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)
- 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: 50 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*

PLASMA LYSIS & SOLUBILIZATION:

1. Dilute plasma 10-fold with 2% SDS in 50 mM $NH_4Bicarb$
2. Quantify diluted plasma using suitable quantification technique (ensure method is not sensitive to SDS such as 2-D Quant Kit from GE Healthcare)
3. Reduce proteins using 10 mM DTT for 30 min at 37°C (diluted into sample from stock solution)
4. Alkylate proteins using 30 mM IAA for 30 min in the dark (diluted into sample from stock solution)

5. Quench IAA by adding a further 10 mM DTT (diluted into sample from stock solution)
6. Dilute your sample containing 20 µg of protein to a minimum of 50 µl if required using MS grade water

MICROPARTICLE EQUILIBRATION:

1. 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.*
2. Transfer 10 µl MagReSyn® HILIC (200 µg) microparticles to 2 ml Protein Lo-Bind tube.
3. Place the tube on a magnetic separator and allow 5-10 sec for the microparticles to clear.
4. Remove the shipping solution by aspiration with a pipette and discard.
5. 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.
6. Place the tube on the magnetic separator and allow the microparticles to clear.
7. Remove the equilibration solution by aspiration with a pipette and discard.
8. Repeat steps **3 – 7**.
9. Resuspend beads in 50 µl of ***Binding Buffer***

SAMPLE BINDING AND WASHING:

10. Add 50 µl of reduced and alkylated plasma sample to the bead suspension. **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.*
11. Incubate for 30 min at room temperature with continuous mixing (e.g. slow vortexing) to ensure adequate sample and microparticle interaction.
12. Place the tube on the magnetic separator and allow the microparticles to clear. Remove and discard the unbound fraction by aspiration with a pipette.
13. Wash the beads with 200 µl of ***Wash Buffer*** and mix for 60 sec with gentle agitation.
14. Place the tube on a magnetic separator and allow 5-10 sec for the microparticles to clear. Remove the supernatant and discard.
15. Repeat steps **13** and **14**.

ON-BEAD PROTEIN DIGESTION:

16. Perform on-bead digestion by adding in 20 µl of 50 mM Ammonium Bicarbonate pH 8.0 containing 2 ug sequencing grade Trypsin (1:10 enzyme:protein ratio) for 4 hrs at 37 °C. Ensure sufficient mixing to keep the particles in solution during digestion to ensure good particle liquid interaction.
17. Place the tube on a magnetic separator and allow 5-10 sec for the microparticles to clear.
18. Remove peptide solution and place in a 0.5 ml Eppendorf LoBind tube.
19. *OPTIONAL: Elute peptides with 20 to 100 µl of 1% TFA for 5 minutes to improve recovery. **NOTE**: This step should be evaluated first to determine whether this results in an increase in sample coverage, and the possible elution of contaminants under these acidic conditions such as SDS. For example, SDS can be quantified using the method of Arand, Friedberg and Oesch, 1992.*
20. *Place the tube on a magnetic separator and allow 5-10 sec for the microparticles to clear.*
21. *Remove supernatant and combine with eluate from **18**.*
22. Analyse supernatant by LC-MSMS

NOTES:

- *Samples can be vacuum or freeze-dried to reduce the volume of the sample from frozen at low temperature.*
- *Sample can be desalted with C18 SPE or on-line with a C18 trap cartridge used in a typical pre-concentration LCMS set-up*