Nanotrap Protein Enrichment Affinity Kits for Protein Enrichment and Biomarker Discovery from Plasma Samples

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Product: Nanotrap® Protein Enrichment Affinity Kit

Introduction

Mass-spectrometry-based proteomic analysis of biological samples is a powerful tool for the identification of potential biomarkers. Plasma is a common sample type used in such proteomic analyses but can be challenging because of its complex composition and the presence of highly abundant proteins. For example, the 20 most abundant proteins account for 97% of the total plasma protein mass, which can mask the detection of lower abundance proteins.[1] Methods utilizing membrane filtration, fractionation, precipitation, or immunodepletion have long been used to improve the detection of moderate and low abundance proteins, but these methods are time-consuming and labor-intensive, among other drawbacks.[2,3,4] More recently, magnetic beads have been introduced for proteomic sample preparation, including those that rely on protein surface aggregation, ethanol-driven solvation capture, charge-based capture, or the use of the protein corona effect.

Here we introduce a new sample preparation tool for plasma-based proteomics that is simple, easy-to-use, and versatile–Nanotrap Protein Enrichment Affinity Kits. Nanotrap Protein Enrichment Affinity Kits use the Nanotrap magnetic hydrogel particle technology to capture and concentrate low abundance, low molecular weight proteins and peptides while simultaneously excluding higher molecular weight proteins.[4,5] The unique nature of Nanotrap Protein Particles, which are hydrogel particles functionalized with chemical affinity capture molecules, improves the detection of low-abundance proteins and peptides from plasma samples with a simple, 30-minute enrichment step prior to protein digestion.

Customer Benefits

- **Nanotrap Protein Enrichment Affinity Kits improve protein identification by three-fold compared to neat plasma samples.**
- **Nanotrap Protein Enrichment Affinity Kits have three different Nanotrap® Protein Particle chemistries available with unique protein binding profiles that can be used in combination or individually.**

Nanotrap Protein Enrichment Kits enable flexibility to choose which method is best for your targets. The user can select from three different Nanotrap Protein Particle chemistries, each with its own unique protein binding profile, which can be used individually or in combination with each other. For example, a plasma sample can be processed using any one of the three Nanotrap Protein Particle types, which we call the 1-particle method, as illustrated in **Figure 1A**. Or multiple aliquots from the sample can be processed in parallel using multiple Nanotrap Protein Particle types, which we call the 3-particle method or the 2-particle method, as illustrated in **Figures 1B and 1C**. If sample volumes are limited, or if there is a desire to limit the number of sample processing and LC-MS/MS runs per sample, all three Nanotrap Protein Particle types can be used together in a single aliquot from a sample, which we call the combined particle method, as illustrated in **Figure 1D**.

In this application note, we demonstrate how to use Nanotrap Protein Enrichment Kits to manually process plasma samples collected in K2EDTA blood collection tubes. We compare the number of unique protein

identifications obtained from a plasma sample using different Nanotrap Protein Particle workflows, as compared to the same plasma sample processed without using the Nanotrap Protein Enrichment Kit. We also compare overlap of unique protein identifications for these different workflows.

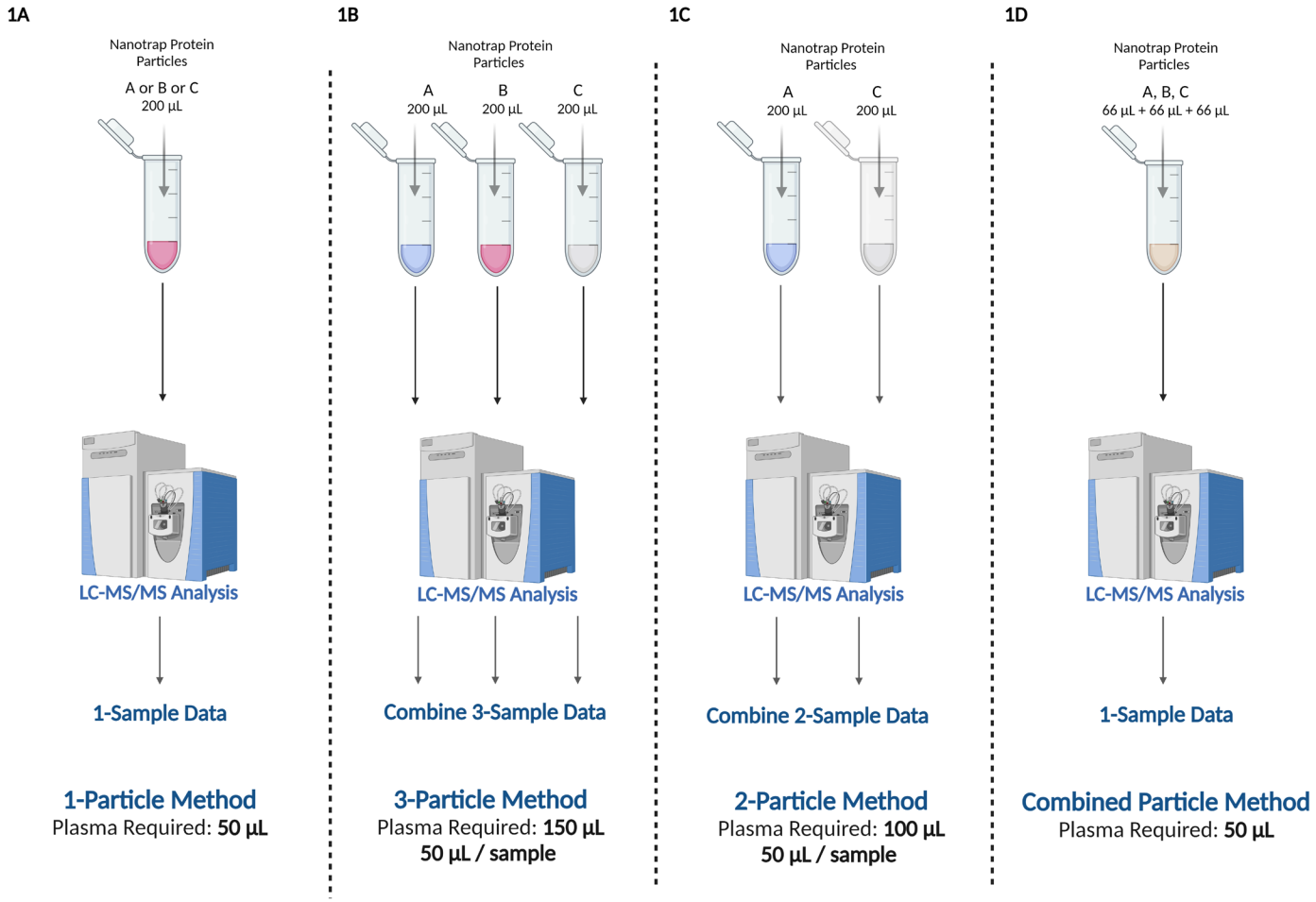


Figure 1A-D (left to right): Comparison of different Nanotrap PEAK workflows highlighting their plasma requirements and protein identification capabilities through LC-MS/MS analysis.

The figure outlines four workflow options for using Nanotrap Protein Enrichment Affinity Kits in protein discovery. In each workflow, Nanotrap Protein Particles and Nanotrap® Buffer 4 are added to the plasma sample for protein enrichment. Protein enrichment is followed protein digestion, peptide cleanup, and LC-MS/MS analysis. **1A:** The 1-particle method uses one of the three Nanotrap Protein Particle types with 50 µL of plasma. **1B:** The 3-particle method uses all three Nanotrap Protein Particle types, each with 50 µL of plasma, for a total of 150 µL. **1C:** The 2-particle method uses Nanotrap Protein A Particles and Nanotrap Protein C Particles, each with 50 µL of plasma, for a total of 100 µL of plasma. **1D:** The combined particle method uses all three Nanotrap Protein Particle Types in a single 50 µL plasma sample.

Methods

Product	Company Name	SKU
Nanotrap Protein Enrichment Kit - Discovery	Ceres Nanosciences	34200
Nanotrap Protein Enrichment Kit - A	Ceres Nanosciences	34210
Nanotrap Protein Enrichment Kit - B	Ceres Nanosciences	34220
Nanotrap Protein Enrichment Kit - C	Ceres Nanosciences	34230
Rapid Digestion–Trypsin/Lys-C Kits	Promega	VA1061
myBlock Mini Digital Dry Bath	Southern Labware	BSH200
Mini Centrifuge	Scientific Industries	WZ-MF6000
DynaMag™-2 Magnet	Thermo Fisher Scientific	12321D
Eppendorf™ Protein LoBind™ Tubes, 2 mL	Fisher Scientific	05-414-207
Vortex-Genie® 2 mixer	Sigmal-Aldrich	Z258423
Head for vortex mixer for 2 mL tubes	Millipore Sigma	CLS480101
Ammonium bicarbonate, BioUltra, ≥99.5%	Sigma-Aldrich	09830
500 nM Bond-Breaker™ TCEP Solution, Neutral pH	Thermo Fisher Scientific	77720
Pierce™ Alkylating Reagents, Iodoacetamide, No-Weigh™ Format	Thermo Fisher Scientific	A39271

Methods

Sample Preparation: Plasma Isolation From Whole Blood

Whole blood from healthy human donors was drawn into BD Vacutainer® K2EDTA anticoagulant. The whole blood was stored at +4 C for one day post-draw. Plasma was collected by centrifuging the BD Vacutainer® tubes at 1,300 g for 10 minutes, per the tube manufacturers' recommendations.

Reagent Preparation

A 5% solution of Trifluoracetic acid (TFA) was made by adding 5 µL of 99% TFA to 95 µL of HPLC-grade water. A 50 mM solution of ammonium bicarbonate, pH 7.8-8.2, was made by adding 0.197 grams of ammonium bicarbonate to 50 mL of deionized water.

Nanotrap PEAK Sample Processing Method

K2EDTA human plasma samples were thawed at room temperature and centrifuged at 5000 ×g for 2 minutes to remove debris. A 50 µL aliquot of plasma was transferred to a clean 2 mL Eppendorf™ Protein LoBind Tube and 150 µL of Nanotrap® Buffer 4 was added to dilute the sample. Prior to use, Nanotrap Protein Particles were resuspended until homogenous by vortexing briefly. For the "3-particle method" and "2-particle method," 200 µL of Nanotrap Protein Particles (A, B, or C) were added to each 50 µL plasma sample. When utilizing the combined-particle ethod, 66 µL of each Nanotrap Protein Particle type were added directly to a single 50 µL plasma sample along with 150 µL of Nanotrap Buffer 4 . The sample was vortexed for one minute after the addition of each particle type. The mixture was vortexed to ensure complete resuspension of the Nanotrap Particles and incubated for 30 minutes at room temperature on a vortex mixer to facilitate the protein enrichment. After 30 minutes, the Nanotrap Particles were pelleted using a Dynamag™-2 Magnet, and the supernatant was removed.

The Nanotrap Particles were washed twice with 500 µL of deionized water. Supernatent was removed using a Dynamag-2 Magnet. The Nanotrap Particle pellet was then resuspended in 100 µL of 50 mM ammonium bicarbonate (pH 7.8 - 8.2), followed by the addition of 1 µL of 500 nM Bond-Breaker™ TCEP Solution. The sample tubes were incubated at 37°C for 30 minutes using a Mini Digital Dry Bath. After 30 minutes, the sample tubes were removed from the Mini Digital Dry Bath and set in a rack on a laboratory bench to cool to ambient temperature. Once the sample tubes were cooled to ambient temperature, one unit of Pierce™ Alkylating Reagents, Iodoacetamide, No-Weigh™ Format was resuspended in 132 µL of 50 mM ammonium bicarbonate. Next, 5 µL of the freshly prepared iodoacetamide solution was added to each sample tube, with a subsequent 30-minute incubation at room temperature, protected from light.

After 30 minutes, the sample tubes were placed on a Dynamag-2 Magnet and the supernatant was discarded. The resulting Nanotrap Particle pellet was washed with 500 µL of 50 mM ammonium bicarbonate, followed by two 500 µL deionized water washes. The protease was prepared by resuspending 100 µg of Promega Rapid Trypsin/Lys-C Mix in 100 µL of Promega Resuspension Buffer to a final concentration of 1 mg/mL. Next, 35 µL of Promega Rapid Digest Buffer, 10 µL of deionized water, and 5 µL of the resuspended protease were added to the Nanotrap Particle pellet and vortexed. The sample tube was incubated at 70°C for 2 hours in a Mini Digital Dry Bath. The reaction was terminated by adding 2 µL of 5% TFA, after which the digested peptides were separated from the Nanotrap Particles using the Dynamag-2 Magnet. The final peptide mixture was desalted utilizing ZipTip™ Pipette Tips with 0.6 µL C_{18} resin. The desalted peptides were concentrated using a SpeedVac and then stored at -80°C until ready for analysis using LC-MS/MS.

Neat Samples Processing Method

As a comparison method, neat plasma samples were processed from the same lots of human K2EDTA plasma. Aliquots of each plasma sample were thawed at room temperature and centrifuged at 5000x g for 2 minutes. A 50 µL sample was mixed in a fresh tube with 150 µL of Promega Rapid Digest Buffer. Following this, 5 µL of resuspended Promega Trypsin/Lys-C Mix (1 mg/mL) was added to the sample. The mixture was briefly vortexed and then incubated at 70°C for two hours in a Mini Digital Dry Bath. After protein digestion, the reaction was terminated by adding 5 µL of 5% TFA. The final peptide mixture was desalted utilizing ZipTip Pipette Tips with

0.6 µL C_{18} resin. The desalted peptides were concentrated using a SpeedVac and then stored at -80°C until ready for analysis using LC-MS/MS.

LC-MS/MS Analysis

The desalted peptide samples were reconstituted in 20 µL of 0.1% formic acid for subsequent mass spectrometry analysis. All liquid chromatography-tandem mass spectrometry (LC-MS/MS) experiments were conducted on a Thermo Scientific Orbitrap Exploris™ 480 Mass Spectrometer, coupled with a nanospray EASY-nLC™ 1200 high-performance liquid chromatography (HPLC) system. Peptide separation was achieved using an Acclaim™ PepMap™ 100 C18 reversed-phase HPLC column, featuring a 3 µm particle size, 75 µm inner diameter, and 70 mm length. The mobile phase consisted of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in 80% acetonitrile (mobile phase B). Following sample injection, peptides were eluted using a linear gradient of 5% to 40% B over 90 minutes, followed by a rapid increase to 100% B over an additional 2 minutes at a flow rate of 300 nL/min.

The Orbitrap Exploris 480 was operated in data-dependent acquisition mode. A full MS scan was performed (m/z 300-1500) at a resolution of 60,000, followed by MS/MS scans of the most abundant ions. Ions were dynamically selected for fragmentation by higher-energy collisional dissociation with a collision energy of 27%. The "EASY-IC™," "Peptide Monoisotopic Precursor Selection," and "Dynamic Exclusion" (15-second duration) features were enabled, with only precursor ions of charge states +2 to +4 selected for fragmentation.

Tandem mass spectra were analyzed using Thermo Scientific™ Proteome Discoverer v.2.3 software, with searches performed against the NCBI human database. The SEQUEST algorithm was used with full tryptic cleavage constraints and dynamic methionine oxidation as a variable modification. Mass tolerances were set to 2 ppm for precursor ions and 0.02 Da for fragment ions. A 1% false discovery rate threshold was applied to report peptide-spectrum matches from the database search.

Results

Nanotrap PEAK Enriches Proteins from Plasma Samples

First, we processed the plasma sample using each of the three different Nanotrap Protein Particle types individually, as illustrated by the 1-particle method in **Figure 1A**, and compared the numbers of proteins identified to the same sample using the neat sample processing method. These results are all presented in **Figure 2**. All of the Nanotrap Protein Particles significantly improved the number of proteins identified compared to the neat samples, with Nanotrap Protein C Particles resulting in a 2.6-fold improvement, and Nanotrap Protein B Particles and Nanotrap Protein A Particles each resulting in 1.9-fold improvements. Further improvements are seen with multi-particle methods. When the data from samples processed in parallel using all three Nanotrap Protein Particles are combined, as illustrated by the 3-particle method in **Figure 1B**, the total number of unique proteins identified is 3.3-fold greater than those identified in the neat sample. Combining the data from Nanotrap Protein A Particles and Nanotrap Protein C Particles, as illustrated in the 2-particle method in **Figure 1C**, resulted in a 3.2-fold improvement in total unique proteins identified. The combined particle method, illustrated in **Figure 1D**, resulted in a 2.8-fold improvement in unique proteins identified compared to the neat sample.

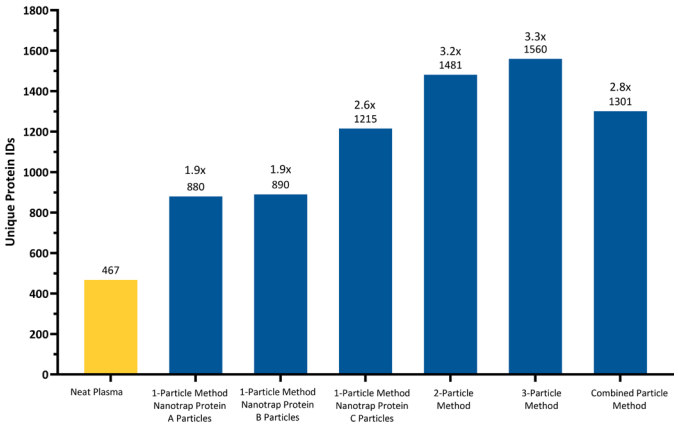


Figure 2: Comparison of unique proteins identified using different Nanotrap Protein Particle workflows

The figure compares the number of unique protein identifications (protein IDs) for each Nanotrap Protein Particle method and the fold-improvement in protein IDs for each method as compared to the neat sample. For the plasma sample used in this experiment, there were 467 unique proteins identified using the neat sample processing method. The 1-particle methods increased protein IDs by 1.9x (Nanotrap Protein A Particles), 1.9x (Nanotrap Protein B Particles), and 2.6x (Nanotrap Protein C Particles). Further improvements are seen with multi-particle methods. The 2-particle method increased protein IDs by 3.2x, the 3-particle method increased protein IDs by 3.3x, and the combined particle method increased protein IDs by 2.8x. Data end labels for each bar have the fold-improvement vs. neat plasma above the number of unique protein IDs. Each bar is an average of two biological replicates (i.e. two different 50 µL of plasma processed in replicate from enrichment through LC-MS/MS). Coefficients of variation for each set of replicates was less than 10%.

As shown in the Venn diagram in **Figure 3**, Nanotrap Protein C Particles enabled the largest number of unique proteins to be identified (33.9% of the total number of identified unique proteins), followed by Nanotrap Protein A Particles (6.6% of the total number of identified unique proteins), and Nanotrap Protein B Particles (5.1% of the total number of identified unique proteins). Fewer than 40% of the proteins identified were present across samples processed by each of the three particle types, demonstrating that each Nanotrap Protein Particle type has a unique binding profile.

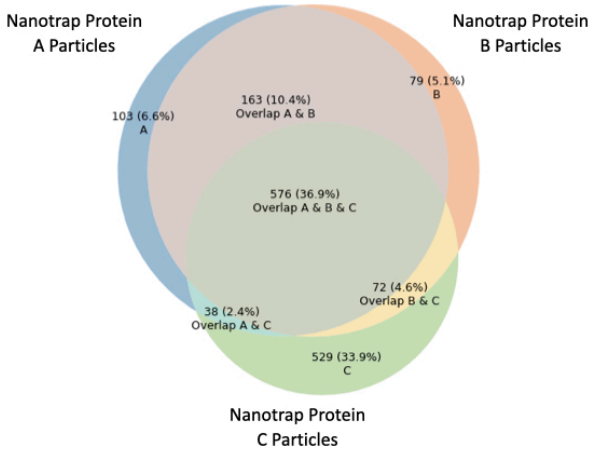


Figure 3: Venn diagram illustrating the distribution and overlap of unique protein identifications for the 1-particle method for each of the three Nanotrap Protein Particle types

The Venn diagram illustrates the overlap of unique protein identifications (protein IDs) for the 1-particle method using each of the three different Nanotrap Protein Particle types. Of the 1560 total proteins identified using the 1-particle method with each of the three Nanotrap Protein Particle types, 576 (36.9% of total) were detected in each method. Nanotrap Protein C Particles facilitated the identification of the largest number of unique proteins, with 529 proteins (33.9% of total). Nanotrap Protein A Particles facilitated the identification of 103 unique proteins (6.6% of total) and Nanotrap Protein B Particles facilitated the identification of 79 unique proteins (5.1% of total). Overlaps between particle pairs include 163 proteins (10.4% of total) shared between Nanotrap Protein A Particles and Nanotrap Protein B Particles, 72 proteins (4.6% of total) shared between Nanotrap Protein B Particles and Nanotrap Protein C Particles, and 38 proteins (2.4% of total) shared between Nanotrap Protein A Particles and Nanotrap Protein C Particles.

Further comparison of the 3-particle method **(Figure 1B)** and the combined particle method **(Figure 1D)** revealed a substantial overlap, with 74.9% of the proteins being identified by both methods. **(Figure 4A)** A comparison of the 2-particle method **(Figure 1C)** showed a 77.3% overlap in protein identification with the combined particle method. **(Figure 4B)** These results demonstrate that the choice of method can be guided by specific research goals, taking into account plasma volumes available for testing and the desired breath of protein coverage.

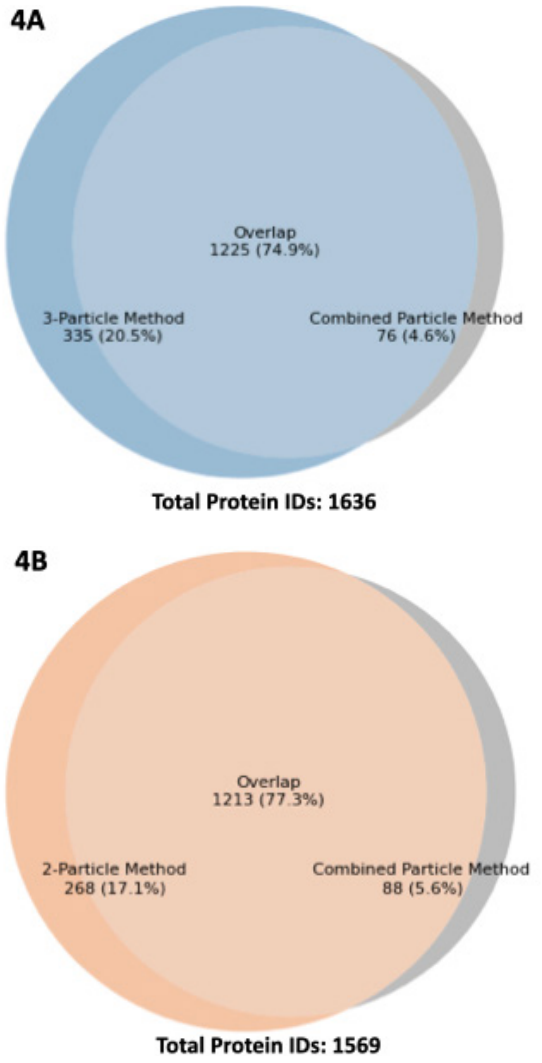


Figure 4A-B (top to bottom): 3-particle method, 2-particle method, and combined particle method have high overlap in unique proteins identified

This figure compares the similarity in protein identification between the 3-particle method, which utilizes 150 µL of human plasma; the 2-particle method, which utilizes 100 µL of plasma; and the combined particle method, which utilizes 50 µL of plasma. **4A:** There is a substantial overlap of 74.9% of the total proteins detected between the 3-particle method and the combined particle method, representing 74.9% of the total proteins detected. **4B:** Similarly, a comparison between the 2-particle method and the combined particle method shows an overlap of 77.3% in protein identification.

Conclusion

Nanotrap Protein Enrichment Kits are simple and easy to use. Mixing a plasma sample with Nanotrap Buffer 4 and the Nanotrap Protein Particle(s) of your choice and incubating for 30 minutes enriches low abundance proteins by more than 2-fold, compared to neat plasma. With multiple kit configurations available, users can choose which method is best for their targets.

In this application note, we showed that the 3-Particle Method, which uses each of the three Nanotrap Protein Particles types with 50 µL of plasma each, for a total sample volume of 150 µL of plasma processed, yielded the highest number of unique proteins, improving the number of proteins identified by 3.3 fold. The 2-Particle Method, which used two Nanotrap Protein Particles and 100 µL of plasma, improved the number of proteins identified by 3.2 fold. The Combined Particle Method, which used all three Nanotrap Protein Particles in a single 50 µL plasma sample, improved the number of proteins identified by 2.8 fold.

Nanotrap Protein Enrichment Kits represent a powerful and versatile tool for protein enrichment from plasma samples, offering significant advantages for biomarker discovery and detection. The use of Nanotrap particles not only increases the number of identifiable proteins but also offers flexibility in sample processing, allowing researchers to tailor their approaches based on specific study requirements, whether it be maximizing protein diversity or conserving sample volume. The particles' hydrogel polymer structure, functionalized with specific chemical baits, ensures high affinity for target analytes, making them an essential tool for overcoming the challenges posed by the complex nature of plasma samples.

References

1. Anderson, N. L. & Anderson, N. G. The human plasma proteome: history, character, and diagnostic prospects. Mol. Cell Proteom. 1, 845–867 (2002).

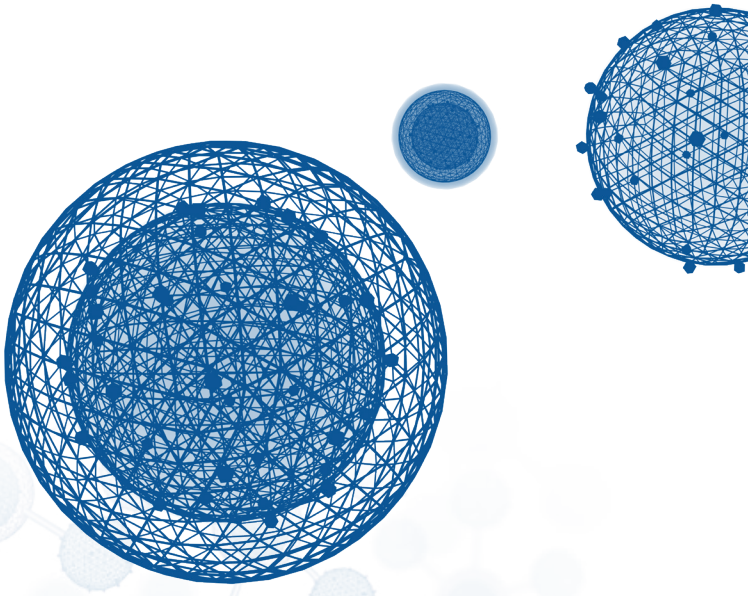
2. Nakayasu, E.S., Gritsenko, M., Piehowski, P.D. et al. Tutorial: best practices and considerations for mass-spectrometry-based protein biomarker discovery and validation. Nat Protoc 16, 3737–3760 (2021). https://doi.org/10.1038/s41596-021-00566-6

3. Yadav, A. K. et al. A systematic analysis of eluted fraction of plasma post immunoaffinity depletion: implications in biomarker discovery. PLoS ONE 6, e24442 (2011).

4. Brianna Kim, Robyn Araujo, Marissa Howard, Ruben Magni, Lance A. Liotta & Alessandra Luchini (2018): Affinity enrichment for mass spectrometry: improving the yield of low abundance biomarkers, Expert Review of Proteomics, DOI: 10.1080/14789450.2018.1450631

5. Shafagati, N., Patanarut, A., Luchini, A., Lundberg, L., Bailey, C., Petricoin, E., Liotta, L., Narayanan, A., Lepene, B., & Kehn-Hall, K. (2014). The use of Nanotrap particles for biodefense and emerging infectious disease diagnostics. *Pathogens and Disease*, 71(2), 164-176. https://doi.org/10.1111/2049-632X.12136

6. Figure 1 images - Created in BioRender, Patnaik, A. (2024). BioRender.com/q93t009



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