

Rapid Sample Prep for N-Glycan Analysis Using High Throughput Micro Chromatography

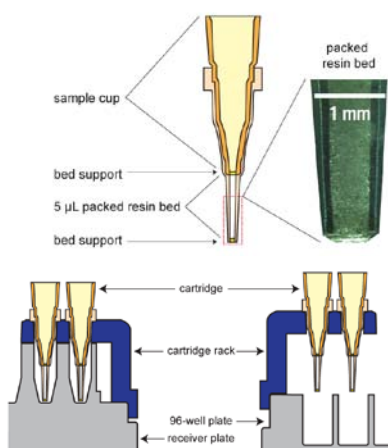
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Introduction

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. Mass screening of N-linked glycosylation in drug discovery is hardly contemplated.

ProZyme, Inc. has developed a new procedure for rapid, quantitative N-glycan sample preparation (GlycoScreen™ Rapid Glycoprotein Sample Preparation System or RGSPS). 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 RGSPS, 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

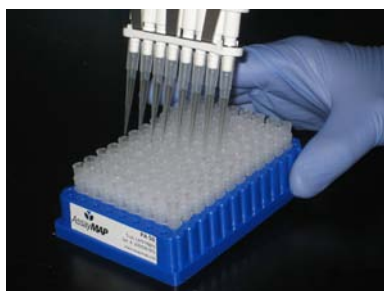


AssayMAP utilizes disposable cartridges containing a 5 µL packed bed with any resin (particle size 20 – 100 µm). The bed is retained by insert-molded bed support filters. A sample cup above the bed can hold up to 200 µL sample or reagent.

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.

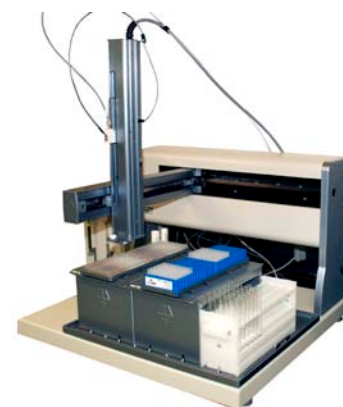
Cartridges are loaded into racks stacked on the appropriate plates, and sample, buffers and other reagents are pipetted into the cartridge cups. Spinning at low speed in a standard microplate centrifuge drives the liquid through the bed. With a 2-plate rotor, up to 192 samples can be processed simultaneously.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

Automation Approaches

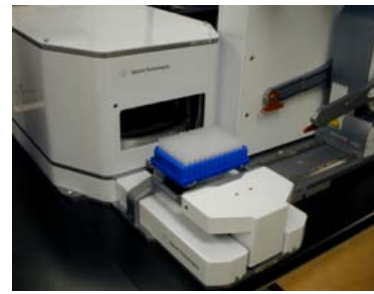
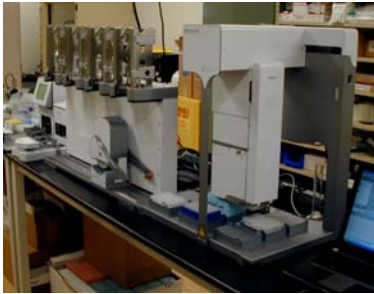


A variety of different approaches may be used to perform AssayMAP protocols. One approach is to run manually with multi-channel pipets. For simple protocols (such as the MAb Titer Assay) up to 192 samples per hour can be processed in this way.

Complex protocols (such as the RGSPS Assay) can be efficiently performed using a single channel automated liquid handler, such as the AssayMAP One Workstation shown here. This low cost approach minimizes human error and improves precision over manual liquid handling methods.

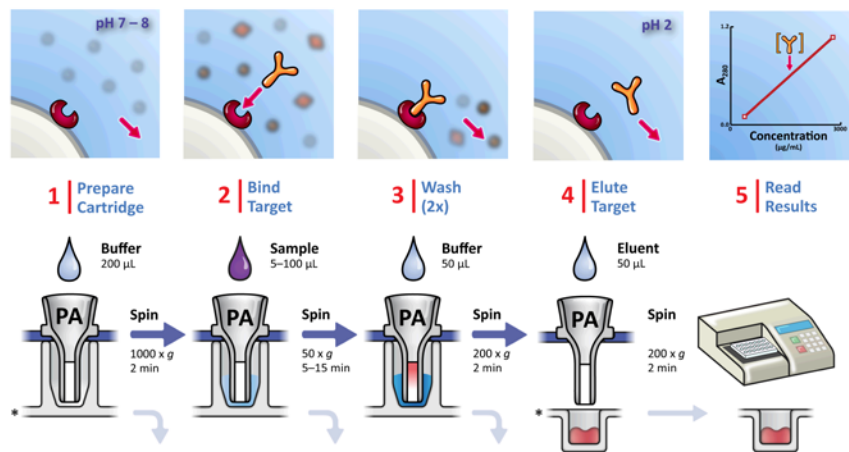


AssayMAP protocols can also be fully automated for applications requiring very high throughput and/or full “walk-away” capability. These photos show an assay being performed at Agilent Automation Solutions, using the Bravo liquid handler, BenchCel plate stacker/handler and VSpin robotic centrifuge.



MAb Titer Assay

Antibody concentration or titer assays are a known bottleneck in cell culture process development. The current “gold standard” method – protein A affinity HPLC – is limited to at most 4 to 12 samples/hour. The AssayMAP MAb Titer assay was developed to

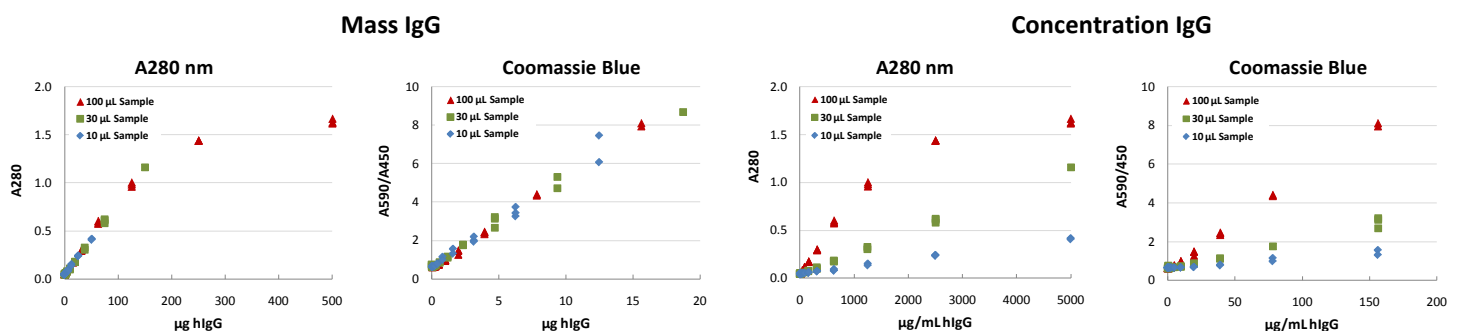


utilize the same method and chemistry as the “gold standard” (*i.e.*, protein A affinity chromatography), with dramatically improved throughput without special equipment. Protein A cartridges are first prepared by simultaneously rewetting the resin and equilibrating in buffer. Sample is then placed in the cup and spun at a low x g to insure complete binding. The cartridge is then washed twice with medium x g spins. The bound, purified IgG is then eluted with low pH buffer using a medium x g spin into a half-area UV microplate.

Readout of the plate is performed in a plate reader at 280 nm. A colorimetric protein assay reagent (such as Coomassie Blue) can be added for ~10X increased sensitivity

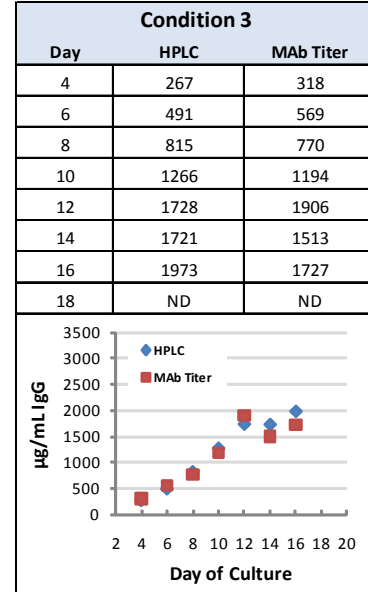
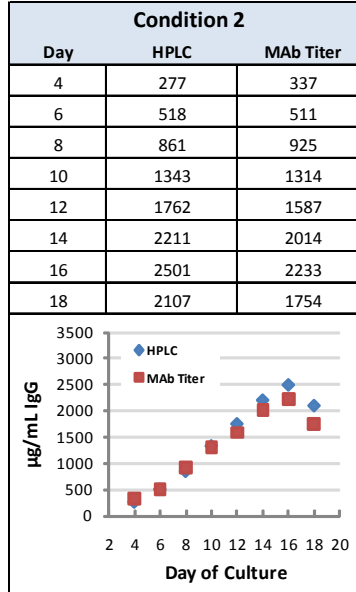
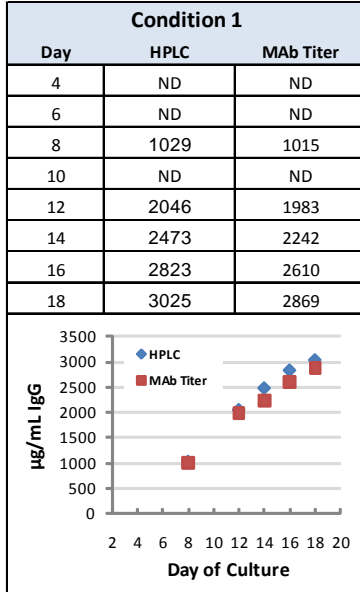
Performance with Standards

The MAb Titer Assay cartridge selectively and quantitatively binds the IgG from the sample (irrespective of its volume) and the bound IgG is eluted into a fixed final volume for readout. The functional assay range is best expressed in terms of mass (μg) of IgG in the sample. The upper limit for high product concentration is determined by the quantitative binding capacity of the cartridge ($\sim 100 \mu\text{g}$) and the sensitivity of the readout method determines the LOD/LOQ at low concentration, as illustrated in the data below. 10, 30 and 100 μL samples with various concentrations of hIgG were run on the cartridges. Readout was done using both $A_{280 \text{ nm}}$ and with Coomassie Blue reagent (using the ratio $A_{590 \text{ nm}}/A_{450 \text{ nm}}$). Plotted vs. μg hIgG, the curves for the three sample volumes overlap. 96-well plates run with the same 25 μg IgG standard sample gave CVs in the range of 4 – 5 %.



Monitoring Cell Culture Optimization

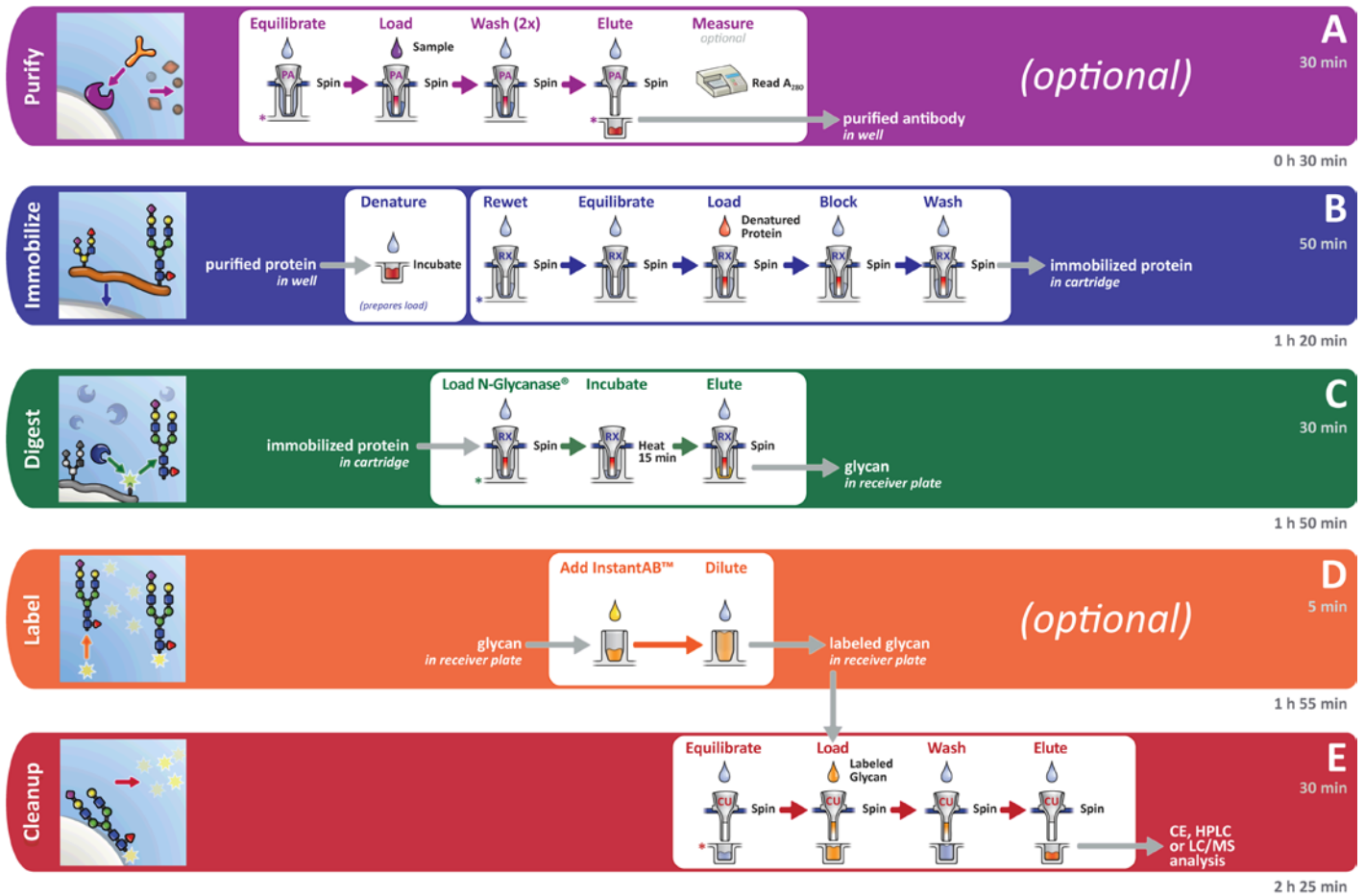
A major application for the MAb Titer assay is monitoring product IgG concentration during cell culture process optimization. The results shown below are from a set of bioreactor runs under three different operating conditions. The product was a monoclonal antibody produced in mammalian cells transfected using the GPEX[®] technology at Catalent Pharma Solutions (Middleton, WI). Periodic samples were taken from the bioreactor, and samples were analyzed using the standard affinity HPLC method at Catalent and by the MAb Titer assay (25 μ L sample, readout by A_{280nm}) at BioSystem Development. Samples and HPLC data provided by Drs. Ian Collins and John Otto of Catalent Pharma Solutions.



N-Glycan Profiling Assay

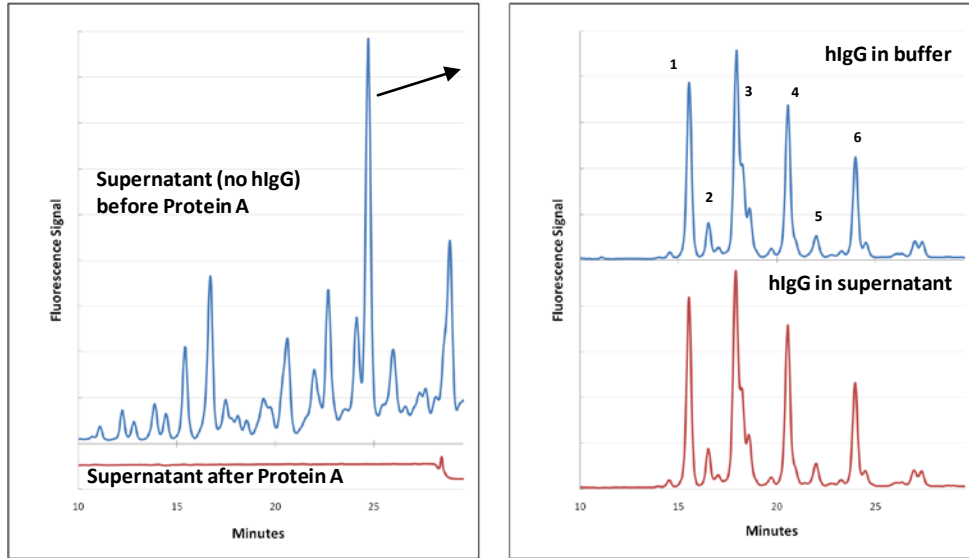
Glycosylation profiles, set during cell culture, critically affect the efficacy and pharmacokinetics of biotherapeutics, including antibodies. Glycan profile analysis is currently performed by CE, HPLC or LC/MS, but sample preparation is a major bottleneck. The target protein must first be purified from the sample. N-glycans are then specifically released by digestion with enzyme, 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.

ProZyme, Inc. has developed a new N-glycan preparation procedure (GlykoScreen™ Rapid Glycoprotein Sample Preparation System or RGSPS) that has been implemented on the AssayMAP platform. The procedure optionally begins with purification of antibody from crude samples using the MAb Titer Assay described previously. Depending upon the the automation approach used, fluorescently labeled N-glycans can be produced for up to 192 samples in less than 3 hours. The protocol is summarized here:



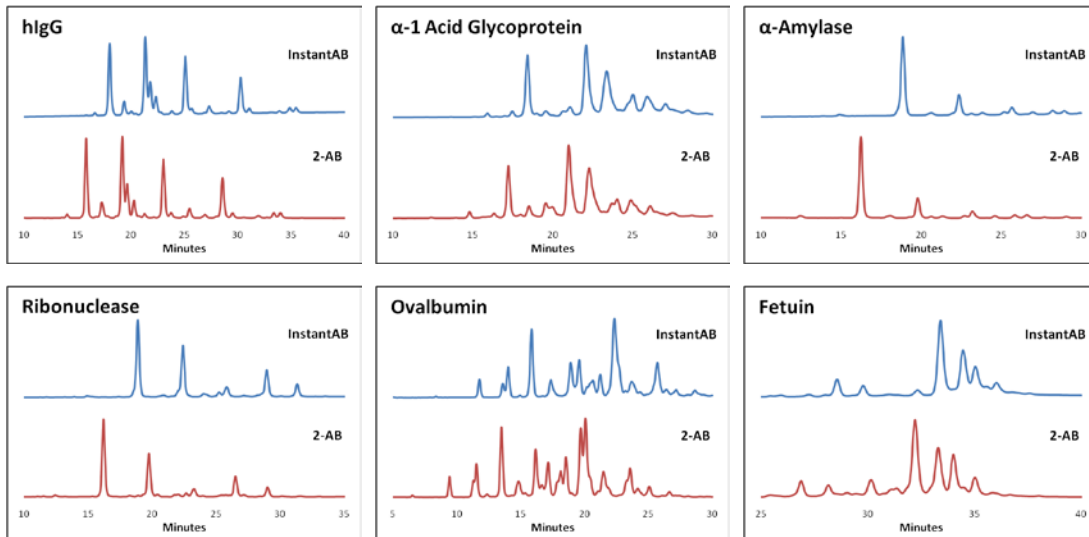
N-Glycan Profiles of IgG from Cell Culture

The HPLC chromatograms below left show the results of running the RGSPS protocol on CHO cell culture supernatant containing no antibody, with or without the protein A purification step. On the right are runs on 25 µg purified human IgG, either in buffer or spiked into the culture supernatant, using the protein A purification. The prepared N-glycans were analyzed by HPLC using a GlykoSep™ N Plus column and a binary gradient of ammonium formate buffer and acetonitrile. The protein A purification is highly effective at removing the non-IgG glycoproteins in the supernatant, but does not affect the N-glycan analysis of the hIgG. CHO cell culture supernatant was provided by Dr. Marty Vanderlaan of Genentech.



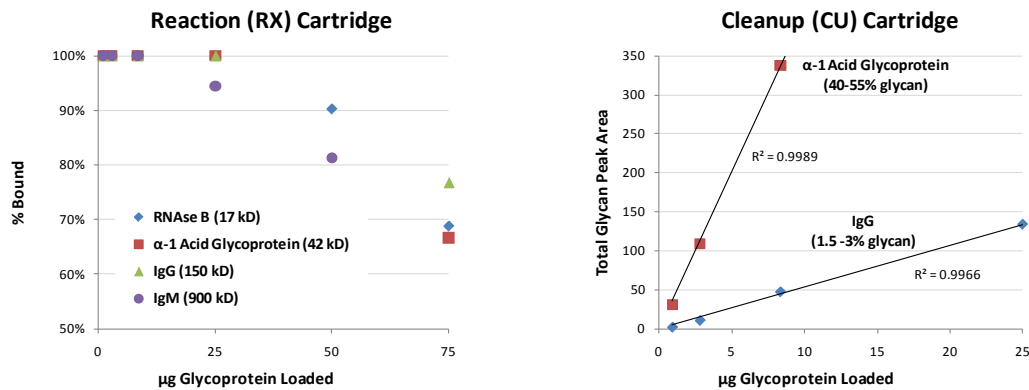
Comparison with Standard Methods

The chromatograms below show comparisons between the RGSPS method and a conventional sample preparation method (using standard 2-AB dye) for a number of well-characterized glycoproteins. The labeled N-glycan samples were run on HPLC as described previously 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.

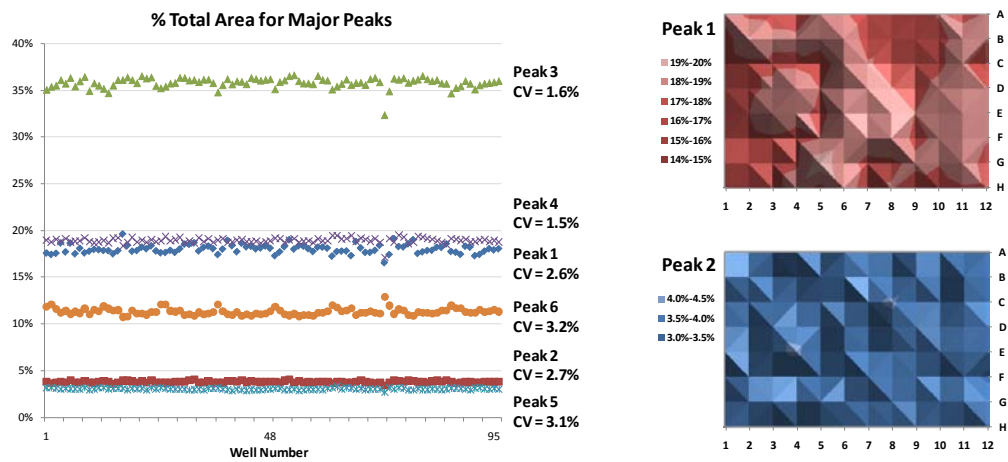


Capacity, Sensitivity & Reproducibility

The RGSPS protocol can produce N-glycan profiles quantitatively from up to 25 µg of glycoprotein (including those with very high N-glycan content). Even with low N-glycan content proteins such as IgG, accurate profiles can be obtained with ≤1 µg (data not shown).



Reproducibility of an IgG sample run 96 times in a single run is shown below. CVs for percent of total peak area for the major peaks are <4%. Plate maps show no position bias.



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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