FlowCellect™ GFP-LC3 Reporter Autophagy Assay Kit

Catalog No. FCCH100170 and FCCH100181

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

USA & Canada Phone: +1(800) 437-7500 Fax: +1 (951) 676-9209
Australia +61 3 9839 2000
www.millipore.com

Introduction

Autophagy is an intracellular catabolic pathway which causes cellular protein and organelle turnover, and is associated with a diverse range of diseases including Alzheimer's disease, aging-related processes, cancers, and Crohn's disease. It is a tightly regulated process that plays a normal part in cell growth, development, and cellular homeostasis. Autophagy functions as a housekeeping mechanism through disposal of aging and/or dysfunctional proteins and organelles by sequestering and priming these proteins for lysosomal degradation. Increasing evidence suggests that autophagy, in addition to apoptosis, can contribute to cell death and greatly influence general cell health. Malfunctions of the autophagy process are hypothesized to influence cell health, cellular longevity and the ability of cells to function at full capacity.

During autophagy, LC3 protein is translocated from the cytoplasm to the autophagosome where it is targeted to the lysosome for degradation. The process of autophagy can be categorized into four distinct stages (See figure 1):

1) *Induction and LC3 Translocation:* The process is initiated by external/internal stimuli (e.g. nutrient depletion);
2) *Autophagosome formation*: Unwanted cytosolic proteins and aging organelles are sequestered by a double membrane vesicle, i.e.—"autophagosome". Formation of this vesicle is coordinated by complexes of Atg proteins (Atg5 and Atg12) that are conjugated, enabling the recruitment of LC3;
3) *Lysosomal docking and fusion:* LC3 protein regulates traffic between autophagosome to lysosome. (LC3-I is cytoplasmic; LC3-II is lipidated and sequestered into autophagosomal membrane);
4) *Degradation:* Fusion with the lysosome and subsequent breakdown of the autophagic vesicle and its contents.

Millipore's FlowCellect™ GFP-LC3 Reporter Autophagy Assay Kits provide a quantitative solution for the study of autophagy and potency evaluation of autophagy inducers using flow cytometry. This kit has four unique features:

- The use of selective permeabilization solution discriminates between cytosolic LC3 from autophagic LC3 by extracting the soluble cytosolic proteins, while protecting LC3 which has been sequestered into the autophagosome;
- A monomeric GFP is used as a reporter to facilitate the translocation of the fusion protein, as other forms of GFP form dimers and aggregate when over-expressed in the cells, thus making it difficult to be extracted from cytoplasm and impossible to measure translocation by flow cytometry;
- Since autophagy is a constitutive cellular degradation process, the use of an autophagy detection reagent (Autophagy Reagent A) will prevent the lysosomal degradation of LC3, allowing its quantification by flow cytometry;
- The monomeric GFP used in our fusion protein to LC3 is attached on the 5' end (N-terminal fusion), protecting the GFP from Atg4 cleavage, thus, allowing its visualization within the autophagosomes (figure 3).

To assist in autophagy study, two host cell lines are offered: either CHO (Chinese Hamster Ovary) or U20S (human osteosarcoma). The CHO reporter cell line is ideal for flow cytometry applications, while U20S is suitable for both imaging and flow cytometry instruments.

The ability to measure and quantify autophagy enables the user to screen and rank order autophagy inducers or inhibitors, monitor cell culture health and protein turnover rate. These benefits enable one to study the mechanisms of protein degradation, and can aid in identifying new autophagy targets and pathways leading to aging and neurodegenerative diseases.

The GFP-LC3 reporter cell lines and autophagy enabling reagents are optimized on guava® bench top flow cytometers and provide a complete solution for autophagy analysis which is reliable, quantitative, and completely validated. Moreover, these kits can be used on any flow cytometer or imaging device by following the same protocol, offering the same benefits. The GFP-LC3 reporter cell line provided in the kits has been carefully chosen to ensure optimal performance, alleviating the need for any additional validation of the kit reagents.

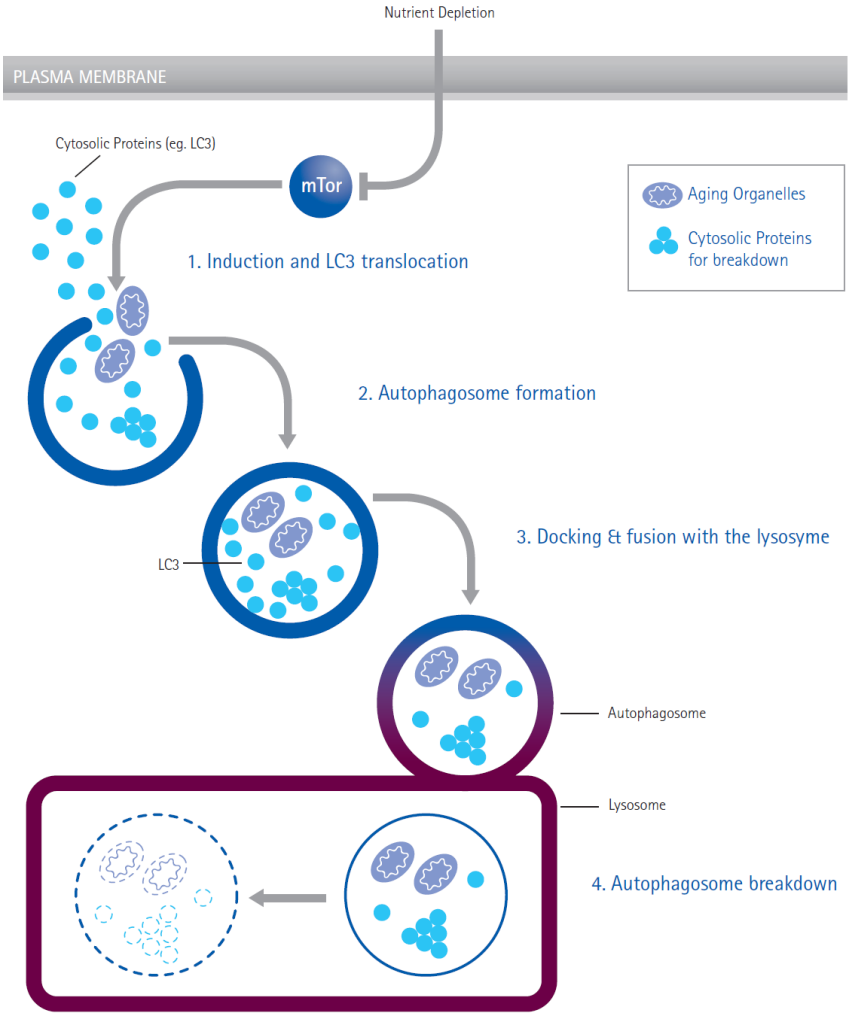


Figure 1. Autophagy: Four Stages of Autophagy Autophagy can be induced by nutrient depletion or inhibition of mTOR pathway. During autophagy, cytosolic proteins and aging organelles are sequestered by a double membrane vesicle to form autophagosomes. One of the hallmarks of autophagy is translocation of LC3 from the cytoplasm to the autophagosome. Autophagosome then fuses with the lysosome to cause the breakdown of autophagosome vesicle and its contents, including LC3. This process can be visualized using either a GFP-LC3 fusion protein and/or an anti-LC3 antibody.

Test Principle

Discrimination between cytosolic and autophagosome associated LC3 is achieved by monitoring the translocation of GFP-LC3 fusion protein using flow cytometry. This kit provides the reagent for the disruption of the cell plasma membrane using a proprietary selective permeabilization solution (figure 2). The selective permeabilization solution will extract cytosolic GFP-LC3 by flushing away during washing steps. GFP-LC3 translocated into the autophagosome is protected from the extraction and remains intact inside autophagosome, thereby allowing its fluorescence to be measured by flow cytometry or imaging. Another attractive feature is the GFP-LC3 reporter cell lines provided in the kits contain a monomeric GFP instead of the traditional GFP which exists as a dimer. The monomeric format is chosen to minimize undesirable multimerization and aggregation of the fusion proteins. Forced dimerization common to normal GFP results in aggregates which are not optimal for measurements of translocation events by flow cytometry or other applications. Since autophagy is a constitutive cellular degradation process, the use of an autophagy detection reagent (Autophagy Reagent A) is also added to prevent the lysosomal degradation of GFP-LC3, and ensure that it is retained for fluorescence detection. .

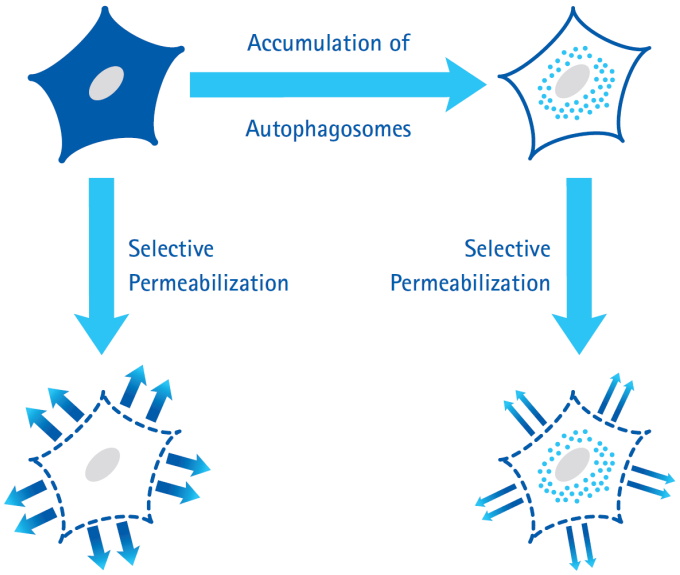


Figure 2. Selective Permeabilization helps discriminate cytosolic from autophagic LC3. Discrimination between GFP-cytosolic LC3-I from autophagosome associated GFP-LC3-II is achieved by disruption of cell PM by using an autophagy enabling solution (Autophagy Reagent B). This selective permeabilization will "release" cytosolic LC3 by flushing away during washing steps. LC3-II trapped in the autophagosome remains intact and fluorescence can be measured.

In various case studies, we have validated the useful application of a GFP-LC3 reporter by screening well known autophagy inducers and inhibitors, rapamycin and dynasore, respectively. And in addition to hit identification, we have further characterized the activity of other various small molecules such as STF-62247 and PI-103 which are well known autophagy inducers by performing dose response curves to derive EC_{50} values.

Millipore's GFP-LC3 Reporter Autophagy Assay Kits include one immortalized GFP-LC3 reporter cell line to measure and track the levels of LC3 within the cell. The autophagy detection reagents and cell line have been optimized together to ensure the ability to measure and discriminate between cytosolic and lipidated LC3 to accurately measure the autophagic process. Sufficient enabling reagents are provided to perform 100 tests. Detailed assay instructions are included to assist in analysis.

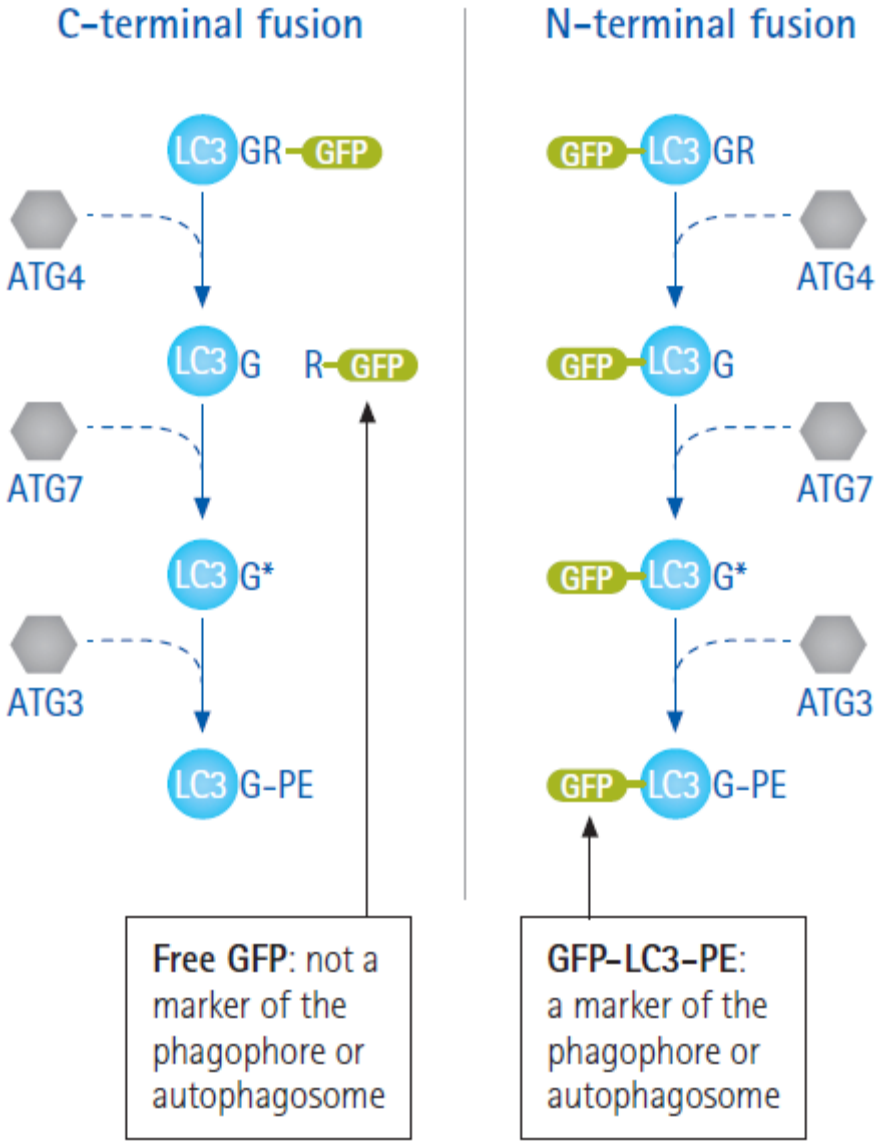


Figure 3. A Comparison between N-Terminal versus C-Terminal fusion of GFP to LC3
According to Klionsky (2011), the location of GFP fusion to LC3 is critical for measurement of LC3 translocation to serve as a marker for autophagosomes. If GFP is fused to the C-Terminal (or 3' end), following Atg4 cleavage GFP is removed and subsequently GFP is now lost. But GFP fusion to the N-Terminal (or 5' end) will retain GFP, making this construct a suitable marker to track autophagic activity.

Case Study #1(optional): Assessment of autophagic activity by autophagosomes using both an autophagy inducer (Rapamycin) and inhibitor (Dynasore)

A case study was conducted to evaluate the effects of both Rapamycin and Dynasore treatments on autophagy.

Rapamycin is an mTOR inhibitor and has been indicated to induce autophagy. mTOR is a member of the PI3-kinase family and is a central modulator of cell growth in response to environmental signals. It plays a critical role in transducing proliferative signals by activating downstream protein kinases that are required for both ribosomal biosynthesis and translations. 2000 Nobel Laureate Paul Greengard has demonstrated that a small molecule enhancer of Rapamycin - SMER28, decreases levels of amyloid-β (Ab) peptide, which is a hallmark of Alzheimer's disease. Autophagy is one major cellular pathway leading to the removal of such proteins. By targeting mTOR, rapamycin mimics the cellular starvation response by inhibiting signals required for cell cycle progression, cell growth, and proliferation and leads to the activation of autophagy (figure 6).

Dynasore is a cell-permeable inhibitor of dynamin which has been indicated to inhibit autophagy. Dynamin is essential for clathrin-dependent coated vesicle formation. Dynamin is required for membrane budding at a late stage during the transition from a fully formed pit to a pinched off vesicle. Dynamin may also fulfill other roles during earlier stages of vesicle formation. Dynasore acts as a potent inhibitor of endocytic pathways known to depend on dynamin by rapidly blocking coated vesicle formation within seconds of dynasore addition. As a result, Dynasore will inhibit autophagosome formation, which in effect, will inhibit autophagy (figure 7).

Case Study #2 (optional): Deep dive small molecule evaluation by performing dose response curves for EC_{50} determination by flow cytometry

Another case study was conducted to perform comprehensive analysis of small molecule autophagy inducers, STF-62247 and PI-103. STF-62247 and PI-103 have been indicated as autophagy inducers, and by using our selective permeabilization method along with the GFP-LC3 reporter cell line we were able to utilize this assay as a viable screening tool. Since structure-activity relationships (SAR) of small molecules are critical in identifying selective autophagy inducers; the level of LC3-II was determined by flow cytometry as indicated by the mean fluorescence intensity of the signal relative to the baseline negative control. In figure 8, STF-62247 and PI-103 were incubated for 8 hours in a 12 point, half-log dose dependent manner. From these values, a dose response curve is developed and EC_{50} values determined. By implementing this method autophagy compounds can be rank ordered to help complement any SAR campaigns during drug development. This data clearly illustrates the wide dynamic range of the reporter cell line as well validates the effective use of the autophagy enabling solutions.

For Research Use Only; Not for use in diagnostic procedures

Kit Components

- GFP-LC3 reporter cell line (stored in -80℃ for short term; in liquid nitrogen for long term):
    - CHO cell background for **FCCH100170** (Part No. CF200095) One vial containing 5 million cells.

      or,
    - U20S cell background for **FCCH100181** (Part No. CF200096) One vial containing 5 million cells

CF200097: Autophagy Detection Reagent Pack (stored at 2℃ - 8℃) :

- Autophagy reagent A: (Part No. CS208212) One vial (lyophilized)
- Autophagy reagent B: (Part No. CS208215) One vial containing 1 mL
- 5X Assay Buffer: (Part No. CS202124) One bottle containing 55 mL

Materials Not Supplied

1. Test tubes for sample preparation and storage
2. Tissue culture reagents, i.e. HBSS, PBS w/o Ca^{2+} or Mg^{2+}, cell dislodging buffers, etc.
3. Pipettors with corresponding tips capable of accurately measuring 10 – 1000 µL
4. Tabletop centrifuge capable of achieving 300 x g
5. Mechanical vortex
6. Flow Cytometer
7. Deionized water (for reagent dilutions)
8. Rapamycin reagent (EMD Chemicals; Part No. 553210)
9. Dynamin Inhibitor I, Dynasore reagent (EMD Chemicals; Part No. 324410)
10. STF-62247, Autophagy Inducer (EMD Chemicals; Part No. 189497)
11. PI-103, ATP-competitive inhibitor of PI3-K and mTOR (EMD Chemicals; Part No. 528100)

Precautions

- The instructions provided have been designed to optimize the kit's performance. Deviation from the kit's instructions may result in suboptimal performance and may produce inaccurate data.
- Some assay components included in the kit may be harmful. Please refer to the MSDS sheet for specific information on hazardous materials (MSDS forms can be found on the web page or by contacting Millipore technical services).
- During storage and shipment, the autophagy enabling reagents may condense within the vial. For maximum recovery of the product, centrifuge original vial prior to removing cap.
- Cell line must be stored either in -80℃ (short term) or liquid nitrogen (long term) upon receipt. Keeping cells warmer than -20℃ can compromise the integrity of the product.
- Do not use reagents beyond the expiration date of the kit.

Storage

This is a dual storage kit. All reagents must be stored at 2 - 8ºC, and the cell line must be stored at either -80℃ (short term) or in liquid nitrogen (lo ng term).

All kit components are stable up to six (6) months from date of receipt if stored and handled correctly. **Please avoid repeated changes in temperature as this will affect the integrity of the product.**

Preparation of Reagents

1. **Autophagy Reagent A**
   This material is supplied in a lyophilized vial. Prior to use, reconstitute the contents of the vial in 250 µL deionized water.

   Note: It is recommended to aliquot multiple vials and maintain them stored at -20℃. Avoid exposure of the reagent to repeated freeze and thaw cycles.

2. **Autophagy Reagent B**
   Autophagy reagent B is supplied at 10X concentration and should be diluted to 1X with deionized water prior to use. Prepared 1X Autophagy Reagent B is stable up to one year. Store at 2 - 8ºC.

3. **Assay Buffer**
   Assay Buffer is supplied at 5X concentration and should be diluted to 1X with deionized water prior to use. Prepared 1X Assay Buffer is stable up to one year. Store at 2 - 8ºC

Assay Instructions

Storage and Handling / Cell Propagation and Maintenance
Immediately upon receipt, thaw cells or place cells in-80℃ (short term storage) or in liquid nitrogen (long term storage)

1. Thaw cells rapidly by removing from liquid nitrogen by immediately immersing in a 37ºC water bath. Immediately after thaw, sterilize the exterior of the vial with 70% ethanol. Transfer contents of the vial to a T-75 flask containing 20-25 mL growth media. Place the flask in a humidified incubator at 37ºC with 5% CO_2.
2. After 24 hours, all live cells will be attached. Viability of the cells is expected to be 80-90%. At this time, replace growth media to remove any residual DMSO and return to incubator.
3. When cells have reached this stage and are approximately 80% confluent, passage the cells as follows: Remove media and wash once with HBSS without Ca^{++} and Mg^{++} (10 mL/T-75). Add 1 mL of a mild enzyme to dissociate adherent cells from the flask (Accutase; Cat. No. SCR005 from EMD Millipore or 0.05% trypsin/0.2 g/L EDTA; Cat No. SM-2002-C). Place in humidified incubator at 37ºC with 5% CO_2 until cells begin to round up and detach (5-10 minutes). Gently tap the side of the flask to dislodge the cells. Neutralize cell dissociating enzyme by addition of 4 mL growth media per 1 mL cell dissociating enzyme.
4. Cell passage is typically at 1:30 every 3-4 days. Passage ratios may be varied according to requirements of the investigator. Although the reporter cell line can be continually be propagated, indefinite cell propagations are done at the discretion of the researcher. It is recommended to bank frozen vials stored in liquid nitrogen for long term storage prior to use.

Cell Culture Media

CF200095 (CHO): Growth Media

- F12-K media containing 2 mM L-glutamine
- 10% heat-inactivated FBS (EMD Millipore, Cat. No. ES-009-B) NOTE: Must heat-inactive before use.
- 1X Penicillin/Streptomycin (from 100X stock; EMD Millipore, Cat. No. TMS-AB2-C)
- 250 μg/mL Gentamicin Sulfate (G418)

CF200095 (CHO): Plating Media

- F12-K media containing 2 mM L-glutamine
- 10% heat-inactivated FBS
- 1X Penicillin/Streptomycin

CF200096 (U20S): Growth Media

- DMEM with 4.5 g/L glucose and 4 mM glutamine (EMD Millipore, Cat. No. SLM-020-A)
- 10% heat-inactivated FBS (EMD Millipore, Cat. No. ES-009-B) NOTE: *Must heat-inactive before use.*
- 1X Non-essential amino acids (from 100X stock; EMD Millipore, Cat. No. TMS-001-C)
- 10mM HEPES (from 1 M HEPES, EMD Millipore, Cat. No. TMS-003-C)
- 1X Pen-Strep (from 100X stock; EMD Millipore, Cat. No. TMS-AB2-C)
- 250 μg/mL Genetecin/G-418

CF200096 (U20S): Plating Media

- DMEM with 4.5 g/L glucose and 4 mM glutamine
- 10% heat-inactivated FBS
- 1X Non-essential amino acids
- 10mM HEPES
- 1X Pen-Strep

I. General Assay Protocol (To monitor autophagosomes)

1. Seed approximately 3 to 4 million cells (CHO) or 4 to 5 million cells (U20S) into each of two T-75 flasks overnight in a humidified 37℃ incubator with 5% CO_2. Cells should be at about 80% confluent the next day.

2. Once the cells are 80% confluent, aspirate and wash cells in both T-75 flasks with 10 mL of 1X HBSS. To one flask, label it as "untreated" and add 20 mL Earle's Balanced Salt Solution (EBSS). To the other flask, label it as "treated" and add 20 mL of EBSS + 20 µL of Autophagy Reagent A. Incubate both flasks in a humidified incubator at 37ºC with 5% CO_2 for 2 hours.

3. Aspirate media and wash both flasks with 10mL of 1x HBSS. Detach and transfer cells to labeled conical tubes.

4. Determine cell numbers by using ViaCount or a hemacytometer and note cell viability. Healthy cell cultures are greater than 90% viable.

5. Spin down cells at 300 x g for 5 minutes at room temperature and aspirate media.

6. Resuspend each sample to 2 million cells per mL in 1X Assay Buffer.

7. Add 50 µL of cell suspension into the well of a "V" bottomed 96-well plate (100K cells/well) if using a guava HT instrument. NOTE: Refer to instrument user manual for instrument-compatible plates. If not using a guava HT instrument, add 100 µL of cell suspension into sample tubes.

8. Wash cells by adding 150 µL of a 1X Assay Buffer into each well and spin at 300 x g for 5 minutes.

9. Add 100 µL of 1X Autophagy Reagent B to each well, followed by gentle resuspension to ensure proper distribution, and immediately spin at 300 x g for 5 minutes.

10. Wash once with 1X Assay Buffer to remove residual 1X Autophagy Reagent B and spin at 300 x g for 5 minutes.

11. Resuspend each well in 200 µL of 1X Assay Buffer (or 500 µL of 1X Assay Buffer in sample tubes) and acquire data.

Results are tested using Guava easyCyte systems. However, the kit can be used on any flow cytometer with the correct laser and filter configurations. Simply make adjustments (e.g. increased cell density) to control for higher reagent usage required in sheath fluid instruments.

II. General Protocol for Compound Screening (optional)

a. Compound Hit Identification:

1. Seed 30K cells into a 96-well plate overnight in a humidified 37℃ incubator with 5% CO_2. Cells should be at about 80-90% confluent the next day.

2. Dilute each compound to a final concentration 10 μM in plating media or media preferred by the investigator (NOTE: Starting sample concentration must be determined at the researcher's own discretion).

3. Remove plating media from each well and discard.

4. Add 200 μL of the diluted compounds to the appropriate wells (or sample tubes) and incubate to the desired time point (e.g. 1 to 8 hour incubation; to be determined by the researcher).

5. Thirty minutes before the end of the scheduled time point, dilute Autophagy Reagent A at a 1:10 dilution in plating media (e.g. 10 μL Autophagy Reagent A into 90 μL plating media).

6. Add 20 μL of the diluted Autophagy Reagent A (in step #5) to each well/sample.

7. Incubate the cells for 1 hour at 37℃.

8. Aspirate plating media and wash cells once with 200 μL of 1X HBSS.

9. Add 100 μL of a mild enzyme (e.g. Accutase) to each well.

10. Allow cells to incubate at 37℃ for 5 minutes t o detach cells.

11. During the incubation step, add 100 μL of plating media to a guava compatible "V"-bottomed 96-well plate.

12. After cell incubation, gently resuspend cells in the 96-well plate to dislodge cells, followed by transferring cells to the "V"-bottomed 96-well plate for a total of 200 μL in each well.

13. Spin at 300 x g for 5 minutes at room temperature and discard supernatant.

14. Add 100 μL of 1X Autophagy Reagent B to each well and immediately spin at 300 x g for 5 minutes. Carefully discard supernatant from each well.

15. Wash once with 1X Assay Buffer to remove residual 1X Autophagy Reagent B and spin at 300 x g for 5 minutes. Carefully discard supernatant from each well.

16. Resuspend cells in each well with 200 μL 1X Assay Buffer.

17. Acquire samples.

b. Compound Dose Response Curves

1. Seed 30K cells into a 96-well plate overnight in a humidified 37℃ incubator with 5% CO_2. Cells should be at about 80-90% confluent the next day.

2. Dilute each compound to a final concentration of 10 μM in plating media or media preferred by the investigator (NOTE: Starting sample concentration must be determined at the researcher's own discretion).

3. Serially dilute each compound of interest in preparation for cell treatment and determination of dose response curves.

4. Aspirate media from each well and discard (Optional: Wash once with 1X HBSS and aspirate to remove any residual growth media from cells).

5. Add 200 μL of the diluted compounds to the appropriate wells and incubate to the desired time point.

6. Thirty minutes before the end of the scheduled time point, dilute Autophagy Reagent A at a 1:10 dilution in plating media (e.g. 10 μL Autophagy Reagent A into 90 μL plating media). .

7. When the desired time point is reached, add 20 μL of the diluted Autophagy Reagent A (in step #6) to each well.

8. Incubate the cells for 1 hour at 37℃.

9. Aspirate plating media and wash cells once with 200 μL of 1X HBSS.

10. Add 100 μL of a mild enzyme (e.g. Accutase) to each well.

11. Allow cells to incubate at 37℃ for 5 minutes to detach cells.

12. During the incubation step, add 100 μL of plating media to a guava compatible "V"-bottomed 96-well plate.

13. After cell incubation, gently resuspend cells in the 96-well plate to dislodge cells, followed by transferring cells to the "V"-bottomed 96-well plate for a total of 200 μL in each well.

14. Spin at 300 x g for 5 minutes at room temperature and discard supernatant.

15. Add 100 μL of 1X Autophagy Reagent B to each well and immediately spin at 300 x g for 5 minutes. Carefully discard supernatant from each well.

16. Wash once with 1X Assay Buffer to remove residual 1X Autophagy Reagent B and spin at 300 x g for 5 minutes. Carefully discard supernatant from each well.

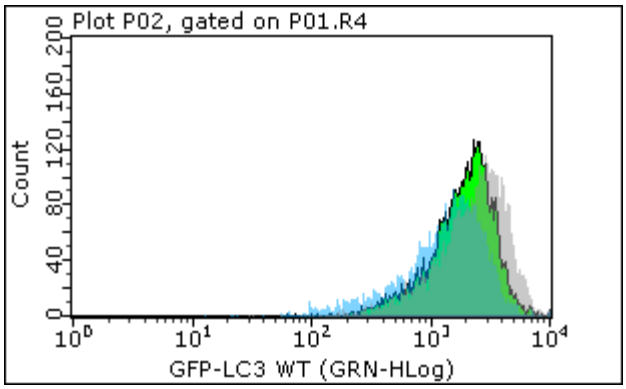
17. Resuspend cells in each well with 200 μL 1X Assay Buffer.

18. Acquire samples.

Sample Data

Reporter Assay: Flow cytometry detection of LC3 translocation via autophagosomes by addition of a lysosome inhibitor (using GFP-LC3 reporter)

A. Without Selective Permeabilization



B. With Selective Permeabilization

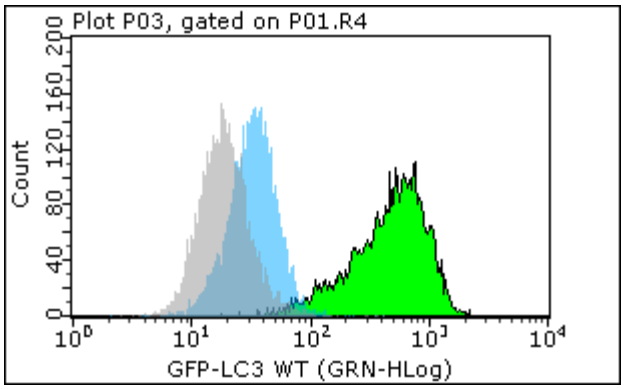
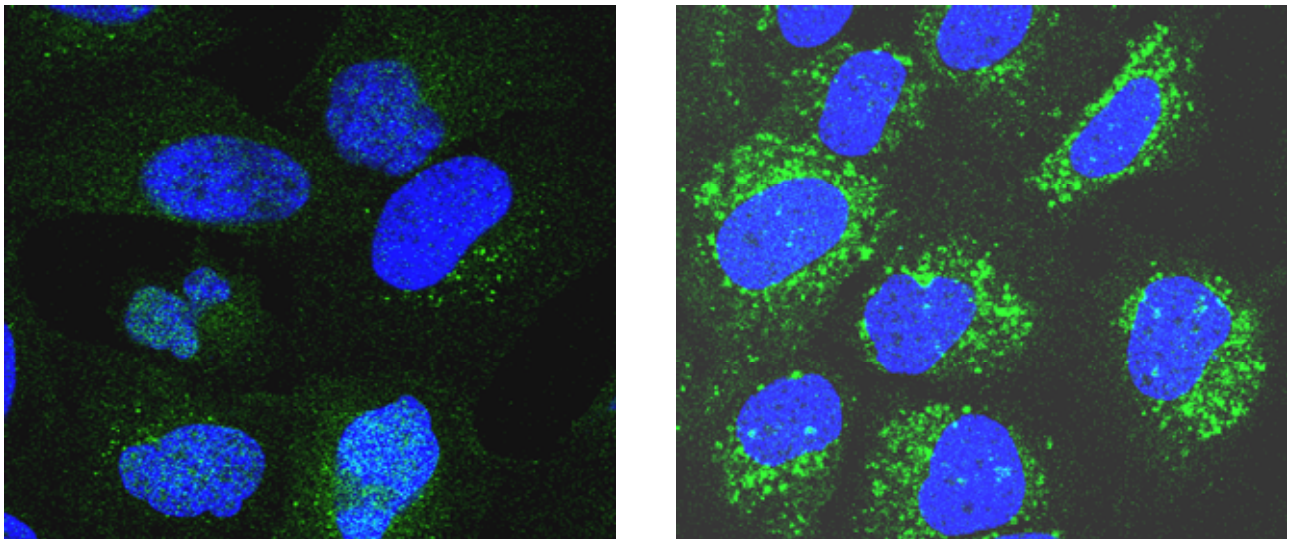


Figure 4. GFP-LC3 stable reporter cell line for detecting the rate of autophagy and for drug screening. In (A), without Selective Permeabilization, no shift of GFP-LC3 level is detected using flow cytometry before and after starvation (induction of autophagy). The position of the histograms indicates the high level of GFP-LC3 expression in the cytoplasm.
In (B), with Selective Permeabilization, the GFP-LC3 level remains high in autophagosomes when starved in the presence of lysosome inhibitor (green); even without the inhibitor, a slight shift is observed when starved (blue). All the cytosolic GFP-LC3 is washed away if no autophagy is induced by starvation (gray).

Legend:
Non-starved (control for no autophagy)
Starved in the absence of lysosome inhibitor
Starved in the presence of lysosome inhibitor

A. Control (Un-induced cells)

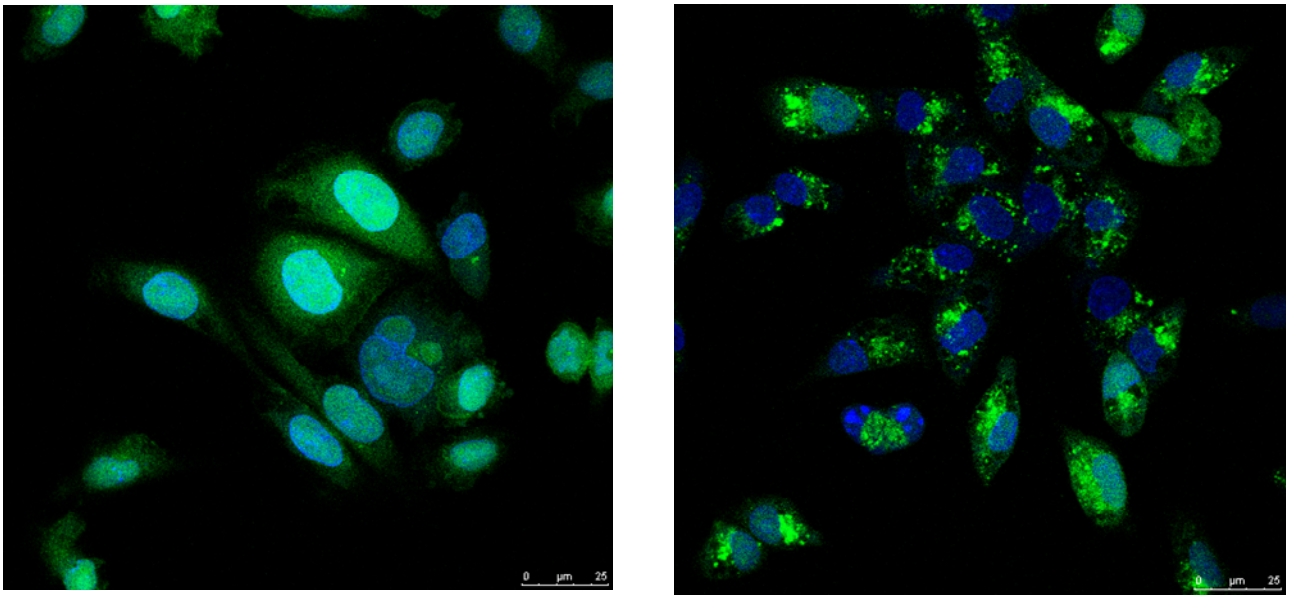
B. Induced Autophagy



U20S cells

A. Control (Un-induced cells)

B. Induced Autophagy



CHO cells

Figure 5. Image analysis of GFP-LC3 reporter cell line Cells are nutrient-deprived to induce autophagy and then treated with autophagy reagent A to prolong the signal for detection of translocated GFP-LC3 in the autophagosomes, which is a hallmark of the autophagic process (B). As illustrated, GFP-LC3 puncta is visualized as green dots. Control cells which are uninduced are shown in (A) which give a more diffuse fluorescence.. Cell nuclei stained with Dapi (blue).

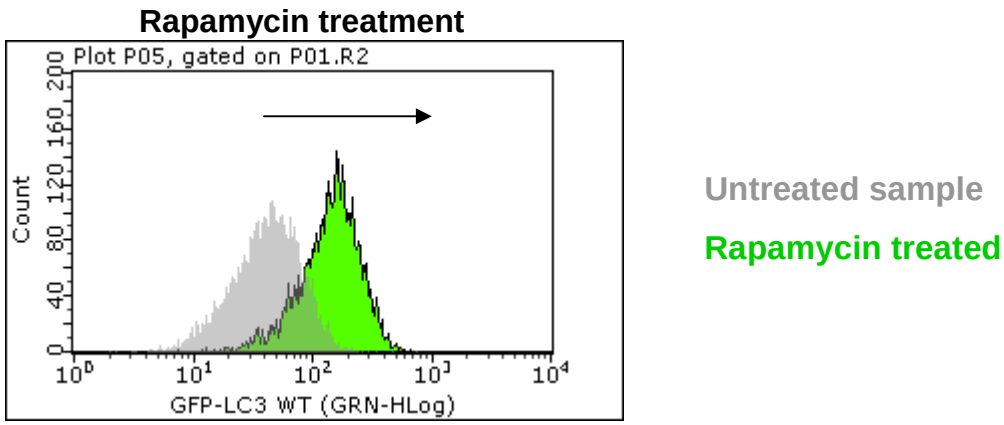


Figure 6. Rapamycin induces Autophagy through the mTOR pathway. Rapamycin is an inhibitor of the mTOR pathway, and by targeting mTOR, rapamycin mimics the cellular starvation response and leads to activation of autophagy as illustrated by the right shift of the histogram (green). Cells were treated with 400 nM Rapamycin for 48 hours prior to data acquisition.

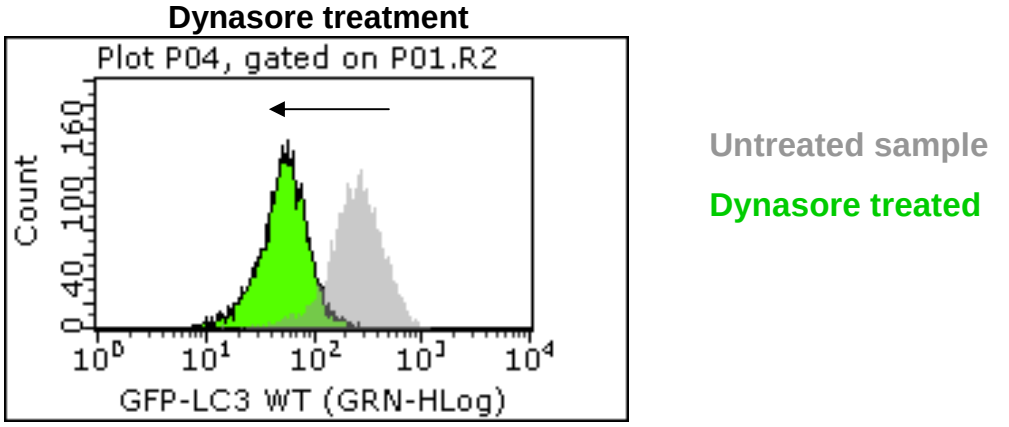
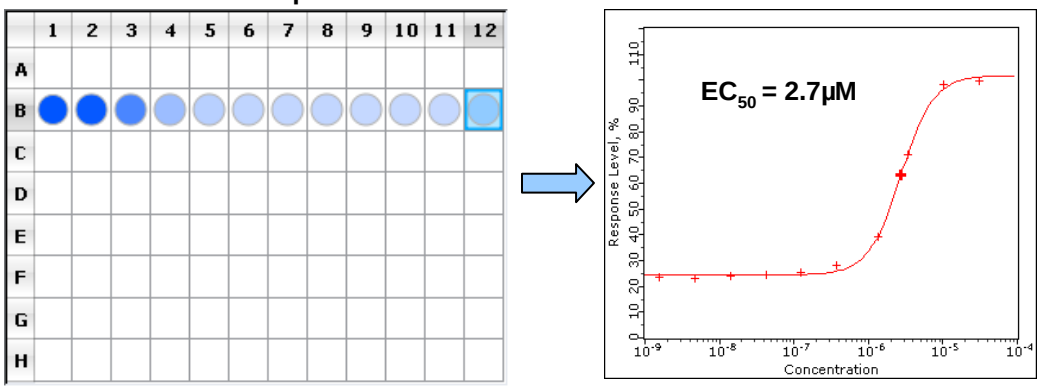


Figure 7. Dynasore inhibits Autophagy by inhibition of autophagosome formation. Dynasore is a cell-permeable inhibitor of dynamin. Dynamin is essential for clathrin-dependent coated vesicle formation. Dynasore acts as a potent inhibitor of endocytic pathways known to depend on dynamin by rapidly blocking coated vesicle formation within seconds of dynasore addition. As a result, Dynasore will inhibit autophagosome formation, which in effect, will inhibit autophagy as illustrated by the left shift of the histogram (green). Cells were treated with 80 µM Dynasore for 3 hours prior to data acquisition.

96-well Plate Heat map
(12 pt, dose response assay)

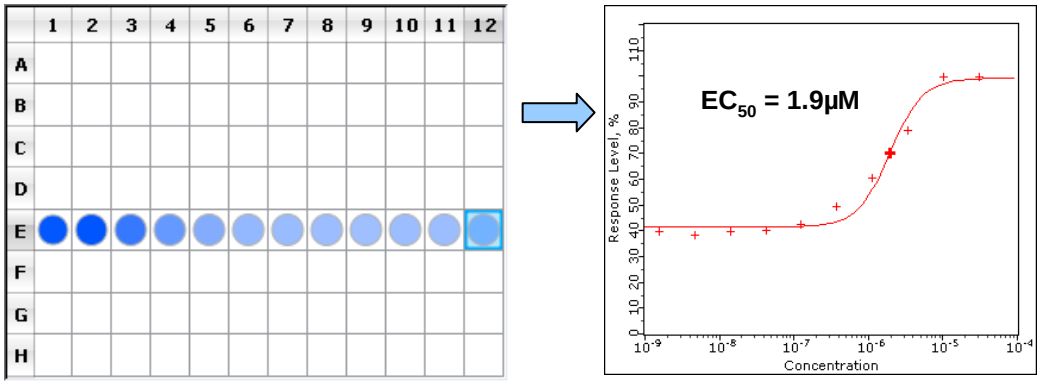
Dose Response Curve

A. STF-62247 Dose response curve



STF-62247

B. PI-103 Dose Response Assay



PI-103

Figure 8. Detailed small molecule evaluation by performing dose response curves for EC50 determination by flow cytometry using the InCyte™ Software Module STF-62247 and PI-103 have been indicated as autophagy inducers, and by using our selective permeabilization method along with the GFP-LC3 reporter cell line we were able to utilize this assay as a viable screening tool. STF-62247 (A) and PI-103 (B) were incubated for 8 hours in a 12 point, half-log dose dependent manner.
By implementing this method autophagy compounds can be rank ordered to help complement any SAR campaigns during drug development. This data clearly illustrates the wide dynamic range of the reporter cell line as well validates the effective use of the autophagy enabling solutions.

Technical Hints

- If minor precipitate is detected in the 10X Wash Buffer, place the bottle in a warm water bath for 15 minutes, followed by mixing the contents on a mechanical vortex.
- Confirm that the cell line is greater than 80% viability prior to assay and is maintained under drug selection media to ensure ideal expression GFP-LC3 over time.
- For drug treatments, all incubation times and sample concentrations must be optimized at the researcher's own discretion. Some guidelines for drug treatment are provided in this kit but can be modified to suit the researcher's individual experimental needs.
- Do not mix or interchange reagents from various kit lots.

Troubleshooting

Assay Step	Potential Problem	Experimental Suggestions
Reagent Preparation	Precipitation found in 10X Wash buffer	• If storing at -20ºC, precipitate can form in the 10X wash buffer. Prior to use, place bottle in a 37ºC water bath, swirling the contents occasionally. If this does not remove the precipitate completely, allow 10X Wash Buffer to sit at room temperature overnight.
Acquisition	Acquisition rate decreases dramatically	This usually indicates that the fluid pathway on the instrument may be blocked. This can be alleviated by the following: • Decreasing number of cells for analysis. Guava flow cytometers have the capacity of analyzing a steady stream of 300 – 500 cells per microliter. Using cell concentrations in excess of the recommended level can essentially block the normal flow, causing disruption during the assay. Decrease the number of cells being analyzed by diluting the sample to approximately 0.5 million cells per milliliter (5 x10^5 cells per ml). • Adherent cells can result in cellular clumping. Using a stronger enzyme for dissociation such as the guava ViaCount CDR Cell Dispersal Reagent (Cat. No. 4700-0050) or trypsin during cell harvesting should help keep cells in single suspension. Alternatively, using a cell strainer can help disrupt cell clumping if needed. • After many uses, it is possible that the fluid system on any standard flow cytometer will require cleaning. Run standard cleaning procedures to clean the fluid system during or after an assay. This will prevent any material from forming where the steady flow stream takes place.
Cellular Analysis	A loss or lack of signal	• Cell numbers may need to increase. Cell loss is common during washing steps in the assay procedure. A substantial decrease in cell numbers can lead to a loss of signal. Make sure that cell density remains at approximately 0.5 million cells per milliliter during analysis.
Cellular Analysis	Variability in day to day experiments	• Monitor experimental cell cultures to ensure that cell viability and cell numbers being analyzed are consistent. Any drop in cell numbers or viability can influence experimental results. • When using any guava easyCyte™ instrument for flow analysis, make sure that a quality check on the instrument (e.g. calibration) is performed on a daily basis prior to use. (**See Analytical Sensitivity and Detection Limits Section for Guava Check standards*)

For Technical Service, please visit www.millipore.com/techservice.

References

1. Shvets, E., *et al*. (2008). Utilizing flow cytometry to monitor autophagy in living mammalian cells. *Autophagy*; 4(5): 621-8.
2. Zhang, L., *et al.* (2007). Small molecule regulators of autophagy identified by an image-based high-throughput screen. *Proc Natl Acad Sci U S A*;104(48):19023-8.
3. Degtyarev, M., *et al.* (2008). Akt inhibition promotes autophagy and sensitizes PTEN-null tumors to lysosomotropic agents. *J Cell Biol*.;183(1):101-16.
4. Mizushima, N., *et al.* (2004). Methods for monitoring autophagy. *Int J Biochem Cell Biol*.;36(12):2491-502.
5. Fleming, A., *et al.* (2010). Chemical modulators of autophagy as biological probes and potential therapeutics. *Nat Chem Biol*.;7(1):9-17.
6. Tsien, R., (1998). The Green Fluorescent Protein. *Annu. Rev. Biochem.*; 67:509–44.
7. Tian, Y., *et al.* (2011). A small-molecule enhancer of autophagy decreases levels of Aβ and APP-CTF via Atg5-dependent autophagy pathway. *FASEB J*.;25(6):1934-42.
8. Turcotte, T., *et al.* (2008). A molecule targeting VHL-deficient Renal Cell Carcinoma that induces autophagy. *Cancer Cell*; 14(1):90-102.
9. Klionsky, D. J., et al. (2011). For the last time, it is GFP-Atg8, not Atg8-GFP (and the same goes for LC3). Autophagy; 7(10):1093-4.

Related Products

1. FlowCellect™ RFP-LC3 Reporter Autophagy Assay Kit (U20S) (Catalog No. FCCH100183)
2. FlowCellect™ Autophagy LC3 antibody-based Assay Kit (Catalog No. FCCH100171)
3. FlowCellect™ Bivariate Cell Cycle Kit for DNA Replication Analysis (Catalog No. FCCH025102)
4. FlowCellect™ Bivariate Cell Cycle Kit for G2/M Analysis (Catalog No. FCCH025103)
5. FlowCellect ™ Multi-Color DNA Damage Response Kit (Catalog No. FCCH025104)
6. FlowCellect™ Histone H2A.X Phosphorylation Assay Kit (Catalog No. FCCS100182)
7. FlowCellect™ Cell Cycle Checkpoint H2A.X DNA Damage Kit (Catalog No. FCCH025142)
8. FlowCellect™ Cell Cycle Checkpoint ATM DNA Damage Kit (Catalog No. FCCH025143)
9. FlowCellect™ PI3K-mTOR Signaling Cascade Mapping Kit (Catalog No. FCCS025210)
10. FlowCellect™ PI3K/MAPK Dual Pathway Activation and Cancer Marker Detection kit (Catalog No. FCCS025100)

SCHEDULE C

LICENSED PRODUCT LABELING

This product is the subject of one or more issued and/or pending U.S. Patent Applications and foreign equivalents licensed to Merck Millipore (EMD Millipore in North America). The purchase of this product includes the non-transferable right of the purchaser to use the purchased amount of the product and components of the product for research use only conducted by the purchaser. The purchaser has no right, and shall not, use this product for (a) use in or administration to humans, (b) any use for any clinical or therapeutic purposes, (c) any use for the development of drugs or diagnostics, and (d) any other purposes beside research use.

The purchaser cannot sell, distribute or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for any commercial purpose. Such prohibited commercial purposes include (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data to others in exchange for consideration; (3) use of the product or its components (or any derivatives thereof) for therapeutic, diagnostic or prophylactic purposes (including as part of a device, chip, assay or other product); or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. The purchase cannot modify or create derivatives of this product. The purchaser cannot use this product or its components in humans or as food for humans or animals.

The purchaser shall comply with all applicable laws in its use and handling of (a) this product (b) its components or (c) materials made using this product or its components, and shall keep all of the foregoing under reasonable safe and secure conditions so as to prevent unauthorized use or access.

Purchasers other than academic institutions or not-for-profit entities shall only have the right to use the purchased amount of this product and components of the product for a six (6) month evaluation period after the date of purchase. Such commercial research purchasers shall not, and shall have no rights to, use this product and its components beyond the six (6) month evaluation period unless and until such purchasers have obtained from Merck Millipore (EMD Millipore in North America) or its designee formal permission to conduct research beyond the six (6) month period (and pay the fees associated with such permission) at the time of initial purchase or prior to the end of the six (6) month period, in accordance with Merck Millipore's (EMD Millipore in North America) policies and procedures for obtaining such permission. In the absence of purchasing such permission, such commercial research purchasers shall promptly return or destroy any remaining amounts of the product or components at the end of such six (6) month period.

If the purchaser is not willing to accept the limitations and obligations set forth in this label, the purchaser shall not use the product and shall promptly return it to Merck Millipore (EMD Millipore in North America) for a full refund. For information on other issues of this product beyond those permitted above, contact: Intellectual Property Department (951-676-8080, ext. 274), provided that purchaser is hereby put on notice that it can be given no assurances or representations that any such other uses can be permitted or authorized or rights to such other uses will be available.

Warranty

Millipore Corporation (“Millipore”) warrants its products will meet their applicable published specifications when used in accordance with their applicable instructions for a period of one year from shipment of the products. **MILLIPORE MAKES NO OTHER WARRANTY, EXPRESSED OR IMPLIED. THERE IS NO WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE.** The warranty provided herein and the data, specifications and descriptions of Millipore products appearing in Millipore’s published catalogues and product literature may not be altered except by express written agreement signed by an officer of Millipore. Representations, oral or written, which are inconsistent with this warranty or such publications are not authorized and if given, should not be relied upon.

In the event of a breach of the foregoing warranty, Millipore’s sole obligation shall be to repair or replace, at its option, the applicable product or part thereof, provided the customer notifies Millipore promptly of any such breach. If after exercising reasonable efforts, Millipore is unable to repair or replace the product or part, then Millipore shall refund to the Company all monies paid for such applicable Product. **MILLIPORE SHALL NOT BE LIABLE FOR CONSEQUENTIAL, INCIDENTAL, SPECIAL OR ANY OTHER DAMAGES RESULTING FROM ECONOMIC LOSS OR PROPERTY DAMAGE SUSTAINED BY ANY COMPANY CUSTOMER FROM THE USE OF ITS PRODUCTS.**

Unless otherwise stated in our catalog or other company documentation accompanying the product(s), our products are intended for research use only and are not to be used for any other purpose, which includes but is not limited to, unauthorized commercial uses, in vitro diagnostic uses, ex vivo or in vivo therapeutic uses or any type of consumption or application to humans or animals.