SensoLyte® Homogeneous AMC Caspase - 3/7 Assay Kit *Fluorimetric*

Revision# 1.2	*Last Updated: July 2021*
Catalog #	**AS-71118**
Kit Size	500 Assays (96-well plate)

- ***Convenient Format:*** All essential assay components are included.
- ***Optimized Performance:*** Optimal conditions for the detection of caspase-3/7 activity.
- ***Enhanced Value:*** Less expensive than the sum of individual components.
- ***High Speed:*** Minimal hands-on time.
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	Caspase-3/7 substrate Peptide sequence=Ac-Asp-Glu-Val-Asp-AMC Ex/Em=354 nm/442 nm upon caspase-3/7 cleavage	270 µL
Component B	AMC, fluorescence reference standard Ex/Em=354 nm/442 nm	10 mM DMSO solution, 20 µL
Component C	Ac-DEVD-CHO, a known caspase-3/7 inhibitor (5 mM DMSO solution	15 µL
Component D	Assay Buffer	30 mL
Component E	DTT	1 M, 1 mL
Component F	10X Lysis Buffer	20 mL

Other Materials Required (but not provided)

- 96-well microplate: Black tissue culture microplates with or without clear bottom.
- Fluorescence microplate reader: Capable of detecting emission at 440±20 nm with excitation at 350 ±20 nm.

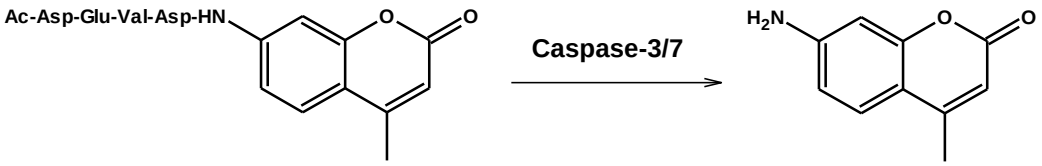
Storage and Handling

- Store all kit components at -20°C
- Protect Components A and B from light

Introduction

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. Central to the execution phase of apoptosis are the two closely related caspase-3 and caspase-7. They share common substrate specificity and structure, but differ completely in the sequence of their respective N-terminal regions. Both caspase-3 and caspase-7 have substrate selectivity for the amino acid sequence Asp-Glu-Val-Asp (DEVD).

The SensoLyte® Homogeneous AMC Caspase-3/7 Assay Kit uses Ac-DEVD-AMC as the fluorogenic indicator for assaying caspase-3/7 activities. Upon caspase-3/7 cleavage, Ac-DEVD-AMC generates the AMC fluorophore that has bright blue fluorescence and can be detected at excitation/emission=354 nm/442 nm (**Scheme 1**). A bi-functional assay buffer lyzes the cells and provides optimal conditions for measuring enzymatic activity. Thus, this kit can measure caspase-3/7 activity in cell culture directly in a 96-well plate without a time-consuming cell extraction step. In cases where cells are cultured in larger plates or flasks, a lysis buffer and a protocol for cell lysate preparation are also conveniently included in the kit. This kit can be used for high throughput screening of apoptosis inducers and inhibitors.



Weakly Fluorescent at shorter wavelength

Strongly Fluorescent at longer wavelength

Scheme 1. Proteolytic cleavage of Ac-DEVD-AMC.

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. Screen apoptosis inducers or inhibitors using cell culture.

1. Prepare apoptotic cells.

Note: The following description is for seeding cells in a 96-well plate. If cells are cultured in plates larger than 96-well plates (e.g. 6-well plate or 10 cm plate), it is necessary to prepare the cell extract. Please refer to Appendix III for details.

1.1 Seed $1X10^{3-6}$ cells per well in a microplate. Add test compounds and then culture cells in a 37°C incubator for the desired exposure time. The suggested volume for a 96-well plate is 100 µL of cells plus 50 µL of test compounds for a total volume of 150 µL/well. Set up the following controls at the same time:

- Positive control contains cells and a known apoptosis inducer.

- Negative control contains cells but no test compounds.
- Vehicle control contains cells and the vehicle used to deliver test compounds.
- Non-cell control contains growth medium but no cells.
- Test compound control contains growth medium and test compound. Some test compounds have strong autofluorescence and may give false results.

Note: Bring up the total volume of all the controls to 150 µL/well (96-well plate) using growth medium.

2. Prepare working solutions.

Note: Warm all kit components until thawed at room temperature before starting the experiments.

2.1 Caspase-3/7 substrate solution: Prepare DTT-containing assay buffer by adding 40 µL of 1 M DTT (Component E) per mL of assay buffer (Component D). Dilute caspase-3/7 substrate (Component A) 1:100 in this DTT-containing assay buffer. Mix the reagents well.

Table 1. Caspase-3/7 substrate solution for one 96-well plate (100 assays).

Components	Volume
Caspase-3/7 substrate (Component A)	50 µL
1 M DTT (Component E)	200 µL
Assay buffer (Component D)	5 mL
Total volume	5 mL

Note: Prepare fresh substrate solution for each experiment.

3. Initiate enzymatic reaction.

3.1 Retrieve plates from the 37°C incubator if using cells cultured in a microplate. Or if using cell lysate (refer to **Appendix III**), dispense cell extract at 150 µL/well (96-well plate).

3.2 Add 50 µL/well of caspase-3/7 substrate solution into each well. Mix the reagents completely by shaking on a plate shaker for 30-60 sec at 100-200 rpm. Avoid bubbles.

Note: It is not necessary to remove the culture medium from 96-well plates. The caspase-3/7 substrate solution is a dual function solution, it lyzes cells and supports optimal caspase-3/7 activity. The 10X lysis buffer (Component F) is only for preparing cell extract from plate larger than 96-well plates, for example, 6-well plate or 10 cm plate. Please refer to **Appendix III** for details.

3.3 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=354 nm/442 nm continuously and record data every 5 min for 30 to 60 min.
- For end-point reading: Incubate the reaction at room temperature for 30 to 60 min on a plate shaker at 100-200 rpm. Keep plate away from direct light. Measure fluorescence intensity at Ex/Em=354 nm/442 nm.

Note: If the caspase-3/7 activity is low in your samples, incubation time can be extended up to 18 hr before taking the end-point reading.

3.4 Data analysis: Refer to Appendix I.

Protocol B. Screen caspase-3/7 inducers or inhibitors using purified caspase-3/7.

1. Prepare working solutions.

Note: Warm all kit components until thawed at room temperature before starting the experiments.

1.1 Assay buffer: Add 1 M DTT (Component E) 1:50 into the assay buffer. Use this DTT-containing assay buffer in **all** the following steps.

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

Components	Volume
1 M DTT (Component E)	200 µL
Assay buffer (Component D)	10 mL
Total volume	10 mL

Note: Prepare fresh DTT-containing assay buffer for each experiment.

1.2 Caspase-3/7 substrate solution: Dilute caspase-3/7 substrate (Component A) 1:100 in assay buffer. Mix the reagents well.

Table 2. Caspase-3/7 substrate solution for one 96-well plate (100 assays).

Components	Volume
Caspase-3/7 substrate (Component A)	50 µL
DTT-containing Assay buffer	5 mL
Total volume	5 mL

Note: Prepare fresh substrate solution for each experiment.

1.3 Caspase-3/7 diluent: Dilute caspase-3/7 to an appropriate concentration in assay buffer.

Note: Prepare enzyme diluent immediately before use. Do not vortex enzyme. Prolonged storage of diluent or vigorous vortexing will denature the enzyme. Keep enzyme on ice.

1.4 Test compound: Dilute test compounds with deionized water or an appropriate vehicle.

1.5 Inhibitor: Ac-DEVD-CHO (Component C) is a known caspase-3/7 inhibitor. Dilute Ac-DEVD-CHO 5-fold in assay buffer.

2. Set up enzymatic reaction.

2.1 Add test compounds and caspase-3/7 diluent into microplate. The suggested total volume of test compound and caspase-3/7 diluent is 50 µL (96-well plate.

2.2 Set up the following controls at the same time:

- Positive control contains caspase-3/7 diluent without test compound.
- Inhibitor control contains caspase-3/7 diluent and known inhibitor, Ac-DEVD-CHO (10 µL/well).
- Vehicle control contains caspase-3/7 diluent and vehicle used to deliver test compound.
- Test compound control contains assay buffer and test compound. Some test compounds have strong autofluorescence and may give false results.
- Substrate control contains assay buffer.

Note: Bring up the total volume of all the controls to 50 µL/well (96-well plate) using assay buffer.

3. Pre-incubation.

3.1 Incubate the plate at the desired temperature (e.g. 25°C or 37°C) for 10-15 min. Also incubate the caspase-3/7 substrate solution at the same temperature.

4. **Initiate the enzymatic reaction.**

4.1 Add 50 µL of caspase-3/7 substrate solution into the wells. Mix the reagents completely by shaking on a plate shaker for 30-60 sec at 300-400 rpm.

4.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=354 nm/442 nm continuously and record data every 5 min for 30 to 60 min.
- For end-point reading: Incubate reaction at room temperature for 30 to 60 min on a plate shaker at 100-200 rpm. Keep plate away from direct light. Measure fluorescence intensity at Ex/Em=354 nm/442 nm.

4.3 Data analysis: Refer to Appendix I.

Appendix I: Data Analysis

- The fluorescence reading from the non-cell control well or substrate control well is the background fluorescence. This background reading has to be subtracted from the readings of the other wells to get the relative fluorescence unit (RFU).
- For kinetic reading:
  - Plot data as RFU versus time for each sample.
  - Determine the range of initial time points during which the reaction is linear. 10-15% conversion appears to be the optimal range.
  - Obtain the initial reaction velocity (Vo) in RFU/min. Determine 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 reading:
  - 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.

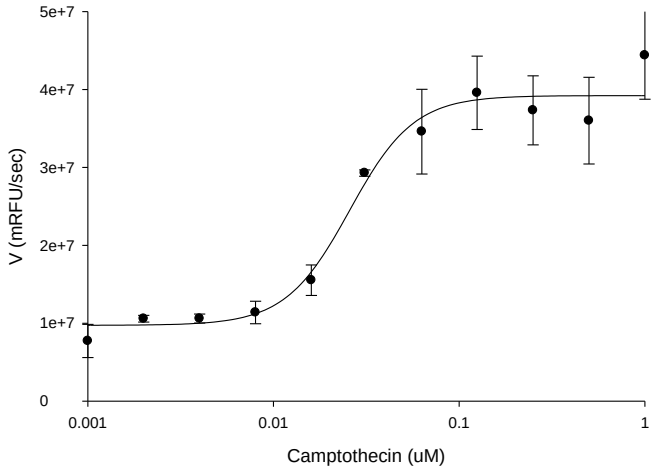


Figure 1. Dose-response curve of Camptothecin.

$1X10^5$/well Jurkat cells were treated with Camptothecin for 5 h. 50 µL/well of AMC caspase substrate solution was added to apoptotic cells and incubated at room temperature for 30 min. The kinetic fluorescence signal was measured by a fluorescence microplate reader (FlexStation II384, Molecular Device, CA) with Ex/Em=354 nm/442 nm, cutoff 430 nm. EC_{50}= 0.026±0.003 µM.

Appendix II: Instrument Calibration

- AMC fluorescence reference standard: Dilute 10 mM AMC (Component B) to 60 µM in deionized water. Do 2-fold serial dilutions to obtain 30, 15, 7.5, 3.75, 1.88, 0.94 µM AMC solutions, include a water blank. Add 50 µL/well of the serially diluted AMC solutions from 60 µM to 0 µM into the plate.
- Add 50 µL/well of caspase-3/7 substrate solution (refer to Protocol B Step 1 for preparation). Mix the reagents by shaking the plate gently for 3 to 5 sec.

  Note: The caspase-3/7 substrate solution should be added into the reference standard to normalize for the fluorescence inner filter effect.
- Measure the fluorescence intensity of reference standard and substrate control wells at Ex/Em=354 nm/442 nm. Adjust the sensitivity of the microplate reader until satisfactory signals can be read. Use the same setting of sensitivity in the enzymatic reaction in protocols A and B.
- The fluorescence reading from the wells containing 0 µM AMC 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).
- Plot AMC fluorescence reference standard as RFU (relative fluorescent unit) versus concentration.

  Note: The final concentration of AMC reference standard solutions are 30, 15, 7.5, 3.75, 1.88, 0.94, 0.47 and 0 µM. The resulting reference standard curve is used to calibrate for the variation of different instruments and for different batches of experiments. It can also serve as an indicator of the amount of caspase enzymatic reaction final product. The concentration of AMC in your samples can be extrapolated from the RFU of the reference standard curve.

Appendix III

Prepare cell extract if culturing cells in plates larger than a 96-well plate, e.g., 6-well plate or 10 cm plate.

- Seed at least $1X10^6$ cells per well. Add an appropriate amount of apoptosis-inducing test compound to the cells. Culture cells at 37°C incubator for the desired exposure time.
- Set up the following controls at the same time.
  - Positive control contains cells and known apoptosis inducer.
  - Negative control contains cells but no test compounds.
  - Vehicle control contains cells and the vehicle used to deliver test compounds.
  - Non-cell control contains growth medium but no cells.
  - Test compound control contains growth medium and test compound. Some test compounds have strong autofluorescence and may give false positive results.
- Prepare 1X lysis buffer by adding 1 mL of 10X lysis buffer (Component F) to 9 mL of deionized water.
- Suspension cells are collected by centrifugation at 500 X g for 5 min. For adherent cells, simply aspirate the growth medium.
- Add an appropriate amount of lysis buffer to cells or cell pellet, e.g. 300 µL 1X lysis buffer for one well of a 6-well plate. Scrape off the adherent cells or resuspend the cell pellet, and collect the cell suspension in a microcentrifuge tube.
- Rotate the cell suspension on a rotating apparatus for 30 min at 4°C.
- Centrifuge the cell suspension at 2500 X g for 10 min at 4°C.
- Collect the supernatant. You may store the supernatant at -80°C for future assay.
- Add 150 µL/well (96-well plate) of supernatant and controls.
- Proceed to Step 2 (protocol A) for caspase-3/7 assay.

References

1. Thornberry, N. A. et al. *Science* **281**, 1312 (1998).
2. Reed, J. C. *J. Clin. Oncol.* **17**, 2941 (1999).