Revised: 09–January–2003

EnzChek® Caspase-3 Assay Kit #1

***Z-DEVD–AMC Substrate* (E-13183)**

Quick Facts

Storage upon receipt:
- –20°C
- Protect Components A and G from light

Ex/Em: 342/441 nm (cleaved substrate)

Introduction

Apoptosis, or programmed cell death, plays a critical role in development as well as in several different disease states.[1] This process is both biochemically and morphologically distinct from the process of necrosis. In contrast to necrotic cells, apoptotic cells are characterized morphologically by compaction of the nuclear chromatin, shrinkage of the cytoplasm and production of membrane-bound apoptotic bodies. Biochemically, apoptosis is characterized by fragmentation of the genome and cleavage or degradation of several cellular proteins.[1]

Recently, members of the caspase (CED-3/ICE) family of proteases have been found to be crucial mediators of the complex biochemical events associated with apoptosis.[1-3] In particular, the activation of caspase-3 (CPP32/apopain), which has a substrate specificity for the amino acid sequence Asp-Glu-Val-Asp (DEVD) and cleaves a number of different proteins, including poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, protein kinase Cδ and actin, has been shown to be important for the initiation of apoptosis.[3,4]

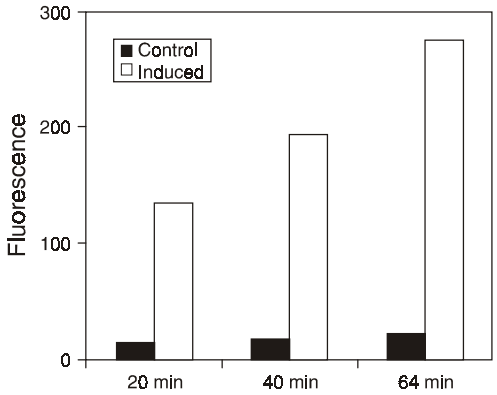

Figure 1. *Detection of protease activity in Jurkat cells (T-cell leukemia, human) using the EnzChek Caspase-3 Assay Kit #1 with Z-DEVD–AMC substrate. Cells were either treated with 10 µM camptothecin for four hours at 37°C to induce apoptosis (induced) or left untreated (control). Both induced and control cells were then harvested, lysed and assayed as described in the kit protocol. Reactions were carried out at room temperature and fluorescence was measured in a fluorescence microplate reader using excitation at 360 ± 20 nm and emission detection at 460 ± 20 nm after the indicated amount of time.*

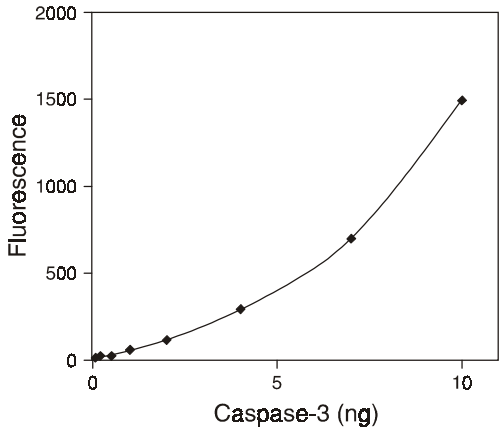

Figure 2. *Detection of caspase-3 activity. Increasing amounts of purified active human (recombinant) caspase-3 (PharMingen) were allowed to react with 100 µM Z-DEVD–AMC in 1X Reaction Buffer for ~45 minutes at room temperature. Fluorescence was measured in a fluorescence microplate reader using excitation at 360 ± 17.5 nm and emission detection at 465 ± 17.5 nm. Background fluorescence (386 arbitrary units), determined for a no-enzyme control, has been subtracted from each value.*

The EnzChek® Caspase-3 Assay Kit #1 allows the detection of apoptosis by assaying for increases in caspase-3 and other DEVD-specific protease activities (e.g., caspase-7). The basis for the assay is the 7-amino-4-methylcoumarin-derived substrate Z-DEVD–AMC (where Z represents a benzyloxycarbonyl group), which is weakly fluorescent in the UV range (excitation/emission ~330/390 nm), but which yields a bright blue–fluorescent product (excitation/emission ~342/441 nm) upon proteolytic cleavage. The kit can be used to continuously monitor the activity of caspase-3 and closely related proteases in cell extracts (Figure 1) and purified enzyme preparations (Figure 2) using a fluorescence microplate reader or fluorometer. In addition to the Z-DEVD–AMC substrate, the EnzChek Caspase Assay Kit #1 contains the reversible aldehyde inhibitor Ac-DEVD-CHO,[4] as well as the reference standard 7-amino-4-methylcoumarin (AMC). The Ac-DEVD-CHO inhibitor can be used to confirm that the observed fluorescence signal in both induced and control cell populations is due to the activity of caspase-3–like proteases. The reference standard is included to allow quantitation of the amount of AMC released in the reaction.

Materials

Kit Components

- **Z-DEVD–AMC substrate** (MW = 767.7, Component A), 4 mg
- **Dimethylsulfoxide (DMSO)** (Component B), 1.3 mL
- **20X Cell Lysis Buffer** (Component C), 1.5 mL of 200 mM TRIS, pH 7.5, 2 M NaCl, 20 mM EDTA, 0.2% TRITON™ X-100
- **5X Reaction Buffer** (Component D), 20 mL of 50 mM PIPES, pH 7.4, 10 mM EDTA, 0.5% CHAPS
- **Dithiothreitol (DTT)** (MW = 154.2, Component E), 100 mg
- **Ac-DEVD-CHO inhibitor** (MW = 502.5, Component F), 0.2 mg
- **7-Amino-4-methylcoumarin (AMC) reference standard** (MW = 175.2, Component G), 0.5 mg

Each kit provides sufficient reagent for performing ~500 assays using a reaction volume of 100 µL per assay.

Storage

Upon receipt, the kit should be stored at -20°C.

Stock Solution Preparation

1.1 Prepare a 10 mM stock solution of the Z-DEVD–AMC substrate: Bring the vial of Z-DEVD–AMC (Component A) and the vial of DMSO (Component B) to room temperature. Add 520 µL of DMSO directly to the vial of Z-DEVD–AMC. After use, the substrate stock solution should be stored desiccated at -20°C, protected from light.

1.2 Prepare a 1 M DTT stock solution by adding 650 µL of deionized water (dH_2O) directly to the vial of DTT solid (Component E). This stock solution should be stored frozen at -20°C.

1.3 If desired, prepare a 1 mM stock solution of the Ac-DEVD-CHO inhibitor: Bring the vial of Ac-DEVD-CHO (Component F) and the vial of DMSO (Component B) to room temperature. Add 400 µL of DMSO directly to the vial of Ac-DEVD-CHO. This inhibitor can be used to confirm the correlation between signal detection and caspase-3–like protease activity. After use, the inhibitor stock solution should be stored desiccated at -20°C.

1.4 If desired, prepare a 10 mM stock solution of the AMC reference standard: Bring the vial of AMC reference standard (Component G) and the vial of DMSO (Component B) to room temperature. Add 285 µL of DMSO directly to the vial of AMC. This solution can be used to prepare a standard curve to determine the moles of product produced in the caspase-3–containing reactions. After use, the reference standard stock solution should be stored at -20°C, protected from light.

Protocol

The following procedure is designed for use with a fluorescence microplate reader. For use with a standard fluorometer, volumes should be increased accordingly. For best results, we recommend using the lysate of at least 1×10^6 cells for each reaction.

2.1 Induce apoptosis in cells using the desired method. A negative control should be prepared by incubating cells in the absence of inducing agent. If desired, additional samples and controls can be prepared for subsequent incubation with the inhibitor Ac-DEVD-CHO (see step 2.6).

2.2 Harvest the cells after the desired length of time and wash in phosphate-buffered saline (PBS). If desired, cell pellets may be stored frozen at -80°C for analysis at a later time.

2.3 Prepare a 1X Cell Lysis Buffer working solution: Add 50 µL of the 20X Cell Lysis Buffer (Component C) to 950 µL of dH_2O. This 1 mL volume is sufficient for ~20 assays.

2.4 Resuspend each cell sample or control in 50 µL of the 1X Cell Lysis Buffer. For optimal lysis, we recommend subjecting the cells to a freeze–thaw cycle. For example, freeze the cells in a dry ice–ethanol bath for ~5 minutes and then thaw. Alternatively, cells can be lysed by incubating on ice for ~30 minutes.

2.5 While the cells are being lysed, prepare a solution of 2X Reaction Buffer: Add 400 µL of the 5X Reaction Buffer (Component D) and 10 µL of 1 M DTT (prepared in step 1.2) to 590 µL of dH_2O. This 1 mL volume is sufficient for performing ~20 assays.

Note: The 5X Reaction Buffer, and the 2X Reaction Buffer made from it, may contain micellular material in suspension. This is normal and will not adversely affect the reactions. Always mix these reagents well immediately before sampling.

2.6 Centrifuge the lysed cells to pellet the cellular debris. For example, centrifuge at 5000 rpm for 5 minutes in a microcentrifuge. Transfer 50 µL of the supernatant from each sample to individual microplate wells. Use 50 µL of the 1X Cell Lysis Buffer as a no-enzyme control to determine the background fluorescence of the substrate.

2.7 If desired as an additional control, add 1 µL of the 1 mM Ac-DEVD-CHO inhibitor stock solution (prepared in step 1.3) to selected samples. Cover and incubate at room temperature for 10 minutes. The remaining samples (without inhibitor) should be stored on ice during this time. If desired, 1 µL of DMSO (without inhibitor) can be added to the remaining no-inhibitor samples to act as a control for the DMSO added to the inhibitor-containing samples; these control samples should be incubated for the same length of time and at the same temperature as the inhibitor-containing samples.

2.8 Prepare a 2X substrate working solution by mixing 20 µL of the 10 mM Z-DEVD–AMC substrate (prepared in step 1.1) with 980 µL of the 2X Reaction Buffer (prepared in step 2.5).

2.9 Add 50 µL of the 2X substrate working solution to each sample and control.

2.10 Cover the microplate and incubate the samples at room temperature for approximately 30 minutes.

2.11 If desired, prepare an AMC standard curve: Dilute the appropriate amount of 10 mM AMC stock solution (prepared in step 1.4) into 1X Reaction Buffer (prepared by diluting the 5X reaction buffer five-fold in dH_2O) to yield AMC solutions rang-

ing in concentration from 0–100 µM. Pipet 100 µL of each standard into empty microplate wells at any time prior to measuring the fluorescence.

2.12 Measure the fluorescence (excitation/emission ~342/441 nm) using appropriate excitation and emission filters or settings. Because the assay is continuous, measurements can be made at multiple time points.

References

1. Immunol Cell Biol 76, 1 (1998); **2.** Science 281, 1312 (1998); **3.** Trends Biochem Sci 22, 388 (1997); **4.** Nature 376, 37 (1995).

Product List

Current prices may be obtained from our Web site or from our Customer Service Department.

Cat #	Product Name	Unit Size
E-13183	EnzChek® Caspase-3 Assay Kit #1 *Z-DEVD-AMC substrate* *500 assays*	1 kit

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Further information on Molecular Probes' products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

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