**Kingfisher Protocol for Membrane Particle (EV) Capture and Protein Digestion using MagReSyn SAX Beads (SAX KFKF Protocol)**

* Materials:
  1. Samples
     + Plasma (Human plasma is typically ~60-85 µg/µL)
  2. Resyn Biosciences MagResyn SAX particles
     + Supplied as 20 mg/mL in 20% ethanol
  3. HALT Protease and Phosphatase Inhibitor Single-Use Cocktail (100X)
  4. Kingfisher Flex
  5. 2 sets Kingfisher 96 well plates, deep-well (1 set = 8 plates + 1 comb = 8 positions)
  6. Bis Tris Propane (BTP) Buffers
     + 2X Binding Buffer
       - Binding Buffer (100 mM Bis Tris Propane pH 6.3, 150 mM NaCl, 10 mL)
         * 5 mL 200 mM Bis-Tris Propane, pH 6.3 in 15 mL Falcon
         * 375 µL 4 M NaCl
         * 4.625 mL mQH2O
     + Equilibration/Wash Buffer
       - Equilibration/Wash Buffer (50 mM Bis Tris Propane pH 6.5, 150 mM NaCl, 50 mL)
         * 12.5 mL 200 mM Bis-Tris Propane, pH 6.5 in 50 mL Falcon
         * 1.875 mL 4 M NaCl
         * 35.625 mL mQH2O
  7. 20% SDS
  8. 1 M Tris, pH 8.5
  9. 500 mM 2-iodoacetamide (IAA). (Aliquots in R2D2 -20°C freezer)
     + Working concentration 15 mM
  10. TCEP (Bond-Breaker TCEP Solution, Neutral pH, 500 mM concentration)
      + Working concentration 10 mM
  11. 500 mM 1,4-dithiothreitol (DTT). (Aliquots in R2D2 -20°C freezer)
      + Working concentration 10 mM
  12. 100% acetonitrile (ACN)
  13. 70% ethanol (EtOH)
  14. 95% acetonitrile (ACN)
  15. Enolase (400 ng/mL stock solution)
  16. Pierce porcine trypsin (20 or 100 mg vial)
      + Working ratio (20:1) (protein:enzyme)
  17. 10% trifluoroacetic acid (TFA)
      + Working concentration 0.1%, 0.5% or 1%
  18. QC Standard (10 µL aliquots): (200 fmol/µL PRTC, 800 fmol/µL BSA).
      + Add 30 µL 0.1% formic acid for a total of 40 µL working solution (50 fmol/µL PRTC, 200 fmol/µL BSA) (inject 3 µL for 150 fmol PRTC/sample and 600 fmol/µL BSA/sample)
  19. Pierce Retention Time Calibrant (PRTC) (5 pmol/µL stock solution)
      + Dilute 5 pmol/µL PRTC (stock) to working concentration of 50 fmol/µL (inject 3 µL for 50 fmol/sample
* Methods:
  1. Thaw plasma samples on ice and add HALT. (Use HALT without phosphatases if proceeding to phosphopeptide enrichment.)
  2. Label Kingfisher 96 well plates, deep-well.

Set 1: KF1

* Plate 1: SAX Bead Capture
* Plate 2: Bead Equilibration
* Plate 3: Membrane Particle Capture
* Plate 4-6: Bead Wash
* Plate 7: Protein Denature/Reduction
* Plate 8: 96 well comb

Set 2: KF2

* Plate 1: KF1 Plate 7
* Plate 2-4: Acetonitrile Wash
* Plate 5-6: Ethanol Wash
* Plate 7: Digestion/Elution
* Plate 8: 96 well comb
  1. **EQUILIBRATE SAX BEADS:** **Kingfisher (KF1 plates 1, 2)** 
     + Add 500 µl BTP Equilibration/Wash Buffer into each well of KF1 Plate 1 and 2.
     + Add 25 µl MagResyn SAX beads into each well of KF1 Plate 1. Mix by gently pipetting up and down.
  2. **DILUTE PLASMA WITH BINDING BUFFER:** **Kingfisher (KF1 plate 3)**
     + Save 5 µL each plasma sample for Step 8 (Manual Reduction of Total Plasma Samples) to add to KF2 plates (label “Total Plasma”)
     + Add 1 volume BTP Binding Buffer to 1 volume plasma. Mix by inverting 5 times and bring to room temperature.
       - Note: Plasma volumes can be between 10 – 200 µL. Currently, we routinely use 100 µL.
     + Add diluted plasma/Binding Buffer into each well of KF1 Plate 3.
  3. **WASH BOUND PARTICLES ON BEADS:** **Kingfisher (KF1 plates 4, 5, 6)**
     + Add 500 µL BTP Equilibration/Wash Buffer into each well of KF1 Plates 4, 5, and 6.
  4. **REDUCTION**: **Kingfisher (KF1 plate 7)**
     + Add 100 mL 100 mM Tris, pH 8.5/2% SDS, 800 ng enolase (2 µL stock), and 2 mL TCEP (10 mM) into each well of KF1 Plate 7.
  5. **Run KF1 method: (SAX\_KF1\_Transfer\_current)**
     + Method takes ~2.5 hours. While waiting for this, proceed to Step 8 and process the Total Plasma samples.
     + Remove all plates from Kingfisher.
     + Allow Plate 7 to cool completely and continue to Step 9.
  6. **Manual Reduction of Total Plasma Samples**
     + For each Total Plasma sample, make 1/10 dilution with 100 mM Tris, pH 8.5/2% SDS to pipet from (5 mL plasma + 45 mL Tris/SDS)
       - Note: Do not try to pipet 1 mL plasma. This sample will be used to determine enrichments.
     + Combine 10 mL (equivalent to 1 mL plasma) and add 90 mL Tris/SDS.
     + Add 2 mL enolase (800 ng) and 2 mL TCEP (10 mM) to each tube.
* Heat tubes at 37°C for 60 min on Thermomixer with light shaking (650 rpm).
* Allow tubes to cool completely.
  1. **Alkylation/IAA Quenching (Manual in KF plate)**
* Add IAA to Total Plasma samples in tubes and to each well in KF1 Reduction Plate from Step 7.
* Add 500 mM IAA to each tube/well for a final concentration of 15 mM.
  + Pipette up and down gently
  + Incubate at room temperature in the dark for 30 minutes
* Add 500 mM DTT to each tube/well for a final concentration of 10 mM to inactivate free IAA.
* Incubate at room temperature for 15 min.
  1. **PRECIPITATE PROTEIN:** **Kingfisher (KF2 plate 1, previously KF1 plate 7)** 
     + Add 25 mL of SAX beads (at their stock concentration of 20 mg/mL) to each of Total Plasma samples in tubes.
* Mix by pipetting up and down gently.
  + - Transfer to separate wells in the KF1 Reduction Plate (now, KF2 Protein Precipitation Plate) from the Plasma Particle samples already in there. This is to prepare for second Kingfisher method.
    - Add volume of 100% ACN to each well to reach a 70% final concentration, to precipitate proteins. Mix by pipetting up and down gently.
    - Incubate for 10 minutes at room temperature.
  1. **WASH PRECIPITATED PROTEINS ON BEADS:** **Kingfisher (KF2 plates 2, 3, 4)**
     + Add 1 mL 95% Acetonitrile into each well of KF2 Plates 2, 3, and 4.
  2. **WASH PRECIPITATED PROTEINS ON BEADS:** **Kingfisher (KF2 plates 5, 6)**
     + Add 1 mL 70% Ethanol into each well of KF2 Plates 5 and 6.
  3. **Run KF2 method: (SAX\_KF2\_current)**
     + Method (modified from Julia Robbins’ protocol) takes ~2 hours.
     + There will be a pause after the washes (~35-40 minutes) during which the trypsin digestion Buffer will be added to KF2 Plate 7 (Digestion/Elution).
     + Adjust total volume added to each well depending on peptide concentration preferred.
       - 200 µL 50 mM Tris, pH 8.5 per well with various amounts of trypsin depending on range of protein in sample with an approximate final working ratio (20:1) (protein:enzyme).
         * 3.75 µg trypsin:~75 µg total plasma
         * 7.5 µg trypsin:~150 µg EVs
     + Add **KF2 Plate 7** to Kingfisher and continue method.
     + Remove all plates from Kingfisher.
  4. **QUENCH DIGESTION**
* Collect samples out of KF2 Plate 7 into low-bind Eppendorf tubes.
* Add 10% TFA to quench (to 0.5% final concentration).
* Freeze peptide samples (-80°C).
  1. **MS ANALYSIS OF PEPTIDES**
* Microfuge samples to remove particulates (10 min, RT, max g on microfuge)
* Dilute 10 µL QC aliquot with 30 µL 0.1% formic acid.
* Dilute PRTC stock (5 pmol/µL) to working concentration (500 nmol/µL) with 1/10 dilution with 0.1% TFA.
* Each sample from the SP3 prep is ~100 µL. Pipet 50 µL to separate tube and add 5 µL 500 nmol/µL to achieve 50 nmol/µL (total 150 nmol PRTC per 3 µL injection).
* Pipet 15 µL of each peptide/PRTC sample and place in autosampler vials.
* Note: Make sure there are no bubbles at bottom of vial.
* Pipet 5 µL from each EV sample and pool. Place 30 µL in autosampler vial for narrow window injections (Chromatogram Library).
* Freeze peptide/PRTC samples (-80°C).
* Queue on Eclipse.
  1. Randomization of samples.