TMRE Mitochondrial Membrane Potential Assay Kit

Item No. 701310

Customer Service 800.364.9897 * **Technical Support** 888.526.5351
www.caymanchem.com

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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
600331	Cell-Based Assay TMRE (0.5 mM)	1 vial/100 µl	-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: 734-971-3641
Email: techserv@caymanchem.com
Hours: M-F 8:00 AM to 5:30 PM EST

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 and used before the expiration date indicated on the outside of the box. Store the TMRE dye and FCCP at or below -20°C protected from light. Multiple freeze-thaw cycle should be avoided.

Materials Needed But Not Supplied

1. A plate reader capable of measuring fluorescence excitation at 530 nm and emission at 580 nm, preferably with temperature control.
2. Adjustable and multichannel pipettes.
3. A black 96-well tissue culture treated plate with clear bottom.
4. Cells (*e.g.* HepG2) and appropriate culture media.
5. Water-jacketed cell incubator at 37°C with 5% CO_2 (may vary with cell lines).
6. A T75 tissue culture flask with vented cap.
7. Hemocytometer or other cell counting device.
8. 15 ml Polypropylene tubes and reagent troughs (avoid polystyrene for TMRE).
9. Distilled water.
10. Plate washer (optional).

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.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Equilibrated Cell Culture Medium**

   For each 96-well plate to be assayed, 25 ml of cell culture medium (not provided) should be pre-warmed to 37°C before use. If the medium does not contain a buffer (*e.g.* HEPES), place the culture medium in a T75 flask with a vented cap and equilibrate in a 37°C CO_2 incubator for at least 30 minutes before use. This will ensure a stable pH and temperature throughout the experiment. Serum and antibiotics supplements are not required for this assay due to the short incubation time. TMRE, FCCP, test compounds, and vehicle controls should be diluted freshly in this equilibrated medium immediately before use.

2. **Cell-Based Assay TMRE - (Item No. 600331)**

   This vial contains 0.5 mM TMRE in DMSO. Thaw the dye completely and vortex before use. The optimal dye concentration (typically between 10 and 200 nM) should be pre-determined in a pilot experiment as described below. Dilute the dye in the equilibrated medium to 2x of the final assay concentration immediately before adding to the cells. Unused, undiluted dye should be aliquoted for storage at -20°C and protected from light. Avoid multiple freeze-thaw cycles. Diluted dye cannot be stored.

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

   This vial contains 20 mM FCCP in DMSO to be used as a control for disrupting the inner membrane potential of mitochondria. Dilutions of FCCP should be made in the equilibrated medium immediately before adding to the cells. For most cell lines, 30 µM FCCP is sufficient for mitochondria membrane depolarization.

4. **Assay Buffer**

   Dissolve each Cell-Based Assay Buffer Tablets (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.

NOTE

- TMRE is light sensitive. All staining procedures must be performed without direct exposure to intense light. Therefore, incubations need to be done 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 fluorescence signal will increase proportionally with cell density until the cells reach confluency. The fluorescence signal will also increase proportionally with TMRE dye concentration until a self-quenching concentration is reached. The optimal experiment setup will have a sub-quenching concentration of TMRE with a nearly confluent cell density. The dye concentrations between 5 and 500 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. Below is a suggested plate map for the pilot experiment.

		15.6 nM TMRE		31.3 nM TMRE		62.5 nM TMRE		125 nM TMRE		250 nM TMRE		500 nM TMRE		
		1	2	3	4	5	6	7	8	9	10	11	12	
No Cells	A													Control
	B													10 µM FCCP
50,000/well	C													Control
	D													10 µM FCCP
100,000/well	E													Control
	F													10 µM FCCP
150,000/well	G													Control
	H													10 µM FCCP

Figure 1.

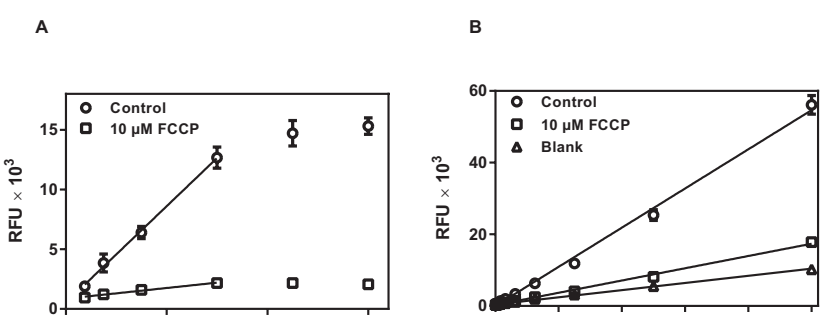


Figure 2. Sample data from assay optimization experiments. HepG2 cells were treated with FCCP or control medium for 30 minutes and then incubated with TMRE for 30 minutes as described in the assay protocol. (A) Saturation of signal was observed when HepG2 cells were plated above 150,000 cells per well (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

Cell Preparation

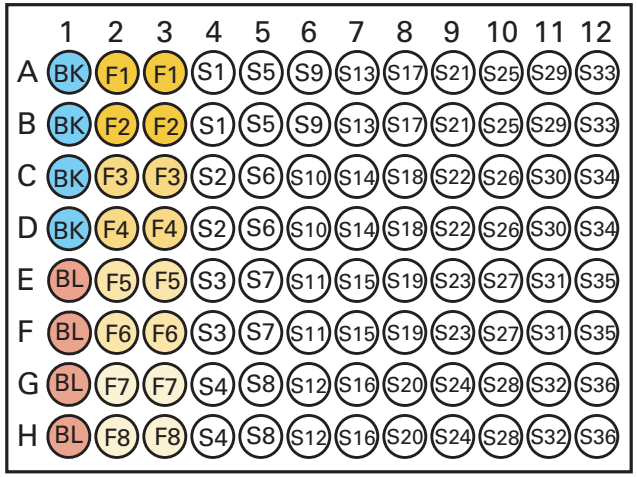
1. Seed cells in black 96-well clear bottom plate at the desired cell density in 100-200 µl medium per well. Leave four wells without cells (see plate map below). Dispense the same volume of medium only into those blank wells.

   Make sure the cell suspension is homogenous before dispensing onto the 96-well plate. Typically for HepG2 cells, 100,000 to 150,000 cells per well will reach near confluency within 24 hours after plating which is ideal for TMRE assay.

2. Let the cells sit for 30 minutes inside the hood until they adhere to the bottom of the plate. This will allow for a more even distribution of cells in the wells.

3. Incubate overnight using the appropriate culture conditions.

Plate Set Up



BK - Blank wells without cells
BL - Baseline (Maximal TMRE Uptake)
F1-F8 - FCCP serial dilutions
S1-S36 - Samples in duplicates

Figure 3. Sample plate map. There is no specific layout for the assay. In the sample plate map above, wells A1 to D1 are blanks (no cells but with culture medium) for background (non-specific binding) signal subtraction. Wells E1 to H1 are control cells in medium (or vehicle control). Columns 2 and 3 are for serial dilutions of FCCP control in duplicate.

Performing the Assay

1. Check the cells on plate to make sure they have reached the same desired density in all wells.
2. Prepare dilutions of testing compounds and FCCP control in Equilibrated Cell Culture Medium (page 6).
3. Carefully remove the culture media from the plate and replace with 100 µl of Equilibrated Cell Culture Medium per well containing vehicle, FCCP serial dilutions or testing compounds as described in Plate Set Up.
4. Immediately place the plate back into the incubator and incubate for 30 to 60 minutes.
5. Immediately before use, prepare TMRE at 2x of pre-determined optimal final concentration in Equilibrated Cell Culture Medium.
6. Remove the plate from incubator and apply 100 µl of 2x TMRE 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 200 µl of 1x Assay Buffer to each well.
10. Equilibrate the plate at room temperature for 15 to 30 minutes.
11. Read the plate in a fluorescent plate reader (excitation/emission = 530/580 nm).

Pipetting Hints

- Use different tips to pipette each reagent.
- Avoid introducing bubbles to the well(s).
- Do not expose the pipette tip to the reagent(s) already in the well.

ANALYSIS

Calculations

1. Subtract the value of the blank (BK) wells from all other wells. These values are mainly due to the non-specific binding of TMRE to the polystyrene plate.
2. The net fluorescence signal from the baseline (BL) wells is the maximum amount of TMRE is accumulated inside the mitochondria. The average value from these wells can be considered as 100% TMRE uptake.
3. The percentage TMRE fluorescence signal relative to the baseline vehicle control is calculated using the equation indicated below.

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

4. To determine an IC_{50} value for each compound, plot the % fluorescence 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.

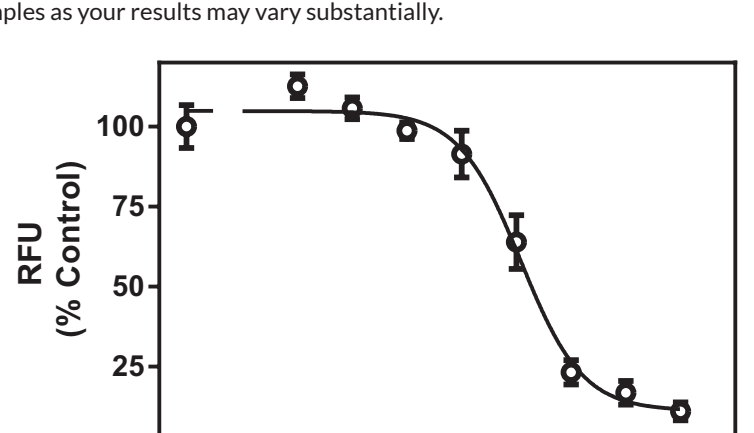


Figure 4. A typical concentration response curve for FCCP. HepG2 cells were seeded on a 96-well plate at 120,000 cells/well the day before performing the assay. The assay was performed as described and TMRE at 10 nM final concentration was used in this experiment. The calculated EC_{50} value is 1.1 µM.

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.