PROTOCOL NAME: Clean-up of acetone precipitated proteins from urine for mass spectrometry analysis using magnetic HILIC SPE

PROTOCOL ID: HILIC-RAPOBD_URINE_2

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ReSyn Biosciences®

INTRODUCTION:

Acetone precipitation of proteins in urine can be used for efficient removal of salts and other contaminants, and the normalization of protein quantity allows for more reproducible downstream processing. Solubilization of acetone precipitated proteins using SDS allows for almost complete re-solubilization of proteins. However, the SDS must be completely removed prior to LC MS analysis using the MagReSyn® HILIC workflow described here

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

- MagReSyn® HILIC – Catalogue Number MR-HLC002
- Eppendorf® LoBind microcentrifuge tubes, 0.5, 1.5 and 2 ml.
- Magnetic Separator or Magnetic bead handling station (e.g. KingFisher™)
- Pipettes
- Sodium dodecyl sµlphate (SDS)
- Iodacetamide (IAA)
- Dithiothreitol (DTT)
- Acetonitrile (MeCN)
- Ammonium acetate (NH_4Ac)
- Ammonium bicarbonate ($NH_4Bicarb$)
- Peptide quantification kit (e.g. Pierce™ Colorimetric or Fluorometric Peptide Assay)
- MS grade water
- LC grade acetone

REAGENT PREPARATION:

- Ice-cold acetone (-20 °C)
- DTT Stock: 1M DTT (prepare fresh)
- IAA Stock: 1M IAA (prepare fresh, light sensitive)
- Protein resuspension buffer: 2% SDS
- 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
- Digestion Buffer: 25 mM $NH_4Bicarb$, pH 8.0

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

METHOD:

NOTES*:*

- *The current methods are suitable for manual preparation or may be fully automated on magnetic bead handling systems such as KingFisher™ or similar. Protocols are available on request.*
- *The current protocol is sufficient for the clean-up of 30 µg of urine sample and can be scaled up or down by keeping a protein to bead ratio of 1:10.*
- *Urine contains contaminates that interfere with most standard protein quantification approaches. A suitable kit for protein quantification is considered the QuantiChrom™ Total Protein Assay Kit, by BioAssay Systems. Alternatively total protein content can be quantified after precipitation by acetone with a standard BCA assay kit that is compatible with SDS. However, our preferred approach is to estimate the quantity of MagReSyn® HILIC based on the average protein content of urine from a 'healthy' sample (~100 µg.ml^{-1}).*

URINE PREPARATION:

1. Centrifuge urine sample at 2 000 x g for 10 min at 4 °C to remove particulates
2. Prepare 1-2ml aliquots and store at -80 °C

EXTRACTION AND SOLUBILIZTOIN OF URINARY PROTEINS:

3. Thaw the sample of urine slowly on ice
4. Transfer 300 µl urine to a 2ml Eppendorf® LoBind tube.
5. Add 1.7 ml ice-cold acetone and incubate at -20 °C for 1 h
6. Centrifuge samples to pellet precipitated urine at 12 000 x g for 1 h at 4 °C
7. Gently decant acetone or carefully aspirate with a pipette.
   NOTE. *Ensure that the residual acetone on the tube is completely removed before proceeding to the next step. The tube can be left open to air dry, or be placed in a dry-bath at 70 °C for 30 - 60 seconds. We recommend that you take care not to dry the pellet as this can result in poor sample re-solubilisation, reducing protein recovery.*
8. To the semi dry pellet add 100 µl 2% SDS. Vortex vigorously to ensure pellets are resuspended.
9. Sonicate using an ultra sonicator bath for 5 min
10. Chemically reduce the proteins using a final concentration of 10 mM DTT for 15 min at 70 °C followed by 15 min at 40 °C.
11. Cool the samples to RT, and alkylate proteins using a final concentration of 30 mM IAA for 30 min (incubated in the dark)
12. Quench the residual IAA by adding a further 10 mM DTT
13. Your sample is now ready for on-bead protein capture, clean-up and digestion.

PROTEIN CAPTURE, CLEAN-UP AND ON-BEAD DIGESTION:

MICROPARTICLE EQUILIBRATION:

14. Resuspend 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.*
15. Transfer 15 µl MagReSyn® HILIC (300 µg) microparticles to 2 ml Protein Lo-Bind tube.
    NOTE: *We estimate that 15 µl MagReSyn® HILIC (300 µg) is sufficient to capture proteins from 300 µl urine*.
16. Place the tube on a magnetic separator and allow 5-10 sec for the microparticles to clear.
17. Remove the shipping solution by aspiration with a pipette and discard.
18. Wash the microparticles by resuspending in 200 µl of ***Equilibration Buffer*** (refer materials) with agitation (e.g. gentle vortex mixing) for 15-30 sec.
19. Place the tube on the magnetic separator and allow the microparticles to clear.
20. Remove the equilibration solution by aspiration with a pipette and discard.
21. Repeat steps **11 – 13**.
    NOTE: *only remove the 2nd equilibration solution from the microparticles once the sample of urine is ready to be added. This will ensure that microparticles do not dry and can be readily resuspended in the sample.*

PROTEIN CAPTURE AND WASHING**:**

22. Mix reduced and alkylated sample (from step 11) with 100 µl ***Binding Buffer*** in 1:1 (v/v) ratio.
    NOTE: *in order to ensure efficient microparticle mixing and protein binding the total volume (sample plus binding buffer) should be in the range of 100-200 µl.*
23. Transfer this solution to the equilibrated microparticles from step 19.
    NOTE: *if automating your workflow please refer to the volumetric parameters of your instrument to ensure compatibility of your clean-up protocol, and adjust as necessary to fall within the parameters. We recommend a minimum volume of 5 µl of HILIC beads are used to ensure good bead recovery for both manual and automated sample preparation.*
24. Incubate for 30 min at room temperature with continuous mixing (e.g. slow vortexing) to ensure adequate sample and microparticle interaction.
25. Place the tube on a magnetic separator and allow the microparticles to clear. Remove and discard the unbound fraction by aspiration with a pipette.
26. 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 potential contaminants bound on the tube walls are not re-introduced in the sample during subsequent digestion steps.*
27. Place the tube on a magnetic separator and allow 5-10 sec for the microparticles to clear. Remove the supernatant and discard.
28. Repeat the wash steps described in **24** and **25**.

ON-BEAD PROTEIN DIGESTION:

29. Perform on-bead digestion by adding 200 µl of ***Digestion Buffer*** containing 0.3 µg sequencing grade LysC (1:100 enzyme:protein ratio) and 1.5 µg sequencing grade Trypsin (1:30 enzyme:protein ratio) and incubate for 4 hrs at 50 °C. Ensure sufficient mixing to keep the particles in suspension during digestion.
30. Place the tube on a magnetic separator and allow 5-10 sec for the microparticles to clear.
31. Remove peptide solution and place in a 0.5 ml Eppendorf LoBind tube.
    OPTIONAL*: To improve peptide recovery use optional elution with 50 to 100 µl of 1% TFA for 5 minutes (with adequate mixing) and subsequently pool the supernatant with digest solution from step **29**.*
32. Vacuum or freeze-dry the samples and resuspend in 20 to 40 µl LCMS solvent e.g. 2% ACN with 0.2% formic acid
33. 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 concentration in the presence of peptides such as the one described in Arand, Friedberg and Oesch, 1992.*
34. Analyse the digested sample by LC-MSMS.
    NOTE*: if utilising a nano LCMS set-up without a trap-elute option, we recommended to desalt the digest using a standard C18 desalting workflow*