PROTOCOL NAME: HILIC Clean-Up of Proteins with On-Bead Reduction, Alkylation & Digestion

IMPORTANT NOTE: This protocol is under development. Due to improved reduction and alkylation efficiency RAPOBD is currently considered the preferred protocol.

PROTOCOL ID: HILIC_POBRAD_4

DATE LAST MODIFIED: 28 May 2018

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

- Eppendorf LoBind microcentrifuge tubes
- Benzonaze® (Merck)
- Magnetic Separator or Magnetic bead handling station (e.g. KingFisher™)
- Pipettes
- Dithiothreitol
- Iodoacetamide
- Ammonium acetate (NH_4Ac)
- Acetonitrile (ACN)
- Ammonium formate (MS Grade)
- Trypsin (Sequencing grade)
- Formic Acid
- BCA Assay (or suitable protein quantification method)
- MS Grade Water

REAGENT PREPARATION:

- Cell Lysis buffer: e.g. 20 mM Tris-HCl pH 8.0 with 1% SDS (refer application note, ASMS 2017 and HUPO 2017 poster for compatible detergents/lysis reagent additives)
- 1M DTT stock solution (prepare fresh) – 100 µl
- 1 M IAA stock solution (prepare fresh) – 100 µl
- HILIC equilibration buffer: 15% Acetonitrile (ACN) in 100 mM Ammonium acetate (NH_4Ac) pH 4.5 (400 mM NH_4Ac stock used for equilibration and binding buffer)
- 2 X HILIC DTT Binding Buffer: 20mM DTT, 30% ACN in 200 mM NH_4Ac pH 4.5
- Alkylation Buffer: 40mM IAA, 15% ACN in in 100 mM NH_4Ac pH 4.5
- HILIC Wash Buffer: 95% ACN
- Digestion Buffer: 50mM Ammonium formate pH 8.2

PROTEIN EXTRACTION:

1. Pellet mammalian cells at 200-500 g for 10 min or bacteria at 3000-5000 g for 10 min
2. Resuspend cells in lysis buffer (volume may vary according to pellet size), ~200 µl for pellet of 0.5 million cells
3. Sonicate cells on ice, 9 pulses (~10 sec per pulse)
4. Incubate cell lysate with 25 units, (1 µl of stock – 2500 units in 100 µl) of Benzonase®, per 0.5 million cells at 37°C for 30 min
5. Centrifuge at 15 000 g for 10 min to clear cell debris
6. Repeat step 5 if buffy layer still evident

7. Collect supernatant and determine concentration of proteins using BCA assay (or alternate suitable protein assay)
8. Dilute protein solution if necessary (~1mg.ml^{-1} stock suitable for HILIC protocol)
9. Perform sample clean-up using HILIC protocol.

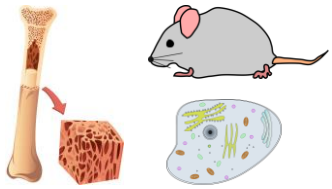
HILIC CLEAN-UP, ON-BEAD REDUCTION, ALKYLATION & DIGESTION:

1. Resuspend MagReSyn® HILIC (20 mg.ml^{-1} formulation) thoroughly by vortex mixing for 3 seconds to ensure a homogenous suspension.
2. Transfer 25 µl (500 µg) MagReSyn® to a new 2ml microcentrifuge tube. A ratio of 10:1 beads to protein is recommended, i.e. 500 µg (25 µl) beads per 50 µg of protein.
3. Place the tube on the magnetic separator and allow the microparticles to clear.
4. Remove the shipping solution by aspiration with a pipette and discard.
5. Wash and equilibrate the microparticles in 250 µl equilibration buffer (15% ACN, 100 mM ammonium acetate pH 4.5), with gentle agitation for 20-30 sec
6. Place the tube on the magnetic separator and allow the microparticles to clear. Remove the wash buffer by aspiration with a pipette and discard.
7. Repeat steps 5 and 6.
8. After removal of the binding buffer, MagReSyn® HILIC is ready for the binding of protein samples.
9. Mix the protein sample containing 50 µg total protein, with and equal volume of reducing binding buffer (20mM DTT, 30% ACN in 200 mM NH_4Ac pH 4.5). We recommend a minimum volume of 25 µlof protein solution, i.e. final binding volume of 50 µl. (***NOTE****: For automated protocols we recommend a minimum final volume of 100* µl)
10. Add this mixture to the pre-equilibrated MagReSyn® HILIC and mix thoroughly by pipette mixing.
11. Allow proteins to bind to microparticles for 30 min. Mix gently and continuously to ensure good sample microparticle interaction during the binding procedure. Excessive mixing can results in HILIC microparticles drying on tube side walls leading to poor protein recovery.
12. Place the tube on the magnetic separator and allow the microparticles to clear. Remove the supernatant by pipette aspiration (***NOTE:*** *Supernatant may be stored and run on a gel to determine whether all protein bound to the beads*).
13. Resuspend the beads in 50 µl alkylation buffer (40mM IAA, 15% ACN in 100 mM NH_4Ac pH 4.5), and mix by vortexing for 15 seconds.
14. Allow alkylation to proceed for 30 min. Mix gently and continuously in the dark. Excessive mixing can results in HILIC microparticles drying on tube side walls leading to poor protein recovery.
15. Resuspend the beads in a minimum of 200 µl wash buffer (95% ACN), and mix by gentle vortexing for 1 minute.
16. Recover the microparticles on the magnetic separator. Remove the supernatant by pipette aspiration.
17. Resuspend the beads in 200 µl wash buffer (95% ACN), and mix by gentle vortexing for 1 minute. Transfer microparticle suspension to a new tube to avoid potential carry over of detergent/contaminants from the tube.
18. Recover the microparticles on the magnetic separator. Remove the wash supernatant by pipette aspiration.
19. Resuspend microparticles with adsorbed protein mix in 50 to 100 µl total volume Digestion Buffer, e.g. 50 mM ammonium formate pH 8.0, containing suitable digestion enzyme. In the case of sequencing grade trypsin we recommend a protein to enzyme ratio of 10:1.
20. Incubate sample at suitable temperature and time period as per enzyme specifications. In the case of sequencing grade trypsin we recommend incubation at 37°C for 4 hr. Mix continuously to ensure microparticles remain in suspension. Excessive mixing can results in HILIC microparticles drying on tube side walls leading to poor peptide recovery.
21. Recover the microparticles on the magnetic separator; aspirate the supernatant containing peptides with a pipette.
22. **OPTIONAL**: If not using MS grade ammonium formate, desalt or clean-up using e.g. MagReSyn® HILIC peptide clean-up protocol.
23. **RECOMMENDED**: Transfer the beads to a 0.5 ml Protein LoBind Eppendorf tube and apply to a magnetic separator. This will remove any beads that may have carried over from the manual or automated processing. Application to a magnetic plate separator can similarly be used to remove residual beads.

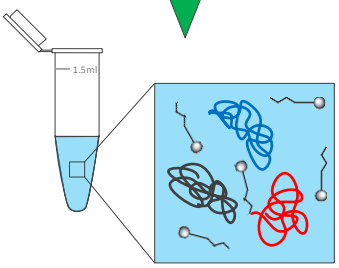
24. **OPTIONAL**: Vacuum dry/lyophilize samples if concentration of peptides is required for low concentration samples.
25. Make peptide solution up to 2% ACN and 0.2% formic acid and proceed to LC-MSMS analysis. If samples have been dried resuspend peptides in 2% acetonitrile with 0.2% formic acid and proceed to LC-MSMS analysis.

Protocol: Protein Clean-up with On-Bead Reduction Alkylation and Digestion (POBRAD)

Protein Preparation



Protein extraction, solubilization & clarification

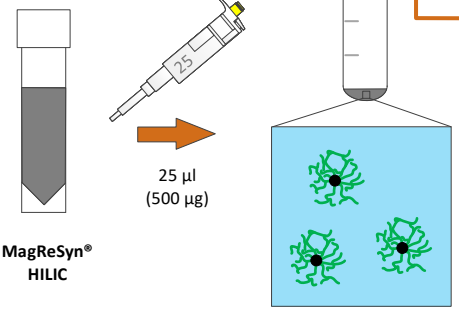


50 µg Protein (X volume)

Equivalent Vol 2X HILIC DTT Binding Buffer (20 mM DTT, 30% ACN, 200 mM NH_4Ac)

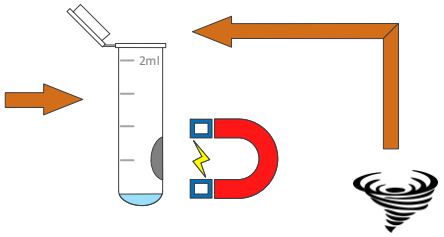
Transfer to equilibrated MagReSyn® HILIC

Bead Preparation



MagReSyn® HILIC

25 µl (500 µg)



Aspirate liquid & discard

Vortex 30 sec

Resuspend in 250 µl Equilibration Buffer (15% ACN in 100 mM NH_4Ac)

Repeat for 2 Equilibrations

HILIC Protein Clean-Up Workflow

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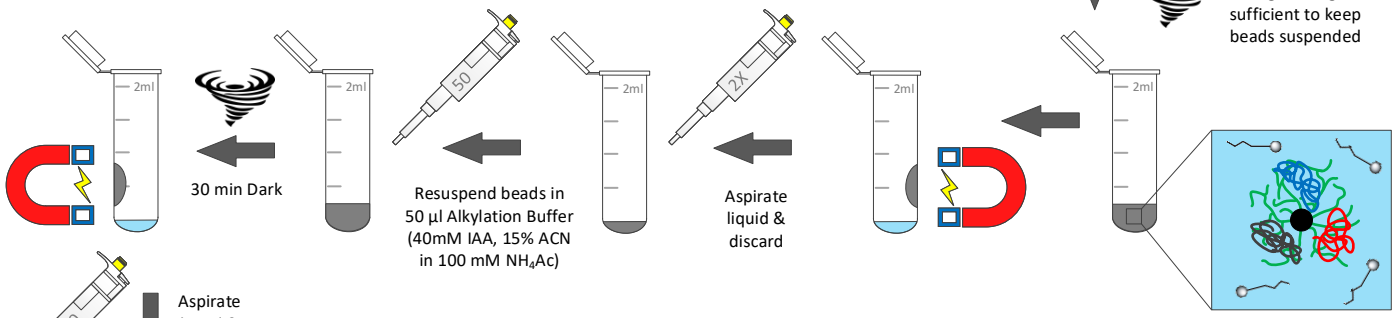


Bind for 30 minutes with gentle agitation, sufficient to keep beads suspended

Aspirate liquid & discard

Resuspend beads in 50 µl Alkylation Buffer (40mM IAA, 15% ACN in 100 mM NH_4Ac)

30 min Dark

Aspirate liquid & discard

Resuspend beads in 200 µl Wash Buffer (95% ACN)

Vortex 1 min

Transfer suspension to a new tube

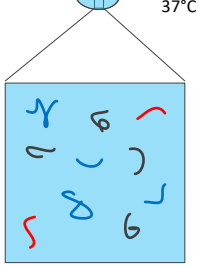
Resuspend beads in 200 µl Wash Buffer (95% ACN)

Vortex 1 min

Aspirate liquid & discard

Resuspend beads in 50 to 100 µl of Digestion Buffer

37°C for 4 Hours

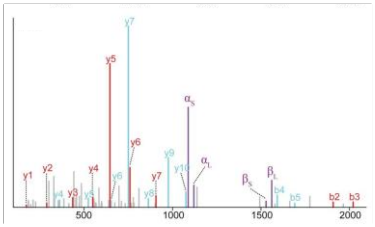


Peptide Solution

Transfer peptides to MS sample vial

NOTE: *Due to the possible inefficiency of magnetic bead handling stations/ separators we recommend sample centrifugation prior to loading for LC-MS/MS.*

LC-MS/MS analysis



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