Amplite™ Fluorimetric Peroxidase Assay Kit

Near Infrared Fluorescence

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 11553 (500 assays)	Keep in freezer Avoid exposure to light	Fluorescence microplate readers

Introduction

Horseradish Peroxidase (HRP) is a small molecule (MW ~40 KD) that is widely used in a variety of biological detections. HRP conjugates are extensively used as secondary detection reagents in ELISAs, immuno-histochemical techniques, Northern, Southern and Western blot analyses. Due to its small size, it rarely causes steric hindrance problem with the antibody/antigen complex formation. It is usually conjugated to an antibody in a 4:1 ratio. Meanwhile, HRP is inexpensive compared to other labeling enzymes.

We offer this quick HRP assay in a one-step, homogeneous, no wash assay system. Our Amplite™ Fluorimetric Peroxidase Assay Kit uses Amplite™ IR, our near infrared flurogenic HRP substrate to quantify peroxidase in solution. Amplite™ IR generates a substance that has maximum absorption of 647 nm with maximum emission at 670 nm. This near infrared absorption and fluorescence minimize the assay background that is often caused by the autoabsorption and/or autofluorescence of biological samples that rarely absorb light beyond 600 nm. The kit can be used for ELISAs, characterizing kinetics of enzyme reaction and high throughput screenings, etc. The kit provides an optimized "mix and read" assay protocol that is compatible with HTS liquid handling instruments. It can detect as low as 1 mU/mL HRP (as shown in Figure1). 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 = 600 to 650/650 to 690 nm (maximum Ex/Em = 640 /680 nm) or an absorbance microplate reader at 647±5 nm.

Kit Key Features

Broad Application:	Can be used for quantifying HRP activities in solutions and solid surfaces (e.g, ELISA)
Sensitive:	Detect as low as 1 mU/mL HRP 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.

Kit Components

Components	**Amount**
Component A: Amplite™ IR Peroxidase Substrate	1 vial
Component B: H_2O_2	1 vial (3% stabilized solution, 200 µL)
Component C: Assay Buffer	1 bottle (100 mL)
Component D: Horseradish Peroxidase	1 vial (20 units)
Component E: DMSO	1 vial (1 mL)

Ordering: sales@aatbio.com; Tel: 800-990-8053 or 408-733-1055; Fax: 408-733-1304
Technical Support: support@aatbio.com; Tel: 408-733-1055

Assay Protocol for One 96-Well Plate

Brief Summary

Prepare peroxidase reaction mixture (50 µL) → Add peroxidase standards and/or test samples (50 µL)→ Incubate at room temperature for 30-60 minutes → Monitor fluorescence intensity at Ex/Em = 640/680 nm

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

1. Prepare stock solutions:

1.1 100X Amplite™ IR Peroxidase Substrate stock solution: Add 250 µL of DMSO (Component E) into the vial of Amplite™ IR Peroxidase Substrate (Component A). The stock solution should be used promptly, and any remaining solution should be aliquoted and refrozen at -20 °C.
Note: Avoid repeated freeze-thaw cycles.

1.2 20 U/mL HRP stock solution: Add 1 mL of Assay Buffer (Component C) into the vial of Horseradish Peroxidase (Component D).
Note: The unused HRP stock solution should be divided into single use aliquots and stored at -20 °C.

1.3 20 mM H_2O_2 stock solution: Add 22.7 µL of 3% H_2O_2 (0.88 M, Component B) into 977µL of Assay Buffer (Component C).
Note: The diluted H_2O_2 solution is not stable. The unused portion should be discarded.

2. Prepare peroxidase reaction mixture:

Prepare the peroxidase reaction mixture according to the following table and keep from light:

Table 1. Proxidase Reaction mixture for one 96-well plate (2X)

Components	Volume
Amplite™ IR Peroxidase Substrate stock solution (100X, from Step 1.1)	50 µL
20 mM H_2O_2 stock solution (from Step 1.3)	50 µL
Assay Buffer (Component C)	4.9 mL
Total volume	5 mL

3. Prepare serially diluted HRP standards (0 to 300 mU/mL):

Warning 1: *The component A is unstable in the presence of thiols such as DTT and β-mercaptoethanol. The presence of thiols at concentration higher than 10 uM would significantly decrease the assay dynamic range.*
2: *NADH and glutathione (reduced form: GSH) may interfere with the assay.*

3.1 Add 15 µL of 20 U/mL HRP stock solution (from Step 1.2) into 985 µL of Assay Buffer (Component C) to get 300 mU/mL HRP solution.

3.2 Take 200 µL of 300 mU/mL HRP stock solution to perform 1:3 serial dilutions to get 100, 30, 10, 3, 1, 0.3 and 0 mU/mL serially diluted HRP standards.

3.3 Add serially diluted HRP standards and/or peroxidase-containing test samples into a solid black 96-well microplate as described in Tables 2 and 3.

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

BL	BL	TS	TS	….	….						
PS1	PS1	….	….	….	….						
PS2	PS2										
PS3	PS3										
PS4	PS4										
PS5	PS5										
PS6	PS6										
PS7	PS7										

Note: PS= Peroxidase Standards, BL=Blank Control, TS=Test Samples.

Table 3. Reagent composition for each well

Peroxidase Standards	**Blank Control**	**Test Sample**
Serial Dilutions*: 50 µL	Assay Buffer (Component C): 50 µL	50 µL

**Note: Add the serially diluted peroxidase standards from 0.3 mU/mL to300 mU/mL into wells from PS1 to PS7 in duplicate.*

4. Run HRP assay in supernatants:

4.1 Add 50 µL of peroxidase reaction mixture (from Step 2) into each well of peroxidase standard, blank control, and test samples (see Step 3.3) to make the total peroxidase assay volume of 100 µL/well
Note: For a 384-well plate, add 25 µL of sample and 25 µL of peroxidase reaction mixture into each well.

4.2 Incubate the reaction at room temperature for 30 to 60 minutes, protected from light.

4.3 Monitor the fluorescence increase with a fluorescence plate reader at excitation 600-650 nm (optimal at 640) with emission at 650-690 nm (optimal at 680).
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 647±5 nm. The absorption detection has lower sensitivity compared to fluorescence reading.

Data Analysis

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 peroxidase reactions. A HRP 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.*

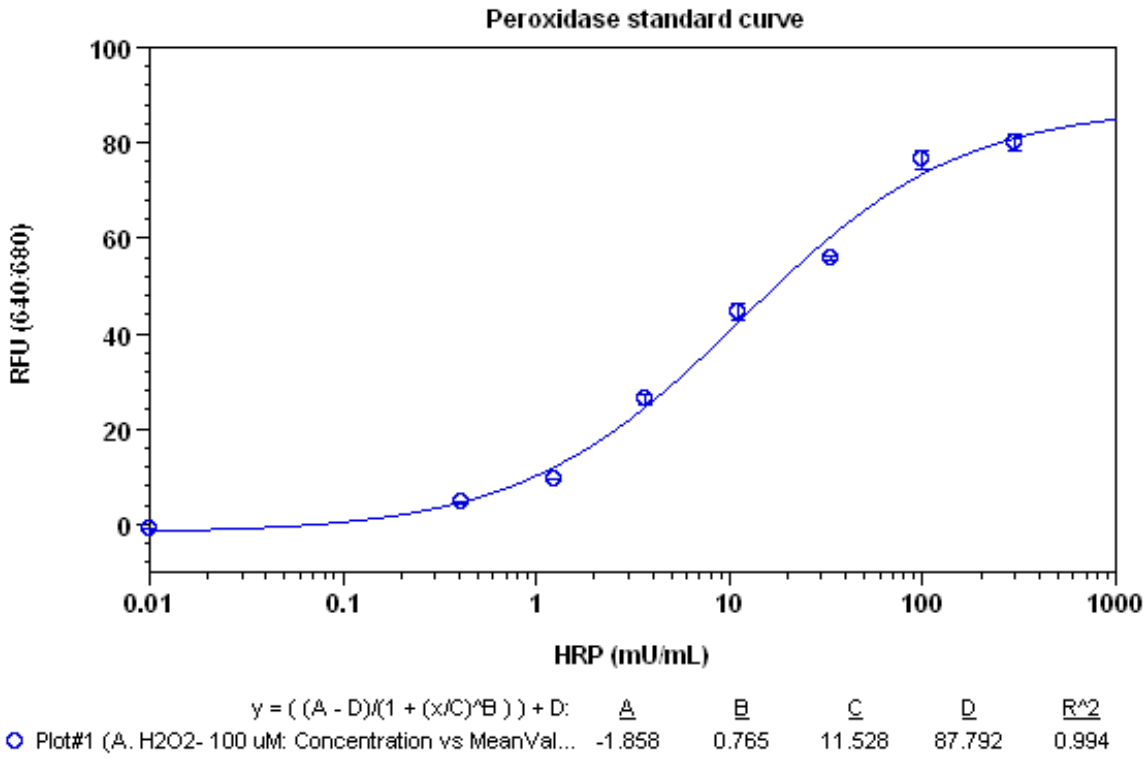

Figure 1. HRP dose response was measured with the Amplite™ Fluorimetric Peroxidase Assay Kit in a black 384-well plate using a Gemini fluorescence microplate reader (Molecular Devices). As low as 1 mU/mL peroxidase can be detected with 30 minutes incubation (n=3).

References

1. Porstmann, B., Porstmann, T., Nugel, E. and Evers, U. (1985). Which of the commonly used marker enzymes gives the best results in colorimetric and fluorimetric enzyme immunoassays: horseradish peroxidase, alkaline phosphatase, ß-galactosidase? *J. Immunol. Meth.* 79**,** 27-37.
2. Wordinger, R.J., Miller, G.W. and Nicodemus, D.S. (1987). *Manual of Immunoperoxidase Techniques, 2nd Edition.* Chicago: American Society of Clinical Pathologists Press, pp. 23-24.
3. Yolken, R.H. (1982). Enzyme immunoassays for the detection of infectious antigens in body fluids: current limitations and future prospects. Rev. Infect. Dis. 4(1)**,** 35-68.
4. Cordell, J.L., et al. (1984). Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J. Histochem. Cytochem***.** 32, 219-229.
5. Passey, R.B., et al. (1977). Evaluation and comparison of 10 glucose methods and the reference method recommended in the proposed product class standard. *Clin. Chem*. 23(1)**,** 131.
6. Hosoda, H., Takasaki, W., Tsukamoto, R. and Nambara, T. (1987). Sensitivity of steroid immunoassays. Comparison of alkaline phosphatase, ß-galactosidase and horseradish peroxidase as labels in a colorimetric assay system. *Chem. Pharm. Bull*. 35, 3336-3342.
7. Samoszuk, M.K., et al. (1989). *Antibody, Immunoconjugates and Radiopharmaceuticals* **2**, 37-46.