

## Product Information

### Protease Fluorescent Detection Kit

Product Code **PF0100**

Storage Temperature  $-20\text{ }^{\circ}\text{C}$

### TECHNICAL BULLETIN

#### Product Description

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.

This kit uses a modification of a published procedure.<sup>1</sup> The described kit procedure detects the trypsin protease control at a concentration of approximately  $0.5\text{ }\mu\text{g/ml}$  ( $5\text{ ng}$  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.

#### Components

Each kit contains sufficient reagents for 200 one ml assays.

Incubation Buffer (Product Code I 7158) 20 mM sodium phosphate with 150 mM sodium chloride, pH 7.6	25 ml
Assay Buffer (Product Code A 8478) 500 mM Tris buffer, pH 8.5	200 ml
FITC-Casein Substrate (Product Code F 0554)	5 ml

0.6 N Trichloroacetic Acid (TCA) Solution  
(Product Code T 0199) 30 ml

Fluorescein Isothiocyanate (FITC) Control  
(Product Code F 7250) 50 mg

Trypsin, Protease Control  
(Product Code T 6567) 20  $\mu\text{g}$

#### Reagents and Equipment Required but Not Provided

- Pipettes
- Fluorimeter cuvettes or black 96 well plates
- Microcentrifuge tubes
- Microcentrifuge
- Appropriate instrument to measure fluorescence (excitation wavelength of 485 nm and an emission wavelength of 535 nm).
- 1 mM Hydrochloric acid (HCl)

#### Precautions and Disclaimer

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

#### Preparation Instructions

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

- 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  $-20\text{ }^{\circ}\text{C}$  and protected from light. Each sample, blank, or control reaction requires  $20\text{ }\mu\text{l}$  of the FITC-Casein Substrate.

- **Fluorescein isothiocyanate (FITC) Control Solution-** The FITC control can be reconstituted in Assay Buffer to the appropriate concentration. This solution should be made fresh.
- **Trypsin Control Solution** - Add 100  $\mu$ l 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  $\mu$ l 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  $\mu$ g/ml.  
Note: The vial of trypsin may also be reconstituted with 100  $\mu$ l of the 1 mM HCl and stored at 2–8 °C for 2 weeks or at –20 °C for at least 4 weeks. 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.

#### **Storage/Stability**

- The FITC-Casein Substrate is light sensitive and should be stored in the dark at –20 °C. If properly stored, the substrate is stable for 2 years.
- The Incubation Buffer is stable for at least 2 years at 2–8 °C.
- The Assay Buffer is stable for at least 2 years at 2–8 °C.
- The 0.6 N TCA solution is stable for at least 2 years at 2–8 °C.
- **Trypsin, Protease Control.** The lyophilized Trypsin, Protease Control, powder is stable for five years, if stored unopened at 2–8 °C. An acidic, reconstituted solution (pH 3.0) can be stored at 2–8 °C for 2 weeks or at –20 °C for at least 4 weeks, and is stable for at least 3 freeze-thaw cycles.
- **Fluorescein Isothiocyanate (FITC) Control.** The FITC control is light sensitive and should be stored desiccated in the dark at 2–8 °C. If properly stored, the powder should be stable for at least 2 years.

#### **Procedure**

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. Modifications to the procedure may include pH adjustments, the addition of metal ions, such as calcium, or a reformulation of the incubation buffer. The researcher must determine the optimal procedure conditions for the protease specific to their application.

1. For each test sample, add 20  $\mu$ l of Incubation Buffer, 20  $\mu$ l of FITC-Casein Substrate, and 10  $\mu$ l 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  $\mu$ l of Incubation Buffer, 20  $\mu$ l of FITC-Casein Substrate, and 10  $\mu$ l of the control sample to a microcentrifuge tube.
3. Prepare a blank sample by adding 20  $\mu$ l of Incubation Buffer, 20  $\mu$ l of FITC-Casein Substrate, and 10  $\mu$ l 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  $\mu$ l of the 0.6 N TCA Solution to each microcentrifuge tube.
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 ( $FLU_{\text{blank}}$ ) from the value of each test sample ( $FLU_{\text{test}}$ ).

#### Cuvettes

1. Pipette 10  $\mu\text{l}$  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  $\mu\text{l}$  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  $\mu\text{l}$  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  $\mu\text{l}$  of the supernatant (step 7) and 200  $\mu\text{l}$  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

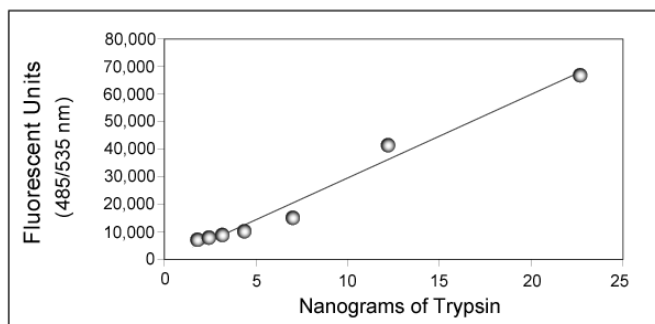
The Trypsin Control Solution can be used to confirm the assay is performing properly, to determine the detection limit, or create a general standard curve. For the assay of a different, specific protease, it is recommended to prepare a control solution containing the specific protease in the appropriate 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  $\mu\text{g/ml}$  would result in the desired 5 ng of trypsin in the assay. A 40-fold dilution of the Trypsin Control Solution (20  $\mu\text{g/ml}$ ) results in a 0.5  $\mu\text{g/ml}$  control solution, i.e. one part of Trypsin Control Solution to 39 parts of Incubation Buffer.

The Trypsin Control Solution (20 µg/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 ( $FLU_{\text{blank}}$ ) from the value of each control sample ( $FLU_{\text{control}}$ ).

**Figure 1.**  
Standard Curve of Trypsin Activity



Typical standard curve for trypsin (Product Code T 6567) using this kit and control samples ranging from 0.15 µg/ml (1.5 ng) to 2.5 µg/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.

#### References

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

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