Mitochondrial PT Pore Assay Kit

Item No. 601430

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. Once opened, please remove components and store as stated below.

Item Number	Item	Quantity/Size	Storage
400146	Cell-Based Assay Calcein AM	1 vial/50 µl	-20°C
601431	Cobalt Chloride Solution	1 vial/100 µl	-20°C
601283	TMRE Dye	1 vial	4°C
701311	FCCP Control	1 vial/25 µl	-20°C
601432	Calcein Quenching Control	1 vial/100 µl	-20°C

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.

NOTE: It is recommended that gloves be worn at all time when working using these components.

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. Hank's Balanced Salt Solution with calcium and magnesium
2. Suggested - Cell viability stains such as DAPI Viability Dye (Item No. 601361) or RedDot™2 Viability Dye (Item No. 601282)

Flow Cytometry:

1. A centrifuge capable of 400 × g with a microplate adapter
2. Polypropylene v-bottom plate or tubes for treating and staining cells
3. A flow cytometer equipped with a blue laser (488 nm) and filters for measuring FITC (525 nm) and PE (575 nm)

Fluorescence Microscopy:

1. A cell culture-treated plate or chamber slide suitable for treating and staining cells
2. A fluorescence microscope with filters sufficient for measuring FITC and PE or TRITC

INTRODUCTION

About This Assay

The mitochondrial permeability pore (mPTP) is a cyclosporin A (CsA)-sensitive and cyclophilin D-dependent pore complex which forms in the inner mitochondrial membrane (IMM) and results from mitochondrial perturbation. Opening of the mPTP allows for larger, divalent ions to permeate the mitochondrial matrix, leading to membrane depolarization, calcium efflux, and subsequent mitochondrial swelling due to increased osmotic concentration.[1,2]

Initially observed in isolated mitochondria, the measurement of mPTP opening was adapted for whole cells using a calcein/cobalt quenching technique developed by Petronilli, *et. al*.[3] The calcein/cobalt technique uses calcein AM to stain the entire cell, followed by a treatment with cobalt chloride to quench the calcein fluorescence outside of the mitochondrial matrix. If the IMM is intact, cobalt is unable to penetrate the IMM and the mitochondrial matrix exhibits green fluorescence. If the IMM is compromised, the calcein fluorescence is quenched and no fluorescence is observed.

This assay utilizes the calcein/cobalt technique multiplexed with TMRE, allowing for the measurement of both membrane integrity and mitochondrial membrane potential ($\Delta_{\psi m}$). This assay has been adapted for use with flow cytometry and fluorescence microscopy, and includes controls for $\Delta_{\psi m}$ depolarization (FCCP) and calcein quenching without the loss of membrane potential (Calcein Quenching Control).

PRE-ASSAY PREPARATION

NOTE: This protocol requires Hank's Balanced Salt Solution supplemented with calcium and magnesium (HBSS) to dilute all reagents unless noted.

Reagent Preparation

1. **Cell-Based Assay Calcein AM (Item No. 400146)**

   This vial contains a 1 mM solution of Calcein AM in DMSO. This vial should be stable for one year when stored at -20°C. Dilute 15 µl into 1.5 ml HBSS to make a Calcein Working Solution (10 µM), which is stable for one hour on ice. Unused portions of the DMSO stock can be aliquoted and stored at -20°C. Multiple freeze-thaw cycles should be avoided.

2. **Cobalt Chloride Solution (Item No. 600431)**

   This vial contains a 1M solution of cobalt chloride in UltraPure water. Store this vial at 4°C for up to one year after the initial thaw. Make a 100 mM Cobalt Chloride Working Solution by diluting 60 µl into 540 µl of HBSS.

3. **TMRE Dye (Item No. 601283)**

   This vial contains a TMRE powder. Reconstitute in 100 µl of DMSO to make a 0.5 mM stock. This reconstituted stock should be stable for six months when stored at -20°C. Unused TMRE Dye can be aliquoted and stored at -20°C. Multiple freeze-thaw cycles should be avoided. Make TMRE Working Solution (20 nM) by diluting the stock solution 1:100 in HBSS and then adding 80 µl of this to 20 ml HBSS.

4. **Calcein Quenching Control (Item No. 600432)**

   This vial contains a proprietary solution used for quenching calcein fluorescence in the mitochondrial matrix, without causing a loss of membrane potential. This vial should be stable for one year when stored at -20°C. To make a Calcein Quenching Control Working Solution, dilute 50 µl into 450 µl HBSS.

5. **FCCP Control (Item No. 701311)**

   This vial contains a 20 mM solution of FCCP in DMSO. This vial should be stable for one year when stored at -20°C. Dilute 10 µl into 200 µl HBSS to make an FCCP Control Working Solution (1 mM). Unused FCCP can be aliquoted and stored at -20°C. Multiple freeze-thaw cycles should be avoided.

ASSAY PROTOCOL

NOTE: We recommend analyzing suspension cells by flow cytometry and adherent cells by fluorescence microscopy. Removing most adherent cell lines from the culture dish by traditional methods is incompatible with this staining protocol.

Flow Cytometry

Compensation Controls:

- TMRE Working Solution - Use TMRE Working Solution as detailed in **Pre-Assay Preparation.** No additional dilution required.
- Calcein Staining Solution - Dilute 5 µl of Calcein Quenching Control Working Solution in 5 ml HBSS.

Protect from light. Solutions are stable for one hour on ice. All solutions should be pre-warmed to 37°C immediately before staining.

mPTP Staining Solution:

Reagent	Working Solution Concentration	Final Concentration	Volume
Calcein	10 µM	10 nM	12 µl
Cobalt Chloride	100 mM	0.4 mM	48 µl
TMRE Working Solution	-	20 nM	12 ml

As prepared above, the amount of mPTP Staining Solution is sufficient for 100 tests at 100 µl/test. Scale volumes up approximately 5-fold if staining is to be performed in tubes. All solutions should be protected from light, and are stable for one hour on ice. All solutions should be pre-warmed to 37°C immediately before staining.

Control Compound Staining Solutions:

Control	Calcein Quenching Control Working Solution	FCCP Control Working Solution	mPTP Staining Solution
Calcein Quencing Control	5 µl	-	495 µl
FCCP Control	-	5 µl	495 µl

Staining solutions should be stable for one hour on ice. Pre-warm staining solutions to 37°C immediately before staining.

Protocol:

Cell staining should be optimal at 1-5 x 10^5 cells/well, however this may vary with cell type. To determine optimal staining density, it is recommended that different cell densities be tested when working with unfamiliar cell types. Always begin with healthy, growing cells, and avoid staining when cells are overgrown.

For the screening of unknown compounds, cells can be treated with compounds up to 12 hours (long term treatment) prior to staining. Multiplexing of viability staining is recommended if effects of compounds on viability are unknown. Depending on laser/filter set availability, DAPI Viability Dye (Item No. 601361) or RedDot™2 Viability Dye (Item No. 601282) are plasma membrane permeability detectors ideally suited for this purpose. A protocol for using these reagents is contained in the **Appendix** on page 17.

Before treatment, reserve untreated cells for compensation (controls unstained, calcein alone and TMRE alone) as well as functional controls (FCCP control and calcein quenching control). We recommend performing these controls and all samples in triplicate.

1. Transfer cells at optimal density to 96-well v-bottom polypropylene plate.
2. Centrifuge plate at 400 x g to pellet cells, and discard supernatant.
3. Add 100 µl of the appropriate single stains to wells reserved for compensation controls.
4. To wells designated as FCCP Control, add 100 µl of the FCCP Staining Solution.
5. To wells designated as Calcein Quenching Control, add 100 µl of the Calcein Quenching Staining Solution.
6. Add 100 µl of mPTP Staining Solution to the remaining cell-containing wells.
7. Incubate plate for 15 minutes at 37°C.
8. Remove plate from incubator and centrifuge plate at 400 x g to pellet cells.
9. Discard supernatant, and resuspend cells in PBS or a buffer suitable for flow cytometry.
10. Detect calcein fluorescence using a typical FITC channel and TMRE using a typical PE channel.
11. Collect at least 20,000 events per sample.

Fluorescence Microscopy

NOTE: Final concentrations of calcein and cobalt differ between microscopy and flow cytometry protocols.

mPTP Staining Solution:

Reagent	Working Solution Concentration	Final Concentration	Volume
Calcein Quenching Control Working Solution	10 µM	1 µM	1.5 ml
Cobalt Chloride Working Solution	100 mM	4 mM	600 µl
TMRE Working Solution	-	20 nM	12.9 ml

As prepared above, the amount of mPTP Staining Solution is sufficient for a 12 well plate at 1 ml/well. Scale volumes as necessary for different size vessels. All solutions should be protected from light, and should be stable for one hour on ice. All solutions should be pre-warmed to 37°C immediately before staining.

Control Compounds:

Calcein Quenching Control Working Solution - use as detailed in the **Pre-Assay Preparation** section on page 8. No additional dilution required.

FCCP Control Working Solution - use as detailed in the **Pre-Assay Preparation** section on page 8. No additional dilution required.

Protocol:

Cell staining should be optimal at densities near confluency, however this may vary with cell type. Avoid staining when cells are at 100% confluency or overgrown. Should nuclear staining be desired for microscopic identification of individual cells, a protocol for using Cell-Based Assay Hoechst Dye (purchased separately, Item No. 600332) can be found in the **Appendix** on page 17.

For the screening of unknown compounds, cells can be treated prior to staining for up to 12 hours (long term treatment). Before treatment, designate at least one well each for FCCP mitochondrial depolarization control and calcein quenching positive control. These control wells should contain cells, but not be treated with compounds.

1. Plate, culture, and treat cells according to your typical experimental protocol.
2. Remove cell culture medium and wash cells with PBS.
3. Remove PBS and add 1 ml of mPTP Staining Solution to all wells except the control wells.
4. To wells designated for FCCP control, add 990 µl mPTP Staining Solution and 10 µl of the FCCP Control Working Solution.
5. To wells designated for Calcein quenching positive control, add 990 µl mPTP Staining Solution and 10 µl of Calcein Quenching Control Working Solution.
6. Incubate plate for 15 minutes at 37°C.
7. Remove plate from incubator and aspirate staining solution.
8. Gently wash cells with HBSS and add a suitable volume of HBSS for imaging.
9. Visualize calcein with a filter set typically used for FITC and TMRE with a filter set typically used for PE (approximately ex/em 485/535 and 545/576, respectively).

ANALYSIS

Performance Characteristics

Flow Cytometry

Compensation controls:

Make dot plots of TMRE *versus* calcein. Compensate as necessary to bring the calcein single-stained control cells to a mean fluorescence intensity in the TMRE channel equivalent with unstained cells. As these cells are not treated with cobalt, they should be substantially brighter than any other sample. TMRE single-stained cells should show minimal spectral overlap in the calcein channel.

Control Compounds:

Make dot plots of TMRE *versus* calcein as shown in Figure 1 on page 15. Establish quadrants based on vehicle-treated cells so that:

Vehicle-treated - TMRE positive, calcein positive (upper right quadrant)

FCCP-treated - TMRE negative, calcein positive (lower right quadrant)

Calcein Quenching Control-treated - TMRE positive, calcein negative (upper left quadrant)

Fluorescence Microscopy

Control Compounds:

Vehicle-treated cells should have colocalized fluorescence for TMRE and calcein.

FCCP-treated cells should exhibit calcein fluorescence with little or no TMRE fluorescence.

Calcein Quenching Control-treated cells should exhibit TMRE fluorescence without calcein fluorescence.

Sample Data

Flow Cytometry

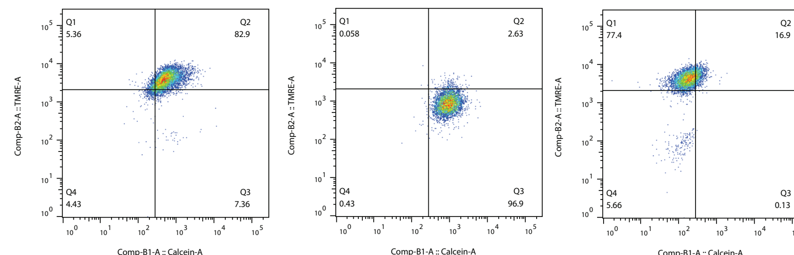

Figure 1. THP-1 cells treated with vehicle, FCCP, or Calcein Quenching Control. Treatment with vehicle or the control compounds FCCP and Calcein Quenching Control results in cell populations in the upper right, lower right, and upper left quadrants, respectively. The total loss of membrane integrity would decrease both the TMRE and calcein fluorescence, resulting in a population of cells in the lower left hand quadrant.

Fluorescence Microscopy

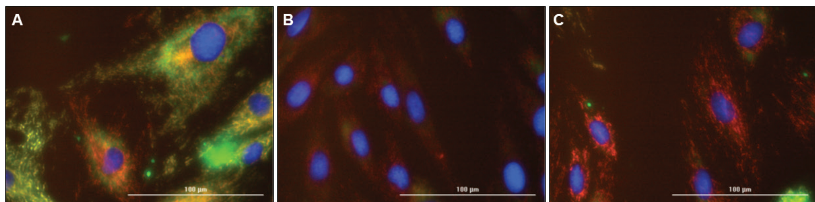

Figure 2. H9c2 cells treated with vehicle, FCCP or Calcein Quenching Control. Treatment of vehicle or control compounds results in the following: Colocalized fluorescence of TMRE and calcein with vehicle treatment (A), Loss of TMRE fluorescence with FCCP treatment (B), and loss of calcein fluorescence with Calcein Quenching Control treatment (C). The total loss of membrane integrity that occurs with mPTP opening would decrease fluorescence for both TMRE and calcein. Opening of the mPTP is defined based on sensitivity to Cyclosporin A (not provided; Item No. 12088). Nuclei were stained with Cell-Based Assay Hoechst Dye (not provided, Item No. 600332). Cells were imaged using the Cytation™ 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Inc.).

RESOURCES

Appendix

1. **RedDot™2 Viability Dye (Item No. 601282), purchased separately**

   This vial contains 50 µl of RedDot™2, a product of Biotium, Inc. To use for fluorescence microscopy or flow cytometry, dilute 1:200 in PBS, pH 7.4, and add 100-500 µl per 1 x 10^5-1 x 10^6 cells. Assay immediately using excitation of 633 nm and emission around 700 nm.

2. **DAPI Viability Dye (Item No. 601361), purchased separately**

   This vial contains 100 µl of 100X DAPI. To use for fluorescence microscopy or flow cytometry, dilute 1:100 in PBS, pH 7.4, and add 100-500 µl per 1 x 10^5-1 x 10^6 cells. Assay immediately using excitation between 350-405 nm and emission around 450 nm.

3. **Cell-Based Assay Hoechst Dye (Item No. 600332), purchased separately**

   This vial contains 50 µl of 20 mM Hoechst dye in water. Dilute the contents of this vial 1:5,000 in mPTP Staining Solution to yield a final concentration of ~4 µM. Follow the staining procedure for mPTP Staining Solution found on page 13. Hoechst Dye fluorescence can be visualized using a DAPI filter set (or an excitation/emission wavelength between 330-370 nm/420-500 nm, respectively).

Troubleshooting

Problem	Possible Causes	Recommended Solutions
No fluorescence or minimal fluorescence is detected	A. Cell density is too low or too high B. Cell are not viable C. Filter sets are not optimal for calcein and TMRE D. Inner mitochondrial membrane has been permeabilized	A. Perform a cell density titration to determine the optimal density for your cell type B. Stain with a cell viability dye as suggested in the protocol C. Contact instrument manufacturer to determine optimal filter sets

References

1. Bernardi, P., Vassanelli, S., Veronese, P., *et al.* Modulation of the mitochondrial permeability transition pore. Effect of protons and divalent cations. *J. Biol. Chem.* **267(5)**, 2934-2939 (1992).
2. Zulian, A., Schiavone, M., Giorgio, V., *et al.* Forty years later: Mitochondria as therapeutic targets in muscle diseases. *Pharmacol. Res.* **113(Pt A)**, 563-573 (2016).
3. Petronilli, V., Miotto, G., Canton, M., *et al.* Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. *Biophys. J.* **76(2)**, 725-734 (1999).

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.

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