Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit

Catalog no. A22188

Table 1. Contents and storage information.

Material	Amount	Storage *	Stability
Amplex® Red reagent (MW = 257.25) (Component A, blue cap)	5 vials, each containing 154 µg of reagent	• ≤–20°C • Dessicate • Protect from light	When stored as directed, the kit components are stable for at least 6 months.
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 †		
Hydrogen peroxide (H_2O_2) (MW = 34.01) (Component E, red cap)	200 µL of a stabilized ~3% solution‡		
* The kit can be stored under the conditions listed. For optimal storage conditions of individual components, refer to the labels on the vials.			
† 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. ‡ Actual concentration is indicated on the label.			
Number of assays: Each kit provides sufficient reagents for approximately 500 assays using a fluorescence or absorbance microplate reader and reaction volumes of 100 µL per assay.			
Approximate fluorescence excitation and emission maxima: 571/585 nm for resorufin, the product of the Amplex® Red reaction.			

Introduction

The Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit 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 excitation and emission maxima of approximately 571 nm and 585 nm, respectively (Figure 1), and because the extinction coefficient is high (58,000 ± 5,000 $cm^{-1}M^{-1}$), you can perform the assay 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).

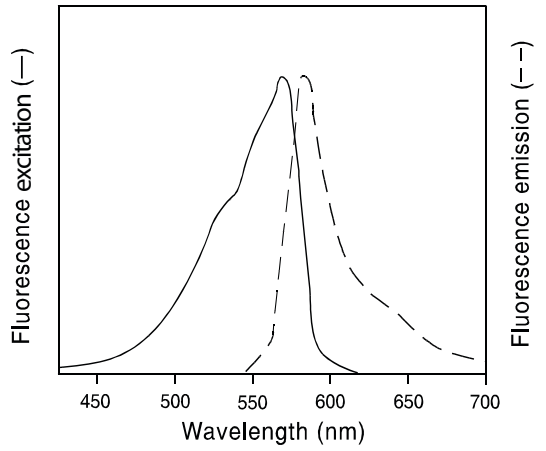

Figure 1. Normalized excitation and emission spectra of resorufin, the product of the Amplex® Red reaction.

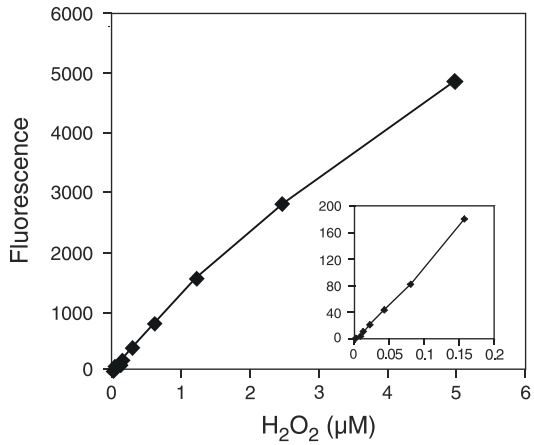

Figure 2. Detection of H_2O_2 using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit. Reactions containing 50 µM Amplex® Red reagent, 0.1 U/mL HRP and the indicated amount of H_2O_2 in 50 mM sodium phosphate buffer, pH 7.4, were incubated for 30 minutes at room temperature. Fluorescence was then measured with a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. Background fluorescence, 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.

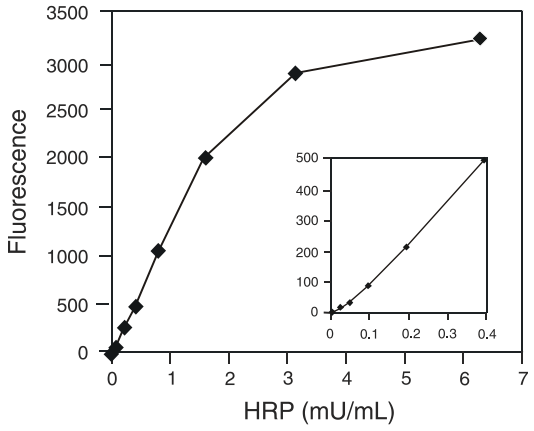

Figure 3. Detection of HRP using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit. Reactions containing 50 µM Amplex® Red reagent, 1 mM H_2O_2 and the indicated amount of HRP in 50 mM sodium phosphate buffer, pH 7.4, were incubated for 30 minutes at room temperature. Fluorescence was then measured with a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. Background fluorescence, 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.

Before You Begin

Using the Amplex® Red Reagent

- The Amplex® Red reagent is air sensitive. Once you open a vial of Amplex® Red, use the reagent on the same day.
- The Amplex® Red reagent is unstable in the presence of thiols such as dithiothreitol (DTT) and 2-mercapto-ethanol. 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 fluoroscence excitation and emission 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, perform the reactions at pH 7–8. The provided Reaction Buffer is pH 7.4.
- Protect the Amplex® Red reagent from light.
- Allow all reagents in the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit to completely warm to room temperature before opening.

Caution DMSO is hazardous; avoid contact with skin and eyes and do not swallow. Handle reagents containing DMSO using equipment and practices appropriate for the hazards posed by such materials.

Preparing Stock Solutions

10 mM Amplex® Red reagent stock solution

1.1 Allow one vial of Amplex® Red reagent (Component A, blue cap) and DMSO (Component B, green cap) to warm to room temperature.

1.2 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. Use the Amplex® Red reagent stock solution on the same day it is prepared.

1X Reaction Buffer

1.3 Add 4 mL of 5X Reaction Buffer (Component C, white cap) to 16 mL of deionized water. 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.

10 U/mL Horseradish Peroxidase (HRP) stock solution

1.4 Dissolve the contents of the vial of HRP (Component D, yellow cap) in 1.0 mL of 1X Reaction Buffer. After the assay, divide any unused HRP stock solution into single-use aliquots and store frozen at –20°C.

20 mM Hydrogen Peroxide (H_2O_2) working solution

1.5 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, you can prepare a 20 mM H_2O_2 working solution 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.

Note that although the ~3% H_2O_2 stock solution has been stabilized to slow its degradation, the 20 mM H_2O_2 working solution prepared in this step will be less stable and should be used within a few hours of preparation.

Stability of Solubilized Reagents

We have shown in our laboratory that Amplex® Red, Amplex® UltraRed, and HRP solutions are stable for at least six months if stored correctly. The recommended storage conditions for these reagents are minimal exposure to light, air, and freeze thaw cycles. We also recommend using only high quality and fresh solvents. Despite these measures, we cannot guarantee their storage stability. Pink coloring in Amplex® or Amplex® UltraRed reagents is an early indicator of compromised material.

Experimental Protocols

The following procedures are designed for use with a fluorescence or absorbance 96-well microplate reader. To use with a standard fluorometer or with different sized microplates, adjust the volumes accordingly.

H_2O_2 Assay

The following protocol describes the H_2O_2 assay 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 1.5) into 1X Reaction Buffer (prepared in step 1.3) 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 two-fold lower (*e.g.,* 0 to 5 µM).

2.2 If you are not using a standard curve, 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. Use a volume of 50 µL for each reaction. A variable dilution will be required depending on the total H_2O_2 present in the sample.

In the first trial, serially dilute the samples 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 1.2)
- 100 µL of 10 U/mL HRP stock solution (prepared in step 1.4)
- 4.85 mL of 1X Reaction Buffer (prepared in step 1.3)

This 5 mL volume is sufficient for ~100 assays. Note that the final concentration of each component will be two-fold 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), you may measure fluorescence or absorbance 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.

Measuring H_2O_2 Released from Cells

You can use the Amplex® Red reagent 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. If desired, you can add an activator, such as phorbol 12-myristate 13-acetate (PMA), to the reaction mixture. Each reaction has a volume of 100 µL.

Note: Krebs–Ringer phosphate (KRPG) consists of 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.

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

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

3.4 Start the reaction. Add 20 µL of ~1.5×10^4 cells suspended in KRPG buffer to the 100 µL reaction mixture prepared in step 3.1 and warmed in step 3.3. If a negative control is required, add 20 µL of KRPG buffer without cells to a separate 100 µL warmed reaction mixture.

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 1.4) into 1X Reaction Buffer (prepared in step 1.3) 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. Note that the HRP concentrations will be two-fold lower in the final reaction volume.

4.2 If you are not using a standard curve, prepare positive and negative controls. For a positive control, dilute the 10 U/mL HRP stock solution (prepared in step 1.4) to 2 mU/mL in 1X Reaction Buffer (prepared in step 1.3). Use 1X Reaction Buffer without HRP as a negative control.

4.3 Dilute the peroxidase-containing samples in 1X Reaction Buffer. Use a volume of 50 µL for each reaction. A variable dilution will be required depending on the total peroxidase present in the sample.

In the first trial, serially dilute the samples 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 mM H_2O_2. Mix the following:

- 50 µL of 10 mM Amplex® Red reagent stock solution (prepared in step 1.2)
- 500 µL of 20 mM H_2O_2 working solution (prepared in step 1.5)
- 4.45 mL of 1X Reaction Buffer (prepared in step 1.3)

This 5 mL volume is sufficient for ~100 assays. Note that the final concentration of each component will be two-fold 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), you may measure fluorescence or absorbance at multiple time points to follow the kinetics of the reactions.

4.8 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.

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 website or from our Customer Service Department.**

Cat. no.	Product Name	Unit Size
A22188	Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit *500 assays*	1 kit
Related Products		
A12222	Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine)	5 mg
A22177	Amplex® Red reagent *packaged for high-throughput screening*	10 x 10 mg
A33855	Amplex® Red/UltraRed stop reagent *500 tests*	set of 5 vials
A36006	Amplex® UltraRed reagent	5 x 1 mg
R363	resorufin, sodium salt *reference standard*	100 mg

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