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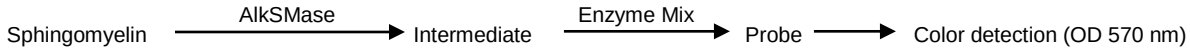
Alkaline Sphingomyelinase Activity Assay Kit (Colorimetric)

rev 08/20

(Catalog # K987-100; 100 assays; Store at -20°C)

Introduction:

I. Sphingomyelinase (SMase, E.C. 3.1.4.12) cleaves sphingomyelin to produce phosphocholine and ceramide. Both of these molecules can act as second messengers and regulate downstream events including apoptosis, cell proliferation, and sterol homeostasis. The sphingomyelinase family of enzymes includes acid sphingomyelinase, found in the lysosome and also secreted, neutral sphingomyelinase, which is enriched in the brain, and alkaline sphingomyelinase (AlkSMase), which acts in the intestinal tract. Unlike the neutral variety (nSMase), AlkSMase shows optimal activity at higher pH and does not require Mg^{2+} for enzymatic activity. AlkSMase, also referred to as NPP7, because of its homology to the Nucleotide Pyrophosphatase/Phosphodiesterase family of enzymes, has been implicated in prevention of tumor formation. Its activity is decreased in some intestinal cancers, as wells as instances of chronic colitis. Alkaline Sphingomyelinase may also play a role in cholesterol absorption. BioVision's Alkaline Sphingomyelinse Colorimetric Assay Kit provides a simple, high throughput adaptable means to identify and quantify alkaline sphingomyelinase activity in a variety of samples without influence from neutral or acid sphingomyelinase. Upon incubation with substrate, the hydrolysis produces an intermediate that reacts with the probe, generating a colorimetric signal. The assay can detect as low as 0.5 µU of alkaline sphingomyelinase activity.



II. Applications:

- Measurement of AlkSMase/NPP7 activity in various tissues/cell extracts
- Characterization of AlkSMase activity of purified recombinant enzyme

III. Sample Type:

- Animal tissues: liver, intestine, etc.
- Purified Enzyme Preparations

IV. Kit Contents:

Components	K987-100	Cap Code	Part Number
AlkSMase Assay Buffer	25 ml	WM	K987-100-1
AlkSMase Developer Buffer	5 ml	NM	K987-100-2
AlkSMase Probe (in DMSO)	200 µl	Red	K987-100-3
AlkSMase Substrate (Lyophilized)	1 vial	Blue	K987-100-4
AlkSMase Enzyme Mix I (Lyophilized)	1 vial	Green	K987-100-5
AlkSMase Enzyme Mix II (Lyophilized)	1 vial	White	K987-100-6
Choline Standard (Lyophilized)	1 vial	Yellow	K987-100-7
AlkSMase Positive Control (Lyophilized)	1 vial	Orange	K987-100-8

V. User Supplied Reagents and Equipment:

- 96-well clear plate with flat bottom, multi-well spectrophotometer, Eppendorf tubes (0.6 or 1.5 ml)

VI. Storage Conditions and Reagent Preparation:

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

- **AlkSMase Assay Buffer and Developer Buffer:** Warm to room temperature (RT) before use. Store at -20ºC.
- **AlkSMase Probe:** Store at -20°C. Warm to RT before use. Use within two months.
- **Substrate:** Reconstitute with 550 µl **Assay Buffer**. Gently pipette to dissolve completely. Store at -20°C. Use within two months.
- **Enzyme Mix I:** Reconstitute with 220 µl **Developer Buffer**. Store at -20°C. Keep on ice while in use. Use within two months.
- **Enzyme Mix II:** Reconstitute with 1.1 ml **Developer Buffer**. Store at -20°C. Keep on ice while in use. Use within two months.
- **Choline Standard:** Reconstitute with 100 µl **Assay Buffer** to generate 50 mM Choline stock. Store at -20°C. Use within two months.
- **AlkSMase Positive Control:** Add 55 µl **AlkSMase Assay Buffer** to the Positive Control and mix thoroughly. Aliquot and store at –20°C. Keep on ice while in use. Use within two months.

VII. Alkaline Sphingomyelinase Assay Protocol:

1. **Sample Preparation:** Add 50 µl of AlkSMase Assay Buffer per 10 mg of sample (wet weight or cell pellet). *See note (a) below*. Homogenize on ice using a Dounce homogenizer (Cat. # 1998). Centrifuge at 10,000 X g for 5 min. at 4°C. Collect the supernatant. Add 5-10 µl of supernatant into an Eppendorf tube (0.6 or 1.5 ml) and adjust the volume to 50 µl with AlkSMase Assay Buffer. For each sample, prepare identical background control reaction in a separate tube. For Positive Control: add 5 µl of AlkSMase Positive Control into a Eppendorf tube and adjust the final volume to 50 µl with AlkSMase Assay Buffer.

   Notes:

   a. We recommend adding Protease Inhibitor Cocktail (BV Cat.# K271-500) at a 2X final concentration while preparing the samples.

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

   c. For Unknown Samples, we recommend doing a pilot experiment testing several doses to ensure that readings are within the range of the Standard Curve.

   d. For samples exhibiting significant background (i.e. tissue lysates), prepare parallel sample reactions without the substrate (sphingomyelin) as Background Controls.

e. We recommend filtration of small molecules that may interfere with the assay. This can be accomplished by concentrating with BioVision 10k spin column (Cat.# 1997). Spin a desired volume to concentrate the protein, then dilute the ultraconcentrate back to the original volume with fresh **AlkSMase Assay Buffer**.

2. Substrate Mix: Add 5 µl of the Resuspended **AlkSMase Substrate** to each reaction tube containing Positive Control or Sample. Add 5 µl AlkSMase Assay Buffer to the Background Control for each Sample tested.

	Sample Activity Reaction Mix	Sample Background Reaction Mix
AlkSMase Reaction	50 µl	50 µl
AlkSMase Assay Buffer	-	5 µl
AlkSMase Substrate	5 µl	-

Incubate all reactions at 37°C for 30-60 minutes. For a Positive Control, run assay for 1 hr. After desired length of time, boil all samples at ≥ 98°C for 20 minutes to stop any enzymatic activity. Place the tubes on ice for 5 minutes. Spin down the tubes briefly and add 50 µl of each sample to an individual well in a 96-well clear flat-bottom plate.

Note: For Samples with low AlkSMase Activity, longer incubation times (> 1 hr.) may be required.

3. Standard Curve Preparation: Dilute Choline Standard to 0.5 mM by adding 10 µl of 50 mM Choline Standard into 990 µl AlkSMase Assay Buffer. Add 0, 2, 4, 6, 8 and 10 µl of the diluted Choline Standard into a series of wells in a 96-well plate to generate 0, 1, 2, 3, 4, and 5 nmol per well of Choline Standard. Bring the total volume in each well to 50 µl with Assay Buffer.

4. Development: Mix enough reagents for the number of assays to be performed, including Standards. For each well, prepare 50 µl Mix containing:

	Reaction Mix
AlkSMase Developer Buffer	36 µl
AlkSMase Enzyme Mix I	2 µl
AlkSMase Enzyme Mix II	10 µl
AlkSMase Probe	2 µl

Mix and add 50 µl of the Reaction Mix to each well containing the Choline Standards, Positive Control and Test Samples. Mix well.

5. Measurement: Incubate plate for 60 min. at 37°C and read absorbance at 570 nm.

6. Calculation: Subtract 0 Standard reading from all readings. Plot the Choline Standard Curve. If Sample Background Control reading is significant, subtract the Background Control reading from its paired Sample reading. Calculate the alkaline sphingomyelinase activity of the Test Sample: $\Delta OD = OD_{final} - OD_{initial}$. Apply the ΔOD to the Choline Standard Curve to get B nmol of choline generated during the reaction time ($\Delta t = t_2 - t_1$).

Sample Alkaline Sphingomyelinase Activity = B/(Δt X V) x D = nmol/min/ml = mU/ml

Where: **B** = Choline amount from Standard Curve (nmol).
$\Delta OD = OD_{final} - OD_{initial}$
$\Delta t = t_{final} - t_{initial}$ (min.)
V = sample volume added into the reaction well (ml)
D = Dilution Factor

Unit Definition: One unit of NPP7/alkaline sphingomyelinase is the amount of enzyme that generates 1.0 nmol of choline per min. at pH 9.0 at 37°C.

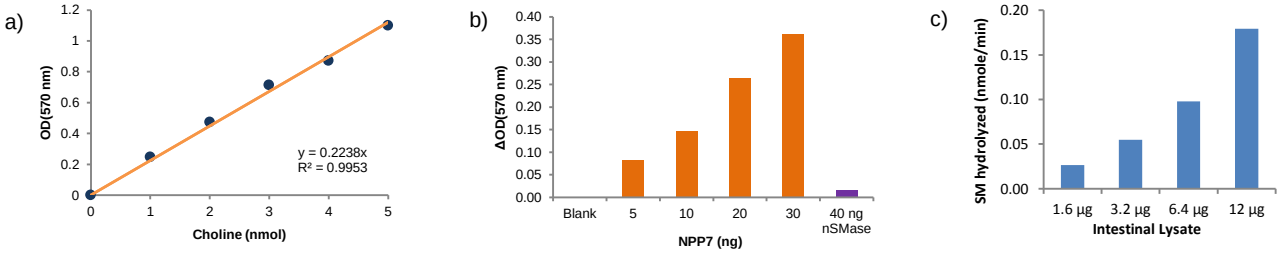


Figure: Choline Standard Curve; (b) Alkaline Sphingomyelinase activity of NPP7. Hydrolysis was allowed to proceed for 1 hr. Neutral Sphingomyelinase (nSMase, purple bar) showed minimal activity after the same incubation time. (c) Activity determination of intestinal tissue lysate. For this experiment, 100 mg rat intestine was used, following Alkaline Sphingomyelinase Activity Assay Kit protocol with 2X protease inhibitor. Lysate was assayed and specific activity was determined to be 0.016 nmol/min/µg lysate.

VIII. RELATED PRODUCTS:

Choline/Acetylcholine Quantification Colorimetric/Fluorometric Kit (K615)
Sphingomyelinase Activity Fluorometric Assay Kit (K574)
Sphingomyelinase Activity Colorimetric Assay Kit (K599)
Triglyceride Quantification Colorimetric/Fluorometric Kit (K622)
Free Fatty Acid Colorimetric /Fluorometric Assay Kit (K612)
Acid Sphingomyelinase Activity Colorimetric Assay Kit (K598)
Phosphatidylcholine Colorimetric/Fluorometric Kit (K576)
Sphingosne Kinase 1 (SPK1) Antibody (3883)
Sphingosne Kinase 2 (SPK2) Antibody (3884)
Sphingosine Kinase Inhibitor, SKI-I (2046)
Isocitrate Dehydrogenase Activity Assay Kit (K756)
Aconitase Activity Colorimetric Assay Kit (K716)

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