SensoLyte® Homogeneous AFC Caspase-3/7 Assay Kit

Revision# 1.2	*Last Updated: July 2021*
Catalog #	**AS-71114**
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 Ex/Em=380 nm/500 nm	270 µL
Component B	AFC, fluorescence reference standard Ex/Em=380 nm/500 nm	10 mM, 20 µL
Component C	Caspase-3/7 inhibitor	5 mM, 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 are recommended.
- Fluorescence microplate reader: Capable of detecting emission at 500 ±30 nm with excitation at 380±30 nm

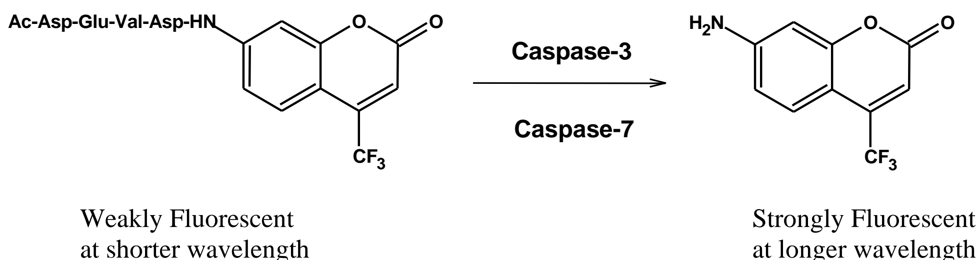
Storage and Handling

- Store Components A, B, C, and E at -20°C. Protect Component A from light and moisture.
- Keep Components A and B away from light.
- For convenience, Components D and F can be stored at 4°C.

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 have substrate selectivity for the amino acid sequence Asp-Glu-Val-Asp (DEVD).

The SensoLyte® Homogeneous AFC Caspase-3/7 Assay Kit uses Ac-DEVD-AFC as the fluorimetric indicator caspase-3/7 activity assays. Upon caspase-3/7 cleavage, Ac-DEVD-AFC generates the AFC fluorophore which has bright blue fluorescence and can be detected at excitation/emission=380 nm/500 nm (**Scheme 1**). A bi-functional assay buffer lyses 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. For preparation of cell lysate of cells cultured in large plates or flasks, lysis buffer and procedure are conveniently included in the kit. This kit can be used for high throughput screening of apoptosis inducers and inhibitors.



Scheme 1. Proteolytic cleavage of Ac-DEVD-AFC

Protocol

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

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 other than a 96-well plate (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 culture cells in a 37°C incubator for the desired exposure period. The suggested volume for a 96-well plate is 100 µL of cells and 50 µL of test compounds with a total volume of 150 µL per well.

1.2 Set up the following controls at the same time:

- Positive control contains cells and any known apoptosis inducers

- 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 auto fluorescence and may give false results.

Note: Bring the total volume of all the controls to 150 µL using growth medium.

2. Prepare working solutions.

Note: Warm all the kit components to 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) to 1 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)	4.75 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. If cell lysates are being used (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 the microplate. The caspase-3/7 substrate solution is a dual function solution; it lyses 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=380 nm/500 nm continuously and record data every 5 min for 30 min to 60 min.

For end-point reading: Incubate reaction at room temperature for 30 min to 60 min on a plate shaker at 100-200 rpm. Keep the plate away from direct light. Measure fluorescence intensity at Ex/Em=380 nm/500 nm.

Note: If the caspase-3/7 activity is low in your samples, you may extend the incubation up to 18 hrs before 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 the kit components to room temperature before starting the experiments.

   1.1 Assay buffer: Add 1 M DTT (Component E) 50-fold 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)	9.8 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	4.95 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 vortex will denature the enzyme. Keep the enzyme on ice.

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

   1.5 Inhibitor: Dilute caspase-3/7 inhibitor (Component C) 5-fold in assay buffer.

2. **Set up enzymatic reaction.**

   2.1 Add test compounds and caspase-3/7 diluent into a microplate. The suggested total volume of test compound and caspase-3/7 diluent is 50 µL/well. The suggested volume of inhibitor is 10 µL/well.

   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 inhibitor
   - Vehicle control contains caspase-3/7 diluent and vehicle used to deliver test compound.
   - Test compound control contains assay buffer and test compound.
   - Substrate control contains assay buffer.

   Note: Bring up the total volume of all the controls to 50 µL/well (96-well plate) with 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=380 nm/500 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=380 nm/500 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. The readings from other wells need to be subtracted by this background fluorescence 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.

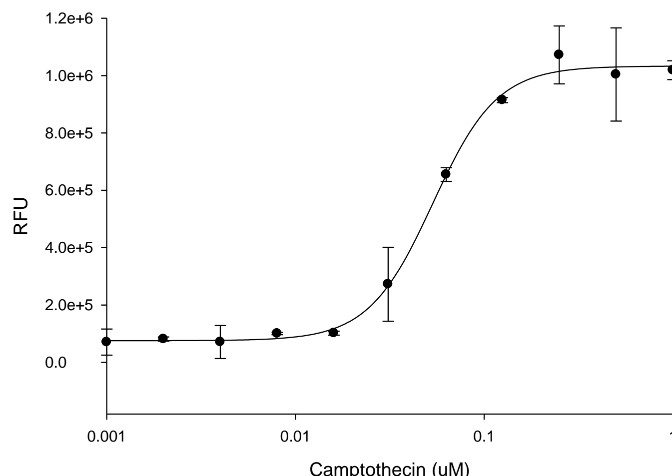


Figure 1. Dose response curve of Camptothecin

$1X10^5$/well Jurkat cells were treated with Camptothecin for 5 hrs. 50 µL/well of AFC caspase substrate solution was added to apoptotic cells and incubated at room temperature for 30 min. Endpoint fluorescence signal was measured by a fluorescence microplate reader (Flex Station II384, Molecular Device, CA) with Ex/Em=380 nm/500 nm, cutoff 495 nm. EC_{50}= 0.054±0.002 µM.

Appendix II: Instrument Calibration

- AFC fluorescence reference standard: Dilute 10 mM AFC (Component B) to 60 µM in deionized water. Do 2-fold serial dilutions to obtain 30, 15, 7.5, 3.75, 1.88, and 0.94 µM AFC solutions, include water blank. Add 50 µL/well of the serially diluted AFC 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: Caspase-3/7 substrate solution should be added into the reference standard to normalize the fluorescence inner filter effect.
- Measure the fluorescence intensity at Ex/Em=380 nm/500 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 AFC 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 AFC fluorescence reference standard as RFU (relative fluorescent unit) versus concentration to get a linear curve.

  Note: The final concentration of AFC 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 different batches of experiments. It can also serve as an indicator of the amount of final product in the caspase enzymatic reaction. The concentration of AFC in you samples can be extrapolated using the RFU of your samples on reference standard curve.

Appendix III

Prepare cell extract if culturing cells in plates larger than 96-well plates, 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 in a 37°C incubator for the desired exposure period.
- 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 auto fluorescence 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 6-well plate. Scrape off the adherent cells or resuspend the cell pellet, and collect the cell suspension in a micro centrifuge tube.
- Rotate the cell suspension on a rotating apparatus for 30 min at 4°C.
- Centrifuge the cell suspension at 2,500 X g for 10 min at 4°C.
- Collect the supernatant (supernatant may be stored at -80°C for future experiments).
- Add 150 µL/well (96-well plate) of supernatant and controls.
- Continue to **Step 2 in protocol A** for the caspase-3/7 assay.

References

1. Thornberry, N.A. and Lazebnik Y., *Science* **281**, 1312-1316 (1998)
2. Reed, J.C. *J. Clin. Oncol.* **17**, 2941-2953 (1999)