ELISA PRODUCT INFORMATION & MANUAL

Caspase-9 Assay Kit (Fluorometric)

NBP2-54822

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

www.novusbio.com - P: 303.730.1950 - P: 888.506.6887 - F: 303.730.1966 - technical@novusbio.com

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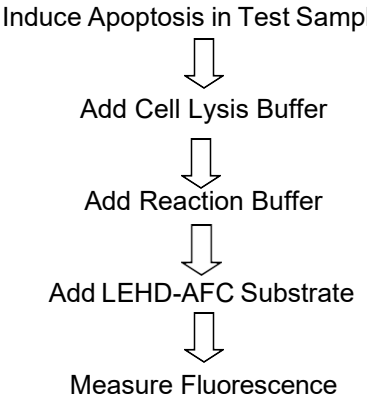
1. Overview

Activation of ICE-family proteases/Caspases initiates apoptosis in mammalian cells. Novus Biologicals's Caspase-9 Assay Kit (Fluorometric) provides a simple and convenient means for assaying the activity of Caspases that recognize the sequence LEHD.

The assay is based on detection of cleavage of substrate LEHD-AFC (AFC: 7-amino-4-trifluoromethyl coumarin). LEHD-AFC emits blue light (λ_{max} = 400 nm); upon cleavage of the substrate by Caspase-9 or related Caspases, free AFC emits a yellow-green fluorescence (λ_{max} = 505 nm), which can be quantified using a fluorometer or fluorescence microtiter plate reader.

2. Protocol Summary



3. Components and Storage

A. Kit Components

Item	Quantity
Cell Lysis Buffer	100 mL
2X Reaction Buffer	4 x 2 mL
LEHD-AFC (1 mM)	500 µL
DTT (1 M)	400 µL

* Store kit at -20°C.

- Protect LEHD-AFC from light.
- Store Cell Lysis Buffer, 2X Reaction Buffer at +4°C after opening.

Additional Materials Required

- Microcentrifuge
- Pipettes and pipette tips
- Fluorometric microplate reader or Fluorometer
- 96-well plate
- Orbital shaker

4. Assay Protocol

1. Induce apoptosis in cells by desired method. Concurrently incubate a control culture *without* induction.
Note: This product detects proteolytic activity. Do not use protease inhibitors in the sample preparation step as it might interfere with the assay.

2. Count cells and pellet 1-5 x 10^6 cells or use 50-200 µg cell lysates if protein concentration has been measured.

3. Re-suspend cells in 50 µl of chilled Cell Lysis Buffer. Incubate cells on ice for 10 minutes.

4. Aliquot enough 2X Reaction Buffer for the number of assays to be performed. Add DTT to the 2X Reaction Buffer immediately before use (10 mM final concentration: add 10 µl of 1.0 M DTT stock per 1 ml of 2X Reaction Buffer).
Add 50 µl of 2X Reaction Buffer (containing 10 mM DTT) to each sample. Add 5 µl of the 1 mM LEHD-AFC substrate (50 µM final concentration) and incubate at 37°C for 1-2 hour.

5. Read samples in a fluorometer equipped with a 400 nm excitation filter and 505 nm emission filter. You may also perform the entire assay directly in a 96 well plate. Fold-increase in Caspase-9 activity can be determined by comparing these results with the level of the un-induced control.

5. Factors to consider for caspase activity assays

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 show 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
Assay not working	Cells did not lyse completely	Re-suspend the cell pellet in the lysis buffer and incubate as described in the datasheet
	Experiment was not performed at optimal time after apoptosis induction	Perform a time-course induction experiment for apoptosis
	Plate read at incorrect wavelength	Ensure you are using appropriate reader and filter settings (refer to datasheet)
	Old DTT used	Always use freshly thawed DTT in the cell lysis buffer
High Background	Increased amount of cell lysate used	Refer to datasheet and use the suggested cell number to prepare lysates
	Increased amounts of components added due to incorrect pipetting	Use calibrated pipettes
	Incubation of cell samples for extended periods	Refer to datasheet and incubate for exact times
	Use of expired kit or improperly stored reagents	Always check the expiry date and store the individual components appropriately
	Contaminated cells	Check for bacteria/ yeast/ mycoplasma contamination

Problem	Reason	Solution
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 datasheet for appropriate cell number
	Use of samples stored for a long time	Use fresh samples or aliquot and store and use within one month for the assay
	Incorrect setting of the equipment used to read samples	Refer to datasheet and use the recommended filter setting
	Allowing the reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
Unexpected results	Measured at incorrect wavelength	Check the equipment and the filter setting
	Cell samples contain interfering substances	Troubleshoot if it interferes with the kit (run proper controls)
General Issues	Improperly thawed components	Thaw all components completely and mix gently before use
	Incorrect incubation times or temperatures	Refer to datasheet & verify the correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
	Air bubbles formed in the well/tube	Pipette gently against the wall of the well/tubes
	Substituting reagents from older kits/ lots	Use fresh components from the same kit
	Use of a different 96-well plate	Fluorescence: Black plates; Absorbance: Clear plates

Samples with erratic readings	Uneven number of cells seeded in the wells	Seed only equal number of healthy cells (correct passage number)
	Samples prepared in a different buffer	Use the cell lysis buffer provided in the kit
	Adherent cells dislodged and lost at the time of experiment	Perform experiment gently and in duplicates/triplicates; apoptotic cells may become floaters
	Cell/ tissue samples were not completely homogenized	Use Dounce homogenizer (increase the number of strokes); observe efficiency of lysis under microscope
	Samples used after multiple freeze-thaw cycles	Aliquot and freeze samples, if needed to use multiple times
	Presence of interfering substance in the sample	Troubleshoot as needed
	Use of old or inappropriately stored samples	Use fresh samples or store at correct temperatures until use