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ab186029 Cellular Reactive Oxygen Species Detection Assay Kit (Deep Red Fluorescence)

For the detection of Reactive Oxygen Species (ROS) in live cells.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Cellular Reactive Oxygen Species Detection Assay Kit (Deep Red Fluorescence) (ab186029) is a sensitive fluorometric one-step assay to detect intracellular reactive oxygen species (ROS) such as superoxide and hydroxyl radical in live cells. The assay uses our proprietary ROS Deep Red dye to quantify ROS: the dye is cell-permeable and reacts with ROS present in the cell to generate a deep red fluorescent signal (Ex/Em = 650/675 nm).

The assay can be performed in 1 hour and can be detected by fluorescence microscopy, flow cytometry, microplate fluorometry or high-content imaging. It can be easily adapted to use in 384-well microplate format.

Reactive oxygen species (ROS) are natural by-products of the normal metabolism of oxygen. The two major sources of cellular ROS are Complex I (NADH dehydrogenase ubiquinone-ubiquinol reductase) and Complex III (ubiquinol cytochrome c reductase), both part of the mitochondrial electron transport chain. These two complexes generate ROS particularly when electron transport is slowed by high mitochondrial membrane potential ($\Delta\psi m$). The major product of ROS in mitochondrial is in the form of superoxide and hydroperoxyl radical. Superoxide generated in complex III occurs in the presence of slow electron transport which allows for the ubisemiquinone anion radical to react with oxygen dissolved in the membrane. The exact source of superoxide generated by complex I is less known and it is believed to be due to electron leakage from its iron-sulfur clusters.

Low levels (or optimum levels) of ROS play an important role in signaling pathways. However, when ROS production increases and overwhelms the cellular antioxidant capacity, it can induce macromolecular damage (by reacting with DNA, proteins and lipids) and disrupt thiol redox circuits. In the first instance, damage can lead to apoptosis or necrosis. Disruption of thiol redox circuits can lead to aberrant cell signaling and dysfunctional redox control.

2. Protocol Summary

Grow cells in appropriate medium

Treat cells to induce ROS

Stain cells with ROS Deep Red Dye Working Solution

Incubate cells for 30 – 60 minutes at 37°C

Monitor changes in fluorescence intensity (Ex/m = 650/675 nm)

Determine ROS change as percentage of control
after background subtraction

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- If applicable, please refer to the current Safety Data Sheet (SDS) provided with this product for safety, handling, and disposal information. The most up to date and current versions are available on our website https://www.abcam.com/en-us

4. Storage and Stability

Store kit at -20°C in the dark immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage Condition (Before prep)	Storage Condition (After prep)
Assay Buffer	20 mL	-20°C	-20°C
DMSO	100 µL	-20°C	-20°C
ROS Deep Red Dye (lyophilized)	1 vial	-20°C	-20°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Fluorometric microplate reader (bottom read mode) or flow cytometer capable of measuring fluorescence at Ex/Em = 650/675 nm
- PBS or HHBS buffer
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- General tissue culture supplies
- Sterile 96 well plate with clear flat bottom, preferably black (if performing assay in microplate format). Use a poly-D-lysine coated plate for suspension cells

8. Technical Hints

- **This kit is sold based on number of tests. A "test" simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 Assay Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 DMSO:

Ready to use as supplied. Warm by placing in a 37°C bath for 1 – 5 min to thaw the DMSO solution before use.

9.3 ROS Deep Red Dye:

Reconstitute dye by adding 40 µL of DMSO (step 9.2) to the vial to prepare **1000X ROS Deep Red Stain Stock Solution**. Mix well by pipetting up and down. Equilibrate Stock Solution to room temperature before use.

Aliquot unused Stock Solution so that you have enough volume to perform the desired number of assay. Store at - 20°C with the cap sealed tightly away from light. Avoid freeze-thaw cycles. Use within 2 months.

Δ Note: ROS Deep Red Stock Solution can be diluted 5-fold to 200X in DMSO for convenience. 200X stock solution can be aliquoted and stored as initial solution (Step 9.3).

10. Assay Procedure – Microplate format

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate.
- Prepare all reagents and samples as directed in the previous sections.
- Each cell line should be evaluated on an individual basis to determine the optimal cell density.
- The protocol described here is for 96-well microplate format. You can adapt the protocol for 384-well format by dividing working volumes by half.

10.1 Grow cells:

10.1.1 Adherent cells: plate cells overnight in growth medium at 10^4 – 4 x 10^4 cells/90 µL per well.

Δ Note: for 384 well plate, use 2,5 x 10^3 – 10^4 cells/20 µL per well.

10.1.2 Suspension cells: on the day of the assay, centrifuge cells from the culture medium and resuspend the cell pellet in culture medium at 5 x 10^4 – 10^5 cells/90 µL per cell in a poly-D-lysine coated plate.

Δ Note: for 384 well plate, use 10^4 – 2.5 x 10^4 cells/20 µL per well.

10.1.3 Centrifuge plate at 800 rpm for 2 minutes with brake off.

10.2 Prepare ROS Deep Red Stain working solution:

10.2.1 Add 20 µL of 1000X ROS Deep Red Stock Solution (Step 9.3) to 10 mL of Assay Buffer and mix well.

Δ Note: ROS Deep Red Stain **working solution** is stable for at least 2 hours at room temperature.

10.3 Run ROS assay:

10.3.1 Treat cells with 10 µL of 10X test compounds in PBS or HHBS. For untreated cells, add 10 µL of compound buffer.

Δ Note: for 384 well plate, add 5 µL of 5X test compounds.

10.3.2 Induce ROS production by incubating cell plate at room temperature or in a 37°C/5% CO_2 incubator for at least 15 minutes, or required time for your compound.

Δ Note: for example, incubate HeLa cells for 30 minutes when treated with 100 µM TBHP (tert-butyl-hydroperoxide).

10.3.3 Add 100 µL/well of ROS Deep Red Working Solution into the cell plate.

Δ Note: for 384 well plate, add 25 µL/well.

10.3.4 Incubate cells in a 37°C/5% CO_2 incubator for 30 – 60 minutes.

10.3.5 Monitor fluorescence increase at Ex/Em = 650/675 nm (cut off = 665 nm) with bottom read mode.

11. Assay Procedure – Flow cytometry

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate.
- Prepare all reagents and samples as directed in the previous sections.

11.1 Grow cells:

11.1.1 Prepare cells at a density of 5 x 10^5 – 10^6 cells/mL.

Δ Note: Each cell line should be evaluated on an individual basis to determine the optimal cell density.

11.2 Run ROS assay:

11.2.1 Treat cells with test compounds in PBS or HHBS. For untreated cells, add equivalent amount of compound buffer.

11.2.2 Induce ROS production by incubating cell plate at room temperature or in a 37°C/5% CO_2 incubator for at least 15 minutes, or required time for your compound.

Δ Note: for example, incubate HeLa cells for 30 minutes when treated with 100 µM TBHP (tert-butyl-hydroperoxide).

11.2.3 Add 1 µL of 1000X ROS Deep Red Stock Solution (Step 9.3) to 1 mL of cells and mix well.

If using 200X ROS Deep Red Stock Solution, add 5 µL to 1 mL of cells.

11.2.4 Incubate cells in a 37°C/5% CO_2 incubator for 30 – 60 minutes.

11.2.5 Monitor fluorescence increase with a flow cytometer at Ex/Em = 650/675 nm (FL4 channel).

12. Data Analysis

FLUORESCENCE MICROPLATE MEASUREMENT

- Subtract blank readings from all measurements (control and treated)
- Using fluorescent intensity, determine fold change between control and treated cells.

FLOW CYTOMETRY MEASUREMENT

- Establish appropriate FSC vs SSC gates to exclude debris and cell aggregates.
- Using mean fluorescent intensity, determine fold change between control and treated cells.

13. Typical data

Data provided for **demonstration purposes** only.

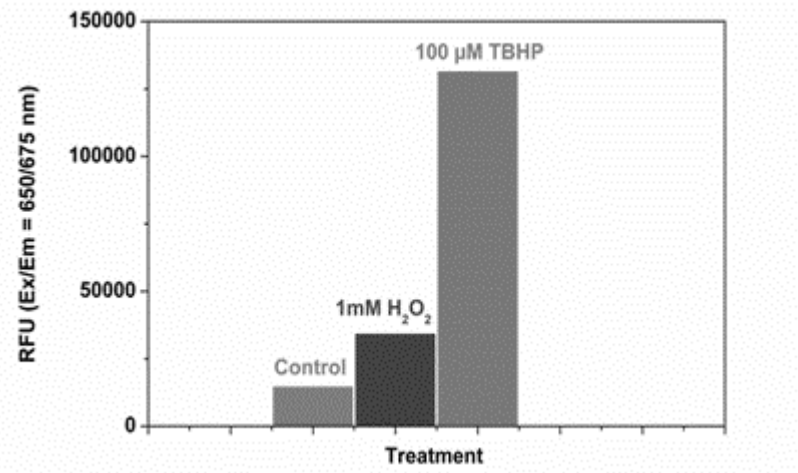

Figure 1. Detection of ROS in HeLa cells. HeLa cells were seeded overnight at 1.5 x 10^4 cells/90 µL per well in a Costar black wall/clear bottom 96-well plate. Cells were untreated (control) or treated with 1 mM H_2O_2 or 100 µM TBHP (ter-butyl hydroperoxide) for 30 minutes at 37°C. The ROS Deep Red stain solution (100 µl/well) was added and cells were incubated in a 37°C/5% incubator for 1 hour. The fluorescence signal was monitored at Ex/Em = 650/675 nm (cut off 665 nm) with bottom read mode using FlexStation (Molecular Devices).

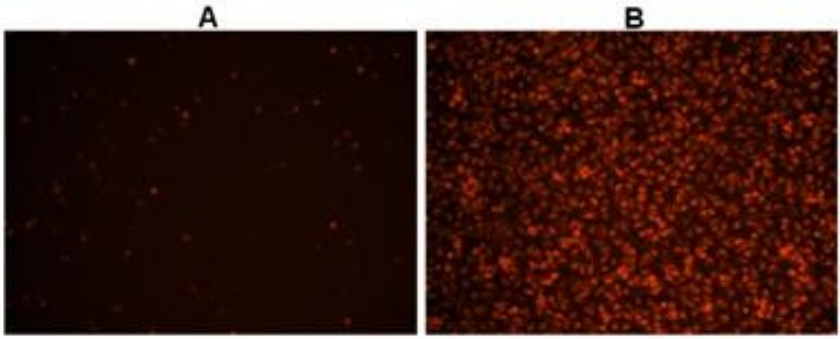

Figure 2. HeLa cells were stained were stained with ROS Deep Red stain solution in a Costar black wall/clear bottom 96-well plate following protocol for microplate assay. A: Untreated control cells. B: Cells treated with 100 µM TBHP (ter-butyl hydroperoxide) for 30 minutes before staining.

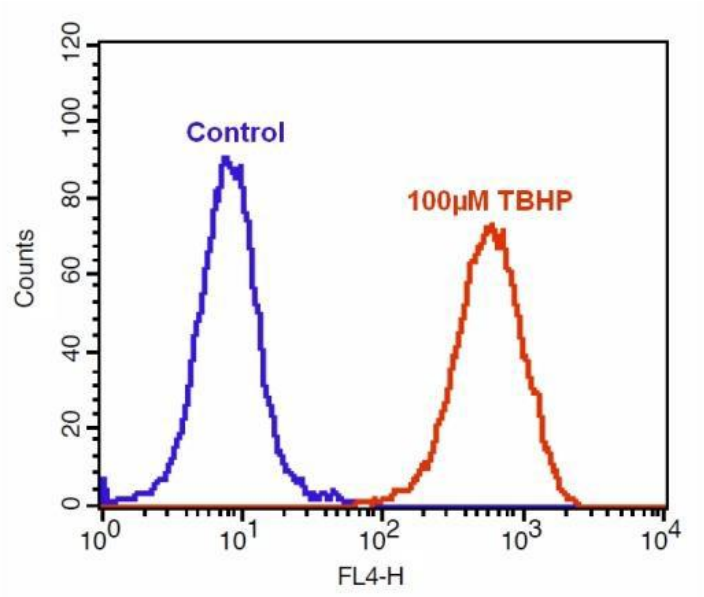

Figure 3. Detection of ROS in Jurkat cells. Jurkat cells were left untreated (blue) or treated with 100 µM TBHP (ter-butyl hydroperoxide) (red) for 30 minutes at 37°C. The ROS Deep Red stain solution was added and cells were incubated in a 37°C/5% incubator for 1 hour. Fluorescent intensities were measured with a FACScalibur flow cytometer using FL4 channel.

14. FAQs

Q. Do I need to do any washing steps?

A. No washing steps are required for the staining procedure. However, we do recommend to change the culture media before addition of the test compounds.

Q. Can I fix my cells?

A. The dye is not compatible with fixation. The dye is for live cells and in solution assays.

15. Notes

Technical Support

For all technical or commercial enquiries please go to:
https://www.abcam.com/en-us/contact-us
https://www.abcam.cn/contact-us (China)
https://www.abcam.co.jp/contact-us (Japan)