ab139484
Autophagy Detection Kit

Instructions for Use

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

This product is for research use only and is not intended for diagnostic use.
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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, a known inducer of autophagy, is included as a positive control 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

\[ \downarrow \]

Quantify Using Flow Cytometry

OR

Detect Using Fluorescence Microscopy

OR

Detect Using a Fluorescence Microplate Reader

4. Components and Storage

A. Kit Contents

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green Detection Reagent</td>
<td>50 µl</td>
<td>≤ -20°C</td>
</tr>
<tr>
<td>Nuclear Stain</td>
<td>50 µl</td>
<td>≤ -20°C</td>
</tr>
<tr>
<td>Autophagy Inducer (Rapamycin)</td>
<td>25 nmol</td>
<td>≤ -20°C</td>
</tr>
<tr>
<td>10X Assay Buffer</td>
<td>30 ml</td>
<td>≤ -20°C</td>
</tr>
</tbody>
</table>
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 ≤-20°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.

A. Reagent Preparation

1. Positive Control
   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.

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.
3. **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 \( \mu L \) Green Detection Reagent to 1 mL cell culture medium without Phenol Red Indicator, supplemented with 5% FBS. For each sample to be stained, 250 \( \mu L \) 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 \( \mu L \) of Green Detection Reagent and 1 \( \mu L \) Nuclear Stain into 1 mL cell culture medium without Phenol Red Indicator, supplemented with 5% FBS. For each sample to be stained, 100 \( \mu L \) of Microplate Dual Detection Reagent will be used.

B. **Cell Preparations**
Positive control cells should be pretreated with the Autophagy Inducer (Rapamycin) for 6-18 hours. Response to Rapamycin 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.
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 (Rapamycin and mock-treated cells accordingly).

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 15-30 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. 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.
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. Fixed Cell Analysis By Fluorescence/Confocal Microscopy

1. Grow cells overnight on microscopy slides or in suspension to approximately 70% confluency for adherent cells or a density of $1 \times 10^6$/mL for suspension cells.

2. Induce autophagy in the test sample using supplied Autophagy Inducer Rapamycin (positive control) or your test reagents. Make sure you set a negative control of cells where autophagy was not induced or mock-induced with the solvent.


4. Carefully wash cells with 1X Assay Buffer (autophagic cells tend to get loose and can be easily washed away if precautions not taken). Aspirate the rest of buffer or remove buffer by gentle tapping of slides. *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.*

5. Apply the 100 $\mu$L of Microscopy Dual Detection Reagent to both treated and untreated cells. Stain 15-30 minutes at 37°C.

6. Carefully wash cells 2 times for 1 minute each using 1X Assay Buffer.
7. Fix cells by adding freshly made 4% formaldehyde for 20 minutes at room temperature.
8. Wash with 1X Assay Buffer 3 times for 5 minutes each and coverslip the slide.
9. 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.
10. Optionally, Nuclear Stain can be added to the Detection Reagent. The nuclear signal can be imaged using a DAPI filter set.

D. 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 37°C, 5% CO₂.

   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.

3. Prepare positive control cells by incubating with the diluted Autophagy Inducer (Rapamycin) (recommended starting concentration is 500 nM), for 18 hours under normal tissue culture conditions.
4. At the end of the treatment, trypsinize (adherent cells), or collect cells by centrifugation (suspension cells). Samples should contain $1 \times 10^5$ to $1 \times 10^6$ cells per mL.

5. 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.

6. Resuspend each live cell sample in 250 $\mu$L of indicator free cell culture medium containing 5% FBS.

7. Add 250 $\mu$L 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.

8. After treatment, collect the cells by centrifugation and wash with 1X Assay Buffer. Resuspend the cell pellets in 500 $\mu$L of fresh 1X Assay Buffer.

9. Analyze the samples in green (FL1) or orange (FL2) channel of a flow cytometer.
E. Fixed Cell Analysis by Flow Cytometry

1. Grow the cells according to a standard tissue culture practice.

   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.

3. Prepare positive control cells by incubating them with the diluted Autophagy Inducer, Rapamycin with a recommended starting concentration is 500 nM, for 18 hours under normal tissue culture conditions.

4. At the end of the treatment, trypsinize (adherent cells), or collect cells by centrifugation (suspension cells). Samples should contain 1x10^5 to 1x10^6 cells per mL.

5. 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.

6. Resuspend each live cell sample in 250 μL of indicator free cell culture medium containing 5% FBS.

7. Add 250 μL of the diluted Green dye staining 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.

8. After treatment, collect the cells by centrifugation and wash with 1X Assay Buffer.

9. Fix cells with freshly made 4% formaldehyde for 20 minutes at room temperature.

10. Wash with 1X Assay buffer 3 times.

11. Resuspend the cell pellets in 500 μL of fresh 1X Assay Buffer.

12. Analyze the samples in the green (FL1) or orange (FL2) channel of a flow cytometer.

F. Live Cell Analysis by Fluorescence Microplate Reader

The procedure described below was developed using the HepG2 cell line. HepG2 cells were cultured in DMEM supplemented with 10% FBS, seeded into 96-well plates at 20,000 cells per well (100 μL) and incubated overnight.

Positive control cells should be pretreated with the Autophagy Inducer, Rapamycin, for 6~18 hours. Recommended starting concentration of Rapamycin is 500 nM. Response to Rapamycin 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 Flow Cytometry other solvent used to reconstitute or dilute an inducer or inhibitor) for an equal length of time under similar conditions.
1. Seed cells in 96-well microplates, using 100 μL cells/well, the day before the experiment, and allow cells to attach overnight. Cells should reach 70 – 80% confluency the next day.

2. After overnight incubation, treat cells by adding 100 μL/well of 500 nM Rapamycin for 16 - 18 hours with increasing doses of test compounds of interest for up to 18 hours.

3. After treatment, carefully wash cells 2 times using 1X Assay Buffer.

   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. Add 150 μL of Microplate Dual Detection reagent to each well for compound treated cells and incubate for 30 minutes.

5. Wash cells 2 times with 1X Assay Buffer (see Note above) to remove excess dye and then add 80 μL of 1X Assay Buffer to each well.

6. Analyze the plate with a fluorescence microplate reader. It is recommended to acquire data as soon after completing the assay as feasible. The Green detection reagent can be read with a FITC filter (Excitation 480 nm, Emission 530), and the 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, without significant loss of blue signal, indicates 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.](image-url)
B. Typical Outputs:

**Fluorescence/Confocal Microscopy**

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.](image)
Typical results of autophagy detection using this Green Detection Reagent is presented in Figure 3. Nuclear Stain is used to localize cellular nuclei.

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) overnight. Untreated cells do not display green staining while rapamycin-treated cells display intense punctuate structures.

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.

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 4 below shows the typical results of flow cytometry-based analysis of cell populations ab139484 Autophagy Detection Kit. Control uninduced and 0.5 μM Rapamycin-treated Jurkat (acute T-Cell leukemia) cells were used. After 18 hours treatment, cells were
loaded with Green Detection Reagent, then washed and analyzed by flow cytometry. Results are presented by histogram overlays. Control cells were stained only faintly, displaying low fluorescence signal intensity. In the samples treated with 0.5 μM Rapamycin for 18 hours, the Green Detection Reagent signal increases about 2-fold, indicating that Rapamycin causes an increase in autophagic vesicles in Jurkat cells.

Figure 4: 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.
Overnight incubation of HepG2 cells with Rapamycin, an inhibitor of mTOR kinase, results in an increase in Green Detection Reagent signal (Figure 5). 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 6). 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 7). 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 autophagosome. Cellular exposure to concentrations of 10 μM or greater resulted in an increase in Green Detection Reagent signal (Figure 8).
Figure 5: Effect of Rapamycin on Green Detection Reagent signal.

Figure 6: Effect of Starvation on Green Detection Reagent signal.

Figure 7: Effect of Tamoxifen on Green Detection Reagent signal.

Figure 8: Effect of Verapamil on Green Detection Reagent signal.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Target</th>
<th>Effect</th>
<th>μM used</th>
<th>Induction Time (hrs)</th>
<th>Cell Line</th>
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<tbody>
<tr>
<td>Starvation</td>
<td>Inhibits mammalian target of rapamycin (mTOR)</td>
<td>Activates autophagy</td>
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<td>1~4</td>
<td>HeLa, HepG2, Jurkat</td>
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<td>Rapamycin</td>
<td>Inhibits mammalian target of rapamycin (mTOR)</td>
<td>Activates autophagy</td>
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<td>PP242</td>
<td>ATP-competitive inhibitor of mTOR</td>
<td>Activates autophagy</td>
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<td>18</td>
<td>HeLa</td>
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<tr>
<td>Lithium</td>
<td>Inhibits IMPase and reduce inositol and IP3 levels; mTOR-independent</td>
<td>Activates autophagy</td>
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<td>HeLa, Jurkat</td>
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<td>Trehalose</td>
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<td>Activates autophagy</td>
<td>50,000</td>
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<td>HeLa, Jurkat</td>
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<td>Bafilomycin A1</td>
<td>Inhibits Vacuolar-ATPase</td>
<td>Inhibits autophagy</td>
<td>6~9*10^-2</td>
<td>18</td>
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<td>Chloroquine</td>
<td>Alkalizes Lysosomal pH</td>
<td>Activates autophagy</td>
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<td>HeLa, Jurkat</td>
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<td>Tamoxifen</td>
<td>Increases the intracellular level of ceramide and abolishes the inhibitory effect of PI3K</td>
<td>Activates autophagy</td>
<td>4~10</td>
<td>6~18</td>
<td>HeLa, HepG2, Jurkat</td>
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<tr>
<td>Verapamil</td>
<td>Ca^{2+} channel blocker; reduces intracytosolic Ca^{2+} levels; mTOR-independent</td>
<td>Activates autophagy</td>
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<td>Hydroxychloroquine</td>
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<td>Loperamide</td>
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<td>HeLa</td>
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<td>Clonidine</td>
<td>Imidazoline-1 receptor agonist; reduces cAMP levels; mTOR-independent</td>
<td>Activates autophagy</td>
<td>100</td>
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<td>HeLa</td>
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<td>MG-132</td>
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<td>Activates autophagy</td>
<td>2~5</td>
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<td>Induce aggresome</td>
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<td>SK-N-SH</td>
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Table 1: Treatments that influence autophagy, validated using ab139484.
## 8. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay not working</td>
<td>Assay buffer at wrong temperature</td>
<td>Assay buffer must not be chilled – needs to be at RT</td>
</tr>
<tr>
<td>Protocol step missed</td>
<td>Protocol step missed</td>
<td>Re-read and follow the protocol exactly</td>
</tr>
<tr>
<td>Plate read at incorrect wavelength</td>
<td>Plate read at incorrect wavelength</td>
<td>Ensure you are using appropriate reader and filter settings</td>
</tr>
<tr>
<td>Unsuitable microtiter plate for assay</td>
<td>Unsuitable microtiter plate for assay</td>
<td>Fluorescence: Black plates (clear bottoms); Luminescence: White plates; Colorimetry: Clear plates. If critical, protocol will indicate whether to use flat- or U-shaped wells</td>
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<tr>
<td>Unexpected results</td>
<td>Measured at wrong wavelength</td>
<td>Ensure you are using appropriate reader and filter settings</td>
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<tr>
<td>------------------------------------</td>
<td>------------------------------------------------------</td>
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<tr>
<td>Unsuitable sample type</td>
<td></td>
<td>Refer to datasheet for details about incompatible samples</td>
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<tr>
<td>Sample readings are outside linear range</td>
<td></td>
<td>Concentrate/ dilute samples to be in linear range</td>
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</table>

<table>
<thead>
<tr>
<th>Samples with inconsistent readings</th>
<th>Unsuitable sample type</th>
<th>Refer to datasheet for details about incompatible samples</th>
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<td>Too many freeze/thaw cycles</td>
<td>Aliquot samples to reduce the number of freeze/thaw cycles</td>
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<tr>
<td></td>
<td>Samples are too old or incorrectly stored</td>
<td>Use fresh made samples and store at recommended temperature until use</td>
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<tr>
<td>Lower/higher readings in samples and standards</td>
<td>Not fully thawed kit components</td>
<td>Wait for components to thaw completely and gently mix prior use</td>
</tr>
<tr>
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<tr>
<td></td>
<td>Reagents sitting for extended periods on ice</td>
<td>Try to prepare a fresh reaction mix prior to each use</td>
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<tr>
<td></td>
<td>Incorrect amounts used</td>
<td>Check pipette is calibrated correctly (always use smallest volume pipette that can pipette entire volume)</td>
</tr>
<tr>
<td>Standard curve is not linear</td>
<td>Not fully thawed kit components</td>
<td>Wait for components to thaw completely and gently mix prior use</td>
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<tr>
<td></td>
<td>Pipetting errors when setting up the standard curve</td>
<td>Try not to pipette too small volumes</td>
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<tr>
<td>Incorrect pipetting when setting up the reaction mix</td>
<td>Always prepare a master mix</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>Air bubbles in wells</td>
<td>Air bubbles will interfere with readings; try to avoid producing air bubbles and always remove bubbles prior to reading plates</td>
<td></td>
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</tbody>
</table>