

Sample Preparation for N-Glycosylation Analysis of Therapeutic Monoclonal Antibodies by Electrophoresis

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Abstract

There are a considerable number of biopharmaceuticals that have been approved for clinical use in the past decade. Over half of these new generation drugs are glycoproteins, such as monoclonal antibodies or other recombinant glycoproteins, which are mostly produced in mammalian cell lines. The linked carbohydrate moieties affect not only their physicochemical properties and thermal stability but also crucial features like receptor-binding activity, circulating half-life, as well as immunogenicity. The structural diversity of these attached glycans can be manifested in altered monosaccharide composition and linkages/positions among the monosaccharide building blocks. In addition, as more and more biosimilar products hit the market, understanding the effects of their glycosylation modification has become a recent target in efficacy and safety issues. To ensure consistent quality of these products, glycosylation profiles have to be monitored and controlled in all steps of the manufacturing process, i.e., from clone selection to lot release. In this paper, we describe some of the recently introduced and commonly used sample preparation techniques for capillary electrophoresis (CE)-based profiling and structural elucidation of N-glycans. The presented protocols include protein A affinity partitioning of monoclonal antibodies (mAbs), enzymatic release of the N-linked glycans, labeling of the liberated carbohydrates, reaction mixture purification techniques to remove the excess labeling reagent, and high-resolution and rapid capillary electrophoresis-laser-induced fluorescence (CE-LIF)-based profiling of the labeled and purified N-glycans.

Key words Biopharmaceuticals, Therapeutic monoclonal antibody, N-glycan analysis, Fluorophore labeling, Capillary electrophoresis

1 Introduction

Glycosylation is one of the most important and complex posttranslational modifications on the majority of new biotherapeutic drugs. Glycoproteins are typically produced as mixtures of different glycoforms having the same polypeptide backbone but differing in glycosylation site specificity (macroheterogeneity) and in the structures at a same site (microheterogeneity) [1]. It is well known that these attached carbohydrate chains are very important from the point of view of quality by design (QbD) in biopharmaceutical

manufacturing because of their significant influence on activity and efficacy. Since glycan biosynthesis and processing are exquisitely responsive to the host cell type and growth environment, changes may occur in the glycosylation profile of different production batches of the innovative drug but also in their follow-up versions (biosimilars) [2]. Meanwhile, there have been reports of adverse events caused by nonhuman glycosylation moieties due to the presence of immunogenic residues, such as α -1,3-Gal or N-glycolylneuraminic acid [3]. Therefore, it is crucial to maintain the carbohydrate distribution profile of glycosylated biopharmaceuticals for efficient and safe use [4, 5]. Advances are continually being made in the biotechnology industry to minimize glycan heterogeneity and improve the sensitivity and specificity of the analytical assays used to identify and quantify possible glycosylation changes. In spite of the fact that several analytical techniques and assays have already been implemented and validated for the characterization of these complex molecules, rapid and comprehensive profiling and quantitation of all glycoforms are still a challenging task. One of the most powerful bioseparation techniques for detailed N-linked glycosylation analysis of biotherapeutics is capillary electrophoresis combined with laser-induced fluorescent (CE-LIF) detection [6–10]. Different CE-based assays can be used to characterize the drug during the manufacturing process, troubleshoot production problems, and demonstrate biosimilarity or comparability.

In this protocol, we introduce some of the most efficient sample preparation methods for CE-LIF analysis of complex carbohydrates. Usual protocols start with affinity partitioning of the formulated or crude monoclonal antibody product using protein A resins. This step is essential for the complete removal of all additives and formulation ingredients, which can affect glycan labeling or the consequent CE-LIF analysis. Next, we describe some of the commonly used in-solution N-glycan release method using peptide-N-glycosidase F (PNGase F). The affinity partitioning and glycan release steps are followed by fluorophore labeling of the liberated glycans and the removal of the excess labeling material. Finally, we describe the optimal capillary electrophoresis analysis parameters for the labeled N-linked glycans in respect to regular profiling (high resolution) and rapid screening (low resolution).

2 Materials

Always use HPLC grade or ultrapure water (18 M Ω cm at 25 °C) for all solutions and buffers in all procedures. In addition, all reagents should be microbiology or HPLC grade unless otherwise stated. The use of powder-free, nitrile gloves for all sample handling procedures is important too. Ensure that all glass, plasticware,

and solvents are free of glycosidases and possible environmental carbohydrate contaminations. All procedures should be performed using appropriate personal safety protection, laboratory coat, eyeglasses, and nitrile gloves. The labeling reactions should be performed in a fume hood.

2.1 Protein A Affinity Partitioning Using PhyTip 200+ Columns

1. Crude or formulated mAb sample.
2. 1× PBS buffer, pH 7.2.
3. 200+ PhyTip protein A columns with 5 μ L bed volume (Phynexus Inc., San Jose, CA).

All PhyTip protein A columns are supplied with buffers and reagents including:

Capture buffer: provided for those situations where additional buffer is needed to supplement sample volume and ensure correct capture pH.

Wash buffer I: phosphate buffer solution, pH 7.4.

Wash buffer II: saline solution.

Enrichment buffer: phosphate buffer solution, pH 2.5.

Neutralization buffer: Tris buffer solution, pH 9.0.

4. Rainin PureSpeed (Rainin Instrument LLC, Oakland, CA, USA) electronic semiautomated multichannel pipette (8 or 12 channels).
5. 96-well plate, 0.5 mL/well capacity.
6. 0.2-mL flat cap PCR tubes.
7. 100 mM sodium carbonate buffer (pH 9.0) to replace the neutralization buffer.
8. 20 mM sodium carbonate buffer, pH 7.0.
9. 10 % acetic acid in water to replace the enrichment buffer.
10. 10 kDa cutoff spin filters (Nanosep 10k Omega, Pall, Port Washington, NY, USA).
11. Microcentrifuge equipped with a rotor suitable for 2.0-mL microfuge tubes and capable to provide 17,200 $\times g$.
12. Centrifugal vacuum evaporator (e.g., SpeedVac).

2.2 In-Solution N-Glycan Release and APTS Labeling of N-Glycans

1. 50 mM dithiothreitol (DTT) in water.
2. 50 mM iodoacetamide (IAM) in water.
3. 20 mM NaHCO₃, pH 7.0.
4. Peptide-N-glycosidase F (ProZyme, Hayward, CA, USA).
5. 0.2-mL flat cap PCR tubes.
6. PCR thermocycler or other general microvial-based heating devices capable to provide stable 65 and 37 °C temperature.
7. 15 % acetic acid in water.

8. 8-Aminopyrene-1,3,6-trisulfonic acid (APTS) (Beckman Coulter, Brea, CA, USA).
9. 1 M sodium cyanoborohydride in tetrahydrofuran (THF).
10. 10 mg/mL APTS solution in 15 % acetic acid.
11. 10 kDa cutoff spin filters (Nanosep 10 k Omega, Pall, Port Washington, NY, USA).
12. Microcentrifuge equipped with a rotor suitable for 2.0-mL microfuge tubes and capable to provide $17,200 \times g$.
13. Centrifugal vacuum evaporator.
14. Vortex mixer.
15. Pipettors and disposable pipette tips (P5/P10, P200, and P1000).
16. Miscellaneous labware for buffers and dilutions.

**2.3 APTS Cleanup
Using 1000+ PhyTip
with 20 μ L Normal
Phase Resin**

1. 1000+ PhyTip normal phase columns with 20 μ L bed volume.
2. Rainin multichannel 100–1000- μ L pipettor (8 channels).
3. Acetonitrile (100 %, HPLC grade).
4. 20 % acetonitrile in water.
5. 95 % acetonitrile in water.
6. 96-deep-well plate 2 mL/well.
7. Centrifugal vacuum evaporator.
8. 0.2-mL flat cap PCR tubes.

**2.4 Sample
Preparation Using
GlykoPrep Rapid
N-Glycan Preparation
Platform**

1. GlykoPrep Digestion Module (ProZyme) includes:
 - Digestion (RX) cartridges (24 cartridges).
 - Immobilization reagent set.
 - Denaturation reagent.
 - Blocking reagent.
 - Digestion reagent set.
 - N-Glycanase.
 - 25 \times digestion buffer.
 - Finishing reagent.
 - Aluminum sealing film.
2. GlykoPrep APTS Cleanup Module (ProZyme) includes:
 - 5 \times APTS sample loading buffer.
 - Cleanup (CU) cartridges (24 cartridges).
3. Acetonitrile (HPLC grade).
4. Microcentrifuge (capable of 50–1,000 $\times g$) and rotor suitable for 1.5-/2.0-mL microcentrifuge vials.
5. Heater and heating block accommodating 0.2-mL PCR tubes.
6. Centrifugal vacuum evaporator.

7. Vortex mixer.
8. Pipettors and disposable pipette tips (P5/P10, P200, and P1000).
9. Miscellaneous labware for buffers and dilutions.

2.5 CE-LIF Analysis of APTS-Labeled N-Glycans

1. Beckman Coulter PA 800 *plus* Pharmaceutical Analysis System, equipped with LIF detection (solid-state 488-nm laser) and 32 Karat software v.8.0 or higher.
2. Carbohydrate separation buffer (Beckman Coulter).
3. N-CHO-coated capillary, 50 μm ID (Beckman Coulter).
4. Sample vials.
5. 2.0-mL plastic vials.
6. Vial caps.
7. Capillary cartridge equipped with N-CHO-coated capillary with a total length of 60.2 cm (effective length 50.2 cm).

3 Methods

3.1 Protein A Affinity Partitioning of mAbs

1. Dilute the crude or formulated mAb sample to 2 mg/mL using the PBS buffer, pH 7.2 (*see Note 1*).
2. Transfer 220 μL of capture buffer to the first row of the 96-well plate.
3. Transfer 100 μL of diluted mAb sample and 120 μL of *capture buffer* to each well in the second row. Mix the sample gently by pipetting.
4. Fill up the wells in the third row with 220 μL of *wash buffer I*.
5. Fill up the wells in the fourth row with 220 μL of *wash buffer II*.
6. Fill up the wells in the fifth row with 25 μL of 10 % acetic acid.
7. Conditioning (first row): 200 μL of capture buffer over the resin bed for one cycle at a flow rate of 250 $\mu\text{L}/\text{min}$.
8. Capture (second row): capture the IgG by passing 200 μL of sample solution through the resin bed in four cycles at a flow rate of 100 $\mu\text{L}/\text{min}$.
9. Purification (third and fourth row): pass 100 μL of protein A wash buffer I through the resin bed in one cycle at a flow rate of 250 $\mu\text{L}/\text{min}$ followed by a second wash with 200 μL wash buffer II, passing through the resin bed in one cycle at a flow rate of 250 $\mu\text{L}/\text{min}$. It is essential to use wash buffer II as it exchanges the pH 7.4 buffer of wash buffer I and in doing so ensures effective low pH elution during the enrichment step.
10. Enrichment (fifth row): elute the captured IgG with 15 μL 10 % acetic acid solution (pH \sim 2.5) passed through the resin bed in four cycles at a flow rate of 100 $\mu\text{L}/\text{min}$. Neutralize the sample by the addition of 175 μL of 100 mM sodium carbonate buffer (pH 9.0).

11. Wash the membrane of the 10 kDa cutoff spin filter with 250 μL of water for 15 min at $17,200\times g$.
12. Pipette the sample onto the membrane and centrifuge for 15 min at $17,200\times g$.
13. The sample is washed three times with 250 μL of water by spinning for 15 min at $17,200\times g$ (*see Note 2*).
14. The desalted proteins are recovered from the membrane by inversion of the cartridge—to a new tube—and centrifugation at $1,320\times g$ for 4 min (*see Note 3*).
15. To increase the recovery, invert the cartridge again—back to the original position—pipette 50 μL of water onto the membrane, vortex it for 1 min, invert the cartridge, and centrifuge at $1,320\times g$ for 4 min. Repeat this step two times (*see Note 4*).
16. After the desalting step, dry the recovered mAb sample in the centrifugal vacuum evaporator (heat setting turned to the off position) using continuous vacuum.

3.2 In-Solution N-Glycan Release

1. Dissolve the dry protein samples in 45 μL of 20 mM sodium carbonate buffer, pH 7.0.
2. Add 5 μL of 50 mM dithiothreitol (DTT) to the sample and incubate at 65 °C for 15 min.
3. After the incubation step, add 5 μL of 50 mM iodoacetamide (IAM) to the sample and keep in the dark for 10 min at room temperature (RT).
4. Add 2 μL 2.5 U/mL of N-glycanase solution to the sample and incubate for 2 h to overnight at 37 °C.
5. Wash the membrane of the 10 kDa cutoff spin filter with 250 μL of water for 15 min at $17,200\times g$.
6. Pipette the sample onto the membrane and centrifuge for 15 min at $17,200\times g$ (*see Note 5*).
7. Wash the membrane again with 50 μL of water for 15 min at $17,200\times g$.
8. Collect the flow through and transfer it to a 0.2-mL clean PCR tube.
9. Dry the clean released glycans in a centrifugal vacuum evaporator (heat setting turned to off position).

3.3 N-Glycan Release Using GlykoPrep Digestion (RX) Cartridges

1. Dissolve the dry protein sample in 50 μL of 20 mM sodium carbonate buffer, pH 7.0.
2. Add 50 μL of denaturation reagent to the protein solution. Mix well by pipetting up and down several times.
3. Incubate at room temperature for at least 5 min.
4. Nest the cartridge in a 0.5-mL screw cap microtube.

5. Pipette 50 μL of 100 % acetonitrile into the sample cup of the RX cartridge.
6. Place the tube in a centrifuge and spin at $300\times g$ for 3 min.
7. Pipette 150 μL of denaturation reagent into the sample cup of each cartridge.
8. Spin at $1,000\times g$ for 2 min.
9. Empty the flow through by lifting each RX cartridge and removing the liquid collected in the microtube below. Dispose off this liquid and return the cartridge to the tube.
10. Load 100 μL of the denatured mAb sample into the sample cup of each RX cartridge.
11. Spin at $50\times g$ until all sample cups are empty (~15 min).
12. Empty the flow through as described in **step 9**.
13. Pipette 50 μL of blocking reagent into the sample cup of each RX cartridge.
14. Spin at $300\times g$ for 3 min.
15. Pipette 50 μL of digestion buffer (1 \times concentrated) into the sample cup of each RX cartridge.
16. Spin at $300\times g$ for 3 min.
17. Prepare the digestion and elution assembly by nesting each PCR tube into 0.5-mL tubes and nesting that within a 2.0-mL microcentrifuge tube.
18. Transfer the RX cartridge into the corresponding digestion and elution assemblies and return to the centrifuge. Dispose the microtubes and the flow through.
19. Pipette 10 μL of enzyme solution (2.5 μL of N-glycanase and 7.5 μL of 1 \times digestion buffer) into the sample cup of the RX cartridge.
20. Spin at $300\times g$ for 3 min; *do not* discard the flow through.
21. Transfer the PCR tubes with the RX cartridges to a 45 $^{\circ}\text{C}$ PCR heat block and incubate for 30 min.
22. Remove the PCR tubes with RX cartridges from the heat block and return to the digestion and elution assembly.
23. Pipette 15 μL of finishing reagent into the sample cup of each RX cartridge.
24. Spin at $300\times g$ for 3 min.
25. Remove RX cartridge from the PCR tube. The eluted N-glycans are now in the PCR tubes; *do not discard*.
26. Open the PCR tubes, return them to the digestion assembly (minus the RX cartridge), and dry the N-glycans in a centrifugal vacuum evaporator (heat setting turned to the off position) for 30 min or until fully dry.

3.4 APTS Labeling of the Released N-Glycans

1. Add 4 μL of 10 mg/mL APTS (in 15 % acetic acid) to the dried sugars.
2. Add 2 μL of 1 M NaBH_3CN (in THF) to the sample (*see Note 6*).
3. Incubate the reaction mixture at 37 °C overnight. For nonsilylated glycoproteins, incubation at 55 °C for 2 h is sufficient.

3.5 Removal of Excess APTS Using Normal Phase Columns

1. Add 100 μL of water to the labeled samples and mix it by pipetting up and down several times. Transfer the mixture to the third row of the plate (Fig. 1) and add 900 μL acetonitrile to it. Mix the sample well again.
2. Prepare the elution (20 % acetonitrile in water) and the washing solutions (95 % acetonitrile in water) and fill up the 96-deep-well plate as shown in Fig. 1 (*see Note 7*).
3. Insert the 1000+ DPA-6S normal phase PhyTip columns (20 μL bed volume) to the multichannel pipettor.
4. Perform conditioning in the first row (Fig. 1) by passing 900 μL of elution solution through the resin bed four times.
5. Equilibrate the columns by passing 900 μL of washing solution over the resin bed for four times in the second row in Fig. 1.

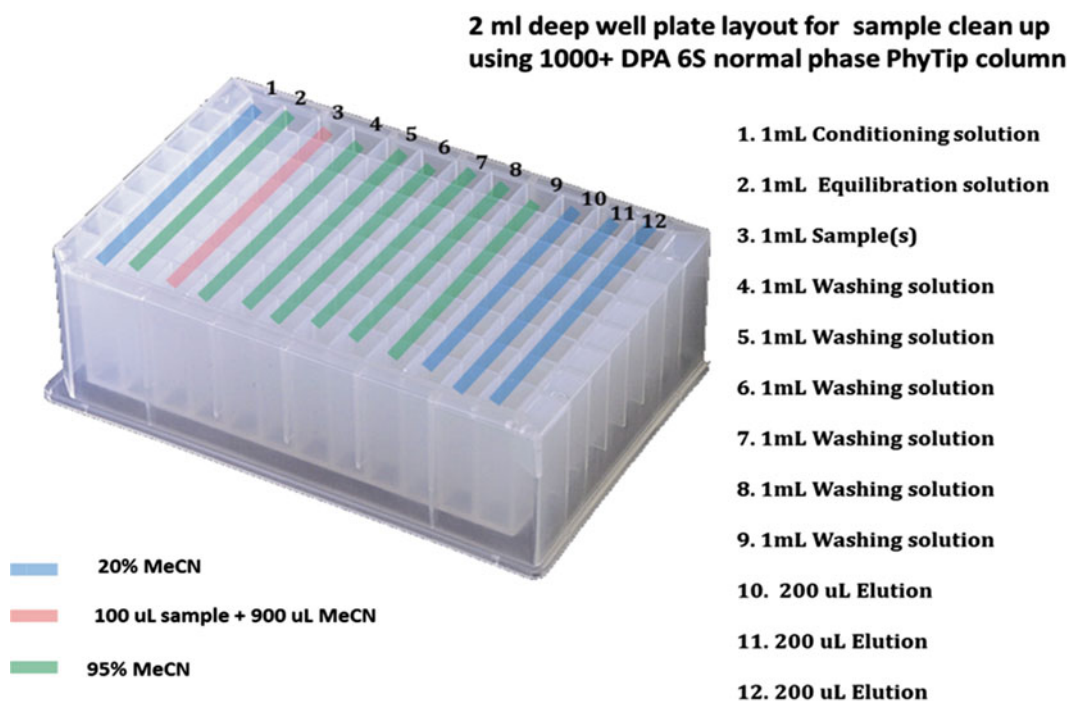


Fig. 1 Reagent distribution in a deep-well plate for labeling reagent cleanup

6. Load the labeled glycans to the columns by pipetting 900 μL of sample mixture through the resin bed ten times (Fig. 1, third row).
7. Wash off the salts and excess labeling reagent by pipetting 900 μL of washing solution through the resin bed ten times. Repeat this step six times, always using fresh solution in a new row (Fig. 1, fourth to ninth row).
8. Elute the APTS-labeled glycans by passing 180 μL elution solution through the resin bed three times. Repeat this step in the next two rows (Fig. 1, 10th to 12th row).
9. Collect the eluted sample from the elution positions to a 2.0-mL tube and dry it in a centrifugal vacuum evaporator (heat setting turned to the off position).
10. Dissolve the dried samples in 25 μL HPCE grade water and proceed to capillary electrophoresis analysis.

3.6 Cleanup of the Fluorophore- Labeled Glycans by APTS Cleanup Modules

1. Prepare 5 mL of APTS sample load buffer by adding 1 mL of 5 \times APTS sample load buffer to a small, glass graduated cylinder. Bring the volume up to 5 mL with 100 % acetonitrile.
2. Dissolve the dried and labeled glycan samples in 200 μL of APTS sample load buffer. Pipette it up and down a couple of times to mix well.
3. Add 200 μL of APTS sample load buffer to each N-glycan sample in the PCR tubes. Pipette up and down to mix.
4. Transfer each N-glycan sample into the sample cup of a CU cartridge. Place the CU cartridge into a 0.5-mL tube by nesting within a 2.0-mL microcentrifuge tube.
5. Spin at 300 $\times g$ for 3 min or until the sample cup of each CU cartridge is empty.
6. Discard the flow through and spin at 300 $\times g$ for 3 min again.
7. Pipette 200 μL of APTS sample load buffer into the sample cup of each CU cartridge.
8. Spin at 300 $\times g$ for 3 min.
9. Discard the flow through and repeat **steps 6 and 7** again.
10. Place the CU cartridge to a clean 0.2-mL PCR tube and nest this back to the 2.0-mL microcentrifuge tube, which is already containing the 0.5-mL tube.
11. Pipette 25–50 μL of ultrapure water into the sample cup of each CU cartridge.
12. Spin at 1,000 $\times g$ for 3 min. The PCR tube now contains the purified labeled N-glycans; *do not discard*.
13. N-Glycan samples are now ready for capillary electrophoresis analysis. If not analyzed immediately, store sealed at $-20\text{ }^{\circ}\text{C}$ in the dark.

3.7 CE-LIF Analysis of APTS-Labeled N-Glycans

3.7.1 Preparing the Glucose Ladder Standard (G20)

1. Weigh and dissolve 5 mg of glucose ladder standard (G20, supplied with the N-CHO Carbohydrate Kit) in 80 μ L deionized water in 1.5-mL microcentrifuge tube. Sonicate if necessary.
2. Aliquot at least ten 2 μ L portions of the glucose ladder standard solution to 0.5-mL microcentrifuge vials and dry them in a centrifugal vacuum evaporator. The dried glucose ladder can be stored at room temperature or used immediately.
3. Label the ladder standard as described under Subheading 3.4 and proceed with sample cleanup.
4. Transfer the APTS-labeled samples and standards to a 0.2-mL sample tube (Beckman Coulter), nest the tubes to 2-mL plastic vials, and cap them tight. Place the sample vials to the sample tray and record their actual positions in the methods timetable.
5. Fill the appropriate reagents into vials as follows:
 - 1.5 mL of ultrapure water into H₂O vial (four vials).
 - 1.5 mL of carbohydrate separation buffer into Gel-R vial (one vial).
 - 1.3 mL of carbohydrate separation buffer into Gel-S vial (two vials).
 - 0.8 mL of ultrapure water into waste vial (one vial).

Place the vials in the buffer tray and set up their actual positions in the methods timetable.

6. Set up the initial detection and separation parameters as follows:
 - Detection: laser-induced fluorescence.
 - Wavelength excitation, 488 nm; emission, 520 nm.
 - Data rate: 4 Hz.
 - Dynamic range: 100 RFU (relative fluorescence units).
 - Filter setting: normal.
 - Peak width: 16–25.

3.7.2 Performing the High-Resolution CE-LIF Analysis

1. Rinse the capillary with buffer for 3 min at 30 psi from gel buffer (Gel-R vial for rinse) vial to waste vial.
2. Inject the sample at 0.5 psi for 5 s from sample vial to buffer vial (Gel-S vial for separation).
3. Wait for 0.2 min with vials filled with ultrapure water. This step dips the capillary in water to protect against sample carryover. Change the rinse water vials if they are contaminated.
4. Separation step: 20 min from gel buffer vial to gel buffer vial (Gel-S vial for separation). The applied voltage should be 30 kV, with REVERSED polarity (anode at the detection side) with 0.17 min ramp time.

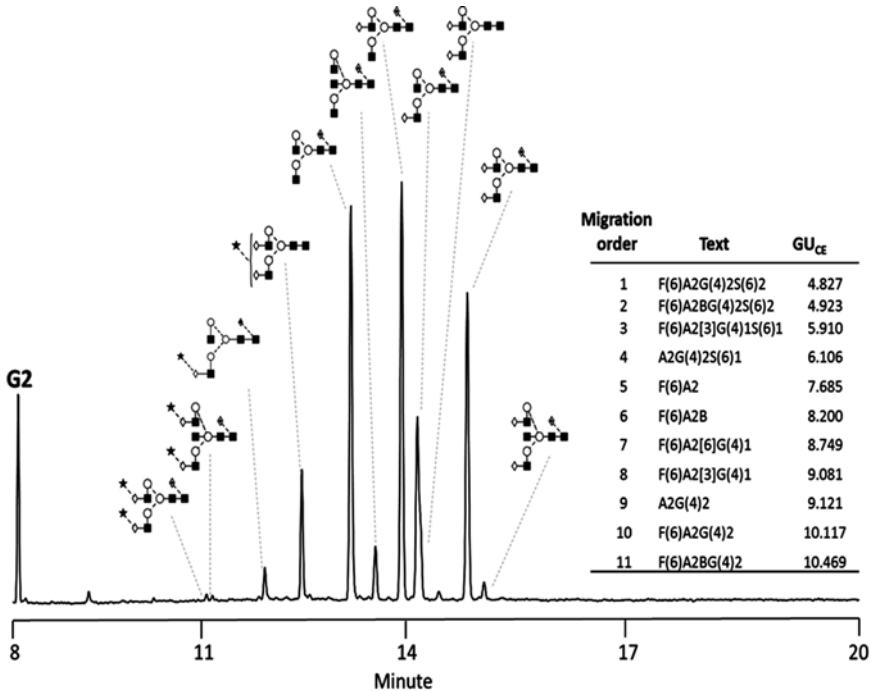


Fig. 2 High-resolution capillary electrophoresis profiling of IgG N-glycans. Conditions: capillary, N-CHO neutral-coated capillary (effective length, 50 cm; total length, 60 cm); separation buffer, N-CHO carbohydrate separation buffer; applied electric field, 500 V/cm; separation temperature, 25 °C; pressure injection, 6.89 kPa for 5 s. The different glycans have been represented with cartoons based on the Oxford symbol notation with embedded linkage information [11]

5. Autozero at 1.0 min.
6. End at 20.0 min.
7. An example of a high-resolution CE-LIF analysis of APTS-labeled IgG N-glycans performed on 50 cm separation length is shown in Fig. 2 with the corresponding glycan structures of the peaks.

3.7.3 Performing the Rapid CE-LIF Analysis for High-Throughput Screening

1. Rinse the capillary with buffer for 3 min at 30 psi from gel buffer (Gel-R vial for rinse) vial to waste vial.
2. Inject the sample at 0.5 psi for 5 s from sample vial to buffer vial (Gel-S vial for separation). When injecting from the 10 cm effective length side of the capillary, make sure that the tray layout has changed accordingly.
3. Wait for 0.2 min with vials filled with ultrapure water. This step dips the capillary in water to protect against sample carryover. Change the rinse water vials if they are contaminated.
4. Separation step: 5 min from gel buffer vial to gel buffer vial (Gel-S vial for separation). The applied voltage should be 30 kV, with NORMAL polarity (cathode at the detection side) with 0.17 min ramp time.

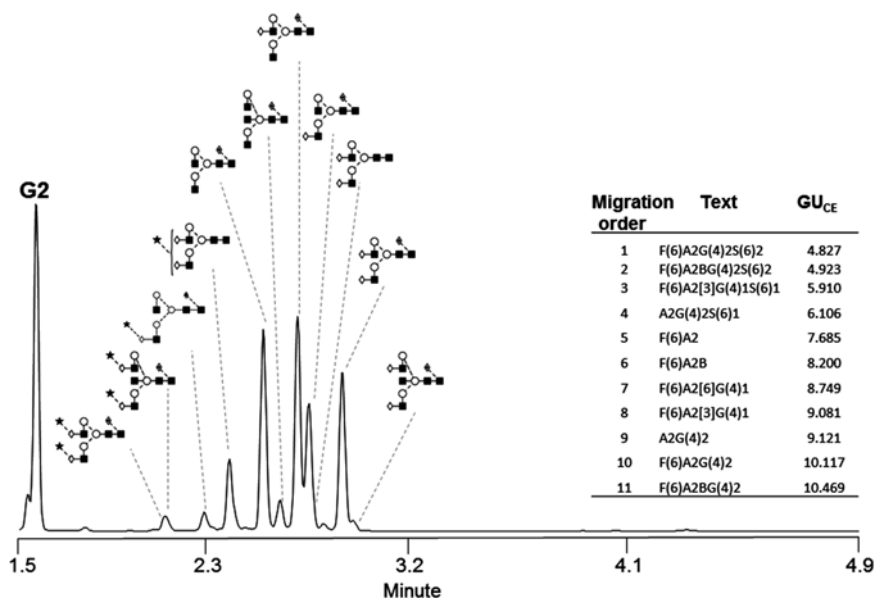


Fig. 3 Rapid capillary electrophoresis profiling of IgG N-glycans. Conditions: same as in Fig. 2 with the effective separation length of 10 cm

5. Autozero at 1.0 min.
6. End at 5.0 min.
7. An example of a rapid CE-LIF analysis of APTS-labeled IgG N-glycans performed on 10 cm separation length is shown in Fig. 3 with the corresponding glycan structures of the peaks.

4 Notes

1. If the sample concentration is less than 2 mg/mL, pre-concentrate before starting the procedure.
2. Make sure that all the liquid passed through the membrane after the last step. If not, spin it again.
3. Never use higher g -forces for the inverted cartridge as it can destroy the membrane.
4. When vortexing, always hold the filtration device vertically.
5. *Do not* discard the flow through because it contains the released N-glycans.
6. Always work with NaBH₃CN in the fume hood!
7. Always prepare fresh acetonitrile solutions.

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