

Image-iT™ LIVE Mitochondrial Transition Pore Assay Kit (I35103)

Quick Facts

Storage upon receipt:

- $\leq -20^{\circ}\text{C}$
- Protect from light
- Desiccate
- Avoid freeze-thaw cycles

Ex/Em:

- 494/517 nm (calcein)
- 579/599 nm (MitoTracker® Red CMXRos stain)
- 350/461 nm (Hoechst 33342 stain)

Number of assays: 100, based on labeling volumes of 1.0 mL

permeability transition pore opening than assays relying on mitochondrial membrane potential alone. Cells are loaded with the acetoxymethyl ester of calcein dye, calcein AM, which passively diffuses into the cells and accumulates in cytosolic compartments, including the mitochondria. Once inside cells, intracellular esterases cleave the acetoxymethyl esters to liberate the very polar fluorescent dye calcein, which does not cross the mitochondrial or plasma membranes in appreciable amounts over relatively short periods of time. The fluorescence from cytosolic calcein is quenched by the addition of CoCl_2 , while the fluorescence from the mitochondrial calcein is maintained. As a control, cells that have been loaded with calcein AM and CoCl_2 can also be treated with an ionophore, ionomycin, to allow entry of excess Ca^{2+} into the cells to trigger mitochondrial pore activation and subsequent loss of green mitochondrial calcein fluorescence. The ionomycin response can be blocked with cyclosporine A, a compound reported to prevent mitochondrial transition pore formation by binding cyclophilin D. The Image-iT LIVE Mitochondrial Transition Pore Assay Kit has been tested with HeLa and bovine pulmonary artery endothelial (BPAE) cells.

Introduction

The mitochondrion plays a vital role in the processes of apoptotic and necrotic cell death. The mitochondrial permeability transition pore is a nonspecific channel formed by components from the inner and outer mitochondrial membranes, and appears to be involved in the release of mitochondrial components during cell death. The Image-iT™ LIVE Mitochondrial Transition Pore Assay Kit is based on published experimentation for mitochondrial transition pore opening.^{1,2} This assay employs calcein AM, a colorless and nonfluorescent esterase substrate, and CoCl_2 , a quencher of calcein fluorescence, to selectively label mitochondria.

In a healthy cell, the inner mitochondrial membrane is responsible for maintaining the electrochemical gradient that is essential for cellular respiration and oxidative phosphorylation. As Ca^{2+} is taken up and released by mitochondria, a low conductance permeability transition pore appears to flicker between open and closed states.³ During cell death, the opening of the mitochondrial permeability transition pore dramatically alters the permeability of mitochondria. Continuous pore activation results from mitochondrial Ca^{2+} overload, oxidation of mitochondrial glutathione, increased levels of reactive oxygen species in mitochondria, and other pro-apoptotic conditions.⁴ Cytochrome *c* release from mitochondria and loss of mitochondrial membrane potential are observed subsequent to continuous pore activation.

The Image-iT LIVE Mitochondrial Transition Pore Assay Kit provides a more direct method of measuring mitochondrial

Materials

Kit Contents

- **Calcein, AM** (Component A), 5 vials, each containing 50 μg of lyophilized reagent
- **MitoTracker Red CMXRos** (Component B), 50 μg
- **Hoechst 33342** (Component C), 400 μL of a 1.0 mM solution in water
- **Ionomycin** (Component D), one vial containing 37 μg lyophilized reagent
- **Cobalt (II) chloride hexahydrate** (Component E), 200 μL of a 1.0 M solution in water
- **DMSO** (Component F), 2 vials, each containing 500 μL

Storage and Handling

Upon receipt, store desiccated and protected from light at $\leq -20^{\circ}\text{C}$. Before opening each vial, allow the product to warm to room temperature. When stored properly, components should be stable for up to 6 months. Avoid freeze-thaw cycles.

Spectral Characteristics

The approximate excitation/emission peaks of calcein after hydrolysis are 494/517 nm, the approximate excitation/emission peaks of MitoTracker Red CMXRos dye are 579/599 nm, and the approximate excitation/emission peaks of Hoechst 33342 dye are 350/461 nm, respectively. Calcein, MitoTracker Red CMXRos dye, and Hoechst 33342 dye can be observed using standard filter sets.

Experimental Protocol

The reagents in the Image-iT LIVE Mitochondrial Transition Pore Assay Kit are used to follow mitochondrial transition pore opening following a given experimental treatment, such as induction of apoptosis. The following protocol describes the preparation of calcein-stained cells, including the appropriate use of CoCl_2 (to quench cytosolic fluorescence) and ionomycin (to facilitate mitochondrial calcium overload and subsequent pore activation). The protocol was optimized using HeLa and bovine pulmonary artery endothelial (BPAE) cells grown on coverslips, but can be used with other cell types. To achieve optimal results, experimental parameters such as incubation times and reagent concentration should be adjusted depending on cell type and culture conditions used. This protocol can also be adapted for use in conjunction with other probes.

Buffer Requirements and Recommendations

This assay was developed using Hanks' Balanced Salt Solution (HBSS) with sodium bicarbonate, calcium, and magnesium that also included HEPES (10 mM), L-glutamine (2 mM) and succinate (100 μM) to support healthy mitochondrial function in live cells. This protocol is compatible with common buffers used in live-cell imaging, but the buffer used for the optional ionomycin positive control must contain Ca^{2+} in order to trigger mitochondrial transition pore activation.

Preparation of Stock Solutions

Aliquots of stock solutions can be frozen at $\leq -20^\circ\text{C}$ for up to 6 months.

1.1 Prepare a 1.0 mM calcein AM stock solution. Dissolve the contents of one vial of calcein AM (Component A) in 50 μL of DMSO (Component F) for a final concentration of 1.0 mM. Once prepared, the 1.0 mM calcein AM stock solution should be used within a short time period and should not be frozen and thawed repeatedly. Multiple vials of calcein AM are provided with this kit to ensure ample material for multiple experiments.

1.2 Prepare a 200 μM MitoTracker Red CMXRos stock solution. Dissolve the contents of one vial of Mitotracker Red CMXRos dye (Component B) in 470 μL of DMSO (Component F) for a final concentration of 200 μM .

1.3 Prepare a 500 μM ionomycin stock solution (optional). To the Component D vial, add 100 μL of DMSO (Component F) and mix well.

Labeling Protocol

2.1 Prepare the labeling solution. Combine 1.0 μL of each of the following: 1.0 mM calcein AM stock solution, 200 μM MitoTracker Red CMXRos stock solution, 1.0 mM Hoechst 33342 dye (Component C), and 1.0 M CoCl_2 (Component E). Add to 996 μL of the modified HBSS prepared above and warm to 37°C protected from light.

2.2 Label cells. Wash cells twice in the modified HBSS buffer, aspirate the buffer from the cells, and apply a sufficient amount of labeling solution to cover the cells adhering to a coverslip. Incubate for 15 minutes at 37°C , protected from light.

2.3 Wash cells. Wash cells in warm modified HBSS buffer to remove residual dye and minimize background, and aspirate buffer.

2.4 (Optional) Prepare a positive control sample. Ionomycin (prepared in step 1.3) is supplied as a positive control for Ca^{2+} -mediated pore opening. Prepare a 0.5–1.0 μM ionomycin solution by making a 1000-fold to 500-fold dilution of 500 μM ionomycin (Component D) in the modified HBSS buffer. To a previously labeled sample, add a sufficient amount of the 0.5–1.0 μM ionomycin solution to cover the cells. As cells experience Ca^{2+} overload from ionomycin treatment, mitochondrial calcein signal should be lost very quickly while MitoTracker Red CMXRos stain signal is preserved.

2.5 Prepare cells for viewing. Mount the cells in warm buffer.

References

1. Biophys J 76, 725 (1999); 2. Biofactors 8, 263 (1998); 3. Am J Physiol Cell Physiol 279, C852 (2000); 4. Biochem J 341, 233 (1999).

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
I35103	Image-iT™ LIVE Mitochondrial Transition Pore Assay Kit *for microscopy*	1 kit

Contact Information

Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Paisley, United Kingdom. All others should contact our Technical Service Department in Eugene, Oregon.

Please visit our website — probes.invitrogen.com — for the most up-to-date information.

Molecular Probes, Inc.

29851 Willow Creek Road, Eugene, OR 97402
Phone: (541) 465-8300 • Fax: (541) 335-0504

Customer Service: 6:00 am to 4:30 pm (Pacific Time)

Phone: (541) 335-0338 • Fax: (541) 335-0305 • probesorder@invitrogen.com

Toll-Free Ordering for USA:

Order Phone: (800) 438-2209 • Order Fax: (800) 438-0228

Technical Service: 8:00 am to 4:00 pm (Pacific Time)

Phone: (541) 335-0353 • Toll-Free (800) 438-2209
Fax: (541) 335-0238 • probestech@invitrogen.com

Invitrogen European Headquarters

Invitrogen, Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Phone: +44 (0) 141 814 6100 • Fax: +44 (0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com

Molecular Probes products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Please read the Material Safety Data Sheet provided for each product; other regulatory considerations may apply.

Limited Use Label License

For research use only. Not intended for any animal or human therapeutic or diagnostic use. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Molecular Probes, Inc., Business Development, 29851 Willow Creek Road, Eugene, OR 97402. Tel: (541) 465-8300. Fax: (541) 335-0504.

Several Molecular Probes products and product applications are covered by U.S. and foreign patents and patents pending. All names containing the designation ® are registered with the U.S. Patent and Trademark Office.

Copyright 2007, Molecular Probes, Inc. All rights reserved. This information is subject to change without notice.