

## SIGMA QUALITY CONTROL TEST PROCEDURE

### Enzymatic Assay of PROTEASE IMPURITY using Fluorescein Isothiocyanate-Casein

#### PRINCIPLE:



Abbreviations:

FITC-Casein = Fluorescein Isothiocyanate-Casein

FITC = Fluorescein Isothiocyanate

#### CONDITIONS:

Part A: T = 37°C, pH 7.6,

Part B: T = 25°C, pH 8.5, Excitation = 490 nm,  
Emission = 525 nm, Light path = 1 cm

**METHOD:** Fluorometric Stopped Reaction

#### REAGENTS:

- A. 20 mM Sodium Phosphate Buffer with 150 mM Sodium Chloride, pH 7.6 at 37°C  
(Prepare 200 ml in deionized water using Sodium Phosphate, Dibasic, Anhydrous, Sigma Prod. No. S-0876, and Sodium Chloride, Sigma Prod. No. S-9625. Adjust to pH 7.6 at 37°C with 1 M HCl.)
- B. 0.5% (w/v) Fluorescein Isothiocyanate-Casein Solution (FITC-C)  
(Prepare 1.0 ml in Reagent A using Casein-Fluorescein Isothiocyanate, Sigma Prod. No. C-3777. **KEEP FROM LIGHT!**)
- C. Protease Enzyme Solution  
(Immediately before use, prepare a solution containing 10 µg/ml in cold Reagent A using Protease Type VIII, Sigma Prod. No. P-5380.)
- D. 10% (v/v) Trichloroacetic Acid Solution (TCA)  
(Prepare 10 ml in deionized water using Trichloroacetic Acid, 6.1 N Solution, Sigma Stock No. 490-10.)

**Enzymatic Assay of PROTEASE IMPURITY  
using Fluorescein Isothiocyanate-Casein**

**REAGENTS:**

- E. 500 mM Tris HCl Solution, pH 8.5 at 25°C  
(Prepare 500 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 8.5 at 25°C with 5 M HCl.)
- F. 0.001% (w/v) Fluorescein Isothiocyanate Standard Solution (FITC)  
(Prepare 1 ml in Reagent E using Fluorescein Isothiocyanate, Sigma Prod. No. F-7250.  
**KEEP FROM LIGHT!**)
- G. Sample Solution (Sample)  
(Prepare solution in cold deionized water as per the requested assignment.)

**PROCEDURE:**

Part A:

To prepare Protease Standards (PS), pipette (in milliliters) the following reagents into suitable containers:

	<u>PS 1</u>	<u>PS 2</u>	<u>PS 3</u>	<u>PS 4</u>
Reagent A (Buffer)	1.90	0.90	0.70	0.50
Reagent C (Protease)	0.10	0.10	0.30	0.50

Mix by swirling.

Pipette (in milliliters) the following reagents into suitable microcentrifuge tubes:

	<u>Test</u>						<u>PS</u>
	<u>Test</u>	<u>Blank</u>	<u>PS 1A</u>	<u>PS 2A</u>	<u>PS 3A</u>	<u>PS 4A</u>	<u>Blank</u>
Reagent A (Buffer)	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Reagent B (FITC-C)	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Equilibrate to 37°C. Then add:

Reagent G (Sample)	0.01	----	----	----	----	----	----
PS 1	----	----	0.01	----	----	----	----
PS 2	----	----	----	0.01	----	----	----
PS 3	----	----	----	----	0.01	----	----
PS 4	----	----	----	----	----	0.01	----

**Enzymatic Assay of PROTEASE IMPURITY  
using Fluorescein Isothiocyanate-Casein**

**PROCEDURE:** (continued)

Immediately mix by swirling and incubate at 37°C for exactly 60 minutes. Then add:

	Test						PS
	<u>Test</u>	<u>Blank</u>	<u>PS 1A</u>	<u>PS 2A</u>	<u>PS 3A</u>	<u>PS 4A</u>	<u>Blank</u>
Reagent D (TCA)	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Reagent G (Sample)	----	0.01	----	----	----	----	----
PS 4 (Protease Standard)	----	----	----	----	----	----	0.01

Mix by swirling and incubate at 37°C for exactly 60 minutes. Centrifuge all tubes for 15 minutes.<sup>1</sup>

**Part B:**

Pipette (in milliliters) the following reagents into suitable containers:

Reagent E (Tris)	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Supernatant-Test	0.10	----	----	----	----	----	----
Supernatant-Test Blank	----	0.10	----	----	----	----	----
Supernatant-PS-1A	----	----	0.10	----	----	----	----
Supernatant-PS-2A	----	----	----	0.10	----	----	----
Supernatant-PS-3A	----	----	----	----	0.10	----	----
Supernatant-PS-4A	----	----	----	----	----	0.10	----
Supernatant-PS Blank	----	----	----	----	----	----	0.10

Mix by swirling and transfer the solutions to suitable cuvettes. Record the fluorescence intensity at the excitation wavelength of 490 nm and the emission wavelength of 525 nm for all the containers in a suitable fluorometer at 25°C.

Prepare the FITC (FS) standards by pipetting (in milliliters) the following reagents into suitable containers:

	FS				PS
	<u>FS 1</u>	<u>FS 2</u>	<u>FS 3</u>	<u>FS 4</u>	<u>Blank</u>
Reagent E (Tris)	10.00	10.00	10.00	10.00	10.00
Reagent F (FITC)	0.01	0.05	0.07	0.10	----

Mix by swirling and transfer to suitable cuvettes. Record the fluorescence intensity at the excitation wavelength of 490 nm and the emission wavelength of 525 nm for all the containers in a suitable fluorometer at 25°C.

**Enzymatic Assay of PROTEASE IMPURITY  
using Fluorescein Isothiocyanate-Casein**

**CALCULATIONS:**

F = Fluorescence at the excitation wavelength of 490 nm and the emission wavelength of 525 nm

$\Delta F_{FS}$  Standard =  $F_{FS}$  Standard -  $F_{FS}$  Blank

$\Delta F_{PS}$  Standard =  $F_{PS}$  Standard -  $F_{PS}$  Blank

**SPECIFICATIONS:**

The change in fluorescence for the sample must be less than the 10 mg fluorescein isothiocyanate (FITC) standard and the 5 ng protease standard. The detection limit for this assay is  $2.56 \times 10^{-5}$   $\mu$ moles FITC released per minute.

**FINAL ASSAY CONCENTRATIONS:**

In a 0.05 ml reaction mix, the final concentrations are 16 mM sodium phosphate, 120 mM sodium chloride and 0.2% (w/v) casein-fluorescein isothiocyanate. The units of protease will vary.

**NOTES:**

1. At this point, 0.10 ml of the supernatant from all samples and standards (FITC standard must be included) can be pipetted into 4 dram vials, wrapped in foil and placed in the cooler for up to a week before diluting with 10 ml of Reagent E (Tris) and read on the fluorometer.
2. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.

**Sigma warrants that the above procedure information is currently utilized at Sigma and that Sigma products conform to the information in Sigma publications. Purchaser must determine the suitability of the information and products for its particular use. Upon purchase of Sigma products, see reverse side of invoice or packing slip for additional terms and conditions of sale.**