Revised: 01–October–2004

Amplex® Red Acetylcholine/Acetylcholinesterase Assay Kit (A12217)

Quick Facts

Storage upon receipt:

- –20°C
- Desiccate
- Protect from light

Abs/Em of reaction product: 571/585 nm

Introduction

The Amplex® Red Acetylcholine/Acetylcholinesterase Assay Kit provides an ultrasensitive method for continuously monitoring acetylcholinesterase (AChE) activity or for detecting acetylcholine (ACh) in a fluorescence microplate reader or fluorometer. Potential uses for this kit include screening for AChE inhibitors and measuring the release of ACh from synaptosomes. In the assay, AChE activity is monitored indirectly using 10-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H_2O_2.[1] First, AChE converts the acetylcholine substrate to choline. Choline is in turn oxidized by choline oxidase to betaine and H_2O_2, the latter of which, in the presence of horseradish peroxidase, reacts with Amplex Red reagent in a 1:1 stoichiometry 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.

Experiments with purified AChE from electric eel indicate that the Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit can detect AChE levels as low as 0.002 U/mL using a reaction time of one hour (Figure 2). By providing an excess of AChE in the assay, the kit can also be used to detect acetylcholine levels as low as 0.3 µM, with a range of detection from 0.3 µM to 100 µM acetylcholine (Figure 3).

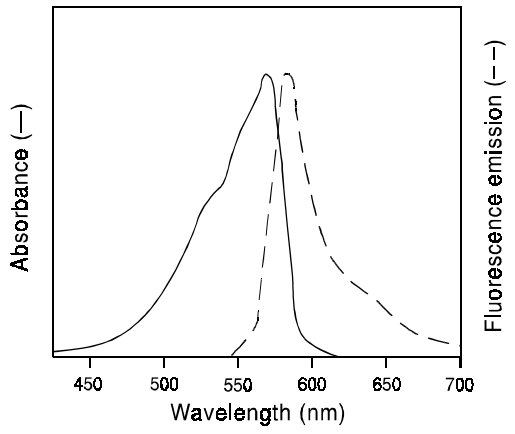

Figure 1. *Normalized absorption and fluorescence emission spectra of resorufin, the product of the Amplex Red reagent.*

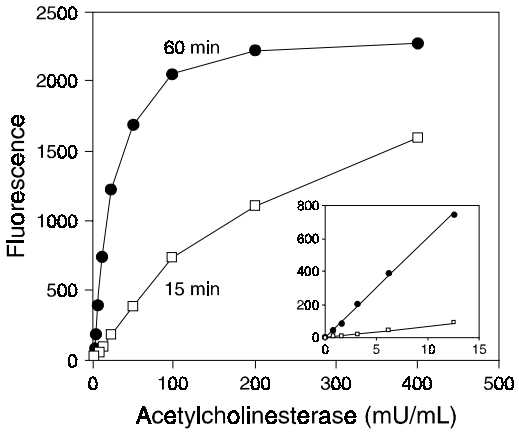

Figure 2. *Detection of electric eel acetylcholinesterase activity using the Amplex Red reagent–based assay. Each reaction contained 50 µM acetylcholine, 200 µM Amplex Red reagent, 1 U/mL HRP, 0.1 U/mL choline oxidase and the indicated amount of acetylcholinesterase in 1X Reaction Buffer. Reactions were incubated at room temperature. After 15 and 60 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 560 ± 10 nm and fluorescence detection at 590 ± 10 nm.*

Materials

Kit Contents

- **Amplex Red reagent** (MW = 257, Component A), five vials, each containing 1 mg
- **Dimethylsulfoxide (DMSO)**, anhydrous (Component B), 1.3 mL
- **Horseradish peroxidase** (Component C), 200 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 20°C
- **Hydrogen peroxide (H_2O_2)** (Component D), 500 µL of a stabilized ~3% solution; the actual concentration is indicated on the component label
- **5X Reaction Buffer** (Component E), 28 mL of 250 mM Tris-HCl, pH 8.0
- **Choline oxidase from *Alcaligenes* sp.** (Component F), 12 U, where 1 unit is defined as the amount of choline oxidase that will form 1.0 µmole of H_2O_2 due to oxidation of 1 µmole of choline to betaine aldehyde per minute at pH 8.0 at 37°C

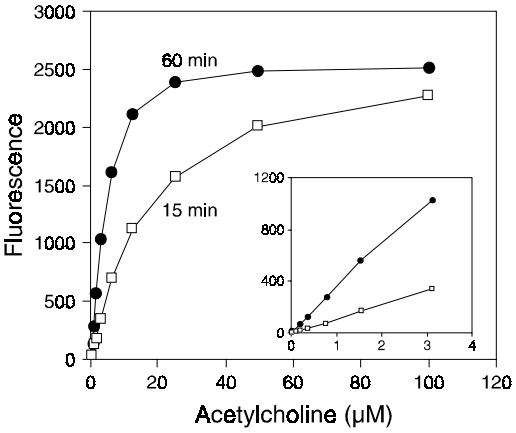

Figure 3. *Detection of acetylcholine using the Amplex Red reagent–based assay. Each reaction contained 200 µM Amplex Red reagent, 1 U/mL HRP, 0.1 U/mL choline oxidase, 0.5 U/mL acetylcholinesterase and the indicated amount of acetylcholine in 1X Reaction Buffer. Reactions were incubated at room temperature. After 15 and 60 minutes, fluorescence was measured with a fluorescence microplate reader using excitation at 560 ± 10 nm and fluorescence detection at 590 ± 10 nm.*

- **Acetylcholine chloride** (MW = 181.7, Component G), ~100 mg
- **Acetylcholinesterase from electric eel** (Component H), 60 U, where one unit is defined as the amount of enzyme that will hydrolyze 1.0 µmole of acetylcholine to choline and acetate per minute at pH 8.0 at 37°C

Each kit provides sufficient reagents for approximately 500 assays using a fluorescence microplate reader and reaction volumes of 200 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 multiwell plate scanner. For use with a standard fluorometer, volumes must be increased accordingly. Please 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 8).

Stock Solution Preparation

1.1 Prepare an ~20 mM stock solution of the Amplex Red reagent: Allow one vial of the 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 (1 mg) in 200 µL DMSO. Each vial of Amplex Red reagent is sufficient for approximately 100 assays of 200 µL each. This stock solution should be stored frozen at -20°C, protected from light.

1.2 Prepare a 1X working solution of Reaction Buffer by adding 5 mL of 5X Reaction Buffer stock solution (Component E) to 20 mL of deionized water (dH_2O). This 25 mL volume of 1X Reaction Buffer is sufficient for approximately 100 assays of 200 µL each, with a 5 mL excess for making stock solutions and dilutions.

1.3 Prepare a 200 U/mL stock solution of horseradish peroxidase (HRP) by dissolving the contents of the vial of HRP (Component C) in 1.0 mL 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 20 U/mL stock solution of choline oxidase by dissolving the contents of the vial of choline oxidase (Component F) in 600 µL of 1X Reaction Buffer. After use, the remaining solution should be divided into small aliquots and stored frozen at -20°C.

1.6 Prepare a 100 mM solution of acetylcholine in dH_2O. For example, dissolve 5 mg of acetylcholine chloride (Component G) in 275 µL of dH_2O. This solution should be made fresh before each set of experiments. Because acetylcholine hydrochloride is hygroscopic, the remaining solid should be stored desiccated at -20°C. Please note that the concentration of acetylcholine in the stock solution should be considered approximate, since the acetylcholine hydrochloride may have varying amounts of water in the solid.

1.7 Prepare a 100 U/mL stock solution of acetylcholinesterase by dissolving the contents of the vial of acetylcholinesterase (Component H) in 600 µL of 1X Reaction Buffer. After use, the remaining solution should be divided into small aliquots and stored frozen at -20°C.

Acetylcholine Assay

The following protocol describes the assay of acetylcholine in a total volume of 200 µL per microplate well. The volumes recommended here are sufficient for ~100 assays.

2.1 Prepare an acetylcholine standard curve: Dilute the appropriate amount of 100 mM acetylcholine stock solution (prepared in step 1.6) into 1X Reaction Buffer to produce acetylcholine concentrations of 0 to 100 µM. Use 1X Reaction Buffer without acetylcholine as a negative control. A volume of 100 µL will be used for each reaction. Please note that the acetylcholine concentrations will be twofold lower in the final reaction volume.

2.2 Dilute the acetylcholine-containing samples in 1X Reaction Buffer. A volume of 100 µL will be used 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 100 µL of the diluted samples and controls into separate wells of a microplate.

2.5 Prepare a working solution of 400 µM Amplex Red reagent containing 2 U/mL HRP, 0.2 U/mL choline oxidase and 1 U/mL acetylcholinesterase by adding 200 µL of Amplex Red reagent stock solution (prepared in step 1.1), 100 µL of the HRP stock solution (prepared in step 1.3), 100 µL of the choline oxidase stock solution (prepared in step 1.5) and 100 µL of the acetylcholinesterase stock solution (prepared in step 1.7) to 9.5 mL of 1X Reaction Buffer. This 10 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 100 µL of the Amplex Red reagent/HRP/choline oxidase/acetylcholinesterase working solution to each microplate well containing the samples and controls.

2.7 Incubate the reactions for 30 minutes or longer at room temperature, 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-acetylcholine control.

Acetylcholinesterase Assay

The following protocol provides a guideline for using the Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit to measure acetylcholinesterase activity. The volumes recommended here are sufficient for ~100 assays, each containing a volume of 200 µL.

3.1 Dilute the acetylcholinesterase-containing samples in 1X Reaction Buffer. A volume of 100 µL will be used for each reaction.

3.2 Prepare a positive control by diluting the 100 U/mL acetylcholinesterase stock solution (prepared in step 1.7) into 1X Reaction Buffer to produce a 0.2 U/mL acetylcholinesterase solution. Use 1X Reaction Buffer without acetylcholinesterase as a negative control. A volume of 100 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 100 µL of the diluted samples and controls into separate wells of a microplate.

3.5 Prepare a working solution of 400 µM Amplex Red reagent containing 2 U/mL HRP, 0.2 U/mL choline oxidase and 100 µM acetylcholine by adding 200 µL of Amplex Red reagent stock solution (prepared in step 1.1), 100 µL of the HRP stock solution (prepared in step 1.3), 100 µL of choline oxidase stock solution (prepared in step 1.5) and 10 µL of acetylcholine stock solution (prepared in step 1.6) to 9.59 mL of 1X Reaction Buffer. This 10 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 100 µL of the Amplex Red reagent/HRP/choline oxidase/acetylcholine working solution to each microplate well containing the samples and controls.

3.7 Incubate the reactions for 30 minutes or longer at room temperature, 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-acetylcholinesterase control.

References

1. Anal Biochem 253, 162 (1997); **2.** J Immunol Methods 202, 133 (1997).

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

Cat #	Product Name	Unit Size
A12217	Amplex® Red Acetylcholine/Acetylcholinesterase 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

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