Amplex® UltraRed Reagent

Catalog no. A36006

Table 1. Contents and storage information.

Material	Amount	Storage	Stability
Amplex® UltraRed reagent (MW = ~300)	5 vials, each containing 1 mg	• ≤–20˚C • Desiccate • Protect from light	When stored as directed, the product is stable for at least 1 year.
Number of assays: Sufficient material is supplied for 3,400 reactions in 96-well microplates at 100 µL per well, based on the protocol below.			
Approximate fluorescence excitation and emission maxima: 568/581 nm for the reaction product.			
Recommended instrument settings: 490–550 nm/580–590 nm (see Step 4.9).			

Introduction

The Amplex® UltraRed reagent is a sensitive and robust tool for detection in ELISA formats. The reagent is a fluorogenic substrate for horseradish peroxidase (HRP) that reacts with hydrogen peroxide (H_2O_2) in a 1:1 stoichiometric ratio to produce Amplex® UltroxRed, a brightly fluorescent and strongly absorbing reaction product (excitation/emission maxima ~568/581 nm) (Figure 1). Because the Amplex® UltroxRed product has long-wavelength spectra, there is little interference from the blue or green autofluorescence found in most biological samples. Superiority of fluorescence-based detection in ELISAs is known[1], and with a high extinction coefficient, good quantum efficiency, and resistance to auto-oxidation, the fluorogenic Amplex® UltraRed reagent delivers greater overall signal enhancement and a broader assay range than colorimetric reagents such as TMB (Figures 2 a & b). In addition, Amplex® UltroxRed has a lower pK_a value than the reaction products for similar fluorogenic substrates such as Amplex® Red, giving Amplex® UltraRed utility across a broader pH range (Figure 3). For HRP-based ELISA applications, this routinely results in at least two-fold greater sensitivity compared to Amplex® Red (Figure 4).

In addition to ELISA visualization, Amplex® UltraRed can be used as a very sensitive assay for H_2O_2 (Figure 5). In combination with excess HRP, the reagent may be used to detect H_2O_2 released from biological samples, including cells, or generated in enzyme-coupled reactions. Any target of interest that may be associated with an oxidase enzyme reaction can be detected and quantified with Amplex® UltraRed reagent. For example, excess glucose oxidase may be used to react with D-glucose to form D-gluconolactone and H_2O_2. Because H_2O_2 reacts with Amplex® UltraRed in the presence of HRP in a 1:1 stoichiometry to generate Amplex® UltroxRed, the reagent can be used as an assay for glucose. Likewise, using the same system in the presence of excess D-glucose, Amplex® UltraRed can be used as an assay for glucose oxidase activity.

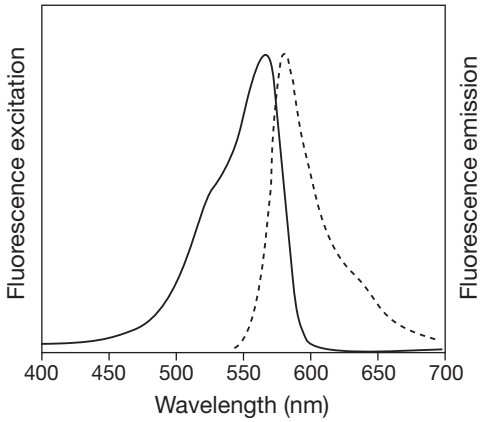

Figure 1. Normalized absorption and fluorescence emission spectra for the Amplex® UltraRed reagent reaction product.

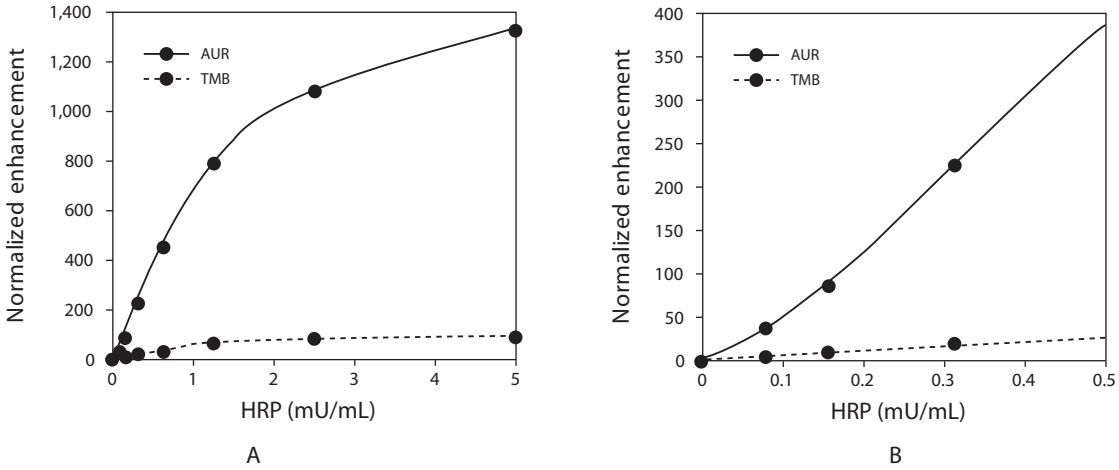

Figure 2. Titration of HRP using Amplex® UltraRed (solid line) and TMB (dashed line). Triplicate samples of HRP were assayed at concentrations of 0.078 mU/mL to 5 mU/mL in the presence of 50 µM Amplex® UltraRed and 1 mM hydrogen peroxide or 1X TMB Liquid Substrate System for ELISA (Sigma T-0440) (panel A). Panel B shows the same data at low concentrations of HRP. Reactions were incubated for 15 minutes at room temperature and read using a Molecular Devices SpectraMax M5 microplate reader. TMB reactions were stopped with addition of 1 M HCl, and optical density measurements were taken at 450 nm. Amplex® UltraRed measurements were taken using fluorescence Ex/Em settings of 490/585 nm. Normalized enhancement is equal to signal divided by background, and may vary.

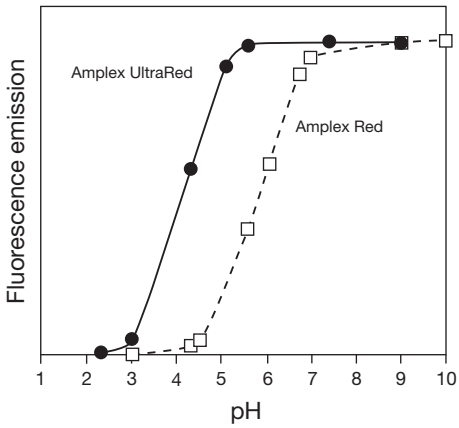

Figure 3. Fluorescence signal as a function of pH for reaction products of Amplex® UltraRed and Amplex® Red. Fluorescence intensities were measured using Ex/Em settings of ~570/585 nm.

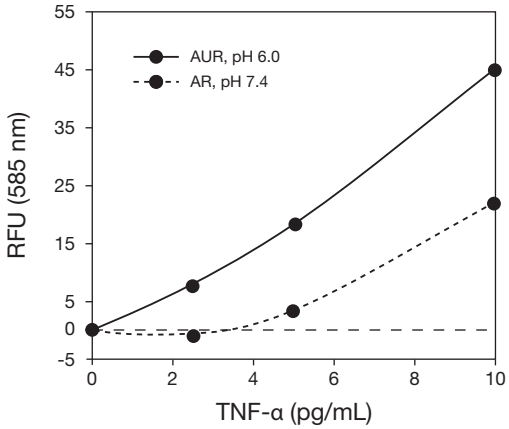

Figure 4. Sensitivity of the Amplex® UltraRed reagent in ELISA detection. Triplicate samples of TNF-α were assayed at concentrations of 2.5 pg/mL to 10.0 pg/mL according to the standard TNF-α Human ELISA kit protocol (Cat. no. KHC3011) using Amplex® UltraRed (solid lines) in 50 mM sodium citrate (pH 6.0) and Amplex® Red (dashed lines) in PBS (pH7.4). The concentration of reagent used in both cases was 50 µM in the presence of 1 mM hydrogen peroxide. Reactions were incubated for 15 minutes at room temperature and fluorescence was measured at Ex/Em 490/585 nm using a Molecular Devices SpectraMax M5 microplate reader. Background fluorescence has been subtracted.

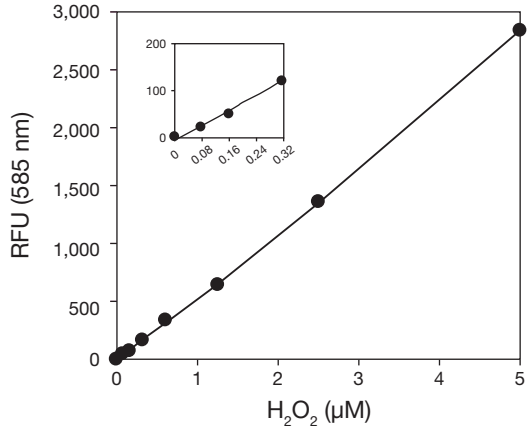

Figure 5. Detection of H_2O_2 using the Amplex® UltraRed reagent. Reactions containing 50 µM Amplex® UltraRed reagent, 0.1 U/mL HRP, and the indicated amount of H_2O_2 were incubated in 50 mM sodium citrate (pH6.0) for 30 minutes at room temperature and fluorescence was measured at Ex/Em 490/585 nm using a Molecular Devices SpectraMax M5 microplate reader. The inset, using the same data set, highlights the sensitivity of the assay at low levels of H_2O_2. Background fluorescence has been subtracted.

Before you Begin

Materials Required but Not Provided

- Fresh, anhydrous DMSO
- Stabilized hydrogen peroxide (H_2O_2)
- Reaction buffer
- 96-well microplates
- Deionized water
- Fluorescence microplate reader (see Step 3.5 for settings)
- *Optional*: Horseradish peroxidase (HRP)
- *Optional*: Standards for oxidase-coupled enzyme reactions
- *Optional*: Amplex® Red/UltraRed stop reagent (Cat. no. A33855)
- *Optional*: Absolute ethanol

Caution

No data are currently available addressing the mutagenicity or toxicity of the Amplex® UltraRed reagent or the oxidized reaction product.

Use caution when handling DMSO stock solutions, as DMSO is known to facilitate the entry of organic molecules into tissues.

Handling Amplex® UltraRed Reagent

Upon receipt, store the Amplex® UltraRed reagent frozen at –20°C, **protected from light**. Allow the reagent to equilibrate to room temperature before use.

The Amplex® UltraRed reagent is sensitive to air; keep all vials containing the reagent tightly capped when not in use, and prepare all necessary solutions promptly after opening vials.

Determining Appropriate Buffer for Analyte System

- For enzyme-coupled systems, choose a pH near the reported optimum of most relevant enzyme(s).
- For detecting H_2O_2, buffers with pH 6–7.5 work best (*e.g.*, 50–100 mM sodium citrate, pH 6.0, or HEPES, pH 7.0).
- Because HRP has a pH optimum near 6.0 in the reaction with Amplex® UltraRed, reaction conditions at pH 6.0 may result in slightly greater sensitivity and signal enhancement over a shorter incubation period (5–15 minutes). Buffers that contain Tris-HCl or have pH ≥ 8.0 contribute to increased background, and may result in reduced sensitivity at similar timepoints.
- Many cell lysates have been shown to stabilize background signal from Amplex® UltraRed.

Preparing Stock Solutions

Amplex® UltraRed stock solution

1.1 Prepare a 10 mM stock solution of Amplex® UltraRed reagent by adding 340 µL of fresh, high-quality DMSO to one vial of Amplex® UltraRed reagent. Vortex well to dissolve.

Protect this solution from light and moisture. Store remaining solution in the dark with desiccant at –20°C for future use. When stored properly, this solution is stable for at least 6 months.

Amplex® Red/UltraRed stop solution (optional)

1.2 Prepare Amplex® Red/UltraRed stop solution by dissolving the contents of one vial of stop reagent (Cat. no. A33855) in 1.45 mL of ethanol. Vortex briefly to mix.

1.3 Transfer 1.0 mL of this solution to an empty vial or reservoir with a capacity of ≥ 2 mL, and add an equal volume (*i.e.*, 1.0 mL) of deionized water for a 1:1 final dilution.

Based on the protocol below, this 2.0 mL volume of stop solution is sufficient to stop 100 detection reactions of 100 µL. After reconstitution, the stop reagent is stable for approximately one month when stored at 2–6°C, protected from light. The stop solution is colorless. Appearance of amber coloration is indicative of decomposition.

Horseradish Peroxidase (HRP) stock solution

1.4 Prepare a 10 U/mL stock solution of HRP in a suitable reaction buffer (see **Determining Appropriate Buffer for Analyte System**). After performing the assay, divide any unused stock solution into single-use aliquots and store frozen at –20°C.

Hydrogen Peroxide (H_2O_2) stock solution

1.5 To prepare a 20 mM H_2O_2 stock solution, dilute 22.7 µL of stabilized 3.0% peroxide in 977 µL of reaction buffer (see **Determining Appropriate Buffer for Analyte System**). Adjust dilutions based on actual concentration.

Although most 3.0% H_2O_2 solutions are stabilized to slow degradation, the 20 mM solution prepared in this step will be less stable and should be used promptly.

Other components of enzyme couples system of interest

1.6 Use enzymes employed in oxidase-coupled reaction systems in excess of targeted analyte. Typically, final assay concentrations of 1–5 U/mL are sufficient. You may prepare stock solutions at concentrations ≥ 100 U/mL for convenience.

Preparing Substrate

2.1 To prepare 10 mL of substrate mixture, add the following to 10 mL of reaction buffer (*e.g.*, 50 mM sodium citrate, pH 6.0; see **Determining Appropriate Buffer for Analyte System**):

- 50 µL of 10 mM Amplex® UltraRed stock solution (Step 1.1)
- 22.7 µL of 3% stabilized hydrogen peroxide (adjust volume, if necessary, based on actual concentration).

Protect the reaction mixture from light and use within 4 hours or preparation.

Note: You may prepare Amplex® UltraRed, Amplex® Red, and TMB in the same manner. Optimal buffer composition and pH may vary.

Stability of Solubilized Reagents

We have shown in our laboratory that 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® UltraRed reagent is an early indicator of compromised material.

Experimental Protocols

ELISA Protocol

The following procedure is designed for use with a fluorescence microplate reader. The procedure has been optimized for use with 96-well microplates and reaction volumes of 100 µL per assay.

3.1 After completing relevant ELISA binding incubations, shake the plate contents into a sink and wash three times with a suitable wash buffer. You may adjust the stringency of the assay by washing more or fewer times, or by incubating/agitating the wash buffer in the assay wells.

Invert the plate on a paper towel and firmly tap to remove any remaining buffer after the final wash.

Note: Protect the plate from excessive exposure to light from this point onward.

3.2 Add 100 µL of substrate mixture (Step 2.1) to each assay well using a multichannel or repeat pipettor.

3.3 Cover the plate and incubate at room temperature, **protected from light**, until you are ready to measure the fluorescence. For most reactions, a 15–30 minute incubation is sufficient. You may also read the plate continuously for up to one hour or longer, if needed.

3.4 *Optional*: If desired, you may add 20 µL of Amplex® stop solution (Step 1.3) to each assay well. The time-dependent fluorescence signal increase will cease immediately and the fluorescence signal level will remain stable for at least 3 hours, if the plate is covered and protected from light at room temperature. Add stop solution to all wells, including any reagent controls.

Note: It is very important to add the stop solution to reagent controls to take into account the ~17% dilution of the samples by the addition of the stop solution, and also to compensate for the fluorescence quenching effect (typically < 5%) of the stop solution on the Amplex® UltraRed reagent oxidation products.

3.5 Measure the fluorescence in a microplate reader. Excitation/emission maxima are 568/581 nm. Wavelength settings of 530/590 nm work well on most instruments.

Note: Optimal wavelength settings may vary slightly between instruments. If excitation at 530 nm results in signal saturation when the emission is read at 590 nm, you may lower the excitation wavelength to 490–525 nm.

Peroxide/Enzyme-Coupled Assay Protocol

The following protocol describes the general detection of H_2O_2 using Amplex® UltraRed. For guidelines pertaining to specific analytes, see our current line of Amplex® Red kits. In all cases, you can use Amplex® UltraRed in place of Amplex® Red.

4.1 Prepare stock solutions of Amplex® UltraRed reagent, Amplex® Red/UltraRed stop solution (optional), HRP, H_2O_2, and/or other components of the enzyme-coupled system of interest (see **Preparing Stock Solutions**).

4.2 Prepare a standard curve for H_2O_2 or other analyte of interest:

- Dilute the appropriate amount of H_2O_2 solution (Step 1.5) into reaction buffer (see **Determining Appropriate Buffer for Analyte System**) to produce H_2O_2 concentrations of 0 to 10 µM.

  The final H_2O_2 concentrations in the assay will be two-fold lower (0 to 5 µM). Each assay well requires 50 µL of standard or sample, based on this protocol. We recommend triplicates for each standard and sample.

- Concentrations for analytes in enzyme-coupled systems should be loosely based on those used in the H_2O_2 assay, since oxidase enzymes produce peroxide in a 1:1 stoichiometry. For example, a suitable concentration range for glucose is 0 to 50 µM. You can readily prepare glucose stock solutions at concentrations ≥ 100 mM.

4.3 If you are not using a standard curve, prepare positive and negative controls:

- For a positive control, dilute H_2O_2 to 10 µM in reaction buffer.
- For a negative control, use reaction buffer only, without H_2O_2 or other analyte.

4.4 Pipet 50 µL of the standard curve samples (Step 4.2), controls (Step 4.3), and experimental samples into individual wells of a microplate.

4.5 Prepare a working solution of Amplex® UltraRed/HRP. To prepare enough working solution to perform 100 assays for detection of H_2O_2, combine the following:

- 50 µL of 10 mM Amplex® UltraRed reagent stock solution (Step 1.1)
- 100 µL of 10 U/mL HRP (Step 1.4)
- 4.85 mL of reaction buffer (see **Determining Appropriate Buffer for Analyte System**)

4.6 Add 50 µL of Amplex® UltraRed/HRP working solution (Step 4.5) to each microplate well containing standards, controls, and samples to initiate the reaction.

For detecting other targets in an enzyme-coupled system, include additional components as necessary. For example, when detecting glucose, in addition to 50 µL of 10 mM Amplex® UltraRed reagent stock solution and 100 µL of 10 U/mL HRP, add 100 µL of glucose oxidase to 4.75 mL of reaction buffer.

Note: Reaction conditions that result in extremely high levels of H_2O_2 can produce lower fluorescence than moderately high levels because excess H_2O_2 further oxidizes the UltroxRed reaction product to a non-fluorescent form.

4.7 Cover the plate and incubate at room temperature, **protected from light,** until until you are ready to measure the fluorescence. For most reactions, a 15–30 minute incubation is sufficient. You may also read the plate continuously for up to one hour.

4.8 *Optional*: If desired, you may add 20 µL of Amplex® stop solution (Step 2.2) to each assay well. The time-dependent fluorescence signal increase will cease immediately and the fluorescence signal level will remain stable for at least 3 hours, if the plate is covered and protected from light at room temperature. Add stop solution to all wells, including any reagent controls.

Note: It is very important to add the stop solution to reagent controls to take into account the ~17% dilution of the samples by the addition of the stop solution, and also to compensate for the fluorescence quenching effect (typically < 5%) of the stop solution on the Amplex® UltraRed reagent oxidation products.

4.9 Measure the fluorescence in a microplate reader. Excitation/emission maxima are 568/581 nm. Wavelength settings of 530/590 nm work well on most instruments.

Note: Optimal wavelength settings may vary slightly between instruments. If excitation at 530 nm results in signal saturation when the emission is read at 590 nm, you may lower the excitation wavelength to 490–525 nm.

Reference

1. Anal Biochem 345, 227 (2005).

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

Cat. no.	Product Name	Unit Size
A36006	Amplex® UltraRed reagent	5 × 1 mg
Related Product		
A33855	Amplex® Red/UltraRed stop reagent *500 tests*	set of 5 vials

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