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Premo™ Autophagy Tb/GFP TR-FRET LC3B Antibody Kit, Catalog nos. A14292 or A14294

Kit components	Amount A14294	Amount A14292	Storage	Handling
Tb-anti-LC3B Antibody	6 µg	36 µg	4°C	Protect from light
LanthaScreen® 6X Cellular Assay Lysis Buffer	6 mL	50 mL	4°C	On day of assay, add protease inhibitor and antibody
Chloroquine	1 mL	1 mL	4°C	Protect from light
Instrument Control Terbium TR-FRET	1 kit	1 kit	4°C	Protect from light
Number of assays: Sufficient reagent is supplied for: A14294 333 assays (96-well format) up to 1,000 assays (384-well format) A14292 2,000 assays (96-well format) up to 6,000 assays (384-well format)				
Instrument setup: www.invitrogen.com/instrumentsetup.				

Premo™ Autophagy Tb/GFP TR-FRET LC3B Expression Kit, Catalog nos. A14070 or A14071

Kit components	Amount A14070	Amount A14071	Storage	Handling
LC3B-GFP BacMam 2.0	1 mL	2 × 3 mL	4°C	• DO NOT FREEZE • Use sterile technique • Protect from light
Tb-anti-LC3B Antibody	6 µg	36 µg	4°C	Protect from light
LanthaScreen® 6X Cellular Assay Lysis Buffer	6 mL	50 mL	4°C	On day of assay, add protease inhibitor and antibody
Chloroquine	1 mL	1 mL	4°C	Protect from light
Instrument Control Terbium TR-FRET	1 kit	1 kit	4°C	Protect from light
Number of assays: Sufficient reagent is supplied for: A14070 333 assays (96-well format)* up to 1,000 assays (384-well format)* A14071 2,000 assays (96-well format)* up to 6,000 assays (384-well format)* *calculations assume use of easy-to-transduce cells. For other cell types the number of assays will vary.				
Instrument setup: www.invitrogen.com/instrumentsetup.				

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I. Product Overview

How does the Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay work?

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 (Figure 1). 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.

The **Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay** measures autophagy in cells expressing green fluorescent protein (GFP)-tagged LC3B, using a Terbium (Tb)-based Time Resolved-Fluorescence Energy Transfer (TR-FRET) immunoassay approach that quantifies autophagosome-associated LC3B-II levels. 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 (TR-FRET donor) binds GFP-tagged LC3B-II (TR-FRET acceptor). For more information on Tb-based TR-FRET (LanthaScreen® Terbium) detection, visit www.invitrogen.com/lanthascreen.

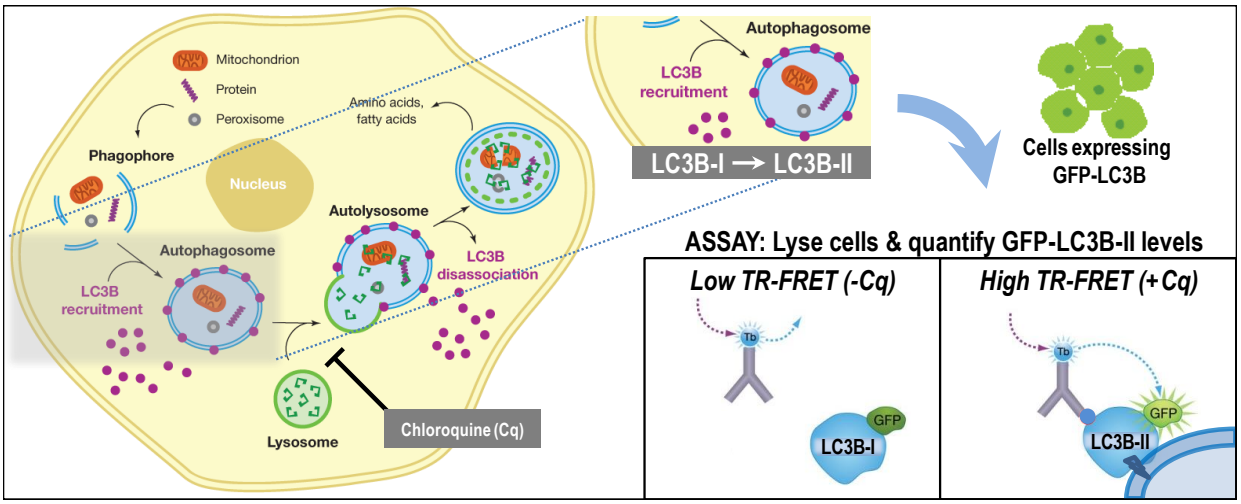

Figure 1. Mechanism of Action for the Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay. Autophagy marker protein LC3B normally resides in the cytosol (LC3B-I) but upon induction of autophagy becomes recruited to autophagosomal membranes (LC3B-II). Lysosomal inhibitor chloroquine can be used to block autophagosome 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 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.

What is the difference between the Antibody Kit and the Expression Kit?

The **Premo™ Autophagy Tb/GFP TR-FRET LC3B Antibody Kit** provides Tb-labeled LC3B detection antibody for performing the assay with a cell line already expressing GFP-LC3B. Note that cells expressing GFP-tagged LC3 isoforms (LC3A, LC3C) and/or species sequences that differ from the validated human LC3B sequence (RefSeq NP_073729.1) recognized by the detection antibody may not be suitable for this assay.

The **Premo™ Autophagy Tb/GFP TR-FRET LC3B Expression Kit** utilizes BacMam Technology to express a GFP-tagged version of the autophagosomal marker protein LC3B and also includes Tb-labeled detection antibody to perform the assay. Note that the GFP tag is at the N-terminal end of LC3B, allowing for LC3B to be lipidated at the C-terminus and embedded into autophagosomal membranes. 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 on BacMam technology and compatible cell types, visit www.invitrogen.com/bacmam.

What is a typical assay workflow using the Antibody Kit?

A typical workflow (Figure 2) using the Antibody Kit involves plating cells already expressing GFP-tagged LC3B in 96- or 384-well format and incubating overnight to allow the cells to adhere. 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 the 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 and the Tb/GFP TR-FRET is read with a suitable fluorescence plate reader.

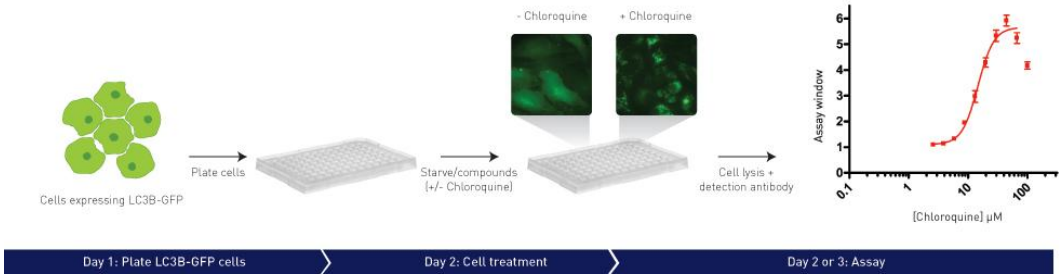

Figure 2. Representative Assay Workflow using the Premo™ Autophagy Tb/GFP TR-FRET LC3B Antibody Kit.

What is a typical assay workflow using the Expression Kit?

A typical workflow (Figure 3) using the Expression Kit involves mixing the cells of interest with LC3B-GFP BacMam, plating the cells/BacMam mixture in 96- or 384-well format, and incubating overnight to allow for GFP-tagged LC3B expression. 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 the 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 and the Tb/GFP TR-FRET is read with a suitable fluorescence plate reader.

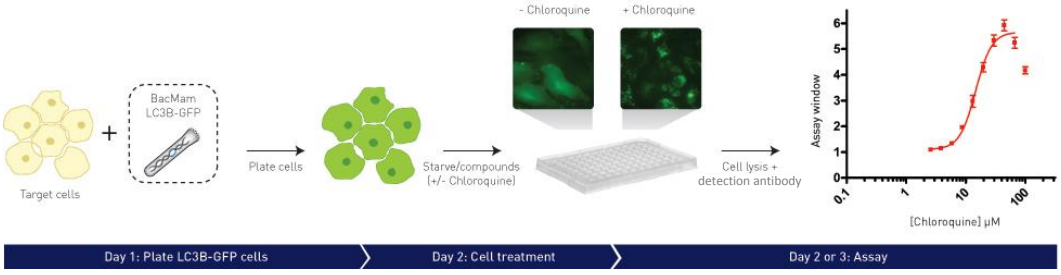

Figure 3. Representative Assay Workflow using the Premo™ Autophagy Tb/GFP TR-FRET LC3B Expression Kit.

What kind of data do you get from the Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay?

The Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay provides highly quantitative data on the autophagy activity of cells based on the selective detection of autophagosome-associated, GFP-tagged LC3B-II levels using a Tb/GFP TR-FRET immunoassay approach. Time Resolved-Fluorescence Resonance Energy Transfer (TR-FRET) is a preferred fluorescent assay format since it is less susceptible to compound interference than other assay formats.

In the Premo™ Autophagy Assay (e.g., Figure 4), two raw TR-FRET emission signals are measured from each assay well: one for the terbium-labeled detection antibody (the TR-FRET donor) and a second emission signal corresponding to the amount of GFP-tagged LC3B-II detected (the TR-FRET acceptor). The raw emission values obtained for each well are typically combined together by dividing the TR-FRET acceptor emission value by the donor emission value to report out a TR-FRET Emission Ratio. For comparing experiments, the data can be further normalized and expressed as Assay Window values by dividing the Emission Ratio calculated for a given test condition by the Emission Ratio determined for the untreated baseline control.

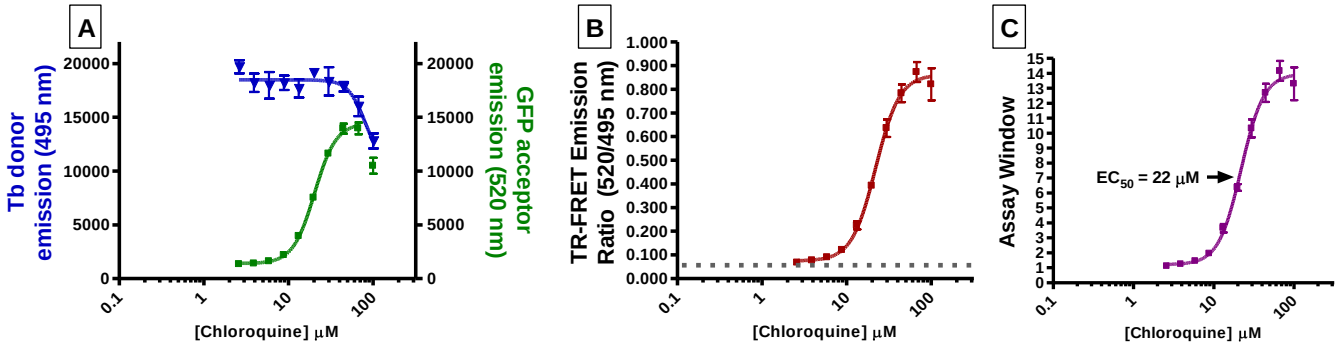

Figure 4. Representative data using the Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay. U-2OS osteosarcoma cells were transduced overnight with 0.5% LC3B-GFP BacMam virus in 384-well format. Next day, a serial dilution of lysosomal inhibitor chloroquine was added to the wells and incubated for ~24 hours prior to applying lysis/detection antibody and reading TR-FRET using a PerkinElmer EnVision® plate reader (with TRF laser light source). **A,** the raw TR-FRET emission values obtained for the Tb donor (at 495 nm) and GFP acceptor (at 520 nm) were plotted as the mean ± standard deviation (n = 4). **B,** to simplify the data presentation, the raw emission values were combined to generate TR-FRET Emission Ratios by dividing the GFP acceptor emission (520 nm) obtained for each well by the corresponding Tb donor emission (495 nm) value for that well and then plotting the Emission Ratios as the mean ± standard deviation (n = 4). The untreated cells baseline control ratio is depicted as a dotted line. **C,** to further normalize the data, Assay Window values were calculated by dividing the chloroquine-treated Emission Ratio values by the average Emission Ratio determined for the untreated baseline control. Curve fitting and EC_{50} calculation were performed with GraphPad Prism® software using a nonlinear regression equation for variable slope sigmoidal dose-response.

Notes: the Tb donor emission signal (495 nm) remains relatively constant (consistent with equal loading of lysis/detection antibody in the wells), except for at the highest two chloroquine concentrations which appear to negatively affect the donor signal. The GFP acceptor signal (520 nm) increases in a chloroquine dose-dependent fashion, correlating with increased autophagosome accumulation and associated LC3B-II levels resulting from blocking turnover in lysosomes.

What controls should I use before I begin setting up my assay?

Controls for checking plate reader setup

Proper plate reader setup is critical to experimental success. For more information about settings recommended for your specific instrument and to purchase filters, visit www.invitrogen.com/instrumentsetup (also see section V Optimizing plate reader settings).

Prior to setting up an assay, we strongly recommend checking your plate reader setup for Tb-based TR-FRET (LanthaScreen® Terbium) detection using the **Instrument Control Terbium TR-FRET kit** (Catalog no. A14138) included with the Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay kits. 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.

Controls needed for running the assay

Appropriate assay controls are important in assay design. The following controls are recommended:

1. **Cell-free control** – wells containing assay media without any cells. These wells are used to determine the assay background as this may vary based on media conditions and instrument setup. If desired, the cell-free control can be used to perform background subtraction after calculating the TR-FRET Emission Ratio for each well. However, background subtraction should be done only if the Emission Ratio of the cell-free control is significantly lower than the untreated cells control.

2. **Untreated cells control** – wells of untreated cells serve as the assay baseline control. This control is also used for normalizing the data to compute treated/untreated Assay Window values to compare results from different experiments (see Figure 4 for example data).

3. **Chloroquine-treated cells control** – wells of cells treated with lysosomal inhibitor chloroquine. Chloroquine blocks autophagosome turnover in lysosomes serving as a positive assay control. Test a dilution series of Chloroquine to determine the optimal concentration as it may depend on the cell type and/or assay medium conditions. Final assay concentrations of ~20 – 60 µM typically work well.

What type of plates should be used for this assay?

Only white opaque tissue culture-treated plates (96-well or 384-well format) should be used for this assay. Do not read Tb/GFP TR-FRET using clear or black plates. If samples are not setup in the right plate type the lysates can be prepared and then transferred to the correct white opaque assay plate immediately prior to reading the Tb/GFP TR-FRET.

II. Storage and Handling

How light sensitive are the Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay kit components?

Avoid unnecessary long term exposure of the reagents to bright lights. Ambient light exposure is acceptable

What if I accidentally left the kit components at room temperature overnight?

Short-term exposure of components to room temperature will have minimal effect on function. If components are left at room temperature long term replacing the reagent is recommended.

What if I accidentally froze the kit components, can I still use them?

LC3B-GFP BacMam 2.0: Freezing of BacMam reagents is not recommended as baculoviruses are not stable when freeze-thawed.

Tb-anti-LC3B Antibody: A single freeze-thaw of the detection antibody should have minimal impact on its performance. Avoid multiple freeze-thaws.

Chloroquine: Chloroquine can be frozen.

Instrument Control Terbium TR-FRET kit: The instrument control tubes can be frozen.

LanthaScreen® 6X Cellular Assay Lysis Buffer: The lysis buffer can be frozen.

Since the LC3B-GFP BacMam is a virus, are there any specialized handling requirements?

Standard laboratory handling precautions (e.g., personal protection equipment) used for other routine cell culture methodologies are recommended. There are no specialized handling requirements necessary for using the LC3B-GFP BacMam 2.0 reagent. BacMam technology involves the use of modified baculovirus to efficiently deliver and transiently express genes in many different mammalian cell-types. Baculoviruses are insect viruses that do not replicate in mammalian cells and are therefore considered safe (i.e., classified as BSL1). For more information, refer to www.invitrogen.com/bacmam.

III. Usage

Can I measure "autophagic flux" with the Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay?

Autophagy is a dynamic process that consists of upstream autophagy induction events leading to autophagosome formation and later downstream degradation of the engulfed cargo following fusion with lysosomes (Figures 1 and 5). The cycle of autophagy induction to turnover in lysosomes is referred to as "autophagic flux" and can be modulated by various cellular stress conditions. Autophagic Flux can be explained by using a sink analogy (Figure 5), where water flowing into the sink and then down the drain represents autophagosome formation followed by turnover in lysosomes. Under basal autophagy conditions the amount of autophagosome formation is so low that it is rapidly turned over without any significant accumulation. Conditions that induce autophagy (e.g., starvation, mTOR inhibitors) can turn up the volume of autophagosome formation high enough to outpace turnover. Autophagosome accumulation can also occur by blocking turnover (e.g., with lysosomal inhibitor chloroquine) without any increase in induction. When an autophagy inducer is combined with a turnover inhibitor, then higher autophagosome levels accumulate than by either treatment alone. Therefore, by measuring autophagosome-associated LC3B-II levels in the presence and absence of a turnover inhibitor, the effects of a particular treatment on autophagic flux can be estimated.

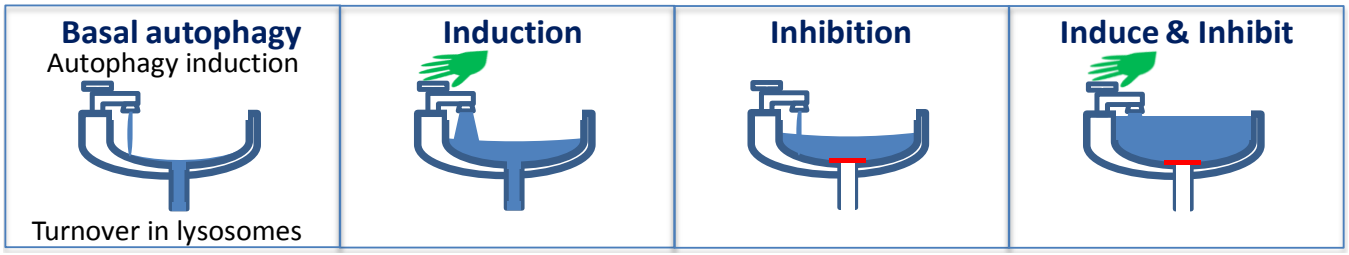

Figure 5. Describing autophagic flux (i.e., the flow of autophagosomes from formation to turnover in lysosomes) using a sink analogy.

The Premo™ Autophagy Tb/GFP TR-FRET LC3B immunoassay is an endpoint assay which precludes its use for direct, real-time monitoring of autophagic flux. However, the assay can be used to indirectly measure effects of test conditions on autophagic flux. This is achieved by performing two parallel assays: one assay performed in the presence of an inhibitor that blocks autophagosome turnover (e.g., lysosomal inhibitor chloroquine), and the second assay performed without the inhibitor. These two data sets are combined to calculate percent autophagic flux as follows. For each test condition, the TR-FRET Emission Ratio obtained without lysosomal inhibitor chloroquine is subtracted from the Ratio obtained with chloroquine. The baseline control difference is also determined by subtracting the TR-FRET Emission Ratio obtained for the untreated cells control from the chloroquine-treated cells control. The test condition difference is then divided by the baseline control difference. The percent autophagic flux estimates the degree to which a particular test condition is affecting autophagy induction or blocking turnover (e.g., Figure 6).

An alternative approach to assess the effects of test conditions on autophagic flux is to perform a time-course in which the effects of the test condition on autophagosome/LC3B-II levels is compared to control conditions (e.g., Figure 7).

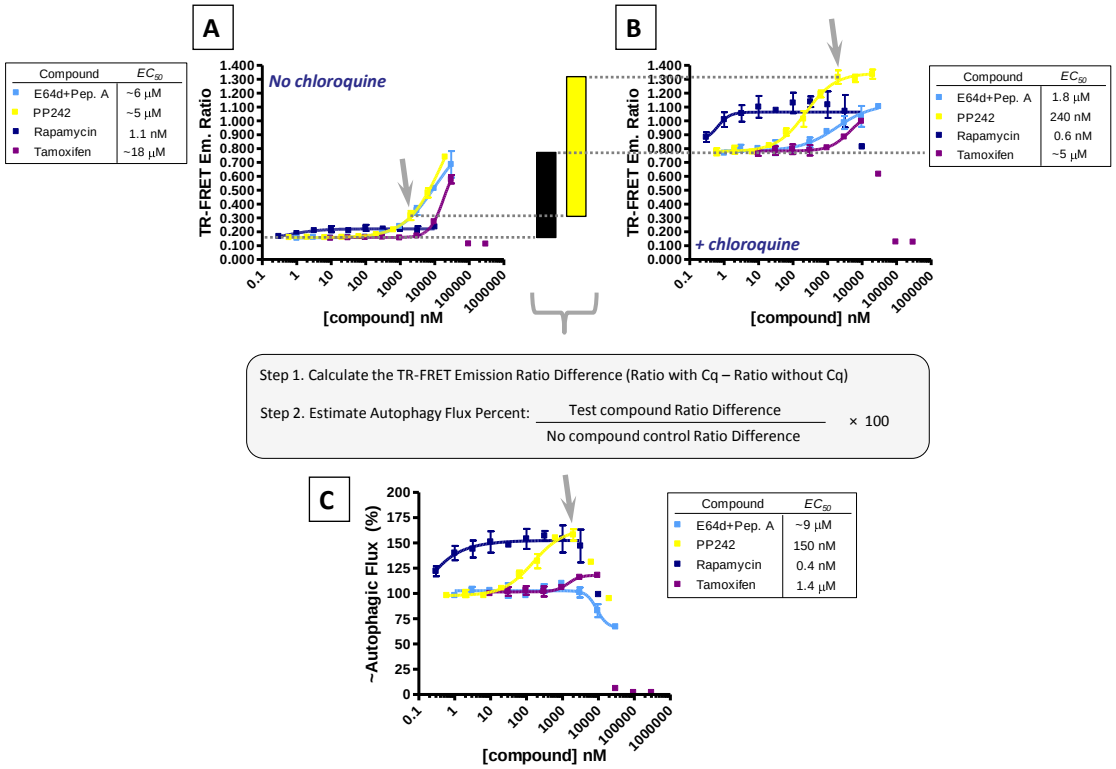

Figure 6. Measuring changes in autophagic flux. **A** and **B,** GripTite™ 293 cells stably expressing GFP-tagged LC3B were harvested and resuspended in nutrient rich growth media with or without an ~EC_{50} concentration of chloroquine. The cells were plated into two 384-well assay plates containing serial dilutions of selected compounds and incubated overnight. Next day, lysis/detection antibody was added to the wells and Tb/GFP TR-FRET was read. **C,** to estimate changes in flux, the TR-FRET Emission Ratio Difference was calculated by subtracting the Emission Ratio obtained for each test condition without chloroquine from the Emission Ratio obtained in the presence of chloroquine. The TR-FRET Emission Ratio Difference obtained for each condition was then divided by the Difference obtained with the no compound baseline control. In this data set, protease inhibitor combination E64d and Pepstatin A resulted in decreased flux, consistent with an overlapping mechanistic role with chloroquine in blocking downstream autophagosomal turnover in lysosomes. In contrast, known autophagy activators Tamoxifen (increases intracellular ceramide levels) and mTOR inhibitors PP242 and Rapamycin elicited increased flux consistent with upstream autophagy induction.

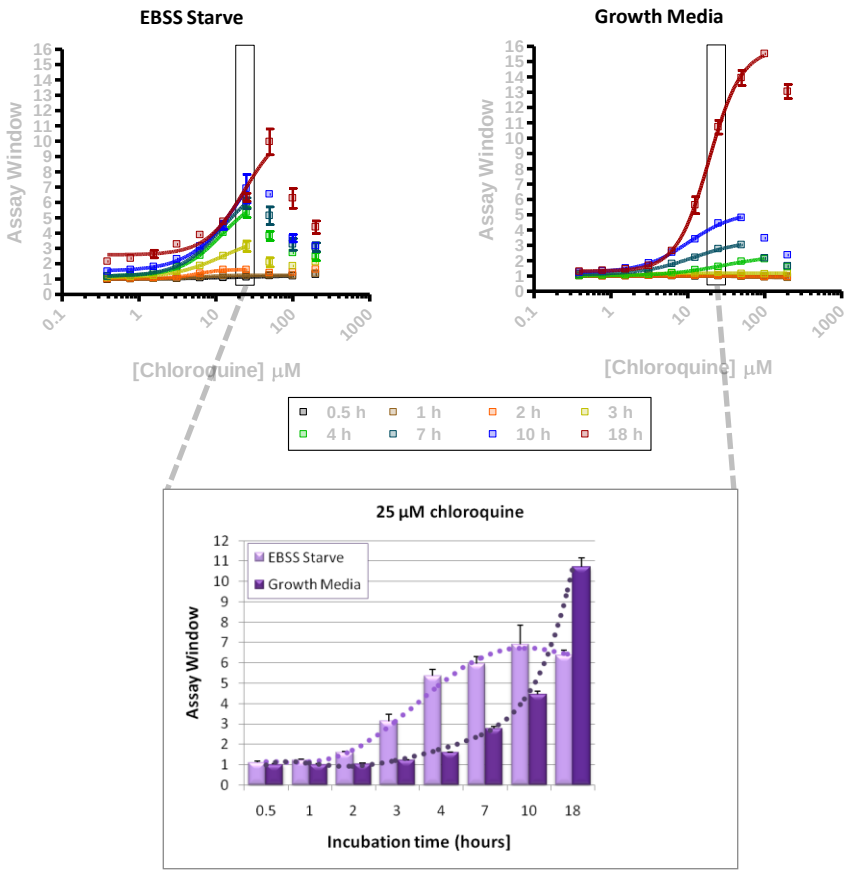

Figure 7. Time course comparison of autophagosome accumulation with starvation-induced autophagy versus nutrient-rich basal autophagy conditions. **A** and **B,** GripTite™ 293 cells stably expressing GFP-tagged LC3B were plated in 384-well format and allowed to adhere overnight in nutrient rich growth media (DMEM without phenol red + 10% dFBS + supplements). The cells were either left in growth medium or starved by replacing the media with Earle's Balanced Salt Solution (EBSS). A dilution series of lysosomal inhibitor chloroquine was applied to block autophagosome turnover. At designated times, lysis/detection antibody was added to the wells and the Tb/GFP TR-FRET was read. Assay Window values were plotted as the mean ± standard deviation (n = 4). **C,** the Assay Window values obtained at 25 µM chloroquine were separately plotted to highlight the autophagosome accumulation differences between growth media and starve (EBSS) conditions over the time course.

How does the Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay compare to other methods?

The Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay uses a highly quantitative TR-FRET immunoassay approach to measure the autophagic activity of cells. Following expression of GFP-tagged LC3B and the desired cell treatment, the assay step involves a single addition of lysis/detection antibody solution prior to reading the TR-FRET. In comparison, traditional methods for monitoring autophagy have relied primarily upon qualitative/semi-quantitative imaging (e.g., imaging the formation of fluorescent protein-tagged LC3B/autophagosome "spots" in cells) and immunoblotting techniques (e.g., Figure 8, Western blot detection of LC3B-I and LC3B-II levels). These traditional approaches typically involve several steps to go from sample to result (Figure 9).

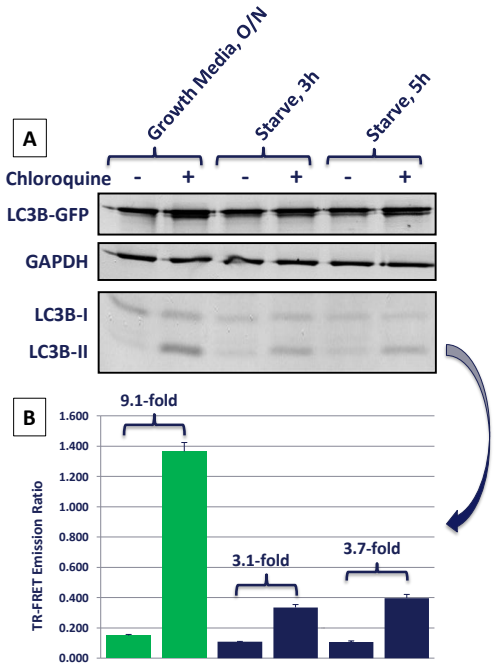

Figure 8. The Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay generates highly quantitative data. **A,** GripTite™ 293 cells stably expressing GFP-tagged LC3B were plated in 96-well format and incubated overnight in growth media with or without 25 µM chloroquine. Following overnight incubation, additional sets of cells were starved for 3 or 5 hours in the presence or absence of 25 µM chloroquine. Cell lysates were prepared and immunoblotted with a GFP antibody (top panel) to detect GFP-tagged LC3B, a GAPDH antibody (middle panel) to confirm equal sample loading, and an LC3B antibody (Sigma, bottom panel) that detects both LC3B-I and LC3B-II. **B,** a parallel set of samples was quantified using the Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay, with the results correlating closely with the relative amounts of LC3B-II detected by western blotting.

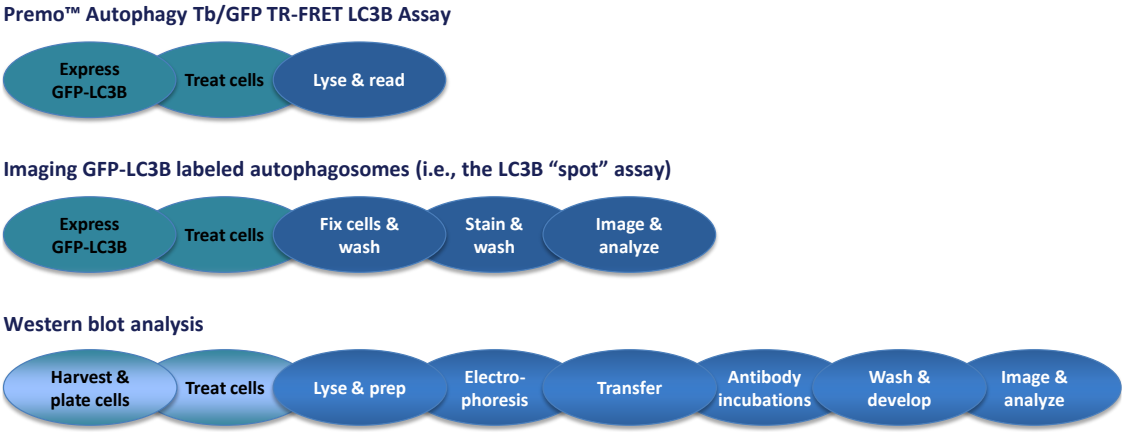

Figure 9. The Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay requires a single lyse and read step to go from sample to results.

IV. Methods Optimization

What parameters do I need to consider optimizing for best assay performance?

Due to the variety of cell types in which the Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay may be used, we recommend testing the following key parameters with your cell line of interest to identify the best assay conditions:

1. Expression level of GFP-tagged LC3B (Figures 10 and 11)
2. Cell plating density (Figure 12)
3. Test compound concentrations and treatment times (Figure 7)

Note that while the lysis/antibody detection portion of the assay should only be performed in an opaque white plate, it can be helpful during optimization to also plate some cells in a parallel clear-bottom plate for visually monitoring the optimization parameters (e.g., GFP expression, uniform distribution of plated cells in the wells, obvious morphological effects of treatments on cell viability).

Additional aspects to keep in mind include the selection of assay media (Figures 7 and 13) and to a lesser degree the lysis/detection antibody incubation time prior to reading the assay plate (Figure 14). *Proper instrument setup (see section V below) is also critical for experimental success and should not be overlooked.*

How do I determine the optimal amount of LC3B-GFP BacMam reagent to add to my cells?

The expression level of GFP-tagged LC3B in the cells can have a big impact on assay performance. Too much expression can be toxic to the cells while too little expression may result in suboptimal assay windows and reduced assay sensitivity.

Performing a BacMam reagent titration experiment is the best method to determine the optimal percentage of virus (i.e., the volume of BacMam reagent per volume of cell media) for transduction of your cell line of interest. For initial testing in a given cell-type, testing a final concentration range of 0.1 – 10 % BacMam reagent is recommended. Select the lowest percentage of BacMam reagent that yields the largest Assay Window (Figure 10).

BacMam Enhancer (Catalog no. PV5835, supplied at 1000X) can improve the expression of BacMam-delivered targets, particularly with difficult-to-transduce cell types (Figure 11). 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).

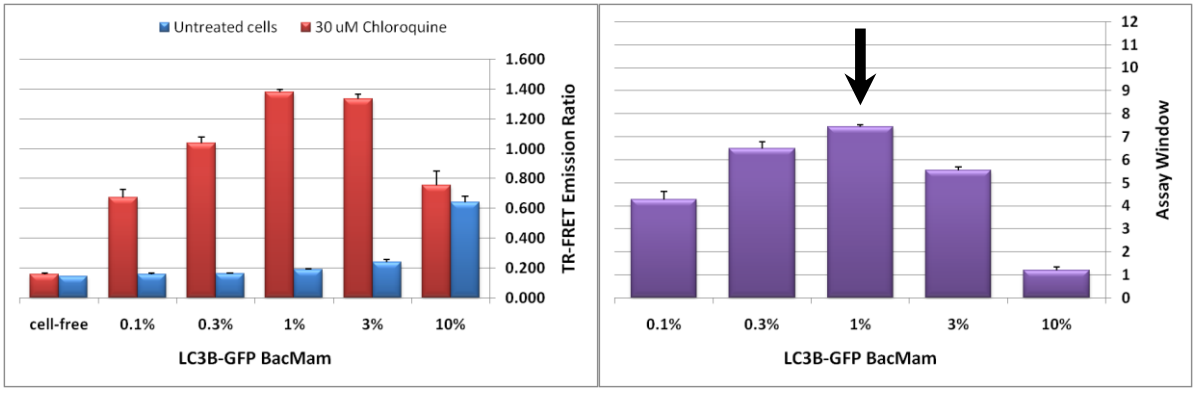

Figure 10. GFP-tagged LC3B expression can be optimized by performing an LC3B-GFP BacMam titration experiment. U2OS cells were transduced with a titration of LC3B-GFP BacMam in 96-well format. Next day, the cells were incubated overnight in growth media with or without 30 µM chloroquine and then lysis/detection antibody was applied to the wells and TR-FRET was measured on a PerkinElmer Envision® plate reader (with flashlamp light source). The TR-FRET Emission Ratio and normalized Assay Window values were plotted as the mean ± standard deviation (n = 6). In this example, a final concentration of 1% LC3B-GFP BacMam would be recommended as it gave the largest Assay Window.

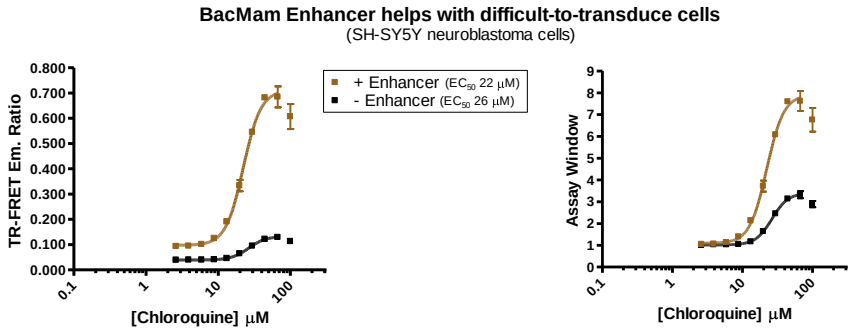

Figure 11. BacMam Enhancer can improve expression of BacMam delivered targets, especially when using cells that are difficult-to-transduce by BacMam. SH-SY5Y neuroblastoma cells were transduced overnight with 5% LC3B-GFP BacMam ± 0.5X final concentration of BacMam Enhancer (supplied at 1000X stock). Next day, cells were harvested, resuspended in serum-free media (DMEM/F12 without phenol red), plated in 384-well format, and treated overnight with a dilution series of chloroquine. Next day, lysis/detection antibody was applied to the wells and Tb/GFP TR-FRET was measured on a PerkinElmer Envision® plate reader (with TRF laser light source) and plotted as the mean ± standard deviation (n = 3). In this example, inclusion of BacMam Enhancer during the transduction approximately doubled the Assay Window.

What is the optimal incubation time with the GFP-LC3B BacMam reagent?

Maximum expression of recombinant protein using BacMam is usually observed between 24 – 48 hours post transduction, depending on cell type.It is not necessary to remove the BacMam reagent prior to proceeding with the cell treatment and assay.

What is the optimal cell number per well to use for the assay?

The number of cells plated per well will affect assay performance and should be optimized (Figure 12). To minimize day-to-day variability, be sure to use the same growth conditions (e.g., similar harvest density, similar passage numbers).

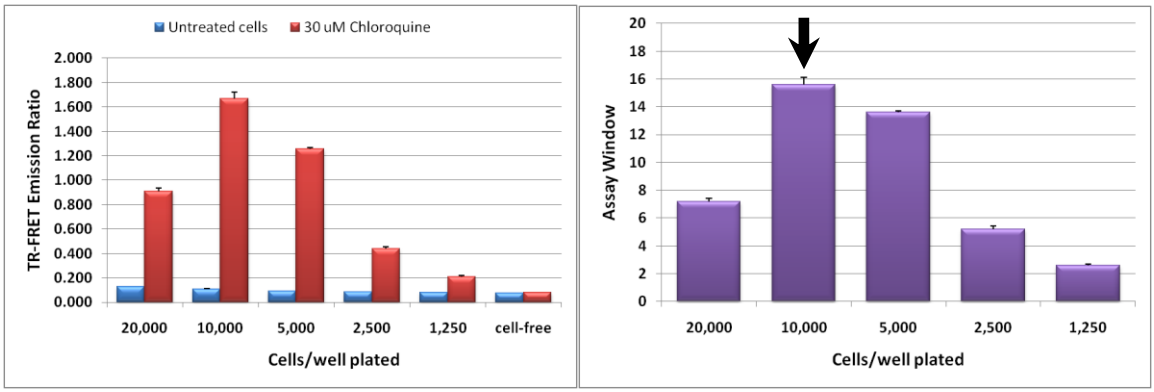

Figure 12. The number of cells plated per well affects assay performance. GripTite™ 293 cells stably expressing GFP-tagged LC3B were plated at varying cell plating densities in 384-well format and incubated overnight in nutrient rich assay media (Opti-MEM® I without phenol red + 10% dialyzed FBS + supplements). Next day, cells were left untreated or treated with 30 µM chloroquine overnight. On day 3, lysis buffer/detection antibody was applied to the wells and Tb/GFP TR-FRET was measured on a PerkinElmer Envision® plate reader (with TRF laser light source) and plotted as the mean ± standard deviation (n = 3). In this example, the largest Assay Window was obtained by using a cell plating density of ~10,000 cells/well in 384-well format.

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.

How does a different assay medium affect the assay?

The media used for the setting up the assay can have a significant impact on assay outcome, due to its influence on variables such as GFP-tagged LC3B expression levels, cell growth and proliferation within the assay time-course, as well as directly modulating autophagic activity via differences in nutrient levels (Figures 7 and 13). 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.

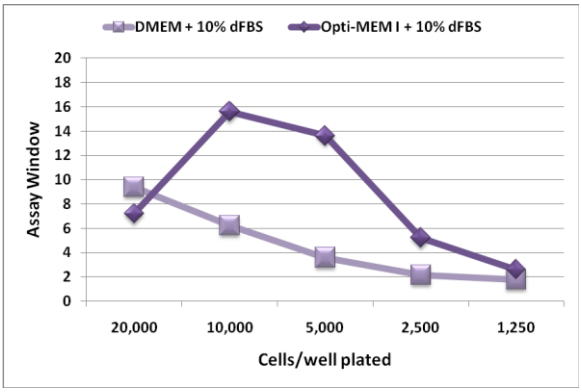

Figure 13. Assay Media affects assay performance. GripTite™ 293 cells stably expressing GFP-tagged LC3B were plated at varying cell plating densities in 384-well format and incubated overnight in nutrient rich assay media (Opti-MEM® I without phenol red + 10% dFBS + supplements) or growth media (DMEM without phenol red + 10% dFBS + supplements). Next day, cells were left untreated or treated with 30 µM chloroquine overnight. On day 3, lysis buffer/detection antibody was applied to the wells and Tb/GFP TR-FRET was measured on a PerkinElmer Envision® plate reader (with TRF laser light source) and plotted as the mean ± standard deviation (n = 3). In this example, the largest Assay Windows were achieved by using Opti-MEM® I-based Assay Media with this cell-type.

What is the optimal incubation time with the lysis/Tb-anti-LC3B detection antibody?

The incubation time of lysis buffer/detection antibody prior to reading the assay plate can have a modest effect on assay performance (Figure 14). Incubation for 1 – 2 hours at room temperature prior to reading the plate is recommended to ensure adequate lysis and detection. Longer incubation times may also be used as long as evaporation is minimized. For best assay reproducibility, using consistent incubation times is recommended.

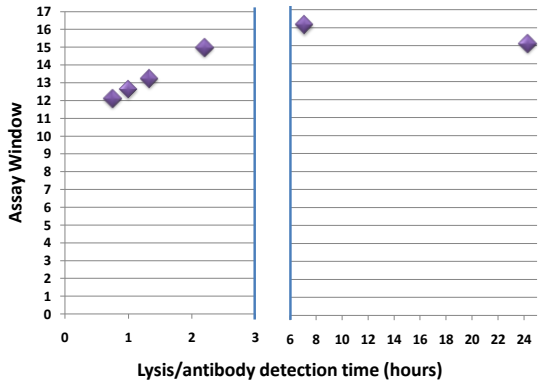

Figure 14. Incubation time of lysis buffer/antibody prior to reading the plate can have a modest effect upon assay performance. U-2OS cells were transduced with 1% LC3B-GFP BacMam reagent in 384-well format overnight. Next day, cells were left untreated or treated with 50 µM chloroquine overnight. On day 3, lysis buffer/detection antibody was applied to the wells and Tb/GFP TR-FRET was measured on a PerkinElmer Envision® plate reader (with TRF laser light source) at several different times and the average Assay Window was calculated (n = 6).

Can I cryopreserve the LC3B-GFP BacMam transduced cells for future use or for making a large batch for library screening?

Cells can be cryopreserved in large batches 24 – 48 hours post BacMam transduction for later use (Figure 15).

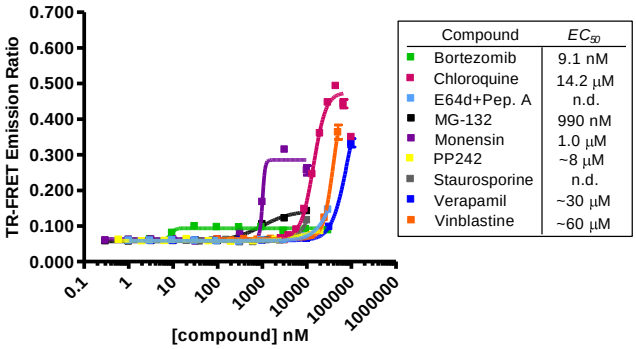

Figure 15. Cells can be transduced with LC3B-GFP BacMam and cryopreserved for later use. U-2OS cells were transduced with 0.25% LC3B-GFP BacMam reagent for ~24 hours and aliquots of the cells were cryopreserved. At a later date, a vial of the transduced cells was thawed and resuspended in growth media. The cells were plated onto a 384-well assay plate containing serial dilutions of a small panel of test compounds and incubated overnight prior to adding lysis buffer/detection antibody and reading TR-FRET on a PerkinElmer Envision® plate reader (with TRF laser light source) and plotted as the mean ± standard deviation (n = 2).

V. Optimizing plate reader settings

What does someone need to know about setting up their plate reader for this assay?

Terbium-based TR-FRET (LanthaScreen® Terbium) technology requires specific plate reader settings that are critical to experimental success. For detailed information about settings recommended for your specific instrument and to purchase filters, visit www.invitrogen.com/instrumentsetup. Please note that not all fluorescence plate readers are capable of performing time-resolved fluorescence. In addition, we do not recommend using monochromator-based instruments with the Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay as the sensitivity of these plate readers is generally not sufficient to detect the Tb/GFP TR-FRET signal (Figure 16).

Prior to setting up an assay, we strongly recommend verifying your plate reader setup for Tb-based TR-FRET (LanthaScreen® Terbium) detection using the **Instrument Control Terbium TR-FRET kit** (Catalog no. A14138) included with the Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay kits. The HIGH/LOW fold-change should be 2 – 4, depending on the plate reader used (Figure 17). Values near or below 2 may indicate that the instrument is not setup properly and/or lacks enough sensitivity for Tb-based TR-FRET.

For technical assistance, please contact Drug Discovery Technical Support at drugdiscoverytech@lifetech.com or 760-603-7200, extension 40266).

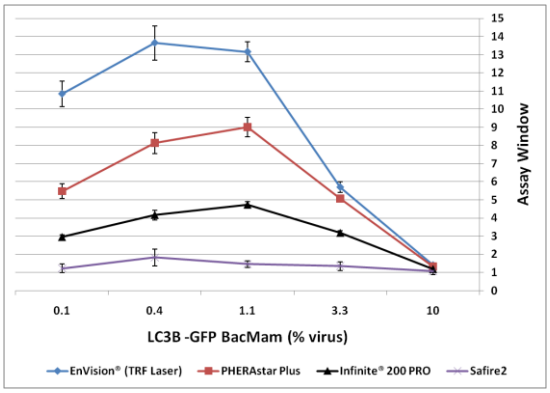

Figure 16. Assay performance testing on different TR-FRET plate readers: PerkinElmer Envision®, BMG LABTECH PHERAstar *Plus*, Tecan Infinite® 200 PRO, and Tecan Safire². U-2OS cells were transduced with a titration of LC3B-GFP BacMam in 384-well format. Next day, cells were incubated overnight in growth media with or without 50 µM chloroquine and then lysis/detection antibody was applied to the wells and TR-FRET was measured on four different plate readers. Assay Window values were plotted as the mean ± standard deviation (n = 6). Note that the Tecan Safire² gave unsatisfactory performance and is a monochromator-based plate reader whereas the other three readers are filter-based.

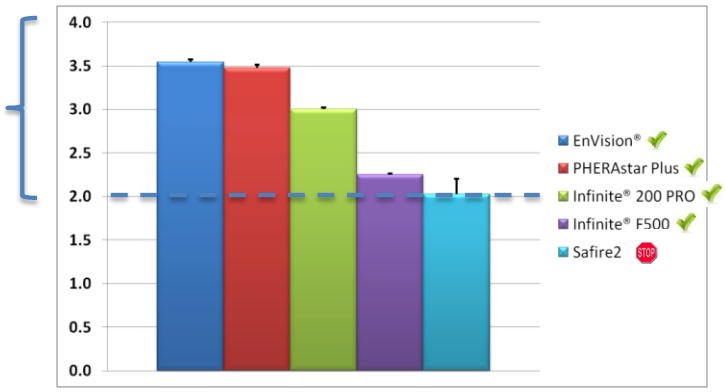

Figure 17. Verification of Plate reader setup using the instrument Control Terbium TR-FRET kit. Several wells of the HIGH and the LOW Instrument Control were plated and then the Tb/GFP TR-FRET was read. The HIGH/LOW-fold change was calculated and plotted here. In this example, the monochromator-based Tecan Safire² plate reader did not meet the >2 cut-off, suggesting it lacks enough sensitivity for performing Tb/GFP TR-FRET-based cellular assays.

How important is it that I use the exact filter set specified for my plate reader?

The emission filter wavelengths and their respective bandwidth requirements specified for your plate reader are exact and should not be substituted (see www.invitrogen.com/instrumentsetup). For example, do not use a 520 nm filter with a 10 nm bandwidth instead of the specified 25 nm bandwidth; substitution with the smaller 10 nm bandwidth will yield very poor to unusable raw GFP acceptor emission signals (also see Figure 4). Note that Tb-based TR-FRET (LanthaScreen® Terbium) emission filters may not come standard with your plate reader and may need to be purchased separately.

There is some flexibility with the excitation filter (typically 340 nm with 30 or 60 nm bandwidth) and appropriate excitation filters often come standard with TR-FRET compatible fluorescence plate readers and are more commonly available.

What plate reader settings may need to be optimized?

Depending on your specific plate reader, the following settings may need to be optimized for best assay performance:

Number of flashes: the number of flashes to use can affect the magnitude of the signal detected. For instruments with a flash lamp light source (most common) we recommend using 200 flashes for the TR-FRET GFP acceptor channel (520 nm) and 100 flashes for

the TR-FRET Tb donor channel (495 or 490 nm). Fewer flashes can be used when using a TRF laser light source (typically 20 and 10 flashes).

Plate/focal height: certain instruments (e.g., PerkinElmer EnVision®, BMG LABTECH PHERAstar) allow for checking and optimizing the focal height which can differ significantly by plate type (e.g., 96-well vs. 384-well) and also by the assay volume in the well. Once proper setup is established using the Instrument Control Terbium Kit, we recommend that any further refinements be made using an actual assay control (e.g., chloroquine-treated control; also see Section I Controls needed for running the assay).

Gain: some plate readers (e.g., Tecan Infinite® F200 PRO, Infinite® F500) allow for adjusting the gain setting used by selecting a specific well (usually the well expected to have the highest signal) or set of wells to optimize the gain against. In general, we recommend selecting "optimal" gain and then making any additional manual adjustments to the gain only if needed. **Important:** if you choose to plate the Instrument Controls on the same assay plate used to run a Premo™ Autophagy LC3B assay we recommend reading the plate twice: first with the gain setting optimized against the assay wells (e.g., chloroquine-treated control) and not against the Instrument Controls, and then a second time with gain setting optimization performed against the HIGH Instrument Control. The reason for this is that in certain cases the raw signal intensities for the assay wells may be sufficiently different from the raw signals for the Controls so as to alter the gain setting selected by the reader's optimization routine, which directly affects instrument sensitivity.

REFERENCES:

Carlson, C. B., Mashock, M.J., and Bi, K. (2010) BacMam-enabled LanthaScreen cellular assays for PI3K/Akt pathway compound profiling in disease-relevant cell backgrounds. *J. Biomol. Screen. 15, 327-334.*

Klionsky et al. (2008) Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy 4, 151-175.*

Kost, T. A., Condreay, J. P., Ames, R. S., Rees, S., and Romanos, M. A. (2007) Implementation of BacMam virus gene delivery technology in a drug discovery setting. *Drug Disc. Today 12:396-403.*

Mizushima, N., Yoshimori, T., and Levine, B. (2010) Methods in mammalian autophagy research. *Cell 140:313-326.*

Robers, M. B., Horton, R. A., Bercher, M. R., Vogel, K. W., and Machleidt, T. (2008) High-throughput cellular assays for regulated post-translational modifications. *Anal. Biochem. 372:189–197.*