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Reactive Oxygen Species (ROS) Detection Assay Kit

rev 07/19

(Catalog # K936-100,-250; 100-250 assays; Store at -20°C; Protect from light)

I. Introduction:

Constant generation of low levels of reactive oxygen species (ROS) and free radicals is a basic feature of all living cells. Low levels of ROS play an essential role in signaling pathways, whereas increased levels under oxidative stress, results in damage to nucleic acids, proteins and membrane lipids. Accumulation of ROS during oxidative stress is also associated with aging, apoptosis or necrosis, and is implicated in pathological conditions such as vascular diseases, diabetes, renal ischemia, arteriosclerosis, pulmonary disorders, inflammatory diseases, and cancer. Cellular activity of ROS is offset by antioxidants, numerous repair systems, and replacement of damaged DNA. Probes for measuring intracellular ROS levels provide important tools to study oxidative stress inducers and effects of antioxidant therapies. **BioVision's ROS Detection Assay Kit** is designed for the detection of hydroxyl, peroxyl, or other reactive oxygen species in live cells. We utilize H2DCFDA, a unique cell-permeable fluorogenic probe compatible with phenol red, FBS and BSA to detect reactive oxygen species in live cells. Upon the cell entry, H2DCFDA is modified by cellular esterases to form a non-fluorescent H2DCF. Oxidation of H2DCF by intracellular ROS yields highly a fluorescent product that can be detected by FACS, microplate reader, or fluorescence microscope (Ex/Em 495/529 nm). The fluorescence intensity is proportional to the ROS levels. Our kit provides a simple and specific assay for the real-time measurement of global levels of ROS in living cells. We include sufficient reagents to perform 100 assays and a common ROS inducer as control for the measurement of ROS levels or antioxidant activity with high sensitivity, specificity and accuracy.

II. Applications:

- Measurement of intracellular levels of ROS
- Screening compounds with a stress inducing and/or antioxidant function

III. Sample Type:

- Suspension or adherent cells cultures

IV. Kit Contents:

Components	K936-100	K936-250	Cap Code	Part Number
	100 assays	250 assays		
ROS Assay Buffer	25 ml	65 ml	WM/NM	K936-XXX-1
ROS Label (1000X)	10 µl	25 µl	Green	K936-XXX-2
ROS Inducer (250X)	20 µl	20 µl	Yellow	K936-XXX-3

V. User Supplied Reagents and Equipment:

- Tissue culture vessels and appropriate culturing media; flow cytometry vessels
- Phosphate Buffered Saline (PBS, pH 7.4)
- Fluorescence microscope, Flow cytometer (FL-1 channel) and Microplate reader capable of measuring Ex/Em 495/529 nm spectra
- 96-well microplates (black and/or clear)

VI. Storage Conditions and Reagent Preparation:

Some reagents are sensitive to light and air exposure. *Do not to keep the vials open for long time periods*. Briefly centrifuge small vials prior to opening. Read entire protocol before performing the assay.

- **ROS Assay Buffer:** Upon receipt place and store at 4°C. *Always use sterile techniques for handling*. Equilibrate to 37°C before use.
- **ROS Label (1000X):** Due to light-induced auto-oxidation **always store at -20°C** protected from light, avoid multiple freeze/thaw cycles. Prior to labeling, dilute the stock solution at 1:1000 in pre-warmed Assay Buffer or culture media to a 1X final working concentration. Do not store the 1X reagent for future use. Stable for 6 months.
- **ROS Inducer (250X):** Store at 4°C protected from light. Warm to room temperature before use. Prepare fresh 1X working solution in pre-warmed Assay Buffer or culture media prior to experiment. Do not store the 1X reagent for future use.

VII. ROS Detection Assay Protocol:

The following protocols, developed with Jurkat (suspension) and HeLa (adherent) cells, provide general guidelines and should be modified depending on application, cell line and sensitivity required. Growth conditions, cell density and other factors may affect ROS labeling; therefore, cells should not be overly dense during the experiment; do not exceed 5 X 10^5 cells/ml of complete growth medium. We suggest testing several ROS Probe concentrations to find best conditions for your cell type and experimental design. **The assay volume is 100 µl in a 96-well tissue culture plate.** Adjust volumes accordingly for other plate formats. Equilibrate all materials and prepared reagents to correct temperature prior to use. **Note:** For testing ROS-inducing compounds in cell cultures, we recommend labeling cells prior to treatment. Detection of ROS in these experimental conditions prevents removal of ROS species during wash steps.

1. Detection of ROS in Suspension and Adherent Cells by Flow Cytometry:

a. Grow cells (adherent or suspension) in appropriate media to obtain at least of 3 x 10^4 cells per assayed conditions; positive, negative and experimental controls, and test compound(s). Ensure that adherent cells are sub-confluent. Account for cell loss during the processing. **Negative control** – unlabeled cells not exposed to ROS Inducer or treatment, **Positive control** – cells incubated with 1X ROS Label only, **Experimental control** – labeled cells treated with 1X ROS Inducer.

b. Harvest the suspension cells by centrifugation at 300 x g for 5 min at room temperature. *Use these setting throughout the entire protocol for both cell types*. Fully detach adherent cells (e.g. trypsinize and quench with media) and harvest by centrifugation. Re-

suspend the cell pellets in culture media with 1X ROS Label. Ensure a single cell suspension by gently pipetting up and down and incubate for 30 minutes at 37°C protected from light.

c. Upon completion, spin down the cells and remove the media. **DO NOT** wash the cells. Treat the cells with compound(s) of interest for desired time period directly in culture media, ROS Assay Buffer supplemented with 10% FBS, or culture media without phenol red. Include appropriate controls. If using ROS Inducer as an **experimental control**, dilute the stock to 1X and treat the cells for 1 hour prior to analyses.

d. Adjust the cell concentration so at least 1 x10^4 cells should be analyzed per experimental condition. Gently pipette cells up/down to ensure single cell suspension and analyze on flow cytometer in FL-1 channel. Establish forward and side scatter gates from negative control cells to exclude debris and cellular aggregates. Mean fluorescence intensity in Ex/Em = 495/529 nm can be quantified and compared between untreated cells and cells treated with test compounds, or between different cell types.

2. Detection of ROS in Suspension and Adherent Cells by Microplate Assay:

a. Seed 2.5 x10^4 adherent cells per well in 96-well plate to obtain ~ 70-80% confluency on the day of experiment. Allow cells to adhere overnight. Grow suspension cells so that approximately 1.5 x 10^5 cells per well are available. Next day, remove the media and wash the adherent cells in 100 µl of ROS Assay Buffer. Collect suspension cells by centrifugation and wash once in PBS. Discard the wash.

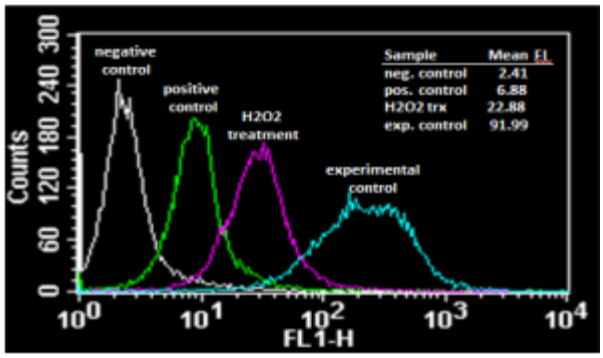
b. Add 100 µl of 1X ROS Label diluted in ROS Assay Buffer per well into adherent cells or re-suspend the pelleted cells at 1.5 x 10^6 cells/ml. Incubate for 45 min at 37°C in the dark.

c. For adherent cells: remove the ROS Label, add 100 µl of ROS Assay Buffer or PBS and measure fluorescence immediately, or treat the cells with 100 µl of diluted test compound(s) for desired period of time. Include appropriate controls as well as blank wells (media or buffer only). For suspension cells: wash the cells by centrifugation in ROS Assay Buffer, maintain the same cell concentration. Seed 100,000 labeled cells per well in 100 µl volume and measure the ROS or treat the cells with test compound(s) in ROS Assay Buffer supplemented with 10% FBS or media without phenol red for appropriate time. If using ROS Inducer as an **experimental control**, dilute the ROS inducer stock to 1X and treat the cells for 1 hour prior to analyses.

d. Measure fluorescence at Ex/Em= 495/529 nm in end point mode in presence of compounds and controls. Determine change in fluorescence after background subtraction.

3. For **Fluorescence microscope** analysis: seed the cells directly onto glass slides or tissue culture plates to ensure~50-70% confluency on the day of the experiment. Follow the kit protocol and upon completion observe the cells immediately using emission filter appropriate for fluorescein.

A.



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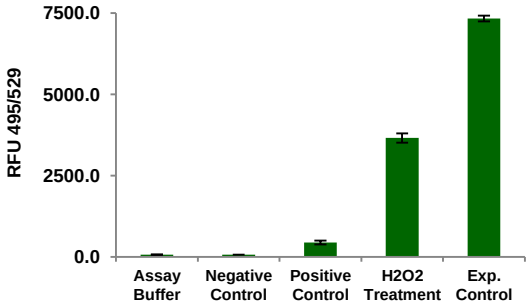

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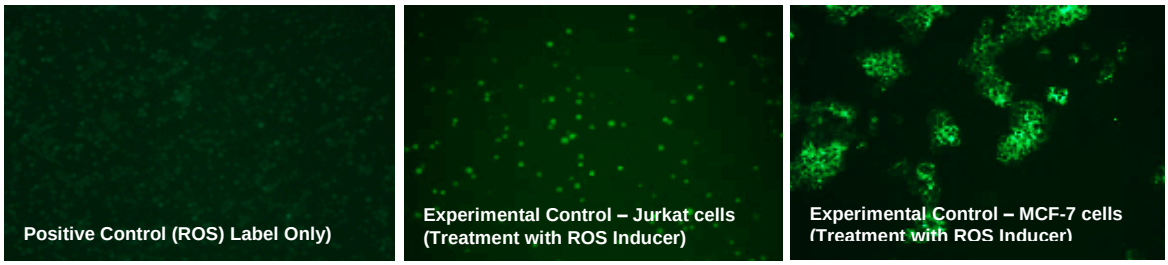

Figures: Analysis of oxidative stress in live cells based on ROS staining. Live cells labeled and treated with ROS Inducer and 100 µM of H_2O_2 according to the kit protocol. **A.** 2 x10^4 Jurkat cells per condition were analyzed by FACS. Plotted intensity values show a significant increase in ROS production between treatments and controls. **B.** 1x10^5 Jurkat cells were analyzed on a plate reader. Mean+/- standard deviation plotted for 3 replicates per condition. **C.** Profiling of ROS formation by Fluorescence Microscopy in Jurkat and MCF-7 live cells.

VIII. RELATED PRODUCTS:

Ascorbic Acid Colorimetric/Fluorometric Assay Kit (K661)
Catalase Activity Colorimetric/Fluorometric Assay Kit (K773)
EZCell™ Glutathione Detection Kit (Blue Fluorescence) (K504)
Hydrogen Peroxide Colorimetric/Fluorometric Assay Kit (K265)
Cardiolipin Assay Kit (Fluorometric) (K944)
Ethanol Colorimetric/Fluorometric Assay Kit (K620)
Glutathione Colorimetric Assay Kit (K261)
Nitric Oxide Colorimetric Assay Kit (K262)

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