



Enabling Discovery in Life Science®

CASPASE-3 Cellular Assay Kit PLUS

Designed to measure caspase-3 and caspase-3-like (cellular DEVDase) activity in cell extracts.

Instruction Manual

BML-AK703

For research use only

USE FOR RESEARCH PURPOSES ONLY

Unless otherwise specified expressly on the packaging, all products sold hereunder are intended for and may be used for research purposes only and may not be used for food, drug, cosmetic or household use or for the diagnosis or treatment of human beings. Purchase does not include any right or license to use, develop or otherwise exploit these products commercially. Any commercial use, development or exploitation of these products or development using these products without the express written authorization of Enzo Life Sciences, Inc. is strictly prohibited. Buyer assumes all risk and liability for the use and/or results obtained by the use of the products covered by this invoice whether used singularly or in combination with other products.

LIMITED WARRANTY; DISCLAIMER OF WARRANTIES

These products are offered under a limited warranty. The products are guaranteed to meet all appropriate specifications described in the package insert at the time of shipment. Enzo Life Sciences' sole obligation is to replace the product to the extent of the purchasing price. All claims must be made to Enzo Life Sciences, Inc., within five (5) days of receipt of order. THIS WARRANTY IS EXPRESSLY IN LIEU OF ANY OTHER WARRANTIES OR LIABILITIES, EXPRESS OR IMPLIED, INCLUDING WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, AND NONINFRINGEMENT OF THE PATENT OR OTHER INTELLECTUAL PROPERTY RIGHTS OF OTHERS, AND ALL SUCH WARRANTIES (AND ANY OTHER WARRANTIES IMPLIED BY LAW) ARE EXPRESSLY DISCLAIMED.

TRADEMARKS AND PATENTS

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

✦ **CASPASE-3 Cellular Assay Kit PLUS – BML-AK703** ✦

BACKGROUND

Caspase-3 (also known as CPP32, apopain and Yama) is a member of the interleukin-1 β 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}. The caspase-3 is activated during apoptotic signaling events by upstream proteases including caspase-6, caspase-8 (FLICE)⁴ and cytotoxic T-cell-derived granzyme B⁵. Caspase-3 is one of the principal caspase activities found in apoptotic cells⁶. Targets of caspase-3 cleavage include poly(ADP-ribose) polymerase (PARP)⁷, nuclear lamins⁸, gelsolin⁹ and others. Caspase-3 is a potential therapeutic target¹⁰.

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. L. Faleiro *et al. EMBO J.* 1997 **16** 2271
7. J. Schlegel *et al. J. Biol. Chem.* 1996 **271** 1841
8. Y.A. Lazebnik *et al. Proc. Natl. Acad. Sci. USA* 1995 **92** 9042
9. S. Kothakota *et al. Science* 1997 **278** 294
10. D.W. Nicholson *Nature Biotechnol.* 1996 **14** 297

The *CASPASE-3 Cellular Assay Kit PLUS* is a complete assay system designed to measure caspase-3 and caspase-3-like (cellular DEVDase) activity in cell extracts. It comes in a convenient 96-well format with all reagents necessary for preparing cell extracts, measuring caspase activity colorimetrically and/or fluorometrically and calibrating the assay. Plus, *caspase-3 enzyme* is included for use as a positive control and testing cell extracts for endogenous inhibitors. If the kit is being used to screen agents for their effect on the cellular caspase activity, the purified enzyme may also provide a ready means to check for direct effects on caspase-3.

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-AK703 KIT

BML-SE169-9090 CASPASE-3 ENZYME (HUMAN, RECOMBINANT)

FORM: 10 U/ μ L in assay buffer. One U=1 pmol/min at 30°C, 200 μ M DEVD-pNA. Purity >95% by SDS-PAGE

STORAGE: -70°C; AVOID FREEZE/THAW CYCLES!

QUANTITY: 300 U, (30 μ L)

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)

FORM: 50 μ M in assay buffer. $A_{405\text{nm}}=0.525 \text{ cm}^{-1}$

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 μ M 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 μ L

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: -70°C

QUANTITY: 20 mL

BML-KI117-0030 CELL LYSIS BUFFER

(50 mM HEPES, pH 7.4, 0.1% CHAPS, 5 mM DTT, 0.1 mM EDTA)

FORM: Liquid in white screw-cap plastic bottle

STORAGE: -70°C

QUANTITY: 30 mL.

80-2410 ½-VOLUME MICROPLATES

1 clear, 1 black, 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 $>10 \mu\text{L}$)

Ice bucket to keep reagents cold until use

Cultured cells induced for apoptosis

Centrifuge

Phosphate buffered saline

EXPERIMENTAL METHODS

To prepare cell extracts:

1. Grow cell cultures and induce apoptosis as desired. (See enclosed list of apoptosis inducers or contact Enzo Life Sciences technical service for assistance.). Appropriate controls may include untreated cells, cells treated with an inactive chemical analog of the apoptosis-inducer or simply the “time-zero” sample from an apoptosis induction time course.

The number of cells required for an experiment must be determined by the user. A sufficient quantity is needed to assay caspase-3 activity, plus additional material to determine protein concentration (optional). As a guide, here is an example of data obtained with U937 cells:

- Cell density (at lysis)= $\sim 2 \times 10^7$ cells/mL
- Protein concentrations=1-3 mg/mL
- 10 μL assay sample= $\sim 2 \times 10^5$ cells or 10-30 μg protein

- DEVD-pNA cleavage assays: 10 μ L samples; 37°C; OD's at 405 nm after 30 min.-control=0.004, apoptotic=0.22

It is desirable to have enough extract to perform duplicate assays, add an inhibitor-treated control, determine protein concentration, etc. Thus, for U937 cells, a minimum of 10^6 cells in 50 μ L for each test condition is suggested. Other cell types may have other requirements.

2. Count cells and harvest by centrifugation (e.g.: 1000 x g, 4°C, 10 min). Wash cells 1x with phosphate buffered saline (PBS). If the cells have been treated with a reagent which may interfere with the subsequent caspase assay (e.g. a potential caspase inhibitor), it may be desirable to wash cells more extensively prior to lysis.

3. Resuspend cells to desired concentration (e.g.: 2×10^7 /mL) with ice-cold CELL LYSIS BUFFER. Incubate 5 min on ice bath. If cell lysis is incomplete, additional detergent may be added to the CELL LYSIS BUFFER to assist membrane solubilization/destabilization. Addition of Tween 20, NP-40 or Triton X-100 to a final concentration of 0.1% is compatible with the subsequent caspase assay.

4. Centrifuge at 10,000xg, 10 min @ 4°C.

5. Save supernatant (cytosolic extract) and hold on ice bath until use. Alternatively, the extracts may be quickly frozen and stored at -70°C for later use.

Note on storage: Store all components at -70°C for the highest stability, except store the microplate at RT. The caspase-3 enzyme component (BML-SE169-9090) must be handled with particular care in order to retain maximal enzymatic activity. Thaw 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 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 several tubes and store at -70°C.

To start assay:

1. Thaw all kit components and hold on ice bath until use. All kit components are highly stable for several hours on an ice bath. If using frozen cell extracts, handle in the same manner as caspase-3.

2. Dilute inhibitor (Ac-DEVD-CHO; Note: Briefly warm to RT to thaw) 1/200 in assay buffer as follows. Add 1 μ L inhibitor to 200 μ L assay buffer, in a separate tube. This solution contains 0.5 μ M Ac-DEVD-CHO and will serve as a 5x stock for preparing inhibitor-treated controls.

3. For colorimetric assay (at A405nm, using clear microplate): Dilute substrate (Ac-DEVD-pNA) in Assay Buffer to 2x the desired final concentration. For example, dilute Ac-DEVD-pNA to 400 μ M (final 200 μ M).

4. For fluorimetric assay (at Ex:360 nm/Em:460 nm, using black microplate): Dilute substrate (Ac-DEVD-AMC) in Assay Buffer to 2x the desired final concentration. For example, Ac-DEVD-AMC to 60 μ M (final 30 μ M).

5. Equilibrate the substrate dilution to assay temperature, e.g. 37°C.

6. Dilute caspase-3, 1/5 in assay buffer to required quantity (15 μ L=30 U are needed per well). Example: Add 10 μ L caspase-3 to 40 μ L assay buffer to make 3 samples.
7. Add assay buffer to each well of the 1/2-volume microplate as required. The final volume of each reaction will be 100 μ L. Table 1 lists examples with the reagent volumes needed for a variety of assay types.

The “Blank” and “Cell Extract” samples are essential for determining cellular activity. Two additional controls are highly recommended: 1) “Inhibitor-Treated Cell Extract” to measure nonspecific hydrolysis of DEVD-pNA or DEVD-AMC (see Figure 5, DEVD-CHO-insensitive activity); 2) “Purified Caspase-3” which provides a positive control and standard with which to compare cellular activities (see Figure 1).

The two “Test Sample” assays listed in Table 1 illustrate a format that may be useful for inhibitor screening/drug discovery work. Endogenous inhibitory activity in cell extracts may be assessed by comparing the rate obtained in the “Cell Extract/Purified Caspase-3” assay with the rates obtained separately with the cell extract and purified enzyme.
8. Allow the microplate to equilibrate to assay temperature (e.g.: 37°C). NOTE: *The assays illustrated in Figs. 1-5 were performed at 37°C. Similar data were obtained at 25°C, but rates of DEVD-pNA cleavage were ~2/3 of those obtained with the same samples at 37°C.*
9. Add 10 μ L of cell extracts and/or 15 μ L caspase-3 (diluted in step 4) to the appropriate wells (see Table 1). DO NOT ADD CELL EXTRACTS OR CASPASE-3 TO BLANKS!
10. Add test sample and/or 20 μ L of inhibitor (diluted in step 2; final concentration=0.1 μ M) to the appropriate wells (see Table 1).
11. Incubate plate at assay temperature for 10 min (or as desired) to allow inhibitor/enzyme interaction.
12. Start reaction by the addition of 50 μ L Ac-DEVD-pNA substrate or 50 μ L Ac-DEVD-AMC substrate (equilibrated to reaction temperature, e.g.: 30°C). Final substrate concentration=200 μ M with the pNA Substrate and 30 μ M with the AMC Substrate.

TABLE 1. ASSAY MIXTURE EXAMPLES

SAMPLE	Assay buffer	Cell Extract	Caspase-32 U/ μ l	Inhibitor	Substrate
Blank	50 μ L	0	0	0	50 μ L
Cell Extract	40 μ L	10 μ L	0	0	50 μ L
Inhibitor-Treated	20 μ L	10 μ L	0	20	50 μ L
Purified Caspase-	35 μ L	0	15 μ L	0	50 μ L
*Test Sample/Cell	X μ L	10 μ L	0	Y μ L	50 μ L
*Test	X μ L	0	15 μ L	Y μ L	50 μ L
Cell Extract/	25 μ L	10 μ L	15 μ L	0	50 μ L

*Test sample refers to an experimental inhibitor/activator. 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 μ L.

13. Read plate continuously, at $A_{405\text{nm}}$ 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.

14. Perform data analysis (see below).

NOTE: Retain microplate for future use of unused wells.

DATA ANALYSIS

PLOTTING

1. Plot data as $A_{405\text{nm}}$ or Arbitrary Fluorescence Units (AFU) versus time for each sample.
2. For each sample determine the length of the initial time period, over which the plot of OD (or AFU) vs. time remains linear, and there is sufficient ΔOD (or ΔAFU) to obtain an accurate slope. The initial pNA substrate concentration (200 μM DEVD-pNA) is saturating for caspase-3 ($K_m = 10 \mu\text{M}$). For many samples the rate of DEVD-pNA cleavage will remain constant for 2 hrs. or more. Highly active samples, however, can reduce the substrate concentration to sub-saturating levels in substantially less time. In such cases, choose data from the earlier, linear portion of the time course for use in the slope calculation of Step 3. (For an example, see the "Etop.-4" data in Fig. 1). Since the AMC substrate is used at a sub-saturating concentration (30 μM), extra care must be taken in choosing data that lies within the linear portion of the reaction progress curve.
3. Obtain the slope of the line, fitted to the linear portion of the data, using an appropriate linear regression program.
4. Average the slopes of replicate 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 since the slope will be nearly 0.
6. To find the activity of the samples expressed as pmol substrate/min:

Determine microplate reader conversion factor:

- a) Add 100 μL (assay volume) calibration standard (50 μM p-nitroaniline in assay buffer) to 2 wells of the $\frac{1}{2}$ -volume microplate.
- b) Determine the average $A_{405\text{nm}}$ using 100 μL (assay volume) assay buffer as a blank.
- c) Calculate the conversion factor. The calculation is based on the concentration of p-nitroaniline in the calibration standard (50 μM). The extinction coefficient for p-nitroaniline in assay buffer is 10,500 $\text{M}^{-1}\text{cm}^{-1}$.

conversion factor ($\mu\text{M}/\text{mOD}$) =

$50 \mu\text{M} \div \text{average } A_{405\text{nm}} \text{ from step b}$

d) Calculate the activity as pmol/min:

$$\begin{aligned} \text{activity (pmol/min)} &= \\ &\text{slope (mOD/min)} \times \text{conversion factor } (\mu\text{M/mOD}) \\ &\times \text{assay vol } (\mu\text{L}) \end{aligned}$$

Assay volume is 100 μl for the standard assay (Table 1). NOTE: If a different volume is used, be sure to perform steps a) to d) using the actual assay volume.

Sample activity calculation:

$$\text{conversion factor} = 50 \mu\text{M} \div 294 \text{ mOD} = 0.170 \mu\text{M/mOD}$$

Example: sample for which the slope of the $A_{405 \text{ nm}}$ vs. time plot is 3.4 (mOD/min)

activity =

$$3.4 \text{ (mOD/min)} \times 0.170(\mu\text{M/mOD}) \times 100(\mu\text{l}) = 58 \text{ pmol/min}$$

7. Specific Activity Calculations

a) To normalize activities with respect to cell number, divide the values calculated in step 6 by the number of cells suspended in 10 μl of lysis buffer. (see Experimental Methods, steps 1-3):

$$\begin{aligned} \text{Sp. Act. (pmol/min/cell \#)} &= \\ &\text{activity (pmol/min)} / \# \text{ of cells per well} \end{aligned}$$

b) To calculate specific activities with respect to total protein, determine the protein content of each cell extract. Divide activities by the protein content of the corresponding sample (see Figures 1 and 2):

$$\begin{aligned} \text{Sp. Act. (pmol/min}/\mu\text{g protein)} &= \\ &\text{Activity (pmol/min)} / \mu\text{g protein} \end{aligned}$$

NOTE: *The cell lysis buffer is compatible with Coomassie dye binding (Bradford) and bicinchoninic acid (BCA) based protein assays under appropriate conditions. For the BCA assay, cell extract samples must be diluted ≥ 10 -fold in water. Increased backgrounds may be exhibited with the BCA assays due to the increased DTT concentration. Cell extracts diluted ≥ 10 -fold with water are considered compatible according to the manufacturer (i.e. Pierce BCA Protein Assay Reagent). Be sure to use cell lysis buffer in the standards and maintain equal amounts in all samples.*

*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 Cellular Activity Assay Kit* can be used to confirm apoptosis in cell extracts by detecting caspase-3 and caspase-3-like (cellular DEVDase) activity, one of the principal caspase activities found in apoptotic cells.

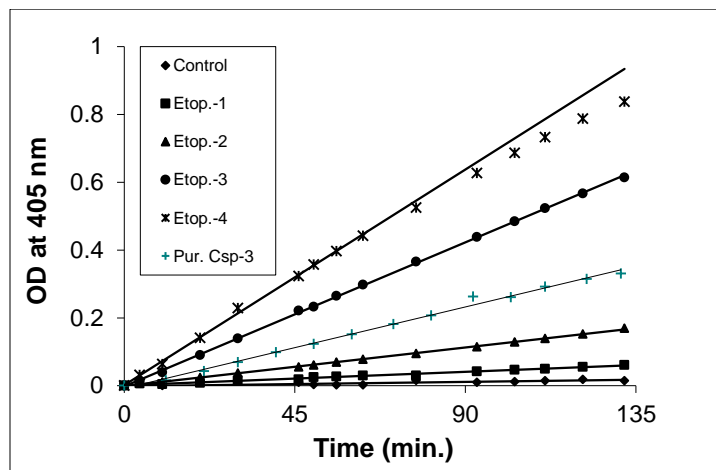


Figure 1. Cleavage of DEVD-pNA by Cell Extracts and Purified Caspase-3. U937 cells (a human histiocytic lymphoma) were induced to undergo apoptosis by treatment with 50 μM etoposide. Extracts were prepared as described in "EXPERIMENTAL METHODS". Etop.-1, -2 etc. indicate hours of etoposide treatment. "Control" cell extract was prepared from untreated cells. The "Pur. Csp-3" sample contained 30U of Caspase-3 enzyme. Assay temperature: 37°C

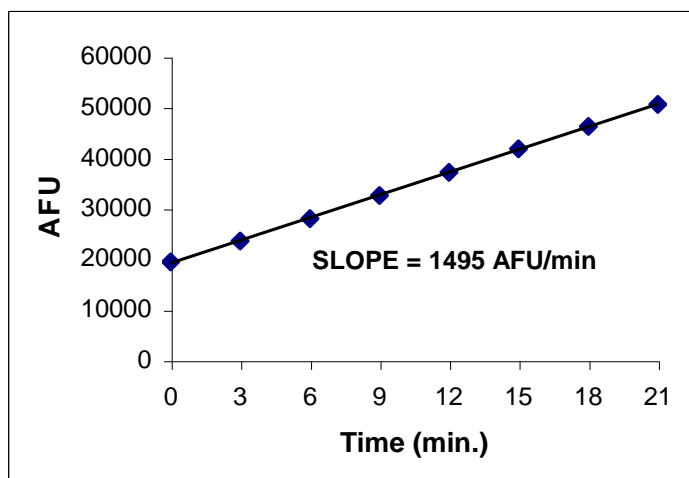


Figure 2. Cleavage of DEVD-AMC by Cell Extracts. Jurkat cells (human cervical cancer cell line) were treated with 2 $\mu\text{g}/\text{mL}$ of human TRAIL ligand [Enzo Life Sciences catalog# BML-SE721] for 90 min to induce apoptosis. Cell extracts were prepared and assayed according to the AK-703 kit protocol. 10 μL Jurkat cell extract, [DEVD-AMC] = 30 μM , 37°C.

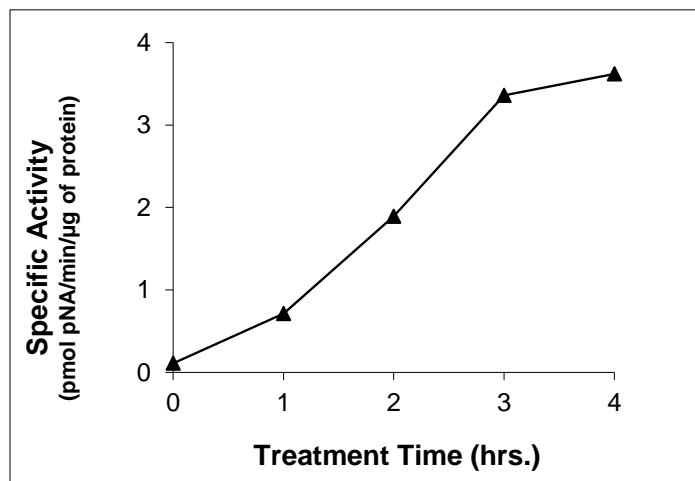


Figure 3. Induction of DEVDase Activity in Etoposide-Treated U937 Cells. DEVD-pNA cleavage rates were calculated from the slopes of the best-fit lines in Fig. 1. Protein content of each sample was determined with a BCA assay. After 3 hr of treatment, ~50% of cells were morphologically apoptotic (2 or more membrane “blebs” per cell).

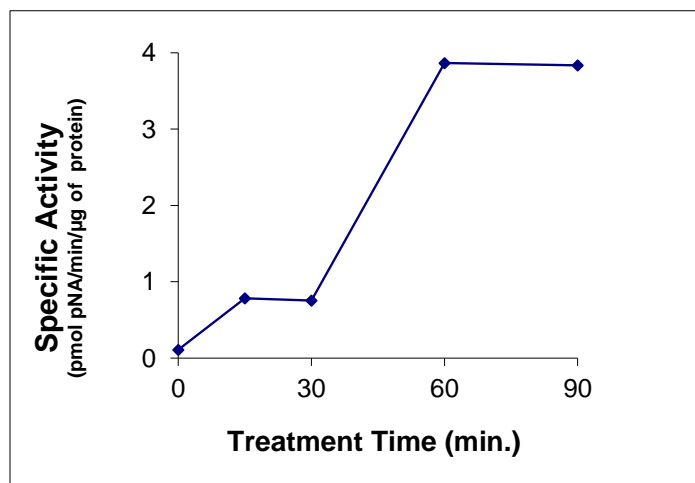


Figure 4. Induction of DEVDase Activity in TNF- α -Treated U937 Cells. U937 cells were treated with 2 ng/mL tumor necrosis factor - α (TNF- α) plus 0.5 $\mu\text{g}/\text{mL}$ cycloheximide. After 1 hr. of treatment, ~50% of cells were morphologically apoptotic.

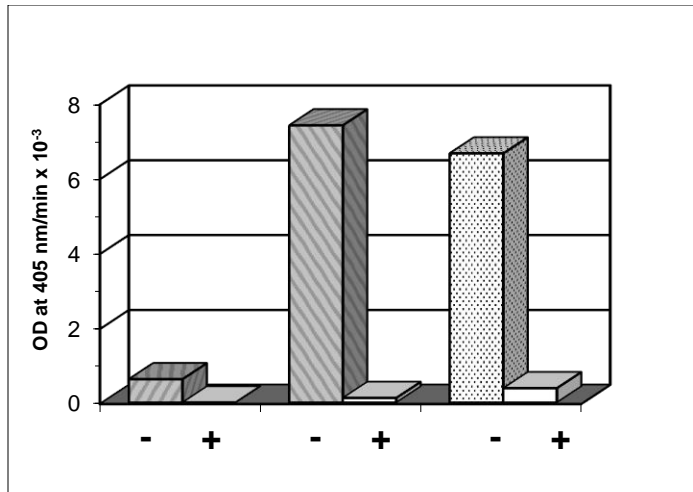


Figure 5. DEVD-CHO Inhibition of Caspase-3-Like Activity in Cell Extracts. Extracts were prepared from untreated (control), etoposide and TNF- α treated U937 cells as described in “EXPERIMENTAL METHODS” and Figs. 1-3. Prior to assay of DEVD-pNA cleavage, extracts were preincubated 10 min, 37°C (+) or (-) 0.1 μ M DEVD-CHO.

Literature Citations of the Caspase-3 Cellular Activity Assay Kit Plus.

- M.E. Reyland *et al. J. Biol. Chem.* 1999 **274** 19115
L. Zhu *et al. J. Biol. Chem.* 1999 **274** 33267
L. Li *et al. Mol. Cell. Biol.* 1999 **19** 8547
T. Bellido *et al. J. Biol. Chem.* 2000 **275** 26328
K. Kinoshita *et al. Mol. Cell. Biol.* 2000 **20** 4680
H. Han *et al. J. Biol. Chem.* 2001 **276** 26357
E. Hamilton *et al. J. Biol. Chem.* 2001 **276** 9029
J. Munger and B. Roizman *Proc. Natl. Acad. Sci. USA* 2001 **98** 10410
S.R. Danielson *et al. J. Biol. Chem.* 2002 **277** 5810

Notes

Notes



www.enzolifesciences.com

Enabling Discovery in Life Science®

Global Headquarters

Enzo Life Sciences Inc.

10 Executive Blvd
Farmingdale, NY 11735

(p) 1-800-942-0430

(f) 1-631-694-7501

(e) info-usa@enzolifesciences.com

Enzo Life Sciences (ELS) AG

Industriestrasse 17, Postfach
CH-4415 Lause / Switzerland

(p) +41/0 61 926 89 89

(f) +41/0 61 926 89 79

(e) info-ch@enzolifesciences.com

Please visit our website at www.enzolifesciences.com for additional contact information.