Product Manual

CYTO-ID® Autophagy Detection Kit

Catalog No. ENZ-51031

ENZ-51031-K200

200 flow cytometry assays

250 fluorescence microscopy assays

3 x 96-well microplate assays

ENZ-51031-0050

50 flow cytometry assays

60 fluorescence microscopy assays

1 x 96-well microplate assays

NOTE: This version contains a change to shipping condition of product.

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Product Manual

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The Cyto-ID® Autophagy Detection Kit is a member of the CELLestial® product line, reagents and assay kits comprising fluorescent molecular probes that have been extensively benchmarked for live cell analysis applications. CELLestial® reagents and kits are optimal for use in demanding cell analysis applications involving confocal microscopy, flow cytometry, microplate readers and HCS/HTS, where consistency and reproducibility are required.

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Product Manual

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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.

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. Enzo Life Sciences' CYTO-ID® Autophagy Detection Kit 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 488nm-excitable green fluorescent detection reagent supplied in the CYTO-ID® 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.

The conventional way of monitoring autophagic activity is to measure the increased numbers of autophagosomes in cells responding to induction. However, the autophagosome formation is an intermediate stage in the whole dynamic autophagy process. The accumulation of autophagosomes can represent either an increased generation of autophagosomes or the blocked conversion to autolysosomes. To distinguish these two sources of autophagosomes, monitoring autophagic flux provides a meaningful way, in which the balance of the autophagosome generation and clearance will be measured [1-4].

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 autophagic activity at the cellular level.

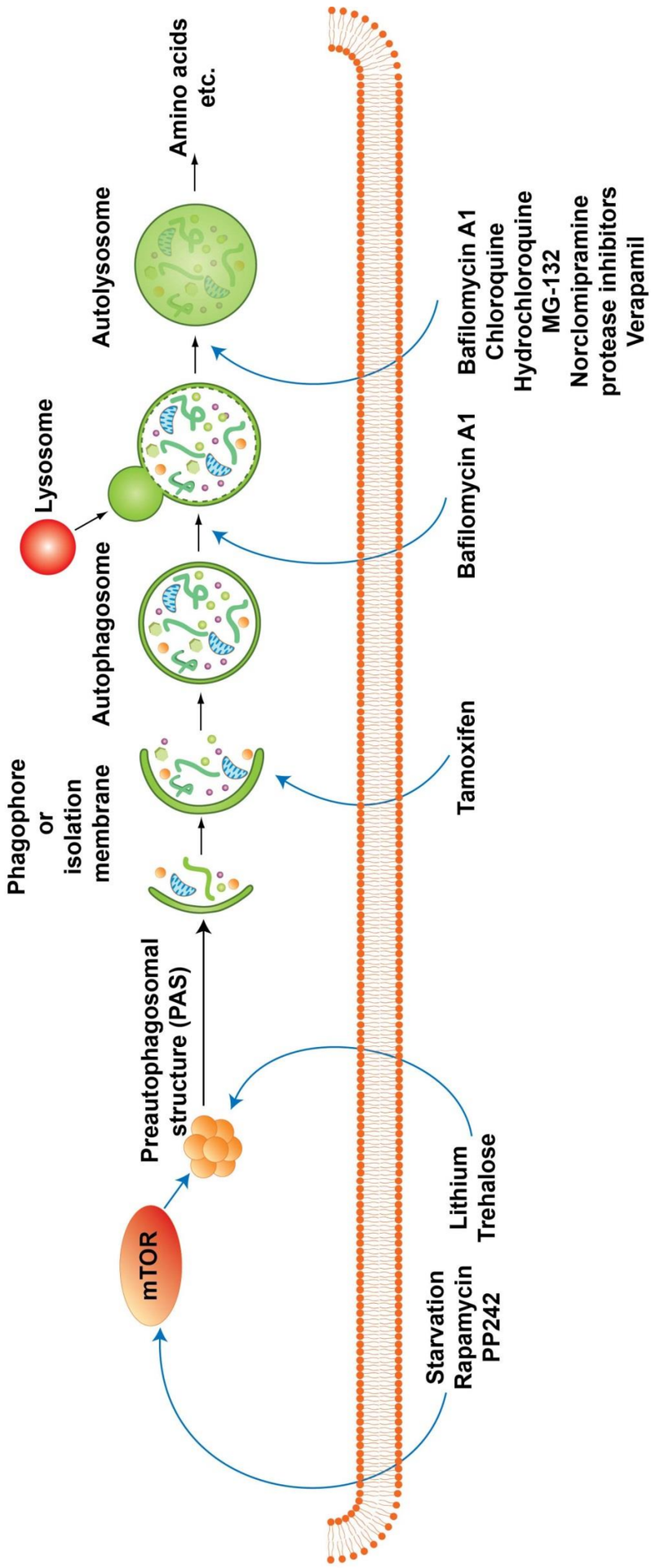

Figure 1. Schematic depiction of autophagy.[4] Cytosolic material is sequestered by an expanding membrane sac, the phagophore, resulting in the formation of a double-membrane vesicle, an autophagosome. The outer membrane of the autophagosome subsequently fuses with the lysosome, and the internal material is degraded in the autolysosome. Various regulators of autophagy are also depicted in the diagram.

SAFETY WARNINGS AND PRECAUTIONS

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Handle with care

- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.


Protect from light

- To avoid photobleaching, perform all manipulations in low light environments or protected from light by other means.
- The CYTO-ID® Green Detection Reagent and the Autophagy Inducer (Rapamycin) contain DMSO which is readily absorbed through the skin. DMSO is harmful if ingested or absorbed through the skin and may cause irritation to the eyes. Observe appropriate precautions when handling these reagents.

REAGENTS PROVIDED AND STORAGE


Avoid freeze / thaw cycles

All reagents are shipped on blue ice (-20°C). Upon receipt, the kit should be stored at -20°C, protected from light. When stored properly, these reagents are stable for at least twelve months. **Avoid repeated freezing and thawing.**

Reagents provided in the **ENZ-51031-0050** kit are sufficient for approximately 50 flow cytometry, 60 fluorescence microscopy or 1 x 96-well microplate assays.

Reagents provided in the **ENZ-51031-K200** kit are sufficient for approximately 200 flow cytometry, 250 fluorescence microscopy or 3 x 96-well microplate assays.

Reagent	ENZ-51031-0050	ENZ-51031-K200
CYTO-ID® Green Detection Reagent	13 µL	50 µL
Hoechst 33342 Nuclear Stain	13 µL	50 µL
Rapamycin (Autophagy Inducer)	25 nmol	25 nmol
10X Assay Buffer	8 mL	30 mL
Chloroquine Control	7.5 µmol	7.5 µmol

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 (e.g. Sigma Prod. No. D5921)
- EBSS (Sigma Prod. No. E2888)
- FBS (Fetal Bovine Serum)
- Glass microscope slides
- Glass cover slips of appropriate size
- 96-well tissue culture microplate with black wall and clear bottom

METHODS AND PROCEDURES

The procedures described in this manual assume that the user is familiar with the basic principles and practices of flow cytometry and is able to run samples according to the operator's manual pertaining to the instrument being used

Gently hand mix or vortex

NOTE: *Allow all reagents to thaw at room temperature before starting with the procedures. Once thawed, 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 Controls

a) Rapamycin

Rapamycin, a lipophilic macrolide antibiotic, is a widely used inhibitor of the mammalian target of Rapamycin (mTOR) and a well-established inducer of autophagy in a diverse range of cell lines from yeast to mammalian cells including neuron-like cells. Rapamycin-induced autophagy is characterized by the accumulation of autophagic vacuoles and the stimulation of autophagic flux.

Rapamycin included in the kit is supplied lyophilized (25 nmol). Before using, 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. Unused stock Rapamycin may be stored in small aliquots at -20°C for one month.

b) Chloroquine

Chloroquine is an anti-inflammatory drug used in the treatment or prevention of malaria. It suppresses inflammation by increasing lysosomal pH, thereby inhibiting lysosomal activity.

Chloroquine provided in the kit may be used in combination with Rapamycin or starvation in monitoring autophagic flux. It is supplied lyophilized (7.5 µmol) and should be centrifuged briefly to gather the material at the bottom of the tube. Reconstitute the lyophilized material in 125 µL 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 may be stored in small aliquots at -20°C for one month.

c) Rapamycin and Chloroquine as positive control

Some positive control cells (e.g., Jurkat cells) should be treated with Rapamycin and Chloroquine for 16-18 hours.

Recommended starting concentration of Rapamycin is 500 nM, Chloroquine is 10 µM. 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, deionized water, media or other solvent used to reconstitute or dilute an inducer or inhibitor) for an equal length of time under similar conditions.

d) Starvation and Chloroquine as positive control

Some positive control cells (e.g., HeLa cells) should be starved in EBSS media in the presence of Chloroquine for 3-5 hours. Recommended starting concentration of Chloroquine is 10 µM. 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, deionized water, media or other solvent used to reconstitute or dilute Chloroquine) for an equal length of time under similar conditions.

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. CYTO-ID® Green Detection Reagent

For optimal staining, the concentration of the CYTO-ID® Green dye 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.

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 CYTO-ID® Green Detection Reagent and 1 µL of Hoechst 33342 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.*

Important Information

- *The Hoechst 33342 Nuclear Stain can be diluted further if its staining intensity is much stronger than that of the CYTO-ID® Green Detection Reagent. When staining BFP- or CFP-expressing cells, the Hoechst 33342 Nuclear Stain should be omitted due to its spectral overlap with these fluorescent proteins.*
- *CYTO-ID® 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 CYTO-ID® Green Detection Reagent to 1 mL 1X Assay Buffer or cell culture medium without Phenol Red Indicator, supplemented with 5% FBS. For each sample to be stained, 250 µL of diluted CYTO-ID® 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 analyzed as follows: Add 1 µL of CYTO-ID® Green Detection Reagent and 1 µL Hoechst 33342 Nuclear Stain into 1 mL 1X Assay Buffer or 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.

B. 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.

Important Information

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 Section A, Step 1*).
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 (*see Section A, Step 3*) to cover each sample of monolayer cells.


Protect from light

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 (*see Section A, Step 2*). 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.


Microscopy

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.

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

1. Cells should be cultured to a density not to exceed $1x10^6$ cells/mL. Ensure that cells are in the log phase of growth before starting an experiment.


Important Information

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

2. Collect the cells by centrifugation (5 minutes, 1000 rpm at room temperature). Resuspend the cells to a density of $1x10^6$/mL.


Centrifuge

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 Section A, Step 1*).
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 (*see Section A, Step 3*) 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. An optional fixation step may be included at this step. Incubate for 20 minutes with 4% formaldehyde. Wash 3 times with 1X Assay Buffer.
9. Apply a drop of the cell suspension onto a glass microscope slide and overlay with a coverslip.
10. 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).


Microscopy

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 37ºC, 5% CO_2.


Important Information

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/mL.*

2. 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 A, Step 1*)
3. 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/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 1X Assay Buffer or indicator free cell culture medium containing 5% FBS.
6. Add 250 µL of the diluted CYTO-ID® Green stain solution (*see Section A, Step 3*) 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.

E. Live Cell Analysis by Fluorescence Microplate Reader

The CYTO-ID® Autophagy Detection Kit 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 A, Step 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 A, Step 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 CYTO-ID® 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% CO_2.

   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 A, Step 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 CYTO-ID® Green stain solution (*see Section A, Step 3*). Incubate the cells for 30 minutes at 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.
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.

APPENDICES

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 2**). Consult the microscope or filter set manufacturer for assistance in selecting optimal filter sets for your microscope.

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

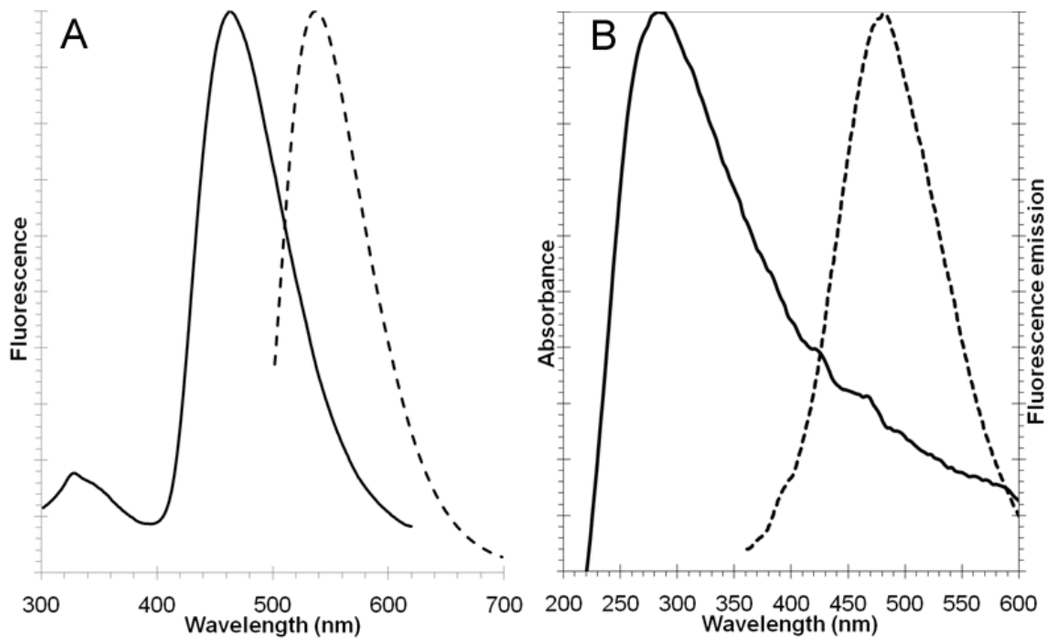

Figure 2. Excitation and fluorescence emission spectra (463/534 nm) for CYTO-ID® Green dye (panel A). Spectra were determined in 10 mM sodium acetate buffer, pH 4 with 3 mg/mL BSA. Absorbance and Fluorescence emission spectra (350/461 nm) for Hoechst 33342 (panel B) were determined in 1X Assay Buffer.

Typical Results

A number of methods have been devised to investigate the autophagy pathway and the steps involved in the maturation of autophagosomes to autolysosomes, acid hydrolase-rich organelles in which the sequestered cytoplasmic material is ultimately degraded.[4-6] For example, monodansylcadaverine (MDC) has been determined to be a useful probe for the analysis of the autophagic process by fluorescence microscopy.[5] However, this probe requires 365nm UV illumination and thus, is not compatible with 488nm excitation sources commonly implemented in flow cytometry. The CYTO-ID® Autophagy Detection Kit employs a 488nm-excitable green-emitting fluorescent probe to highlight the various vacuolar components of the autophagy pathway.

It should be noted that unlike the lysomotrophic dyes, MDC, LysoTracker® Red and Acridine Orange, which primarily detect lysosomes, the CYTO-ID® Green autophagy dye only weakly stains lysosomes, while serving both as a selective marker of autolysosomes and earlier autophagic compartments. This staining pattern differs markedly from that achieved with Lyso-ID® Red dye as well, which detects autolysosomes generated by Chloroquine and bafilomycin A treatment, but not vacuoles associated with other stimuli, such as serum starvation.(7)

Fluorescence Microscopy

Under physiological conditions, autophagy is a constitutive self-degradative process involved both in basal turnover of cellular components and as an induced response to nutrient starvation in eukaryotes. During autophagy, portions of the cytoplasm are sequestered by elongation of double-membrane structures called phagophores, which form vesicles called autophagosomes. These vesicles then fuse with lysosomes to form autolysosomes, where their contents are degraded by acidic lysosomal hydrolases for subsequent recycling (**Figure 1**). A prominent mammalian protein known to specifically associates with the autophagosome membrane is LC3-II.

When CYTO-ID® Green autophagy detection dye 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 the CYTO-ID® Green autophagy dye-labeled vesicles co-localizes with LC3, a specific autophagosome marker (**Figure 3**). Transfected HeLa cells expressing RFP-LC3 were treated with either vehicle or 100 nm Rapamycin overnight. The cells were then stained with CYTO-ID® Green dye. Rapamycin induces an increase in CYTO-ID® Green dye fluorescence intensity in punctuate structures that co-localize with RFP-LC3.

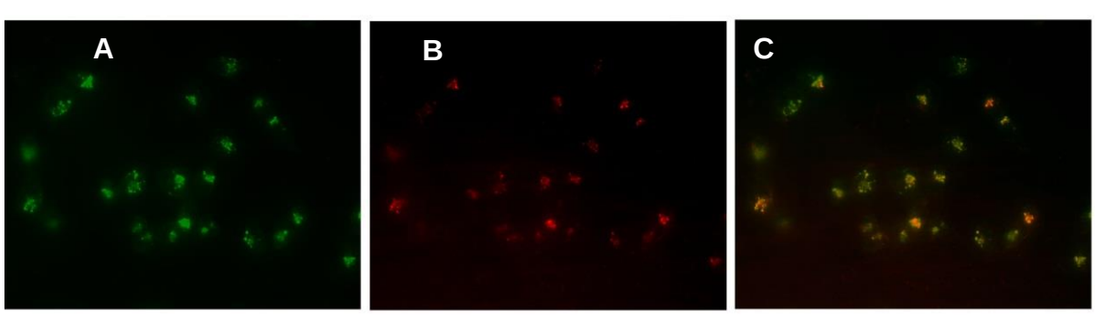

Figure 3. CYTO-ID® Green dye mostly co-localizes with RFP-LC3 protein. Transfected HeLa cells expressing RFP-LC3 were treated with 0.1 µM Rapamycin (a typical autophagy inducer) overnight. Panel A: CYTO-ID® Green staining; Panel B: RFP-LC3; Panel C: Composite images.

In addition, an enhancement in the fluorescence emission intensity of the CYTO-ID® dye occurs upon compartmentalization with the lamellar membrane structures associated with autophagic vesicles. Oeste, *et al.*, have reported the dye's selectivity for autophagic vesicles with BAECs transduced with RFP-LC3.

Typical results of autophagy detection using this CYTO-ID® Green Autophagy Detection Reagent are presented in **Figure 4** and **Figure 5**.

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 CYTO-ID® Green dye 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 the CYTO-ID® Autophagy Detection Kit (**Table 1**). Lithium induces autophagy through inhibition of inositol mono-phosphatase (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.

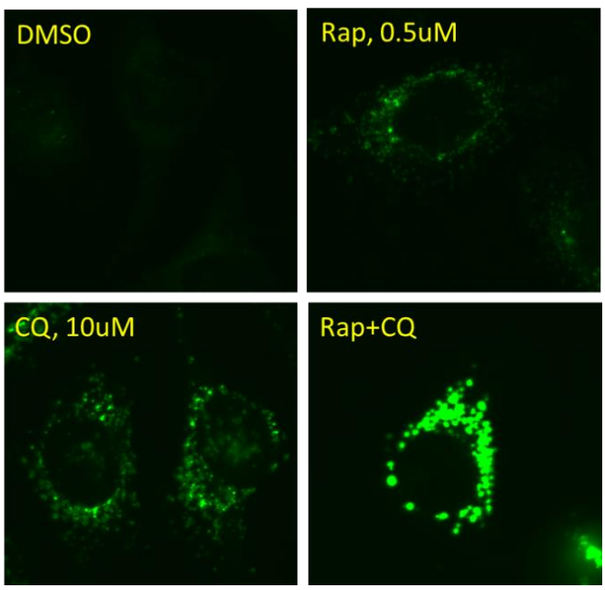

Figure 4. CYTO-ID® Green dye typically accumulates in spherical vacuoles in the perinuclear region of the cells, in foci distributed throughout the cytoplasm, or in both locations, depending upon the cell type under investigation. HeLa cells were treated with 0.5 µM Rapamycin (a typical autophagy inducer) with or without 10 µM Chloroquine for 18h. Untreated cells do not display green staining while some of Rapamycin-treated cells display 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

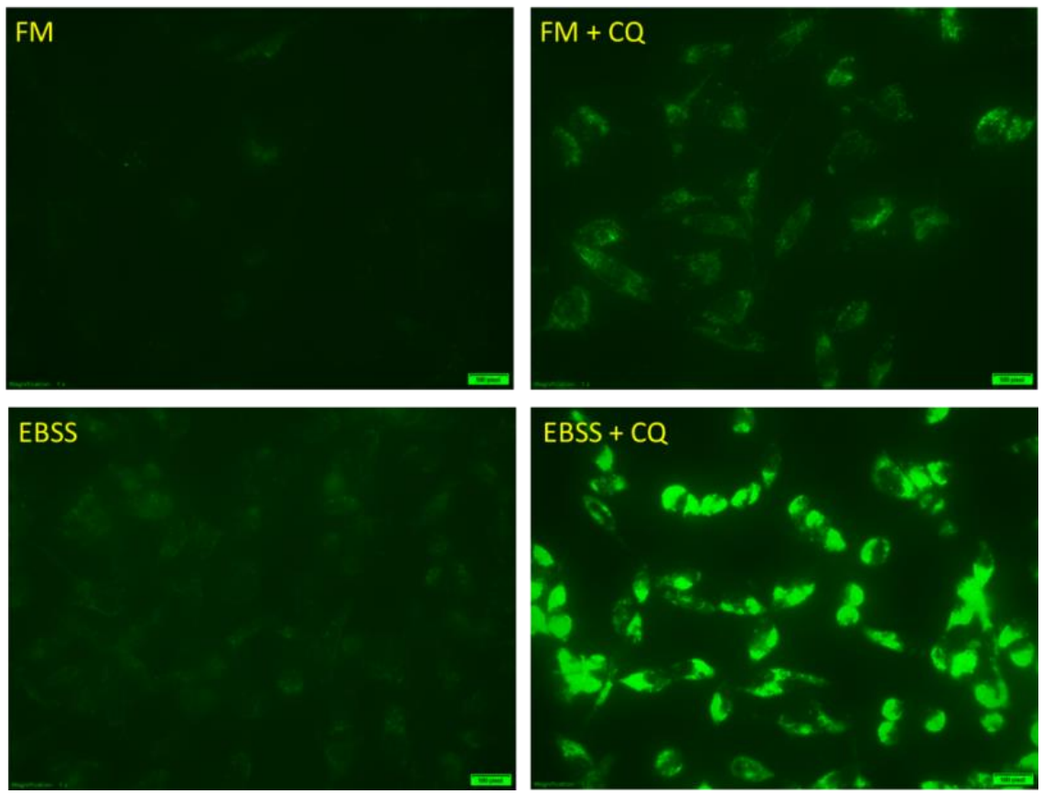

Figure 5. HeLa cells were cultured or starved in full media (FM) or starvation media EBSS with or without 40 µM Chloroquine (CQ) for 4h. Cells grown in FM do not display green staining while the addition of Chloroquine or culturing in EBSS displayed punctuate structures. Cells starved in EBSS in the 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 CYTO-ID® 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 ubiquitin-proteasome 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.[9]

Flow Cytometry

Figure 6 show the typical results of flow cytometry-based analysis of cell populations using the CYTO-ID® Autophagy Detection kit.

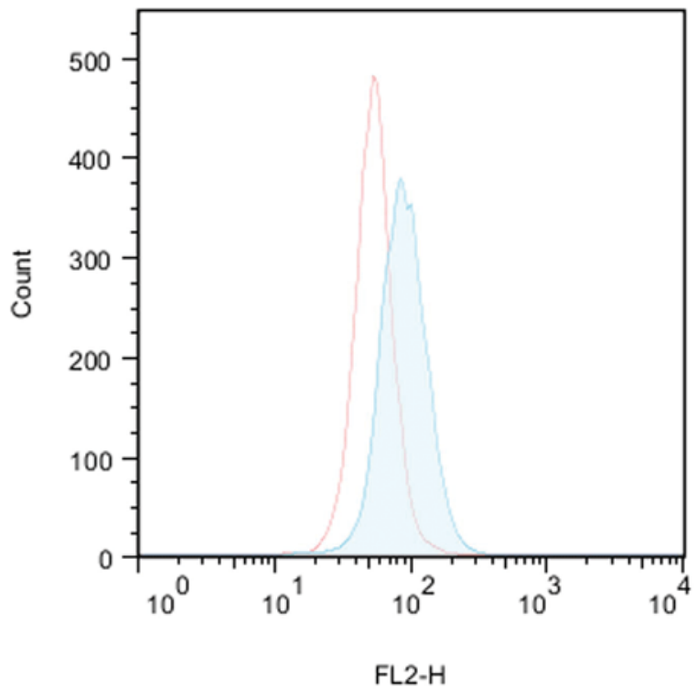

Figure 6. Flow cytometry-based profiling of autophagy with CYTO-ID® Autophagy Detection Kit: Control (red-lined peak) uninduced and 10 µM Tamoxifen (ALX-550-095) treated (blue-filled peak) Jurkat cells (T-cell leukemia) were used. After 18 hours treatment, cells were loaded with CYTO-ID® Green Detection Reagent, then analyzed without washing by flow cytometry. Results are presented by histogram overlays. Control cells were stained as well but mostly display low fluorescence. In the samples treated with 10 µM Tamoxifen for 18 hours, CYTO-ID® Green dye signal increases about 2-fold, indicating that Tamoxifen causes an increase in autophagy in Jurkat cells.

Fluorescence Microplate

Overnight incubation of HepG2 cells with Rapamycin, an inhibitor of mTOR kinase, results in an increase in CYTO-ID® dye signal (**Figure 7**). Likewise ATP-competitive inhibitors of mTOR such as PP242 will also increase CYTO-ID® dye signal (**Table 1**). Amino acid starvation for as little as 1 hour demonstrates an increase in CYTO-ID® dye 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 8**). Tamoxifen, which increases the intracellular level of ceramide and abolishes the inhibitory effect of PI3K, can increase CYTO-ID® dye signal at concentrations above 1 µM with a 16 hour exposure (**Figure 9**). 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 CYTO-ID® dye signal (**Figure 10**)[10].

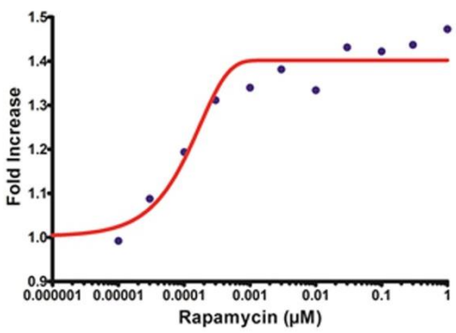

Figure 7. Effect of Rapamycin on Cyto-ID® dye signal.

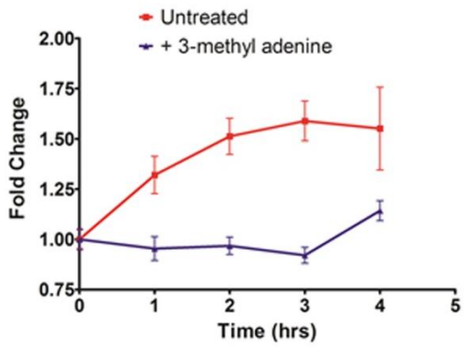

Figure 8. Effect of Starvation on Cyto-ID® dye signal.

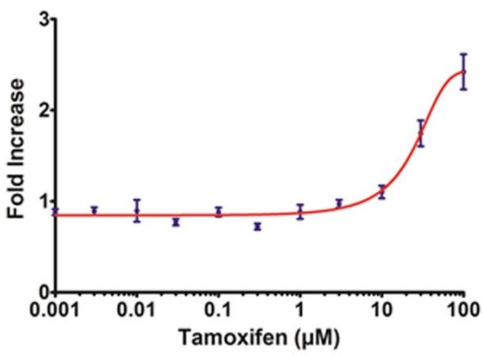

Figure 9. Effect of Tamoxifen on Cyto-ID® dye signal.

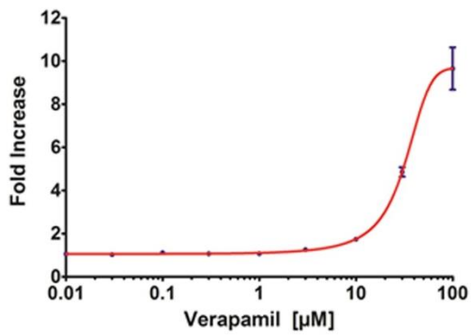

Figure 10. Effect of Verapamil on Cyto-ID® dye signal.

Autophagy Treatments

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 lysosome function	$6\text{-}9\times10^{-3}$	18	HeLa, Jurkat
Chloroquine	Alkalinizes Lysosomal pH	Inhibits lysosome function	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 lysosome function	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 with CYTO-ID® Autophagy Detection Kit.

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TROUBLESHOOTING GUIDE

Problem	Potential Cause	Suggestion
Low CYTO-ID® Green dye staining in all treatments, including positive control.	CYTO-ID® Detection Reagent is photobleaching	Use mounting medium that prevents photo-bleaching. Optimize handling of the samples for fluorescence microscopy.
	A low concentration of the CYTO-ID® 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 CYTO-ID® Green dye staining observed in negative control sample.	Cell cultures overgrown.	Suspension cells should not exceed a density $1x10^6$ 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.

Problem	Potential Cause	Suggestion
The number of CYTO-ID® 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.
CYTO-ID® 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.

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