Protease Activity Fluorometric Assay Kit (#BN00994)

(Catalog #BN00994; 100 assays; Store kit at –20°C)

I. Introduction:

Proteases are naturally present in all organisms. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades. Proteases can either break specific peptide bonds (*limited proteolysis*), depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids (*unlimited proteolysis*). The activity can be a destructive change (abolishing a protein's function), an activation of a function (preform to mature form) or it can be a signal in a signaling pathway. Assay Genie's Protease Activity Assay Kit is designed for the quantitative determination of proteases present in the protein sample. The assay uses fluorescein isothiocyanate (FITC)-labeled casein as a general protease substrate. The fluorescein label on the FITC-Casein is highly quenched. Upon digestion by proteases present in the sample the FITC-Casein substrate is cleaved into smaller peptides which abolish the quenching of the fluorescence label. The fluorescence of the FITC-labeled peptide fragments is measured at Ex/Em=485/530 nm. The kit is supplied with our Mass Spectrometry Grade (MSG), chemically stabilized Trypsin for use as a general protease control. However, other protease standard controls can also be used. This kit is easy to use and can detect <500 pg/well Trypsin present in the sample.

II. Kit Contents:

Components	100 assays	Cap Color	Part Number
Protease Assay Buffer	25 ml	WM	BN00994-1
Protease Substrate (lyophilized)	1 vial	Red	BN00994-2
FITC Standard (25 µM)	200 µl	Yellow	BN00994-3
Positive Control (lyophilized)	1 vial	Green	BN00994-4

III. Reagent Preparation and Storage Conditions:

Substrate: Reconstitute with 220 µl dH_2O. Pipette up and down to completely dissolve. Store at -20°C. Use within two months.

Positive Control: Reconstitute with 100 µl Assay Buffer. Pipette up and down to completely dissolve. Aliquot and store at -20°C. Use within two months. Avoid freeze/thaw cycles.

IV. Protease Assay Protocol:

1. Standard Curve Preparations:

Add 0, 2, 4, 6, 8, 10 µl FITC Standard into a series of standards wells. Adjust the final volume to 100 µl/well with Assay Buffer to generate 0, 0.05, 0.1, 0.15 0.2, and 0.25 nmol/well of the FITC Standard.

2. Sample and Positive Control Preparations:

Tissues or cells can be extracted with 4 volumes of the Assay Buffer, centrifuge to remove insoluble material and get a clear extract. Prepare test samples at 50 µl/well with Assay Buffer in a 96-well plate. Serum can be directly added into sample wells, and the volume adjusted to 50 µl/well with Assay Buffer. We suggest using several doses of your sample to ensure the readings are within the linear range. For Positive Control, add 5 µl Positive Control solution to wells and adjust volume to 50 µl/well with Assay Buffer. Include a reagent background control which only contains 50 µl of Assay Buffer.

3. Reaction Mix:

Mix enough reagents for the number of assays to be performed. For each well, prepare 50 µl of Reaction Mix containing:

Assay Buffer 48 µl
Substrate 2 µl

Add 50 µl of the Reaction Mix to each well containing Positive Controls, reagent background control and test samples. Mix well. **(DO NOT ADD TO STANDARDS)**

4. Measurement: Read Ex/Em=485/530 nm R_1 at T_1 then read R_2 at T_2 after incubating the reaction at 25°C for 60 min, protected from light (or incubate longer if the sample activity is low). The fluorescence of the unquenched FITC generated by proteolytic digestion of the substrate is $\Delta RFU = R_2 - R_1$.

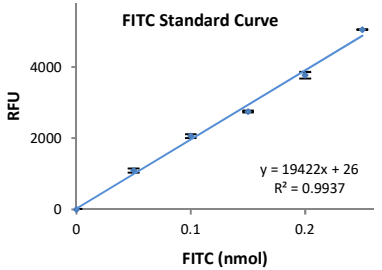
Note: A. It is essential to read R_1 and R_2 in the reaction linear range. It will be more accurate if you read the reaction kinetics, then choose R_1 and R_2 in the reaction linear range. **B.** Since the assay is a fluorescence quenching assay, the background reading is high, but sample reading are consistent.

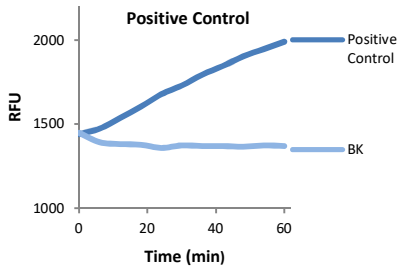
5. Calculation: Subtract 0 Standard from all Standard readings. Plot the FITC Standard Curve. Apply the ΔRFU to the FITC Standard Curve to get B nmol of FITC (amount of unquenched FITC generated between T_1 and T_2 in the reaction wells).

Protease Activity = $\frac{B}{(T2-T1)\times V}$ **× Sample Dilution Factor = nmol/min/ml = mU/ml**

Where: **B** is the FITC amount from FITC Standard Curve (in nmol).
T_1 is the time of the first reading (R_1) (in min).
T_2 is the time of the second reading (R_2) (in min).
V is the pretreated sample volume added into the reaction well (in ml).

Unit Definition: One unit is defined as the amount of protease that cleaves the substrate, to yield an amount of fluorescence equivalent to 1.0 µmol of unquenched FITC per minute at 25°C.





FOR RESEARCH USE ONLY! Not to be used on humans.

GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	• Use of ice-cold assay buffer	• Assay buffer must be at room temperature
	• Omission of a step in the protocol	• Refer and follow the data sheet precisely
	• Plate read at incorrect wavelength	• Check the wavelength in the data sheet and the filter settings of the instrument
	• Use of a different 96-well plate	• Fluorescence: Black plates (clear bottoms) ; Luminescence: White plates ; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type	• Refer data sheet for details about incompatible samples
	• Samples prepared in a different buffer	• Use the assay buffer provided in the kit or refer data sheet for instructions
	• Cell/ tissue samples were not completely homogenized	• Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope
	• Samples used after multiple free-thaw cycles	• Aliquot and freeze samples if needed to use multiple times
	• Presence of interfering substance in the sample	• Troubleshoot if needed
	• Use of old or inappropriately stored samples	• Use fresh samples or store at correct temperatures until use
Lower/ Higher readings in Samples and Standards	• Improperly thawed components	• Thaw all components completely and mix gently before use
	• Use of expired kit or improperly stored reagents	• Always check the expiry date and store the components appropriately
	• Allowing the reagents to sit for extended times on ice	• Always thaw and prepare fresh reaction mix before use
	• Incorrect incubation times or temperatures	• Refer datasheet & verify correct incubation times and temperatures
	• Incorrect volumes used	• Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	• Use of partially thawed components	• Thaw and resuspend all components before preparing the reaction mix
	• Pipetting errors in the standard	• Avoid pipetting small volumes
	• Pipetting errors in the reaction mix	• Prepare a master reaction mix whenever possible
	• Air bubbles formed in well	• Pipette gently against the wall of the tubes
	• Standard stock is at an incorrect concentration	• Always refer the dilutions in the data sheet
	• Calculation errors	• Recheck calculations after referring the data sheet
	• Substituting reagents from older kits/ lots	• Use fresh components from the same kit
Unanticipated results	• Measured at incorrect wavelength	• Check the equipment and the filter setting
	• Samples contain interfering substances	• Troubleshoot if it interferes with the kit
	• Use of incompatible sample type	• Refer data sheet to check if sample is compatible with the kit or optimization is needed
	• Sample readings above/below the linear range	• Concentrate/ Dilute sample so as to be in the linear range
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		