

**Live/Dead Assay for Cell Viability**  
**AfCS Procedure Protocol PP0000002300**  
**Version 1, 01/21/02**

This assay is used to measure cell viability. It is a two-color fluorescence assay that simultaneously determines:

- Live cell number—Live cells have intracellular esterases that convert nonfluorescent, cell-permeable calcein acetoxymethyl (calcein AM) to the intensely fluorescent calcein. Cleaved calcein is retained within cells.
- Dead cell number—Dead cells have damaged membranes; the ethidium homodimer-1 (EthD-1) enters damaged cells and is fluorescent when bound to nucleic acids. EthD-1 produces a bright red fluorescence in damaged or dead cells.

This protocol is designed for use with the GEMINI XS Microplate Spectrofluorometer, a multi-well plate scanner with dual excitation/emission capabilities, but the assay is also adaptable for flow cytometry and fluorescence microscopy.

Live and dead cell numbers are calculated from standard curves with known numbers of live and dead cells. The data from this assay are typically presented as

- Percent live cells—calculated as the number of live cells divided by the number of total cells at each time point.
- Percent survival—calculated as the number of live cells at each time point divided by the number of live cells at 0 hr.

### **Cell Culture**

1. Experimental manipulations are performed under the conditions and for the times to be tested. If the culture medium does not contain phenol red, serum, or high concentrations of protein, cells can be plated directly in black-wall, clear-bottom 96-well plates (see below), which are used for the Live/Dead assay. The AfCS cultures B cells in Supplemented Iscove's Modified Dulbecco's Medium, which contains 100 µg/ml of bovine serum albumin (BSA), and which may contain phenol red (SIMDM) or may be phenol-red—free (SIMDM-PRF). This concentration of BSA does not interfere with the assay. If interfering substances must be removed prior to assay, see *Alternate Protocol—For Medium Containing Phenol Red, Serum, and/or High Concentrations of Protein*, below. For viability assays, the AfCS cultures B cells in 96-well Costar ultralow tissue culture plates to minimize adhesion of cells to the dish. The remainder of this protocol assumes that cells are cultured in SIMDM-PRF in Costar ultralow 96-well plates. After culture, cells are transferred to black-wall, clear-bottom 96-well plates for assay.
2. B cells are plated in at least four replicate wells for each condition to be tested.

### **Preparation for the Assay**

3. Prepare the calcein acetoxymethyl/ethidium homodimer-1, 2X (2X calcein AM/EthD-1) reagent fresh at the time of the assay. The assay requires 100 µl of

this reagent per well or about 10 ml for an entire 96-well plate. Also prepare 1 ml of each fluorescent reagent separately: calcein acetoxymethyl, 2X (2X calcein AM) and ethidium homodimer-1, 2X (2X EthD-1)

4. Prepare cells for the standard curve. Isolate B cells from one spleen at each time point using AfCS Protocol *Isolation of Resting B Lymphocytes from One or More Mouse Spleens*, PP0000000100. Count fresh cells and dilute to  $5 \times 10^6$  live cells/ml (Note: because viability is typically 95%, we ignore the 5% dead cells in this preparation; this does not interfere significantly with the standard curve. See also Molecular Probes Live/Dead Assay product information). Each plate to be assayed requires 2 ml of cells at this concentration.
5. Prepare the live and dead cells for standard curves. For live cells, take 1 ml of cells at  $5 \times 10^6$  cells/ml. For dead cells, take 1 ml of cells at  $5 \times 10^6$  cells/ml, add 20  $\mu$ l of 5% saponin, mix, and let stand for 10 min. This will permeabilize the cell membranes and permit EthD-1 staining of the nuclei.

### Live/Dead Assay

6. Mark a black-wall, clear-bottom culture plate; the four left columns are reserved for the standard curve (32 wells) and the remaining 64 wells are used for the experimental samples.
7. The linear range of the Live/Dead Assay is approximately 500 to  $10^6$  cells.
8. It is important that cells be distributed evenly on the bottom of the well, since the plate reader monitors fluorescence at the bottom. Cells in suspension must be allowed to settle on the bottom of the wells prior to reading the assay in the plate scanner. The cells settle to the bottom when incubated with the fluorescent probes (see below).
9. Transfer cultured cells that are to be assayed from the 96-well ultralow tissue culture plate to the labeled black-wall, clear-bottom assay plate with a multichannel pipette. The cells in the tissue culture plate should be pipetted up and down 3 to 4 times to suspend the cells homogeneously before they are transferred to the assay plate. Add phosphate buffered saline with calcium and magnesium (PBS with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ), if necessary, to bring the total volume of each well to 100  $\mu$ l.
10. Make the standard curve.

	Live		Dead		Samples							
	1	2	3	4	5	6	7	8	9	10	11	12
A	BI	BI	BI	BI								
B	5	5	5	5								
C	2.5	2.5	2.5	2.5								
D	1.25	1.25	1.25	1.25								
E	0.63	0.63	0.63	0.63								
F	0.31	0.31	0.31	0.31								
G	5	5	5	5								
H	5	5	5	5								

This chart is a schematic diagram of the standard curve in a 96-well plate. Each number in the table equals the cell number  $\times 10^{-5}$ . Add 100  $\mu\text{l}$  of PBS with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  to row A, columns 1 through 4, which is the blank (BI), and 100  $\mu\text{l}$  of PBS with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  to rows C through F, columns 1 through 4. Add 200  $\mu\text{l}$  of live cells to rows B, G, and H, columns 1 and 2, and 200  $\mu\text{l}$  of dead cells to rows B, G, and H, columns 3 and 4. Using a multichannel pipette, transfer 100  $\mu\text{l}$  of cells from row B, columns 1 through 4, to row C, columns 1 through 4. Mix the cells in row C and repeat the transfer to row D. Continue through rows E and F and finally discard the extra 100  $\mu\text{l}$  of cells from row F. Each row now has 100  $\mu\text{l}$  of the correct cell concentration (or the blank in row A). To determine if the medium alone gives a signal, this can be measured as a sample.

11. Add 100  $\mu\text{l}$  of 2X calcein AM/EthD-1 reagent to each well, with the following exceptions: add 100  $\mu\text{l}$  of the 2X calcein AM reagent to row G, columns 1 through 4; add 100  $\mu\text{l}$  of 2X EthD-1 reagent to row H, columns 1 through 4.
12. Place the plate in the incubator at 37° C, 5%  $\text{CO}_2$ , for 30 min.
13. This whole process should take about 5 to 6 min.

### Reading the Plate

14. The AfCS has used the SPECTRAmax GEMINI XS with SOFTmax PRO 3.2.1 software (Molecular Devices) and is currently using the FLEXstation System (Molecular Devices) with SOFTmax PRO 3.2.1 and 4.0.1. Other plate readers can be used following the manufacturer's instructions.
15. Using the GEMINI XS, the plate is read in well-scan mode, using the cross-pattern mode, which reads five different points on the bottom of each well. The excitation/emission wavelengths are 485 nm/515 nm for calcein AM and 525/590 nm for EthD-1.
16. Turn on the machine and set the temperature to 37° C at least 10 min prior to reading the plate.
17. Generate a protocol file using the SOFTmax PRO software, following the instructions included with the software.
18. After the 30-min incubation with 2X calcein AM/EthD-1 (step 12 above), place the plate in the reader and run the program to collect the data.

### Data Analysis

19. Live and dead cell numbers are collected directly from the SOFTmax PRO software. The program calculates the standard curves for live and dead cells automatically. Check to make sure that the fit for the standard curve is correct. Check the conditions of calcein AM alone (row G, columns 1 through 4) and EthD-1 alone (row H, columns 1 through 4) to ensure that spectral overlap of calcein AM into the channel set for EthD-1 and EthD-1 into the channel set for calcein AM is minimal.
20. Once satisfied that the standard curve is correct, calculate percentage of live cells and dead cells. Typically, these experiments are done at multiple time points; calculate percentage of live cells at each time point and the percent survival as follows:

Percent live (dead) at time X hr = [live (dead) cells at t = x]/[total cells at t = x]  
Percent survival at time X hr = [live cells at t = x]/[live cells at t = 0 hr]

### **Alternate Protocol—For Medium Containing Phenol Red, Serum, and/or High Concentrations of Protein**

1. If cells must be cultured in medium containing phenol red, serum, or high concentrations of protein, these reagents must be removed before the assay can be performed.
2. To remove these additives, transfer the volume of cells to be assayed to a microfuge tube and add 300  $\mu$ l of magnetic cell sorting buffer (MACS buffer).
3. Centrifuge all tubes for 3 min at 400 x g.
4. Gently aspirate the supernatant from the cells using a vacuum aspirator.
5. Resuspend the cells in 100  $\mu$ l of PBS with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  and transfer to the black-wall, clear-bottom assay plate.
6. Continue with the remainder of the protocol.

### **Reagents and Materials**

Calcein acetoxymethyl (calcein AM), 2 mM: AfCS Solution Protocol ID PS0000000300

Ethidium homodimer-1 (EthD-1), 2 mM: AfCS Solution Protocol ID PS0000000400

Supplemented Iscove's Modified Dulbecco's Medium, with phenol red (SIMDM): AfCS Solution Protocol ID PS0000005600

Supplemented Iscove's Modified Dulbecco's Medium, phenol-red free (SIMDM-PRF): AfCS Solution Protocol ID PS0000005700

96-well ultralow tissue culture plates (Costar): Fisher Biotech; catalog no. 07-200-603

96-well black-wall, clear-bottom assay plates (Costar): Fisher Biotech; catalog no. 07-200-565

Calcein acetoxymethyl/Ethidium homodimer-1, 2X (2X Calcein AM/EthD-1): AfCS Solution Protocol ID PS0000000600

Calcein acetoxymethyl, 2X (2X calcein AM): AfCS Solution Protocol ID PS0000000500

Ethidium homodimer-1, 2X (2X EthD-1): AfCS Solution Protocol ID PS0000000700

Saponin, 5% (5% Saponin): AfCS Solution Protocol ID PS0000000800

Phosphate buffered saline with calcium and magnesium (PBS with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ): AfCS Solution Protocol ID PS0000004200

SPECTRAmax GEMINI XS Microplate Spectrofluorometer: Molecular Devices; catalog no. 0200-3940

FLEXstation System: Molecular Devices; catalog no. 0200-4000

Magnetic cell sorting buffer (MACS buffer): AfCS Solution Protocol ID PS0000000100

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**Date:** 02/15/02

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