Amplex® Red Sphingomyelinase Assay Kit

Table 1. Contents and storage information.

Material	Amount	Concentration	Storage†	Stability
Amplex® Red reagent, (Component A, MW = 257)*	5 vials, each containing 0.26 mg	NA	• ≤–20°C • Desiccate • Protect from light	When stored as directed the kit is stable for at least 6 months
Dimethylsulfoxide (DMSO, Component B)	0.7 mL	NA		
Horseradish peroxidase (Component C)	200 U‡	NA		
Hydrogen peroxide (H_2O_2, Component D, MW = 34)	500 µL	~3% (stabilized)§		
5 X Reaction buffer (Component E)	28 mL	0.5 M Tris-HCl, 50 mM $MgCl_2$, pH 7.4		
Choline oxidase from *Alcaligenes* sp. (Component F)	12 U**	NA		
Alkaline phosphatase (Component G, from calf intestine)	500 U††	NA		
Sphingomyelin (Component H)	300 µL	100 mM solution in ethanol		
Triton® X-100 (Component I),	0.7 mL	20% solution in water		
Sphingomyelinase from *Bacillus cereus* (Component J)	1 U‡‡	NA		

*The Amplex® Red reagent is somewhat air sensitive. PROTECT IT FROM LIGHT. †The entire kit can be stored under the conditions listed. For optimal storage conditions of individual kit components, refer to the labels on the vials. ‡One unit is defined as the amount of enzyme that will form 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C. §See component label for exact concentration. **One unit is defined as the amount of choline oxidase that will form 1.0 µmole of H_2O_2 due to oxidation of 1 µmole of choline to betaine aldehyde per minute at pH 8.0 at 37°C. ††One unit is defined as the amount of enzyme that will hydrolyze 1 µmole of 4-nitrophenyl phosphate per minute in 1 M diethanolamine buffer, pH 9.8, at 37°C. ‡‡One unit is defined as the amount of sphingomyelinase that will hydrolyse 1 µmole of TNPAL–sphingomyelin per minute at pH 7.4 at 37°C. NA = Not applicable.

Number of Assays: Each kit provides sufficient reagents for approximately 500 assays using a fluorescence microplate reader and reaction volumes of 200 µL per assay.

Approximate fluorescence excitation and emission maxima: 571/585 nm for the reaction product.

Introduction

The Amplex® Red Sphingomyelinase Assay Kit provides a sensitive method for measuring sphingomyelinase activity *in vitro* using a fluorescence microplate reader or fluorometer. In this enzyme-coupled assay, sphingomyelinase activity is monitored indirectly using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex® Red reagent), a sensitive fluorogenic probe for H_2O_2.[1] First, sphingomyelinase hydrolyses the sphingomyelin to yield ceramide and phosphorylcholine. After the action of alkaline phosphatase, which hydrolyses phosphorylcholine, choline is oxidized by choline oxidase to betaine and H_2O_2. Finally, H_2O_2, in the presence of horseradish peroxidase, reacts with Amplex® Red reagent in a 1:1 stoichiometry to generate the highly fluorescent product, resorufin.[1,2] Because resorufin has absorption and fluorescence emission maxima of approximately 571 nm and 585 nm, respectively (Figure 1), there is little interference from autofluorescence in most biological samples. The kit can be used to continuously assay sphingomyelinase enzymes with near-neutral pH optima, whereas sphingomyelinase enzymes with acidic pH optima can be assayed in a simple two-step procedure. Experiments with purified sphingomyelinase from *Bacillus cereus* indicate that the Amplex® Red sphingomyelinase Assay Kit can detect sphingomyelinase levels as low as 80 µU/mL using a reaction time of one hour (Figure 2). The kit is useful for detecting sphingomyelinase activity in cell extracts 3 or for screening sphingomyelinase inhibitors.

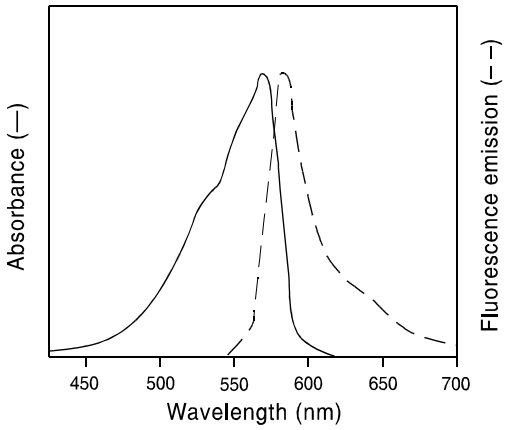

Figure 1. Normalized absorption and fluorescence emission spectra of resorufin, the product of the Amplex® Red reagent.

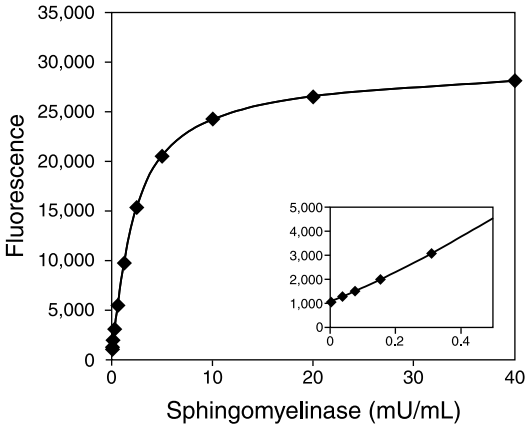

Figure 2. Detection of sphingomyelinase using the Amplex® Red reagent–based assay. Each reaction contained 50 µM Amplex® Red reagent, 1 U/mL HRP, 0.1 U/mL choline oxidase, 4 U/mL of alkaline phosphatase, 0.25 mM sphingomyelin and the indicated amount of *Bacillus cereus* sphingomyelinase in 1X Reaction Buffer. Reactions were incubated at 37°C for 30 minutes. Fluorescence was measured with a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm.

Experimental Protocol

The following procedure is designed for use with a fluorescence multi-well plate scanner. For use with a standard fluorometer, volumes must be increased accordingly. Please note that the product of the Amplex® Red reaction is unstable in the presence of thiols such as dithiothreitol (DTT) or 2-mercaptoethanol. For this reason, the final DTT or 2-mercaptoethanol concentration in the reaction should be less than 10 µM.

The absorption and fluorescence of resorufin are pH-dependent. Below the pK_a (~6.0), the absorption maximum shifts to ~480 nm and the fluorescence quantum yield is markedly lower. In addition, the Amplex® Red reagent is unstable at high pH (>8.5). For these reasons, the reaction should be performed at pH 7–8. For assaying sphingomyelinase enzymes at moderately acidic pH, the reaction can be performed in two steps (see *Two-Step Sphingomyelinase Assay*, below).

Stock Solution Preparation

1.1 Prepare a 10 mM stock solution of the Amplex® Red reagent: Allow one vial of Amplex® Red reagent (Component A) and DMSO (Component B) to warm to room temperature. Just prior to use, dissolve the contents of the vial of Amplex® Red reagent (0.26 mg) in 100 µL DMSO. Each vial of Amplex® Red reagent is sufficient for approximately 100 assays, with a final reaction volume of 200 µL per assay. This stock solution should be stored frozen at –20°C, protected from light.

1.2 Prepare a 1X working solution of Reaction Buffer by adding 5 mL of 5X Reaction Buffer stock solution (Component E) to 20 mL of deionized water (dH_2O). This 25 mL volume of 1X Reaction Buffer is sufficient for approximately 100 assays of 200 µL each, with a 5 mL excess for making stock solutions and dilutions.

1.3 Prepare a 200 U/mL stock solution of horseradish peroxidase (HRP) by dissolving the contents of the vial of HRP (Component C) in 1.0 mL of 1X Reaction Buffer. After use, the remaining solution should be divided into small aliquots and stored frozen at –20°C.

1.4 Prepare a 20 mM H_2O_2 working solution by diluting the ~3% H_2O_2 stock solution (Component D) into the appropriate volume of dH_2O. The actual H_2O_2 concentration is indicated on the component label. For instance, a 20 mM H_2O_2 working solution can be prepared from a 3.0% H_2O_2 stock solution by diluting 23 µL of 3.0% H_2O_2 into 977 µL of dH_2O. Please note that although the ~3% H_2O_2 stock solution has been stabilized to slow degradation, the 20 mM H_2O_2 working solution will be less stable and should be used promptly.

1.5 Prepare a 20 U/mL stock solution of choline oxidase by dissolving the contents of the vial of choline oxidase (Component F) in 600 µL of 1X Reaction Buffer. After use, the remaining solution should be divided into small aliquots and stored frozen at –20°C.

1.6 Prepare a 400 U/mL stock solution of alkaline phosphatase by dissolving the contents of the vial of alkaline phosphatase (Component G) in 1.25 mL of 1X Reaction Buffer. After use, the remaining solution should be divided into small aliquots and stored frozen at –20°C.

1.7 Prepare a 2% working solution of Triton X-100 by diluting the 20% solution of Triton X-100 (Component I) in 1X Reaction Buffer. For instance, a 2% working solution of Triton X-100 can be prepared by diluting 130 µL of 20% Triton X-100 into 1.17 mL of 1X Reaction Buffer

1.8 Prepare a 5 mM sphingomyelin working solution by diluting the 100 mM sphingomyelin solution (Component H) into 2% Triton X-100 solution prepared in step 1.7. For instance, a 5 mM sphingomyelin working solution can be prepared from a 100 mM sphingomyelin solution by diluting 60 µL of 100 mM sphingomyelin into 1.14 mL of 2% Triton X-100 solution. The resulting solution will be turbid.

1.9 If desired, prepare a 10 U/mL stock solution of sphingomyelinase by dissolving the contents of the vial of sphingomyelinase (Component J) in 100 µL of 1X Reaction Buffer. This sphingomyelinase solution can be used as a positive control. After use, the remaining solution should be divided into small aliquots and stored frozen at –20°C.

Continuous Sphingomyelinase Assay

The following protocol describes the assay of sphingomyelinase in a total volume of 200 µL per microplate well. The volumes recommended here are sufficient for ~100 assays.

2.1 Dilute the sphingomyelinase–containing samples in 1X Reaction Buffer. A volume of 100 µL will be used for each reaction.

2.2 Prepare a positive control by diluting the 10 U/mL sphingomyelinase stock solution (prepared in step 1.9) into 1X Reaction Buffer to produce a 0.04 U/mL sphingomyelinase solution. Use 1X Reaction Buffer without sphingomyelinase as a negative control. A volume of 100 µL will be used for each reaction.

2.3 Prepare another positive control by diluting the 20 mM H_2O_2 working solution to 10 µM in 1X Reaction Buffer.

2.4 Pipet 100 µL of the diluted samples and controls into separate wells of a microplate.

2.5 Prepare a working solution of 100 µM Amplex® Red reagent containing 2 U/mL HRP, 0.2 U/mL choline oxidase, 8 U/mL of alkaline phosphatase and 0.5 mM sphingomyelin by adding:

- 100 µL of Amplex® Red reagent stock solution (prepared in step 1.1)
- 100 µL of HRP stock solution (prepared in step 1.3)
- 100 µL of choline oxidase stock solution (prepared in step 1.5)
- 200 µL of alkaline phosphatase stock solution (prepared in step 1.6)
- 1.0 mL sphingomyelin working solution (prepared in step 1.8)
- 8.5 mL of 1X Reaction Buffer.

Note that this solution may be slightly turbid in appearance due to the sphingomyelin. This 10 mL volume is sufficient for ~100 assays. Final concentrations of each component will be twofold lower in the final reaction volume.

2.6 Begin the reactions by adding 100 µL of the Amplex® Red reagent/HRP/choline oxidase/alkaline phosphatase/sphingomyelin working solution to each microplate well containing the samples and controls.

2.7 Incubate the reactions for 30 minutes or longer at 37°C, protected from light. Because the assay is continuous (not terminated), fluorescence may be measured at multiple time points to follow the kinetics of the reactions.

2.8 Measure the fluorescence in a fluorescence microplate reader using excitation in the range of 530–560 nm and emission detection at ~590 nm (see Figure 1).

2.9 For each point, correct for background fluorescence by subtracting the values derived from the no-sphingomyelinase control.

Two-Step Sphingomyelinase Assay (for Sphingomyelinase Enzymes with Acidic pH Optima)

Some sphingomyelinase enzymes have acidic pH optima. To assay these enzymes, you may wish to perform a two-step assay in which the sphingomyelinase reaction is performed at a lower pH, (e.g., in 50 mM sodium acetate, pH 5.0) and then the pH is raised to 7.0–8.0 (e.g., by adding an equal volume of 100 mM Tris-HCl, pH 8.0) to allow detection with the Amplex® Red reagent. The following protocol can be used as a guideline for performing a two-step

assay. The volumes recommended here are sufficient for ~100 assays, using a final reaction volume of 200 µL per assay.

3.1 Dilute the sphingomyelinase–containing samples in the reaction buffer of your choice (e.g., 50 mM sodium acetate, pH 5.0). Use reaction buffer without sphingomyelinase as a negative control. A volume of 100 µL will be used for each reaction.

3.2 Add 10 µL of the 5 mM sphingomyelin solution (prepared in step 1.8) to each sample or negative control.

3.3 Incubate the first-step reactions at 37°C for the desired length of time (e.g., one hour).

3.4 If desired, prepare a positive control by diluting the 10 U/mL sphingomyelinase stock solution (prepared in step 1.9) into 1X Reaction Buffer (prepared in step 1.2) to produce a 0.4 U/mL sphingomyelinase solution (pH optimum ~7.4). A volume of 100 µL will be used for each reaction.

3.5 Prepare another positive control by diluting the 20 mM H_2O_2 working solution to 10 µM in 1X Reaction Buffer.

3.6 Pipet 100 µL of the diluted controls into separate wells of a microplate.

3.7 Add 10 µL of the 5 mM sphingomyelin solution (prepared in step 1.8) to each positive control.

3.8 Prepare a working solution of 100 µM Amplex® Red reagent containing 2 U/mL HRP, 0.2 U/mL choline oxidase and 8 U/mL alkaline phosphatase by adding:

- 100 µL of Amplex® Red reagent stock solution (prepared in step 1.1)
- 100 µL of HRP stock solution (prepared in step 1.3)
- 100 µL of choline oxidase stock solution (prepared in step 1.5)
- 200 µL of alkaline phosphatase stock solution (prepared in step 1.6)
- 9.5 mL of high–pH buffer (e.g., 100 mM Tris-HCl, pH 8.0).

This 10 mL volume is sufficient for ~100 assays. Concentrations of each component will be twofold lower in the final reaction volume.

3.9 Begin the second step reactions by adding 100 µL of the Amplex® Red reagent/HRP/choline oxidase/alkaline phosphatase working solution to each microplate well containing the samples and controls.

3.10 Incubate the reactions for 30 minutes or longer at 37°C, protected from light.

3.11 Measure the fluorescence in a fluorescence microplate reader using excitation in the range of 530–560 nm and emission detection at ~590 nm (see Figure 1).

3.12 For each point, correct for background fluorescence by subtracting the values derived from the no-sphingomyelinase control.

References

1. Anal Biochem 253, 162 (1997); **2.** J Immunol Methods 202, 133 (1997); **3.** J Biol Chem 276, 11775 (2001).

Product List **Current prices may be obtained from our website or from our Customer Service Department.**

Cat #	Product Name	Unit Size
A12220	Amplex® Red Sphingomyelinase Assay Kit *500 assays*	1 kit
A22177	Amplex® Red reagent *packaged for high-throughput screening*	10 x 10 mg
A12222	Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine)	5 mg
A33855	Amplex® Red/UltraRed stop reagent *500 tests* *set of 5 vials*	1 set
A36006	Amplex® UltraRed reagent	5 x 1 mg

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