PROTOCOL NAME: HILIC Clean-Up of Peptides Post Protein Digestion

PROTOCOL ID: HILIC_PEPCLU_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
- Magnetic Separator or Magnetic bead handling station (e.g. KingFisher™)
- Pipettes
- Ammonium acetate (NH_4Ac)
- Ammonium bicarbonate (ABC)
- Ammonium formate (AFM)
- Acetonitrile (ACN)
- Formic Acid
- BCA Assay (or suitable protein quantification method)
- MS Grade Water

REAGENT PREPARATION:

- HILIC Equilibration Buffer: 90% Acetonitrile in 20 mM Ammonium Formate pH 2.5 (prepared from 400 mM Ammonium Formate Stock Solution diluted with ACN and water in a ratio of 5:90:5)
- HILIC wash buffer: 95% ACN
- Ammonium Formate Stock Solution: 400 mM, pH 2.5
- HILIC Elution Buffer: 50 mM Ammonium Bicarbonate pH 8.0 *or* 50 mM Ammonium Formate pH 8.5. **NOTE**: *We have not observed any difference in elution using formate or bicarbonate on standards (tryptic digest of standard proteins used in protocol development). Formate may be preferred due to the availability of MS grade reagent, with application in native MS and as a common solvent additive.*

METHOD – HILIC CLEAN-UP OF PEPTIDES:

NOTE*: The current protocol is suitable for peptide clean-up from a tryptic digest of a sample from 50 µg of total protein. The protocol has ONLY been evaluated on a protein digest of standard proteins (Tryptic digest of Casein and BSA), and evaluation for clean-up from complex cell lysates is currently underway. Some optimization may be required to achieve highly-pure peptides from crude lysates. This protocol is not intended to be limiting, and the peptide clean-up protocol may be optimized to achieve the desired clean-up of peptides.*

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) of 20 $mg.ml^{-1}$ HILIC microparticles to a 2.0 ml LoBind Eppendorf tube
3. Place the tube on the magnetic separator and allow for the microparticles to clear.
4. Remove the shipping solution by aspiration with a pipette and discard.
5. Wash and equilibrate the microparticles in 500 µl HILIC Equilibration Buffer (90% ACN in 20 mM Ammonium formate pH 2.5), with gentle agitation for 20-30 sec
6. Place the tube on the magnetic separator and allow for the microparticles to clear. Discard the HILIC Equilibration Buffer.
7. Repeat steps 5 and 6.

8. Prepare Peptide Binding Sample by adding ACN and Ammonium Formate Stock Solution in the ratio of 10:85:5 Peptide:ACN:Ammonium Formate. As an example of suitable volumes consult the following table:

Peptide Volume (µl)	ACN Volume (µl)	AFM (400 mM, pH 2.5, µl)	Final Volume (µl)
10.0	85.0	5.0	100
25.0	212.5	12.5	250
50.0	425.0	25.0	500
100.0	850.0	50.0	1000

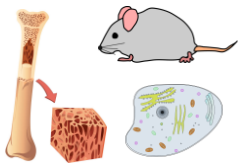
NOTE: *Variation of the final ACN concentration provides the potential to fractionate components in peptide samples such as glycans, glycopeptides, and phosphopeptides. Please consult the HILIC product page on our website for recent advances/protocols if available.*

9. Transfer the Peptide Binding Sample to the pre-equilibrated MagReSyn® HILIC microparticles.
10. Allow peptides 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 peptide recovery.
11. 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 quantified to estimate peptide binding*).
12. Resuspend the beads in a minimum of 200 µl wash buffer (95% ACN), and mix by gentle vortexing for 1 min.
13. Recover the microparticles on the magnetic separator. Remove the supernatant by pipette aspiration.
14. Resuspend the beads in 200 µl wash buffer (95% ACN), and mix by gentle vortexing for 1 min. Transfer microparticle suspension to a new tube to avoid potential carry over of detergent/contaminants from the tube.
15. Recover the microparticles on the magnetic separator. Remove the wash supernatant by pipette aspiration.
16. Elute peptides using 20 - 50 µl Elution Buffer for 15 min with sufficient agitation to ensure beads remain in suspension.
17. Recover the microparticles on a magnetic separator. Remove the supernatant containing peptides and transfer to 0.5 ml LoBind centrifuge tube.
18. Centrifuge at 14 000 g for 10 min and transfer supernatant to new 0.5 ml LoBind tube.
19. Add ACN and Formic acid as desired prior to MS analysis.

NOTE: *KingFisher™ Duo and Flex protocols are available on request by e-mail to info@resynbio.com*

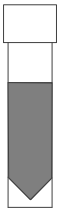
Protocol: Peptide Clean-up (PEPCLU)

Peptide Preparation



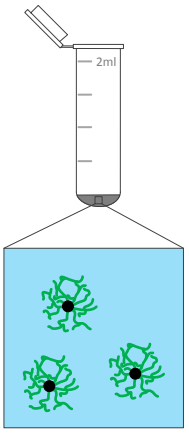
Protein extraction, solubilization & clarification

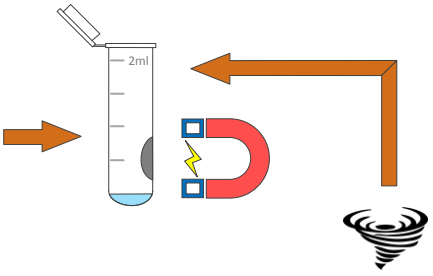
Bead Preparation



MagReSyn® HILIC

25 µl (500 µg)



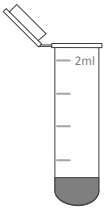


Vortex 30 sec

Aspirate liquid & discard

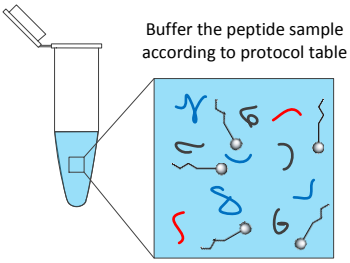
Resuspend in 500 µl Equilibration Buffer (90% ACN, 20 mM NH_4Formate)

Repeat for 2 Equilibrations

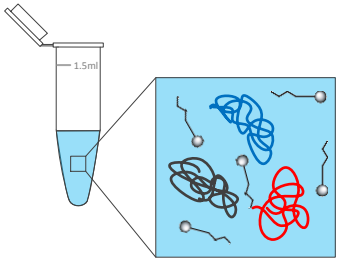


Transfer to equilibrated MagReSyn® HILIC

Buffer the peptide sample according to protocol table

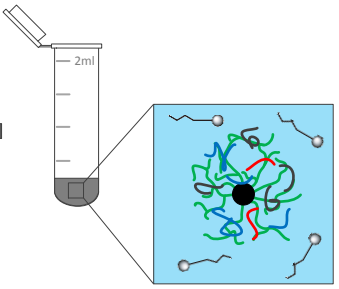


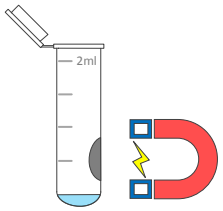
Protein digestion



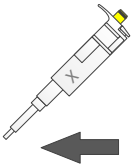
HILIC Peptide Clean-Up Workflow

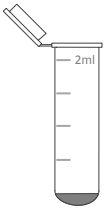
Bind for 30 minutes with gentle agitation, sufficient to keep beads suspended



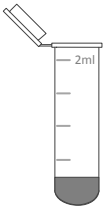


Aspirate liquid & discard

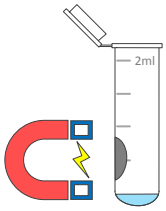


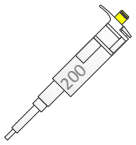


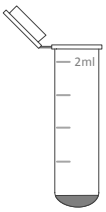
Resuspend beads in 200 µl Wash Buffer (95% ACN)



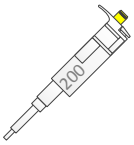
Vortex 1 min

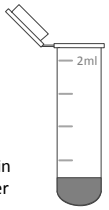




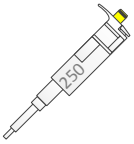


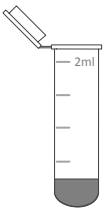
Aspirate liquid & discard





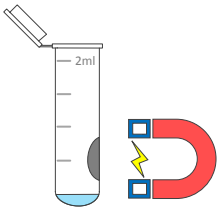
Resuspend beads in 200 µl Wash Buffer (95% ACN)

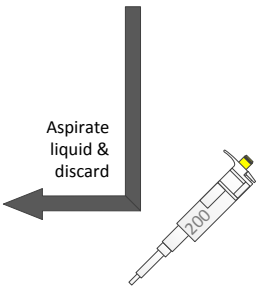




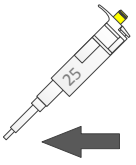
Transfer suspension to a new tube

Vortex 1 min





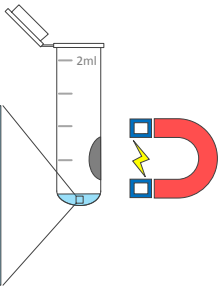
Aspirate liquid & discard



Resuspend beads in 25 to 50 µl of Elution Buffer (50mM NH_4Formate)

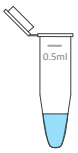
NOTE: *This step provides a possible point to elute using a stepwise ACN gradient for sample fractionation based on hydrophilicity.*

Vortex 15 min

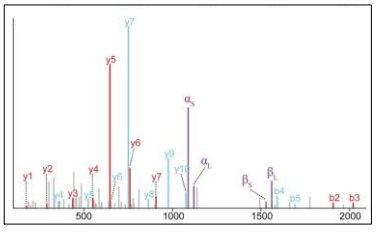


Peptide Solution

Transfer peptides to MS sample vial



LC-MS/MS analysis



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