OncoImmunin, Inc.

Application Note #1: Flow Cytometry Analysis
Updated 11/15/04 for PhiPhiLux®-G_2D_2

Substrate composition and fluorescence characteristics:

- ☞ Each substrate molecule contains a peptide homodoubly labeled with a fluorophore. The cleaved substrate has the following excitation and emission peaks: λ_{ex}=552 nm and λ_{em}=580 nm. (The fluorescence of the intact, *i.e.*, precleaved, fluorogenic protease substrate is not completely quenched (*vide infra*))
- ☞ The main peptide amino acid sequence is DEVDGI where the $P_1$and P_1' residues are in green and blue, respectively.

Components: The caspase 3 substrate reagent kit Catalog # A304R2G-3 contains 4 vials and 1 bottle of 60 ml flow cytometry dilution buffer. The caspase 3 substrate reagent kit Catalog # A304R2G-6 contains 8 vials and 2 bottles of 60 ml flow cytometry dilution buffer. Each vial contains at least 565 µl of 10 µM substrate in RPMI 1640 medium with 25 mM HEPES. The entire unopened kit can be stored at room temperature or 4 ^{0}C. If any of the substrate-containing vials are opened, then store at -10 to -20 ^{0}C. Prior to restoring, vials should be lightly centrifuged to remove any liquid from caps. Upon thawing: shake until the solution appears homogeneous.

Possible additional reagent needed: 250 µl of fetal calf serum (FCS).

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.**

1. Treat target cells with chosen apoptosis-inducing reagent (apoptogen) 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 0.5% (v/v) (0.1% is preferable). (As mentioned in E below the FL1 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 ml microcentrifuge tubes, centrifuge and then remove ***all culture medium*** in order to minimize subsequent substrate dilution. Suggestions: (i) Avoid high speed table top centrifuges; use centrifugation conditions similar to normal handling of cells. (ii) A gentle vacuum suction equipped with a fine tip, e.g., a pipette tip, is suggested.
3. To each of the centrifuged cell pellets, add 75 µl of 10 µM substrate solution (add 8 µl 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 flicking tubes with finger tips. ***Do not vortex tubes containing cells as apoptotic cells can be "fragile".***
4. Incubate tubes at 37 ^{0}C for 60 minutes before flow cytometric analysis. (See C below.) Keep substrate at physiological pH: avoid direct light to substrate as well as exposure to extremes of pH.

Sample preparation for and measurement by flow cytometry:

5. Wash cells once by adding 1 ml of flow cytometry dilution buffer, centrifuging, and removing all medium and buffer. Loosen cell pellets by flicking tubes with finger tips and then resuspending loosened pellets in 1 ml of fresh dilution buffer. ***Do not vortex tubes containing cells, but rather flick tubes with finger tips to resuspend cells. Apoptotic cells can be fragile. (See below.)*** All samples should be analyzed within 60 to 90 minutes after the end of the 37ºC incubation.
6. Recommended flow cytometer settings: excitation with a 488 nm laser line and detection in the FL2 channel of a BD or similar instrument. Set the peak channel for cells from control cell population (absence of apoptogen (with vehicle, if appropriate)) in the first decade. Then run apoptogen-treated cell populations.
7. After collecting data by the preceding procedure, one can delete PI-positive cells if *ca.* 10 µl of a 5-10 µg/ml propidium iodide (PI) solution is added and samples are rerun on the flow cytometer (final PI concentration of *ca.* 200 ng/ml) with a dot plot of FL2 *vs.* FL3 (See D). Reanalysis should be *within 5-15 minutes of PI addition.*

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 the cells taken directly from culture with cells in log phase be included in the assay to test whether there is any high cell density-induced apoptosis.

B. In certain settings, one may be able to use the substrate at a concentration lower than 9 µM (addition of FCS to a final concentration of 10% v/v would lower the substrate concentration to 9 µM (*vide supra*). ***However, the kit has been formulated with the substrate concentration at 10 µM for optimal performance under most conditions.***

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C. Viable cell uptake of the substrate reaches a near maximum between 20 and 30 minutes at 37^0C in most cell types. However, as substrate uptake may vary with cell type and specific conditions, incubation times should be optimized.

D. It is recommended that in order to identify PI-negative apoptotic and uninduced cell populations analysis first be carried out in the absence of PI followed by a second round of data collection after PI addition. ***Sample dilution volume should be the same for all samples.*** A comparison between the dot plots (FL2 *vs.* FL3) of these two sets of samples will enable easy identification of PI-negative populations as those cells that remain in the same location in the two sets of dot plots. Thus, if a gate is placed around the PI-negative cells and cells within this gate are then be plotted in an FL1 histogram, this methodology will result in analysis of both uninduced and apoptotic PI-negative cells.

E. 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 apoptotic 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 substrates have been recognized and cleaved by their cognate proteases, the cleaved fragments are largely retained on the side of the membrane where the proteases reside. Thus, cleaved substrate fragments generate a positive signal in PI-negative cells and once a cell looses its membrane integrity as indicated by PI positivity, 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 fluoresence than cells which have not been exposed to substrates (See Figure 3 in reference 14.) 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. Generally, cells in samples with a high percentage of PI-positive or TUNEL assay positive cells will be in late apoptosis and/or necrosis. Use of PhiPhiLux®-G_2D_2 is optimal with samples containing a low percentage of PI-positive cells.

In most cases it is recommended to lower the apoptogen concentration rather than simply looking for an earlier time point. Appropriate time points for analysis of PhiPhiLux®-G_2D_2-positive cells should be such that a large percentage of PI-negative cells is present: ideally, samples should contain **both** uninduced and caspase$^+$/PI$^-$ cells. Conditions under which only a single population is observable should be avoided. Examination of cells under a fluorescence microscope may assist in determining exact apoptosis inducing conditions.

Application Note #2 : Fluorescence Microscopy

Updated 11/12/04

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 cells are in default death; these should be used as positive controls. In the rare cases where there is no default death, treatment with an established apoptogen, e.g., 1 µM Staurosporine, is recommended for generating positive controls.

I. **Standard (nonconfocal) fluorescence microscope application**

1. Treat target cells with chosen apoptosis-inducing reagent (apoptogen) 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 0.5% (v/v) (0.1% is preferable). (As mentioned in E above for flow cytometry possible changes for caspase-negative cells with and without vehicle should be determined to avoid false conclusions.)
2. (a) For suspension cells aliquot cells into 1.5 to 2.0 ml microcentrifuge tubes, centrifuge and then remove ***all culture medium*** in order to minimize subsequent substrate dilution. A gentle vacuum suction equipped with a fine tip, e.g., a pipette tip, is suggested. (b) For adherent cells, remove all culture medium.
5. (a) For suspension cells, to each of the centrifuged cell pellets, add 75 µl of 10 µM substrate solution (add 8 µl of FCS, if 10% FCS is appropriate). The cell number should be between 0.5 and 1 million per sample (See A and B above). Mix suspension containing cells and substrate by flicking tubes with finger tips. ***Do not vortex tubes containing cells as apoptotic cells can be "fragile".*** (b) For adherent cells, add enough substrate solution to completely cover the monolayer or individual cells. As with suspension cells, be sure to remove all medium before addition of substrate-containing solution to minimize dilution of substrate. (For adherent cells cell culture dishes with glass coverslips attached to the bottom side is recommended. (Contact OncoImmunin, Inc. for the source of such cell culture dishes.))
4. Incubate suspension cell samples in tubes or adherent cells at 37 ºC for 30 to 60 minutes. Exact incubation times are cell type and inducer specific. Keep substrate at physiological pH: avoid direct light to substrate as well as exposure to extremes of pH.
5. Immediately following incubation with PhiPhiLux®-G_2D_2 substrate solution: (a) for suspension cells dilute with 1 ml of the Flow Cytometry Buffer or any physiological buffer, e.g., PBS, centrifuge, and replace the supernatant with 1 ml of fresh buffer. Repeat cell washing (typically two to four more times) depending on fluorescence microscope's lamp power and detection capability. Check cells after each wash under the fluorescence microscope to see if the background fluorescence is dark enough to distinguish between untreated control and treated cells (b) for adherent cells remove PhiPhiLux®-G_2D_2 substrate solution and wash gently with buffer. Perform a similar number of cell washing cycles as recommended for suspension cell samples. After each cycle observe the background fluorescence level under the fluorescence microscope. Take special care in washing adherent cells since apoptotic cells are generally more easily detached from the plate than nonapoptotic cells. Therefore, it is recommended that all washes be saved until positive cells are recognizable.
6. Recommended microscopy settings are rhodamine filters.

II. **Confocal microscope application**

Due to the presence of pinholes in confocal instruments and their optical filtering effect washing steps can be deleted. Thus, one can have the substrate present throughout the experiment or add the substrate at any time during a given experiment.

The substrate concentration appropriate for this type of experiment should be less than the stock concentration of 10 µM. It is recommended that a series of substrate dilutions ranging from 5 down to 1µM be carried out.

When using a confocal microscope, setting the appropriate detector gains and laser power settings is essential. Generally, in any cell culture, there is a small number of apoptotic cells, *i.e.* cells in default death. Use of the latter in combination with healthy control cells can serve as limits for setting the dynamic range. A good starting point is to use the signal from the default death cells as 90% of the maximum and the healthy cells as the negative or lower signal level.

Nota bene: at various substrate concentrations, a lower pixel signal intensity for the intracellular domain of healthy cells compared with those pixel intensities of the extracellular domain will often be observed. This is due to the intact cells' membrane permeability barrier. When a given caspase activity is activated followed by cleavage of the cognate protease substrate, the intracellular pixel intensity level will be elevated above the extracellular pixel intensity due to dequenching of the substrate. If the pixel intensity of the cytoplasmic area only reaches the extracellular intensity level, then one cannot unequivocally declare at that time point that this fluorescence intensity increase is due to the caspase activity alone as a mere membrane permeability increase of given cells alone could account for this increase. However, the intensity increase above that associated with the background substrate solution can only be possible if the fluorescence of the fluorophores conjugated to the substrate is dequenched by the target caspase's actions on the intact substrate by cleaving the substrate into two fragments.

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