



EnzoLyte™ AnaRed™ Caspase-7 Assay Kit

Catalog #	71123
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 a time-consuming cell extraction step. It uses a sensitive fluorogenic substrate, Z-DEVD-AnaRed™, whose signal can be monitored at excitation/emission=595 nm/635 nm. It is also suitable to measure caspase-7 activity in cell extracts and to screen for caspase-7 inducers and inhibitors. The kit provides sufficient materials 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 events¹, ranging from normal fetal development to diseases, such as cancer², 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™ AnaRed™ Caspase-7 Assay Kit uses Z-DEVD-AnaRed™ as the fluorogenic indicator for assaying caspase-7 activities. Upon caspase-7 cleavage, Z-DEVD-AnaRed™ generates the AnaRed™ fluorophore that has bright red fluorescence and can be detected at excitation/emission=595 nm/635 nm. This kit can measure caspase-7 activity in cell culture directly in a 96-well or 384-well plate without a time-consuming cell extraction step. Since caspase-7 shares the same substrate specificity with caspase-3, this kit can also detect caspase-3 activity in the cell culture and is unable to differentiate between these two caspases. The kit is suitable for high throughput screening of apoptosis inducers and inhibitors.

KIT COMPONENTS, STORAGE AND HANDLING

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

- Component A:** Caspase-7 substrate (270 µL)
Peptide sequence= Z-DEVD-AnaRed™
Ex/Em=595 nm/635 nm upon caspase-7 cleavage
- Component B:** AnaRed™, fluorescence reference standard (1 mM DMSO solution, 50 µL)
Ex/Em=595 nm/635 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 635 ±20 nm with excitation at 595 ±20 nm.

STANDARD OPERATION PROTOCOL

Note 1: For fluorescence instrument calibration, please refer to Appendix II. This is recommended for 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 a 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 details.

- Seed 1×10^4 cells per well in a microplate. Add test compounds and then culture cells in a 37°C incubator for the desired exposure period. 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. Suggested volumes for a 384-well plate is 50 µL of cells and 10 µL of test compounds with a total volume of 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 150 µL for a 96-well plate or 60 µL for a 384-well plate by growth medium.

2. Prepare working solutions.

Note: Warm all kit components until thawed to 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 the 37°C incubator.
- Add 50 µL of caspase-7 substrate solution into each well of a 96-well plate. Or add 20 µL of caspase-7 substrate solution into each well of a 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 measuring fluorescence intensity at Ex/Em=595 nm/635 nm continuously and record 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. Keep the plate from direct light, then measure fluorescence intensity at Ex/Em=595 nm/635 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.

Protocol B. Screen caspase-7 inducers or inhibitors using purified caspase-7

1. Prepare working solutions.

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

- Assay buffer: Add 1 M DTT (component E) 1:100 into diluted assay buffer. Use this DTT-containing assay buffer in **all** the following steps.
Note: Prepare fresh DTT-containing assay buffer for each experiment.
- Caspase-7 substrate solution: Dilute caspase-7 substrate (component A) 1:200 in assay buffer. Mix the reagents well.
Note: Prepare fresh substrate solution for each experiment.
- Caspase-7 diluent: Dilute caspase-7 to appropriate concentration in assay buffer.
Note: Prepare enzyme diluent right before use. Do not vortex enzyme. Prolonged storage of diluted enzyme or vigorously vortexing will denature the enzyme.
- Test compound: Dilute test compounds with deionized water or appropriate vehicle.
- Known inhibitor: Ac-DEVD-CHO (component C) is a known caspase-7 inhibitor. Dilute Ac-DEVD-CHO 1:10 in assay buffer.

2. Set up enzymatic reaction.

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

Note: Match the total volume of all the controls to 50 μ L for 96-well plate or 20 μ L for 384-well plate by assay buffer.

3. Pre-incubation.

- Incubate the plate at the desired temperature for enzymatic reaction (e.g. 25°C or 37°C) for 10-15 min. In the mean time, also incubate the caspase-7 substrate solution at the same temperature.

4. Initiate the enzymatic reaction.

- Add 50 μ L of caspase-7 substrate solution into the wells of a 96-well plate. Or add 20 μ L of caspase-7 substrate solution into the wells of a 384-well plate. Mix the reagents completely by shaking the plate gently for 30-60 seconds.
- Measure fluorescence signal:
 - For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=595 nm/635 nm continuously and record 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. Keep the plate from direct light. Then measure fluorescence intensity at Ex/Em=595 nm/635 nm

Appendix I: Data analysis.

- The fluorescence reading from the non-cell control well or substrate control well is the background fluorescence. This background reading has to be subtracted from the readings of the other wells. This reading is the 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_0) 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 the concentration of test compounds.
 - A variety of data analyses can be done, e.g., determining inhibition %, EC_{50} , IC_{50} , etc.

Appendix II: Instrument calibration.

- AnaRed™ fluorescence reference standard: Dilute 1 mM AnaRed™ (component B) to 20 μ M in deionized water. Do 1:2 serial dilutions to give the concentrations of 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0 μ M AnaRed™ solutions. Add 50 μ L/well of the serially diluted AnaRed™ solutions from 20 μ M to 0 μ M into a 96-well plate or 20 μ L/well into a 384-well plate.
- Add 50 μ L/well of caspase-7 substrate solution (refer to Protocol B step 1 for preparation) into a 96-well plate or 20 μ L/well into a 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 the reference standard to normalize for the fluorescence inner filter effect.

- Measure the fluorescence intensity of the reference standard and substrate control wells at Ex/Em=595 nm/635 nm. Adjust the sensitivity of the microplate reader until satisfactory signals can be read. Use the same sensitivity setting in the enzymatic reaction in protocols A and B.
- The fluorescence reading from the wells containing 0 μ M AnaRed™ 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 AnaRed™ fluorescent reference standard as RFU (relative fluorescent unit) versus concentration.

Note: The final concentration of AnaRed™ reference standard solutions are 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16 and 0 μ M. This reference standard curve is used to calibrate for the variation of the different instruments and for the different batches of experiments. The reference standard can also serve as an indicator of the amount of final product in the caspase enzymatic reaction.

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 1×10^6 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 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.
- Suspension cells are collected by centrifugation at $500 \times g$ for 5 minutes. For adherent cells, simply aspirate the growth medium.
- Add an appropriate amount of lysis buffer to cells or cell pellet, e.g. $300 \mu\text{L}$ 1X lysis buffer for one well of 6-well plate. Scrape off the adherent cells or resuspend 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 $2500 \times g$ for 10 min at 4°C .
- Add $150 \mu\text{L}$ /well of supernatant and controls to the 96-well plate. Or add $60 \mu\text{L}$ /well to the 384-well plate.
- Continue to **Step 2 in protocol A** for caspase-7 assay.

REFERENCES

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