ChromoFluor96
For fluorescent detection of caspases

Product Number **CF96-101**
Storage Temperature –20 °C

ProductInformation

TECHNICAL BULLETIN

Product Description

Each 96-well plate contains 3 fluorescent caspase substrates, allowing for quick detection of caspase-3, -8 and -9 in experimental samples.

The assay uses dilute enzyme samples (in 190 µl) that are added to the 10 µl of fluorescent substrate already impregnated in the plate wells (see instructions under "samples & blanks"). The fluorimeter is adjusted and set to read for up to 3 hours at 5 minute intervals.

The 7-amino-4-trifluoromethylcoumarin (AFC) detecting group is a fluorogenic synthetic compound. The AFC-labeled substrate is hydrolyzed by the enzyme at a rapid rate and liberates a product at a concentration that is easily measured using a fluorimeter.

Components

Each 96-well plate includes:

- Two rows (A&B) impregnated with 10 µl of 10 mM N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin (Ac-DEVD-AFC).
- Two rows (D&E) impregnated with 10 µl of 10 mM N-Acetyl-Ile-Glu-Thr-Asp-7-amido-4-trifluoromethylcoumarin (Ac-IETD-AFC).
- Two rows (G&H) impregnated with 10 µl of 10 mM N-Acetyl-Leu-Glu-His-Asp-7-amido-4-trifluoromethylcoumarin (Ac-LEHD-AFC).
- Two rows (C&F) impregnated with 10 µl of a serial dilution of 1 mM 7-amido-4-trifluoromethylcoumarin standard (AFC).

Preparation Instructions

Reagents necessary, but not supplied

- Dimethyl sulfoxide (DMSO) Product Code D5879
- PIPES free acid (1,4-piperazinediethanesulfonic acid) Product Code P1851
- HEPES free acid [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)] Product Code H4034
- EDTA free acid (ethylenediaminetetraacetic acid) Product Code EDS
- CHAPS (3-((3-cholamidopropyl)dimethylammonio)-1-propane sulfonate) Product Code C5849
- DTT (dithiothreitol) Product Code D0632
- Protease Inhibitor Cocktail For use with mammalian cell and tissue extracts. Product Code P8340
- Caspase Inhibitors (see Related Products)
- Phosphate buffered saline, pH 7.4. Product Code P3813
- Centrifuge
- 15 ml centrifuge tubes
- Microfuge
- Microfuge tubes
- Fluorimeter/fluorescent plate reader

Solution Preparation

1000 X Inhibitor (A-peptide-FMK) Stock Solutions
Add 1.0 ml of DMSO to an l mg vial of inhibitor, molar concentration = 1/MW. Thoroughly dissolve the contents of the vial. Store DMSO stock solutions at –20 °C.

25 X Reaction Buffer, [250 mM PIPES, pH7.4, 50 mM EDTA, 2.5% CHAPS, 125 mM DTT]
Dissolve the following components in filtered, deionized water and adjust the pH 7.4 and the volume to 100 ml.
PIPES - 7.55 G
EDTA - 1.46 G
CHAPS - 2.5 G
DTT - 1.93 G
Keep the solution on ice while preparing experiments. Store solution at –20 °C.

Cell Lysis Buffer [10 mM HEPES, pH 7.4,2 mM EDTA, 0.1% CHAPS, 5mM DTT, containing Protease Inhibitors]
Dissolve the following components in filtered, deionized water and adjust the pH 7.4 and the volume to 10 ml.
HEPES - 23.8 mg
EDTA - 5.8 mg

CHAPS - 10 mg
DTT - 7.7 mg
Protease Inhibitor Cocktail, Product Code P8340 – 100 µl
Keep the solution on ice while preparing experiments. Store solution at –20 °C.

Prepare cell lysates from suspension cultures

1. Cell lysates may be prepared in advance and stored frozen at –70 °C until use.
2. Obtain the desired cells number in culture.
3. Transfer cells into a 15 ml centrifuge tubes and centrifuge and pellet at 400 x g.
4. Decant the culture supernatant and resuspend the cells at 10^6 cells/ml in phosphate buffered saline.
5. Transfer the cell suspension in 1 ml aliquots into microcentrifuge tubes and centrifuge for one minute at full speed.
6. Decant the supernatant and disperse the cell pellets by agitating the bottom of the tubes.
7. Add 100 µl of lysis buffer to each pellet and vortex gently.
8. Freeze and thaw the sample three or four times by transferring from an isopropanol-dry ice bath to a 37 °C water bath; do not vortex between freeze thaw steps.
9. Centrifuge the lysed cells at 4 °C for 30 minutes at full speed.
10. Transfer the supernatant to a clean tube and keep on ice if the assay is to be performed within the hour, otherwise, store the cell extracts at –70 °C and minimize freeze-thaw cycles.

Preparation of cell lysates from adherent cultures

1. Cell lysates may be prepared in advance and stored frozen at –70 °C until use.
2. Adherent cells can be grown in 6-well plates and lysates from duplicate wells may be combined at the end of this section.
   Note: Adherent cultures undergoing apoptosis may release into the media. These floating cells should be recovered for the assay.
3. Transfer culture media from each well into an appropriate centrifuge tube. Cover the adherent layer in the culture dish with phosphate buffered saline (PBS). Pellet the culture supernatant, which contains the floating cells, for 4 minutes at 400 x g. Decant the supernatant, resuspend in about 1 ml of PBS.
4. Transfer the suspension into a microcentrifuge tube and pellet at full speed. Discard PBS and resuspend the cell pellet in 100 µl lysis buffer.
5. Aspirate the PBS from the adherent cell monolayer in the culture dish.
6. Transfer the lysis solution from the microcentrifuge tube back to its corresponding culture well.
7. Rock the plate to coat the entire surface and release the cells using a rubber policeman.
8. Transfer the cells and lysis solution back to the microcentrifuge tube.
9. Freeze and thaw the sample three or four times by transferring from an isopropanol-dry ice bath to a 37 °C water bath; do not vortex between freeze thaw steps.
10. Centrifuge the lysed cells at 4 °C for 30 minutes at full speed.
11. Transfer the supernatant to a clean tube and keep on ice if the assay is to be performed within the hour, otherwise, store the cell extracts at –70 °C and minimize freeze-thaw cycles.

Samples and Blanks

Using the following guide, mix the reaction components to prepare a Blank and the Samples in bulk in separate containers. Alternatively, the addition of components can be made directly in the ChromoFluor96 well plate. The final total volume added to each sample or blank well will be 190 µl. Substrates are already in the plate as 10µl of a 10 mM solution in DMSO. The total reaction volume in the plate will be 200 µl.

	Component	**Volume**
Blank	Water	182 µl
	25X Reaction Buffer	8 µl
Sample	Water	182 µl – L
	25X Reaction Buffer	8 µl
	Cell lysate*	L

*The cell lysate volume (L) is dependent on the caspase activity in the rest cell culture and determines the volume of water to be added. The minimum volume of lysate required to detect baseline caspase activity may be determined by preparing a series of samples with increasing amounts of lysate. Enough lysate should be used to detect a measurable activity within 3 hours after mixing the components.

Positive Controls

Positive control samples can be made by using recombinant enzyme or lysates from cells induced to undergo apoptosis by a variety of methods.

Inhibition

Caspase activity can be blocked using peptide-FMK inhibitors added directly to live cells. Dilute the inhibitor stock solution (20 mM in DMSO) at least 1,000-fold into the cell culture media, e.g., add 5 µl of inhibitor stock to 5 ml cell media and incubate at least 15-30 minutes prior to preparing lysates. Alternatively, dilute the inhibitor stock at least 100-fold into samples containing crude cell extracts or purified enzyme and incubate for 15 minutes prior to adding to the plate. Nonspecific inhibition increases with inhibitor concentration; specific inhibition may require further dilution.

Storage/Stability

Store at –20 °C for up to eight months. Storage in "frost-free" freezers is not recommended.

If plate is not completely used, it can be returned to the freezer for future use. An additional plate sealer is supplied for this purpose.

Procedure

1. Under low light, equilibrate plate to room temperature before use.
2. Prepare the fluorescence detector according to the manufacturers directions. Set the fluorimeter to the setting of Excitation 390-400 nm and Emission 510-540 nm. Set the instrument gain so the signal is about 20% full scale using the "Blank". For example, if the instruments maximum signal = 10,000 units, set the gain so the signal reads for 2000 for the blank. If subsequent sample measurements indicate very low activity, increasing the instrument gain should improve the sensitivity.
3. Add 190 µl of Sample, Positive Control or Blank prepared in bulk in the previous section to the appropriate wells. Alternatively, add individual components to wells according to the volumes given in the guide.
4. For the AFC standard curve, add 190 µl of blank buffer to row C appropriate wells. When the plate is used a second time, add the blank buffer to row F.
5. Zero the instrument using a buffer blank without substrate. These are row C or F, wells 11 and 12.
6. Record the time and signal for the blank and enzyme samples immediately following mixing of the reaction components in the plate. Read the standard curve and use this first reading for the curve. Repeat measurements every 5 minutes until the enzyme sample signal nears the instrument's maximum or until 3 hours have elapsed. Alternatively, because the reaction will vary with temperature, incubate the plate and cell lysate for 15-30 minutes before starting to read. You may use this time to read the standard curve, since it is not affected by temperature. Keep the sample temperature constant, e.g. 37°C between readings and use the same temperature for samples that will be compared.

Results

Compare the fluorescence or absorbance rate of change (R) to obtain measure of relative activity for different samples measured under equivalent conditions. To determine R, plot Δs versus Δt and calculate the slope (Δs/Δt).

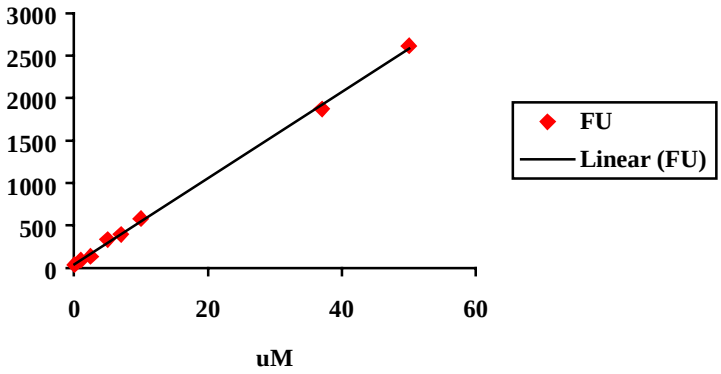
$\Delta s = [S(t_1)-B(t_1)]- S(t_0)]$, $\Delta t= (t_1-t_0)$, R= slope of ΔS versus Δt

Δs = the fluorescence or absorbance signal change over the time interval Δt,
t_1= time at measurement,
t_0= time at mixing of components,
S= sample signal, and
B=blank signal.

The blank measurement is required to remove signal caused by baseline substrate hydrolysis and instrument drift.
Note: If the activity is very high R may begin to decrease at later time points. In this case use R from data corresponding to the early (linear) part of the reaction.

To plot the AFC standard curve, plot the standard curve from the serially diluted AFC in rows C & F. Plot the Fluorescence intensity verus concentration. The concentration of AFC in each well is recorded in the table below. You will get a linear standard curve. Plot the fluorescence generated from the measurement of the reaction cells and interpolate the concentration of free AFC generated from the reaction.

Well Number of Rows C & F	**AFC conc./200 µl**
1	25 µM
2	12.5 µM
3	6.25 µM
4	3.12 µM
5	1.56 µM
6	0.781 µM
7	0.390 µM
8	0.195 µM
9	0.0976 µM
10	0.0488 µM
11	0 µM
12	0 µM



Related Products

C0605 N-CBZ-Asp(OMe)-Glu(OMe)-Val-Asp(OMe) fluoromethyl ketone (Z-DEVD-FMK)

C1230 N-CBZ-Ile-Glu(OMe)-Thr-Asp(OMe) fluoromethyl ketone (Z-IETD-FMK)

C1355 N-CBZ-Leu-Glu(OMe)-His-Asp(OMe) fluoromethyl ketone (Z-LEHD-FMK)

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