ELISA PRODUCT INFORMATION & MANUAL

Caspase-9 Assay Kit (Fluorometric)

NBP2-54865

Enzyme-linked Immunosorbent Assay for quantitative detection. For research use only.
Not for diagnostic or therapeutic procedures.

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1. Overview

Activation of caspases plays a central role in apoptosis. Novus Biologicals's Caspase-9 Assay Kit (Fluorometric) provides a convenient means for sensitive detection of activated caspase-9 in living cells. The assay utilizes the caspase-9 inhibitor LEHD-FMK conjugated to FITC (FITC-LEHD-FMK) as a fluorescent marker. FITC-LEHD-FMK is cell permeable, nontoxic, and irreversibly binds to activated Caspase-9 in apoptotic cells.

2. Protocol Summary

Induce Apoptosis in Cell Samples

Add FITC-LEHD-FMK

Analyze by Flow Cytometry

OR

Detect by Fluorescence Microscopy

OR

Measure Fluorescence in Microplate Reader

3. Components and Storage

A. Kit Components

Item	Quantity
FITC-LEHD-FMK	100 µL
Wash Buffer	2 x 100 mL
Z-VAD-FMK	10 µL

* Store kit at -20°C.

B. Additional Materials Required

- Microcentrifuge
- Pipettes and pipette tips
- Fluorescent microplate reader or microscope
- Black microtiter plate
- Orbital shaker

4. Assay Protocol

1. Sample Preparation:

a) Induce apoptosis in cells (1 x 10^6/ml) by desired method. Concurrently incubate a control culture *without* induction.

An additional **negative control** can be prepared by adding the caspase inhibitor Z-VAD-FMK at 1 µl/ml to an induced culture to inhibit Caspase-9 activation.

Note:

This product detects proteolytic activity. Do not use protease inhibitors in the sample preparation step as it might interfere with the assay.

b) Aliquot 300 µl each of the induced and control cultures into eppendorf tubes.

c) Add 1 µl of FITC-LEHD-FMK into each tube and incubate for 0.5-1 hour at 37°C incubator with 5% CO_2.

d) Centrifuge cells at 3000 rpm for 5 minutes and remove supernatant.

e) Re-suspend cells in 0.5 ml of Wash Buffer, and centrifuge again.

f) Repeat Step e.

g) Proceed to Step 2, 3 or 4 depending on methods of analysis.

2. Quantification by Flow Cytometry:

For flow cytometric analysis, re-suspend cells in 300 µl of Wash buffer. Keep samples on ice. Analyze samples by flow cytometry using the FL-1 channel.

3. Detection by Fluorescence Microscopy:

For fluorescence microscopic analysis, re-suspend cells in 100 µl Wash buffer. Put one drop of the cell suspension onto a microslide and cover with a coverslip.

Observe cells under a fluorescence microscope using FITC filter. Caspase positive cells appear to have brighter green signals, whereas caspase negative control cells show much weaker signal.

4. Analysis by Fluorescence Plate Reader:

For analysis with fluorescence plate reader, re-suspend cells in 100 µl Wash Buffer and then transfer the cell suspension to each well in the black microtiter plate.

Measure the fluorescence intensity at Ex. = 485 nm and Em = 535 nm. For control, use wells containing unlabeled cells.

5. Factors to consider when assaying caspases

Three major factors need to be taken into account when using caspase activity assays:

1. The substrate in a particular assay is not necessarily specific to a particular caspase.

   Cleavage specificities overlap so reliance on a single substrate/assay is not recommended. Other assays, such as Western blot or use of fluorescent substrates e.g. FRET assays should be used in combination with caspase activity assays.

2. The expression and abundance of each caspase in a particular cell type and cell line will vary.

3. As the activation and cleavage of caspases in the cascade will change over time, you should consider when particular caspase will be at its peak concentration e.g. after 3 hours, after 20 hours etc.

The table below shows the known cross-reactivities with other caspases.

Classification of caspases based on synthetic substrate preference, does not reflect the real caspase substrate preference *in vivo* and may provide inaccurate information for discriminating amongst caspase activities. Thus, caution is advised in applying the intrinsic

tetrapeptide preferences to predict the targets of individual caspases.

Apoptotic Initiator Caspases

Caspase	**Cleavage motif**	**Inhibitor motif**	**Cross-reactivity with other caspase:**									
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Caspase 2	VDVAD				Y				Y			
Caspase 8	IETD	IETD, LETD			Y			Y				Y
Caspase -9	LEHD				Y			Y		Y		Y
Caspase 10	AEVD				Y				Y	Y?		

6. Troubleshooting

Problem	Reason	Solution
High Background	Cell density is higher than recommended	Refer to datasheet and use the suggested cell number
	Increased volumes of components added	Use calibrated pipettes accurately
	Incubation of cell samples for extended periods	Refer to datasheets and incubate for exact times
	Use of extremely confluent cells	Perform assay when cells are at 80-95% confluency
	Contaminated cells	Check for bacteria/ yeast/ mycoplasma contamination
Lower signal levels	Cells did not initiate apoptosis	Determine the time-point for initiation of apoptosis after induction (time-course experiment)
	Very few cells used for analysis	Refer to data sheet for appropriate cell number
	Incorrect setting of the equipment used to read samples	Refer to datasheet and use the recommended filter setting
	Use of expired kit or improperly stored reagents	Always check the expiry date and store the components appropriately

Problem	Reason	Solution
Erratic results	Uneven number of cells seeded in the wells	Seed only healthy cells (correct passage number)
	Adherent cells dislodged at the time of experiment	Perform experiment gently and in duplicates or triplicates for each treatment
	Incorrect incubation times or temperatures	Refer to datasheet & verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly