Live/Dead Cell Staining Kit II

PromoKine

Instruction Manual

Product Name	Product Description	Size	Catalog Number
Live/Dead Cell Staining Kit II	Fluorometric detection of viable and dead cells.	300 assays	PK-CA707-30002

Introduction

PromoKine's Live/Dead Cell Staining Kit II provides a two-color fluorescent staining of live (green) and dead cells (red) using two probes and is suited for animal live and dead cells. Calcein-AM stains live cells green, while EthD-III stains dead cells red. These probes 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 multi-well plate scanners and flow cytometers. The assay principles are general and applicable to most eukaryotic cell types, including adherent cells and certain tissues, but not to bacteria or yeast. This fluorescence-based method can also be used for assessing cell viability in place of trypan blue exclusion, ^{51}Cr release and similar methods for determining cell viability and cytotoxicity. EthD-III (PK-CA707-40050 and PK-CA707-40051) is a proprietary DNA stain that is a superior alternative to Ethidium homodimer I (EthD-I) offered by competitors. EthD-III is spectrally similar to EthD-I but has higher DNA binding affinity and stains DNA with 30% brighter fluorescence.

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 25-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.

Note that Calcein AM-based assays can be used in adherent or suspension cultures of eukaryotic cells (3) and certain tissues (4), but cannot be used in yeast or bacteria. A bacterial viability/cytotoxicity assay is also available at PromoKine (PK-CA707-30027).

Kit Contents

Calcein-AM (4 mM in anhydrous DMSO), 2 x 50 µl

EthD-III (2 mM in DMSO/H_2O 1:4 [v/v]), 2 x 150 µl

Assay number is based on 0.5 ml staining volume at 2 µM Calcein-AM/4 µM EthD-III. Number of assays may vary depending on the staining volume and optimal dye concentrations for your application. 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 Stability

Store kit at -20°C, desiccated and protected from light. Product is stable until the indicated expiry date on the kit label when stored as recommended. Note: aqueous solutions of Calcein-AM are susceptible to hydrolysis. Working solutions of Calcein-AM diluted in buffer should be used within one day of preparation. Working solutions of EthD-III in buffer can be stored at -20°C, protected from light, for at least one year.

Cell Staining Protocol

For fluorescence microscopy:

1. Warm the dye stock solutions to room temperature. Prepare a staining solution of 2 µM Calcein-AM/4 µM EthD-III by adding 5 µl of 4 mM Calcein-AM and 20 µl of 2 mM EthD-III to 10 ml of PBS or other serum-free buffer or medium. Vortex to ensure thorough mixing.

   Note: Volumes may be scaled proportionally as needed.

   Note: Optimal dye concentrations may vary depending on cell type. In general it is best to use the lowest dye concentration that gives sufficient signal. Typical staining concentrations range between 0.1 µM and 10 µM for Calcein-AM and EthD-III.

   Note: Aqueous solutions of Calcein-AM are susceptible to hydrolysis. Working solutions of Calcein-AM diluted in buffer should be used within one day of preparation. Working solutions of EthD-III can be stored at -20°C, protected from light, for at least 1 year.
2. Wash the cells twice with serum-free buffer or medium to remove serum esterase activity. For suspension cells, pellet cells by centrifugation, remove the supernatant, and resuspend in wash buffer; repeat once.
3. Dead cell controls can be prepared by treating cells with 0.1% saponin or 0.1-0.5% digitonin for 10 minutes.
4. For adherent cells, add a sufficient volume of Calcein-AM/EthD-III staining solution to cover the cell monolayer. For suspension cells, resuspend the washed cell pellet in staining solution at or below the typical cell density of a confluent culture.
5. Incubate the cells for 30-45 minutes at room temperature.
6. Image the labeled cells by fluorescence microscopy. Calcein and EthD-III can be viewed simultaneously with a conventional fluorescein long pass filter, or the dyes can be imaged separately; calcein can be viewed with a standard fluorescein bandpass filter and EthD-III can be viewed with filters for rhodamine, propidium iodide or Texas Red®.

   Optional: The staining solution can be removed and replaced with fresh buffer or medium or your choice prior to imaging. For suspension cells, pellet the cells by centrifugation, remove the staining solution, and resuspend the cells in fresh buffer or medium.

For flow cytometry:

1. Stain cells in suspension (or trypsinized adherent cells in suspension) according to the protocol for fluorescence microscopy.
2. Pellet the cells by centrifugation and resuspend in your preferred buffer for flow cytometry analysis.
3. Analyze calcein fluorescence in the fluorescein channel, and EthD-III fluorescence in the channel for either propidium iodide or Texas Red®.

For fluorescence microplate reader:

1. Grow adherent cells or aliquot suspension cells in well of a 96-well microplate.

   Note: The range of detection for cells is usually between 200-500 and 10^6 cells per well of a 96-well plate.

   Note: Dead cells can be obtained for use as a control by treatment with 0.1% saponin or 0.1-0.5% digitonin for about 10 minutes.
2. Prepare a staining solution of 2 µM Calcein-AM/4 µM EthD-III. Warm the dye stock solutions to room temperature. Add 20 µl of 2 mM EthD-III and 5 µl of 4 mM Calcein-AM to 10 ml of PBS or other serum-free buffer or medium. Vortex to mix well.

   Note: The 10 ml of staining solution is sufficient for one 96-well microplate; volumes may be scaled proportionally as needed.

   Note: Optimal dye concentrations may vary depending on cell type. In general it is best to use the lowest dye concentration that gives sufficient signal. Typical staining concentrations range between 0.1 µM and 10 µM for Calcein-AM and EthD-III.

   Note: Aqueous solutions of Calcein-AM are susceptible to hydrolysis. Working solutions of Calcein-AM diluted in buffer should be used within one day of preparation. Working solutions of EthD-III can be stored at -20°C, protected from light, for at least 1 year.
3. Wash the cells in serum-free buffer or medium to remove serum esterase activity. For adherent cells in a 96-well plate, wash with 100 µl buffer per well. For suspension cells, pellet the cells by centrifugation in the plate and then resuspend the cells in 100 µl serum-free medium or buffer; repeat once.
4. Add 100 µl serum-free buffer to each well. For suspension cells, resuspend in 100 µl serum-free buffer per well.
5. Add 100 µl of the Calcein-AM/EthD-III working solution to each well. This results in a final volume of 200 µl per well, and final concentrations of 1 µM Calcein-AM and 2 µM EthD-III. Pipet gently up and down, or shake the plate on an orbital shaker to mix well.
6. Incubate the samples at room temperature for 30–45 minutes.
7. Measure fluorescence using a microplate reader. Calcein can be detected using settings for fluorescein, while EthD-III can be detected using settings for rhodamine or Texas Red®. See spectral properties for optimal excitation/emission wavelengths.

   Note: Relative fluorescence values (RFU) can be compared between samples to measure changes in the number of live or dead cells in a sample relative to a reference sample. See below for alternative methods of data analysis.

Determining the percentage of live and dead cells in a population

The following controls can be used to determine the percentage of live or dead cells in a population. These include dead cell controls, healthy cell controls, and cell-free controls. Dead cell controls can be prepared by treating cells with 0.1% saponin or 0.1-0.5% digitonin for about 10 minutes.

1. Prepare working solution and stain cells as described in the microplate protocol above. In addition, prepare 1 ml each of separate solutions of 2 μM Calcein-AM alone and 4 μM EthD-III alone to stain the controls as indicated below.
2. Measure fluorescence of experimental and control samples:

   A: Fluorescence at 645 nm in the experimental cell sample, labeled with Calcein-AM and EthD-1 = $F(645)_{sam}$

   B: Fluorescence at 530 nm in the experimental cell sample, labeled with Calcein-AM and EthD-1 = $F(530)_{sam}$

   C: Fluorescence at 645 nm in a sample where all the cells are dead, labeled with EthD-1 only = $F(645)_{max}$

   D: Fluorescence at 645 nm in a sample where all the cells are dead, labeled with Calcein-AM only = $F(645)_{min}$

   E: Fluorescence at 530 nm in a sample where the majority of cells are alive, labeled with EthD-1 only = $F(530)_{min}$

   F: Fluorescence at 530 nm in a sample where the majority of cells are alive, labeled with Calcein-AM only = $F(530)_{max}$

   G: Fluorescence at 530 nm of the cell-free sample with or without dye added = $F(530)_0$

   H: Fluorescence at 645 nm of a cell-free sample with or without dye added = $F(645)_0$

3. Calculate the percentages of live and dead cells from the fluorescence readings:

% Live Cells = (B - E) ÷ (F - E)

% Dead Cells = (A - D) ÷ (C - D)

Determining absolute numbers of live and dead cells in a population

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 of each dye is linearly related to the total number of live or dead cells present in the sample, respectively.

References

1. Principles and Methods of Toxicology, Third Edition, A.W. Hayes, Ed., Raven Press (1994) pp. 1231–1258.
2. Papadopoulos NG, et al. J Immunol Methods 177, 101 (1994).
3. Vaughan PJ, et al. J Neurosci 15, 5389 (1995).
4. Poole CA, et al. J Cell Sci 106, 685 (1993).

Intended Use

For *in vitro* research use only. Not for diagnostic or therapeutic procedures.

PromoCell GmbH

Sickingenstr. 63/65
69126 Heidelberg
Germany

Email: info@promokine.info
www.promokine.info

North America
Phone: 1 – 866 – 251 – 2860 (toll free)
Fax: 1 – 866 – 827 – 9219 (toll free)

Deutschland
Telefon: 0800 – 776 66 23 (gebührenfrei)
Fax: 0800 – 100 83 06 (gebührenfrei)

France
Téléphone: 0800 90 93 32 (ligne verte)
Téléfax: 0800 90 27 36 (ligne verte)

United Kingdom
Phone: 0800 – 96 03 33 (toll free)
Fax: 0800 – 169 85 54 (toll free)

Other Countries
Phone: +49 6221 – 649 34 0
Fax: +49 6221 – 649 34 40

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