

## Amplex® Red Glucose/Glucose Oxidase Assay Kit

**Table 1.** Contents and storage information.

Material	Amount	Concentration	Storage	Stability
Amplex® Red reagent (Component A, blue cap, MW = 257)	5 vials, 154 µg of reagent each	NA	<ul style="list-style-type: none"> <li>• ≤-20°C</li> <li>• Desiccate</li> <li>• Protect from light</li> </ul>	When stored as directed, kit components should remain stable for at least 6 months.
DMSO, anhydrous (Component B, green cap)	700 µL	NA		
5X reaction buffer (Component C, white cap)	28 mL	0.25 M sodium phosphate, pH 7.4		
Horseradish peroxidase (Component D, yellow cap)	10 U *	NA		
Glucose oxidase (Component E, orange cap)	100 U †	NA		
D-Glucose (Component F, black cap, MW = 180)	~1 g	NA		
Hydrogen peroxide (H <sub>2</sub> O <sub>2</sub> ) (Component G, red cap)	200 µL	In a stabilized ~3% solution ††		

\* 1 unit (U) 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.

† 1 unit (U) is defined as the amount that will oxidase 1.0 µmole of β-D-glucose to D-gluconolactone and H<sub>2</sub>O<sub>2</sub> per minute at pH 5.1 and 30°C. †† The actual concentration is indicated on the label.

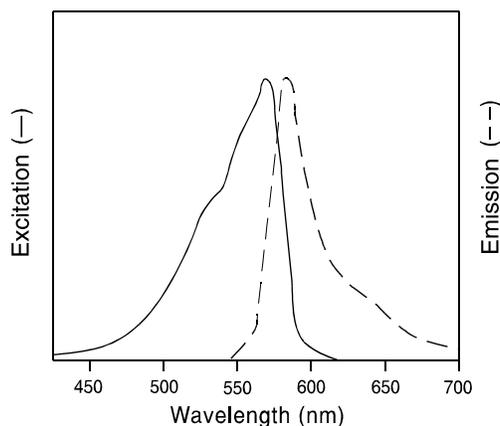
**Number of labelings:** Each kit provide sufficient reagents for approximately 500 assays using either a fluorescence or absorbance microplate reader and reaction volumes of 100 µL per assay.

**Approximate fluorescence excitation/emission maxima:** 571/585 nm for reaction product

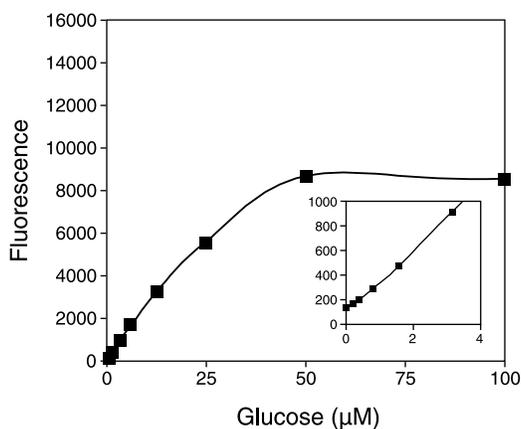
### Introduction

The Amplex® Red Glucose/Glucose Oxidase Assay Kit (A22189) provides a sensitive one-step method for detecting glucose or glucose oxidase. The Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) is a colorless, stable, and extremely versatile peroxidase substrate. Because peroxidase- and glucose oxidase-mediated reactions can be coupled,<sup>1,2</sup> it is possible to measure glucose oxidase activity or the release of glucose by any glucosidase enzyme—for instance, β-glucosidase and glucocerebrosidase—in either a continuous or discontinuous assay. This assay should also be very useful for quantitation of glucose levels in foods, fermentation media, and bodily fluids.

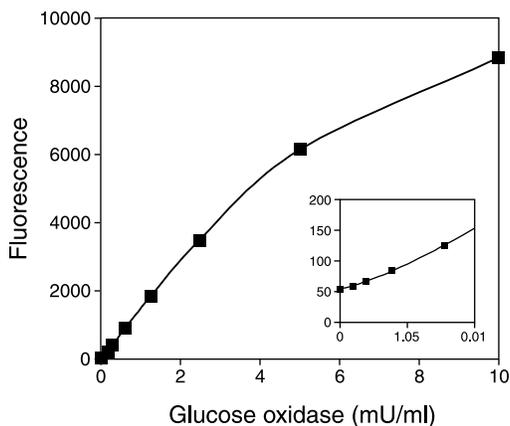
In the assay, glucose oxidase reacts with D-glucose to form D-gluconolactone and H<sub>2</sub>O<sub>2</sub>. In the presence of horseradish peroxidase (HRP), the H<sub>2</sub>O<sub>2</sub> then reacts with the Amplex<sup>®</sup> Red reagent in a 1:1 stoichiometry to generate the red-fluorescent oxidation product, resorufin.<sup>3</sup> Resorufin has fluorescence excitation and emission maxima of approximately 571 nm and 585 nm, respectively (Figure 1), and because the extinction coefficient is high (54,000 cm<sup>-1</sup>M<sup>-1</sup>), the assay can be performed either fluorometrically or spectrophotometrically. Furthermore, at these long wavelengths, there is little interference from autofluorescence found in most biological samples. With the Amplex<sup>®</sup> Red Glucose/Glucose Oxidase Assay Kit, we have detected as little as 3 μM D-glucose (Figure 2) and 0.05 mU/mL glucose oxidase (Figure 3).



**Figure 1.** Normalized fluorescence excitation and emission spectra of resorufin, the product of the Amplex<sup>®</sup> Red reaction.



**Figure 2.** Detection of glucose using the Amplex<sup>®</sup> Red Glucose/Glucose Oxidase Assay Kit (A22189). Reactions containing 50 μM Amplex<sup>®</sup> Red reagent, 0.1 U/mL HRP, 1 U/mL glucose oxidase and the indicated amount of glucose in 50 mM sodium phosphate buffer, pH 7.4, were incubated for 37°C. Fluorescence was then measured with a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. Background fluorescence (201 arbitrary units), determined for a no-glucose control reaction, has been subtracted from each value. The inset shows the sensitivity and linearity of the assay at low levels of glucose.



**Figure 3.** Detection of glucose oxidase using the Amplex® Red Glucose/Glucose Oxidase Assay Kit (A22189). Reactions containing 50  $\mu$ M Amplex® Red reagent, 0.1 U/mL HRP, 10 mM glucose and the indicated amount of glucose oxidase in 50 mM sodium phosphate buffer, pH 7.4, were incubated for 30 minutes at 37°C. Fluorescence was measured with a fluorescence microplate reader using excitation at  $530 \pm 12.5$  nm and fluorescence detection at  $590 \pm 17.5$  nm. Background fluorescence (19 arbitrary units), determined for a no-glucose oxidase control reaction, has been subtracted from each value. The inset shows the assay's sensitivity at low levels of glucose oxidase.

## Before You Begin

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

### Amplex® Red Reagent Restrictions

Please note the following restrictions on the use of the Amplex® Red reagent. The Amplex® Red reagent is unstable in the presence of thiols such as dithiothreitol (DTT) and 2-mercaptoethanol. The final concentration of DTT or 2-mercaptoethanol in the reaction should be no higher than 10  $\mu$ M. The Amplex® Red reagent is also unstable at high pH (>8.5). Furthermore, the absorption and fluorescence of the reaction product, resorufin, are pH-dependent. Below the  $pK_a$  ( $\sim 6.0$ ), the absorption maximum shifts to  $\sim 480$  nm and the fluorescence quantum yield is markedly lower. For these reasons, the reactions should be performed at pH 7–8. The provided reaction buffer, pH 7.4, is recommended.

### Preparing the Stock Solutions

- 1.1 Prepare a 10 mM stock solution of Amplex® Red reagent.** Allow one vial of Amplex® Red reagent (Component A, blue cap) and DMSO (Component B, green cap) to warm to room temperature. Just prior to use, dissolve the contents of the vial of Amplex® Red reagent in 60  $\mu$ L of DMSO. Each vial of Amplex® Red reagent is sufficient for approximately 100 assays, with a final reaction volume of 100  $\mu$ L per assay.
- 1.2 Prepare 1X Reaction Buffer.** Add 4 mL of 5X Reaction Buffer (Component C, white cap) to 16 mL of deionized water ( $dH_2O$ ). This 20 mL volume of 1X Reaction Buffer is sufficient for approximately 100 assays of 100  $\mu$ L each with a 10 mL excess for making stock solutions.

- 1.3 Prepare a 10 U/mL stock solution of horseradish peroxidase (HRP).** Dissolve the contents of the vial of HRP (Component D, yellow cap) in 1 mL of 1X Reaction Buffer. After the assay, any remaining unused solution should be divided into single-use aliquots and stored frozen at  $\leq -20^{\circ}\text{C}$ .
- 1.4 Prepare a 100 U/mL glucose oxidase stock solution.** Dissolve the contents of the vial of glucose oxidase (Component E, orange cap) in 1.0 mL of 1X Reaction Buffer. This stock solution should be stored frozen at  $\leq -20^{\circ}\text{C}$ .
- 1.5 Prepare a 400 mM (72 mg/mL) glucose stock solution.** Weigh out a portion of glucose (Component F, black cap), and dissolve it in the appropriate amount of 1X Reaction Buffer
- 1.6 Prepare a 20 mM H<sub>2</sub>O<sub>2</sub> working solution.** Dilute the ~3% H<sub>2</sub>O<sub>2</sub> stock solution (Component G, red cap) into the appropriate volume of 1X Reaction Buffer. The actual concentration of H<sub>2</sub>O<sub>2</sub> is indicated on the label. For instance, a 20 mM H<sub>2</sub>O<sub>2</sub> working solution can be prepared from a 3.0% (0.88 M) H<sub>2</sub>O<sub>2</sub> stock solution by diluting 22.7  $\mu\text{L}$  of 3.0% H<sub>2</sub>O<sub>2</sub> into 977  $\mu\text{L}$  of 1X Reaction Buffer.

**Note:** Although the ~3% H<sub>2</sub>O<sub>2</sub> stock solution has been stabilized to slow degradation, the 20 mM H<sub>2</sub>O<sub>2</sub> working solution prepared in this step will be less stable and should be used promptly.

## Experimental Protocol

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The following procedure is designed for use with a fluorescence or absorbance microplate reader. For use with a standard fluorometer, volumes must be increased accordingly.

### Glucose Assay

The following protocol describes the assay of glucose in a total volume of 100  $\mu\text{L}$  per microplate well. The volumes recommended here are sufficient for ~100 assays. The kit provides sufficient material for ~500 assays.

- 2.1 Prepare a glucose standard curve.** Dilute the appropriate amount of the 400 mM glucose stock solution (prepared in step 1.5) into 1X Reaction Buffer to produce glucose concentrations of 0 to 200  $\mu\text{M}$ , each in a volume of 50  $\mu\text{L}$ . Be sure to include a no-glucose control. Final glucose concentrations will be twofold lower (e.g., 0 to 100  $\mu\text{M}$ ).
- 2.2 If no standard curve is to be used, prepare positive and negative controls.** For a glucose-positive control, dilute the 400 mM glucose stock solution (prepared in step 1.5) to 200  $\mu\text{M}$  in 1X Reaction Buffer. For an H<sub>2</sub>O<sub>2</sub>-positive control, dilute the 20 mM H<sub>2</sub>O<sub>2</sub> working solution (prepared in step 1.6) to 10  $\mu\text{M}$  in 1X Reaction Buffer. For a negative control, use 1X Reaction Buffer without H<sub>2</sub>O<sub>2</sub>.
- 2.3 Dilute the glucose-containing samples in 1X Reaction Buffer.** A volume of 50  $\mu\text{L}$  will be used for each reaction. A variable dilution will be required depending on the total glucose present in the sample. In the first trial the samples should be serially diluted to determine the optimal amount of sample for the assay.

**Note:** Extremely high levels of glucose (e.g., 500  $\mu\text{M}$ , final concentration) can produce lower fluorescence than moderately high levels (e.g., 100  $\mu\text{M}$ ), because excess H<sub>2</sub>O<sub>2</sub> resulting from the reaction of glucose with glucose oxidase can oxidize the reaction product, resorufin, to nonfluorescent resazurin.

- 2.4 Load the samples.** Pipet 50  $\mu\text{L}$  of the standard curve samples, controls, and experimental samples into individual wells of a microplate.

**2.5 Prepare a working solution of 100  $\mu\text{M}$  Amplex<sup>®</sup> Red reagent, 0.2 U/mL HRP and 2 U/mL glucose oxidase.** Mix the following:

- 50  $\mu\text{L}$  of 10 mM Amplex<sup>®</sup> Red reagent stock solution (prepared in step 1.1)
- 100  $\mu\text{L}$  of 10 U/mL HRP stock solution (prepared in step 1.3)
- 100  $\mu\text{L}$  of 100 U/mL glucose oxidase stock solution (prepared in step 1.4)
- 4.75 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.

**2.6 Begin the reactions.** Add 50  $\mu\text{L}$  of the Amplex<sup>®</sup> Red reagent/HRP/glucose oxidase working solution to each microplate well containing the standards, controls, and samples.

**2.7 Incubate the reactions.** Incubate at room temperature for 30 minutes, 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.

**2.8 Measure the fluorescence or absorbance.** Use a microplate reader equipped for excitation in the range of 530–560 nm and fluorescence emission detection at ~590 nm, or for absorbance at ~560 nm (see Figure 1).

**2.9 Correct for background fluorescence or absorbance.** For each point, subtract the value derived from the no-glucose control.

**Glucose Oxidase Assay**

The following protocol describes the assay of glucose oxidase in a total volume of 100  $\mu\text{L}$  per microplate well. The volumes here are sufficient for ~100 assays. The kit provides sufficient material for ~500 assays.

**3.1 Prepare a glucose oxidase standard curve.** Dilute the appropriate amount of 100 U/mL glucose oxidase stock solution (prepared in step 1.4) into 1X Reaction Buffer (prepared in step 1.2) to produce glucose oxidase concentrations of approximately 0 to 10 mU/mL glucose oxidase, each in a volume of 50  $\mu\text{L}$ . Be sure to include a no-glucose oxidase control. Please note that final glucose oxidase concentrations will be twofold lower (e.g., 0 to 5 mU/mL).

**3.2 If no standard curve is to be used, prepare positive and negative controls.** For a glucose oxidase-positive control, dilute the 100 U/mL glucose oxidase stock solution (prepared in step 1.4) to 10 mU/mL in 1X Reaction Buffer. For an  $\text{H}_2\text{O}_2$  positive control, dilute the 20 mM  $\text{H}_2\text{O}_2$  working solution to 10  $\mu\text{M}$  in 1X Reaction Buffer. For a negative control, use 1X Reaction Buffer without  $\text{H}_2\text{O}_2$ .

**3.3 Dilute the glucose oxidase-containing samples in 1X Reaction Buffer.** A volume of 50  $\mu\text{L}$  will be used for each reaction. A variable dilution will be required depending on the total glucose oxidase present in the sample. In the first trial, the samples should be serially diluted to determine the optimal amount of sample for the assay. Note that extremely high levels of glucose oxidase (e.g., 50 mU/mL, final concentration) can produce lower fluorescence than moderately high levels (e.g., 10 mU/mL), because excess  $\text{H}_2\text{O}_2$  resulting from the reaction of glucose with glucose oxidase can oxidize the reaction product, resorufin, to nonfluorescent resazurin.

**3.4 Load the samples.** Pipet 50  $\mu\text{L}$  of the standard curve samples, controls and experimental samples into individual wells of a microplate.

**3.5 Prepare a working solution of 100  $\mu$ M Amplex<sup>®</sup> Red reagent, 0.2 U/mL HRP, and 100 mM glucose.** Mix the following:

- 50  $\mu$ L of 10 mM Amplex<sup>®</sup> Red reagent stock solution (prepared in step 1.1)
- 100  $\mu$ L of 10 U/mL HRP stock solution (prepared in step 1.3)
- 1.25 mL of 400 mM glucose stock solution (prepared in step 1.5)
- 3.60 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.

**3.6 Begin the reactions.** Add 50  $\mu$ L of the Amplex<sup>®</sup> Red reagent/HRP/glucose working solution to each microplate well containing the standards, controls, and samples.

**3.7 Incubate the reactions.** Incubate at room temperature for 30 minutes, 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.

**3.8 Measure the fluorescence or absorbance.** Use a microplate reader equipped for excitation in the range of 530–560 nm and fluorescence emission detection at ~590 nm (see Figure 1), or for absorbance at ~560 nm.

**3.9 Correct for background fluorescence or absorbance.** For each point, subtract the value derived from the no–glucose oxidase control.

## References

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1. Proc SPIE-Intl Soc Opt Eng 3606, 119 (1999); 2. Anal Biochem 253, 162-168 (1997); 3. J Immunol Methods 202, 133 (1997).

## Product List

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

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Cat #	Product Name	Unit Size
A22189	Amplex <sup>®</sup> Red Glucose/Glucose Oxidase Assay Kit *500 assays* .....	1 kit
A12222	Amplex <sup>®</sup> Red reagent .....	5 mg
A22177	Amplex <sup>®</sup> Red reagent *packaged for high-throughput screening* .....	10 x 10 mg
A36006	Amplex <sup>®</sup> UltraRed reagent.....	5 x 1 mg
R363	resorufin, sodium salt *reference standard* .....	100 mg

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