Caspase-9 Fluorometric Assay Kit

Introduction

Cysteine-containing aspartate proteolytic enzymes (Caspase) are a family of cysteine proteases that play important roles in apoptosis, necrosis, and inflammation. Caspase-9 is a priming type of caspase that initiates endogenous apoptosis, which is apoptosis of the mitochondrial pathway. When cells are stressed, Cytochrome c is released into the cytoplasm and binds to the apoptotic protein activator 1 (Apaf-1) to form a complex. Caspase-9 was then recruited into this complex, forming the apoptotic bodies. In apoptotic bodies, caspase-9 is activated by self-cleavage. Activated caspase-9 further activates downstream effector caspases, such as caspase-3 and caspase-7.

Caspase-9 Fluorometric Assay Kit provides a convenient and simple way for detecting the LEHD-dependent caspase activity. LEHD-AFC (AFC:7-amino-4-trifluoromethyl coumarin) emits blue light (λmax = 400 nm); while cleavage of LEHD-AFC by Caspase-9 or related caspases, free AFC emits a yellow-green fluorescence (λmax = 505 nm), which can be quantified by using a microplate reader or a fluorometer. Comparison of the fluorescence of AFC from an apoptotic sample with an uninduced control determines the fold increase in Caspase-9 activity.

Components and Storage

Components	K2018-25T	K2018-100T	K2018-200T	K2018-400T
Cell Lysis Buffer	25 mL	100 mL	100 mL	100 mL
2X Reaction Buffer	2 mL	4 X 2 mL	16 mL	32 mL
LEHD-AFC (1 mM)	125 µL	500 µL	2 X 0.5 mL	2 X 1 mL
DTT (1 M)	100 µL	400 µL	400 µL	400 µL

Store the kit at -20°C, stable for 6 months. LEHD-AFC (1 mM) should be stored away from light.

Protocol

1. **Preparation before the experiment:**

   1) Equilibrate Cell Lysis Buffer and 2X Reaction Buffer to room temperature before use. Thaw LEHD-AFC (1 mM) in ice before use. Once thawed, aliquot LEHD-AFC (1 mM) to avoid repeated freeze/thaw cycles.

   2) Add 10 µL DTT (1 M) per 1 mL of 2X Reaction Buffer to make the 2X Reaction Buffer (containing DTT).

2. **Sample Preparation:**

Try to use fresh samples. If this is not possible in time, it is recommended to complete the sample preparation step before storing at -80°C. And thaw and mix them on ice before use.

1) **For cell samples:**

a) Prepare two groups of cells. One experimental group is treated according to the experimental design and the other is an untreated control group.

b) Collect 1-5 x 10^6 cells after treatment. Wash the cells one time with pre-chilled PBS.

***Note:** When using adherent cells for testing, if there are suspended cells after treatment in the experimental group, the suspended cells should be collected and tested together.

c) Resuspend cells in 50 μL of pre-chilled Cell Lysis Buffer. Incubate in ice for 10 min.

d) After incubation, centrifuge samples at 12,000 rpm for 2-5 min, carefully transfer the supernatant to a new EP tube and place on ice for later use.

e) Take a small amount of supernatant (1-2 μL) to measure the protein concentration Cpr using the Bradford method. To ensure the accuracy of the experiment, it is recommended to achieve a protein concentration of 1-4 mg/mL.

2) **For tissue samples:**

a) Harvest 50-100 mg of tissue samples from the experimental group and the untreated control group. Wash cells with pre-chilled PBS once.

b) Add 500 μL of pre-chilled Cell Lysis Buffer to homogenize tissue on ice. Incubate in ice for 10 min.

c) After incubation, centrifuge at 12,000 rpm for 2-5 min, carefully transfer the supernatant to a new EP tube and place on ice for later use.

d) Take a small amount of supernatant (1-2 μL) to measure the protein concentration Cpr using the Bradford method. To ensure the accuracy of the experiment, it is recommended to achieve a protein concentration of 1-4 mg/mL.

3. **Caspase enzyme assay:**

1) Refer to the following table to prepare the groups. This assay can be performed directly in a black 96-well plate. Or transfer the samples to a black 96-well plate before detection.

Sample group (experimental group and untreated control group)	50 μL supernatant (If the volume is less than 50 μL, adjust volume to 50 μL with Cell Lysis Buffer)
Negative control group	50 μL Cell Lysis Buffer

2) Add 50 μL of 2X Reaction Buffer (containing DTT) to each well.

3) Add 5 μL of 1 mM LEHD-AFC to each well. Incubate at 37°C in the dark for 1-2 h.

*Note: The negative control group does not need to add VDVAD-AFC substrate.

4) Measure the Fluorescence signal by microplate reader at Ex/Em = 400/505 nm. Or measure the signal using a fluorometer.

*Note: This kit detects fluorescence, so a black plate is recommended for microplate readers.

4. **Analysis:** The following formula can be used to calculate the enzyme activity

$$Caspase\ activity\ (\%) = \frac{F_{\text{experimental group}} - F_{\text{Negative control group}}}{Cpr_{\text{experimental group}}} \div \frac{F_{\text{untreated control grou}} - F_{\text{Negative control group}}}{Cpr_{\text{untreated control grou}}} \times 100\%$$

Note: F is the fluorescence signal value for each group; Cpr is the protein concentration; If the sample set is diluted with Cell Lysis Buffer, the F of the original sample needs to be corrected first, that is, ($F_{\text{experimental group}}$ - $F_{\text{negative control group}}$) × a, a is the dilution coefficient of the original sample.

Note

Problems	Cause	Solution
Assay not working	• Cells did not lyse completely • Experiment was not performed at optimal time after apoptosis induction • Plate read at incorrect wavelength • Old DTT used	• Resuspend the cell pellet in the lysis buffer and incubate as described in the datasheet • Perform a time-course induction experiment for apoptosis • Check the wavelength listed in the datasheet and the filter settings of the instrument • Always use freshly thawed DTT in the cell lysis buffer
High Background	• Increased amount of cell lysate used • Increased amounts of components added due to incorrect pipetting • Incubation of cell samples for extended periods • Use of expired kit or improperly stored reagents • Contaminated cells	• Refer to datasheet and use the suggested cell number to prepare lysates • Use calibrated pipettes • Refer to datasheet and incubate for exact times • Always check the expiry date and store the individual components appropriately • Check for bacteria/ yeast/ mycoplasma contamination
Lower signal levels	• Cells did not initiate apoptosis • Very few cells used for analysis • Use of samples stored for a long time • Incorrect setting of the equipment used to read samples • Allowing the reagents to sit for extended times on ice	• Determine the time-point for initiation of apoptosis after induction (time-course experiment) • Refer to datasheet for appropriate cell number • Use fresh samples or aliquot and store and use within one month for the assay • Refer to datasheet and use the recommended filter setting • Always thaw and prepare fresh reaction mix before use
Samples with	• Uneven number of cells seeded in the wells	• Seed only equal number of healthy cells (correct

erratic readings	• Samples prepared in a different buffer • Adherent cells dislodged and lost at the time of experiment • Cell/ tissue samples were not completely homogenized • Samples used after multiple freeze-thaw cycles • Presence of interfering substance in the sample • Use of old or inappropriately stored samples	passage number) • Use the cell lysis buffer provided in the kit • Perform experiment gently and in duplicates/ triplicates; apoptotic cells may become floaters • Use Dounce homogenizer (increase the number of strokes); observe efficiency of lysis under microscope • Aliquot and freeze samples, if needed to use multiple times • Troubleshoot as needed • Use fresh samples or store at correct temperatures until use
Unanticipated results	• Measured at incorrect wavelength • Cell samples contain interfering substances	• Check the equipment and the filter setting • Troubleshoot if it interferes with the kit (run proper controls)
General issues	• Improperly thawed components • Incorrect incubation times or temperatures • Incorrect volumes used • Air bubbles formed in the well/tube • Substituting reagents from older kits/ lots • Use of a different 96-well plate	• Thaw all components completely and mix gently before use • Refer to datasheet & verify the correct incubation times and temperatures • Use calibrated pipettes and aliquot correctly • Pipette gently against the wall of the well/tubes • Use fresh components from the same kit • Fluorescence: Black plates; Absorbance: Clear plates

Note: The most probable cause is listed under each section. Causes may overlap with other sections.

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