



Enabling Discovery in Life Science®

Cyto-ID™ Autophagy/Apoptosis/Necrosis Detection Kit

for flow cytometry

Instruction Manual

Cat. No. ENZ-51041-K100 100 Assays

For research use only.

Rev. 1.1.0 May 2011

Notice to Purchaser

The Cyto-ID™ Autophagy/Apoptosis/Necrosis 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.

This product is manufactured and sold by ENZO LIFE SCIENCES, INC. for research use only by the end-user in the research market and is not intended for diagnostic or therapeutic use. Purchase does not include any right or license to use, develop or otherwise exploit this product commercially. Any commercial use, development or exploitation of this product or development using this product without the express prior written authorization of ENZO LIFE SCIENCES, INC. is strictly prohibited.

Limited Warranty

These products are offered under a limited warranty. The products are guaranteed to meet appropriate specifications described in the package insert at the time of shipment. Enzo Life Sciences' sole obligation is to replace the product to the extent of the purchase price. All claims must be made to Enzo Life Sciences, Inc. within five (5) days of receipt of order.

Trademarks and Patents

Enzo, CELLestial and Cyto-ID are trademarks of Enzo Life Sciences, Inc. Several of Enzo's products and product applications are covered by US and foreign patents and patents pending.

Contents

I. Introduction	1
II. Reagents Provided and Storage.....	2
III. Additional Materials Required	2
IV. Safety Warnings and Precautions.....	2
V. Methods and Procedures	3
A. REAGENT PREPARATION.....	3
B. ASSAY PROTOCOL.....	4
C. FLOW CYTOMETRY COMPENSATION	6
VI. Appendices	6
A. FILTER SET SELECTION.....	6
B. EXPECTED RESULTS	7
VII. References	9
VIII. Troubleshooting Guide	10

I. Introduction

Programmed cell death (PCD) can be classified into three major types: apoptosis, autophagic cell death, and necrosis.¹⁻⁴ The hallmarks of apoptotic cell death (PCD type 1) include exposure of phosphatidyl serine on the extracellular face of the plasma membrane, activation of caspases, disruption of mitochondrial membrane potential, cell shrinkage, DNA fragmentation and DNA condensation. Autophagic cell death (PCD type 2) is a degradative process involving sequestration of cellular components in double-membrane vesicles that fuse with lysosomes to form autophagolysosomes. Nuclear changes in the form of chromatin condensation occur only late in this process, if at all. Autophagy is a survival strategy in situations of stress, but can also lead to non-apoptotic cell death. Necrotic cell death (PCD type 3) is characterized by cell swelling, as well as destruction of the plasma membrane and subcellular organelles, without nuclear fragmentation and condensation. Necrotic cell death is considered a heterogeneous phenomenon including both programmed and accidental cell death. Necrosis is often defined in a negative manner, as a type of cell death that involves rupture of the plasma membrane without the hallmarks of apoptosis and without massive autophagic vacuolization.² Typically, the three forms of PCD have been defined on the basis of morphological criteria. Due to the overall complexity of the cited death pathways, a sensitive and quantifiable biochemical assay capable of distinguishing between apoptosis, autophagic cell death and necrosis is fundamental to the further understanding of these pathophysiological processes.

The Cyto-ID™ Autophagy/Apoptosis/Necrosis Detection Kit is a three-parameter assay utilizing an argon laser excitable, green-emitting fluorophore to highlight the various vacuolar components of the autophagy pathway, a violet laser-excitable, blue fluorophore conjugate of Annexin V to measure phosphatidyl serine exposure in early stage apoptosis, and an argon laser excitable, cell-impermeable red fluorescent DNA intercalation dye to measure membrane disintegration in late-stage apoptosis/necrosis. The assay has been validated using a range of conditions known to modulate autophagy, apoptosis and necrosis pathways. The assay provides a rapid, information-rich read-out of the three principal PCD pathways by flow cytometry and should facilitate better drug activity profiling and clearer kinetic analysis of these fundamental processes in living cells.

II. Reagents Provided and Storage

All reagents are shipped on wet ice. Upon receipt, store the Apoptosis Inducer and Cyto-ID™ Green Autophagy Detection Reagent at -20°C , and all other reagents store at 4°C , protected from light.

Reagent	100 Reactions
Apoptosis Detection Reagent (Annexin V-Atlantic Blue)	500 μL
Cyto-ID™ Green Autophagy Detection Reagent	25 μL
Necrosis Detection Reagent (Red)	600 μL
Apoptosis Inducer (Staurosporine)	50 nmol
10X Binding Buffer	6 mL
EBSS (Earle's Balanced Salt Solution; starvation medium)	25 mL

III. Additional Materials Required

- A flow cytometer that is equipped with both 405 nm and 488 nm lasers for fluorescent dye excitation
- CO_2 incubator (37°C), tissue culture plasticware
- Calibrated, adjustable precision pipettors, preferably with disposable plastic tips
- 5 mL round bottom polystyrene culture tubes for holding cells during staining and assay procedure
- Adjustable speed centrifuge with swinging buckets
- Anhydrous DMSO
- PBS
- Deionized water
- RPMI culture medium with 10% fetal bovine serum

IV. Safety Warnings and Precautions

- This product is for research use only and is not intended for diagnostic purposes.
- Some components of this kit contain hazardous substances. Reagents can be harmful if ingested or absorbed through the skin and may cause irritation to the eyes. 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, in amber microcentrifuge tubes or protected from light by other means.

V. Methods and Procedures

The procedures described in this manual assumes 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. Apoptosis Inducer (Staurosporine)

The Apoptosis Inducer (Staurosporine) is supplied as a lyophilized powder (50 nmoles) and should be reconstituted in 50 μ L DMSO for a 1 mM stock solution. It is recommended that induction occur near the half maximal effective concentration (EC_{50}) and that the final percent DMSO in the assay not exceed 0.2%.

2. 1X Binding Buffer

Prepare 10 mL of 1X Binding Buffer per sample by diluting 1 mL of 10X Binding Buffer into 9 mL of deionized water. Batch preparation is recommended for large numbers of samples.

3. Detection Reagent

For optimal staining, the concentration of each detection reagent 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.

For Cyto-ID™ Green Autophagy Detection Reagent, prepare a sufficient amount of detection reagent for the number of samples to be assayed as follows: dilute 5 μ L Cyto-ID™ Green Autophagy Detection Reagent in 95 μ L media or buffer of choice. The volumes recommended are sufficient for twenty assays and must be scaled accordingly.

Note: *No dilution required for Apoptosis and Necrosis detection reagents.*

4. Controls

It is recommended that positive apoptosis control Jurkat samples be pretreated with the Apoptosis Inducer (Staurosporine) at a final concentration of 1 μM for 2~4 hours. Apoptosis may vary significantly and is dependent upon the length of induction, inducer concentration, cell type, cell line and exposure time. Negative apoptotic 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. After the incubation period, wash the cells with PBS, and resuspend them in 1X Binding Buffer (see section A-2, page 3) at a concentration of $\sim 0.5 \times 10^6 - 1 \times 10^6$ cells/mL.

Positive autophagy control cells are induced with amino acid starvation. Autophagy positive control Jurkat cells are washed two times with PBS to remove the medium and resuspended in EBSS (starvation medium) at a concentration of $\sim 0.5 \times 10^6 - 1 \times 10^6$ cells/mL for 1~3 hours at 37°C in an atmosphere of 95% air and 5% CO₂. Negative autophagy control Jurkat cells are also washed two times with PBS and resuspended in RPMI culture medium with 10% fetal bovine serum at a concentration of $\sim 0.5 \times 10^6 - 1 \times 10^6$ cells/mL for an equal length of time under similar conditions.

Positive necrosis control Jurkat cells can be induced with 100 mM methanol overnight at 37°C in an atmosphere of 95% air and 5% CO₂. Necrosis may vary significantly and is dependent upon the length of induction, inducer concentration, cell type, cell line and exposure time. After the incubation period, wash the cells with PBS, and resuspend them in 1X Binding Buffer (see section A-2, page 3) at a concentration of $\sim 0.5 \times 10^6 - 1 \times 10^6$ cells/mL.

B. ASSAY PROTOCOLS

Cells should be maintained via standard tissue culture practices. Always make sure that cells are healthy and in the log phase of growth before using them for an experiment.

1. Grow cell line of choice in the appropriate medium. Adherent cells should be dislodged from the plates using standard methods and used in suspension for the assay.

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

2. Treat cells with the compound of interest and negative control cells with vehicle. At the end of the treatment, centrifuge at 400 x g for 5 minutes to pellet the cells. Discard the supernatant. Wash the cells with PBS, centrifuge as before, and discard the supernatant.
3. Re-suspend cells in 1X Binding Buffer (see section A-2, page 3) and centrifuge as before. Discard the supernatant and re-suspend the cells in 1X Binding Buffer to a density of $\sim 0.5 \times 10^6 - 1 \times 10^6$ cells/mL.

4. Transfer 500 μL of each cell suspension (from step B-3) to separate 5 mL culture tubes. To each tube, add 5 μL of diluted Cyto-ID™ Green Autophagy Detection Reagent (see section A-3, page 3), 5 μL of Apoptosis Detection Reagent and 5 μL of Necrosis Detection Reagent.
5. Gently vortex the cells and incubate for 15 min at RT (25°C) in the dark.
6. After the incubation period, keep the samples on ice.
7. As soon as possible (within one hour of staining), analyze the stained cells by flow cytometry using a 488 nm laser with the FL-1 channel for Cyto-ID™ Green Autophagy Detection Reagent, and the FL-3 channel for the Necrosis Detection Reagent.

Also, samples should be analyzed using a violet diode laser excitation 405 nm with FL-4 channel (450 nm bandpass or equivalent) for Apoptosis Detection Reagent.

8. Obtain the mean fluorescence intensity (MFI) values for treated and untreated cells.

For apoptosis induced samples, the percentage of apoptotic cells after treatment with 1 μM staurosporine for 4 hours is greater than 40%.

The expected ratio of MFI for apoptotic cells to MFI for viable cells is greater than 30.

9. Calculate the autophagy activity factor (AAF) for the autophagy positive control cells (cells treated with the starvation medium, EBSS).

AAF is calculated as follows:

$$\text{AAF} = 100 \times [(\text{MFI}_{\text{treated}} - \text{MFI}_{\text{control}}) / \text{MFI}_{\text{treated}}]$$

whereby:

$\text{MFI}_{\text{treated}}$ = mean fluorescence intensity values for control and treated samples

$\text{MFI}_{\text{control}}$ = mean fluorescence intensity values for treated samples

The expected AAF value using the 1 hour starvation treatment is greater than 30.

C. FLOW CYTOMETRY COMPENSATION

Because there is very little spectral overlap between the three dyes, very little compensation is required. If you would like to perform compensation, the following are the suggested compensation controls for Flow Cytometry:

- unstained cells
- cells stained with Autophagy Detection Reagent (without Apoptosis and Necrosis Detection Reagents)
- cells Stained with Apoptosis Detection Reagent (without Autophagy and Necrosis Detection Reagents)
- cells Stained with Necrosis Detection Reagent (without Autophagy and Apoptosis Detection Reagents)

NOTE: Only certain software programs allow the compensation correction for non-adjacent channels.

NOTE: It is important to use the brightest positive single stained samples for proper compensation correction.

VI. APPENDICES

A. FILTER SELECTION

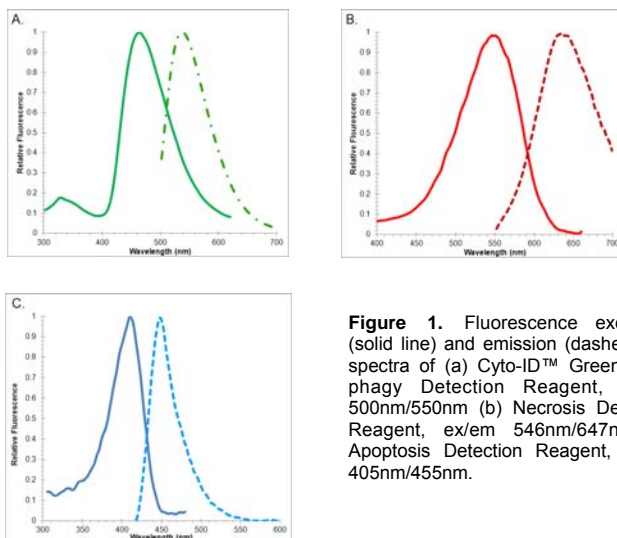


Figure 1. Fluorescence excitation (solid line) and emission (dashed line) spectra of (a) Cyto-ID™ Green Auto-phagy Detection Reagent, ex/em 500nm/550nm (b) Necrosis Detection Reagent, ex/em 546nm/647nm (c) Apoptosis Detection Reagent, ex/em 405nm/455nm.

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. Please consult the microscope or filter set manufacturer for assistance in selecting optimal filter sets for your microscope.

B. EXPECTED RESULTS

1. Autophagy Induction

The most well-known inducer of autophagy is nutrient starvation, both in cultured cells and in intact organisms, ranging from yeast to mammals. We evaluated the incorporation of Cyto-ID™ Green Autophagy dye in cells where autophagy was induced by amino acid deprivation. Jurkat cells were incubated in complete growth media with 10% FBS (control cells) or in EBSS (Earle's Balanced Salt Solution, starved cells) at 37°C for one hour. Results are presented using histogram overlay graphs (Figure 2). Control Jurkat cells were stained but displayed low Cyto-ID™ Green Autophagy dye fluorescence. The Cyto-ID™ Green Autophagy dye signal increases about 2-fold after 1 hour starvation (panel B), indicating that starvation causes an increase in autophagic vesicles in Jurkat cells. The AAF value is about 50. However, the starvation treated cells have similar percentage of apoptotic and necrosis cells to the basal level of apoptosis seen in the control cells (panels A and C).

Earl's Buffer Starvation for 1 Hour

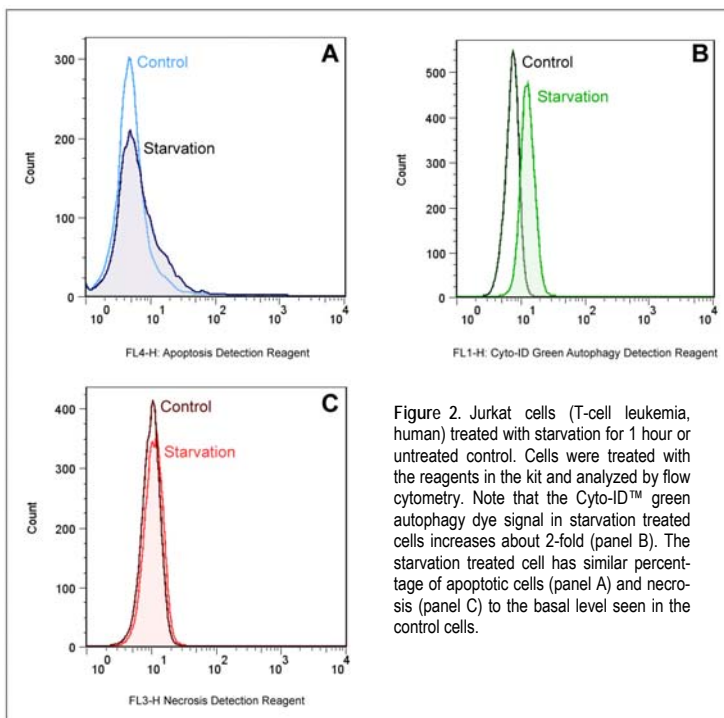


Figure 2. Jurkat cells (T-cell leukemia, human) treated with starvation for 1 hour or untreated control. Cells were treated with the reagents in the kit and analyzed by flow cytometry. Note that the Cyto-ID™ green autophagy dye signal in starvation treated cells increases about 2-fold (panel B). The starvation treated cell has similar percentage of apoptotic cells (panel A) and necrosis (panel C) to the basal level seen in the control cells.

2. Apoptosis Induction

Control and Staurosporine-induced Jurkat cells (T-Cell leukemia) were stained with the Apoptosis Detection Reagent (Figure 3). Cells that were not induced for apoptosis, with the exception of a small percentage (up to 5%) as expected in routine cultures of untreated cells, are negative with the Apoptosis Detection Reagent (panel A). At least 60% of population of cells induced with 1 μM Staurosporine for 4 hours demonstrate positive with the Apoptosis Detection Reagent (panel A) and with a minor increase of necrosis population of (panel C). The Cyto-ID™ Green Autophagy dye signal remained at control levels after staurosporine treatment (panel B).

Apoptosis Induction with 1 μM Staurosporine for 4 hours

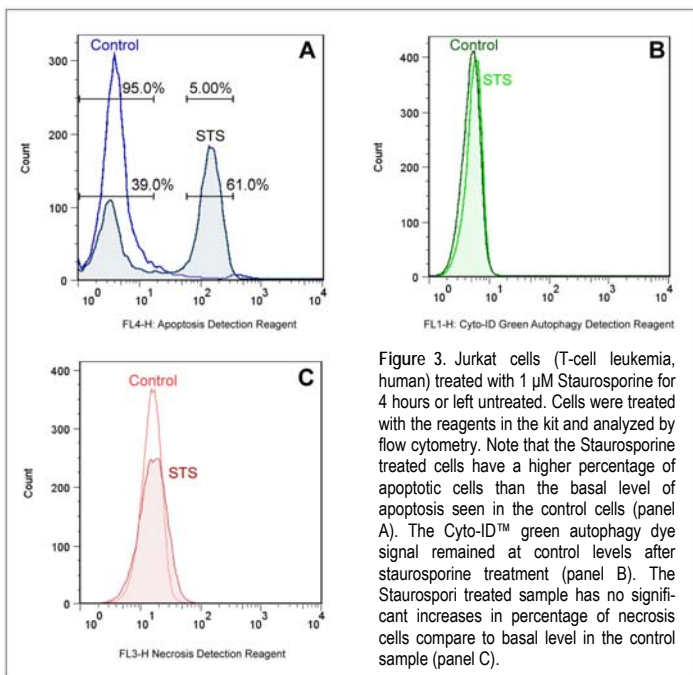


Figure 3. Jurkat cells (T-cell leukemia, human) treated with 1 μM Staurosporine for 4 hours or left untreated. Cells were treated with the reagents in the kit and analyzed by flow cytometry. Note that the Staurosporine treated cells have a higher percentage of apoptotic cells than the basal level of apoptosis seen in the control cells (panel A). The Cyto-ID™ green autophagy dye signal remained at control levels after staurosporine treatment (panel B). The Staurosporine treated sample has no significant increases in percentage of necrosis cells compare to basal level in the control sample (panel C).

3. Necrosis Induction

Typical results of necrosis induction with Jurkat cells are shown in Figure 4. Results are presented using histogram overlay graphs. Control populations of Jurkat cells stained but not induced are negative for apoptosis, autophagy or necrosis detection reagents. Cells treated overnight with 100 mM methanol demonstrate cells positive for both apoptosis and necrosis detection reagent (panels A and C). However, the Cyto-ID™ Green Autophagy dye signal remained at control levels after treatment with 100 mM methanol (panel B).

Necrosis Induction with 100 mM Methanol Overnight

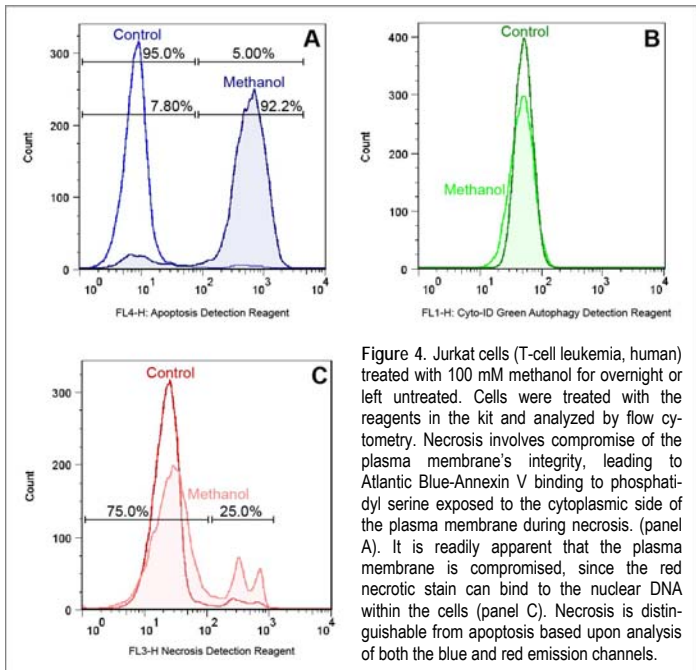


Figure 4. Jurkat cells (T-cell leukemia, human) treated with 100 mM methanol for overnight or left untreated. Cells were treated with the reagents in the kit and analyzed by flow cytometry. Necrosis involves compromise of the plasma membrane's integrity, leading to Atlantic Blue-Annexin V binding to phosphatidyl serine exposed to the cytoplasmic side of the plasma membrane during necrosis. (panel A). It is readily apparent that the plasma membrane is compromised, since the red necrotic stain can bind to the nuclear DNA within the cells (panel C). Necrosis is distinguishable from apoptosis based upon analysis of both the blue and red emission channels.

VII. References

1. Kroemer G, El-Deiry WS, Golstein P, Peter ME, et al (2005) Classification of Cell Death: Recommendations of the Nomenclature Committee on Cell Death. *Cell Death & Differ.* 12 Suppl 2:1463-7.
2. Diez-Fraile, A., Lammens, T. and D'Herde, K. (2009) "Apoptotic, Autophagic and Necrotic Cell Death Types in Pathophysiological Conditions: Morphological and Histological Aspects." In: *Phagocytosis of Dying Cells: From Molecular Mechanisms to Human Diseases*, D.V. Krysko, P. Vandenabeele (eds.), Springer Science.
3. Edinger, A. and Thompson, C. (2004) Death by Design: Apoptosis, Necrosis and Autophagy. *Current Opinion in Cell Biology* 16: 663-669.
4. Kroemer G et al. (2009) "Classification of cell death: recommendations of the Nomenclature Committee on Cell Death" *Cell Death Differ.* Cell Death Differ. Jan;16(1):3-11.

VIII. Troubleshooting Guide

Problem	Potential Cause	Suggestions
Little or no apoptosis observed or detected	No or few apoptotic cells present in sample	Use the Apoptosis Inducer, provided in the kit, as a positive control to induce apoptosis and optimize the Inducer concentration and time of treatment.
	Incorrect buffer used during sample processing	Calcium and magnesium ions are required for binding of the Apoptosis Detection Reagent to PS. Binding is reversible, so divalent cations must be present throughout the entire assay. Use the Binding Buffer provided in the kit.
		Completely remove wash buffer by aspiration as to not dilute the binding buffer or inducer.
Significant apoptosis detected in negative control cells.	Assay is inappropriate for the cells or cell line used.	Some cells (<i>i.e.</i> , megakaryocytes, platelets, some myeloid lineage cells) are known to display large amounts of PS on their cell surfaces. Peripheral Blood Mononuclear Cells (PBMCs) coated with platelets, therefore, may be positive for the Apoptosis Detection Reagent. Use a different method to monitor apoptosis.
	Cells were damaged or permeabilized during the assay	Permeable cells (advanced apoptotic or necrotic), yield an increase in apoptosis detection via cytoplasmic leaflet of the plasma membrane. Trypsinization, scraping or disaggregation of adherent cells may result in false positives due to cell damage, arising from disruption of the PS-containing plasma membrane. Use gentler cell preparation methods and optimize the Inducer concentration and time of treatment.
	Assay measurement or analysis was delayed.	Analysis must be performed quickly, following dye labeling in order to avoid post-label autophagy/apoptosis. Stained samples should be kept on ice until an assay is run.

Problem	Potential Cause	Suggestions
Low Cyto-ID™ Green Auto-phagy dye staining in all treatments, including positive control.	A low concentration of the Cyto-ID™ Green 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 .
Cyto-ID™ Green Autophagy 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 Autophagy 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.
Small apoptotic population detected and high necrotic population detected	Cells are no longer apoptotic and have progressed towards necrotic	High doses of an agent can often lead to necrosis, while lower doses of the same agent may lead to apoptosis. Often times, apoptosis and necrosis simply represent two extremes of biochemically overlapping cell death pathways. Lower the dose of the test agent.
High variability of results observed between and among samples on the same day or in different experiments from day to day	Inconsistencies in cell treatment, labeling and FCM data analysis were introduced.	All reagents should be prepared in batches. An untreated negative control and if possible an independent positive control should be included. Proper controls should be included in each experiment and gating of samples should be performed uniformly especially the gating out of debris.



www.enzolifesciences.com

Enabling Discovery in Life Science®

NORTH/SOUTH AMERICA

ENZO LIFE SCIENCES INTERNATIONAL, INC.

5120 Butler Pike
Plymouth Meeting, PA 19462-1202

USA

T 1-800-942-0430/(610) 941-0430

F (610) 941-9252

E info-usa@enzolifesciences.com

GERMANY

ENZO LIFE SCIENCES GMBH

Marie-Curie-Strasse 8

DE-79539 Lörrach

Germany

T +49/0 7621 5500 526

Toll Free 0800 664 9518

F +49/0 7621 5500 527

E info-de@enzolifesciences.com

www.enzolifesciences.com

UK & IRELAND

ENZO LIFE SCIENCES (UK) LTD.

Palatine House

Matford Court

Exeter EX2 8NL

UK

T 0845 601 1488 (UK customers)

T +44/0 1392 825900 (from overseas)

F +44/0 1392 825910

E info-uk@enzolifesciences.com

www.enzolifesciences.com

SWITZERLAND & REST OF EUROPE

ENZO LIFE SCIENCES AG

Industriestrasse 17, Postfach

CH-4415 Lausen

Switzerland

T +41/0 61 926 89 89

F +41/0 61 926 89 79

E info-ch@enzolifesciences.com

www.enzolifesciences.com

BENELUX

ENZO LIFE SCIENCES BVBA

Frankrijklei 33

BE-2000 Antwerpen, Belgium

T +32/0 3 466 04 20

F +33/0 437 484 239

E info-be@enzolifesciences.com

www.enzolifesciences.com

FRANCE

ENZO LIFE SCIENCES

c/o Covalab s.a.s.

13, Avenue Albert Einstein

FR -69100 Villeurbanne

France

T +33 472 440 655

F +33 437 484 239

E info-fr@enzolifesciences.com

www.enzolifesciences.com