Chapter 8

High-Throughput Analysis of the Plasma *N*-Glycome by UHPLC

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Abstract

The understanding of glycosylation alterations in health and disease has evolved significantly and glycans are considered to be relevant biomarker candidates. High-throughput analytical technologies capable of generating high-quality, large-scale glycoprofiling data are in high demand. Here, we describe an automated sample preparation workflow and analysis of *N*-linked glycans from plasma samples using hydrophilic interaction liquid chromatography with fluorescence detection on an ultrahigh-performance liquid chromatography (UHPLC) instrument. Samples are prepared in 96-well plates and the workflow features rapid glycoprotein denaturation, enzymatic glycan release, glycan purification on solid-supported hydrazide, fluorescent labeling, and post-labeling cleanup with solid-phase extraction. The development of a novel approach for plasma *N*-glycan analysis and its implementation on a robotic platform significantly reduces the time required for sample preparation and minimizes technical variation. It is anticipated that the developed method will contribute to expanding high-throughput capabilities to analyze protein glycosylation.

Key words *N*-linked glycosylation, Plasma, Glycan analysis, Ultrahigh-performance liquid chromatography, Robotics, Automation, High-throughput

1 Introduction

The growing interest in glycomics has provided an impetus for the development of high-throughput workflows for sample preparation and analysis. The biological relevance of glycans has been exemplified in many processes including protein folding, secretion and degradation, cell signaling, immune response, to name but a few [1–4]. Changes in glycosylation play a pivotal role in many diseases, such as cancer, autoimmune and infectious diseases as well as congenital disorders of glycosylation [5, 6]. Therefore, exploiting disease-associated alterations in glycosylation represents an excellent target for the discovery and development of biomarkers. The identification of potential glycan-based targets can improve the diagnostic value of protein biomarkers currently used

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by increasing their sensitivity and specificity since many cancer associated proteins display clear shifts in their glycan profiles.

Glycomics has lagged behind other -omics disciplines, in part due to the complexity of glycans, which pose more significant analytical challenges compared to genomics or proteomics. The ongoing drive to elucidate these complex structures facilitates the development and great advancement of current glycomics technologies. Deciphering the glycome is a challenging task, in which glycoanalytical methods such as high or ultrahigh-performance liquid chromatography (HPLC, UHPLC), mass spectrometry (MS), capillary electrophoresis (CE), nuclear magnetic resonance (NMR), lectin and glycan arrays are aiding in the characterization of the complete glycome [7–13].

One of the most widely reported and well-known methods for glycan quantification is chromatographic separation with fluorescence detection [14]. The standard procedure includes enzymatic de-glycosylation of glycoproteins, labeling of released glycans with a fluorescent tag and cleanup procedures to remove excess reagents followed by separation on an HPLC instrument, recently replaced by UHPLC [9, 15]. Previously in our laboratory, a reproducible, quantitative, and robust method was developed for *N*-glycan analysis that involved glycoprotein immobilization in polyacrylamide [8, 16]. This method was successfully employed in many studies [17, 18] but immobilization in a gel block hampered the possibility for automation. Several sample preparation protocols have been published to date [19–23]. However, most of them have focused on analyzing antibodies, especially IgG.

Glycoprofiling of serum or plasma *N*-glycome is more challenging due to the higher complexity and overall heterogeneity of plasma glycoproteins. Although plasma glycoprofiling has substantial relevance and delivers a comprehensive view of released glycans from all plasma glycoproteins, it lacks information about the original glycan attachment sites and the identity of the carrier protein. Nevertheless, plasma glycoprofiling represents a recognized aim that can deliver interesting biological insights for further exploration.

While glycan analysis capabilities continue to improve and new approaches are developed, the sample preparation is still a labor-intensive and time consuming process. The manual workup is suitable for small sample sets but in order to meet requirements for large scale glycoprofiling, automated workflows are necessary. To that end, we have developed a high-throughput 96-well platform which is automated through the use of a robotic liquid handling system and applicable for IgG and plasma glycoprofiling (Fig. 1) [21, 24]. The method described here is an updated version and represents an advance in technology available for glycan analysis and should facilitate more routine analysis and large-scale studies.

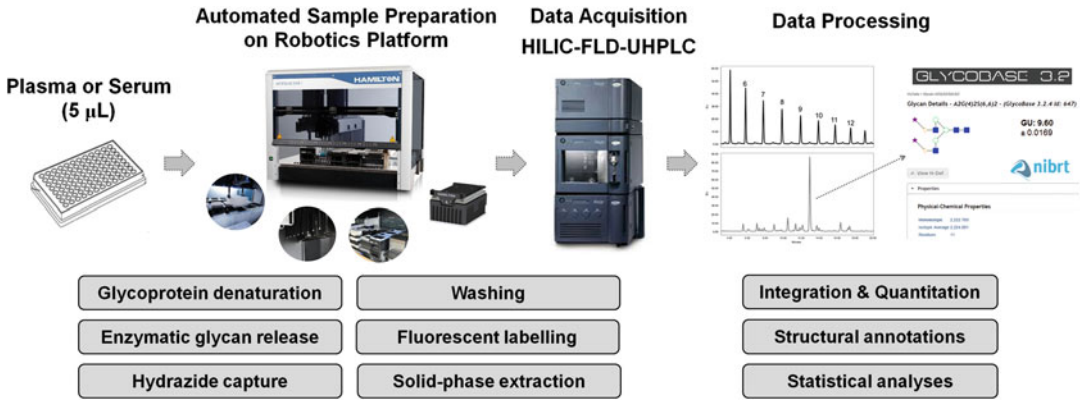


Fig. 1 Overview of the high-throughput workflow for sample preparation and analysis

2 Materials

2.1 Robotic Platform

Hamilton Robotics StarLet liquid handling workstation (Hamilton, Reno, NV) and safety cabinet with its key components: (1) eight independent pipetting channels with liquid-level detection and anti-droplet control, (2) robotic vacuum manifold, (3) plate transport tool, (4) temperature-controlled orbital shaker, (5) Hamilton Robotics Venus one software (*see* **Note 1**).

2.2 Sample Preparation

1. Denaturation solution—100 mM ammonium bicarbonate, 12 mM dithiothreitol (DTT).
2. 96-well V-bottom PP plate (cat.no.: 651201, Greiner Bio-One, Frickehausen, Germany).
3. Alkylating agent—120 mM iodoacetamide solution (IAA). This solution is light-sensitive and must be kept in the dark.
4. Trypsin (cat. no.: M150, Amresco, Solon, OH).
5. Greiner multiwell plate sealers (cat no.: A5596, Sigma-Aldrich).

2.3 PNGase F Digestion

1. Recombinant peptide-N-glycosidase F (PNGaseF; EC 3.5.1.52) expressed in *E. coli* (GKE-5006D, Prozyme, Glyco *N*-Glycanase).
2. PNGaseF solution—1.0 M ammonium bicarbonate.

2.4 Hydrazide-Assisted Glycan Cleanup

1. 96-well chemically inert filter plate with hydrophobic polytetrafluoroethylene (PTFE) membrane, 0.45 µm pore size (cat no.: MSRPN0450, Millipore, Billerica, MA).
2. Ultralink hydrazide resin (cat no.: 53149, ThermoFisher Scientific, Waltham, MA).

3. Methanol (MeOH), acetonitrile (MeCN), water (H_2O), and acetic acid (CH_3COOH) of the highest purity available.
4. 2.0 M Guanidine (CH_5N_3), triethylamine ($(C_2H_5)_3N$) (Sigma-Aldrich), acetic anhydride ($(CH_3CO)_2O$) (Sigma-Aldrich).

2.5 2AB Labeling

1. Dimethylsulfoxide (DMSO, cat no.: D8779, Sigma-Aldrich).
2. Acetic acid (CH_3COOH, cat no.: A6283, Sigma-Aldrich).
3. Sodium cyanoborohydride ($NaBH_3CN$, cat no.: 156159, Sigma-Aldrich).
4. 2-aminobenzamide (2-AB, anthranilamide, cat no.: A89804, Sigma-Aldrich).

2.6 Solid-Phase Extraction (SPE)

1. Polypropylene square well 2 mL/well plates (cat no.: AHO-7194, Phenomenex, Torrance, CA).
2. HyperSep™ Diol SPE cartridges (cat no.: 60108, ThermoFisher Scientific).
3. MeOH, MeCN, H_2O of the highest purity available.
4. AcroPrep™ Advance 96-Well filter plates with hydrophilic polypropylene membrane, 0.45 μm pore size (cat no.: 8084, Pall, Port Washington, NY).

2.7 HILIC-FLD- UHPLC

1. UHPLC instrument (Acquity UPLC H-class) consisting of a binary solvent manager, sample manager, and fluorescent detector.
2. Hydrophilic interaction liquid chromatography (HILIC) column for glycan separation, BEH Glycan column, 2.1 × 150 mm, 1.7 μm BEH particles (cat no.: 186004742, Waters, Milford, MA).
3. Empower 3 chromatography workstation software (Waters).
4. Dextran Calibration Ladder Standard (cat no.: 186004742, Waters).
5. 50 mM ammonium formate, pH 4.4 as solvent A and MeCN as solvent B.

3 Methods

3.1 Glycoprotein Denaturation

1. Plasma (or serum) aliquots of 5 μL per sample are dispensed into a 96-well V-bottom PP plate (*see* **Notes 2** and **3**).
2. Denaturation solution (55 μL per well, 100 mM ammonium bicarbonate, 12 mM DTT) is added and the plate is covered and placed on a robotic heater shaker and incubated at 65 °C with agitation at 700 rpm for 30 min.
3. The plate is left to cool down for 10 min and then iodoacetamide solution (10 μL per well, 120 mM) is added, the plate

is covered with an anti-evaporation lid and incubated at room temperature with agitation at 700 rpm for 30 min.

4. A trypsin solution (10 μL of a 40,000 U/mL solution per well) is added, the plate is covered with a foil seal and placed on a robotic heater shaker at 37 °C with agitation at 700 rpm for 120 min. Next, the temperature is increased to 105 °C and incubation is continued for 10 min.
5. The plate is left to cool to room temperature, briefly spun down and the seal removed.

3.2 N-glycan Release

1. To release glycans from glycoproteins prepare a PNGaseF solution (0.5 mU in 1.0 M ammonium bicarbonate).
2. PNGaseF solution (10 μL) is added and the plate is sealed with an anti-evaporation lid and incubated at 37 °C with agitation at 700 rpm for 120 min.

3.3 Hydrazide-Mediated Glycan Cleanup

1. A 96-well chemically inert filter plate is washed with 100 μL of MeOH.
2. Ultralink hydrazide resin (40 μL of a suspension in water) is dispensed to each well.
3. The resin is sequentially washed with MeOH (200 μL), H_2O (200 μL), and MeCN (200 μL).
4. To seal the membranes plate is placed on a heater (80 °C, 10 min).
5. MeCN–acetic acid (98:2) is added to the resin (180 μL), followed by 20 μL of the glycan solution (glycans released with PNGaseF, the remaining glycan sample is kept as a stock).
6. The filter plate is incubated with shaking at 700 rpm at 80 °C for 60 min.
7. MeCN–acetic acid (98:2) is added to the resin (50 μL) and shaking is continued at the same temperature (80 °C) for 10 min to disrupt resin aggregates.
8. The resin is washed sequentially with MeOH (2 × 300 μL per well), 2.0 M guanidine (2 × 200 μL), H_2O (2 × 200 μL), trimethylamine–MeOH (1:99, 2 × 200 μL), and MeOH (2 × 200 μL).
9. Fresh MeOH (180 μL) and acetic anhydride (20 μL) are added and the plate is incubated for 30 min with agitation at 700 rpm at ambient temperature.
10. Excess reagent is removed by filtration and the resin beads are washed sequentially with MeOH, H_2O, and MeCN (400 μL).
11. MeCN–acetic acid (98:2, 175 μL) and H_2O (25 μL) are sequentially added and the plate is incubated at 70 °C with agitation at 700 rpm for 90 min.

3.4 Fluorescent Labeling

1. 2-AB fluorescent labeling mix (50 μL, 350 mM 2-aminobenzamide, 1.0 M sodium cyanoborohydride in acetic acid–dimethyl sulfoxide (30:70) is dispensed into each well (*see* **Note 4**).
2. The plate is incubated at 65 °C with agitation at 800 rpm for 120 min. [AU1]
3. The labeling reaction is quenched by the addition of 400 μL MeCN–H_2O (95:5).

3.5 Glycan Solid-Phase Extraction

1. The suspension after 2-AB labeling is transferred to a 2 mL collection plate, the beads are left to settle and 200 μL of the supernatant is aspired and dispensed back into the filter plate.
2. The suspension is mixed several times and transferred back into the 2 mL collection plate. This cycle is repeated once more to ensure a quantitative transfer of the resins.
3. HyperSep Diol SPE cartridges are washed with 1 mL MeCN–H_2O (95:5), 1 mL H_2O, and 1 mL MeCN–H_2O (95:5).
4. The supernatants of the quenched reaction mixtures without the beads are transferred onto the SPE cartridges (900 μL).
5. Typically 5 min incubation leads to complete drainage of the solvent.
6. The SPE cartridges are washed three times with 750 μL MeCN–H_2O (95:5) to remove excess of 2-AB.
7. A collection plate is placed inside the robotic vacuum manifold and the SPE cartridges are washed under vacuum with 200 μL H_2O/MeCN (80:20), with an intermittent incubation period of 5 min to elute the retained glycans on the SPE cartridges.
8. Following glycan elution, samples are concentrated to dryness in a vacuum evaporator.

3.6 HILIC-FLD- UHPLC

1. Prepare fresh solvents for UHPLC separation: solvent A (50 mM ammonium formate solution, pH 4.4) and solvent B (MeCN) (*see* **Note 5**).
2. Set up the UHPLC system: prime the system, connect the column and turn on fluorescence detector.
3. Set up the instrument method in Empower software with the following parameters, flow: 0.56 mL/min, a linear gradient of 70–53% MeCN in 30 min separation run: $t = 0$ min, 70% solvent B; $t = 24.81$ min, 53% solvent B; $t = 25.5$ min, 30% solvent B and $t = 26.55$ min, 70% solvent B.
4. Adjust the settings of the fluorescence detector: excitation wavelength 330 nm and emission wavelength 420 nm, data units: emission, date rate: 2 pts/s and PTM gain 20.0.
5. Run a blank sample of H_2O.

6. Run 2-AB labeled dextran ladder (standard) that is be used for calibration, dissolve sample in 30 μL MeCN–H_2O (70:30) (*see* **Note 6**).
7. Run your samples of interest, bring the volume of your 2-AB labeled glycan samples to 30 μL with MeCN–H_2O (70:30) (*see* **Note 7**).

3.7 Data Analysis in Empower 3 Software

1. All samples are located in the sample sets; to process them you should view them as "Channels."
2. Highlight all the samples that you wish to process by holding "Ctrl" and clicking on each sample or to highlight all samples in the sample set click "E" in the top left corner and right click on highlighted samples and select "Alter sample."
3. Make sure to include a dextran standard, which is used to calibrate the runs. The GU value is calculated by fitting a fifth order polynomial distribution curve to the dextran ladder, then using this curve to allocate GU values from retention times.
4. Ensure that Sample Type is correct, narrow standard for dextran and broad unknown for samples.
5. Highlight the dextran line, go to "Edit" and select "Amount." In the Molecular Weight column enter 40,000 in line 1, all the way up to 150,000. Click OK and the window will close. Click "Save" in Alter Sample window.
6. To process samples, go back to "Channels" and right click on processed samples and select "Process."
7. Ensure that the "Clear Calibration" box is always ticked. Select "Use specified processing method" and choose appropriate method from the drop-down list. When you process a large set of samples, it is advisable to create your own processing method that will facilitate integration in a semiautomatic manner.
8. To analyze your processed samples go to "Channels" and right-click on highlighted samples and select "View as"-> "Results."
9. Open all samples in Review, first check the integration of dextran making sure that the peaks are integrated from peak 4 to 15 and delete any others.
10. To calibrate dextran, go to "Process" and select "Calibrate."
11. For each profile, once you have integrated as appropriate go to "Process" and select "Quantitate."
12. Always make sure to integrate the samples in the same manner, the same number of peaks to ensure proper comparison.
13. When finished, go to "File" and select "Save"-> "All."

14. All data can be exported, including peak list with % area, GU value, and other parameters that are of interest, to Excel file by creating Export method.
15. An excel spreadsheet can be then used for further statistical analysis of your samples.
16. For preliminary structural annotation, glycan structures are assigned by their GU value to the peaks and they can by matched to a database developed at NIBRT (http://glycobase.nibrt.ie). Glycobase is a tool that allows computer-assisted preliminary structural assignments [25] and to further elucidate the glycan structures, exoglycosidase digestions can be performed (*see* Chapter 18 for more details) (*see* **Note 8**).

4 Notes

1. Further increase in throughput can be achieved by converting the method to a 384-well plate format, it is possible with an optional 384 channel pipetting head available from Hamilton.
2. When performing glycoprofiling of many samples, make sure that all samples are of the same type, either plasma or serum to ensure high quality data and consistent results. There is a significant difference in the relative percentage area for several glycan structures, mainly biantennary digalactosylated monosialylated glycan (A2G2S1) between plasma and serum [26].

   The difference appears to be largely attributable to the presence of fibrinogen in plasma samples whereby a lack of this glycoprotein is observed in serum (Fig. 2).
3. To obtain more information about low abundant proteins, the depletion of high-abundant plasma proteins can be performed by affinity chromatography which significantly improves detection of low-level glycoproteins.
4. The protocol uses 2-AB as a fluorescent tag for glycans, however it might be also possible to take an advantage of AQC labeling agent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) that has been recently applied for IgG analysis [27]. In this case, a hydrazide-mediated cleanup step is neither necessary nor possible. We have tested AQC label for analysis of

Fig. 2 (continued) available in Saldova et al. [29]. *Asterisk* denotes peaks glycan structures that in part may be originating from fibrinogen (**b**) Fibrinogen *N*-glycan profile with annotated structures as described in Adamczyk et al. [26]. (**c**) Serum sample from gastric cancer patient, some structures that were previously reported to be significantly altered with disease progression are shown, namely IgG G0 (FA2), IgG G1 (FA2G1), and sLe^x epitope (A3F1G3S3) [30, 31]. The structural symbols for the *N*-glycans and their linkages are shown in the boxes below the chromatograms

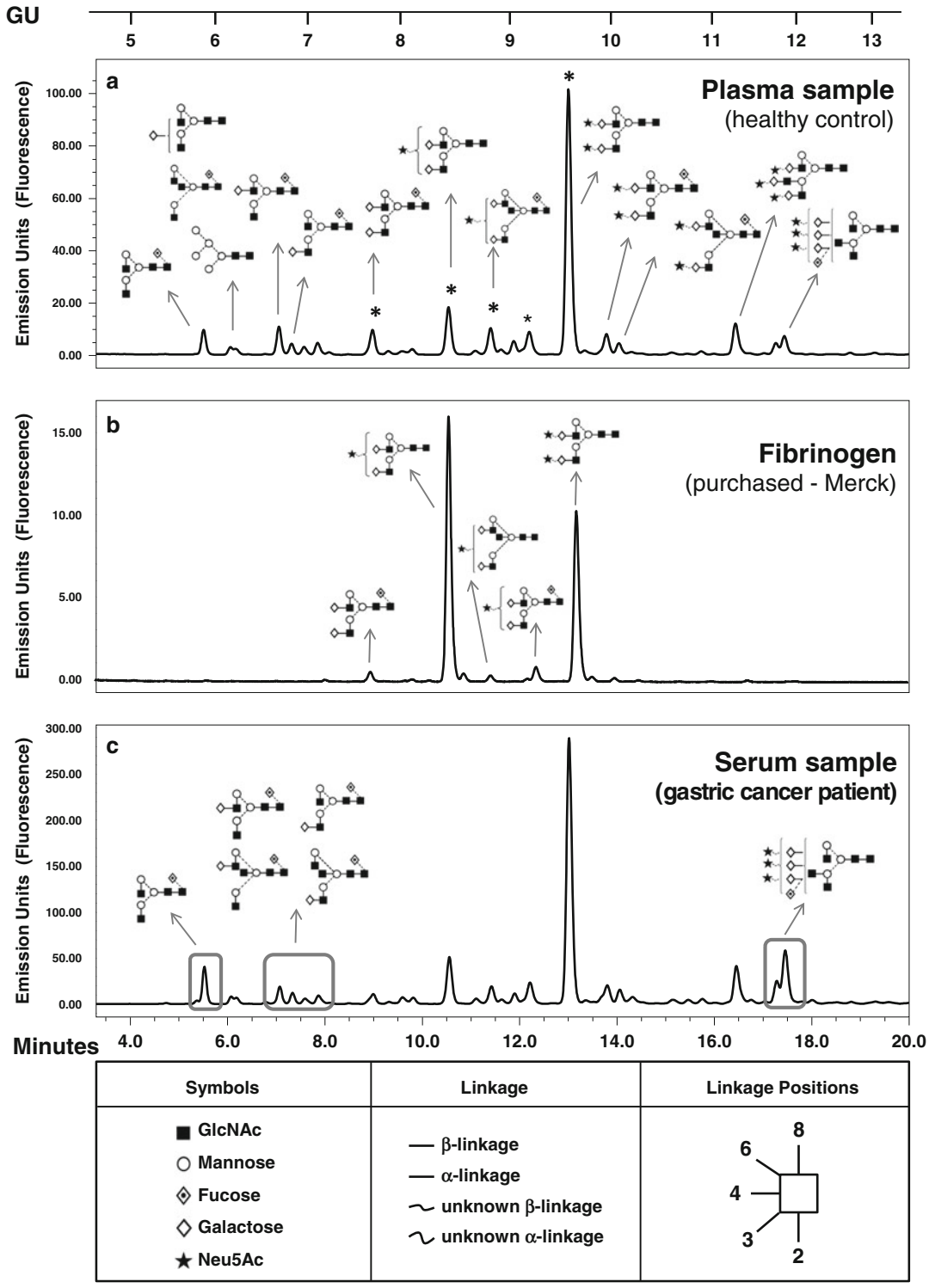
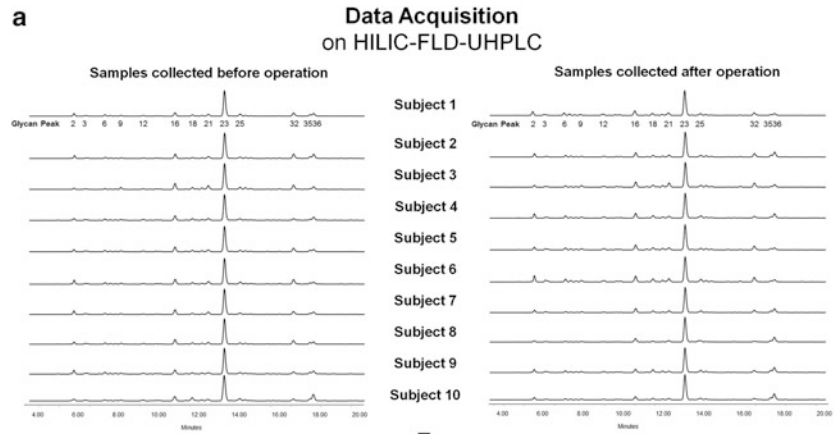


Fig. 2 HILIC-FLD-UHPLC chromatograms of 2AB labeled *N*-glycans (**a**) A typical plasma *N*-glycan profile; contains more than 160 glycans, some of the major structures are represented on the figure and abbreviated according to Harvey et al. [28]. The detailed description including monosaccharide sequences and linkage information is



b

Sample Integration
to obtain % areas for all peaks

	1_Pre	2_Pre	3_Pre	4_Pre	5_Pre	6_Pre	7_Pre	8_Pre	9_Pre	10_Pre	1_Post	2_Post	3_Post	4_Post	5_Post	6_Post	7_Post	8_Post	9_Post	10_Post
GP1	0.11	0.14	0.08	0.19	0.27	0.16	0.22	0.26	0.22	0.15	0.14	0.12	0.14	0.22	0.38	0.23	0.13	0.13	0.14	0.17
GP2	3.73	3.88	1.74	2.71	3.28	5.03	5.19	3.26	5.33	2.96	5.03	4.34	2.35	5.25	3.55	6.53	3.49	1.99	3.91	3.47
GP3	1.54	1.52	1.32	1.84	2.2	1.68	2.05	2.18	2.33	1.73	2.24	1.5	1.84	1.92	2.55	2.9	1.72	1.21	2.18	1.31
GP4	0.05	0.06	0.06	0.11	0.16	0.06	0.06	0.16	0.07	0.05	0.08	0.05	0.08	0.1	0.19	0.09	0.05	0.05	0.04	0.06
GP5	0.1	0.24	0.14	0.17	0.24	0.15	0.1	0.24	0.28	0.19	0.14	0.22	0.21	0.17	0.27	0.21	0.09	0.1	0.19	0.2
GP6	2.07	1.71	1.26	2.11	1.99	2.08	1.61	1.98	2.62	1.6	3.39	2.09	1.9	2.99	2.7	3.18	1.34	1.07	2.3	1.49
GP7	0.99	0.79	0.51	0.75	0.92	1.02	0.69	0.92	1.06	0.92	1.9	1.28	0.82	1.12	1.36	1.56	0.64	0.48	1.15	0.92
GP8	0.62	0.69	0.72	0.83	0.87	0.68	0.6	0.87	1.21	1.05	0.94	0.77	0.94	0.93	1.16	1.16	0.76	0.46	1.28	0.68
GP9	1.4	1.13	2.7	1.56	1.62	1.46	1.46	1.61	1.47	1.49	1.6	1.19	2.35	1.53	1.76	1.92	1.38	1.12	1.49	1.09
GP10	0.18	0.18	0.19	0.36	0.32	0.2	0.22	0.32	0.22	0.12	0.29	0.15	0.28	0.29	0.44	0.29	0.17	0.16	0.14	0.12
GP11	0.08	0.13	0.12	0.15	0.16	0.1	0.09	0.16	0.16	0.1	0.14	0.12	0.17	0.13	0.22	0.15	0.08	0.08	0.1	0.09
GP12	1.38	1.19	1.3	1.9	1.65	1.33	1.08	1.64	1.62	1.66	2.58	1.59	1.77	2.11	2.35	2.17	1.07	0.79	1.77	1.14
GP13	0.26	0.27	0.43	0.4	0.41	0.3	0.26	0.41	0.47	0.23	0.39	0.29	0.49	0.42	0.53	0.48	0.31	0.15	0.57	0.21
GP14	0.76	1.05	1.11	0.85	0.99	0.91	0.83	0.97	1.07	1.21	1.08	1.04	1.4	0.89	1.17	1.14	0.75	0.68	0.89	0.88
GP15	0.73	0.65	0.81	0.66	0.77	0.86	0.64	0.78	0.82	0.95	1.07	0.92	0.81	0.9	0.97	1.11	0.59	0.46	0.99	0.78
GP16	5.91	6.69	8.68	7.78	7.73	7.44	7.86	7.68	6.59	6.65	7.27	6.45	7.7	7.14	7.36	7.47	7.15	6.69	5.01	5.17
GP17	0.51	0.75	0.7	0.59	1.03	0.47	0.44	1.03	0.81	0.81	0.68	0.78	1.11	0.58	1.32	0.7	0.52	0.57	0.77	0.63
GP18	2.23	1.72	2.94	3.03	2.43	2.57	2.2	2.43	2.22	4.93	3.3	2.53	3.17	3.84	3.37	4.12	3	1.71	4.31	2.47
GP19	0.65	0.57	1.16	0.69	0.7	0.57	0.45	0.69	0.45	0.74	0.69	0.59	0.94	0.66	0.86	0.55	0.48	0.29	0.69	0.49
GP20	1.33	1.16	2.23	1.29	1.75	1.32	1.06	1.73	1.71	1.7	1.69	1.41	2.33	1.63	2.28	2.13	1.04	0.75	2.17	1.24
GP21	3.83	3.89	6.38	3.93	3.69	4.87	4.93	3.67	2.89	2.77	4.79	3.5	6.95	3.5	3.81	4.9	3.64	3.32	2.58	2.72
GP22	0.18	0.14	0.19	0.17	0.21	0.18	0.25	0.21	0.15	0.22	0.17	0.14	0.19	0.19	0.24	0.18	0.19	0.2	0.23	0.17
GP23	42.03	37.9	37.18	42.54	40.55	38.64	41.69	40.39	40.44	35.74	37.95	36.68	34.97	35.76	36.58	31.66	41.91	45.97	35.83	39.86
GP24	0.97	0.88	0.91	0.97	1.07	0.89	1.17	1.07	0.95	0.65	0.91	0.75	0.89	0.88	1.15	0.79	0.9	1.1	0.98	0.94
GP25	4.08	2.93	3.84	3.95	2.68	3.81	3.25	2.67	3.15	4.58	3.49	3.35	3.53	4.54	2.87	3.96	3.91	4.63	4.11	3.75
GP26	1.28	1.8	2.76	1.17	1.76	1.27	0.87	1.73	1.82	1.86	1.55	2	2.59	1.56	2.12	1.8	0.9	0.89	1.92	1.7
GP27	1.2	1.65	2	1.35	1.41	1.61	1.56	1.39	1.32	1.18	1.28	1.53	1.72	1.47	1.5	1.59	1.37	1.11	1.22	0.96
GP28	0.86	1.05	0.77	0.83	1	0.91	0.79	0.98	1.1	0.65	0.84	0.94	0.83	0.79	0.97	0.81	0.76	0.59	0.73	0.58
GP29	0.47	0.63	0.4	0.64	0.41	0.39	0.47	0.4	0.6	0.61	0.43	0.65	0.4	0.71	0.37	0.35	0.56	0.76	0.6	0.61
GP30	0.67	0.92	1.26	0.51	0.66	1.12	0.82	0.65	0.43	0.43	0.68	0.76	1.33	0.55	0.69	1.04	0.56	0.32	0.39	0.33
GP31	0.28	0.24	0.23	0.23	0.18	0.29	0.29	0.17	0.14	0.17	0.33	0.21	0.3	0.2	0.2	0.27	0.23	0.2	0.16	0.25
GP32	6.3	7.6	6.9	3.71	6.68	7.92	5.86	6.77	4.51	3.36	4.84	6.02	6.59	3.36	6.19	6.39	4.84	2.27	3.93	3.04
GP33	0.39	0.57	0.56	0.46	0.3	0.41	0.5	0.3	0.3	0.65	0.33	0.63	0.58	0.57	0.29	0.38	0.53	0.64	0.46	0.67
GP34	0.52	0.44	0.59	0.29	0.29	0.56	0.39	0.29	0.28	0.22	0.34	0.37	0.53	0.28	0.29	0.41	0.34	0.21	0.28	0.25
GP35	2.71	2.82	1.19	1.96	2.63	2.08	2	2.7	2.74	2.65	1.39	2.93	1.07	2	1.96	1.38	2.7	2.41	3.02	3.15
GP36	5.15	7.32	4.51	5.94	4.27	3.8	4.79	4.35	5.46	9.55	3.43	8.08	4.2	7.01	3.53	3.34	7.34	9.82	8.11	10.6
GP37	0.43	0.38	0.24	0.3	0.23	0.33	0.3	0.23	0.33	0.34	0.25	0.37	0.24	0.34	0.21	0.27	0.36	0.49	0.43	0.42
GP38	0.5	0.57	0.44	0.52	0.24	0.33	0.4	0.24	0.42	0.66	0.3	0.63	0.43	0.66	0.23	0.27	0.59	0.9	0.66	0.76
GP39	0.53	0.57	0.25	0.45	0.42	0.35	0.41	0.43	0.5	0.55	0.38	0.52	0.32	0.53	0.43	0.37	0.46	0.54	0.64	0.61
GP40	0.51	0.56	0.33	0.25	0.32	0.49	0.42	0.34	0.21	0.29	0.37	0.35	0.47	0.22	0.32	0.47	0.36	0.24	0.32	0.34
GP41	0.77	0.78	0.3	0.48	0.56	0.54	0.56	0.6	0.47	0.6	0.45	0.57	0.38	0.5	0.49	0.48	0.61	0.64	0.7	0.71
GP42	0.5	0.54	0.23	0.31	0.29	0.33	0.37	0.32	0.24	0.55	0.29	0.4	0.29	0.33	0.25	0.3	0.48	0.42	0.47	0.78
GP43	0.73	0.77	0.22	0.57	0.41	0.33	0.49	0.46	0.45	1.23	0.35	0.66	0.25	0.62	0.3	0.27	0.9	1.23	1.07	1.91
GP44	0.33	0.36	0.09	0.33	0.18	0.12	0.22	0.21	0.25	0.86	0.16	0.36	0.14	0.38	0.15	0.14	0.57	1.39	0.77	1.64
GP45	0.14	0.17	0.04	0.16	0.08	0.04	0.09	0.09	0.1	0.38	0.08	0.17	0.07	0.22	0.07	0.07	0.21	0.78	0.29	0.65

c

Statistical analysis
to compare data sets and test significant differences

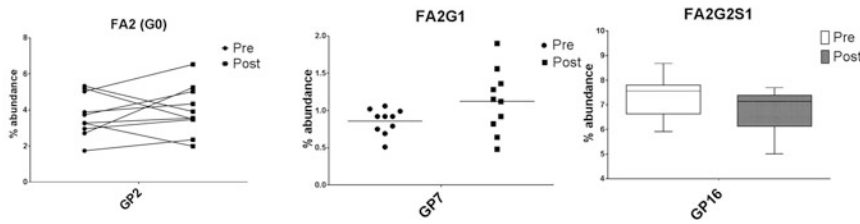


Fig. 3 Examples of the data output from a glycoprofiling experiment. (**a**) HILIC-FLD-UHPLC profiles of gastric cancer serum samples, *on the left* samples collected before operation and *on the right* samples collected after operation when patients are classified cancer-free (**b**) Analysis of the data in Empower results in the table with % area for all integrated peaks for both sets of patients (**c**) Statistical analysis to compare data sets using different approaches for data analysis, interpretation and presentation of the data (for example Boxplots, Heat Plots, Cluster Analysis, Principal Component Analysis)

plasma samples using manual in-gel-block method [8] and the obtained data were of good quality. However, before embarking on large-scale analysis more thorough tests and optimization should be performed.

5. Reagents used should be of the highest grade commercially available, especially for preparing buffers for HILIC-FLD-UHPLC analysis.
6. It is recommended to run a dextran ladder standard followed by the samples, for calibration and to check the performance of the instrument. The dextran should be run once every 24 h or every 48 samples.
7. The workflow presented in this book chapter has many applications; one of them is glycoprofiling of disease samples and comparing different stages of the disease. An example of glycoprofiling of serum samples, originating from gastric cancer patients, pre- and post-surgery is illustrated on Fig. 3.
8. The automated sample preparation workflow presented in this book chapter can be interfaced with other downstream analytical technologies, including mass spectrometry (MS) and capillary electrophoresis (CE), for rapid characterization and quantitation of oligosaccharides.

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