

Amplite® Fluorimetric Peroxidase (HRP) Assay Kit *Near Infrared Fluorescence*

 Catalog number: 11553
 Unit size: 500 Tests

Component	Storage	Amount
Component A: Amplite™ IR Peroxidase Substrate	Freeze (< -15 °C), Minimize light exposure	1 vial
Component B: H ₂ O ₂	Freeze (< -15 °C), Minimize light exposure	1 vial (3% stabilized solution, 200 µL)
Component C: Assay Buffer	Freeze (< -15 °C)	1 bottle (100 mL)
Component D: Horseradish Peroxidase	Freeze (< -15 °C), Minimize light exposure	1 vial (20 units)
Component E: DMSO	Freeze (< -15 °C)	1 vial (0.5 mL)

OVERVIEW

Peroxidase is a small molecule (MW ~40 KD) that can usually be conjugated to an antibody in a 4:1 ratio. Due to its small size, it rarely causes steric hindrance problem with antibody/antigen complex formation. Peroxidase 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. HRP conjugates are extensively used as secondary detection reagents in ELISAs, immuno-histochemical techniques and Northern, Southern and Western blot analyses. We offer this quick (10 min) HRP assay in a one-step, homogeneous, no wash assay system. This kit uses Amplite® IR, our near infrared fluorogenic HRP substrate. 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 screening of oxidase inhibitors, etc. The kit provides an optimized 'mix and read' assay protocol that is compatible with HTS liquid handling instruments.

AT A GLANCE

Protocol Summary

1. Prepare HRP standards and/or test samples (50 µL)
2. Add Peroxidase working solution (50 µL)
3. Incubate at room temperature for 30 - 60 minutes
4. Monitor fluorescence intensity at Ex/Em = 640/680 nm (Cutoff = 665 nm)

Important Thaw all the kit components at room temperature before starting the experiment. 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. NADH and glutathione (reduced form: GSH) may interfere with the assay.

KEY PARAMETERS

Absorbance microplate reader

Absorbance 647 ± 5 nm
 Recommended plate Clear bottom

Fluorescence microplate reader

Excitation 640nm
 Emission 680 nm
 Cutoff 665 nm
 Recommended plate Solid black

PREPARATION OF STOCK SOLUTIONS

Unless otherwise noted, all unused stock solutions should be divided into single-use aliquots and stored at -20 °C after preparation. Avoid repeated freeze-thaw cycles.

1. Amplite™ IR Peroxidase Substrate stock solution (100X)

Add 250 µL of DMSO (Component E) into the vial of Amplite™ IR Peroxidase Substrate (Component A) to make 100X Amplite™ IR Peroxidase Substrate stock solution.

2. HRP standard solution (20 U/mL)

Add 1 mL of Assay Buffer (Component C) into the vial of Horseradish Peroxidase (Component D) to make 20 U/mL HRP standard solution.

3. H₂O₂ stock solution (20 mM)

Add 22.7 µL of 3% H₂O₂ (0.88 M, Component B) into 977 µL of Assay Buffer (Component C) to make 20 mM H₂O₂ stock solution. **Note:** The diluted H₂O₂ solution is not stable. The unused portion should be discarded.

PREPARATION OF STANDARD SOLUTION

For convenience, use the Serial Dilution Planner:

<https://www.aatbio.com/tools/serial-dilution/11553>

HRP standard

Add 15 µL of 20 U/mL HRP standard solution into 985 µL of Assay Buffer (Component C) to get 300 mU/mL HRP standard solution (SD7). Take 300 mU/mL HRP standard solution (SD7) and perform 1:3 serial dilutions to get serially diluted HRP standards (SD6 - SD1) with Assay Buffer (Component C).

PREPARATION OF WORKING SOLUTION

Add 50 µL of 100X Amplite™ IR Peroxidase Substrate stock solution and 50 µL of 20 mM H₂O₂ stock solution into 4.9 mL of Assay Buffer (Component C) to make Peroxidase working solution. Keep from light.

SAMPLE EXPERIMENTAL PROTOCOL

Table 1. Layout of HRP standards and test samples in a solid black 96-well microplate. SD= HRP Standards (SD1 - SD7, 0.41 to 300 mU/mL), BL=Blank Control, TS=Test Samples.

BL	BL	TS	TS
SD1	SD1
SD2	SD2
SD3	SD3		
SD4	SD4		
SD5	SD5		
SD6	SD6		
SD7	SD7		

Table 2. Reagent composition for each well.

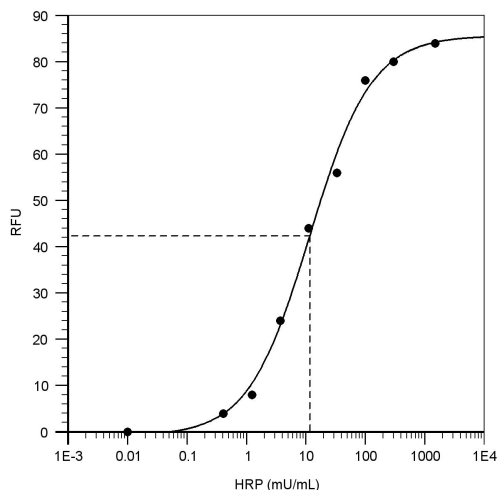
Well	Volume	Reagent
SD1 - SD7	50 µL	Serial Dilutions (0.41 to 300 mU/mL)
BL	50 µL	Assay Buffer (Component C)
TS	50 µL	test sample

1. Prepare HRP standards (SD), blank controls (BL), and test samples (TS) according to the layout provided in Tables 1 and 2. For a

384-well plate, use 25 μ L of reagent per well instead of 50 μ L.

2. Add 50 μ L of Peroxidase working solution to each well of HRP standard, blank control, and test samples to make the total Peroxidase assay volume of 100 μ L/well. For a 384-well plate, add 25 μ L of Peroxidase working solution into each well instead, for a total volume of 50 μ L/well.
3. Incubate the reaction at room temperature for 30 to 60 minutes, protected from light.
4. Monitor the fluorescence increase with a fluorescence plate reader at Excitation = 600 - 650 nm, Emission = 650 - 690 nm (optimal Ex/Em = 640/680 nm, Cutoff = 665 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 647 ± 5 nm. The absorption detection has lower sensitivity compared to fluorescence reading.

EXAMPLE DATA ANALYSIS AND FIGURES



HRP dose response was measured with Amplitude™ Fluorimetric Peroxidase Assay Kit in a solid black 384-well plate using a Gemini fluorescence microplate reader (Molecular Devices).

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Figure 1. HRP dose response was measured with Amplitude™ Fluorimetric Peroxidase Assay Kit in a solid black 384-well plate using a Gemini fluorescence microplate reader (Molecular Devices).

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