SensoLyte® Homogeneous AFC Caspase-8 Assay Kit *Fluorimetric*

Catalog #	72088-200
Kit Size	200 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect caspase-8 activity.
- ***Enhanced Value:*** It provides enough reagents to perform 200 assays in a 96-well format.
- ***High Speed:*** Minimal hands-on time.
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	Ac-IETD-AFC, caspase-8 substrate, Ex/Em=380 nm/500 nm upon cleavage	10 mM, 200 µL
Component B	AFC (7-amido-4-trifluoromethylcoumarin), fluorescence reference standard, Ex/Em=380 nm/500 nm	6 mM, 10 µL
Component C	Inhibitor of caspase-8, Ac-IETD-CHO	5 mM, 10 µL
Component D	Assay Buffer	20 mL
Component E	DTT	1 M, 1 mL

Other Materials Required (but not provided)

- Caspase-8 source: Cell culture, the purified enzyme.
- 96-well microplate: Black tissue culture microplates with clear bottom.
- Fluorescence microplate reader: Capable of detecting emission at 500 nm with excitation at 380 nm.

Storage and Handling

- Store all kit components at -20°C.
- Protect Components A and B from light and from moisture.
- Component D can be stored at room temperature for convenience.

Introduction

Apoptosis is programmed cell death which leads to the elimination of damaged, unwanted, or unneeded cells without releasing harmful substances into the surrounding area. Apoptosis is involved in a variety of physiological and pathological events,[1] ranging from normal fetal development to diseases, such as cancer,[2] organ failure and neurodegenerative diseases. Caspases are proteases, which play essential roles in apoptosis and inflammation. There are two types of apoptotic caspases: initiator caspases and effector caspases. Caspase-8, an initiator enzyme, also known as Mch5, MACH and FLICE, accepts signals from death receptors (FAS, TRAIL, TNF) and activates downstream effector caspases through proteolytic cleavage.[3,4] Activated effector caspases induce apoptosis through cleavage of other cellular substrates such as PARP and DFF.

Caspase-8 has substrate specificity for the amino acid sequence Ile-Glu-Thr-Asp (IETD). The SensoLyte® Homogeneous AFC Capase-8 Assay Kit uses Ac-IETD-AFC as the fluorogenic indicator to measure caspase-8 activity. Upon cleavage of the substrate by caspase-8, free AFC (7-amido-4-trifluoromethylcoumarin) emits a yellow-green fluorescence, which can be quantified at excitation/emission=380 nm/500 nm. A bi-functional assay buffer in this kit is optimized for cell lysis and measurement of the enzyme activity. This kit can assay caspase-8 activity in cells grown in a 96-well plate or in larger plates or flasks. It is also adaptable for high throughput screening of apoptosis inducers and inhibitors.

Protocol

Note 1: For standard curve, please refer to Appendix II (optional).
Note 2: Please use Protocol A or B based on your needs.

Protocol A. Protocol for Cells Cultured in a 96-Well Plate

1. Prepare apoptotic cells.

Note: The following homogeneous assay procedure is for cells grown directly in a 96-well plate. If using plates larger than 96-well plates (e.g. 10-cm plate), please use protocol B.

1.1 Seed $1X10^{4-6}$ cells per well in a 96-well plate. Add test compounds and then incubate cells in a 37°C incubator for the desired exposure period. The suggested volume for a 96-well plate is 100 µL of medium.

Set up the following controls at the same time:

- Positive control contains cells and a known apoptosis inducer.
- Negative control contains cells without apoptosis inducer.
- Vehicle control contains cells and the vehicle used to deliver test compounds.

1.2 Bring up the total volume of all the controls to 100 µL using growth medium.

2. Prepare working solutions.

Note: Warm all kit components until thawed to room temperature before starting the experiment.

2.1 Assay buffer: Prepare fresh assay buffer for each experiment according to Table 1. Use the DTT-containing assay buffer in all the subsequent steps.

Table 1. Assay buffer for one 96-well plate (100 assays)

Components	Volume
Assay buffer (Component D)	9.6 mL
1 M DTT (Component E)	400 µL
Total volume	10 mL

2.2 Caspase-8 substrate solution: Dilute caspase-8 substrate (Component A) 1:10 in assay buffer. Prepare fresh substrate solution for each experiment.

Table 2. Caspase-8 substrate solution for one 96-well plate (100 assays)

Components	Volume
Caspase-8 substrate (Component A)	100 µL
Assay buffer	900 µL
Total volume	1 mL

2.3 Caspase-8 inhibitor (Ac-IETD-CHO): Dilute 5 mM inhibitor solution (Component C) 1:100 in assay buffer. Use inhibitor solution for inhibitor control wells only.

3. Prepare cell lysates and set up the enzymatic reaction.

3.1 Retrieve plates from the 37°C incubator. Add 90 µL/well of assay buffer. Add 90 µL/well of the caspase inhibitor solution (refer to step 2.3 for preparation) to inhibitor control wells. Mix the reagents thoroughly.

Note: It is not necessary to remove the culture medium from the 96-well plates. The assay buffer is a dual function solution; it lyses cells and supports optimal caspase activity.

3.2 Incubate the cells 30 min. at room temperature on a plate shaker at 100-200 rpm.

3.3 Establish the following control wells, as deemed necessary:

- Substrate control contains growth medium (100 µL/well).
- Test compound control contains test compound in growth medium (100 µL/well). Some test compounds have strong autofluorescence and may give false results.

3.4 Bring up the total volume of all the controls to 190 µL using assay buffer.

4. Run the enzymatic reaction.

4.1 Add 10 µL of caspase-8 substrate solution into each well. Mix the reagents thoroughly.

4.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=380 nm/500 nm continuously and record data every 5 min. for 60 min.
- For end-point reading: Incubate the reaction for 60 min. on a plate shaker at 100-200 rpm. Keep plate from direct light. Measure fluorescence intensity at Ex/Em=380 nm/500 nm.

Note: If the caspase-8 activity is low in your samples, incubation time can be extended up to 18 h before taking the end-point reading.

4.3 For data analysis: Refer to Appendix I. A sample data for Protocol A is shown in Figure 1.

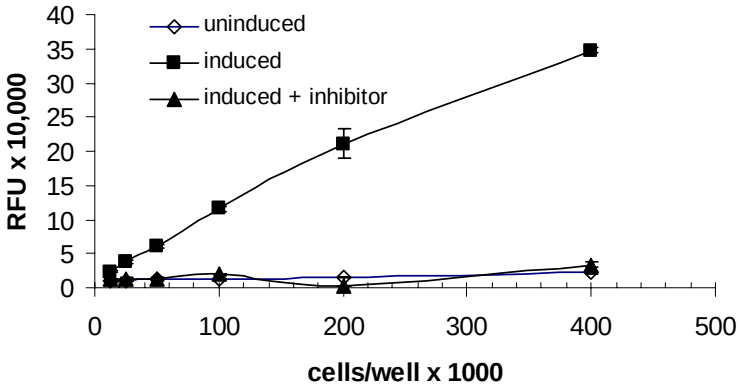

Figure 1. Jurkat cells were seeded to 96-well plates. Apoptosis was induced with 1 μg/mL staurosporine for 3 h. The identical population of cells after induction of apoptosis was incubated with caspase-8 specific inhibitor Ac-IETD-CHO at 25 μM final concentration. Fluorescence readings were taken 60 min. after addition caspase-8 substrate (Flexstation 384II, Molecular Devices). Each point represents the average of 3 replicates.

Protocol B. Protocol for Cells Cultured in Plates Larger than 96-well Plates (e.g., 10-cm dish)

1. Prepare apoptotic cells.

Note: The following assay procedure is for cells cultured in plates larger than 96-well plates (e.g. 10-cm plate).

1.1 Seed an appropriate amount of cells. Add apoptosis-inducing test compounds to the cells. Culture cells in a 37°C incubator for the desired exposure time.

Set up the following controls at the same time:

- Positive control contains cells and a known apoptosis inducer.
- Negative control contains cells without apoptosis inducer.
- Vehicle control contains cells and the vehicle used to deliver test compounds.

2. Prepare working solutions.

Note: Warm all kit components until thawed to room temperature before starting the experiment.

2.1 Assay buffer: Dilute 1 M DTT (Component E) 1:50 in assay buffer (Component D). Use this DTT-containing assay buffer in all the following steps. Prepare fresh assay buffer for each experiment.

2.2 Caspase-8 substrate solution: Dilute caspase-8 substrate (Component A) 1:100 in assay buffer. Prepare fresh substrate solution for each experiment.

2.3 Caspase-8 inhibitor (Ac-IETD-CHO): Dilute 5 mM inhibitor solution (Component C) 1:10 in assay buffer.

3. Prepare cell lysates and set up the enzymatic reaction.

3.1 Retrieve cells from the 37°C incubator. Suspension cells are collected by centrifugation at 900 X g for 10 min. For adherent cells, simply aspirate the growth medium.

3.2 Add an appropriate amount of lysis buffer to cells, e.g. 50 μL of assay buffer for approximately 1-2X10^6 cells. Resuspend the cells thoroughly.

3.4 Incubate cells on ice for 10 min.

3.5 Aliquot prepared cell lysates to the 96-well plate (45 μL/well). Add 5 μL/well of the caspase inhibitor solution (refer to Step 2.3 for preparation) to inhibitor control wells and 5 μL/well of assay buffer to all other wells. Incubate 10 –30 min. at room temperature on a plate shaker at 100-200 rpm.

3.6 Establish the following control wells, as deemed necessary:

- Substrate control contains assay buffer only
- Test compound control contains test compound in assay buffer. Some test compounds have strong autofluorescence and may give false results.

3.7 Bring up the total volume of all the controls to 50 μL using assay buffer.

4. Run the enzymatic reaction.

4.1 Add 50 μL of caspase substrate solution into the wells. Mix the reagents completely by shaking the plate gently for 30 sec.

4.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=380 nm/500 nm continuously and record data every 5 min. for 60 min.
- For end-point reading: Incubate the reaction for 60 min. Keep plate from direct light. Measure fluorescence intensity at Ex/Em=380 nm/500 nm.

4.3 For methods of data analysis: Refer to Appendix I. A sample data for Protocol B is shown in Figure 2.

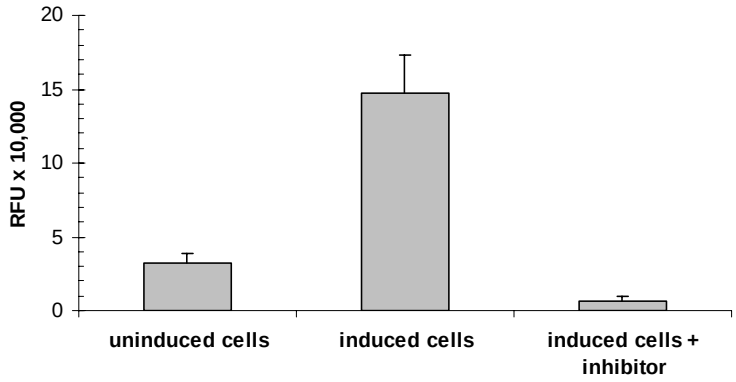

Figure 2. Jurkat cells were seeded to 20-cm dishes. Apoptosis was induced with 1 μg/mL staurosporine for 3 h. The identical population of cells after induction of apoptosis was incubated with caspase-8 specific inhibitor Ac-IETD-CHO at 25 μM final concentration. Cell lysates were prepared according to the procedure described above and aliquoted in 96-well plate. Fluorescence readings were taken 60 min. after addition caspase-8 substrate (Flexstation 384II, Molecular Devices). Each point represents the average of 3 replicates.

Appendix I. Data Analysis

- All fluorescence readings are expressed in relative fluorescence units (RFU). The fluorescence reading from the substrate control well is used as the background fluorescence. This background reading should be subtracted from the readings of the other wells containing substrate. After subtraction of the background fluorescence, the signal obtained from an apoptotic cells should be compared with the results of uninduced control cells.
- For kinetic analysis:
  - Plot data as RFU versus time for each sample. To convert RFUs to the concentration of the product of the enzymatic reaction, please refer to Appendix II for establishing a fluorescence reference standard.
  - Determine the range of initial time points during which the reaction is linear. Typically, the first 10-15% of the reaction will be the optimal range.
  - Obtain the initial reaction velocity (V_o) in RFU/min. by determining the slope of the linear portion of the data plot.
  - A variety of data analyses can be done, e.g., determining inhibition %, $EC_{50,}$ IC_{50}, K_m, K_i, etc.
- For endpoint analysis:
  - Plot data as RFU versus concentration of test compounds.
  - A variety of data analyses can be done, e.g., determining inhibition %, EC_{50}, IC_{50}, etc.

Appendix II. Instrument Calibration

- AFC fluorescence reference standard: Dilute 6 mM AFC (Component B) to 60 µM in growth medium (for Protocol A) or in assay buffer (for Protocol B). Do 2-fold serial dilutions to get concentrations of 30, 15, 7.5, 3.75, 1.9, 0.95 µM, include growth medium or an assay buffer blank. Add 100µL/well (Protocol A) or 50 µL/well (Protocol B) of these serially diluted AFC solutions.
- Add 100µL/well (Protocol A) or 50 µL/well (Protocol B) of the diluted caspase-8 substrate solution (refer to Protocol B, step 2.2 for preparation).
  Note: The caspase-8 substrate solution is added to the AFC reference standard to normalize for the intrinsic substrate fluorescence. If multiple concentrations of substrate are used, this step must be performed for each concentration.
- Measure the fluorescence intensity of the reference standard and substrate control wells at Ex/Em=380 nm/500 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot the AFC fluorescent reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 3.
- The final concentrations of AFC reference standard are 30, 15, 7.5, 3.75, 1.9, 0.95, 0.475, and 0 µM. This reference standard is used to calibrate the variation of different instruments and different experiments. It is also an indicator of the amount of final product of the enzymatic reaction.

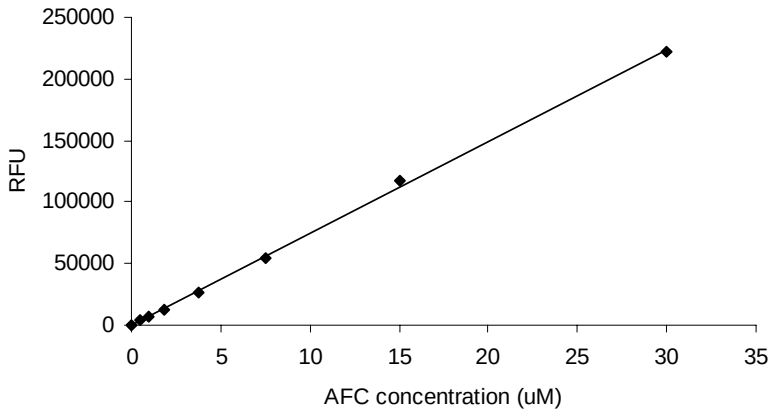

Figure 3. AFC reference standard, AFC was serially diluted in assay buffer containing substrate, and the fluorescence was recorded at Ex/Em=380 nm/ 500 nm. (Flexstation 384II, Molecular Devices)

References

1. Thornberry, NA. and Lazebnik, Y. *Science* **281**, 1312 (1998).
2. Reed, JC. *J. Clin. Oncol* **17**, 2941 (1999).
3. Muzio, M. et al. *J. Biol. Chem* **272**, 2952 (1997).
4. Takahashi, A. et al. *Oncogene* **14**, 2741 (1997).