

Caspase-8 Colorimetric Assay Kit

Introduction

Cysteine-dependent aspartate-directed proteases (Caspases) are a family of cysteine proteases that play important roles in apoptosis, necrosis, and inflammation. Sequential activation of caspases plays an important role in cell apoptosis. Caspase-8 is a caspase protein that cleaves and activates Caspase-3 and is involved in the programmed cell death induced by Fas and different apoptotic stimuli. Caspase-8 was detected in the insoluble fraction of the affected brain region in Huntington disease patients, which suggested it plays an important role in neurodegenerative diseases.

Caspase-8 Colorimetric Assay Kit provides a convenient and simple way for detecting the IETD-dependent caspase activity. When cleavage of IETD from the labeled substrate IETD-p-nitroaniline (IETD-pNA) by Caspase-8 or related caspases, the free pNA light emission can be quantified by using a microtiter plate reader or a spectrophotometer at 405 or 400 nm. Comparison of the absorbance of free pNA from an apoptotic sample with an uninduced control determines the fold increase in Caspase-8 activity.

Components and Storage

Components	K2013-25 25 assays	K2013-100 100 assays	K2013-200 200 assays	K2013-400 400 assays	Part Number
Cell Lysis Buffer	25 ml	100 ml	100 ml	100 ml	K2013-C-1
2 X Reaction Buffer	2 ml	4 x 2 ml	16 ml	32 ml	K2013-C-2
IETD-pNA (4 mM)	125 μ l	500 μ l	2 x 0.5 ml	2 x 1 ml	K2013-C-3
DTT (1 M)	100 μ l	400 μ l	400 μ l	400 μ l	K2013-C-4

Store kit at -20°C. (Store Cell Lysis Buffer and 2X Reaction Buffer at 4°C after opening).

All reagents are stable for 6 months under proper storage conditions.

Protocol

1. General Considerations

Aliquot enough 2 X Reaction Buffer for the number of assays to be performed. Add DTT to the 2 X Reaction Buffer immediately before use (10 mM final concentration: add 10 μ l of 1.0 M DTT stock per 1 ml of 2 X Reaction Buffer). After thawing, store the Cell Lysis Buffer and 2X Reaction Buffer at 4°C.

Protect IETD-pNA from light.

2. Assay Procedure

- (1) Induce apoptosis in cells by desired method. Concurrently incubate a control culture without induction.
- (2) Count cells and pellet $1-5 \times 10^6$ cells.
- (3) Resuspend cells in 50 μ l of chilled 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 (optional).
- (7) Dilute 100-200 μ g protein to 50 μ l Cell Lysis Buffer for each assay.
- (8) Add 50 μ l of 2X Reaction Buffer (containing 10 mM DTT) to each sample. Add 5 μ l of the 4 mM IETD-pNA substrate (200 μ M final conc.). 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- μ l micro quartz cuvette (Sigma).

You may also perform the entire assay in a 96-well plate.

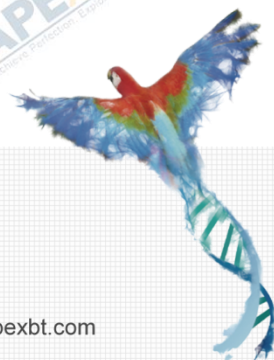
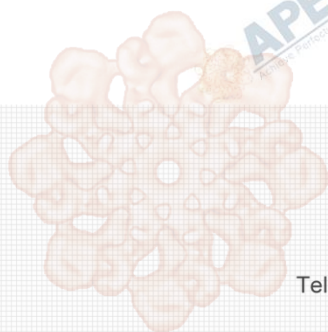
Fold-increase in FLICE activity can be determined by comparing the results of treated samples with the level of the uninduced control.

Tips: Background reading from cell lysates and buffers should be subtracted from the readings of both induced and the uninduced samples before calculating fold increase in FLICE activity.

Notes

Problems	Cause	Solution
Assay not working	<ul style="list-style-type: none">Cells did not lyse completelyExperiment was not performed at optimal time after apoptosis inductionPlate read at incorrect wavelengthOld DTT used	<ul style="list-style-type: none">Resuspend the cell pellet in the lysis buffer and incubate as described in the datasheetPerform a time-course induction experiment for apoptosisCheck the wavelength listed in the datasheet and the filter settings of the instrumentAlways use freshly thawed DTT in the cell lysis buffer
High Background	<ul style="list-style-type: none">Increased amount of cell lysate usedIncreased amounts of components added due to incorrect pipettingIncubation of cell samples for extended periodsUse of expired kit or improperly stored reagentsContaminated cells	<ul style="list-style-type: none">Refer to datasheet and use the suggested cell number to prepare lysatesUse calibrated pipettesRefer to datasheet and incubate for exact timesAlways check the expiry date and store the individual components appropriatelyCheck for bacterial/ yeast/ mycoplasma contamination
Lower signal levels	<ul style="list-style-type: none">Cells did not initiate apoptosisVery few cells used for analysisUse of samples stored for a long timeIncorrect setting of the equipment used to read samplesAllowing the reagents to sit for extended times on ice	<ul style="list-style-type: none">Determine the time-point for initiation of apoptosis after induction (time-course experiment)Refer to datasheet for appropriate cell numberUse fresh samples or aliquot and store and use within one month for the assayRefer to datasheet and use the recommended filter settingAlways thaw and prepare fresh reaction mix before use
Samples with erratic readings	<ul style="list-style-type: none">Uneven number of cells seeded in the wellsSamples prepared in a different bufferAdherent cells dislodged and lost at the time of experimentCell/ tissue samples were not completely homogenized	<ul style="list-style-type: none">Seed only equal number of healthy cells (correct passage number)Use the cell lysis buffer provided in the kitPerform experiment gently and in duplicates/triplicates; apoptotic cells may become floatersUse Dounce homogenizer (increase the number of

	<ul style="list-style-type: none"> • Samples used after multiple freeze-thaw cycles • Presence of interfering substance in the sample • Use of old or inappropriately stored samples 	<ul style="list-style-type: none"> strokes); observe efficiency of lysis under microscope • Aliquot and freeze samples, if needed to use multiple times • Troubleshoot as needed • Use fresh samples or store at correct temperatures until use
Unanticipated results	<ul style="list-style-type: none"> • Measured at incorrect wavelength • Cell samples contain interfering substances 	<ul style="list-style-type: none"> • Check the equipment and the filter setting • Troubleshoot if it interferes with the kit (run proper controls)
General issues	<ul style="list-style-type: none"> • Improperly thawed components • Incorrect incubation times or temperatures • Incorrect volumes used • Air bubbles formed in the well/tube • Substituting reagents from older kits/ lots • Use of a different 96-well plate 	<ul style="list-style-type: none"> • Thaw all components completely and mix gently before use • Refer to datasheet & verify the correct incubation times and temperatures • Use calibrated pipettes and aliquot correctly • Pipette gently against the wall of the well/tubes • Use fresh components from the same kit • Fluorescence: Black plates; Absorbance: Clear plates
<p>Note: The most probable cause is listed under each section. Causes may overlap with other sections.</p>		



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