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Technical Datasheet

EZAssay™ Mitochondrial Membrane Potential Assay Kit with JC-1

Product Code: CCK079

1. Introduction

One of the distinctive feature of the early marker of programmed cell death (PCD) is the disruption of active mitochondria. This include changes in the membrane potential and alterations in the oxidation–reduction potential of the mitochondria. During this PCD mitochondrial permeability transition pore (MPTP) opens which allow passage of ions and small molecules. Thus the equilibration of ions changes leads to the decoupling of the respiratory chain and the release of cytochrome c into the cytosol. JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine-iodide), a membrane permeant cationic dye, is widely used in apoptosis studies to monitor mitochondrial and cell health. JC-1 dye being positively charged permeable in the cell and accumulates and forms aggregates in the mitochondrial membrane due to electronegative interior of healthy cells. Excitation with 488nm wavelength results in red fluorescence. Loss of mitochondrial membrane potential is a hallmark for apoptosis. It is an early event preceding phosphatidylserine externalization on the plasma membrane and coinciding with caspase activation caspase-3/7. In healthy cells the JC-1 dye accumulates in mitochondrial membrane resulting in red fluorescence at 590 nm. However, in necrotic and apoptotic cells due to disruption of mitochondrial membrane aggregates of JC-1 leaches out in cytoplasm which gives green fluorescence at 530 nm of JC-1 monomer. The JC-1 kit provides a step-by-step protocol and ready-to-use reagents for performing assays for flow cytometry, fluorescence microscopy, or fluorometric microplate reader. JC-1 is a ratio metric probe, the ratio of green and red fluorescence emitted by JC-1 is used to analyse the apoptotic nature of the cell. This ratio increases as the membrane potential decreases. JC-1 dye can be used as an indicator of mitochondrial potential in a variety of cell types. It also helps in to monitor the effects of some pharmacological agents, such as anesthetics that alter mitochondrial function.

2. About the Assay

The JC-1 Cell Assay kit is designed for determination of mitochondrial health of cells. This kit is based on the quantitative measurement of potential dependent presence of JC-1 dye accumulated in mitochondria. Consequently, mitochondrial depolarization is indicated by an increase in the green/red fluorescence intensity ratio. The potential-sensitive color shift is due to concentration-dependent formation of red fluorescent J-aggregates.

3. Applications

- **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

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

Code	Description	Quantity	Storage
CCK079(A)	JC-1 staining solution	2 X 30 µl	-20°C
CCK079(B)	Dimethyl sulfoxide (DMSO)	2.0 ml	RT
CCK079(C)	Assay buffer 10X	15.0 ml	RT
CCK079(D)	Molecular biology grade water	10.0 ml	RT

5. Materials required but not provided in the kit

- Cells in appropriate medium without phenol red.
- Adjustable pipettes and a repeat pipetter.
- 96-black well with transparent bottom 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 JC-1 Cell Assay Kit.

Assay controls

Include appropriate assay controls i.e.

1. Cell control (medium with cells but without the experimental drug/ compound)
2. Vehicle control (medium containing the experimental drug or compound with cells)
3. Positive control (medium with cells + 5-20µl of CCK079 (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 the measurement of JC-1 aggregates or monomers formed. Therefore, 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 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 fluorescence

Fluorescence can be read with a filter Ex-488nm, Em-530nm and 590nm.

7. Directions for use

Users are advised to review entire procedure before starting the assay

7.1 Preparation of working CCK079(C)

1ml of CCK079(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 confluence is reached. 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.)*

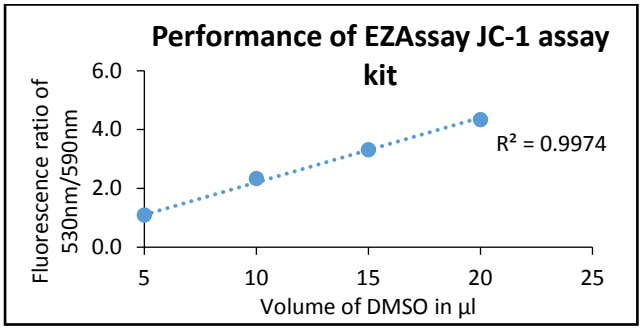
7.3 Assay procedure

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 or Part (B) in test wells.
6. Incubate the plate for desired period of time at 37ºC with 5% CO_2.
7. Thaw one bottle of CCK079 (A) and equilibrate at room temperature just before use.
   Note: Reagent in one bottle is sufficient for one 96-well plate.
8. Take 25µl of CCK079 (A) dye. Add in 4.5 ml of CCK079 (D) and mix properly. After dissolution add, 0.5 ml of CCK079(C).
9. To this add 5.0 ml of complete media and label it as JC-1 homogenous solution.(Please refer to Table No. 1) *Note: CCK079 (A) is light sensitive hence, make sure that experiments are carried out in dark.*

Table No.1

CCK079 (A)	CCK079 (D)	CCK079 (C)	Complete media	JC-1 homogeneous solution
25 µl	4.5 ml	0.5 ml	5.0 ml	10.0 ml
50 µl	9.0 ml	1.0 ml	10.0 ml	20.0 ml

10. Remove media from wells by aspiration.
11. Add 100µl JC-1 homogenous solution to each well including controls and mix the dye uniformly by rotating the plate (clockwise and anti-clockwise).
12. Wrap the plate with aluminum foil to avoid exposure to light.
13. Return the plate to 5% CO_2 incubator at 37°C for standardized incubation period between 25-30 minutes.
    Note: Incubation time with JC-1 varies for different cell lines. Users should optimize the time empirically.
14. After incubation remove the culture media and wash the cells with working CCK079 (C) 2 -3 times.
15. After washing add 100 µl of working CCK079 (C).
16. Measure the fluorescence reading at emission spectra of -530nm and 590nm, using excitation wavelength of 488nm.
17. Determine the average values from triplicate readings and subtract from this value the average value for blank (i.e. cell control).
    Specific fluorescence = fluorescence (530nm)/ (590nm).
    Plot the graph of fluorescence versus cell density at 530nm and 590nm wavelength of fluorescence.



BHK-21 cells were treated with DMSO and change in mitochondrial membrane potential was determined by JC-1 reagent provided in EZAssay JC-1 assay kit, in a transparent flat-bottom black 96-well microtiter plate. The sensitivity of JC-1 dye with respect to change in volume of DMSO has been determined by plotting the graph of normalized fluorescence of 530nm vs 590nm excited with 488nm wavelength.

8. Storage and shelf life

- Store CCK079 (A) dye at -20°C once received.
- Repeated freezing and thawing of CCK079 (A) may result in loss in activity of the reagent and increased background absorbance.
- Use before expiry date given on the label.

9. 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: JC-1 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: JC-1 works on adherent as well as suspension cell lines.

- **Troubleshooting points:**

Use the following troubleshooting guidelines for technical assistance.

Problem	Cause	Solution
Inappropriate readings	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
	Improper selection of the filter	Choose appropriate filters
Color change in JC-1 reagent	Exposure of the reagent to light	Wrap the reagent bottles and culture plates with aluminum foil
Very high fluorescence values	Microbial contamination	Discard. Repeat the assay with new media and reagents
	Too much signal in JC-1 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 JC-1	Refer to the pharmacological properties of the compound
	Microbial contamination	Discard. Repeat the assay with new media and reagents.