

# Evaluating Protein Glycosylation in Limited-Quantity Samples by HPAE-PAD

Deanna Hurum, Lipika Basumallick, and Jeffrey Rohrer  
Thermo Fisher Scientific, Sunnyvale, CA, USA

## Key Words

Prostate-Specific Antigen (PSA), Transferrin, N-Glycans, O-Glycans, Dionex CarboPac Column

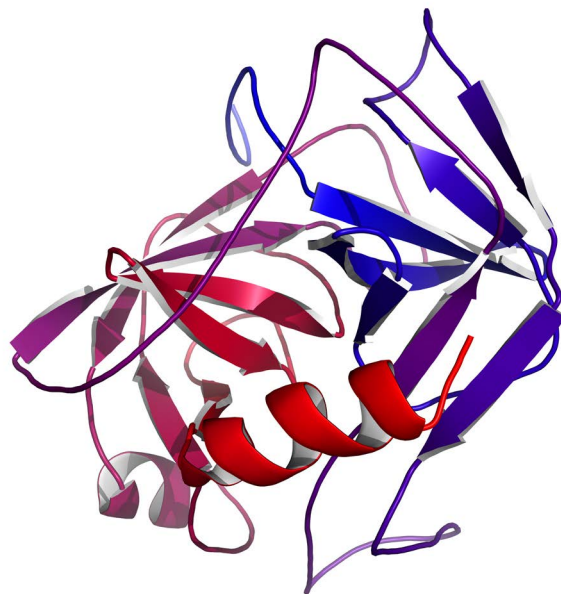
## Introduction

Changes in protein glycosylation are frequently studied in cancer research to identify potential biomarkers. Factors investigated include differences in overall oligosaccharide (glycan) content, monosaccharide content changes, differences in sialylation amount and sialylation linkages, the degree of fucosylation, and differences in glycan branching.

PSA is an example of a well known glycoprotein cancer marker. The glycosylation of this protein is of importance as researchers seek to understand the changes that occur in this protein during carcinogenesis and tumor growth to provide a greater understanding of the disease. PSA is a 28.7 kD protein with a single glycosylation site at Asn-69 (Asn-45 after cleavage of the signal peptide and propeptide regions).<sup>1</sup> It is typically 8% carbohydrate by mass.<sup>2</sup>

Recent investigations that study glycan changes in PSA for the purpose of understanding changes that occur between benign and cancerous states have identified a number of potential glycan biomarkers. Key differences investigated include sialylation and sialyl linkages,<sup>3,4</sup> fucosylation,<sup>5</sup> and the elevated presence of O-glycans which are involved in regulation of cellular growth and function.<sup>6</sup> Further investigations into the mechanisms of O-glycan activity have recently been suggested for tumor growth as well as apoptosis regulation.<sup>7-8</sup>

Many methods are currently available for identification and quantification of glycans. Chromatographic methods include gas chromatography (GC), GC-mass spectrometry (MS), high-performance liquid chromatography (HPLC), LC-MS, capillary electrophoresis, and HP anion exchange chromatography with pulsed amperometric detection



(HPAE-PAD). For many of these methods, glycan derivatization is a common step to enhance detection sensitivity and improve chromatographic retention and resolution. For GC methods, derivatization is needed to increase the volatility of the glycans for GC separation.

In contrast to these methods, HPAE-PAD does not require modification of the glycans before analysis. As is true of many biomarkers, the amounts of glycoproteins available for research are limited, adding additional challenges to work assessing changes in glycosylation. The sensitivity of PAD allows this analysis to be performed with sample quantities as low as 3–10 µg of protein. Specificity is ensured by both the separation and the electrochemical detection that has been optimized for carbohydrate analysis.

In this study, the glycosylation of human transferrin (as a model of PSA) and PSA are investigated by HPAE-PAD using <10 µg of protein. Two methods are utilized to evaluate protein glycosylation. One method is used to investigate the N-linked oligosaccharides and glycan sialylation. A second method evaluates the potential presence of O-linked glycans by monitoring monosaccharide composition. By using a combination of acid hydrolysis and enzymatic digestion steps prior to HPAE-PAD analysis, information about the glycosylation, as well as linkages within the glycans, is determined.

These methods provide a cost-effective option for investigating glycans without the derivatization required by many other methods. Electrochemical detection determines the glycans directly without the potential loss of sialylation or linkage rearrangement that may occur during analysis with other methods. HPAE-PAD provides a direct-detection method without the additional time and reagent costs needed for methods that rely on derivatization for effective separation and detection.

### Goal

To develop HPAE-PAD methods to evaluate glycoprotein glycosylation using <10 µg amounts of protein

### Equipment

- Thermo Scientific™ Dionex™ ICS-3000/5000+ system, including:
  - SP Single Pump or DP Dual Pump
  - DC Detector/Chromatography Compartment
  - Dionex AS or AS-AP Autosampler
  - EG Eluent Generator
  - ED Electrochemical Detector (without Cell, P/N 079830)
  - ED Cell with Reference Electrode and Spacer Block (P/N AAA-061756)
  - Disposable Electrodes for Carbohydrates (P/N 060139): for oligosaccharide analysis
  - Gold on PTFE Disposable Electrode (P/N 066480): for monosaccharide analysis
  - pH-Ag/AgCl Reference Electrode (P/N 061879)
  - 10 µL PEEK™ Sample Loop (P/N 042949)
- Thermo Scientific™ Savant™ SPD131DDA SpeedVac™ Concentrator
- Fisher Scientific™ accuSpin™ Micro17 Microcentrifuge (Fisher Scientific P/N 13-100-675)
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software was used for all data acquisition and processing.

### Additional Equipment for Monosaccharide Determination Using Eluent Generation

- Thermo Scientific Dionex EGC III KOH Eluent Generator Cartridge (P/N 074532)
- Thermo Scientific Dionex CR-ATC Continuously Regenerated Anion Trap Column (P/N 060477)
- EG Vacuum Degas Conversion Kit (P/N 063353)

### Additional Equipment for Protein Sample Concentration Determination

- Thermo Scientific™ NanoDrop™ 2000c Spectrophotometer (P/N ND-2000c)

### Consumables

- Vial Kit, 0.3 mL Polypropylene with Caps and Septa (P/N 055428)
- Vial Kit, 1.5 mL Polypropylene with Caps and Septa (P/N 079930)
- Thermo Scientific™ Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with Nylon Membrane (1000 mL, 0.2 µm pore size, Fisher Scientific P/N 09-740-46)
- Polypropylene Microcentrifuge Screw Cap Tubes, 1.5 mL (Sarstedt® P/N 72.692.005)
- 10 KD MWCO Centrifugal Filters, Amicon® Ultra-0.5 mL (Millipore P/N UFC501024)
- 30 KD MWCO Centrifugal Filters, Amicon Ultra-0.5 mL (Millipore P/N UFC503024)

### Reagents and Standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistivity or better
- Sodium Hydroxide, 50% w/w (Fisher Scientific P/N SS254-500)
- Sodium Acetate, anhydrous, electrochemical grade (P/N 059326)
- Hydrochloric Acid, 6N, 1 mL ampules (Thermo Scientific P/N 24308)
- Sodium Chloride (Fisher Scientific P/N S671-500)
- Sodium Azide (Fisher Scientific P/N BP922I-500)
- G0 (NGA2) (Fisher Scientific P/N NC0145854)
- G1F (NA2G1F) (Fisher Scientific P/N NC9603959)
- MAN-6 (Fisher Scientific P/N NC9071406)
- G2bF (NA2FB) (Fisher Scientific P/N NC0145860)
- MAN-9 (Fisher Scientific P/N 50-355-869)
- A1F (Fisher Scientific P/N NC9603958)
- A2F (Fisher Scientific P/N NC9698597)
- PNGase F, 15,000 units (equivalent to 500,000 U/mL). A unit is defined as the amount of enzyme required to remove >95% of carbohydrate from 10 µg of denatured RNase B in 1 h at 37 °C in a total reaction volume of 10 µL (Fisher Scientific P/N 50-811-832). Enzyme is supplied with: 10 × glycoprotein denaturing buffer (5% SDS, 10% β-mercaptoethanol), 10 × G7 buffer (0.5 M sodium phosphate, pH 7.5 at 25 °C), and 10% NP-40.

- Neuraminidase, 2000 units (equivalent to 50,000 U/mL). One unit is defined as the amount of enzyme required to cleave >95% of terminal  $\alpha$ -Neu5Ac from 1 nmol Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc-7-amino-4-methyl-coumarin (AMC) in 5 min at 37 °C in a total reaction volume of 10  $\mu$ L (Fisher Scientific P/N 50-811-834, cloned from *Clostridium perfringens* and overexpressed in *E. coli*).
- $\alpha$ 2-3 Neuraminidase, 2500 units (equivalent to 50,000 U/mL). One unit is defined as the amount of enzyme required to cleave >95% of the  $\alpha$ -Neu5Ac from 1 nmol of AMC in 1 h at 37 °C in a total reaction volume of 10  $\mu$ L (Fisher Scientific P/N 50-811-844, cloned from *Salmonella typhimurium* LT2 and overexpressed in *E. coli*).
- $\alpha$ -L-Fucosidase from bovine kidneys, 1 unit (13 units/mg). One unit will hydrolyze 1.0  $\mu$ mole of p-nitrophenyl  $\alpha$ -L-fucoside to p-nitrophenol and L-fucose per min at pH 5.5 at 25 °C (Sigma-Aldrich® P/N F5884).

### Samples

- Human Transferrin, 10 mg/mL as supplied (Fisher Scientific P/N NC9583465)
- Human Transferrin (Sigma-Aldrich P/N T8158)
- Human Prostate Specific Antigen, 5.9 mg/mL as supplied (Lee Biosolutions P/N 497-11)
- Human Transferrin, spiked with porcine mucin (Sigma-Aldrich P/N M-2378)
- Pierce™ Human Polyclonal IgG (Thermo Scientific P/N PA1-31154)

### Conditions: Oligosaccharides

Columns:	Thermo Scientific™ Dionex™ CarboPac™ PA200 Guard, 3 × 50 mm (P/N 062895) Dionex CarboPac PA200 Analytical, 3 × 250 mm (P/N 062896)
Eluents:	A) DI water B) NaOH, 100 mM C) Sodium acetate, 200 mM, in 100 mM NaOH
Eluent Gradient:	50–100 mM NaOH from 0 to 30 min, 100 mM NaOH from 30 to 35 min, 0–200 mM NaOAc in 100 mM NaOH from 35 to 50 min, 200 mM NaOAc from 50 to 60 min; equilibration at 50 mM NaOH for 15 min before injection
Flow Rate:	0.5 mL/min
Inj. Volume:	5 $\mu$ L (partial loop)
Temperature:	30 °C (column and detector compartments)
Detection:	Pulsed amperometric, disposable Au electrode, carbohydrate certified
Background:	~18 nC (using the carbohydrate 4-potential waveform, Table 1) <sup>9</sup>
Noise:	~50 pC
System Backpressure:	~2900 psi

Table 1. Carbohydrate 4-potential waveform for the ED.

Time (s)	Potential (V)	Gain Region*	Ramp*	Integration
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	-2.0	Off	On	Off
0.42	-2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	-0.1	Off	On	Off
0.50	-0.1	Off	On	Off

\*Settings required in the Dionex ICS-3000/5000\* system but not used in older Dionex systems

Reference electrode in Ag mode (Ag/AgCl reference)

### Preparation of Solutions and Reagents

#### Eluent Solutions: Oligosaccharides

##### NaOH, 100 mM

Add 5.2 mL of 50% w/w NaOH to 994.8 mL of degassed deionized water to prepare 1 L of 100 mM NaOH. Proportion the 100 mM hydroxide solution with DI water to produce the described hydroxide gradient.

##### Sodium acetate, 200 mM, in 100 mM NaOH

Dissolve 16.4 g of sodium acetate in 400 mL of DI water. Once the solid has dissolved, dilute the solution with an additional 400 mL of DI water. Filter degas the solution through a 0.2  $\mu$ m nylon filter unit and transfer the solution to a 1 L polypropylene volumetric flask. Pipette 5.2 mL of 50% w/w NaOH into the volumetric flask. Fill the flask to the mark with degassed DI water. Promptly transfer the solution to the Dionex ICS-5000\* system and maintain an inert headspace of ultrahigh purity (UHP)-grade nitrogen or UHP helium gas. See Dionex (now part of Thermo Scientific) Technical Note 71 for detailed information on eluent preparation.<sup>10</sup>

#### Reagents

##### Sodium chloride, 100 mM

Dissolve 74.75 mg of sodium chloride, anhydrous, in 12.75 g of DI water.

##### Sodium azide, 1 mg/mL

Dissolve 13.25 mg of sodium azide in 13.25 g of DI water.

##### Sodium acetate buffer, 25 mM, pH 5

Dissolve 2.50 g of sodium acetate in 100.27 g (100.27 mL) of DI water to prepare a 0.3 M sodium acetate stock solution. Dilute 22.5 mL of glacial acetic acid with 77.5 mL of DI water to prepare a 4.0 M acetic acid stock solution. Add 17.0 mL of 0.3 M sodium acetate and 0.45 mL of 4.0 M acetic acid to a 250 mL volumetric flask. Dilute to the mark with DI water.

##### Human transferrin, 4 mg/mL

Gently dissolve 8.3 mg/mL human transferrin in 2.0 mL of DI water. If the transferrin is supplied dissolved in a buffer, dilute a 10 mg/mL solution by adding 200  $\mu$ L of transferrin solution to 300  $\mu$ L of DI water to prepare a 4 mg/mL solution.

**Mucin, 2.5 mg/mL**

Gently dissolve 13.14 mg of porcine mucin in 1.3 mL of DI water to prepare a viscous suspension of 10 mg/mL mucin. Dilute 250  $\mu$ L of the 10 mg/mL suspension to a total of 1 mL with DI water to prepare a nominal 2.5 mg/mL solution of mucin.

**Conditions: Monosaccharides<sup>13</sup>**

Columns:	Thermo Scientific™ Dionex™ AminoTrap,™* 3 × 30 mm (P/N 060146) Dionex CarboPac PA20 Analytical, 3 × 150 mm (P/N 060142)
Eluent:	10 mM potassium hydroxide (KOH)
Eluent Source:	Dionex EGC III KOH Cartridge with Dionex CR-ATC Continuously Regenerated Anion Trap Column
Flow Rate:	0.5 mL/min
Inj. Volume:	5 $\mu$ L (partial loop)
Temperature:	30 °C (column and detector compartments)
Detection:	Pulsed amperometric, disposable Au on PTFE electrode
Background:	~30 nC (using the carbohydrate 4-potential waveform, Table 1)
Noise:	~20 pC
System Backpressure:	~2200 psi

\*Installed before the Dionex CarboPac PA20 column

The waveform used for monosaccharide analysis is the same as applied for oligosaccharides (Table 1).

**Eluent Solutions: Monosaccharides**

Generate the KOH eluent on line by pumping high-quality degassed DI water through the Dionex EGC III KOH cartridge. The Chromeleon CDS software will track the amount of KOH used and calculate the remaining cartridge lifetime.

If the Dionex ICS-3000/5000+ system does not have eluent generation and the degas conversion kit has not been installed, eluents for monosaccharide analysis can be manually prepared. See Dionex (now part of Thermo Scientific) Technical Note 71 for detailed information on eluent preparation.<sup>10</sup>

**Stock Standard Solutions: Monosaccharide Standards**

Dissolve the contents of one Thermo Scientific™ Dionex™ MonoStandard™ 100 nmol vial in 1.0 mL of DI water and mix to prepare a stock standard solution containing 0.1 mM (100 pmol/ $\mu$ L) of each monosaccharide. Dilute this stock to prepare standards at concentrations of 0.05  $\mu$ M (0.25 pmol), 0.10  $\mu$ M (0.50 pmol), 0.50  $\mu$ M (2.5 pmol), 1.0  $\mu$ M (5.0 pmol), 5.0  $\mu$ M (25 pmol), and 10  $\mu$ M (50 pmol). For example, add 100  $\mu$ L of the stock solution to 900  $\mu$ L of DI water to prepare a 10  $\mu$ M standard that equates to 50 pmol when 5  $\mu$ L are injected.

Immediately freeze unused stock standard at <-10 °C.

**Sample Preparation: Oligosaccharides**

Sample preparation was performed by modified versions of previously described protocols.<sup>11-13</sup>

**PNGase F digestion with denaturing: large scale**

The PNGase F solution is supplied as 30  $\mu$ L of solution. Add 270  $\mu$ L of DI water to the PNGase F solution to prepare 300  $\mu$ L of 50,000 U/mL enzyme.

**Digestion control sample**

Add 752  $\mu$ L of DI water to a 1.5 mL microcentrifuge vial. Add 96.0  $\mu$ L of 100 mM sodium chloride to match the saline content of many buffered protein solutions. Add 57  $\mu$ L of 1 mg/mL sodium azide to match the preservative concentration in many buffered protein solutions. Add 13  $\mu$ L of denaturant provided with the PNGase F.

**Protein samples**

Add 688  $\mu$ L of DI water to a 1.5 mL microcentrifuge vial. Add 64.0  $\mu$ L of 4 mg/mL human transferrin solution. If not already present, add 96.0  $\mu$ L of 100 mM sodium chloride and 57  $\mu$ L of 1 mg/mL sodium azide as with the digestion control sample described above. Add 13  $\mu$ L of denaturant provided with the PNGase F.

Prepare a mucin sample by replacing the 64.0  $\mu$ L of human transferrin with 10  $\mu$ L of mucin solution and 54  $\mu$ L of DI water.

Prepare a transferrin sample spiked with mucin by reducing the amount of DI water by 10  $\mu$ L and adding 10  $\mu$ L of mucin solution.

Incubate each sample at 100 °C for 10 min to denature protein.

After protein denaturation, treat each sample as follows:

- Add 9.6  $\mu$ L of NP40 to protect the PNGase F from denaturation.
- Add 9.6  $\mu$ L of 10 × G7 followed by 22.4  $\mu$ L of 10-fold diluted PNGase F. Gently mix the solution.
- Incubate the samples at 37 °C for 20 h (overnight).

**PNGase F digestion with denaturing: small scale****Digestion control sample**

Add 44.7  $\mu$ L of DI water to a 1.5 mL microcentrifuge vial. Add 9.0  $\mu$ L of 100 mM sodium chloride to match the saline content of buffered protein solutions. Add 3.6  $\mu$ L of 1 mg/mL sodium azide to match the preservative concentration in many buffered protein solutions. Add 0.8  $\mu$ L of denaturant provided with the PNGase F.

**PSA samples**

Add 56.8  $\mu$ L of DI water to a 1.5 mL microcentrifuge vial. Add 3.0  $\mu$ L of 5.9 mg/mL human PSA solution. Add 0.8  $\mu$ L of denaturant as provided with the PNGase F. Incubate each sample at 100 °C for 10 min.

After protein denaturation, add 0.70  $\mu$ L of NP40 to each sample. Then:

- Add 0.70  $\mu$ L of G7 followed by 1.4  $\mu$ L of 10-fold diluted PNGase F to each sample.
- Briefly centrifuge the sample to ensure the solutions mix.
- Incubate the samples at 37 °C for 20 h.

### Separation of released glycans from protein

Glycans were separated from the remaining protein by a two-step process:

- Remove detergents and salts remaining from the PNGase F digest by ultracentrifugation in a 10 kD MWCO filter. Transfer the retentate (proteins and glycans) from this filter to a 30 kD MWCO filter with an additional 40  $\mu$ L of DI water.
- Centrifuge the solution to separate the glycans (filtrate) from the proteins in the retentate. Confirm protein removal by UV absorbance at 280 nm using a 2  $\mu$ L sample and a NanoDrop 2000c spectrophotometer.

Glycan retention by the filter was confirmed by recovery experiments of glycans released from human transferrin by PNGase F. These samples were released without denaturing so that the glycan recovery could be determined by injections of PNGase F-released transferrin glycans prior to filtration followed by injections of the filtrate and retentate. Although some glycans pass through the filter and are detected in the filtrate (typically between 7–23%), greater than 76% of the transferrin glycans were retained. The loss of transferrin glycans from the retentate did not appear to be correlated with the size or charge, suggesting nonselective loss (data not shown).

This method provided the best recoveries for these limited-quantity samples compared to other methods to desalt, remove detergents, and remove the proteins prior to HPAE-PAD analyses.

Precautionary Note: This method has been tested and applied for proteins with primarily branched sialylated glycans. For proteins that are known to contain neutral glycans, other methods are available and should be followed.<sup>11-12</sup>

#### Centrifugal filter preparation

Assemble the filter unit by inserting the filter into the provided centrifuge tube. Load each filter with 500  $\mu$ L of DI water and centrifuge at 14,000  $\times$ g for 10 min. Invert the filter and reinsert it into the centrifuge tube. Centrifuge briefly at 2000  $\times$ g to remove the retentate. Discard the water wash. Repeat this process a second time to ensure removal of any humectants present in the filter.

#### PNGase F digest sample preparation

For each sample, load 60  $\mu$ L (or in the case of the small-scale protein digest, load the entire sample) into a 10 kD MWCO filter unit. Centrifuge the sample at 14,000  $\times$ g for 15 min. Collect the retentate (20  $\mu$ L) for further preparation. Transfer the retentate into a 30 kD MWCO filter unit. Add an additional 40  $\mu$ L of DI water to the filter unit with the retentate. Centrifuge the sample for 15 min at 14,000  $\times$ g for 15 min. Collect the filtrate for injection and further work. The retentate contains the PNGase F and the protein of interest. This may be saved for other analyses if desired.

### $\alpha$ 2-3/2-6 Neuraminidase digestion of released glycans

Dilute the 40  $\mu$ L solution of neuraminidase 1:10 by adding 360  $\mu$ L of 25 mM sodium acetate, pH 5.0, to prepare a solution of 5000 U/mL. Add 5.0  $\mu$ L of the diluted neuraminidase to 5.0  $\mu$ L of the glycans isolated as described above. Incubate the samples at 37 °C for 1 h. Transfer the samples to a 300  $\mu$ L autosampler vial and keep at 6 °C until injection. Store unused neuraminidase at –40 °C.

#### $\alpha$ 2-3 Neuraminidase digestion of released glycans

In a 300  $\mu$ L autosampler vial, add 0.5  $\mu$ L of  $\alpha$ 2-3 neuraminidase to 10  $\mu$ L of glycans isolated as described in the PNGase F digest sample preparation section. Ensure that the enzyme and sample are well mixed by gently tapping the vial to move all solution to the bottom of the vial and remove any air bubbles that may be present. Alternatively, centrifuge the sample briefly. Incubate the samples at 37 °C for 1 h. Keep the samples at 6 °C until injection.

#### $\alpha$ -L-Fucosidase digestion of released glycans

Add 2000  $\mu$ L of DI water to the supplied fucosidase suspension of 1 unit in 80  $\mu$ L of 3.2 M  $(\text{NH}_4)_2\text{SO}_4$ , 10 mM  $\text{NaH}_2\text{PO}_4$ , and 10 mM citrate (pH 6.0) to prepare a suspension of 0.04 U/mL in 120 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.38 mM  $\text{NaH}_2\text{PO}_4$ , and 0.38 mM citrate. Add 5  $\mu$ L of this diluted fucosidase suspension to 5  $\mu$ L of PSA PNGase F digest and incubate the solution for 20 h at 37 °C. Store samples at –40 °C, if necessary, before analysis.

### Sample Preparation: Monosaccharides Acid hydrolysis

Open a fresh ampule of 6 N hydrochloric acid.

Add 100  $\mu$ L of 6 N hydrochloric acid to a 1.5 mL microcentrifuge vial. Add 5  $\mu$ L of released glycans from PSA to the hydrochloric acid. Similarly, add 5  $\mu$ L of glycans released from transferrin, 5  $\mu$ L of glycans released from transferrin spiked with mucin, and 5  $\mu$ L of mucin solution to individual 1.5 microcentrifuge vials containing 100  $\mu$ L of 6 N hydrochloric acid. Prepare two control samples: 5  $\mu$ L of PNGase control that has been prepared in the same manner as the samples, and 5  $\mu$ L of DI water. For comparison to the released glycans, hydrolyze samples of 0.5  $\mu$ L of PSA (2.9  $\mu$ g of protein) in 100  $\mu$ L of 6 N hydrochloric acid.

Hydrolyze samples for 4 h at 100 °C. After hydrolysis, dry the samples in a SpeedVac concentrator equipped with an acid trap. Dissolve the samples in 30  $\mu$ L of DI water prior to analysis.

Note: These conditions are optimized for release of amino sugars and will significantly degrade other monosaccharides. See Thermo Scientific Technical Note 40 for alternate hydrolysis methods.<sup>13</sup>



## Precautions

- Carbohydrates have limited stability unless sterility is maintained. Store solutions and samples at  $-40^{\circ}\text{C}$ . Avoid multiple freeze/thaw cycles to preserve the carbohydrates.
- Thoroughly remove glycerol or polyethylene glycol from filters before use to avoid interferences in HPAE-PAD analyses. Similarly, be sure to use glycerol- (glycerin-) free PNGase F.
- The buffers used during the enzymatic digests can lead to column overload due to high salt concentrations. If larger sample volumes are injected, this must be considered and control experiments are recommended to evaluate column loading. Desalting of PNGase F digests may be necessary if sample preparation methods other than those described in this work are used.
- When using a Dionex ICS-3000/5000+ EG Eluent Generator, it is critical to install the Vacuum Degass Conversion Kit (P/N 063353). This degasser will remove gasses generated by the EG and help maintain a stable baseline. This kit is not necessary when preparing eluents manually.

## Health Precaution

When working with human- or animal-derived fluids, appropriate Biosafety Level protocol must be followed. For guidance, see the Center for Disease Control publication *Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5<sup>th</sup> Edition*.<sup>14</sup>

## Results and Discussion

Human PSA was analyzed following the scheme in Figure 1. Human transferrin was used as a model protein to assess the method following PNGase F digestion. Although human transferrin is a larger protein—with a MW of 77 kD compared to PSA at 28.7 kD—it has two glycosylation sites leading to a similar total glycosylation amount of ~6–9% by weight compared to 8% for PSA.<sup>2,15</sup> In both cases, the glycans are dominated by mono- and disialylated biantennary glycans with minor amounts of neutral glycans,<sup>5,15</sup> although higher-order branched glycans have been identified in human transferrin.<sup>16</sup>

Transferrin was analyzed as a model protein to determine monosaccharide content as well as to profile the *N*-linked glycans. The monosaccharide content of PSA was determined for both the intact protein and for the isolated *N*-glycans. A selection of *N*-linked glycan structures commonly investigated in human proteins is shown in Table 2. For the sake of brevity, one glycan, G0, is shown afucosylated. However, glycans may be present fucosylated or afucosylated. Carbohydrate structure schematics are based on the nomenclature of the Center for Functional Glycomics.<sup>17</sup>

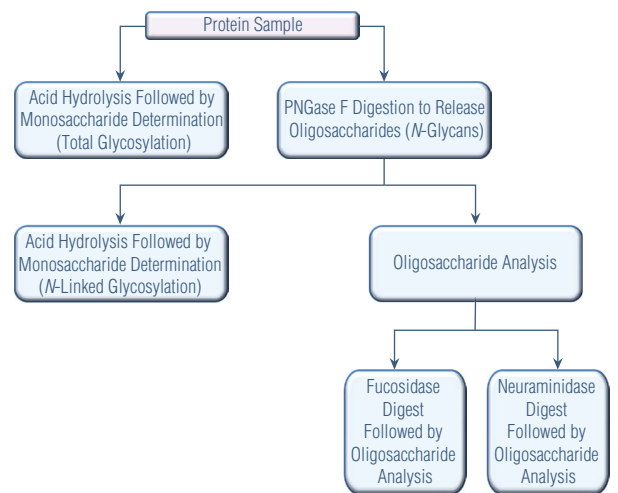


Figure 1. Schematic of glycan analysis workflow for PSA samples.

Table 2. A selection of commonly investigated glycans and their nomenclature. Afucosylated glycans of the shown fucosylated structures, as well as other branched glycans, may be found on glycoproteins. Structures adapted from Center for Functional Glycomics (CFG) standards.

Glycan (Oxford)	mAb Acronym	Structure (Adapted from CFG)
NGA2F (FA2G0)	G0F	
NA2G1F (FA2G1)	G1F	
NA2F (FAG2)	G2F	
NA2FB (FABG2)	G2bF	
G2FA1 (FA2G2S1)	A1F	
G2FA2 (FAG2S2)	A2F	
NGA2 (A2G0)	G0	
Man3	M3	
Man5	M5	
Man6	M6	

- *N*-acetylglucosamine (GlcNAc)
- ▲ Fucose (Fuc)
- Mannose (Man)
- Galactose (Gal)
- ◆ *N*-acetylneuraminic acid (Neu5Ac)

## Comparative Monosaccharide Determination

As an indicator of *O*- and *N*-glycosylation differences, the amounts of galactosamine (GalN) and glucosamine (GlcN) were determined by hydrochloric acid hydrolysis of both the intact proteins and released glycans after protein PNGase F digestion, followed by glycan separation from the remaining protein. To test the viability of this approach, glycans were released from human transferrin via PNGase F and then hydrolyzed with HCl. At the same time, human transferrin that had been spiked with porcine mucin was treated similarly. Porcine mucin primarily contains abundant *O*-glycans with potential *N*-linked glycan sites that have been identified.<sup>18</sup> The addition of mucin to transferrin will simulate changes in *O*-glycosylation (three sites) or contamination of a sample with 8% (by weight) of a highly *O*-linked glycoprotein.

Figure 2 shows the separation of monosaccharides released from transferrin by hydrochloric acid to investigate the amino sugars present. As shown in Chromatogram 2C, the transferrin digest contained primarily glucosamine with no detectable galactosamine. When mucin was treated similarly, there was much less carbohydrate content overall; however, there were significant amounts of galactosamine and glucosamine (Chromatogram 2A). When the mixed transferrin/mucin sample was analyzed, glucosamine and galactosamine were both present, indicating that the presence of mucin-type glycans, and therefore *O*-linked glycans, can be identified (Chromatogram 2B).

By repeating this process for two samples of PSA—one that has been treated with PNGase F and one that has not—the potential presence of *O*-glycans can be determined. Figure 3 illustrates the separation of monosaccharides present in PSA and PSA that has been treated with PNGase F. In both cases, both glucosamine and galactosamine were present in roughly the same molar ratio of 0.07. This suggests that for this PSA sample, the galactosamine was not a result of *O*-glycosylation but is associated with the PSA *N*-glycans, or that there was a contaminating protein with *O*-glycosylation that is not removed during the sample preparation to separate protein from released *N*-glycans.

## Oligosaccharide Analysis

In order to investigate the neutral glycans that may be present and to better identify the neutral glycans after neuraminidase digestion, a two-part gradient method was developed. The first portion of this gradient is a 50–100 mM sodium hydroxide gradient that focuses on separating the neutral glycans that may be present. The second portion of the gradient is a 0–200 mM acetate gradient in 100 mM sodium hydroxide. This portion of the gradient elutes the larger oligomannose species and the charged glycans. If individual neutral glycans are not of interest, a simpler method of a sodium acetate gradient in 100 mM NaOH can be used, as has been previously published using human transferrin<sup>11</sup> and IgG,<sup>12</sup> as examples. This method separates the charged glycans well, providing a rugged method for profiling the sialylated glycans.

Columns: Dionex CarboPac PA20, 3 × 150 mm  
Dionex AminoTrap, 3 × 30 mm  
Eluent: 10 mM KOH, 10 min 100 mM KOH wash followed by 10 min of equilibration at 10 mM KOH  
Eluent Source: Dionex EGC III Cartridge and Dionex CR-ATC Column  
Flow Rate: 0.5 mL/min  
Inj. Volume: 5 µL  
Temp: 30 °C  
Samples: Hydrochloric acid hydrolyzate of PNGase F digests of  
A) mucin, B) human transferrin spiked with mucin,  
C) human transferrin, and D) monosaccharide standards

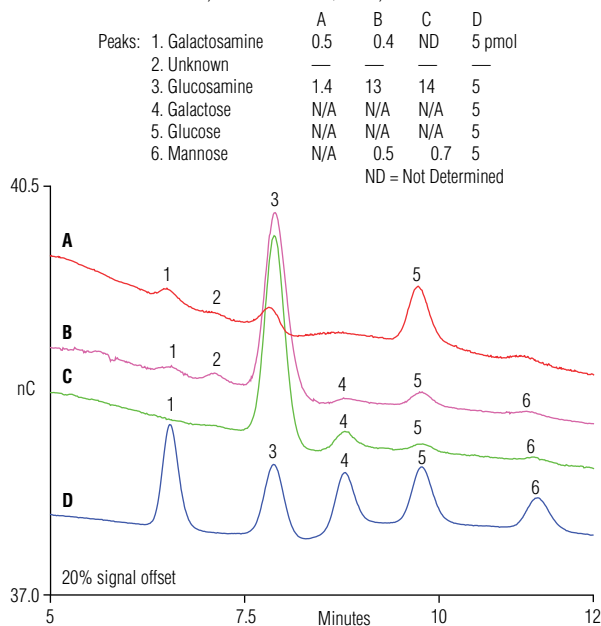


Figure 2. Separation of transferrin acid hydrolysates on a Dionex CarboPac PA20 column. The presence of contaminating mucin as an *O*-glycan source can be identified by the presence of galactosamine in Chromatogram B.

Columns: Dionex CarboPac PA20, 3 × 150 mm  
Dionex AminoTrap, 3 × 30 mm  
Eluent: 10 mM KOH, 10 min 100 mM KOH wash followed by 10 min of equilibration at 10 mM KOH  
Eluent Source: Dionex EGC III Cartridge and Dionex CR-ATC Column  
Flow Rate: 0.5 mL/min  
Inj. Volume: 5 µL  
Temp: 30 °C  
Samples: Hydrochloric acid hydrolyzates of A) 0.67 µg PSA, B) PSA glycans after PNGase F release (equivalent to 0.09 µg of protein), and C) monosaccharide standards

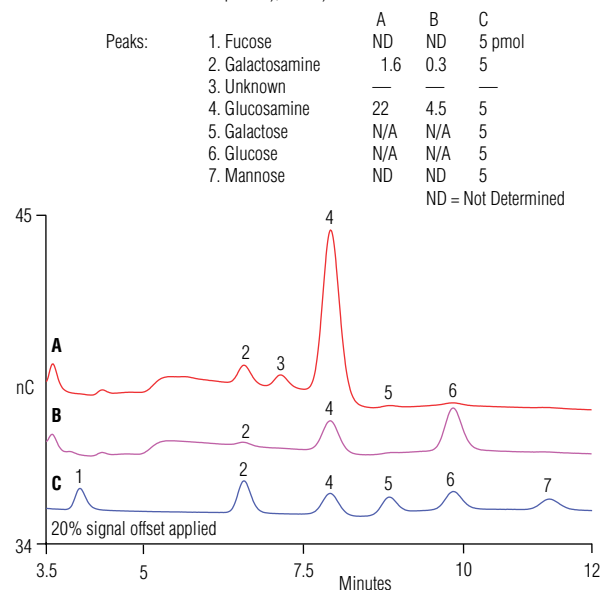


Figure 3. Separation of PSA acid hydrolysates on a Dionex CarboPac PA20 column. Both the total protein hydrolysis and the PSA-*N*-linked glycans have a similar ratio of galactosamine/glucosamine of 0.07. The hydrolysis conditions here do not allow reliable quantification of galactose and glucose.

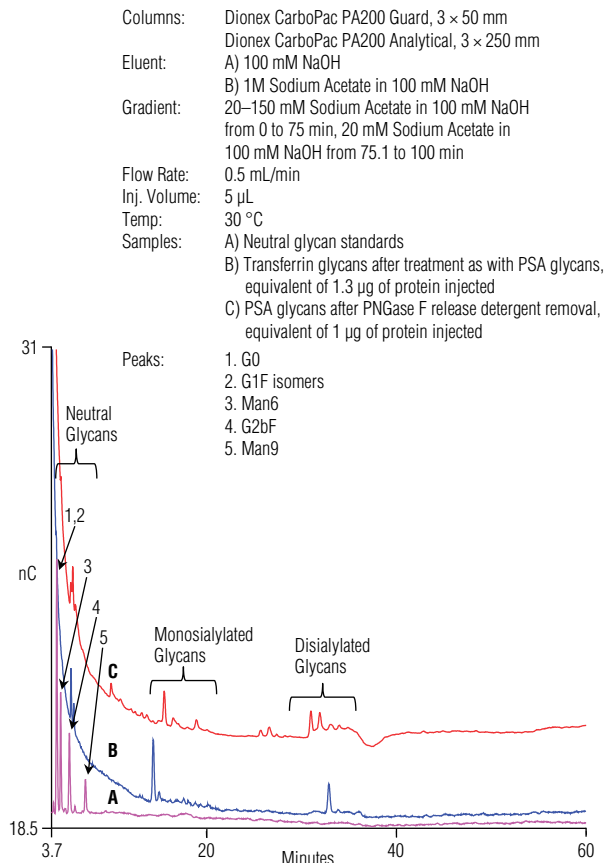


Figure 4. Charged glycan profiling using the Dionex CarboPac PA200 column with a 20–150 mM sodium acetate gradient in 100 mM NaOH. Neutral glycan standards are at a concentration of 5 pmol each. The same neutral glycan standards are also shown in Figure 5.

Figure 4 illustrates the separation of charged glycans with a 20–150 mM acetate gradient in 100 mM sodium hydroxide. In Chromatogram 4A, a standard of neutral glycans has been injected for comparison. The neutral glycans were partially resolved but elute in a region prone to interferences from other reagents in digested samples. Chromatograms 4B and 4C show PSA and transferrin glycans, respectively. The similarity between the glycans of the two proteins is evident in this profile; however, they are not identical.

For both proteins, monosialylated and disialylated glycans were present. The distribution within these classes of glycans show some differences, including a peak present in PSA PNGase F digests that eluted between chromatographic regions associated with the mono- and disialylated glycans, potentially a glycan modified with sulfate or phosphate that will be discussed further in the description of Figure 8. For both proteins, identification of neutral glycans was hindered by the presence of a large weakly retained peak. If charged glycans are of primary interest, the separation with an acetate gradient, as illustrated in Figure 4, provides superior resolution of charged glycans.

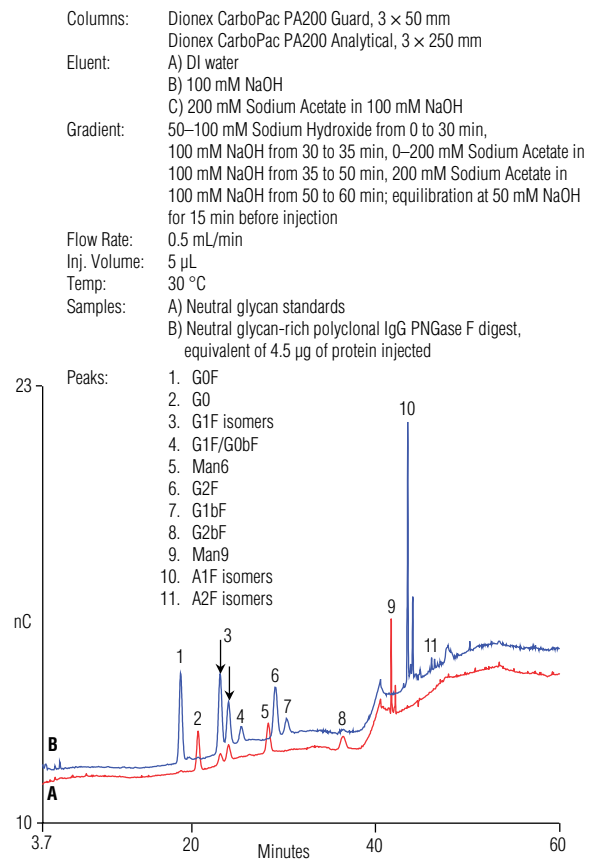


Figure 5. Improved resolution for neutral glycan profiling using the Dionex CarboPac PA200 column with a hydroxide gradient followed by an acetate gradient. Note the improved resolution between neutral glycans compared to the method shown in Figure 4. Human IgG is shown here due to its high proportion of neutral glycans compared to the other glycoproteins investigated for this study.

Separation of neutral glycans was evaluated using multiple gradients, including weak acetate gradients with constant hydroxide concentration<sup>19</sup> and methods that combine isocratic and gradient separations within a method.<sup>20</sup> The final approach chosen for this work—a hydroxide gradient followed by an acetate gradient—was selected based on glycan resolution, the method analysis time, and eluent preparation convenience.

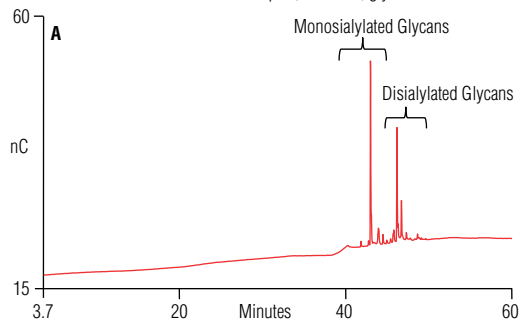
The method illustrated in Figure 5, which used a hydroxide gradient followed by an acetate gradient, allowed excellent separation of the neutral glycans, as illustrated by the chromatogram of a PNGase F digest of polyclonal human IgG. Chromatogram 5A shows the same neutral glycan standard illustrated in Chromatogram 4A. With the method described here, these glycans are well resolved and retained past the void, reducing the potential for interferences from other reagents used during sample digestion and preparation.



Glycans released by PNGase F were identified by comparison to known standards and by enzymatic digestion to determine core glycan structures. The work described here was performed with minimal protein to meet the need to conduct multiple experiments with a total protein sample of 10  $\mu\text{g}$ . Greater amounts of protein, if available, can be digested by PNGase F and analyzed with better sensitivity. If the protein concentration in the digest is increased, the amount of PNGase F must also be increased.

The conditions used for this work were selected based on control experiments with human transferrin that would allow efficient glycan release with minimal denaturant and enzyme. PNGase F and other enzyme digest protocols are available for greater protein amounts.<sup>11</sup> Figure 6

Columns: Dionex CarboPac PA200 Guard, 3  $\times$  50 mm  
Dionex CarboPac PA200 Analytical, 3  $\times$  250 mm  
Eluent: A) DI water  
B) 100 mM NaOH  
C) 200 mM Sodium Acetate in 100 mM NaOH  
Gradient: 50–100 mM Sodium Hydroxide from 0 to 30 min, 100 mM NaOH from 30 to 35 min, 0–200 mM Sodium Acetate in 100 mM NaOH from 35 to 50 min, 200 mM Sodium Acetate in 100 mM NaOH from 50 to 60 min; equilibration at 50 mM NaOH for 15 min before injection  
Flow Rate: 0.5 mL/min  
Inj. Volume: 5  $\mu\text{L}$   
Temp: 30  $^{\circ}\text{C}$   
Sample: PNGase F digestion of human transferrin  
Note: Denaturation was not used for these samples; therefore, glycan release is not complete.



Columns: Dionex CarboPac PA200 Guard, 3  $\times$  50 mm  
Dionex CarboPac PA200 Analytical, 3  $\times$  250 mm  
Eluent: A) 100 mM NaOH  
B) 1 M Sodium Acetate in 100 mM NaOH  
Gradient: 20–150 mM Sodium Acetate in 100 mM NaOH from 0 to 75 min, 20 mM Sodium Acetate in 100 mM NaOH from 75.1 to 100 min  
Flow Rate: 0.5 mL/min  
Inj. Volume: 5  $\mu\text{L}$   
Temp: 30  $^{\circ}\text{C}$   
Sample: PNGase F digestion of human transferrin  
Note: Denaturation was not used for these samples; therefore, glycan release is not complete.

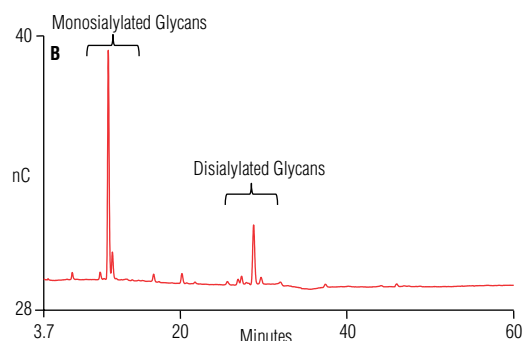


Figure 6. Illustration of typical baseline for injections of human transferrin samples. The equivalent of 4  $\mu\text{g}$  of protein is injected in both chromatograms. Note the improved resolution of charged glycans in Chromatogram B for samples that do not typically contain neutral glycans.

shows examples of human transferrin digests to illustrate the improved signal-to-noise ratio by methods optimized for neutral (Chromatogram 6A) and charged (Chromatogram 6B) glycans by injecting released glycans from the equivalent of 4  $\mu\text{g}$  of protein.

As shown in Figure 7, neutral, monosialylated, and disialylated glycans were present in the PSA sample. These digests were treated with  $\alpha$ 2-3 neuraminidase in order to investigate the linkages of the terminal sialic acids as well as measure the released sialic acid relative to the amount of protein used in the initial digest (Chromatogram 8B). After the  $\alpha$ 2-3 neuraminidase treatment, 3.6 mol Neu5Ac/mol protein were released. At the same time, peaks correlating to G1F, G2F, and G2 increased in relative peak area, suggesting that the Neu5Ac had been present in a  $\alpha$ 2-3 linkage on the G1F and G2F termini (Chromatogram 9B).

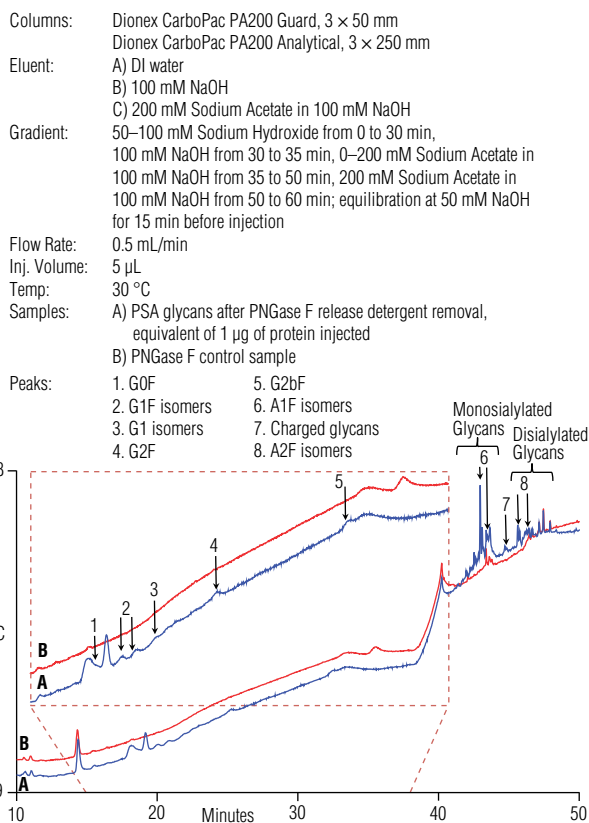


Figure 7. Released PSA oligosaccharides separated on a Dionex CarboPac PA200 column. The elution region of neutral *N*-linked glycans is expanded in the inset. Charged glycans are further expanded in Figure 8.

Further digestion of this sample with a  $\alpha$ 2-3/ $\alpha$ 2-6 neuraminidase released an additional 2.8 mol Neu5Ac/mol protein, removed most of the peaks present in the charged glycan region of the chromatogram, and increased the relative peak area of G2F (Figure 9). This suggests that the Neu5Ac present on remaining glycans are  $\alpha$ 2-6 linked and present as A2F and A1F. Additionally, a single peak persisted that was resistant to sialidase digestion. The glycan eluting at this position is potentially a charged glycan that is not sialylated but may be modified with phosphate or sulfate (Peak 2 in Figure 8).

Columns: Dionex CarboPac PA200 Guard, 3  $\times$  50 mm  
Dionex CarboPac PA200 Analytical, 3  $\times$  250 mm  
Eluent: A) DI water  
B) 100 mM NaOH  
C) 200 mM Sodium Acetate in 100 mM NaOH  
Gradient: 50–100 mM Sodium Hydroxide from 0 to 30 min, 100 mM NaOH from 30 to 35 min, 0–200 mM Sodium Acetate in 100 mM NaOH from 35 to 50 min, 200 mM Sodium Acetate in 100 mM NaOH from 50 to 60 min; equilibration at 50 mM NaOH for 15 min before injection  
Flow Rate: 0.5 mL/min  
Inj. Volume: 5  $\mu$ L  
Temp: 30  $^{\circ}$ C  
Samples: A) PSA glycans after PNGase F release  
B) Glycans after  $\alpha$ 2-3 neuraminidase digestion, equivalent of 1.6  $\mu$ g of protein injected  
C) Glycans after  $\alpha$ 2-3/2-6 neuraminidase digestion; equivalent of 0.8  $\mu$ g of protein injected  
D) A1F and A2F glycan standards  
Peaks: 1. A1F isomers  
2. Neuraminidase-resistant charged glycan  
3. A2F isomers

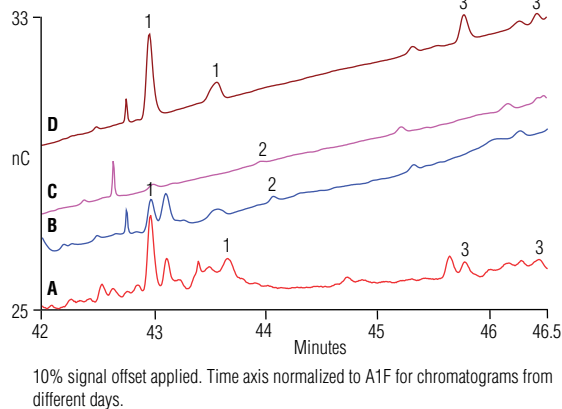


Figure 8. Separation of charged glycans after PNGase F digest followed by neuraminidase digestions. A charged glycan standard of A1F and A2F is shown in Chromatogram D. Note the dramatic reduction of charged glycans after neuraminidase treatment, with the exception of peak 2.

Columns: Dionex CarboPac PA200 Guard, 3  $\times$  50 mm  
Dionex CarboPac PA200 Analytical, 3  $\times$  250 mm  
Eluent: A) DI water  
B) 100 mM NaOH  
C) 200 mM Sodium Acetate in 100 mM NaOH  
Gradient: 50–100 mM Sodium Hydroxide from 0 to 30 min, 100 mM NaOH from 30 to 35 min, 0–200 mM Sodium Acetate in 100 mM NaOH from 35 to 50 min, 200 mM Sodium Acetate in 100 mM NaOH from 50 to 60 min; equilibration at 50 mM NaOH for 15 min before injection  
Flow Rate: 0.5 mL/min  
Inj. Volume: 5  $\mu$ L  
Temp: 30  $^{\circ}$ C  
Samples: A) PSA PNGase F-released glycans  
B) PSA glycans after  $\alpha$ 2-3 neuraminidase, equivalent of 1.6  $\mu$ g of protein injected  
C) PSA glycans after  $\alpha$ 2,3/2-6 neuraminidase, equivalent of 0.8  $\mu$ g of protein injected  
Peaks: 1. G0F 5. G2F  
2. G0 6. G2  
3. Neu5Ac 7. G2bF  
4. G1F isomers

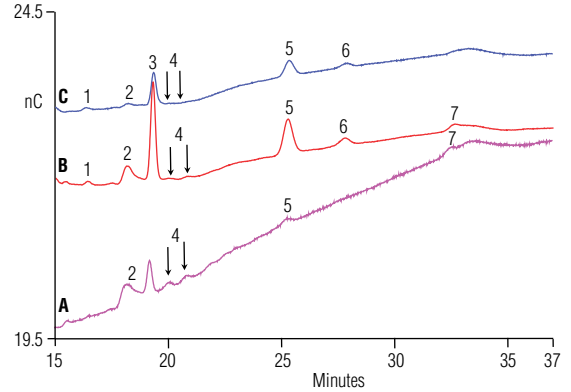


Figure 9. Separation of neutral glycans after PNGase F digest followed by neuraminidase digestions. Note the increase in the relative amounts of neuraminidase and G2F (Peak 5), confirming the presence of A1F and A2F as dominant sialylated glycan species. An increase in G2 (Peak 6) is also observed, indicating the presence of afucosylated sialylated glycans.

Fucosidase digestion of the released glycans further indicated the presence of G2F in the PNGase F-released glycans. Peaks in Chromatogram 10B (G1F isomers, G2F) were removed after fucosidase digestion and a corresponding relative increase in G2 observed, as shown in Chromatogram 10A. Although G1F isomers were present in the released glycans, it is not an abundant glycan, and the combination of dilution during the enzyme digest and the presence of isomers reduced overall sensitivity.

Columns: Dionex CarboPac PA200 Guard, 3 × 50 mm  
Dionex CarboPac PA200 Analytical, 3 × 250 mm  
Eluent: A) DI water  
B) 100 mM NaOH  
C) 200 mM Sodium Acetate in 100 mM NaOH  
Gradient: 50–100 mM Sodium Hydroxide from 0 to 30 min, 100 mM NaOH from 30 to 35 min, 0–200 mM Sodium Acetate in 100 mM NaOH from 35 to 50 min, 200 mM Sodium Acetate in 100 mM NaOH from 50 to 60 min; equilibration at 50 mM NaOH for 15 min before injection  
Flow Rate: 0.5 mL/min  
Inj. Volume: 5  $\mu$ L  
Temp: 30 °C  
Samples: A) Neutral PSA glycans after fucosidase digestion, equivalent of 0.5  $\mu$ g of protein injected  
B) Neutral PSA glycans after PNGase F release, equivalent of 1.0  $\mu$ g of protein injected  
Peaks: 1. G0F 5. G2F  
2. G0 6. G2  
3. Neu5Ac 7. G2bF  
4. G1F isomers

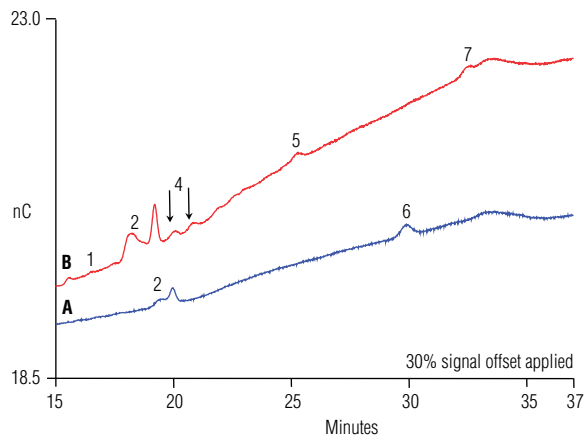


Figure 10. Neutral PSA glycans treated with  $\alpha$ -L-fucosidase. Note the shifting of the G2F peak to later retention correlating with G2. Note that data collected on different days may show retention time shifts with different eluent preparations. For this work, G0 and other neutral glycan standards were used to confirm peak identity (data not shown).

The expected presence of G1 isomers was not confirmed. Peak assignments were made after a corresponding fucosidase digestion of a known sample and standards containing G1F and G2F. Similarly, peaks from charged glycans shifted to longer retention times after fucosidase digestion, suggesting that the majority of the sialylated glycans were also fucosylated, as shown in Figure 11. In this figure, the major mono- and disialylated peaks shift to longer retention times, which is consistent with the behavior observed when treating sialylated glycan standards with fucosidase.

Columns: Dionex CarboPac PA200 Guard, 3 × 50 mm  
Dionex CarboPac PA200 Analytical, 3 × 250 mm  
Eluent: A) DI water  
B) 100 mM NaOH  
C) 200 mM Sodium Acetate in 100 mM NaOH  
Gradient: 50–100 mM Sodium Hydroxide from 0 to 30 min, 100 mM NaOH from 30 to 35 min, 0–200 mM Sodium Acetate in 100 mM NaOH from 35 to 50 min, 200 mM Sodium Acetate in 100 mM NaOH from 50 to 60 min; equilibration at 50 mM NaOH for 15 min before injection  
Flow Rate: 0.5 mL/min  
Inj. Volume: 5  $\mu$ L  
Temp: 30 °C  
Samples: A) Charged PSA glycans after fucosidase digestion, equivalent of 0.5  $\mu$ g of protein injected  
B) A1F and A2F standards  
C) Charged PSA glycans after PNGase F release, equivalent of 1.0  $\mu$ g of protein injected  
Peaks: 1. A1F isomers  
2. A1 isomers  
3. A2F isomers  
4. A2 isomers

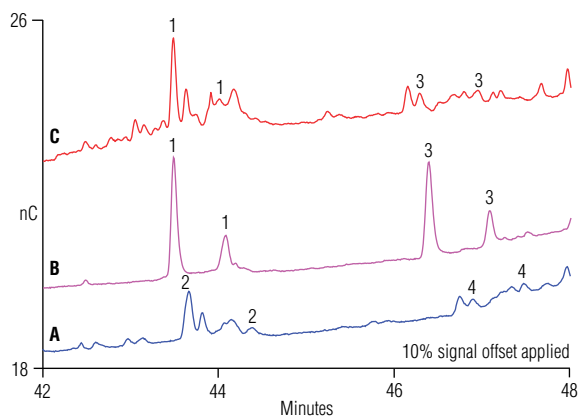


Figure 11. Charged PSA glycans treated with  $\alpha$ -L-fucosidase. Note the shift and reduced number of peaks after fucosidase digest (Chromatogram A), compared to peaks present before fucosidase digestion (Chromatogram C), indicating that both fucosylated and afucosylated sialylated glycans are present in PSA. A fucosylated and sialylated standard (Chromatogram B) is included for comparison.

Identified glycans from seminal plasma PSA were compared to those previously determined by Tabares et al., with the comparative results presented in Table 3.<sup>4</sup> Although the results are not identical, some trends are consistent. In both studies, charged glycans greatly dominated the population of glycans on the protein. These glycans were mostly fucosylated, as indicated by the increase in the concentration of G2F when the glycans were treated with neuraminidase, as well as peak shifting to later retention times after fucosidase digestion. Potential desialylation of the protein during handling is always a risk. The samples investigated seem to be less sialylated than those previously studied; however, there is much debate in the literature regarding the PSA glycan composition.<sup>3-5, 21,22</sup>

### Conclusion

By combining HPAE-PAD analysis with acid hydrolysis and enzymatic digestion of protein glycans, information about the glycan identity as well as terminal carbohydrate linkage isomers can be determined from small amounts of protein (0.5–1.6 µg per injection). This study investigated human PSA to evaluate the potential presence of O-linked glycans and provided a detailed set of experiments to identify the N-linked glycans present on the protein. The methods described here directly determine the carbohydrates present, without additional labeling steps that are often needed for other analysis methods, thus saving time and reagent costs.

### Acknowledgement and References

PSA samples were generously donated by Lee Biosolutions as part of the Association of Biomolecular Research Facilities (ABRF) study glycoprotein research group (gPRG) 2012. The authors would like to thank the ABRF gPRG group for organization of the study.

1. The UniProt Consortium, Reorganizing the Protein Space at the Universal Protein Resource (UniProt), *Nucleic Acids Res.* 2012, 40, D71-D75, Human PSA (KLK3\_HUMAN) <http://www.uniprot.org/uniprot/P07288> (accessed Jan 25, 2013).
2. Belanger, A.; van Halbeek, H.; Graves, H.C.; Grandbois, K.; Stamey, T.A.; Huang, L.; Poppe, I.; Labrie, F. Molecular Mass and Carbohydrate Structure of Prostate Specific Antigen: Studies for Establishment of an International PSA Standard. *Prostate* 1995, 27, 187–197.
3. Li, Y.; Tian, Y.; Rezai, T.; Prakash, A.; Lopez, M.F.; Chan, D.W.; Zhang, H. Simultaneous Analysis of Glycosylated and Sialylated Prostate-Specific Antigen Revealing Differential Distribution of Glycosylated Prostate-Specific Antigen Isoforms in Prostate Cancer Tissues. *Anal. Chem.* 2011, 83, 240–245.
4. Tabares, G.; Radcliffe, C.M.; Barrabés, S.; Ramírez, M.; Alexandre, R.N.; Hoesel, W.; Dwek, R.A.; Rudd, P.M.; Peracaula, R.; De Llorens, R. Different Glycan Structures in Prostate-Specific Antigen from Prostate Cancer Sera in Relation to Seminal Plasma PSA. *Glycobiology* 2006, 16, 132–145.

Table 3. PSA glycans identified in this work compared to those noted in previously published work.

mAb Acronym	% Present Found by Current Study (by Peak Area)	% Present in Seminal PSA (Tabares et al.) <sup>4</sup>	Structure (Adapted from CFG)
G0	2.5		
Neutral afucosylated glycan	5		
G1F	1.5		
G2F	5		
G2bF	9		
	33 (monosialylated glycans, excluding A1F)	6	
A1		18	
A1F	10	25	
Sialidase-resistant charged glycan	5		
A2F+A2	29	51	

- N-acetylglucosamine (GlcNAc)
- ▲ Fucose (Fuc)
- Mannose (Man)
- Galactose (Gal)
- ◆ N-acetylneuraminic acid (Neu5Ac)

5. Fukushima, K.; Satoh, T.; Baba, S.; Yamashita, K. α1,2-Fucosylated and β-N-Acetylgalactosaminylated Prostate-Specific Antigen as an Efficient Marker of Prostatic Cancer. *Glycobiology* 2012, 20, 452–460.
6. Yi, W.; Clark, R.M.; Mason, D.E.; Keenan, M.C.; Hill, C.; Goddard, W.A.; Peters, E.C.; Triggers, E.M.; Hsieh-Wilson, L.C. Phosphofructokinase 1 Glycosylation Regulated Cell Growth and Metabolism. *Science* 2012, 337, 975–980.
7. Valenzuela, H.F.; Pace, K.E.; Cabrera, P.V.; White, R.; Porvari, K.; Kaija, H.; Vihko, P.; Baum, L.G. O-Glycosylation Regulated LNCaP Prostate Cancer Cell Susceptibility to Apoptosis Induced by Galectin-1. *Cancer Res.* 2007, 67, 6155–6162.
8. Hart, G.W.; Housley, M.P.; Slawson, C. Cycling of O-linked β-N-Acetylglucosamine on Nucleocytoplasmic Proteins. *Nature* 2007, 446, 1017–1022.
9. Dionex (now part of Thermo Scientific) Application Update 141: Improved Long-Term Stability of N-Acetylneuraminic Acid and N-Glycolylneuraminic Acid Peak Area Responses Using Waveform A, a Quadruple Potential Waveform. Sunnyvale, CA, 2000. [Online] [www.dionex.com/en-us/webdocs/6511-AU141\\_LPN1225.pdf](http://www.dionex.com/en-us/webdocs/6511-AU141_LPN1225.pdf) (accessed Jan 22, 2013).

10. Dionex (now part of Thermo Scientific) Technical Note 71: Eluent Preparation for High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection. Sunnyvale, CA, 2007. [Online] [www.dionex.com/en-us/webdocs/58087-TN71-Eluent-Prep-HPAE-PAD-16Sept2009-LPN1932-02.pdf](http://www.dionex.com/en-us/webdocs/58087-TN71-Eluent-Prep-HPAE-PAD-16Sept2009-LPN1932-02.pdf) (accessed Jan 22, 2013).
11. Dionex (now part of Thermo Scientific) Application Update 176: Preparation of Peptide N-Glycosidase F Digests for HPAE-PAD Analysis. Sunnyvale, CA, 2010. [Online] [www.dionex.com/en-us/webdocs/88108-AU176-IC-PNGaseF-HPAEPAD-07Sep2010-LPN2576-R2.pdf](http://www.dionex.com/en-us/webdocs/88108-AU176-IC-PNGaseF-HPAEPAD-07Sep2010-LPN2576-R2.pdf) (accessed Jan 22, 2013).
12. Dionex (now part of Thermo Scientific) Application Note 215: Separation of Asparagine-Linked (N-Linked) Oligosaccharides from Human Polyclonal IgG Using the CarboPac PA200 Column. Sunnyvale, CA, 2009. [Online] [www.dionex.com/en-us/webdocs/69880-AN215-IC-Oligosaccharides-PolyclonalIgG-07Jan09-LPN2141-R2.pdf](http://www.dionex.com/en-us/webdocs/69880-AN215-IC-Oligosaccharides-PolyclonalIgG-07Jan09-LPN2141-R2.pdf) (accessed Jan 22, 2013).
13. Thermo Scientific Technical Note 40: Glycoprotein Monosaccharide Analysis Using HPAE-PAD with Eluent Generation. Sunnyvale, CA, 2012. [Online] [www.dionex.com/en-us/webdocs/5052-TN40-IC-Glycoprotein-Monosaccharide-23May2012-LPN1632-01.pdf](http://www.dionex.com/en-us/webdocs/5052-TN40-IC-Glycoprotein-Monosaccharide-23May2012-LPN1632-01.pdf) (accessed Jan 22, 2013).
14. *Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition, 2009*; DHHS Publication No. (CDC) 21-1112; Department of Health and Human Services, Public Health Service Centers for Disease Control and Prevention, National Institutes of Health, U.S. Government Printing Office: Washington, DC, 2009 [Online] [www.cdc.gov/biosafety/publications/bmbl5/bmbl.pdf](http://www.cdc.gov/biosafety/publications/bmbl5/bmbl.pdf) (accessed Jan 22, 2013).
15. Spik, G.; Bayard, B.; Fournet, B.; Strecker, G.; Bouquelet, S.; Montreuil, J. Studies on Glycoconjugates. LXIV Complete Structure of Two Carbohydrate Units of Human SeroTransferrin. *FEBS Lett.* **1975**, *50*, 296–299.
16. Fu, D.; von Halbeek, H.; N-Glycosylation Site Mapping of Human Serotransferrin by Serial Lectin Affinity Chromatography, Fast Atom Bombardment-Mass Spectrometry, and <sup>1</sup>H Nuclear Magnetic Resonance Spectroscopy. *Anal. Biochem.* **1992**, *206*, 53–63.
17. Symbol and Text Nomenclature for Representation of Glycan Structure. GFC Functional Glycomics Gateway, ISSN 1752-184X, May 2, 2012. [Online] <http://www.functionalglycomics.org/static/consortium/Nomenclature.shtml> (accessed Jan 22, 2013).
18. The UniProt Consortium, Reorganizing the Protein Space at the Universal Protein Resource (UniProt). *Nucleic Acids Res.* **2012**, *40*, D71–D75, Porcine Mucin [www.uniprot.org/uniprot/P12021](http://www.uniprot.org/uniprot/P12021) (accessed Jan 24, 2013).
19. Zheng, T.; Rohrer, J.; Rao, S. N-Linked Oligosaccharide Separation: New Method Developed to Improve the Resolution of Neutral and Sialylated Glycans. *Genetic Engineering and Biotechnology News* **2010**, *30* [Online] [www.genengnews.com/gen-articles/n-linked-oligosaccharide-separation/3300/?page=1](http://www.genengnews.com/gen-articles/n-linked-oligosaccharide-separation/3300/?page=1) (accessed Jan 24, 2013).
20. Grey, C.; Edebrink, P.; Krook, M.; Jacobsson, S.P. Development of a High Performance Anion Exchange Chromatography Analysis for Mapping of Oligosaccharides, *J. Chromatogr., B* **2009**, *877*, 1877–1832.
21. Peracaula, R.; Tabarés, G.; Royle, L.; Harvey, D.J.; Dwek, R.A.; Rudd, P.M.; de Llorens, R. Altered Glycosylation Pattern Allows the Distinction between Prostate-Specific Antigen (PSA) from Normal and Tumor Origins. *Glycobiology* **2003**, *13*, 457–470.
22. Tajiri, M.; Ohyama, C.; Wada, Y. Oligosaccharide Profiles of the Prostate Specific Antigen in Free and Complexed Forms from the Prostate Cancer Patient Serum and in Seminal Plasma: A Glycopeptide Approach. *Glycobiology* **2008**, *18*, 2–8.

[www.thermofisher.com/dionex](http://www.thermofisher.com/dionex)

©2016 Thermo Fisher Scientific Inc. All rights reserved. Amicon is a registered trademark of Merck KGaA Darmstadt, Germany. PEEK is a trademark of Victrex PLC. Sarstedt is a registered trademark of Sarstedt AG & Co. Sigma-Aldrich is a registered trademark of Sigma-Aldrich Co. LLC. All other trademarks are the property of Thermo Fisher Scientific Inc. and its subsidiaries. This information is presented as an example of the capabilities of Thermo Fisher Scientific Inc. products. It is not intended to encourage use of these products in any manners that might infringe the intellectual property rights of others. Specifications, terms and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representative for details.

<b>Australia</b> +61 3 9757 4486	<b>Denmark</b> +45 70 23 62 60	<b>Japan</b> +81 6 6885 1213	<b>Switzerland</b> +41 62 205 9966
<b>Austria</b> +43 1 333 50 34 0	<b>France</b> +33 1 60 92 48 00	<b>Korea</b> +82 2 3420 8600	<b>Taiwan</b> +886 2 8751 6655
<b>Belgium</b> +32 53 73 42 41	<b>Germany</b> +49 6126 991 0	<b>Netherlands</b> +31 76 579 55 55	<b>UK/Ireland</b> +44 1442 233555
<b>Brazil</b> +55 11 3731 5140	<b>India</b> +91 22 6742 9494	<b>Singapore</b> +65 6289 1190	<b>USA and Canada</b> +847 295 7500
<b>China</b> +852 2428 3282	<b>Italy</b> +39 02 51 62 1267	<b>Sweden</b> +46 8 473 3380	

**Thermo**  
SCIENTIFIC

Part of Thermo Fisher Scientific