molecular probes® by life technologies™

Premo™ Autophagy Tb/GFP TR-FRET LC3B Expression Kit

Catalog no. A14070 or A14071 **Shipping:** 4°C **Storage:** 4°C

Protocol part no. MAN0005547 **Rev. date:** 18 October 2011

Table of Contents

Kit Contents and Handling

Component	Part no.	Amount	Storage	Handling
LC3B-GFP BacMam 2.0	A14295 A14300	1 mL 2 × 3 mL	4°C	• DO NOT FREEZE • Use sterile technique • Protect from light
Tb-anti-LC3B Antibody	A14072 A14299	6 µg 36 µg	4°C	Protect from light
LanthaScreen® 6X Cellular Assay Lysis Buffer	A12891 A14298	6 mL 50 mL	4°C	On day of assay, add protease inhibitor* and antibody
Chloroquine	A14301	1 mL	4°C	Protect from light
Instrument Control Terbium TR-FRET	A14138	1 kit	4°C	Protect from light

*See **Materials Required but Not Provided** on page 4.

Product Use

For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

Overview

Autophagy is an essential cell health maintenance process used to recycle no longer needed or damaged protein complexes and organelles. During autophagocytosis, cytoplasmic cargo (e.g., protein complexes, organelles) is engulfed in membranes which fuse to lysosomes that digest the cargo. Autophagy marker protein LC3B normally resides in the cytosol (LC3B-I) but upon induction of autophagy becomes lipidated and recruited to autophagosomal membranes (LC3B-II). Accordingly, LC3B-II levels can serve as a measure of autophagy activity. Lysosomal inhibitor chloroquine can be used to block autophagosome turnover leading to accumulation of LC3B-II, rendering it a useful tool compound when assessing the autophagy activities of cells following various treatments. The autophagy activities of cells expressing GFP-tagged LC3B can be measured using a TR-FRET immunoassay approach.

The **Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay** measures autophagy in cells expressing green fluorescent protein (GFP)-tagged LC3B using a Tb-based TR-FRET immunoassay approach (Figure 1). The assay step involves a single addition of cell lysis buffer containing detection antibody and is read using rapid, highly quantitative fluorescence plate readers in 96- or 384-well plate format. The **Antibody Kit** provides detection antibody for performing the assay with a cell line already expressing GFP-LC3B. The **Expression Kit** includes detection antibody and also allows for expressing N-terminal GFP-tagged human LC3B in many different cell-types using BacMam Technology.

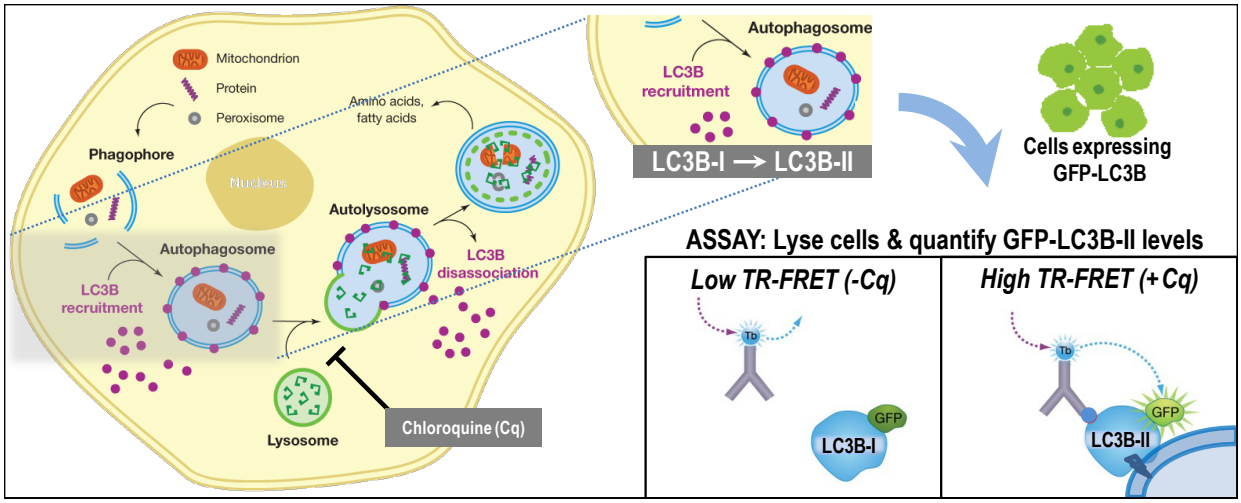

Figure 1 Mechanism of Action for the Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay. The assay step involves a single addition of cell lysis buffer containing Tb-labeled detection antibody selective for LC3B-II. Relative levels of LC3B-II are quantified using a fluorescence plate reader capable of detecting the Tb/GFP TR-FRET that occurs when the Tb-labeled detection antibody binds GFP-tagged LC3B-II.

Terbium-based TR-FRET detection

Time-resolved fluorescence energy transfer (TR-FRET) combines the benefits of detecting specific proximity-driven events via FRET with the increased assay sensitivity and lower fluorescence background obtained with time-resolved fluorescence. In Tb-based TR-FRET (LanthaScreen® detection) the long half-life terbium lanthanide serves as the FRET donor fluorophore whose excitation leads to energy transfer when in proximity to a suitable acceptor fluorophore, which then emits light at its specific wavelength. Applications include cellular assays where the use of GFP-tagged fusion proteins as FRET acceptors in combination with Tb-labeled antibodies circumvents the need to use complex antigen-capturing reagents, thereby providing a simpler, high-throughput plate reader alternative to commonly used analytical methods such as Western blot and ELISA. For more information, visit **www.invitrogen.com/lanthascreen**.

BacMam Technology

Whereas GFP-tagged LC3B can be delivered to cells via multiple methods (e.g., stable cell line generation, transient transfection, electroporation), BacMam technology is a convenient approach that uses a modified baculovirus to efficiently deliver and transiently express genes in many different mammalian cell-types. For more information, visit **www.invitrogen.com/bacmam**.

BacMam technology has several advantages over traditional transient gene expression methods, including:

- High viral transduction efficiency across a broad range of cell types, including primary and stem cells
- Little-to-no microscopically observable cytopathic effects
- Reproducible and titratable target gene expression
- Compatible with simultaneous delivery of multiple genes.

Assay Workflow

Day 1: Cells are transduced with BacMam reagent overnight (e.g., 18–24 hours), resulting in expression of GFP-tagged LC3B.

Day 2: Cells are left untreated or treated (e.g., starvation, application of autophagy modulating compounds) to influence their autophagy activity (i.e., autophagosome levels) for the desired time-point (e.g., several hours or overnight).

Day 2 or 3: Following treatment, cells are lysed in the presence of a Tb-labeled LC3B-II selective detection antibody and the levels of autophagosome-associated LC3B-II are measured using a Tb TR-FRET-compatible plate reader.

Low TR-FRET generally indicates little or no detectable activity whereas high TR-FRET indicates high autophagy activity.

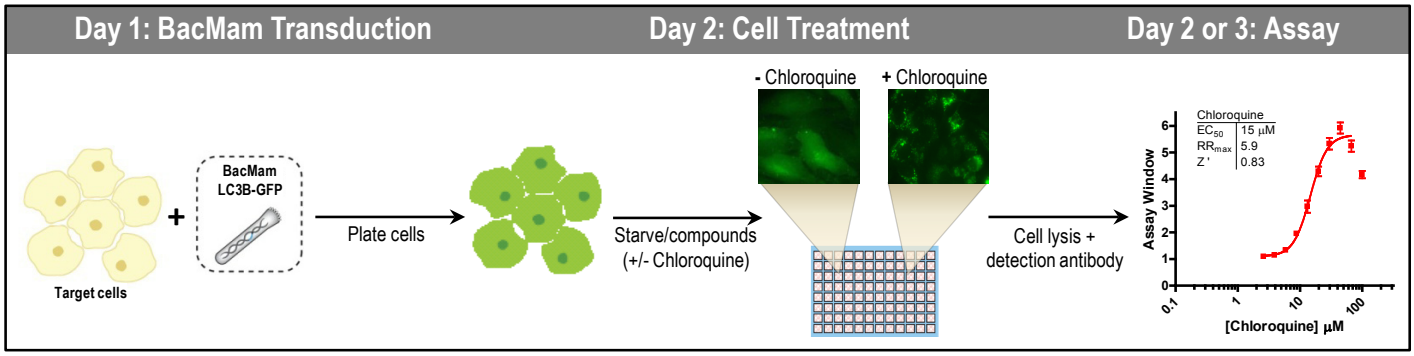

Figure 2 Representative Assay Workflow. Cells are mixed with LC3B-GFP BacMam and plated in 96- or 384-well format. Next day, cells are treated to induce autophagy (e.g., starve, mTOR inhibitors) leading to conversion of cytosolic LC3B-I to autophagosome-associated LC3B-II. Lysosomal inhibitor chloroquine can be used to block autophagosome turnover leading to accumulation of LC3B-II. At desired time-point (e.g., several hours, overnight), cells are lysed in the presence of a detection antibody that exhibits strong selectivity for LC3B-II. To measure autophagy activity, relative levels of LC3B-II are quantified using a fluorescence plate reader capable of detecting the Tb/GFP TR-FRET that occurs when the Tb-labeled detection antibody binds GFP-tagged LC3B-II.

Before Starting

Materials Required but Not Provided

Materials	Recommended Source	Cat. no.
Fluorescence plate reader with Tb TR-FRET (LanthaScreen®) capability Tb-based TR-FRET (LanthaScreen®) technology requires specific instrument settings that are critical to experimental success. Also note that for this assay we do not recommend using monochromator-based instruments as the sensitivity of these instruments is generally not sufficient to detect the TR-FRET signal.	For instrument set-up guidance, refer to **www.invitrogen.com/instrumentsetup**	
Cell Line of Interest (Visit **www.invitrogen.com/bacmam** for more information regarding BacMam compatible cell types.)	Various	Various
Assay Medium (Commonly used growth or starve media are compatible with the TR-FRET readout. Where possible, avoid media containing phenol red or wash it out immediately prior to the lysis/antibody detection step as its presence may result in reduced assay performance.)	Various	Various
Protease Inhibitor Cocktail	Sigma	P8340
Assay Plates (white opaque plates; do not use black plates with this assay)		
White opaque tissue culture-treated 96-well plates, or	Corning	3917
white opaque tissue culture-treated 384-well plates	Corning	3570

U-2 OS Cell Culture Reagents (optional, if using U-2 OS cells)

U-2 OS cells transduce exceptionally well with BacMam viruses, and we recommend their use as a control cell line.

Media/Reagents	Recommended Source	Part no.
U-2 OS cells	ATCC	HTB-96
Earle's Balanced Salt Solution (EBSS)	Invitrogen	14155-063
McCoy's 5A Medium	Invitrogen	16600-108
Opti-MEM® I (without phenol red)	Invitrogen	11058-021
Fetal Bovine Serum (dialyzed recommended)	Invitrogen	26400-036
Nonessential Amino Acids (NEAA)	Invitrogen	11140-050
Sodium Pyruvate	Invitrogen	11360-070
Penicillin/Streptomycin (antibiotic)	Invitrogen	15140-122
HEPES Buffer Solution (1 M)	Invitrogen	15630-080
Dulbecco's Phosphate-buffered Saline (PBS) without Ca^{2+} and Mg^{2+}	Invitrogen	14190-136
Trypsin/EDTA	Invitrogen	25300-062

Preparing Recommended Media

Media components	U-2 OS Growth Medium	Nutrient-rich Assay Medium	Starve Medium
McCoy's 5A Medium	500 mL	—	—
Opti-MEM® I	—	500 mL	—
Earle's Balanced Salt Solution (EBSS)	—	—	500 mL
Fetal Bovine Serum	50 mL	50 mL	—
Nonessential Amino Acids (NEAA)	5 mL	5 mL	—
Sodium Pyruvate (100X)	5 mL	5 mL	—
Penicillin/Streptomycin (100X)	5 mL	5 mL	—
HEPES Buffer Solution (1 M)	12.5 mL	—	10 mL
$CaCl_2$ and $MgCl_2$ (*optional*: improves cell adherence)	—	—	2 mM final

Guidelines for Optimizing Tb/GFP TR-FRET Cellular Assays

First-time Terbium TR-FRET users

Prior to setting up an assay, we strongly recommend checking your plate reader setup for Tb-based TR-FRET (LanthaScreen®) detection using the Instrument Controls included with this kit. For more information about settings recommended for your specific instrument and to purchase filters, visit **www.invitrogen.com/instrumentsetup**. For technical assistance, contact Drug Discovery Technical Support at **drugdiscoverytech@lifetech.com** or 760-603-7200, extension 40266).

Important assay parameters for optimization

Due to the variety of cell types in which this product may be used, further assay optimization may be required for best results. Key parameters to consider optimizing with your cell line include:

- Expression level of the GFP-fusion protein (i.e., GFP-tagged LC3B in this case)
- Cell plating density (i.e., the number of cells per well)
- Test compound concentrations and treatment times

Note that for end-users who are used to imaging autophagosomes labeled with GFP-tagged LC3B (i.e., the GFP-LC3B "spot" assay), the expression level and cell plating density required for optimal Tb TR-FRET assay performance may be higher than that used for imaging. Treatment times may also be longer to enable accumulation of higher LC3B-II levels for better assay windows.

Guidelines for Working with BacMam Reagent

- For first time users of BacMam reagent, we recommend including a control cell line which transduces exceptionally well, such as U-2 OS (ATCC® HTB-96).
- Although many cell types can be transduced efficiently using the protocol described here for U-2 OS cells, some cell types (e.g., CHO, MEF) are hard-to-transduce by BacMam and may require the alternate protocol described below. Visit **www.invitrogen.com/bacmam** for more information regarding BacMam compatible cell types.
- For best results use healthy, well-maintained cells when performing BacMam transductions. To minimize day-to-day variability, be sure to use the same growth conditions (e.g., similar harvest density, similar passage numbers).
- BacMam reagent is compatible with commonly used growth media and supplements. We recommend transducing cells in complete/nutrient-rich growth medium (e.g., typically containing 10% FBS) for optimal transduction and GFP-fusion protein expression. Once adequate GFP-fusion protein expression is achieved (usually within 18–24 hours following transduction), the media can be exchanged with fresh media or starve media as desired. Where possible, avoid media containing phenol red or wash it out immediately prior to the lysis/antibody detection step, because the presence of phenol red may result in reduced assay performance.
- BacMam Enhancer (Cat. no. PV5835, supplied at 1000X) is not required for this BacMam 2.0 reagent. However, it can be useful to improve the expression of BacMam-delivered targets, particularly with hard-to-transduce cell types. When using the Enhancer, we recommend testing at least two concentrations (e.g., 0.5X and 1X final) in comparison to a control without Enhancer to identify the best concentration for your cell-type (i.e., the concentration that yields robust GFP-tagged LC3B expression with little or no detectable toxicity).

Titration of LC3B-GFP BacMam Reagent

Since assay performance depends upon effective expression of GFP-tagged LC3B, we strongly recommend performing a titration of the BacMam reagent to determine the optimal percentage of BacMam (i.e., volume of virus to volume of cell media) for the transduction in your cell background of interest. For initial testing in a given cell-type, we recommend testing a final concentration range of 0.1 – 10 % BacMam reagent as outlined in the reference protocol provided below. Select the lowest percentage of BacMam reagent that yields the largest assay window.

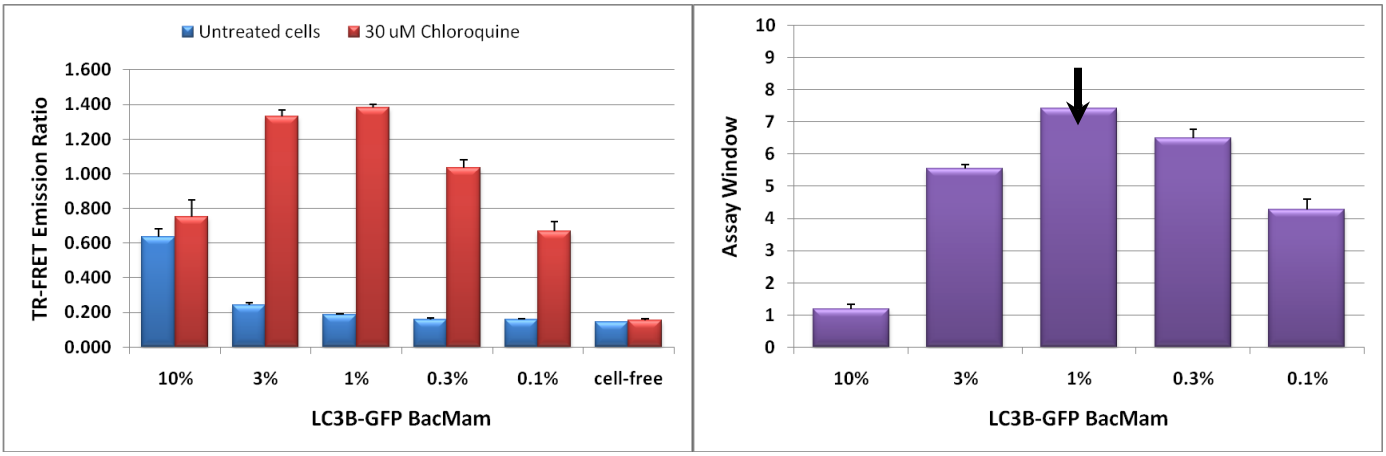

Figure 3 LC3B-GFP BacMam Titration Example. As outlined in the Quick Reference Protocol (see next page), U-2 OS cells were harvested, resuspended in nutrient-rich Assay Medium, and transduced with a dilution series of LC3B-GFP BacMam reagent in 96-well format. Next day, cells were left untreated or treated with 30 µM chloroquine overnight (3 replicate wells per variable). Next day, the assay was performed by adding lysis/detection antibody to the cells and reading Tb/GFP TR-FRET using a PerkinElmer® EnVision® plate reader. **Left panel**: The TR-FRET Emission Ratio was calculated for each well and the mean and standard deviation for each BacMam concentration was plotted. **Right panel**: The data was converted to normalized Assay Window values by dividing the Emission Ratio of the chloroquine-treated cells by the Emission Ratio of the untreated cells. Under these conditions, the largest assay window was obtained with U-2 OS cells transduced with 1% LC3B-GFP BacMam.

Assay Protocols

Quick Reference Protocol

The following example protocol was established using U-2 OS cells (ATCC HTB-96), which we recommend as a control cell line for first-time users of this kit. This protocol allows for testing a titration of the LC3B-GFP BacMam reagent in the presence or absence of chloroquine or other test compound. Conditions such as the number of cells to plate and chloroquine concentration/treatment time may need to be further optimized for other cell types.

Table 1. Assay Setup guide

Plate format	Number of tests A14070	Number of tests A14071	Total volume	Plating volumes/well
96-well	333	2000	90 µL/well	60 µL BacMam/cells (e.g., 18,000 U-2 OS cells/well) 15 µL 5X Chloroquine or test compound 15 µL Complete 6X lysis buffer + antibody*
384-well	1000	6000	30 µL/well	20 µL BacMam/cells (e.g., 6,000 U2-OS cells/well) 5 µL 5X Chloroquine or test compound 5 µL Complete 6X lysis buffer + antibody*

* Complete recipe: 1 mL 6X lysis buffer + 30 µL Protease Inhibitor + 6 µL Tb-Anti-LC3B Antibody. Scale as needed.

BacMam Transduction (Day 1)

1. Grow cells in appropriate Growth Medium to ~60–90% confluence.
2. Harvest cells and resuspend in Assay Medium (typically phenol-red-free Growth Medium or Opti-MEM® I with 10% FBS).
   - e.g., prepare ≥3 mL of U-2 OS cells at 3.3×10^5 cells/mL
3. Prepare four 3-fold serial dilutions of BacMam reagent in Assay Medium.
   - e.g., add 50 µL undiluted BacMam reagent to 100 µL Assay Medium, mix, then dilute 50 µL into 100 µL media, repeat two more times
4. Add BacMam reagent to cells to create a titration range of ~10% to 0.1% v/v final concentration.
   - e.g., for ~10% final, add 50 µL undiluted BacMam reagent to 450 µL cells
   - e.g., for remaining titrations, combine 50 µL of each BacMam reagent dilution from step 3 with 450 µL cells
5. Plate the volume listed in Table 1 of BacMam reagent /cells to white opaque assay plate as suggested in Figure 4.
6. Incubate the cells at 37ºC and 5% CO_2 for 16–24 hours to allow for expression of GFP-tagged LC3B.

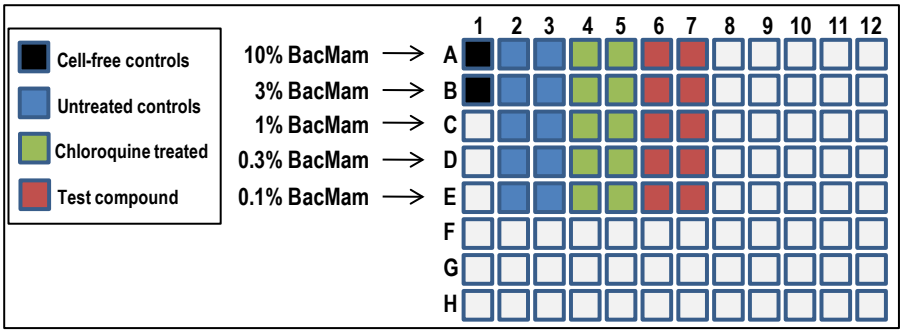

Figure 4 Suggested plate layout for initial BacMam reagent titration assay (two replicates per variable). Scale or modify as desired.

Cell Treatment (Day 2)

1. *Optional*: remove BacMam media and replace with fresh nutrient-rich Assay Medium or with Starve Medium.
2. Prepare Chloroquine and test compound at 5X final desired concentration in Assay or Starve Medium.
   - e.g., for 1X Chloroquine = 30 µM, prepare 5X Chloroquine = 150 µM by adding 1 µL of 30 mM Chloroquine to 200 µL of media.
3. Plate the volume listed in Table 1 of 5X Chloroquine or test compound; also plate media for cell-free and untreated controls.
4. Incubate the plate at 37ºC and 5% CO_2 for the desired treatment time.
   - e.g., for U-2 OS we recommend 4–8 hours if using Starve Medium, overnight if using nutrient-rich Assay Medium.

TR-FRET Assay (Day 2 or 3)

1. Prepare Complete 6X Lysis Buffer (supplemented with Protease Inhibitor and Antibody) as outlined in Table 1.
2. Lyse cells by adding the appropriate volume listed in Table 1.
3. Incubate plate for 1–2 hours at room temperature, protected from light.
4. Read plate on a Tb TR-FRET-compatible plate reader (visit **www.invitrogen.com/instrumentsetup**).
5. Analyze data by calculating the TR-FRET Emission Ratio for each well (divide GFP acceptor by Tb donor emission values).

Detailed Protocol

For first-time Terbium TR-FRET (LanthaScreen®) users, we strongly recommend checking your plate reader setup for Tb-based TR-FRET detection using the Instrument Controls included with this kit prior to setting up the assay (see page 12**).**

STEP 1: BacMam Transduction

A. Cells types that are easy-to-transduce by BacMam (e.g., U-2 OS cells, HEK293T)

In this 1-day protocol, cells are incubated with BacMam reagent at the time of plating.

1. Begin with a healthy, sub-confluent cell culture grown under normal Growth Medium conditions (e.g., U-2 OS cells grown to 60–90% confluence). To minimize day-to-day transduction variability, use consistent growth conditions (e.g., similar harvest density, similar passage numbers).

   Note: For many cell-types, cryopreserved cells can be transduced immediately following thaw to save culturing time. Similarly, many cell-types are amenable to being transduced (e.g., in batch format) with BacMam followed by cryopreservation of "pre-transduced" stock cell vials for later thaw and plating of the cells for the assay.

2. Harvest the cells, count them, and resuspend them in Growth Medium or nutrient-rich Assay Medium appropriate for your particular cell line. For many cell types, a concentration of 2.5×10^5–5×10^5 cells/mL is optimal for plating.

   Note: BacMam reagent is compatible with commonly used growth media and supplements. We recommend transducing cells in complete/nutrient-rich growth medium (e.g., typically containing 10% FBS) for optimal transduction and GFP-fusion protein expression. Once adequate GFP-fusion protein expression is achieved (usually within 16–24 hours following transduction) the media can be exchanged with fresh media or starve media as desired. Where possible, avoid media containing phenol red or wash it out immediately prior to the lysis/antibody detection assay step as its presence may result in reduced assay performance.

3. Add LC3B-GFP BacMam reagent to the cells. A typical final concentration of the LC3B-GFP BacMam reagent is 0.1–10% (volume of reagent to volume of cell media). Mix gently by inversion.

   Note: We strongly recommend testing a titration of the BacMam reagent to identify the optimal concentration to use with your cells for best assay performance. The Quick Reference Protocol provides an example of how to do this.

4. Plate the BacMam/cells mixture in a white opaque assay plate. See Table 1 for recommended plating volumes.

5. *Optional:* Plate remaining BacMam/cells in a parallel clear-bottom plate for image analysis of GFP expression.

   Note: If the experiment is performed manually, briefly spin the assay plate at $30 \times g$ for 1 minute.

6. Incubate the cells in a humidified incubator at 37ºC and 5% CO_2 for 16–24 hours to allow for transduction and expression of GFP-tagged LC3B.

7. Proceed to **STEP 2: Cell Treatment**, page 11.

B. Cells types that are hard-to-transduce by BacMam (e.g., CHO, MEF)

In this 3-day protocol, cells are allowed to adhere to the tissue-culture dish before performing the transduction, and then plated afterwards for the assay.

Day 1: Prepare cells for transduction

1. Begin with a healthy, sub-confluent cell culture grown under normal Growth Medium conditions.
2. Harvest the cells, count them, and plate the cells in Growth Medium such that they will be sub-confluent the next day. For many cell types (with a ~24-hour doubling time), a seeding density of approximately 2×10^4–4×10^4 cells/cm^2 (e.g., ~10,000 cells/well in 96-well format) will result in ~50–80% confluence 24 hours after seeding.

   Note: When starting out, we recommend plating cells in 96-well format in order to minimize reagent consumption during optimization of the BacMam transduction. We recommend setting up two parallel plates: a white opaque assay plate and a second clear-bottom visualization plate. Once optimal conditions are determined for your cell type, the transduction can be scaled up to larger cultures as desired.
3. Incubate the cells in a humidified incubator at 37ºC and 5% CO_2 for 16–24 hours to allow them to fully adhere.

Day 2: Transduce the cells

4. Determine the volume of BacMam/PBS Solution necessary to cover the adherent cells. We recommend ~150 µL of BacMam/PBS Solution (prepared in the next step) for every 1 cm^2 of surface area (see Table 2).

Table 2. Recommended volumes for BacMam/PBS transduction

Plate format:	384-well	96-well	48-well	24-well	12-well	6-well
~Surface area (cm^2/well):	0.1	0.3	1	2	4	10
BacMam reagent/PBS Solution:	**15 µL**	**50 µL**	**150 µL**	**300 µL**	**600 µL**	**1500 µL**
Growth Medium:	30 µL	100 µL	300 µL	600 µL	1200 µL	3000 µL

5. Prepare a dilution of the BacMam Reagent in Phosphate Buffered Saline (PBS) containing Ca^{2+} and Mg^{2+} (Cat. no. 14040-133). We recommend testing a range of BacMam Reagent dilutions—10%, 3%, and 1% (v/v) as a starting point (e.g., add 5 µL of BacMam Reagent to 45 µL of PBS to prepare a 10% dilution for 96-well format).
6. Gently wash the cells once with PBS containing Ca^{2+} and Mg^{2+}.
7. Remove the PBS from Step 6, and gently add the solution of the BacMam/PBS Solution prepared in Step 5 to the cells. Incubate the cells at room temperature (20–25ºC) for 2–4 hours, protected from light.
8. Following the incubation, remove the diluted BacMam Reagent from the cells and add an appropriate volume of Growth Medium to the cells (see Table 2).
9. *Optional:* Add BacMam Enhancer (Cat. no. PV5835) to the Growth Medium. Enhancer can improve expression of BacMam-delivered targets. When using Enhancer, we recommend testing at least two concentrations (e.g., 0.5X and 1X final concentrations) in comparison to a control without Enhancer to identify the best concentration for your cell-type (i.e., the concentration that yields robust GFP-tagged LC3B expression with little or no detectable toxicity).
10. Incubate the cells in a humidified incubator at 37ºC and 5% CO_2 for 16–24 hours to allow for expression of GFP-tagged LC3B.

Day 3: Plate the transduced cells for the assay

11. *Optional:* Analyze GFP expression levels by fluorescence microscopy using standard FITC filter sets.

12. Harvest the transduced cells, being careful not to over-trypsinize the cells as this can result in poor viability and reduced assay performance.

    Note: If the transduction was performed in a white opaque 96-well assay plate, then there is no need to harvest the cells and re-plate them. Simply replace the media with 60 µL/well of Assay Medium and proceed to **STEP 2: Cell Treatment.**

13. Resuspend the cells in Growth Medium or nutrient-rich Assay Medium appropriate for your particular cell line. For many cell types, a concentration of 2.5×10^5–5×10^5 cells/mL is optimal for plating.

    Note: Where possible, avoid media containing phenol red or wash it out immediately prior to the lysis/antibody detection assay step as its presence may result in reduced assay performance.

14. Plate the transduced cells in a white opaque assay plate. See Table 1 for recommended plating volumes.

    Note: If the experiment is performed manually, briefly spin the assay plate at 30 × *g* for 1 minute.

15. Incubate the cells in a humidified incubator at 37ºC and 5% CO_2 for 16–24 hours to allow them to adhere.

STEP 2: Cell Treatment

There are many cell treatment options for modulating autophagy. This protocol provides guidance on testing compounds such as lysosomal inhibitor chloroquine, which blocks the turnover of autophagosomes.

1. *Optional:* remove the media from the cells and add fresh nutrient-rich Assay Medium or Starve Medium (see Table 1 for plating volumes).

2. Prepare cell treatments at 5X final desired concentration in Assay or Starve Medium as follows:

   a. As applicable, prepare Control Medium containing a 5X concentration of the same solvent/vehicle (e.g., DMSO) used for the test compound (e.g., if desired 1X final concentration is 0.1%, prepare a 0.5% solution). Since solvent may affect the assay, try to keep the amount of solvent consistent in all of the assay wells.

   b. Prepare a 5X concentration of Chloroquine in Control Medium. The optimal amount of Chloroquine to use will depend on the cell type and/or Assay Medium, but 5X concentrations of ~100–300 µM (1X in assay of 20–60 µM) typically work well. We recommend testing a dilution series of Chloroquine to check your conditions.

   c. Prepare a 5X concentration of the test compound in Assay or Starve Medium. Since compound effects on autophagic activity will be both concentration and incubation time-dependent, we recommend testing a range of concentrations.

   Note: 5X Test Compound can also be prepared in medium containing 5X Chloroquine for comparing effects on autophagy activity in the presence and absence of lysosomal inhibition. Under optimized conditions, such comparisons can indirectly measure a compound's effect on autophagic flux (i.e., discriminate upstream autophagy induction from downstream inhibition of autophagosomal turnover in lysosomes).

3. Add the plating volume listed in Table 1 of 5X Chloroquine or test compound to the appropriate assay plate wells. Also, plate an equivalent volume of Control Medium for the cell-free and untreated controls.

   Note: If the experiment is performed manually, briefly spin the assay plate at 30 × *g* for 1 minute after compound addition.

4. Incubate the cells in a humidified incubator at 37°C and 5% CO_2 for the desired treatment time.

   Note: The cell treatment time prior to the lysis/antibody detection step may need to be optimized for your particular cell type and/or assay condition. For example, we recommend incubating chloroquine-treated U-2 OS cells for 4–8 hours if using Starve Medium or overnight (18–24 hours) if using nutrient-rich Assay Medium to ensure robust autophagosome accumulation for detection over background levels.

STEP 3: Assay

1. Following the cell treatment, prepare (fresh on day of assay) **Complete 6X Lysis/Detection Antibody:**

   a. Dispense the volume of LanthaScreen® 6X Cellular Assay Lysis Buffer needed.

   b. Add protease inhibitor cocktail at a 1:33 dilution (e.g., add 30 µL per 1000 µL buffer). Mix gently.

   c. Add Tb-anti-LC3B Antibody at a concentration of 6 nM (e.g., add 6 µL of 1 µM stock antibody per 1000 µL).

   d. Mix gently to minimize forming bubbles and store on ice until ready to use.

   Note: Scale the volume to prepare as follows: multiply the number of assay wells to be lysed × lysis volume/well × 1.2 scaling factor (see Table 3 example). This ensures some extra volume to offset potential losses during dispensing. The scaling factor may need to be adjusted based on your dispensing setup.

Table 3. Calculation example for preparing Complete 6X Lysis/Detection Antibody

Plate Format	Number of wells to lyse	Lysis volume/well	Scaling Factor	6X Lysis Buffer (L)	Protease Inhibitor (L/33)	Tb-anti-LC3B Antibody (L × 0.006)	Total volume
96-well	*32 wells × 15 µL/well × 1.2 =*			576 µL	17.5 µL	3.5 µL	*597 µL*
384-well	*32 wells × 5 µL/well × 1.2 =*			192 µL	5.8 µL	1.2 µL	*199 µL*

2. Remove the assay plate from the incubator and add Complete 6X Lysis/Detection Antibody to the wells.

   Note: If the experiment is performed manually, briefly spin the assay plate at 30 × *g* for 1 minute after adding lysis buffer.

3. Incubate the covered plate at room temperature in the dark for ~1–2 hours.

   Note: The assay plate may be also be stored at 4°C overnight (if evaporation is minimized) prior to reading. Allow the plate to warm to room temperature prior to reading.

4. Proceed to reading the plate, as described in the next section.

Terbium TR-FRET Detection

Instruments and Filters

Tb-based TR-FRET (LanthaScreen®) technology requires specific instrument settings that are critical to experimental success. For this assay we do not recommend using monochromator-based instruments as the sensitivity of these instruments is generally not sufficient to detect the TR-FRET signal. For more information about settings recommended for your specific instrument and to purchase filters, visit **www.invitrogen.com/instrumentsetup**. For technical assistance, contact Drug Discovery Technical Support at **drugdiscoverytech@lifetech.com** or 760-603-7200, extension 40266).

Testing Terbium TR-FRET Detection Using the Instrument Controls

To test your instrument set-up for performing terbium-based TR-FRET cellular assays:

1. Add 60 µL/well of the HIGH control to empty assay plate wells for 96-well format (or 20 µL/well for 384-well format). We recommend plating a minimum of 3 replicates.
2. Add 60 µL/well of the LOW control to empty assay plate wells for 96-well format (or 20 µL/well for 384-well format). We recommend plating a minimum of 3 replicates.
3. Read the plate, as described in the next section.
4. For each well, calculate the TR-FRET Emission Ratio (e.g., 520 nm/495 nm) by dividing the acceptor emission value (e.g., 520 nm) by the donor emission value (e.g., 495 nm).
5. Average the Emission Ratios for the HIGH control, and separately average the Emission Ratios for the LOW control.
6. Determine the HIGH to LOW fold-change by dividing the average Emission Ratio for the HIGH control by the average Emission Ratio for the LOW control.

   Note: The HIGH/LOW fold-change should be 2–4, depending on the plate reader used. Values below 2 may indicate that the instrument is not setup properly and/or lacks enough sensitivity for Tb-based TR-FRET.

Reading the Assay Plate

1. Let the assay plate warm to room temperature before reading, if necessary.
2. Set the fluorescence plate reader to top/time-resolved read mode (allow the lamp in the plate reader to warm up for at least 10 minutes before making measurements).
3. Remove the lid/seal and read the plate using the LanthaScreen® Tb TR-FRET instrument-specific filter selection guidelines provided at **www.invitrogen.com/instrumentsetup**.

   Note: Filter bandwidths are critical and cannot be approximated. For instruments using a flash lamp light source, we strongly recommend using 200 flashes for the acceptor channel (e.g., 520 nm) and 100 flashes for the terbium channel (e.g., 495 nm). We also recommend performing plate height (or focal height) optimization for best results.

Data Analysis

1. For each well, calculate the TR-FRET Emission Ratio (e.g., 520 nm/495 nm) by dividing the acceptor emission value (e.g., 520 nm) obtained for that well by the donor emission value (e.g., 495 nm) obtained for that well. Do **not** average the 520 nm or 495 nm readings and then take the ratio.

   Note: A common practice employed with TR-FRET data is to multiply the Emission Ratio values by 10,000. This is done to convert the Emission Ratio values from decimals to integers for easier graphing and data visualization.

   Note: In general, we do not recommend performing background subtraction with TR-FRET data since care must be taken to not inadvertently distort the results. However, in certain cases it may be desirable to perform background subtraction by subtracting the Emission Ratio value obtained for the cell-free control from the Emission Ratios of the untreated and treated cell wells.
2. *Optional:* Convert the TR-FRET Emission Ratio data into normalized Assay Window values, by dividing each Emission Ratio value determined for a cell treatment by the value calculated from the untreated control cells. (See Figure 3 for example data.)

Troubleshooting Guide

Observation	Potential Solutions
Weak/no expression of GFP-tagged LC3B in the cell line of interest as observed in a clear-bottom visualization plate.	Confirm that your fluorescence microscope is configured appropriately for detection of GFP/FITC.
	Perform a BacMam titration to find the optimal concentration for your cell background.
	Confirm that no contamination (e.g., looks cloudy) of the BacMam Reagent has occurred.
	For first-time users, we recommend the standard transduction protocol using U-2 OS cells at 60–90% confluence. Overly confluent or unhealthy cells will not transduce efficiently.
	If the standard protocol works for U-2 OS cells but not for your cells, try the protocol for hard-to-transduce cells by BacMam and include BacMam Enhancer (Cat. no. PV5835).
>50% expression of GFP-fusion is observed, but weak/no detectable TR-FRET signal over background is detected.	Confirm that the fluorescence plate reader is configured appropriately for Terbium TR-FRET detection using the Instrument Controls. Note that filter bandwidth requirements are exact. When using an instrument with a flash lamp light source, the number of flashes is important. Plate or focal height optimization may also need to be optimized. For more information about your specific instrument and to purchase filters, visit **www.invitrogen.com/instrumentsetup**. Contact Drug Discovery technical support for more information.
	For first-time users, we recommend following the detailed protocol using U-2 OS cells as a control cell line for the BacMam transduction, followed by cell treatment with chloroquine overnight in nutrient-rich Assay Medium prior to lysis/antibody detection.
	Image the cells in a clear-bottom plate. Ensure that cells are adhered to the bottom of the plate and are not expressing very high levels of GFP. Moderately green cells are desirable. Excessive expression of GFP-tagged LC3B may be deleterious to cell health. Alternatively, check to make sure that the cell plating density is not too low (i.e., low numbers of cells plated per well will result in low signals); test a cell seeding titration to optimize the number of cells plated per well.
	Compare test conditions in the absence and presence of chloroquine. Lysosomal inhibitor chloroquine can serve as a positive control compound for the assay since it blocks autophagosome turnover, leading to LC3B-II accumulation and larger TR-FRET signals. However, the chloroquine concentration to use and total incubation time may need to be optimized for your cell type and/or assay media condition.
Day-to-day fluctuations in assay window are observed.	Be sure to use cells with the same growth conditions (e.g., similar harvest density and passage number).
	Take care to count and plate similar numbers of cells per well each day.
	Use consistent cell treatment/assay conditions (e.g., same concentrations, incubation times).

Purchaser Notification

Limited Use Label License: Research Use Only

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact outlicensing@lifetech.com or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

Limited Use Label License No. 21: Bac-to-Bac® and Bac-to-Bac® HT

This product is sold under patent license from Monsanto for research purposes only and no license for commercial use is included. Requests for licenses for commercial manufacture or use should be directed to Director, Monsanto Corporate Research, 800 N. Lindbergh, St. Louis, Missouri 63167.

Limited Use Label License No. 127: GFP with Heterologous Promoter

This product is sold under license from Columbia University. Rights to use this product are limited to research use only, and expressly exclude the right to manufacture, use, sell or lease this product for use for measuring the level of toxicity for chemical agents and environmental samples in cells and transgenic animals. No other rights are conveyed. Not for human use or use in diagnostic or therapeutic procedures. Inquiry into the availability of a license to broader rights or the use of this product for commercial purposes should be directed to Columbia Innovation Enterprise, Columbia University, Engineering Terrace-Suite 363, New York, New York 10027.

Limited Use Label License No. 168: Lanthanide Chelates

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell 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 Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (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; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use, or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500.

Limited Use Label License No. 198: Fluorescent Proteins and Stable Cell Lines Expressing Such Proteins

The purchase of this product conveys to the buyer the nontransferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for profit entity). No rights are conveyed to modify or clone the gene encoding GFP contained in this product. The buyer cannot sell or otherwise transfer (a) this product, (b) its components, or (c) materials made by the employment of this product or its components to a third party or otherwise use this product or its components or materials made by the employment of this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the employment of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (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; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Life Technologies Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that none of this product, or any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Life Technologies Corporation is willing to accept return of the product with a full refund. For information on purchasing a license to use this product for purposes other than those permitted above, contact Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008. Phone (760) 603-7200 or outlicensing@lifetech.com.

Limited Use Label License No. 272: Humanized GFP

This product is sold for research use only. Not for therapeutic or diagnostic use in humans.

Limited Use Label License No. 306: Baculovirus Vectors

This product is for research use only by those researchers in laboratories of academic, government, industrial and/or clinical institutions engaged in the investigation of biological or biochemical processes, or research and development of biological products. This product is not to be used in the manufacture, use or sale of human or animal diagnostic, therapeutic or prophylactic products.

Limited Use Label License No. 308: WPRE Element

This product contains the Woodchuck Post-transcriptional Regulatory Element ("WPRE") which is the subject of intellectual property owned by The Salk Institute for Biological Studies, and licensed to Life Technologies Corporation. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell 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 Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (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; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; and/or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. In addition, any use of WPRE outside of this product or the product's authorized use requires a separate license from the Salk Institute. Life Technologies will not assert a claim against the buyer of infringement of patents owned by Life Technologies and claiming this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product or for a Commercial Purpose. If the purchaser is not willing to accept the limitations of this limited use statement, Life Technologies is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008, Phone (760) 603-7200. Fax (760) 602-6500, or The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, Attn.: Office of Technology Management, Phone: (858) 453-4100 extension 1275, Fax: (858) 546-8093.

Limited Use Label License No. 332: BacMam Virus Use

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer solely in accordance with the accompanying product literature or manual. Purchase of this product does not convey a license to expand, amplify, or otherwise propagate the provided viral particles or to otherwise modify or alter the virus by any means.

For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.