SensoLyte® AMC Caspase Profiling Kit
Fluorimetric

Revision#1.1	*Last updated:2/6/17*
Catalog #	**AS-71120**
Kit Size	Two 96-well plates

- ***Convenient Format:*** All essential assay components are included.
- ***Optimized Performance:*** Optimal conditions for assaying the activity of caspase.
- ***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	AMC-based caspase substrates. Peptide sequence: refer to **Appendix I** Table 2. Ex/Em=354 nm/442 nm upon cleavage	Two 96-well plates pre-coated with 8 different caspase substrates.
Component B	AMC, fluorescence reference standard Ex/Em=354nm/442 nm	10 mM DMSO solution, 20 µL
Component C	Assay Buffer, add DTT before use	40 mL
Component D	DTT	1 M, 1 mL
Component E	Caspase dilution buffer	20 mL
Component F	10X Lysis Buffer	20 mL

Other Materials Required (but not provided)

- Fluorescence microplate reader: Capable of excitation at 354±30nm and emission at 442±30nm.

Storage and Handling

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

Introduction

Apoptosis is a programmed, cell-autonomous death process. It plays important roles 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. During apoptosis, caspases execute the disassembly of the cellular components by proteolytic cleavage of a variety of substrates, such as poly-(ADP ribose) polymerase (PARP)[3], DNA-dependent protein kinase (DNA-PK), topoisomerases, and protein kinase C (PKC)δ.[4] At least ten caspases have been discovered. Some of caspases identify and cleave a specific peptide substrate, while others recognize the same peptide substrate[4].

The SensoLyte® AMC Caspase Profiling Kit contains two 96-well plates pre-coated with a series of AMC-based peptide substrates (**Appendix I**, Table 2) as fluorogenic indicators for assaying caspase activities. This kit provides a convenient platform for profiling substrate specificity of caspases. AMC-based substrates are widely used to monitor caspase activity and can be monitored at the wavelength of excitation/emission = 354 nm/442 nm

Protocol

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

1. Prepare working solutions.

1.1 Assay buffer: Add 20 µL 1 M DTT (Component D) per mL assay buffer (Component C). Use this DTT-containing assay buffer in all the following steps.

Note: Use freshly prepared DTT-containing assay buffer for each experiment.

1.2 Prepare caspase substrate plate: Add 50 µL assay buffer to wells from columns 3 to 12 on pre-coated plate (Component A, refer to **Table 2**). Leave columns 1 and 2 empty for setting up the fluorescence reference standard later. Completely dissolve the substrate by shaking the plate on a plate shaker at 100-200 rpm for 5 minutes.

Table 1. Layout of peptide substrates on the 96-well microplate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	RS1	RS1	SB 1	SB 1	SB 1	SB 1	SB 1	SB 1	SB 1	SB 1	SB 1	SB 1
B	RS2	RS2	SB 2	SB 2	SB 2	SB 2	SB 2	SB 2	SB 2	SB 2	SB 2	SB 2
C	RS3	RS3	SB 3	SB 3	SB 3	SB 3	SB 3	SB 3	SB 3	SB 3	SB 3	SB 3
D	RS4	RS4	SB 4	SB 4	SB 4	SB 4	SB 4	SB 4	SB 4	SB 4	SB 4	SB 4
E	RS5	RS5	SB 5	SB 5	SB 5	SB 5	SB 5	SB 5	SB 5	SB 5	SB 5	SB 5
F	RS6	RS6	SB 6	SB 6	SB 6	SB 6	SB 6	SB 6	SB 6	SB 6	SB 6	SB 6
G	RS7	RS7	SB 7	SB 7	SB 7	SB 7	SB 7	SB 7	SB 7	SB 7	SB 7	SB 7
H	RS8	RS8	SB 8	SB 8	SB 8	SB 8	SB 8	SB 8	SB 8	SB 8	SB 8	SB 8

Note: RS=Reference standard, SB=Substrates 1 to 8. Please refer to **Appendix I Table 2** for substrate sequence.

1.3 AMC fluorescence reference standard: Dilute 10 mM AMC (Component B) to 50 µM in deionized water. Do 2-fold serial dilutions with deionized water to obtain 25, 12.5, 6.25, 3.125, 1.56, 0.78, and 0.39 µM AMC solutions. Take 50 µL of the serially diluted

AMC solutions from 50 μM to 0.39 μM, and add into each reference standard well on the 96-well plate (RS1 to RS8, refer to **Table 1**).

1.4 Prepare caspase:
When using purified caspase: Dilute caspase to an appropriate concentration in caspase dilution buffer (Component E). Each well in a 96-well microplate will need 50 μL of diluted enzyme. Calculate an appropriate amount of caspase diluent for all your substrate wells.

When using caspase-containing biological sample: Refer to **Appendix II** for sample preparation. Each well in a 96-well microplate will need 50 μL of sample.

Note: Warm the caspase samples to room temperature before doing the following enzymatic reaction.

2. Calibrate the fluorescence microplate reader.

2.1 Add 50 μL of assay buffer to the reference standard wells. Mix the reagents by shaking the plate gently for 30 seconds.

2.2 Measure the fluorescence intensity of the 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 subsequent enzymatic reactions.

3. Initiate the enzymatic reaction.

3.1 Add 50 μL caspase dilution buffer (Component E) if purified enzyme is used, or 50 μL 1X lysis buffer (diluted Component F) if biological sample is used, to selected substrate-containing wells to serve as the substrate control. The fluorescence reading from the substrate control well is the background fluorescence.

3.2 Add 50 μL caspase diluent or biological samples into selected substrate-containing wells. Mix the reagents by shaking the plate gently for 30 seconds. Immediately start measuring fluorescence intensity at Ex/Em=354 nm/442 nm continuously and record data every 5 minutes for 30 minutes.

3.3 Data analysis: Refer to **Perform Data Analysis** section.

Perform Data Analysis:

- The fluorescence reading from the substrate control well is the background fluorescence. The readings from other wells need to be subtracted with this background fluorescence.
- Plot AMC fluorescent reference standard as RFU (relative fluorescent unit) versus concentration as shown in **Figure 1**.

  Note: The final concentrations of the AMC reference standard solutions are 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39 and 0.195 μM. The resulting reference standard curve is used to calibrate the variation of different instruments and different batch of experiments. It can also serve as an indicator of the amount of final product of the caspase enzymatic reaction.
- Plot data as RFU versus time for each sample if you recorded kinetic reading.
- 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 (V_0) in RFU/min. Determine the slope of the linear portion of the data plot.
- A variety of data analysis can be done, e.g., determining inhibition %, IC_{50}, K_m, K_i, etc.

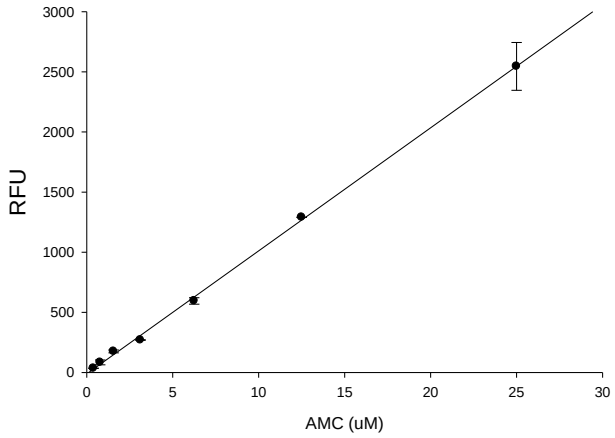

Figure 1. AMC reference standard calibration curve.

AMC was diluted in assay buffer. 100 µL of AMC of each concentration was added into a 96-well microplate. The fluorescence signal was measured by a fluorescence microplate reader (FLx800, Bio-Tek Instruments) with a filter set of Ex/Em=360±40 nm/460±40 nm. (mean ± S.D.; n= 2 independent samples).

Appendix I

Table 2: Caspase substrate sequences in the 96-well plate.

Substrate No.	Substrate Name	Substrate Sequence
SB1	Caspase-1 substrate	Ac-YEVD-AMC
SB2	Caspase-1 substrate	Ac-WEHD-AMC
SB3	Caspase-1/4 substrate	Ac-YVAD-AMC
SB4	Caspase-3 substrate	Ac-DMQD-AMC
SB5	Caspase-3/7 substrate	Ac-DEVD-AMC
SB6	Caspase-6 substrate	Ac-VEID-AMC
SB7	Caspase-8 substrate	Ac-IETD-AMC
SB8	Caspase-9 substrate	Ac-LEHD-AMC

Appendix II

1. Prepare caspase-containing sample from cell extract.

- Induce apoptosis in cell culture with the desired method.
- 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 1X lysis buffer to cells or cell pellet, e.g. 300 µL 1X lysis buffer per well of a 6-well plate. Scrape off the adherent cells or re-suspend the cell pellet, and then 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.

2. Prepare caspase-containing sample from tissue.

- Prepare 1X lysis buffer by adding 1 mL of 10X lysis buffer (Component F) to 9 mL of deionized water.
- Tissues samples should be homogenized in 1X lysis buffer, and then centrifuged for 15 min at 10,000x g at 4°C. The supernatant, which contains caspase, can be frozen at -70 °C until use.

References

1. Thornberry, N. A. et al. *Science* **281**, 1312-1316 (1998).
2. Reed, J. C. *J. Clin. Oncol.* **17**, 2941-2953 (1999).
3. Lazebnik, Y. A. et al. *Nature* **371**, 346-347 (1994).
4. Villa, P. et al. *Trends Biochem. Sci.* **22**, 388-393 (1997).