Improved reproducibility of automated sample preparation using HILIC magnetic beads automated by Hamilton Robotics

RESYN BIOSCIENCES

Stoyan Stoychev, Justin Jordaan
ReSyn Biosciences Pty Ltd, Johannesburg, South Africa

Michelle du Plessis, Charné Ross, Elizabeth Ross, Liam Bell
Centre for Proteomics and Genomics Research, Cape Town, South Africa

Jane Charlton
Separations, Johannesburg, South Africa

CPGR CENTRE FOR PROTEOMIC & GENOMIC RESEARCH

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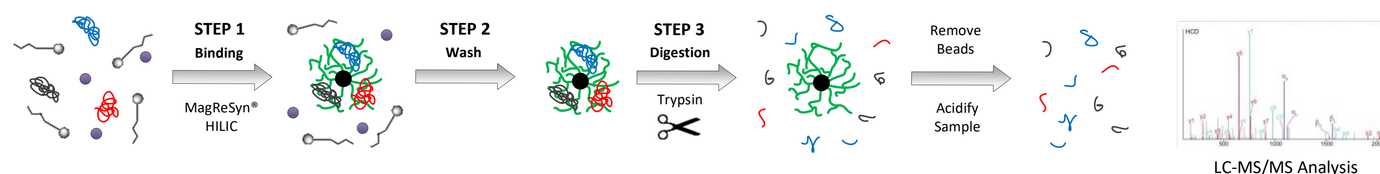
ABSTRACT

We demonstrate the integration of MagReSyn® HILIC ferromagnetic beads with a Hamilton Microlab® STAR™ liquid handling station for the automation of proteomics sample preparation, including all steps from protein capture, clean-up (from a wide range of detergents, denaturants, salts and polymers), with on-bead trypsin digestion for mass spectrometry analysis. The method is suitable for the preparation of 96 samples in parallel with little to no hands-on time, with results comparable to that of sample processing using FASP. The sample preparation takes less than 8 hours (<4 min per sample) with hands-on time of less than 1 hour.

INTRODUCTION

- Efficient and robust sample preparation is critical for dealing with proteomics samples as the field moves towards clinical applications.
- These methods and workflows are required to be high-throughput, transferable and reproducible for robust sample analysis by MS.
- Automation is key to achieve these methods, and magnetics is preferred due the versatility, linear scalability, inter-operability of steps, while the strong **ferromagnetic** property of MagReSyn® speeds up workflows and reduces sample loss during automated handling.
- Towards these goals we describe the full automation of HILIC SPE sample preparation on a Hamilton Microlab® STAR™.

HILIC SPE WORKFLOW



STEP 1: Reduced and alkylated protein lysate, solubilised in detergent such as SDS (1-5%), is captured on MagReSyn® HILIC beads by addition of 1:1 sample:binding buffer (30% ACN in 200mM Amm Acetate pH 4.5). **NOTE**: *Reduction and alkylation can also be performed on bead by adding TCEP and CAA in the binding buffer.*
STEP 2: Contaminants such as detergents are removed by washing with 95% Acetonitrile.
STEP 3: Trypsin digestion is performed on-bead for 4 hours at 37°C with a 1:10 trypsin to protein ratio releasing peptides into solution that are suitable for subsequent MS analysis (**OPTIONAL**: *Alternate digestion methods include 1 hour at 47°C using Trypsin, or sequential digestion using LysC & Trypsin*). **NOTE**: *Optional elution using 1% TFA can be used to improve peptide elution post digestion (if extraction is performed in detergents other than SDS, we recommend testing for elution of extraction components before implementing this step).*
This method has been shown to be suitable for the removal of a range of common MS contaminants.

Contaminant	Concentration
SDS	20%
SDC	1%
Triton	2%
Tween 20	2%
NP-40	2%
Urea	8M
NaCl	1M
GdHCl	5M
Glycine	0.2M
PEG2000	5%
Glycerol	20%

METHODS

eFASP was performed according to Erde *et al*. 2014 (adapted from Wiśniewski *et al*, 2009), using **100µg** of protein. Protein samples (**50µg**) for HILIC SPE were reduced and alkylated using 10mM TCEP followed by 10mM MMTS (methyl methanethiosulfonate). The HILIC SPE workflow was automated on a Hamilton Microlab® STAR™ as described below. An additional offline C18 desalting step was performed with eFASP samples, but was not required with HILIC SPE samples. Analysis was performed using a Q-Exactive™ Orbitrap™ mass spectrometer (ThermoFisher Scientific, USA) coupled to a Dionex™ UltiMate™ 3000 nanoRSLC equipped with C18 trap column (Acclaim™ PepMap™ 100) and Waters nanoEase ZenFit™ M/Z CSH C18 analytical column over a 60min gradient.

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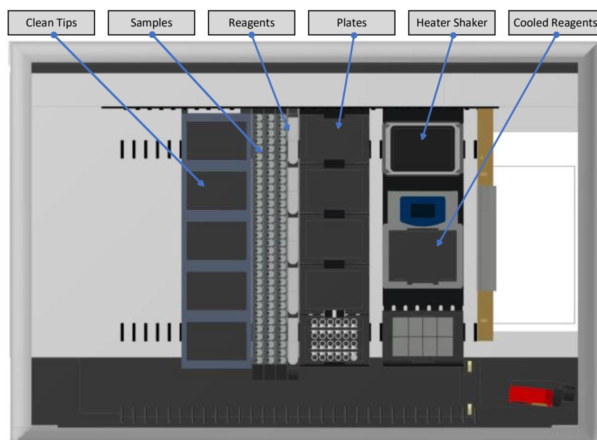
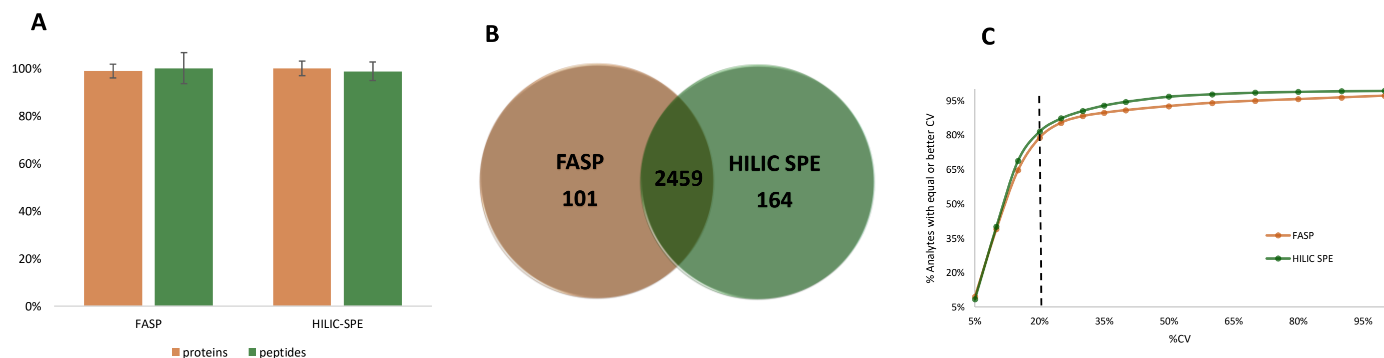
AUTOMATION ON HAMILTON MICROLAB® STAR™



Procedure	Steps	Details	Time (96 Samples)
Normalisation	Transfer samples Add diluent	50µg of protein to Lobind plate Make sample to 50µl with 50mM TEAB	10 min
Binding	Add beads	250µg per 50µg sample (5:1 beads to protein) Beads in 30% ACN 200mM Amm Acetate pH 4.5, **NOTE**: More recently we have seen a 10:1 bead to protein can further increase protein recovery.	35 min
	Shaking incubation	900 rpm for 30 minutes	
	Incubation on magnet	1 minute	
Wash	Remove supernatant & Wash	500µl of 95% ACN	60 min
	Shaking incubation	900 rpm for 1 minute	
	Incubate on magnet	30 seconds	
	Cycle	x4 wash cycles performed	
Digest	Add diluent	175µl of 50mM TEAB	260 min
	Add cold enzyme	25µl of 0.2µg/ul Trypsin (1:10)	
	Heated incubation (4 hrs)	4 hrs at 37°C @ 900 rpm	
	Incubate on magnet Transfer eluate	30 seconds 200µl	
Bead Elution (*Optional step to improve recovery, evaluate to test for elution of possible captured contaminants*)	Add diluent	50µl of 1% TFA	15 min
	Shaking incubation	900 rpm for 5 minutes	
	Incubate on magnet	30 seconds	
	Transfer eluate	50µl Pooled with digest eluate	
		Total Time	**380 min**
		Time per Sample	**< 4 min per sample**

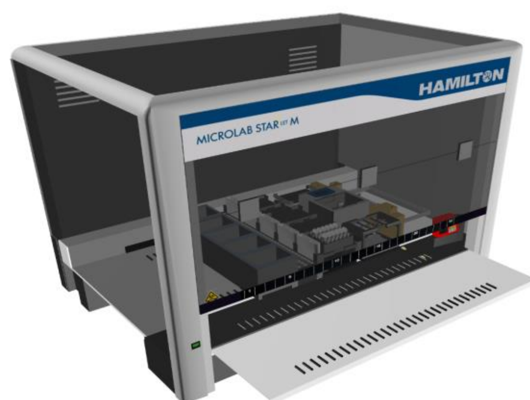
RESULTS AND DISCUSSION

- FASP and HILIC SPE showed similar results (**A** below) with an average (four technical replicates) of 2265 proteins (11700 peptides) identified with the FASP method, with CV's of 3 and 7% respectively, and automated HILIC SPE resulting in an average of 2292 proteins (11550 peptides) with CV's of 3 and 4% respectively).
- Protein overlap analysis (**B**) revealed that ~95% of the identified proteins were found in both the automated HILIC SPE and FASP methods.
- Both methods showed high reproducibility (**C**) with ~80% of the quantified peptides having CV's below 20%.
- Gene ontology analysis showed no discernible selectivity for any biological pathways (data not shown).
- Sample processing with FASP took approximately 21 hours to process 24 samples (inclusive of 18 hours for trypsin digestion), while the automated HILIC SPE took ~8 hours to process 96 samples in parallel (inclusive of a 4-hour digestion) with little to no manual intervention.



CONCLUSIONS

- The fully automated HILIC SPE workflow performed similarly to FASP, the most referenced method for clean-up and digestion of proteins.
- Automation of the workflow on a HAMILTON Microlab® STAR™ provided seamless sample preparation including magnetic bead and liquid handling.
- The automated workflow provided the same coverage as FASP, using half the starting protein quantity.
- Offline C18 desalting was not required for HILIC SPE prepared samples as they were of sufficient purity for LC MS analysis using a pre-concentration setup.
- The features of the automated MagReSyn® HILIC SPE workflow, including broad contaminant compatibility and increased throughput, make it desirable for implementation in laboratories moving to clinical sample analysis.
- Implementation of robust automated workflows improves laboratory resource management, improves reproducibility, enabling inter-laboratory research.



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Ver 1: Dec 2019