Revised: January 28, 2013

Product Information

Caspase-8 IETD-R110 Fluorometric and Colorimetric Assay Kit

Catalog Number: 30011-1, 30011-2

Unit Size:
30011-1: 25 assays
30011-2: 100 assays
Assays based on 96-well format

Kit Contents

Component	30011-1	30011-2
Cell Lysis Buffer	30 mL 99917	100 mL 99918
Assay Buffer	1 X 1.25 mL 99919	4 X 1.25 mL 99919
Enzyme Substrate $(Ac\text{-}IETD)_2$-R110 (1 mM)	125 uL 30011C1	500 uL 30011C2
Enzyme Inhibitor Ac-IETD-CHO (5 mM)	5 uL 99928	20 uL 99929
R110 (80 uM)	1 mL 99906	1 mL 99906

Storage and Handling

Store at –20°C and avoid multiple freeze-thaw cycles. The kit is stable for at least 6 months from date of receipt when stored as recommended.

Spectral Properties: Ex/Em: 496/520 nm

Product Description

Caspase-8 is the most upstream caspase in the CD95/Fas apoptotic pathway and is activated by the signaling pathway for CD95/Fas and TNF (1). Caspase-8 IETD-R110 Fluorometric and Colorimetric Assay Kit provides a simple assay system for fast and highly sensitive detection of caspase-8 activity (see note below). The fluorogenic and chromogenic substrate (Ac-IETD)2-R110 contains two IETD tetrapeptides and is completely hydrolyzed by the enzyme in two successive steps. Cleavage of the first IETD peptide results in the monopeptide Ac-IETD-R110 intermediate, which has absorption and emission wavelengths similar to those of R110 but has only about 10% of the fluorescence of the latter (2). Hydrolysis of the second IETD peptide releases the dye R110, leading to a substantial fluorescence increase.

Although fluorometric detection of R110 is preferred due to superior sensitivity, absorbance-based measurements also can be used. In fact, the extinction coefficient of R110 is 10 times higher than that of p-nitroaniline (pNA), a dye commonly used in chromogenic substrates. Therefore, R110-based substrates are significantly more sensitive than pNA-based substrates, even by colorimetric detection. The intensity of the fluorescent or colorimetric signal generated from the assay is proportional to the caspase-3 activity present in the sample.

The assay kit includes the competitive caspase-8 inhibitor Ac-IETD-CHO for use as a negative control. Also, R110 is provided in the kit for generating a standard curve, which can be used for quantifying caspase activity.

Note: While caspase-8 preferentially cleaves the consensus sequence IETD compared to other substrate sequences (3), other caspases such as caspase-3 also can cleave IETD efficiently (4). Overlapping caspase substrate recognition limits the usefulness of caspase substrate peptides for distinguishing between different caspase activities in cell lysates.

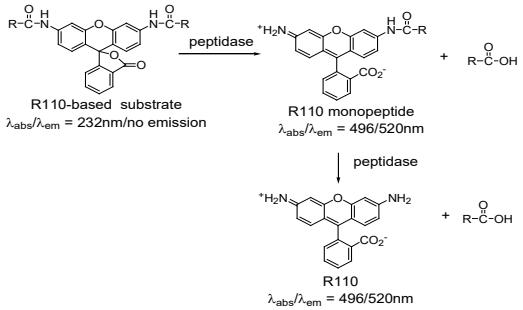

Figure 1. Two-step cleavage of R110-based substrates by peptidases to release green fluorescent R110 dye.

Protocol

The following protocol is designed for use in 96-well plates with a total assay volume of 100 uL per well. Volumes can be scaled proportionally as needed.

A. General Considerations

We recommend performing three control reactions:

1) Negative control using untreated cells
2) Positive control using cells treated with an apoptosis inducing agent
3) Inhibitor control using induced cells and Caspase-8 inhibitor.

B. Assay Procedure

1. Plate adherent cells in black 96-well plates. Suspension cells can be plated in flasks or plates.
2. Induce apoptosis in cells by desired methods. Remember to include untreated wells as controls.
3. Cell lysis:

   For suspension cells:

   a) Aliquot equal numbers of cells into microcentrifuge tubes or wells of a black 96-well plate. 500-1,000,000 cells per sample can be used for fluorometric detection (10,000-100,000 cells is optimal for Jurkat cells), while 1,000,000 cells per sample is required for colorimetric detection.

   b) Centrifuge cells at 400 xg for 5 minutes and aspirate supernatant. For fluorometric detection, it is not necessary to centrifuge cells as long as cells are suspended in less than 10 uL medium. Optional: after this step, you may freeze the cell pellets at -70°C to assay at a later time.

   c) Resuspend the cell pellets in 50 uL of chilled Cell Lysis Buffer.

   For adherent cells:

   a) Aspirate culture medium from each well of the 96-well plate. Add 50 uL chilled Cell Lysis Buffer per well.
4. Incubate cells in Lysis Buffer on ice for 10 minutes.
5. Centrifuge cell lysates in a microcentrifuge tube at maximum speed for 5 minutes at 4°C to pellet insoluble cell debris. Transfer the supernatants to new microcentrifuge tubes.

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6. Add 50 uL of Assay Buffer to each sample and mix well.

   Optional: to verify that the signal detected by the kit is due to Caspase-8 activity, incubate an induced sample with caspase-3 inhibitor before adding substrate. Add 1 uL of Enzyme Inhibitor Ac-IETD-CHO (5 mM) to each inhibitor control sample. Incubate on ice for 30 minutes or room temperature for 15 minutes along with the other samples. Proceed to step 7.

7. Add 5 uL of 1 mM Enzyme Substrate to each sample and mix well. Incubate samples at 37°C for 30-60 minutes (up to 3 hours maximum).

8. Measure fluorescence with 470 nm excitation and 520 nm emission. For colorimetric measurement, measure absorbance at 495 nm.

C. R110 Reference Standard (Optional)

1. Dilute R110 (80 uM) to 20 uM in Cell Lysis Buffer. Perform 1:2 serial dilutions to obtain concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, and 0.156 uM R110. Use Cell Lysis Buffer for the 0 uM (blank) sample. Add 100 uL/ well of the serially diluted R110 solutions from 20 uM to 0 uM into a 96-well plate.

2. Measure the fluorescence intensity of the standards at Ex/Em=470 nm/520 nm. Subtract the fluorescence reading from the blank (0 uM R110) from each fluorescence value to calculate relative fluorescence units (RFU).

3. Plot RFU versus R110 concentration to generate a standard curve.

   Note: The kinetics of fluorescence generation due to substrate cleavage are not linear because the two-step cleavage of the substrate generates an intermediate and an end-product with different fluorescence intensities (see Product Description). Therefore, the R110 standard can be used to quantitate the amount of R110 generated at the endpoint of the assay, but cannot be used for kinetic studies.

References

1. Porter AG, Janicke RU. (1999) Emerging roles of caspase-3 in apoptosis. Cell Death Differ 6(2):99-104.
2. Hug H, Los M, Hirt W, Debatin KM. (1999) Rhodamine 110-linked amino acids and peptides as substrates to measure caspase activity upon apoptosis induction in intact cells. Biochemistry 38(42):13906-11.
3. Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT, Nicholson DW. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem. 1997 Jul 18;272(29):17907-11.
4. McStay GP, Salvesen GS, Green DR. Overlapping cleavage motif selectivity of caspases: implications for analysis of apoptotic pathways. Cell Death Differ. 2008 Feb;15(2):322-31.

Related Products

Catalog number	Product
30012	Caspase-8 IETD-R110 Fluorometric HTS Assay Kit
30008	Caspase-3 DEVD-R110 Fluorometric and Colorimetric Assay Kit
30009	Caspase-3 DEVD-R110 Fluorometric HTS Assay Kit
30029	NucView 488 Caspase-3 Substrate Kit for Live Cells
30067	Dual Apoptosis Assay Kit with NucView 488 & CF594-Annexin V
30062	NucView 488 and MitoView 633 Apoptosis Kit
30065	Apoptosis & Necrosis Quantitation Kit Plus
30066	Apoptotic, Necrotic & Healthy Cells Quantitation Kit Plus
30001	JC-1 Mitochondrial Membrane Detection Kit
70055	MitoView 633 mitochondrial membrane potential dye
30063	CF488A TUNEL Assay Apoptosis Detection Kit
30064	CF594 TUNEL Assay Apoptosis Detection Kit