Product Manual

Caspase Fluorometric (AMC) Substrate/Inhibitor *QuantiPak*™

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

Instruction Manual
BML-AK005

For research use only

Product Manual

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Caspase Fluorometric (AMC) Substrate/Inhibitor *QuantiPak*™
✦BML-AK005✦

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 (7-amino-4-methylcoumarin). It is designed to add versatility to the Enzo Life Sciences *Caspase-3 Cellular Activity Assay Kit PLUS* (BML-AK703), *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 fluorescent assay of the caspases is based on the cleavage a 7-amino-4-methylcoumarin (AMC) dye from the C-terminus of the peptide substrates. Cleavage of the dye from the substrate increases its fluoresence intensity at 460 nm. There is a linear correlation between the fluorescence 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 selectivity's 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

V.J. Kidd *Annu. Rev. Physiol.* 1998 **60** 533
G.M. Cohen *Biochem. J.* 1997 **326** 1
S. Nagata *Cell* 1997 **88** 355
M.D. Jacobson et al. *Cell* 1997 **88** 347
M. Leist and P. Nicotera *Exper. Cell. Res.* 1998 **239** 183
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
Z. Han *et al. J. Biol. Chem.* 1997 **272** 13432

THE PROTOCOLS DESCRIBED BELOW ARE INTENDED FOR USE IN 96-WELL MICROTITER-PLATES, AND FLUORESCENCE EMISSION KINETICS FOLLOWED USING A MICROPLATE READER CAPABLE OF FLUORESCENCE EXCITATION 360 NM AND EMISSION 460 NM, APPROXIMATELY. 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. PROTECT FROM LIGHT!

NOTE: See individual items for lot numbers.

#BML-P411-9091 Ac-DEVD-AMC, fluorescent substrate

QUANTITY: 1 mg net peptide/vial.
MOLECULAR WEIGHT: 676.0
PURITY: >95% by HPLC analysis.
PRESENTATION: Lyophilized solid in screw-cap microfuge tube.

BML-P401-9091 Ac-YVAD-AMC, fluorescent substrate

QUANTITY: 1 mg net peptide/vial.
MOLECULAR WEIGHT: 665.7
PURITY: >95% by HPLC analysis.
PRESENTATION: Lyophilized solid in screw-cap microfuge tube.

BML-P432-9091 Ac-IETD-AMC, fluorescent substrate

QUANTITY: 1 mg net peptide/vial.
MOLECULAR WEIGHT: 674.1
PURITY: >95% by HPLC analysis.
PRESENTATION: Lyophilized solid in screw-cap microfuge tube.

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

QUANTITY: 0.1 mg 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: 0.1 mg 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: 0.1 mg net peptide/vial.
MOLECULAR WEIGHT: 502.4
PURITY: >95% by HPLC analysis.
PRESENTATION: Lyophilized solid in screw-cap microfuge tube.

BML-KI144-0001 7-amino-4-methylcoumarin (AMC), calibration standard

QUANTITY: 1 mg net weight/vial.
MOLECULAR WEIGHT: 175.2
PURITY: >95% by TLC analysis.
PRESENTATION: Lyophilized solid in screw-cap microfuge tube.

OTHER MATERIALS NEEDED

Microtiter-plate reader capable of measuring fluorescence at approximate excitation 360nm and emission 460nm, or a fluorometer.

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, except use DMSO for the # BML-KI144/AMC vial. Vortex vigorously to solubilize:

TABLE 1. RECONSTITUTION OF VIAL CONTENTS

Cat#/Description	Add quantity ASSAY BUFFER	Final conc. (mg/ml)	(mM)
BML-P411/Ac-DEVD-AMC	730µl	1.3	2
BML-P401/Ac-YVAD-AMC	730µl	1.3	2
BML-P432/Ac-IETD-AMC	730µl	1.3	2
BML-P410/Ac-DEVD-CHO	1ml	0.1	0.2
BML-P403/Ac-YVAD-CHO	1ml	0.1	0.2
BML-P430/Ac-IETD-CHO	1ml	0.1	0.2
BML-KI144/AMC	531µl (DMSO)	1.75	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, except use DMSO for the KI-144/AMC.

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

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:

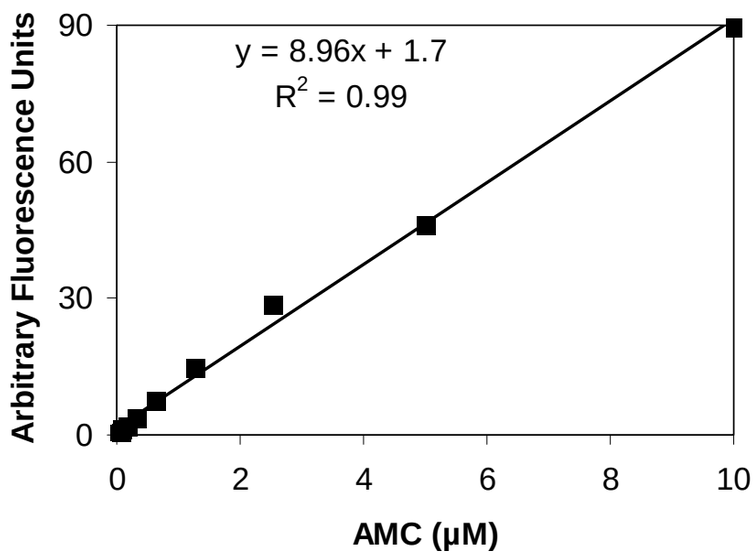
- Cell density (at lysis)=~2 x 10^7 cells/ml
- Protein concentrations=1-3 mg/ml
- 10µl assay sample=~2 x 10^5 cells or 10-30µg protein

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.

FLUORESCENCE STANDARD CURVE/INSTRUMENT SET-UP

1. Dilute 7-amino-4-methylcoumarin (AMC) calibration standard (BML-KI144) directly to 50µM in ASSAY BUFFER.
2. Prepare a serial dilution of the AMC Calibration Standard in ASSAY BUFFER to a final volume of 100µl (or the actual caspase assay volume). Be sure to use the exact or identical type of microtiter plate as will be used in the subsequent caspase assays.
3. Determine optimal fluorescence plate reader instrument settings to obtain the best combination of sensitivity, linearity and range. Do this by trial and error. Use a plot of arbitrary fluorescence units (AFU) versus AMC concentration to visualize results. See Figure 1 for an example.
4. Use the standard curve and the optimal instrument settings when performing the caspase assays, below.

Figure 1. Fluorescence Standard Curve



CASPASE FLUOROMETRIC 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. Include the fluorescence standard curve as described above as a reference standard.
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 emission in a fluorescence 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 relative fluorescence is linear. See Figure 2 for an example.
6. Perform data analysis (see below).

TABLE 2. ASSAY MIXTURE EXAMPLES

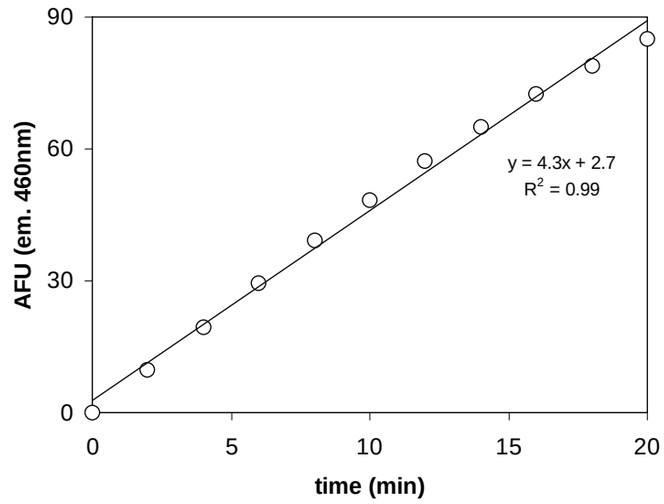
SAMPLE	Assay buffer	Cell Extract	Purified Caspases	-CHO Inhibitor[1] (1µM)	Substrate (2 mM)
Blank	90µl	0	0	0	10µl
Cell Extract	80µl	10µl	0	0	10µl
Inhibitor-Treated Cell Extract	70µl	10µl	0	10µl	10µl
Purified Caspase	5µl	0	15µl	0	10µl
Test Sample[2]/ Cell Extract	Xµl	10µl	0	Yµl	10µl
Test Sample[2]/ Purified Caspase	Xµl	0	15µl	Yµl	10µl
Cell Extract/ Purified Caspase	65µl	10µl	15µl	0	10µl

1. Dilute the -CHO peptide aldehyde inhibitors (#P410-9091, 403-9091, and 430-90901) 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.

<u>NOTE</u>: 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!

Figure 2. Caspase-3 Assay
Ac-DEVD-AMC Substrate



DATA ANALYSIS

PLOTTING

1. Plot data as arbitrary fluorescence units (AFU) versus time for each sample.
2. For each sample determine the initial time period over which the plot of AFU versus time remains linear, and there is sufficient ∆fluorescence 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:

5. Calculate the activity as pmol/min:

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

   NOTE: The conversion factor is the 1/slope of the AMC Calibration Standard (KI-144) versus AFU (µM), described above (see Figure 1 for an example). 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.

EXAMPLE: Sample for which the slope of the AFU vs. time plot is 4.3 (AFU/min). See Figure 2.

specific activity =

4.3 (AFU/min) x 1/8.96 (µM/AFU) x 100(µl) = 48.0 pmol/min

Enzo Life Sciences PRODUCTS OF INTEREST

Please contact Enzo Life Sciences Technical Services for the latest products and information. Below are products useful for caspase assays and may be of interest to you.

CAT#	DESCRIPTION
BML-SE169	Caspase-3 Enzyme
BML-SE172	Caspase-8 Enzyme
BML-SE760	Casputin Reagent-Selective Caspase Inhibitor
BML-AK703	Caspase-3 Cellular Activity Assay Kit PLUS
BML-AK700	Caspase-3 Assay Kit for Drug Discovery
BML-AK715	Caspase-8 Kit for Drug Discovery

Please also see the Enzo Life Sciences List of Apoptosis Inducers.

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