SensoLyte® ADHP Peroxidase Assay Kit
Fluorimetric

Revision Number:1.1	*Last Revised: October 2014*
Catalog #	**AS-71111**
Kit Size	500 Assays (96-well) or 1250 Assays (384-well)

- ***Convenient Format:*** Complete kit includes all the assay components.
- ***Optimized Performance:*** Optimal conditions for detecting peroxidase.
- ***Enhanced Value:*** Less expensive than the sum of individual components.
- ***High Speed:*** Minimal hands-on time.
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	ADHP	10 mM, 250 µL
Component B	H_2O_2	1 vial
Component C	Assay buffer	60 mL

Other Materials Required (but not provided)

- Microplate: Black, flat-bottom, 96-well plate.
- Fluorescence microplate reader: Capable of detecting emission at 590 nm with excitation at 530-560 nm.

Storage and Handling

- Store Component A at -20°C and keep from direct light.
- Store the rest of the components at 4°C

Introduction

Cellular peroxidases play an important role in protecting cell from oxidative injury. Horseradish peroxidase (HRP) conjugates are extensively used as secondary detection reagents in ELISA.

The SensoLyte® ADHP Peroxidase Assay Kit provides a convenient, highly sensitive fluorescent assay for detecting peroxidase activity in solution, cell extract, and ELISA. Non-fluorescent ADHP (10-Acetyl-3, 7-dihydroxyphenoxazine) can be oxidized to the strongly fluorescent resorufin in presence of H_2O_2 by peroxidases, such as horseradish peroxidase (HRP), eosinophil peroxidase[1] and myeloperoxidase.[1] Resorufin signal (Ex/Em=530-560nm/590nm) can easily be read with a fluorescence microplate reader.

Protocol

Note 1: Warm all kit components to room temperature before starting the experiment.
Note 2: Choose Protocol A or B based on your needs.

Protocol A. Detecting peroxidase activity in biological samples

1. Prepare stock solution (for first time use only).

1.1 H_2O_2 stock solution: Add 100 µL of deionized water into one vial of H_2O_2 (Component B). Store this stock solution tightly capped at 4°C.

2. Prepare peroxidase-containing samples.

2.1 Add 50 µL/well of samples to 96-well plate or 20 µL/well to 384-well plate.
Note: Exceeding large amount of peroxidase may further convert fluorescent resorufin to non-fluorescent resazurin and lead to reduce fluorescent signal. It is necessary to test first with different sample dilutions.

3. Prepare ADHP reaction mixture.

3.1 Prepare ADHP reaction mixture fresh according to the following Table and keep from light.

Table 1. ADHP reaction mixture for one 96-well plate (100 assays).

Components	Volume
ADHP (Component A)	50 µL
H_2O_2 stock solution (Component B)	10 µL
Assay buffer (Component C)	4.94 mL
Total volume	5 mL

4. Initiate the enzymatic reaction:

4.1 Add 50 µL/well (96-well plate) or 20 µL/well (384-well plate) of ADHP reaction mixture. Mix the reagents by gently shaking plate for 30 sec.

4.2 Measure signals:

- For kinetic reading: Immediately start measuring fluorescence, Ex/Em=530-560 nm/590 nm. Record data every 5 min. for 15 to 30 min.
- For end-point reading: Incubate reaction at the desired temperature for 15-30 min, then measure fluorescence, Ex/Em=530-560 nm/590 nm.

Protocol B. Detecting horseradish peroxidase (HRP) activity in ELISA

Note: For the preparation of ELISA plate, please refer to Appendix I.

1. Prepare stock solution (for first time use only).

1.1 H_2O_2 stock solution: Add 100 µL of deionized water into one vial of H_2O_2 (Component B). Store this stock solution tightly capped at 4°C.

2. Prepare ADHP reaction mixture.

2.1 Prepare ADHP reaction mixture fresh according to the following Table and keep from light.

Table 1. ADHP reaction mixture for one 96-well plate (100 assays).

Components	Volume
ADHP (Component A)	50 µL
H_2O_2 stock solution (Component B)	2 µL
Assay buffer (Component C)	10 mL
Total volume	10 mL

3. Detect HRP activity.

3.1 Add 100 µL/well (96-well plate) or 50 µL/well (384-well plate) of ADHP reaction mixture. Mix the reagents by gently shaking the plate for 30 sec.

3.2 Measure signal: Incubate reaction at the desired temperature for 15-30 min, then measure fluorescence, Ex/Em=530-560 nm/590 nm.

Appendix: General ELISA protocol

1. Required buffers:

1. Coating buffer: 1.59 g of Na_2CO_3 and 2.93 g of $NaHCO_3$ in 1L of deionized H_2O. pH is 9.6 without adjustment.
2. Phosphate-buffered saline (PBS): 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 ml of deionized H_2O. Adjust pH to 7.2-7.4 with HCl or NaOH. Add H_2O to 1L.
3. Blocking buffer: Add 10 g of bovine serum albumin (BSA) and 0.2 mL of Tween®-20 into 1 L of PBS.
4. EIA buffer: Add 1 g of bovine serum albumin (BSA) and 0.2 mL Tween®-20 into 1 L of PBS.
5. Wash buffer: Add 0.2 mL of Tween®-20 into 1 L of PBS.

2. Required ELISA microplate:

Use black high-binding ELISA plates for better signal to noise ratio.

3. ELISA procedures.

1. Coating: Add 100 µL of peptide-conjugate (PP-BSA) to each well of the 96-well plate at a concentration of 10 µg/mL in coating buffer. Seal plate with Para film® and incubate at 4°C overnight.
2. Washing: Discard solution and wash plate with 300 µL of wash buffer per well three to five times. Soak plate during the last wash step for 5 min. Pat dry on paper towel.
3. Blocking: Add 200 µL/well of blocking buffer and incubate for 1h at room temperature.
4. Washing: Repeat Step 2.
5. Add the primary antibody: Dilute anti-peptide antibody in EIA buffer to an appropriate concentration. Add 100µL/well of the diluted antibody and incubate at room temperature for 1h on a plate shaker.
6. Washing: Repeat Step 2.
7. Add the secondary antibody: Dilute HRP conjugated secondary antibody in EIA buffer to an appropriate concentration (1:5000 to 1:100,000 dilution). Add 100 µL/well of diluted secondary antibody and incubate at room temperature for 1h on a plate shaker.
8. Washing: Repeat Step 2.
9. Detection: The plate is now ready for the ADHP detection (refer to Protocol B).

References

1. Mohanty, JG. et al. *J. Immunol. Methods* **202**, 133 (1997).