Marker Gene Technologies, Inc

Product Information Sheet

MarkerGene™ Live: Dead/Cytotoxity Assay Kit

Product M0795

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MGT

NOTE: The following information is given as a viable methodology for use of the MarkerGene™ Live:Dead/Cytotoxicity Assay Kit. The user may determine their own best conditions for use dependent on the specific conditions present in their experiment.

I. OVERVIEW

Analysis of cell viability in cell culture is an important means of evaluating *in vitro* drug or environmental effects in cell-mediated cytotoxicity assays or for monitoring cell proliferation and health in cell culture. Our sensitive Live:Dead Assay Kit can be used with mammalian cells in culture, and provides quick, accurate and quantitative analysis of cell viability using the two compounds, carboxyfluorescein di-acetate (M0011) and propidium iodide (M0793) in a protocol that allows staining of up to 1000 microplates of cells. Analyses by fluorescence microscopy, flow cytometry or by using standard microtiterplate formats are easily performed and the red and green fluorescence emissions are readily resolved.

The determination of live and dead cells can be measured by intracellular esterase activity and plasma membrane integrity. Carboxyfluorescein di-acetate and propidium iodide are optimal dyes for this application. Assessing cell viability with this fluorescence-based method can replace similar methods for determining cell cytotoxicity and viability including trypan blue exclusion, and ^{51}Cr release, which tend to be slower, more expensive, and less sensitive indicators of cytotoxic events.

The enzymatic conversion of the cell-permeant, non-fluorescent carboxyfluorescein di-acetate (CFDA) to the intensely fluorescent carboxyfluorescein (CF) is an indicator of intracellular esterase activity, a characteristic of live cells. The carboxyfluorescein dye is retained within live cells, producing a green fluorescence, with excitation and emission at ~475nm and 517nm, respectively. Cells with damaged membranes allow the entrance of propidium iodide, which undergoes a fluorescence enhancement upon binding to nucleic acids, promoting a red fluorescence in dead cells with excitation and emission at ~493nm and 630nm, respectively. The intact plasma membrane of live cells excludes propidium iodide. Determination of cell viability with this kit depends on these specific biochemical properties; cytotoxic events having no affect on these properties might not be accurately measured by this method. With this assay technique, background fluorescence levels are inherently low because the dyes are virtually non-fluorescent before interacting with cells. The protocol also describes methods of preparing “dead” cells as controls for more accurate analysis.

II. MATERIALS

A.) 1 **Fluorescent Reagent A:** Two vials of 4mM solution of carboxyfluorescein diacetate (CFDA, M0011) in anhydrous DMSO, 40µL each.

B.) 2 **Fluorescent Reagent B:** Two vials of 2mM solution of propidium iodide (PI, M0793) in 1:4 DMSO: H_2O, 150µL each.

Storage and Handling. Fluorescent reagents should be handled with care, kept cold when not in use, and stored frozen (-20°C). In case of contact with skin or eyes, wash thoroughly with soap and cold-water. Reagents should be stable for at least 6 months following purchase. High background fluorescence readings for blank samples will indicate decomposition.

III. MICROPLATE READER PROTOCOL TO EVALUATE PERCENTAGE OF LIVE/DEAD CELLS

For optimal sensitivity using a microplate reader, it is recommended that the fluorophores should be excited using optical filter sets ideal for their respective absorbances. The fluorescence emissions used in this assay are 630 and 517 nm respectively.

IIIa. Prepare cells for assay: Cells are usually grown for 2-4 days to reach exponentially growth prior transfer to the multiwell plates. For adherent cells, carefully transfer to sterile tissue culture 96-well microplates. Allow the cells to recover overnight. After 24 hours, wash the cells with D-PBS several times (enough volume of D-PBS added to cover the bottom of the wells during each wash). This is done to stop or slow esterase activity present in serum-supplemented media that can promote non-specific hydrolysis of the live cell stain, carboxyfluorescein di-acetate. Then a small volume of D-PBS (100 µL) is added to each well containing cells in the microplate. For non-adherent cells, cells are washed with 2x-4x volumes of D-PBS and centrifuged to expel esterase activity. Add non-adherent cells to microplate wells with enough buffer to reach even distribution. Count the cells before transfer.

Note: to reduce dilution, small buffer volumes are preferred (i.e 100µL/well). At this point, cells can be treated with test drugs or compounds as needed. Alternately, the drug or test samples can be added synchronous with Live:Dead reagent staining. The minimum number of cells per well should be at least ~200-500 / well using a 96-well format. The maximum number of cell is ~ 1 x 10^5 / Cwell.

IIIb. Prepare Live/Dead staining reagents: Remove Live:Dead reagents from freezer and let them warm to room temperature. Add 20µL of the 2mM propidium iodide solution 2 and 5µL of 4mM carboxyfluorescein diacetate

solution 1 to 10mL of D-PBS in a screw cap test tube or vial — this gives a 2µM carboxyfluorescein diacetate AND 4µM propidium iodide working solution. Also add 20µL of the 2mM propidium iodide solution 2 to 10mL of D-PBS in a screw cap test tube or vial — this gives a 2µM carboxyfluorescein diacetate working solution. Finally add 5µL of 4mM carboxyfluorescein diacetate solution 1 to 10mL of D-PBS in a screw cap test tube or vial — to give a 4µM propidium iodide working solution. Vortex each working solution to ensure thorough mixing. Add 100µl of the working solutions to separate test wells of the cell plate. Pipette these solutions carefully to avoid cell damage. This will give a total of 200µL per well containing a final concentration of 1µM carboxyfluorescein diacetate and 2µM propidium iodide or the individual dyes at these concentrations. Incubate for 30 minutes at room temperature. The optimal incubation time interval can be varied from 30-50 minutes as necessary.

When using the 2µM carboxyfluorescein diacetate and 4µM propidium iodide working solution, the ratio of the fluorescence at 630nm (CF) to the fluorescence at 517 nm (PI) will give you the ratio of live:dead cells in the sample. For more accurate readings or for cytotoxicity testing of drugs or other samples, see the calculations in Section IIIc.

This above protocol is useful for most applications. It may be necessary, however, to determine the optimal dye concentrations for best results. In that case, while maintaining a constant cell seeding density, vary concentrations of propidium iodide solution from 0.1-10µM while taking measurements every 10-15 minutes. Similarly, to determine the optimum concentration of carboxyfluorescein di-acetate that gives negligible staining of dead cells, you may need to vary CFDA concentration from 0.1-10µM. Next, to find the optimum cell density concentration, you will need to keep carboxyfluorescein di-acetate and/or propidium iodide concentration constant and vary cell concentration as necessary if the signal is too high or low. Once the optimum staining concentrations are defined, use the determined fluorophore and cell concentrations should be used to perform future assays.

IIIc. Calculating percentage of Live/Dead cells: Below is the example of how to calculate percentage of live or dead cells with this Live:Dead Assay Kit when evaluating the cytotoxicity of a drug.

1.) Fluorescence at 630nm in drug-treated cell sample labeled with carboxyfluorescein di-acetate and propidium iodide. **(F1)**

2.) Fluorescence at 517nm in drug-treated cell sample labeled with carboxyfluorescein di-acetate and propidium iodide. **(F2)**

3.) Fluorescence at 630nm in a dead cell sample labeled only with propidium iodide. **(F3).** This dead cell sample can be prepared by treating the cells with 0.1% saponin or 0.1-0.5% digitonin for 10 minutes.

4.) Fluorescence at 630nm in a dead cell sample labeled only with carboxyfluorescein di-acetate. **(F4)**

5.) Fluorescence at 517nm in live cell sample labeled only with propidium iodide. **(F5**). This live cell sample is not treated with any drug.

6.) Fluorescence at 517nm in live cell sample labeled only with carboxyfluorescein di-acetate. **(F6)**

7.) Fluorescence at 517nm **(F7)** and 630nm **(F8)** of cell-free sample with or without dye (for measuring background fluorescence). Note: both dyes should not fluoresce without cells.

A percentage of live and dead cells can be expressed at 517nm and diminished fluorescence signal at longer wavelengths. Intense fluorescence at 630nm and minimal fluorescence at ~517nm characterizes dead cells. Background fluorescence readings (**F7** and **F8**) should be subtracted from all values before the live:dead calculations.

$$\%\ \text{Live cells} = \frac{\mathbf{F2 - F5}}{\mathbf{F6 - F5}} \times 100\% \qquad \%\ \text{Dead Cells} = \frac{\mathbf{F1 - F4}}{\mathbf{F3 - F4}} \times 100\%$$

IV. PROTOCOL FOR FLUORESCENCE MICROSCOPY

Carboxyfluorescein and propidium iodide can be viewed separately or together depending on the filters used. For simultaneous viewing, use a conventional fluorescein longpass filter; to view only carboxyfluorescein, use a fluorescein bandpass filter and for propidium iodide, use filters with the same name (see table below for more information).

Culture adherent cells on sterile glass coverslips until acceptable cell densities are reached (usually grown for 2-3 days). Non-adherent cells can also be used. Remove media and wash adherent cells with ~1000 volumes of D-PBS to hinder or remove serum esterase activity. If non-adherent cells are used, wash with ~1000 volumes of D-PBS, sediment by centrifugation, and place an aliquot of cell suspension on a coverslip. Allow cells to settle at 37°C to glass surface. Place coverslips in small petri dishes (2 per dish) and cover with 100-150µL of Live:Dead working solution (see microplate reader protocol to make working solution). Incubate for 30-45 minutes. After incubation, add ~10µL of Live:Dead working solution or D-PBS to a microscope slide, and carefully invert and mount coverslip on slide. View with fluorescence microscope.

OMEGA FILTERS+	CHROMA FILTERS+	NOTES
XF25, XF26, XF115	11001, 41012, 71010	LONGPASS AND DUAL EMISSION FILTERS USEFUL FOR SIMULTANEOUS VIEWING OF FLUORESCEIN AND PROPIDIUM IODIDE STAINS
XF100, XF23	31001, 41001	BANDPASS FILTERS FOR VIEWING FLUORESCEIN ALONE (EX 494, EM 518)
XF32, XF43, XF102, XF108, XF103, XF35	31002, 31004, 41002, 41004, 41005, 31005	BANDPASS FILTERS FOR VIEWING PROPIDIUM IODIDE ALONE (EX 536, EM 617)

Omega filters are supplied by Omega Optical, Inc.; Chroma filters are supplied by Chroma Technology Corp.

V. FLOW CYTOMETRY USE

Culture cells in suspension or treat adherent cells with trypsin to produce a suspension. For analysis by flow cytometry, wash cell suspensions by centrifugation and resuspension with D-PBS. Centrifuge again and resuspend in Live:Dead working solution (see microplate reader protocol to make working solution). Positive results have been confirmed for staining 1 x 10^6 cells (HL-60 cells grown in suspension or D5 cells treated with trypsin) with ~200µL of a D-PBS solution containing 1µM carboxyfluorescein di-acetate and 8µM propidium iodide. Control experiments should be included in experiments performed to ensure appropriate cell staining and cells should be examined by fluorescence microscopy.

VI. NOTES

The suggested tissue culture buffer, D-PBS is prepared as follows: (200mg/L KCl, 200mg/L KH_2PO_4, 8000mg/L NaCl, and 1150mg/L Na_2HPO_4). In addition, any standard saline buffer or even serum free media can be used in place of this assay buffer, but any color additives (e.g. phenol red) should be checked for potential fluorescence interference.

Saponin or digitonin solutions for preparing “dead” cell standards are not provided in this kit.

REFERENCES

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7.) Riordan HD, Riordan NH, Meng X, Zhong J, Jackson JA. (1994) "Improved microplate fluorometer counting of viable tumor and normal cells." Anticancer Res. **14**: 927-931.

M0795 KIT CONTENTS			
DESCRIPTION	**QUANTITY**	**PART NO.**	**STORAGE**
REAGENTS			
1 Fluorescent Reagent A	2 x 40µL	0795-001	FL, F
2 Fluorescent Reagent B	2 x 150µL	0795-002	FL, F, G
DOCUMENTATION			
MSDS Sheets	3		
Product Information Sheet	1		

Notes: F=store at or below -18°C; R=store at room temperature; C=store cold (4°C); L=light sensitive; D=store desiccated; FL=flammable; G=wear protective clothing/gloves/safety glasses when using; B=avoid breathing dust/fumes.

Contact and Support

For questions or comments on this or any product from Marker Gene Technologies, Inc., you may contact us by phone or via our website. We welcome customer feedback and we make every effort to improve our products based on input from our clients.

To ask a question or make a comment or suggestion, you can call us at **1-888-218-4062** or fax to **541-342-1960**.

For more information on our products and services, please visit our website at www.markergene.com, where you can find:

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