Rapid and automatable magnetic microparticle-based methods for clinical proteogenomic studies

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PROTEOMICS WORKFLOW OVERVIEW

TRADITIONAL FASP URINARY PROTEOMICS WORKFLOW

Urine Sample Collection (1.5 ml) → Centrifugation & Clarification → Storage (-80° C) → Acetone Precipitation (1 hr) → Reduction and Alkylation (1 hr) → Protein Clean-up (FASP – 2 hr for 16 samples) → In solution digestion (18 hr) → Desalting C18 (30 min for up to 96 samples) → Drying → LCMS Analysis

TOTAL TIME: **22.5 HR**
LABOUR: **3.5 HR**
THROUGHPUT: **16 (PPTN)**
TIME PER SAMPLE: **1.5 HR**
AUTOMATABLE: **NO**

RESEARCH AIMS

- RAPID
- AUTOMATED
- HT COMPATIBLE
- URINE COMPATIBLE
- VENDOR INDEPENDENT
- VERSATILE WORKFLOWS
- >96 SAMPLES TO MS in <5HR

NORGEN BIOTEK SAMPLE COLLECTION KIT

Urine Sample Collection (30 ml) → Add to Norgen sample collection beads → Storage (-80° C) → SPE for clean-up (3 hr for 20 samples) → Reduction and Alkylation (1 hr) → In solution digestion (18 hr) → Desalting C18 (30 min for up to 96 samples) → Drying → LCMS Analysis

TOTAL TIME: **22.5 HR**
LABOUR: **4.5 HR**
THROUGHPUT: **20**
TIME PER SAMPLE: **1.1 HR**
AUTOMATABLE: **NO**

MAG HILIC URINARY PROTEOMICS WORKFLOW

Urine Sample Collection (1.5 ml) → Centrifugation & Clarification → Storage (-80° C) → Acetone Precipitation (1 hr) → Reduction and Alkylation (1 hr: on-bead optimization in progress) → Protein Clean-up (HILIC, 20 min) → On bead digestion (4 hr) → Drying → LCMS Analysis

TOTAL TIME: **6.2 HR**
LABOUR: **1.25 HR**
THROUGHPUT: **16 (PPTN)**
TIME PER SAMPLE: **<25 MIN**
AUTOMATABLE: **PART**

MAG SAX URINARY PROTEOMICS WORKFLOW

Urine Sample Collection (0.8 ml) → Centrifugation & Clarification (future work: option to remove) → Storage (-80° C) → Reduction and Alkylation (1 hr: on-bead optimization in progress) → Protein Clean-up (SAX, 20 min) → On bead digestion (4 hr) → Drying → LCMS Analysis

TOTAL TIME: **4.2 HR**
LABOUR: **15 MIN**
THROUGHPUT: **96**
TIME PER SAMPLE: **<3 MIN**
AUTOMATABLE: **YES**

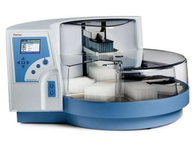
INTRODUCTION

- Urine is considered an attractive source for biomarker studies due to non-invasive collection and availability of large quantities
- Urinary DNA, RNA and proteins are prone to degradation, and have a high background contamination
- Methods with minimal processing steps are sought, which are high-throughput and automatable to deal with large patient cohorts
- Hyper-porous magnetic microparticle technology is ideally suited to high-throughput sample preparation owing to:
  - High capacity allowing for miniaturization
  - High specificity due to increase in functional group density
  - Strong magnetic moment (ferromagnetic), reduces sample loss and speeds up workflows due to fast bead recovery (2-5s)
  - Compatibility with a wide variety of liquid handling stations with magnetic bead capturing stand
- The current work focuses on the capture and profiling of the urinary proteome, with possible modification of the protocols for extension to the capture of RNA and DNA from urine
- We compare two magnetic bead workflows and benchmark against a traditional method as well as an existing commercial kit
- Although our aim is to fully automate mass spectrometry workflows (independent of the magnetic bead handling station), the protocols are also suitable for manual preparation with the ability to perform parallel sample processing using a magnetic stand.
- All experiments were performed on a KingFisher™ Duo (ThermoFisher) magnetic bead handling station (protocols available on request)

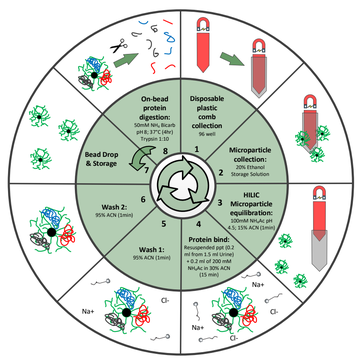
PROTEOMICS METHODS

IMPROVING LIFE THROUGH RESEARCH

Urine from consenting adult males (ethics ref. CSIR 58/2013 & Uni. Witwatersrand 120612) was used for method optimization. All urine was collected midstream, clean-catch, into sterile urine collection containers (PlastPro Scientific, Gauteng, South Africa) and immediately transported to the laboratory on ice. Biological variability was reduced by pooling urine samples for the optimization.



Automation of magnetic clean-up workflows can be achieved with KingFisher magnetic bead handlings stations. Conditions used for HILIC SPE of proteins (**left**) and SAX clean-up using alkaline conditions (**right**). On bead digestion takes place at position 8 (heating element). Elution with 1% TFA increased recovery.



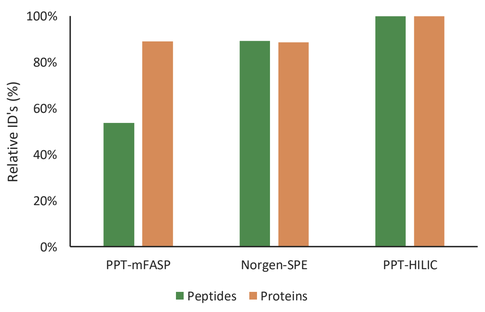
FASP was performed according to the optimised method described by Dwivedi *et al* (2016). The Norgen kit was used as per the manufacturer's instructions. Samples were analysed using a SCIEX TripleTOF 6600 coupled to a Dionex nanoRSLC using 45 minute gradients (initial comparison performed in microflow, while SAX vs Norgen performed with nanoflow). Data was searched using Sciex Protein Pilot (Shilov *et al*., 2007) against a Uniprot Swiss Prot *H. sapiens* database supplemented with sequences of common contaminant proteins. A 1% FDR cut-off was applied at the PSM, peptide and protein levels.

GENOMICS METHODS

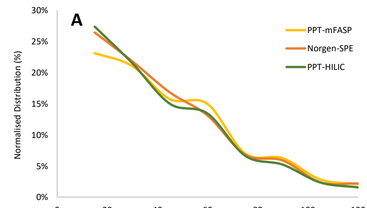
Plasmid DNA from *E. coli* was selected as a model system for determining conditions required for isolation of genetic material, especially considering DNA in urine is highly fragmented, making it difficult to quantify. Further, *E. coli* lysate also contains high RNA content making it a suitable model for all genetic material. Briefly *E. coli* DH10B transfected with plasmid (pBluescript SK) and grown in Enpresso B medium according to the manufacturers instructions (BioSilta). Cells were grown to an OD of 10, washed in saline, and lysed using 1% SDS in 0.2M NaOH. The lysate was neutralised (equivalent volume of 1M K-Acetate pH 5.5) and cell extract was removed by centrifugation. RNAse was added to a final concentration of 100 ug per ml for 30 minutes for DNA purification. Genetic material was captured using magnetic SAX beads and eluted from the SAX with 1.25 M NaCl in 50 mM Tris pH 8.5 and 15% isopropanol for 2 min. DNA was precipitated using 0.7 volumes of isopropanol (recovery by centrifugation at 15000xg). The pellet was washed with 70% ethanol (-20°C), air dried and resuspended in ultrapure water.

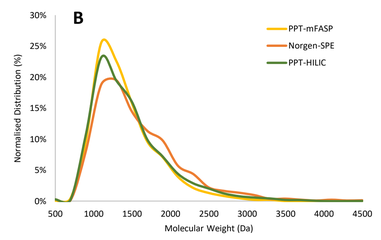
RESULTS & DISCUSSION

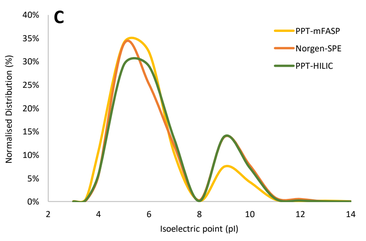
Urine was processed using traditional sample processing technique (FASP), a commercial Norgen Biotek sample collection kit, and magnetic bead workflow (refer to graphical abstract). The comparative protein and peptide coverage by LC-MSMS is illustrated (**right**). The magnetic HILIC SPE workflow showed the highest peptide recovery resulting in the highest urinary proteome coverage. Relative peptide ID's are normalised to 2567 peptides (HILIC SPE) and 471 proteins (HILIC SPE). Although providing the lowest peptide numbers (~50% of HILIC), the traditional method using FASP showed similar coverage to the Norgen kit based method, both of which were approximately 15% lower than the magnetic bead HILIC SPE workflow.

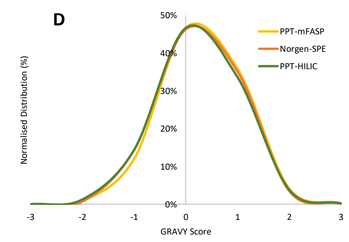


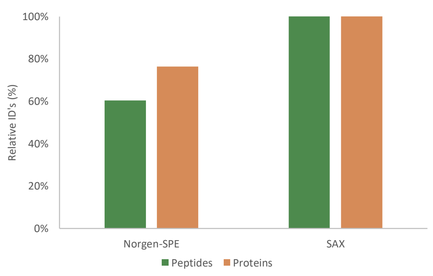
Proteins and peptides identified in the various workflows were analysed for bias with respect to hydrophobicity, protein and peptide MW, and peptide pI. Hydrophobicity analysis (**D**) indicated no bias for any of the workflows, but as expected some loss of lower molecular weight proteins was observed for FASP for protein clean-up (**A**). The use of Norgen Biotek Kit for SPE showed a slight bias for higher molecular weight peptides the cause for this is not yet known (**B**). SPE using either the Norgen Kit or HILIC beads indicated some selectivity for proteins with increased iso-electric point (**C**).



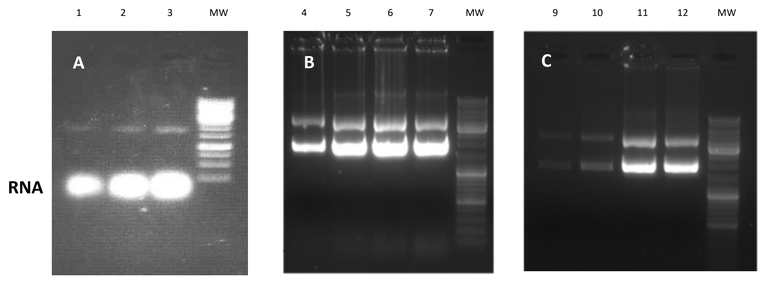








In order to fully automate the urinary proteomics workflow we conceptualised a proteomics workflow using SAX to isolate all protein and peptides from urine. Proteins were isolated directly from 0.8 ml of urine using MagReSyn® SAX with protein extraction for 15 min in optimized buffers. Eluted digested peptides were analysed by MS. The graph above shows comparative identifications for the methods with Peptide ID's relative to 6688, and Protein ID's relative to 1027.



Binding of bacterial plasmid DNA (or RNA) was used to estimate capacity of magnetic SAX. Agarose gel (**A**) relates to the binding of genetic material without the addition of RNAse showing a clear preference for the binding of RNA. Gel (**B**) illustrates samples treated with RNAse and capture of plasmid (increasing concentration with standard bead quantity), and gel (**C**) indicates relative quantity of plasmid left in the sample (confirmation of extraction) from (**B**). The DNA quality was further analysed by UV-VIS spectroscopy. The estimated capacity beads was 15 $\mu g.mg^{-1}$ for plasmid DNA and 95 $\mu g.mg^{-1}$ for RNA. The higher capacity for the RNA was attributed to possible molecular sieving of the polymer microparticles, allowing the smaller RNA to penetrate the beads. We intend to apply these techniques for the isolation of genetic material from urine with automation of processes where possible.

The purity and quality of the plasmid DNA was assessed using UV-VIS spectroscopy ratios of 260/280nm and 260/230nm. The results indicated highly pure plasmid was being prepared from the crude *E. coli* lysate.

	260/280nm	260/230nm
Experiment 1	1.99	2.49
Experiment 2	2.16	2.45
Experiment 3	2.09	2.45
MagReSyn® SAX (average)	**2.08**	**2.46**

CONCLUSIONS & FUTURE WORK

- This work aims to improve urinary proteome profiling by increasing throughput and introducing automated sample processing since traditional workflows are not suitable for large sample cohorts and are labour intensive
- The magnetic HILIC workflow resulted in higher coverage than FASP and the Norgen sample collection kit, but still requires manual processing (precipitation) prior to clean-up to concentrate the sample and remove interfering salts.
- The HILIC and Norgen Kit workflows appeared to show no bias for selection of peptides when compared to FASP
- Magnetic SAX is preferred since it allows for high-throughput automation of the sample processing and removes the necessity for precipitation, with data comparing favourably to samples extracted with the Norgen Kit
- Initial evaluation of magnetic SAX for DNA and RNA isolation shows promise for using this bead for urinary genomic studies, but currently requires a precipitation step after capturing of the DNA or RNA
- We anticipate that this **precipitation may be performed on-bead** (similar to PAC or SP3, Batth *et al*., 2019 and Hughes *et al*., 2019) and intend to evaluate this for the full automation of DNA and RNA isolation and clean-up from urine

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