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Caspase-3, 7 Activity Fluorometric Assay Kit (250 Assays)

(Cat. # J7130)

Caspase-3, 7 Activity Fluorometric Assay Kit can be used for assaying caspase-3, 7 activities in cell/tissue extracts in a 96-well plate format. Each of the supplied fluorogenic substrate and the pan caspase inhibitor Z-VAD-FMK is sufficient for 250 x 100 µl reactions. The supplied TRAIL-treated cell lysate is used as a positive control, sufficient for 5 X 100 µl reactions.

Description

Death ligands and various stresses can activate initiator caspase-8/10 and caspase-9 to induce extrinsic and intrinsic apoptosis, respectively. Activated initiator caspases activate downstream executor caspase-3/7, which cleave cellular proteins to commit apoptosis. In addition, extrinsic and intrinsic apoptosis can cross-talk.

Caspase-3, 7 Activity Fluorometric Assay Kit can be used for assaying caspase-3, 7 activities in cell/tissue extracts in a 96-well plate format. Caspase-3, 7 cleave the supplied fluorogenic substrate. The fluorescence of released AFC can be monitored using a plate reader or a fluorometer at the excitation/emission wavelength of 400 nm/505 nm, respectively. The 96-well format assay can be completed in 2-4 hours.

Ac-DEVD-AFC is a fluorogenic substrate of caspase-3/7.

Z-VAD-FMK is a pan caspase inhibitor, used to inhibit caspases in reactions to obtain background signals.

TRAIL-treated HCT116 cell lysates contain activated caspase-8/9/3/7, used as a positive control.

AFC is used for generating a standard curve for quantitating the amount of AFC produced in reactions.

Components

• 1000X Ac-DEVD-AFC (25 mM in DMSO)	25 µl
• 1000X Z-VAD-FMK (25 mM in DMSO)	25 µl
• TRAIL-treated HCT116 cell lysates (5 mg/ml)	25 µl
• AFC (0.1 mM in DMSO)	250 µl
• Tris (2 M), pH 7.1 at 37°C	1 ml
• DTT (200 mM)	1 ml

1X Reaction Buffer (not included): 20 mM Tris, pH 7.1 at 37°C, 150 mM NaCl, and 2 mM DTT

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Protocol for assaying caspase-3/7 activity using whole cell extracts

The following protocol has been used to test J7110, J7120 and J7130. Users are strongly recommended to optimize conditions for your experimental needs.

[Prepare TRAIL-treated cell extracts]

1. Two dishes (100 mm) of HCT116 cells were grown in DMEM supplemented with 10% fetal bovine serum to ~ 90% confluence. Cells were changed to fresh media with 50 ng/ml recombinant 6xHis-TRAIL for 4 hours. Cells were then harvested, washed twice with ice-cold 1X PBS, and kept in a 15 ml conical tube.
2. Resuspend the cell pellet in 1 ml ice-cold cell lysis buffer (20 mM Tris, pH 7.2, 300 mM NaCl, 2 mM βME and 10% Glycerol). Briefly sonicate cells using a 550 Sonic Dismembrator (Fisher Scientific). Settings: power output: 2, 20 seconds/cycle for three cycles, put the tube on ice for 2 min between cycles. Cells can also be lysed using a homogenizer.
   Note: No protease inhibitors were added in the cell lysis buffer.
3. Transfer cell lysates into a 1.5 ml centrifuge tube. Centrifuge using a desktop centrifuge at 16,000 X*g* for 15 min under 4 ^{0}C.
4. Transfer the supernatants to a 1.5 ml centrifuge tube. Measure the supernatant concentration using the Bradford assay. We obtained a total of 7 mg cell extracts (7 mg/ml), dilute to 5 mg/ml. Snap freeze using liquid nitrogen and store the supernatants at -80 ^{0}C for future use.
   Note: We recommend a cell extract concentration at ~ 5 mg/ml. Users are strongly recommended to prepare their cell/tissue extracts accordingly to ensure sufficient caspase activities to be measured.

[Set up a plate reader]

5. We use a BioTek Synergy II plate reader with exciting and emission filters at 400±40 nm and 508±30 nm, respectively, to determine AFC fluorescence. For a time-course kinetic assay, we use the following parameters: 30 min reading time, 1 min/reading, sensitivity setting 65, and probing from the bottom of a black clear bottom 96-well plate. Warm up the instrument to 37 ^{0}C.
   Note: Each brand of plate reader is different, reading parameters may change accordingly. Users should test the sensitivity setting, cell lysate concentration, and incubation time to optimize conditions. Both clear bottom (can be detected form both the top and the bottom) and solid bottom (detected from the top) *black* plate*s* are suitable for fluorescence detection.

[Caspase-9/3/7 activity assay]

6. Prepare 2X substrate and inhibitor in reaction buffer (*Users should prepare the appropriate amounts depending on your needs*)
   1X reaction buffer (NOT PROVIDED): 20 mM Tris, pH7.1 at 37°C, 150 mM NaCl, and 2 mM DTT. 100X Tris and 100X DTT were provided for making reaction buffer. Warm up 1X reaction buffer in a 37 ^{0}C water bath.
   2X Ac-DEVD-AFC (substrate): Add 1 μl supplied substrate into 499 μl warmed 1X reaction buffer. Vortex to mix and keep warm (37 ^{0}C).

2X Z-VAD-FMK (inhibitor): Add 1 µl supplied inhibitor into 499 µl warmed 1X reaction buffer. Vortex to mix and keep warm (37 ^{0}C).

7. Set up reactions (triplicates for each setting)

   Setting 1 (no caspase inhibitor)

   Reaction 1-3: Mixing 25 µg TRAIL-treated HCT116 cell lysate (5 µl) + 45 ul 1X reaction buffer.

   Setting 2 (with caspase inhibitor)

   Reaction 4-6: Mixing 25 µg TRAIL-treated HCT116 cell lysate (5 µl) + 45 ul 2X Z-VAD-FMK prepared in Step 6.

   Keep all reactions at 37 ^{0}C for 10 min.

   Note: Users are strongly recommended to do cell/tissue lysate concentration titration experiments to determine the appropriate amount to be used. Too high concentrations of caspases in cell/tissue extracts may consume up the substrate rapidly. In this case, you can dilute cell/tissue extracts. If the signal is too weak, you can use more cell/tissue lysates and increase your plate reader sensitivity.

8. Measure caspase activities.

   Transfer each reaction in Step 7 into a well of a black clear bottom 96-well plate that is pre-warmed at 37 ^{0}C in the plate reader. Use an 8-channel pipettor to add 50 µl 2X Ac-DEVD-AFC substrate prepared in Step 6. Start reading immediately for a 30 min kinetic assay.

   Note: Arranging reactions with the same substrate in a row or a column of the 96-well plate can facilitate substrate adding using a multiple channel pipettor.

9. Data processing.

   The slope of the linear region of each curve in a reaction added Ac-DEVD-AFC can be used for reflecting the relative caspase-3/7 activities, respectively. The average slope value from three reactions with Z-VAD-FMK should be subtracted as the background signal.

   To quantitate the amount of AFC produced in each reaction, an AFC standard curve can be plotted by measuring the fluorescence of a series of AFC compound concentration: add 0, 2, 4, 6, 8 and 10 µl of 100 µM AFC (PROVIDED in the kit) into a series of wells in a 96-well black plate, then add 100, 98, 96, 94, 92 and 90 µl of 1X reaction buffer to make a final volume of 100 µl in each well, that generates 0, 200, 400, 600, 800 and 1000 pmol/well of AFC standard. Reading parameters should be the same as those used in your experiments.

10. Representative activity curves.

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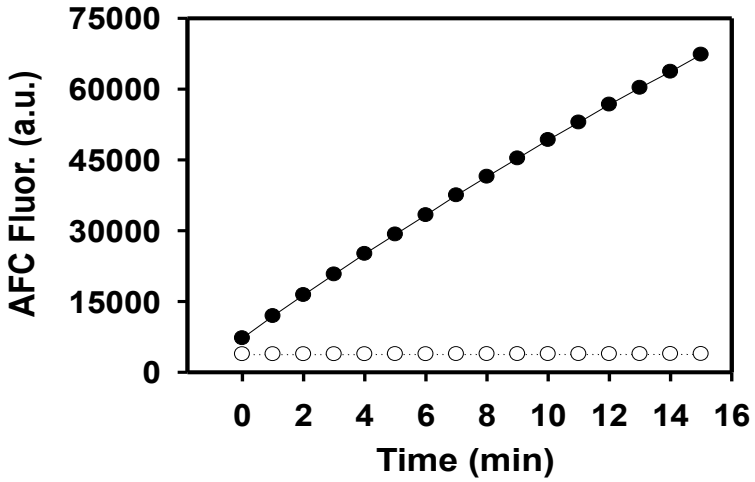

Representative time-course reactions described in this protocol. HCT116 cells were treated with 50 ng/ml 6xHis-TRAIL for 4 hours to activate extrinsic apoptosis. 25 µg cell lysates were incubated with (open circles) or without (solid circles) 20 µM Z-VAD-FMK (a pan caspase inhibitor) for 10 min at 37 ^{0}C. 25 µM Ac-DEVD-AFC was added to initiate the reaction. AFC fluorescence was recorded with a plate reader using excitation/emission filter set at 400±40/508±30 nm, respectively.