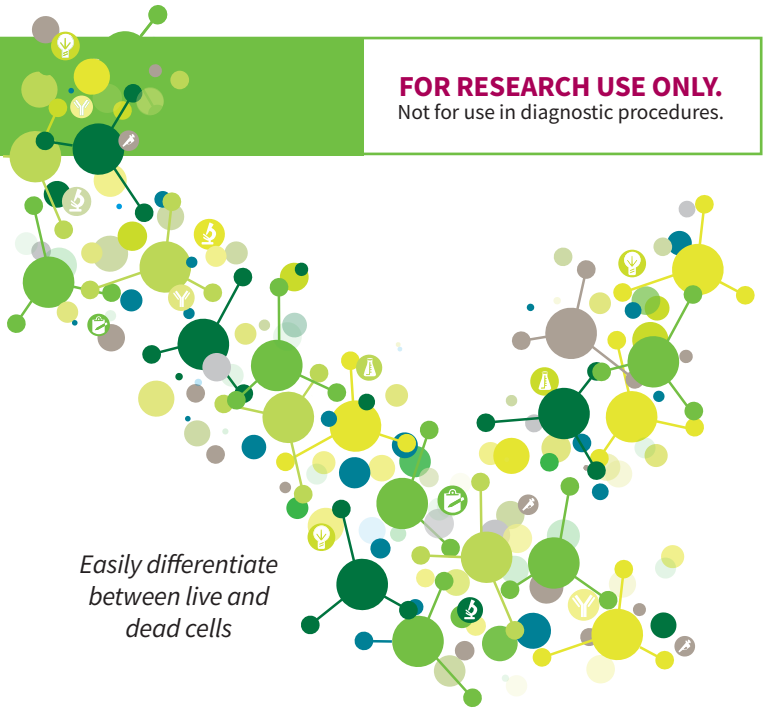
Basic Calcein AM Cell Viability Kit

Catalog #9153

FOR RESEARCH USE ONLY.
Not for use in diagnostic procedures.



Easily differentiate between live and dead cells

1. INTRODUCTION

Assessment of cell viability is a critical step during the evaluation of novel drug treatments and therapies for potential cytotoxic properties. With cell viability assessment playing a central role in countless research and environmental safety studies, there is an ever present need for simple, straightforward analysis methods capable of distinguishing between live and dead cells. The Basic Calcein AM Cell Viability kit developed by ImmunoChemistry Technologies, LLC (ICT) allows for easy and simultaneous differentiation of live and dead cells within a single sample.

To use Calcein AM, simply add the reagent directly to the cell sample, incubate, and analyze (no wash steps necessary). Calcein AM is a membrane permeant, fluorogenic reagent widely recognized for its utility in assessing the relative cell viability status of different cell populations. Calcein AM's overall hydrophobic nature allows it to readily traverse the lipid bilayer structure of the cell membrane in a concentration gradient-dependent manner. Once inside the cell, the hydrophobic and non-fluorescent Calcein AM is quickly hydrolyzed by intracellular esterases that are active in live cells. This leads to the cleavage and removal of two non-polar acetoxymethyl ester (AM) groups. Once the AM groups have been cleaved, the resulting polar (hydrophilic) and now fluorescence-capable Calcein dye molecule is efficiently retained within the confines of the cell membrane. Polar dye molecules will naturally be excluded from passive diffusion back out of the cell again due to the hydrophobic lipid bilayer composition of the cell membrane. Dead cells lack active esterases and do not cleave Calcein AM.

The large quantum yield of Calcein dyes enables them to be readily detected within widely used applications such as flow cytometers, fluorescence plate readers, and fluorescence microscopes. The degree of fluorescence correlates with relative cell viability status[1-3]. For microscopy usage, Hoechst 33342 is included with the kit to concurrently label nuclei after labeling with Calcein.

Calcein optimally excites at 494 nm with maximal emission at 517 nm. Hoechst 33342 can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm.

2. KIT CONTENTS

- 1 vial of Calcein AM Reagent, #6696
- 1 vial of Hoechst 33342, 200 µg/mL (1 mL), #639
- 1 bottle of 10X Cellular Assay Buffer (60 mL), #6695

3. STORAGE

- Store the unopened Calcein AM (Pack 2) at ≤-20°C until the expiration date.
- Store the remaining unopened kit components (Pack 1) at 2-8°C until the expiration date.

Once reconstituted with DMSO, use Calcein AM immediately, or aliquot and store at ≤-20°C for 6 months protected from light. Avoid repeated freeze thaw cycles.

4. SAFETY DATA SHEETS (SDS)

Safety data sheets are available online at www.immunochemistry.com or by calling 1-800-829-3194 or 952-888-8788.

5. RECOMMENDED MATERIALS

- DMSO, up to 250 µL (50 µL per vial to reconstitute Calcein AM, and more to create controls)
- diH_2O (540 mL per bottle to dilute 10X Cellular Assay Buffer)
- Phosphate buffered saline (PBS) pH 7.4, up to 100 mL, to dilute Calcein AM and handle cells
- FBS and/or BSA to add to the buffer when handling cells
- Cultured cells treated with the experimental conditions ready to be analyzed
- 90% ethanol or 3% formaldehyde to create live/dead controls for Calcein AM (See Figure 1)
- Hemocytometer
- Centrifuge at <200 x g
- FACS tubes (for flow cytometry analysis)
- Slides and coverslips (for microscopy analysis)
- Black 96-well microtiter plate, flat bottom, non-treated, non-sterile (ICT catalog #266). If using a bottom reading instrument, use a plate with black walls and a clear bottom. If cutluring cells in the plate, use a black sterile tissue culture plate. Plates only required for fluorescence plate reader analysis.
- 15 mL polypropylene centrifuge tubes (1 per sample)
- Ice bath (if using 3% formaldehyde to create dead cell control population)

6. DETECTION EQUIPMENT

The assay can be analyzed with a:

- Flow cytometer
- Fluorescence microscope
- Fluorescence plate reader

Use filter pairings that best approximate these settings:

- Calcein AM optimally excites at 494 nm and has a peak emission at 517 nm (use FL-1 channel for flow cytometry). Hoechst optimally excites at 365 nm and has a peak emission at 480 nm.

7. EXPERIMENTAL PREPARATION

Staining cells with Calcein AM can be completed within a few hours. However, Calcein AM is used with living cells, which require periodic maintenance and cultivation several days in advance. In addition, once the proper number of cells has been cultivated, time must be allotted for the experimental treatment, which may vary. The recommended sample size is 400 µL cells at 5 x 10^5 cells/mL.

Create cell populations, such as:

a. Cells that were exposed to the experimental treatment.

b. A negative control population of cells that received a placebo treatment.

Culture cells to a density optimal for the specific experimental protocol. Cell density should not exceed 10^6 cells/mL. Cells cultivated in excess of this concentration may begin to naturally enter apoptosis. An initial experiment may be necessary to determine how much Calcein AM to use.

• Calcein AM is NOT compatible with fixative.

8. CONTROLS

Create experimental samples and control cell populations:

a. Treated experimental population(s): cells exposed to the experimental condition(s).

b. Positive control for Calcein AM (Live cells): mock-treated cells grown in a normal culture environment (Figures 1A, 2A, and 3).

c. Negative control for Calcein AM (Dead cells): cells treated with ethanol or formaldehyde to create a dead cell population. For example, briefly expose cells to 90% ethanol for 30-60 seconds at 37°C (Figure 1B, 2B, and 3), or expose cells to 3% formaldehyde for 30 minutes on ice, then wash the cells 1X with PBS, and resuspend the sample in Cellular Assay Buffer.

A common pool of cells should be used to generate the positive and negative control populations for Calcein AM, and should contain similar quantities of cells. For example, if labeling with Calcein AM, make 4 populations:

1&2. Unlabeled: live and dead cells

3&4. Calcein AM-labeled: live and dead cells

9. PREPARATION OF 1X CELLULAR ASSAY BUFFER

ICT's Cellular Assay Buffer (catalog #6695) is an isotonic solution used to stabilize cells when staining with Calcein AM. It contains mammalian proteins to stabilize cells, and sodium azide to retard bacterial growth (1X Cellular Assay Buffer contains 0.01% w/v sodium azide). Alternative solutions including cell culture media containing FBS and other additives may be used to stabilize/dilute cells instead of 1X Cellular Assay Buffer.

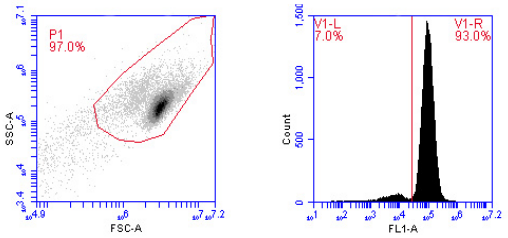
1. 10X Cellular Assay Buffer may form precipitates during cold storage. If this happens, gently warm it until all crystals have dissolved. Do not boil.
2. Dilute 10X Cellular Assay Buffer 1:10 in diH_2O. For example, add 60 mL 10X Cellular Assay Buffer to 540 mL diH_2O for a total of 600 mL.

- 1X Cellular Assay Buffer may be stored at 2-8°C and used within 1 week or frozen and used within 6 months.

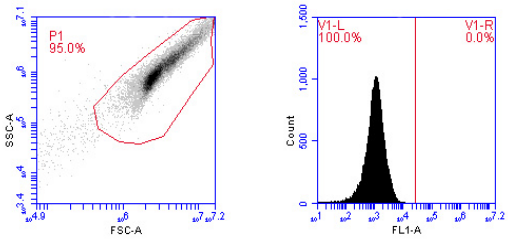
Figure 1: Live and Dead Controls Stained with Calcein AM

ICT's Calcein AM reagent was used to assess viability in Jurkat cells. Live cells exhibiting green fluorescence (right side of each histogram) can easily be distinguished from dead cells (unstained, left side of each histogram). The forward and side scatter graphs are also shown. An overlay of the live and dead cells is shown in panel C.

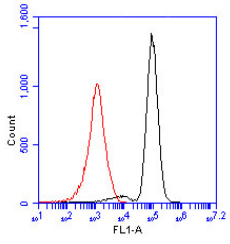
In this example, Jurkat cells were grown to 5 x 10^5 cells/mL and split into two populations. One population was mock-treated (A) while the other population was killed by exposure to 90% ethanol for 60 seconds (B). Cells that were exposed to ethanol (B) were treated with a 5-fold larger volume of PBS to stop the ethanol surface denaturation process. Cells were pelleted by centrifugation (200 x g for 5 minutes) and resuspended in PBS. Cells were stained with 1 µM Calcein AM for 60 minutes at 37°C. Then cells were analyzed using an Accuri C6 flow cytometer equipped with a FL-1 99% attenuation filter. The majority (93.0%) of the live cells stained positive with Calcein AM (A, right), while 0.0% of ethanol-killed cells stained positive (B, right). Data courtesy of Dr. Kristi Strandberg (ICT 226:87-92).



A: Live/Mock-treated Cells: 7.0 % Dead (left) 93.0 % Live (right)



B: Ethanol-killed Cells: 100.0% Dead (left) 0.0 % Live (right)



C: Overlay
Live Cells: black
Dead Cells: red

10. PREPARATION OF CALCEIN AM

Calcein AM is supplied as a lyophilized powder that may be slightly visible as an iridescent sheen inside the vial. Protect from light and use gloves when handling. Once diluted in aqueous buffer, Calcein AM solution must be used immediately; prepare it just before staining.

1. Reconstitute the vial of Calcein AM with 50 µL DMSO to form the stock solution at 2 mM. The stock solution should be colorless to light yellow. Once reconstituted in DMSO, it may be aliquoted and stored at ≤-20°C for 6 months protected from light. Avoid repeated freeze/thaw cycles.
2. Immediately prior to addition to the samples and controls, dilute the 2 mM Calcein AM stock solution 1:5 by adding 200 µL PBS. This creates a 400 µM Calcein AM solution. If staining at a final concentration of 10 µM, then this solution is ready to use.
3. If staining at a final concentration of 1 µM, further dilute the 400 µM Calcein AM solution 1:10 in PBS. The resulting solution is ready to use.

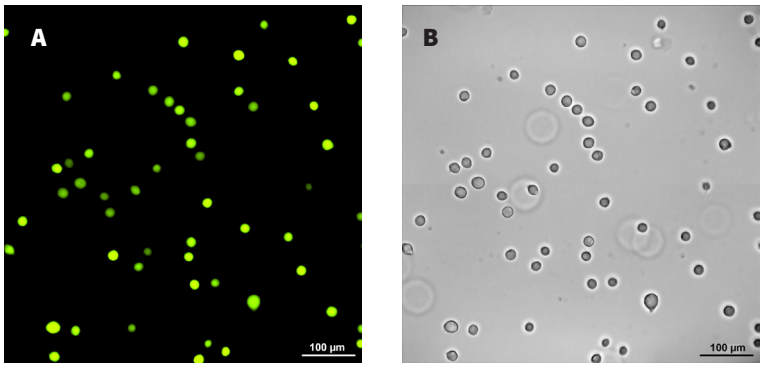
These amounts are recommendations, however, the sample size and Calcein AM staining concentration needed may vary based on the experimental conditions and the instrument used for analysis. Each investigator should adjust the amount of Calcein AM to accommodate the particular cell line and research conditions.

Figure 2: Microscopy Analysis of Live Jurkat Cells Stained with Calcein AM

Mock-treated Jurkat suspension cells were stained with 1 µM Calcein AM for 60 minutes at 37°C to detect live cells. The majority of the cells imaged were considered to be live and healthy.

Panel A reveals green fluorescence-stained live cells. Panel B shows a corresponding differential interference contrast (DIC) image, which reveals cell morphology.

Microscope images were obtained using a Nikon Eclipse 90i microscope with a Hamamatsu Flash 4.0 camera. Data courtesy of Dr. Kristi Strandberg (ICT 226:95).



Mock-treated **DIC**

11. PREPARATION OF HOECHST 33342

Hoechst 33342 is a cell-permeant nuclear stain that emits blue fluorescence when bound to double stranded DNA. It is used to stain the nuclei of living or fixed cells, to distinguish condensed pyknotic nuclei in apoptotic cells, and for cell cycle studies.

Hoechst 33342 is provided ready-to-use at 200 µg/mL. Hoechst 33342 can be used with Calcein AM to label the DNA of live and dead cells. To use, add to samples at 0.5% v/v, and incubate 5 minutes at 37°C. For example, add 2 µL of Hoechst to a 400 µL suspension cell sample.

When bound to DNA, it has a maximum absorbance at 350 nm and a maximum emission at 480 nm. It is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm.

- Hoechst 33342 contains a low concentration of Bis benzimide H 33342 trihydrochloride which is below the threshold for reporting on the safety data sheet (SDS). It is a suspected mutagen at high concentrations. Prolonged skin contact may cause redness and irritation. Because of the small quantity of product, the health hazard is small. See SDS for further information.

12. CELL STAINING PROTOCOL

Prepare experimental and control cell populations. Ideally, the cell concentration should be 3-5 x 10^5 cells/mL. The concentration should not exceed 10^6 cells/mL, as cells cultivated in excess of this concentration may begin to naturally enter apoptosis. Cells may need to be concentrated to 2-5 x 10^6 cells/mL after staining as microscopy and fluorescence plate reader analysis methods (Sections 14 and 15, respectively) require high cell concentrations.

Flow cytometry can analyze samples at 3-5 x 10^5 cells/mL.

When using a fluorescence plate reader to analyze adherent cells, culture cells on tissue culture plates with clear bottoms and black walls. Culture cells to approximately 90% confluency.

1. Expose cells to the experimental or control conditions. If analyzing with a flow cytometer, set aside two populations to create instrument controls with Calcein AM positive and negative controls (Section 8, and Figure 1).
2. Transfer 390 µL cells into fresh tubes.
3. To stain at 10 µM, add 10 µL of the reconstituted Calcein AM solution that was diluted 1:5 in PBS (Section 10) to 390 µL cells, forming a final volume of 400 µL. To stain at 1 µM, add 10 µL of the reconstituted Calcein AM solution that was subsequently diluted 1:10 in PBS (Section 10) to 390 µL cells, forming a final volume of 400 µL. If different cell volumes were used, adjust Calcein AM volume proportionally. Mix by gently flicking the tubes.
4. Incubate cells at 37°C protected from light. The incubation period may range from 30 minutes to several hours and should be optimized for each cell line and experimental condition. As cells may settle on the bottom of the tubes, gently resuspend them by swirling cells every 15 minutes to ensure an even distribution of Calcein AM throughout the staining process.

a. To analyze using a flow cytometer, refer to Section 13.

b. To analyze using a microscope, refer to Section 14.

c. To analyze using a fluorescence plate reader, refer to Section 15.

- **Wash steps are not required, as any Calcein AM that has not been cleaved by intracellular esterases will be non-fluorescent.**

13. FLOW CYTOMETRY ANALYSIS

Follow Section 12 (Steps 1-4). Sample flow cytometry data is shown in Figure 1.

5. Samples are ready for analysis. No further processing is required. To analyze the samples, measure green fluorescence (Calcein) on the FL-1 channel.

a. Live cells fluoresce green due to the presence of active esterases capable of cleaving Calcein AM.

b. Dead cells do not fluoresce green due to the absence of active esterases, leaving Calcein AM in its uncleaved (and non-fluorescent) form.

14. MICROSCOPY ANALYSIS

Follow Section 12 (Steps 1-4). Sample microscopy data is shown in Figure 2.

5. Optional Hoechst 33342 staining: If dual staining with Hoechst, add Hoechst at 0.5% v/v and incubate for 5 minutes at 37°C. For example, add 2 µL of Hoechst to a 400 µL suspension cell sample.
6. When ready to view cells, place 1 drop of cell suspension onto a microscope slide and cover with a coverslip.
7. Observe cells under a fluorescence microscope using excitation at 470-490 plus a >520 nm long pass filter. Live cells fluoresce green. Observe Hoechst staining using a UV-filter with excitation at 365 nm and emission at 480 nm.

15. FLUORESCENCE PLATE READER ANALYSIS

Follow Section 12 (Steps 1-4). Sample fluorescence plate reader results are shown in Figure 3.

5. Determine the concentration and compare the cell density of each sample. The live/mock-treated population may have more cells than the killed population. Adjust the volume of the cell suspensions to equalize the cell density. When ready to read, cells should be between 2-5 x 10^6 cells/mL.
6. Pipette 100 µL cell suspension per well into a black-opaque-well, fluorescence plate reader-compatible, microtiter plate. It is recommended that each sample be tested in duplicate (at a minimum).

• **Do NOT use clear plates. Avoid bubbles.**

7. Perform an endpoint read. If possible, set the excitation wavelength to 494 nm and the emission wavelength to 517 nm.

a. Live cells fluoresce green due to the presence of active esterases capable of cleaving Calcein AM.

b. Dead cells do not fluoresce green due to the absence of active esterases, leaving Calcein AM in its uncleaved (and non-fluorescent) form.

16. REFERENCES AND CITATIONS

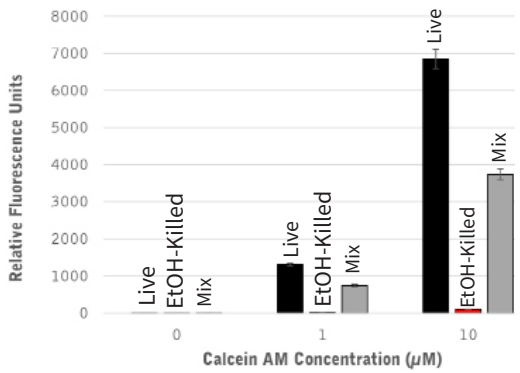
1. Mueller, H., Kassack, M. U. & Wiese, M. Comparison of the usefulness of the MTT, ATP, and calcein assays to predict the potency of cytotoxic agents in various human cancer cell lines. J Biomol Screen 9, 506-515, doi:10.1177/1087057104265386 (2004).
2. Papadopoulos, N. G. et al. An improved fluorescence assay for the determination of lymphocyte-mediated cytotoxicity using flow cytometry. J Immunol Methods 177, 101-111 (1994).
3. Uggeri, J. et al. Calcein-AM is a detector of intracellular oxidative activity. Histochem Cell Biol 122, 499-505, doi:10.1007/s00418-004-0712-y (2004).

Figure 3: Fluorescence Plate Reader Analysis of Live and Dead Jurkat Cells Stained with Calcein AM

Jurkat suspension cells were live/mock-treated (black bars) or killed by 60 second exposure to 90% ethanol (red bars). A mix of the untreated and ethanol-treated cells is represented by gray bars.

Jurkat suspension cells were stained with 0, 1, and 10 µM Calcein AM for 60 minutes to detect live cells. Samples were concentrated so that each sample contained ≥ 2 x 10^6 cells/mL. Samples were loaded into wells of a black bottom 96-well plate in triplicate (100 µL per well) and then analyzed using a Molecular Devices SpectraMax M5e fluorescence plate reader. The excitation and emission were 494 nm and 517 nm, respectively.

Cells stained with 0 µM Calcein AM showed little fluorescence. Cells stained with 1 µM Calcein AM showed an increase in fluorescence signal. Cells stained with 10 µM Calcein AM showed even greater signal. Ethanol-killed cells had little or no fluorescence. Live/mock-treated cells had the greatest fluorescence. A mixture of live and dead cells showed intermediate levels of fluorescence. Data courtesy of Dr. Strandberg (ICT 227:1-2).



Thank you for using our Basic Calcein AM Cell Viability Kit! If you have any questions, or would like to share your data, please contact us at 1-800-829-3194 or 952-888-8788, or send an email to help@immunochemistry.com.

ImmunoChemistry TECHNOLOGIES LLC

BRIGHT MINDS, BRIGHT SOLUTIONS.

ImmunoChemistry Technologies, LLC gratefully acknowledges the significant contributions made by one of its founders, Brian W. Lee, Ph.D in the development of this product, including the creation and illustration of its strategy and protocol.

ImmunoChemistry Technologies, LLC
9401 James Ave. S. #155, Bloomington, MN 55431 USA 952-888-8788, 800-829-3194