Echelon Biosciences Inc.

Neutral Sphingomyelinase (nSMase) Activity Assay Kit

K-1800 (96 tests)

Support: echelon@echelon-inc.com

Description: 96-well absorbance assay for the detection and quantification of Neutral Sphingomyelinase activity

Materials Provided

Catalog #	Description	Amount
---	Microtiter plate	1 plate
---	Microtiter plate seal	1 seal
K-1801	5X SMase Enzyme Mix	2.4 mL
K-3106	DAOS	150 uL
K-1802	Choline Oxidase	120 uL
K-1803	Sphingomyelin (Suspension)	1.3 mL
K-1804	Sphingomyelinase (Standard is not stable once diluted)	1 vial
K-1805	SMase Reaction Buffer (50 mM Tris, 10 mM $MgCl_2$, 0.66 mM $CaCl_2$, pH 7.4)	30 mL
K-1806	Choline Chloride (80 µg/mL)	450 uL

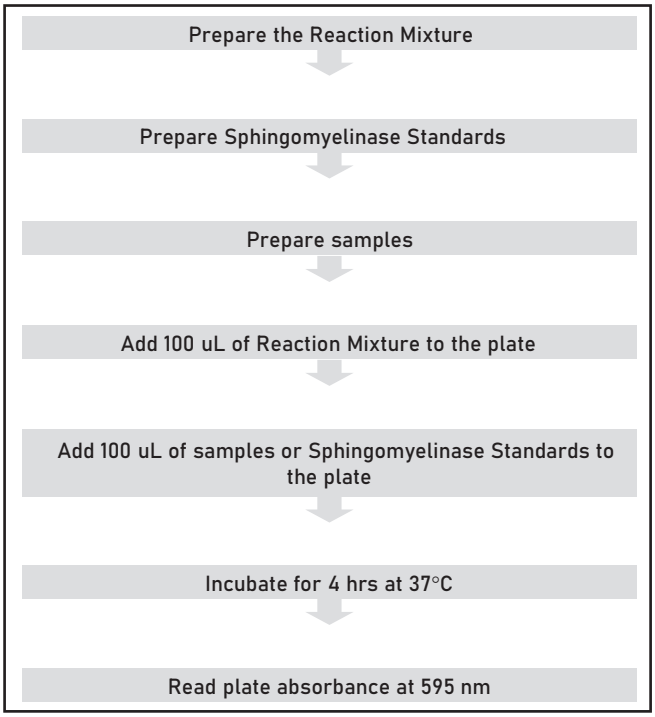
Additional Materials Provided by User:

- Microplate reader capable of reading absorbance at 595 nm.

Storage:

Store kit at -20°C upon receipt. Stored correctly, the kit is stable for 6 months.

Quick Protocol



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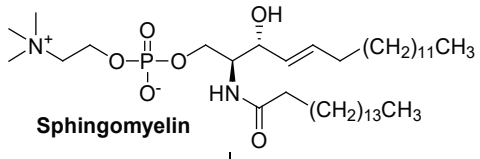
Assay Design

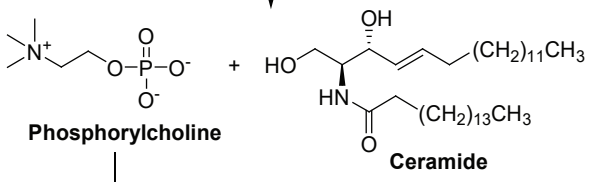
The Neutral Sphingomyelinase (nSMase) Assay Kit provides a sensitive and homogenous method to measure the activity of nSMase in vitro. In this enzyme coupled assay, (1) nSMase catalyzes the hydrolysis of sphingomyelin into phosphorylcholine and ceramide. (2) Alkaline phosphatase catalyzes the hydrolysis of phosphorylcholine followed by the oxidation of choline to hydrogen peroxide which is catalyzed by choline oxidase. DAOS plus 4-Aminoantipyrine (4AAP) in the presence of hydrogen peroxide and peroxidase results in the oxidative coupling of DAOS and 4AAP to form a blue chromogen that is detected by measuring the absorbance of light at 595 nm. The nSMase Assay Kit can measure the activity of nSMase at concentrations as low as 78 µU/mL in 4 hours (see assay notes). The kit provides all necessary reagents to measure the nSMase activity of 40 samples ran in duplicate.

1)



Sphingomyelin

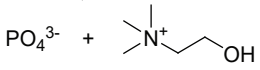
Sphingomyelinase



Phosphorylcholine + **Ceramide**

2)

Alkaline Phosphatase



Choline

Choline Oxidase

H_2O_2 + **Betaine**

4-aminoantipyrine + **DAOS**

Peroxidase

Blue dye (OD 595 nm)

Assay Kit Notes

1. The nSMase kit is designed for one-time use only. Collect enough samples to ensure the kit is fully utilized. If the assay kit is to be run twice, remember to only prepare enough reaction mixture (step1) needed for the day's experiment. A reduction in signal may be observed in the second run, and a vial of Sphingomyelinase Standard (K-1804) may need to be purchased separately, in addition to the kit.
2. High concentrations of glycerol will inhibit the colorimetric reaction.
3. Ensure all samples are free from debris before adding to the plate.
4. The plate can be read multiple times with no significant loss in signal levels.
5. If a lower sensitivity is required (>1 mU/mL) a 2 hour incubation is sufficient.
6. If a greater sensitivity is required (0.078 mU/mL) an overnight incubation is recommended.
7. Choline Chloride (K-1806) is a positive control for the Reaction Mixture prepared in the Assay Procedure section step 1. To test the Reaction Mixture add 100 uL of Choline Chloride to 100 uL of reaction mixture. Then follow steps 7-8. Average Absorbance at 595 nm of the Choline Choride control should be at least 10 times greater than the Blank control.
8. DMSO does not affect nSMase activity or the assay at ≤10 µL/reaction (5%).
9. Preparation of "No Sphingomyelin" control. This control is used when determining Sphingomyelinase activity in cell lysate when a null cell line control is not available.

   Prepare Negative Control Buffer by mixing:
   a. 449 µL: Ethanol
   b. 26 µL: Triton X 100
   c. 825 µL: Sphingomyelinase Reaction Buffer (K-1805)

   To prepare No Sphingomyelin control, substitute 0.6 mL Negative Control Buffer (prepared above) for Sphingomyelin in the Reaction Mixture (step 1c of the Assay Procedure).

   Prepare two Reaction Mixtures; one with Sphingomyelin (K-1803) and one with Negative Control Buffer (prepared above):
   a. 1.2 mL: 5X SMase Enzyme Mix (K-1801)
   b. 60 µL : DAOS (K-3106)
   c. 0.6 mL: Sphingomyelin (K-1803) or Negative Control Buffer (prepared above). Vortex Sphingomyelin before use. Sphingomyelin is provided as a suspension.
   d. 43 µL : Choline Oxidase (K-1802)
   e. 4.1 mL: SMase Reaction Buffer (K-1805)

   Proceed with step 2 of Assay procedure.

   Note: Two reaction mixtures will need to be prepared; one with Sphingomyelin and one without. The "No Sphingomyelin" control is to be added for each concentration and cell type when using cell lysate. The No Sphingomyelin control should be subtracted from each reaction before the enzyme activity is calculated from the standard curve.

Assay Procedure

Please read this entire section and the assay notes section before beginning the assay. Bring Sphingomyelin (K-1803), Shpingomyelin Reaction Assay Buffer (K-1805), and Choline Chloride (K-1806) to room temperature prior to use.

1. Prepare the Reaction Mixture as described below then proceed immediately to the next step. The Reaction Mixture has a limited stability. If you are not running the whole assay, the Reaction Mixture volumes will need to be adjusted (See Assay Notes #1). If using cell lysate, please see Assay Note #9.
   a. 2.4 mL : 5X SMase Enzyme Mix (K-1801)
   b. 120 µL : DAOS (K-3106)
   c. 1.2 mL : Sphingomyelin (K-1803) Vortex before use. Sphingomyelin is provided as a suspension.
   d. 86 µL : Choline Oxidase (K-1802)
   e. 8.2 mL : Sphingomyelinase Reaction Buffer (K-1805)

2. Calculate the amount of SMase Reaction Buffer (K-1805) to

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add to the vial of Sphingomyelinase (K-1804) by using the following equation. This will make a stock solution of 0.02 U/mL of Sphingomyelinase Standard. The Sphingomyelinase Standard is not stable once it's diluted. See Assay Note #1.

(_____ U/mL / 0.02 U/mL) -1 = _____ uL

3. Further dilute the 0.02 U/mL Sphingomyelinase Standard, prepared in step 2, by serial diluting in SMase Reaction Buffer (K-1805) as shown in Table 1.

4. Prepare sample dilutions (if needed) in provided Sphingomyelinase Reaction Buffer (K-1805).

5. Add 100 uL of Reaction Mixture, prepared in step 1, to each well of microtitter plate.

6. Add 100 uL of samples, Choline Chloride, or Sphingomyelinase Standard, prepared in step 3 and 4, to microtiter plate according to Table 2.

7. Cover and incubate the microtitter plate at 37 °C for 4 hours with shaking. The plate can be incubated for 2 hours or overnight resulting in reduced or enhanced sensitivity respectively. (Please see Assay Notes # 5 and 6)

8. Read Absorbance at 595 nm using a microplate reader.

Data Analysis

Calculate unknowns from Sphingomyelinase Standard curve using non-linear curve fit. See Figure 1.

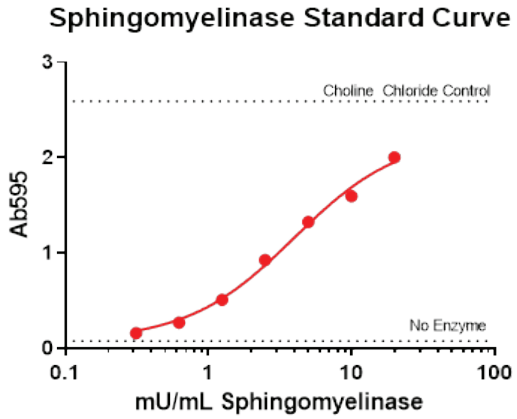

Figure 1

Sphingomyelinase standard curve was generated using non-linear regression [log (agonist) vs response – variable slope (four parameters)] analysis with GraphPad Software.

Table 1, SMase Standards

Row	Standard Curve (mU/mL)	uL stock or previous dilution	uL SMase Reaction Buffer (K-1805)
A	20 mU/mL	500 µL (0.02 U/mL; prepared in step 2)	0 µL
B	10 mU/mL	250 µL (0.02 U/mL; prepared in step 2)	250 µL
C	5 mU/mL	250 µL (10 mU/mL)	250 µL
D	2.5 mU/mL	250 µL (5 mU/mL)	250 µL
E	1.25 mU/mL	250 µL (2.5 mU/mL)	250 µL
F	0.625 mU/mL	250 µL (1.25 mU/mL)	250 µL
G	0.313 mU/mL	250 µL (0.625 mU/mL)	250 µL
H	0 mU/mL	0	250 µL

Table 2, Suggested Detection Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	20 mU/mL	20 mU/mL	1	1	9	9	17	17	25	25	33	33
B	10 mU/mL	10 mU/mL	2	2	10	10	18	18	26	26	34	34
C	5 mU/mL	5 mU/mL	3	3	11	11	19	19	27	27	35	35
D	2.5 mU/mL	2.5 mU/mL	4	4	12	12	20	20	28	28	36	36
E	1.25 mU/mL	1.25 mU/mL	5	5	13	13	21	21	29	29	37	37
F	0.625 mU/mL	0.625 mU/mL	6	6	14	14	22	22	30	30	38	38
G	0.313 mU/mL	0.313 mU/mL	7	7	15	15	23	23	31	31	39	39
H	0 mU/mL	0 mU/mL	8	8	16	16	24	24	32	32	Choline Chloride	Choline Chloride

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Support Protocol

Cell Lysis

The number of cells and volume of sample used in the assay should be determined for each cell type. Since the assay detects choline, which is present in cell lysate, a "No Sphingomyelin" control or appropriate null cell line control should be included for each concentration and cell type used. See assay note #9 for preparation of a "No Spingomyelin" control.

1. Aspirate media from cells
2. Add 37 °C PBS (enough to cover cells) to remove residual medium. Aspirate off PBS.
3. Cover cells with Ice cold Lysis buffer (recipe: 1% Triton X 100, 150 mM NaCl and 25 mM Tris-HCl, pH 7.4) and transfer lysate to an appropriate sized centrifuge tube.
4. Sonicate*
5. Centrifuge lysate at 12,000 rpm for 5 min.
6. Collect supernatant.
7. Use in assay according to assay procedure.

*This step is dependent on sample size and strength of sonicator and should be determined by user.

References

1. Liu, H., X. Wu, et al. (2013). "Fatty acid synthase causes drug resistance by inhibiting TNF-alpha and ceramide production." J Lipid Res 54(3): 776-785.
2. Qin, J., E. Berdyshev, et al. (2012). "Neutral sphingomyelinase 2 deficiency increases hyaluronan synthesis by up-regulation of hyaluronan Synthase 2 through decreased ceramide production and activation of Akt." J Biol Chem

Related Products

Products	Catalog Number
Assays and Reagents	
Acid Sphingomyelinase Assay Kit	K-3200
Sphingomyelin Beads	P-B0SM
Ceramide Beads	P-BCer
SphingoBeads Sample Pack	P-B00Ss

Products	Catalog Number
Lipids	
N-Stearoyl Ceramide 1-phosphate	S-5018
N-Biotin Ceramide 1-phosphate	S-500B
N-NBD Ceramide 1- Phosphate	S-500N6
Biotin Sphingomyelin	S-400B

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