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Proteomics

High-throughput plasma proteomics for translational biomarker discovery using PreOmics sample preparation and the Orbitrap Astral mass spectrometer

Authors

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Goal

Evaluate and compare the analytical performance of PreOmics™ sample preparation kits —iST-BCT, ENRICH-iST, and ENRICHplus—in combination with the Thermo Scientific™ Orbitrap™ Astral™ Mass Spectrometer for blood plasma sample analysis. This study aims to demonstrate analytical reproducibility, plasma proteome depth of coverage, and the detection of biologically relevant protein signatures in translational studies using clinically relevant biological samples, highlighting the feasibility of this end-to-end workflow for biomarker discovery and clinical research applications.

Introduction

Blood plasma proteomics is a large pillar of biomarker discovery and clinical research due to its minimally invasive collection and the rich biological information plasma contains. This includes signaling molecules and tissue-derived factors that reflect physiological and pathological states.[1] Mass spectrometry (MS) offers a powerful, unbiased approach to analyze the plasma proteome, enabling insights into disease diagnosis, monitoring, and precision medicine. However, the inherent complexity of plasma and its vast dynamic range of protein concentrations spanning approximately 12 orders of magnitude pose significant analytical challenges, as high-abundance proteins frequently obscure the detection of low-abundance proteins that often hold key biological relevance.[2,3]

Advances in sample preparation and MS technologies have been critical to overcome these challenges and meet the demands of translational and clinical research. PreOmics has developed streamlined and reproducible sample preparation solutions—namely InterStageTip™ (iST) and ENRICH technologies that facilitate scalable plasma proteome profiling using liquid chromatography-mass spectrometry (LC/MS). Coupled with the Thermo Scientific™ Vanquish™ Neo UHPLC System and the Orbitrap Astral mass spectrometer, these technologies enhance proteome coverage through protein enrichment strategies, higher sensitivity, and faster throughput for biomarker discovery applications.

In this study, we evaluated three PreOmics™ sample preparation workflows—iST-BCT, ENRICH-iST, and ENRICHplus on a small, matched disease cohort comprising healthy, Alzheimer's disease, lung cancer, and colorectal cancer patients (Figure 1). Here, we demonstrate the synergistic performance of PreOmics sample preparation workflows with the Orbitrap Astral mass spectrometer, highlighting the depth of plasma proteome coverage, quantitative precision, and capability to uncover biologically relevant protein signatures and disease-associated pathways in clinically relevant and representative disease as compared to healthy plasma samples.

Plasma proteomics sample preparation methodology

Plasma proteomics relies on workflows that balance simplicity, coverage, and reproducibility. Here, we employ three different complementary strategies for evaluation:

iST-BCT (Neat Plasma)—a minimal-processing approach that preserves the full spectrum of plasma proteins while reducing sample loss and preparation-induced modifications. It is low-cost, highly reproducible, and fully automatable for large-scale studies. However, low-abundance proteins may be masked by highly abundant proteins, limiting proteome depth.

ENRICH-iST—a selective enrichment workflow that enhances access to the low-abundance plasma proteome while retaining a balanced representation of proteins. It provides greater depth and biological insight compared with neat plasma, without requiring sample fractionation. The method remains simple, flexible, and fully automatable for high-throughput studies.

ENRICHplus—an advanced enrichment strategy that further extends coverage into regions of the plasma proteome typically inaccessible with neat plasma or ENRICH-iST, including tissue leakage and signaling proteins. It maximizes low-abundance protein detection, improves biomarker discovery potential, and supports scalable, fractionation-free analysis. Like the other methods, it is fully automatable and suited for large-cohort studies.

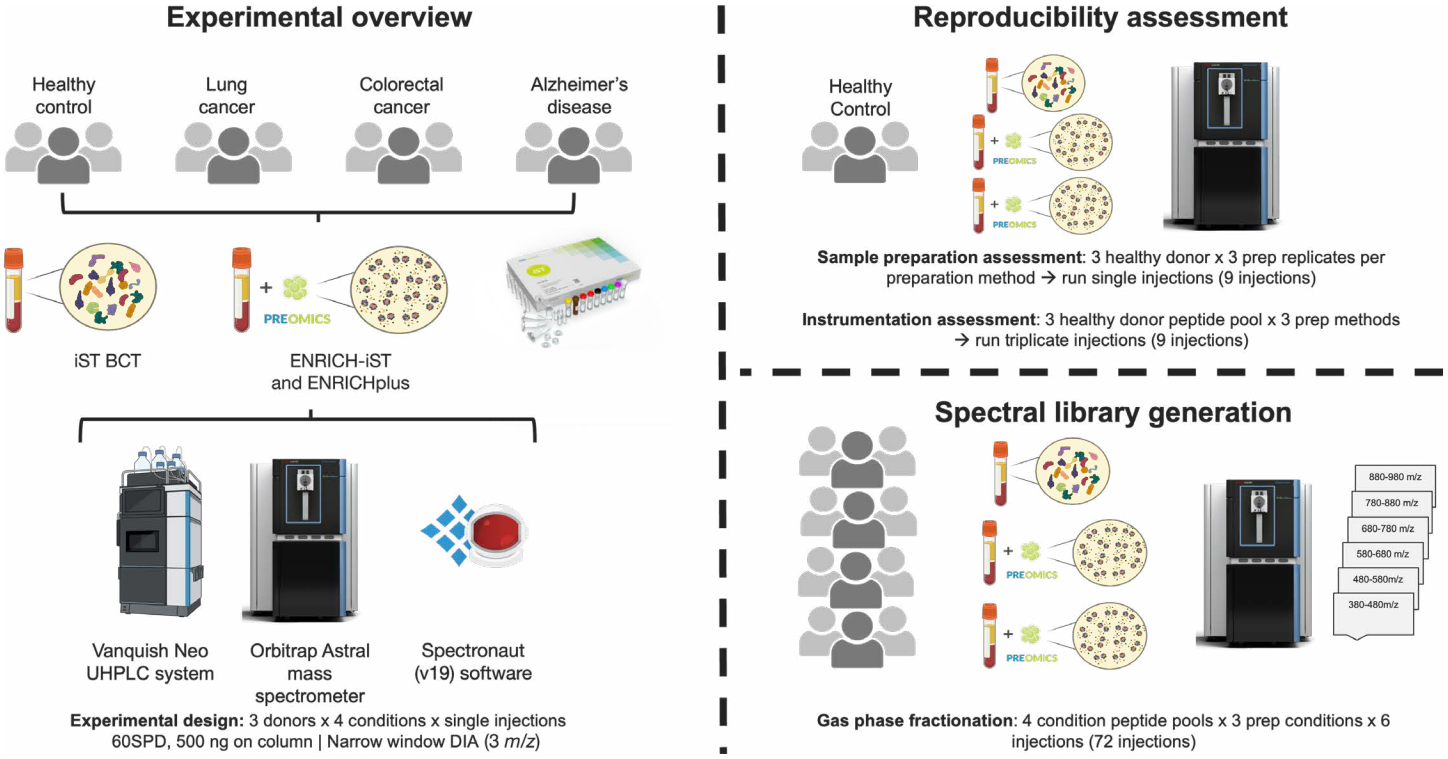


Figure 1. Experimental design. A multi-tiered experiment was developed to evaluate: (1) sample preparation reproducibility, (2) quantitative analytical measurement reproducibility, (3) the impact of gas phase fractionation (GPF) in spectral library generation, and (4) the capabilities of detecting differential biological signal in representative clinical research plasma samples.

Experimental

Common consumables

- Water with 0.1% formic acid (FA) (v/v), Optima™ LC-MS grade, Fisher Chemical™ (Part No. LS118-500)
- 80% Acetonitrile (ACN), 20% water with 0.1% formic acid, Optima™ LC-MS, Fisher Chemical™ (Part No. LS122500)
- Formic acid, 99.0+%, Optima™ LC-MS grade, Fisher Chemical™ (Part No. A117-50)
- Thermo Scientific™ Pierce™ Quantitative Fluorometric Peptide Assay Kit (Part No. 23290)
- Thermo Scientific™ SureSTART™ 9 mm Screw Caps (Part No. 6PSC9STB1)
- Thermo Scientific™ SureSTART™ 0.2 mL TPX Screw Top Microvial with Glass Insert (Part No. 60180-1655)
- PreOmics™ iST-BCT 96x sample preparation kit (P.O.00099)
- PreOmics™ ENRICH-iST 96x sample preparation kit (P.O.00164)
- PreOmics™ ENRICHplus 96x HT sample preparation kit (P.O.00216)

LC analytical and trap columns

- Thermo Scientific™ EASY-Spray™ HPLC Column, 2 µm C18, 1 50 µm × 15 cm (Part No. ES906)
- Thermo Scientific™ PepMap™ Neo Trap Cartridge, 5 µm C18, 300 µm × 5 mm (Part No. 174500)

Instrumentation

- Thermo Scientific™ Savant™ SpeedVac™ Concentrator
- Vanquish Neo UHPLC system
- Orbitrap Astral mass spectrometer
- Eppendorf™ ThermoMixer™ C temperature-controlled plate shaker

Sample preparation

K_2EDTA human plasma samples from healthy controls and age- and gender-matched patients with lung cancer, colorectal cancer, and Alzheimer's disease were procured from BioIVT. Single-spun plasma was used for healthy controls, lung cancer, and Alzheimer's disease samples, while double-spun plasma was used for colorectal cancer samples, based on sample availability. Before performing sample preparation, all plasma samples underwent an additional centrifugation step to remove remaining sample cellular debris and other insoluble components (7,500 x g, 10 min, 4°C). Plasma samples were then prepared using the iST-BCT, ENRICH-iST, or ENRICHplus sample preparation kits following the manufacturer's protocol recommendations. For iST-BCT, 1.5 µL of plasma was used, for ENRICH-iST, 20 µL of plasma was used, and for ENRICHplus, 50 µL of plasma was used. All sample preparation methods were digested for 3 hours using the ThermoMixer C. Purified peptides were then dried down using the Savant SpeedVac. After sample evaporation, samples were resuspended in 98% Water, 2% Acetonitrile, 0.1% Formic Acid, quantified using the Pierce Quantitative Fluorometric Peptide Assay kit, then normalized to 100 ng/µL prior to loading onto the LC-MS.

LC-MS analysis

All LC-MS runs for all plasma matched iST-BCT, ENRICH-iST, and ENRICHplus peptides were analyzed using a Vanquish Neo UHPLC system in trap and elute configuration, paired with an Orbitrap Astral mass spectrometer. Peptides were separated on the Vanquish Neo UHPLC system using an EASY-Spray HPLC column, and chromatographic gradients were formed using 0.1% formic acid in water for mobile phase A and 0.1% formic acid in 80% acetonitrile for mobile phase B. Liquid chromatography parameters and gradient settings can be found in Table 1. Mass spectrometer source parameters and scan parameters can be found in Table 2. GPF MS^1 and MS^2 parameters can be found in Table 3.

Table 1. HPLC gradient, configuration, and parameters.

60SPD			
Gradient	Time (min)	% Mobile phase B	Flow (µL/min)
	0	10	2.0
	0.3	10	2.0
	0.6	10	0.8
	13.6	22.5	0.8
	20.5	35.0	0.8
	20.9	55.0	2.0
	20.95	99.0	2.0
	22.35	99.0	2.0
LC parameters	LC configuration	Trap and elute	
	Fast loading/ Equilibration mode	Pressure control	
	Loading/ Equilibration/Wash pressure	Max pressure	
	Equilibration factor	3	
	Sampler temperature	7 °C	
	Mobile phase A/Weak wash	0.1% formic acid in water	
	Mobile phase B/Strong wash	0.1% Formic Acid in 80% Acetonitrile	
	Zebra wash	Enabled	
	Zebra wash cycles	4	
	Analytical column temperature	50°C	
Column specifications	Analytical column	EASY-Spray HPLC column, 2 µm C18, 150 µm × 15 cm (Part No. ES906)	
	Trap column	PepMap Neo Trap cartridge, 5 µm C18 300 µm x 5 mm, (Part No. 174500)	

Table 2. Orbitrap Astral MS parameters. (A) Global source and mass spectrometer parameters. (B) MS^1 full scan experiment parameters. (C) MS^2 DIA scan experiment parameters.

A

Global parameters (source and MS)	
Positive ion voltage	2100 Volts
Ion transfer tube temperature	290 °C
Expected peak width	10 seconds
Default charge state	2
Lock mass correction	Off

B

MS^1 full scan experiment parameters	
Orbitrap resolution	240 K
Scan Range (*m/z*)	380-980
RF lens (%)	40
Normalized AGC target (%)/Absolute AGC value	500% / 5.00e6
Maximum injection time	5 milliseconds
Microscans	1

C

MS^2 DIA scan experiment parameters	
Precursor mass range (*m/z*)	380–980
Isolation window (*m/z*)	3
Window placement optimization	On
AGC target	Custom
Normalized AGC target (%)/Absolute AGC value	500% / 5.00e4
Maximum injection time	7 milliseconds
DIA scan range (*m/z*)	150–2,000
HCD collision energy (%)	25
RF lens (%)	40
Loop control	Time
Time	0.6 seconds

Table 3. Orbitrap Astral MS GPF scan parameters. (A) MS full scan GPF full scan parameters. (B) MS^2 DIA GPF experiment parameters.

A

MS^1 full scan GPF experiment parameters	
Orbitrap resolution	240 K
Scan range (*m/z*)	380–480; 480–580; 580–680; 680–780; 780–880; 880–980
RF lens (%)	40
Normalized AGC target (%)/ Absolute AGC value	500% / 5.00e6
Maximum injection time	5
Microscans	1

B

MS^2 DIA GPF experiment parameters	
Precursor mass range (*m/z*)	380–480; 480–580; 580–680; 680–780; 780–880; 880–980
Isolation window (*m/z*)	1
Window placement optimization	On
AGC target	Custom
Normalized AGC target (%)/ Absolute AGC value	500% / 5.00e4
Maximum injection time	18 milliseconds
DIA scan range (*m/z*)	150–2,000
HCD collision energy (%)	25
RF lens (%)	40
Loop control	Time
Time	0.6 seconds

LC-MS data processing and analysis

All LC-MS data were processed using the Biognosys™ Spectronaut™ (v19) software with default settings. All sample preparation methods were searched separately to minimize any signal inferences made across different sample preparation workflows. A human UniProt database downloaded on 2024-07-29 containing verified sequences without isoforms (20,435 entries) was used for all analyses. All results were processed and filtered with a 1% precursor and 1% protein group false discovery rate (FDR). Exported output files were imported to RStudio (2023.09.0 Build 463) with R (v4.3.1) for downstream data analysis and visualization.

Results

Plasma proteome depth of coverage and analytical precision

The healthy control sample pool comprised three healthy donors, from which 1,124, 2,027, and 3,178 average protein groups corresponding to 6,789, 12,840, and 24,711 average peptides were identified, respectively, from iST-BCT, ENRICH-iST, and ENRICHplus across triplicate technical injections (Figure 2). With respect to measurement precision across sample preparation workflows, peptide coefficients of variation (%CV) were the following: iST-BCT: 12.0%; ENRICH-iST: 13.6%; ENRICHplus: 17.4%. For protein groups, measurement precision was 9.5%, 9.5%, and 10.5% CV for iST-BCT, ENRICH-iST, and ENRICHplus, respectively (Figure 2).

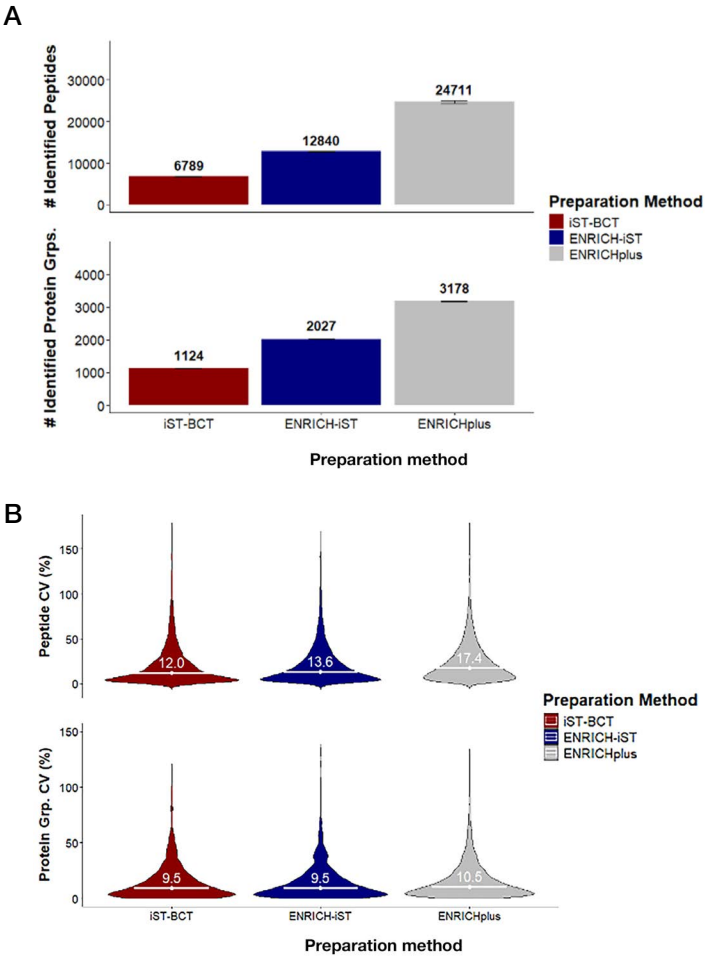
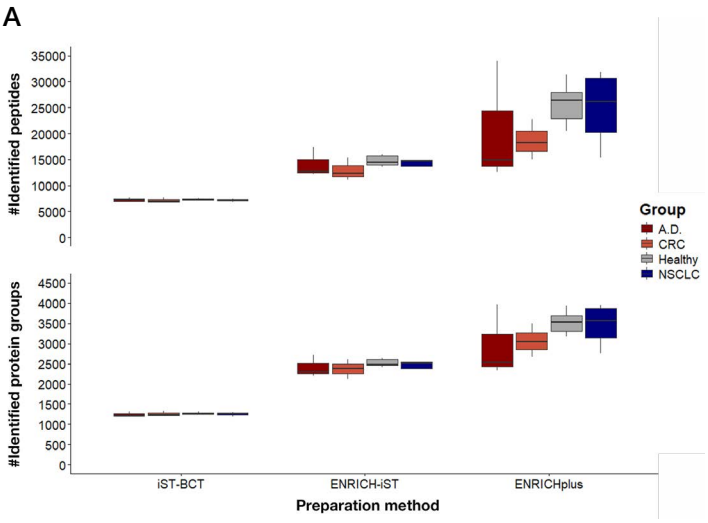


Figure 2. Evaluation of analytical measurement reproducibility. (A) Peptide and protein group identification performance and identification reproducibility across sample preparation methods from triplicate technical injections of a healthy donor peptide pool. (B) Peptide and protein group quantity coefficients of variation (%CV) across sample preparation workflows from triplicate technical injections of a healthy donor peptide pool.

Across the entire study, including all biological samples, 1,413, 2,972, and 4,438 protein groups were identified at least once using iST-BCT, ENRICH-iST, and ENRICHplus, respectively (Figures 3 and 4). Some subtle variations were noted across biological conditions, with most variability within and across biological groups found using ENRICHplus (Figure 3), suggesting that ENRICHplus may be most sensitive to picking up differences in individual samples based on pre-analytical factors, much like other deep discovery-based enrichment sample preparation workflows.[4]



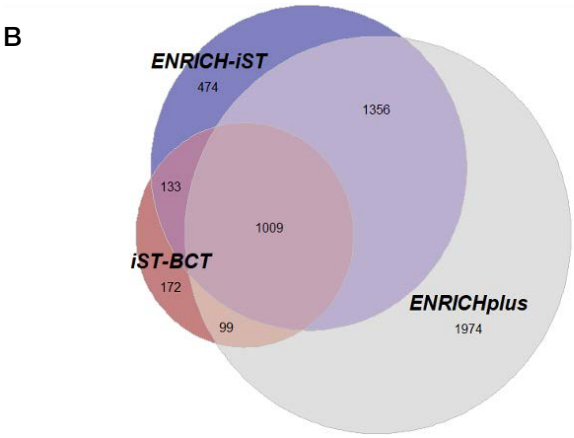


Figure 3. Overview of identifications across biological samples and complementarity among sample-preparation methods. (A) Peptides and protein group identifications across various biological samples and sample preparation methods. (B) Protein group overlaps and complementarity across all identified protein groups identified using each sample preparation method.

To evaluate the dynamic range of protein quantification achieved, protein groups identified were ranked by average protein group quantity across all samples, log transformed and plotted on a logarithmic scale. As shown in the rank plots, protein groups with low-to-mid quantities were more prevalent in the ENRICH-based workflows relative to neat plasma with iST-BCT (Figure 4). When all partially overlapping or fully overlapping protein groups were removed and the original ranking was preserved, low-to-mid quantity protein groups remained, demonstrating that lower abundant proteins have higher uniqueness across the workflows, with greater number of lower abundant proteins being detected in the ENRICH-based workflows (Figure 4).

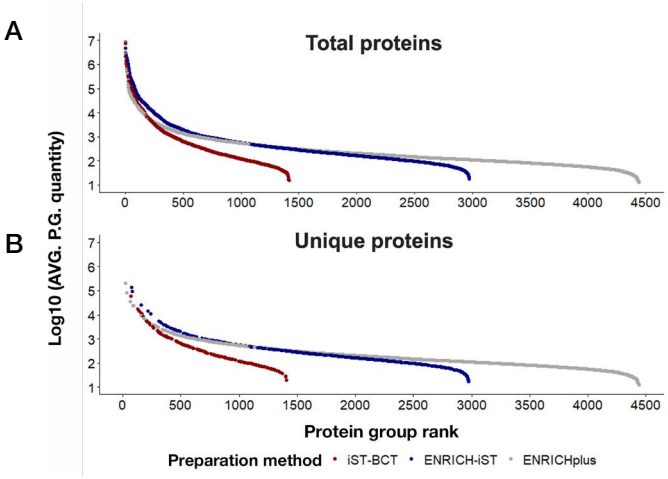


Figure 4. Protein Group Rank plots across sample preparation methods. (A) Protein group rank plot across each sample preparation method. (B) A protein group rank plot with all overlapping protein groups identified across multiple methods removed, demonstrating unique protein groups for each sample preparation method with conserved original ranks based on log transformed protein group quantities.

GPF spectral library performance and utility

By utilizing GPF on peptide pools from each biological condition, gains in spectral library composition from spectral libraries built with directDIA™ search archives in combination with GPF chromatogram libraries increased the number of peptides in spectral libraries from 11.4–20.6% depending on the sample preparation type, with the largest gains observed with iST-BCT. Across protein groups, 14.5–33.6% gains were observed, with the largest gains observed in the iST-BCT spectral library (Figure 5). When using the combined directDIA search archive + GPF spectral libraries in their respective sample preparation specific DIA searches, 3.7–16.5% increase in peptide identifications were observed, corresponding to 4.4–17.5% protein group increases. Overall, sample-specific GPF demonstrates value by increasing the number of peptides and protein groups in the spectral library, as well as increasing identifications across biological samples at the peptide and protein level, increasing proteomic depth of coverage. This further supports what has been shown in literature.[5]

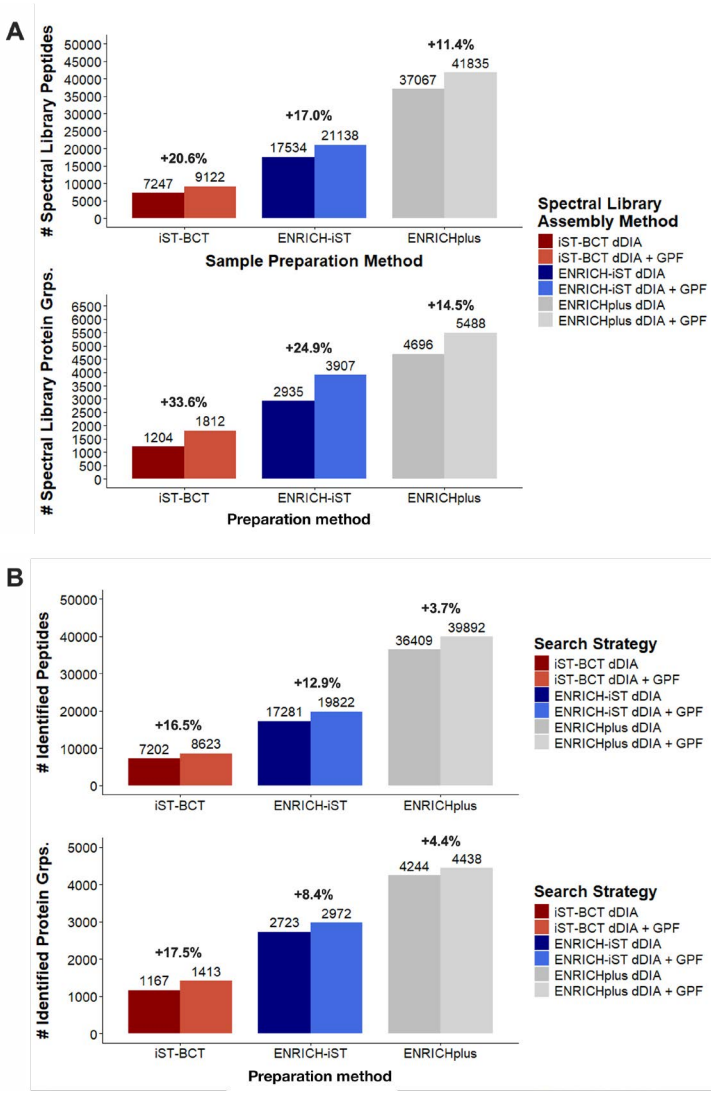


Figure 5. Identification increases in GPF spectral libraries and search implementation. (A) Peptides and protein groups in spectral library using library-free and GPF-assisted libraries across sample preparation methods. (B) Peptide and protein group identifications across experiments, including biological samples using library-free and GPF-assisted libraries across sample preparation methods.

Biological profiling across sample groups and disease states

To demonstrate the feasibility of using iST-BCT, ENRICH-iST, and ENRICHplus in combination with the Orbitrap Astral mass spectrometer for determining biological differences between healthy and disease states, non-small cell lung cancer vs. healthy samples were further evaluated. With Benjamini-Hochberg FDR adjusted differential abundance testing from unpaired T-tests, 157, 355, and 548 differentially abundant proteins were detected using iST-BCT, ENRICH-iST, and ENRICHplus, respectively (Figure 6). The expanded proteome depth using ENRICH technology allowed for the detection of more differentially abundant candidates, demonstrating the power of gaining more biologically relevant information in addition to increased plasma proteome depth of coverage. When comparing all differential candidates across all sample preparation workflows, 24 were mutually shared among iST-BCT, ENRICH-iST, and ENRICHplus (Figure 7). Additionally, 65 differential candidates were shared between iST-BCT and ENRICH-iST, 55 between iST-BCT and ENRICHplus, and 118 between ENRICH-iST and ENRICHplus.

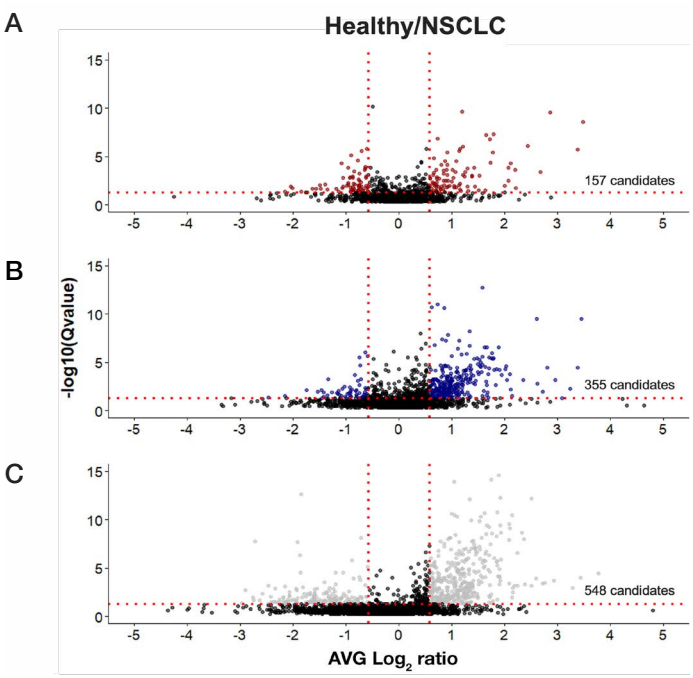


Figure 6. Volcano plots for Non-small cell lung cancer (NSCLC) across sample preparation methods. (A) iST-BCT (B) ENRICH-iST (C) ENRICHplus.

To assess the presence of enrichment bias that may alter the preservation of biological signals, we calculated fold change Pearson correlations across differential candidate overlaps from iST-BCT, ENRICH-iST, and ENRICHplus using $\log_2$ fold change values. For the 24 differentially abundant candidates identified across all three sample preparation workflows, the $\log_2$ fold change values from ENRICH-iST and iST-BCT showed a strong Pearson correlation of 0.96. In contrast, iST-BCT vs. ENRICHplus showed a correlation of 0.71, and ENRICH-iST vs. ENRICHplus showed a correlation of 0.72 (Figure 7). These results demonstrate that ENRICH-iST has very similar fold changes to iST-BCT, highlighting its ability to conserve biological information with minimal impact from enrichment. The Pearson correlations for ENRICHplus with iST-BCT and ENRICH-iST were 0.71 and 0.72, respectively, indicating its capability to preserve biological information while identifying many more differentially abundant candidates. This is due to the added proteomic depth of coverage and enhanced detection of low-abundance protein groups afforded by the unique physicochemical properties of the enrichment.

To further characterize the relationships among the workflows, we evaluated partial intersections where differential candidates were shared by two of the three workflows. Overall, iST-BCT and

ENRICH-iST had the highest Pearson correlation of 0.94 for all overlapping candidates and a correlation of 0.7 with ENRICHplus, suggesting that biological differences are conserved across both workflows, but more accurately in ENRICH-iST. ENRICH-iST and ENRICHplus had the highest number of differential candidate overlaps, with 118 overlaps, but had a Pearson correlation of 0.56, suggesting that each method may have different enrichment properties.

Furthermore, all sample preparation workflows identified similar classes of enriched pathways in non-small cell lung cancer across gene ontology, KEGG metabolic pathways, and Reactome pathways, further supporting the preservation of biological signals with ENRICH technology (Figure 8). Overall, detecting overlapping differential candidates highlights that changes at the biological level are preserved using enrichment techniques. The correlation between the overlaps validates that enrichment workflows not only enhance the depth of coverage of the plasma proteome but also provide accurate quantitation that reflects true biological differences.

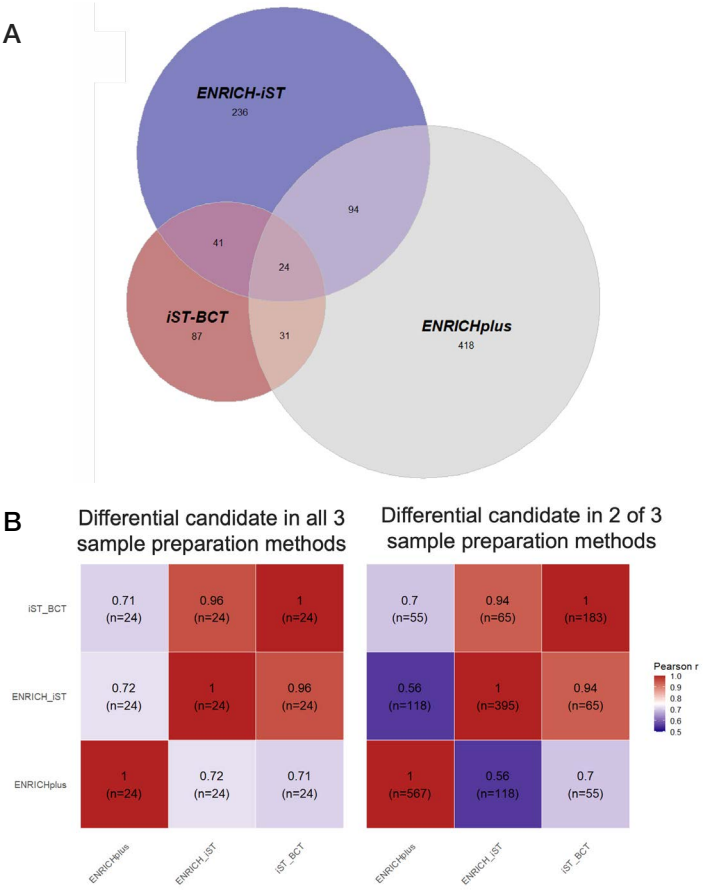


Figure 7. Non-small cell lung cancer differential candidate overlap evaluation. (A) Differential candidate protein group overlaps. (B) Log_2 fold change Pearson correlation matrices for complete (identified as a differential candidate in all 3 sample preparation types) or partial intersections (identified as a differential candidate in 2 of 3 sample preparation types).

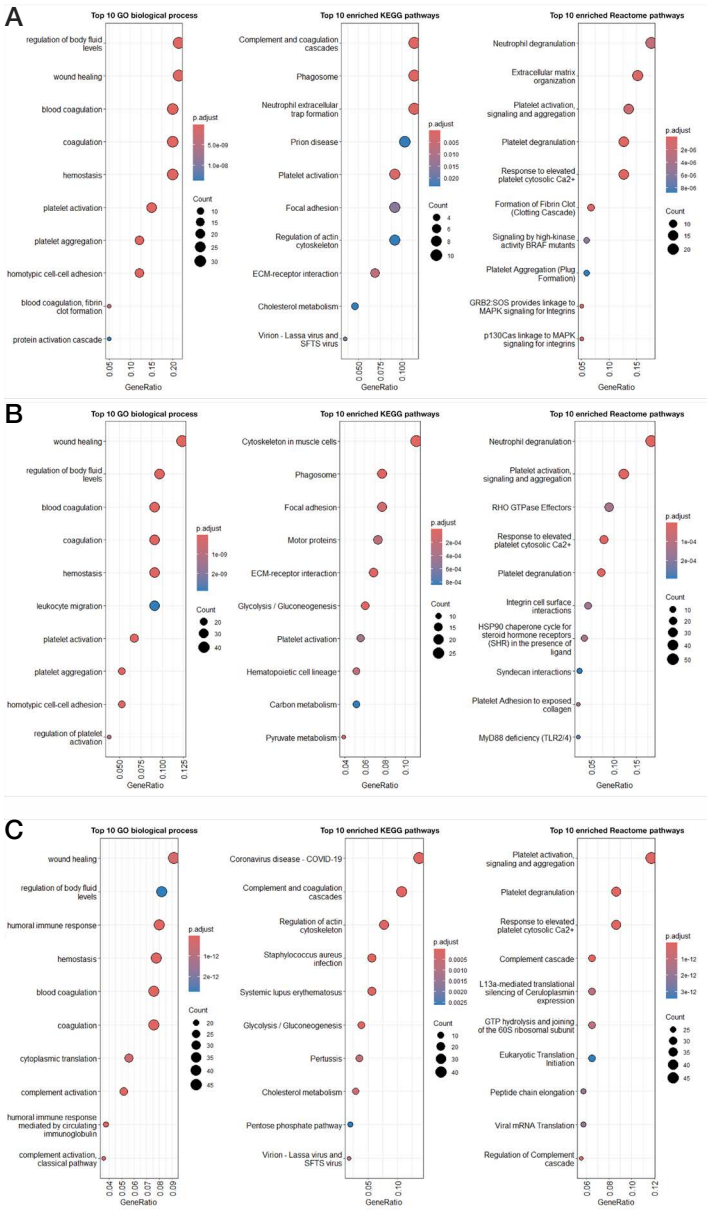


Figure 8. Non-small cell lung cancer differential candidate pathway enrichment across sample preparation methods. Gene Ontology (GO), KEGG, and Reactome pathway enrichment analyses were performed using Thermo Fisher's exact test, comparing the proportion of proteins in lung cancer candidate lists from iST-BCT (A), ENRICH-iST (B), and ENRICHplus (C) plasma preparations to background proportions. P-values were adjusted using the Benjamini-Hochberg method to control the FDR, with significance thresholds set at $p < 0.05$ and $q < 0.05$.

Conclusion

- Deep plasma proteome coverage: Identified 1,413, 2,972, and 4,438 protein groups using iST-BCT, ENRICH-iST, and ENRICHplus, respectively
- High quantitative precision: Peptide CVs of 12.0% (iST-BCT), 13.6% (ENRICH-iST), and 17.4% (ENRICHplus); protein group CVs of 9.5% (iST-BCT and ENRICH-iST) and 10.5% (ENRICHplus)
- Enhanced measurable dynamic range: ENRICH workflows detect more low-to-mid quantity protein groups, improving identification of lower abundant proteins
- Improved plasma proteome depth from GPF: Increased peptide identifications up to 16.5% and protein groups up to 17.5%, demonstrating utility of building sample-specific chromatogram libraries
- Preserved biological signatures: Detected 157, 355, and 548 differentially abundant proteins with high Pearson correlations (up to 0.96) in NSCLC vs. Healthy comparison, indicating preserved biological differences and quantitative accuracy with enrichment methods

Overall, the integration of PreOmics kits with the Vanquish Neo UHPLC system and Orbitrap Astral mass spectrometer significantly enhances plasma proteome coverage and measurement precision. This combination preserves critical biological information and ensures accurate quantitation of true biological differences. The powerful synergy between these technologies enables the detection of a greater number of differentially abundant proteins and improves the identification of low-abundance proteins. By coupling PreOmics sample preparation kits with the Orbitrap Astral mass spectrometer, researchers gain access to an end-to-end, robust, and scalable workflow that is ideally suited for translational biomarker discovery in complex clinical research plasma samples.

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