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Cyto-ID™ Autophagy Detection Kit

for flow cytometry and fluorescence microscopy

Instruction Manual

Cat. No. ENZ-51031-K200

For research use only.

Rev. 1.3 April 2011

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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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Contents

I. 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 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 upregulated, 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, neurodegenerative 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 and flow cytometry. 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 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. Tamoxifen, 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 this organelle. This live cell analysis kit provides a convenient approach for the analysis of the regulation of autophagy at the cellular level.

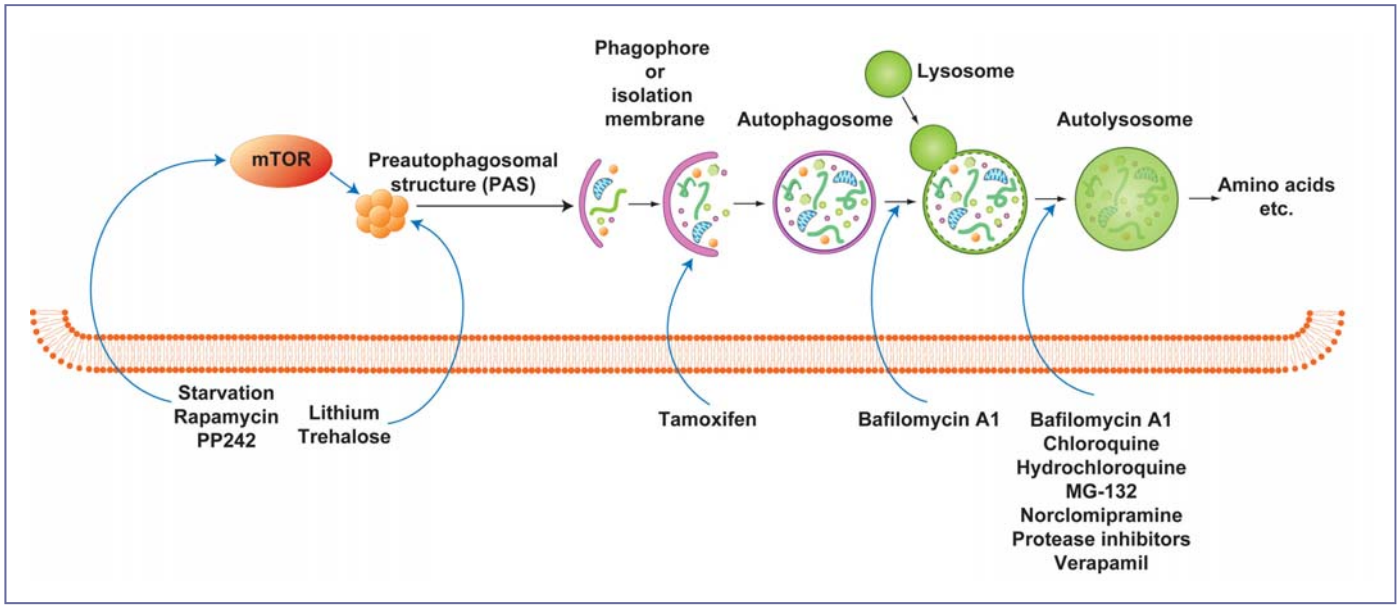

Figure 1. Schematic depiction of autophagy.[3] Cytosolic material is sequestered by an expanding membrane sac, the phagophore, resulting in the formation of a double-membrane vesicle, an autophagosome. The outer membrane of the autophagosome subsequently fuses with the lysosome, and the internal material is degraded in the autolysosome. Various regulators of autophagy are also depicted in the diagram

II. Reagents Provided and Storage

All reagents are shipped on dry ice. 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 kit are sufficient for approximately 200 assays for flow cytometry application or 125 assays for fluorescence microscopy.

Reagent	Quantity
Cyto-ID™ Green Detection Reagent	25 µL
Hoechst 33342 Nuclear Stain	50 µL
Autophagy Inducer (Tamoxifen, 50 mM)	20 µL
10X Assay Buffer	30 mL

III. Additional Materials Required

- Flow cytometer equipped with 488 nm laser source
- Standard fluorescence microscope
- 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
- Glass microscope slides
- Glass cover slips (18 x 18 mm)

IV. Safety Warnings and Precautions

- This product is for research use only and is not intended for diagnostic purposes.
- The Cyto-ID™ Green Detection Reagent and the Autophagy Inducer (Tamoxifen) 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 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.
- To avoid photobleaching, perform all manipulations in low light environments or protected from light by other means.

V. 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.

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

Tamoxifen is a drug that is widely used in the treatment of breast cancer. It competes with estrogen for receptor binding and prevents the transcription of estrogen-responsive genes, which results in reduced cell growth. Tamoxifen is thought to stimulate autophagy by increasing intracellular levels of ceramide and abolishing the inhibitory effect of the class-I PI3K pathway. Tamoxifen-induced autophagy is characterized by the accumulation of autophagic vacuoles and the stimulation of autophagic flux.

The Tamoxifen included in the kit is supplied as a 50mM solution. To use it as a positive control, dilute the Tamoxifen to 5-20 µM into your culture medium. 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. 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. **For fluorescence microscopy**, prepare a sufficient amount of **Dual Detection Reagent** for the number of samples to be assayed as follows: For every 1 mL of 1X Assay Buffer (see preparation in step 2, above) or cell culture medium, add 2 µL of Cyto-ID™ Green Detection Reagent and 1 µL of Hoechst 33342 Nuclear Stain.

NOTE: *(a) The dyes may be combined into one staining solution or each may be used separately, if desired.*

(b) The Hoechst 33342 Nuclear Stain can be diluted further if its staining intensity is much stronger than that of the Cyto-ID™ Green dye.

(c) Some cells require serum to remain healthy. Add serum to the detection reagent and wash solutions. Serum improves staining. Typical amounts of serum to add range from 2% to 10%.

(d) *When staining BFP- or CFP-expressing cells, the Hoechst 33342 Nuclear Stain should be omitted due to its spectral overlap with these fluorescent proteins.*

b. **For flow cytometry**, for each sample to be stained, dilute 1 µL Cyto-ID™ Green Detection Reagent to a final volume of 4 mL with media or buffer of choice.

B. Cell Preparations

Positive control cells should be pretreated with the Autophagy Inducer (Tamoxifen) for 6~18 hours. Response to Tamoxifen 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.

C. Live Cell Analysis By Fluorescence/Confocal Microscopy (Adherent Cells)

1. Grow cells on 18 x 18 mm coverslips, or tissue culture treated slides, inside a Petri dish filled with the appropriate culture medium. When the cells have reached 50% ~ 70% level of confluence, carefully remove the medium.

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

2. Dispense 100 µL of Dual Detection Reagent (see section A-3a, page 4) to cover the monolayer cells.
3. Protect samples from light and incubate for 30 minutes at 37°C.
4. Carefully wash the cells with 100 µL of 1X Assay Buffer (see section V-A2). Remove excess buffer and place coverslip upside down on microscope slide.
5. 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. Optionally, image the nuclear signal using a DAPI filter set.

D. 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.

   IMPORTANT: *Cells should be healthy and not overcrowded since results of the experiments will depend significantly on the cells' overall condition. A sufficient volume of cells should be centrifuged at 400 x g for 5 minutes, yielding a working cell count of 1 x 10^5 cells/sample.*

2. Centrifuge cells for 5 minutes at 400 x *g* at room temperature (RT) to obtain a cell pellet.
3. Carefully remove the supernatant by aspiration and dispense 100 µL of Dual Detection Reagent (from step 4A-3a, page 4) to cover the cell pellet.
4. Protect samples from light and incubate for 30 minutes at 37°C.
5. *(Optional)* Wash the cells with 100 µL 1X Assay Buffer. Remove excess buffer. Re-suspend cells in 100 µL 1X Assay Buffer.
6. Apply the cell suspension to a glass microscope slide and overlay with a coverslip.
7. 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. Optionally, image the nucleus using a DAPI filter set.

E. 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% CO_2.

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

2. Treat cells with compound of interest and negative control cells with vehicle.
3. Prepare positive control cells by incubating with the diluted Autophagy Inducer (Tamoxifen) (5-20 µM, see section V-A1, page 4) for 18 hours under normal tissue culture conditions.
4. At the end of the treatment, trypsinize (adherent cells), or collect cells (suspension cells). Samples may contain 1 x 10^5 to 1 x 10^6 cells per mL.
5. Centrifuge at 400 x *g* for 5 minutes to pellet the cells. Re-suspend in media, 1X Assay Buffer, or other buffer of choice and centrifuge as before.

6. Resuspend each live cell sample in 0.5 mL of freshly diluted Cyto-ID™ Green Detection Reagent (see step A-3b, page 5). Incubate for 30 minutes at room temperature or 37°C in the dark. It is important to achieve a monodisperse cell suspension at this step by gently pipetting up and down repeatedly.
7. Analyze the samples in the green (FL1) or orange (FL2) channel of a flow cytometer. No washing is required prior to the flow cytometry analysis.

VI. APPENDICES

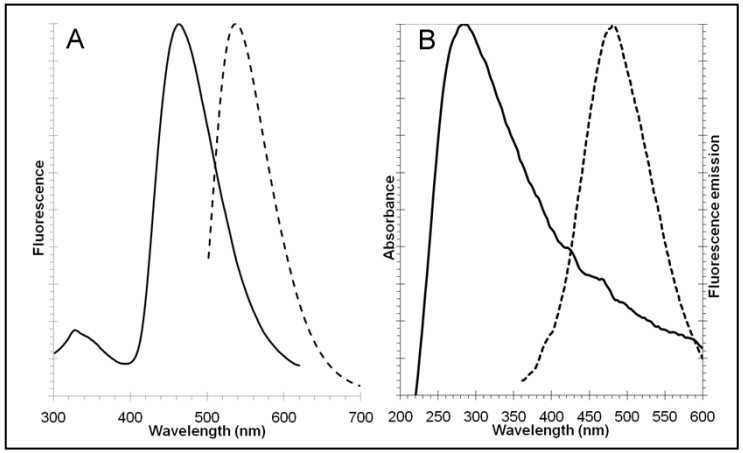

Figure 2. Excitation and fluorescence emission spectra (463/534nm) for Cyto-ID™ Green dye (panel A). Spectra were determined in 10 mM sodium acetate buffer, pH 4 with 3 mg/mL BSA. Absorbance and Fluorescence emission spectra (350/461nm) for Hoechst 33342 (panel B) were determined in 1X Assay Buffer.

A. Fluorescence Channel Selection for Data Collection

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.

B. Expected 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.[1-3] For example, monodasylcadaverine (MDC) has been determined to be a useful probe for the analysis of the autophagic process by fluorescence microscopy.[1] However, this probe requires 365 nm UV illumination and thus, is not compatible with 488 nm excitation sources commonly implemented in flow cytometry. The Cyto-ID™ Autophagy Detection Kit employs a 488 nm-excitable green-emitting fluorescent probe to highlight the various vacuolar components of the autophagy pathway. It should be noted that unlike the lysomotrophic dyes, 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 autophagosomes generated by chloroquine and bafilomycin A_1 treatment, but not vacuoles associated with other stimuli, such as serum starvation.[4]

1. 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 (Fig. 1, page 2). 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 dis-

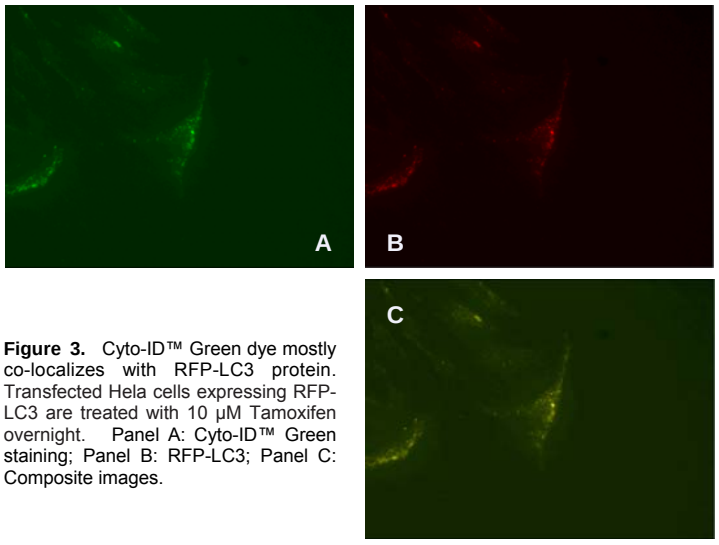

Figure 3. Cyto-ID™ Green dye mostly co-localizes with RFP-LC3 protein. Transfected Hela cells expressing RFP-LC3 are treated with 10 µM Tamoxifen overnight. Panel A: Cyto-ID™ Green staining; Panel B: RFP-LC3; Panel C: Composite images.

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
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
Trehalose	Unknown, mTOR-independent	Activates autophagy	50,000	6	HeLa
Bafilomycin A1	Inhibits Vacuolar-ATPase	Inhibits autophagy	$6~9*10^{-3}$	18	HeLa
Chloroquine	Alkalinizes Lysosomal pH	Inhibits autophagy	10	18	HeLa
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	9	18	HeLa
Hydroxychloroquine	Alkalinizes Lysosomal pH	Inhibits autophagy	10	18	HeLa
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	4~10	18	HeLa, Jurkat
Norclomipramine	Alkalinizes Lysosomal pH	Inhibits autophagy	5-20	18	HeLa

Table 1. Treatments that influence autophagy, validated with Cyto-ID™ Autophagy Detection Kit

tributed 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 10 µM Tamoxifen overnight. The cells were then stained with Cyto-ID™ Green dye. Tamoxifen induces an increase in Cyto-ID™ Green dye fluorescence intensity in punctuate structures that co-localize with RFP-LC3.

Besides Tamoxifen 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 signaling pathways. A popular target is mTOR, which is a potent suppressor of autophagy. Rapamycin, an inhibitor of mTOR and ATP-competitive inhibitors of mTOR such as PP242 increase Cyto-ID™ Green dye signal (Table I, page 9).

Several mTOR-independent autophagy activators have also been validated using the Cyto-ID™ Autophagy Detection Kit (Table I). 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 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.[5]

2. Flow Cytometry

Figure 4 on page 11 shows the typical results of flow cytometry-based analysis of cell populations using the Cyto-ID™ Autophagy Detection kit. Control uninduced and 10 µM Tamoxifen-treated Jurkat (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 display low fluorescence. In the samples treated with 10 µM Tamoxifen for 18 hours. The Cyto-ID™ Green dye signal increases about 2-fold, indicating that Tamoxifen causes an increase in autophagic vesicles in Jurkat cells.

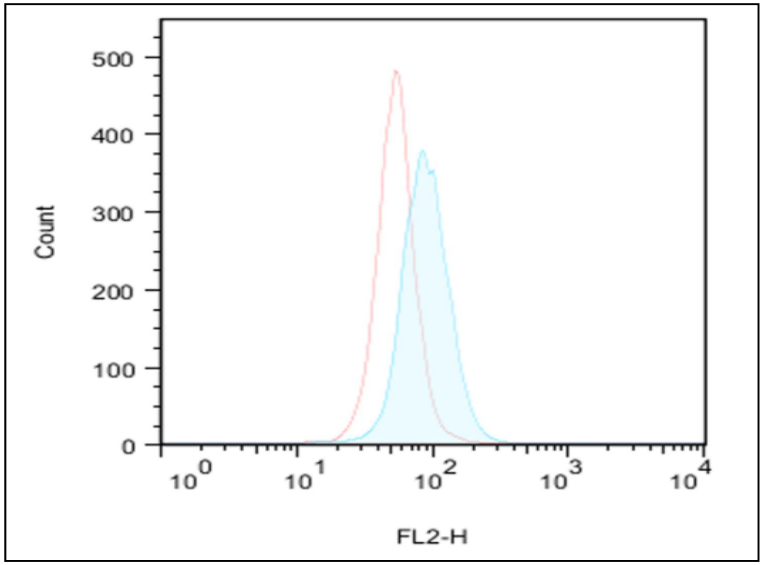

Figure 4. Flow cytometry-based profiling of Cyto-ID™ Autophagy Detection Kit: Control uninduced and 10 µM Tamoxifen-treated Jurkat cells(T-Cell leukemia) were used. After 18 hours treatment, cells were loaded with Cyto-ID™ Green Detection Reagent, then analyzed without washing by flow cytometry. Results 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.

VII. References

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2. Klionsky *et al* (2008) “Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes” Autophagy 4:2, 151-175
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4. Coleman *et al*. (2010) “A live-cell fluorescence microplate assay suitable for monitoring vacuolation arising from drug or toxic agent treatment.” J Biomol Screen. 15(4):398-405.
5. Rossi *et al*. (2009) “Desmethylclomipramine induces the accumulation of autophagy markers by blocking autophagic flux.” J Cell Sci 122: 3330-3339.

VIII. Troubleshooting Guide

Problem	Potential Cause	Suggestion
Low Cyto-ID™ Green dye staining in all treatments, including positive control.	A low concentration of the Cyto-ID™ Detection Reagent was used or the reagent was incubated with the cells for an insufficient length of time.	Either increase the reagent concentration or increase the incubation time .
High Cyto-ID™ Green dye staining observed in negative control sample.	Cell cultures overgrown.	Suspension cells should be cultured to a density not to exceed 1 x 10^6 cells and adherent cells should be approximately 50% - 70% confluent.
	Growing cells in media for too long will cause depletion of nutrients.	Change media 4 ~ 8 hours before the experiment.
	Pathogen infection (Mycoplasm, etc.).	Obtain fresh cultures from reputable cell repository.
Cyto-ID™ Green dye stained cells are too low to be readily quantified.	Cell density is either too low or cells are lost during process.	Increase cell density and gently aspirate supernatant during wash steps.
Cyto-ID™ Green dye staining fails to stain in fixed and/or permeabilized cells.	The dye is only suitable for live-cell staining.	Use the dye only for live-cell analysis.
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.	Some cells require serum to remain healthy.	Add serum to the detection reagent and wash solutions. Serum improves staining. Typical amounts of serum to add range from 2% to 10%.
Tamoxifen-treated cells appear dead or are no longer attached to the plate surface.	The EC_{50} of Tamoxifen may differ with different cell lines.	Try lowering the dose of Tamoxifen.

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