

Video Article

Use of the Protease Fluorescent Detection Kit to Determine Protease Activity

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Abstract

The Protease Fluorescent Detection Kit provides ready-to-use reagents for detecting the presence of protease activity. This simple assay to detect protease activity uses casein labeled with fluorescein isothiocyanate (FITC) as the substrate.

Protease activity results in the cleavage of the FITC-labeled casein substrate into smaller fragments, which do not precipitate under acidic conditions. After incubation of the protease sample and substrate, the reaction is acidified with the addition of trichloroacetic acid (TCA). The mixture is then centrifuged with the undigested substrate forming a pellet and the smaller, acid soluble fragments remaining in solution. The supernatant is neutralized and the fluorescence of the FITC-labeled fragments is measured.

The described kit procedure detects the trypsin protease control at a concentration of approximately 0.5 µg/ml (5 ng of trypsin added to the assay). This sensitivity can be increased with a longer incubation time, up to 24 hours. The assay is performed in microcentrifuge tubes and procedures are provided for fluorescence detection using either cuvettes or multiwell plates.

Video Link

The video component of this article can be found at <https://www.jove.com/video/1514/>

Protocol

Preparation Instructions

The Incubation Buffer, Assay Buffer, and 0.6 N TCA Solution are provided as ready-to-use solutions.

The FITC-Casein Substrate - It is recommended to aliquot the FITC-Casein Substrate into smaller volumes upon arrival to avoid repeated freeze-thaw cycles. If the FITC-Casein Substrate is subjected to repeated freeze-thaw cycles, a slight increase in the background will occur, thereby, lowering the sensitivity. The aliquots should be stored at -20 °C and protected from light. Each sample, blank, or control reaction requires 20 µl of the FITC -Casein Substrate. Freeze/thaw as well as vigorous mixing or shaking can cause the FITC to separate from the Casein resulting in a higher background reading and causing less substrate to be available for protease cleavage.

The Fluorescein isothiocyanate (FITC) Control Solution- The FITC control can be reconstituted in Assay Buffer to the appropriate concentration. This solution should be made fresh and protected from light.

Final the Trypsin Control Solution - Add 100 µl of 1 mM HCl to the vial of Trypsin, Protease Control (Product Code T 6567). Mix briefly to ensure the trypsin is dissolved. Add 900 µl of the Incubation Buffer and mix well. Alternatively, other buffers may be used if desired for the assay. The final working concentration of the trypsin is 20 µg/µl. When ready to prepare the Trypsin Control Solution, combine an aliquot of the acidic trypsin solution with the correct amount of the Incubation Buffer (1 part acidified trypsin to 9 parts buffer). Storing the trypsin under acidic conditions increases the stability of the trypsin. The trypsin control solution is temperature sensitive and is not stable at low concentrations. Please review the kit's technical bulletin for storage and stability of these prepared solutions.

Procedure

1. It is easiest to prepare controls, blanks, and samples all at one time. For each test sample, add 20 µl of Incubation Buffer, 20 µl of FITC - Casein Substrate, and 10 µl of the test sample to a microcentrifuge tube. For test samples with high protease activity, sample dilution may be required.
2. Prepare appropriate control samples (see Control Samples) by adding 20 µl of Incubation Buffer, 20 µl of FITC -Casein Substrate, and 10 µl of the control sample to a microcentrifuge tube.
3. Prepare a blank sample by adding 20 µl of Incubation Buffer, 20 µl of FITC-Casein Substrate, and 10 µl of ultrapure water to a microcentrifuge tube.

4. Gently mix each tube and incubate at 37 °C in the dark for 60 minutes. Be careful not to mix too vigorously, as excessive turbulence may cause high fluorescence background and reduce the sensitivity of the assay.
Note: Incubation time may be extended up to 24 hours to increase sensitivity. Be careful not to exceed 24 hours as the FITC-Casein may begin to degrade, leading to high fluorescence background.
5. After incubation add 150 µl of the 0.6 N Trichloroacetic Acid Solution to each microcentrifuge tube. TCA is very corrosive and should be handled while wearing the appropriate protective equipment.
6. Gently mix and incubate at 37 °C in the dark for 30 minutes.
7. Centrifuge the tubes for 10 minutes at 10,000 x g. The supernatant contains the acid soluble, FITC labeled fragments and is used for the fluorescence measurement.

Fluorescence Measurements

These methods can be scaled up or down according to the requirements of the instrumentation available. For comparison to a standard curve prepared with the appropriate control samples, subtract the fluorescence reading of the blank sample (FLUblank) from the value of each test sample (FLUtest).

Cuvettes

1. Pipette 10 µl of the supernatant (step 7) and 1 ml of the Assay Buffer into a suitable cuvette and mix gently. Note: The solution of the supernatant and Assay Buffer may be stored in the dark at 2-8 °C for up to 24 hours before measuring the fluorescence.
2. Record the fluorescence intensity with excitation at 485 nm and monitoring the emission wavelength of 535 nm.

Multiwell Plates

1. Pipette 10 µl of the supernatant (step 7) and 1 ml of the Assay Buffer into a suitable tube or vial and mix gently. Note: The solution of the supernatant and Assay Buffer may be stored in the dark at 2-8 °C for up to 24 hours before measuring the fluorescence.
2. Transfer 200 µl to a well of a black 96 well plate. Record the fluorescence intensity with excitation at 485 nm and monitoring the emission wavelength of 535 nm.
Or
1. Pipette 2 µl of the supernatant (step 7) and 200 µl of the Assay Buffer into a well of a black 96 well plate. Note: The solution of the supernatant and Assay Buffer may be stored in the dark at 2-8 °C for up to 24 hours before measuring the fluorescence.
2. Record the fluorescence intensity with excitation at 485 nm and monitoring the emission wavelength of 535 nm.

Control Samples

The Trypsin Control Solution can be used to confirm the assay is performing properly, to determine the detection limit, or create a general standard curve. For the assay of a different, specific protease, it is recommended to prepare a control solution containing the specific protease in the appropriate incubation buffer.

The limit of detection of the assay is the amount of protease that produces a significant fluorescence reading above the value obtained with the blank sample. The limit of detection will vary depending on the sensitivity of the instrumentation. Serial dilutions of the Trypsin Control Solution may be used to generate the control solutions.

A reading equal to 120% of the value obtained with the blank sample is considered significant. Routinely, a limit of detection of 5 ng of trypsin was obtained with this procedure. It is recommended that at least one 5 ng trypsin control be run with each assay. A trypsin control solution with a concentration of 0.5 µg/ml would result in the desired 5 ng of trypsin in the assay. A 40-fold dilution of the Trypsin Control Solution (20 µg/ml) results in a 0.5 µg/ml control solution, i.e. one part of Trypsin Control Solution to 39 parts of Incubation Buffer.

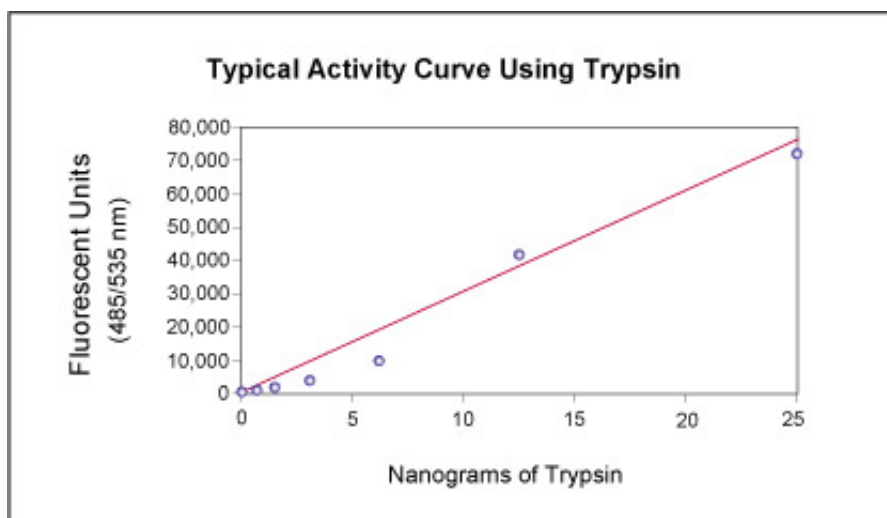


Figure 1 (Standard Curve of Trypsin Activity): The Trypsin Control Solution (20 $\mu\text{g/ml}$) may also be used to generate a standard curve by making serial dilutions (see Figure 1). The fluorescence reading of each control sample was corrected by subtracting the fluorescence reading of the blank sample (FLUblank) from the value of each control sample (FLUcontrol).

Typical standard curve for trypsin (Product Code T 6567) using this kit and control samples ranging from 0.15 $\mu\text{g/ml}$ (1.5 ng) to 2.5 $\mu\text{g/ml}$ (25 ng). This curve was generated by following the described procedure, with an incubation time of 30 minutes for step 4. Fluorescence measurements were made in a multiwell plate.

Fluorescein isothiocyanate is provided as a control for possible instrument calibration (see appropriate manufacturer's instructions) or determination of the linearity range of the FITC signal.

Discussion

We have just shown an example assay using the Protease Fluorescent Detection Kit to detect protease activity in a biological sample. This kit has been optimized to detect a diverse range of proteases found in physiological applications. It is suitable for detection of serine, cysteine, metallo, and aspartic proteases; however, modifications may be required to detect some specific proteases.

Disclosures

The author is an employee of Sigma Aldrich that produces reagents and tools used in this article.

References

1. Twining, S.S., Fluorescein Isothiocyanate-Labeled Casein Assay for Proteolytic Enzymes. *Anal. Biochem.*, 143, 30-34 (1984).