ENRICH-iST: Solving the dynamic range challenge for efficient high-throughput plasma proteomic analyses

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Introduction

Blood plasma is widely used in biomarker discovery, as it is easily obtainable and is a valuable source of information on an individual's health status[1]. Over the past decade, LC-MS-based proteomics has emerged as a powerful tool for identifying and quantifying proteins in plasma. However, the high dynamic range (up to 12 orders of magnitude) and the heterogeneity and complexity of plasma samples pose significant challenges for LC-MS-based proteomics, limiting access to the full proteome. Consequently, results lack robustness and reproducibility, workflows are complex and tedious, and low abundance proteins are masked by high abundance proteins.

Various techniques have been developed for sample preparation of plasma to reduce the dynamic range[2, 3]. Fractionating plasma samples or enriching specific groups of proteins can provide a deeper understanding of the plasma proteome compared with unprocessed plasma, but require extensive hands-on and measurement time[4]. Conventional depletion of high abundance proteins, such as albumin and immunoglobulins, can also uncover low abundance proteins that were previously masked. However, using immunodepletion kits or columns is often time-consuming and incompatible with high-throughput techniques. Immunoaffinity-based depletion

Keywords

Proteomics, plasma & serum analysis, depletion, enrichment, high-throughput sample preparation, iST technology, LC-MS, timsTOF

Key takeaways

- Streamlined enrichment of low abundance plasma proteins coupled with iST proteomic sample preparation technology for greater proteomic depth
- Automatable high-throughput processing of up to 96 raw samples/day for ready-to-measure peptides
- Fast, easy-to-use and standardized protocol for high reproducibility and less hands-on time
- Species-independent and flexible technology compatible with serum samples
- Optimized for low input samples (20 µL) for large-scale proteomic studies

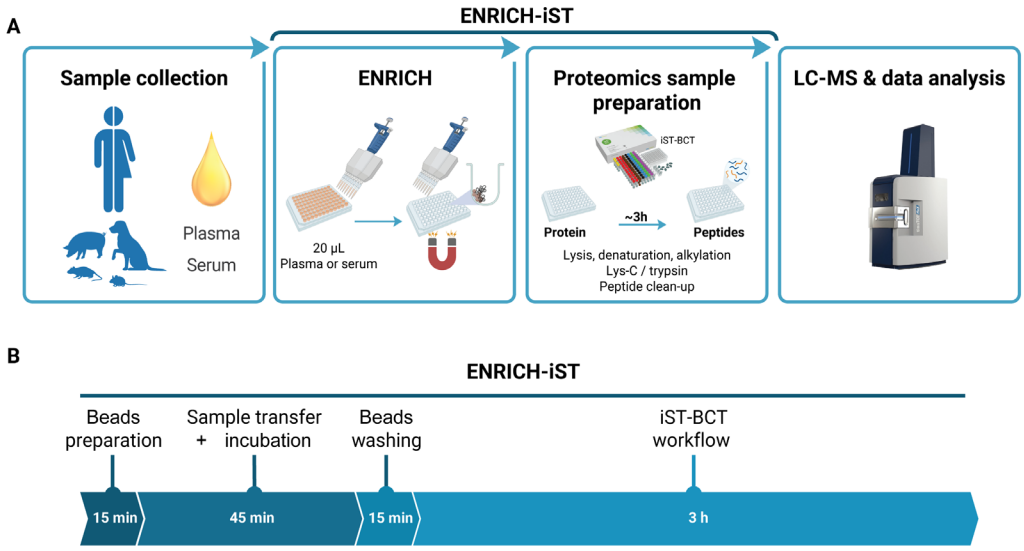

Figure 1 | Overview of the ENRICH-iST workflow

High-throughput LC-MS-based proteomic workflow for blood plasma and serum samples coupling the novel ENRICH technology for dynamic range compression with the proven iST-BCT sample preparation. Schematic depiction of the experimental workflow (A) with protocol times for individual steps (B).

is species-specific, which limits the sample sources. This approach can also carry the risk of potential cross-reactivity of antibodies or co-depletion of proteins along with carrier proteins like albumin, affecting the precision of the detection of proteins of interest[5].

The complete ENRICH-iST workflow provides an easy-to-use, yet robust solution to the dynamic range challenge of plasma samples through biologically unbiased enrichment of low abundance proteins onto paramagnetic particles (EN-BEADS). This reduces the dynamic range while conserving analytical depth and proteome coverage. PreOmics' streamlined iST-BCT approach includes proteomic sample preparation and peptide clean-up. This easy-to-automate three-step protocol is optimized for biological fluids in terms of alkylation rate and minimal artificial modifications, and allows for high-throughput processing of up to 96 samples in parallel. ENRICH-iST is compatible with human, mouse and rat samples.

ENRICH-iST offers an efficient, flexible and robust solution for plasma and serum proteomic sample preparation.

ENRICH-iST's effectiveness, reproducibility and handling for thorough and streamlined plasma proteome analysis was compared with an iST-BCT sample preparation workflow without an enrichment step ("neat" sample) as well as with a commercially available depletion technique.

Materials and Methods*

Studies were performed using commercially available plasma and serum samples from human, mouse and rat specimens from the following suppliers: Sigma Aldrich, BioIVT and Diaserve Laboratories GmbH. Plasma samples were collected with EDTA or citrate as anticoagulants, as indicated in the results section. Human citrated plasma from Sigma (P9523) was reconstituted from powder with LC-MS grade water to the indicated volume before use.

Sample preparation

Plasma and serum samples were processed with the ENRICH-iST Kit 96x according to the protocol[6]. For protein enrichment onto paramagnetic beads, 20 µL of plasma or serum were incubated with previously washed EN-BEADS for 30 minutes (30 °C, 1,200 rpm) under dedicated buffer conditions using EN-BIND. Proteins bound to EN-BEADS were washed three times and proteins were further processed using the iST-BCT workflow optimized for biofluids.

For plasma sample preparation using antibody-based depletion, the 14 most abundant proteins were removed using a commercially available kit with resin-bound antibodies. 10 µL of each sample was incubated with the bead-slurry (RT, 1,000 g for 20 min) and depleted plasma collected by centrifugation using spin columns. 50 µL of the collected flowthrough (approximately 100 µg of protein) was prepared following the iST-BCT protocol.

*for protocol details please see reference 6 and 7

The fast, three-step iST-BCT protocol was carried out with neat plasma and serum as well as with samples following the enrichment or depletion step. As per the protocol, samples were denatured, reduced and alkylated and then digested using a trypsin/LysC mix (37 °C, 1200 rpm, 1 h). Once digestion was stopped, the peptides were purified from hydrophobic and hydrophilic contaminants using a cartridge-based clean-up. After elution, peptides were dried and stored at -20 °C until LC-MS analysis.

LC-MS/MS analysis and data analysis

Peptides were resuspended in LC-LOAD (PreOmics GmbH), and 300 ng peptide mix was analyzed using the EASY-nLC™ 1200 system (Thermo Fisher Scientific) coupled to a timsTOF-HT mass spectrometer (Bruker Daltonics) in DIA-PASEF mode using a 30-minute gradient.

Raw files were analyzed using DIA-NN[8] V1.8 in library-free mode and searched against the UniProt FASTA database of Homo sapiens, Rattus norvegicus and Mus musculus (Swiss-Prot entries; downloaded 2022-02-14). The false discovery rate was set to 1% on the precursor level and evaluated against decoy precursors. Enzyme specificity was set as C-terminal to arginine and lysine, using trypsin as protease, and a maximum of one missed cleavage was allowed in the database search. Statistical analysis was performed using Perseus (V 1.6.15.0).

Results and Discussion

The new ENRICH-iST workflow allows reliable high-throughput plasma proteome analysis

A major challenge when working with plasma samples for LC-MS-based proteomics studies is its high dynamic range with low abundance proteins of interest being masked by high abundance proteins. Current approaches to overcoming this hurdle typically utilize fractionation of the samples or depletion of the most abundant proteins to reduce the dynamic range. However, the resulting protocols are tedious, lack reproducibility, need extensive optimization and are difficult to automate.

The novel ENRICH-iST approach provides a streamlined and easy-to-use plasma sample preparation workflow that combines biologically unbiased enrichment of low abundance proteins to reduce the dynamic range with highly reliable iST-BCT sample preparation that is optimized for biological fluids. The resulting workflow (Figure 1) needs only a small amount of starting material (20 µL of plasma or serum) and allows fast and automatable sample preparation of up to 96 samples in parallel within less than a working day. The included iST-sample preparation process guarantees clean peptides and high-quality mass spectrometry data. The 96-well format makes it readily adaptable to any automation platforms with magnetic racks.

The ENRICH-iST workflow is species-independent and improves proteome characterization for plasma and serum samples

Because of the high dynamic range in neat plasma, the protein information obtained is limited, as proteins of high abundance mask low abundance proteins. The ENRICH technology compresses the dynamic range of protein concentrations by binding low abundance proteins on paramagnetic ENBEADS using specified buffer conditions. ENRICH-iST works with both plasma and serum samples. For both sample types, the ENRICH-iST workflow results in a substantial increase in peptide and protein identification compared with non-enriched, neat samples (Figure 2A). A direct comparison of protein and peptide identification in human neat plasma and serum samples and in samples prepared using the ENRICH-iST workflow reveals an improvement in identification of peptides and proteins of 1.5 – 2.2-fold. The increase was seen in plasma samples treated with either citrate or EDTA anti-coagulants.

As the ENRICH technology is not antibody-based, it can be used with samples from non-human species, such as mouse or rat (Figure 2B). Comparisons of neat plasma and enriched plasma samples from mice and rats show an increase in identified proteins of 1.3 to 1.8-fold. External collaborators have carried out comparisons with dog and pig samples (data not shown) and shown similar outcomes. This confirms that ENRICH-iST is species-independent and offers a high level of flexibility.

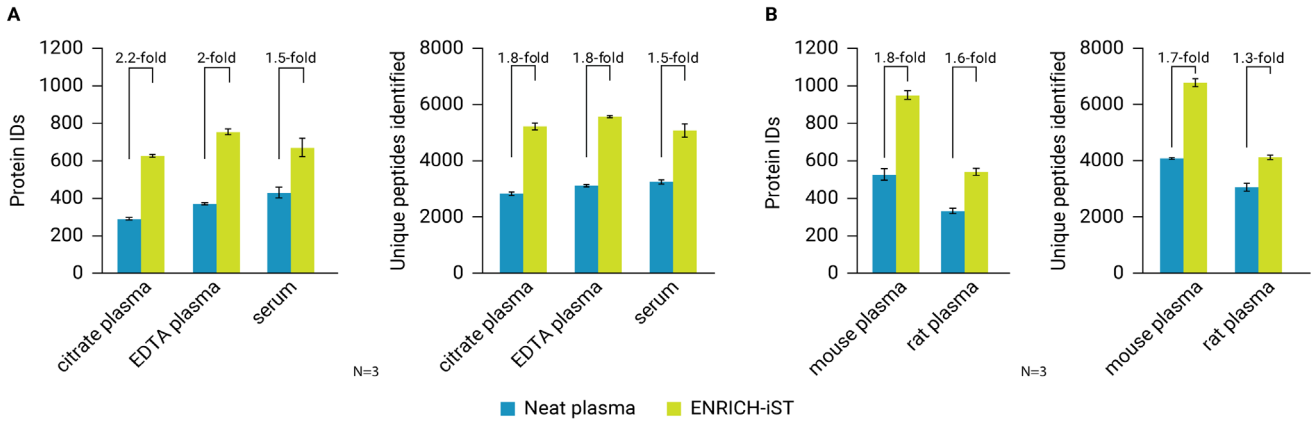

Figure 2 | Comparison of protein and peptide identifications

For both human plasma and serum samples a significant increase in peptide and protein identification was observed when comparing neat plasma and serum with enriched samples (A). There is also an increase in identified proteins compared with neat plasma when using the ENRICH-iST workflow with mouse and rat plasma samples (B).

The ENRICH-iST workflow exhibits remarkable robustness and reproducibility

The robustness of a workflow is essential for the reliable execution of larger studies. The data must be comparable and reproducible, independent of the day the study is conducted and of the operator involved carrying out the work.

The robustness of the ENRICH-iST workflow was assessed by conducting technical replicates with human EDTA plasma. Both the intra-/inter-day and the intra-/inter-operator variabilities were evaluated. Coefficients of variation (CV) for protein intensities (LFQ) within a day and by one operator were less than 12%. The CVs increased only slightly to around 15% for replicates performed by two different operators or across two days (Figure 3A, B), highlighting the robustness of the ENRICH-iST workflow.

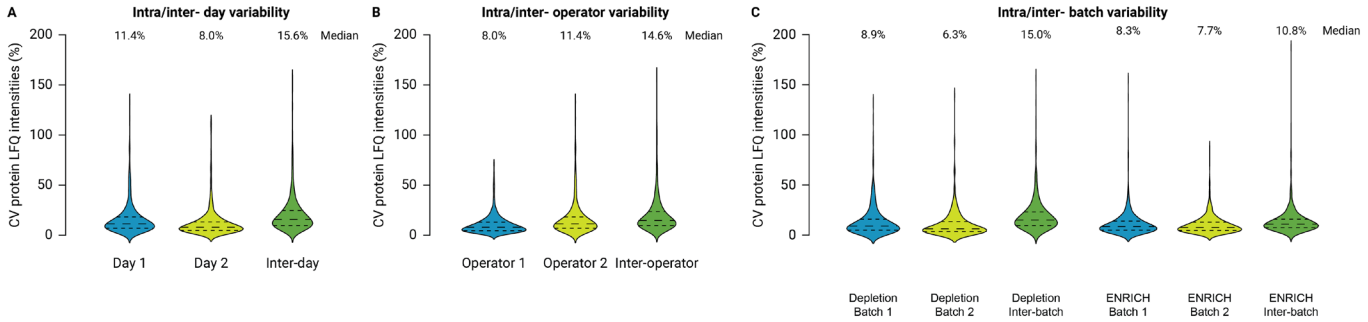

Figure 3 | Technical variability

Quadruplicates of ENRICH-iST using human EDTA plasma were performed across two days (A) or by two different operators (B). CVs were calculated for proteins with at least three valid values. The batch effect (C) was compared between ENRICH-iST and a commercially available depletion solution. Triplicates were performed for two batches of each workflow. CVs were calculated for proteins with at least two valid values. Samples were measured on the same day using identical settings.

For large sample cohorts and long-term studies, it is crucial to have consistent results across different kit batches. Batch effects of ENRICH-iST and a commercially available depletion solution were compared using two sets of two different kit batches. The analysis revealed that the replicates prepared using the same kit batch of ENRICH-iST or the commercially available depletion solution exhibited comparable CVs ranging from 6-9%. However, ENRICH-iST demonstrated lower inter-batch variability (CV=11%) compared with a commercially available depletion approach (CV=15%) (Figure 3C). The exceptional precision of ENRICH-iST makes it an ideal workflow for preparing and analyzing proteomic samples from large sample cohorts.

Superior analytical depth and proteome coverage

A commercially available depletion solution and the ENRICH-iST workflow were compared using neat EDTA plasma processed only with iST-BCT sample preparation without enrichment. The abundance ranking showed that while all three workflows covered an intensity range (MAXLFQ intensity) of approximately four orders of magnitude, the ENRICH-iST and depletion workflow increased the numbers of identified proteins by over two-fold compared with neat plasma indicating a highly efficient reduction of the dynamic range (Figure 4A). Matching the identified proteins with the plasma proteome database (Human Protein Atlas) revealed a superior coverage for the ENRICH-iST workflow over neat and depleted samples with identified proteins covering almost the entire dynamic range of the database (Figure 4B). Considering the CV as indicator of precision, ENRICH-iST shows much higher precision over the entire abundance range, whereas a decrease in precision towards low abundance proteins can be detected for the depletion workflow. This shows that the ENRICH-iST workflow provides enhanced dynamic range compression and confirms improved performance for the identification and quantification of low abundance proteins.

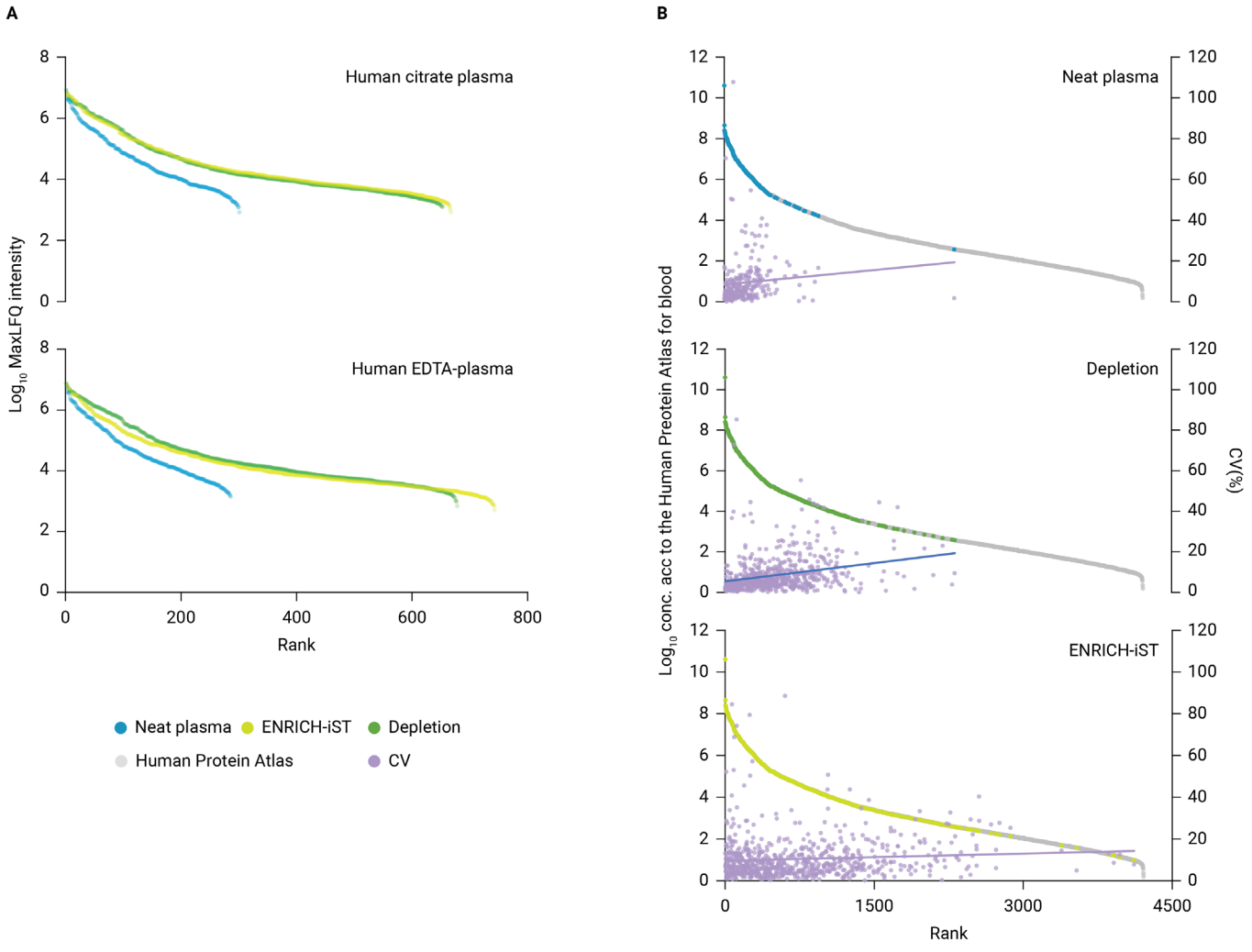

Figure 4 | Proteome depth covered by different workflows

(A) LFQ intensities of quantified protein-groups comparing neat plasma, a commercially available depletion approach (Depletion) as well as the ENRICH-iST workflow. (B) Distribution of protein-groups quantified from human EDTA-plasma according to the protein concentrations from the Human Protein Atlas for plasma (grey scatters, primary vertical axis). The purple scatters reveal coefficient of variation of individual protein groups quantified for neat plasma, a commercially available depletion approach (Depletion) and the ENRICH-iST workflow (secondary vertical axis).

Conclusions

A streamlined, robust and automatable plasma and serum sample preparation workflow is essential for creating reliable research, for example cohort studies. In this context, enhanced characterization of liquid biopsy samples to provide deeper insight in pathogenic disease mechanisms is crucial for biomarker discovery.

The ENRICH-iST workflow provides a fast, easy-to-use, standardized, and automatable protocol for high-throughput proteome profiling of blood-derived biofluidic samples. It offers streamlined enrichment of low abundance plasma and serum proteins. Coupled with the proven iST-BCT sample preparation technology, up to 96 raw samples per day can be processed to ready-to-measure peptides with minimal hands-on time. The workflow is optimized for low sample volumes, using just 20 µL of starting material, and is compatible with standard automation platforms using magnetic racks. Compared with conventional commercially available depletion methods, the ENRICH-iST workflow provides superior robustness, excellent analytical depth and improved proteome coverage with high precision. Analyses of mouse and rat plasma confirm its compatibility with other mammalian species, creating a flexible workflow for a wide range of sample types.

Overall, the ENRICH-iST workflow delivers a straightforward answer to the dynamic range challenge in blood-derived biofluids by enriching low abundance proteins onto paramagnetic beads, enabling true high-throughput, and providing an ideal workflow for the preparation and analysis of small to larger sample cohorts in a wide array of basic and preclinical research.

PREOMICS

Products

Product	Manufacturer	Product Code
ENRICH-iST 8x	PreOmics GmbH	P.O.00163
ENRICH iST kit 96x	PreOmics GmbH	P.O.00164
ENRICH iST kit 96x HT	PreOmics GmbH	P.O.00165

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