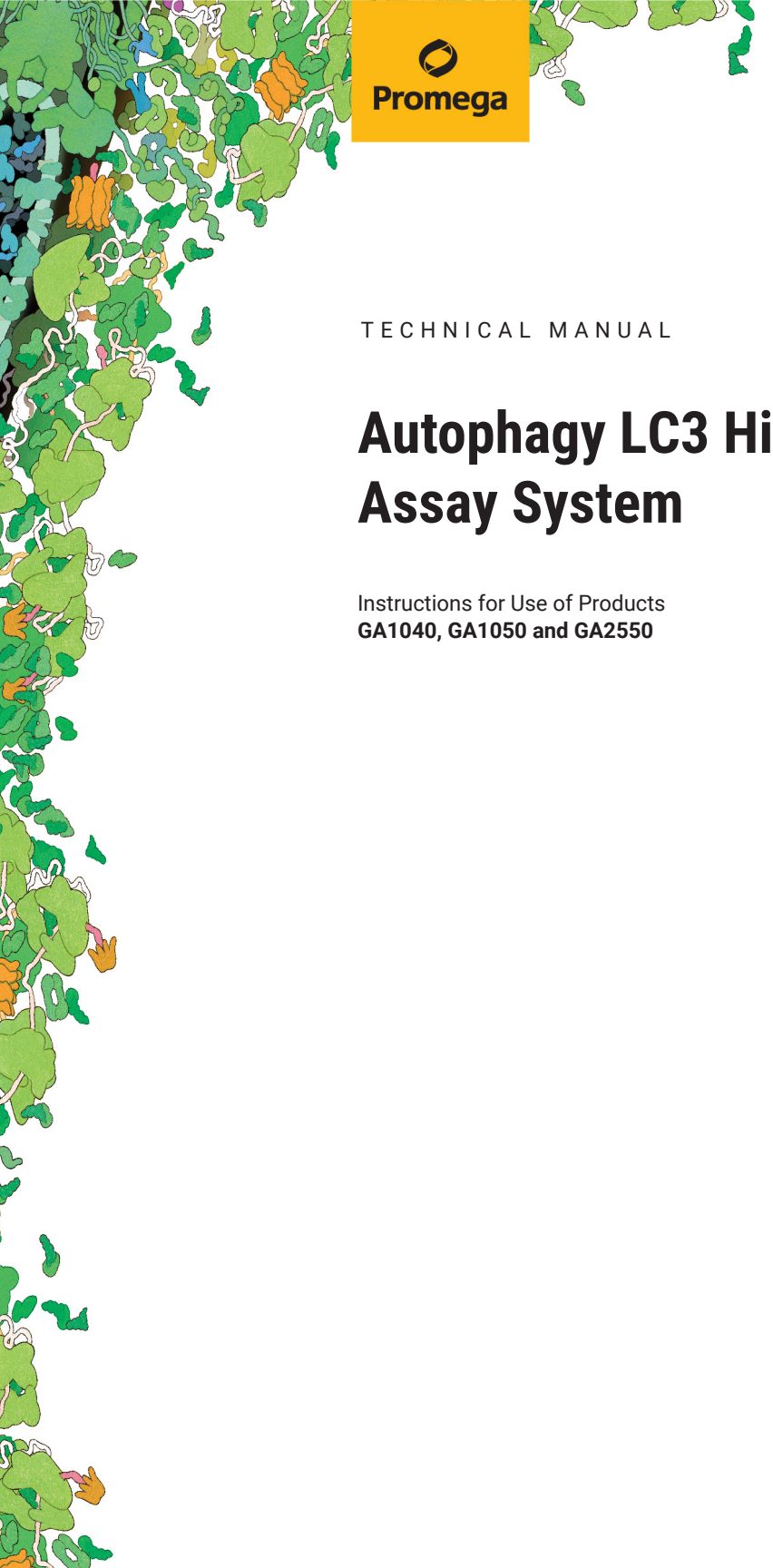

TECHNICAL MANUAL

# Autophagy LC3 HiBiT Reporter Assay System

Instructions for Use of Products
**GA1040, GA1050 and GA2550**

Revised 9/25
TM535

# Autophagy LC3 HiBiT Reporter Assay System

**All technical literature is available at: www.promega.com/protocols/**
**Visit the website to verify that you are using the most current version of this Technical Manual.**
**Email Promega Technical Services if you have questions on use of this system: techserv@promega.com**

## 1. Description

Autophagy is an important intracellular pathway for the degradation of superfluous or harmful subcellular materials, thereby playing a critical role in maintenance of cell health under normal and stress conditions. The Autophagy LC3 HiBiT Reporter Assay System[a-d] provides a homogeneous, bioluminescent, plate-based method for quantitative assessment of autophagy, including simple and reliable discrimination between inducers and inhibitors of the pathway. You can utilize the Autophagy LC3 HiBiT Reporter Vector to stably express the reporter at low levels in a chosen cell line or take advantage of ready-to-use reporter-expressing clonal lines: HEK293 Autophagy LC3 HiBiT Reporter Cell Line and U2OS Autophagy LC3 HiBiT Reporter Cell Line. Following cell treatment with test compounds, a simple add-mix-measure protocol using the Nano-Glo® HiBiT Lytic Detection System produces a luminescent signal directly proportional to the level of autophagy reporter present. The bright NanoBiT® luciferase technology allows low levels of autophagy reporter expression to produce strong signal-to-background values (>100) while maintaining responsiveness to changes in autophagy. The luminescent signal is highly stable (half-life >3 hours), enabling measurement of multiple assay plates in a single study.

When autophagy is induced, cytosolic LC3-I (as well as expressed Autophagy LC3 HiBiT Reporter) is recruited to phagophores where it becomes conjugated to phosphatidylethanolamine, thereby forming LC3-II. Upon phagophore membrane elongation and closure to form the mature autophagosomal vesicle, a significant fraction of tethered LC3 protein becomes captured within the lumen along with various cargo materials (Figure 1). Subsequently, autophagosomes fuse with lysosomes to form autolysosomes, leading to the ultimate degradation of both cargo material and the captured LC3 protein (and Autophagy LC3 HiBiT Reporter protein). The capture of cargo material, its trafficking to the autolysosome and the ultimate degradation of this material is referred to as autophagic flux. Therefore, changes in the total level of LC3 protein (or Autophagy HiBiT LC3 Reporter) can be used to monitor changes in autophagic flux (1).

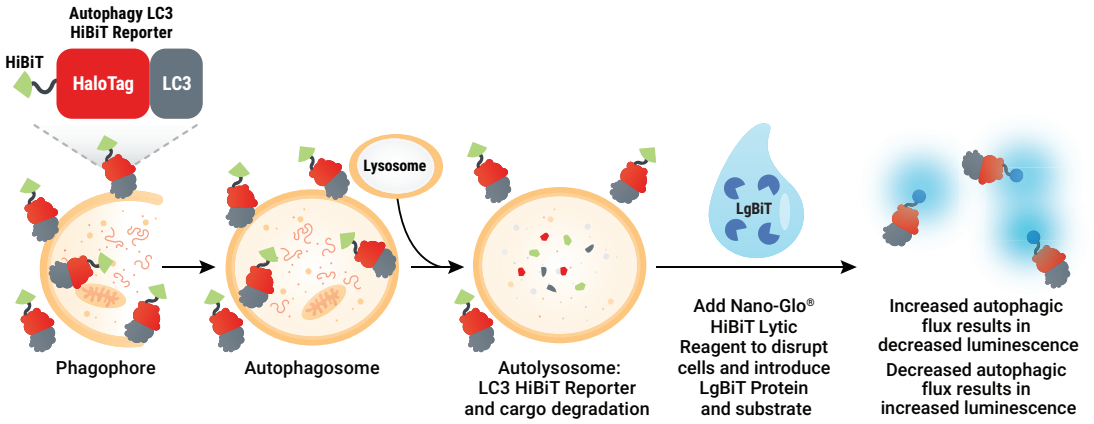


**Figure 1. Principle of the Autophagy LC3 HiBiT Reporter Assay.** Like endogenous LC3 protein, the Autophagy LC3 HiBiT Reporter expressed in cells becomes targeted to phagophores upon autophagy induction. The reporter molecules captured within the lumen of autophagosomes are subsequently degraded upon autolysosome formation. After cell treatment, the level of intact Autophagy LC3 HiBiT Reporter is indicated by luminescent signal following application of the NanoGlo® HiBiT Lytic Detection System.


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The Autophagy LC3 HiBiT Reporter consists of human LC3B, the HiBiT tag and an intervening spacer region consisting of HaloTag® protein tag (Figure 2). The use of HaloTag® and HiBiT protein tags allow for the reporter to be used in three complementary assay modalities to effectively assess changes in autophagic activity. The three modalities can be used on their own or in parallel experiments to confirm results and understand where in the autophagic pathway a treatment is acting.

The first assay modality is a simple, quantitative, plate-reader based format enabled by the HiBiT reporter tag. When a lytic detection reagent containing the substrate furimazine and LgBiT (the large subunit used in NanoLuc® Binary Technology) is added to cells expressing the autophagy reporter, HiBiT binds tightly to LgBiT ($K_D$ = 0.7nM) to form a bright, luminescent enzyme. The luminescent signal is proportional to the amount of HiBiT-tagged reporter in the cell lysate over seven orders of magnitude. An increase in autophagic flux will accelerate the degradation of the autophagy reporter, resulting in decreased luminescent signal. In contrast, inhibition of autophagy (whether early or late in the pathway) will increase reporter levels and luminescent signal. Therefore, the mechanism of action of a putative autophagy modulator can be verified by cotreatment with a reference inhibitor, such as bafilomycin A1 (Baf A1).

The second assay modality uses the HiBiT tag for protein blotting, to visualize the conversion of LC3-I to the lipidated LC3-II. The third assay modality uses the HaloTag® portion of the reporter in conjunction with fluorogenic ligands to visualize the subcellular localization of the LC3-based reporter within the cell.

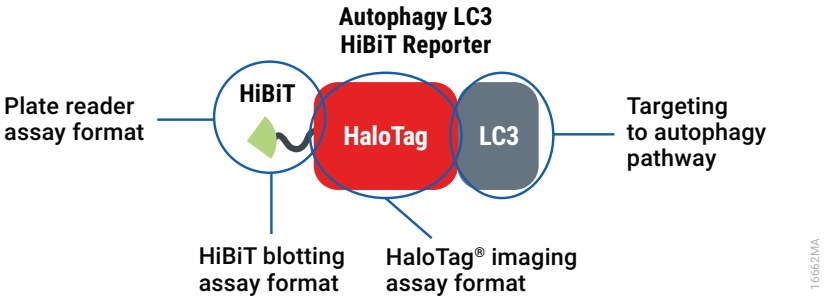


**Figure 2. Autophagy Reporter Components.** The three components of the autophagy reporter—LC3, HaloTag and HiBiT—create a versatile reporter system based on LC3, a well-accepted marker of autophagic activity.

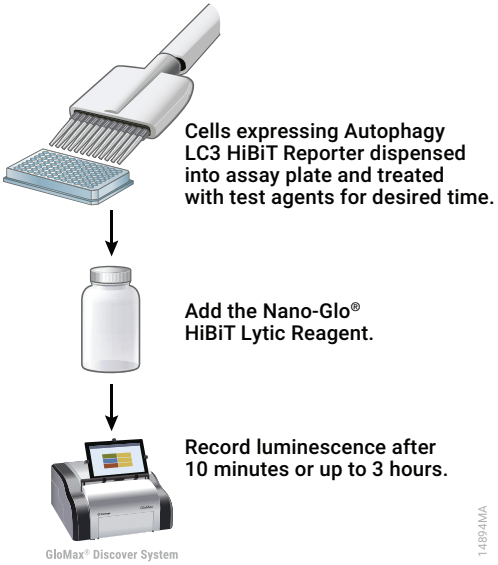


**Figure 3. Autophagy LC3 HiBiT Reporter Assay protocol.** Cells stably expressing the Autophagy LC3 HiBiT Reporter can be plated into microplate assay formats and treated with modulators of autophagic activity or test agents as desired. Subsequently, Nano-Glo® HiBiT Lytic Reagent is added and luminescence measured, with altered assay signal reflecting changes in autophagic flux and related reporter degradation.

## 2. Product Components and Storage Conditions

| PRODUCT | SIZE | CAT.# |
|---|---|---|
| **HEK293 Autophagy LC3 HiBiT Reporter Cell Line and Detection System** | **1 each** | **GA1040** |

Not for Medical Diagnostic Use. Includes:

- 1 vial HEK293 Autophagy LC3 HiBiT Reporter Cells, 2 × $10^6$ cells/ml (1.0ml per vial)
- 1 kit Nano-Glo® HiBiT Lytic Detection System, 10ml (available separately, Cat.# N3030):
  - 10ml Nano-Glo® HiBiT Lytic Buffer
  - 0.2ml Nano-Glo® HiBiT Lytic Substrate
  - 0.1ml LgBiT Protein

| PRODUCT | SIZE | CAT.# |
|---|---|---|
| **U2OS Autophagy LC3 HiBiT Reporter Cell Line and Detection System** | **1 each** | **GA1050** |

Not for Medical Diagnostic Use. Includes:

- 1 vial U2OS Autophagy LC3 HiBiT Reporter Cells, 2 ×$10^6$ cells/ml (1.0ml per vial)
- 1 kit Nano-Glo® HiBiT Lytic Detection System, 10ml (available separately, Cat.# N3030):
  - 10ml Nano-Glo® HiBiT Lytic Buffer
  - 0.2ml Nano-Glo® HiBiT Lytic Substrate
  - 0.1ml LgBiT Protein

| PRODUCT | SIZE | CAT.# |
|---|---|---|
| **Autophagy LC3 HiBiT Reporter Vector and Detection System** | **1 each** | **GA2550** |

Not for Medical Diagnostic Use. Includes:

- 1 vial Autophagy LC3 HiBiT Reporter Vector (20µg)
- 1 kit Nano-Glo® HiBiT Lytic Detection System, 10ml (available separately, Cat.# N3030):
  - 10ml Nano-Glo® HiBiT Lytic Buffer
  - 0.2ml Nano-Glo® HiBiT Lytic Substrate
  - 0.1ml LgBiT Protein

**Storage Conditions:** Upon arrival, immediately transfer cell vials to at or below –140°C (freezer or liquid nitrogen vapor phase) for long-term storage. Do not store cell vials submerged in liquid nitrogen. Do not store cell vials at –80°C as this will negatively impact cell viability and cell performance. Store the Nano-Glo® HiBiT Lytic Detection System at –20°C. Do not thaw Detection System components above 25°C. The Nano-Glo® HiBiT Lytic Buffer may be stored at 4°C for 1 year or at room temperature for 3 months and can be frozen and thawed at least ten times without any change in performance. The Nano-Glo® HiBiT Lytic Substrate and LgBiT Protein will not freeze at –20°C. Do not store LgBiT protein directly on dry ice. Protect Nano-Glo® HiBiT Lytic Substrate from prolonged exposure to light. The Autophagy LC3 HiBiT Reporter Vector should be stored at –30°C to –10°C.

**WARNING:** Do not use cryotubes in the liquid phase of liquid nitrogen. Improper use may trap liquefied nitrogen inside the vial and lead to pressure buildup, resulting in possible explosion or biohazard release. Use appropriate safety procedures when handling and disposing of the cryotubes.

Follow institutional guidelines for handling, including use of personal protective equipment (PPE), and waste disposal for biohazardous material. The HEK293 and U2OS Autophagy LC3 HiBiT Reporter Cells are considered Risk Group 1.

## 3. Before You Begin

Please read through this entire technical manual to become familiar with the components and assay procedures before beginning your experiments. Cell thawing, propagation and banking should be performed exactly as described in Section 4.B, 4.C and 4.D.

### 3.A. Instrumentation and Multiwell Plate Recommendations

The Autophagy LC3 HiBiT Reporter Assay Systems require a plate-reading instrument capable of measuring luminescence. This can be achieved by using a preprogrammed setting on the GloMax® Discover System. For optional multiplexing with the CellTox™ Green Cytotoxicity Assay, a multimodal plate reading instrument (e.g., GloMax® Discover) capable of measuring luminescence as well as green fluorescence ($485nm_{Ex}$/$525–530nm_{Em}$) is required. Alternatively, a separate plate reader for optional fluorescence measurements can be used.

All experiments should be conducted using sterile, white, tissue culture-treated 96- or 384-well plates to minimize luminescence and fluorescence cross talk between wells. White, clear-bottom plates are acceptable; however, solid white bottom plates will produce higher luminescent and fluorescent intensity values and less cross talk between assay wells.

### 3.B. Materials to Be Supplied by the User

- cell culture medium for HEK293 Autophagy LC3 HiBiT Reporter Cell Line:
  - 90% DMEM Medium (e.g., GIBCO®, Cat.# 11995)
  - 10% fetal bovine serum (e.g., Seradigm, Cat.# 1500-050)
  - 500µg/ml G418 (Geneticin; e.g., GIBCO®, Cat.# 10131)
- cell culture medium for U2OS Autophagy LC3 HiBiT Reporter Cell Line:
  - 90% McCoy's 5A Medium (e.g., GIBCO®, Cat.# 16600)
  - 10% fetal bovine serum (e.g., Seradigm, Cat.# 1500)
  - 250µg/ml G418 (Geneticin; e.g., GIBCO®, Cat.# 10131)
- DMSO (e.g., Corning, Cat.# 25-950-CQC)
- DPBS (e.g., GIBCO®, Cat.# 14190)
- 0.05% Trypsin (e.g., GIBCO®, Cat.# 25300) for use with HEK293 Autophagy LC3 HiBiT Reporter Cells
- 0.25% Trypsin (e.g., GIBCO®, Cat.# 25200) for use with U2OS Autophagy LC3 HiBiT Reporter Cells
- Trypan blue solution (e.g., Sigma, Cat.# T8154)
- sterile, white, tissue culture-treated 96-well plates (e.g., Costar®, Cat.# 3917 solid white bottom or Costar®, Cat.# 3903 white clear bottom) or 384-well plates (e.g., Corning, Cat.# 3570)
- plate reader(s) minimally capable of measuring luminescence (e.g., GloMax® Discover System, Cat.# GM3000) and, if performing optional multiplexing with CellTox™ Green Cytotoxicity Assay, green fluorescence ($485nm_{Ex}$/$525–530nm_{Em}$)
- reference autophagy inducer **Note:** For HEK293 and U2OS Autophagy LC3 HiBiT Reporter Cells, we recommend using PP242 (e.g., Selleckchem, Cat.# S2218) as the reference inducer.
- reference autophagy inhibitor **Note:** For HEK293 and U2OS Autophagy LC3 HiBiT Reporter Cells, we recommend using Baf A1 (e.g., Alfa Aesar, Cat.# J67193) as the reference inhibitor.
- **Optional control:** Parental cells not expressing autophagy reporter. **Note:** Typically, parental-cell controls are not necessary once a stable Autophagy LC3 HiBiT Reporter Cell Line has been established; background signal for no-cell control is roughly equivalent to parental-cell control.

## 4. Preparation of the HEK293 and U2OS Autophagy LC3 HiBiT Reporter Cells

### 4.A. Overview of the Autophagy LC3 HiBiT Reporter Cell Lines

HEK293 Autophagy LC3 HiBiT Reporter or U2OS Autophagy LC3 HiBiT Reporter Cell Lines are clonal cell lines stably expressing the Autophagy LC3 HiBiT Reporter at low-to-moderate levels under the control of a constitutive promoter. For these cell lines, both reporter expression and functional responsiveness have been shown to be stable for at least 40 passages. For stable expression of the Autophagy LC3 HiBiT Reporter in other cell types, we recommend using the Autophagy LC3 HiBiT Reporter Vector for transfection and selection of stable cell lines described in Section 10.A.

### 4.B. Cell Thawing and Initial Cell Culture

1. Prepare 40ml of initial cell culture medium by adding 4ml of FBS to 36ml of medium (DMEM for HEK293 Autophagy LC3 HiBiT Reporter Cells; McCoy's 5A for U2OS Autophagy LC3 HiBiT Reporter Cells) prewarmed to 37°C. This initial cell culture medium **(no G418)** will be used for culturing the cells immediately after thawing.
2. Transfer 9ml of prewarmed initial cell culture medium to a sterile 15ml conical tube.
3. Remove one vial of HEK293 or U2OS Autophagy LC3 HiBiT Reporter Cells from storage at –140°C (or liquid $N_2$ vapor phase) and thaw in a 37°C water bath with gentle agitation (do not invert) until just thawed (typically 3 minutes).
4. Transfer all the cells (approximately 1ml) to the 15ml conical tube containing 9ml of prewarmed initial cell culture medium.
5. Centrifuge at 156 × *g* for 10 minutes at room temperature.
6. Carefully aspirate the medium and resuspend the cell pellet in 5ml of prewarmed cell culture medium (no antibiotics).
7. Transfer the 5ml cell suspension to a tissue culture flask containing additional cell culture medium (T75 flask with 10ml cell culture medium for HEK293 Autophagy LC3 HiBiT Reporter Cells; T150 flask with 25ml cell culture medium for U2OS Autophagy LC3 HiBiT Reporter Cells) and place the flask horizontally in a 37°C, 5% $CO_2$ incubator.
8. Incubate the cells for approximately 48–72 hours before passaging.

   **Note:** Do not allow cells to become greater than 70–80% confluent prior to passaging.

### 4.C. Cell Maintenance and Propagation

For cell maintenance and propagation starting from the next cell passage, use cell culture medium containing antibiotic (500µg/ml G418 for HEK293 Autophagy LC3 HiBiT Reporter Cells; 250µg/ml G418 for U2OS Autophagy LC3 HiBiT Reporter Cells). Once established in steady culture, cell viability is typically >95%. For best results, do not allow cells to become greater than 70–80% confluent prior to passaging. Seed cells at the recommended cell seeding densities (cells/cm$^2$) listed in the following table when passaging every 2, 3 or 4 days:

| | Every 2 days | Every 3 days | Every 4 days |
|---|---|---|---|
| **HEK293 Autophagy LC3 HiBiT Reporter Cells** | $4.0–4.6 \times 10^4$ | $1.7–2.1 \times 10^4$ | $0.8–1.0 \times 10^4$ |
| **U2OS Autophagy LC3 HiBiT Reporter Cells** | $1.5–1.7 \times 10^4$ | $0.9–1.1 \times 10^4$ | $0.4–0.5 \times 10^4$ |

1. On the day of cell passage, aspirate the cell culture medium and wash the cells with DPBS (10ml per T75 flask; 20ml per T150 flask).
2. Add trypsin (0.05% for HEK293 Autophagy LC3 HiBiT Reporter Cells; 0.25% for U2OS Autophagy LC3 HiBiT Reporter Cells) to each flask (2ml to each T75 flask; 4ml to each T150 flask) and place in a 37°C, 5% $CO_2$ incubator for 3–5 minutes or until the cells round up and detach from the bottom of the flask.
3. Neutralize the trypsin by adding cell culture medium to each flask (8ml to each T75 flask; 16ml to each T150 flask). Transfer the cell suspension to a sterile 50ml conical centrifuge tube.
4. Centrifuge at 156 × *g* for 10 minutes at room temperature.
5. Carefully aspirate the medium and resuspend the cell pellet in 5–10ml of prewarmed cell culture medium by gentle mixing with a pipette to create a homogeneous cell suspension.
6. Count the cells and determine percent viability by Trypan blue staining. Calculate the cell numbers needed for replating based on desired cell seeding density per area and destination flask size and number.
7. Add an appropriate amount of cell culture medium (with G418) to destination flasks to achieve the desired total culture volume per flask (be sure to take into account the volume of cell suspension to be added). We suggest that you maintain a consistent ratio of culture volume to culture surface area (e.g., 15ml volume per T75 flask or 30ml volume per T150 flask).
8. Transfer the appropriate volume of cell suspension to new flasks to achieve the desired cell seeding density and total culture medium volume.
9. Place the flasks in a 37°C, 5% $CO_2$ incubator. Incubate the cells for approximately 48–96 hours (depending on plating density) before passaging again.

### 4.D. Cell Freezing and Banking

1. On the day of cell freezing, make new cell freezing medium and keep on ice.

   **Note:** Freezing medium is initial cell culture medium **(no G418)** plus 10% DMSO.

2. Aspirate the cell culture medium and wash the cells with DPBS (10ml per T75 flask; 20ml per T150 flask).

3. Add trypsin (0.05% for HEK293 Autophagy LC3 HiBiT Reporter Cells; 0.25% for U2OS Autophagy LC3 HiBiT Reporter Cells) to each flask (2ml to each T75 flask; 4ml to each T150 flask) and place in a 37°C, 5% $CO_2$ incubator for 3–5 minutes or until the cells round up and detach from the bottom of the flask.

4. Neutralize the trypsin by adding cell culture medium to each flask (8ml to each T75 flask; 16ml to each T150 flask). Gently mix the cells with a pipette to create a homogeneous cell suspension, then transfer the cell suspension to a sterile 50ml conical centrifuge tube.

5. Count the cells and determine percent viability by Trypan blue staining.

6. Centrifuge at 156 × *g* for 10 minutes at room temperature.

7. Gently resuspend the cell pellet in ice-cold cell freezing medium to a final cell density of $2\times10^6$–$1\times10^7$ viable cells/ml. Combine the cell suspensions in a single tube and dispense 0.5–1ml aliquots into 1.2ml or 2.0ml cryovials.

8. Freeze the cells using a controlled-rate freezer (preferred) or with a freezing container or Styrofoam® rack in a –80°C freezer overnight. Transfer the vials to storage at –140°C or below (e.g., liquid $N_2$ vapor phase) for long-term storage. Do not store cells long-term at –80°C.

## 5. Detection of Autophagy Inducers and Inhibitors using Autophagy LC3 HiBiT Reporter Cells

**Autophagy Assay modes of use:**

- General detection of autophagy inducers and inhibitors
- Optimized detection of autophagy inhibitors
- Confirmation of mechanism of action (see Section 6)

This section provides basic protocol guidance for quantitative assessment of changes in autophagic flux in response to test compounds, known autophagy modulators and various cell treatment conditions. If you are testing specifically for autophagy inhibition, you may want to induce autophagic activity, thereby lowering the luminescent signal to allow more capacity for signal increase by an autophagy inhibitor. This may be done by cotreatment with a strong autophagy inducer (reference inducer), such as PP242. In this case, please follow the alternative instructions for optimized detection of autophagy inhibitors provided in this section. To further confirm whether the test compound is a true autophagy inducer or inhibitor, we recommend that you perform a secondary screen as described in Section 6.

Following treatment of cells stably expressing the Autophagy LC3 HiBiT Reporter with reference and test compounds or other treatment conditions, the Nano-Glo® HiBiT Lytic Detection System can be used to quantify the relative amount of Autophagy LC3 HiBiT Reporter present in cells at the time of reagent application and cell lysis. Changes in luminescent signal represent changes in autophagic flux. Induction of autophagy results in decreased reporter levels and decreased luminescent signal, while inhibition of autophagy results in increased reporter levels and increased luminescent signal.

The Nano-Glo® HiBiT Lytic Detection System measures the total amount of Autophagy LC3 HiBiT Reporter in a sample using a detergent-containing buffer to lyse cell membranes. The assay is compatible with most commonly used cell culture media containing 0–10% serum and has been tested with DMEM, RPMI 1640, McCoy's 5A, MEMα, Opti-MEM® I, F-12 and $CO_2$-independent media. While the reagent has been designed to give a signal half-life of greater than 3 hours at 22°C, different combinations of medium and serum may affect the background, signal or signal decay rate. Additional information can be found in the *Nano-Glo® HiBiT Lytic Detection System Technical Manual* #TM516.

### 5.A. Plating of Autophagy LC3 HiBiT Reporter Cells for Assay

For plating into multiwell microplates for treatment and assay, use Autophagy LC3 HiBiT Reporter Cells passaged or re-fed with cell culture medium (either with or without G418 antibiotic) within 1–3 days prior to plating. Cells should be maintained at less than 70–80% confluency. For best results, ensure that cell culture medium has not passed expiration date and has not been subject to repeated warming periods prior to use. Cell numbers and volumes provided below are for 96-well assay plates; use proportional cell numbers and volumes for 384- and 1536-well formats.

1. Trypsinize, pellet, resuspend in cell culture medium and count cells expressing Autophagy LC3 HiBiT Reporter as described in Section 4.C.
2. Generate a volume of diluted cells necessary for the desired number of assay wells. Allow for nonrecoverable void volumes in tubes and reagent reservoirs. Typically, 7ml of cell suspension supports plating 60 wells of a 96-well plate. Dilute cells with additional cell culture medium to the indicated concentrations:
   a. 250,000 cells/ml for HEK293 Autophagy LC3 HiBiT Reporter Cell Line
   b. 100,000 cells/ml for U2OS Autophagy LC3 HiBiT Reporter Cell Line
3. Dispense 80µl of cell dilution into each of the inner-60 wells of sterile, white, tissue culture-treated 96-well plates (e.g., Costar® Cat.# 3917 solid white bottom or Costar® Cat.# 3903 clear bottom). For outer wells, dispense cell culture medium without cells.
   a. 20,000 cells/well HEK293 Autophagy LC3 HiBiT Reporter Cells
   b. 8,000 cells/well U2OS Autophagy LC3 HiBiT Reporter Cells

   **Notes:**
   a. Use an opaque, white tissue-culture plate to minimize cross talk between wells and absorption of the emitted light. Ensure that the plates used are compatible with the plate-reader.
   b. Confluent cells can exhibit increased basal autophagic activity and modified compound responsiveness. Using the prescribed cell densities at plating will maintain subconfluency throughout a typical 5–24-hour compound treatment initiated the day after cell plating. Plate fewer cells for longer treatments.
   c. Maintain cell dispersion during dispensing by using cells immediately after dilution or by mixing the cell dilution during large-scale plating when cell settling may occur. Use of an electronic multichannel pipette and a sterile reagent reservoir to deliver cells can reduce pipetting time and replicate error.
4. Place plates with cells in a 37°C, 5% $CO_2$ incubator and allow cells to attach overnight prior to treatment.

   **Note:** For suspension cells, plating overnight before treatment may not be necessary.

### 5.B. Protocol for Autophagy LC3 HiBiT Reporter Cell Treatment

1. Prepare working solutions in prewarmed (22–37°C) cell culture medium. For general detection of autophagy inducers and inhibitors, see Table 1. For optimized detection of autophagy inhibitors, see Table 2. Scale volumes as necessary.

   **Notes:**

   For HEK293 and U2OS Autophagy LC3 HiBiT Reporter Cells, we recommend using a final concentration of 2µM PP242 as the reference inducer and 50nM Baf A1 as the reference inhibitor. Be aware that rapamycin is only a moderate autophagy inducer in most cell types.

   Working solutions can include either single concentrations or serial dilutions of test or reference compounds. A known strong autophagy inducer (reference inducer) and/or strong autophagy inhibitor (reference inhibitor), depending on assay objectives, should be included in every assay as positive control(s).

**Table 1. 5X Working Solutions for General Detection of Autophagy Inducers and Inhibitors.**

| | Vehicle | 1,000X Test Compound | 1,000X Reference Inducer | 1,000X Reference Inhibitor | Cell Culture Medium | Total Volume |
|---|---|---|---|---|---|---|
| **Vehicle Dilution** | 2µl | | | | 398µl | 400µl |
| **5X Test Compound** | | 2µl | | | 398µl | 400µl |
| **5X Reference Inducer** | | | 2µl | | 398µl | 400µl |
| **5X Reference Inhibitor** | | | | 2µl | 398µl | 400µl |

**Table 2. 10X Working Solutions for Optimized Detection of Autophagy Inhibitors**

| | Vehicle | 1,000X Test Compound | 1,000X Reference Inducer | 1,000X Reference Inhibitor | Cell Culture Medium | Total Volume |
|---|---|---|---|---|---|---|
| **Vehicle Dilution** | 5µl | | | | 495µl | 500µl |
| **10X Test Compound** | | 2µl | | | 198µl | 200µl |
| **10X Reference Inducer** | | | 10µl | | 990µl | 1000µl |
| **10X Reference Inhibitor** | | | | 2µl | 198µl | 200µl |

2. Treat replicate assay wells containing Autophagy LC3 HiBiT Reporter Cells in 80µl cell culture medium with 20µl treatment volumes as specified in the following tables. For general detection of autophagy inducers and inhibitors, see Table 3. For optimized detection of autophagy inhibitors, see Table 4.

   **Note:** To ensure complete mixing and reduce well-to-well variability, gently shake the plate (e.g., 10 seconds at 150–200rpm) immediately following completion of treatment additions.

**Table 3. Treatment Volumes for General Detection of Autophagy Inducers and Inhibitors.**

| | Cell Volume | Vehicle Dilution | 5X Test Compound | 5X Reference Inducer | 5X Reference Inhibitor | Total Well Volume |
|---|---|---|---|---|---|---|
| **Vehicle** | 80µl cells | 20µl | | | | 100µl |
| **Test Compound** | 80µl cells | | 20µl | | | 100µl |
| **Reference Inducer** | 80µl cells | | | 20µl | | 100µl |
| **Reference Inhibitor** | 80µl cells | | | | 20µl | 100µl |
| **No-Cell Background Control** | 80µl culture medium only | 20µl | | | | 100µl |

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | | | | | | | | |
| B | | | | | PP242 treatment concentrations (final) | | | | | | | |
| C | No-cell control | Vehicle | 19nM | 39nM | 78nM | 156nM | 312nM | 625nM | 1250nM | 2500nM | 5000nM | |
| D | | | | | | | | | | | | |
| E | | | | | Test compound treatment concentrations (final) | | | | | | | |
| F | | Ref. | 0.004X | 0.008X | 0.016X | 0.031X | 0.062X | 0.125X | 0.25X | 0.5X | 1X | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

**Figure 4. Example 96-well plate layout for dose-response screening of autophagy inducers and inhibitors.** Dose-dependent treatment of Autophagy LC3 HiBiT Reporter Cell Lines with reference inducer, PP242, are shown in Figures 5.A and 5.B. You can perform similar control experiments to determine whether the Autophagy Assay is working as expected.

## 5.B. Protocol for Autophagy LC3 HiBiT Reporter Cell Treatment (continued)

**Table 4. Treatment Volumes for Optimized Detection of Autophagy Inhibitors.**

| | Cell Volume | Vehicle Dilution | 10X Test Compound | 10X Reference Inducer | 10X Reference Inhibitor | Total Well Volume |
|---|---|---|---|---|---|---|
| **Vehicle** | 80µl | 20µl | | | | 100µl |
| **Test Compound** | 80µl | 10µl | 10µl | | | 100µl |
| **Reference Inducer** | 80µl | 10µl | | 10µl | | 100µl |
| **Reference Inhibitor** | 80µl | 10µl | | | 10µl | 100µl |
| **Reference Inducer + Test Compound** | 80µl | | 10µl | 10µl | | 100µl |
| **Reference Inducer + Reference Inhibitor** | 80µl | | | 10µl | 10µl | 100µl |
| **No-Cell Background Control** | 80µl culture medium only | 20µl | | | | 100µl |

**Note:** For combination treatments, test compounds (or reference inhibitor) should be added together with or just before the reference inducer to avoid significant reporter degradation prior to test compound addition. However, if a slow mechanism of action is expected for test compounds, you can pretreat cells with test compounds (or reference inhibitor) for a longer period of time before adding the reference inducer.

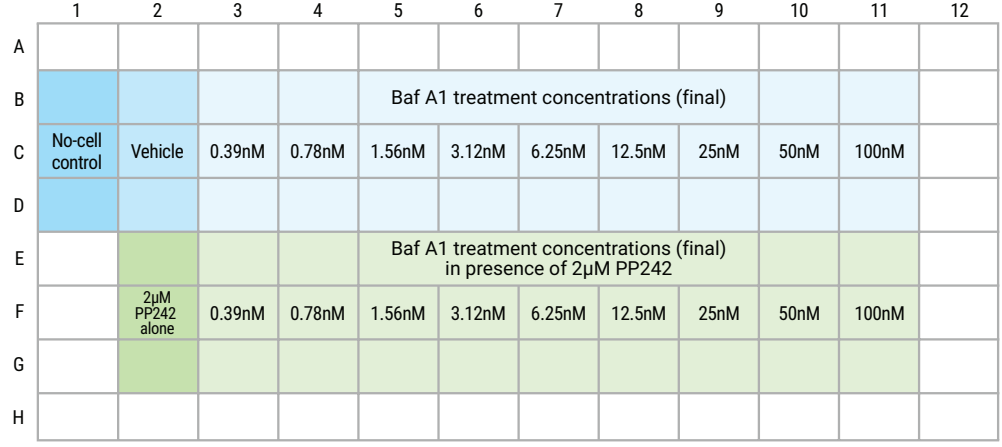


**Figure 5. Example 96-well plate layout for dose-response screening of autophagy inhibitors.** Dose-dependent treatments of Autophagy LC3 HiBiT Reporter Cell Lines with reference inhibitor, Baf A1, without or with a fixed concentration of reference inducer, PP242, are shown in Figures 5.C and 5.D. You can perform a similar control experiment to determine whether the Autophagy Assay is working as expected.

3. Return assay plate(s) to a 37°C, 5% $CO_2$ incubator for the desired treatment time.

   **Note:** A treatment time of 5–8 hours with 2µM PP242 is generally sufficient for substantial degradation of the autophagy reporter in HEK293 and U2OS Autophagy LC3 HiBiT Reporter Cells. Overnight (or longer) treatment times can be used to generate a greater assay response or when test compounds have a slower mechanism of action or less efficacy.

4. At the end of the desired treatment time, determine reporter levels as described in Sections 5.C and 5.D.

### 5.C. Preparing the Nano-Glo® HiBiT Lytic Reagent

Calculate the amount of Nano-Glo® HiBiT Lytic Reagent needed to perform the desired experiments. This volume is usually equal to the total amount of medium in wells plus any extra required for dispensing. Dilute the LgBiT Protein 1:100 and the Nano-Glo® HiBiT Lytic Substrate 1:50 into an appropriate volume of room-temperature Nano-Glo® HiBiT Lytic Buffer in a new tube. Mix by inversion.

For example, if 10ml of Nano-Glo® HiBiT Lytic Reagent is needed, transfer 10ml of Nano-Glo® HiBiT Lytic Buffer to a 50ml centrifuge tube and add 100µl of LgBiT Protein and 200µl of Nano-Glo® HiBiT Lytic Substrate.

**Notes:**

a. If the Nano-Glo® HiBiT Lytic Substrate or LgBiT Protein has collected in the cap or on the sides of the tube, briefly centrifuge the tubes.

b. The LgBiT Protein stock contains glycerol, which prevents it from freezing at –20°C. The solution viscosity may make accurate pipetting difficult. Pipet slowly and avoid excess solution clinging to the outside of the pipette tip. Use a positive displacement pipette, if possible.

c. Because luciferase activity is temperature-dependent, the temperature of the samples and reagents should be kept constant while measuring luminescence. We recommend equilibrating reagents to room temperature. For ease of use, store the Nano-Glo® HiBiT Lytic Buffer at room temperature at least a day before experiments.

d. We recommend preparing the Nano-Glo® HiBiT Lytic Reagent fresh for each use. Once reconstituted, the reagent will lose about 10% activity over 8 hours and about 30% activity over 24 hours at room temperature. Unused reconstituted reagent may be stored at –80°C, –20°C or 4°C for later use, although there will be some loss of performance relative to freshly prepared reagent. At 4°C, the reconstituted reagent should lose less than 10% activity over 24 hours.

### 5.D. Detecting Autophagy LC3 HiBiT Reporter in Mammalian Cell Lines

1. Remove plates containing mammalian cells expressing Autophagy LC3 HiBiT Reporter from the incubator and equilibrate to room temperature for 10–15 minutes.
2. Add a volume of Nano-Glo® HiBiT Lytic Reagent equal to the volume present in each well and mix. For optimal results, mix the samples by placing the plate on an orbital shaker (300–400rpm) for 2–10 minutes. For HEK293 and U2OS Autophagy LC3 HiBiT Reporter Cells, 2 minutes of orbital shaking is sufficient to recover reporter and reduce variability between replicates.
3. Wait at least 10 minutes total, including shaking time, for equilibration of LgBiT and HiBiT binding in the lysate. Measure luminescence using settings specific to your instrument. When using 96-well plates on the GloMax® instruments, we recommend integration times of 0.5–2 seconds. The luminescence intensity will usually decay with a signal half-life of greater than 3 hours.

   **Note:** For 3D cell culture models, longer shake and total incubation times may be required for optimal recovery and detection of Autophagy LC3 HiBiT Reporter. Use untreated control microtissues to determine the optimal protocol for your particular 3D model. Increase shake or total incubation times until maximal signal and acceptable variability are obtained.
4. Refer to Section 7, Table 8, for guidance on general interpretation of assay results.

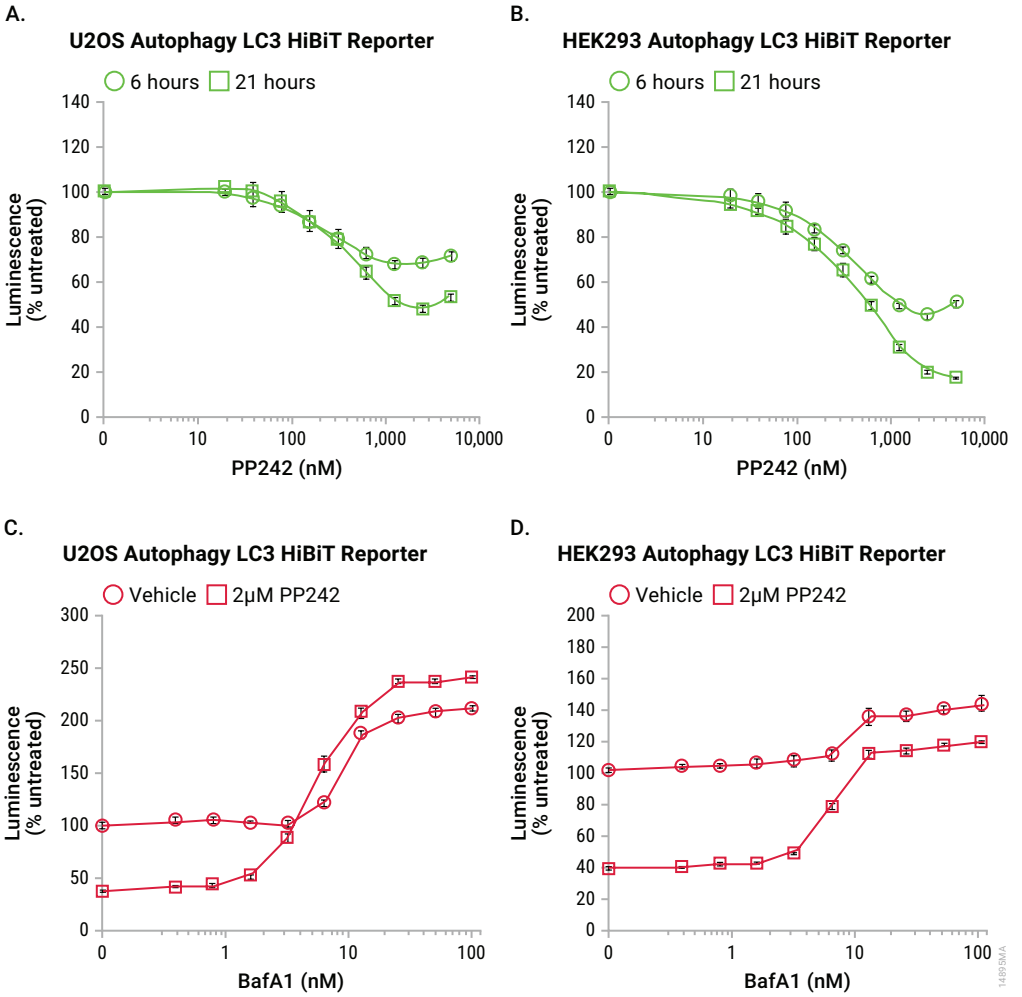


**Figure 6. Autophagy inducer and inhibitor dose-dependent evaluation.** U2OS (Panels A and C) and HEK293 (Panels B and D) Autophagy LC3 HiBiT Reporter Cells were plated at 8,000 and 20,000 cells per well, respectively. After cells were allowed to attach overnight, treatment was performed in triplicate. **Panels A and B.** Cells were treated with increasing concentrations of the reference inducer, PP242, for the indicated times. **Panels C and D.** Cells were treated with increasing concentrations of a reference inhibitor, Baf A1, without or with 2µM PP242 for 21 hours (Panel C) or 6 hours (Panel D). Luminescent signal was normalized to untreated control for each study. Treatment with PP242 significantly increased autophagy and decreased luminescent signal to allow more capacity for signal increase by cotreatment with the autophagy inhibitor, Baf A1.

### 6. Confirmation of Mechanism of Action (MOA) for Potential Autophagy Inducers and Inhibitors

Cotreatment of cells with a suspected modulator of autophagy with a strong autophagy inhibitor (reference inhibitor) can be used to confirm mechanism of action (MOA) for the test compound. We recommend using 50nM Baf A1 as the reference inhibitor, as it strongly inhibits autophagy in most cell types. If the assay signal resulting from cotreatment with test compound plus reference inhibitor is similar to reference inhibitor treatment alone, you can conclude that the test compound modulates autophagy. You can also multiplex with the CellTox™ Green Cytotoxicity Assay to assess cell health in real-time during treatment. The CellTox™ Green Dye binds DNA upon loss of cell membrane integrity and produces a fluorescent signal proportional to cytotoxicity.

**Note:** Adherent cells must be plated the day prior to treatment. For important guidance on plating of Autophagy LC3 HiBiT Reporter Cells into appropriate multiwell plates for subsequent treatment and assay, see Section 5.A.

**Protocol for MOA confirmation using Autophagy LC3 HiBiT Reporter Cells**

1. Prepare 10X working solutions in prewarmed (22–37°C) cell culture medium according to Table 6. Scale volumes as necessary.

   **Table 6. 10X Working Solutions for MOA Confirmation.**

| | Vehicle | 1,000X Test Compound | 1,000X Reference Inducer | 1,000X Reference Inhibitor | Cell Culture Medium | Total Volume |
|---|---|---|---|---|---|---|
| **Vehicle Dilution** | 5µl | | | | 495µl | 500µl |
| **10X Test Compound** | | 2µl | | | 198µl | 200µl |
| **10X Reference Inducer** | | | 2µl | | 198µl | 200µl |
| **10X Reference Inhibitor** | | | | 10µl | 990µl | 1000µl |

   **Note:** 10X working solutions can include either single concentrations or multiple dilutions of test compounds. However, a strong autophagy inducer (reference inducer) known to be effective in the particular target cells (e.g., 2µM PP242 for HEK293 and U2OS Autophagy LC3 HiBiT Reporter Cells) should be included in every assay as a positive control. Be aware that rapamycin is only a moderate autophagy inducer in most cell types.

2. **Optional:** Multiplexing with CellTox™ Green Cytotoxicity Reagent: To enable same-well monitoring of cell death due to compound treatments, 1.25X CellTox™ Green Cytotoxicity Reagent can be added in cell culture medium at the time of cell dispension into assay plates or added later. Alternatively, all 10X working solutions can be prepared in cell culture medium containing 10X CellTox™ Green Cytotoxicity Reagent.

   **Note:** For further information and protocol guidance on the use of CellTox™ Green Cytotoxicity Assay for real-time and endpoint determination of cytotoxicity, please refer to the *CellTox™ Green Cytotoxicity Assay Technical Manual* #TM375.

3. Treat replicate assay wells containing Autophagy LC3 HiBiT Reporter Cells in 80µl cell culture medium with 20µl total treatment volumes according to Table 7.

   **Note:** To ensure complete mixture and reduce well-to-well variability, gently shake the plate (e.g., 10 seconds at 150–200rpm) immediately following completion of treatment additions.

**Table 7. Treatment Volumes Added Per Well for Test Compound MOA Confirmation.**

| | Cell Volume | Vehicle Dilution | 10X Test Compound | 10X Reference Inducer | 10X Reference Inhibitor | Total Well Volume |
|---|---|---|---|---|---|---|
| **Vehicle** | 80µl | 20µl | | | | 100µl |
| **Test Compound** | 80µl | 10µl | 10µl | | | 100µl |
| **Reference Inducer** | 80µl | 10µl | | 10µl | | 100µl |
| **Reference Inhibitor** | 80µl | 10µl | | | 10µl | 100µl |
| **Reference Inhibitor + Test Compound** | 80µl | | 10µl | | 10µl | 100µl |
| **Reference Inhibitor + Reference Inducer** | 80µl | | | 10µl | 10µl | 100µl |
| **No-Cell Background Controls** | 80µl culture medium only | 20µl | | | | 100µl |
| **(Optional) Reference Inducer +Test Compound** | 80µl | | 10µl | 10µl | | 100µl |

**Note:** The reference inhibitor should be added with or just before test compounds (or reference inducer) in cotreatment wells to avoid significant reporter degradation prior to reference inhibitor addition. However, if the MOA of the reference inhibitor is expected to be slow, you can pretreat cells with the reference inhibitor for a longer period of time before test compound addition. See example results of MOA confirmation in Figure 6.

4. Return assay plate(s) to a 37°C, 5% $CO_2$ incubator for the desired treatment time.

   **Note:** 5–8 hours of treatment with 2µM PP242 is generally sufficient for substantial autophagic degradation of the autophagy reporter in HEK293 and U2OS Autophagy LC3 HiBiT Reporter Cells. Overnight (or longer) treatment times can be used to generate a greater assay response or when test compounds have a slower mechanism of action.

5. **Optional:** If CellTox™ Green Cytotoxicity Reagent was previously added, measure fluorescence ($485nm_{Ex}$/$525–530nm_{Em}$). Repeat measurements can be made in real-time, although the assay plate should be quickly returned to the 37°C, 5% $CO_2$ incubator after each intermediate reading. It is critical that these cytotoxicity readings are taken prior to the addition of the Nano-Glo® HiBiT Lytic Reagent.

6. At the end of the desired treatment time, determine autophagy reporter levels by adding 100µl Nano-Glo® HiBiT Lytic Reagent, shaking the assay plate (>2 minutes, 300–400rpm), then reading luminescence at >10 minutes as described in Sections 5.C and 5.D.

   **Note:** In rare cases, a test compound may interfere with assay chemistry and influence luminescence readings. To address this possibility, include a control in which the test compound is added to Autophagy LC3 HiBiT Reporter Cells immediately before or after addition of the Nano-Glo® HiBiT Lytic Reagent. If the luminescent signal in this control is altered relative to corresponding vehicle control, you may conclude that the compound interferes with assay chemistry.

7. Refer to Section 7, Table 9, for guidance on interpretation of MOA confirmation results.

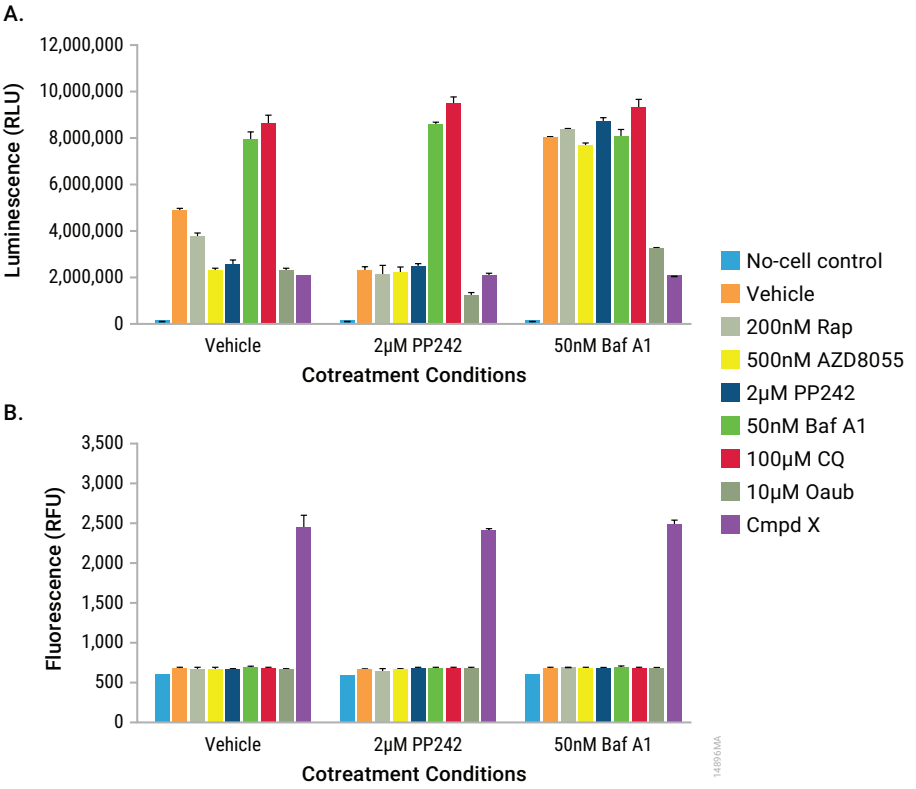


**Figure 7. Confirmation of MOA by Baf A1 blockade and cytotoxicity assessment.** U2OS Autophagy LC3 HiBiT Reporter Cells were plated into all-white, 96-well plates at 8,000 cells per well in growth medium containing 1.25X CellTox™ Green Dye. Cells were allowed to attach overnight and then treated in triplicate for 21 hours with vehicle or the indicated test compounds (Rapamycin, AZD8055, PP242, Baf A1, Chloroquine, Ouabain and Compound X) either alone or in cotreatments with the reference inducer (2µM PP242) or reference inhibitor (50nM Baf A1). **Panel A.** Nano-Glo® HiBiT Lytic Reagent was added and luminescence measured after 10 minutes. Average RLU was determined from triplicate wells. **Panel B.** Prior to lytic reagent addition, cytotoxicity was assessed by measuring fluorescence ($485nm_{Ex}$/$525–530nm_{Em}$) of triplicate assay wells. Only Compound X caused apparent cell death during the 21-hour treatment time.

## 7. Interpretation of Results

To interpret results, average the luminescent RLU values corresponding to autophagy reporter levels from replicate treated assay wells. If multiplexing was done with the CellTox™ Green Cytotoxicity Assay, average the fluorescent RFU values obtained from replicate treated assay wells. For primary screens, graph results and interpret according to Table 8. Figure 6 and Table 9 show an example of how to interpret data in a secondary screen to confirm MOA of various test compounds.

**Table 8. General Interpretation of Assay Results.**

| Change in luminescent signal (RLU) of test compound treatment compared to vehicle only | Indicated effect of test compound on autophagy |
|---|---|
| RLU ↓ | Potential inducer |
| RLU ↑ | Potential inhibitor |
| No change | No effect |

**Table 9. Interpretation of MOA Confirmation Results Shown in Figure 6 Example.**

| | RLU change compared to vehicle only | Effect on autophagy | Is RLU of compound + Baf A1 similar to Baf A1 alone? | (Optional) Is there significant cell death? | Confirmed MOA of test compound on autophagy |
|---|---|---|---|---|---|
| **Rapamycin** | ↓ | Potential inducer | Yes | No | Confirmed inducer |
| **AZD8055** | ↓ | Potential inducer | Yes | No | Confirmed inducer |
| **PP242** | ↓ | Potential inducer | Yes | No | Confirmed inducer |
| **Baf A1** | ↑ | Potential inhibitor | Yes | No | Confirmed inhibitor |
| **Chloroquine** | ↑ | Potential inhibitor | Yes | No | Confirmed inhibitor |
| **Ouabain** | ↓ | Potential inducer | No | No | Nonspecific |
| **Compound X** | ↓ | Potential inducer | No | Yes | Nonspecific/cytotoxic |

**Note:** If a test compound used alone or in cotreatment with a reference inhibitor results in a significant increase in CellTox™ Green Cytotoxicity Assay fluorescence readings, the test compound has likely caused cell death during treatment. To distinguish the test compound effect on autophagy from general cytotoxicity, repeat the test with shorter treatment times or lower compound concentrations.

## 8. References


1. Mizushima, N. *et al.* (2010) Methods in mammalian autophagy research. *Cell* **140**, 313–26.
2. Dixon, A.S. *et al.* (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol.* **11**, 400–8.


## 9. Related Products

### Cell Health Assays

| Product | Size | Cat.# |
|---|---|---|
| CellTox™ Green Express Cytotoxicity Assay* | 200µl | G8731 |
| CellTox™ Green Cytotoxicity Assay | 10ml | G8741 |
| CellTiter-Glo® 2.0 Assay | 10ml | G9241 |
| CellTiter-Glo® 3D Cell Viability Assay | 10ml | G9681 |
| RealTime-Glo™ MT Cell Viability Assay | 100 reactions | G9711 |

*1,000X solution for direct addition to cell culture prior to plating or with treatment prior to dosing.

### Apoptosis Assays

| Product | Size | Cat.# |
|---|---|---|
| Caspase-Glo® 3/7 Assay | 10ml | G8091 |
| RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay | 100 assays | JA1011 |

### Multimode Plate Readers

| Product | Size | Cat.# |
|---|---|---|
| GloMax® Discover System | 1 each | GM3000 |
| GloMax® Explorer System | 1 each | GM5000 |

### Transfection Reagents

| Product | Size | Cat.# |
|---|---|---|
| FuGENE® HD Transfection Reagent | 1ml | E2311 |
| ViaFect™ Transfection Reagent | 0.75ml | E4981 |

**Plasmid Purification**

| Product | Size | Cat.# |
|---|---|---|
| PureYield™ Plasmid Miniprep System | 100 preps | A1223 |

## 10. Appendix

### 10.A. Establishing Autophagy LC3 HiBiT Reporter Cell Lines in Additional Cell Types

The Autophagy LC3 HiBiT Reporter Vector enables investigators to study autophagy in a range of cell types. This vector enables stable low-to-moderate expression of Autophagy LC3 HiBiT Reporter under the control of the constitutive HSV-TK promoter in mammalian cells. The vector also includes a Neo-Kan Resistance gene for G418 antibiotic resistance. Clonal cell lines stably expressing Autophagy LC3 HiBiT Reporter can be generated using standard transfection and clonal line selection approaches that are suitable for the cell type of interest. Mixed stable populations, although generally not considered optimal practice, may be suitable for some research purposes. Because transient transfection protocols may perturb autophagy, use of the vector in transient transfection assays to study autophagy may produce suboptimal results and is not encouraged.

If desired for individual lab use and not for further distribution, the Autophagy LC3 HiBiT Reporter Vector can also be used to transform bacteria for subsequent plasmid amplification and DNA purification. However, be aware that certain vectors may be unstable in large-scale plasmid amplifications. To increase stability of the Autophagy LC3 HiBiT Reporter Vector during scale-up production for subsequent mammalian cell transfections, we recommend use of *E. coli* specifically designed for the propagation of unstable plasmid DNA sequences. Plasmid DNA purification should be performed in a manner consistent with the production of transfection quality plasmid material to ensure optimal transfection in mammalian cells.

### 10.B. Special Considerations for Nutrient Deprivation Studies

In nutrient deprivation (starvation) studies, phenol red-free buffers such as EBSS or HBSS are frequently utilized to generate a nutrient-deprived state and induce autophagic flux. However, phenol red dye can absorb light and partially reduce measured luminescence. This effect is inconsequential for most Autophagy LC3 HiBiT Reporter assays; however, results will be confounded if phenol red dye is present in control cell culture medium but not in nutrient-deficient buffer. Therefore, when performing nutrient-deprivation studies, use phenol red-free cell culture medium for the control (e.g., Opti-MEM® with L-Glutamine; without phenol red). Alternatively, ensure that phenol red concentrations are the same in all assay wells, including those with nutrient-deficient buffer.

The presence or absence of FBS may also impact assay chemistry and luminescence. Typical nutrient-deficient buffers (EBSS, HBSS) do not contain serum (FBS), while cell culture media frequently contain 10% FBS. For optimal assay performance when studying nutrient deprivation effects, consider substituting dialyzed FBS for standard FBS in medium and add the same concentration of dialyzed FBS to the nutrient-deficient buffer used. Equivalent use of dialyzed FBS in both cell culture medium and nutrient-deficient buffer eliminates potential influences of FBS variation. This will also avoid introducing small molecule nutrients into the nutrient-deficient buffer which would occur with standard FBS addition.

## 11. Summary of Changes

The following change was made to the 9/25 revision of this document:

1. Removed expired patent statement.

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[c]U.S. Pat.No. 9,797,890 and other patents pending.

[d]HEK293 cells were obtained under license from Advec, Inc.



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