



SensoLyte[®] Green Protease Assay Kit

Fluorimetric

Revision Number:1.1	Last Revised: October 2014
Catalog #	AS-71124
Kit Size	500 Assays (96-well plate)

- **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.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	Protease substrate: HiLyte Fluor [™] 488-labeled casein. Ex/Em=488 nm/520 nm upon cleavage	280 μ L
Component B	Trypsin	5 U/ μ L, 100 μ L
Component C	2X Assay buffer	30 mL

Other Materials Required (but not provided)

- 96-well microplate: Black, flat bottom 96-well plate with non-binding surface.
- Fluorescence microplate reader: Capable of excitation at 488 nm and detecting emission at 520 nm.

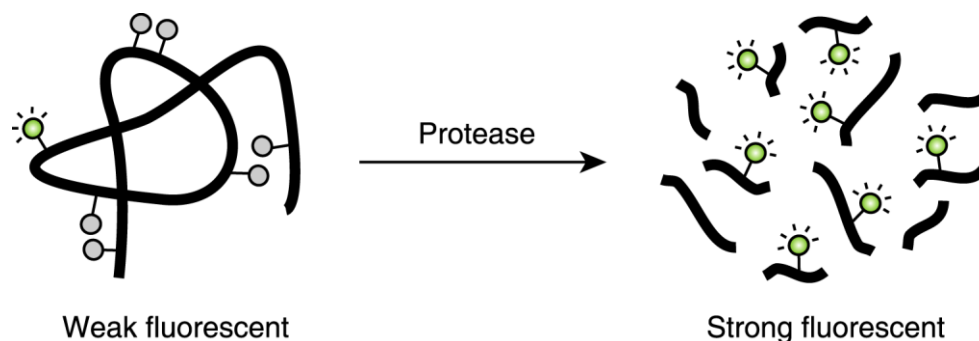
Storage and Handling

- Store Components A and B at -20°C
- Protect Component A from light and moisture
- Component C can be stored at room temperature for convenience

Introduction

Protease assay is widely used in the investigation of protease inhibitors and detection of protease activity in samples for quality inspection purpose.¹⁻³

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.

Protocol

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

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

Protocol A. Screening protease inhibitor using a purified enzyme.

1. Prepare working solutions.

- 1.1 1X assay buffer: Add 5 mL de-ionized water to 5 mL of 2X assay buffer (Component C).
- 1.2 Protease substrate solution: Dilute protease substrate (Component A) 100-fold in 1X assay buffer. You will need 50 μ L of protease substrate solution per assay in 96-well plate Prepare it according to **Table 1**.

Table 1. Protease substrate solution for one 96-well plate (100 assay).

Components	Volume
Protease substrate (20X, Component A)	50 μ L
1X Assay buffer	4.95 mL
Total volume	5 mL

Note 1: 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 proteases other than above, please refer to **Appendix** for appropriate assay buffer formula.

Note 2: Dilute substrate according to the amount needed. Save the rest of the 20X substrate stock solution for future experiments.

1.3 Trypsin diluents: Dilute trypsin (Component B) 40-fold in 1X assay buffer. This amount of enzyme is enough for a full 96-well plate. If not using the entire plate, adjust the amount of enzyme to be diluted accordingly.

2. Set up the enzymatic reaction.

2.1 Add test compounds and diluted protease solution to the microplate wells. For one well of a 96-well plate, the suggested volume of trypsin solution is 40 μ L and 10 μ L of test compound.

2.1 Simultaneously establish the following control wells, as deemed necessary:

- Positive control contains the diluted trypsin without test compound.
- Vehicle control contains trypsin and vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control contains 1X assay buffer and test compound. Some test compounds have strong autofluorescence and may give false results.
- Substrate control contains 1X assay buffer.

2.3 Using the assay buffer bring the total volume of all controls to 50 μ L.

3. Initiate the enzymatic reaction.

3.1 Add 50 μ L protease substrate solution to the positive control, vehicle control, test sample wells, and substrate control wells. Mix the reagents well by shaking the plate gently for 30 sec.

3.2 Measure fluorescence signal:

For kinetics reading: Immediately start measuring fluorescence intensity at Ex/Em=490 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=490 nm/520 nm.

3.3 Data analysis: Refer to **Data Analysis** section.

Protocol B. Measuring protease activity in test samples.

1. Prepare working solutions.

- 1.1** Protease substrate solution: Dilute protease substrate (Component A) 100-fold in 2X assay buffer (Component C) according to Table 1. Prepare fresh substrate solution for each experiment.

Table 1. Protease substrate solution for one 96-well plate (100 assays).

Components	Volume
Protease substrate (100 X, Component A)	50 μ L
2X assay buffer	4.95 mL
Total volume	5 mL

Note 1: 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.

Note 2: Dilute substrate according to the amount needed. Save the rest of the 100x substrate stock solution for future experiments.

- 1.2** Trypsin diluents: If using trypsin as a positive control, dilute the enzyme (Component B) 50-fold in deionized water. Add 50 μ l of the diluted enzyme into each of the positive control well.

Note: Prepare trypsin diluents immediately before use. Do not vortex the trypsin solutions. Prolonged storage or vigorous agitation of the diluted trypsin will cause denaturation. Store the trypsin solution on ice.

2. Set up the protease assay.

- 2.1** Add 50 μ l of test sample into microplate wells (96-well plate).

Note: Use Deionized water to dilute the samples

- 2.2** Simultaneously establish the following control wells

- Positive control contains trypsin diluents
- Substrate control contains de-ionized water

Note1: Bring the total volume of all controls to 50 μ l/well for a 96-well plate.

Note 2: If less than 50 μ L of protease-containing biological sample is used, add dH₂O to bring volume up to 50 μ L.

3. Initiate the enzymatic reaction.

- 3.1** Add 50 μ L of protease substrate solution to all the wells in the assay plate. Mix the reagents by shaking the plate gently for 30 seconds.

- 3.2** Measure fluorescence signal:

For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=490 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=490 nm/520 nm.

- 3.3** Data analysis: Refer to the **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.

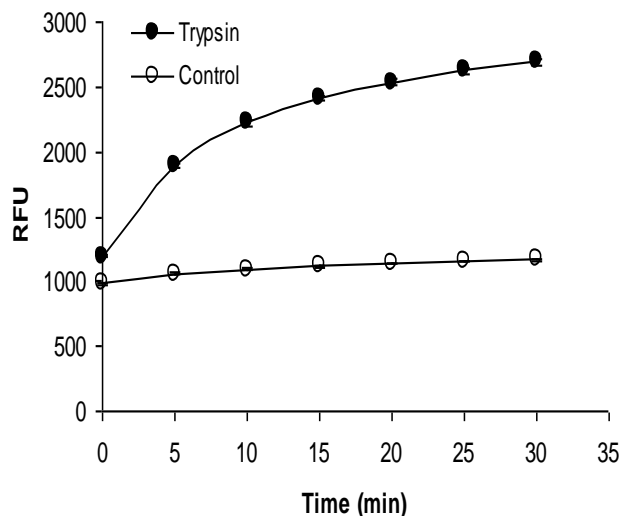


Figure 1. Proteolytic cleavage of HiLyte Fluor™ 488-labeled casein by trypsin. Fluorescence signal was measured by fluorescence microplate reader (Flexstation 384II, Molecular Devices) with Ex/Em=490 nm/520 nm.

Appendix I

Protease	1X Assay Buffer*
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

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

References

1. Wiesner, R. et al. *Anal Biochem* **121**, 290 (1982).
2. Sevier, E. D. et al. *Anal Biochem* **74**, 592 (1976).
3. Spencer, PW. et al. *Anal Biochem* **64**, 556 (1975).