Product Name: Acid Sphingomyelinase Assay Kit

Product Number: K-3200

Kit Components:

<table>
<thead>
<tr>
<th>Part #</th>
<th>Description</th>
<th>Storage</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-3201</td>
<td>Acid Sphingomyelinase (aSMase) Standard</td>
<td>-20 ºC</td>
<td>2 vials</td>
</tr>
<tr>
<td>K-3202</td>
<td>Acid Sphingomyelinase (aSMase) Substrate</td>
<td>-20 ºC</td>
<td>2 vials</td>
</tr>
<tr>
<td>K-3203</td>
<td>Substrate Buffer</td>
<td>-20 ºC</td>
<td>1 bottle</td>
</tr>
<tr>
<td>K-3204</td>
<td>Stop Buffer</td>
<td>RT / -20 ºC</td>
<td>1 bottle</td>
</tr>
<tr>
<td>96 well plate</td>
<td></td>
<td>RT / -20 ºC</td>
<td>1 each</td>
</tr>
<tr>
<td>Microtiter Plate Seal</td>
<td></td>
<td>RT / -20 ºC</td>
<td>2 each</td>
</tr>
</tbody>
</table>

Each kit provides sufficient reagents for 96 samples assay (including standards).

Researcher must provide:
- Fluorescence microtiter plate reader capable of reading at 360 nm excitation and 460 emission.
- Microcentrifuge tubes (0.5 mL or 1.5 mL)
- Pipettes (20 µL, 200 µL, and 1,000 µL)
- 37 ºC incubator

Storage:
Upon receipt, the kit should be stored at −20 ºC. 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. Acid Sphingomyelinase (aSMase) Standard (K-3201) can handle 1-2 freeze/defrost cycles.

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.

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 Mg$^{2+}$-dependent sphingomyelinase (nSMase), neutral Mg$^{2+}$-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). The Echelon Acid Sphingomyelinase 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. The kit provides all necessary reagents to measure the acid sphingomyelinase activity of 40 samples ran in duplicate.
**Assay Procedure (duplicate points):**

*This assay is not compatible with some common cell lysis buffers. Sonication or freeze-thaw cycle is recommended in preparing cell lysate samples. See Assay Notes and Cell Lysate Preparation for additional information.*

1. Bring the assay kit to room temperature before use. **Except Standard (K-3201) and Substrate (K-3202) which should be stored at -20 °C until use. Make sure the Substrate Buffer (K-3203) is clear before using. If precipitation occurs, heat at 37 °C until clear.**

2. Add 150 µL of the substrate buffer (K-3203) to the aSMase standard (K-3201). This is the 10 U/mL standard and will be used to generate a standard curve of enzyme activity. Use the same buffer as samples to dilute aSMase standard to avoid matrix effects. If the sample buffer is not available, use substrate buffer as diluent. **Please see assay note #1 for non-compatible buffers and matrix effects.**

   Use the following table to make the standard dilution series:

<table>
<thead>
<tr>
<th>aSMase Standard Points</th>
<th>µL of aSMase Standard (K-3201) or µL of previous dilution</th>
<th>µL Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Units / mL</td>
<td>pmol / hour</td>
<td>Sample 1</td>
</tr>
<tr>
<td>10</td>
<td>200</td>
<td>Sample 2</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>Sample 3</td>
</tr>
<tr>
<td>2.5</td>
<td>50</td>
<td>Sample 4</td>
</tr>
<tr>
<td>1.25</td>
<td>25</td>
<td>Sample 5</td>
</tr>
<tr>
<td>0.625</td>
<td>12.5</td>
<td>Sample 6</td>
</tr>
<tr>
<td>0.3125</td>
<td>6.25</td>
<td>Sample 7</td>
</tr>
<tr>
<td>0.15625</td>
<td>3.125</td>
<td>Sample 8</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

3. Load 20 µL of diluted standards or samples per well using the suggested template as a guide.

4. Then, add 30 µL Substrate Buffer (K-3203) per well. **Samples can be run in single, duplicate, or triplicate as desired. Adjust volumes accordingly.**

5. Meanwhile, thaw the aSMase Substrate (K-3202) at 37 °C and mix well. If substrate is not clear, heat to 60 °C until clear (less than 1 minute, repeat if necessary). **Precipitation will severely reduce enzyme activity.** Use within 15 minutes of thawing.

6. Dilute the aSMase Substrate 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. Use within 15 minutes.

7. Add 50 µL of diluted substrate (step 4) to each well of the 96-well plate.

8. Incubate plate at 37 °C for 3 hours with shaking. (see assay notes for other incubation options)
9. Add 50 µL Stop Buffer (K-3204) to each well of the 96-well plate. Incubate for 10 minutes at room temperature with shaking. Protect from light. **Make sure the Stop Buffer is clear before adding.** If precipitation occurs, heat at 37 °C until clear.

10. Read plate at 360 nm excitation and 460 nm emission.

## Cell Lysate Preparations (Optional):

### Sonication

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>ATCC#</th>
<th># of Cells Used</th>
<th>Protein Concentration by Bradford Reagent</th>
<th>Protein Loaded as Samples</th>
<th>ASMase Activity (pmol/hr/µg) by 1 mM PMSF H$_2$O STD Curve</th>
<th>Substrate Buffer STD Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH 3T3</td>
<td>CRL-1658</td>
<td>1 x 10$^6$</td>
<td>0.191 mg/mL</td>
<td>1.910 µg</td>
<td>21.753</td>
<td>18.37*</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>HTB-26</td>
<td>1.45 x 10$^6$</td>
<td>0.081 mg/mL</td>
<td>0.811 µg</td>
<td>28.569</td>
<td>24.09*</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>HTB-132</td>
<td>1.45 x 10$^6$</td>
<td>0.056 mg/mL</td>
<td>0.558 µg</td>
<td>17.842</td>
<td>14.99*</td>
</tr>
</tbody>
</table>

### Freez-thaw

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>ATCC#</th>
<th># of Cells Used</th>
<th>Protein Concentration by Bradford Reagent</th>
<th>Protein Loaded as Samples</th>
<th>ASMase Activity (pmol/hr/µg) by 1 mM PMSF H$_2$O STD Curve</th>
<th>Substrate Buffer STD Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>HTB-26</td>
<td>1.45 x 10$^6$</td>
<td>0.283 mg/mL</td>
<td>2.834 µg</td>
<td>19.441</td>
<td>16.421*</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>HTB-132</td>
<td>1.45 x 10$^6$</td>
<td>0.483 mg/mL</td>
<td>4.833 µg</td>
<td>7.999</td>
<td>6.753*</td>
</tr>
</tbody>
</table>

* Standards and samples should be diluted in similar buffers to reduce potential buffer effects.

### Reference:

**Assay Notes:**

1. The assay is not compatible with some common cell lysis buffer (Figure 1). Sonication or freeze-thaw cycle is recommended for preparing cell lysate samples. Avoid non-compatible components if lysis buffer is used (Figure 2).

![Lysis Buffer interference](image1)

**Figure 1:** The assay is not compatible with some common cell lysis buffers. Cell Lysis buffer and RIPA buffer eliminate the assay signal. See recipies below and Figure 2 for incompatable lysis buffers and additives.

**Cell Lysis Buffer:**
- 20 mM Tris-HCl (pH 7.5)
- 150 mM NaCl
- 1 mM Na<sub>2</sub>EDTA
- 1% Triton
- 2.5 mM sodium pyrophosphate
- 1 mM β-glycerophosphate
- 1 mM Na<sub>3</sub>VO<sub>4</sub>
- 1 µg/mL leupeptin

**RIPA Buffer:**
- 50 mM Tris-HCl (pH 7.5)
- 1 mM EDTA
- 150 mM NaCl
- 0.1% SDS
- 0.5% deoxycholic acid
- 1% Igepal CA-630

![Non-compatible components in lysis buffer](image2)

**Figure 2:** Sonication or freeze-thaw cycle is recommended for preparing cell lysate samples. Avoid non-compatible components if lysis buffer is used

2. 10 to 30 µg protein per 20 µL sample is recommended. Sample protein concentration can be changed depending on the acid sphingomyelinase activity within the sample.
3. Stop Buffer is necessary for fluorescence detection.
4. Minimum of 5 pmol hydrolyzed substrate is needed for fluorescence detection.
5. The plate can be read multiple times with no significant loss in activity.
6. If a lower sensitivity is required (> 200 pmole/hour) a 2 hour incubation is sufficient.
7. Overnight incubation (17 hours) will result in greater sensitivity (< 3.125 pmol/hour). However, higher coefficient variation might occur.

**Related Products:**
- Sphingomyelinase Activity Assay Kit (K-1800)
- Neutral Sphingomyelinase Assay Service (T-1800)
- Biotin Sphingomyelin (S-400B)