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

Catalog no. A33851

Table 1. Contents and storage information.

Material	Amount	Storage*	Stability
Amplex® UltraRed Reagent (Component A)	5 × 180 µg	• ≤–20˚C • Desiccate • Protect from light	When stored as directed this kit is 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-mouse IgG (H+L), horseradish peroxidase (HRP) conjugate (Component D)	2 × 100 µg		
Amplex® Red/UltraRed stop reagent (Component E)	25 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		
Approximate fluorescence excitation/emission maxima: Amplex® UltroxRed product**:** 568/581 in nm.			
Recommended instrument settings: 490–550 nm/580–590 nm.			

Introduction

The Amplex® ELISA Development Kit for Mouse IgG provides a comprehensive set of components for creating a fluorescence-based ELISA using a mouse primary antibody. The assay is based on Amplex® UltraRed reagent, a fluorogenic substrate for horseradish peroxidase (HRP) that reacts with 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, we routinely detect 75 pg of antigen (Figure 2). Using TMB (a common colorimetric reagent) in the same sandwich ELISA format, the assay was 25-fold less sensitive.

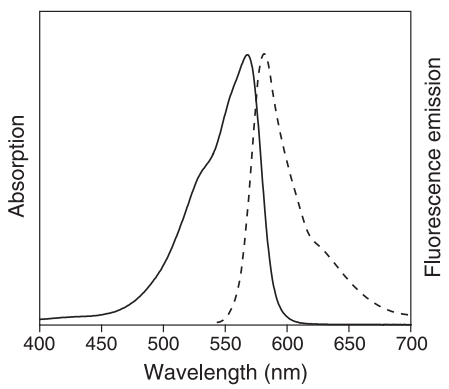

Figure 1. Normalized absorption and fluorescence emission spectra for the Amplex® UltroxRed product.

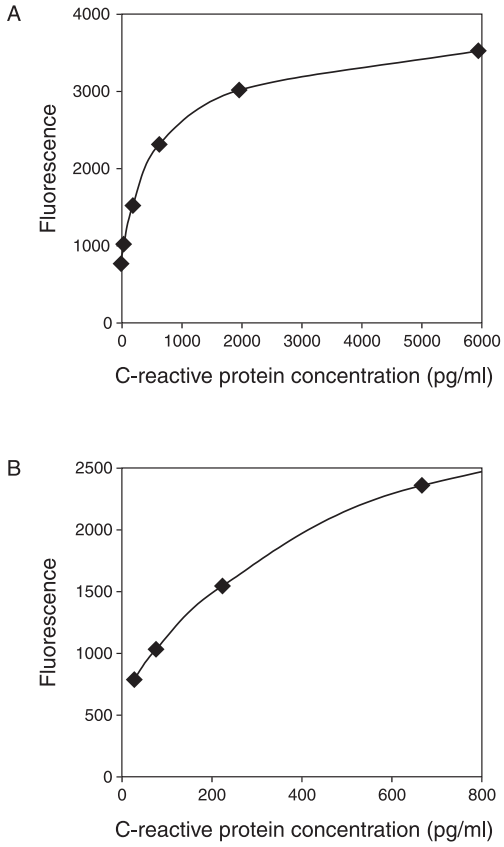

Figure 2. Detection range of C-reactive protein (CRP) using the Amplex® ELISA Development Kit for Mouse IgG. The sandwich ELISA was carried out as described in the protocol using a rabbit anti-CRP capture antibody, C-reactive protein in a concentration range from 6000 pg/mL to 0.10 pg/mL, and a mouse monoclonal 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 75 pg/mL or 7.5 pg/well (based on a well volume of 100 µL in the sandwich ELISA).

Before Starting

Materials Required but Not Provided

- Mouse-derived antibody against target antigen
- Capture antibody against target antigen (e.g., rabbit)
- Antigen (for generating a standard curve – see protocol step 2.6)
- Single-channel and multichannel pipets (1 µL to 1 mL range)
- Fluorescence microplate reader equipped with filters for 530 nm excitation and 590 nm emission (recommended instrument settings are 490–550 nm/580–590 nm)

Caution

DMSO (Component B), provided as a solvent in this kit, is known to facilitate entry of organic molecules into tissues. Handle reagents containing DMSO using equipment and practices appropriate for the hazards posed by such materials. Dispose of the reagents in compliance with all pertaining local regulations.

Experimental Protocols

Preparing Stock Solutions

1.1 Prepare 500 mL 1X PBS by adding 50 mL 10X PBS (Component C) to 450 mL distilled and deionized water. This stock of 1X PBS is used to prepare other buffers as well as in the final Amplex® UltraRed reaction.

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

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

1.4 Reconstitute the goat anti–mouse IgG HRP conjugate (Component D) by adding 0.5 mL PBS-BSA directly to a vial, to create a 200 µg/mL stock solution. 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 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 Amplex® Red/UltraRed stop solution by resuspending the dried Amplex® stop reagent (Component E) with 1 mL 1 M NaOH, and once resuspended, adding it to 9 mL 1X PBS. This solution is stable for one month at 4°C when protected from light. If this solution begins to turn amber, discard the solution.

Assay Protocol

The following protocol describes a typical sandwich ELISA designed for use with a fluorescence microplate reader in a 96-well format.

This protocol is supplied as a convenience and may be replaced with any standard sandwich ELISA protocol at your discretion. However, follow steps 2.11–2.18 of this protocol.

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.

Note: Any capture antibody can be used, but it must not cross-react with the goat anti–mouse IgG or the mouse anti-target antibodies used for detection.

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

Note: Overnight deposition of capture antibody provides optimal detection. If desired, 8 hours of room-temperature deposition can be followed by overnight blocking of the plate with 1X PBS-BSA at 4°C.

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

2.4 To each well of the microplate, add 200 µL 1X PBS-BSA (prepared in step 1.3). Incubate the plate for at least four hours at room temperature, or overnight at 4°C (preferred).

2.5 Discard plate contents into the sink and wash wells three times with 200 µL 1X PBST.

2.6 Add 100 µL 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.

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.

2.7 Incubate the plate for one hour at room temperature.

2.8 Discard plate contents into the sink and wash wells three times with 200 µL 1X PBST.

2.9 Prepare 10 mL 50 ng/mL secondary capture antibody in 0.1X PBS-BSA and add 100 µL to each well of the microplate.

2.10 Incubate the plate for 30 minutes at room temperature.

2.11 Discard plate contents into the sink and wash wells three or more times with 200 µL 1X PBST.

2.12 Prepare 10 mL 50 ng/mL goat anti–mouse IgG HRP by adding 2.5 µL of the stock goat anti–mouse IgG HRP solution (prepared in step 1.4) to 10 mL 0.1X PBS-BSA. Add 100 µL of this solution to each well of the microplate.

2.13 Incubate the plate for 30 minutes at room temperature.

2.14 Discard plate contents into the sink and wash wells three times with 200 µL 1X PBST. Protect plate from light at all times from this point onward.

Note: The stringency of the assay may be adjusted by washing more or fewer times with PBST, or by incubating or agitating PBST in the wells for a time during the wash steps. 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.

2.15 Prepare 10 mL of reaction mixture by adding 50 µL of the Amplex® UltraRed stock solution and 22.7 µL of 3% H_2O_2 (adjusting if necessary for the actual hydrogen peroxide concentration, check the label on Component F for actual H_2O_2 concentration) to 10 mL 1X PBS. Protect the reaction mixture from light and use within 4 hours.

2.16 Using a multichannel pipet, 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.

2.17 Incubate the plate at room temperature **protected from light** (whenever possible) 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 or longer, if needed.

If desired, 20 µL of Amplex® Red/UltraRed stop solution (prepared in step 1.6) may be added 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.

Note: It is very important to add the stop solution to reagent controls to take into account the ~17% dilution of the samples by the addition of the stop solution, and also to compensate for the fluorescence quenching effect (typically < 5%) of the stop solution on the Amplex® UltraRed reagent oxidation products.

2.18 Read the microplate wells using filters for 530 nm (excitation) and 590 nm (emission).

Note: Optimal wavelength settings may vary slightly between instruments. If excitation at 530 nm results in signal saturation when the emission is read at 590 nm, you may lower the excitation wavelength to 490–525 nm.

Reference

1. J Biomol Screen 4, 67–73 (1999).

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

Cat. no.	Product Name	Unit Size
A33851	Amplex® ELISA Development Kit for Mouse IgG *with Amplex® UltraRed reagent* *500 assays*	1 kit
Related Product		
A33852	Amplex® ELISA Development Kit for Rabbit IgG *with Amplex® UltraRed reagent* *500 assays*	1 kit

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