EarlyTox Live/Dead Assay Kit

The EarlyTox™ Live/Dead Assay Kit enables the detection of both the live and dead populations of mammalian cells based on the integrity of the cell membrane using a fluorescence microplate reader.

Table 1-1: Available Kits

Assay Kit	Explorer Kit	Bulk Kit
EarlyTox Live/Dead Assay Kit	R8340	R8341

Contents

Product manufactured by Molecular Devices, LLC.
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Molecular Devices, LLC is ISO 9001 registered.

Chapter 1: About the EarlyTox Live/Dead Assay Kit

The EarlyTox Live/Dead Assay Kit provides a two-color fluorescence assay that detects both live and dead cells for mammalian cell types. The key features of this kit are the following:

- Optimized for fluorescence microplate readers, such as SpectraMax® readers
- Simple workflow – direct measurement in wells with or without medium removal
- Increased sample throughput with microplate format
- Preconfigured protocol in SoftMax® Pro Software

Assay Principles

The kit contains two markers for live or dead cells based on the integrity of the cell membrane. Calcein AM is a widely used live-cell marker. The non-fluorescent calcein AM permeates the intact cell membrane and is converted into calcein, the fluorescent form, by intracellular esterases. The number of live cells are therefore indicated by the intensity of green fluorescence in the cytosol, with excitation at 495 nm and emission at 530 nm.

EthD-III is virtually non-fluorescent and impermeable to an intact plasma membrane. In the event of compromised cell membrane integrity that is associated with cell death, EthD-III enters cells and binds to nucleic acids, resulting in bright red fluorescence in dead cells, with excitation at 530 nm and emission at 645 nm. Cytotoxic events that do not affect cell membrane integrity might not be accurately assessed using this method. Cell viability assays for mammalian cells using similar fluorescent dyes have been demonstrated in many publications.[1,2,3,4,5,6]

Chapter 2: Materials and Equipment

Kit Components

Table 2-1: Components of the EarlyTox Live/Dead Assay Kits

Item	Explorer Kit (R8340)	Bulk Kit (R8341)
Calcein AM, 4 mM in DMSO	1 x 30 µL	3 x 50 µL
Ethidium Homodimer-III, 2 mM in DMSO:H2O 1:4 (v/v)	1 x 60 µL	2 x 150 µL

- The Explorer kit is sufficient for two 96-well microplates.
- The Bulk kit is sufficient for ten 96-well microplates.

The number of microplates is based on the example protocol that is detailed in this document.

Storage and Handling

Reagents in this kit should be stored sealed, desiccated, protected from light, and frozen at –20°C. Allow the reagents to warm up to room temperature before opening. Before refreezing, seal all stock solutions tightly. Calcein AM is susceptible to hydrolysis when exposed to moisture. If the color of Calcein AM stock solution turns orange, discard the tube. EthD-III is stable and insensitive to moisture. Stock solutions of EthD-III in DMSO/H2O or other aqueous media can be stored frozen at –20°C. When stored as directed, the kit is stable for at least 6 months from the date it is received.

WARNING! Reagents can contain chemicals that are harmful. Exercise care when handling reagents as described in the related safety data sheet (SDS or MSDS). The safety data sheet is available in the Knowledge Base on the Molecular Devices support web site: www.moleculardevices.com/support.

Materials Required but Not Provided

Table 2-2: Reagents and Supplies

Item	Suggested Vendor
Black-walled, clear bottomed microplates (96-well)	Corning 3904 or equivalent
Phosphate-Buffered Saline (PBS)	Corning 21-030 or equivalent

Compatible Molecular Devices Microplate Readers

- SpectraMax® i3x Multi-Mode Detection Platform
- SpectraMax® M2 and M2e Multi-Mode Microplate Readers
- SpectraMax® M3 Multi-Mode Microplate Reader
- SpectraMax® M4 Multi-Mode Microplate Reader
- SpectraMax® M5 and M5e Multi-Mode Microplate Readers
- SpectraMax® Paradigm® Multi-Mode Detection Platform
- FlexStation® 3 Benchtop Multi-Mode Microplate Reader
- Gemini™ EM and XPS Fluorescence Microplate Readers
- FilterMax™ F3 and F5 Multi-Mode Microplate Readers

Chapter 3: Assay Protocols

Example of Assay Protocol

The following was used for a viability assay of HeLa cells in the 96-well format. The optimal assay conditions for different cell types and microplate formats can vary. Molecular Devices recommends testing a range of dye concentrations (1 to 10 µM), incubation times, and temperatures (room temperature or 37°C) to determine the optimal conditions for your assay system.

Working Solution Preparation

Use the following procedure to prepare 6 µM calcein AM and 6 µM EtD-III:

1. Remove the calcein AM and EthD-III reagent stock solutions from the freezer and allow them to warm to room temperature.
2. Add 30 µL of the 2 mM EthD-III and 15 µL of 4 mM calcein AM to 10 mL of PBS, and then vortex to ensure thorough mixing.

   Note: 10 mL of this working solution is sufficient for one 96-well microplate.

3. Optionally, if the percentage of live or dead cells in the population needs to be determined, for live-only and dead-only controls, prepare 1 mL of 6 µM calcein AM only solution and 1 mL of 6 µM EthD-III only solution.

CAUTION: Because aqueous solutions of calcein AM are susceptible to hydrolysis (see Storage and Handling on page 4), use aqueous working solutions within one day.

Assay Setup

1. Plate 20000 cells in 100 µL culture medium per well in a black-walled, clear-bottomed, 96-well microplate. Incubate in a 37°C, 5% CO_2 incubator overnight. If needed, include wells without cells as a background control.
   Molecular Devices recommends seeding cells at sufficient density to form a confluent.
   - For suspension cells, the recommended cell density is 40000 to 200000 cells per well.
   - For adherent cells, the recommended cell density is 20000 to 80000 cells per well.
2. Prepare samples of live cells and dead cells for control. Typically, dead cell controls can be prepared by treatment of live cells with 0.1% saponin or 0.1 to 0.5% digitonin, or another method of your choosing, for about 10 minutes.
3. Treat cells using the methods of your choosing for cell viability assays. Remember to include untreated wells as controls.
4. Add 100 µL of the calcein AM/EthD-III working solution to each well, which results in a final volume of 200 µL per well and a final concentration of 3 µM calcein AM and 3 µM EthD-III.
5. Incubate the samples at room temperature for 30 to 60 minutes, protected from light.
6. Measure fluorescence in a fluorescence microplate reader with the following setup:
   - For live cells: with excitation at 495 nm and emission at 530 nm.
   - For dead cells: with excitation at 530 nm and emission at 645 nm.

Fluorescence Microplate Reader Setup with SoftMax Pro Software

Table 3-1 displays the typical fluorescence microplate reader settings. In SoftMax® Pro Software, use the preconfigured EarlyTox Live/Dead Assay protocol that is available in the protocol library.

Table 3-1: EarlyTox Live/Dead Assay protocol

Parameter	Setting
Read Mode	Fluorescence
Read Type	Endpoint
Wavelengths	Lm1: Ex = 495 nm, Em = 530 nm Lm2: Ex = 530 nm, Em = 645 nm
PMT and Optics	PMT Gain: Automatic Flashes per read: 6 Read From Bottom*

*Bottom read is preferred for cell-based assays, but top read can be used with microplate readers that do not have the bottom read option.

Chapter 4: Data Analysis Examples

Example Results

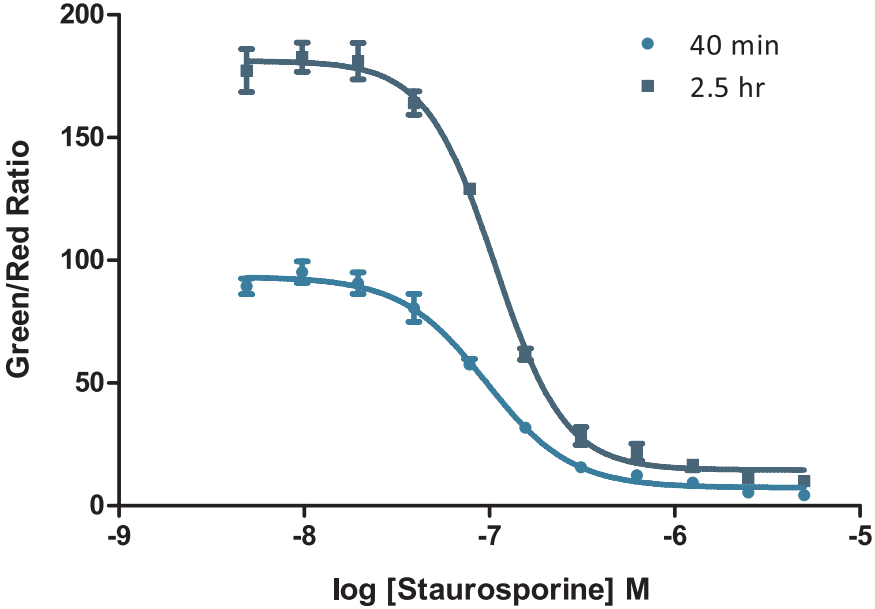

Figure 4-1: Cell viability assay: HeLa cells treated with staurosporine

HeLa at 20000 cells/well were plated in a 96-well microplate and grown overnight. Cells were then treated with staurosporine for approximately 24 hours at 37°C. 100 µL of the working solution of 6 µM Calcein AM and 6 µM of EthD-III (2x concentrations) were added directly to each well (n = 4). The samples were incubated at room temperature for 40 minutes or 2.5 hours. Fluorescence was measured on a SpectraMax M5 microplate reader, and read from the bottom. The concentration curves were plotted using the ratio of green (530 nm) over red (645 nm) RFUs at the Y-axis.

Assay Optimization Tips

- The example protocol was developed using HeLa cells, and can be adapted for use with other cell types. Assay signals can be optimized by adjusting the cell number and concentrations of the two dyes, generally between 1 and 10 µM.
- The example protocol described is a homogeneous protocol that does not replace the cell medium or use a wash step to simplify workflow and avoid disturbing cells. If needed, cell medium can be replaced with PBS prior to the addition of reagents to reduce background fluorescence. These manipulations, however, might result in inconsistent well-to-well signals because of disturbance or loss of cells.
- Try measuring the signals with both top and bottom read to determine the best instrument settings.
- The Well Scan read type might improve the data quality and consistency.

Determining the Percentage of Live and Dead Cells in a Population

To determine the percentage of live or dead cells in the population, set up your controls as described in Table 4-1.

Table 4-1: Fluorescence measurements for determining the percentage of live and dead cells in a population

Sample	Label Added
Experimental cells	Calcein AM and EthD-III
All (or mostly) live cells	EthD-III only
All (or mostly) live cells	Calcein AM only
All dead cells	EthD-III only
All dead cells	Calcein AM only
Cell-free control (optional)	Dye optional*

*You can set up the cell-free control with or without dye added.

Measure the fluorescence in the experimental cell samples and the control cell samples using both of the following wavelength pairs:

- Excitation at 495 nm and emission at 530 nm
- Excitation at 530 nm and emission at 645 nm

To calculate the percentage of live and dead cells in the experimental cells samples, see the following:

- Calculating the Percentage of Live Cells on page 9
- Calculating the Percentage of Dead Cells on page 10

Calculating the Percentage of Live Cells

Calculate the percentage of live cells from the fluorescence readings at the emission wavelength 530 nm according to the following formula:

$$\% \text{ Live Cells} = \frac{A - B}{C - B} \times 100$$

Where:

A. *A* is fluorescence at the emission wavelength 530 nm in the experimental cell sample, labeled with calcein AM and EthD-III.

B. *B* is fluorescence at the emission wavelength 530 nm in a sample where most of the cells are alive, labeled with EthD-III only.

C. *C* is fluorescence at the emission wavelength 530 nm in a sample where most of the cells are alive, labeled with calcein AM only.

Optionally, to subtract background fluorescence, measure the fluorescence of the cell-free control at the emission wavelength 530 nm. Then, subtract the background fluorescence measured at the emission wavelength 530 nm from values A, B, and C.

Calculating the Percentage of Dead Cells

Calculate the percentage of dead cells from the fluorescence readings at the emission wavelength 645 nm according to the following formula:

$$\% \text{ Dead Cells} = \frac{D - E}{F - E} \times 100$$

Where:

D. *D* is fluorescence at the emission wavelength 645 nm in the experimental cell sample, labeled with calcein AM and EthD-III.

E. *E* is fluorescence at the emission wavelength 645 nm in a sample where all the cells are dead, labeled with calcein AM only.

F. *F* is fluorescence at the emission wavelength 645 nm in a sample where all the cells are dead, labeled with EthD-III only.

Optionally, to subtract background fluorescence, measure the fluorescence of the cell-free control at the emission wavelength 645 nm. Then, subtract the background fluorescence measured at the emission wavelength 645 nm from values D, E, and F.

Chapter 5: Obtaining Support

Molecular Devices is a leading worldwide manufacturer and distributor of analytical instrumentation, software and reagents. We are committed to the quality of our products and to fully supporting our customers with the highest possible level of technical service.

Our support web site, www.moleculardevices.com/support, has a link to the Knowledge Base with technical notes, software upgrades, safety data sheets, and other resources. If you do not find the answers you are seeking, follow the links to the Technical Support Service Request Form to send an email message to a pool of technical support representatives.

You can contact your local representative or contact Molecular Devices Technical Support by telephone at 800-635-5577 (U.S. only) or +1 408-747-1700. In Europe call +44 (0) 118 944 8000.

Please have the product name, part number, and lot number available when you call.

EarlyTox Product Family

Table 5-1: EarlyTox Product Family: Available Kits

Assay Kit	Explorer Kit	Bulk Kit
EarlyTox Live/Dead Assay Kit	R8340	R8341
EarlyTox Live Cell Assay Kit	R8342	R8343
EarlyTox Glutathione Assay Kit	R8344	R8345
EarlyTox Caspase-3/7 R110 Assay Kit	R8346	R8347
EarlyTox Caspase-3/7-D NucView 488 Assay Kit (DMSO Formulation)	R8348	R8349
EarlyToxCaspase-3/7 NucView 488 Assay Kit (PBS Formulation)	R8350	R8351

References

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2. Papadopoulos NG, Dedoussis GV, Spanakos G, Gritzapis AD, Baxevanis CN, Papamichail M. "An improved fluorescence assay for the determination of lymphocyte-mediated cytotoxicity using flow cytometry." *J Immunol Methods* **177**, 101 (1994).

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4. Jacobsen MD, Weil M, Raff MC. "Role of Ced-3/ICE-family proteases in staurosporine-induced programmed cell death." *J Cell Biol* **133**, 1041 (1996).

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6. Wang XM, Terasaki PI, Rankin GW Jr, Chia D, Zhong HP, Hardy S. "A new microcellular cytotoxicity test based on calcein AM release." *Hum Immunol* **37**, 264 (1993).

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