H_2DCFDA-Cellular ROS Assay Kit

(1000 assays)

Zellbio GmbH (Germany)

CAT No. ZX-11104-1000

www.zellx.de

Application:

Measurement of Reactive Oxygen Species (ROS) levels within the cell cytosol

!!! Caution: This product is for Research Use Only. Not for *in-vitro* Diagnostics !!!

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Please read this insert completely prior to using the product.

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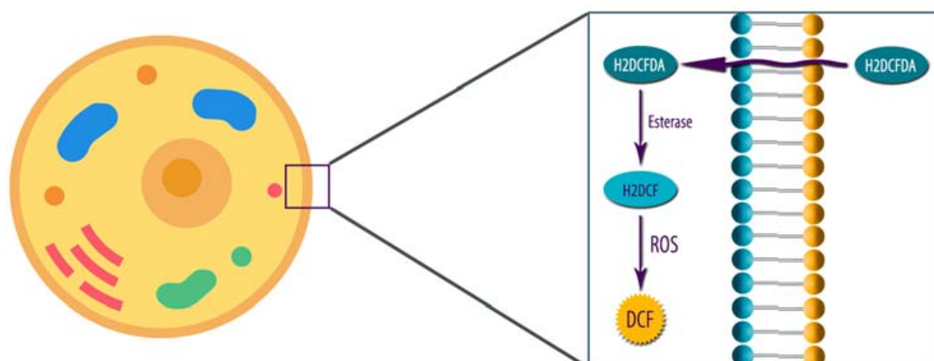
Background

Reactive Oxygen Species (ROS) are chemically-reactive free radicals derived from molecular oxygen. Living cells constantly generate low levels of ROS and free radicals which work as both intra- and intercellular messengers. High levels of ROS, however, damages the biological components of the cells, including proteins, lipids, and DNA. Additionally, oxidative stress reactions can interfere with many essential biological processes such as phagocytosis (immunity and inflammation), cell respiration (mitochondria), metal metabolism, lipid synthesis, lysosomes, xenobiotic biotransformation of organic compounds and cellular signaling. Accumulation of ROS is also associated with aging, apoptosis or necrosis, and is implicated in pathological conditions such as vascular diseases, diabetes, renal ischemia, arteriosclerosis, pulmonary disorders, inflammatory diseases, and cancer.

ZellX® Cellular ROS Assay Kit uses the H_2DCFDA (2',7'-Dichlorodihydrofluorescein diacetate), a fluorogenic dye to measure the activity of hydroxyl, peroxyl and other reactive oxygen species (ROS) in living cells. Firstly, H_2DCFDA diffuses inside the cells and subsequently intracellular esterase cleaves the acetyl groups on H_2DCFDA to yield the non-fluorescent compound H_2DCF, which is rapidly oxidized by ROS to the highly fluorescent 2',7'-Dichlorodihydrofluorescein (DCF). The fluorescence intensity is proportional to the ROS levels within the cell cytosol.



Intended use

Measurement of Reactive Oxygen Species (ROS) levels within the cell cytosol.

Materials supplied in the Kit

Component	*Quantity*
H_2DCFDA (20 mM)	250 µL
ROS Inducer Concentrate (200X)	250 µL
Assay Buffer Concentrate (10X)	25 mL

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Storage instruction

All reagents should be stored at 4 °C until the expiration date of the kit, except H_2DCFDA (20 mM) which need to be aliquoted and store it at -20 °C to -80 °C in the dark. Avoid multiple freeze/thaw cycles.

Materials required but not supplied

Fluorescence microscope, Flow cytometer (FL-1 channel) and Microplate reader capable of measuring Ex/Em 485/530 nm

Phosphate Buffered Saline (PBS, pH 7.4)

Sterile 96-well plate (for reading with fluorescent microplate reader, black area 96-well plates must be used)

Precautions

This kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

General remarks

- Equilibrate all kit components to room temperature (RT) before use.
- The instruction must be strictly followed.
- Pipette tips should not be used more than once to avoid cross contamination.
- Reagents of different batches should not be mixed or used after their expiration dates.
- For ROS measurement only live cells must be used; fixed samples are not compatible with this assays.

Reagent preparation

i. **Assay Buffer:** Prepare a 1:10 dilution of Assay Buffer Concentrate with diH2O (1 part Assay Buffer Conc. with 9 parts diH2O), and mix well.

ii. **ROS Inducer Working solution:** Prepare a 1:200 dilution of Assay Buffer Concentrate with Assay Buffer (1 part ROS Inducer Conc. with 199 parts Assay Buffer), and mix well. (The ROS Inducer Working solution consists of 44 µM H_2O_2)

- Other ROS inducing agents such as doxorubicin, idarubicin, or antimycin (not included) can be used as positive controls based on the experiment setup.

iii. **H_2DCFDA working solution:** Prepare 1:800 dilution of H_2DCFDA with Assay Buffer (1 part H_2DCFDA with 799 parts 1x Assay Buffer). **The stock concentration of H_2DCFDA is 20 mM, making a working concentration of 25 µM after dilution**. Mix well by vortexing (15 to 30 seconds) and protect from

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light. H_2DCFDA working solution must be used immediately after preparation. The exact H_2DCFDA working solution concentration depends on the cell type and must be evaluated by the end user.

Assay Procedure

Each assay must include:

- A **Negative control**: unlabeled cells not exposed to ROS Inducer or treatment
- A **Positive control**: cells only incubated with H_2DCFDA
- An **Experimental control**: labeled cells treated with H_2O_2
- A **Blank control**: media/buffer without cells (To analyze with a fluorescence microplate reader, it is necessary to include a **Blank control**).

Suspension Cells to be analyzed by a **Fluorescence Microplate Reader**

1. Cultivate cells to have approximately 1-2 x 10^5 cells per well on the day of the experiment.
2. Collect cells in a test tube and wash by centrifugation once in PBS.
3. Discard the supernatant and resuspend the cell pellet in 100 µL of H_2DCFDA working solution and incubate at 37°C for 30-60 minutes in the dark.
4. Wash cells by centrifugation with Assay Buffer.
5. Discard the supernatant and resuspend cells in 100 µL complete media (without phenol red) containing 10% FBS.
6. Treat the cells with 100 µL double concentrated (2X) target ROS inducing agents or (2X) H_2O_2 Working solution as a positive control and incubate at 37° C for appropriate time. The appropriate incubation time may vary for different cell types and ROS inducers, and must be evaluated by the end user. **DO NOT wash after treatment.**
7. Read the fluorescent intensity at 530 nm with the excitation at 485 nm.
8. Subtract blank control readings from all measurements and determine fold change from assay control (diluent treated cells if performing toxicity studies).

Adherent Cells to be analyzed by a **Fluorescence Microplate Reader**

1. One day before the experiment, seed the cells in black area 96-well plates at a density of 2-3 x 10^4 cells per well and incubate in a CO_2 incubator at 37°C overnight.
2. Remove the media and add 100 µL/well of Assay Buffer.
3. Remove the Assay Buffer and resuspend the cell pellet in 100 µL of H_2DCFDA working solution and incubate at 37°C for 30-60 minutes in the dark.

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4. Remove DCFDA Solution and add 100 µL/well of PBS.
5. Remove the Assay Buffer and treat the cells with 100 µL target ROS inducing agents or H_2O_2 Working solution as a positive control and incubate at 37° C for appropriate time. The appropriate incubation time may vary depending on the cell type and ROS inducer, and must be evaluated by the end user. **DO NOT wash after treatment**.
6. Read the fluorescent intensity at 530 nm with the excitation at 485 nm.
7. Subtract blank control readings from all measurements and determine fold change from assay control (diluent treated cells if performing toxicity studies).

Flow Cytometry

1. Cultivate cells to have approximately 2-4 x 10^4 cells per each assay condition on the day of experiment
2. Collect the cells and ensure single cell suspension by gently pipetting up and down in case of suspension cells or by fully detaching (e.g. trypsinizing and quenching with media) in case of adherent cells.
3. Wash the cells by centrifugation with PBS.
4. Discard the supernatant and resuspend the cell pellet in 100 µL of H_2DCFDA working solution and incubate at 37°C for 30-45 minutes in the dark.
5. Remove the DCFDA Solution and wash cells by centrifugation with Assay Buffer.
6. Discard the supernatant and treat the cells with the test ROS inducing agents or H_2O_2 Working solution as a positive control and incubate at 37° C for appropriate time. The appropriate incubation time may vary depending in the cell type and ROS inducer, and must be evaluated by the end user. **DO NOT wash after treatment**.
7. Ensure single cell suspension by gently pipetting the cells up and down.
8. Analyze approximately 10,000 cells immediately by FL1 (DCF exited by 488 nm laser and detected at 535 nm filter). Exclude debris and cell aggregates by forward and side scatter gates.
9. Determine fold change between control and treated samples, using mean fluorescent intensity.

Fluorescent Microscopy

1. Cultivate cells to ensure ~50-70% confluency on appropriate container for live cell imaging. **Avoid phenol red as it increases the background fluorescence signal.**
2. Wash cells with Assay Buffer and remove the supernatant.

3. Add 100 µL of H_2DCFDA working solution to the cells and incubate at 37°C for 30-60 minutes in the dark.
4. Remove the H_2DCFDA Solution and wash cells with Assay Buffer.
5. Perform live cell microscopy with FITC or TRITC filter.
6. Visually score cells for brightness and compare between control and samples or use image analysis methods to compare signals on digital photographs of the cells.

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