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

PROTOCOL ID: HILIC_PEPCLU_12

DATE LAST MODIFIED: 25 October 2021

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 formate (MS Grade)
- Ammonium bicarbonate (optional replacement for ammonium formate)
- 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% Acetonitrile (5% aqueous)
- *Ammonium Formate Stock Solution*: 400 mM, pH 2.5
- *HILIC Elution Buffer* (**All reagents should be LCMS grade**): 50 mM Ammonium Formate pH 8.5, or water followed by 0.5% formic acid. **NOTE**: *Elution with water followed by formic acid is recommended for an LCMS setup that does not include an online trap column.*

METHOD – HILIC CLEAN-UP OF PEPTIDES:

NOTES: *The current protocol is suitable for peptide clean-up from a tryptic digest of a sample from 50 µg of total protein. This protocol is not intended to be limiting, due to the possible range of contaminants and variations in source material, the clean-up protocol may require optimization to achieve complete clean-up of peptides.*

IMPORTANT NOTE: *This protocol is suitable for clean-up of peptides from a range of contaminants (refer poster HUPO 2018 at www.resynbio.com). To ensure peptide-bead interaction the sample* ***must be contaminated****. At a minimum your sample should contain 50 mM Tris buffer (from Tryptic protein digestion) to ensure binding of the peptides to the beads. Please contact info@resynbio.com for more details on suitable contaminants. If you sample is insufficiently contaminated you will have to spike with buffer for clean-up.*

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. Add 10 µl of *HILIC Equilibration Buffer* (or sufficient to ensure beads are covered and do not dry out which may lead to clumping and poor peptide recovery). **NOTE**: *It is advisable to prepare samples in advance, ready for binding to the beads immediately after their equilibration to avoid possible bead drying.*
9. Acidify peptide solution with neat formic acid to a final concentration of 0.5%. (**NOTE***: Binding of peptides is pH sensitive and low peptide binding may be a direct result of the incorrect pH)*
10. Prepare Peptide Binding Sample by adding ACN and *Ammonium Formate Stock Solution* in the ratio of 5:90:5 Peptide:ACN:Ammonium Formate. As an example of suitable volumes consult the following table:

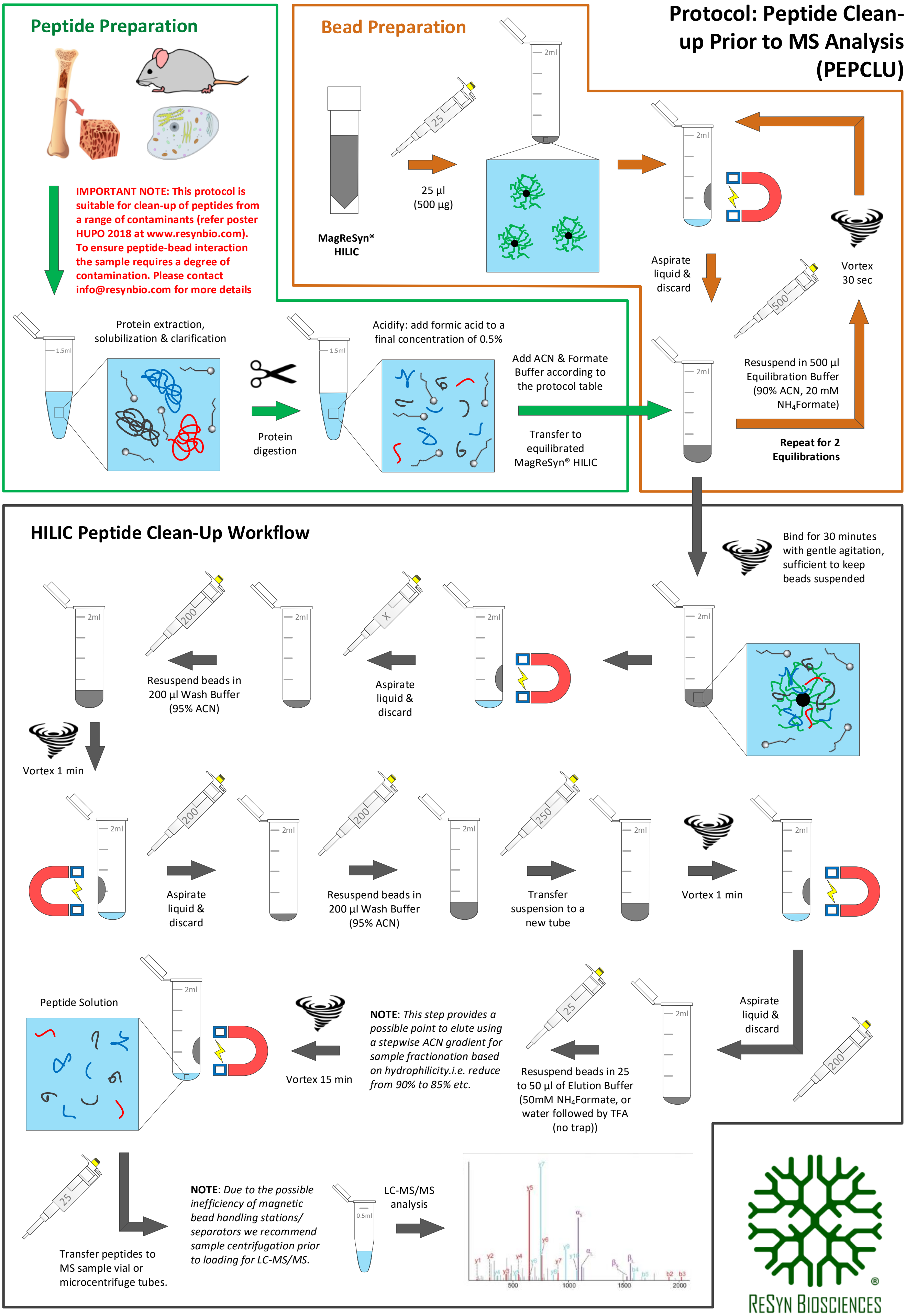
Peptide Volume (µl)	ACN Volume (µl)	AFM (400 mM, pH 2.5, µl)	Final Volume (µl)
5	90	5	100
10	180	10	200
25	450	25	500
50	900	50	1000
100	1800	100	2000

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.*

11. Transfer the Peptide Binding Sample to the pre-equilibrated MagReSyn® HILIC microparticles.
    NOTE: *order of addition of components is important, sample and binding buffer must be pre-mixed before addition to the equilibrated magnetic HILIC beads, to prevent the beads clumping in solution. Certain buffers, such as HEPES, may result in bead clumping. If bead clumping is observed try alternate buffers such as TEAB, Ammonium bicarbonate, or TRIS, or remove ammonium formate from the binding buffer (as this can exacerbate the aggregation). If bead clumping is observed at any point, sonication (high power) in a water bath for 10 to 20 seconds* may reduce this clumping.
    NOTE: *When replacing AFM in the table above (to circumvent clumping), use digestion buffer as a replacement to achieve the correct acetonitrile concentration.*
12. 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 result in HILIC microparticles drying on tube side walls leading to poor peptide recovery.
13. 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*).
14. Resuspend the beads in a minimum of 200 µl wash buffer (95% ACN), and mix by gentle vortexing for 1 min.
15. Recover the microparticles on the magnetic separator. Remove the supernatant by pipette aspiration.
16. 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.
17. Recover the microparticles on the magnetic separator. Remove the wash supernatant by pipette aspiration.
18. Elute peptides using 20 - 50 µl Elution Buffer for 15 min with sufficient agitation to ensure beads remain in suspension.
19. For increased recovery, or when using sequential elution with water and formic acid, repeat steps 16 and 17. For maximum yield, place sample in bath sonicator during this elution incubation.
20. Recover the microparticles on a magnetic separator. Remove the supernatant containing peptides and transfer to 0.5 ml LoBind centrifuge tube.
21. Centrifuge at 14 000 g for 10 min and transfer supernatant to new 0.5 ml LoBind tube. (**NOTE**: *this step is recommended due to possible inefficiences in magnetic bead separators and/or handling stations. For automated protocols a secondary magnetic separation may be included as a possible alternative*)
22. 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 Prior to MS Analysis (PEPCLU)



ReSyn Biosciences

Protocol ID: PEPCLU_VIS_07