SensoLyte® AFC Caspase Profiling Kit
Fluorimetric

Revision Number:1.1	Last Revised: October 2014
Catalog #	**AS-71116**
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	Caspase microplate: pre-coated with 8 different caspase substrates. Peptide sequence: Appendix I, Table 2. Ex/Em=380 nm/500 nm upon cleavage	2 96-well plates (8 x 12 strips)
Component B	AFC, fluorescence reference standard Ex/Em=380 nm/500 nm	10 mM, 20 µL
Component C	Assay buffer	40 mL
Component D	DTT	1 M, 1 mL
Component E	10X Lysis Buffer	20 mL

Other Materials Required (but not provided)

- 96-well microplate or 384-well microplate: Black tissue culture microplate with or without clear bottom
- Fluorescence microplate reader: Capable of detecting excitation at 380 ±30 nm with emission at 500±30 nm.

Storage and Handling

- Store all components at -20°C
- Keep Components A and B away from light

Introduction

Apoptosis is a programmed, cell-autonomous death process. It plays important roles in 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 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 these caspases identify and cleave a specific peptide substrate, while others recognize the same peptide substrate[4].

The SensoLyte® AFC Caspase Profiling Kit contains two 96-well plates pre-coated with a series of AFC-based peptide substrates (Appendix I, Table 2) for use as fluorogenic indicators in assaying caspase activities. This kit provides a convenient platform for profiling substrate specificity of caspases. AFC-based substrates are widely used to monitor caspase activity at the wavelength of excitation/emission = 380 nm/500 nm.

Protocol

Note 1: Thaw all kit components to room temperature before starting the experiments.
Note 2: For instrument calibration, refer to Appendix II

1. Prepare working solutions.

Note: The following description is for seeding cells in a 96- or 384-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 Assay buffer: Add 20 µL of 1 M DTT (Component D) per 1 mL of assay buffer (Component C).

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

1.2 Prepare caspase substrate plate: Add 50 µL DTT-containing assay buffer to wells starting from columns 3 to 12 on the pre-coated plate (Component A, refer to Table 1). Leave columns 1 and 2 empty for setting up the fluorescence reference standard (refer to Appendix II) 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. Refer to Appendix I, Table 2 for substrate sequence.

1.3 Prepare caspase:

- When using purified caspase: Dilute caspase to an appropriate concentration in DTT-containing assay buffer.
- When using caspase-containing biological sample: Refer to Appendix III for sample preparation.

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

2. Initiate the enzymatic reaction.

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

2.2 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=380 nm/500 nm continuously and record data every 5 minutes for 30 minutes.

Appendix I: Peptide sequences

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

Substrate No.	Cat No.	Substrate Name	Substrate Sequence
SB1	25271	Caspase-1 substrate	Ac-YVAD-AFC
SB2	25285	Caspase-1 substrate	Ac-WEHD-AFC
SB3	25264	Caspase-2 substrate	Ac-VDVAD-AFC
SB4	25270	Caspase-8 substrate	Ac-IETD-AFC
SB5	25273	Caspase-3/7 substrate	Ac-DEVD-AFC
SB6	21685	Caspase-3/7 substrate	Z-DEVD-AFC
SB7	25276	Caspase-9 substrate	Ac-LEHD-AFC
SB8	25272	Caspase-6 substrate	Ac-VEID-AFC

Appendix II: Instrument Calibration

- AFC fluorescence reference standard: Dilute 10 mM AFC (Component B) to 60 μM in deionized water. Perform 2-fold serial dilutions to obtain 30, 15, 7.5, 3.75, 1.88, 0.94 μM AFC solutions, include a water only sample (0 μM). Add 50 μL/well of the serially diluted AFC solutions from 60 μM to 0 μM into the reference standard wells (refer to Table 1).
- Add 50 μL/well of assay buffer. Mix the reagents by shaking the plate gently for 3 to 5 seconds.
- Measure the fluorescence intensity of the reference standards 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 of the standard operation protocol.
- Plot AFC fluorescent reference standard as RFU (relative fluorescent unit) versus concentration as shown in Figure 1.

  Note: The final concentrations of the AFC reference standards are 30, 15, 7.5, 3.75, 1.88, 0.94, 0.47 and 0 μM. This reference standard curve is used to calibrate for the variation of different instruments and the different batches of experiments. It also can serve as an indicator of the amount of caspase enzymatic reaction final product.

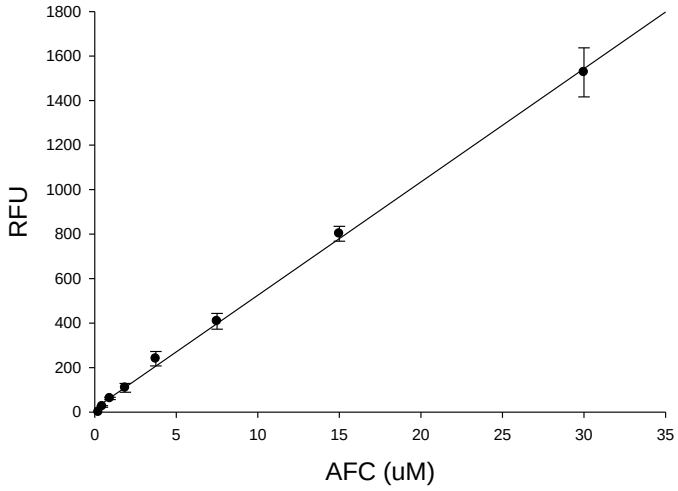

Figure 1. AFC reference standard. AFC was serially diluted in 1X assay buffer. 100 μL of AFC at each concentration was added and fluorescence was recorded at Ex/Em=360±40 nm/460±40 nm (FLx800, Bio-Tek Instruments). Samples were done in duplicates.

Appendix III

Prepare caspase-containing sample from cell extract.

- Induce apoptosis in cell culture using a method of your choice.
- 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 minutes. 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 for one well of 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.

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