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TITLE:

Immunoglobulin G N-Glycan Analysis by Ultra-Performance Liquid Chromatography

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glycomics, immunoglobulin G, *N*-glycan, ultra-performance liquid chromatography

SUMMARY:

Immunoglobulin G (IgG) *N*-glycan is characterized using hydrophilic interaction chromatography UPLC. In addition, the structure of IgG *N*-glycan is clearly separated. Presented here is an introduction to this experimental method so that it can be widely used in research settings.

ABSTRACT:

Glycomics is a new subspecialty in omics system research that offers significant potential in discovering next-generation biomarkers for disease susceptibility, drug target discovery, and precision medicine. Alternative IgG *N*-glycans have been reported in several common chronic diseases and suggested to have great potential in clinical applications (i.e., biomarkers for diagnosis and prediction of diseases). IgG *N*-glycans are widely characterized using the method of hydrophilic interaction chromatography (HILIC) ultra-performance liquid chromatography (UPLC). UPLC is a stable detection technology with good reproducibility and high relative quantitative accuracy. In addition, the structure of IgG *N*-glycan is clearly separated, and glycan composition and relative abundance in plasma are characterized.

INTRODUCTION:

N-glycosylation of human proteins is a common and essential post-translational modification[1] and may help predict the occurrence and development of diseases relatively accurately. Due to the complexity of its structure, it is expected that there are more than 5,000 glycan structures, providing great potential as diagnostic and predictive biomarkers for diseases[2]. *N*-glycans attached to immunoglobulin G (IgG) have been shown to be essential for IgG's function, and IgG *N*-glycosylation participates in the balance between the pro- and anti-inflammatory systems[3].

Differential IgG *N*-glycosylation is involved in disease development and progression, representing both a predisposition and functional mechanism involved in disease pathology. The inflammatory role of IgG *N*-glycosylation has been associated with aging, inflammatory diseases, autoimmune diseases, and cancer[4].

With the development of detection technology, the following methods are most widely used in high throughput glycomics: hydrophilic interaction chromatography (HILIC) ultra-performance liquid chromatography with fluorescence detection (UPLC-FLR), multiplex capillary gel electrophoresis with laser induced fluorescence detection (xCGE-LIF), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), and liquid chromatography electrospray mass spectrometry (LC-ESI-MS). These methods have overcome previous shortcomings of low flux, unstable results, and poor sensitivity specificity[5,6].

UPLC is widely used to explore the association between IgG *N*-glycosylation and certain diseases (i.e., ageing[7], obesity[8], dyslipidemia[9], type II diabetes[10], hypertension[11], ischemic stroke[12], and Parkinson's disease[13]). Compared to the other three abovementioned methods, UPLC has the following advantages[5,14]. First, it provides a relative quantitative analysis method, and the data analysis that involves total area normalization improves the comparability of each sample. Second, the cost of equipment and required expertise are relatively low, which makes it easier to implement and transform glycosylation biomarkers into clinical applications. Presented here is an introduction to UPLC so it can be more widely used.

PROTOCOL:

All the subjects included in the protocol have been approved by the Ethics Committee of the Capital Medical University, Beijing, China[12]. Written informed consent was obtained from each subject at the beginning of the study.

1. IgG isolation

1.1. Prepare the chemicals including binding buffer (phosphate buffered saline, PBS): 1x PBS (pH = 7.4), neutralizing buffer: 10x PBS (pH = 6.6–6.8), eluent: 0.1 M formic acid (pH = 2.5), neutralizing solution for eluent: 1 M ammonium bicarbonate, stored buffer: 20% ethanol + 20 mM Tris + 0.1 M NaCl (pH = 7.4), cleaning solution for protein G: 0.1 M NaOH + 30% propan-2-ol.

NOTE: The level of pH is critical in this protocol. The elution of IgG requires a very low pH, and there is a risk of the loss of sialic acids due to acid hydrolysis. Therefore, elution occurs within seconds, and the pH is quickly restored to neutrality, preserving the integrity of IgG and the sialic acids.

1.2. Prepare the samples: thaw the frozen plasma sample then centrifuge at 80 x *g* for 10 min, and leave the protein G monolithic plate and the abovementioned chemicals for 30 min at room temperature (RT).

1.3. Transfer a 100 µL sample (which can be used to detect 2x to prevent the first failure) into a 2 mL collection plate (here, a total of six standard samples, one control sample [ultra-pure water], and 89 plasma samples were designed for 96 well plates and randomly assigned to the plate).

1.4. Dilute the samples with 1x PBS by 1:7 (v/v).

1.5. Clean a 0.45 µm hydrophilic polypropylene (GHP) filter plate with 200 µL of ultra-pure water (repeat 2x).

1.6. Transfer the diluted samples into the filter plate and filter the samples into the collection plate using a vacuum pump (control vacuum pressure at 266.6–399.9 Pa).

1.7. Preparation of the protein G monolithic plates

1.7.1. Discard the storage buffer.

1.7.2 Clean the monolithic plates with 2 mL of ultra-pure water, 2 mL of 1x PBS, 1 mL of 0.1 M formic acid, 2 mL of 10x PBS, 2 mL of 1x PBS (sequentially), and remove flowing liquid using a vacuum pump.

1.8. Transfer the filtered samples to the protein G monolithic plate for IgG binding and cleaning, then clean the monolithic plates with 2 mL of 1x PBS (repeat the cleaning 2x).

1.9. Elute IgG with 1 mL of 0.1 M formic acid and filter the samples into the collection plate by vacuum pump, then add 170 µL of 1 M ammonium bicarbonate into the collection plate.

1.10. Detect IgG concentration using an absorption spectrophotometer (optimal wavelength = 280 nm).

1.10.1. Open the software and select the protein-CY3 mode.

1.10.2. Draw 2 µL of ultra-pure water and load it into the screen, then click **Blank** in the software to clear the screen (repeat 1x).

1.10.3. Draw 2 µL of ultra-pure water and load it into the screen, then click **Sample** in the software to detect the ultra-pure water.

1.10.4. Draw 2 µL of IgG sample and load it into the screen, then click **Sample** in the software to detect the sample.

1.10.5. Draw 2 µL of ultra-pure water and load it into the screen, then click **Blank** in the software to clear the screen.

1.10.6 Close the software.

NOTE: The formula for calculating IgG concentration is as follows:

$$C_{IgG} = \text{absorbance} \times \text{extinction coefficient (13.7)} \times 1000\ \mu g/mL$$

1.11 Put the extracted IgG to dry in an oven at 6 0°C and preserve the extracted IgG (300 µL extracted IgG for 4 h).

1.11.1. Remove 300 µL of extracted IgG if the concentration is greater than 1,000 µg/mL.

1.11.2. Remove 350 µL of extracted IgG if the concentration is between 500–1,000 µg/mL.

1.11.3. Remove 400 µL of extracted IgG if the concentration is between 200–500 µg/mL.

1.11.4. Remove 600 µL of extracted IgG if the concentration is smaller than 200 µg/mL.

NOTE: The concentration of IgG should be preferably >200 µg/mL for subsequent detection. The average amount of IgG should be preferably >1,200 µg, which can be tested 2x in case the first test fails.

1.12. Cleaning the protein G monolithic plate for reuse

1.12.1. Wash the plate with 2 mL of ultra-pure water, 1 mL of 0.1 M NaOH (for removing precipitated proteins), 4 mL of ultra-pure water, and 4 mL of 1x PBS (sequentially), then remove flowing liquid using a vacuum pump.

1.12.2. Wash the plate with 2 mL of ultra-pure water, 2 mL of 30% propan-2-ol (for removing bound hydrophobic proteins), 2 mL of ultra-pure water, and 4 mL of 1x PBS (sequentially), then remove flowing liquid using a vacuum pump.

1.12.3. Wash the plate with 1 mL of buffer (20% ethanol + 20 Mm Tris + 0.1 M NaCl) and add 1 mL of buffer (20% ethanol + 20 Mm Tris + 0.1 M NaCl) to the plate, then leave the plate at 4 °C.

2. Glycan release

2.1. Prepare the dried IgG and store the chemicals including 1.33% SDS, 4% Igepal (store away from light), and 5x PBS at RT.

2.2. Prepare PNGase F enzyme by diluting 250 U enzyme with 250 µL of ultra-pure water.

2.3. Denaturation of IgG

2.3.1. Add 30 µL of 1.33% SDS and mix by vortexing, transfer the sample into a 65 °C oven for

10 min, then remove it from the oven and let rest for 15 min.

2.3.2. Add 10 µL of 4% Igepal and place it on the shaking incubator for 5 min.

2.4. Removal and release of glycans

2.4.1. Add 20 µL of 5x PBS and 30–35 µL of 0.1 mol/L NaOH to regulate a pH of 8.0, and mix by vortexing. Add 4 µL of PNGase F enzyme and mix by vortexing. Then, incubate for 18–20 h in a 37 °C water bath.

2.4.2. Put the released glycans to dry in an oven at 60 °C for 2.5–3.0 h.

2.4.3. Save the released glycans at -80 °C until further measurement.

NOTE: This step is critical. The key to glycan release is improving the activity of the PNGase F enzyme to maximize its efficiency.

3. Glycan labeling and purification

3.1. Prepare the 2-aminobenzamide (2-AB) labeling reagent with 0.70 mg of 2-AB, 10.50 µL of acetic acid, 6 mg of sodium cyanoborohydride (NaBH3CN), and 24.50 µL of dimethyl sulfoxide (DMSO) (total volume = 35 µL). Then, add acetic acid, 2-AB, and NaBH3CN into the DMSO in order.

3.2. Label the glycans using 35 µL of 2-AB labeling reagent, transfer the labeled glycans to the oscillator for 5 min, transfer to the oven for 3 h at 65 °C, then transfer to RT for 30 min.

NOTE: The entire glycan labeling step must be performed while protected from light.

3.3. Pretreat a 0.2 µm GHP filter plate with 200 µL of 70% ethanol, 200 µL of ultra-pure water, and 200 µL of 96% acetonitrile (4 °C), then remove waste using a vacuum pump.

3.4. Purification of 2-AB labeled glycan

3.4.1. Add 700 µL of 100% acetonitrile to the 2-AB labeled glycan and transfer to a shaking incubator for 5 min.

3.4.2. Centrifuge at 134 x *g* for 5 min (4 °C).

3.4.3 Transfer the sample to a 0.2 µm GHP filter plate for 2 min and remove the filtrate (flowing liquid) using a vacuum pump.

3.5. Wash 2-AB labeled glycan with 200 µL of 96% acetonitrile (4 °C) and remove the filtrate (flowing liquid) using a vacuum pump 5x–6x.

3.6. Elute 2-AB labeled glycan with 100 µL of ultra-pure water 3x.

3.7. Transfer the 2-AB labeled glycan into an oven to dry at 60 °C for 3.5 h.

3.8. Save the labeled *N*-glycans at -80 °C until further measurement.

4. Hydrophilic interaction chromatography and analysis of glycans

4.1. Conditioning of UPLC instruments and preparation of mobile phases

4.1.1. Prepare mobile phases including solvent A: 100 mM ammonium formate (pH = 4.4), solvent B: 100% acetonitrile, solvent C: 90% ultra-pure water (10% methanol), and solvent D: 50% methanol (ultra-pure water).

4.1.2. Open the software to control the mobile phases.

4.1.3. Wash UPLC instruments at flow rate of 0.2 mL/min (50% solvent B and 50% solvent C) balancing for 30 min, then at a flow rate of 0.2 mL/min (25% solvent A and 75% solvent B) balancing for 20 min, then a flow rate of 0.4 mL/min balancing.

4.2. Dissolve the labeled *N*-glycans with 25 µL of a mixture of 100% acetonitrile and ultra-pure water at a 2:1 ratio (v/v). Then, centrifuge at 134 × *g* for 5 min (4 °C) and load 10 µL of the labeled *N*-glycans into the UPLC instruments.

4.3. Separate the labeled *N*-glycans at flow rate of 0.4 mL/min with a linear gradient of 75% to 62% acetonitrile for 25 min. Then, perform an analytical run by dextran calibration ladder/glycopeptide column on a UPLC at 60 °C (here, samples were kept at 4 °C prior to injection).

4.4. Detect *N*-glycan fluorescence at excitation and emission wave lengths of 330 nm and 420 nm, respectively.

4.5. Integrate the glycans based on peak position and retention time.

4.6. Calculate the relative value of each Glycan Peak (GP)/ all Glycan Peaks (GPs) (percentage, %) as follows: GP1: GP1/GPs*100, GP2: GP2/GPs*100, GP3: GP3/GPs*100, etc.

REPRESENTATIVE RESULTS:

As shown in **Figure 1**, IgG *N*-glycans were analyzed into 24 initial IgG glycan peaks (GPs) based on peak position and retention time. The *N*-glycan structures are available through mass spectrometry detection according to a previous study (**Table 1**)[15]. To ensure that the results were comparable, total area normalization was applied, in which the amount of glycans in each peak was expressed as a percentage of the total integrated area.

To assess the repeatability and stability of the method, the standard sample was tested in parallel six times. As shown in **Table 2**, the coefficient of variation (CV) of 24 GPs ranged from 1.84%–16.73%, 15 (62.50%) of which were below 10%. GPs with relatively small proportions (≤1.16%) showed high measurement errors (more than 10% of CV). In addition, the IgG *N*-glycan profiles combined from 76 individuals (**Figure 2**) indicated that the position of GP was stable, shape of GP was similar, and integration for the samples maintained the same intervals. The above results indicate that the method is stable and repeatable.

As shown in **Table 3**, an additional 36 derived traits describing the relative abundances of galactosylation, sialylation, bisecting GlcNAc, and core fucosylation were calculated by the remaining 24 directly measured glycans. For example, G2/G0 (GP12/GP2) reflected the level of galactosylation (di-/a-) without core fucosylation and bisecting GlcNAc. G2/G1 (GP14/[GP8 + GP9]) reflected the level of galactosylation (di-/mono-) with core fucosylation and without bisecting GlcNAc. Finally, G1/G0 ([GP10 + GP11]/GP6) reflected the level of galactosylation (mono-/a-) with core fucosylation and bisecting GlcNAc. These calculations of derived glycans follow a principle, to see the change of only one glycosylation trait.

FIGURE AND TABLE LEGENDS:

Figure 1: UPLC chromatogram of one individual. IgG *N*-glycans were analyzed into 24 initial IgG glycan peaks (GPs) based on peak position and retention time. GP8 represents the sum of GP 8a and GP 8b. GP16 represents the sum of GP 16a and GP 16b. The structure of glycans in each chromatographic peak and the average percentage of individual structures are shown in **Table 1** and **Table 2**.

Figure 2: UPLC chromatogram of 76 individuals. The IgG *N*-glycan profiles were combined from 76 individuals to demonstrate the repeatability and stability of the method.

Table 1: Structure of the 24 initial IgG glycans.

Table 2: Precision of the method. The standard sample is tested in parallel six times to assess the repeatability and stability of the method.

Table 3: Calculation of the derived glycans.

DISCUSSION:

UPLC serves as a relative quantitative analysis method[5,15]. The results indicate that UPLC is a stable detection technology with good reproducibility and relative quantitative accuracy. The amount of glycans in each peak is expressed as a percentage of the total integrated area using UPLC, which is the relative value. The relative quantification improves the comparability of test samples. In addition, 96 well protein G plates are used to purify IgG with 96 samples at one time for high throughput detection. The ability of protein G to bind IgG is greater than that of protein A, as described in previous studies[15,16].

In addition, the structures of IgG *N*-glycan are clearly separated. The derived IgG glycans describe the level of galactosylation, sialylation, bisecting GlcNAc, and core fucosylation, which are calculated by the initial IgG *N*-glycans. In a previous study, some derived glycans (FB^n, $FBG0^n$ / $G0^n$, FB^n/ F^{ntotal}, B^n / (F^n + FB^n), $FBG2^n$ / $FG2^n$, $FG2^n$ / ($BG2^n$ + $FBG2^n$) , $BG2^n$ / ($FG2^n$ + $FBG2^n$)) could reflect the change in multiple glycosylation levels but did not reflect the level of specific glycosylation[15].

Recently, a large-scale study showed that IgG galactosylation (referred to as Gal-ratio) can serve as a promising biomarker for cancer screening in multiple cancer types[17]. The distribution of IgG galactosylation is measured by calculating the relative intensities of agalactosylated (G0) *vs.* monogalactosylated (G1) and digalactosylated (G2) *N*-glycans according to the formula (G0/[G1 + G2 x 2]). The Gal-ratio reflects the level of galactosylation with core fucosylation and without bisecting GlcNAc. Therefore, multiple initial IgG *N*-glycans were combined with a derived glycan, representing the level of a specific glycosylation trait. The calculations of derived glycans follow a principle that explores the changes in only one glycosylation trait fixed over other glycosylation trait.

In the present protocol, pH is important for maintaining the stability of IgG and glycan structures, especially for stabilizing terminal sialylation. Therefore, the pH value of the solution must be strictly controlled, and the pH of the solution exposed to IgG must be restored as well as glycans kept at a neutral pH level. In addition, the glycan release step is critical in this protocol. The key to glycan release is improving the activity of the PNGase F enzyme to maximize its efficiency. For example, it was found that 18–20 h is optimal for PNGaseF digestion. This step needs to be fully reacted.

There are some limitations of this technique. The method used cannot differentiate glycans released from the Fab and the Fc portions of IgG. Glycans from Fab and Fc are known to be different. With the developments in glycoproteomics, detection techniques can measure the levels of IgG combining *N*-glycans to explore the role of IgG *N*-glycans and *N*-glycosylation in diseases. The cost of equipment is relatively low; however, the cost per sample is rather high.

In summary, this protocol introduces UPLC so that it can be widely used. Comprehensive valuation and standardization of the analytical methods are needed before significant amounts of time and resources are invested in large-scale studies. As UPLC becomes more widely used, the effects of IgG *N*-glycans and *N*-glycosylation on certain diseases can be more accurately determined, and glycosylation biomarkers can be used for clinical applications.

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DISCLOSURES:

The authors have nothing to disclose.

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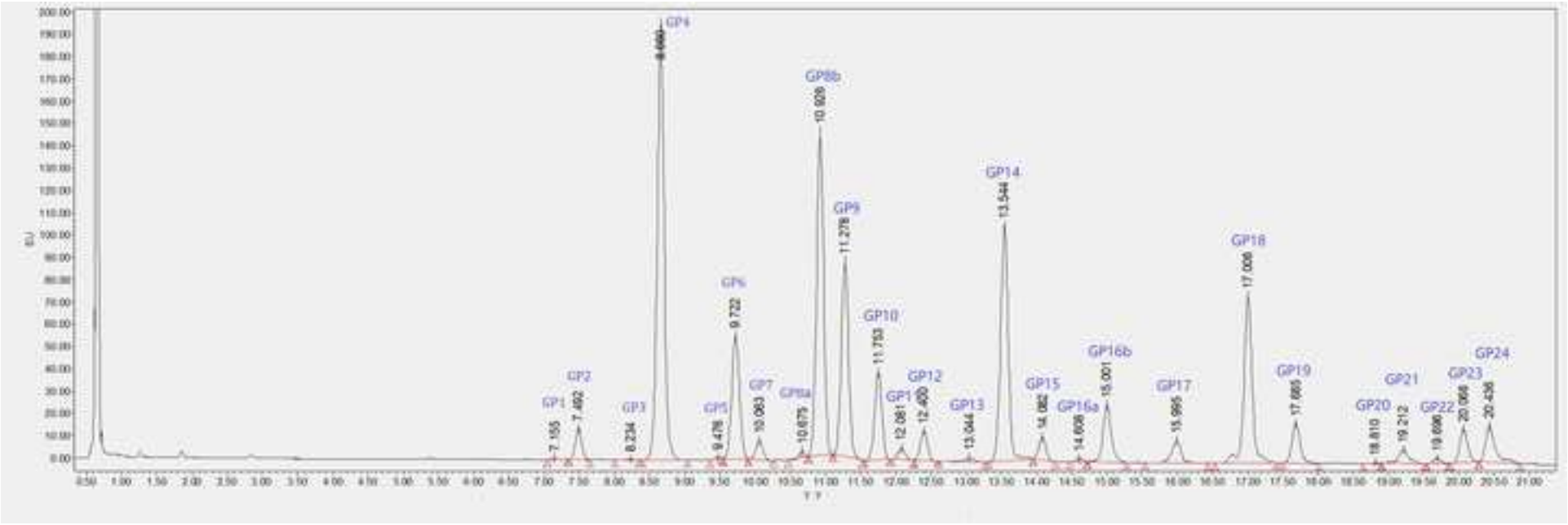
Figure

Click here to access/download;Figure;Figure 1.jpg



Figure

Click here to access/download;Figure;Figure 2.jpg

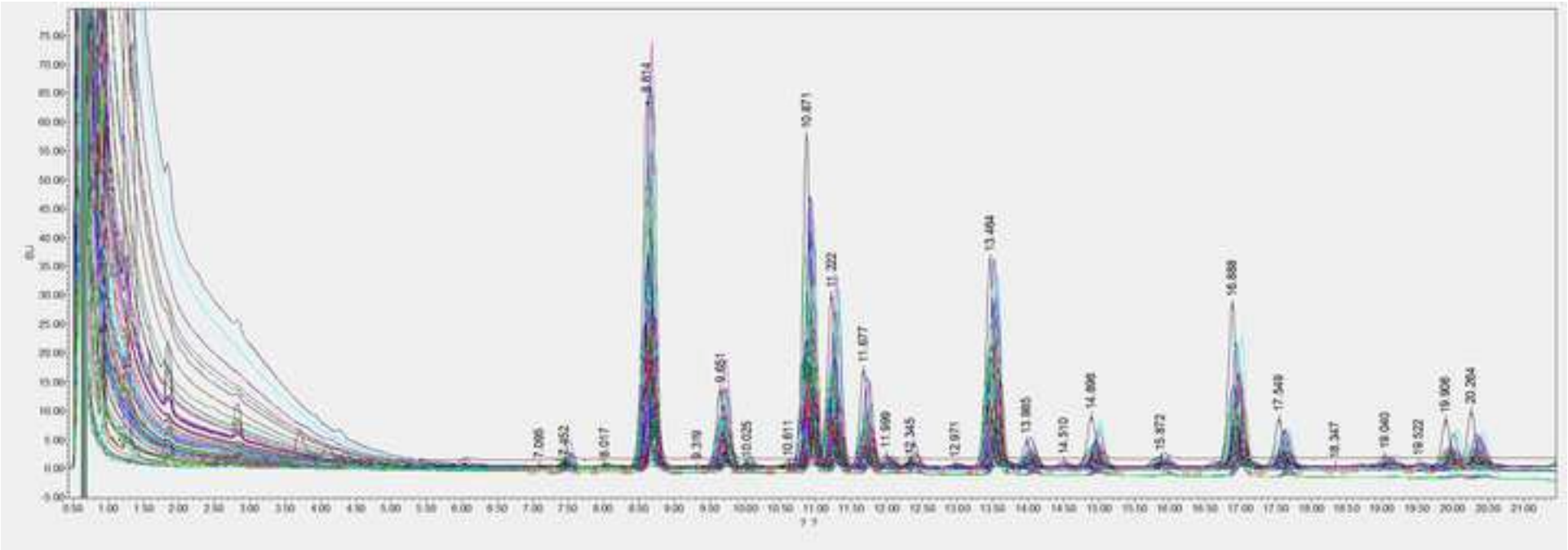
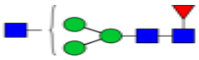
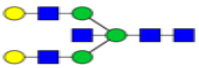
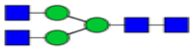
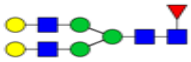
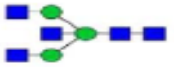
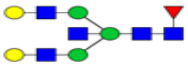
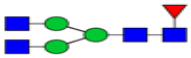
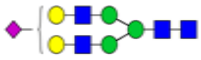
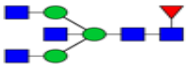
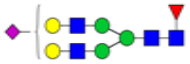
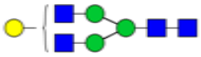
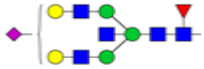
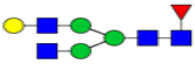
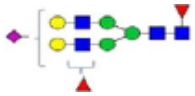
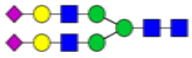
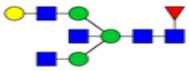
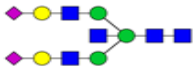
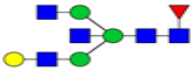
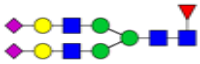
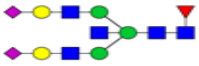


Table 1. The structures of the initial IgG glycans

Glycan peak	Peak composition	Abbreviation	Glycan peak	Peak composition
GP1		FA1	GP13	
GP2		A2	GP14	
GP3		A2B	GP15	
GP4		FA2	GP16	
GP5		M5	GP17	
GP6		FA2B	GP18	
GP7		A2G1	GP19	
GP8		A2BG1	GP20	
GP9		FA2G1	GP21	
GP10		FA2BG1	GP22	
GP11		FA2BG1	GP23	
GP12		A2G2	GP24	

GP: glycan peak; F: fucose; A: number of antenna's attached to the core sequence (existi
NAcetylglucosamine (GlcNAc) and three mannose residues); B: bisecting GlcNac; G: galactos
Structural schemes are defined as follows: blue square: GlcNac; green circle: mannose; red trian
antennary fucose; yellow circle: galactose; purple rhomb: sialic acid.

Abbreviation
A2BG2
FA2G2
FA2BG2
FA2G1S1
A2G2S1
FA2G2S1
FA2BG2S1
FA2FG2S1
A2G2S2
A2BG2S2
FA2G2S2
FA2BG2S2

ng of two
e; S: sialic acid.
gle: core fucose/

Table 2. The precision of the method

Glycan peak	Mean (SD)	CV (%)	Glycan peak	Mean (SD)	CV (%)
GP1	0.23 (0.017)	7.28	GP13	0.50 (0.043)	8.64
GP2	0.24 (0.034)	13.97	GP14	19.54 (0.36)	1.84
GP3	0.96 (0.16)	16.73	GP15	1.16 (0.14)	11.63
GP4	19.52 (0.58)	2.98	GP16	2.79 (0.19)	6.93
GP5	0.17 (0.024)	13.86	GP17	1.29 (0.13)	9.78
GP6	3.19 (0.26)	8.22	GP18	13.16 (0.51)	3.85
GP7	0.29 (0.032)	11.51	GP19	2.12 (0.21)	9.76
GP8	16.43 (0.62)	3.77	GP20	0.12 (0.018)	14.91
GP9	8.97 (0.52)	5.74	GP21	1.05 (0.17)	16.09
GP10	2.97 (0.22)	7.34	GP22	0.18 (0.025)	14.16
GP11	0.30 (0.041)	13.82	GP23	2.14 (0.14)	6.45
GP12	1.27 (0.026)	2.08	GP24	1.43 (0.13)	9.12

CV: Coefficient of Variation; GP: Glycan peak; SD: Standard deviation.

Table 3 The calculation of derived glycans

Derived	Formulas	Derived	Formulas
Galactosylation		**Fucosylation**	
G2/G0	GP12/GP2	F1/F0	GP4/GP2
	GP14/GP4		GP6/GP3
	GP15/GP6		(GP8+ GP9)/GP7
G2/G1	GP12/GP7		GP14/FGP12
	GP14/(GP8+ GP9)		GP15/GP13
	GP15/(GP10+ GP11)		GP18/GP17
G1/G0	GP7/GP2		GP23/GP21
	(GP8+ GP9)/GP4		GP24/GP22
	(GP10+ GP11)/GP6		
Sialylation		**Bisecting GlcNAc**	
S2/S0	GP21/GP12	B1/B0	GP3/GP2
	GP22/GP13		GP6/GP4
	GP23/GP14		(GP10+GP11)/ (GP8+ GP9)
	GP24/GP15		GP13/GP12
S2/S1	GP21/GP17		GP15/GP14
	GP23/GP18		GP19/GP18
	GP24/GP19		GP22/GP21
S1/S0	GP17/GP12		GP24/GP23
	GP18/GP13		
	GP16/GP14		
	GP19/GP15		

GP: glycan peak; F: fucose; B: bisecting GlcNac; G: galactose; S: sialic acid.

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2-aminobenzamide, 2-AB	Sigma, China		
96-well collection plate	AXYGEN		
96-well filter plate	Pol	0.45 um GHP	
96-well monolithic plate	BIA Separations		
96-well plate rotor	Eppendorf Co., Ltd, Germany	T_1087461900	
Acetic acid	Sigma, China		
Acetonitrile	Huihai Keyi Technology Co., Ltd, China		
Ammonium bicarbonate	Shenggong Biological Engineering Co., Ltd, China		
Ammonium formate	Beijing Minruida Technology Co., Ltd.		
Constant shaking incubator/rocker	Zhicheng analytical instrument manufacturing co., Ltd, China	ZWY-10313	
Dextran Calibration Ladder/Glycopeptide	Watts technology Co., Ltd, China	BEH column	
Dimethyl sulfoxide (DMSO)	Sigma, China		
Disodium phosphate	Shenggong Biological Engineering Co., Ltd, China		
Electric ovens	Tester instruments Co., Ltd	202-2AB	
Empower 3.0	Waters technology Co., Ltd, America		
Ethanol	Huihai Keyi Technology Co., Ltd, China		
Formic acid	Sigma, China		
GlycoProfile 2-AB Labeling kit	Sigma, China		
HCl	Junrui Biotechnology Co., Ltd, China		

High-speed centrifuge	Eppendorf Co., Ltd, Germany	5430
Igepal	Sigma, China	
Low temperature centrifuge	Eppendorf Co., Ltd, Germany	
Low temperature refrigerator	Qingdao Haier Co., Ltd	
Manifold 96-well plate	Watts technology Co., Ltd, China	186001831
Methanol	Huihai Keyi Technology Co., Ltd, China	
Milli-Q pure water meter	Millipore Co., Ltd, America	Advantage A10
NaOH	Shenggong Biological	
PH tester	Sartorius Co., Ltd, Germany	PB-10
Phosphate buffered saline, PBS	Shenggong Biological	
Pipette	Eppendorf Co., Ltd, Germany	4672100, 0.5-10μl & 10-100μl & 20-200μl & 1000μl
PNGase F enzyme	Sigma, China	
Potassium dihydrogen phosphate	Shenggong Biological	
Propan-2-ol	Huihai Keyi Technology Co.,	
SDS	Sigma, China	
Sodium chloride	Shenggong Biological	
Sodium cyanoborohydride (NaBH3CN)	Sigma, China	
Spectrophotometer	Shanghai Yuanxi instrument Co., Ltd	B-500
Transfer liquid gun	Smer Fell Science and Technology	4672100
Tris	Amresco, America	
Ultra-low temperature refrigerator	Thermo Co., Ltd, America	MLT-1386-3-V; MDF-382E

Ultra-performance liquid chromatography	Watts technology Co., Ltd, China	Acquity MLtraPerformance LC
Vacuum Pump	Watts technology Co., Ltd, China	725000604
Volatilizing machine/Dryer	Eppendorf Co., Ltd, Germany	T_1087461900
Vortex	Changzhou Enpei instrument Co.,	NP-30S
Water-bath	Tester instruments Co., Ltd	DK-98-IIA
Weighing balance	Shanghai Jingke Scientific Instrument Co., Ltd.	MP200B

JoVE

Manuscript ID: JoVE60104

Ref: Title: IgG *N*-glycans analysis by ultra-performance liquid chromatography

Di Liu[1*], Xizhu Xu[2*], Yuejin Li[2], Jie Zhang[1], Xiaoyu Zhang[1], Qihuan Li[1], Haifeng Hou[2], Dong Li[2], Wei Wang [1,2,3], Youxin Wang[1].

Dear editors,

Based on your insightful and helpful comment, we have revised the manuscript accordingly. Our specific responses to the comments are listed below.

Thank you again for your encouragement and consideration. We hope that this revised manuscript will be acceptable for publication in ***JoVE***.

Sincerely yours,

Youxin Wang, PhD
Professor, School of Public Health
Capital Medical University, Beijing100069, P.R. China
Email: sdwangyouxin@163.com/wangy@ccmu.edu.cn

Editorial comments:

1. Numerous grammatical errors especially in terms of verb usage and tensing are present. After you may the requested edits, **please employ a professional language editing service.**

Answer:

We are so sorry that the previous manuscript performed numerous grammatical errors especially in terms of verb usage and tensing. We have we have sought the help of native English speaker for language and grammatical corrections and tried our best to revise the manuscript to make it clear and easy to understand.

2. I have highlighted 2.75 pages for filming.

Answer:

Thanks for your revision. We have confirmed that.

3. melt the frozen plasma sample… use "thaw" by "melt"?

Answer:

Yes, what we mean is to thaw the frozen plasma sample. As you advised, we have changed "melt" to "thaw".

4. Were the plates purchased with the protein G coating? If not, how were they prepared? What is the protein G concentration?

Answer:

We are sorry that we did not elaborate on this issue. The protein G monolithic plates are purchased with the protein G coating.

5. "Prepare the samples: thaw the frozen plasma sample and centrifuge at 80 × g for 10 minutes and put the Protein G monolithic plate and the buffer for 30 minutes at room temperature" … Which buffer?

Answer:

We are sorry that we did not elaborate on this issue. The buffer should be replaced by the above solvent. We have changed it (lines 85-86).

6. Draw and load into the cuvette?

Answer:

We are sorry that we did not elaborate on this issue. What we mean is draw 2 ul ultra-pure water and load it into the screen. We have changed it.

7. "Dry the extracted IgG by under vacuum and preserve the extracted IgG (300 ul extracted IgG for 4 hours)." I changed the previously unclear phrasing. Please verify if this is correct.

Answer:

Thanks for your suggestion. Actually, we put the extracted IgG to dry by oven at 60°C. We have changed it.

8. “Add 30 ul 1.33% SDS and transfer the sample into a 65°C oven for 10 minutes. Then take it out of the oven and let it rest for 15 minutes” I changed the word "transfer" to "add", please check if correct. Is the SDS mixed properly with the sample? How? Do you vortex? Is the SDS removed after this?

Answer:

Yes. We have confirmed this correction is right. Thanks for your suggestion. The above solutions are mixed by vortex. In addition, we have added the vortex in our material table. Actually, the SDS wasn’t removed after this. We have changed it (lines 172-173).

9. “Add 10 ul 4% Igepal and put it on the rocker for 5 minutes” Correct?

Answer:

Yes. We have confirmed this correction is right. Actually, the rocker you mentioned is shaking incubator.

10. “Add 20 ul 5×PBS and add 30-35 ul 0.1mol/L NaOH to regulate Ph=8.0. Transfer 4 ul PNGase” I changed the word "transfer" to "add", please check if correct. Mix by vortex? Use Add by transfer?

Answer:

Yes. We have confirmed this correction is right. The above solutions are mixed by vortex. We have changed “transfer” to “add”, too (lines 179-181).

11. “Drying the released glycans for 2.5-3.0 hours” Dry how? Under a fume hood? In an oven? At what temperature? How are the released glycans separates from the rest of the material? Please be more clear with your descriptions. Several steps are vague or imprecise.

Answer:

Thanks for your suggestion. Actually, we put the released glycans to dry by oven at 60°C. We have changed it. The released glycans are separated by the PNGase F enzyme. We have changed the steps to make them clear.

12. Unclear why you say this “currently available”?

Answer:

We are sorry that we did not elaborate on this issue. We have deleted this.

13. “Labeling glycans by 35 ul 2-AB labeling reagent, transfer it on the oscillator for 5 minutes, then transfer it in the oven for 3 hours at 65°C and transfer it for 30 minutes at room temperature;” Do you quantify this “glycans”? If so, how? What is “it”?

Answer:

We are sorry that we did not elaborate on this issue. In fact, the process of UPLC is to quantify the glycans. What we mean is the labelled glycans. We have changed it (lines 196-198).

14. “Add 700 ul 100% acetonitrile and transfer it on the shaking incubator for 5

minutes;" Add to the filter plate or the glycan with 2-AB? Unclear. What is it?

Answer:

We are sorry that we did not elaborate on this issue. What we mean is adding 700 ul 100% acetonitrile to the 2-AB labeling glycans and transferring them on the shaking incubator. We have changed it (lines 207-208).

15. "Transfer the sample to 0.2 um GHP filter plate for 2 minutes and remove waste by vacuum pump;" Waste is the residue in the filter or the flow through?

Answer:

We are sorry that we did not elaborate on this issue. What we mean is the flowing liquid.

16. "Wash 2-AB labeled glycan with 200 ul 96% acetonitrile (4℃) and remove waste by vacuum pump for 5-6 times;" Waste is the residue in the filter or the flow through?

Answer:

We are sorry that we did not elaborate on this issue. What we mean is the flowing liquid.

17. "Drying the 2-AB labeled glycan for 3.5 hours." Dry how? Under a fume hood? In an oven? At what temperature?

Answer:

Thanks for your suggestion. Actually, we put the 2-AB labeled glycans to dry by oven at 60°C. We have changed it (line 220).

18. Remove this from "Empower 3.0" the text and add it to the table of materials.

Answer:

Thanks for your suggestion. We have removed it from the text and added it to the table of materials.

19. "Dissolve the labeled *N*-glycans with 25 ul mixture of 100% acetonitrile and ultra-pure water by 2:1 (v/v) and take 10 ul the labeled *N*-glycans into UPLC instruments;" Do you vortex? Load?

Answer:

Thanks for your suggestion. We dissolved the labeled *N*-glycans with 25 ul mixture of 100% acetonitrile and ultra-pure water by 2:1 (v/v) then centrifuge them at 134 × g for 5 minutes (4℃). We have changed "take" to "load".

20. "Separate the labeled *N*-glycans at flow rate of 0.4 ml/min with linear gradient of 75-62% acetonitrile in a 25 minutes analytical run by dextran calibration ladder/Glycopeptide column on a waters acquity UPLC at 60℃ (The samples were kept at 4 ℃ prior to injection);" Language needs clarification.

Answer:

Thanks for your suggestion. We have revised this part (lines 242-244).

21. “Detect *N*-glycans by FLR fluorescence detector set with excitation and emission wave lengths of 330 and 420 nm respectively;” Remove the product name, add it to the table of materials.

Answer:

Thanks for your suggestion. We have removed it from the text and added it to the table of materials.

22. Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.

Answer:

Thanks for your suggestion. We have revised this part (lines 279-295).

23. Add legends for these.

Answer:

Thanks for your suggestion. We have added the information.

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Author(s): Di Liu, Xizhu Xu, Jie Zhang, Xiaoyu Zhang, Qihuan Li, Haifeng Hou, Dan Li, Wei Wang, Youxin Wang

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