Biotium, Inc.
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Viability/Cytotoxicity Assay Kit
For
Animal Live & Dead Cells

Catalog Number: 30002 (>300 assays)

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Introduction

Viability/Cytotoxicity Assay Kit for Animal Live & Dead Cells (30002) provides a two-color fluorescence staining on both live and dead cells using two probes that measure two recognized parameters of cell viability — intracellular esterase activity and plasma membrane integrity [1-2]. The kit is suitable for use with fluorescence microscopes, fluorescence multiwell plate scanners and flow cytometers and other fluorescence detection systems. The assay principles are general and applicable to most eukaryotic cell types, including adherent cells [3] and certain tissues [4], but not to bacteria (see #30027 for bacteria cells) or yeast. This fluorescence-based method of assessing cell viability can be used in place of trypan blue exclusion, 51Cr release and similar methods for determining cell viability and cytotoxicity. It is generally faster, less expensive, safer and a more sensitive indicator of cytotoxic events than alternative methods. EthD-III shares similar properties with EthD-I and is 40% brighter compared to EthD-I. Validity of the LIVE/DEAD Viability/Cytotoxicity assay for animal cell applications has been established by several publications [5-13].

Principle of the Method

Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm). EthD-III enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~530 nm/~635 nm). EthD-III is excluded by the intact plasma membrane of live cells. The determination of cell viability depends on these physical and biochemical properties of cells. Cytotoxic events that do not affect these cell properties may not be accurately assessed using this method. Background fluorescence levels are inherently low with this assay technique because the dyes are virtually nonfluorescent before interacting with cells.

Kit Components

Component A, **Calcein AM**, two vials, 50 µL each, 4 mM in anhydrous DMSO.
Component B, **EthD-III**, two vials, 150 µL each, 2 mM in DMSO/H2O 1:4 (v/v). At the recommended reagent concentrations and volumes, this kit contains sufficient material to perform ≥1000 tests using a fluorescence microscope or fluorescence microplate reader or ≥300 tests using a flow cytometer.

Storage and Handling of Reagents

Reagents in this kit should be stored sealed, desiccated, protected from light and frozen at -20°C. Allow the reagents to warm up to room temperature for 30 min and centrifuge briefly before opening. Before refreezing, seal all stock solutions tightly. Calcein AM is susceptible to hydrolysis when exposed to moisture. If the color of Calcein AM stock solution turns orange, discard the tube. Prepare aqueous working solutions containing Calcein AM immediately prior to use, and use within 8 hours. EthD-III is stable and insensitive to moisture. Stock solutions of EthD-III in DMSO/ H_2O or other aqueous media can be stored frozen at -20°C for at least one year.

Fluorescence Microscopy Protocol

Prepare Standard Working Solution (2 µM calcein AM, 4 µM EthD-III)

Best results are obtained by adjusting the dye concentrations to achieve distinct labeling of live cells with calcein AM and of dead cells with EthD-III. The optimal concentrations are likely to vary depending on the cell type. In general it is best to use the lowest dye concentration that gives sufficient signal. The range of titration is within 0.1 to 10 µM for both calcein AM and EthD-III. The standard staining solution is suitable for NIH3T3, PtK2 and MDCK.

1.1 Remove the Calcein AM and EthD-III reagent stock solutions from the freezer and allow them to warm to room temperature for 30 min.

1.2 Add 20 µL of the supplied 2 mM EthD-III stock solution to 10 mL of PBS, vortexing to ensure thorough mixing. This gives an approximately 4 µM EthD-III solution.
1.3 Combine the reagents by transferring 5 µL of the supplied 4 mM calcein AM stock solution to the 10 mL EthD-III solution. Vortex the resulting solution to ensure thorough mixing.
1.4 The resulting approximately 2 µM calcein AM and 4 µM EthD-III working solution is then added directly to cells.
1.5 Note that aqueous solutions of calcein AM are susceptible to hydrolysis (see *Storage and Handling of Reagents*). Aqueous working solutions should therefore be used within one day.

Prepare the Cells and Perform the Viability Assay

1.6 Adherent cells may be cultured on sterile glass coverslips or chamber slides as either confluent or subconfluent monolayers. Nonadherent cells may also be used.
1.7 Wash the cells prior to the assay to remove serum esterase activity. Wash adherent cells gently with 500–1000 volumes of PBS and aspirate out the supernatant. Wash non-adherent cells in a test tube with 500–1000 volumes of PBS, sediment by centrifugation and aspirate out the supernatant.
1.8 Add sufficient amount of Calcein AM/EthD-III standard staining solution or your optimal staining solution to cover cell monolayer.
1.9 Incubate the cells for 30–45 minutes at room temperature.
2.0 For adherent cells, aspirate out Calcein AM/EthD-III solution incubation, add about 10 µL of the fresh Calcein AM/EthD-III solution or PBS to a clean microscope slide or coverslip, and mount a coverslip on a slide. Seal the coverslip with fingernail polish to prevent evaporation.
2.1 View the labeled cells under the fluorescence microscope.

Optical Filter Selection

Calcein and EthD-III can be viewed simultaneously with a conventional fluorescein long pass filter. The fluorescence from these dyes may also be observed separately; Calcein can be viewed with a standard fluorescein bandpass filter and EthD-III can be viewed with filters for propidium iodide or Texas Red. Lists of various filters are as follows.
Longpass and dual emission filters useful for simultaneous viewing of calcein and EthD-III stains
Omega Filters: XF25, XF26, XF115
Chroma Filters: 11001, 41012, 71010
Bandpass filters for viewing calcein alone
Omega Filters: XF22, XF23
Chroma Filters: 31001, 41001
Bandpass filters for viewing EthD-III alone
Omega Filters: XF32, XF43, XF102, XF108
Chroma Filters: 31002, 31004, 41002, 41004

Fluorescence Microplate Protocol

Prepare 2X Standard Working Solution (2 µM calcein AM, 4 µM EthD-III)

Best results are obtained by adjusting the dye concentrations to achieve distinct labeling of live cells with calcein AM and of dead cells with EthD-III. The optimal concentrations are likely to vary depending on the cell type. In general it is best to use the lowest dye concentration that gives sufficient signal. The range of titration is within 0.1 to 10 µM for both calcein AM and EthD-III. 10 mL 2X standard staining solution is suitable for staining mouse leukocytes for one 96-well microplate.
2.1 Remove the Calcein AM and EthD-III reagent stock solutions from the freezer and allow them to warm to room temperature for 30 min.
2.2 Add 20 µL of the supplied 2 mM EthD-III stock solution to 10 mL of PBS, vortexing to ensure thorough mixing. This gives an approximately 4 µM EthD-III solution.
2.3 Combine the reagents by transferring 5 µL of the supplied 4 mM calcein AM stock solution to the 10 mL EthD-III solution. Vortex the resulting solution to ensure thorough mixing.
2.4 The resulting approximately 2 µM calcein AM and 4 µM EthD-III working solution.

2.5 Prepared 1 mL 2 uM calcein AM only solution and 1 mL 4 uM EthD-III only solution if the percentage of live or dead cells in the population needs to be obtained.
2.6 Note that aqueous solutions of calcein AM are susceptible to hydrolysis (see *Storage and Handling of Reagents*). Aqueous working solutions should therefore be used within one day.

Prepare the Cells for the Microplate Reader

The minimum detectable number of cells per well is usually between 200 and 500. The maximum usable number of cells per well is on the order of 10^6.

Adherent Cells

2.6 Culture adherent cells and nonadherent cells in a multiwell plate or culture dish. Treat cells with proliferation or cytotoxic agents. Prepare samples of live cells and dead cells for control. Dead cells can be obtained by treatment with 0.1% saponin or 0.1-0.5% digitonin for about 10 minutes.
2.7 Wash the cells with 500–1000 volumes of PBS. For adherent cells, add 100uL PBS to at least cover the bottom of the well. For nonadherent cells, the wash can be done in a test tube and then resuspend cells in PBS. Distribute 100 µL of cell-containing buffer to each well of a microplate. The cell samples are washed to remove esterase activity generally present in serum-supplemented growth media that could cause an increase in extracellular fluorescence due to hydrolysis of calcein AM.

Fluorescence Measurements Using a Microplate Reader

In order to obtain the greatest sensitivity using a plate reader, we recommend exciting the fluorophores using optical filters optimal for their respective absorbances. Calcein is well excited using a fluorescein optical filter (485 ± 10 nm) whereas EthD-III is compatible with a typical rhodamine optical filter (530 ± 12.5 nm). The fluorescence emissions should be acquired separately as well, calcein at 530 ± 12.5 nm, and EthD-III at 645 ± 20 nm.
2.8 Arrange following controls for your experimental samples if the percentage of live or dead cells in the population needs to be obtained. For measuring the relative increase in the number of live or dead cell number, these controls may not be required. These controls include a cell-free control (G and H below), live cell control (E and F below) and dead cell control (C and D below).
2.9 Add an additional 100 µL of the Calcein AM/EthD-III working solution, yielding 200 µL per well containing 1 µM calcein AM and 2 µM EthD-III. Incubate the samples at room temperature for 30–45 minutes.
2.10 Measure the fluorescence in the experimental and control cell samples using the appropriate excitation and emission filters:

A. Fluorescence at 645 nm in the experimental cell sample, labeled with calcein AM and EthD-1 = $\mathbf{F(645)_{sam}}$
B. Fluorescence at 530 nm in the experimental cell sample, labeled with calcein AM and EthD-1 = $\mathbf{F(530)_{sam}}$
C. Fluorescence at 645 nm in a sample where all the cells are dead, labeled with EthD-1 only = $\mathbf{F(645)_{max}}$
D. Fluorescence at 645 nm in a sample where all the cells are dead, labeled with calcein AM only = $\mathbf{F(645)_{min}}$
E. Fluorescence at 530 nm in a sample where all (or nearly all) cells are alive, labeled with EthD-1 only = $\mathbf{F(530)_{min}}$
F. Fluorescence at 530 nm in a sample where all (or nearly all) cells are alive, labeled with calcein AM only = $\mathbf{F(530)_{max}}$
G. Fluorescence at 530 nm of the cell-free sample with or without dye added = $\mathbf{F(530)_0}$
H. Fluorescence at 645 nm of a cell-free sample with or without dye added = $\mathbf{F(645)_0}$

Interpretation of the Results

The relative numbers of live and dead cells can be expressed in terms of percentages or as absolute numbers of cells (described in *Determining Absolute Numbers of Live and Dead Cells)* at about 530 nm and limited fluorescence signal at longer wavelengths. Dead cells are

characterized by intense fluorescence at > 600 nm and little fluorescence around 530 nm. Background fluorescence readings ($F(530)_0$ and $F(645)_0$) may be subtracted from all values of F(530) and F(645) respectively prior to calculation of results. The percentage of live cells can be calculated from the fluorescence readings defined above as:

$$\% \text{ Live Cells} = \frac{B - E}{F - E}$$

The percentage of dead cells can be calculated from the fluorescence readings defined above as:

$$\% \text{ Dead Cells} = \frac{A - D}{C - D}$$

Determining Absolute Numbers of Live and Dead Cells

The absolute number of live and dead cells in a sample can be obtained by constructing a standard curve of cell number versus fluorescence at ~530 nm and at ~645 nm. The fluorescence intensity is linearly related to the total number of cells present in the sample.

Flow Cytometry

The assay can easily be adapted for use in flow cytometry. Cells cultured in suspension, or trypsined adherent cells in suspension, can be stained analogously to the staining described for fluorescence microscopy (see *Fluorescence Microscopy Protocol*). For flow cytometry analysis, the cell suspensions (control cells or cells treated with a cytotoxic agent) are washed by centrifugation and resuspension in PBS, pelleted again and resuspended in the working solution of calcein AM and EthD-III for 30-45 min before the analysis.

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