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ab288313 – JC1 - Mitochondrial Membrane Potential Assay Kit

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For the measurement of mitochondrial membrane potential by fluorescence plate reader

This product is for research use only and is not intended for diagnostic use.

Table of Contents

1. Overview

JC1 - mitochondrial membrane potential (Δψm) kit uses tetraethylbenzimidazolylcarbocyanine iodide (JC-1), a cationic dye that accumulates in energized mitochondria to measure the mitochondrial membrane potential.

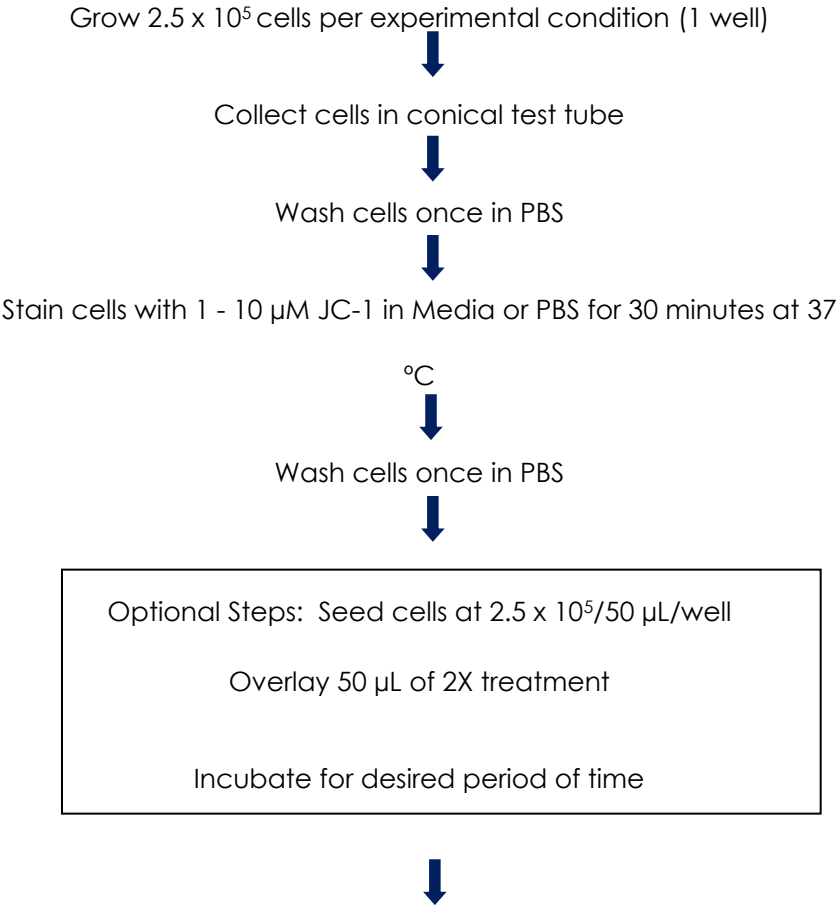
At low concentrations (due to low Δψm) JC-1 is predominantly a monomer that yields green fluorescence with emission of 530±15 nm. At high concentrations (due to high Δψm) the dye aggregates yielding a red to orange colored emission (590±17.5 nm). Therefore, a decrease in the aggregate fluorescent count is indicative of depolarization whereas an increase is indicative of hyperpolarization. The accompanying FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) is an ionophore uncoupler of oxidative phos-phorylation. Treating cells with FCCP eliminates mitochondrial membrane potential and JC1 staining. JC1 is suitable for the labeling of mitochondria in live cells and it is not compatible with fixation.

Depolarization can be found in the presence of ionophores that could induce nonselective cation channels or become selective mobile ionic carriers. Protonophores such as FCCP and CCCP induce reversal of the ATPase, as a compensatory mechanism that tries to maintain Δψm, which will deplete ATP even in the presence of a normal glycolytic pathway. Hyperpolarization could be found in the presence of ATPase inhibition, inadequate supply of ADP, increased supply of NADH, apoptosis due to oxidative stress and potentially proton slippage due to cytochrome c oxidase dephosphorylation. In either scenario, OXPHOS uncoupling ensues.

Membrane potential (Δψm) is highly interlinked to many mitochondrial processes. The Δψm controls ATP synthesis, generation of ROS, mitochondrial calcium sequestration, import

of proteins into the mitochondrion and mitochondrial membrane dynamics. Conversely, Δψm is controlled by ATP utilization, mitochondrial proton conductance, respiratory chain capacity and mitochondrial calcium. Hence pharmacological changes in Δψm can be associated with a multitude of other mitochondrial pathological parameters which may require further independent evaluation.

2. Suspension cell assay summary – microplate



3. Adherent cell assay summary – microplate

Harvest 3-4x10^6 cells

↓

Seed cells at 1.5x10^4 cells/well on a 96 well plate

↓

Allow cells to attach overnight

↓

Wash cells once in PBS

↓

Stain cells with 20 µM JC-1 in PBS or Media for 10 minutes at 37°C

↓

Wash cells twice in PBS

↓

Optional Steps: Add 100 µL/well of treatment

Incubate for desired period of time

↓

Read signal at Ex475±20 nm / Em530 ± 15 nm and 590 ± 17.5 nm

(Buffer or compound must be present in the wells during the reading of the signal. Do not allow wells to dry out)

4. Precautions

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

5. Storage and stability

Store kit as described below immediately upon receipt.

JC-1 is stable for 12 - 18 months if stored in the dark at -20°C.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 11.

6. Materials supplied

Item	Amount	Storage **temperature**
JC-1	15 x 32 µg	-20°C
5X Opti-Klear™ Live Cell Imaging Buffer (sterile)	30 mL	+4°C
DMSO (cell culture tested)	1 mL	-20°C
50 mM FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone)	10 µL	-20°C

7. Materials required, not supplied

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Fluorescence plate reader. JC-1 may also be detected with similar settings to those used to detect rhodamine (excitation/emission wavelengths: 540/570 nm) or texas red (excitation/emission wavelengths: 590/610 nm)
- General tissue culture supplies
- PBS (1.4 mM KH2PO4, 8 mM Na2HPO4, 140 mM NaCl, 2.7 mM KCl, pH 7.4)
- Fetal Bovine Serum (FBS)
- Sterile, tissue culture treated, clear bottom, dark sided 96-well microplates.
- Multichannel pipette (50 – 300 µL)
- Optional:
    - Test compounds/diluents of interest
    - Uncouplers include CCCP (carbonyl cyanide 3-chlorophenylhydrazone), 2', 4' Dinitrophenol
    - 96-Well deep sided, clear bottom, dark sided microplates with lids

8. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in the results

9. Technical hints

- Clear bottom, dark sided microplates are recommended with this assay. Clear sided microplates have not been tested with this kit
- While working with JC-1 inside the biological safety cabinet, turn the fluorescent lights off to prevent quenching of the dye during handling.
- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions

10. Reagent preparation

Equilibrate all reagents to room temperature (18-25°C) prior to use.

The sample volumes below are sufficient for 96 100 µL tests; adjust volumes as needed for the number of wells used in your experiment.

10.1 1 mM JC-1 Stock Solution

Allow the JC-1 vial and the DMSO to warm to room temperature. Reconstitute the JC-1 by adding 50 µL DMSO to a vial of JC-1.

Any unused 1 mM JC-1 solution should stored at -20 °C.

10.2 1X Opti-Klear™ Live Cell Imaging Buffer (sterile)

Dilute 5X Opti-Klear™ Live Cell Imaging Buffer 1:5 in sterile DI water to prepare a 1X working solution

10.3 Working JC-1 Solution

To prepare the Working JC-1 Solution, add the appropriate volume of 1 mM JC-1 Solution to previously warmed PBS or phenol red free media. As an example, to generate a 1 µM JC-1 Solution, mix 10 µL 1 mM JC-1 with 10 mL of 1X PBS. Mix thoroughly and gently. If necessary, centrifuge the 1 µM solution at 13,000 x g for 3 minutes to sediment non-soluble particles.

The exact concentration of JC-1 required will depend on the cell line being used and must be determined on an individual basis by the end user. Typical working concentrations, along with recommended seeding densities (steps 11.1.6 & 11.2.2) for certain cell lines are shown in the table below:

Typical working concentrations		
Sample Type	Seeding Density per Well	JC-1 Concentration (µM)
HepG2	15,000	20
HL60	200,000	10
HeLa	6,000	1
HDFN	6,000	1

11. Assay procedure

- Equilibrate all materials and prepared reagents to 37°C prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.

11.1. Fluorescent Microplate Measurement (Suspension Cells, e.g. HL60 cells)

11.1.1. Grow HL60 cells in glucose based media so that approximately $2.5x10^7$ cells are available on the day of the experiment per plate

11.1.2. If performing toxicity assays, dilute compounds of interest in media without phenol red to 2X of final desired concentration for the experiment. A 96-well deep well microplate may be use in this step. Include a depolarization control (100 µM FCCP) and a normal control (vehicle or diluent of choice).

11.1.3. Collect cells and wash by centrifugation once in 1X PBS.

11.1.4. Resuspend cells in 10 mL of the Working JC-1 Solution and incubate at 37°C for 30 minutes in the dark.

11.1.5. Wash cells by centrifugation with 10 mL of PBS.

11.1.6. Resuspend cells in 1X Opti-Klear™ Live Cell Imaging Buffer.

11.1.7. Seed in a 96-well dark plate at 200,000 stained cells/50uL/well. Include blank wells (with non-stained cells).

11.1.8. Observe fluorescence staining in the presence of compounds, media, or buffer by plate

reader, flow cytometer, or epi-fluorescence microscope at Ex. 485/Em. 530 for monomer and Ex. 535/Em. 590 for aggregates. (FITC and rhodamine filter sets, or equivalents are acceptable)

11.2. Fluorescent Microplate Measurement (Adherent Cells, e.g. HepG2 cells)

11.2.1 Seed adherent cells with $1.5x10^4$ cells per well in a 96-well dark plate. Allow to attach overnight.

11.2.2 If performing toxicity assay, dilute compounds of interest in complete media without phenol red. Include a depolarization control (100uM FCCP) and a normal control (vehicle or diluent of choice).

11.2.3 Wash cells seeded in 96-well dark plate with 100uL/well 1X PBS.

11.2.4 Add 100uL/well of Working JC-1 solution and incubate for 15-20 minutes at 37°C in the dark.

11.2.5 Aspirate the working JC-1 solution and wash plate once with 1X PBS. If a media change would disrupt sensitive drug dosing formulations, an alternative protocol allows adding the Working JC-1 at 2X concentration directly to cells in complete media. Note: some background will be present, but the signal to noise is enough for most analyses.

11.2.6 Observe fluorescence staining in the presence of compounds, media, or buffer by plate reader, flow cytometer, or epi-fluorescence microscope at Ex. 485 ± 11nm/Em. 530 ± 15 nm for monomer and Ex. 535 ± 17.5 nm/Em. 590 ±

17.5 nm for aggregates. (FITC and rhodamine filter sets, or equivalents are acceptable).

12. Calculations

Subtract background (A590 of non-stained cells) from test signal and express signal as percentage from control (untreated healthy cells). If both monomer and aggregate forms are measured, a ratio between the two measurements may be obtained and plotted. Data obtained with the JC-1 assay gives a relative measure of mitochondrial membrane potential as a percentage of control and cannot be used for absolute measurements of membrane potential in millivolts. Decrease in JC-1 signal may indicate either mitochondrial depolarization or cell death and must be interpreted in parallel with a cytotoxicity assay (such as the ATP detection kit ab113849). The data in Figure 1 below shows the uncoupling effect of FCCP acute treatment on HL60 cells as measured with the JC-1 stain and read on a fluorescent plate reader.

13. Typical data

Data provided for **demonstration purposes only**.

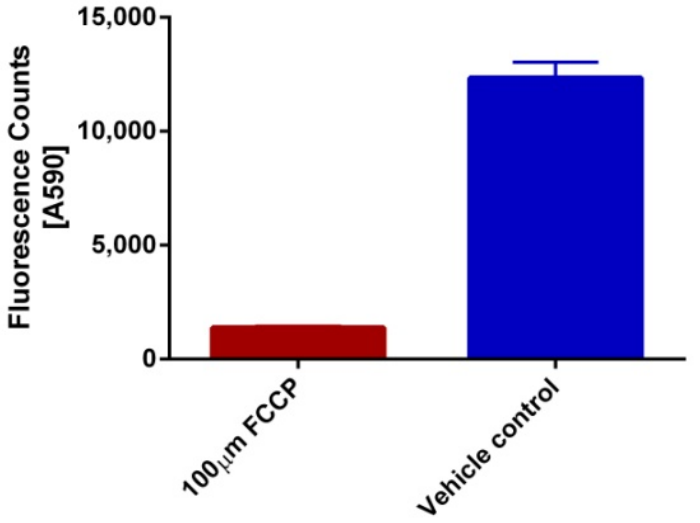


Figure 1. JC-1 assay result in HL60 cells treated with FCCP. HL60 cells were seeded and labeled according to section 11.1 of the protocol. Cells were then treated for 4 hours with 100 µM FCCP or vehicle/diluent control (DMSO). Cells were read on a Perkin Elmer-Wallac 1420 Victor 2 Multilabel plate reader. Mean and standard deviation is plotted for 3 replicates from each condition.

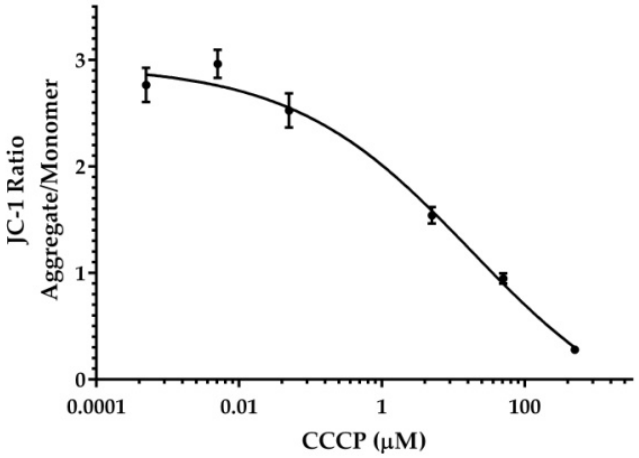


Figure 2. JC-1 assay result in HepG2 cells treated with CCCP. HepG2 cells were seeded and labeled according to section 11.2 of the protocol. Cells were then treated for 4 hours with a titration series of CCCP (carbonyl cyanide 3-chlorophenylhydrazone) and both monomer and aggregate forms were read on a Perkin Elmer-Wallac 1420 Victor 2 Multilabel plate reader. Mean and standard deviation of aggregate/monomer ratios is plotted for 12 replicates for each concentration. IC_{50} of CCCP in HepG2 cells was calculated at 8.7 µM

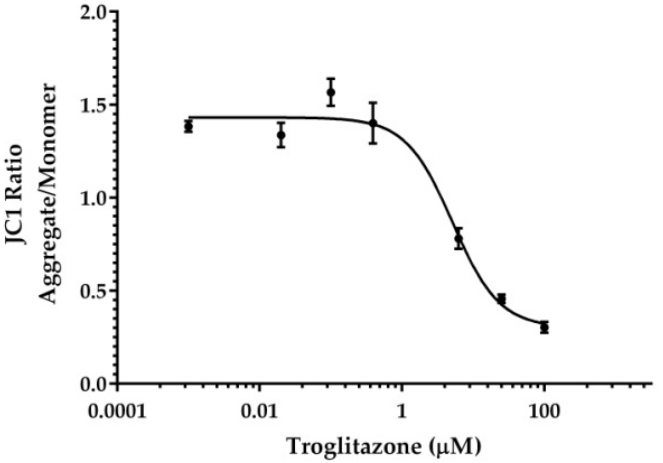


Figure 3. JC-1 assay result in HL60 cells treated with Troglitazone. HL60 cells were seeded and labeled according to section 11.1 of the protocol. Cells were then treated for 4 hours with a titration series of the thiazolidinedione Troglitazone and both monomer and aggregate forms were read on a Perkin Elmer-Wallac 1420 Victor 2 Multilabel plate reader. Mean and standard deviation of aggregate/monomer ratios is plotted for 3 replicates for each concentration. IC_{50} of Troglitazone in HL60 cells was calculated at 1.2 µM

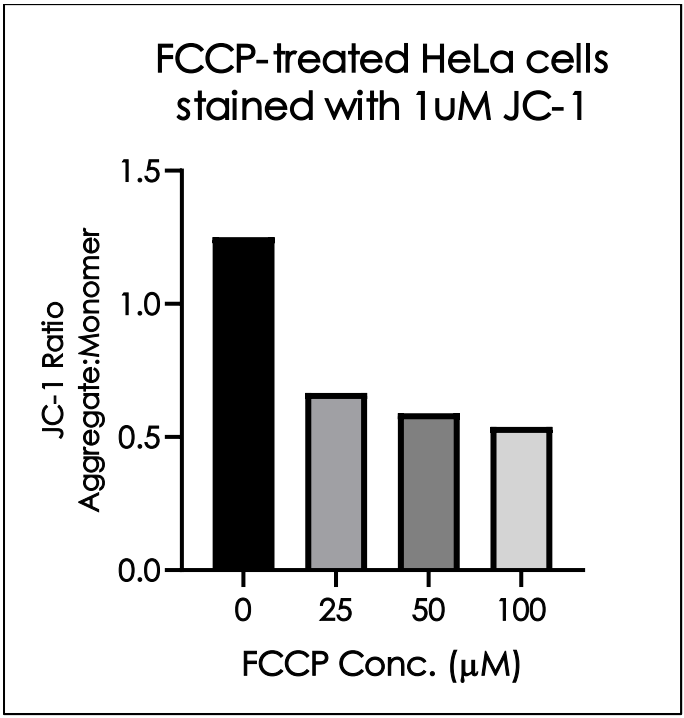


Figure 4. HeLa cells plated at 12000 cells/well. Next day, aspirated media and replaced with complete media containing 0, 25, 50, or 100uM FCCP for 30 minutes. Drug solution aspirated from wells and cells were washed once with serum free media. A staining solution of 1uM JC-1 in serum free media was added to cells and incubated for 15 minutes at 37°C in the dark. Staining solution was aspirated, cells were washed once with 1X PBS, and bathed in 1X Opti-klear. Measured fluorescence on Tecan plate reader using Ex. 485/Em. 530 for monomer and Ex. 535/Em. 590 for aggregates.

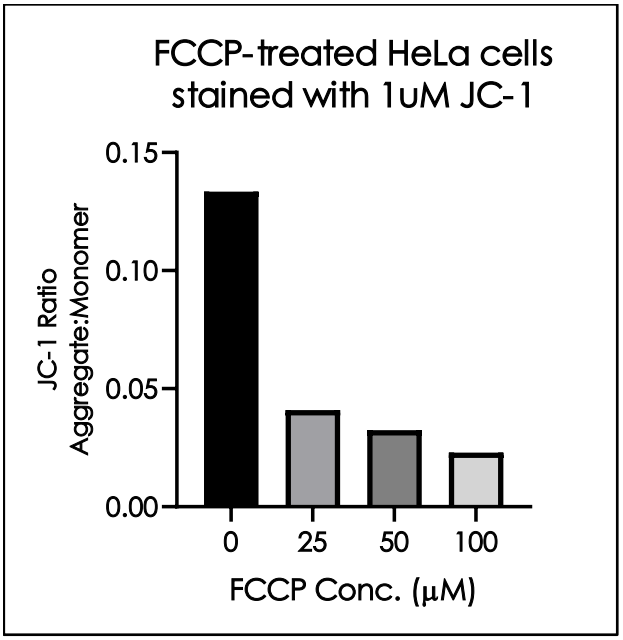


Figure 5. HeLa cells plated at 12000 cells/well. Next day, aspirated media and replaced with complete media containing 0, 25, 50, or 100uM FCCP for 30 minutes. Drug solution aspirated from wells and cells were washed once with serum free media. A staining solution of 1uM JC-1 in serum free media was added to cells and incubated for 15 minutes at 37°C in the dark. Staining solution was aspirated, cells were washed once with 1X PBS, and bathed in 1X Opti-Klear. Plate was analyzed using the CX5 CellInsight SpotDetector bioapp

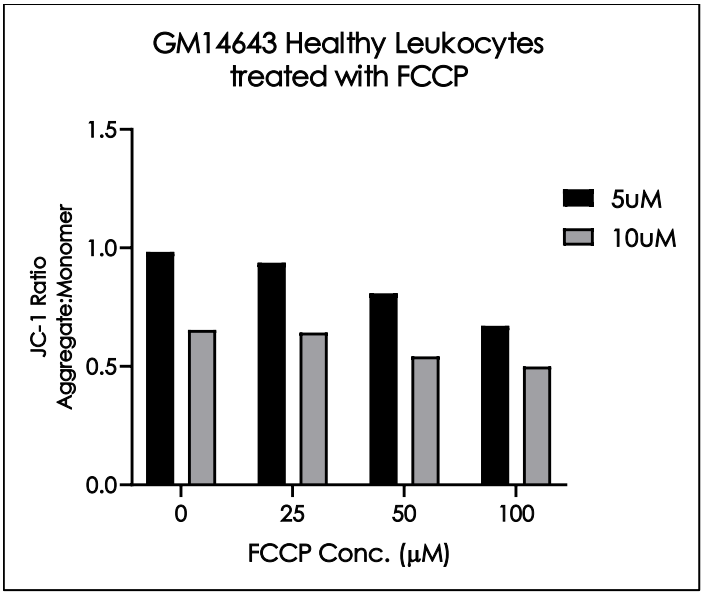


Figure 6. GM14643 healthy leukocytes were treated with 0, 25, 50, or 100uM FCCP in complete media for 30 minutes. Cells were collected by centrifugation and washed once with serum free media. Cells were resuspended with a staining solution of 5uM or 10uM JC-1 in serum free media and incubated for 30 minutes at 37°C in the dark. Cells were collected by centrifugation, washed once with 1XPBS, and resuspended in 1X Opti-Klear. Cells were then seeded in a 96-well optical bottom plate at a density of 200,000 cells/50uL/well. Fluorescence was measured on Tecan plate reader using Ex. 485/Em. 530 for monomer and Ex. 535/Em. 590 for aggregates.

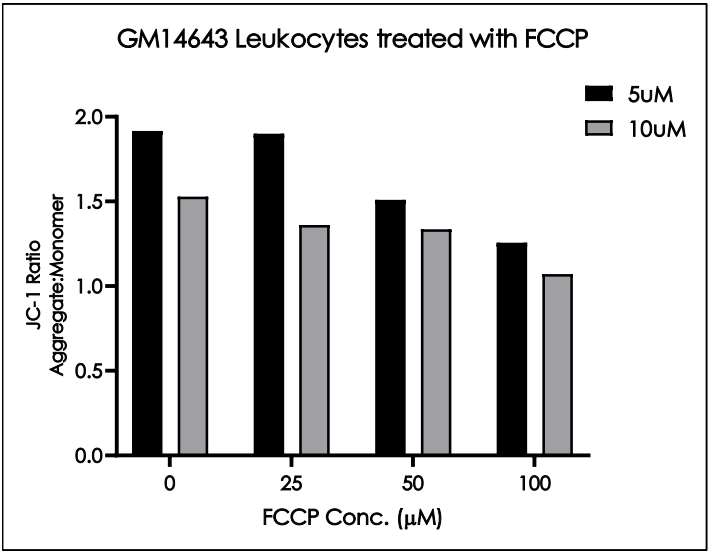


Figure 7. GM14643 healthy leukocytes were treated with 0, 25, 50, or 100uM FCCP in complete media for 30 minutes. Cells were collected by centrifugation and washed once with serum free media. Cells were resuspended with a staining solution of 5uM or 10uM JC-1 in serum free media and incubated for 30 minutes at 37°C in the dark. Cells were collected by centrifugation, washed once with 1XPBS, and resuspended in 1X Opti-Klear. Cells were then seeded in a 96-well optical bottom plate at a density of 200,000 cells/50uL/well. Plate was analyzed using the CX5 CellInsight SpotDetector bioapp

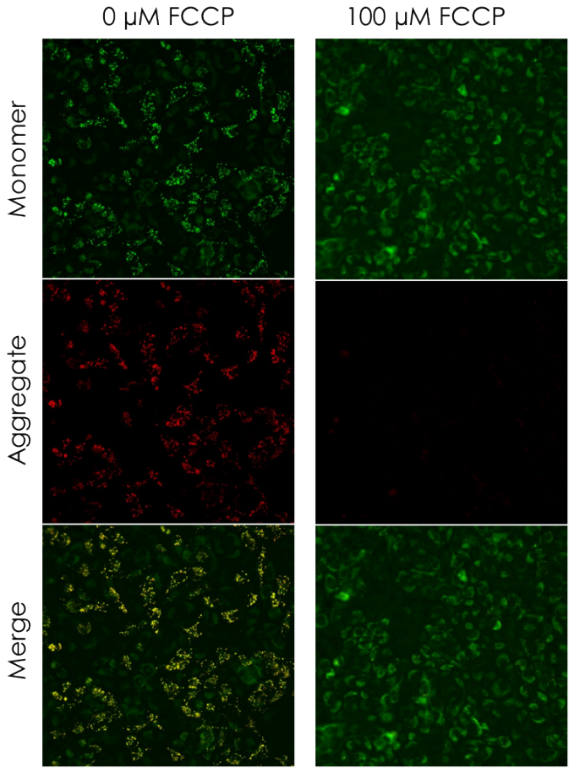


Figure 8. HeLa cells plated at 12000 cells/well. Next day, aspirated media and replaced with complete media containing 0 or 100uM FCCP for 30 minutes. Drug solution aspirated from wells and cells were washed once with serum free media. A staining solution of 1uM JC-1 in serum free media was added to cells and incubated for 15 minutes at 37°C in the dark. Staining solution was aspirated, cells were washed once with 1X PBS, and bathed in 1X Opti-Klear. Plate was imaged using the CX5 CellInsight.

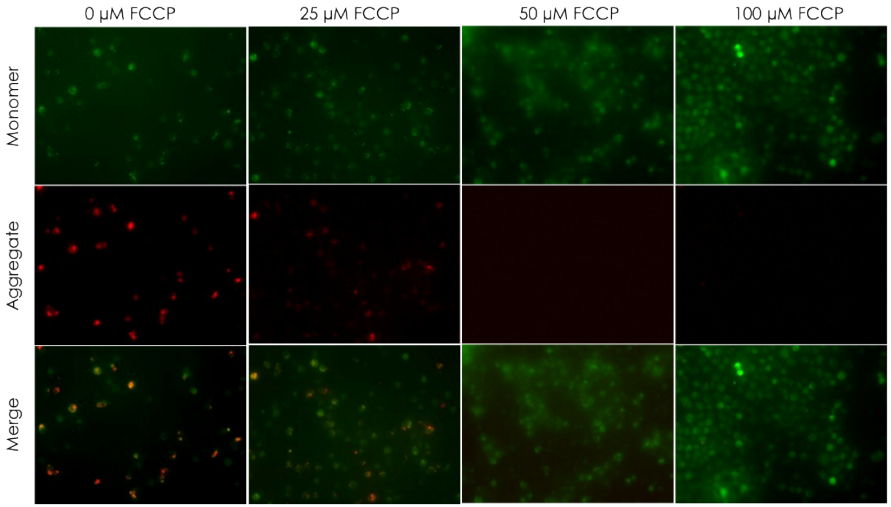
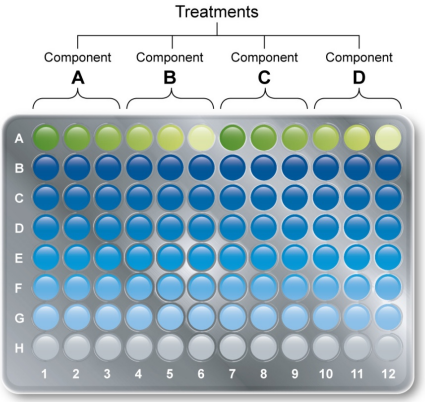


Figure 9. GM14643 healthy leukocytes were treated with 0, 25, 50, or 100uM FCCP in complete media for 30 minutes. Cells were collected by centrifugation and washed once with serum free media. Cells were resuspended with a staining solution of 5uM or 10uM JC-1 in serum free media and incubated for 30 minutes at 37°C in the dark. Cells were collected by centrifugation, washed once with 1XPBS, and resuspended in 1X Opti-Klear. Cells were then seeded in a 96-well optical bottom plate at a density of 200,000 cells/50uL/well. Plate was imaged using the CX5 CellInsight.

This assay may be used for screening pharmacological depolarization of mitochondria in any cell line. Depending on the microplate template (see figure 4) either 3 or 4 compounds may be tested in triplicate dose response per plate.

A.



B.

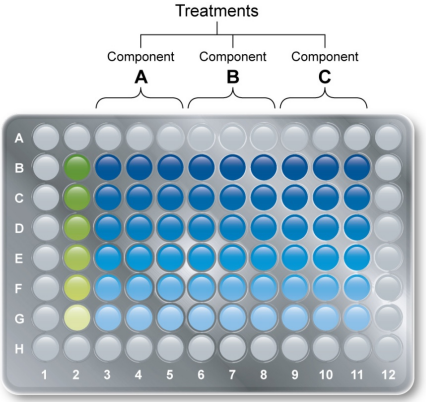


Figure 10. Suggested assay templates. Two assay examples are shown above. The example (A) allows for screening of four compounds in dose response. Row A contains the vehicle control to determine maximal signal in the absence of compound. Row H contains non-stained cells to determine background fluorescence. The example (B) only allows for screening of three compounds in dose response with perimeter wells as the background fluorescence and column 2 as the

vehicle titration control. Column 1, column 12, row A and row H contain non-stained cells.

14. Frequently asked questions

Q. Is this kit designed to do a cell treatment before or after JC-1 incubation?

A. It is essential to read the JC-1 signal in the presence of compound (or treatment) because changes in membrane potential can be short lived and reversible as soon as the treatment is removed from the cells

Q. I am already growing some cells on coverslips, will I be able to use these cells with the assay?

A. Any mammalian live cell may be stained with JC-1 provided that the staining concentration has been optimized. When cells are seeded on coverslips, two major changes must be done to the protocol in order to obtained the correct results:

1. Volumes will have to be adjusted from 100µL per well to 500 - 100 µL per well depending on whether the cells are seeded on a 6-well plate or on a 24-well plate. Due to the change in volume, only 10 to 20 tests can be run using this kit under these conditions.
2. Coverslips must be ideally imaged on an inverted fluorescence microscope or a microscope with a lens that can be immersed in water, using a Texas Red filter. In the absence of these instruments, the coverslip could be mounted with the buffer provided on top of an imaging chamber gasket. In this later case, imaging must be prompt to prevent bleaching of the dye.

Q. I am using primary cells and I'm afraid that 10 minutes without media is too much. Can I mix the JC-1 with the growing meda rather than the supplied buffer?

A. Yes, the JC-1 may be mixed with the growing media. However caution must be taken as the phenol red present in most media formulations may cause increased background. Furthermore, we have also observed higher background on RPMI media in comparison to DMEM media.

Q. Is fixation of the cells with paraformaldehyde in the wells possible, after live imaging, for storage purposes?

A. Fixation of the cells will interfere with the dye signal. Once the signal has been obtained by live imaging or fluorescent read out, cells cannot be stored for future use.

Q. I am planning a drug treatment for 18 hours; can I run FCCP in parallel for 18 hours? Will the signal dissipate after 4 hours?

A. We do not recommend treating cells with FCCP for more than 4 hours as this will generate excessive toxicity and results will represent depolarization but rather general cell toxicity. Furthermore incubations for longer than 4 hours after JC-1 staining may lead to dissipation of the signal. In this case, we suggest to reverse the protocol and treat first prior to staining. The following protocol should be follow in this scenario:

3. Dilute compounds of interest in complete media without phenol red. Make four times the volume required.
4. Treat suspension or adherent cells for the desired period of time. If treating cells for microplate measurements, treat with 100 µL per well.
5. Include blank wells with no cells but with compound at the same concentration used for treatment.
6. Include at least 2 depolarized control wells, to be reserved for FCCP treatment, containing cells but none of the test compounds.

7. 4 hours prior to completion of treatment, dilute FCCP to 10X of final concentration (1 mM) and spiked 10X FCCP into the reserved depolarized control wells by adding 11 µL per well.
8. 1 hour prior to completion of the treatment, dilute JC-1 at 2X of the final concentration desired in the same media used for treatment (containing experimental compounds) and warm at 37°C.
9. 10 – 30 minutes prior to completion of the treatment, overlay 2X JC-1 dilution on top of the treated cells. If treating cells for microplate measurements, overlay 100 µL of 2X JC-1 dilution per well.
10. Incubate JC-1 and compounds for the desired period of time (10 – 30 minutes).
11. Once incubation is completed, wash the wells twice with 100 µL per well of culture media containing compounds. Leave last wash in the wells.
12. Transfer the plate to the microplate reader and read according to the protocol.

15. NOTES

Technical Support

For all technical or commercial enquiries please go to:
www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)