SensoLyte® Red Protease Assay Kit
Fluorimetric

Revision Number: 1.1	*Last updated: October 2014*
Catalog #	**AS-71140**
Kit Size	500 Assays

- ***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: 5(6)-TAMRA -labeled casein. Ex/Em=546 nm/575 nm upon cleavage	280 µL
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, flat bottom 96-well plate with non-binding surface.
- Fluorescence microplate reader: Capable of excitation at 546 nm and detecting emission at 575 nm.

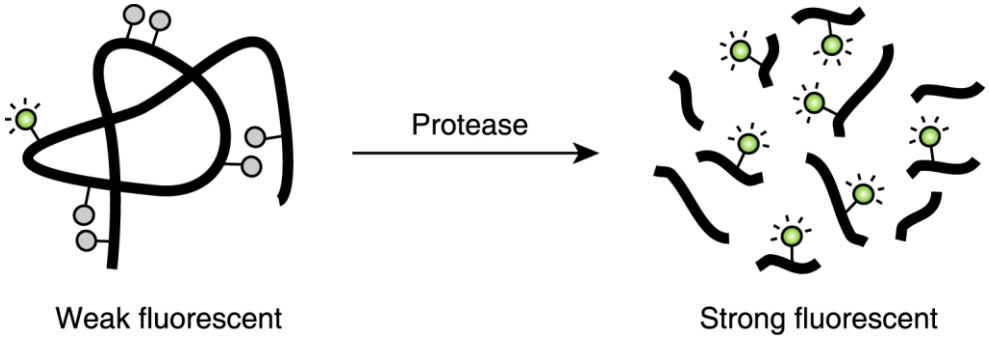
Storage and Handling

- 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[1-3] is widely used for the investigation of protease inhibitors and detection of protease activity in samples for quality inspection purpose.

The SensoLyte® Red Protease Assay Kit uses casein that is heavily labeled with 5(6)-TAMRA, a pH-insensitive red fluorophore, resulting in almost total quenching of its fluorescence. Proteolytic cleavage of this quenched casein-5 (6)-TAMRA conjugate yields brightly red fluorescence, which can be continuously monitored at excitation/emission= 546 nm/575 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. The SensoLyte® Red Protease Assay 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. The 384-well or 1536-well format can be used as well with minor modifications.



Scheme 1. Proteolytic cleavage of 5(6)-TAMRA™ -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. Screen protease inhibitor using purified enzyme.

1. **Prepare working solutions.**
   1.1 1X assay buffer: Add 5 mL of 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 μL 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 μL
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 100X 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 μL and 10 μL 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 90 μL.

3. Initiate the enzymatic reaction.

3.1 Add 50 μL protease substrate solution to positive control, vehicle control, test compound control, and substrate control wells. Mix the reagents well by shaking the plate gently for 30 seconds.

3.2 Measure fluorescence signal:

For kinetic reading: Immediately start to measure fluorescence intensity at Ex/Em=546 nm/575 nm continuously and record the data every 3 minutes for 30 to 60 minutes.

For end-point reading: Incubate the reaction at the desired temperature for 30 to 60 minutes, and keep from light. Then measure fluorescence intensity at Ex/Em=546 nm/575 nm.

3.3 Data analysis: refer to Data Analysis section.

Protocol B. Measure protease activity in test samples.

1. Prepare working solutions.

1.1 Protease substrate solution: Dilute protease substrate 100-fold in 2X assay buffer (Component C). 50 μL of protease substrate solution per assay in 96-well plate is required. Prepare it according to **Table 1**.

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

Components	Volume
Protease substrate (100 X, Component A)	50 µL
2X Assay buffer (Component C)	4.95
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 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 µl 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 µl 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 diluent
- Substrate control contains deionized water

Note 1: Bring the total volume of all controls to 50 µl/well for a 96-well plate.
Note 2: If less than 50 µL of protease-containing biological sample is used, add dH_2O to bring volume up 50µL.

3. Initiate the enzymatic reaction.

3.1 Add 50 µL 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 to measure fluorescence intensity at Ex/Em=546 nm/575 nm continuously and record the data every 5 minutes for 30 to 60 minutes.

For end-point reading: Incubate the reaction at the desired temperature for 30 to 60 minutes, and keep from light. Then measure fluorescence intensity at Ex/Em=546 nm/575 nm.

3.3 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) in RFU/min. Determine the slope of a line fit to the linear portion of the data plot using an appropriate method.
- A variety of data analysis can be done, e.g., determining inhibition %, IC_{50}, K_m, K_i, etc.

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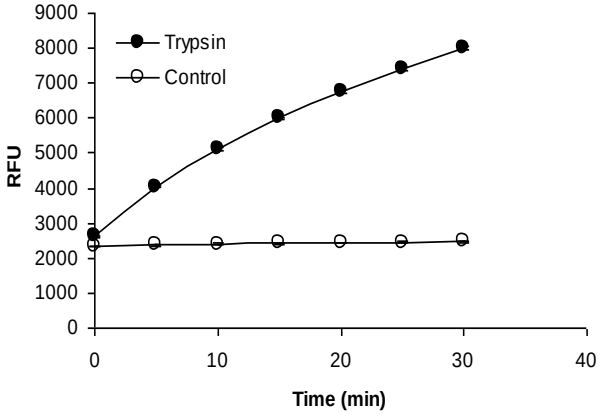

Figure 1. Proteolytic cleavage of 5(6)-TAMRA-labeled casein by trypsin. The fluorescence signal was measured by fluorescence microplate reader (Flex station 384II, Molecular Devices) with Ex/Em=546 nm/575 nm.

Appendix

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, you need to prepare 1X assay buffer. For protocol B, you need to prepare 2X assay buffer.

References

1. Wiesner, R. and W. Troll, *Anal.Biochem.* **121**, 290-294 (1982).
2. Sevier, ED. *Anal.Biochem.* **74**, 592-596 (1976).
3. Spencer, PW. et al. *Anal.Biochem.* **64**, 556-566 (1975).