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Automated, High-Throughput IgG-Antibody Glycoprofiling Platform

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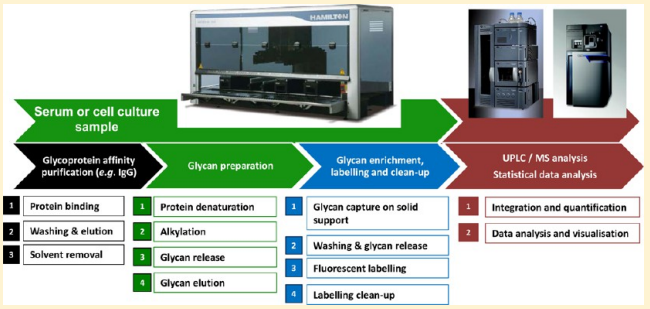
Supporting Information

ABSTRACT: One of today's key challenges is the ability to decode the functions of complex carbohydrates in various biological contexts. To generate high-quality glycomics data in a high-throughput fashion, we developed a robotized and low-cost *N*-glycan analysis platform for glycoprofiling of immunoglobulin G antibodies (IgG), which are central players of the immune system and of vital importance in the biopharmaceutical industry. The key features include (a) rapid IgG affinity purification and sample concentration, (b) protein denaturation and glycan release on a multiwell filtration device, (c) glycan purification on solid-supported hydrazide, and (d) glycan quantification by ultra performance liquid chromatography. The sample preparation workflow was automated using a robotic liquid-handling workstation, allowing the preparation of 96 samples (or multiples thereof) in 22 h with excellent reproducibility and, thus, should greatly facilitate biomarker discovery and glycosylation monitoring of therapeutic IgGs.



Biomolecular glycosylation, often referred to as the third language of biology, has fundamental roles in many biological recognition events. The diversity and complexity of glycans provide a wealth of information in biological systems, and one of today's key challenges is to understand in detail which glycans contribute to specific biological functions. Both the number and the structural diversity of glycans found in mammalian systems are significant analytical challenges for determining detailed glycan structural profiles in complex biological systems. Glycans undergo rapid structural changes in response to biological stimuli, providing a unique opportunity to identify and exploit glycan biomarkers for disease states, disease progression, and response to therapy. In addition, a comprehensive structural picture of glycan diversity can provide insights into potential biological recognition events and regulatory mechanisms for glycan biosynthesis. Challenges in structural analysis include the large number of glycan classes, the complexity of sample workup, and the efficient exploitation of analytical tools that are available for structural interrogation.[1] Various approaches are now available for sensitive analysis of *N*-glycans (i.e., those that are linked to proteins via the amino acid asparagine). Analytical techniques include chromatographic separations with fluorescence detection, mass spectrometry, and a combination of both.[2] However, few methods have been developed that can dissect complex mixtures of structural isomers and accurately quantify them in a high-throughput format. The availability of affordable high-throughput platforms for glycan profiling could provide new insights into regulated glycan expression in human disease. Even in a medium-throughput format, profiling of glycan structures, in combination with human genome-wide association studies (GWAS), has given tremendous insights into the regulation of glycan expression in human populations.[3,4] Here, complex sets of glycomics and single nucleotide polymorphism (SNP) data are analyzed to understand the details of glycan structural changes that occur in complex biological systems.[5] Profiling immunoglobulin G (IgG) *N*-glycosylation, in particular, has been very informative in the context of various GWA studies, linking glycosylation with genetic information in human populations.[6] However, due to the large sizes of GWAS sample sets, the glycans in thousands of samples must be quantified in a rapid and cost-effective fashion to make the approach viable. Moreover, IgG *N*-glycosylation profiling plays a vital role in the development of therapeutic antibodies because very specific glycosylation patterns are desired to modulate antibody receptor binding and to maintain antibody properties such as appropriate pharmacokinetics. Thus, methods are needed to rapidly screen cell-culture-derived IgG to identify clones that produce the desired pattern. Ultra performance liquid chromatography (UPLC) has become a well-established, robust analytical technique to obtain high-resolution separation of *N*-linked glycans released from glycoproteins.[7] Tagging the glycans with a fluorescent label such as 2-aminobenzamide (2-AB) allows their detection at femtomole levels. The major advantages of UPLC analysis of fluorescently labeled glycans are the high reproducibility, exact relative glycan quantification, effective separation of structural isomers, and ease-of-use. Although UPLC is a powerful analytical technique, it relies on efficient sample preparation workflows, but the methods developed to date are either very

Received: July 7, 2013
Accepted: August 6, 2013
Published: August 6, 2013

 © 2013 American Chemical Society dx.doi.org/10.1021/ac402068r | *Anal. Chem.* 2013, 85, 8841−8849

labor-intensive and low-throughput or extremely expensive. For example, two robotized *N*-glycoprofiling assays have recently become commercially available,[8,9] but reagent and consumable costs can be prohibitively high even when processing a small number of samples. High-throughput glycan screening assays using DNA sequencers or microchips in conjunction with well-plate parallel processing have been reported, but the resulting information content and resolution were low in comparison to UPLC.[10,11] Thus, new and efficient sample preparation workflows for UPLC-based glycan analysis are urgently required to enable automation, decrease costs, and increase throughput, which prompted us to design an automatable sample preparation workflow and implement it on a liquid-handling platform.

EXPERIMENTAL SECTION

All chemical reagents and solvents were purchased from Sigma-Aldrich. Solid-supported hydrazide, as well as Protein A and Protein G affinity purification plates, was obtained from Fisher. Solvinert filter plates were from Merck-Millipore, and Acroprep filter plates were from Pall. Samples were prepared on a Hamilton Robotics StarLet liquid-handling platform. The instrument is equipped with eight software-controlled pipettes, a vacuum manifold, and an automated heater shaker. Samples were analyzed on a Waters Acquity H-Class UPLC instrument.

The following workflow was implemented as a robotic program on Hamilton Robotics Venus One software.

Native Human Serum (NHS) Samples. One hundred adult serum samples from apparently healthy male and female adult blood donors were pooled and used as a source of IgG for subsequent glycan analysis (courtesy of the U.K. Blood Transfusion Service).

Cell Culture of Chinese Hamster Ovary (CHO) Cells. CHO cells (CHO DP-12: ATCC CRL-12444) engineered to express anti-human interleukin-8 (IL-8) IgG and adapted for serum-free and suspension conditions were a generous gift from Prof. Nigel Jenkins (NIBRT). Cells were routinely cultured in CDCHO media (Gibco) supplemented with 4 mM L-glutamine (Gibco), 1% penicillin/streptomycin, and 100 nM methotrexate in an atmosphere of 5% CO_2 and at 37 °C. Cells were regularly counted, and viability analysis was performed using a hemocytometer with trypan blue exclusion. When cell density reached between 7×10^5 and 1×10^6 cells/mL, cells were harvested by centrifugation (120*g*, 5 min, 18 °C), and the conditioned culture medium was collected and used as a source of recombinant IgG for subsequent glycan analysis.

Glycoprotein Affinity Purification. Note: Washing refers to solvent addition to solid supports in a filter plate, intermittent incubation (if noted), and subsequent solvent removal by vacuum filtration on a robotic vacuum manifold or centrifuge filtration.

A 96-well IgG affinity purification plate (Thermo Scientific, 50 μL Protein G agarose resin per well) was preconditioned by washing three times with 500 μL binding buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4). Aliquots of pooled native human serum (50 μL per sample) were diluted with an equal volume of binding buffer and filtered through a 96-well filter plate (Pall AcroPrep, 1 μm pore size glass fiber membrane) by centrifuge filtration. The filtrates were added to the Protein G plate, which was agitated on a robotic shaker at 700 rpm at room temperature for 30 min. Chinese hamster ovary cell culture IgG was isolated by first filtering the cell culture supernatants (400 μL per sample) through a 96-well filter plate (Pall AcroPrep, 1 μm pore size glass fiber membrane) and then transferring the filtrate to a preconditioned 96-well IgG affinity purification plate (Thermo Scientific, 50 μL Protein A agarose resin per well). Buffer was removed by vacuum filtration, and the Protein G or Protein A resins were washed five times with washing buffer (500 μL per well, 0.1 M sodium phosphate, 0.15 M sodium chloride, 1% Triton-X, pH 7.4) with intermittent agitation at 700 rpm for 1 min at room temperature. Next, the resins were washed five times with binding buffer (500 μL per well) to remove residual detergent. A receiver plate was prepared by dispensing neutralization buffer (50 μL per well, 1 M Tris-hydrochloride, pH 9.0) into a 96-well 2 mL collection plate, which was placed into the robotic vacuum manifold. Elution buffer (200 μL per well, 0.2 M glycine-hydrochloride, pH 2.5) was added to the Protein G or Protein A resins followed by incubation at room temperature for 2 min without agitation. IgG was eluted by vacuum filtration and collected. This cycle was repeated once more, and the collection plate was briefly centrifuged (4000*g*, 1 min). Half of the filtrate was transferred to a 96-well ultrafiltration plate (Pall Acroprep, Omega membrane, 10 kDa nominal molecular weight limit), and the solvent was removed by vacuum filtration (24 mmHg, typically 30 min) or centrifuge filtration (3700*g*, room temperature, typically 15 min). The flow-through was discarded, the remaining filtrate was added to the ultrafiltration plate, and solvent was removed by vacuum (24 mmHg, typically 60 min) or centrifuge filtration (3700*g*, room temperature, typically 30 min). The retentate was washed with water (20 μL per well).

Glycoprotein Denaturation and Glycan Release. Denaturation buffer (50 μL per well, 100 mM ammonium bicarbonate, 50 mM dithiothreitol, 0.1% sodium dodecyl sulfate) was dispensed into the ultrafiltration plate, which was placed on a robotic heater shaker and fully covered and insulated with an antievaporation lid. This assembly was incubated at 65 °C with agitation at 700 rpm for 20 min. After cooling to room temperature, an iodoacetamide solution (100 mM, 10 μL per well) was added, and the ultrafiltration plate was covered with an antievaporation lid and incubated at room temperature with agitation at 700 rpm for 30 min. Excess reagents, together with solvent, were removed by vacuum (24 mmHg, typically 30 min) or centrifuge filtration (3700*g*, 15 min, room temperature), and the samples were washed with water (20 μL per well). Water (40 μL per well) and PNGase F (Prozyme Glyco *N*-Glycanase, code GKE-5006D, 10 μL per well, 0.5 mU in 1 M ammonium bicarbonate, pH 8.0) were sequentially added, and the ultrafiltration plate was insulated with an antievaporation lid and incubated at 40 °C with agitation at 700 rpm for 2 h. During this time, the Protein G or Protein A resin was regenerated by washing six times with regeneration buffer (500 μL per well, 0.2 M glycine/HCl, 0.5% Triton-X, pH 2.5), five times with elution buffer (500 μL per well), and finally four times with binding buffer (500 μL per well). Glycans were recovered from the ultrafiltration plate by centrifuge filtration (3700*g*, 15 min, room temperature). The ultrafiltration membranes were washed with water (20 μL per well), and the filtrate was combined with the first filtrate to obtain a final volume of 50−60 μL per sample.

Hydrazide-Mediated Glycan Cleanup. Each well of a 96-well chemically inert filter plate (Millipore Solvinert, hydrophobic polytetrafluoroethylene membrane, 0.45 μm pore size) was washed with 100 μL of methanol (MeOH). UltraLink hydrazide resin (50 μL of a suspension in water, Thermo

dx.doi.org/10.1021/ac402068r | *Anal. Chem.* 2013, 85, 8841−8849

Scientific) was dispensed to each well. The resin was sequentially washed with MeOH, H_2O, and acetonitrile (MeCN), and the plate was placed on a heater (70 °C, 10 min) to seal the membranes. One hundred eighty microliters of MeCN/acetic acid (98:2) was added to the resin, followed by 20 μL of the glycan solution. The filter plate was incubated with shaking at 700 rpm at 70 °C for 45 min. Fifty microliters of MeCN/acetic acid (98:2) was added, and shaking was continued at the same temperature for 10 min to disrupt resin aggregates. The resin was washed sequentially with MeOH, guanidine, H_2O, triethylamine/MeOH (1:99), and MeOH (200 μL per well). Fresh MeOH (180 μL) and acetic anhydride (20 μL) were added, and the plate was incubated for 10 min with agitation at 700 rpm. Excess reagent was removed by filtration, and the resins were washed sequentially with MeOH, H_2O, and MeCN. Acetic acid/MeCN (2:98, 180 μL) and H_2O (20 μL) were sequentially added, and the plate was incubated at 70 °C with agitation at 700 rpm for 60 min. Fluorescent labeling mix (50 μL, 350 mM 2-aminobenzamide, 1 M sodium cyanoborohydride in acetic acid/dimethyl sulfoxide (30:70)) was dispensed into each well, and the plate was incubated at 70 °C with agitation at 700 rpm for 120 min.

Glycan Solid-Phase Extraction. The labeling reaction was quenched by the addition of 200 μL of MeCN/H_2O (95:5). The suspension was transferred to a 2 mL collection plate containing 800 μL of MeCN/H_2O (95:5) per well, the beads were left to settle, and 200 μL of the supernatant was aspirated and dispensed back into the filter plate. After extensive mixing, the suspension was transferred back into the collection plate. This cycle was repeated once more to ensure a quantitative transfer of the resins.

HyperSep Diol SPE cartridges (Thermo Scientific) were washed with 1 mL of MeCN/H_2O (95:5), 1 mL of H_2O, and 1 mL of MeCN/H_2O. Next, the beads were suspended and transferred onto the SPE cartridges. A 10 min incubation typically led to complete drainage of the solvent by gravity. The SPE cartridges were washed three times with 700 μL of MeCN/H_2O (95:5). A collection plate was placed inside the robotic vacuum manifold, and the SPE cartridges were washed twice with 200 μL of H_2O/MeCN (80:20), with an intermittent incubation period of 10 min. The samples were concentrated to dryness in a vacuum evaporator (typically 4−6 h for 96 samples). The samples were dissolved in 30 μL of MeCN/H_2O (70:30) and filtered (Pall Acroprep GHP membrane, 0.45 μm pore size). A 10 μL aliquot of the filtrate was analyzed by UPLC.

Ultra Performance Liquid Chromatography (UPLC). Separation of 2-AB-derivatized *N*-glycans was carried out by UPLC with fluorescence detection on a Waters Acquity UPLC H-Class instrument consisting of a binary solvent manager, sample manager, and fluorescence detector under the control of Empower 3 chromatography workstation software (Waters, Milford, MA, USA). The HILIC separations were performed using a Waters Ethylene Bridged Hybrid (BEH) Glycan column (150 × 2.1 mm i.d., 1.7 μm BEH particles) with 50 mM ammonium formate (pH 4.4) as solvent A and MeCN as solvent B. The separation was performed using a linear gradient of 70−53% MeCN at 0.56 mL/min in 16.5 min for IgG separation. An injection volume of 10 μL sample prepared in 70% v/v MeCN was used throughout. Samples were maintained at 5 °C prior to injection, and the separation temperature was 40 °C. The fluorescence detection excitation/emission wavelengths were λ_{ex} = 330 nm and λ_{em} = 420 nm, respectively. The system was calibrated using an external standard of hydrolyzed and 2-AB-labeled glucose oligomers to create a dextran ladder, as described previously.[12] A fifth-order polynomial distribution curve was fitted to the dextran ladder to assign glucose unit (GU) values from retention times (using Empower software from Waters).

RESULTS AND DISCUSSION

Current Glycoprotein Immobilization Strategies. Accurate relative glycan quantification and reproducible separation can only be achieved if the glycans released from the glycoproteins are free from contaminants that may lead to artifacts and poor assay reproducibility. The most widely used method for releasing *N*-glycans from glycoproteins is in-solution treatment with peptide *N*-glycosidase F (PNGase F).[13] Not only does this require optimization for different glycoproteins on an individual basis, but undesired species in the solution such as buffer, detergents, and serum contaminants may interfere with chemical and biochemical reactions.[14] More recent developments exploit glycoprotein immobilization strategies to allow the efficient removal of buffers, reagents, and endogenous carbohydrates by washing the immobilized glycoproteins prior to enzymatic glycan release. Immobilization can be achieved by capturing glycoproteins onto PVDF membranes.[15] Although this could be automated on a liquid-handling platform, the method is plagued by reproducibility problems because protein binding is nonselective due to nonquantitative binding of the applied material.[14]

An elegant alternative is to trap a glycoprotein in sodium dodecyl sulfate (SDS)-polyacrylamide gels.[14] SDS gels capture proteins quantitatively, have a high-immobilization capacity, and allow the efficient removal of small molecule species. In this so-called in-gel-block (IGB) method, glycoproteins such as IgG are purified by affinity purification using solid-supported Protein A or Protein G. Next, the samples are concentrated by evaporation under vacuum, and the glycoprotein is immobilized in polyacrylamide gels. In this "gold standard" *N*-glycan analysis process, glycoprotein immobilization is followed by the removal of small molecules through washing and filtration, enzymatic glycan release using PNGase F, fluorescent glycan labeling, sample cleanup, and quantitative UPLC/HPLC profiling followed by quantitation and optional structural assignment of the glycans by database matching. This method allows the preparation of 96 glycoprotein samples in 3 days. Advantages of in-gel digestion include its robustness against impurities, which may interfere with digestion, but the gel may prevent efficient glycan release and recovery. Although the in-gel digestion protocol can be performed by hand in a high-throughput format in 96-well plates, automation is challenging and the workflow is time-consuming. A significant problem is the necessity to perform multiple sample concentration steps. The removal of aqueous solvents by vacuum evaporation, for example, after affinity purification and after enzymatic glycan release, is not only time- and energy-intensive but also difficult to automate.

Development of an Alternative Method. The main advantage of the in-gel-block method is the possibility of conducting protein denaturation, buffer exchange, and enzymatic glycan release in one and the same gel block in a 96-well format. Robotizing the in-gel-block method proved to be challenging, and thus, an alternative method was sought allowing the full automation of all biochemical operations.

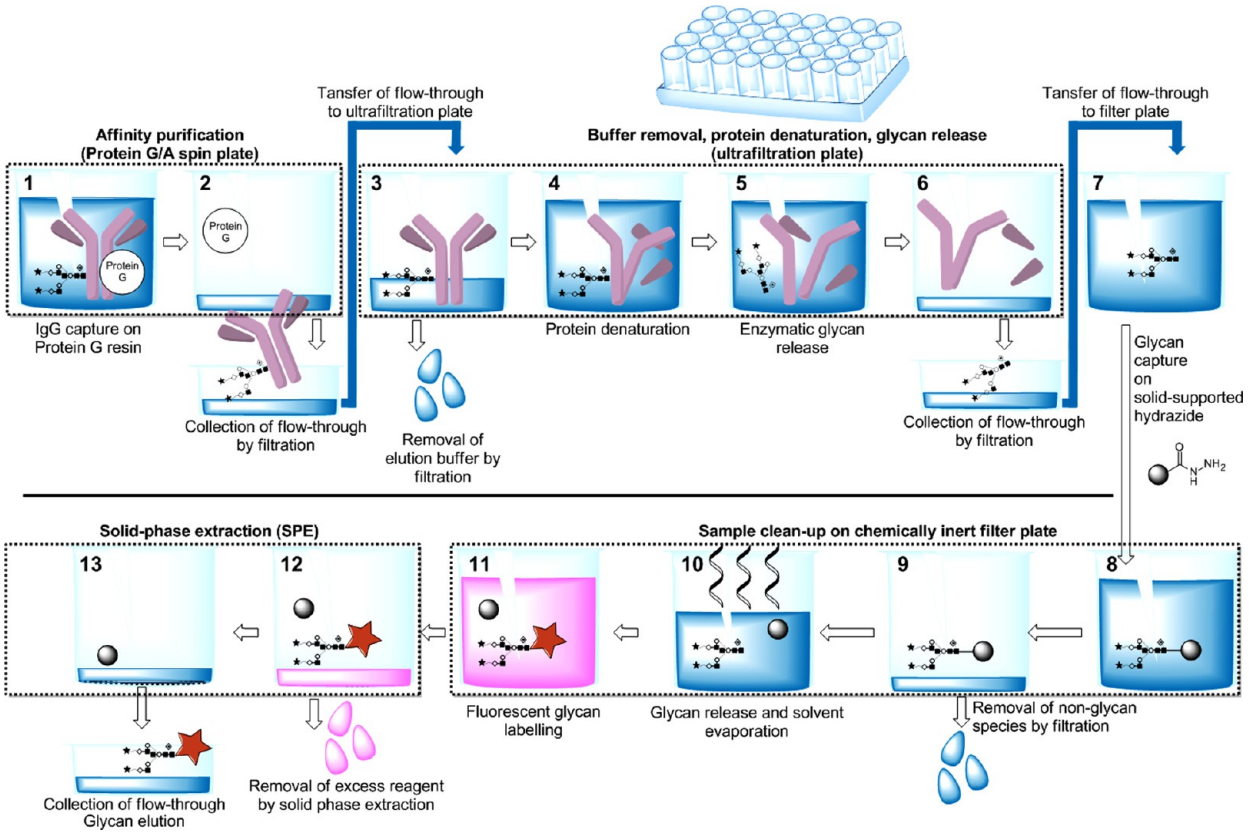


Figure 1. Sample preparation workflow, consisting of glycoprotein affinity purification, protein denaturation, enzymatic glycan release, glycan immobilization on solid supports, removal of contaminants, glycan release, labeling, and solid-phase extraction.

Buffer exchange after glycoprotein affinity purification (1), glycoprotein denaturation (2), and enzymatic glycan release and separation (3) are performed in sequence; therefore, the use of multiwell ultrafiltration plates was an ideal choice for all processes. A number of sample preparation workflows for proteomics and glycoproteomics utilizing individual ultrafiltration spin cartridges have been reported to date,[16−19] but none of them has been translated to a high-throughput, automated format that can be used for quantitative glycan profiling.

Ultrafiltration plates allow the quantitative retention of glycoproteins above the nominal molecular weight limit (NMWL) of the ultrafiltration membrane, and thus, they permit rapid buffer exchange and removal of small molecule species. After protein denaturation, excess reagents such as detergents and alkylating agents can be removed by ultrafiltration. Once glycans have been enzymatically released from the protein, they can be recovered by simple filtration because released IgG *N*-glycans have a size well below the NMWL of common ultrafiltration membranes (Figure 1).

To develop the high-throughput sample preparation method, a suitable ultrafiltration membrane and multiwell plate type were selected. Tested membrane materials were regenerated cellulose and polyethersulfone membranes, both with a 10 kDa NMWL to retain IgG, which has a molecular weight of 150 kDa. As expected, the regenerated cellulose membranes exhibited comparatively low filtration flow rates at 4000*g* relative centrifugal force (rcf) and were deemed impractical, whereas the polyethersulfone membranes maintained high flow rates even after multiple filtration cycles. This membrane type had already been extensively used and tested for glycan sequencing in our laboratory.[12] Next, a number of plate designs were tested for assay compatibility and automation-friendliness. Multiwell ultrafiltration plates are commercially available in 96-well robotics-compatible formats to allow sample filtration on a robotic vacuum manifold or in a centrifuge. It was particularly important to identify a plate that could be incubated with agitation at temperatures of up to 70 °C for efficient protein denaturation. The selected design maintained its ultrafiltration capability even after extended incubation periods. To minimize evaporation of water from the ultrafiltration plate during incubation and to ensure an even temperature distribution across the plate, we constructed a robotics-compatible antievaporation lid that could be used on a robotic heater−shaker and yet fully enclosed the ultrafiltration device and the heater plate. The antievaporation lid is an insulated aluminum enclosure and features a multiwell condenser on the inside to trap evaporating liquid. Both the ultrafiltration plate and the antievaporation lid can be moved with a robotic arm to automatically assemble and disassemble the reaction plate on top of a software-controlled robotic shaker. With the key hardware components in place, all protein manipulation operations could now be efficiently performed on a liquid-handling robot, which is schematically shown in Figure 2A. The robot is fully software-controlled and was equipped with eight robotic pipettes with individual liquid level and pressure sensors (Figure 2B), pipet tip racks, plate carriers, reagent reservoirs, a software-controlled vacuum manifold (Figure 2C), a temperature- controlled orbital shaker (Figure 2D), and a plate-transport tool to move multiwell plates between positions and operate the vacuum manifold.

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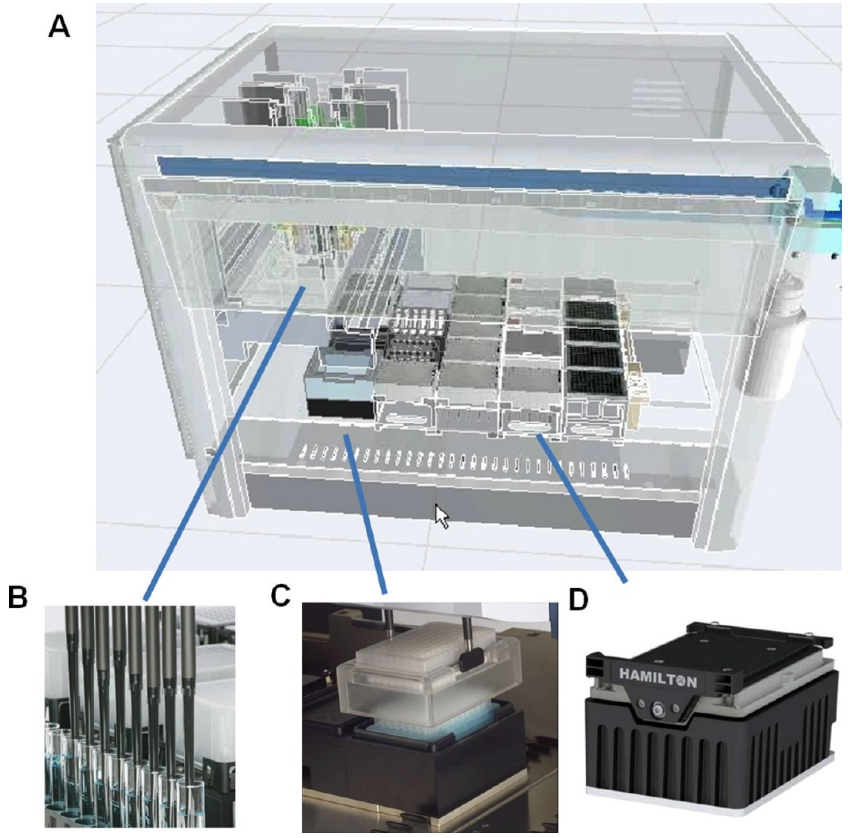

Figure 2. (A) Schematic of the Hamilton liquid-handling workstation and its key components, including (B) pipetting channels with liquid-level detection and antidroplet control, (C) software-controlled robotic vacuum manifold and plate-transport tool, and (D) temperature-controlled orbital shaker. Panels (B–D) are courtesy of Hamilton Robotics.

A robust robotic program with automatic error recovery was created to implement the workflow shown schematically in Figure 1. The program starts with the isolation of a glycoprotein of interest. Human IgG (subclasses 1–4), typically from 20 to 50 μL of human or animal serum or cell culture supernatant, was affinity-purified using a robotics-compatible 96-well filter plate containing solid-supported Protein G (Figure 1, steps 1 and 2). Protein A was used for the isolation of IgG derived from Chinese hamster ovary cell cultures. After IgG elution, samples were transferred to a 96-well ultrafiltration plate, and all subsequent biochemical operations were performed on the same device (Figure 1, steps 3–6). First, the elution buffer was removed by filtration (Figure 1, step 3), followed by protein denaturation, cysteine reduction, and alkylation (Figure 1, step 4). Next, excess reagents and buffer were removed by filtration, and fresh buffer, together with PNGase F, was added to release *N*-glycans from the protein (Figure 1, step 5). Glycans were recovered by filtration, retaining the enzyme on the filter (Figure 1, step 6). The filter unit, thus, served as a combined filter and reaction vessel for solvent removal, buffer exchange, chemical protein modification, and glycan release. To minimize incubation times and to reduce assay costs, the enzymatic glycan release was performed in a minimal volume of solvent and under vigorous agitation at 37 °C. We tested different incubation times and enzyme concentrations and observed that the glycans were released efficiently even when using small amounts of enzyme. After 2 h of incubation time, all enzyme concentrations between 2 and 600 μU/μL led to identical glycan yields, indicating that glycan release on the ultrafiltration plate is very efficient. The minimum PNGase F incubation time was determined by conducting a time-course experiment with incubation times between 5 min and 2 h. The obtained glycan profiles were highly consistent, and minor time-dependent changes in glycan quantities were observed only for two sialylated glycans (FA2G2S2, FA2BG2S2) when the incubation time was less than 30 min at an enzyme concentration of 10 μU/μL (see Supporting Information (SI), Figure S2). As the IgG affinity purification resin can be regenerated after use, glycan release was performed as a parallel process on the liquid-handling robot during the 2 h regeneration step.

Fluorescence glycan labeling after solvent removal by evaporation in a vacuum concentrator frequently led to inconsistent UPLC chromatograms (see SI, Figure S1). This could have arisen from the presence of impurities such as buffer salts or residual detergent, which in turn could lead to side reactions or incomplete reduction of imines during the glycan labeling step. Thus, a sample cleanup procedure was incorporated into the program before fluorescence labeling (Figure 1, steps 8–11). To avoid time-consuming solid-phase extraction and aqueous solvent removal, we chose to use solid-supported hydrazide, which under acid catalysis is known to selectively react with reducing carbohydrates forming stable covalent hydrazone bonds so that all reducing carbohydrates are transiently linked to solid supports, and any non-carbohydrate species can easily be removed by a simple washing step. The carbohydrates can be released at-will from the solid supports by incubation in the presence of water and catalytic amounts of acid and can then be fluorescently labeled (Figure 3).

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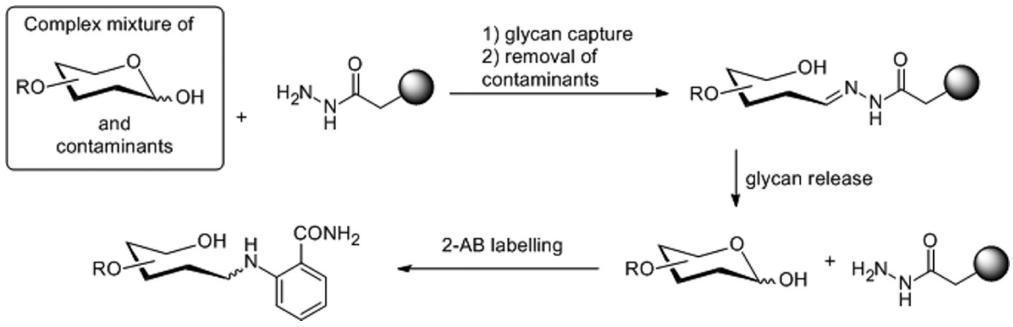

Figure 3. Solid-supported hydrazide-mediated sample cleanup. Reducing-end glycans are reacted with solid-supported hydrazides to form hydrazones, capturing glycans on solid supports. Contaminants such as excess reagents and buffer salts are removed by filtration. Next, glycans are released by acid catalysis in the presence of water. Finally, glycans are fluorescently labeled with 2-AB.

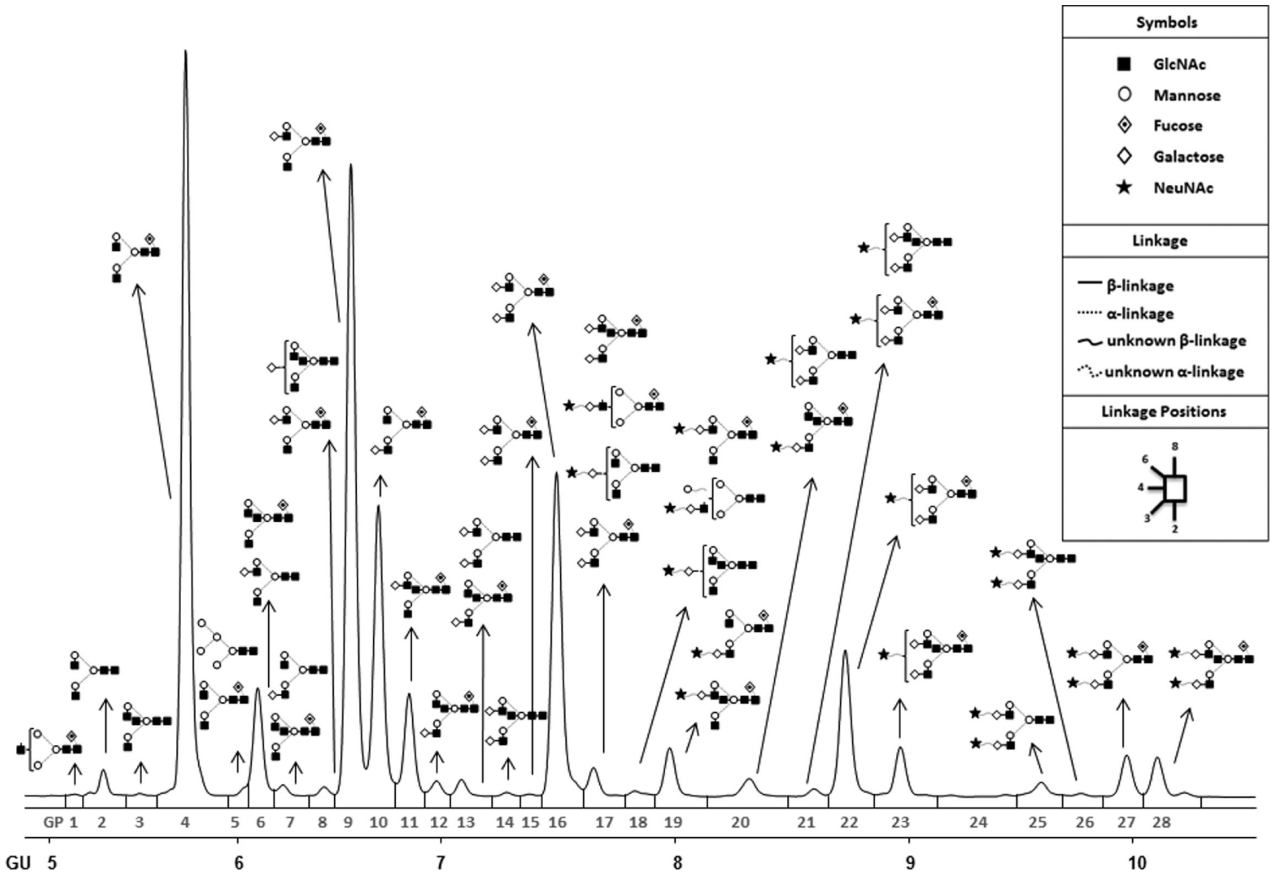

Figure 4. Representative human IgG *N*-glycosylation chromatogram and peak assignments, resulting from the automated sample preparation method. IgG from native human serum was isolated and processed on the liquid-handling workstation, as described in the Experimental Section, followed by glycan analysis by UPLC with fluorescence detection. The peaks corresponded to those assigned in Pucic et al.[4] GP: glycan peak number, GU: glucose units.

Importantly, solid-supported chemical reactions are also programmed on the liquid-handling robot, enabling full automation for this part of the workflow, as well. An elegant protocol developed by Nishimura and co-workers was adapted here.[20] This so-called "glycoblotting" method relies on a bespoke solid-supported glycoblotting hydrazide polymer to isolate glycans from complex analyte mixtures, but reagent costs are very high. In the search for an affordable alternative, we replaced the glycoblotting polymer with competitively priced UltraLink hydrazide resin, which is a hydrazide-loaded form of 3M-Emphaze biosupport medium. This material is commercially available in bulk quantities and is normally used as an affinity support for immobilizing glycoproteins through oxidized carbohydrate groups.[21] The solid support is a polymer with an average particle diameter of 60 μm, a surface area of 350 m^2/g, and with 25% of the total surface area having pore size diameters of more than 30 nm. Hydrazide loading (15 μmol per mL suspended solid support) is ≈30-fold lower than that of the glycoblotting polymer and could potentially lead to reduced glycan recovery. However, in contrast to the glycoblotting protocol, we conducted all solid-supported reactions with vigorous agitation on a temperature-controlled robotic orbital shaker. Thus, the solid supports were kept in suspension during the reactions, which maximized reaction

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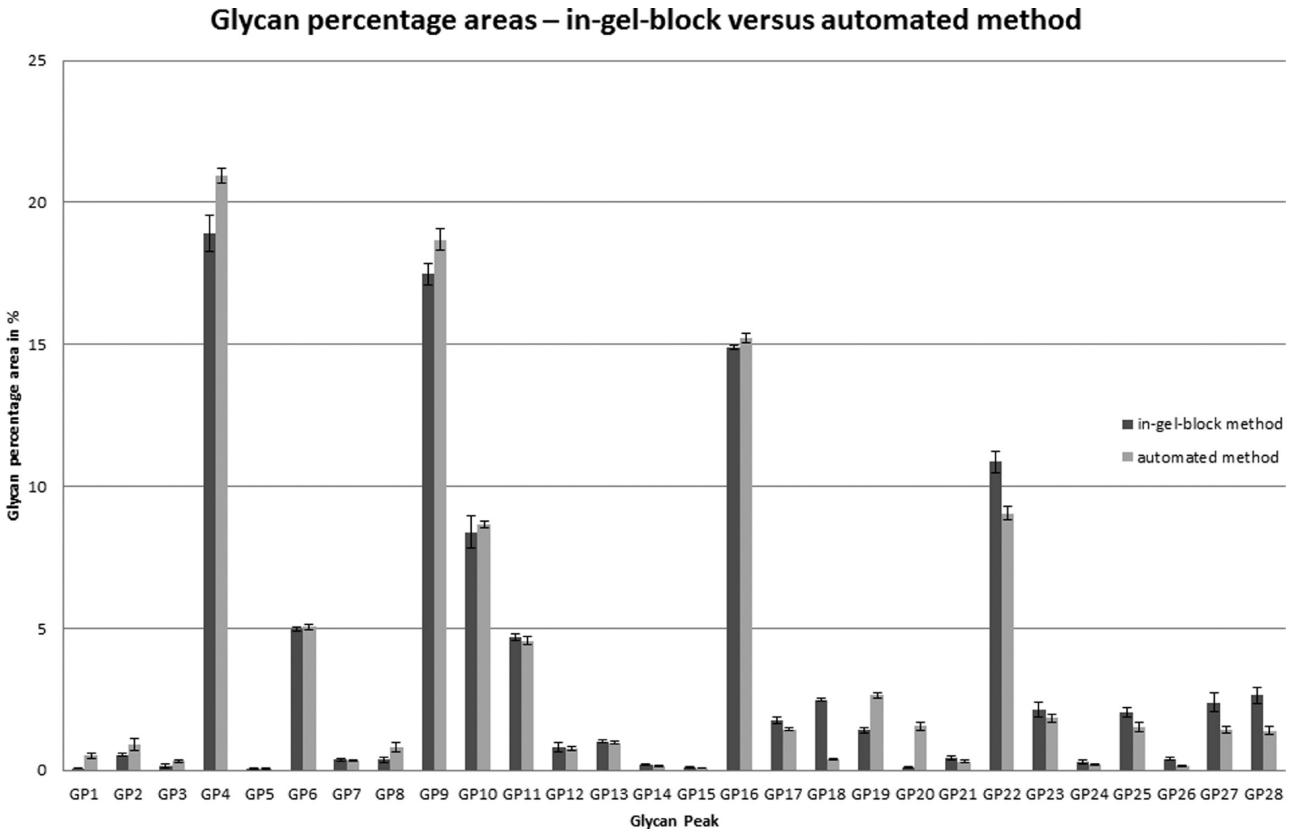

Figure 5. Area comparison of the 28 glycan peaks from human IgG between the automated assay and the in-gel-block (IGB) method. Samples were prepared on the robotic platform or using the IGB method in quadruplicate on 4 different days followed by UPLC analysis. GP: glycan peak number.

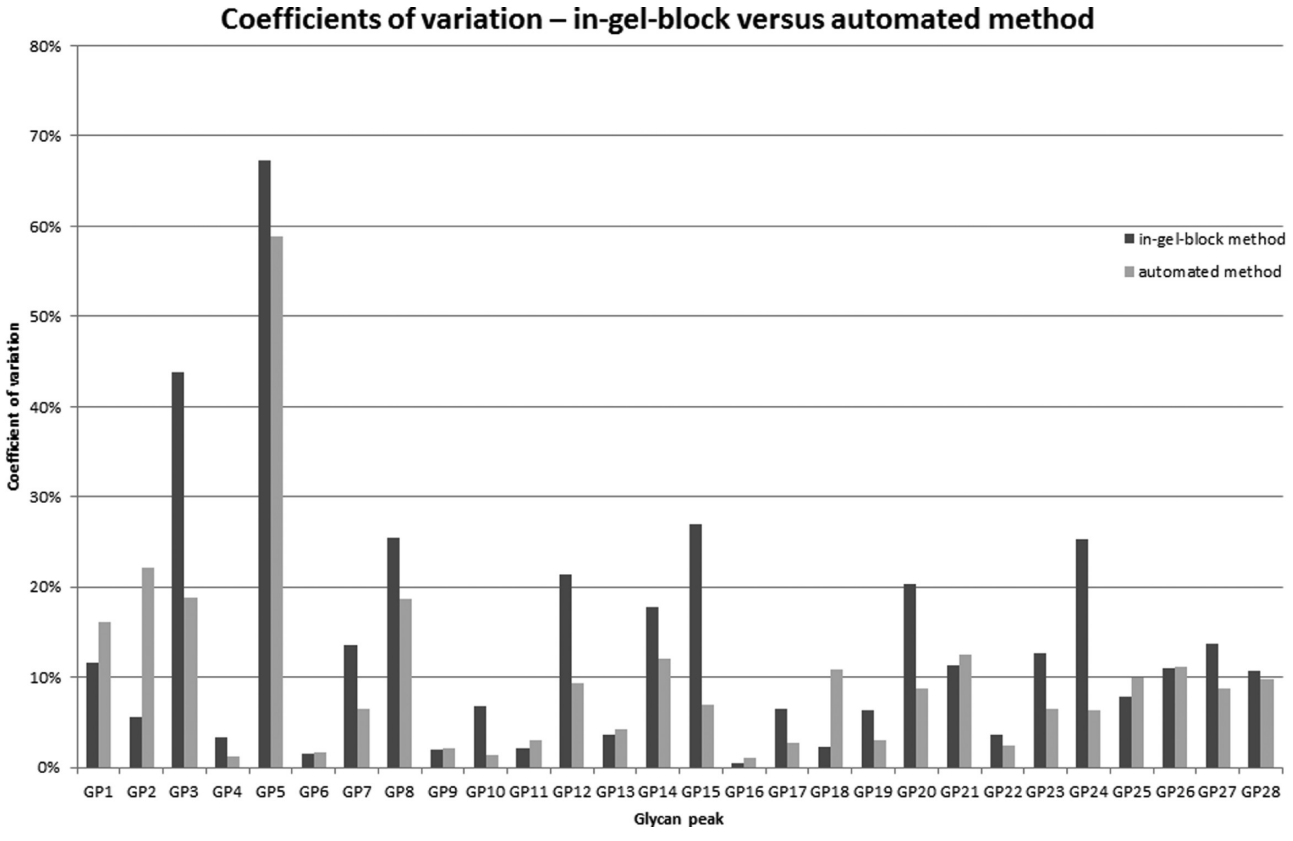

Figure 6. Comparison of the glycan area's coefficients of variation between the automated assay and the in-gel-block (IGB) method. Samples were prepared automatically or manually with the IGB method in quadruplicate on 4 different days and analyzed by UPLC. GP: glycan peak number.

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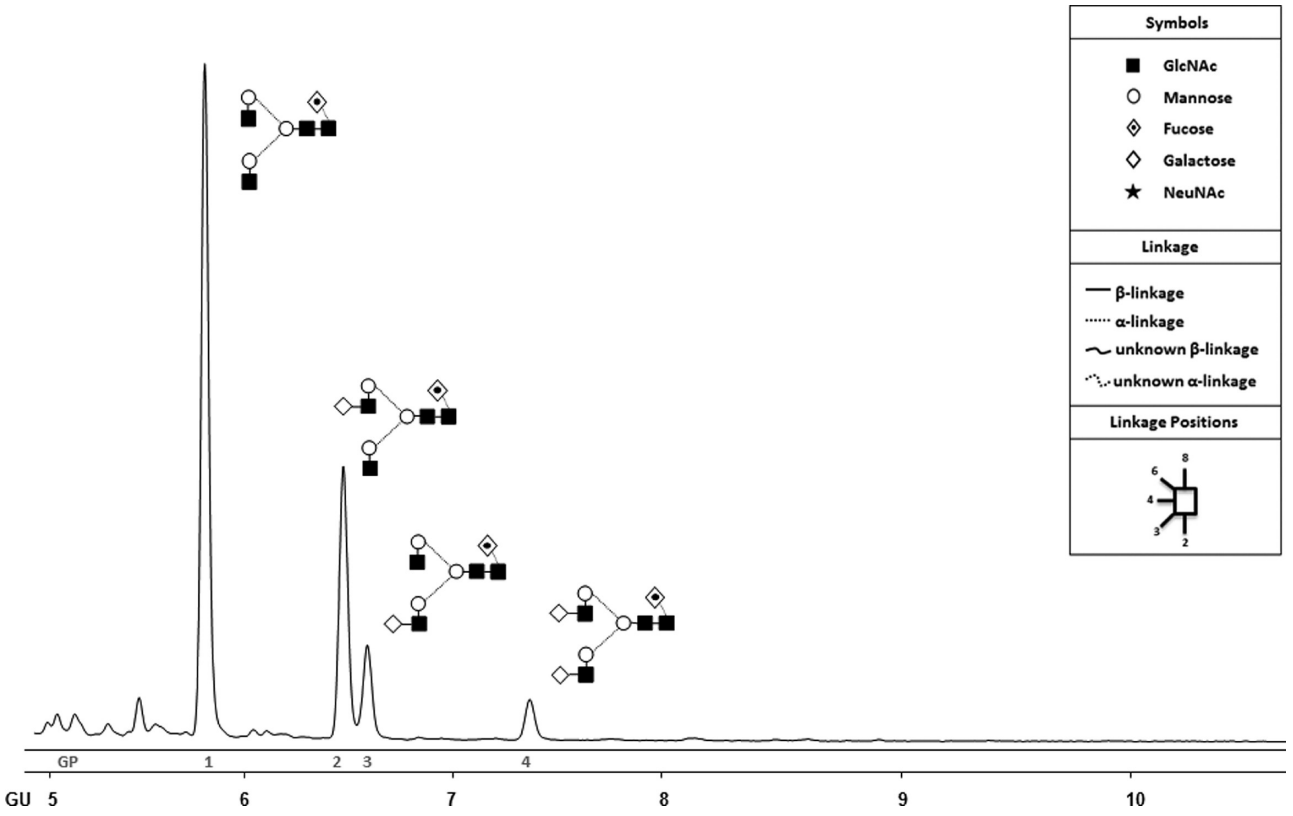

Figure 7. Representative CHO cell-derived anti-human IL-8 IgG *N*-glycosylation chromatogram and peak assignments, resulting from the automated sample preparation method. Anti-human IL-8 IgG from CHO cells was isolated and processed on the liquid-handling workstation followed by glycan analysis by UPLC with fluorescence detection. GP: glycan peak number, GU: glucose units.

rates and routinely led to between 80 and 100% glycan recovery. Given that a 50 μL aliquot of human serum contains a maximum of 20 nmol IgG *N*-glycans, we chose to utilize 50 μL UltraLink hydrazide resin per sample (i.e., an almost 40-fold excess or an equivalent of 0.75 μmol hydrazide). All solid-supported reactions were conducted in organic solvent in a chemically inert filter plate with hydrophobic polytetrafluoroethylene membranes. These membranes proved to be ideal as they could be permeabilized by the addition of methanol and impermeabilized by solvent removal and evaporation. After glycan capture, washing, and release, glycans were fluorescently labeled with 2-AB, a carbonyl-reactive fluorophore. The reactions were quenched, and the reaction mixtures were transferred to a solid-phase extraction plate to remove excess labeling reagent under normal-phase conditions (Figure 1, steps 12 and 13). Reproducible results could only be obtained upon quantitative transfer of the resin to the solid-phase extraction plate. Glycans were found to strongly bind noncovalently to the 3M-Emphaze resin upon addition of aprotic organic solvent, but they could be easily released by addition of protic solvent, preferably water. After water-mediated glycan elution, the samples were concentrated and analyzed by UPLC. The robotic program has been built to run unsupervised overnight with a processing time of 22 h for 96 samples or multiples of 96 samples if the liquid-handling workstation is equipped with hard- and software for parallel plate processing. Typical sample volumes that can be processed are between 20 and 50 μL of serum or between 300 and 500 μL of cell culture supernatant. This range was found to be a good compromise between maintaining sufficient signal-to-noise ratios in the fluorescence chromatograms and maintaining sufficient flow rates on the ultrafiltration plates when used in a plate centrifuge (maximum rcf of 4000*g*) or on a robotic vacuum manifold (maximum pressure of 24 mmHg). Typical consumable costs are ≈10 times less than the current methods on the market, making the procedure attractive for large-scale patient screening campaigns.

Method Verification. To verify our protocol, serum samples from pooled native human serum were prepared on 4 different days on the Hamilton Star workstation and also manually using the in-gel-block method. The samples were then analyzed on a UPLC system with fluorescence detection and equipped with a BEH HILIC glycan column. A representative chromatogram resulting from the automated sample preparation method, together with the corresponding peak assignments, is shown in Figure 4.

The chromatograms obtained using the robotic method showed 28 integratable peaks, in accordance with previously acquired data.[4] Furthermore, the glycan peak areas were very similar to those of the IGB method, with only a slight bias toward smaller glycans in the robotic method (Figure 5). The coefficients of variation between the samples prepared with the automated method were below 10% for all major peaks (i.e., those peaks with a relative percentage area above 1%). In addition, the coefficients of variation of the automated method were lower than those of the IGB method for most glycan peaks (Figure 6). Sample crossover was checked by processing water blanks alongside serum samples. The UPLC profiles indicated that sample crossover was below the UPLC detection limit.

Cell-culture-derived IgG *N*-glycans were prepared with the same automated assay but using a Protein A instead of a Protein G affinity resin for IgG affinity purification. Due to a

lower IgG concentration in the cell culture supernatant compared to serum, a sample loading of 500 μL of cell culture supernatant was used. A representative chromatogram for anti-human IL-8 IgG is shown in Figure 7 and depicts the expected four peaks FA2, FA2[6]G1, FA2[3]G1, and FA2G2. To date, thousands of IgG samples have been prepared in our laboratory using the robotic platform. Moreover, the analysis is not restricted to that of IgG from human sera and works equally well for IgG from animal species.

CONCLUSION

Most glycan sample preparation workflows developed to date are impacted considerably by low sample throughput, lack of automation, and extremely high consumable costs, significantly hampering the urgently needed progress in glycomics research. We addressed these key issues and developed a concise, automated workflow for IgG *N*-glycan separation and quantification. To the best of our knowledge, this is the first low-cost, automated high-throughput assay for UPLC-based IgG glycomics and should enable efficient profiling of immunoglobulin glycans for biopharmaceuticals development, genome-wide association studies, and clinical disease research, all of which are active research areas in our laboratory.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Louise Unwin for technical assistance and acknowledge EU FP7 program HighGlycan, Grant No. 278535, for funding this work.

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