Autophagy/Cytotoxicity Dual Staining Kit (Cell-Based) (BN00691)

(Catalog BN00691); 50 assays; Store at -20°C)

I. Introduction:

Autophagy or autophagocytosis ("self-eating") refers to a process of degradation of cytoplasmic components within lysosomes, a unique process mediated by autophagosomes. Autophagy consists of several sequential steps: sequestration, transport to lysosomes, degradation and eventual re-utilization of degradation products. Autophagosomes engulf a portion of cytoplasm, thus autophagy is generally thought to be a nonselective degradation system regulated by many different cellular signaling pathways. Autophagy functions as a stress response upregulated by nutrient and energy starvation, oxidative stress, or other harmful conditions (such as damage to organelles, protein aggregation and infection by pathogens). Dysfunction of autophagy is associated with many human cancers and neurodegenerative diseases. Assay Genie's Autophagy/Cytotoxicity Dual Staining Kit (Cell-Based) enables detection and monitoring of autophagy and autophagic cell death in cultured cells. The kit contains two fluorescent probes: a membrane permeable selective autophagy stain and a cell death marker. An autophagy-inducing positive control reagent, which increases autophagy staining and serves as an experimental control. This easy-to-use non-radioactive kit allows researchers to study the regulation of autophagy and cytotoxicity at the cellular level by Fluorescence Microscopy and Flow Cytometry in cultured cells.

II. Applications:

- Staining for autophagy and cell death.
- Screening of compounds capable of inducing or inhibiting autophagy function.

III. Sample Type:

- Suspension or adherent cells cultures

IV. Kit Contents:

Components	BN00691	Cap Code
Assay Buffer (10X)	25 ml	NM
Autophagy Stain (500X)	100 µl	Orange
Cell Death Marker (500X)	100 µl	Red
Positive Control Reagent (10000X)	30 µl	Yellow

V. User Supplied Reagents and Equipment:

- 1X PBS
- Tissue culture plates and media
- Fluorescence microscope with UV/Blue and Green/Red fluorescence filters
- Flow cytometer with UV/violet (355/405 nm) and blue (488 nm) laser lines

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protected from light. Briefly centrifuge small vials prior to opening. Read entire protocol before performing the assay.

- **Assay Buffer (10X):** Dilute 10X Assay 10 times in ddH_2O to obtain a 1X Assay Buffer. Keep on ice while in use.
- **Autophagy Stain (1000X), Cell Death Marker (500X) and Positive Control Reagent (10000X):** Warm to room temperature before use. Store at -20°C, avoid repeated freeze/thaw cycles.

VII. Autophagy/Cytotoxicity Dual Staining Protocol:

This protocol was developed for Jurkat cells and can be adjusted for any cell type. The assay volume is 1 ml; however, optimal conditions depend on the cell type. Reagents, buffer, and the number of cells should be adjusted accordingly for different plate formats.

1. Sample Preparation:

a. Obtain suspension or adherent cell culture of desired density and incubate the cells for 8-12 hours in appropriate medium supplemented with 10% FBS at 37°C with 5% CO_2.

b. Next day, remove the media and replace with 2 ml of fresh complete medium containing either vehicle or the test compound at desired concentration. Pellet the **suspension cells** at 300 x g for 5 minutes at room temperature prior to media removal**.** For positive autophagy **experimental control:** First, dilute 10-fold for Positive Control Reagent (10000X) by taking 5 µl of Positive Control Reagent into 45 µl 1X Assay Buffer, then add 2 µl of diluted Positive Control Reagent (1000X) directly into 2 ml of culture media and incubate the cells for 24 hour at 37°C with 5% CO_2, or time required by your experimental protocol.

c. **For adherent and suspension cells:** Terminate the experiment and harvest the cells. Wash the cells twice in 1 ml ice-cold 1X Assay Buffer. Pellet the cells at 300 x g for 5 min at room temperature prior to removal of media and washes**.**

d. Re-suspend cell pellets in 2 ml of 1X Assay Buffer; and split each cell suspension equally into 2 vials (one for Autophagy Staining, one for cell death staining).

e. **Autophagy Staining:** Add 2 µl of 500X Autophagy Stain into 1 ml of cell suspension. Incubate for 10 min at 37°C, 5% CO_2.

f. Spin down the cells at 300 x g for 5 min at room temperature, remove the media. Wash cells twice with 1X PBS. Re-suspend cells in 1 ml of 1X Assay Buffer. Cells are ready to be analyzed by flow cytometer or fluorescence microscope for autophagy staining.

g. **Cell Death Staining:** Add 2 µl of Cell Death Marker (500X) into the second tube containing 1 ml of cell suspension and incubate at room temperature for 2 min. Cells are ready to be analyzed by flow cytometer or fluorescence microscope for cell death.

2. FACS Acquisition and Analysis: Select the main cell population in the FSC vs. SSC plot to exclude dead cells and cellular debris from unstained cells (Negative Control Cells). Within the main cell population, mean fluorescence intensity in the UV/violet laser channel

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(Autophagy Staining) and FL-2 channel (Cell Death) can be quantified and compared between untreated cells and cells treated with test compounds, or between different cell types.

Notes:

- Trypsin can be used to collect the adherent cells prior to FACS analysis.
- The assay can be used to measure and compare both autophagy staining and cell death in various cell types.

3. **Fluorescence Microscope Analysis:** Observe the cells and capture images under the fluorescence microscope using a UV/blue (Ex = 340-380 nm, Em ≥ 460 nm) filter set to observe autophagy staining and a green/red (Ex = 535-555 nm, Em ≥ 590 nm) filter set to observe cell death, respectively.

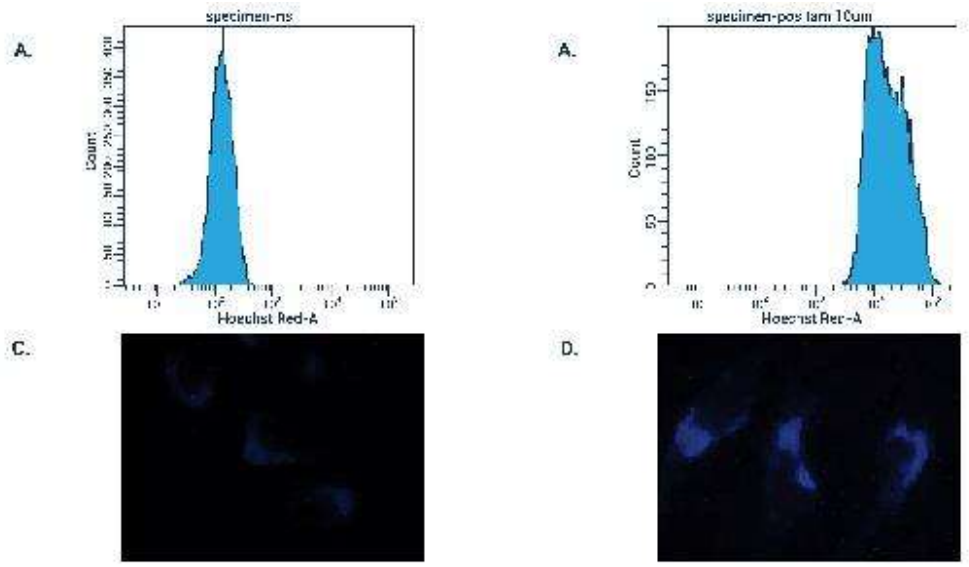

Figure: Autophagy/Cytotoxicity Staining in Jurkat and HeLa cells. 1x10^6 Jurkat or 1x10^5 HeLa cells were treated with vehicle or Positive Control Reagent for 24 h followed by Autophagy Staining according to kit's protocol. Histograms from flow analysis of untreated cells (Basal Autophagy; **A**) and Experimental Control Cells (treated with Positive Control; **B**). Fluorescence microscope images of Basal Autophagy **(C)** and Experimental Control Cells **(D)**. Increased blue fluorescence induced by treatment with Positive Control Reagent compared to the Basal Autophagy levels confirms intracellular accumulation of fluorescent stain in the autophagosomes.

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