

Figure 10.49 A-12222 Amplex Red reagent.

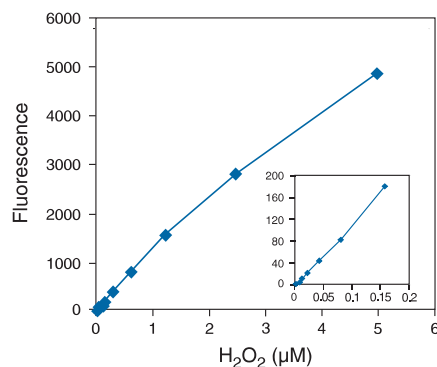


Figure 10.51 Detection of H_2O_2 using the Amplex Red Hydrogen Peroxide Peroxidase Assay Kit (A-22188). Reactions containing $50 \mu\text{M}$ Amplex Red reagent, 1 U/mL HRP and the indicated amount of H_2O_2 in 50 mM sodium phosphate buffer, $\text{pH } 7.4$, were incubated for 30 minutes at room temperature. Fluorescence was measured with a fluorescence-based microplate reader using excitation at $530 \pm 12.5 \text{ nm}$ and fluorescence detection at $580 \pm 25 \text{ nm}$. Background fluorescence (24 units), determined for a no- H_2O_2 control reaction, was subtracted from each value. The inset shows the sensitivity and linearity of the assay at low levels of HRP.

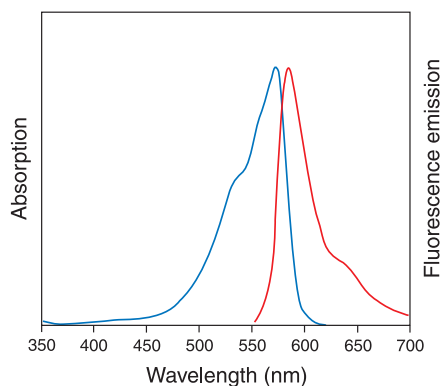


Figure 10.52 Absorption and fluorescence emission spectra of resorufin (R-363) in $\text{pH } 9.0$ buffer.

Our Amplex Red technology always uses natural substrates for the enzyme being measured, not synthetic pseudo-substrates.

10.5 Substrates for Oxidases, Including Amplex Red Kits

Oxidases, the most useful of which is undoubtedly horseradish peroxidase (HRP), are important enzymes that are used in a wide variety of bioassays. Peroxidase activity is also present in many cells. Reagents for quantitation of peroxidase and the activity of a variety of other oxidases are described in this section; reagents for detecting the activity of cellular peroxidases and the oxygen radicals produced by these peroxidases are described in Section 16.1 and Section 19.2. Antibody, protein G, avidin and streptavidin conjugates of horseradish peroxidase are listed in the price list of this section and described in Section 7.3 and Section 7.6. Tyramide signal amplification (TSA) technology (Section 6.2) makes extensive use of peroxidase-conjugated reagents and fluorescent dye- or hapten-labeled tyramides to deposit a detectable product at the site of enzymatic activity (Figure 6.6). Our exclusive Zenon One technology (Section 7.2) includes the Zenon One Horseradish Peroxidase Labeling Kit (Z-25054), which permits the rapid and quantitative formation of HRP-labeled complexes of any whole mouse IgG_1 antibody. This product is described in detail in Section 7.2.

We have used our extremely versatile Amplex Red reagent — the most stable and sensitive fluorogenic substrate known for horseradish peroxidase — to develop a variety of novel fluorogenic and chromogenic assays for enzymes that produce hydrogen peroxide. Furthermore, these coupled assays permit the ultrasensitive quantitation of a diverse assortment of analytes, including glucose, galactose, cholesterol, glutamic acid, xanthine (or hypoxanthine), uric acid, choline and acetylcholine, as well as hydrogen peroxide. Our patented P_i Per Phosphate Assay Kit and P_i Per Pyrophosphate Assay Kit (P-22061, P-22062; Section 10.3) also utilize our exclusive Amplex Red technology for the continuous assays of enzymes that produce either inorganic phosphate or pyrophosphate.

Peroxidases

Amplex Red Reagent: A Stable Substrate for Peroxidase Detection

The best fluorogenic substrate known for peroxidase and any other oxidase that produces H_2O_2 is the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine; A-12222, A-22177; Figure 10.49), which is a sensitive and stable probe for H_2O_2 . In the presence

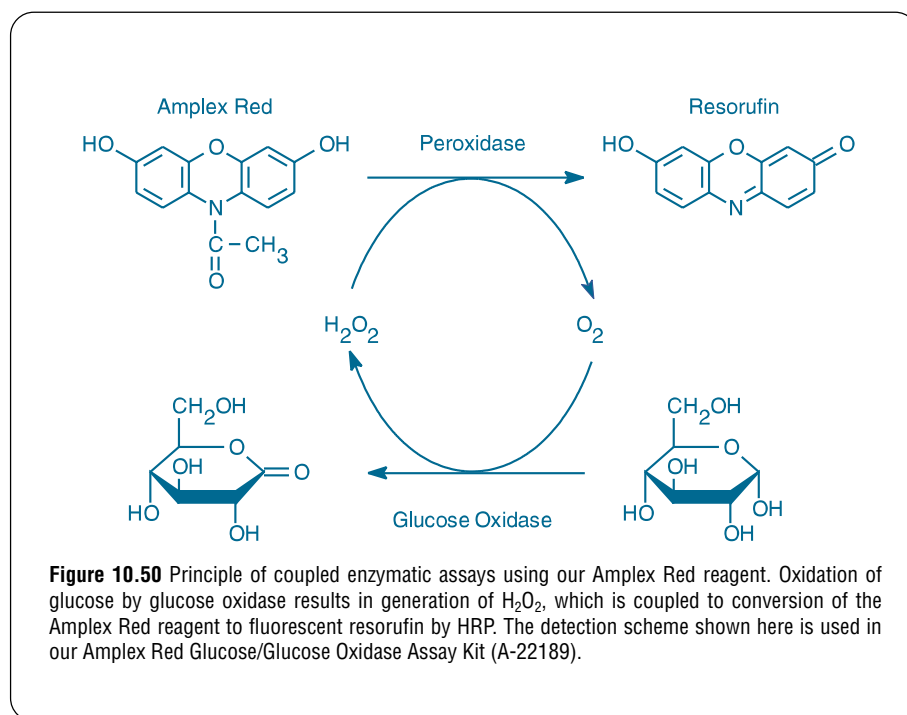


Figure 10.50 Principle of coupled enzymatic assays using our Amplex Red reagent. Oxidation of glucose by glucose oxidase results in generation of H_2O_2 , which is coupled to conversion of the Amplex Red reagent to fluorescent resorufin by HRP. The detection scheme shown here is used in our Amplex Red Glucose/Glucose Oxidase Assay Kit (A-22189).

of horseradish peroxidase (HRP), the Amplex Red reagent reacts with a 1:1 stoichiometry with H_2O_2 to produce highly fluorescent resorufin¹ (R-363, Figure 10.50, Section 10.1). Unlike most peroxidase substrates, the Amplex Red reagent is very stable in the presence of H_2O_2 , except when a peroxidase is present. The sensitivity of the Amplex Red reagent in detecting the activity of D-amino acid oxidase has been reported to be 5- to 25-times better than that of the QuantaBlu fluorogenic peroxidase substrate,² with a lower limit for detection of D-alanine of 2 pmol.² The Amplex Red reaction can be used to routinely detect as little as 10 picomoles of H_2O_2 in a 100 μ L volume (50 nM, Figure 10.51), at least a tenfold greater sensitivity than that attained with the commonly used scopoletin assay for H_2O_2 .^{1,3} In the scopoletin assay, HRP catalyzes conversion of the fluorescent scopoletin to a nonfluorescent product. Unlike scopoletin, the Amplex Red reagent is a fluorogenic substrate with very low background fluorescence. Consequently, assays using Amplex Red as the substrate result in an increase in fluorescence, not as a decrease — an inherently superior method for enzymatic assays. Other advantages of the Amplex Red reaction over scopoletin-based H_2O_2 assays include high chemical stability of the Amplex Red reagent and its fluorescent product, resorufin, and the long-wavelength spectra of resorufin. Because resorufin has excitation and fluorescence emission maxima (Figure 10.52) of approximately 570 nm and 585 nm (versus 360 nm and 460 nm, respectively, for scopoletin), there is much less interference from autofluorescence in most biological samples.

Because H_2O_2 is produced in many different enzymatic reactions, the Amplex Red reagent can be used in coupled enzymatic reactions to detect the activity of many different enzymes or, when the substrate concentration is limited, to assay solutions for metabolically active constituents such as glucose, acetylcholine and cholesterol (see below). Advantages of Amplex Red reagent-based assays include the following:

- The Amplex Red reagent is a fluorogenic substrate with extremely low background color or fluorescence
- Stock solutions of the Amplex Red reagent are chemically stable
- The fluorescent product, resorufin, is also stable
- The long-wavelength spectra of resorufin (excitation/emission ~570/585 nm, Figure 10.52) result in little interference from autofluorescence in most biological samples

The Amplex Red reagent has been used to detect the release of H_2O_2 from activated human leukocytes,^{1,3} to measure the activity of monoamine oxidase in cow brain tissue,⁴ to demonstrate the extracellular production of H_2O_2 produced by UV light stimulation of human keratinocytes⁵⁻⁷ and to measure L-glutamate in food samples.⁸ The Amplex Red reagent can be purchased separately in a single 5 mg vial (A-12222) or packaged as a set of 10 vials, each containing 10 mg of the substrate, for high-throughput screening applications (A-22177). The Amplex Red reagent is also utilized as the detection reagent in our many Amplex Red Assay Kits, including the:

- Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (A-22188)
- Amplex Red Catalase Assay Kit (A-22180)
- Amplex Red Monoamine Oxidase Assay Kit (A-12214)

- Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit⁸ (A-12221)
- Amplex Red Glucose/Glucose Oxidase Assay Kit (A-22189)
- Amplex Red Galactose/Galactose Oxidase Assay Kit (A-22179)
- Amplex Red Neuraminidase (Sialidase) Assay Kit (A-22178)
- Amplex Red Cholesterol Assay Kit (A-12216)
- Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit (A-12217)
- Amplex Red Phosphatidylcholine-Specific Phospholipase C Assay Kit (A-12218, Section 18.4)
- Amplex Red Phospholipase D Assay Kit (A-12219, Section 18.4)
- Amplex Red Sphingomyelinase Assay Kit (A-12220, Section 13.3)
- Amplex Red Uric Acid/Uricase Assay Kit (A-22181)
- Amplex Red Xanthine/Xanthine Oxidase Assay Kit (A-22182)
- Amplex Red ELISA Kits #1 and #2 (A-22170, A-22171)

Most of the Amplex Red kits are further discussed in this section; however, some are only presented in the sections listed above. The Amplex Red reagent and kits containing the Amplex Red reagent are sold for noncommercial use and for high-throughput screening applications only.

Amplex Red ELISA Kits

Molecular Probes' Amplex Red ELISA Kits offer an extremely sensitive fluorometric or colorimetric detection method for horseradish peroxidase (HRP)–amplified enzyme-linked immunosorbent assays (ELISAs). The Amplex Red ELISA Kit #1 (A-22170) contains an HRP goat anti–mouse IgG conjugate, which can be used for the ELISA detection of any mouse IgG antibody. The Amplex Red ELISA Kit #2 (A-22171) contains the versatile protein G conjugate of HRP, which can be used for the ELISA detection of IgGs from most commonly used species, including human, mouse, rabbit, goat, sheep, cow and horse. The Amplex Red reagent provided in these kits is a highly sensitive and stable probe for the detection of HRP activity. As described above, the Amplex Red reagent reacts with H_2O_2 in the presence of HRP to form the fluorescent product resorufin^{1,3} (R-363, Section 10.1, Figure 10.50). Because resorufin also has strong absorption, the assay can be performed either fluorometrically or spectrophotometrically. The Amplex Red ELISA Kit #1 with the HRP goat anti–mouse IgG antibody conjugate has detection limits of as little as 10 pg/microplate well of a mouse monoclonal IgG antibody by fluorometry or 50 pg/microplate well by colorimetry (Figure 7.44). The Amplex Red ELISA Kit #2 with HRP protein G has detection limits of as little as 1 ng/microplate well of a mouse monoclonal IgG antibody by fluorometry or 3 ng/microplate well by colorimetry.

The Amplex Red ELISA Kits contain:

- The Amplex Red reagent
- DMSO
- A concentrated reaction buffer
- Hydrogen peroxide
- A horseradish peroxidase (HRP) conjugate of goat anti–mouse IgG antibody (in Kit #1, A-22170) or protein G (in Kit #2, A-22171)
- Detailed ELISA protocols

The Amplex Red ELISA Kits provide sufficient reagents for approximately 1000 ELISAs using either a fluorescence or absorbance microplate reader and reaction volumes of 100 μL per assay. Our HRP conjugates of goat anti-mouse IgG antibody (G-21040, Section 7.3) and protein G (P-21041, Section 7.3) are also available separately.

Other Substrates for Peroxidase Assays

Although HRP is an important enzyme for both histochemistry and ELISAs, fluorogenic peroxidase substrates have not been extensively used for its detection. Fluorogenic peroxidase substrates such as the dihydrofluoresceins (also known as fluoresceins) (D-399, C-400, C-13293), dihydrocalcein AM (D-23805, Figure 15.4), dihydrorhodamines (D-632, D-633, D-23806; Section 19.3) and dihydroethidium (hydroethidine; D-1168, D-11347, D-23107; Section 19.2) are converted to highly fluorescent products in the presence of the enzyme and hydrogen peroxide. Because these substrates are insufficiently stable for routine use in ELISA assays, Molecular Probes has converted the dihydrofluoresceins to diacetates. When used in intracellular applications, the acetates are cleaved by endogenous esterases, releasing the intact substrate. However, when used for *in vitro* assays, an esterase or a mild base must first be added to cleave the acetates, releasing the substrate. The dihydrofluoresceins have been used to measure peroxidase activity⁹ and to detect hydroperoxide formation.^{10–13} In addition to being a reagent for derivatization of aldehydes and ketones (Section 3.2) and detection of nitric oxide (Section 19.3), NBD methylhydrazine (*N*-methyl-4-hydrazino-7-nitrobenzofurazan, M-20490) has been reported to be useful as a fluorogenic peroxidase substrate, with a sensitivity limit for detection of H_2O_2 of about 75 nM.¹⁴

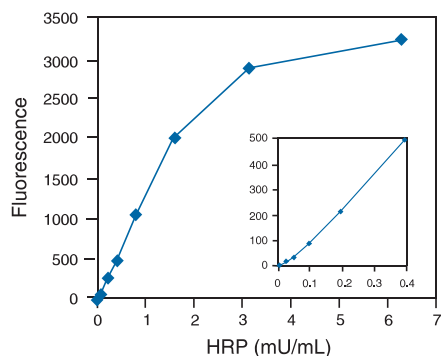


Figure 10.53 Detection of HRP using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (A-22188). Reactions containing 50 μM Amplex Red reagent, 1 mM H_2O_2 and the indicated amount of HRP in 50 mM sodium phosphate buffer, pH 7.4, were incubated for 30 minutes at room temperature. Fluorescence was measured with a fluorescence-based microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. Background fluorescence (3 units), determined for a no-HRP control reaction, was subtracted from each value. The inset shows the sensitivity of the assay at very low levels of HRP.

Luminol and MCLA: Chemiluminescent Peroxidase Substrates

Nonisotopic immunoassays utilizing peroxidase conjugates and the chemiluminescent horseradish peroxidase substrate luminol (L-8455) have provided a rapid and sensitive method for quantitating a wide variety of analytes, including cholesterol,¹⁵ digoxin¹⁶ and acetylcholine.¹⁷ Addition of trace amounts of luciferin (L-2911, L-2912, L-2916; Section 10.6) has been shown to considerably enhance the sensitivity in the assay of thyroxine, digoxin, α -fetoprotein and other analytes.¹⁸ A method that employs luminol has been developed for the quantitation of very limiting samples of human DNA from single hairs, saliva, small blood stains and paraffin-embedded and fixed tissue sections. Using a biotinylated oligodeoxynucleotide probe to membrane-immobilized DNA, a streptavidin-horseradish peroxidase conjugate and luminol, researchers have detected 150 pg of human DNA.¹⁹

MCLA (M-23800) is principally utilized as a superoxide-sensitive chemiluminescent probe (Section 19.2). MCLA has also been utilized for the determination of both horseradish peroxidase²⁰ and myeloperoxidase.^{21–23}

Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit

The Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (A-22188) provides a simple, one-step assay for detecting H_2O_2 or the activity of horseradish peroxidase using a fluorescence-based microplate reader or fluorometer (Figure 10.53). Using the reagents in the kit one can also assay the activity of other oxidases that produce H_2O_2 , such as NADPH oxidase.²⁴ This assay has been used to detect H_2O_2 release from activated human leukocytes.³ In the scopoletin assay, HRP catalyzes the conversion of fluorescent scopoletin to a nonfluorescent product. Unlike scopoletin, the Amplex Red reagent is a fluorogenic substrate with very low background fluorescence. Other advantages of the Amplex Red reaction over scopoletin-based H_2O_2 assays include high chemical stability of the Amplex Red reagent and its fluorescent product, resorufin, and the long wavelength spectra of resorufin. Because resorufin has excitation and fluorescence emission maxima (Figure 10.52) of approximately 570 nm and 585 nm (versus 360 nm and 460 nm, respectively, for scopoletin), there is much less interference from autofluorescence in most biological samples. The Amplex Red Hydrogen Peroxide Assay Kit contains the principal detection reagents required to assay many other oxidases that produce H_2O_2 , such as NADPH oxidase.²⁴ Included in the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit are:

- The Amplex Red reagent
- Horseradish peroxidase (HRP)
- H_2O_2 for use as a positive control
- DMSO and a concentrated reaction buffer
- A detailed protocol

Each kit provides a sufficient amount of each reagent for approximately 500 assays using a reaction volume of 100 μL per assay.

Catalase

The Amplex Red Catalase Assay Kit (A-22180) provides an ultrasensitive, yet simple, assay for measuring catalase activity. Catalase is a heme-containing redox protein found in nearly all animal and plant cells as well as in aerobic microorganisms. In eukaryotic cells it is concentrated in the peroxisomes. Catalase is an important enzyme because H_2O_2 is a powerful oxidizing agent that is potentially damaging to cells. By preventing excessive buildup of H_2O_2 , catalase allows important cellular processes that produce H_2O_2 as a by-product to take place safely.

In the assay, catalase first reacts with H_2O_2 to produce water and oxygen (O_2). Next, the Amplex Red reagent reacts with a 1:1 stoichiometry with any unreacted H_2O_2 in the presence of horseradish peroxidase to produce the highly fluorescent oxidation product, resorufin. Therefore, as catalase activity increases, the signal from resorufin decreases (Figure 10.54). The results are typically plotted by subtracting the observed fluorescence from that of a no-catalase control. Using this kit, it is possible to detect catalase in a purified system at levels as low as 50 mU/mL.

Resorufin has absorption and fluorescence emission maxima of approximately 563 nm and 587 nm, respectively (Figure 10.52). Because the absorbance is strong, the assay can be performed either fluorometrically or spectrophotometrically. The Amplex Red Catalase Assay Kit contains

- The Amplex Red reagent
- Horseradish peroxidase (HRP)
- Catalase
- Hydrogen peroxide
- DMSO and a concentrated reaction buffer
- A detailed protocol

Each kit provides sufficient reagents for approximately 400 assays using either a fluorescence or absorbance microplate reader and reaction volumes of 100 μ L per assay.

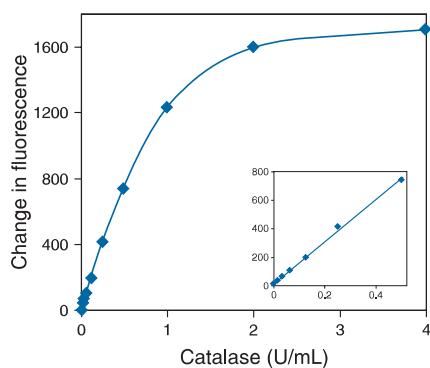


Figure 10.54 Detection of catalase using the Amplex Red Catalase Assay Kit (A-22180). Reactions contained the indicated amount of catalase and 20 μ M H_2O_2 in 1 \times reaction buffer and was incubated for 30 minutes. The final reaction containing 50 μ M Amplex Red reagent and 0.2 U/mL HRP and was incubated at 37°C. After 30 minutes, fluorescence was measured in a fluorescence-based microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. The change in fluorescence is reported as the observed fluorescence intensity subtracted from that of a no-catalase control.

Glucose and Glucose Oxidase

Glucose oxidase is widely used for glucose determination and, when conjugated to antibodies, for use in enzyme immunoassays (EIAs). Molecular Probes has found that the Amplex Red reagent can be utilized for the ultrasensitive detection of both glucose and glucose oxidase. In this enzyme-coupled assay, glucose oxidase reacts with glucose to form gluconolactone and H_2O_2 . The H_2O_2 is then detected using the Amplex Red reagent peroxidase substrate (Figure 10.50). The Amplex Red Glucose/Glucose Oxidase Assay Kit (A-22189) can be used to detect glucose levels as low as 185 ng/mL (1 μ M; Figure 10.23), and is at least tenfold more sensitive than assays using *o*-dianisidine as the peroxidase substrate. The same kit can be used to detect glucose oxidase levels as low as 1×10^{-5} U/mL (Figure 10.55). This Amplex Red Kit can be used with a fluorescence microplate reader or fluorometer and contains:

- The Amplex Red reagent
- Horseradish peroxidase (HRP)
- H_2O_2 for use as a positive control
- DMSO and a concentrated reaction buffer
- D-Glucose
- Glucose oxidase
- A detailed protocol

The kit provides a sufficient amount of each reagent for approximately 500 assays using a reaction volume of 100 μ L per assay.

Galactose and Galactose Oxidase

The Amplex Red Galactose/Galactose Oxidase Assay Kit (A-22179) provides the reagents and a general protocol for the assay of terminal galactosylated proteins, galactose-producing

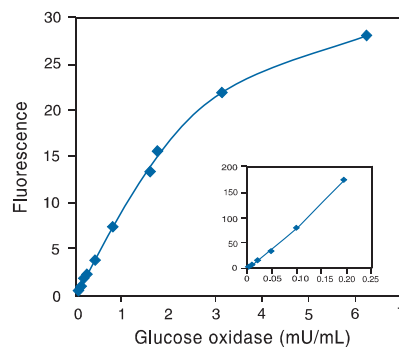


Figure 10.55 Detection of glucose oxidase using the Amplex Red Glucose/Glucose Oxidase Assay Kit (A-22189). Reactions containing 50 μ M Amplex Red reagent, 1 U/mL HRP, 50 mM glucose and the indicated amount of glucose oxidase in 50 mM sodium phosphate buffer, pH 7.4, were incubated for 30 minutes at room temperature. Fluorescence was measured with a fluorescence-based microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. Background fluorescence (19 units) determined for a no-glucose oxidase control reaction was subtracted from each value. The inset shows the assay's sensitivity at low levels of glucose oxidase (0–0.2 mU/mL).

enzymes and for the assay of galactose oxidase. Unlike glucose oxidase, galactose oxidase can produce H_2O_2 from either free galactose or from polysaccharides — including glycoproteins in solution or on cell surfaces — in which galactose is the terminal residue, producing an aldehyde moiety on the 6-position of the galactose (Figure 10.13). We have used our Amplex Red galactose oxidase assay for the quantitative assay of mucin-type glycoproteins by using a method similar to one described by Kinoshita and collaborators.²⁵ The Amplex Red Galactose/Galactose Oxidase Assay Kit (A-22179) contains:

- The Amplex Red reagent
- DMSO
- Galactose
- Galactose oxidase from *Dactylium dendroides*
- Horseradish peroxidase (HRP)
- Hydrogen peroxide
- A 5× reaction buffer
- A detailed protocol

Sufficient reagents are provided for approximately 400 assays using either an absorption- or fluorescence-based microplate reader and reaction volumes of 100 μ L per assay. The Amplex Red fluorescent galactose/galactose oxidase assay can accurately detect free galactose concentration as low as 4 μ M (Figure 10.14) and 2 mU/mL of galactose oxidase activity (Figure 10.15). Because of the high absorbance of resorufin, the absorptimetric assay has only slightly lower sensitivity. The Amplex Red Neuraminidase (Sialidase) Assay Kit (A-22178) utilizes this galactose oxidase-coupled chemistry for continuous assay of neuraminidase-catalyzed hydrolysis of fetuin, a sialoglyconjugate. This product is described in detail in Section 10.2.

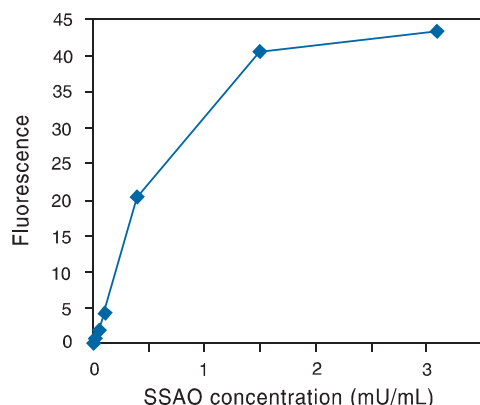


Figure 10.56 Detection of plasma amine oxidase (an SSAO) activity using the Amplex Red Monoamine Oxidase Assay Kit (A-12214) and benzylamine as the substrate. Each reaction contained 1 mM benzylamine, 1 U/mL HRP, 200 μ M Amplex Red reagent and the indicated amount of SSAO in 50 mM potassium phosphate, pH 7.4. Reactions were incubated at room temperature for 15 minutes. Fluorescence was measured with a fluorescence-based microplate reader using excitation at 560 ± 10 nm and fluorescence detection at 590 ± 10 nm.

Cholesterol and Cholesterol Oxidase

The Amplex Red Cholesterol Assay Kit (A-12216) provides what may be the most sensitive assay available for cholesterol in serum and other complex mixtures. The assay provided in this kit can detect as little as 5 ng/mL (5×10^{-4} mg/dL) cholesterol (Figure 13.32) and can accurately measure the cholesterol content in the equivalent of 0.01 μ L of human serum.²⁶ In the assay, cholesterol is detected by an enzyme-coupled reaction. 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 the Amplex Red reagent. The Amplex Red cholesterol assay is continuous, requires no separation or wash steps and, except potentially at extremely high concentrations, is insensitive to bilirubin contamination. These characteristics make the assay particularly well suited for 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, by providing an excess of cholesterol in the reaction, the assay can be used to sensitively detect the activity of cholesterol oxidase. This kit can be used with a fluorescence microplate reader or fluorometer and contains:

- The Amplex Red reagent
- Horseradish peroxidase (HRP)
- H_2O_2 for use as a positive control
- DMSO and a concentrated reaction buffer
- Cholesterol oxidase
- Cholesterol esterase
- Cholesterol for preparation of a standard curve
- A detailed protocol

Each kit provides a sufficient amount of reagent for approximately 500 assays using a reaction volume of 100 μ L per assay.

Monoamine Oxidase

Monoamine oxidase, which inactivates several primary, secondary and tertiary amines via oxidative transamination, serves to regulate tissue levels of amine neurotransmitters and dietary amines. The Amplex Red Monoamine Oxidase Assay Kit (A-12214) provides a simple fluorometric method for the continuous measurement of amine oxidase activity in tissue homogenates or purified preparations. We have found that the assay is able to sensitively detect both monoamine oxidase (MAO) activity and semicarbazide-sensitive amine oxidase (SSAO) activity and is useful for performing both end-point and continuous measurements of amine oxidase activity. The assay is able to detect both MAO-A and MAO-B from cow brain tissue using as little as 200 μ g of total protein per sample⁴ and has been used to measure plasma amine oxidase (an SSAO) activity levels as low as 1.2×10^{-5} U/mL using a commercially available enzyme (Figure 10.56).

To facilitate discrimination of MAO-A and MAO-B activity, two MAO substrates and two MAO inhibitors are included in the

kit. *p*-Tyramine is a substrate for both MAO-A and MAO-B, whereas benzylamine is a substrate for MAO-B.²⁷ Both *p*-tyramine and benzylamine are also substrates for SSAO enzymes. Clorgyline is a specific inhibitor of MAO-A activity and pargyline is a specific inhibitor of MAO-B activity.^{28,29} The potential applications of this kit include the measurement of amine oxidase activity in normal and diseased tissues, blood samples and other biological fluids, the screening of drugs as possible MAO inhibitors or substrates and the determination of kinetic constants for different amine oxidase substrates. Each kit contains:

- The Amplex Red reagent
- Horseradish peroxidase (HRP)
- H₂O₂ for use as a positive control
- DMSO and a concentrated reaction buffer
- Benzylamine, a substrate for MAO-B and SSAO enzymes
- *p*-Tyramine, a substrate for MAO-A, MAO-B and SSAO enzymes
- Clorgyline, a specific inhibitor of MAO-A activity
- Pargyline, a specific inhibitor of MAO-B activity
- Resorufin for use as a reference standard
- A detailed protocol

Each kit provides a sufficient amount of each reagent for approximately 500 assays using a reaction volume of 200 μ L per assay.

Glutamic Acid and Glutamate Oxidase

The Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit (A-12221) provides an ultrasensitive method for continuously detecting glutamic acid³⁰ or for monitoring glutamate oxidase activity in a fluorescence microplate reader or fluorometer.⁸ In this assay, L-glutamic acid is oxidized by glutamate oxidase to produce α -ketoglutarate, NH₃ and H₂O₂. L-Alanine and L-glutamate-pyruvate transaminase are also included in the reaction. Thus, the L-glutamic acid is regenerated by transamination of α -ketoglutarate, resulting in multiple cycles of the initial reaction and a significant amplification of the H₂O₂ produced. Hydrogen peroxide reacts with the Amplex Red reagent in a 1:1 stoichiometry in a reaction catalyzed by horseradish peroxidase (HRP) to generate the highly fluorescent product resorufin^{1,3} (R-363, Section 10.1). Because resorufin has absorption/emission maxima of 563/587 nm (Figure 10.5), there is little interference from autofluorescence in most biological samples

If the concentration of L-glutamic acid is limiting in this assay, then the fluorescence increase is proportional to the initial L-glutamic acid concentration. The Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit allows detection of as little as 10 nM L-glutamic acid in purified systems using a 30-minute reaction time (Figure 16.35). If the reaction is modified to include an excess of L-glutamic acid, then this 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 μ U/mL (Figure 16.36).

The Amplex Red reagent has been used to quantitate the activity of glutamate-producing enzymes in a high-throughput assay for drug discovery.³⁰ Each Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit contains:

- The Amplex Red reagent
- Horseradish peroxidase (HRP)
- H₂O₂
- DMSO and a concentrated reaction buffer
- L-Glutamate oxidase from *Streptomyces* sp.
- L-Glutamate-pyruvate transaminase from pig heart
- L-Glutamic acid
- L-Alanine
- A detailed protocol

Each kit provides sufficient reagents for approximately 200 assays using a fluorescence microplate reader and a reaction volume of 100 μ L per assay.

Acetylcholine, Acetylcholinesterase and Choline Oxidase

Acetylcholine, the neurotransmitter released from the nerve terminal at neuromuscular junctions, binds to the acetylcholine receptor and opens its transmitter-gated ion channel (Section 16.3). The action of acetylcholine (ACh) is regulated by acetylcholinesterase (AChE), which hydrolyzes ACh to choline and acetate. The Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit (A-12217) provides an ultrasensitive method for continuously monitoring AChE activity or for detecting ACh in a fluorescence microplate reader or fluorometer. The kit can also be used for the assay of free choline, a newly declared “essential nutrient,” in foods.³¹ 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 the Amplex Red reagent (see above). 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 HRP, reacts with the Amplex Red reagent to generate the red-fluorescent product, resorufin (Figure 10.52). 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 16.27). We have been able to detect acetylcholinesterase activity from a tissue sample with total protein content as low as 200 ng/mL or 20 ng/well in a microplate assay.³² By providing an excess of AChE in the assay, the kit can also be used to detect acetylcholine levels as low as 0.3 μ M, with a range of detection from 0.3 μ M to ~100 μ M acetylcholine (Figure 16.28). Each kit contains:

- The Amplex Red reagent
- Horseradish peroxidase (HRP)
- H₂O₂ for use as a positive control
- DMSO and a concentrated reaction buffer
- Choline oxidase
- Acetylcholine (ACh)
- Acetylcholinesterase (AChE)
- A detailed protocol

Each kit provides a sufficient amount of each reagent for approximately 500 assays using a reaction volume of 200 μ L per assay.

Xanthine and Xanthine Oxidase

The Amplex Red Xanthine/Xanthine Oxidase Assay Kit (A-22182) provides an ultrasensitive method for detecting xanthine or hypoxanthine or for monitoring xanthine oxidase activity. In the assay, xanthine oxidase catalyzes the oxidation of purine nucleotides, hypoxanthine or xanthine, to uric acid and superoxide. In the reaction mixture, the superoxide spontaneously degrades to H₂O₂, which in the presence of HRP reacts stoichiometrically with the Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin. Resorufin has absorption and fluorescence emission maxima of approximately 563 nm and 587 nm, respectively, and because the extinction coefficient is high (54,000 cm⁻¹M⁻¹), the assay can be performed either fluorometrically or spectrophotometrically.

In healthy individuals, xanthine oxidase is present in appreciable amounts only in the liver and jejunum. However, in various liver disorders the enzyme is released into circulation. Therefore, determination of serum xanthine oxidase level serves as a sensitive indicator of acute liver damage such as jaundice. Previously, researchers have utilized chemiluminescence or absorbance to monitor xanthine oxidase activity. Using the Amplex Red Xanthine/Xanthine Oxidase Assay Kit, it is possible to detect xanthine oxidase in a purified system at levels as low as 0.1 mU/mL by fluorescence (Figure 19.3). The kit can also be used to detect as little as 200 nM hypoxanthine or xanthine (Figure 19.4).

The Amplex Red Xanthine/Xanthine Oxidase Assay Kit (A-22182) contains:

- The Amplex Red reagent
- Horseradish peroxidase (HRP)
- Hydrogen peroxide
- DMSO and a concentrated reaction buffer
- Xanthine oxidase
- Xanthine
- Hypoxanthine
- A detailed protocol

Each kit provides sufficient reagents for approximately 400 assays using either an absorbance or fluorescence microplate reader and reaction volumes of 100 µL per assay.

Uric Acid and Uricase

Serum uric acid is the end product of purine metabolism in the body tissues and is cleared through the kidneys by glomerular filtration. Most animals can metabolize uric acid to more readily excreted products, but humans lack the necessary enzyme, urate oxidase (uricase), as a result of the presence of two “nonsense mutations” in the human gene for uricase. Increased uric acid levels may result from leukemia, polycythemia, ingestion of foods high in nucleoproteins (e.g., liver and kidney) or impaired renal function. Gout results from the deposit of uric acid in body joints.

The Amplex Red Uric Acid/Uricase Assay Kit (A-22181) provides an ultrasensitive method for detecting uric acid or for monitoring uricase activity. In the assay, uricase catalyzes the conversion of uric acid to allantoin, H₂O₂ and carbon dioxide. In the presence of HRP the H₂O₂ reacts stoichiometrically with Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin. Resorufin has absorption and fluorescence emission maxima of approximately 563 nm and 587 nm, respectively, and because the extinction coefficient is high (54,000 cm⁻¹M⁻¹), the assay can be performed either fluorometrically or spectrophotometrically. Previous literature reports colorimetric detection limits at 3.6 µM, whereas the Amplex Red Uric Acid/Uricase Assay Kit can be used to detect as little as 100 nM uric acid in a purified system. The kit can also be used to detect a little as 0.2 mU/mL uricase in a purified system.

The Amplex Red Uric Acid/Uricase Assay Kit (A-22181) contains:

- The Amplex Red reagent
- Horseradish peroxidase (HRP)
- Hydrogen peroxide
- DMSO and a concentrated reaction buffer
- Uricase
- Uric acid
- A detailed protocol

Each kit provides sufficient reagents for approximately 400 assays using either an absorbance or fluorescence microplate reader and reaction volumes of 100 µL per assay.

The full citations and, in most cases, links to PubMed for all references in this Handbook are available at our Web site (www.probes.com/search).

References

1. Anal Biochem 253, 162 (1997); 2. Anal Biochem 287, 196 (2000); 3. J Immunol Methods 202, 133 (1997); 4. Anal Biochem 253, 169 (1997); 5. J Invest Dermatol 112, 751 (1999); 6. Free Radic Biol Med 27, 1197 (1999); 7. J Invest Dermatol 110, 966 (1998); 8. Anal Chim Acta 402, 47 (1999); 9. Anal Biochem 11, 6 (1965); 10. J Clin Invest 87, 711 (1991); 11. J Lab Clin Med 117, 291 (1991); 12. Anal Biochem 187, 129 (1990); 13. Anal Biochem 134, 111 (1983); 14. Angew Chem Int Ed Engl 39, 1453 (2000); 15. Biochim Biophys Acta 1210, 151 (1994); 16. Clin Chem 31, 1335 (1985); 17. J Neurochem 39, 248 (1982); 18. Nature 305, 158 (1983); 19. Nucleic Acids Res 20, 5061 (1992); 20. J Biolumin Chemilumin 9, 355 (1994); 21. Cell Mol Neurobiol 18, 565 (1998); 22. Methods Enzymol 233, 495 (1994); 23. Anal Biochem 199, 191 (1991); 24. J Biol Chem 275, 15749 (2000); 25. Anal Biochem 284, 87 (2000); 26. J Biochem Biophys Methods 38, 43 (1999); 27. Methods Enzymol 142, 617 (1987); 28. Anal Biochem 244, 384 (1997); 29. Biochem Pharmacol 18, 1447 (1969); 30. Anal Biochem 284, 382 (2000); 31. Science 281, 794 (1998); 32. Proc SPIE-Intl Soc Opt Eng 3926, 166 (2000).

Data Table — 10.5 Substrates for Oxidases, Including Amplex Red Kits

Cat #	MW	Storage	Soluble	Abs	EC	Em	Solvent	Product	Notes
A-12222	257.25	FF,D,A	DMSO	280	6,000	none	pH 8	R-363 *	1
A-22177	257.25	FF,D,A	DMSO	280	6,000	none	pH 8	R-363 *	1
C-400	531.30	F,D	DMSO, EtOH	290	5,600	none	MeCN	see Notes	2
C-13293	498.39	F,D	DMSO, EtOH	290	5,500	none	MeCN	see Notes	3
D-399	487.29	F,D	DMSO, EtOH	258	11,000	none	MeOH	see Notes	2
D-23805	1068.95	F,D	DMSO	285	5,800	none	MeCN	C-481 †	
L-8455	177.16	D,L	DMF	355	7,500	411	MeOH	see Notes	4
M-20490	209.16	F,L	MeCN	487	24,000	none	MeOH	see Notes	5
M-23800	291.74	FF,D,LL,AA	DMSO	430	8,400	546	MeOH	see Notes	6

For definitions of the contents of this data table, see “How to Use This Book” on page viii.

* See Section 10.1. † See Section 14.3.

Notes

1. This substrate is used for peroxidase-coupled detection in our Amplex Red Assay kits.
2. Dihydrofluorescein diacetates are colorless and nonfluorescent until both the acetates are hydrolyzed and the products are subsequently oxidized to fluorescein derivatives. The materials contain less than 0.1% of oxidized derivative when initially prepared. The end products from C-400 and D-399 are 2',7'-dichlorofluorescein derivatives with spectra similar to C-368 (Section 21.3).
3. Difluorodihydrofluorescein diacetates are colorless and nonfluorescent. Acetate hydrolysis and subsequent oxidation generate a fluorescent 2',7'-difluorofluorescein derivative with spectra similar to O-6146 (Section 21.3).
4. This compound emits chemiluminescence (Em = 425 nm) upon oxidation in basic aqueous solutions.
5. Peroxidase-catalyzed oxidation of NBD methylhydrazine generates fluorescent *N*-methyl-4-amino-7-nitrobenzofuran, Abs = 470 nm, Em = 547 nm in pH 5.8 aqueous buffer (Angew Chem Int Ed Engl 39, 1453 (2000)).
6. Generates chemiluminescence (Em = 455 nm) upon reaction with superoxide.

Product List — 10.5 Substrates for Oxidases, Including Amplex Red Kits

Cat #	Product Name	Unit Size
A-12217	Amplex [®] Red Acetylcholine/Acetylcholinesterase Assay Kit *500 assays*	1 kit
A-22180	Amplex [®] Red Catalase Assay Kit *400 assays*	1 kit
A-12216	Amplex [®] Red Cholesterol Assay Kit *500 assays*	1 kit
A-22170	Amplex [®] Red ELISA Kit #1 *with goat anti-mouse IgG, horseradish peroxidase conjugate* *1000 assays*	1 kit
A-22171	Amplex [®] Red ELISA Kit #2 *with protein G, horseradish peroxidase conjugate* *1000 assays*	1 kit
A-22179	Amplex [®] Red Galactose/Galactose Oxidase Assay Kit *400 assays*	1 kit
A-22189	Amplex [®] Red Glucose/Glucose Oxidase Assay Kit *500 assays*	1 kit
A-12221	Amplex [®] Red Glutamic Acid/Glutamate Oxidase Assay Kit *200 assays*	1 kit
A-22188	Amplex [®] Red Hydrogen Peroxide/Peroxidase Assay Kit *500 assays*	1 kit
A-12214	Amplex [®] Red Monoamine Oxidase Assay Kit *500 assays*	1 kit
A-22178	Amplex [®] Red Neuraminidase (Sialidase) Assay Kit *400 assays*	1 kit
A-12218	Amplex [®] Red Phosphatidylcholine-Specific Phospholipase C Assay Kit *500 assays*	1 kit
A-12219	Amplex [®] Red Phospholipase D Assay Kit *500 assays*	1 kit
A-12222	Amplex [®] Red reagent (10-acetyl-3,7-dihydroxyphenoxazine)	5 mg
A-22177	Amplex [®] Red reagent *packaged for high-throughput screening*	10 x 10 mg
A-12220	Amplex [®] Red Sphingomyelinase Assay Kit *500 assays*	1 kit
A-22181	Amplex [®] Red Uric Acid/Uricase Assay Kit *400 assays*	1 kit
A-22182	Amplex [®] Red Xanthine/Xanthine Oxidase Assay Kit *400 assays*	1 kit
C-400	5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H ₂ DCFDA) *mixed isomers*	25 mg
C-13293	5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate (carboxy-H ₂ DFFDA) *mixed isomers*	5 mg
D-22185	Diaminobenzidine (DAB) Histochemistry Kit #1 *with goat anti-mouse IgG-HRP*	1 kit
D-22186	Diaminobenzidine (DAB) Histochemistry Kit #2 *with goat anti-rabbit IgG-HRP*	1 kit
D-22187	Diaminobenzidine (DAB) Histochemistry Kit #3 *with streptavidin-HRP*	1 kit
D-399	2',7'-dichlorodihydrofluorescein diacetate (2',7'-dichlorofluorescein diacetate; H ₂ DCFDA)	100 mg
D-23805	dihydrocalcein, AM *special packaging*	20 x 50 µg
G-21040	goat anti-mouse IgG (H+L), horseradish peroxidase conjugate	1 mg
L-8455	luminol (3-aminophthalhydrazide)	25 g
M-20490	<i>N</i> -methyl-4-hydrazino-7-nitrobenzofurazan (NBD methylhydrazine)	25 mg
M-23800	2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one, hydrochloride (MCLA)	5 mg
P-22061	P _i Per™ Phosphate Assay Kit *1000 assays*	1 kit
P-22062	P _i Per™ Pyrophosphate Assay Kit *1000 assays*	1 kit
P-21041	protein G, horseradish peroxidase conjugate	1 mg