3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

Sialic Acid Quantitation Kit

Catalog Number **SIALICQ**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product Description
The Sialic Acid Quantitation Kit provides a rapid and accurate determination of total *N*-acetylneuraminic acid (NANA), also known as sialic acid. The content of NANA is often related to the efficacy of many glycoproteins of pharmaceutical interest and must be constantly monitored during the manufacturing process. In addition, the kit can provide a simple and reliable method for the quantitation of NANA in sera and specific glycoconjugates to study disease states and virulence potentials in many pathogens.

The kit contains all reagents needed to quantitate the amount of NANA either free, or in glycoproteins, cell surface glycoproteins, polysialic acids, and capsular polysaccharides consisting of only polysialic acid.

Components
Sufficient reagents are provided to perform 25 assays, including control samples. Each assay will measure 1–200 nmoles of NANA.

N-Acetylneuraminic Acid Aldolase 25 µL
Catalog Number A0849

α-(2→3,6,8,9)-Neuraminidase 25 µL
(Sialidase A)
Catalog Number N8271

L-Lactic Dehydrogenase 25 µL
Catalog Number L9889

β-NADH, Disodium Salt 3 vials
(5 mg/vial)
Catalog Number N8129

Fetuin, Bovine 0.5 mg
Catalog Number F4301

0.01 M *N*-Acetylneuraminic Acid (NANA) 200 µL
Catalog Number A0974

1.0 M Tris-HCl, pH 7.5 1 mL
Catalog Number T3946

Sialidase Buffer 500 µL
(250 mM sodium phosphate, pH 5.0)
Catalog Number S7189

Precautions and Disclaimer
For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability
The kit is shipped with a cold pack and can be used for at least one year if stored at 2–8 °C.
DO NOT FREEZE.

Notes: Upon receipt, remove and store β-NADH, Disodium Salt vials (N8129) at –20 °C.

For optimal performance, β-NADH solutions should be prepared fresh daily. Prepared solutions should be discarded if the A_{340} drops 20% or the solution turns yellow. Keep unopened tubes in the sealed bag with desiccant stored at –20 °C.

Procedures
NANA is a negatively charged, non-reducing sugar, making its direct analysis more difficult than conventional sugars. However, quantitation can be accomplished with an enzymatic method.
N-acetylneuraminic acid aldolase catalyzes the reversible reaction:

N-Acetylneuraminic acid ↔ *N*-Acetylmannosamine + Pyruvic acid

The pyruvic acid can be reduced to lactic acid by β-NADH and lactic dehydrogenase:

Pyruvic Acid + β-NADH ↔ Lactic Acid + β-NAD

Under the proper conditions, the first forward reaction predominates, and when coupled with β-NADH reduction of pyruvic acid, the reaction goes to completion. β-NADH oxidation can be accurately measured spectrophotometrically.

Most forms of NANA found in nature are complexed in glycoconjugates. NANA can be released through the action of neuraminidase and can be detected as free NANA. The kit includes α-(2→3,6,8,9)-neuraminidase and procedures for the quantitation of total NANA in:

- Glycoproteins
- Cell surface glycoproteins
- Polysialic acids
- Capsular polysaccharides consisting only of polysialic acid

This kit is not suitable for use with glycolipids or gangliosides.

In the past, release of NANA from glycoconjugates has been limited by the effectiveness of the neuraminidase used and has led to under reporting of total NANA yields. α-(2→3,6,8,9)-Neuraminidase cleaves all NANA linkages, including α-2→8 and α-2→9 linkages, as well as branched NANA. Variants of NANA such as *N*-glycolyl or *O*-acylneuraminic acid are also cleaved. Digestion with α-(2→3,6,8,9)-neuraminidase is therefore the only reliable method of generating total free NANA from glycoconjugates without introducing losses typically associated with chemical hydrolysis.

The accuracy of the procedures used with this kit depends on:
1. the quantitative generation of NANA for analysis
2. the completion of the reaction to form pyruvic acid
3. the completion of the reaction to form lactic acid and the subsequent measurement of the oxidation of β-NADH

Capacity: Each reaction will measure 1–200 nmoles of NANA. If this level is exceeded, β-NADH will be exhausted from the reaction mix.

Sensitivity: The millimolar extinction coefficient of β-NADH is 6.22 at 340 nm. Therefore, each nmole of NANA will cause a drop of 0.00622 absorbance units in a 1 mL reaction, a quantity reliably measured on a digital spectrophotometer.

Setting up the Assay

First, obtain an approximation of the amount of NANA in the sample to be quantitated either from the literature or from gel analysis after neuraminidase treatment. Otherwise run a series of dilutions to determine the quantity of sample to be assayed. In order to meet the volumetric requirements of the assay, diluted samples may require concentration.

Two control samples have been supplied to perform positive controls before proceeding with analysis of unknowns. In the assay of free NANA, use the *N*-Acetylneuraminic Acid Solution (Catalog Number A0974) as a control. Addition of 10 µL to the reaction is equivalent to ~100 nmoles. In the assay of glycoproteins, use the Bovine Fetuin (Catalog Number F4301) as a control. When the entire sample is digested with α-(2→3,6,8,9)-neuraminidase, the amount of NANA should be ~48 nmoles.

1. Assay of Free NANA

Reagent Preparation
- NANA Sample - The sample may be in solution or lyophilized. An amount equivalent to 1-200 nmoles of NANA is added to the reaction.
- NANA Solution (Catalog Number A0974) - Provided as a control. Addition of 10 µL to the reaction should result in a reading of ~100 nmoles.
- Tris Reaction Buffer - Dilute the 1 M Tris-HCl (Catalog Number T3946) 40-fold with distilled water to make a 25 mM solution
- β-NADH Solution - Just prior to use, add 640 µL of Tris Reaction Buffer to one 5 mg vial of β-NADH (Catalog Number N8129) to make a 0.01 M solution (A_{340} = 62.2). Prepare solutions fresh daily. Prepared solutions should be discarded if the A_{340} drops 20% or the solution turns yellow.
- *N*-Acetylneuraminic Acid Aldolase (Catalog Number A0849) – ready-to-use
- L-Lactic Dehydrogenase (Catalog Number L9889) – ready-to-use

Procedure
1. Add the sample or control to Tris Reaction Buffer to give a final volume of 980 µL.
2. Pipette the reaction mixture into a cuvette and blank the spectrophotometer. Add 20 µL of the β-NADH Solution and mix by inversion several times.
3. Read and record the initial β-NADH absorbance. Initial A_{340} should read ~1.25.

4. Return the reaction mixture to the original tube. Add 1 µL of *N*-Acetylneuraminic Acid Aldolase and 1 µL of Lactic Dehydrogenase and mix by inversion several times.
5. Incubate in a 37 °C water bath for a minimum of 1 hour.
6. Pipette the reaction mixture back into the cuvette. Read and record the final A_{340}. Calculate the nmoles of NANA.

Calculation:

$$\text{nmoles NANA} = \frac{(A_{340\ \text{Initial}} - A_{340\ \text{Final}}) \times 1{,}000}{6.22}$$

2. Assay of Multiple Samples for free NANA

For processing many samples, it may be more convenient to premix sufficient Tris Reaction Buffer, β-NADH Solution, *N*-Acetylneuraminic Acid Aldolase, and Lactic Dehydrogenase to make a reaction mix for all of the samples plus a blank tube. Aliquot 1 mL of the reaction mix into each tube and add the NANA sample. Incubate in a 37 °C water bath for a minimum of 1 hour. Read the blank (no NANA added) and then read the sample tubes. Subtract the sample readings from the blank value to calculate the nmoles of NANA.

This method may be adapted to a multiwell plate format with a plate reader that measures absorbance at 340 nm. Include several blank wells for calculating the starting absorbance. Since the calculation of nmoles of NANA is based on a 1 mL sample size, the final calculation should be multiplied by the volume (in mL) used in each well (multiply by 0.1 for a 100 µL [0.1 mL] sample size).

3. Assay of Glycoprotein or Polysialic Acid

The sample of interest is digested with a broad-spectrum neuraminidase to release free NANA. Free NANA is converted to pyruvic acid by *N*-acetylneuraminic acid aldolase and then treated with lactic dehydrogenase to form lactic acid with the oxidation of β-NADH to β-NAD.

Reagent Preparation

- Sample of protein or polysialic acid after digestion with α-(2→3,6,8,9)-neuraminidase should yield 1–200 nmoles of NANA.
- Bovine Fetuin (Catalog Number F4301) - When the entire provided sample is digested with α-(2→3,6,8,9)-neuraminidase the amount of free NANA released is ~48 nmoles.
- Tris Reaction Buffer - Dilute the 1 M Tris-HCl (Catalog Number T3946) 40-fold with distilled water to make a 25 mM solution.
- β-NADH Solution - Just prior to use, add 640 µL of Tris Reaction Buffer to one 5 mg vial of β-NADH (Catalog Number N8129) to make a 0.01 M solution (A_{340} = 62.2). Prepare solutions fresh daily. Prepared solutions should be discarded if the A_{340} drops 20% or the solution turns yellow.
- α-(2→3,6,8,9)-Neuraminidase (Catalog Number N8271) – ready-to-use
- *N*-Acetylneuraminic Acid Aldolase (Catalog Number A0849) – ready-to-use
- L-Lactic Dehydrogenase (Catalog Number L9889) – ready-to-use
- Sialidase Buffer (250 mM sodium phosphate, pH 5.0, Catalog Number S7189) – ready-to-use

Procedure

1. Dissolve the glycoprotein or polysialic acid in 40 µL of distilled water and then add 10 µL of Sialidase Buffer.
2. Add 1 µL of α-(2→3,6,8,9)-Neuraminidase and incubate for at least 3 hours in a 37 °C water bath.
3. Add 930 µL of Tris Reaction Buffer to the tube. Note: If the glycoprotein precipitates, centrifuge the sample to remove the precipitate.
4. Pipette the reaction mixture into a cuvette and blank the spectrophotometer. Add 20 µL of the β-NADH Solution and mix by inversion several times.
5. Read and record the initial β-NADH absorbance. Initial A_{340} should read ~1.25.
6. Return the reaction mixture to the original tube. Add 1 µL of *N*-Acetylneuraminic Acid Aldolase and 1 µL of Lactic Dehydrogenase and mix by inversion several times.
7. Incubate in a 37 °C water bath for a minimum of 1 hour.
8. Pipette the reaction mixture back into the cuvette. Read and record the final A_{340}. Calculate the nmoles of NANA.

Calculation

$$\text{nmoles NANA} = \frac{(A_{340\ \text{Initial}} - A_{340\ \text{Final}}) \times 1{,}000}{6.22}$$

4. Assay of Whole Cells

Whole cells containing cell surface glycoproteins or capsular polysaccharides consisting only of polysialic acid are killed by heat treatment to prevent metabolism of the free NANA. The cells are treated with a broad-spectrum neuraminidase to release NANA and then removed by centrifugation. Free NANA in the supernatant is converted to pyruvic acid by *N*-acetylneuraminic acid aldolase, and then treated with lactic dehydrogenase to form lactic acid with the oxidation of β-NADH to β-NAD.

Reagent Preparation

- Sample – The number of cells should be chosen such that the amount of NANA released is between 1 and 200 nmoles.
- Tris Reaction Buffer – Dilute the 1 M Tris-HCl (Catalog Number T3946) 40-fold with distilled water to make a 25 mM solution.
- β-NADH Solution – Just prior to use, add 640 µL of Tris Reaction Buffer to one 5 mg vial of β-NADH (Catalog Number N8129) to make a 0.01 M solution (A_{340} = 62.2). Prepare solutions fresh daily. Prepared solutions should be discarded if the A_{340} drops 20% or the solution turns yellow.
- α-(2→3,6,8,9)-Neuraminidase (Catalog Number N8271) – ready-to-use
- *N*-Acetylneuraminic Acid Aldolase (Product Code A0849) – ready-to-use
- L-Lactic Dehydrogenase (Catalog Number L9889) – ready-to-use
- Sialidase Buffer (250 mM sodium phosphate, pH 5.0, Catalog Number S7189) – ready-to-use

Procedure

1. Wash cells in Tris Reaction Buffer and centrifuge.
2. Resuspend the cell pellet in 80 µL of distilled water and 20 µL of Sialidase Buffer. Kill the cells by heating to 90 °C for 5 minutes. Cool to room temperature.
3. Add 1 µL of α-(2→3,6,8,9)-Neuraminidase and incubate overnight at 37 °C.
4. Centrifuge cells and pipette the supernatant into another tube.
5. Bring the volume to 980 µL with Tris Reaction Buffer.
6. Pipette the reaction mixture into a cuvette and blank the spectrophotometer. Add 20 µL of the β-NADH Solution and mix by inversion several times.
7. Read and record the initial β-NADH absorbance. Initial A_{340} should read ~1.25.
8. Return the reaction mixture to the original tube. Add 1 µL of *N*-Acetylneuraminic Acid Aldolase and 1 µL of Lactic Dehydrogenase and mix by inversion several times.
9. Incubate in a 37 °C water bath for a minimum of 1 hour.
10. Pipette the reaction mixture back into the cuvette. Read and record the final A_{340}. Calculate the nmoles of NANA.

Calculation

$$\text{nmoles NANA} = \frac{(A_{340\ \text{Initial}} - A_{340\ \text{Final}}) \times 1{,}000}{6.22}$$

Troubleshooting Guide

Pipetting errors - Duplicate or triplicate samples should be assayed for a statistically meaningful determination.

Sample with high absorbance at 340 nm - Reasonable levels of absorbance at 340 nm will not interfere with the determination as the sample absorbance is set to zero prior to addition of β-NADH. High levels require precipitation and removal of the digested protein with subsequent analysis of free NANA.

Dilute samples - The amount of NANA added to the reaction may be too low to be measured in the assay (<1 nmole).

Presence of α-keto acids - Samples may contain α-keto acids other than the pyruvic acid generated from NANA by *N*-acetylneuraminic acid aldolase. When reduced by lactic dehydrogenase and β-NADH, they will result in an incorrect high reading. If their presence is a possibility, run a control reaction omitting the *N*-acetylneuraminic acid aldolase.

Lactic dehydrogenase contamination in the sample - Small amounts are not significant as long as the initial absorbance is read immediately. Significant contamination results in a rapid drop in the initial absorbance at 340 nm before addition of lactic dehydrogenase. Wash the cuvette thoroughly between assays, as any carryover of lactic dehydrogenase will result in a low initial absorbance reading.

Incomplete digestion by α-(2→3,6,8,9)-neuraminidase - Neuraminidase digestion may not be complete if too much sample is added to the reaction or insufficient incubation time is allowed. The amount of NANA should be determined in a time course experiment to ascertain that the digestion has gone to completion.

References

1. Cornfield, A.P. *et al.*, The specificity of viral and bacterial sialidases for α-(2-3) and α-(2-6)-linked sialic acids in glycoproteins. Biochim. Biophys. Acta, **744**, 121-126 (1983).
2. Dwek, R.A. *et al.*, Analysis of glycoprotein-associated oligosaccharides. Ann. Rev. Biochem., **62**, 65-100 (1993).
3. Kobata, A., Use of endo- and exoglycosidases for structural studies of glycoconjugates. Anal. Biochem., **100**, 1-14 (1979).
4. Kolisis, F.N., An immobilized bienzyme system for assay of sialic acid. Biotechnol. Appl. Biochem., **8**, 148-152 (1986).
5. Prime, S.J. *et al.*, Oligosaccharide sequencing based on exo- and endoglycosidase digestion and liquid chromatographic analysis of the products. J. Chromatogr., **A 720**, 263-274 (1996).
6. Sugahara, K. *et al.*, Enzymatic assay of serum sialic acid. Clin. Chim. Acta, **108**, 493-498 (1980).
7. Uchida, Y. *et al.*, Enzymatic properties of neuraminidases from *Arthrobacter ureafaciens*. J. Biochem., (Tokyo), **86**, 573-585 (1979).

SS,DW,SG,GL,RBG,PC,LCM,MAM 03/19–1