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1. Purpose

This procedure describes a method for the analysis of Fc glycans on antibodies and Fc fusion proteins. N-linked oligosaccharides are released from the proteins under non-denaturing conditions using PNGase F, labeled with the fluorophore APTS and analyzed using capillary electrophoresis (CE), which allows for the separation of APTS-labeled glycans based primarily on their size, with laser induced fluorescence (LIF) detection.

2. Scope

Process Analytics/Quality Control (PA/QC) personnel will perform this procedure. Other Biopharmaceutical Development Program (BDP) personnel may use this protocol for development or in-process analysis.

3. Authority and Responsibility

3.1. The Director, PA/QC has the authority to define this procedure.

3.2. The Director, PA/QC is responsible for assignment of this procedure.

3.3. The Supervisor of PA/QC is responsible for ensuring personnel are trained on this procedure and that this training is documented to Biopharmaceutical Quality Assurance (BQA).

3.4. PA/QC personnel are responsible for the performance of this procedure.

3.5. BQA has the authority to review and approve/disapprove all test data relative to its conformance to the stated product specifications.

3.6. BQA is responsible for quality oversight of this operation.

4. Equipment

- Beckman Coulter P/ACE MDQ Capillary Electrophoresis System MEF 72400.
- Beckman Coulter LIF Detector and Power Supply MEF 72401.
- Water System - Sartorius arium pro MEF LWPS-008-B or equivalent.
- Waterbath - VWR MEF 78150 or equivalent.
- Centrifuge - Eppendorf 5415R MEF 78670 or equivalent.
- Centrifugal Vacuum Drier - Savant AES1010 SpeedVac (MEF 73040) or equivalent.
- Heating Block – VWR MEF 78710 or equivalent.
- Beckman Coulter DU800 Spectrophotometer (MEF 80400) or equivalent.

5. Reagents and Materials

- Beckman Coulter Carbohydrate Labeling and Analysis Kit (BDP PN 22051).

  Kit includes items that can be ordered separately including:

N-CHO Coated Capillary	BDP PN 22052
Carbohydrate Separation Buffer	BDP PN 22053
L6 - Labeling Dye (APTS)	BDP PN 22054

- New England Biolabs (NEB) PNGase F Kit (BDP PN 30958).
- Human IgG1, K, Sigma BDP PN 30690.
- Murine IgG1, K, Sigma BDP PN 30695.
- MAb4 Antibody Glycan Reference Panel, QA Bio BDP PN 30963.
- Sodium cyanoborohydride, 1.0M solution in tetrahydrofuran (THF) Aldrich BDP PN 30487.
- Amicon Ultra Centrifugal Filters, 10K, Millipore BDP PN 30982.
- 0.5 mL Microfuge Tubes (Eppendorf), BDP PN 21369.
- 1.5 mL Microfuge Tubes (Eppendorf), BDP PN 20595.

6. Procedure

Information is collected on the worksheets associated with this procedure and ***SOP 22914 - General Operation of the Beckman Coulter P/ACE MDQ Capillary Electrophoresis System*.**

6.1. *N*-Glycans are released from the resulting peptides/glycopeptides via digestion with peptide *N*-glycosidase F (PNGase F). Oligosaccharides released by PNGase F are separated from proteins using centrifugal filtration with a 10,000 Dalton MWCO. This protocol can also be performed following denaturation of the protein – record details in the laboratory notebook.

6.1.1. For samples with concentrations greater than 1 mg/mL, transfer 50 µg (range 25 to 300 µg) of test article in phosphate buffer to 500 µL microfuge tube. Include samples of HuIgG1, K; MuIgG1, K; and blank controls.

6.1.2. Add water and NEB G7 10X Buffer (5 µL) to a final volume of 48 µL.

6.1.3. Add 2 µL of NEB PNGase F.

6.1.4. Vortex to mix. Incubate in a 37°C waterbath overnight (acceptable range 5 to 24 hour).

6.1.5. Centrifuge briefly.

6.1.6. Prepare centrifugal ultrafilters by adding 500 µL of water to each and centrifuging for 15 minutes. Dispose of water. This step is not critical, but it removes the glycerin preservative from the ultrafilter membrane.

6.1.7. Transfer samples and controls to prepared centrifugal ultrafilter devices with 10,000 MWCO. Rinse each tube with 100 µL of water and add to samples.

6.1.8. Centrifuge at 13,000 RPM for thirty (30) minutes at 4ºC. The glycans released by PNGase F treatment will pass through the filters while the proteins will be retained.

6.1.9. Transfer the filtrates containing the glycans to 500 µL microfuge tubes.

6.1.10. Vacuum dry the samples using the Savant SpeedVac (or equivalent) for 3 hours at the “Full Vacuum” setting with the radiant heater (RC) turned off. Include the Glucose Ladder sample and a sample of the MAb4 standard.

6.2. APTS Labeling

6.2.1. Prepare the APTS labeling reagent.

To a vial of APTS labeling dye (L6).

6.2.1.1. Add 48 µL of Labeling dye solvent (15% acetic acid – L3).

6.2.1.2. Vortex for 5 seconds or until completely dissolved.

6.2.1.3. This is adequate for twenty (20) samples, but formulated reagent can be stored at -35º to -15°C for up to two (2) weeks.

6.2.2. Prepare the Glucose Ladder.

6.2.2.1. Weigh and dissolve 5 mg of the Glucose Ladder Standard (G20) in 80 µL of water in a 1.5 mL microfuge tube. Sonicate if necessary.

6.2.2.2. Aliquot at least ten 2 µL portions of the Glucose Ladder Standard solution into 500 µL microfuge tubes and dry them in a centrifugal vacuum evaporator. The dried glucose ladder can be stored at -20°C for two (2) years or used immediately.

6.2.3. Performing the Labeling Reaction

6.2.3.1. Add 2 µL of 1 M sodium cyanoborohydride/THF to the dried oligosaccharide or control samples. Avoid introducing moisture into the sodium cyanoborohydride.

6.2.3.2. Add 2 µL of APTS Labeling Reagent to the sample.

6.2.3.3. Incubate at 55°C for 90 minutes.

6.2.3.4. Add 46 µL of water to stop the reaction.

6.2.3.5. These samples can be stored at 2º to 8°C for two (2) weeks or at -20°C for six (6) months before analysis.

6.2.4. To prepare the samples for CE analysis add 50 µL of 1/10th strength Carbohydrate Separation Buffer to each sample. Mix well and transfer into CE pcr vials or micro vials for analysis in the CE.

6.3. Capillary Electrophoresis

6.3.1. Operation of the Beckman Coulter P/ACE MDQ capillary electrophoresis system is described in ***SOP 22914 - General Operation of the Beckman Coulter P/ACE MDQ Capillary Electrophoresis System***, and the associated worksheets. Only specifics of the carbohydrate analysis are described in this procedure.

6.3.2. Preparation of the capillary cartridge is described in ***SOP 22911 - Capillary Cartridge Assembly for the Beckman-Coulter P/ACE MDQ Capillary Electrophoresis Unit***. Be sure to pre-rinse a new capillary for 10 minutes at 30 psi pressure with DDI water and then rinse for ten (10) minutes at 30 psi pressure with Carbohydrate Separation Buffer prior to the first run.

6.3.3. Turn on the system and permit the LIF laser to warm up for at least thirty (30) minutes.

6.3.4. Preparing the buffer trays. The following quantities of vials are appropriate for 20 to 40 samples but may be increased or decreased depending on the number of samples to be analyzed. Fill 2-mL CE vials with the appropriate volumes of each reagent.

- 1.5 mL of DDI water per H_2O vial (8 vials).
- 1.5 mL of Carbohydrate Separation Buffer per Gel-R vial (2 vials).
- 1.3 mL of Carbohydrate Separation Buffer per Gel-S vial (4 vials).
- 0.8 mL of DDI water per Waste vial (2 vials).

6.3.5. Place the reagent vials on the inlet and outlet buffer trays as indicated in the figure below. During the runs salts migrate from one vial of gel buffer to the other, changing the buffer composition therefore the method program is set to increment to fresh buffer vials after 20 runs. Ensure that the trays are filled with the appropriate number of vials to manage the number of runs that are to be performed. The H_2O vials are used to clean the capillary tips and are also incremented after 20 runs to prevent contamination.

Figure 1. Buffer Tray Configuration

Inlet Buffer Tray

						6
H_2O						5
H_2O						4
						3
H_2O	Gel-R	Gel-S				2
H_2O	Gel-R	Gel-S				1
A	B	C	D	E	F	

Outlet Buffer Tray

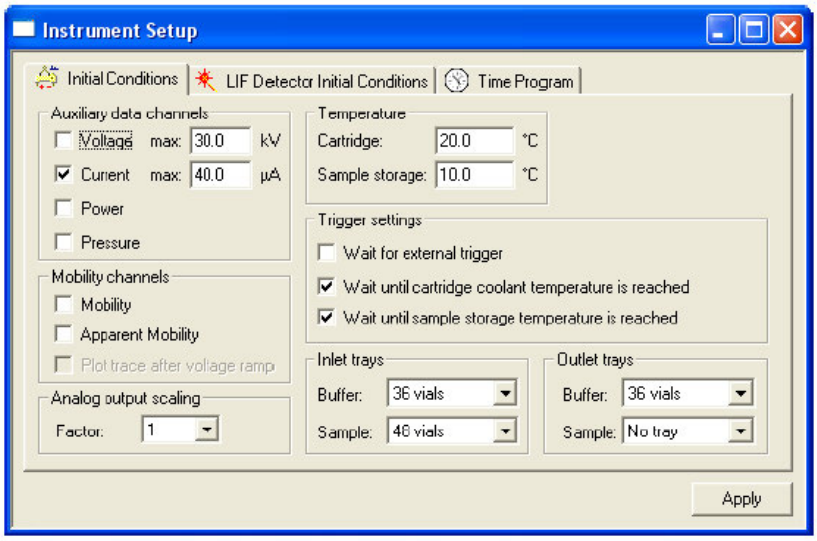
						6
H_2O						5
H_2O						4
						3
H_2O	Waste	Gel-S				2
H_2O	Waste	Gel-S				1
A	B	C	D	E	F	

6.3.6. Sample Vial Setup. Transfer samples to 200 µL vials or microvials and place into the inlet sample tray. For best quantitative results, perform one injection per vial, introducing replicates in separate vials.

6.3.7. Initial Conditions for running the samples (Instrument Setup Menu)

Capillary Temperature:	20°C
Sample Storage Temperature:	10°C
Auxiliary Data Channel:	Current

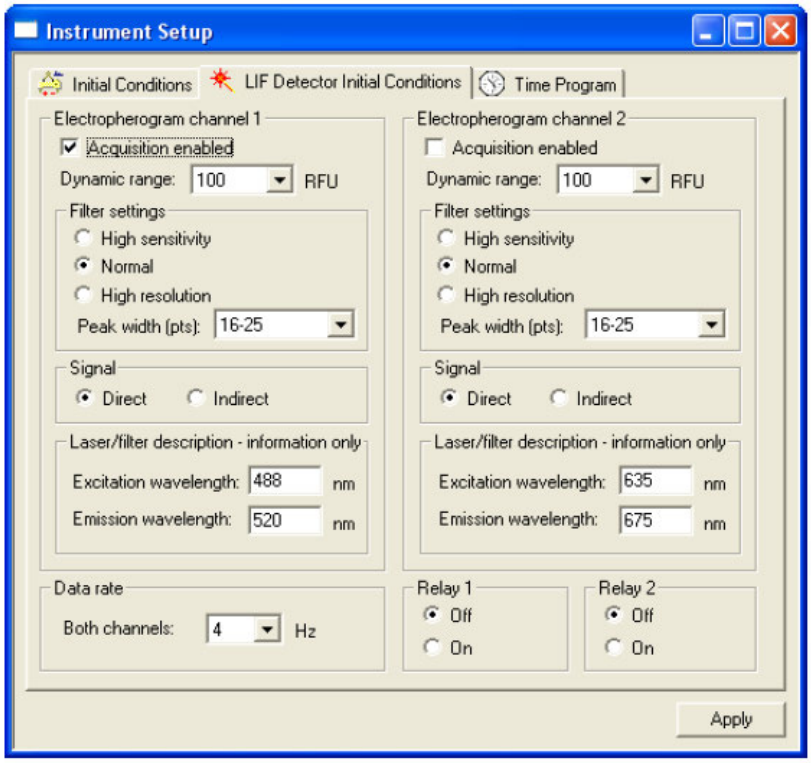
Figure 2. Instrument Setup – Initial Condition Tab



6.3.7.1. LIF Detector Initial Conditions (Instrument Setup Menu).

Detection:	Laser Induced Fluorescence
Wavelength:	Excitation – 488 nm, Emission – 520 nm
Data Rate:	4 Hz
Dynamic Range:	100 RFU (relative fluorescence units)
Filter Setting:	Normal
Peak Width:	16-25

Figure 3. Instrument Setup – LIF Detector Initial Condition Tab



6.3.7.2. Time Program

6.3.7.2.1. Rinse the capillary with buffer for 3 minutes at 30 psi from BI:B1 (Gel-R buffer) to waste at vial BO:B1.

6.3.7.2.2. Inject the sample at 0.5 psi from sample vial to buffer vial BO:C1. A 3 to 20 sec at 0.5 psi injection is recommended.

6.3.7.2.3. Wait 0.2 minutes with vials BI:A4 and BO:A4. This step dips the capillary in water to protect against sample carryover.

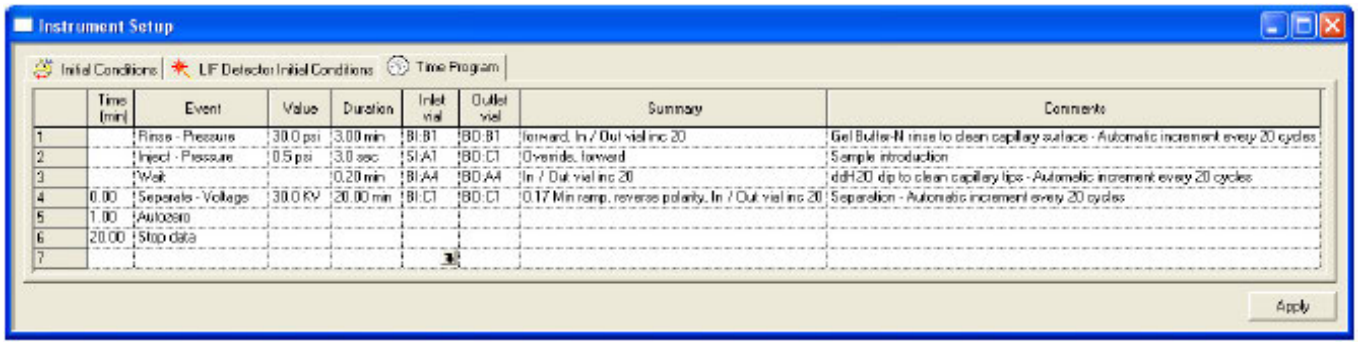
6.3.7.2.4. Separation step – 20 minutes from vial BI:C1 to vial BO:C1 (Gel-S buffer). The constant voltage should be 30 kV, with reverse polarity and a 0.17 ramp time.

6.3.7.2.5. Autozero at 1.0 minute.

6.3.7.2.6. End at 20.0 minutes.

6.3.7.2.7. In the time program the vials are set to increment to fresh vials after 20 runs.

Figure 4. Instrument Setup – Time Program Tab



	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	30.0 psi	3.00 min	BI:B1	BO:B1	forward, In / Out vial inc 20	Gel Buffer-N rinse to clean capillary surface - Automatic increment every 20 cycles
2		Inject - Pressure	0.5 psi	3.0 sec	SI:A1	BO:C1	Override, forward	Sample introduction
3		Wait		0.20 min	BI:A4	BO:A4	In / Out vial inc 20	ddH2O dip to clean capillary tips - Automatic increment every 20 cycles
4	0.00	Separate - Voltage	30.0 KV	20.00 min	BI:C1	BO:C1	0.17 Min ramp, reverse polarity, In / Out vial inc 20	Separation - Automatic increment every 20 cycles
5	1.00	Autozero						
6	20.00	Stop data						
7								

6.4. System Shutdown and Capillary Storage

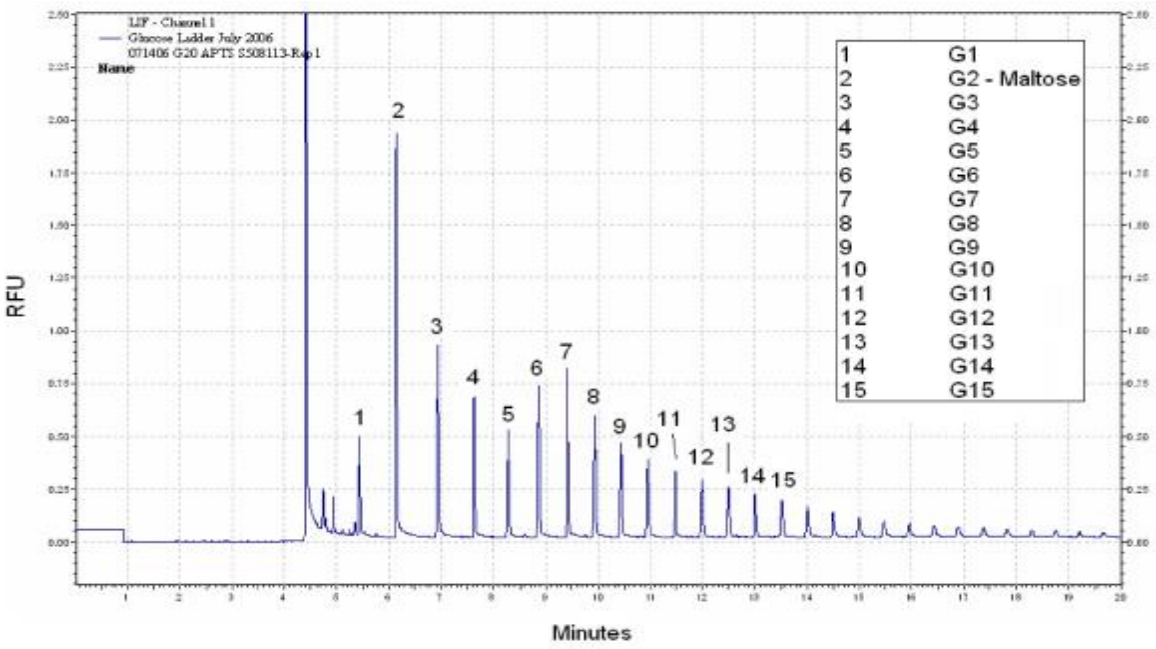
6.4.1. For short term storage – Perform a 3-minute, 30 psi rinse with water. The capillary may be stored on the instrument with the capillary ends immersed in water. Whenever the capillary has not been used for 3 hours or longer, rinse the capillary by performing a 3-minute, 30 psi rinse with water before performing a separation.

6.4.2. For long term storage - Perform a 3-minute, 30 psi rinse with water and then with Carbohydrate Separation Buffer for 3 minutes. Remove the capillary (with cartridge) and place in a cassette box with the capillary ends placed in vials of DDI water. Store the cartridge box at 2°C to 8°C in an upright position. Whenever returning the capillary to use, rinse the capillary by performing a 3-minute, 30 psi rinse with water and then a 10-minute rinse with Carbohydrate Separation Buffer before performing a separation.

7. Evaluation of Results

7.1. The test mixture containing the APTS-labeled glucose oligomers (Glucose Ladder) consists of at least 20 individual oligomers. An example electropherogram of this test mix is below.

Figure 5. Electropherogram – Glucose Ladder



7.2. Analyze sample electropherograms for the G0F, G1F, and G2F glycans and include the analysis with the report.

8. Documentation

8.1. Documentation for a QC test request will include the SOP worksheets, a printout of the method file as well as the electropherograms for each sample.

8.2. All protocols, raw data, computer records, completed forms and the original copy of the final report will be maintained by BQA Documentation.

8.3. Generate and maintain all documentation relevant to this procedure according to ***SOP 21409 - Good Documentation Practices*****.**

9. References

9.1. **SOP 21409** *Good Documentation Practices*

9.2. **SOP 22914** *General Operation of Beckman Coulter P/ACE MDQ Capillary Electrophoresis System*

9.3. **SOP 22911** *Capillary Cartridge Assembly for the Beckman-Coulter P/ACE MDQ Capillary Electrophoresis Unit*

9.4. Beckman Coulter Application Guide A51969AA, PA 800 plus Pharmaceutical Analysis System: Carbohydrate Labeling and Analysis

9.5. **Form 16137-01** CE Glycan Analysis: Datasheet and Checklist

9.6. **Form 16137-02** Reagents, Materials and Equipment

10. Attachments

10.1. **Attachment 1** Beckman Coulter Bulletin AIBA A1986A "CE Separation of N-Linked Oligosaccharides Released from Recombinant Monoclonal Antibody

11. Change Summary

[redacted]

Attachment 1

Beckman Coulter Bulletin AIBA A1986A "CE Separation of N-Linked Oligosaccharides Released from Recombinant Monoclonal Antibody

A-1986A

APPLICATION INFORMATION

Protein Characterization

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

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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 IgG_2, was analyzed for N-linked carbohydrates.

BECKMAN COULTER

Attachment 1 (Continued)

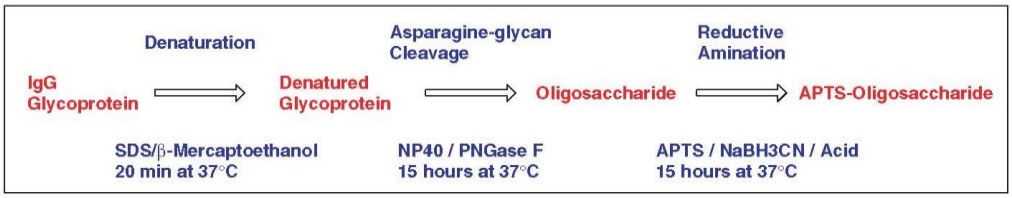

Figure 1. *Process profile for PNGase F digestion and APTS derivatization of IgG glycoproteins.*

Background

An illustration of the step-by-step process for preparing recombinant mouse IgG_2 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 oligosaccharades 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 animation 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 oligosaccharade (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.

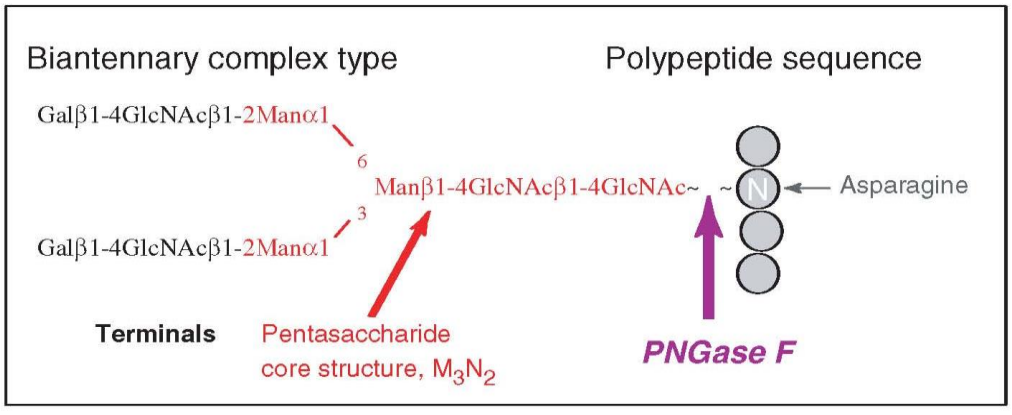

Figure 2. *Schematic diagram of N-Glycosidase, the PNGase F amidase that cleaves between asparagine residues and the innermost GlcNAc of complex N-linked oligosaccharides from glycoproteins.*

Attachment 1 (Continued)

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 ± 10 nm. A neutral coated capillary 50 µm 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 µA. 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 IgG_2 was prepared from ascites fluid obtained within Beckman Coulter. PNGase F, sodium dodecyl sulfate (SDS), ß–mercaptoethanol (ß-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 IgG_2

Approximately 250 µg 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 ß-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 µL of PNGase F (500,000 U/mL) was used to digest 250 µg of IgG_2. 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 µL of a 0.1 M APTS solution in aqueous glacial acetic acid (15 %) and 2 µL 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.

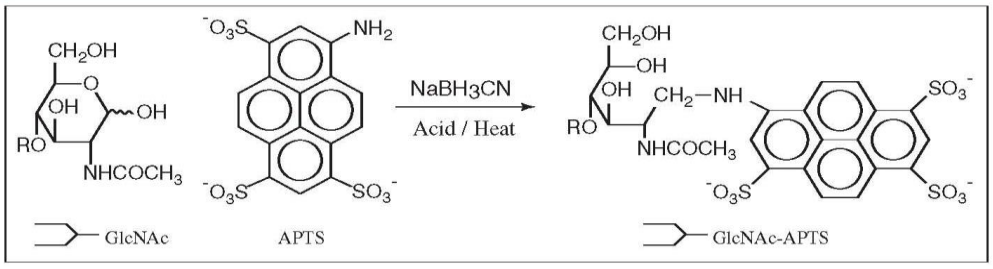

Figure 3. *Reductive amination of the oligosaccharide with APTS (8-aminopyrene-1,3,6-trisulfonate) with presents of acid catalysis and temperature.*

Attachment 1 (Continued)

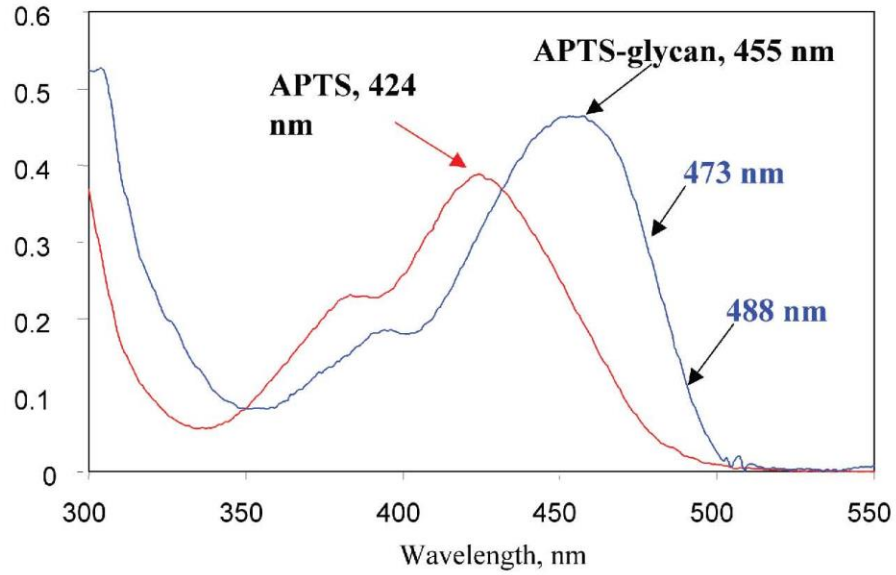

Figure 4. *The excitation spectra of APTS and APTS-labeled glycan adducts.*

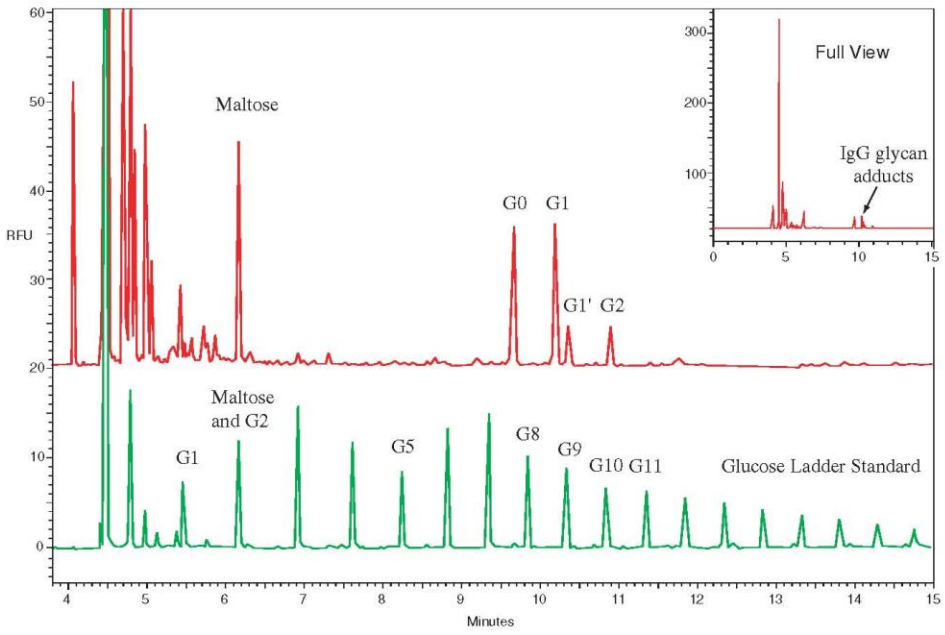

Figure 5. *Electropherograms of profile of APTS-derivatized N-linked oligosaccharides obtained from PNGase F-catalyzed hydrolysis of recombinant monoclonal antibody, mouse IgG_2 (in red) and APTS-detivatized glucose ladder standard (in green). The insert shows a full view of the electropherogram.*

Attachment 1 (Continued)

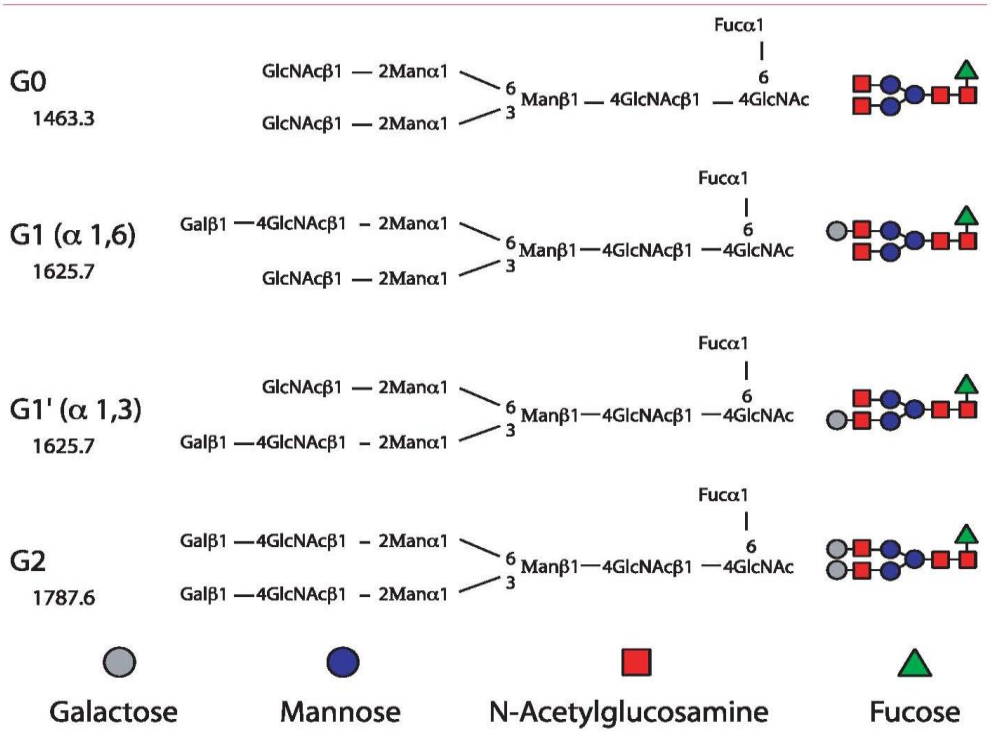

Figure 6. *Schematic representation of four glycans structure. Biantennary N-linked oligosaccharides present on the recombinant monoclonal antibody IgG, which can be resolved by CE-LIF after APTS labeling.*

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 IgG_2, is shown as upper trace in Figure 5. The electrophoresis of four IgG_2 glycans G0, G1, G1′ and G2 (Figure 6) are correlated with the relative positions of the standard maltooligosaccharide peaks. The glycan G2 peak of IgG_2 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 oligosaccaharides released from IgG_2.

A schematic illustration of the glycans observed on the IgG_2 is represented in Figure 6. All glycans are fucosylated with the same branched core structure, three manose and two N-acetylglucosamines (M_3N_2) 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, IgG_2.

5

Attachment 1 (Continued)

References

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