

Protein Characterization

CE SEPARATION OF N-LINKED OLIGOSACCHARIDES RELEASED FROM RECOMBINANT MONOCLONAL ANTIBODY

Vita F. Knudson and Michael H. Simonian
Biomedical Research Division
Beckman Coulter Inc, Fullerton, CA USA

Introduction

Complex carbohydrates are integral components of glycoproteins that play vital functional roles in biological systems. Glycoproteins are involved in cell stability and adhesion, antibody recognition, and microorganism binding and serve as cell-surface markers.⁽¹⁻²⁾ The assessment of oligosaccharide microheterogeneity is an important analytical task in immunology, modern biomedical technology and food products.

Glycoprotein glycosylation is divided into two classes, referred to as N- and O-glycosylation. The best studied mode of glycosylation is the formation of an N-glycosidic linkage to asparagines in the polypeptide chain. O-glycosylation occurs at serine and/or threonine residues in the polypeptide chain and tends to be shorter and simpler structure than N-linked. Variations in N-linked sugars create different glycoforms that play an important role in bioactivity and immunogenicity. Microheterogeneity of glycosylation sites is important in many properties of therapeutic proteins including structure analysis, solubility, stability, and protease resistance.⁽³⁻⁴⁾ The importance of the glycan structure in therapeutic use of monoclonal antibodies is well documented.⁽⁵⁾

A whole branch of glycobiology is involved in releasing oligosaccharides from glycoproteins for their subsequent separation and characterization. Profiling and quantitative analysis of glycans has been performed with gas chromatography,⁽⁶⁾ anion-exchange chromatography,⁽⁷⁾ size-exclusion chromatography,⁽⁸⁾ and high-concentration polyacrylamide gel electrophoresis.⁽⁹⁻¹⁰⁾ Mass spectrometry⁽¹¹⁻¹²⁾ and nuclear magnetic resonance spectroscopy⁽¹³⁻¹⁴⁾ are indispensable tools for the structural analysis of carbohydrates. Capillary electrophoresis (CE) and capillary gel electrophoresis have been widely used for complex carbohydrate separation⁽¹⁵⁻¹⁶⁾ because of enhanced separation efficiency and shorter analysis times. CE-based carbohydrate analysis can be applied easily to determine molar ratio, degree of polymerization of oligosaccharides, and to detect changes in the extent or nature of the oligosaccharide distribution (fingerprinting).

This application bulletin describes the isolation, separation, and profiling analysis of complex oligosaccharides by CE using the ProteomeLab™ PA 800 Protein Characterization System. A typical recombinant monoclonal antibody, mouse IgG₂, was analyzed for N-linked carbohydrates.



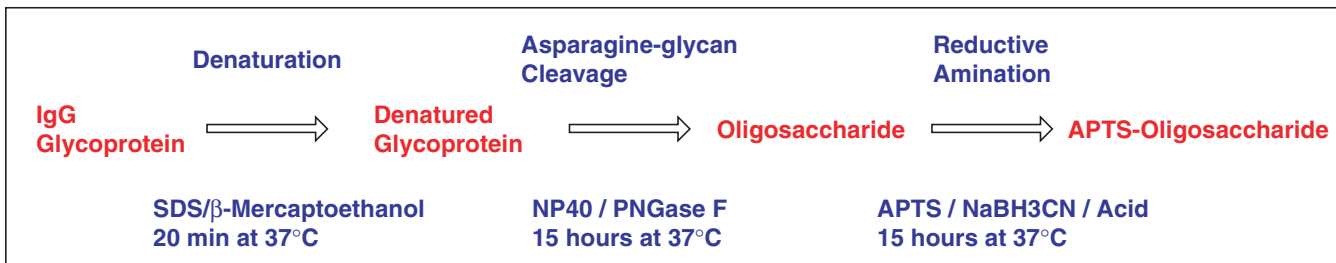


Figure 1. Process profile for PNGase F digestion and APTS derivatization of IgG glycoproteins.

Background

An illustration of the step-by-step process for preparing recombinant mouse IgG₂ for analysis is shown in Figure 1. Below is a description of the steps in this process.

The first step in the analysis of N-linked glycoproteins begins with the release of oligosaccharides from glycoproteins by enzymatic or chemical reaction. Peptide-N-glycosidase F (PNGase F) is one of the most widely used enzymes for the deglycosylation of glycoproteins. PNGase F is an amidase which cleaves between asparagine residues and the innermost N-acetylglucosamine (GlcNAc) of complex N-linked oligosaccharides from glycoproteins (Figure 2). This highly specific endoglycosidase virtually hydrolyzes all N-linked glycans from glycoproteins.⁽¹⁷⁻¹⁹⁾

The second step is labeling by reductive amination. The labeling of oligosaccharides with 8-aminopyrene-1,3,6-trisulfonate (APTS) has proven ideal for analysis by providing both fluorescence and mobility to the analyte.⁽²⁰⁻²²⁾

Acid catalysis of the reductive amination of oligosaccharides with APTS was used to improve labeling efficiency.⁽²³⁾ The stoichiometry of labeling is such that one molecule of APTS fluorophore is attached to each molecule of oligosaccharide (Figure 3). The labeling efficiency is a function of the total quantity of sugar used in the reaction, temperature of labeling and desialylation kinetic processes.⁽²⁴⁾ The addition of a quantitation as an internal standard is a good technique to determine the labeling efficiency of an unknown sample.

The optimal excitation wavelength of the APTS labeled oligosaccharides is close to 488 nm, the wavelength of the Argon-ion laser. Figure 4 shows spectra of APTS and APTS labeled glycan adducts. At 488 nm, the signal of APTS is minimum compared to signal of the APTS labeled sugar adducts.

The third step is the separation of the labeled oligosaccharides by CE with laser-induced fluorescence detection. The separation occurs in 15 minutes with high resolution of the glycans released from glycoproteins.

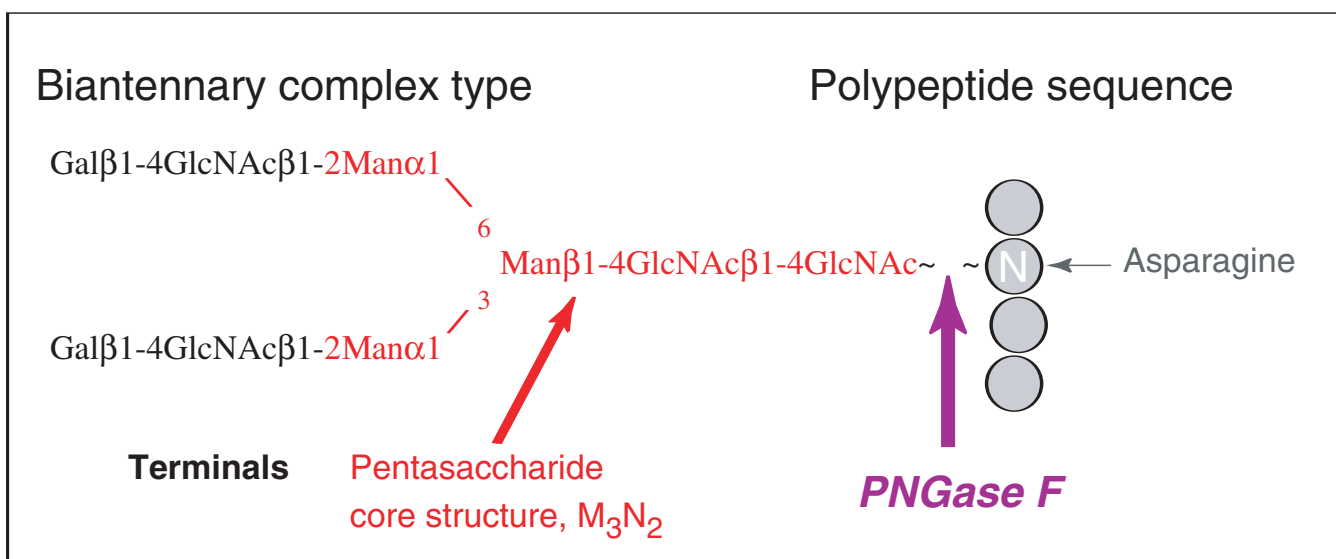


Figure 2. Schematic diagram of N-Glycosidase, the PNGase F amidase that cleaves between asparagine residues and the innermost GlcNAc of complex N-linked oligosaccharides from glycoproteins.

Materials and Methods

Instrumentation

The CE-based carbohydrate analysis is performed on a ProteomeLab™ PA 800 System using LIF detection with an excitation wavelength of 488 nm and an emission band-pass filter of 520 nm \pm 10 nm. A neutral coated capillary 50 μ m I.D x 50.2 cm (40 cm effective length to detector) was used to reduce electroosmotic flow and minimize surface interactions. The separation was performed at constant voltage of -30 KV with the anode at the detector. The current was stable at -14 μ A. Capillary temperature was controlled at 25°C. Sample introduction was accomplished using an applied pressure of 0.5 psi for 8 sec. The capillary was rinsed with the separation gel buffer prior to sample introduction.

Materials

The ProteomeLab Carbohydrate Labeling and Analysis Kit was used. This kit contains glucose ladder standard, maltose quantitation marker, dye solvent (15% acetic acid), separation gel buffer, APTS and a neutral coated capillary. Monoclonal IgG₂ was prepared from ascites fluid obtained with Beckman Coulter. PNGase F, sodium dodecyl sulfate (SDS), β -mercaptoethanol (β -ME), Nonidet NP40 and sodium phosphate buffer were all purchased from New England BioLab (Beverly, MA). 1M sodium cyanoborohydride in tetrahydrofuran (THF) was purchased from Sigma-Aldrich (St. Louis, MO).

Sample Preparation

Denaturing of IgG₂

Approximately 250 μ g of purified IgG sample was dried in a speed vacuum centrifuge and then dissolved in releasing enzyme buffer, 50 mM

sodium phosphate buffer at pH 7.5. Samples were denatured in 0.1% SDS solution with 50mM β -ME at 37°C for 20 min.

Enzymatic Release of N-linked Oligosaccharides from Glycoproteins

The sample cleavage was performed by PNGase F in 0.75 % NP40 detergent at 37°C overnight in water bath. The required amount of releasing enzyme may vary depending on the glycoprotein being studied. 4 μ L of PNGase F (500,000 U/mL) was used to digest 250 μ g of IgG₂. The released N-linked oligosaccharides were separated from the protein by adding three volumes of cold ethanol to precipitate the protein. The protein was pelleted by centrifugation for 8 min at 10,000 g (Microfuge® 18 Centrifuge with F241.5P rotor). The supernatant fraction with oligosaccharides was saved and dried for reductive amination (labeling). Reconstituted quantitation control marker, 2 nM maltose, was added as an internal labeling control at the ethanol precipitation step.

Reductive Amination

The released oligosaccharide sample, lyophilized standard of glucose ladder and maltose quantitation control standard were labeled with APTS by reductive amination. To the carbohydrate samples, 2 μ L of a 0.1 M APTS solution in aqueous glacial acetic acid (15 %) and 2 μ L of freshly prepared 1 M aqueous sodium cyanoborohydride in THF were added. The final reaction mixture contains 50 mM APTS. Samples were incubated in a 37°C water bath for approximately 15 hours. To stop the reaction, a 30-fold dilution with CE grade water was added to the samples. The samples were ready for electrophoretic separation.

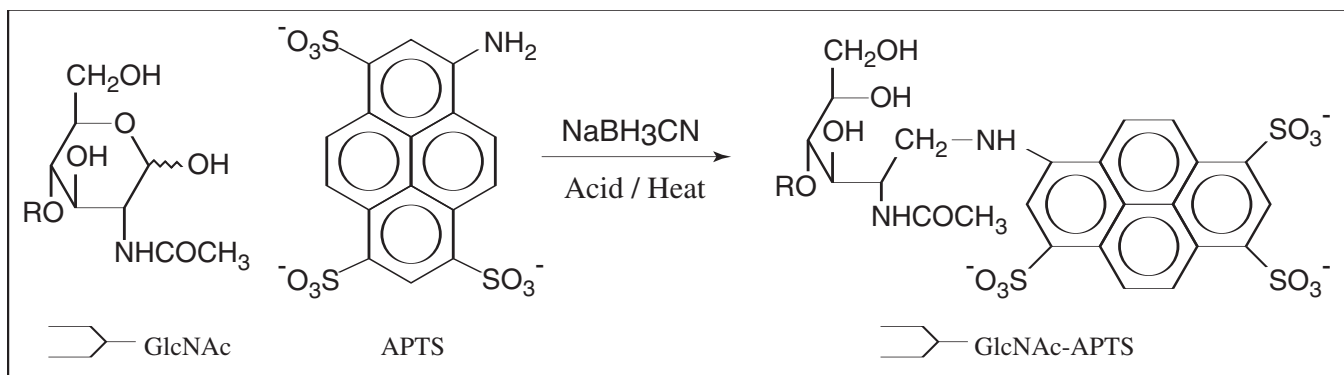


Figure 3. Reductive amination of the oligosaccharide with APTS (8-aminopyrene-1,3,6-trisulfonate) with presents of acid catalysis and temperature.

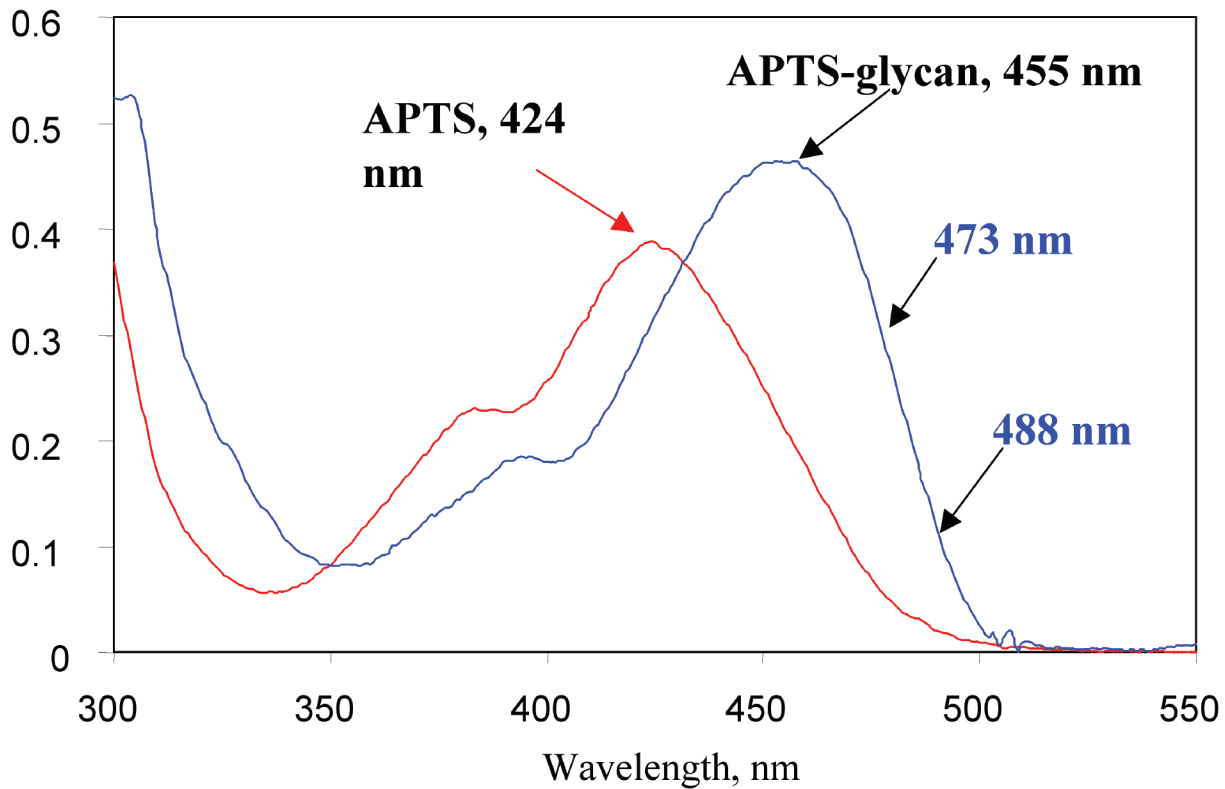


Figure 4. The excitation spectra of APTS and APTS-labeled glycan adducts.

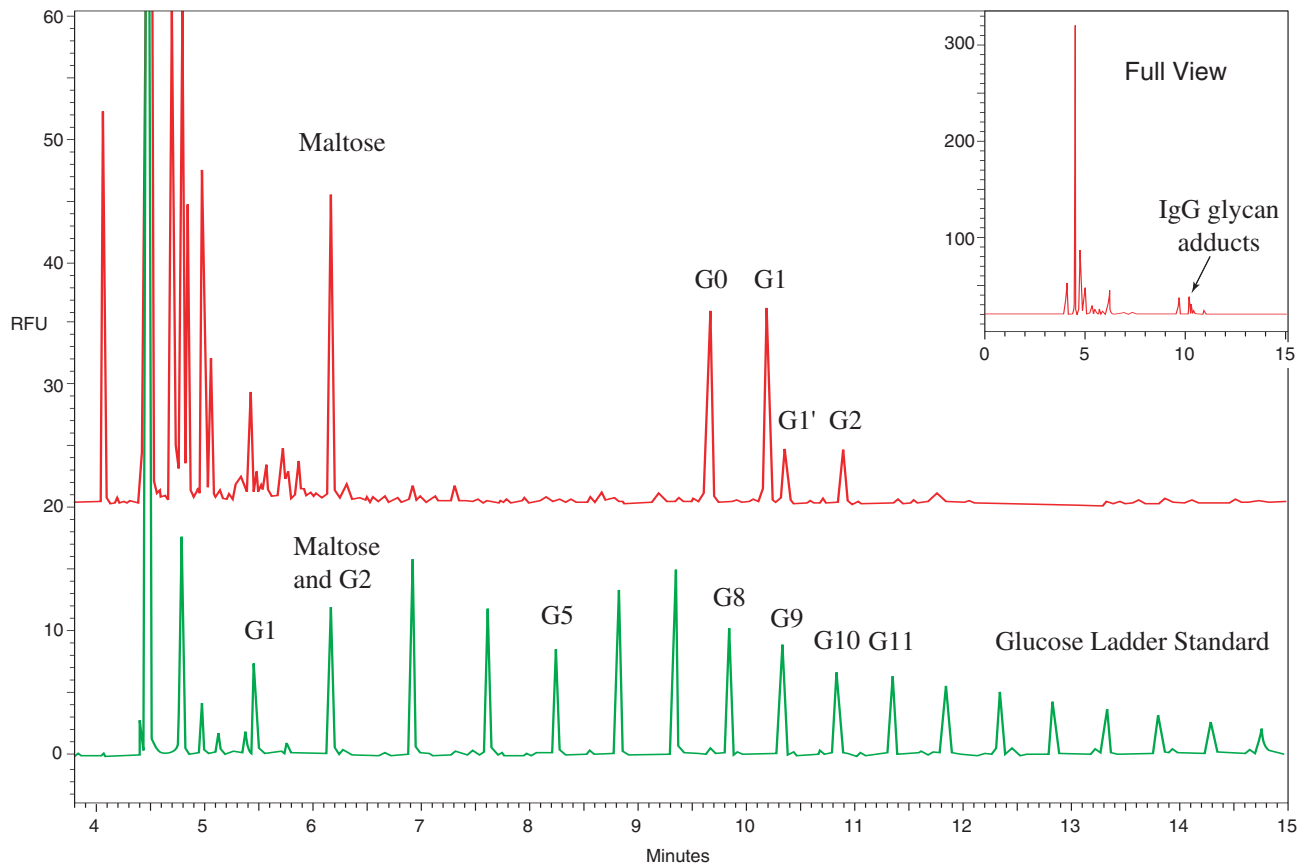


Figure 5. Electropherograms of profile of APTS-derivatized N-linked oligosaccharides obtained from PNGase F-catalyzed hydrolysis of recombinant monoclonal antibody, mouse IgG₂ (in red) and APTS-derivatized glucose ladder standard (in green). The insert shows a full view of the electropherogram.

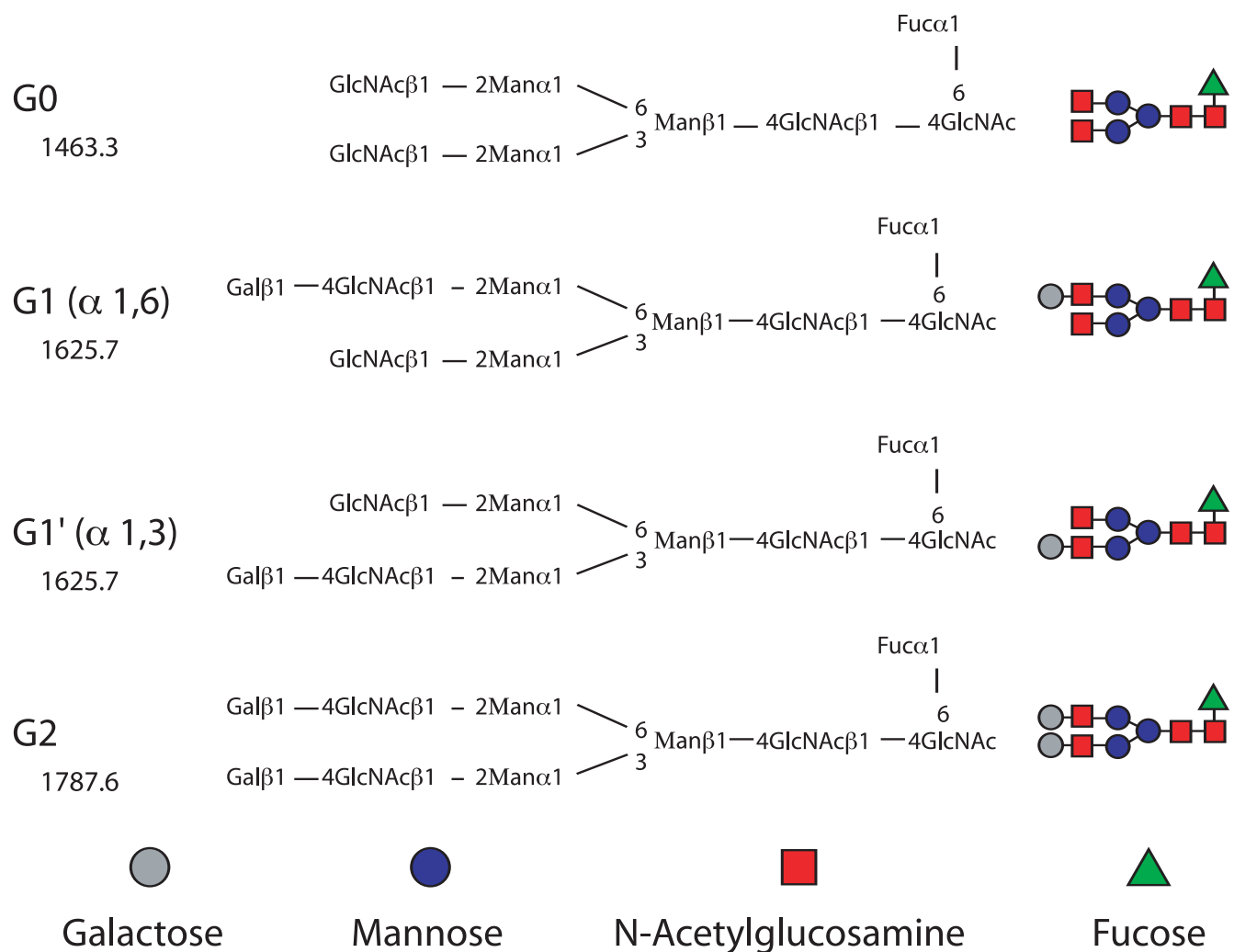


Figure 6. Schematic representation of four glycans structure. Biantennary N-linked oligosaccharides present on the recombinant monoclonal antibody IgG, which can be resolved by CE-LIF after APTS labeling.

Results and Discussion

The separation of APTS-derivatized glucose ladder standard (malto-oligosaccharide ladder) is shown in Figure 5. The numbers, G1, G2...G10, G11 above the peaks of the lower trace represent the degree of polymerization of the glucose standard size marker. The maltose peak co-migrates with similar velocity to the maltotriose G2 peak of the glucose ladder. The electropherogram of the profile of APTS-derivatized N-linked oligosaccharides released from recombinant monoclonal antibody, mouse IgG₂, is shown as upper trace in Figure 5. The electrophoresis of four IgG₂ glycans G0, G1, G1' and G2 (Figure 6) are correlated with the relative positions of the standard maltooligosaccharide peaks. The glycan G2 peak of IgG₂ is migrating with similar velocity to the maltodecaose peak of glucose ladder providing oligosaccharide size identification. The maltose peak with a concentration of 2 nM represents the internal quantitation control

marker of the labeling efficiency of the analyzed N-linked oligosaccharides released from IgG₂.

A schematic illustration of the glycans observed on the IgG₂ is represented in Figure 6. All glycans are fucosylated with the same branched core structure, three manose and two N-acetylglucosamines (M₃N₂) and vary only in their terminals (degalactosylated and partially galactosylated). After APTS derivatization all released glycans have the same charge and are separated by CE-LIF based on their size and/or structure. Even positional isomers may be resolved using this approach as illustrated by the separation of glycan isoforms G1 and G1', which have the same structure and number of monosaccharide units.

In summary, this paper demonstrates an application of the ProteomeLab Carbohydrate Labeling and Analysis assay for the separation and analysis of N-linked oligosaccharides released from the mouse monoclonal antibody, IgG₂.

References

1. Wyss, D. F., Wagner, G. *Curr. Opin. Biotechnol.* **7**, 409-416 (1996).
2. Hounsell, E. F., Davies, M. J., Renouf, D. V. *Glycoconjugates J.* **13**, 19-26 (1996).
3. Goochee, C. F., Gramer, M. J., Anderson, D. F., Bahr, J. B. *Am. Chem. Soc.* **9**, 1347-1355 (1991).
4. Goochee, C. F., Gramer, M. J., Anderson, D. F., Bahr, J. B. *Am. Chem. Soc.* **2**, 199-240 (1992).
5. Parekh, R. B., Dwek, R. A., Sutton, B. J., *et al.* *Nature (London)* **316**, 452-457 (1985).
6. Karlsson, N. G., Karlsson, H., and Hansson, G. C. *Glycoconjugate J.* **12**, 69-76 (1995).
7. Hardy, M. R. and Townsend, R. R. *Proc. Natl. Acad. Sci. USA* **85**, 3289-3293 (1988).
8. Yamashita, K., Mizuoichi, T. and Kobata, A. *Methods Enzymol.* **83**, 105-126 (1982).
9. Jackson, P. *Anal. Biochem.* **216**, 243-252 (1994).
10. Westfall, D. A., Flores, R. R., Negrete, G. R., Martinez, A. O. and Haro, L.S. *Analytical Biochemistry* **265**, 232-237 (1998).
11. Reinhold, V. N., Reinhold, B. B., Chan, S. *Methods Enzymol.* **271**, 377-402 (1996).
12. Kovasik, V., Hirsch, J., Zoric, S., Grutzmacher, H. F. *Rapid Commun. Mass Spectrom.* **10**, 1661-1667 (1996).
13. Sugahara, K., Nadanaka, S., Takeda, K., Kojima, T. *Eur. J. Biochem.* **271**, 12555-12561 (1996).
14. Larnkjaer, A., Hansen, S. H., Ostergaard, P. B. *Carbohydr.* **266**, 37-52 (1996)
15. Guttman, A. *Nature* **380**, 461-462 (1996).
16. Chiesa, C. and O'Neil, R. *Electrophoresis* **15**, 1132-1140 (1994).
17. Stefansson, M. and Novotny, M. *Carbohydr. Res.* **258**, 1-9 (1994).
18. Chiesa, C., O'Neil, R., Horvath, C., Oefner, P. *Capillary Electrophoresis in Analytical Biotechnology*, Righetti, P., CRC Press: Boca Raton, p. 277-430 (1996).
19. O'Neil, R. A. *J. Chromatogr.* **720**, 201-215 (1996).
20. Takahashi, N., Marumatsu, T. *CRC Handbook of Endoglycosidases and Glycosamidases*. CRC Press: Boca Raton (1992)
21. Chen, F.-T. A., Dobashi, T. S., Evangelista, R. A. *Glycobiology, Vol. 8, no 11*, 1045-1052, (1998).
22. Chen, F.-T. A., Evangelista, R. A. *Electrophoresis* **19**, 2639-2644 (1998).
23. Evangelista, R. A., Guttman, A., Chen, F.-T. A. *Electrophoresis* **17**, 347-351 (1996).
24. Guttman, A., Evangelista, R. A., Chen, F.-T. A. *Electrophoresis* **17**, 412-417 (1996).

* All trademarks are the property of their respective owners.



Developing innovative solutions in Systems Biology.

Innovate SIMPLIFY Automate

Beckman Coulter, Inc. • 4300 N. Harbor Boulevard, Box 3100 • Fullerton, California 92834-3100
Sales & Service: 1-800-742-2345 • Telex: 678413 • Fax: 1-800-643-4366 • www.beckmancoulter.com

Worldwide Biomedical Research Division Offices:

Australia (61) 2 9844-6000 Canada (905) 819-1234 China (86) 10 6515 6028 Eastern Europe, Middle East, North Africa (41) 22 994 07 07
France 01 49 90 90 00 Germany 49 21 513335 Hong Kong (852) 2814 7431/2814 0481 Italy 02-953921 Japan 03-5404-8359
Mexico (55) 560-57770 Netherlands 0297-230630 Singapore (65) 6339 3633 South Africa, Sub-Saharan Africa (27) 11-805-2014/5 Spain 91 3836080
Sweden 08-564 85 900 Switzerland 0800 850 810 Taiwan (886) 2 2378 3456 Turkey 90 216 309 1900 U.K. 01494 441181 U.S.A. 1-800-742-2345