ROS®

DCFDA Cellular reactive oxygen species (ROS) Assay Kit
Instructions

Quick Facts

1. Storage conditions before opening: **4 ℃/ Protect from light.**
2. Ex/Em (nm) for fluorescence microplate reader：**485/530.**
3. Filter for fluorescent microscope or flow-cytometry：**FITC.**
4. Recommended DCFDA concentration & stain time：**20 μM & 30 mins.**
5. **The well plate with clear flat bottom and black sides is suggested.**

Introdution

Reactive oxygen species (ROS) include a number of active molecules and free radicals produced in the metabolic process of the cells. ROS are both necessary and harmful to organisms. ROS are involved in cell growth, proliferation, development and differentiation, aging and apoptosis, as well as many physiological and pathological processes. ROS also damage DNA and RNA and oxidize proteins and lipids. Excessive ROS will lead to oxidation stress and oxidative damage of cells, further promoting the development of many diseases, such as, cancer, cardiovascular diseases and diabetes.

2,7-Dichlorofluoroscin Diacetate (DCFDA) is a widely-used, cell-permeable redox sensitive fluorescent probe that is oxidized by ROS to yield the highly fluorescent product 2,7-dichlorofluoroscein (DCF) (Fig. 1). This Assay Kit uses DCFDA as a fluorescent probe for the detection of ROS generation. The aqueous solution of H_2O_2, is included as a positive control for ROS generation. *N*-acetyl-cysteine is also included as an antioxidant control.

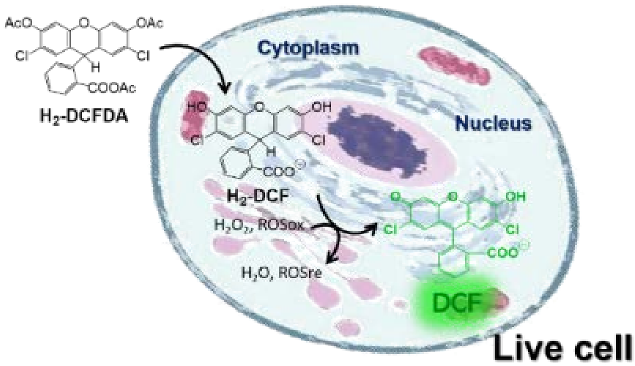


Fig. 1. Mechanism of DCFDA assay.

Shipping and Storage Information

Composition	Shipping	Storage
1. Ⓐreagent：DCFDA (5 mg)	Under 4 °C with protection from light.	6 months under4 °C with protection from light.
2. Ⓑreagent：10X buffer (50 mL)		
3. Ⓒreagent：DMSO (1 mL)		
4. Ⓓreagent：8.8 M H_2O_2 (500 μL)		
5. Ⓔreagent：N-acteyl Cysteine (50 mg)		

Materials Not Supplied

1. Microplate reader or flow cytometer or fluorescence microscope capable of measuring fluorescence at Ex/Em = 485/535 nm (use similar settings to those used to detect FITC).
2. Sterile, tissue culture treated well plate with clear flat bottom and black sides.
3. Test cell lines/compounds/diluents of interest.

Assay procedures：

Reagent preparation

1. **Preparation of DCFDA stock solution:** H2DCFDA is supplied as powder (5 mg，Ⓐreagent) and should be reconstituted in 500μl DMSO (Ⓒreagent) to yield a 20mM stock solution. Upon reconstitution, divided into portions. The stock solution should be stored at -20°~ -80°C in the dark. Gently mix before use. Avoid multiple freeze/thaw cycles. This stock solution is stable for about 3 months.
2. **Prepare 1X Assay Buffer**：Warm Ⓑreagent at 37 ℃ for 1 hour or 60 ℃ for 30 mins. (Ⓑreagent could be gel-like in the refrigerator.) Add 10 mL 10X Assay Buffer (Ⓑreagent) to 90 mL deionized water. Mix gently and thoroughly, cold down to room temperature.
3. **Preparation of DCFDA working solution**：Add the appropriate volume of 20 mM H2DCFDA stock solution (procedure 1.) to 1X Assay Buffer. Vortex to evenly disperse the dye. For example, to make a 20 μM 10mL working solution, add 10 μL of 20 mM DCFDA stock solution to 10 mL 1X Assay Buffer.

Note：This solution is unstable and must be used immediately for staining cells in culture. Any unused staining working solution should be discarded immediately after use.

4. **Prepare H_2O_2 working dilutions**：Dilute 8.8 M H_2O_2 (Ⓓ reagent) solution with 1X Assay Buffer into 1 mM. Do not store diluted solutions. This dilution may be used as a positive control in the assay. For example, to make a 1 mM 8.8 mL dilution H_2O_2 solution, add 1 μL Ⓓ reagent to 8.8 mL 1X Assay Buffer. Mix gently and thoroughly.

Note：This dilution should be used immediately. Any unused dilution should be discarded immediately after use.

5. **Prepare N-acetyl Cysteine Working Reagent**：Weigh and dissolve 10 mg N-acetyl Cysteine (Ⓔreagent) with 200 uL 1X Assay Buffer , to make a 300 mM N-acetyl Cysteine working solution.

Note：This solution should be used immediately. Any unused solution should be discarded immediately after use.

Assay protocol (for 96-well plate)

A. Culture cells in the media best suited for your cell line. Carefully aspirate off the culture media and wash the cells by adding 150 μL of 1X Assay Buffer.
B. Carefully aspirate off 1X Assay Buffer. Stain the cell by adding 150 uL DCFDA working solution (procedure 3.).

Note：The optimal DCFDA working concentration for your application must be empirically determined. Generally, the working concentration is between 10~50 μM.

C. Add 10 μl N-acetyl Cysteine working solution (procedure 5.) to designated negative control wells. Add 10 µl test solution to experimental control wells. Use 1X Assay Buffer (procedure 2.) for the test solution.

D. Cover plate and incubate for 30 mins at 37°C - *protected from light.*
E. Add 10 µl H_2O_2 working dilutions (procedure 4.) to designated positive control wells. control wells.

Note：The negative and positive control were optional, not necessary.

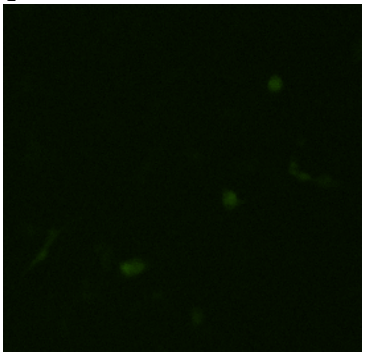
F. Cover plate and incubate for 60 mins at 37°C - *protected from light.*
G. Carefully aspirate Staining Buffer and add 150 µl of 1X Assay Buffer (procedure 2.).
H. The intensity of the signal can be easily measured using a fluorescence microplate reader at Ex/Em = 485/530 nm, or a fluorescent microscope with FITC filter or a flow-cytometry in FL1 channel.

Troubleshooting

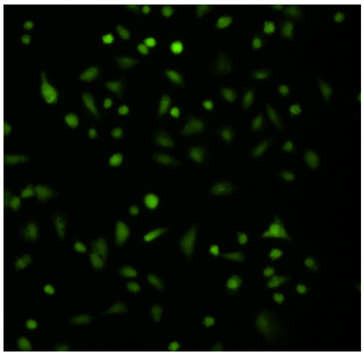
Problem	Possible Causes	Recommended Solution
No fluorescence or minimal fluorescence is detected.	Cells are not at sufficient density.	Determine optimal cell density before performing experiment.
	Gain is not optimized.	Adjust gain to optimize signal.

Example of Results

Negative Control (20mM NAC)



Positive Control (2mM H_2O_2)



(Evaluation of ROS production under fluorescence microscopy in B4G12 cells stain 30 mins with 20 µM H2-DCFDA.).

References

Under development.

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