Amplite™ Fluorimetric Peroxidase Assay Kit

Red Fluorescence

Ordering Information:	*Storage Conditions:*	*Instrument Platform:*
Product Number: 11552 (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. Additionally, HRP is inexpensive compared to other labeling enzymes. The major disadvantage associated with peroxidase is their low tolerance to many preservatives such as sodium azide that inactivates peroxidase activity even at low concentration.

We offer this quick (10 min) HRP assay in a one-step, homogeneous, no wash assay system. This kit uses our fluorogenic Amplite™ Red HRP substrate to quantify peroxidase in solutions. 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. The Kit can detect as low as 10 µU/mL of HRP (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 = 540±10/590 ±10 nm (maximum Ex/Em = 540/590 nm) or an absorbance microplate reader at 576±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 10 µU/mL of 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™ Red 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)

Assay Protocol for One 96-Well Plate

Brief Summary

Prepare peroxidase reaction mixture (50 µL) → Add peroxidase standards or test samples (50 µL) → Incubate at room temperature for 10-30 min → Read fluorescence intensity at Ex/Em = 540/590 nm

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

1. Prepare stock solutions:

1.1 Amplite™ Red peroxidase substrate stock solution (100X): Add 250 µL of DMSO (Component E) into the vial of Amplite™ Red 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, and keep from light.

1.2 20 U/mLHRP stock solution: 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.

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™ Red 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 serial peroxidase (0 to 10 mU/mL) standard solutions:

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

3.1 Add 1 µL of 20 U/mL HRP stock solution (from Step 1.2) in 1999 µL of assay buffer (Component C) to get 10 mU/mL peroxidase solution.

3.2 Take 200 µL of 10 mU/mL HRP stock solution to perform 1:3 serial dilutions to get 3, 1, 0.3, 0.1, 0.03, 0.01 and 0 standard peroxidase solutions.

3.3 Add peroxidase standards and peroxidase-containing test samples into a 96-well solid black 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 Standard	**Blank Control**	**Test Sample**
Serial dilutions* (50 µL)	Assay buffer (Component C): 50 µL	50 µL

Note 1. Add the serially diluted peroxidase standards from 0.01 mU/mL to10 mU/mL into wells from PS1 to PS7 in duplicate.
Note 2. High levels of HRP (e.g., >100 mU/mL final concentration) may cause reduced fluorescence signal due to the over oxidation of Amplite™ Red (to non-fluorescent one).

4. Run HRP assay in supernatants reaction:

4.1 Add 50 µL of peroxidase reaction mixture (from Step 2) to each well of the 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 15 to 30 minutes, protected from light.

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

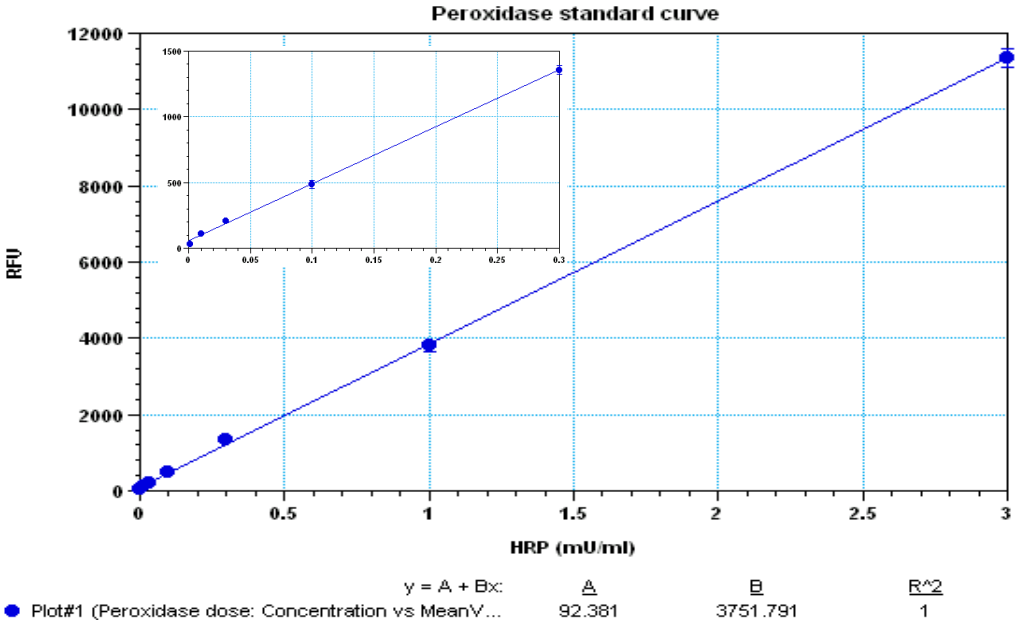

Figure 1. HRP dose response was measured with the Amplite™ Fluorimetric Peroxidase Assay Kit in a 384-well black plate using a Gemini fluorescence microplate reader (Molecular Devices). As low as 10 µU/mL of peroxidase can be detected with 30 minutes incubation time (n=3). The insert shows the low levels of HRP detection.

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.