Universal Unbiased pre-MS Clean-Up Using Magnetic HILIC Microparticles for SPE

Stoyan Stoychev[a], Previn Naicker[a], Sipho Mamputha[a], Fezile Khumalo[a], Isak Gerber[a], Chris van der Westhuyzen[a], Justin Jordaan[b]

[a] *Council for Scientific and Industrial Research, Pretoria, South Africa*
[b] *Rhodes University, Grahamstown, South Africa*
E-mail: sstoychev@csir.co.za

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-MS/MS 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).

Methods

An outline of the sample preparation workflow tested in this study is illustrated in ***Figure 1***. HCT 116 (colon carcinoma) cell extract was solubilized using 2% SDS. Salts and detergent were removed by magnetic HILIC SPE beads (ReSyn Biosciences), as well magnetic carboxyl (SP3, GE Healthcare, refer Hughes *et al.*, for method), and FASP, followed by trypsin digestion. For the magnetic particles, the clean-up and digestion steps were automated using a magnetic bead handling station (KingFisher™ Duo). FASP-based sample clean-up and digestion were performed as per the manufacturer's instructions (Expedeon). Samples were analysed using an AB Sciex TripleTOF 6600 coupled to a Dionex nanoRSLC. 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 and Mann, 2008) and Perseus (Tyanova *et al.*, 2016) software packages.

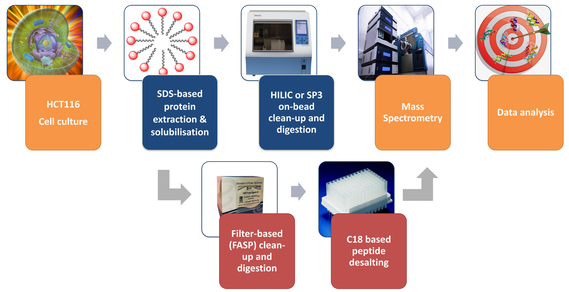


Figure 1: Diagrammatic representation of FASP, HILIC SPE and SP3 workflows.

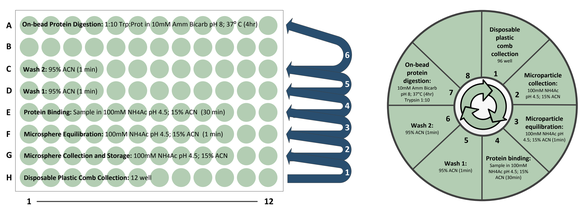


Figure 2: Automated sample preparation workflow, using magnetic HILIC functional microparticles for SPE. Particles are returned and stored in Row H (KF Duo - Left) or position 2 (KF Flex - Right) post workflow.

Automated sample clean-up and digestion was performed on KingFisher™ magnetic 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 heads with disposable plastic comb preventing sample cross-contamination when mixing, recovering and moving of magnetic particles. ***Figure 2*** illustrates KingFisher™ Duo and Flex systems configured for automated sample clean-up and digestion. The automated protocols were optimized to efficient pick-up, mixing and transfer of HILIC microparticles without significant protein loss or microparticle carry-over. Kingfisher import-ready protocols are available upon request (contact author). The protocol takes less then 45 min with an additional 240 min digestion time. With the ability to process up to 96 samples in parallel, the HILIC SPE and SP3 protocols are far more time efficient compared to FASP with close to 7 fold improvement in throughput (***Figure 3***).

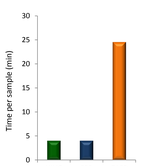


Figure 3: Sample preparation time

Results & Discussion

Total ion chromatographs of HCT 116 lysates extracted using 2% SDS and processed using HILIC SPE, SP3 or FASP methods are illustrated in ***Figure 4***. The HILIC SPE workflow showed significantly increased peptide recovery as indicated by 2x higher TIC as compared to SP3 and FASP. Each workflow was tested in triplicate using 50 µg total protein as starting material. Resulting peptides were first de-salted on Acclaim PepMap C18 trap (100 µm × 2 cm) for 2 min at 10 µl.min^{-1} using 2% acetonitrile/0.2% formic acid, than separated on Acclaim PepMap C18 RSLC column (300 µm × 15 cm, 3 µm particle size). Peptide elution was achieved using a flow-rate of 8 µl.min^{-1} with a 60 min gradient, 5-60% 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 500 ms followed by 50 MS2 scans, acquired from *m/z* 100-1800 at 50 msec each, for a total scan time of 3.0 sec.

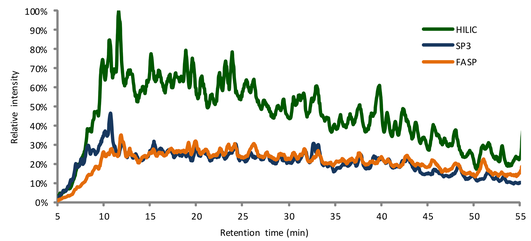


Figure 4: Comparative TIC for samples prepared using the HILIC, SP3 and FASP workflows.

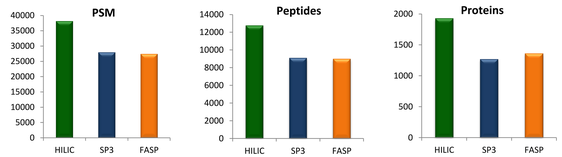


Figure 5: Identified PSM (0.1% FDR cut-off), peptides (1% FDR cut-off) and unique proteins (1% FDR, ≥1 unique peptide) are reported for HILIC SPE, SP3 and FASP workflows. The higher peptide recovery, as indicated by the TIC signal (Figure 4) in the case of HILIC translated in 30 % increase in matched PSM, peptides and proteins as compared to SP3 and FASP.

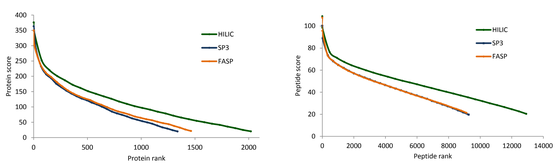


Figure 6: Peptide and protein score distribution as a function of rank indicates that samples processed with HILIC magnetic microparticles result in higher peptide and protein scores particularly for low abundance (low rank) species.

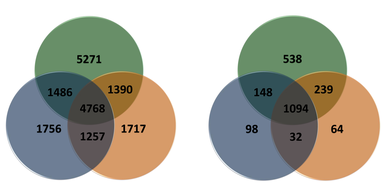


Figure 7: Venn diagram indicating peptide and protein overlap between the three clean-up workflows used in this study. The superior performance of the HILIC SPE workflow (green) translates into more then 5000 peptides and 500 proteins uniquely identified in the HILIC SPE prepared samples, over SP3 (blue), and FASP (orange).

Results & Discussion Cont…

Scatter plots of peptide intensities (A – C) based on the MQ calculated log(LFQ) values (***Figure 8***) confirm that HILIC SPE resulted in approximately double the recovery as compared to SP3 and FASP methods, as 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 (D – F) based on the MaxQuant calculated log(LFQ) values (A – C), normalized using total ion signal, show high correlation of protein abundance values.

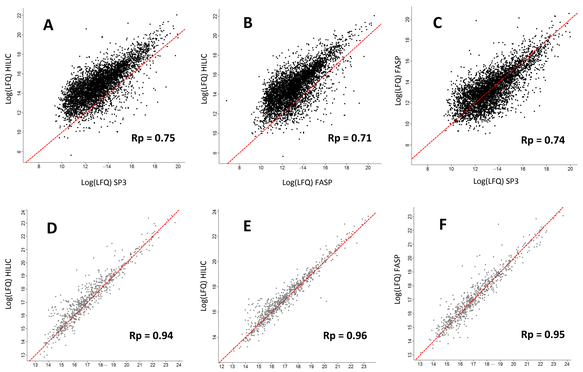
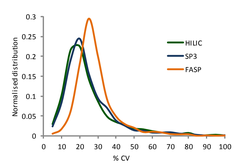


Figure 8: Data dependant runs were processed using MaxQuant where MS1 Label Free Quantitation (LFQ) was performed on triplicate runs from each of the three sample preparation methods. The peptide (A – C) and protein correlations (D – F) were calculated using the Pearson method (Rp).

Figure 9 (Right): The CV distributions of peptides with <1% FDR were plotted for each method demonstrating that HILIC and SP3 workflows show significantly lower variance, indicating improved reproducibility when compared to FASP.

To assess any possible bias in the sample preparation, the data was analysed with respect to several criteria including the charge state, molecular mass, isolelectric point (pI) and hydropathicity (GRAVY score). The results are illustrated in ***Figure 10*** below and show no major bias for any of the workflows.



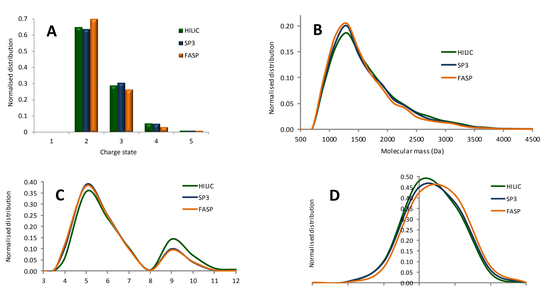


Figure 10: Analysis of possible sample bias for clean-up using the various strategies. The peptide distribution in terms of (A) Charge state, (B) Molecular mass, (C) Isoelectric point (pI) and Hydropathicity (GRAVY score) suggests no major bias in the identified peptides between the three methods with perhaps the only significant difference observed in terms of pI where SPE with HILIC functionalized microparticles retain ~5% more peptides with increased pI.

Results & Discussion Cont…

In a final assessment of 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, with lack of any significant bias.

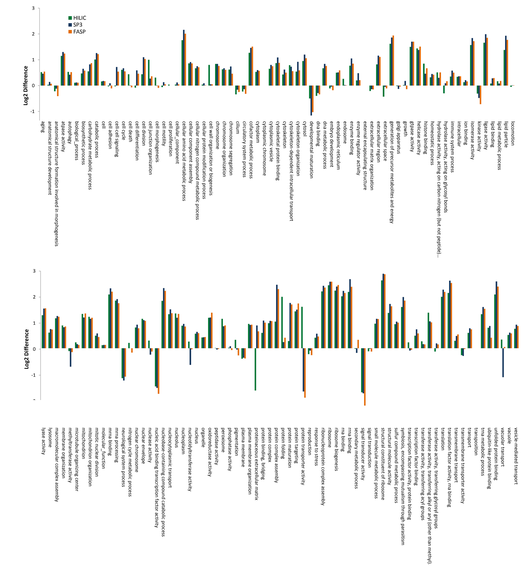


Figure 11: Assessment of Bias by GOEA analysis of cleaned samples.

Conclusions

This work describes an automated workflow for quantitative proteomic sample preparation using MagReSyn® HILIC magnetic microparticles after efficient detergent based solubilisation using SDS. The performance of the HILIC based workflow was compared to the most commonly used methods of FASP and SP3, where the 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. The protocol, including on-bead tryptic digestion, was automated using a Thermo KingFisher™ magnetic bead handling station, allowing for high throughput processing of up to 96 samples in parallel with over 7 fold increase in throughput, as well as significantly improved reproducibility, compared to FASP. The sample clean-up was achieved without significant bias with respect to the properties of the peptides or identified proteins. This workflow provides an improved and robust method for the clean-up of samples prior to MS analysis. We are evaluating the compatibility of the HILIC workflow for clean-up from other common solubilization and denaturation reagents.

References

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