Amplite™ Fluorimetric Sphingomyelinase Assay Kit
Red Fluorescence

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 13621 (200 assays)	Keep in freezer and avoid exposure to light	Fluorescence microplate readers

Introduction

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 mode or in 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.

Kit Key Features

Broad Application:	Used for quantifying acidic sphingomyelinase in blood, cell extracts and solutions.
Sensitive:	Detect as low as of 0.15 mU/mL sphingomyelinase in solution.
Continuous:	Easily adapted to automation without a separation step.
Convenient:	Formulated to have minimal hands-on time.

Kit Components

Components	**Amount**
Component A: Enzyme Mix	2 bottles (lyophilized powder)
Component B: Sphingomyelin	1 vial (100 µL)
Component C: Amplite™ Red	1 vial (lyophilized powder)
Component D: SMase Reaction Buffer	1 bottle (10 mL)
Component E: Assay Buffer	1 bottle (20 mL)
Component F: Sphingomyelinase Standard	0.2 unit (lyophilized powder)
Component G: DMSO	1 vial (200 µL)

Assay Protocol for One 96-well Plate

Brief Summary

Prepare sphingomyelin working solution (50 µL) → Add SMase standards and/or SMase test samples (50 µL) → Incubate at 37 °C for 1-2 hours→ Add sphingomyelinase assay mixture (50 µL) → Incubate at RT for 1-2 hours → Monitor fluorescence intensity at Ex/Em = 540/590 nm (cut off at 570 nm)

Note: Thaw one vial (or bottle) of each kit component at room temperature before starting your experiment.

1. Prepare sphingomyelin working solution:

Add 50 µL of Sphingomyelin (Component B) into 5 mL SMase Reaction Buffer (Component D), and mix well.
Note: The sphingomyelin working solution should be used promptly.

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2. Prepare sphingomyelinase standards and/or sphingomyelinase-containing samples:

2.1 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 stock solution.
Note: The unused sphingomyelinase standard stock solution should be aliquoted and stored at -20°C.

2.2 Add 1 µL of 10 units/mL sphingomyelinase standard stock solution (from Step 2.1) into 1000 µL assay buffer (Component E) to generate a 10 mU/mL sphingomyelinase standard.
Note: Diluted sphingomyelinase standard stock solution is unstable, should be used within 4 hours.

2.3 Take 500 µL of 10 mU/mL sphingomyelinase standard to perform 1 to 2 serial dilutions to get 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, and 0 mU/mL serially diluted sphingomyelinase standards.

2.4 Add the serially diluted sphingomyelinase standards and/or sphingomyelinase-containing test samples into a solid black 96-well microplate as shown in Tables 1 and 2.
Note: Treat your cells or tissue samples as desired.

Table 1 Layout of sphingomyelinase standards and test samples in a solid black 96-well microplate

BL	BL	TS	TS	….	….						
SMase 1	SMase 1	….	….	….	….						
SMase 2	SMase 2										
SMase 3	SMase 3										
SMase 4	SMase 4										
SMase 5	SMase 5										
SMase 6	SMase 6										
SMase 7	SMase 7										

Note: SMase = Sphingomyelinase Standards, BL = Blank Control, TS = Test Samples.

Table 2 Reagent composition for each well

Sphingomyelinase Standards	**Blank Control**	**Test Sample**
Serial Dilutions: 50 µL	Assay buffer: 50 µL	50 µL

Note: Add the serially diluted sphingomyelinase standards from 0.078 to5 mU/mL into wells from SMase 1 to SMase 7 in duplicate.

2.5 Add 50 µL of sphingomyelin working solution (from Step 1) into each well of sphingomyelinase standards, blank control and test samples (from Step 2.4).

2.6 Incubate the reaction mixture at 37 °C for 1-2 hours.

3. Prepare 200X Amplite™ Red stock solution:

Add 80 µL of DMSO (Component G) into the vial of Amplite™ Red (Component C) to make 200X Amplite™ Red stock solution.
Note 1: The unused Amplite™ Red stock solution should be aliquoted and stored at -20°C (kept from light).

Note 2: 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.

4. Prepare sphingomyelinase assay mixture:

4.1 Add 5 mL of Assay Buffer (Component E) into the bottle of Enzyme Mix (Component A), and mix them well.

4.2 Add 25 µL of 200X Amplite™ Red stock solution (from Step 3) into the bottle of Enzyme Mix solution (from Step 4.1) to make the sphingomyelinase assay mixture before starting the assay.
Note: The sphingomyelinase assay mixture should be used promptly and kept from light; longer storage is likely to cause high assay background.

5. Run sphingomyelinase assay:

5.1 Add 50 µL of sphingomyelinase assay mixture (from Step 4.2) into each well of sphingomyelinase standards, blank control, and test samples (from Step 2.4) to make the total sphingomyelinase assay volume of 150 µL/well. *Note: For a 384-well plate, add 25 µL of sample, 25 µL of sphingomyelin working solution, and 25 µL of sphingomyelinase assay mixture into each well.*

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

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

Data Analysis

The fluorescence in blank wells (with the assay buffer only) is used as a control, and is subtracted from the values for those wells with the sphingomyelinase reactions. A sphingomyelinase standard curve is shown in Figure 1.

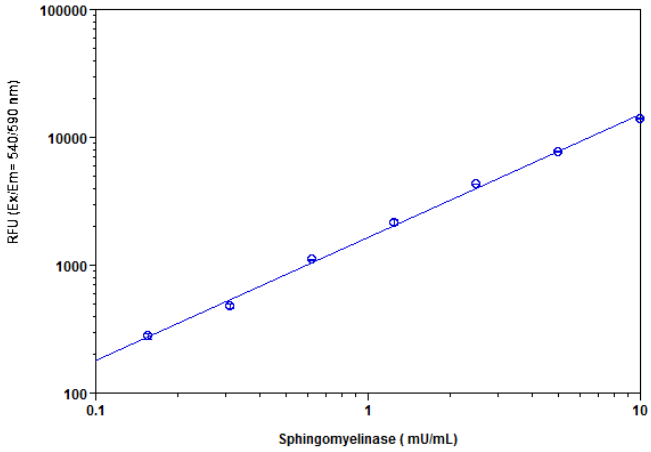

Figure 1 Sphingomyelinase dose response was measured on a 96-well black plate with Amplite™ Fluorimetric Sphingomyelinase Assay Kit using a Gemini fluorescence microplate reader (Molecular Devices). As low as 0.15 mU/mL sphingomyelinase can be detected with 60 minutes incubation (n=3). *Note: The fluorescence background increases with time. It is important to subtract the fluorescence intensity value of the blank wells for each data point.*

References

1. Kentaro Hanada, et al. (2000). "Neutral sphingomyelinase activity dependent on Mg2+ and anionic phospholipids in the intraerythrocytic malaria parasite Plasmodium falciparum". Biochem. J. (2000) 346, 671-677.
2. Bin Liu, et al. (1998). "Purification and Characterization of a Membrane Bound Neutral pH Optimum Magnesium-dependent and Phosphatidylserine-stimulated Sphingomyelinase from Rat Brain". The Journal of Biological Chemistry, (1998) 273(51), 34472–34479