Product Manual

Caspase-8 Drug Discovery Kit

Catalog #: BML-AK715

A complete assay system designed to screen caspase-8 inhibitors.

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Product Manual

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Carefully note the handling and storage conditions of each kit component.

INTRODUCTION

Caspase-8 (also known as FLICE, MACH and Mch5) is a member of the interleukin-1β converting enzyme (ICE) family of cysteine proteases. It is implicated as the apical signaling protease in Fas-induced apoptosis. The enzyme is composed of 18 and 11 kDa subunits derived from a common proenzyme. The N-terminal prodomain of the zymogen comprises two “death effector domains” homologous to those of FADD (Mort1)[1-3]. Activation of the Fas-receptor (Apo-1/CD95), by ligand binding, causes FADD to bind the receptor[4,5]. Procaspase-8 binds the receptor-bound FADD, presumably via interactions between their homologous domains[1,2]. Binding of the proenzyme leads to its proteolytic processing and activation, possibly by autocatalysis or by interaction with a related caspase (e.g. FLICE2/Mch4)[6-8]. An event which lies downstream from caspase-8 activation is proteolytic activation of the caspase-3 proenzyme, possibly through direct cleavage by caspase-8[8,9]. The substrate IETD-pNA is based on the recognition sequence for proteolytic activation in procaspase-3. Those tetrapeptide substrates, which incorporate the IETD sequence, are among those cleaved most efficiently by caspase-8[10] (see also Fig. 3).

The CASPASE-8 Drug Discovery Kit is a complete assay system designed to screen caspase-8 inhibitors. It contains both a colorimetric substrate (IETD-pNA) and a fluorogenic substrate (IETD-AMC). Cleavage of the p-nitroanilide (pNA) from the colorimetric substrate increases absorption at 405nm. The fluorescent assay is based on the cleavage of 7-amino-4-methylcoumarin (AMC) dye from the C-terminus of the peptide substrate. Cleavage of the dye from the substrate increases its fluorescence intensity at 460 nm. The assays are performed in a convenient, 96-well microtiter-plate format. The kit is useful to screen inhibitors of caspase-8, a potential therapeutic target. An inhibitor, IETD-CHO (aldehyde), is included for use as a control.

MATERIALS SUPPLIED

BML-SE172-5000 CASPASE-8 ENZYME (HUMAN, RECOMBINANT)

FORM: 100 U/µl in assay buffer. One U=1 pmol/min@30°C, 200 µM IETD-pNA. Purity >90% by SDS-PAGE
STORAGE: -80°C; AVOID FREEZE/THAW CYCLES!
QUANTITY: 5000 U (50 µl)

BML-P431-9090 pNA SUBSTRATE (Ac-IETD-pNA; MW=639)

FORM: 2 mM (1.3 mg/ml) in assay buffer
STORAGE: -80°C
QUANTITY: 1 ml

BML-KI106-0001 CALIBRATION STANDARD (p-nitroaniline; MW=138)

FORM: 50 µM in assay buffer. A_{405nm}=0.525 cm^{-1}
STORAGE: -80°C
QUANTITY: 1 ml

BML-P432-9090 AMC SUBSTRATE (Ac-IETD-AMC; MW=674)

FORM: 0.75 mM (0.5 mg/ml) in assay buffer
STORAGE: -80°C
QUANTITY: 1 ml

BML-KI107-0001 AMC CALIBRATION STANDARD

(7-amino-4-methylcoumarin; MW=175)
FORM: 30 µM in assay buffer
STORAGE: -80°C
QUANTITY: 1 ml

BML-P430-9090 INHIBITOR (Ac-IETD-CHO; MW=502.5)

FORM: 0.1 mM (0.05 mg/ml) in DMSO
STORAGE: -80°C
QUANTITY: 50 µl

BML-KI111-0020 ASSAY BUFFER

(50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol)
FORM: Liquid in screw-cap plastic bottle
STORAGE: -80°C
QUANTITY: 20 ml

80-2404 1/2 VOLUME MICROPLATE

1 clear, 96-well
STORAGE: Ambient

Storage temp

STORAGE & STABILITY

Store all components except the microtiter plate at -80°C for the highest stability. The caspase-8 enzyme component, BML-SE172, must be handled particularly carefully in order to retain maximum enzymatic activity. Defrost it quickly in a RT water bath or by rubbing between fingers, then immediately store in an ice bath. The remaining, unused enzyme should be quickly refrozen by placing at -80°C. The enzyme is stable to at least 4 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the caspase-8 into separate tubes and store at -80°C.

ADDITIONAL MATERIALS NEEDED

- Microplate reader capable of measuring A405 to ≥3-decimal accuracy, or fluorescence at wavelengths of approximately 360nm (excitation)/ 460nm (emission).
- Pipette or multi-channel pipette capable of pipetting 10-100 µl accurately (note: dilution of reagents can be made to increase the minimal volume to >10 µl).
- Ice bucket to keep reagents cold until use.

SAFETY WARNINGS & PRECAUTIONS

Important/ Warning

1. Wear appropriate personnel protective apparel. Avoid contact with clothes and exposed skin. In case of accidental skin exposure, flush with water immediately. Consult a physician if required.
2. Use a safety pipetting device for all pipetting. Never pipet by mouth.
3. Interpretation of the results is the sole responsibility of the user.

PROCEDURE

To start assay:

1. Defrost all kit components and hold on ice bath until use. All components are highly stable for several hours in an ice bath.
2. Dilute inhibitor (Ac-IETD-CHO) 1/200 in assay buffer. (Note: Bring the tube of Ac-IETD-CHO to room temperature to thaw.) Example: Add 1 µl inhibitor to 200 µl assay buffer, in a separate tube.
3. Dilute caspase-8, 1/50 in assay buffer to required quantity (25 µl is needed per well). For example, dilute 5 µl caspase-8 into 245 µl assay buffer.
4. Dilute the Ac-IETD-pNA or Ac-IETD-AMC substrate in Assay Buffer to 2X the desired final concentration. For example, dilute the Ac-IETD-pNA to 400µM (final 200µM) or Ac-IETD-AMC to 150µM (final 75µM). Equilibrate the dilution to assay temperature, e.g. 30°C.
5. Add assay buffer to each desired well of the 1/2 volume microtiter plate as follows:

   Blank (no caspase-8)=50 µl

   Control (no inhibitor)=25 µl

   Inhibitor (will receive 25 µl diluted IETD-CHO)=0 µl
6. Test sample=varies (see Table 1)
7. Allow microtiter-plate to equilibrate to assay temperature (e.g.: 30°C).
8. Add 25 µl caspase-8 (diluted in step 3) to the control, inhibitor and test sample wells. Final amount of caspase-8 will be 50 U per well. DO NOT ADD CASPASE-8 TO BLANKS!
9. Add 25 µl inhibitor (diluted in step 2) to the inhibitor wells only! Final inhibitor concentration=0.1 µM.

10. Add desired volume of test sample(s) to appropriate well(s). See Table 1.
11. Incubate plate for 10 min at reaction temperature (or as desired) to allow inhibitor/enzyme interaction.
12. Start reaction by the addition of 50 µl Ac-IETD-pNA substrate or 50 µl Ac-IETD-AMC substrate (equilibrated to reaction temperature, e.g.: 30°C). Final substrate concentration=200 µM with the pNA Substrate and 75 µM with the AMC Substrate.
13. Read plate continuously, at A_{405nm} for the pNA substrate or fluorescence for the AMC substrate, in a microtiter-plate reader. For example, record data at 1 min. intervals for a total of 10 to 60 min.
14. Perform data analysis (see below).

NOTE: Retain microplate for future use of unused wells!

TABLE 1. ASSAY MIXTURE EXAMPLES.

Sample	Assay buffer	Caspase-8 (2 U/µl)	Inhibitor	Substrate
Blank	50 µl	0	0	50 µl
Control	25 µl	25 µl	0	50 µl
Inhibitor	0 µl	25 µl	25 µl	50 µl
Test sample*	X µl	25 µl	Y µl	50 µl

*Test sample is the experimental inhibitor. Dissolve/dilute inhibitor into assay buffer and add to appropriate wells at desired volume “Y”. Adjust volume “X” to bring the total volume to 100 µl.

DATA ANALYSIS

Plotting

1. Plot data as A_{405nm} or Arbitrary Fluorescence Units (AFU) versus time for each sample.
2. Determine the time points in which the reaction is linear. Typically, points from 1 to 15 min are sufficient.
3. Obtain the slope of a line fit to the data using an appropriate routine.
4. Average the slopes of duplicate samples.

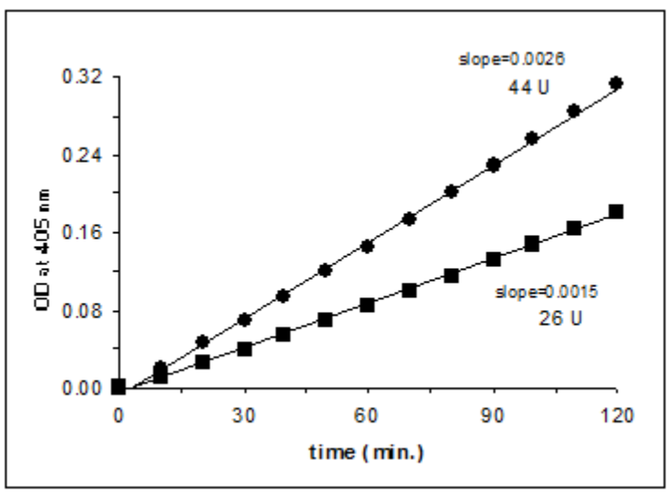

Figure 1. Caspase-8 activity is stable for at least 2 hrs. under the conditions of the IETD-pNA cleavage assay. [IETD-pNA]=200 µM; 25°C

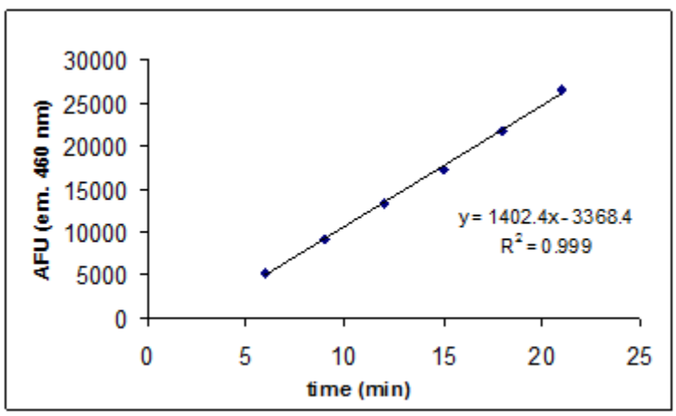

Figure 2. Assay of Caspase-8 activity using the AMC substrate. 30 U /well; [IETD-AMC]=75 µM; 37°C

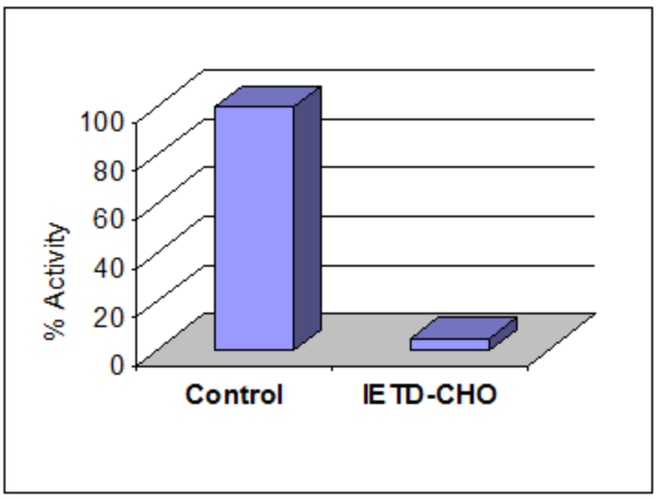

Figure 3. Inhibition of Caspase-8 by Ac-IETD-CHO. The enzyme was incubated with the inhibitor for 10 minutes prior to addition of substrate. 30 U/well; [IETD-CHO]=0.1µM; [IETD-pNA]=200µM; 25°C

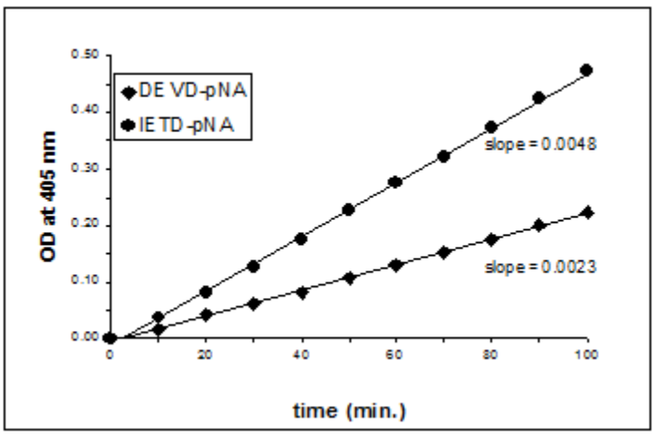

Figure 4. Caspase-8 cleaves IETD-pNA more rapidly than DEVD-pNA. 82 U/well; [Substrate]=200 µM; Temp=25°C

Data Reduction

1. If the blank has a significant slope, subtract this number from all samples. Under normal circumstances this will not be necessary as the slope will be nearly 0.

2. <u>To find % remaining activity in presence of inhibitor</u>:
   % activity remaining (plus inhibitor) =
   (slope of + inhibitor sample/control slope) x 100

3. <u>To find the activity of the samples expressed as pmol substrate/min</u>:
   Determine microtiter-plate reader conversion factor:

   a) Add 100 µl of pNA Calibration Standard (50 µM concentration) to 2 wells of the 1/2 volume microtiter plate.

b) Determine the average A_{405nm} using 100 µl assay buffer as a blank.

c) Calculate the conversion factor. The calculation is based on the concentration of calibration standard of 50 µM (A_{405nm}=0.525 cm^{-1}):

conversion factor (µM/OD) =50/average A_{405nm} from step b)

d) Calculate the activity as pmol/min:

activity (pmol/min) =
slope (OD/min) x conversion factor (µM/OD) x assay vol (µl)

The assay vol in µl = 100 for the standard assay.

Example calculation for activity:

conversion factor = 50 µM/0.294 OD = 170 µM/OD

activity of a control sample =
1.76E-03 (OD/min) x 170(µM/OD) x 100(µl) = 30 pmol/min

***A note about the AMC calibration standard**

The exact AMC concentration range that will be useful for preparing a standard curve will vary depending on the fluorimeter model, the gain setting and the exact excitation and emission wavelengths used. The AMC standard, as provided (30 µM), may yield off-scale readings in some cases. We recommend diluting some of the standard to a relatively low concentration with Assay Buffer (0.5 or 1.0 µM) and then measuring the fluorescence of 100 µl. The estimate of AFU/µM obtained with this measurement; together with the observed range of values obtained in the enzyme assays can then be used to plan an appropriate series of dilutions for a standard curve.

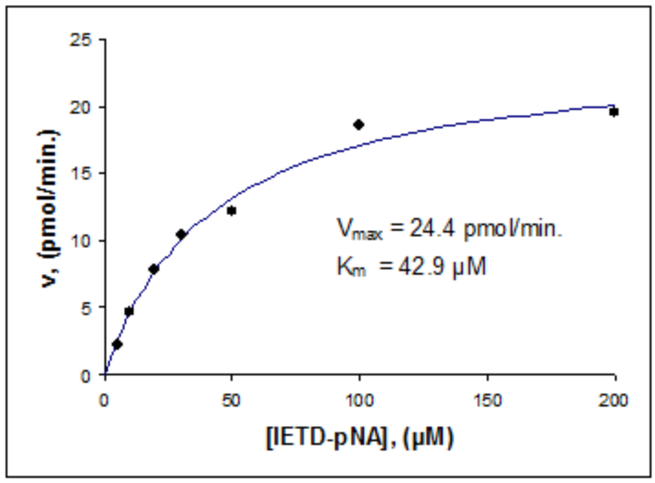

Figure 5. Kinetics of IETD-pNA cleavage by Caspase-8. 20 U/well; 25°C. Rates were obtained from the slopes of the initial, linear portion of plots of A_{405} vs. time. Each point is the mean of four determinations. Curve and kinetic parameters derive from a non-linear least squares fit to the Michaelis-Menten equation (Marquadt algorithm).

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NOTES

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