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Technical Datasheet

EZAssay™ Reactive oxygen species Assay Kit (FRAP)

Product Code: CCK078

1. Introduction

Reactive oxygen species (ROS) are produced inside the cell as a metabolic byproduct of oxidation process. When ROS production goes above threshold level, it causes oxidative stress and damages the cellular integrity. ROS contribute to various cell signaling process and induces various disease conditions like Parkinson's and Alzheimer's disease, aging, cancer etc. Presence of ROS in cellular environment can be detected using cell permeable fluorogenic dye. Dye is sensitive towards hydroxyl, peroxyl and other reactive oxygen species (ROS) activity within the cell. Fluorogenic dye can be detected by cytation, spectrofluorimeter or flow cytometer. The excitation spectra is 488nm and while emission spectra is 528nm.

2. About the Assay

EZAssay™ ROS Kit is designed for determination of reactive oxygen species activity within a cell using a cell permeable fluorogenic dye. Once fluorogenic dye diffuses inside the cells it gets deacetylated to non-fluorescent compound due to esterases. In presence of ROS, non fluorescent dye immediately gets oxidized. Fluorogenic dye is easily detectable using wavelength of excitation-488nm and Emission-528nm. The fluorescent intensity produced is directly proportional to ROS level in the cells.

3. Applications

- **Cell metabolism:** Comparative quantification of changes in cellular activity of with or without treatment of any compound or drug.
- **Cell cytotoxicity**: Evaluation of effect of inhibitors or inducers of apoptosis.
- **Drug discovery**: High-throughput screening of various anti-cancer, anti-inflammatory drugs.

4. Kit contents

Reagents supplied in the kit are sufficient for 200 assays (two- 96-microwell plates).

Code	Description	Quantity	Storage
CCK078(A)	ROS staining reagent	2 x 20 µl	-20°C
CCK078(B)	Active oxygen positive control (Tert-butyl hydrogen peroxide)	0.5 ml	RT
CCK078(C)	Washing buffer	100.0 ml	RT

5. Materials required but not provided in the kit

- Cells in appropriate medium without phenol red.
- Adjustable pipettes and a repeat pipetter.
- Transparent flat-bottom black 96-well microtiter plate for culturing the cells.
- 96-well plate reader capable of measuring the fluorescence at 450nm and >600nm.

6. General guidelines

It is important to optimize experimental factors like cell density, incubation time, media composition and concentration of the agents under investigation prior to use of ROS Assay Kit.

Assay controls

Include appropriate assay controls i.e.

1. Cell control (medium with cells but without the experimental drug /compound and fluorogenic dye).
2. Vehicle control (medium containing the experimental drug or compound with cells).
3. Positive control (medium with cells + 1-10µl of CCK078 (B) per well)

Accuracy

- Perform the assay in triplicates or more to obtain statistically significant data.

- Accuracy of the assay depends on pipetting skills of the personnel. Inappropriate addition and mixing practices may result in erroneous and false-positive or false-negative results.
- Use of a repeating pipetter is recommended to pipette reagents. This saves time and helps to maintain more precise incubation times.
- Pipette tip should be equilibrated with the reagent before use. This is carried out by slowly filling up the tip with reagent and gently expelling the contents several times.
- Care should be taken so that no bubbles are introduced into the wells during pipetting or mixing of the reagents.

Culture Medium

Phenol red may interfere with fluorogenic dye. Therefore, the cell culture media used for this assay should not contain phenol red.

Temperature

Temperature affects the performance of the assay because of its effect on enzymatic rates. It is crucial to run the assay at a uniform temperature to ensure reproducibility across a single plate or among stacks of several plates. Since fluorescence readings measured at room temperature, it is important to ensure adequate equilibration of assay plates after removal from a 37ºC incubator to avoid differential temperature gradients. Stacking large numbers of assay plates in close proximity should be avoided to ensure complete temperature equilibration.

Measurement of absorbance

Fluorescence can be read with a filter Ex-488nm, Em-528nm

Directions for use

Users are advised to review entire procedure before starting the assay

7.1 Preparation of working CCK078(C)

1ml of CCK078 (C) + 9ml of cell culture grade Water

7.2 Preparation of cells

Always use freshly harvested cells for assay. Seed the cells in a cell culture flask or dish in an amount appropriate for the assay and incubate at 37ºC in a 5% CO_2 environment. Allow the cells to grow for up to 24 hours or till confluency reaches. Harvest the cells and use for assay.

***Note**: The quantity of the cell suspension to be seeded in the medium depends upon doubling time of individual cell lines and seeding density to be used in the assay.*

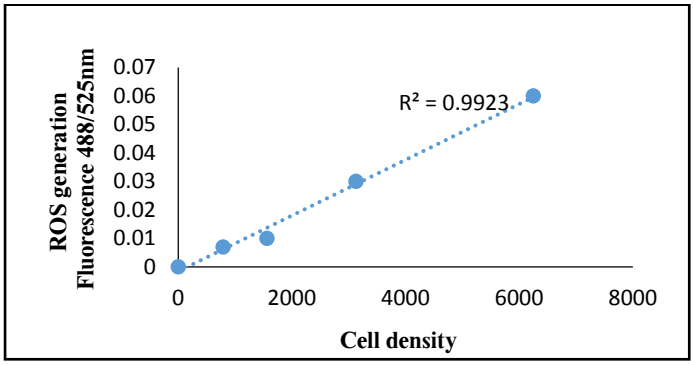
7.3 Assay procedure

1. Harvest the cells as explained in section 7.2.
2. Adjust appropriate cell density as per the user requirement and type of cells being used.
3. Seed 100µl cells/well in a 96 well black well plate with transparent bottom.
4. Include appropriate controls as mentioned in section 6 (assay controls)
5. Add test molecule in test wells.
6. Incubate the plate for desired period of time at 37ºC with 5% CO_2.
7. Thaw one bottle of CCK078 (A) and equilibrate at room temperature just before use.

   Note: Reagent in one bottle is sufficient for one 96-well plate.
8. Take 12.5 µl of CCK078 (A) and add in 10ml of medium (without serum) and make a homogenous solution.

   Note: CCK078(A) is light sensitive hence, make sure that experiments are carried out in dark.
9. Remove the media from wells.
10. Add 100µl of homogenous solution of fluorogenic dye to each well except in cell control and mix the dye uniformly by rotating the plate (clockwise and anti-clockwise).
11. Wrap the plate with aluminum foil to avoid exposure to light.
12. Return the plate to the 5% CO_2 incubator at 37°C for standardized incubation period between 20-30 min.
13. After incubation remove the culture media and wash the cells with CCK078(C) at least 2 -3 times.
14. After washing add 100 µl of CCK078(C) and read the fluorescence using wavelength of excitation-488nm, Emission-528nm.
15. Determine the average values from triplicate readings and subtract from this value the average value for blank (i.e. cell control).

    Specific fluorescence= fluorescence 488nm/528nm. Plot the graph of fluorescence versus cell density.



CHO cells with different cell density were treated with DCFDA dye, in a transparent flat-bottom black 96-well microtiter plate. The sensitivity of DCFDA dye with respect to change in cell number has been determined by plotting the graph of normalized fluorescence values versus cell density per well.

7. Storage and shelf life

- Store CCK078 (A) dye at -20ºC once received. Repeated freezing and thawing of CCK078 (A) may result in loss in activity of the reagent and increased background absorbance.
- Use before expiry date given on the table.

8. Advantages

Time saving: Fluorescence can be measured without involving solubilization with organic solvent.

Easy reagent preparation: Ready to mix reagents offer ease of reagent preparation.

Reproducibility: Entire assay can be performed in a single plate. Cells and reagents need not be transferred. This facilitates reproducibility of the results.

Sensitivity and accuracy: DCFDA dye strongly correlates with the metabolic activity of the cells. This allows use of low cell densities.

Safety: No radioisotopes are involved

Fast: Use of multi-well black transparent flat bottom 96 well plates allows the processing of large number of samples.

Flexibility: DCFDA works on adherent as well as suspension cell lines.

- **Troubleshooting points:**

Use the following troubleshooting guidelines for technical assistance

Problem	Cause	Solution
Color change in DCFDA reagent	Exposure of the reagent to light	Wrap the reagent bottles and culture plates with aluminum foil
Inappropriate readings	Improper selection of the filter	Choose appropriate filters
	Cross contamination due to inaccurate pipetting technique or inaccurate equipment	Perform the assay using automated electronic pipettes for seeding the cell suspension and adding the reagents
Very high fluorescence values	Microbial contamination	Discard. Repeat the assay with new media and reagents
	Too much signal in DCFDA signal due to high cell densities	Repeat the assay with reduced cell densities
Random fluorescence values/ poor consistency of replicates	Test compound under study is responsible for improper response of the cells to DCFDA	Refer to the pharmacological properties of the compound
	Microbial contamination	Discard. Repeat the assay with new media and reagents.