

REAGENTS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at $\leq -20^{\circ}\text{C}$ in a manual defrost freezer.

REAGENT	DESCRIPTION	STORAGE OF OPENED MATERIAL
IETD-AFC Substrate	500 μL of 1 mM IETD substrate peptide conjugated to 7-amino-4-trifluoromethyl coumarin (AFC) (protect from light).	Store at $\leq -20^{\circ}\text{C}$ 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 $2-8^{\circ}\text{C}$.
2X Reaction Buffer 8	4 vials (2.0 mL/vial) of 2X Reaction Buffer 8.	

INTENDED USE

The purpose of this kit is to determine the increased enzymatic activity of the Caspase-8 class of proteases in apoptotic cells by fluorometric 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-8, also known as FLICE, MACH, and Mch5 (6), is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the cascade of events associated with apoptosis. Eight different isoforms of Caspase-8 have been described, each with different functions (7-9). Only the proteins of isoforms a and b have been detected intracellularly (10). Caspase 8, a CrmA sensitive caspase, is considered an upstream caspase. Activation of Caspase-8 releases two active subunits, p18 and p10, into the cytosol which activate additional caspases that cleave other apoptosis-related substrates such as poly ADP-ribose polymerase (PARP), the 70 kD protein of the U1-ribonucleoprotein and a subunit of the DNA dependent protein kinase (11).

PRINCIPLE OF TEST

Cells that are suspected 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 fluorescent reporter molecule 7-amino-4-trifluoromethyl coumarin (AFC). The cleavage of the peptide by the caspase releases the fluorochrome that, when excited by light at 400 nm wavelength, emits fluorescence at 505 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the fluorescence signal detected with a fluorimeter or a fluorescent microplate reader.

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:** *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 μ L of cold Lysis Buffer per 1×10^6 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 equipped with fluorescence detection capabilities.
4. Each reaction requires 50 μ L of cell lysate (i.e. derived from 2×10^6 cells or 100-200 μ 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 μ L of 2X Reaction Buffer 8. Prior to using the 2X Reaction Buffer 8, add 10 μ L of fresh DTT stock per 1 mL of 2X Reaction Buffer 8.
6. To each reaction well, add 5 μ L of Caspase-8 fluorogenic substrate (IETD-AFC).
7. Incubate the plate at 37 °C for 1-2 hours.
8. Read the plate on a fluorescent microplate reader using filters that allow light excitation at a wavelength of 400 nm and can collect emitted light at a wavelength of 505 nm.
9. Additional controls that should be included in this assay are a) no cell lysate and b) no substrate. A recombinant Caspase-8 enzyme is available for use as a positive control or standard (R&D Systems, Catalog # 705-C8). 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 IETD-AFC 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-8 substrate.

REFERENCES

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