On-bead HILIC digest

In preparation for the HILIC magnetic bead workflow, the HILIC beads (ReSyn Biosciences, HLC010) were aliquoted into a new tube and the shipping solution removed. Beads were then washed with 250µl wash buffer (15% ACN, 100mM Ammonium acetate (Sigma 14267) pH 4.5) for one minute. This was repeated once. The beads were then resuspended in loading buffer (30% ACN, 200mM Ammonium acetate pH 4.5). A total of 50 µg of protein (or the entire sample) from each tube was transferred to a 96-well protein LoBind plate (Merck, 0030504.100). Protein was reduced with tris (2-carboxyethyl) phosphine (TCEP; Sigma 646547) which was added to a final concentration of 10mM TCEP and incubated at 30˚C for two hours (urea containing samples) or 60 ˚C for one hour (all other samples). Samples were cooled to room temperature and then alkylated with methylmethanethiosulphonate (MMTS; Sigma 208795) which was added to a final concentration of 10mM MMTS and incubated at room temperature for 15 minutes. HILIC magnetic beads were added at an equal volume to that of the sample and a ratio of 5:1 total protein. The plate was then incubated at room temperature on the shaker at 900RPM for 30 minutes for binding of protein to beads. After binding, the beads were washed four times with 500µl of 95% ACN for one minute. For digestion Trypsin (Promega PRV5111), made up in 50mM TEAB was added at a ratio of 1:10 total protein and the plate was incubated at 37˚C on the shaker for four hours. After digestion, the supernatant containing peptides was removed and dried down. Samples were then resuspended in LC loading buffer: 0.1% FA, 2.5% ACN.

LCMS

LCMS analysis was conducted with a Q-Exactive quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) coupled with a Dionex Ultimate 3000 nano-UPLC system. Data was acquired using: Xcalibur v4.1.31.9, Chromeleon v6.8 (SR13), Orbitrap MS v2.9 (build 2926) and Thermo Foundations 3.1 (SP4). Peptides were dissolved in 0.1% Formic Acid (FA; Sigma 56302), 2% Acetonitrile (ACN; Burdick & Jackson BJLC015CS) and loaded on a C18 trap column (PepMap100, 300 µm × 5 mm × 5 µm). The solvent system employed was solvent A: LC water (Burdick and Jackson BJLC365); 0.1% FA and solvent B: ACN, 0.1% FA. Samples were trapped onto the column at 2% solvent B and washed for 3 minutes before the valve was switched and peptides eluted onto the analytical column as described hereafter. Chromatographic separation was performed with a Waters nanoEase (Zenfit) M/Z Peptide CSH C18 column (186008810, 75 µm × 25 cm × 1.7 µm) as described below.

1224MASS:

The multi-step gradient for peptide separation was generated at 300 nL/min as follows: time change 117 min, gradient change: 5 – 40% Solvent B, time change 5 min, gradient change 40

– 80% Solvent B. The gradient was then held at 80% solvent B for 10 minutes before returning it to 2% solvent B and conditioning the column for 15 minutes.

1264MASS:

The multi-step gradient for peptide separation was generated at 300 nL/min as follows: time change 5 min, gradient change: 2 – 5% Solvent B, time change 40 min, gradient change 5 – 18% Solvent B, time change 10 min, gradient change 18 – 30% Solvent B, time change 2 min, gradient change 30 – 80% Solvent B. The gradient was then held at 80% solvent B for 10 minutes before returning it to 2% solvent B and conditioning the column for 15 minutes.

All data acquisition was performed using Proxeon stainless steel emitters (Thermo Fisher TFES523). The mass spectrometer was operated in positive ion mode with a capillary temperature of 320°C. The applied electrospray voltage was 1.95 kV. Details of data acquisition are shown in the table below.

Full Scan	
Resolution	70,000 (@ *m/z* 200)
AGC target value	1e6
Scan range	350-2000 m/z
Maximal injection time (ms)	50
Data-dependent MS/MS	
Inclusion	Off
Resolution	17,500 (@ *m/z* 200)
AGC target value	5e4
Maximal injection time (ms)	50
Loop Count	10
Isolation window width (Da)	2
NCE (%)	27
Data-dependent Settings	
Underfill ratio (%)	1
Charge exclusion	Unassigned, 1, 7, 8, >8
Peptide match	preferred
Exclusion isotopes	on
Dynamic exclusion (s)	60