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Agilent AdvanceBio Total Sialic Acid Quantitation Kit
(formerly ProZyme)

User Manual

Notices

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Introduction

Sialic acid serves a critical role in mediating the effectiveness of recombinant therapeutic glycoproteins. The presence (or absence) of sialic acid on the non-reducing terminal of N- or O-glycans can dramatically affect the pharmacokinetics of the protein, as well as its immunogenicity. It is therefore essential that sialic acid on protein therapeutics be maintained and controlled at the highest possible level.

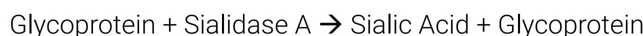
Agilent AdvanceBio Total Sialic Acid Quantitation Kit (formerly ProZyme) represents a sensitive, high-throughput approach to sialic acid quantitation, based on a coupled enzyme reaction, converting enzymatically-released sialic acid to hydrogen peroxide, which reacts with a dye stoichiometrically, generating intense fluorescence or absorbance signal. This approach allows enzymatic release of sialic acid, conversion, detection and quantitation to be performed in a single well for fast and simple processing.

Use of the AdvanceBio Total Sialic Acid Quantitation Kit offers several advantages:

- Rapid quantitation of total sialic acid released from intact proteins by Sialidase A
- Broad range of detection of sialic acid levels, from 40 to 1,000 pmol of sialic acid per sample using fluorescence detection or 500 to 4,000 pmol of sialic acid for absorbance detection
- Minimal (if any) degradation of sialic acid due to enzymatic release
- Automation-friendly: please contact Agilent for assistance, we are happy to provide guidance on how the kit can be implemented on your automation platform of choice.

Kit chemistry

Step 1: Release of Sialic Acid (30 minutes)


Glycoprotein + Sialidase A → Sialic Acid + Glycoprotein

Step 2: Detection of Released Sialic Acid (60 minutes)

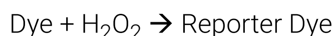
N-Acetylneuraminic aldolase catalyzes the reversible reaction:

Sialic Acid ⇔ Mannosamine + Pyruvic Acid

Then pyruvate oxidase catalyzes the reaction:

Pyruvic Acid → Acetylphosphate + H_2O_2

Finally, H_2O_2 forms a 1:1 complex with the Dye to form a fluorescent Reporter Dye that may be read by fluorescence or absorbance detection.


Dye + H_2O_2 → Reporter Dye

Kit Components

The Total Sialic Acid Kit (GS48-SAQ and GS96-SAQ) contain the following reagents and standards.

Table 1 Kit Components

Module	Component	Units GS48-SAQ	Units GS96-SAQ	Storage
Total Sialic Acid Kit GS48-SAQ and GS96-SAQ	100 μM N-acetylneuraminic acid (NANA, NeuAc) Sialic Acid Standard, 1 mL	1	2	-20 °C
	Bovine Fetuin Control, 0.4 mg, lyophilized	1	2	-20 °C
	SAQ Dye, lyophilized	1	2	-20 °C
	Horseradish Peroxidase, lyophilized	1	2	-20 °C
	DMSO	1	2	-20 °C - 4 °C
	Conversion Reagent, lyophilized	1	2	-20 °C
	SAQ Buffer A	1	2	-20 °C - 4 °C
	SAQ Buffer B	1	2	-20 °C - 4 °C
	SAQ Buffer C	1	2	-20 °C - 4 °C
	Sialidase A	1	2	4 °C
	96-Well Clear Bottom Microplate	1	2	-20 °C - RT
	Sealing Film	1	2	-20 °C - RT

NOTE **Ten wells will be used for standards (five standards run in duplicates), leaving 38 wells for samples in GS48-SAQ kit and 86 wells in GS96-SAQ kit.**

NOTE **The 96-well Clear Bottom Microplate shipped with the kit is Greiner μClear Bottom 96-well Black Microplate (Greiner 655096, VWR 82050-754): non-sterile, medium binding polystyrene plate with black wells and clear/transparent bottoms, 340 μL capacity.**

Equipment and Reagents Provided By User

- Laboratory oven or block heater capable of 37 °C incubation.
- Heat block with a flat surface to accept a 96-well skirted plate (for example, VWR 13259-295 Modular Heating Block for Titer Plate).
- Plate reader for measurement by fluorescence detection (530 nm excitation, 590 nm emission), or absorbance (530 nm).
- Optional: Plate reader with 37 °C temperature control (no need for the heater and block).

Sample Considerations

- Samples that can be measured by the kit include glycoproteins, glycopeptides, glycolipids, polysialic acids, or whole cells.
- The dynamic range of this assay is 40 pmol to 1,000 pmol (fluorescence detection) and 500 pmol to 4,000 pmol (absorbance detection). Sample concentration may need to be adjusted to assure the signal falls within the range.
- Some samples can contain free sialic acid. A negative control (sample blank) containing sample and all reaction components except for Sialidase A may be included in each run to account for signal from free sialic acid in samples.
- Low levels of intrinsic glycoprotein fluorescence (or absorbance) will not interfere with sialic acid quantitation, as the negative control will be subtracted before determining the amount of sialic acid.
- Samples should be in water, PBS, or similar buffer. Ideally the samples should not be in a high molarity buffer to ensure the correct reaction pH.
- Sialidase digestion may not be complete if too much sample is added to the reaction, insufficient incubation time or temperature is allowed, or the sialic acid is sterically hindered from Sialidase A.
- An alternative expedited protocol is available, see **"Performing the assay - expedited protocol"** on page 13 for details and considerations.
- Some O-acetylated sialic acids may be poor substrates for the N-acetylneuraminic aldolase, so may not give an accurate value for the sialic acid content. The presence of O-acetyl groups should be confirmed by DMB derivatization followed by HPLC analysis (Agilent p/n GKK-407). If present, de-O-acetylation of the sample may be carried out by mild base hydrolysis[1] prior to treatment with the converting enzymes.

If you have questions on the kit protocol, please contact **https://www.agilent.com/en/contact-us/page**.

Kit Capacity

The kit contains sufficient reagents for 48 or 96 data points (GS48-SAQ or GS96-SAQ).

Each sialic acid standard curve uses 10 data points (5-point standard curve in duplicate).

Each sample uses 6data points for triplicate analysis (sample and sample blanks), or 4 data points for duplicate analysis. Use of a Bovine Fetuin Control glycoprotein is optional (see **Table 3** on page 10 for reconstitution instructions).

Table 2 Kit Capacity for a single run. Each run uses 10 data points for the sialic acid standard curve

Kit	Total Data Points	Standard Curve Data Points	Data Points Remaining for Samples	No. Samples (Triplicate Analyses with Blanks)	No. Samples (Duplicate Analyses with Blanks)	No. Samples (Singlicate Analyses with Blanks)
GS48-SAQ	48	10	38	6	9	19
GS96-SAQ	96	10	86	14 (2 x 7)	21	43

Protocol

Getting started

Set a heater or oven containing 96-well titer plate heat block to 37 °C.

Have items on hand:

- 96-Well Clear Bottom Microplate
- Sealing film
- Appropriate pipettes
- Ice bucket
- DI water

Preparing working solutions

Prepare the working solutions listed in **Table 3** below.

Table 3 Working solution instructions. GS48-SAQ kit contains one of each reagent vial, GS96-SAQ contains two of each vial. For GS96-SAQ, reconstitute one vial for ≤ 48 tests

Working Solution	Instructions	Notes
Conversion Reagent Solution	Add 1.8 mL of SAQ Buffer A to lyophilized Conversion Reagent vial. Cap and vortex to dissolve.	30 µL required per sample in Conversion and Developer Mix. Maintain on ice short-term, store at 4 °C for up to two weeks after reconstitution, avoid freezing.
HRP Solution	Add 0.9 mL of SAQ Buffer A to lyophilized Horseradish Peroxidase. Cap and vortex to dissolve.	15 µL required per sample in Conversion and Developer Mix. Maintain on ice short-term, store at 4 °C for up to two weeks after reconstitution, avoid freezing.
SAQ Dye Solution	Add 0.3 mL of DMSO to lyophilized SAQ Dye. Cap and vortex to dissolve.	5 µL required per sample in Conversion and Developer Mix. Store short term at room temperature and long term at 4 °C.
Bovine Fetuin Control (optional control glycoprotein)	Supplied as 0.4 mg protein, lyophilized. For fluorescence detection, reconstitute in 1.6 mL DI water for a working solution of 0.25 mg/ml. For absorbance detection, reconstitute in 0.4 mL DI water for a working solution of 1.0 mg/ml.	Add 10 µL per well for use as a control protein. Store long term at long term at -20 °C. Expected range is 0.20 - 0.29 nmol of sialic acid per µg fetuin.

Performing the assay

NOTE **An expedited one-pot protocol is also an option, see "Performing the assay - expedited protocol" on page 13 for details and considerations.**

Sialidase digest

NOTE **Triplicate or duplicate samples and negative controls (sample blanks) for each protein are recommended. See "FAQs" on page 18 for suggested starting amounts for various glycoproteins.**

1 Add 10 µL of sample glycoprotein per well to the 96-well Clear Bottom Microplate.

NOTE **If necessary, up to 30 µL sample may be used for dilute glycoprotein samples. If using more than 10 µL sample, adjust the amount of water added in step 4 so that the final reaction volume is 50 µL.**

2 Add 10 µL of Sialidase A (for positive digest) or 10 µL of DI water (for negative controls) to each well containing glycoprotein.

3 Add 10 µL of SAQ Buffer B to each well.

4 Add 20 µL of DI water to each well and mix well using a pipette. Cover with lid and incubate at 37 °C for 30 minutes.

NOTE **Longer incubation times up to overnight may be substituted. For longer incubation times use foil film to seal plate.**

5 After incubation is complete, remove and uncover the plate.

Preparing standards

6 Prepare 0 pmol standard (blank) by mixing 120 µL of SAQ buffer B and 480 µL of DI water.

7 Prepare 1,000 pmol standard for fluorescence detection (a) or 4,000 pmol standard for absorbance detection (b):

   a Prepare 400 µL of 1,000 pmol/50 µL standard by mixing 80 µL of 100 µM Sialic Acid Standard with 80 µL of SAQ Buffer B and 240 µL of DI Water.

   b Prepare 200 µL of 4,000 pmol/50 µL standard by mixing 160 µL of 100 µM Sialic Acid Standard with 40 µL of SAQ Buffer B.

NOTE **Short term room temperature storage of sialic acid standards is acceptable. For longer term storage of Sialic Acid Standard stock solution, return to -20 °C.**

8 Prepare 120 µL of each standard using a 1:1 serial dilution of the 1,000 or 4,000 pmol standard with the 0 pmol blank. Do not use the supplied 96-Well Clear Bottom Microplate for the dilution series, use a separate plate or microtubes.

NOTE **The following standards will be required for fluorescent measurement: 1,000, 500, 250, 125, and 0 pmol. Additional dilutions may be performed below 125 pmol to verify the limit of quantitation with a given plate reader.**

NOTE **The following standards will be required for absorbance measurement: 4,000, 2,000, 1,000, 500, and 0 pmol.**

Conversion and Color development

9 Prepare the Conversion and Developer Mix immediately before use by combining Conversion Reagent Solution, HRP Solution, SAQ Buffer C and SAQ Dye as described in **Table 4**.

Table 4 Conversion and Developer Mix (prepare immediately prior to use)

Number of Wells	Conversion Reagent Solution (µL)	HRP Solution (µL)	SAQ Buffer C (µL)	SAQ Dye (µL)	Total (µL)	Number of Wells
n	30/sample	15/sample	5/sample	5/sample	55	n
16	480	240	80	80	880	16
36	1080	540	180	180	1980	36
48	1440	720	240	240	2640	48

NOTE **Background fluorescence will slowly increase once the Conversion and Developer Mix is prepared. Keep the Mix on ice if not used right away. For best results, use the Mix within 30 minutes of preparation.**

10 Add 50 µL of each standard to empty wells of the plate in duplicate.

11 Add 50 µL of Conversion and Developer Mix (**Table 4**) to each well. Mix well with a pipette.

12 Cover with lid and incubate at 37 °C for 60 minutes.

13 Proceed to Reading the Plate and Analysis

NOTE **After incubation, read the plate as soon as possible. The background fluorescence will slowly increase as the plate sits after incubation. Reading the plate within 30 minutes is recommended.**

Performing the assay - expedited protocol

The following expedited protocol reduces assay time by combining the Sialidase A reaction with the conversion and development reactions in the same incubation step, as opposed to using two separate incubation steps for Sialidase A digestion and conversion/development reactions, as in the main protocol.

The expedited one-pot protocol may be appropriate for samples that do not require Sialidase A digestion as a distinct step. The user will have more control over the Sialidase digest if the main two-step protocol is followed since digestion time can be changed, and the pH of the reaction is similar to GS300/GF57 kits offered by Agilent in the past. Results are equivalent to the main protocol for Bovine Fetuin Control glycoprotein, if in doubt other glycoproteins may be tested in both protocols to ensure equivalent data is obtained. Please contact Agilent for further information.

Preparing standards

1 Prepare working solutions and the appropriate standards for fluorescence or absorbance as described in the Standard Protocol above.

Sialidase digestion, conversion and color development

NOTE **Triplicates and triplicate blanks for each protein are recommended.**

2 Add 10 µL of sample glycoprotein per well to the 96-well Clear Bottom Microplates Plate.

3 Add 10 µL of Sialidase A (for positive digest) or 10 µL of DI water (for negative controls) to each well containing glycoprotein.

4 Add 10 µL of SAQ Buffer B to each well.

5 Add 20 µL of DI water to each well and mix well using a pipette.

6 Prepare the Conversion and Developer Mix immediately before use by combining Conversion Reagent Solution, HRP Solution, SAQ Buffer C and SAQ Dye as described in **Table 5**.

Table 5 Conversion and Developer Mix (prepare immediately prior to use)

Number of Samples	Conversion Reagent Solution (µL)	HRP Solution (µL)	SAQ Buffer C (µL)	SAQ Dye (µL)	Total (µL)	Number of Samples
n	30/sample	15/sample	5/sample	5/sample	55	n
16	480	240	80	80	880	16
36	1080	540	180	180	1980	36
48	1440	720	240	240	2640	48

NOTE **Background fluorescence will slowly increase once the Conversion and Developer Mix is prepared. Keep the Mix on ice if not used right away. For best results, use the mixture within 30 minutes of preparation.**

7 Add 50 µL of Conversion and Developer Mix to each well. Mix well with a pipette.

8 Cover with lid and incubate at 37 °C for 60 minutes.

9 Proceed to **“Reading and Data Analysis”** on page 15.

NOTE

After incubation, read the plate as soon as possible. The background fluorescence will slowly increase as the plate sits after incubation. Reading the plate within 30 minutes is recommended.

Reading and Data Analysis

Reading the plate

The plate can be read immediately after incubation.

- For fluorescence detection, use wavelengths of 530 nm excitation and 590 nm emission.

NOTE **Filter-based instruments: compatible filters include 530DF30 and 590DF35 (Omega Optical, Brattleboro, VT, USA) or equivalent. Monochromator instruments: 5 nm slit width suggested.**

- For absorbance, use 530 nm.

Data analysis

Data analysis includes plotting the standard curve and calculating results.

1. Fit the results from the standards and blanks to a linear model to determine slope and intercept.
2. Determine the concentration of each sample and negative control using the formula:

   Sialic Acid (pmol) = ($Signal_{sample}$ - Intercept) ÷ Slope

   where:

   Slope = Slope of the standard curve

   Intercept = Intercept of the standard curve

   Signal = Fluorescence intensity or absorbance
3. Examine the results for the samples. The readings should fall within the linear portion of the standard curve. Otherwise, rerun the assay adjusting the amounts of the samples.
4. Average the replicate readings for each sample and the negative control. Subtract the negative control average from the sample average to determine total bound sialic acid in the sample.

NOTE **When using the Bovine Fetuin Control protein as a positive control, the expected range is 0.20 - 0.29 nmol of sialic acid per µg fetuin (200 - 290 nmol/mg, 9.6 - 13.9 mol/mol).**

For fluorescence detection, use 10 µL of 0.25 mg/ml (2.5 µg fetuin), for absorbance detection use 10 µL of 1 mg/ml (10 µg) fetuin, see **Table 3** on page 10.

Appendix A

Sialic acid release by acid hydrolysis

Acid hydrolysis may be used to confirm that Sialidase A treatment gives complete release of sialic acid. Although enzymatic release may be performed on the day of the assay, acid-catalyzed release will require several additional hours of digestion and sample preparation. Acid hydrolysis should be performed on a day prior to use of the Rapid Sialic Acid Quantitation Kit, and the samples frozen until processing.

Acid hydrolysis

NOTE **Triplicates and triplicate blanks for each protein are recommended.**

1 Samples may be dry or suspended in 10 µL pure water or buffer. We recommend using 500 µL screw-capped polypropylene tubes, fitted with rubber o-ring seals.

NOTE **If drying samples, use a SpeedVac with the heat setting turned to the “Off” position.**

2 If using dry samples, resuspend with 10 µL pure water.

3 Add 40 µL of 14.5% (v/v) Acetic Acid to the 10 µL Sample triplicates. Add 40 µL water to the 10 µL Negative control triplicates.

4 Seal the Sample tubes and place in a heating block set at 80 °C for three hours (but not the corresponding negative controls).

NOTE **Do not heat the negative controls because sialic acid is labile and may be hydrolyzed.**

5 Remove the vials and cool to room temperature.

6 Dry the samples and negative controls using a SpeedVac with the heat setting turned to the “off” position.

7 Prepare a solution of 5x diluted SAQ Buffer B by diluting 100 µL of SAQ Buffer B with 400 µL of DI water (scale accordingly based on number of wells).

8 Suspend the samples and negative controls in 50 µL of 5x diluted SAQ Buffer B and transfer to a microplate.

9 Store samples frozen in covered plate prior to use.

Troubleshooting

Non-linear or Variable standard curve

In fluorescence mode the gain may need to be adjusted to bring the signal within the dynamic range of the detector. Reduce the gain if the signal saturates the detector for higher points on the standard curve.

High background in sample blanks and standard curve

Degradation of the SAQ Dye causes higher signal for all results including blanks. The readings may not be linear above a certain level. Breakdown of the dye will occur over time after it is mixed with other reagents in the Conversion and Developer Mix, so this should be prepared just prior to use. Store the reconstituted SAQ Dye solution according to product recommendations (up to two weeks at 4 °C).

Low or No signal from samples

Several possible causes include:

- The sample may not be sialylated, or the level of sialylation is below the sensitivity of the assay. Samples can be concentrated by evaporation or using a molecular weight cut off filter.
- Possible pH difference in reaction due to sample buffer. Check pH of samples (after sialidase A digestion) and reagents, which should be between 6.8 and 7.0. Dilute or dialyze sample, or perform buffer exchange.
- The sample may have lost sialic acid prior to 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 pH range 5.0 to 8.5 at temperatures below 30 °C.
- The sialic acid may not be completely released. Longer Sialidase A incubation times can be evaluated.

Unexpectedly high signal from samples

Several possible causes include:

- The sample may have of endogenous sialic acid and/or α-keto acids that contribute to higher readings. Include a negative control (all reactants except the Sialidase A); subtract the measured value from the sample.
- Degradation of the dye causes higher numbers for the samples and the negative controls. The readings may not be linear above a certain level. Blanks in standard curve will also show high signal if this is the root cause.
- If the fluorescence is due to soluble glycoprotein, precipitate the protein with three volumes of cold 100% ethanol (after sialic acid release), centrifuge to remove the pellet, and dry down the supernatant. Finish the assay as described.
- Hydrogen peroxide may be present in the sample. It has been reported that protein samples stored for long periods undergo glycation, non-enzymatic addition of glucose or lactose to lysine residues, and that this reaction is accompanied by the accumulation of hydrogen peroxide in the protein solution.

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 non-reducing 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 6 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 6 Examples of starting concentrations and amounts of glycoprotein used with GS96-SAQ (fluorescence detection)

Glycoprotein	Concentration (mg/ml)	Sample Volume (µL)	Sample Mass (µg)	MW (kDa)	pmol Protein
Fetuin	0.25	10	2.5	48	52
MabThera	10	10*	100	145	690
Enbrel	0.25	10	2.5	150	16.7
Zaltrap	0.5	10	5	115	43
Orencia	0.5	10	5	92	54
EPO alfa	1	10	10	30.4	329

*For glycoproteins with lower sialylation such as monoclonal antibodies, up to 30 µL sample may be used with the kit. See **"Getting started"** on page 10.

Q. What is the difference between this kit (GS96-SAQ) and GS300 and GF57 GlykoScreen Rapid Sialic Acid Quantitation Kits previously offered?

A. The principle behind the chemistry of GS96-SAQ is the same as GS300 and GF57 sialic acid quantitation kits. The main difference is that GS96-SAQ buffer components have been optimized to allow for a two-step protocol: 1) Sialidase A digestion, followed by 2) conversion and development. This is opposed to the a four-step protocol in GS300 and GF57. The improvements made to GS96-SAQ makes the kit more robust and user friendly. For easy-to-digest samples we have also developed a one-step protocol (Section 3): sample digestion, conversion and detection can be performed in a single well for simple processing unless an extended digestion with Sialidase A is required.

Q. What is the source of the NeuAc Sialic Acid Standard used in the kit?

A. The 100 µM N-acetylneuraminic acid standard is quantitatively prepared from N-Acetylneuraminic acid, United States Pharmacopeia (USP) Reference Standard using calibrated, NIST-traceable lab equipment.

Resources and References

Visit Agilent's website for additional information, downloadable posters, publications and tech notes:

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Reference

1 Reuter, G. and R. Schauer. etermination of Sialic Acids. In Meth Enzymol 230 Academic Press, New York, pp. 168 199 (1994).

Technical Assistance

Agilent is committed to developing rapid, automatable methods for glycan analysis. Call us to discuss products in development.

If you have any questions or experience difficulties regarding any aspect of our products, please contact us at **https://www.agilent.com/en/contact-us/page**.

Agilent values customer opinions and we encourage you to contact us. We welcome your suggestions about product performance or new applications and techniques.

Ordering Information

Table 7 Kits and Modules

Product Code	Description
GS48-SAQ	AdvanceBio Total Sialic Acid Quantitation Kit, 48 wells
GS96-SAQ	AdvanceBio Total Sialic Acid Quantitation Kit, 96 wells
GK80040	Sialidase A
WS0377	100 µM Sialic Acid Standard, 1 mL

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