Product Manual

Sphingomyelin Assay Kit

Catalog Number

STA- 601 **96 assays**

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

Introduction

Phospholipids are important structural lipids that are the major component of cell membranes and lipid bilayers. Phospholipids contain a hydrophilic head and a hydrophobic tail which give the molecules their unique characteristics. Most phospholipids contain one diglyceride, a phosphate group, and one choline group. Sphingomyelin (ceramide phosphorylcholine) is a sphingolipid found in eukaryotic cell membranes and lipoproteins. Sphingomyelin usually consists of a ceramide and phosphorylcholine molecule where the ceramide core comprises of a fatty acid bonded via an amide bond to a sphingosine molecule. There is a polar head group which is either phosphphoethanolamine or phosphocholine. Sphingomyelin represents about 85% of all sphingolipids and makes up about 10-20% of lipids within the plasma membrane. Sphingomyelin is involved in signal transduction and is highly concentrated in the myelin sheath around many nerve cell axons. The plasma membranes of many cells are rich with sphingomyelin. Sphigolipids are synthesized in a pathway that originates in the ER and is completed in the Golgi apparatus. Many of their functions are done in the plasma membranes and endosomes.

Sphingomyelin is converted to ceramide via sphingomyelinases. Ceramides have been implicated in signaling pathways that lead to apoptosis, differentiation and proliferation. Sphingomyelins have been implicated in the pathogenesis of atherosclerosis, inflammation, necrosis, autophagy, senescence, stress response as well as other signaling disease states. Niemann-Pick disease is an inherited disease where deficiency of sphingomyelinase activity results in sphingomyelin accumulating in cells, tissues, and fluids. Other sphingolipid diseases are Fabry disease, Gaucher disease, Tay-Sachs disease, Krabbe disease and Metachromatic leukodystrophy.

Cell Biolabs' Sphingomyelin Assay Kit is a simple fluorometric assay that measures the amount of sphingomyelin present in plasma or serum, tissue homogenates, or cell suspensions in a 96-well microtiter plate format. Each kit provides sufficient reagents to perform up to 96 assays, including blanks, sphingomyelin standards and unknown samples. Sample sphingomyelin concentrations are determined by comparison with a known sphingomyelin standard.

Assay Principle

Cell Biolabs' Sphingomyelin Assay Kit measures the sphingomyelin present within serum, plasma, or tissue samples. The assay is based on the enzyme driven reaction that will detect sphingomyelin via the enzymatic activity of sphingomyelinase, alkaline phosphatase, and choline oxidase. First, sphingomyelinase hydrolyzes sphingomyelin into phosphorylcholine and the lipid ceramide. Alkaline phosphatase breaks down phosphorylcholine into choline, which is then oxidized by choline oxidase to produce hydrogen peroxide. The hydrogen peroxide is then detected with a highly specific fluorescence probe. Horseradish peroxidase catalyzes the reaction between the probe and hydrogen peroxide, which bind in a 1:1 ratio. Samples are compared to a known concentration of sphingomyelin standard within the 96-well microtiter plate format. Samples and standards are incubated for 60 minutes and then read with a standard 96-well fluorometric plate reader (Figure 1).

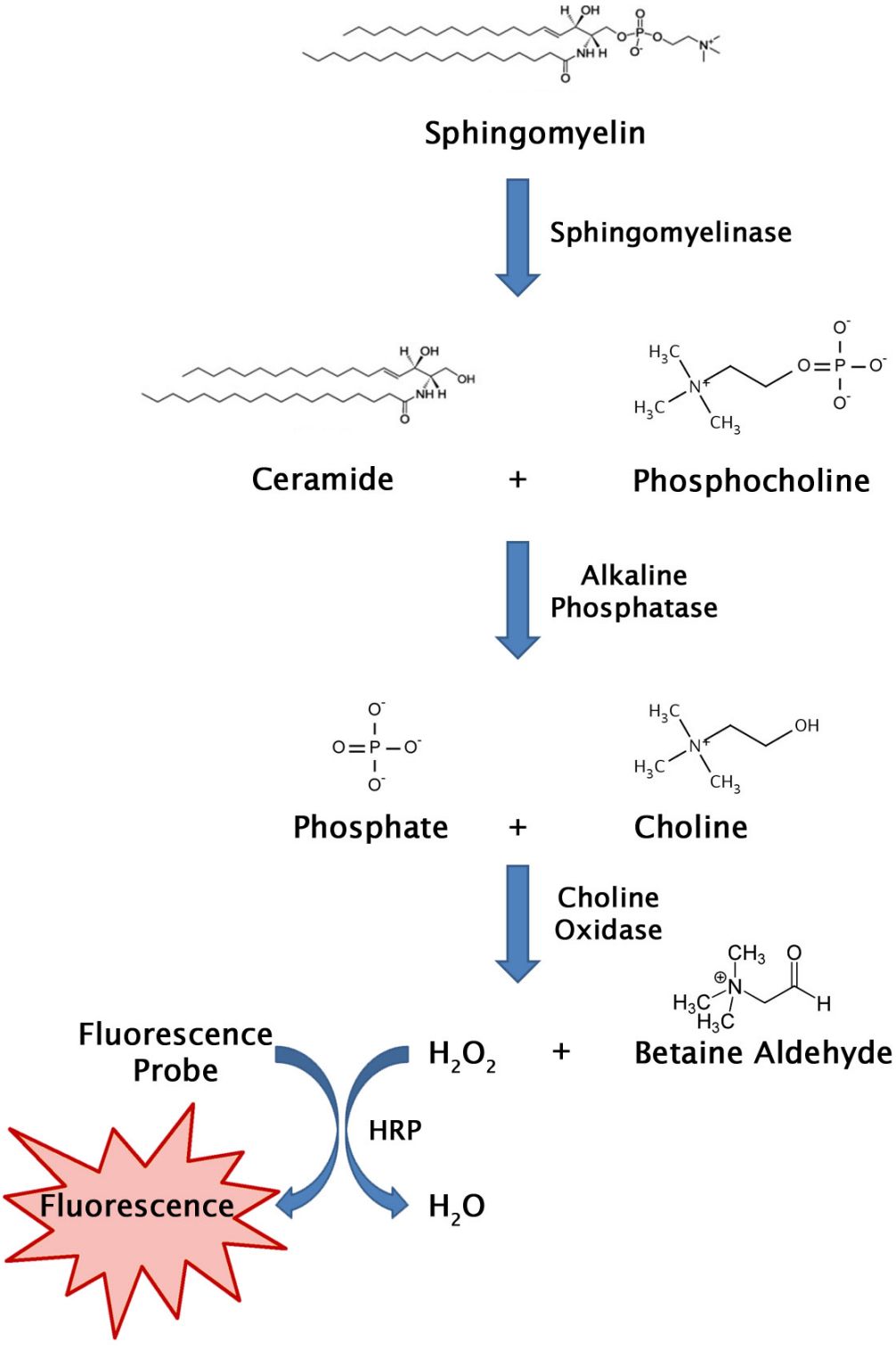

Figure 1. Sphingomyelin Assay Principle

Related Products

1. STA-361: Human ApoAI and ApoB Duplex ELISA Kit
2. STA-368: Human ApoB-100 ELISA Kit
3. STA-369: OxiSelect™ Human Oxidized LDL ELISA Kit (MDA-LDL Quantitation)
4. STA-384: Total Cholesterol Assay Kit (Colorimetric)
5. STA-390: Total Cholesterol Assay Kit (Fluorometric)
6. STA-391: HDL and LDL/VLDL Cholesterol Assay Kit
7. STA-394: HDL Cholesterol Assay Kit
8. STA-396: Serum Triglyceride Quantification Kit (Colorimetric)
9. STA-398: Free Glycerol Assay Kit (Colorimetric)
10. STA-600: Phosphatidylcholine Assay Kit (Fluorometric)

Kit Components

Box 1 (shipped at room temperature)

1. 96-well Microtiter Plate (Part No. 234501): One 96-well clear bottom black plate.
2. Assay Buffer (10X) (Part No. 260002): One 25 mL bottle.
3. Fluorescence Probe (100X) (Part No. 260003): One 100 µL tube in DMSO.
4. HRP (Part No. 234402): One 100 µL tube of 100 U/mL HRP solution in glycerol.
5. Standard Diluent (10X) (Part No. 260006): One 1 mL tube.

Box 2 (shipped on blue ice packs)

1. Sphingomyelin Standard (Part No. 260101): One 25 µL tube of a 10,000 mg/dL solution in ethanol.
2. Sphingomyelinase (400X) (Part No. 260102): One 25 µL tube.
3. Choline Oxidase (Part No. 260005): One 50 µL tube.
4. Alkaline Phosphatase (1000X) (Part No. 260103): One 10 µL tube.

Materials Not Supplied

1. Distilled or deionized water
2. Samples for testing and extraction reagents
3. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips
4. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
5. Multichannel micropipette reservoir

6. Fluorescence microplate reader capable of reading excitation in the 530-570 nm range and emission in the 590-600 nm range.
7. Superoxide dismutase (optional)

Storage

Upon receipt, store the Sphingomyelin Standard, Fluorescence Probe, HRP, Alkaline Phosphatase and Choline Oxidase at -20ºC. The Fluorescence Probe is light sensitive and must be stored accordingly. Avoid multiple freeze/thaw cycles. Store the remaining kit components at 4ºC.

Preparation of Reagents

- 1X Assay Buffer: Warm the Assay Buffer (10X) to room temperature prior to using. Dilute the Assay Buffer (10X) with deionized water by diluting the 25 mL Buffer with 225 mL deionized water for 250 mL total. Mix to homogeneity. Store the 1X Assay Buffer at 4ºC up to six months.
- 1X Standard Diluent: Warm the Standard Diluent (10X) to room temperature prior to using. Dilute the Standard Diluent (10X) with deionized water by diluting the 1 mL Diluent with 9 mL deionized water for 10 mL total. Vortex to homogeneity. Store the 1X Standard Diluent at 4ºC up to six months.
- Reaction Reagent: Prepare a Reaction Regent by diluting the Choline Oxidase 1:200, Alkaline Phosphatase 1:1000, HRP 1:500, Fluorescence Probe 1:100, and Sphingomyelinase 1:400 in 1X Assay Buffer. (eg. For 50 assays, combine 25 µL of Choline Oxidase, 5 µL Alkaline Phosphatase, 10 µL of HRP, 50 µL Fluorescence Probe, and 12.5 µL Sphingomyelinase with 1X Assay Buffer to 5 mL total solution). Mix thoroughly and protect the solution from light. For best results, place the Reaction Reagent on ice and use within 30 minutes of preparation. Do not store the Reaction Reagent solution.

  Note: The Fluorescence Probe is light sensitive and must be stored accordingly.

Preparation of Samples

Samples should be assayed immediately or stored at -80ºC prior to performing the assay. Optimal experimental conditions for samples must be determined by the investigator. The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design. A set of serial dilutions is recommended for samples to achieve optimal assay results and minimize possible interfering compounds. Run proper controls as necessary. Always run a standard curve with samples.

- Tissues or Cell Suspensions: Homogenize 250 mg of sample (wet tissue or cell pellet) in 4.5 mL of chloroform/methanol (2:1, v/v). Centrifuge to remove debris. After centrifugation, incubate the homogenate at room temperature for 1 hour on an orbital shaker. Induce phase separation by adding 1.25 mL dH_2O. Incubate 10 minutes at room temperature and centrifuge at 1000 x g for 10 minutes. Collect the lower (chloroform) organic phase and re-extract the upper phase with 2 mL of solvent mixture whose composition is $CHCl_3$/MeOH/water (86:14:1, v/v/v). Combine organic phases and dry in a vacuum centrifuge. Dissolve in 200 µL $CHCl_3$/MeOH/water (60:30:4.5, v/v/v) for storage. Before sphingomyelin assay, samples must be diluted at least 1:50 to 1:400 with Assay Buffer.
- Serum: Collect blood without using an anticoagulant. Allow blood to clot for 30 minutes at room temperature. Centrifuge at 2000 x g and 4ºC for 10 minutes. Remove the serum layer and store on

ice. Take care to avoid disturbing the white buffy layer. Aliquot samples for testing and store remaining solution at -80ºC. Perform serum dilutions in 1X Assay Buffer. Serum samples must be diluted at least 1:50 to 1:400 with Assay Buffer. This will provide values within the range of the standard curve.

- Plasma: Collect blood with heparin or citrate and centrifuge at 1000 x g and 4ºC for 10 minutes. Remove the plasma layer and store on ice. Take care to avoid disturbing the white buffy layer. Aliquot samples for testing and store remaining solution at -80ºC. Perform plasma dilutions in 1X Assay Buffer. Plasma samples must be diluted at least 1:50 to 1:400 with Assay Buffer. This will provide values within the range of the standard curve.

  Notes:

  1. *Samples with NADH concentrations above 10 µM and glutathione concentrations above 50 µM will oxidize the probe and could result in erroneous readings. To minimize this interference, it is recommended that superoxide dismutase (SOD) be added to the reaction at a final concentration of 40 U/mL.*
  2. *Avoid samples containing DTT or β-mercaptoethanol since the fluorescence probe is not stable in the presence of thiols (above 10 µM).*
  3. *Choline can generate high background if present in samples. If choline may be present, run a background control without Sphingomyelinase or Alkaline Phosphatase. Subtract this value from sample reading values.*

Preparation of Sphingomyelin Standard Curve

1. Prepare fresh sphingomyelin standards by first diluting a portion of the 10,000 mg/dL Sphingomyelin Standard stock solution 1:100 in 1X Standard Diluent. (eg. Add 5 µL of Sphingomyelin Standard in 495 µL Standard Diluent). Vortex thoroughly. This provides a 100 mg/dL concentration. Use this 100 mg/dL solution to prepare a series of the remaining sphingomyelin standards according to Table 1 below.

Tubes	100 mg/dL Sphingomyelin Standard (µL)	1X Assay Buffer (µL)	Resulting Sphingomyelin Concentration (mg/dL)
1	100	400	20
2	250 of Tube #1	250	10
3	250 of Tube #2	250	5
4	250 of Tube #3	250	2.5
5	250 of Tube #4	250	1.25
6	250 of Tube #5	250	0.625
7	250 of Tube #6	250	0.313
8	0	250	0

Table 1. Preparation of Sphingomyelin Standards.

Note: Do not store diluted sphingomyelin standard solutions.

Assay Protocol

Each sphingomyelin standard and sample should be assayed in duplicate or triplicate. A freshly prepared standard curve should be used each time the assay is performed.

1. Add 10 µL of the diluted sphingomyelin standards or samples to the 96-well microtiter plate.
2. Add 100 µL of the prepared Reaction Reagent to each well and mix the well contents thoroughly.
3. Cover the plate wells to protect the reaction from light. Incubate the plate for 60 minutes at 37ºC.
4. Read the plate with a fluorescence microplate reader equipped for excitation in the 530-570 nm range and for emission in the 590-600 nm range.
5. Calculate the concentration of sphingomyelin within samples by comparing the sample RFU to the sphingomyelin standard curve.

Example of Results

The following figures demonstrate typical Sphingomyelin Assay results. One should use the data below for reference only. This data should not be used to interpret or calculate actual sample results.

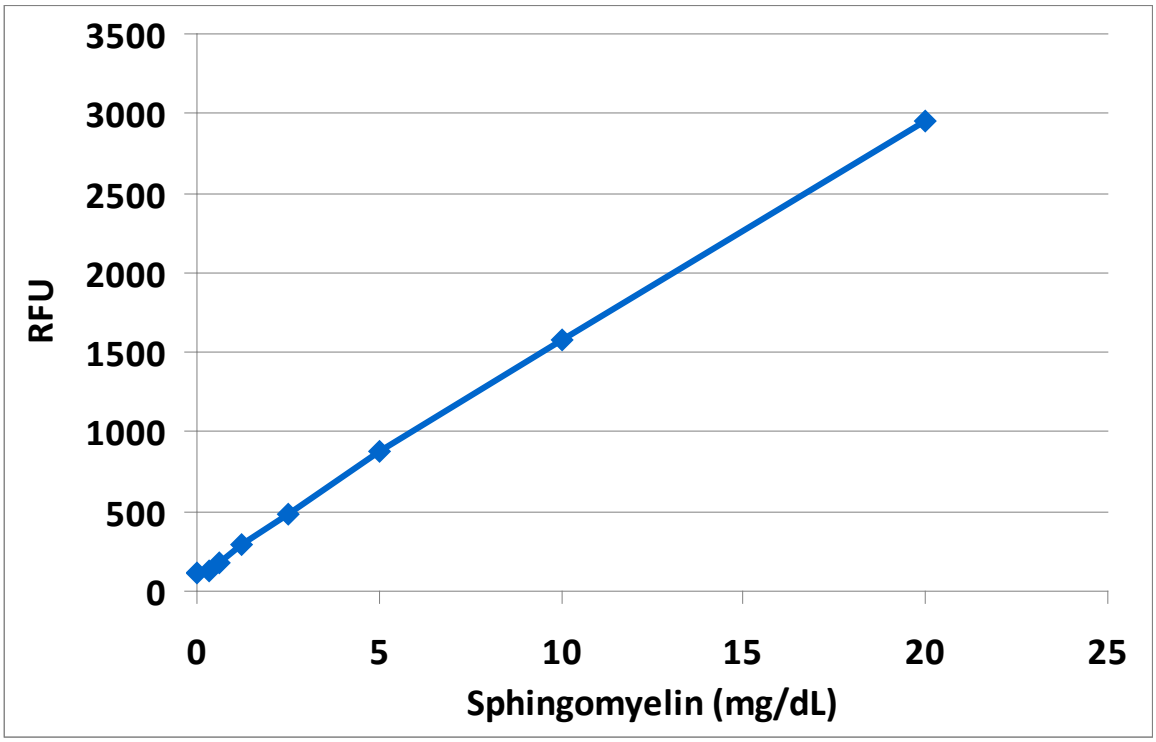

Figure 2: Sphingomyelin Standard Curve.

Calculation of Results

1. Calculate the average fluorescence values for every standard, control, and sample. Subtract the average zero standard value from itself and all standard and sample values. This is the corrected fluorescence.
2. Plot the corrected fluorescence for the standards against the final concentration of the sphingomyelin standards from Table 1 to determine the best curve. See Figure 2 for an example standard curve.

3. Determine the sphingomyelin concentration of the samples with the equation obtained from the linear regression analysis of the standard curve. Substitute the corrected fluorescence values for each sample. Remember to account for dilution factors.

$$\textbf{Sphingomyelin (mg/dL)} = \left[\frac{\textbf{Sample corrected fluorescence}}{\textbf{Slope}}\right] \textbf{ x Sample dilution}$$

References

1. Hojjati, M.R., et al. (2006) *J. Lipid Res*. **47(3)**: 673-676.
2. Jiang, X.-C., et al. (2000) *Arterioscler. Thromb. Vasc. Biol.* **20(12)**: 2614-2618.
3. Riboni, L., et al. (1997) *Prog. Lipid Res.* **36**: 153-195.
4. Testi, R. (1996) *Trends Biochem. Sci.* **21**: 468-471.

Recent Product Citations

1. Jacob, S. et al (2017). Assessment of plasma biomarkers for their association with Multiple Sclerosis progression. *Journal of Neuroimmunology.* **305**:5-8. http://dx.doi.org/10.1016/j.jneuroim.2017.01.008.
2. Winkler, E.A. et al. (2014). Blood-spinal cord barrier disruption contributes to early motor-neuron degeneration in ALS-model mice. *PNAS* **111**:E1035-E1042.

Warranty

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Contact Information

Cell Biolabs, Inc.
7758 Arjons Drive
San Diego, CA 92126
Worldwide: +1 858 271-6500
USA Toll-Free: 1-888-CBL-0505
E-mail: tech@cellbiolabs.com
www.cellbiolabs.com