

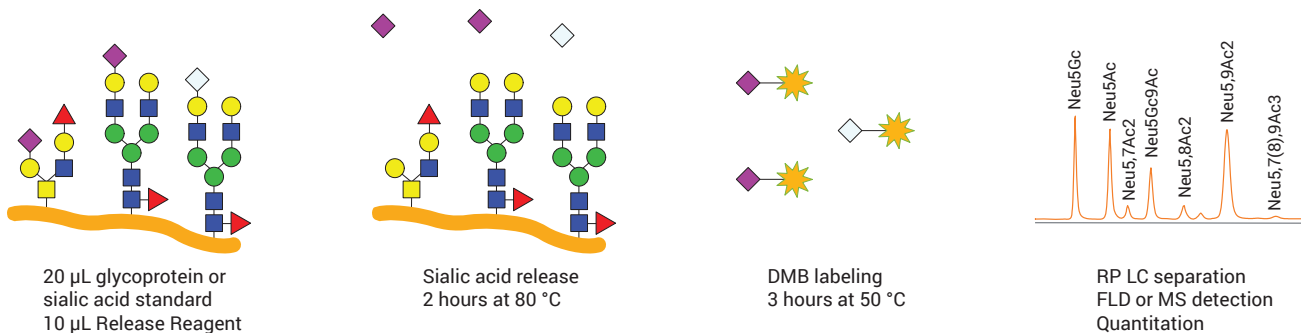
# Sialic Acid Analysis of Biotherapeutic Glycoproteins Using AdvanceBio Sialic Acid Profiling and Quantitation Kit and LC/FLD/MS



## Sialic acid analysis simplified and standardized

The composition of glycans present on biotherapeutic glycoproteins can affect immunogenicity, pharmacokinetics, and pharmacodynamics.<sup>1</sup> Glycans are carbohydrates composed of monosaccharides arranged into many different possible oligosaccharide structures based on composition and linkage position. Depending on the molecule and the application, terminal sialic acid may reduce the rate of clearance, reduce antibody-dependent cellular cytotoxicity (ADCC) activity, or can be anti-inflammatory. Two forms commonly found in biotherapeutics are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Neu5Ac is usually the predominant species, while Neu5Gc is not synthesized by humans and its presence on biotherapeutics can be immunogenic. Therefore, it is essential to monitor the absolute quantity of sialic acid, as well as the levels of different sialic acid species present in therapeutic glycoproteins.

The Agilent AdvanceBio Sialic Acid Profiling and Quantitation kit (part number GS24-SAP) offers improved sensitivity for proteins with low levels of sialylation such as monoclonal antibodies with a single N-glycosylation site in the Fc region. The kit includes all the reagents necessary to prepare fluorescently labeled sialic acid samples in about 5 hours. Paired with the Agilent InfinityLab Poroshell 120 EC-C18 column, both profiling and quantitation of sialic acids can be accomplished quickly and easily by LC/FLD or LC/MS.



**Figure 1.** Sialic acid release and DMB labeling workflow followed by separation and detection using FLD or MS.

## End-to-end sialic acid analysis solution designed and manufactured by Agilent

In this guide, you'll find the consumables you need to get started with sialic acid analysis. Many of these consumables were used to assess the sialic acids present in rituximab (Rituxan, a monoclonal antibody or mAb), etanercept (Enbrel, a Fc fusion protein), cetuximab (Erbix, a monoclonal antibody), and NISTmAb.<sup>2</sup> The streamlined workflow provides a method to determine both absolute and relative quantities of Neu5Ac and Neu5Gc, covering both profiling and quantitative analysis by LC/FLD and LC/MS.

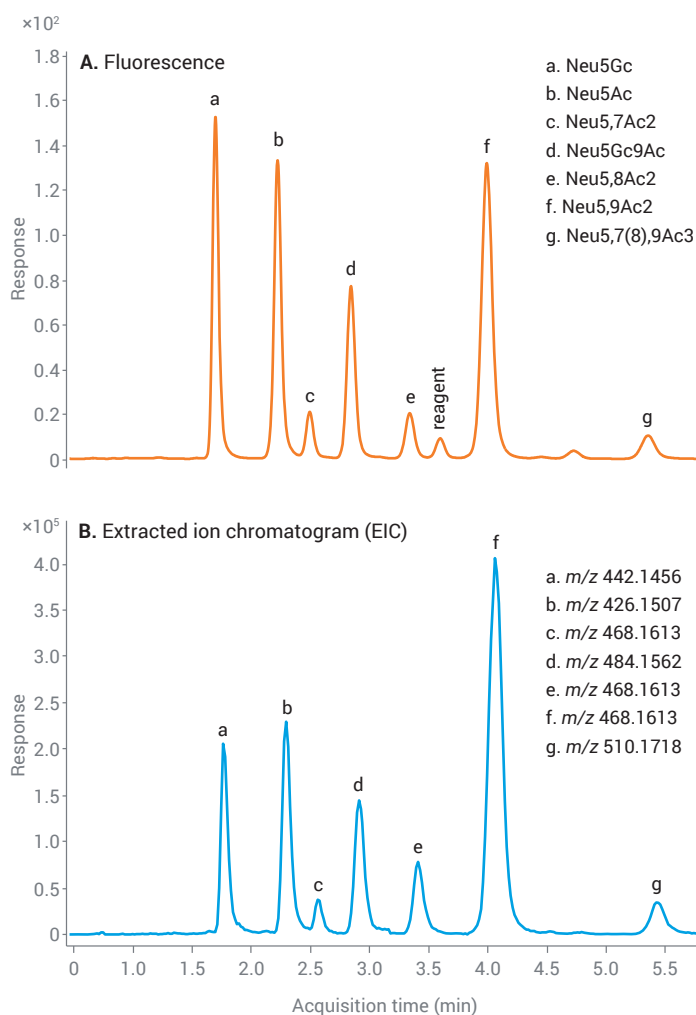
This AdvanceBio sialic acid analysis workflow guide provides ordering information for:

- The AdvanceBio Sialic Acid Profiling and Quantitation sample preparation kit. This kit includes the necessary reagents to release and label sialic acids, as well as a qualitative sialic acid reference panel and quantitative NANA and NGNA standards.
- Liquid chromatography columns for separation of glycans by reversed phase liquid chromatography (RPLC).
- Solvents and reagents.
- Vials and caps.

### Boost your sialic acid analysis productivity with:

- Samples ready for LC/FLD or LC/MS analysis in about 5 hours.
- An automatable 96-well plate format.
- No glass ampoules, all screw-cap containers.
- No sample drying required before sample input.
- Sialic acids separated by RPLC in only 10 minutes using an Agilent InfinityLab Poroshell 120 column EC-C18 column.
- A well-known DMB (1,2-diamino-4,5-methylenedioxybenzene) dye.

Examples of LC/FLD and LC/MS (EIC) data for sialic acids present in the included sialic acid reference panel. For further information including LC and MS parameters and example analyses of sialic acids in biotherapeutics see Reference 2.



	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.75	0.01	1.55
G0	1.47	0.02	1.18
G0F	46.82	0.07	0.15
Man5	1.21	0.01	0.83
G1[6]	0.75	0.02	2.67
G1F[6]	31.21	0.11	0.35
G1F[3]	9.27	0.05	0.54
G2F	7.04	0.04	0.51
G2FS1[6]	0.67	0.02	2.29
G2FS1[3]	0.37	0.06	15.98
G2FS2	0.45	0.03	6.67

**Figure 2:** UHPLC chromatogram of DMB-labeled SARP. (A) Fluorescence (B) extracted ion chromatogram of DMB-labeled sialic acid species  $[M+H]^+$ .

**Table 1:** Agilent 1290 Infinity II UHPLC HILIC/FLD conditions.

Parameter	Value				
Instrument	Agilent 1290 Infinity II LC System				
Column	Agilent InfinityLab PoroShell 120EC-318, 2.1 x 7.5 mm, 2.7 µm (p/n 697775-902)				
Column Temperature	30 °C				
Mobile Phase	A) Methanol:acetonitrile:water (4:8:88) B) acetonitrile				
Gradient Program	Time (min)	%A	%B	Flow rate (mL/min)	
	0.00	100	0	0.4	Isocratic
	6.00	100	0	0.4	Elution
	6.25	20	80	0.4	
	7.30	20	80	0.4	Wash
	7.50	100	0	0.4	Re-equilibrium
10.00	100	0	0.4		
Injection Volume	10 µL				
Detection	Agilent 1250 Infinity II FLD λ <sub>EX</sub> 373 nm λ <sub>EM</sub> 448 nm				

**Table 2:** Agilent 6545XT AdvanceBio LC/Q-TOF parameters.

6545XT AdvanceBio LC/Q-TOF	
Source	Dual AJS ESI
Gas Temperature	350 °C
Drying Gas Flow	11 L/min
Nebulizer	15 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Vcap	1,400 V
Nozzle Voltage	1,800 V
Fragmentor	120 V
Skimmer	65 V
Mass Range (MS)	<i>m/z</i> 400 to 1,000
Mass Range (MS/MS)	<i>m/z</i> 100 to 500
Acquisition Mode	High resolution (4 GHz)

## Getting started with the AdvanceBio Sialic Acid Profiling and Quantitation kit<sup>3</sup>

### Sialic acid sample considerations

- Samples that the kit measures include glycoproteins, glycopeptides, glycolipids, polysialic acids, serum, plasma, tissue, or whole cells.
- The dynamic range of this assay is 1 to 2,000 pmol sialic acid per well. The sample concentration may need to be adjusted to assure that the signal falls within the range.
- Samples can be concentrated by drying and reconstituting in a smaller volume of DI water before use. Sample can be dried directly in the analysis wells or prepared in a separate tube.

### Incubation hardware

During the sample preparation using the AdvanceBio Sialic Acid Profiling and Quantitation kit (GS24-SAP), the samples are heated to 80 °C during the acid release step and labeled with DMB at 50 °C. To heat the samples in the 96-well plate provided, we recommend using a thermal cycler or two independent dry block heaters. Suggestions are provided below.

Incubation Hardware (non-Agilent)	Part No.
96-well Thermal Cycler capable of 50 °C and 80 °C setpoints	Various
Dry Block Heater, 4 Block (Qty 2) (Troemner)	HB4DG
Modular Heating Blocks (Qty 2) (VWR)	13259-260

### Data analysis and reporting

The data was analyzed with Agilent OpenLab CDS 2.3 and MassHunter Qualitative Analysis 10.0 software. Neu5Gc and Neu5Ac were quantified using the calibration curves.

**Note:** Agilent now offers MassHunter 11 with OpenLab ECM XT data analysis software that is 21 CFR Part 11 compliant.

## References

1. Antibody Glycosylation and its impact on the Pharmacokinetics and Pharmacodynamics of Monoclonal Antibodies and Fc-Fusion Proteins. *J. Pharm. Sci.* 2015, 104(6), 1866-1884.
2. An Improved Workflow for Profiling and Quantitation of Sialic Acids in Biotherapeutics [5994-2352EN](#), 2020.
3. Agilent AdvanceBio Sialic Acid Profiling and Quantitation Kit, User Manual, [5994-2800EN](#).

## Easy selection and ordering information

To order items listed in the tables below from the Agilent online store, add items to your Favorite Products list by clicking on the MyList link in the header. Then, enter the quantities for the products you need, Add to Cart, and proceed to checkout. Your list will remain under Favorite Products for your use with future orders.

If this is your first time using Favorite Products, you will be asked to enter your email address for account verification. If you have an existing Agilent account, you will be able to log in. However, if you don't have a registered Agilent account, you will need to register for one. This feature is valid only in regions that are e-commerce enabled. All items can also be ordered online by clicking on the individual part numbers or through your regular sales and distributor channels.

## MyList of AdvanceBio Sialic Acid Profiling and Quantitation consumables

Description	Part No.
<b>Sample Preparation</b>	
AdvanceBio Sialic Acid Profiling and Quantitation kit, 24 ct	GS24-SAP
<b>Columns</b>	
InfinityLab Poroshell 120 EC-C18, 2.1 x 75 mm, 2.7 µm, narrow bore	697775-902
InfinityLab Poroshell 120 EC-C18, 2.1 x 5 mm, 2.7 µm, guard column, 3/pk	821725-911
<b>Standards</b>	
Bovine Fetuin, heat-treated (0.4 mg)†*	WS0021
Agilent-NISTmAb, 1 x 25 µL	5191-5744
Agilent-NISTmAb, 4 x 25 µL	5191-5745
<b>Vials, Caps, and Reagents</b>	
Vial, screw style, 2 mL, polypropylene, certified for use in PFAS-related applications, 100/pk†	5191-8150
Vial insert, 300 µL, polypropylene, polymer feet, 100/pk†	5182-0549
9 mm, screw style clear polypropylene cap, 100/pk†	5191-8151
InfinityLab ultrapure LC/MS acetonitrile (1 L)	5191-4496
InfinityLab ultrapure LC/MS water (1 L)	5191-4498
InfinityLab ultrapure LC/MS methanol (1 L)	5190-6896

## Additional supplies

Description	Part No.
<b>MyList of Optional Items for microplate sealer†*</b>	
Peelable aluminum plate seal	24210-001
Filter microplate, 96-well, polypropylene with 0.45 µm polyvinylidene fluoride membrane, 300 L/well, 50/pk	200981-100
<b>MyList of Column Fittings and Connectors</b>	
Agilent InfinityLab Quick Connect Fitting (for connection on column inlet)	5067-5965
Agilent InfinityLab Quick Connect Capillary MP35N 0.12 x 105 mm (for Quick Connect fitting)	5500-1578
Agilent InfinityLab Quick Turn Fitting (for connection on column outlet)	5067-5966
Quick Turn Capillary MP35N 0.12 x 280 mm (for Quick Turn fitting)	5500-1596
Mounting tool for quick turn fittings	5043-0915
<b>MyList of Solvent Filtration Supplies**</b>	
InfinityLab Solvent filtration assembly	5191-6776
InfinityLab solvent filtration flask, glass, 2 L	5191-6781
Filter membrane, Nylon 47 mm, pore size 0.2 µm, 100/pk	5191-4341
Filter membrane, Regenerated Cellulose 47 mm, pore size 0.2 µm, 100/pk	5191-4340
Solvent bottle glass filter, solvent inlet, 20 µm	5041-2168
<b>MyList of Solvent Handling Supplies</b>	
InfinityLab Stay Safe cap starter kit	5043-1222
InfinityLab solvent bottle, clear, 1 L	9301-6524
InfinityLab solvent bottle, amber, 1 L	9301-6526
Solvent bottle, clear, 2 L	9301-6342
Solvent bottle, amber, 2 L	9301-6341
InfinityLab Stay Safe Purging Bottle	5043-1339
InfinityLab waste can, GL45, 6 L with Stay Safe cap (Charcoal filter 5043-1193 not included)	5043-1221
InfinityLab charcoal filter with time strip, 58 g (use with 5043-1221)	5043-1193

† Not available for online sale. Contact your Agilent Representative for ordering information.

\* Click [here](#) for more information about the Agilent PlateLoc Thermal Microplate sealer.

\*\* If using solvents other than those listed in this table, use the InfinityLab Solvent Filtration assembly prior to analysis.

† Agilent recommends using a 250 µL vial insert with the 2 mL polypropylene vial to minimize dead volume.

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