Amplex® Red Cholesterol Assay Kit

Catalog no. A12216

Table 1. Contents and storage information.

Material	Amount	Storage	Stability
Amplex® Red reagent, MW = ~257 (Component A)	2 vials, each containing 1 mg	• ≤–20°C • Desiccate • Protect from light	When stored as directed, the kit components are stable for at least 6 months.
Dimethylsulfoxide (DMSO), anhydrous (Component B)	1.3 mL		
Horseradish peroxidase (HRP) (Component C)	200 U*		
Hydrogen peroxide (H_2O_2), MW = 34 (Component D)	500 µL of a stabilized ~3% solution; the actual concentration is indicated on the component label		
5X Reaction Buffer (Component E)	20 mL of 0.5 M potassium phosphate, pH 7.4, 0.25 M NaCl, 25 mM cholic acid, 0.5% Triton® X-100		
Cholesterol oxidase, from *Streptomyces* (Component F)	50 U†		
Cholesterol esterase, from *Pseudomonas* (Component G)	50 U‡		
Cholesterol reference standard, MW = 387 (Component H),	100 µL of 2 mg/mL cholesterol		
Resorufin, sodium salt, MW = 235 (Component I)	470 µg		

Number of assays: Each kit provides sufficient reagents for approximately 500 assays using a fluorescence microplate reader and reaction volumes of 100 µL per assay, based on the protocol below.

Approximate fluorescence excitation/emission maxima: Amplex® Red reagent**:** ~571/585 in nm.

* 1 unit = 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 = the amount of enzyme that will oxidize 1.0 µmole of cholesterol to 4-cholesten-3-one per minute at pH 7.5 at 25°C.

‡ 1 unit = the amount of enzyme that will will hydrolyze 1.0 µmole of cholesteryl oleate to cholesterol and oleic acid per minute at pH 7.0 in the presence of taurocholate.

Introduction

The Amplex® Red Cholesterol Assay Kit provides a simple fluorometric method for the sensitive quantitation of cholesterol using a fluorescence microplate reader or fluorometer. Because a large portion of cholesterol in blood is in the form of cholesteryl esters, the assay is based on an enzyme-coupled reaction that detects both free cholesterol and cholesteryl esters. Cholesteryl esters are hydrolyzed by cholesterol esterase into cholesterol, which is then oxidized by cholesterol oxidase to yield H_2O_2 and the corresponding ketone product. The H_2O_2 is then detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex® Red reagent), a highly sensitive and stable probe for H_2O_2.[1] In the presence of horseradish peroxidase (HRP), Amplex® Red reagent reacts with H_2O_2 with a 1:1 stoichiometry to produce highly fluorescent 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.

The Amplex® Red cholesterol assay can detect cholesterol at a concentration of 200 nM (80 ng/mL) or lower (Figure 2) and can accurately measure the cholesterol content in the equivalent of 0.01 µL of human serum.[3] Because the assay is continuous and requires no separation steps, the procedure is particularly well suited to the rapid and direct analysis of cholesterol in blood and food samples using automated instruments. By performing reactions in the presence and absence of cholesterol esterase, the assay is also potentially useful for determining the fraction of cholesterol that is in the form of cholesteryl esters within a sample. In addition, the assay can be adapted to detect the activity of cholesterol oxidase by providing an excess of cholesterol in the reaction.[3]

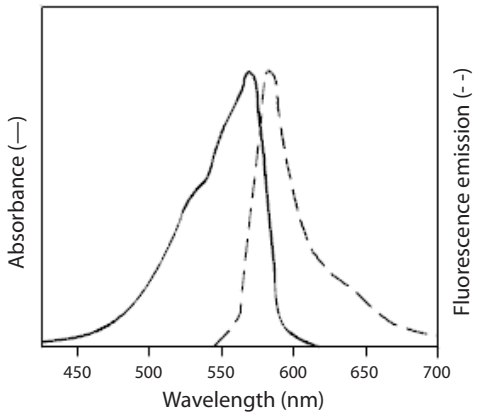

Figure 1. Normalized absorption and fluorescence emission spectra of resorufin, the product of the Amplex® Red reagent.

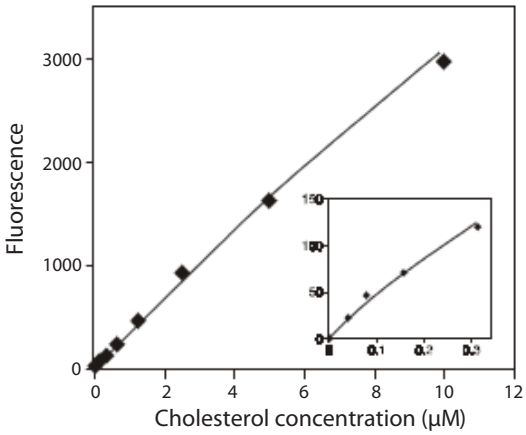

Figure 2. Detection of cholesterol using the Amplex® Red reagent–based assay. Each reaction contained 150 µM Amplex® Red reagent, 1 U/mL HRP, 1 U/mL cholesterol oxidase, 0.1 µM cholesterol esterase and the indicated amount of the cholesterol in 1X Reaction Buffer. Reactions were incubated at 37°C for 30 minutes. Fluorescence was measured with a fluorescence microplate reader using excitation at 560 ± 10 nm and fluorescence detection at 590 ± 10 nm. Background fluorescence (340 arbitrary units), determined for the no-cholesterol control reaction, has been subtracted from each value.

Before Starting

Materials Required But Not Provided

- Deionized water (dH_2O)

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.

Caution

DMSO is known to facilitate the entry of organic molecules into tissues. Handle reagents containing DMSO (e.g., Amplex® UltraRed reagent stock solution in DMSO) using equipment and practices appropriate for the hazards posed by such materials. Dispose off the reagents in compliance with all pertaining local regulations.

Preparing Solutions

20 mM Amplex® Red reagent stock solution

1.1 To prepare a 20 mM stock solution of Amplex® Red reagent, allow one vial of Amplex® Red reagent (Component A) and the DMSO (Component B) to warm to room temperature. Immediately 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 250 assays, with a final reaction volume of 100 µL per assay. Store the stock solution frozen at ≤–20°C, **protected from light**.

1X Reaction Buffer working solution

1.2 To prepare a 1X working solution of Reaction Buffer, add 2.5 mL of 5X Reaction Buffer stock solution (Component E) to 10 mL of deionized water (dH_2O). This 12.5 mL volume of 1X Reaction Buffer is sufficient This 12.5 mL volume of 1X Reaction Buffer is sufficient for approximately 100 assays of 100 µL each, with a 2.5 mL excess for making stock solutions and dilutions.

200 U/mL horseradish peroxidase (HRP) stock solution

1.3 To prepare a 200 U/mL stock solution of horseradish peroxidase (HRP), dissolve the contents of the vial of HRP (Component C) in 1 mL of 1X Reaction Buffer. After use, divide the remaining solution into small aliquots and store frozen at ≤–20°C.

20 mM H_2O_2 working solution

1.4 To prepare a 20 mM H_2O_2 working solution, dilute 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: Although the ~3% H_2O_2 stock solution has been stabilized to slow its degradation, the 20 mM H_2O_2 working solution is less stable and should be used promptly.

200 U/mL cholesterol oxidase stock solution

1.5 To prepare a 200 U/mL solution of cholesterol oxidase, dissolve the entire vial of cholesterol oxidase (Component F) in 250 µL of 1X Reaction Buffer. After use, divide the remaining solution into small aliquots and store frozen at ≤–20°C.

Note: The cholesterol oxidase solution may appear cloudy. This does not interfere with the assay.

200 U/mL cholesterol esterase stock solution

1.6 To prepare a 200 U/mL stock solution of cholesterol esterase, dissolve tthe entire vial of cholesterol esterase (Component G) in 250 µL of 1X Reaction Buffer. Cholesterol esterase is added to the reaction to allow detection of cholesterol in the form of cholesteryl esters. After use, divide the remaining solution into small aliquots and store frozen at ≤–20°C.

2 mM resorufin stock solution

1.7 To prepare a 2 mM stock solution of resorufin, add 1 mL dH_2O directly to the vial of resorufin solid (Component I). This solution can be used to prepare a standard curve to determine the moles of product produced in the Amplex® Red reaction. Store the resorufin stock solution frozen at ≤–20°C, **protected from light**.

Experimental Protocols

General Considerations

- The following procedure is designed for use with a fluorescence multiwell plate reader. For use with a standard fluorometer, volumes must be increased accordingly.
- 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 no higher 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.
- We recommend using the included Reaction Buffer (pH 7.4) for optimal performance of the Amplex® Red reagent.

Cholesterol Assay

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

2.1 Prepare a cholesterol standard curve: Dilute the appropriate amount of 2 mg/mL (5.17 mM) cholesterol reference standard (Component H) into 1X Reaction Buffer to produce cholesterol concentrations of 0 to 8 µg/mL (0 to ~20 µM). Use 1X Reaction Buffer without cholesterol as a negative control. A volume of 50 µL will be used for each reaction.

Note: The cholesterol concentrations will be two-fold lower in the final reaction volume. The cholesteryl esters are digested by cholesterol esterase to free cholesterol, which is then detected in the enzyme-coupled reaction with Amplex® Red reagent. The solution has been calibrated to yield the equivalent of 2 mg/mL cholesterol.

2.2 Dilute the cholesterol-containing samples in 1X Reaction Buffer. A volume of 50 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 50 µL of the diluted samples and controls into separate wells of a microplate.

2.5 Prepare a working solution of 300 µM Amplex® Red reagent containing 2 U/mL HRP, 2 U/mL cholesterol oxidase, and 0.2 U/mL cholesterol esterase by adding 75 µL of Amplex® Red reagent stock solution (prepared in step 1.1), 50 µL of the HRP stock solution (prepared in step 1.3), 50 µL of the cholesterol oxidase stock solution (prepared in step 1.5), and 5 µL of the cholesterol esterase stock solution (prepared in step 1.6) to 4.82 mL of 1X Reaction Buffer. This 5 mL volume is sufficient for ~100 assays.

Note: Final concentrations of each component will be two-fold lower in the final reaction volume.

2.6 Begin the reactions by adding 50 µL of the Amplex® Red reagent/HRP/cholesterol oxidase/cholesterol esterase 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), you may measure fluorescence at multiple time points to follow the kinetics of the reactions.

2.8 If desired, prepare a resorufin standard curve: Dilute the appropriate amount of 2 mM resorufin stock solution in 1X Reaction Buffer to yield resorufin solution ranging from 0 to 20 µM resorufin. Pipet 100 µL of each resorufin standard into individual (empty) wells of a microplate at any time prior to measuring fluorescence.

2.9 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).

2.10 For each point, correct for background fluorescence by subtracting the values derived from the no-cholesterol control.

References

1. Anal Biochem 253, 162 (1997); **2.** J Immunol Methods 202, 133 (1997); **3.** J Biochem Biophys Methods 38, 43 (1999).

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

Cat. no.	Product Name	Unit Size
A12216	Amplex® Red Cholesterol 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 × 10 mg
A36006	Amplex® UltraRed reagent	5 × 1 mg

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