ab138886

Hydrogen Peroxide Assay Kit – (Fluorometric-Near Infrared)

Instructions for Use

For detecting hydrogen peroxide in solutions and cell extracts by using our proprietary AbIR Peroxidase Indicator

This product is for research use only and is not intended for diagnostic use.

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1. 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.

ab138886 uses our unique AbIR Peroxidase Indicator substrate to quantify hydrogen peroxide in solutions and cell extracts. AbIR Peroxidase Indicator generates the fluorescence that is pH-independent from pH 4 to 10. It is a superior alternative to ADHP for the detections that require low pH where ADHP has reduced fluorescence. In addition, AbIR Peroxidase Indicator generates a product that has maximum absorption at 647 nm with maximum emission at 670 nm. This near infrared fluorescence minimizes the assay background that is often caused by the autofluorescence of biological samples. It can also be used to detect a variety of oxidase activities through enzyme-coupled reactions.

ab138886 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 3 picomoles of H_2O_2 in a 100 µL assay volume (30 nM). The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation without a separation step. Its signal can be easily read by either a fluorescence microplate reader at Ex/Em = ~640/680 nm or an absorbance microplate reader at ~650 nm. Due to its long emission wavelength, this kit has low interference from biological samples.

Kit Key Features

- **Broad Application:** Can be used for quantifying hydrogen peroxide in solutions, in cell extracts, in live cells and for detecting a variety of oxidase activities through enzyme-coupled reactions.

- **Sensitive:** Detect as low as 30 nanomoles of H_2O_2 in solution.

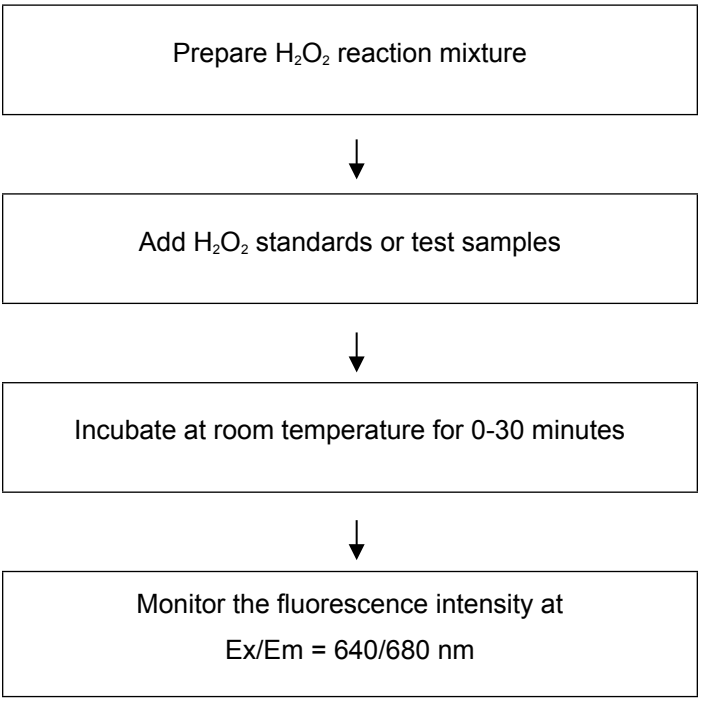
- **Continuous:** Easily adapted to automation without a separation step.

- **Convenient:** Formulated to have minimal hands-on time. No wash is required.

- **Non-Radioactive:** No special requirements for waste treatment.

2. Protocol Summary

Summary for One 96-well Plate



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

3. Kit Contents

Components	Amount
Component A: AbIR Peroxidase Indicator	1 vial
Component B: H_2O_2 (3 % stabilized solution)	1 x 200 µL
Component C: Assay Buffer	1 x 100 ml
Component D: Horseradish Peroxidase	1 x 20 U
Component E: DMSO	1 x 0.5 ml

4. Storage and Handling

Store H_2O_2 at +4C. Keep all other components at -20°C. Avoid exposure to light.

5. Additional Materials Required

- 96-well or 384-well solid, black microplates: Tissue culture microplates with white wall and clear bottom
- Fluorescence microplate reader
- HHBS (1X Hank's with 20 mM Hepes Buffer, pH 7.0)

6. Assay Protocol

Note: This protocol is for one 96 - well plate.

A. Prepare Stock Solutions

1. **AbIR Peroxidase Indicator stock solution (100X):** Add 250 µL of DMSO (Component E) into the vial of AbIR Peroxidase Indicator (Component A). The stock solution should be used promptly. Any remaining solution needs to be aliquoted and refrozen at -20 °C.

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

2. **Peroxidase stock solution (20 U/ml):** Add 1 ml of Assay Buffer (Component C) into the vial of Horseradish Peroxidase (Component D).

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

3. **H_2O_2 stock solution (20 mM):** Add 22.7 µL of 3% H_2O_2 (0.88M, Component B) into 977µL of Assay Buffer (Component C).

   Note: The diluted H_2O_2 stock solution is not stable. The unused portion should be discarded.

B. Prepare H_2O_2 reaction mixture

Prepare the H_2O_2 reaction mixture according to the following table, protect from light.

Components	Volume
AbIR Peroxidase Indicator stock solution (100X, from Step A)	50 µL
Peroxidase stock solution (20 U/ml, from Step A)	200 µL
Assay Buffer (Component C)	4.75 mL
Total Volume	5 mL

Table 1. H_2O_2 reaction mixture for one 96-well plate (2X)

C. Prepare serial dilutions of H_2O_2 standard (0 to 10 µM):

Warning 1: Ab IR Peroxidase Substrate (Component A) is unstable in the presence of thiols such as DTT and β mercaptoethanol. If the final concentration of the thiols is higher than 10 uM, it would significantly decrease the assay dynamic range.

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

1. Add 1 µL of 20 mM H_2O_2 stock solution (from Step A.3) into 1999 µL of Assay Buffer (Component C) to get 10 µM H_2O_2 standard.

2. Take 200 µL of 10 µM H_2O_2 standard to perform 1:3 serial dilutions to get 3, 1, 0.3, 0.1, 0.03, 0.01 and 0 µM serial dilutions of H_2O_2 standard.

3. Add serial dilutions of H_2O_2 standard and H_2O_2-containing test samples into a solid black 96-well microplate as described in Tables 2 and 3.

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

Table 2. Layout of H_2O_2 standards and test samples in a solid black 96-well microplate.

Note: HS= H_2O_2 *Standards, BL=Blank Control, TS=Test Samples.*

H_2O_2 Standard	**Blank Control**	**Test Sample**
Serial dilutions*: 50 µL	Assay Buffer (Component C): 50 µL	50 µL

Table 3. Reagent composition for each well.

**Note 1: Add the serially diluted* H_2O_2 *standards from 0.01 µM to 10 µM into wells from HS1 to HS7 in duplicate.*

D. Run H_2O_2 Assay in supernatants reaction:

1. Add 50 µL of H_2O_2 reaction mixture (from Step B) into each well of H_2O_2 standard, blank control and test samples (see Table 3) to make the total volume of 100 µL/well.

   Note: For a 384-well plate, add 25 µL of sample and 25 µL of assay reaction mixture into each well.

2. Incubate the reaction for 0 to 30 minutes at room temperature, protected from light.

3. Monitor the fluorescence intensity with a fluorescence plate reader at Ex/Em = 640/680 nm.

   Note 1: AbIR Peroxidase Indicator substrate is easy to be self-oxidized, so read the fluorescence as soon as the H_2O_2 reaction mixture was added to increase the signal to noise ratio.

   Note 2: 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 650 nm. The absorption detection has lower sensitivity compared to the fluorescence reading.

E. Run H_2O_2 Assay for cells:

ab138886 can be used to measure the release of H_2O_2 from cells. The following is a suggested protocol that can be modified to meet the specific research needs.

1. The H_2O_2 reaction mixture should be prepared as Step B except that Assay Buffer (Component C) should be replaced with the media 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.

2. Prepare cells in a 96-well plate (50 - 100 µL/well), and activate the cells as desired.
   Note: The negative controls (media alone and non-activated cells) are included for measuring the background fluorescence.

3. Add 50 µL of H_2O_2 reaction mixture (from Step E.1) into each well of cells, and H_2O_2 standards (from Step C).
   Note: For a 384-well plate, add 25 µL of cells and 25 µL of H_2O_2 reaction mixture into each well.

4. Incubate the reaction at room temperature for 0 to 30 minutes, protected from light.Monitor the fluorescence intensity with a fluorescence plate reader at Ex/Em = 640/680 nm

7. Data Analysis

The fluorescence in blank wells (with assay buffer only) is used as a control, and is subtracted from the values for those wells with H_2O_2 reactions. An H_2O_2 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.

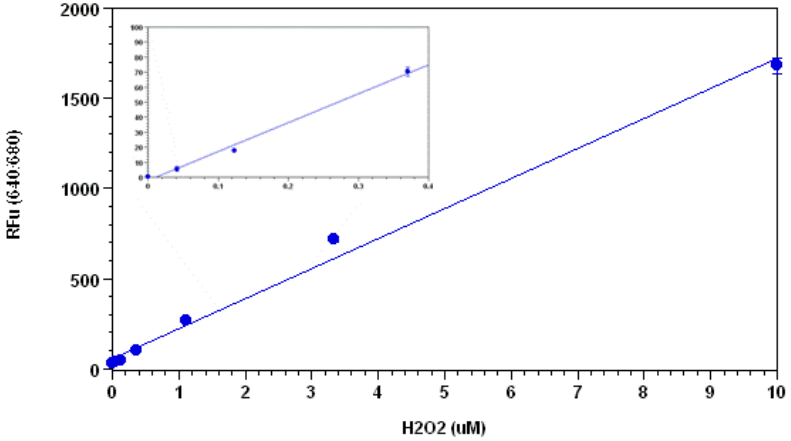


Figure 1. H_2O_2 dose response was measured on a 96-well black plate with Hydrogen Peroxide Assay Kit (Fluorometric-Near Infrared), using a fluorescence microplate reader (Molecular Devices). As low as 0.03 µM H_2O_2 can be detected with 1 minute incubation time (n=3). The insert shows the low levels of H_2O_2 detection.

8. Troubleshooting

Problem	Reason	Solution
Assay not working	Assay buffer at wrong temperature	Assay buffer must not be chilled - needs to be at RT
	Protocol step missed	Re-read and follow the protocol exactly
	Plate read at incorrect wavelength	Ensure you are using appropriate reader and filter settings (refer to datasheet)
	Unsuitable microtiter plate for assay	Fluorescence: Black plates (clear bottoms); Luminescence: White plates; Colorimetry: Clear plates. If critical, datasheet will indicate whether to use flat- or U-shaped wells
Unexpected results	Measured at wrong wavelength	Use appropriate reader and filter settings described in datasheet
	Samples contain impeding substances	Troubleshoot and also consider deproteinizing samples
	Unsuitable sample type	Use recommended samples types as listed on the datasheet
	Sample readings are outside linear range	Concentrate/ dilute samples to be in linear range

Samples with inconsistent readings	Unsuitable sample type	Refer to datasheet for details about incompatible samples
	Samples prepared in the wrong buffer	Use the assay buffer provided (or refer to datasheet for instructions)
	Samples not deproteinized (if indicated on datasheet)	Use the ***10kDa spin column (ab93349)*** or ***Deproteinizing sample preparation kit (ab93299)***
	Cell/ tissue samples not sufficiently homogenized	Increase sonication time/ number of strokes with the Dounce homogenizer
	Too many freeze-thaw cycles	Aliquot samples to reduce the number of freeze-thaw cycles
	Samples contain impeding substances	Troubleshoot and also consider deproteinizing samples
	Samples are too old or incorrectly stored	Use freshly made samples and store at recommended temperature until use
Lower/ Higher readings in samples and standards	Not fully thawed kit components	Wait for components to thaw completely and gently mix prior use
	Out-of-date kit or incorrectly stored reagents	Always check expiry date and store kit components as recommended on the datasheet
	Reagents sitting for extended periods on ice	Try to prepare a fresh reaction mix prior to each use
	Incorrect incubation time/ temperature	Refer to datasheet for recommended incubation time and/ or temperature
	Incorrect amounts used	Check pipette is calibrated correctly (always use smallest volume pipette that can pipette entire volume)

Problem	Reason	Solution
Standard curve is not linear	Not fully thawed kit components	Wait for components to thaw completely and gently mix prior use
	Pipetting errors when setting up the standard curve	Try not to pipette too small volumes
	Incorrect pipetting when preparing the reaction mix	Always prepare a master mix
	Air bubbles in wells	Air bubbles will interfere with readings; try to avoid producing air bubbles and always remove bubbles prior to reading plates
	Concentration of standard stock incorrect	Recheck datasheet for recommended concentrations of standard stocks
	Errors in standard curve calculations	Refer to datasheet and re-check the calculations
	Use of other reagents than those provided with the kit	Use fresh components from the same kit

For further technical questions please do not hesitate to contact us by email (technical@abcam.com) or phone (select "*contact us*" on www.abcam.com for the phone number for your region).

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