EnzoLyte™ AMC Caspase-7 Assay Kit

Catalog #	71119
Unit Size	1 kit
Kit Size	500 assays (96-well) or 1250 assays (384-well)

This kit is optimized to detect caspase-7 activity in cell culture directly without time-consuming cell extraction. It uses a sensitive fluorogenic substrate, Ac-DEVD-AMC, whose signal can be monitored at excitation/emission=354 nm/442 nm. It is also suitable to measure caspase-7 activity in cell extracts and to screen capsase-7 inducers and inhibitors. The kit provides sufficient material to perform 500 assays in a 96-well format and 1250 assays in a 384-well format. The kit has the following features:

- ***Convenient Format:*** All essential assay components are included.
- ***Optimized Performance:*** Optimal conditions for the detection of caspase-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.

USA and Canada Ordering Information

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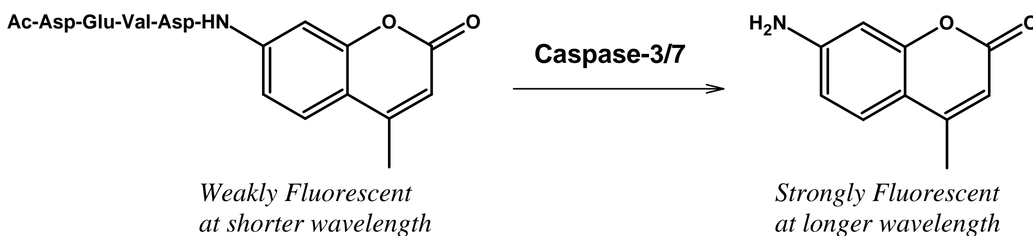
International Ordering Information

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

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. Similar to caspase-3, caspase-7 has substrate selectivity for the amino acid sequence Asp-Glu-Val-Asp (DEVD).

The EnzoLyte™ AMC Capase-7 Assay Kit uses Ac-DEVD-AMC as the fluorogenic indicator for assaying caspase-7 activities. Upon caspase-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**). This kit can measure caspase-7 activity in cell culture directly in a 96-well or 384-well plate without time-consuming cell extraction. Since caspase-3 shares the same substrate specificity with caspase-7, this kit can also detect caspase-3 activity in the cell culture and unable to differentiate these two caspases. The kit is suitable for high throughput screening of apoptosis inducers and inhibitors.



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

KIT COMPONENTS, STORAGE AND HANDLING

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

Component A: Caspase-7 substrate (270 μL)
Peptide sequence=Ac-Asp-Glu-Val-Asp-AMC
Ex/Em=354 nm/442 nm upon caspase-7 cleavage

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

Component C: Ac-DEVD-CHO, a known caspase-7 inhibitor (5 mM DMSO solution, 15 μL)
Peptide sequence=Ac-Asp-Glu-Val-Asp-CHO

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 or 384-well microplate: Black tissue culture microplate with or without clear bottom.

Fluorescence microplate reader: Capable of detecting emission at 440±20 nm with excitation at 350 ±20 nm.

STANDARD OPERATION PROTOCOL

Note 1: For fluorescence instrument calibration, please refer to Appendix II. This is recommended for the first-time users.

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 96 or 384-well plate. If you culture cells in plate larger than 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 detail.

- Seed $1X10^{4-6}$ cells per well in microplate. Add test compounds and then culture cells at 37°C incubator for the desired exposure period. Suggested the total volume of cell and test compound for 96-well plate is 150 µL. Suggested the total volume of cell and test compound for 384-well plate is 60 µL.
- 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 results.

Note: Match the total volume of all the controls to 100 µL for 96-well plate or 40 µL for 384-well plate by growth medium.

2. Prepare working solutions.

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

- Caspase-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-7 substrate (component A) 1:100 in this DTT-containing assay buffer. Mix the reagents well.

  Note: Prepare fresh substrate solution for each experiment.

3. Initiate enzymatic reaction.

- Retrieve the cells from 37°C incubator.
- Add 50 µL of caspase-7 substrate solution into each well of 96-well plate. Or add 20 µL of caspase-7 substrate solution into each well of 384-well plate. Mix the reagents completely by shaking on a plate shaker for 30-60 seconds at 300-400 rpm.
- Measure fluorescence signal:

  For kinetic reading: Immediately start to measure fluorescence intensity at Ex/Em=354 nm/442 nm continuously and record the data every 5 minutes for 30 to 60 minutes.

  For end-point reading: incubate the reaction at room temperature for 30 to 60 minutes on a plate shaker at 300-400 rpm. Kept the plate from direct light. Then measure fluorescence intensity at Ex/Em=354 nm/442 nm.

  Note: If the caspase 7 activity is low in your samples, you may extend the incubation up to 18 hr before the end-point reading.

- Data analysis: refer to Appendix I.

<u>Protocol B.</u> Screen caspase-7 inducers or inhibitors using purified caspase-7.

1. **Prepare working solutions.**
   <u>Note</u>: Warm up all the kit components until thawed at room temperature before starting the experiments.

   - <u>Assay buffer</u>: Add 1 M DTT (component E) 1:100 into assay buffer. Use this DTT-containing assay buffer in ***all*** the following steps.
     <u>Note</u>: Prepare fresh DTT-containing assay buffer for each experiment.
   - <u>Caspase-7 substrate solution</u>: Dilute caspase-7 substrate (component A) 1:200 in assay buffer. Mix the reagents well.
     <u>Note</u>: Prepare fresh substrate solution for each experiment.
   - <u>Caspase-7 diluent</u>: dilute caspase-7 to appropriate concentration in assay buffer.

     <u>Note</u>: Prepare enzyme diluent right before use. Don't vortex enzyme. Prolonged storage of dilutent or vigorously vortex will denature the enzyme. Preserve the enzyme on ice.
   - <u>Test compound:</u> Dilute test compounds with deionized water or appropriate vehicle.
   - <u>Known inhibitor:</u> Ac-DEVD-CHO (component C) is a known caspase-7 inhibitor. Dilute Ac-DEVD-CHO 1:5 in assay buffer.

2. **Set up enzymatic reaction.**
   - Add test compounds and caspase-7 diluent into microplate. The suggested total volume of test compound and caspase-7 diluent is 50 µL for 96-well plate. The suggested total volume for 384-well plate is 20 µL.
   - Set up the following controls at the same time:
     - ➢ <u>Positive control</u> contains caspase-7 diluent without test compound.
     - ➢ <u>Inhibitor control</u> contains caspase-7 diluent and known inhibitor, Ac-DEVD-CHO (10 µL for 96-well plate or 4 µL for 384-well plate).
     - ➢ <u>Vehicle control</u> contains caspase-7 diluent and vehicle used to deliver test compound.
     - ➢ <u>Test compound control</u> contains assay buffer and test compound. Some test compounds have strong autofluorescence and may give false results.
     - ➢ <u>Substrate control</u> contains assay buffer.

   <u>Note:</u> Match the total volume of all the controls to 50 µL for 96-well plate or 20 µL for 384-well plate by assay buffer.

3. **Pre-incubation.**
   - Incubate the plate at desired temperature (e.g. 25°C or 37°C) for 10-15 min. Meanwhile, incubate the caspase-7 substrate solution at the same temperature.

4. **Initiate the enzymatic reaction**.
   - Add 50 µL of caspase-7 substrate solution into the wells of 96-well plate. Or add 20 µL of caspase-7 substrate solution into the wells of 384-well plate. Mix the reagents completely by shaking on a plate shaker for 30-60 seconds at 300-400 rpm.
   - Measure fluorescence signal:

     <u>For kinetics reading</u>: Immediately start to measure fluorescence intensity at Ex/Em=354 nm/442 nm continuously and record the data every 5 minutes for 30 to 60 minutes.

For end-point reading: incubate the reaction at room temperature for 30 to 60 minutes on a plate shaker at 300-400 rpm. Kept the plate from direct light. Then measure fluorescence intensity at Ex/Em=354 nm/442 nm.

- Data analysis: refer to Appendix I.

Appendix I: Data analysis.

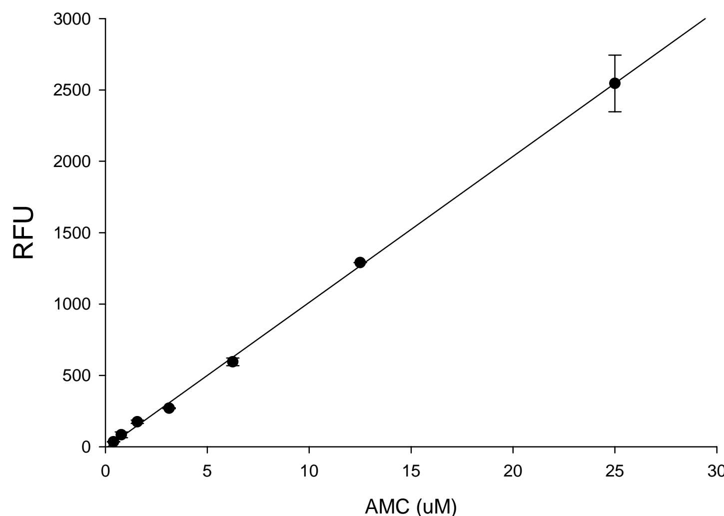
- The fluorescence reading from the non-cell control well or substrate control well is the background fluorescence. The readings from other wells need to be subtracted with this background fluorescence. This reading is relative fluorescence unit (RFU).
- For kinetics reading:
  - ➢ Plot data as RFU versus 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 (V) 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 %, $EC_{50,}$ IC_{50}, K_m, K_i, etc.
- For endpoint reading:
  - ➢ Plot data as RFU versus the concentration of test compounds.
  - ➢ A variety of data analysis can be done, e.g., determining inhibition %, EC_{50}, IC_{50}, etc.

Appendix II: Instrument calibration.

- AMC fluorescence reference standard: Dilute 10 mM AMC (component B) to 60 µM in deionized water. Do 1:2 serial dilutions to give the concentrations of 30, 15, 7.5, 3.75, 1.88, 0.94, and 0 µM AMC solution. Add 50 µL/well of serial diluted AMC solutions from 60 µM to 0 µM into the 96-well plate or 20 µL/well into 384-well plate.
- Add 50 µL/well of caspase-7 substrate solution (refer to Protocol B step 1 for preparation) into the 96-well plate or 20 µL/well into 384-well plate. Mix the reagents by shaking the plate gently for 3 to 5 seconds.

  Note: The caspase-7 substrate solution should be added into reference standard to normalize 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 satisfying signals can be read. Use the same setting of sensitivity in the enzymatic reaction in protocol 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 fluorescent reference standard as RFU (relative fluorescent unit) versus concentration as **Figure 1**.

  Note: The final concentration of AMC reference standard is 30, 15,7.5,3.75,1.88,0.94, 0.47 and 0



µM. This reference standard is used to calibrate the variation of different instruments and different batch of experiments. It also can be served as an indicator of the amount of final product of the caspase enzymatic reaction.

Figure 1. The AMC reference standard calibration curve.
AMC was diluted in 1X assay buffer containing 50 µM of Caspase-7 substrate. 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 III

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

- Seed at least $1X10^6$ cells per well. Add appropriate amount of apoptosis-inducing test compound to the cells. Culture cells at 37°C incubator for desired exposure period.
- 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.
- Suspended cells are collected by centrifugation at 500 X g for 5 minutes. For adherent cells, simply aspirate the growth medium.
- Add 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 resuspense 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.
- Add 150 µL/well of supernatant and controls to the 96-well plate. Or add 60 µL/well to the 384-well plate.
- Continue to **Step 2 in protocol A** for caspase-7 assay.

REFERENCES

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