

# Red Hydrogen Peroxide Assay Kit

Catalog #: ENZ-51004

500 Assays (for 5 x 96-well plates)

**Storage Conditions:** Keep in freezer. Avoid exposure to light.

**Instrument Platform:** Fluorescence microplate readers

## Kit Key Features

|                           |  |
|---------------------------|--|
| <b>Broad Application:</b> | Can be used for quantifying hydrogen peroxide in solutions, in cell extracts and in live cells; and can also be used for detecting a variety of oxidase activities through enzyme-coupled reactions. |
| <b>Sensitive:</b>         | Detect as low as 10 picomoles of H <sub>2</sub> O <sub>2</sub> in solution.  |
| <b>Continuous:</b>        | Easily adapted to automation without a separation step.  |
| <b>Convenient:</b>        | Formulated to have minimal hands-on time. No wash is required.   |
| <b>Non-Radioactive:</b>   | No special requirements for waste treatment.   |

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Please read entire booklet before proceeding with the assay.



Please contact Enzo Life Sciences Technical Support if necessary.

## TABLE OF CONTENTS

|                                     |   |
|-------------------------------------|---|
| Introduction .....                  | 1 |
| Safety Warnings & Precautions ..... | 1 |
| Kit Components .....                | 2 |
| Assay Protocol .....                | 2 |
| Results .....                       | 6 |
| Standard Curve .....                | 6 |
| References.....                     | 7 |
| Contact Information.....            | 9 |

## INTRODUCTION

Hydrogen peroxide ( $H_2O_2$ ) is a reactive oxygen metabolic by-product that serves as a key regulator for a number of oxidative stress-related states. It is involved in a number of biological events that have been linked to asthma, atherosclerosis, diabetic vasculopathy, osteoporosis, a number of neurodegenerative diseases and Down's syndrome. Perhaps the most intriguing aspect of  $H_2O_2$  biology is the recent report that antibodies have the capacity to convert molecular oxygen into hydrogen peroxide to contribute to the normal recognition and destruction processes of the immune system. Measurement of this reactive species will help to determine how oxidative stress modulates a variety of intracellular pathways.

This Red Hydrogen Peroxide Assay Kit uses our non-fluorescent Red peroxidase substrate to quantify hydrogen peroxide in solutions and cell extracts. It can also be used to detect a variety of oxidase activities through enzyme-coupled reactions. The kit is an optimized “mix and read” assay that is compatible with HTS liquid handling instruments. It provides a sensitive, one-step fluorometric assay to detect as little as 10 picomoles of  $H_2O_2$  in a 100  $\mu$ l assay volume (30nM, Figure 1). The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and readily adapted to automation. Its signal can be easily read by either a fluorescence microplate reader at Ex/Em = ~540/590nm or an absorbance microplate reader at ~570nm.

## SAFETY WARNINGS & PRECAUTIONS

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



Handle  
with care



Protect  
from light

- Some components of this kit contain hazardous substances. Reagents can be harmful if ingested or absorbed through the skin and may cause irritation to the eyes. 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.

## KIT COMPONENTS

| Component                                  | Amount                                  |
|--|---|
| Component 1: Red Peroxidase Substrate      | 1 vial                                  |
| Component 2: H <sub>2</sub> O <sub>2</sub> | 1 vial (3% stabilized solution, 200 µl) |
| Component 3: Assay Buffer                  | 1 bottle (100 ml)                       |
| Component 4: Horseradish Peroxidase        | 1 vial (20 units)                       |
| Component 5: DMSO                          | 1 vial (1 ml)                           |

## ASSAY PROTOCOL FOR ONE 96-WELL PLATE

### Brief Summary

Prepare H<sub>2</sub>O<sub>2</sub> reaction mixture (50 µl) →  
 Add H<sub>2</sub>O<sub>2</sub> standards or test samples (50 µl) →  
 Incubate at room temperature for 10-30 minutes →  
 Monitor fluorescence intensity at Ex/Em = 540/590nm

**Note:** Thaw all the kit components at room temperature before starting the experiment.

### 1. Prepare stock solutions.

- 1.1. 100X Red peroxidase substrate stock solution: Add 250µl of **DMSO** (Component 5) into the vial of **Red Substrate** (Component 1). The stock solution should be used promptly; any remaining solution should be aliquoted and refrozen at -20°C.

**Note:** Avoid repeated freeze-thaw cycles and protect from light.

- 1.2. 20 U/mL Peroxidase stock solution: Add 1 ml of **Assay Buffer** (Component 3) into the vial of **Horseradish Peroxidase** (Component 4).

**Note:** The unused **HRP solution** should be divided into single use aliquots and stored at -20°C.

- 1.3. 20 mM H<sub>2</sub>O<sub>2</sub> stock solution: Add 22.7µl of 3% H<sub>2</sub>O<sub>2</sub> (0.88 M, Component 2) into 977µl of Assay Buffer (Component 3).

**Note:** The diluted H<sub>2</sub>O<sub>2</sub> solution is not stable. The unused portion should be discarded.

## 2. Prepare H<sub>2</sub>O<sub>2</sub> reaction mixture:

Prepare the H<sub>2</sub>O<sub>2</sub> reaction mixture according to the following table and keep from light:

**Table 1:** H<sub>2</sub>O<sub>2</sub> Reaction mixture for one 96-well plate (2X)

| Reagent   | Volume  |
|---|---------|
| Red Peroxidase Substrate Stock Solution (100X, from Step 1.1) | 50 µl   |
| 20U/ml Peroxidase Stock Solution (from Step 1.2)              | 200 µl  |
| Assay Buffer (Component 3)                                    | 4.75 ml |
| Total Volume  | 5 ml    |

## 3. Prepare serial dilutions of H<sub>2</sub>O<sub>2</sub> Standard (0-10µM)

**Warning 1:** *The Component 1 is unstable in the presence of thiols such as DTT and β-mercaptoethanol. Thiols higher than 10µM (final concentration) would significantly decrease the assay dynamic range.*

**Warning 2:** *NADH and glutathione (reduced form: GSH) may interfere with the assay.*

- 3.1. Add 1 µl of 20mM H<sub>2</sub>O<sub>2</sub> solution (from Step 1.3) into 1999 µl of Assay Buffer (Component 3) to get a 10µM H<sub>2</sub>O<sub>2</sub> standard.
- 3.2. Take 200 µl of 10µM H<sub>2</sub>O<sub>2</sub> standard to perform 1:3 serial dilutions to get 3, 1, 0.3, 0.1, 0.03, 0.01 and 0µM serial dilutions of H<sub>2</sub>O<sub>2</sub> standard.
- 3.3. Add serial dilutions of H<sub>2</sub>O<sub>2</sub> standard and H<sub>2</sub>O<sub>2</sub>-containing test samples into a solid black 96-well microplate as described in Tables 2 and 3.

**Table 2:** Layout of H<sub>2</sub>O<sub>2</sub> standards and test samples in a solid black 96-well microplate

|     |     |      |      |      |      |  |  |  |  |  |  |  |  |  |  |
|-----|-----|------|------|------|------|--|--|--|--|--|--|--|--|--|--|
| BL  | BL  | TS   | TS   | .... | .... |  |  |  |  |  |  |  |  |  |  |
| HS1 | HS1 | .... | .... | .... | .... |  |  |  |  |  |  |  |  |  |  |
| HS2 | HS2 |      |      |      |      |  |  |  |  |  |  |  |  |  |  |
| HS3 | HS3 |      |      |      |      |  |  |  |  |  |  |  |  |  |  |
| HS4 | HS4 |      |      |      |      |  |  |  |  |  |  |  |  |  |  |
| HS5 | HS5 |      |      |      |      |  |  |  |  |  |  |  |  |  |  |
| HS6 | HS6 |      |      |      |      |  |  |  |  |  |  |  |  |  |  |
| HS7 | HS7 |      |      |      |      |  |  |  |  |  |  |  |  |  |  |

**Note:** HS= H<sub>2</sub>O<sub>2</sub> Standards; BL=Blank Control; TS=Test Samples

**Table 3:** Reagent composition for each well

| H <sub>2</sub> O <sub>2</sub> Standard | H <sub>2</sub> O <sub>2</sub> Standard | H <sub>2</sub> O <sub>2</sub> Standard |
|--|--|--|
| Blank Control                          | Blank Control                          | Blank Control                          |

**\*Note:** Add the serially diluted H<sub>2</sub>O<sub>2</sub> standards from 0.01μM to 10μM into wells from HS1 to HS7 in duplicate. High concentration of H<sub>2</sub>O<sub>2</sub> (e.g., > 100μM, final concentration) may cause reduced fluorescence signal due to the overoxidation of Red Peroxidase Substrate (to a non-fluorescent product).

#### 4. Run H<sub>2</sub>O<sub>2</sub> assay in supernatants reaction

- 4.1. Add 50 μl of H<sub>2</sub>O<sub>2</sub> reaction mixture (from Step 2) into each well of H<sub>2</sub>O<sub>2</sub> standard, blank control, and test samples (see Step 3.3) to make the total H<sub>2</sub>O<sub>2</sub> assay volume of 100 μl/well.

**Note:** For a 384-well plate, add 25 μl of sample and 25 μl of H<sub>2</sub>O<sub>2</sub> reaction mixture in each well.

- 4.2. Incubate the reaction at room temperature for 15 to 30 minutes, protected from light.
- 4.3. Monitor the fluorescence increase with a fluorescence plate reader at Ex/Em = 540 ± 10 / 590 ± 10nm (optimal Ex/Em = 540/590nm).

**Note:** The contents of the plate can also be transferred to a white clear bottom plate and read by an absorbance microplate reader at the wavelength of 576 ± 5nm. The absorption detection has lower sensitivity compared to fluorescence

## 5. Run H<sub>2</sub>O<sub>2</sub> assay for Cells

**NOTE:** The Red Hydrogen Peroxide Assay Kit can be used to measure the release of H<sub>2</sub>O<sub>2</sub> from cells. The following is a suggested protocol that can be modified to meet the specific research needs.

5.1. The H<sub>2</sub>O<sub>2</sub> reaction mixture should be prepared as Step 2 except that the Assay Buffer (Component 3) should be replaced with the media that is used in your cell culture system. Suggested media including (a) Krebs Ringers Phosphate Buffer (KRPB); (b). Hanks Balanced Salt Solution (HBSS); or (c) Serum-free media.

5.2. Prepare cells in a 96-well plate (50-100µl/well), and activate the cells as desired.

**Note:** The negative controls (media alone and non-activated cells) are included for measuring background fluorescence.

5.3. Add 50 µl of H<sub>2</sub>O<sub>2</sub> reaction mixture (from Step 5.1) into each well of cells and H<sub>2</sub>O<sub>2</sub> standards (from Step 3.3).

**Note:** For a 384-well plate, add 25 µl of cells and 25 µl of H<sub>2</sub>O<sub>2</sub> reaction mixture into each well.

5.4. Incubate the reaction at room temperature for 15 to 30 minutes, protected from light.

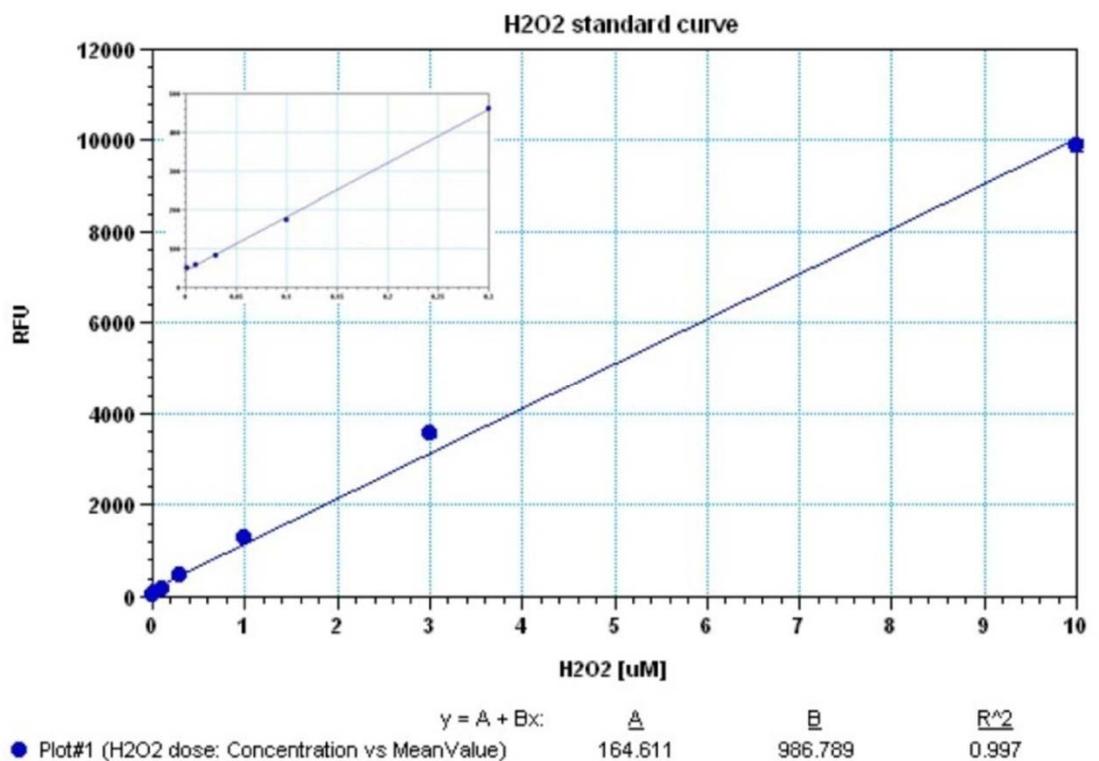
5.5. Monitor the fluorescence increase with a fluorescence plate reader at Ex/Em= 540 ± 10/590 ± 10nm (optimal Ex/Em = 540/590nm).

## RESULTS

### H<sub>2</sub>O<sub>2</sub> Standard Curve

The fluorescence in blank wells (with the assay buffer only) is used as a control, and is subtracted from the values for those wells with the H<sub>2</sub>O<sub>2</sub> reactions. A H<sub>2</sub>O<sub>2</sub> standard curve is shown in Figure 1.

**Note:** The fluorescence background increases with time, thus it is important to subtract the fluorescence intensity value of the blank wells for each data point.



**Figure 1:** H<sub>2</sub>O<sub>2</sub> dose response was measured in a 384-well black plate with the Red Hydrogen Peroxide Assay Kit using a Gemini fluorescence microplate reader (Molecular Devices). As low as 0.03 μM H<sub>2</sub>O<sub>2</sub> can be detected with 30 minutes incubation (n=3). The insert shows the low levels of H<sub>2</sub>O<sub>2</sub> detection.

## REFERENCES

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# Product Manual

## NOTES

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