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Amine-Reactive OxyBURST® Reagents

D-2935 OxyBURST® Green H_2DCFDA, succinimidyl ester
H-13292 OxyBURST® Green H_2HFFDA, succinimidyl ester

Quick Facts

Storage upon receipt:

- –20°F
- Protect from light
- Protect from air

Abs/Em of oxidation product:

- 495/527 nm (D-2935)
- 508/529 nm (H-13292)

Introduction

Molecular Probes offers amine-reactive OxyBURST® Green H_2DCFDA and OxyBURST Green H_2HFFDA succinimidyl esters, which can be used to prepare oxidation-sensitive conjugates of a wide variety of biomolecules and particles, including antibodies, antigens, peptides, proteins, dextrans, bacteria, yeast and polystyrene microspheres.

Our original amine-reactive OxyBURST Green reagent, the succinimidyl ester of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), is a chemically reduced and acetylated form of the highly fluorescent dye 2′,7′-dichlorofluorescein (DCF). Following conjugation to amines, the acetates of this reagent can be removed by treatment with hydroxylamine to yield the H_2DCF conjugate. Because the H_2DCF moiety is nonfluorescent until oxidized to dichlorofluorescein, conjugates of H_2DCF provide a means of detecting the oxidative burst in phagocytic cells.[1-3]

Molecular Probes' other amine-reactive OxyBURST Green reagent, the succinimidyl ester of 2′,4,5,6,7,7′-hexafluorofluorescein diacetate (H_2HFFDA), yields conjugates that have lower background fluorescence and are more stable than conjugates of the OxyBURST Green H_2DCFDA reagent. Like conjugates of OxyBURST Green H_2DCFDA, conjugates of OxyBURST Green H_2HFFDA require removal of the acetate groups by treatment with hydroxylamine before being used as a probe for oxidative burst. Oxidation of the conjugates can be detected by monitoring the increase in fluorescence with a flow cytometer, fluorometer or fluorescence microscope using wavelengths or filters similar to those used with fluorescein (FITC). In fluorescence microscopy, because the dyes are susceptible to photooxidation, low light conditions should be used whenever possible.

Materials

OxyBURST Green H_2DCFDA, succinimidyl ester (D-2935) has a molecular weight of 584.4, and OxyBURST Green H_2HFFDA, succinimidyl ester (H-13292) has a molecular weight of 623.4. The reagents are provided as solids in unit sizes of 5 mg.

Upon receipt, store the unopened products at -20°C. These products are air sensitive and packed under argon. Do not dissolve until immediately prior to use. PROTECT FROM LIGHT.

Experimental Protocol

The following procedure was designed for the conjugation of bovine serum albumin (MW = 66,000) and should be modified based on the application and sensitivity required. Of particular importance for determining the optimal fluorescence signal intensity is the molar ratio of amine-reactive reagent to protein.

Materials Required but Not Provided

- 0.1 M sodium bicarbonate buffer (pH ~8.3) or any suitable buffer for your protein that does not contain amines or thiols (e.g., do not use buffers containing Tris, glycine, β-mercaptoethanol or dithiothreitol). A pH range of 7.5–8.5 is optimal for reaction of the succinimidyl ester.
- High quality, anhydrous dimethylformamide (DMF) or dimethylsulfoxide (DMSO).
- 1.5 M hydroxylamine, pH 8.5. This reagent should be freshly prepared before use (step 1.8).
- Gel filtration column (Sephadex® G-25 or equivalent) equilibrated with the buffer of your choice. A column of approximately 10 mm × 40 mm is required for 10 mg of protein.

Protein Conjugation

1.1 Calculate the amount of amine-reactive OxyBURST reagent necessary for labeling the protein of interest. Generally, ratios of 10–20 moles of reactive dye per mole of protein are optimal, although large proteins may require higher molar ratios and very small proteins may require lower molar ratios. If possible, we recommend doing the conjugation with several molar ratios of dye to protein to ensure that an optimal degree of labeling is achieved.

1.2 Dissolve ~10 mg of protein in 1 mL of 0.1 M sodium bicarbonate, pH 8.3, or buffer of choice (see above). The protein concentration in the reaction should be 5–20 mg/mL. Concen-

trations below 5 mg/mL may require a higher molar ratio of amine-reactive reagent to protein.

1.3 To minimize oxidation of the OxyBURST reagent during the reaction, flood the protein sample tube with argon and then cap (note **A**).

1.4 Immediately before use, dissolve some of the amine-reactive OxyBURST reagent in DMF or DMSO at a concentration of 10 mg/mL. Briefly sonicate in an ultrasonic water bath or vortex. To avoid hydrolysis of the succinimidyl ester or oxidation, *do not store the amine-reactive OxyBURST reagent in solution for more than a few minutes*.

1.5 While stirring or gently shaking the protein solution, slowly add the required amount of amine-reactive OxyBURST reagent. Immediately cap the tube.

1.6 Incubate the reaction, protected from light, for 1 hour at room temperature with continuous stirring or gentle shaking.

1.7 If any precipitate is present after the incubation period, centrifuge the sample at 1000 × g for 10 minutes and transfer the supernatant to a new tube. Flood this tube with argon and cap.

1.8 To hydrolyze the acetyl groups, add 0.1 mL of 1.5 M hydroxylamine, pH 8.5, to the reaction mixture. Incubate for 1 hour at room temperature with stirring.

1.9 Separate the conjugate from the unreacted reagent and hydroxylamine on a gel filtration column equilibrated with PBS or buffer of choice. The column and eluted fractions should be protected from light. Protein conjugate–containing fractions can be identified by measuring the absorbance at 280 nm. The conjugate-containing fractions should be pooled into a container and stored, protected from light, under argon or nitrogen at 4°C. Alternatively, the conjugate can be lyophilized and stored under argon or nitrogen at -20°C to further minimize oxidation. Pronounced increase in color during storage is indicative of oxidation, and the product may no longer be suitable for use. Stability of the conjugate varies for the type of protein and the storage conditions.

Determining the Degree of Labeling

2.1 It is preferable to determine the concentration of the protein–dye conjugate using a standard protein quantitation assay. The Lowry method is usually a good choice because the test can be performed using an absorbance wavelength of 750 nm, where conjugates of the OxyBURST reagents do not appreciably absorb. Alternatively, an approximate value for the protein concentration can be estimated from the initial amount of protein used, minus 10–20% to account for loss during the conjugation and purification procedures.

2.2 To measure the amount of dye in the conjugate, oxidize a known amount of conjugate with a horseradish peroxidase/H_2O_2 system and then measure the absorbance of the solution (note **B**). Once oxidized, the amount of dye present can be determined using the extinction coefficients found in Table 1. The average degree of substitution can be estimated by using the following formula:

$$\frac{A}{\epsilon} \times \frac{\text{MW protein}}{\text{mg protein/mL}} = \frac{\text{moles dye}}{\text{moles protein}}$$

where A is the absorbance value of the oxidized dye at the absorption maximum wavelength andϵ is the molar extinction coefficient of the oxidized dye at the absorption maximum.

Table 1. *Spectral properties of oxidized OxyBURST reagent–protein conjugates.*

Oxidized OxyBURST Reagent	Abs * (nm)	Em * (nm)	ϵ ($cm^{-1}M^{-1}$) † pH 8.0
2′,7′-dichlorofluorescein (DCF)	495	527	~68,000
2′,4,5,6,7,7′-hexafluorofluorescein (HFF)	508	527	~70,000

* Absorption (Abs) and fluorescence emission (Em) maxima. † Molar extinction coefficient determined at pH 8.0.

Adaptation for Conjugation of Other Materials

For conjugation to substrates other than proteins, e.g. water insoluble biopolymers, polystyrene microspheres, bacteria or yeast, the procedure described above can be used with minor modifications. We recommend starting with 1 mg of amine-reactive OxyBURST Green reagent to 10 mg of substrate. This ratio will likely need to be optimized for the specific application. In addition, removal of unreacted reagent (step 1.9) should be accomplished by multiple washes in which the substrate is pelleted by centrifugation rather than removal by column chromatography.

Notes

[**A**] The OxyBURST Green reagent conjugates are subject to oxidation by air, which yields a pronounced increase in color and fluorescence. A small amount of oxidation typically occurs during the conjugation and purification procedures, but can be minimized by working under an inert gas such as argon. Although nitrogen can be used, argon is preferred due to its higher density.

[B] For instance, dilute the conjugate with pH 6.0 buffer to make 1.5 mL of a 1–2 mg/mL solution. Measure the absorbance at the absorbance maximum listed in Table 1. Add 20 µL of 50 mg/mL urea hydrogen peroxide and 20 µL of 10 mg/mL horseradish peroxidase. Read the absorbance again once the value has stabilized.

References

1. J Immunol Methods 130, 223 (1990); **2.** J Cell Phys 1556, 428 (1993); **3.** Immunology 83, 507 (1994).

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

Cat #	Product Name	Unit Size
D-2935	2′,7′-dichlorodihydrofluorescein diacetate, succinimidyl ester (OxyBURST® Green H_2DCFDA, SE)	5 mg
H-13292	2′,4,5,6,7,7′-hexafluorodihydrofluorescein diacetate, succinimidyl ester (OxyBURST® Green H_2HFFDA, SE)	5 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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