



**Caspase-8 IETD-R110  
Fluorometric & Colorimetric Assay Kit.**

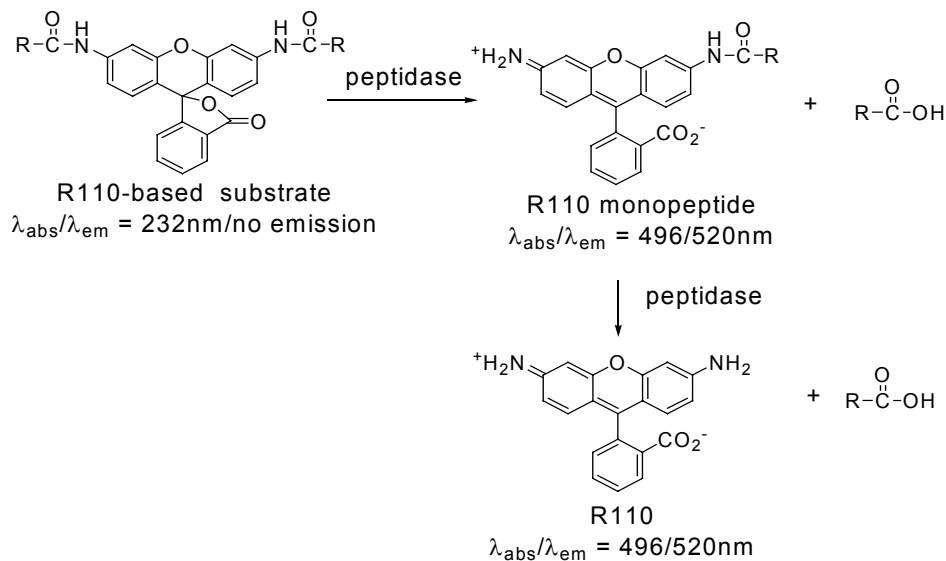
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## I. Description

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, 2). Caspase-8 IETD-R110 Fluorometric and Colorimetric Assay Kit provides a simple assay system for fast and highly sensitive detection of caspase-8 activity either by fluorescence or absorbance in mammalian cells. The fluorogenic and chromogenic substrate (Ac-IETD)<sub>2</sub>-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 mono-peptide Ac-IETD-R110 intermediate, which has absorption and emission wavelengths similar to those of R110 ( $\lambda_{\text{abs}}/\lambda_{\text{em}}=496/520$  nm) but has only about 10% of the fluorescence of the latter (3-4). Hydrolysis of the second IETD peptide releases the dye R110, leading to a substantial fluorescence increase.



Although fluorometric detection of the end products is preferred due to the superior sensitivity, detection by absorbance is also feasible as well. 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-8 activity present in the sample.

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

## II. Kit Components

<b>25 assays</b>	<b>100 assays</b>
<u>(30011-1)</u>	<u>(30011-2)</u>
30 mL	100 mL
1.25 mL	5 mL
125 $\mu$ L	500 $\mu$ L
5 $\mu$ L	20 $\mu$ L
1 mL	4 mL

**Cell Lysis Buffer**

**Assay Buffer**

**Enzyme Substrate (Ac-IETD)<sub>2</sub>-R110 (1 mM)**

**Enzyme Inhibitor Ac-IETD-CHO (5 mM)**

**R110 (80  $\mu$ M)**

### III. Storage Condition

Caspase-8 IETD-R110 Fluorometric and Colorimetric Assay Kit should be stored at  $-20^{\circ}\text{C}$  or below. The components of the kit are stable at  $-20^{\circ}\text{C}$  for six months. Avoid frequent freeze-thaw cycles.

### IV. Features

**Fast:** Fast enzyme kinetics. Enzyme reaction time can be as short as 30 min.

**Sensitive:** The enzymatic reaction forms intensely yellow colored and highly green fluorescent rhodamine 110 (R110) products. The long wavelengths of 110 excitation and emission minimize cellular autofluorescence.

**Versatile:** Compatible with both fluorometric and colorimetric detection systems.

### V. Assay for Detection of Caspase-8 Activity in Cell Culture

#### A. General Considerations

We recommend performing three control reactions:

- 1) a negative control on uninduced cells.
- 2) a control on induced cells treated with Caspase-8 inhibitor.
- 3) a positive control for Caspase-8 induction.

#### B. Assay Procedure

1. Induce apoptosis in cells by desired methods. Remember to incubate concurrent culture without induction.

2. For suspension cells:

a) Count cells and aliquot equal number of cells into each microcentrifuge tubes or wells in a 96-well plate. The range of cells for effective fluorometric detection is 500-1,000,000 cells per sample (10,000-100,000 cells per sample is optimal for the caspase assay using Jurkat cells), while 1,000,000 cells per sample are required for colorimetric detection.

b) Centrifuge cells at 400xg for 5 min and aspirate supernatant. For fluorometric detection, it is not necessary to centrifuge cells as long as cells are suspended in <10 uL medium.

**Note:** After this step, you may freeze the cell pellets at  $-70^{\circ}\text{C}$  and assay at a later time.

c) Flip the tube or shake the 96-well plate to loosen the cell pellets, and resuspend cells in 50 uL of chilled Lysis Buffer.

For attached cells:

a) Aspirate cell medium in each well of the 96-well plate where cells were plated and induced.

b) Add 50 uL of chilled Lysis Buffer into each well of a 96-well plate.

3. Incubate cells in Lysis Buffer on ice for 10 min.

4. Centrifuge cell lysates in a microcentrifuge at a maximum speed for 5 min at  $4^{\circ}\text{C}$  to precipitate cellular debris. Transfer the supernatants to new microcentrifuge tubes.

5. **[Optional]** To verify that the signal detected by the kit is due to Caspase-8 activity, incubate an induced sample with caspase-8 inhibitor before adding substrate. This can be accomplished by adding 50 uL Assay Buffer and 1 uL of Caspase-8 inhibitor (5 mM) to 50 uL of supernatant from a sample obtained in Step 4. Incubate on ice for 30 min or RT for 15 min with the other samples. Proceed to Step 6, where you will add Assay Buffer to the remaining samples.

6. Add 50 uL of Assay Buffer to each reaction.

7. Add 5  $\mu$ L of 1mM Caspase-8 Substrate (IETD-R110; 50  $\mu$ M final concen.) to each sample, Incubate at 37°C from 30 min-1hr (or up to 3 hours maximum) in an incubator.

8. Read in a fluorometer with 470 nm excitation filter and 520 nm emission filter for optimal sensitivity or read in a spectrophotometer or colorimetric plate reader at 495 nm.

9. Use R110 if necessary for generating a standard curve to calculate amount of substrate conversion.

### **C. R110 Reference Standard (Optional)**

1. Dilute R110 (80 $\mu$ M) to 20  $\mu$ M in Cell Lysis Buffer. Do 1:2 serial dilutions to give the concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 and 0  $\mu$ M R110 solutions. Add 100  $\mu$ L/ well of the serially diluted R110 solutions from 20  $\mu$ M to 0  $\mu$ M into a 96-well plate.

2. Measure the fluorescence intensity of the reference standard at Ex/Em=470 nm/520 nm. The fluorescence reading from the wells containing 0  $\mu$ M R110 solution is the background fluorescence. The readings from other wells need to be subtracted by this background fluorescence to get the relative fluorescence unit (RFU).

3. Plot R110 fluorescence reference standard as RFU versus concentration.

**Note:** This reference standard curve is used to calibrate for the variation of the different instruments and for the different batches of experiments. Since the proteolytic cleavage of (IETD)<sub>2</sub>-R110 has two steps, first to IETD-R110 and then to R110, and both the intermediate and final products have fluorescence, the R110 reference standard **cannot** serve as an indicator of the amount of final product of the caspase enzymatic reaction.

### **VI. References**

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2. Kruidering M, Evan GI. Caspase-8 in apoptosis: the beginning of "the end"? *IUBMB Life*. 2000 Aug;50(2):85-90.
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4. Hug H, Los M, Hirt W, Debatin KM. Rhodamine 110-linked amino acids and peptides as substrates to measure caspase activity upon apoptosis induction in intact cells. *Biochemistry*. 1999 Oct 19;38(42):13906-11.