

PROTOCOL NAME: HILIC Clean-Up of Reduced & Alkylated Proteins with on Bead Digestion

PROTOCOL ID: HILIC_RAPOBD_5

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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 (DTT)
- Iodoacetamide (IAA)
- Ammonium acetate (NH₄Ac)
- Acetonitrile (ACN)
- Ammonium formate (MS Grade)
- Trypsin (Sequencing grade)
- Formic Acid
- Ammonium bicarbonate (AmmBic)
- Tris-HCl
- 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)
- 1 M 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 Binding Buffer: 30% ACN in 200 mM NH₄Ac pH 4.5
- HILIC Wash Buffer: 95% ACN
- Digestion Buffer: 50mM Ammonium formate pH 8.2. or 50mM AmmBic pH 8.0, or 50mM Tris pH 8.0

PROTEIN EXTRACTION, REDUCTION & ALKYLATION:

NOTE: This protocol is only a recommendation and may be adapted for alternate starting. As an example, the protocol may be applied to tissues by increasing the SDS to 4% in the lysis buffer. For more information please contact info@resynbio.com

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 Benzonaze®, 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 protein using BCA assay (or alternate suitable protein assay)
8. Reduce proteins using DTT to a final of 10 mM for 45 min at RT
9. Alkylate proteins using IAA to a final of 40 mM for 45 min in the dark
10. Quench IAA with DTT to a final of 20 mM

11. Dilute protein solution if necessary (~1 mg.ml⁻¹ stock suitable for HILIC protocol)
12. Perform sample clean-up using HILIC protocol.

HILIC CLEAN-UP AND ON-BEAD 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 2 ml 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 2 x binding buffer (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.
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 a minimum of 200 µl wash buffer (95% ACN), and mix by gentle vortexing for 1 min.
14. Recover the microparticles on the magnetic separator. Remove the wash supernatant by pipette aspiration.
15. 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.
16. Recover the microparticles on the magnetic separator. Remove the wash supernatant by pipette aspiration.
17. Resuspend microparticles with adsorbed protein mix in 50 - 100 µl total volume of digestion buffer, e.g. 50 mM ammonium formate pH 8.2, containing suitable digestion enzyme (e.g. Trypsin). In the case of sequencing grade trypsin we recommend a protein to enzyme ratio of 20:1.
18. 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 and gently to ensure microparticles remain in suspension. Excessive mixing can result in HILIC microparticles drying on tube side walls leading to poor peptide recovery.
19. Recover the microparticles on the magnetic separator; aspirate the supernatant containing peptides with a pipette.
20. **OPTIONAL:** If ammonium formate (not MS grade), AmmBic or Tris are used for digestion, desalt or clean-up using e.g. MagReSyn® HILIC peptide clean-up protocol or perform C18 based desalting
21. **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.
22. **OPTIONAL:** Vacuum dry/lyophilize samples if concentration of peptides is required for low concentration samples.
23. 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.

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