

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

PROTOCOL ID: HILIC_POBRAD_1

DATE LAST MODIFIED: 3 October 2017

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

- Eppendorf LoBind microcentrifuge tubes
- Benzonase® (Merck)
- Magnetic Separator or Magnetic bead handling station (e.g. KingFisher™)
- Pipettes
- Dithiothreitol
- Iodoacetamide
- Ammonium acetate (NH₄Ac)
- Acetonitrile (ACN)
- Trizma Base *or* Ammonium Bicarbonate
- 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 (NH₄Ac) pH 4.5 (400 mM NH₄Ac stock used for equilibration and binding buffer)
- 2 X HILIC DTT Binding Buffer: 20mM DTT, 30% ACN in 200 mM NH₄Ac pH 4.5
- Alkylation Buffer: 40mM IAA, 15% ACN in 100 mM NH₄Ac pH 4.5
- HILIC Wash Buffer: 95% ACN
- Digestion Buffer: 20mM Tris-HCl *or* 50mM Ammonium Bicarbonate pH 8.0

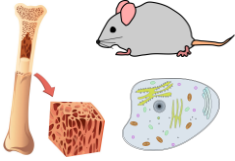
PROTEIN EXTRACTION:

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.

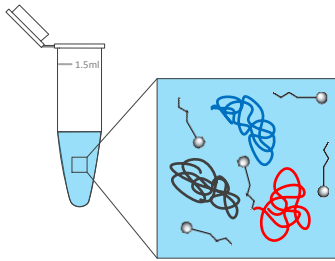
HILIC CLEAN-UP, ON-BEAD REDUCTION, ALKYLATION & DIGESTION:

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 an equal volume of reducing binding buffer (20mM DTT, 30% ACN in 200 mM NH₄Ac pH 4.5). We recommend a minimum volume of 25 µl of 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 result 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 NH₄Ac 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 result 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 bicarbonate 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 result 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:** Desalt or clean-up using e.g. MagReSyn® HILIC peptide protocol.
23. Vacuum dry/lyophilize samples as required.
24. Resuspend peptides in 2% acetonitrile with 0.2% formic acid and proceed to LC-MSMS analysis.

Protein Preparation



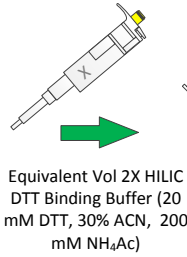
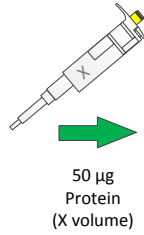
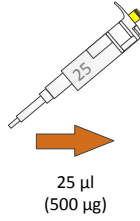
Protein extraction,
solubilization &
clarification



Bead Preparation

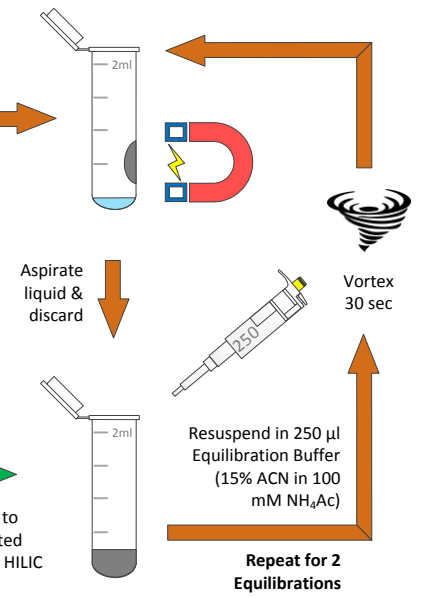


MagReSyn®
HILIC



Transfer to
equilibrated
MagReSyn® HILIC

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



HILIC Protein Clean-Up Workflow

