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Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (A22188)

Quick Facts

Storage upon receipt:

- –20°C
- Desiccate
- Protect from light

Abs/Em of reaction product: 571/585 nm

Introduction

The Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (A22188) contains a sensitive, one-step assay that uses the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to detect hydrogen peroxide (H_2O_2) or peroxidase activity. The Amplex Red reagent, in combination with horseradish peroxidase (HRP), has been used to detect H_2O_2 released from biological samples, including cells,[1–4] or generated in enzyme-coupled reactions.[5–7] Furthermore, Amplex Red reagent can be used as an ultrasensitive assay for peroxidase activity when H_2O_2 is in excess.

In the presence of peroxidase, the Amplex Red reagent reacts with H_2O_2 in a 1:1 stoichiometry to produce 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. This reaction has been used to detect as little as 10 picomoles of H_2O_2 in a 100 µL volume (50 nM; Figure 2) or 1×10^{-5} U/mL of HRP (Figure 3).

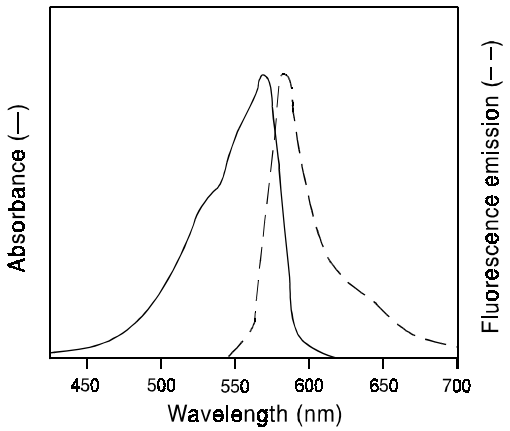

Figure 1. *Normalized absorption and fluorescence emission spectra of resorufin, the product of the Amplex Red reaction.*

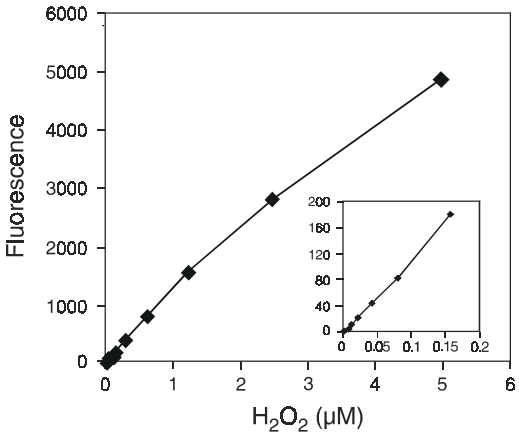

Figure 2. *Detection of H_2O_2 using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit. Reactions containing 50 µM Amplex Red reagent, 0.1 U/mL HRP and the indicated amount of H_2O_2 in 50 mM sodium phosphate buffer, pH 7.4, were incubated for 30 minutes at room temperature. Fluorescence was then measured with a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. Background fluorescence (24 arbitrary units), determined for a no-H_2O_2 control reaction, has been subtracted from each value. The inset shows the sensitivity of the assay at very low levels of H_2O_2.*

Materials

Kit Components

- **Amplex Red reagent** (MW = 257, Component A, blue cap), five vials, each containing 154 µg of reagent
- **Dimethylsulfoxide (DMSO)**, anhydrous (Component B, green cap), 700 µL
- **5X Reaction Buffer** (Component C, white cap), 28 mL of 0.25 M sodium phosphate, pH 7.4
- **Horseradish peroxidase** (Component D, yellow cap), 10 U, where 1 unit (U) is defined as the amount of enzyme that will form 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0 and 20°C
- **Hydrogen peroxide (H_2O_2)** (MW = 34, Component E, red cap), 200 µL of a stabilized ~3% solution; the actual concentration is indicated on the label

Each kit provides sufficient reagents for approximately 500 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

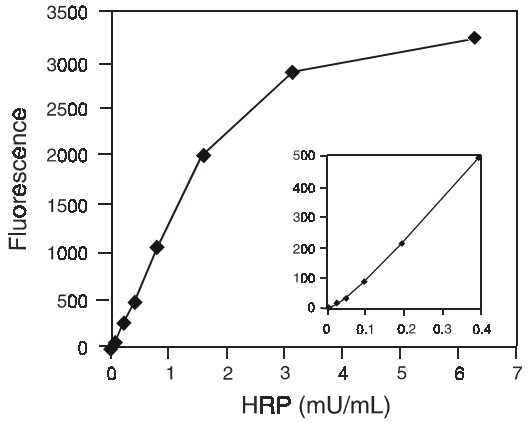

Figure 3. *Detection of HRP using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit. Reactions containing 50 µM Amplex Red reagent, 1 mM H_2O_2 and the indicated amount of HRP in 50 mM sodium phosphate buffer, pH 7.4, were incubated for 30 minutes at room temperature. Fluorescence was then measured with a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. Background fluorescence (3 arbitrary units), determined for a no-HRP control reaction, has been subtracted from each value. The inset shows the sensitivity of the assay at very low levels of HRP.*

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 microplate reader. To use with a standard fluorometer, volumes must be increased accordingly.

Please note the following restrictions on the use of the Amplex Red reagent. The Amplex Red reagent is unstable in the presence of thiols such as dithiothreitol (DTT) and 2-mercaptoethanol. The final concentration of DTT or 2-mercaptoethanol in the reaction should be no higher than 10 µM. The Amplex Red reagent is also unstable at high pH (>8.5). Furthermore, the absorption and fluorescence of the reaction product, 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. For these reasons, the reactions should be performed at pH 7–8. The provided Reaction Buffer is pH 7.4.

Stock Solution Preparation

1.1 Prepare a 10 mM Amplex Red reagent stock solution. Allow one vial of Amplex Red reagent (Component A, blue cap) and DMSO (Component B, green cap) to warm to room temperature. Just prior to use, dissolve the contents of the vial of Amplex Red reagent in 60 µL of DMSO. Each vial of Amplex Red reagent is sufficient for approximately 100 assays, with a final reaction volume of 100 µL per assay.

1.2 Prepare 1X Reaction Buffer. Add 4 mL of 5X Reaction Buffer (Component C, white cap) to 16 mL of deionized water (dH_2O). This 20 mL volume of 1X Reaction Buffer working solution is sufficient for approximately 100 assays of 100 µL each with 10 mL excess for making stock solutions.

1.3 Prepare a 10 U/mL horseradish peroxidase (HRP) stock solution. Dissolve the contents of the vial of HRP (Component D, yellow cap) in 1.0 mL of 1X Reaction Buffer. After the assay, any unused HRP stock solution should be divided into single-use aliquots and stored frozen at -20°C.

1.4 Prepare a 20 mM H_2O_2 working solution. Dilute the ~3% H_2O_2 (Component E, red cap) into the appropriate volume of 1X Reaction Buffer. The actual concentration of H_2O_2 is indicated on the label. For instance, a 20 mM H_2O_2 working solution can be prepared from a 3.0% (0.88 M) H_2O_2 stock solution by diluting 22.7 µL of 3.0% H_2O_2 into 977 µL of 1X Reaction Buffer. 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 prepared in this step will be less stable and should be used promptly.

H_2O_2 Assay

The following protocol describes the assay of H_2O_2 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 ~500 assays.

2.1 Prepare an H_2O_2 standard curve. Dilute the appropriate amount of 20 mM H_2O_2 working solution (prepared in step 1.4) into 1X Reaction Buffer to produce H_2O_2 concentrations of 0 to 10 µM, each in a volume of 50 µL. Be sure to include a no-H_2O_2 control. Final H_2O_2 concentrations will be twofold lower (e.g., 0 to 5 µM).

2.2 If no standard curve is to be used, prepare positive and negative controls. For a positive control, dilute the 20 mM H_2O_2 working solution to 10 µM in 1X Reaction Buffer. For a negative control, use 1X Reaction Buffer without H_2O_2.

2.3 Dilute the H_2O_2-containing samples in 1X Reaction Buffer. A volume of 50 µL will be used for each reaction. A variable dilution will be required depending on the total H_2O_2 present in the sample. In the first trial the samples should be serially diluted to determine the optimal amount of sample for the assay. Note that extremely high levels of H_2O_2 (e.g., 100 µM, final concentration) can produce lower fluorescence than moderately high levels (e.g., 25 µM), because excess H_2O_2 can oxidize the reaction product, resorufin, to nonfluorescent resazurin.

2.4. Load the samples. Pipet 50 µL of the standard curve samples, controls and experimental samples into individual wells of a microplate.

2.5 Prepare a working solution of 100 µM Amplex Red reagent and 0.2 U/mL HRP. Mix the following:

- 50 µL of 10 mM Amplex Red reagent stock solution (prepared in step 1.1)
- 100 µL of 10 U/mL HRP stock solution (prepared in step 1.3)
- 4.85 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. Add 50 µL of the Amplex Red reagent/HRP working solution to each microplate well containing the standards, controls and samples.

2.7 Incubate the reactions. Incubate at room temperature for 30 minutes, 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 or absorbance. Use a microplate reader equipped for excitation in the range of 530–560 nm and fluorescence emission detection at ~590 nm (see Figure 1), or for absorbance at ~560 nm.

2.9 Correct for background fluorescence or absorbance. For each point, subtract the value derived from the no-H_2O_2 control.

Measurement of H_2O_2 Released from Cells

The Amplex Red reagent can be used to detect the release of H_2O_2 from activated human leukocytes. To use the Amplex Red H_2O_2 assay for this type of experiment, the protocol devised by Mohanty and colleagues,[2] summarized here, may be useful.

3.1 Prepare a reaction mixture. The mixture should contain 50 µM Amplex Red reagent and 0.1 U/mL HRP in Krebs–Ringer phosphate (KRPG; 145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, 5.5 mM glucose, pH 7.35). If desired, an activator, such as phorbol 12-myristate 13-acetate (PMA), can be added to the reaction mixture. A volume of 100 µL will be used for each reaction.

3.2 Prepare the samples. Pipet 100 µL of the reaction mixture into each microplate well.

3.3 Prewarm the reaction mixture. Prewarm at 37°C for ten minutes.

3.4 Start the reaction. Add ~1.5×10^4 cells in 20 µL of KRPG. For a negative control, add 20 µL of KRPG alone.

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

3.6 Return the plate to the incubator. Continue to measure the fluorescence at selected time points over the desired time period.

Peroxidase Assay

The following protocol describes the assay of peroxidase in a total volume of 100 µL per microplate well. The volumes here are sufficient for ~100 assays. The kit provides sufficient material for ~500 assays.

4.1 Prepare a peroxidase standard curve. Dilute the appropriate amount of 10 U/mL HRP stock solution (prepared in step 1.3) into 1X Reaction Buffer to produce HRP concentrations of approximately of 0 to 2 mU/mL HRP, each in a volume of 50 µL. Be sure to include a no-HRP control. Please note that the HRP concentrations will be twofold lower in the final reaction volume.

4.2 If no standard curve is to be used, prepare positive and negative controls. For a positive control, dilute the 10 U/mL HRP stock solution (prepared in step 1.3) to 2 mU/mL in 1X Reaction Buffer. Use 1X Reaction Buffer without HRP as a negative control.

4.3 Dilute the peroxidase-containing samples in 1X Reaction Buffer. A volume of 50 µL will be used for each reaction. A variable dilution will be required depending on the total peroxidase present in the sample. In the first trial the samples should be serially diluted to determine the optimal amount of sample for the assay. Note that extremely high levels of HRP (e.g., 100 mU/mL, final concentration) can produce lower fluorescence than moderately high levels (e.g., 1 mU/mL), because excess HRP can oxidize the reaction product, resorufin, to nonfluorescent resazurin.

4.4 Load the samples. Pipet 50 µL of standard curve samples, controls and experimental samples into individual wells of a microplate.

4.5 Prepare a working solution of 100 µM Amplex Red reagent containing 2.0 mM H_2O_2. Mix:

- 50 µL of 10 mM Amplex Red reagent stock solution (prepared in step 1.1)
- 500 µL of 20 mM H_2O_2 working solution (prepared in step 1.4)
- 4.45 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.

4.6 Begin the reactions. Add 50 µL of the Amplex Red reagent/H_2O_2 working solution to each microplate well containing the standards, controls and samples.

4.7 Incubate the reactions. Incubate at room temperature for 30 minutes, protected from light. Because the assay is continuous (not terminated), fluorescence or absorbance may be measured at multiple time points to follow the kinetics.

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

4.9 Correct for background fluorescence or absorbance. For each point, subtract the value derived from the no-HRP control.

References

1. Anal Biochem 253, 162 (1997); **2.** J. Immunol Methods 202, 133 (1997); **3.** J Neurochem 79, 266 (2001); **4.** Am J Physiol Lung Cell Mol Physiol 281, L993 (2001); **5.** Mol Hum Reprod 7, 237 (2001); **6.** Anal Biochem 287, 196 (2000); **7.** J Invest Dermatol 112, 751 (1999).

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

Cat #	Product Name	Unit Size
A22188	Amplex® Red Hydrogen Peroxide/Peroxidase 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
R363	resorufin, sodium salt *reference standard*	100 mg

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