Autophagy Assay, Red

Catalog #9156 & #9157

FOR RESEARCH USE ONLY.
Not for use in diagnostic procedures.

Easily monitor autophagy in live cells using flow cytometry

1. INTRODUCTION

Autophagy is a conserved lysosomal recycling process by which cells break down their own components such as proteins, lipids, and carbohydrates. Autophagy plays a critical role in maintaining homeostasis by preventing the accumulation of damaged organelles by disassembling unnecessary or dysfunctional cells and cellular components[1]. Autophagy occurs at low levels in the cell under normal conditions and can be rapidly upregulated during times of starvation or stress. Such degradation activities serve to provide nutrients (amino acids, nucleotides, fatty acids, etc.) and energy during periods of elevated bioenergetic demands[1,2]. Another function of autophagy is to assist with the detection and destruction of intracellular pathogens (viruses, bacteria, and parasites)3. Dysregulation of autophagy has been associated with many disease states including cancer, infection, and degenerative diseases[2].

Autophagy is a dynamic process typically divided into three stages (Figure 1)[1]. During stage one, cytoplasmic components targeted for degradation are sequestered within a double-membrane phagophore (also called the isolation membrane). This results in the formation of double-membrane vesicle called the autophagosome. During stage two, the autophagosome fuses with the lysosome to form the autophagolysosome or autolysosome. Degradation of the autophagosomal contents occurs during stage three[1,4].

ICT's Autophagy Assay, Red enables researchers to detect and monitor the *in vitro* development of autophagy in living cells. Autophagy Probe, Red is a cell-permeant aliphatic molecule that fluoresces brightly when inserted in the lipid membranes of autophagosomes and autolysosomes. Autophagy Probe, Red can be readily detected by flow cytometry (Figure 2) with optimal excitation at 590 nm and peak emission at 620 nm. Autophagy Probe, Red is for research use only. Not for use in diagnostic procedures.

2. KIT CONTENTS

Trial size kit (#9156) contains:

- 1 vial of Autophagy Probe, Red (50 tests/vial), #6701
- 1 bottle of 10X Cellular Assay Buffer (15 mL), #6694
- 1 bottle of Fixative (6 mL), #636

Standard size kit (#9157) contains:

- 4 vials of Autophagy Probe, Red (50 tests/vial), #6701
- 1 bottle of 10X Cellular Assay Buffer (60 mL), #6695
- 1 bottle of Fixative (6 mL), #636

3. STORAGE

- Store the unopened Autophagy Probe, Red (Pack 2) at ≤-20°C until the expiration date.
- Store the remaining unopened kit components (Pack 1) at 2-8°C until the expiration date.

Once reconstituted with DMSO, use Autophagy Probe, Red immediately, or aliquot and store at ≤-20°C for 6 months protected from light. Avoid repeated freeze thaw cycles. .

4. SAFETY DATA SHEETS (SDS)

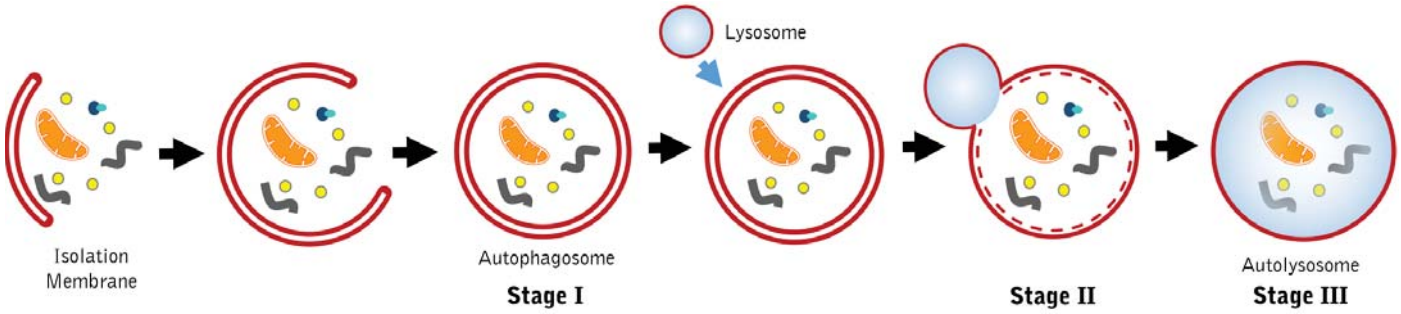
Safety data sheets are available online at www.immunochemistry.com or by calling 1-800-829-3194 or 952-888-8788.

5. RECOMMENDED MATERIALS

- DMSO to reconstitute Autophagy Probe, Red (100 µL per vial). Additional DMSO is needed to create controls.
- DiH_20, 135-540 mL to dilute 10X Cellular Assay Buffer
- Phosphate buffered saline (PBS) pH 7.4, 400 µL per vial to dilute Autophagy Probe, Red
- Cultured cells treated with the experimental conditions ready to be analyzed
- Reagents to induce autophagy such as Rapamycin (Invivogen, #tlrl-rap, CAS #53123-88-9), and to inhibit autophagy such as Chloroquine (Sigma-Aldrich, #C6628, CAS #50-63-5)
- Hemocytometer
- Centrifuge at <200 x g
- FACS tubes (for flow cytometry analysis)
- 15 mL polypropylene centrifuge tubes (1 per sample)

Figure 1: Cellular Process of Autophagy

Autophagy is an intracellular degradation process during which cytosolic organelles and materials are enclosed within an isolation membrane to form an autophagosome. The outer membrane of the autophagosome fuses with the lysosome. The sequestered material is subsequently degraded within the autolysosome.



6. DETECTION EQUIPMENT

The assay can be analyzed with a flow cytometer. Use filter pairings that best approximate these settings:

- Autophagy Probe, Red reagent optimally excites at 590 nm and has a peak emission at 620 nm (use a green/yellow laser for flow cytometry).

7. EXPERIMENTAL PREPARATION

Staining cells with Autophagy Probe, Red can be completed within a few hours. However, Autophagy Probe, Red is used with living cells, which require periodic maintenance and cultivation several days in advance. In addition, once the proper number of cells has been cultivated, time must be allotted for the experimental treatment, which may vary. The recommended sample size is 0.5 mL cells at 5 x 10^5 cells/mL.

Create cell populations, such as:

a. Cells that were exposed to the experimental treatment.

b. A negative control population of cells that received a placebo treatment.

Culture cells to a density optimal for the specific experimental protocol. Cell density should not exceed 106 cells/mL. Cells cultivated in excess of this concentration may begin to naturally enter apoptosis. An initial experiment may be necessary to determine how much Autophagy Probe, Red reagent to use.

8. CONTROLS

Create experimental samples and control cell populations:

a. Treated experimental population(s): cells exposed to the experimental condition(s).

b. Positive control for Autophagy Probe, Red (cells induced to initiate autophagy): Numerous treatments exist for activating autophagy (such as starvation in Earl's Balanced Salt Solution, or exposure to Rapamycin which mimics starvation by inhibiting mTOR) or inhibiting fusion of autophagosome with lysosomes and lysosomal degradation (exposure to Chloroquine). Figure 2 shows Jurkat cells that had been treated with 0.5 µM Rapamycin and 10 µM Chloroquine for 18 hours.

c. Negative control for Autophagy Probe, Red (untreated cells): cells mock-treated with DMSO.

A common pool of cells should be used to generate the positive and negative control populations for Autophagy Probe, Red, and should contain similar quantities of cells. For example, if labeling with Autophagy Probe, Red, make 4 populations:

1&2. Unlabeled: induced and untreated cells

3&4. Autophagy Probe, Red-labeled: induced and untreated cells

9. PREPARATION OF 1X CELLULAR ASSAY BUFFER

ICT's Cellular Assay Buffer (catalog #6694 or #6695) is an isotonic solution used to stabilize cells when analyzing by flow cytometry. ICT's Cellular Assay Buffer contains mammalian proteins to stabilize cells, and sodium azide to retard bacterial growth (1X Cellular Assay Buffer contains 0.01% w/v sodium azide). Alternative solutions including cell culture media containing FBS and other additives may be used instead of 1X Cellular Assay Buffer. When selecting a solution, take care to avoid prolonged incubation in a nutrient-depleted buffer, as this can induce starvation leading to an unintentional induction of autophagy.

1. 10X Cellular Assay Buffer may form precipitates during cold storage. If this happens, gently warm it until all crystals have dissolved. Do not boil.
2. Dilute 10X Cellular Assay Buffer 1:10 in diH_2O. For example, add 15 mL 10X Cellular Assay Buffer to 135 mL diH_2O for a total of 150 mL (#6694), add 60 mL 10X Cellular Assay Buffer to 540 mL diH_2O for a total of 600 mL (#6695).

1X Cellular Assay Buffer may be stored at 2-8°C and used within 1 week or frozen and used within 6 months.

10. PREPARATION OF AUTOPHAGY PROBE, RED REAGENT

Autophagy Probe, Red is supplied as a lyophilized powder that may be slightly visible as an iridescent purple sheen inside the vial. Protect from light and use gloves when handling. Once diluted in aqueous buffer, Autophagy Probe, Red solution must be used immediately; prepare it just before staining.

1. Reconstitute the vial of Autophagy Probe, Red with 100 µL DMSO to form the 250X stock solution. The stock solution should be bluish-purple. Once reconstituted in DMSO, it may be aliquoted and stored at ≤-20°C for 6 months protected from light. Avoid repeated freeze/thaw cycles.

2. Immediately prior to addition to the samples and controls, dilute Autophagy Probe, Red reagent 1:5 by adding 400 µL PBS to each vial to form the 50X Autophagy Probe, Red solution. Use 50X Autophagy Probe, Red within 30 minutes of dilution into aqueous buffers.

These amounts are recommendations, however, the sample size and Autophagy Probe, Red staining concentration needed may vary based on the experimental conditions used. Each investigator should adjust the amount of Autophagy Probe, Red to accommodate the particular cell line and research conditions.

11. FIXATIVE

ICT's Fixative is a formaldehyde solution designed to cross-link and aggregate intracellular components. If the stained cell populations cannot be evaluated immediately after labeling with Autophagy Probe, Red, add Fixative at a ratio of 1:5-1:10. For example, to use Fixative at 1:10, add 100 µL Fixative to 900 µL cells. Never add Fixative until all the staining and final wash steps have been completed. Fixed cells may be stored on ice or at 4°C for up to 16 hours, protected from light.

ICT's Fixative will not interfere with the red fluorescent label. Do not use absolute ethanol- or methanol-based fixatives, as they may inactivate the Autophagy Probe, Red label.

- **Danger:** Fixative contains formaldehyde <10% and methanol <5% and is harmful. Avoid contact with skin, eyes, and clothing by wearing lab coat, gloves, and safety glasses. In case of exposure, immediately flush eyes or skin with water. See SDS for further information.

12. FLOW CYTOMETRY ANALYSIS

The recommended sample size is 0.5 mL. The recommended cell concentration is 3-5 x 10^5 cells/mL. Sample flow cytometry results shown in Figure 2.

1. Expose cells to the experimental or control conditions. Set aside two populations to create positive and negative instrument controls with Autophagy Probe, Red (Section 8, and Figure 2).
2. Optional wash step: depending on the experimental conditions used to induce autophagy, it may be necessary to wash cells and then resuspend them in fresh cell culture medium prior to staining.
3. Transfer 490 µL cells into fresh tubes.
4. Add 10 µL 50X Autophagy Probe, Red solution, forming a final volume of 500 µL. Autophagy Probe, Red can be added directly to the cell culture medium for the staining process. If different cell volumes were used, add Autophagy Probe, Red at a ratio of 1:50. Mix by gently flicking the tubes. The amount of Autophagy Probe, Red should be optimized for each cell line and experimental condition.
5. Incubate cells at 37°C protected from light. The incubation period may range from 30 minutes to several hours and should be optimized for each cell line and experimental condition. As cells may settle on the bottom of the tubes, gently resuspend them by swirling cells every 20 minutes to ensure an even distribution of Autophagy Probe, Red throughout the staining process.
6. Centrifuge at 200 x g for 10 minutes at room temperature.
7. Carefully remove and discard supernatants. Resuspend in 0.5 mL of 1X Cellular Assay Buffer and mix gently. If desired, cells can be washed using fresh cell culture medium or alternate buffers such as PBS containing BSA. Take care that the wash steps are completed quickly so as not to cause unanticipated cellular stress by prolonged exposure to nutrient-deprived buffers.
8. Repeat wash steps until samples have been washed 3 times. After final centrifugation, resuspend the pellet in 0.5 mL 1X Cellular Assay Buffer.
9. Cells are ready for analysis. An optional fixation step may be performed at this time. Add the provided Fixative at a v/v ratio of 1:5-1:10. For example, add 50 µL of Fixative to 450 µL of sample. To analyze the samples, measure fluorescence using a green/yellow laser equipped with an appropriate filter.

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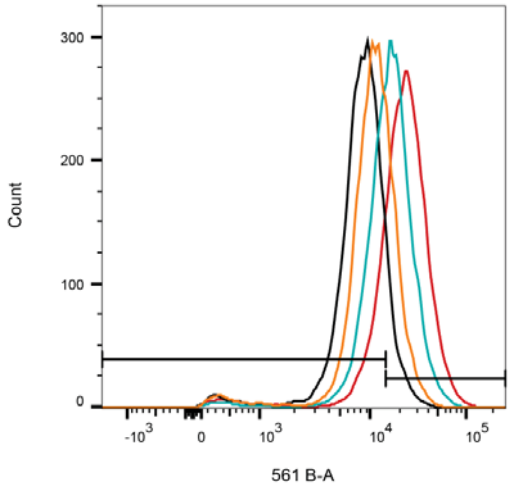
Figure 2: Flow Cytometry Results

ICT's Autophagy Assay Kit, Red was used to assess the induction of autophagy in Jurkat cells. Cells were either untreated (Black) or treated with 0.5 µM Rapamycin (Orange), 10 µM Chloroquine (Blue), or both 0.5 µM Rapamycin and 10 µM Chloroquine (Red) for 18 hours. After staining with Autophagy Probe, Red for 60 minutes, cells were washed and analyzed by flow cytometry (BD LSRFortessa Special Order flow cytometer equipped with a green/yellow laser (561 nm excitation) and a 610/20 emission filter). An overlay of the histograms is shown on the right. A table displaying the median fluorescence signal, % negative, and % positive cells is shown below. Treatment with Rapamycin or chloroquine increased the fluorescence signal detected compared to the untreated control. Combined treatment of rapamycin and chloroquine further increased the fluorescence signal detected. Data courtesy of Dr. Kristi Strandberg (ICT 228:37-40).



Treatment	Median (561 B-A)	% Negative	% Positive
Untreated (DMSO)	8658	86.0	14.0
Rapamycin	11146	67.8	32.2
Chloroquine	16004	36.5	63.5
Rapamycin + Chloroquine	22237	17.9	82.1

13. REFERENCES AND CITATIONS

1. N. Mizushima, M. Komatsu, Autophagy: renovation of cells and tissues. Cell 147, 728-741 (2011).
2. B. Levine, G. Kroemer, Autophagy in the pathogenesis of disease. Cell 132, 27-42 (2008).
3. B. Levine, N. Mizushima, H. W. Virgin, Autophagy in immunity and inflammation. Nature 469, 323-335 (2011).
4. P. Hundeshagen, A. Hamacher-Brady, R. Eils, N. R. Brady, Concurrent detection of autolysosome formation and lysosomal degradation by flow cytometry in a high-content screen for inducers of autophagy. BMC Biol 9, 38 (2011).

Thank you for using our Autophagy Assay, Red.
If you have any questions, or would like to share your data, please contact us at 1-800-829-3194 or 952-888-8788, or send an email to help@immunochemistry.com.

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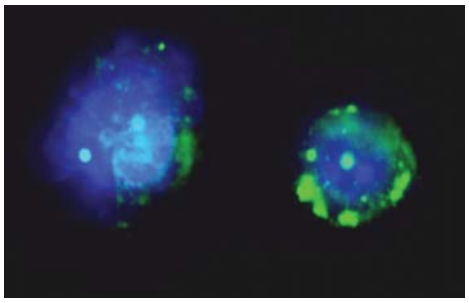
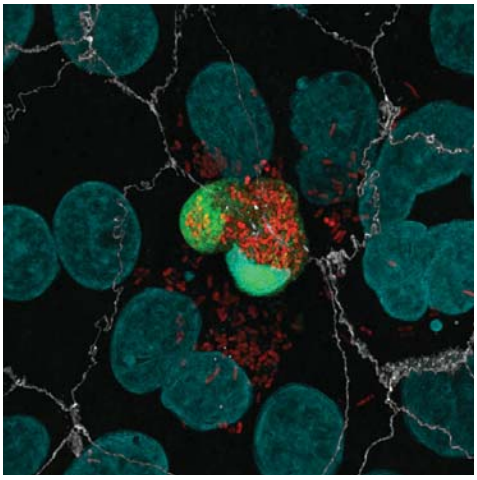


Apoptotic neuroblastoma cells fluoresce green after staining with FAM-FLICA® Poly Caspase Assay (#92). Hoechst 33342 nucleic acid stain (blue in image) is included in the kit as well as propidium iodide vital stain.



Pyroptotic human colorectal cells infected with Salmonella typhimurium (which is expressing mCherry and fluoresces red) fluoresce green after staining with FAM-YVAD-FLICA included in Pyroptosis/ Caspase-1 Assay, Green (#9145). Hoechst 33342 nucleic acid stain (blue in image) and Nigercin (Caspase-1 inducing reagent for generating a positive control) are also included in this kit.

The contents of a FAM-FLICA® Poly Caspase Assay Kit (# 92), standard size.

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ImmunoChemistry Technologies, LLC gratefully acknowledges the significant contributions made by one of its founders, Brian W. Lee, Ph.D in the development of this product.