Product Information

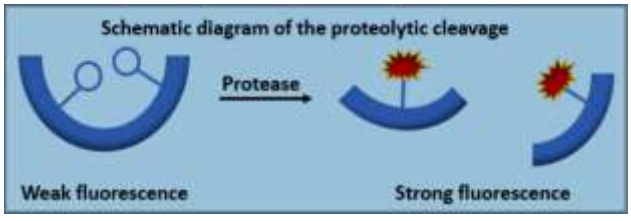
Red Protease Detection Kit

CS0013

Product Description

Proteases play significant roles in regulating important physiological processes[1]. This has led to the development of various methods to test for the presence of proteases, to screen protease inhibitors as potential drug molecules, as well as evaluation of general protease contamination in various samples[2-4].

The Red Protease Detection Kit provides a simple and rapid method for protease detection. In this kit, proteolysis is measured with the use of 5(6)-tetramethylrhodamine (TAMRA)-labeled casein as a substrate. When the TAMRA-labeled casein substrate is intact, its fluorescence is almost totally quenched. Upon proteolytic cleavage, fluorescence of the TAMRA-labeled peptide fragments increases proportionally to protease activity in the sample. The fluorescence of TAMRA-labeled peptide fragments is measured at λ_{ex} / λ_{em}=535 nm / 595 nm.



The TAMRA-labeled casein substrate has a pH-independent fluorescence. This makes the substrate applicable for a wide range of pH samples, as well as convenient and time-saving, since no separation steps are required. This kit allows a high signal-to-noise ratio that increases detection resolution and sensitivity.

This kit is designed for a 96-well plate format. Trypsin is provided in the kit as a reference protease for calibration.

Precautions and Disclaimer

This product is for R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Components

This kit contains sufficient reagents for 500 tests in 96-well plate format.

Component	Component Number	Amount
Trypsin (1 mg/mL)	CS0013A	100 µL
Protease Assay Buffer × 2	CS0013B	30 mL
Substrate	CS0013C	280 µL

Component Information

Trypsin (Component No. CS0013A): 1 mg/mL solution

- To avoid freeze/thaw cycles, it is recommended to prepare aliquots, and to store the aliquots at −20 °C.
- Keep on ice while in use.

Protease Assay Buffer × 2 (Component No. CS0013B):

- Upon thawing, store at 2-8 °C.
- Equilibrate to room temperature before use.

Substrate (Component No. CS0013C): Store at −20 °C.

- To avoid freeze/thaw cycles, it is recommended to prepare aliquots, and store the aliquots at −20 °C, protected from light.
- Keep on ice while in use.

Reagents and Equipment Required, But Not Provided

- 96-well black flat-bottom plate
- Fluorescence (λ_{ex} = 535 nm / λ_{em} = 595 nm) plate reader
- Ultrapure water
- If applicable, an appropriate protease standard of known specific activity (see Sample Preparation, the "Optional: Trypsin or a specific protease standard for Activity Standard Curve" section)

Storage/Stability

- The kit is shipped on dry ice. The unopened kit is stable for 2 years as supplied.
- Upon receipt, store all components at –20 °C, protected from light.
- Upon thawing, the Protease Assay Buffer × 2 should be stored at 2–8 °C.
- It is recommended to aliquot other opened unused components, and store at –20 °C.

Procedure

General Notes

- Briefly centrifuge vials before opening.
- Samples can be run either in duplicate or in triplicate.
- Trypsin can serve as a general standard to compare overall protease activity between different samples. However, for proteases other than trypsin, optimum conditions are likely to differ. To measure accurately such activity of a specific protease different from trypsin, use known amounts of that protease for the standard curve.
- For simple detection of protease activity or contamination, a standard curve may not be required, mostly for samples that contain a single protease, or a mixture of unknown proteases.

Sample preparation

- All assays (samples, standards, and controls) require 50 µL of sample for each reaction (or well). Therefore, a sample volume of 50 µL must be reached.
- When required, samples should be diluted in ultrapure water or specific buffer.
- If the activity is unknown, several dilutions may be required to determine the optimal concentration range.

1. Trypsin (Component No. CS0013A):

If using trypsin as a qualitative positive control, dilute the Trypsin stock solution provided in the kit 100-fold to 800-fold in ultrapure water. For example:

- Step 1 (100-fold dilution): Add 10 µL trypsin stock solution to 990 µL ultrapure water, to a concentration of 10,000 ng/mL
- Optional, Step 2 (8-fold dilution): Take 50 µL from Step 1 solution into 350 µL ultrapure water, to a concentration of 1250 ng/mL
- It is recommended to prepare the dilutions just before the test is performed. Do not vortex the trypsin solution. Work with the trypsin solution on ice.

Optional: Trypsin or a specific protease standard for Activity Standard Curve

i. For quantitation of a purified protease sample, a standard curve may be generated using the Trypsin supplied in the kit, or another enzyme of choice of known specific activity, containing relevant characteristics to the protease in the tested sample.

ii. Determine the range of enzyme response. Titrate at least 5 concentrations of enzyme, including a negative control that contains only the utilized buffer. **Figure 1** shows a sample Trypsin standard curve.

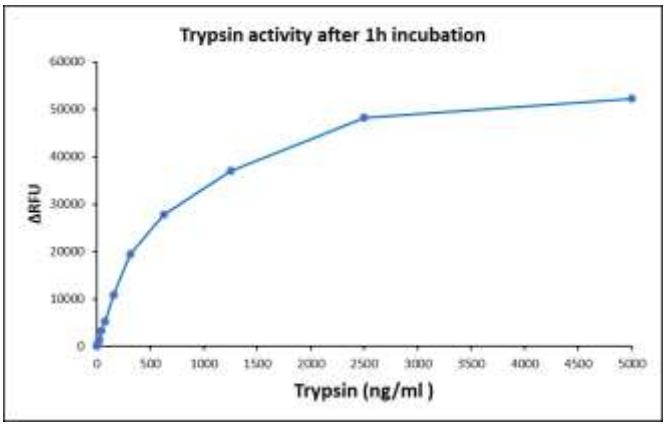


Figure 1. Trypsin standard curve preparation with the Red Protease Detection Kit (Cat. No. CS0013). The fluorescence signal was measured with a filter fluorometer at λ_{ex} / λ_{em} = 535 nm / 595 nm.

2. Protease Assay Buffer × 2 (Component No. CS0013B):

Protease Assay Buffer × 2 (pH ~7.5) provided in the kit is suitable mainly for proteases in the physiological pH range of chymotrypsin and proteinase K, for example. However, if activation compounds or other pH are required, a different specific buffer should be separately prepared.

3. Substrate (Component No. CS0013C):

Dilute the substrate stock solution provided in the kit 100-fold in Protease Assay Buffer × 2. For example:

Add 6 µL substrate into 594 µL Protease Assay Buffer × 2.

Assay Reaction

It is recommended to run samples or standards (when used) either in duplicate or in triplicate.

1. Transfer 50 µL of the sample dilutions into a 96-well plate. If less than 50 µL of the protease-containing sample is used, add ultrapure water or specific buffer to reach a total volume of 50 µL.
2. Prepare a negative control that contains only ultrapure water and/or any other diluent used for the protease-containing sample. Transfer 50 µL into a 96-well plate.
3. Prepare a qualitative positive control that contains trypsin diluted 100-fold (for final concentration of 5000 ng/mL per well) to 800-fold (for a final concentration of 625 ng/mL per well) in ultrapure water, or the appropriate enzyme that is more relevant to the protease in the tested sample. Transfer 50 µL into the 96-well plate.

   Optional:

   - Prepare a standard curve using the Trypsin supplied in the kit, or other relevant enzyme with specific activity for quantitation of purified protease in the sample.
   - Transfer 50 µL from all the standard dilutions into the 96-well plate.
4. Initiate the enzymatic reaction, by adding 50 µL of prepared substrate solution to each of the Sample and Control (positive, negative) wells.
5. Mix gently by shaking the plate for 30 seconds.
6. Measure the fluorescence intensity increase at λ_{ex} / λ_{em} = 535 nm / 595 nm:
   - For kinetic reading: Immediately start measuring fluorescence intensity continuously and record data every 5-10 minutes for 30-60 minutes, protected from light.
   - For end-point reading: Incubate the reaction at the desired temperature for 30-60 minutes, protected from light. Then measure the fluorescence intensity.

Results

Calculations

1. Average the RFU readings of each duplicate sample and each triplicate sample.
2. The negative control is the background fluorescence. Subtract the negative control value from all sample values.
3. Plot the subtracted fluorescence units (Δ RFU) versus time for each sample (see **Figure 2** as an example).
4. Determine the linear part of the plot from which different type of analysis can be generated.
5. When using a standard curve, the results can be expressed as fluorescent change (Δ RFU) vs protease concentration, such as shown in **Figure 1**.

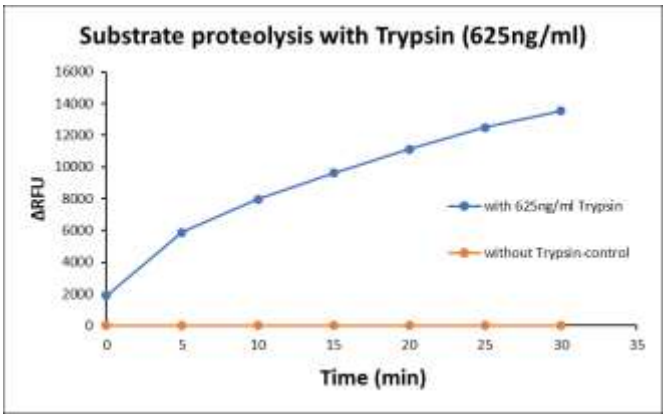


Figure 2. Trypsin protease activity was analyzed by Red Protease Detection Kit (Cat. No. CS0013). Substrate was incubated either with 625 ng/mL Trypsin, or without Trypsin as a control. The fluorescence signal was measured with a filter fluorometer at λ_{ex} / λ_{em} = 535 nm / 595 nm.

Sensitivity

Calculate the Signal/Noise ratio at different Trypsin concentrations:

Average RFU reading at "X" ng/mL of Trypsin divided by the Average RFU reading at the corresponding negative control (**Table 1**).

Table 1. Calculated Signal/Noise (S/N) ratio at different Trypsin concentrations.

Trypsin Concentration	Fold-increase of S/N after 1 hour incubation
5000 ng/mL	12.5
1250 ng/mL	9.0
625 ng/mL	7.0
156 ng/mL	3.3
78 ng/mL	2.1
39 ng/mL	1.7
19 ng/mL	1.3

References

1. Gurumallesh, P. *et al*., *Int. J. Biol. Macromol*., **128**, 254-267 (2019).
2. Twining, S. S., *Anal. Biochem*., **143(1)**, 30-34 (1984).
3. Wiesner, R., and Troll, W., *Anal. Biochem*., **121(2)**, 290-294 (1982).
4. Anson, M. L., *J. Gen. Physiol*., **22(1)**, 79-89 (1938).

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