

## Caspase-6 Fluorometric 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-6 is a member of the caspase family. Caspase-6 is processed by caspases 7, 8 and 10, and can also undergo self-processing. Caspase-6 acts as a downstream enzyme in the caspase activation cascade and interacts with Caspase 8.

Caspase-6 Fluorometric Assay Kit provides a convenient and simple way for detecting the VEID-dependent caspase activity. VEID-AFC (AFC:7-amino-4-trifluoromethyl coumarin) emits blue light ( $\lambda_{max} = 400 \text{ nm}$ ); while cleavage of the substrate VEID-AFC by Caspase-6 or related caspases, free AFC emits a yellow-green fluorescence ( $\lambda_{max} = 505 \text{ nm}$ ), which can be quantified by using a fluorescence microtiter plate reader or a fluorometer. Comparison of the fluorescence of free AFC from an apoptotic sample with an uninduced control determines the fold increase in Caspase-6 activity.

### Components and Storage

Components	K2014-25 25 assays	K2014-100 100 assays	K2014-200 200 assays	K2014-400 400 assays	Part Number
Cell Lysis Buffer	25 ml	100 ml	100 ml	100 ml	K2014-C-1
2 X Reaction Buffer	2 ml	4 x 2 ml	16 ml	32 ml	K2014-C-2
VEID-AFC (1 mM)	125 $\mu\text{l}$	500 $\mu\text{l}$	2 x 0.5 ml	2 x 1 ml	K2014-C-3
DTT (1 M)	100 $\mu\text{l}$	400 $\mu\text{l}$	400 $\mu\text{l}$	400 $\mu\text{l}$	K2014-C-4

Store at  $-20^{\circ}\text{C}$ .

### 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  $\mu\text{l}$  of 1.0 M DTT stock per 1 ml of 2 X Reaction Buffer). Protect VEID-AFC from light.

#### 2. Assay Procedure

(1) Induce apoptosis in cells by desired method. Concurrently incubate a control culture without induction.

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(2) Count cells and pellet  $1-5 \times 10^6$  cells or use 50-250  $\mu\text{g}$  cell lysates if protein concentration has been measured.

(3) Resuspend cells in 50  $\mu\text{l}$  of chilled Cell Lysis Buffer.

(4) Incubate cells on ice for 10 minutes.

(5) Add 50  $\mu\text{l}$  of 2X Reaction Buffer (containing 10 mM DTT) to each sample.

(6) Add 5  $\mu\text{l}$  of the 1 mM VEID-AFC substrate (50  $\mu\text{M}$  final concentration) and incubate at  $37^\circ\text{C}$  for 1-2 hour.

(7) Read samples in a fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter. For a plate-reading set-up, transfer the samples to a 96-well plate. You may also perform the entire assay directly in a 96-well plate.

Fold-increase in caspase-6 activity can be determined by comparing the results of treated samples with the level of the uninduced control.



## Notes

Problems	Cause	Solution
Assay not working	<ul style="list-style-type: none"> <li>Cells did not lyse completely</li> <li>Experiment was not performed at optimal time after apoptosis induction</li> <li>Plate read at incorrect wavelength</li> <li>Old DTT used</li> </ul>	<ul style="list-style-type: none"> <li>Resuspend the cell pellet in the lysis buffer and incubate as described in the datasheet</li> <li>Perform a time-course induction experiment for apoptosis</li> <li>Check the wavelength listed in the datasheet and the filter settings of the instrument</li> <li>Always use freshly thawed DTT in the cell lysis buffer</li> </ul>
High Background	<ul style="list-style-type: none"> <li>Increased amount of cell lysate used</li> <li>Increased amounts of components added due to incorrect pipetting</li> <li>Incubation of cell samples for extended periods</li> <li>Use of expired kit or improperly stored reagents</li> <li>Contaminated cells</li> </ul>	<ul style="list-style-type: none"> <li>Refer to datasheet and use the suggested cell number to prepare lysates</li> <li>Use calibrated pipettes</li> <li>Refer to datasheet and incubate for exact times</li> <li>Always check the expiry date and store the individual components appropriately</li> <li>Check for bacteria/ yeast/ mycoplasma contamination</li> </ul>
Lower signal levels	<ul style="list-style-type: none"> <li>Cells did not initiate apoptosis</li> <li>Very few cells used for analysis</li> <li>Use of samples stored for a long time</li> <li>Incorrect setting of the equipment used to read samples</li> <li>Allowing the reagents to sit for extended times on ice</li> </ul>	<ul style="list-style-type: none"> <li>Determine the time-point for initiation of apoptosis after induction (time-course experiment)</li> <li>Refer to datasheet for appropriate cell number</li> <li>Use fresh samples or aliquot and store and use within one month for the assay</li> <li>Refer to datasheet and use the recommended filter setting</li> <li>Always thaw and prepare fresh reaction mix before use</li> </ul>
Samples with erratic readings	<ul style="list-style-type: none"> <li>Uneven number of cells seeded in the wells</li> <li>Samples prepared in a different buffer</li> <li>Adherent cells dislodged and lost at the time of experiment</li> <li>Cell/ tissue samples were not completely homogenized</li> <li>Samples used after multiple freeze-thaw cycles</li> <li>Presence of interfering substance in the sample</li> <li>Use of old or inappropriately stored samples</li> </ul>	<ul style="list-style-type: none"> <li>Seed only equal number of healthy cells (correct passage number)</li> <li>Use the cell lysis buffer provided in the kit</li> <li>Perform experiment gently and in duplicates/triplicates; apoptotic cells may become floaters</li> <li>Use Dounce homogenizer (increase the number of strokes); observe efficiency of lysis under microscope</li> <li>Aliquot and freeze samples, if needed to use multiple times</li> <li>Troubleshoot as needed</li> <li>Use fresh samples or store at correct temperatures until use</li> </ul>
Unanticipated results	<ul style="list-style-type: none"> <li>Measured at incorrect wavelength</li> <li>Cell samples contain interfering substances</li> </ul>	<ul style="list-style-type: none"> <li>Check the equipment and the filter setting</li> <li>Troubleshoot if it interferes with the kit (run proper controls)</li> </ul>
General issues	<ul style="list-style-type: none"> <li>Improperly thawed components</li> <li>Incorrect incubation times or temperatures</li> <li>Incorrect volumes used</li> <li>Air bubbles formed in the well/tube</li> <li>Substituting reagents from older kits/ lots</li> <li>Use of a different 96-well plate</li> </ul>	<ul style="list-style-type: none"> <li>Thaw all components completely and mix gently before use</li> <li>Refer to datasheet &amp; verify the correct incubation times and temperatures</li> <li>Use calibrated pipettes and aliquot correctly</li> <li>Pipette gently against the wall of the well/tubes</li> <li>Use fresh components from the same kit</li> <li>Fluorescence: Black plates; Absorbance: Clear plates</li> </ul>

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



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