Amplite™ Fluorimetric Alkaline Phosphatase Assay Kit

Near Infrared Fluorescence

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 11954 (500 assays)	Keep in freezer, Avoid exposure to light	Fluorescence microplate readers

Introduction

Alkaline phosphatase is widely used in various biological assays (in particular, immunoassays) and ELISA-based diagnostics. AAT Bioquest's Amplite™ Fluorimetric Alkaline Phosphatase Assay kit uses our SunRed™-based substrate. The weakly fluorescent SunRed™ phosphate is sensitive to phosphatase-induced hydrolysis, giving the SunRed™ fluorophore that possesses intense red fluorescence. Upon phosphatase-induced hydrolysis, the SunRed™ phosphate solution has its absorption blue-shifted more than 100 nm. The maximum absorption of SunRed™ fluorophore at 633 nm makes this substrate an ideal NIR probe that can be readily detected with many fluorescence instrument systems often equipped with Cy5 settings.

Based on the near infrared fluorescence of SunRed™ fluorophore, the signal can be easily read by a fluorescence microplate reader at Ex/Em = ~620/660 nm. The kit has been used for the high throughput screening of protein phosphatase inhibitors due to its low interference from biological sample. It can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation without a separation step.

Kit Components

Components	**Amount**
Component A: SunRed™ Substrate (light sensitive)	1 vial
Component B: Assay Buffer	1 bottle (25 mL)
Component C: Alkaline Phosphatase Standard	1 vial (lyophilized powder, 10 units)

Assay Protocol for One 96-well Plate

Brief Summary

Prepare assay reaction mixture (50 µL) → Add alkaline phosphatase standards and/or test samples (50 µL) → Incubate at RT or 37 °C for 30 to 120 minutes → Monitor fluorescence intensity at Ex/Em = 620/660 nm

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

1. Prepare 250X SunRed™ Substrate stock solution:

Add 100 µL of double sterile H_2O into the vial of SunRed™ Substrate (Component A). The stock solution should be used promptly. Any remaining solution should be make single used aliquots and frozen at -20 °C.

Note: Avoid repeated freeze and thaw cycles.

2. Prepare assay reaction mixture:

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

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

Components	**Volume**
250X SunRed™ Substrate stock solution(from Step 1)	20 µL
Assay Buffer (Component B)	5 mL
Total volume	5 mL

Note: Prepare fresh reaction mixture for each experiment.

3. Prepare serially diluted alkaline phosphatase standards (0 to 100 mU/mL):

3.1 Add 100 µL of distilled H_2O with 0.1% BSA (H_2O-0.1% BSA) to alkaline phosphatase standard (Component C, 10 units) to generate a 100 units/mL alkaline phosphatase standard solution.
Note: The alkaline phosphatase standard solution is not stable. Unused standard solution should be aliquoted and stored at -20 °C. Avoid repeated freeze and thaw cycles.

3.2 Add 10 µL of 100 units/mL alkaline phosphatase standard solution (from Step 3.1) to 990 µL of H_2O-0.1%BSA to generate a 1,000 mU/mL alkaline phosphatase standard solution.

3.3 Take 100 µL of 1,000 mU/mL alkaline phosphatase standard solution (from Step 3.2) to perform 1:3 serial dilutions to get 300, 100, 30, 10, 3, 1, 0.3, and 0 mU/mL serially diluted alkaline phosphatase standards.

3.4 Add serially diluted alkaline phosphatase standards and/or alkaline phosphatase containing test samples into a solid black 96-well microplate as described in Tables 2 and 3.
Note: Prepare cells or tissue samples as desired. Discarded unused serially diluted alkaline phosphatase standards.

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Table 2 Layout of Alkaline Phosphatase Standards and test samples in a solid black 96-well microplate

BL	BL	TS	TS	….	….						
AS1	AS1	….	….	….	….						
AS2	AS2										
AS3	AS3										
AS4	AS4										
AS5	AS5										
AS6	AS6										
AS7	AS7										

Note: AS = Alkaline Phosphatase Standards; BL=Blank Control; TS=Test Samples.

Table 3. Reagent composition for each well:

Alkaline Phosphatase Standards	**Blank Control**	**Test Sample**
Serial dilutions*: 50 µL	H_2O-0.1% BSA: 50 µL	50 µL

Note: Add the serially diluted alkaline phosphatase standards from 100 to 0.01 mU/mL into wells from AS1 to AS7 in duplicate.

4. Run alkaline phosphatase assay in supernatants:

4.1 Add 50 µL of assay reaction mixture (from Step 2) to each well of alkaline phosphatase standard, blank control, and test samples (see Step 3.4, Table 3) to make the total alkaline phosphatase assay volume of 100 µL/well.
Note: For a 384-well plate, add 25 µL of sample and 25 µL of assay reaction mixture into each well.

4.2 Incubate the reaction for 30 to 120 minutes at the desired temperature, protected from light.

4.3 Monitor the fluorescence increase with a fluorescence plate reader at Ex/Em = 620/660 nm (cut off 630 nm).

5. Run alkaline phosphatase assay in cells:

5.1 Treat the cells as desired.

5.2 Remove the growth medium completely from the cell plate.
Note: It is important to remove the growth medium completely from the cell plate due to the interference of the growth medium with the SunRed™ Substrate.

5.3 Make 1:1 dilution of the 5 mL assay reaction mixture (from Step 2, Table 1) with 5 mL distilled H_2O.

5.4 Add 100 µL (96-well plate) or 50 uL(384-well plate) of 1:1 diluted assay reaction mixture (from Step 5.3) into each cell well (from Step 5.2).

5.5 Incubate the reaction for 30 to 60 minutes at the desired temperature, protected from light.

5.6 Monitor the fluorescence increase with a fluorescence plate reader at Ex/Em = 620/660 nm (cut off 630 nm).

Data Analysis

The fluorescence in blank wells (with equal volume of assay reaction mixture and H_2O-0.1%BSA only) is used as a control, and is subtracted from the values for those wells with alkaline phosphatase reactions. An alkaline phosphatase standard curve is shown in Figure 1. *Note: The fluorescence background increases with time due to spontaneous hydrolysis, thus it is important to subtract the fluorescence intensity value of the blank wells for each data point.*

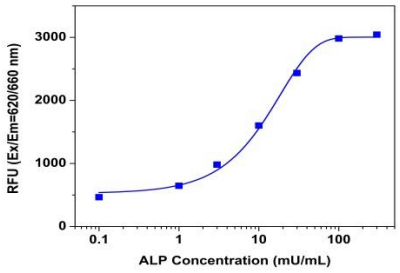

Figure 1. Alkaline phosphatase dose response was measured with the Amplite™ Fluorimetric Alkaline Phosphatase Assay Kit in a solid black 96-well plate using a Gemini microplate reader (Molecular Devices). As low as 3 mU/mL alkaline phosphatase can be detected with 60 minutes incubation (n=3).

References

1. Zhu X, Jiang C. (2006) 8-Quinolyl phosphate as a substrate for the fluorimetric determination of alkaline phosphatase. Clin Chim Acta.
2. Ali AT, Penny CB, Paiker JE, Psaras G, Ikram F, Crowther NJ. (2006) The effect of alkaline phosphatase inhibitors on intracellular lipid accumulation in preadipocytes isolated from human mammary tissue. Ann Clin Biochem, 43, 207.
3. Lee DH, Lim BS, Lee YK, Yang HC. (2006) Effects of hydrogen peroxide (H_2O_2) on alkaline phosphatase activity and matrix mineralization of odontoblast and osteoblast cell lines. Cell Biol Toxicol, 22, 39.
4. Ali AT, Penny CB, Paiker JE, van Niekerk C, Smit A, Ferris WF, Crowther NJ. (2005) Alkaline phosphatase is involved in the control of adipogenesis in the murine preadipocyte cell line, 3T3-L1. Clin Chim Acta, 354, 101.