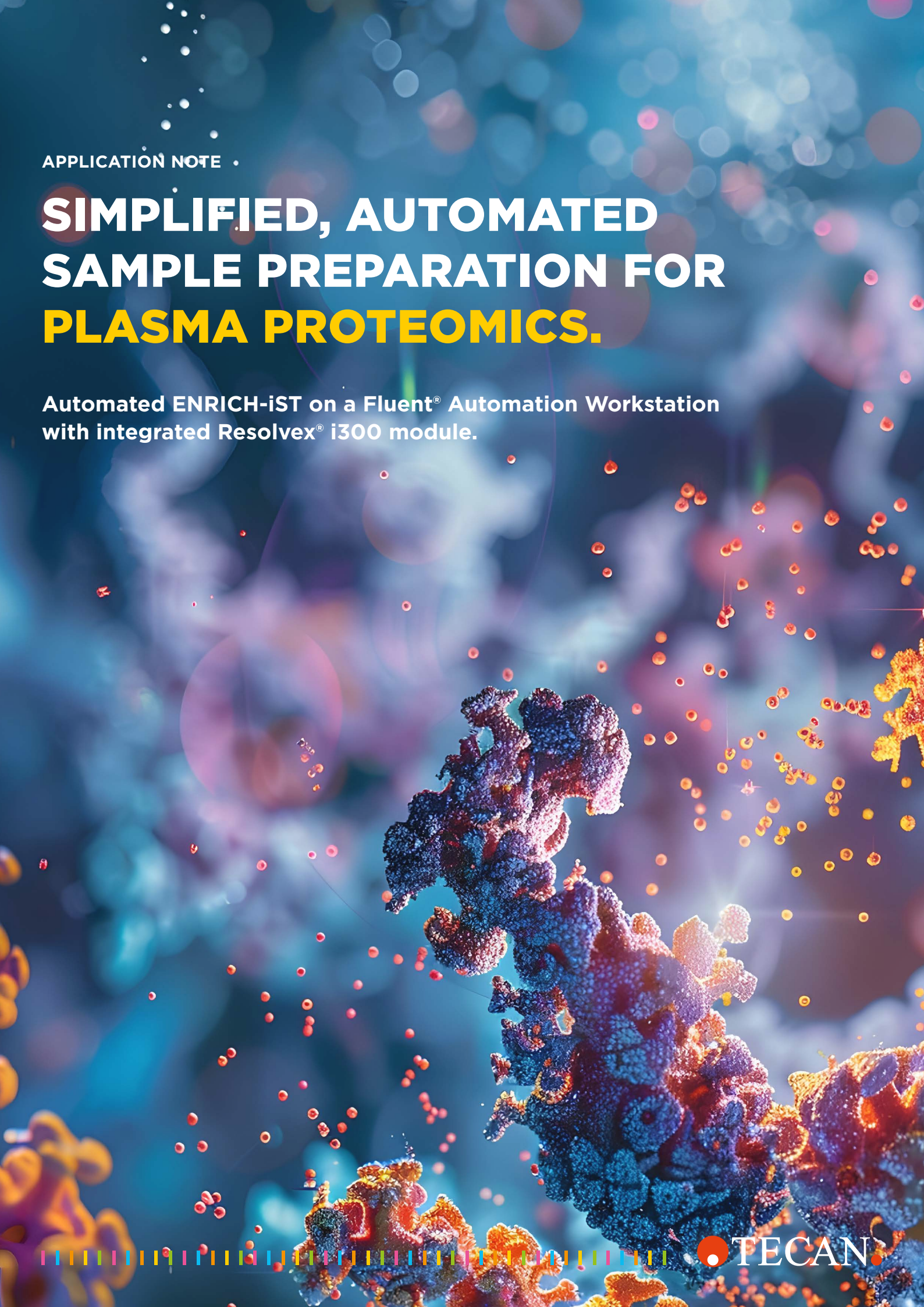
APPLICATION NOTE

SIMPLIFIED, AUTOMATED SAMPLE PREPARATION FOR PLASMA PROTEOMICS.

Automated ENRICH-iST on a Fluent® Automation Workstation with integrated Resolvex® i300 module.



TECAN

INTRODUCTION.

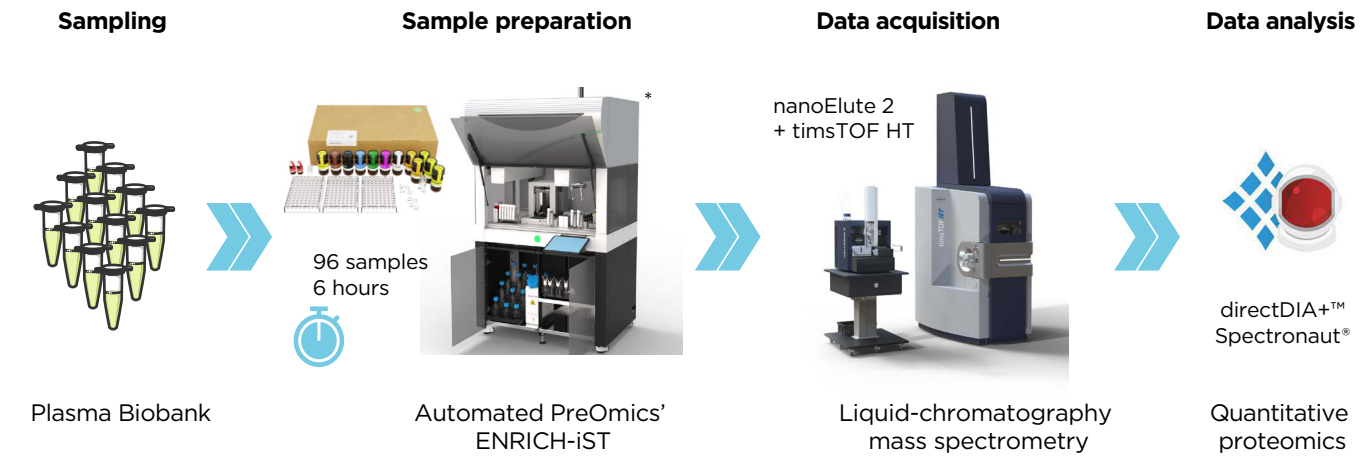
The plasma proteome provides a comprehensive snapshot of an individual's physiological state and is widely used to study human health and disease. The ready availability and informative nature of plasma make it an invaluable resource for the discovery of new biomarker proteins. Mass spectrometry (MS) is the gold standard for proteome analysis, due to its unparalleled sensitivity, specificity and ability to identify and quantify thousands of proteins simultaneously. Despite its immense potential, MS-based plasma proteomics faces several significant challenges, including the complexity and dynamic range of the plasma proteome. The wide dynamic range – up to 12 orders of magnitude – limits access to the full proteome, as low abundance proteins are masked by more abundant species. Sophisticated sample preparation techniques are therefore required to enrich low abundance proteins and ensure in-depth and accurate proteome profiling. However, manual processing of large sample cohorts requires considerable hands-on time, which can slow down the speed of studies and creates the risk of human error. Automation addresses these challenges to ensure sufficient throughput for large cohorts, offering both scalability and reproducible results.

PreOmics' ENRICH-iST workflow offers a standardized and simplified solution for high throughput and in-depth plasma proteome profiling. By capturing low abundance proteins onto paramagnetic beads, the ENRICH technology efficiently breaks the dynamic range while preserving valuable biological information. Upon enrichment, proteins are digested and purified for MS analysis using the iST workflow steps.

An automated ENRICH-iST workflow using the Fluent Automation Workstation and standalone Resolvex A200 positive pressure processor was previously compared with manual processing, demonstrating similar performance in terms of identification and quantification.[1] To further enhance the efficiency of the plasma sample preparation workflow, the ENRICH-iST process has now been fully automated by combining the Resolvex i300 module with the Fluent workstation. This allows direct peptide clean-up and drying on the Fluent system using integrated positive pressure processing, buffer dispensing and peptide sample dry-down by evaporation. This streamlined process starts with plasma samples and yields dried peptides. The automated workflow was rigorously evaluated through a multi-day analysis to assess its reliability and robustness, and further tested in a study examining age-related plasma proteome differences between two age groups.

METHODS.

The overall plasma proteomic analysis strategy is described in Figure 1. Briefly, plasmas samples were processed using the automated ENRICH-iST (PreOmics) sample preparation kit on the Fluent system. The samples were then subjected to liquid chromatography-MS (LC-MS) on the nanoElute-timsTOF HT system (Bruker) for in-depth proteomic characterization. Protein identification and quantification were achieved with the Spectronaut (Biognosys) software.



*Example picture for Fluent with integrated Resolvex i300

Figure 1: Plasma proteomic workflow. Plasma samples were processed with the automated ENRICH-iST workflow using a Fluent with integrated Resolvex i300. Samples were analyzed with dia-PASEF® acquisition on the timsTOF HT system. Data analysis was conducted with directDIA+ using Spectronaut.

Fluent configuration.

The ENRICH-iST 96x HT (PreOmics) sample preparation kit, supplemented with the iST-REG-PSI Buffer Add-on kit (PreOmics) for positive pressure-based peptide clean-up was automated on the Fluent workstation. The Fluent was equipped with an eight-channel Air Flexible Channel Arm™ (AirFCA), an independent Robotic Gripper Arm™ (RGA), integrated on-deck heating/shaking (Qinstruments BioShake D30-T elm), and a magnetic bead capture module (Alpaqua Magnum FLX®). The Resolvex i300 was also integrated onto the Fluent deck to provide positive pressure processing, dispensing and active evaporation functionalities. This setup enabled comprehensive end-to-end automation, from plasma samples to dried peptides.

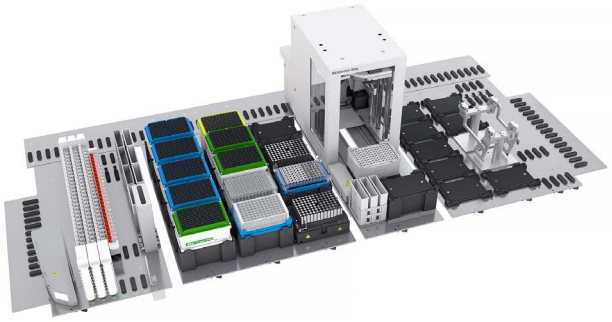


Figure 2: Fluent worktable configuration for ENRICH-iST. Magnetic bead-based enrichment, lysis, digestion and clean-up of plasma proteins was fully automated. Peptide desalting and subsequent dry-down was executed using the Resolvex i300 module.

Sample collection.

Whole blood samples were collected using EDTA as an anticoagulant. Following collection, samples were centrifuged at 2,000 x g (plasma used for multi-day study) or 5,500 x g (plasma used for the age cohort), and the plasma fractions were transferred to new tubes for subsequent storage.

Study design.

For the multi-day study, 24 replicates from one plasma sample (Donor A) were prepared as 60 µl aliquots, and stored at -80 °C until the day of the experiment. In total, three experiments were processed with the same plate layout on three consecutive days. Each experiment involved processing a full 96-well plate containing eight sample replicates distributed across the plate, plus 88 blank samples. For each day, five sample replicates and three adjacent blank wells were analyzed by LC-MS.

For the aging cohort, two age groups of male donors without a disease diagnosis were analyzed. The 'young' age group consisted of 18-27 year old donors and the 'older' age group consisted of 58-67 year old donors. Three samples per age group were provided by the blood donation center. For each donor, five replicates of 60 µl were provided for automated sample preparation, and processed together in a single sample preparation run.

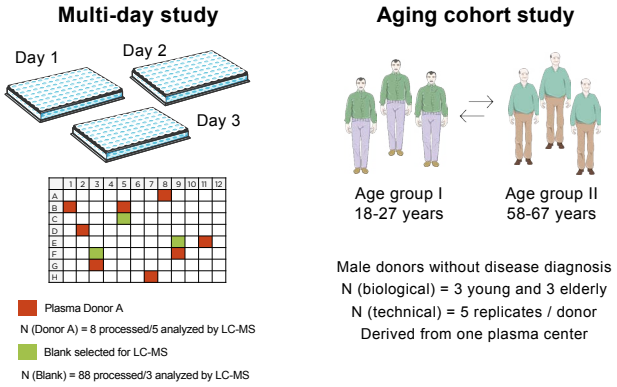


Figure 3: Overview of study design. **(Left panel):** For the multi-day study, the same plate layout was prepared on three consecutive days, each using 8 replicates of a single patient sample (Donor A). On each day, 5 sample replicates and 3 adjacent blank wells were analyzed by LC-MS. **(Right panel):** For the aging cohort study, samples were collected from 3 donors per age group, and five replicates from each donor were prepared using the automated workflow in a single run.

Sample preparation.

For both studies, 20 µl plasma per sample was subjected to automated ENRICH-iST processing. Briefly, the buffer, reagents and samples were prepared by the operator, and loaded onto the Fluent worktable. The ENRICH step was performed using the Fluent platform's liquid handling capabilities. The iST clean-up and peptide dry-down were conducted on the integrated Resolvex i300 module. Dried samples were then resuspended in LC-LOAD, normalized to the peptide concentration using absorbance measurements at 280 nm, and subjected to LC-MS/MS analysis.

LC-MS/MS analysis.

Peptide samples were separated on the nanoElute2 nanoflow liquid chromatography system coupled to a timsTOF HT via a CaptiveSpray ionization source (Bruker). 300 ng samples were injected in 1 µl of LC-LOAD, and the separation was performed using a 40 min acetonitrile gradient. The dia-PASEF acquisitions used a window placement scheme consisting of 10 TIMS ramps with 3 mass ranges per ramp – spanning from 400 to 1000 m/z – and a mobility range of 0.8 to 1.3 1/K0, with a cycle time of 1.17 seconds, including one MS1 frame.

Data analysis.

All dia-PASEF data were elaborated using Spectronaut® (v18, Biognosys), selecting the library-free directDIA+ workflow. Factory default settings were applied, and raw files were searched against the UniProt FASTA database of Homo sapiens (Swiss-Prot entries, downloaded 2022-02-14).

Materials.

The full configuration of the Fluent, its modules and its worktable accessories is available upon request. The consumables in Table 1 were used to process the samples.

Table 1: Consumables overview.

Purpose	Consumable	Supplier
Sample preparation kit	ENRICH-iST 96x HT (P.O.00165) iST-REG-PSI Buffer Add-on (P.O.00109)	PreOmics
Assay/ collection plates	Eppendorf™ Protein LoBind® Plate, 96/500 µl (0030504100)	Eppendorf
Reagent storage	2 ml screw-cap tube (72.694.700)	Sarstedt
Buffer storage	25 and 100 ml troughs (30055743, 10613049)	Tecan
Pipette tips	Tecan Pure Disposable Tips (50, 200 and 1,000 µl), (30057811, 30057814, 30057816)	Tecan

RESULTS AND DISCUSSION.

Manual vs. automated sample preparation.

Performance of the automated ENRICH-iST workflow was evaluated by comparing the results from manual and automated preparation, as outlined in a previous application note [1]. Results showed that the automation of the ENRICH-iST preparation technology on a Fluent workstation next to a stand-alone Resolvex A200 was successful, demonstrating high identification rates and reproducible quantitative performance. In the subsequent experiments reported here, the standalone Resolvex A200 and third party vacuum concentrator have been replaced with the integrated Resolvex i300 module, allowing fully automated iST-based peptide clean-up and peptide drying to minimize manual steps and reduce overall workflow time.

Multi-day analysis.

The multi-day analysis aimed to determine the intra-day reproducibility and inter-day repeatability to assess the overall robustness of the automated ENRICH-iST protocol. Plasma from a single donor was processed in three independent experiments on consecutive days with each experiment following the same plate layout. The total ion current (TIC) overlay of plasma sample from all three days indicated consistently clean sample preparation, resulting in reproducible chromatographic separation irrespective of the day (Figure 4A). Additionally, comparison of total protein abundance between plasma samples, adjacent blank samples, and an LC-LOAD buffer reference confirmed the absence of cross-contamination in the automated set-up (Figure 4B).

Investigation of the identified precursors and protein groups across the three runs revealed consistently high identification rates and reproducible proteome coverage. The number of precursors and protein groups reached >12,000 precursors and >1,100 protein groups across the three experiments (Figure 4C). When comparing the identified protein species across the three experiments, the results showed a 96 % overlap, demonstrating deep and reproducible coverage of the plasma proteome (Figure 4D). To further evaluate the quantitative precision, the coefficient of variation (%CV) for the protein abundance was calculated and compared with a pooled plasma sample for reference. To assess the variance of the LC-MS instrumentation, the pooled plasma peptide sample was injected and analyzed five times consecutively, resulting in a median 10 %CV. Intra-day analysis for the individual plasma samples revealed median %CVs of 15, 14 and 16 % for each experiment, respectively. This demonstrates that the factors involved in sample processing contribute low single digit variance to the overall median %CV (Figure 4E). Moreover, the inter-day comparison showed a 16 %CV across all experiments, underscoring the quantitative reliability of the automated workflow for plasma profiling.

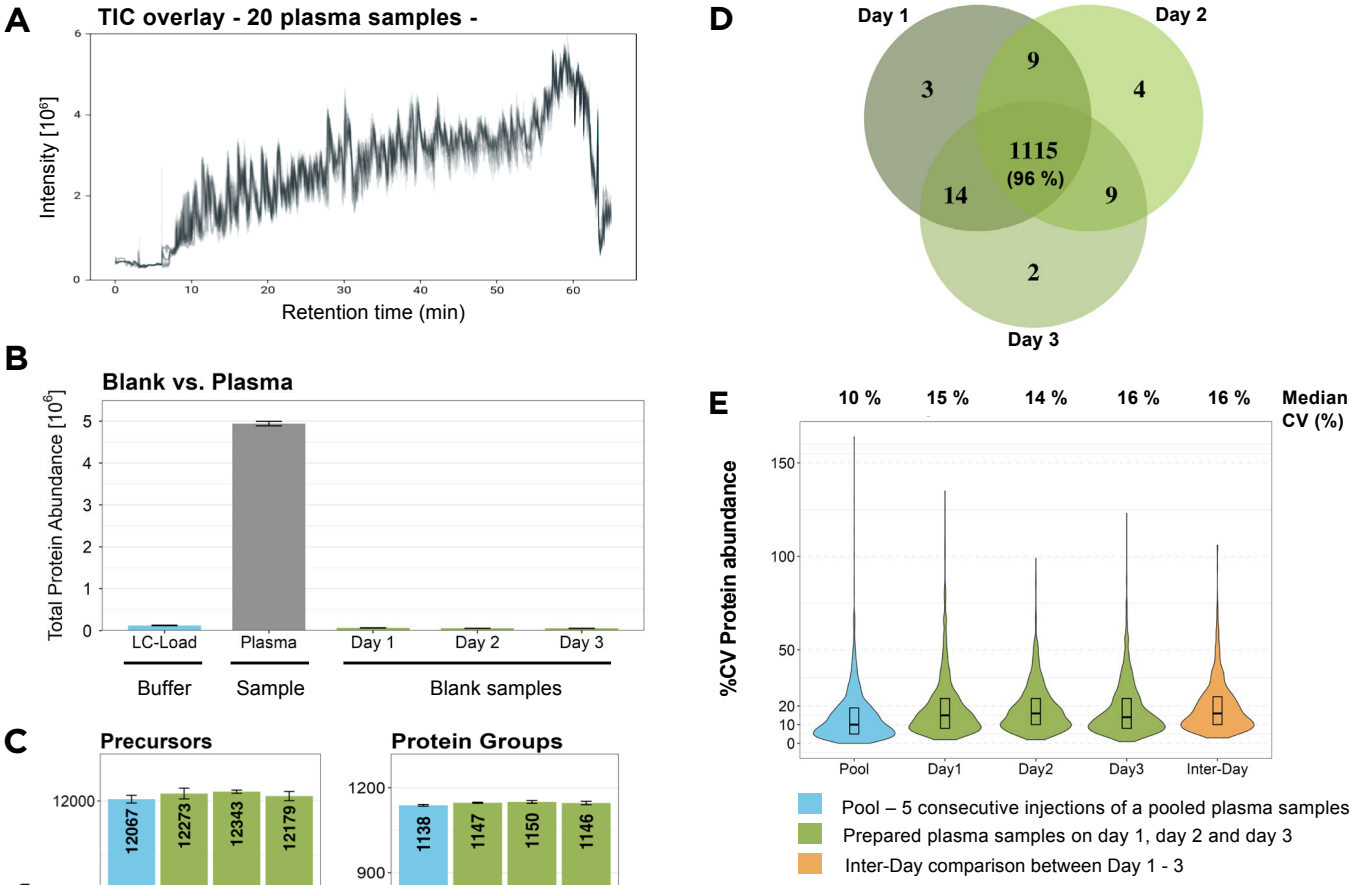


Figure 4: Multi-day analysis. **(A)** Overlay chromatogram (TIC over retention time) for 20 plasma samples - 5 replicates for each day plus 5 injections of pooled peptide sample. **(B)** Comparison of total protein abundance in LC load buffer (reference), plasma samples, and blank samples for days 1-3. **(C)** Mean and standard deviation of identified precursors and protein groups. Pooled sample (blue) is a pool of plasma samples from all days, injected five times consecutively. **(D)** Overlay of identified protein species for one plasma sample processed on three consecutive days. **(E)** Coefficient of variation (%CV) of protein abundance. Blue: Pooled sample origin as in 4C. Green: Intra-day variance. Orange: inter-day across all three days.

Aging cohort analysis.

The automated ENRICH-iST workflow was applied to a cohort scenario by analyzing age-dependent plasma proteome differences across two healthy age groups. The mean number of protein groups identified (~1,700 protein group IDs) was remarkably consistent across the six individuals analyzed, further demonstrating the robustness of the automated ENRICH-iST workflow (Figure 5A). To find age-dependent differences, a differential analysis between the two age groups was performed, highlighting multiple proteins that are differentially regulated in young or older donors (Figure 5B). Eight of the differentially regulated proteins have been reported to be age-regulated (marked in red), including GDF15 – an established aging biomarker – and the top hit CHI3L1, which has been shown to be elevated as aging progresses.[3, 4]

CONCLUSION.

This application note demonstrates the successful automation of the ENRICH-iST sample preparation technology using a Fluent Automation Workstation with an integrated Resolvex i300 module. The integration of the on-deck Resolvex i300 positive pressure module, equipped with a dispenser and active evaporation, enables complete end-to-end automation of the sample preparation process, speeding up plasma sample preparation and minimizing manual steps. The total time from plasma to dried peptides is spanning 6 hours, enabling the preparation of 96 samples within a single working day. Additionally, when combined with fast dia-PASEF acquisition, this set-up forms a high throughput pipeline that merges standardized, automated sample preparation with rapid sample acquisition for deep plasma proteome profiling. The

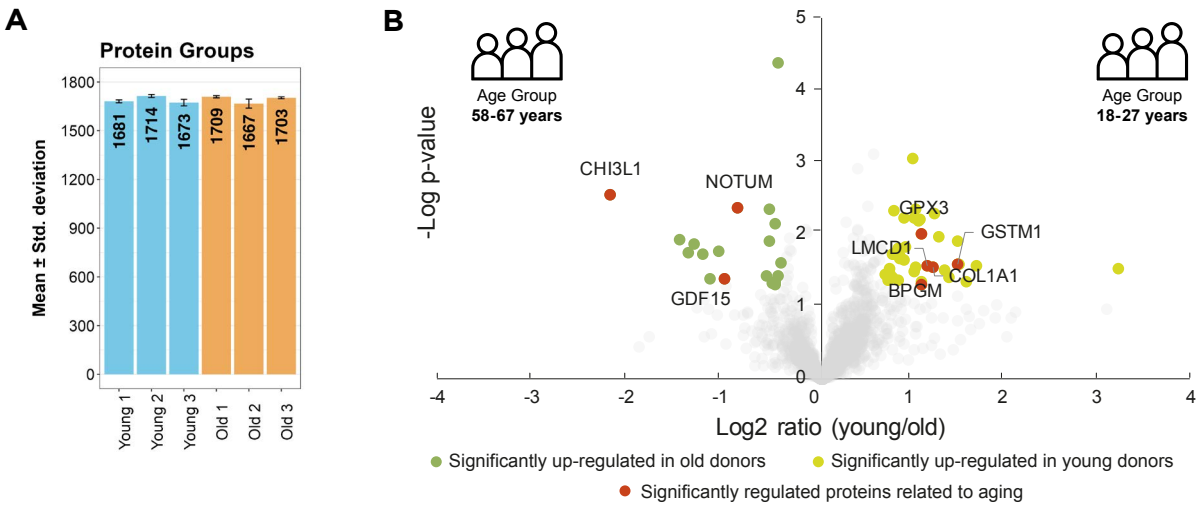


Figure 5: Aging cohort. **(A)** Mean and standard deviation of protein group identifications per donor. **(B)** Volcano plot analysis showing differences between young and older donors, revealing potential aging markers. The log2 fold changes are plotted against the -log10 p-value. Proteins in dark green are significantly upregulated in older donors (p-value ≤0.05 according to t-test and significant A outlier test). Proteins in light green are significantly upregulated in young donors (p-value ≤0.05 according to t-test and significant A outlier test). Significantly regulated proteins that have been reported to be related to aging are marked in red.

results show that the automated ENRICH-iST process combines profound proteomic characterization with reproducible and precise quantification across multiple experiments, demonstrating the robustness of the workflow. Overall, this automated ENRICH-iST workflow offers a practical and simplified solution for the challenging field of plasma proteome preparation, making it possible to gain valuable insights into molecular mechanisms and perform biomarker discovery in both small and large cohorts.

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Note: Resolvex i300 is currently under development and not available for sale. The product characteristics might be subject to change.

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