Product Information Sheet (M1319-005)
MarkerGene™ Fluorescent Protease Assay Kit
(Product M1319)
Note: The following information is given as a viable methodology for use of MarkerGene™ Fluorescent Protease Assay Kit. The user may determine their own best conditions for use dependent on the specific conditions present in their experiment.

I. Overview

Direct fluorescence-based assays for detecting metallo-, serine, acid or sulfhydryl proteases are important in medical, biochemical and cell biology research. Analysis of low levels of protease activity is important in biochemical quality control testing, for analysis of protease inhibitors or cofactors, as well as for basic research application in biology and molecular biology. Several fluorescence-based methods have been developed for detecting protease activity including the fluorescein thiocarbamoyl (FTC)-casein protease assay, in which unhydrolyzed protein must be precipitated with trichloroacetic acid, separated by centrifugation, transferred for measurement and then pH-adjusted to optimize the fluorescence signal. The MarkerGene™ Fluorescent Protease Assay Kit avoids these time-consuming separation steps by taking advantage of the self-quenching of fluorescein when heavily coupled to protein. This kit uses the conjugated protein, FITC-Casein (Product M1315) as a substrate. Casein is a naturally occurring protein in milk that is suitable as a general substrate for a myriad of proteases. Labeled with multiple fluorescent dyes, the substrate exhibits significant fluorescence quenching. Protease-catalyzed hydrolysis releases highly fluorescent-labeled peptides; the accompanying increase in fluorescence is proportional to protease activity and can be conveniently measured in a continuous assay format using a fluorometer equipped with an appropriate (fluorescein) filter set (EX/EM= 490/520 nm). This kit has demonstrated sensitivity of less than 10 mU/mL enzyme. Extensive protease cleavage of the substrate can result in fluorescence increases of greater than 10-fold. In addition to utility for detecting protease contamination of culture media and other experimental samples, the assay can be used to continuously measure the kinetics of a variety of exo- and endopeptidases or to measure the total substrate turnover at a fixed time following addition of the enzyme. Among the enzymes that can be monitored using this method are elastase, chymotrypsin, thermolysin, trypsin, papain, pepsin, cathepsin D and elastase. The kit contains enough substrate for 100 assays and control experiments (96-well microtiterplate, 100 µL reaction volume) and also contains reference standards and a detailed protocol for use. See the references below for more information and applications.
II. MATERIALS

A.) Substrate: 5 mg FITC-Casein. Prepare 1mg/mL solution for use in the assay protocol below.

B.) Reference Standard: 1 mg Fluorescein-5-thiourea (FTU). Dilute to desired concentration using reaction buffer.

C.) Reaction Buffer: This buffer contains 100mM Sodium Phosphate, 150mM Sodium Chloride, pH 7.6.

F.) Storage and Handling: The substrate and reference standard included in this kit should be stored at -20°C when not in use. Protect solutions of the substrate and reference standard from light. Substrate solutions are best when prepared fresh, prolonged storage in solution or repeated freeze/thaw cycles may result in denaturation of the protein and/or increased background fluorescence.

III. ENZYME ASSAY

It is recommended that a calibration curve be generated using concentrations of the Reference Standard in a range from 0 to the highest concentration of substrate reagent used for all assays. Several concentrations of the Reference Standard (M1319-002) can be made by dilution in reaction buffer (M1319-003).

In addition, purified enzyme assays may be performed using several enzyme concentrations in the approximate or estimated range of the enzyme concentration expected for the unknown sample. To normalize data, each enzyme reaction should be subtracted from a blank (no enzyme) sample.

1.) It is recommended that samples to be assayed are diluted at least 1:1 in reaction buffer (Product M1319-003). Presence of solid material in sample may affect fluorescence readings. Solid materials should be removed by centrifugation prior to measurement if possible. Keep samples in an ice bath until needed.

2.) Purified enzyme concentrations should be prepared fresh by diluting enzyme in reaction buffer (Product M1319-003). Keep samples in an ice bath until needed.
3.) To a 96-well microtiter plate, add diluted samples and purified enzyme solutions to wells in triplicate (50 µL/well). Also include in triplicate wells for blanks and reference standards (50 µL reaction buffer/well).

4.) If a standard curve is desired to produce kinetic data, reference standard may be diluted to several concentrations and added to wells. Prepare standard curve by making several serial dilutions of reference standard (Product No. M1319-002) in reaction buffer (Product No. M1319-003) and adding to wells in triplicate (100 µL/well). Reference standard may also be used for optimizing reading conditions of fluorometer (see note (2) below).

5.) Dissolve substrate (Product No. M1219-001) in reaction buffer (5mL) (Product No. M1219-003) to yield a 2X substrate solution. Prepare this solution just prior to performing the assay (see note (1) below). Add 2X substrate reagent (50 µL/well) to all wells, except those containing reference standard.

6.) Read fluorescence (EX/EM = 490/520 nm) in a microtiter plate reader, using appropriate filters. Wells containing reference standard can be used to optimize reading conditions. If kinetic data is desired, readings may be taken beginning immediately after addition of the substrate reagent, and at several defined time points thereafter. If only an endpoint reading is needed, plate can be incubated at desired temperature for the desired time, and fluorescence recorded following incubation. Incubation times of 30-40 mins. are generally adequate to detect enzyme concentrations as low as 10 mU/mL (see note (3) below). Longer incubation times can be employed if greater sensitivity is desired.

7.) Average the readings of triplicate samples. Subtract fluorescence of blanks from that of each sample in order to normalize data. (See Note (1) below).

8.) A calibration curve may be generated using purified enzyme samples by plotting normalized fluorescence vs. time (log-log).

9.) Using the calibration curve generated in step 8, determine the activity (concentration) of the enzyme in the sample, and extrapolate this data to determine the original enzyme concentration.
Note (1.) Substrate reagent should be prepared just prior to performing the assay to prevent denaturation of substrate, which could result in increased background fluorescence and/or reduce sensitivity of the assay. Fluorescence of the unhydrolyzed protein is not fully quenched; some background fluorescence will always persist. This background fluorescence may necessitate the need for blank wells as recommended in the assay protocol.

Note (2.) Use of reference standard wells may be useful in optimizing reading conditions of the microtiter plate reader.

Note (3.) This kit has demonstrated sensitivity of less than 10mU/mL enzyme using subtilisin A (*Bacillus subtilis*), with 40-minute incubation at room temperature. Sensitivity may vary depending on specific protease enzyme and incubation conditions.

![Subtilisin A (B. subtilis) Activity Measured using M1319](image)

**Figure 1:** Several dilutions of Subtilisin A from *Bacillus subtilis* (SIGMA P5380) were prepared in reaction buffer (Product M1319-003). Each preparation was added in triplicate (50µL) to wells on a 96-well microtiter plate (clear, flat bottom). A 1mg/mL substrate reagent was prepared by diluting FITC-Casein (Product M1319-001) in reaction buffer and was added to wells (50 µL/well). Fluorescence was recorded using a Perkin-Elmer HTS 7000 BioAssay Reader, with 485nm excitation filter and 535nm emission filter. Fluorescence readings were taken at 1-minute intervals for 40 minutes. Fluorescence values of blank (50 µL substrate reagent added to 50 µL reaction buffer) were subtracted at each time point.
Proteinase K Activity Measured using M1319

Figure 2: Proteinase K (100µg/mL) was prepared in reaction buffer (Product M1319-003). The preparation was added in triplicate (50µL) to wells on a 96-well microtiter plate (clear, flat bottom). A 1mg/mL substrate reagent was prepared by diluting FITC-Casein (Product M1319-001) in reaction buffer and was added to wells (50 µL/well). Fluorescence was recorded using a Perkin-Elmer HTS 7000 BioAssay Reader, with 485nm excitation filter and 535nm emission filter. Fluorescence readings were taken at 1-minute intervals for 40 minutes. Fluorescence values of blank (50 µL substrate reagent added to 50 µL reaction buffer) were subtracted at each time point.

Activity of Trypsin (0.025%) Measured Using M1319

Figure 3: Trypsin (0.05% in 1X Phosphate Buffered Saline, 530µM EDTA) (GIBCO 1500-054) was diluted 1:1 in reaction buffer (Product M1319-003) to yield a 0.025% trypsin solution. The preparation was added in triplicate (50µL) to wells on a 96-well microtiter plate (clear, flat bottom). A 10mg/mL substrate reagent was prepared by diluting FITC-Casein (Product M1319-001) in reaction buffer and was added to wells (50 µL/well). Fluorescence was recorded using a Perkin-Elmer HTS 7000 BioAssay Reader, with 485nm excitation filter and 535nm emission filter. Fluorescence readings were taken at 1-minute intervals for 40 minutes. Fluorescence values of blank (50 µL substrate reagent added to 50 µL reaction buffer) were subtracted at each time point.
# M1319 Kit Contents

<table>
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<tr>
<th>Description</th>
<th>Quantity</th>
<th>Part No.</th>
<th>Storage</th>
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<tbody>
<tr>
<td><strong>Reagents</strong></td>
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<tr>
<td>Substrate (FITC-Casein)</td>
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<td>Reaction Buffer (100mM Sodium Phosphate, 150mM NaCl, pH 7.6)</td>
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</tbody>
</table>

Notes: F=store at or below -20°C; C=store cold (4°C); L=light sensitive; R=read protocol instructions carefully prior to use.
REFERENCES

4.) Folin, O., Ciocalteu, V., "On tyrosine. and tryptophane determinations in proteins."(1929) J. Biol. Chem. 73, 627