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A Geno Technology, Inc. (USA) brand name

CasPASE™ Apoptosis Fluorometic Assay

(Cat. # 786-200A to 786-206A; 786-200B to 786-206B)

INTRODUCTION

CasPASE™ Apoptosis Fluorometric Assay provides a simple method for assaying the activities of various caspases (proteases) (Caspase 1-10 & 13), a key early indicator of apoptosis in mammalian cells. The assay is based on the detection of cleavage of a synthetic substrate, which has 7-amino-4-trifluromethyl coumarin (AFC) at the C-terminal. When liberated from the peptide, AFC produces an optical change that can be detected as the fluorescence at 500-550nm with the use of a fluorometer. The reaction is selectively and irreversibly inhibited by the peptide Z-VAD-FMK (fluoromethyl ketone). Comparison of the fluorescence from an iapoptotic sample with a control sample allows one to determine the fold-increase in the caspase activity. The activity of the caspases can also be quantified by using a standard curve established with the free dye (AFC).

ITEM(S) SUPPLIED

Description	**50 Assay**	**100 Assay**
CasPASE™ Lysis Buffer	15ml	2 x 15ml
2X CasPASE™ Assay Buffer	5ml	5ml
DTT [1M, 100µl]	1 vial	1 vial
Substrate Solution [1mM][γ]	0.25ml	2 x 0.25ml
Free Dye (AFC), [8mM]	0.2ml	0.2ml
Inhibitor (Z-VAD-FMK) [1mM]	0.1ml	2 x 0.1ml

[γ] The different substrate solutions supplied with individual kits are as follows:

Cat. #	**Description** Substrate supplied	**Size**
786-200A 786-200B	**CasPASE™-1, 4, 5 Assay** Ac-WEHD-AFC substrate	50 Assays 100 Assays
786-201A 786-201B	**CasPASE™-2 Assay** Ac-VDVAD-AFC substrate	50 Assays / 100 Assays
786-202A 786-202B	**CasPASE™-3, 7,10 Assay** Ac-DEVD-AFC substrate	50 Assays / 100 Assays
786-203A 786-203B	**CasPASE™-6 Assay** Ac-VEID-AFC substrate	50 Assays / 100 Assays
786-204A 786-204B	**CasPASE™-8 Assay** Ac-LETD-AFC substrate	50 Assays / 100 Assays
786-205A 786-205B	**CasPASE™-9 Assay** Ac-LEHD-AFC substrate	50 Assays / 100 Assays
786-206A 786-206B	**CasPASE™-13 Assay** Ac-LEED-AFC substrate	50 Assays / 100 Assays

STORAGE CONDITION

The kit is shipped in blue ice. Upon arrival, store all the reagents at -20°C. When used properly, the substrates are stable for 6 months and other components for up to 1 year.

ADDITIONAL ITEMS NEEDED

- Centrifuge
- 96-well plates or Reaction Tube

PREPARATION BEFORE USE

Preparation of Kit Reagents

1. Allow the reagents to thaw into liquid form. Centrifuge the substrate, free dye and the inhibitor vials to collect the reagent solution at the bottom of the vial. Protect from light and humidity. Allow the reagents to reach RT before opening the vial.
2. Dissolve the supplied DTT in 90µl DI water (final volume 100µl) to give 1M concentration. Store at -20°C.

Preparation of Cell Lysate

The following procedure is provided only as a suggestion.

1. Culture 10^7 cells under the appropriate conditions. Suspend cells in PBS or serum-free medium. For the attached cells, remove the cells from culture plate and suspend in PBS or serum-free medium. Pellet cells by centrifugation at 600xg for 5-6 minutes. Remove the supernatant cells and re-suspend the cells in PBS. If necessary, make cell counts. Re-pellet cells as before, remove and discard the supernatant. Lyse the cells by adding an appropriate volume of chilled Lysis Buffer - e.g., 100µl Lysis each 1-5x 10^6 cells. Vortex gently to suspend cells.
2. Lyse the cells by freezing and thawing, 4-5 times. Do not vortex between freezes and thaws. [Alternatively, after adding the Lysis Buffer, lyse the cells by passing the cell suspension 10-15 times through a 21gauge needle].

Preparation of Tissue Lysate

1. Homogenize 3-5mg tissue in 100µl Lysis Buffer.
2. Centrifuge the lysate for 30 minutes at full speed in a microfuge at 4°C. Collect the supernatant for the assay.

Preparation of CasPASE™ Assay Buffer

Immediately before use, transfer an appropriate volume of 2X CasPASE™ Assay Buffer in a tube. Add the DTT (1M) solution to the CasPASE™ Assay Buffer to achieve 5-10mM final concentration (e.g. Add 5-10µl of 1.0M DTT per 1ml of 2X CasPASE™ Assay Buffer). DO NOT ADD THE DTT TO THE STOCK SOLUTION.

Assay Controls

Prepare a negative control reaction with cells not treated with the apoptosis-inducing stimulus.

ASSAY PROTOCOL

First read the section "Preparation Before Use". The assay may be performed in a 96 well microplate or cuvette, using a fluorometer.

Set up the assay in duplicate and arrange the appropriate blanks and controls, such as a non-apoptotic cell lysate (negative control). A blank should be prepared to measure the substrate background and instrument drift.

1. Transfer 50µl of 2X CasPASE™ Assay Buffer (**containing DTT**) into each tube.
2. Add 50µl of cell lysate into each tube.

   NOTE: *For each assay, use lysate (50µl) obtained from at least 1x10^6 cells for fluorescence measurement. The use of fewer cells than this may reduce the observed increase in the caspase activity.*
3. Add 5µl of 1mM AFC-conjugated Substrate (50µM final concentration).
4. Mix the content of the tube and take a reading at zero time point (t = 0).
5. Close the assay tubes and incubate at 20-37°C.
6. Measure the reaction every 30-60 minutes or until the measurements are significantly different from those at t=0.

Component	Blank	Test Sample	Negative Control
2X Assay Buffer	100µl	50µl	50µl
Test Sample/Lysate	---	50 µl	---
AFC-Substrate	5µl	5µl	5µl
Negative Control/Lysate	---	---	50µl

Inhibition of Caspase Activity (Optional)

In order to establish non specific protease activity, a control should be run with or without the caspase specific inhibitor (Z-VAD-FMK) supplied with the kit.

1. Reaction tubes should be prepared as described above (e.g., 50µl lysate and 50µl of 2X Assay buffer).
2. Add 1 µl of the Inhibitor (10µM final conc.), mix and incubate the reaction at 20-37°C for 30 minutes to complete the inhibition.
3. Add 5µl of 1mM conjugated substrate (50µM final concentration).
4. Mix the content of the tube and take a reading at zero time point (t = 0).
5. Incubate the assay tubes at 20-37°C.
6. Measure the reaction every 30-60 minutes until the sample measurements are complete.

Component	Blank	Test Sample
2X Assay Buffer	100µl	50µl
Test Sample/Lysate	---	50µl
Inhibitor	---	0.5-1µl

FLUORESCENT DETECTION OF THE CASPASE ACTIVITY

Read sample in a fluorometer setting at 360-390nm EXCITATION and 510-550nm EMISSION filter. Zero the detection scale using 1x Assay Buffer. Insert the most concentrated solution (4µM AFC) and adjust the setting, e.g. adjust the gain to obtain a signal near the maximum scale. If the blank signal is more than 50% of the full-scale signal, the substrate may have degraded.

Comparison of the fluorescence from an induced/apoptotic sample with an uninduced or inhibited control allows one to determine the fold-increase in protease activity.

CASPASE ACTIVITY CALCULATION

1. Generate a dye (AFC) calibration curve and determine the slope.
2. Dilute AFC solution in CasPASE™ Lysis Buffer and prepare 0, 10, 20, 40, and 80µM stock solutions.
3. Mix 190µl of CasPASE™ Lysis Buffer with each 10µl of stock solutions, as follows:

   10µl of 0µM AFC + 190µl of Lysis Buffer = 0.0nmole AFC
   10µl of 10µM AFC + 190µl of Lysis Buffer = 0.1nmole AFC (0.5µM)
   10µl of 20µM AFC + 190µl of Lysis Buffer = 0.2nmole AFC (1µM)
   10µl of 40µM AFC + 190µl of Lysis Buffer = 0.4nmole AFC (2µM)
   10µl of 80µM AFC + 190µl of Lysis Buffer = 0.8nmole AFC (4µM)
4. Plot nmole AFC (x-axis) Vs (FU) fluorescence unit (y-axis), and determine the slope i.e., FU/nmole AFC.
5. Calculate the rate of increase in fluorescence for each sample as follows:

   ΔF / minute = $[\Delta F_{sample} - \Delta F_{blank}]$ / minute
   (i.e., change in fluorescence over the length of reaction time minus the change in the fluorescence over the same length of reaction for the blank).
6. Calculate unit of caspase activity using the following formula:

   Units caspase = ΔF / minute x (calibration curve slope) $^{-1}$

Example

Slope of calibration plot = 2500 (i.e., per nmole of AFC yields 2500 fluorescence units)
Sample: in 60 minutes fluorescence change from 0 to 800 FU, i.e., rate of fluorescence increase = 800/60.
Units Caspase in sample = (800/60) x (1/2500) = 0.00533 nmole/minute.

CITATIONS

1. Lee, R.X. et al (2012) Anticancer Res. 32:3103
2. Soong, G. et al (2012) J. Infect. Dis. 205:1571
3. Zhang, A. et al (2006) Am. J. Physiol. Renal. Physiol. 291:F1332

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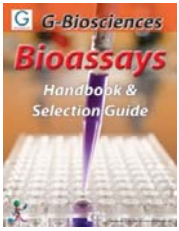

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