



Sphingomyelin Assay Kit

Catalog Number KA1373

96 assays

Version: 04

Intended for research use only

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Introduction

Background

Sphingomyelin (ceramide phosphorylcholine) is an important lipid component of cell membranes and lipoproteins. It consists of a ceramide moiety linked *via* a phosphodiester bond to phosphorylcholine. Sphingomyelinases are a family of enzymes that can hydrolyze sphingomyelin into ceramide and phosphorylcholine (see Figure 1).¹ Ceramides have been implicated as key mediators in signaling pathways, with outcomes as diverse as cell proliferation, differentiation, growth arrest, and apoptosis.^{1,2} An inherited deficiency of acid sphingomyelinase activity results in the sphingomyelin storage disorder Niemann-Pick disease.³ This disease results in the accumulation of sphingomyelin in cells, tissues, and fluids. Since sphingomyelin has been implicated in the pathogenesis of several diseases, including atherosclerosis, sensitive and reliable techniques for its quantification are of considerable importance.⁴⁻⁶

Principle of the Assay

Sphingomyelin Assay Kit provides a specific, sensitive, and convenient method for quantifying sphingomyelin in plasma or serum. In this assay, sphingomyelinase is first used to hydrolyze sphingomyelin to phosphorylcholine and ceramide. Alkaline phosphatase then generates choline from the phosphorylcholine and the newly formed choline is used to generate hydrogen peroxide in a reaction catalyzed by choline oxidase. Finally, with peroxidase as a catalyst, hydrogen peroxide reacts with DAOS and 4-aminoantipyrine to generate a blue color with an optimal absorption at 595 nm (see Figure 2).⁷

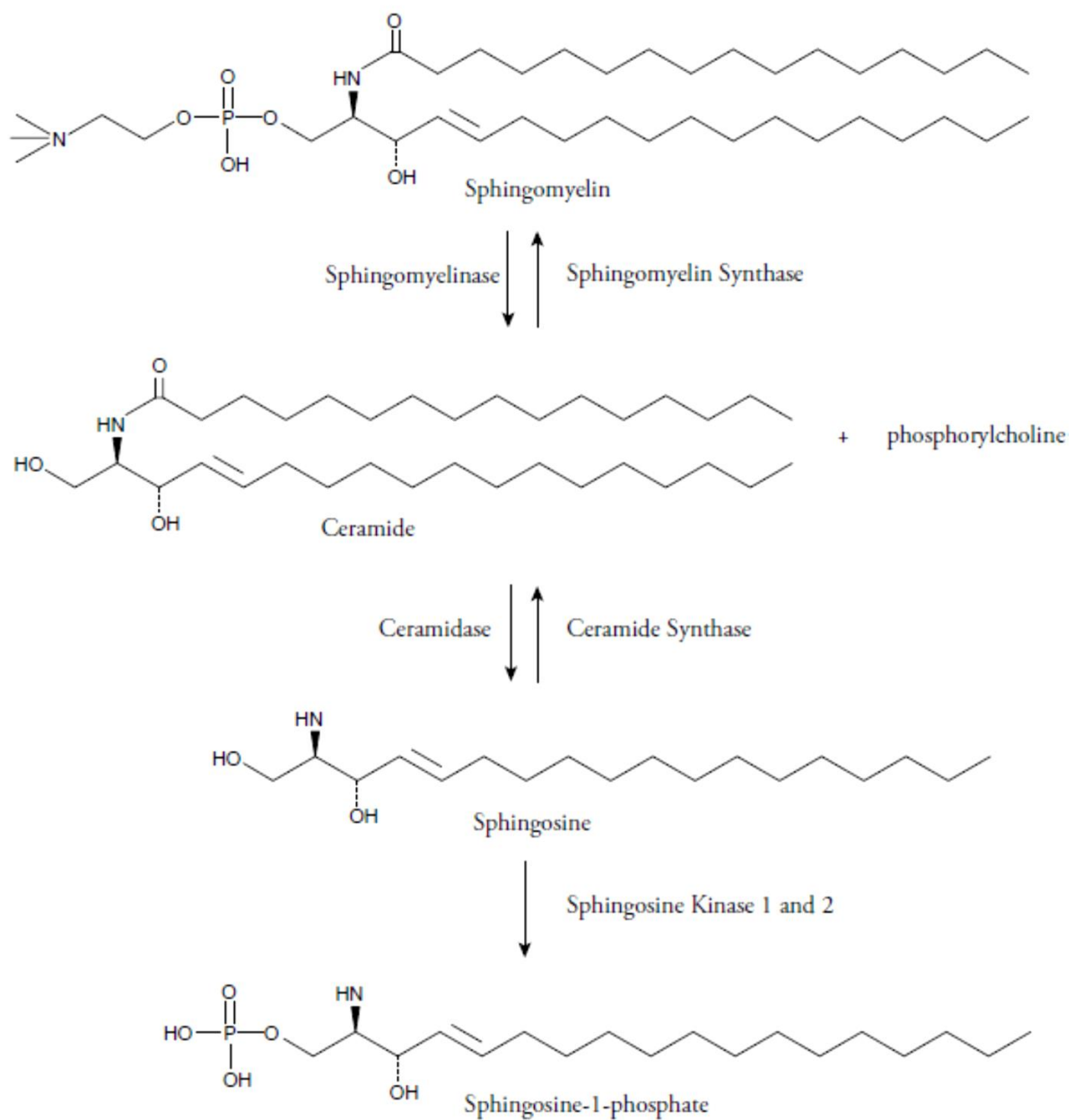


Figure 1. Ceramide/Sphingosine Metabolism

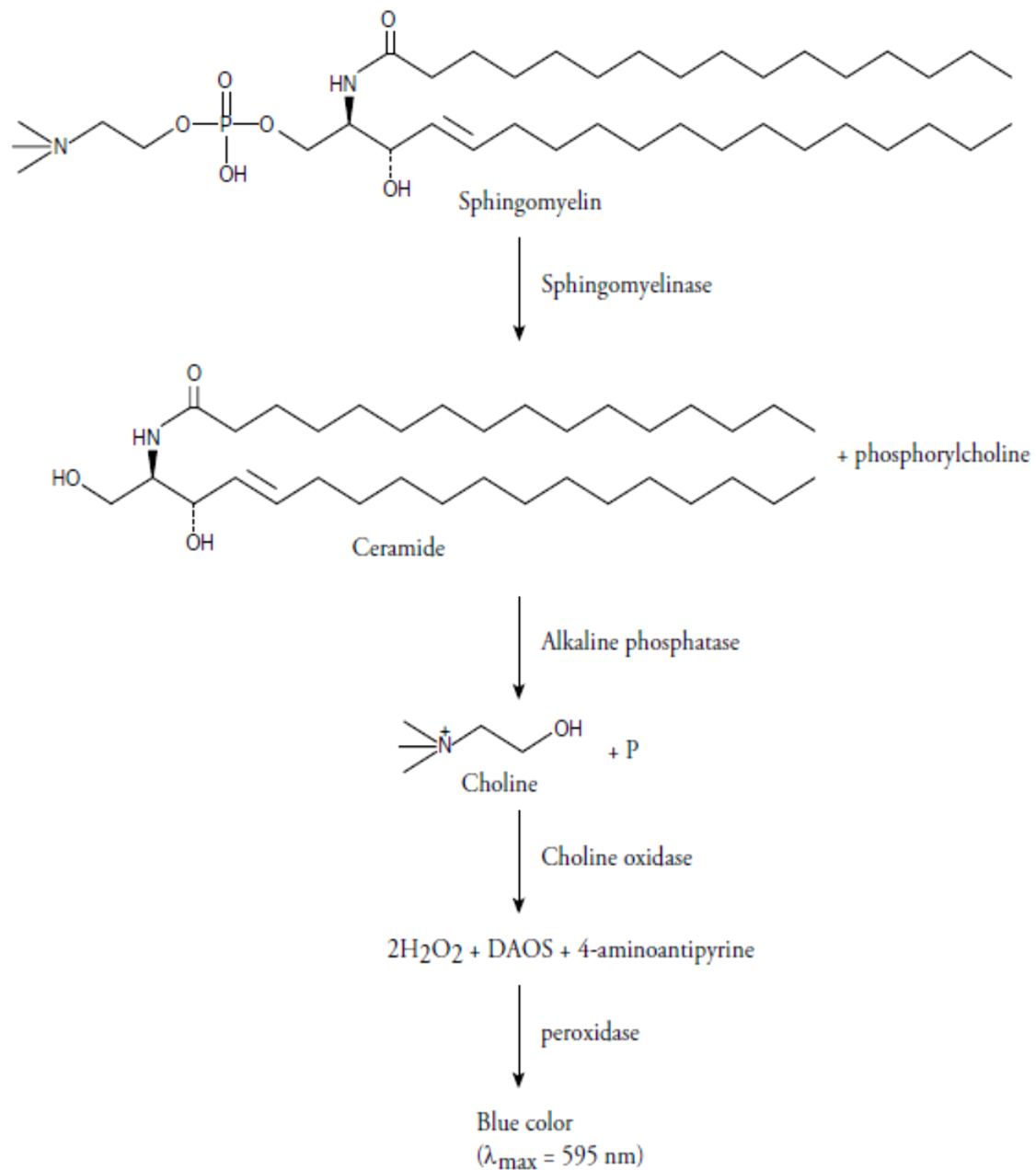


Figure 2. Assay Scheme

General Information

Materials Supplied

List of component

Component	Amount
SM Buffer (10X)	1 vial
SM Color Detector	2 vials
SM Enzyme Mixture	2 vials
SM Alkaline Phosphatase	1 vial
Sphingomyelinase	1 vial
Sphingomyelin Standard	1 vial
SM Detergent Solution	1 vial
96-Well Plate (Colorimetric Assay)	1 plate
96-Well Cover Sheet	1 cover

Storage Instruction

This kit will perform as specified if stored at 4°C and used before the expiration date indicated on the outside of the box.

Materials Required but Not Supplied

- ✓ A plate reader capable of measuring absorbance between 585-600 nm.
- ✓ Adjustable pipettes and a repeat pipettor.
- ✓ A source of pure water; glass distilled water or HPLC-grade water is acceptable.

Precautions for Use

- ✓ Please read these instructions carefully before beginning this assay.
- ✓ For research use only. Not for human or diagnostic use.
- ✓ Pipetting Hints
 - It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
 - Before pipetting each reagent, equilibrate the pipette tip in that reagent (i.e., slowly fill the tip and gently expel the contents, repeat several times).
 - Do not expose the pipette tip to the reagent(s) already in the well.

Assay Protocol

Reagent Preparation

- SM Buffer (10X) - Dilute 3 mL of SM Buffer concentrate with 27 mL of HPLC-grade water. This final buffer (50 mM Tris-HCl, pH 8.0, containing 0.66 mM CaCl_2) should be used for reconstituting the Color Reagent, Enzyme Mixture, Sphingomyelinase, and for diluting the Sphingomyelin Standard. When stored at 4°C, this diluted buffer is stable for at least six months.
- SM Color Detector - Each vial contains a lyophilized powder of DAOS (N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline) and 4-aminoantipyrine. Reconstitute the Color Detector in 3 mL of diluted SM Buffer. The reconstituted Color Detector is stable for eight hours at room temperature.
- SM Enzyme Mixture - Each vial contains a lyophilized powder of choline oxidase and horseradish peroxidase. Prior to use in the assay, reconstitute the vial contents with 1 mL of diluted SM Buffer. Store the reconstituted Enzyme Mixture on ice until ready to use. The reconstituted Enzyme Mixture is stable for 24 hours at 4°C.
- SM Alkaline Phosphatase - The vial contains a solution of alkaline phosphatase. It is ready to use as supplied.
- Sphingomyelinase - The vial contains a lyophilized powder of sphingomyelinase from *Bacillus cereus*. Reconstitute the vial contents with 1 mL of diluted SM Buffer. Store the enzyme on ice until ready to use. Unused enzyme should be frozen at -20°C. It will be stable for one month.
- Sphingomyelin Standard - The vial contains a lyophilized powder of sphingomyelin see Standard Preparation).
- SM Detergent Solution - The vial contains a Triton X-100 solution. It is ready to use as supplied.
- Standard Preparation
Weight out 5 mg of Sphingomyelin Standard into a glass test tube or vial that can hold 10 mL of solution. Dissolve the 5 mg of Standard in 10 mL of SM Detergent Solution to obtain a 50 mg/dL SM Stock Solution. Vortex until dissolved. The reconstituted SM Stock is stable for 24 hours at 4°C. Take seven clean glass test tubes and mark them A-G. Add the amount of SM Stock Standard and SM Buffer (dilute) to each tube as described in Table 1 (below). *NOTE: Bubbles may form in some of the standards, they will disperse in a few minutes and not affect the assay in any way. Diluted standards are stable for four hours.*

Tube	SM Stock Standard (μL) (50 mg/dL)	SM Buffer (μL)	SM Concentration (mg/dL)
A	0	500	0
B	50	450	5
C	100	400	10
D	200	300	20
E	300	200	30
F	400	100	40
G	500	0	50

Table 1. Sphingomyelin standards to be assayed along with plasma and serum samples.

✓ **Plate Set Up**

There is no specific pattern for using the wells on the plate. A typical layout of sphingomyelin standards and samples to be measured in duplicate is given in Plate Layout. We suggest you record the contents of each well on the template sheet.

• **General Information**

- ✓ The final volume of the assay is 110 μL in all wells.
- ✓ The incubation temperature is 22°C.
- ✓ It is not necessary to use all the wells on the plate at one time.
- ✓ It is recommended that the standards and samples be assayed at least in duplicate.
- ✓ Twenty-seven samples can be assayed in triplicate or forty-one in duplicate.
- ✓ Monitor the absorbance at 585-600 nm using a plate reader.

Sample Preparation

✓ **Plasma**

The typical concentration of sphingomyelin in human plasma is 25-60 mg/dL.^{5,6}

1. Collect blood using an anticoagulant such as heparin, EDTA, or citrate.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice. If not assaying the same day, freeze at -80°C. The plasma sample will be stable for one month while stored at -80°C.
3. Plasma does not need to be diluted before assaying.

✓ **Serum**

The typical concentration of sphingomyelin in human serum is 25-60 mg/dL.⁴

1. Collect blood without using an anticoagulant.
2. Allow blood to clot for 30 minutes at 25°C.
3. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The

serum sample will be stable for one month while stored at -80°C.

4. Serum does not need to be diluted before assaying.

Assay Procedure

1. Preparation of Reaction Mixture - To the SM Color Detector add the 1 mL of SM Enzyme Mixture, 0.5 mL of Sphingomyelinase, 10 μ L of SM Alkaline Phosphatase, and 490 μ L of diluted buffer for a final volume of 5 mL. This is enough Reaction Mixture to assay 50 wells. Prepare a second batch of Reaction Mixture using another set of reagent vials for additional samples. The Reaction Mixture is stable for 24 hours at 4°C.
2. Sphingomyelin Standard Wells - add 10 μ L of standard (tubes A-G) per well in the designated wells on the plate.
3. Sample Wells - add 10 μ L of sample (either undiluted plasma or serum) to two or three wells. *NOTE: The amount of sample added to the well should always be 10 μ L.*
4. Initiate the reactions by adding 100 μ L of Reaction Mixture to each well.
5. Carefully shake the microwell plate for a few seconds to mix. Cover with plate cover.
6. Incubate the plate on a shaker for 60 minutes at room temperature. Read the absorbance at a wavelength between 585-600 nm using a plate reader.

Data Analysis

Calculation of Results

1. Determine the average absorbance of each standard and sample.
2. Subtract the absorbance of standard A from itself and all other standards and samples to yield the corrected absorbance value (CAV).
3. Graph the CAV of the standards as a function of the final sphingomyelin concentration (mg/dL) from Table 1. See Figure 3 for a typical standard curve.
4. Calculate the sphingomyelin concentration of the original samples using the equation obtained from the linear regression of the standard curve by substituting the CAV for each sample into the equation. *NOTE: The sphingomyelin concentration is calculated back to the original sample and not what is in the well.*

$$\text{Sphingomyelin (mg/dL)} = \frac{(\text{CAV}) - (\text{y-intercept})}{\text{Slope}}$$

The standard curve presented here is an example of the data typically provided with this kit; however, your results will not be identical to these. You must run a new standard curve - do not use these to determine the values of your samples.

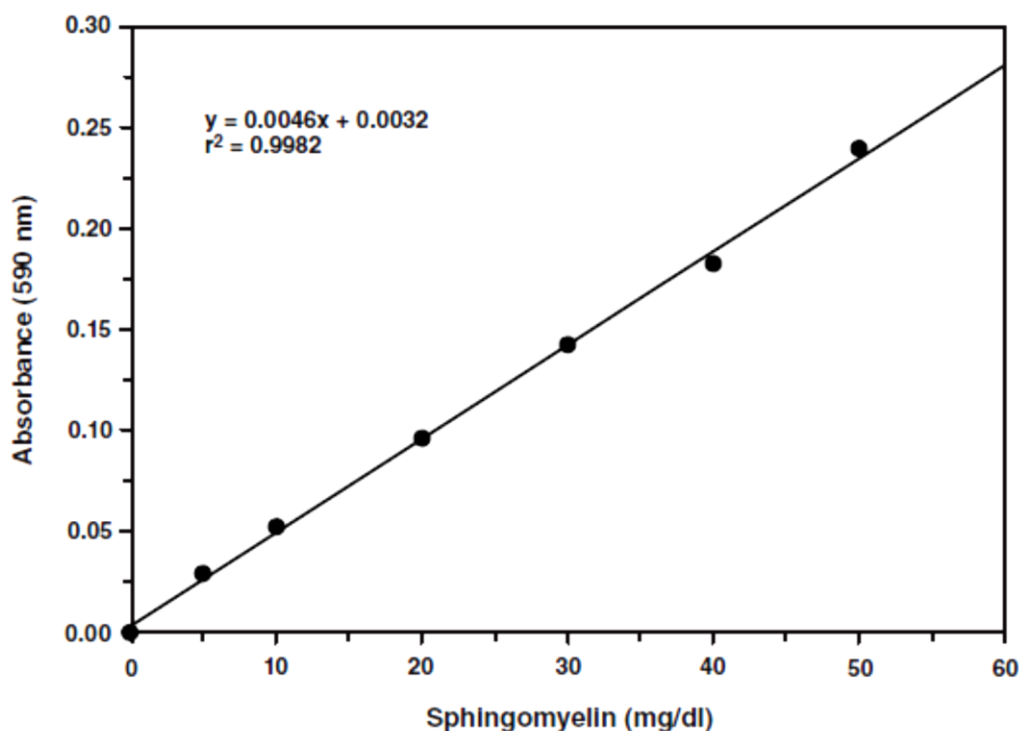


Figure 4. Sphingomyelin standard curve

Performance Characteristics

✓ Precision

When a series of 16 human plasma and serum samples were assayed on the same day, the intra-assay coefficient of variation was 3.9% and 2.9%, respectively. When a series of 16 human plasma and serum samples were assayed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 2.5% and 2.4%, respectively.

✓ Assay Range

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 5-50 mg/dL sphingomyelin.

Resources

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No color development in any of the wells	The reaction mixture was not prepared correctly	Make sure to follow the directions when preparing the reaction mixture and re-assay
No color development in the standard wells	The standards were not diluted correctly	Make sure to follow the directions when preparing the standards and re-assay

References

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Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard A	Standard A	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
B	Standard B	Standard B	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
C	Standard C	Standard C	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
D	Standard D	Standard D	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
E	Standard E	Standard E	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
F	Standard F	Standard F	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
G	Standard G	Standard G	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample