RayBio® FLICE/Caspase-8 Colorimetric Assay Kit

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RayBio® FLICE/Caspase-8 Colorimetric Assay Kit Protocol

(Cat#: 68CL-Casp8-S)

RayBiotech, Inc.

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I. INTRODUCTION

Activation of ICE-family proteases/caspases initiates apoptosis in mammalian cells. The **FLICE/Caspase-8 Colorimetric Assay Kit** provides a simple and convenient means for assaying the activity of caspases that recognize the sequence IETD. The assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labeled substrate IETD-*p*NA. The *p*NA light emission can be quantified using a spectrophotometer or a microtiter plate reader at 400- or 405-nm. Comparison of the absorbance of *p*NA from an apoptotic sample with an uninduced control allows determination of the fold increase in FLICE activity.

II. REAGENTS

Components	68CL-Casp8-S25	68CL-Casp8-S100	68CL-Casp8-S200	68CL-Casp8-S400	Part Number
	25 assays	100 assays	200 assays	400 assays	
Cell Lysis Buffer	25 ml	100 ml	100 ml	100 ml	Item A
2X Reaction Buffer	2 ml	4 x 2 ml	16 ml	32 ml	Item B
IETD-*p*NA (4 mM)	125 µl	0.5 ml	2 x 0.5 ml	2 x 1 ml	Item C
DTT (1 M)	100 µl	0.4 ml	0.4 ml	0.4 ml	Item D
Dilution Buffer	25 ml	100 ml	200 ml	400 ml	Item E

III. ASSAY PROTOCOL

A. General Considerations:

- 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).
- After thawing, store the Cell Lysis Buffer and dilution Buffer at 4°C.
- Protect IETD-*p*NA from light.

B. 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 µg 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-*p*NA 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), or dilute sample to 1 ml with Dilution Buffer and using regular cuvette (note: Dilution of the samples proportionally decreases the reading).

   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.

   Note: 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.

IV. STORAGE AND STABILITY

Store kit at –20˚C (Store Cell Lysis Buffer, 2X Reaction Buffer, and Dilution Buffer at 4˚C after opening). All reagents are stable for at least 6 months.

V. GENERAL TROUBLESHOOTING GUIDE

Problems	Cause	Solution
Assay not working	• Cells did not lyse completely	• Resuspend 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	• Check the wavelength listed in the datasheet and the filter settings of the instrument
	• 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
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
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
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)
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
Note: The most probable cause is listed under each section. Causes may overlap with other sections.		

This product is for research use only.