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ER-ID™ Red Assay Kit (GFP-Certified™)

for detection of endoplasmic reticulum by microscopy

Instruction Manual

Cat. No. ENZ-51026-K500 500 assays

For research use only.

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Notice to Purchaser

The ER-ID™ Red Assay Kit (GFP-Certified™) is a member of the CELLestial® product line, reagents and assay kits comprising fluorescent molecular probes that have been extensively benchmarked for live cell analysis applications. CELLestial® reagents and kits are optimal for use in demanding cell analysis applications involving confocal microscopy, flow cytometry, microplate readers and HCS/HTS, where consistency and reproducibility are required.

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I. Introduction

Enzo Life Sciences' ER-ID™ Red Assay Kit (GFP-Certified™) contains a novel endoplasmic reticulum-selective dye suitable for live cell, or detergent-permeabilized aldehyde-fixed cell staining. Micromolar concentrations of ER-ID™ Red dye are sufficient for staining mammalian cells, as validated with human cervical carcinoma cell line, human T-lymphocyte cell line, Jurkat, HeLa and human bone osteosarcoma epithelial cell line, U2OS.

One important application of ER-ID™ Red dye is in fluorescence co-localization imaging with green fluorescent protein (GFP)-tagged proteins, a powerful approach for determining the targeting of molecules to intracellular compartments and for screening of their associations and interactions. However, to date, photoconversion of red fluorescent dyes to green fluorescent ones 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 upon concentration 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] Consequently, ER-ID™ Red dye, a new red-emitting, cell-permeable small molecule organic probe that spontaneously localizes to live or fixed endoplasmic reticula, was developed. ER-ID™ Red dye can be readily used in combination with other common UV and visible light excitable organic fluorescent dyes and various fluorescent proteins in multicolor imaging and detection applications. The dye emits in the Texas Red region of the visible light spectrum, and is highly resistant to photobleaching, concentration quenching and photoconversion.

The ER-ID™ Red Assay Kit (GFP-Certified™) 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.

II. Reagents Provided and Storage

All reagents are shipped on dry ice. Upon receipt, the kit should be stored upright 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 kit are sufficient for approximately 500 assays using either live, adherent cells or cells in suspension.

Reagent	Quantity
ER-ID™ Red Detection Reagent	50 µL
Hoechst 33342 Nuclear Stain	50 µL
10X Assay Buffer	15 mL

III. Additional Materials Required

- Standard fluorescence microscope
- Calibrated, adjustable precision pipetters, preferably with disposable plastic tips
- Adjustable speed centrifuge with swinging buckets (for suspension cultures)
- Glass microscope slides
- Glass cover slips
- Deionized water
- Anhydrous DMSO (optional)
- Growth medium (*e.g.,* Dulbecco's Modified Eagle Medium, D-MEM)
- Paraformaldehyde (optional, for fixation)
- Triton X-100 (optional, for permeabilization)

IV. Safety Warnings and Precautions

- This product is for research use only and is not intended for diagnostic purposes.
- The ER-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.
- 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.

V. 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.*

A. 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. 3.7% Formaldehyde Solution

The following procedure is for preparation of 10 mL of 3.7% formaldehyde solution: Dilute 0.37 gram paraformaldehyde to a final volume of 10 mL with 1X Assay Buffer. Mix well.

3. 0.1% Triton X-100 (optional)

The following procedure is for preparation of 10 mL of 1% Triton X-100 solution: Dilute 10 µL Triton X-100 to a final volume of 10 mL with 1X Assay Buffer or 1X Assay Buffer containing 2% serum. Mix well.

4. Dual Detection Reagent

The concentration of ER-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 milliliter of 1X Assay Buffer (see preparation in step V-A1) or 1X Assay Buffer containing 2% serum, add 1 µL of ER-ID™ Red Detection Reagent and 1 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) The Hoechst 33342 Nuclear Stain can be diluted further if its staining intensity is much stronger than the red endoplasmic reticulum stain, ER-ID™ Red.

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

B. Staining Live, Adherent Cells

1. Grow cells on cover slips, or tissue culture treated slides, 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 section V-A2, page 3) 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.
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 endoplasmic reticulum. Optionally, image the nucleus using a DAPI filter set and the GFP-tagged protein using a GFP/FITC filter set.

C. 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 (see section V-A2, page 3) to cover the dispersed cell pellet.
3. Protect samples from light and incubate for 15 to 30 minutes at 37°C.
4. (Optional) 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 endoplasmic reticulum. Optionally, image the nucleus using a DAPI filter set and the GFP-tagged protein using a GFP/FITC filter set.

D. Staining of Aldehyde-Fixed and Detergent-Permeabilized Live Cells with ER-ID™ Red Dye

The ER-ID™ Red dye is capable of staining fixed and permeabilized cells. Fixation and permeabilization makes it possible to probe for other intracellular structures by conventional immunofluorescence labeling methods.

1. Wash the cells in 1x Assay Buffer (from step A-1, page 3).
2. Carefully remove the buffer covering the cells, and replace it with freshly prepared 3.7% formaldehyde solution.
3. Incubate the cells at 37°C for 10 minutes.
4. After fixation, rinse the cells several times in 1X Assay Buffer.
5. (Optional) If cells are to be labeled with an antibody, permeabilization step is recommended to enhance the antigen's accessibility. This is done by incubating the fixed cells in 0.1% Triton X-100 (from step A-3, page 3) at room temperature for 1 minute.
6. Following permeabilization, rinse the cells with 1X Assay Buffer.
7. Perform staining as recommended for adherent or suspension cells (see sections B or C).

NOTE: *When performing standard immunofluorescence staining protocols using a fluorescein- or coumarin-labeled antibodies, or equivalent, administer post-fixation according to manufacturer instructions.*

E. Aldehyde Fixation and Detergent Permeabilization of Stained Live Cells

Live cells stained with ER-ID™ Red dye may be fixed with formaldehyde and permeabilized with Triton X-100. Fixation and permeabilization makes it possible to probe for other intracellular structures by conventional immunofluorescence labeling methods. The ER-ID™ Red dye is retained following fixation and permeabilization using the protocol described below.

1. Wash the ER-ID™ Red-stained cells with 1x Assay Buffer (from step A-1, page 3).
2. Carefully remove the buffer covering the cells, and replace it with freshly prepared 3.7% formaldehyde solution.

3. Incubate the cells at 37°C for 10 minutes.
4. After fixation, rinse the cells several times in 1X Assay Buffer.
5. (Optional) If the cells are to be labeled with an antibody, permeabilization step is recommended to enhance the antigen's accessibility. This is done by incubating the fixed cells in 0.1% Triton X-100 (from step A-3, page 3) at room temperature for 1 minute.

Note: *The staining will not be as intense after fixation of cells. It is recommended to fix prior to staining cells (Section D).*

VI. APPENDICES

A. 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.

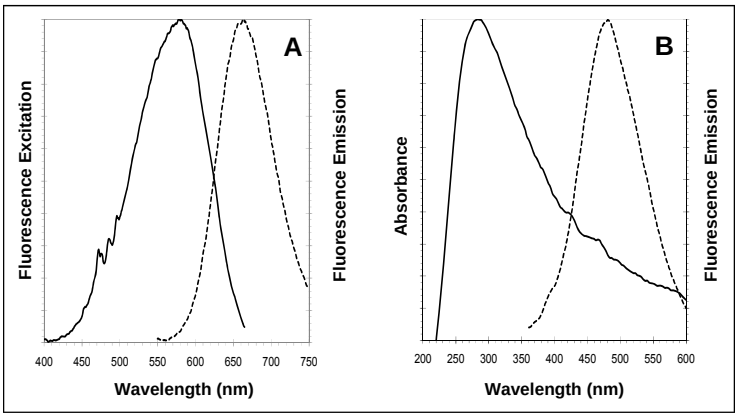

Figure 1. Fluorescence excitation and emission spectra for ER-ID™ Red dye (panel A) and absorbance and fluorescent emission spectra for Hoechst 33342 dye (panel B). All spectra were determined in 1X Assay Buffer.

B. RESULTS

Endoplasmic reticula are subcellular organelles found in eukaryotic cells, responsible for sorting most of the proteins and lipids of the cell. In addition to being a live cell-permeable dye, ER-ID™ Red dye is also partially retained during or after cell fixation and detergent permeabilization.

ER-ID™ Red dye has been shown to co-localize with EGFP-calreticulin chimeric protein in a transduced HeLa cell line. Typically, intense red fluorescent staining of the endoplasmic reticulum in the perinuclear region of mammalian cells is readily apparent using ER-ID™ Red dye. The ER-ID™ Red dye co-localizes with the EGFP-calreticulin signal, demonstrating selectivity for endoplasmic reticula.

VII. References

1. Freundt, Czapiga and Lenardo (2007) "Photoconversion of Lysotracker Red to a green fluorescent molecule" *Cell Res.* 17(11):956-958.
2. Nadrigny, Li, Kemnitz, Ropert, Koulakoff, Rudolph, Vitali, Giaume, Kirchhoff and Oheim (2007) "Systematic colocalization errors between acridine orange and EGFP in astrocyte vesicular organelles" *Biophys J.* 93(3):969-980.
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 Orange™) 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.

VIII. Troubleshooting Guide

Problem	Potential Cause	Suggestion
Endoplasmic reticula are not sufficiently stained.	Very low concentration of ER-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 endoplasmic reticula.
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 endoplasmic reticulum 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%.

Enzo Life Sciences

www.enzolifesciences.com

Enabling Discovery in Life Science®

North/South America
ENZO LIFE SCIENCES INTERNATIONAL, INC.
5120 Butler Pike
Plymouth Meeting, PA 19462-1202
USA
Tel. 1-800-942-0430 / (610) 941-0430
Fax (610) 941-9252
info-usa@enzolifesciences.com

Benelux
ENZO LIFE SCIENCES BVBA
Melkerijweg 3
BE-2240 Zandhoven / Belgium
Tel. +32/0 3 466 04 20
Fax +32/0 3 466 04 29
info-be@enzolifesciences.com

UK & Ireland
ENZO LIFE SCIENCES (UK) LTD.
Palatine House
Matford Court
Exeter EX2 8NL / UK
Tel. 0845 601 1488 (UK customers)
Tel. +44/0 1392 825900 (overseas)
Fax +44/0 1392 825910
info-uk@enzolifesciences.com

Switzerland & Rest of Europe
ENZO LIFE SCIENCES AG
Industriestrasse 17, Postfach
CH-4415 Lausen / Switzerland
Tel. + 41/0 61 926 89 89
Fax + 41/0 61 926 89 79
info-ch@enzolifesciences.com

Germany
ENZO LIFE SCIENCES GmbH
Marie-Curie-Strasse 8
DE-79539 Lörrach / Germany
Tel. +49/0 7621 5500 526
Fax +49/0 7621 5500 527
info-de@enzolifesciences.com

For Local Distributors please visit our Website.