Revised: 24–February–2003

RediPlate™ 96 EnzChek® Caspase-3 Assay Kit (R-35100)

Quick Facts

Storage upon receipt:

- ≤ –20°C
- Desiccate
- Protect from light

Ex/Em of reaction product: 496/520 nm

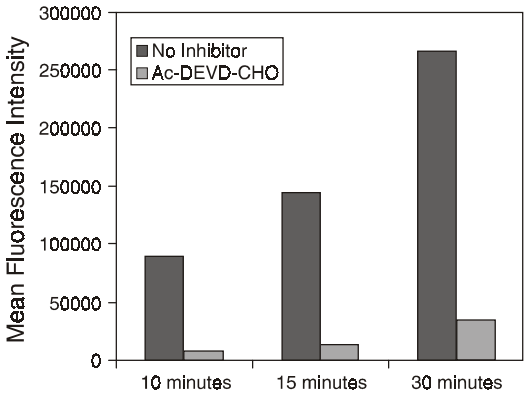

Figure 1. *Detection of protease activity in Jurkat cells (T-cell leukemia, human) using the RediPlate 96 EnzChek Caspase-3 Assay Kit. Cells were treated with 10 µM camptothecin for 4 hours at 37°C to induce apoptosis, then harvested and lysed according to the kit protocol. The cell lysate was separated into two samples, one of which was treated with the included Ac-DEVD–CHO inhibitor. Assay reactions on both the inhibited and the uninhibited samples were carried out at 37° C, and fluorescence was measured in a microplate reader (excitation/emission 485/535 nm).*

Introduction

Molecular Probes' RediPlate™ 96 EnzChek® Caspase-3 Assay Kit [1] provides a fast, simple and direct fluorescence-based assay for detecting caspase-3 activity in cell lysates and purified samples. Unlike other microplate assays, this kit provides the necessary reagents already predispensed into a 96-well microplate. Simply reconstitute the fluorescent substrate in the assay wells, add the desired sample to the wells, and incubate. The resulting signal can be quantitated in any standard fluorescence-based microplate reader.

Members of the caspase (CED-3/ICE) family of proteases have been identified as crucial mediators of the complex biochemical events associated with apoptosis.[2-4] Caspase-3 (CPP32/apopain) is a key effector in the apoptotic pathway, amplifying the signal from initiator caspases (such as caspase-8) and signifying full commitment to cellular disassembly. The active protease recognizes the amino acid sequence Asp-Glu-Val-Asp (DEVD); in addition to cleaving other caspases in the enzyme cascade, caspase-3 has been shown to cleave poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, protein kinase Cδ and actin.[4-5]

The RediPlate 96 EnzChek Caspase-3 Assay Kit detects apoptosis by assaying for increases in caspase-3 and other DEVD-specific protease activities such as caspase-7 (Figure 1). The basis for the assay is rhodamine 110 bis-(*N*-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD–R110). This substrate is a bisamide derivative of rhodamine 110 (R110) containing DEVD peptides covalently linked to each of R110's amino groups, thereby suppressing the dye's visible absorption and its fluorescence. Upon enzymatic cleavage, the nonfluorescent bisamide substrate is converted in a two-step process, first to the fluorescent monoamide and then to the even more fluorescent R110. Both of these hydrolysis products exhibit spectral properties similar to those of fluorescein, with peak excitation and emission wavelengths of 496 nm and 520 nm, respectively.

The RediPlate 96 EnzChek Caspase-3 Assay Kit includes one microplate and all necessary buffers and reagents for performing the assay. Also included is the reversible aldehyde inhibitor Ac-DEVD–CHO (supplied in a separate vial), which 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.[4] To ensure the integrity of the fluorogenic components, the microplate is contained in a resealable foil packet. The microplate consists of twelve removable strips, each with eight wells. Eleven of the strips (88 wells) are preloaded with the Z-DEVD–R110 substrate. The remaining strip, marked with blackened tabs, contains a dilution series of free R110 that may be used as a fluorescence reference standard.

Materials

Kit Contents

- **RediPlate 96 EnzChek caspase-3 assay microplate** (Component A), one microplate
- **RediPlate caspase-3 2X reaction buffer** (Component B), 12 mL of 20 mM PIPES, pH 7.4, 4 mM EDTA and 0.2% CHAPS
- **Dithiotheitol (DTT)** (MW =154.2, Component C), 50 mg
- **Ac-DEVD–CHO inhibitor** (MW=502.5, Component D), 50 µg
- **Dimethylsulfoxide (DMSO)** (Component E), 200 µL
- **20X Cell lysis buffer** (Component F), 1.0 mL of 200 mM TRIS, pH 7.5, 2 M NaCl, 20 mM EDTA, 0.2% TRITON™ X-100

Storage and Handling

Store the RediPlate 96 EnzChek Protease Assay Kit at –20°C or below, desiccated and protected from light. When stored properly, the kit components should remain stable for at least six months.

Experimental Protocol

The following protocols describe the assay for protease or protease inhibitor activity in total volumes of 100 µL per microplate well. Each RediPlate 96 Caspase-3 Assay Kit contains one 96-well microplate with 88 wells (11 strips) intended for assays and 8 wells (1 strip, with blackened tabs) for a fluorescence reference standard curve. Because each strip is removable, one can perform as many or as few assays as needed.

RediPlate 96 Microplate Preparation

1.1 Allow the kit components to warm to room temperature. Remove the RediPlate Kit from the freezer and allow it to warm to room temperature. DO NOT OPEN THE FOIL PACKET UNTIL IT IS WARM. The plate (Component A) will typically take ~20 minutes to warm. Because the reaction buffer (Component B) may take longer than 20 minutes to thaw at room temperature, place the vial of buffer in a warm water bath to accelerate thawing. After thawing, the buffer may be stored at 2–6°C, for convenience.

1.2 Remove any extra strips. Determine the number of strips required; carefully cut through the self-adhesive sealing film with a razor blade and remove any extra strips that are to be used at a later date. Return these to the protective foil bag. All of the strips, with the exception of the control strip with blackened tabs, contain equivalent amounts of the protease substrate. Empty strip holders from previously purchased RediPlate 96 kits are useful for storing extra assay strips.

Fluorescence Reference Standards

The fluorescence reference standards serve as controls for instrument-to-instrument variation and for day-to-day variation in single instrument performance. In an experiment using the Z-DEVD–R110 substrate, the signal obtained will be a combination of the fluorescence from R110 (liberated by two cleavage events) and the less intense fluorescence from the monoamide form of the substrate (the result of a single cleavage event). As a result, the signal obtained from the reference standard cannot be used to quantitate the amount of R110 released by enzymatic cleavage.

Table 1. *Reference standard for the RediPlate 96 Caspase-3 Assay Kit.*

Well	Amount of R110 (picomoles)
A	2500
B	1000
C	200
D	40
E	8
F	1.6
G	0.2
H	0

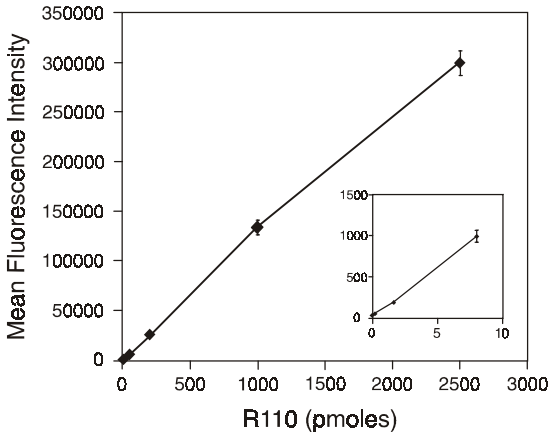

Figure 2. *Fluorescence reference standards of the RediPlate 96 EnzChek Caspase-3 Assay Kit. 100 µL of the 1X reaction buffer was added to each well containing the R110 reference standards from five kits, and fluorescence was measured in a fluorescence-based microplate reader (excitation/emission 485/535 nm). The background fluorescence (determined from well H) was subtracted from each value, and the mean values of the five determinations were plotted with an error bar indicating ± one standard deviation. The inset shows an enlargement of the results obtained with R110 amounts between 0 and 8 picomoles.*

2.1 Prepare the fluorescence standards. Add 100 µL of 1X reaction buffer (prepared as in step 6.1, below) to each well of the control strip of the RediPlate 96 microplate and mix by pipetting. Complete resuspension will take ~1 minute after the addition of buffer. Blackened tabs differentiate this strip from strips containing the Z-DEVD–R110 substrate. The control strip contains a dilution series of the R110 reference standard (Table 1). Well A has the highest concentration of the reference standard; well H contains no reference dye.

2.2 Measure the fluorescence. The fluorescence standard samples are typically measured for fluorescence along with the samples from the protease or protease-inhibitor assays (step 4.11). The data obtained should be similar to that shown in Figure 2.

Stock Solution Preparation

3.1 Prepare a DTT stock solution. Prepare a 1 M DTT stock solution by adding 325 µL of deionized water (dH_2O) directly to the vial of DTT solid (Component C). This stock solution should be stored frozen at –20°C.

3.2 Prepare an inhibitor stock solution (optional). If desired, prepare a 1 mM stock solution of the Ac-DEVD–CHO inhibitor. Bring the vial of Ac-DEVD–CHO (Component D) and the vial of DMSO (Component E) to room temperature. Add 100 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.

Preparation of Cell Lysates

4.1 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 4.6). For best results, we recommend using the lysate of at least 1×10^6 cells for each reaction.

4.2 Harvest the cells. After apoptosis induction is complete, wash the cells in phosphate-buffered saline (PBS). If desired, cell pellets may be stored frozen at –80°C for analysis at a later time.

4.3 Prepare a 1X cell lysis buffer working solution. Add 50 µL of the 20X cell lysis buffer (Component F) to 950 µL of dH_2O. This 1 mL volume is sufficient for ~20 assays.

4.4 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.

4.5 Centrifuge the lysed cells to pellet the cellular debris. Centrifuge at 5000 rpm for 5 minutes in a microcentrifuge. Recover 50 µL of supernatant from each sample. NOTE: If the samples are to be assayed for caspase-3 immediately, the supernatants may be transferred directly to separate wells of the RediPlate containing reconstituted substrate (steps 5.1–5.3). However, if the samples are to be incubated plus and minus the inhibitor Ac-DEVD–CHO (step 4.6, below), the 50 µL supernatant samples must be transferred to separate user-supplied tubes. DO NOT use the RediPlate microplate for these incubations.

4.6 Add inhibitor to desired samples (optional). If desired as an additional control, add 1 µL of the 1 mM Ac-DEVD–CHO inhibitor stock solution (prepared in step 3.2) 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, Component E) 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.

Assay of Cell Lysates

5.1 Add DTT to the 2X reaction buffer. Add 10 µL of 1 M DTT (prepared in step 3.1) to 990 µL of 2X reaction buffer (Component B). This 1 mL volume is sufficient for performing ~20 assays. NOTE: The 2X reaction buffer may contain micelles in suspension. This is normal and will not adversely affect the reactions. Always mix well immediately before using.

5.2 Add reaction buffer to the desired number of wells. NOTE: This step may be performed during the incubation portion of step 4.4 or 4.6. Dispense 50 µL of 2X reaction buffer plus DTT (prepared in step 5.1) to each RediPlate 96 well needed for experimental samples and controls. Pipette up and down 2–3 times per well to fully resuspend the substrate included in the well. The concentration of the reconstituted Z-DEVD–R110 substrate is now 50 µM but will be 25 µM in the final assay volume. Be sure to prepare at least one well to serve as a no-cell control for determining the background fluorescence of the substrate.

5.3 Add the samples to the microplate wells. Transfer 50 µL of each supernatant (step 4.5, or if inhibitor was added, step 4.6) from each sample to individual wells. Add 50 µL of the 1X cell lysis buffer to the well(s) serving as the no-cell control.

5.4 Cover and incubate. Cover the microplate wells and incubate the samples at room temperature for approximately 30 minutes, protected from light. Because the assay is continuous, the fluorescence can be monitored at multiple time points.

5.5 Measure the fluorescence. R110 has fluorescence excitation/emission at ~496/520 nm and can be detected with filters or settings appropriate for fluorescein. Because the assay is continuous, measurements can be made at multiple time points.

Assay of Purified Enzyme

6.1 Prepare a solution of 1X reaction buffer plus DTT. Add 500 µL of the 2X reaction buffer (Component B) and 5 µL of 1 M DTT (prepared in step 3.1) to 495 µL of dH_2O. This 1 mL volume is sufficient for performing ~20 assays.

6.2 Add reaction buffer to the desired number of wells. Dispense 50 µL of 1X reaction buffer plus DTT prepared in step 6.1 to each RediPlate 96 well needed for experimental samples and controls. Pipette up and down 2–3 times per well to fully resuspend the substrate included in the well. The concentration of the reconstituted Z-DEVD–R110 substrate is now 50 µM but will be 25 µM in the final assay volume. Be sure to prepare at least one well to serve as a no-enzyme control for determining the background fluorescence of the substrate.

6.3 Prepare a 2X enzyme solution. Dilute the enzyme to 2X the desired final concentration in 1X reaction buffer plus DTT. A 50 µL volume of the 2X enzyme solution is required for each experimental well.

6.4 Start the reaction. Start the reaction(s) by adding 50 µL of the 2X enzyme solution to each experimental well. To the no-enzyme control well(s), add 50 µL of 1X reaction buffer plus DTT. Incubate the reactions at the optimal temperature for the enzyme being assayed, protected from light.

6.5 Monitor the fluorescence. Because the assay is continuous, measurements can be taken at multiple time points. R110 has fluorescence excitation/emission at 496/520 nm and can be detected with filters or settings appropriate for fluorescein.

6.6 Correct for background fluorescence. For each experimental sample, subtract the value derived from the no enzyme control(s).

References

1. This product is covered by US patent 4,557,862 or 4,460,893 and is sold for nonclinical research and development purposes only. **2.** Immunol Cell Biol 76, 1 (1998); **3.** Science 281, 1312 (1998); **4.** Trends Biochem Sci 22, 388 (1997); **5.** 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
R-35100	RediPlate™ 96 EnzChek® Caspase-3 Assay Kit *Z-DEVD-R110 substrate* *one 96-well microplate*	1 kit

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