

# Sample Preparation Scale-up for Deep N-glycomic Analysis of Human Serum by Capillary Electrophoresis and CE-ESI-MS

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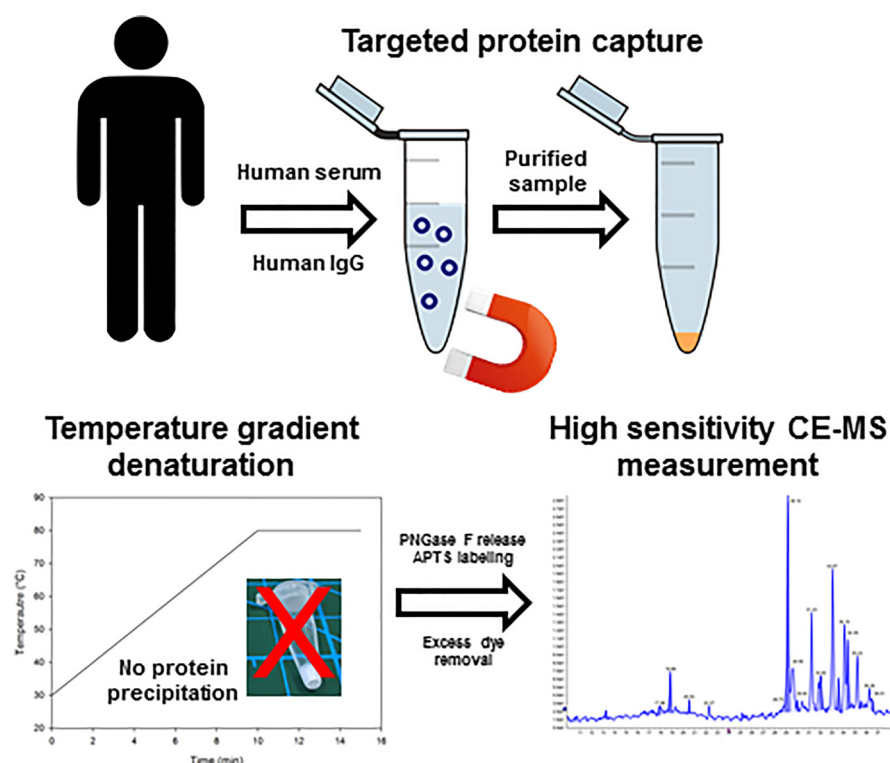
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## In Brief

An efficient sample preparation workflow was developed to facilitate deep N-glycomics analysis of human serum by capillary electrophoresis also accommodating the higher sample concentration requirement of CE-ESI-MS. The temperature gradient denaturing protocol prevented protein precipitation when larger amounts of samples were processed. The decrease in the free sugar content in serum alleviated endoglycosidase inhibition. This new workflow enabled deep CE-LIF analysis of the human serum N-glycome and provided the appropriate amount of material for CE-ESI-MS analysis in negative ionization mode.

## Graphical Abstract



## Highlights

- Efficient sample preparation workflow for deep N-glycomics analysis from serum.
- Temperature gradient denaturing protocol to prevent protein precipitation.
- Decrease of free sugar content in serum enhanced PNGase F digestion efficiency.
- Modified evaporative labeling method increased fluorophore labeling yield.

# Sample Preparation Scale-Up for Deep N-glycomic Analysis of Human Serum by Capillary Electrophoresis and CE-ESI-MS\*<sup>§</sup>

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**We introduce an efficient sample preparation workflow to facilitate deep N-glycomics analysis of the human serum by capillary electrophoresis with laser induced fluorescence (CE-LIF) detection and to accommodate the higher sample concentration requirement of electrospray ionization mass spectrometry connected to capillary electrophoresis (CE-ESI-MS). A novel, temperature gradient denaturing protocol was applied on amine functionalized magnetic bead partitioned glycoproteins to circumvent the otherwise prevalent precipitation issue. During this process, the free sugar content of the serum was significantly decreased as well, accommodating enhanced PN-Gase F mediated release of the N-linked carbohydrates. The liberated oligosaccharides were tagged with aminopyrene-trisulfonate, utilizing a modified evaporative labeling protocol. Processing the samples with this new workflow enabled deep CE-LIF analysis of the human serum N-glycome and provided the appropriate amount of material for CE-ESI-MS analysis in negative ionization mode. *Molecular & Cellular Proteomics* 18: 2524–2531, 2019. DOI: 10.1074/mcp.TIR119.001669.**

Human serum contains glycoproteins across a very wide range of concentrations from pg/ml to the mg/ml level (1). Albumin and immunoglobulins together represent more than three quarters of the total protein content, and several abundant glycoproteins are present in a high concentration range such as haptoglobin,  $\alpha$ 1-antitrypsin and transferrin (1). Aside from these abundant proteins, most serum glycoproteins represent less than 1% of the serum proteins, thus their analysis requires either a higher starting sample volume or targeted fractionation by immunoaffinity based preconcentration methods (2). Most human serum proteins are co- and post-translationally modified, including the attached carbohydrates (3), which reportedly affect a great number of their physiological and pathological properties (4). Consequently, the analysis of the carbohydrate moieties of glycoproteins is of great

importance in the biomedical (e.g. glycobiomarkers) (5) and biopharmaceutical (e.g. effector function of therapeutic mAbs) (6) fields, both requiring robust high sensitivity methods applicable in large scale processing. The presence of various glycoforms at a given site (microheterogeneity) or the occupancy of a potential glycosylation site (macroheterogeneity) represents additional analytical challenges.

Modern glycoanalytical techniques such as HPLC, capillary electrophoresis and mass spectrometry require very efficient sample preparation methods to achieve high sensitivity for deep glycomics analysis. Most of these techniques start with a denaturing step to unfold the glycoproteins in order to allow access for the endoglycosidase enzymes to reach their substrates and release the attached carbohydrate chains. However, with traditionally used denaturation methods with larger than 10  $\mu$ l of serum samples, precipitation occurs (7), necessitating more sophisticated methods for higher sample volumes. In addition, during the endoglycosidase digestion step, the presence of various monosaccharides or low degree of polymerization (DP)<sup>1</sup> sugars in the samples (e.g. glucose, maltose, etc., in blood) (8) and most interestingly, the released Man3GlcNAc2 core containing sugar structures act as inhibitors (9, 10). Finally, fluorophore tagging of the released glycans for high sensitivity detection requires high performance derivatization processes, such as the recently introduced evaporative labeling approach (11).

Analysis of the released and labeled N-linked carbohydrates are most frequently accomplished by the well-established liquid phase separation techniques such as chromatography (12, 13), capillary (14) or multicapillary (15) electrophoresis and in less extent by 2D electrophoresis (16) or microchips (17, 18). Mass spectrometry based glycoanalytical techniques, on the other hand, are also widely utilized including matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) and electrospray ionization (ESI) based methods (19, 20), usually requiring scale-up to provide appropriate

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sample concentration for the analysis. Electrospray ionization may cause degradation of sialylated and core fucosylated glycan structures (21) or can even lead to structural rearrangement (22) during the ionization process, necessitating the use of orthogonal analytical techniques for comprehensive qualitative analysis. For deep N-glycomic studies of human serum samples, all the above mentioned glycoanalytical methods need efficient sample preparation methods capable of generating enough analyte for the technique.

In this article we introduce a novel serum sample preparation protocol that is scalable up to dozens of  $\mu\text{L}$ s of serum sample without any precipitation issues during the denaturing step. The workflow includes amine bead-based capture of the serum glycoproteins in conjunction with a novel temperature gradient denaturation process to avoid precipitation and allow preparation of samples containing as much as 1.5 mg protein in 50  $\mu\text{L}$ . An added benefit of this approach is the removal of the high content of free blood sugars from the sample matrix, which has been shown to inhibit PNGaseF digestion efficiency. The sample preparation method was tested on hlgG1 and human serum samples.

#### EXPERIMENTAL PROCEDURES

**Chemicals and Reagents**—Acetic acid (glacial), ammonium acetate (7.5 M in solution), isopropanol, sodium-cyanoborohydride (1 M in THF), tetrahydrofuran, water (HPLC grade), acetonitrile and all chemicals used for SDS-PAGE analysis were obtained from Sigma Aldrich (St. Louis, MO). The human immunoglobulin sample (hlgG1) was from Molecular Innovations (Novi, MI). Human serum sample was kindly provided by the Medical School of the University of Debrecen (Debrecen, Hungary) with all required ethical permissions and patient consent forms. The Fast Glycan Labeling and Analysis Kit, including the tagging dye of 8-aminopyrene-1,3,6-trisulfonic acid (APTS), HR-NCHO separation gel-buffer system and magnetic beads for excess dye removal were from Sciex (Brea, CA). The PNGase F enzyme was from Asparia glycomics (San Sebastian, Spain). The amine functionalized magnetic beads were from Chemicell (Berlin, Germany).

**Sample Preparation**—The detailed sample preparation workflow is described in Scheme 1. Briefly, glycoproteins from 50  $\mu\text{L}$  of undiluted human serum or 50  $\mu\text{L}$  of 30 mg/ml of hlgG1 solution were captured by 20  $\mu\text{L}$  (50 mg/ml) of amine functionalized magnetic beads suspended in the mixture of 50  $\mu\text{L}$  of 1 M sodium-cyanoborohydride (in THF) and 1.0 ml of 0.1x PBS according to the manufacturer's protocol. After removing the supernatant, the beads were washed with 1.0 ml of HPLC grade water, followed by re-suspension in 20  $\mu\text{L}$  of HPLC grade water. The captured proteins were *in situ* denatured on the beads by temperature gradient denaturation in the presence of 10  $\mu\text{L}$  of premixed denaturation solution (Sciex Fast Glycan Kit, supplemented with 12.5% glycerol) by increasing the temperature with a rate of 5.0  $^{\circ}\text{C}/\text{min}$  from 30  $^{\circ}\text{C}$  to 80  $^{\circ}\text{C}$  with an additional 5.0 min isotherm incubation step at 80  $^{\circ}\text{C}$ . The denaturation step was fol-

lowed by endoglycosidase digestion, again on the beads, by the addition of 2.0  $\mu\text{L}$  of PNGase F enzyme (1.5 IUB unit/ $\mu\text{L}$ ) to the reaction mixture and incubating at 50  $^{\circ}\text{C}$  for 1.0 h, to assure complete removal of all N-linked glycan structure types. At the end of the glycan release process, the remaining polypeptides were fixed onto the beads by the addition of 120  $\mu\text{L}$  ice-cold acetonitrile. The supernatant, containing the released sugars, was dried at 60  $^{\circ}\text{C}$  in a vacuum centrifuge (2500 rpm, 1.0 h). The dry samples were labeled with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) fluorescent dye using our recently published evaporative labeling protocol (11) with a slight modification of 1.0 h at 50  $^{\circ}\text{C}$  with closed lid, followed by 1.0 h at 55  $^{\circ}\text{C}$  with open lid. After the labeling step, the excess APTS dye was removed using the magnetic beads from the Fast Glycan Sample Preparation and Analysis kit (Sciex) and the purified samples were analyzed by CE-LIF and CE-ESI-MS methods.

#### SCHEME 1. Sample preparation scale-up protocol for deep N-glycomic analysis of human serum samples by capillary electrophoresis and CE-ESI-MS.

Sample: 50  $\mu\text{L}$  of human serum/50  $\mu\text{L}$  of hlgG1 (30–50 mg/ml) solution.

1. Protein capture by amine functionalized magnetic beads
  - [1] Add 50  $\mu\text{L}$  of sample into a 1.5 ml Eppendorf tube.
  - [2] Add 50  $\mu\text{L}$  of 1 M  $\text{NaBH}_3\text{CN}$  (in THF) to the sample.
  - [3] Vortex the sample at maximum speed for 5 s.
  - [4] Wait for 1 min.
  - [5] Add 1.0 ml of 0.1x PBS solution.
  - [6] Add 20  $\mu\text{L}$  of well suspended amine functionalized magnetic beads (50 mg/ml; 1.0  $\mu\text{m}$  diameter).
  - [7] Wait for 30 min at room temperature (RT).
2. Bead wash
  - [8] Remove the supernatant after pulling down the magnetic beads by a magnetic stand.
  - [9] Reconstitute the beads in 500  $\mu\text{L}$  of HPLC grade water.
  - [10] Vortex the sample at maximum speed for 10 s.
  - [11] Apply a quick (2–3 s) spin-down in a benchtop microfuge.
  - [12] Wait for 5 min at RT.
  - [13] Remove the supernatant while the vial is on the magnetic stand.
3. Temperature gradient denaturation
  - [14] Prepare the denaturation solution by supplementing 70  $\mu\text{L}$  of Fast Glycan Kit denaturation solution with 10  $\mu\text{L}$  of glycerol (Denaturation solution - Fast Glycan Kit: 50  $\mu\text{L}$  of D4 + 10  $\mu\text{L}$  D1 + 10  $\mu\text{L}$  of D3 reagents. Other similar purpose denaturation solutions from other vendors may be used with the glycerol supplement).
  - [15] Add 20  $\mu\text{L}$  of HPLC grade water to the beads.
  - [16] Apply a quick (2–3 s) spin-down in a benchtop microfuge to remove any sample from the wall of the Eppendorf vial.
  - [17] Add 10  $\mu\text{L}$  of denaturing solution to the sample (do not touch the beads with the pipette tip).
  - [18] Vortex the sample at maximum speed for 10 s.
  - [19] Apply a quick (2–3 s) spin-down in a benchtop microfuge.

<sup>1</sup> The abbreviations used are: DP, degree of polymerization; APTS, 8-aminopyrene-1,3,6-trisulfonic acid, trisodium salt; CE-LIF, capillary electrophoresis with laser induced fluorescence detection; TIC, total ion count; XIC, extracted ion count; hlgG1, human immunoglobulin G1; BST, bracketing standard; MS, mass spectrometry; ESI, electrospray ionization; PBS, phosphate-buffered saline; PNGase F, peptide N-glycosidase F; RT, room temperature.

- [20] Denature the sample for 15 min using the following temperature gradient: 30 °C to 80 °C using 5 °C/min heating program followed by 5.0 min incubation at 80 °C
4. On-bead PNGase F digestion
  - [21] Apply a quick (2–3 s) spin-down in a benchtop microfuge.
  - [22] Add 50  $\mu$ l of HPLC grade water after the denaturation step.
  - [23] Add 2.0  $\mu$ l of PNGase F enzyme (1.5 IUB mU/ $\mu$ l).
  - [24] Vortex the sample at maximum speed for 5 s.
  - [25] Apply a quick (2–3 s) spin-down in a benchtop microfuge.
  - [26] Incubate the sample at 50 °C for 1.0 h.
5. Pre-labeling steps
  - [27] Apply a quick (2–3 s) spin-down in a benchtop microfuge.
  - [28] Add 120  $\mu$ l of ice-cold acetonitrile.
  - [29] Keep the sample at –20 °C for 15 min.
  - [30] Centrifuge the sample at 10,700  $\times g$  for 5 min.
  - [31] Transfer the sample supernatant (200  $\mu$ l) into a new 0.5 ml Eppendorf vial.
  - [32] Dry the sample in a SpeedVac for 60 min at 60 °C.
6. Evaporative APTS labeling
  - [33] Prepare the labeling stock solution of 9.0  $\mu$ l of 20 mM APTS (in 20% acetic acid), 1.0  $\mu$ l of 1 M NaBH<sub>3</sub>CN (in THF) and 10  $\mu$ l of additional THF per sample.
  - [34] Add 20  $\mu$ l of labeling solution to the dry sample (wash down the sample from the Eppendorf wall with the labeling solution).
  - [35] Vortex the sample at maximum speed for 10 s.
  - [36] Apply spin-down for 10 s in a benchtop microfuge.
  - [37] Incubate the sample for 1 h at 50 °C with the lid closed.
  - [38] Incubate the sample for another hour at 55 °C with open lid (or until the sample completely dried).
  - [39] Alternative labeling: Incubate sample at 37 °C overnight with the lid opened.
7. Excess dye removal
  - [40] Reconstitute the dry sample with 20  $\mu$ l of Fast Glycan Kit magnetic beads in water (concentrated from 200  $\mu$ l of M1 Process solvent by storage solution replacement with water on a magnetic stand).
  - [41] Vortex the sample at maximum speed for 10 s (wait for 3 min for complete reconstitution after addition of the beads, then vortex again).
  - [42] Add 180  $\mu$ l of acetonitrile to the sample (do not vortex after this step).
  - [43] Wait for 1 min at RT.
  - [44] Remove the supernatant after placing the vial on a magnetic stand.
  - [45] Repeat steps [41] to [43] three more times using 20  $\mu$ l of HPLC grade water and 180  $\mu$ l of acetonitrile per the Fast Glycan Kit user manual.

### 8. Sample elution

- [46] Add 50  $\mu$ l of HPLC grade water after the last supernatant removal step
- [47] Vortex the sample at maximum speed for 10 s.
- [48] Apply a quick (2–3 s) spin-down in a benchtop microfuge.
- [49] Place the sample on the magnetic stand for at least 1 min.
- [50] Transfer 45  $\mu$ l of sample (avoid magnetic bead transfer) into a new 200  $\mu$ l PCR tube.
- [51] Store the sample at 4 °C until analyzed.
- [52] Use 5.0  $\mu$ l of sample for up to 5 consecutive injections in a CE nanoVial for CESI-MS measurements.

### Capillary Electrophoresis

**CE-LIF**—A PA800 Plus Pharmaceutical Analysis System (Sciex) equipped with solid state laser induced fluorescence detection ( $\lambda_{\text{ex}} = 488 \text{ nm}/\lambda_{\text{em}} = 520 \text{ nm}$ ) was used for all capillary gel electrophoresis separations employing the HR-NCHO separation gel buffer in 50 cm and 20 cm effective length (60 cm and 30 cm respective total lengths) 50  $\mu$ m ID bare fused silica capillaries. The applied electric potential was 30 kV in reversed polarity mode (cathode at the injection side) and the separation temperature was set at 30 °C. A three-step sample injection protocol was applied including (1) 5.0 s water pre-injection at 3.0 and 5.0 psi, for the 30 cm and the 60 cm capillaries, respectively, (2) 1.0 kV to 6.0 kV for 1.0 s to 3.0 s sample injection, based on capillary length and sample concentration, specified in the respective figure captions, and (3) 1.0 kV for 1.0 s bracketing standard injection (maltose and maltopentadecaose). The 32Karat (version 10.1) software package (Sciex) was used for data acquisition and processing.

**CE-MS**—All CE-MS analyses were accomplished by using a CESI 8000 Plus High Performance Separation - ESI Module, connected to a 6500+ QTRAP mass spectrometer (both from Sciex) using a bare fused silica OptiMS capillary cartridge (91 cm total length, 30  $\mu$ m ID, 150  $\mu$ m OD). The mass spectrometer friendly background electrolyte contained 10 mM of ammonium acetate, 0.1% acetic acid (pH 4.5) and 20% isopropanol. The separations were carried out at 20 kV applied voltage in reversed polarity mode at 20 °C with the application of 2.0 psi forward pressure on both the separation and the conductive lines. Here, a two-step sample injection protocol was applied including: (1) 1.0 psi for 5.0 s water pre-injection (2) 10 kV for 20 s sample injection.

**Mass Spectrometry**—The 6500+ QTRAP mass spectrometer (Sciex) was used in negative ionization mode for all CE-ESI-MS experiments with a nanosource interface utilizing an etched sprayer-end bare fused silica (BFS) column OptiMS cartridge. The ESI voltage was set to –1,600 V for 1.0 min to stabilize the spray and then decreased to –1,400 V for high sensitivity detection. The orifice plate temperature was 80 °C and the curtain gas pressure was 5.0 psi to ensure the stability of the electrospray at the ultralow flowrate (20.4 nL/min) of the system. MRM scan targeted the doubly charged APTS labeled target glycan masses at 15 msec time. The results were evaluated and visualized with the PeakView software package (version 2.2, Sciex).

**SDS-PAGE**—A Cleaver nanoPAC-300 (Warwickshire, UK) slab gel electrophoresis system was used for all SDS-PAGE analyses. The separation gel had a 10% upper stacking section and a 12% lower separation section. The stacking gel contained 0.375 ml of 40% acrylamide (containing 2.67% N,N'-methylenebisacrylamide), 0.375 ml of 1 M Tris-HCl (pH 6.8), 30  $\mu$ l of 10% APS, 30  $\mu$ l of 10% SDS, 3.0  $\mu$ l of TEMED and 2.2 ml of water. The separation gel contained 1.8 ml



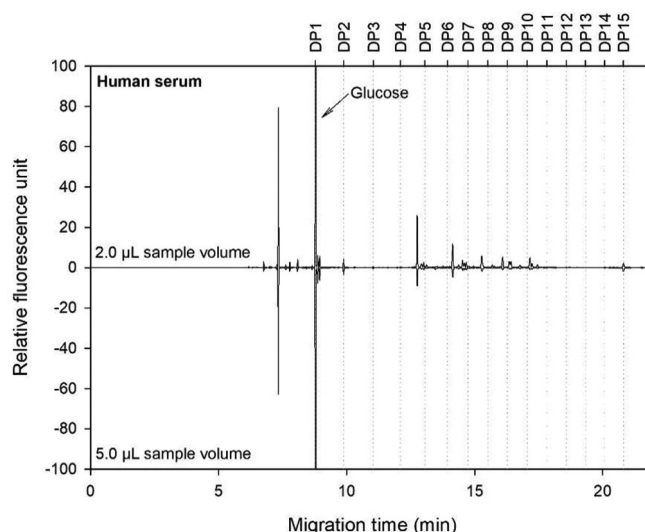
of 40% acrylamide solution (containing 2.67% N,N'-methylenebisacrylamide), 1.5 ml of 1.5 M Tris-HCl (pH 8.8), 60  $\mu$ l of 10% APS, 60  $\mu$ l of 10% SDS, 3.0  $\mu$ l of TEMED and 2.6 ml of water. Prior to SDS-PAGE analysis, the samples were heat denatured at 95 °C for 5.0 min in a 1:1 volume ratio of sample buffer containing 4.0 ml of glycerol, 1.0 ml of 2-mercaptoethanol, 1.2 g of SDS, 5.0 ml of 1 M Tris-HCl (pH 6.8) and 0.03 g of bromophenol blue. The separation buffer contained 36.0 g of Tris, 172.8 g glycine and 120 ml of 10% SDS dissolved in 3.0 L of water. Electrophoresis was carried by applying 150 V constant voltage, generating 75 mA current. The separated proteins were stained overnight with Coomassie blue (1.0 g of Coomassie blue R250 was dissolved in the mixture of 300 ml of methanol, 650 ml of water and 50 ml of glacial acetic acid) followed by destaining in a mixture of 300 ml of methanol, 650 ml of water and 50 ml of glacial acetic acid. The developed gel images were digitalized by a Nikon camera.

## RESULTS

The novel sample preparation protocol introduced in this paper can be scaled up to support deep N-glycomics analysis of the human serum by CE-LIF and the higher sample concentration requirement of CE-ESI-MS. Scaling up was especially important for CE-ESI-MS, as neither the released glycans nor the APTS tag ionized well in the electrospray process. Furthermore, with APTS labeling, negative ionization mode had to be applied resulting lower signal intensity compared with positive ion mode operation.

**Temperature Gradient Denaturation**—For deep glycomics analysis of human serum by CE-LIF and to assure adequate sample amounts to analyze the APTS labeled N-glycans by CE-ESI-MS, a significant increase in sample concentration was necessary. Current sample preparation methods for CE-LIF and CE-ESI-MS start with high glycoprotein concentrations (>10 mg/ml). However, the use of >10  $\mu$ l sample volume (corresponding to ~100  $\mu$ g protein) causes precipitation in most instances during the denaturing step, compromising the downstream sample preparation steps of enzymatic glycan release and fluorophore tagging. For example, we observed that hlgG1 precipitated at and above 20 mg/ml (10  $\mu$ l sample volume; 200  $\mu$ g protein) concentration (supplemental Fig. S1). To alleviate this issue, a novel temperature gradient denaturation method was developed. The temperature was increased from 30 °C to 80 °C with 5.0 °C/min rate followed by an additional 5.0 min incubation at 80 °C. This approach alleviated protein precipitation at concentrations up to 35 mg/ml (10  $\mu$ l sample volume; 350  $\mu$ g protein) that would otherwise represent a serious problem with traditional isothermal temperature denaturation.

**Endonuclease (PNGase F) Digestion**—An interesting phenomenon was observed during the released N-glycan analysis starting from 2.0  $\mu$ l and 5.0  $\mu$ l human serum samples. Albeit no precipitation was observed in either case during the denaturation step, starting with the higher sample volume (5.0  $\mu$ l) resulted in smaller peak intensities. Fig. 1 compares the CE-LIF analysis traces of PNGase F released and APTS labeled N-glycans prepared from 2.0  $\mu$ l and 5.0  $\mu$ l human



**FIG. 1. CE-LIF separation of the PNGase F released and APTS labeled human serum N-glycan pool from 2.0  $\mu$ l (upper trace) and 5.0  $\mu$ l (lower trace) initial sample volumes.** The DP numbers at the top of the figure represent the glucose units (GU) of the maltooligosaccharide ladder. Separation conditions: 50 cm effective (60 cm total), 50  $\mu$ m ID bare fused silica capillary column filled with HR-NCHO gel buffer. Applied voltage: 30 kV. Separation temperature: 30 °C. Injection sequence: (1) 5.0 psi/5.0 s water; (2) 6.0 kV/3.0 s sample and 3) 1.0 kV/1.0 s bracketing standard.

serum. As one can observe, the signal intensities were almost three times higher when the smaller (2.0  $\mu$ l) sample volume was used for sample preparation.

First, we considered that these counterintuitive results were caused by the very high amount of free serum sugar (e.g. glucose as highlighted in Fig. 1) content competing with the released sugars during the APTS labeling step. In order to prove this assumption, increasing amounts of glucose were added to 10  $\mu$ l of 10 mg/ml hlgG1 test protein samples after PNGase F digestion but before APTS labeling (Fig. 2A). The addition of glucose before the APTS labeling step resulted in no apparent changes in the signal intensity of the serum N-glycome peaks, as one can observe in Fig. 2A. The inset in the upper right corner depicts 4.3% peak area RSD for the FA2G2 peak (structural abbreviations followed the nomenclature suggested in (23)). It is important to note that the APTS concentration used in the reaction mixture was enough to facilitate complete labeling of all human serum N-glycans in the sample and the high amount of added glucose. Therefore, in a second experiment, the extra glucose was added to the hlgG1 sample before PNGase F digestion, which on the other hand, caused significant decrease in the signal intensities of all peaks, as shown in Fig. 2B.

As a first approximation, we considered that the increasing glucose concentration in the reaction mixture probably inhibited the PNGase F enzyme reaction, as was earlier suggested by Suzuki *et al.* for mannose residues (9). The continuous decrease in peak areas compared with the reference trace is delineated in Fig. 3 for five major hlgG1 glycans.

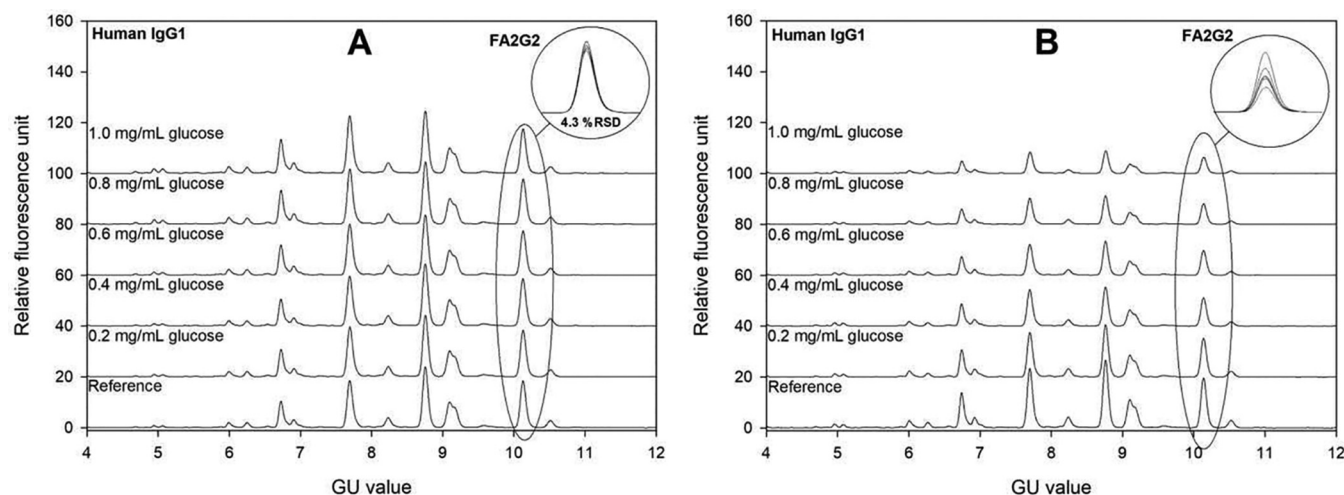


FIG. 2. Effect of the increasing amount of glucose on the APTS labeling reaction (A) and PNGase F glycan release reaction (B) on the resulting peak intensities. The insets show the peak intensity changes of the FA2G2 structure. The CE-LIF separation conditions were the same as in Fig. 1, except for the capillary length (20 cm effective, 30 cm total, 50  $\mu$ m ID) and the injection sequence: (1) 3.0 psi/5.0 s water; (2) 1.0 kV/1.0 s sample and (3) 1.0 kV/1.0 s bracketing standard.

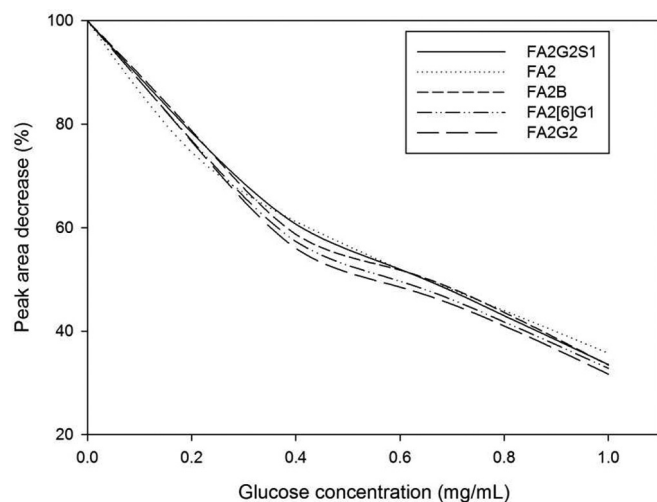


FIG. 3. Effect of the increasing glucose concentration in the reaction mixture during the PNGase F digestion reaction on the resulting peak areas of five major hlgG1 glycan structures (sialylated - FA2G2S1, galactosylated - FA2G2, FA2[6]G1, non-galactosylated - FA2, and bisecting - FA2B).

**Targeted Protein Capture by Amine Functionalized Magnetic Beads**—In order to avoid the above discussed issues, *i.e.* precipitation during the denaturation step above 35 mg/ml (350  $\mu$ g protein in 10  $\mu$ l) of hlgG1 sample concentration and to remove the high amount of PNGase F inhibiting free sugars (mainly glucose) from the human serum samples, an amine functionalized magnetic microparticle mediated workflow was applied. Glycoproteins were captured on the surface of 20  $\mu$ l of well suspended amine functionalized beads in PBS buffer in the presence of sodium-cyanoborohydride. After the capture step, the beads were pulled down by a magnetic stand, the supernatant was removed and the beads were washed with HPLC grade water.

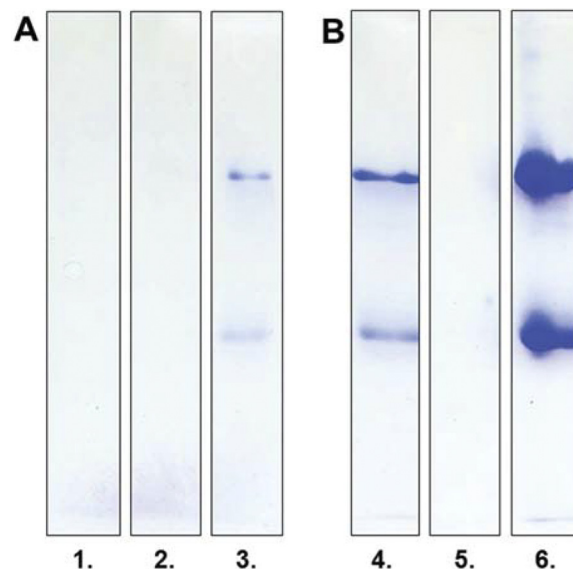


FIG. 4. SDS-PAGE analysis of the efficiency of the amine functionalized magnetic bead capture for 10  $\mu$ l of 10 mg/ml hlgG1 (A) and 50  $\mu$ l of 30 mg/ml (B) hlgG1. Lanes: 1 and 4: non-bound overflow, 2 and 5: wash step; 3 and 6: protein elution with 50 mM citric acid (pH 3.0).

The efficiency of the amine functionalized magnetic bead capture step was evaluated by using 10  $\mu$ l of 10 mg/ml (100  $\mu$ g protein) and 50  $\mu$ l of 30 mg/ml (1.5 mg protein) hlgG1 samples, respectively. In both instances, 10  $\mu$ l of samples were analyzed after the capture and wash steps by SDS-PAGE as well as after elution with 50 mM of citric acid (pH 3.0). Fig. 4 shows that the captured protein amount by the amine functionalized magnetic beads was only limited by the amount of beads applied. In the case of 10  $\mu$ l of 10 mg/ml (100  $\mu$ g protein) hlgG1 sample, the entire amount of proteins was captured on the surface of the beads (Fig. 4, lack of any

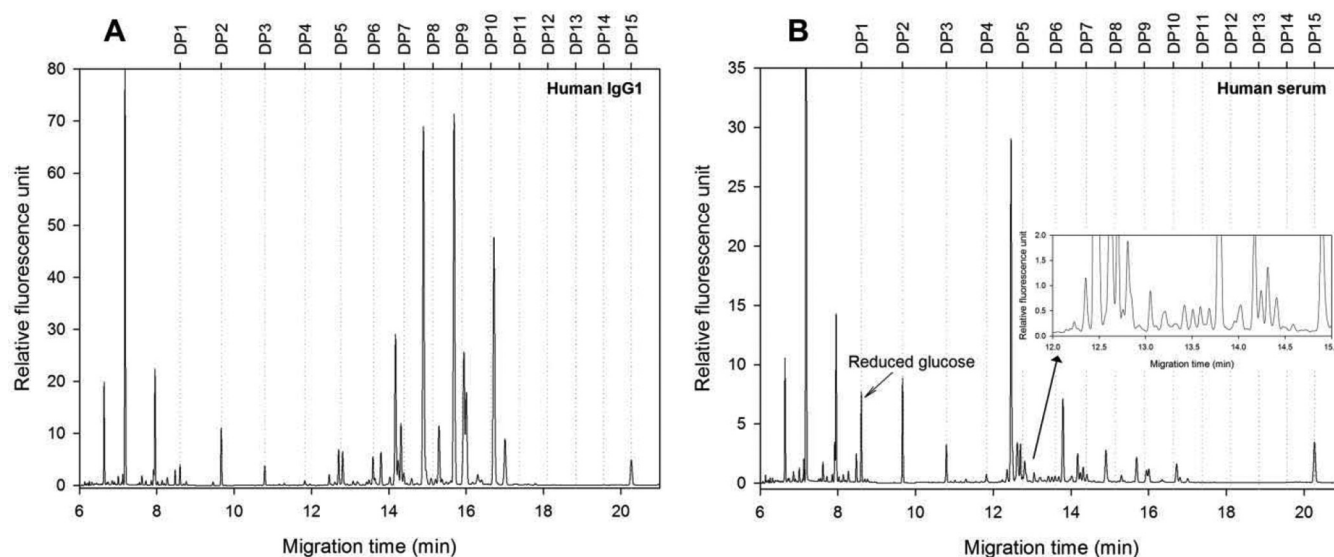


FIG. 5. CE-LIF analysis of PNGase F released and APTS labeled N-linked glycans of human hlgG1 (A) and human serum (B). A zoomed-in view of the lower abundant serum N-glycans are shown in the inset of Panel B. Samples were prepared according the optimized amine functionalized magnetic bead mediated sample preparation and temperature gradient denaturation protocol starting from a large amount of hlgG1 (1.5 mg) and a high volume of human serum (50  $\mu$ l) samples. Separation conditions were the same as in Fig. 1, except the samples were injected by 1.0 kV/1.0 s (injection sequence, step 2).

TABLE I  
Reproducibility of the sample preparation and analysis workflow of Scheme 1

Measurement		%RSD	
		Intra-day reproducibility	Inter-day reproducibility
CE-LIF	Hardware performance (peak area)	0.92	1.35
	hlgG1 sample preparation (peak area)	3.40	5.42
	Human serum sample preparation (peak area)	7.83	9.63
CESI-MS	Peak area (total)	6.11	8.53
	Migration time	1.42	2.31

signal in Lane 1). Also, the wash step in this instance did not remove any proteins, as shown by the band-free Lane 2. In the citric acid eluate, both the light and heavy hlgG1 chains were visible as depicted by the two corresponding bands in Lane 3. On the other hand, the overloading effect was readily apparent when 50  $\mu$ l of 30  $\mu$ g/ml (1.5 mg protein) hlgG1 sample was injected as its components (heavy and light chains) were still visible in the flow through after the bead capture step (Lane 4). More importantly, the wash step did not remove any bound proteins (Lane 5) and like Lane 3, Lane 6 showed that the citric acid eluted the light and heavy chain bands.

**Sample Preparation Protocol**—Based on the above described improvements, a novel sample preparation protocol was established for the N-glycosylation analysis of biological samples, such as human serum, including denaturation, PNGase F digestion and APTS labeling (Scheme 1). Combining the amine functionalized magnetic bead based protein capture with temperature gradient denaturation and our previously reported evaporative labeling technique, samples with as high as 30 mg/ml concentration and 50  $\mu$ l sample volume

(1.5 mg total protein)—e.g. hlgG1 and human serum—were successfully prepared for N-glycosylation analysis without any precipitation issues. The protocol also provided efficient removal of all PNGase F inhibiting free sugars, such as the high amount of glucose present in human serum samples.

**Capillary Electrophoresis with Laser Induced Fluorescence Detection (CE-LIF)**—The released and APTS labeled N-glycans from hlgG1 and human serum samples were first analyzed by CE-LIF. As Fig. 5 shows, high intensity signals were observed for both samples by using the optimized sample preparation protocol of Scheme 1. The applied amine functionalized magnetic bead-based protein capture protocol in conjunction with the temperature gradient denaturation approach enabled the use of 25 times greater amount of serum sample (50  $\mu$ l instead of 2.0  $\mu$ l, Fig. 1) without any protein precipitation issue. Also, the large amount of glucose removed from the serum sample (highlighted in Fig. 5B) greatly reducing the inhibition of PNGase F digestion efficiency. Thus, 1.0 kV for 1.0 s (1 kVs) electrokinetic injection from the processed 50  $\mu$ l serum sample following the workflow of Scheme 1 resulted in similar peak intensities that was observed from

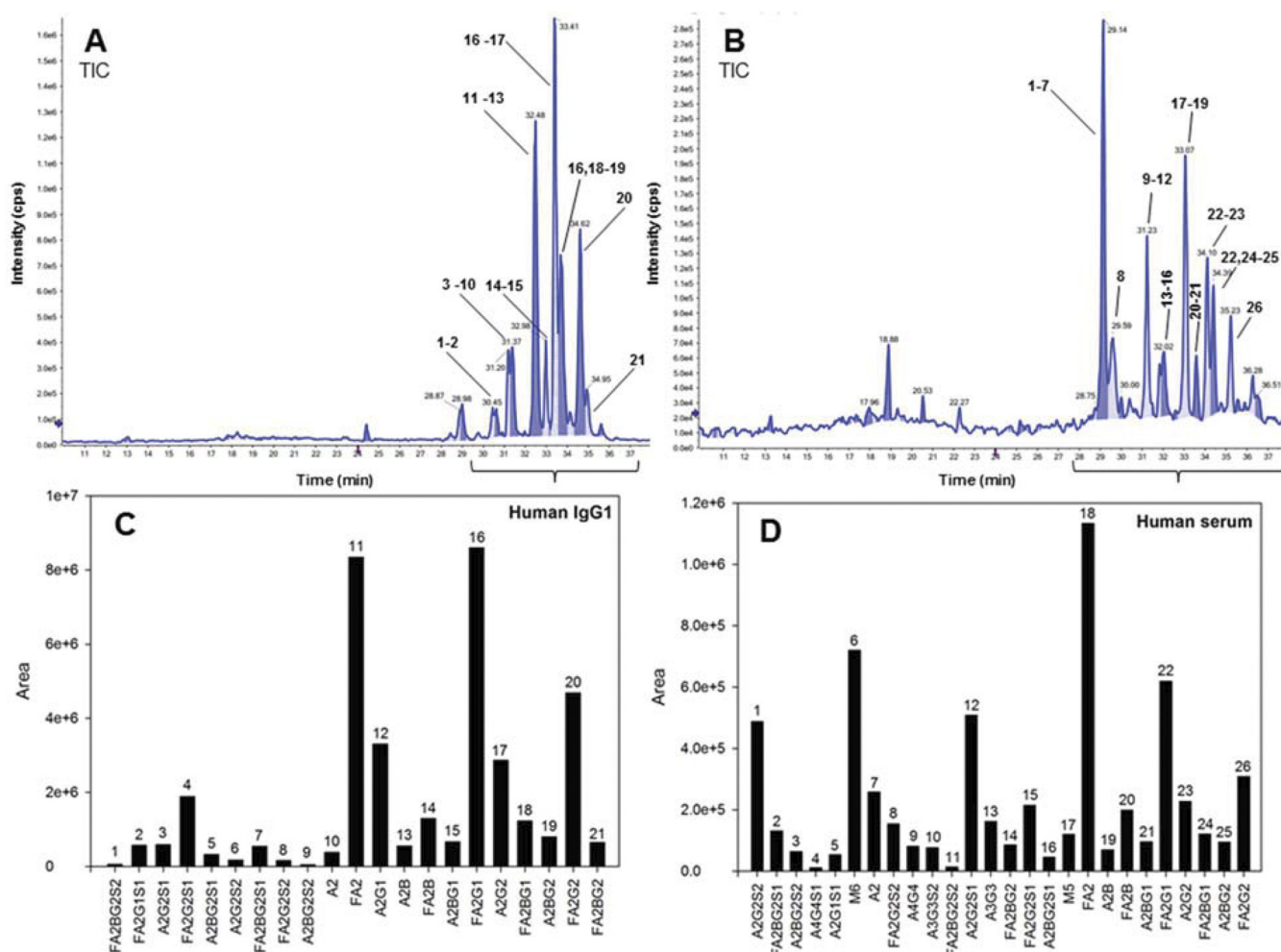


FIG. 6. CE-ESI-MS TICs and the corresponding areas of the identified structures of the released N-linked glycans from human IgG1 (A and C) and human serum (B and D), respectively. Conditions: 91 cm effective length (30  $\mu$ m ID) bare fused silica capillary column (OptiMS) filled with 10 mM AmAc buffer (pH 4.5 with 10% isopropyl alcohol). Applied voltage: 20 kV with 2.0 psi forward pressure. Separation temperature: 20  $^{\circ}$ C. Injection sequence: (1) 1.0 psi/5.0 s water; (2) 10 kV/20 s sample.

2.0  $\mu$ l sample volume using 6.0 kV/3.0 s (18 kV) injection parameters with the traditional sample preparation protocol. It is apparent from the inset in Fig. 5B that this scale up protocol provided enough material for deep N-glycomic analysis of the human serum, *i.e.* revealing peaks that would be otherwise lost in the baseline noise. The corresponding reproducibility of the sample preparation and analysis methods are shown in Table I. Please note that the higher % RSD values from the human serum sample were probably attributed to the tendency of the amine functionalized magnetic beads to stick to the wall of the reaction vials during the sample preparation process.

**Capillary Electrophoresis with Electrospray Ionization Mass Spectrometry (CE-ESI-MS)**—The PNGase F released and APTS labeled N-glycans of hlgG1 and human serum samples, prepared by the sample preparation protocol detailed in Scheme 1, were also analyzed by CE-ESI-MS resulting in high intensity MS data despite of the low ionization efficiency of the negatively charged sugar-APTS conjugates in negative ion-

ization mode (Fig. 6). Panels A and B in Fig. 6 show the total ion electropherograms of the released and APTS labeled N-glycan pools of hlgG1 and serum samples, respectively. The corresponding glycan structures with their integrated peak area values are shown in Panels C and D. Detailed structural information of all identified glycans are listed in supplemental Table S1.

## DISCUSSION

Deep N-glycomic analysis by CE-LIF and CE-ESI-MS of APTS labeled samples necessitated the use of a novel, highly efficient and precipitation free sample preparation protocol when high concentrations of glycoproteins were used. The temperature gradient denaturation step introduced in this paper alleviated precipitation issues, and decreased of the concentration of free serum sugars, which latter otherwise inhibited the endoglycosidase mediated glycan release reaction. Using the enclosed workflow enabled high sensitivity CE-LIF



analysis of the human serum N-glycome and provided adequate sample concentration for CE-ESI-MS analysis even in negative ionization mode.

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