Intracellular ROS Assay Kit (Fluorometric)

Intracellular ROS Assay Kit (Fluorometric) measures ROS or antioxidant activity in cultured cell.

Catalog number: ARG81192

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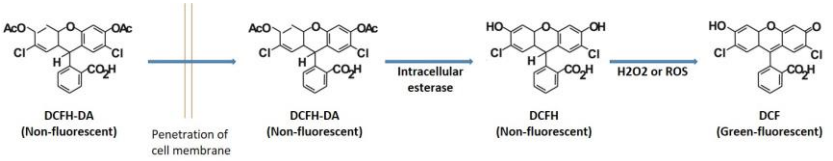
INTRODUCTION

Reactive oxygen species (ROS) are chemically reactive chemical species containing oxygen. Examples include peroxides, superoxide, hydroxyl radical, and singlet oxygen. In a biological context, ROS are formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. However, during times of environmental stress (e.g., UV or heat exposure), ROS levels can increase dramatically. This may result in significant damage to cell structures. Cumulatively, this is known as oxidative stress. ROS are also generated by exogenous sources such as ionizing radiation. [Wikipedia: Reactive oxygen species]

PRINCIPLE OF THE ASSAY

This Intracellular ROS Assay Kit (Fluorometric) is a cell-based assay for measuring antioxidant or ROS activity in cells. Cells are cultured in a 96-well cell culture plate and then pre-incubated with 2', 7'-Dichlorodihydrofluorescin diacetate (DCFH-DA). DCFH-DA is a non-fluorescent, cell-permeable compound. After DCFH-DA diffused into cells and it is deacetylated by intracellular esterases to non-fluorescent 2', 7'-Dichlorodihydrofluorescin (DCFH). The unknown antioxidant or ROS samples are then added to the cells. , the cellular DCFH is rapidly oxidized to highly fluorescent 2', 7'-Dichlorodihydrofluorescein (DCF) by ROS. After a brief incubation, the cells can be read on a standard fluorescence plate reader. The ROS or antioxidant content in unknown samples is determined by comparison with the predetermined DCF standard curve.



MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8 °C. Use the kit before expiration date.

Component	Quantity	Storage information
20X DCFH-DA (20mM)	500 µl	-20°C (Protect from light)
DCF Standard (1mM)	100 µl	-20°C (Protect from light)
Hydrogen Peroxide (8.821 M H2O2)	100 µl	4°C (Protect from light)
2X Cell Lysis Buffer	20 ml	4°C

DCFH-DA and DCF Standard should be aliquoted and stored protect from light at -20°C. Avoid multiple freeze/thaw cycles.

MATERIALS REQUIRED BUT NOT PROVIDED

- Sterile DPBS for cell washes and buffer dilutions
- Hank's Balanced Salt Solution (HBSS)
- Cell culture medium (ie: DMEM with/without 10% FBS)
- 96-well black or fluorescence microtiter plate
- Fluorescent microplate reader capable of reading 480 nm (excitation) and 530 nm (emission)
- Pipettes and pipette tips
- Multichannel micropipette reservoir

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- It is highly recommended assaying the controls, standards and samples at least in duplicates.
- Change pipette tips between the addition of different reagent or samples.

REAGENT PREPARATION

- 1X DCFH-DA: Dilute the 20X DCFH-DA stock solution to 1X in cell culture media, preferably without FBS. Stir or vortex to homogeneity. Prepare only enough for immediate applications.

  Notes:

  1. 1X DCFH-DA/media solution contains 5% methanol. For cells that are sensitive to methanol, we recommend instead preparing a 0.1X (100 µM) solution of DCFH-DA in cell culture media.
  2. Due to light-induced auto-oxidation, DCFH-DA solutions at any concentration must be protected from light.

- Hydrogen Peroxide (H_2O_2): Prepare hydrogen peroxide dilutions in cell culture medium (e.g.: DMEM) or sterile DPBS as needed. Do not store diluted solutions. Hydrogen Peroxide could be used as a positive control in the assay, or as a cell treatment. The concentration of hydrogen peroxide for the assay depending on the cell type user used. It is suggested that user should check suitable concentration from references or do a pretest treating with different concentration of hydrogen peroxide and observe the green cells by fluorescence microscope with

480 nm/530 nm filter. (for example: 1mM H_2O_2 treated HeLa for 30 min could be used as positive control)

- Standards: Prepare a 1:10 serial dilution of DCF standards with cell culture media in the concentration range of 0.01 nM – 10,000 nM by diluting the 1 mM DCF Standard stock solution in cell culture media. The cell culture media serves as zero standard (0 nM)

Dilution table for preparation of DCF standards were as below:

Standard	DCF Conc. (nM)	DCF Standard (µl)	Culture Medium (µl)
S1	10,000	10 µl	990
S2	1,000	100 µl of S1	900
S3	100	100 µl of S2	900
S4	10	100 µl of S3	900
S5	1	100 µl of S4	900
S6	0.1	100 µl of S5	900
S7	0.01	100 µl of S6	900
S8	0	1,000 µl	1,000

Mix 100 µl of each DCF standard and 100 µl of the 2X Cell Lysis Buffer for further analysis when cell cultured in clear cell culture plates.

ASSAY PROCEDURE

Prepare and mix all reagents thoroughly before use. Samples, controls and standards should be assayed in at least duplicates. Controls and standards should be run alongside experimental samples.

DCF Dye penetration:

1. Culture cells in either a clear or black 96-well cell culture plate. Plate cells overnight in growth medium at 1-4 x10^4 cells/well/100 µL for a 96-well

plate.

Note: If using a black plate, choose an appropriate plate based on your fluorometer's reader (i.e. choose a clear bottom black plate for bottom readers).

2. Remove media from all wells and discard. Wash cells gently with DPBS or HBSS for 2-3 times.
3. Add 100 µl of 1X DCFH-DA/media solution to cells. Incubate at 37˚C for 30-60 minutes.
4. Remove 1X DCFH-DA/media solution. Repeat step 2 using multiple washes with DPBS or HBSS. Remove the last wash and discard.
5. Treat DCFH-DA loaded cells with desired oxidant or antioxidant in 100 µL medium. Treat appropriate H_2O_2 in two wells as positive control, and keep 2 wells treated buffer only as negative control.
6. Incubate the plate at 37°C and 5% CO_2 for appropriate time.

Quantitation of Fluorescence

Fluorescence can be analyzed on an inverted fluorescence microscope or by flow cytometry using excitation and emission wavelengths of 480 nm and 530 nm, respectively.

For fluorescence Plate Reader:

1. Assays performed in black cell culture fluorometric plates:

   Plate may be read immediately for kinetic analysis or after 1 hour for static analysis. Plates read for kinetic analysis may be read in increments of 1 and 5 minutes up to 1 hour or more as necessary. Read the fluorescence with a fluorometric plate reader at 480 nm/530 nm.
2. Assays performed in clear cell culture plates:

a. After treatment with desired oxidant or antioxidant, carefully remove treatment media from all wells and discard.
b. Wash cells gently with DPBS or HBSS 2-3 times. Remove the last wash and discard.
c. Add 100 µl of medium to each well. Add 100 µL of the 2X Cell Lysis Buffer, mix thoroughly and incubate 5 minutes.
d. Transfer 150 µL of the cell lysate mixture from step c and Cell Lysis buffer mixed standard (refer to REAGENT PREPARATION) to a 96-well plate suitable for fluorescence measurement. Read the fluorescence with a fluorometric plate reader at 480 nm/530 nm.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of controls and samples.
2. Using linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Use the mean absorbance value for each sample determine the corresponding concentration from the standard curve.
4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.

EXAMPLE OF RESULTS

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.