ab65608

Caspase 9 Assay Kit (Colorimetric)

Instructions for Use

For the rapid, sensitive and accurate measurement of Caspase 9 activity in cell and tissue lysates.

This product is for research use only and is not intended for diagnostic use.

PLEASE NOTE: With the acquisition of BioVision by Abcam, we have made some changes to component names and packaging to better align with our global standards as we work towards environmental-friendly and efficient growth. You are receiving the same high-quality products as always, with no changes to specifications or protocols.

Version 4a, Last Updated 14 June 2023

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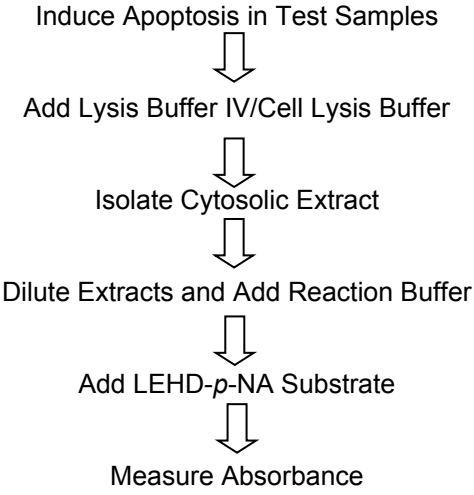
1. Overview

Activation of ICE-family proteases/caspases initiates apoptosis in mammalian cells. Abcam's Caspase 9 Assay Kit provides a simple and convenient means for assaying the activity of caspases that recognize the sequence LEHD. The assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*-NA) after cleavage from the labeled substrate LEHD-pNA/LEHD-*p*-NA. The *p*-NA light emission can be quantified using a spectrophotometer or a microtiter plate reader at 400- or 405 nm.

2. Protocol Summary



3. Components and Storage

A. Kit Components

Item	Quantity
Lysis Buffer IV/Cell Lysis Buffer	100 mL
2X Reaction Buffer I/2X Reaction Buffer	4 x 2 mL
LEHD-pNA/LEHD-*p*-NA (4 mM)	500 µL
DTT I/DTT (1 M)	400 µL
Dilution Buffer II/Dilution Buffer	100 mL

* Store kit at -20°C.

- Protect LEHD-pNA/LEHD-*p*-NA from light.
- Store Lysis Buffer IV/Cell Lysis Buffer, 2X Reaction Buffer I/2X Reaction Buffer, and Dilution Buffer II/Dilution Buffer at 4°C after opening.
- All reagents are stable for 6 months under proper storage conditions.

B. Additional Materials Required

- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric microplate reader or spectrophotometer
- 96 well plate
- Micro-quartz and regular cuvettes
- 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 2-5 x 10^6 cells.
3. Re-suspend cells in 50 µl of chilled Lysis Buffer IV/Cell Lysis Buffer and incubate cells on ice for 10 minutes.
4. Centrifuge for 1 min in a microcentrifuge (10,000 x g).
5. Transfer supernatant (cytosolic extract) to a fresh tube and put on ice.
6. Assay protein concentration.
7. Dilute 100-200 µg protein to 50 µl Lysis Buffer IV/Cell Lysis Buffer for each assay.
8. Aliquot enough 2X Reaction Buffer I/2X Reaction Buffer for the number of assays to be performed. Add DTT I/DTT to the 2X Reaction Buffer I/2X Reaction Buffer immediately before use (10 mM final concentration: add 10 µl of 1.0 M DTT I/DTT stock per 1 ml of 2X Reaction Buffer I/2X Reaction Buffer).

Add 50 µl of 2X Reaction Buffer I/2X Reaction Buffer (containing 10 mM DTT I/DTT) to each sample. Add 5 µl of the LEHD-pNA/4 mM LEHD-*p*-NA substrate (200 µM final conc.) and incubate at 37°C for 1-2 hour.

9. Read samples at 400- or 405-nm in a microtiter plate reader, or spectrophotometer using a 100-µl micro-quartz cuvette, or dilute sample to 1 ml with Dilution Buffer II/Dilution Buffer and using regular cuvette. You may also perform the assay in a 96-well plate.

Fold-increase in Caspase 9 activity can be determined by comparing the results of treated samples with the level of the un-induced control.

Notes:

a) Dilution of the samples proportionally decreases the reading.

b) Background reading from cell lysates and buffers should be subtracted from the readings of both induced and the un-induced samples before calculating fold increase in Caspase 9 activity.

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 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
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

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
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

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
Unanticipated 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)

For further technical questions please do not hesitate to contact us by email (technical@abcam.com) or phone (select "*contact us*" on www.abcam.com for the phone number for your region).

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus

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