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 2–8°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.

<table>
<thead>
<tr>
<th>Item</th>
<th>Qty</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic Release Reagent Pack</td>
<td></td>
</tr>
<tr>
<td>Sialidase A™ (1 U, 200 μl)</td>
<td>4 ea</td>
</tr>
<tr>
<td>5x Reaction Buffer B (1 ml)</td>
<td>(GK80040)</td>
</tr>
<tr>
<td>Conversion Reagents (lyophilized)</td>
<td>1 vial</td>
</tr>
<tr>
<td>Conversion Reaction Buffer (3 ml)</td>
<td>1 bottle</td>
</tr>
<tr>
<td>Horseradish Peroxidase (lyophilized)</td>
<td>1 vial</td>
</tr>
<tr>
<td>HRP Buffer (500 μl)</td>
<td>2 vials</td>
</tr>
<tr>
<td>Sialic Acid Standard Solution (1 ml)</td>
<td>1 vial</td>
</tr>
<tr>
<td>Dye (lyophilized)</td>
<td>1 vial</td>
</tr>
<tr>
<td>DMSO (300 μl)</td>
<td>2 ampules</td>
</tr>
</tbody>
</table>

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 pipetors 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.

NOTE: The following suggestions and data are based on information we believe to be reliable. They are offered in good faith, but without guarantee, as conditions and methods of use of our products are beyond our control. We recommend that the prospective user determine the suitability of our materials and suggestions before adopting them on a commercial scale. Suggestions for use of our products or the inclusion of descriptive material from patents and the citation of specific patents in this publication should not be understood as recommending the use of our products in violation of any patent or as permission to license to use any patents of ProZyme, Inc.

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:

\[
\text{Sialic Acid} \rightleftharpoons \text{Mannosamine} + \text{Pyruvic Acid}
\]

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

\[
\text{Pyruvic Acid} \rightarrow \text{Acetylphosphate} + \text{H}_2\text{O}_2
\]

Under the proper conditions, the forward aldolase reaction
predominates; and when coupled with \(\text{H}_2\text{O}_2\) 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 \( \mu M \) 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 $\text{H}_2\text{O}_2$; 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 $\text{H}_2\text{O}_2$.

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 27).

- 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 27) for special sample preparation.

- Presence of endogenous sialic acid or $\alpha$-keto acids in the samples
  
  Samples may contain endogenous sialic acid and/or $\alpha$-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%.

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**PROTOCOLS**

Outline of the procedure:

1. **Sample preparation**
   Purified glycoproteins/peptides or glycolipids containing up to 1,000 pmol of sialic acid; samples may be dried or dissolved in 10 - 20 μl of pure water or buffer.

2. **Sialic acid release**
   Sialic acid is released from the samples by enzymatic cleavage.

3. **Conversion to hydrogen peroxide**
   Reagents are added to quantitatively convert sialic acid to hydrogen peroxide.

4. **Color development**
   A dye complex is formed in the presence of horseradish peroxidase, yielding intense fluorescence.

5. **Data Analysis**
   Values are compared to the standard curve and the quantity of sialic acid calculated.
Preparation of Reagents

Reagents

Conversion Reagents (supplied with the kit)
Conversion Reaction Buffer (supplied with the kit)
Horseradish Peroxidase (supplied with the kit)
HRP Buffer (supplied with the kit)
Sialidase A™ (supplied as GK80040 with the kit)
Cold water

NOTE: Cold water is specified in order to keep these dilute solutions as cold as possible.

Procedure

NOTE: Prepare just prior to use (~2 hours). Reconstituted Conversion Reagent and Reconstituted Horseradish Peroxidase may be frozen (-20°C) for later use; avoid multiple freeze-thaw cycles. Reconstituted Sialidase A should be maintained at 2-8°C.

1. Thaw Conversion Reaction Buffer and HRP Buffer, maintain on ice.

NOTE: Following multiple freeze-thaw cycles a white precipitate in the Conversion Reaction Buffer may be observed. This is a salt by-product arising from the special formulation of this buffer and does not influence the performance of the kit. If precipitation is observed the buffer should be passed through a 0.2 or 0.45 µm filter and then used as described.

2. Preparing Reconstituted Sialidase A

Add 270 µl of water to 700 µl of Sialidase A to give a total of 970 µl (10 µl/well = 97 wells). Mix well. This is now Reconstituted Sialidase A.

3. Preparing Reconstituted Conversion Reagent

a. Add 1.0 ml of Conversion Reaction Buffer to the Conversion Reagents and mix well.

b. Transfer to a larger container (~6-ml capacity). Add an additional 1.5 ml of Conversion Reaction Buffer for a total volume of 2.5 ml (25 µl/well = 90+ wells).

c. Mix well and store on ice. Allow this solution to hydrate for a minimum of 2 hours prior to use in the assay. This is now ready-to-use Reconstituted Conversion Reagent.

4. Preparing Reconstituted Horseradish Peroxidase

a. Add 350 µl HRP Buffer to the Horseradish Peroxidase.
b. Mix well, transfer to a larger container (~6-ml capacity), and add an additional 600 µl of HRP Buffer. Add an additional 4.750 ml of cold water for a total of 5.7 ml (60 µl/well = 90+ assays).

c. Mix well and maintain on ice. This is now ready-to-use Reconstituted Horseradish Peroxidase.

### Sialic Acid Release

#### Reagents and Supplies

- 96-well microplate (black or white for fluorescence detection)
- Samples should contain no more than 1,000 pmols, either dried or in up to 10 µl (dilute buffer or water), and should be free of other glycosylated proteins.

**NOTE:** The total reaction volume should be no greater than 25 µl. If sample volumes are >10 µl, then concentrate the samples or reduce the dilution of the Sialidase A, which may be used directly from the vial.

- Sample blanks (in duplicate)
- Reconstituted Sialidase A
- 5x Reaction Buffer B (supplied as GK80040 with the kit)
- Water

### Procedure

1. Place samples (10 µl or less) into the wells of a 96-well microplate.
2. Add 5 µl of 5x Reaction Buffer B to each sample.
3. Add 10 µl of Reconstituted Sialidase A to each sample, but not to the sample blanks.
4. If needed, add water to bring the total volume of each well to 25 µl.
5. Seal the microplate to minimize evaporation.
6. Incubate at 37°C for 30 minutes.

**NOTE:** The optimal digestion time should be determined with a time course experiment and with varying amounts of sialidase.

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

**NOTE:** Total assay time may be shortened by proceeding directly to the conversion reaction as the enzymes will not interfere with each other. The procedure has been split into separate steps to allow the user more flexibility.
Sialic Acid Standard

Prepare a series of Sialic Acid Standard Solution samples to generate a standard curve and quantitate the samples.

Reagents

Sialic Acid Standard Solution (supplied with the kit)
Water (at room temperature)

Procedure

For determinations between 200 and 1,000 pmol

1. Thaw the Sialic Acid Standard Solution at room temperature. Mix thoroughly.
2. Dilute 200 μl with 300 μl of water.
3. Add these volumes to duplicate wells:
   - 25 μl (1000 pmol)
   - 20 μl (800 pmol)
   - 15 μl (600 pmol)
   - 10 μl (400 pmol)
   - 5 μl (200 pmol)
   - 0 μl (water blank)
4. Add water to each well to bring to a total of 25 μl (including the water blank).
5. Vigorously mix the solution on a microplate agitator for about 15 seconds.
6. Process the Sialic Acid Standard samples with the rest of the samples.

For determinations between 25 and 200 pmol

NOTE: The relative error may be greater at these low levels.

1. Thaw the Sialic Acid Standard Solution at room temperature. Mix thoroughly.
2. Dilute 40 μl with 460 μl of water.
3. Add these volumes to duplicate wells:
   - 25 μl (200 pmol)
   - 20 μl (160 pmol)
   - 15 μl (120 pmol)
   - 10 μl (80 pmol)
   - 5 μl (40 pmol)
   - 0 μl (water blank)
4. Add water to each well to bring to a total of 25 μl (including the water blank).
5. Vigorously mix the solution on a microplate agitator for about 15 seconds.
6. Process the Sialic Acid Standard samples with the rest of the samples.
Conversion to Hydrogen Peroxide

Reagents
Reconstituted Conversion Reagent (prepared the same day or thawed and maintained on ice)

Procedure
1. Add 25 μl of the Reconstituted Conversion Reagent to each sample.
2. Seal to minimize evaporation.
3. Agitate the microplate on a microplate shaker (set at medium) for about 15 seconds.
4. Incubate at 37°C for 30 minutes.

NOTE: Avoid excessive or extensive agitation, which can hasten the breakdown of hydrogen peroxide.

Fluorescence Development

Reagents
Reconstituted Horseradish Peroxidase (prepared the same day or thawed and maintained on ice)
Dye (supplied with the kit)
DMSO (supplied with the kit)
Water

Procedure
1. Prepare the Dye Solution:
   a. Gently tap the Dye to settle the contents on the bottom of the vial.
   b. Break open the ampules of DMSO and add 475 μl to the Dye. Mix the Dye/DMSO Solution well, collecting any residue from the cap and walls of the tube by brief centrifugation of the solution.
   c. Just prior to use, add 475 μl of cold water to give a total volume of 950 μl of Dye Solution (10 μl/well = 90+ assays).
NOTE: When fewer than 90 samples are processed, the Dye should be maintained in DMSO at -20°C to minimize degradation (some degradation of the Dye will occur, even at -20°C, and results in higher readings in the water blank). Aliquots may be removed, an equal amount of cold water added and then used as directed.

2. Add 10 μl of the Dye Solution to each reaction. Vigorously mix the solution on a microplate agitator for about 15 seconds.

NOTE: Precise pipetting of the Dye Solution is important because small deviations can greatly affect the signal, especially in the sample blank.

3. Add 60 μl of the Reconstituted Horseradish Peroxidase to each reaction. Vigorously mix the solution on a microplate agitator for about 15 seconds.

4. Cover the microplate with aluminum foil to exclude light and leave at room temperature for 10 minutes.

5. Read the results within 30 minutes.

NOTE: The unreacted Dye will degrade over time, increasing the readings in samples and blanks. Eventually, the response will no longer be linear.

### Data Analysis

#### Evaluation of the Assay

1. Generate a standard curve:
   a. Average the replicate readings for the Sialic Acid Standard samples, including the water blanks.
   b. Subtract the average value of the water blank from the Sialic Acid Standard average values (including itself).
   c. Plot the values and determine the slope.

   The results should be linear (standard curves are shown in TechNote TNGS300.1).

2. 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.

3. Examine the results for the water blank. It should be 5% or less of the reading for the highest concentration of the Sialic Acid Standard.
Calculating Results

1. Average the replicate readings for each sample and the sample blanks. Calculate the relative error and standard deviation among the replicates.

2. Calculate the result for each sample using the average of the replicates:

\[
\text{Sialic Acid (pmol)} = \frac{\text{FI}_{\text{sample}} - \text{FI}_{\text{blank}}}{\text{slope}}
\]

where:
- \( \text{slope} \) = slope of the standard curve (FI/pmol)
- \( \text{FI} \) = fluorescence intensity

TROUBLESHOOTING

Accuracy of the method 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 \( \text{H}_2\text{O}_2 \); 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).

As with any assay, it is important to first run a standard curve, establish a linear range, and then restrict sample readings to within that linear range.

Fluorescence is a relative measurement. Sample readings are only as meaningful and accurate as the standard and blank used to calibrate the instrument, and sample readings must be obtained at the same time, on the same fluorometer, and at the same temperature.

Standard curve is non-linear and variable.

1. Extensive agitation following addition of the Reconstituted Conversion Reagent (page 22) may have hastened the breakdown of hydrogen peroxide. This is usually reflected in a highly irregular and/or flattened standard curve.
2. The Dye complex may have failed to form properly because:
   - The Dye was not properly dispersed into the sample volume before the addition of the Reconstituted Horseradish Peroxidase, or
   - The Fluorescence Development step was delayed (>30 minutes) and the hydrogen peroxide became unstable; proceed to Fluorescence Development immediately.

3. Precise pipetting is critical; pipetting errors are the most common cause of assay variability.

4. Fluorescence is dependent on temperature. Ensure that all reagents are equilibrated to ambient temperature and read immediately.

Standard curve is linear and reproducible but variability of samples is high.

Evaluate reproducibility by generating a standard curve with the Sialic Acid Standard Solution; the assay was performed correctly if the standard curve is linear. If the samples alone generate unexpected readings, then refer to the sections titled “Unexpectedly low readings in the sample” and “Unexpectedly high readings in the sample” below.

Unexpectedly low readings in the sample

1. The sample may not be sialylated, or the level of sialylation is below the sensitivity of the assay.

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. Avoid excessive agitation following the addition of the Reconstituted Conversion Reagent, which can hasten the breakdown of hydrogen peroxide.

5. The Conversion Reagents, shipped lyophilized, were not fully hydrated before use. Allow a minimum of 2 hours after reconstitution before use.

6. The Conversion Reagents work maximally over a narrow pH range; extremes of pH or highly buffered samples may depress results.

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.

10. Fluorescence is inversely proportional to temperature. Samples are sitting in the cuvette well of the fluorometer too long (or for variable intervals) before reading, and steadily heat up from the internal circuitry.

**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.

4. Hydrogen peroxide is initially 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.
1. Hydrogen peroxide may be present in the sample buffers.

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.
TRADEMARKS AND TRADENAMES

ProZyme®, Glyko®, Sialidase A™ and GlykoScreen™ are trademarks of ProZyme, Inc., San Leandro, CA, USA.

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

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

REFERENCES


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


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

http://www.prozyme.com/glyko/enzymes.html#sialidase
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ProZyme is committed to developing rapid, high-throughput methods for glycan analysis. Call us to discuss products currently in development.

If you have any questions or experience difficulties regarding any aspect of our products, please contact us:

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PHONE (510) 638-6900
FAX (510) 638-6919
E-MAIL info@prozyme.com
WEB www.prozyme.com

ProZyme values customers’ opinions and considers customers an important source for information regarding advanced or specialized uses of our products. We encourage you to contact us. We welcome your suggestions about product performance or new applications and techniques.

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