Sphingomyelinase Assay Kit

Item No. 10006964

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Materials Supplied

Item Number	Item	Quantity
700221	SMase Assay Buffer (5X)	1 vial
700222	Sphingomyelinase Phosphorylcholine Standard	2 vials
700223	SMase Enzyme Mixture	2 vials
700224	SMase Alkaline Phosphatase	1 vial
700225	SMase Sphingomyelin Substrate	1 vial
700001	DMSO Assay Reagent	1 vial
700227	SMase Fluorometric Substrate	2 vials
700228	SMase Acid Solution	1 vial
700229	SMase Positive Control	1 vial
400091	Half Volume 96-Well Plate (black)	1 plate
400012	96-Well Cover Sheet	1 cover

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 975-3999. We cannot accept any returns without prior authorization.

WARNING: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A fluorometer with the capacity to measure fluorescence using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm
2. Adjustable pipettes and a repeat pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Sphingomyelinase (SMase) is a hydrolase enzyme that is involved in sphingolipid metabolism. SMase is a member of the DNase I superfamily of enzymes and is responsible for the breakdown of sphingomyelin into phosphorylcholine and ceramide. The activation of SMase has been suggested as a major route for the production of ceramide in response to cellular stress.[1] The five distinct types of SMases are classified according to their cation requirements, cellular localization, and pH optima. The types are: Lysosomal Acid SMase, secreted zinc-dependent Acid SMase, a membrane-bound magnesium-dependent Neutral SMase, a cytosolic magnesium-independent Neutral SMase, and an Alkaline SMase.[2-6] Loss of acidic SMase activity due to a mutation in the acid SMase (ASM) gene results in type A and B Niemann-Pick disease.[4]

SMases are important in many physiological and pathophysiological processes, including: 1) lysosomal digestion of sphingomyelin, which is important for normal neuronal and vascular function; 2) ceramide-mediated signal transduction, leading to cytokine-induced apoptosis, cellular differentiation, and various immune and inflammatory responses; 3) lipoprotein aggregation within the vessel wall, which is a key event in atherogenesis, and 4) intracellular cholesterol trafficking and metabolism.[7-10]

About This Assay

Cayman's Sphingomyelinase Assay provides a simple, reproducible, and sensitive tool for assaying neutral and acidic sphingomyelinase activity from tissue homogenates, cell lysates, serum, saliva, and urine. The SMase Assay utilizes a coupled enzymatic reaction to monitor SMase activity (see Figure 1, on page 7). SMase hydrolyzes sphingomyelin to yield ceramide and phosphorylcholine. Alkaline phosphatase hydrolyzes phosphorylcholine forming choline. Choline is then oxidized by choline oxidase to yield betaine and H_2O_2. Finally, H_2O_2, in the presence of horseradish peroxidase (HRP), reacts with 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) in a 1:1 stoichiometry to generate the highly fluorescent product resorufin.[11,12] Resorufin fluorescence is analyzed with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

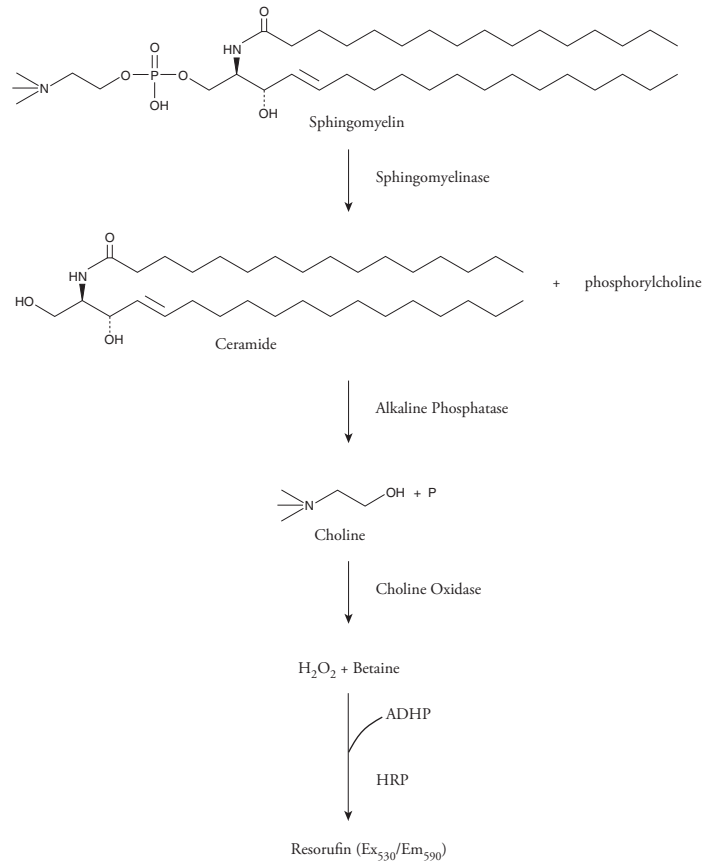

Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. SMase Assay Buffer (5X) - (Item No. 700221)

The vial contains 10 ml of 500 mM Tris-HCl, pH 7.4, containing 50 mM $MgCl_2$. Thaw the Assay Buffer at room temperature. Dilute the contents of the vial with 40 ml of HPLC-grade water. This final 1X buffer (100 mM Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$) should be used in the assay and for diluting reagents. When stored at 4°C, this diluted Assay Buffer is stable for at least six months.

2. Sphingomyelinase Phosphorylcholine Standard - (Item No. 700222)

Each vial contains a lyophilized powder of phosphorylcholine that will be used to prepare the standard curve (see **Standard Preparation** on page 15).

3. SMase Enzyme Mixture - (Item No. 700223)

The vials contain a lyophilized mixture of HRP and choline oxidase. Dissolve the contents of one vial in 500 µl of 1X Assay Buffer. The reconstituted enzyme mixture is stable for two hours at 4°C. One vial is enough enzyme mixture to assay 60 wells. If additional wells are being utilized, then reconstitute the second vial. This reconstituted enzyme mixture will be used to prepare the 'Developer Solution' (see **Performing the Total SMase Assay** on pages 16-17).

4. SMase Alkaline Phosphatase - (Item No. 700224)

The vial contains 150 µl of an alkaline phosphatase solution. Thaw the enzyme on ice. It is then ready to use as supplied to prepare the 'Developer Solution' (see **Performing the Total SMase Assay** on pages 16-17). Store unused enzyme at -20°C.

5. SMase Sphingomyelin Substrate - (Item No. 700225)

Each vial contains 300 µl of sphingomyelin in a Triton X-100 solution and is ready to use as supplied. Thaw and store the Sphingomyelin Substrate on ice while preparing the reagents for the assay. Store unused substrate at -20°C.

If assaying for:

1. **Total SMase** - dilute 100 µl of the Sphingomyelin Substrate with 900 µl of 1X Assay Buffer and mix until homogeneous. This diluted Substrate Solution is sufficient to assay 60 wells. *NOTE: This assay will measure both neutral and acidic SMase activities.*
2. **Acidic SMase** - dilute 100 µl of the Sphingomyelin Substrate with 900 µl of SMase Acid Solution (Item No. 700228) and mix until homogenous. This diluted Substrate Solution is sufficient to assay 60 wells. *NOTE: The acidic assay provides conditions for maximal activity of acidic SMases. Non-acidic SMases should have minor activity under these conditions.*

6. DMSO Assay Reagent - (Item No. 700001)

The vial contains 1 ml of dimethylsulfoxide (DMSO). The reagent is ready to use as supplied. Once thawed, DMSO Assay Reagent can be stored at room temperature.

7. SMase Fluorometric Substrate - (Item No. 700227)

The vials contain a clear lyophilized powder of ADHP (10-Acetyl-3,7-dihydroxyphenoxazine). It will be used to prepare the 'Developer Solution' (see **Performing the Total SMase Assay** on pages 16-17). Once reconstituted, ADHP is stable for 45 minutes. After 45 minutes, increased background fluorescence will occur. One vial is sufficient to assay 60 wells. Use the second vial if utilizing the entire plate.

8. SMase Acid Solution - (Item No. 700228)

Each vial contains 20 ml of 50 mM sodium acetate, pH 5.0. Thaw the Acid Solution at room temperature. The solution is ready to use as prepared. When stored at -20°C, this acid solution is stable for at least six months.

9. SMase Positive Control - (Item No. 700229)

The vial contains a lyophilized powder of sphingomyelinase. Add 500 µl of 1X Assay Buffer to the vial and vortex. Store the enzyme on ice. The reconstituted SMase is stable for one month at -20°C.

A. Prior to assaying in the Total SMase assay, dilute 10 µl of SMase with 990 µl of 1X Assay Buffer.

-OR-

B. Prior to assaying in the Acidic SMase assay, dilute 10 µl of SMase with 990 µl of Acid Solution.

Both diluted enzymes are stable for one hour on ice.

Sample Preparation

Serum

1. Collect blood without using an anticoagulant.
2. Allow blood to clot for 30 minutes at 25°C.
3. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The serum sample will be stable for one month while stored at -80°C.
4. Serum should be diluted 1:5 to 1:10 with either the diluted Assay Buffer or the SMase Acid Solution before assaying.

Saliva

1. Collect saliva in a clean tube.
2. Transfer saliva to micro-centrifuge tubes and centrifuge the saliva at least 14,000 x g (to remove the cheek cells) for five minutes at 4°C.
3. Transfer the supernatant to new micro-centrifuge tubes.
4. Repeat steps 2 and 3. Store the saliva on ice. If not assaying the same day, freeze at -80°C. The saliva sample will be stable for one month while stored at -80°C.
5. The saliva should be diluted 1:5 to 1:10 with either the diluted Assay Buffer or the SMase Acid Solution before assaying.

Urine

1. Collection of urine does not require any special treatment. If not assaying the same day, freeze at -80°C.
2. Urine should be diluted 1:5 to 1:10 with either the diluted Assay Buffer or the SMase Acid Solution before assaying.

NOTE: Sphingomyelinase values from urine samples can be standardized using Cayman's Creatinine Assay Kit (Item No. 500701).

Tissue Homogenate

Sphingomyelinase activity has been detected in brain, kidney, and liver.[13,14]

1. Weigh tissue and then mince into small pieces.
2. Homogenize 350-400 mg of minced tissue in 2 ml of either the diluted Assay Buffer or the SMase Acid Solution.
3. Centrifuge at 800 x g for 10 minutes at 4°C.
4. Transfer the supernatant to another tube and sonicate the supernatant 20X at one second bursts.
5. If not assaying on the same day, freeze the supernatant at -80°C until use. The sample will be stable for one month.
6. Before performing the assay, further dilute the supernatant to a protein concentration of 500-1,000 µg/ml with either cold diluted Assay Buffer or cold SMase Acid Solution.

Cultured Cell Samples

Sphingomyelinase activity is reported to be found in fibroblasts, macrophages, murine microglial cells, monocytes, CHO, and COS-7 cells.[4,15]

1. Aspirate off media.
2. Add PBS and aspirate off to remove any residual medium.
3. Add enough PBS to cover cells.
4. Scrape off cells (~30 x 10^6) with scraper and add to a centrifuge tube.
5. Centrifuge cells at 800 x g for 10 minutes at 4°C.
6. Discard supernatant.
7. Resuspend cell pellet in 1-2 ml of either cold diluted Assay Buffer or cold SMase Acid Solution.
8. Sonicate the cell suspension 20X at one second bursts.
9. Centrifuge cell suspension at 20,000 x g for 10 minutes at 4°C.
10. Aliquot supernatant to vials and freeze at -80°C until use.
11. Resuspend pellet in 0.5-1 ml of either cold diluted Assay Buffer or cold SMase Acid Solution and freeze at -80°C until use.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. We suggest that each sample, positive control, and standard be assayed at least in duplicate; triplicate is recommended, along with background wells for each sample. A typical layout of samples to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 27).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S1	S5	S5	S9	S9	S13	S13	S17	S17
B	B	B	B1	B1	B5	B5	B9	B9	B13	B13	B17	B17
C	C	C	S2	S2	S6	S6	S10	S10	S14	S14	S18	S18
D	D	D	B2	B2	B6	B6	B10	B10	B14	B14	B18	B18
E	E	E	S3	S3	S7	S7	S11	S11	S15	S15	S19	S19
F	F	F	B3	B3	B7	B7	B11	B11	B15	B15	B19	B19
G	G	G	S4	S4	S8	S8	S12	S12	S16	S16	S20	S20
H	+	+	B4	B4	B8	B8	B12	B12	B16	B16	B20	B20

A-G = Standards
S1-S20 = Sample Wells
B1-B20 = Background Sample Wells
+ = Positive Control Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 130 µl in all wells.
- Use the diluted Assay Buffer in the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples be assayed at least in triplicate, but it is the user's discretion to do so.
- The assay is performed at 37°C.
- Monitor the fluorescence with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

Standard Preparation

Reconstitute the contents of a vial of Sphingomyelinase Phosphorylcholine Standard with 1 ml of 1X Assay Buffer to obtain a 100 µM Phosphorylcholine Stock Solution. Place vial on ice. Take seven clean test tubes and label them A-G. Add the amount of 100 µM Stock Solution and either Assay Buffer or Acid Solution to each tube as described in Table 1. We recommend that you store these diluted standards on ice for no more than one hour.

1. If assaying for **Total SMase** activity, standards are prepared in 1X Assay Buffer.
2. If assaying for **Acidic SMase** activity, standards are prepared in acid solution.

Tube	100 µM Phosphorylcholine Stock Solution (µl)	Assay Buffer (µl) or Acid Solution (µl)	Phosphorylcholine Concentration (µM)
A	0	200	0
B	10	190	5
C	20	180	10
D	40	160	20
E	60	140	30
F	80	120	40
G	100	100	50

Table 1. Preparation of phosphorylcholine standards to be used in either the Acidic or Total SMase assays

Performing the Total SMase Assay

1. Prepare the 'Developer Solution' as follows immediately before adding to the wells: Add 5 ml of 1X Assay Buffer to a tube. Add the 500 µl of diluted Enzyme Mixture to the tube. Add 60 µl of the SMase Alkaline Phosphatase to the tube and vortex. Add 50 µl of DMSO Assay Reagent to one vial of the SMase Fluorometric Substrate and mix until dissolved. Then add 390 µl of 1X Assay Buffer and vortex. Add all of the resuspended SMase Fluorometric Substrate solution to the Developer Solution and vortex. This is enough 'Developer Solution' for 60 wells. Prepare additional 'Developer Solution' as needed.
2. **Standard Wells** - Add 10 µl of standard (tube A-G) per well in the designated wells on the plate (see **Sample Plate Format**, Figure 2, page 13).
3. **Positive Control Wells** - Add 10 µl of the Positive Control in the designated wells on the plate (see **Sample Plate Format**, Figure 2, page 13).
4. **Sample Wells** - Add 10 µl of sample to at least three wells. To obtain reproducible results, the amount of sphingomyelinase added to the wells should fall within the range of the assay standard curve. When necessary, samples should be diluted with Assay Buffer.
5. **Sample Background Wells** - Add 10 µl of sample and 20 µl of 1X Assay Buffer to at least three wells.
6. Add 100 µl of the 'Developer Solution' to all wells being used.
7. To initiate the reaction, add 20 µl of the diluted Sphingomyelin Substrate to all standard wells, positive control wells, and sample wells. **DO NOT** add the diluted Sphingomyelin Substrate to the sample background wells. Cover the plate and incubate for 30 minutes at 37°C.
8. Remove the plate cover and read using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

Performing the Acidic SMase Assay

1. **Sample Wells** - Add 10 µl of sample to at least three wells. To obtain reproducible results, the amount of sphingomyelinase added to the wells should fall within the range of the assay standard curve. When necessary, samples should be diluted with the SMase Acid Solution.
2. **Sample Background Wells** - Add 10 µl of sample and 20 µl of the SMase Acid Solution to at least three wells.
3. **Standard Wells** - Add 10 µl of standard (tube A-G) per well in the designated wells on the plate (see **Sample Plate Format**, Figure 2, page 13).
4. **Positive Control Wells** - Add 10 µl of the Positive Control in the designated wells on the plate (see **Sample Plate Format**, Figure 2, page 13).
5. To initiate the reaction, add 20 µl of the diluted Sphingomyelin Substrate (**prepared with SMase Acid Solution**) to all standard wells, positive control wells, and sample wells. **DO NOT** add the diluted Sphingomyelin Substrate to the sample background wells.
6. Cover the plate with the plate cover and incubate at 37°C for thirty minutes.
7. Prepare the 'Developer Solution' as follows within five to ten minutes prior to adding to the wells: Add 5 ml of 1X Assay Buffer to a tube. Add the 500 µl of diluted Enzyme Mixture to the tube. Add 60 µl of the SMase Alkaline Phosphatase to the tube and vortex. Add 50 µl of DMSO Assay Reagent to one vial of the SMase Fluorometric Substrate and mix until dissolved. Then add 390 µl of 1X Assay Buffer and vortex. Add all of the resuspended SMase Fluorometric Substrate solution to the buffer solution and vortex. This is enough 'Developer Solution' for 60 wells. Prepare additional 'Developer Solution' as needed.
8. Add 100 µl of the 'Developer Solution' to all wells being used, including sample, sample background, positive control, and standards. Cover, and incubate for 30 minutes at 37°C.
9. Remove the plate cover and read using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

ANALYSIS

Calculations

1. Determine the average fluorescence of each standard, positive control, sample, and sample background.
2. Subtract the fluorescence value of the sample background from the fluorescence of the sample wells to yield the corrected fluorescence (CF).
3. Determine the average fluorescence of the standards. Subtract the fluorescence value of standard A (0 µM) from itself and all other standards. This is the corrected fluorescence.
4. Plot the corrected fluorescence values (from step 3 above) of each standard as a function of the final concentration of phosphorylcholine from Table 1. See Figure 3, on page 19, for a typical standard curve.
5. Calculate the sphingomyelinase activity using the following equation. One unit is defined as the amount of enzyme that will cause the formation of 1 nmol of phosphorylcholine per minute at 37°C.

$$\text{Sphingomyelinase Activity (nmol/min/ml)} = \frac{\text{CF/30 minutes}}{\text{Slope from std curve (CF/µM)}} \times \text{Sample dilution}$$

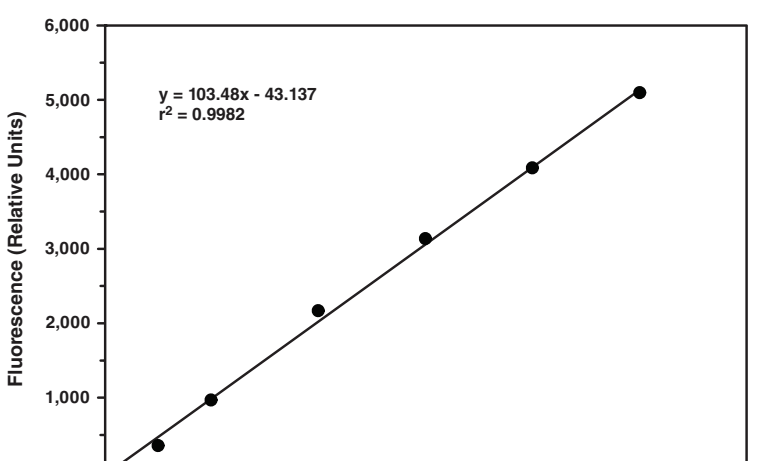

Figure 3. Phosphorylcholine standard curve

Performance Characteristics

Precision:

When a series of 48 sphingomyelinase measurements were performed on the same day, the intra-assay coefficient of variation was 4.68%. When a series of 16 sphingomyelinase measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 4.07%.

Assay Range:

Under the standardized conditions for the assay described in this booklet, sphingomyelinase activity as low as 0.06 nmol/min/ml can be detected without further dilution or concentration.

Interferences

The following reagents were tested for interference in the assay:

Reagent		Will Interfere (Yes or No)
Buffers/Detergent	Borate	No
	HEPES	No
	Phosphate	No
	Triton X-100 (1%)	No
Chelators	EDTA (1 mM)	No
	EGTA (1 mM)	No
Protease Inhibitors	Pepstatin A (10 µg/ml)	Yes
	PMSF (200 µM)	No
	Leupeptin (10 µg/ml)	Yes
	Antipain (0.1 mg/ml)	No
	Chymostatin (10 µg/ml)	Yes
Solvents	DMSO (10 µl)	No
	Ethanol (10 µl)	Yes
	Methanol (10 µl)	Yes
Others	BSA (1%)	No
	Glycerol (10%)	No
	β-Mercaptoethanol (5 mM)	Yes

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No sphingomyelinase activity was detected above background in the sample	A. The sphingomyelinase activity is too low to detect B. The sample does not contain sphingomyelinase, or the sample contains something that is interfering	A. Re-assay the sample using a lower dilution B. Check the interference section for possible interferences (see page 18)
Sample fluorescence was above the highest point in standard curve	A. The sphingomyelinase activity was too high in the sample B. The sample was too concentrated	Dilute samples with 1X Assay Buffer (or Acid Solution) and re-assay. *NOTE: Remember to account for the dilution factor when calculating sphingomyelinase concentration.*
The fluorometer exhibited 'MAX' values for the wells	The GAIN setting is too high	Reduce the GAIN and re-read

References

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Related Products

Cholesterol Assay Kit - Item No. 10007640
Cholesterol Cell-Based Detection Assay Kit - Item No. 10009779
$FABP_4$ Inhibitor/Ligand Screening Assay Kit - Item No. 10010231
β-Hydroxybutyrate (Ketone Body) Assay Kit - Item No. 700190
LDL Uptake Cell-Based Assay Kit - Item No. 10011125
oxLDL-β_2GPI (human) ELISA Kit - Item No. 10007893
Lipid Hydroperoxide (LPO) Assay Kit - Item No. 705002
Liver X Receptor β Transcription Factor Assay Kit - Item No. 10011119
PAF Acetylhydrolase Assay Kit - Item No. 760901
PAF Acetylhydrolase Inhibitor Screening Assay Kit - Item No. 10004380
Phagocytosis Assay Kit (IgG FITC) - Item No. 500290
Phosphatidylcholine Assay Kit - Item No. 10009926
PPARα Transcription Factor Assay Kit - Item No. 10006915
PPARα, δ, γ Complete Transcription Factor Assay Kit - Item No. 10008878
PPARδ Transcription Factor Assay Kit - Item No. 10006914
PPARγ FP-Based Ligand Screening Assay Kit - Green - Item No. 10007685
PPARγ Transcription Factor Assay Kit - Item No. 10006855
Sphingomyelin Assay Kit - Item No. 10009928
SREBP-1 Transcription Factor Assay Kit - Item No. 10010854
SREBP-2 Transcription Factor Assay Kit - Item No. 10007819

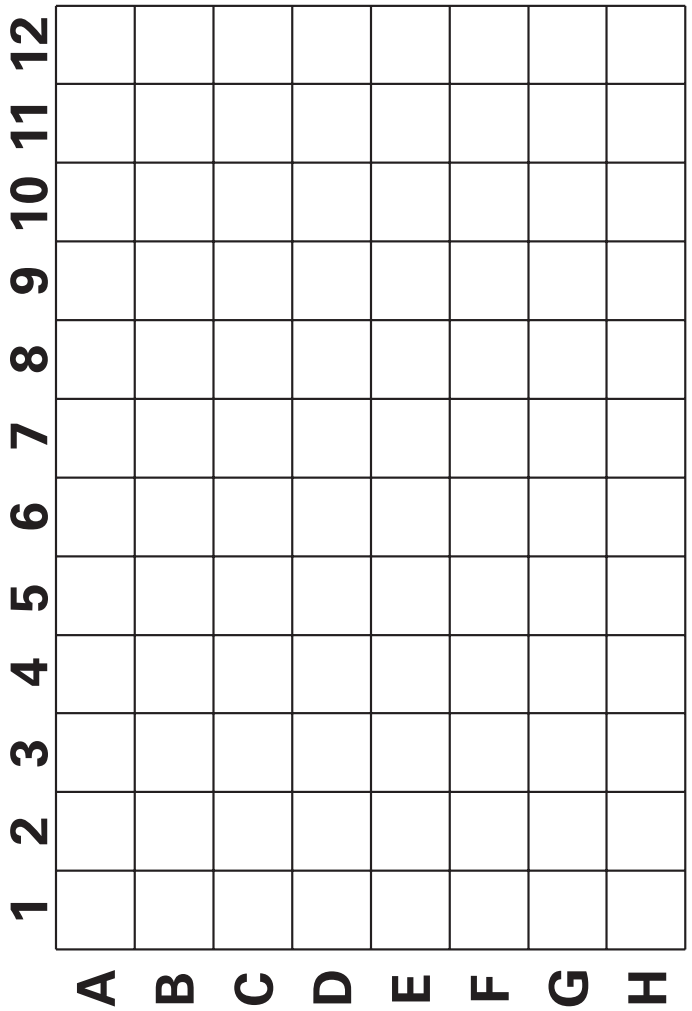
Warranty and Limitation of Remedy

Cayman Chemical Company makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. Cayman **warrants only** to the original customer that the material will meet our specifications at the time of delivery. Cayman will carry out its delivery obligations with due care and skill. Thus, in no event will Cayman have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if Cayman is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of Cayman, its directors or its employees.

Buyer's **exclusive remedy** and Cayman's sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.



NOTES