R&D SYSTEMS
a bio-techne brand

Caspase-9 Colorimetric Assay

Catalog Number: BF10100
Size: 100 Tests

REAGENTS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at ≤ -20 °C in a manual defrost freezer.

REAGENT	DESCRIPTION	STORAGE OF OPENED MATERIAL
LEHD-pNA Substrate	500 µL of 4 mM LEHD substrate peptide conjugated to p-nitroaniline **(protect from light).**	Store at ≤ -20 °C for up to 6 months after initial use. Avoid repeated freeze-thaw cycles.
DTT	400 µL of a 1 M solution of dithiothreitol (DTT).	
Lysis Buffer	100 mL of Lysis Buffer.	May be stored for up to 6 months at 2-8 °C.
Reaction Buffer 9	4 vials (2.0 mL/vial) of 2X Reaction Buffer 9.	
Dilution Buffer	100 mL of Dilution Buffer.	

INTENDED USE

The purpose of this kit is to determine the increased enzymatic activity of the Caspase-9 class of proteases in apoptotic cells by colorimetric reaction.

BACKGROUND INFORMATION

Apoptosis was originally described as a mechanism of controlled or physiological cell death (1). It is associated with the regulation of cellular homeostasis in organs and the elimination of damaged cells or of cells with deleterious reactivities from the host. Apoptosis is very common in tissues with intense hematopoietic activity (e.g. bone marrow and thymus) and in organs with high proliferative activity. Additionally, apoptosis has been implicated in the progression of a number of pathological conditions, including AIDS, cancer, and autoimmune diseases (2, 3).

Apoptosis is characterized by a variety of cellular changes including loss of membrane phospholipid asymmetry (4), chromatin condensation, mitochondrial swelling and DNA cleavage (5). The end result of these changes is a form of cell death that avoids the normal inflammatory response associated with necrosis.

Caspase-9, also known as ICE-LAP6 and Mch6, is an upstream proenzyme in the cascade of enzymatic reactions required to induce cellular apoptosis (6, 7). After the release of mitrochondrial cytochrome c, caspase-9 is activated following its association with the protein complex of Apaf1 and cytochrome c (8). Active caspase-9 in turn activates pro-caspase-3 promoting the manifestation of some of the more classical features of apoptosis. Activation of caspase-9 can be regulated through protein phosphorylation events (9). The optimal cleavage recognition sequence for caspase-9 is LEHD (10). The ability of caspase-9 to associate with both pro-apoptotic and anti-apoptotic proteins as well as its regulatory function during embryonic development suggests that caspase-9 is a key regulator of apoptosis *in vivo*.

PRINCIPLE OF TEST

Cells that are suspected to or have been induced to undergo apoptosis are first lysed to collect their intracellular contents. The cell lysate can then be tested for protease activity by the addition of a caspase-specific peptide that is conjugated to the color reporter molecule p-nitroaniline (pNA). The cleavage of the peptide by the caspase releases the chromophore pNA, which can be quantitated spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the color reaction.

bio-techne.com
info@bio-techne.com
techsupport@bio-techne.com
North America TEL 800 343 7475
Europe • Middle East • Africa TEL +44 (0)1235 529449
China info.cn@bio-techne.com TEL +86 (21) 52380373
Rest of World bio-techne.com/find-us/distributors TEL +1 612 379 2956

SAMPLE PREPARATION

Aliquot enough Reaction Buffer 9 for the number of assays to be performed. Add DTT to Reaction Buffer 9 immediately before use. For a 10 mM final concentration, add 10 µL of 1 M DTT stock per 1 mL of Reaction Buffer 9.

1. Cells that have been induced to undergo apoptosis are collected by centrifugation in a conical tube at 250 x g for 10 minutes (**Note:** Counting the cells before pelleting them is recommended). The supernate is gently removed and discarded while the cell pellet is lysed by the addition of the Lysis Buffer. The amount of Lysis Buffer to be added to the pellet is determined by the number of cells present (this can be estimated from the number of cells initially cultured). Add 25 µL of cold Lysis Buffer per 1 x 10^6 cells.
2. The cell lysate is incubated on ice for 10 minutes and then centrifuged at 10,000 x g for 1 minute. Transfer the supernate to a new tube and keep on ice. This should yield a cell lysate with an approximate protein concentration of 2-4 mg/mL. The protein content of the cell lysate can be estimated using a protein determination assay that is compatible with detergents present in the Lysis Buffer (*e.g.* BCA Protein Assay, Pierce Chemical Co., Catalog # 23225).
3. The enzymatic reaction for caspase activity is best carried out in a 96 well flat bottom microplate that can be read with a microplate reader.
4. Each reaction requires 50 µL of cell lysate (i.e. derived from 2 x 10^6 or 100-200 µg of total protein). If larger volumes of cell lysate are necessary to meet the above requirements, the total reaction volume may be scaled up (*i.e.* the volume of each reagent added should be proportionally increased).
5. Each reaction also requires 50 µL of 2X Reaction Buffer 9. Prior to using the 2X Reaction Buffer 9, add 10 µL of fresh DTT stock per 1 mL of 2X Reaction Buffer 9.
6. To each reaction well add 5 µL of caspase-9 colorimetric substrate (LEHD-pNA).
7. Incubate the plate at 37 °C for 1-2 hours.
8. Read the plate on a microplate reader using a wavelength of 405 nm. If readings are not in the linear range of the instrument, repeat the assay by diluting the cell lysates with the Dilution Buffer.
9. Additional controls that should be included in this assay are a) no cell lysate and b) no substrate. The total reaction volume must be kept constant and therefore distilled water can be used to replace the volume normally occupied by either the cell lysate or the substrate reagent.
10. For comparative analysis, the above assay should be repeated with non-induced cells.

This protocol may require modification, depending upon final utilization.

The results are best expressed as fold increase in caspase activity of apoptotic cells over that of non-induced cells. If the background controls (reactions where no cell lysate is added or where no LEHD-pNA substrate is added) give a substantial reading, it is recommended that these values be subtracted from the experimental results prior to calculating the fold increase. Please note that other proteases may show some affinity for caspase-9 substrate.

REFERENCES

1. Kerr, J.F.R. *et al.* (1972) Br. J. Cancer. **26**:239.
2. Ameisen, J.C. *et al.* (1995) Trends Cell Biol. **5**:27.
3. Thompson, C.B. (1995) Science **267**:1456.
4. Vermes, I. *et al.* (1995) J. Immunol. Meth. **184**:39.
5. Darzynkiewicz, Z. *et al.* (1992) Cytometry **13**:795.
6. Duan, H. *et al.* (1996) J. Biol. Chem. **271**:16720.
7. Srinivasula, S.M. *et al.* (1996) J. Biol. Chem. **271**:27099.
8. Li, P. *et al.* (1997) Cell **91**:479.
9. Cardone, M.H. *et al.* (1998) Science **282**:1318.
10. Thornberry, N.A. *et al.* (1997) J. Biol. Chem. **272**:17907.