molecular probes®
by life technologies™

CellROX® Green and CellROX® Orange Flow Cytometry Assay Kits

formulated for flow cytometry *for oxidative stress detection*

Catalog nos. C10492, C10493

Table 1 Contents and storage

Material	Amount	Concentration	Storage*	Stability
CellROX® Green (Cat. no. C10492) or CellROX® Orange (Cat. no. C10493) 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® Red Dead Cell Stain (Component B)	1 × 100 µL	5 µM 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® Green ROS detection reagent: 508/525 nm (oxidized product); CellROX® Orange ROS detection reagent: 545/565 nm (oxidized product); SYTOX® Red Dead Cell stain: 640/658 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® Green and CellROX® Orange Flow Cytometry Assay Kits (Cat. nos. C10492 and C10493) are specifically formulated for flow cytometry and provide the key reagents necessary for the detection of ROS in live cells.

The cell-permeable CellROX® reagent (Component A) 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® ROS detection reagent, each 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 red-fluorescent, cell-impermeant SYTOX® Red Dead Cell stain (Component B). 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® Green reagent, the CellROX® Orange reagent, and the SYTOX® Red Dead Cell stain have minimal spectral overlap with fluorophores excited by other laser lines, allowing easy multiplexing with other reagents. Moreover, the CellROX® Green reagent retains signal following formaldehyde fixation and permeabilization (the CellROX® Orange reagent is not compatible with 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, page 3. 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® Green and CellROX® Orange reagents following oxidation and of the SYTOX® Red Dead Cell stain 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® ROS detection reagents exhibit an increase in fluorescence upon oxidation. Similarly, the SYTOX® Red Dead Cell stain exhibits a fluorescence enhancement of greater than 500-fold upon binding DNA. The absorption and fluorescence emission maxima of the CellROX® reagents and the SYTOX® Dead Cell stain/DNA complexes are given in Table 2 (page 3).

Figure 1 Fluorescence excitation (solid lines) and emission (dashed lines) spectra of CellROX® ROS detection reagents following oxidation and the SYTOX® Dead Cell stains bound to DNA. **(A)** CellROX® Green reagent, **(B)** CellROX® Orange reagent, **(C)** SYTOX® Red Dead Cell stain.

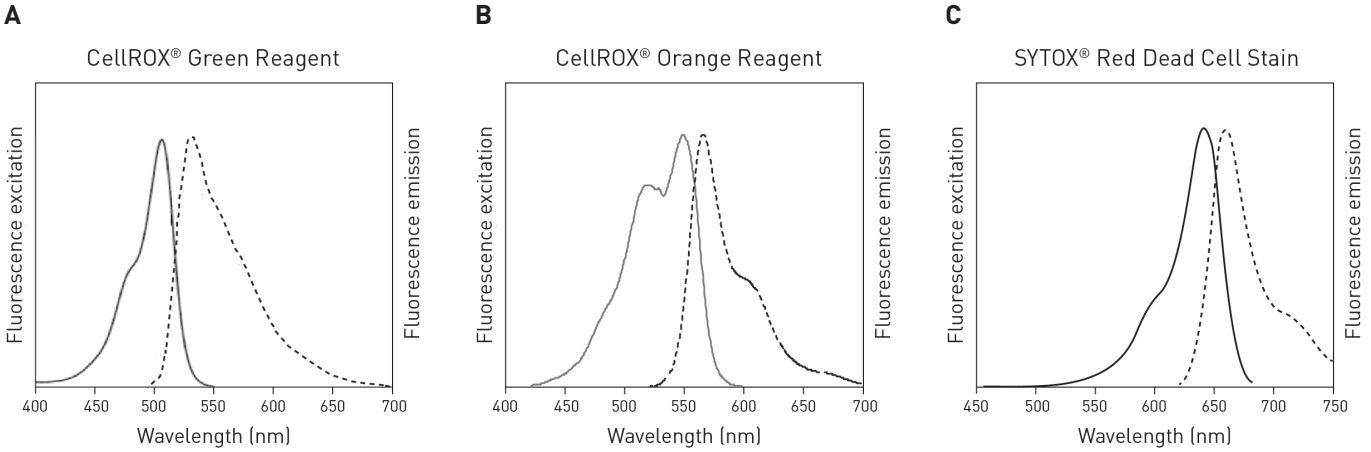


Table 2 Characteristics of the CellROX® ROS detection reagents for flow cytometry

	CellROX® Deep Red Flow Cytometry Assay Kit	CellROX® Green Flow Cytometry Assay Kit	CellROX® Orange Flow Cytometry Assay Kit
Catalog no.	C10491	C10492	C10493
ROS detection reagent	CellROX® Deep Red reagent	CellROX® Green reagent	CellROX® Orange reagent
ROS detection reagent Ex/Em maxima	644/665 nm	508/525 nm	545/565 nm
Cellular localization of stain	Cytoplasm	Nucleus and mitochondria	Cytoplasm
Formaldehyde fixable?	Yes	Yes	No
Detergent compatible?	No	Yes	No
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® ROS detection reagents are 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 before labeling with the **(A)** CellROX® Green reagent or the **(B)** CellROX® Orange reagent. Stained samples were analyzed on a Becton Dickinson LSRII Cytometer equipped with a 488-nm laser for excitation of CellROX® Green reagent and a 530/30 BP filter for collection of fluorescence emission or a 532-nm laser for excitation of CellROX® Orange reagent and a 585/42 BP filter for collection of fluorescence emission. Note that cells treated with the oxidant TBHP (red) have increased staining with CellROX® ROS detection reagent as compared to cells pretreated with the antioxidant NAC (blue) and control cells (green).

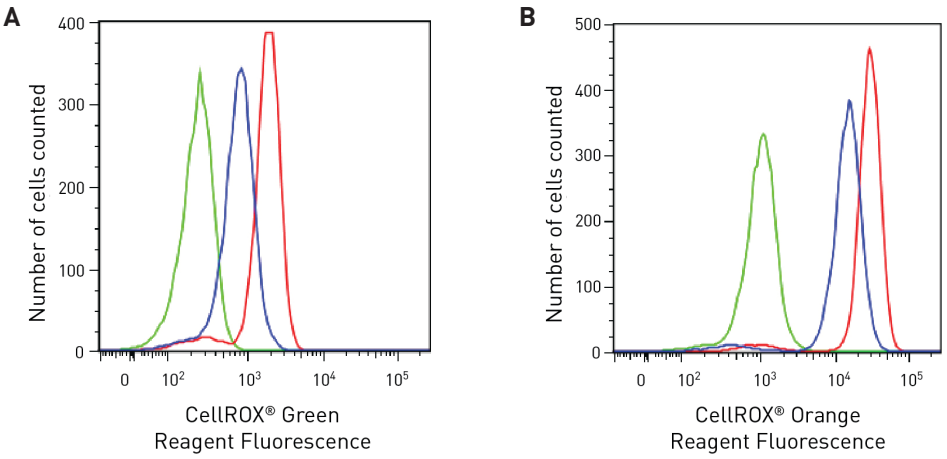
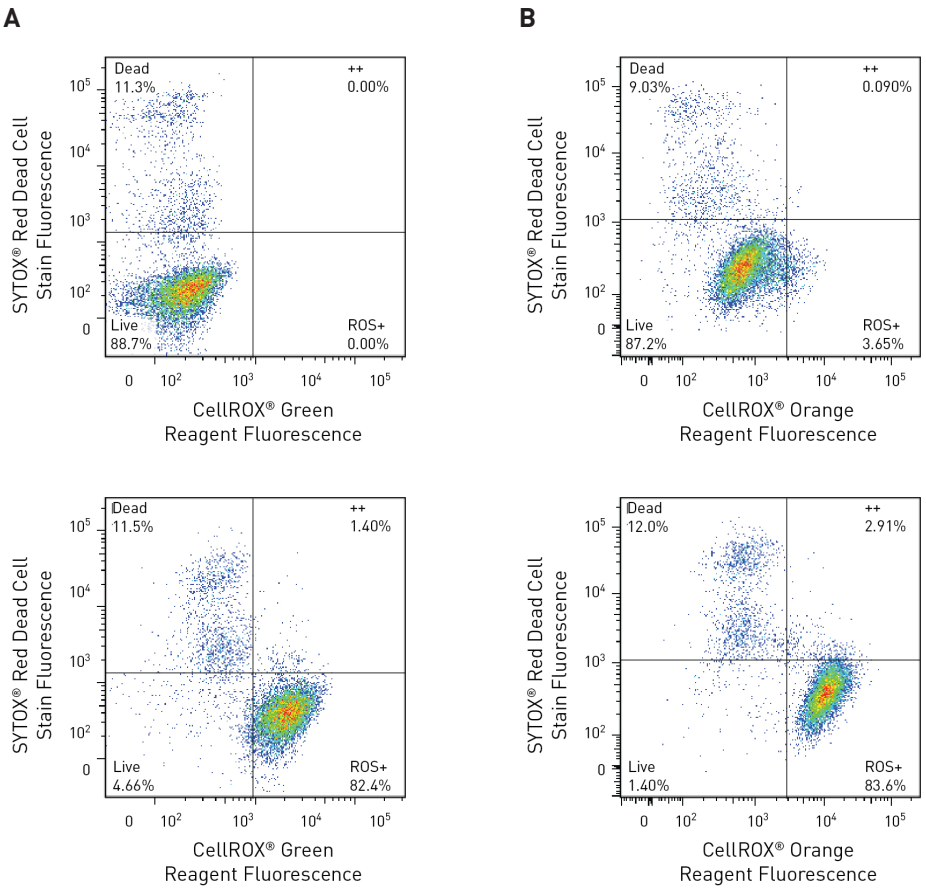


Figure 3 CellROX® Green and CellROX® Orange reagents can be used in conjunction with the SYTOX® Red Dead Cell Stain to differentiate live stressed cells from dead cells. Jurkat cells (T-cell leukemia, human) were treated with PBS (top panels) or 200 µM TBHP (bottom panels) for 30 minutes before labeling with the CellROX® Green (Cat. no. C10492) or the CellROX® Orange (Cat. no. C10493) Flow Cytometry Assay Kit. Stained samples were analyzed on a Becton Dickinson LSRII Cytometer equipped with the lasers and filter sets as described below. Note that the treated cells (bottom panels) have a higher percentage of cells under oxidative stress than the basal level of ROS observed in control cells.

(A) 488-nm and 639-nm lasers were used for the for excitation of CellROX® Green and SYTOX® Red fluorescence. Fluorescence emission was collected using a 530/30BP and a 665/40BP filter for CellROX® Green and SYTOX® Red fluorescence, respectively.

(B) 532-nm and 639-nm lasers were used for the excitation of CellROX® Orange and SYTOX® Red fluorescence. Fluorescence emission was collected using a 585/42BP and a 660/20BP filter for CellROX® Orange and SYTOX® Red fluorescence, respectively.



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® Flow Cytometry Assay Kits. 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 acids 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 CellROX® Flow Cytometry Assay Kits should be stored frozen at –20°C, upright, desiccated, and protected from light. Before refreezing, seal the vials tightly. 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® reagent included in each kit is specifically formulated for flow cytometry and may be frozen and thawed up to five times. When stored properly the reagents are stable for at least 6 months from the date of receipt. Each 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. Each CellROX® Flow Cytometry Assay 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 by incubating the cells in the absence of the ROS inducing agent or by incubating the cells with the 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. For example, add 4 µL of the 50 mM intermediate TBHP solution per mL cells for a final concentration of 200 µM TBHP.

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.

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

4. Briefly centrifuge the vial of CellROX® reagent (Component A) before opening the vial. Add the CellROX® 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® 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® 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® reagent and the SYTOX® Red Dead Cell stain (Component B). Samples treated with TBHP may be used for single-color compensation controls.

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

6. During the final 15 minutes of staining, add 1 µL of the 5 µM SYTOX® Red Dead Cell stain solution in DMSO per 1 mL of the appropriate samples and mix gently. The final labeling concentration of stain is 5 nM. Do not wash samples after the addition of the SYTOX® Red Dead Cell stain.

7. Immediately analyze the samples by flow cytometry, using 488-nm excitation for the CellROX® Green or 532-nm for the CellROX® Orange reagent, and 639-nm excitation for the SYTOX® Red stain. Collect fluorescence emission with a 530/30 BP or a 585/42 BP filter (or equivalents) for emission from the CellROX® Green and CellROX® Orange reagents, respectively, and a 660/20 BP (or equivalent) for emission from the SYTOX® Red stain. 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® Green or CellROX® Orange fluorescence. In this sample a positive population corresponding to the cells under oxidative stress should be visible on a histogram of the fluorescence from the CellROX® reagent. Fluorescence of the CellROX® reagent should 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 techniques, distinct cell populations should be visible on a dual parameter dot plot of CellROX® reagent fluorescence versus SYTOX® Red stain fluorescence (see Figure 3, page 4).

Multicolor staining

The CellROX® Green reagent, the CellROX® Orange reagent, and the SYTOX® Red 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, apply the CellROX® reagent to cells grown in complete growth medium under normal growth conditions, followed with application of the other dyes following manufacturer's instructions. Samples stained with the CellROX® Green reagent alone may be treated with formaldehyde fixative and detergent following staining and retain signal; the CellROX® Orange reagent is not compatible with fixation and permeabilization. Apply SYTOX® Red Dead Cell stain as the last step in multicolor staining of live, non-fixed cells and do not wash or fix samples stained with the SYTOX® Red Dead Cell 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
C10492	CellROX® Green Flow Cytometry Assay Kit *formulated for flow cytometry*	1 kit
C10493	CellROX® Orange Flow Cytometry Assay Kit *formulated for flow cytometry*	1 kit
Related Products		
C10491	CellROX® Deep Red Flow Cytometry Assay Kit *formulated for flow cytometry*	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
L23101	LIVE/DEAD® Fixable Green Dead Cell Stain Kit *for 488 nm excitation* *200 assays*	1 kit
L23102	LIVE/DEAD® Fixable Red Dead Cell Stain Kit *for 488 nm excitation* *200 assays*	1 kit
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
L34957	LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit *for 405 nm excitation* *200 assays*	1 kit
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

Purchaser Notification

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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