Advanced Calcein AM

Cell Viability Kit; Catalog #9154

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

Simultaneously detect live, membrane-compromised, and necrotic cells

1. INTRODUCTION

The Advanced Calcein AM Cell Viability kit developed by ImmunoChemistry Technologies, LLC (ICT) combines Calcein AM with 7-aminoactinomycin D (7-AAD) to allow for easy and simultaneous labeling of live, membrane compromised, and dead cells within a single sample.

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.

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 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. Because Calcein alone will detect cells that are alive, but some of which could possess compromised cell membrane structure and thus be in the process of dying, it is possible to obtain an overly positive picture of the overall health status of the cell population.

Loss of cell membrane integrity, often indicative of necrosis or late stage apoptosis, can be detected using the vital staining dye, 7-AAD, a red fluorescing live/dead stain. This dye easily penetrates cell membrane-compromised cells, binding tightly to GC rich regions of DNA[4-8]. Combining these two different types of fluorescent cell-status-indicator reagents within a single test allows for better resolution of the live and dead cell populations by making it possible to identify the percentage of cells that are 7-AAD positive versus 7-AAD negative within the green fluorescing Calcein positive cell populations.

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. 7-AAD can be efficiently excited at 485-495 nm, but exhibits an optimal emission well into the red fluorescence range (647 nm). This significant difference in fluorescence emission wavelength between the green fluorescing Calcein and the red fluorescing 7-AAD live/dead dye simplifies flow cytometer gating and compensation. Live cells retaining hydrolyzed Calcein are monitored on the FL-1 channel, while membrane compromised or dead cells that have taken up 7-AAD are monitored using the FL-3 channel. Combining the Calcein AM cell viability dye with a membrane integrity dye like 7-AAD makes it easy to distinguish between live, live but membrane-compromised, and dead cells within a single sample.

Learn more about all of ICT's products at www.immunochemistry.com or call 1-800-829-3194.

2. KIT CONTENTS

- 1 vial of Calcein AM Reagent, #6696
- 2 vials of 7-Aminoactinomycin D (7-AAD) vital dye (0.26 mg vial), #6163
- 1 vial of Hoechst 33342 (1mL, 200 µg/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 and 7-AAD 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 1,000 µL (50 µL per vial to reconstitute Calcein AM, 260 µL per vial to reconstitute 7-AAD, and more to create controls)
- DiH_20 (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% ETOH or 3% formaldehyde to create live/dead controls for Calcein AM and 7-AAD staining (Figures 1 and 2)
- Hemocytometer
- Centrifuge at <200 x g
- FACS tubes (for flow cytometry analysis)
- 15 mL polypropylene centrifuge tubes (1 per sample)
- Ice bath (if using 3% formaldehyde to create dead cell control population)
- Hot water bath (if using 56°C water bath to create dead cell control population)

6. DETECTION EQUIPMENT

The assay can be analyzed with a:

- Fluorescence microscope
- Flow cytometer

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). Some flow cytometers may require use of a FL-1 99% attenuation filter.
- 7-AAD optimally excites at 546 nm. It has a peak emission at 647 nm (use FL-3 channel), but displays adequate emission properties when excited within a blue (488-492 nm) light source.
- Hoechst optimally excites at 365 nm and has a peak emission at 480 nm.

7. EXPERIMENTAL PREPARATION

Staining cells with Calcein AM and 7-AAD can be completed within a few hours. However, Calcein AM and 7-AAD are 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.

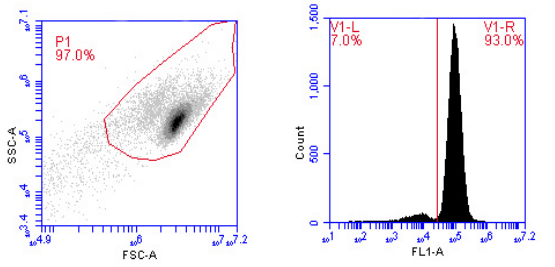
Culture cells to a density optimal for the specific experimental protocol. Cell density should not exceed 10^6 cells/mL. Cells cultivated in excess of this concentration may begin to naturally enter apoptosis. An initial experiment may be necessary to determine how much Calcein AM to use.

Figure 1: Live and Dead Controls Stained with Calcein AM

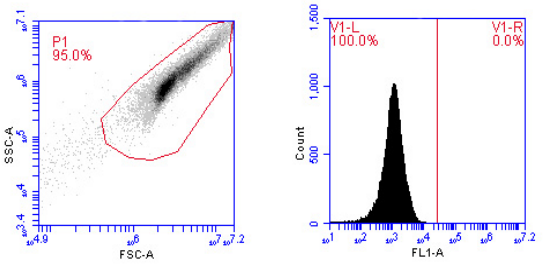
ICT's Calcein AM reagent was used to assess viability in Jurkat cells. Using Calcein AM, live cells exhibiting green fluorescence (right side of each histogram) can easily be distinguished from dead cells (unstained, left side of each histogram). The forward and side scatter graphs are also shown.

To create a positive control for Calcein AM, a population of healthy/untreated cells is needed (Section 8). If analyzing with a flow cytometer, it will be used to compensate the instrument.

In this example, Jurkat cells were grown to 5 x 10^5 cells/mL and split into two populations. One population (A, top) was untreated while the other population (B, bottom) was killed by exposure to 90% ethanol for 60 seconds. 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 1 hour at 37°C and 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: Untreated/Live Cells: 7.0 % Dead (left) 93.0 % Live (right)



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

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): non-treated cells grown in a normal culture environment (Figure 1).

- **The Calcein AM positive control also serves as the negative control for 7-AAD.**

c. Positive control for 7-AAD (Dead cells): cells treated with ethanol, formaldehyde, or heat to create a dead cell population (Section 9 and Figure 2).

- **The 7-AAD positive control also serves as the negative control for Calcein AM.**

A common pool of cells should be used to generate the positive control population for Calcein AM (which is also the negative control for 7-AAD) and the negative control population for Calcein AM (which is also the positive control for 7-AAD) and should contain similar quantities of cells. For example, if labeling with Calcein AM and 7-AAD stain, make 8 populations:

1&2. Unlabeled: live and dead cells

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

5&6. Calcein AM-labeled and 7-AAD-labeled: live and dead cells

7&8. 7-AAD-labeled: live and dead cells

9. PREPARATION OF DEAD CELLS (7-AAD POSITIVE CONTROL/CALCEIN AM NEGATIVE CONTROL)

Prior to commencing the experiment, determine a reproducible method for obtaining a population of dead/killed cells to use as a positive control for 7-AAD staining. This can easily be achieved using many different techniques. For example, briefly expose cells to 90% ethanol for 30-60 seconds at 37°C (Figure 2), expose cells to 3% formaldehyde for 30 minutes on ice, or incubate cells in a 56°C hot water bath for 45 minutes.

10. PREPARATION OF 1X CELLULAR ASSAY BUFFER

ICT's Cellular Assay Buffer (catalog #6694) is an isotonic solution used to stabilize cells when staining with Calcein AM and 7-AAD. 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 stain cells instead of the 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.

11. 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.

12. PREPARATION OF 7-AAD

Detection of cell membrane integrity loss, indicative of necrosis or late stage apoptosis, is detected using the red fluorescent live/dead stain, 7-AAD. This vital dye works by penetrating cell membrane-compromised cells and tightly binding to GC rich regions of DNA. 7-AAD is supplied as a lyophilized powder that may be slightly visible as a red sheen inside the vial. Protect from light and use gloves when handling.

1. Reconstitute each vial of 7-AAD with 260 µL DMSO to create a stock concentrate at 1 mg/mL. Mix by swirling or tilting the vial, allowing the DMSO to travel around the base of the amber vial until the reagent is completely dissolved. At room temperature, the reagent should be dissolved within a few minutes forming a red solution.

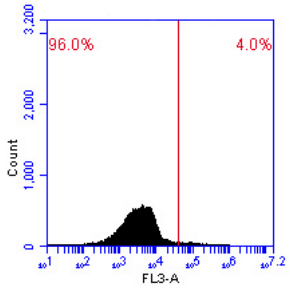
Figure 2: Live and Dead Controls Stained with 7-AAD

Detection of cell membrane integrity loss, indicative of necrosis or late stage apoptosis, can be done using the red fluorescent live/dead stain, 7-AAD. This vital dye works by penetrating cell membrane-compromised cells and tightly binding to GC rich regions of the DNA.

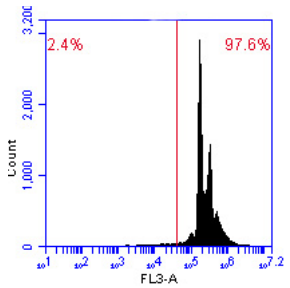
Using 7-AAD, live cells (unstained, left side of each histogram) can easily be distinguished from dead/membrane compromised cells exhibiting red fluorescence (right side of each histogram).

To create a positive control for 7-AAD, a population of dead or killed cell is needed (Section 9). If analyzing with a flow cytometer, it will be used to compensate the instrument.

In this example, Jurkat cells were grown to 5 x 10^5 cells/mL and split into two populations. One population (A) was left untreated while the other population (B) was treated with 90% ethanol for 60 seconds. 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 then stained with 7-AAD for 10 minutes on ice, and analyzed using an Accuri C6 flow cytometer in FL-3. Only 4% of untreated cells (A) are dead compared with 97.6% of the treated cells (B). Data courtesy of Dr. Kristi Strandberg, ICT 226:30-31.



A: Untreated Cells:
96.0 % Live (left)
4.0 % Dead (right)



B: Ethanol-Treated
(Killed) Cells:
2.4 % Live (left)
97.6 % Dead (right)

2. If storing the stock concentrate for future use, prepare small aliquots (50 µL, for example) to avoid freeze-thaw cycles. The stock concentrate will be stable for 6 months when protected from light and stored at or below -20°C.
3. When ready to stain cells, use 7-AAD at 1:200 dilution. For example, add 2 µL per 400 µL cells.

13. 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 and 7-AAD to label the nuclei of live, dying, and apoptotic cells.

When bound to nucleic acids, 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.

14. 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. Just prior to staining, cells may need to be concentrated to 2-5 x 10^6 cells/mL as microscopy analysis methods (Section 15) require high cell concentrations. Start with a larger volume of cells at 3-5 x 10^5 cells/mL (which is a typical density for cell culture) and then concentrate cells and resuspend to 390 µL per sample when ready for Calcein AM staining.

1. Expose cells to the experimental or control condition.
   a. If analyzing with a flow cytometer, set aside four populations to create instrument controls with Calcein AM positive and negative controls, and 7-AAD-positive and 7-AAD-negative cells (Sections 8-9, and Figures 1-2).
   b. If analyzing with a fluorescence microscope, concentrate cells to 2-5 x 10^6 cells/mL just prior to Calcein AM staining. Fluorescence microscopy may require an excess of 2 x 10^6 cells/mL to obtain 5-20 cells per image field. Flow cytometry can analyze samples at 3-5 x 10^5 cells/mL. Sample fluorescent microscopy results are shown in Figures 3, 4, and 5.
2. Transfer 390 µL cells into fresh tubes.
3. To stain at 10 µM, add 10 µL of the reconstituted Calcein AM solution that had been diluted 1:5 in PBS to 390 µL cells, forming a final volume of 400 µL. To stain at 1 µM, add 10 µL of the Calcein AM solution that had been further diluted 1:10 in PBS to 390 µL cells, forming a final volume of 400 µL. If different cell volumes were used, add Calcein AM appropriately. 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 20 minutes to ensure an even distribution of Calcein AM throughout the staining process.

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

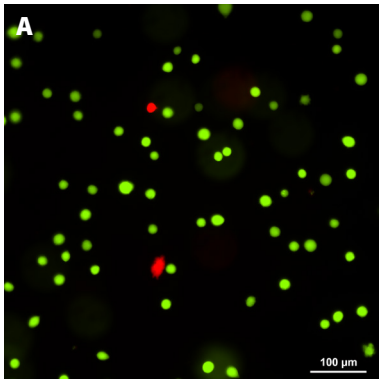
5. Stain with 7-AAD at a final concentration of 5 µg/mL (4 µM). This can be accomplished by:
   a. Adding the stock solution directly to the cell culture at 1:200; e.g. add 2 µL stock to 400 µL cell suspension.
   b. Or by diluting the stock concentrate 1:10 to form the working solution, and then adding the working solution to the cells at 1:20. For example:
      1. Add 50 µL 7-AAD stock concentrate to 450 µL PBS or sterile media to form the working solution.
      2. Mix by inverting or vortexing the vial at RT.
      3. Store on ice up to 2 hours.
      4. Add the working solution to the cell suspension at 1:20; e.g. add 20 µL diluted 7-AAD working solution into 380 µL cell suspension.
6. Incubate for 10-30 minutes on ice while protecting from light.
   a. To analyze using a microscope, refer to Section 15.
   b. To analyze by flow cytometer, refer to Section 16.

Figure 3: Microscopy Analysis of Live Jurkat Cells Stained with Calcein AM and 7-AAD

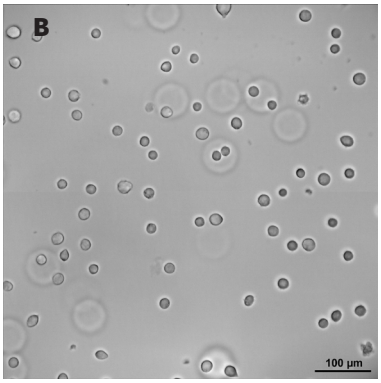
Untreated Jurkat suspension cells were dually stained with 1 µM Calcein AM for 60 minutes at 37°C to detect live cells. Cells were then stained with 4 µM 7-AAD for 10 minutes on ice to detect membrane-compromised or dead cells.

The majority of the cells imaged were considered to be live and healthy. Panel A reveals green fluorescence-stained live cells. Occasional dead cells were encountered, and stained red due to 7-AAD uptake. 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).



Untreated



DIC

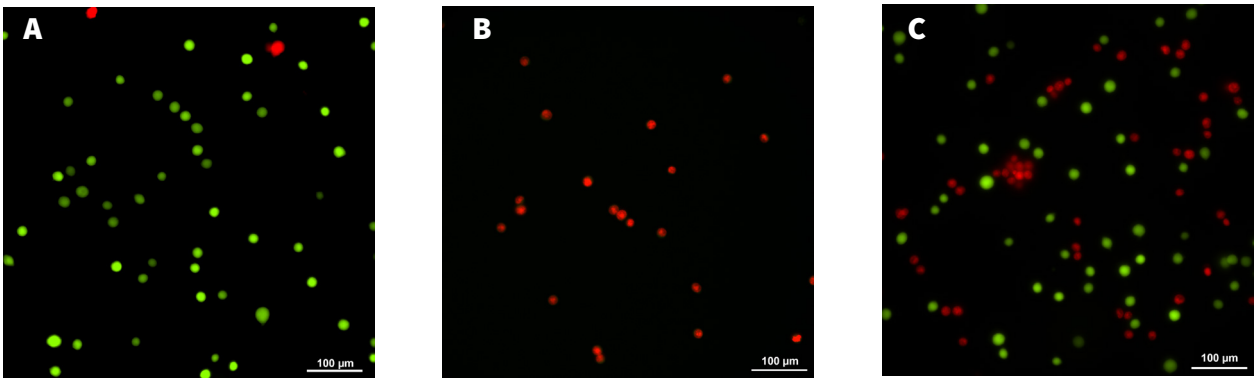
Figure 4: Comparison of Live and Dead Jurkat Cells Stained with Calcein AM and 7-AAD

Live (untreated) and dead (killed by 60 second exposure to 90% ethanol) Jurkat suspension cells were dually stained with 1 µM Calcein AM for 60 minutes at 37°C to detect live cells. Cells were then stained with 4 µM 7-AAD for 10 minutes on ice to detect membrane-compromised or dead cells.

Panel A reveals green fluorescence-stained live, untreated cells. Occasional dead cells were encountered, and stained red due to 7-AAD uptake. Panel B shows ethanol-killed cells that had been stained with both Calcein AM and 7-AAD. As there were only dead cells present in this sample type, all of the cells present stained red due to 7-AAD uptake, and no green cells were visible as Calcein AM remained in its non-fluorescent/uncleaved form. Panel C shows the staining results of a 50/50 mixture of the untreated and ethanol-killed cell populations.

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.

In Figure 7, a similar experiment was performed and cells were analyzed via flow cytometry to quantitate live, dead, and membrane compromised cells.



Untreated **Ethanol-Killed** **Mixture of A and B**

15. MICROSCOPY ANALYSIS

Follow Section 14 (Steps 1-6).

- **Calcein AM is not compatible with fixative. View stained cells immediately.**

7. Once ready to view cells, place 1 drop of cell suspension onto a microscope slide and cover with a coverslip.
8. Observe cells under a fluorescence microscope using excitation at 470-490 plus a >520 nm long pass filter (Figures 3, 4, and 5).
   - Live cells fluoresce green.
   - Necrotic or dead cells containing nucleic acid-bound 7-AAD fluoresce red.
   - Live cells with compromised membrane integrity fluoresce green and red.

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

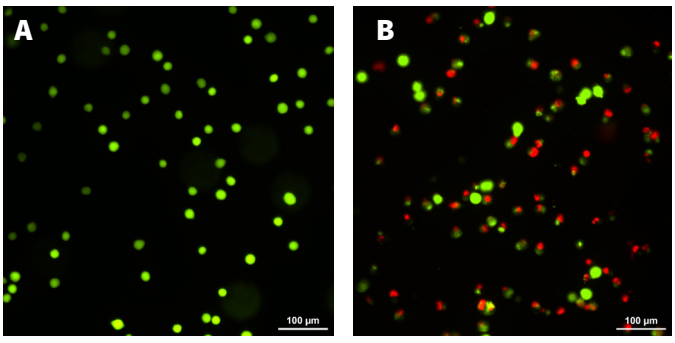
Figure 5: Microscopy Analysis of Apoptotic Jurkat Cells Stained with Calcein AM and 7-AAD

Jurkat cells were A) treated with placebo (non-induced treatment with DMSO), or B) treated with 1 µM staurosporine for 4 hours to induce apoptosis via caspase activity. Cells were then dually stained with 1 µM Calcein AM for 60 minutes at 37°C to detect live cells. Cells were then stained with 4 µM 7-AAD for 10 minutes on ice to detect membrane-compromised or dead cells.

Fluorescence microscopy revealed that all of the non-induced cells fluoresced green only, indicating they were live with intact cell membranes (A). The majority of the staurosporine-treated cells were live (fluoresced green), although many cells fluoresced both green and red indicating they had compromized membranes, and were likely in the late stage of apoptosis (B). Some of the staurosporine-treated cells fluoresced red only, indicating they were dead (B).

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.

In Figure 8, a similar experiment was performed and cells were analyzed via flow cytometry to quantitate live, dead, and membrane compromised cells.



Non-induced **Induced**

16. FLOW CYTOMETRY ANALYSIS

Follow Section 14 (Steps 1-6).

To address compensation issues and set up the flow cytometer, prepare 2 instrument control populations (live and dead):

Cells stained with only Calcein AM (Section 11 and Figure 1):

- Live cells (untreated).
- Killed cells (cell membrane compromised).

Cells stained with only 7-AAD (Section 12 and Figure 2):

- Live cells (untreated).
- Killed cells (cell membrane compromised).

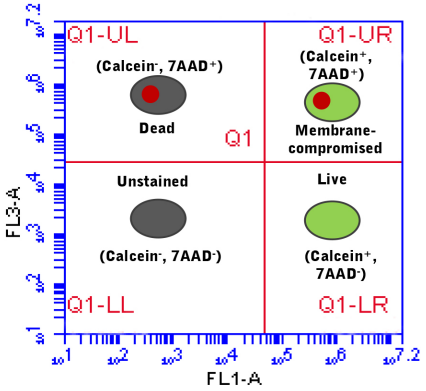
These controls are needed to adjust the instrument PMT's to separate 7-AAD-positive from 7-AAD-negative samples and to compensate for bleed-over of the red 7-AAD signal from FL-3 into FL-1. They will also help to clearly differentiate the Calcein-positive population from the Calcein AM-negative population and compensate bleed-over of the green Calcein signal from FL-1 into FL-3.

7. Set up the instrument compensation.
   a. Read the 7-AAD-positive and 7-AAD-negative controls to compensate bleed-over of the red 7-AAD signal from FL-3 into FL-1.
   b. Read the Calcein-positive and Calcein AM-negative controls to compensate bleed-over of the green Calcein signal from FL-1 into FL-3.
8. To read the samples for bicolor analysis:
   a. Measure green fluorescence (Calcein) on the FL-1 channel.
   b. Measure red fluorescence (7-AAD) on the FL-3 channel.
   c. Generate a log FL-1 versus log FL-3 dot plot (Figures 7 and 8).
   d. This will reveal 4 populations of cells: (Figure 6):
      1. Live cells fluoresce green due to the presence of active esterases capable of cleaving Calcein AM, but do not fluoresce red as their intact cell membranes exclude 7-AAD (LR, lower right).
      2. Live; membrane-compromised cells: fluoresce green due to the presence of active esterases capable of cleaving Calcein AM, and fluoresce red due to compromised membrane integrity allowing 7-AAD to enter cell and intercalate with nucleic acids (UR, upper right).
      3. Dead; red fluorescing cells are 7-AAD positive dead cells. These cells lack active esterases and have compromised cell membranes (UL, upper left).
      4. Unstained cells, no fluorescence (LL, lower left). Few cells are expected to fall into this population. A high percentage of cells in the lower left quadrant could indicate that a problem occurred during the staining process such as too low of a staining concentration.

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

Figure 6: Quantitate Live, Membrane-compromised, and Dead Cell Populations

1. Live Cells: fluoresce green due to the presence of active esterases capable of cleaving Calcein AM, but do not fluoresce red as their intact cell membranes exclude 7-AAD (LR, lower right).
2. Live, Membrane-compromised Cells: fluoresce green due to the presence of active esterases capable of cleaving Calcein AM, and fluoresce red due to compromised membrane integrity allowing 7-AAD to enter cell and intercalate with nucleic acids (UR, upper right).
3. Dead cells fluoresce red due to compromised membrane integrity allowing 7-AAD to enter cell and intercalate with nucleic acids, but lack active esterases and do NOT fluoresce green (UL, upper left).
4. Unstained cells, no fluorescence (LL, lower left).



Density Plot Key

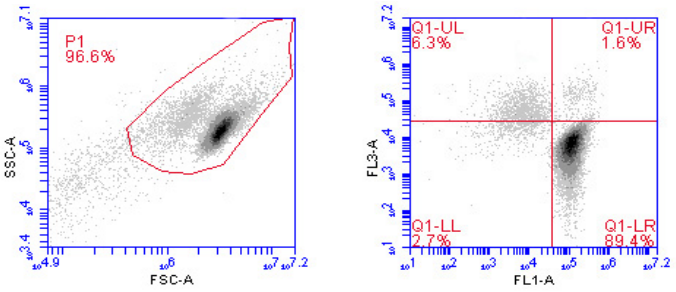
Figure 7: Flow Cytometry Analysis of Live and Dead Cells

ICT's Advanced Calcein AM Cell Viability Kit was used to assess viability in Jurkat cells. The forward and side scatter graphs are shown on the left and density plots are shown on the right. Using Calcein AM and 7-AAD, live cells exhibiting green fluorescence (LR, lower right quadrant of density plot) can easily be distinguished from the green and red fluorescing membrane compromised cells (UR, upper right quadrant of density plot), and dead cells exhibiting red fluorescence only (UL, upper left quadrant of density plot).

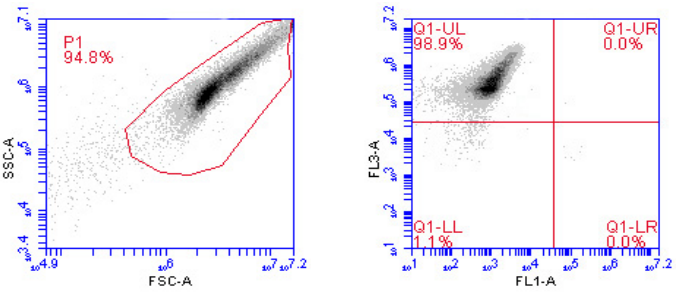
Healthy, untreated cells served as the positive control for Calcein AM and a negative control for 7-AAD. To create a positive control for 7-AAD and a negative control for Calcein AM, cells were killed by brief exposure to ethanol (Section 9). These samples were used to compensate the instrument.

Three populations of Jurkat cells were analyzed. Cells were grown to 5 x 10^5 cells/mL and split into two populations. One population (A) was untreated while the other population (B) was killed by exposure to 90% ethanol for 60 seconds. 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. A third population was created by combining the untreated (live) and ethanol-treated (dead) cells (C).

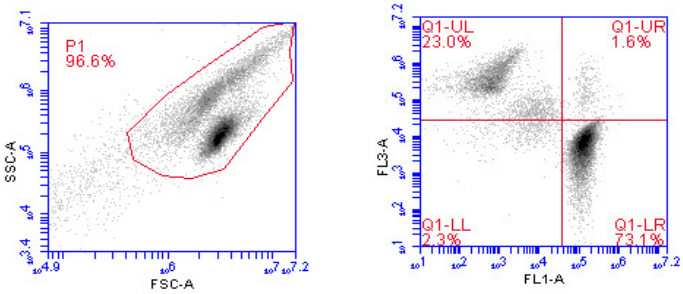
Jurkat suspension cells were first stained with 1 µM Calcein AM for 60 minutes at 37°C to detect live cells. Cells were then stained with 4 µM 7-AAD for 10 minutes on ice to detect membrane-compromised or dead cells. Cells were then analyzed using an Accuri C6 flow cytometer equipped with a FL-1 99% attenuation filter. The majority of the live cells stained positive with Calcein(A; LR, lower right, 89.4%), while 0.0% of ethanol-killed cells stained positive for Calcein (B; LR, lower right). The majority of the ethanol-killed cells stained positive for 7-AAD (B: UL, upper left, 98.9%). A mixture of live and ethanol-killed cells (C) shows both live (LR, 73.1%) and killed (UL, 23.0%). Data courtesy of Dr. Kristi Strandberg(ICT 226:87-92).



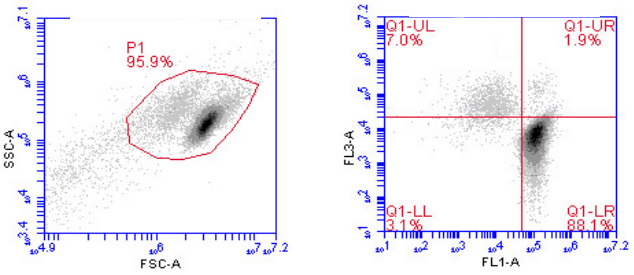
A: Untreated/Live Cells



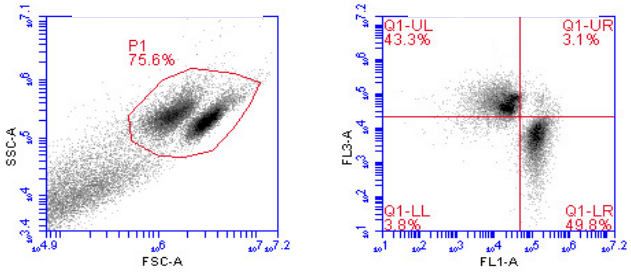
B: Ethanol-Killed/Dead Cells



C: Mixture of Live and Dead Cell Populations



A: Noninduced Cells



B: Induced Cells

Figure 8: Flow Cytometry Analysis of Apoptotic Cells

Jurkat cells were treated with a placebo (non-induced treatment with DMSO; A) or treated with 1 µM staurosporine for 4 hours to induce apoptosis (B). Cells were first stained with 1 µM Calcein AM and then 4 µM 7-AAD. Cells were analyzed using an Accuri C6 flow cytometer equipped with a FL-1 99% attenuation filter.

Compensation was set using cell populations stained individually with either Calcein AM or 7-AAD (data not shown, but similar to Fig. 7 A-B). Calcein AM was analyzed on the 99% attenuated FL-1 channel, and 7-AAD was analyzed on the FL-3 channel. Forward and side scatter density plots (left) and dual stained density plots (right) are shown. The density plot key is shown in Figure 6.

Flow cytometry can be used to quantitate populations of live, membrane-compromised, and dead cells. Live cells fluoresce green due to the ability of intracellular esterases to cleave Calcein AM into the green fluorescent molecule, Calcein (lower right quandrant of density plot A). Dually stained green and red fluorescing cells represent the population of membrane compromised Jurkat cells (most likely in mid to late stage apoptosis); these cells have active intracellular esterases and compromised cell membranes (upper right quadrant of density plot B). Necrotic cells fluoresce red (upper left, A and B). Data courtesy of Dr. Strandberg, ICT 226:87-92.

In Figure 5, a similar experiment was performed and images were taken using a fluorescence microscope. Data courtesy of Dr. Kristi Strandberg (ICT 226:87-92).

17. 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).
4. Murata, S., Herman, P. & Lakowicz, J. R. Texture analysis of fluorescence lifetime images of nuclear DNA with effect of fluorescence resonance energy transfer. Cytometry 43, 94-100 (2001).
5. Gill, J. E., Jotz, M. M., Young, S. G., Modest, E. J. & Sengupta, S. K. 7-Amino-actinomycin D as a cytochemical probe. I. Spectral properties. J Histochem Cytochem 23, 793-799 (1975).
6. Lecoeur, H., Ledru, E., Prevost, M. C. & Gougeon, M. L. Strategies for phenotyping apoptotic peripheral human lymphocytes comparing ISNT, annexin-V and 7-AAD cytofluorometric staining methods. J Immunol Methods 209, 111-123 (1997).
7. Madhavarao, M. S., Chaykovsky, M. & Sengupta, S. K. N7-Substituted 7-aminoactinomycin D analogues. Synthesis and biological properties. J Med Chem 21, 958-961 (1978).
8. Sengupta, S. K., Tinter, S. K., Lazarus, H., Brown, B. L. & Modest, E. J. 7-substituted actinomycin D analogs. Chemical and growth-inhibitory studies. J Med Chem 18, 1175-1180 (1975).

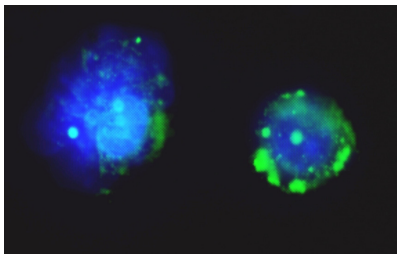
RELATED PRODUCTS:

To detect the intracellular process of apoptosis via activated caspases, use our FLICA® kits. These caspase assays are available in green, red, and far-red fluorescence for the preferred detection of individual active caspase enzymes or pan-caspase activity.

Visit www.immunochemistry.com/products for more information.

- *Label the active apoptotic process*
- *Distinguish apoptosis from necrosis*
- *Whole cell analysis*
- *Counterstain with common fluorophores*

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



Above: Apoptotic neuroblastoma cells fluoresce green after staining with FAM-FLICA® Poly Caspase Assay (#92). Hoechst 33342 nucleic acid stain (blue in image) is included in the kit as well as propidium iodide vital stain.

At left: SR-FLICA® Poly Caspase Assay Kit, standard size (#917).

Thank you for using our ADVANCED 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
immunochemistry.com