KORNBERG SEQNOSTICS
Lyoner Strasse 10, Frankfurt 60528, Germany | Email: contact @kornbergseqnostics.com

Total Sialic Acid Quantitation Kit
Cat No: KS9012-48 & KS-9012-96

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.
Kornberg Total Sialic Acid Quantitation Kit 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 Gly-*SA* 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

Principle:

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

Components Provided:

Components	Cat No KS9012-48	Cat No KS9012-96
100 uM N-acetylneuraminic acid (NANA, NeuAc) Sialic Acid	1	2
Bovine Fetuin Control, 0.4 mg,	1	2
SAQ Dye, lyophilized	1	2
Horseradish Peroxidase,	1	2
DMSO	1	2
Conversion Reagent,	1	2
SAQ Buffer A	1	2
SAQ Buffer B	1	2
SAQ Buffer C	1	2
Sialidase A	1	2
96-Well Clear Bottom	1	2

Note: 10 wells will be used for standards (5 standards run in duplicates), leaving 38 wells for samples in KS9012- 48 kit and 86 wells in KS9012-96 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 ul capacity.

Materials Provided by the End-User:

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

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 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 paragraph “Expedited Procedure” 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. If present, de-O-acetylation of the sample may be carried out by mild base hydrolysis (1) prior to treatment with the converting enzymes.

Pack Size:

The kit contains sufficient reagents for 48 data points. Each sialic acid standard curve uses 10 data points (5-point standard curve in duplicate). Each sample uses 6 data points for triplicate analysis (sample and sample blanks), or 4 data points for duplicate analysis. Use of a Bovine Fetuin Control glycoprotein is optional. 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. of Samples (triplicate analysis with blanks)	No of Samples (duplicate analysis with blanks)	No. of Samples (singlicate analysis with blank)
KS9012-48	48	10	38	6	9	19
KS9012-96	96	10	86	14(2x7)	21	43

Reagent Preparation:

1. Conversion Reagent Solution: Add 1.8 ml of SAQ Buffer A to lyophilized Conversion Reagent vial. Cap and vortex to dissolve.
   Note: 30 ul required per sample in Conversion and Developer Mix. Maintain on ice short-term, store at 4°C for up to 2 weeks after reconstitution, avoid freezing
2. HRP Solution: Add 0.9 ml of SAQ Buffer A to lyophilized Horseradish Peroxidase. Cap and vortex to dissolve.
   Note: Maintain on ice short-term, store at 4°C for up to 2 weeks after reconstitution, avoid freezing.
3. SAQ Dye Solution: Add 0.3 ml of DMSO to lyophilized SAQ Dye.
   Note: Store short term at room temperature and long term at 4°C.
4. 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.
   Note: Store long term at long term at -20°C. Expected range is 0.20 – 0.29 nmol of sialic acid per µg fetuin.
5. Conversion and developer mix: Prepare the Conversion & Developer Mix immediately before use by combining Conversion Reagent Solution, HRP Solution, SAQ Buffer C and SAQ Dye as described in below table:

Conversion and Developer Mix (prepare immediately prior to use). Number of wells	**Conversion Reagent Solution (ul)**	**HRP Solution (ul)**	**SAQ Buffer C (ul)**	**SAQ Dye (ul)**	**Total (ul)**	**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.

Standard Preparation:

1. 0 pmol standard: Add 120 ul of SAQ buffer B in 480 ul of DI water and mix
2. 1,000 pmol / 50 ul for fluorescence detection (a): Add 80 ul of 100 uM Sialic Acid Standard with 80 ul of SAQ Buffer B and 240 ul of DI Water.
3. 4,000 pmol / 50 ul for absorbance detection (b): Add 160 ul of 100 uM Sialic Acid Standard with 40 ul 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.

Prepare 120 ul 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.*

Assay Procedure:

Triplicate or duplicate samples and negative controls (sample blanks) for each protein are recommended.

1. Add **10 ul** of **sample glycoprotein** per well to the 96-well Clear Bottom Microplate. *Note: If necessary, up to 30 ul sample may be used for dilute glycoprotein samples. If using more than 10 ul sample, adjust the amount of water added in step 4 so that the final reaction volume is 50 ul.*

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

3. Add **10 ul** of **SAQ Buffer B** to each well.

4. Add **20 ul** 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.

6. Add **50 ul** of each **standard** to empty wells of the plate in duplicate.

7. Add **50 ul** 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 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.*

10. For fluorescence detection, use wavelengths of 530 nm excitation and 590 nm emission and For absorbance, use 530 nm. *Note: Filter-based instruments: compatible filters include 530DF30 and 590DF35 (Omega Optical, Brattleboro, VT, USA) or equivalent. Monochromator instruments: 5 nm slit width suggested.*

Expedited Procedure:
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
2 Add 10 ul of sample glycoprotein per well to the 96-well Clear Bottom Microplates Plate.
3 Add 10 ul of Sialidase A (for positive digest) or 10 µL of DI water (for negative controls) to each well containing glycoprotein.
4 Add 10 ul of SAQ Buffer B to each well.
5 Add 20 ul 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 below.
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 ul 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 Calculation of Results.

Numberof Samples	Conversion Reagent Solution (ul)	HRP Solution (ul)	SAQBufferC (ul)	SAQDye(ul)	Total(ul)	Numberof 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: 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.

Calculation of 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) = (Signalsample - 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 ug fetuin (200 – 290 nmol/mg, 9.6 – 13.9 mol/mol).

   For fluorescence detection, use 10 ul of 0.25 mg/ml (2.5 ug fetuin), for absorbance detection use 10 ul of 1 mg/ml (10 ug) fetuin,

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 ul pure water or buffer. We recommend using 500 ul 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 ul pure water.

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

   Control in triplicates.

4. Seal the Sample tubes and place in a heating block set at 80°C for 3 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 ul of SAQ Buffer B with 400 ul of DI water (scale accordingly based on number of wells).

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

9. Store samples frozen in covered plate prior to use.

KORNBERG SEQNOSTICS
Lyoner Strasse 10, Frankfurt 60528, Germany | Email: contact @kornbergseqnostics.com

KORNBERG SEQNOSTICS

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 & 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 2 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 - 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 3 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.