ab139484

Autophagy Detection Kit

Instructions for Use

For detection of autophagy in live cells by fluorescence microscopy, flow cytometry and fluorescence microplate assay.

View kit datasheet: www.abcam.com/ab139484
(use www.abcam.cn/ab139484 for China, or www.abcam.co.jp/ab139484 for Japan)

This product is for research use only and is not intended for diagnostic use.

Version: 4b Last Updated: 10 October 2024

Table of Contents

1. Introduction

When subjected to certain hostile conditions that threaten survival, such as when extracellular nutrients are limiting, eukaryotic cells employ a lysosome-mediated intracellular bulk degradation pathway for digesting their own cellular contents by a process referred to as autophagy. Various cytoplasmic constituents, including organelles and long-lived proteins, are sequestered into double-membraned autophagosomes, which subsequently fuse with lysosomes where their contents are degraded. Under physiological conditions, autophagy plays a variety of important roles including maintenance of the amino acid pool during starvation, damaged protein and organelle turnover, prevention of neurodegeneration, tumor suppression, cellular differentiation, clearance of intracellular microbes and regulation of innate and adaptive immunity. Autophagy is considered to be a dynamic, multi-step process which can be regulated at several steps, in both a positive and negative manner. Autophagic activity is typically low under basal conditions, but can be markedly up-regulated, both in cultured cells and intact organisms, by a variety of physiological stimuli such as nutrient starvation, hypoxia, energy depletion, endoplasmic reticulum stress, elevated temperature, high density growth conditions, hormonal stimulation, pharmacological agent treatment, innate immune signaling, and in diseases such as viral, bacterial or parasitic infections as well as various protein aggregopathies (*e.g.,* Alzheimer's, Huntington's and Parkinson's disease), heart disease and acute pancreatitis.

Autophagy can be suppressed in certain other diseases, including particular types of cancers, neuro-degenerative disorders, infectious diseases, and inflammatory bowel disorders. A reduction in autophagic function is also considered a characteristic of the aging process.

2. Product Overview

A conventional fluorescent probe, monodansylcadaverine (MDC), has served as a useful fluorescent marker for lysosomal/ autophagic vacuoles. However, it is known to generate high background and weak fluorescent signal. ab139484 has been optimized for detection of autophagy in live cells by fluorescence microscopy, flow cytometry and fluorescence microplate assay. The assay provides a rapid, specific and quantitative approach for monitoring autophagic activity at the cellular level. The 488 nm-excitable green fluorescent detection reagent supplied in the Autophagy Detection Kit becomes brightly fluorescent in vesicles produced during autophagy and has been validated under a wide range of conditions known to modulate autophagy pathways. Rapamycin and starvation are well known inducers of autophagy. Chloroquine is a lysosomal inhibitor. Rapamycin and Chloroquine are included as positive controls in the kit. A nuclear counterstain is provided in the kit as well to highlight cellular nuclei. This live cell analysis kit provides a convenient approach for the analysis of the regulation of autophagy at the cellular level.

3. Assay Summary

Incubate Cells with Green Detection Reagent and Nuclear Stain

Quantify Using Flow Cytometry

OR

Detect Using Fluorescence Microscopy

OR

Detect Using a Fluorescence Microplate Reader

4. Components and Storage

A. Kit Contents

Item	Quantity	Storage Temperature
Green Detection Reagent	50 µl	-80°C
Nuclear Stain	50 µl	-80°C
Autophagy Inducer (Rapamycin)	25 nmol	-80°C
10X Assay Buffer	30 ml	-80°C
Chloroquine	7.5 µmol	-80°C

Reagents provided in the kit are sufficient for approximately 200 flow cytometry, 250 fluorescence microscopy or 3 x 96-well microplate assays.

B. Storage and Handling

Upon receipt, the kit should be stored at -80°C, protected from light. Avoid repeated freezing and thawing.

C. Additional Materials Required

- Flow cytometer equipped with 488 nm laser source
- Standard fluorescence microscope
- Fluorescence microplate reader
- Tubes appropriate for holding cells for the flow cytometer
- Calibrated, adjustable precision pipetters, preferably with disposable plastic tips
- Adjustable speed centrifuge with swinging buckets (for suspension cultures)
- Deionized water
- Anhydrous DMSO
- Total growth medium suitable for cell type
- Indicator-free cell growth medium
- FBS (Fetal Bovine Serum)
- Glass microscope slides
- Glass cover slips of appropriate size
- 96-well tissue culture microplate with black wall and clear bottom.

5. Pre-Assay Preparation

NOTE: Allow all reagents to thaw at room temperature before starting with the procedures. Upon thawing, gently hand-mix or vortex the reagents prior to use to ensure a homogenous solution. Briefly centrifuge the vials at the time of first use, as well as for all subsequent uses, to gather the contents at the bottom of the tube.

1. Reagent Preparation

1. Positive Controls

a) Autophagy Inducer (Rapamycin) included in the kit is supplied lyophilized. To use it as a positive control, resuspend lyophilized Rapamycin in 50 µL of DMSO. Resulting 500 µM stock solution can be further diluted in cell culture medium to a desired concentration (recommended starting concentration of Rapamycin as a positive control is 500 nM). However, the optimal final concentration is cell-dependent and should be determined experimentally for each cell line being tested. The agent has been validated in HeLa, HepG2 and Jurkat cells.

b) Chloroquine provided in the kit may be used in combination with Rapamycin or starvation in monitoring autophagic flux. Chloroquine included in the kit is supplied lyophilized (7.5 µmoles) and should be centrifuged briefly to gather the material at the bottom

of the tube. Reconstitute the lyophilized material in 125µl deionized water for a 60 mM stock solution. Depending on the applications and specific cell lines, it is recommended that treatment with the agent will be performed using 10-120 µM final concentration in order to observe changes in autophagic flux. Unused stock Chloroquine/ Rapamycin may be stored in small aliquots at -20°C for one month.

2. **1X Assay Buffer**

   Allow the 10X Assay Buffer to warm to room temperature. Make sure that the reagent is free of any crystallization before dilution. Prepare enough 1X Assay Buffer for the number of samples to be assayed by diluting each milliliter (mL) of the 10X Assay Buffer with 9 mL of deionized water.

2. Green Detection Reagent

For optimal staining, the concentration of Green Detection Reagent for optimal staining will vary depending upon the application. Suggestions are provided to use as guidelines, though some modifications may be required depending upon the particular cell type employed in the application. Refer to sections below for details on the preparation of the staining solution for specific applications. Prepare sufficient amount of the staining solution for the number of samples to be assayed.

a) Fluorescence microscopy application:

Prepare a sufficient amount of Microscopy Dual Detection Reagent for the number of samples to be assayed as follows: For every 1 mL of 1X Assay Buffer or complete cell growth medium, add 2 µL of Green Detection Reagent and 1 µL of Nuclear Stain. If 1X Assay Buffer is used, supplement it with 5% FBS.

Note: The dyes may be combined into one staining solution or each may be used separately, if desired. The Nuclear Stain can be diluted further if its staining intensity is much stronger than that of the Green Detection Reagent. When staining BFP- or CFP-expressing cells, the Nuclear Stain should be omitted due to its spectral overlap with these fluorescent proteins.

The green dye emits in the green region of the visible light spectrum and is thus not compatible with GFP.

b) **Flow cytometry application**:
Make a dye stain solution by diluting 1 µL Green Detection Reagent to 1 mL cell culture medium without Phenol Red Indicator, supplemented with 5% FBS. For each sample to be stained, 250 µL of diluted Green dye staining solution will be used.

c) **Fluorescence microplate application**: Prepare a sufficient amount of Microplate Dual Detection Reagent for the number of samples to be assays as follows: Add 1 µL of

Green Detection Reagent and 1 µL Nuclear Stain into 1 mL cell culture medium without Phenol Red Indicator, supplemented with 5% FBS. For each sample to be stained, 100 µL of Microplate Dual Detection Reagent will be used.

3. Cell Preparations

- Autography Inducer (Rapamycin) and Chloroquine as positive control - Positive control cells (e.g. Jurkat cells) should be pretreated with the Autophagy Inducer (Rapamycin) and Chloroquine for 16-18 hours. Response to Rapamycin and Chloroquine is time and concentration dependent and may also vary significantly depending upon cell type and cell line. Negative control cells should be treated with a vehicle (DMSO, media or other solvent used to reconstitute or dilute an inducer or inhibitor) for an equal length of time under similar conditions.
- Starvation and Chloroquine as positive control - Positive control cells (e.g. HeLa cells) should be starved in EBSS media in the presence of Chloroquine for 3-5 hours. Response to starvation and Chloroquine is time and concentration dependent and may also vary significantly depending upon cell type and cell line. Negative control cells should be incubated in the complete culture media with a vehicle (DMSO, media or other solvent used to reconstitute or dilute Chloroquine) for an equal length of time under similar conditions.

6. Assay Protocol

A. Live Cell Analysis by Fluorescence/Confocal Microscopy (Adherent Cells)

1. Grow cells on coverslips or tissue culture treated slides. When the cells have reached 50% ~ 70% level of confluence, carefully remove the medium.

   NOTE: Cells should be healthy and not overcrowded as results of the experiments will depend significantly on the cells' condition.

2. Treat the cells with the testing reagent according to your experimental procedure. It is highly recommended to set up positive and negative controls within the same experiment (see Pre-Assay Preparation)
3. Post-treatment, remove the medium with the testing reagents and positive control and wash the cells twice with 1X Assay buffer.

   NOTE: Be careful during washing procedure since autophagic cells can be easily dislodged from the slides. To preserve the cells, 2% - 5% FBS also may be added to the assay buffer at this point.

4. Dispense 100 µL of Microscopy Dual Detection Reagent to cover each sample of monolayer cells.

5. Protect samples from light and incubate for 30 minutes at 37°C.
6. Carefully wash the cells with 100 µL of 1X Assay Buffer. Remove excess buffer and place coverslip on microscope slide.
7. An optional fixation step may be included at this step. Incubate for 20 minutes with 4% formaldehyde. Wash 3 times with 1X Assay Buffer.
8. Analyze the stained cells by wide-field fluorescence or confocal microscopy (60X magnification is recommended). Use a standard FITC filter set for imaging the autophagic signal. Optionally, image the nuclear signal using a DAPI filter set.

B. Live Cell Analysis by Fluorescence/Confocal Microscopy (Suspension Cells)

1. Cells should be cultured to a density not to exceed 1 x 10^6 cells/mL. Ensure that cells are in the log phase of growth before starting an experiment.

   NOTE: Cells should be healthy and not overcrowded as results of the experiments will depend significantly on the cells' condition.

2. Collect the cells by centrifugation (5 min, 1000 rpm at room temperature). Resuspend the cells to a density of 1 x 10^6/ml.

3. Treat the cells with the testing reagent according to your experimental procedure. It is highly recommended to set up positive and negative controls within the same experiment (see Pre-Assay Preparation).
4. Post-treatment, remove the medium with the testing reagents and positive control and wash the cells twice with 1X Assay buffer.
5. Carefully remove the supernatant and dispense 100 µL of Microscopy Dual Detection Reagent solution to cover the cell pellet. Resuspend the pellet by gently pipetting up and down.
6. Protect samples from light and incubate for 30 minutes at 37°C.
7. Wash the cells with 1X Assay Buffer. Remove excess buffer and re-suspend cells in 100 µL 1X Assay Buffer.
8. Apply a drop of the cell suspension onto a glass microscope slide and overlay with a coverslip.
9. Analyze the stained cells by wide-field fluorescence or confocal microscopy (60X magnification recommended). Use a standard FITC filter set for imaging the autophagic signal. Image the nucleus using a DAPI filter set (optional). Image the nucleus using a DAPI filter set (optional).

C. Live Cell Analysis by Flow Cytometry

1. Cells should be maintained via standard tissue culture practice. Grow cells overnight to log phase in a humidified incubator at 37ºC, 5% CO_2.

   NOTE: Cells should be healthy and not overcrowded as results of the experiments will depend significantly on the cells' condition.

2. Treat cells with compound of interest according to experimental protocol. Prepare negative control cells using vehicle treatment. Prepare positive control cells (See Pre-Assay Preparation).
3. At the end of the treatment, trypsinize (adherent cells), or collect cells by centrifugation (suspension cells). Samples should contain 1 x 10^5 to 1 x 10^6 cells per mL
4. Centrifuge at 1000 rpm for 5 minutes to pellet the cells. Wash the cells by re-suspending the cell pellet in cell culture medium, 1X Assay Buffer, or other buffer of choice and collect the cells by centrifugation.
5. Resuspend each live cell sample in 250 µL of indicator free cell culture medium containing 5% FBS.
6. Add 250 µL of the diluted Green stain solution to each sample and mix well. Incubate for 30 minutes at room temperature or 37°C in the dark. It is important to achieve a mono-disperse cell suspension at this step by gently pipetting up and down repeatedly.

7. After treatment, collect the cells by centrifugation and wash with 1X Assay Buffer. Resuspend the cell pellets in 500 µL of fresh 1X Assay Buffer.
8. An optional fixation step may be included at this step. Incubate for 20 minutes with 4% formaldehyde (or 10% formalin). Wash 3 times with 1X Assay Buffer.
9. Analyze the samples in green (FL1) or orange (FL2) channel of a flow cytometer.

D. Live Cell Analysis by Fluorescence Microplate Reader

ab139484 has been shown to work for microplate readers. However, the conditions used for microscopy and flow cytometry may require additional optimization depending on cell line and end user applications.

For adherent cells

The procedure described below was developed using HepG2 and HeLa cells for which it is recommended that cells be seeded on plates at a density of 2.5x10^5 to 3.0x10^5 cells/mL, using 100 µL cells/well. Any cell number and plate coating requirements should be optimized for the chosen cell model.

1. Seed cells in 96-well microplates, using 100 µL cells/well, the day before the experiment, and allow cells to attach overnight under standard tissue culture practices. Cells should reach about 90% confluency to form a uniform

monolayer in the well at the end of the experiment. NOTE: Cells should be healthy and not overcrowded as results of the experiments will depend significantly on the cells' overall condition.

2. After overnight incubation, treat cells with compound of interest according to experimental protocol. Prepare negative control cells using vehicle treatment. It is highly recommended to set up positive and negative controls within the same experiment (see Section 5.1.1).
3. After the treatment, carefully remove the medium and dispense 100 µL of 1X Assay Buffer to each well. NOTE: Be careful during washing procedure since autophagic cells can be easily dislodged from the plate. To preserve the cells, 2% - 5% FBS also may be added to the assay buffer at this point.
4. Carefully remove all the buffer and dispense 100 µL of dual color detection solution (see Section 5.1.3) to each well.
5. Protect the sample from light and incubate at 37°C for 30 minutes.
6. Wash cells twice with 200 µL of 1X Assay Buffer (see Note above) to remove excess dye and then add 100 µL of 1X Assay Buffer to each well.
7. Analyze the plate with a fluorescence microplate reader. It is recommended to acquire data as soon after completing the assay as possible. The Green Detection Reagent can be read with a FITC filter (Excitation ~480 nm, Emission ~530),

and the Hoechst 33342 Nuclear Stain can be read with a DAPI filter set (Excitation ~340, Emission ~480). If the blue nuclear counterstain signal decreases by more than 30%, the compound is considered generally cytotoxic. Increases in the green autophagy signal after normalization with blue signal indicate the accumulation of the probe within the cells arising from an increase in autophagic vesicles.

For suspension cells

1. Culture the cells via standard tissue culture practice. Grow cells overnight to log phase in a humidified incubator at 37ºC, 5% CO2. NOTE: Cells should be healthy and not overcrowded since results of the experiments will depend significantly on the cells' overall condition. Cell density should not exceed 1x10^6 cell/mL.
2. Collect the cells by centrifugation (5 minutes, 1000 rpm at room temperature). Resuspend the cells to a density of 1x10^6 cells/mL.
3. Treat cells with compound of interest according to experimental protocol. Prepare negative control cells using vehicle treatment. It is highly recommended to set up positive and negative controls within the same experiment (see Section 5.1.1).
4. At the end of the treatment, collect cells by centrifugation. Samples should contain 1x10^5 to 1x10^6 cells/mL. Wash the cells by re-suspending the cell pellet in cell culture

medium, 1X Assay Buffer, or other buffer of choice and collect the cells by centrifugation. It is recommended that each suspension cell sample is tested in triplets. Following procedures are described accordingly.

5. Resuspend each cell sample in 400 µl of Green Detection Reagent (see Section 5.1.3). Incubate the cells for 30 minutes at 37°C in the dark. It is important to achieve a monodisperse cell suspension at this step by gently pipetting up and down repeatedly.
6. Wash the cells with 1X Assay Buffer. Remove excess buffer and re-suspend cells in 1X Assay Buffer. Count the cells and adjust the cells to a density of 5x10^5 cells/mL. If the number of the cells with testing reagent decreases by more than 30% compared to control, the compound is considered generally cytotoxic.
7. Add 100 µL/well of the above cell suspension (e.g., 5x10^4 cells/well) to a 96-well microplate in triplicate, and analyze the cells with a fluorescence microplate reader. It is recommended to acquire data as soon after completing the assay as possible. The stain can be read with a FITC filter (Excitation ~480 nm, Emission ~530nm). Nuclear counterstain with Hoechst 33342 is optional for suspension cells, as the cell number has been normalized before adding to each well. Increases in the green autophagy signal indicate the accumulation of the probe within the cells arising from an increase in autophagic vesicles.

7. Data Analysis

A. Fluorescence Channel Selection

The selection of optimal filter sets for a fluorescence microscopy application requires matching the optical filter specifications to the spectral characteristics of the dyes employed in the analysis (see Figure 1).

For flow cytometry, fluorescence channel FL1 (green) or FL2 (orange) is recommended for analysis of the Green Detection Reagent staining using a 488 nm laser source.

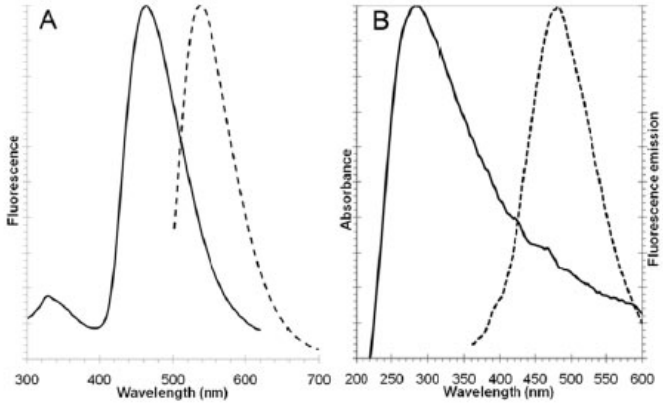

Figure 1. Excitation and fluorescence emission spectra (463/534 nm) for Green Detection Reagent (panel A). Spectra were determined in 10 mM sodium acetate buffer (pH 4) with 3 mg/ml BSA. Absorbance and fluorescence emission spectra (350/461 nm) for Nuclear Stain (panel B) were determined in 1X Assay Buffer.

B. Typical Outputs:

Fluorescence/Confocal Micoscropy

When the Green Detection Reagent is incorporated into cells, the accumulation of this fluorescent probe is typically observed in spherical vacuoles in the perinuclear region of the cell, in foci distributed throughout the cytoplasm, or in both locations, depending upon the cell type under investigation. A population of Green Detection Reagent-labeled vesicles co-localizes with LC3, a specific autophagosome marker (Figure 2). Transfected HeLa cells expressing RFP-LC3 were treated with either vehicle or 100 nM Rapamycin overnight. The cells were then stained with Green Detection Reagent. Rapamycin induces an increase in Green Detection Reagent fluorescence intensity in punctuate structures that co-localize with RFP-LC3.

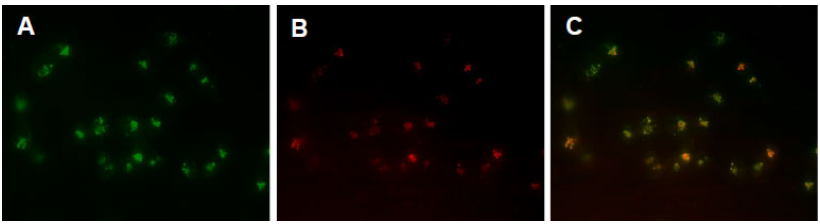

Figure 2: Green Detection Reagent mostly co-localizes with RFP-LC3 protein. Transfected HeLa cells expressing RFP-LC3 were treated with 0.1 μM Rapamycin (a typical autophagy inducer) overnight. Panel A: Green Detection Reagent; Panel B: RFP-LC3; Panel C: Composite images.

Besides Rapamycin treatment, there are several other approaches known to induce autophagy. One of the most potent known

physiological inducers of autophagy is starvation. Autophagy induction can be observed with the Green Detection Reagent within 1 hour of serum removal in both the HepG2 and HeLa cell lines. Another approach to activate autophagy is through the modulation of nutrient-sensing signal pathways. Several mTOR-independent autophagy activators have also been validated using ab139484 Autophagy Detection Kit (Table 1). Lithium induces autophagy through inhibition of inositol monophosphatase (an mTOR-independent pathway). Trehalose and small-molecule enhancers of rapamycin (SMERs) also induce autophagy by mechanisms that are not well understood. Two FDA-approved compounds that induce autophagy in an mTOR-independent manner, Loperamide hydrochloride and Clonidine, also substantially increase green fluorescent signal in the assay.

Typical results of autophagy detection using this Green Detection Reagent is presented in Figure 3 and 4.

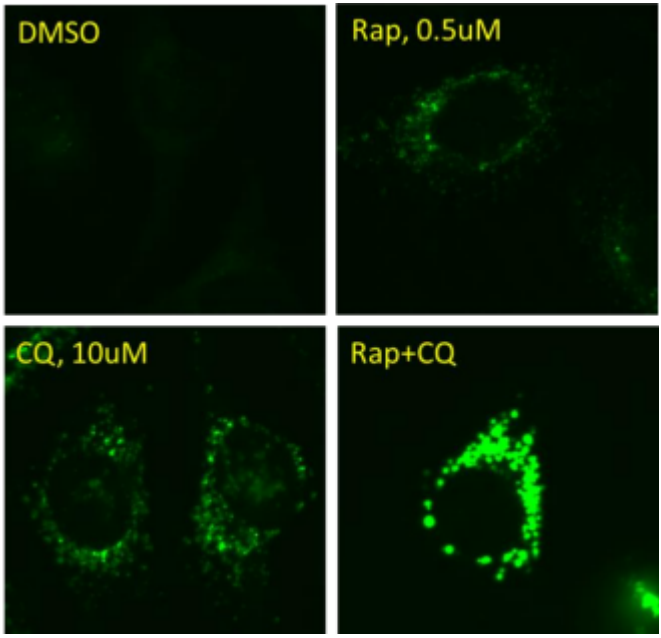

Figure 3. Green Detection Reagent typically accumulates in spherical vacuoles in the perinuclear region of the cells, in foci distributed throughout the cytoplasm, or in both locations, depending upon the cell type under investigation. HeLa cells were treated with 0.5 μM Rapamycin (a typical autophagy inducer) with or without 10 μM Chloroquine for 18h. Untreated cells do not display green staining while rapamycin-treated cells display intense punctuate structures. Cells treated with Chloroquine uniformly showed green fluorescent vesicles and the addition of both Rapamycin and Chloroquine substantially increased the green fluorescent signals.

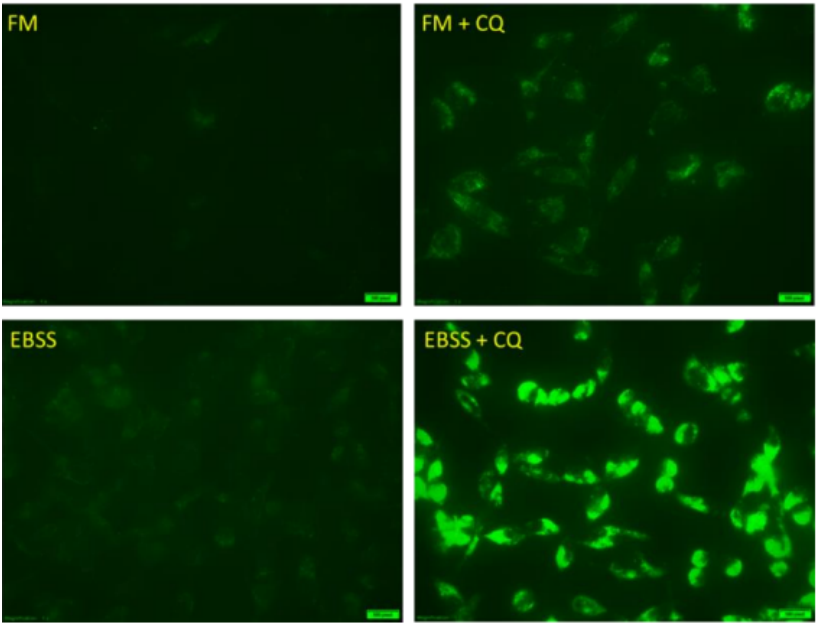

Figure 4: HeLa cells were cultured or starved in full media (FM) or starvation media EBSS with or without 40 μM Chloroquine (CQ) for 4h. Cells grown in FM do not display green staining while the addition of Chloroquine or culturing in EBSS displayed punctuate structures. Cells starved in EBSS in the presence of Chloroquine showed very bright green fluorescent signals.

Bafilomycin A1 is a selective inhibitor of vacuolar (V)-type ATPases, which results in elevated lysosomal pH. Chloroquine, verapamil, norclomipramine and hydroxychloroquine are small molecule modulators that passively diffuse into the lysosome and become trapped upon protonation. All these agents also cause an increase in lysosomal pH, which inhibits lysosome function and blocks fusion of

the autophagosome with the lysosome. The agents generate a positive signal in the Autophagy Detection Assay.

Furthermore, MG-132, a potent cell-permeable and selective proteasome inhibitor, has been shown to induce autophagy as demonstrated with the described assay. The ubiquitinproteasome system (UPS) and autophagy serve as two complementary, reciprocally regulated protein degradation systems. Blockade of UPS by MG-132 is well known to activate autophagy.

Flow Cytometry

Figure 5 below shows the typical results of flow cytometry-based analysis of cell populations ab139484 Autophagy Detection Kit.

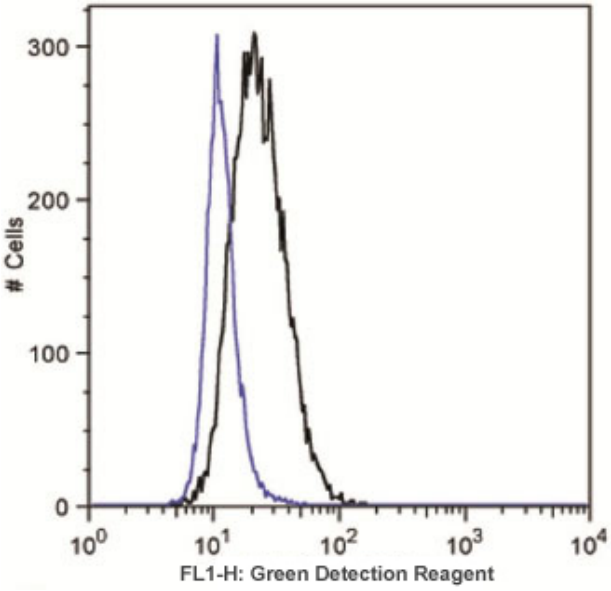

Figure 5: Flow cytometry-based profiling of ab139484: Jurkat cells (acute T-Cell leukemia), uninduced or treated overnight with 0.5 µM Rapamycin (a typical autophagy inducer) were loaded with Green Detection Reagent, then washed and analyzed by flow cytometry. Results are presented as histogram overlay. Control cells (blue solid line) were stained as well but mostly display low fluorescence. In the samples treated with 500 nM Rapamycin for 18 hours (black solid line), Green dye signal increases about 2-fold, indicating that Rapamycin induced autophagy in Jurkat cells.

Fluorescence Microplate Reader

Overnight incubation of HepG2 cells with Rapamycin, an inhibitor of mTOR kinase, results in an increase in Green Detection Reagent signal (Figure 6). Likewise ATP-competitive inhibitors of mTOR such as PP242 will also increase Green Detection Reagent signal (Table 1). Amino acid starvation for as little as 1 hour demonstrates an increase in Green Detection Reagent signal as compared to the untreated control. This effect is maximal by 2 hours, remaining constant for a total of 4 hours. Starvation beyond 4 hours resulted in significant loss of cells during wash steps. (Figure 7). Tamoxifen, which increases the intracellular level of ceramide and abolishes the inhibitory effect of PI3K, can increase Green Detection Reagent signal at concentrations above 1 µM with a 16 hour exposure (Figure 8). Verapamil is a small molecule that passively diffuses into the lysosome and becomes trapped upon protonation. Verapamil causes an increase in lysosomal pH, which inhibits lysosome function and blocks fusion with the autophagasome. Cellular exposure to concentrations of 10 µM or greater resulted in an increase in Green Detection Reagent signal (Figure 9).

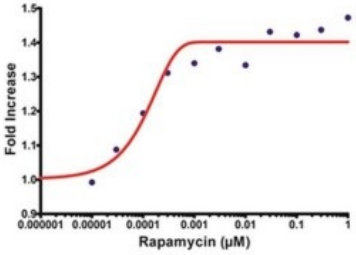

Figure 6. Effect of Rapamycin on Cyto-ID® dye signal.

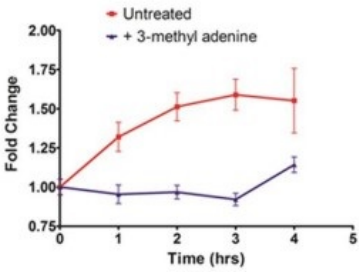

Figure 7. Effect of Starvation on Cyto-ID® dye signal.

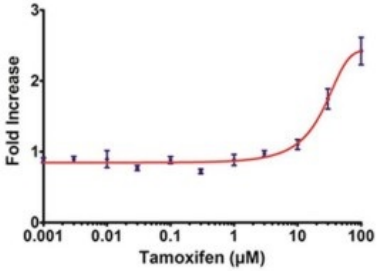

Figure 8. Effect of Tamoxifen on Cyto-ID® dye signal.

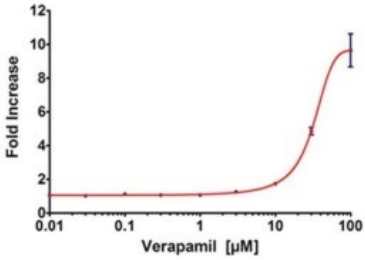

Figure 9. Effect of Verapamil on Cyto-ID® dye signal.

Treatment	Target	Effect	µM used	Induction Time (hrs)	Cell Line
Starvation	Inhibits mammalian target of rapamycin (mTOR)	Activates autophagy	N/A	1~4	HeLa, HepG2, Jurkat
Rapamycin	Inhibits mammalian target of rapamycin (mTOR)	Activates autophagy	0.2	6~18	HeLa, Jurkat
PP242	ATP-competitive inhibitor of mTOR	Activates autophagy	1	18	HeLa
Lithium	Inhibits IMPase and reduce inositol and IP_3 levels; mTOR-independent	Activates autophagy	10,000	18	HeLa, Jurkat
Trehalose	Unknown, mTOR-independent	Activates autophagy	50,000	6	HeLa, Jurkat
Bafilomycin A1	Inhibits Vacuolar-ATPase	Inhibits autophagy	$6~9*10^{-5}$	18	HeLa, Jurkat
Chloroquine	Alkalinizes Lysosomal pH	Inhibits autophagy	10~50	18	HeLa, Jurkat
Tamoxifen	Increases the intracellular level of ceramide and abolishes the inhibitory effect of PI3K	Activates autophagy	4~10	6~18	HeLa, HepG2, Jurkat
Verapamil	Ca^{2+} channel blocker; reduces intracytosolic Ca^{2+} levels; mTOR-independent	Activates autophagy	40~100	18	HeLa, Jurkat
Hydroxychloroquine	Alkalinizes Lysosomal pH	Inhibits autophagy	10	18	HeLa, Jurkat
Loperamide	Ca^{2+} channel blocker; reduces intra-cytosolic Ca^{2+} levels; mTOR-independent	Activates autophagy	5	18	HeLa
Clonidine	Imidazoline-1 receptor agonist; reduces cAMP levels; mTOR-independent	Activates autophagy	100	18	HeLa
MG-132	Selective proteasome inhibitor	Activates autophagy	2~5	18	HeLa, Jurkat
Norclomipramine	Alkalinizes Lysosomal pH	Inhibits autophagy	5-20	18	HeLa
Epoxomicin	Selective proteasome inhibitor	Induce aggresome	0.5	18	HeLa
Velcade®	Selective proteasome inhibitor	Induce aggresome	0.5	18	HeLa
Amyloid beta peptide 1-42	Induce oxidative stress	Induce aggresome	25	18	SK-N-SH

Table 1: Treatments that influence autophagy, validated using ab139484.

8. Troubleshooting

Problem	Potential cause	Suggestion
Low green dye staining in all treatments, including positive control.	Detection Reagent is photobleaching	Use mounting medium that prevents photo-bleaching. Optimize handling of the samples for fluorescence microscopy.
	A low concentration of the Detection Reagent was used	Increase the reagent concentration (500X dilution of the dye is recommended)
	The incubation time with the dye reagent was insufficient	Increase the incubation time

	Concentration and/or time of treatment with autophagy inducer(s) is not optimized	The optimal final concentration of autophagy inducers (including positive control Rapamycin) is cell-dependent and should be determined experimentally for each cell line being tested.
High green dye staining observed in negative control sample.	Cell cultures overgrown.	Suspension cells should not exceed a density 1x106 cells/mL and adherent cells should be approximately 50 - 70% confluent.
	Cell culture medium was depleted of nutrients.	Change media 4 ~ 8 hours before the experiment.

	Pathogen infection (Mycoplasma, etc.).	Obtain fresh cultures from reputable cell repository.
The number of green dye stained cells in the sample is too low to be readily quantified after assay.	Cell density/number was too low in the sample before the assay	Increase density/number of the cells in the sample
	Majority of the cells were lost during assay.	Autophagic cells may be loosely attached, so all staining and washing procedures should be performed gently. FBS or BSA (2-10%) may be added to the assay buffer.
Green dye staining fails to stain fixed and/or permeabilized cells.	The dye is only suitable for live-cell staining.	Use the dye for live-cell staining only. Cells can be fixed post staining as described.

Precipitate is observed in the 10X Assay Buffer	Precipitate forms at low temperatures.	Allow solution to warm to room temperature or 37°C, then vortex to dissolve all precipitate.
Cells do not appear healthy by microscopic examination post-assay.	Some cells require serum to remain healthy.	Add serum of BSA (2-10%) to the detection reagent and wash solutions.
Positive control (Rapamycin-treated) cells appear to be dead or are no longer attached to the plate surface.	The concentration and/or time of treatment are not optimized	The optimal final concentration of positive control (Rapamycin) is cell-dependent and should be determined experimentally for each cell line being tested.

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)