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High-Throughput Profiling of the Serum N-Glycome on Capillary Electrophoresis Microfluidics Systems: Toward Clinical Implementation of GlycoHepatoTest

Dieter Vanderschaeghe,[†,‡] Ákos Szekrényes,[§] Christian Wenz,[∥] Marcus Gassmann,[∥] Natasha Naik,[⊥] Maggie Bynum,[⊥] Hongfeng Yin,[⊥] Joris Delanghe,[†] Andras Guttman,[§] and Nico Callewaert*[,†,#]

Unit for Molecular Glycobiology, Department for Molecular Biomedical Research, VIB, Technologiepark 927, B-9052 Ghent, Belgium, Department of Clinical Chemistry, Microbiology, and Immunology, Ghent University, De Pintelaan 185, B-9000 Ghent, Belgium, Horvath Laboratory of Bioseparation Sciences, Institute of Medical Chemistry, University of Debrecen, Nagyerdei krt 98, Elmeleti tomb 1 em 111, HU-4032 Debrecen, Hungary, Agilent Technologies, R&D and Marketing GmbH & Co. KG, Hewlett-Packard-Strasse 8, 76337 Waldbronn, Germany, Agilent Laboratories, 5301 Stevens Creek Blvd, Santa Clara, California 95051, and Laboratory for Protein Biochemistry and Biomolecular Engineering, Department of Biochemistry and Microbiology, Ghent University, K.L.-Ledeganckstraat 35, B-9000 Ghent, Belgium

We developed a 3 h procedure for preparing serum N-glycans and labeling them with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) by sequential addition of reagents to the serum and incubation in a polymerase chain reaction (PCR) thermocycler. Moreover, we succeeded in analyzing these samples by capillary electrophoresis on three commercial microfluidics-based platforms: the MCE-202 MultiNA, the 2100 Bioanalyzer, and a modified prototype of the eGene system which were originally designed for nucleic acid separation and detection. Although these instruments use short separation channels, our technical improvements made it possible to reliably measure the N-glycans constituting GlycoHepatoTest. This test comprises a panel of biomarkers that allows follow-up of liver fibrosis patients starting from the early stage. In this way and for the first time, we demonstrate a clinical glycomics assay on an affordable, robust platform so that clinical chemistry laboratories can exploit glycomics in the diagnosis and monitoring of chronic liver disease. Another potential application is the rapid screening of the N-glycosylation of recombinant glycoproteins produced for pharmaceutical use.

Chronic liver disease affects about 10% of people worldwide and is mainly caused by viral infection with hepatitis B or C virus, alcohol consumption, or nonalcoholic steatohepatitis (NASH). The continuous necrosis of hepatocytes overstretches the liver's capacity for regeneration. Consequently, fibrotic scar tissue forms and replaces functional liver tissue.[1] Liver fibrosis can progress to cirrhosis,[2] which is the major cause of death and morbidity in chronic liver disease and increases the risk of developing hepatocellular carcinoma (HCC) at least 20-fold.[3] Often, many years lie between mild fibrosis and the establishment of cirrhosis and/or HCC. Over the last 10 years, many biomarkers[4] have been discovered and designed to replace or complement liver biopsy, which is the best current method for assessing fibrogenesis and inflammation in the liver. Besides the high cost of liver biopsy, sampling error, and interobserver variability, this invasive technique is associated with considerable risks.[5,6] Therefore, noninvasive biomarkers are needed to monitor the progression of liver fibrosis from the early stage onward in order to adjust the treatment of nonresponders to therapy. Moreover, compensated (early stage) cirrhosis is not clinically overt and is difficult to diagnose. The detection of this high-risk group using biomarkers would make it possible to start treatment early, and these patients can also be screened more intensively for HCC by ultrasound and computed tomography scanning. This should make it possible to detect HCC in these patients when surgical tumor removal is still effective.

We previously found that a single N-glycan profile of total serum protein (N-glycome) contains information to follow-up

* To whom correspondence should be addressed. Phone: +32 9 331 36 30. Fax: 32 9 331 36 09. E-mail: Nico.Callewaert@dmbr.vib-UGent.be.

† VIB.

‡ Department of Clinical Chemistry, Microbiology, and Immunology, Ghent University.

§ University of Debrecen.

∥ Agilent Technologies, R&D and Marketing GmbH & Co. KG.

⊥ Agilent Laboratories.

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chronic liver disease patients and to specifically diagnose liver cirrhosis at an early stage. This panel of biomarkers, designated GlycoHepatoTest, consists of two biomarkers: GlycoFibroTest[7] and GlycoCirrhoTest.[8] These markers have been validated in different etiologies of chronic liver disease[9,10] (and unpublished data). To implement GlycoHepatoTest in routine clinical practice, the technology should be robust, cost-effective, and sufficiently simple for nonexperts.

One difficulty in the analysis of N-glycans is that they do not contain good chromophores. Therefore, we are labeling them with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) by reductive amination to increase sensitivity and to provide the analytes with a charge.[11] To detect and separate the APTS-labeled sugars, a multicapillary DNA sequencer is used.[12] However, because these systems are expensive and have a limited range of applications, they are found only in larger molecular diagnostic laboratories. Capillary electrophoresis (CE) platforms, which are approximately 1 order of magnitude cheaper, are used instead of classical agarose gels mainly to separate and size DNA and RNA fragments, especially in regulated laboratories, such as food microbiology, clinical microbiology, and forensics. In this paper, we demonstrate the separation of the serum N-glycome on the MCE-202 MultiNA system (Shimadzu, Japan), the 2100 Bioanalyzer (Agilent, Germany), and a modified prototype of the eGene apparatus (eGene, CA; recently acquired by Qiagen, Germany). These systems have a blue light emitting diode (LED), making them suitable for detecting APTS-labeled sugars. However, their fluorescence detection is at least 4 orders of magnitude less sensitive than DNA sequencers that use laser induced fluorescence (argon-ion laser). Moreover, the separation capillaries in these instruments are only 2.3, 1.2, and 10 cm long, respectively, as compared to 36 cm for the previously used ABI 3130 DNA sequencer. Therefore, the challenge was to develop a brief method to prepare APTS-labeled N-glycans from serum, enabling high-resolution separation and detection on these next-generation microfluidics systems, and this is what we discuss in this paper.

EXPERIMENTAL SECTION

Sera. Serum samples from one healthy volunteer, 15 chronic liver disease patients, and three HCC patients were obtained from an outpatient clinic (Ghent University Hospital, Belgium). Informed consent was given, and the protocol was approved by the Ethics Committee of Ghent University Hospital. The liver fibrosis stage was assessed according to the METAVIR scoring system.[2]

Optimization of the PNGase F reaction. Serum from a healthy volunteer (3 μL) was diluted to 5 μL with 10 mM NH_4HCO_3, pH 8.3, containing 3.5% sodium dodecyl sulfate (SDS). The sample was then incubated in a polymerase chain reaction (PCR) thermocycler at 95 °C for 5 min, followed by 5 min at 4 °C. It was then combined with an equal volume of 10 mM NH_4HCO_3, pH 8.3, containing 2% Igepal (or Nonidet-P40), and different amounts of recombinant peptide N-glycosidase F (PNGase F; see Supporting Information) were added. The reaction was incubated at 37 °C for 10 min in a PCR thermocycler, and every minute, 1 μL of the digest was removed and incubated in 5 μL of Milli-Q water at 80 °C, to immediately inactivate the enzyme. The samples were then evaporated to dryness in a thermocycler at 80 °C with the tubes and lid open for a maximum of 20 min. Finally, the N-glycans were labeled by adding 1 μL of a 1:1 mixture (v/v) of 20 mM APTS (Molecular Probes, USA) in 1.2 M citric acid and 1 M $NaCNBH_3$ in dimethylsulfoxide (DMSO). After overnight incubation at 37 °C, reactions were quenched by adding 200 μL of Milli-Q water. The signal intensity of the N-glycans on an ABI 3130 DNA sequencer (see below) is an estimate of the extent of the deglycosylation reaction mediated by PNGase F.

Optimization of the Sialidase Reaction. Three microliters of serum from a healthy volunteer was combined with 2 μL of 10 mM NH_4HCO_3, pH 8.3, containing 3.5% SDS. The sample was incubated in a PCR thermocycler at 95 °C for 5 min, followed by 5 min at 4 °C, and then, it was combined with 5 μL of 10 mM NH_4HCO_3, pH 8.3 (+2% Igepal), containing 2.5 IUBMB mU (optimized concentration) of PNGase F and incubated for 10 min at 37 °C. Then, 5 μL of NH_4Ac buffer (pH 5; 50, 75, or 100 mM) containing different quantities of recombinant sialidase (see Supporting Information) was added. At different time points, samples were transferred from 37 to 80 °C to inactivate the enzyme. One microliter of the sialidase digest in 5 μL of Milli-Q water was then evaporated to dryness. Finally, 1 μL of a 1:1 mixture (v/v) of 20 mM APTS in 1.2 M citric acid and 1 M $NaCNBH_3$ in DMSO was added and left overnight at 37 °C. Finally, the samples were quenched with 200 μL of ultrapure water and analyzed on an ABI 3130 DNA sequencer (see below).

Effect of Temperature, Time, and APTS Concentration on N-Glycan Labeling. Serum from a cirrhosis patient was used to prepare a master mix of sialidase digest following the optimized assay described here (Figure 1). Thus, 3 μL of serum was combined with 2 μL of 10 mM NH_4HCO_3, pH 8.3, containing 3.5% SDS, and incubated at 95 °C for 5 min, followed by 5 min at 4 °C. Then, 5 μL of 10 mM NH_4HCO_3, pH 8.3 (+2% Igepal), containing 2.5 IUBMB mU of PNGase F was added and incubated for 10 min at 37 °C. The N-glycans were desialylated completely by adding 5 μL of 100 mM NH_4Ac, pH 5, buffer containing 25 mU of sialidase and incubating for 15 min at 37 °C. Such prepared samples were combined as a master mix, and for each labeling condition tested, 15 μL of this mix was evaporated to dryness. N-Glycans were labeled by adding 5 μL of a 1:1 mixture (v/v) of APTS in 1.2 M citric acid and 1 M $NaCNBH_3$ in DMSO. Final APTS concentrations of 10, 25, 50, and 100 mM were tested at different incubation temperatures (37, 50, and 70 °C) and labeling times (0.5, 1, 2, 4, and 14 h). The reactions were quenched by adding 25 μL of ultrapure water. Note that we did not include overnight incubation at 70

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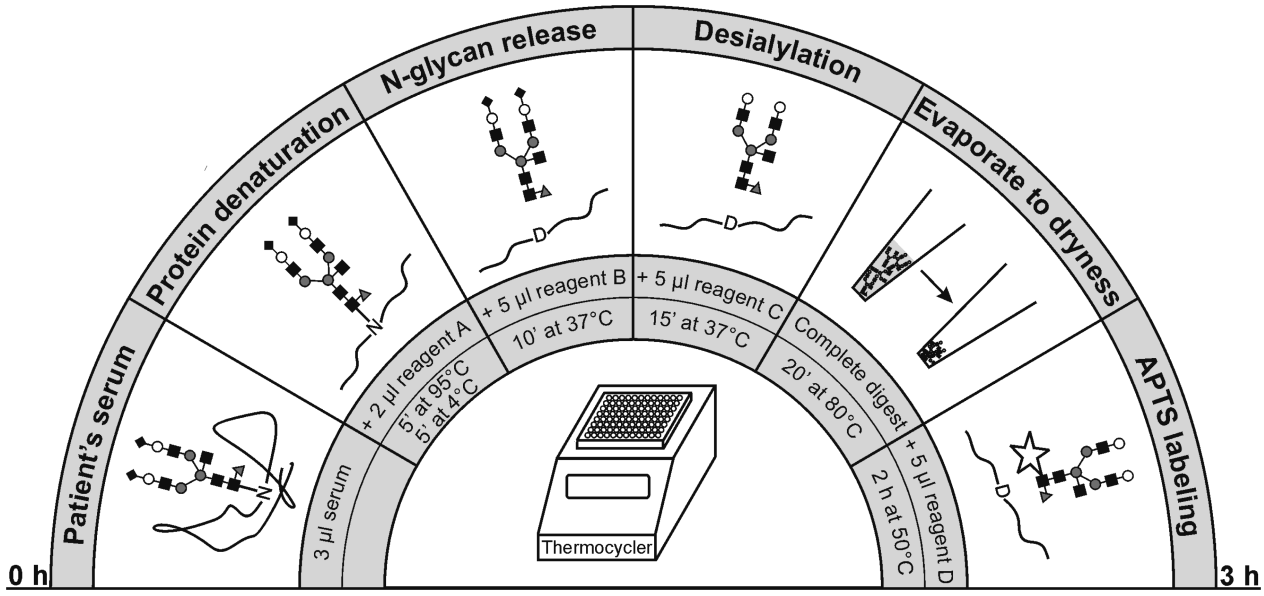

Figure 1. Optimized procedure for preparing APTS-labeled N-glycans from serum. Serum is diluted in 10 mM NH_4HCO_3, pH 8.3, containing 3.5% SDS (reagent A) to allow the PNGase F (reagent B) to specifically remove the N-glycans from the proteins to which they are attached. After desialylation, the glycans are labeled with 5 μL of a 1:1 mixture (v/v) of 200 mM APTS in 1.2 M citric acid and 1 M $NaCNBH_3$ in dimethyl sulfoxide (reagent D). All reactions are performed in a 96-well plate and incubated in a thermocycler. Reagent B: 2.5 IUBMB mU of recombinantly produced PNGase F in 10 mM NH_4HCO_3, pH 8.3, containing 2% Igepal. Reagent C contains 25 mU of recombinantly produced $\alpha(2\rightarrow3,6,8)$-sialidase in 100 mM NH_4Ac, pH5.

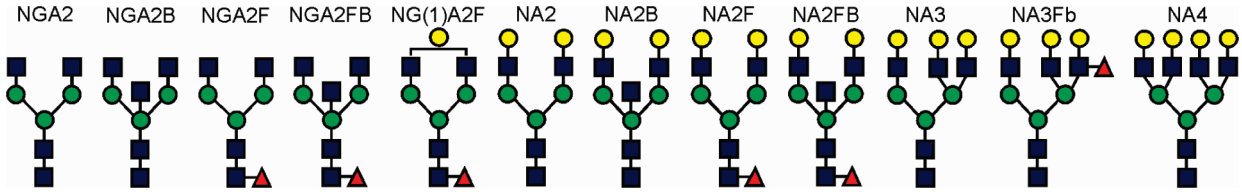

Figure 2. Nomenclature and structures of the most abundant N-glycans in the N-glycome profile (after desialylation). [yellow ●: galactose, ■: *N*-acetylglucosamine, green ●: mannose, ▲: fucose; symbols are those suggested by the Consortium for Functional Glycomics (http://glycomics.scripps.edu/CFGnomenclature.pdf)].

°C due to excessive evaporation and that overnight incubation at 50 °C was done only for 25 and 100 mM APTS.

N-Glycan Analysis on an ABI 3130 DNA Sequencer. N-Glycan samples were diluted in Milli-Q water (5000 fold or more) to obtain a dilution that yielded an electropherogram in which none of the peaks were off-scale. The diluted samples were analyzed by DSA-FACE (DNA sequencer-asssisted fluorophore-assisted carbohydrate electrophoresis) using an ABI 3130 DNA sequencer (Applied Biosystems, USA) with four capillaries (e.l. 36 cm, i.d. 50 μm) and an argon-ion laser for laser induced fluorescence detection. The standard settings for DNA fragment analysis were applied as described earlier.[12] The peaks that were detected in all samples were quantified using the sequencer's GeneMapper software. The peak height intensities were normalized to the total intensity of the measured peaks (Figure 2 for nomenclature). To determine the level of sialylation, the amount of sialylated N-glycan branches was normalized to the total amount of N-glycan branches present in the N-glycome.

N-Glycan Profiling on MCE-202 MultiNA. The MCE-202 MultiNA (Shimadzu, Japan) is equipped with a blue LED (470 nm; 20 mA). Fluorescence detection uses a long-pass filter with a cut-on wavelength of 525 nm. The apparatus can be equipped with four microchips in which samples are analyzed by CE. The rectangular microchannels (88 × 50 μm) are made using deep reactive ion etching. The undiluted sample is introduced into the separation channel (e.l. 2.3 cm) via double-T injection (Figure S-1, Supporting Information) in which the sample and waste arms are offset by 100 μm. Before the analysis, the chips were automatically rinsed with ultrapure water before the running buffer was pumped into the microchips. The commercially available DNA-500 and RNA separation buffers (Shimadzu, Japan) were used with the corresponding settings of the system, unless mentioned differently in the text. CE settings that were used for the DNA-500 buffer were V1 = 280 V, V2 = 510 V, V3 = 320 V, and V4 = 0 V during a 50 s injection of the sample (numbers refer to the different vials as shown in Figure S-1, Supporting Information). The sample was separated for 185 s where V1 = 250 V, V2 = 250 V, V3 = 0 V, and V4 = 1000 V. The CE settings that were used for the RNA buffer were V1 = 120 V, V2 = 260 V, V3 = 150 V, and V4 = 0 V during a 30 s injection of the sample (numbers refer to the different vials as shown in Figure S-1, Supporting Information). The sample was separated for 130 s where V1 = 210 V, V2 = 220 V, V3 = 0 V, and V4 = 820 V. The N-glycan structures corresponding to the peaks were identified by running N-glycan standards (Dextra Laboratories, United Kingdom) and by enzymatic digestion with exoglycosidases[8] that cleave specific sugar residues, such as $\beta(1\rightarrow4)$-galactosidase, $\beta(1\rightarrow2,3,4,6)$-*N*-acetylhexosaminidase, and $\alpha(1\rightarrow2,3,4,6)$-fucosidase.

N-Glycan Analysis on 2100 Bioanalyzer. The 2100 Bioanalyzer (Agilent, Germany) has both a red laser and a blue LED, but only the latter is suitable for detection of APTS-labeled N-glycans. Therefore, we adapted the standard assays on the system accordingly. Depending on the kit tested, the samples were diluted 1:5 (Agilent Small RNA kit) or 1:1 in marker solution (Agilent prototype kit), which is provided with these kits. The microchip contains 16 vials in which separation buffer or sample (up to 11 or 12) is added according to the instructions of the kit. Each vial is connected to an electrode that is connected to an independent power supply. Microchannels interconnect these vials in such a way that the samples are introduced with a cross-injector. The effective length of the separation channel is 12 mm (width is 36 μm). The standard CE settings recommended for the different kits were used. Samples were separated at 300−500 V/cm for 100−130 s (depending on the kit).

Software Deconvolution on 2100 Bioanalyzer. Peak-fitting software (PeakFit, SeaSolve Software Inc., CA, USA) was used to fit the peaks from the serum glycans. Signal intensity data from the blue channel of the Bioanalyzer was first imported into Excel, where the region of serum glycan elution was determined (between t = 35 and t = 60 s), and the remaining data was truncated for the purposes of peak fitting. This Excel data was then opened in PeakFit. The "AutoFit and Subtract Baseline" feature was used to subtract the baseline (second Deriv Zero, Tol% = 2.4%, Best Fit). The peaks were then fitted using the "AutoPlace and Fit Peaks−Second Derivative Method" (Baseline = Best, D2, Tol% = 2.9%, Smoothing Sm% = 3.6%, Peak Type = All Peak Fns, Gauss Area). The fitted data was analyzed in "Review Fit", and numerical area parameters were exported for the peaks ("Numeric"); the relative area parameters were used to estimate relative glycan proportions.

N-Glycan Profiling on eGene Unit. The eGene CarbCE multicapillary gel electrophoresis unit (eGene), a prototype system equipped with a blue LED, was operated as previously described.[13] Briefly, a 12-capillary (e.l. 10 cm with i.d. 20 μm) CarbCE cartridge was used in conjunction with a buffer modified (25 mM Li-acetate, pH 4.75) DNA separation gel buffer (eGene). APTS-labeled samples (see above) were reconstituted in 10 μL of ultrapure water and subject to size-exclusion chromatography on Sephadex G-10 resin.[12] The obtained samples were reconstituted in 30 μL of ultrapure water and electrokinetically injected for 10 s at 2000 V and separated at 2000 V for 1800 s. A four-capillary cartridge (e.l. 30 cm, i.d. 50 μm) was also tested in a similar way, except that the sample was separated at 6000 V for 2000 s. An APTS-labeled maltooligosaccharide ladder was run in parallel as a migration standard.

Safety Considerations: Labeling N-glycans with APTS requires the addition of $NaCNBH_3$ in acidic conditions, which releases toxic HCN. Therefore, one should work in a hood or well-ventilated place.

RESULTS AND DISCUSSION

Optimization of the Serum N-Glycome Sample Preparation. The serum N-glycome sample preparation protocol for clinical laboratories (Figure 1) is not difficult to perform, and it should not take longer than half a day; so, results can be obtained on the same day. It consists of five steps. The glycoproteins are denatured by heat and SDS to enable PNGase F to reach the glycosylation sites, and the N-glycans are specifically removed from their denatured carrier proteins using PNGase F. Subsequently, the sialic acids are cleaved from the glycans to simplify the CE profile, and the solutions are dried to prepare the samples for the final step: labeling of the reducing termini of the N-glycans using the APTS fluorophore. The label contains three sulfonic acid groups, which provide the charge needed for electrophoretic separation of the N-glycans. Moreover, only one APTS molecule can bind to an N-glycan molecule, which makes quantification easy. To speed up the enzymatic reactions, we produced recombinant PNGase F and sialidase in *Escherichia coli* (*E. coli)* in order to use large amounts of the enzymes at low cost. We found that 2.5 IUBMB mU of PNGase F can completely remove the N-glycans from the glycoproteins in 3 μL of serum in a volume of 10 μL in less than 10 min (results not shown). Moreover, 25 mU of sialidase should be added to the optimized PNGase F digest to complete the reaction within 10 min at its optimal pH of 5.0 (100 mM NH_4Ac pH 5, results not shown).

Derivatization of N-Glycans with APTS. The sialidase digest (15 μL) was evaporated to dryness in the PCR thermocycler with the tubes and thermocycler lid open (max. 20 min at 80 °C). If we assume that a liter of serum contains 30 g of glycoproteins with an average size of 50 kDa and between one to three N-glycosylation sites per protein, we estimate that at least 2−5 nmol of N-glycans are present in the tube. To optimize the labeling, we added 5 μL of APTS solution as described in Experimental Section, at concentrations that result in the presence of 50, 125, 250, or 500 nmol of APTS. The effects of APTS concentration, temperature, and incubation time were examined on two analyzers: ABI3130 DNA sequencer and MCE-202 MultiNA apparatus. The latter is more suitable for estimating the labeling yield, because the double-T injection allows steady-state injection of the APTS-labeled glycans in the presence of high amounts of free label and salts. In contrast, injection competition occurs in the traditional electrokinetic system of the ABI3130 sequencer, i.e., two samples with the same labeling yield but different salt and APTS concentrations will give different signal intensities. Figure 3 illustrates that the yield, estimated using the peak height of NA2 (see Figure 2 for nomenclature), increases in relation to the final APTS concentration. Moreover, derivatization is clearly accelerated at higher temperatures. Note that we did not include overnight labeling at 70 °C or at higher temperatures because of excessive evaporation. The highest yield was obtained after overnight incubation at 37 or 50 °C with a final APTS concentration of 100 mM in the sample. However, we do not know if this achieves complete labeling, but a high concentration of the labeling agent is evidently necessary to obtain a high yield and higher temperature accelerates the reaction. To enable reliable quantification of scarce N-glycans and to allow for some variability in labeling yield from sample to sample, an NA2 signal on the Shimadzu equipment of at least 150 mV is needed. Moreover, the labeling procedure should be rapid enough for clinical assays, i.e., not longer than half a day. We conclude that labeling for 2 h at 50 °C with a final APTS concentration of 100 mM meets these criteria.

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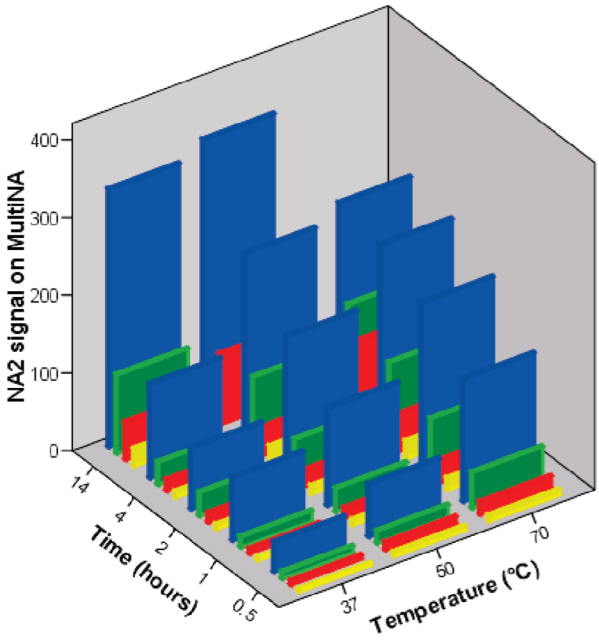

Figure 3. N-Glycan signal on a MCE-202 MultiNA system in relation to APTS derivatization conditions. Bars represent different APTS concentrations: 10 mM (yellow), 25 mM (red), 50 mM (green), and 100 mM (blue).

Effect of Labeling Conditions on N-Glycan Profile. Derivatization conditions affect not only the yield (Figure 3) but also the N-glycome profile (Figure 4, panel A). The N-glycan profiles look different for standard labeling (overnight at 37 °C, 10 mM APTS[7]), optimized labeling (2 h at 50 °C, 100 mM APTS), and 4 h incubation at 70 °C (Figure 4, panel A). The peak height of NA2, and to a lesser extent NA3 and NA3F, increases significantly under stronger labeling conditions, whereas the peak heights of the other N-glycans decrease (Table S-1, Supporting Information, and Figure 4, panel B). Temperature and incubation time seem to be the major determining factors, whereas the APTS concentration has a less pronounced effect. This indicates that different N-glycans have differential labeling kinetics under these conditions. Although the reason is unclear, it seems that structures that are not modified with an α(1,6)-fucose at their reducing *N*-acetylglucosamine (GlcNAc) are consistently more difficult to label with APTS. Chemical defucosylation under the more aggressive labeling conditions is highly unlikely because the relative abundance of NA2F was decreased by only 2%, whereas the product of such defucosylation (NA2) was increased by more than 8%. The situation is different for the N-glycans that are modified only with a bisecting GlcNAc but not with both a core fucose and a bisecting GlcNAc. They were hardly detectable after labeling at higher temperatures and for longer times. They seem to lose this modification gradually with increasing labeling time, and increasing the temperature from 50 to 70 °C also has a major impact (Figure 4, panel B). Therefore, the concentration of the product of this chemical removal of the bisecting GlcNAc (NGA2) increases more than would be expected only from improved labeling. Interestingly, glycans with a bisecting GlcNAc combined with an α(1,6)-fucose are much more stable. Their relative abundance in the profile decreased under stronger labeling conditions, but this follows the quantitative trend seen for core-fucosylated glycans without a bisecting GlcNAc modification. For example, the ratio between NA2F and NA2FB remained constant (mean = 2.4; $\sigma = 0.08$), which would not be the case if NA2FB had lost a substantial part of its bisecting GlcNAc. The values for the GlycoHepatoTest biomarker panel depend on the derivatization conditions, because GlycoFibroTest [log (NGA2FB/NA3)] and GlycoCirrhoTest [log (NA2FB/NA3)] are all ratios between a core-fucosylated and a noncore-fucosylated glycan (Figure 4, panel C). The biomarker values that are obtained depend strongly on the incubation temperature and on the labeling time (Figure 4, panel C), but these can be accurately controlled in PCR thermocyclers. As mentioned, the label concentration at a given temperature and the labeling time are much less important. Therefore, small pipeting errors in adding the label will have negligible effects, especially because the sample is evaporated to dryness before adding the label. In conclusion, use of the validated biomarkers of GlycoHepatoTest demands that serum N-glycome sample preparation should rigorously follow the same standardized protocol used in the labeling step. This is best ascertained by including a reference sample in each clinical sample batch, or at least when validating a new laboratory's setup, with the definition of acceptance criteria for that reference sample.

Glycomics on Next-Generation Microfluidics Systems. To profile APTS-labeled N-glycans, we tested CE-based analyzers used for separating and sizing DNA and RNA fragments. These systems are significantly cheaper than the DNA sequencers that we used for GlycoHepatoTest.[12] The separation is also based on capillary electrophoresis, and their use of a blue LED (470 nm) with a fluorescence detector (>520 nm) makes their detection system compatible with the APTS fluorophore. The MCE-202 MultiNA system (Shimadzu) allows high-throughput analysis because it processes a 96-well plate in less than 3 h (including washing steps). The samples can be automatically mixed with internal markers to correct for migration differences. First, we compared the commercial DNA-500 and RNA buffer from Shimadzu at the highest separation voltage (1500 V), because this results in the best resolution (Figure 5, panel A). The RNA buffer is clearly superior for separating N-glycans. Moreover, we do not need to remove the large amounts of salts and free label in the sample in order to efficiently inject the samples. This is due to the double-T injector-based geometry of the microchips, which enables unbiased injection of every sample analyte independently of its electrophoretic mobility (Figure S-1, Supporting Information). The sample can be injected for a long time because of the large sample size, and so, every analyte can reach a steady-state concentration in the sample plug, which is separated afterward. However, we found that the injection time should be long enough for multiantennary N-glycans, which migrate slowly. The optimal injection time was 40 s for both the DNA-500 and RNA buffer (results not shown). Moreover, we determined the GlycoHepatoTest biomarker values for 18 chronic liver disease patients (three for each METAVIR fibrosis stage and three HCC patients) on both the ABI3130 DNA sequencer (Genemapper software) and the MultiNA system (manually). We found a good linear correlation ($R^2 \geq 0.9$ for all three biomarkers) between the two systems (Figure 6 and Table S-2, Supporting Information).

The 2100 Bioanalyzer from Agilent makes use of a disposable microchip rather than a reusable chip (e.g., as in the MCE-202 MultiNA). However, the microchip has to be filled manually with

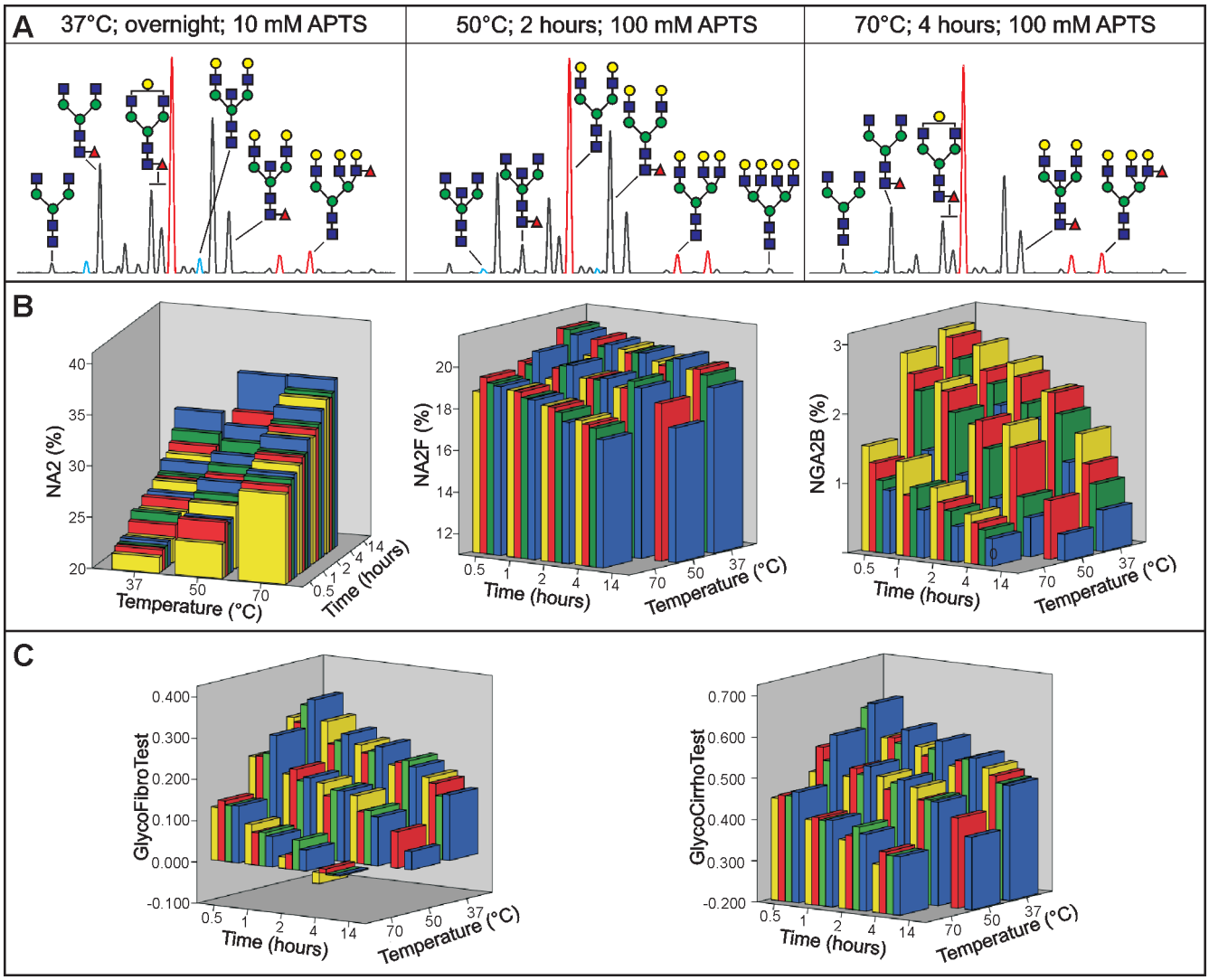

Figure 4. Effect of APTS labeling on N-glycome profile. (A) DSA-FACE profile obtained after three different labeling conditions were used. Peaks depicted in red represent N-glycans that are more abundant at higher temperature and longer incubation time, whereas the blue peaks disappear due to loss of their bisecting GlcNAc. (B) N-Glycans without an α(1,6)-fucose are increased under stronger labeling conditions (left panel) in contrast to those with this modification (e.g., NA2F). Glycans with a bisecting GlcNAc show degradation (right panel). (C) GlycoHepatoTest biomarker panel in function of APTS labeling conditions. Bars represent different APTS concentrations: 10 mM (yellow), 25 mM (red), 50 mM (green), and 100 mM (blue). [yellow ●: galactose, ■: *N*-acetylglucosamine, green ●: mannose, ▲: fucose; symbols are those suggested by the Consortium for Functional Glycomics (http://glycomics.scripps.edu/CFGnomenclature.pdf)].

running buffer using a chip priming station. Moreover, samples and marker solution have to be pipetted manually into the chip. Eleven or 12 samples can be analyzed in 30−45 min (depending on the kit used). The system employs cross-injection that is very similar to that of the MCE-202 MultiNA (Figure S-1, Supporting Information), except that there is no offset and, thus, a straight cross. This results in smaller injection plugs and potentially higher resolution profiles. We tested the same serum sample using buffers from Agilent and the CE settings for these kits (Figure 5, panel B). The highest resolution was obtained using a prototype buffer. For this system too, we did not have to clean up the samples to remove free label and salts. The result is very similar with that obtained for the RNA buffer on the MCE-202 MultiNA, as illustrated in Figure 5, except that NA2B is easier to detect on the MultiNA. However, the resolution is inferior to that of the ABI 3130 sequencer (Figure 4), which uses capillaries with an effective length of 36 cm, whereas the microchips of Shimadzu and Agilent have a separation length of only 1 to 2 cm. Surprisingly, the MCE-202 MultiNA and 2100 Bioanalyzer are still able to resolve most of the N-glycans in just a few minutes. To determine the biomarkers of GlycoHepatoTest, we should be able to reliably quantify the peak heights of NGA2FB, NA2FB, NA3, and NA3F (Figure 2 for nomenclature). This is possible with these microfluidics systems, but NA2F and NA2FB are not baseline resolved (Figure 5). Software deconvolution of these two peaks is possible (Figure 7), and chemical optimization of the separation buffers could improve the resolution further. Another improvement could be gained by increasing the separation channel length on the same chip surface.

The last microfluidics-based CE platform that we tested was an eGene apparatus modified by the addition of a blue LED and associated optics. This instrument can be equipped with a disposable cartridge containing 12 capillaries for simultaneous analysis of samples in less than half an hour. Automation and 96-well plate design assures high-throughput analysis. We analyzed samples using the buffer modified DNA separation solution.[13] Due

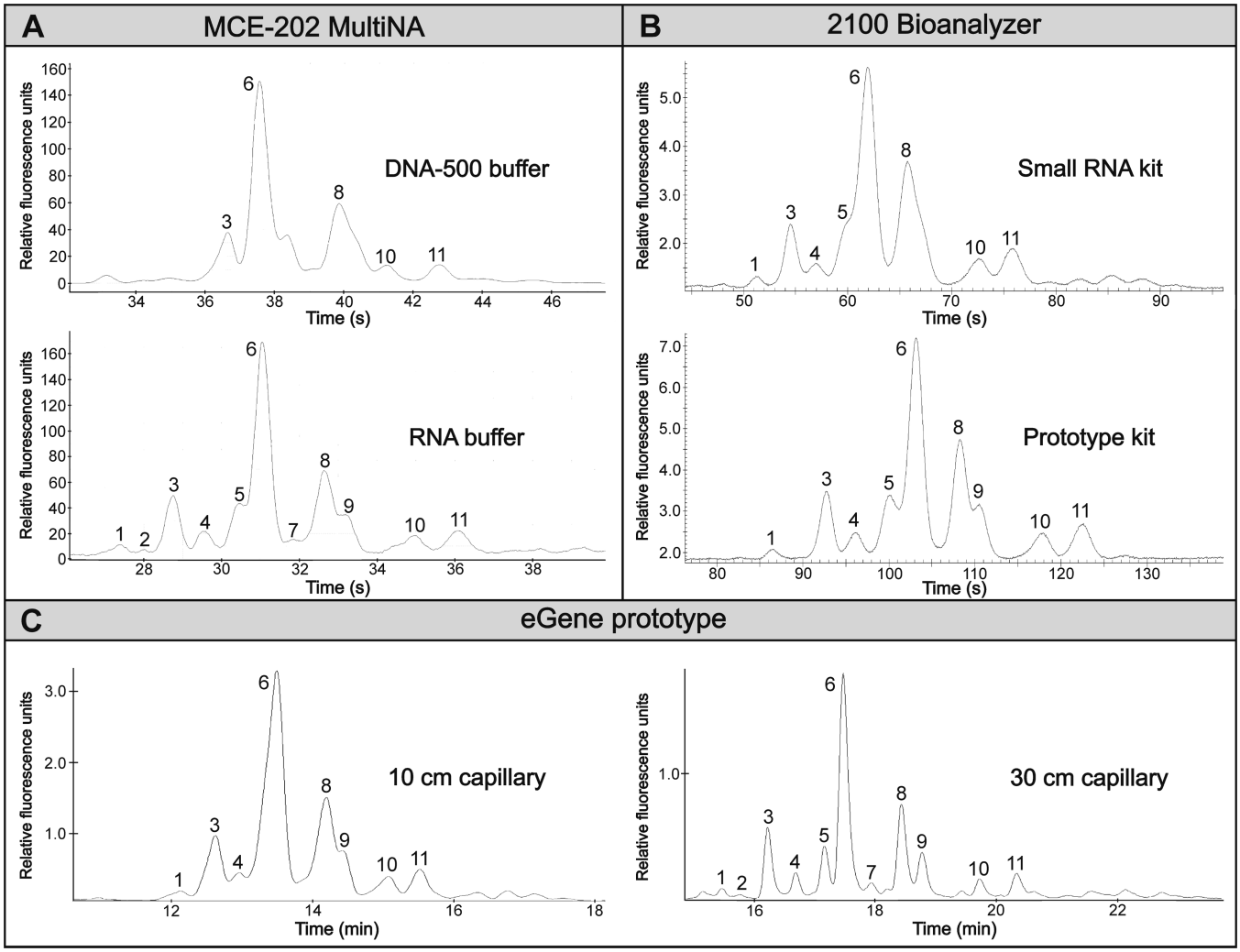

Figure 5. Serum N-glycome analysis on the MCE-202 MultiNA (A), the 2100 Bioanalyzer (B), and the eGene prototype system (C). APTS-labeled N-glycans were prepared from serum following the optimized procedure (Figure 1). Subsequently, they were separated by capillary electrophoresis on three different analyzers [see Experimental Section for settings; note that we applied the optimal injection time (40 s) and separation voltage (1500 V) to the MultiNA system]. 1, NGA2; 2, NGA2B; 3, NGA2F; 4, NGA2FB; 5, NG1A2F; 6, NA2; 7, NA2B; 8, NA2F; 9, NA2FB; 10, NA3, and 11, NA3Fb. See Figure 2 for nomenclature of the N-glycans.

to the large amount of the labeling dye (APTS) still in the reaction solution, electrokinetic sample injection was challenging. In other words, the presence of large quantities of free APTS and salts, which have a high electrophoretic mobility, suppressed the injection of APTS-labeled N-glycans and resulted in a biased analysis. To overcome these problems, most of these interfering ions were removed by size exclusion chromatography on Sephadex G-10. This extra cleanup step can be performed by a semiautomated liquid handling system (PhyNexus, CA) using pipet tips that can be filled with Sephadex G-10.[14] The resultant CE profiles (Figure 5, panel C) were very similar to those obtained by other microfluidics systems and could be improved using 30 cm capillaries (Figure 5, panel C).

CONCLUSION

It is generally believed that studying glycosylation requires complex and state-of-the-art technology, such as mass spectrometry and nuclear magnetic resonance.[14] This certainly holds true for most of the research questions concerning glycosylation, but in the context of chronic liver disease diagnostics, our results clearly show that high-throughput profiling of serum-derived N-glycans is possible in clinical chemistry laboratories. Our optimized procedure for preparing APTS-labeled N-glycans from serum can be performed in 96-well plates in less than half a day and involves only addition of reagents and incubation in a PCR thermocycler. Moreover, the required enzymes can be produced in *E. coli*, making strict quality control possible. To obtain a sufficient amount of labeled glycans, we had to use a large amount of APTS and incubate at higher temperatures, as guided by other studies.[11] We found that glycans with an α(1→6)-fucose at the reducing GlcNAc had faster derivatization kinetics than sugars without this modification. Differential labeling has been described for different monosaccharides,[15] but as far as we know, this has not been studied before for complex N-glycans with the same reducing terminus. The reason for this observation is not clear, but the α(1,6)-fucose residue on the GlcNAc at the reducing

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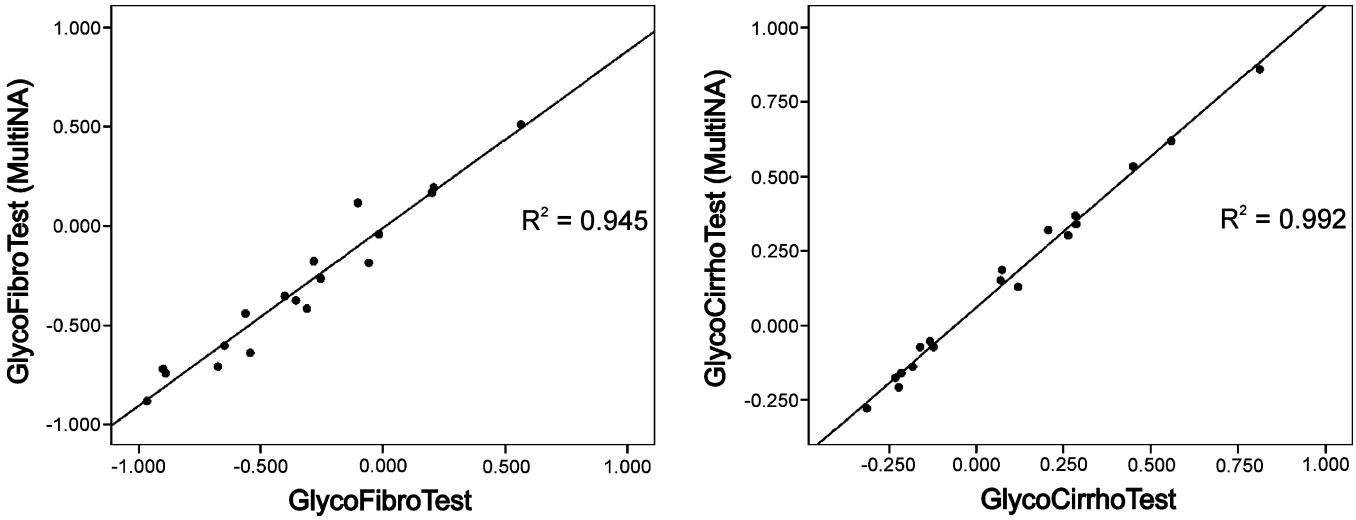

Figure 6. GlycoHepatoTest biomarker panel determined on an ABI 3130 DNA sequencer and MCE-202 MultiNA platform. GlycoFibroTest [log(NGA2FB/NA3)] and GlycoCirrhoTest [log(NA2FB/NA3)] were determined for 18 different serum samples [three samples per METAVIR fibrosis stage and three HCC samples were prepared following the optimized protocol (see Figure 1 of main text)] on both the ABI 3130 DNA sequencer and MCE-202 MultiNA. Graphs represent scatter plots, and the best fitting linear curve with its correlation coefficient is given (SPSS 15.0 software).

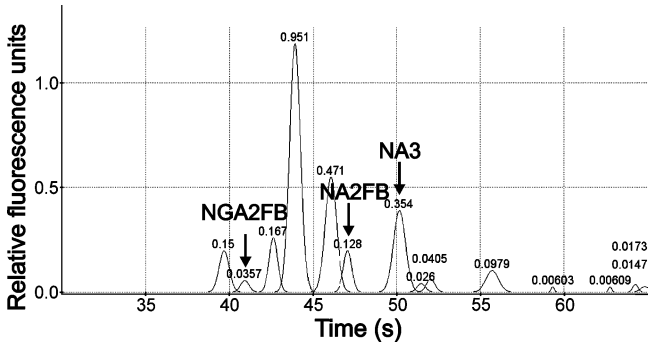

Figure 7. Software deconvolution on 2100 Bioanalyzer. Peak-fitting software (Peakfit) was used to fit the peaks from the serum glycans (see Experimental Section). The relative area parameters are given, estimating the relative glycan abundances. Arrows indicate the N-glycans constituting GlycoHepatoTest. See Figure 2 for nomenclature of the N-glycans.

terminus might alter the steric environment of the reducing terminus or perhaps promote the open-chain form of the GlcNAc. Consequently, these data show that reproducibility of the N-glycan profile requires strict adherence to the procedure.

The processed sample contains more than 150 μM of the analytes, making it suitable for detection by cheaper blue LED-based detectors used in commercial microfluidics CE systems. Although the separation channels are very short in the MCE-202 MultiNA and 2100 Bioanalyzer systems (about 1 to 2 cm), they yield comparable N-glycan profiles in which most of the glycans are resolved. However, NA2FB (Figure 2) is not baseline resolved, but software deconvolution can be used to reliably quantify this peak. The eGene prototype, when equipped with 30 cm capillaries, had a resolution resembling that of an ABI 3130 DNA sequencer (except that isomers were not resolved) and was comparable with the MultiNA and BioAnalyzer systems when supplied with 10 cm capillaries. Please note, however, that an extra sample preparation step was required to remove the excess of free label in order to allow sufficient injection of the APTS-labeled glycans on this analyzer. In conclusion, all three systems are suitable for GlycoHepatoTest analysis and can be used to profile the N-glycans of abundant proteins (e.g., recombinant IgGs).

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SUPPORTING INFORMATION AVAILABLE

A detailed description of the recombinant production of PNGase F and sialidase in *E. coli* and an explanatory figure of the double-T injection mechanism. Extra results on the effect of labeling conditions on the N-glycan profile and the correlation between the ABI 3130 DNA sequencer and MCE-202 MultiNA system. This material is available free of charge via the Internet at http://pubs.acs.org.

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