An EasyPep Magnetic Solution for Automated Proteomics Sample Preparation

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Abstract

Purpose: Develop a new EasyPep Magnetic sample preparation workflow using magnetic clean-up beads for automated proteomics sample preparation.

Methods: The Thermo Scientific™ EasyPep™ Magnetic MS Sample Prep Kit contains pre-formulated buffers, MS-grade enzyme mix, magnetic beads for peptide clean-up, and an optimized protocol to generate MS-compatible peptide samples.

Results: The EasyPep Magnetic solution enables rapid and efficient processing of cultured mammalian cells, plasma, and tissues for MS analysis in less than 3 hours. The kit is optimized to process protein samples from 1-1500 µg with a high yield of peptides in less than 3 hours that can be directly injected into liquid chromatography-mass spectrometry (LC-MS) without the need for SpeedVac.

Introduction

Magnetic methods are becoming more widely adopted for proteomics sample preparation due to the ability to automate protein clean-up. However, these methods face challenges related to sample and reagent compatibility, different magnetic bead options, and lack of standardized procedures. To address these challenges, we introduce a new EasyPep Magnetic sample preparation workflow utilizing a novel magnetic bead for streamlined and automated proteomics sample preparation. Compared to other magnetic resins, our resin exhibits excellent compatibility with a wider range of solvents (water, acetone, acetonitrile, methanol), pH (pH 3-12), and is free from leachables. We optimized the peptide binding and elution for different sample input amounts (1µg-1.5mg) and preparation of up to 96 samples using KingFisher and Hamilton automated systems in under 3 hours. Validation studies using mammalian cells, plasma, and tissue samples (10-100µg) demonstrated exceptional digestion efficiency with a missed cleavage rate of ~10% and complete cysteine reduction and alkylation. Notably, the eluted peptides can be directly injected into liquid chromatography-mass spectrometry (LC-MS), eliminating the conventional and time-consuming SpeedVac drying and reconstitution steps. Our sample preparation process exhibits high robustness and reproducibility with peptide and protein identifications having less than 5% CV and quantification of protein abundances showing less than 10% CV. The EasyPep Magnetic workflow was applied to the quantitative analysis of colon tumor/normal formalin-fixed, paraffin-embedded (FFPE) tissues, helping ensure exceptional quantification reproducibility and validation against known protein markers. To enhance the sample throughput of our automation solution, we integrated tandem mass tag (TMT™) for multiplexed proteome analysis.

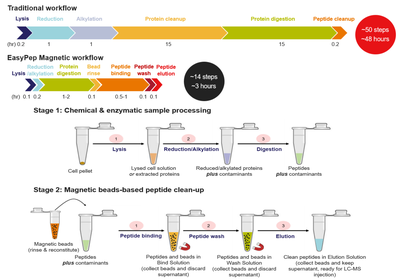

Figure 1. Schematic of EasyPep Magnetic proteomic sample preparation workflow. The workflow is optimized to process protein samples with a high yield of peptides that are MS-ready to inject without the need for SpeedVac within 3 hours. The EasyPep Magnetic MS Sample Prep Kit can process samples manually or through automation using an instrument such as the Thermo Scientific™ KingFisher™ Apex system.

Materials and methods

Various samples including mammalian cells, plasma, tissue, and bacteria were prepared following the EasyPep Magnetic MS Sample Preparation Kit protocol. A Thermo Scientific™ Vanquish™ Neo UPLC system equipped with a Thermo Scientific™ EASY-Spray™ Pepmap™ Neo (75 µm x 50 cm) coupled to a Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer or a Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer was used for the analysis of prepared peptides with data-independent acquisition (DIA) method. A Thermo Scientific™ Dionex™ Ultimate™ 3000 Nano LC system (75 µm x 50 cm) coupled to a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer using a 120 min gradient was used for analysis of FFPE peptides with data-dependent acquisition (DDA) method and TMT/TMTpro-labeled peptides. Raw files were analyzed using Thermo Scientific™ Proteome Discoverer™ (PD) 3.0, Spectronaut 18, and CHIMERYS™ search algorithm on PD 3.1.

Results

Benchmark of EasyPep Magnetic vs. AccelerOme Automated

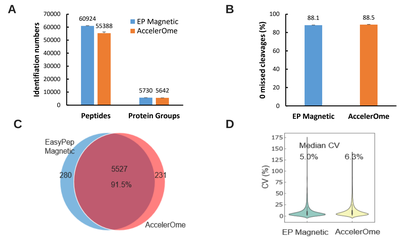

Figure 2. Benchmark of EasyPep Magnetic method against AccelerOme Automated method. (A) Comparable protein IDs; (B) High digestion efficiency (>88% zero peptide missed cleavages) using 1-hour digestion; (C) High overlaps of quantifiable proteins (>91%); (D) Good quantification reproducibility with median CV of protein abundances for both methods (<10%). In this experiment, digest from 25 µg of HeLa cell lysate was analyzed by Exploris 480 with DIA method.

Benchmark of EasyPep Magnetic vs. SP3

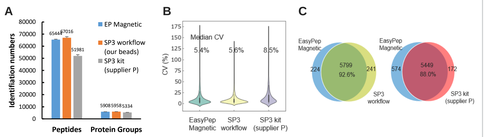

Figure 3. Benchmark of EasyPep Magnetic method against SP3 method using the same magnetic beads we developed and using an SP3 kit from a competitor. (A) The IDs were comparable between the EasyPep Magnetic method and the SP3 method using the same magnetic beads; >25% peptides and >10% proteins were observed compared to the SP3 kit method (supplier P). (B) The median CVs of protein abundances were <10% for all methods, indicating good quantification reproducibility. (C) The overlaps of quantifiable proteins between the EasyPep Magnetic and SP3 methods using the same magnetic beads and using the SP3 kit were ~93% and ~88%, respectively. In this experiment, digest from 50 µg of HeLa cell lysate was analyzed by Exploris 480 with DIA method. The digestion time was 1 hour for the EasyPep Magnetic method and 5 hours for the SP3 methods.

Sample Compatibility

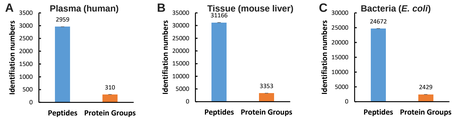

Figure 4. Performance of the EasyPep Magnetic for processing samples including (A) plasma, (B) tissue, and (C) bacteria. Reproducible peptide and protein identifications could be achieved for complex protein samples. The input amount was 25 µg per sample and digest was analyzed by Exploris 480 with DIA method.

Sample Preparation Range (1 µg-1.5 mg)

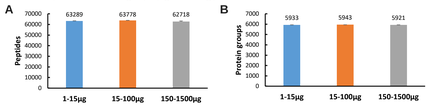

Figure 5. Performance of sample preparation for microgram (1-15µg) and milligram (0.15-1.5mg) sample range. Consistent peptide (A) and protein (B) identification numbers were obtained across the whole sample prep range. HeLa cell lysate and Exploris 480 with DIA method were used in this experiment.

KingFisher Automated Magnetic Beads-based Peptide Cleanup

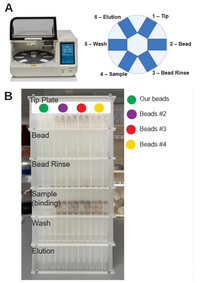

Table 1. KingFisher plate setup protocol

Plate #	Plate Name	Content	Volume
1	Tip plate		
2	Bead plate	20 µL Magnetic Beads in 80 µL Binding solution	100 µL
3	Bead wash plate	Binding solution	200 µL
4	Sample plate	Peptides in Binding solution	250 µL (15-30 µg); 500 µL (30-60 µg) and 1000 µL (60-150 µg)
5	Wash plate	Wash solution	500 µL
6	Elution plate	Elution solution	50-150 µL

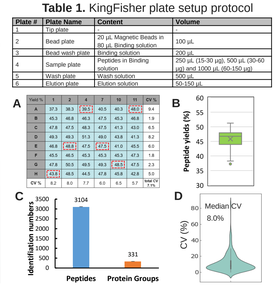

Figure 6. Automated EasyPep Magnetic solution using a KingFisher system for sample preparation. (A) The deck layout of a KingFisher™ Apex Magnetic Particle Processor system. (B) Evaluation of the compatibility of multiple magnetic beads. Our magnetic beads show the best compatibilities with plastic, organic solvents, and pH, while significant surface adsorption loss was observed using the other commercial magnetic beads (Beads #2, #3, #4), especially during the peptide binding step.

Figure 7. KingFisher automated system for high-throughput plasma sample preparation. (A) Peptide yield from each plate well. The CVs per row and column were <10%. Six samples marked in red were randomly selected for LC-MS analysis. (B) Peptide yield distribution. The average peptide yield was 47% and the CV of 48 samples was 7%. (C) IDs. ~3100 unique peptides and ~330 proteins per sample were identified with CVs <5%. (D) Protein quantification. The median CV of protein abundances across 6 samples was <10%, indicating good quantification reproducibility. Plasma (25 µg) digest was analyzed by Exploris 480 with DIA method.

Hamilton Automated Sample Processing

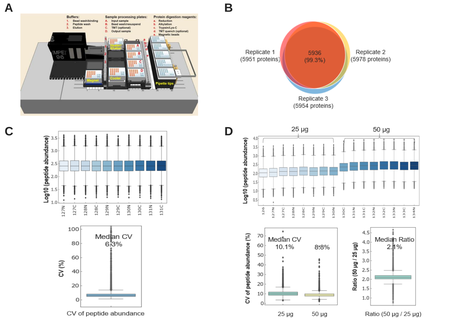

Figure 8. Hamilton automated system for end-to-end sample preparation. In this experiment, 25 µg and 50 µg of HeLa cell lysates were processed using a Hamilton automated system for both label-free (Exploris 480 with DDA method) and TMT/TMTpro-labeling (Q Exactive Plus) quantification. (A) The deck layout of the Hamilton system for end-to-end label-free and TMT/TMTpro-labeling sample processing. (B) Overlap of identified protein groups among three replicates for label-free protein quantification. (C) TMT quantification. Peptide abundances across all TMT channels were distributed consistently and the CV of peptide abundances was <10%. (D) TMTpro quantification. Peptide abundances across all TMTpro channels within the same input protein amount were distributed consistently. CVs of peptide abundances within the same input protein amount were <10%. The fold of peptide abundances between the 50 µg and the 25 µg input proteins was ~2 (theoretical ratio: 2:1). The results demonstrate excellent quantification precision and accuracy.

Quantitative Analysis of Prepared FFPE Tissues

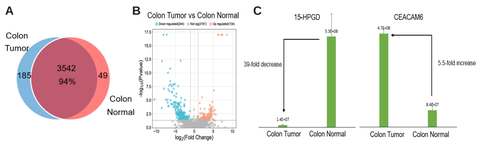

Figure 9. Quantitative analysis for EasyPep Magnetic prepared colon tumor and colon normal FFPE tissues. In this experiment, paraffin was removed using xylene and sequential ethanol washes. Q Exactive Plus with DDA method was used for FFPE digest analysis. (A) Overlap of quantifiable proteins between colon tumor and colon normal FFPE tissues. A total of ~3,700 quantifiable proteins were identified with ~94% overlap between colon tumor and normal FFPE tissues. (B) Volcano plot. 134 proteins were upregulated and 244 proteins were downregulated with >2-fold in colon tumor tissue compared to normal tissue. (C) Validation with known specific biomarkers. 15-HPGD protein, acting as a tumor suppressor and often suppressed in colorectal cancer, shows a 39-fold decrease in colon tumors compared to normal colon tissues. CEACAM6 protein, significantly upregulated in colon cancer tissues and closely associated with poor prognosis, shows a 5.5-fold increase in colon tumors compared to normal colon tissues.

Orbitrap Astral Analysis of 96 Plasma Samples

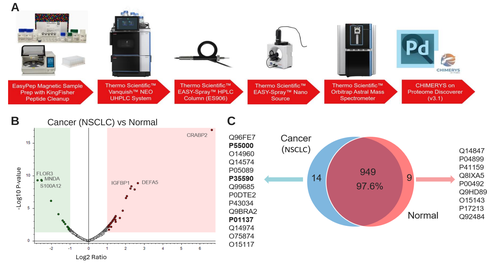

Figure 10. EasyPep Magnetic coupled with KingFisher automated system for preparing 96 plasma samples. The samples comprise plasma from 24 individual donors, with 12 classified as normal and 12 as non-small cell lung cancer (NSCLC) cases, each with 4 replicates. Plasma digests were injected randomly using a short gradient, allowing the analysis of 60 samples per day. Data analysis was conducted using the Astral analysis system with a data-independent acquisition (DIA) method, by analysis using CHIMERYS on PD3.1. Panel A depicts the Astral analysis system. Panel B presents the volcano plot displaying significantly differentially expressed proteins between NSCLC and normal groups, including known protein markers. Panel C illustrates the unique proteins identified from NSCLC and normal plasma donors, respectively. These analyzed proteins are consistent across all 12 individual donors from the NSCLC or normal groups.

Conclusions

- The EasyPep Magnetic sample preparation workflow enables rapid and efficient processing of mammalian cells, tissues, plasma, and other samples for MS-based proteomics.
- The EasyPep Magnetic MS sample prep kit is compatible with automation platforms such as KingFisher and Hamilton systems for high-throughput proteomic sample preparation.
- Our comprehensive EasyPep Magnetic solution can help maximize laboratory productivity while significantly improving the speed and reproducibility of high-quality proteomics sample preparation.

References

1. Hughes, C.S., Moggridge, S., Müller, T. *et al*. Single-pot, solid-phase-enhanced sample preparation for proteomics experiments. *Nat Protoc* 14, 68-85 (2019).
2. Waas M, Pereckas M, Jones Lipinski RA, Ashwood C, Gundry RL. SP2: Rapid and automatable contaminant removal from peptide samples for proteomic analyses. *J Proteome Res*. 18 (4), 1644-1656 (2019).

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