SialiQuant™ Sialic Acid Quantitation Kit

Part Number KE-SIALIQ
Certification of Analysis Lot Number 706.1A
Kit Storage Kits should be stored at 4°C.

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

In this 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 sialic acid found in nature are complexed in glycoconjugates. Sialic acid can be released through the action of sialidase and can be detected as free sialic acid. The kit includes Sialidase Au and procedures for the quantitation of total sialic acid in:

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

Enzymes

N-Acetylneuraminic Acid Aldolase
25 µL - ~100 U/ml
Sialidase Au (*A. ureafaciens*)
25 µL - 5 U/ml
L-Lactic Dehydrogenase
25 µL - 1000 U/ml
β-NADH, Disodium salt
25 mg
Fetuin, Bovine
0.5 mg
N-Acetylneuraminic Acid
0.01 M - 100 µL
Tris-HCl
1.0 M, pH 7.5 - 1 ml
Sialidase Buffer
250 mM sodium phosphate, pH 5 - 500 µL

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

KE-SIALIQ

Sialic Acid Quantitation Kit

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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

digital spectrophotometer

Note: The calculations in this kit are based on a 1 ml reaction volume using a cuvette with a 1 cm pathlength. If different volumes or pathlength cuvettes are used, the results must be corrected (see troubleshooting section).

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 from gel analysis after sialidase 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 solution as a control. Addition of 10 µl to the reaction is equivalent to approximately 100 nmoles. In the assay of glycoproteins, use the bovine fetuin as a control. When the entire sample is digested with Sialidase Au, 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.
- NANA Sialic Acid Control - Addition of 10 µl 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 µl 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 the A340 drops 20% or the solution turns yellow.
- N-Acetylneuraminic Acid Aldolase
- L-Lactic Dehydrogenase

Procedure

1. Add the sample or control to Tris Reaction Buffer to a final volume of 980 µL.
2. Pipette the reaction mixture into 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 A340 should read approximately 1.25.
4. Return reaction mixture to the original tube. Add 1 µL each of Nacetylneuraminic 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 A340. Calculate the nmoles of sialic acid.

Calculation

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

Method 2- HT 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 blank 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 30 minutes. Read the blank (no sialic acid added) and then read the sample tubes. Subtract the sample readings from the blank 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 readerv correction factor.

Method 3- Assay of Glycoprotein or Polysialic Acid

The sample of interest is digested with a broad spectrum sialidase to release 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 of β-NAD.

Reagent Preparation

- Sample of protein or polysialic acid after digestion with Sialidase Au should yield 1 - 200 nmoles of sialic acid.
- Bovine Fetuin - When the entire provided sample is digested with Sialidase Au 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.
- β-NADH Solution - Just prior to use, add 640 µl 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 the A_{340} drops 20% or the solution turns yellow.
- Sialidase Au
- N-Acetylneuraminic Acid Aldolase
- L-Lactic Dehydrogenase
- Sialidase Buffer

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 Sialidase Au 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 mix into cuvette and blank the spectrophotometer. Add 20 µL β-NADH Solution and mix by inverting several times.
5. Read and record the initial β-NADH absorbance. Initial A340 should read approximately 1.25.
6. Return the reaction mix to the original tube. Add 1 µL each of Nacetylneuraminic 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 A340. Calculate the nmoles of sialic acid.

Calculation

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

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 a broadspectrum 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 µl 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 the A_{340} drops 20% or the solution turns yellow.
- Sialidase Au
- N-Acetylneuraminic Acid Aldolase
- L-Lactic Dehydrogenase
- Sialidase Buffer (250 mM sodium phosphate, pH 5.0)

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 Sialidase Au 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 and equilibrate to 37 °C.
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 A340 should read approximately 1.25.
8. Return reaction mix to tube. Add 1 µL each of N-acetylneuraminic acid Aldolase andLactic Dehydrogenase and mix by inversion several times
9. Incubate in a 37°C water bath for a minimum of 1 hour.
10. Pipette the reactin mixture back into the cuvette. Read and record the final A340. Calculate the nmoles of sialic acid.

Calculation

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

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 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).

Presence of α-keto acids - Samples may contain α-keto acids other than the pyruvic acid generated from sialic acid 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 Sialidase Au - Sialidase digestion may not be complete if too much sample is added to the 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 determinind the factor.

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

References

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