EnzoLyte™ AMC Caspase Profiling Kit
Fluorimetric

Catalog #	71120
Unit Size	1 kit
Kit Size	Two 96-well plates

This kit contains two 96-well plates pre-coated with a series of well-designed synthetic peptide substrates as fluorogenic indicators for assaying caspase activities. The fluorescence of AMC can be monitored at the wavelength of Ex/Em=354 nm/442 nm. Ample materials are provided to perform 96 assays in a 96-well format. The kit has the following features:

- ***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.

USA and Canada Ordering Information

AnaSpec Corporate Headquarter
2149 O'Toole Ave.
San Jose, CA 95131
Toll-Free: 800-452-5530
Tel: 408-452-5055
Fax: 408-452-5059
E-mail: service@anaspec.com
Internet: www.anaspec.com

International Ordering Information

A list of international distributors is available at www.anaspec.com.

INTRODUCTION

Apoptosis is a programmed, cell-autonomous death process. It 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. During apoptosis, the caspases executes 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 EnzoLyte™ 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. It 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

KIT COMPONENTS, STORAGE AND HANDLING

Note: Store all components at -20℃, and keep components A and B away from light.

Component A: Two 96-well plates pre-coated with 10 different caspase substrates.
Peptide sequence: refer to **Appendix I** Table 2.
Ex/Em=354 nm/442 nm upon cleavage

Component B: AMC, fluorescence reference standard (10 mM DMSO solution, 20 µL)
Ex/Em=354nm/442 nm

Component C: 2X Assay buffer (20 mL), add DTT before use.

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±30 nm and emission at 442±30 nm.

STANDARD OPERATION PROTOCOL

Note 1: Warm all the kit components until thawed to room temperature before starting the experiments.

1. Prepare working solutions

- Assay buffer: add 20 µL 1 M DTT (component D) per mL 2X assay buffer (component C). Use this DTT-added 2X assay buffer in all the following steps.
- 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. The 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 10. Please refer to **Appendix I Table 2** for substrate sequence.*

- AMC fluorescence reference standard: Dilute 10 mM AMC (component B) to 50 µM in deionized water. Do 1:2 serial dilutions with deionized water to give the concentrations of 25, 12.5, 6.25, 3.125, 1.56, 0.78, and 0.39 µM AMC solution. Take 50 µL of serial diluted AMC solutions from 50 µM to 0.39 µM, and add it into each reference standard well on the 96-well plate (RS1 to RS8, refer to **Table 1**).
- Prepare caspase:
  If you use purified caspase: Dilute caspase to a concentration of 50 to 10 nM in caspase dilution buffer (component E). Each well in a 96-well microplate will need 50 µL of diluted enzyme. Calculate appropriate amount of caspase diluent for all your substrate wells.
  If you use 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 up the caspase samples to room temperature before the following enzymatic reaction.

2. Calibrate the fluorescence microplate reader

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

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

  Note: The final concentration of AMC reference standard is 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39 and 0.195 μM. This reference standard 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 a line fit to the linear portion of the data plot using an appropriate method.
- A variety of data analysis can be done, e.g., determining inhibition %, IC_{50}, K_m, K_i, etc.

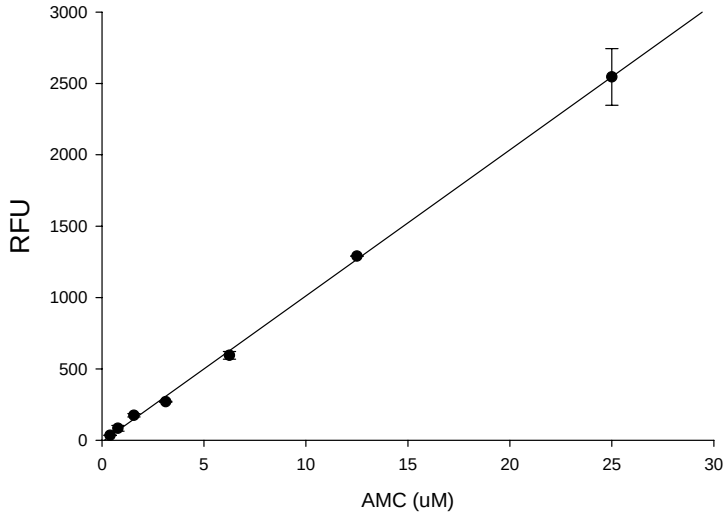

Figure 1. The AMC reference standard calibration curve.
AMC was diluted in 1X assay buffer. 100 μL of AMC at each concentration was added into a 96-well microplate. The fluorescence signal was measured by a fluorescence microplate reader (FLx800, Bio-Tek Instruments) with a filter set of Ex/Em=360±40 nm/460±40 nm. (mean ± S.D.; n= 2 independent samples).

Appendix I

Table 2: The caspase substrate sequences in 96-well plate.

Substrate No.	Cat. No.	Substrate Name	Substrate Sequence
SB1	25267	Caspase-1 substrate	Ac-YEVD-AMC
SB2	25275	Caspase-1 substrate	Ac-WEHD-AMC
SB3	25261	Caspase-1/4 substrate	Ac-YVAD-AMC
SB4	25266	Caspase-3 substrate	Ac-DMQD-AMC
SB5	25262	Caspase-3/7 substrate	Ac-DEVD-AMC
SB6	25256	Caspase-6 substrate	Ac-VEID-AMC
SB7	25257	Caspase-8 substrate	Ac-IETD-AMC
SB8	25286	Caspase-9 substrate	Ac-LEHD-AMC

Appendix II

1. Prepare caspase-containing sample from cell extract.

- Induce apoptosis in cell culture with 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 minutes. For adherent cells, simply aspirate the growth medium.
- Add 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.

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 centrifuge 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. N. A. Thornberry and Y. Lazebnik, *Science* 281, 1312-1316 (1998).
2. J. C. Reed, *J.Clin.Oncol.* 17, 2941-2953 (1999).
3. Y. A. Lazebnik, S. H. Kaufmann, S. Desnoyers, G. G. Poirier, W. C. Earnshaw, *Nature* 371, 346-347 (1994).
4. P. Villa, S. H. Kaufmann, W. C. Earnshaw, *Trends Biochem.Sci.* 22, 388-393 (1997).