Amplex® Red Glutamic Acid/Glutamate Oxidase Assay Kit

Table 1. Contents and storage information.

Material	Amount	Storage	Stability
Amplex® Red reagent* (MW = 257, Component A)	2 vials, each containing 0.26 mg	• ≤–20˚C • Desiccate • Protect from light	When stored as directed, the kit is stable for at least 6 months.
Dimethylsulfoxide (DMSO), anhydrous (Component B)	0.7 mL		
Horseradish peroxidase (Component C)	10 U†		
Hydrogen peroxide (H_2O_2, a stabilized ~3% solution [the actual concentration is indicated on the component label]) (MW = 34, Component D)	500 µL		
5X Reaction buffer (0.5 M Tris-HCl, pH 7.5, Component E)	10 mL		
L-Glutamate oxidase, recombinant from *E. coli* (Component F)	1 U‡		
L-Glutamate–pyruvate transaminase from pig heart (Component G)	10 U§		
L-Glutamic acid, monosodium salt, monohydrate (MW = 187.1, Component H)	20 mg		
L-Alanine (MW = 89.1, Component I), 20 mg	20 mg		

* The Amplex® Red reagent is somewhat air sensitive. Once a vial of Amplex® Red reagent is opened, use the reagent promptly. PROTECT THE AMPLEX® RED REAGENT FROM LIGHT. † 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. ‡ 1 unit is defined as the amount of L-glutamate oxidase that will form 1.0 µmole of α-ketoglutaric acid from L-glutamic acid per minute at pH 7.4 at 30°C. § 1 unit is defined as the amount of L-glutamate–pyruvate transaminase that will convert 1 µmole of α-ketoglutarate to L-glutamate per minute at pH 7.6 at 37°C in the presence of L-alanine.

Number of assays: Each kit provides sufficient reagents for approximately 200 assays using a fluorescence microplate reader and reaction volumes of 100 µL per assay.

Approximate fluorescence excitation and emission maxima: 571/585 nm for the reaction product.

Introduction

The Amplex® Red Glutamic Acid/Glutamate Oxidase Assay Kit provides an ultrasensitive method for continuously detecting glutamic acid or for monitoring glutamate oxidase activity in a fluorescence microplate reader or fluorometer. In the assay, L-glutamic acid is oxidized by glutamate oxidase to produce α-ketoglutarate, NH_3 and H_2O_2. L-Alanine and L-glutamate–pyruvate transaminase are included in the reaction to regenerate L-glutamic acid by transamination of α-ketoglutarate, resulting in multiple cycles of the initial reaction and a significant

amplification of the H_2O_2 produced. The hydrogen peroxide reacts with 10-acetyl-3,7-dihydroxyphenoxazine (Amplex® Red reagent) in a 1:1 stoichiometry in the reaction catalyzed by horseradish peroxidase (HRP) to generate the highly fluorescent product, resorufin.[1,2] 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.

In the Amplex® Red Glutamic Acid/Glutamate Oxidase Assay, if the concentration of L-glutamic acid is limiting, the fluorescence increase is proportional to the initial L-glutamic acid concentration. Using the kit, one can detect L-glutamic acid in a purified system at levels as low as 40 nM in a 30 minute reaction (Figure 2). As an example, Amplex® Red Glutamic Acid/Glutamate Oxidase Assay Kit has been successfully applied to measuring L-glutamate in food samples.[3] If the reaction is modified to include an excess of L-glutamic acid, the kit can be used to continuously monitor glutamate oxidase activity. For example, purified L-glutamate oxidase from *Streptomyces* can be detected at levels as low as 40 µU/mL (Figure 3).

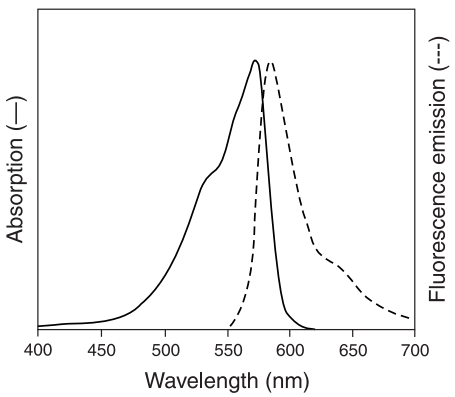

Figure 1. Normalized absorption and fluorescence emission spectra of resorufin, the product of the Amplex® Red reagent.

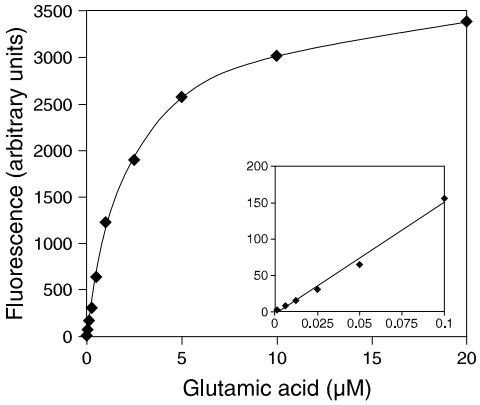

Figure 2. Detection of L-glutamic acid using the Amplex® Red reagent–based assay. Each reaction contained 50 µM Amplex® Red reagent, 0.125 U/mL HRP, 0.04 U/mL L-glutamate oxidase, 0.25 U/mL L-glutamate–pyruvate transaminase, 100 µM L-alanine and the indicated amount of L-glutamic acid in 1X reaction 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.

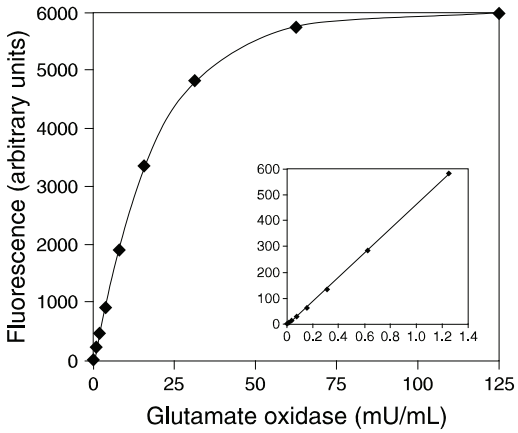

Figure 3. Detection of L-glutamate oxidase using the Amplex® Red reagent–based assay. Each reaction contained 50 µM Amplex® Red reagent, 0.125 U/mL HRP, 0.25 U/mL L-glutamate–pyruvate transaminase, 20 µM L-glutamic acid, 100 µM L-alanine and the indicated amount of Streptomyces L-glutamate oxidase in 1X reaction buffer. Reactions were incubated at 37°C. After 60 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. The inset represents data from a separate experiment for lower L-glutamate oxidase concentrations and incubation time of 60 minutes.

The following procedure is designed for use with a fluorescence multi-well plate scanner. For use with a standard fluorometer, increase volumes accordingly. 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 less than 10 µM.

The absorption and fluorescence 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 reaction should be performed at pH 7–8, for example by using the provided reaction buffer (pH 7.5).

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 DMSO. Each vial of Amplex® Red reagent is more than sufficient for approximately 100 assays, with a final reaction volume of 100 µL per assay. Store this stock solution frozen at –20°C, protected from light.

1.2 Prepare a 1X working solution of reaction buffer 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 100 µL of 1X reaction buffer. After use, divide the remaining solution into small aliquots and store 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. 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 5 U/mL stock solution of L-glutamate oxidase by dissolving the contents of the vial of L-glutamate oxidase (Component F) in 200 µL of 1X reaction buffer. After use, divide the remaining solution into small aliquots and store frozen at –20°C.

1.6 Prepare a 100 U/mL solution of L-glutamate–pyruvate transaminase by dissolving the contents of the vial of L-glutamate–pyruvate transaminase (Component G) in 100 µL of 1X reaction buffer. After use, divide the remaining solution into small aliquots and store frozen at –20°C.

1.7 Prepare a 200 mM solution of L-glutamic acid by dissolving the contents of the vial of L-glutamic acid (Component H) in 534 µL of 1X reaction buffer. After use, store the remaining solution frozen at –20°C.

1.8 Prepare a 200 mM stock solution of L-alanine by dissolving the contents of the vial of L-alanine (Component I) in 1.12 mL of 1X reaction buffer. After use, store the remaining solution frozen at –20°C.

Glutamic Acid Assay

The following protocol describes the assay of L-glutamic acid in a total volume of 100 µL per microplate well. The volumes recommended here are sufficient for ~100 assays.

2.1 Prepare a L-glutamic acid standard curve: Dilute the appropriate amount of 200 mM L-glutamic acid stock solution (prepared in step 1.7) into 1X reaction buffer to produce L-glutamic acid concentrations of 0 to 20 µM. Use 1X reaction buffer without L-glutamic acid as a negative control. A volume of 50 µL will be used for each reaction. Note that the L-glutamic acid concentrations will be two-fold lower in the final reaction volume.

2.2 Dilute the L-glutamic acid-containing samples in 1X reaction buffer. Use a volume of 50 µL for each reaction.

2.3 Prepare a positive control by diluting the 20 mM H_2O_2 working solution 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 0.25 U/mL HRP, 0.08 U/mL L-glutamate oxidase, 0.5 U/mL L-glutamate–pyruvate transaminase, and 200 µM L-alanine by adding:

- 50 µL of the Amplex® Red reagent stock solution (prepared in step 1.1)
- 12.5 µL of the HRP stock solution (prepared in step 1.3)
- 80 µL of the L-glutamate oxidase stock solution (prepared in step 1.5)
- 25 µL of the L-glutamate–pyruvate transaminase stock solution (prepared in step 1.6)
- 5 µL of the L-alanine stock solution (prepared in step 1.8)
- 4.83 mL of 1X reaction buffer

This 5 mL volume is sufficient for ~100 assays. Note that final concentrations 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/glutamate oxidase/glutamate–pyruvate transaminase/alanine working solution to each microplate well containing the samples and controls.

2.7 Incubate the reactions for 30 minutes or longer at 37°C, **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.

2.8 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–glutamic acid control.

Glutamate Oxidase Assay

The following protocol provides a guideline for using the Amplex® Red Glutamic Acid/Glutamate Oxidase Assay Kit to measure L-glutamate oxidase activity. The volumes recommended here are sufficient for ~100 assays, each containing a volume of 100 µL.

3.1 Dilute the L-glutamate oxidase-containing samples in 1X reaction buffer. A volume of 50 µL will be used for each reaction.

3.2 Prepare a positive control by diluting the 5 U/mL L-glutamate oxidase stock solution (prepared in step 1.5) into 1X reaction buffer to produce a 0.04 U/mL L-glutamate oxidase solution. Use 1X reaction buffer without L-glutamate oxidase as a negative control. A volume of 50 µL will be used for each reaction.

3.3 Prepare a second positive control by diluting the 20 mM H_2O_2 working solution 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 0.25 U/mL HRP, 0.5 U/mL L-glutamate–pyruvate transaminase, 40 µM L-glutamic acid, and 200 µM L-alanine by adding:

- 50 µL of the Amplex® Red reagent stock solution (prepared in step 1.1)
- 12.5 µL of the HRP stock solution (prepared in step 1.3)
- 25 µL of the L-glutamate–pyruvate transaminase stock solution (prepared in step 1.6)
- 1 µL of the L-glutamic acid stock solution (prepared in step 1.7)
- 5 µL of the L-alanine stock solution (prepared in step 1.8)
- 4.91 mL of 1X reaction buffer

This 5 mL volume is sufficient for ~100 assays. Note that 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/glutamate–pyruvate transaminase/glutamic acid/alanine 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 may be measured at multiple time points to follow the kinetics of the reactions.

3.8 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).

3.9 For each point, correct for background fluorescence by subtracting the values derived from the no–glutamate oxidase control.

References

1. Anal Biochem 253, 162 (1997); **2.** J Immunol Methods 202, 133 (1997); **3.** Anal Chim Acta 402, 47 (1999).

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

Cat. no.	Product Name	Unit Size
A12221	Amplex® Red Glutamic Acid/Glutamate Oxidase Assay Kit *200 assays*	1 kit
A12222	Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine)	5 mg
A22177	Amplex® Red reagent *packaged for high-throughput screening*	10 × 10 mg
A36006	Amplex® UltraRed reagent	5 × 1 mg

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