PROTOCOL NAME: HILIC Clean-Up of proteins with on-bead reduction, alkylation and digestion

PROTOCOL ID: HILIC_POBRAD_6

DATE LAST MODIFIED: 6 August 2019

IMPORTANT NOTE: This protocol is currently under development and it should be noted that the protocol is less efficient than reduction and alkylation prior to binding of proteins for clean-up.

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
- (2-carboxyethyl)phosphine (TCEP)
- Chloroacetamide (CAA)
- Ammonium acetate (NH_4Ac)
- 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)
- 0.5 M TCEP stock solution (prepare fresh) – 100 µl
- 0.5 M CAA 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 Binding Buffer: 30% ACN, 10mM TCEP, 10mM CAA in 200 mM NH_4Ac 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 material, and/or improved efficiency. 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 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 protein using BCA assay (or alternate suitable protein assay)

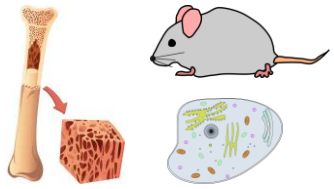
8. Dilute protein solution if necessary (~1 mg.ml^{-1} stock suitable for HILIC protocol)
9. Perform sample clean-up with on-bead reduction and alkylation using HILIC protocol.

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 and equal volume of 2 x binding buffer (30% ACN supplemented with 10mM TCEP and CAA in 200 mM NH_4Ac 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 as well as reduce and alkylate 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.

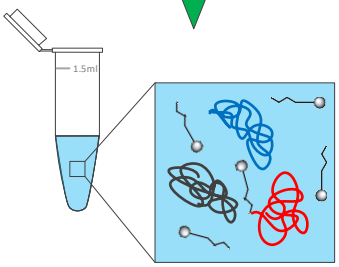
Protocol: Protein Clean-up with On-Bead Reduction Alkylation and Digestion (POBRAD)

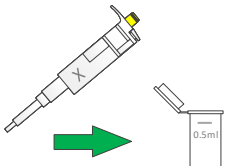
Protein Preparation



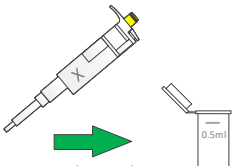
Protein extraction, solubilization & clarification

Reduce & alkylate your protein according to standard protocols





50 µg Protein (X volume)



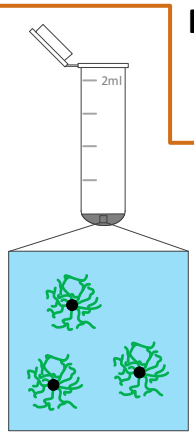
Equivalent Vol 2X HILIC Binding Buffer (30% ACN, 200 mM NH_4Ac, 10mM each TCEP and CAA)

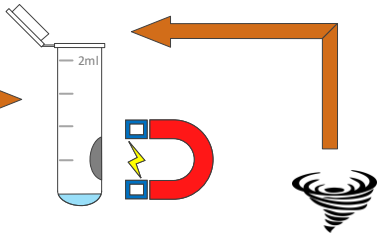
Transfer to equilibrated MagReSyn® HILIC

Bead Preparation

MagReSyn® HILIC

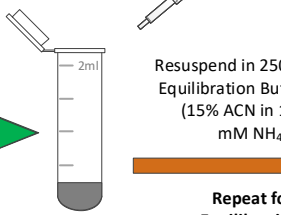
25 µl (500 µg)





Vortex 30 sec

Aspirate liquid & discard

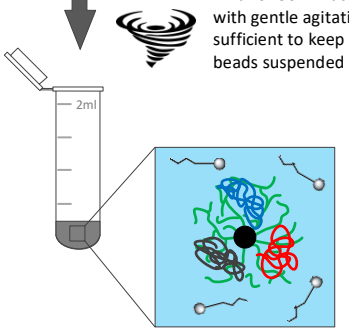


Resuspend in 250 µl Equilibration Buffer (15% ACN in 100 mM NH_4Ac)

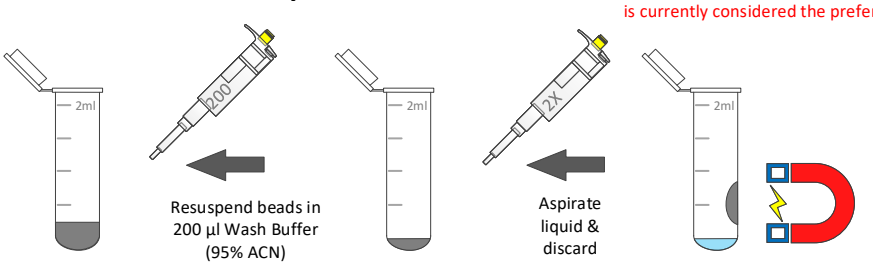
Repeat for 2 Equilibrations

HILIC Protein Clean-Up Workflow

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



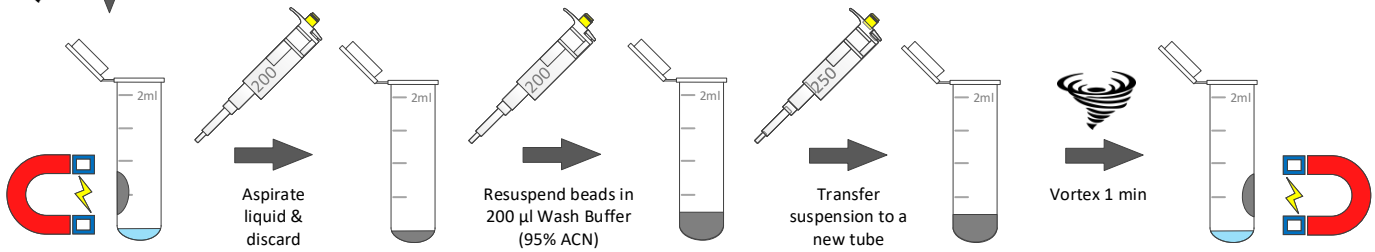
Bind for 30 minutes with gentle agitation, sufficient to keep beads suspended



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

Aspirate liquid & discard

Vortex 1 min

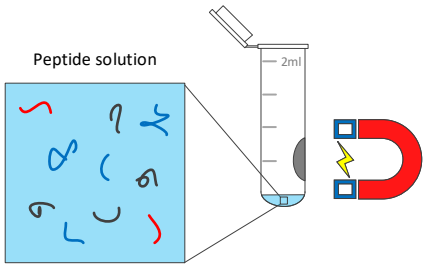


Aspirate liquid & discard

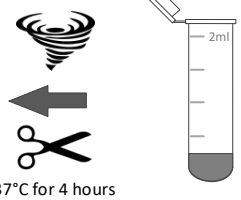
Resuspend beads in 200 µl Wash Buffer (95% ACN)

Transfer suspension to a new tube

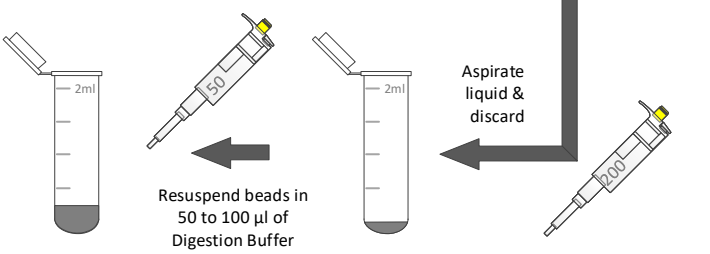
Vortex 1 min



Peptide solution

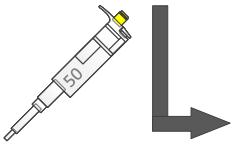


37°C for 4 hours



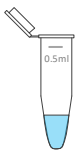
Resuspend beads in 50 to 100 µl of Digestion Buffer

Aspirate liquid & discard

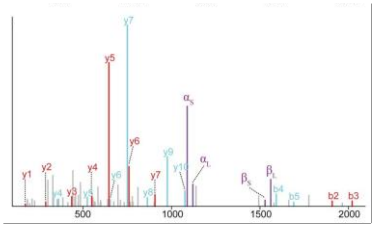


Transfer peptides to MS sample vial

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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