

# N-Glycan Analysis: Better Together

Agilent and ProZyme sample preparation workflow



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## Push Your Glycoprotein Analysis to New Levels of Performance

The structure of N-linked glycans can affect the immunogenicity, pharmacokinetics, and pharmacodynamics of therapeutic proteins such as monoclonal antibodies (mAbs) and Fc fusion proteins. That's why N-glycan characterization is essential to the biotherapeutic development process.<sup>1,2</sup>

A common N-glycan characterization method involves labeling enzymatically released glycans to enable fluorescence detection (FLD) and to enhance ionization for mass spectrometry (MS). These labeled glycans are separated by liquid chromatography (LC) or capillary electrophoresis (CE), with relative quantitation enabled by FLD and mass confirmation by MS detection. Hydrophilic interaction liquid chromatography (HILIC) is the most popular separation technique for labeled N-glycans.

## End-to-end workflows for N-glycan sample preparation and analysis

With our integration of ProZyme glycoanalysis products, Agilent now offers several sample preparation options for N-glycans to support your LC/FLD/MS and CE workflows (Figure 1):

- Gly-X, our newest product. Shortening sample preparation time to one hour, it uses a five-minute PNGase F digestion and InstantPC—a glycosylamine labeling dye with high FLD and MS signal. Labeling with 2-AB and APTS is also available with a workflow time of about two hours.
- GlykoPrep, an earlier generation spin-based kit. It is based on AssayMAP cartridges introduced in 2012 with a workflow time of three to five hours.
- Tools to support more traditional methods.

These modular workflows are available with several different glycan labels, and are supported by a wide portfolio of labeled and unlabeled glycan standards and libraries.

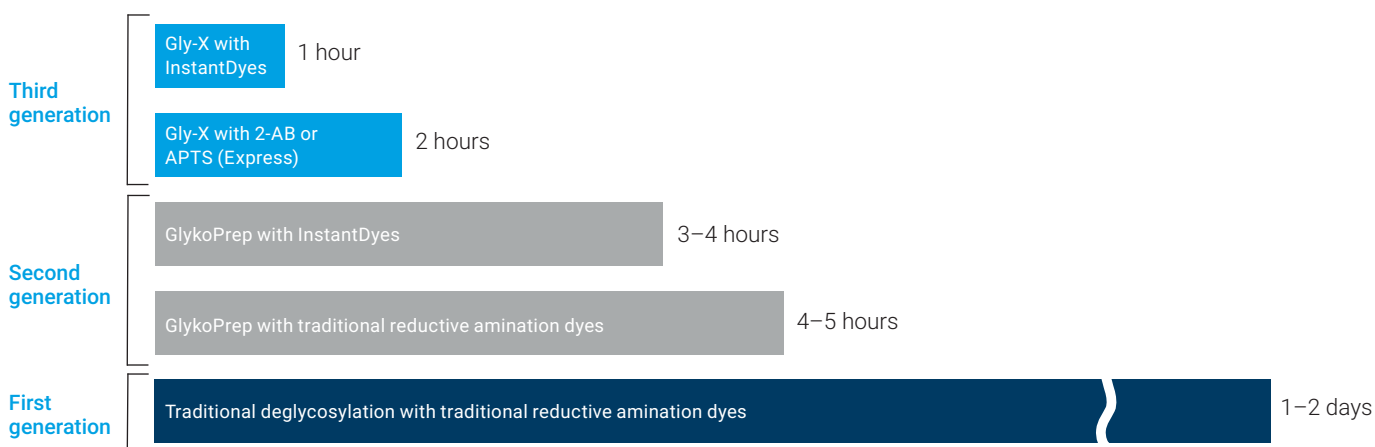


Figure 1. The evolution of N-glycan sample preparation (times are shown on the right).

Together with AdvanceBio Glycan Mapping HILIC columns and instrumentation for LC and MS, Agilent now supports your entire released glycan workflow.

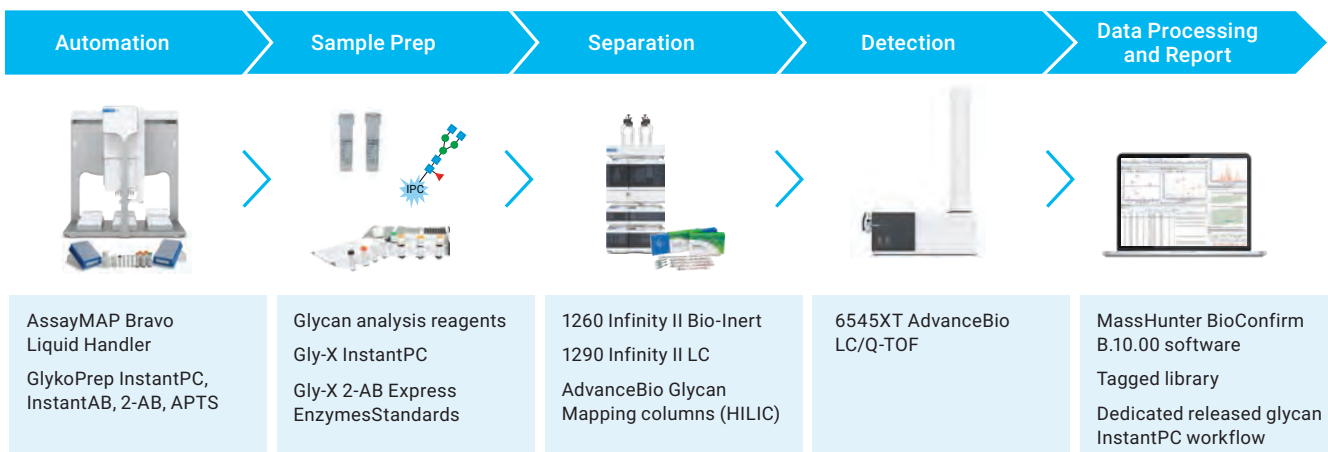


Figure 2. Agilent released N-glycan workflow elements.

# Gly-X N-Glycan Sample Preparation

Gly-X is a next-generation N-glycan preparation platform that provides a rapid, simplified, in-solution workflow. It uses a five-minute PNGase F digestion—combined with InstantPC dye and an efficient vacuum plate cleanup step—to remove excess label and denaturant. That means your samples can be ready for UHPLC with high-signal FLD and/or MS detection in 60 minutes or less (Figure 3).

Do you want to continue using the 2-aminobenzamide (2-AB) label to compare with historical data? The Gly-X platform supports 2-AB labeling with 2-AB Express, where glycans are immobilized on the cleanup matrix before labeling, which eliminates the need for dry down.

CE separations of N-glycans are also supported by the APTS (8-Aminopyrene-1,3,6-trisulfonic acid) Express kit. 2-AB Express and APTS Express workflows take about two hours, due to the additional hour required for labeling via heated reductive amination reaction.

Gly-X N-glycan sample preparation workflow

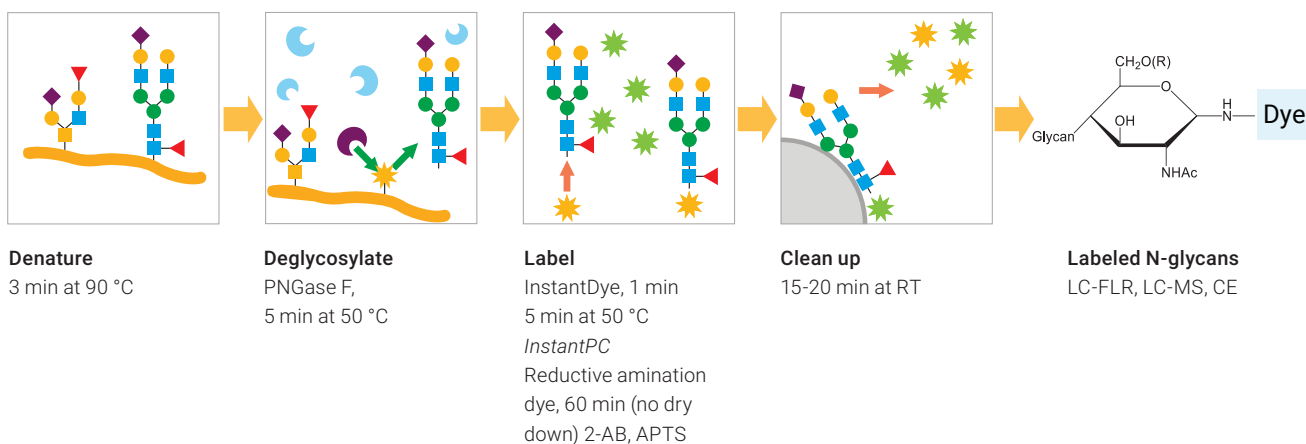


Figure 3. Gly-X N-glycan sample preparation workflow. The recommended starting sample amount is 1 to 40 µg, which is a higher maximum sample amount than similar workflows. You may also be able to use more protein, depending on the molecule. For further sample considerations, please see the individual Gly-X product manuals.

## Gly-X sample preparation is robust and reproducible

All reagents—including N-glycanase (PNGase F) enzyme—are supplied, except for common reagents such as acetonitrile, formic acid, and water. Using a 96-well format for the vacuum-driven cleanup plate, anywhere from 1 to 96 samples may be processed per run. The used wells of the cleanup plate are sealed before storage at room temperature. Partially used reagents may be returned to the appropriate storage conditions for re-use (see instruction manuals for details). Separation of InstantPC labeled N-glycans from rituximab is shown in Figure 4, along with relative percent area data.

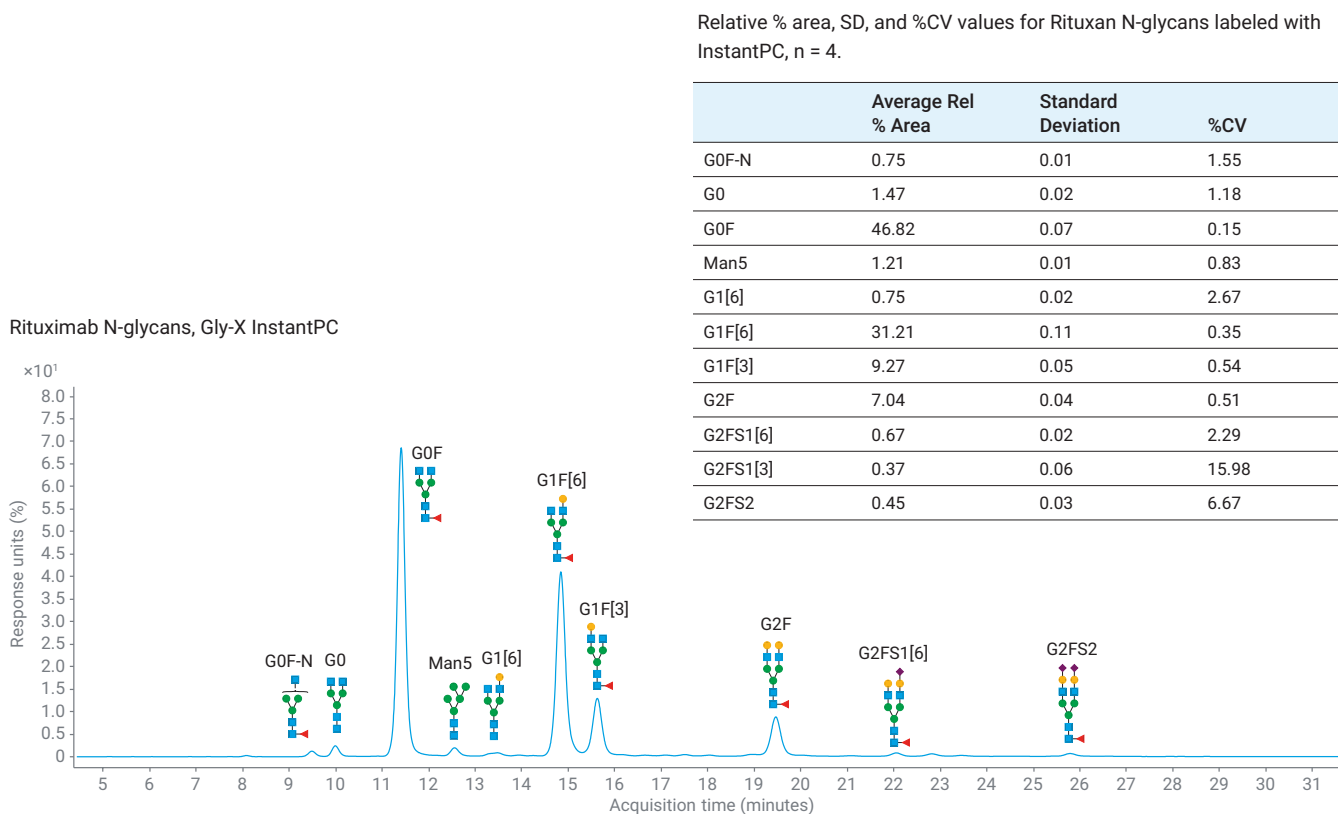


Figure 4. HILIC-UHPLC fluorescence profile of Rituxan N-glycans prepared with Gly-X InstantPC. N-Glycan relative percent areas are shown in the table. Data from application note [5994-1348EN](#).

To learn more about Gly-X, consult these resources or visit our [Gly-X webpage](#).

Publication	Document	Title
<a href="#">5994-1348EN</a>	Application note	Streamlined Workflows for N-Glycan Analysis of Biotherapeutics Using Agilent AdvanceBio Gly-X InstantPC and 2-AB Express Sample Preparation with LC/FLD/MS
<a href="#">5994-0682EN</a>	Application note	Preparation of Released N-Glycan Samples from Monoclonal Antibodies Using Agilent AdvanceBio Gly-X 2-AB Express for LC-Fluorescence Analysis
<a href="#">5994-0944EN</a>	Application note	Development of a Rapid APTS Sample Preparation Workflow for N-Glycan Release and Labeling
<a href="#">5994-1231EN</a>	User manual	Agilent AdvanceBio Gly-X N-Glycan Prep with InstantPC Kit (p/n GX24-IPC and GX96-IPC)
<a href="#">5994-1228EN</a>	User manual	Agilent AdvanceBio Gly-X N-Glycan Prep with 2-AB Express Kit (p/n GX24-2AB and GX96-2AB)
<a href="#">5994-1229EN</a>	User manual	Agilent Gly-X N-Glycan Rapid Release and Labeling with APTS Express Kit (p/n GX96-APTS)

# GlykoPrep N-Glycan Sample Preparation

Released in 2012, ProZyme GlykoPrep solid phase cartridges were the first platform to use “instant” glycan labeling (Figure 5). The cartridges streamlined and standardized N-glycan sample preparation in both spin and automation (AssayMAP Bravo) formats. Reproducibility was demonstrated in two interlaboratory studies using LC<sup>3</sup> and CE<sup>4</sup>. Although GlykoPrep has been superseded by Gly-X, we continue to support existing GlykoPrep customers.

GlykoPrep N-glycan sample preparation workflow

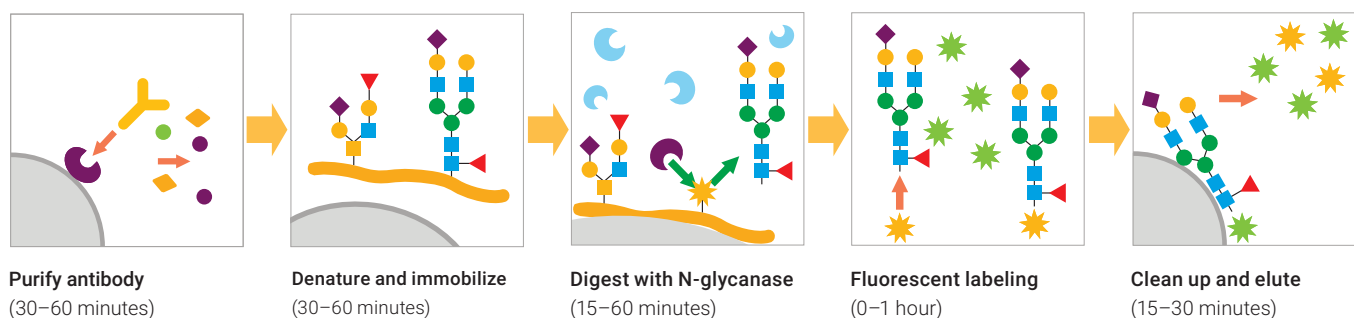


Figure 5. GlykoPrep N-glycan sample preparation workflow. Many labeling options are available, including InstantPC, 2-AB, and InstantAB, APTS.

To learn more about GlykoPrep, consult these resources or visit our [GlykoPrep webpage](#).

Publication	Document	Title
<a href="#">5994-0942EN</a>	Application note	Comparison of Common Fluorescent Labels for LC/MS Analysis of Released N-Linked Glycans
<a href="#">5991-8550EN</a>	Application note	A Comprehensive Approach for Monoclonal Antibody N-linked Glycan Analysis from Sample Preparation to Data Analysis
<a href="#">5991-6958EN</a>	Application note	Comparison of Relative Quantification of Monoclonal Antibody N-Glycans Using Fluorescence and MS Detection
<a href="#">5991-0871EN</a>	Application note	Analysis of Monoclonal Antibody N-Glycans by Fluorescence Detection and Robust Mass Selective Detection Using the Agilent LC/MSD XT



## Traditional Methods for N-Glycan Sample Preparation

Older N-glycan sample preparation workflows include native or denaturant-driven digestion with PNGase F, purification of released glycans, labeling with a fluorophore, and purification of labeled glycans. These workflows take 1 to 2 days, and are not suitable for high-throughput applications or automation.

With the introduction of Gly-X, we can help you transition from a 2-AB workflow to faster sample preparation techniques with higher throughput. However, we will continue to support traditional workflows with a range of glycan labeling and cleanup tools.

To learn more about our products for traditional glycan preparation, consult these resources or visit our [traditional glycan preparation webpage](#).

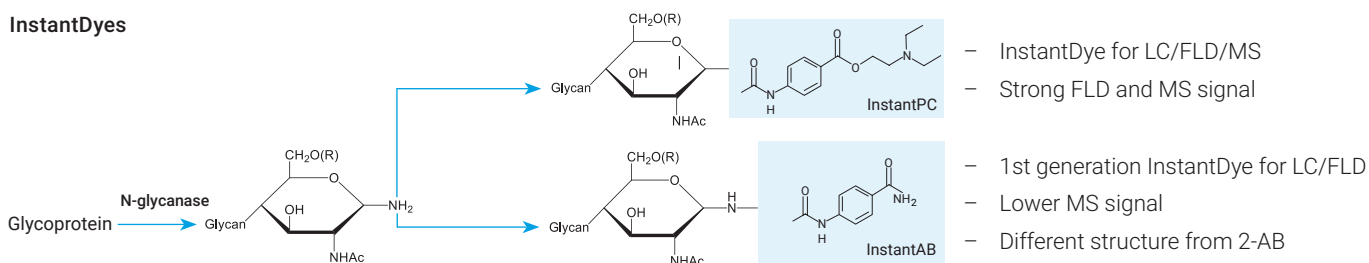
Publication	Document	Title
<a href="#">5994-1056EN</a>	Data sheet	AdvanceBio N-Glycanase (PNGase F) $\geq 2.5$ U/mL (p/n GKE-5006)
<a href="#">GKK-804</a>	User guide	2-AB-plus Labeling Kit (p/n GKK-804)
<a href="#">GKI-4756</a>	User guide	GlycoClean R Cartridges (p/n GKI-4756)
<a href="#">GKI-4025</a>	User guide	GlycoClean H Cartridges (p/n GKI-4025)
<a href="#">GKI-4726</a>	User guide	GlycoClean S Cartridges (p/n GKI-4726)
<a href="#">5991-9561</a>	User guide	AdvanceBio N-Glycan Sample Preparation Kit (p/n 5190-8005)



## Glycan labels

You can label N-glycans with InstantDyes such as InstantPC, or traditional dyes such as 2-AB for HILIC, or APTS for CE, summarized in Figure 6. Keep in mind that reductive amination requires a heated labeling reaction of an hour or more, so sample preparation will take longer. Glycan labeling dyes have differences in UHPLC-HILIC retention time and selectivity, although retention order remains relatively consistent.<sup>5</sup>

### InstantDyes



### Reductive Amination

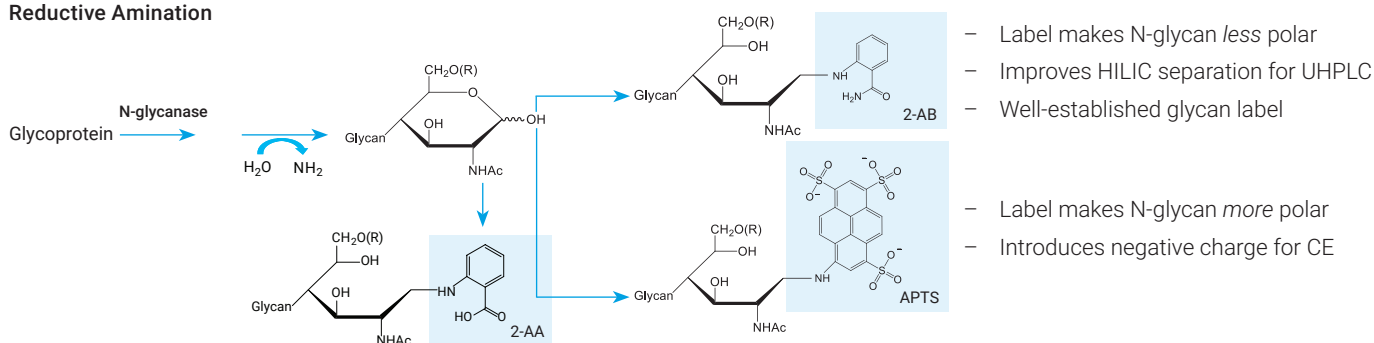


Figure 6. Glycan labeling options.

In addition to high FLD signal in LC, InstantPC contains a tertiary amine that generates high MS signal in positive mode. Unlabeled glycans, as well as traditional labels for glycan analysis such as 2-AB and 2-AA, ionize poorly in MS.

InstantPC had the highest FLD signal of the glycan labels, shown in Figure 7. The second-best label for fluorescence was procainamide, which is added to glycans by reductive amination—a more time-intensive workflow.

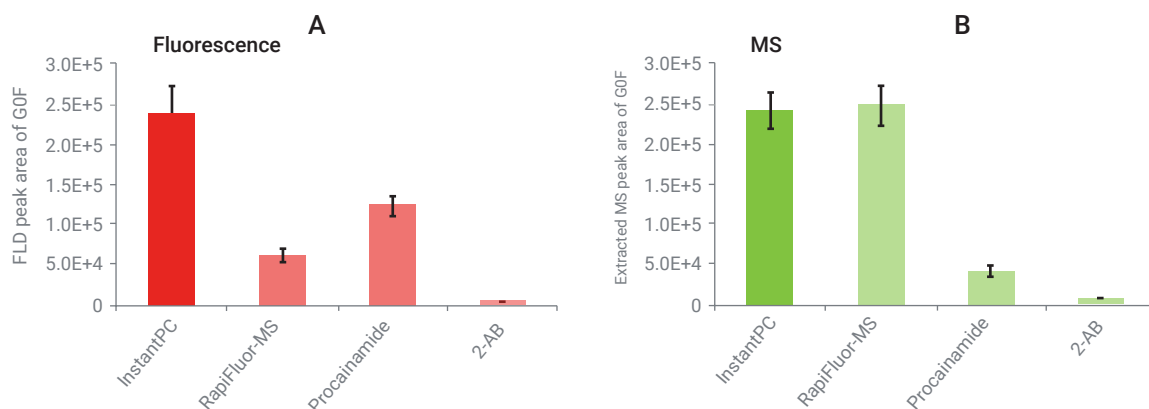


Figure 7. Comparison of FLD (A) and MS (B) response of glycan labels. Glycans from equal amounts of glycoprotein samples were labeled with InstantPC, RapiFluor-MS, procainamide, and 2-AB according to manufacturer instructions, and measured by UHPLC. Bars represent the peak area of G0F N-glycan species.





## N-Glycan Standards

We offer purified glycan standards in both labeled and unlabeled forms. Supported labels include InstantPC, 2-AB, 2-AA, and APTS. All can be used as qualitative standards for procedures such as LC/FLD, LC/MS, CE/LIF, and CE/MS.

Many common glycan types seen in biotherapeutics are covered, including complex biantennary neutral and sialylated, high mannose, and alpha gal. You can also choose from several glycan libraries for glycoproteins such as:

- Human IgG
- Bovine RNase B
- Bovine fetuin
- Human  $\alpha$ 1-acid glycoprotein (AGP)
- Recombinant monoclonal IgG (mAb) made in Chinese hamster ovary (CHO) cells
- Triantennary and tetraantennary sialylated N-glycan libraries

In addition, we offer both  $\alpha$ (2,3) and  $\alpha$ (2,6) sialylation within these standards.

- The  $\alpha$ (2,3) sialic acid linkage is found on glycoproteins produced in CHO cells.<sup>6</sup> Glycans with  $\alpha$ (2,3) sialylation have shorter HILIC retention times than isomeric N-glycans with  $\alpha$ (2,6) sialic acid linkages.<sup>7</sup>
- The  $\alpha$ (2,6) sialic acid linkage is found on glycoproteins such as human intravenous immunoglobulin (IVIG).<sup>8</sup>



For a list of individual N-glycan standards, libraries, and glucose unit (GU) ladders, along with structures and alternative nomenclatures, see publication [5994-0999EN](#).

To explore our full range of N-glycan standards, visit the [Agilent website](#).



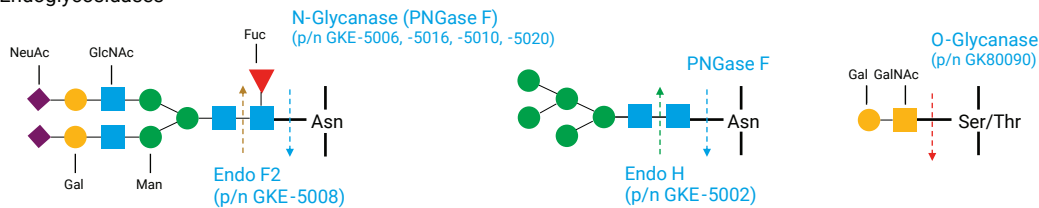
## Glycoenzymes

Agilent now offers a variety of glycoenzymes to support your released glycan and other analytical workflows. A selection of our enzymes is shown in Figure 8. Please visit the [Agilent website](#) for our full range.

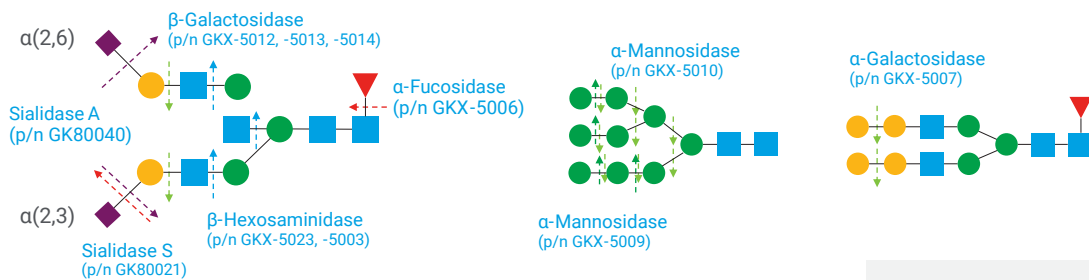
- Endoglycosidases cleave within a glycan structure. N-glycanase (PNGase F, technically an asparagine amidase) is widely used to study released glycans and to generate de-N-glycosylated protein, because it releases most intact N-glycans.
- Exoglycosidases cleave exposed or “terminal” monosaccharide residue from glycans. Commonly used exoglycosidases include galactosidase for degalactosylation and sialidase (neuraminidase) for desialylation of released glycans, glycoproteins, or cells.
- Glycosyltransferases catalyze the addition of a specific monosaccharide to an existing glycan structure, either free or while attached to a protein, sugar, or lipid. Each enzyme type adds a specific monosaccharide. Applications include modifying glycans to a glycoprotein protein in vitro in order to “glycoengineer” a desired glycan profile. Our sialyltransferases and galactosyltransferases also include the appropriate sialic acid or galactose donor substrates.

## Specificities for selected endoglycosidases (A), exoglycosidases (B), and glycosyltransferases (C)

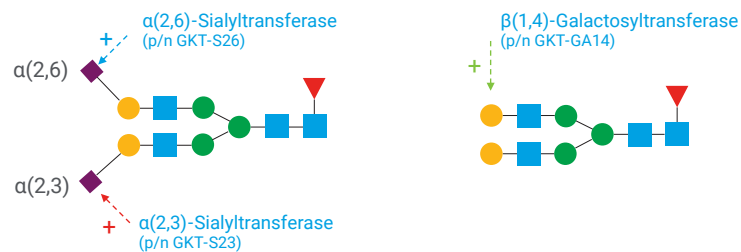
### A. Endoglycosidases



### B. Exoglycosidases



### C. Glycosyltransferases



### Glycan structure key

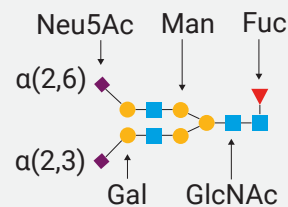


Figure 8. Specificities for selected A) endoglycosidases; B) exoglycosidases; and C) glycosyltransferases.

Glycan cartoons follow the recommendations of the Consortium for Functional Glycomics<sup>9</sup> (CFG) and were drawn using GlycoWorkbench 2.14.<sup>10</sup>

Neu5Ac = N-acetylneuraminic acid

GalNAc = N-acetylgalactosamine

Gal = galactose

Asn = asparagine

Man = mannose

Ser = serine

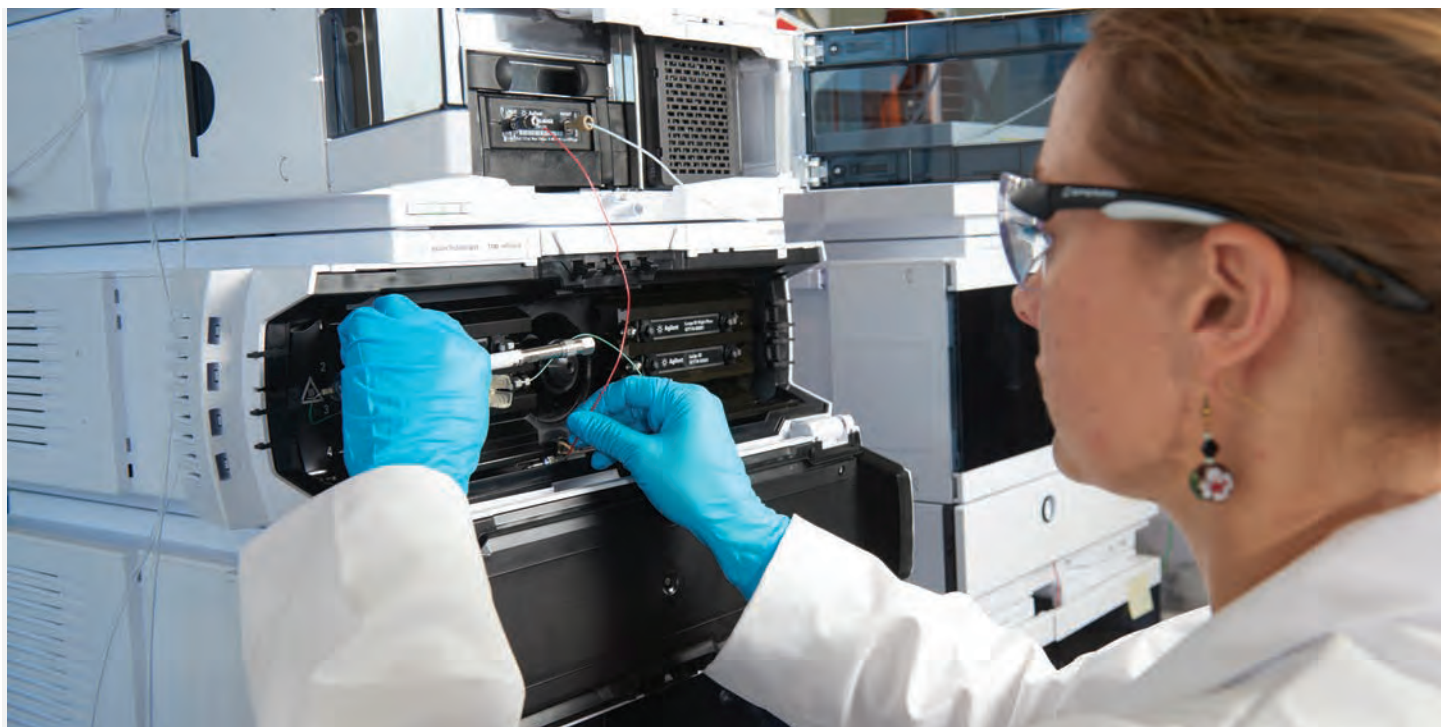
GlcNAc = N-acetylglucosamine

Thr = threonine

Fuc = fucose

To learn more about glycoenzymes, consult these resources or visit our [glycobiology enzymes webpage](#).

Publication	Document	Title
5994-1225EN	Data sheet	Sialidase A (p/n GK80040)
GKT-S23	Data sheet	$\alpha(2,3)$ -Sialyltransferase (p/n GKT-S23)
GKT-S26	Data sheet	$\alpha(2,6)$ -Sialyltransferase (p/n GKT-S26)
GKT-GA14	Data sheet	$\beta(1,4)$ -Galactosyltransferase (p/n GKT-GA14)



## AdvanceBio Glycan Mapping Columns Deliver High Speed and Resolution

In the race to discover and develop the next promising biotherapeutic, or develop a comparable biosimilar, you cannot compromise on analytical accuracy and efficiency. Reducing process development time, making procedural changes, and controlling costs are just a few of the challenges you face every day.

Once you have generated your labeled N-glycan samples using Gly-X sample prep, use Agilent AdvanceBio Glycan Mapping columns for HILIC separation of labeled N-glycans. These amide-based columns have a unique hydrophilic bonding and are available in two configurations:

- 1.8  $\mu\text{m}$  fully porous for speed and performance (1200 bar)
- 2.7  $\mu\text{m}$  superficially porous for resolution at lower pressures (600 bar)

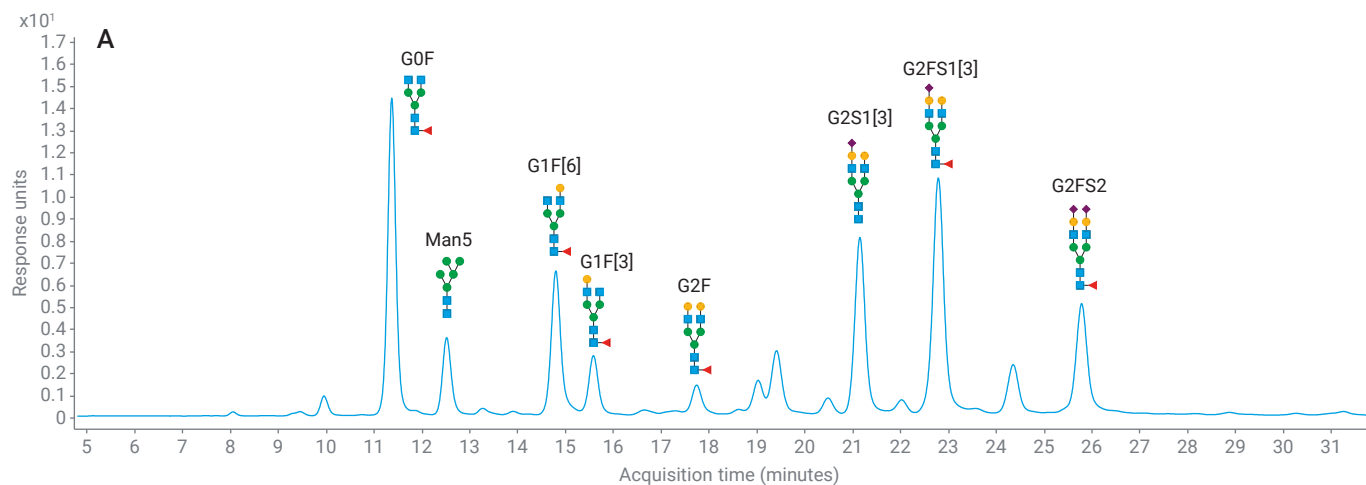
HILIC methods for N-glycan separation using the AdvanceBio Glycan Mapping columns are within the literature in Tables 1 and 2, and separation of InstantPC labeled N-glycans from Enbrel on a 2.1  $\times$  150 mm, 1.8  $\mu\text{m}$  column (p/n 859700-913) using a 60-minute method is shown in Figure 9.



AdvanceBio Glycan Mapping columns deliver consistent, exceptional performance for separating and characterizing peptides, proteins, antibodies, conjugates, new biological entities, and biopharmaceuticals. Many column lengths are available.

## AdvanceBio Glycan Mapping HILIC columns help you overcome difficult glycan analysis challenges

Enbrel, InstantPC FLD



Enbrel, InstantPC TIC

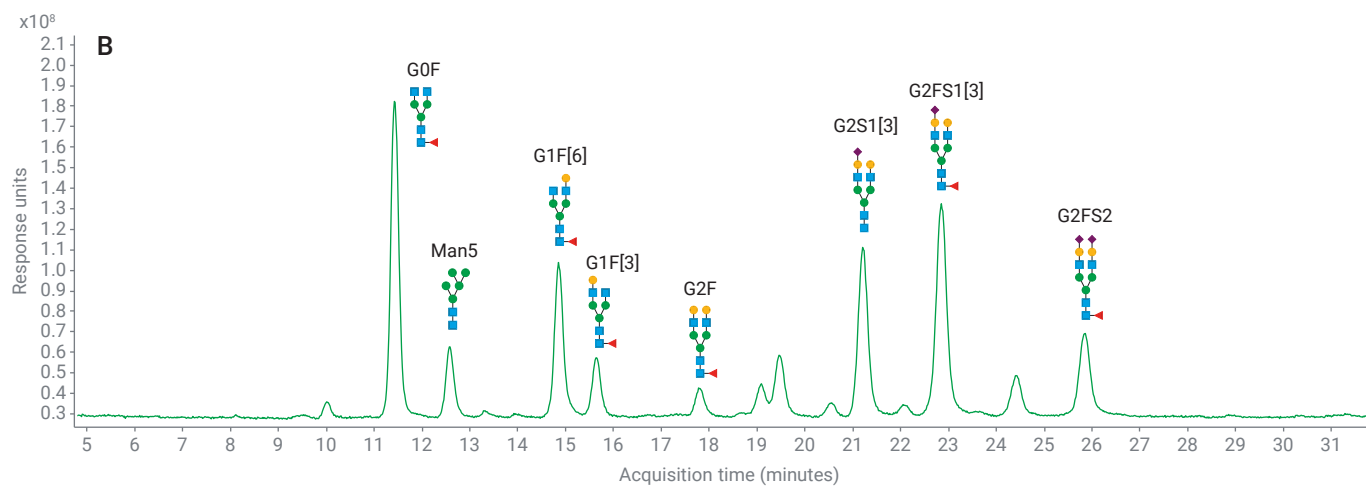


Figure 9. Separation of InstantPC N-glycans from Enbrel (etanercept) on a  $2.1 \times 150$  mm,  $1.8 \mu\text{m}$  column (p/n 859700-913) using a 60-minute method. A) Fluorescence detection, B) MS detection.

To learn more about AdvanceBio Glycan Mapping columns, consult these resources or visit our [Glycan Mapping column webpage](#).

Publication	Document	Title
<a href="#">5994-1469EN</a>	Application note	Separation of a Critical Pair of N-Glycans Using a Quality by Design (QbD) Approach
<a href="#">5994-0372EN</a>	Application note	Glycopeptide Characterization for Various Monoclonal Antibodies Using the Agilent 6545XT AdvanceBio LC/Q-TOF
<a href="#">5994-8796EN</a>	Application note	Profiling Glycosylation of Monoclonal Antibodies at Three Levels Using the Agilent 6545XT AdvanceBio LC/Q-TOF
<a href="#">5994-8550EN</a>	Application note	A Comprehensive Approach for Monoclonal Antibody N-linked Glycan Analysis from Sample Preparation to Data Analysis



## Analytical Services to Support Your N-Glycan Analysis

Insights from the experts can help ensure the success of your drug development program. Agilent offers services for released glycan analysis with reporting options including:

- LC/FLD for relative percent area
- LC/MS for glycan peak ID
- Using standards and exoglycosidases to confirm glycan peak assignment
- Generating glycovariants using exoglycosidases and glycosyltransferases to suit the needs of your study

If you have limited instrumentation or personnel, or are under tight time constraints, no problem. We can partner with you to develop and implement a glycan analysis method with a quick data turnaround time. What's more, we perform many of our analytical services with off-the-shelf products, allowing for easier method transfer when you choose to bring the analysis into your lab.



## References

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## Agilent CrossLab services

CrossLab is an Agilent capability that integrates services and consumables to support workflow success and important outcomes like improved productivity and operational efficiency. Through CrossLab, Agilent strives to provide insight in every interaction to help you achieve your goals. CrossLab offers method optimization, flexible service plans, and training for all skill levels. We have many other products and services to help you manage your instruments and your lab for best performance.



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