PROTOCOL NAME: Mag-Net, Enrichment of membrane-bound vesicles from Plasma using MagReSyn® SAX for analysis by LC-MSMS

PROTOCOL ID: Mag-Net EV ENRICHMENT FROM PLASMA **DATE LAST MODIFIED:** May 2025

INTRODUCTION

Researchers at the University of Washington Genome Sciences, in collaboration with ReSyn Biosciences, have developed a robust, inexpensive, and simple magnetic bead-based method, Mag-Net, to enrich EV particles from plasma while simultaneously depleting abundant plasma proteins. EV capture, in the presence of similarly sized lipoprotein particles, is based on electrostatic interactions between MagReSyn® SAX microparticle and negatively charged phosphoplipids, such as phosphatidylserine, located on EV membrane surfaces. In addition, EV capture is thought to be enhanced by unique size exclusion properties of the hyper-porous MagReSyn® backbone. The end-to-end, plasma to LCMS, workflow seamlessly incorporates all steps including EV capture, abundant plasma protein depletion, EV lysis, reduction, alkylation as well as PAC-based EV protein on-bead aggregation, washing and digestion, transitioning from plasma to mass spectrometry ready to analyse peptides in <4 hours. Ultimately Mag-Net provides a high-throughput and cost-effective deep-dive of the plasma proteome.

Please contact info@resynbio.com if you have any queries relating to this protocol as well as to obtain methods for semi-automated sample processing on a KingFisher™ magnetic handling station. This protocol is not and should not be construed as an endorsement of any product, rather it is made available by the authors of the associated publication to assist in the reproducibility of the method for researchers to achieve inter-lab reproducibility.

REQUIREMENTS

- MagReSyn® SAX – supplied as 20 mg.ml^{-1} in 20% ethanol
- Intelli-Mixer™ or similar mixing device
- Magnetic stand or magnetic handling station
- Incubator with adjustable temperature

STOCK REAGENT PREPARATION

(Reagents and chemicals should be of analytical grade or better, preferably MS grade)

- *Optional*: cOmplete™ mini- EDTA free protease inhibitor and PhosSTOP™ Phosphatase inhibitor/ HALT™ Protease and Phosphatase Inhibitor Single-Use Cocktail (100X)
- 4M NaCl (dissolve 1.17g in 5mL ultrapure water (mQH2O))
- 200 mM Bis-Tris Propane, pH 6.3
- TCEP Stock: 0.5M (prepare fresh – 71.66 mg in 1 mL ultrapure water (mQH2O), or commercial pre-prepared TCEP stock solution such as Bond-Breaker® Thermo Fisher catalogue number 77720)
- IAA Stock: 0.5M (prepare fresh, light sensitive – 92.48 mg in 1 mL ultrapure water (mQH2O)
- 20% SDS (10 g in 50 mL in ultrapure water (mQH2O)
- 1M Tris, pH 8.5 (0.485g in 40mL ultrapure water (mQH2O)
- Porcine trypsin (Pierce 20 or 100µg vial – 20:1 protein : enzyme)
- 10% Trifluoroacetic acid (TFA)
- Acetonitrile (100% - ACN)
- *Optional*: Enolase (400 ng.µL^{-1} stock solution)

WORKING REAGENT PREPARATION

- 2 x EV binding buffer (10mL of 100 mM Bis Tris Propane pH 6.3, 150 mM NaCl)
    - 5 mL 200 mM Bis-Tris Propane, pH 6.3 in 15 mL Falcon
    - 375µL 4M NaCl
    - 4.625mL mQH2O

- Optional: Protease and Phosphatase inhibitors

- Equilibration/Wash Buffer (50ml of 50mM Bis Tris Propane pH 6.3, 150mM NaCl)
  - 12.5mL of 200mM Bis-Tris Propane, pH 6.3 in 50 mL conical centrifugation tube
  - 1.875mL of 4M NaCl
  - 35.625mL mQH2O

- Lysis and Reduction Mix (100µL 50mM Tris, pH 8.5, 1% SDS, 800ng enolase, 10mM TCEP, **prepare fresh**)
  - Make 10mL stock 50mM Tris, pH 8.5, 1% SDS (500µL 1M Tris, 500µL 20% SDS, 9mL mQH2O)
  - Add TCEP to a final to 10mM (2 µL from stock)
  - Optional: add 800ng enolase (2 µL from stock)

METHODS

A: CAPTURE OF EXTRACELLULAR VESICLES (EVs), DEPLETION OF HIGH ABUNDNCE PLASMA PROTEINS, EV LYSIS, REDUCTION AND ALKYLATION OF EV PROTEINS

Plasma preparation:

Note: Mag-Net has been validated using plasma collected based on the Early Detection Research Network (EDRN) Standard Operating Procedure (SOP) for collection of EDTA plasma as described below, the procedure can be found online at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2655764/, refer to Supplementary Material 2. Alternative plasma collection SOPs may be used, but we recommend that aspects such as the SAX bead to plasma ratio are empirically optimized for EV capture, and to minimize recovery of high abundance plasma proteins.

1. Collect Blood using EDTA Collection Tubes (eg. BD vacutainers catalogue# 366450)
2. After collection, gently mix the blood by inverting the tube 8 to 10 times. Store vacutainer tubes upright at 4°C until centrifugation. **Samples should be centrifuged within one hour of blood collection**.
3. Centrifuge blood samples in a horizontal rotor (swing-out head) for 15 minutes, 1,200g at room temperature.
4. Carefully collect the plasma layer, as close as possible to the buffy coat layer without disturbing it. Aliquot 100µL and store at -80C.

SAX Beads equilibration:

5. Add 12.5µL of MagReSyn® SAX beads to a 2ml round-bottom tube (sufficient to process 50µL plasma; keep ratio of 1:4 bead to plasma if scaling down the protocol).

Note: 1:4 ratio is optimal for the plasma collection protocol described above, but optimization of this ratio may be required when using alternate plasma preparation protocols. As an example, for plasma preparations that have undergone filtration or multiple rounds of centrifugation, the plasma volume may need to be increased up to 10X. We recommend reducing the bead volume to 5 µL and increasing the volume of plasma used up to 500 µL.

6. Add 200µL BTP Equilibration/Wash Buffer (50 mM Bis Tris Propane pH 6.3, 150 mM NaCl, 50mL)
7. Mix gently for 30 sec
8. Place tubes on magnetic separator
9. Remove the equilibration solution. (Repeat the equilibration, Steps 7-9)

Plasma dilution with binding buffer (1:1):

10. Mix 50µL plasma with 50µL bind buffer supplemented with HALT™ inhibitors.

Note: keep the ratio 1:1 plasma to bind buffer if scaling up the volume of plasma

Note: the method can be scaled down to lower input plasma volumes, however the total volume needs to be increased by adding wash buffer to a final of ~100 µL e.g. for 10µL plasma, add 10µl bind buffer followed by 80µL wash buffer.

11. Mix gently in the Intelli-Mixer™ 30 sec.

Note: nutator, vortex or thermomixer are suitable alternatives but mixing should be gentle in order to minimize EV rupture. With a 2ml round-bottom tube 600-800 rpm should be sufficient to keep beads in suspension.

Binding of extracellular vesicles (EVs):

12. Add diluted plasma to equilibrated SAX beads.
13. Place tube in Intelli-Mixer™ and mix gently for 30 minutes.

Note: nutator, vortex or thermomixer are suitable alternatives but mixing should be gentle to minimize EV rupture, and reduce SAX beads from adhering to tube walls.
Recommended: the use of Eppendorf LoBind® tubes reduces SAX bead adherence to tube walls.

14. Place tubes on magnetic separator.
15. Remove flow-through containing unbound soluble plasma proteins.

Note: the unbound sample can be kept for further non-depleted plasma analysis.

Deplete high abundance plasma proteins:

16. Add 500µL BTP Equilibration/Wash Buffer (50mM Bis Tris Propane pH 6.3, 150mM NaCl)
17. Wash by gently mixing particle bound beads for 5 minutes in Intelli-Mixer™

Note: nutator, vortex or ThermoMixer® are suitable alternatives but mixing should be gentle in order to minimize EV rupture as well as SAX beads from adhering to tube walls

18. Place tubes on magnetic separator
19. Discard the wash solution
20. Repeat steps (16-19) twice for a total of 3 wash steps

Vesicle lysis and protein reduction:

21. Add 100µL of lysis and reduction mix (100µL 50 mM Tris, pH 8.5, 1% SDS, 10mM TCEP (2µL))

Note: 800 ng (2µL of 400ng.µL^{-1}) Yeast Enolase can be added to the lysis and reduction mix

22. Incubate for 60 minutes at 37°C with gentle mixing

Note: nutator, vortex or thermomixer are suitable alternatives but mixing should be gentle in order to minimize EV rupture as well as SAX beads from adhering to tube walls

Vesicle protein alkylation:

23. Add 3µL IAA (500mM stock) to a final of 15mM.
24. Incubate in the dark for 30 minutes whilst mixing,

B: PROTEIN AGGREGATION CAPTURE WITH ON-BEAD DIGESTION

Protein capture:

27. Add 240µL ACN to a final of ~70% to induce on-bead protein precipitation
28. Pipette mix the bead ACN protein mixture between 5-10 times.
29. Incubate samples at RT for 10 minutes without mixing.
30. Move sample tube to magnetic separator and remove excess supernatant

Recommended: proceed to wash without pausing at this point, to avoid potential of beads drying out.

Washing (contaminant removal):

Recommended: Perform all the washes without mixing whilst the beads are captured on a magnetic separator.

31. Add and, after 10-30 seconds, remove 500µL of 95% ACN.
32. Repeat step 31 twice for a total of 3 washes with 95% ACN

Digestion

33. Add 200µL of 25mM ammonium bicarbonate and trypsin to the beads

Note: estimate the enzyme required based on the volume of SAX beads, rather than plasma volume: for every 12.5µl beads we recommend using 3.75 µg of Trypsin. To further enhance digest efficiency 1µg LysC can be added per 12.5µl SAX beads. These can be scaled linearly based on the volume of beads used e.g. for 5µl SAX beads use 1.5 ug Trypsin and 400ng LysC, irrespective of the plasma volume.

Note: If you notice beads tending to adhere to the tube walls, use an alternate digest buffer of 25mM Tris-HCl pH 8 can be used to reduce this adherence

35. Incubate for 2 hrs at 47°C whilst gently mixing using an Intelli-Mixer™

Note: vortex or thermomixer can be used as alternatives to mix the beads. Ensure beads mixing is not too vigorous in order to reduce beads from adhering to tube side wall

Recommended: the use of Eppendorf LoBind® tubes reduces SAX beads adheres to tube walls.

36. Quench digestion by adding 10% TFA to a 0.5% final

37. Remove beads using a magnetic separator and transfer supernatant to a new 0.5mL tube.
Recommended: Centrifuge the tubes containing supernatant at 10,000 xg for 5 minutes prior to LCMS analysis to remove possible residual magnetic beads and particulates.
38. Freeze peptide samples (-80°C).

*Note***:** it is advisable to perform peptide desalting if LCMS set-up is being performed without a pre-concentration trap column.

Note: Peptides can be quantified prior to LCMS analysis to determine optimal loading e.g. using a Pierce quantitative colorimetric peptide assay kit.