AssayMAP breakthroughput

PROzyme®

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A New Platform for High Throughput N-Glycan Sample Preparation

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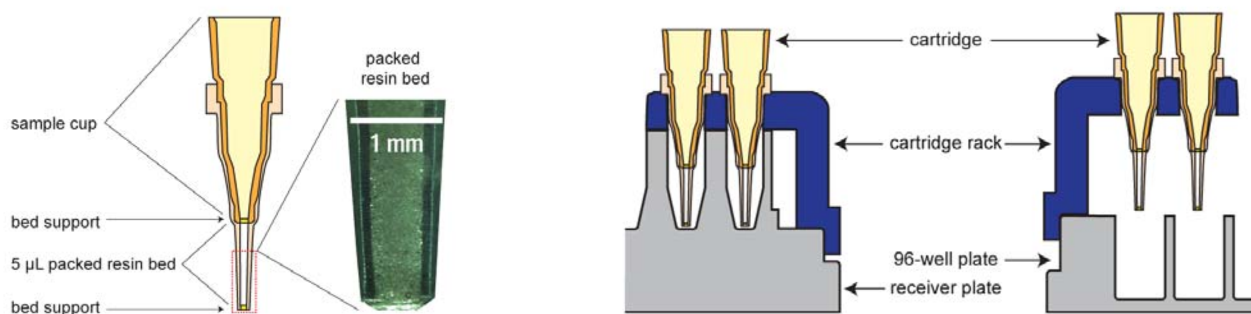
Introduction

The distribution of N-linked glycans plays a critical role in the pharmacology of therapeutic proteins, potentially affecting immunogenicity, pharmacokinetics and pharmacodynamics. While prompt information is highly valuable for bioreactor control, strain selection, comparability studies and biomarker discovery, the manual, complex and multi-day sample preparation usually limits analysis to only the most critical decision points. With the advent of QbD, there is a growing need for dramatically increased throughput of N-glycan profiling.

ProZyme, Inc. and BioSystem Development, LLC have jointly developed the new GlykoPrep™ platform for rapid, quantitative N-glycan sample preparation. This assay has been implemented on the AssayMAP® platform, which enables microliter-scale separation and enzymatic digestion in a high-throughput format compatible with microplate liquid handling. Using GlykoPrep, fluorescently labeled N-glycans can be produced from up to 192 crude supernatant samples in less than 3 hours, ready for analysis by CE, HPLC or LC/MS.

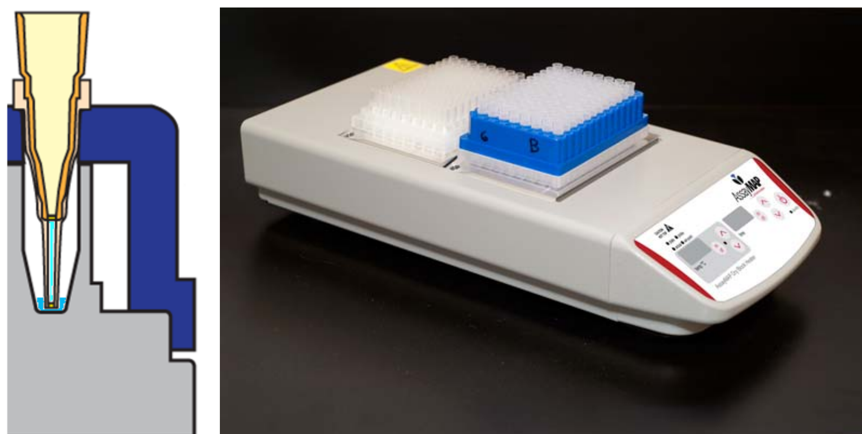
AssayMAP Cartridge Technology

AssayMAP utilizes disposable cartridges containing a 5 µL packed bed with any resin (particle size 20 – 100 µm). The bed is retained by insert-molded bed support filters. A sample cup above the bed can hold up to 200 µL sample or reagent.



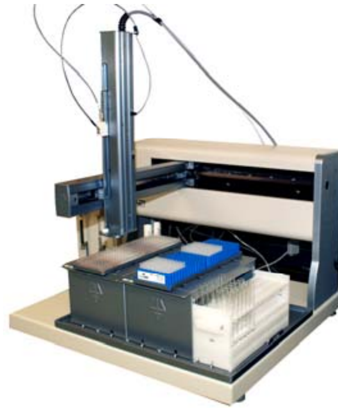
AssayMAP cartridges are used in special molded racks which stack on either a standard microplate to collect eluted product for analysis or a special "receiver plate", which keeps the cartridge tip immersed in liquid during operation to prevent the bed from drying out.

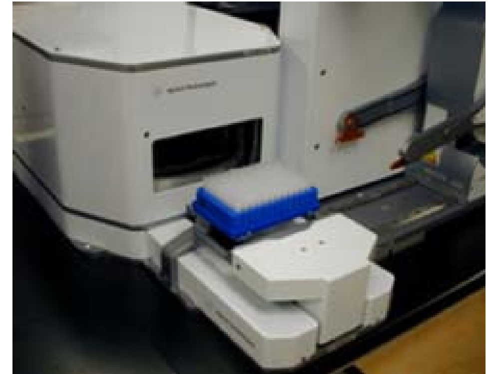
Cartridges are loaded into racks stacked on the appropriate plates, and sample, buffers and other reagents are pipetted into the cartridge cups. Spinning at low speed in a standard microplate centrifuge drives the liquid through the bed. With a 2-plate rotor, up to 192 samples can be processed simultaneously. Fewer samples can be processed by loading any number of individual cartridges in the rack.



In addition to separations, the AssayMAP cartridges can also be used for enzymatic digestion. A special aluminum heater block (designed to fit in standard dry-block lab heaters) fits the receiver plates to provide rapid heating of the liquid inside the well, which in turn heats the liquid in the packed bed. This system enables elevated temperature incubation of enzyme reactions, which is critical for providing complete deglycosylation in a short period in the GlykoPrep protocol.

A variety of different approaches may be used to perform AssayMAP protocols. One is to run manually with multi-channel pipets. Up to 192 samples can be processed in 3 hours using manual pipetting. Another approach is to automate the pipetting using a single channel automated liquid handler, such as the Gilson GX271 shown here. This low cost approach minimizes human error and improves precision over manual liquid handling. AssayMAP protocols can also be fully automated using standard laboratory robotics systems for applications requiring very high throughput and/or full “walk-away” capability.

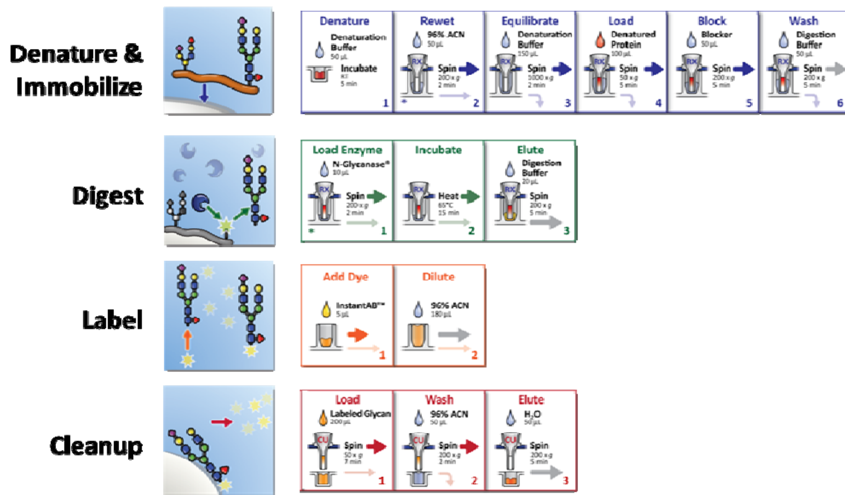




GlykoPrep Protocol

N-Glycan profile analysis is currently performed by CE, HPLC or LC/MS, but sample preparation is a major bottleneck. The target protein must first be purified from the sample. N-glycans are then specifically released by digestion with the enzyme PNGase F (N-Glycanase®), separated from the glycoprotein, and often fluorescently labeled and cleaned up (to remove excess dye) for analysis. This sample prep is usually done manually, can take several days, and is cumbersome with a large number of samples.

The GlykoPrep system was developed to make the cumbersome sample preparation procedure much faster, more efficient and suitable for automation. The procedure optionally begins with purification of antibody from crude samples using AssayMAP purification cartridges. The overall protocol is summarized here:

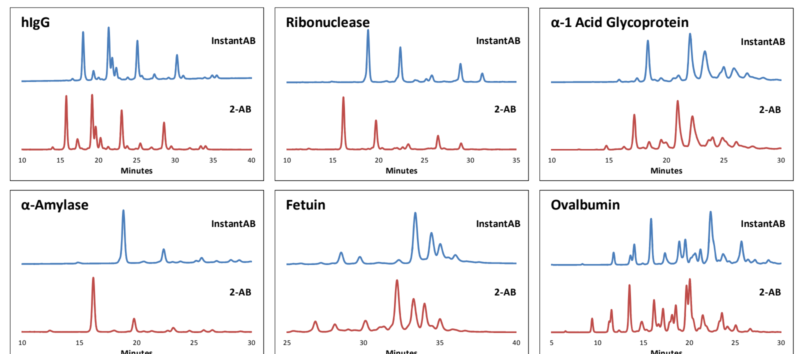


Several features distinguish the GlykoPrep system from standard methods:

- Integrated purification and sample preparation
- Glycoprotein denatured and immobilized on solid phase, which greatly enhances effectiveness of enzymatic digestion and separates released glycans from glycoprotein. Deglycosylated glycoprotein can be recovered for further analysis.
- “Instant” labeling chemistry eliminates drying steps and helps preserve sialic acids
- Integrated hydrophilic interaction cleanup of labeled N-glycans

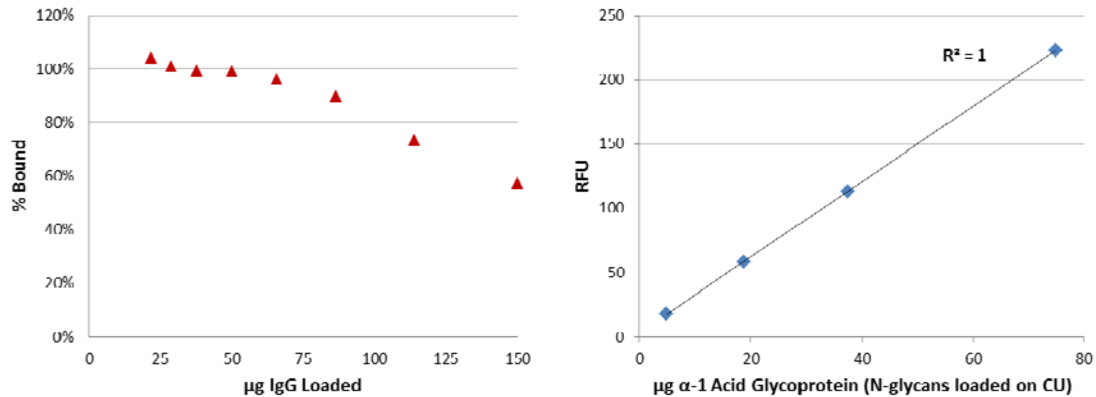
Comparison with Standard Methods

The chromatograms below show comparisons between the GlykoPrep method with Instant AB™ label and a conventional sample preparation method (using standard 2-AB label) for a number of well-characterized glycoproteins. The labeled N-glycan samples were run on HPLC using a GlykoSep™ N Plus column and a binary gradient of ammonium formate buffer and acetonitrile with the signal normalized. These proteins cover a broad range of molecular weights, types of N-glycans and glycan content. Although the retention times shift slightly due to the different dye structures, the results between the two methods are comparable.



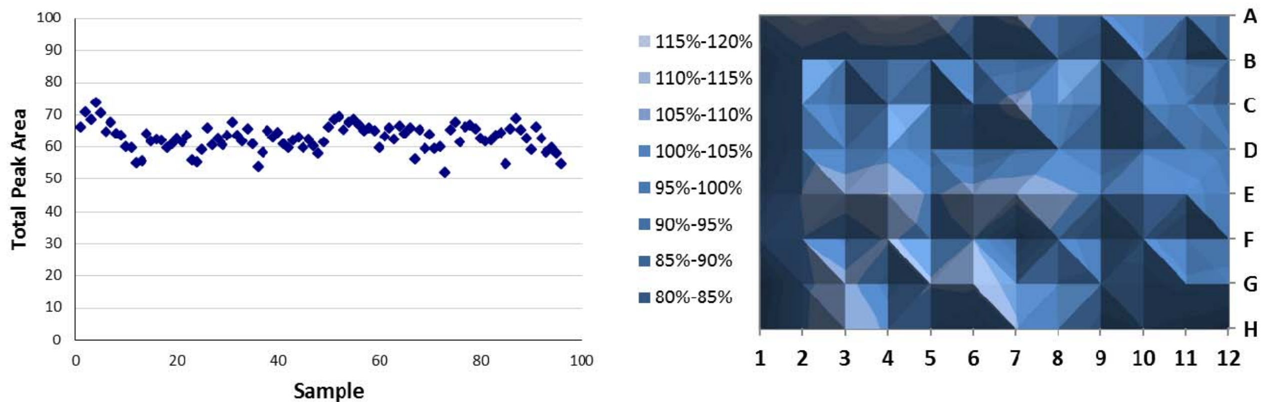
Capacity, Sensitivity & Reproducibility

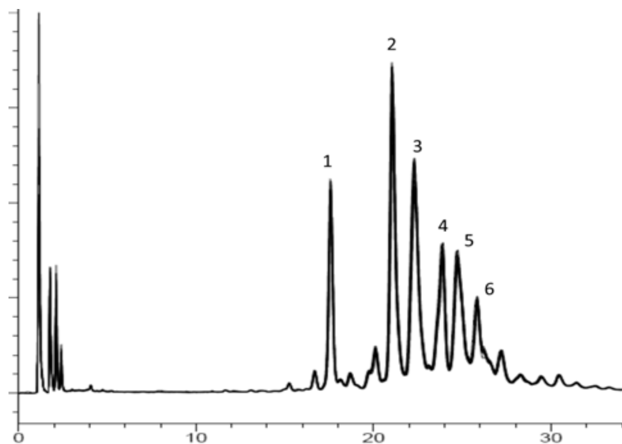
The GlykoPrep protocol can produce N-glycan profiles quantitatively from up to 50 µg of glycoprotein (including those with very high N-glycan content). The data below show the breakthrough capacity for the RX reaction cartridge for IgG (a relatively high MW glycoprotein) and the total peak area obtained from the CU cleanup cartridge as a function of load for α-1 acid glycoprotein (which has an N-glycan content of 45-55%). Lower MW proteins show similar capacity on the RX; IgM (~1 M kD) has a capacity of ~20 µg (data not shown). Even with low N-glycan content proteins (such as IgG) accurate N-glycan HPLC profiles can be obtained with ≤1 µg protein (data not shown).



To test the reproducibility of the GlykoPrep protocol, 96 identical samples (a full plate) of
25 µg α-1 acid glycoprotein were run through the protocol at one time. The data below show the total glycan peak area from the HPLC chromatograms, as well as a map of the plate, expressed as % of the total area. The intra-assay %CV for the 96-well run was 6.4%. Inter-assay and inter-operator runs (n=24 each) showed %CVs of 8 – 10%. Despite the complex protocol with multiple steps, the assay is quantitative.

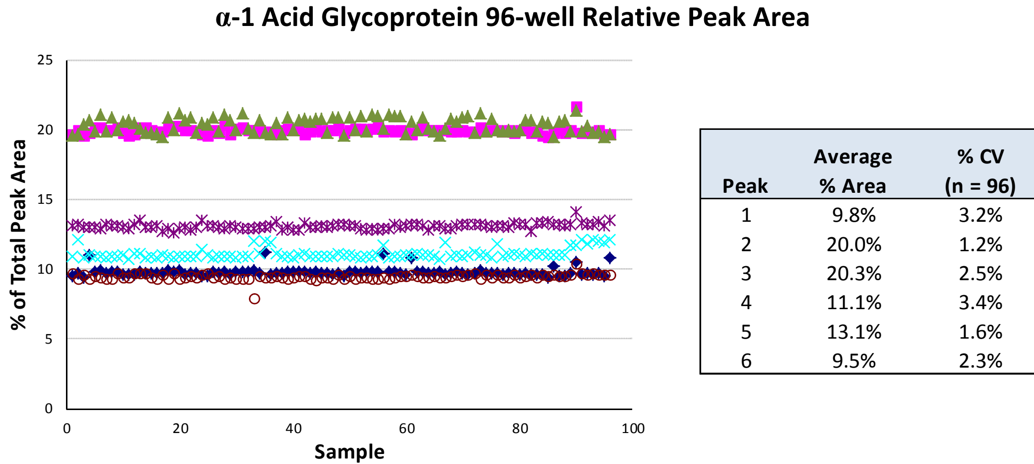
α-1 Acid Glycoprotein 96-well Total Peak Area





12 stacked chromatograms from runs 24 - 36

Reproducibility of the % of the total peak area for the 6 major peaks in the chromatogram from the run described above is shown below. CVs for percent of total peak area are <3.5%.

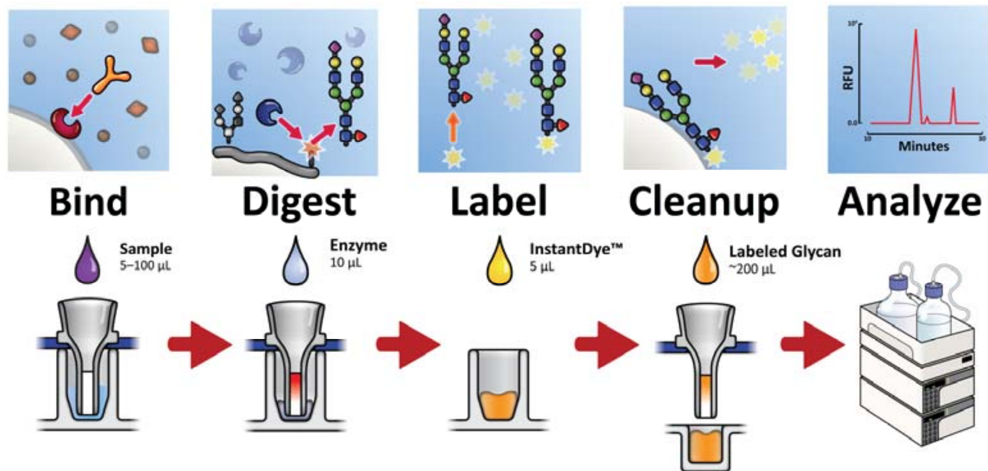


Peak	Average % Area	% CV (n = 96)
1	9.8%	3.2%
2	20.0%	1.2%
3	20.3%	2.5%
4	11.1%	3.4%
5	13.1%	1.6%
6	9.5%	2.3%

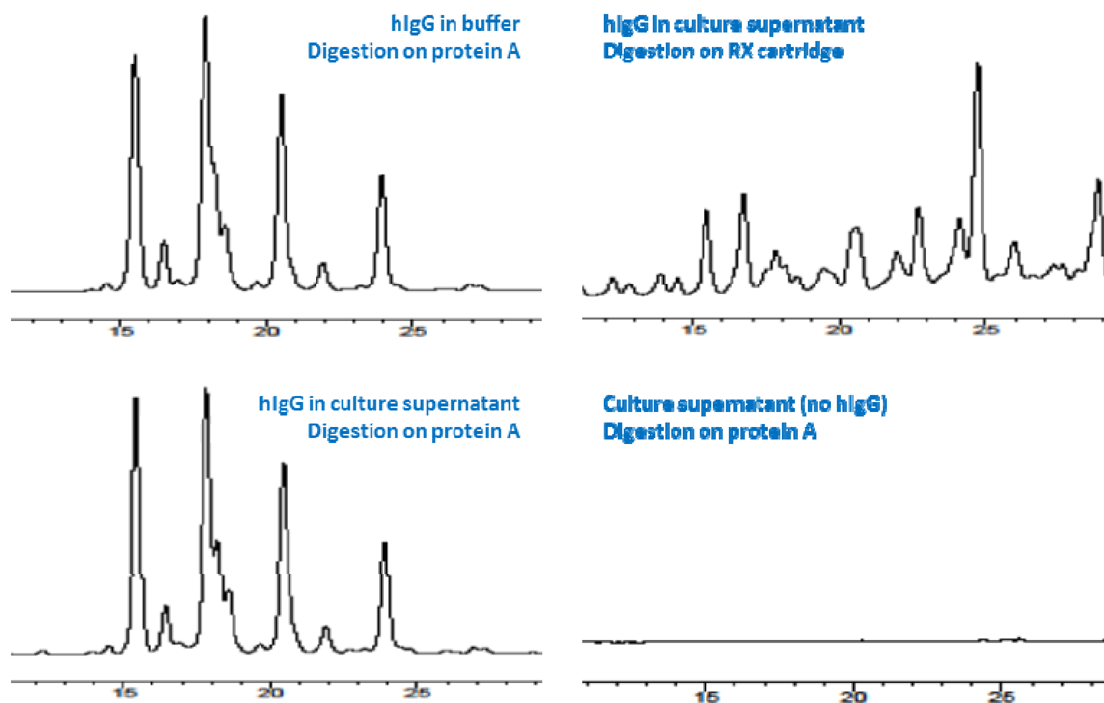
Combined Purification & Digestion for MAbs

The basic GlykoPrep protocol (shown at left) requires that the target glycoprotein be purified before starting the procedure. For applications in cell culture process development and analysis of clinical trial patient samples, such a purification step is a critical element in the workflow. AssayMAP cartridges packed with various kinds of affinity media can be used as an initial bind/wash/elute purification step, with the benefit of accurate and precise target glycoprotein quantitation included.

For MAbs, this combined purification and N-glycan sample preparation workflow can be simplified even further. The protein A binding site on IgG is well removed from the N-glycosylation sites located in the Fc region. In addition, IgG is relatively “easy” to digest with PNGase F, so that denaturation is not required for rapid and complete deglycosylation. Thus if protein A is used to selectively bind the MAb from a crude cell culture sample, the enzymatic digestion can be carried out directly on the protein A bound MAb, thus combining purification and digestion in a single step. An overview of the protocol is shown here:



The data below show comparative HPLC chromatograms of labeled N-glycans produced by the protocol. When the protein A purification and digestion was run, the profiles for 25 µg loads of hIgG were identical whether the protein was spiked into buffer or a reference non-IgG-containing cell culture supernatant. When the IgG spiked into supernatant was applied directly to the RX reaction cartridge (which binds all proteins) for the standard GlykoPrep method, the glycans from non-IgG proteins in the supernatant completely swamped out the IgG profile. On the other hand, when culture supernatant with no IgG was run with the protein A purification and digestion method, no glycans were detected.



Conclusion

The ability to obtain hundreds of N-glycan profiles per day directly from crude supernatant samples has good potential to increase the effectiveness of strain development and cell culture optimization, and opens the door to other applications in drug discovery.

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