

# Comparison of Relative Quantification of Monoclonal Antibody N-glycans Using Fluorescence and MS Detection

## Application Note

Biotherapeutics & Biologics

### Authors

Oscar Potter and Gregory Staples  
Agilent Technologies, Inc.  
Santa Clara, California, USA

### Abstract

This application note describes the analysis of monoclonal antibody N-glycosylation using a novel instant mass tag (InstantPC) that permits detection using both fluorescence and mass spectrometry (MS). We have optimized a HILIC separation method for the purpose of comparing the two detection methods. Our results indicate that fluorescence and MS relative quantification of InstantPC-labeled glycans is highly similar. The sample prep procedure was conducted in quadruplicate for two different samples, and the results showed outstanding reproducibility with low RSDs even for minor components. The high MS sensitivity afforded by the InstantPC label facilitates identification of unknown glycans using accurate mass and tandem MS.



**Agilent Technologies**

## Introduction

Monoclonal antibodies (mAbs) are modified by N-glycans during biosynthesis in cell culture. Typical mAbs contain two N-glycosylation sites, one in each of the Fc regions of the molecule. Some mAbs contain additional glycosylation sites, including N- or O-glycosylation in the Fab region. Glycans can affect the function of the mAb, so it is important to monitor the glycosylation profile using appropriate analytical methods.

Popular methods for glycan analysis involve NMR, CE-LIF, HPLC with fluorescence detection (FLD), and more recently, LC/MS. Both CE-LIF and HPLC-FLD require that the glycans are labeled with a dye to permit optical detection. Conventionally, the dyes that have been used also increase the ionization efficiency of glycans in comparison to the unlabeled species, but only to the point where the most abundant compositions can be detected using MS. More recently, a novel dye (InstantPC from ProZyme Inc., depicted in Figure 1) has been developed, which moderately improves fluorescence activity and greatly improves ionization efficiency for MS analysis. Using such a tag, researchers can now use MS (in the form of accurate mass or tandem MS) for identification of glycans from LC separations. Furthermore, they have the option of relative quantification using MS rather than fluorescence detection.

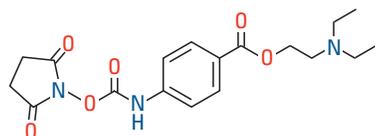


Figure 1. Diagram of InstantPC (ProZyme, Inc), an amine reactive instant label for fluorescence and MS detection of glycans.

This application note investigates the performance of InstantPC in the context of relative quantification of N-glycans released from two mAb preparations. Quadruplicate samples of the mAb samples were processed using the InstantPC kit from ProZyme, Inc. The samples were then separated by HILIC on a UHPLC system using FLD and MS detection. The LC separation conditions were optimized for maximum chromatographic separation. In doing so, the goal was to decrease the number of overlapping peaks that would otherwise not be discernable using FLD detection alone. As a result, we were able to compare the relative quantification results from the two detection methods for nearly all significant glycan structures. Accurate mass and tandem MS spectra were acquired for all glycan compositions, and were used for identification of the glycans present in the mAb preparations. Figure 2 shows the entire workflow.

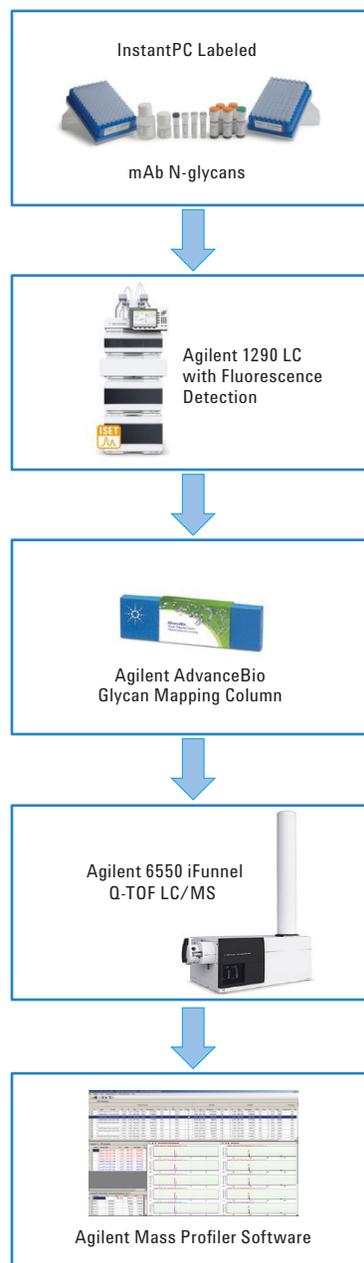


Figure 2. Workflow used for identification and quantification of InstantPC-labeled N-glycans from mAbs.

## Experimental

The Agilent LC/MS System used in this work comprised the following modules:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity High Performance Autosampler (G4226A) with an Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Fluorescence Detector (G1321B)

## MS system

Agilent 6550 iFunnel Q-TOF LC/MS system with dual-nebulizer AJS source

## Columns

Agilent AdvanceBio Glycan Mapping column, 2.1 × 150 mm, 1.8 µm connected to a second AdvanceBio Glycan Mapping column, 2.1 × 100 mm

## Software

- Agilent PCDL Manager (Version B.07.00 Build 7024.0) and Agilent Mass Profiler (Version B.07.01 Build 99.0)
- Agilent MassHunter Workstation Software, Version B.05.01, Build 5.01.5125.1

## Solvents and samples

All reagents and solvents used were of the highest purity available.

## Chromatographic conditions

Parameter	Value
Mobile phase A	50 mM ammonium formate pH 4.4
Mobile phase B	Acetonitrile
Gradient	Time (min) %B
	0 75
	32 69
	48 60
	48.5 25
	50.5 25
	52 75
Autosampler temperature	4 °C
FLD	Ex. 285 Em. 345
Injection	2 µL in 20 % DMSO (1 µg of IgG equivalent)
Column temperature	40 °C
Flow rate	0.4 mL/min

## Results and Discussion

FLD chromatograms from both mAb 1 and mAb 2 revealed that each molecule was modified by a very similar set of glycoforms, as shown in Figure 3. Some structures have been annotated in the figures, and represented by symbols according to the guidelines of the Consortium for Functional Glycomics (CFG) [1].

Using accurate mass and tandem mass spectrometry information, FLD peaks were assigned to glycan compositions in the form:

$H_xN_xF_xSg_x + \text{Core}$

H = galactose or mannose, N = N-acetylglucosamine, F = fucose, Sg = N-glycolylneuraminic acid, and Core = trimannosyl core common to all N-glycans.

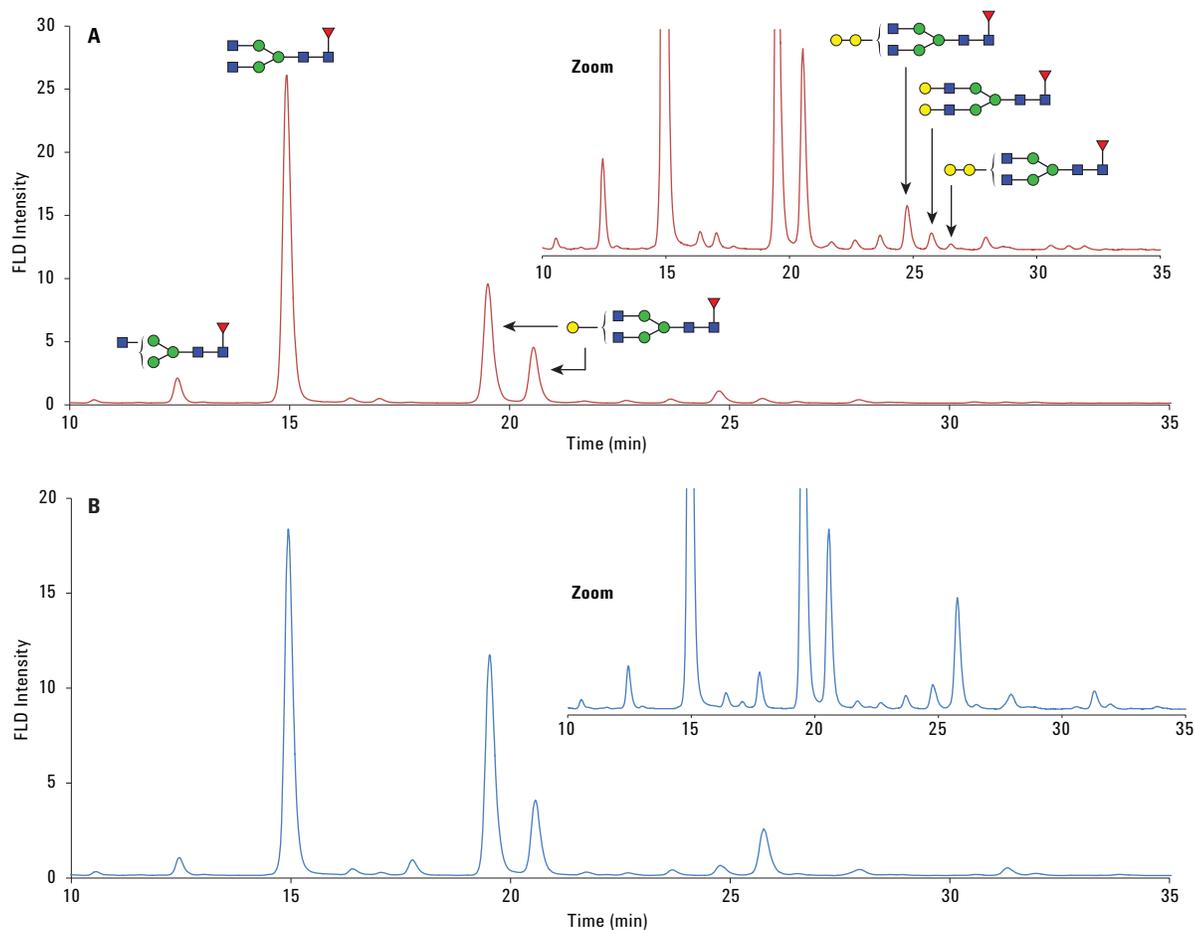


Figure 3. FLD chromatograms of InstantPC-labeled N-glycans released from mAb 1 and mAb 2. A) FLD chromatogram for mAb 1. B) FLD chromatogram for mAb 2.

Based on peak area from the FLD chromatograms, each composition was quantified as a relative sum percentage based on the total FLD area for all compositions. The results are shown as a histogram in Figure 4.

Overall, 21 glycan compositions were quantified based on the FLD data. The criteria used for inclusion in the FLD quantification were a relative abundance of 0.1 % or greater,

and reasonable resolution from neighboring peaks. One exception was the pair H2N1F1Sg1 + Core/H2N3F1 + Core. These coeluting compositions were abundant enough to merit inclusion in the FLD quantification. Therefore, the FLD signal area from this peak was divided into two portions according to the relative abundance of each as determined by MS.

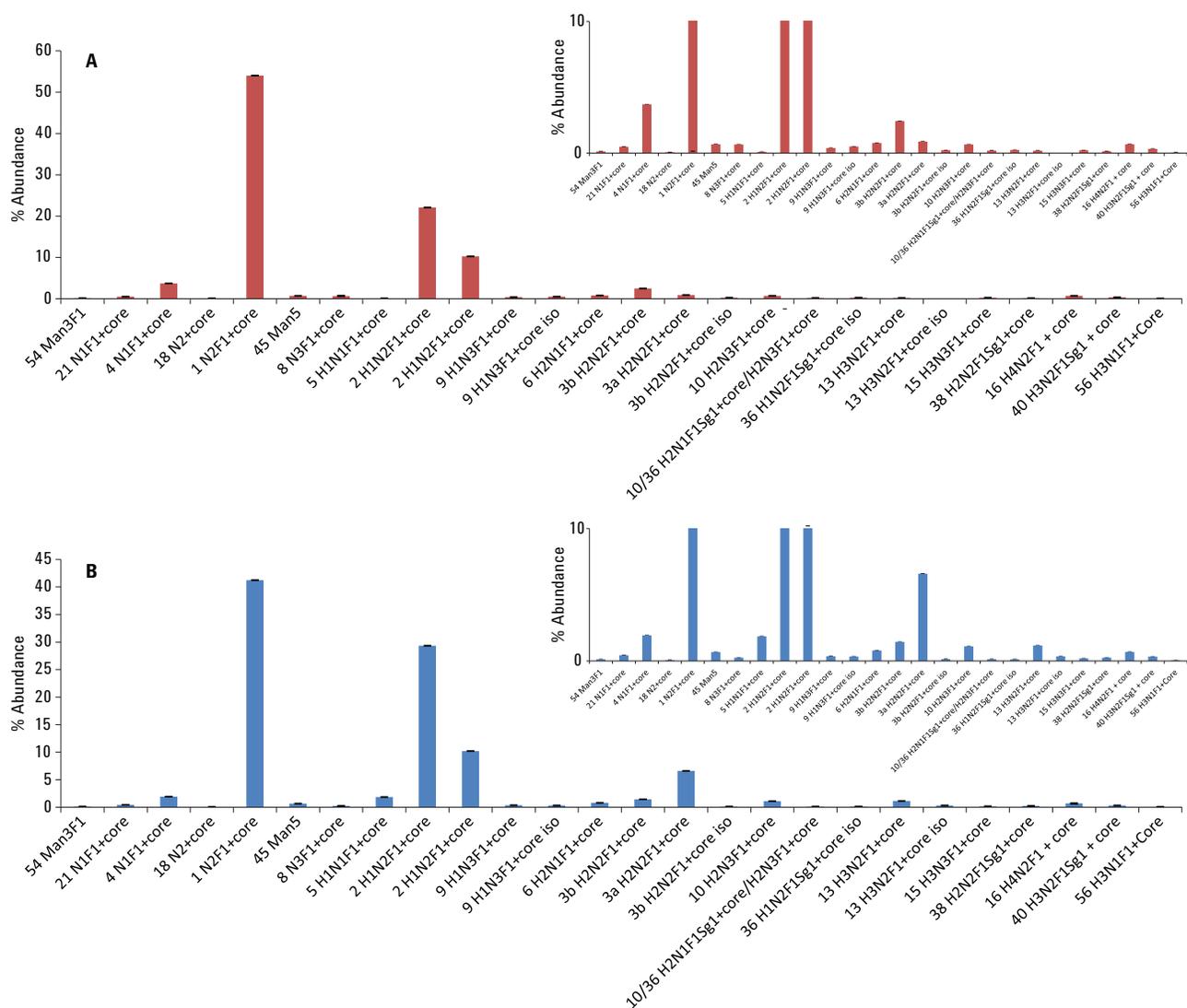


Figure 4. A) Relative FLD quantification of mAb 1 glycans. B) Relative FLD quantification of mAb 2 glycans. Error bars represent  $\pm$  standard deviation of quadruplicates having gone through the entire workflow. Integration of FLD signals was performed using Agilent MassHunter Qualitative Analysis Software. Insets show the same data zoomed to better display components with <10 % relative abundance.

InstantPC imparts high ionization efficiency to N-glycans. Thus, it is possible to perform relative quantification using the peak area from extracted ion chromatograms from MS detection. To assess this possibility, we compared FLD chromatograms with ion chromatograms. Figure 5 shows that the FLD and MS chromatograms were highly similar. There is a corresponding MS peak for every FLD peak that was detected.

Encouraged by the high similarity seen in Figure 5, we performed relative quantification of glycans from mAb 1 and mAb 2 based on the MS data. Ion chromatograms for each feature (defined as a mass-retention time pair, which includes signals from all charge states and adducts) were created using Agilent Mass Profiler software. In this case, no lower threshold for detection was imposed. The features determined using Mass Profiler were identified using a Personal Compound Database (PCD) constructed for these experiments.

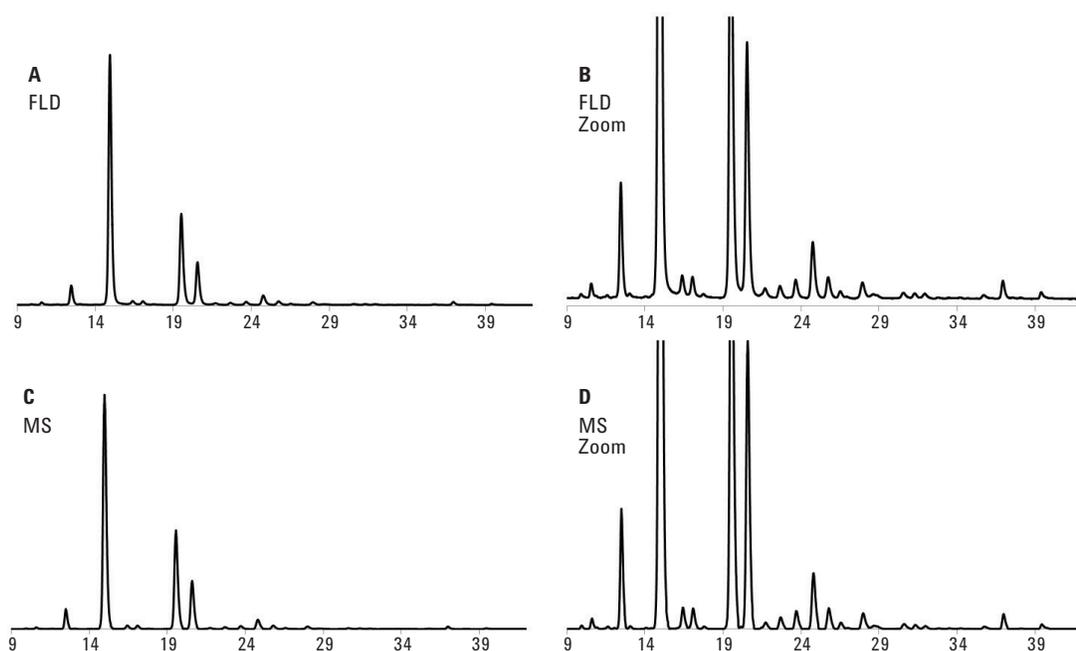


Figure 5. Comparison of FLD and MS chromatograms for mAb 1. A) FLD chromatogram of mAb 1 glycans. B) Zoom of FLD chromatogram of mAb 1 glycans. C) MS chromatogram of mAb 1 glycans. D) Zoom of MS chromatogram of mAb 1 glycans.

The PCD contains accurate mass and retention time information for mAb glycans. The database was constructed based on a combination of tandem MS information from the current work in addition to knowledge of glycan biosynthetic rules. Figure 6 shows an example of the utility of tandem MS for assigning glycan compositions. In particular, the example shown in Figure 6B illustrates a common case where mass

alone may be insufficient for assignment of composition, due to the fact that the mass of NeuGc + fucose is isobaric with that of NeuAc + galactose. Tandem MS resolves the ambiguity, because the presence of the fragment ion at  $m/z$  673 provides strong evidence that the structure contains an antenna with NeuGc.

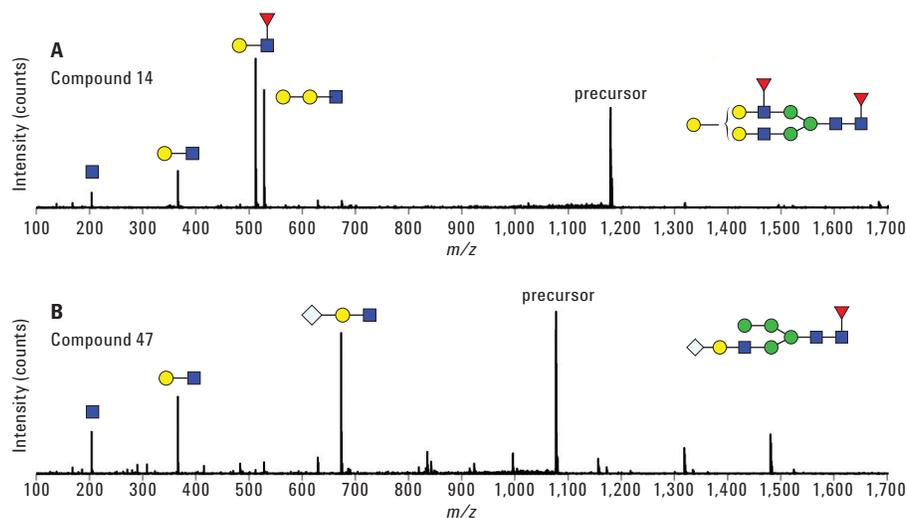


Figure 6. Tandem MS data were acquired for all glycans. MS/MS aided in compound identification when accurate mass was insufficient. The two examples above are consistent with gal-gal and outer arm fucose (A) and NeuGc (B) modifications.

Figure 7 shows the results of the MS-based quantification of mAb 1 and mAb 2 glycans.

As a result of the mass selectivity provided by Q-TOF detection, it was possible to quantify more compositions

than from the FLD detection. In this case, a total of 35 compositions were quantified. The average RSD was 3.2 % for mAb 1 and 3.9 % for mAb 2 for all features independent of abundance. For those features equal to or greater than 0.1 % relative abundance, RSDs were 2.7 % and 3.4 % respectively.

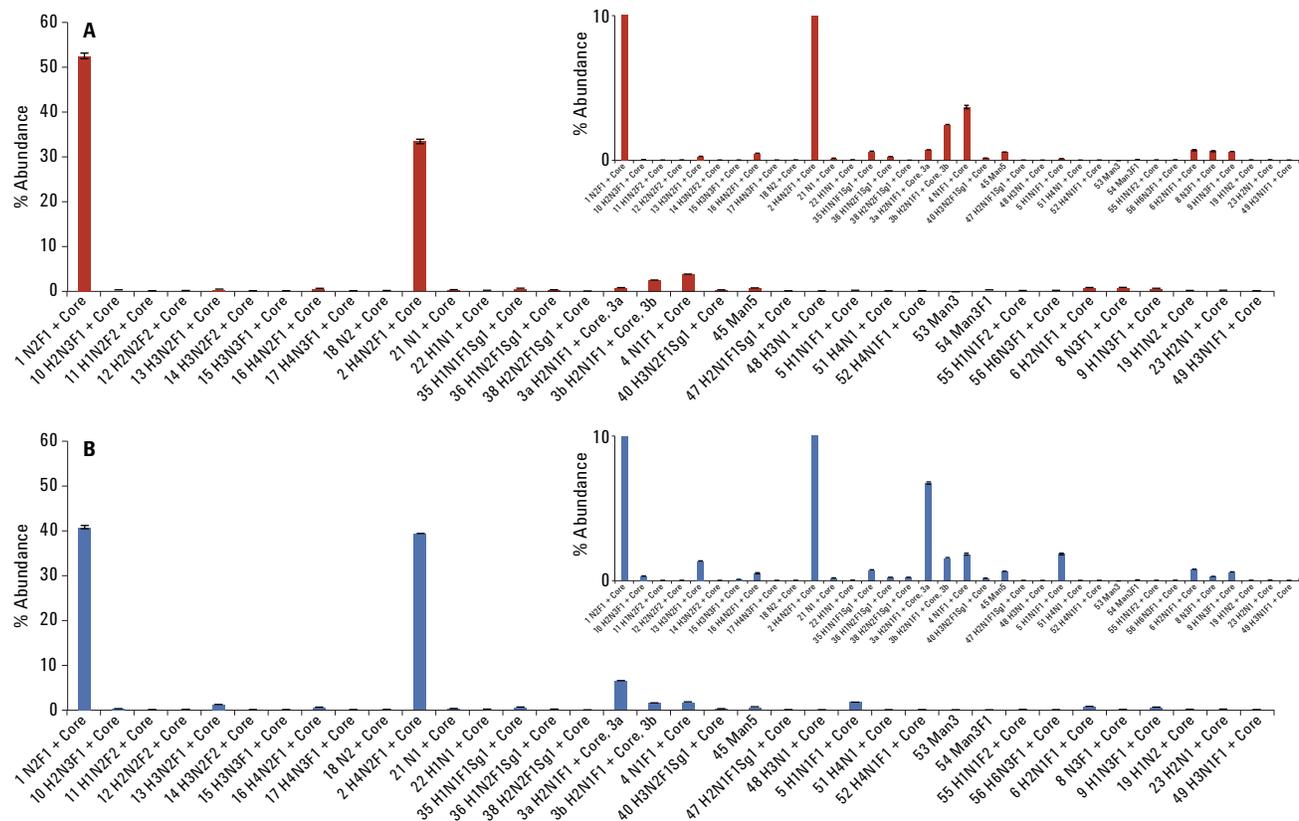


Figure 7. Relative MS quantification of InstantPC labeled N-glycans released from mAb 1 and mAb 2. A) Relative MS quantification of mAb 1 glycans. B) Relative MS quantification of mAb 2 glycans. Error bars represent  $\pm$  standard deviation of quadruplicates having gone through the entire workflow. Insets show the same data zoomed to better display components with <10 % relative abundance.

Finally, we directly compared the relative quantification of glycans from mAb 1 and mAb 2 using FLD and MS. Figure 8 shows the results from each method plotted on a single histogram.

## Conclusion

As shown in Figure 8, the relative quantification results from FLD and MS were highly similar. Some small differences in the results from the two methods can be explained by the different numbers of compositions quantified in the two methods (21 from FLD, 35 from MS). Based on the results of this study, the combination of Prozyme's InstantPC label and an Agilent LC/MS system provides the researcher with the capability to perform MS-based quantification of glycans from mAbs. Still, FLD will likely remain a gold standard detection method for this compound class. In that case, high quality Q-TOF MS data greatly facilitate peak assignment by offering accurate mass and tandem mass information for each of the InstantPC-labeled glycans detected using FLD.

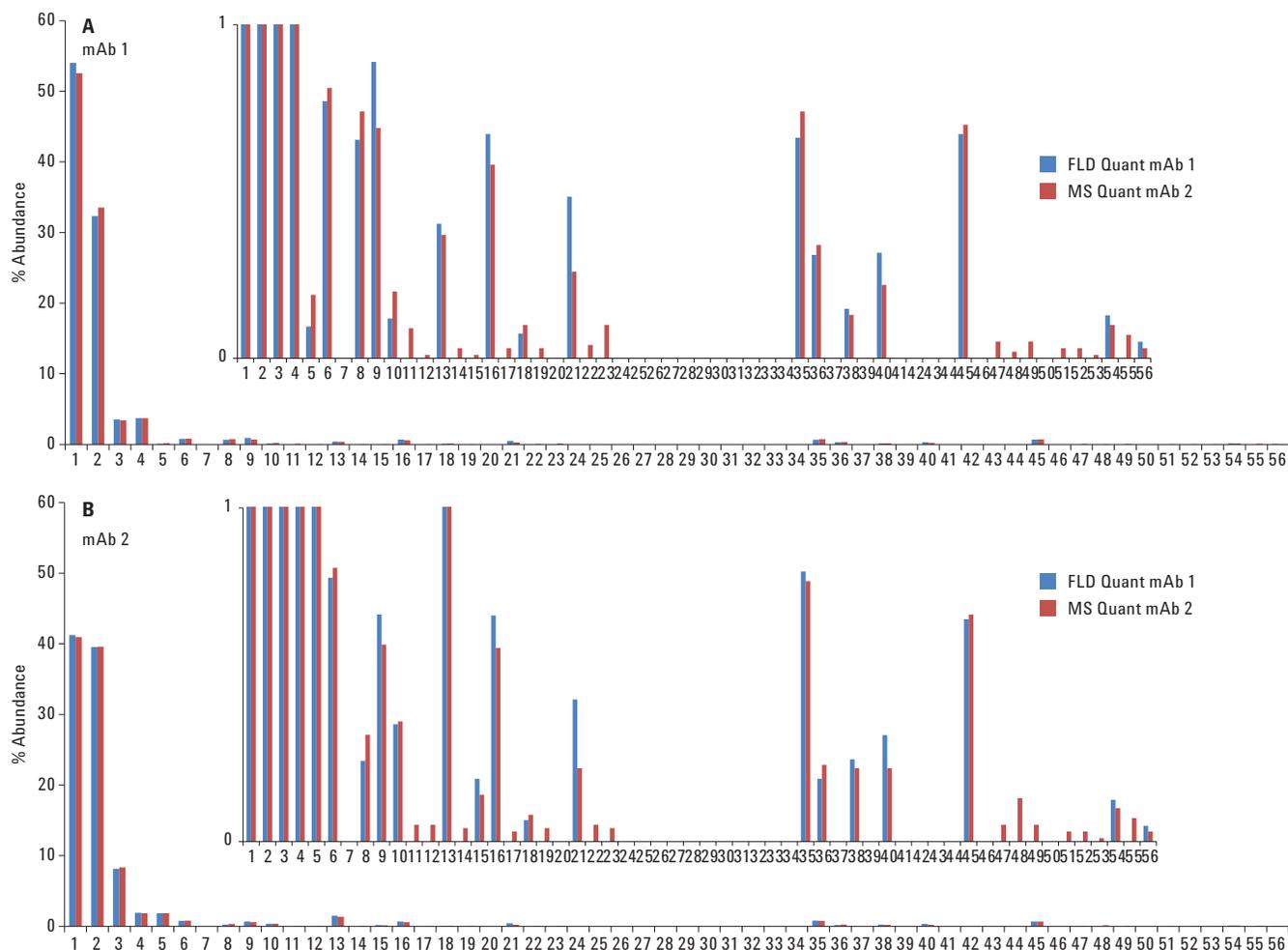


Figure 8. Comparison of fluorescence and relative MS abundance (area sum percentage) of InstantPC-labeled N-glycans from mAb 1 and mAb 2. The X-axis represents individual glycan compositions quantified in the study.

## Acknowledgements

We would like to thank NIST for providing the two mAb samples used in this work.

## Reference

1. <http://glycomics.scripps.edu/CFGnomenclature.pdf>

## For More Information

These data represent typical results. For more information on our products and services, visit our Web site at [www.agilent.com](http://www.agilent.com)

[www.agilent.com](http://www.agilent.com)

For Research Use Only. Not for use in diagnostic procedures.

This information is subject to change without notice.

© Agilent Technologies, Inc., 2016

Printed in the USA

June 29, 2016

5991-6958EN



**Agilent Technologies**