

Mag-Net HP Kit for deep plasma proteome profiling via LC-MSMS

INTRODUCTION

Researchers at the University of Washington Genome Sciences, in collaboration with our team ReSyn Biosciences, have developed a robust, inexpensive, and simple magnetic bead-based method, termed Mag-Net™, to enrich extracellular vesicle (EVs) particles from plasma, while simultaneously depleting abundant plasma proteins. This method has since been improved with the development of the Mag-Net™ HP kit, utilising a new bead mixture and optimised buffer formulations. Ultimately, the Mag-Net™ HP workflow provides a high-throughput and cost-effective deep-dive of the plasma proteome.

Before you begin: Between 5-20 µL of plasma is sufficient for this protocol when using the plasma collection protocol described by the EDRN network below (see plasma preparation section). However, higher volumes may be required when using alternate plasma preparation protocols (e.g. 20-50 µL may be required for platelet-poor plasma prepared with higher centrifugation speeds). It is best practice to test the volume of plasma required for maximal proteomic depth on a pooled sample prior to preparing all individual study samples.

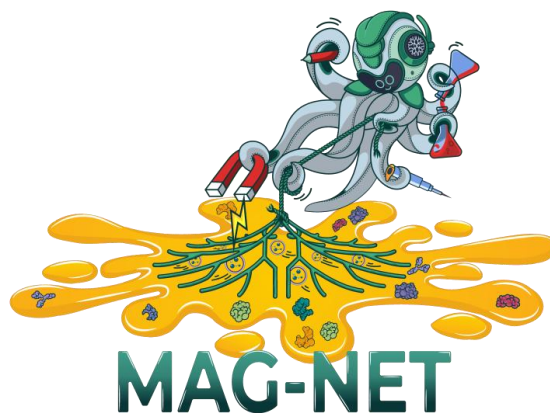
This kit is suitable for manual or automated processing of up to 96 samples (one microplate) on a magnetic bead handling station or a liquid handling robotic system fitted with a magnetic module (or deck) for isolation of magnetic beads. The recommended volumes of buffers in this protocol are optimised for use in KingFisher™ deep-well plates, but may be reduced when using lower volume plates suitable for alternate automation systems. Please contact info@resynbio.com if you would like to obtain the protocols and .bdz files for automated processing on a KingFisher™ magnetic bead handling station.

REQUIREMENTS NOT SUPPLIED IN THE KIT

- LC-MS grade Acetonitrile
- LC-MS Water
- TEAB or alternative digestion buffer
- Sequencing grade Trypsin (Lys-C optional)
- Trifluoroacetic acid (TFA)

REAGENTS PROVIDED IN THE KIT – 96 REACTIONS

-  MagReSyn® Mag-Net™ HP beads (0.72 mL)
-  Bind Buffer (5.5 mL)
-  Wash Buffer (100 mL)
-  Lysis Buffer (8 mL)
-  TCEP (~ 11.5 mg)
-  2-Chloroacetamide (~ 7.5 mg)
-  Protein Binding Buffer (24 mL)



STOCK REAGENT PREPARATION

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

-  TCEP Stock: Resuspend contents of TCEP vial using 100 µL of Lysis Buffer. Once resuspended add the 100 µL of TCEP stock back to the Lysis Buffer stock. Final concentration of TCEP in the Lysis Buffer is ~ 5 mM.
-  2-Chloroacetamide Stock: Resuspend contents of 2-Chloroacetamide vial using 100 µL of Lysis Buffer. Once resuspended add the 100 µL of 2-Chloroacetamide stock back to the Lysis Buffer stock. Final concentration of 2-Chloroacetamide in the Lysis Buffer is ~ 10 mM.
- TCEP and 2-Chloroacetamide should be added freshly to the Lysis Buffer on the day of sample preparation. Amounts of TCEP and 2-Chloroacetamide supplied are suitable for the total 96 samples that may be prepared with each kit. Alternatively if all 96 samples are not prepared at the same time, relevant stock solutions of TCEP and 2-Chloroacetamide should be made freshly and supplemented to the appropriate volume of Lysis Buffer at 5 mM and 10 mM, respectively.
 - Acetonitrile (95% - MeCN) – 1 mL required per sample
 - 1X Digestion buffer – 50mM TEAB or alternative (Dilute 1M TEAB pH 8.5; Sigma-Aldrich catalogue number T4708) 20X using LC-MS water) ~200 µL is required per sample
 - 5% TFA – 45 µL required per sample

MAG-NET™ HP PROTOCOL

Please refer to Figure 1 below for how to set up the KingFisher™ plate for automated sample processing.

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, refer to [Supplementary Material 2](#). Alternative plasma collection SOPs may be used, however the optimal volume of plasma to use may need to be optimised.

1. Collect Blood using EDTA Collection Tubes (e.g. 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) for 15 minutes, 1,200xg at room temperature.
4. Carefully collect the plasma layer, as close as possible to the buffy coat layer without disturbing it, aliquot and store at -80°C.

MagReSyn® Mag-Net™ HP Bead equilibration

-  5. Add 5 µL of **MagReSyn® Mag-Net HP** beads per sample to the appropriate plate wells (this is sufficient to process up to 50 µL of plasma)
Recommended: the use of polystyrene plates should be avoided. Polypropylene plates, or ideally Eppendorf LoBind® plates, reduce the adherence of Mag-Net™ HP beads to the walls during extended processing steps.
-  6. Add 400 µL Wash Buffer – reduce volumes accordingly if using lower volume plates required for alternate automation systems, to the maximum recommended plate volume.
- 7. Mix for 1 minute
- 8. Transfer beads/separate beads on magnet and discard the supernatant

Plasma dilution with Bind Buffer (1:1)

-  9. Mix 5 µL of plasma with 5 µL Bind Buffer, and add 90 µL of the **Wash Buffer** for a final volume of 100 µL.
Note: keep the ratio 1:1 of plasma to **Bind Buffer** if scaling up the volume of plasma is required, i.e. for 50 µL of plasma use 50 µL of Bind buffer, and always adjust the volume to a final of 100 µL using the **Wash Buffer**. The method may be scaled for higher plasma input volumes, the minimum volume of 100 µL for this dilution step needs to be maintained by adjusting the volume of Wash Buffer.

Enrichment of extracellular vesicles (EVs), and depletion of high abundance plasma proteins

10. Add the diluted plasma to the equilibrated Mag-Net™ HP beads
11. Mix for 30 minutes
12. Transfer beads/separate beads on magnet and remove supernatant



13. Remove flow-through containing unbound soluble plasma proteins
14. Add 400 μL **Wash Buffer** – reduce volumes accordingly for low volume plates
15. Mix for 1 minute
16. Transfer beads/separate beads on magnet and discard the supernatant

Vesicle lysis and protein solubilization



17. Add 75 μL of **Lysis Buffer** (supplemented with 5mM TCEP and 10mM CAA)
18. Incubate for 60 minutes at room temperature with mixing

Binding of solubilized proteins



19. Add 225 μL of **Protein Binding Buffer** to the reduced and alkylated protein lysate
20. Mix for 30 minutes
21. Transfer beads/separate beads on magnet and discard supernatant

Protein washing (Position 5 and 6 on KingFisher™ diagram):

22. Add 500 μL of 95% MeCN – reduce volumes accordingly for low volume plates
23. Mix for 1 minute
24. Transfer beads/separate beads on magnet and discard supernatant
25. Repeat steps 22-24 for a total of 2 washes with 95% MeCN

Protein digestion

26. Add 200 μL of digestion mix containing 50 mM TEAB pH 8.5 (or alternative buffer) and proteolytic enzymes to the beads (trypsin alone or trypsin in combination with Lys-C)
Note: Protein recoveries are dependent on the plasma source used. For plasma collected using the EDRN protocol, protein recoveries are approximately 10 μg when starting with 20 μL of plasma. We recommend using 500 ng of Trypsin (optional: add 125 ng of Lys-C per digest). The proteolytic enzymes may be pre-mixed with 50 mM TEAB pH 8.5 to create a digestion mix, or dispensed separately to achieve a total digest volume of 200 μL .
 Incubate for 2 hours at 47°C whilst gently mixing or alternatively 4 hours at room temperature
Note: The volume of the solution may be reduced following the digestion step due to evaporation at elevated temperature. For example, in a KingFisher™ system the volume may reduce from 200 μL to ~180 μL .
27. Quench digestion by adding 5% TFA to a 1% final (addition of approximately 45 μL of 5% TFA)
28. Mix for a further 5 minutes
29. Transfer beads/separate beads on magnet and **recover supernatant** into a new microplate well
Recommended: Place plate with eluted peptides on a 96-well pin magnet and transfer eluates to a new 0.5 mL protein LoBind® tube or preferred plate for storage.
30. Freeze and store peptide samples at -80°C for later analysis.

Notes for LC MS Analysis

31. It is advisable to perform peptide desalting if LCMS is being performed without a pre-concentration trap column. If using an Evosep One upfront of the mass spectrometer, proceed with Evotip loading (you do not have to dry the peptides to reduce the volume which may be required for alternate LC systems depending on limitations of the injection volumes).
32. Peptides can be quantified prior to LCMS analysis to determine optimal loading, alternatively pool the peptides and inject 10-20 μL onto your LCMS system, thereafter, adjust the loads across all samples depending on the desired total ion chromatogram for your particular LCMS method.

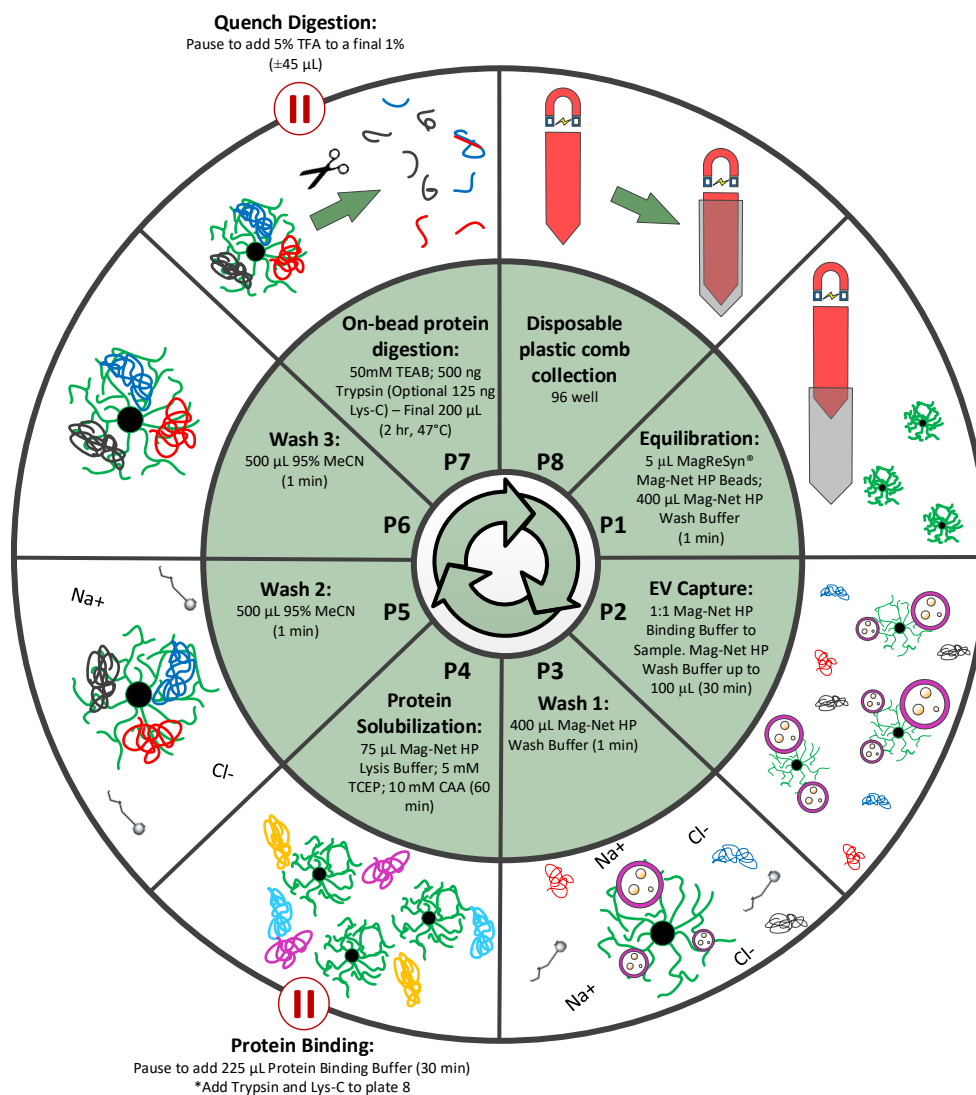


Figure 1: Overview of the automated Mag-Net HP workflow using the KingFisher Flex layout as an example.

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