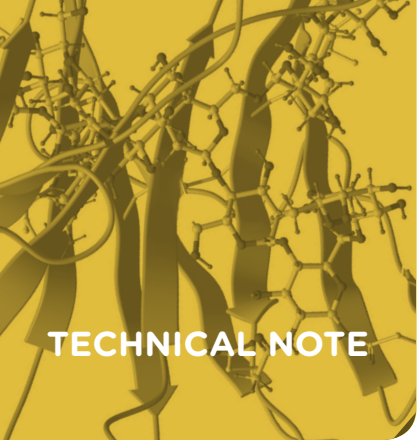

TECHNICAL NOTE

PROzyme

Gly-Q

Gly-X

GlykoPrep

Glyko Enzymes

Glyko Standards

InstantPC

InstantAB

InstantQ

2-AB

APTS

PhycoLink

PhycoPro

RPE & APC Conjugates

Streptavidins

High Throughput Analysis of N-Glycans Released from Biosimilar Glycoproteins

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SUMMARY

- Analysis of enzymatically-released N-glycans with hypdrophilic interaction liquid chromatography typically involves the addition of a fluorescent dye
- Gly-X with InstantPC N-glycan sample preparation is used to rapidly prepare InstantPC-labeled N-glycans from 4 monoclonal antibodies
- InstantPC-labeled N-Glycans are separated by hydrophilic interaction liquid chromatography (HILIC) and detected by fluorescence and mass spectrometry (MS)
- Fluorescence and MS properties of InstantPC dye allow for robust quantitation and identification of N-glycan species
- MS1 and MS2 are utilized to characterize glycans including those containing potentially immunogenic non-human Galα1,3-Gal (alpha-Gal) epitope and N-glcyolylneuraminic acid (NGNA)

Keywords

Biotherapeutic
Rituximab
Cetuximab
Trastuzumab
Fluorescent Dye
N-Glycan Labeling
HILIC
Mass spectrometry
Alpha gal
UHPLC
InstantPC

INTRODUCTION

Monoclonal antibodies (mAbs) account for around half of all marketed biopharmaceuticals, and are also the focus of investigations into follow-on biosimilar and biobetter molecules (1, 2). The structure of mAb N-linked glycans can potentially affect immunogenicity, pharmacokinetics and pharmacodynamics (3), making the characterization of N-glycans an essential part of the biotherapeutic development process. Analysis of N-glycans typically involve the labeling of enzymatically-released glycans with a tag to allow for fluorescence (FLR) detection; a process that often requires numerous hours or days to complete. In addition to fluorescence detection, mass spectrometry is also often utilized. Unfortunately, many of the commonly-used fluorescent tags are limited with regard to MS sensitivity. Here we present a workflow for rapid sample preparation of N-glycans released from mAbs using ProZyme's Gly-X platform with InstantPC label, coupled with analysis using Thermo Fisher Scientific LC-MS instrumentation and consumables.

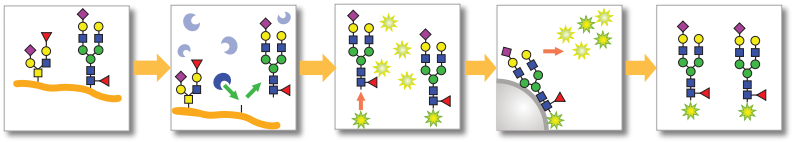

Figure 1: Gly-X with InstantPC N-glycan sample preparation workflow.

HILIC Chromatography

N-Glycans labeled with InstantPC were separated with hydrophilic interaction liquid chromatography (HILIC). Injection volumes were 1 µl eluent for FLR and MS1 experiments, and 10 µl eluent diluted with appropriate organic solvents for MS2. FLR and MS1 data were interpreted with Chromeleon (Thermo Fisher Scientific).

Instrument: Dionex Ultimate 3000 UHPLC system equipped with Dionex Ultimate 3000 XRS Autosampler and fluorescence detector.

Column: Accucore-150-Amide-HILIC (150 mm x 2.1 mm), particle size: 2.6 µm (Thermo Fisher Scientific), column temperature 45 °C, gradient details listed in Table 1.

MS Conditions

All MS experiments were performed on a Thermo Scientific Q Exactive mass spectrometer in positive mode ESI, operated at full mass scan: m/z 400-2000 at a resolution of 70,000 with automatic gain control (AGC) at 3 x10^6 with maximum IT: 150 ms. Data dependent MS2 was acquired for the top 5 ions at a resolution of 17,500, the AGC target was set at 1 x10^5, injection time was 300 ms. Isolation window was set at 2 m/z. Stepped collision energy was applied at 28, 30 and 32. Xcalibur software (Thermo Fisher Sceintific) was used for data acquisition and MS2 spectra were interpreted manually and with SimGlycan 5.6 software (Premier Biosoft).

METHODS AND MATERIALS

Materials:

1. MabThera (rituximab), lot #B60055B01
2. Trastuzumab biosimilar (Epirus)
3. Erbitux (cetuximab), lot #10C00061B
4. mAb R (Roche)

Sample Preparation

N-glycan samples were prepared using ProZyme's Gly-X N-Glycan Rapid Release and Labeling with InstantPC Kit (Figure 1) using 40 µg material. The workflow uses a 5-minute in-solution digestion with PNGase F to release N-glycans, followed by labeling with InstantPC (Figure 2) to allow for enhanced FLR and MS performance (4), and excess label is removed with a vacuum-driven cleanup plate, with a total sample preparation time of around 1 hour.

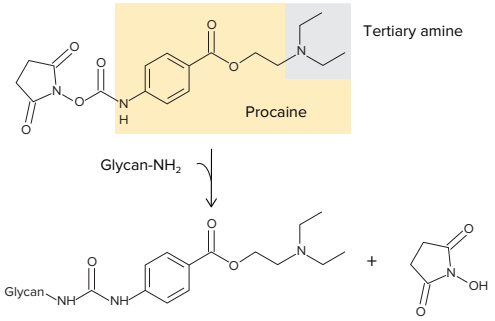

Figure 2: InstantPC (instant procaine) dye structure and conjugation with glycosylamine. Mass of InstantPC-Labeled Glycan = Mass of Glycan (free reducing end) + C14N3O2H19 (261.14773 Da, monoisotopic).

Time (min)	Flow (ml/min)	% A: 100 % acetonitrile (ACN), LC-MS grade	% B: 50 mM ammonium formate, pH 4.4
0	0.4	73.0	27.0
45.0	0.4	59.0	41.0
45.1	0.4	45.0	55.0
48.9	0.4	45.0	55.0
49.0	0.4	73.0	27.0
58.0	0.4	73.0	27.0

Table 1: HILIC gradient details.

RESULTS

HILIC-FLR analysis of InstantPC N-glycans with MS1

- Major N-glycan species from were separated for rituximab (Figure 3), trastuzumab biosimilar (Figure 4), cetuximab (Figure 5) and mAb R (Figure 6).
- Figures 3–6A shows the total ion chromatogram (TIC), Figures 3–6B show the FLR trace. The high FLR and MS response of InstantPC in positive mode enhanced by the tertiary amine (4) allows these two profiles to be compared, and e.g. Man5 to be identified from mass spectra (Figure 7).
- Table 2 shows reproducibility of the sample preparation for relative % area of glycan peaks for rituximab (n=5).
- The sample prep gives consistent results for 16–128 µg rituximab for flexibility of input amount (Figure 8).
- The low pressure of the 2.6 µm column allows use with HPLC as well as UHPLC systems.

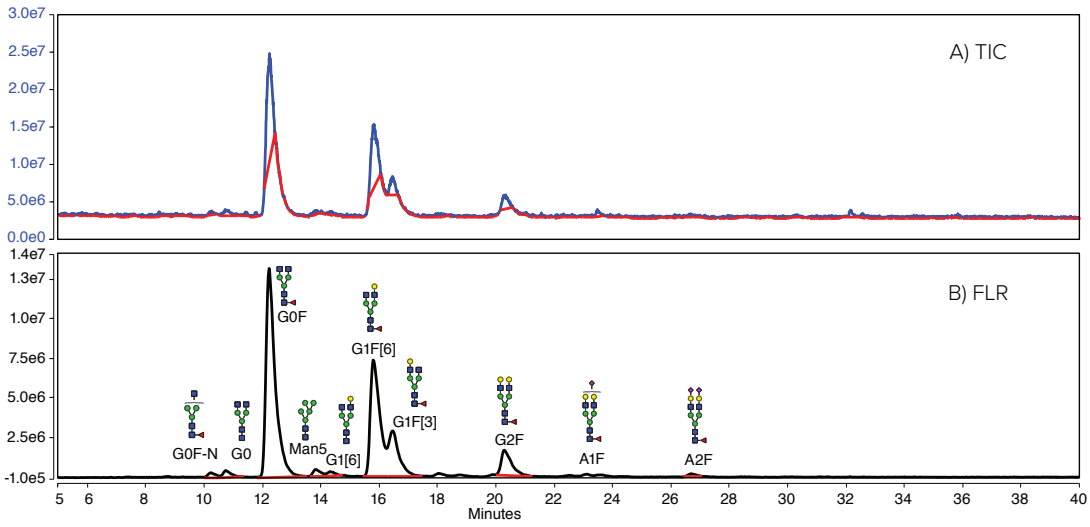

Figure 3: Rituximab N-glycans labeled with InstantPC and separated by HILIC. (A) fluorescence detection (FLR), (B) total ion chromatogram (TIC).

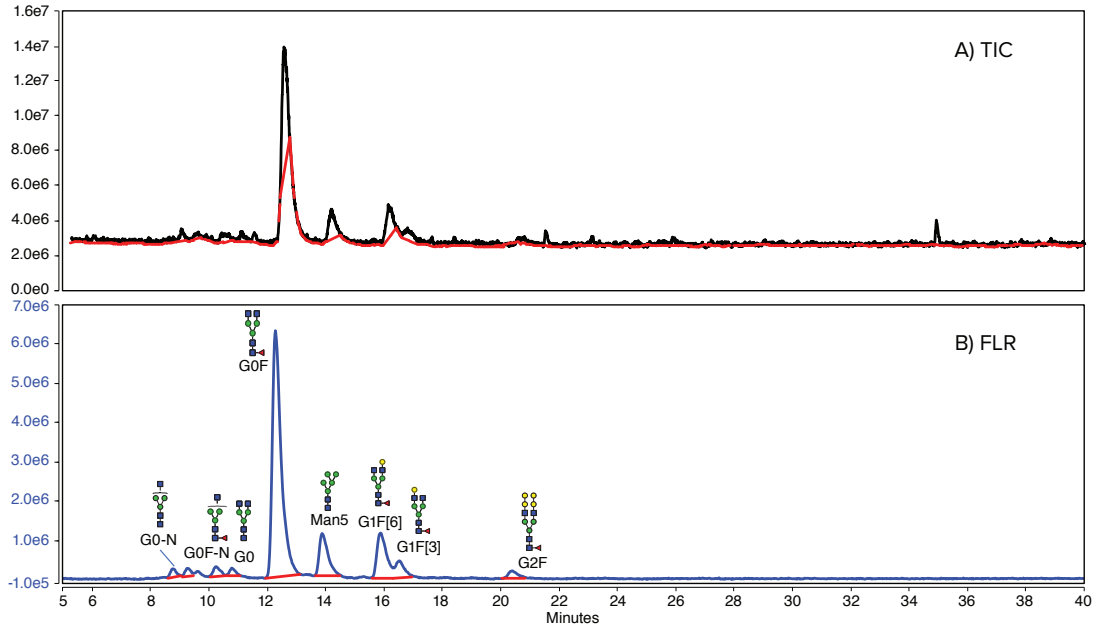

Figure 5: Trastuzumab biosimilar N-glycans labeled with InstantPC and separated by HILIC. (A) fluorescence detection (FLR), (B) total ion chromatogram (TIC).

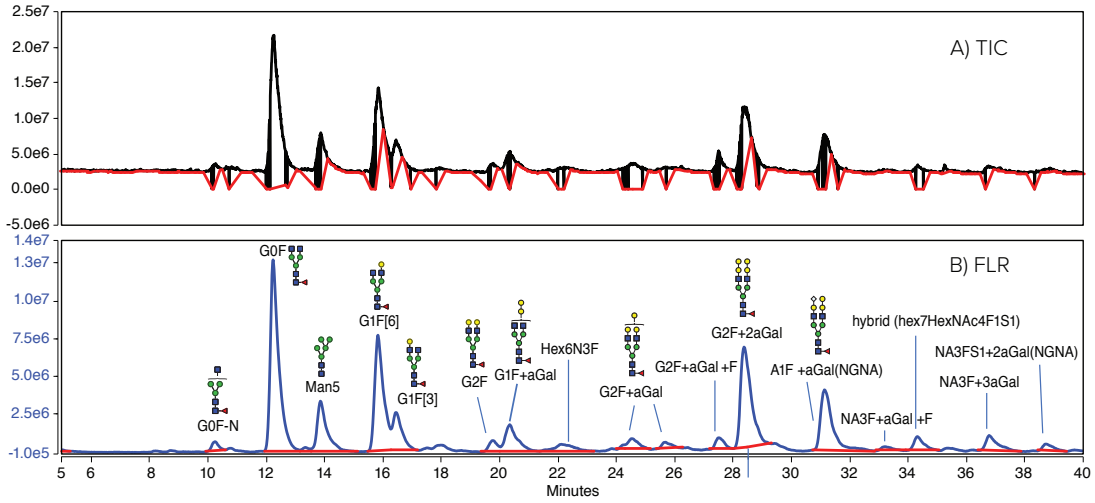

Figure 4: Cetuximab N-glycans labeled with InstantPC and separated by HILIC. (A) fluorescence detection (FLR),(B) total ion chromatogram (TIC).

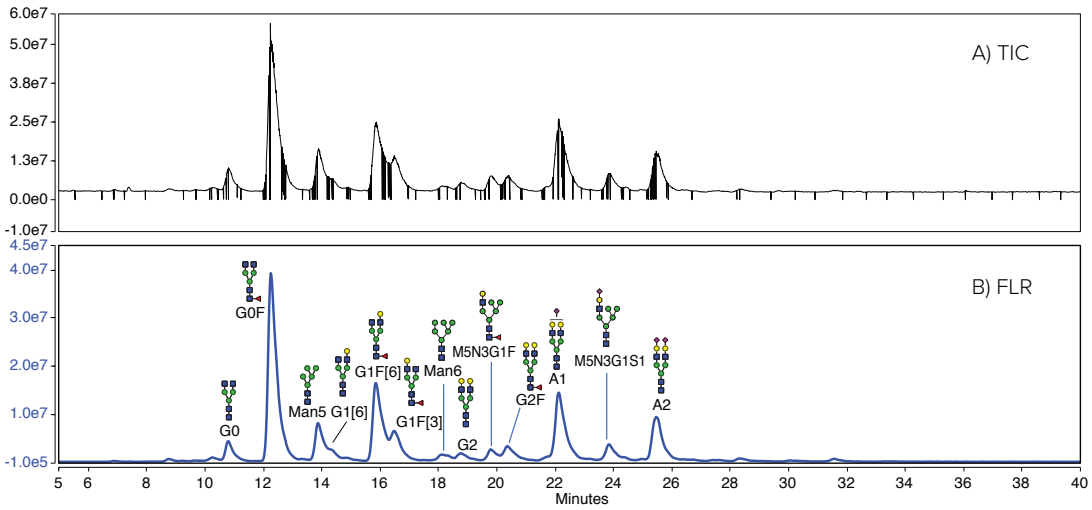

Figure 6: mAb R N-glycans labeled with InstantPC and separated by HILIC. (A) fluorescence detection (FLR), (B) total ion chromatogram (TIC).

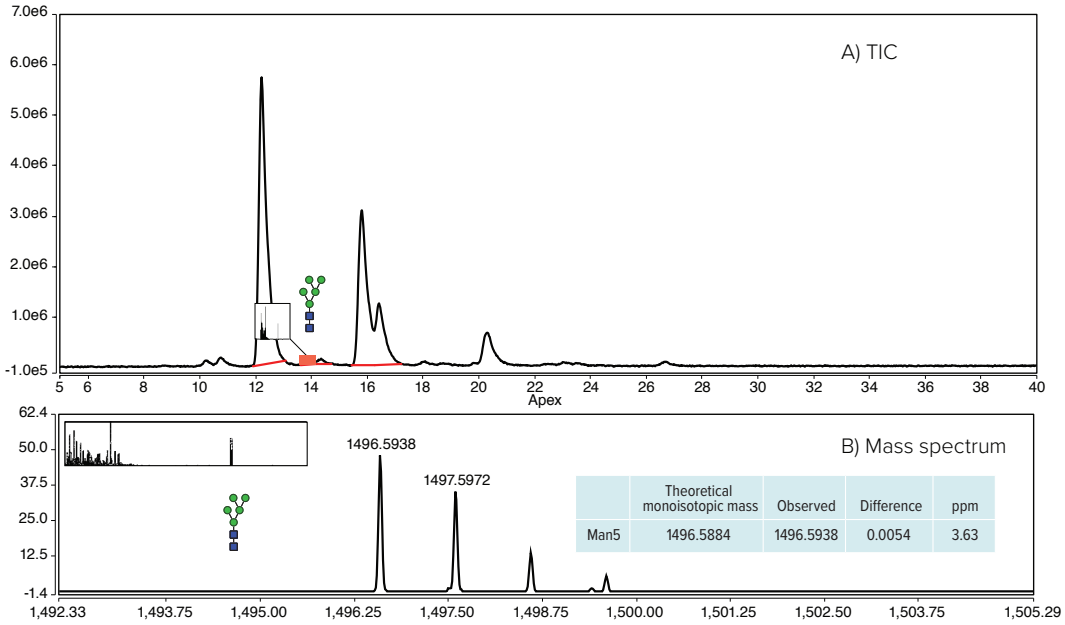

Figure 7: Identification of Man5 from rituximab. A) TIC, B) mass spectrum. Error (ppm) = (observed mass - theoretical mass)/theoretical mass x 10^6.

Structure Key:

Galactose
Mannose
Fucose
N-Acetylglucosamine
N-Acetylneuraminic acid (NANA)
N-Glycoylneuraminic acid (NGNA)

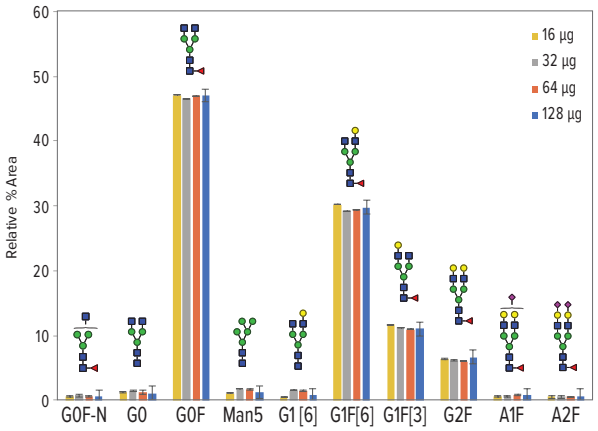

Figure 8: N-Glycan relative % area (FLR) for 16–128 µg of rituximab. Error bars represent %CV (n=3).

Glycan	Average	%CV
G0F-N	0.80	5.00
G0	1.61	6.65
G0F	49.45	1.60
Man5	1.33	8.10
G1[6]	0.86	18.27
G1F[6]	27.51	1.06
G1F[3]	11.24	5.56
G2F	6.08	5.61
A1F	0.49	34.83
A2F	0.62	8.33

Table 2: Relative % area for N-glycans released from rituximab (n=5).

HILIC separation of InstantPC N-glycans with MS2

- Figures 9 and 10 show a workflow to characterize glycan species from cetuximab and mAb R, from FLR trace to extracted ion chromatogram (XIC) to MS2 analysis in positive mode.
- Note: [3] arm position of NGNA on A1F +aGal(NGNA) is based on (5).
- Alpha-gal and NGNA-containing structures such as A1F + aGal(NGNA) in cetuximab (Figure 9) are most likely from the Fab region (6), where Erbitux produced in the mouse myeloma SP2/0 cell line is known to contain the alpha-gal N-glycan epitope.
- Hybrid-type N-glycan M5N3G1S1 is identified on mAb R (Figure 10).

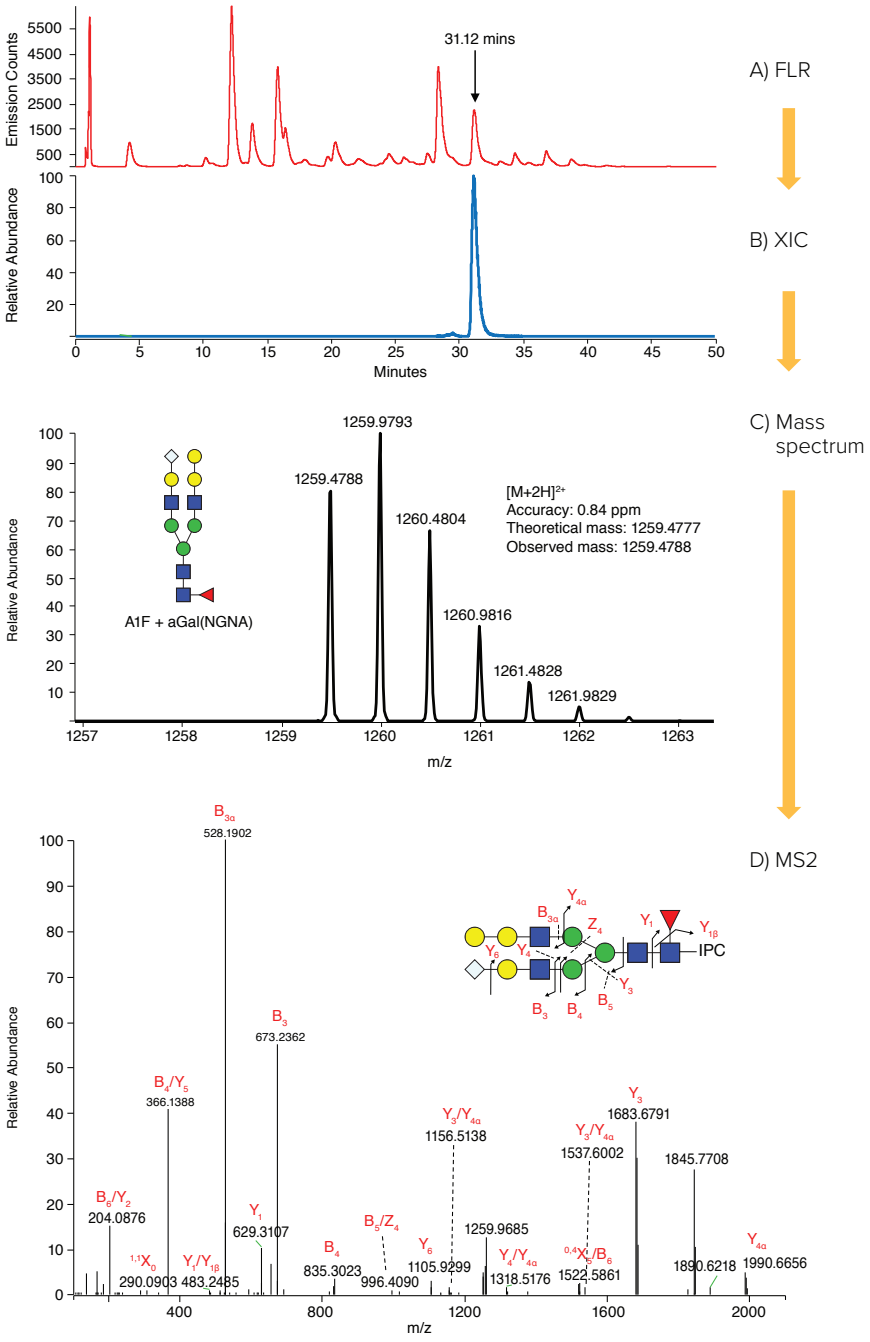

Figure 9: Characterization workflow for alpha-gal and NGNA-containing N-glycan A1F +alpha Gal(NGNA) from cetuximab with MS2.

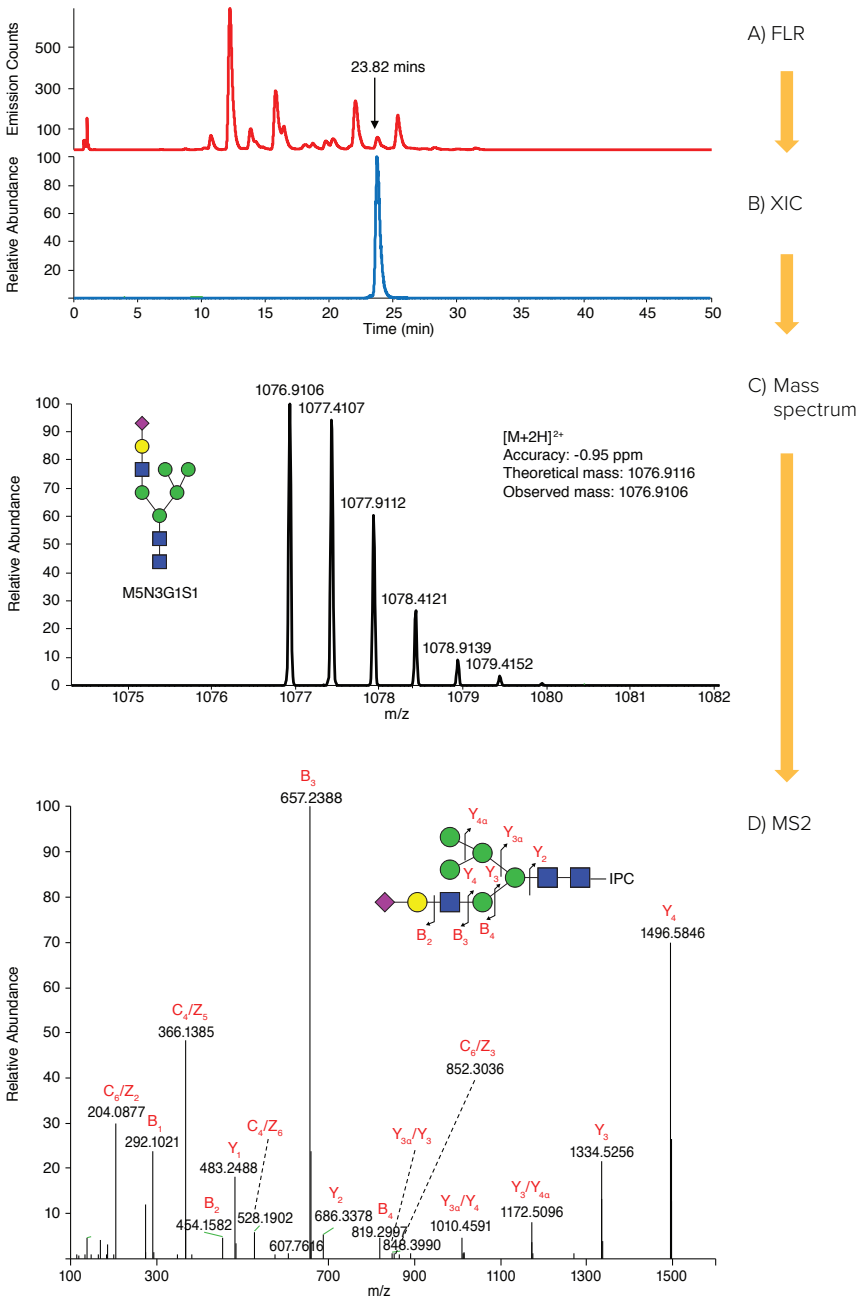

Figure 10: Characterization workflow for hybrid-type N-glycan M5N3G1S1 from mAb R with MS2.

CONCLUSIONS

1. Gly-X N-glycan sample preparation with InstantPC can be completed in around 45 minutes.
2. InstantPC N-glycan dye offers FLR and MS sensitivity.
3. HILIC separation by Accucore-150-Amide column separates major N-glycan species.
4. FLR may be used for reproducible relative quantification, MS1 spectra to assign glycan ID.
5. MS2 using the Q Exactive may be used to further characterize exotic N-glycans on the Fab region such as hybrid-type glycans and those containing potentially immunogenic epitopes such as alpha-gal and NGNA.

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