

Fc OxyBURST[®] Assay Reagents

F-2902 Fc OxyBURST[®] Green assay reagent
O-13291 OxyBURST[®] H₂HFF Green BSA

Quick Facts

Storage upon receipt:

F-2902

- 4°C
- Do not freeze
- Protect from light
- Protect from air

O-13291

- -20°C
- Desiccate
- Protect from light
- Protect from air

Introduction

When soluble or surface-bound IgG immune complexes interact with Fc receptors on phagocytic cells, a number of host defense mechanisms are activated. These include phagocytosis and activation of an NADPH oxidase-mediated oxidative burst. Dichlorodihydrofluorescein diacetate (H₂DCFDA, D-399), a cell-permeant fluorogenic probe that localizes in the cytosol, has frequently been used to monitor this oxidative burst;¹ however, its fluorescence response is limited by the diffusion rate of the reactive oxygen species from the phagovacuole into the cytosol.

Molecular Probes' Fc OxyBURST Green reagent (F-2902) was developed in collaboration with Elizabeth Simons of Boston University to permit measurement of the kinetics of Fc receptor-mediated internalization and the subsequent oxidative burst directly in the phagovacuole.² The Fc OxyBURST Green assay reagent consists of bovine serum albumin (BSA) that has been covalently linked to dichlorodihydrofluorescein (H₂DCF) and then complexed with purified rabbit polyclonal anti-BSA IgG antibodies. When these immune complexes bind to Fc receptors, the nonfluorescent H₂DCF molecules are internalized within the phagovacuole and subsequently oxidized to green fluorescent dichlorofluorescein (DCF). Fc OxyBURST Green assay reagent has been found to produce >8 times more fluorescence than does H₂DCFDA at 60 seconds and >20 times more at 15 minutes following internalization of the immune complex (Figure 1). Furthermore, unlike H₂DCFDA, the Fc OxyBURST Green assay reagent does not require intracellular esterases for activation, making this reagent particularly suitable for detecting the oxidative burst in cells with low esterase activity such as monocytes.³

Several reports have described the use of the Fc OxyBURST Green assay reagent to study the oxidative burst in phagovacuoles. Neutrophils from patients with chronic granulomatous disease, a genetic deficiency known to disable NADPH oxidase-mediated oxidative bursts, were observed to bind but not oxidize the Fc OxyBURST Green assay reagent² (Figure 2). Using microfluorometry to detect the Fc OxyBURST Green response, researchers were able to simultaneously monitor oxidative activity and membrane currents in voltage-clamped human mononuclear cells.⁴ The Fc OxyBURST Green assay reagent has also been employed to assess the effect of manganese-based superoxide dismutase mimetics on superoxide generation in human neutrophils.⁵

We also offer OxyBURST Green H₂HFF BSA (O-13291), a sensitive fluorogenic reagent for detecting extracellular release of oxidative products in a spectrofluorometer or a fluorescence microscope. OxyBURST Green H₂HFF BSA consists of bovine serum albumin coupled to dihydro-2',4,5,6,7,7'-hexafluorofluorescein (H₂HFF), a new reduced dye with improved stability. This reagent provides up to 1000 times greater sensitivity than conventional methods based on spectrophotometric detection of superoxide dismutase-inhibitable reduction of cytochrome c^{6,7} and allows researchers to take advantage of the sample stirring and thermostating capabilities available in many spectrofluorometers. Because OxyBURST Green H₂HFF BSA is a protein conjugate, it is supe-

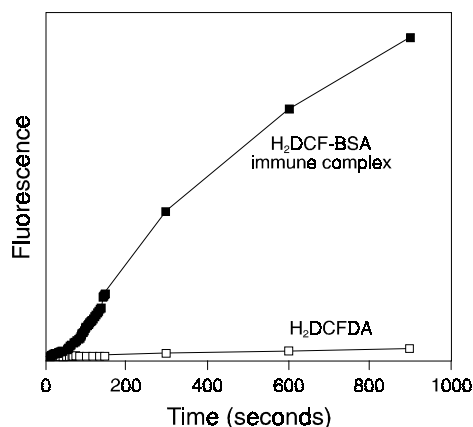


Figure 1. Fluorescence emission of human neutrophils challenged either with Molecular Probes' Fc OxyBURST Green assay reagent (H₂DCF BSA immune complexes, F-2902) or with unlabeled immune complexes in the presence of dichlorodihydrofluorescein diacetate (H₂DCFDA, D-399). The Fc OxyBURST Green assay reagent generates significantly more fluorescence than does the more commonly used H₂DCFDA. Data provided by Elizabeth Simons, Boston University [*J Immunol Methods* 130, 223 (1990)].

rior to low molecular weight probes such as dihydrotetramethylrosamine and dihydrorhodamine 123, which are cell permeant and therefore do not exclusively detect extracellular oxidants.

Materials

Contents

The Fc OxyBURST Green assay reagent is provided in a unit size of 500 μ L, as a concentrated suspension of 3.0 mg protein per mL in phosphate-buffered saline (PBS) containing 2 mM sodium azide, and packaged under argon. Once diluted, the azide does not appear to interfere with cellular oxidation. Sufficient immune complex (IC) is provided for ~25 flow cytometric tests assuming a volume of 0.5 mL per test at a subsaturating concentration ratio of 120 μ g ICs/mL to 2.0×10^6 cells/mL.

OxyBURST Green H₂HFF BSA is supplied in lyophilized form, packaged under argon in five vials, each containing 1 mg.

Storage

The Fc OxyBURST assay reagents should be stored refrigerated at 4°C and protected from light. DO NOT FREEZE. Keep in a tightly capped container and, if possible, under nitrogen or argon gas to avoid oxidation prior to use. During prolonged storage there may be some reduction in the maximum fluorescence signal.

Store OxyBURST Green H₂HFF BSA frozen (-20°C), desiccated and protected from light, under argon or nitrogen in a sealed container.

Stability

The Fc OxyBURST IC is slowly oxidized by molecular oxygen and is also susceptible to oxidation catalyzed by illumination in a fluorescence microscope. When stored as described above (see *Storage*), the Fc OxyBURST Green assay reagent is stable in concentrated form for at least six months. The reduced Fc OxyBURST Green assay reagent is essentially colorless and nonfluorescent in solution; storage under the above-specified conditions should produce no visible color changes in the sample. The oxidized reagent is highly colored and fluoresces bright green. A visibly distinct color change after prolonged storage is indicative of deterioration. Note, however, that a small amount of oxidized substrate (typically about 5% of the total oxidizable dye) is normal and acceptable in the conjugates when they are shipped. This “pre-oxidized” substrate is useful for measuring receptor binding at 4°C (see steps 4.1–4.3). Dilute aqueous substrate solutions that have been left on the laboratory bench at room temperature without azide should be discarded at the end of the work day.

OxyBURST Green H₂HFF BSA, when stored as described above is stable for at least six months.

Materials Required but Not Provided

- Sterile, tissue-culture grade, phosphate-buffered saline (PBS) for diluting the ICs (e.g. pyrogen-free, endotoxin-free, calcium-free and magnesium-free PBS; available from any company that sells tissue-culture media).
- PBS/glucose buffer, composed of 125 mM NaCl, 8 mM Na₂HPO₄, 2 mM NaH₂PO₄, 5 mM KCl and 5 mM glucose, pH 7.4, for storing cells prior to an experiment.
- Sterile, pyrogen-free and endotoxin-free Krebs’ Ringer’s PBS (KRP buffer), which contains phosphate-buffered saline, pH 7.4 with 1.0 mM Ca²⁺, 1.5 mM Mg²⁺ and 5.5 mM glucose, the final medium for cells exposed to ICs.

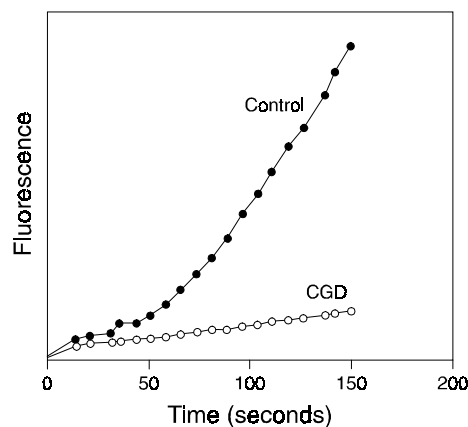


Figure 2. Oxidative bursts of human neutrophils from a healthy donor (control) compared to those from a patient with chronic granulomatous disease (CGD), as detected using the Fc OxyBURST Green assay reagent (F-2902). Data provided by Elizabeth Simons, Boston University [*J Immunol Methods* 130, 223 (1990)].

Fc OxyBURST Characteristics

The labeled antigen consists of BSA covalently bound to a reduced dye, H₂DCF. Unbound dye is removed from the preparation using chromatographic techniques. The anti-BSA antibodies are rabbit polyclonal IgG obtained from an IgG fraction of antisera pooled from different rabbits, which has been purified by ammonium sulfate precipitation and ion-exchange chromatography. Further purification also occurs during preparation of the insoluble ICs; non-specific IgG antibodies are removed during washing of the ICs. The insoluble complexes of antibody and antigen–substrate are formed at the equivalence point on their immunoprecipitation curve. At the equivalence point, the molar ratio of IgG to BSA–H₂DCF is 4:1; on a weight basis, the ratio is 9:1. The concentrated insoluble ICs are formed in tissue-culture grade PBS, pH 7.3 \pm 0.1, without added calcium, magnesium, glucose or pH indicator dye. Sodium azide at 2 mM has been added as a preservative.

Fc OxyBURST Flow Cytometry Assay Procedure

The flow cytometry protocol and data presented here are derived from the research of Dr. Elizabeth Simons and her colleagues.²

Preparation of Immune Complexes

1.1 Vortex the suspension of insoluble ICs to make as uniform a suspension as possible. Avoid forming air bubbles, which can denature proteins.

1.2 Make the dilutions of the ICs with PBS buffer (lacking azide). Selecting the final concentrations of ICs depends on many variables, including the cell type, the number of Fc receptors/cell, the incubation conditions and the event that the researcher wants to study. For example, it has been reported that the oxidative burst of human polymorphonuclear leukocytes produced about a half maximal fluorescence signal when 2×10^6 cells/mL were stimulated with insoluble ICs at a concentration of 120 μ g/mL.² Maximal activation was elicited with an IC concentration of 360 μ g/mL.

Preparation of Cells

2.1 This Fc OxyBURST assay is appropriate for cells containing Fc receptors and an oxidative burst, such as neutrophils and monocytes.

2.2 If necessary, isolate and purify the cells and determine their concentration (cells/mL).

2.3 The cells should be suspended in cold (4°C), glucose-containing, calcium- and magnesium-free PBS and kept on ice until use.

2.4 Shortly before use, adjust the final concentration to 2×10^6 cells/mL by diluting into KRP buffer.

2.5 Pre-warm (37°C) the cells for uptake and oxidative burst studies or pre-cool (4°C) the cells for receptor-binding without uptake (see steps 4.1 to 5.5). **Do not pre-warm the ICs, as this will cause them to clump.**

Flow Cytometry Preparation

3.1 Select the optical filters that are appropriate for the fluorogenic substrate. The Fc OxyBURST Green assay reagent produces fluorescence similar to fluorescein, with excitation and emission maxima of ~490 nm and ~520 nm, respectively.

3.2 Calibrate the flow cytometer following the manufacturer's recommendations.

3.3 Measure the intrinsic autofluorescence of the cells without the ICs. In published measurements with resting human neutrophils, cellular autofluorescence was low and did not interfere with the assay.²

3.4 If available, set up a mixing chamber for applying samples to the flow cytometer. The mixing chamber is recommended, but not required. Continuous stirring during the application of the sample to the flow cytometer produces a more uniformly distributed suspension of ICs; cells are challenged more uniformly and a higher fluorescence signal is achieved sooner.

Detecting Receptor Binding Without Uptake

At 4°C, where phagocytosis and the subsequent oxidative bursts are blocked, the small amount of substrate present in the Fc OxyBURST Green reagent can be used to demonstrate IC binding to Fc receptors. Inaccuracies will arise, however, if the reduced substrate is allowed to oxidize prior to assay. Inaccuracies can also arise if the cellular sample has been significantly pre-activated before measurement.

4.1 Separately, pre-cool the suspension of ICs and the cells in KRP buffer (from step 2.4) to 4°C.

4.2 Add the desired amount of ICs, in a relatively small volume, to the pre-cooled suspension of cells.

4.3 Collect time-sequential fluorescence data (e.g. immediately and again every 5 seconds for a minimum of 2.5 minutes).

Receptor Binding, Uptake and Oxidative Burst

5.1 Warm all sterile experimental solutions to 37°C for 15–20 minutes.

5.2 Warm the cells in KRP buffer (from step 2.4) to 37°C (10–20 minutes depending on volume).

5.3 Take initial baseline readings.

5.4 Add the desired amount of ICs, in a relatively small volume, to the pre-warmed suspension of cells.

5.5 Collect time-sequential fluorescence data (e.g., immediately and again every 5 seconds for a minimum of 2.5 minutes).

OxyBURST Green H₂HFF BSA Assay Procedure

Stock Solution

Prepare a 1 mg/mL stock solution of OxyBURST Green H₂HFF BSA by dissolving the contents of one vial in 1.0 mL PBS. This stock solution should be stored at 4°C, protected from light and, if possible, under nitrogen or argon. The addition of 2 mM sodium azide is recommended.

Fluorometric Assay

In a typical assay, neutrophils (2×10^6 cells/mL) are incubated in KRP in the presence of 10 µg/mL OxyBURST H₂HFF Green BSA in a fluorescence cuvette for 2 minutes at 37°C and then stimulated by addition of the chemotactic peptide fMLP in the presence of cytochalasin B, or by phorbol myristate acetate (PMA). Oxidative product (primarily H₂O₂) release in the stirred thermostatted sample is detected by a continuous fluorescence increase excited at 488 nm and detected at 530 nm over a period of about 2 minutes. The rate of fluorescence increase is proportional to the amount of oxidative species generated. Neutrophils in which the oxidative burst response is impaired (e.g. by chelation of intracellular Ca²⁺) show a negligible fluorescence increase over the same period of time.

References

1. J Immunol 130, 1910 (1983); 2. J Immunol Methods 130, 223 (1990); 3. J Leukoc Biol 43, 304 (1988); 4. J Biol Chem 270, 8328 (1995); 5. J Biol Chem 269, 18535 (1994); 6. J Clin Invest 61, 1081 (1978); 7. J Biol Chem 255, 1874 (1980).

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

Cat #	Product Name	Unit Size
F-2902	Fc OxyBURST® Green assay reagent *25 assays* *3 mg/mL*	500 µL
O-13291	OxyBURST® Green H ₂ HFF BSA *special packaging*	5 x 1 mg

Contact Information

Further information on Molecular Probes' products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

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