Lysosomal Intracellular Activity Assay Kit (Cell-Based) (#BN00690)

(Catalog # BN00690; 50 assays; Store at -20°C)

I. Introduction:

Lysosomes are membrane-bound organelles important for various cellular processes. They contain hydrolytic enzymes that are utilized in the metabolism of some biomolecules. The extracellular cargo (e.g. nutrients toxins) binds to the cell membrane and is subsequently transported into membrane-bound endosomes for further degradation by lysosomes while intracellular components are transported to lysosomes through autophagy. Lysosomal dysfunction is associated with many human conditions such as aging and neurodegenerative disease. Although the intracellular activity of lysosome is difficult to measure in living cells, Assay Genie has developed a proprietary Lysosome-Specific Self-Quenched Substrate which has low background fluorescence, high signal to background ratio and is pH insensitive. The substrate, acting as endocytic cargo, can be taken up by cells and degraded within an endo-lysosomal vesicle. The fluorescent signal is recovered from the Self-Quenched Substrate. The fluorescence signal, generated by degradation, is proportional to the intracellular lysosomal activity and can be measured using a fluorescence microscopy and/or flow cytometry. Lysosomal Intracellular Activity Assay Kit (Cell-Based) includes Cytochalasin D, a cell-permeable inhibitor of endocytosis that serves as an experimental control. This easy-to-use, non-radioactive kit allows imaging and accurate measurement of de-quenching substrate in cultured cells.

II. Applications:

- Measurement of lysosomal intracellular activity.
- Elucidation of the mechanisms of endocytic pathway in living cells.
- Screening compounds with anti-lysosomal intracellular activity.

III. Sample Type:

- Suspension or adherent cells cultures

IV. Kit Contents:

Components	BN00690	Cap Code	Part Number
Assay Buffer (50X)	1.8 ml	Brown	BN00690-1
Self-Quenched Substrate	1 vial	Orange	BN00690-2
Cytochalasin D (100X)	50 µl	Yellow	BN00690-3

V. User Supplied Reagents and Equipment:

- 1X PBS
- Tissue culture plates and media
- Fluorescence microscope
- Flow cytometer with excitation filter at 488 nm wavelength

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 (50X):** Dilute 50X Assay Buffer 50 times in 1X PBS to obtain a 1X Assay Buffer. Keep on ice while in use.
- **Self-quenched substrate:** Re-constitute the vial with 1 ml of 1X PBS. Mix well. Aliquot and store at -20°C, avoid repeated freeze/thaw.
- **Cytochalasin D:** Warm to room temperature before use. Aliquot and store at -20°C, avoid repeated freeze/thaw.

VII. Lysosomal Intracellular Activity Assay Protocol:

This protocol was developed for U937 suspension cells and can be adjusted for any cell type. The cell culture density was 1 x 10^6 cells/ml and an assay volume of 1 ml; however, optimal conditions depend on the cell type. Reagents, buffer, and the number of cells should be adjusted accordingly for different plates.

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. **For adherent and suspension cells:** Next day, remove the media and replace with fresh complete medium containing either vehicle (positive control) or the test compound at desired concentration. For experimental control (Cytochalasin D treatment): dilute the 100X Cytochalasin D stock directly into the media to obtain the 1X final concentration. Incubate the cells for 1 hour, or time required by your experimental protocol, at 37°C with 5% CO_2. **For suspension cells:** Pellet the cells at 300 x g for 5 minutes at room temperature prior to media removal**.**

c. **For adherent and suspension cells:** Upon completion, remove the media and replace with fresh aliquots supplemented with 0.5% FBS. Add vehicle (positive control) or test compound at the same concentration as in step 1b. For experimental control: add Cytochalasin D to 1X final concentration. Add 15 µl of Self-Quenched Substrate per 1 ml of media into the positive control, experimental control and tested compound cells. Incubate the cells for 1 hour, or the time required for your specific cell line, at 37°C with 5% CO_2. **For suspension cells:** Pellet the cells at 300 x g for 5 minutes at room temperature prior to media removal**.**

d. **For adherent and suspension cells:** Terminate the experiment and harvest the cells. Wash the cells twice in 1 ml ice-cold 1X Assay Buffer containing the tested compound at the same concentration as in step 1b. **For suspension cells:** Pellet the cells at 300 x g for 5 minutes at room temperature prior to media and washes removal**.**

e. Re-suspend cell pellets in 1 ml of 1X PBS containing the tested compound at the same concentration as in step 1b. Cells are ready to be analyzed on flow cytometer (488 nm excitation laser).

2. **FACS acquisition and analysis**: select the main cell population in the FSC vs SSC plot to exclude dead cells and cellular debris. Within the main cell population, mean fluorescence intensity in FL1 can be quantified and compared between untreated cells and cells treated with test compounds or between different cell types to distinguish different levels of released fluorescence from Self-Quenched Substrate.

   Notes:

   a. Trypsin can be used to collect the adherent cells prior to FACS analysis.

   b. The assay can be used to measure & compare the lysosomal intracellular activity in various cell types.

3. **Fluorescence microscope analysis:** To visualize the fluorescence of released Self-Quenched Substrate, observe the cells under fluorescence microscope with 488 nm excitation filter.

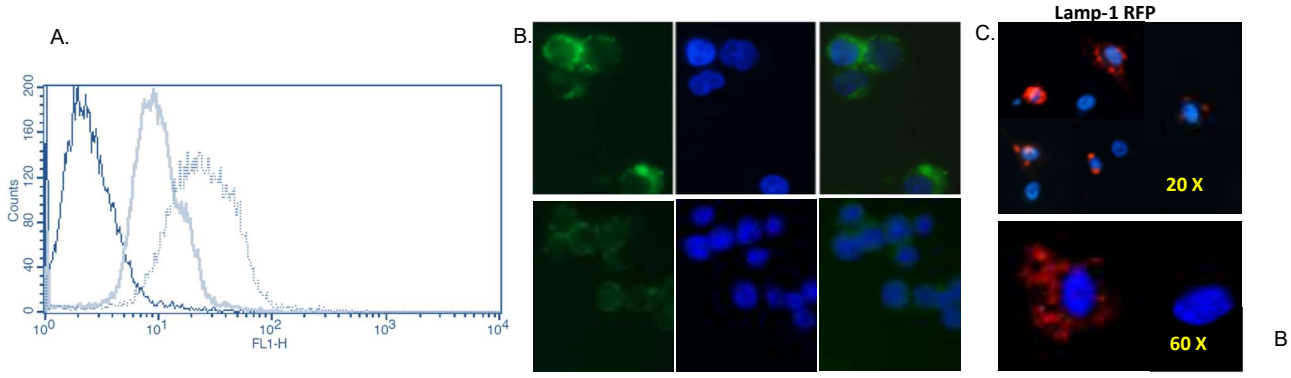

Figure: Release of self-Quenched Substrate **in U937 cells**. 1x10^6 U937 cells were pretreated with vehicle or 1X Cytochalasin D for 1 hr. After pre-treatment, cells were incubated with Self-Quenched Substrate and the same concentration of Cytochalasin D for additional hour in medium supplemented with 0.5% FBS according to kit's protocol. **Panel A:** Comparison of histograms from flow analysis showing the inhibition of De-Quenching of Substrate by Cytochalasin D. Unstained cells (black); experimental control (green) in the presence of 1X Cytochalasin D; Positive control (pink) without 1X Cytochalasin D. **Panel B:** Images of U937 cells obtained using fluorescence microscope. Top: positive control cells treated Self-quenched substrate only. Bottom: negative control cells treated with 1X Cytochalasin D. U937 cells showing the release of Self-quenched substrate in the endocytotic vesicle (punctured pattern). **Panel C:** Lysosomal staining with Lysosomal Associated Membrane Protein 1 (Lamp-1 RFP, a lysosomal marker). Cells were stained with nuclear dye for 10 min, washed with 1X PBS and mounted on the slide. Images were taken using a fluorescence microscope with a 60X objective lens.

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