ENRICH-iST enables deep and quantitative plasma proteomics: Insights from a controlled quantitative experiment

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Introduction

Blood is a highly informative biofluid for biomarker discovery, containing a complex proteome that spans cytokines and tissue-derived proteins indicative of disease. However, MS-based plasma proteomics remains challenging due to the wide dynamic range of protein concentrations, where highly abundant proteins can mask low-abundance targets.[1,2] The ENRICH technology, a bead-based enrichment strategy, reduces the effective concentration range, enhancing the accessibility to low-abundance proteins in the plasma proteome for LC–MS analysis.[2]

While enrichment strategies like ENRICH-iST improve proteome depth, they can perturb the original concentration relationships among proteins, which has important implications for quantitative accuracy. Quantitative performance is evaluated by precision and three levels of accuracy: relative accuracy (faithful measurement of fold-changes), relative linearity (consistency of fold-changes across dilutions), and absolute linearity and sensitivity (recovery of true concentrations within LOD/LOQ). For biological insights, relative changes and systematic trends are often most critical, with log-linear responses preferred and calibration functions applied when absolute quantification is needed.[3]

In this study, a Controlled Quantitative Experiment (CQE) was established to benchmark ENRICH-iST against a neat plasma workflow, assessing proteome coverage, reproducibility, and quantitative accuracy (Figure 1). The results demonstrate how ENRICH-iST enhances plasma proteomics while maintaining reliable quantitative performance.

Keywords

Proteomics, plasma samples, low-abundance protein, dynamic range, biomarkers, ENRICH-iST, LC-MS analysis, timsTOF

Key takeaways

Superior proteome coverage and reproducibility: ENRICH-iST consistently quantified more proteins than neat plasma across all dilution conditions with CVs <20%, delivering superior reproducibility and depth.

Preserved quantitative accuracy and linearity: ENRICH-iST maintained fold-change accuracy and delivered high linearity than neat plasma, enabling many more proteins to pass stringent quantitative criteria.

Extended dynamic range without compromising sensitivity: ENRICH-iST quantified 1.7× more proteins across the dilution series and retained sensitivity even at 1% plasma dilution, supporting reliable detection of low-abundance biomarker proteins.

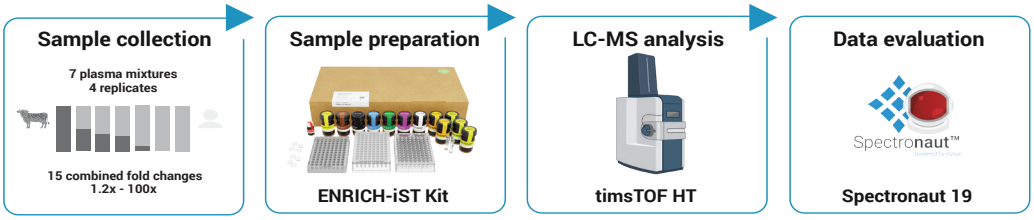

Figure 1 | Experimental design of the controlled quantitative experiment using the ENRICH-iST workflow. Bovine plasma was spiked into human plasma at seven ratios. ENRICH-iST, a bead-based sample preparation workflow for enriching low-abundance proteins, was compared to the neat workflow (not shown). Peptides were analyzed on a nanoElute® 2 – timsTOF HT in dia-PASEF® mode, and data were processed in Spectronaut® 19 using bovine and human spectral libraries from pure samples.

Materials and Method

Controlled quantitative experiment design

K_2EDTA bovine plasma (Neo Biotech) was spiked into pooled K_2EDTA human plasma (Diaserve Laboratories) at seven volume ratios (bovine:human): 1:0, 1:1, 1:1.5, 1:2, 1:9, 1:99, and 0:1, each in quadruplicate.[3]

Sample preparation

For neat plasma preparation, 2 µL of plasma were processed using the iST-BCT protocol (PreOmics). Samples were denatured, reduced, and alkylated in a single step, followed by digestion with a Trypsin/LysC mix (37°C, 1200 rpm, 1 h). Peptides were purified using a cartridge-based cleanup, dried, and stored until LC–MS analysis.

For ENRICH-iST preparation, 20 µL of plasma were processed using the ENRICH-iST 96x HT protocol (PreOmics) on a KingFisher Flex instrument (Thermo Fisher Scientific), including enrichment, lysis, and digestion. Peptides were purified following the same steps previously described for neat plasma preparation.

All peptide eluates were dried in a SpeedVac concentrator and stored at −80°C until reconstitution in LC-Load buffer prior to LC–MS analysis.

LC–MS/MS analysis and data analysis

Peptides were resuspended in LC-LOAD buffer to a concentration of 300 ng/µL. For LC–MS/MS analysis, 300 ng of peptides were injected onto a nanoElute® 2 HPLC system (Bruker) equipped with an Aurora™ Ultimate CSI 25 × 75 C18 UHPLC column (IonOpticks), operated with a 30-min gradient and coupled to a timsTOF HT mass spectrometer (Bruker) in dia-PASEF® mode.

For each workflow and species, spectral libraries were generated from pure plasma measurements in Spectronaut® 19 using Pulsar, searched against either the Homo sapiens or Bos taurus Swiss-Prot FASTA database (release 2024-02; isoforms excluded). All raw files from each workflow were subsequently processed in Spectronaut® 19 against both bovine and human spectral libraries, using maxLFQ quantification and prototypic peptides only.

Statistical analyses and data visualization were conducted in R using Shiny applications. MaxLFQ intensity values were normalized to either sample sum or median. Fifteen observed fold-changes were calculated by combining different dilution conditions. Accuracy error was defined as $|\log_2FC\_\{observed\} - \log_2FC\_\{expected\}| / \log_2FC\_\{expected\}$. Linearity of fold-change measurements was assessed by Pearson correlation between observed and expected fold-changes of bovine proteins. Limits of detection (LOD) and quantification (LOQ) were estimated from the dilution series using the matrix-matched model[4] approach (https://github.com/sjust-seerbio/matrix-matched-calcurves).

Results and Discussion

High proteome coverage with high precision

As part of the quantitative performance assessment, the precision of the ENRICH-iST workflow was evaluated using mixed-species spike-in experiments on the timsTOF HT system. Across all dilution ratios, ENRICH-iST quantified more than 3,000 proteins in total and consistently outperformed neat plasma digestion. At each spike-in ratio, ENRICH-iST identified more proteins at a given CV threshold, reflecting superior reproducibility. For example, at a 1:1 spike-in ratio, ENRICH-iST quantified 1,466 proteins compared with 692 for neat plasma, with median protein-level CVs below 20% across four replicates. These results highlight the high repeatability of the ENRICH-iST workflow relative to neat plasma (Figure 2).

Fold-change accuracy maintained with ENRICH-iST

To assess accuracy and linearity, bovine-specific proteins were selected, as their broader dilution range enabled more reliable estimations than the human samples. Relative fold-change accuracy was evaluated by comparing observed fold-changes in protein intensities between spike-in ratios with the corresponding expected fold-changes derived from protein concentrations. In total, 15 pairwise comparisons were performed across six spike-in ratios. Representative examples for five dilution pairs (2:1, 2.5:1, 3:1, 4:1, and 5:1) are shown in Figures 3A and 3B. For both ENRICH-iST and neat plasma digestion, the median fold-changes closely followed the expected ratios, with values generally falling within the interquartile range. Importantly, ENRICH-iST consistently quantified more proteins at any chosen fold-change accuracy threshold compared with neat plasma (Figure 3C). These results demonstrate that ENRICH-iST not only preserves fold-change accuracy across a wide dilution range but also expands the number of reliably quantified proteins relative to a neat plasma workflow.

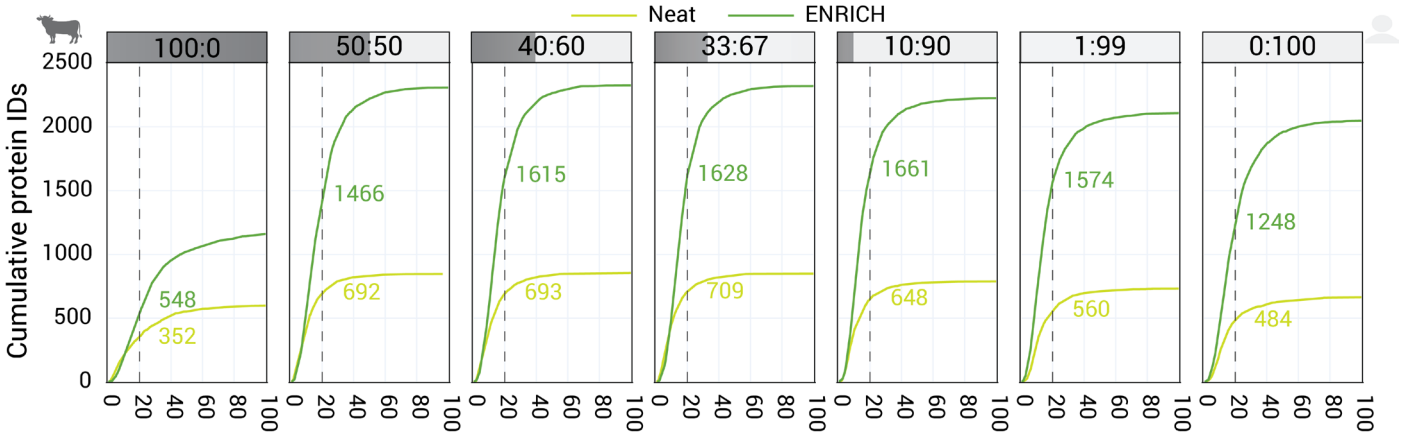

Figure 2 | Number of proteins identified at different coefficient of variation (CV) thresholds for each spiked-in ratio. The x-axis represents the CV and y-axis shows the cumulative number of bovine and human proteins quantified in at least two replicates. Proteins were considered reproducibly quantified when CVs were below the 20% cutoff.

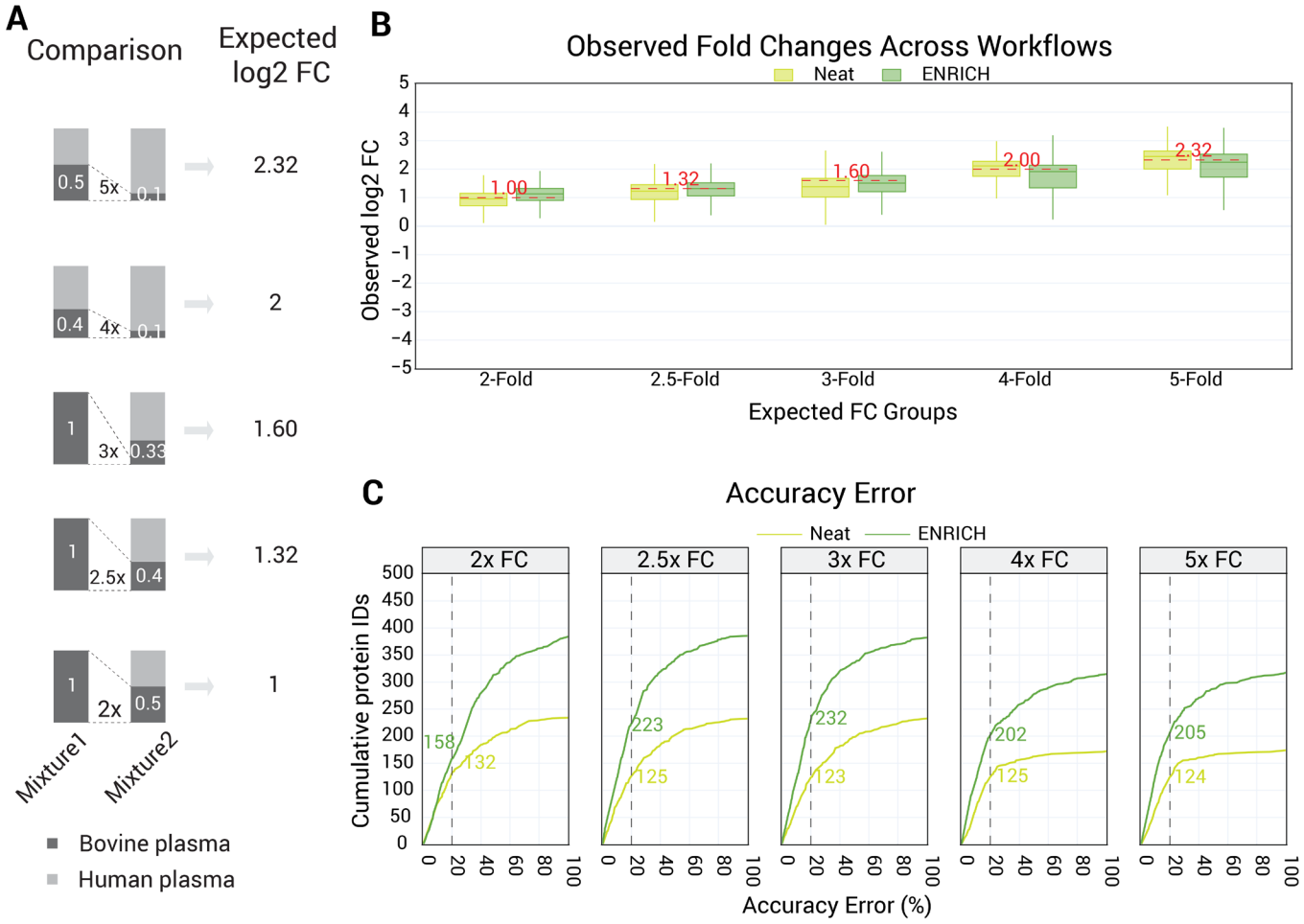

Figure 3 | Comparison of fold-change accuracy performance between ENRICH-iST and neat plasma workflows. (A) Five representative sample pairs with the expected $\log_2$ fold-changes of bovine proteins. (B) Observed fold-changes of bovine proteins across the five comparisons. Horizontal dashed lines mark the expected $\log_2$ fold-changes. Boxplots show the distribution of observed values (median, interquartile range, whiskers at 1.5× IQR; outliers omitted). (C) Cumulative number of bovine proteins at different accuracy thresholds for each expected fold-change. Dashed lines indicate proteins within 20% accuracy error.

Comparable linearity delivered by ENRICH-iST

Protein fold-change accuracy can be affected not only by compression or inflation of the dynamic range during enrichment, but also by downstream workflows and LC–MS acquisition itself. Since such effects are unavoidable in enrichment- and LC–MS–based plasma proteomics, it is particularly important to maintain a linear correlation between observed and expected log fold-changes, especially in targeted proteomics applications such as biomarker monitoring.

To assess response linearity, Pearson correlation coefficients were calculated between the observed and expected log fold-changes for all proteins detected in each assay, providing an estimate of how accurately protein regulation across biosamples can be captured. Across all 15 spiked-in comparisons, the ENRICH-iST workflow demonstrated high linearity, with a median Pearson correlation of 0.93 versus 0.96 for the neat plasma workflow (Figure 4A). Furthermore, ENRICH-iST quantified more proteins at any given linearity threshold. For example, at a stringent Pearson correlation cutoff of ≥0.9, ENRICH-iST consistently quantified 235 bovine-unique proteins across all dilutions, compared with 157 proteins detected by the neat plasma workflow (Figure 4B).

To validate the potential of ENRICH-iST for targeted proteomics and biomarker applications, the quantitative performance of human biomarker proteins mapped to bovine orthologs was assessed. A total of 8 FDA-approved pharmacogenomic biomarkers were detected in both workflows. The observed $\log_2$ fold-changes were plotted against the expected $\log_2$ fold-changes (Figure 4C), showing good linearity in both workflows, except for Q9XSG3 (IDHC), identified as a common outlier likely due to the low abundance of its proteotypic peptides.

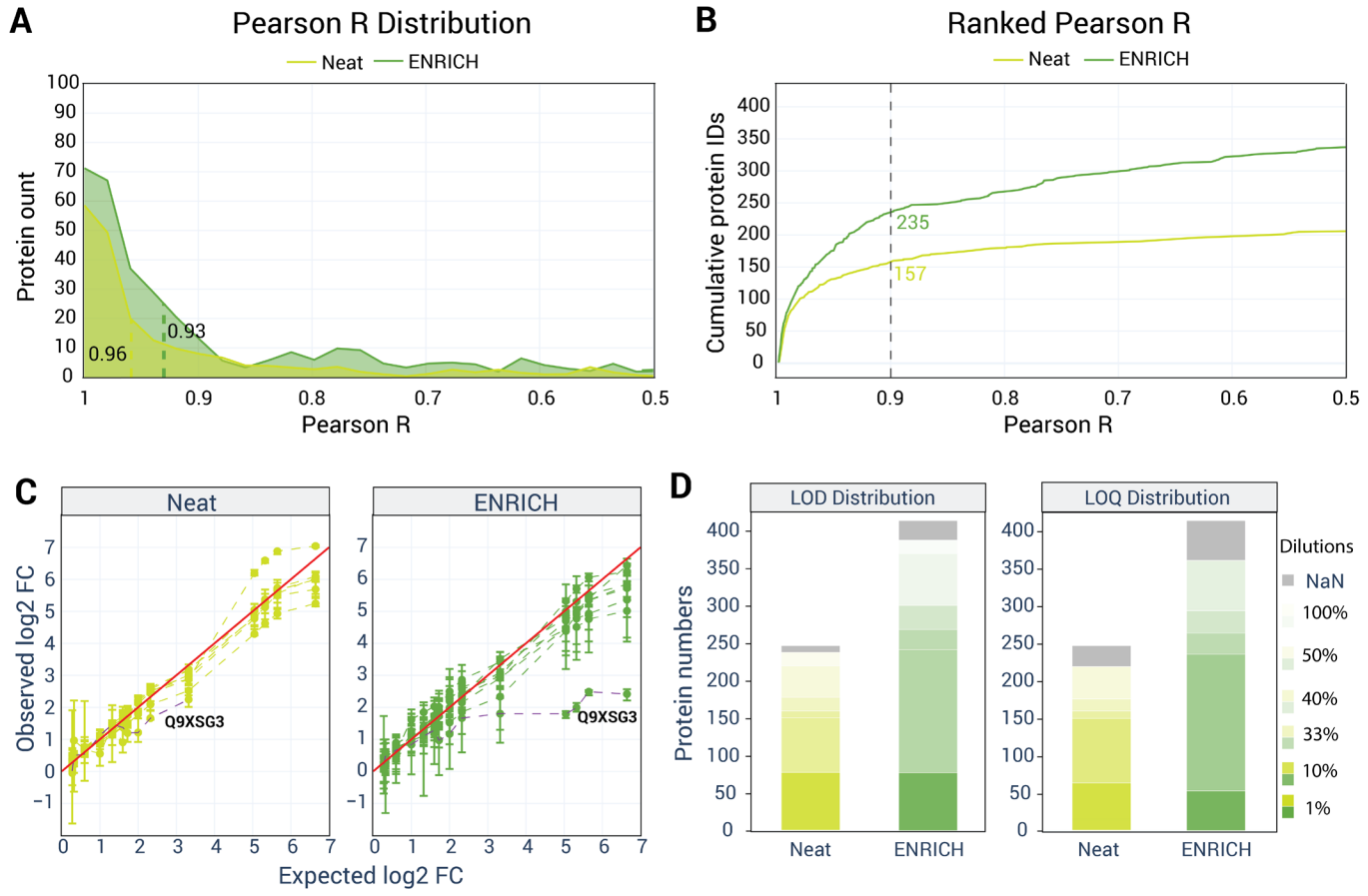

Figure 4 | Comparison of fold-change linearity between ENRICH-iST and neat workflows. (A) Density plots show the distribution of Pearson correlation coefficients across the two workflows. (B) Cumulative number of bovine proteins identified at different correlation thresholds. The x-axis shows the Pearson correlation (truncated at 0.5), and the y-axis shows the cumulative number of proteins at each correlation level. Dashed lines indicate proteins with a correlation ≥0.90. (C) Linearity of FDA-approved pharmacogenomic biomarkers mapped to bovine proteins by gene symbol. Scatter plots show observed versus expected $\log_2$ fold-changes for eight quantified biomarkers, with dashed lines connecting estimated fold-changes across dilutions. The purple dashed line highlights the common outlier Q9XSG3. (D) Distribution of bovine proteins with calculated limits of detection (LOD) and quantification (LOQ). Bars represent the number of proteins per workflow, stratified by the percentage of dilution levels at which an LOD or LOQ could be assigned (1%, 10%, 33%, 40%, 50%, 100%, or not assigned [NaN]).

Matrix-matched calibration curves across six bovine plasma dilutions (100%, 50%, 40%, 33%, 10%, 1%) were used to assess absolute linearity and sensitivity of the workflows. Protein counts were determined at the limit of detection (LOD) and the limit of quantification (LOQ), with LOQ restricted to proteins showing a correlation coefficient of $R \geq 0.9$ over the dilution series to ensure linear quantitative response. Across the tested dilution range, ENRICH-iST detected and quantified 1.7-fold more proteins than neat plasma. At the lowest 1% dilution, ENRICH-iST achieved comparable detection and quantification to the neat workflow, demonstrating that sensitivity was maintained even under extreme dilution conditions (Figure 4D).

In summary, ENRICH-iST preserves linearity comparable to neat plasma while substantially increasing overall protein coverage and sensitivity, thereby extending the dynamic range of plasma proteomics and enabling robust biomarker quantification.

Conclusion

Plasma proteomics is inherently challenging due to the wide dynamic range of protein concentrations, where highly abundant proteins can mask signals from low-abundance biomarkers of clinical relevance. Enrichment strategies, such as ENRICH-iST, have been developed to reduce this complexity and improve access to low-abundance proteins. While these approaches increase proteome depth, they must also maintain quantitative accuracy and linearity to support reliable biological interpretation.

In this study, ENRICH-iST was systematically benchmarked against a neat plasma workflow using a controlled quantitative experiment, with a focus on fold-change accuracy, relative and absolute linearity, and sensitivity as key performance indicators. The results demonstrate that ENRICH-iST delivers quantitative performance comparable to neat plasma while achieving substantially greater proteome coverage. Across fifteen pairwise comparisons, ENRICH-iST maintained strong correlations between observed and expected fold-changes and consistently quantified more proteins at stringent linearity thresholds. Matrix-matched calibration curves further showed that ENRICH-iST quantified 1.7-fold more proteins across the tested dilution range, extending the dynamic range without compromising accuracy or sensitivity.

Overall, these findings establish ENRICH-iST as a robust workflow that combines depth with quantitative reliability, enabling comprehensive and accurate plasma proteomics for biomarker discovery.

Products

Product	Manufacturer	Product Code
iST-BCT 96x	PreOmics GmbH	P.O.00099
ENRICH-iST 96x HT	PreOmics GmbH	P.O.00165

Ordering information

http://www.preomics.com/quote

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