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LIVE/DEAD® Cell-Mediated Cytotoxicity Kit (L7010)

Quick Facts

Storage upon receipt:
- 4°C for Component B
- Protect from light

Introduction

The LIVE/DEAD® Cell-Mediated Cytotoxicity Kit is based directly on procedures developed by Kroesen and colleagues for measuring natural killer (NK), lymphokine-activated killer (LAK) and T cell–mediated cellular cytotoxicity by fluorescence microscopy.[1] This two-color fluorescence assay allows direct assessment of cell-mediated cytotoxicity over the course of many hours and yields cytotoxicity measurements that correlate well with those obtained using conventional ^{51}Cr release assays. In order to distinguish target cells from effector cells, cultures of target cells are labeled overnight with 3,3′-dioctadecyloxacarbocyanine ($DiOC_{18}(3)$ or "DiO"), a green fluorescent membrane stain. Target cells are then washed free of $DiOC_{18}(3)$ and combined in various proportions with effector cells. After the target cells have incubated with the effector cells, the membrane-impermeant nucleic acid counterstain propidium iodide is added to label any cells with compromised plasma membranes. Because the target cells retain the green fluorescent membrane stain, both live and dead effector cells and live and dead target cells are readily discriminated in the fluorescence microscope. Dead target cells have coincident green-membrane and red-nucleus staining while dead effector cells have only red-nucleus staining. This procedure has also been utilized successfully for analyzing cell-mediated cytotoxicity by flow cytometry.[2] At the recommended reagent dilutions and volumes, the LIVE/DEAD Cell-Mediated Cytotoxicity Kit contains sufficient material to perform ~2000 assays by microscopy or ~200 assays by flow cytometry.

Materials

Kit Contents

- **$DiOC_{18}(3)$** (Component A), 400 µL of a 3 mM solution in anhydrous DMSO
- **Propidium iodide** (Component B), 1.1 mL of a 3.75 mM solution in water

Storage and Handling

The $DiOC_{18}$ (3) solution (Component A) may be stored, protected from light, at room temperature, 4°C or ≤–20°C. The propidium iodide solution (Component B) should be stored, protected from light, at 4°C — if stored at ≤–20°C, the propidium iodide may come out of solution, but the precipitate will readily dissolve at room temperature. Allow reagents to warm to room temperature and centrifuge briefly before opening the vials. After each use, seal the vials tightly. When stored properly, these stock solutions are stable for at least one year.

CAUTION: Propidium iodide is a known mutagen and should be used with appropriate care. To our knowledge, there is no available toxicity or mutagenicity data for $DiOC_{18}(3)$. DMSO stock solutions should be handled with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues. We strongly recommend the use of double gloves when handling DMSO stock solutions.

Spectral Properties of the Dyes

$DiOC_{18}(3)$ has fluorescence excitation and emission maxima of 484 nm and 501 nm, respectively, measured in methanol; the spectra do not change appreciably upon association with membranes. Propidium iodide bound to nucleic acids has fluorescence excitation and emission maxima of 536 nm and 617 nm, respectively.

Protocol for Analysis by Fluorescence Microscopy

Labeling of Target Cells

1.1 Determine the approximate number of target cells in the culture flask or in suspension.

1.2 The amount of $DiOC_{18}(3)$ staining solution required depends on the number of target cells to be labeled; prepare enough staining solution to provide 1 mL of solution per 5×10^5 cells. To make the staining solution, add 4 µL of the $DiOC_{18}(3)$ stock solution (Component A) per mL of complete culture medium (1:250 dilution) and mix well.

1.3 Remove culture medium from target cells and replace with the staining solution prepared in step 1.2.

1.4 Incubate cells in staining solution overnight under normal culture conditions.

Cell-Mediated Cytotoxicity Assay

2.1 Rinse target cells free of staining solution and wash once in phosphate-buffered saline solution (PBS).

2.2 Resuspend the target cells to 2×10^4 cells/mL in complete culture medium.

2.3 Prepare suspensions of effector cells in complete culture medium to yield the desired effector:target (E:T) ratios when mixed with an equal volume of the target-cell suspension (e.g., 2×10^4 effector cells/mL for an E:T ratio of 1:1; 1×10^5 effector cells/mL for an E:T ratio of 5:1).

2.4 Mix a 0.5 mL aliquot of each effector-cell suspension with 0.5 mL of target-cell suspension. In addition, mix 0.5 mL of complete culture medium (without cells) with 0.5 mL of the target-cell suspension as a control for spontaneous cell death.

2.5 Incubate the cell mixtures for a period of time that is appropriate for the particular effector cell type used (typically 4, 8, 12 or 24 hours).

2.6 Concentrate cell mixtures by centrifugation at 250 × g for 10 minutes.

2.7 The amount of propidium iodide counterstaining solution required depends on the number of 1 mL samples to be labeled; prepare enough counterstaining solution to allow 50 µL of solution per sample. To make the counterstaining solution, add 2 µL of the propidium iodide stock solution (Component B) per mL of PBS (1:500 dilution) and mix well.

2.8 Resuspend the cell pellets in 25–50 µL of counterstaining solution prepared in step 2.7.

2.9 After incubating the cells in counterstaining solution at room temperature for 5 minutes, trap 15 µL of cell mixture between a slide and an 18–22 mm^2 coverslip and seal with wax or other nontoxic sealant.

2.10 Observe the cells in the fluorescence microscope using one of the filter sets recommended below.

Cell Counting by Fluorescence Microscopy

For each E:T ratio used, scan at least 200 green fluorescent–labeled (target) cells and record the number that are also labeled in the nuclear region with orange-red propidium iodide fluorescence. Cells that show both green and red fluorescence are counted as dead target cells. Dead effector cells will have orange-red nuclei, but their membranes will not have green fluorescence.

Data Analysis

The percentage of dead target cells in the presence of effector cells (+ effectors) corrected for spontaneous target-cell death in the absence of effector cells (– effectors) is calculated according to the following equation, where G = green; G+R = both green and red. Corrected % cytotoxicity is equal to:

$$\left[\left(\frac{\#\text{G} + \text{R cells}}{\#\text{G cells}}\right)_{+\text{effectors}} - \left(\frac{\#\text{G} + \text{R cells}}{\#\text{G cells}}\right)_{\text{-effectors}}\right] \times 100$$

Selection of Optical Filter Sets

The fluorescence from both live and dead bacteria stained with the LIVE/DEAD Cell-Mediated Cytotoxicity Kit may be viewed simultaneously with any standard fluorescein long-pass filter set. Alternatively, the fluorescent live and dead cells may be viewed separately with fluorescein and either rhodamine or Texas Red® bandpass filter sets. A summary of the fluorescence microscope filter sets recommended for use with the LIVE/DEAD Cell-Mediated Cytotoxicity Kit is shown in Table 1.

Protocol for Analysis by Flow Cytometry

The following protocol for using the LIVE/DEAD Cell-Mediated Cytotoxicity Kit with flow cytometry has been adapted from the published results of Chang and co-workers.[2] In the study, natural killer lytic activity of human peripheral blood mononuclear cells was assessed using K562, a human erythroleukemic cell line, as target cells; additional details can be found in that publication. The protocol presented here serves as an example; optimal conditions for different experimental situations may need to be empirically determined.

Labeling of Target Cells

3.1 Determine the number of target cells in the culture flask or in suspension.

3.2 The amount of $DiOC_{18}(3)$ staining solution required depends on the number of target cells to be labeled; 10 µL of the $DiOC_{18}(3)$ stock solution (component A) is used for 1×10^6 cells.

3.3 Wash target cells in PBS and resuspend in PBS at a concentration of 1×10^6 cells/mL.

Table 1. *Characteristics of common filters suitable for use with the LIVE/DEAD Cell-Mediated Cytotoxicity Kit.*

Omega Filters*	Chroma Filters*	Notes
XF25, XF26, XF115	11001, 41012, 71010	Longpass and dual emission filters useful for simultaneous viewing of $DiOC_{18}(3)$ and propidium iodide stains
XF22, XF23	31001, 41001	Bandpass filters for viewing $DiOC_{18}(3)$ alone
XF32, XF43 XF102, XF108	31002, 31004 41002, 41004	Bandpass filters for viewing propidium iodide alone

***** Catalog numbers for recommended bandpass filter sets for fluorescence microscopy. Omega® filters are supplied by Omega Optical Inc. (www.omegafilters.com). Chroma filters are supplied by Chroma Technology Corp. (www.chroma.com).

3.4 For each 1 mL volume of target cells to be stained, dispense a 10 µL volume of the $DiOC_{18}(3)$ stock solution (Component A) into the bottom of a conical, 15 mL centrifuge tube. Forcefullly add 1 mL of the target cells to disperse the dye.

3.5 Incubate the cells in the staining solution for 20 minutes at 37°C.

3.6 Wash the cells twice with PBS and resuspend in complete culture medium at a concentration of 1×10^6 cells/mL.

Cell-Mediated Cytotoxicity Assay

4.1 Prepare suspensions of effector cells in fresh culture medium to yield the desired effector:target (E:T) ratios, for example 40:1, 20:1, 10:1 and 5:1, when mixed with target cells as described below.

4.2 Mix 130 µL volumes of the effector cells with 10 µL aliquots of the target cells from step 3.6. Thus, for a 5:1 ratio, the 130 µL volume should contain 5×10^4 effector cells, as there will be 1×10^4 target cells. Prepare control samples containing effector cells alone and containing target cells alone.

4.3 Prepare a sufficient amount of propidium iodide counterstaining solution to have at least 130 µL per assay. To make the counterstaining solution, add 40 µL of the propidium iodide stock solution (Component B) per mL of complete culture medium and mix well.

4.4 Add 130 µL of the counterstaining solution prepared in step 4.3 to each cell mixture from step 4.2 and gently mix. Pellet the cells by centrifugation at 1000 × g for 30 seconds — do not discard the supernatants. Incubate at 37°C for 30 minutes to 2 hours, or longer if appropriate.[2]

4.5 After the incubation period, tap the tubes gently to dislodge the pellets and then vortex to complete the resuspension immediately before analysis in the flow cytometer.

Analysis in the Flow Cytometer

The following recommendations for instrument setup and data acquisition have been developed using a Becton Dickinson FACS® Vantage™ flow cytometer. Although detector configuration and filter combinations vary among flow cytometers, comparable filter combinations used with other instruments should work equally well.

5.1 Both propidium iodide and $DiOC_{18}(3)$ are excited with an argon-ion laser tuned to 100 mW at 488 nm.

5.2 Red fluorescence from propidium iodide–stained cells (membrane-compromised cells) is deflected by a 610 nm shortpass dichroic, passed through a 630 ± 10 nm bandpass filter and detected by photomultiplier tube PMT-3. Green fluorescence from $DiOC_{18}(3)$-stained cells (target cells) is collected through a 560 nm shortpass dichroic, passed through a 530 ± 15 nm bandpass filter and detected by photomultiplier tube PMT-1.

5.3 A two-parameter cytogram, log(PMT-1) versus log(PMT-3), is plotted to discriminate the different cell types in the experiment. Live target cells (DiO^+, PI^-) are represented as signals detected in the lower righthand quadrant, whereas membrane-compromised target cells (DiO^+, PI^+) are represented as signals in the upper right-hand quadrant. Live effector cells (DiO^-, PI^-) are represented as signals in the lower lefthand quadrant, and membrane-compromised effector cells (DiO^-, PI^+), in the upper lefthand quadrant. A threshold on forward angle light scatter is set to ~10% of the signal range to exclude debris from the analysis.

5.4 Control samples, as recommended in step 4.2, will help in interpreting the experimental results. Independent reference standards, such as the fluorescent microspheres provided in our CompenFlow™ Flow Cytometry Compensation Kit (C7301), are recommended for calibrating the flow cytometer and optimizing instrument performance and reproducibility.

References

1. J Immunol Methods 156, 47 (1992); **2.** J Immunol Methods 166, 45 (1993).

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
L7010	LIVE/DEAD® Cell-Mediated Cytotoxicity Kit *for animal cells* *2000 assays*	1 kit

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Molecular Probes, Inc.
29851 Willow Creek Road, Eugene, OR 97402
Phone: (541) 465-8300 ● Fax: (541) 335-0504

Customer Service: 6:00 am to 4:30 pm (Pacific Time)
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Phone: (541) 335-0353 ● Toll-Free: (800) 438-2209
Fax: (541) 335-0238 ● tech@probes.com

Molecular Probes Europe BV
Poortgebouw, Rijnsburgerweg 10
2333 AA Leiden, The Netherlands
Phone: +31-71-5233378 ● Fax: +31-71-5233419

Customer Service: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5236850 ● Fax: +31-71-5233419
eurorder@probes.nl

Technical Assistance: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5233431 ● Fax: +31-71-5241883
eurotech@probes.nl

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