3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

# Product Information

**Intracellular Hydrogen Peroxide Assay Kit**
Green Fluorescence

Catalog Number **MAK164**
Storage Temperature –20 °C

## TECHNICAL BULLETIN

### Product Description

Hydrogen peroxide, a reactive oxygen species produced through the metabolism of molecular oxygen, serves as both an intracellular signaling messenger and a source of oxidative stress. Hydrogen peroxide is generated in cells via multiple mechanisms such as the NOX-mediated ROS production by neutrophils and macrophages (respiratory burst) or by the dismutase of superoxide anions produced as a result of electron leak during mitochondrial respiration. Abnormal hydrogen peroxide production contributes to oxidative cell damage and the progression of diseases such as asthma, atherosclerosis, osteoporosis, and neurodegeneration.

The Intracellular Hydrogen Peroxide Assay Kit provides a simple and reproducible method to quantify hydrogen peroxide levels in living cells and in a variety of other samples such as cellular extracts. This kit utilizes a unique cell-permeable sensor that generates a fluorescent product ($\lambda_{ex}$ = 490/$\lambda_{em}$ = 520 nm) after reaction with hydrogen peroxide that can be analyzed by a fluorescent microplate reader, flow cytometer, or fluorescent microscope. This assay is compatible with high-throughput handling systems.

### Components

The kit is sufficient for 200 assays in 96 well plates.

| | |
|---|---|
| Fluorescent Peroxide Sensor<br>Catalog Number MAK164A | 1 vl |
| Hydrogen Peroxide, 3% (0.88 M) solution<br>Catalog Number MAK164B | 0.2 mL |
| Assay Buffer<br>Catalog Number MAK164C | 20 mL |
| DMSO<br>Catalog Number MAK164D | 0.2 mL |

### Reagents and Equipment Required but Not Provided.

- 96 well flat-bottom plate – It is recommended to use black plates with clear bottoms for fluorometric assays.
- Fluorescence multiwell plate reader or fluorescent microscope

### Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

### Storage/Stability

The kit is shipped on wet ice and storage at –20 °C, protected from light, is recommended.

### Preparation Instructions

Briefly centrifuge vials before opening. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

Allow all reagents to come to room temperature before use.

Fluorescent Peroxide Sensor – Reconstitute with 50 µL of DMSO. Mix well by pipetting. Aliquot and store at –20 °C, protected from light. Stock solution should be used promptly upon preparation or thawing. Remaining stock solution should be immediately frozen.

Hydrogen Peroxide Solution – Add 22.7 µL of 3% $H_2O_2$ solution to 977 µL of Assay Buffer to prepare a 20 mM $H_2O_2$ stock solution immediately before use in assay. Diluted solution is not stable and unused portion should be discarded.

**Procedure**
All samples and standards should be run in duplicate.

Hydrogen Peroxide Assay in Live Cells:

1. Plate the cells, both samples and controls, and activate as desired. Following incubation, wash cells 1× with PBS. It is strongly suggested to include a positive control well (for example, cells treated with 100 µM $H_2O_2$).

2. Prepare the Fluorescent Peroxide Sensor working solution according to the scheme in Table 1. 100 µL of the working solution is required for each reaction (well).

**Table 1.**
Fluorescent Peroxide Sensor Working Solution

| Reagent | Volume |
|---|---|
| Fluorescent Peroxide Sensor | 0.5 µL |
| Assay Buffer | 125 µL |

3. Add 100 µL of the Fluorescent Peroxide Sensor working solution to each of the wells. Mix well and incubate the plate for 5–60 minutes.
   Note: For 384 well plates, add 25 µL of the Fluorescent Peroxide Sensor working solution to each of the wells.

4. Following the incubation, wash the cells with PBS.

5. Measure the fluorescence intensity at ($\lambda_{ex}$ = 490/ $\lambda_{em}$ = 525 nm) using a fluorescence plate reader, flow cytometer, or fluorescence microscope.

Hydrogen Peroxide Assay for samples in 96 well plates:
Hydrogen Peroxide Standards:
Add 22.7 µL of 3% $H_2O_2$ solution to 977 µL of Assay Buffer to prepare a 20 mM $H_2O_2$ stock solution. Add 50 µL of the 20 mM stock solution to 950 µL of Assay buffer to get a 1,000 µM working solution. Further dilute the 1,000 µM working solution to prepare 300, 100, 30, 10, 3, 1, 0.3, and 0 µM standards. Add 50 µL of the prepared standards to the appropriate wells in the 96 well plate.
Note: Diluted solution is not stable and unused portion should be discarded.

Sample Preparation:
Add up to 50 µL of sample to wells. Bring samples to a final volume of 50 µL with Assay Buffer.
Note: For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the standard curve.

Assay Reaction

1. Set up the Fluorescent Peroxide Sensor working solution according to the scheme in Table 2. 50 µL of the working solution is required for each reaction (well).

**Table 2.**
Fluorescent Peroxide Sensor Working Solution

| Reagent | Volume |
|---|---|
| Fluorescent Peroxide Sensor | 20 µL |
| Assay Buffer | 5 mL |

Note: The Fluorescent Peroxide Sensor working solution is enough for one plate. The amount of Fluorescent Peroxide Sensor working solution prepared can be scaled if necessary. The Fluorescent Peroxide Sensor working solution is not stable and best used within 2 hours.

2. Add 50 µL of the Fluorescent Peroxide Sensor working solution to each of the wells (samples, standards, and controls). Mix well and incubate the plate at room temperature for 15–30 minutes. Protect the plate from light during the incubation.
   Note: For 384 well plates, add 25 µL of the Fluorescent Peroxide Sensor working solution to each of the wells.

3. Measure the fluorescence intensity at ($\lambda_{ex}$ = 490/ $\lambda_{em}$ = 525 nm) using a fluorescence plate reader.

**Results**
Calculations
The background blank for the assay is the value obtained for the 0 (blank) Hydrogen Peroxide standard. Correct for the background by subtracting the blank value from all readings. Background values can be significant and must be subtracted from all readings.

The Hydrogen Peroxide concentration for the samples can be determined from the standard curve.

**Troubleshooting Guide**

| Problem | Possible Cause | Suggested Solution |
|---|---|---|
| Assay not working | Cold Reagents | Assay Buffer must be at room temperature |
| | Omission of step in procedure | Refer and follow Technical Bulletin precisely |
| | Plate reader at incorrect wavelength | Check filter settings of instrument |
| | Type of 96 well plate used | For fluorometric assays, use black plates with clear bottoms |
| Samples with erratic readings | Cell/Tissue culture samples were incompletely homogenized | Repeat the sample homogenization, increasing the length and extent of homogenization step. |
| | Samples used after multiple freeze-thaw cycles | Aliquot and freeze samples if needed to use multiple times |
| | Presence of interfering substance in the sample | If possible, dilute sample further |
| | Use of old or inappropriately stored samples | Use fresh samples and store correctly until use |
| | Improperly thawed components | Thaw all components completely and mix gently before use |
| Lower/higher readings in samples and standards | Allowing the reagents to sit for extended times on ice | Prepare fresh Fluorescent Peroxide Sensor working solution before each use |
| | Incorrect incubation times or temperatures | Refer to Technical Bulletin and verify correct incubation times and temperatures |
| | Incorrect volumes used | Use calibrated pipettes and aliquot correctly |
| | Use of partially thawed components | Thaw and resuspend all components before preparing the reaction mix |
| | Pipetting errors in preparation of standards | Avoid pipetting small volumes |

LS,MAM 04/14-1