Caspase-6 Colorimetric Assay Kit (BN00044)

(Catalog BN00044 -25, -100, -200, -400, Store kit at –20˚C)

I. Introduction:

Activation of ICE-family proteases/caspases initiates apoptosis in mammalian cells. The **Caspase-6 Colorimetric Assay Kit** provides a simple and convenient means for assaying the activity of caspases that recognize the sequence VEID. The assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labeled substrate VEID-*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-6 activity.

II. Kit Contents:

Components	BN00044 25 assays	BN00044 100 assays	BN00044 200 assays	BN00044 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
VEID-*p*NA (4 mM)	125 µl	0.5 ml	2 x 0.5 ml	2 x 1ml
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-6 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).
- After thawing, store the Cell Lysis Buffer, 2X Reaction Buffer, and dilution Buffer at 4˚C.
- Protect VEID-*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 100-250 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 VEID-*p*NA substrate (200 µM final conc.). Incubate at 37˚C for 1-2 hour (or longer time if desired).
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 entire assay in a 96-well plate.

Fold-increase in Mch2 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-6 activity.

IV. Storage and Stability:

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.

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

Problems	Cause	So
Assay not working	• Cells did not lyse completely	• P
	• Experiment was not performed at optimal time after apoptosis induction	• P
	• Plate read at incorrect wavelength	• C
	• Old DTT used	• A
High Background	• Increased amount of cell lysate used	• P
	• Increased amounts of components added due to incorrect pipetting	• U
	• Incubation of cell samples for extended periods	• P
	• Use of expired kit or improperly stored reagents	• A

	• Contaminated cells	• 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 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	• Seed only equal number of healthy cells (correct 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.		