

---

# Amplite™ Universal Fluorimetric Protease Activity Assay Kit

## *\*Red Fluorescence\**

Ordering Information	Storage Conditions	Instrument Platform
Product Number: 13501 (500 assays)	Store at -20 °C and keep from light Components C and D 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 new drug development targets.

Our Amplite™ Universal Fluorimetric Protease Activity Assay Kit is an ideal choice to perform routine protease assays for the isolation of proteases, or for identifying the presence of contaminating proteases in samples. The kit uses a red fluorescent casein conjugate that 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 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. Its signal can be easily read at Ex/Em = 540 /590 nm. This kit has been used for screening protease inhibitors in a HTS mode.

### **Kit Key Features**

<b>Convenient Format:</b>	Include all the key assay components.
<b>Optimized Performance:</b>	Optimal conditions for the detection of generic protease activity.
<b>Continuous:</b>	Easily adapted to automation without a separation step.
<b>Convenient:</b>	Formulated to have minimal hands-on time.

### 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 = 540/590 nm**

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

---

### 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  $\mu\text{L}$ /well of protease substrate solution for 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/ $\mu\text{L}$ , Component B) at 1:50 in de-ionized water to get a concentration of 0.1 U/ $\mu\text{L}$ .

### 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 $\mu\text{L}$	Trypsin dilution: 50 $\mu\text{L}$	Protease-containing samples: 50 $\mu\text{L}$
Total volume: 50 $\mu\text{L}$	Total volume: 50 $\mu\text{L}$	Total volume: 50 $\mu\text{L}$

*Note: If less than 50  $\mu\text{L}$  of protease-containing biological sample is used, add ddH<sub>2</sub>O to make a total volume of 50  $\mu\text{L}$ .*

### 3. Run the enzymatic reaction:

3.1 Add 50  $\mu\text{L}$  of protease substrate solution (from Step 1.1) into each well of the assay plate. Mix the reagents well.

3.2 Monitor the fluorescence increase with a fluorescence plate reader at Ex/Em = 540/590 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  $\mu\text{L}$ ) → Add substrate control, positive control, vehicle control or test samples (90  $\mu\text{L}$ ) → Incubate for 0 minute (for kinetic reading) or 30 minutes -1 hour (for end point reading) → Monitor fluorescence intensity at Ex/Em = 540/590 nm

### 1. Prepare working solutions:

1.1 Make 1X assay buffer: Add 5 mL of de-ionized water to 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  $\mu\text{L}$ /well of protease substrate solution for 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.

- 1.3 **Protease dilution:** Dilute the protease in 1X assay buffer to a concentration of 500-1000 nM. Each well will need 10  $\mu$ L of protease diluent. 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										
....	....										

Note 1: SC=Substrate Control, PC= Positive Control, VC=Vehicle Control, TS=Test Samples.

Note 2: It is 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 $\mu$ L	1X assay buffer: 80 $\mu$ L Protease dilution: 10 $\mu$ L	Vehicle*: X $\mu$ L 1X assay buffer: (80-X) $\mu$ L Protease dilution: 10 $\mu$ L	Test compound: X $\mu$ L 1X assay buffer: (80-X) $\mu$ L Protease dilution: 10 $\mu$ L
Total volume: 90 $\mu$ L	Total volume: 90 $\mu$ L	Total volume: 90 $\mu$ L	Total volume: 90 $\mu$ L

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  $\mu$ L of protease substrate solution to the positive control (PC), vehicle control (VC), and test sample (TS) wells. Mix the reagents well.
- 3.2 Monitor the fluorescence intensity with a fluorescence plate reader at Ex/Em = 540/590 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.

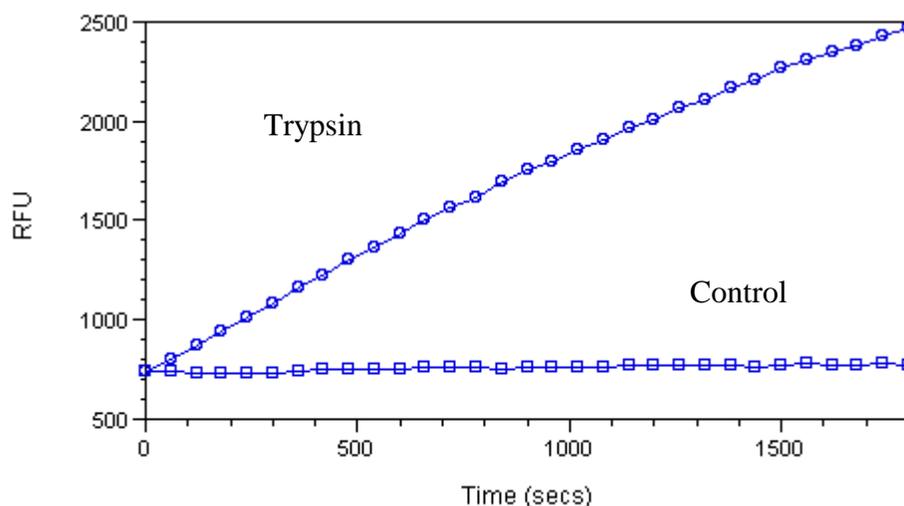
**4. Data analysis:**

Refer to **Data Analysis** section.

**Data Analysis**

The fluorescence in the substrate control wells are used as a control, and subtracted from the values of sample wells with the enzymatic reactions.

- Plot data in the format of 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 ( $V_0$ ) in RFU/min. Determine the slope of the linear portion of the data plot.
- A variety of data analyses can be run, e.g., inhibition percentage,  $IC_{50}$ ,  $K_m$ ,  $K_i$ , etc.



**Figure 1.** Trypsin protease activity was analyzed by using Amplite™ Universal Fluorimetric Protease Activity Assay Kit. Protease substrate was incubated with 3 units of 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 Gemini fluorescence microplate reader (Molecular Devices ) with a filter set of Ex/Em = 540/590 nm. Samples were done in triplicate.

## 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 marinum (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.