

## PRODUCT DATA SHEET

**Product:** Ac-VEID-AFC (Fluorogenic caspase-1, 3, 6 substrate)

**Cat. No.:** AC-007 (10 mg)

**Chemical Name:**

Ac-Val-Glu-Ile-Asp-AFC

**Molecular Weight:** 727.69

**Description:**

Peptide substrate labeled at the carboxy end with AFC (7-amino-4-trifluoromethyl coumarin). Designed to measure Caspase-6 activity *in vitro*.

**Introduction:**

Caspase-6 (also known as Mch2) is a member of the caspase family of cysteine proteases involved in apoptosis. It is a member of the Group III caspases (6, 8, and 9) which prefer the (L/V)EXD sequence as a substrate. Caspase-6 prefers a hydrophobic amino acid at P4, along with caspases-1 and -4, as opposed to the preference for Asp seen with caspases-2, -3, and -7. This is at odds with the gene sequence alignment that predicts Caspase-6 is more closely related to caspases-3 and -7 than to caspase-1. The preference by Caspase-6 for  $\beta$ -branched amino acids in P4 fits well with the one known natural substrate, lamin A, and distinguishes it from caspases-1 and -4. Reconstitution experiments indicate that Caspase-6 activates caspases-3 and -7 and is therefore part of the proteolytic cascade that initiates apoptosis.

**Principal:**

A synthetic peptide substrate, Ac-Val-Glu-Ile-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 Caspase-6 activity. Caspase-6 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). Caspase-6 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.

**Specificity:**

Highly specific substrate for Caspase-6. May be weak substrate for Caspases-1 and -3.

**Applications:**

For *in vitro* assays of Caspase-6 activity. Can be used with purified or partially purified enzymes or possibly with crude cell lysates (if the Caspase-6 Inhibitor 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 standard curve of fluorescence versus  $\mu$ moles AFC.

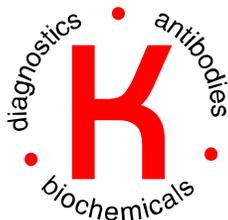
**Samples:** Can be either purified or partially purified enzyme preparations. Application to crude cell lysates has not been confirmed. If crude cell lysates are to be assayed, the non-specific protease background must be determined using our Caspase-6 Inhibitor (Cat. No. AB-007).

**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.

1. Prepare:

- 20 mM fluorogenic caspase-1, 3, 6 substrate (Ac-Val-Glu-Ile-Asp-AFC) stock solution in DMSO. Dilute 1:10 in DMSO.
- 20 mM Caspase-6 Inhibitor (Z-VEID-FMK) stock solution in DMSO. Dilute 1:10 in DMSO.
- Caspase-6 buffer: 100 mM HEPES, 10% sucrose, 10 mM DTT, 0.5 mM EDTA, adjust to pH 7.5 w/ conc. NaOH.



## PRODUCT DATA SHEET

**Cat. No.: AC-007**

**Page 2 of 2**

2. Prepare several dilutions of sample using Caspase-6 buffer (1/10, 1/100, 1/1000).

3. Ideally, each sample dilution should be assayed using three different reactions:

Group 1: Substrate only (blank)

Group 2: Sample + inhibitor + substrate (background protease activity)

Group 3: Sample + substrate (enzyme assay)

4. Prepare standard curve for AFC fluorescence using known amounts of AFC. (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  $\mu$ L Caspase-6 buffer, 10  $\mu$ L 2.0 mM inhibitor solution, vortex, then add 20  $\mu$ L sample. Mix gently, incubate at 4°C for 30 min and then at 37°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  $\mu$ L Caspase-6 buffer, 10  $\mu$ L 2.0 mM substrate solution.

6b. Group 2 reactions: Add 10  $\mu$ L 2.0 mM substrate solution.

6c. Group 3 reactions: Add 470  $\mu$ L Caspase-6 buffer, 10  $\mu$ L 2.0 mM substrate solution, vortex, then add 20  $\mu$ L sample.

7. Groups 1, 2, and 3 : Mix gently, incubate at 4°C for 30 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  $\Delta$ FU for each sample dilution at  $T_1$  as follows:

$$\Delta\text{FU} = [\text{Group 3 FU at } T_1 - \text{Group 1 FU at } T_1] - [\text{Group 3 FU at } T_0 - \text{Group 1 FU at } T_0]$$

10. Calculate enzyme activity in samples for  $T_1$ . If activity is low, assay should be run for a longer time (up to 24 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 Caspase-6 activity = 1  $\mu$ mol of free AFC/min.

Units Caspase-6 =  $[(\Delta\text{FU}/\text{min}) / (\text{std. curve slope})] \times [1 \text{ Unit} / (1 \times 10^{-6} \mu\text{moles AFC}/\text{min})]$

Example calculation:

Dilute an 80  $\mu$ M AFC DMSO stock solution in Caspase-6 buffer to give 0.5 mL final volumes as follows:

1 in 50 dilution =  $8 \times 10^{-4}$   $\mu$ moles AFC

2 in 50 dilution =  $16 \times 10^{-4}$   $\mu$ moles AFC

3 in 50 dilution =  $24 \times 10^{-4}$   $\mu$ moles AFC.

Plot the results with x axis =  $\mu$ mole AFC and y axis = Fluorescence Units (FU).

An example curve gives a slope of  $8 \times 10^{-6}$   $\mu$ moles AFC/FU.

For a  $\Delta\text{FU} = 7.8 (T_1 - T_0)$ ;  $T_1 = 60 \text{ min}$

Units Caspase-6 =  $(7.8/60) \times (8 \times 10^{-6}) \times (1 \times 10^6) = 1.04$

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

### **Storage and Stability:**

Store fluorogenic caspase-1, 3, 6 substrate in a desiccator at -20°C. The Caspase-6 Fluorogenic Substrate has a shelf life of up to 3 years if stored at -20°C. DMSO stock solutions have a shelf life of 1 year if stored at 4°C.

### **References:**

1. Fernandes-Alnemri, T. *et al.* (1995). *Cancer Res.* 55: 2737-42.
2. Takahashi, A., *et al.* (1997). *Oncogene* 14(23): 2741-2752.
3. Thornberry, N. *et al.* (1997). *J. Biol. Chem.* 272(29): 17907-17911.

### **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.