



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

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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 phospholipids, 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)

- 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
- cOmplete™ mini- EDTA free protease inhibitor (1 tablet) + PhosSTOP™ (1 tablet) per 7ml binding buffer.

- 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 ABUNDANCE 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 recovery of EV's, 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 25μL of MagReSyn® SAX beads to a 2ml tube (sufficient to process 100μL plasma; for 10 to 50μL plasma use 12.5μL beads).
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.
Recommended: Do not use phosphatase inhibitors if proceeding to phosphopeptide enrichment. These interfere with IMAC based phosphopeptide binding. The high concentration of detergent used during EV lysis (step 21) will ensure phosphatases are deactivated.
Note: the method can be scaled down to 10μL plasma as input but the volume needs to be increased by adding PBS e.g. for 10μL plasma, add 40μL PBS and 50μL binding buffer containing protease inhibitors.
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.

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 in order to minimize EV rupture as well as 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 250µL ACN to a final of ~70% in order to induce on-bead protein precipitation (add 240.3µL ACN to the sample)
28. Pipette mix the bead ACN protein mixture between 5-10 times.
29. Incubate samples at RT for 10 minutes without mixing.

Washing (contaminant removal):

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

30. Add and, after 10-30 seconds, remove 500µL of 95% ACN.
31. Repeat step 30 twice for a total of 3 washes with 95% ACN
32. Add and, after 10-30 seconds, remove 500µL of 70% EtOH
33. Repeat step 32 twice for a total of 3 washes with 70% EtOH

Digestion:

34. Add 200µL of 25mM ammonium bicarbonate and trypsin (1:20 enzyme to protein ratio).
Note: If beads tend to coat tube walls, an alternate digest buffer of 25mM Tris-HCl pH 8 can be used to reduce bead adherence to tube walls
35. Incubate for 2 hrs at 47°C whilst gently mixing in 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.

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