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Cell-Check™ Viability/Cytotoxicity Kit for Animal Cells

Catalog Number: A017

Table 1. Kit Components and Storage

Material	Amount	Concentration	Storage	Stability
Calcein AM (Component A)	**100 µL**	**4 mM**	**-20 °C**	**The product is stable for one year when stored as directed.**
Propidium Iodide (Component B)	**100 µL**	**8 mM**	**-20 °C**	

Number of assays: 1,000 tests using a fluorescence microscope or 100 tests using a flow cytometer.
Approximate fluorescence excitation/emission maxima, in nm: Calcein**:** 494/517; Propidium iodide: 528/617, bound to DNA.
Note: Calcein AM may hydrolyze if exposed to moisture.

Introduction

The Cell-Check™ Viability/Cytotoxicity Kit for Animal Cells 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. 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 and certain tissues, but not to bacteria or yeast. It is generally faster, less expensive, safer and a more sensitive indicator of cytotoxic events than alternative methods.

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/~520 nm). Propidium iodide (PI) enters cells with damaged membranes and undergoes a 30-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (Ex/Em ~528 nm/~617 nm). PI 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.

Fluorescence Microscopy Protocol

1. Working Solution Preparation (2 µM calcein AM, 4 µM PI)

Best results are obtained by adjusting the dye concentrations to achieve distinct labeling of live cells with calcein AM and of dead cells with PI. 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 PI.

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

1.2 Add 5 µL of 8 mM PI 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 PI solution.

1.3 Combine the reagents by transferring 5 µL of 4 mM calcein AM stock solution (Component A) to the 10 mL PI solution. Vortex the resulting solution to ensure thorough mixing.

1.4 The resulting working solution (2 µM calcein AM and 4 µM PI) is then added directly to cells.

Note: the aqueous solutions of calcein AM are susceptible to hydrolysis. Aqueous working solutions should therefore be used within one day.

2. Cell Preparation

2.1 Adherent cells may be cultured on sterile glass coverslips or chamber slides. Nonadherent cells may be cultured in disposable petri dishes or other suitable containers.

2.2 Wash the cells prior to the assay to remove serum esterase activity. Wash adherent cells gently with Dulbecco's phosphate-buffered saline (D-PBS). Wash non-adherent cells in a test tube with tissue culture grade D-PBS, 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.

2.3 (Optional) Treat the cells with cytotoxic agents as required at certain time period.

3. Perform the Viability Assay

3.1 Add 100-150 µL of calcein AM / PI working solution (prepared in step 1.4) 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.
3.2 Incubate the cells for 30–45 minutes at room temperature.
3.3 Following incubation, add about 100 µL of D-PBS to a clean microscope slide, and wash the coverslip with D-PBS.
3.4 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.
3.5 View the labeled cells under the fluorescence microscope.

Optical Filter Selection

Calcein and PI 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 PI can be viewed with filters for PI or Texas Red. Typical characteristics of some appropriate filters are summarized in Table 1.

Table 1. Characteristics of common filters suitable for use with Calcein and PI

Omega Filters	Chroma Filters	Notes
XF25, XF26, XF115	11001, 41012, 71010	*Longpass and dual emission filters useful for simultaneous viewing of calcein and PI*
XF22, XF23	31001, 41001	*Bandpass filters for viewing calcein alone*
XF32, XF43, XF102, XF108	31002, 31004, 41002, 41004	*Bandpass filters for viewing PI alone*

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 absorbance. Calcein is well excited using a fluorescein

optical filter (485 ± 10 nm) whereas PI 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 PI at 645 ± 20 nm.

Prepare the Cells for the Microplate Reader

4.1 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. 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.

4.2 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.

4.3 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 dilution of cytotoxic agents and other reagents.

4.4 (Optional) Treat the cells with cytotoxic agents as required at certain time period.

4.5 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 PI. 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.

5.1 Remove the Calcein AM and PI reagent stock solutions from the freezer and allow them to warm to room temperature. Select appropriate filters and settings on the plate reader.

5.2 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).

5.3 Using samples of dead cells, determine the saturating concentration of PI (the lowest concentration that still yields maximal fluorescence). Try from 0.1 to 10 µM of PI, 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 PI saturates the binding sites in a sample of 120,000 killed mouse leukocytes.

5.4 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).

5.5 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.

5.6 The reagent concentrations determined in steps 5.3 and 5.5 are optimal for the viability assay.

Perform the Cell Viability Measurements Using a Microplate Reader

6.1 Prepare 2X working solution of calcein AM and PI according to steps 1.1-1.4 using the optimal concentration determined in steps 5.3-5.5.

6.2 Prepare the samples of experimental cells (A and B below) and of live and dead cell controls (C through F below) according steps 4.1-4.5. For measuring the relative increase in the number of live or dead cell number, these controls may not be required. These controls include live cell control (E and F below) and dead cell control (C and D below).

6.3 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 PI. Label the control samples as indicated with either calcein AM or PI.

6.4 Add an equal volume of 2X working solution of calcein AM and PI to each well. (e.g., add 100 μL of 2X working solution to each well containing 100 μL of cells)

6.5 Incubate the samples at room temperature for 30–45 minutes.

6.6 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 PI = **$F(645)_{sam}$**
B. Fluorescence at 530 nm in the experimental cell sample, labeled with calcein AM and PI= **$F(530)_{sam}$**
C. Fluorescence at 645 nm in a sample where all the cells are dead, labeled with PI 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 all (or nearly all) cells are alive, labeled with PI only = **$F(530)_{min}$**
F. Fluorescence at 530 nm in a sample where all (or nearly all) cells are alive, labeled with calcein AM only = **$F(530)_{max}$**

6.7 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:

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

Flow Cytometry Protocol

7.1 Allow all reagents to come to room temperature.

7.2 Make an 80-fold dilution of calcein AM (Component A) in DMSO to make a 50 μM working solution (i.e., add 2 μL of Component A to 158 μL DMSO). The working solution should be used within one day.

7.3 Prepare a 1 mL suspension of cells with 0.1 to 5 × 10^6 cells/mL for each assay. Cells may be in culture medium or buffer.

7.4 Add 2 μL of 50 μM calcein AM working solution and 1 μL of the 8 mM PI stock to each milliliter of cells. Mix the sample.

7.5 Incubate the cells for 15–20 minutes at room temperature, protected from light.

7.6 As soon as possible after the incubation period (within 1–2 hours), analyze the stained cells by flow cytometry using 488 nm excitation and measuring green fluorescence emission for calcein (i.e., 530/30 bandpass) and red fluorescence emission for PI (i.e., 610/20 bandpass). Gate on cells to exclude debris. Using single color stained cells, perform standard compensation. The population should separate into two groups: live cells will show green fluorescence and dead cells will show red fluorescence.