**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 H₂O₂.¹ First, AChE converts the acetylcholine substrate to choline. Choline is in turn oxidized by choline oxidase to betaine and H₂O₂, 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.¹² 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).

**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 µmole of purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C
- **Hydrogen peroxide (H₂O₂)** (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 H₂O₂ due to oxidation of 1 µmole of choline to betaine aldehyde per minute at pH 8.0 at 37°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.

**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.

For this reason, the final DTT or 2-mercaptoethanol concentration in the reaction should be less than 10 µM.

**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.

**Stock Solution Preparation**

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.

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 (dH2O). 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.

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.

4. Prepare a 20 mM H2O2 working solution by diluting the ~3% H2O2 stock solution (Component D) into the appropriate volume of dH2O. The actual H2O2 concentration is indicated on the component label. For instance, a 20 mM H2O2 stock solution can be prepared from a 3.0% H2O2 stock solution by diluting 23 µL of 3.0% H2O2 into 977 µL of dH2O. Please note that although the ~3% H2O2 stock solution has been stabilized to slow degradation, the 20 mM H2O2 working solution will be less stable and should be used promptly.

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.

6. Prepare a 100 mM solution of acetylcholine in dH2O. For example, dissolve 5 mg of acetylcholine chloride (Component G) in 275 µL of dH2O. 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.

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 H₂O₂ 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) into 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 H₂O₂ 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) into 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.

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**


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<td>Amplex® Red reagent (10-acetyl-3, 7-dihydroxyphenoxazine)</td>
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