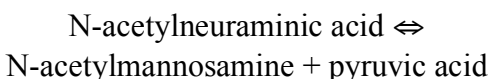


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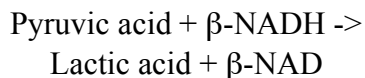
N-Acetylneuraminic Acid Quantitation Kit

Contains all the reagents needed to quickly and accurately quantitate sialic acids, including N-acetylneuraminic acid (NANA) and N-glycolylneuraminic acid (NGNA). This kit includes reagent for 25 assays, each assay measuring from 1-200 nmoles of sialic acid.

In this method, N-acetylneuraminic acid Aldolase catalyzes the reversible reaction:



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



The first forward reaction, when coupled with β -NADH reduction of pyruvic acid, goes to completion. β -NADH oxidation can be accurately quantitated spectrophotometrically.

Most forms of sialic acid found in nature are complexed in glycoconjugates. They can be released through the action of sialidase (neuraminidase) and can be detected as free sialic acid.

The kit includes $\alpha(2-3,6,8,9)$ neuraminidase and procedures for the quantitation of total sialic acid in:

- Glycoproteins
- Cell surface glycoproteins
- Polysialic acids

- Capsular polysaccharides consisting only of polysialic acid

Product Code: GE 57

Kit Contents

N-acetylneuraminic acid Aldolase 100 U/ml
25 μ L

$\alpha(2-3,6,8,9)$ Neuraminidase 5 U/mL, 25 μ L
(recombinant from *Arthrobacter ureafaciens*)

Lactic Dehydrogenase 500 U/mL, 25 μ L

β -NADH, Disodium salt 30 mg

Bovine Fetuin 0.5 mg

N-acetylneuraminic acid 0.01 M, 100 μ L

Tris-HCl pH 7.5 1 M, 1 mL

Sialidase buffer 5X 500 μ L
(250 mM Sodium Phosphate pH 5.0)

Storage: Store at 4°C. Do not freeze.

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

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Capacity

Each reaction will measure 1 - 200 nmoles of sialic acid. 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 sialic acid will cause a drop of 0.00622 absorbance units in a 1 mL reaction, a quantity reliably measured on a digital spectrophotometer.

Additional Required Equipment

A digital spectrophotometer is needed. Calculations in this kit are based on a 1 mL reaction volume using a cuvette with 1 cm pathlength. If different volumes or pathlength cuvettes are used, the results must be corrected (see troubleshooting).

Setting up the Assay

First, obtain an approximation of the amount of sialic acid in the sample to be quantitated either from the literature or 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 sialic acid, use the N-acetylneuraminic acid as a control. Addition of 10 μ L to the reaction is equivalent to approximately 100 nmoles. In the

assay of glycoproteins, use the bovine fetuin as control. When the entire sample is digested with α (2-3,6,8,9) Neuraminidase, the amount of sialic acid should be approximately 60 nmoles.

The accuracy of the procedures used with this kit depends on:

1. The quantitative generation of sialic acid 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.

Method 1 - Assay of Free Sialic Acid

Reagent Preparation

- Sialic acid Sample - The sample may be in solution or lyophilized. An amount equivalent to 1 – 200 nmoles of sialic acid is added to the reaction.
- Sialic Acid Control - Addition of 10 μ L to the reaction should result in a reading of approximately 100 nmoles.
- Tris Reaction Buffer – Dilute the 1 M Tris-HCl 40-fold with distilled water to make a 25 mM solution.
- β -NADH Solution - Just prior to use, add 640 μ L of Tris Reaction Buffer to 5 mg of β -NADH to make a 0.01 M solution ($A_{340} = 62.2$). Prepare solutions fresh daily. Prepared solutions should be discarded if A_{340} drops 20%, or solution turns yellow.
- N-acetylneuraminic acid Aldolase

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- L-Lactic Dehydrogenase

Procedure

1. Add the sample or control to Tris Reaction Buffer to a final volume of 980 μ L.
2. Pipette the reaction mixture into cuvette and blank the spectrophotometer. Add 20 μ L 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 approximately 1.25.
4. Return reaction mixture to the original tube. Add 1 μ L each of N-acetylneuraminic acid Aldolase and 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 sialic acid.

Calculation

Read and record the A_{340} Final. Calculate the nmoles of NANA:

nmoles sialic acid =

$$\frac{(A_{340} \text{ Initial} - A_{340} \text{ Final}) \times 1000}{6.22}$$

Method 2 – Multiple Tube Assay for Free Sialic Acid

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 control tube. Aliquot 1 mL of the reaction mix into each tube and add the sialic acid sample. Incubate in a 37°C water bath for 1 hour. Read the control (no sialic acid added) and then read the sample tubes. Subtract the sample readings from the control value to calculate the nmoles of sialic acid.

This method may be adapted to a microtiter plate format with a plate reader that measures absorbance at 340 nm. Include several control wells for calculating the starting absorbance. See Troubleshooting Guide for microtiter reader correction factor.

Method 3 - Assay of Glycoprotein or Polysialic Acid

The sample of interest is digested with a broad-spectrum sialidase to release the free sialic acid. Free sialic acid 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 by $\alpha(2-3,6,8,9)$ Neuraminidase should contain 1 - 200 nmoles of sialic acid.
- Bovine Fetuin – When the entire provided sample is digested with $\alpha(2-3,6,8,9)$ Neuraminidase the amount of free sialic acid released is approximately 60 nmoles.
- Tris Reaction Buffer - Dilute the 1 M Tris-HCl 40-fold with distilled water to make a 25 mM solution.

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- β -NADH Solution - Just prior to use, add 640 μ L of Tris Reaction Buffer to 5 mg of β -NADH to make 0.01 M solution ($A_{340} = 62.2$). Prepare solutions fresh daily. Prepared solutions should be discarded if A_{340} drops 20%, or solution turns yellow.
- $\alpha(2-3,6,8,9)$ Neuraminidase
- N-acetylneuraminic acid Aldolase
- L-Lactic Dehydrogenase
- Sialidase Buffer

Procedure

1. Dissolve the glycoprotein or polysialic acid in 40 μ L of distilled water and then add 10 μ L Sialidase Buffer.
2. Add 1 μ L $\alpha(2-3,6,8,9)$ Neuraminidase and incubate for at least 3 hours in a 37°C water bath.
3. Add 930 μ L of Tris Reaction Buffer to the tube.

NOTE: If glycoprotein precipitate forms, centrifuge sample to remove precipitate.

4. Pipette the reaction mix into cuvette and blank the spectrophotometer. Add 20 μ L β -NADH Solution and mix by inverting several times.
5. Read and record the initial β -NADH absorbance. Initial A_{340} should read approximately 1.25.
6. Return the reaction mix to the original tube. Add 1 μ L each of N-acetylneuraminic acid Aldolase and

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 mix back into the cuvette. Read and record the final A_{340} . Calculate the nmoles of sialic acid.

Calculation

nmoles sialic acid =

$$\frac{(A_{340} \text{ Initial} - A_{340} \text{ Final}) \times 1000}{6.22}$$

Method 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 sialic acid. The cells are treated with sialidase to release sialic acid, and then removed by centrifugation. Free sialic acid 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 sialic acid released is between 1 and 200 nmoles.
- Tris Reaction Buffer - Dilute the 1 M Tris-HCl 40 fold with distilled water to make a 25 mM solution.
- β -NADH Solution - Just prior to use, add 640 μ L of Tris Reaction Buffer to 5mg of β -NADH to make a 0.01 M solution

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($A_{340} = 62.2$). Prepare solutions fresh daily. Prepared solutions should be discarded if A_{340} drops 20%, or the solution turns yellow.

- $\alpha(2-3,6,8,9)$ Neuraminidase
- N-acetylneuraminic acid Aldolase
- L-Lactic Dehydrogenase
- Sialidase Buffer

Procedure

1. Wash cells in Tris Reaction Buffer and centrifuge.
2. Resuspend the cell pellet in 80 μL of distilled water and 20 μL Sialidase Buffer. Kill the cells by heating to 90°C for 5 minutes. Cool to room temperature.
3. Add 1 μL of $\alpha(2-3,6,8,9)$ 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 mix into a cuvette and blank the spectrophotometer. Add 20 μL β -NADH solution and mix by inversion several times.
7. Read and record the initial β -NADH absorbance. Initial A_{340} should read approximately 1.25.
8. Return reaction mix to tube. Add 1 μL each of N-acetylneuraminic acid Aldolase and 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 sialic acid.

Calculation

nmoles sialic acid =

$$\frac{(A_{340} \text{ Initial} - A_{340} \text{ Final}) \times 1000}{6.22}$$

Troubleshooting Guide

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

Samples 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 sialic acid.

Dilute samples – The amount of sialic acid added to the reaction may be too low to be measured in the assay (<1 nmole). Sample should be concentrated prior to assay.

Presence of α -keto acids – Samples may contain α -keto acids other than the pyruvic acid which is generated from sialic acids 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.

Incomplete digestion by $\alpha(2-3, 6, 8, 9)$ Neuraminidase – Sialidase digestion may not be complete if too much sample is added to the

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reaction or insufficient incubation time is allowed. The amount of sialic acid should be determined in a time course experiment to confirm that the digestion has gone to completion.

Pathlength and Volume corrections – For pathlengths different from 1 cm, multiply the results by a factor of 1 divided by the new pathlength in cms. For example, for a 5 mm pathlength, multiply the nanomoles of sialic acid by 2. For microtitre plate readers, you must determine the factor by reading a standard solution of NADH using a 1 cm cuvette and the microtiter plate reader and empirically determine the factor.

For reaction volumes different from 1 mL, multiply the result by the volume in mL. For example, multiply by 0.1 for a 0.1 mL reaction volume.

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