

A High-Throughput Platform for Preparation of APTS-Labeled N-Glycans: Improving the Accuracy, Reproducibility and Time-to-Results of N-Glycan Profiling

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Abstract

Analysis of APTS-labeled glycan conjugates using capillary electrophoresis (CE) is a sensitive, robust and fast approach to characterize N-linked sugar structures. However, this method has not been widely implemented in screening large numbers of samples, due in part to the lack of commercially available tools for automated sample preparation and analysis. Figure 1 presents an overview of a glycoanalysis platform comprising rapid sample preparation coupled with CE-based glycan analysis, which includes: 1) an automatable format with an optional purification module to allow direct screening of monoclonal antibody (MAB), cell-culture samples; 2) quantitative deglycosylation and separation of N-glycans; 3) complete glycan labeling for laser-induced fluorescence (LIF) detection; 4) efficient glycan sample cleanup and desalting to reduce excess reagent peaks; and 5) N-glycan profiling and glucose unit (GU) value-based structural prediction with a high degree of accuracy. The platform enables the generation of up to 96, high-quality results overnight.

The GlykoPrep® N-Glycan Sample Preparation protocol features optional purification of MABs, enzymatic deglycosylation, APTS labeling, cleanup and standardization of N-glycans optimized for downstream CE-LIF analysis. Using proprietary reagents, the optimized labeling reagents provide complete derivatization with only one-hour incubation time, without degradation of important labile groups, such as sialic acid and core or outer-arm fucose. After labeling, the remaining free APTS is efficiently (>99.9%) removed and the sample eluted in water, enabling the potential for mass spectrometry confirmation of atypical peaks. Spike-in, Lower and Upper Internal Mobility Standards are used to normalize glycan migration times further reducing variability between runs; GU assignment based on normalized glycan migration times showed significant improvement in precision compared to GU assignment without use of the Internal Mobility Standards. It is envisioned that this unique glycoanalysis solution will be important for the standardization and expansion of N-glycan profiling in such applications as clone selection and cell-culture optimization.

Methods

Deglycosylation, labeling and purification of APTS-labeled N-glycans. For each sample, 50 µg of protein was deglycosylated using the GlykoPrep Digestion Module (ProZyme product code G996-RX). The glycans were eluted in water, dried and labeled with APTS using a developmental GlykoPrep APTS Labeling Module, where the labeling solution was added to the glycans and incubated for 1 hour on a GlykoPrep Incubation Block (70°C setting, 52°C well temperature), then purified using a developmental GlykoPrep APTS Cleanup Module, where APTS-labeled N-glycans were eluted in 50 µL of HPLC-grade water. The labeled glycans were analyzed by CE using a PA 800 plus Pharmaceutical Analysis System (Beckman Coulter®), equipped with an N-CHO capillary equilibrated with Carbohydrate Separation Buffer. Hydrodynamic injections were 0.5 psi for 3.0 sec, and the separation voltage was constant, 30 kV for the 50-cm capillary.

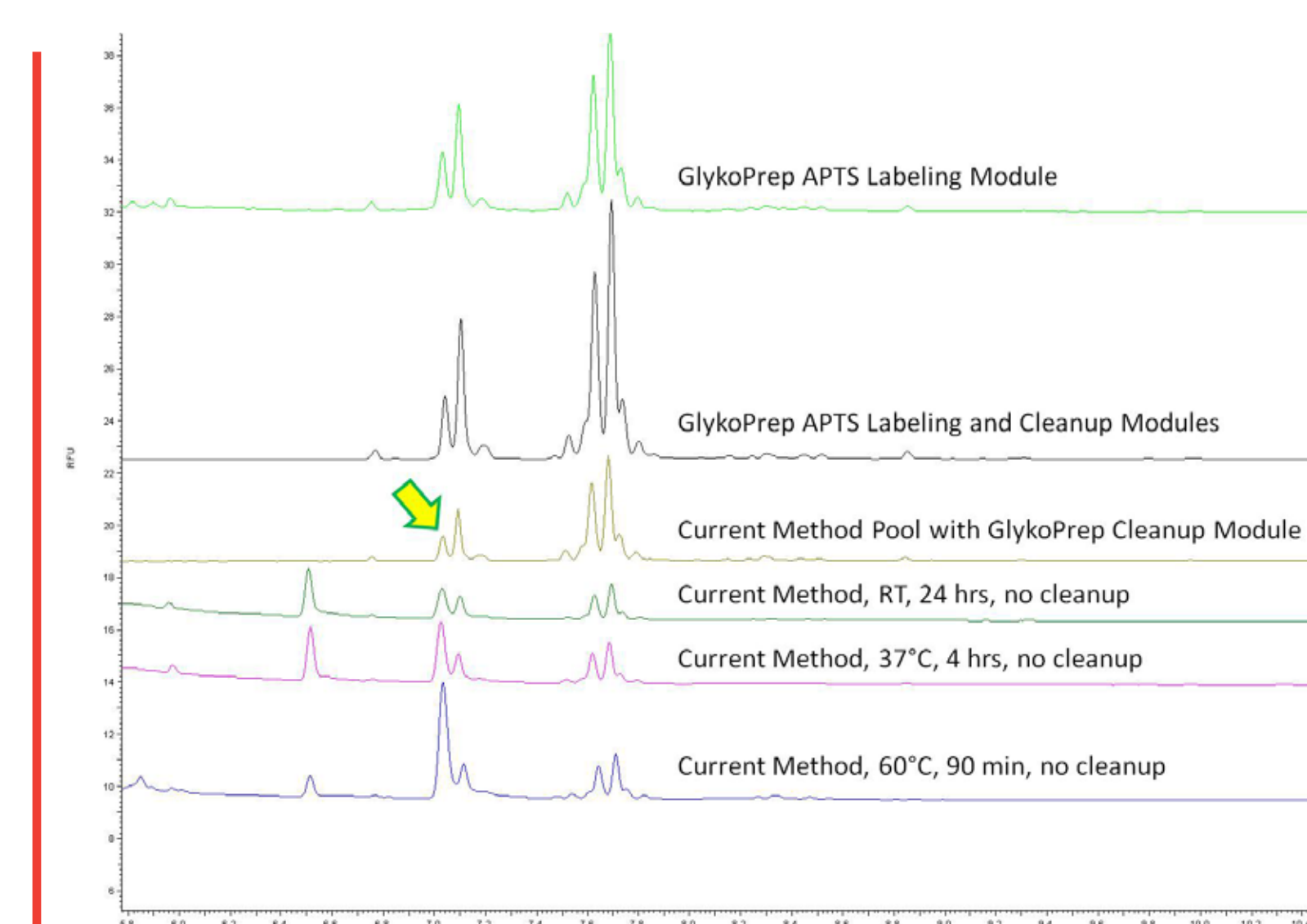


Figure 2. Fetuin N-glycans Labeled with APTS under Various Conditions with and Without Cleanup.

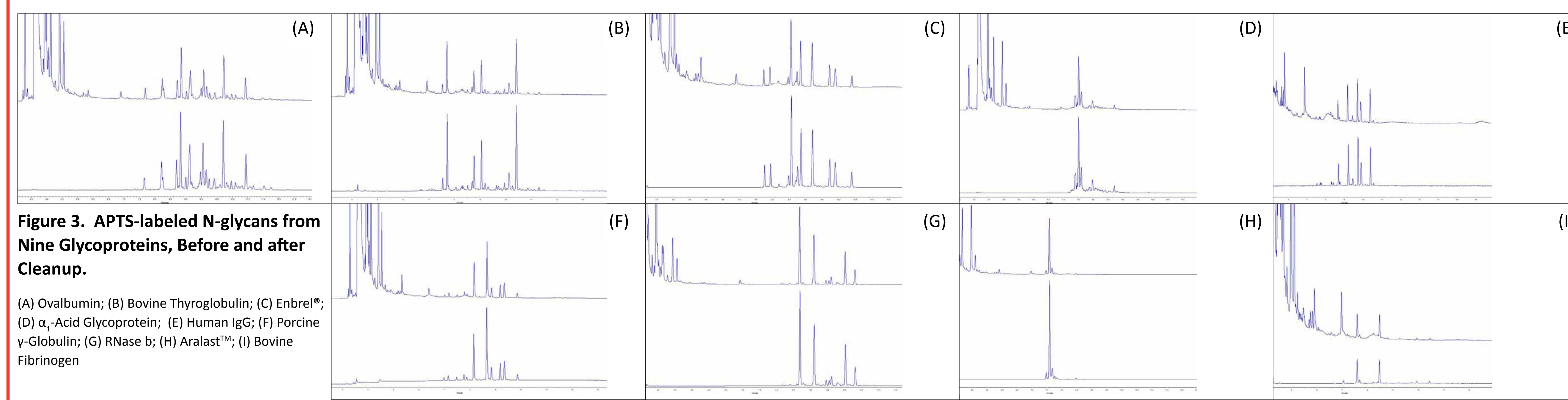


Figure 3. APTS-labeled N-glycans from Nine Glycoproteins, Before and after Cleanup.

(A) Ovalbumin; (B) Bovine Thyroglobulin; (C) Enbrel®; (D) α-Acid Glycoprotein; (E) Human IgG; (F) Porcine γ-Globulin; (G) RNase b; (H) Aralast™; (I) Bovine Fibrinogen

Comparison of common APTS labeling conditions with the GlykoPrep APTS Labeling Module. Five samples of bovine fetuin (5x50 µg) were deglycosylated using the GlykoPrep Digestion Module. The glycans were eluted in water, pooled, aliquoted into six samples and dried. Two of the samples were labeled using the developmental GlykoPrep APTS Labeling Module. After labeling, the samples were separated into two portions: one analyzed by CE without purification and one purified using the developmental GlykoPrep APTS Cleanup Module prior to CE analysis. The remaining three samples were labeled using Current Methods (Carbohydrate Labeling and Analysis Kit from Beckman Coulter) with each sample incubated at one of the three recommended conditions: 60°C for 90 minutes; 37°C for 4 hours; or RT for 24 hours. The samples were analyzed by CE individually, then pooled and purified using the developmental GlykoPrep APTS Cleanup Module prior to CE analysis.

Use of the Lower and Upper Internal Mobility Standards to assign GU. A maltodextrin ladder was labeled with APTS and purified from the unreacted dye. Similarly, maltose (Dp2, GU=2) was labeled and purified to produce a Lower Internal Mobility Standard. An Upper Internal Mobility Standard was optimized to migrate slower than most common glycans. N-glycans from Human IgG (hIgG) were APTS labeled, purified and the Internal Mobility Standards added. The N-glycans were analyzed by CE in ten replicates and GU assignment calculated based on peak migration times alone or relative migration times using the Internal Mobility Standards.

Differential exo-glycosidase treatment of APTS-labeled fetuin glycans. N-glycans from 150 µg of bovine fetuin (3x50 µg) were prepared using the GlykoPrep Digestion Module and labeled with APTS using the developmental GlykoPrep APTS Labeling and

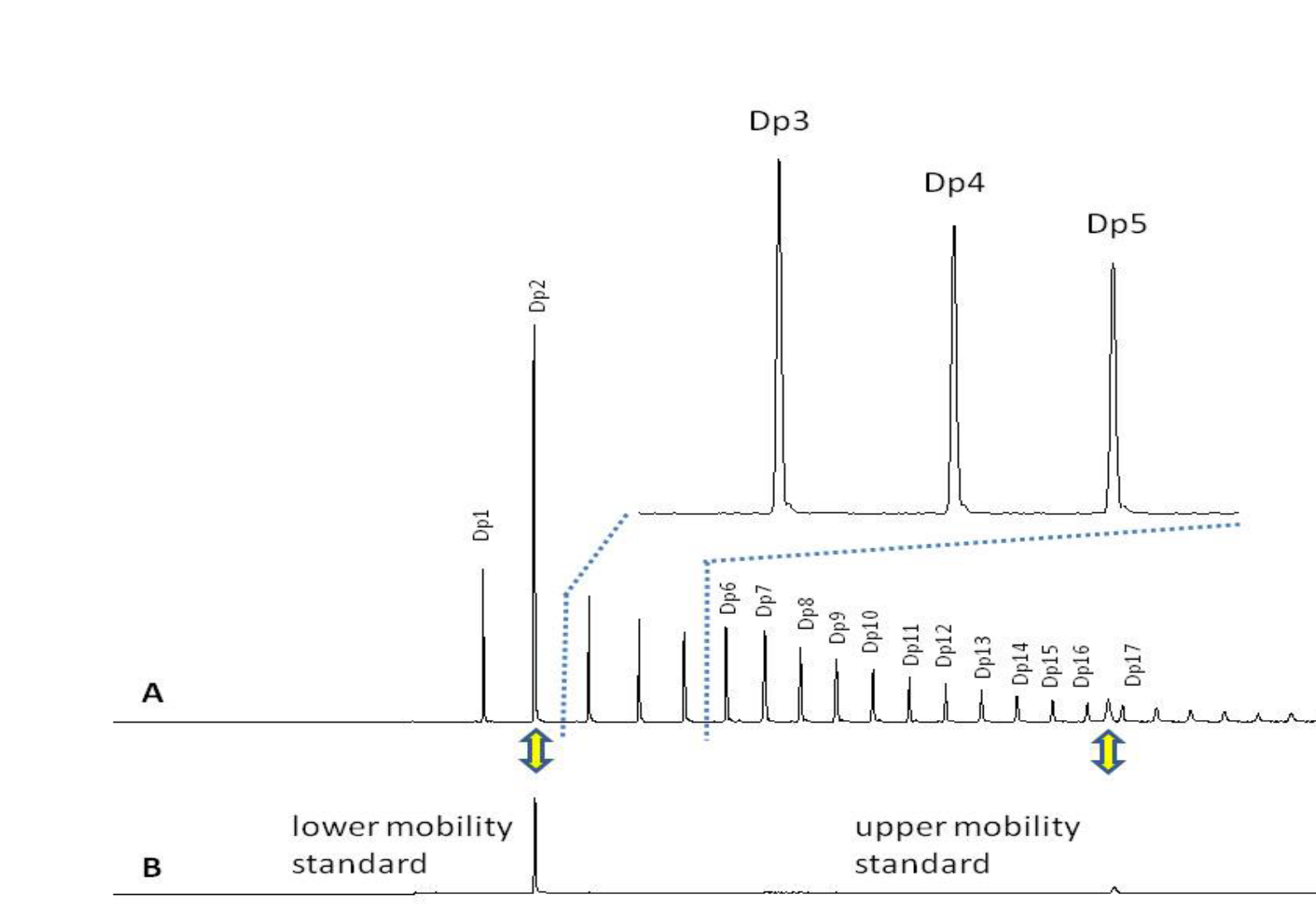


Figure 4. A) Maltodextrin-APTS Ladder with Upper Internal Mobility Standard. B) Internal Mobility Standards. Inset shows peak resolution for APTS-labeled tri-, tetra- and pentamer (Dp3, Dp4, Dp5) dextran oligomers. The Internal Mobility Standards (lower trace) contain APTS-labeled Dp2 and the Upper Mobility Standard.

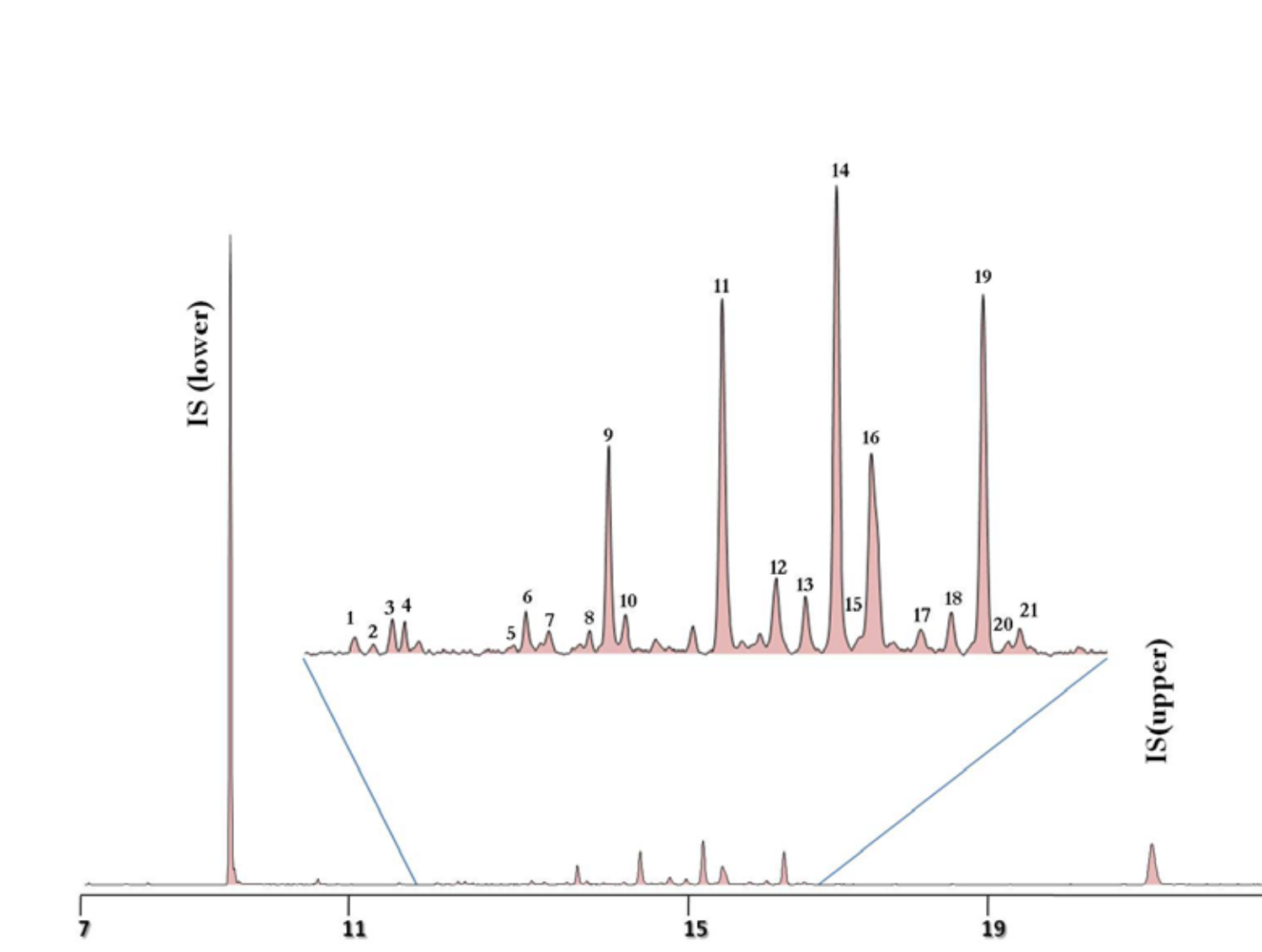


Figure 5. CE Profile of N-glycans from Human IgG with Internal Mobility Standards.

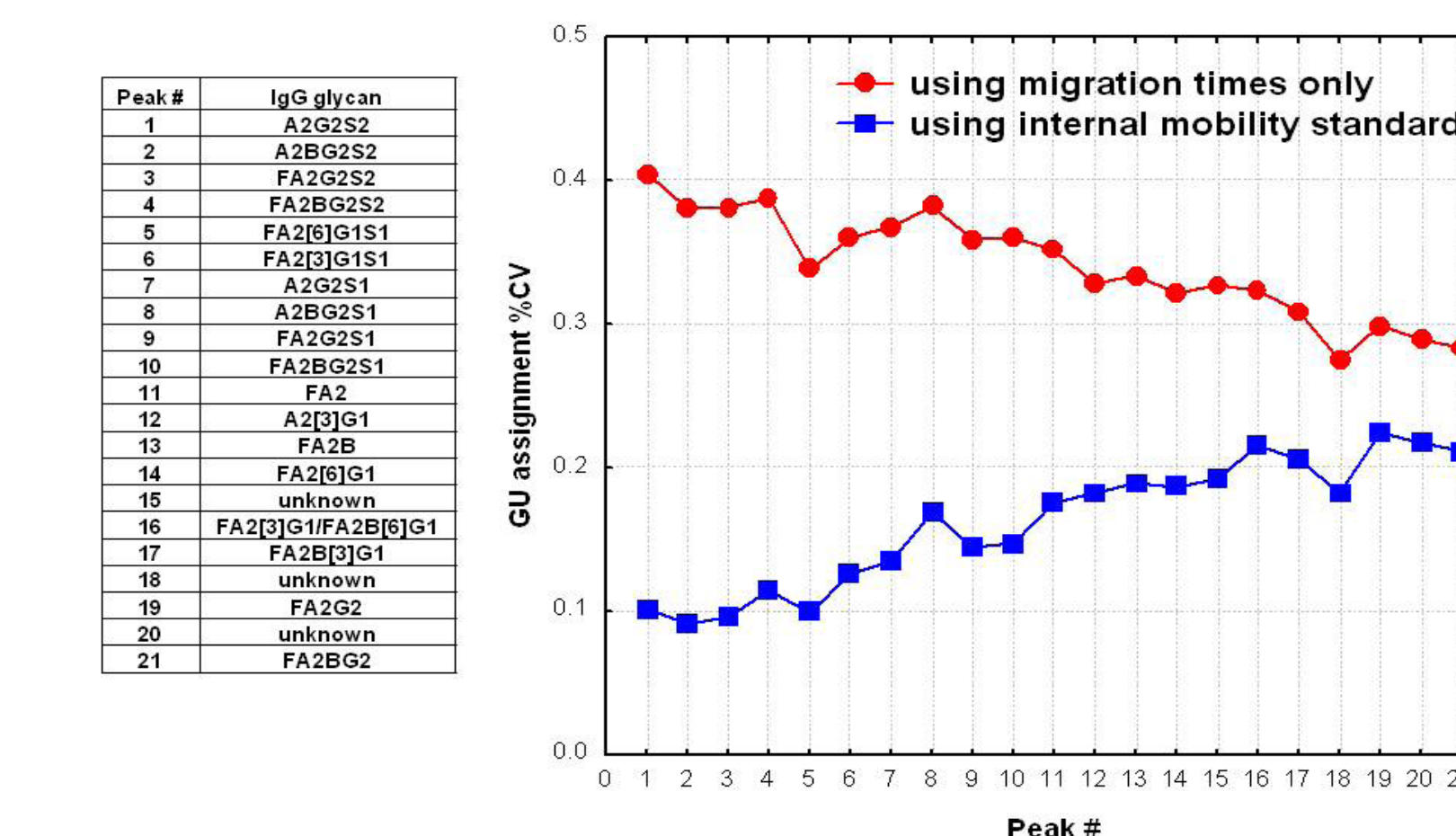


Figure 6. Improvement in the Precision of GU Assignment Using Internal Mobility Standards.

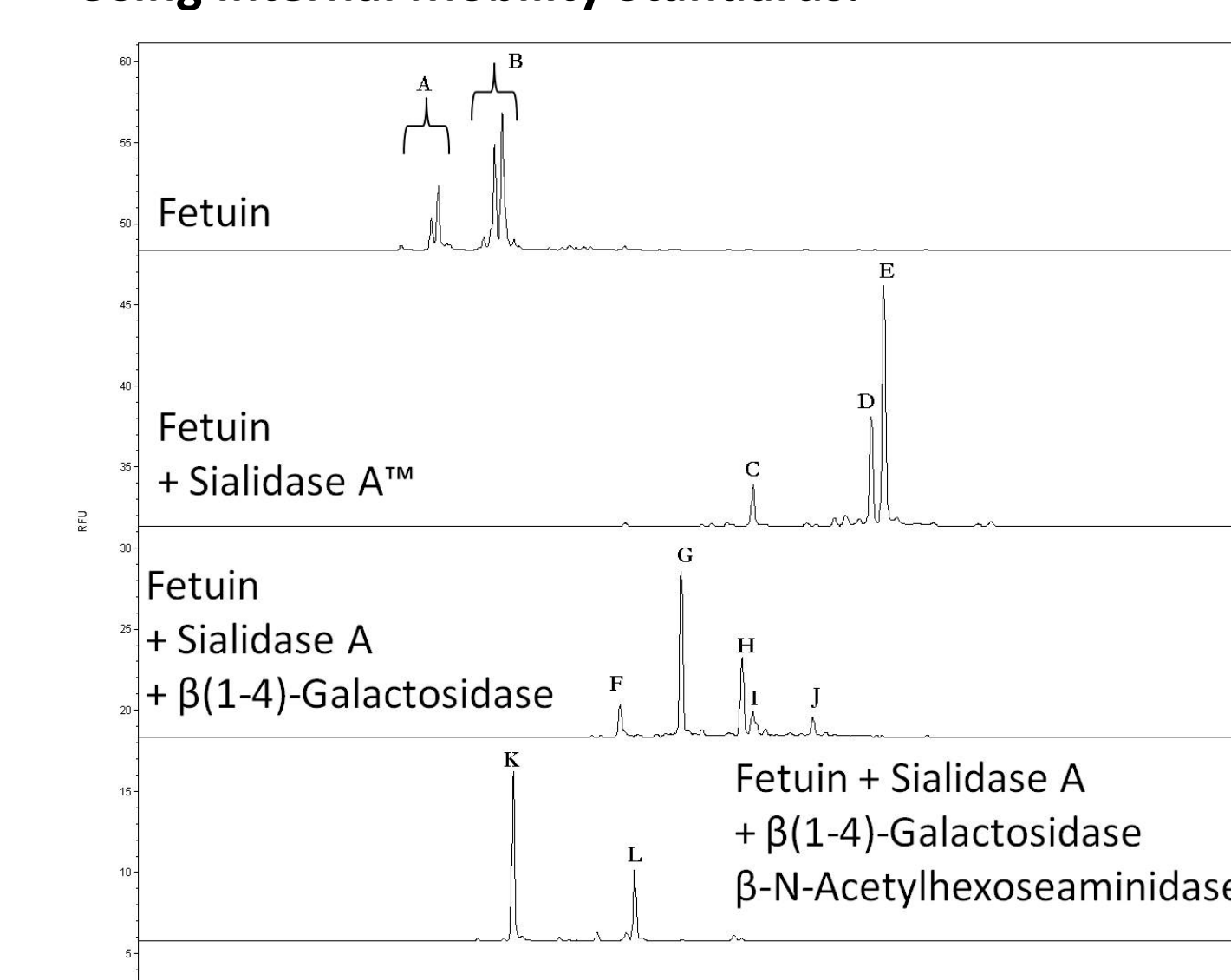


Figure 7. Treatment of APTS-labeled N-glycans with Exo-glycosidases Allows Oligosaccharide Sequencing, Verification and Validation.

A) Tetrasialylated triantennary; B) Trisialylated triantennary; C) A2G2; D) A3G(4)3; E) A3G(3,4,4)3; F) A2; G) A3; H) A3[4]G(3)1; I) A3[6]G(3)1; J) A3G(3)2; K) M3; L) A1G(3)1

Cleanup Modules. The N-glycans were then pooled and digested using the FACE® N-Linked Oligosaccharide Sequencing Kit (ProZyme product code GK90300), utilizing a combination of Sialidase A™, β(1-4)-Galactosidase and/or β-N-Acetylhexosaminidase. Digested samples were analyzed by CE and compared to undigested APTS-labeled N-glycans.

Results

GlykoPrep APTS labeling outperforms Current Methods and preserves highly sialylated structures. Bovine fetuin was used as a model protein because it contains highly sialylated N-glycans. The N-glycans were isolated using the GlykoPrep Digestion Module and eluted pooled and aliquoted prior to APTS labeling to ensure that each labeling condition contained equal amounts of starting material. Comparison of methods in Figure 2 shows that the GlykoPrep APTS Labeling and Cleanup Modules give more intense peaks, yet do not induce desialylation (evidenced by the absence of peaks >9 minutes on the CE profile). An artifact at ~7 minutes (arrow) increases with increasing temperature when using Current Methods. This artifact co-migrates with the tetrasialylated peak at 7 minutes. This artifact, however, is removed upon cleanup (brown trace).

Cleanup efficiently removes free APTS and retains labeled glycans. Nine standard glycoproteins were chosen to produce a variety of glycoforms. Figure 3 shows CE profiles of APTS-labeled N-glycans prepared with the developmental GlykoPrep APTS Cleanup Module. In each profile, the top trace is an unpurified sample, and the lower trace is purified using the developmental GlykoPrep APTS Cleanup Module. Equal amounts of labeled glycan were purified and the results show that recovery based on peak area differences is greater than 100%. This is likely due to reduced viscosity of the purified samples. In all cases, removal of APTS is complete, which significantly improves the baselines.

No significant differences were found in the relative abundance for the hIgG N-glycans before and after cleanup (data not shown). Future work will include detailed comparisons using a statistically significant number of replicates.

APTS Ladder and Internal Mobility Standards. Figure 4A shows the APTS-labeled ladder containing the Lower and Upper Internal Mobility Standards. The inset shows a zoomed region to illustrate peak integrity. Figure 4B shows the Internal Mobility Standards well separated from the migration of typical glycans.

Improved reproducibility of GU assignment using the Internal Mobility Standards. The N-glycans from hIgG (Figure 5) were analyzed in ten

replicates with the Internal Mobility Standards. GU-assignment of the 21 hIgG peaks were calculated with and without normalization to the Internal Mobility Standards. Percent CV's of the GU assignments for each peak are shown in Figure 6. Peak identity of CE_{GU} of hIgG is based on previous work.¹

Purified APTS-labeled glycans are ideal substrates for oligosaccharide sequencing by exo-glycosidases. Tetra- and tri-sialylated triantennary structures are the predominant N-glycans in bovine fetuin (Figure 7). Digestion shows the products when the sialylated structures are treated with a combination of Sialidase A, β(1-4)-Galactosidase and/or β-N-Acetylhexosaminidase.

Conclusion

1. The developmental GlykoPrep APTS Labeling Module provides rapid and complete labeling in one hour while preserving sialylated structures.
2. The developmental GlykoPrep APTS Cleanup Module results in quality baselines and elimination of co-migrating artifacts from the APTS labeling reaction, allowing a more accurate interpretation of the CE profile.
3. Internal Mobility Standards and the APTS-labeled Maltodextrin Ladder improve overall reproducibility as well as the precision of GU assignments.
4. APTS-labeled N-glycans can be sequenced with exo-glycosidases for structural elucidation.

References

1. Mittermayr, S., Bones, J., Doherty, M., Guttman, A., Rudd, P.M., Multiplexed Analytical Glycomics: Rapid and Confident IgG N-Glycan Structural Elucidation. *Journal of Proteome Research* 2011, 10 (8), 3820-3829.

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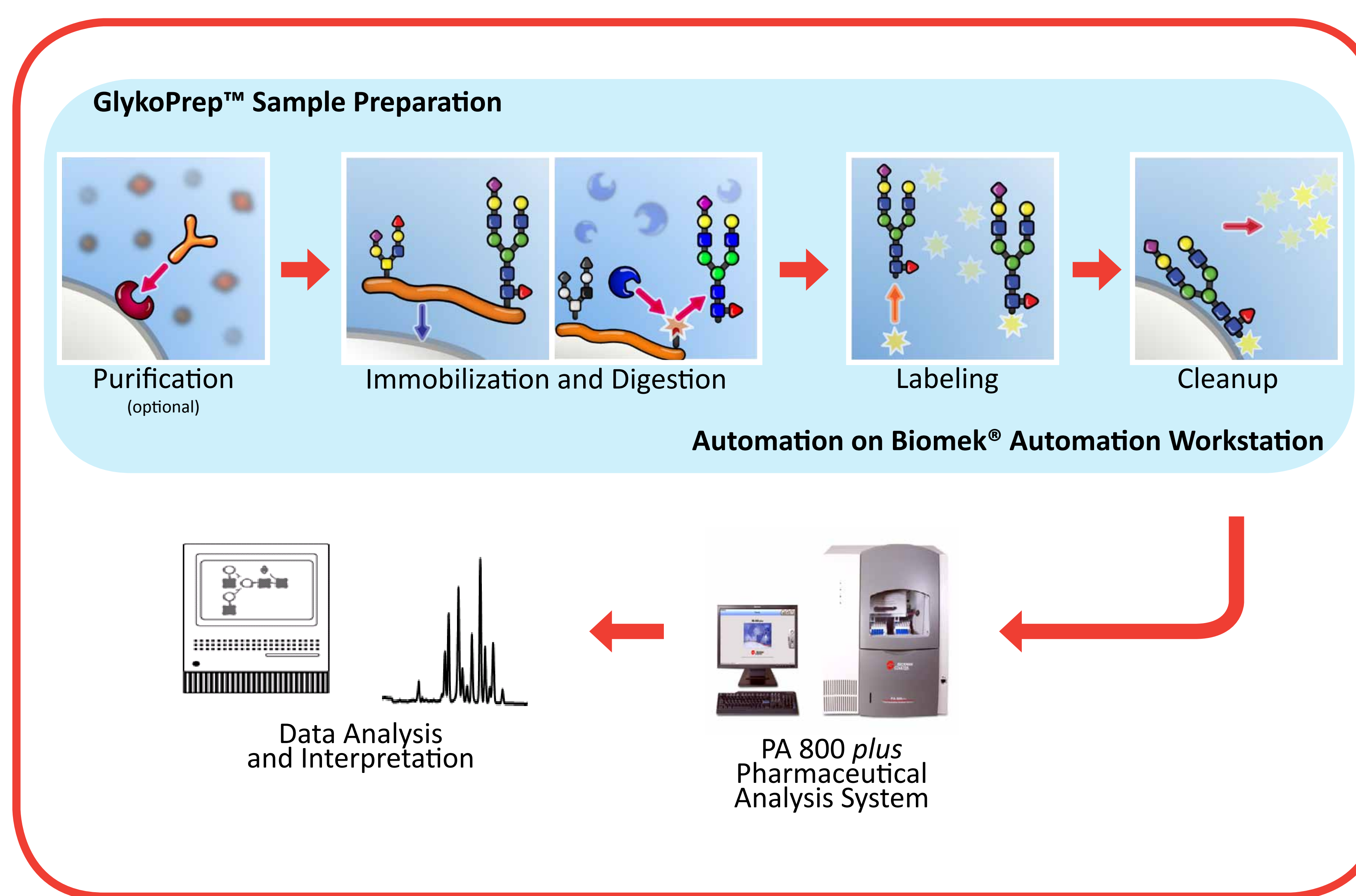


Figure 1. Glycoanalysis Platform Overview.