Amplite™ Fluorimetric Sphingomyelin Assay Kit

Red Fluorescence

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 13625 (100 assays)	Keep in freezer and avoid exposure to light	Fluorescence microplate readers

Introduction

Sphingomyelin (SM) is largely found in the exoplasmic leaflet of the cell membrane, primarily in nervous tissue. It plays an important role in signal transduction. Sphingomyelin accumulates abnormally in Niemann-Pick disease and Abetalipoproteinemia.

Our Amplite™ Fluorimetric Sphingomyelin Assay Kit provides the most sensitive method for detecting neutral SM activity or screening SM 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 amount of SM in blood, cell extracts or other solutions. The fluorescence intensity of Amplite™ Red is proportional to the formation of phosphocholine, therefore to the amount of SM. Amplite™ Red enables the assay readable by either a fluorescence reader or an absorbance reader. The kit is an optimized "mix and read" assay that can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation without a separation step.

Kit Key Features

Broad Application:	Used for quantifying sphingomyelin in blood, cell extracts and solutions.
Sensitive:	Detect as low as 1 µM sphingomyelin 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	1 bottle (lyophilized powder)
Component B: Sphingomyelinase	1 vial (lyophilized powder)
Component C: Amplite™ Red	1 vial (lyophilized powder)
Component D: SMase Reaction Buffer	1 bottle (20 mL)
Component E: Assay Buffer	1 bottle (5 mL)
Component F: 50 mM Sphingomyelin	1 vial (20 µL)
Component G: DMSO	1 vial (200 µL)

Assay Protocol for One 96-well Plate

Brief Summary

Prepare SMase working solution (50 µL) → Add sphingomyelin standards or test samples (50 µL) → Incubate at 37 °C for 1 - 2 hours → Add sphingomyelin assay mixture (50 µL) → Incubate at RT for 0.5 - 2 hours → Monitor fluorescence intensity at Ex/Em = 540/590 nm (cut off at 570 nm)

Note: Thaw kit components at room temperature before starting your experiment.

1. Prepare Sphingomyelinase (SMase) working solution:

1.1 Prepare 100X SMase stock solution by adding 50 µL of PBS with 0.1% BSA into the vial of Sphingomyelinase (Component B).

Note: The unused 100X SMase stock solution should be aliquoted and stored at -20°C

Ordering: sales@aatbio.com; Tel: 800-990-8053 or 408-733-1055; Fax: 408-733-1304
Technical Support: support@aatbio.com; Tel: 408-733-1055

1.2 Prepare SMase working solution by adding the whole content (50 µL) of 100X SMase stock solution (from Step 1.1) into 5 mL of SMase Reaction Buffer (Component D), and mix well.
Note: The SMase working solution should be used promptly.

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

2.1 Add 2 µL of 50 mM Sphingomyelin (Component F) into 1000 µL of SMase Reaction Buffer (Component D) to get a 100 µM Sphingomyelin standard solution.

2.2 Take 200 µL of 100 µM Sphingomyelin standard solution to perform 1:3 serial dilutions to get 30, 10, 3, 1, 0.3, 0.1 and 0 µM serially diluted sphingomyelin standards.

2.3 Add the sphingomyelin standards and sphingomyelin-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 sphingomyelin standards and test samples in a solid black 96-well microplate

BL	BL	TS	TS	….	….						
SM 1	SM 1	….	….	….	….						
SM 2	SM 2										
SM3	SM3										
SM4	SM4										
SM5	SM5										
SM6	SM6										
SM 7	SM 7										

Note: SM = Sphingomyelin Standards, BL = Blank Control, TS = Test Samples.

Table 2 Reagent composition for each well

Sphingomyelin Standards	**Blank Control**	**Test Sample**
Serial Dilutions: 50 µL	SMase Reaction Buffer: 50 µL	50 µL

Note: Add the serially diluted sphingomyelin standards from 0.1 to100 µM into wells from SM 1 to SM 7 in duplicate.

2.4 Add 50 µL of SMase working solution (from Step 1.2) into each well of sphingomyelin standards, blank control and test samples (from Step 2.3).

2.5 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 sphingomyelin assay mixture:

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

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

5. Run sphingomyelin assay:

5.1 Add 50 µL of sphingomyelin assay mixture (from Step 4.2) into each well of sphingomyelin standards, blank control, and test samples (from Step 2.5) 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 sphingomyeliase working solution and 25 µL of sphingomyelin 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 sphingomyelin reactions. A sphingomyelin standard curve is shown in Figure 1.

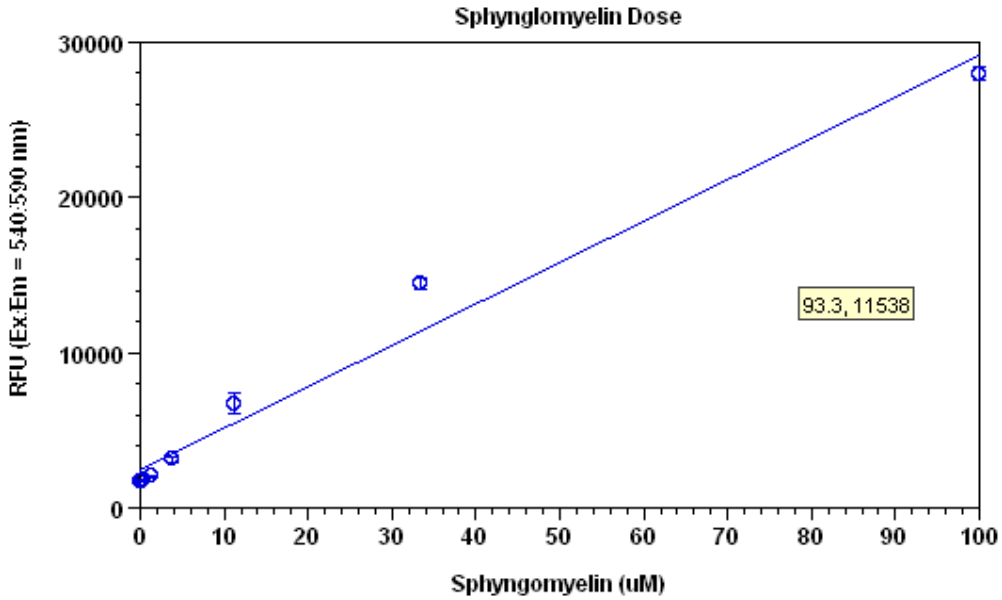

Figure 1 Sphingomyelin dose response was measured on a solid black 96-well plate with Amplite™ Fluorimetric Sphingomyelin Assay Kit using a Gemini fluorescence microplate reader (Molecular Devices). As low as 1 µM sphingomyelin can be detected with 60 minutes incubation (n=3). *Note: The fluorescence 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