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

Catalog no. A14292 or A14294 **Shipping:** 4°C **Storage:** 4°C

Protocol part no. MAN0005546 **Rev. date:** 18 October 2011

Table of Contents

Kit Contents and Handling

Component	Part no.	Amount	Storage	Handling
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.

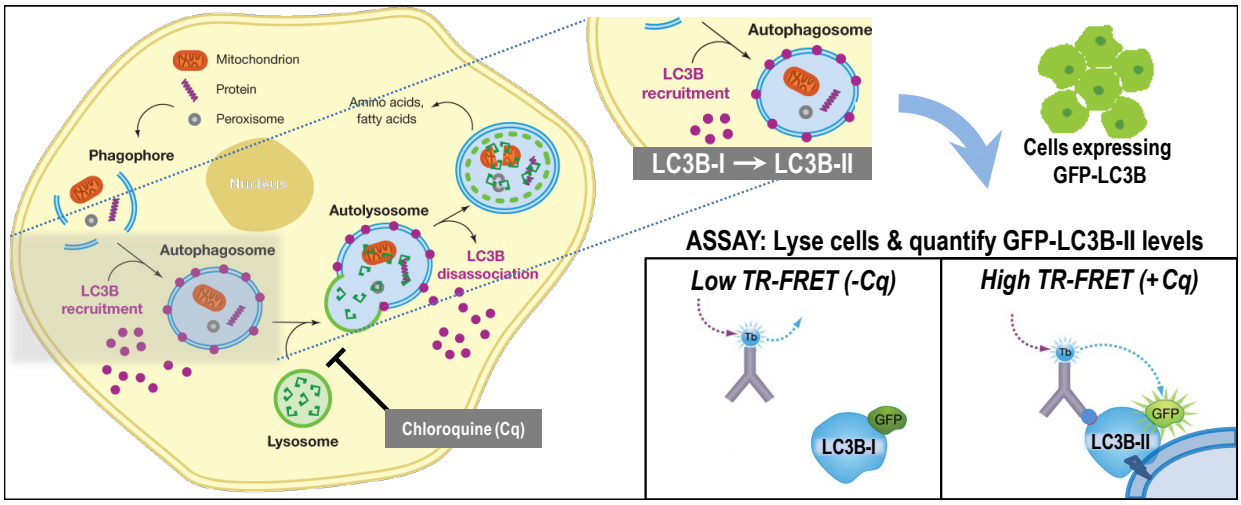

Figure 1 Mechanism of Action for the Premo™ Autophagy Tb/GFP TR-FRET LC3B Assay. The assay step involves a single addition of cell lysis buffer containing Tb-labeled detection antibody selective for LC3B-II. Relative levels of LC3B-II are quantified using a fluorescence plate reader capable of detecting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

Assay Workflow

Day 1: Cells expressing GFP-tagged LC3B are plated overnight (e.g., 16–24 hours) to allow cells to adhere.

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.

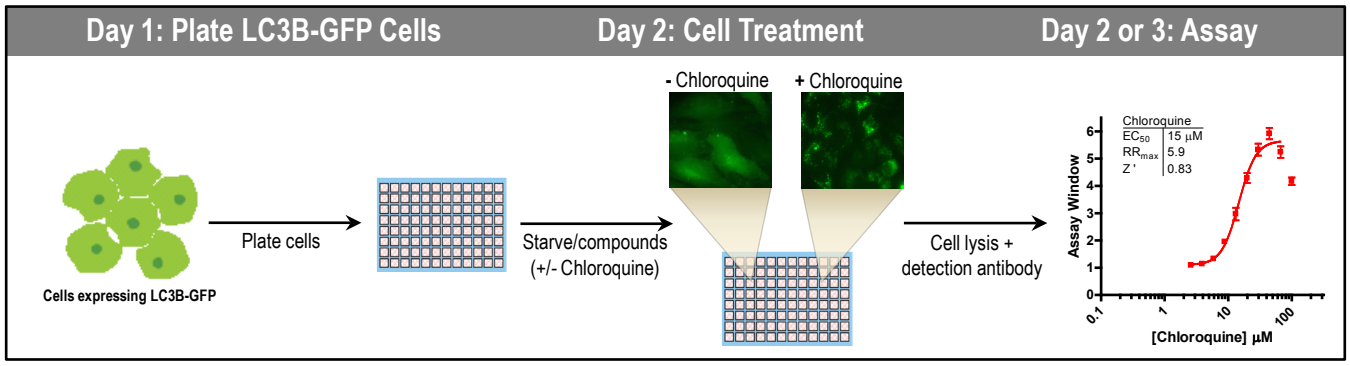

Figure 2 Representative Assay Workflow. Cells expressing GFP-tagged LC3B are plated in 96- or 384-well format. Next day, cells are treated to induce autophagy (e.g., starve, mTOR inhibitors) leading to conversion of cytosolic LC3B-I to autophagosome-associated LC3B-II. Lysosomal inhibitor chloroquine 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 Expressing GFP-tagged LC3B (stable line or transiently expressing cells)	Various	Various
Alternatively, the Premo™ Autophagy Sensor LC3B-GFP (BacMam 2.0) reagent can be used to express GFP-tagged LC3B in many different cell types.	Invitrogen	P36235
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

Preparing Recommended Media

Media components	Recommended Source	Part no.	Nutrient-rich Assay Medium	Starve Medium
Opti-MEM® I (without phenol red)	Invitrogen	11058-021	500 mL	—
Earle's Balanced Salt Solution (EBSS)	Invitrogen	14155-063	—	500 mL
Fetal Bovine Serum (dialyzed recommended)	Invitrogen	26400-036	50 mL	—
Nonessential Amino Acids (NEAA, 100X)	Invitrogen	11140-050	5 mL	—
Sodium Pyruvate (100X)	Invitrogen	11360-070	5 mL	—
Penicillin/Streptomycin (100X)	Invitrogen	15140-122	5 mL	—
HEPES Buffer Solution (1 M)	Invitrogen	15630-080	—	10 mL
$CaCl_2$ and $MgCl_2$ (*optional*: improves cell adherence)	Various	Various	—	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:

- 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.

Titration of GFP-LC3B cells plated per well

Assay performance is influenced by the number of GFP-LC3B expressing cells plated per well. Therefore, we recommend performing a titration of the number of cells plated with your cells as outlined in the example protocol below. Select the lowest cell plating density that yields the largest assay window.

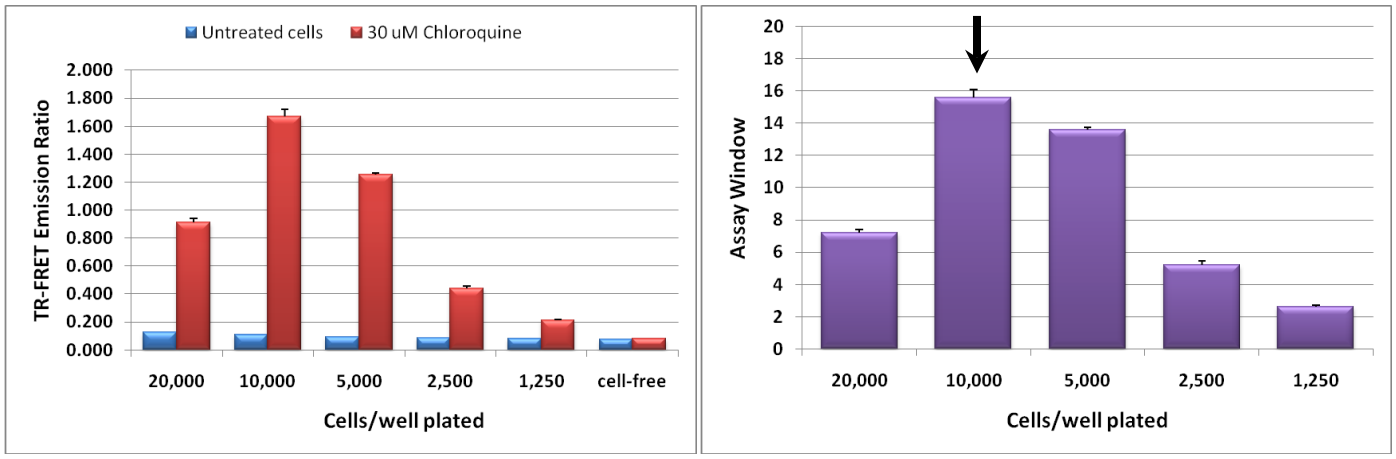

Figure 3 GFP-LC3B Cell Plating Titration Example. As outlined in the Quick Reference Protocol, GripTite™ 293 cells stably expressing GFP-LC3B were harvested, resuspended in nutrient-rich Assay Medium, and plated in 384-well format. Next day, cells were left untreated or treated with 30 µM chloroquine 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 cell plating density 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 when cells were plated at 10,000 cells/well.

Assay Protocols

Quick Reference Protocol

This example protocol allows for testing a titration of the number of GFP-LC3B expressing cells plated per well in the presence or absence of chloroquine or other test compound. Depending on cell-type, the chloroquine concentration/treatment time may need to be further optimized. 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.

Table 1. Assay Setup guide

Plate format	Number of tests A14294	Number of tests A14292	Total volume	Plating volumes/well
96-well	333	2000	90 µL/well	60 µL GFP-LC3B expressing cells 15 µL 5X Chloroquine or test compound 15 µL Complete 6X lysis buffer + antibody*
384-well	1000	6000	30 µL/well	20 µL GFP-LC3B expressing cells 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.

Plate GFP-LC3B Cells (Day 1)

1. Grow cells expressing GFP-LC3B 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 ≥ 1 mL of cells at 1×10^6 cells/mL
3. Prepare four 2-fold serial dilutions of the cells in Assay Medium.
   - e.g., add 0.5 mL of 1×10^6 cells/mL to 0.5 mL Assay Medium, mix, then dilute 0.5 mL cells + 0.5 mL media, repeat two more times
4. Plate the volume listed in Table 1 of the 1×10^6 cells/mL and the four dilutions to white opaque assay plate as per Figure 4.
5. Incubate the cells at 37°C and 5% CO_2 for 16–24 hours to allow cells to adhere.

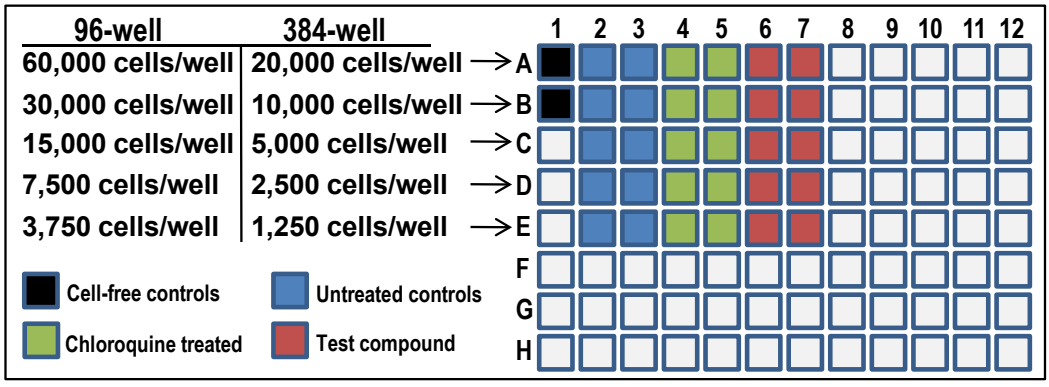

Figure 4 Suggested plate layout for initial cell titration testing (two replicates per variable). Scale or modify as desired.

Cell treatment (Day 2)

1. *Optional*: remove plating 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 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., several 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 (page 10).

STEP 1: Plate GFP-LC3B Cells

1. Begin with a healthy, sub-confluent cell culture grown under normal Growth Medium conditions (e.g., 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 also be plated immediately following thaw to save culturing time.

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

   Note: We recommend testing a titration of the cell plating density to identify the optimal number of cells per well to use for best assay performance. The Quick Reference Protocol provides an example of how to do this.

4. *Optional:* Plate remaining cells in a parallel clear-bottom plate for visually monitoring the cells.

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

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

   Note: The incubation time prior to cell treatment can be adjusted as needed, or in some cases omitted entirely (i.e., immediately begin cell treatment at time of plating the GFP-LC3B expressing cells).

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. In general, we recommend incubating chloroquine-treated 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: We recommend scaling 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, please contact Drug Discovery Technical Support at **drugdiscoverytech@lifetechnologies.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 distort the results. However, in certain cases it may be desirable to perform background subtraction by subtracting the Emission Ratio 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 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 performed. For more information about your specific instrument and to purchase filters, visit **www.invitrogen.com/instrumentsetup**. Contact Drug Discovery technical support for more information.
	Use only white opaque plates with this assay. Black or clear-bottom plates will not work well.
	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. Alternatively, the Premo™ Autophagy Sensor LC3B-GFP (BacMam 2.0, Cat. no. P36235) reagent has been confirmed to work with this assay and can be used to express GFP-tagged LC3B in many different cell types. For further details, please visit **www.lifetechnologies.com** and search for A14070 to download a protocol for using BacMam reagent to setup the assay. Protocols are located under the "How to Use" tab on the product page.
	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. The concentration of chloroquine to use and incubation time may need to be optimized for your cell type.
	Check to make sure that the cell plating density used 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 for best assay performance.
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).

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