PROTOCOL NAME: Preparation and HILIC SPE clean-up of SDS extracted plasma proteins for MS analysis

PROTOCOL ID: HILIC-PLASMA

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INTRODUCTION:

The high concentration of proteins in plasma can be efficiently extracted and solubilized using SDS. However, the SDS has to be completely removed prior to LC MS analysis. This protocol describes the efficient sample preparation of plasma samples with clean-up of SDS using MagReSyn® HILIC. 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 sulphate (SDS)
- Trizma base (Tris)
- Iodacetamide (IAA)
- Dithiothreitol (DTT)
- Acetonitrile (ACN)
- Ammonium acetate (NH_4Ac)
- Ammonium bicarbonate (NH_4Bicarb)
- Protein quantification method (e.g. 2D Quant Kit or BCA Assay)
- MS grade water

REAGENT PREPARATION:

- DTT Stock: 1M DTT (prepare fresh)
- IAA Stock: 1M IAA (prepare fresh, light sensitive)
- Plasma dilution buffer: 2% SDS in 50mM Tris-HCl pH 8
- Equilibration Buffer: 15% ACN, 100 mM NH4Ac pH 4.5 (diluted from 1M NH_4Ac stock)
- Binding Buffer: 30% ACN, 100 mM NH4Ac pH 4.5
- Wash Buffer: 95% Acetonitrile
- Trypsin Digestion Buffer: 25 mM NH_4Bicarb

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 20 µg of Plasma sample, but can be scaled up or down by keeping a protein to bead ratio of 1:10.*
- ***OPTIONAL**: Perform abundant protein depletion or SAX-based protein fractionation prior the HILIC SPE clean-up in order to increase proteome coverage.*

PLASMA LYSIS & SOLUBILIZATION:

1. Dilute 5-20 µl plasma 25-fold with 2% SDS in 50 mM Tris-HCl pH 8
   NOTE*: abundant protein depletion and/or SAX based protein fractionation was performed do not dilute the sample further but proceed from step 2. below*
2. Quantify diluted plasma using suitable quantification technique (ensure method is not sensitive to SDS such as the Pierce™ BCA Protein Assay Kit from ThermoFisher Scientific or the 2-D Quant Kit from GE Healthcare)
3. Reduce proteins using a final concentration of 10 mM DTT for 30 min at 60°C
4. Alkylate proteins using a final concentration of 30 mM IAA for 30 min in the dark
5. Quench IAA by adding a further 10 mM DTT
6. Transfer 20 µg plasma sample to a new eppendorf tube
   Note: *if the sample volume is less than 50 µl additional Plasma dilution buffer (2% SDS in 50mM Tris-HLC pH 8) should be added.*

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 re-suspending 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 **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 microparticles do not air dry and can be easily resuspended when the sample is added.*

SAMPLE BINDING AND WASHING:

15. Mix 20 µg (50 µl) of reduced and alkylated plasma sample from step 6. With 50 µl Binding Buffer (30% ACN, 100 mM NH4Ac pH 4.5) in 1:1 (v/v) ratio
    NOTE: *in order to ensure efficient microparticle mixing and protein binding the total volume (sample plus bind buffer) should be in the range of 100-200 µl.*
16. Add the plasma – 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. 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.*
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 steps **19** and **20**.

ON-BEAD PROTEIN DIGESTION:

22. Perform on-bead digestion by adding 200 µl of 25 mM Ammonium Bicarbonate pH 8.0 containing 0.2 µg sequencing grade LysC (1:100 enzyme:protein ratio) and 1 µg sequencing grade Trypsin (1:20 enzyme:protein ratio) and incubate for 2 hrs at 47 °C. Ensure sufficient mixing to keep the particles in solution during 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 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 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*

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