Enabling Discovery in Life Science®

Caspase-3 Drug Discovery Kit

A complete assay system designed to screen caspase-3 inhibitors.

Instruction Manual
BML-AK700

For research use only

12/23/2011

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TRADEMARKS AND PATENTS

Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending.

✦CASPASE-3 Drug Discovery Kit – BML-AK700✦

BACKGROUND

Caspase-3 (also known as CPP32, apopain and Yama) is a member of the interleukin-1β converting enzyme (ICE) family of cysteine proteases. The enzyme is composed of 17 and 12 kDa subunits derived from a common proenzyme, pro-caspase-3[1,2,3]. Caspase-3 is activated during apoptotic signaling events by upstream proteases including caspase-6, caspase-8 (FLICE)[4] and cytotoxic T-cell-derived granzyme B[5]. Targets of caspase-3 cleavage include poly(ADP-ribose) polymerase (PARP)[6], nuclear lamins[7] and others.

The *CASPASE-3 Assay Kit for Drug Discovery* is a complete assay system designed to screen caspase-3 inhibitors. It contains both a colorimetric substrate (DEVD-pNA) and a fluorogenic substrate (DEVD-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 microplate format. The kit is useful to screen inhibitors of caspase-3, a potential therapeutic target[8]. An inhibitor, DEVD-CHO (aldehyde), is also included as a prototypic control inhibitor[1]. The DEVD amino acid sequence is derived from the caspase-3 cleavage site in PARP.

REFERENCES:

1. D.W. Nicholson *et al. Nature* 1995 **376** 37
2. T. Fernandes-Alnemri *et al. J. Biol. Chem.* 1994 **269** 30761
3. M. Tewari *et al. Cell* 1995 **81** 801
4. S.M. Srinivasula *et al. Proc. Natl. Acad. Sci. USA* 1996 **93** 14486
5. A.J. Darmon *et al. Nature* 1995 **377** 446
6. J. Schlegel *et al. J. Biol. Chem.* 1996 **271** 1841
7. Y.A. Lazebnik *et al. Proc. Natl. Acad. Sci. USA* 1995 **92** 9042
8. D.W. Nicholson *Nature Biotechnol.* 1996 **14** 297

PLEASE READ ENTIRE BOOKLET BEFORE PROCEEDING WITH THE ASSAY. CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF EACH KIT COMPONENT. PLEASE CONTACT ENZO LIFE SCIENCES TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.

COMPONENTS OF BML-AK700 KIT

BML-SE169-5000 CASPASE-3 ENZYME (HUMAN, RECOMBINANT)
FORM: 100 U/µl in assay buffer. One U=1 pmol/min@30°C, 200 µM DEVD-pNA. Purity >95% by SDS-PAGE
STORAGE: -70°C; AVOID FREEZE/THAW CYCLES!
QUANTITY: 5000 U (50 µl)

BML-P412-9090 pNA SUBSTRATE (Ac-DEVD-pNA; MW=637)
FORM: 2 mM (1.3 mg/ml) in assay buffer
STORAGE: -70°C
QUANTITY: 1 ml

BML-KI106-0001 pNA CALIBRATION STANDARD
(p-nitroaniline;MW=138.1)
FORM: 50 µM in assay buffer. A_{405nm}=0.525 cm^{-1}
STORAGE: -70°C
QUANTITY: 1 ml

BML-P411-9090 AMC SUBSTRATE (Ac-DEVD-AMC; MW=676)
FORM: 0.3 mM (0.20 mg/ml) in assay buffer
STORAGE: -70°C
QUANTITY: 1 ml

BML-KI107-0001 AMC CALIBRATION STANDARD
(7-amino-4-methylcoumarin; MW=175)
FORM: 30 µM in assay buffer
STORAGE: -70°C
QUANTITY: 1 ml

BML-P410-9090 INHIBITOR (Ac-DEVD-CHO; MW=502)
FORM: 0.1 mM (0.05 mg/ml) in DMSO (dimethylsulfoxide)
STORAGE: -70°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)
STORAGE: -70°C
QUANTITY: 20 ml

80-2404 ½ -VOLUME MICROPLATE
1 clear, 96-well
STORAGE: Room temperature

OTHER MATERIALS REQUIRED

Microplate reader capable of measuring A_{405} to ≥3-decimal accuracy, or fluorescence at wavelengths of approximately 360nm (excitation)/ 460nm (emission)
Pipettor or multi-channel pipettor 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

EXPERIMENTAL METHODS

Note on storage: Store *all components except the microplate at -70°C for the highest stability. The caspase-3 enzyme component, BML-SE169, must be handled particularly carefully in order to retain maximal enzymatic activity. Defrost it quickly in a RT water bath or by rubbing between fingers, then immediately store on an ice bath. The remaining unused enzyme should be quickly refrozen by placing at -70°C. The enzyme is stable to freeze/thaw cycles x 4. To minimize the number of freeze/thaw cycles, aliquot the caspase-3 into separate tubes and store at -70°C.*

To start assay:

1. Defrost all kit components and hold on ice bath until use. All components are highly stable for several hours on an ice bath.
2. Dilute inhibitor (Ac-DEVD-CHO; Note: Briefly warm to RT to thaw) 1/200 in assay buffer as follows. Add 1 µl inhibitor into 200 µl assay buffer, in a separate tube.
3. Dilute caspase-3, 1/50 in assay buffer to required quantity (25 µl are needed per well). For example, 5 µl caspase-3 into 245 µl assay buffer, in a separate tube.

4. Dilute the Ac-DEVD-pNA or Ac-DEVD-AMC substrate in Assay Buffer to 2x the desired final concentration. For example, dilute Ac-DEVD-pNA to 400µM (final 200µM) or Ac-DEVD-AMC to 60µM (final 30µM). Equilibrate the dilution to assay temperature, e.g. 37°C.

5. Add assay buffer to each desired well of the ½-volume microplate as follows:
   - Blank (no caspase-3)=50 µl
   - Control (no inhibitor)=25 µl
   - Inhibitor (will receive 25 µl diluted DEVD-CHO)=0 µl
   - Test sample=varies (see Table 1)
6. Allow microplate to equilibrate to assay temperature (e.g.: 30°C).
7. Add 25 µl caspase-3 (diluted in step 3) to the control, inhibitor and test sample wells. Final amount of caspase-3 will be 50 U per well. DO NOT ADD CASPASE-3 TO BLANKS!
8. Add 25 µl inhibitor (diluted in step 2) to the inhibitor wells only! Final inhibitor concentration=0.1 µM.
9. Add desired volume of test sample to appropriate wells. See Table 1.
10. Incubate plate for 10 min at reaction temperature (or as desired) to allow inhibitor/enzyme interaction.
11. Start reaction by the addition of 50 µl Ac-DEVD-pNA substrate or 50 µl Ac-DEVD-AMC substrate (equilibrated to reaction temperature, e.g.: 30°C). Final substrate concentration=200 µM with the pNA Substrate and 30 µM with the AMC Substrate.
12. Read plate continuously, at A_{405nm} for the pNA substrate or fluorescence for the AMC substrate, in a microplate reader. For example, record data at 1 min. intervals for a total of 10 to 60 min.
13. Perform data analysis (see below).

NOTE: Retain microplate for future use of unused wells!

TABLE 1. ASSAY MIXTURE EXAMPLES.

Sample	Assay buffer	Caspase-3 (2 U/µl)	Inhibitor	Substrate	Total Volume
Blank	50 µl	0	0	50 µl	100 µl
Control	25 µl	25 µl	0	50 µl	100 µl
Inhibitor	0 µl	25 µl	25 µl	50 µl	100 µl
Test sample*	X µl	25 µl	Y µl	50 µl	100 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.

DATA REDUCTION

5. 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.
6. To find inhibitor % remaining activity:
   % inhibitor activity remaining=
   (inhibitor slope/control slope) x 100

7. To find the activity of the samples expressed as pmol substrate/min:
   Determine microplate reader conversion factor:
   a) Add 100 µl of pNA Calibration Standard (50 µM concentration) to 2 wells of the ½-volume microplate. The extinction coefficient for p-nitroaniline at 405 nm is ~10,500 $M^{-1}cm^{-1}$. Typically, 100 µl of the 50 µM standard, in a ½ volume well, produces an A_{405} of about 0.3 (or 300 mOD)
   b) Determine the average A_{405nm} using 100 µl assay buffer as a blank.
   c) Calculate the conversion factor.

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

   d) Calculate the activity as pmol/min:
   activity (pmol/min) =
   slope (mOD/min) x conversion factor(µM/mOD)
   x assay vol (µl)

   The assay vol in µl = 100 for the standard assay. NOTE: If a different volume is used, be sure to perform steps a) to d) using the actual assay volume.

Sample activity calculation:

conversion factor = 50 µM/294 mOD = 0.170 µM/mOD

Example: sample for which the slope of the $A_{405\ nm}$ vs. time plot is 1.7 (mOD/min)
activity =
1.7 (mOD/min) x 0.170(µM/mOD) 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.

APPLICATION EXAMPLES

The *CASPASE-3 Assay Kit for Drug Discovery* can be used to study enzyme regulation and kinetics, cleave target substrates and screen inhibitors of caspase-3.

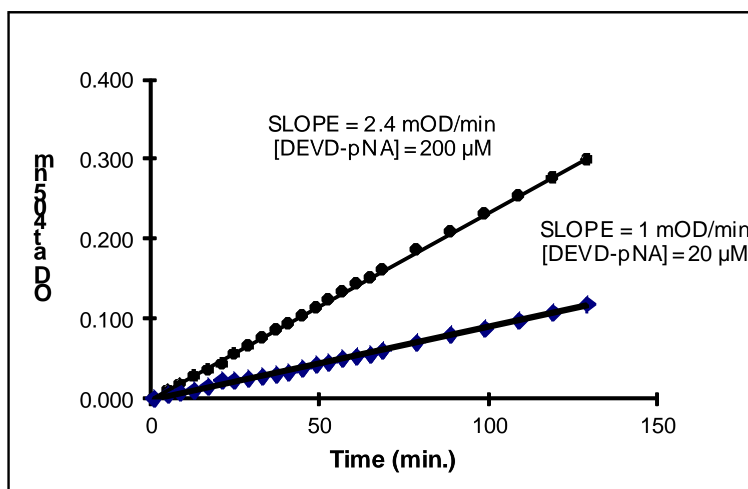

Figure 1. Caspase-3 activity is stable for at least 120 min. under the conditions of the DEVD-pNA cleavage assay. [DEVD-pNA]=200 µM and 20 µM; 25°C

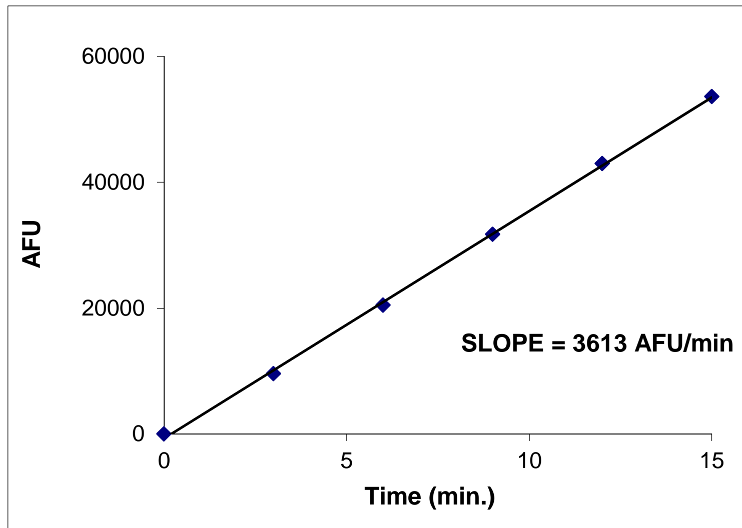

Figure 2. Assay of Caspase-3 activity using the AMC substrate. 30 U /well; [DEVD-AMC]=30 μM; 25°C

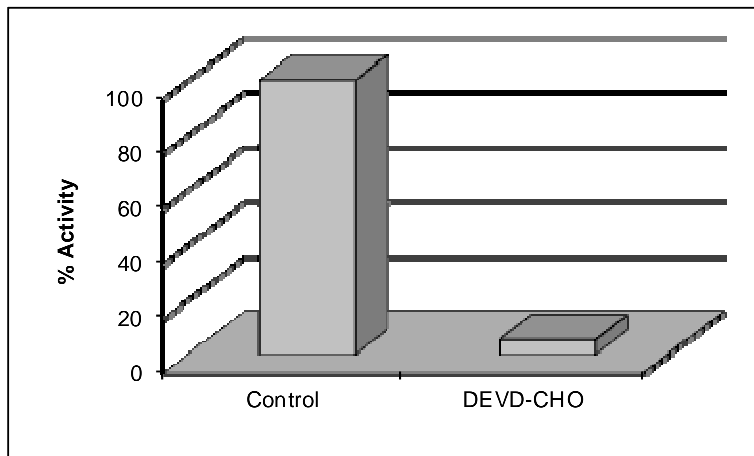

Figure 3. Inhibition of Caspase-3 by Ac-DEVD-CHO. The enzyme was incubated with the inhibitor for 10 minutes prior to addition of substrate. 30 U/well; [DEVD-CHO]=0.1 μM; [DEVD-pNA]=200 μM; 25°C

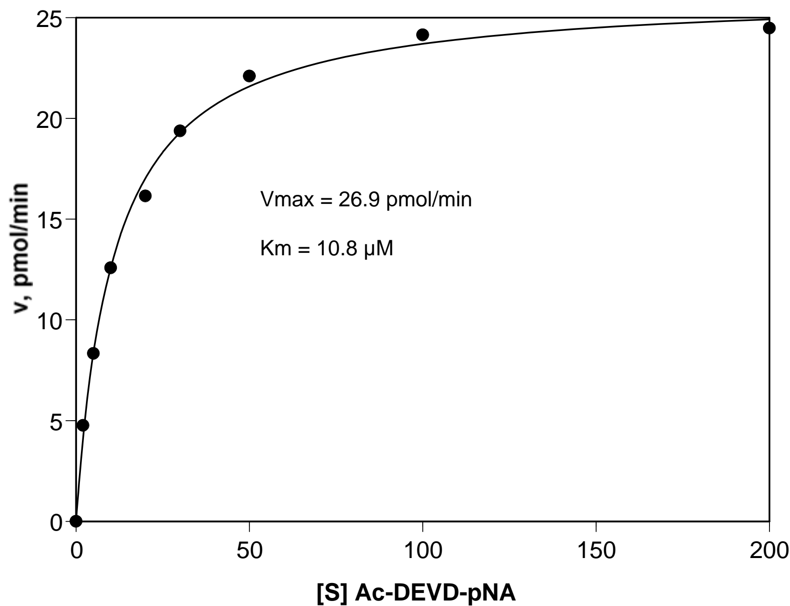

Figure 4. Kinetics of DEVD-pNA cleavage by Caspase-3. 25 U/well; 25°C. Rates were obtained from the slopes of the initial, linear portion of plots of A_{405} vs. time. Curve and kinetic parameters derive from a non-linear least squares fit to the Michaelis-Menten equation (Marquadt algorithm).

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