Image-iT™ LIVE Green Reactive Oxygen Species Detection Kit (I36007)

Quick Facts

Storage upon receipt:

- 2–6ºC
- Do not freeze
- Desiccate
- Protect from light

Ex/Em:

Carboxy-H_2DCFDA	495/529 nm
Hoechst 33342	350/461 nm

Note: Carboxy-H_2DCFDA is air sensitive; activity of this reagent (in powder form) is best preserved by storage at 2–6°C with minimum exposure to air.

Introduction

The Image-iT™ LIVE Green Reactive Oxygen Species (ROS) Detection Kit provides the key reagents necessary for the detection of ROS in live cells. The assay is based on 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA), a reliable fluorogenic marker for ROS in live cells [1,2] (see Figure 1 for the mechanism of this reaction). In addition to carboxy-H_2DCFDA, the kit provides the common inducer of ROS production *tert*-butyl hydroperoxide (TBHP), as a positive control,[3–6] and the blue-fluorescent, cell-permeant nucleic acid stain Hoechst 33342. Using this combination of dyes according to the optimized protocol provided here, oxidatively stressed and nonstressed cells are reliably distinguished by fluorescence microscopy.

Generation of ROS is inevitable for aerobic organisms, and, in healthy cells, occurs at a controlled rate. Under conditions of oxidative stress, ROS production is dramatically increased, resulting in subsequent alteration of membrane lipids, proteins, and nucleic acids. Oxidative damage of these biomolecules is associated with a variety of pathological events including atherosclerosis, carcinogenesis, ischemic reperfusion injury, neurodegenerative disorders [7,8] and with aging.[9,10]

Materials

Contents

- **Component A: 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA)**, 5 vials each containing 275 µg
- **Component B: Hoechst 33342**, 400 µL of a 1 mM solution
- **Component C: *tert*-butyl hydroperoxide (TBHP) solution**, 50 µL of a 7.78 M solution
- **Component D: Dimethylsulfoxide (DMSO)**, 500 µL

Storage and Handling

Upon receipt, store the kit at 2–6˚C, desiccated, protected from light. It is important that the TBHP solution (Component C) not be frozen. The carboxy-H_2DCFDA (Component A) is packaged with an oxygen scavenging pouch, which will extend the

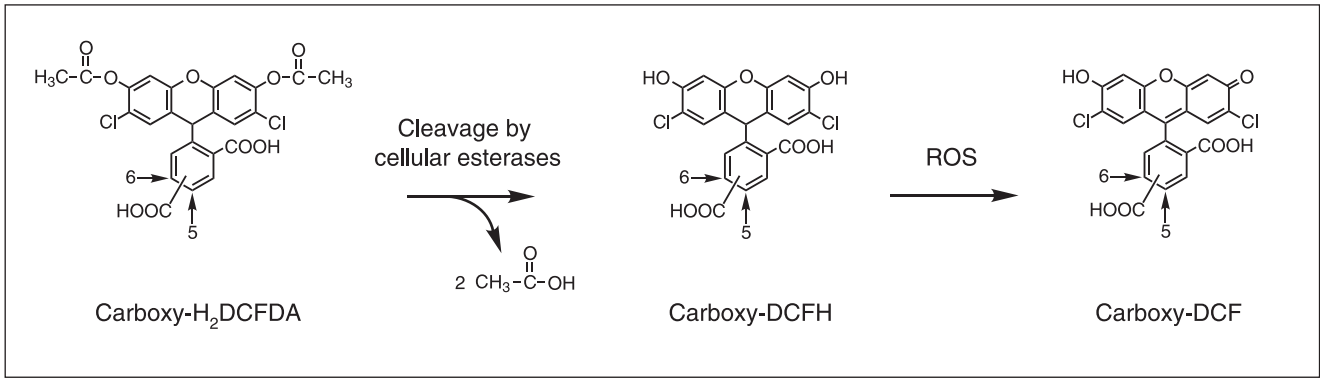

Figure 1. The nonfluorescent 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) permeates live cells and is deacetylated by nonspecific intracellular esterases. In the presence of nonspecific ROS (produced throughout the cell, particularly during oxidative stress) the reduced fluorescein compound is oxidized and emits bright green fluorescence.

shelf life of the product. After removing vials for use, quickly return the unused vials to the pouch and seal the pouch to preserve the activity of the reagent. Solutions of carboxy-H_2DCFDA in DMSO can be divided into aliquots and stored at ≤–20˚C.

Spectral Characteristics

The oxidation product of carboxy-H_2DCFDA has excitation/emission maxima of approximately 495/529 nm and can be observed using standard fluorescein filter sets. Employing neutral density filters and limiting the exposure of the stained sample to light are effective in minimizing background of carboxy-H_2DCFDA. Hoechst 33342 has excitation/emission maxima of approximately 350/461 nm and can be observed using filter sets appropriate for DAPI.

Materials Recommended but Not Provided

While this kit is suitable for use with most live-cell buffering systems, we recommend Hank's balanced salt solution with calcium and magnesium (HBSS/Ca/Mg, available from Gibco (14025-092)) for optimal results.

Experimental Protocols

The protocol was developed using live bovine pulmonary artery endothelial cells (BPAEC) and MRC5 human lung fibroblasts adhering to coverslips, but is amenable for use with other cell types. An additional protocol is provided for the use of TBHP as a positive control for the induction of ROS, which, if desired, must be performed **before** labeling with carboxy-H_2DCFDA.

Labeling with Carboxy-H_2DCFDA

1.1 Prepare a 10 mM carboxy-H_2DCFDA stock solution. Add 50 µL of DMSO (Component D) to one vial of carboxy-H_2DCFDA (Component A, 275 µg) to make a 10 mM stock solution. Vortex the vial until the powder is completely dissolved.

1.2 Prepare 25 µM carboxy-H_2DCFDA working solution. Add 5.0 µL of the 10 mM carboxy-H_2DCFDA stock solution (prepared in step 1.1) to 2.0 mL of warm HBSS/Ca/Mg or other suitable buffer.

1.3 Wash cells. Gently wash cells once with warm HBSS/Ca/Mg or other suitable buffer.

1.4 Label cells. Apply a sufficient amount of the 25 µM carboxy-H_2DCFDA working solution (prepared in step 1.2) to cover the cells adhering to the coverslip(s). Incubate for 30 minutes at 37°C, protected from light.

1.5 (Optional) Counterstain with Hoechst 33342. Hoechst 33342 should be added at a final concentration of 1.0 µM to the carboxy-H_2DCFDA staining solution during the last 5 minutes of the incubation in step 1.4. The Hoechst 33342 stain (Component B) is supplied as a 1.0 mM solution, so for every 1.0 mL of carboxy-H_2DCFDA working solution used in step 1.4, add 1.0 µL of Hoechst 333342 stain.

1.6 Wash cells. Gently wash the coverslips three times in warm HBSS/Ca/Mg or other suitable buffer.

1.7 Mount in warm buffer and image immediately. Best results are obtained when imaging takes place immediately after washing and mounting the sample. Molecular Probes recommends the use of neutral density filters to circumvent limitations traditionally observed with reduced fluorescein dyes, which are susceptible to photooxidation as well as photobleaching. If the cells were labeled with Hoechst, we recommend using a Hoechst (or DAPI) filter set and neutral density filter(s) to assist with locating the cells on the coverslip, followed by a fluorescein filter set for imaging. This strategy minimizes photobleaching of fluorescein due to prolonged exposure to blue wavelengths of light.

Induction of Cellular ROS Production with TBHP

2.1 Prepare a 100 mM stock solution of TBHP. Add 1.0 µL of TBHP (Component C, 7.78 M) to 77 µL of high-purity water to make a 100 mM stock solution. Slow pipetting of the viscous TBHP solution is recommended.

2.2 Make 100 µM working solution of TBHP. Dilute the 100 mM TBHP stock (prepared in step 2.1) 1:1000 in appropriate complete growth media to produce a 100 µM working solution. For example, to make 1.0 mL of 100 µM TBHP working solution, add 1.0 µL 100 mM TBHP to 1.0 mL of complete media.

2.3 Induce ROS production in cells. Apply a sufficient amount of the 100 µM TBHP working solution (prepared in step 2.2) to the cells adhering to the coverslip(s). Incubate the coverslip(s) at 37˚C and 5% CO_2. During development of the product using BPAE and MRC5 cells, a 60–90 minute incubation period was required. Appropriate incubation periods for ROS production in other cell lines should be determined empirically. After TBHP induction, label the cells with carboxy-H_2DCFDA starting with step 1.1, above.

2.4 Wash cells. Gently wash the coverslips twice in warm HBSS/Ca/Mg or other suitable buffer. After washing, label the cells with carboxy-H_2DCFDA starting with step 1.1, above.

References

1. J Natl Cancer Inst 91, 1138 (1999); **2.** Am J Physiol Heart Circ Physiol 279, H2424 (2000); **3.** Cancer Res 61, 1392 (2001); **4.** Am J Physiol 272, C1286 (1997); **5.** Histochem Cell Biol 120, 319 (2003); **6.** Lipids 36, 57 (2001); **7.** Free Radic Biol Med 31, 164 (2001); **8.** J Cell Mol Med 6, 175 (2002); **9.** Ann N Y Acad Sci 908, 219 (2000); **10.** Mitochondrion, 2, 361 (2003).

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

Cat #	Product Name	Unit Size
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