Glycome mapping on DNA sequencing equipment

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Published online 27 June 2006; doi:10.1038/nprot.2006.60

Here we provide a detailed protocol for the analysis of protein-linked glycans on DNA sequencing equipment. This protocol satisfies the glyco-analytical needs of many projects and can form the basis of 'glycomics' studies, in which robustness, high throughput, high sensitivity and reliable quantification are of paramount importance. The protocol routinely resolves isobaric glycan stereoisomers, which is much more difficult by mass spectrometry (MS). Earlier methods made use of polyacrylamide gel–based sequencers, but we have now adapted the technique to multicapillary DNA sequencers, which represent the state of the art today. In addition, we have integrated an option for HPLC-based fractionation of highly anionic 8-amino-1,3,6-pyrenetrisulfonic acid (APTS)-labeled glycans before rapid capillary electrophoretic profiling. This option facilitates either two-dimensional profiling of complex glycan mixtures and exoglycosidase sequencing, or MS analysis of particular compounds of interest rather than of the total pool of glycans in a sample.

INTRODUCTION

Labeling carbohydrates by reductive amination with the fluorophore APTS for capillary electrophoretic (CE) separation was introduced in 1995 (ref. 1). This label facilitated sensitive detection by the stable argon ion lasers commonly used in commercial analytical equipment, while maintaining the good separation characteristics that had been previously achieved by labeling with the naphthalene analog of APTS (8-aminonaphtalene-1,3,6-trisulphonic acid, or ANTS), introduced in 1990 (ref. 2).

The detection limit of laser-induced fluorescence (LIF) of APTS-labeled sugars in CE is in the low femtomolar range. Taking into account the fact that the minimum practical sample volume for most CE instruments is ~10 µl, 1–10 fmol of glycan should be sufficient to obtain a CE-LIF profile; however, a large excess of the APTS label (10 mM range) is needed to drive the reductive amination-based glycan-to-label coupling to completion[3], and injecting the undiluted labeling mix would overload the capillaries with free label. Consequently, the labeling mixture is diluted 500–1,000-fold before injection, which effectively decreases the overall sensitivity of the method by three orders of magnitude. Thus, 1–10 pmol of glycan is needed before the labeling step. This overall sensitivity is unfortunately not much better than that in fluorophore-assisted carbohydrate electrophoresis (FACE)[2], which uses much less expensive PAGE gels and fluorescence imaging on standard UV transilluminators.

We have solved this problem by developing a parallel and robust cleanup step using size-exclusion chromatography over Sephadex G10 packed in 96-well plates[4]. This simple step removes >90% of the free APTS label and effectively desalts the sample, while recovering >70% of the labeled glycans in a nonbiased way with respect to glycan size, provided that the glycans have more than two monosaccharide residues. The labeling mix therefore has to be diluted only tenfold to obtain acceptable CE-LIF results, effectively regaining two orders of magnitude in sensitivity. This means that 10–100 fmol of unlabeled glycans is sufficient, thereby breaking the picomole barrier for glycan analysis by CE. This Sephadex G10 cleanup step is an essential part of the integrated protocol that we describe here.

For detection at the low femtomole level, the problem of achieving more sensitive integrated glycoconjugate analysis has now shifted to preparation of the glycans themselves. Assuming that one would like to detect an *N*-glycan species representing 1% of the *N*-glycan pool present on a given target glycoprotein and has the above technology to hand with a detection limit of 10 fmol, enough glycan can be derived from 1 pmol of that protein (~50 ng for an average protein). At this level, loss by adsorption to vessel walls is important and it becomes imperative to minimize the number of tube transfers of the material, to miniaturize the reaction volumes, and to ensure reagent purity.

We have found an excellent complement to our more sensitive CE-LIF analysis in the deglycosylation method developed by scientists at the Department of Analytical Chemistry at Genentech[5]. The critical improvement of their method over earlier peptide *N*-glycosidase F (PNGaseF)-based *N*-glycan preparation methods lies in first denaturing the glycoprotein and dot-blotting it on a polyvinylidene fluoride (PVDF) membrane–lined 96-well filtration plate. Most nonproteinaceous contaminants in the sample are thus removed, and all reagents used subsequently are removed by extensive washing without any tube transfers. Consequently, the *N*-glycans are released with PNGaseF in a minimal volume of low-molarity buffer. On vacuum evaporation, they are ready for APTS labeling. This method of *N*-glycan preparation is limited by the binding capacity of the PVDF membrane in one well of the 96-well plate. On the basis of our years of experience using this method, up to ~10 µg of an 'average' glycoprotein (a few hundred picomoles) will bind to such a well. Obviously, if the glycoproteins of interest are contaminated with other proteins, those nontarget proteins will occupy part of the binding capacity of the membrane, thereby reducing the amount of the glycoprotein of interest bound to that membrane. This is particularly important when the target glycoproteins are stabilized with albumin or when analyzing total cellular lysates, which contain an excess of nonglycosylated proteins that bind to PVDF more effectively than do glycoproteins, aggravating the situation. In addition, one should be aware of the fact that PVDF may bind different proteins with varying efficiency. We have not noted any differences in the glycan profile of human serum, however, whether it is prepared by this method or by the in-solution method described below.

TABLE 1 | Exoglycosidases typically used in the sequencing of glycans[a]

Enzyme	Source	Specificity	Amount per digest
α-Sialidase	*Arthrobacter ureafaciens*	α2,6 > α2,3 > α2,8-bound sialic acid	2 mU
α-Sialidase	Recombinant from *Salmonella typhimurium*	α2,3-bound sialic acid	5 mU
β1,4-Galactosidase	Recombinanant from *Streptococcus pneumonia*	β1,4-bound galactose	0.4 mU
α-Fucosidase	Bovine kidney	α1,2-, α1,3-, α1,4- or α1,6-bound fucose	2 mU
α1,3/4-Fucosidase	Almond meal	α1,3- or α1,4-bound fucose	1 μU
β-*N*-Acetylhexosaminidase	Jack bean	β-bound HexNAc	10 mU
α-Mannosidase	Jack bean	α1,2-, α1,3- or α1,6-bound mannose	20 mU
α1,2-Mannosidase	*Trichoderma reesei*	α1,2-bound mannose	0.33 μg

[a]Exoglycosidases that are typically used in the sequencing of glycans are listed, together with their source, specificity and the typical amount used in a digest. Use the same amount for deglycosylation reactions in 2 or 10 μl (Step 8Ciii). This list is not comprehensive.

In the 'problem' cases mentioned above, and also if one wishes to derive the *N*-glycans from more than ~10 μg of glycoprotein, the more 'classical' in-solution PNGaseF digestion method can be used. In this method, the protein is first denatured by heating in the presence of SDS, after which a molar excess of a nonionic detergent (typically Nonidet-P40) is added. The nonionic detergent protects the PNGaseF enzyme from denaturation by SDS. However, these detergents hamper downstream analysis of the released glycans, and a miniaturized, porous, graphitized carbon solid-phase extraction method (modified from ref. 6) is included in this protocol to remove proteins and detergents from the glycan sample before further processing.

This protocol not only allows fingerprinting of glycan mixtures, it often also facilitates rapid obtainment of structural information on the glycans of interest. These experiments need highly purified glycosidases that selectively hydrolyze glycosidic bonds linking specific carbohydrate moieties in a stereospecific way. Most of the enzymes used to obtain structural information on *N*-and *O*-glycans are exoglycosidases; that is, they selectively remove monosaccharide residues from the nonreducing termini of a glycan molecule. By analyzing the electrophoretic mobility of a glycan before and after digestion with increasingly complex mixtures of such enzymes, one can often deduce the structure of the glycan or part of it. This works especially well for glycans derived from cell types whose relevant glycosylation pathways are well characterized (mammalian, insect, plant, and some yeasts and fungi). A nonexhaustive list of commonly used exoglycosidases is given in **Table 1**.

Exoglycosidase array sequencing can be performed on mixtures of glycans if one is interested in the main components and these components are present in distinct amounts. In these cases, peak intensity is useful in tracing the peak of a specific compound throughout the sequencing reactions. Because this approach is clearly suboptimal, however, we have developed a method of HPLC separation for APTS-labeled glycans. The labeled glycans are highly anionic (three sulfonic acid groups on the label); therefore, adding an ion pairing reagent (triethylamine) to the HPLC buffer mask these charges, upon which the separation behavior is again determined more by the glycan structure (hydrophobicity) in normal-phase HPLC (i.e., smaller glycans eluting earlier than larger ones). Because multicapillary DNA sequencers allow the rapid analysis of many samples, one can collect fractions over the whole HPLC gradient and then rapidly profile all of them in a two-dimensional mapping approach. The glycans in such HPLC fractions can be sequenced by using exoglycosidases as described above, but they can be also analyzed by MS. Although HPLC profiling is one of the techniques currently used for the analysis of glycans (and this protocol can be used in such profiling), this is not our prime intention in this protocol. The different separation chemistries of the HPLC and CE methods described can be advantageous in two-dimensional mapping, but both the sensitivity and throughput of the former are much lower.

In our hands, methods for matrix-assisted laser desorption-ionization (MALDI) of APTS-labeled glycans[7,8] are not sufficiently robust to be generally applicable. In addition, the sulfonic acid

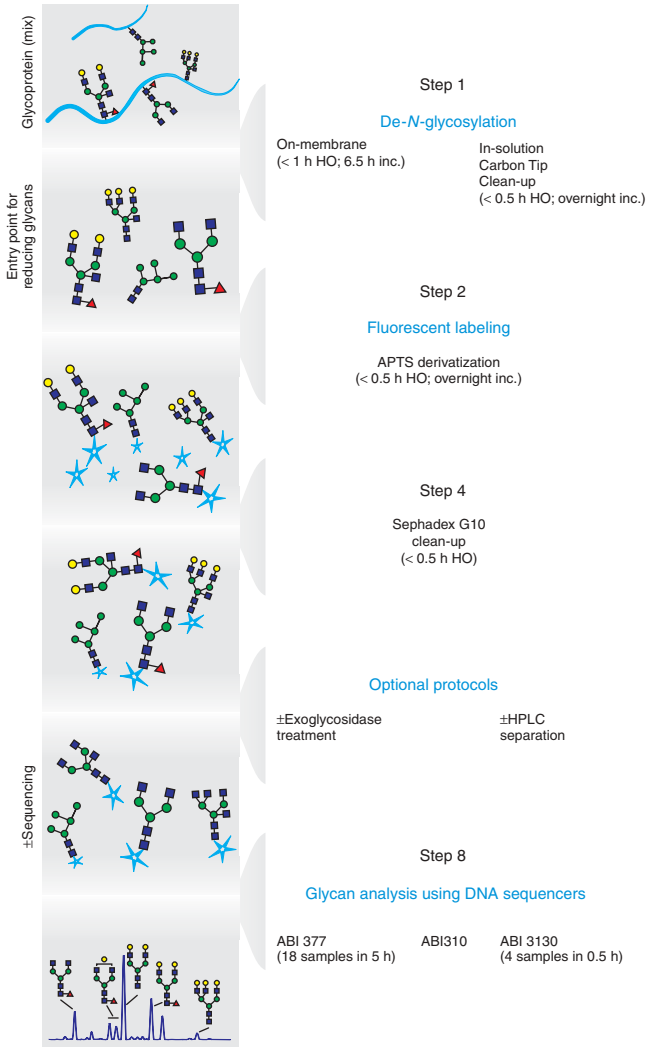

Figure 1 | Protocol workflow. The step numbers of the PROCEDURE are indicated. An estimation of the hands-on (HO) and incubation (inc.) times needed are given in parentheses.

groups on the label are labile in the reflectron, thereby limiting the mass resolution and accuracy of these measurements. Much better results are obtained with electron-spray ionization MS. On a Q-Trap mass spectrometer (Applied Biosystems), 100 fmol μl^{-1} of APTS-labeled glycan is sufficient to obtain good quality tandem mass spectrometry (MS/MS) fragmentation data in the negative-ion mode in an off-line setting (ref. 9; and K. Sandra, personal communication). Owing to the strongly anionic character of the label, only fragment ions that retain this label, and thus contain the 'reducing terminus' of the glycan, are detected. This simplifies the spectra and their interpretation.

In this protocol, CE-LIF analysis is done on the current generation of (multi)capillary DNA sequencing equipment commonly used in molecular biology laboratories (**Fig. 1**), as an extension of our previous use of polyacrylamide gel-based DNA sequencers (**Fig. 1**). We have optimized the analytical parameters for the latest ABI 3130 instrument to enable both DNA and glycans to be analyzed in the same automated run of the instrument, thereby allowing full and unhindered double use.

Because of the strict 1:1 stoichiometry of the APTS labeling of glycans, this protocol facilitates straightforward quantification of the analytes. On the ABI 3130 instrument, the linear range stretches from 0.1 to 15 fmol μl^{-1} of labeled glycan (in the sample from which one injects), thus the labeled glycans have to be diluted appropriately. Owing to the very good reproducibility of the method, even small differences (10%) in glycan abundance can be detected reliably. Because of the electrokinetic loading system, only very small amounts of sample are loaded, which means that most of the sample remains for other analyses (e.g. exoglycosidase sequencing, MS).

In the past, many researchers have optimized or introduced techniques for the analysis of glycans (refs. 10,11, and references therein). The implementation of glycan analytical methods on DNA sequencers adds the power of high throughput to glycobiology and enables us to address glycobiological research issues from an 'omics' perspective. One can envisage the comparative analysis of, for example, the glycan patterns of thousands of blood samples, thousands of mutant clones of pathogens and collections of carbohydrate-active enzyme variants, as well as the effect of drug libraries on glycosylation-dependent phenomena within a reasonable timescale and cost. The presence of sialic acids on the glycans adds an extra negative charge on the structures, which is a disadvantage in many CE-based methods. Using this protocol, we can obtain readable electropherograms, most probably owing to the use of borate in the separation buffer. However, sialic acids add to the enormous heterogeneity of the glycans, and a desialylation reaction may thus add to the readability in complex mixtures.

This protocol has been successfully applied to the analysis of different recombinant glycoproteins, including microgram amounts of recombinant human EPO produced in *Leishmania tarentolae*[12], IgG purified at the microgram scale from small-scale culture of human IgG-producing NS0 cells[13], and bovine rhodopsin purified from glyco-engineered HEK293S cells[14]. In addition, we have used this protocol for rapid high-throughput screening of yeast and fungal clones to evaluate the efficiency of glycan engineering in these organisms[15].

In the field of diagnostics, we have generated *N*-glycome profiles of more than 1,000 human, murine and rat sera, and developed high-performance diagnostic biomarkers for liver cirrhosis[16] and hepatocellular carcinoma (Liu, X. *et al.*, manuscript in preparation).

All steps in this protocol, including the *N*-glycan preparation and exoglycosidase options, are optimized for high-throughput applications. When using CE-based DNA sequencers, the hands-on time of the analysis is very low. With the four-capillary ABI 3130, four samples are run in half an hour, and automation is easy with 96- or 384-well plates. Obviously, improved throughput can be reached with the existing equipment and more capillaries. The methods of *N*-glycan preparation also mainly involve enzyme incubation steps and are optimized for high-throughput applications. With the on-membrane method, no more than 1 h of hands-on time is needed for 96 samples. The optional method using HPLC is not normally included in this protocol, and is incorporated only when more structural data of complex mixtures are needed.

MATERIALS

REAGENTS

- Acetonitrile (Biosolve)
- Acrylamide:bisacrylamide (19:1; Bio-Rad)
- Ammonium acetate (Merck)
- Ammonium persulfate (Sigma)
- DTT (Sigma)
- Almond meal fucosidase (Prozyme)
- *Arthrobacter ureafaciens* sialidase (Roche)
- Bovine kidney fucosidase (Prozyme)
- Jack bean β-*N*-acetylhexosaminidase (Prozyme)
- Jack bean α-mannosidase (Sigma)
- *Salmonella typhimurium* sialidase (Takara)
- *Streptococcus pneumoniae* β-galactosidase (Prozyme)
- *Trichoderma reesei* α-1,2-mannosidase (expressed and purified in our laboratory[17])
- Iodoacetic acid (Sigma)
- Methanol (Biosolve)
- $NaCNBH_3$ (Acros) **! CAUTION** In acidic conditions, $NaCNBH_3$ releases HCN gas; work in a hood or a well-ventilated place
- Nonidet P40 (New England Biolabs)
- PNGaseF (New England Biolabs) ▲ **CRITICAL** Different suppliers use different unit definitions for PNGaseF. One IUBMB milliunit equals one Prozyme unit and 65 New England Biolabs units.
- Polyvinylpyrrolidone 360 (Sigma)
- Sephadex G10 (Amersham)
- Sodium acetate (Sigma)
- *N,N,N′,N′*-Tetramethylethylenediamine (TEMED; ICN)
- Trifluoroacetic acid (TFA; Sigma)

EQUIPMENT

- Adhesive tape for 96-well plates (Millipore)
- Capillary (36 cm for ABI 310) or capillary array (36 cm for ABI 3130; Applied Biosystems)
- GeneScan (ABI 377 and ABI 310) or GeneMapper (ABI 3130) software (Applied Biosystems)
- Genetic analyzer (ABI 377, ABI 310 or ABI 3130, Applied Biosystems)
- Multiscreen-ImmobilonP (Millipore)
- Multiscreen-Durapore 96-well filtration plates (Millipore)
- Multiscreen column loader system, 100 µl (Millipore)
- NuTip type P20 carbon solid-phase extraction pipette tips (Glygen)
- Reaction plates, 96-well (Applied Biosystems)
- Vacuum manifold for filtration plates (Millipore)

REAGENT SETUP

On-membrane denaturing buffer: 8 M urea (Merck), 360 mM Tris-HCl (pH 8.6), 3.2 mM EDTA.

In-solution denaturing buffer: 0.2 M Tris-HCl (pH 8), 1% SDS and 1% β-mercaptoethanol (BDH).

APTS labeling solution: 1:1 mixture of 20 mM APTS (Molecular Probes) in 1.2 M of citric acid (Calbiochem) and 1 M of $NaCNBH_3$ in DMSO (Aldrich)

! CAUTION In acidic conditions, $NaCNBH_3$ releases HCN gas; work in a hood or a well ventilated place

HPLC solvent composition Solvent A: 0.1% acetic acid (Merck) in acetonitrile; solvent B: 0.2% acetic acid in water plus 0.2% triethylamine (Merck).

TABLE 2 | Reagents needed for glycan electrophoresis.

	ABI 377	ABI 310	ABI 3130
Size standard	GeneScan-500 ROX (Applied Biosystems)	GeneScan-500 ROX (Applied Biosystems)	GeneScan-500 LIZ (Applied Biosystems)
Polymer	19:1 mixture of acrylamide:bisacrylamide (Bio-Rad)	POP-6 (Applied Biosystems), three times less concentrated then specified by the manufacturer	POP-7 Performance Optimized Polymer (Applied Biosystems)
Running buffer	89 mM Tris, 89 mM borate (Sigma), 2.2 mM EDTA (= standard DNA sequencing buffer)	Running buffer for ABI 310 (Applied Biosystems)	Running buffer for ABI 3130 with EDTA (Applied Biosystems)
Other	Deionized formamide (Sigma)	Deionized formamide (Sigma)	

Glycan analysis The reagents needed for glycan electrophoresis depend on the DNA sequencer equipment, as described in **Table 2**:

EQUIPMENT SETUP

Normal phase HPLC of APTS-labeled glycans Any make of HPLC instrument will do. We use a Dionex system equipped with a GS50 pump, a FP-1520 fluorescence detector (Jasco) and a Foxy Jr. fraction collector (Isco). Samples are injected through a rheodyne injection valve or an AS50 autosampler onto a TSK-GEL amide-80 column (25 × 4.6 mm internal diameter; 5-μm particle size; Tosoh Bioscience), preceded by a TSK-GEL amide-80 Guard column (1 × 4.6 mm internal diameter; 5-μm particle size; Tosoh Bioscience). Set up an HPLC method using the HPLC solvents (see REAGENT SETUP). The gradient described in **Table 3** was optimized for separation of glycans containing up to 30 glucose units. For purification of a single glycan, rather than a complex mixture, a faster gradient can be used.

TABLE 3 | Gradient for separation of glycans containing up to 30 glucose units.

Time (min)	% Solvent B	Action
0	22	Inject
10	22	Start collecting 0.5-ml fractions
95	60	
105	70	
105.1	90	
115	90	
115.1	22	
125	22	Flow = stop

PROCEDURE

Preparation of *N*-glycan samples

1| Two methods for preparing *N*-glycan samples are described: 96-well on-membrane deglycosylation (Step A), and in-solution deglycosylation (Step B). Choose Option A for *N*-glycan preparation if <10 μg of protein are available. This protocol also efficiently removes salt, detergents and other contaminants that may influence PNGaseF activity. Choose Step B for *N*-glycan preparation if large amounts of protein are used, or when an excess of nonglycosylated protein is present. These methods have worked well in our hands. However, other approaches such as in-gel deglycosylation[18] have been described.

Glycan samples from other biological or commercial sources can be used providing that the reducing end is available for the reductive amination reaction and no compounds that inhibit this reaction are present (aromatic amines, high salt concentration). This protocol focuses on an integrated workflow for the analysis of PNGaseF-released *N*-linked glycans; however, its sections that deal with glycan microscale labeling, cleanup, (exo)glycosidase sequencing, HPLC separation and DNA sequencer–based profiling are also suitable for analyzing other types of reducing glycan (starch fragments[19], lignocellulosic biomass hydrolysates[20], glycosaminoglycan-derived disaccharides[21] and glycolipid headgroups[22]), as long as they withstand the acid-catalyzed labeling conditions. Nevertheless, the methods for preparing samples of these types of glycans need to be miniaturized further to match the very high analytical sensitivity of this protocol towards glycans.

(A) 96-Well on-membrane deglycosylation

(i) Denature the glycoproteins by adding a minimum of two volumes (preferably not exceeding 300 μl) of on-membrane denaturing buffer (see REAGENT SETUP). Incubate at 50 °C for 1 h.

(ii) Wet the PVDF membranes at the bottom of the wells of a Multiscreen-ImmobilonP plate with 300 μl of methanol. Only treat the membranes in the wells that you want to use; seal the others with adhesive tape for 96-well plates. Throughout this protocol, liquids are removed from the wells by applying a vacuum under the plate, unless specified otherwise.

(iii) Wash the membranes three times with 300 μl of water and once with 50 μl of on-membrane denaturing buffer.

(iv) Load the denatured glycoprotein (from Step 1Ai) in preconditioned wells containing 15 μl of on-membrane denaturing buffer. If high volumes cannot be avoided in Step 1Ai, reload with additional sample solution.

▲ **CRITICAL STEP** It is very important to regulate the vacuum under the plate carefully so that the liquid is passed through the membrane in ~1 min. Alternatively, the sample solution can be removed by centrifugation. For optimal results, optimize the centrifugation conditions to allow complete removal of liquid from the wells in 5 min. Using our centrifuge equipment, this corresponds to a 5-min spin at 30*g*.

(v) Wash the membranes twice with 50 µl of on-membrane denaturing buffer.
(vi) Reduce the bound protein by adding 50 µl of 0.1 M DTT in on-membrane denaturing buffer (see REAGENT SETUP). Incubate at 37 °C for 1 h.
▲ CRITICAL STEP Prepare the DTT solution immediately before use
(vii) Wash the wells three times with 300 µl of water.
(viii) Carboxymethylate the proteins by adding 50 µl of freshly prepared 0.1 M iodoacetic acid in on-membrane denaturing buffer (see REAGENT SETUP). Incubate at room temperature (20–25 °C) for 30 min in the dark.
▲ CRITICAL STEP Prepare iodoacetic acid solution immediately before use
(ix) Wash the wells three times with 300 µl of water.
(x) Block the remaining protein binding capacity of the membranes by incubation with 100 µl of 1% polyvinylpyrrolidone 360 in water at room temperature for 1 h.
■ PAUSE POINT The plate can be left overnight at 4 °C with the blocking solution on the membranes if you cannot proceed immediately
(xi) Wash the wells three times with 300 µl of water.
(xii) Add 1.25 IUBMB milliunits of PNGaseF in 50 µl of 10 mM Tris-acetate (pH 8.3) and seal the plate to avoid evaporation. Digestion is typically complete after 3 h of incubation at 37 °C.
▲ CRITICAL STEP We refer to IUBMB units of PNGaseF. Note that different suppliers use other unit definitions (see REAGENTS). Also note that plant glycoproteins with typical α-1,3-core fucosylation are resistant to PNGaseF activity, in which case we refer to other methods[23,24] that use peptide *N*-glycanase A.
(xiii) Transfer the incubation mix from the wells to 200-µl reaction tubes or 96-well reaction plates. To avoid loss of sample, use a (multichannel) micropipette to do so.
(xiv) Evaporate the mixture to dryness (vacuum). Note that it is not necessary to remove the PNGaseF before APTS labeling, because its presence does not influence the electropherogram in the size range of 3–25 glucose units. If desired, Step 1Bv–1Bix from the in-solution deglycosylation option can be used to remove the protein.
■ PAUSE POINT Dried samples can be stored at −20 °C for at least 1 yr

(B) In-solution deglycosylation

(i) Dissolve the dried glycans in 100–200 µl of in-solution denaturing buffer (see REAGENT SETUP).
(ii) Incubate in a boiling water bath for 10 min.
(iii) When the mixture reaches room temperature, add Nonidet P40 to a final concentration of 1%, followed by 10 IUBMB milliunits of PNGaseF. Incubate overnight at 37 °C.
▲ CRITICAL STEP We refer to IUBMB PNGaseF units. Note that different suppliers use other unit definitions (see REAGENTS). Also note that plant glycoproteins with typical α-1,3-core fucosylation are resistant to PNGaseF activity, in which case we refer to other methods[23,24] that use *N*-glycanase A
(iv) Evaporate the reaction mixture to dryness (vacuum).
(v) Condition a NuTip P20 carbon tip with 20 µl of 60% acetonitrile plus 0.05% TFA, followed by 20 µl of 0.05% TFA in water.
(vi) Resuspend the dried glycans in 20 µl of 0.05% TFA in water and load them on the conditioned NuTip by 20 aspirate-expel cycles using a micropipette.
(vii) Wash the NuTip three times with 20 µl of 0.05% TFA in water; each wash involving five aspirate-expel cycles.
(viii) Elute the glycans two times with 10 µl of 60% acetonitrile plus 0.05% TFA; each step involving ten aspirate-expel cycles.
(ix) Evaporate to dryness (vacuum).
■ PAUSE POINT Dried samples can be stored at −20 °C for at least 1 yr

APTS derivatization reaction

2| Add 1 µl of APTS labeling solution to the dried glycans. Vortex carefully and spin briefly. Incubate the tubes or the plate overnight at 37 °C, preferably in a PCR thermocycler with a heated lid to avoid evaporation.
! CAUTION $NaCNBH_3$ in acidic conditions releases HCN gas; work in a hood or a well ventilated place

3| Quench the reaction by adding 4 µl of water.

96-Well Sephadex G10 postderivatization cleanup

4| Pack the wells of a Multiscreen-Durapore membrane-lined 96-well plate with Sephadex G10 to a height of 1.2 cm.
▲ CRITICAL STEP Plates are packed by using a 100-µl Multiscreen column loader system. After loading the first 100 µl of resin, wet it with 100 µl of water. Repeat this loading and swelling step once more. This gives microcolumns of ~200 µl of packed resin easily and reproducibly, which is not the case when preswollen slurry is loaded in the plates. Whole plates should be prepared at once and can be stored at room temperature. Immediately before use, re-swell the resin with 150 µl of water.

5| Centrifuge the microcolumns to dryness immediately before loading samples (10 s, 750*g*).

6| Load the samples obtained in Step 3 on the microcolumns and elute three times, each time adding 10 μl of water and centrifuging for 10 s at 750*g*. Collect the eluates in a tapered-well microtiter plate.
▲ **CRITICAL STEP** Centrifugation conditions may need to be optimized for different types of centrifuge. Use the highest acceleration and deceleration speed available on your centrifuge. Successful cleanup can be measured against a blank labeling mix and is hallmarked by an 80–90% reduction in absorbance at 424 nm before and after cleanup (dilute a pre- and postcleanup blank reaction mix to an equal volume in PBS buffer)

7| Evaporate the elution mixture to dryness (vacuum) and resuspend in the appropriate amount of water. For samples prepared by the 96-well on-membrane deglycosylation option, this is typically 5 μl. For samples prepared by the in-solution PNGaseF digestion, the volume should be adjusted according to the concentration. Steps 6 and 7 can be repeated if greater purity is desired.
■ **PAUSE POINT** APTS-labeled glycans are stable when stored at −20 °C; we have successfully reanalyzed samples that have been stored for >1 yr

Glycan analysis

8| At this point, you can either perform glycan analysis directly (Step 9), or perform exoglycosidase digestion (Step A) or HPLC separation (Step B) first. Take Step A if you want to simplify the electropherogram or for partial sequencing of the labeled glycans. Take Step B when analyzing complex mixtures or when MS or exoglycosidase sequencing of complex mixtures is desired. This approach typically follows *N*-glycan preparation by in-solution PNGaseF digestion, because more starting material is needed for detection with HPLC fluorescence detectors, for which the detection limit is ~1 pmol.

(A) Exoglycosidase digestion

(i) Transfer batches of 0.8 μl (if analyzing by ABI 377 and ABI 310) or 0.4 μl (ABI 3130) of the cleaned-up derivatized *N*-glycans (from Step 7) to small reaction tubes or tapered-well microtiter reaction plates for treatment with exoglycosidase arrays.
(ii) Add 20 mM sodium acetate (ABI 377) or 20 mM ammonium acetate (ABI 310 and ABI 3130), pH 5.5, to a final volume of 10 μl (ABI 377) or 2 μl (ABI 310 and ABI 3130).
▲ **CRITICAL STEP** Electrokinetic loading of the capillary-based DNA sequencer is sensitive to salt in the loading sample; therefore, minimal volumes of volatile buffers should be used
(iii) Add the appropriate amount of a single exoglycosidase or mixture thereof (see **Table 1** for a list of typically used enzymes and the amounts needed) and incubate overnight at 37 °C.
(iv) Evaporate the mixture to dryness (vacuum).

(B) Normal-phase HPLC of APTS-labeled glycans

(i) Equilibrate the TSK-GEL Amide-80 column (see EQUIPMENT SETUP) first with 90% HPLC solvent B (a two-solvent system is used) for 10 min, and then with 22% HPLC solvent B for 10 min. Maintain a flow rate of 1 ml min^{-1} throughout the separation. Elution is followed by fluorescence detection (excitation, 455 nm; emission, 512 nm).
(ii) Inject the APTS-labeled glycans dissolved in 22% HPLC solvent B.
(iii) Run the HPLC method and start collecting 0.5-ml fractions at 10 min.
▲ **CRITICAL STEP** This gradient was optimized for separation of glycans of up to 30 glucose units. For purifying a single glycan, rather than separating a complex mixture, a faster gradient can be used.
(iv) Evaporate the fractions to dryness (vacuum).

9| Perform glycan analysis using an ABI 377 (Step A) or ABI 310 (Step B) DNA sequencer, or an ABI 3130 multicapillary sequencer (Step C).

(A) Glycan analysis on an ABI 377 DNA sequencer (slab-gel)

(i) To 1 μl of glycans that have been derivatized, digested and/or collected by HPLC fractionation, add 0.5 μl of ROX-labeled GeneScan 500 standard mixture for internal standardization and 0.5 μl of deionized formamide for easy sample loading.
(ii) Prepare the polyacrylamide gel. Use standard 36-cm well-to-read length glass plates and 0-2 mm thick spacers. The gel contains 10% of a 19:1 mixture of acrylamide:bisacrylamide and is prepared with the standard DNA-sequencing buffer. Polymerization is catalyzed by adding 200 μl of freshly made 10% ammonium persulfate in water and 20 μl of TEMED per 50 ml of gel. Use a 36-well shark tooth format comb.
(iii) Prerun the gel at 3,000 V for 1 h. Here, and throughout the running step, keep the temperature of the system at 30 °C by connecting an external circulating cooling bath (can be connected to the sequencer according to the ABI 377 DNA sequencer user bulletin 'Modifications for subambient temperature operations').
▲ **CRITICAL STEP** Do not use lower temperatures in order to avoid condensation on the glass plates as this can result in arching and may damage the instrument
(iv) Thoroughly rinse the wells formed by the comb with sequencing buffer.

(v) Load 1.8 μl of the APTS-derivatized glycans (from Step 8A).
▲ CRITICAL STEP Due to the rapid diffusion of low molecular mass carbohydrates, we skip one gel lane between each two samples to avoid cross-contamination and to ease the lane tracking process later. In the 36-well sequencing format, this allows analysis of 18 samples per run.
(vi) Run the electrophoresis at 3,500 V for 5 h.
(vii) Analyze the data with the GeneScan software. Use the same fluorescence-overlap correction matrix used for DNA sequencing with BigDye dye terminators. The fluorescence of APTS-derivatized carbohydrates and rhodamine-labeled oligonucleotides are readily resolved. For strictly quantitative purposes, switch off the 'multiplexing' option of the analysis software. This means that the fluorescence-overlap correcting matrix is not applied, yielding data that represent the truly measured baseline-subtracted intensities. Multiplexing can give differences in measurement of a few per cent.

(B) Glycan analysis on an ABI 310 DNA sequencer

(i) Dilute 1 μl of glycans that have been APTS-labeled, digested and/or collected by HPLC fractionation in 12 μl of deionized formamide. Add 0.5 μl of ROX-labeled GeneScan 500 standard mixture for internal standardization.
(ii) Load the samples in an ABI 310 DNA sequencer equipped with a standard 36-cm capillary filled with POP-6 polyacrylamide linear polymer, diluted three-fold more than the concentration specified by the manufacturer for DNA genotyping.
(iii) Run the sequencer with the following parameters:

Parameter	Value
Temperature	30 °C
Gel pumping	10 s
Prerun	No prerun
Injection voltage	15 kV
Injection time	10 s
Run voltage	20 kV
Run time	20 min

(iv) Analyze the data with the GeneScan software as described in Step 8Evii.

(C) Glycan analysis on the multicapillary sequencers ABI 3130 and ABI 3130XL

(i) Dilute 0.4 μl of glycans, which have been APTS-labeled, digested and/or collected by HPLC fractionation in 10 μl of ultrapure water dispensed in the wells of a 96-well reaction plate. Samples should be arranged to permit simultaneous loading of 4 (ABI 3130) or 16 (ABI 3130XL) capillaries (see the loading schedule in the manual). Add water to blank wells.
(ii) Load the plate into an ABI 3130 or ABI 3130XL sequencer equipped with a standard 36-cm capillary array filled with the POP-7 polyacrylamide linear polymer. Use the running buffer supplied by the manufacturer for DNA sequencing. As an internal standard, LIZ-labeled GeneScan standard, or any APTS-labeled glycan, can be used.
(iii) Run the sequencer with the following parameters:

Parameter	Value
Oven temperature	60 °C
Prerun voltage	15 kV
Prerun time	180 s
Injection voltage	1.2 kV
Injection time	16 s
Run voltage	15 kV
Run time	1,000 s

▲CRITICAL STEP If the APTS-derivatized glycan concentration is low, increase the injection time rather than injection voltage to minimize the impact on the final electropherogram.
(iv) Analyze the data with the GeneMapper software. The factory-provided settings for amplified fragment length polymorphism (AFLP) analysis can be used.

● TIMING

See **Figure 1**.

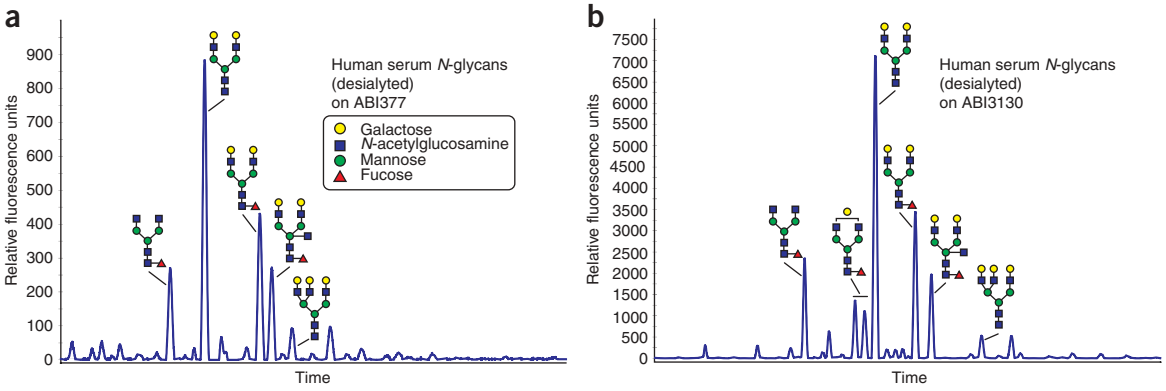

Figure 2 | Comparison of gel- and CE-based glycan analysis on DNA sequencing equipment. *N*-glycans were prepared from the total glycoprotein pool present in 5 μl of human serum and then desialylated to simplify the profile. (**a**) Derivatized glycans were separated using the ABI 377 equipment. (**b**) Typical electropherogram obtained from an ABI 3130 apparatus. The different mobility properties in both systems can be clearly seen, as well as the improved resolving power of the ABI 3130. About 100-fold less material was used in **b** than in **a**, illustrating the higher sensitivity of the CE system. Symbols used for glycans are those suggested by the Consortium for Functional Glycomics (http://glycomics.scripps.edu/CFGnomenclature.pdf).

ANTICIPATED RESULTS

We have presented a workflow (**Fig. 1**) that allows detailed glycan analysis with high sensitivity, resolution and throughput. We have optimized conditions for glycan analysis on three types of DNA-sequencer: the gel-based ABI 377 and the capillary-based ABI 310 and ABI 3130. In **Figure 2**, results obtained from ABI 377 and ABI 3130 are compared: both electropherograms show the *N*-glycans derived from human serum glycoproteins. We have performed a desialylation reaction before analysis to obtain a profile with lower complexity. Where possible, the capillary systems are preferentially chosen because higher sensitivity (>100-fold higher for the ABI 3130 versus the ABI 377) and resolving power (note the separation of the isomeric structures in **Fig. 2**) are obtained on these instruments; in addition, the hands-on workload is much lower and the throughput is higher.

In some cases, getting an *N*-glycan profile is not sufficient, and exoglycosidase digestions of the derivatized glycans can be used to obtain structural information. This type of glycan sequencing has been used before in combination with other methods, such as MS or FACE. Here, we show an example of the sequencing of a pure glycan standard: biantennary nonsialylated core fucosylated complex structure with a bisecting *N*-acetylglucosamine (**Fig. 3**). When doing this analysis, one should be well aware of the specificities of the enzymes that are used. Here, for example, the

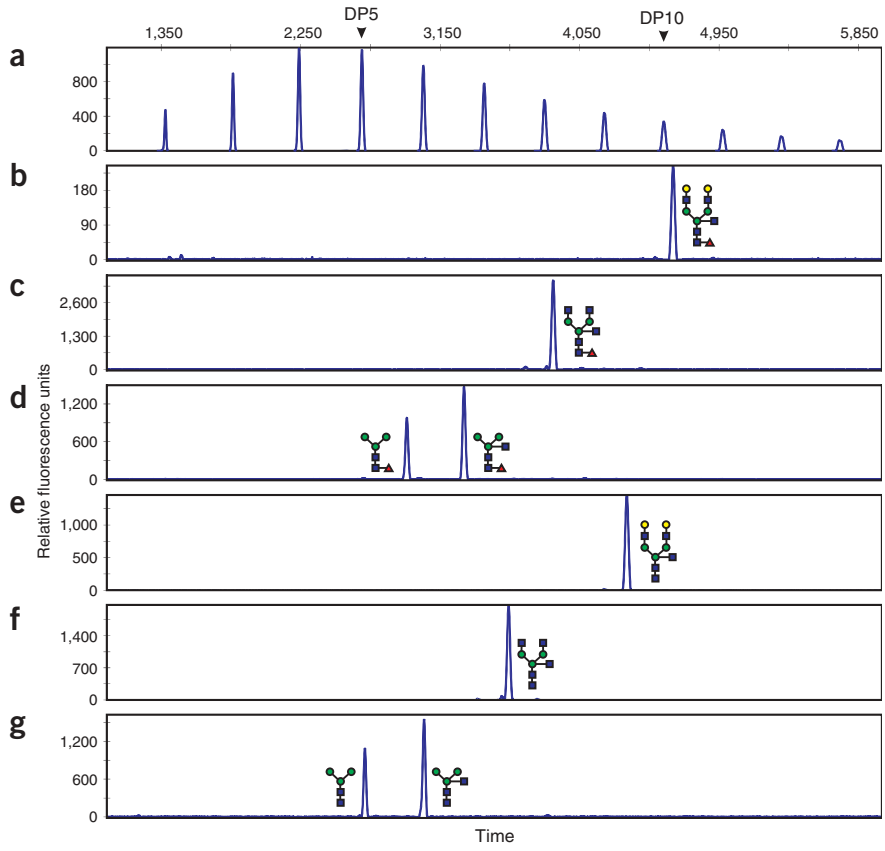

Figure 3 | Exoglycosidase sequencing of a pure biantennary nonsialylated core fucosylated complex structure with a bisecting *N*-acetylglucosamine standard. (**a**) Separation of an APTS-labeled oligomaltose hydrolysate that serves as a reference. The number of glucose units (DP, degree of polymerization) in these structures is indicated. (**b**) Nondigested standard. (**c**) Standard digested with *S. pneumonia* β-galactosidase. (**d**) Standard digested with jack bean β-*N*-acetylhexosaminidase.(**e**) Standard digested with bovine kidney α-fucosidase. (**f**) Standard digested simultaneously with the β-galactosidase and the α-fucosidase. (**g**) Standard digested simultaneously with the β-galactosidase, the β-*N*-acetylhexosaminidase and the α-fucosidase. In **d** and **g**, it can clearly be seen that the hexosaminidase fully removes the branch *N*-acetylglucosamine residues, whereas the bisecting residue is more resistant under these conditions. Symbols for glycans are as in **Figure 2**.

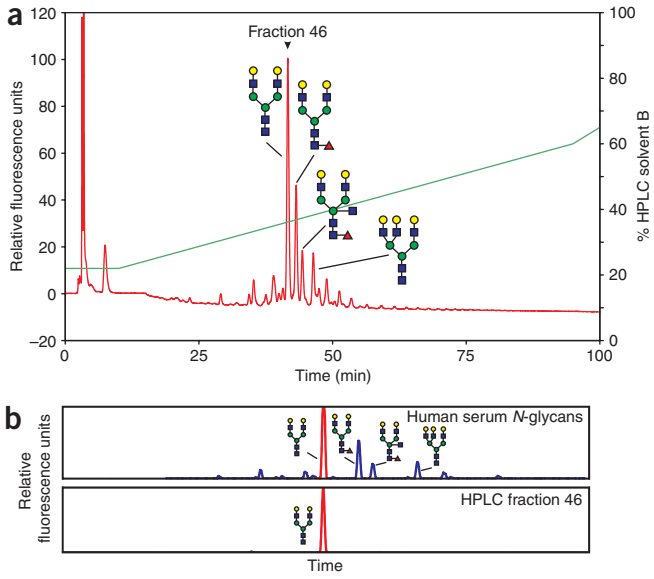

Figure 4 | NP-HPLC separation of human serum *N*-glycans. *N*-glycans were prepared from 5 µl of human serum glycoproteins, labeled with APTS and treated with *A. ureafaciens* sialidase. (**a**) One-twentieth of the final solution was loaded on a TSK-GEL Amide-80 column and separation was obtained using a gradient as specified in the PROCEDURE. Fractions of 0.5 ml were collected and analyzed with the ABI 3130 sequencer (1/100 volume was vacuum-dried and resuspended in water). (**b**) For fraction 46, this analysis shows purification from the complex mixture of *N*-glycans (top) to near homogeneity of the complex biantennary *N*-glycan (bottom). Symbols for glycans are as in **Figure 2**.

jack bean β-hexosaminidase has lower activity towards the bisecting *N*-acetylglucosamine than towards the branch residue.

Exoglycosidase digestions of complex mixtures may be hard to analyze. We have therefore optimized an optional HPLC step for the separation of such mixtures. As an example, we show the separation profile of desialylated APTS-labeled *N*-glycans prepared from total human serum glycoproteins (**Fig. 4a**). The complex biantennary galactosylated *N*-glycan was obtained at near homogeneity, as clearly demonstrated by glycan analysis using the ABI 3130 sequencer (**Fig. 4b**).

ACKNOWLEDGMENTS We thank A. Van Hecke for technical assistance and A. Bredan for manuscript editing. W. Declercq critically read the protocol. We thank M. Aebi (ETH Zurich) for the use of the ABI 310 System. W.L. is a postdoctoral fellow with the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT-Vlaanderen; grant IWT/OZM/040636). N.C. is supported by a Marie Curie Excellence Grant of the European Union (Framework Programme 6). This work was further supported by the Fund for Scientific Research Flanders (FWO-Vlaanderen; G005201) and a grant from Ghent University (BOF No. 01106205).

AUTHOR CONTRIBUTIONS N.C. and W.L. designed and optimized the technology and wrote the manuscript. R.C. participated in the design of the method.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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