

## Amplex® Red Monoamine Oxidase Assay Kit (A12214)

### Quick Facts

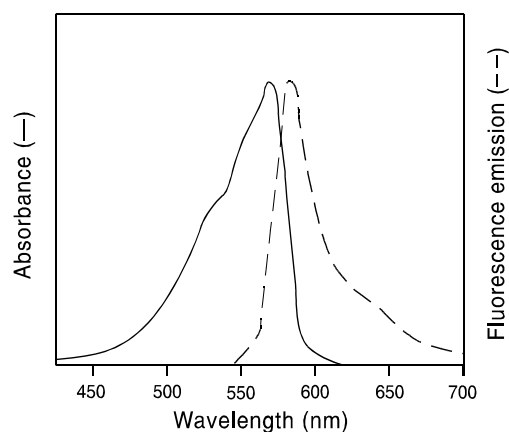
#### Storage upon receipt:

- $\leq -20^{\circ}\text{C}$
- Desiccate
- Protect from light

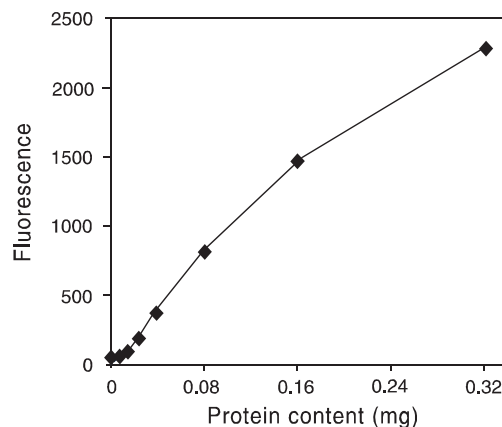
**Abs/Em of reaction product:** 571/585 nm

### Introduction

The Amplex® Red Monoamine Oxidase Assay Kit provides a one-step fluorometric method for the continuous measurement of amine oxidase activity using a fluorescence microplate reader or fluorometer. We have found that the assay is able to sensitively detect both monoamine oxidase (MAO) activity and semicarbazide-sensitive amine oxidase (SSAO) activity. The assay is based on the detection of  $\text{H}_2\text{O}_2$  in a horseradish peroxidase-coupled reaction using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a highly sensitive and stable probe for  $\text{H}_2\text{O}_2$ .<sup>1</sup> Advantages of the Amplex Red reagent over other  $\text{H}_2\text{O}_2$ -sensitive probes include the Amplex Red reagent's stability, the stability of the reaction product, resorufin, and the long wavelength spectra of resorufin. 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.



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



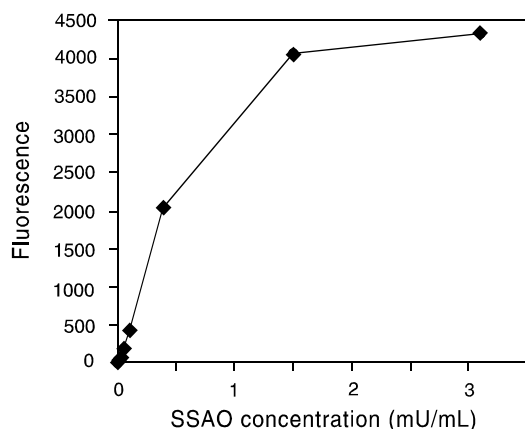
**Figure 2.** Detection of MAO activity in cow brain tissue using the Amplex Red reagent-based assay. Each reaction contained 1 mM benzylamine, 1 U/mL HRP, 200  $\mu\text{M}$  Amplex Red reagent and the indicated amount of brain homogenate in 50 mM potassium phosphate, pH 7.4. Reactions were incubated at room temperature for 60 minutes. Fluorescence was measured with a fluorescence microplate reader using excitation at  $560 \pm 10$  nm and fluorescence detection at  $590 \pm 10$  nm.

The Amplex Red monoamine oxidase assay is useful for performing both end-point and continuous measurements of amine oxidase activity. The Amplex Red assay has been found to detect monoamine oxidase activity from cow brain tissue using as little as 20  $\mu\text{g}$  of total protein per sample (Figure 2) and has also been used to measure plasma amine oxidase (SSAO) activity levels as low as  $1.2 \times 10^{-5}$  U/mL using a commercially available enzyme<sup>2</sup> (Figure 3). To facilitate discrimination of MAO-A and MAO-B activity, two MAO substrates and two MAO inhibitors are included in the kit. *p*-Tyramine is a substrate for both MAO-A and MAO-B, whereas benzylamine is a substrate for MAO-B.<sup>3</sup> Both *p*-tyramine and benzylamine are also substrates for SSAO enzymes. Clorgyline is a specific inhibitor of MAO-A activity and pargyline is a specific inhibitor of MAO-B activity.<sup>3,5</sup> The potential applications of this kit include the measurement of amine oxidase activity in normal and diseased tissues, blood samples and other biological fluids, the screening of drugs as possible MAO inhibitors or substrates and the determination of kinetic constants for different amine oxidase substrates.

### Materials

#### Kit Contents

- Amplex Red reagent (MW = 257, Component A), five vials, each containing 1 mg
- Dimethylsulfoxide (DMSO), anhydrous (Component B), 1.3 mL



**Figure 3.** Detection of plasma amine oxidase (SSAO) activity using the Amplex Red reagent-based assay and benzylamine as the substrate. Each reaction contained 1 mM benzylamine, 1 U/mL HRP, 200  $\mu$ M Amplex Red reagent and the indicated amount of SSAO (Sigma, catalog no. M4636) in 50 mM potassium phosphate, pH 7.4. Reactions were incubated at room temperature for 15 minutes. Fluorescence was measured as described in Figure 2.

- Horseradish peroxidase (Component C), 200 U, where 1 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
- Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Component D), 500  $\mu$ L of a stabilized ~3% solution; the actual concentration is indicated on the component label
- 5X Reaction Buffer (Component E), 28 mL of 0.25 M sodium phosphate, pH 7.4
- Benzylamine hydrochloride (MW = 144, Component F), 17.2 mg
- p-Tyramine hydrochloride (MW = 174, Component G), 20.8 mg
- Clorgyline hydrochloride (MW = 309, Component H), MAO-A inhibitor, 154  $\mu$ g
- Pargyline hydrochloride (MW = 196, Component I), MAO-B inhibitor, 98  $\mu$ g
- Resorufin, sodium salt (MW = 235, Component J), 470  $\mu$ g

Each kit provides sufficient reagents for approximately 500 assays using a fluorescence microplate reader and reaction volumes of 200  $\mu$ L per assay.

### Storage and Handling

Upon receipt, the kit should be stored frozen at  $\leq -20^{\circ}\text{C}$ , protected from light. Stored properly, the kit components should remain stable for at least six months. Allow reagents to warm to room temperature before opening vials. The Amplex Red reagent is somewhat air sensitive. Once a vial of Amplex Red reagent is opened, the reagent should be used promptly. **PROTECT THE AMPLEX RED REAGENT FROM LIGHT.**

## 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 no higher than 10  $\mu$ M.

The absorption and fluorescence of resorufin are pH-dependent. Below the  $\text{pK}_a$  (~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. We recommend using the included Reaction Buffer (pH 7.4) for optimal performance of the Amplex Red reagent.

### Stock Solution Preparation

**1.1** Prepare an ~20 mM stock solution of the Amplex Red reagent by allowing one vial of the Amplex Red reagent (Component A) and the DMSO (Component B) to warm to room temperature. Just prior to use, dissolve the contents of one vial of Amplex Red reagent (1 mg) in 200  $\mu$ L DMSO. Each vial of Amplex Red reagent provides sufficient material for approximately 100 assays of 200  $\mu$ L each. This stock solution should be stored frozen at  $\leq -20^{\circ}\text{C}$ , 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<sub>2</sub>O). This 25 mL volume of 1X Reaction Buffer is sufficient for approximately 100 assays of 200  $\mu$ L 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 entire 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  $\leq -20^{\circ}\text{C}$ .

**1.4** Prepare a 20 mM H<sub>2</sub>O<sub>2</sub> working solution by diluting the ~3% H<sub>2</sub>O<sub>2</sub> stock solution (Component D) into the appropriate volume of 1X Reaction Buffer. The actual H<sub>2</sub>O<sub>2</sub> concentration is indicated on the component's label. For instance, a 20 mM H<sub>2</sub>O<sub>2</sub> working solution can be prepared from a 3.0% H<sub>2</sub>O<sub>2</sub> stock solution by diluting 23  $\mu$ L of 3.0% H<sub>2</sub>O<sub>2</sub> into 977  $\mu$ L of 1X Reaction Buffer. Please note that although the ~3% H<sub>2</sub>O<sub>2</sub> stock solution has been stabilized to slow degradation, the 20 mM H<sub>2</sub>O<sub>2</sub> working solution will be less stable and should be used promptly.

**1.5** Prepare 100 mM stock solutions of the two amine oxidase substrates (benzylamine and tyramine, Components F and G) by adding 1.2 mL dH<sub>2</sub>O directly to the individual substrate vials. Substrate stock solutions should be stored at  $\leq -20^{\circ}\text{C}$ .

**1.6** Prepare 0.5 mM stock solutions of each of the MAO inhibitors (clorgyline and pargyline, Components H and I) by adding 1.0 mL dH<sub>2</sub>O directly to the individual vials. Inhibitor stock solutions should be stored at  $\leq -20^{\circ}\text{C}$ .

**1.7** If desired, prepare a 2 mM stock solution of resorufin by adding 1 mL dH<sub>2</sub>O directly to the vial of resorufin solid (Component J). This solution can be used to prepare a standard curve to determine the moles of product produced in the Amplex Red reaction. This stock solution should be stored frozen at  $\leq -20^{\circ}\text{C}$ , protected from light.

## Amine Oxidase Assay

The following protocol can be used as a guide for adapting the Amplex Red MAO assay for your application. In actual practice, incubation times, substrate concentrations and inhibitor concentrations will need to be optimized for the particular experimental conditions. The two amine oxidase substrates included in the kit are interchangeable in the protocol, depending on the desired application. Benzylamine is a substrate of MAO-B, and *p*-tyramine is a substrate of both MAO-A and MAO-B.<sup>3</sup> Both of these compounds are substrates for plasma amine oxidase (SSAO). The MAO-A inhibitor, clorgyline, and the MAO-B inhibitor, pargyline, can also be included to help confirm the identity of the enzyme responsible for amine oxidase activity.<sup>4</sup> Alternatively, these inhibitors can be used as controls in inhibitor screening applications. H<sub>2</sub>O<sub>2</sub> can be used as a positive control in the reaction. In addition, resorufin can be used to prepare a standard curve to determine the moles of product produced in the Amplex Red reaction.

**2.1** Dilute the amine oxidase-containing samples in 1X Reaction Buffer. A volume of 100  $\mu$ L will be used for each reaction. One of the MAO inhibitors (at a concentration of 1  $\mu$ M) can be included, if desired, by adding the equivalent of 0.2  $\mu$ L of the 0.5 mM inhibitor stock solution (prepared in step 1.6) to each 100  $\mu$ L volume of diluted sample and preincubating the sample (e.g. 30 minutes at room temperature). Please note that amine oxidase and inhibitor concentrations will be twofold lower in the final reaction volume.

**2.2** Prepare a positive control by diluting the 20 mM H<sub>2</sub>O<sub>2</sub> working solution to 10  $\mu$ M in 1X Reaction Buffer. Use 1X Reaction Buffer without H<sub>2</sub>O<sub>2</sub> as a negative control.

**2.3** Pipet 100  $\mu$ L of the diluted amine oxidase samples and controls into separate wells of a microplate.

**2.4** Prepare a working solution of 400  $\mu$ M Amplex Red reagent containing 2 U/mL HRP and 2 mM substrate by adding 200  $\mu$ L of Amplex Red reagent stock solution (prepared in step 1.1), 100  $\mu$ L of the HRP stock solution (prepared in step 1.3) and 200  $\mu$ L of substrate stock solution (prepared in 1.5) to 9.5 mL 1X Reaction Buffer. This 10 mL volume is sufficient for ~100 assays. Note that final concentrations of each component will be twofold lower in the final reaction volume.

**2.5** Begin the reactions by adding 100  $\mu$ L of the Amplex Red reagent/HRP/substrate working solution to each microplate well containing the amine oxidase samples and controls.

**2.6** Incubate the reactions for 30 minutes or longer at room temperature, 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.

**2.7** If desired, prepare a resorufin standard curve: Dilute the appropriate amount of 2 mM resorufin stock solution in 1X Reaction Buffer to yield resorufin solution ranging from 0 to 20  $\mu$ M resorufin. Pipet 200  $\mu$ L of each resorufin standard into individual (empty) wells of a microplate at any time prior to measuring fluorescence.

**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-amine oxidase control.

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## References

1. Anal Biochem 253, 162 (1997); 2. Anal Biochem 253, 169 (1997); 3. Methods Enzymol 142, 617 (1987); 4. Anal Biochem 244, 384 (1997); 5. Biochem Pharmacol 18, 1447 (1969).

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## Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
A12214	Amplex® Red Monoamine Oxidase Assay Kit *500 assays*	1 kit
A12222	Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine)	5 mg
A22177	Amplex® Red reagent *packaged for high-throughput screening*	10 x 10 mg
A36006	Amplex® UltraRed reagent	5 x 1 mg

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