Product Manual

MITO-ID® Red Detection Kit (GFP CERTIFIED®)

GFP-Certified® Mitochondrial Detection for microscopy

Catalog #: ENZ-51007

ENZ-51007-500 500 assays

ENZ-51007-0100 100 assays

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Product Manual

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Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

INTRODUCTION

Enzo Life Sciences' MITO-ID® Red Detection Kit (GFP-Certified® Mitochondrial Detection) contains a novel mitochondria-selective dye suitable for live-, detergent-permeabilized- and aldehyde-fixed-cell staining. Conventional fluorescent stains for mitochondria — such as JC-1 (Catalog No. ENZ-52304), rhodamine 123 (Catalog No. ENZ-52307) and tetra-methylrhodamine ethyl ester (TMRE, Catalog No. ENZ-52309) — are readily sequestered by actively respiring mitochondria. However, these mitochondrial stains are subsequently leached out of cells once the mitochondria's membrane potential dissipates. This characteristic severely limits their use in experiments in which cells must be treated with nonionic detergents, aldehyde fixatives or other agents that affect the energetic state of the mitochondria. Sub-micromolar concentrations of MITO-ID® Red dye are sufficient for staining mammalian cells. This has been validated with a human cervical carcinoma cell line, HeLa; a human T-lymphocyte cell line, Jurkat; and human bone osteosarcoma epithelial cell line, U2OS.

One important application of MITO-ID® Red dye is in fluorescence co-localization imaging with green fluorescent protein (GFP)-tagged proteins. This is a powerful approach for determining the targeting of molecules to intracellular compartments, and for screening of associations and interactions between these molecules. However to date, photoconversion of red fluorescent dyes to green and metachromatic artifacts wherein fluorescent dyes emit both in the red and green regions of the spectrum have led to spurious results in GFP co-localization experiments.[1,2] Additionally, many organelle-targeting probes photobleach rapidly, are subject to quenching when concentrated in organelles, are highly toxic, or only transiently associate with the target organelle, requiring imaging within a minute or two of dye addition.[3,4]

Enzo's MITO-ID® Red dye, a new red-emitting, cell-permeable small organic probe molecule that spontaneously localizes to live or fixed mitochondria, was developed to overcome the above problems. The MITO-ID® Red dye can be readily used in combination with other common UV and visible light excitable fluorescent dyes and various fluorescent proteins in multi-color imaging and detection applications. It emits in the Texas Red region of the visible light spectrum, and is highly resistant to photobleaching, concentration quenching and photoconversion.

The MITO-ID® Red Detection Kit (GFP-Certified® Mitochondrial Detection) is specifically designed for use with GFP-expressing cell lines, as well as cells expressing blue, cyan or yellow fluorescent proteins (BFPs, CFPs, YFPs). Additionally, the kit is

suitable for use with live or post-fixed cells in conjunction with probes, such as labeled antibodies, or other fluorescent conjugates displaying similar spectral properties as fluorescein or coumarin. A nuclear counterstain is provided to highlight this organelle as well.

SAFETY WARNINGS & PRECAUTIONS

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Handle with care

- This product is for research use only and is not intended for diagnostic purposes.
- The MITO-ID® Red Detection Reagent contains DMSO which is readily absorbed through the skin. It is harmful if ingested or absorbed through the skin and may cause irritation to the eyes. Observe appropriate precautions when handling.

Avoid freeze / thaw cycles

- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.
- To avoid photobleaching, perform all manipulations in low light environments or protected from light by other means.

REAGENTS PROVIDED AND STORAGE

All reagents are shipped on dry ice. Upon receipt, the kit should be stored at ≤-20°C, protected from light. When stored properly, these reagents are stable for at least twelve months. Avoid repeated freezing and thawing.

Reagents provided in the ENZ-51007-500 kit are sufficient for approximately 500 assays and the ENZ-51007-0100 are sufficient for approximately 100 assays using either live, adherent cells or cells in suspension.

Reagent	ENZ-51007-500	ENZ-51007-0100
MITO-ID® Red Detection Reagent	10 µL	2 µL
Hoechst 33342 Nuclear Stain	50 µL	13 µL
10X Assay Buffer	15 mL	3 mL

Reagants require separate storage conditions.

OTHER MATERIALS NEEDED

1. Standard fluorescence microscope
2. Calibrated, adjustable precision pipetters, preferably with disposable plastic tips
3. Adjustable speed centrifuge with swinging buckets (for suspension cultures)
4. Glass microscope slides
5. Glass cover slips
6. Deionized water
7. Anhydrous DMSO (optional)
8. Growth medium (e.g., Dulbecco's Modified Eagle Medium, D-MEM)
9. Formaldehyde (optional, for fixation protocol)
10. Triton X-100 (optional, for permeabilization protocol)
11. Antifade Mounting Media (hardset) or Antifade Mounting Media with DAPI (hardset) (ENZ-53002-M010 or ENZ-53003-M010, optional)

METHODS AND PROCEDURES

***NOTE**: Allow all reagents to thaw at room temperature before starting with the procedures. Upon thawing, gently hand-mix or vortex the reagents prior to use to ensure a homogenous solution. Briefly centrifuge the vials at the time of first use, as well as for all subsequent uses, to gather the contents at the bottom of the tube.*

REAGENT PREPARATION

1. 1X Assay Buffer

Allow the 10X Assay Buffer to warm to room temperature. Make sure that the reagent is free of any crystallization before dilution. Prepare enough 1X Assay Buffer for the number of samples to be assayed by diluting each milliliter (mL) of the 10X Assay Buffer with 9 mL of deionized water.

2. Dual Detection Reagent

The concentration of MITO-ID® Red dye for optimal staining will vary depending upon the application. Suggestions are provided to use as guidelines, though some modifications may be required depending upon the particular cell type employed and other factors such as the permeability of the dye to the cells or tissues. To reduce potential artifacts from overloading of the cells, the concentration of the dye should be kept as low as possible.

Prepare sufficient amount of Dual Detection Reagent for the number of samples to be assayed as follows: For every 10 mL of 1X Assay Buffer (see preparation in step 1) or cell culture medium, add 1 µL of MITO-ID® Red Detection Reagent and 10 µL of Hoechst 33342 Nuclear Stain.

NOTE:

(a) The dyes may be combined into one staining solution or each may be used separately, if desired.

(b) An intermediate 10-fold dilution of the MITO-ID® Red Detection Reagent can be made in DMSO if larger staining volumes are not needed. The intermediate dilution is stable for at least 4 weeks if stored at -20°C.

(c) The Hoechst 33342 Nuclear Stain can be diluted further if its staining intensity is much stronger than the red Mitochondrial stain, MITO-ID® Red.

(d) When staining BFP- or CFP-expressing cells, the Hoechst 33342 Nuclear Stain should be omitted due to its spectral overlap with these fluorescent proteins.

STAINING LIVE, ADHERENT CELLS

1. Grow cells on cover slips inside a Petri dish filled with the appropriate culture medium. When the cells have reached the desired level of confluence, carefully remove the medium.
2. Dispense sufficient volume of Dual Detection Reagent (see Reagent Prep section 2, page 5) to cover the monolayer cells (~100 µL of labeling solution for cells grown on an 18 X 18 mm coverslip).
3. Protect samples from light and incubate for 15-30 minutes at 37°C.
4. Wash the cells with 100 µL 1X Assay Buffer. Remove excess buffer and place coverslip on slide.

   Analyze the stained cells by wide-field fluorescence or confocal microscopy (60X magnification recommended). Use a standard Rhodamine or Texas Red filter set for imaging the mitochondria. Optionally, image the nucleus using a DAPI filter set and the GFP-tagged protein using a GFP/FITC filter set.

STAINING LIVE CELLS GROWN IN SUSPENSION

1. Centrifuge cells for 5 minutes at 400 x g at room temperature (RT) to obtain a cell pellet.
2. Carefully remove the supernatant by aspiration and dispense sufficient volume of Dual Detection Reagent (Reagent Prep section 2, page 5) to cover the dispersed cell pellet.
3. Protect samples from light and incubate for 15 to 30 minutes at 37°C.
4. Wash the cells with 100 µL 1X Assay Buffer. Remove excess buffer. Resuspend cells in 100 µL 1X Assay Buffer, then apply the cells to a glass slide and overlay with a coverslip.
5. Analyze the stained cells by wide-field fluorescence or confocal microscopy (60X magnification recommended). Use a standard Rhodamine or Texas Red filter set for imaging the mitochondria. Optionally, image the nucleus using a DAPI filter set and the GFP-tagged protein using a GFP/FITC filter set.

ALDEHYDE FIXATION AND DETERGENT PERMEABILIZATION OF LIVE CELLS STAINED WITH MITO-ID® RED DYE

***NOTE**: It is NOT recommended to stain with both MITO-ID® Red and the Hoechst nuclear counterstain if they are to be fixed. The recommended method of staining fixed cells is to stain after the cells have been fixed.*

1. Stain the cells as described in the preceding section, using a 2,500 fold dilution of the MITO-ID® Red dye instead of the 10,000 fold dilution. After staining with MITO-ID® Red dye, wash the cells in fresh, pre-warmed growth medium or 1X Assay Buffer.
2. Carefully remove the growth medium, or 1X Assay Buffer covering the cells, and replace it with freshly prepared medium or buffer containing 3.7% formaldehyde.

   ***NOTE**: If the growth medium contains serum, the formaldehyde solution should also be prepared in growth medium containing serum.*
3. Incubate the cells at 37°C for 15 minutes.
4. After fixation, wash the cells several times in PBS or 1X Assay Buffer.
5. If the cells are to be subsequently labeled with an antibody, a permeabilization step is usually required to enhance the antigen's accessibility. Incubate the fixed cells in PBS or 1X Assay Buffer containing 0.1% Triton X-100 at room temperature for no more than 5 minutes.
6. Following permeabilization, rinse the cells in PBS or 1X Assay Buffer.

   ***NOTE**: If desired, standard immunofluorescence staining protocols using fluorescein- or coumarin-based antibody conjugates, or equivalent, may be performed after fixation and permeabilization steps. Antifade compounds using p-phenylenediamine are not recommended. If an antifade is desired, we recommend Enzo's Antifade Mounting Media (hardset) (ENZ-53002-M010) or Antifade Mounting Media with DAPI (hardset) (ENZ-53003-M010).*

   Try to view the samples as soon as possible after staining for sharper staining.
7. If antifade is desired, remove all liquid. Add one drop of antifade (warmed to room temperature), then carefully add a coverslip over sample, being careful not to introduce air bubbles. For sharper images, the sample may be viewed

immediately, or for long term storage, the sample may be allowed to dry overnight before sealing the coverslip.

STAINING OF ALDEHYDE-FIXED AND DETERGENT PERMEABILIZED CELLS

The MITO-ID® Red dye is capable of staining already fixed and permeabilized cells. It is not recommended to stain fixed cells with both MITO-ID® Red dye and the Hoechst nuclear counterstain.

1. Fixation and permeabilization should be performed as described in the preceding section.
2. Perform staining as recommended for adherent or suspension cells (the two preceding sections) using a 2,500 fold dilution of the MITO-ID® Red dye instead of the 10,000 fold dilution.

   ***NOTE**: If desired, standard immunofluorescence staining protocols using fluorescein- or coumarin-based antibody conjugates, or equivalent, should be performed before Staining with MITO-ID® Red. Antifade formulations using p-phenylenediamine are not recommended. If an antifade agent is desired, we recommend Enzo's Antifade Mounting Media (hardset) (ENZ-53002-M010) or Antifade Mounting Media with DAPI (hardset)(ENZ-53003-M010).*

   Try to view the samples as soon as possible after staining for sharper staining.
3. If an antifade agent is desired, remove all liquid. Add one drop of antifade reagent (warmed to room temperature), then carefully add a coverslip over sample, being careful not to introduce air bubbles. For sharper images, the sample may be viewed immediately, or for long term storage, the sample may be allowed to dry overnight before sealing the coverslip.

APPENDICES

FILTER SET SELECTION

The selection of optimal filter sets for a fluorescence microscopy application requires matching the optical filter specifications to the spectral characteristics of the dyes employed in the analysis. Consult the microscope or filter set manufacturer for assistance in selecting optimal filter sets for your microscope

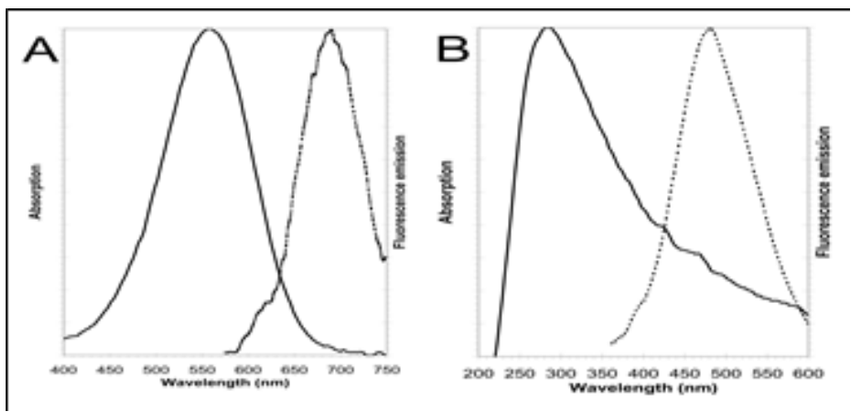

Figure 1. Absorption and fluorescence emission spectra, ex/em 558/690 nm, for MITO-ID® Red (A) and Hoechst 33342, ex/em 350/461 nm (B) dyes. All spectra were determined in 1X Assay Buffer.

RESULTS

Mitochondria are subcellular organelles found in eukaryotic cells, often representing as much as 10% of the total cell volume. Although conventional fluorescent stains for mitochondria, such as JC-1, rhodamine 123 and tetramethylrhodamine, are readily sequestered by functioning mitochondria, they are subsequently leached out of the cells once the mitochondrial membrane potential is dissipated. MITO-ID® Red dye accumulates in the mitochondria regardless of the mitochondrial membrane potential. The dye selectively stains mitochondria of living cells and is relatively insensitive to mitochondrial membrane potential uncouplers of phosphorylation, such as CCCP (carbonyl cyanide 3-chlorophenylhydrazone), as well as ion-channel perturbing drugs, such as valinomycin. In addition to being a live cell-permeable dye, MITO-ID® Red dye is also retained during or after cell fixation and detergent permeabilization.

MITO-ID® Red dye has been shown to co-localize with EGFP-cytochrome C oxidase chimeric protein in the HeLa-TurboGreen-mitochondria cell line (HeLa-mitoGFP, MarinPharm GmbH, Luckenwalde, Germany). Typically, intense red fluorescent staining of the mitochondrial network in the perinuclear region of mammalian cells is readily apparent using MITO-ID® Red dye, often with a more grain-like structure in the sub-plasma membrane region. MITO-ID® Red dye co-localizes with the EGFP-cytochrome C oxidase signal, demonstrating selectivity for mitochondria.

REFERENCES

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3. Minamikawa, Sriratana, Williams, Bowser, Hill and Nagley (1999) Chloromethyl-X-rosamine (MitoTracker Red) photosensitises mitochondria and induces apoptosis in intact human cells. Journal of Cell Science 112, 2419-2430.
4. Scorrano, Petronilli, Colonna, Di Lisa and Bernardi (1999) Chloromethyltetramethylrosamine (Mitotracker OrangeÔ) Induces the Mitochondrial Permeability Transition and Inhibits Respiratory Complex I: Implications for the mechanism of cytochrome c release” J. Biol Chem. 274, 35, 24657–24663.

TROUBLE SHOOTING

Problem	Potential Cause	Suggestion
Mitochondria are not sufficiently stained.	Very low concentration of MITO-ID® Red dye was used or dye was incubated with the cells for an insufficient length of time.	Either increase the labeling concentration or increase the time allowed for the dye to accumulate in the mitochondria once the cells have been transferred to fresh medium.
MITO-ID® Red dye is non-specifically binding to the surface.	We have noted that certain cell culture surfaces with associated gaskets can absorb the dye.	Grow cells on glass coverslips, or try another type of slide.
Precipitate is seen in the 10X Assay Buffer.	Precipitate forms at low temperatures.	Allow solution to warm to room temperature or 37°C, then vortex to dissolve all precipitate.
Blue nuclear counterstain is too bright compared to the red mitochondrial stain.	Different microscopes, cameras and filters may make some signals appear very bright.	Reduce the concentration of the nuclear counterstain or shorten the exposure time.
Cells do not appear healthy	Some cells require serum to remain healthy.	Add serum to stain and wash solutions. Serum does not affect staining. Normal amounts of serum added range from 2% to 10%.

NOTES

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