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Instruction manual for:

Cellular Reactive Oxygen Species (ROS) Assay Kit

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About This Kit

ROS molecules are a class of highly reactive molecules that contain oxygen (and nitrogen) and, are the products of normal oxygen consuming metabolic process in the body. These unstable molecules easily react with other molecules (i.e., Nucleic acids, Proteins, Lipids) within cells and result in detrimental changes of many cell components.

This kit utilizes the widely used fluorogenic H_2DCF-DA probe. The H_2DCF-DA is a cell-permeable probe that easily diffuses into the cell, where intracellular esterases cleave off its diacetate (DA) moiety and renders the molecule (H_2DCF) sensitive to oxidation by ROS. In its oxidized form (i.e., dichlorofluorescein (DCF)), the probe is highly fluorescent and can be detected using fluorescence microscopy, microplate fluorometry or flow cytometry.

Kit Components

Item Label	Quantity	Storage
ROS Buffer Essentials	2 Tablets	2-8°C
Fluorescent Substrate	~5 mg	2-8°C
Substrate Diluent	1 mL	2-8°C
Hydrogen Peroxide	0.2 mL	2-8°C
DCF Standard (1 mM)	0.2 mL	2-8°C
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Storage and Stability

- This kit will perform as specified if **stored at 2-8°C**.
- Use before the **expiration date** indicated on the box.
- Aliquot components in working volumes before storing at the recommended temperature.
- Avoid repeated freeze-thaws of prepared reagents.

Kit Performance

- **Sensitivity:** 10 pM
- **Application:** This kit is designed exclusively for live cells*; fixed samples are not compatible.*

Materials Required (Not Provided)

- Double distilled water (ddH_2O)
- Cell culture medium
- General tissue culture labware
- 96-well black plate with clear flat bottom
- V-bottom, 96-well microplate (in case of suspension cells)
- 1.5 ml sterile microcentrifuge tubes (in case of suspension cells)

Required Instrumentation

- Fluorescent plate reader with filter sets capable of measuring excitation wavelength between 480-500 nm and emission wavelength between 510-550 nm.
- Flow cytometer equipped with Blue Laser (488 nm) and filter for measuring FITC (530/30 nm) (in case of suspension cells).
- Centrifuge with microplate adapter capable of >400 × g (in case of suspension cells).
- CO_2 Incubator
- Laminar Hood

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Warning and Precautions

- It is recommended that gloves, lab coat, and protective eyewear be worn at all times.
- In case of contact with skin or eyes, wash thoroughly with soap and cold water.
- These components should be considered hazardous and disposed of in accordance with established safety procedures.

Reagent Preparation

Note: Equilibrate all materials and prepared reagents to room temperature just prior to use and gently agitate.

Note: Avoid direct exposure to light.

Note: Vortex for about 15 to 30 seconds (avoid excessive vortexing).

1) **1X ROS Buffer:** Dissolve one tablet of ROS Buffer Essentials in 100 mL of ddH_2O to make 1X ROS Buffer. Unused 1X ROS Buffer can be stored at 4°C for several months.

2) **Fluorescent Reagent:** Reconstitute the content of the Fluorescent Substrate vial in 500 µL of Substrate Diluent (DMSO) to yield a 20 mM stock solution of Fluorescent Reagent. This solution is stable for at least 2 months if stored at -20°C. Avoid freeze-thaw cycles.

3) **DCF Staining Solution:** In a separate tube, dilute 15 µL of the prepared Fluorescent Reagent in 10 ml cell culture media, preferably without FBS, and stir or vortex for homogeneity. This solution (30 µM) is unstable and must be used immediately for staining cells in culture (2 hours at room temperature). Prepare only enough for immediate applications and any unused DCF Staining Solution should be discarded.

Note: The exact concentration of H_2DCF-DA required will depend on the cell line being used but a general starting range would be 10 - 50 µM. Exact concentrations have to be determined on an individual basis by the end user.

Note: Due to light-induced auto-oxidation, both prepared Fluorogenic Substrate reagent and DCF Staining Solution must be protected from light.

4) **ROS Inducing Reagent:** Add 10 µl of Hydrogen Peroxide reagent to 1.16 mL 1X ROS buffer and mix well to obtain 100 mM concentration of ROS Inducing Reagent. Use 100 mM ROS Inducing Reagent as external positive stimulator within the assay at desired concentration (200 - 500 µM recommended). Diluted Hydrogen Peroxide last for 2 to 4 hours at room temperature. Prepare enough for immediate use.

Standard Preparation

➢ ROS content in unknown samples or the effect of antioxidant or free radical compounds on H_2DCF-DA can be measured against the fluorescence of the provided DCF standard.

1. Take six clean microtubes and label them A-F.
2. Add the amount of DCF standard (1mM) stock solution and 1X ROS Buffer to each tube as described below (Concentration range: 0 – 10 µM).

Tube	DCF Standard (µl)	1X ROS Buffer (µl)	Final Conc. Of DCF (nM)
A	10	990	10,000
B	100 of Tube #A	900	1000
C	100 of Tube #B	900	100
D	100 of Tube #C	900	10
E	100 of Tube #D	900	1
F	-	1000	0

3. Transfer 100 µL of the prepared DCF standards to a 96-well plate suitable for fluorescence measurement.
4. Measure the fluorescence intensity using an excitation wavelength between 480-500 nm and an emission wavelength between 510-550 nm.

Things to Note

1. Black plate with clear bottom for an enhanced fluorescence measurement is recommended.
2. The optimal cell density should be evaluated for each cell line.
3. It is recommended that all samples and controls be assayed at least in duplicate.

Assay Protocol

For Adherent Cells

1. Culture desired number of cells in a 96-well culture plate. Ensure that cells are healthy and not overgrown. *(NOTE: When working with unfamiliar cell lines, we recommend performing a seeding titration as cell types can vary in size and volume).*
2. Treat cells and incubate at 37ºC/5% CO_2 for required time as you planned. Designate wells as positive (H_2O_2) control, non-treated controls, and blank (untreated cells not loaded with dye to examine cellular autofluorescence).
3. Carefully aspirate off the culture media and add 100 µL of 1X ROS Buffer.
4. Carefully discard 1X ROS Buffer from wells.
5. Add 100 µL of DCF Staining Solution to all wells except blank wells.
6. Cover the plate and incubate for 60 min at 37°C - *protected from light*.
7. Following 60 min incubation, add 100 µL of ROS Inducing Reagent with desired concentration to designated positive control wells and incubate for an additional 20 minutes at 37°C - *protected from light*.
8. Carefully aspirate DCF Staining Solution and add 100 µL of 1X ROS Buffer.
9. Carefully discard the Buffer from wells and add 100 µL of 1X ROS Buffer again.
10. Measure the fluorescence intensity using an excitation wavelength between 480-500 nm and an emission wavelength between 510-550 nm.
11. Subtract background fluorescence of the blank wells from all other values.

For Suspension Cells

1. Culture cells per desired protocol in media best suited for your cell line in a V-bottom, 96-well microplate. *(NOTE: When working with unfamiliar cell lines, we recommend performing a seeding titration as cell types can vary in size and volume).*

Note: If a V-bottom 96-well plate or centrifuge microplate adapter is unavailable, use 1.5 mL microcentrifuge tubes instead.

2. Treat cells and incubate at 37ºC/5% CO_2 for required time as you planned. Designate wells/tubes as positive (H_2O_2) control, non-treated controls, and blank (untreated cells not loaded with dye to examine cellular autofluorescence).
3. Centrifuge the plate/tubes at 400 x g for 2 minutes to pellet cells.
4. Without disrupting the cell pellet, carefully remove the culture media and wash with 100 µL of 1X ROS Buffer.
5. Centrifuge the plate/tubes at 400 × g for 2 minutes to pellet cells.
6. Without disrupting the cell pellet, carefully remove off the 1X ROS Buffer, and add 100 µL of DCF Staining Solution to each well/tube.

7. Cover the plate and incubate for 60 min at 37°C - *protected from light*.
8. Following 60 min incubation, add 100 µL of ROS Inducing Reagent with desired concentration to designated positive control wells/tubes and incubate for an additional 20 min at 37°C - *protected from light*.
9. Centrifuge the plate/tubes at 400 × g for 2 minutes to pellet cells.
10. Carefully remove DCF Staining Solution and wash cells with 100 µL of 1X ROS Buffer.
11. Centrifuge the plate/tubes at 400 × g for additionally 2 minutes to pellet cells again.
12. Carefully discard the Buffer from wells/tubes and add 100 µL of 1X ROS Buffer again.
13. Readout:
    a. **Plate reader -** Transfer cells to a black, tissue culture-treated 96-well plate. Place the assay plate on fluorescent plate reader and measure the fluorescence using an excitation wavelength between 480-500 nm and an emission wavelength between 510-550 nm.
    b. **Flow cytometer readouts -** Transfer cells to tubes appropriate for your flow cytometer. DCFDA is typically excited with a 488 nm laser and emits in the FITC channel. Collect at least 20,000 events.

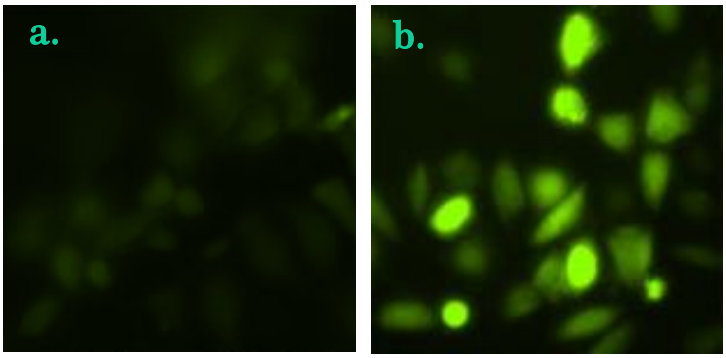
Note: Plate can be monitored for kinetic analysis in microplate reader for up to 1 hour and more if required. Clear cell culture plates are not good candidate for this purpose.

Note: Presence of fluorescence can also be visualized by fluorescence microscopy as pictured below (**Fig. 1**).

Fig. 1: DCF Fluorescence in H_2O_2 treated cells after 1 hour under inverted fluorescence microscope.

(**a.**) Blank

(**b.**) 1 hour post H_2O_2 treatment



Troubleshooting

Problem	Possible Causes	Recommended Solutions
No fluorescence or minimal fluorescence is detected	Cells are not at sufficient density	Conduct seeding titrations to determine optimal cell density before performing experiment
	Gain is not optimized	Adjust gain
High background fluorescence readings for blank samples	Decomposition of fluorescent substrate	Replace substrate with new stock