funakoshi
FRONTIERS IN LIFE SCIENCE

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Hyp-Stamp <H_2O_2-Responsive Protein Labeling Reagent>

Catalog NO. FDV-0052

Research use only, not for human or animal therapeutic or diagnostic use.

Product Background

Reactive **O**xygen **S**pecies (ROS) are reactive molecules produced in a wide range of biological processes. ROS are mainly produced during oxidative stress and damage lipids, proteins, and DNAs, which are concerned with cellular senescence and cause various diseases, such as cancer, inflammation, cardiovascular diseases, and neurodegenerative diseases. On the other hand, ROS are also generated in normal metabolic processes and play essential roles in cellular functions by regulating many signaling pathways. Hydrogen peroxide (H_2O_2) is one of the ROS and exhibits mild reactivity and chemical stability compared with other ROS. Therefore, H_2O_2 is an ideal cellular signaling molecule that can move and diffuse with sufficient distance. H_2O_2 regulates various physiological processes, including inflammation reactions and growth factor stimulation. For these reasons, investigating the H_2O_2 behavior in diverse types of cells has been an important topic in life science.

Researchers conventionally use H_2O_2-responsive fluorescent probes that turns "ON" by reacting with H_2O_2 to analyze the cellular H_2O_2. Although they are valuable tools for observing H_2O_2 generation in living cells, they diffuse to other regions in the cell after reacting with H_2O_2, which makes it difficult to observe the exact localization of H_2O_2 generation over time. In addition, the fluorescent probes cannot be used for multistaining with antibodies because they are removed in cell fixation process.

"Hyp-Stamp" is an H_2O_2-responsive protein labeling reagent. Hyp-Stamp responds to cellular H_2O_2 and accomplishes fluorescein labeling of proteins lolalized nearby H_2O_2. Since Hyp-Stamp-mediated fluorescein-labeled proteins remain at the original site, and after cell or tissue fixation, users can observe the H_2O_2 localization in fixed cells or fixed tissues by fluorescent microscope. Further, performing multistaining of the fluorescein-labeled proteins with antibodies for the protein of user interest is compatible. In addition, proteins localized nearby cellular H_2O_2 can be analyzed and identified using proteomics methodologies. Hyp-Stamp is a novel and innovative reagent for monitoring the behavior of cellular H_2O_2 and can be used complementary to conventional fluorescent probes.

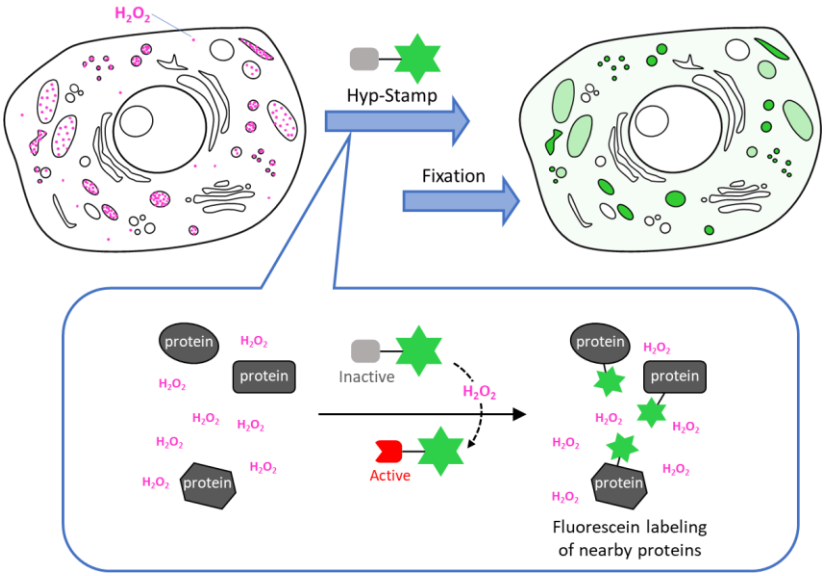


Figure 1. Overview of H_2O_2 monitoring with Hyp-Stamp

Product principle

"Hyp-Stamp" is a reagent developed by Prof. Hamachi and co-workers at Kyoto University.[1] Hyp-Stamp consists of a protein labeling moiety and a Fluorescein moiety. Before reacting with H_2O_2, the protein labeling moiety is protected with boronic acid ester, which does not react to proteins in this "inactivate form." The protein labeling portion converts to its "active form" (quinone methide form) by reacting with cellular H_2O_2, removing the protecting group. The "active form" moiety rapidly reacts with the nucleophilic groups of proteins that exist nearby cellular H_2O_2.

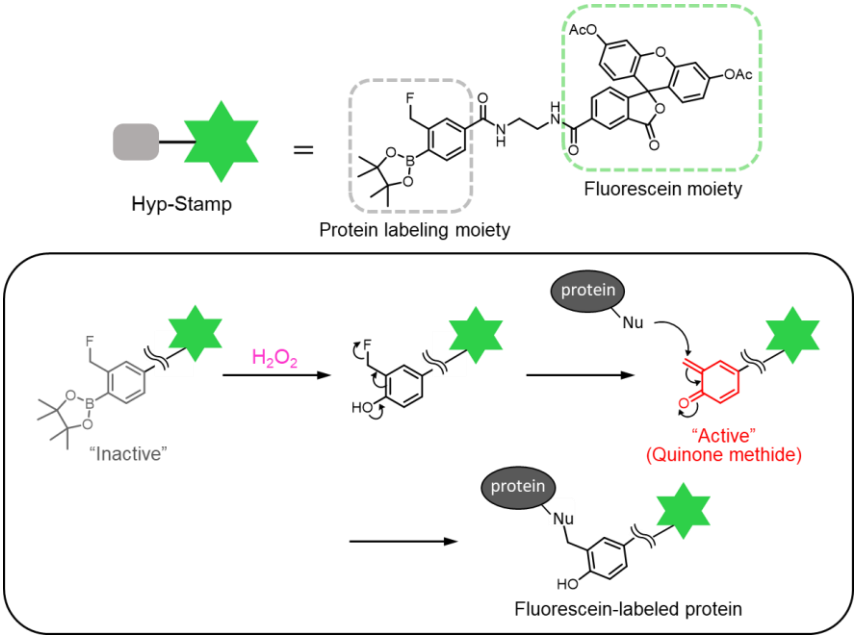


Figure 2. Structure of Hyp-Stamp and its reaction mechanism

Conventional H_2O_2-responsive fluorescent probes can observe H_2O_2 generation in living cells while they become undetectable in fixed cells because they are removed in the fixation process. On the other hand, Hyp-Stamp can visualize the local site of H_2O_2 after fixation of the cells, while it cannot observe H_2O_2 localization in living cells before fixation because of the overwhelmed fluorescence derived from the excess unreacted Hyp-Stamp.

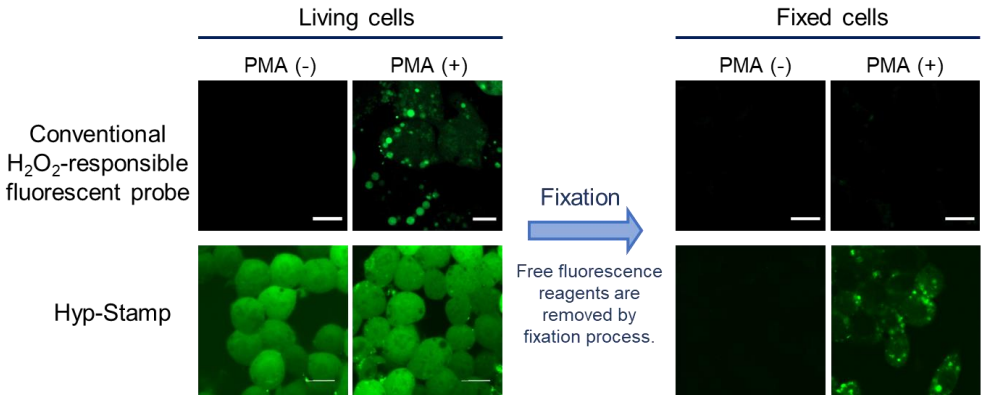


Figure 3. Fluorescent images of RAW264.7 cells in which H_2O_2 was generated by immune stimulation

Description

Catalog Number: FDV-0052

Size: 100 µg

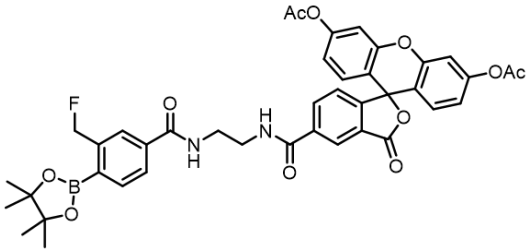
Formulation: $C_{41}H_{38}BFN_2O_{11}$

Molecular weight: 764.56 g/mol

Solubility: Soluble in DMSO

Ex/Em: 495 nm/515 nm

*FITC filter sets are available.



Note: This reagent contains a diacetylfluorescein (FDA), which promotes cell-permeability and exhibits no fluorescence. A strong green fluorescence emission occurs when the FDA is cleaved by intracellular esterases and converted to fluorescein. Hyp-Stamp can be slowly hydrolyzed in an aqueous medium. Thus, we recommend to prepare the Hyp-Stamp aqueous solution, use it immediately, and avoid storing it.

Reconstitution and Storage

Reconstitution:	Stock solution in 100% DMSO.
Storage:	Store powder at -20ºC.
	After reconstitution in DMSO, make aliquots and store at -20 °C.
	Avoid repeated freeze-thaw cycles. Protect from light.

Applications

- Cell or tissue imaging after fixation of sample for observing H_2O_2 localization
- SDS-PAGE with fluorescent detection
- Western blotting with antibodies of interest following immunoprecipitation with anti-fluorescein antibody
- Proteomics by MS spectrometry (LC-MS/MS) following immunoprecipitation with anti-fluorescein antibody

How to use

General procedure for cellular H_2O_2 imaging

1. Culture cells on appropriate dishes.
2. Remove the culture medium and wash cells several times.
3. Stimulate cells with any reagents such as oxidative stress inducer or immune stimulant and incubate cells for the appropriate time.
4. Incubate cells with 5 µM of Hyp-Stamp diluted in serum-free medium for 30 min.
   NOTE: Empirically optimize and determine the concentration of Hyp-Stamp for your experiments.
5. The cells are fixed with chilled methanol or formaldehyde solution, washed with PBS, and imaged by fluorescence microscope.
   NOTE: You may need to optimize cell fixation conditions, including selecting a fixative solution, time, and temperature for your experiments.

Western blotting of Hyp-Stamp treated cell lysate

1. Stimulate cells with any reagent and treat with Hyp-Stamp, as described above.
2. The treated cells are washed several times with PBS and lysed in lysis buffer, such as RIPA buffer, containing a protease inhibitor cocktail.
3. Apply the samples to SDS-PAGE and electrotransfer onto PVDF membranes.
4. Treat the membranes with anti-fluorescein antibodies to detect fluorescein-labeled proteins.

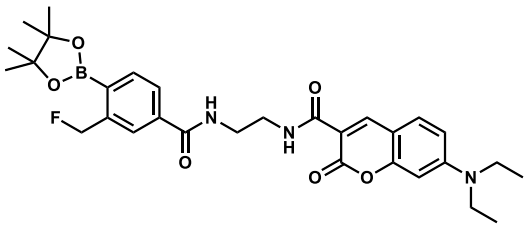
Preparation of the lysate of Hyp-Stamp treated cells and enrichment of fluorescein-labeled proteins

1. Stimulate cells with any reagent and treat with Hyp-Stamp, as described above.
2. The treated cells are washed several times with PBS and lysed in lysis buffer, such as RIPA buffer, containing a protease inhibitor cocktail.
3. Centrifuge the lysates and collect the supernatant.
4. Small molecules, including an excess Hyp-Stamp, are removed from the lysates with any appropriate process (i.e., dialysis, ultrafiltration, and protein extraction).
5. The resulting protein solutions are immunoprecipitated with anti-fluorescein antibodies.

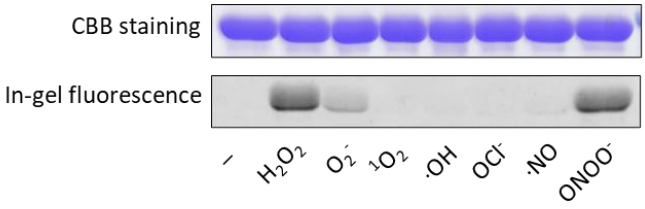
Reference data

Ractivity of the protein-binding moiety of Hyp-Stamp with several reactive oxygen species (ROS) and reactive nitrogen species (RNS)

200 μM of several ROS and RNS were added to a PBS (−) solution of BSA (10 μM) containing 50 μM of Hyp-Stamp related compound having the same protein-binding moiety as Hyp-Stamp (see structure below), and the mixture was incubated for 30 min. The samples were applied to a 12.5% SDS–PAGE gel and imaged by Coomassie Brilliant Blue (CBB) and in-gel fluorescence. As a result, the protein binding moiety was found to selectively react with H_2O_2 compared with other reactive species, except for peroxynitrite ($ONOO^-$), which was also able to trigger the BSA labeling. In general, the cellular concentration of $ONOO^-$ is much lower than H_2O_2. Nevertheless, the control experiments using $ONOO^-$ generation inhibitor such as N^G-nitro-L-arginine methyl ester (L-NAME) are recommended to determine whether the specific analysis of H_2O_2 by Hyp-Stamp with eliminating the influence of $ONOO^-$.

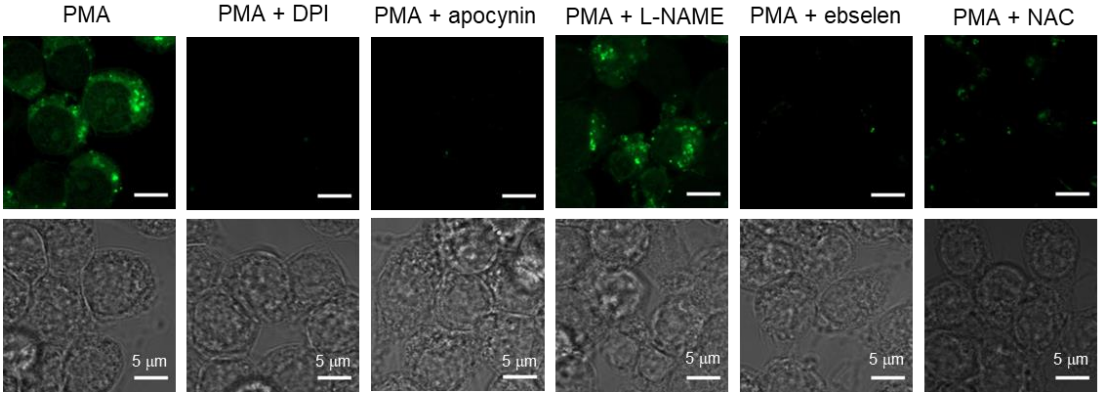


The structure of Hyp-Stamp related compound



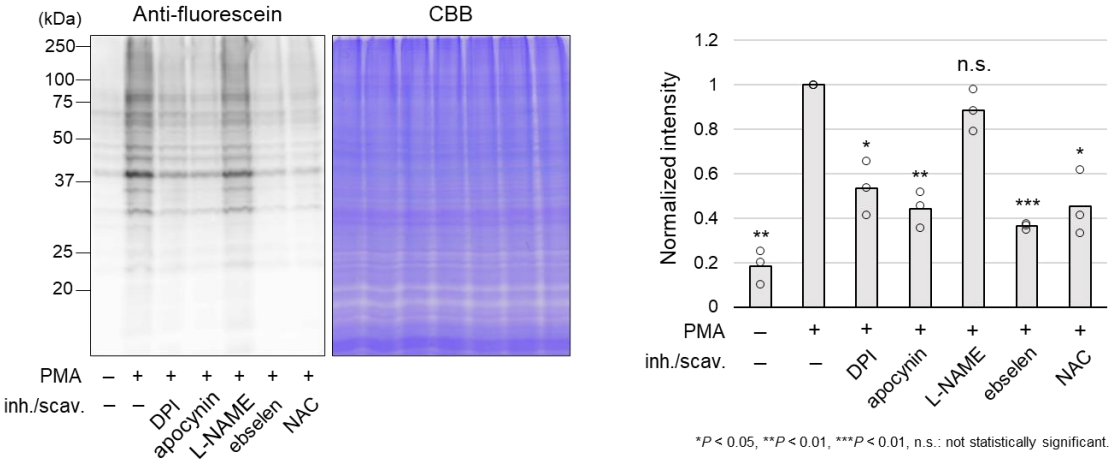
Imaging of the H_2O_2 localization by immunostimulation in macrophage cells

RAW264.7 cells (murine macrophage cell line) were immunostimulated (treated with 1 μg/mL PMA) in the presence of various inhibitors or scavengers of reactive species, including 10 μM diphenyleneiodonium (DPI), an NADPH oxidase inhibitor, 5 mM apocynin, an NADPH oxidase inhibitor, 5 mM L-NAME, an inhibitor of the production of ·NO and $ONOO^-$, 5 μM ebselen, an H_2O_2 scavenger, and *N*-acetylcysteine (NAC), an antioxidant, and incubated for 30 minutes. After adding 5 μM Hyp-Stamp, the cells were cultured for 30 minutes. Confocal laser scanning microscope imaging was performed after cell fixation with chilled methanol. Fluorescent vesicles that appeared upon immunostimulation were either disappeared or decreased by adding inhibitors or scavengers except for L-NAME.



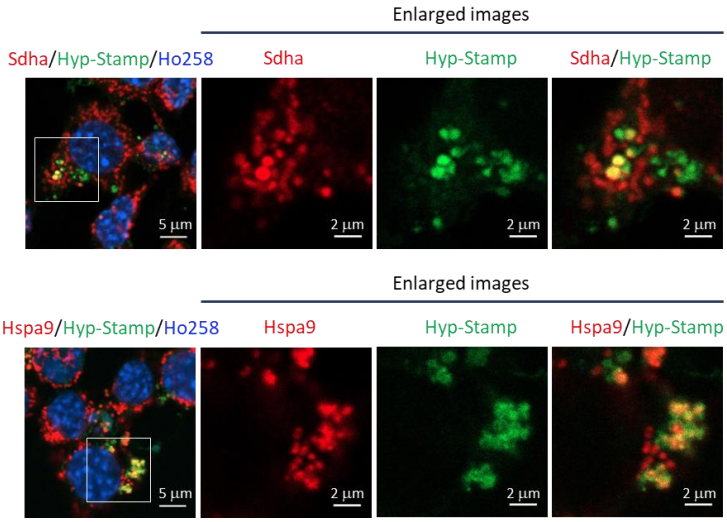
Western blot analysis of the immunostimulation in macrophage cells

RAW264.7 cells were immunostimulated (treated with 1 μg/mL PMA) in the presence of various inhibitors or scavengers described above and incubated for 30 minutes. Afterward, 5 μM Hyp-Stamp was added, and the cells were cultured for 30 minutes. Fluoresein-labeled proteins were assessed by western blot using an anti-fluorescein antibody. The band intensity and the number of labeled proteins increased upon the immunostimulation, and this was decreased by adding inhibitors or scavengers except for L-NAME.



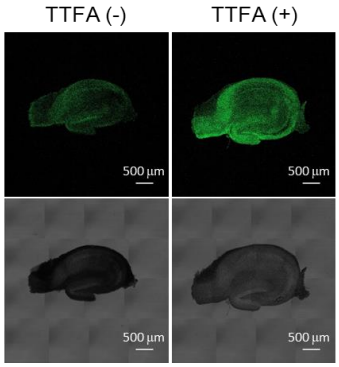
Multistainig of the fluorescein-labeled proteins with antibodies in immunostimulated macrophage cells

RAW264.7 cells were immunostimulated (treated with 1 μg/mL PMA) and incubated for 30 minutes. Afterward, 5 μM Hyp-Stamp was added, and the cells were cultured for 30 minutes. Then, cells were fixed with cold methanol or 4% formaldehyde and immunostained with mitochondrial proteins Sdha and Hspa9. Confocal laser scanning microscope imaging shows the immunofluorescence against Sdha and Hspap partially merged with fluorescein-labeled vesicles.



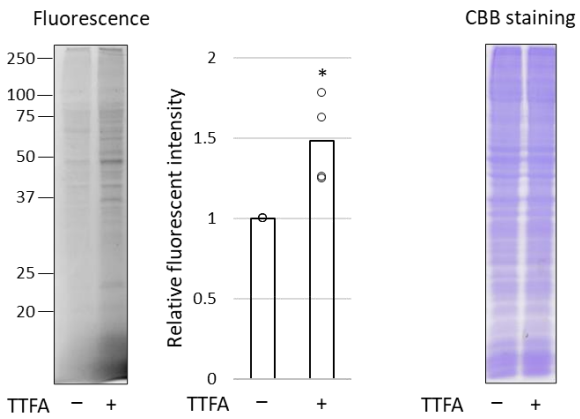
Imaging of the H_2O_2 localization in mouse hippocampal slices

Murine hippocampal slices were treated with mitochondrial Complex II inhibitor TTFA (1 mM, 30 min) to induce oxidative stress, then 5 μM Hyp-Stamp was added and incubated for 1 hour. The slices were fixed with 4% formaldehyde and permeabilized with cold methanol, followed by fluorescent imaging with a confocal laser scanning microscope. Localization of TTFA-triggered H_2O_2 was observed in the hippocampal slices.



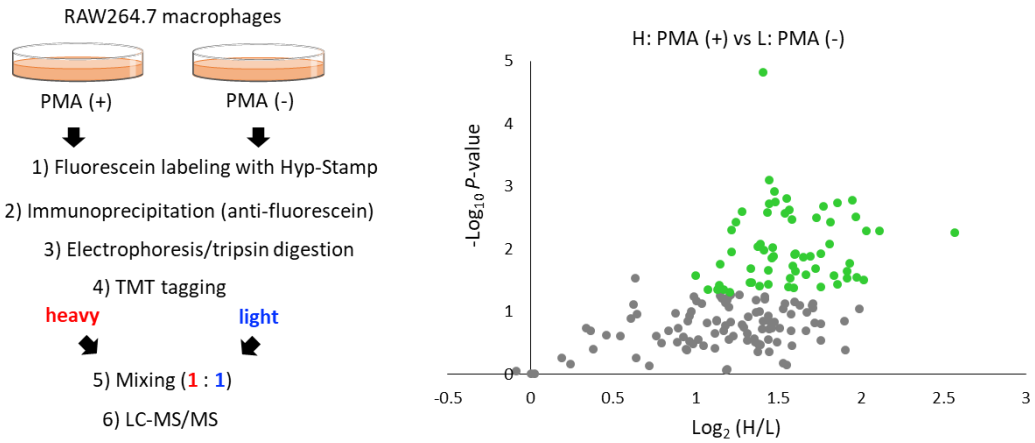
Analysis of total fluorescein-labeled proteins under oxidative stress of mitochondria

HeLa cells were treated with mitochondrial Complex II inhibitor TTFA (1 mM, 30 min) to induce oxidative stress, then 5 μM Hyp-Stamp was added and incubated for 30 min. After preparing a cell lysate, samples are applied to an SDS-PAGE, imaged by Coomassie Brilliant Blue (CBB), and in-gel fluorescence. Results revealed the amount of fluorescein-labeled protein increased by TTFA treatment, while TTFA did not affect the amount of total protein.



Identification of proteins existing nearby H_2O_2 generating site by proteomics methodology.

5 μM Hyp-Stamp was added to RAW264.7 cells in which immunostimulation (treated with 1 μg/mL PMA for 30 minutes) was given (+) or not (-), and the cells were cultured for 30 minutes. After preparing the cell lysates, immunoprecipitation was performed with an anti-fluorescein antibody to enrich fluorescein-labeled proteins. Proteins obtained by immunoprecipitation were applied to in-gel trypsin digestion to produce peptide solutions. After modifying the PMA(+) and PMA(-) samples with TMT heavy tag and TMT light tag, respectively, the two samples were mixed in a 1:1 ratio and subjected to LC-MS/MS analysis to investigate changes between samples comprehensively. Figures show the experimental scheme of proteomics analysis (left) and volcano plot of protein signal changes by PMA(+)/(-) (right). In the plot, proteins with $\log_2$(PMA added/not added) > 1, and *P* value < 0.05 are shown in green dots. Mitochondrial proteins such as Sdha and many vesicle proteins were identified, suggesting that these proteins exited nearby H_2O_2 generated by immunostimulation.



Reference

1. Zhu *et al., J. Am. Chem. Soc.*, **142**, 15711–15721 (2020) Imaging and Profiling of Proteins under Oxidative Conditions in Cells and Tissues by Hydrogen-Peroxide-Responsive Labeling.

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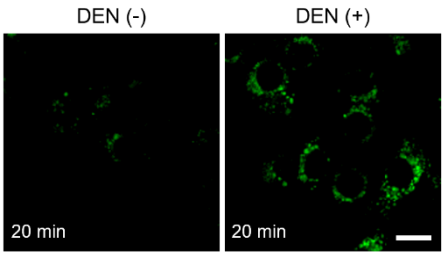
Related products

LipiRADICAL Green <Lipid Radical Detection Reagent>

LipiRADICAL Green is a specific fluorescent dye for lipid-derived radicals which are the most upstream factor of lipid peroxidation (LPO). LipiRADICAL Green can be applied into both *in vitro* assay and cell-based assay to monitor lipid radical productions.

Catalog No. FDV-0042
Size 0.1 mg
Features

- Recommended Ex/Em:~480 nm / 520 nm
- Enable to detect very unstable lipid-derived radicals
- Compatible with *in vitro* assay and in cell-based assay
- An innovative reagent for comprehensive identification of lipid-derived radicals by lipidomics

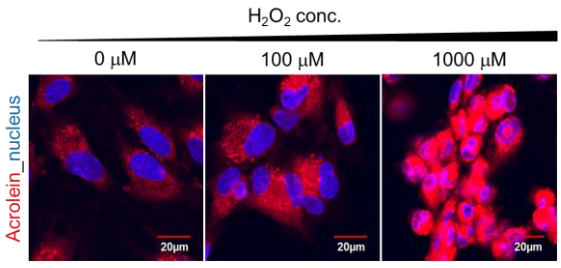


AcroleinRED <Cell-based Acrolein Detection Reagent>

Acrolein is a LPO downstream aldehyde and one of the most toxic oxidative stress marker. AcroleinRED is the world first cell-based acrolein detection reagent.

Catalog No. FDV-0022
Size 0.5 mg
Features

- Easy and quick protocol
- Enable to monitor acrolein production under live cells with various stimulations

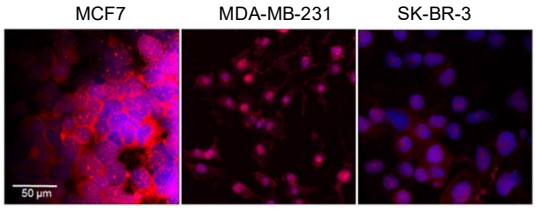


PolyamineRED <Indracellular Polyamine Detection Reagent>

Polyamines are one of the essential class of metabolites in al living organisms and show an anormous number of biological functions. PolyamineRED is the world first reagent for detecting intracellular polyamines without any pre-treatment and cell lysis.

Catalog No. FDV-0020
Size 0.5 mg
Features

- Easy and quick protocol
- Enable to detect linear primary alkylamines selectively.
- Recommended Ex/Em: 560 nm / 585 nm
  TAMTA filter sets are available.



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