TMRE Mitochondrial Membrane Potential Assay Kit

Item No. 701310

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. After opening kit, store individual components as stated below.

Item Number	Item	Quantity/Size	Storage
601283	TMRE Dye	1 vial	-20°C
701311	FCCP control (20 mM)	1 vial/25 µl	-20°C
10009322	Cell-Based Assay Buffer Tablet	3 tablets	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3640

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the Materials Supplied section, on page 3, and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. Microplate assay: Plate reader capable of measuring fluorescence excitation at 530 nm and emission at 580 nm, and a black 96-well tissue culture treated plate with clear bottom.
2. Fluorescence microscopy: Microscope with a typical RFP filter set and optically clear tissue culture plates/slides.
3. Flow cytometry: Flow cytometer with a PE-compatible channel (488/575 or equivalent) and plates or tubes appropriate for staining, centrifuging and analyzing cells.
4. Cells and appropriate culture media.
5. DMSO
6. Distilled water

INTRODUCTION

About This Assay

Cayman's TMRE Mitochondrial Membrane Potential Assay Kit utilizes tetramethylrhodamine ethyl ester (TMRE), a cell-permeable, cationic dye which accumulates in the mitochondrial matrix based on mitochondrial membrane potential ($\Delta\psi_M$). This assay, which is suitable for high-throughput screening, includes the mitochondrial uncoupler FCCP as a positive control for membrane depolarization. Cells can be analyzed by this kit with fluorescence plate readers, fluorescence microscopes, or flow cytometers.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Assay Buffer**

   Dissolve each Cell-Based Assay Buffer Tablet (Item No. 10009322) in 100 ml of distilled water. Assay Buffer should be pre-warmed to 37°C before use. Unused buffer can be stored at 4°C for one year.

2. **TMRE 2X working stock**

   Reconstitute the vial of TMRE (Item No. 601283) in 100 µl DMSO to make a 0.5 mM stock. Dilute dye in your cell culture medium to 2X immediately before adding to the cells. Unused stock dye should be aliquoted for storage at -20°C and protected from light. Avoid multiple freeze-thaw cycles. Diluted dye cannot be stored.

   Recommended: For plate reader and imaging assays, the optimal dye concentration (typically between 10 and 250 nM) should be pre-determined in a pilot experiment as described below

3. **FCCP control - (Item No. 701311)**

   This vial contains 20 mM FCCP in DMSO to be used as a control for disrupting the mitochondrial membrane potential. Make a 100 µM working stock by diluting 5 µl into 1 ml of pre-warmed Assay Buffer.

NOTE

TMRE is light sensitive. All staining procedures must be performed without direct exposure to intense light. Therefore, incubations need to be performed in the dark.

Assay Optimization

An optimization experiment should be performed for each cell line to determine the optimal cell seeding density and dye concentration. The fluorescent signal will increase proportionally with cell density until the cells reach confluence. The fluorescent signal will also increase proportionally with TMRE dye concentration until a self-quenching concentration is reached. An example experimental setup is shown below, but cell seeding densities will vary with cell type. Dye concentrations between 5 and 250 nM should be examined. The optimal dye concentration and cell density should produce a signal at least three fold higher than the non-specific binding background without cells. Follow the relevant protocol below for staining and data acquisition.

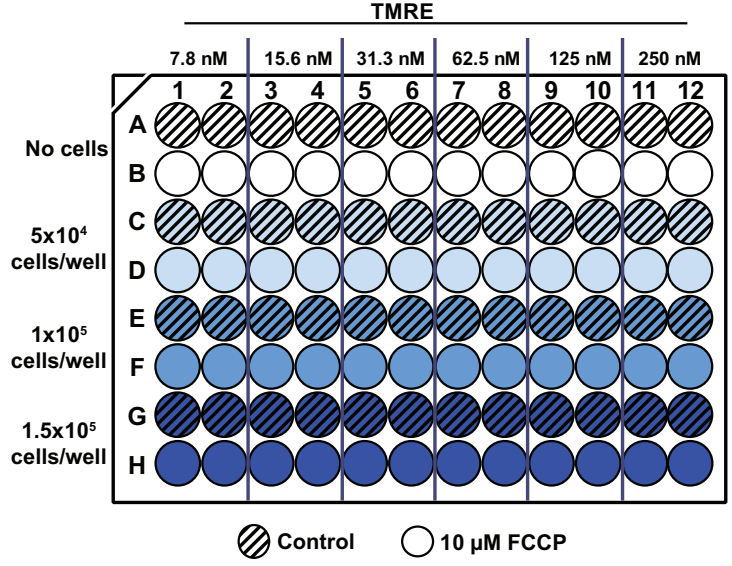

Figure 1. Example of an assay optimization plate map for fluorescence plate reader or fluorescence imaging. Note a wide range of cell densities and TMRE concentrations, including wells with no cells at each TMRE concentration.

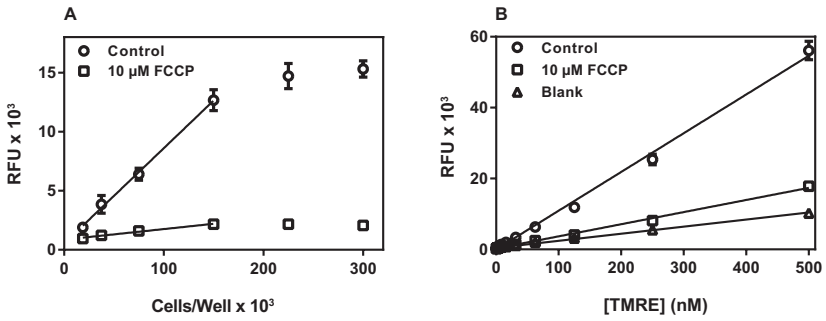

Figure 2. Sample data from assay optimization experiments. HepG2 cells were treated with FCCP or control medium for 30 minutes and then incubated with TMRE for 30 minutes as described in the assay protocol. (A) Saturation of signal was observed when HepG2 cells were plated above 150,000 cells per well (50 nM TMRE). (B) At a sub-confluent cell density, TMRE up to 500 nM showed a proportional increase in fluorescence signal indicating there is no observable self-quenching with this range of TMRE dye concentrations

ASSAY PROTOCOL

Performing the Assay

Fluorescence Plate Reader/Fluorescence Microscope

1. Seed cells in black 96-well clear bottom plate at the pre-determined cell density in 100-200 µl medium per well. Leave four wells without cells. Dispense the same volume of medium only into those blank wells.

   NOTE: Letting the cells sit for 30 minutes inside the hood until they adhere to the bottom of the plate will allow for a more even distribution of cells in the wells.
2. Incubate overnight (or until cells have reached the proper density) using the appropriate culture conditions.
3. Dilute and add test conditions as required by your experimental design.
4. For FCCP control wells, dilute the 100 µM working stock prepared on page 6 1:10 directly into your cell culture medium for a 10 µM treatment, or prepare 10X stocks and add to your culture medium.
5. Incubate for 10 to 30 minutes at 37°C.
6. Add equal volume of 2X TMRE, determined during the optimization phase and prepared on page 6, to every well in the plate.
7. Incubate for 30 minutes at 37°C.
8. Aspirate the media and wash the cells gently twice with 200 µl of 1X Assay Buffer.
9. Add 100 µl of 1X Assay Buffer to each well.
10. Equilibrate the plate at room temperature for 15 to 30 minutes.
11. Acquire data:
    a. Fluorescence plate reader: Read the plate in a fluorescence plate reader (excitation/emission = 530/580 nm).
    b. Microscopy: Image the cells using typical RFP filter sets.

Flow Cytometer

NOTE: Flow cytometry is an ideal method for evaluating mitochondrial membrane potential in suspension cells or mixed populations.

1. Treat cells as required by your experimental design. Be sure to reserve cells for no treatment and FCCP controls.
2. At the end of the treatment period, collect cells into tubes or a v-bottom polypropylene plate for staining at a concentration of 0.5-5x10^6 cells/ml.
3. For FCCP controls, dilute the 100 µM working stock prepared on page 6 1:10 directly into your cell culture medium for a 10 µM treatment, or prepare 10X stocks and add to your culture medium.
4. Incubate for 10 to 15 minutes at 37°C.
5. Centrifuge at 250xg and aspirate supernatant.
6. Resuspend in 100 µl Assay Buffer.
7. Dilute 5 µl of the 0.5 mM TMRE stock into 12.5 ml of Assay Buffer to make a 200 nM (2X) working stock.

   NOTE: Flow cytometry works well with a wider range of TMRE concentrations than other applications, but empirical determination of the optimal TMRE concentration for your cell line may be required.
8. Add 100 µl 2X TMRE to each well.

   NOTE: If cell surface markers are to be stained, fluorophore-conjugated antibodies may be added to the TMRE dye solution. This protocol is not compatible with fixation for intracellular staining.
9. Incubate at RT for 15 to 30 minutes.
10. Centrifuge at 250xg for 5 minutes, aspirate staining solution and replace with 200 µl Assay Buffer.
11. Collect data using the typical PE channel on your flow cytometer.

ANALYSIS

Calculations

1. Subtract the RFU value of the blank wells from all other wells. These values are mainly due to the non-specific binding of TMRE to the polystyrene plate.
2. The average value from control (vehicle/untreated) wells can be considered as 100% TMRE uptake.
3. The percentage TMRE fluorescent signal relative to the control is calculated using the equation indicated below.

$$\text{Relative Fluorescence Signal (\%)} = \frac{\text{Sample RFU - Blank RFU}}{\text{Control RFU - Blank RFU}} \times 100$$

4. To determine an IC_{50} value for each compound, plot the % fluorescent signal as a function of test compound concentration.

Performance Characteristics

Sample Data

The data shown below are an examples of data obtained with this kit. Your results will not be identical to these. Do not use these data to directly compare your samples as your results may vary substantially.

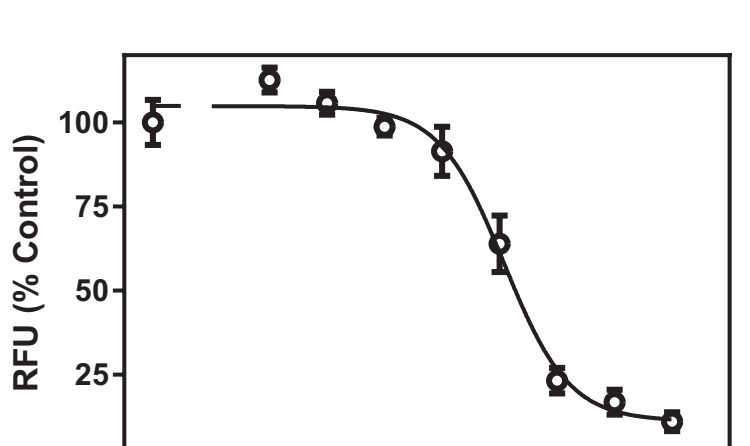

Figure 3. A typical concentration response curve for FCCP. HepG2 cells were seeded on a 96-well plate at 120,000 cells/well the day before performing the assay. The assay was performed as described and TMRE at 10 nM final concentration was used in this experiment.

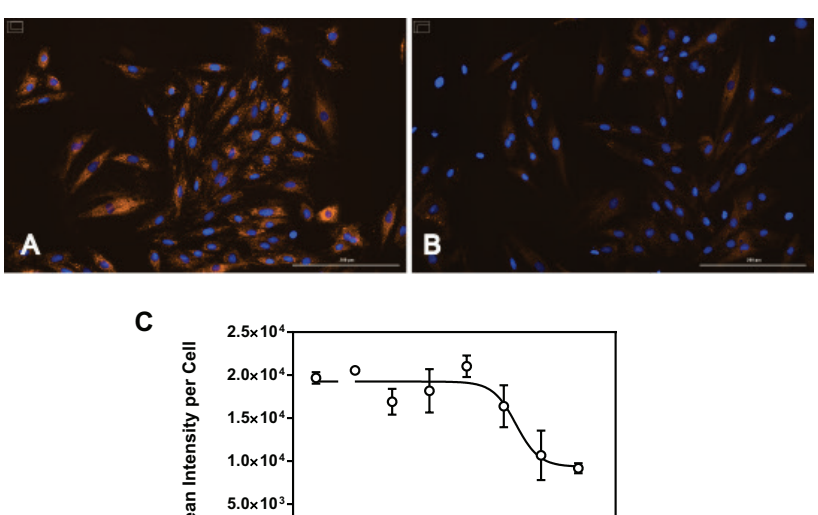

Figure 4. Mitochondrial membrane potential can be quantified by imaging. H9C2 cells were plated and allowed to grow to about 70% confluence. They were treated with FCCP at the concentrations indicated and stained with TMRE at 25 nM (A, no FCCP and B, 10 µM FCCP) or 100 nM (C) and Hoechst dye (Item No. 600332) as described in the kit booklet. Images were captured using BioTek's Cytation™ 5 Multi-Mode Imaging Plate Reader in 3x3 montage at 20X with DAPI and RFP LED/filter sets. The mean intensity of TMRE per cell was quantified using Gen5 software.

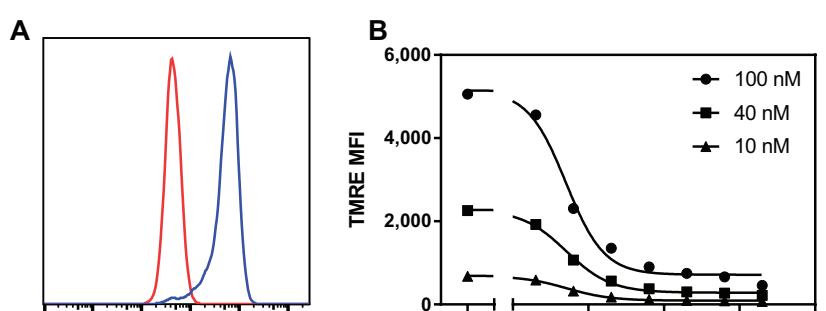

Figure 5. TMRE fluorescence can be quantified by flow cytometry. Jurkat cells were treated with serial dilutions of FCCP and stained with the indicated concentrations of TMRE as described in the kit booklet. (A) A representative histogram is shown with untreated cells (blue) and 20 µM FCCP treatment (red). Geometric mean fluorescence intensities were calculated and are shown (B) for each TMRE concentration.

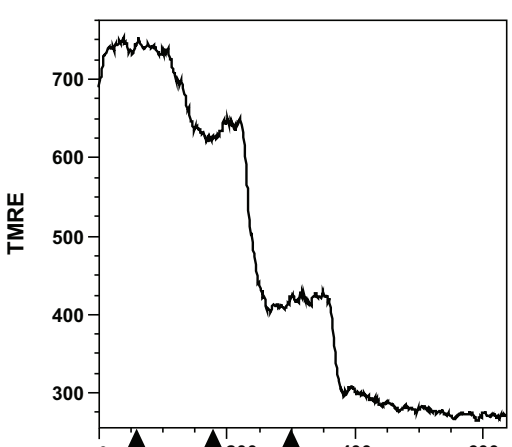

Figure 6. The kinetics of membrane potential change, as measured by flow cytometry. Jurkat cells were stained with 100 nM TMRE and run on Miltenyi's MACSQuant X flow cytometer to visualize the time parameter. At the indicated times (arrows), FCCP was added to the final concentrations of 0.1, 1 and 10 µM (left to right). Drop in membrane potential is rapid and dependent on the concentration of FCCP.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; Dispersions of duplicates/ triplicates.	A. Incomplete aspiration or pipetting error B. Cells lost during pipetting C. Bubble in the well(s) D. Uneven distribution or low density of cells E. Insufficient washing after dye loading F. Temperature gradient across the plate	A. Aspirate thoroughly and dispense consistently across the plate B. Pipette slower and angle the pipette tip towards the edge of the well C. Carefully tap the side of the plate with your finger or centrifuge to remove bubbles D. Make sure the cell suspension is homogenous and sufficient cells are seeded on the well E. Increase the volume or number of washes F. Minimize temperature fluctuation of the medium and the cell plate
Weak fluorescence signal above background	A. TMRE dye concentration is too low B. Cell density is too low C. Toxicity of test compound D. TMRE dye concentration is too high leading to excessive background and self-quenching E. Cells added to blank wells	A. Make sure the dye is completely thawed and diluted properly B. Increase the cell density C. Try lower concentration of test compound D. Lower the concentration of the dye E. Make sure no cells are added to blank well for background subtraction
Fluorescence signal change over time	A. Temperature change over time B. Release of non-specifically bound TMRE dye from the wall	A. Equilibrate the plate inside the plate reader for 15-30 minutes before scanning B. Increase the number of washes and/or equilibration time before reading

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.