RAPID SIALIC ACID QUANTITATION KIT

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

- broad range of detection, 40 pmol - 1,000 pmol
- 96-well microplate format
- sample digestion, conversion, detection and quantitation performed in a single well for fast and simple processing
- enzymatic cleavage allows rapid analysis (~75 minutes or less) with minimal, if any, degradation of sialic acid
- sufficient for ~90 data points

Product Code: GS300

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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)	4 ea
5x Reaction Buffer B (1 ml)	(GK80040)
Conversion Reagents (lyophilized)	1 vial
Conversion Reaction Buffer (3 ml)	1 bottle
Horseradish Peroxidase (lyophilized)	1 vial
HRP Buffer (500 µl)	2 vials
Sialic Acid Standard Solution (1 ml)	1 vial
Dye (lyophilized)	1 vial
DMSO (300 µl)	2 ampules

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
Microplate 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
Containers (2 ea, ~6-ml capacity)

Reading the fluorescent results:

1. Filter-based instruments
   Black or white, 96-well microplate with sealers
   Fluorescence microplate reader (ex 530 nm, em 590 nm)
   Optimized filter set, 530DF30 and 590DF35 (Omega Optical, Brattleboro, VT, USA or equivalent)

2. 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.

NOTE: Use of a multichannel pipettor facilitates the addition of reagents to microplates In order to insure a sufficient volume of reagents in the reservoir, 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]).

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

Sialic acid serves a critical role in mediating the effectiveness of recombinant therapeutic proteins. The presence (or absence) 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.

Standard Methods

Although an extraordinarily wide variety of methods for sialic acid quantitation have been developed, the most frequently employed method is analysis by high-performance liquid chromatography of pre-column derivatized, fluorescently labeled samples. HPLC analysis (such as ProZyme's product GKK-407) is advantageous because it offers an extremely high level of sensitivity (femtomole range), and allows the identification of individual sialic acid species. However, the method is cumbersome and time consuming, particularly when analysis of multiple samples is required. Moreover, the method is not readily adapted to a high-throughput modality.

Rapid Sialic Acid Quantitation

ProZyme's Rapid Sialic Acid Quantitation Kit represents a sensitive, high-throughput 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. This approach 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 or less if cleavage and conversion are performed at the same time). A comparison of the steps and time requirement for the DMB-HPLC procedure *vs.* the Rapid Sialic Acid Quantitation procedure, as well as results of analyses on typical glycoproteins, may be found in TechNote TNGS300.1 *An Enzyme-based Sialic Acid Quantitation Assay for Rapid Screening of Therapeutic Glycoproteins During Process Development: A Potential Process Analytical Technology.*

The recommended procedure for Rapid Sialic Acid Quantitation employs enzymatic digestion to release sialic acid from the glycoprotein. Digestion with Sialidase A is advantageous, compared with acid hydrolysis, because it is rapid and releases sialic acid under moderate conditions. However, not all sialic acids are equally accessible to the enzyme, so it may be necessary to qualify a specific protein substrate by optimizing the conditions for cleavage (amount of enzyme and/or the time of incubation) that give maximal values.

Use of the Rapid Sialic Acid Quantitation Kit offers a number of advantages over standard procedures:

- rapid quantitation of total sialic acid released from intact proteins as well as quantitation of free sialic acid
- broad range of detection of sialic acid levels, from 40 - 1,000 pmol of sialic acid per sample using fluorescence detection
- enzymatic cleavage allows rapid analysis with minimal, if any, degradation of sialic acid

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 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.

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 Rapid Sialic Acid Quantitation Kit.

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 25 µl reaction volume with the substrate protein, Sialidase A and its digestion buffer. 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 the research version of this kit (ProZyme product code GF57) for confirmation of the amount of sialic acid in the samples using acid.

Using the Kit

Types of Samples

Samples to be measured may be glycoproteins/peptides, glycolipids, polysialic acid, serum, tissue or whole cells.

Detection Methods

Quantitation of sialic acid can be performed by measuring fluorescence (see Figure 1 for spectral characteristics of the selected dye).

Fluorescence measurements may be obtained using a filter-based, fluorescence microplate reader with black, 96-well microplate; excitation was at 530 nm and emission at 590 nm (using an optimized filter set, 530DF30 and 590DF35, Omega Optical, Brattleboro, VT, USA). The Sialic Acid Standard gave a linear response in the range of 100 - 1,000 pmol using the standard protocol; greater sensitivity may be obtained with white microplates (see TechNote TNGS300.1).

The use of a monochromator-based microplate 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.

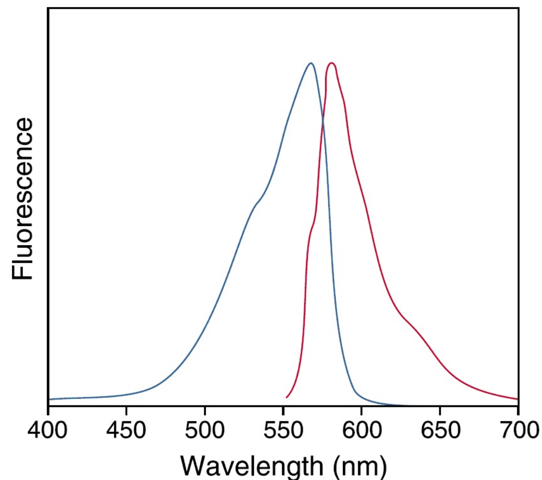

Figure 1 - Spectral properties of the detection dye (blue is excitation, red is emission).

Setting up the Assay

Quantities to be analyzed should contain no greater than 1,000 pmols of sialic acid. Obtain an approximation of the amount in the sample to be quantitated from the literature, gel analysis after sialidase treatment, or a preliminary assay of serial dilutions of the sample.

Assay Standards and Controls

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

A Sialic Acid Standard Solution, 1 ml of 100 µ*M* sialic acid, has been provided for generation of a standard curve (minimum of 6 samples in duplicate).

Capacity of the Kit

This kit contains sufficient reagents to process ~90 data points; the Sialic Acid standard will account for 12 of these when performed in duplicate. Samples may be assayed in triplicate for a statistically meaningful determination (3 sample assays plus 3 blanks).

Quicker Results

Total assay time may be shortened by proceeding directly from sialidase cleavage to the conversion reaction without an incubation step in between because the enzymes will not interfere with each other. The procedure has been split into separate steps to allow the user more flexibility.

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 may 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 fluorescence in the same range as the assay

  Low levels of 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 fluorescence may require precipitation and removal of the digested protein before proceeding with quantitation (see Troubleshooting, page 26).

- Dilute samples

  The amount of sialic acid added to the reaction may be too low to be accurately measured in the assay (<40 pmol). See Troubleshooting (page 26) 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.

- 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. A protocol has been provided in the research version of this kit (ProZyme product code GF57) for confirmation of the amount of sialic acid in the samples using acid.

Capacity of the assay - Each reaction will measure up to 1,000 pmol of sialic acid. 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. Detection as low as 200 pmols of sialic acid may be made with a relative error of about 5%.

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.

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. The assay was performed correctly if the standard curve is linear.

   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 22).

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 the dilution of the sialidase is sufficient to give complete cleavage under the conditions of incubation.

   Microplates may require more 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 Fluorescence Development step was delayed (>30 minutes) and the H_2O_2 became unstable. Proceed to Fluorescence 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. The samples may have significant fluorescence in the range of the assay. Include a sample blank (all reactants except the sialidase); subtract the blank value from the sample value.

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.

High or variable background

Hydrogen peroxide that may be present in the sample or buffers. 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.

TRADEMARKS AND TRADENAMES

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

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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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