



## SensoLyte® Green Protease Assay Kit *\*Fluorimetric\**

<b>Catalog #</b>	<b>71124</b>
<b>Unit Size</b>	1 Kit
<b>Kit Size</b>	500 Assays

This kit is optimized to detect generic protease activities (e.g. trypsin, chymotrypsin, thermolysin, proteinase K, protease XIV, and elastase) using casein that is heavily labeled with HiLyte Fluor™ 488, a pH-insensitive green fluorophore. It provides ample materials to perform 500 assays in a 96-well format. The protocol can be readily modified to run assays in a 384-well format. The kit has the following features:

- **Convenient Format:** Complete kit including all the assay components.
- **Optimized Performance:** Optimal conditions for the detection of generic protease activity.
- **Enhanced Value:** Less expensive than the sum of individual components.
- **High Speed:** Minimal hands-on time.
- **Assured Reliability:** Detailed protocol and references are provided.

### USA and Canada Ordering Information

#### **AnaSpec Corporate Headquarter**

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Toll-Free: 800-452-5530  
Tel: 408-452-5055  
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E-mail: [service@anaspec.com](mailto:service@anaspec.com)  
Internet: [www.anaspec.com](http://www.anaspec.com)

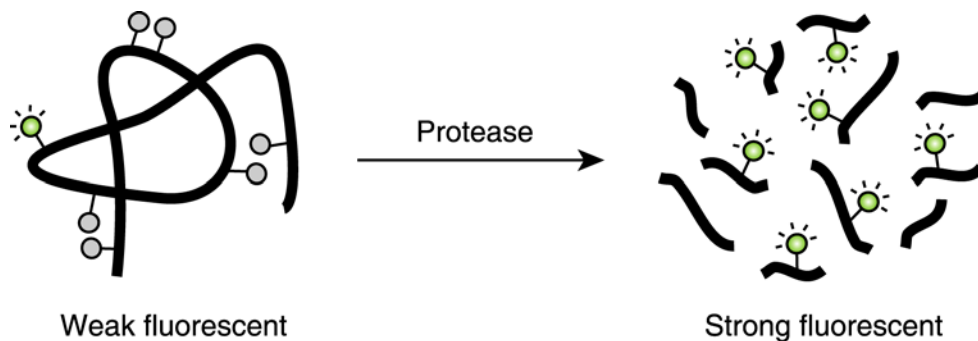
### International Ordering Information

A list of international distributors is available at [www.anaspec.com](http://www.anaspec.com).

## **INTRODUCTION**

Protease assay is widely used in the investigation of protease inhibitors and detection of protease activity in samples for quality inspection purpose.<sup>1-3</sup>

The SensoLyte® Green Protease Assay Kit uses casein that is heavily labeled with HiLyte Fluor™ 488, a pH-insensitive green fluorophore, resulting in almost total quenching of its fluorescence. Proteolytic cleavage of this quenched casein-HiLyte Fluor™ 488 conjugate yields brightly green fluorescence, which can be continuously monitored at excitation/emission= 488 nm/520 nm (see **Scheme 1**). The increase in fluorescence intensity is directly proportional to protease activity. Casein is the major protein of bovine milk, which is more similar to physiological substrates for proteases than synthetic peptides. This kit does not require any separation steps and can be used to continuously measure the kinetics of a variety of exopeptidases and endopeptidases in acidic and basic buffer. The assays are performed in a convenient 96-well microplate format. 384-well or 1536-well format can be used as well with minor modifications.



**Scheme 1.** Proteolytic cleavage of HiLyte Fluor™ 488-labeled casein.

## **KIT COMPONENTS, STORAGE AND HANDLING**

*Note: Store Components A and B at -20 °C, and keep A from light. Components C and D can be stored at room temperature for convenience.*

**Component A:** Protease substrate (280 µL)  
HiLyte Fluor™ 488 -labeled casein; Ex/Em=488 nm/520 nm upon cleavage

**Component B:** Trypsin (5 U/µL, 100 µL)

**Component C:** 2X Assay buffer (30 mL)

### ***OTHER MATERIALS REQUIRED (BUT NOT PROVIDED)***

**96-well microplate:** Black microplate provides better signal/noise value.

**Fluorescence microplate reader:** Capable of excitation at 488 nm and detecting emission at 520 nm.

## **PROTOCOL**

*Note 1: Warm up all the kit components until thawed at room temperature before starting the experiments.*

*Note 2: Please choose Protocol A or B based on your needs.*

### **Protocol A. Measuring protease activity in test samples.**

#### **1. Prepare working solutions.**

- **Protease substrate solution:** Dilute protease substrate (Component A) 1:100 in 2X assay buffer (Component C). 50  $\mu\text{L}$  of protease substrate solution per assay in a 96-well plate is required.

*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.*

- **Trypsin diluent:** Dilute trypsin (5 U/ $\mu\text{L}$ , Component B) 50 fold 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 samples in a 96-well microplate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	SC	SC										
B	PC	PC										
C	TS	TS										
D	...	...										
E												
F												
G												
H												

*Note: SC=substrate control, PC=positive control, TS=test sample.*

**Table 2.** Reagent composition for each well.

Substrate Control		Positive Control		Test Sample	
De-ionized water	50 $\mu\text{L}$	Trypsin diluent	50 $\mu\text{L}$	Protease-containing sample	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 bring volume up 50  $\mu\text{L}$ .*

#### **3. Initiate the enzymatic reaction.**

- Add 50  $\mu\text{L}$  protease substrate solution to all the wells in the assay plate. Mix the reagents by shaking the plate gently for 30 seconds.
- Measure fluorescence signal:
  - For kinetic reading:** Immediately start measuring fluorescence intensity at Ex/Em=488 nm/520 nm continuously and record data every 5 minutes for 30 minutes.
  - For end-point reading:** Incubate the reaction at the desired temperature for 30 to 60 minutes, and keep from light. Measure fluorescence intensity at Ex/Em=488 nm/520 nm.

#### **4. Data analysis:** Refer to the **Data Analysis** section.

## **Protocol B. Screening protease inhibitor using a purified enzyme.**

### **1. Prepare working solutions.**

- **1X assay buffer:** Add 5 mL de-ionized water to 5 mL of 2X assay buffer (Component C).
- **Protease substrate solution:** Dilute protease substrate (Component A) 1:20 in 1X assay buffer. You will need 10 µL of protease substrate solution per assay in 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.*
- **Protease diluent:** Dilute the protease in 1X assay buffer to a concentration of 500-1000 nM. Each well will need 10 µL of protease diluent. Prepare an appropriate amount for all your test samples, positive control and vehicle control wells.

### **2. Add reagents prepared in Step 1 into a 96-well microplate according to Tables 1 and 2.**

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	SC	SC	TS	TS	...	...						
B	PC	PC	...	...								
C	VC	VC										
D												
E												
F												
G												
H												

*Note 1:* SC=substrate control, PC=positive control, VC=vehicle control, TS=test sample.

*Note 2:* We recommend testing at least three different concentrations of each test compound. All the samples should be done in duplicates or triplicates.

**Table 2.** Reagent composition for each well.

<b>Substrate Control</b>		<b>Positive Control</b>	
1X assay buffer	90 µL	1X assay buffer	80 µL
		Protease diluent	10 µL
Total volume	90 µL	Total volume	90 µL
<b>Vehicle Control</b>		<b>Test Sample</b>	
Vehicle*	X µL	Test compound	X µL
1X assay buffer	(80-X) µL	1X assay buffer	(80-X) µL
Protease diluent	10 µL	Protease diluent	10 µL
Total volume	90 µL	Total volume	90 µL

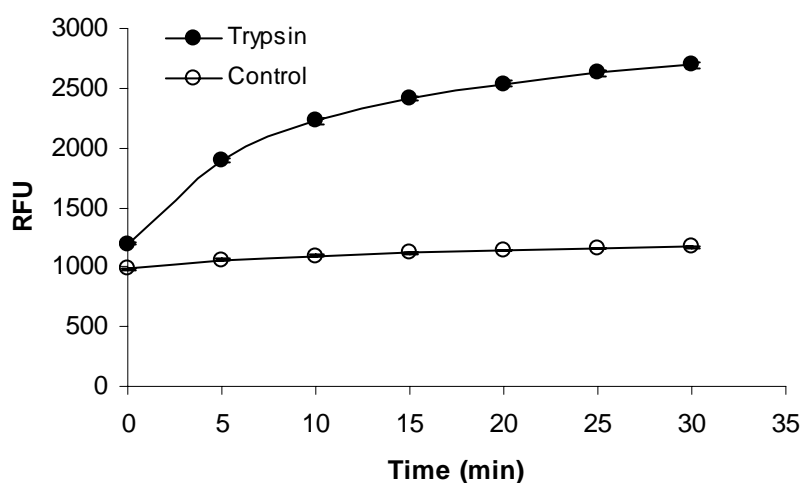
*Note:* \*For each volume of test compound added into a well, the same volume of solvent used to deliver test compound needs to be checked for the effect of vehicle on the activity of protease.

### **3. Initiate the enzymatic reaction.**

- Add 10 µL protease substrate solution to the positive control (PC), vehicle control (VC), and test sample (TS) wells. Mix the reagents well by shaking the plate gently for 30 sec.
- Measure fluorescence signal:  
**For kinetics reading:** Immediately start measuring fluorescence intensity at Ex/Em=488 nm/520 nm continuously and record data every 3 minutes for 30 minutes.  
**For end-point reading:** Incubate reaction at the desired temperature for 30 to 60 minutes, and keep from light. Then measure fluorescence intensity at Ex/Em=488 nm/520 nm.
- Data analysis: Refer to **Data Analysis** section.

## Data Analysis

- The fluorescence reading from the substrate control well is the background fluorescence. The readings from other wells need to be subtracted with this background fluorescence.
- Plot data as relative fluorescence unit (RFU) versus time for each sample (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 done, e.g., determining inhibition %,  $IC_{50}$ ,  $K_m$ ,  $K_i$ , etc.



**Figure 1.** Proteolytic cleavage of HiLyte Fluor<sup>TM</sup> 488-labeled casein by trypsin. HiLyte Fluor<sup>TM</sup> 488-labeled casein was cleaved by 1 unit trypsin in assay buffer. The control wells had HiLyte Fluor<sup>TM</sup> 488-labeled casein, but without trypsin. Fluorescence signal was measured starting from Time 0, when trypsin was added, by a fluorescence microplate reader (Flexstation 384II, Molecular Devices) with a filter set of Ex/Em=485±20 nm/528±20 nm. Samples were done in duplicates.

## **Appendix I**

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

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\* For Protocol A, 2X assay buffer is needed. For Protocol B, 1X assay buffer is needed.

## **REFERENCES**

1. Wiesner, R. and W. Troll, Anal. Biochem. 121, 290 (1982).
2. Sevier, ED., Anal.Biochem. 74, 592 (1976).
3. Spencer, PW. et al. Anal.Biochem. 64, 556 (1975).