PROTOCOL NAME: Preparation and HILIC SPE clean-up of urinary proteins for MS analysis

PROTOCOL ID: urine-HILIC

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INTRODUCTION:

This protocol describes the efficient sample preparation of urine samples with clean-up using MagReSyn® HILIC for bottom-up proteomics.

Please contact info@resynbio.com if you have any queries relating to this protocol.

MATERIALS: All reagents and chemicals should be of analytical grade or better, and preferably MS grade.

Reagent or Equipment	Description and catalogue number
MagReSyn® HILIC	MR-HLC002 (https://resynbio.com/hilic-order/)
Acetonitrile (MeCN)	Pierce™ 51101
Ammonium acetate (NH_4Ac)	Fluka A1542-500g
Ammonium bicarbonate (NH_4HCO_3)	Fluka 40867-50g
Dithiothreitol (DTT)	VWR 0281-25G
Iodoacetamide (IAA)	MERCK I1149-25G
Protein LoBind tubes	Eppendorf 0030108450
Magnetic bead handling station for automated protocol	KingFisher Duo or Flex
Magnetic separator for manual protocol	DynaMag™-2 Magnet Catalog number: 12321D
MS grade water	Pierce™ 51140
Peptide quantification method	Pierce™ Colorimetric (23275) or Fluorometric (23290) Peptide Assay
Adjustable Pipettes	
Sodium dodecyl sµlphate (SDS)	MERCK L4509-1Kg
Urea	MERCK U5378-1Kg

Note: chemicals suggested above have been tested for use with this protocol however chemicals of equivalent grade may be used.

REAGENT PREPARATION:

- DTT Stock: 1M DTT in LCMS grade H_2O (prepare fresh)
- IAA Stock: 1M IAA in LCMS grade H_2O (prepare fresh, light sensitive)
- Urea sample buffer: 8M urea, 2% SDS (prepare fresh)
- Equilibration Buffer: 15% ACN, 100 mM NH_4Ac pH 4.5 (diluted from 1M NH_4Ac stock)
- Binding Buffer: 30% ACN, 100 mM NH_4Ac pH 4.5
- Wash Buffer: 95% Acetonitrile in LCMS grade H_2O
- Trypsin Digestion Buffer: 50mM NH_4HCO_3 LCMS grade H_2O

NOTE: *Buffers can be stored at 4°C for up to 2 weeks.*

METHOD:

NOTES:

- The method has been developed and benchmarked using a Kingfisher™ magnetic handling station. If you do not have access to a magnetic handling station, you may follow the protocol detailed below (manual format).
- It is advisable to freshly prepare all buffers.
- Urine samples should be collected using a standard collection procedure. It is advisable to collect first-morning, clean-catch urine samples for clinical analysis. Samples should be kept on ice until processing by centrifugation to remove debris (800 x g, 10 min). Aliquots must be stored at -80 °C if not being used immediately.

URINE PREPARATION

1. Thaw urine sample on ice
2. Transfer 100 µl urine to a fresh 2ml LoBind® Eppendorf tube.
3. Add 300 µl urea sample buffer and vortex mix for 10 -15 sec.
4. Reduce proteins using a final concentration of 10 mM DTT for 30 min at room temperature.
5. Alkylate proteins using a final concentration of 30 mM IAA for 30 min in the dark at room temperature.
6. Quench IAA by adding a further 10 mM DTT

MICROPARTICLE EQUILIBRATION:

7. Re-suspend MagReSyn® HILIC thoroughly by vortex mixing or inversion to ensure a homogenous suspension. **NOTE**: *When multiple samples are being prepared, ensure that you maintain a homogeneous suspension by mixing regularly, for example by inversion or pipetting the micro particle mixture up/down before transferring the required volume.*
8. Transfer 10 µl MagReSyn® HILIC (200 µg) microparticles to 2 ml Protein Lo-Bind tube.
9. Place the tube on a magnetic separator and allow 5-10 sec for the microparticles to clear.
10. Remove the shipping solution by aspiration with a pipette and discard.
11. Wash the microparticles by resuspending in 200 µl of ***Equilibration Buffer*** (refer above) with agitation (e.g. gentle vortex mixing) for 15-30 sec.
12. Place the tube on the magnetic separator and allow the microparticles to clear.
13. Remove the equilibration solution by aspiration with a pipette and discard.
14. Repeat steps **12 – 14**.

    NOTE: *only remove the 2nd equilibration solution from the microparticles once the sample is ready to be added (see step 15. below). This will ensure that microparticles do not air dry and can be easily resuspended when the sample is added.*

SAMPLE BINDING AND WASHING:

15. Mix (from step 6) reduced and alkylated sample with (416 µl) Binding Buffer (30% ACN, 100 mM NH4Ac pH 4.5) in 1:1 (v/v) ratio.
16. Transfer the urea sample buffer – bind buffer mix to the equilibrated microparticles from step 14. **NOTE**: *if automating the workflow refer to the volumetric parameters of your instrument to ensure compatibility of your clean-up.*
17. Incubate for 30 min at room temperature with continuous mixing (e.g. slow vortexing) to ensure adequate sample and microparticle interaction.
18. Place the tube on the magnetic separator and allow the microparticles to clear. Remove and discard the unbound fraction by aspiration with a pipette.
19. Wash the microparticles with 200 µl of ***Wash Buffer*** and mix for 60 sec with gentle agitation.

    OPTIONAL: the microparticle mixture can be transferred to a new 2ml Protein Lo-Bind tube to ensure contaminants left on the tube side walls are not re-introduced in the sample during subsequent digest steps
20. Place the tube on a magnetic separator and allow 5-10 sec for the microparticles to clear. Remove the supernatant and discard.
21. Repeat the wash steps described in STEP **19** and **20**.

ON-BEAD PROTEIN DIGESTION:

22. Perform on-bead digestion by adding 200 µl of 50 mM Ammonium Bicarbonate pH 8.0 containing 1 µg sequencing grade Trypsin (1:30 enzyme:protein ratio) and incubate for 2 hrs at 47 °C. Ensure sufficient mixing to keep the particles in solution during digestion.
    OPTIONAL: 0.25 µg sequencing grade LysC can also be added to the digest buffer to improve digestion.
23. Place the tube on a magnetic separator and allow 5-10 sec for the microparticles to clear.
24. Remove peptide solution and place in a 0.5 ml Eppendorf LoBind tube.
    OPTIONAL: To improve peptide recovery, it is possible to incubate the microparticles with 50 to 100 µl of 1% TFA for 5 minutes whilst mixing and subsequently to pool the supernatant with digest solution from step 29.
25. Vacuum or freeze-dry the samples and re-suspend in 20-40 µl LCMS solvent e.g. 2% ACN with 0.2% formic acid
26. Perform a colorimetric Peptide Assay to determine peptide recovery and adjust the loads for LC-MSMS analysis.
    OPTIONAL: the efficiency of SDS removal by the HILIC method can be evaluated using a colorimetric assay that can measure SDS concertation in the presence of peptides such as the one described in Arand, Friedberg and Oesch, 1992.
27. Analyse digest by LC-MSMS.
    ***NOTE**: if utilising a nano LCMS set-up without a trap-elute option it is recommended to further desalt the digest using standard C18 desalting workflows*