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

<table>
<thead>
<tr>
<th>Component</th>
<th>30011-1</th>
<th>30011-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Lysis Buffer</td>
<td>30 mL</td>
<td>100 mL</td>
</tr>
<tr>
<td></td>
<td>99917</td>
<td>99918</td>
</tr>
<tr>
<td>Assay Buffer</td>
<td>1 X 1.25 mL</td>
<td>4 X 1.25 mL</td>
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<tr>
<td></td>
<td>99919</td>
<td>99919</td>
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<tr>
<td>Enzyme Substrate</td>
<td>125 uL</td>
<td>500 uL</td>
</tr>
<tr>
<td>(Ac-IETD)2-R110 (1 mM)</td>
<td>30011C1</td>
<td>30011C2</td>
</tr>
<tr>
<td>Enzyme Inhibitor</td>
<td>5 uL</td>
<td>20 uL</td>
</tr>
<tr>
<td>Ac-IETD-CHO (5 mM)</td>
<td>99928</td>
<td>99929</td>
</tr>
<tr>
<td>R110 (80 uM)</td>
<td>1 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td></td>
<td>99906</td>
<td>99906</td>
</tr>
</tbody>
</table>

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.

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, 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.
   For adherent 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.
      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. Incubahe 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 quantify the amount of R110 generated at the endpoint of the assay, but cannot be used for kinetic studies.

References