molecular probes®
by life technologies™

CellROX® Deep Red Flow Cytometry Assay Kit

formulated for flow cytometry *for oxidative stress detection*

Catalog no. C10491

Table 1 Contents and storage

Material	Amount	Concentration	Storage*	Stability
CellROX® Deep Red Reagent (Component A)	1 × 25 µL	2.5 mM stabilized solution in DMSO	• ≤–20°C • Desiccate • Store vial upright • Protect from light	When stored as directed, the kit contents are stable for at least 6 months from the date of receipt.
SYTOX® Blue Dead Cell Stain (Component B)	1 × 100 µL	1 mM stabilized solution in DMSO		
N-acetylcysteine (Component C) *antioxidant for negative control*	2 × 10 mg	250 mM (when resuspended in PBS)		
Dimethylsulfoxide (DMSO) (Component D)	200 µL	NA		
Tert-butyl hydroperoxide, 70% in water (Component E) *for positive control*	1 × 50 µL	7.78 M		
*Before refreezing, seal the vials tightly. NA = not aplicable.				
Number of reactions: Sufficient material is supplied for approximately 100 reactions, based on the protocol below.				
Fluorescence excitation and emission maxima: CellROX® Deep Red reagent: 644/665 nm (oxidized product); SYTOX® Blue Dead Cell stain: 444/480 nm (bound to nucleic acid); see Figure 1 (page 2).				

Introduction

Generation of reactive oxygen species (ROS), which is inevitable for aerobic organisms, occurs at a controlled rate in healthy cells. Under conditions of oxidative stress, production of ROS 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,[1,2] and with aging.[3,4]

The CellROX® Deep Red Flow Cytometry Assay Kit is specifically formulated for flow cytometry and provides the key reagents necessary for the detection of ROS in live cells.

The cell-permeable CellROX® Deep Red reagent is essentially non-fluorescent while in a reduced state, but exhibits a strong fluorogenic signal upon oxidation, providing a reliable measure of reactive oxygen species (ROS) in live cells. In addition to the CellROX® Deep Red reagent, the kit provides the common inducer of ROS production tert-butyl hydroperoxide (TBHP) as a positive control,[5–8] the antioxidant N-acetylcysteine (NAC) as a negative control,[9,10] and the blue-fluorescent, cell-impermeant SYTOX® Blue Dead Cell stain.

Using this combination of dyes according to the optimized protocol provided here, oxidatively stressed and non-stressed cells are reliably distinguished from dead cells by flow cytometry (Figures 2 and 3, page 3). The CellROX® Deep Red reagent and the SYTOX® Blue Dead Cell stain have minimal spectral overlap with fluorophores excited by other laser lines, allowing easy multiplexing with other reagents. Moreover, the CellROX® Deep Red reagent retains its signal following formaldehyde fixation and permeabilization.

Life Technologies offers a wide selection of products for detection of oxidative stress by flow cytometry. For more information on the CellROX® ROS Detection reagents, refer to Table 2, below. For more information on other products available to study cell health, visit www.lifetechnologies.com/flowcytometry.

Spectral characteristics

The fluorescence absorption and emission spectra of the CellROX® Deep Red reagent following oxidation and the SYTOX® Blue Dead Cell stain bound to DNA are shown in Figure 1, below. These spectra were obtained in 10 mM Tris, 1 mM EDTA, pH 8 in the presence of double-stranded DNA. The CellROX® Deep Red reagent exhibits an increase in fluorescence upon oxidation. Similarly, the SYTOX® Blue Dead Cell stain exhibits a fluorescence enhancement of greater than 500-fold upon binding DNA. The absorption and fluorescence emission maxima of the CellROX® Deep Red reagent and the SYTOX® Blue Dead Cell stain/DNA complexes are 644/665 nm and 444/480 nm, respectively.

Figure 1 Fluorescence excitation (solid lines) and emission (dashed lines) spectra of **(A)** CellROX® Deep Red reagent following oxidation and **(B)** the SYTOX® Blue Dead Cell stain bound to DNA.

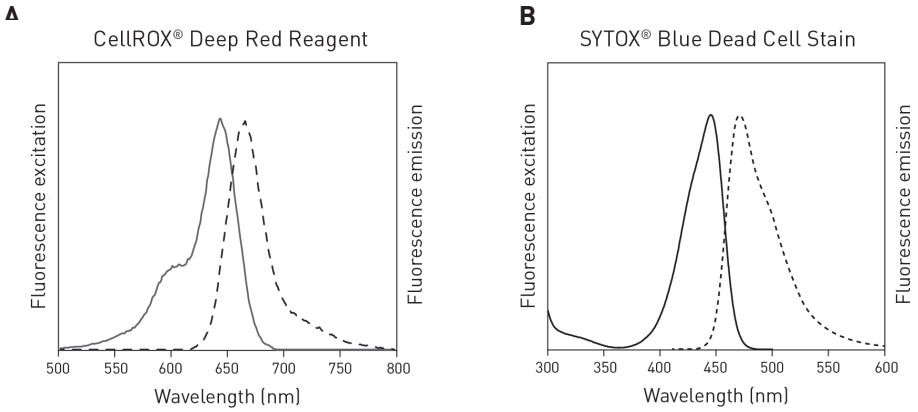


Table 2 Characteristics of the CellROX® ROS detection reagents for flow cytometry

	CellROX® Deep Red Flow Cytometry Assay Kit	CellROX® Orange Flow Cytometry Assay Kit	CellROX® Green Flow Cytometry Assay Kit
Catalog no.	C10491	C10492	C10493
ROS detection reagent	CellROX® Deep Red reagent	CellROX® Orange reagent	CellROX® Green reagent
ROS detection reagent Ex/Em maxima	644/665 nm	545/565 nm	508/525 nm
Cellular localization of stain	Cytoplasm	Cytoplasm	Nucleus and mitochondria
Formaldehyde fixable?	Yes	No	Yes
Detergent compatible?	No	No	Yes
Suggested dead cell stain	SYTOX® Blue Dead Cell Stain	SYTOX® Red Dead Cell Stain	SYTOX® Red Dead Cell Stain
Dead cell stain Ex/Em maxima	444/480 nm	640/658 nm	640/658 nm

Figure 2 ROS levels detected by CellROX® Deep Red reagent is decreased in TBHP-treated Jurkat cells with pre-treatment of cultures using NAC. Jurkat cells (T-cell leukemia, human) were incubated with 1 mM NAC for 1 hour prior to treatment with 1× PBS or 200 µM TBHP for 30 minutes, and then labeled with the CellROX® Deep Red reagent. Stained samples were analyzed on a Becton Dickinson LSRII Cytometer equipped with a 639-nm laser for excitation of CellROX® Deep Red ROS detection reagent and a 665/40 BP filter for collection of fluorescence emission. Note that the cells treated with the oxidant TBHP (red) have increased staining with the CellROX® Deep Red reagent as compared to the cells pre-treated with the antioxidant NAC (blue) and the control cells (green).

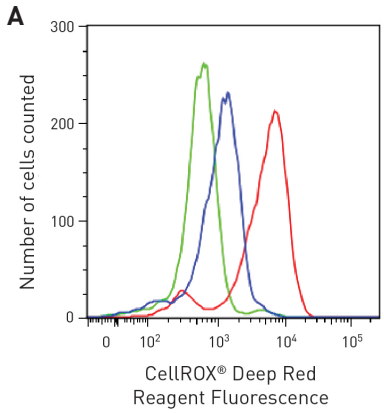
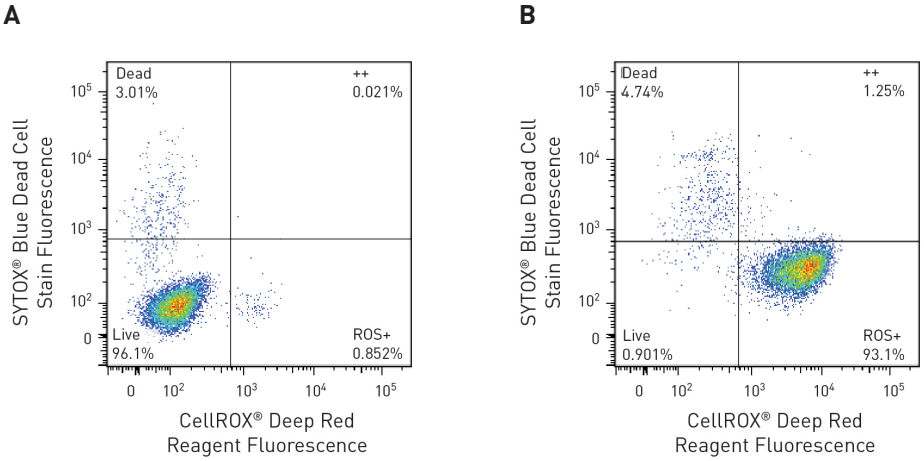


Figure 3 CellROX® Deep Red reagent can be used in conjunction with the SYTOX® Blue Dead Cell stain to differentiate live stressed cells from dead cells. Jurkat cells (T-cell leukemia, human) were treated with **(A)** PBS or **(B)** 200 µM TBHP for 30 minutes before labeling with the CellROX® Deep Red Flow Cytometry Assay Kit. Stained samples were analyzed on a Becton Dickinson LSRII Cytometer equipped with 405-nm and 639-nm lasers for the excitation of SYTOX® Blue and CellROX® Deep Red fluorescence, respectively. Fluorescence emission was collected using a 450/50BP and a 665/40BP filter for SYTOX® Blue and CellROX® Deep Red fluorescence, respectively. Note that the treated cells (panel B) have a higher percentage of cells under oxidative stress than the basal level of ROS observed in control cells (panel A).



Before Starting

Materials required but not provided

- Cells of interest in a single cell suspension (appropriate sample concentrations range from 10^4–10^6 cells/mL)
- Appropriate suspension buffer (e.g., complete medium)
- *Optional:* Inducing agent appropriate for the cell model used, if different from TBHP (e.g., lipopolysaccharide, menadione, angiotensin II, nefazodone, etc.)
- *Optional:* Control sample (no treatment)

Caution

No data are available addressing the mutagenicity or toxicity of the reagents within the CellROX® Deep Red Flow Cytometry Assay Kit. Components A, B, and D contain DMSO, and should be handled with particular caution because DMSO is known to facilitate the entry of organic molecules into tissues. Component B binds to nucleic acid and should be treated as a potential mutagen and used with appropriate care. As with all nucleic acid stains, solutions containing this reagent should be disposed of according to local regulations.

Storage and handling

- Upon receipt, the contents of the CellROX® Deep Red Flow Cytometry Assay Kit should be stored frozen at –20°C, upright, desiccated, and protected from light.
- The CellROX® reagents are sensitive to exposure to light and air; care should be taken not to keep the vials open for long periods of time.
- The CellROX® Deep Red reagent included in the kit may be frozen and thawed up to five times. Before refreezing, seal the vials tightly.
- When stored properly, the reagents are stable for at least 6 months from the date of receipt.
- The CellROX® Deep Red Flow Cytometry Assay Kit kit contains sufficient material to assay approximately 100 samples utilizing the method outlined below.

Experimental Protocols

Staining procedure

The following procedure was developed using the Jurkat T-cell leukemia cell line, but it can be adapted for any cell type. Growth medium, cell density, cell type variations, and other factors may influence staining. In initial experiments, test a range of stain concentrations to determine the optimal stain concentration for the given cell type, buffer, and experimental conditions.

1. Harvest the cell sample(s). Adjust the cell concentration of the sample(s) to ~5×10^5 cells/mL in complete growth medium (e.g., RPMI, Cat. no. 22400; DMEM, Cat. no. 11995). If an adherent cell line is used, ensure that the cells are sub-confluent. **Staining of cells in phosphate buffered saline (PBS) is not recommended.**

2. Induce ROS in cells using the desired method.

3. Prepare positive and negative controls. The kit contains tert-butyl hydroperoxide (TBHP) to induce oxidative stress and N-acetylcysteine (NAC) to increase the antioxidant capability of the cell. You can prepare the negative control either by incubating the cells in the absence of the ROS inducing agent or by incubating the cells with antioxidant.

   a. Reconstitute one vial containing 10 mg of NAC (Component C) with 245 µL of PBS to make a 250 mM solution.

b. Prepare a 50 mM intermediate dilution of TBHP (Component E) by adding 3.22 µL of the 70% stock (7.78 M) to 496.8 µL of PBS or complete media.

c. *Negative control:* Prepare a negative control by incubating the cells with NAC **before** treatment with TBHP. Add NAC to the negative control sample and incubate for 1 hour under normal growth conditions (e.g., 37°C, 5% CO_2).

Although the suggested final concentration of NAC for use is 200–5000 µM, the optimal final concentration is cell-dependent and should be determined experimentally for each cell line being tested.

d. *Positive control:* Create a positive control by adding 100–400 µM of TBHP to a sample of cells. Ensure that the same concentration of TBHP is used in both positive and negative controls.

e. Following the 1 hour incubation with NAC, add TBHP to the negative control cells **from step 3c**. Although the suggested concentration of TBHP is 100–400 µM, the optimal final concentration is cell-dependent and should be determined experimentally for each cell line being tested. For example, add 4 µL of the 50 mM intermediate TBHP solution per mL cells for a final concentration of 200 µM TBHP.

f. Incubate the samples **from step 3d and 3e** for 30–60 minutes under normal growth conditions before staining with the CellROX® Deep Red reagent (see below).

4. Briefly centrifuge the vial of CellROX® Deep Red reagent (Component A) before opening the vial. Add the CellROX® Deep Red reagent at a final concentration of 500–1000 nM to the samples and/or appropriately induced cells, and incubate for 30–60 minutes at 37°C, protected from light.

a. It is best to prepare an intermediate dilution of the CellROX® Deep Red reagent in DMSO (Component D). Mix the intermediate dilution well by pipetting up and down, and then add the specified amount of the diluted solution to the cells, so that the final concentration of the reagent incubated with the cells is 500–1000 nM. For example, combine 1 µL of CellROX® Deep Red reagent with 9 µL of DMSO to make a 250 µM solution; use 2 µL of this intermediate solution to stain 1 mL of the cell suspension for a final concentration of 500 nM.

Note: We recommend that you prepare and acquire single stained compensation controls using the CellROX® Deep Red reagent and the SYTOX® Blue Dead Cell stain (Component B). Samples treated with TBHP may be used for single-color compensation controls.

5. *Optional:* Wash the cells once with PBS or other appropriate buffer using 3× the staining volume (e.g., wash 1 mL of stained cells with 3 mL of PBS or buffer). Note that washing is not required following staining with the CellROX® Deep Red reagent.

6. During the final 15 minutes of staining, add 1 µL of the 1 mM SYTOX® Blue Dead Cell stain solution in DMSO (Component B) per 1 mL of the appropriate samples and mix gently. The final labeling concentration of the SYTOX® Blue Dead Cell stain is 1 µM. Do not wash the samples after the addition of the SYTOX® Blue Dead Cell stain.

7. Immediately analyze the samples by flow cytometry, using 405-nm excitation for the SYTOX® Blue Dead Cell stain and 635-nm excitation for the CellROX® Deep Red reagent. Collect fluorescence emission with 450/50 BP and 665/40 BP filters (or equivalents) from the SYTOX® Blue Dead Cell stain and the CellROX® Deep Red ROS detection reagent, respectively. Do not allow the staining reaction to proceed further than 120 minutes.

a. The positive control from step 3d may be used to adjust instrument settings for the CellROX® Deep Red fluorescence. In this sample a positive population corresponding to cells under oxidative stress should be visible on a histogram of the CellROX® Deep Red fluorescence. Fluorescence of the CellROX® Deep Red reagent will be decreased in the negative control sample treated with both TBHP and NAC, as compared to the positive control (see Figure 2, page 3).

b. Following application of standard fluorescence compensation technique, distinct cell populations should be visible on a dual parameter dot plot of CellROX® Deep Red fluorescence versus SYTOX® Blue fluorescence (see Figure 3, page 3).

Multicolored staining

The CellROX® Deep Red reagent and the SYTOX® Blue Dead Cell stain have minimal spectral overlap with fluorophores excited by other laser lines, and can be combined with other dyes. If used in combination with other reagents for multicolor applications, first apply the CellROX® Deep Red reagent to cells grown in complete growth medium under normal growth conditions, and then apply the other dye(s) following manufacturer's instructions. Samples stained with the CellROX® Deep Red reagent alone may be treated with a formaldehyde fixative following staining and still retain signal. Apply the SYTOX® Blue Dead Cell stain as the last step in the multicolor staining of live, non-fixed cells, and do not wash or fix the samples stained with the SYTOX® Blue stain prior to flow cytometric analysis.

References

1. Free Radic Biol Med 31, 164 (2001); **2.** J Cell Mol Med 6, 175 (2002); **3.** Ann N Y Acad Sci 908, 219 (2000); **4.** Mitochondrion, 2, 361 (2003); **5.** Cancer Res 61, 1392 (2001); **6.** Am J Physiol 272, C1286 (1997); **7.** Histochem Cell Biol 120, 319 (2003); **8.** Lipids 36, 57 (2001); **9.** Cell Death and Differentiation 9, 1007 (2002); **10.** Cell Mol Life Sci 60, 6 (2003).

Product List

Current prices may be obtained at www.lifetechnologies.com or from our Customer Service Department.

Catalog no.	Product Name	Unit Size
C10491	CellROX® Deep Red Flow Cytometry Assay Kit *formulated for flow cytometry*	1 kit
Related Products		
C10492	CellROX® Green Flow Cytometry Assay Kit *formulated for flow cytometry*	1 kit
C10491	CellROX® Orange Flow Cytometry Assay Kit *formulated for flow cytometry*	1 kit
C10422	CellROX® Deep Red Reagent *for oxidative stress detection*	5 × 50 µL
C10443	CellROX® Orange Reagent *for oxidative stress detection*	5 × 50 µL
C10444	CellROX® Green Reagent *for oxidative stress detection*	5 × 50 µL
C10448	CellROX® Reagent Variety Pack *for oxidative stress detection*	1 kit
A36003	APF (Hydroxyl Radical, Hypochlorite or Peroxynitrite Sensor)	470 µL
C6827	CM-H_2DCFDA (5-(and-6)-chloromethyl- 2',7'-dichlorodihydrofluorescein diacetate, acetyl ester) *mixed isomers* *special packaging*	5 × 50 µg
D399	H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate (2',7'-dichlorofluorescin diacetate)	100 mg
H36004	HPF (Hydroxyl Radical and Peroxynitrite Sensor)	470 µL
I36007	Image-iT® LIVE Green Reactive Oxygen Species Detection Kit	1 kit
L10119	LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit *for 633 or 635 nm excitation* *200 assays*	1 kit
L10120	LIVE/DEAD® Fixable Far Red Dead Cell Stain Kit *for 633 or 635 nm excitation* *200 assays*	1 kit
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L23105	LIVE/DEAD® Fixable Blue Dead Cell Stain Kit *for UV excitation* *200 assays*	1 kit
L34955	LIVE/DEAD® Fixable Violet Dead Cell Stain Kit *for 405 nm excitation* *200 assays*	1 kit
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L34959	LIVE/DEAD® Fixable Yellow Dead Cell Stain Kit *for 405 nm excitation* *200 assays*	1 kit
L34960	LIVE/DEAD® Fixable Dead Cell Stain Sampler Kit *for flow cytometry* *320 assays*	1 kit
S10274	SYTOX® AADvanced™ Dead Cell Stain Kit *for flow cytometry* *for 488 nm excitation* *500 tests*	1 kit
S10349	SYTOX® AADvanced™ Dead Cell Stain Kit *for flow cytometry* *for 488 nm excitation* *100 tests*	1 kit
S34857	SYTOX® Blue dead cell stain *for flow cytometry* *1000 assays* *1 mM solution in DMSO*	1 mL
S34859	SYTOX® Red dead cell stain *for 633 or 635 nm excitation* *5 µM solution in DMSO*	1 mL
S34860	SYTOX® Green dead cell stain *for flow cytometry* *30 µM* *1000 tests*	1 mL
S34861	SYTOX® Orange dead cell stain *for flow cytometry* *250 µM* *1000 tests*	1 mL
S34862	SYTOX® Dead Cell Stain Sampler Kit *for flow cytometry* *50 tests per vial*	1 kit

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These high-quality reagents and materials must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Read the Safety Data Sheet provided for each product; other regulatory considerations may apply.

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