Amplite™ Fluorimetric Sphingomyelinase Assay Kit *Red Fluorescence*

Catalog number: 13621
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)	1 bottle (20 mL)
Component F: Sphingomyelinase Standard	Freeze (<-15 °C), Dessicated, Avoid Light	0.2 unit (lyophilized powder)
Component G: DMSO	Freeze (<-15 °C)	1 vial (200 µL)

OVERVIEW

Five types of sphingomyelinase (SMase) have been identified based on their cation dependence and pH optima of action. They are lysosomal acid SMase, secreted zinc-dependent acid SMase, magnesium-dependent neutral SMase, magnesium-independent neutral SMase, and alkaline SMase. Among the five types, the lysosomal acidic SMase and the magnesium-dependent neutral SMase are considered major candidates for the production of ceramide in the cellular response to stress. Our Amplite™ Fluorimetric Sphingomyelinase Assay Kit provides the most sensitive method for detecting neutral 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. Amplite™ Red enables the assay readable either in fluorescence intensity or absorption mode. The kit is an optimized "mix and read" assay that can be used for real time monitoring of Smase activities. Our kit 13622 has been developed for monitoring acid SMase activity.

AT A GLANCE

Protocol summary

1. Prepare Sphingomyelin working solution (50 µL)
2. Add SMase standards and/or SMase test samples (50 µL)
3. Incubate at 37°C for 1 - 2 hours
4. Add Sphingomyelinase working solution (50 µL)
5. Incubate at RT for 1 - 2 hours
6. Monitor fluorescence intensity at Ex/Em = 540/590 nm (Cutoff = 570 nm)

Important Thaw one vial (or bottle) of each kit component at 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. Sphingomyelinase standard solution (10 U/mL):

Add 20 µL of PBS with 0.1% BSA into the vial of Sphingomyelinase Standard (Component F) to make a 10 units/mL Sphingomyelinase standard solution.

2. Amplite™ Red stock solution (200X):
Add 80 µL of DMSO (Component G) into the vial of Amplite™ Red (Component C) to make 200X Amplite™ Red stock solution. Keep from light.

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. pH 7.4 is recommended for the assay buffer.

PREPARATION OF STANDARD SOLUTION

Sphingomyelinase standard

For convenience, use the Serial Dilution Planner:
https://www.aatbio.com/tools/serial-dilution/13621

Add 1 µL of 10 units/mL Sphingomyelinase standard solution into 1000 µL Assay Buffer (Component E) to generate a 10 mU/mL Sphingomyelinase standard solution. Take 10 mU/mL Sphingomyelinase standard solution and perform 1:2 serial dilutions to get serially diluted Sphingomyelinase standards (SMase7 - SMase1).

Note Diluted Sphingomyelinase standard solution is unstable. Use within 4 hours.

PREPARATION OF WORKING SOLUTION

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

Note The Sphingomyelin working solution should be used promptly.

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

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SAMPLE EXPERIMENTAL PROTOCOL

Table 1. Layout of Sphingomyelinase standards and test samples in a solid black 96-well microplate. SMase = Sphingomyelinase Standards (SMase1 - SMase7, 0.078 to 5 mU/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 Dilutions (0.078 to 5 mU/mL)
BL	50 µL	Assay Buffer
TS	50 µL	test sample

1. Prepare Sphingomyelinase standards (SMase), blank controls (BL), and test samples (TS) according to the layout provided 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 to each well of Sphingomyelinase standard, blank control, and test samples to make the total Sphingomyelin assay volume of 100 µL/well. 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 1 - 2 hours.

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

5. Incubate the 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 (Cutoff = 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 Sphingomyelinase 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

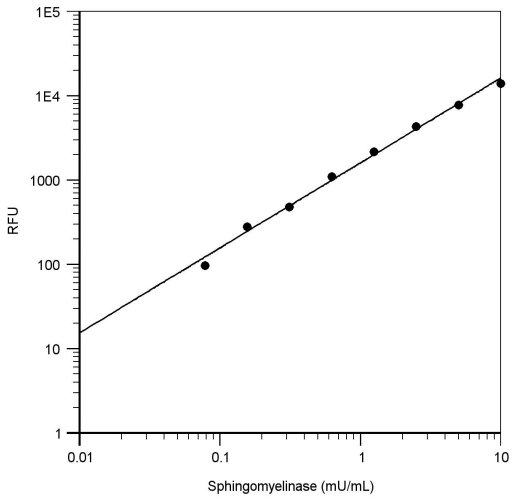

Figure 1. Sphingomyelinase dose response was measured on a solid black 96-well plate with Amplite™ Fluorimetric Sphingomyelinase Assay Kit using a Gemini fluorescence microplate reader (Molecular Devices).

DISCLAIMER