

## Product Information

### Protease Fluorescent Detection Kit

Catalog Number **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  $\sim 5\text{ }\mu\text{g/mL}$  (50 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 (Catalog Number I7158) 20 mM sodium phosphate with 150 mM sodium chloride, pH 7.6	25 mL
Assay Buffer (Catalog Number A8478) 500 mM Tris buffer, pH 8.5	200 mL
FITC-Casein Substrate (Catalog Number F0554)	5 mL

Trichloroacetic Acid (TCA) Solution 6.1 N trichloroacetic acid (Catalog Number T0699)	30 mL
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Fluorescein Isothiocyanate (FITC) Control (Catalog Number F7250)	50 mg
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Trypsin, Protease Control (Catalog Number T6567)	20 $\mu\text{g}$
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### 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

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.

### Preparation Instructions

The Incubation Buffer and Assay Buffer are 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  $\mu\text{L}$  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  $\mu\text{L}$  of 1 mM HCl to the vial of Trypsin, Protease Control (Catalog Number T6567). Mix briefly to ensure the trypsin is dissolved. Add 900  $\mu\text{L}$  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  $\mu\text{g}/\text{mL}$ .

**Note:** The vial of trypsin may also be reconstituted with 100  $\mu\text{L}$  of the 1 mM HCl and stored at 2–8  $^{\circ}\text{C}$  for 2 weeks or at –20  $^{\circ}\text{C}$  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.

**0.6 N TCA Solution:** This kit comes with T0699 (6.1 N TCA Solution). 0.6 N TCA Solutions may be prepared by a 10-fold dilution of the desired volume of T0699 with ultrapure water. It is not necessary to dilute the entire volume of T0699 for immediate use of the kit.

**Storage/Stability**

- The FITC-Casein Substrate is light sensitive and should be stored in the dark at –20  $^{\circ}\text{C}$ . If properly stored, the substrate is stable for 2 years.
- The Incubation Buffer is stable for at least 2 years at 2–8  $^{\circ}\text{C}$ .
- The Assay Buffer is stable for at least 2 years at 2–8  $^{\circ}\text{C}$ .
- The TCA solution is stable for at least 2 years at 2–8  $^{\circ}\text{C}$ .
- Trypsin, Protease Control: The lyophilized Trypsin, Protease Control, powder is stable for five years, if stored unopened at 2–8  $^{\circ}\text{C}$ . An acidic, reconstituted solution (pH 3.0) can be stored at 2–8  $^{\circ}\text{C}$  for 2 weeks or at –20  $^{\circ}\text{C}$  for at least 4 weeks, and is stable for at least 3 freeze-thaw cycles.
- The Fluorescein Isothiocyanate (FITC) Control is light-sensitive and should be stored desiccated in the dark at 2–8  $^{\circ}\text{C}$ . 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. Researchers must determine the optimal procedure conditions for the protease specific to their application.

1. For each test sample, add 20  $\mu\text{L}$  of Incubation Buffer, 20  $\mu\text{L}$  of FITC-Casein Substrate, and 10  $\mu\text{L}$  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  $\mu\text{L}$  of Incubation Buffer, 20  $\mu\text{L}$  of FITC-Casein Substrate, and 10  $\mu\text{L}$  of the control sample to a microcentrifuge tube.
3. Prepare a blank sample by adding 20  $\mu\text{L}$  of Incubation Buffer, 20  $\mu\text{L}$  of FITC-Casein Substrate, and 10  $\mu\text{L}$  of ultrapure water to a microcentrifuge tube.
4. Gently mix each tube and incubate at 37  $^{\circ}\text{C}$  in the dark for 60 minutes. Be careful not to mix too vigorously, as excessive turbulence may cause a 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 a high fluorescence background.
5. After incubation, add 150  $\mu\text{L}$  of the 0.6 N TCA Solution to each microcentrifuge tube.
6. Gently mix and incubate at 37  $^{\circ}\text{C}$  in the dark for 30 minutes.
7. Centrifuge the tubes for 10 minutes at 10,000  $\times g$ . 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 that the assay is performing properly, to determine the detection limit, or to 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 (LOD) of the assay is the amount of protease that produces a significant fluorescence reading above the value obtained with the blank sample. The LOD 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 LOD of 50 ng of trypsin has been obtained with this procedure. It is recommended that at least one 50 ng trypsin control be run with each assay. A trypsin control solution with a concentration of 5  $\mu\text{g/mL}$  would result in the desired 50 ng of trypsin in the assay. A 4-fold dilution of the Trypsin Control Solution (20  $\mu\text{g/mL}$ ) results in a 5  $\mu\text{g/mL}$  control solution, i.e., one part of Trypsin Control Solution to 3 parts of Incubation Buffer. The Trypsin Control Solution (20  $\mu\text{g/mL}$ ) may also be used to generate a standard curve by making serial dilutions.

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(1)**, 30-34 (1984).

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