Journal of Biological Methods | **2015** | Vol. 2(2) | e19
DOI: 10.14440/jbm.2015.52

PROTOCOL

Isolation and quantification of N-glycans from immunoglobulin G antibodies for quantitative glycosylation analysis

Venkata S. Tayi*, Michael Butler

Department of Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada

*Corresponding author: Venkata S. Tayi, Email: venkata.tayi@gmail.com

Competing interests: The authors have declared that no competing interests exist.

Abbreviations used: 2-AB, 2-Aminobenzamide; ADCC, Antibody-dependent cell-mediated cytotoxicity; CDC, Complement-dependent cytotoxicity; CE, Capillary electrophoresis; HILIC, Hydrophilic interaction liquid chromatography; HPLC, High performance liquid chromatography; IgG, Immunoglobulin G; mAb, monoclonal antibody; MS, Mass spectrometry; PNGase F, Peptide-N-glycosidase F; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Received March 16, 2015; Revision received April 30, 2015; Accepted May 25, 2015; Published June 22, 2015

Abstract N-glycosylation is one of the critical quality attributes for the therapeutic monoclonal antibodies. Characterization of N-glycans of monoclonal antibodies provides valuable information about its therapeutic efficacy. We present a non-invasive method of isolating N-glycans from Immunoglobulin G based antibodies for glycosylation analysis. The method consists of purification of antibodies from biological solution and release of N-glycans with peptide-N-glycosidase F in a single consolidated process using a mini affinity ligand column (e.g. protein-A column). The method is highly reproducible with average coefficient of variation of 0.012 in the glycoform percentage distributions between the replicates. The method provides quantification of the molar yield of glycans as a function of molar concentration of antibody in a single analysis. To our knowledge, this is the first time this approach was used to detect and quantify any macro-heterogeneity of N-glycosylation in monoclonal antibody samples. This fairly rapid and very cost-efficient method would be of great interest for academic labs and biopharmaceutical industries.

Keywords: 2-aminobenzamide (2-AB), HILIC-HPLC, monoclonal antibody (mAb), N-glycans, PNGase F, protein-A

BACKGROUND

Monoclonal antibodies (mAbs) represent the major fraction of approved bio-therapeutics for the treatment of cancer, autoimmune disorders and inflammatory diseases [1]. N-linked glycosylation of immunoglobulin G (IgG) antibodies is an important biomarker that defines the therapeutic efficacy of mAbs or immune state of the human body. The structure and the terminal sugar type of the N-glycans play a critical role in modulating the pharmacokinetic properties and effector functions of antibodies. Appropriate glycosylation at a conserved site in the C_H2 domain of Fc region is essential for effector functions, including antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). For example, the absence of core fucose was found to enhance ADCC activity of mAb dramatically [2]. The presence of galactose as terminal sugar in N-glycans of IgGs enhances their CDC activity [3,4], binding affinity to FcγRIIIa receptors [5] and ADCC activity [6]. On the other hand, antibodies with terminal sialic acid in their N-glycans have reduced ADCC activity [7] and become anti-inflammatory [8]. In addition, mAbs with the presence of a particular sugar type or linkage (e.g. N-glycolylneuraminic acid, galactose-α(1,3)-galactose epitope) in their glycans can trigger immunogenic responses in humans [9,10]. Deglycosylated [11] or hemi-glycosylated [12] mAbs exert no effector functions, less thermally stable and prone to aggregation.

N-glycan distribution (micro-heterogeneity) in the mAbs is influenced by various bioprocess parameters such as the choice of production cell line, operating culture conditions and availability of nutrients [13]. Either the depletion of glucose [14] or the replacement of glucose with alternate carbon sources [15] could influence the site occupancy (macro-heterogeneity) of glycosylation in mAb. Characterization of both micro- and macro-heterogeneity of N-glycans of mAbs is very important to understand their therapeutic efficacy and to optimize bioprocess parameters to produce effective mAbs. Glycosylation characterization of antibodies can be achieved either by mass spectrometry analysis of peptides bearing N-glycans or by the analysis of released glycans [16]. The later method requires isolating N-glycans from glycoproteins using PNGase F digestion [17], fluorescent labelling of glycans and analysis by high performance liquid chromatography (HPLC) or capillary electrophoresis (CE). Hydrophilic interaction liquid chromatography (HILIC) based HPLC separation of labeled glycans is the most prominent method of analysing free glycans [18]. Some advantages include separation of glycans with the same mass but with different structures (e.g. isomers with galactose on either [3] or [6] mannose arm of bi-antennary structure, α(2-3) or α(2-6) linkage of sialic acid). The development of ultra-performance liquid chromatography (UPLC) systems and efficient columns made this method very reliable in separation of glycans with high resolution.

The traditional way of isolating N-glycans from mAb is by PNGase

F digestion of either the heavy chain bands following SDS-PAGE gel separation of reduced mAb samples [19] or by direct in-solution reduction and digestion of purified mAb. The immobilization of either the enzyme or the mAb on to a solid support provides the advantage of increased efficiency of digestion and easy handling of reagents. Based on this approach, high throughput robotic platform methods have been developed using PNGase F immobilized micro fluidic chips [20,21] or immobilizing mAb on to multi-well plates [22,23]. However, the affordability of high-throughput robotic platform based analysis of N-glycans in academic laboratories and small scale biopharmaceutical industries is limited. In addition, quantifying the glycan concentration in combination with the mAb concentration for the analysis of both micro- and macro-heterogeneity of glycosylation has not been addressed previously.

We present a relatively rapid, highly reproducible and cost-efficient method of isolating N-glycans of mAbs directly from cell culture supernatant using readily available mini affinity chromatography (e.g. Protein-A) columns (**Fig. 1**). The method involves purification of mAbs and PNGase F digestion of their N-glycans in a single consolidated process. The optimization and efficiency of the method was demonstrated by 2-aminobenzamide (2-AB) labelling and HILIC-based HPLC analysis of released N-glycans. This high yield and non-invasive method provides complete recovery of N-glycans thus allowing quantification of both antibody and glycan concentrations in a single analysis. We have devised a strategy to detect and quantify any macro-heterogeneity present in mAb product and demonstrated using the mAb samples produced under glucose-starvation conditions.

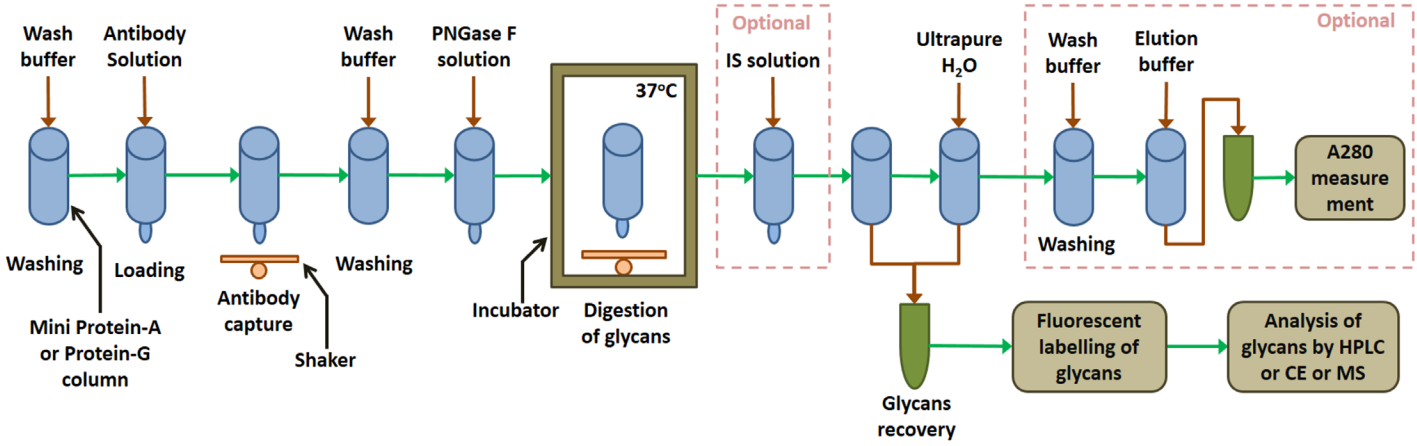


Figure 1. Schematic representation of the steps involved in the process of isolation and characterization of N-glycans from antibodies. The steps in the dashed-line boxes are optional and only needed to quantity the molar yield of glycans from antibodies. IS: internal standard; HPLC, high performance liquid chromatography; CE, capillary electrophoresis; MS, mass spectrometry, A280, absorbance at 280 nm.

MATERIALS

Reagents

- ✓ TRIS (Fisher Scientific, Cat. # BP152)
- ✓ Sodium phosphate monobasic (NaH_2PO_4) (Fisher Scientific, Cat. # BP329)
- ✓ Sodium phosphate dibasic (Na_2HPO_4) (Fisher Scientific, Cat. # BP332)
- ✓ Glycine (Fisher Scientific, Cat. # BP381)
- ✓ Acetonitrile (Fisher Scientific, Cat. # A998)
- ✓ HCl (Fisher Scientific, Cat. # AC124210010)
- ✓ Ammonium hydroxide (NH_4OH) (Fisher Scientific, Cat. # AC255210010)
- ✓ Formic acid (Fisher Scientific, Cat. # AC270480010)
- ✓ Acetic acid (Fisher Scientific, Cat. # AC222140010)
- ✓ Dimethyl sulfoxide (DMSO) (Fisher Scientific, Cat. # AC397600010)
- ✓ 2-Aminobenzamide (2-AB) (Sigma-Aldrich, Cat. # A89804)
- ✓ Sodium cyanoborohydride (NaBH3CN) (Sigma-Aldrich, Cat. # 156159)
- ✓ Maltotetraose (DP4) (Sigma-Aldrich, Cat. # 47877)
- ✓ PNGase F (Promega Corporation, Cat. # V4831)
- ✓ 2-AB-Dextran ladder (Waters Corporation, Cat. # 186006841)
- ✓ Note: Unlabelled dextran standard can be purchased from other sources and 2-AB labeled stock can be prepared using the procedure presented in section 4 of this protocol

Recipes

Phosphate buffer

To make 500 ml phosphate buffer, add 1.14 g Na_2HPO_4 into 400 ml of ultrapure H_2O. Add 0.24 g NaH_2PO_4 into 100 ml of ultrapure H_2O. Mix both solutions. pH should be 7.4 ± 0.05 without adjustment.

2-AB labeling solution

To make 400 µl 2-AB labeling solution, add 20 mg 2-aminobenzamide into 200 µl solvent (150 µl glacial acetic acid + 350 µl Dimethyl sulfoxide). Add 25 mg $NaBH_3CN$ into 200 µl solvent (as above). Combine equal volumes of both solutions. Volume can be adjusted based on the amount of ingredient weighted.

CAUTION: $NaBH_3CN$ should be handled in a fume hood. Less toxic 2-picoline borane with same molar concentration can be used as alternative to $NaBH_3CN$.

Elution buffer (100 mM glycine-HCl)

To make 100 ml Elution buffer, add 0.75 g glycine into 90 ml of ultrapure H_2O. Adjust pH to 2.7 with 1 N HCl and final volume to 100 ml with ultrapure water.

Neutralizing buffer (1 M TRIS-HCl)

To make 100 ml (1 M) neutralizing buffer, add 12.12 g TRIS into 90 ml of ultrapure H_2O. Adjust pH to 9.0 with 1 N HCl and final volume to 100 ml with ultrapure water.

Buffer A (50 mM ammonium formate solution)

To make 2 L buffer A, add 3.9 ml formic acid into 1.9 L ultrapure H_2O. Adjust pH to 4.4 with ammonium hydroxide and final volume to 2 L with ultrapure H_2O.

Equipment

- ✓ Protein-A HP SpinTrap columns (GE Healthcare Life Sciences, Cat. # 28-9031-32)
- ✓ HyperSep Diol SPE cartridges (Thermo Fisher Scientific, Cat. # 60108-571)
- ✓ Nanosep 10K Omega filters (Pall Corporation, Cat. # OD010C33)
- ✓ 2 ml sample tubes (Corning Life Sciences, Cat. # MCT-200-C)
- ✓ 1.5 ml sample tubes (Corning Life Sciences, Cat. # MCT-150-C)
- ✓ 0.5 ml tube rack (Eppendorf, Cat. # 022364243)
- ✓ 1.5 ml tube rack (Eppendorf, Cat. # 022364227)

PROCEDURE

1. Antibody capture and glycan cleavage
   - 1.1. Remove the plug at the bottom of new Protein A column, place it in 2 ml sample tube and centrifuge at lowest possible speed on micro-centrifuge to remove storage solution.
   - 1.2. Wash the column by adding 600 µl of phosphate buffer and centrifuge to remove the buffer.
   - 1.3. Plug the bottom of the column and add antibody solution.
   - 1.4. Incubate at room temperature for 15 min on a rocker or orbital shaker.
   - 1.5. Remove the plug and centrifuge to remove the solution.
   - 1.6. Repeat the steps 1.3–1.5, if necessary, to load desired amount of antibody (50–100 µg).
   - 1.7. Wash the columns 3 times with 600 µl of phosphate buffer.
   - 1.8. Plug the bottom of column and add 150 µl of phosphate buffer and 10–20 units of PNGase F (1–2 µl).
   - 1.9. Incubate the columns at 37°C on a rocker or rotary shaker for 24 h.

   HINTS: (i) Columns can be filled with only 600 µl of solution at a time. Antibody solution can be concentrated with 30K filters to avoid multiple loading steps to reach desired antibody concentration. (ii) 100 pM antibody is sufficient for decent detection of glycans on HPLC. mAb concentration should be at least 100 µg/ml for accurate quantification of eluted antibody using Nanodrop™ spectrophotometer. For the cases of quantifying antibody, capture at least 50 µg and elute with total of 400 µl elution buffer. (iii) More PNGase F can be added to reduce the incubation time. One unit of Promega's PNGase F will catalyze the deglycosylation of 1 nmol of denatured Ribonuclease B in one min at 37°°C. When using PNGase F from other sources, amount should match these units.

2. Glycans collection
   - 2.1. Add 1 or 2 nmol of DP4 standard to the protein-A column containing released glycans and mix gently (optional).
   - 2.2. Remove the plug and place the column immediately in fresh 2 ml tube.
   - 2.3. Centrifuge to collect the filtrate containing glycans.
   - 2.4. Plug the bottom of the column and add 250 µl of ultrapure water, place the cap and rinse the column by gently inverting the column with hand.
   - 2.5. Remove the plug and collect the filtrate into the same tube as in step 2.3.
   - 2.6. Plug the bottom of the column and add 600 µl of phosphate buffer and keep it aside for eluting antibody as described in section 3.
   - 2.7. Filter the glycans through 10K filter and collect the filtrate into 1.5 ml micro-centrifuge tube.
   - 2.8. Dry the glycans in a vacuum concentrator.

   HINT: Wash 10K filter at least once with 500 µl ultrapure water.

3. Elution of antibody (optional)

3.1. Take the column from step 2.6 and wash it three times with phosphate buffer.

3.2. Plug the bottom of column and add 300 µl of elution buffer.

3.3. Gently mix by shaking with hand.

3.4. Collect the filtrate into a 2 ml tube.

3.5. Repeat steps 19 and 20 with 100 µl of elution buffer and collect the filtrate in the same tube as in step 3.4.

3.6. Neutralize the eluted antibody with neutralizing buffer to pH ~7.2.

3.7. Store it to quantify later by absorbance at 280 nm using UV-Vis spectrophotometer.

HINT: Eluted antibody can be stored at 4°C for short-term and -20°C for long term.

NOTE: The isolated glycans can be labeled with 2-aminobenzamide (2-AB) or 2-Aminobenzoic acid (2-AA) for HPLC/UPLC analysis or with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) for capillary electrophoresis analysis. Here we present 2-AB labeling and cost-effective method of cleaning up labeled glycans.

4. 2-AB labeling and clean-up of glycans

4.1. Add 5 µl of 2-AB solution to the dried glycans tube from step 2.8 and mix.

4.2. Incubate at 65°C for 2 h.

4.3. Wash the HyperSep™ Diol cartridge with 1 ml of ultrapure water.

4.4. Condition the cartridge with 4 ml of acetonitrile (1 ml each time).

4.5. Add 40 µl of acetonitrile to the labeled glycans, mix the solution and transfer the glycans on to the top of the cartridge.

4.6. Let it sit for 15 min to allow the glycans to bind.

4.7. Wash the cartridge with 6 ml of acetonitrile (1 ml at each time).

4.8. Elute the glycans with 3 × 400 µl of ultrapure water and collect them into 1.5 ml micro-centrifuge tube.

4.9. Dry the labeled glycans in a vacuum concentrator.

HINTS: (i) Dried glycans can be stored at -20°C. (ii) Glycan clean-up can be performed by placing HyperSep™ Diol cartridges on Eppendorf rack and applying positive pressure of air so that liquid flow rate is ≤ 2 ml/min. Glycans during elution can be collected in sample tubes placed on another Eppendorf rack stacked underneath. Alternatively, Vacuum Manifold designed for cartridges could be purchased.

NOTE: The 2-AB labeled glycans can be analyzed using HPLC or UPLC system. The guidelines provided below are for the Water HPLC system equipped with 1525 µ Binary Pump, 2707 auto-sampler and 2475 fluorescent detector. The column heater housing Waters X-Bridge BEH amide column (3.5 µm, 4.6 × 250 mm, Part # 186004870) was set at 30°C. The data acquisition and analysis was done using Empower 2.0 software. The flow rate and gradient for mobile phase A (Buffer A) and mobile phase B (Acetonitrile) are presented in **Table 1**. The settings for fluorescent detector are presented in **Table 2**.

5. Analysis of glycans by HILIC-HPLC

5.1. Add 20 µl of ultrapure water to each glycan sample from step 4.9 and mix well.

5.2. Prepare samples with HPLC vials by mixing 6 µl of sample with 24 µl of acetonitrile in HPLC auto-sampler tubes.

5.3. Prepare 30 µl of dextran ladder standard in HPLC vial with 80% of acetonitrile.

5.4. Place the vials and run the HPLC with the above mentioned settings.

5.5. Integrate the dextran ladder standard chromatogram with empower software and obtain retention times for at least 12 glucose unit (GU) polymers.

5.6. Fit the GU value as a function of retention time (t) of dextran standard data with a polynomial equation: $GU = a \times t + b \times t^2 + c \times t^3$.

5.7. Integrate the chromatograms of samples and record the retention times and peak areas for each sample as outlined in **Table 3**.

5.8. Calculate the GU values for each peak and assign structure based on reference GU values from Glycobase database (https://glycobase.nibrt.ie)

5.9. If needed, perform exo-glycosidase digestion of the labeled glycan sample and rerun on HPLC to confirm the structures assigned.

5.10. If internal standard is used, record the area of internal standard and sum of the areas of all N-glycans of a sample as outlined in **Table 4**

5.11. Perform the calculations for the samples as outlined in **Table 4** to quantify the molar yield of glycan units per molar quantity of antibody sample.

ANTICIPATED RESULTS

Kinetics of glycan release

The kinetics of PNGase F digestion of N-glycans from an antibody molecule provides valuable information for optimization of time and enzyme concentration. The PNGase F enzyme mediated kinetics of digestion of N-glycans from two monoclonal antibodies of different sizes and structure, EG2-hFc or aIL8-hFc, were investigated. EG2-hFc is a chimeric heavy chain only monoclonal antibody of 80 KDa [24], whereas aIL8-hFc is an IgG-1 type humanized monoclonal antibody of 146 KDa [25]. The details on antibodies and their production are provided in Supplementary Materials and Methods. Similar molar quantities (0.8 nmol) of both antibodies were used. The rate of digestion of the N-glycans by PNGaseF from EG2-hFc and aIL8-hFc antibodies immobilized to protein-A are presented in **Figure 2A** and **2B**, respectively. The antibody samples eluted from protein-A columns after PNGase F digestion were run in SDS-PAGE to visualize the extent of deglycosylation as a function of time and PNGase F concentration. The corresponding SDS-PAGE images of EG2-hFc and aIL8-hFc are presented in **Figure 2C** and **2D**, respectively. The molecular weight of N-glycan unit is typically range from 1.7 to 2.6 KDa depending on the extent of galactosylation and sialylation. At a PNGase F concentration of 50 units/ml, the rate of digestion of N-glycans from EG2-hFc is at least 4-fold higher than of aIL8-hFc. Though both antibodies share similar Fc fragments from human IgG-1, the presence of light chains in aIL8-hFc might makes it difficult for PNGase F to access and cleave the N-glycans from this antibody. A 5-fold increase in PNGase F concentration enhanced the rate of digestion of N-glycans 2-fold in the case of aIL8-hFc. The majority of mAbs and human antibodies are of similar size as aIL8-hFc and would be expected to show similar kinetics. Though higher enzyme concentration should rapidly release the glycans and reduce the isolation time, the PNGase F concentration and incubation time should be chosen based on the antibody type and the balanced choice between time and cost. Higher incubation temperature may also be used to enhance the rate of isolation of N-glycans by PNGase F.

Glycan recovery efficiency and reproducibility

The efficiency of recovering N-glycans and reproducibility of the glycoform distributions by this method was investigated. The glycans were extracted from 0.8 nmol of EG2-hFc and 0.4 nmol of aIL8-hFc. In both cases, 67 units/ml of PNGase F was used and incubated at 37°C for 24 h. The profiles of N-glycans extracted from EG2-hFc and aIL8-hFc are presented in **Figure 3A** and **3B**, respectively. The total yield of glycans recovered is presented as an inset bar graph within each figure as the average molar units of N-glycans per mole of antibody. Each antibody molecule consists of 2 units of N-glycans and hence the total yield has a maximum theoretical value of 2. Each N-glycan unit is labeled with one 2-AB molecule at the reducing end of GlcNAc released from N-glycosylation site. Hence, the number of N-glycans is proportional to the total area of labeled N-glycan units. The calculated yields of glycans from EG2-hFc and aIl8-hFc were 97% and 94%, respectively. These results along with SDS-PAGE analysis (**Fig. 2A-2D**) demonstrate that complete recovery of N-glycans is obtained with the current method without the need of reducing the mAb. EG2-hFc and aIl8-hFc have two distinct glycosylation profiles. EG2-hFc has a glycosylation profile with highly galactosylated and sialylated N-glycans. The reproducibility of the glycan distribution was also very high with average coefficient of variation of 0.012 in the glycoform percentage distributions between the replicates.

Mini protein-G columns can also be used as an alternative to protein-A columns (**Table S1**). The method was also tested to isolated N-glycans from polyclonal IgGs in human serum (**Table S2**).

Table 1. Flow rate and gradient for 1525 µ binary pump.

Time (min)	Flow (ml/min)	Buffer A (%)	Buffer B (%)
0	0.86	20	80
48	0.86	50	50
49	0.86	100	0
54	0.86	100	0
56	0.86	20	80
63	0.86	20	80
64*	0	20	80

* Run time is for final sample only to stop the pumps. Actual run time for each sample is 63 min.

Table 2. Settings for 2475 Fluorescent detector.

Parameter	Set value
Mode	2D
Excitation	330 nm
Emission	420 nm
EUFS	10000
Gain	1
Time constant	1.5

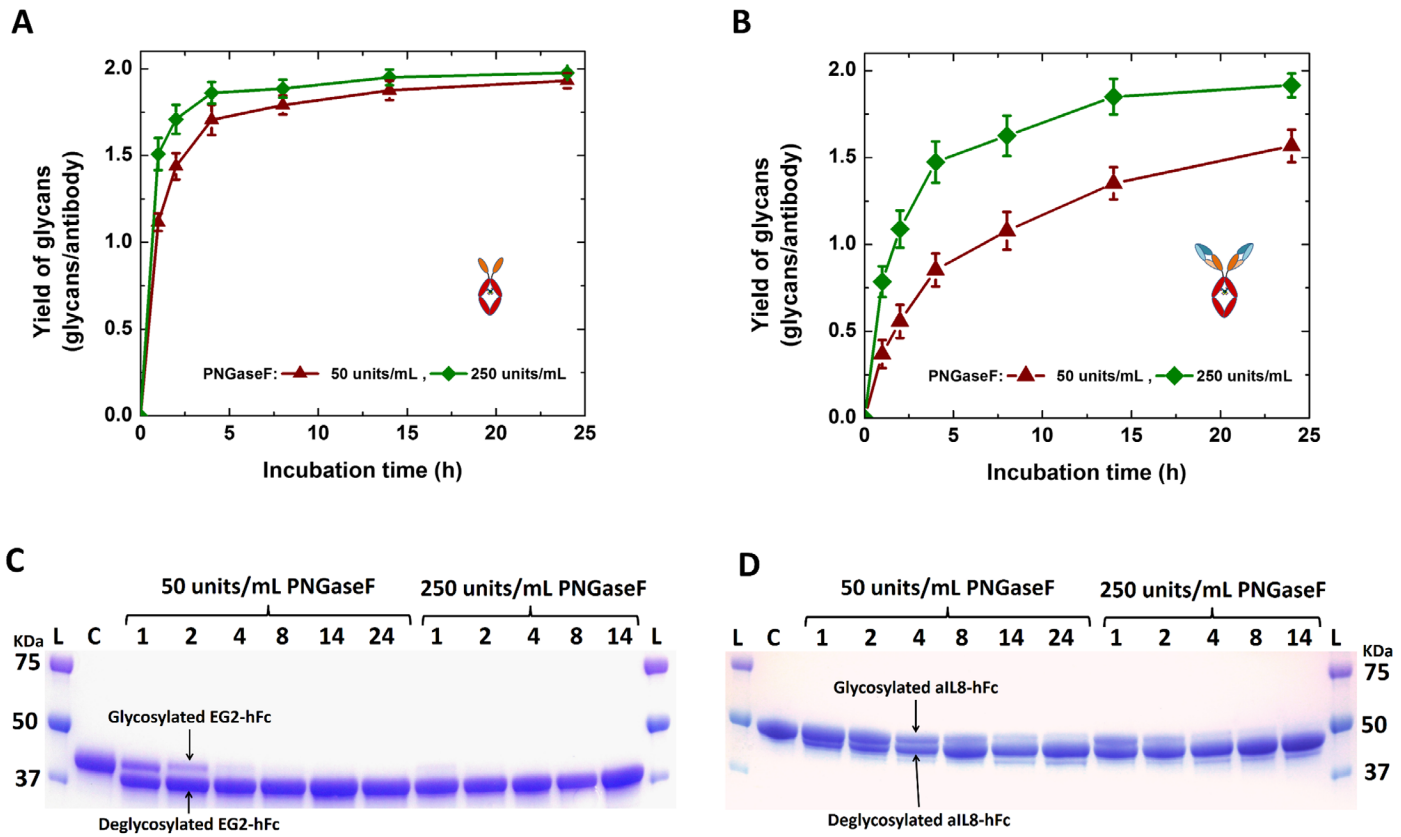
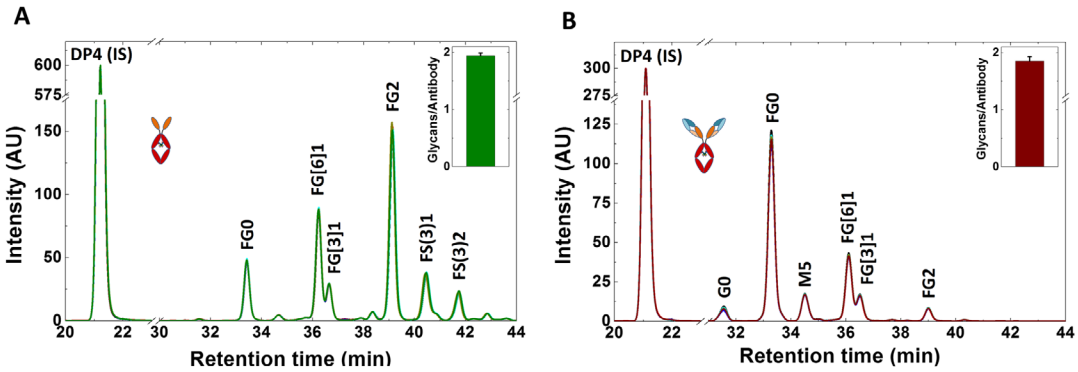


Figure 2. Kinetics of PNGase F digestion of N-glycans from monoclonal antibodies immobilized onto protein-A. A and **B.** Quantities of glycans released by the PNGase F digestion of immobilized EG2-hFc (A) and aIL8-hFc monoclonal antibodies (B), as a function of digestion time with two different PNGase F concentrations. **C** and **D.** SDS-PAGE analysis under reducing condition of corresponding EG2-hFc (C) and aIL8-hFc (D) samples eluted from Protein-A column after digestion with PNGase F for indicated hours (top line). L, Protein ladder, C, Control sample (t = 0).



Glycan	G0	FG0	M5	FG[6]1	FG[3]1	FG2	FS(3)1	FS(3)2
Structure								
EG2-hFc (A)	N.D.	11.6±0.1	N.D.	21.3±0.2	6.83±0.07	36.9±0.2	11.7±0.1	6.14±0.14
aIL8-hFc (B)	4.26±0.16	53.7±0.3	8.19±0.08	19.6±0.2	7.47±0.05	3.97±0.04	N.D.	N.D.

N-Acetylglucosamine ● Mannose ● Galactose ▲ Fucose ◆ N-Acetylneuraminic acid N.D.: Not determined (<1%)

Figure 3. N-glycan profiles of EG2-hFc (A) and aIL8-hFc (B) obtained by Protein-A method. The glycans were labeled with 2-AB and analysed by HILIC-HPLC method. Each figure consists chromatograms of 6 replicates from three independent analyses, normalized with the average area of internal standard. The mole to mole ratio of recovered glycans is shown in the inset of each panel. The table represents corresponding percentage glycan distribution in each antibody. The error represents S.D. of the values from six replicates.

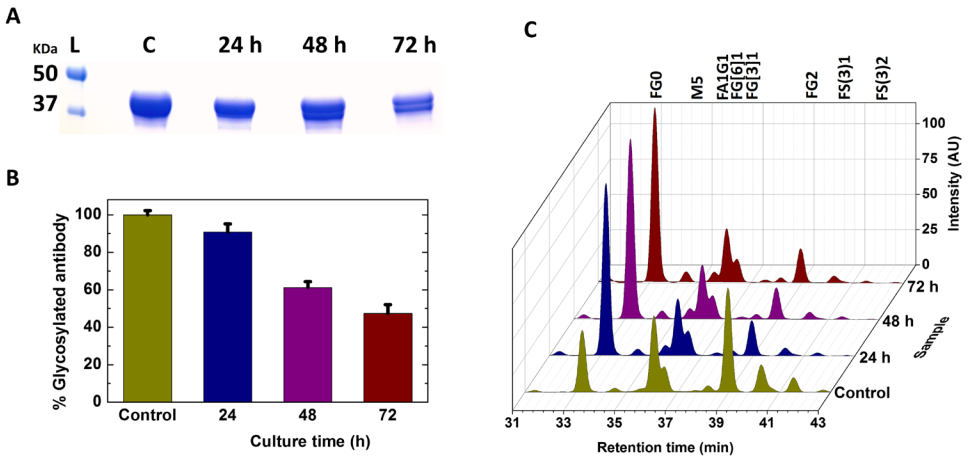


Figure 4. Application of the current glycan analysis method to detect and quantify macro-heterogeneity of N-glycosylation in monoclonal antibodies. A. SDS-PAGE analysis of the purified mAb samples harvested at 24, 48 and 72 h of culture after transferring into 5 mM glucose medium. **B.** Quantification of macro-heterogeneity in the corresponding samples using the current method. **C.** N-glycan distribution profiles of the corresponding samples. The data represents the average of two culture samples. L: Protein ladder, C: Control sample.

Table 3. Calculations and structure assignment of N-glycans of a given sample (EXAMPLE).

Peak No	Retention time (min) t	Peak Area	% area of the peak	GU Value [a × t3 + b × t^2 + c × t]	Structure

Table 4. Calculations for yield of glycans from captured mAb (EXAMPLE).

Sample No	Area of IS p	Total area of N-glycans q	Amount of IS added (nmol) r	Quantity of N-glycans (nmol) Gly = q*r/p	mAb captured (µg) x	Mol. Wt. of mAb (KDa) y	mAb captured (nmol) Ab = x/y	Glycans yield (glycans/mAb) = Gly/Ab

Quantifying macro-heterogeneity

We have tested the ability of the current method to quantify glycosylation macro-heterogeneity of mAb samples produced by CHO-EG2 cells in limited glucose media. The methods (Supplementary Materials and Methods), cell culture performance and the antibody production profiles are provided in **Figure S2**. **Figure 4A** represents SDS-PAGE analysis of reduced EG2-hFc samples along with a control antibody sample produced under nutrient-rich onditions (exponential phase). It can be visualized that starting from 24 h there was an increased production of non-glycosylated mAb with time. The fraction of non-glycosylated mAb in the product was quantified with the current method (**Fig. 4B**). From this analysis, we can observe that only 9% of non-glycosylated antibody was produced in the first 24 h whereas 30% and 14% of non-glycosylated mAb was produced on day 2 and 3, respectively. The corresponding N-glycan profiles of these samples are shown in the **Figure 4C**. These profiles along with macro-heterogeneity levels (**Fig. 4B**) indicate that majority of glycosylated mAb was produced until the glucose is exhausted (within 24 h) with reduced galactosylation (compared to control) and later most of the mAb produced is non-glycosylated. Hence, this method would be useful to obtain quantitative information on the extent of both micro- and macro-heterogeneity of glycosylation together along with the kinetics of antibody production under nutrient-limited culture conditions.

TROUBLESHOOTING

1. Samples contain only internal standard peak with no trace of glycans.
   - 1.1 No antibody captured: Check pH of mAb solution.
   - 1.2 Antibody captured: Check PNGase F activity or verify the reaction buffer pH.
   - 1.3 Antibody with uncommon Fc sequence or N-glycan structures: Verify with a glycan analysis method involving reduction of antibody (in-solution or gel-based method)
2. Neither internal standard nor glycans peaks were detected.
   - 2.1 Glycans were not labeled: Check the labeling solution.
   - 2.2 Glycans were lost during processing: Check the glycan clean-up cartridges.

HINT: Though quantification of the yield of glycans is optional, it is recommended to use internal glycan standard and quantify the antibody captured as a routine process to troubleshoot any problems associated with a sample.

Acknowledgments

We thank Dr. Yves Durocher (NRC-BRI, Montreal, Canada) for providing the CHO-EG2 cell line. We also thank Vincent Jung, Evonne Liu and Melissa Pheneuf for assistance and Dr. Maureen Spearman for valuable discussions. The work is funded by Natural Sciences and Engineering Research Council of Canada through a strategic grant (MabNet).

References

1. Walsh G (2014) Biopharmaceutical benchmarks 2014. Nat Biotechnol 32: 992-1000. doi: 10.1038/nbt.3040. PMID: 25299917
2. Shields RL, Lai J, Keck R, O'Connell LY, Hong K, et al. (2002) Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. J Biol Chem 277: 26733-26740. doi: 10.1074/jbc.M202069200. PMID: 11986321
3. Hodoniczky J, Zheng YZ, James DC (2005) Control of Recombinant Monoclonal Antibody Effector Functions by Fc N-Glycan Remodeling in Vitro. Biotechnol Prog 21: 1644-1652. doi: 10.1021/bp050228w. PMID: 16321047
4. Boyd PN, Lines AC, Patel AK (1995) The effect of the removal of sialic acid, galactose and total carbohydrate on the functional activity of Campath-1H. Molecular Immunology 32: 1311-1318. PMID: 8643100
5. Houde D, Peng Y, Berkowitz SA, Engen (2010) Post-translational Modifications Differentially Affect IgG1 Conformation and Receptor Binding. Molecular & Cellular Proteomics 9: 1716-1728. doi: 10.1074/mcp.M900540-MCP200. PMID: 20103567
6. Kumpel BM, Wang Y, Griffiths HL, Hadley AG, Rook GAW (1995) The biological activity of human monoclonal IgG anti-D is reduced by beta-galactosidase treatment. Human Antibodies and Hybridomas 6: 82-88. PMID: 8597627
7. Scallon BJ, Tam SH, McCarthy SG, Cai AN, Raju TS (2007) Higher levels of sialylated Fc glycans in immunoglobulin G molecules can adversely impact functionality. Molecular Immunology 44: 1524-1534. doi: 10.1016/j.molimm.2006.09.005. PMID: 17045339
8. Kaneko Y, Nimmerjahn F, Ravetch JV (2006) Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. Science 313: 670-673. doi: 10.1126/science.1129594. PMID: 16888140
9. Ghaderi D, Taylor RE, Padler-Karavani V, Diaz S, Varki A (2010) Implications of the presence of N-glycolylneuraminic acid in recombinant therapeutic glycoproteins. Nat Biotechnol 28: 863-867. doi: 10.1038/nbt.1651. PMID: 20657583
10. Chung CH, Mirakhur B, Chan E, Le_Q-T Berlin J (2008) Cetuximab-Induced Anaphylaxis and IgE Specific for Galactose-α-1,3-Galactose. New England Journal of Medicine 358: 1109-1117. doi: 10.1056/NEJMoa074943. PMID: 18337601
11. Zheng K, Bantog C, Bayer R (2011) The impact of glycosylation on monoclonal antibody conformation and stability. MAbs 3: 568-576. doi: 10.4161/mabs.3.6.17922. PMID: 22123061
12. Ha S, Ou Y, Vlasak J, Li Y, Wang S, et al. (2011) Isolation and characterization of IgG1 with asymmetrical Fc glycosylation. Glycobiology 21: 1087-1096. doi: 10.1093/glycob/cwr047. PMID: 21470983
13. Hossler P, Khattak SF, Li ZJ (2009) Optimal and consistent protein glycosylation in mammalian cell culture. Glycobiology 19: 936-949. doi: 10.1093/glycob/cwp079. PMID: 19494347
14. Liu B, Spearman M, Doering J, Lattová E, Perreault H, et al. (2013) The availability of glucose to CHO cells affects the intracellular lipid-linked oligosaccharide distribution, site occupancy and the N-glycosylation profile of a monoclonal antibody. J Biotechnol 170: 17-27. doi: 10.1016/j.jbiotec.2013.11.007. PMID: 24286971
15. Moellering BJ, Tedesco JL, Townsend RR, Hardy MR, Scott RW (1989) Electrophoretic differences in a mAb expressed in three media. BioPharm International 3: 30-38.
16. Huffman JE, Pucic-Bakovic M, Klaric L, Hennig R, Selman MHJ (2014) Comparative Performance of Four Methods for High-throughput Glycosylation Analysis of Immunoglobulin. G in Genetic and Epidemiological Research. Molecular & Cellular Proteomics 13: 1598-1610. doi: 10.1074/mcp.M113.037465. PMID: 24719452
17. Mann AC, Self CH, Turner GA (1994) A general method for the complete deglycosylation of a wide variety of serum glycoproteins using peptide-N-glycosidase-F. Glycosylation Disease 1: 253-261.
18. Melmer M, Stangler T, Schiefermeier M, Brunner W, Toll H, et al. (2010) HILIC analysis of fluorescence-labeled N-glycans from recombinant biopharmaceuticals. Anal Bioanal Chem 398: 905-914. doi: 10.1007/s00216-010-3988-x. PMID: 20640408
19. Royle L, Radcliffe CM, Dwek RA, Rudd PM (2006) Detailed structural analysis of N-glycans released from glycoproteins in SDS-PAGE gel bands using HPLC combined with exoglycosidase array digestions. Methods Mol Biol 347: 125-143. doi: 10.1385/1-59745-167-3:125. PMID: 17072008
20. Krenkova J, Lacher NA, Svec F (2009) Multidimensional system enabling deglycosylation of proteins using a capillary reactor with peptide-N-glycosidase F immobilized on a porous polymer monolith and hydrophilic interaction liquid chromatography–mass spectrometry of glycans. Journal of Chromatography A 1216: 3252-3259. doi: 10.1016/j.chroma.2009.02.036. PMID: 19268959
21. Bynum MA, Yin H, Felts K, Lee YM, Monell CR (2009) Characterization of IgG N-Glycans Employing a Microfluidic Chip that Integrates Glycan Cleavage. Sample Purification, LC Separation, and MS Detection. Analytical Chemistry 81: 8818-8825. doi: 10.1021/ac901326u. PMID: 19807107
22. Royle L, Campbell MP, Radcliffe CM, White DM, Harvey DJ, et al. (2007) HPLC-based analysis of serum N-glycans on a 96-well plate platform with dedicated database software. Anal Biochem 376: 1-12. doi: 10.1016/j.ab.2007.12.012. PMID: 18194658
23. Doherty M, Bones J, McLoughlin N, Telford JE, Harmon B, et al. (2013) An automated robotic platform for rapid profiling oligosaccharide analysis of monoclonal antibodies directly from cell culture. Anal Biochem 442: 10-18. doi: 10.1016/j.ab.2013.07.005. PMID: 23872006
24. Bell A, Wang ZJ, Arbabi-Ghahroudi M, Chang TA, Durocher Y, et al. (2009) Differential tumor-targeting abilities of three single-domain antibody formats. Cancer Lett 289: 81-90. doi: 10.1016/j.canlet.2009.08.003. PMID: 19716651
25. Gonzalez TN, Leong SR, Presta LG (2000) Humanized anti-IL-8 monoclonal antibodies. USPTO: 6133426, Genentech, Inc., U.S.A.

Supplementary information

Supplementary Materials and Methods.

Figure S1. Linear response of glycan yield as a function of % glycosylated antibody in the antibody sample mixture (n = 4).

Figure S2. CHO-EG2 cells culture performance and EG2-hFc production profiles after seeding into BioGro-CHO medium containing only 5 mM glucose (n = 2).

Table S1. Composition of N-glycans of EG2-hFc sample obtained by using either protein-A or Protein-G columns for isolating glycans (n = 2).

Table S2. Composition of N-glycans of IgGs from human serum (Sigma-Aldrich Cat# H4522) obtained by current method using either protein-A or Protein-G columns (n = 2).

Supplementary information of this article can be found online at http://www.jbmethods.org.