ab112152

Protease Activity Assay Kit (Fluorometric - Green)

Instructions for Use

For detecting Protease activity in biological samples or to screen protease inhibitors using our proprietary green fluorescence probe

This product is for research use only and is not intended for diagnostic use.

Table of Contents

1. 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.

ab112152 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. ab112152 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.

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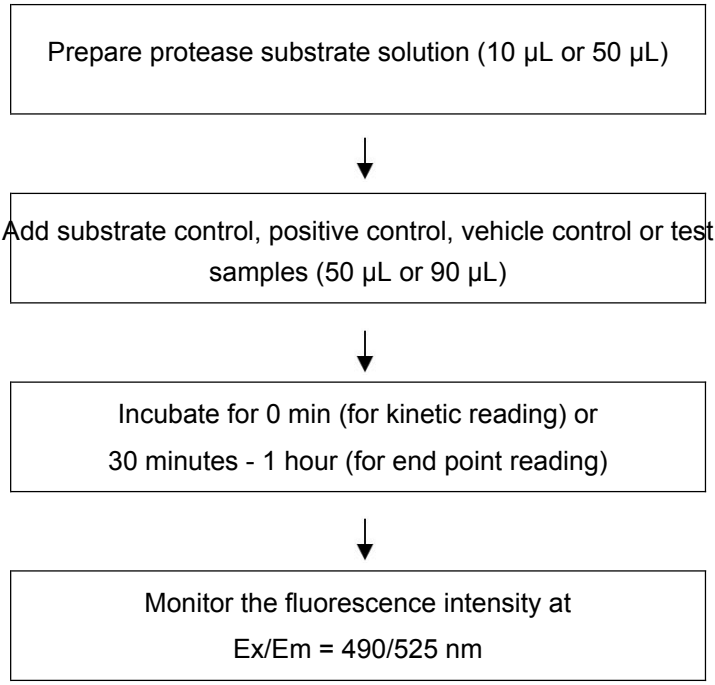
Kit Key Features

- **Convenient Format**: Include all the key assay components
- **Optimized Performance:** Optimized 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.

2. Protocol Summary

Summary for One 96-well Plate (see each individual protocol for full details)



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

3. Kit Contents

Components	Amount
Component A: Protease Substrate (Light Sensitive)	300 µL
Component B: Trypsin 5U/ µL	100 µL
Component C: 2X Assay Buffer	30 mL

4. Storage and Handling

Store at -20 °C and keep from light.

Component C can be stored at 4 °C.

5. Assay Protocol

Note: This protocol is for one 96 - well plate.

Please choose Protocol I or II according to your needs.

Protocol I: Measurement of Protease Activity in Samples

A. Preparation of Working Solutions:

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.

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.

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

SC	SC	.....	.....
PC	PC	....	....
TS	TS	....	....
.....	.....		
.....	.....		
.....	.....		
.....	.....		
.....	.....		

Table 1. Layout of the substrate control, positive control, and test samples in a 96-well microplate. *Note: SC=Substrate Control, PC =Positive Control, TS=Test Samples.*

Identifier	Contents	Volume
Substrate Control	De-ionized water:	50 µL
Positive Control	Trypsin dilution	50 µL
Test Sample	Protease-containing samples	50 µL

Table 2. Reagent composition for each well.

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

C. Run the Enzyme Assay

1. Add 50 µL of protease substrate solution (from Step A.1) to all the wells in the assay plate. Mix the reagents well

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.

D. Data Analysis

Refer to the Data Analysis section.

Protocol II: Screening Protease Inhibitors (Purified Enzyme)

A. Preparation of Working Solutions:

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

2. Make protease substrate solution: Dilute Protease Substrate (Component A) at 1: 20 in 1X assay buffer (from Step A.1). Use 10 µL/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

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

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

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

Table 1. Layout of appropriate controls (as desired) and test samples in a 96-well microplate.

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.

Identifier	Contents	Total Volume
Substrate Control	1XAssay Buffer	90 µL
Positive Control	1X assay buffer: 80 µL Protease dilution: 10 µL	90 µL
Vehicle Control	Vehicle*: X µL 1X assay buffer: (80-X) µL Protease dilution: 10 µL	90 µL
Test Sample	Test compound: X µL 1X assay buffer: (80-X) µL Protease dilution: 10 µL	90 µL

Table 2. Reagent composition for each well.

*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.*

C. Run the Enzyme Reaction:

1. Add 10 µL of protease substrate solution (from Step A.2) into the wells of positive control (PC), vehicle control (VC), and test sample (TS). Mix the reagents well.

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

D. Data Analysis

Refer to the Data Analysis section.

6. 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.

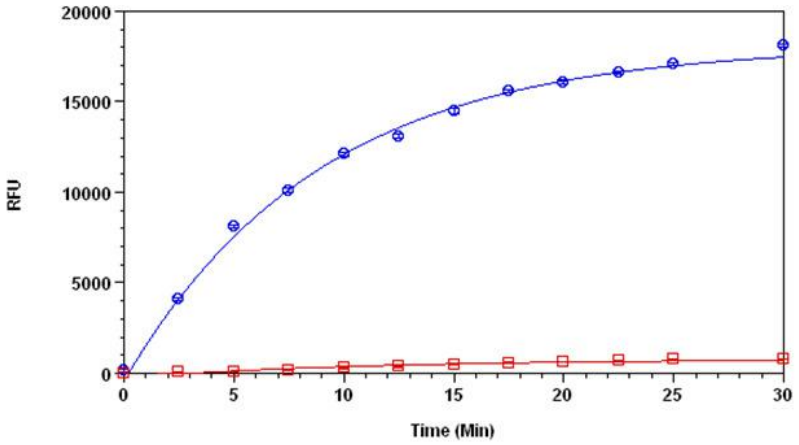


Figure 1. Trypsin protease activity was analyzed using ab112152. Protease substrate was incubated with 1 unit trypsin in the kit assay buffer. The control wells had protease substrate only (without trypsin). The fluorescence signal was measured starting from time 0 when trypsin was added using a microplate reader with a filter set of Ex/Em = 490/525 nm. Samples were done in triplicates.

7. 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 I, 2X assay buffer is needed. For Protocol II, 1X assay buffer is needed.*

8. Troubleshooting

Problem	Reason	Solution
Assay not working	Assay buffer at wrong temperature	Assay buffer must not be chilled - needs to be at RT
	Protocol step missed	Re-read and follow the protocol exactly
	Plate read at incorrect wavelength	Ensure you are using appropriate reader and filter settings (refer to datasheet)
	Unsuitable microtiter plate for assay	Fluorescence: Black plates (clear bottoms); Luminescence: White plates; Colorimetry: Clear plates. If critical, datasheet will indicate whether to use flat- or U-shaped wells
Unexpected results	Measured at wrong wavelength	Use appropriate reader and filter settings described in datasheet
	Samples contain impeding substances	Troubleshoot and also consider deproteinizing samples
	Unsuitable sample type	Use recommended samples types as listed on the datasheet
	Sample readings are outside linear range	Concentrate/ dilute samples to be in linear range

Problem	Reason	Solution
Samples with inconsistent readings	Unsuitable sample type	Refer to datasheet for details about incompatible samples
	Samples prepared in the wrong buffer	Use the assay buffer provided (or refer to datasheet for instructions)
	Samples not deproteinized (if indicated on datasheet)	Use the ***10kDa spin column (ab93349)*** or ***Deproteinizing sample preparation kit (ab93299)***
	Cell/ tissue samples not sufficiently homogenized	Increase sonication time/ number of strokes with the Dounce homogenizer
	Too many freeze-thaw cycles	Aliquot samples to reduce the number of freeze-thaw cycles
	Samples contain impeding substances	Troubleshoot and also consider deproteinizing samples
	Samples are too old or incorrectly stored	Use freshly made samples and store at recommended temperature until use
Lower/ Higher readings in samples and standards	Not fully thawed kit components	Wait for components to thaw completely and gently mix prior use
	Out-of-date kit or incorrectly stored reagents	Always check expiry date and store kit components as recommended on the datasheet
	Reagents sitting for extended periods on ice	Try to prepare a fresh reaction mix prior to each use
	Incorrect incubation time/ temperature	Optimize the incubation time and/or temperature
	Incorrect amounts used	Check pipette is calibrated correctly (always use smallest volume pipette that can pipette entire volume)

Standard curve is not linear	Not fully thawed kit components	Wait for components to thaw completely and gently mix prior use
	Pipetting errors when setting up the standard curve	Try not to pipette too small volumes
	Incorrect pipetting when preparing the reaction mix	Always prepare a master mix
	Air bubbles in wells	Air bubbles will interfere with readings; try to avoid producing air bubbles and always remove bubbles prior to reading plates
	Concentration of standard stock incorrect	Recheck datasheet for recommended concentrations of standard stocks
	Errors in standard curve calculations	Refer to datasheet and re-check the calculations
	Use of other reagents than those provided with the kit	Use fresh components from the same kit

Technical Support

For all technical or commercial enquiries please go to:
www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)