Caspase-9 Fluorometric Assay Kit (BN00056)

(Catalog BN00056 -25, -100, -200, -400; Store kit at –20˚C)

I. Introduction:

Activation of ICE-family proteases/caspases initiates apoptosis in mammalian cells. The **Caspase-9 Fluorometric Assay Kit** provides a simple and convenient means for assaying the activity of caspases that recognize the sequence LEHD. The assay is based on detection of cleavage of substrate LEHD-AFC (AFC: 7-amino-4-trifluoromethyl coumarin). LEHD-AFC emits blue light (λmax = 400 nm); upon cleavage of the substrate by caspase-9 or related caspases, free AFC emits a yellow-green fluorescence (λmax = 505 nm), which can be quantified using a fluorometer or fluorescence microtiter plate reader. Comparison of the fluorescence of AFC from an apoptotic sample with an uninduced control allows determination of the fold increase in caspase-9 activity.

II. Kit Contents:

Components	BN00056 25 assays	BN00056 100 assays	BN00056 200 assays	BN00056 400 assays
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	0.5 ml	2 x 0.5 ml	2 x 1 ml
DTT (1 M)	100 µl	0.4 ml	0.4 ml	0.4 ml

III. Caspase-9 Assay Protocol:

A. General Considerations

- Aliquot enough 2X Reaction Buffer for the number of assays to be performed. Add DTT to the 2X Reaction Buffer immediately before use (10 mM final concentration: add 10 µl of 1.0 M DTT stock per 1 ml of 2X Reaction Buffer).
- Protect LEHD-AFC from light.
- We recommend using a flat bottom, opaque, white or black 96-well plate for enhanced sensitivity.

B. Assay Procedure

1. Induce apoptosis in cells by desired method. Concurrently incubate a control culture *without* induction.
2. Count cells and pellet 1-5 x 10_6 cells or use 50-200 µg cell lysates if protein concentration has been measured.
3. Resuspend cells in 50 µl of chilled Cell Lysis Buffer. Incubate cells on ice for 10 minutes.
4. Add 50 µl of 2X Reaction Buffer (containing 10 mM DTT) to each sample. Add 5 µl of the 1 mM LEHD-AFC substrate (50 µM final concentration) and incubate at 37˚C for 1-2 hour.
5. Read samples in a fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter. For a plate-reading set-up, transfer the samples to a 96-well plate. You may also perform the entire assay directly in a 96-well plate.

Fold-increase in Caspase-9 activity can be determined by comparing these results with the level of the uninduced control.

IV. Storage and Stability:

Store kit at –20˚C (Store Cell Lysis Buffer and 2X Reaction Buffer at 4˚C after opening). All reagents are stable for 1 year under proper storage conditions.

FOR RESEARCH USE ONLY! Not to be used on humans.

Problems	Cause
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
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
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
Samples with erratic readings	• Uneven number of cells seeded in the wells • 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	• 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.		