Substrate composition and fluorescence characteristics:

Each substrate molecule is composed of a peptide homodoubly labeled with a fluorophore. The cleaved substrate has the following excitation and emission peaks: \( \lambda_{ex}=552 \text{ nm} \) and \( \lambda_{em}=580 \text{ nm}. \) (The fluorescence of the intact, \( i.e., \) precleaved, fluorogenic protease substrate is not completely quenched \( \text{vide infra}. \)) The protease recognition sequence is IETDSG where the P\(_2\) and P\(_1\) residues are in green and blue, respectively.

Components: The caspase 8 substrate reagent kit Catalog # CPL8R2E-5 includes 4 red-topped vials each containing at least 940 \( \mu \text{l} \) of the substrate and 1 bottle with 60 \( \text{ml} \) of flow cytometry dilution buffer. The substrate concentration in each vial is 10 \( \mu \text{M} \) in RPMI 1640 medium with 25 mM HEPES. The entire unopened kit can be stored at room temperature or 4 \( \degree \text{C}. \) If any of the vials are opened, then they should be restored at -10 to -20 \( \degree \text{C}. \) Prior to restoring and/or reopening, vials should be lightly centrifuged to remove any liquid from caps. Possible additional reagent needed: fetal calf serum (FCS).

Analysis by Flow Cytometry

Incubation conditions:

Remove all steps involving fixation or permeabilization from your protocol. Do not fix or permeabilize substrate-exposed cells for labeling with antibody or other reagents.

1. Treat target cells with chosen apoptosis-inducing reagent and/or inhibitor. Two control samples, one with and one without the vehicle (organic cosolvent), should be included in every sample set. The concentration of vehicle should not exceed 0.3\% (v/v) (0.1\% is preferable). (As mentioned below the peak channel as well as possible scatter changes for caspase negative cells with and without vehicle should be determined to avoid false conclusions.)

2. After treatment, aliquot cells into 1.5 to 2.0 \( \text{ml} \) microcentrifuge tubes, centrifuge and then remove all culture medium and buffer.

3. To each of the centrifuged cell pellets, add 75 \( \mu \text{l} \) of 10 \( \mu \text{M} \) substrate solution (add 8 \( \mu \text{l} \) of FCS, if 10\% FCS is appropriate). The cell number should be between 0.5 and 1 million per sample (See A and B below). Mix cell suspensions with substrate by pipetting. Do not vortex tubes containing cells as apoptotic cells can be "fragile".

4. Incubate tubes at 37 \( \degree \text{C} \) for 30-60 minutes before flow cytometric analysis. Keep substrate at physiological pH: avoid exposure of substrate to direct light or extremes of pH.

Sample preparation for and measurement by flow cytometry:

5. Wash cells once by adding 1 \( \text{ml} \) of flow cytometry dilution buffer, centrifuging, and removing all medium and buffer.

6. Loosen cell pellets by flicking tubes with finger tips and then pipetting loosened pellets with 1 \( \text{ml} \) of fresh dilution buffer. Do not vortex tubes. All samples should be analyzed within 60 to 90 minutes after the end of the 37\degree C incubation.

7. Recommended flow cytometer settings: excitation with a 532, 543, 561, or 568 nm laser is preferable but a 488 nm line is acceptable; detection should be in the channel with filters consistent with the 580 nm emission peak of the fluorophore, e.g., the PE channel.

8. Set the peak channel for detection of control cell population (absence of apoptogen (with vehicle, if appropriate)) in the first decade. Then run apoptogen-treated cell populations.

Useful Hints & Warnings:

A. The cell density during incubation with the substrate should be between 0.5 and 1.0 million cells per sample (although lower concentrations are analyzable). It is recommended that a control sample with cells taken directly from a log phase culture be included in the assay to test whether there is significant default apoptotic death background.

B. In certain settings, one may be able to use the substrate at a concentration lower than 9 \( \mu \text{M} \) (Addition of FCS to a final concentration of 10\% v/v would lower the substrate concentration to 9 \( \mu \text{M} \) \( \text{vide supra} \)). However, the kit has been formulated with the substrate concentration at 10 \( \mu \text{M} \) for optimal performance under most conditions. Viable cell uptake of the substrate reaches a near maximum between 20 and 30 minutes at 37\degree C in most cell types. However, substrate uptake may vary with cell type and specific conditions, incubation times should be optimized.

C. If a population of cells with very low fluorescence intensity, \( i.e., \) lower than the uninduced cell population, appears, then more than likely (i) the samples have been overinduced and/or (ii) the final vehicle concentration has lead to toxicity. Therefore, a vehicle control sample should be included.

In order to see the brightest induced cell populations in histograms, cells must retain their membrane integrity. Please note: the principle upon which all OncoImmunin substrates work is that the intact substrates diffuse across all membranes, \( i.e., \) plasma as well as intracellular membranes, by passive diffusion; once the target sequences in the substrates have been recognized and cleaved by their...
cognate proteases, the fragments are largely retained on the side of the membrane where the proteases reside. Thus, cleaved substrate fragments generate a positive signal in cells with intact membranes. Once a cell loses its membrane integrity, the cleaved fragments are free to diffuse out of the cell. Since the fluorescence of intact substrates is not completely quenched, uninduced populations of cells loaded with substrate have higher fluorescence than cells which have not been exposed to substrates. Thus, if the permeability barrier of live cells with intact membranes is lost in cases such as overinduction or exposure to high concentrations of organic cosolvents, then intact as well as cleaved fragments may be lost from induced cells.

In some cases it may be informative to analyze early time points where caspase activation has not yet taken place to see if test compounds themselves induce membrane permeability changes by noting a decrease in the peak channel number.

**Analysis by Fluorescence Microscopy**

**Incubation conditions:**
*Remove all steps involving fixation or permeabilization from your protocol. Do not fix substrate-exposed cells for labeling with antibody or other reagents.*

In most cell cultures a few percent of cells are in default death; these should be used as positive controls. In the rare cases where there is no cognate proteases, the fragments are largely retained on the side of the membrane where the proteases reside. Thus, cleaved substrate fragments generate a positive signal in cells with intact membranes. Once a cell loses its membrane integrity, the cleaved fragments are free to diffuse out of the cell. Since the fluorescence of intact substrates is not completely quenched, uninduced populations of cells loaded with substrate have higher fluorescence than cells which have not been exposed to substrates. Thus, if the permeability barrier of live cells with intact membranes is lost in cases such as overinduction or exposure to high concentrations of organic cosolvents, then intact as well as cleaved fragments may be lost from induced cells.

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