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Hours: S-T 8:00 AM to 3:30 PM

TEB PAZHOUHAN RAZI

(TPR)

Cellular Reactive Oxygen Species (ROS) Assay Kit

(200 Tests)

Storage and Stability

This kit will perform as specified if stored at -20 °C
Use before the expiration date

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About This Kit

The term "ROS" defines a class of endogenous, highly reactive, unstable molecules that contain oxygen (and nitrogen) and, are the products of normal oxygen consuming metabolic process in the body which easily react with other molecules within cells. A buildup of reactive oxygen species (ROS) may cause damage to cellular and intracellular structures, including cell membranes, proteins, DNA and mitochondria. ROS-induced damage can lead to apoptosis or necrosis of cells and has been implicated in the pathogenesis of diverse diseases such as neurodegeneration, diabetes, cancer, and atherosclerosis.

TPR-ROS Assay Kit is based on a fluorometric assay using ROS sensitive probe DCFH-DA. Once DCFH-DA has diffused into cells, it is hydrolyzed into to a non-fluorescent compound (DCFH) by ester hydrolysis enzyme and rapidly oxidized to strong green fluorescence DCF (dichlorofluorescein). The created fluorescence intensity is proportionally related to the level of ROS in cells and can be detected using fluorescence microscopy, microplate fluorometry or flow cytometry (ex 485/em 535).

Kit Components

Item Label	Item	Quantity	Storage
Reagent 1 **(R1)**	Assay Buffer Essentials	1 Tablet	-20℃
Reagent 2 (**R2**)	DCFH-DA Solution	40 µl	-20℃
Reagent 3 (**R3**)	Hydrogen Peroxide (9.8 M)	0.2 ml	-20℃
Reagent 4 (**R4**)	DCF Standard (1 mM)	0.2 ml	-20℃
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Kit Performance

- This kit is designed exclusively for live cells*; fixed samples are not compatible.*

Warning and precautions

- It is recommended that gloves, lab coat, and protective eyewear be worn at all times.
- These components should be considered hazardous and disposed of in accordance with established safety procedures.

Required Materials (Not Provided)

- Cell culture medium
- General tissue culture lab wares
- 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).

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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).
- Centrifuge with microplate adapter capable of >400 × g.
- CO2 Incubator
- Laminar Hood

Preparation of Reagents

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

1) **Assay Buffer Essentials Tablet:** Dissolve tablet in 100 ml of pure water to make *Ready Assay Buffer*. Unused Ready Assay Buffer can be stored at 4°C for several months.

2) **DCF Staining Buffer**: In a separate tube, dilute 15 µl of R2 Reagent in 10 ml cell culture media, preferably without FBS and stir or vortex to homogeneity. This reagent is stable for at least 2 hours at room temperature. Prepare only enough for immediate applications and any unused DCF Staining Buffer should be discarded immediately after use.
Note: Once opened, the R2 stock solution is stable for at least 2 months if stored at -20°C. Avoid freeze-thaw cycles.
Note: Due to light-induced auto-oxidation, both R2 reagent and DCF staining buffer must be protected from light.

3) **R3 Ready Reagent:** Add 10.2 µl of **R3** stock reagent to 1 ml culture media and mix well to obtain 100 mM concentration of *R3 working reagent*. Use 100 mM *R3 Working Reagent* as external positive stimulator within assay at desired concentration (200-500 µM recommended).

Preparation of Standard Curve

➢ ROS content in unknown samples is determined by comparison with the predetermined DCF standard curve.

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

Tube	DCF Standard (µl)	R1 (µl)	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 DCF standard 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

- Black plate with clear bottom for an enhanced fluorescence measurement is recommended.
- The optimal cell density should be evaluated for each cell line.
- 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).

   Note: A minimum of two replicates for each condition recommended.
3. Carefully aspirate off the culture media and add 100 µl of Ready Assay Buffer.
4. Carefully discard Ready Assay Buffer from wells.
5. Add 100 µl of DCF Staining Buffer to all wells except blank wells.
6. Cover plate and incubate for 60 min at 37°C - *protected from light*.
7. Following 60 min incubation, add 100 µl of R3 stimulator 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 Buffer and add 100 µl of Ready Assay Buffer.
9. Carefully discard Ready Assay Buffer from wells and add 100 µl of Ready Assay 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, you can 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 as positive (H_2O_2) control, non-treated controls, and blank (untreated cells not loaded with dye to examine cellular autofluorescence).

Note: A minimum of two replicates for each condition recommended.

3. Centrifuge the plate 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 Ready Assay Buffer.
5. Centrifuge the plate at 400 × g for 2 minutes to pellet cells.
6. Without disrupting the cell pellet, carefully remove off the Assay Buffer, and add 100 µl of DCF Staining Buffer to each well.
7. Cover plate and incubate for 60 min at 37°C - *protected from light*.
8. Following 60 min incubation, add 100 µl of of R3 stimulator with desired concentration to designated positive control wells and incubate for an additional 20 min at 37°C - *protected from light*.
9. Centrifuge the plate at 400 × g for 2 minutes to pellet cells.
10. Carefully remove DCF Staining Buffer and wash cells with 100 µl of Ready Assay Buffer
11. Centrifuge the plate at 400 × g for additionally 2 minutes to pellet cells again.
12. Carefully discard Ready Assay Buffer from wells and add 100 µl of Ready Assay Buffer again.
13. Readout:

a. **Plate reader -** Transfer cells to a black, tissue culture-treated 96-well plate. Place 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: Presence of fluorescence can be observed on fluorescence microscope, also.

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

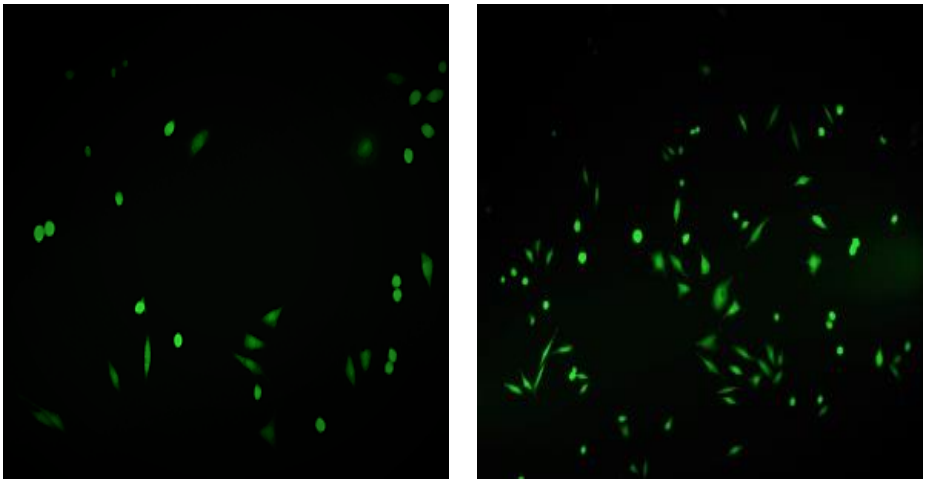

Fig. DCF Fluorescence in H_2O_2 treated cells after 1 hour under inverted fluorescence microscope.

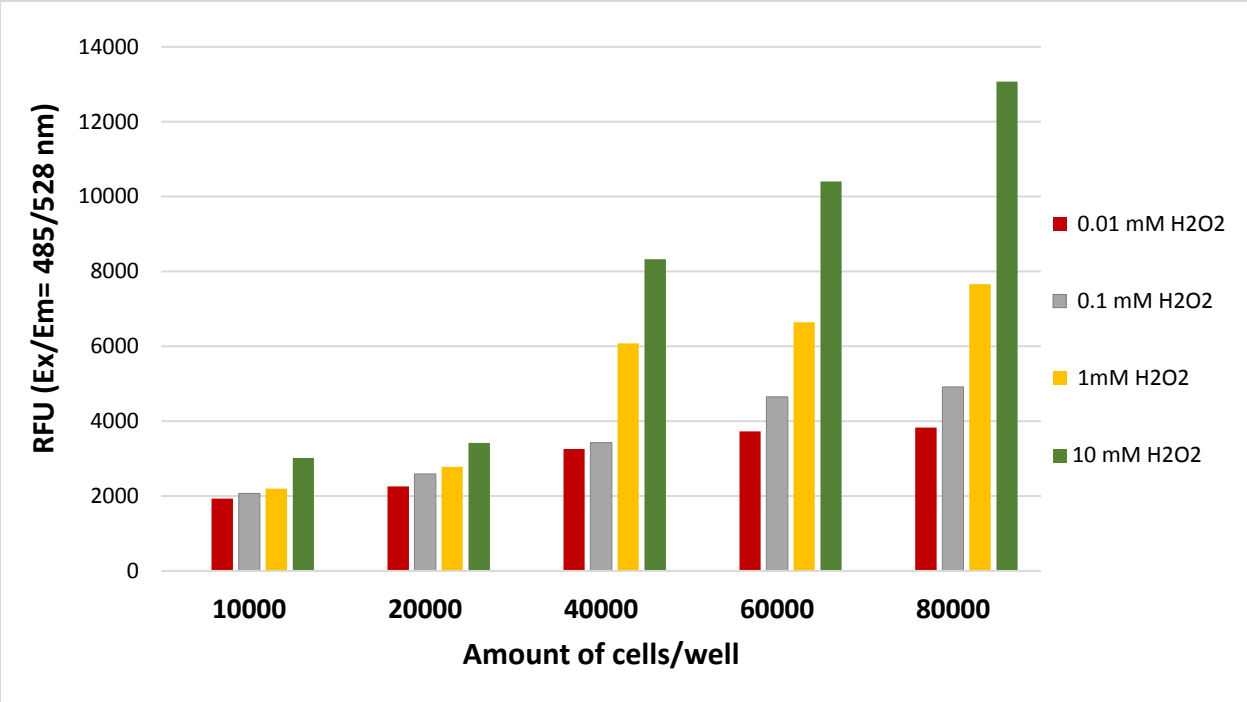

Fig. ROS generated in a typical cell line treated with H_2O_2. Differenet number of cells were plated and treated with various concentrations of H_2O_2 for 20 minutes.

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

References

- Brandt R, Keston AS. Synthesis of diacetyldichlorofluorescin: A stable reagent for fluorometric analysis. *Anal Biochem.* 1965; 11:6-9.
- LeBel CP, Ischiropoulos H, Bondy SC. Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chem. Res. Toxicol.* 1992; 5(2), 227-231.
- Keston AS, Brandt R. The fluorometric analysis of ultramicro quantities of hydrogen peroxide. *Anal Biochem.*1965; 11:1-5.