Amplite™ Fluorimetric Acidic Sphingomyelinase Assay Kit *Red Fluorescence*

Catalog number: 13622
Unit size: 200 Tests

Component	Storage	Amount
Component A: Enzyme Mix	Freeze (<-15 °C), Dessicated, Avoid Light	2 bottles (lyophilized powder)
Component B: Sphingomyelin	Freeze (<-15 °C), Avoid Light	1 vial (100 µL)
Component C: Amplite™ Red	Freeze (<-15 °C), Dessicated, Avoid Light	1 vial (lyophilized powder)
Component D: SMase Reaction Buffer	Freeze (<-15 °C), Avoid Light	1 bottle (10 mL)
Component E: Assay Buffer	Freeze (<-15 °C), Avoid Light	1 bottle (10 mL)
Component F: DMSO	Freeze (<-15 °C), Avoid Light	1 vial (200 µL)

OVERVIEW

Sphingomyelinase (SMase) is an enzyme that is responsible for cleaving sphingomyelin (SM) to phosphocholine and ceramide. Activation of SMases plays an important role in the cellular response such as regulation of cell growth, cell differentiation, cell cycle arrest and programmed cell death. Five types of sphingomyelinase (SMase) have been identified based on their cation dependence and pH optima of action, including lysosomal acid SMase, secreted zinc-dependent acid SMase, magnesium-dependent neutral SMase, magnesium-independent neutral SMase and alkaline SMase. Among them, the lysosomal acidic SMase and the magnesium-dependent neutral SMase are considered to be the major factors for the production of ceramide in cellular stress responses. Our Amplite™ Fluorimetric Acidic Sphingomyelinase Assay Kit provides one of the most sensitive methods for detecting acidic SMase activity or screening its inhibitors. The kit uses Amplite™ Red as a fluorogenic probe to indirectly quantify the phosphocholine produced from the hydrolysis of sphingomyelin (SM) by sphingomyelinase (SMase). It can be used for measuring the SMase activity in blood, cell extracts or other solutions. The fluorescence intensity of Amplite™ Red is proportional to the formation of phosphocholine, therefore to the SMase activity. The kit is an optimized "mix and read" assay compatible with HTS liquid handling instruments.

AT A GLANCE

Protocol summary

1. Prepare sphingomyelin working solution (50 µL)
2. Add acidic SMase standards or SMase test samples (50 µL)
3. Incubate at 37°C for 2 - 3 hours
4. Add sphingomyelinase working solution (50 µL)
5. Incubate at RT for 1 - 2 hours
6. Monitor fluorescence increase at Ex/Em = 540/590 nm (cut off at 570 nm)

Important Thaw 1 vial (or bottle) of each kit component to room temperature before starting your experiment.

KEY PARAMETERS

Instrument:	Fluorescence microplate reader
Excitation:	540 nm
Emission:	590 nm
Cutoff:	570 nm
Recommended plate:	Solid black

PREPARATION OF STOCK SOLUTIONS

Unless otherwise noted, all unused stock solutions should be divided into single-use aliquots and stored at -20 °C after preparation. Avoid repeated freeze-thaw cycles.

1. Amplite™ Red stock solution (200X):
Add 80 µL of DMSO (Component F) into the vial of Amplite™ Red (Component C) to make 200X Amplite™ Red stock solution.

Note The Amplite™ Red is unstable in the presence of thiols (such as DTT and 2-mercaptoethanol). The final concentration of DTT or 2-mercaptoethanol in the reaction should be lower than 10 µM. Amplite™ Red is also unstable at high pH (>8.5). The reactions should be performed at pH 7 – 8. The assay buffer at pH 7.4 is recommended.

PREPARATION OF STANDARD SOLUTION

Acidic sphingomyelinase standard

For convenience, use the Serial Dilution Planner:
https://www.aatbio.com/tools/serial-dilution/13622

Dilute acidic sphingomyelinase stock solution in 20 mM sodium acetate buffer (pH = 5.0, not provided in the kit). We recommend the concentration range from 10 U/mL to 0.5 U/mL.

Note Acidic sphingomyelinase standard (from human placenta) can be obtained from Sigma-Aldrich (S-5383). Diluted acidic sphingomyelinase standard solution is unstable, and should be used within 4 hours.

PREPARATION OF WORKING SOLUTION

1. Sphingomyelin working solution:
Add 50 µL of Sphingomyelin (Component B) to 5 mL of SMase Reaction Buffer (Component D) and mix well.

Note The sphingomyelin working solution should be used promptly.

2. Sphingomyelinase working solution:
Add 5 mL of Assay Buffer (Component E) to the bottle of Enzyme Mix (Component A) and mix well. Add 25 µL of 200X Amplite™ Red stock solution into the bottle of Enzyme Mix solution to make the sphingomyelinase working solution before starting the assay.

Note The sphingomyelinase working solution should be used promptly and kept from light; longer storage is likely to cause a higher assay background.

SAMPLE EXPERIMENTAL PROTOCOL

Table 1. Layout of acidic sphingomyelinase standards and test samples in a solid black 96-well microplate. SMase = Acidic Sphingomyelinase Standards (SMase1-SMase7, 0.5 to 10 U/mL); BL = Blank Control; TS = Test Samples.

BL	BL	TS	TS

SMase1	SMase1	...	...
SMase2	SMase2	...	...
SMase3	SMase3		
SMase4	SMase4		
SMase5	SMase5		
SMase6	SMase6		
SMase7	SMase7		

Table 2. Reagent composition for each well

Well	Volume	Reagent
SMase1 - SMase7	50 µL	Serial Dilution (0.5 to 10 U/mL)
BL	50 µL	20 mM Sodium Acetate Buffer (pH = 5)
TS	50 µL	Test Sample

1. Add the acidic sphingomyelinase standards and sphingomyelinase-containing test samples into a solid black 96-well microplate as shown in Tables 1 and 2. For a 384-well plate, use 25 µL of reagent per well instead of 50 µL.

   Note Treat your cells or tissue samples as desired.

2. Add 50 µL of sphingomyelin working solution into each well of the sphingomyelinase standards, blank control and test samples. Add the diluted acidic sphingomyelinase standards in duplicate. For a 384-well plate, add 25 µL of sphingomyelin working solution into each well instead, for a total volume of 50 µL/well.

3. Incubate the reaction mixture at 37°C for 2 - 3 hours.

4. Add 50 µL of sphingomyelinase working solution into each well of the acidic sphingomyelinase standards, blank control, and test samples to make the total sphingomyelinase assay volume of 150 µL/well. For a 384-well plate, add 25 µL sphingomyelinase assay working solution into each well instead, for the total sphingomyelin assay volume of 75 µL/well.

5. Incubate the enzyme reaction mixture for 1 - 2 hours at room temperature (protected from light).

6. Monitor the fluorescence increase with a fluorescence microplate reader at Ex/Em = 540/590 nm (cut off at 570 nm).

EXAMPLE DATA ANALYSIS AND FIGURES

The reading (RFU) obtained from the blank standard well is used as a negative control. Subtract this value from the other standards' readings to obtain the base-line corrected values. Then, plot the standards' readings to obtain a standard curve and equation. This equation can be used to calculate Sphingomyelin samples. We recommend using the Online Linear Regression Calculator which can be found at:

https://www.aatbio.com/tools/linear-logarithmic-semi-log-regression-online-calculator/

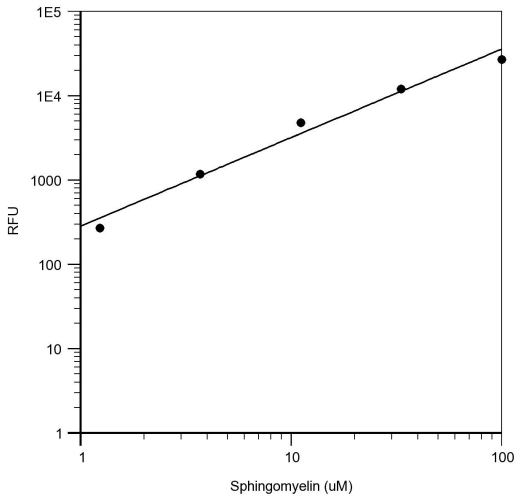

Figure 1. Sphingomyelin dose response was measured on a solid black 96-well plate with Amplite™ Fluorimetric Sphingomyelin Assay Kit using a Gemini fluorescence microplate reader (Molecular Devices).

DISCLAIMER