Amplex® ELISA Development Kit for Rabbit IgG with Amplex® UltraRed Reagent

Catalog no. A33852

Table 1. Contents and storage information.

Material	Amount	Storage*	Stability
Amplex® UltraRed reagent (Component A)	5 vials, each containing 180 µg	• ≤–20˚C • Desiccate • Protect from light	When stored as directed, the kit components are stable for at least 6 months.
Dimethyl sulfoxide (DMSO), anhydrous (Component B)	1.75 mL		
10X Phosphate-buffered saline (PBS) pH 7.2 (Component C)	200 mL		
Goat anti–rabbit IgG (H+L), horseradish peroxidase conjugate (Component D)	2 vials, each containing 100 µg		
Amplex® stop reagent (Component E)	20 mg		
Hydrogen peroxide (H_2O_2), stabilized ~3% solution (Component F)	500 µL		
0.1 M sodium bicarbonate buffer, pH ~9.3 (Component G)	50 mL		
Bovine serum albumin (BSA) (Component H)	1.2 g		
Tween® 20 (Component I)	1.5 mL		
Nunc-Immuno™ MaxiSorp™ U96 plate (Component J)	5 each		
*The kit can be stored under the conditions listed. For optimal storage conditions of individual components, refer to the labels on the vials.			
Number of assays: Sufficient material is supplied for 500 reactions in 96-well microplates at 100 µL per well, based on the protocol below..			
Approximate fluorescence excitation and emission maxima: 568/581 nm for the reaction product.			

Introduction

The Amplex® ELISA Development Kit for Rabbit IgG provides a comprehensive set of components for creating a fluorescence-based ELISA using a rabbit primary antibody. The assay is based on Amplex® UltraRed reagent, a fluorogenic substrate for horseradish peroxidase (HRP) that reacts with hydrogen peroxide (H_2O_2) in a 1:1 stoichiometric ratio to produce Amplex® UltroxRed product, a brightly fluorescent and strongly absorbing reaction product (excitation/emission maxima ~568/581 nm) (Figure 1). Because the Amplex® UltroxRed product has long-wavelength spectra, there is little interference from the blue or

green autofluorescence found in most biological samples. With a high extinction coefficient, good quantum efficiency, and resistance to autooxidation, the fluorescence-based Amplex® UltraRed reagent delivers better sensitivity and a broader assay range than colorimetric reagents. In a sandwich ELISA format using C-reactive protein, it is possible to routinely detect 1 pg/ml of antigen (Figure 2). Using TMB (3,3',5,5'-Tetramethylbenzidine, a common colorimetric reagent) in the same sandwich ELISA format, the assay was 25-fold less sensitive.

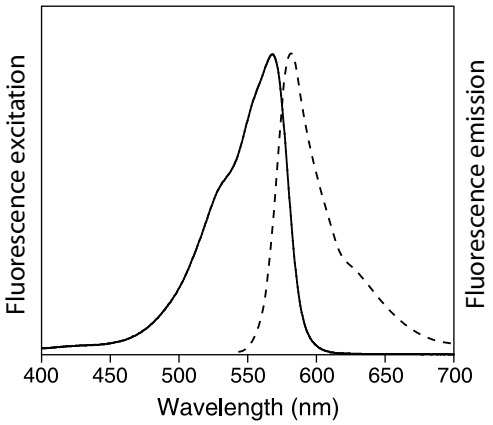

Figure 1. Normalized absorption and fluorescence emission spectra for the Amplex® UltroxRed product.

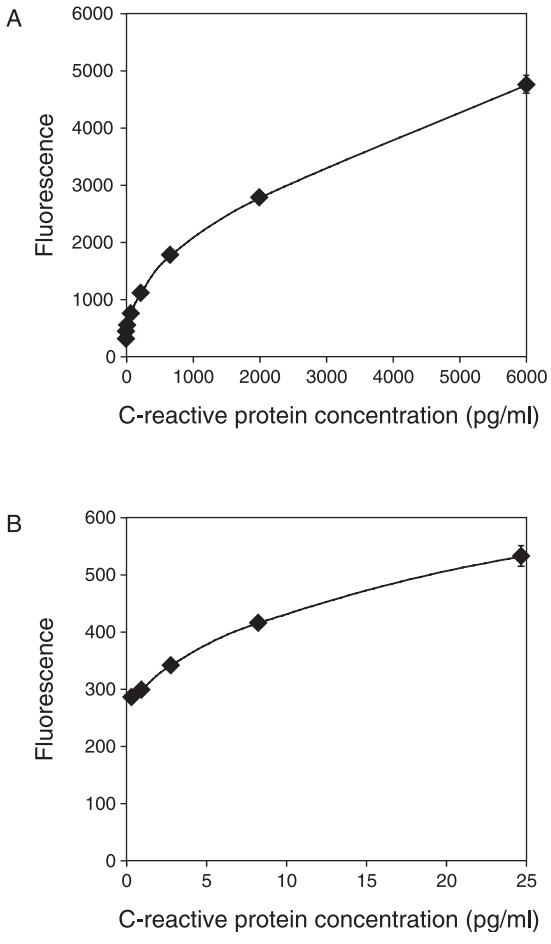

Figure 2. Detection range of C-reactive protein (CRP) using the Amplex® ELISA Development Kit for Rabbit IgG. The sandwich ELISA was carried out as described in the protocol using a mouse anti-CRP capture antibody, C-reactive protein in a concentration range from 6,000 pg/mL to 0.10 pg/mL, and a rabbit polyclonal anti-CRP primary antibody (100 µL per well of a 50 ng/mL solution). The Z' factor[1] analysis of the data obtained gives a lower limit of detection for CRP in this system of 1 pg/mL or 0.1 pg/well (based on a well volume of 100 µL in the sandwich ELISA).

Before you Begin

Materials Required but Not Provided

- Rabbit-derived antibody against target antigen
- Capture antibody against target antigen (e.g., mouse)
- Antigen (for generating a standard curve; see protocol step 2.5)
- Distilled and deionized water
- Single-channel and multichannel pipettes (1 µL to 1 mL range)
- Fluorescence microplate reader capable of excitation/emission settings of 530 nm/590 nm

Caution

No data are currently available addressing the mutagenicity or toxicity of the Amplex® UltraRed reagent (Component A).

DMSO (Component B) is hazardous; avoid contact with skin and eyes and do not swallow. Handle reagents containing DMSO using equipment and practices appropriate for the hazards posed by such materials.

Amplex® stop reagent (Component E) is irritating to eyes, respiratory system, and skin, and may be harmful if swallowed. Avoid prolonged or repeated exposure. If eye or skin contact occurs, wash affected area with water for 15 minutes and seek medical advice. If inhaled, move individual to fresh air and seek medical advice. If swallowed, seek medical advice.

Preparing Stock Solutions

Allow components warm to room temperature before opening the vials and preparing various stock solutions.

1.1 Prepare **500 mL of 1X PBS** by adding 50 mL of 10X PBS (Component C) to 450 mL distilled and deionized water. You will use this stock of 1X PBS in preparing other buffers as well as in the final Amplex® UltraRed reaction.

1.2 Prepare **300 ml of 1X PBST** by adding 300 µL of Tween® 20 (Component I) to 300 mL of 1X PBS. Shake well to mix. This solution is sufficient for 100 assays using the protocol below. Storage at 4˚C is not required, but will not harm this solution.

1.3 Prepare **100 mL of 1X PBS-BSA** by adding 1 g of BSA (Component H) to 100 mL of 1X PBS. Dissolve completely. Store at 4˚C when not in use.

1.4 Prepare a **200 µg/mL stock solution of the goat anti–rabbit IgG HRP conjugate** (Component D) by adding 0.5 mL of PBS-BSA directly to the vial. Store this stock solution at 4˚C after adding thimerosal to a final concentration of 0.02%.

1.5 Prepare a **~10 mM stock solution of Amplex® UltraRed** by adding 60 µL of DMSO (Component B) to one vial of Amplex® UltraRed reagent (Component A). Vortex well to dissolve. Protect from light and moisture.

1.6 Prepare **10 mL of Amplex® stop solution** by resuspending the dried Amplex® stop reagent (Component E) in 1 mL of 1 M NaOH, and once resuspended, adding it to 9 mL of 1X PBS. This solution is stable for one month at 4˚C when protected from light. If this solution begins to turn amber, it is no longer good and should be discarded.

The following procedure describes a typical sandwich ELISA, and is designed for use with a fluorescence microplate reader. The procedure has been optimized for use with 96-well microplates and reaction volumes of 100 µL per assay.

You may replace the protocol with any standard sandwich ELISA protocol at your discretion; however, you must follow steps 2.8–2.14 of this protocol as described.

2.1 Using 0.1 M sodium bicarbonate (Component G), prepare 10 mL of a 10 µg/ml solution of the desired capture antibody and aliquot 100 µL of this solution into each microplate well. You can use any capture antibody but it must **not** cross-react with the goat anti–rabbit IgG or the rabbit anti-target antibodies used for detection.

Incubate at room temperature for at least four hours, or overnight at 4°C (preferred).

Note: Overnight deposition of capture antibody provides optimal detection. If desired, you can incubate the plate at room temperature for 8 hours, and then block with 1X PBS-BSA overnight at 4°C.

2.2 Discard or shake contents of the plate into the sink, and wash the wells three times with 200 µL of 1X PBST (prepared in step 1.2).

2.3 Add 200 µL of 1X PBS-BSA (prepared in step 1.3) to each well of the microplate, and incubate at room temperature for at least four hours, or overnight at 4°C (preferred).

2.4 Shake plate contents into the sink, and wash the wells three times with 200 µL of 1X PBST.

2.5 Add 100 µL of 0.1X PBS-BSA (prepared by diluting the 1X PBS-BSA ten-fold in 1X PBS) to each well of the microplate. Add antigen to the first well of each row and serially dilute across the plate to achieve the desired range of concentrations. Leave the last well of the row as a no-antigen control. Incubate the plate at room temperature for one hour.

Example: Prepare 150 µL of 60 ng/mL antigen in the first well and serially dilute 50 µL into each successive well in that row to make a three-fold dilution series ranging from 6 ng to 0.1 pg antigen.

2.6 Following incubation, shake plate contents into the sink and wash the wells three times with 200 µL of 1X PBST.

2.7 Prepare 10 mL of 50 ng/mL secondary capture antibody in 0.1X PBS-BSA and add 100 µL to each well of the microplate. Incubate at room temperature for 30 minutes.

2.8 Shake plate contents into the sink, and wash the wells three or more times with 200 µL of 1X PBST.

2.9 Prepare 10 mL of 50 ng/mL goat anti–rabbit IgG HRP by adding 2.5 µL of the stock goat anti–rabbit IgG HRP solution (prepared in step 1.4) to 10 mL of 0.1X PBS-BSA. Add 100 µL of this solution to each well of the microplate and incubate at room temperature for 30 minutes.

2.10 Shake plate contents into the sink, and wash the wells three times with 200 µL of 1X PBST. You may adjust the stringency of the assay by washing more or fewer times with PBST, or by incubating or agitating PBST in the wells for a time during the wash steps. **Protect the plate from light at all times from this point onward.**

Note: Antibodies exposed to UV light can produce trace amounts of singlet oxygen, which can interfere with detection of Amplex® UltraRed reagent. Protecting the plate from light after the final wash provides optimal sensitivity for the assay.

2.11 Prepare 10 mL of reaction mixture by adding 50 µL of the 10 mM Amplex® UltraRed stock solution (prepared in step 1.5) and 22.7 µL of 3% H_2O_2 (Component F) to 10 mL of 1X PBS. If necessary, adjust the volume for the actual hydrogen peroxide concentration (check the label on Component F for actual H_2O_2 concentration). Protect the reaction mixture from light and use within 4 hours or preparation.

2.12 Using a multichannel pipette, add 100 µL of the reaction mixture to each assay well.

Note: Adding the reaction mixture (containing Amplex® UltraRed reagent) to the wells initiates the reaction.

2.13 Incubate the plate at room temperature, protected from light, until the fluorescence measurement is taken. For most reactions, a 30 minute incubation is sufficient. The plate can also be read continuously for up to an hour.

If desired, you may add 20 µL of Amplex® stop solution (prepared in step 1.6) to each assay well. This will arrest the reaction, providing a stable signal that may be read for at least two hours if the plate is protected from light and kept at room temperature.

2.14 Measure the fluorescence in a microplate reader using filters for 530 nm (excitation) and 590 nm (emission)

Reference

1. J Biomol Screen 4, 67 (1999).

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

Cat. no.	Product Name	Unit Size
A33852	Amplex® ELISA Development Kit for Rabbit IgG *with Amplex® UltraRed reagent* *500 assays*	1 kit
Related Products		
A33851	Amplex® ELISA Development Kit for Mouse IgG *with Amplex® UltraRed reagent* *500 assays*	1 kit
A33855	Amplex® Red/UltraRed stop reagent *500 tests*	set of 5 vials
A36006	Amplex® UltraRed reagent	5 × 1 mg

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