PhiPhiLux®-G_2D_2

Updated 09/01/09

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: 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 protease recognition 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-5 includes 4 red-topped vials each containing at least 940 µl of the substrate and 1 bottle with 60 ml of flow cytometry dilution buffer. The caspase 3 substrate reagent kit Catalog # A304R2G-8 includes 8 green-topped vials each containing at least 770 µl of the substrate and 2 bottles with 60 ml of flow cytometry dilution buffer. The substrate concentration in each vial is 10 µM 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 vials are opened, then they should be restored at -10 to -20 ^{0}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 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 pipetting. ***Do not vortex tubes containing cells as apoptotic cells can be "fragile".***
4. Incubate tubes at 37 ^{0}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 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 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^{o}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 cells from 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 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.*** Viable cell uptake of the substrate reaches a near maximum between 20 and 30 minutes at 37 ^{0}C in most cell types. However, as 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.

207A Perry Parkway, Suite 6
Gaithersburg, MD 20877
V: 301-987-7881
F: 301-987-7882
OncoImmunin@PhiPhiLux.com
www.PhiPhiLux.com

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 looses 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 fluoresence 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 default death, treatment with an established apoptogen, e.g., 1 µM Staurosporine, is recommended for generating positive controls.

For standard (nonconfocal) fluorescence microscopy

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 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, to each of the centrifuged cell pellets, add 75 µl of the 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 culture dishes with glass coverslips attached to the bottom is recommended. (Contact OncoImmunin, Inc. for the source of such cell culture dishes.))
3. Incubate suspension cell samples in tubes or adherent cells at 37 ^{o}C 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.
4. 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 one to two 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.
5. Recommended microscopy settings are rhodamine filters.

For confocal microscope

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 1 µM 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 are 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 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 that of the extracellular intensity due to dequenching of the substrate. If the pixel intensity of the cytoplasmic area only reaches the extracellular 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.