

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

Horseshradish 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, immunohistochemical 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. It can be used for ELISAs, characterizing kinetics of enzyme reaction and high throughput screenings, etc. This Amplite™ Fluorimetric Peroxidase Assay Kit provides an optimized “mix and read” assay protocol that is compatible with HTS liquid handling instruments. It can detect as low as 10 µU/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 = 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 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 ₂ O ₂	1 vial (3% stabilized solution, 200 µL)
Component C: Assay Buffer	1 bottle (100 mL)
Component D: Horseshradish Peroxidase	1 vial (20 units)
Component E: DMSO	1 vial (1 mL)

Assay Protocol for One 96-Well Plate

Brief Summary

Prepare HRP reaction mixture (50 µL) → Add HRP standards and/or test samples (50 µL) → Incubate at room temperature for 10-30 minutes → Monitor 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 **100X Amplite™ Red peroxidase substrate stock solution:** 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/mL HRP 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 H₂O₂ stock solution:** Add 22.7 µL of 3% H₂O₂ (0.88 M, Component B) into 977 µL of Assay Buffer (Component C).
Note: The diluted H₂O₂ solution is not stable. The unused portion should be discarded.

2. Prepare HRP reaction mixture:

Prepare the HRP reaction mixture according to the following table and keep from light.

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

Components	Volume
100XAmplite™ Red peroxidase substrate stock solution (from Step 1.1)	50 µL
20 mM H ₂ O 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 10 mU/mL):

Warnings 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 µM 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 HRP standard solution.
- 3.2 Take 200 µL of 10 mU/mL HRP standard solution to perform 1:3 serial dilutions to get 3, 1, 0.3, 0.1, 0.03, 0.01 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 HRP 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

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

Note 1: Add the serially diluted HRP standards from 0.01 mU/mL to 10 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:

- 4.1 Add 50 μL of HRP reaction mixture (from Step 2) to each well of HRP standard, blank control, and test samples (see Step 3.3) to make the total HRP assay volume of 100 μL /well.

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

- 4.2 Incubate the reaction mixture 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 = $540 \pm 10/590 \pm 10$ nm (optimal Ex/Em = 540/590).

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 HRP 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.*

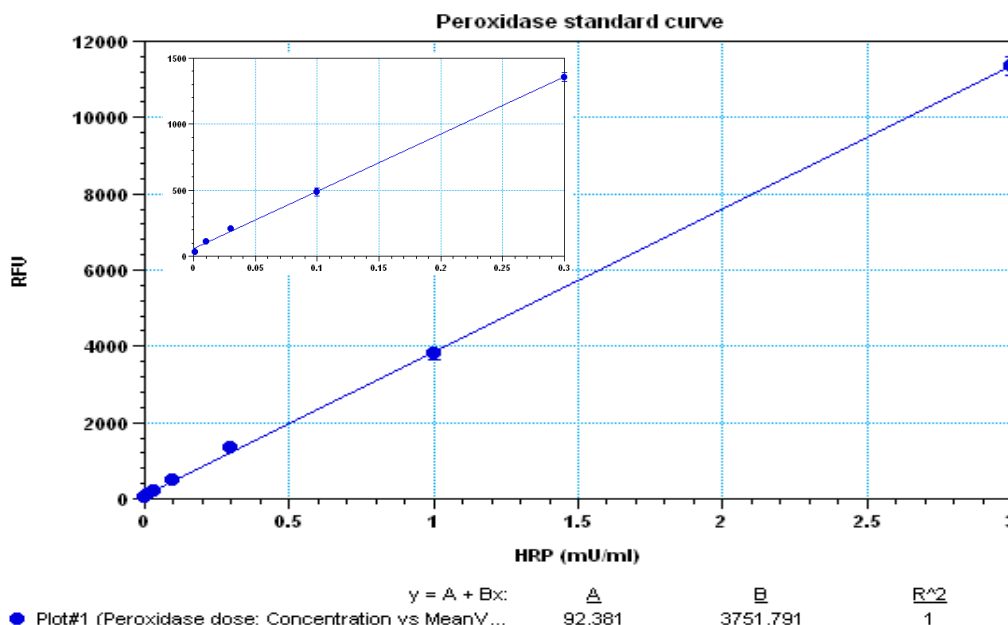


Figure 1. HRP dose response was measured with the Amplitude™ Fluorimetric Peroxidase Assay Kit in a black 384-well black plate using a Gemini fluorescence microplate reader (Molecular Devices). As low as 10 $\mu\text{U}/\text{mL}$ peroxidase can be detected with 30 minutes incubation (n=3). The insert 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, β -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.

Warning: This kit is only sold to end users. Neither resale nor transfer to a third party is allowed without written permission from AAT Bioquest. Chemical analysis of the kit components is strictly prohibited. Please call us at 408-733-1055 or e-mail us at info@aatbio.com if you have any questions.