workflow that integrates sample clean-up and digestion using multi-mode hydrophilic interaction chromatography magnetic microparticles for solid phase extraction (HILIC SPE), followed by on-bead tryptic digestion, and direct LC-MSMS analysis. Automation of the workflow provides processing capability of up to 96 samples (inclusive of digestion) without time consuming offline steps such as centrifugation. We compare the performance of the magnetic HILIC SPE workflow to commonly used universal methods for pre-MS sample clean-up, including Filter Aided Sample Preparation (FASP, Wiśniewski *et al*. 2009) and Single-Pot Solid-Phase-enhanced Sample Preparation (SP3, Hughes *et al*., 2014).

Materials & Buffers for HILIC Sample Preparation

- *Lysis Buffer:* 1% SDS in 50 mM Tris-HCl pH 8.0
- *Equilibration Buffer*: 100 mM ammonium acetate , pH 4.5, 15% acetonitrile
- *Binding Buffer*: 200 mM ammonium acetate , pH 4.5, 30% acetonitrile
- *Wash Buffer*: 95% acetonitrile (5% water)
- *Digestion Buffer*: 50 mM Ammonium bicarbonate pH 8.0
- *DTT Reduction reagent*: 1 M Dithiothreitol
- *IAA Alkylation Reagent:* 1 M Iodoacetamide
- 0.5 ml, 1.5 ml, and 2 ml Eppendorf® LoBind microcentrifuge tubes (recommend for sample storage, clean-up and digestion due to low non-specific binding and low leaching of compounds that may interfere with subsequent mass spectrometry analysis).
- A magnetic separator is required to isolate magnetic microparticles from solution between each step of the protocol.
- Automation of the protocol requires a suitable liquid handling system with magnetic bead isolation unit, or magnetic bead handling station such as a KingFisher™ Duo or Flex.

Methods

Sample Preparation: Protein Extraction, Reduction and Alkylation

An HTC 116 (colon carcinoma) cell pellet (~500,000 cells) was re-suspended in 200 µl *Lysis buffer*. Cell lysis was induced via probe sonication (9 pulses, 10 s per pulse). To digest chromatin, 25 U benzonaze (Merck) was added and the cell lysate was incubated for 30 min at 37°C. The samples were clarified by centrifugation at 14,000 g for 10 min, and the supernatant transferred to a 1.5 ml microcentrifuge tube. Protein concentration was performed using BCA assay as per manufacturers' instructions. Proteins were reduced through addition of *DTT Reduction Reagent* to a final concentration of 10 mM followed by incubation at RT for 45 min. Subsequent alkylation was performed by addition of *IAA Alkylation Reagent* to a final concentration of 30 mM, with incubation at RT for 45 min in the dark. The protein sample was quenched by adding further *DTT Reduction Reagent* to achieve a final concentration of 30 mM DTT. The protein solution was aspirated and stored as 50 µg total protein in 0.5 ml microcentrifuge tubes and stored at -80°C. For each method described below, samples were processed in triplicate.

HILIC: Manual Protein Clean-up and Digestion

MagReSyn® HILIC (20 $mg.ml^{-1}$) magnetic microparticles were resuspended thoroughly by vortex mixing for 3 seconds to ensure a homogenous suspension, and 25 µl (500 µg) was transferred to 2 ml microcentrifuge tube. The tube was placed on a magnetic separator until the magnetic microparticles were captured and the solution was clear (~10 s). The supernatant (storage solution) was removed and discarded. The HILIC microparticles were washed in 250 µl *Equilibration Buffer*, with gentle agitation for 20 s. The microparticles were recovered, and *Equilibration Buffer* was removed. The equilibration step was repeated a further two times. A protein to MagReSyn® HILIC microparticle ratio of 1:10 protein:microparticle is recommended for protein adsorption. Thus 50 µg HTC 116 cell extract (as prepared above) was mixed with and equal volume of *Binding Buffer*, and subsequently added to the equilibrated MagReSyn® HILIC. The suspension was initially mixed by pipetting and protein-bead interaction allowed to progress for a further 30 min at RT with gentle mixing (***NOTE:*** *Mixing gently and continuously is required to ensure good protein microparticle interaction during the binding procedure, excessive mixing can result in HILIC microparticles drying on tube side walls and result in poor protein recovery).* The tube was placed on the magnetic separator until clear and the supernatant removed by pipette aspiration (***NOTE:*** *Supernatant may be stored to determine unbound protein, e.g. by SDS-PAGE)*. The beads were washed with 200 µl *Wash Buffer* and mixed by vortexing for 15 s. The microparticles were recovered and washed once more for a total of two washes. Sequencing grade trypsin (Promega) in 100 µl *Digestion Buffer* was added to the beads containing bound protein to achieve an enzyme to protein ratio of 1:10. Protein digestion was performed at 37°C for 4 hr. (***NOTE:*** *Mix gently and continuously to ensure good sample microparticle interaction).* The microparticles were recovered on a magnetic separator and the supernatant, containing peptides, was transferred to a 0.5 ml microcentrifuge tube. Peptides were vacuum-dried and stored at -80°C until analysis.

HILIC: Automated Protein Clean-up and Digestion

Automated sample clean-up and digestion was performed on a KingFisher™ Duo magnetic handling station (Thermo Scientific, USA), using 96 deep-well microtiter plates, suitable of processing of up to 12 samples in parallel. The system has a 12-pin robotic magnet head with disposable plastic comb. This prevents sample cross-contamination during binding, mixing and transfer steps between wells (*Figure 1*). The KingFisher™ Duo Peltier block (row A) was used for protein digestion at 37°C. Protocol files for The KingFisher™ Duo and Flex (parallel processing of 96 samples) are freely available upon request (info@resynbio.com).

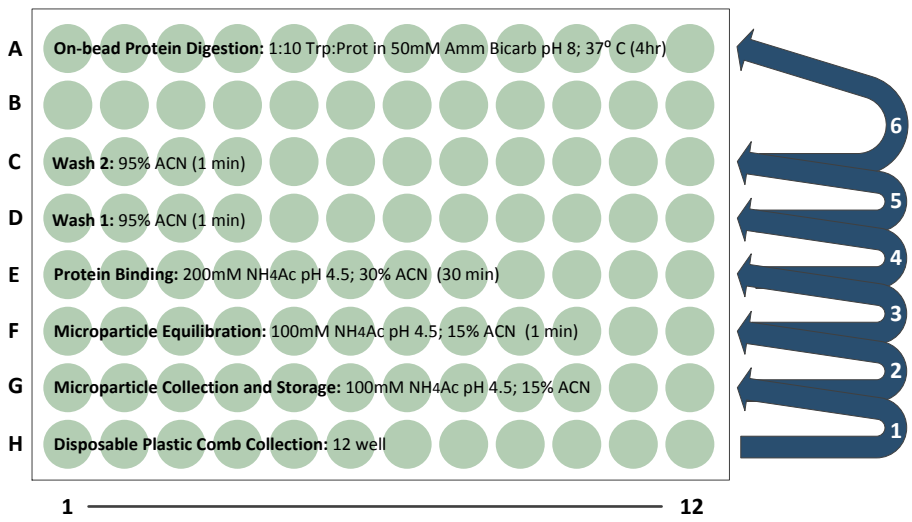


Figure 1: A KingFisher™ Duo magnetic handling station was configured for HILIC based clean-up and protein digestion. MagReSyn® HILIC microparticles are placed in row G and protein samples, pre-mixed with *Binding Buffer*, in row E. The microparticles are collected from row G, transferred to row F for equilibration thereafter transferred to row E for protein binding. Contaminants are removed by two successive washes in rows D and C. Digestion proceeds for 4 hr in row A (peltier system). Microparticles are then removed from the digested protein mixture back to the storage position in row G. Peptide – containing supernatant from row A is recovered and dried in 0.5 ml microcentrifuge tubes.

FASP: Protein Clean-up and Digestion

FASP based sample clean-up and digestion were performed as per manufacturer's instructions (Expedeon, catalogue # 44250). The sample contained 50 µg of total reduced protein lysate, extracted in SDS and reduced as above. The final filtrate containing the digested peptides was acidified with formic acid (final concentration 5%) and samples were desalted using 200 µl C18 Stage Tips (ThermoFisher Scientific catalogue # SP301) as per manufacturers' instructions. Desalted peptides were vacuum-dried and stored at -80°C until analysis by LC-MSMS.

SP3: Protein Clean-up and Digestion

SP3 based sample clean-up and digestion were performed as per method described by Hughes *et al.* in 2014, using a combination of Sera-mag A (GE Healthcare catalogue # 09-981-121) and Sera-mag B (GE Healthcare catalogue # 09-981-123) Speed Beads. The sample containing 50 µg of protein was prepared and reduced as above prior to clean-up (refer HILIC). Trypsin was added in 50 mM ammonium bicarbonate buffer (protein to enzyme ratio 20:1) and samples incubated for 18 hr at 37°C. Peptides were vacuum-dried and stored at -80°C until analysis.

LC-MSMS Analysis

Dried peptide mixtures were resuspended in 50 µl 2% ACN, 0.2% FA. Samples were centrifuged for 14,000 g for 10 min and supernatants transferred to new 0.5 ml microcentrifuge tubes. LC-MSMS analysis was performed using an AB Sciex TripleTOF® 6600 coupled to a Dionex™ 3500 nanoRSLC fitted with a capillary flow selector (1-10 µl.min^{-1}). A volume of 8 µl (~8 µg total protein) from each sample was loaded on an Acclaim® PepMap C18 trap column (100 µm × 2 cm) and desalted for 2.5 min at 10 µl.min^{-1} using 2% ACN, 0.2% FA. Peptides were separated on an Acclaim® PepMap C18 column (300 µm × 15 cm, 3 µm particle size) coupled to the TripleTOF® 6600 MS (AB SCIEX) using a 25 µm electrospray probe. Peptide elution was achieved using a flow-rate of 8 µl.min^{-1} with a gradient of 4-30% solution **B** over 60 min (**A**: 0.1% FA; **B**: 80% ACN with 0.1% FA). Data acquisition mode: one survey scan (360–1500 m/z) with 500 ms accumulation time and 50 product ion scans (100–1800 m/z) each with 50 ms accumulation time. Product ions scans were triggered automatically when multiply charged ions (2^+ to 5^+) mass 360–1500 m/z and intensity ≥ 200 counts were detected. An exclusion window of 30 s was applied, and rolling collision energy with a collision energy spread (CES) of 10 eV was used.

Data Processing

LC-MSMS spectral data was analyzed using PEAKS Studio 6 software (Bioinformatics Solutions Inc., Canada, Ma *et al*., 2003). Spectral data was searched against a Uniprot Swissprot Homo Sapiens, non-redundant, database supplemented with sequences of common protein contaminants. The following search parameters were applied: parent mass tolerance error 20 ppm; fragment mass tolerance error 0.05 Da; up to four missed cleavages per peptide; non-specific cleavages allowed at one end; cysteine carbamidomethylation (fixed modification); methionine oxidation and N-terminal acetylation (variable modification). A false discovery rate of 0.1% was used at the peptide-to-spectrum matches (PSM) and peptide levels. Minimum of one unique peptide was required for protein identification. For MS1 based quantification data was analyzed using MaxQuant (MQ: Cox and Mann, 2008) v 1.5.8.3. The same protein database and search parameters were used as with PEAKS Studio 6 processing; the exception being the maximum miss-cleavages which was set to 2. For Label Free Quantification (LFQ) the default MQ parameters were used. Perseus (Tyanova *et al*., 2016), and Peptide Shaker (Vaudel *et al*., 2015) were used for further analysis (refer *figures 6 and 8* respectively).

Results & Discussion

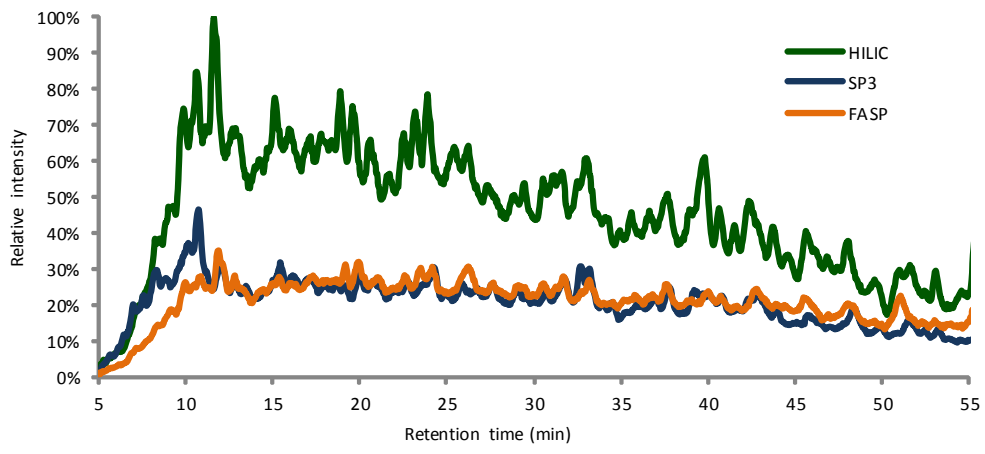


Figure 2: TIC (total ion chromatograms) of HCT 116 lysates extracted with 1% SDS and processed using HILIC SPE, SP3 or FASP methods. The workflow with HILIC SPE showed increased peptide recovery as indicated by 2x higher TIC when compared to SP3 and FASP. Each workflow was tested in triplicate using 50 µg total protein as the starting material. Samples were analyzed in parallel and submitted randomly for LC-MSMS analysis.

Higher peptide recovery, as indicated by the TIC signal (*Figure 2*) in the case of SPE using MagReSyn® HILIC translated in greater than 30% increase in matched PSM, peptides and proteins (*Figure 3*) as well as higher confidence identifications particularly in the low abundance species (*Figure 4*) when compared to data acquired for SP3 and FASP.

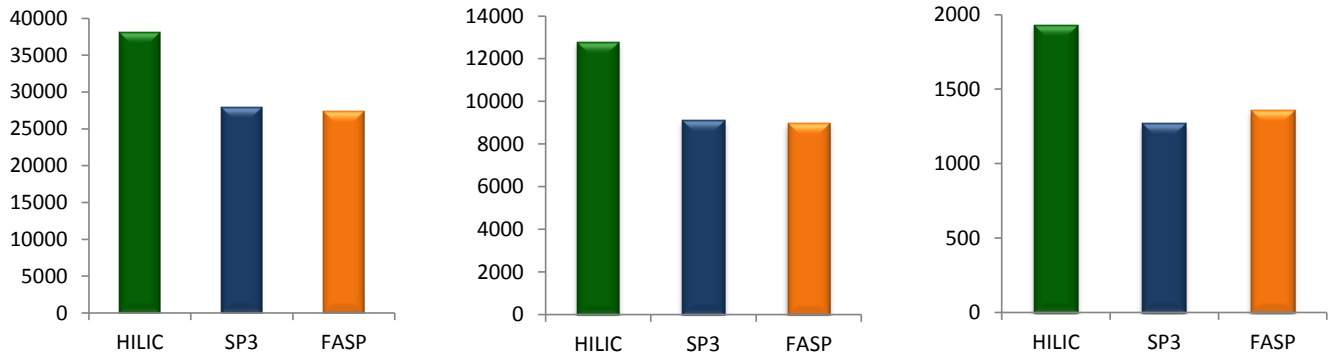


Figure 3: *Left to right*: Identified PSM (0.1% FDR cut-off), peptides (1% FDR cut-off) and, unique proteins (1% FDR, ≥1 unique peptide) reported for HILIC SPE, SP3 and FASP workflows.

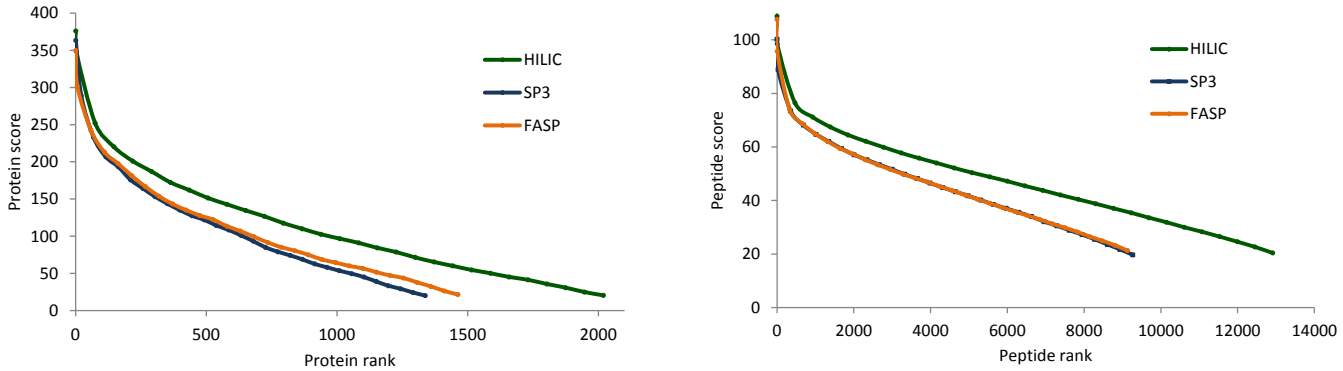


Figure 4: Peptide (left) and protein (right) score distribution as a function of rank indicates that samples processed with HILIC magnetic microparticles result in higher peptide and protein scores particularly for low abundance (low rank) species.

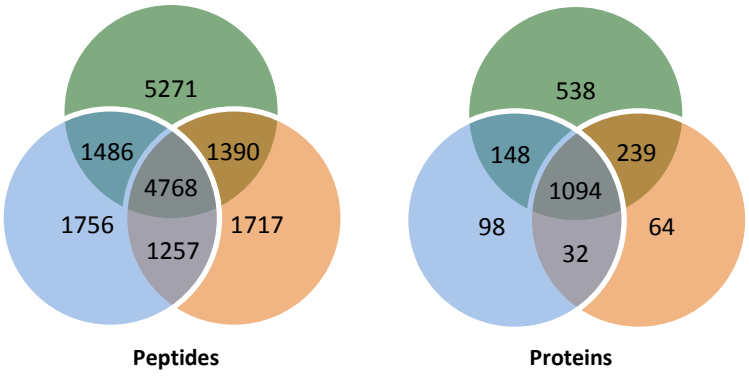
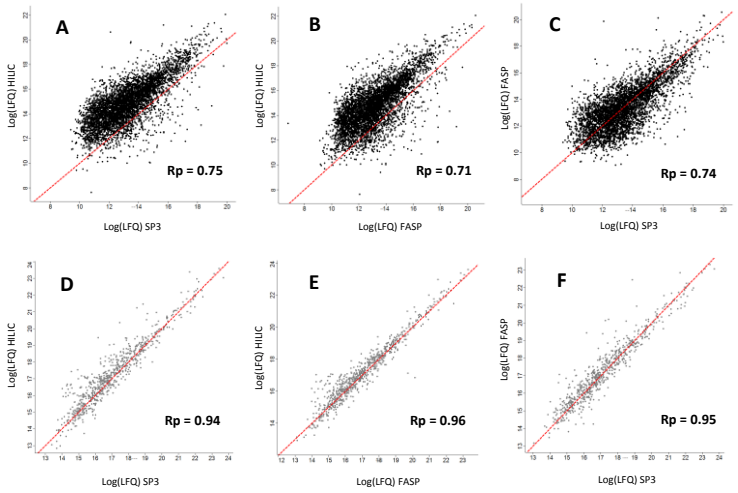


Figure 5 (left): Venn diagram indicating peptide and protein overlap between the three workflows for clean-up used in this study. The superior performance of the HILIC SPE workflow (green) translates into more than 5000 peptides and 500 proteins uniquely identified in the samples, over SP3 (blue), and FASP (orange).

Figure 6 (below): Data dependant runs were processed using MQ v1.3.5.8 where MS1 Label Free Quantitation (LFQ) was performed on triplicate runs from each of the three sample preparation methods. The peptide (A – C) and protein correlations (D – F) were calculated using the Pearson method (Rp).

Scatter plots of peptide intensities (Figure 6. A – C) based on the MQ calculated log(LFQ) values confirm that HILIC SPE resulted in approximately double the recovery as compared to SP3 and FASP, illustrated by the shift from linear fit (red line) in the case of HILIC vs SP3 (A) as well as HILIC vs FASP (B).

Scatter plots of protein intensities (Figure 6. D – F) based on the MQ calculated log(LFQ) values, normalized using total ion signal, show high correlation of the protein abundance values.



To assess any possible bias in the sample preparation, the data was analyzed with respect to several criteria including the charge state, molecular mass, isolelectric point (pI) and hydropathicity (GRAVY score). The results are illustrated in *Figure 7* below and show no major bias for any of the workflows.

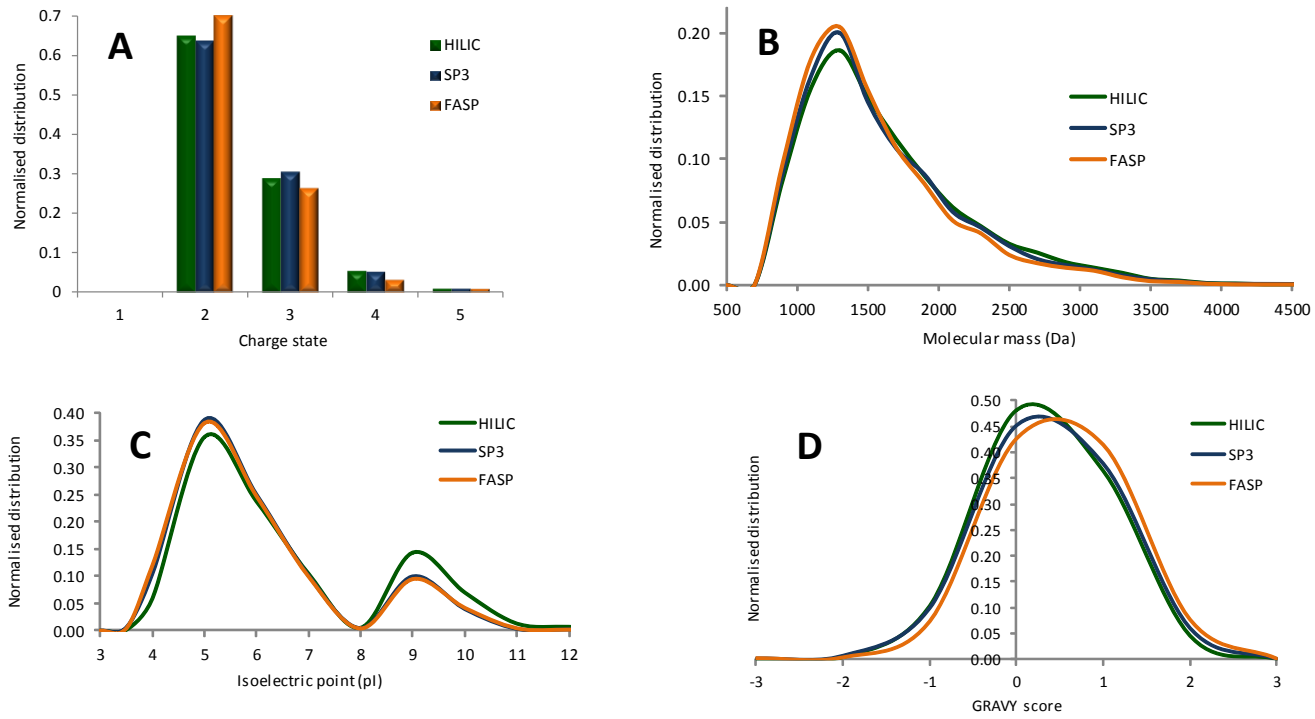
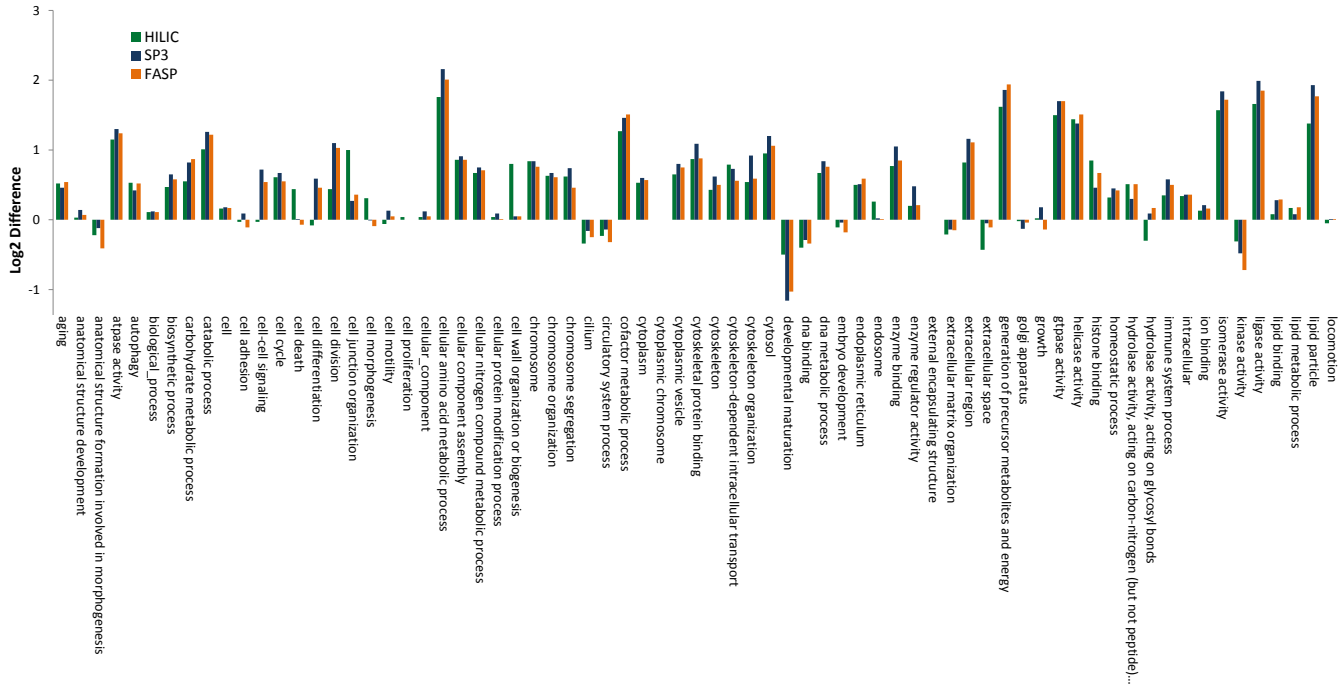


Figure 7: Analysis of possible sample bias for clean-up using the various strategies. The peptide distribution in terms of (A) charge state, (B) molecular mass, (C) isoelectric point (pI) and hydropathicity (GRAVY score) suggests no major bias in the identified peptides between the three methods with perhaps the only significant difference observed in terms of pI where SPE with MagReSyn® HILIC microparticles retain ~5% more peptides with increased pI.

In a final assessment for potential sample bias, the data from the three workflows were subjected to Gene Ontology Enrichment Analysis (GOEA) using validated proteins from each clean-up method. The results indicate a similar Gene Ontology (*Figure 8*), with lack of any significant bias.



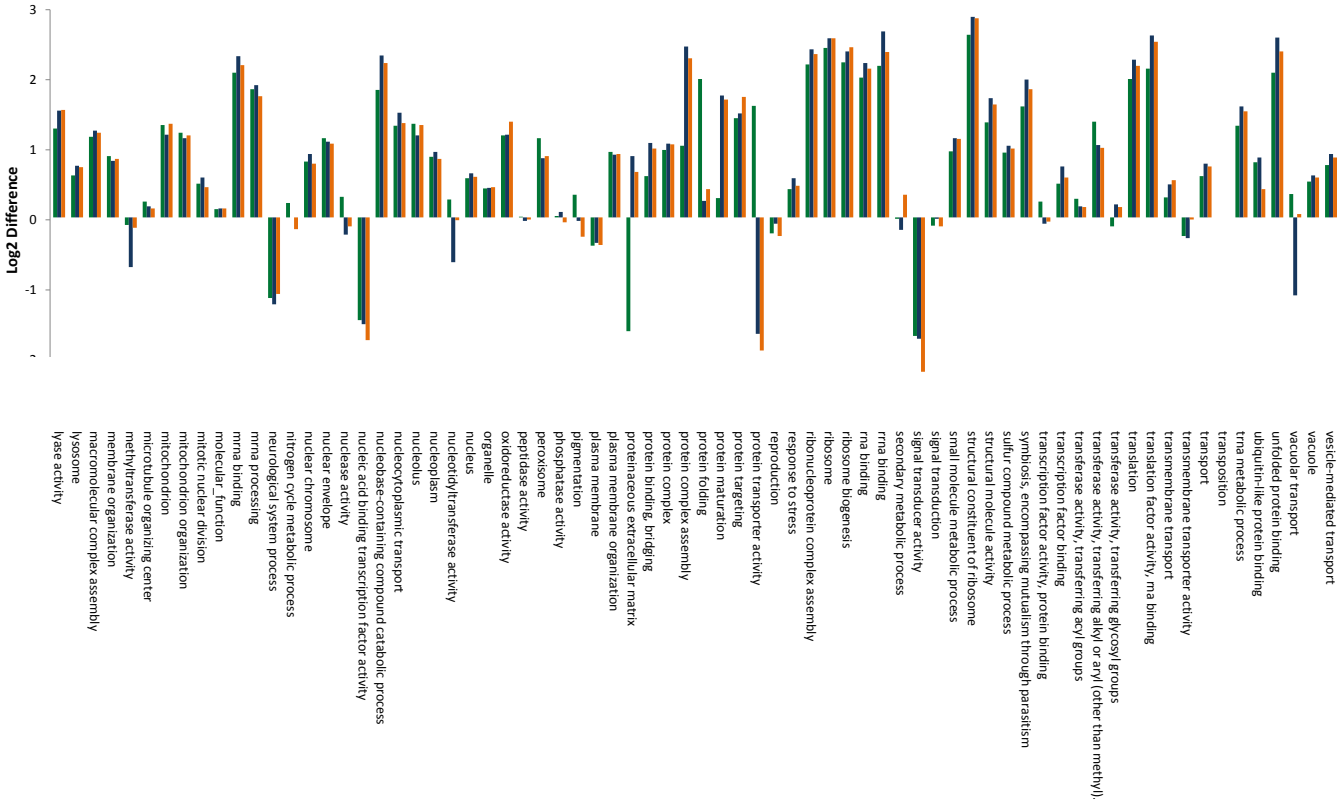


Figure 8: Assessment of potential adsorption bias by GOEA analysis of samples cleaned up with the three methods.

Conclusions

The application note describes an automated workflow for quantitative proteomic sample preparation using MagReSyn® HILIC microparticles after efficient detergent based sample solubilization using SDS. The performance of the HILIC based clean-up workflow was compared to other universal methods, FASP and SP3. The MagReSyn® HILIC SPE workflow resulted in approximately 2 fold increase in peptide recovery, translating to over 30% increase in identified PSMs, peptides, and ultimately unique proteins identified. The protocol, including on-bead tryptic digestion, was automated using KingFisher™ magnetic bead handling stations, allowing for high throughput processing of up to 96 samples in parallel with over 7 fold increase in throughput compared to FASP, as well as significantly improved reproducibility. The sample clean-up was achieved without significant bias with respect to the protein properties. This workflow provides a robust and high-throughput method for the clean-up and digestion of protein samples prior to MS analysis.

Ordering Information

Description	*Product Code*
MagReSyn® HILIC 2 ml	**MR-HLC002**
MagReSyn® HILIC 5 ml	**MR-HLC005**
MagReSyn® HILIC 10 ml	**MR-HLC010**

Acknowledgements

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References

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