



SIALIC ACID QUANTITATION KIT

Rapid quantitation of total sialic acid, either free or released from intact glycoproteins:

- method evaluation with a known glycoprotein (bovine fetuin)
- comparison between enzymatic and acid-catalyzed cleavage
- broad range of detection, 200 pmol - 1,000 pmol (fluorescence) to 1 nmol - 5 nmol (absorbance)
- three assay formats: 96-well microplate format using absorbance or fluorescence, and test tubes/cuvettes with a standard fluorometer
- enzymatic cleavage allows rapid analysis with minimal, if any, degradation of sialic acid

Product Code: GF57

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Storage Conditions

The Kit is shipped with cold packs for next day delivery. Store the Enzymatic Release Reagent Pack at 4°C, and the rest of the kit at -20°C. The kit is warranted to be free of defects for six months from date of shipment.

This product is intended for *in vitro* research use only.

KIT CONTENTS

NOTE: We want successful results for our customers, so please read this entire booklet before starting the experiment.

Item	Qty
Enzymatic Release Reagent Pack	
Sialidase A™ (1 U, 200 µl)	1 ea
5x Reaction Buffer B (1 ml)	(GK80040)
Quantitation Reagents	2 sets
Conversion Reagents (lyophilized)	1 vial
Conversion Reaction Buffer (3 ml)	1 bottle
Horseradish Peroxidase (lyophilized)	1 vial
HRP Buffer (500 µl)	1 vial
Dye (lyophilized)	1 vial
DMSO (300 µl)	1 ampule
Sialic Acid Standard Solution (1 ml)	1 vial
Fetuin Control Protein (lyophilized)	1 vial
Alternate Release Reagent (1.5 ml)	1 ampule

ProZyme offers additional isoforms of Sialidase A, which may improve desialylation with some glycoproteins.

Additional Required Reagents/Equipment

Ultrapure deionized water (Milli-Q® or equivalent), filtered
Plate Shaker or agitator (pipet aspiration of individual wells is also acceptable)

Dry oven or water bath set to 37°C

Laboratory timer

Pipettors & disposable tips (P2/P10/P20)

Ice bucket and ice

Benchtop Centrifuge

Acid-hydrolysis Confirmation

Screw capped polypropylene tubes, fitted with a rubber o-ring seal (500 µl)

Heating Block set to 80°C

centrifugal vacuum evaporator, SpeedVac® or equivalent

Reading the Results:

for absorbance measurements:

Clear, 96-well microplate with plate sealers

Standard microplate reader, 560 nm filter

for fluorescence measurements:

1. 10 x 75 mm glass test tubes and plastic film

Quartz cuvettes (*optional*)

Fluorometer (ex 565 nm, em 585 nm)

2. Black or white, 96-well microplate with plate sealers

a. Fluorescence plate reader (ex 530 nm, em 590 nm)

Optimized filter set, 530DF30 and 590DF35 (Omega

Optical, Brattleboro, VT, USA or equivalent)

- b. Monochromator instruments
Black or white, 96-well microplate with sealers
Fluorescence microplate reader optimized settings:
ex 560 nm, em 590 nm, with a 5 nm slit width.

SAFETY AND HANDLING

Please read the Material Safety Data Sheets (MSDS) included with the kit.

Opening the Component Ampules

Gently tap the ampule to settle the contents on the bottom. To open, hold both the body and the top of the ampule, then gently but firmly snap open at the colored break-ring. Snap away from your body.

Fluids may be pipetted into or out of the ampules with standard pipettors or syringes with slim tips or needles. Be careful to avoid sharp edges around the opening (*be sure to wear gloves and safety glasses during these operations*).

General Laboratory Procedures

Use powder-free gloves for all sample handling procedures. Ensure that all glass, plasticware or solvents are free of glycosidases and environmental carbohydrates.

Minimize exposure of sialic acid-containing samples to elevated temperatures or extremes of pH; high temperatures or low pH will cause degradation of sialic acid.

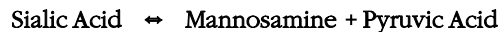
INTRODUCTION

This Kit represents a sensitive approach to sialic acid quantitation, based on a coupled enzyme reaction, converting released sialic acid to hydrogen peroxide, which reacts with a dye stoichiometrically, generating intense fluorescence (or absorbance). It allows enzymatic release of sialic acid, conversion, detection and quantitation to be performed in a single well for fast and simple processing (ready for data analysis in ~75 minutes).

The recommended procedure employs enzymatic digestion to release sialic acid from the glycoprotein. Digestion with Sialidase A is advantageous (compared to acid hydrolysis), because it is faster and releases sialic acid under moderate conditions. However, not all sialic acids are equally accessible to the enzyme, so it may be necessary to optimize cleavage conditions (amount of enzyme and/or the time of incubation) that give maximal values for a specific protein substrate. Acid has been provided in the Kit (Alternative Release Reagent) to allow the user to confirm the degree of enzymatic release.

The Coupled-Enzyme Method

Although there are many direct assays for sialic acid, conversion of sialic acid to hydrogen peroxide by enzymatic means provides one of the most sensitive and gentle methods of analysis. N-acetylneuraminic acid aldolase catalyzes the reversible reaction:



Variants of sialic acid, such as N-glycolyl- and some O-acetyl-neuraminic acids, are also converted to pyruvic acid and the corresponding mannosamine. Pyruvate oxidase then catalyzes the reaction:



Under the proper conditions, the forward aldolase reaction predominates; and when coupled with H_2O_2 generation, the reaction goes to completion. Hydrogen peroxide forms a 1:1 molecular complex with the selected dye when catalyzed by horseradish peroxidase; the complex is intensely fluorescent (or absorbant) and can be readily quantitated.

NOTE: Some O-acetylated sialic acids may be poor substrates for the neuraminyl-aldolase, and may not then 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 (Reuter and Schauer, 1994) prior to treatment with the converting enzymes.

Enzymatic release - Sialidase A is utilized to release the sialic acids from glycoproteins. This sialidase has been selected because it has broad substrate specificity for a wide variety of sialic acid molecules and linkages; and it has a relatively small molecular weight that allows it to access sterically hindered sialic acid residues on the polypeptide core. Sialidase A treatment has been designed as a distinct step so that conditions which give complete release of sialic acid can be optimized and tailored to the specific glycoprotein.

Typically a reaction digest is carried out in about 50 μl reaction volume with the substrate protein, Sialidase A and its digestion buffer. Either a 96-well microplate or test tubes may be utilized depending on the sensitivity of detection required. To identify the optimal cleavage time for the glycoprotein, several samples could be prepared by digesting at 37°C for various incubation times (30 minutes to overnight).

Acid hydrolysis - Sometimes Sialidase A treatment is not sufficient for complete release; the sialic acid residue may be buried and the enzyme sterically hindered. Acid hydrolysis offers an alternative means, which is not sterically limited, for removal of sialic acid. A protocol has been provided in Appendix B for confirmation of the amount of sialic acid in the samples using acid.

Using the Kit

The GF57 Kit is designed to meet a number of requirements:

- introduction to the method using a known glycoprotein.
- comparison of enzymatic *vs.* acid-catalyzed cleavage as a first step in optimizing sialidase cleavage.
- assessment of the value of the method as a sensitive, rapid, automatable assay suitable for product release or process analytical technology (PAT).

The Samples

Samples to be measured may be glycoproteins/peptides, glycolipids, polysialic acid, serum, tissue or whole cells. A suggested method for preparation of complex samples is described in Appendix A - Assay of Whole Cells.

Quantities to be analyzed should contain no greater than 5 nmols of sialic acid when using absorbance detection, and no greater than 1 nmols when using fluorescence detection. Obtain an approximation of the amount of sialic acid in the sample to be quantitated from the literature, gel analysis after sialidase treatment, or a preliminary assay of serial dilutions of the sample.

Sialic Acid Cleavage

Sialic acid must be released from the sample prior to quantitation. Enzymatic release may be performed on the day of the assay. Acid-catalyzed release requires several additional hours of digestion and sample preparation; it should be performed on a day prior to use of the Sialic Acid Quantitation Kit. Samples may then be combined to proceed to the next step, Conversion to Hydrogen Peroxide.

Detection Methods

Quantitation of sialic acid can be performed by measuring absorbance or fluorescence in three assay formats (see Figure 1 for spectral characteristics of the selected dye):

- 96-well microplate format (using absorbance or fluorescence)

- test tubes/cuvettes with a standard fluorometer

Absorbance readings at 560 nm may be obtained using a plate reader with clear, 96-well microplates. Using the standard protocol, the Sialic Acid Standard gave a linear response in the range of 1 - 5 nmol (see TechNote TNGS300.1 *An Enzyme-based Sialic Acid Quantitation Assay for Rapid Screening of Therapeutic Glycoproteins During Process Development: A Potential Process Analytical Technology*).

Individual samples, diluted to 1 ml and transferred to cuvettes, may be read using a xenon, continuous-lamp fluorometer (excitation at 565 nm and emission at 585 nm). Using the standard protocol, the Sialic Acid Standard gave a linear response in the range of 200 - 1,000 pmol.

Fluorescence measurements may be obtained using a filter-based fluorescence plate reader with black microplates; excitation was at 530 nm and emission at 590 nm. The Sialic Acid Standard gave a linear response in the range of 25 - 1,000 pmol using the standard protocol; greater sensitivity may be obtained with white plates (see TechNote TNGS300.1).

The use of a monochromator-based plate reader is still in development, however, we recommend the user evaluate results at different excitation and emission wavelengths in order to assess linearity and self-quenching. Use the widest slit width available and centered at 555 - 565 nm for excitation and at 590 nm and, again at 610 nm, for emission; the setting at the emission max (590 nm) may give higher sensitivity, and the setting at 610 nm may give greater dynamic range.

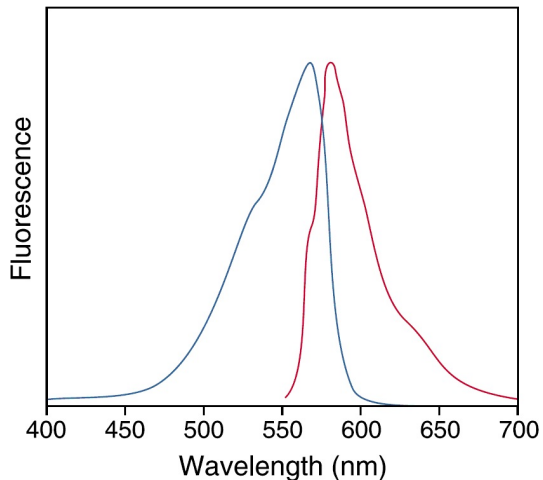


Figure 1 - Spectral properties of the detection dye (blue is excitation, red is emission).

Assay Standards and Controls

Since the samples may contain free sialic acid as a contaminant, a sample blank (in duplicate) should be prepared for each sample, which contains the sample, buffer and water, but omitting the sialidase (or the acid). The sample blanks should be processed similarly to the samples, including incubation.

Two standards have been supplied with the kit:

- A Sialic Acid Standard Solution

One ml of 100 μ M sialic acid standard has been provided for generation of a standard curve (prepare a minimum of 6 samples in duplicate).

- Fetuin Control Protein

Bovine fetuin contains sialylated N- and O-linked oligosaccharides, and is known to contain endogenous sialic acid (Townsend *et al.*, 1997; Spiro, 1960; and Rohrer *et al.*, 1997).

The Fetuin Control Protein (0.5 mg) may be reconstituted in 500 μ l of deionized water. Optimal sialic acid release was obtained when a 10- μ l aliquot was digested with 0.05 U (10 μ l) of Sialidase A for 60 minutes at 37°C.

- For absorbance detection, a 10- μ l aliquot should give a signal corresponding to ~2 nmol of sialic acid.
- For fluorescence detection, the reconstituted Fetuin Control Protein should be diluted further with 3 volumes of water to assure that the result falls within the range of the standard curve. A 10- μ l aliquot should now give a signal corresponding to ~0.5 nmol of sialic acid (see enclosed lot-specific Certificate of Analysis).

Capacity of the Kit

Each set of reagents will yield 30 data points; the standards and control (fetuin) will account for 16 of these when performed in duplicate. The capacity of the kit for each set of reagents for single replicates is seven (1 sample plus 1 sample blank). For duplicates, the number of samples is reduced to 3. Samples may be assayed in triplicate for a statistically meaningful determination. In this case, the total number of samples is 2, as each sample will account for 6 data points (3 sample assays plus 3 blanks).

If both reagent sets are used together, then data points reserved for standards and the control may be used for samples. In this case, the capacity for the total Kit for single replicate samples is 22, for duplicate samples 11, and for triplicates, 7.

NOTE: To double the number of data points, use “half volume” microplates and reduce the volume of reagents per well by half (Molecular Devices, part number 42-000-0117, or Corning, part numbers 3694 [black] or 3693 [white]).

Evaluation of the Assay

The accuracy of the method used in this kit depends on the quantitative generation of free sialic acid; completion of the reaction to form pyruvic acid; completion of the conversion to H_2O_2 ; quantitative formation of the dye complex; and the subsequent measurement of the dye complex. The method is robust when performed according to the supplied protocol (see TechNote TNGS300.1 for assay qualification data).

Accuracy & Reproducibility - Inaccuracies may be introduced due to:

- Quality of the samples

Errors in quantitation of the initial sample have a significant effect on variability, especially when purity, formulation or homogeneity affect the amount used in the assay.

- Pipetting errors

Errors introduced by pipetting of the initial sample can occur. Triplicate samples should be assayed for a statistically meaningful determination.

Small pipetting errors involving the conversion reagents will not affect the outcome of the assay, as they are added in such excess that a several fold increase or decrease will still result in quantitative conversion of sialic acid to H_2O_2 .

Precise pipetting of the Dye Solution is important, as small deviations can greatly affect the signal, especially in the sample blanks.

- Intrinsic glycoprotein absorbance (or fluorescence) in the same range as the assay

Low levels of absorbance (or fluorescence) in the same range as the assay will not interfere with the determination, as the sample blank will be subtracted before determining the amount of sialic acid.

High levels of absorbance (or fluorescence) may require precipitation and removal of the digested protein before proceeding with quantitation (see Tips & Hints, page 30).

- Incomplete digestion by Sialidase A

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 the sialidase.

The amount of sialic acid may be verified using the acid hydrolysis protocol (Appendix B) to evaluate whether the sialidase digestion conditions give complete release.

- Dilute samples

The amount of sialic acid added to the reaction may be too low to be accurately measured in the assay (<200 pmol). See Troubleshooting, page 31 for special sample preparation.

- Presence of endogenous sialic acid or α -keto acids in the samples

Samples may contain endogenous sialic acid and/or α -keto acids other than the pyruvic acid generated from sialic acid by the aldolase. When converted during the assay, they will result in an artificially high reading.

The sample blank (all reactants except sialidase) corrects for free sialic acid and α -keto acids present in the sample.

Capacity of the assay - Each reaction will measure up to 5 nmol of sialic acid if using absorbance detection (or 1 nmol if using fluorescence detection). If this level is exceeded, a non-linear response may be observed because the Dye will be exhausted from the reaction mix.

Sensitivity - Sensitivity of the assay depends upon the assay format and the instrument used to measure the dye complex (absorbance *vs.* fluorescence). Detection as low as 200 pmols of sialic acid may be made with a relative error of about 5%.

APPENDIX A: ASSAY OF WHOLE CELLS

Whole cells containing cell surface glycoproteins, tissue or capsular polysaccharides (consisting only of polysialic acid) should be killed by heat treatment to prevent metabolism of the free sialic acid. The samples should then be treated with Sialidase A to depolymerize the sialic acid, and cells should be removed by centrifugation. Analyze free sialic acid in the supernatant starting with Sialic Acid Standard (page 20).

APPENDIX B: ACID HYDROLYSIS

In order to confirm that Sialidase A treatment gives complete release of sialic acid from the sample, acid hydrolysis may be used to confirm the amount of sialic acid present.

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.

Reagents

Samples (similar to those for enzymatic release).

Sample blanks

Fetuin Control Protein (*optional*, supplied with the kit)

Fetuin Control Protein blank (*optional*, supplied with the kit)

Alternate Release Reagent (supplied with the kit)

Procedure

1. Transfer the samples to 500- μ l, screw-capped polypropylene tubes, fitted with rubber o-ring seals.
2. Dry the samples using a SpeedVac with the heat setting turned to the "off" position.
3. Add 50 μ l of Alternate Release Reagent (add water for the sample blanks).
4. Seal the sample tubes and place in a heating block set at 80°C for 3 hours (but not the Sample blanks).

NOTE: Do not heat the sample blanks because sialic acid is labile and may be hydrolyzed.

5. Remove the vials and cool to room temperature.
6. Dry the samples and sample blanks using a SpeedVac with the heat setting turned to the "off" position.
7. Suspend the samples and sample blanks in 50 μ l of water and transfer to a microplate. Alternatively, place them in 10 x 75 mm test tubes.

NOTE: Choice of plate color and format depends on the choice of detection method (see Detection Methods, page 8).

NOTE: Digested samples at this point may be frozen for several days before proceeding with the assay with no degradation of sialic acid.

8. Proceed to Sialic Acid Standard (page 20).

TIPS & HINTS

Estimate the amount of sample needed for the assay

Since the degree of glycosylation for a given protein may vary widely, this example gives an approximation of the amount of protein required to generate 5 nmol of sialic acid.

You will need to know the approximate level of glycosylation by weight. Assume that the average MW of a typical glycan structure is 1,000 $\mu\text{g}/\mu\text{mol}$.

Therefore, the maximum value for the assay (5 nmol) multiplied by the conversion to weight of an average glycan (1,000 ng/nmol) divided by the % glycosylation (by weight) yields the maximum sample quantity for the assay. For example, the sample quantity for an antibody (2 - 3% glycosylation) should be no greater than 250 μg ; the sample quantity for fetuin (~20% glycosylation) should be no greater than 25 μg .

Troubleshooting

The accuracy of the method used in this kit depends on the quantitative generation of free sialic acid; the completion of the reaction to form pyruvic acid; the completion of the conversion to H_2O_2 ; quantitative formation of the dye complex; and the subsequent measurement of the dye complex. The method is robust when performed according to the supplied protocol (see TechNote TNGS300.1 for assay qualification data).

Assay variability is high

1. Samples may not be homogeneous. Evaluate reproducibility using the Sialic Acid Standard Solution and the Fetuin Control Protein.
2. The Dye complex failed to form properly because the Reconstituted Horseradish Peroxidase was not adequately mixed into the sample.

Unexpectedly low readings

1. The sample may not be sialylated, or the level of sialylation is below the sensitivity of the assay (100 pmol). The assay was performed correctly if the standard curve is linear and the Fetuin Control Protein produced a value similar to the stated value in the Certificate of Analysis.

Several strategies exist for concentrating the amount of sialic acid if it is below the sensitivity of the assay:

- A larger sample volume may be concentrated by gentle drying prior to the Sialic Acid Release (page 18).
 - A large-scale Sialidase A digestion may be concentrated by drying prior to Conversion to Hydrogen Peroxide (page 23).
2. The sample, prior to the assay, may have lost sialic acid. Avoid prolonged exposure of sialylated glycans in aqueous solutions to low pH and/or elevated temperature. In general, glycans in solution should be kept in the pH range 5 - 8.5 at temperatures below 30°C.
 3. The sialidase may not have achieved complete release. Make sure that your sample was incubated with 10 μ l of Reconstituted Sialidase A at 37°C for at least 30 minutes.

Tubes require time to reach 37°C, especially in a convection incubator; use a water bath, if available, and/or increase the incubation time.
 4. The Conversion Reagents, shipped lyophilized, were not fully hydrated before use. Allow a minimum of 2 hours after reconstitution before use.
 5. The Conversion Reagents work maximally over a narrow pH range; extremes of pH or highly buffered samples may depress results.

6. The Dye complex failed to form properly because:
 - The Dye was not properly dispersed into the sample before the addition of the Reconstituted Horseradish Peroxidase.
 - The Color/fluorescence Development step was delayed (>30 minutes) and the H₂O₂ became unstable. Proceed to Color Development immediately.
7. Proteins or other compounds, which react with hydrogen peroxide, will interfere with formation of the Dye complex. These include catalase, ascorbate, bilirubin and hemoglobin.
8. Not all components commonly used for protein formulation have been evaluated with this kit. Add them to the Sialic Acid Standard when analyzing the samples. The standard curve may be altered (lower slope), but may be used if the linearity of response is preserved over the assay range.
9. The sample is too dilute. Concentrate the sample prior to the assay and use the maximal volume of sample suggested.

Unexpectedly high readings in the sample

1. The presence of endogenous sialic acid and/or α -keto acids in the sample contributes to higher readings. Include a sample blank (all reactants except the sialidase); subtract the measured value from the sample.
2. Degradation of the Dye causes higher numbers for the samples and the sample blanks. The readings may not be linear above a certain level. Prepare the Dye Solution just prior to use.
3. Check to see if the sample has high absorbance (or fluorescence) in the range of the assay. Include a sample blank (all reactants except the sialidase); subtract the measured value from the sample.

If the absorbance (or 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.

High or variable background

High or variable background signals may be due to the presence of hydrogen peroxide that may be present in the sample or buffers. Specifically, 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, which will result in a high background signal.

The Fetuin Control Protein value was low

The Sialidase may not have achieved complete release. Make sure that your sample was incubated with 20 μ l of Sialidase A at 37°C for at least 30 minutes. Tubes require time to reach 37°C, especially in a convection incubator; use a 37°C water bath, if available, and/or increase the incubation time.

PRODUCT USE AND WARRANTY

Terms and conditions of sale as well as product warranties may be found at:

<http://www.prozyme.com/pdf/terms-products.pdf>

TRADEMARKS AND TRADENAMES

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SpeedVac® is a registered trademark of Thermo Savant, Inc, New York, NY, USA.

MilliQ® is a registered trademark of Millipore Corporation in the United States and/or other countries.

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

On ProZyme's Website:

TechNote TNGS300.1 *An Enzyme-based Sialic Acid Quantitation Assay for Rapid Screening of Therapeutic Glycoproteins During Process Development: A Potential Process Analytical Technology*

http://www.prozyme.com/pdf/tngs300_1.pdf

Sialidase A™, Sialidase A™-51 & Sialidase A™-66

<http://www.prozyme.com/glyko/enzymes.html#sialidase>

TECHNICAL ASSISTANCE

ProZyme is committed to developing rapid, high-throughput methods for glycan analysis. Call us to discuss products currently in development.

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