molecular probes®
by life technologies™

Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B Kit

Catalog no. P36239

Table 1 Contents and storage

Material	Amount	Concentration	Storage*
RFP-GFP-LC3B (Component A)	1 mL	~1 × 10^8 viral particles/mL	• 2–8°C • Protect from light • DO NOT FREEZE
Chloroquine diphosphate (Component B)	1 mL	30 mM aqueous solution	• ≤25°C • Protect from light
Approximate fluorescence excitation/emission maxima: Emerald GFP: 488/509 in nm; TagRFP: 555/584 in nm.			
* When stored as directed, this kit is stable for at least 6 months.			

Introduction

Autophagy describes the segregation and delivery of cytoplasmic cargo, including proteins and organelles, for degradation by hydrolytic enzymes through the lysosomal machinery. Although first described in 1963, it has only been in the past decade that this pathway has been the subject of intense research to gain further insight into the role basal autophagy plays in cell homeostasis and development (Figure 1, page 2). Efforts are also directed to further elucidate the role of induced autophagy as a cell survival response to stress, microbial infection, and disease (e.g., neurodegeneration, cancer).[1–3]

Single FP (fluorescent protein) chimeras such as the Premo™ Autophagy Sensor LC3B-GFP (Cat. no. P36235) or LC3B-RFP (Cat. no. P36236) are useful for monitoring autophagosome formation, especially when combined with other FP chimeras or fluorescent reagents. However, the use of a tandem FP construct that allows an enhanced dissection of the maturation of the autophagosome to the autolysosome has recently been described.[4,5] By combining an acid-sensitive GFP (i.e., Emerald GFP) with an acid-insensitive RFP (i.e., TagRFP), the change from an autophagosome (neutral pH) to the autolysosome (with an acidic pH) can be visualized by imaging the specific loss of the GFP fluorescence upon acidification of the autophagosome following lysosomal fusion. Upon induction of autophagy, the Premo™ Tandem Autophagy Sensor labels the punctate autophagosomes; these structures are positive for both GFP and RFP. Once the lysosome has fused, the pH drops, which quenches the GFP, making autolysosomes appear red (Figure 2, page 2, and Figure 3, page 3). Combining Premo™ Autophagy Tandem Sensor with the far-red emitting LysoTracker® Deep Red allows for a three-color analysis of the autophagosomal/autolysosomal/lysosomal dynamics.

The Premo™ Autophagy Tandem Sensor combines the selectivity of an LC3B-fluorescent protein (FP) chimera with the transduction efficiency of the BacMam 2.0 technology. BacMam reagents (insect **Bac**ulovirus with a **Mam**malian promoter) are safe to handle (Biosafety Level 1) because they are non-replicating in mammalian cells. They are also non-cytotoxic and ready-to-use. Unlike expression vectors, BacMam reagents enable titratable and reproducible expression and offer high co-transduction efficiency. Recent improvements made to the BacMam system, BacMam 2.0, enable efficient transduction in a wider variety of cells, including neurons and neural stem cells (NSCs).

Each Premo™ Autophagy Sensor Kit includes chloroquine diphosphate for artificially generating autophagosomes. Following treatment with chloroquine diphosphate, normal autophagic flux is disrupted and autophagosomes accumulate as a result of the increased lysosomal pH that inhibits lysosomal fusion with the autophagosomes.

Figure 1 Schematic depiction of the autophagy pathway in a eukaryotic cell. The first step involves the formation and elongation of the isolation membranes or phagophore. The second step entails the expansion and sequestering of the cytoplasm and formation of the double-membrane autophagosome and includes the association of the cytosolic LC3B protein. Fusion of lysosomes with autophagosome to generate the autolysosome is the penultimate step. In the fourth and final phase, the cargo is degraded.

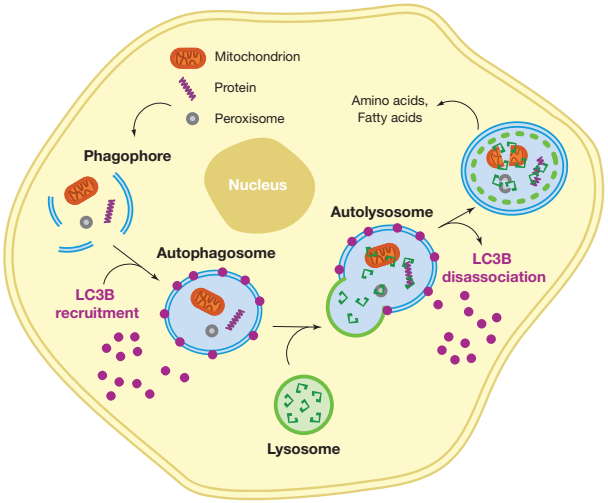


Figure 2 The use of the Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B allows for an enhanced dissection of the maturation of the autophagosome to the autolysosome. By combining an acid-sensitive GFP with an acid-insensitive RFP, the change from autophagosome (neutral pH) to autolysosome (with an acidic pH) can be visualized by imaging the specific loss of the GFP fluorescence, leaving only red fluorescence.

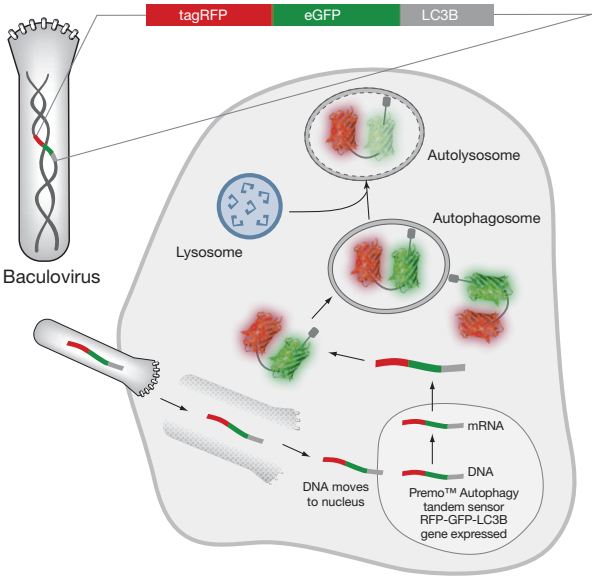
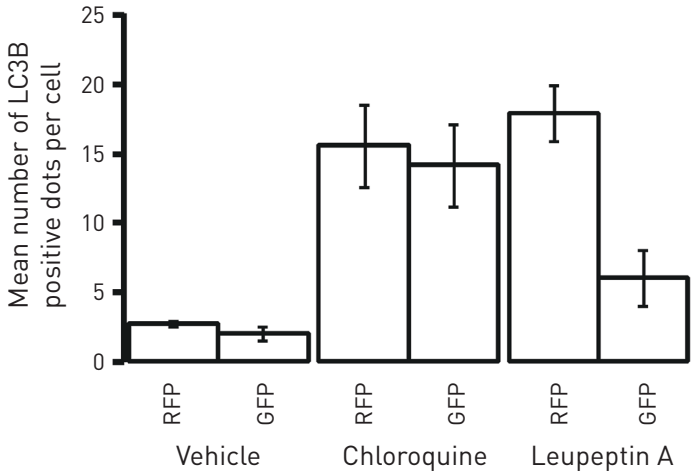


Figure 3 Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B can be used to discriminate between acidic and neutral LC3B-positive vesicles. HeLa cells were transduced with 40 particles per cell of the Premo™ Autophagy Tandem Sensor and cultured for 24 hours. Cells were then incubated in either Vehicle, 90 µM Chloroquine or 200 µM Leupeptin A for an additional 24 hours. Cells were imaged using standard FITC/TRITC filter sets. Chloroquine blocks autophagy through neutralization of lysosomal pH; as a result, fluorescence is seen from both GFP and RFP, reflected in a significantly higher number of GFP and RFP positive spots compared to vehicle treated cells. Leupeptin A blocks autophagy without changing lysosomal pH; consequently, GFP fluorescence is lost in the acidic environment of the autolysosome whereas TagRFP retains its fluorescence. Therefore, a significant increase in the number of RFP positive spots with a decrease in the number of GFP positive spots is observed in Leupeptin A-treated cells.



Before Starting

Materials Required but Not Provided

- Cell culture medium

Guidelines for Working with BacMam Reagents

- The following protocol is based on a 2 mL labeling volume and ~40,000 cells plated in a 35-mm dish or 1 well of a 6-well culture plate, with a PPC (particles per cell) of 30.
- For applications that require a larger number of cells, such as high-content screening (HCS), we recommend transducing the cells in a 10-cm dish or a T-75 flask and increasing the labeling volume to 10 mL with a proportionate increase in the volume of the virus. Following an overnight incubation under normal growth conditions, trypsinize and count cells for distribution to appropriate plates at cell number desired.
- For optimal results you may alter the PPC, cell density, temperature, and incubation time.
- We recommend transducing cells at a confluency of about 70% for best results.
- For first time users of BacMam reagents, we recommend using the exceptionally well-transduced cells like U-2 OS (ATCC CCL-11226).

Experimental Protocols

Protocol

The following protocol was optimized using cells that have been plated. You can also treat the cells in suspension prior to plating.

1.1 Plate the cells at the desired density and allow them sufficient time to adhere. Premo™ Autophagy Sensors work best when used on low-passage-number cells.

1.2 Calculate the volume of the tandem LC3B-FP (Component A) using the equation below:

$$\text{mL of tandem LC3B-FP} = \frac{\text{(number of cells) (PPC)}}{\text{(1} \times 10^8 \text{ Premo™ particles/mL)}}$$

where the number of cells is the estimated total number of cells at the time of cell labeling, PPC (particles per cell) is the number of viral particles per cell, and 1×10^8 is the number of viral particles per mL of the reagent.

For example, to label 40,000 cells with a PPC of 30,

$$\text{mL of tandem LC3B-FP} = \frac{\text{(40,000) (30)}}{\text{(1} \times 10^8 \text{ Premo™ particles/mL)}} = 0.012 \text{ mL (12 µL)}$$

1.3 Mix each LC3B reagent several times by inversion to ensure a homogenous solution. **Do not vortex the LC3B reagent.**

1.4 Add the LC3B reagent directly to the cells in complete cell medium and mix gently.

1.5 Incubate the cells overnight (≥16 hours).

1.6 *Optional*: Treat the control cells with 30–100 µM chloroquine (Component B) for 12–16 hours.

Note: For best results, allow cells 48 hours post-transduction for expression levels to equilibrate.[6,7]

1.7 Image and analyze the cells using the appropriate instrument filter sets for GFP and RFP detection. Autophagosomes are typically located in the perinuclear region.

Notes

A. Premo™ Autophagy Tandem Sensors were designed for use in live-cell imaging of autophagy. The cell-permeant nucleic acid stains Hoechst 33342 and HCS NuclearMask™ Blue stains are spectrally compatible with the Premo™ Autophagy Tandem Sensor fluorescence. Should you prefer fixed cell analysis, the fluorescence from GFP and RFP has been demonstrated to be resistant to fixation with 4% formaldehyde and permeabilization with 0.1% Triton® X-100. Fixation and permeabilization enables processing of labeled cells with antibodies to other cellular targets. We recommend fixed cell format for large sample sizes, such as for HCS. Note that the fixation treatment may need to be optimized, and that certain treatments may change the pH and thus alter the fluorescence intensity of either fluorescent protein.

B. Be aware of bleed-through from one fluorescent protein into the detection channel of another,[6] especially with the excitation of TagRFP by GFP filter sets and the differential brightness of GFP versus TagRFP.

C. GFP and TagRFP have differential photostabilities. Therefore, artifacts caused by photobleaching during time-lapse imaging should be taken into consideration.

D. Drug treatments may be used to calibrate the system. Chloroquine (Component B) is an excellent drug to block autophagic flux, which causes the accumulation of GFP- and TagRFP-positive vesicles. These protocols allow for the extent of bleed-through and bleaching to be examined. Note that Chloroquine inhibits autophagy by neutralizing lysosomal pH. The hydrolases in the lysosome require an acidic environment to function; even mild alkalinization inhibits these enzymes. Conditions that inhibit the enzymes may not be sufficient to alkalinize the autolysosomes sufficiently to rescue GFP fluorescence. For this reason, chloroquine should be used at relatively high concentrations (>90 µM) for at least 12 hours. Leupeptin A or other drugs such as an E64D/Pepstatin A cocktail inhibit lysosomal enzymes without affecting pH and can be used to block autophagy, resulting in an accumulation of autolysosomes that maintain their acidity, thereby quenching GFP fluorescence. We recommend 200 µM Leupeptin A treatment for 18 hours. Under these conditions, cells with predominantly RFP-positive and GFP-negative vesicles are seen. Earle's Balanced Salt Solution (EBSS) nutrient deprivation is also a useful control that allows for the progression from diffuse GFP/RFP fluorescence to GFP/RFP-positive autophagosomes, followed by RFP-positive autolysosomes.

References

1. Molecular Cell Biology 8, 931 (2007); **2.** Genes & Development 21, 2861 (2007); **3.** Drug Discovery 6, 304 (2007); **4.** J Cell Biol 171, 603 (2005); **5.** FEBS Letts 512, 19 (2002); **6.** J Biol Chem 282, 24131 (2007); **7.** Meth Enz 452, 25 (2009); **8.** Meth Enz 452, 181 (2009); **9.** Autophagy 8, 445 (2012).

Product List Current prices may be obtained from our website or from our Customer Service Department.

Cat. no.	Product Name	Unit Size
P36239	Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B Kit	1 kit
Related Products		
P36235	Premo™ Autophagy Sensor LC3B-GFP *BacMam 2.0*	1 kit
P36236	Premo™ Autophagy Sensor LC3B-RFP *BacMam 2.0*	1 kit
P36240	Premo™ Autophagy Sensor GFP-p62	1 kit
P36241	Premo™ Autophagy Sensor RFP-p62	1 kit
C10596	CellLight™ Lysosomes-GFP *BacMam 2.0*	1 mL
C10597	CellLight™ Lysosomes-RFP *BacMam 2.0*	1 mL
C10600	CellLight™ Mitochondria-GFP *BacMam 2.0*	1 mL
C10601	CellLight™ Mitochondria-RFP *BacMam 2.0*	1 mL
H3570	Hoechst 33342, trihydrochloride, trihydrate *10 mg/mL solution in water*	10 mL
H10325	HCS NuclearMask™ Blue	65 µL
L7528	LysoTracker® Red DND-99 *1 mM solution in DMSO* *special packaging*	10 × 50 µL
L7535	LysoTracker® Red DND-26 *1 mM solution in DMSO* *special packaging*	10 × 50 µL
L10382	LC3B Antibody Kit for Autophagy *rabbit polyclonal LC3B* *includes autophagosome inducer*	1 kit
M7510	MitoTracker® Orange CMTMRos *special packaging*	10 × 50 µL
M7512	MitoTracker® Red CMXRos *special packaging*	10 × 50 µL
M7514	MitoTracker® Green FM *special packaging*	10 × 50 µL
M22423	MitoTracker® Deep Red *special packaging*	10 × 50 µL

Purchaser Notification

Corporate Headquarters
5791 Van Allen Way
Carlsbad, CA 92008
USA
Phone: +1 760 603 7200
Fax: +1 760 602 6500
Email: techsupport@lifetech.com

European Headquarters
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF
UK
Phone: +44 141 814 6100
Toll-Free Phone: 0800 269 210
Toll-Free Tech: 0800 838 380
Fax: +44 141 814 6260
Tech Fax: +44 141 814 6117
Email: euroinfo@invitrogen.com
Email Tech: eurotech@invitrogen.com

Japanese Headquarters
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Japan
Phone: +81 3 5730 6509
Fax: +81 3 5730 6519
Email: jpinfo@invitrogen.com

Additional international offices are listed at www.lifetechnologies.com

These high-quality reagents and materials must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Read the Safety Data Sheet provided for each product; other regulatory considerations may apply.

Obtaining Support
For the latest services and support information for all locations, go to www.lifetechnologies.com.

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (techsupport@lifetech.com)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

SDS
Safety Data Sheets (SDSs) are available at www.lifetechnologies.com/sds.

Certificate of Analysis
The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to www.lifetechnologies.com/support and search for the Certificate of Analysis by product lot number, which is printed on the product packaging (tube, pouch, or box).

Disclaimer
LIFE TECHNOLOGIES CORPORATION AND/OR ITS AFFILIATE(S) DISCLAIM ALL WARRANTIES WITH RESPECT TO THIS DOCUMENT, EXPRESSED OR IMPLIED, INCLUDING BUT NOT LIMITED TO THOSE OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, OR NON-INFRINGEMENT. TO THE EXTENT ALLOWED BY LAW, IN NO EVENT SHALL LIFE TECHNOLOGIES AND/OR ITS AFFILIATE(S) BE LIABLE, WHETHER IN CONTRACT, TORT, WARRANTY, OR UNDER ANY STATUTE OR ON ANY OTHER BASIS FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING BUT NOT LIMITED TO THE USE THEREOF.

Limited Product Warranty
Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

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 services of any kind, including, without limitation, 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 Corporation, 5791 Van Allen Way, Carlsbad, California 92008.

Limited Use Label License No. 127: GFP with Heterologous Promoter
Notice to Purchaser: 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. 306: Baculovirus Vectors
Notice to Purchaser: 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

Notice to Purchaser: 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 obtaining additional rights, please contact outlicensing@lifetech.com 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. 321: TagRFP

Notice to Purchaser: This product is for the buyer's internal research use only and may not be used for commercial purposes. No rights are conveyed to modify or clone the gene encoding fluorescent protein contained in this product. The right to use this product specifically excludes the right to validate or screen compounds for commercial purposes. For information on commercial licensing, contact Licensing Department, Evrogen JSC, email: license@evrogen.com.

life technologies™