LIVE/DEAD® Viability/Cytotoxicity Assay Kit

Cat. No. PA-3016

Storage upon receipt: -20°C

Protect from light

Abs/Em:

Calcein = 494/517 nm

Ethidium homodimer-1 (on DNA) = 528/617 nm

Notes: Calcein AM may hydrolyze if exposed to moisture.

Instructions for Use

The LIVE/DEAD® Viability/Cytotoxicity Assay Kit provides a two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes that measure two recognized parameters of cell viability — intracellular esterase activity and plasma membrane integrity. It has been established that calcein AM and ethidium homodimer (EthD-1) are optimal dyes for this application [1-3].

The kit is suitable for use with fluorescence microscopes or fluorescence multiwell plate scanners and easily adaptable for use with flow cytometers and other fluorescence detection systems. The assay principles are general and applicable to most eukaryotic cell types, including adherent cells [4] and certain tissues[5,6] ,but not to bacteria or yeast [3]. This fluorescence-based method of assessing cell viability can be used in place of trypan blue exclusion, Cr_{51} 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. Validity of the LIVE/DEAD Viability/Cytotoxicity assay for animal cell applications has been established by several laboratories. Published applications include measuring the cytotoxic effects of tumor necrosis factor (TNF) [7], β-amyloid protein [8], adenovirus E1A proteins [9], tetrodotoxin (TTX) binding to Na+ channels [10], methamphetamines [11] and mitogenic sphingolipids [12]. The assay has also been utilized to quantitate apototic cell death [13,14] and cell-mediated cytotoxicity [15,16].

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-1 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 ~495 nm/~635 nm). EthD-1 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 Contents

Calcein AM (Component A), two vials, 40 µl each, 4 mM in anhydrous DMSO

Ethidium homodimer-1 (Component B), 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 to room temperature and centrifuge briefly before opening.

Before refreezing, seal all stock solutions tightly. Calcein AM is susceptible to hydrolysis when exposed to moisture. Prepare aqueous working solutions containing calcein AM immediately prior to use, and use within about one day.

Cambrex Bio Science Walkersville, Inc.

www.cambrex.com

biotechserv@cambrex.com

Orders: 800-638-8174 Technical Service: 800-521-0390

Part No. 06867

INST-PA-3016 09/04

CAMBREX

Innovation. Experience. Performance.

EthD-1 is stable and insensitive to moisture. Stock solutions of EthD-1 in

DMSO/ H_2O or other aqueous media can be stored frozen at -20°C for at least one year.

Fluorescence Microscopy Protocol

Select the Optical Filters

Calcein and EthD-1 can be viewed simultaneously with a conventional fluorescein longpass filter. The fluorescence from these dyes may also be observed separately; calcein can be viewed with a standard fluorescein bandpass filter and EthD-1 can be viewed with filters for propidium iodide.

Preparation of the Cells

1.1 Adherent cells may be cultured on sterile glass coverslips as either confluent or subconfluent monolayers (e.g., fibroblasts are typically grown on the coverslip for 2–3 days until acceptable cell densities are obtained). The cells may be cultured inside 35 mm disposable petri dishes or other suitable containers; nonadherent cells may also be used.

1.2 Wash the cells prior to the assay to remove or dilute serum esterase activity generally present in serum-supplemented growth media (serum esterases could cause some increase in extracellular fluorescence by hydrolyzing calcein AM). Wash adherent cells gently with 500–1000 volumes of Dulbecco's phosphate buffered

saline (D-PBS) (note A).

1.3 Wash non-adherent cells in a test tube with 500–1000 volumes of tissue culture–grade D-PBS and sediment by centrifugation. Transfer an aliquot of the cell suspension to a coverslip. Allow cells to settle to the surface of the glass coverslip at 37°C in a covered 35 mm petri dish.

1.4 Treat the cells with cytotoxic agents as required at any time prior to or concurrent with LIVE/DEAD reagent staining.

Determine the Optimal Dye Concentrations

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-1. 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 following method can be used to determine optimal dye concentrations:

2.1 Remove the LIVE/DEAD assay reagents from the freezer and allow them to warm to room temperature.

2.2 Prepare some samples of live cells as well as of dead cells on glass coverslips. Kill the cells using any preferred method (e.g. treatment with 0.1% saponin for 10 minutes, 0.1–0.5% digitonin for 10 minutes, 70% methanol for 30 minutes or complement and the appropriate IgG for 30 minutes).

2.3 Using samples of dead cells, select an EthD-1 concentration that stains the dead cell nuclei bright red without staining the cytoplasm significantly (try from 0.1 to 10 µM EthD-1).

2.4 Using samples of dead cells, select a calcein AM concentration that does not give significant fluorescence in the dead cell cytoplasm (try from 0.1 to 10 µM calcein AM).

2.5 Using samples of live cells, check to see that the calcein AM concentration selected in step 2.4 generates sufficient fluorescence signal in live cells (if not, try a higher concentration).

2.6 The reagent concentrations determined in steps 2.3 and 2.5 are optimal for the viability experiments.

Example Dilution Protocol

This example protocol makes 10 ml of an approximately 2 µM calcein AM and 4 µM EthD-1 solution. We found these dye concentrations to be suitable for NIH 3T3, PtK2 and MDCK cells when incubated at room temperature for 20-40 minutes.

Cultured mouse leukocytes (J774A.1), which have higher esterase activity, require 5-10 times less calcein AM than that required for the three other cell types, but the same amount of EthD-1.

Cambrex Bio Science Walkersville, Inc.

www.cambrex.com

biotechserv@cambrex.com

Orders: 800-638-8174 Technical Service: 800-521-0390

Part No. 06867

INST-PA-3016 09/04

CAMBREX

Innovation. Experience. Performance.

This is an example protocol only; the optimal dye concentrations for any experiment will vary.

3.1 Remove the LIVE/DEAD reagent stock solutions from the freezer and allow them to warm to room temperature.

3.2 Add 20 µl of the supplied 2 mM EthD-1 stock solution (Component B) to 10 ml of sterile, tissue culture–grade D-PBS, vortexing to ensure thorough mixing. This gives an approximately 4 µM EthD-1 solution.

3.3 Combine the reagents by transferring 5 µl of the supplied 4 mM calcein AM stock solution (Component A) to the 10 ml EthD-1 solution. Vortex the resulting solution to ensure thorough mixing.

3.4 The resulting approximately 2 µM calcein AM and 4 µM EthD-1 working solution is then added directly to cells. The final concentration of DMSO is ≤0.1%, a level generally innocuous to most cells.

3.5 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.

Perform the Viability Assay

4.1 Add 100–150 µl of the combined LIVE/DEAD assay reagents, using optimized concentrations, to the surface of a 22 mm square coverslip, so that all cells are covered with solution. Incubations should be performed in a covered dish (e.g., 35 mm disposable petri dish) to prevent contamination or drying of the samples.

4.2 Incubate the cells for 30-45 minutes at room temperature. A shorter incubation time may be used if the dye concentrations or incubation temperature are increased.

4.3 Following incubation, add about 10 µl of the fresh LIVE/DEAD reagent solution or D-PBS to a clean microscope slide.

4.4 Using fine-tipped forceps, carefully (but quickly) invert and mount the wet coverslip on the microscope slide. To prevent evaporation, seal the coverslip to the glass slide (e.g., with clear fingernail polish). Avoid damaging or shearing cells in the preparation of the slides.

4.5 View the labeled cells under the fluorescence microscope.

Fluorescence Microplate Protocol

Select the Optical Filters for the 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-1 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-1 at 645 ± 20 nm.

Preparation of the Cells for the Microplate Reader

5.1 Culture adherent cells in the multiwell plate. Fibroblast cells are typically grown in the wells for 2–3 days until acceptable cell densities are obtained. Wash the cells gently with 500–1000 volumes of Dulbecco's phosphate-buffered saline (D-PBS) prior to the assay (note A). After the last wash, add sufficient D-PBS to at least cover the bottom of the well. The cell samples are washed to remove or to dilute esterase activity generally present in serum-supplemented growth media that could cause an increase in extracellular fluorescence due to hydrolysis of calcein AM.

5.2 Wash relatively nonadherent cells (e.g., leukocytes or other suspended cells) in a test tube with 500–1000 volumes of tissue culture–grade D-PBS and sediment by centrifugation to remove serum esterase activity.

5.3 Add the cells in a sufficient volume of buffer to at least cover the bottom of the wells. In general, for flat-bottomed wells where the total capacity is 250–300 µl, add about 100 µl; for round-bottomed wells where the total capacity is 150–200 µl, add about 70 µl; for conical wells where the total capacity is 100–150 µl, add about 50 µl. Small buffer volumes may be preferred to minimize

Cambrex Bio Science Walkersville, Inc.
www.cambrex.com
biotechserv@cambrex.com
Orders: 800-638-8174 Technical Service: 800-521-0390

Part No. 06867
INST-PA-3016 09/04

dilution of cytotoxic agents and other reagents.

5.4 Treat the cells with cytotoxic agents as required at any time prior to or concurrent with LIVE/DEAD reagent staining.

5.5 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.

Determine the Optimal Dye Concentrations

Best results are obtained by adjusting the dye concentrations to achieve distinct labeling of live cells with calcein AM and dead cells with EthD-1. Changes in optical filters, instrument sensitivity settings and numbers or types of cells may require different dye concentrations. In general, it is best to use the lowest dye concentration that gives sufficient signal.

The following method can be used to determine optimal dye concentrations:

6.1 Remove the LIVE/DEAD reagents from the freezer and allow them to warm to room temperature. Select appropriate filters and settings on the plate reader.

6.2 Prepare samples of live cells as well as of dead cells. Kill the cells using any preferred method (e.g., treatment with 0.1% saponin for about 10 minutes, 0.1–0.5% digitonin for about 10 minutes, 70% methanol for about 30 minutes or complement and the appropriate IgG for about 30 minutes).

6.3 Using samples of dead cells, determine the saturating concentration of EthD-1 (the lowest concentration that still yields maximal fluorescence). Try from 0.1 to 10 µM of EthD-1, while maintaining a constant high cell concentration (about 10^6 cells per ml). Monitor the time course of staining to determine optimum incubation times (try taking measurements every 10–15 minutes). We found a 45-minute incubation in 4 µM EthD-1 saturates the binding sites in a sample of 120,000 killed mouse leukocytes.

6.4 Using samples of dead cells, determine concentrations of calcein AM that give negligible staining of dead cells (try from 0.1 to 5 µM calcein AM).

6.5 Using samples of live cells, determine the concentration of calcein AM that gives fluorescence in live cells sufficient to permit clear detection. If the signal is too low, increase the number of cells or use a slightly higher concentration of the dye.

6.6 The reagent concentrations determined in steps 6.3 and 6.5 are optimal for the viability assay.

Sample Preparation Example for Microplate Reader

Measurements

This example protocol makes 10 ml of the LIVE/DEAD reagents for use in a multiwell plate scanner at 1 µM calcein AM and 2 µM EthD-1 (we found these reagent concentrations to be optimal for mouse leukocytes). The protocol prepares a 2X concentrated reagent stock to allow for a final two-fold dilution upon addition to the wells. Ten milliliters of the stock solution at 100 µl per test gives enough dye solution for one 96-well microplate.

This is an example protocol only; the actual volumes and concentrations used in an experiment will depend on the type of cells and microplates used.

7.1 Remove the LIVE/DEAD reagent stock solutions from freezer and allow them to warm to room temperature.

7.2 Add 20 µl of the supplied 2 mM EthD-1 stock solution (Component B) to 10 ml of sterile, tissue culture–grade D-PBS, vortexing to ensure thorough mixing. This gives an approximately 4 µM EthD-1 solution.

7.3 Transfer a 5 µl aliquot of the supplied 4 mM calcein AM solution in DMSO (Component A) to the 10 ml of 4 µM EthD-1 solution. Vortex or sonicate the resulting solution to ensure thorough mixing. This gives an approximately 2 µM calcein AM and 4 µM EthD-1 working solution.

7.4 Distribute 100 µl of cell-containing buffer to each well. Add an additional 100 µl of the LIVE/DEAD working solution, yielding 200 µL per well containing 1 µM calcein AM and 2 µM EthD-1. The final concentration of DMSO is $\leq$ 0.1%, a level generally innocuous to most cells.

Cambrex Bio Science Walkersville, Inc.
www.cambrex.com
biotechserv@cambrex.com
Orders: 800-638-8174 Technical Service: 800-521-0390

Part No. 06867
INST-PA-3016 09/04

CAMBREX
Innovation. Experience. Performance.

Fluorescence Measurements Using a Microplate Reader

8.1 Prepare the samples of experimental cells (A and B below) and of live and dead cell controls (C through F below).

8.2 The set of control measurements is included to account for sources of background fluorescence, which can then be factored out in subsequent calculations. Treat the experimental and control cell samples identically (i.e., maintain constant cell numbers, reagent concentrations and incubation times and temperatures).

Label the experimental cells with calcein AM and EthD-1. Label the control samples as indicated with either calcein AM or EthD-1. A cell-free control (G and H below) may be included to test for background fluorescence from the cytotoxic agent being tested or from other additives in the medium.

8.3 Add the LIVE/DEAD reagents to the wells to the optimal final concentrations (described in Determine the Optimal Dye Concentrations).

8.4 Incubate the samples for the optimal time interval (described in Determine the Optimal Dye Concentrations), e.g., at room temperature for 30-45 minutes.

8.5 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:

$$\% \text{ Live Cells} = \frac{F(530)_{sam} - F(530)_{min}}{F(530)_{max} - F(530)_{min}} \times 100\%$$

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

$$\% \text{ Dead Cells} = \frac{F(645)_{sam} - F(645)_{min}}{F(645)_{max} - F(645)_{min}} \times 100\%$$

Determining Absolute Numbers of Live and Dead Cells

The total number of cells in a sample can be counted by killing all of the cells (see step 6.2), labeling with a saturating concentration of EthD-1 and measuring fluorescence at > 600 nm. The fluorescence intensity is then linearly related to the total number of cells present in the sample.

This may be done at the end of a set of viability experiments in order to express cell viability in terms of absolute numbers of live and dead cells.

Cambrex Bio Science Walkersville, Inc.
www.cambrex.com
biotechserv@cambrex.com
Orders: 800-638-8174 Technical Service: 800-521-0390

Part No. 06867
INST-PA-3016 09/04

CAMBREX
Innovation. Experience. Performance.

9.1 Perform the cell-viability measurements (described in Fluorescence Measurements Using Microplate Reader).

9.2 Kill all of the cells in the samples (e.g., by adding about 0.1% saponin to each well; add 2–5 µl per well from a 5% saponin stock solution in distilled water).

9.3 Mix by shaking the plate; wait 10 minutes (or until the signal equilibrates).

9.4 Read the EthD-1 fluorescence at ~645 nm. The fluorescence intensity is linearly related to the number of cells in the sample. This value can be compared to a standard curve of numbers of dead cells versus fluorescence intensity, generated separately by using a saturating EthD-1 concentration on known numbers of dead cells in a microplate.

Adaptation for Use in Flow Cytometry

The LIVE/DEAD assay can easily be adapted for use in flow cytometry. Cells cultured in suspension, or adherent cells following treatment with trypsin to produce a 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 D-PBS, pelleted again and resuspended in the working solution of calcein AM and EthD-1. We recommend examining the cells by fluorescence microscopy and performing control experiments to confirm that the cells are stained appropriately (see Fluorescence Microscopy Protocol).

Notes

[A] Any standard saline buffer may be used throughout these protocols. Colored additives like phenol red should be checked, however, to see if they interfere with the fluorescence (see step 8.2). A suggested buffer is sterile tissue culture–grade D-PBS: KCl (200 mg/L), KH_2PO_4 (200 mg/L), NaCl (8000 mg/L), and Na_2HPO_4 (1150 mg/L).

References

1. Principles and Methods of Toxicology, Third Edition, A.W. Hayes, Ed., Raven Press (1994) pp. 1231–1258;
2. J Immunol Methods 177, 101 (1994);
3. J Microbiol Methods 17, 1 (1993);
4. J Neurosci 15, 5389 (1995);
5. J Cell Sci 106, 685 (1993);
6. Pflügers Arch 427, 24 (1994);
7. Cytometry 20, 181 (1995);
8. J Biol Chem 270, 23895 (1995);
9. Biol Chem 270, 7791 (1995);
10. J Neurosci 14, 2464 (1994);
11. J Neurosci 14, 2260 (1994);
12. J Biol Chem 269, 6803 (1994);
13. J Cell Biol 133, 1041 (1996);
14. J Cell Biol 133, 1053 (1996);
15. J Immunol Methods 177, 101 (1994);
16. Hum Immunol 37, 264 (1993).

Cambrex Bio Science Walkersville, Inc.

www.cambrex.com

biotechserv@cambrex.com

Orders: 800-638-8174 Technical Service: 800-521-0390

Part No. 06867

INST-PA-3016 09/04

CAMBREX

Innovation. Experience. Performance.