Version 4f Last updated 10 May 2021

ab190554 Acidic Sphingomyelinase Assay Kit (Fluorometric)

For the accurate detection of acidic sphingomyelinase activity in cell and tissue extracts.

This product is for research use only and is not intended for diagnostic use.

Table of Contents

1. Overview

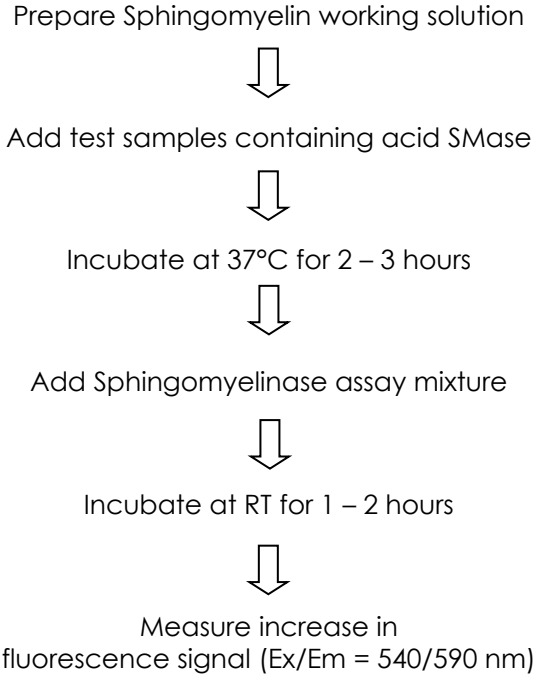
Acidic Sphingomyelinase Assay Kit (Fluorometric) (ab190554) provides one of the most sensitive methods for detecting acidic sphingomyelinase (SMase) activity in cell extracts, or for screening the effect of inhibitors on acid SMase activity. The kit uses our AbRed Indicator as a fluorogenic probe to indirectly quantify the phosphocholine produced from the hydrolysis of sphingomyelin (SM) by sphingomyelinase (SMase). The fluorescence intensity of AbRed is proportional to the formation of phosphocholine, therefore proportional to the SMase activity.

This product can be used for measuring the SMase activity in cell extracts or solutions such as blood. The kit is an optimized "mix and read" assay which is compatible with HTS liquid handling instruments.

This assay is semi-quantitative as it does not contain a SMase standard for calibration. When a known concentration of sphingomyelinase is used, the assay can detect as low as 1 U/mL acidic sphingomyelinase in solution.

Sphingomyelinase (SMase; sphingomyelin phosphodiesterase, EC 3.1.4.12) is responsible for cleaving sphingomyelin (SM) to phosphocholine and ceramide. Activation of SMase plays an important role in cellular responses such as regulation of cell growth, cell differentiation, cell cycle arrest and programmed cell death. Five types of sphingomyelinase have been identified, based on their cation dependence and optimal pH of action: lysosomal acid SMase, secreted zinc-dependent acid SMase, magnesium-dependent neutral SMase, magnesium-independent neutral SMase and alkaline SMase. Among those five types, lysosomal acidic SMase and magnesium-dependent neutral SMase are considered to be the major factors for the production of ceramide in cellular stress responses.

2. Protocol Summary



3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage condition (before prep)	Storage condition (after prep)
Assay Buffer	10 mL	-20°C	-20°C
SMase Reaction Buffer	10 mL	-20°C	-20°C
DMSO	200 µL	-20°C	-20°C
Enzyme Mix (lyophilized)	2 vials	-20°C	-20°C
Sphingomyelin	100 µL	-20°C	-20°C
AbRed Indicator (lyophilized)	1 vial	-20°C	-20°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring fluorescence at Ex/Em = 540/590 nm
- MilliQ water or other type of double distilled water (ddH_2O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom, preferably black
- Cell lysis buffer: we recommend Mammalian Cell Lysis Buffer 5X (ab179835)
- 20mM sodium acetate buffer (pH=5.0)

8. Technical Hints

- **This kit is sold based on number of tests. A "test" simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 Assay Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 SMase Reaction Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.3 DMSO:

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.4 Enzyme Mix:

Reconstitute 1 vial of Enzyme Mix in 5 mL of Assay Buffer. Mix well. Keep on ice while in use. Aliquot enzyme mix so that you have enough reagent to perform the desired number of assays. Store at -20°C.

9.5 Sphingomyelin:

Ready to use as supplied. Equilibrate to room temperature before use. Aliquot sphingomyelin so that you have enough reagent to perform the desired number of assays. Store at -20°C.

Immediately prior to use, prepare **Sphingomyelin Working Solution** by diluting 50 µL of sphingomyelin in 5 mL of SMase Reaction Buffer and mixing well by pipetting up and down. This solution should be use promptly and should not be stored for further use.

Δ Note: There is enough Sphingomyelin Working Solution to assay 1 96 well plate.

9.6 AbRed Indicator:

Reconstitute AbRed Indicator in 80 µL DMSO to make a 200X AbRed Indicator Stock Solution. Equilibrate to room temperature before use. Aliquot 200X AbRed Indicator stock solution so that you have enough reagent to perform the desired number of assays. Store at - 20°C protected from light.

10. Sample Preparation

General sample information:

- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you snap freeze your samples in liquid nitrogen upon extraction and store them immediately at -80°C. When you are ready to test your samples, thaw them on ice and proceed with the Sample Preparation step. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.
- The preparation step described in this section uses Mammalian Cell Lysis Buffer 5X (ab179835). Other general lysis buffer can be used as long as they don't contain Tween-20, or contain <0.1% SDS or <0.1% NP-40.

10.1 Adherent cell samples:

10.1.1 Grow and treat cells as required in your desired culture vessel to about ~80% confluence.
10.1.2 Prepare Mammalian Cell Lysis Buffer 5X (ab179835) as per the product instructions, to obtain 1X Mammalian Lysis Buffer.
10.1.3 Wash cells in PBS to remove residual media.
10.1.4 Lysis cells with 1X Mammalian Lysis Buffer. Table below indicates suggested volumes.

Plate	384-wp	96-wp	48-wp	24-wp	12-wp	6-wp	100 cm^2
Volume	20 µL	50 µL	100 µL	150 µL	200 µL	300 µL	800 µL

10.1.5 Incubate cells at room temperature for 10 – 20 minutes.
10.1.6 Centrifuge lysate at 1500 rpm for 5 minutes.
10.1.7 Transfer supernatant to a new tube.
10.1.8 Keep on ice.

10.2 Suspension cell samples:

10.2.1 Prepare Mammalian Cell Lysis Buffer 5X (ab179835) as per the product instructions, to obtain 1X Mammalian Lysis Buffer.
10.2.2 Wash cells in PBS to remove residual media.
10.2.3 Add 100 µL of 1X Mammalian Lysis Buffer to 1 – 5 x 10^6 cells.

10.2.4 Incubate cells at room temperature for 10 – 20 minutes.
10.2.5 Centrifuge lysate at 1500 rpm for 5 minutes.
10.2.6 Transfer supernatant to a new tube.
10.2.7 Keep on ice.

10.3 Tissue samples:

10.3.1 Prepare Mammalian Cell Lysis Buffer 5X (ab179835) as per the product instructions, to obtain 1X Mammalian Lysis Buffer.
10.3.2 Harvest 20 mg tissue.
10.3.3 Wash tissue with cold PBS.
10.3.4 Homogenize tissue with 400 µL of 1X Mammalian Lysis Buffer. Homogenization can be done using a Dounce homogenizer or pestle, sitting on ice, with 10 – 15 passes.
10.3.5 Centrifuge at 2500 rpm for 5 – 10 minutes.
10.3.6 Transfer supernatant to a new tube.
10.3.7 Keep on ice.

11. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate.
- Prepare all reagents and samples as directed in the previous sections.
- The protocol described in this section is for 1 x 96 well plate. To perform assay in 384 well plate, scale down volumes by 50%.

11.1 Assay Set up:

11.1.1 Set up reaction wells in a solid black 96 well plate:

- Test sample wells = 1 – 50 µL sample (adjust volume to 50 µL/well with 20mM sodium acetate buffer (pH=5.0)).
- Assay blank control wells = 50 µL 20mM sodium acetate buffer (pH=5.0).

11.1.2 Add 50 µL of Sphingomyelin Working Solution (Step 9.5) into each test sample and blank control well.

11.1.3 Incubate the reaction mixture at 37°C for 2 – 3 hours.

11.2 Sphingomyelinase assay procedure:

11.2.1 Prepare Sphingomyelinase assay mixture by adding 25 µL of 200X AbRed Indicator Stock Solution (Step 9.6) to the reconstituted Enzyme Mix (Step 9.4).

Δ Note: The Sphingomyelinase assay mixture should be used promptly and kept from light. Longer storage is likely to cause high assay background.

11.2.2 Add 50 µL of Sphingomyelinase assay mixture to the test sample and black control wells to make a total assay volume of 150 µL.

11.2.3 Incubate the enzyme mixture at room temperature for 1 – 2 hours protected from light.

11.2.4 Monitor fluorescence increase on a microplate reader at Ex/Em = 540/590nm (cut off as 570nm).

12. Additional Procedure – Quantitative assay

To perform a quantitative assay, additional reagents are required:

- Active acid sphingomyelinase protein.
- 20 mM sodium acetate buffer (pH 5.0)

12.1 Dilute sphingomyelinase stock solution in 20 mM sodium acetate buffer (pH 5.0). Recommended concentration range: 0.5 – 10 U/mL.

Δ Note: diluted sphingomyelinase standard solution is unstable, and should not be stored for future use.

12.2 Prepare test samples as described in Section 10.

12.3 Set up reaction wells in a solid black 96 well plate:

- Standard wells: 50 µL standard dilution
- Test sample wells: 1 – 50 µL sample (adjust volume to 50 µL/well with 20 mM sodium acetate buffer pH 5.0).
- Assay blank control wells = 50 µL sodium acetate buffer pH 5.0.

12.4 Add 50 µL of Sphingomyelin Working Solution (Step 9.5) into each standard, test sample and blank control well. Incubate the reaction mixture at 37°C for 2 – 3 hours.

12.5 Follow procedure as described in Step 11.2.

13. Typical Data

Data provided **for demonstration purposes only.**

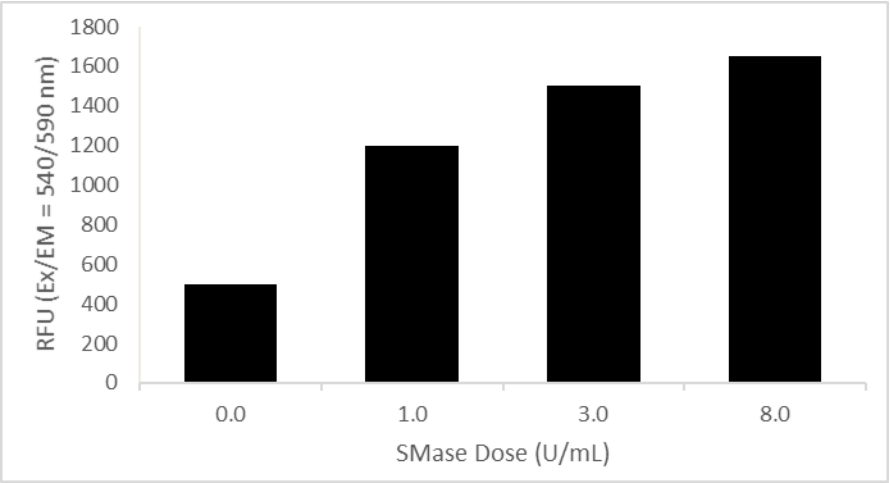


Figure 1. Sphingomyelinase (purified from human placenta) dose response was measured on a 96-well half-are black plate following assay procedure, using a Gemini fluorescence microplate reader (Molecular Devices). 20 µL of SMase solution was incubated with 20 µL of Sphingomyelin Working Solution at 37°C for 3 hours, and then 20 µL of sphingomyelinase assay mixture was added into each well. Signals shown in the figure correspond to the readings at Ex/Em = 540/590 nm (cut off at 570 nm) after 2 hours incubation at room temperature.

14. Troubleshooting

Problem	Reason	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at assay temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different microplate	Colorimetric: clear plates Fluorometric: black wells/clear bottom plates Luminometric: white wells/clear bottom plates
Sample with erratic readings	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

15. Interferences

These chemical or biological materials will cause interferences in this assay causing compromised results or complete failure:

- Tween 20
- SDS: can be used in sample preparation buffer if total amount is <0.1%
- NP 40: can be used in sample preparation buffer it total amount is <0.1%.
- Reducing thiols (DTT or 2-mercaptoethanol): final concentration present in reaction should be < 10 µM.

16. FAQs

17. Notes

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)