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

PROTOCOL ID: urine-HILIC

DATE LAST MODIFIED: 17 April 2024

INTRODUCTION: This protocol describes the efficient preparation of urine samples with clean-up using MagReSyn® HILIC for bottom-up proteomics analysis. Please contact info@resynbio.com if you have any queries relating to this protocol.

MATERIALS: All reagents and chemicals should be analytical grade or better, and preferably MS grade. The chemicals suggested below have been tested for use with this protocol; however, chemicals of equivalent grade may be used.

Reagent or Equipment	Description and catalogue number
MagReSyn® HILIC	MR-HLC002
Acetonitrile (ACN)	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
Tube mixer	Intelli-Mixer™, ThermoMixer®. A nutator or vortex are suitable alternatives, mixing should be gentle, but sufficient to keep the beads in suspension.
Magnetic bead handling station	KingFisher™ Duo, Prime, Flex or Apex for automated protocol
Magnetic separator for manual protocol	e.g. DynaMag™-2 Magnet Catalog number: 12321D (for higher throughput contact us for assessing your magnetic separation requirements)
MS grade water	Pierce™ 51140
Peptide quantification method	Pierce™ Colorimetric (23275) or Fluorometric (23290) Peptide Assay
Adjustable Pipettes	Any
Sodium dodecyl sulphate (SDS)	Merck L4509-1Kg
Urea	Merck U5378-1Kg

REAGENT PREPARATION:

- DTT Stock: 1M DTT in LCMS grade H_2O (prepare fresh)
  - 154.5 mg DTT in 1 ml LCMS grade H_2O
- IAA Stock: 1M IAA in LCMS grade H_2O (prepare fresh, light sensitive)
  - 184.9 mg IAA in 1 ml LCMS grade H_2O
- Sample solubilization buffer: 8M urea, 2% SDS (prepare fresh)
  - 10% SDS stock solution
    - 10g SDS in 80 ml LCMS grade H_2O and thereafter make up to 100 ml with LCMS grade H_2O.
  - 4.80 g of Urea in 2 ml of 10% SDS stock solution. Add a further 4 ml LCMS grade H_2O to dissolve urea **completely,** thereafter, make up to 10 ml using LCMS grade H_2O.
- HILIC Binding Buffer: 30% ACN, 200 mM NH_4Ac pH 4.5 (diluted from 1M NH_4Ac stock)
  - 1M NH_4Ac Stock
    - 3.85 g ammonium acetate in 30 ml LCMS grade H_2O.
    - pH adjust to 4.5 using acetic acid
    - make up to 50 ml final volume using LCMS grade H_2O.
  - Mix 5 ml of 1M NH_4Ac stock, 15 ml of 100% ACN and 30 ml of LCMS grade H_2O.
- HILIC Equilibration Buffer: 15% ACN, 100 mM NH_4Ac pH 4.5
  - dilute HILIC Binding Buffer 1:1 with LCMS grade H_2O.
- HILIC Wash Buffer: 95% ACN in LCMS grade H_2O.
  - 9.5 ml 100% ACN added to 500 µl LCMS grade H_2O.
- Trypsin Digestion Buffer: 50mM NH_4HCO_3 LCMS grade H_2O.
  - 39.53 mg ammonium bicarbonate dissolved in 10 ml LCMS grade H_2O.
- Trypsin stock (1 µg/200 µL), store at -20 °C
  - Dissolve 20 µg trypsin (Pierce Sequencing Grade Modified) in 4 ml trypsin digestion buffer.

METHOD:

IMPORTANT NOTES:

- The method has been developed and benchmarked using KingFisher™ magnetic handling stations (methods available upon request). If you do not have access to a magnetic handling station, you may follow the protocol detailed below (manual format).
- It is recommended to freshly prepare all buffers, but they may be stored at 4°C for up to 2 weeks.
- 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 processed by centrifugation (800 x g, 10 min) to remove debris. 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 sample solubilization 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 (add 4 µl of 1M DTT stock).
5. Alkylate proteins using a final concentration of 30 mM IAA for 30 min in the dark at room temperature (add 12 µl of 1M IAA stock).
6. Quench IAA by adding a further 10 mM DTT (4 µl of 1M DTT stock)

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 microparticle mixture up/down before transferring the required volume.*
8. Transfer 10 µl MagReSyn® HILIC (200 µg) microparticles to a 2 ml Protein LoBind® 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 HILIC Equilibration Buffer 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 **11-13**
    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 the microparticles do not dry out and can be easily resuspended when the sample is added.*

SAMPLE BINDING AND WASHING:

15. Mix the reduced and alkylated sample (from step 6) with (420 µl) HILIC Binding Buffer in 1:1 (v/v) ratio.
16. Transfer the sample and HILIC bind buffer mix (from step 15) 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 HILIC Wash Buffer and mix for 60 sec with gentle agitation.
    OPTIONAL: the microparticle mixture can be transferred to a new 2ml Protein LoBind® 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 Trypsin Stock (1 µg/200 µl, 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.
    NOTE: ~30 µg total protein is estimated to be in 100 µl urine from a pooled sample of healthy donors. Based on quantification using QuantiChrom™ Total Protein Assay. Urinary protein concentration is highly sample specific.
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 Protein LoBind® tube.
    OPTIONAL: To improve peptide recovery, incubate microparticles with 50 µl of 1% TFA for 5 minutes while mixing and subsequently combining the supernatant with digest solution from step 24.
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 loading for LCMS 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, e.g. Arand, Friedberg and Oesch, 1992 (DOI: 10.1016/0003-2697(92)90502-x).
27. Analyse digest by LC-MSMS.
    ***NOTE**: if utilising a nano-LCMS setup without a trap-elute option, it is recommended to further desalt the digest using a standard C18 desalting workflow before analysis.*

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