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Sphingomyelinase Activity Assay Kit (Fluorometric)

rev 08/20

(Catalog # K574-100; 100 assays; Store at -20°C)

I. Introduction:

Sphingomyelinase (SMase) cleaves sphingomyelin to produce phosphocholine and ceramide. The activation of SMase leads to increased production of ceramide, which acts as a lipid second messenger that induces a variety of cell regulatory phenomena such as apoptosis, cell differentiation, cell proliferation and sterol homeostasis. **BioVision's Sphingomyelinase Activity Assay Kit** provides a simple and sensitive method for measuring neutral-SMase enzymatic activity using fluorescence (Ex/Em = 535/587 nm). Neutral sphingomyelinase (N-SMase) is a Mg^{2+} sensitive enzyme that can be activated by a host of physiologically relevant and structurally diverse molecules. In this assay, SMase converts sphingomyelin substrate to phosphocholine and ceramide, which is then further utilized to produce fluorescence. This high-throughput adaptable assay kit can detect sphingomyelinase activity as low as 10 µU/ml in a variety of samples.

II. Application:

- Measurement of SMase activity in various tissues/cells extracts

III. Sample Types:

- Animal tissues: brain, heart, kidney, etc.
- Cell culture: Adherent or suspension cells

IV. Kit Contents:

Components	K574-100	Cap Code	Part Number
SMase Assay Buffer	28 ml	WM	K574-100-1
SMase Probe (DMSO)	0.2 ml	Red	K574-100-2A
SMase Substrate (Lyophilized)	1 vial	Purple	K574-100-3
SMase Enzyme Mix I (Lyophilized)	1 vial	Green	K574-100-4
SMase Enzyme Mix II (Lyophilized)	1 vial	White	K574-100-5
Choline Standard (Lyophilized)	1 vial	Yellow	K574-100-6
SMase Positive Control (Lyophilized)	1 vial	Orange	K574-100-7
SMase Extraction Detergent	1 ml	Amber	K574-100-8

V. User Supplied Reagents and Equipment:

- 96-well clear plate with flat bottom
- Multi-well spectrophotometer

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protected from light. Briefly centrifuge small vials prior to opening. Read the entire protocol before performing the assay.

- **SMase Assay Buffer:** Warm SMase Assay Buffer to room temperature (RT) before use. Store at 4°C or -20°C.
- **SMase Probe:** Store at -20°C. Avoid light exposure. Warm to RT before use. Use within two months.
- **SMase Substrate:** Reconstitute with 110 µl SMase Assay Buffer. Store at -20°C. Keep on ice while in use. Use within two months.
- **SMase Enzyme Mix I:** Reconstitute with 220 µl SMase Assay Buffer. Store at -20°C. Keep on ice while in use. Use within two months.
- **SMase Enzyme Mix II:** Reconstitute with 1.1 ml of SMase Assay Buffer. Store at -20°C. Use within two months.
- **Choline Standard:** Reconstitute with 100 µl SMase Assay Buffer to generate 50 mM Choline Standard stock. Store at -20°C. Use within two months.
- **SMase Positive Control:** Reconstitute with 100 µl of SMase Assay Buffer. Aliquot and store at -20°C. Use within two months.
- **SMase Extraction Detergent:** Store at RT. Vortex quickly before use.

VII. Sphingomyelinase Assay Protocol:

1. **Sample Preparation:** Add 92 µl of SMase Assay Buffer and 8 µl of SMase Extraction Detergent to 10 mg of sample (wet weight or cell pellet). Homogenize on ice using a Dounce homogenizer (BV Cat.# 1998). Centrifuge at 10,000 X g, 4°C for 5 min. Collect the supernatant. Add 5-50 µl of supernatant into desired well(s) in 96-well plate and adjust the volume to 50 µl with SMase Assay Buffer. Add 5-10 µl of SMase Positive Control into desired positive control well(s) and adjust the final volume to 50 µl with SMase Assay Buffer.

   Notes:

   a. We recommend adding Protease Inhibitor Cocktail (Cat.# K271-500) in 1:1000 ratio while preparing the samples.

   b. Cell & tissue lysate can be stored at -80°C for future experiments.

   c. For Unknown Samples, we suggest to do a pilot experiment & test several doses to ensure the readings are within the Standard Curve range.

   d. For samples having high background, prepare parallel well(s) containing the same amount of sample as in the test well. Adjust the volume to 50 µl with SMase Assay Buffer.

2. **Standard Curve Preparation:** Dilute Choline Standard to 0.5 mM by adding 10 µl of 50 mM Choline Standard into 990 µl of SMase Assay Buffer and mix well. Dilute further to 50 µM by adding 10 µl of 0.5 mM Choline Standard into 90 µl of SMase Assay Buffer and

mix well. Add 0, 2, 4, 6, and 8 µl of the diluted 50 µM Choline Standard into a series of wells in 96-well plate. Adjust the volume to 50 µl/well with the SMase Assay Buffer to generate 0, 100, 200, 300 and 400 pmol/well of Choline Standard.

3. **Reaction Mix:** Mix enough reagents for the number of assays (samples, Standards & Positive Control) to be performed. Dilute SMase Probe 1:10 (1 µl Probe in 9 µl SMase Assay Buffer) before use. For each well, prepare 50 µl Reaction Mix containing:

	Reaction Mix	*Background Control Mix
SMase Assay Buffer	35 µl	36 µl
SMase Enzyme Mix I	2 µl	2 µl
SMase Enzyme Mix II	10 µl	10 µl
SMase Substrate	1 µl	---
SMase Probe	2 µl	2 µl

Mix well. Add 50 µl of Reaction Mix to each well containing Choline Standards, Positive Control and samples. Mix well.

* For samples having high background, add 50 µl of Background Control Mix to Sample Background Control well(s). Mix well.

4. **Measurement:** Incubate for 30 min. at 37°C and measure fluorescence (Ex/Em = 535/587 nm).

Note: Incubation time depends on the SMase Activity in the samples. We recommend measuring fluorescence in kinetic mode, and choosing two time points (T_1 and T_2) in the linear range to calculate the SMase activity of the samples. We recommend running the assay for at least 1 hr. in kinetic mode. The Standard Curve can be read in end point mode (i.e. at the end of incubation time).

5. **Calculations:** Subtract 0 Choline Standard reading from all readings. Plot the Choline Standard Curve. If the Sample Background Control reading is significant, subtract Background Control reading from sample readings. Calculate SMase activity of test sample: $\Delta RFU = RFU_2 - RFU_1$. Apply ΔRFU to Standard Curve to get B pmol of Choline generated by SMase during the reaction time ($\Delta T = T_2 - T_1$).

Sample Sphingomyelinase Activity = B/(ΔT X V) x D = pmol/min/ml = µU/ml

Where: **B** = SMase amount from the Standard Curve (pmol)
ΔT = reaction time (min.)
V = sample volume added into the reaction well (ml)
D = sample dilution factor

Sphingomyelinase specific activity can be expressed as mU/mg or as µU/mg of protein.

Unit Definition: One unit of SMase activity is the amount of enzyme that generates 1.0 µmol of Choline per min. at pH 7.4 at 37°C.

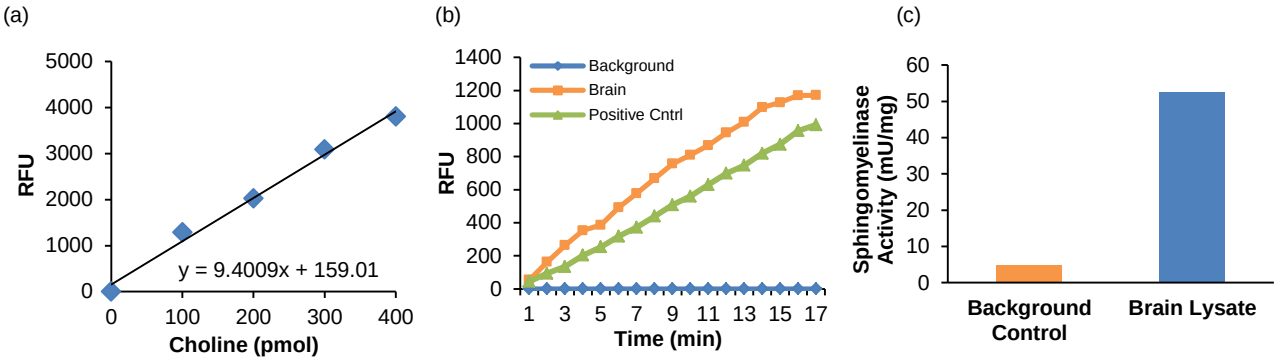

Figure: (a) Choline Standard Curve, (b) SMase activity of brain lysate (0.15 mg) & Positive Control (5 µl) & (c) SMase specific activity in rat brain lysate. Assays were performed following the kit protocol.

VIII. RELATED PRODUCTS:

Choline/Acetylcholine Quantification Colorimetric/Fluorometric Kit (K615)
Free Fatty Acid Quantification Colorimetric/Fluorometric Kit (K612)
HDL and LDL/VLDL Quantification Colorimetric/Fluorometric Kit (K613)
Triglyceride Quantification Colorimetric/Fluorometric Kit (K622)
CETP Antibody (3413) Sphingosine Kinase Inhibitor, SKI-II (2047)
C2-Dihydroceramide (2391) α-Galactosylceramide (2152)
Dounce Tissue Homogenizer (1998)
Sphingomyelinase Activity Colorimetric Assay Kit (K599)
Phosphatidylcholine Colorimetric/Fluorometric Kit (K576)
Sphingosine Kinase 1 (SPK1) Antibody (3883)
Sphingosine Kinase 2 (SPK2) Antibody (3884)
Sphingosine Kinase Inhibitor, SKI-I (2046)
EZLys™ Mammalian Protein Extraction Reagent (8004)
C2 Ceramide (2390)

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