PRODUCT DATA SHEET

Product: Ac-IEPD-AFC (Granzyme B Fluorogenic Substrate)

Cat. No.: **AC-063 (10 mg)**

Chemical Name:
Ac-Ile-Glu-Pro-Asp-AFC

Molecular Weight:
725

Form:
White lyophilized powder

Description:
Peptide substrate labeled at the carboxy end with AFC (7-amino-4-trifluoromethyl coumarin). Designed to measure Granzyme B activity *in vitro.*

Introduction:
Cell-mediated killing by cytotoxic T-lymphocytes (CTLs) is an important immunologic defense against tumor cell proliferation, viral infection, and transplanted tissue. Cell death induced by CTLs is mostly apoptotic and is thought to involve perforin, a pore-forming protein, and the granzymes, a family of serine proteinases, that are present in the cytoplasmic granules of CTLs and natural killer (NK) cells.

Seven serine proteases (Granzyme A, B, C, D, E, F, and G) have been isolated from mouse CTL granules. Two serine proteases (Granzyme A and B) have been isolated from human CTL granules and are homologous to the mouse enzymes.

Granzyme B is the granzyme most specifically found in CTLs and the granzyme shown to cause the most rapid kinetics of cell death. Granzyme B shares an unusual substrate specificity with interleukin-1ß converting enzyme (ICE), another enzyme involved in apoptosis, in that both require an Asp in the P1 position.

Principal:
A synthetic peptide substrate, Ac-Ile-Glu-Pro-Asp, has been labeled with AFC (7-amino-4-trifluoromethyl coumarin) at the carboxy end. AFC is a fluorescent molecule whose release from the substrate can be used to measure Granzyme B activity. Granzyme B enzyme activity in the sample is proportional to the amount of free AFC produced.

When AFC is attached to the peptide substrate, it produces a blue fluorescence upon exposure to UV light (400 nm). Granzyme B enzymatically cleaves the AFC-substrate and releases free AFC, which produces a yellow- green fluorescence at 505 nm when exposed to UV light.

AFC has two advantages over other fluorogenic labels. The wide Stokes' shift between bound and free AFC enables the substrate to be both chromogenic (yellow-green color visible to the naked eye) and fluorogenic (emission at 505 nm). The wide Stoke's shift also makes the assay more sensitive.

Applications:
For *in vitro* assays of Caspase-1 and Caspase-4 activities. Can be used with purified or partially purified enzymes, or possibly with crude cell lysates (if the Caspase-1/ICE Inhibitor 2 is included to determine background protease activity).

Protocol:
Fluorometer calibration: The fluorometer is calibrated using known concentrations of free AFC (Excitation = 400 nm, Emission = 505 nm) to generate a calibration curve of fluorescence versus µmoles AFC.

Samples: Can be either purified or partially purified enzyme preparations. Application to crude cell lysates has not been confirmed. If crude cell lysates are to be assayed, the non-specific protease background must be determined using our specific Granzyme B Inhibitor (Cat. No. AB-002).

General Fluorometric Assay Procedure:
CAUTION: The following procedure is provided only as an example for reference purposes. The user should determine the optimal conditions for their system.

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1. Prepare:
   - 20 mM Granzyme B Fluorogenic Substrate (Ac-Ile-Glu-Pro-Asp-AFC) stock solution in DMSO. Dilute 1:10 in DMSO.
   - 20 mM Granzyme B Inhibitor (Z-AAD-CMK) stock solution in DMSO. Dilute 1:10 in DMSO.
   - HEPES buffer: 0.1 M HEPES, 0.05 M CaCl2, adjust pH to 7.5 with conc. NaOH.
2. Prepare several dilutions of sample using HEPES buffer (1/10, 1/100, 1/1000).
3. Ideally, each sample dilution should be tested in three different reactions:
   Group 1: Substrate only (blank)
   Group 2: Sample + inhibitor + substrate (background protease activity)
   Group 3: Sample + substrate (enzyme assay)
4. Prepare calibration curve for AFC fluorescence by measuring known amounts of AFC in a fluorometer. (Excitation = 400 nm, Emission = 505 nm)
5. Group 2 reactions should be started first since the inhibitor needs to react with the sample before the substrate is added. For Group 2 reactions: Add 460 µL HEPES buffer, 10 µL 2.0 mM inhibitor solution, vortex, then add 20 µL sample. Mix gently, incubate at 30°C for 30 min. to 12 hours (time should be determined by the user). After incubation is finished, proceed with step 6b.

6a. Group 1 reactions: Add 490 µL HEPES buffer, 10 µL 2.0 mM substrate solution, vortex.

6b. Group 2 reactions: Add 10 µL 2.0 mM substrate solution.

6c. Group 3 reactions: Add 470 µL HEPES buffer and 10 µL 2.0 mM substrate solution, vortex, then add 20 µL sample.

7. Groups 1, 2, and 3: Mix gently, incubate at 37°C for 60 minutes, then measure fluorescence for time 0 (T_0).
8. Continue incubation at 37°C for another 60 min. and measure fluorescence for time 1 hr (T_1).
9. Calculate ΔFU for each sample dilution at T_1 as follows:
   ΔFU = [Group 3 FU at T_1 - Group 1 FU at T_1] - [Group 3 FU at T_0 - Group 1 FU at T_0]
10. Calculate enzyme activity in sample for T_1. If activity is low, assay should be run for a longer time (up to 24 hours if necessary). For best results, use the sample dilution giving the highest Group 3 (assay) values and lowest Group 2 (background protease) values.

Unit of Granzyme activity = 1 µmol free AFC/min.
Units Granzyme B = [(ΔFU/min) / (cal. curve slope)] x [1 Unit/ (1 x 10^{-6} µmoles AFC/min.)]

Example calculation:
Dilute an 80 µM AFC DMSO stock solution in HEPES buffer to give 0.5 mL final volumes as follows:
1 in 50 dilution = 8 x 10^{-4} µmoles AFC
2 in 50 dilution = 16 x 10^{-4} µmoles AFC
3 in 50 dilution = 24 x 10^{-4} µmoles AFC.

Plot the results with x-axis = µmole AFC and y-axis = Fluorescence Units (FU).

An example curve gives a slope of 8 x 10^{-6} µmoles AFC/FU.

For a $\Delta FU = 7.8$ (T_1 - T_0); T_1 = 60 min.
Units Granzyme = (7.8/60) x (8 x 10^{-6}) x (1 x 10^{6}) = 1.04

The number of assays that can be run with the 10 mg of substrate provided depends upon the reaction volumes.

Storage:

Store Granzyme B Fluorogenic Substrate in a desiccator at room temperature or 4°C. Granzyme B Fluorogenic Substrate has a shelf-life of up to 1 year. For long term, 4°C is recommended. DMSO stock solutions have a shelf-life of 6 months if stored at -20°C.

Limitations:
For *in vitro* research use only. Not for use in diagnostics or in humans.

Warranty:
No warranties, expressed or implied, are made regarding the use of this product. KAMIYA BIOMEDICAL COMPANY is not liable for any damage, personal injury, or economic loss caused by this product.