Automated HILIC-Enriched Glycopeptide Analysis for High-Throughput, High-Sensitivity Glycoprotoemics using GlycoPASEF® on the timsTOF Ultra-2

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Introduction

Efficient glycopeptide profiling requires an integrated workflow that combines automated sample preparation, optimized enrichment, and high-sensitivity data acquisition. This application note describes a streamlined approach using Hydrophilic Interaction Liquid Chromatography (HILIC)-based glycopeptide enrichment on an AssayMAP Bravo (Agilent) automated liquid handling platform, followed by glyco-PASEF® data acquisition on a timsTOF Ultra 2 mass spectrometer for high-throughput, high sensitivity glycoproteomic analysis. The method was applied to plasma (citrate- and EDTA-anticoagulated) and cell lysates (HeLa and K562), demonstrating significantly increased glycopeptide identifications (by up to 9-fold) and improved reproducibility across replicates. These findings highlight the need for optimized sample preparation and data acquisition strategies for glycoproteomic R&D, offering a scalable solution for fast, precise, and comprehensive interrogation of the N-glycoproteome in both basic and applied research.

Methods

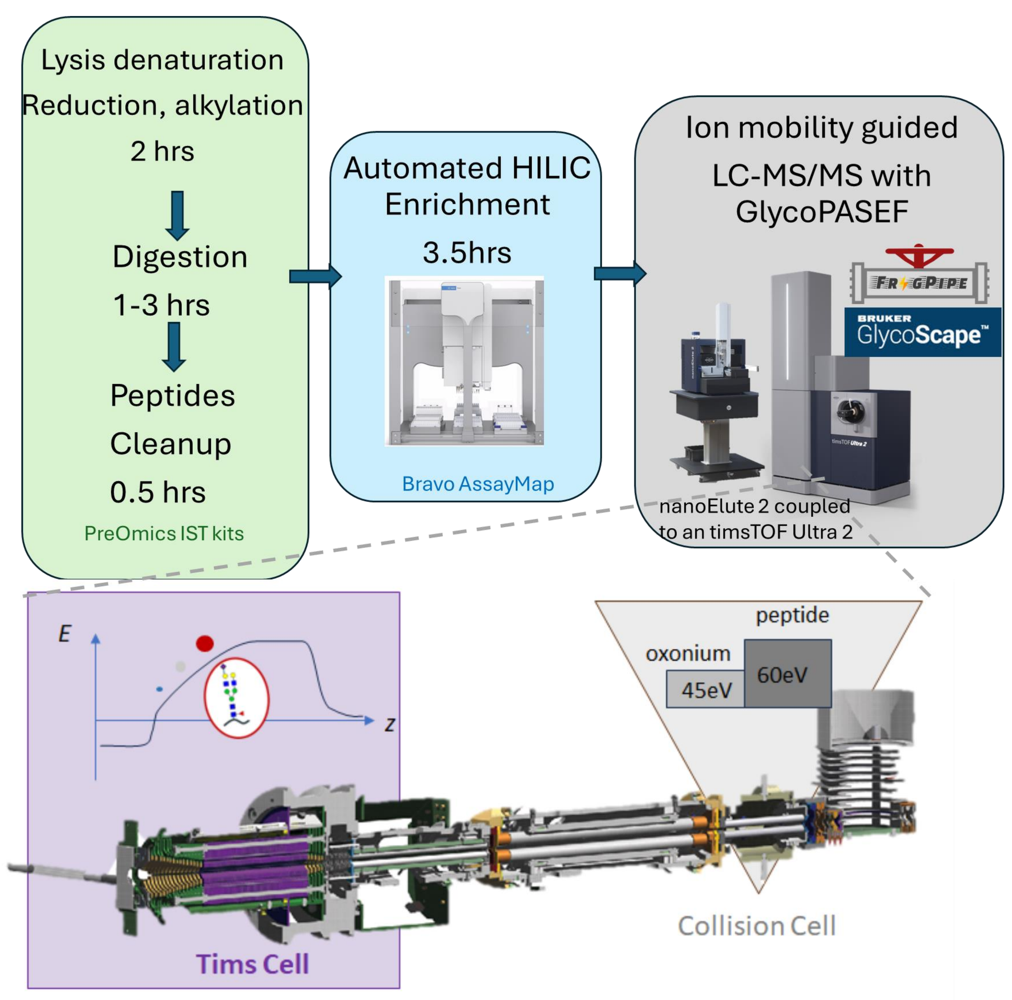


Figure 1. Automated glycoproteomics workflow integrating Hydrophilic Interaction Liquid Chromatography (HILIC) enrichment with ion mobility-guided LC-MS/MS. Samples undergo denaturation, reduction, alkylation, and digestion, followed by automated HILIC glycopeptide enrichment using the Bravo AssayMap with CU cartridges. Enriched peptides are analyzed on a timsTOF Ultra 2 coupled with nanoElute 2, employing GlycoPASEF for ion mobility separation and stepped-energy CID for optimized glycopeptide fragmentation. The datasets were analyzed with real-time analysis platform, GlycoScape and further analyzed with offline MS-Fragger

Results

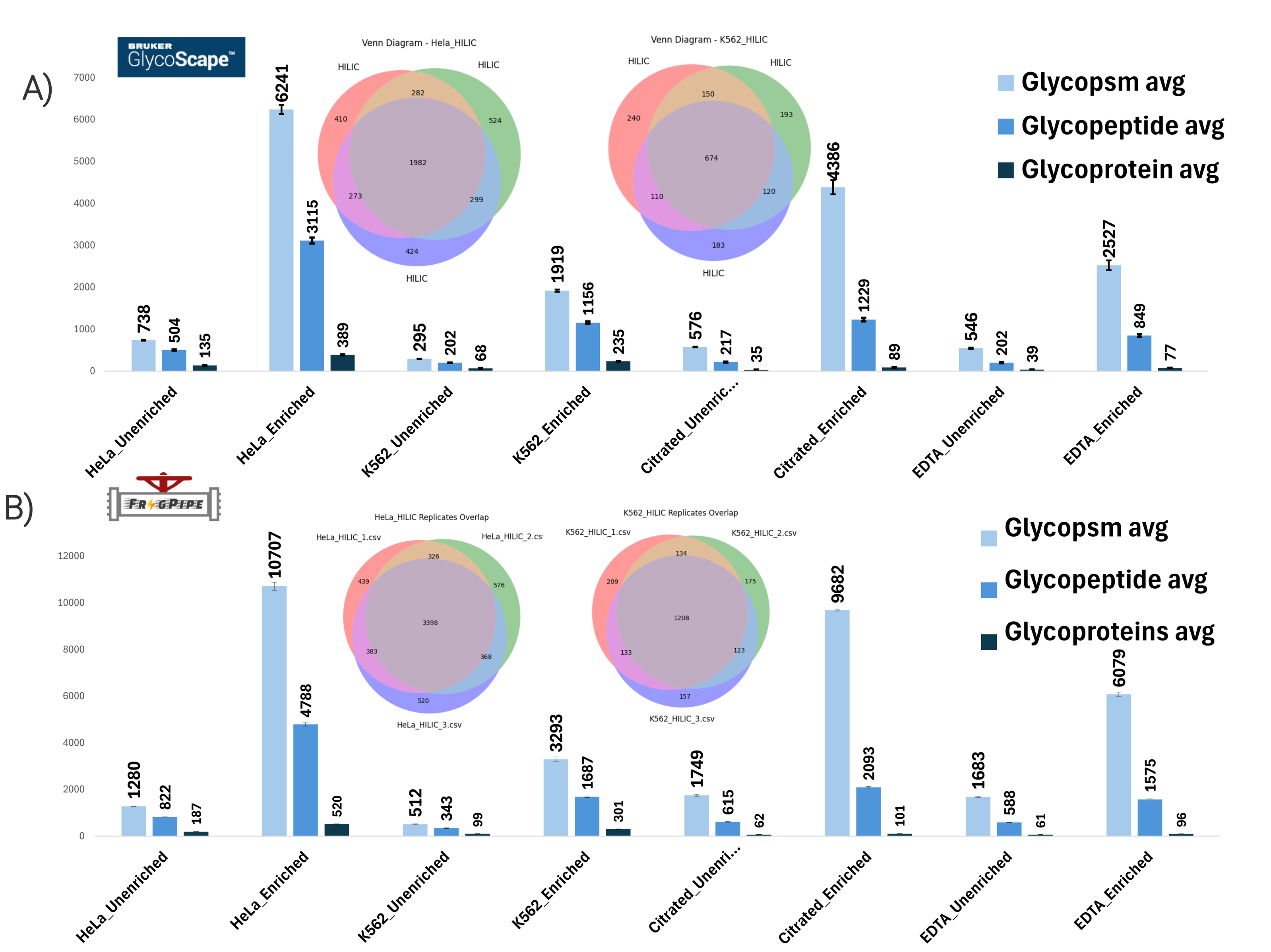


Figure 2. Comparison of Glycoscape and FragPipe Glycoproteomic Analyses Across Sample Conditions(A) Glycoscape: Average counts of GlycoPSMs, glycopeptides, and glycoproteins across unenriched and enriched HeLa, K562, Citrated, and EDTA samples. Venn diagrams depict glycopeptide overlap among replicates for HeLa_HILIC and K562_HILIC.(B) FragPipe: Similar analysis with FragPipe, highlighting identification counts and replicate overlaps for the same sample conditions.

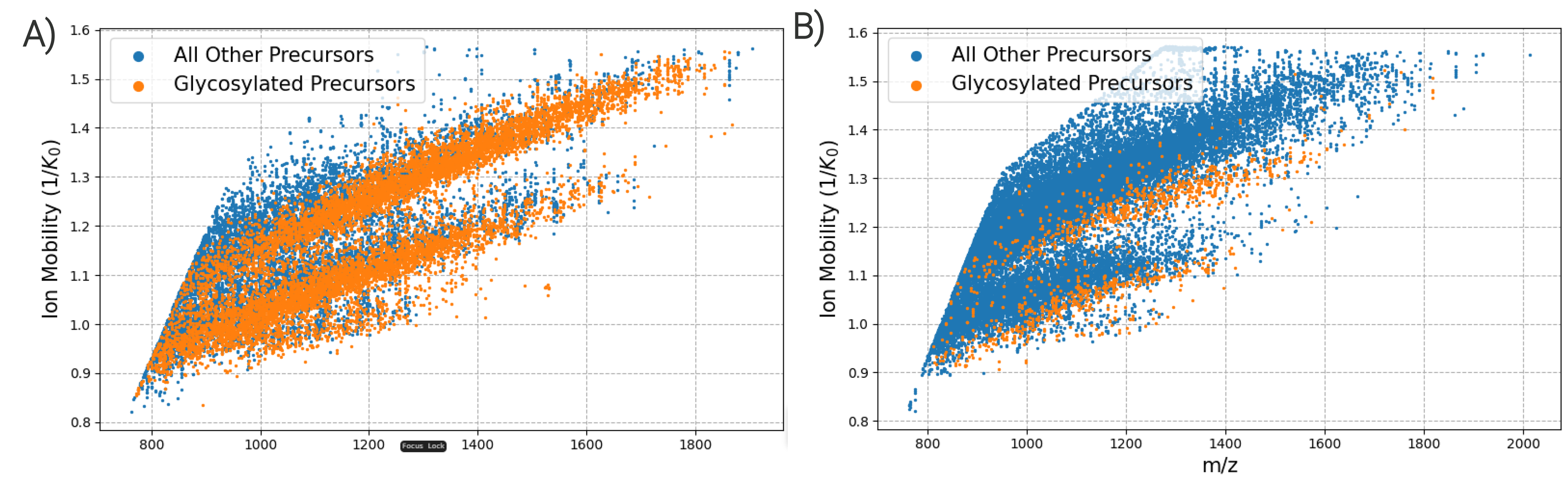


Figure 3. Scatter plots of enriched (A) and (B) not enriched HeLa digest.

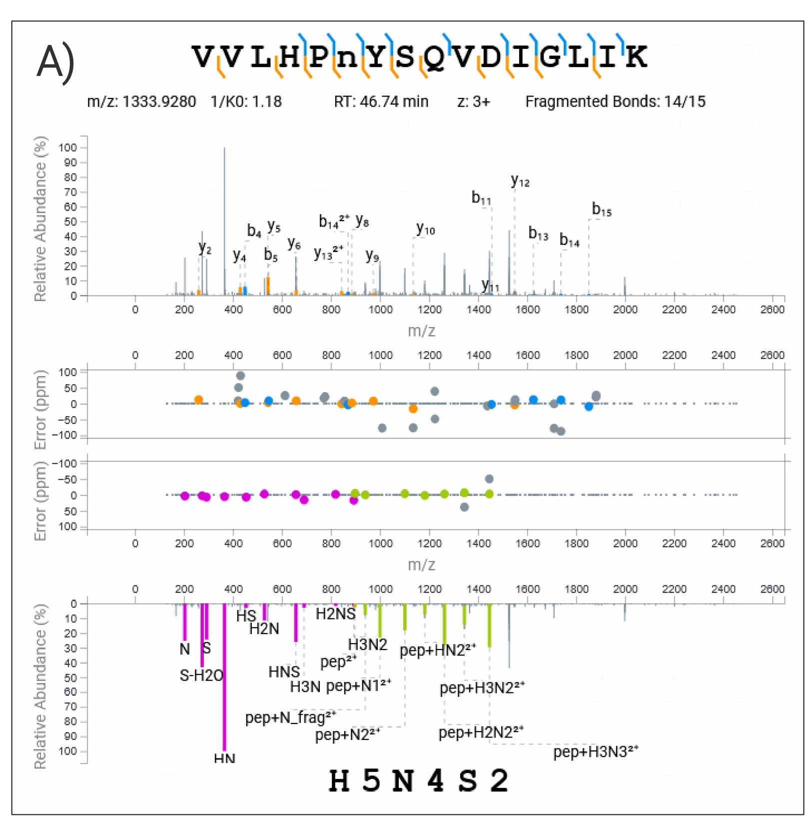
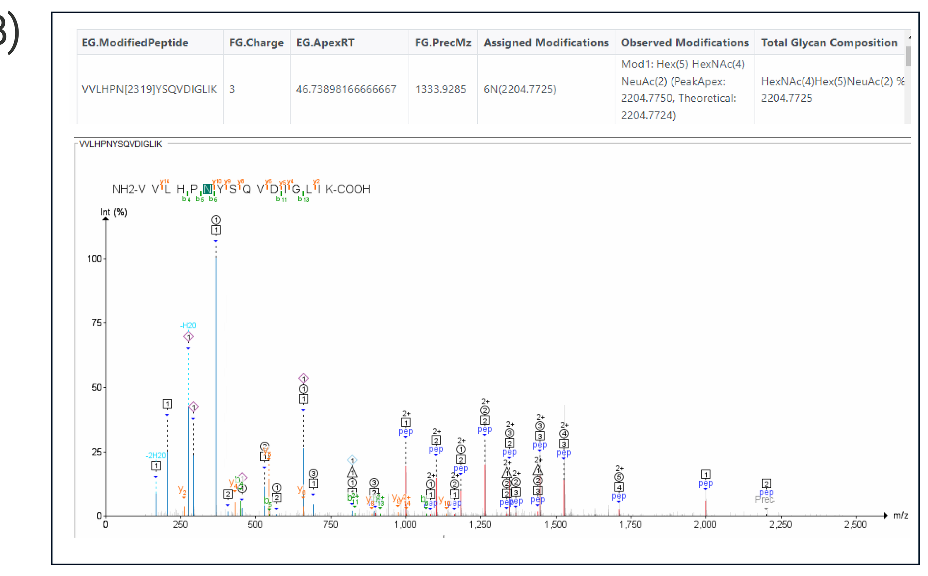
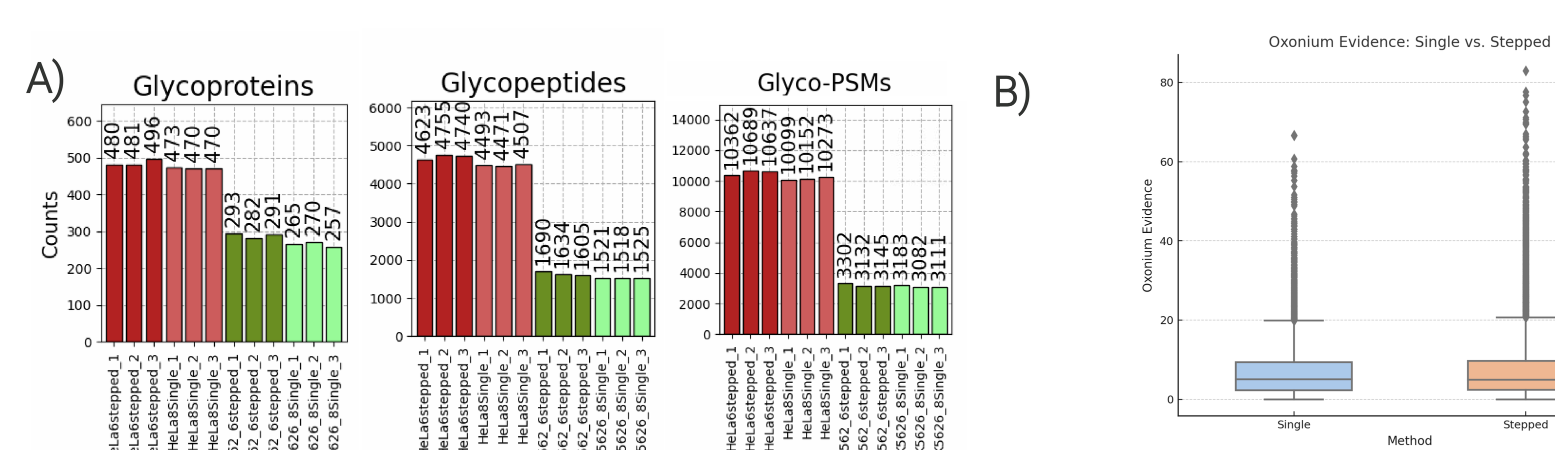



Figure 4. Example glycopeptide spectrum match identified by both GlycoScape and MSFragger. Consistent fragmentation patterns confirm the robustness of the workflow.



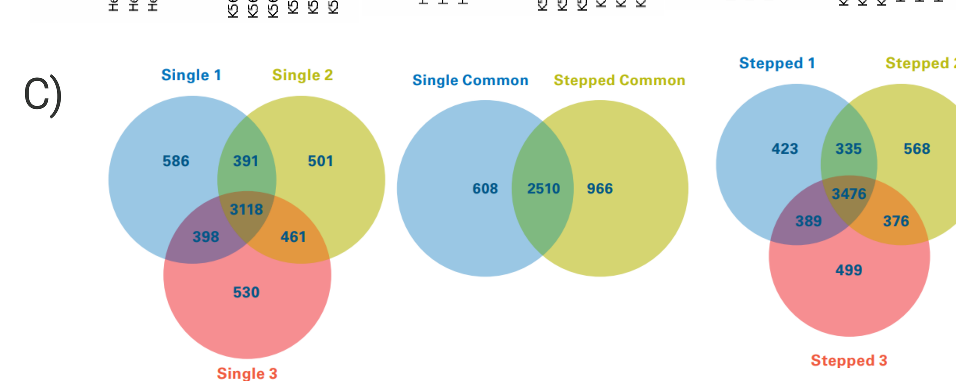


Figure 5. Comparison of Glycoprotein Identifications Between Single and Stepped Fragmentation Methods (A) Counts of GlycoPSMs, glycopeptides, and glycoproteins identified with and without stepped CID. (B) Oxonium evidence under single and stepped CID conditions. (C) Venn diagram showing glycopeptide overlaps across three HeLa replicates,

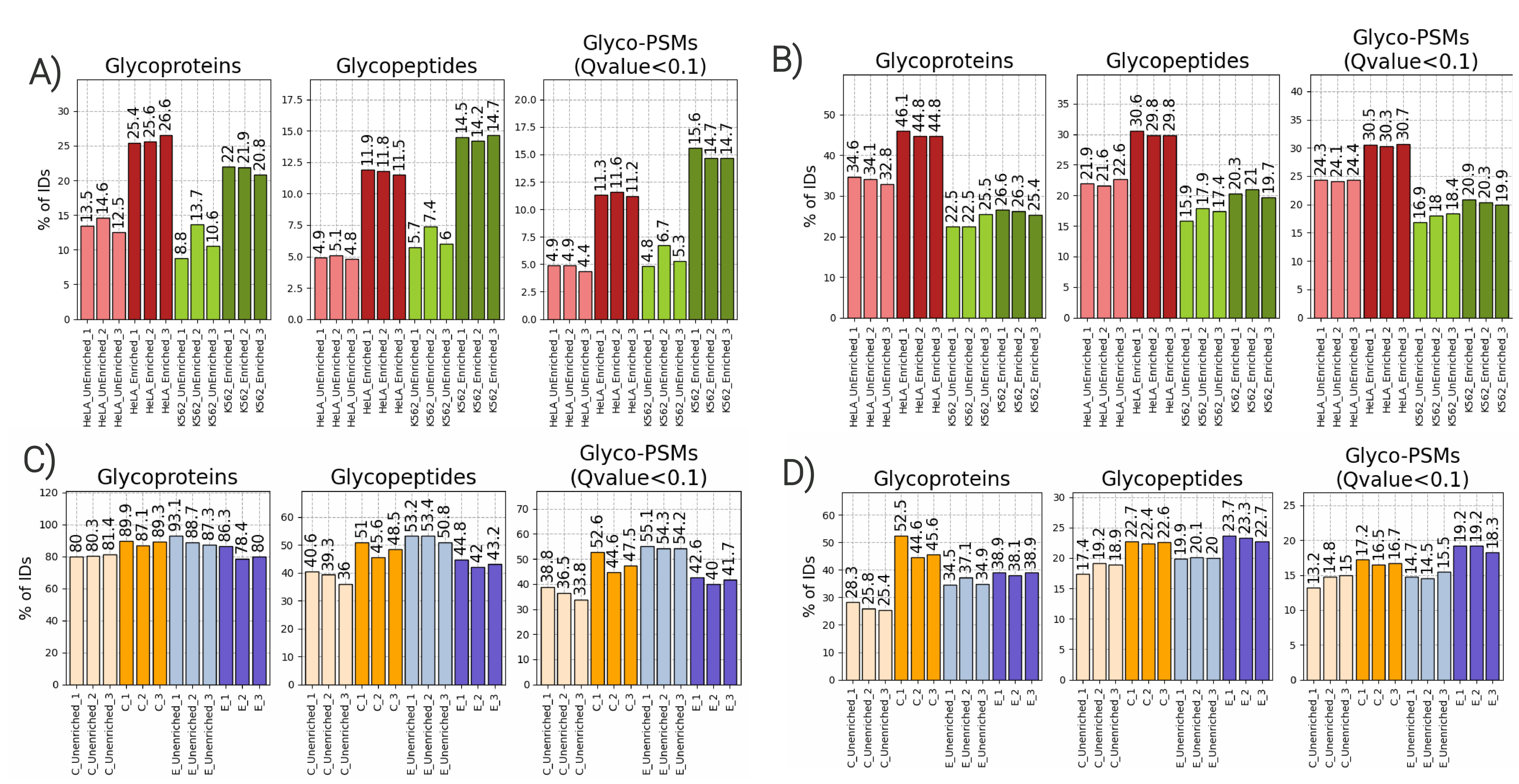


Figure 6. Sialylation (A: HeLa and K562, C: Plasma,) and Fucosylation (B: HeLa and K562, D: Plasma) Profiles in Enriched and Non-Enriched Samples, revealing both global enrichment effects and species-specific glycosylation trends.

Summary

HILIC enrichment significantly improved glycopeptide identifications, with consistent reproducibility across replicates (CV <10%). Stepped-energy CID enhanced fragmentation efficiency, leading to higher glycopeptide coverage. GlycoScape and MSFragger analyses showed comparable improvement results, confirming workflow robustness. Combining automated HILIC enrichment with glyco-PASEF® on timsTOF Ultra 2 offers a scalable and high-throughput workflow for comprehensive glycoproteomic profiling. Enhanced glycopeptide identifications and improved reproducibility make this approach ideal for biomarker discovery and disease characterization.