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Agilent AdvanceBio Sialic Acid Profiling and Quantitation Kit

User Manual

Notices

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Contents

Introduction

Glycans are carbohydrates composed of monosaccharides arranged into many different possible oligosaccharide structures based on composition and linkage position. Sialic acid capping at the nonreducing terminal of N- or O-glycans can serve a key role biological processes and in mediating the effectiveness of therapeutic glycoproteins.[1] The composition of glycans present on biotherapeutic glycoproteins can affect immunogenicity, pharmacokinetics, and pharmacodynamics.[1] 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.[3-5] Two sialic acid species commonly found in biotherapeutics are N-acetylneuraminic acid (NANA or Neu5Ac) and N-glycolylneuraminic acid (NGNA or Neu5Gc). Neu5Ac is usually the predominant species, while Neu5Gc is not synthesized by humans and its presence on biotherapeutics can potentially be immunogenic. Because of this, it is essential to monitor not only the absolute quantity of sialic acid, but also the levels of different sialic acid species present in therapeutic glycoproteins.

The AdvanceBio Sialic Acid profiling and quantitation kit represents a sensitive, high-throughput approach to sialic acid quantitation. Sialic acids are released from glycoproteins using acid hydrolysis, followed by derivatization with the fluorophore 1,2-diamino 4,5-methylenedioxybenzene (DMB), allowing for separation by reversed-phase (RP) liquid chromatography with fluorescence detection (FLD).

The workflow offers both qualitative characterization of sialic acid species using a sialic acid reference panel (SARP), as well as quantitation with picomolar level sensitivity using included NANA and NGNA quantitative standards. The workflow enables reliable and reproducible high-throughput profiling and quantitation of sialic acids, providing a broad detection range and improved sensitivity for molecules with low levels of sialylation versus traditional DMB labeling workflows such as our GKK-407 kit.

Sialic acid species comparison may be made by labeling the included qualitative sialic acid reference panel (SARP, p/n GKRP-2503), which includes the following sialic acid species: Neu5Gc (NGNA); Neu5Ac (NANA); Neu5,7Ac2; Neu5Gc,9Ac; Neu5,9Ac2; Neu5,7(8)9Ac3.

Use of the AdvanceBio Sialic Acid profiling and quantitation kit offers several advantages:

- 96-well format
- Broad range of detection of sialic acid levels, from 1 to 2,000 pmol
- Picomolar sensitivity for proteins with low levels of sialylation
- Quantitative NANA and NGNA standards are included
- Workflow may be completed in five hours including incubation periods
- Automation-friendly: please contact us for assistance, we are happy to provide guidance on how the kit can be implemented on your automation platform of choice.

Kit Workflow

Step 1: Release of sialic acid using acid hydrolysis (2 hours)

→Sialylated Glycoprotein + acid → Sialic Acid + Glycoprotein

Step 2: Labeling of released sialic acid (3 hours)

Samples are now ready for separation by reversed-phase liquid chromatography.

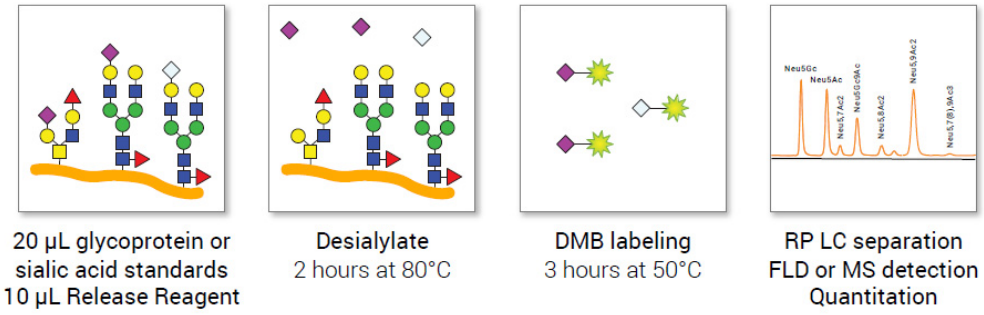

Figure 1. Sialic acid release and DMB labeling workflow.

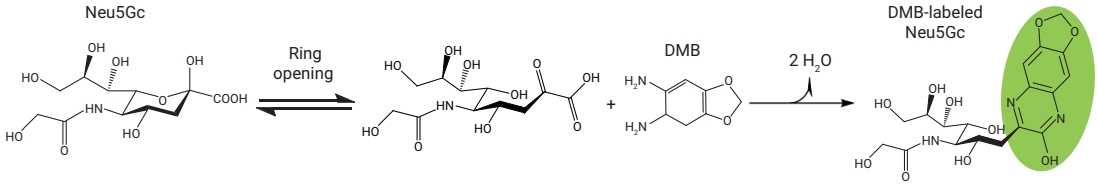

Figure 2. DMB labeling mechanism of sialic acid Neu5Gc.

Kit Components

Table 1 shows the Sialic Acid profiling and quantitation kit components.

Table 1 Kit Components

Module	Component	Units	Storage
AdvanceBio Sialic Acid profiling and quantitation kit GS24-SAP	100 µM N-acetylneuraminic acid (NANA, NeuAc) Sialic Acid Standard, 200 µL	1	–20 °C
	100 µM N-glycolylneuraminic acid (NGNA, NeuGc) Sialic Acid Standard, 200 µL	1	–20 °C
	Sialic Acid Reference Panel, lyophilized	1	–20 °C
	Vial A: Labeling diluent, 300 µL	1	–20 °C
	Vial B: Reductant	2	–20 °C
	Vial C: DMB dye	2	–20 °C
	Vial D: Release reagent, 300 µL	1	–20 °C
	Cap strips	6	–20 °C to RT
	96-well reaction plate	1	–20 °C to RT

Equipment and Reagents Provided By User

- Thermocycler (preferred), or heater with PCR block insert with lid capable of 50 °C and 80 °C incubation and standard heavy-duty aluminum foil such as Reynolds Wrap Heavy Duty or equivalent.
- HPLC or UHPLC with fluorescence detection (373 nm excitation, 448 nm emission)
- Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 75 mm column, p/n 697775-902 or equivalent (for further information see **"Sample Analysis by UHPLC or HPLC"** on page 14.

Sample Considerations

- Samples that can be measured by the kit 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. Sample concentration may need to be adjusted to assure 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.

If you have questions on the kit protocol, please contact Agilent at:
www.agilent.com/en/contact-us/page or **advancebio.glycan@agilent.com**

Kit Capacity

The kit contains sufficient reagents for 24 data points (two sets of 12 reactions).

For routine use, NGNA and NANA standards can be combined (1:1) and used neat to form a single 1,000 pmol standard mixture. Alternatively, standards can be used individually as 2,000 pmol standards. Dilutions with DI water can be prepared to generate a standard curve. When using a single point standard or NGNA/NANA standard mix, the recommendation is triplicates with duplicate blank and optional Sialic Acid Reference Panel.

Table 2 Kit Capacity for a 24-well run using a single level NGNA/NANA standard mixture and triplicate or duplicate sample analysis.

Kit	Total Data Points	Standard NGNA/NANA mix (triplicate analysis	Sialic Acid Reference Panel	Blank (Duplicate Analysis)	Data Points Remaining for Samples	No. Samples (Triplicate Analyses)	No. Samples (Duplicate Analyses)
GS24-SAP	24	3	1	2	18	6	9

Protocol

Getting started

1 Prepare heater:

   a For thermocycler, program a heating cycle to heat to 80 °C for two hours and then to cool and hold at 20 °C. Thermocycler lid should be set between 80 °C and 100 °C.

   b For heat block, set heater containing heat block to 80 °C. Heavy-duty aluminum foil will be required to insulate samples during incubation. If using a heat block, cover capped tube strips with foil entirely, press down foil around the capped tubes and heat block to tightly seal the tubes, and enclose heat block with a lid.

   c When using a dry block heater, it is important to control both well-to-well temperature variation and reagent evaporation/condensation in the well caps. Depending on the block design, interior wells of the heat block may give more consistent temperatures.

2 Remove the kit from –20 °C storage and allow to equilibrate to room temperature. Have items on hand:

- 96-well break-away plate

NOTE **Reactions can be carried out in 0.2 mL flip top PCR tubes if desired.**

- Cap strips
- Appropriate pipettors and pipette tips
- DI water

Performing the assay

Sialic acid release

1 Add 20 µL of sample glycoprotein per well to the 96-well break-away plate.

NOTE **Sample dilution may be required prior to assay. For monoclonal antibodies with low levels of sialylation, up to 20 mg/mL may be used. For highly sialylated proteins, a concentration of 0.25 mg/mL may be appropriate. Target range for sialic acid should be 10 to 2,000 pmol per well.**

WARNING **Samples should be handled in a fume hood.**

WARNING **Wear appropriate PPE when handling the Release Reagent, Vial D, which is corrosive.**

2 Allow Release Reagent (Vial D) to equilibrate to room temperature. Following equilibration, vortex Vial D before use.

3 Add 10 µL of Release Reagent (Vial D) each well containing glycoprotein. Mix by pipette, or by vortex (if vortexing, spin down/tap to collect samples at the bottom of the well before next step).

4 Tightly seal with cap strip and incubate at 80 °C for two hours in a thermocycler followed by cooling and hold step at 20 °C. If using a heat block, tightly cover capped tube strips with foil to reduce condensation and enclose heat block with lid and incubate for two hours at 80 °C.

5 If using a heat block, remove from heat after incubation and allow to cool down to room temperature for at least two minutes. Spin down/tap to collect samples at the bottom of the well before uncapping.

Standards and controls

NOTE

Duplicate or triplicate wells for each quantitative standard are recommended. NGNA and NANA standard can be combined in a single well. A standard curve can be performed using dilution of the standards with DI water. Sialic Acid Reference Panel can be run in singlicate.

1 The NGNA and NANA standards are supplied at a concentration of 100 µM. To create a 2,000 pmol/well standard, use 20 µL of each standard directly without dilution.

2 To create a combined 1,000 pmol/well NGNA + 1,000 pmol/well NANA standard add 10 µL of each standard to a single well.

3 A standard curve can be prepared from either the individual or combined standards by diluting with DI water. For example, prepare a 4x dilution of the 2,000 pmol/well standard to make a 500 pmol/well standard by diluting 20 µL of standard with 60 µL of DI water and mixing by pipette. Repeat the dilution with 20 µL of the 500 pmol/well standard plus 60 µL of DI water to make a 125 pmol/well standard. Prepare additional dilutions as desired.

4 Prepare Sialic Acid Reference Panel (SARP) by diluting lyophilized material with 50 µL of DI water. Use 20 µL of this solution for each assay and freeze remainder for future use.

5 Add 20 µL of standards, SARP, and blanks to empty wells on the cooled PCR strips containing glycoproteins following Sialic Acid Release reaction.

6 Add 10 µL of release reagent (vial D) to all wells containing standards, SARP, and blanks to bring to a total volume of 30 µL and mix using pipette tip.

Prepare DMB labeling mixture

NOTE

Labeling mixture should be used within three hours of preparation.

1 Transfer 140 µL of Labeling diluent (Vial A) to Reductant (Vial B) and mix thoroughly by pipette.

2 Using the same pipette tip, transfer the entire contents of the reconstituted Reductant (Vial B) to the DMB Dye (Vial C) and mix. The labeling mix is now ready for use and is stable for three hours at room temperature.

DMB labeling reaction

1 Using fresh tips, add 10 µL of DMB Labeling mixture (steps **3** and **4**) to each well containing samples, standards, controls, and blanks. Mix with pipette tip after each addition.

2 Tightly seal with cap strip and incubate at 50 °C for three hours in a thermocycler. If using a heat block, tightly cover capped tube strips with foil to reduce condensation and enclose heat block with a lid.

3 Following incubation, allow samples to cool for at least five minutes. Spin down/tap to collect samples at the bottom of the well before uncapping.

WARNING

Samples should be handled in a fume hood.

4 Uncap and bring the final volume of samples to 200 µL (40 µL sample + 160 µL of DI water) and mix well with the same tips.

5 Samples are now ready for analysis by HPLC or UHPLC. Analyze samples immediately or store in the dark at 4 °C for up to three days.

NOTE

Some glycoproteins or their formulations may form precipitates during this reaction. Samples may be filtered using optional Agilent PVDF membrane filter plate (part number 200981-100) or equivalent (order separately).

Sample Analysis by UHPLC or HPLC

The preferred method of analysis for DMB-labeled sialic acids is reversed-phase liquid chromatography with isocratic elution, coupled to fluorescence detection.

Recommended column: Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 75 mm, 2.7 µm (part number 697775-902).

Mobile Phase A: Methanol:Acetonitrile:Water (4:8:88)

Mobile Phase B: Acetonitrile

NOTE **We recommend that mobile phase A is prepared as follows. In a 1 L graduated cylinder, add ~500 mL of water and a stir bar. Add 40 mL of methanol and 80 mL of acetonitrile while stirring. Bring the final volume to 1 L with water.**

A suggested method is shown in **Table 3**. Pressure should be less than 400 bar using this method.

Needle Wash: 50% Acetonitrile in water

Table 3 Suggested LC method for separation of DMB labeled sialic acid samples.

Parameter	Value
Instrument	Agilent 1290 Infinity II LC System
Column	Agilent InfinityLab PoroShell 120 EC-C18, 2.1 × 75 mm, 2.7 µm (part number 697775-902)
Column Temp	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-equilibration 10.00 100 0 0.4
Injection Volume	10 µL (1 to 20 µL acceptable)
Detection	Agilent 1260 Infinity II FLD λ_{Ex} 373 nm, λ_{Em} 448 nm

DMB may also be detected using UV at 370 nm.**7** Increase injection volume to 20 µL for increased sensitivity.

Suggested MS conditions for DMB Labeled Sialic Acids

Agilent Jet Stream ESI source, any MS positive mode, sheath gas 400 °C at 12 L/min, dry gas 350 °C at 11 L/min, nebulizer pressure 15 psig, Vcap 1400 V, Nozzle 1800 V, Fragmentor 120 V, *m/z* range 400 to 1,000.

Table 4 6545XT Q-TOF parameters for analysis of DMB labeled sialic acids.

6545XT 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	*m/z* 400 to 1,000
Scan Rate	1 spectra/sec
Acquisition Mode	High resolution (4 GHz)

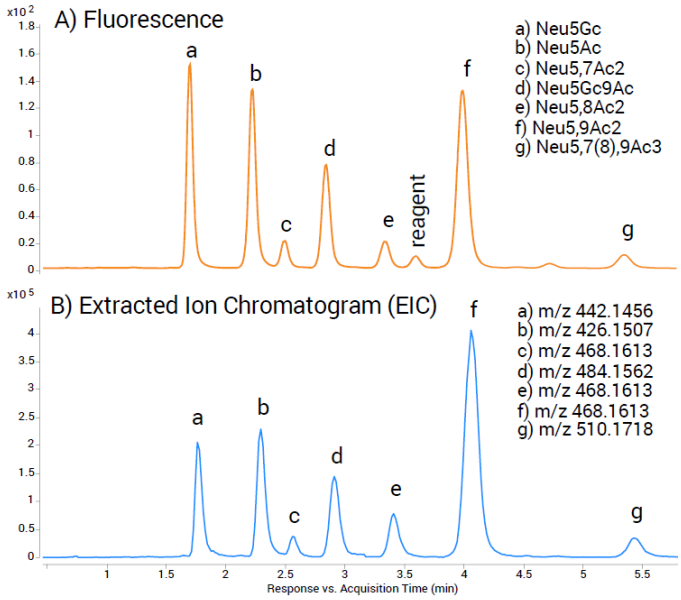

Figure 3. Example separation of sialic acid reference panel (SARP) using the AdvanceBio PoroShell EX-120 C18 column, 2.1 × 75 mm, 2.7 µm, using the conditions listed in **Table 3**.
Detection is by A) fluorescence using parameters in **Table 3** and B) MS using parameters in **Table 4**.

FAQs

Q. How much glycoprotein sample do I need to use with the kit?

A. This will depend on the sialylation level of your glycoprotein, which in turn will depend on the number of N- and O-linked glycosylation sites and the relative amount of sialic acid capping at the nonreducing terminal of the glycans. Samples such as monoclonal IgGs generally have a low level of sialylation, while Fc fusion proteins and fetuin have a much higher level.

Table 5 shows examples for starting amounts of glycoproteins for use with the kit for fluorescence detection to allow signal to be within the range of the standard curve. More protein should be used if using absorbance detection. The optimal amount of starting glycoprotein should be determined by the user, depending on the level of sialylation and the method of detection used.

Table 5 Examples of starting concentrations and amounts of glycoprotein used with GS24-SAP (fluorescence detection).

Glycoprotein	Concentration (mg/ml)	Sample Volume (µL)	Sample Mass (µg)	Mol wt (kDa)	pmol Protein
Fetuin	0.25	20	5	48	104
MabThera	10	20	200	145	1,379
Enbrel	0.25	20	5	150	33
Cetuximab	2	20	40	115	348
NISTmAb	10	20	200	150	1,333
EPO alfa	1	20	20	30.4	658

Q. What is the source of the Neu5Ac and Neu5Gc Sialic Acid Standard used in the kit?

A. The standards are quantitatively prepared from N-Acetylneuraminic acid and N-glycolylneuraminic acid, United States Pharmacopeia (USP) Reference Standard using calibrated, NIST-traceable lab equipment.

Q. Which species of sialic acid should I expect to see in NISTmAb?

A. NISTmAb contains only Neu5Gc (NGNA) and does not contain Neu5Ac (NANA) in any detectable quantity. This is described in application note **5994-2352EN**. While some studies on the NISTmAb**8** have reported the presence of Neu5Ac, the absence of Neu5Ac was confirmed through in-depth analysis by full MS, CID MS2, and SID.**9**

NOTE

NISTmAb material is available from Agilent in convenient 25 µL aliquots (part number 5191-5744) and in a 4 × 25 µL pack (part number 5191-5745).

Q. Can I use a 96-well plate instead of the 8-tube strips included in this kit?

A. Yes, the kit is compatible with other polypropylene 96-well PCR plates with a working volume of 200 µL (300 µL total volume) and matching caps (i.e. 96-well semi-skirted PCR plate part number 4ti-0770 and cap strips part number 4ti-0755 from Brooks Life Sciences)

Q. Is there a path to automation for this kit?

A. Yes, the kit is automation friendly. For best results a thermocycler is recommended for incubations. Plate sealing can be accomplished either through use of an automated plate sealer (Agilent PlateLoc Thermal Microplate Sealer or equivalent) or using an auto sealing lid (BioRad part number MSL2022 or equivalent) For further assistance, please contact us.

Q. What if I see a change in the baseline during LC separation, or a change in retention times of sialic acid species?

A. We recommend you remake the solvent **page 12**, as changes can result due to evaporation. The organics (acetonitrile and methanol) evaporate as the mobile phase sits on the instrument over a couple of days.

Q. What if I see unexpectedly high signal?

A. The protein concentration may be too high. This is dependent on the level of sialylation of the glycoprotein. We recommend diluting the sample to assess what dilution should be used to fall within range of the assay.

Alternatively, the sample may have endogenous sialic acid and/or α-keto acids that contribute to higher readings. Include a negative control (all reactants except the Vial D Release reagent); subtract the measured value from the sample.

Q. What if I see unexpectedly low signal?

A. The protein concentration may be too low. This is dependent on the level of sialylation of the glycoprotein. The sample may not be sialylated, or the level of sialylation is below the sensitivity of the assay. We recommend drying down the samples and resuspending in a lower volume before starting the assay or concentrating by another method such as molecular weight cutoff filter.

Alternatively, the sample may have lost sialic acid before analysis. Avoid prolonged exposure of sialylated glycoproteins in aqueous solutions to low pH and/or elevated temperature. In general, glycans in solution should be kept in the 5.0 to 8.5 pH range at temperatures below 30 °C.

Q. The pressure when using a C18 column for separation is increasing over time, and retention times are changing. What do I do to address this?

A. This may happen if you are using proteins at high concentration. We suggest filtering the DMB-labeled sialic acids before separation using the Agilent PVDF membrane filter plate (part number 200981-100) or equivalent. Use of a PVDF membrane for filtration been shown not to interfere with this assay since the labeled sialic acids do not interact with this material.

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Technical Assistance

Agilent is committed to developing rapid, automatable methods for glycan analysis. We value customer opinions, and encourage you to contact us with your suggestions about product performance or new applications and techniques. You can also call us to discuss products in development.

If you have questions or comments, please contact us at **www.agilent.com/en/contact-us/page** or **advancebio.glycan@agilent.com**.

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