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Product Information

Fluorimetric Hydrogen Peroxide Assay Kit
Red Fluorescence

Catalog Number **MAK165**
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 Fluorescent Hydrogen Peroxide Assay Kit provides a simple and reproducible method to quantify hydrogen peroxide levels in a variety of samples such as cellular extracts and solutions. This kit can also be used to detect hydrogen peroxide released from living cells or produced by oxidase activities. This kit utilizes a peroxidase substrate that generates a red fluorescent product (λ_{ex} = 540/λ_{em} = 590 nm) after reaction with hydrogen peroxide that can be analyzed by a fluorescent microplate reader. This assay is compatible with high-throughput handling systems.

Components

The kit is sufficient for 500 assays in 96 well plates.

Red Peroxidase Substrate Catalog Number MAK165A	1 vl
Hydrogen Peroxide, 3% (0.88 M) solution Catalog Number MAK165B	0.2 mL
Assay Buffer Catalog Number MAK165C	100 mL
Horseradish Peroxidase Catalog Number MAK165D	1 vl
DMSO Catalog Number MAK165E	1 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.

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.

Red Peroxidase Substrate – Reconstitute with 250 µL of DMSO to prepare the Red Peroxidase Substrate stock solution. 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.

Horseradish Peroxidase – Reconstitute with 1 mL of Assay Buffer to prepare a 20 units/mL solution. Mix well by pipetting. Divide into single-use aliquots 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.

Procedure
All samples and standards should be run in duplicate.

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 1 µL of the 20 mM stock solution to 1,999 µL of Assay buffer to get a 10 µM working solution. Further dilute the 10 µM working solution to prepare 10, 3, 1, 0.3, 0.1, 0.03, 0.01, 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. The peroxidase substrate is not stable in the presence of thiols such as DTT and β-mercaptoethanol. Thiol concentrations >10 µM will significantly decrease the assay dynamic range.
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 Master Mix according to the scheme in Table 1. 50 µL of the Master Mix is required for each reaction (well).

Table 1.
Master Mix

Reagent	Volume
Red Peroxidase Substrate Stock	50 µL
20 units/mL Peroxidase Stock	200 µL
Assay Buffer	4.75 mL

Note: The Master Mix is enough for one plate. The amount of Master Mix prepared can be scaled if necessary. The Master Mix is not stable and best used within 2 hours.

2. Add 50 µL of the Master Mix 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.

3. Measure the fluorescence intensity at (λ_{ex} = 540/ λ_{em} = 590 nm) using a fluorescence plate reader. Note: This Assay can be adapted for 384 well plates by adding 25 µL of the appropriate solution at each step.

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.

Hydrogen Peroxide Release Assay for Live Cells:
1. Plate the cells (50 µL/well) for both samples and controls, and activate as desired. It is strongly suggested to include a positive control well (for example, cells treated with 100 µM H_2O_2) and negative control wells (cells only and medium only).

2. Prepare the Master Mix according to the scheme in Table 2. 50 µL of the Master Mix is required for each reaction (well).

Table 2.
Master Mix

Reagent	Volume
Red Peroxidase Substrate Stock	50 µL
20 units/mL Peroxidase Stock	200 µL
Serum Free Medium or buffer	4.75 mL

Note: The Master Mix is enough for one plate. The amount of Master Mix prepared can be scaled if necessary. The Master Mix is not stable and best used within 2 hours. Instead of serum-free medium, a suitable balanced salt solution such as Krebs Ringer Phosphate buffer or Hanks Balanced Salt solution can be substituted.

3. Add 50 µL of the Master Mix to each of the sample and control wells. Mix well and incubate the plate, protected from light, for 15–30 minutes.

4. Measure the fluorescence intensity at (λ_{ex} = 540/ λ_{em} = 590 nm) using a fluorescence plate reader. Note: This Assay can be adapted for 384 well plates by adding 25 µL of the appropriate solution at each step.

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

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