Enabling Discovery in Life Science®

Caspase-2 Drug Discovery Kit

A complete assay system designed to screen caspase-2 inhibitors.

Instruction Manual
BML-AK702

For research use only

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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-2 Assay Kit for Drug Discovery - AK-702✦

BACKGROUND

Caspase-2 is activated during apoptosis in a variety of systems[1-4]. Like caspase-8, it can be recruited to a ligated death-receptor complex via binding to an adaptor protein. The adaptor protein in this case is RAIDD (CRADD)[5,6] whose CARD binds the CARD of the caspase-2 prodomain[5-7]. RAIDD binds to the kinase RIP, which, by way of its binding to the adaptor TRADD, can associate with the tumor necrosis factor receptor 1[5,6,8]. As in the case of other long-prodomain procaspases (–8, –9 & –10), proximity induced by receptor/adaptor oligomerization could act as the driving force behind procaspase-2 autoprocessing and activation[9,10]. On the other hand, there is evidence suggesting that procaspase-2 may be processed and activated by caspase-3[11].

Despite the similarity of its domain structure and receptor interactions, it is unclear whether caspase-2 is an initiator caspase in quite the same sense as caspases –8, –9, and –10. Unlike these caspases, caspase-2 does not directly cleave and activate downstream, effector procaspases. Aside from its own proenzyme, the only two caspase-2 substrates identified thus far are golgin-160[12] and Bid[11,13]. Depending on their tissue of origin, the cells of caspase-2-deficient mice can be either more or less resistant to apoptosis induction than their wild-type counterparts[14]. This would seem to imply that caspase-2 plays more the role of an apoptosis regulator than initiator[14,15]. This role may be played in part via expression of the splicing isoforms caspase-2L (proapoptotic) and caspase-2S (anti-apoptotic)[15]. A possible exception, although perhaps one that proves the rule, is *Salmonella*-induced macrophage apoptosis, where caspase-2 may be acting, with caspase-1, as a co-initiator[16] Recent work indicates that caspase-2L promotes apoptosis by causing release of proapoptotic mitochondrial proteins such as cytochrome c and Smac[11,13,17]. Possible mechanisms include cleavage of Bid[11,13,17], direct, Bid-independent action on the mitochondria[13] and release of an unidentified nuclear factor[17].

The *CASPASE-2 Drug Discovery Kit* is a complete assay system designed to screen caspase-2 inhibitors. Caspase-2 is unique among the caspases in its strong preference for pentapeptides over tetrapeptides as artificial substrates[18] The kit contains both a colorimetric substrate (Ac-VDVAD-pNA; K_m= 53 µM) and a fluorogenic substrate (Ac-VDVAD-AMC; K_m= 80 µM) based on the pentapeptide recognition sequence, VDVAD[18]. Cleavage of the p-nitroaniline (pNA) from the colorimetric substrate increases absorbance at 405 nm. The fluorescent assay is based on the cleavage of 7-amino-4-methylcoumarin (AMC) dye from the C-terminus of the peptide substrate, which increases its fluorescence intensity at 460 nm. The kit is useful to screen inhibitors of caspase-2, a potential therapeutic target. A potent inhibitor, Ac-VDVAD-CHO (aldehyde;K_I= 3.5 nM[18]), is included for use as a control.

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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 AK-702 KIT

SE-175-9091 CASPASE-2 ENZYME (HUMAN, RECOMBINANT)
FORM: 250 U/µl in assay buffer. One U=1 pmol/min@30°C, 200 µM Ac-VDVAD-pNA. Purity >90% by SDS-PAGE.
STORAGE: -70°C; AVOID FREEZE/THAW CYCLES!
QUANTITY: 10,000 U (40 µl)

P461-9090 SUBSTRATE (Ac-VDVAD-pNA; MW=679.8)
FORM: 2 mM (1.4 mg/ml) in assay buffer
STORAGE: -70°C
QUANTITY: 1 ml

KI-106-0001 CALIBRATION STANDARD (p-nitroaniline; MW=138)
FORM: 50 µM in assay buffer.
STORAGE: -70°C
QUANTITY: 1 ml

P462-9090 AMC SUBSTRATE (Ac-VDVAD-AMC; MW=717)
FORM: 0.5 mM (0.36 mg/ml) in assay buffer
STORAGE: -70°C
QUANTITY: 1 ml

KI-107-0001 AMC CALIBRATION STANDARD
(7-amino-4-methylcoumarin; MW=175)
FORM: 30 µM in assay buffer.
STORAGE: -70°C
QUANTITY: 1 ml

P463-9090 INHIBITOR (Ac-VDVAD-CHO; MW=543.8)
FORM: 0.1 mM (0.05 mg/ml) in DMSO (dimethylsulfoxide)
STORAGE: -70°C
QUANTITY: 50 µl

KI-111-0020 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

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)

Pipetman or multi-channel pipetman 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 microtiter plate at -70°C for the highest stability. The caspase-2 enzyme component, SE-175, must be handled with particular care 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 enzyme is stable on ice for the time typically required to set up an experiment (30-60 min.), but does lose activity with prolonged storage on ice (half-life= ~5 hrs.). It is recommended that thawing and dilution of the enzyme be done within as short a time as possible before start of the assay. The remaining, unused enzyme should be refrozen quickly by, for example, snap-freezing in a dry/ice ethanol bath or by placing it at -70°C. The enzyme is stable to at least 4 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the caspase-2 into separate tubes and store at -70°C.*

To start assay:

1. Defrost all kit components, except the enzyme, and hold on ice bath until use. The caspase-2 enzyme can lose activity with prolonged storage on ice and should be handled accordingly (see storage note above). All other components are highly stable for several hours in an ice bath.
2. Dilute inhibitor (Ac-VDVAD-CHO) 1/10 in assay buffer. (Note: Bring the tube of Ac-VDVAD-CHO to room temperature to thaw.) Example: Add 10 µl inhibitor to 90 µl assay buffer, in a separate tube.
3. Dilute substrate (Ac-VDVAD-pNA) 1/5 in assay buffer. (Note: Bring tube of Ac-VDVAD-pNA to room temperature to thaw.) Example: Add 50 µl substrate to 200 µl assay buffer, in a separate tube.
4. Add assay buffer to each desired well of the ½-volume microplate as follows:
   Blank (no caspase-1)=50 µl
   Control (no inhibitor)=35 µl
   Inhibitor (will receive 10 µl diluted VDVAD-CHO)=25 µl
   Test sample=varies (see Table 1, below)
5. Allow microplate to equilibrate to assay temperature (e.g. 30°C).

6. Dilute enough caspase-2, SE-175-9091, 1/50 in assay buffer to produce required quantity (15 µl is needed per well). For example, dilute 5 µl caspase-2 into 245 µl assay buffer.
7. Add 15 µl caspase-2 to the “Control”, “Inhibitor” and “Test Sample” wells. Final amount of caspase-2 will be 75 U per well. DO NOT ADD CASPASE-2 TO BLANKS!
8. Add 10 µl Ac-VDVAD-CHO inhibitor (diluted in step 2) to the “Inhibitor” well only! Final inhibitor concentration=1 µM.
9. Add desired volume of test sample(s) to appropriate well(s). See Table 1, below.
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-VDVAD-pNA substrate (diluted in step 3) or 50 µl Ac-VDVAD-AMC substrate equilibrated to reaction temperature (e.g. 30°C). Enough of each substrate is provided to do 96 assays at 200 µM for the colorimetric substrate or 50 µM for the fluorogenic substrate.
12. 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.
13. Perform data analysis (see below).

NOTE: Retain microplate for future use of unused wells!

TABLE 1. ASSAY MIXTURE EXAMPLES.

Sample	Assay Buffer	Caspase-2 (5 U/µl)	Inhibitor	Substrate
Blank	**50 µl**	**0**	**0**	**50 µl**
Control	**35 µl**	**15 µl**	**0**	**50 µl**
Inhibitor‡	**25 µl**	**15 µl**	**10 µl‡**	**50 µl**
Test sample*	**X µl**	**15 µl**	**Y µl**	**50 µl**

‡Refers to 10 µl of the diluted VDVAD-CHO prepared in step 2.

*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 (X+Y=35 µl).

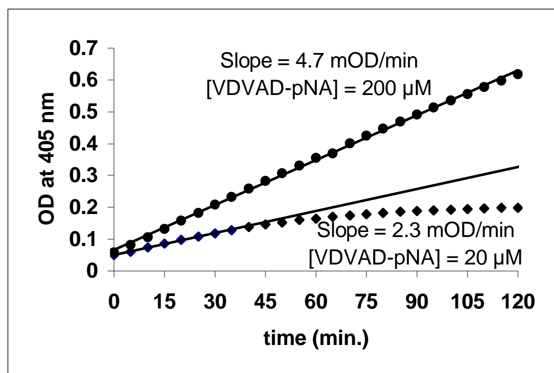

Figure 1. Caspase-2 activity is stable for at least 120 min. At [Ac-VDVAD-pNA]= 20 µm, substrate depletion begins to lower the rate after 35 minutes. 75 U/ well; 30°C.

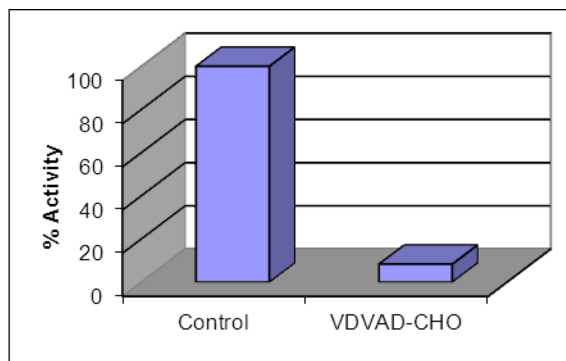

Figure 3. Inhibition of Caspase-2 by Ac-VDVAD-CHO. The enzyme was incubated with the inhibitor for 10 minutes prior to addition of substrate. 75 U/well; [VDVAD-CHO]=1 µM; [VDVAD-pNA]=200 µM; 30°C

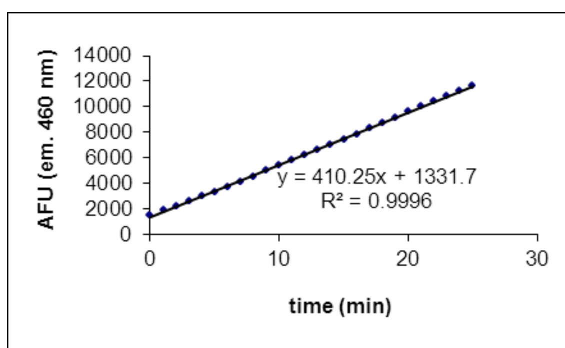

Figure 2. Assay of Caspase-2 activity using the AMC substrate. 30 U /well; [VDVAD-AMC]=50 µM; 25°C.

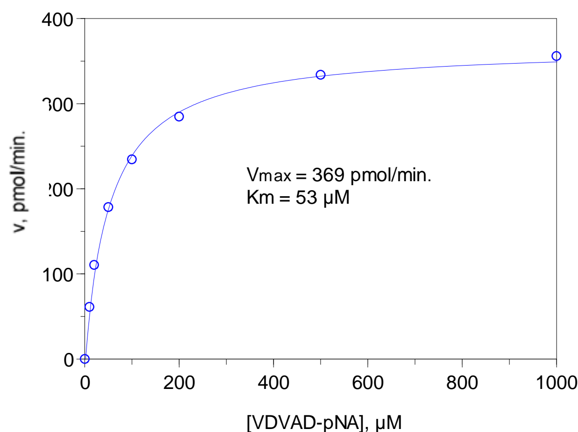

Figure 4. Kinetics of VDVAD-pNA cleavage by Caspase-2. 280 U/well; 30°C. Rates were obtained from the slopes of the initial, linear portion of plots of A_{405} vs. time. Curve and kinetic parameters derive from a non-linear least squares fit to the Michaelis-Menten equation (Marquadt algorithm).

DATA ANALYSIS

Plotting

1. Plot data as A_{405nm} or Arbitrary Fluorescence Units (AFU) versus time for each sample.
2. Determine the time range over which the reaction is linear. Typically, 0 to 15 min works well.
3. Obtain a "best fit" line for the data points and determine the slope.
4. Average the slopes of duplicate samples.

Data Reduction

5. If the blank has a significant slope, subtract this number from the slopes for all samples. Under normal circumstances this will not be necessary, since the slope will be nearly 0.
6. To find % remaining activity in presence of inhibitor:
   % activity remaining (plus inhibitor)=
   (slope of + inhibitor sample/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 calibration standard (p-nitroaniline; 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.

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

conversion factor (µM/OD) =50/average A_{405} 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.

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