# 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<br>25 assays | K2013-100<br>100 assays | K2013-200<br>200 assays | K2013-400<br>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 µl | 500 µl | 2 x 0.5 ml | 2 x 1 ml | K2013-C-3 |
| DTT (1 M) | 100 µl | 400 µl | 400 µl | 400 µl | 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 µl 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 x $10^6$ cells.

(3) Resuspend cells in 50 µl 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 µg protein to 50 µl Cell Lysis Buffer for each assay.

(8) Add 50 µl of 2X Reaction Buffer (containing 10 mM DTT) to each sample. Add 5 µl of the 4 mM IETD-pNA substrate (200 µM 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-µl 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 | • Cells did not lyse completely<br>• Experiment was not performed at optimal time after apoptosis induction<br>• Plate read at incorrect wavelength<br>• Old DTT used | • Resuspend the cell pellet in the lysis buffer and incubate as described in the datasheet<br>• Perform a time-course induction experiment for apoptosis<br>• Check the wavelength listed in the datasheet and the filter settings of the instrument<br>• Always use freshly thawed DTT in the cell lysis buffer |
| High Background | • Increased amount of cell lysate used<br>• Increased amounts of components added due to incorrect pipetting<br>• Incubation of cell samples for extended periods<br>• Use of expired kit or improperly stored reagents<br>• Contaminated cells | • Refer to datasheet and use the suggested cell number to prepare lysates<br>• Use calibrated pipettes<br>• Refer to datasheet and incubate for exact times<br>• Always check the expiry date and store the individual components appropriately<br>• Check for bacteria/ yeast/ mycoplasma contamination |
| Lower signal levels | • Cells did not initiate apoptosis<br>• Very few cells used for analysis<br>• Use of samples stored for a long time<br>• Incorrect setting of the equipment used to read samples<br>• Allowing the reagents to sit for extended times on ice | • Determine the time-point for initiation of apoptosis after induction (time-course experiment)<br>• Refer to datasheet for appropriate cell number<br>• Use fresh samples or aliquot and store and use within one month for the assay<br>• Refer to datasheet and use the recommended filter setting<br>• Always thaw and prepare fresh reaction mix before use |
| Samples with erratic readings | • Uneven number of cells seeded in the wells<br>• Samples prepared in a different buffer<br>• Adherent cells dislodged and lost at the time of experiment<br>• Cell/ tissue samples were not completely homogenized | • Seed only equal number of healthy cells (correct passage number)<br>• Use the cell lysis buffer provided in the kit<br>• Perform experiment gently and in duplicates/triplicates; apoptotic cells may become floaters<br>• Use Dounce homogenizer (increase the number of |

| | | |
|---|---|---|
| | • Samples used after multiple freeze-thaw cycles<br>• Presence of interfering substance in the sample<br>• Use of old or inappropriately stored samples | strokes); observe efficiency of lysis under microscope<br>• Aliquot and freeze samples, if needed to use multiple times<br>• Troubleshoot as needed<br>• Use fresh samples or store at correct temperatures until use |
| Unanticipated results | • Measured at incorrect wavelength<br>• Cell samples contain interfering substances | • Check the equipment and the filter setting<br>• Troubleshoot if it interferes with the kit (run proper controls) |
| General issues | • Improperly thawed components<br>• Incorrect incubation times or temperatures<br>• Incorrect volumes used<br>• Air bubbles formed in the well/tube<br>• Substituting reagents from older kits/ lots<br>• Use of a different 96-well plate | • Thaw all components completely and mix gently before use<br>• Refer to datasheet & verify the correct incubation times and temperatures<br>• Use calibrated pipettes and aliquot correctly<br>• Pipette gently against the wall of the well/tubes<br>• Use fresh components from the same kit<br>• Fluorescence: Black plates; Absorbance: Clear plates |

Note: The most probable cause is listed under each section. Causes may overlap with other sections.

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