Revised: 01–October–2004

Amplex® Red Galactose/Galactose Oxidase Kit (A22179)

Quick Facts

Storage upon receipt:

- –20°C
- Desiccate
- Protect from light

Abs/Em of reaction product: 571/585 mn

Introduction

Galactose oxidase has been an important tool in the detection of galactose in clinical samples [1] and recently, has been used to detect mucin-like glycoproteins in biological fluids.[2] These glycoproteins are a major component of mucus secreted by epithelial and glandular cells and are primarily responsible for the protective properties of the viscoelastic mucous barrier.[3] Abnormal mucin gene expression in cancers has recently generated more interest in mucins as possible diagnostic tumor markers.[4-6] Mucins have also been implicated in the process of cholesterol gallstone formation.[7,8]

The Amplex® Red Galactose/Galactose Oxidase Assay Kit provides an ultrasensitive method for detecting galactose or for monitoring galactose oxidase activity. In the assay, galactose oxidase catalyzes the oxidation of galactose at the C_6 position and generates hydrogen peroxide (H_2O_2). The H_2O_2 then, in the presence of horseradish peroxidase (HRP), reacts with 1:1 stoichiometry with Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin.[9] Resorufin has absorption and fluorescence emission maxima of approximately 571 nm and 585 nm, respectively (Figure 1), and because the extinction coefficient is high (54,000 $cm^{-1}M^{-1}$), the assay can be performed either fluorometrically or spectrophotometrically. With the Amplex Red Galactose/Galactose Oxidase Assay Kit, we have detected as little as 2 mU/mL galactose oxidase (Figure 2) and 4 µM galactose (Figure 3).

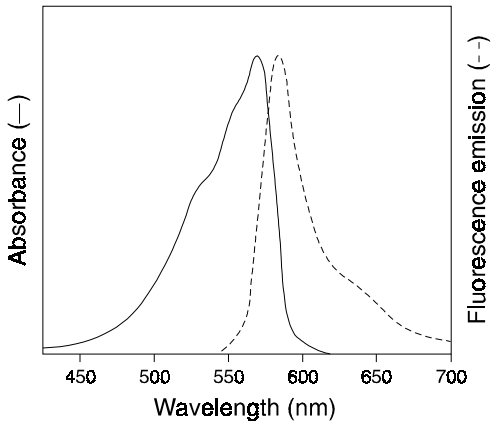

Figure 1. *Normalized absorption and fluorescence emission spectra of resorufin, the product of the Amplex Red reagent.*

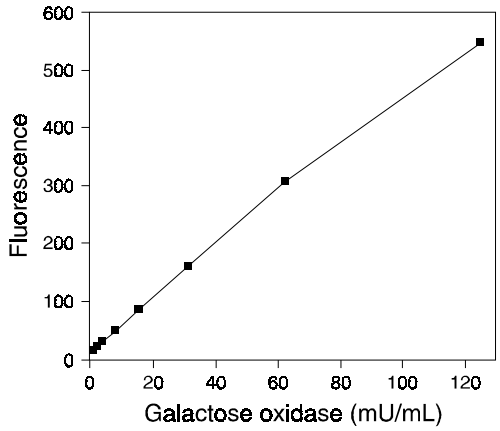

Figure 2. *Detection of galactose oxidase activity using the Amplex Red reagent–based assay. Each reaction contained 50 µM Amplex Red reagent, 0.1 U/mL HRP, 100 µM galactose and the indicated amount of galactose oxidase in 1X Reaction Buffer. Reactions were incubated at 37°C. After 20 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm.*

Materials

Kit Contents

- **Amplex Red reagent** (MW = 257, Component A), two vials, each containing 0.26 mg
- **Dimethylsulfoxide (DMSO)**, anhydrous (Component B), 500 µL
- **Horseradish peroxidase** (Component C), 20 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)** (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), 10 mL of 0.25 M Tris-HCl, pH 7.2, 5 mM $CaCl_2$
- **Galactose oxidase**, from *Dactylium dendroides* (Component F), 100 U, where one unit will produce a change in absorbance of 1.0 at 425 nm per minute at pH 6.0 at 25°C using a peroxidase/*o*-tolidine coupled assay with galactose as the substrate
- **D-Galactose**, (MW = 180, Component G), 100 mg

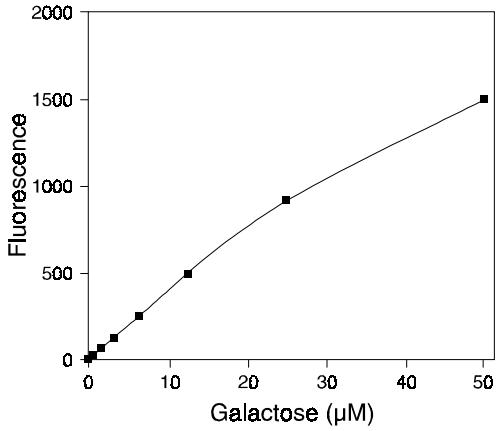

Figure 3. *Detection of galactose using the Amplex Red reagent–based assay. Each reaction contained 50 µM Amplex Red reagent, 0.1 U/mL HRP, 2 U/ml of galactose oxidase and the indicated amount of galactose in 1X Reaction Buffer. Reactions were incubated at 37°C. After 30 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. A background fluorescence 93 units was subtracted from each data point.*

Each Kit provides sufficient reagents for approximately 400 assays using either a fluorescence or absorbance microplate reader and reaction volumes of 100 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 or absorbance multiwell plate scanner. For use with a standard fluorometer or spectrophotometer, volumes must be increased accordingly.

Please note that resorufin, the product of the Amplex Red reaction, is unstable in the presence of thiols such as dithiothreitol (DTT) and 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 reactions should be performed at pH 7–8. We recommend using the included Reaction Buffer (pH 7.2) for optimal performance of the Amplex Red reagent.

Stock Solution Preparation

1.1 Prepare a 10 mM stock solution of 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 of DMSO. Each vial of Amplex Red reagent is sufficient for approximately 200 assays, with a final reaction volume of 100 µL per assay. After use, the remaining stock solution should be immediately stored 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. The 1X Reaction Buffer should be stable for several weeks if stored at 4°C

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 200 µL 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 200 U/mL galactose oxidase stock solution by dissolving the contents of the vial of galactose oxidase (Component F) in 500 µL of 1X Reaction Buffer. After use, the remaining solution should be divided into small aliquots and stored at -20°C.

1.6 Prepare a 20 mM D-galactose stock solution (Component G) by first resuspending the contents of the vial in 1.39 mL of H_2O to make a 400 mM stock. Take 50 µL of the 400 mM stock and add to 950 µL of 1X Reaction Buffer to make the final 20 mM galactose stock solution. The remaining 400 mM stock solution should be divided into small aliquots and stored at -20°C. The 20 mM stock solution can be stored at 4°C for several weeks or at -20°C for long-term stability.

Galactose Oxidase Assay

The following protocol describes the assay of galactose oxidase activity in a total volume of 100 µL per microplate well. The volumes recommended here are sufficient for ~100 assays. The kit provides sufficient material for ~400 assays.

2.1 Prepare a galactose oxidase standard curve by first adding 50 µL of 1X Reaction Buffer to 12 wells of a 96-well microplate. Next, make serial dilutions of the 200 U/mL galactose oxidase stock solution (prepared in step 1.5) in 1X Reaction Buffer to produce galactose oxidase concentrations of 0 to 2000 mU/mL. To do this, add 1 µL of the 200 U/mL galactose oxidase stock solution to the first well and add 49 µL of 1X Reaction Buffer for a total volume of 100 µL in the first well. Mix the contents of the well by pipet and then perform serial two-fold dilutions across the remainder of the microplate wells by pipetting 50 µL from well 1 into well 2, mixing by pipet, and removing 50 µL from well 2 and adding it to well 3. Continue this process across all

wells of the series except the final well. The volume of the last dilution made will be 100 µL, therefore 50 µL must be removed from this well to keep the volumes equal. Note that the galactose oxidase concentrations will be twofold lower in the final 100 µL reaction volumes. The 50 µL of 1X Reaction Buffer in the twelfth well serves as a negative control.

2.2 Dilute the galactose oxidase–containing samples in 1X Reaction Buffer. A volume of 50 µL will be used for each reaction. A variable dilution will be required depending on the total galactose oxidase present in the sample. In the first trial the samples should be serially diluted to determine the optimal amount of sample for assay. Pipet 50 µL into each well.

2.3 If desired, prepare a positive control by diluting the 20 mM H_2O_2 working solution (prepared in step 1.4) to 10 µM in 1X Reaction Buffer. Pipet 50 µL of the 10 µM stock solution into a separate well of the microplate.

2.4 Prepare a 2X working solution of 100 µM Amplex Red reagent containing 0.2 U/mL HRP and 200 µM galactose by mixing:

- 50 µL of the Amplex Red reagent stock solution (prepared in step 1.1)
- 10 µL of the HRP stock solution (prepared in step 1.3)
- 50 µL of the galactose stock solution (prepared in step 1.6)
- 4.89 mL of 1X Reaction Buffer

This 5 mL volume is sufficient for ~100 assays. Note that the final concentration of each component will be twofold lower in the final reaction volume.

2.5 Begin the reaction by adding 50 µL of the Amplex Red reagent/HRP/galactose working solution to each microplate well containing the samples and controls.

2.6 Incubate the reaction for 30 minutes or longer at 37°C, protected from light. Because the assay is continuous (not terminated), fluorescence or absorbance may be measured at multiple time points to follow the kinetics of the reactions.

2.7 Measure the fluorescence or absorbance in a microplate reader using excitation in the range of 530–560 nm and emission detection at ~590 nm or absorbance at ~560 nm (see Figure 1).

2.8 For each point, correct for background fluorescence or absorbance by subtracting the value derived from the no-galactose oxidase control.

Galactose Assay

The following protocol describes the assay of galactose in a total volume of 100 µL per microplate well. The volumes recommended here are sufficient for ~100 assays. The kit provides sufficient material for ~400 assays.

3.1 Prepare a galactose standard curve by first adding 50 µL of 1X Reaction Buffer to 12 wells of a 96-well microplate. Next, make serial dilutions of the 20 mM galactose stock solution (prepared in step 1.6) in 1X Reaction Buffer to produce galactose concentrations of 0 to 240 µM. To do this, add 1.2 µL of 20 mM galactose to the first well and add 48.8 µL of 1X Reaction Buffer for a total volume of 100 µL in the first well. Mix the contents in the well by pipet and then perform serial twofold dilutions across the remainder of the microplate wells by pipetting 50 µL from well 1 into well 2, mixing by pipet, removing 50 µL from well 2 and adding it to well 3. Continue this process across all wells of the series except the final well. The volume of the last dilution made will be 100 µL, therefore 50 µL must be removed from this well to keep the volumes equal. Note that the galactose concentrations will be twofold lower in the final 100 µL reaction volumes. The 50 µL of 1X Reaction Buffer in the twelfth well serves as a negative control.

3.2 Dilute the galactose-containing samples in 1X Reaction Buffer. A volume of 50 µL will be used for each reaction. A variable dilution will be required depending on the total galactose present in the sample. In the first trial the samples should be serially diluted to determine the optimal amount of sample for assay. Pipet 50 µL into each well.

3.3 If desired, prepare a positive control by diluting the 20 mM H_2O_2 working solution (prepared in step 1.4) to 10 µM in 1X Reaction Buffer. Pipet 50 µL of the 10 µM solution into a separate well of the microplate.

3.4 Prepare a 2X working solution of 100 µM Amplex Red reagent containing 0.2 U/mL HRP and 4 U/mL galactose oxidase by mixing:

- 50 µL of the Amplex Red reagent stock solution (prepared in step 1.1)
- 10 µL of the HRP stock solution (prepared in step 1.3)
- 100 µL of the galactose oxidase stock solution (prepared in step 1.5)
- 4.84 mL of 1X Reaction Buffer

This 5 mL volume is sufficient for ~100 assays. Note that the final concentration of each component will be twofold lower in the final reaction volume.

3.5 Begin the reaction by adding 50 µL of the Amplex Red reagent/HRP/galactose oxidase working solution to each microplate well containing the samples and controls.

3.6 Incubate the reaction for 30 minutes or longer at 37°C, protected from light. Because the assay is continuous (not terminated), fluorescence or absorbance may be measured at multiple time points to follow the kinetics of the reactions.

3.7 Measure the fluorescence or absorbance in a microplate reader using excitation in the range of 530–560 nm and emission detection at ~590 nm or absorbance at ~560 nm (see Figure 1).

3.8 For each point, correct for background fluorescence or absorbance by subtracting the value derived from the no-galactose control.

References

1. Anal Chem 36, 2477 (1964); **2.** Anal Biochem 284, 87 (2000); **3.** Annul Rev Physiol 57, 635 (1995); **4.** J Biol Chem 271, 6933 (1996); **5.** Curr Probl Cancer 15, 300 (1991); **6.** In Vivo 7, 607 (1993); **7.** Gastroenterology 104, 1515 (1993); **8.** Hepatology 22, 856 (1995); **9.** 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
A22179	Amplex® Red Galactose/Galactose Oxidase Assay Kit *400 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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