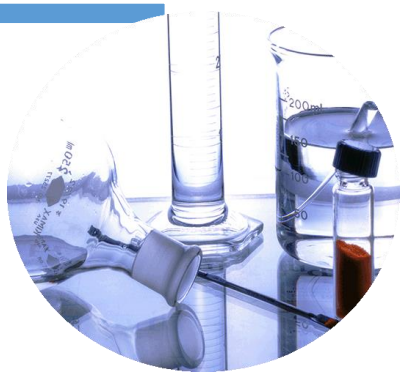
Ros Assay Kit

INSTRUCTION MANUAL

Assays kits



Reactive Oxygen Species Assay Kit

The Ros Assay kit is a ready-to-use assay kit for quantitative measurement of cellular Reactive Oxygen Species (ROS) in cells.

Description	Content	Catalog Number	Number of assays
ROS ASSAY	25mM DFC-DA in DMF (35µL) 10X ROS Buffer (5 mL) Tert-Butyl Hydrogen Peroxide (TBHP) 25 mM (50 µL)	**ROS0300**	3 x 96-well plate

For any technical questions, contact us at tech@ozbiosciences.com

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1. Technology

1.1. Description

Reactive Oxygen Species (ROS) are chemically reactive species containing oxygen. The term "ROS" defines a class of endogenous, highly reactive, oxygen (and nitrogen) –bearing molecules and is generally used to describe a number of reactive molecules and free radicals derived from molecular oxygen. ROS include oxygen ions/O2., free radicals (superoxide/O2– and hydroxyl radicals) and peroxides (hydrogen peroxide/H2O2) and are the products of normal oxygen consuming metabolic process in the body. ROS are key molecules with important cell signaling roles when maintained at proper cellular concentrations; a state of cellular stress generally leading to a dramatic imbalance in ROS levels. If not reversed, this effect termed oxidative stress may lead to modification of other oxygen species, proteins or lipids. An excess production or decreased scavenging of ROS has been implicated in the pathogenesis of diverse diseases such as neurodegeneration, diabetes, cancer, and atherosclerosis.

The ROS Assay Kit (Green Fluorescence) is a cell-based assay designed for measuring Reactive Oxygen Species activity within a cell using the cell-permeable fluorogenic probe 2', 7'-Dichlorodihydrofluorescein diacetate (DCF-DA). Once DCF-DA has diffused into cells it is deacetylated by cellular esterases to a non-fluorescent compound and rapidly oxidized by ROS into DCF. DCF is highly fluorescent and can be detected by microscopy, titration in microplate and also cytometry (ex 485/em 535). The fluorescence intensity that results is proportional to the ROS levels within the cell.
Each ROS Kit provides sufficient reagents for approximately 300 measurements in microplate format and includes Tert-Butyl hydroperoxide (organic peroxide) solution provided as a positive control for ROS generation.

1.2. Storage and shipping condition

Storage: Upon reception, store the DFC-DA tube (avoid multiple freeze/thawing cycles) and the 10X ROS Buffer at -20°C. Store the TBHP tube at 4°C.

Shipping condition: The kit is shipped at +4°C.

2. Applications and Protocols

2.1. General Considerations

- Each ROS Assay Kit is suitable for approximately 300 assays in 96-well plates or 150 assays in 24-well plates
- Prefer using black 96-well/clear bottom plates for fluorescence measurement
- We recommend to always include a positive control sample (cells treated with TBHP for 2-4 hours) to your experiment

2.2. Solution preparation

Allow reagent to reach room temperature before beginning. Avoid direct exposure to light.

1X ROS Buffer.

Dilute the 10X ROS Buffer 1:10 by adding 9 vol. of distilled H2O to 1 vol. of 10 X ROS Buffer. Store the solution at 4°C.

NOTE: This solution can be kept at 4°C for several months.

DCF-DA Solution.

Prepare DCF-DA solution extemporaneously by diluting the DFC-DA (25mM) solution into 1X ROS Buffer according to the table 1 below. We do not recommend preparing DCF-DA solution for less than 10 assays (1 mL) in 96-well plate (5 assays in 24-well plate).

Table 1: Volumes to consider for preparing DCF-DA Solution

96-well plate (100 µl)	24-well plate (200 µL)	DCF-DA 25mM (µL)	1X ROS Buffer (mL)	**Total DCF-DA solution (mL)**
10	5	1	1	**1**
50	25	5	5	**5**
100	50	10	10	**10**

Tert-Butyl Hyrogen Peroxide (TBHP) solution (positive control).

Fresh working solution should be made for every assay and discarded after use. Prepare a 250 µM TBHP working solution by diluting 25 mM TBHP stock solution into your cell culture medium. The final concentration of TBHP to use depends on the cell type and sensitivity: depending on the cell, we recommend to use concentrations between 5 and 50 µM TBHP.

2.3. General protocol for 96-well plate – adherent cells

Cell preparation:

1. Seed cells in a 96-well plate under standard culture conditions.

NOTE: we recommend using a black plate with clear bottom for an enhanced fluorescence measurement.

2. Carry out experiment by adding chemical compounds or biological agents to cells
3. Keep untreated wells for a 2 or 4 hours stimulation with TBHP (positive control)
4. 2 to 4 hours prior the experiment stimulate cells with the desired TBHP concentration

Measuring ROS:

5. Remove culture medium and gently wash cells using 100 µL PBS.
6. Repeat step 5 one time.
7. Stain the cells by adding 100 µL of diluted DCF-DA solution.
8. Incubate cells with DCF-DA solution for 30 min at 37°C in the dark.
9. Remove DCF-DA solution and gently wash cells using 100 µL PBS.
10. Add 100 µL/well PBS and read fluorescence immediately.
11. Measure fluorescence using a fluorescence microtiter plate reader (exc: ˜485nm/ em: ˜535nm).
12. Subtract background fluorescence of the non-treated cells from all other values.
13.

2.4. General protocol for 24-well plate – adherent cells

Follow the same protocol than for 96-well plates; changes only apply on the volumes used:

- Steps 5, 7, 9, 10 : use 200 µL PBS or DCF-DA solutions per well instead of 100 µL

2.5. General protocol for suspension cells

Cell preparation:

1. Cultivate your cells under standard culture conditions.

NOTE: Cells should be healthy and density should not exceed $1x10^6$ cells/mL

2. Carry out experiment by adding chemical compounds or biological agents to cells and prepare supplemental wells for TBHP stimulation
3. 2 to 4 hours prior the experiment stimulate cells with the desired TBHP concentration

Measuring ROS:

4. Harvest sufficient volume of cells to reach ~ $1x10^5$ cells per sample into 1.5 mL tubes
5. Wash cells by centrifugation (5min x 500g) using PBS.
6. Repeat this step one time and remove supernatant.
7. Resuspend the cell pellets in 200 µL of DCF-DA solution.
8. Incubate cells with DCF-DA solution for 30 min at 37°C in the dark.
9. Centrifuge cells (5min x 500g) and remove supernatant containing DCF-DA solution
10. Resuspend cells in 100 µL/tube PBS and place them into black microtiter plate.
11. Measure fluorescence using a fluorescence microtiter plate reader (exc: 485nm/ em: 535nm).
12. Subtract background fluorescence of the non-treated cells from all other values.

NOTE: at step 10, a 20 µL aliquot can be applied onto a microscope slide for fluorescent microscopy visualization. Protect samples from light.

2.6. General protocol for Cytometry analysis

Cell preparation:

1. Cultivate your cells under standard culture conditions and carry out experiment by adding chemical compounds or biological agents to cells.
2. 2 to 4 hours prior the experiment stimulate cells with the desired TBHP concentration

Measuring ROS:

3. Harvest cells and prepare a single cell suspension by (1) pipetting suspension cells or (2) detaching adherent cells using trypsin.
4. Dispose sufficient volume of cells to reach ~ $1x10^5$ cells per sample into 1.5 mL tubes
5. Wash cells by centrifugation (5min x 500g) using PBS.
6. Repeat this step one time and remove supernatant.

7. Resuspend the cell pellets in 200 µL of DCF-DA solution.
8. Incubate cells with DCF-DA solution for 30 min at 37°C in the dark.
9. Centrifuge cells (5min x 500g) and remove supernatant containing DCF-DA solution
10. Resuspend cells in 100 µL/tube PBS.
11. Analyze on flow cytometry using 485 nm laser for excitation and 535 nm for emission.
12. Determine fold change between control and treated samples.

2.4. Performance characteristics

Adherent cells - Fluorescence microscopy

NIH-3T3 and HeLa cells were cultivated under standard culture conditions and kept untreated or stimulated with 5 and 10 µM TBHP for 2 hours prior ROS measurement. After washing procedure, cells were labelled with DCF-DA solution for 30 min at 37°C and ROS production was monitored under fluorescence microscopy.

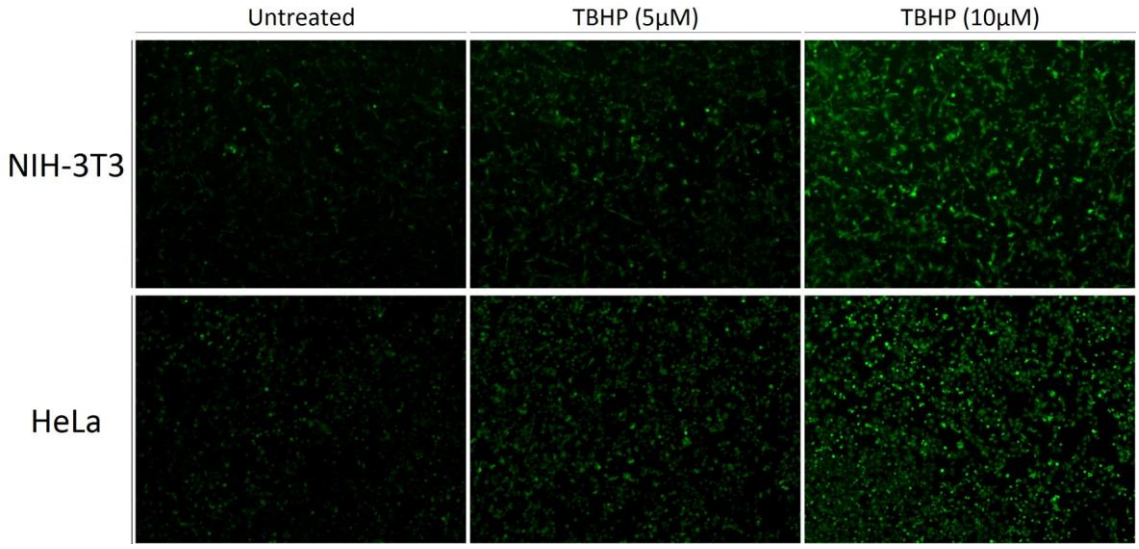

Figure 1: Evaluation of ROS production under fluorescence microscopy in NIH-3T3 and HeLa cells 2 hours after TBHP treatment. Visual fluorescence intensity increases with the dose of TBHP in both NIH-3T3 and HeLa cell lines.

Adherent cells – fluorescence evaluation (plate reader/cytometry)

COS7 cell line was cultivated under standard culture conditions and stimulated with increasing concentrations of TBHP for 2 hours (0 to 50 µM). Cells were then directly processed for ROS measurement using a fluorescence microtiter plate reader (exc: 485nm/ em: 535nm) or detached from their adherent state to perform cytometry analysis. The standard protocol was followed as previously described.

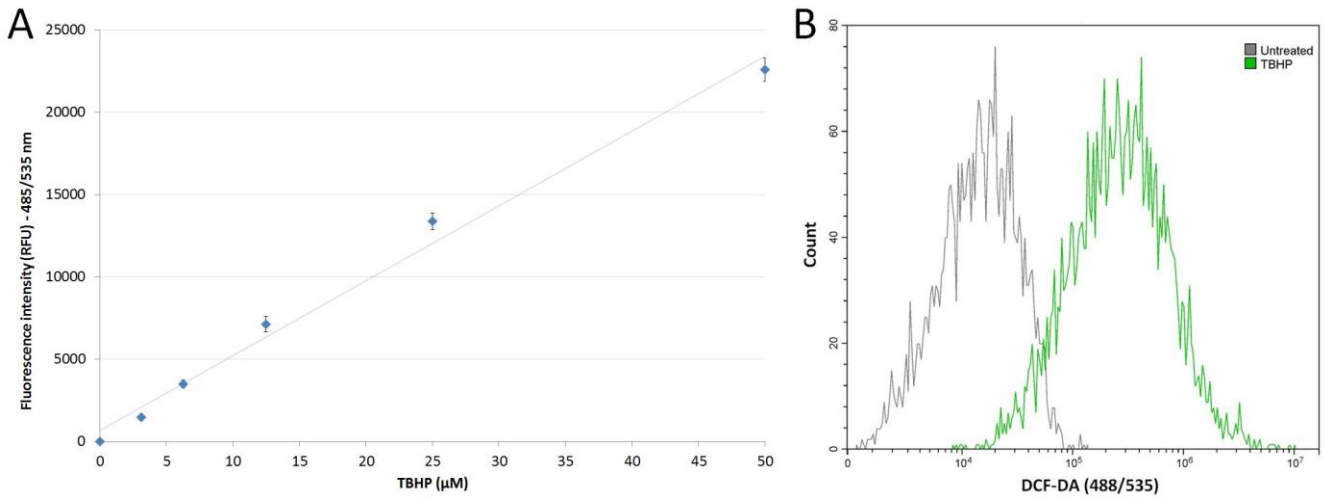

Figure 2: ROS production measurement using ROS assay Kit on COS7 adherent cell line after TBHP stimulation. (A)Fluorescence intensity measured using a fluorescence microtiter plate reader (exc: 485nm/ em: 535nm) demonstrates increasing ROS production proportionally to TBHP concentration. (B) COS7 were treated with 50 µM TBHP and labelled (TBHP) or not (Untreated) with DFC-DA. Ros production was then analysed by flow cytometry. A 15 fold difference between control and TBHP mean fluorescence intensities can be noticed.

Suspension cells – fluorescence evaluation (plate reader/cytometry)

Jurkat T cells were cultivated under standard culture conditions and stimulated with increasing concentrations of TBHP for 2 hours (0-250 µM). Cells were then processed for ROS measurement using a fluorescence microtiter plate reader (exc: 485nm/ em: 535nm) according to the standard protocol for suspension cells.

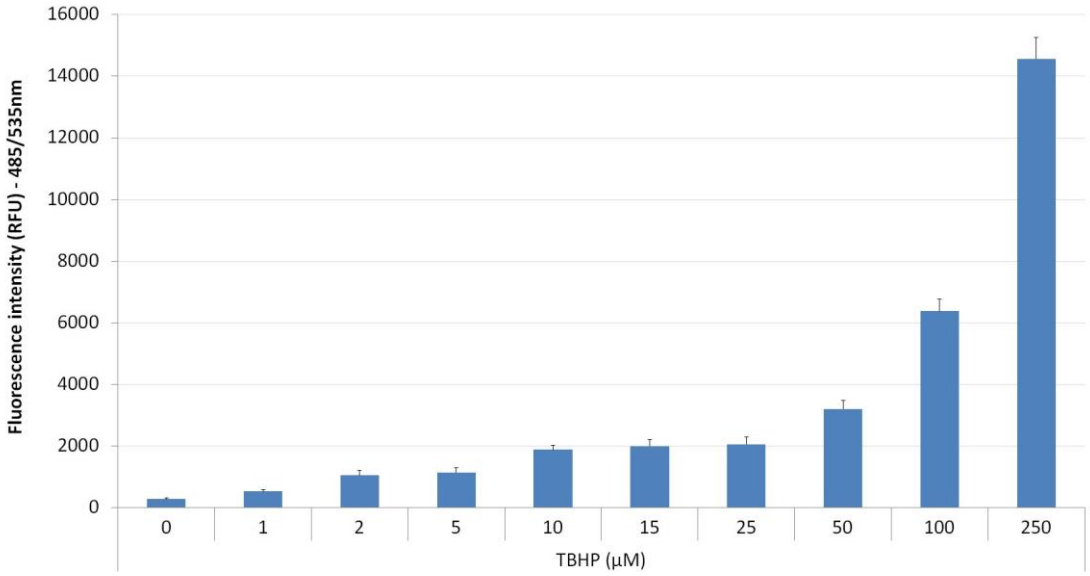

Figure 3: ROS production measurement using DCF-DA solution in Jurkat T suspension cell line after TBHP stimulation. Fluorescence intensity measured using a fluorescence microtiter plate reader (exc: 485nm/ em: 535nm) demonstrates increasing ROS production proportionally to TBHP concentration.

Adherent cells – Antioxidants effect on ROS production

HEK-293 cell line was cultivated under standard culture conditions and stimulated 3 µM TBHP for 2 hours in presence of increasing doses of two antioxidants. Cells were then directly processed for ROS measurement using a fluorescence microtiter plate reader (exc: 485nm/ em: 535nm) or detached from their adherent state to perform cytometry analysis. The standard protocol was followed as previously described.

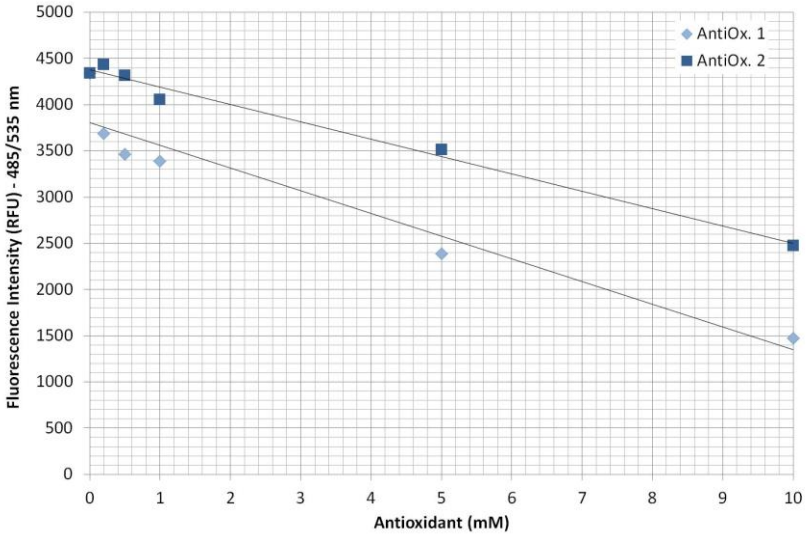

Figure 4: Antioxidants inhibits the production of ROS in adherent cells. ROS assay kit allows to monitoring changes in ROS production depending on antioxidant. Both antioxidants reduce the oxygen species production in a dose dependant manner; however results showed discrepancy between the inhibitory capacities of the antioxidants.

3. Related Products

PLASMIDS PVECTOZ
pVectOZ-LacZ
pVectOZ-SEAP
pVectOZ-GFP
pVectOZ-Luciferase
ASSAY KITS
Luciferase Assay kit
OZBlue Cell viability kit
Bradford – Protein Assay Kit
MTT cell proliferation kit
SEAP Assay Kit
X-Gal Staining Kit
Senescence Kit for Stem Cells
β-Galactosidase assay kits (CPRG/ONPG)

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