

**Enzymatic Assay of SPHINGOMYELINASE¹
from Bacterial Sources
(EC 3.1.4.12)**

PRINCIPLE:

TNPAL-Sphingomyelin + H₂O $\xrightarrow{\text{Sphingomyelinase}}$ Choline Phosphate + TNPAL-N-Acylsphingosine

Abbreviations:

TNPAL-Sphingomyelin = Trinitrophenylaminolauroyl-Sphingomyelin

TNPAL-N-Acylsphingosine = Trinitrophenylaminolauroyl-N-Acylsphingosine

CONDITIONS: T = 37°C, pH = 7.4, A_{330nm}, Light path = 1 cm

METHOD: Colorimetric

REAGENTS:

- A. 500 mM Tris HCl Buffer with 20 mM Magnesium Chloride, pH 7.4 at 37°C
(Prepare 100 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503 and Magnesium Chloride, Hexahydrate, Sigma Prod. No. M-0250. Adjust to pH 7.4 at 37°C with 1 M HCl.)
- B. Trinitrophenylaminolauroyl-Sphingomyelin Substrate² Solution (TNPAL-Sphingomyelin)
(Use Trinitrophenylaminolauroyl-Sphingomyelin, Sigma Prod. No. T-1014. Immediately before use, pipette 0.045 ml (0.05 mg) of T-1014 into a one-dram glass vial on ice. Evaporate the solvent from the material with nitrogen gas. Turn the nitrogen on low, and equip the hose with a 200 µl pipet tip. Insert the pipet tip into the vial about half-way. Hold the vial at a 45E angle and rotate slowly until the substrate is a yellow film on the glass vial. Immediately return the vial to the ice and cap the vial.)
- C. 0.2% (v/v) Triton³ X-100 Solution (Triton X-100)
(Prepare 10 ml in deionized water using Triton³ X-100, Sigma Stock No. X-100.)

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REAGENTS: (continued)

- D. Isopropanol:Heptane:H₂SO₄ Solution (40:10:1) (ISOPRO)
(Prepare 25 ml using Isopropanol, Anhydrous, Sigma Stock No. 405-7, n-Heptane, Sigma Prod. No. H-9629, and Sulfuric Acid, Sigma Prod. No. S-1526.)
- E. Heptane (Heptane)
(Use n-Heptane, Sigma Prod. No. H-9629)
- F. Sphingomyelinase Enzyme Solution
(Immediately before use, prepare a solution containing 1.0 unit/ml in cold Reagent A.)

PROCEDURE:

Pipette (in milliliters) the following reagents into a one-dram vial of Reagent B (TNPAL-Sphingomyelin):

	<u>Test</u>	<u>Blank</u>
Reagent A (Buffer)	0.18	0.18
Reagent C (Triton X-100)	0.20	0.20

Mix by swirling to remove any yellow substrate affixed to the glass. The solution should be clear yellow. Equilibrate at 37°C for exactly 2 minutes. Then add:

Reagent F (Enzyme Solution)	0.01	-----
Reagent A (Buffer)	-----	0.01

Mix by swirling and incubate for exactly 2 minutes. Then add:

Reagent D (ISOPRO)	1.50	1.50
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Put caps on vials and place the vials in ice. Uncap vials right before addition. Then add:

Deionized Water	0.80	0.80
Reagent E (Heptane)	1.80	1.80

Replace the caps and shake the vials vigorously for 5 minutes by hand. Allow the vials to sit at 25°C for 5 minutes. Remove the upper layer and transfer to suitable cuvettes. Record the A_{330nm} for both the Test and Blank using a suitable spectrophotometer.

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CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(A_{330\text{nm}} \text{ Test} - A_{330\text{nm}} \text{ Blank})(2.4)}{(14.0)(2)(0.01)}$$

14.0 = Millimolar extinction coefficient of TNPAL-N-Acylsphingosine at 330 nm

2 = Time of assay in minutes (Unit Definition)

0.01 = Volume (in milliliters) of enzyme used

2.4 = Volume (in milliliters) of organic phase used in assay

$$\text{Units/mg solid} = \frac{\text{Units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{Units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

UNIT DEFINITION:

One unit will hydrolyze 1.0 μ mole of TNPAL-sphingomyelin per minute at pH 7.4 at 37°C.

FINAL ASSAY CONCENTRATION:

In a 0.39 ml reaction mix, the final concentrations are 250 mM Tris, 10 mM MgCl₂, 12% (w/v) TNPAL-sphingomyelin, 1.0% (v/v) Triton³ X-100, and 0.01 unit sphingomyelinase.

REFERENCE:

Gatt, S., Dinur, T., and Barenholz, Y. (1978) *Biochimica et Biophysica Acta* **530**, 503-507

NOTES:

1. This assay is not to be used for Sphingomyelinase, from Human Placenta, Sigma Prod. No. S-5383.
2. The substrate is unstable and the solution should be stored capped and on ice until just prior to running the assay.
3. Triton is a registered trademark of Union Carbide Chemicals and Plastics Co., Inc.

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NOTES: (continued)

4. All products and stock numbers, unless otherwise indicated, are Sigma product and stock numbers.

This procedure is for informational purposes. For a current copy of Sigma's quality control procedure contact our Technical Service Department.