

## Reagents Provided

**LEHD-pNA Substrate:** 500  $\mu$ L of 4 mM LEHD substrate peptide conjugated to p-nitroaniline.

**Cell Lysis Buffer:** 100 mL of Cell Lysis Buffer (narrow-mouth bottle)

**Reaction Buffer:** 4 x 2.0 mL vials of 2X Reaction Buffer

**DTT:** 400  $\mu$ L of a 1 M solution of dithiothreitol

**Dilution Buffer:** 100 mL of Dilution Buffer (wide-mouth bottle)

Upon arrival, store the entire kit at  $-20^{\circ}\text{C}$ . After the first use, only the substrate and DTT solution need to be returned to  $-20^{\circ}\text{C}$  storage. The remaining reagents (buffers) may be stored at  $4^{\circ}\text{C}$ .

## References

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3. Thompson, C.B. (1995) *Science* **267**:1456.
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6. Duan, H. *et al.* (1996) *J. Biol. Chem.* **271**:16720.
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## Intended Use

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 mitochondrial 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 suggest that caspase-9 is a key regulator of apoptosis *in vivo*.

## Principle of the 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-nitroanilide. The cleavage of the peptide by the caspase releases the chromophore p-NA, 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.

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NOT FOR USE IN DIAGNOSTIC OR THERAPEUTIC PROCEDURES.

## ***Sample Preparation***

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: we recommend counting the cells before pelleting them). The supernatant is gently removed and discarded while the cell pellet is lysed by the addition of the Cell Lysis Buffer. The amount of Cell 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  $\mu\text{L}$  of cold Cell Lysis Buffer per  $1 \times 10^6$  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 supernatant 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 Cell 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  $\mu\text{L}$  of cell lysate (*i.e.* derived from  $2 \times 10^6$  or 100 - 200  $\mu\text{g}$  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  $\mu\text{L}$  of 2X Reaction Buffer. Prior to using the 2X Reaction Buffer, add 10  $\mu\text{L}$  of fresh DTT stock per 1 mL of 2X Reaction Buffer.
  6. To each reaction well add 5  $\mu\text{L}$  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 405 nm wavelength light. 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.
- 1) 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.