Caspase Colorimetric Substrate/Inhibitor *QuantiPak*™

Designed to measure or inhibit caspase-3, caspase-1 and caspase-8-like protease activity.

Instruction Manual

BML-AK004

For research use only

✦ Caspase Colorimetric Substrate/Inhibitor *QuantiPak*™✦
BML-AK004

DESCRIPTION/BACKGROUND

This kit contains peptide substrates and aldehyde inhibitors designed to measure or inhibit caspase-3, caspase-1 and caspase-8 -like protease activity (DEVD, YVAD and IETD, respectively) PLUS a calibration dye standard (p-nitroaniline). It is designed to add versatility to the Enzo Life Sciences *Caspase-3 Cellular Activity Assay Kit PLUS* (BML-AK703), *Caspase-1 (BML-AK701), Caspase-3* (BML-AK700) and *Caspase-8* (BML-AK715) *Assay Kit for Drug Discovery*. The components are supplied with purity >95% and lyophilized in convenient screw-cap microfuge tubes. Quantities of reagents are sufficient to perform multiple assays. Individual components may be purchased separately in larger package sizes (please refer to catalog #'s listed beside each component).

The colorimetric assay of the caspases is based on the cleavage a p-nitroaniline dye from the C-terminus of the peptide substrates. Cleavage of the dye from the substrate increases its yellow intensity and absorption at 405nm. There is a linear correlation between the absorption and the amount of dye released within instrument limitations. The -CHO (aldehyde) inhibitors are slowly reversible enzyme inhibitors, which interact at the enzyme substrate binding site. Both substrates and inhibitor selectivities are based on the absolute caspase enzyme substrate cleavage site C-terminal to aspartate (D). The N-terminal 3 amino acids confer selectivity for the various caspase isotypes. Substrate specificity among the various known caspases is not absolute, but rather a preference. The cleavage rate for each substrate and caspase reflects a combination of K_m value and intrinsic velocity rate. Please see bibliography below for review and discussion.

SELECTED BIBLIOGRAPHY

GENERAL REVIEWS ON APOPTOSIS

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A. G. Uren and D.L. Vaux *Pharmacol. Ther.* 1996 **72** 37

CASPASE ASSAYS

N.A. Thornberry *Methods Enzymol.* 1994 **244** 615
H.R. Stennicke and G.S. Salvesen *J. Biol. Chem.* 1997 **272** 25719

CASPASE SUBSTRATE SPECIFICITY

N.A. Thornberry *et al. J. Biol. Chem.* 1997 **272** 17907
R.V. Talanian *et al. J. Biol. Chem.* 1997 **272** 9677
N. Margolin *et al. J. Biol. Chem.* 1997 **272** 7223
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THE PROTOCOLS DESCRIBED BELOW ARE INTENDED FOR USE IN 96-WELL MICROTITER-PLATES WITH ABSORPTION KINETICS FOLLOWED WITH A MICROPLATE READER CAPABLE OF MEASURING OD AT APPROXIMATELY 405 NM. ASSAYS MAY BE ADAPTED TO OTHER FORMATS AT THE USER'S DISCRETION. 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

NOTE ON STORAGE: Store all components desiccated at -20°C. After reconstitution, store at -70°C. Avoid repeated freeze/defrost cycles.

NOTE: See individual items for lot numbers.

BML-P412-9091 Ac-DEVD-pNA, colorimetric substrate

QUANTITY: 1mg net peptide/vial.

MOLECULAR WEIGHT: 638.0

PURITY: >95% by HPLC analysis.

PRESENTATION: Lyophilized solid in screw-cap microfuge tube.

BML-P408-9091 Ac-YVAD-pNA, colorimetric substrate

QUANTITY: 1mg net peptide/vial.

MOLECULAR WEIGHT: 628.6

PURITY: >95% by HPLC analysis.

PRESENTATION: Lyophilized solid in screw-cap microfuge tube.

BML-P431-9091 Ac-IETD-pNA, colorimetric substrate

QUANTITY: 1mg net peptide/vial.

MOLECULAR WEIGHT: 638.6

PURITY: >95% by HPLC analysis.

PRESENTATION: Lyophilized solid in screw-cap microfuge tube.

BML-P410-9091 Ac-DEVD-CHO, inhibitor

QUANTITY: 0.1mg net peptide/vial.

MOLECULAR WEIGHT: 502.5

PURITY: >95% by HPLC analysis.

PRESENTATION: Lyophilized solid in screw-cap microfuge tube.

BML-P403-9091 Ac-YVAD-CHO, inhibitor

QUANTITY: 0.1mg net peptide/vial.

MOLECULAR WEIGHT: 492.0

PURITY: >95% by HPLC analysis.

PRESENTATION: Lyophilized solid in screw-cap microfuge tube.

BML-P430-9091 Ac-IETD-CHO, inhibitor

QUANTITY: 0.1mg net peptide/vial.

MOLECULAR WEIGHT: 524.5

PURITY: >95% by HPLC analysis.

PRESENTATION: Lyophilized solid in screw-cap microfuge tube.

BML-KI145-0001 p-Nitroaniline (pNA), calibration standard

QUANTITY: 1mg net weight/vial.

MOLECULAR WEIGHT: 138.1

PURITY: >95% by TLC analysis.

PRESENTATION: Lyophilized solid in screw-cap microfuge tube.

OTHER MATERIALS NEEDED

Microtiter-plate reader capable of measuring OD405nm or spectrophotometer.

Caspase enzymes or cellular extracts containing active caspases. (*Enzo Life Sciences offers a complete line of active, purified recombinant caspases.*)

Optional: *Caspase-3 Cellular Activity Assay Kit PLUS* (BML-AK703), *Caspase-3* (BML-AK700) and *Caspase-8* (BML-AK715) *Assay Kit for Drug Discovery*.

EXPERIMENTAL METHODS

NOTE: THE FOLLOWING PROCEDURES ARE INTENDED ONLY AS A GUIDELINE. THE OPTIMAL CONCENTRATIONS OF SUBSTRATES AND INHIBITORS, ASSAY VOLUMES, BUFFER COMPOSITION, AND OTHER EXPERIMENTAL CONDITIONS MUST BE DETERMINED BY THE INDIVIDUAL USER. NO WARRANTY OR GUARANTEE OF PERFORMANCE USING THESE PROCEDURES IS MADE OR IMPLIED.

THE EXPECTED RESULTS WILL VARY GREATLY DEPENDING ON EXPERIMENTAL CONDITIONS. THE CELL TYPE AND METHOD OF INDUCTION WILL DICTATE THE TYPE AND EXTENT OF CASPASE ACTIVATION. THE EXPERIMENTAL DESIGN MUST BE DETERMINED BY THE USER FOR THE SPECIFIC APPLICATION INVESTIGATED.

RECONSTITUTION OF VIAL CONTENTS

1. Add 40µl DMSO (dimethylsulfoxide) to each of the 7 kit vials. Replace cap, then vortex vigorously to dissolve vial contents completely.
2. Centrifuge vials in a microfuge briefly to consolidate vial contents.
3. Add a quantity of ASSAY BUFFER to each vial as listed in Table 1 to obtain the final concentrations listed. Vortex vigorously to solubilize:

TABLE 1. RECONSTITUTION OF VIAL CONTENTS

Cat#/Description	Add quantity ASSAY BUFFER	Final conc. (mg/ml)	(mM)
BML-P412/Ac-DEVD-pNA	730µl	1.3	2
BML-P408/Ac-YVAD-pNA	730µl	1.3	2
BML-P431/Ac-IETD-pNA	730µl	1.3	2
BML-P410/Ac-DEVD-CHO	1 ml	0.1	0.2
BML-P403/Ac-YVAD-CHO	1 ml	0.1	0.2
BML-P430/Ac-IETD-CHO	1 ml	0.1	0.2
BML-KI145/pNA	684µl	1.38	10

NOTE: Each vial should be reconstituted in 40µl DMSO, then the indicated volumes of ASSAY BUFFER are to be added to obtain the final concentrations listed.

4. Store unused portions of each vial at -70°C. Shelf-life is >3 months at -70°C.

PREPARATION OF CELL EXTRACTS

1. Grow cell cultures and induce cells as desired. (See Enzo Life Sciences 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 or simply a “time-zero” sample from an induction time course.

   The number of cells required for an experiment must be determined by the user. As a guide, here is an example of data obtained with apoptosis-induced U937 cells:

   - Cell density (at lysis)=~2 x 10^7 cells/ml
   - Protein concentrations=1-3mg/ml
   - 10µl assay sample=~2 x 10^5 cells or 10-30µg protein
   - OD_{405nm} after 30 min @37°C for apoptotic cells: DEVD=0.22; IETD=0.1; YVAD= negligible
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.: 10^7-10^8/ml) with ice-cold CELL LYSIS BUFFER (e.g.: 50 mM HEPES, pH 7.4, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA). 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 (e.g.: Tween 20, NP-40 or Triton X-100 to a final concentration of 0.1%). Mechanical disruption may also be necessary.
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.

CASPASE COLORIMETRIC 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.
2. Prepare samples in a 96-well microtiter-plate as desired. See Table 2 for examples of the reagent volumes needed for a variety of assay types. Use ASSAY BUFFER (e.g.: 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) for final dilutions.
3. Incubate plate at assay temperature for 10 min (or as desired) to allow inhibitor/enzyme interaction.
4. Start reaction by the addition of 10µl substrate (pre-equilibrated to assay temperature).
5. Measure OD_{405nm} in a microplate reader. Record data at 1-10 min intervals for 10 to 120 min. Initial experiments must establish the time course for which the time versus OD is linear.
6. Perform data analysis (see below).

TABLE 2. ASSAY MIXTURE EXAMPLES

SAMPLE	Assay buffer	Cell Extract	Purified Caspases	-CHO Inhibitor[1] (1µM)	Substrate (2 mM)
Blank	90µl	0	0	0	10µl
Cell Extract	80µl	10µl	0	0	10µl
Inhibitor-Treated Cell Extract	70µl	10µl	0	10µl	10µl
Purified Caspase	75µl	0	15µl	0	10µl
Test Sample[2]/Cell Extract	Xµl	10µl	0	Yµl	10µl
Test Sample[2]/Purified Caspase	Xµl	0	15µl	Yµl	10µl
Cell Extract/ Purified Caspase	65µl	10µl	15µl	0	10µl

1. Dilute the -CHO peptide aldehyde inhibitors (#BML-P410-9091, 403-9091, and 430-9091) 1/200 in ASSAY BUFFER prior to addition.
2. 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 100µl.

***NOTE**: Cell extracts, purified caspases, -CHO inhibitors and substrate volumes and concentrations must be determined by the user according to the purpose of the experiment. Table 2 is intended only as a guideline example!*

DATA ANALYSIS

PLOTTING

1. Plot data as OD_{405nm} versus time for each sample.
2. For each sample determine the initial time period over which the plot of OD vs. time remains linear, and there is sufficient ΔOD to obtain an accurate slope. The amount of caspase enzyme and/or cell extract can be increased or decreased as desired to adjust the time scale of the experiments.
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.

SPECIFIC ACTIVITY CALCULATIONS

To find the activity of the samples expressed as pmol substrate/min:

Determine microtiter-plate reader conversion factor:

1. Dilute p-nitroaniline calibration standard (BML-KI145) to 50µM in ASSAY BUFFER.
2. Add 100µl (or the actual assay volume used) of the p-nitroaniline calibration standard (50µM) to 2 wells of the microtiter-plate.
3. Determine the average OD_{405nm} using 100µl (or the actual assay volume used) ASSAY BUFFER as a blank.
4. Calculate the conversion factor. The calculation is based on the concentration of p-nitroaniline in the calibration standard (50µM). The extinction coefficient for p-nitroaniline in the ASSAY BUFFER is 10,500 $M^{-1}cm^{-1}$. This number may vary depending on the composition of the buffer.

   conversion factor (µM/OD) =
   50µM ÷ average OD_{405nm} from step 3

5. Calculate the activity as pmol/min:

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

NOTE: The suggested assay volume is 100µl. If a different volume is used, be sure to perform steps 2 to 5 using the actual assay volume.

Sample activity calculation:

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

Example: Sample for which the slope of the OD_{405nm} vs. time plot is 3.4E-03 (OD/min)

activity =
3.4E-03 (OD/min) x 170(µM/OD) x 100(µl) = 58 pmol/min

DISCUSSION/EXAMPLES

USE OF SUBSTRATES: AN APPLICATION EXAMPLE

In this section we present several experiments which illustrate potential uses of the substrate components of the kit. Specifically, we investigated the substrate specificity and kinetics of recombinant caspases -3 and –8. We then applied this information to the question of whether Ac-IETD-pNA cleavage, in a particular set of apoptotic cell extracts, could be attributed to caspase-8 activity. All data were gathered using ENZO LIFE SCIENCES's recombinant caspase-3 and 8 (Cat.# BML-SE169 and BML-SE172, respectively) and assay kits (BML-AK700, BML-AK703, and BML-AK715).

Figure 1 depicts the relative cleavage activity of caspases –3 and –8 with 200µM of the three substrates provided in this kit. While neither enzyme displays significant activity with the caspase-1 (ICE) substrate, YVAD-pNA, each has substantial activity with both its own preferred substrate and the preferred substrate of the other caspase. Kinetic constants derived from more extensive rate vs. substrate concentration data are summarized in Table 3. Using this information, it is possible to choose a pair of conditions, one DEVD-pNA concentration and one IETD-pNA concentration, which produce highly distinct responses from the two enzymes. For example, the ratio of the cleavage rate at 50µM IETD-pNA to that at 200µM DEVD-pNA is ~0.09 for caspase-3, but ~1.1 for caspase-8.

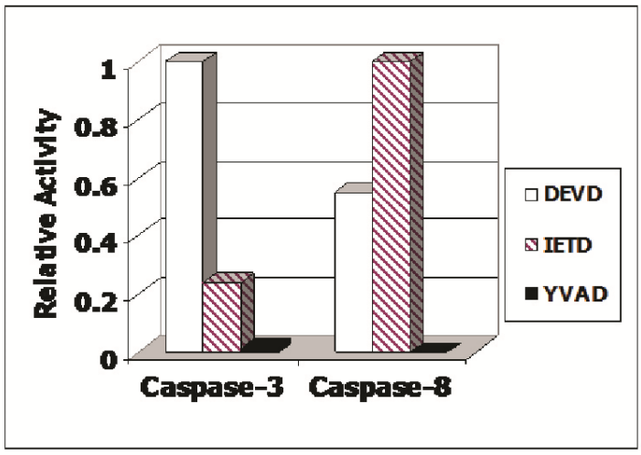

FIGURE 1. Relative Rates of Substrate Cleavage by Recombinant Caspases 3 and 8. Initial rates of pNA release were determined at 25°C with 200µM of the indicated substrates.

TABLE 3. KINETIC CONSTANTS FOR CASPASES 3 & 8

ENZYME	Km DEVD-pNA, µM	Vmax, pmol DEVD-pNA/min. (Rel,)	Km IETD-pNA, µM	Vmax, pmol IETD-pNA/min. (Rel,)
Csp.-3	16	31 (1)	210	14 (0.45)
Csp.-8	43	25 (0.54)	53	46 (1)

U937 cells treated with the death-ligand TRAIL (Cat #BML-SE721) undergo apoptosis. During the first few hours of treatment, cytosolic DEVD-pNA cleavage activity is induced (not shown), as is IETD-pNA cleavage activity (**Figure 2**). We wished to know to what degree IETD-pNA cleavage activity might be attributed to caspase-8 or other "upstream" caspases with similar substrate preferences. We compared the 50µM IETD-pNA/200µM DEVD-pNA activity ratios of the TRAIL-treated cell extracts to those for recombinant caspase-3 and caspase-8 (**Figure 3**). The cleavage ratios of the cell extracts are extremely close, if not identical to the ratio for recombinant caspase-3. Therefore, we conclude that the vast majority of the IETD-pNA cleavage activity in the cell extracts derives from caspase-3 or caspase-3-like enzymes. These experiments illustrate that it would be dangerous to simply assume that the cleavage of a substrate, which is preferred by a particular caspase, necessarily indicates the presence of that caspase.

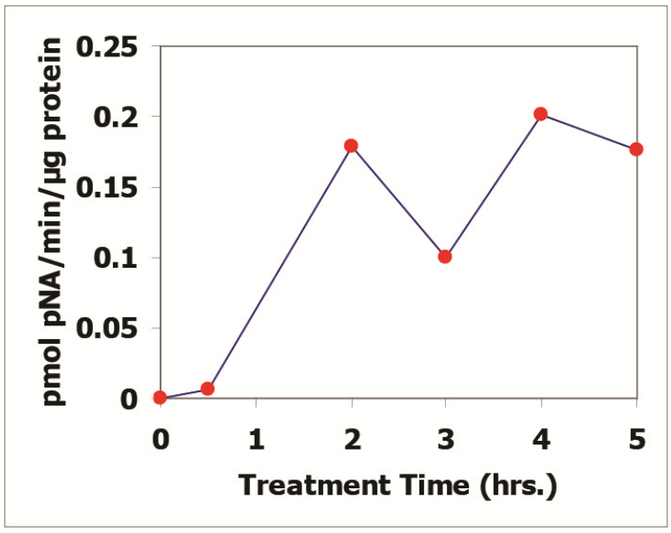

FIGURE 2. Induction of IETD-pNA Cleavage Activity by TRAIL. Apoptosis was induced in U937 cells by treatment with 2µg/ml TRAIL (SE-721). DNA fragmentation was >70% by 5 hrs. Cell extracts prepared with the Enzo Life Sciences AK-703 kit and IETD-pNA cleavage was assayed at 50µM, 25°C.

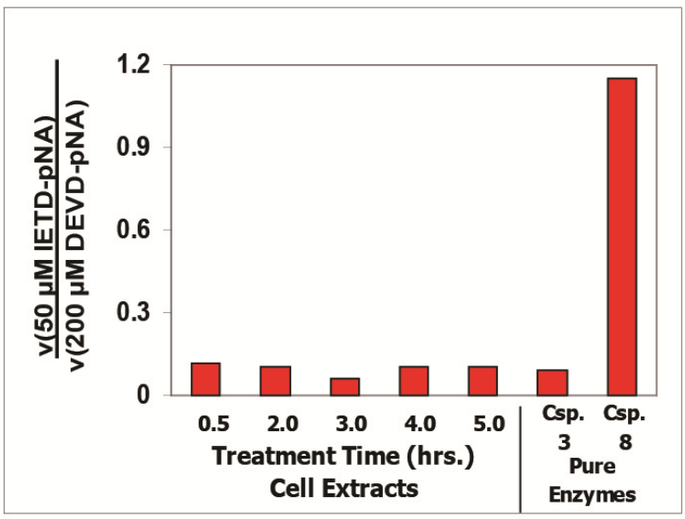

FIGURE 3. IETD/DEVD Cleavage Ratios: Extracts of TRAIL-Treated U937 Cells and Recombinant Caspases 3 & 8. Rates of pNA release were determined at 50µM IETD-pNA and 200µM DEVD-pNA, 25°C.

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