



Autophagy/Cytotoxicity Dual Staining Kit

Catalog Number KA1299

96 assays

Version: 03

Intended for research use only

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Introduction

Background

Autophagy is a critical cellular process that involves the degradation and digestion of intracellular components by the lysosome. This process not only enables cells to efficiently mobilize and recycle cellular constituents, but also prevents the accumulation of damaged organelles, misfolded proteins, and invading microorganisms.¹ Autophagy is a multi-step process that begins with the sequestration of cytoplasmic organelles and proteins. These cellular components are sequestered by a double membrane, forming an autophagosome. The autophagosome then fuses with a lysosome to form an autolysosome, where the cellular material is then degraded.^{2,3} Normal autophagy is essential for survival, differentiation, development, and homeostasis. Dysregulation of autophagy has been implicated in cancer, infection, aging, and degenerative diseases.² While autophagy most often acts to promote cell survival in response to stress, it can also promote cell death. The relationship between autophagy and apoptosis is complex. The two pathways share common stimuli and components, and can regulate the activity of each other.⁴ However, the specific factors and mechanisms that dictate the choice between autophagy and apoptosis remain unclear.

Principle of the Assay

The Autophagy/Cytotoxicity Dual Staining Kit provides a convenient tool for studying the regulation of autophagy and cytotoxicity at the cellular level. The kit employs monodansylcadaverine (MDC), a fluorescent compound that is incorporated into multilamellar bodies by both an ion trapping mechanism and the interaction with membrane lipids,⁵ as a probe for detection of autophagic vacuoles in cultured cells. Propidium iodide is used as a marker of cell death. Tamoxifen, a known inducer of autophagy, is included as a positive control. This kit provides sufficient reagent to effectively treat/stain 960 individual wells of cells when utilized in a 96-well plate format. Lower density plates will still require approximately the same amount of reagent on a per plate basis. Therefore, up to 10 plates worth of cells can be examined irrespective of the number of wells/plate. Exceptions include protocols in which non-adherent cells are utilized.

General Information

Materials Supplied

List of component

Item	Amount
Cell-Based Monodansylcadaverine	1 vial/100 µl
Cell-Based Assay Buffer Tablet	1 vial/5 tablets
Cell-Based Propidium Iodide Solution	1 vial/250 µl
Cell-Based Tamoxifen (100 mM)	1 vial/50 µl

Storage Instruction

This kit will perform as specified if stored as directed and used before the expiration date indicated on the outside of the box.

Item	Storage
Cell-Based Monodansylcadaverine	-20 °C
Cell-Based Assay Buffer Tablet	Room Temperature
Cell-Based Propidium Iodide Solution	4 °C
Cell-Based Tamoxifen (100 mM)	-20 °C

Materials Required but Not Supplied

- ✓ A 6-, 12-, 24-, or 96-well plate.
- ✓ HepG2 cells (can be obtained from ATCC); other cell lines can also be used.
- ✓ A fluorescence microscope equipped with a UV filter and a filter usually designed to detect rhodamine or Texas Red, or a plate reader capable of detecting MDC at excitation and emission wavelengths of 335 nm and 512 nm, respectively, and propidium iodide at excitation and emission wavelengths of 520-570 nm and 570-610 nm, respectively.
- ✓ Adjustable pipettes and a repeat pipettor.

Precautions for Use

- ✓ This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.
- ✓ Please read these instructions carefully before beginning this assay.
- ✓ For research use only. Not for human or diagnostic use.
- ✓ MDC is extremely light sensitive and is photobleached quickly. All staining procedures must be performed without direct exposure to intense light. Therefore, incubations need to be done in the dark.

- ✓ For all assay protocols, it is imperative that samples be analyzed immediately following completion of the staining. It is recommended that MDC staining be measured first and the propidium iodide staining be measured second to avoid depletion of the MDC fluorescence during analysis.

Assay Protocol

Reagent Preparation

Store all buffers at 4°C; they will be stable for about two months.

- **Cell-Based Assay Buffer Preparation**
Dissolve each Cell-Based Assay Buffer Tablet in 100 ml of distilled water. This buffer should be stable for approximately one year at room temperature.
- **Cell-Based Propidium Iodide Solution Preparation**
Prepare a staining solution by diluting the Cell-based Propidium Iodide Solution 1:1,000 in the Assay Buffer prepared above. Mix well. Protect from light.
- **Cell-Based MDC Preparation**
Prepare a staining solution by diluting the Cell-Based MDC 1:1,000 in the Assay Buffer prepared above. Mix well. Protect from light.

Assay Procedure

- **Fluorescence Microscopy**

The following protocol is designed for a 96-well plate. Adjust volumes accordingly for other sizes of plates. Optimal conditions will be dependent on the cell type.

1. Seed a 96-well plate with 5×10^4 cells/well. Grow cells overnight.
2. The next day, treat the cells with experimental compounds or vehicle control for 24-72 hours, or for the period of time used in your typical experimental protocol. To use the included Tamoxifen as a positive control, dilute the Tamoxifen 1:5,000-1:100,000 into your culture medium.
3. After the treatment period, centrifuge the plate for five minutes at 400 x g at room temperature.
4. Aspirate the supernatant.
5. Add 100 µl of the Cell-Based Propidium Iodide solution to each well. Be careful to not disturb the cell layer. Incubate the cells for two minutes at room temperature.
6. Centrifuge the plate for five minutes at 400 x g at room temperature.
7. Aspirate the supernatant.
8. Add 100 µl of Cell-Based Assay Buffer to each well. Be careful to not disturb the cell layer.
9. Centrifuge the plate for five minutes at 400 x g at room temperature.
10. Aspirate the supernatant.
11. Add 100 µl of the Cell-Based MDC solution to each well. Be careful to not disturb the cell layer. Incubate the cells for ten minutes at 37°C.
12. Repeat steps 6-10.
13. Add 100 µl of Cell-Based Assay Buffer to each well. The cells are now ready for analysis by fluorescent microscopy and must be analyzed immediately. Dead cells are stained by propidium iodide and can be detected with a fluorescent filter usually designed to detect rhodamine (excitation/emission = 540/570 nm)

or Texas Red (excitation/emission = 590/610 nm). Autophagic vacuoles stained by MDC can be detected with a UV filter usually designed to detect DAPI.

- Plate Reader Fluorescence Detection

The following protocol is designed for a 96-well plate. Adjust volumes accordingly for other sizes of plates. A 96-well black culture plate should be used for this method. Optimal conditions will be dependent on the cell type.

1. Seed a 96-well black culture plate with 5×10^4 cells/well. Two to three wells with same number of cells should be used as background. Grow cells overnight.
2. The next day, treat the cells with experimental compounds or vehicle control for 24-72 hours, or for the period of time used in your typical experimental protocol. The background wells receive culture medium only. To use the included Tamoxifen as a positive control, dilute the Tamoxifen 1:5,000-1:100,000 into your culture medium.

NOTE: Differences in cell density can significantly affect results. Ensure that experimental compounds used do not significantly inhibit cell proliferation.

3. After the treatment period, centrifuge the plate for five minutes at 400 x g at room temperature.
4. Aspirate the supernatant.
5. Add 100 µl of the Cell-Based Propidium Iodide Solution to each well except the background wells. Be careful to not disturb the cell layer. Incubate the cells for two minutes at room temperature.
6. Centrifuge the plate for five minutes at 400 x g at room temperature.
7. Aspirate the supernatant.
8. Add 100 µl of Cell-Based Assay Buffer to each well. Be careful to not disturb the cell layer.
9. Centrifuge the plate for five minutes at 400 x g at room temperature.
10. Aspirate the supernatant.
11. Add 100 µl of the Cell-Based MDC solution to each well except the background wells. Be careful to not disturb the cell layer. Incubate the cells for ten minutes at 37°C.
12. Repeat steps 6-10.
13. Add 100 µl of Cell-Based Assay Buffer to each well. The cells are now ready for analysis in the plate reader, and must be analyzed immediately. Autophagic vacuole staining intensity can be detected using an excitation wavelength of 335 nm and an emission wavelength of 512 nm. The degree of cell death can be assessed by measuring propidium iodide staining intensity at excitation and emission wavelengths of 536 nm and 617 nm, respectively.

Data Analysis

Performance

Cell Staining

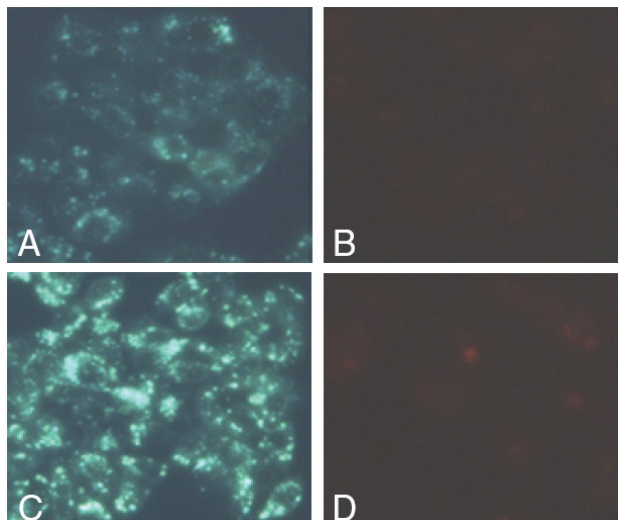


Figure 1. Tamoxifen increases autophagy but not cell death in HepG2 cells as measured by fluorescence microscopy.

HepG2 cells were seeded at a density of 5×10^4 cells/well and incubated overnight in a cell culture incubator at 37°C. The next day, cells were treated with either vehicle or different concentrations of Tamoxifen for 24 hours. On the third day, cells were processed for propidium iodide and MDC staining according to the protocol described. Panel A: MDC staining of HepG2 cells treated with vehicle. There is a basal level of autophagy, indicated by faint silver dot staining of autophagic vacuoles. Panel B: Propidium iodide staining of HepG2 cells treated with vehicle. There are few dead cells with only background staining of propidium iodide. Panel C: MDC staining of HepG2 cells treated with 10 μ M Tamoxifen. Note the increase in fluorescence intensity and numbers of autophagic vacuoles compared to the cells treated with vehicle. Panel D: Propidium iodide staining of HepG2 cells treated with 10 μ M Tamoxifen, showing a similar staining pattern to that of cells treated with vehicle.

Plate Reader Fluorescence Detection

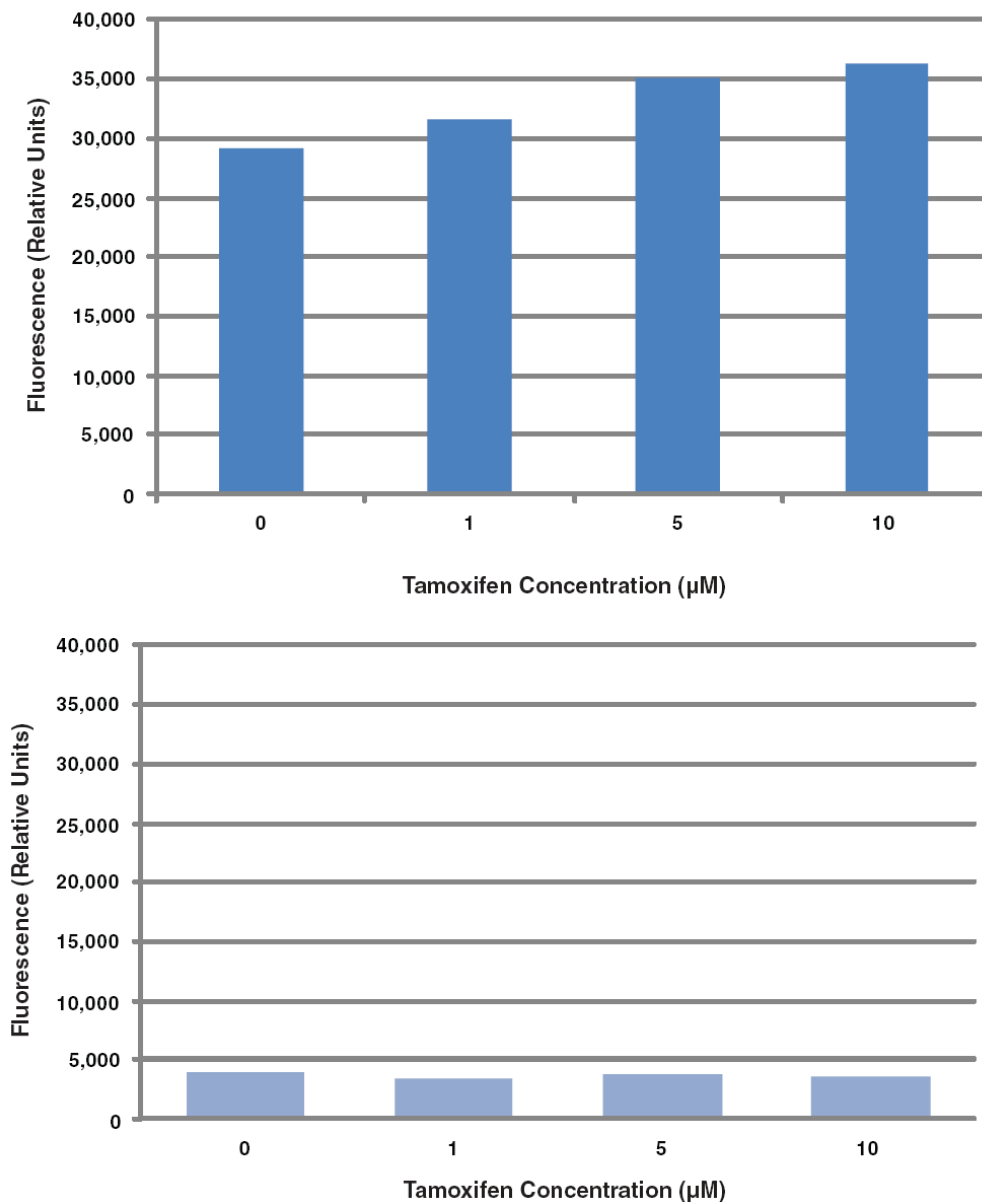


Figure 2. Tamoxifen increases autophagy but not cell death in HepG2 cells.

HepG2 cells were seeded in a 96-well plate at a density of 5×10^4 cells/well in EMEM culture medium and incubated overnight at 37°C. The next day, cells were treated with different concentrations of Tamoxifen and incubated overnight. On the third day, cells were stained with propidium iodide and MDC according to the protocol described and fluorescence was quantified using a plate reader. Top panel: Tamoxifen treatment increased MDC fluorescence intensity, indicating that Tamoxifen caused an increase in autophagy in HepG2 cells. Bottom panel: Tamoxifen treatment did not cause an increase in propidium iodide staining, indicating that at the concentration used here, Tamoxifen did not cause cytotoxicity in HepG2 cells.

Resources

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Low MDC staining in all treatments, including positive control.	Cells are not healthy.	Use only healthy cells.
High level of propidium iodide staining, including in control cells.	Cells are not healthy or are dead.	Use only healthy living cells.
Low fluorescence intensity in both MDC and propidium iodide staining.	A. Cell density too low. B. Cells are lost during processing.	A. Increase cell density. B. Gently aspirate supernatant to ensure most of the cells stay on the plate.

References

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5. Niemann, A., Takatsuki, A., and Elsässer, H.-P. The lysosomotropic agent monodansylcadaverine also acts as a solvent polarity probe. *J. Histochem. Cytochem.* 48(2), 251-258 (2000).

Plate Layout

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