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), Minimize light exposure	2 bottles (lyophilized powder)
Component B: Sphingomyelin	Freeze (< -15 °C), Minimize light exposure	1 vial (100 µL)
Component C: Amplite™ Red	Freeze (< -15 °C), Minimize light exposure	1 vial (lyophilized powder)
Component D: SMase Reaction Buffer	Freeze (< -15 °C), Minimize light exposure	1 bottle (10 mL)
Component E: Assay Buffer	Freeze (< -15 °C)	1 bottle (10 mL)
Component F: DMSO	Freeze (< -15 °C)	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 acidic SMase standards or SMase test samples (50 µL)
2. Add sphingomyelin working solution (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

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.

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 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

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

Acidic sphingomyelinase standard
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

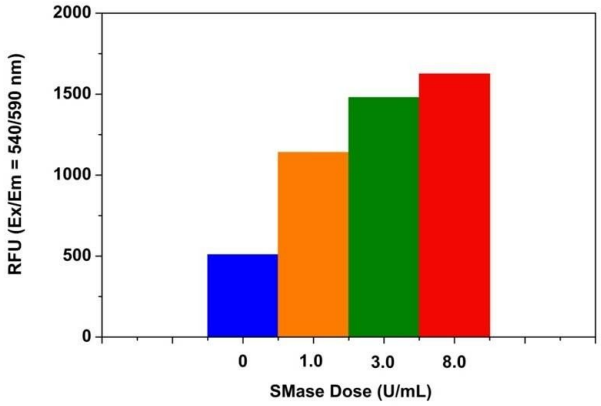

Figure 1. Sphingomyelinase (from human placenta) dose response was measured on a 96-well half-area black plate with Amplite™ Fluorimetric Acidic Sphingomyelinase Assay Kit (13622) using a fluorescence microplate reader. 20 µL of SMase standard or control was incubated with 20 µL of sphingomyelin working solution at 37 °C for 3 hours, and then 20 µL of sphingomyelinase assay mixture was added into each well. The signals shown in the figure are the readings at Ex/Em = 540/590 nm (cut off at 570 nm) after 2 hours incubation at room temperature.

DISCLAIMER

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