Autophagy Assay Red Detection Kit

BIO-RAD

Autophagy Assay Kit

Cat #	Quantity
APO010A	50 Tests

Kit Components

Component	Storage Conditions	Quantity
1 vial Autophagy Probe Red	-20°C	
1 vial fixative	2-8°C	6 ml

Autophagy Assay Kit

Cat #	Quantity
APO010B	200 Tests

Kit Components

Component	Storage Conditions	Quantity
4 vials Autophagy Probe Red	-20°C	
1 vial fixative	2-8°C	6 ml

Both kits are provided in two parts which should be stored at different temperatures:

- Part one contains the Autophagy Probe which should be stored at -20°C. Once reconstituted with DMSO, use immediately or aliquot and store at -20°C for up to 6 months protected from light. Avoid repeat freezing and thawing
- Part two contains the fixative which should be stored at 2-8°C. Do not freeze

Background

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 (Mizushima et al. 2011). 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 (for example amino acids, nucleotides, fatty acids) and energy during periods of elevated bioenergetic demands (Mizushima et al. 2011, Levine et al. 2008). Another function of autophagy is to assist with the detection and destruction of intracellular pathogens (viruses, bacteria and parasites) (Levine et al. 2011). Dysregulation of autophagy has been associated with many disease states including cancer, infection and degenerative diseases (Levine et al. 2008).

Autophagy is a dynamic process typically divided into three stages (Figure 1). During stage one, cytoplasmic components targeted for degradation are sequestered within a double-membrane phagopore (also called the isolation membrane). This results in the formation of a double-membrane vesicle called the autophagosome. During stage two, the autophagosome fuses with the lysosome to form the autolysosome. Degradation of the autophagosomal contents occurs during stage three (Mizushima et al. 2011, Hundeshagen et al. 2011).

Bio-Rad's Autophagy Assay Red Detection Kit 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 (ZE5 Cell Analyzer settings: 561 nm laser; 615/24 or 640/20 filter). Autophagy Probe Red is for research use only, not for use in diagnostic procedures.

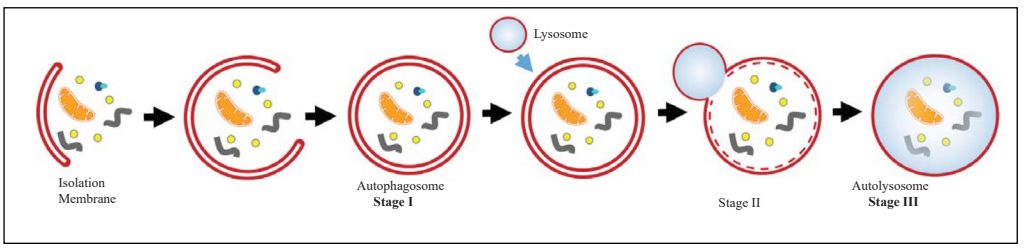

Fig. 1. Cellular process of autophagy. Autophagy is an intracellular degradation process during which cytosolic organelles and materials are enclosed within an isolation membrane to form an autophagosome. The outer membrane of the autophagosome fuses within an isolation membrane to form an autophagosome. The outer membrane of the autophagosome fuses with the lysosome. The sequestered material is subsequently degraded within the autolysosome.

Experimental Set-Up

Staining cells with Autophagy Probe Red can be completed within a few hours. However, Autophagy Probe Red is used with living cells, which may require periodic maintenance and cultivation several days in advance. You may have to allow time for experimental treatment, which may vary. Create cell populations that have been exposed to experimental treatment and cells that have received no treatment and vehicle only. The density of suspension cells during treatment should not exceed 1 x 10^6 cells /ml as cells may begin to enter apoptosis. The recommended staining is 10 µl of 50x probe per 490 µl of cells at a concentration of 5 x 10^5 cells /ml, however this may vary depending on the cells and treatment used. Titration of Autophagy Probe is recommended.

Include appropriate controls for your experiment, such as:

- Unlabeled treated (experimental conditions) and untreated cells
- Labeled treated, untreated and vehicle (for instance DMSO) treated cells
- As a positive control, several treatments can be used. Starvation in Earl's Balanced Salt Solution (EBSS), Hank's Balanced Salt Solution (HBSS) or exposure to Rapamycin (mTOR inhibition) can induce autophagy. Exposure to Chloroquine or Bafilomycin A will inhibit fusion of autophagosome with lysosomes and lysosomal degradation. Figure 2 shows an example of Jurkat cells stained with Autophagy Probe Red with various treatments
- A common pool of cells should be used to generate both the positive and negative control populations for Autophagy Probe Red and should contain similar quantities of cells

Reagent Preparation

Autophagy Probe Red is supplied as a lyophilized powder that may be visible as an iridescent purple sheen on the inside of the vial. Reconstitute with 100 µl of DMSO to form the stock solution (250x). The stock solution should be bluish-purple. Once reconstituted, it can be aliquoted and stored, protected from light, at -20°C for 6 months. Immediately before use, dilute the Autophagy Probe Red 1:5 by adding 400 µl PBS to each 100 µl vial to form the 50x Autophagy Probe Red solution. Use this 50x solution within 30 min of dilution.

A wash buffer composed of PBS containing 5% BSA should be freshly made for each experiment. Alternatively cells can be washed in cell culture media.

Recommended Materials

- DMSO to reconstitute Autophagy Probe Red
- Wash buffer of PBS containing 5% BSA or cell culture media
- Phosphate Buffered Saline (PBS) to dilute 250x Autophagy Probe Red
- Experimentally treated cells
- Rapamycin
- Chloroquine
- FACS tubes
- Flow cytometer with appropriate laser and filters/detectors. For instance, 561 nm laser and 615/24 nm or 640/20 nm filters on ZE5 Cell Analyzer

General Protocol for Suspension Cells

1. Prepare samples (3-5 x 10^5 /ml) and appropriate controls including positive and negative.
2. Optional wash step: depending upon the conditions used to induce autophagy, it may be necessary to wash the cells and resuspend in fresh culture medium prior to staining.
3. Make wash buffer of PBS containing 5% BSA or cell culture media.
4. Reconstitute Autophagy Probe Red with 100 µL DMSO (250x stock).
5. Immediately before use, dilute Autophagy Probe Red 1:5 by adding 400 µL PBS (50x stock).
6. Transfer 490 µl of cells into fresh tubes.
7. Add 10 µL 50x Autophagy Probe Red to each sample (490 µL aliquot of cells). If different volumes are used, add Autophagy Probe Red initially at a ratio of 1:50. The amount of Autophagy Probe Red may need to be optimized depending on experimental conditions and cells used.
8. Incubate at 37°C protected from light. This can be 30 min to several hours depending on experiment. Gently resuspend the cells every 20 min to ensure even distribution of Autophagy Probe Red.
9. Centrifuge cells at 200 g and discard supernatant.
10. Discard the supernatant. Resuspend in 500 µl of wash buffer. Repeat until the samples have been washed 3 times (3x).

11. Resuspend in 500 ml wash buffer and analyze. Samples can be fixed at this point if required. Add the fixative at a ratio of 1:5-1:10.

12. Samples can be analyzed and fluorescence measured using a green/yellow laser equipped with the appropriate filter. Autophagy Probe Red excites at 590 nm and emits at 620 nm.

If adherent cells are to be trypsinized for analysis by flow cytometry, after step 7 trypsinize cells and neutralize the trypsin using standard protocols. Add 2 ml wash buffer, gently mix, incubate for 5 min at RT or 37°C, then centrifuge cells at 300 g and discard supernatant. Repeat this wash step twice. Continue from step 9 of Suspension Cell Protocol.

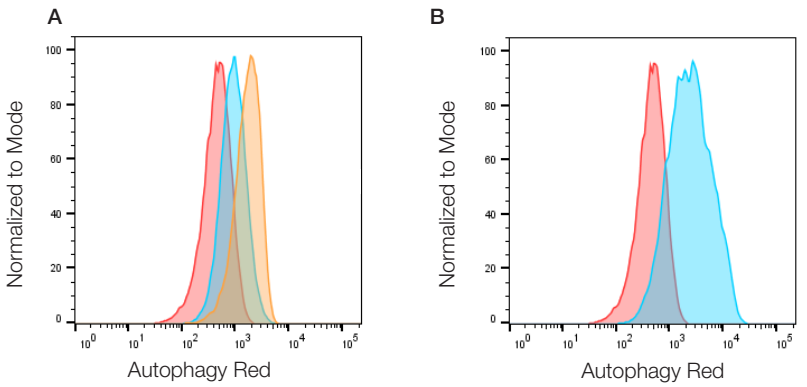

Fig 2. Autophagy induction in Jurkats measured by flow cytometry using Autophagy Probe Red. Jurkat cells were **A**, untreated (red) treated with 0.5 µM Rapamycin for 18 hr (blue) or treated with 0.5 µM Rapamycin for 18 hr and 10 nM Bafilomycin A for 2 hr (orange). **B**, starvation was also used to induce autophagy. Untreated cells (red) and cells incubated in HBSS for 2 hr and 10 nM Bafilomycin A for 2 hr (blue). After staining with Autophagy Probe Red for 30 min, cells were washed and analyzed on the ZE5 Cell Analyzer using the 561 nm laser and 640/20 filter.

Autophagic Flux

Autophagic flux was calculated to determine levels of autophagy and allow comparison between treatments. This was calculated by taking the geometric mean for each treatment subtracting the untreated geometric mean and dividing by the untreated geometric mean. Values for the data in Figure 2 above, is shown in the table below.

Table 1. Autophagic Flux

Treatment	Geo Mean	Autophagic Flux
Untreated	424	0
Rapamycin	877	1.07
Rapamycin & Bafilomycin A	1573	2.71
HBSS (starvation)	2271	4.36

References

Mizushima et al. (2011). Autophagy: renovation of cells and tissues. Cell 147, 728-741.

Levine et al. (2008). Autophagy in the pathogenesis of disease. Cell 132, 27-42.

Levine et al. (2011). Autophagy in immunity and inflammation. Nature 469, 323-335.

Hundeshagen et al. (2011). Concurrent detection of autolysosome formation and lysosomal degradation by flow cytometry in a high-content screen for inducers of autophagy. BMC Biol 9, 38.

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