Amplite® Fluorimetric Peroxidase (HRP) Assay Kit *Red Fluorescence*

Catalog number: 11552
Unit size: 500 Tests

Component	Storage	Amount
Component A: Amplite™ Red Peroxidase Substrate	Freeze (< -15 °C), Minimize light exposure	1 vial
Component B: H2O2	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. 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 HRP working solution (50 µL)
3. Incubate at room temperature for 10 - 30 minutes
4. Monitor fluorescence intensity at Ex/Em = 540/590 nm (Cutoff = 575 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	576 ± 5 nm
Recommended plate	Clear bottom

Fluorescence microplate reader

Excitation	540 nm
Emission	590 nm
Cutoff	575 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™ Red Peroxidase Substrate stock solution (100X)
Add 250 µL of DMSO (Component E) into the vial of Amplite™ Red Peroxidase Substrate (Component A) to make 100X Amplite™ Red Peroxidase Substrate stock solution. The stock solution should be used promptly. Keep from light.

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 of HRP standard solution.

3. H_2O_2 stock solution (20 mM)
Add 22.7 µL of 3% H_2O_2 (0.88 M, Component B) into 977 µL of Assay Buffer (Component C) to make 20 mM H_2O_2 stock solution . **Note:** The diluted H_2O_2 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/11552

HRP standard
Add 1 µL of 20 U/mL HRP standard solution in 1999 µL of Assay Buffer (Component C) to get 10 mU/mL HRP standard solution (SD7). Take 10 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™ Red Peroxidase Substrate stock solution and 50 µL of 20 mM H_2O_2 stock solution into 4.9 mL of Assay Buffer (Component C) to make HRP 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, 0.01 to 10 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 (0.01 to 10 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 µL of reagent per well instead of 50 µL. **Note:** 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).

2. Add 50 µL of HRP working solution to each well of HRP standard, blank control, and test samples to make the total HRP assay volume of 100 µL/well. For a 384-well plate, add 25 µL of HRP working solution into each well instead, for a total volume of 50 µL/well.

3. Incubate the reaction at room temperature for 10 to 30 minutes, protected from light.

4. Monitor the fluorescence increase with a fluorescence plate reader at Excitation = 540 ± 10 nm, Emission = 590 ± 10 nm (optimal Ex/Em = 540/590 nm, Cutoff = 575 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.

EXAMPLE DATA ANALYSIS AND FIGURES

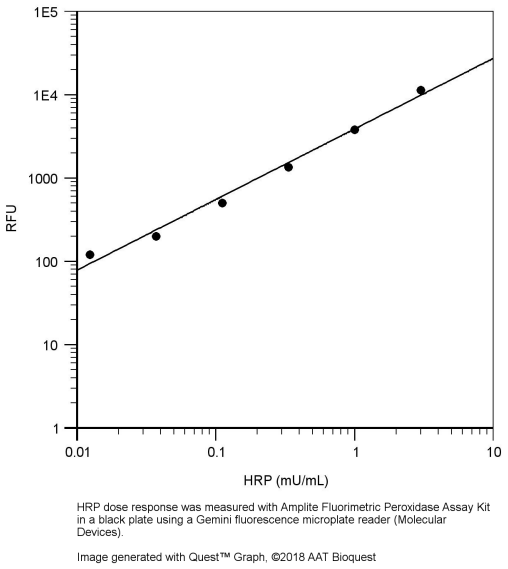

Figure 1. HRP dose response was measured with Amplite Fluorimetric Peroxidase Assay Kit in a black plate using a Gemini fluorescence microplate reader (Molecular Devices).

DISCLAIMER

AAT Bioquest provides high-quality reagents and materials for research use only. For proper handling of potentially hazardous chemicals, please consult the Safety Data Sheet (SDS) provided for the product. Chemical analysis and/or reverse engineering of any kit or its components is strictly prohibited without written permission from AAT Bioquest. Please call 408-733-1055 or email info@aatbio.com if you have any questions.