Echelon Biosciences Inc.

Acid Sphingomyelinase (aSMase) Activity Assay Kit

K-3200 (96 tests)

Support: echelon@echelon-inc.com

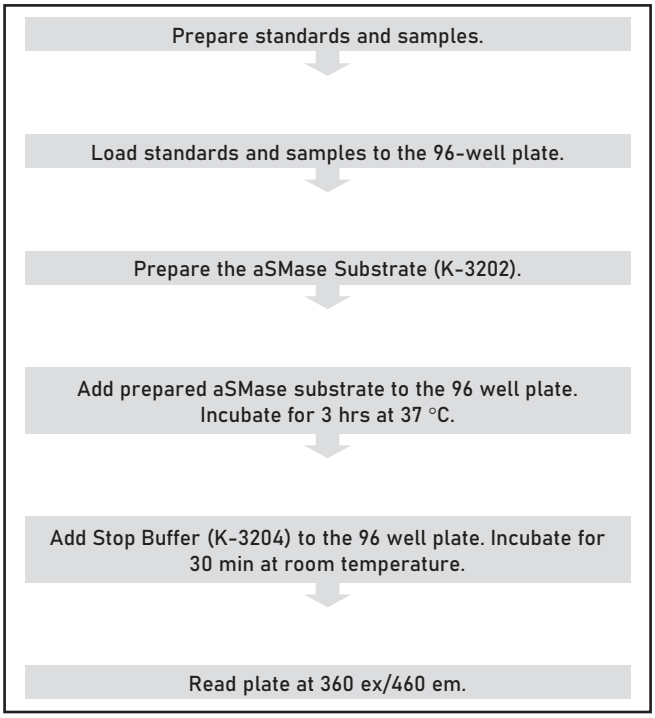
Materials Provided

Catalog #	Description	Amount
K-3205	Standard	2 vials
K-3202	aSMase Substrate	2 vials
K-3203	Substrate Buffer	1 bottle
K-3204	Stop Buffer	1 bottle
---	96 well plate	1 plate
---	Microtiter Plate Seal	2 seals

Additional Materials Provided by User

- Fluorescence microtiter plate reader capable of reading at 360 nm excitation and 460 emission.
- 37 °C Plate Shaker / Incubator
- 70 °C Heat block

Quick Protocol



Storage

Upon receipt, the kit should be stored at –20 °C. Under proper storage conditions, the kit components should remain stable for at least 6 months from date of receipt. Allow the reagents to warm to room temperature before opening vials. Substrate Buffer (K-3203) and Stop Buffer (K-3204) can be stored at room temperature after thawing.

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Background

Sphingomyelinase catalyzes the hydrolysis of sphingomyelin into ceramide and phosphoryl choline; and is involved in programmed cell death (apoptosis), cell differentiation and cell proliferation. Sphingomyelinases are classified into five categories: acid sphingomyelinase (aSMase), secretory sphingomyelinase (sSMase), neutral $Mg2^{+}$-dependent sphingomyelinase (nSMase), neutral $Mg2^{+}$-independent sphingomyelinase and alkaline sphingomyelinase. Acid sphingomyelinase was the first described and best characterized of the sphingomyelinases. A deficiency of lysosomal acid sphingomyelinase leads to rapid neurodegeneration and death due to excessive accumulation of sphingomyelin (Niemann-Pick disease).

Assay Design

Echelon's Acid Sphingomyelinase Activity Assay Kit uses a fluorogenic substrate, specific for Acid Sphingomyelinase, to provide a sensitive and homogenous method to measure the activity of aSMase in vitro from cell lysates or tissue homogenates. The kit provides all necessary reagents to measure the acid sphingomyelinase activity of 40 samples run in duplicate.

Health Hazard Data

The aSMase Substrate (K-3202) and Substrate Buffer (K-3203) contain highly toxic sodium azide and should be handled with caution. Sodium azide can be absorbed into the body by inhalation, ingestion and through the skin causing irritation to the eyes, skin and respiratory tract.

Assay Kit Notes

1. The assay is not compatible with some common lysis buffers components (Table 1). Sonication or freeze-thaw protocols are recommended for preparing cell lysate samples. Avoid non-compatible components if a lysis buffer is used. See support protocol for Cell lysis samples at the end of document.

Table 1, Incompatible Buffer Components

Buffer Component	Concentration
EDTA	≥1 mM
EGTA	≥1 mM
Na+ Pyrophosphate	≥2.5mM
Glycerophosphate	N/A
Na+ vanadate	≥1 mM
Triton X-100	≥1.0% v/v
Deoxycholate	≥0.5% w/v
Igepal CA-630	≥1% v/v
SDS	≥0.1% v/v

2. Cell lysis and Tissue homogenate samples should be titrated in the assay for optimal performance. As a starting point use 10 to 30 µg total protein per data point. Sample protein concentration should be adjusted depending on the acid sphingomyelinase activity within the sample.
3. Tested cell lines and the lysis method used can be found in Table 2.
4. Cell lysate and Tissue Homogenate samples should be prepared on the same day as the assay. Samples with freeze defrost cycles or prolonged storage have not been tested.
5. Stop Buffer is necessary for fluorescence detection.
6. Minimum of 25 ρmol hydrolyzed substrate is needed for fluorescence detection.
7. The plate can be read multiple times with no significant loss in signal.
8. If a lower sensitivity is required (> 200 ρmole/hour) a 2 hour incubation is sufficient.
9. Overnight incubation (17 hours) will result in greater sensitivity (< 3.125 ρmol/hour). However, higher coefficient of variation might occur.
10. To avoid matrix effects keep the buffer compositions between the standards and samples the same.
11. This assay can be adapted for use with 384 well plates. When working with 384 well plates, add 20 uL substrate, standards, and stop solution to each well at the respective steps.

Table 2, Cell Types and Lysis Methods

Lysis Method used	Cell Line	# of Cells Used	Protein/well	aSMase Activity (ρmol/hr/µg)
Sonication	NIH 3T3	1 x 10^6	1.910 µg	21.753
Sonication	MDA-MB-231	1.45 x 10^6	0.811 µg	28.569
Sonication	MDA-MB-468	1.45 x 10^6	0.558 µg	17.842
Freeze-thaw	MDA-MB-231	1.45 x 10^6	2.834 µg	19.441
Freeze-thaw	MDA-MB-468	1.45 x 10^6	4.833 µg	7.999

Assay Procedure

Please read this entire section, Assay Notes, and relevant support protocols before beginning the assay. This protocol has been developed for duplicate reaction points. If singlet or triplicate points are required, the protocol will need to be adjusted accordingly.

1. Turn on a plate shaker / incubator to 37 °C. Warm a heat block, with 1.5 mL tube block, to 70°C. Bring the Substrate Buffer (K-3203), Stop Buffer (K-3204), and Standard (K-3205) to room temperature before use. Place the aSMase Substrate (K-3202) on ice until use. Once defrosted, verify the Substrate Buffer (K-3203) and the Stop Buffer (K-3204) is clear. If precipitation is visible, heat at 37°C until clear. Bring back to room temperature before use.
2. Prepare the fluorescent standard curve by adding 400 µL of Substrate buffer (K-3203) to vial of room temperature Standard (K-3205). Vortex 10 sec. Place at room temperature. This is the 32.8 µM standard. This is stable at room temperature for at least 2 hours. Placing on ice may affect solubility. Serial dilute the 32.8 µM standard 2-fold, 6 times for a total of 7-concentrations using the table below.

Table 3, Preparation of Fluorescent Standard

µM	pmol/well	µL of prepared standard of previous dilution	µL of Substrate buffer (K-3202)
32.8	1,640	150 uL of 32.8 µM standard	--
16.4	820	150 uL of 32.8 µM standard	150 µL
8.2	410	150 uL of 16.4 µM standard	150 µL
4.1	205	150 uL of 8.2 µM standard	150 µL
2.05	102.5	150 uL of 4.1 µM standard	150 µL
1.025	51.25	150 uL of 2.05 µM standard	150 µL
0.5125	25.625	150 uL of 1.025 µM standard	150 µL
0	0	--	150 µL

3. Prepare your samples using Substrate Buffer (K-3203). Please see assay notes for non-compatible buffers, matrix effects, and sample prep suggestions.

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4. Load 50 µL/well standards (step 2) or samples (step 3) to the provided 96-well plate using the suggested template as a guide (Table 4).
5. Thaw the aSMase Substrate (K-3202) in heat block at 70 °C for 2 min. Mix well. If substrate is not clear, repeat until clear. Precipitation will severely reduce enzyme activity.
6. Dilute the aSMase Substrate at 1:40 (80 µL per vial). For the entire plate, add 150 µL aSMase Substrate (K-3202) to 6 mL Substrate Buffer (K-3203). Mix well and keep at room temperature until use. This preparation is stable at room temperature for at least 5 hours.
7. Add 50 µL/well of the diluted substrate (step 6) to the 96-well plate. Cover plate with acetate plate seal and incubate at 37 °C for 3 hours with shaking. See assay notes (7-9) for other incubation options.
8. Add 50 µL/well Stop Buffer (K-3204) to the 96-well plate. Incubate for 30 minutes at room temperature with shaking. Protect from light. Read plate at 360 nm excitation and 460 nm emission.

Quantification of Samples

Generate a best fit curve for the fluorescent standards and interpolate relative sample values. We use Graphpad Prism software for sample analysis. The standard curve can be analyzed using a linear curve. Figure 1 shows a 7-point standard linear curve. Determine the activity of your sample by comparing the RFU of your sample to the fluorescent standard curve. For tissue homogenate and cell lysate samples the samples should be normalized with grams of total protein or tissue.

Figure 1

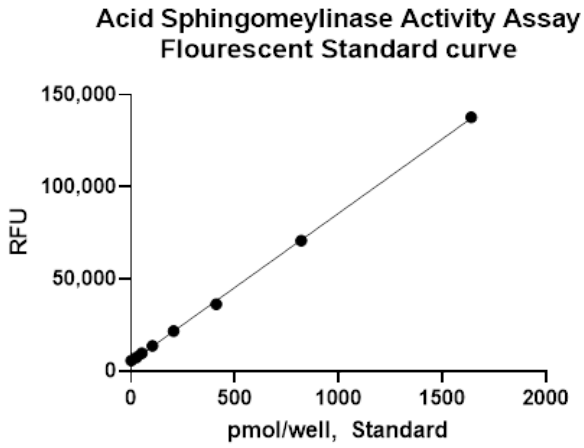

Table 4, Suggested Detection Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	1640 pmol/well	1640 pmol/well	Sample 1	Sample 1	Sample 9	Sample 9	Sample 17	Sample 17	Sample 25	Sample 25	Sample 33	Sample 33
B	820 pmol/well	820 pmol/well	Sample 2	Sample 2	Sample 10	Sample 10	Sample 18	Sample 18	Sample 26	Sample 26	Sample 34	Sample 34
C	410 pmol/well	410 pmol/well	Sample 3	Sample 3	Sample 11	Sample 11	Sample 19	Sample 19	Sample 27	Sample 27	Sample 35	Sample 35
D	205 pmol/well	205 pmol/well	Sample 4	Sample 4	Sample 12	Sample 12	Sample 20	Sample 20	Sample 28	Sample 28	Sample 36	Sample 36
E	102.5 pmol/well	102.5 pmol/well	Sample 5	Sample 5	Sample 13	Sample 13	Sample 21	Sample 21	Sample 29	Sample 29	Sample 37	Sample 37
F	51.25 pmol/well	51.25 pmol/well	Sample 6	Sample 6	Sample 14	Sample 14	Sample 22	Sample 22	Sample 30	Sample 30	Sample 38	Sample 38
G	25.625 pmol/well	25.625 pmol/well	Sample 7	Sample 7	Sample 15	Sample 15	Sample 23	Sample 23	Sample 31	Sample 31	Sample 39	Sample 39
H	0 pmol/ hr	0 pmol/ hr	Sample 8	Sample 8	Sample 16	Sample 16	Sample 24	Sample 24	Sample 32	Sample 32	Sample 40	Sample 40

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Support Protocol

Preparation of Cell Lysate

We suggest sonication or freeze-thaw protocols for lysing cells. These protocols are described in further detail below. Cell lysate buffers (see Assay Note 1) can interfere with the detection of aSMase activity. Prior to running the aSMase Activity Assay, total protein should be determined on each sample and the samples diluted to reflect the same concentration before addition to the assay.

Sonication:

1. Add 500 µL 1 mM PMSF & scrape cells.
2. Sonicate in ice water bath for 10 minutes 3 times.
3. Vortex between each sonication.
4. Centrifuge 10 mintues at 14000 x g.
5. Collect supernatant.
6. Follow steps 1-9 outlined in the Protocol: for the detection of aSMase Activity.

Freeze-thaw:

1. Add 500 µL 1 mM PMSF & scrape cells.
2. Freez-thaw 3 times in liquid nitrogen.
3. Vortex between each freez-thaw cycle.
4. Centrifuge 10 minutes at 14000 x g.
5. Collect supernatant.
6. Follow steps 1-9 outlined in the Protocol: for the detection of aSMase Activity.

Preparation of Tissue Homogenate

This protocol was developed using mouse brain tissue (WT and KO aSMase mice) by Isidora Rovic, M.Sc. at the University of Toronto, Faculty of Medicine; PI: Dr. Andrea Jurisicova. The samples were run in the aSMase Activity Assay at 7 µg total protein. Prior to running the aSMase Activity Assay, total protein should be determined on each sample and the samples diluted to reflect the same concentration before addition to the assay. Since there are no protease inhibitors, samples should be kept on ice, at all times, until they are added to the activity assay.

1. In a 2ml Eppendorf tube place ~50mg of frozen brain tissue.
2. Add 8x volume of ddH2O (ex. For 50 mg tissue add 400µl ddH2O). Keep samples on ice.
3. Immediately homogenize tissue 3x for 15 seconds each, on medium-high power. Keep samples on ice.
4. Freeze-thaw the homogenate once on dry ice.
5. Immediately begin sonication of tissue. Sonicate at medium-high power for 30 seconds, allow 10 second break, and resume for 30 more seconds (total 1 minute). Keep samples on ice. Longer sonication may overheat lysates.
6. After sonication place tissue on ice.
7. Follow steps 1-9 outlined in the Protocol: for the detection of aSMase Activity. For mouse brain lysates, samples were diluted in the sample lysates to 14 µg total protein, and then diluted 1:1 in Substrate Buffer (K-3202) before adding to 96-well plate.

References

1. Biswas R, Trout KL, Jessop F, Harkema JR, Holian A. Imipramine blocks acute silicosis in a mouse model. Particle and Fibre Toxicology. 2017;14(1):36.
2. Lopes Pinheiro MA, Kroon J, Hoogenboezem M, Geerts D, van Het Hof B, van der Pol SM, et al. Acid Sphingomyelinase-Derived Ceramide Regulates ICAM-1 Function during T Cell Transmigration across Brain Endothelial Cells. J Immunol. 2016;196(1):72-9.
3. Chowdhury A, Sarkar J, Chakraborti T, Chakraborti SCCBFR. Role of Spm–Cer-S1P signalling pathway in MMP-2 mediated U46619-induced proliferation of pulmonary artery smooth muscle cells: protective role of epigallocatechin-3-gallate. Cell Biochemistry and Function. 2015;33(7):463-77.
4. Biswas R, Hamilton RF, Jr., Holian A. Role of lysosomes in silica-induced inflammasome activation and inflammation in absence of MARCO. J Immunol Res. 2014;2014:304180. Epub 2014/07/24.
5. Li J, Yu W, Tiwary R, Park SK, Xiong A, Sanders BG, et al. alpha-TEA-induced death receptor dependent apoptosis involves activation of acid sphingomyelinase and elevated ceramide-enriched cell surface membranes. Cancer Cell Int. 2010;10:40.

Related Products

Products	Catalog Number
Assays and Reagents	
Neutral Sphingomyelinase Assay Kit	K-1800
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

Please visit our website at www.echelon-inc.com for more enzyme and lipid products.

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