Amplite™ Colorimetric Peroxidase Assay Kit

Blue Color

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 11551 (500 assays)	Keep in freezer Avoid exposure to light	Absorbance 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.

This kit uses Amplite™ Blue, our chromogenic HRP substrate that is much more sensitive to both H_2O_2 and peroxidase than other chromogenic peroxidase substrates such as TMB, ABTS, OPD and K-Blue. Amplite™ Blue generates a highly absorptive material that has maximum absorption of 664 nm. This near infrared absorption minimizes the background absorption often caused by the auto-absorption of biological samples. The kit provides an optimized "mix and read" assay protocol that is compatible with HTS liquid handling instruments. 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 an absorbance microplate reader at 664±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 3 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™ Blue 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 HRP reaction mixture (50 µL) → Add HRP standards and/or test samples (50 µL) → Incubate at room temperature for 10-30 minutes → Monitor absorbance at 664±5 nm

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

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1. Prepare stock solutions:

1.1 100X Amplite™ Blue peroxidase substrate stock solution: Add 250 µL of DMSO (Component E) into the vial of Amplite™ Blue 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 HRP (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 977 µL of Assay Buffer (Component C).
Note: The diluted H_2O_2 solution is not stable. The unused portion should be discarded.

2. Prepare HRP reaction mixture:

Prepare 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**
Amplite™ Blue 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):

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 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 standard solution.

3.2 Take 200 µL of 300 mU/mL HRP standard 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 HRP standards and/or HRP-containing test samples into a white wall/clear bottom 96-well microplate as described in Tables 2 and 3

Table 2. Layout of HRP standards and test samples in a white wall/clear bottom 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: Add the serially diluted HRP 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 HRP reaction mixture (from Step 2) into 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 at room temperature for 30 to 60 minutes, protected from light.

4.3 Monitor the absorbance with an absorbance plate reader at 664±5 nm.

Data Analysis

The absorbance in blank wells (with the assay buffer only) is used as a control, and is subtracted from the values for those wells with the HRP reactions. A HRP standard curve is shown in Figure 1.

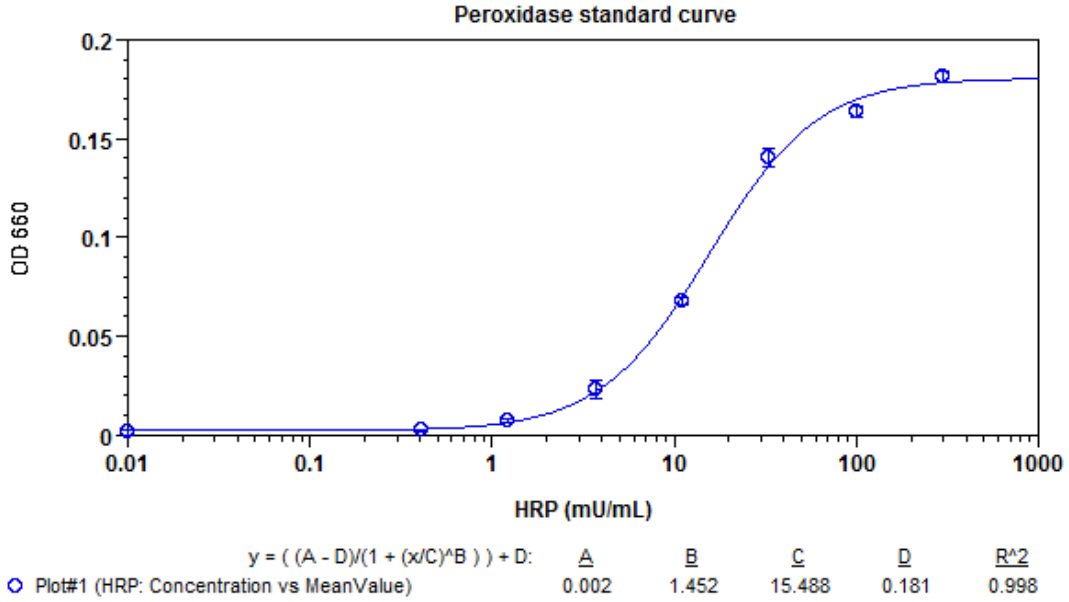

Figure 1 HRP dose response was measured with the Amplite™ Colorimetric Peroxidase Assay Kit in a white wall/clear bottom 96-well plate using a NovoStar absorbance microplate reader (BMG Labtech). As low as 3 mU/mL peroxidase can be detected with 30 minutes incubation (n=3).

References

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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.
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