For research use only

Caspase-9 Colorimetric Assay Kit (BN00060)

(Catalog BN00060 -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 Colorimetric Assay Kit** provides a simple and convenient means for assaying the activity of caspases that recognize the sequence LEHD. The assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labeled substrate LEHD-*p*NA. The *p*NA light emission can be quantified using a spectrophotometer or a microtiter plate reader at 400- or 405-nm. Comparison of the absorbance of *p*NA from an apoptotic sample with an uninduced control allows determination of the fold increase in Caspase-9 activity.

II. Kit Contents:

Components	BN00060 25 assays	BN00060 100 assays	BN00060 200 assays	BN00060 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-*p*NA (4 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
Dilution Buffer	25 ml	100 ml	200 ml	400 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).
- Store kit at –20˚C (Store Cell Lysis Buffer, 2X Reaction Buffer, and Dilution Buffer at 4˚C after opening). All reagents are stable for 6 months under proper storage conditions.
- Protect LEHD-*p*NA from light.

B. Assay Procedure

1. Induce apoptosis in cells by desired method. Concurrently incubate a control culture *without* induction.
2. Count cells and pellet 2-5 x 10_6 cells.
3. Resuspend cells in 50 µl of chilled Cell Lysis Buffer and incubate cells on ice for 10 minutes.
4. Centrifuge for 1 min in a microcentrifuge (10,000 x g).
5. Transfer supernatant (cytosolic extract) to a fresh tube and put on ice.
6. Assay protein concentration.
7. Dilute 100-200 µg protein to 50 µl Cell Lysis Buffer for each assay.
8. Add 50 µl of 2X Reaction Buffer (containing 10 mM DTT) to each sample. Add 5 µl of the 4 mM LEHD-*p*NA substrate (200 µM final conc.) and incubate at 37˚C for 1-2 hour.
9. Read samples at 400- or 405-nm in a microtiter plate reader, or spectrophotometer using a 100-µl micro quartz cuvette (Sigma), or dilute sample to 1 ml with Dilution Buffer and using regular cuvette (note: Dilution of the samples proportionally decreases the reading).

You may also perform the assay in a 96-well plate.

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

Note: Background reading from cell lysates and buffers should be subtracted from the readings of both induced and the uninduced samples before calculating fold increase in Caspase-9 activity.

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Problems	Cause	S
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	• 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.		