Amplite™ Universal Fluorimetric Protease Activity Assay Kit

Green Fluorescence

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 13500 (500 assays)	Store at -20 °C and keep from light Component C can be stored at 4 °C	Fluorescence microplate readers

Introduction

Protease assays are widely used for the investigation of protease inhibitors and detection of protease activities. Monitoring various protease activities has become a routine task for many biological laboratories. Some proteases have been identified as good drug development targets.

Our Amplite™ Universal Fluorimetric Protease Activity Assay Kit is an ideal choice to perform routine assays for the isolation of proteases, or for identifying the presence of contaminating proteases in protein samples. The kit uses a fluorescent casein conjugate which is proven to be a generic substrate for a broad spectrum of proteases (e.g. trypsin, chymotrypsin, thermolysin, proteinase K, protease XIV, and elastase). In the intact substrate, casein is heavily labeled with a green fluorescent dye, resulting in significant fluorescence quenching. Protease-catalyzed hydrolysis relieves its quenching effect, yielding brightly fluorescent dye-labeled short peptides. The increase in fluorescence intensity is directly proportional to protease activity. The assay can be performed in a convenient 96-well or 384-well microtiter plate format and readily adapted to automation. Its signal can be easily read with a fluorescence microplate reader at Ex/Em = 490/525 nm using FITC filter set.

Kit Key Features

Convenient Format :	Include all the key assay components.
Optimized Performance:	Optimal conditions for the detection of generic protease activity.
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: Protease Substrate	1 vial (300 µL), protected from light
Component B: Trypsin	1 vial (100 µL, 5 U/µL)
Component C: 2X Assay Buffer	1 bottle (30 mL)

Assay Protocols for One 96-well Plate

Please choose Protocol A or B according to your needs.

Protocol A: Measure protease activity in test samples

Brief Summary

Prepare protease substrate solution (50 µL) → Add substrate control, positive control or test samples (50 µL) → Incubate for 0 min (for kinetic reading) or 30 minutes - 1 hour (for end point reading) → Monitor fluorescence intensity at Ex/Em = 490/525 nm

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

©2008 by AAT Bioquest®, Inc., 520 Mercury, Sunnyvale, CA 94085. Tel: 408-733-1055
Ordering: sales@aatbio.com; Tel: 800-990-8053 or 408-733-1055; Fax: 408-733-1304
Technical Support: support@aatbio.com; Tel: 408-733-1055

1. Prepare working solutions:

1.1 Make protease substrate solution: Dilute Protease Substrate (Component A) at 1:100 in 2X assay buffer (Component C). Use 50 µL of protease substrate solution per assay in a 96-well plate.
*Note: The 2X Assay Buffer (Component C) is designed for detecting the activity of chymotrypsin, trypsin, thermolysin, proteinase K, protease XIV, and human leukocyte elastase. For other proteases, please refer to **Appendix I** for the appropriate assay buffer formula.*

1.2 Trypsin dilution: Dilute Trypsin (5 U/µL, Component B) at 1:50 in de-ionized water to get a concentration of 0.1 U/µL.

2. Add reagents prepared in step 1 into a 96-well microplate according to Table 1 and Table 2.

Table 1. Layout of the substrate control, positive control, and test samples in a 96-well microplate

SC	SC										
PC	PC										
TS	TS										
….	….										

Note: SC=Substrate Control, PC =Positive Control, TS=Test Samples.

Table 2. Reagent composition for each well

Substrate Control	**Positive Control**	**Test Sample**
De-ionized water: 50 µL	Typsin dilution: 50 µL	Protease-containing samples: 50 µL
Total volume: 50 µL	Total volume: 50 µL	Total volume: 50 µL

Note: If less than 50 µL of protease-containing biological sample is used, add ddH_2O to make a total volume of 50 µL.

3. Run the enzymatic reaction:

3.1 Add 50 µL of protease substrate solution (from Step 1.1) to all the wells in the assay plate. Mix the reagents well.

3.2 Monitor the fluorescence increase with a fluorescence plate reader at Ex/Em = 490/525 nm.
For kinetic reading: Immediately start measuring fluorescence intensity continuously and record data every 5 minutes for 30 minutes.
For end-point reading: Incubate the reaction at a desired temperature for 30 to 60 minutes, protected from light. Then measure the fluorescence intensity.

4. Data analysis:

Refer to **Data Analysis** section**.**

Protocol B: Screening protease inhibitors using a purified enzyme

Brief Summary

Prepare protease substrate solution (10 µL) → Add substrate control, positive control, vehicle control or test samples (90 µL) → Incubate for 0 minute (for kinetic reading) or 30 minutes - 1 hour (for end point reading) → Monitor fluorescence intensity at Ex/Em = 490/525 nm

1. Prepare working solutions:

1.1 Make 1X assay buffer: Add 5 mL de-ionized water into 5 mL of 2X Assay Buffer (Component C).

1.2 Make protease substrate solution: Dilute Protease Substrate (Component A) at 1: 20 in 1X assay buffer (from Step 1.1). Use 10 µL/well of protease substrate solution for a 96-well plate.

©2008 by AAT Bioquest®, Inc., 520 Mercury, Sunnyvale, CA 94085. Tel: 408-733-1055
Ordering: sales@aatbio.com; Tel: 800-990-8053 or 408-733-1055; Fax: 408-733-1304
Technical Support: support@aatbio.com; Tel: 408-733-1055

*Note: The 2X assay buffer (Component C) is designed for detecting the activity of chymotrypsin, trypsin, thermolysin, proteinase K, protease XIV, and human leukocyte elastase. For other proteases, please refer to **Appendix I** for the appropriate assay buffer formula.*

1.3 Protease dilution: Dilute the protease in 1X assay buffer to a concentration of 500-1000 nM. Each well will need 10 µL of protease dilution. Prepare an appropriate amount for all the test samples and extra for the positive control and vehicle control wells.

2. Add reagents prepared in step 1 into a 96-well microplate according to Table 1 and Table 2.

Table 1. Layout of the samples in a 96-well microplate

SC	SC	….	….								
PC	PC	….	….								
VC	VC										
TS	TS										
….	….										

Note1: SC=Substrate Control, PC= Positive Control, VC=Vehicle Control, TS=Test Samples.
Note 2: It's recommended to test at least three different concentrations of each test compound. All the test samples should be done in duplicates or triplicates.

Table 2. Reagent composition for each well

Substrate Control	**Positive Control**	**Vehicle Control**	**Test Sample**
1X assay buffer: 90 µL	1X assay buffer: 80 µL Protease dilution: 10 µL	Vehicle*: X µL 1X assay buffer: (80-X) µL Protease dilution: 10 µL	Test compound: X µL 1X assay buffer: (80-X) µL Protease dilution: 10 µL
Total volume: 90 µL	Total volume: 90 µL	Total volume: 90 µL	Total volume: 90 µL

*Note: *For each volume of test compound added into a well, the same volume of solvent used to deliver test compound needs to be checked for the effect of vehicle on the activity of protease.*

3. Run the enzymatic reaction:

3.1 Add 10 µL of protease substrate solution (from Step 1.2) into the wells of positive control (PC), vehicle control (VC), and test sample (TS). Mix the reagents well.

3.2 Monitor the fluorescence intensity with a fluorescence plate reader at Ex/Em = 490 /525 nm.
For kinetic reading: Immediately start measuring fluorescence intensity continuously and record data every 5 minutes for 30 minutes.
For end-point reading: Incubate the reaction at a desired temperature for 30 to 60 minutes, protected from light. Then measure the fluorescence intensity.

4. Data analysis:

Refer to **Data Analysis** section**.**

Data Analysis

The fluorescence in the substrate control wells is used as a control, and is subtracted from the values for other wells with the enzymatic reactions.

- Plot data as relative fluorescence unit (RFU) versus time for each sample (as shown in **Figure 1**).
- Determine the range of initial time points during which the reaction is linear. 10-15% conversion appears to be the optimal range.
- Obtain the initial reaction velocity (Vo) in RFU/min. Determine the slope of the linear portion of the data plot.

- A variety of data analyses can be done, e.g., determining inhibition %, IC_{50}, K_m, K_i, etc.

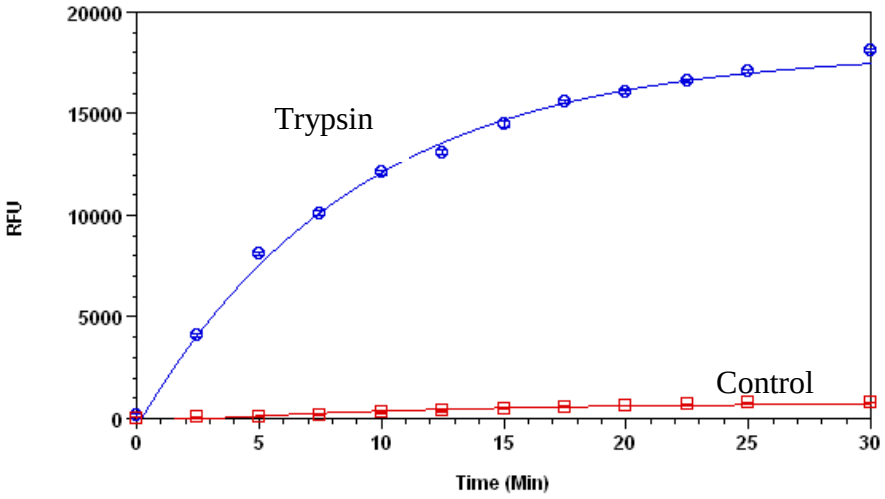

Figure 1. Trypsin protease activity was analyzed by using Amplite™ Universal Fluorimetric kit. Protease substrate was incubated with 1 unit trypsin in the kit assay buffer. The control wells had protease substrate only (without trypsin). The fluorescence signal was measured starting from time 0 when trypsin was added using a NOVOStar microplate reader (BMG Labtech) with a filter set of Ex/Em = 490/525 nm. Samples were done in triplicates.

Appendix I

Protease	**1X Assay Buffer***
Cathepsin D	20 mM Sodium Citrate, pH 3.0
Papain	20 mM sodium acetate, 20 mM cysteine, 2 mM EDTA, pH 6.5
PAE	20 mM sodium phosphate, pH 8.0
Pepsin	10 mM HCl, pH 2.0
Porcine pancreas elastase	10 mM Tris-HCl, pH 8.8
Subtilisin	20 mM potassium phosphate buffer, pH 7.6, 150 mM NaCl

Note: For protocol A, 2X assay buffer is needed. For protocol B, 1X assay buffer is needed.*

References

1. Vineyard D, Zhang X, Lee I. (2006) Transient kinetic experiments demonstrate the existence of a unique catalytic enzyme form in the peptide-stimulated ATPase mechanism of Escherichia coli Lon protease. Biochemistry, 45, 11432.
2. Yadav SC, Pande M, Jagannadham MV. (2006) Highly stable glycosylated serine protease from the medicinal plant Euphorbia milii. Phytochemistry, 67, 1414.
3. Frohbieter KA, Ismail B, Nielsen SS, Hayes KD. (2005) Effects of Pseudomonas fluorescens M3/6 bacterial protease on plasmin system and plasminogen activation. J Dairy Sci, 88, 3392.
4. Chauhan V, Sheikh AM, Chauhan A, Spivack WD, Fenko MD, Malik MN. (2005) Fibrillar amyloid beta-protein inhibits the activity of high molecular weight brain protease and trypsin. J Alzheimers Dis, 7, 37.
5. Lee EH, Kim CS, Cho JB, Ahn KJ, Kim KH. (2003) Measurement of protease activity of live Uronema marinun (Ciliata: Scuticociliatida) by fluorescence polarization. Dis Aquat Organ, 54, 85.
6. Cilenti L, Lee Y, Hess S, Srinivasula S, Park KM, Junqueira D, Davis H, Bonventre JV, Alnemri ES, Zervos AS. (2003) Characterization of a novel and specific inhibitor for the pro-apoptotic protease Omi/HtrA2. J Biol Chem, 278, 11489.

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.