

Extended range characterization of N-Glycans.

Authors

Zhiyong Peng, Ph.D.
James White, Ph.D.

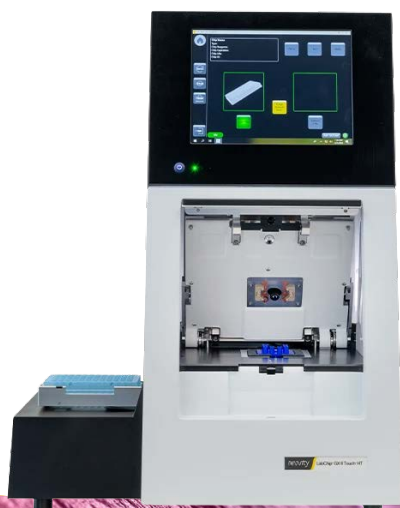
Abstract

Biotherapeutic biomolecules typically contain glycosylation sites, which not only are a key manufacturing attribute but also represent the efficacy and safety of a biotherapeutic product. For example, the functions of monoclonal antibodies (mAbs) are significantly impacted by their glycosylation pattern. Therefore, it is necessary to closely monitor the glycan profile of a biotherapeutic molecule throughout the entire development process, from cell line development through product release.

Capillary electrophoresis (CE) has been widely adopted as an effective method to monitor glycans. In order to meet the productivity efficiency of modern biotherapeutic development and the production process, an automated glycan profiling system should be used. Additional features, such as high-throughput capability, minimal sample consumption, and fast analysis, are key factors to consider when choosing a microfluidic platform.

Based on patented LabChip™ technologies, the LabChip™ GXII Touch™ protein characterization system performs microfluidic capillary electrophoresis on reusable microchips to characterize and analyze therapeutic biomolecules, such as proteins, glycans, and nucleic acids. This system provides industry leading throughput and analysis with accurate sizing and digitized traceable results. It is ideal for quality monitoring and assessment of biotherapeutics.

When it comes to specific glycan screening, an appropriate assay is also required to cover the variety of glycan molecules. N-linked glycans can be neutral or charged, depending on



specific monosaccharide structures. The LabChip™ Extended Range N-Glycan assay allows users to analyze both neutral and sialylated N-linked glycans for automated, high-throughput characterization on the LabChip GXII Touch protein characterization system.

Nomenclature

We use Symbol Nomenclature for Glycans (SNFG) for glycan structures.

Monosaccharide symbols:

- N-acetylglucosamine (GlcNAc)
- N-acetyl-D-galactosamine (GalNAc)
- ◆ N-acetylneuraminic acid (Neu5Ac)
- Galactose
- Mannose
- ▲ Fucose

Methods

The Revvity LabChip GXII Touch HT protein characterization system (Revvity, PN CLS138160) was used to screen and measure the size of glycans. The instrument comes with the LabChip GX Touch software for automatic system control and data acquisition functions. The LabChip GX Reviewer (Version 5.7) software was used to analyze glycan sizing and related statistics separately.

Figure 1 shows the operation interface on the LabChip GXII Touch instrument.

Two categories of glycan samples were used in this study: purified glycan standards and glycans released from glycoproteins. Purified glycan standards, including ten neutral N-linked glycan standards (Man3, Man3f, Man5, G0, G0f, G0fB, G1f, G2, G2f, G3) and four mono- and di-sialylated glycan standards (A1, A1f, A2, A2f) were purchased from Agilent Technologies, Inc. (Agilent, PN GKC-004300, GKC-004301, GKC-004311, GKC-014301, GKC-024300, GKC-024301, GKC-035300, GKC-002300, GKC-002301, GKC-002500, GKC-124301, GKC-224301, GKC-143200, GKC-224300). Two glycoproteins were purchased from the National Institute of Standards and Technology (NIST) and New England Biolabs: NIST Monoclonal Antibody Reference Material (NISTmAb), (NIST, PN RM8671) and Bovine Fetuin (New England Biolabs, PN P6042S). We used the Glycan Profiling Assay Release and Labeling Kit (Revvity, CLS155434) to denature and cleave glycans from these two glycoproteins by peptide-N-Glycosidase F (PNGase F).

Figure 1 shows a typical workflow of LabChip glycan profiling workflow. The LabChip Extended Range N-Glycan Assay protocol (Revvity, Inc., 2020) for the Glycan Profiling Assay Reagent Kit (Revvity, PN 760525) and high resolution LabChip™ microchips (Revvity, PN 760524) were used to prepare microfluidic electrophoresis measurement.

The glycans samples were loaded on a 96 microwell plate and submitted to analysis on the LabChip GXII Touch system. The fully automated data acquisition and analysis is shown in Figure 2.

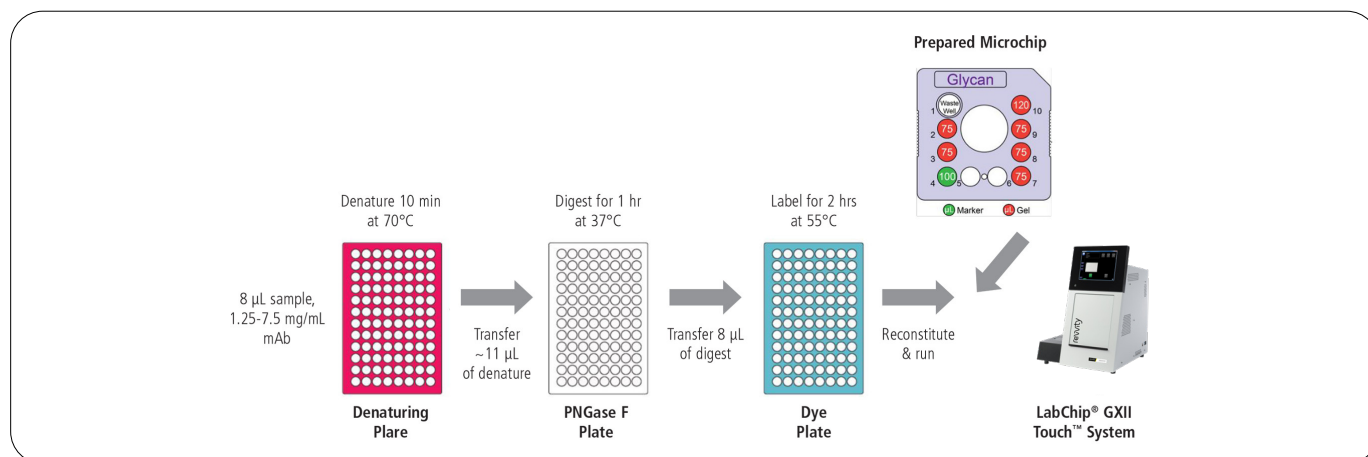


Figure 1: A typical LabChip glycan profiling process workflow. Glycans can be cleaved from denatured glycoproteins, e.g., mAbs, by following PNGase F process, or purified glycans can be labeled directly. The user needs to prepare and load reagent solutions, lower marker and gels, into designated wells in the microchips. The user can analyze up to 192 samples (2×96 microwell plates) with one microchip preparation.
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A typical sample preparation workflow for glycan analysis in LabChip includes protein denaturation, enzyme digestion, and glycan labeling. The Revvity's Glycan Profiling Assay Reagent Kit (PN 760525) was used for rapid digestion, dyeing, and identification of glycans using following steps.

1. Denaturation. 8 μ L of glycoprotein sample was loaded to the microwell on denaturing plate and incubated at 70 °C for 10 minutes to fully denature the protein structure.
2. Enzyme digestion. The denatured sample was transferred to a PNGase F plate and incubated at 37 °C for 1 hour to release all N-linked glycans.
3. Labeling. Transfer 8 μ L of the digested sample was then transferred to the dye plate and incubated at 55 °C for 2 hours or until complete dry. For purified glycan standards, 18 μ L of purified glycan sample was loaded into the dye plate and incubated at 55 °C for 2 hour or until complete dry with lid open.
4. The dried sample was reconstituted into solution by adding 100 μ L of molecular grade water immediately before loading into the LabChip GXII Touch instrument for analysis.

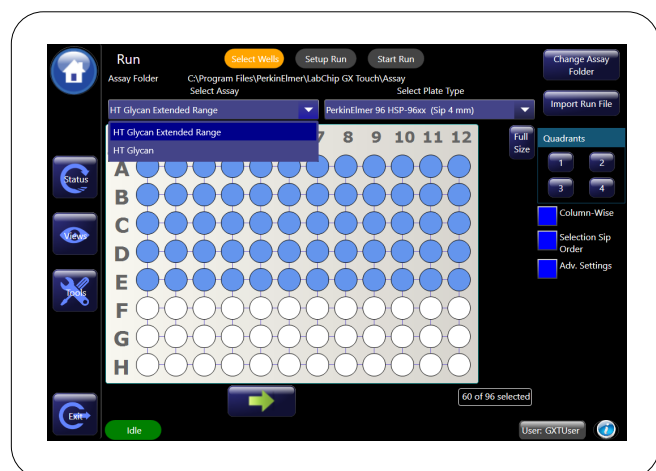


Figure 2: Choose the Glycan Extend Range assay on the Lab-Chip GXII Touch instrument to start the automatic glycan profiling process. It takes less than two hours to acquire data from 96 samples on a standard microwell plate. Analysis results are available in real time during data acquisition. Data can also be analyzed separately on a personal computer using LabChip GX Reviewer software.

Results & discussion

Typical biopharmaceutical products contain multiple glycans in a single sample mixture. It is important to be able to differentiate, detect, and identify these glycan molecules simultaneously in one measurement. Figure 3 shows the representative measurement results of a mixture of 14 glycan standards at three different concentration conditions. Each measurement was completed in about 45 seconds. Depending on their electrical mobility, glycans arrive at the detector at different times and are seen as individual fluorescent signal peaks in the electropherogram. By comparing these peaks with pre-calibrated standard ladders, the LabChip GX Touch software automatically detected and identified glycan molecules. All 14 individual glycans were detected and identified in three mixture conditions. This data shows the LabChip GXII Touch system and LabChip Extended Range N-Glycan assay detect both neutral and sialylated N-linked glycans reliably.

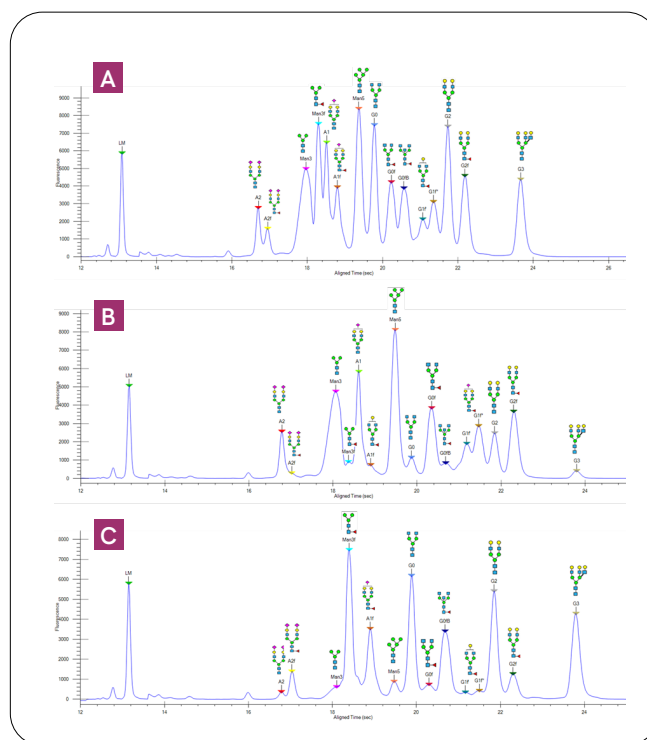


Figure 3: LabChip Extended Range N-Glycan assay detected both neutral and sialylated N-linked glycans. Electropherograms of glycan standards mixture measured in at different mixture concentration conditions: (A) A1, A1f, A2, A2f, Man3, Man3f, Man5, G0, G0f, G0fB, G1f, G2, G2f, G3 with equal concentration of 10 ng/ μ L. (B) A1, A2, Man3, Man5, G0f, G1f, G2f concentration of 10 ng/ μ L; A1f, A2f, Man3f, G0, G0fB, G2, G3 with concentration of 1 ng/ μ L. (C) A1, A2, Man3, Man5, G0f, G1f, G2f with concentration of 1 ng/ μ L; A1f, A2f, Man3f, G0, G0fB, G2, G3 with concentration of 10 ng/ μ L. LM stands for lower marker.

Measuring the level of impurities during the quality control and assessment of biotherapeutic products is critical as certain glycans can trigger an immunogenicity reaction in the human body even at very low abundance. The LabChip Extended Range N-Glycan assay has high sensitivity allowing it to detect even low abundance species. Figure 4 shows the measurement of glycan standards A2 and G2 at both high (80 ng) and low (1 ng) concentrations. G2 and A2 were clearly resolved at a low 1 ng concentration. We used the signal-to-noise ratio (S.N.R.) of individual peaks to quantify the quality of detection in these measurements. With the digitized data format, the electropherogram details can be investigated and analyzed with a few mouse clicks to zoom in the data. This is not possible on conventional gel electrophoresis. The LabChip Extended Range N-Glycan assay can also detect impurities of N-linked glycans.

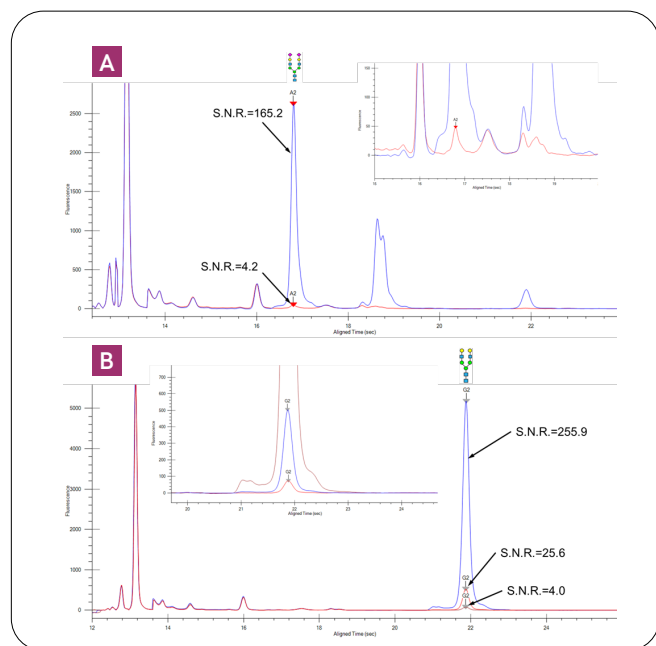


Figure 4: The LabChip Extended Range N-Glycan assay reliably detects glycans at low concentrations: (A) A2 at 80 ng and 1 ng, and (B) G2 at 80 ng, 8 ng, and 1 ng. The quality of each peak is characterized by the signal-to-noise ratio (S.N.R.). Insert shows close-up of data peak under magnified view. The peaks at ~13 seconds are signals of lower marker (not labelled).

Figure 5 shows electropherograms from a glycan standard mixture at different dilutions. 15 glycans were detected and identified at different dilution levels, and the intensities of the individual glycan peaks changed proportionally to input. The LabChip Extended Range N-Glycan array detected and estimated the concentration of neutral and charged glycans with good linearity. This linearity can be used to monitor any trends in the concentrations glycans during the quality assessment of a biotherapeutic product.

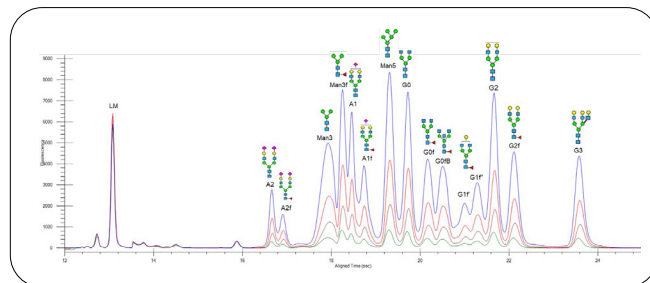


Figure 5: The LabChip Extended Range N-Glycan assay separated, identified, and measured the mixture of standard N-linked glycans at low and high concentrations, 1 ng/μL, 2.5 ng/μL, 5.0 ng/μL, and 10 ng/μL. LM stands for lower marker.

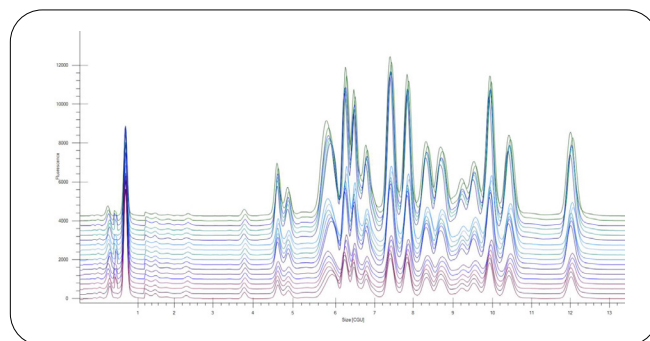


Figure 6: 18 electropherograms were taken from a mixture of N-linked glycan standards at three different concentrations, 2.5 ng/μL, 5.0 ng/μL, and 10 ng/μL. At each concentration, measurements were repeated using six different sample wells. The glycan size was analyzed using the LabChip Extended Range N-Glycan assay module in the LabChip GX Touch software and then quantified in CGU, Caliper Glucose Units.

Table 1: 18 measurements from a mixture of N-linked glycan standards were taken at three different concentrations, 2.5 ng/μL, 5.0 ng/μL, and 10 ng/μL. At each concentration, measurements were repeated from six different sample wells. The LabChip Extended Range N-Glycan assay identified 15 glycan molecules in this mixture: A2, A2f, Man3, Man3f, A1, A1f, Man5, G0, G0f, G0fB, G1f, G1f", G2, G2f and, G3. For each glycan, size was analyzed by the LabChip Extended Range N-Glycan assay module in the LabChip GX Touch software and then quantified in CGU, Caliper Glucose Units. Statistical average and C.V. (coefficient of variance) were calculated to evaluate repeatability of the measurements.

Sample/ Glycan	A2	A2f	Man3	Man3f	A1	A1f	Man5	G0	G0f	G0fB	G1f	G1f"	G2	G2f	G3
Mix 5 ng/μL															
Run #1	4.615	4.879	5.903	6.234	6.467	6.770	7.403	7.839	8.319	8.670	9.218	9.521	9.932	10.414	12.004
Run #2	4.622	4.885	5.895	6.248	6.484	6.794	7.406	7.853	8.319	8.694	9.231	9.535	9.947	10.430	12.022
Run #3	4.618	4.887	5.852	6.235	6.472	6.782	7.402	7.857	8.326	8.702	9.239	9.543	9.955	10.439	12.033
Run #4	4.630	4.876	5.834	6.238	6.479	6.794	7.408	7.854	8.318	8.693	9.229	9.532	9.925	10.414	12.013
Run #5	4.639	4.908	5.854	6.276	6.495	6.805	7.444	7.882	8.368	8.726	9.264	9.569	9.981	10.473	12.079
Run #6	4.637	4.888	5.818	6.273	6.492	6.802	7.422	7.878	8.346	8.722	9.264	9.574	9.976	10.460	12.055
Mix 2.5 ng/μL															
Run #1	4.616	4.880	5.921	6.235	6.468	6.789	7.404	7.841	8.338	8.689	9.219	9.523	9.934	10.416	12.024
Run #2	4.621	4.885	5.894	6.229	6.466	6.775	7.405	7.835	8.318	8.694	9.230	9.534	9.928	10.411	12.021
Run #3	4.614	4.883	5.848	6.211	6.448	6.757	7.395	7.832	8.318	8.694	9.231	9.535	9.928	10.411	12.022
Run #4	4.626	4.890	5.851	6.259	6.482	6.798	7.415	7.862	8.346	8.704	9.242	9.546	9.958	10.450	12.054
Run #5	4.635	4.904	5.832	6.270	6.488	6.798	7.418	7.874	8.359	8.718	9.255	9.559	9.953	10.444	12.049
Run #6	4.638	4.889	5.819	6.274	6.493	6.803	7.423	7.879	8.347	8.724	9.266	9.558	9.977	10.462	12.057
Mix 10 ng/μL															
Run #1	4.610	4.873	5.880	6.226	6.458	6.761	7.393	7.828	8.308	8.676	9.205	9.508	9.901	10.400	11.988
Run #2	4.622	4.868	5.879	6.230	6.467	6.795	7.407	7.836	8.319	8.677	9.214	9.536	9.930	10.413	12.023
Run #3	4.622	4.891	5.841	6.240	6.459	6.788	7.408	7.846	8.333	8.710	9.247	9.552	9.947	10.431	12.044
Run #4	4.618	4.882	5.825	6.247	6.488	6.803	7.418	7.865	8.330	8.705	9.223	9.545	9.939	10.429	12.030
Run #5	4.639	4.907	5.836	6.275	6.494	6.804	7.442	7.880	8.366	8.725	9.262	9.567	9.961	10.453	12.059
Run #6	4.609	4.877	5.789	6.257	6.457	6.767	7.403	7.840	8.325	8.682	9.222	9.531	9.932	10.415	12.005
Average	4.624	4.886	5.854	6.248	6.475	6.788	7.412	7.855	8.333	8.700	9.237	9.543	9.945	10.431	12.032
C.V.	0.22%	0.23%	0.59%	0.32%	0.23%	0.23%	0.19%	0.23%	0.22%	0.21%	0.21%	0.19%	0.21%	0.20%	0.19%

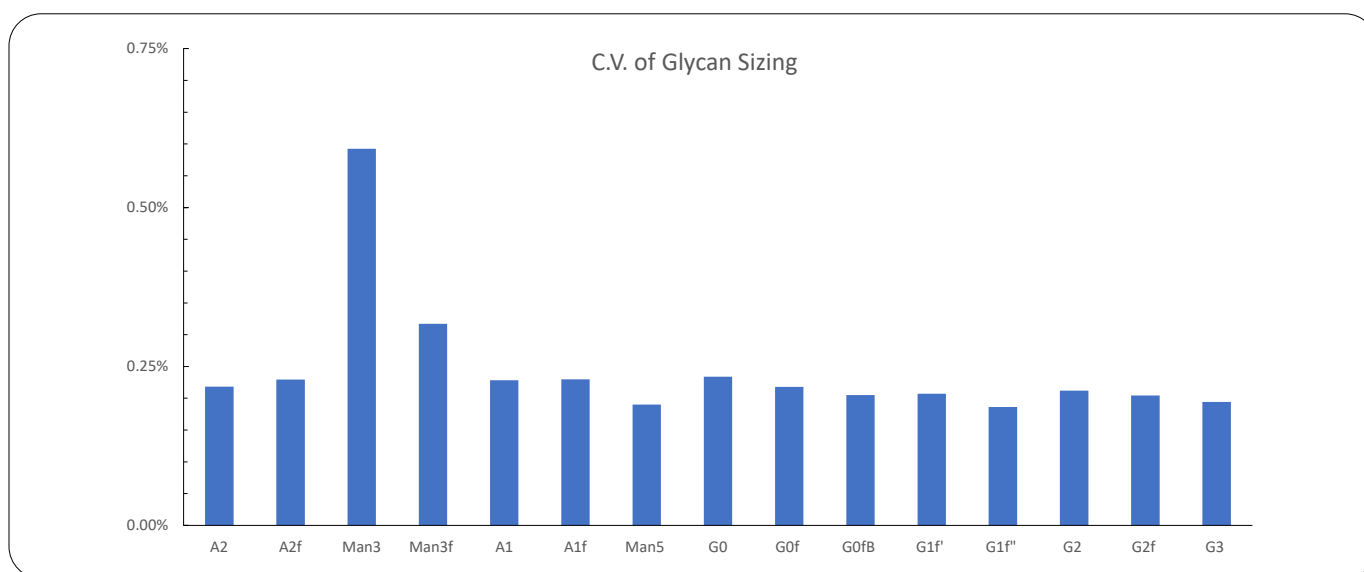


Figure 7: Glycan sizing C.V. (coefficient of variance) of 15 N-linked glycans identified in standard mixtures.

Figure 8 shows electropherograms of glycans deglycosylated from two glycoproteins. The NISTmAb and bovine fetuin were denatured to release glycans using the PGNase F protocol. From NISTmAb, Man3, Man5, G0f, G1f, G1f', G2, and G2f glycans were released and detected. From bovine fetuin, A2, Man3, and Man3f glycans were released and detected.

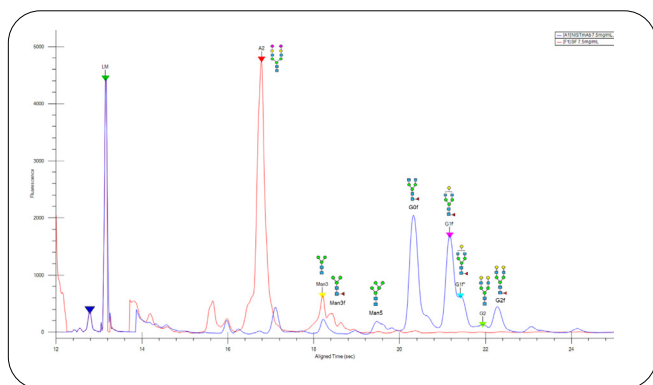


Figure 8: Glycans deglycosylated from two glycoprotein samples were detected and identified using the LabChip® Extend Range N-Glycan assay: Man3, Man5, G0f, G1f, G1f', G2 and G2f in NISTmAb; A2, Man3 and Man3f were detected and identified in the Bovine Fetuin sample.

Conclusions

The LabChip Extended Range N-Glycan assay and LabChip GXII Touch protein characterization system can be used for highly automated, high-throughput. Rapid analytical characterization of both neutral and charged N-linked glycans in biotherapeutic products.

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