Revised: 01–October–2004

Amplex® Red Xanthine/Xanthine Oxidase Assay Kit (A22182)

Quick Facts

Storage upon receipt:

- –20°C
- Desiccate
- Protect from light

Abs/Em of reaction product: 571/585 nm

Introduction

The Amplex® Red Xanthine/Xanthine Oxidase Assay Kit provides an ultrasensitive method for detecting xanthine or hypoxanthine or for monitoring xanthine oxidase activity. In the assay, xanthine oxidase catalyzes the oxidation of purine bases, hypoxanthine or xanthine, to uric acid and superoxide. In the reaction mixture, the superoxide spontaneously degrades to hydrogen peroxide (H_2O_2), and the H_2O_2, in the presence of horseradish peroxidase (HRP), reacts stoichiometrically with Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin.[1] Resorufin has absorption and fluorescence emission maxima of approximately 571 nm and 585 nm, respectively (Figure 1), and because the extinction coefficient is high (54,000 $cm^{-1}M^{-1}$), the assay can be performed either fluorometrically or spectrophotometrically.

In healthy individuals, xanthine oxidase is present in appreciable amounts only in the liver and jejunum. However, in various liver disorders the enzyme is released into circulation. Therefore, determination of serum xanthine oxidase level serves as a sensitive indicator of acute liver damage such as jaundice.[2] Previously researchers have utilized chemiluminescence [3,4] or absorbance [5] to monitor xanthine oxidase activity. Using the Amplex Red Xanthine/Xanthine Oxidase Assay Kit, one can detect xanthine oxidase in a purified system at levels as low as 0.1 mU/mL by fluorescence (Figure 2). The kit can also be used to detect as little as 200 nM hypoxanthine (Figure 3) or xanthine (not shown).

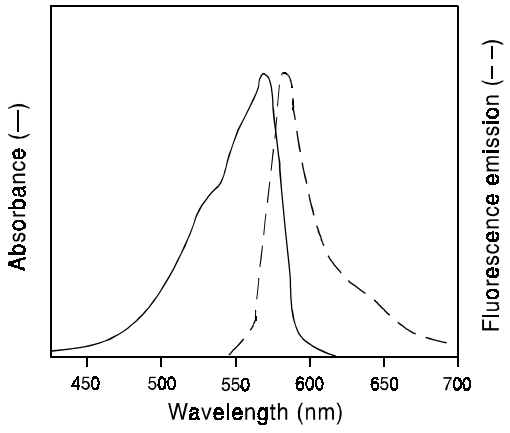

Figure 1. *Normalized absorption and fluorescence emission spectra of resorufin, the product of the Amplex Red reagent.*

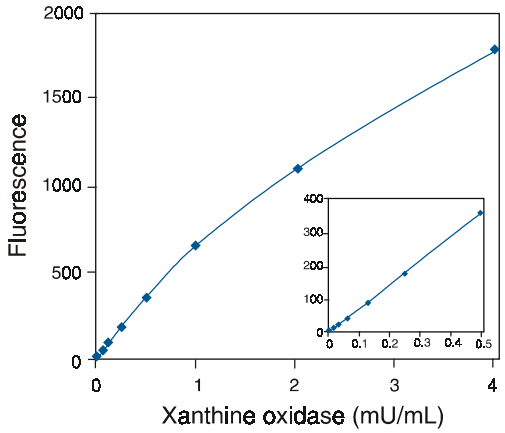

Figure 2. *Detection of xanthine oxidase using the Amplex Red reagent–based assay. Each reaction contained 50 µM Amplex Red reagent, 0.2 U/mL HRP, 0.1 mM hypoxanthine and the indicated amount of xanthine oxidase in 1X Reaction Buffer. After 30 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. A background of 65 fluorescence units was subtracted from each data point.*

Materials

Kit Contents

- **Amplex Red reagent** (MW = 257, Component A), two vials, each containing 0.26 mg
- **Dimethylsulfoxide (DMSO)**, anhydrous (Component B), 500 µL
- **Horseradish peroxidase** (Component C), 20 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 20ºC
- **Hydrogen peroxide (H_2O_2)** (MW = 34, Component D), 500 µL of a stabilized ~3% solution; the actual concentration is indicated on the component label
- **5X Reaction Buffer** (Component E), 20 mL of 0.5 M Tris-HCl, pH 7.5
- **Xanthine oxidase from buttermilk** (Component F), 1 U, where 1 unit is defined as the amount of xanthine oxidase that will form 1 µmole of uric acid from hypoxanthine at 25°C
- **Hypoxanthine** (Component G), 1.0 mL of a 20 mM solution in 40 mM NaOH
- **Xanthine** (Component H), 1.0 mL of a 20 mM solution in 40 mM NaOH

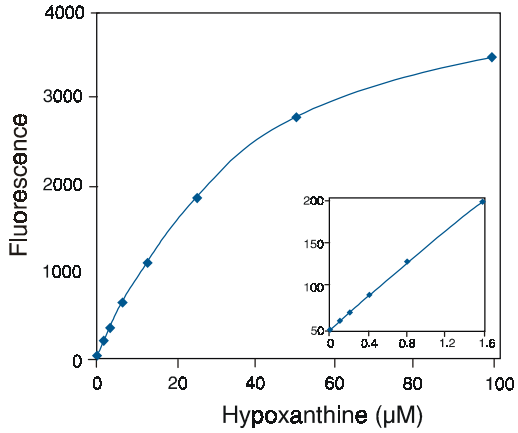

Figure 3. *Detection of hypoxanthine using the Amplex Red reagent–based assay. Each reaction contained 50 µM Amplex Red reagent, 0.2 U/mL HRP, 20 mU/mL xanthine oxidase and the indicated amount of hypoxanthine in 1X Reaction Buffer. Reactions were incubated at 37°C. After 30 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. A background of 54 fluorescence units was subtracted from each data point.*

Each Kit provides sufficient reagents for approximately 400 assays using either a fluorescence or absorbance microplate reader and reaction volumes of 100 µL per assay.

Storage and Handling

Upon receipt, the kit should be stored frozen at -20°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 or absorbance multiwell plate scanner. For use with a standard fluorometer or spectrophotometer, volumes must be increased accordingly.

Please note that resorufin, the product of the Amplex Red reaction, is unstable in the presence of thiols such as dithiotheritol (DTT) and β-mercaptoethanol. For this reason, the final DTT or 2-mercaptoethanol concentration in the reaction should be no higher than 10 µM.

The absorption and fluorescence spectra 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 reactions should be performed at pH 7–8. We recommend using the included Reaction Buffer (pH 7.5) for optimal performance of the Amplex Red reagent.

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 of DMSO. Each vial of Amplex Red reagent is sufficient for approximately 200 assays, with a final reaction volume of 100 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 (Component E) by adding 4 mL of 5X Reaction Buffer stock solution (Component E) to 16 mL of deionized water (dH_2O). This 20 mL volume of 1X Reaction Buffer is sufficient for approximately 100 assays of 100 µL each with a 10 mL excess for making stock solutions and dilutions.

1.3 Prepare a 100 U/mL stock solution of horseradish peroxidase (HRP) by dissolving the contents of the vial of HRP (Component C) in 200 µL 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 10 U/mL stock solution of xanthine oxidase by dissolving the contents of the vial xanthine oxidase (Component F) in 100 µL of dH_2O. After use, the remaining solution should be divided into small aliquots and stored frozen at -20°C.

Xanthine or Hypoxanthine Assay

The following protocol describes the assay of either xanthine or hypoxanthine in a total volume of 100 µL per microplate well. The volumes recommended here are sufficient for ~100 assays. The kit provides sufficient material for ~400 assays.

2.1 Prepare a hypoxanthine or xanthine standard curve: Dilute the 20 mM hypoxanthine (Component G) or xanthine (Component H) in 1X Reaction Buffer to produce hypoxanthine or xanthine concentrations of 0 to 100 µM. Use 1X Reaction Buffer without hypoxanthine or xanthine as a negative control. A volume of 50 µL will be used for each reaction. Please note that the hypoxanthine or xanthine concentrations will be twofold lower in the final reaction volume.

2.2 Dilute the hypoxanthine- or xanthine-containing samples in 1X Reaction Buffer. A volume of 50 µL will be used for each reaction.

2.3 If desired, prepare a positive control by diluting the 20 mM H_2O_2 working solution (prepared in step 1.4) to 10 µM in 1X Reaction Buffer.

2.4 Pipet 50 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 0.4 U/mL HRP and 40 mU/mL xanthine oxidase by mixing:

- 50 µL of the Amplex Red reagent stock solution (prepared in step 1.1)
- 20 µL of the HRP stock solution (prepared in step 1.3)
- 20 µL of the xanthine oxidase stock solution (prepared in step 1.5)
- 4.91 mL of 1X Reaction Buffer.

This 5 mL volume is sufficient for ~100 assays. Note that the final concentration of each component will be twofold lower in the final reaction volume.

2.6 Begin the reactions by adding 50 µL of the Amplex Red reagent/HRP/xanthine oxidase working solution to each microplate well containing the samples and controls.

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

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

2.9 For each point, correct for background fluorescence or absorbance by subtracting the value derived from the no-hypoxanthine or no-xanthine control.

Xanthine Oxidase Assay

The following protocol describes the assay of xanthine oxidase activity in a total volume of 100 µL. The volumes recommended here are sufficient for ~100 assays. The kit provides sufficient material for ~400 assays.

3.1 Prepare a xanthine oxidase standard curve: Dilute the 10 U/mL xanthine oxidase stock solution (prepared in step 1.5) to produce xanthine oxidase concentrations of 0 to 10 mU/mL. Use 1X Reaction Buffer without xanthine oxidase as a negative control. A volume of 50 µL will be used for each reaction. Note that the xanthine oxidase concentrations will be twofold lower in the final reaction volume.

3.2 Dilute the xanthine oxidase–containing samples in 1X Reaction Buffer. A volume of 50 µL will be used for each reaction.

3.3 If desired, prepare a positive control by diluting the 20 mM H_2O_2 working solution (prepared in step 1.4) to 10 µM in 1X Reaction Buffer.

3.4 Pipet 50 µL of the diluted samples and controls into separate wells of a microplate.

3.5 Prepare a working solution of 100 µM Amplex Red reagent containing 0.4 U/mL HRP and either 200 µM hypoxanthine or xanthine by mixing:

- 50 µL of the Amplex Red reagent stock solution (prepared in step 1.1)
- 20 µL of the HRP stock solution (prepared in step 1.3)
- 50 µL of either hypoxanthine (Component G) or xanthine (Component H)
- 4.88 mL of 1X Reaction Buffer

This 5 mL volume is sufficient for ~100 assays. Note that the final concentrations of each component will be twofold lower in the final reaction volume.

3.6 Begin the reactions by adding 50 µL of the Amplex Red reagent/HRP/hypoxanthine or xanthine working solution to each microplate well containing the samples and controls.

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

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

3.9 For each point, correct for background fluorescence or absorbance by subtracting the values derived from the no-xanthine oxidase control.

References

1. J Immunol Methods 202, 133 (1997); **2.** Clin Chim Acta 63, 37 (1975); **3.** J Reprod Fert 97, 441 (1993); **4.** Free Radic Biol Med 8, 121 (1990); **5.** J Biol Chem 273, 7828 (1998).

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

Cat #	Product Name	Unit Size
A12222	Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine)	5 mg
A22177	Amplex® Red reagent *packaged for high-throughput screening*	10 x 10 mg
A22182	Amplex® Red Xanthine/Xanthine Oxidase Assay Kit *400 assays*	1 kit
A36006	Amplex® UltraRed reagent	5 x 1 mg

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