Premo™ Autophagy Sensors

Catalog Numbers P36235, P36236, P36240, P36241, P36239

Pub. No. MAN0026683 Rev. A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from thermofisher.com/support.

Product description

Autophagy describes the sorting and delivery of cytoplasmic cargo, including proteins and organelles, for degradation by hydrolytic enzymes through the lysosomal machinery. 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). 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) (Klionsky, 2007; Mizushima, 2007; Rubinsztein et al., 2007).

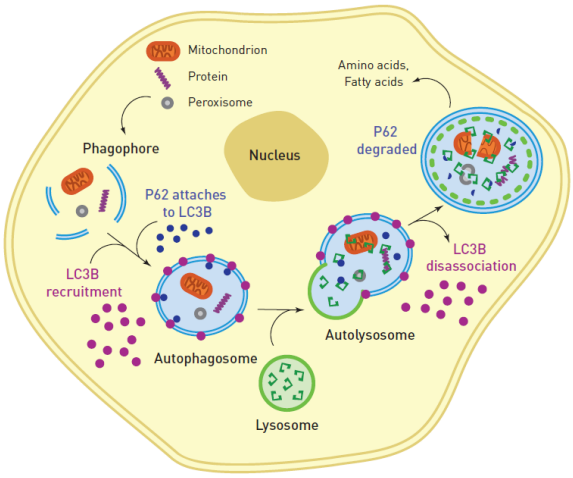

Figure 1 Schematic depiction of the autophagy pathway in a eukaryotic cell.
The first step involves the formation and elongation of the isolation membranes or phagophore. The second step entails the expansion and sequestering of the cytoplasm and formation of the double-membrane autophagosome and includes 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.

The Premo™ Autophagy Sensor and Premo™ Autophagy Tandem Sensor Kits combine the selectivity of an LC3B- or p62-fluorescent protein (FP) chimera or tandem RFP-GFP-LC3B construct with the transduction efficiency of the BacMam 2.0 technology. BacMam reagents (**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. Multiple BacMam reagents can therefore be readily used in the same cell. 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). The two-step protocol for imaging autophagy involves simply adding the BacMam 2.0 chimera or tandem reagent to the cells, incubating overnight, and then visualizing transduction using standard GFP (green fluorescent protein) and/or RFP (red fluorescent protein) settings.

Premo™ Autophagy Sensor LC3B-FP Kits

The LC3B protein plays a critical role in autophagy. Normally, this protein resides in the cytosol, but following cleavage and lipidation with phosphatidylethanolamine, LC3B associates with the phagophore. This localization can be used as a general marker for autophagic membranes (Figure 1).

Each Premo™ Autophagy Sensor LC3B-FP Kit includes the BacMam LC3B-FP, a control BacMam LC3B(G120A)-FP, and chloroquine diphosphate to induce autophagosomes (Figure 2). The mutation on the control BacMam LC3B prevents its cleavage and subsequent lipidation during normal autophagy, and protein localization remains cytosolic and diffuse. 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.

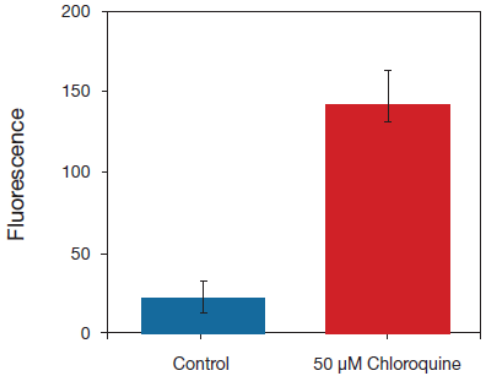

Figure 2 Autophagy detection with Premo™ Autophagy Sensor LC3B-GFP.
HeLa cells were plated into a 96-well plate at 5,000 cells per well and allowed to adhere overnight. Cells were then transduced with LC3B-GFP. 24 hours later, the cells were incubated with 50 µM chloroquine or left untreated (control) for 16 hours. Analysis was performed by quantifying the fluorescence from vesicular structures in the perinuclear region using the Thermo Scientific™ Cellomics™ ArrayScan™ VTI platform with Compartmental Analysis.

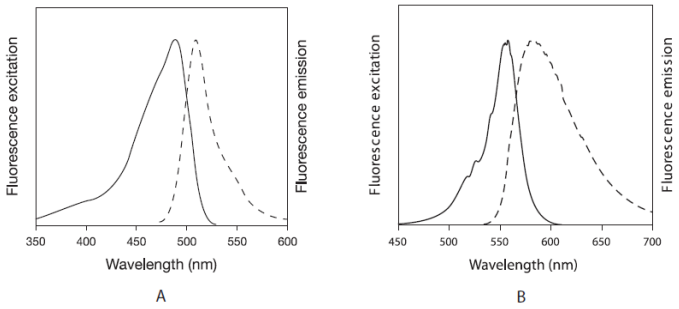

Figure 3 Spectra for Premo™ Autophagy Sensor LC3B-FP Kits.
Fluorescence excitation and emission spectra for Premo™ LC3B-Green Fluorescent Protein (LC3B-GFP, panel A) and Premo™ LC3B-Red Fluorescent Protein (LC3B-RFP, panel B).

Premo™ Autophagy Sensor p62-FP Kits

The p62/SQSTM1 protein is a receptor for cargo destined to be degraded by autophagy, specifically cargo that is ubiquitinated and serves to clear protein aggregates (Bjørkøy et al., 2005). The p62/SQSTM1 protein is able to bind both ubiquitin (Geetha and Wooten, 2002) and the key autophagic marker ATG8/LC3 (Pankiv et al., 2007), thereby facilitating clearance of ubiquitinated proteins via autophagy. Labeling of p62 serves as a useful marker for the induction of autophagy, clearance of protein aggregates, and the inhibition of autophagy.

Each Premo™ Autophagy Sensor p62-FP Kit includes the BacMam p62-FP and chloroquine diphosphate for artificially generating autophagosomes (Figure 4).

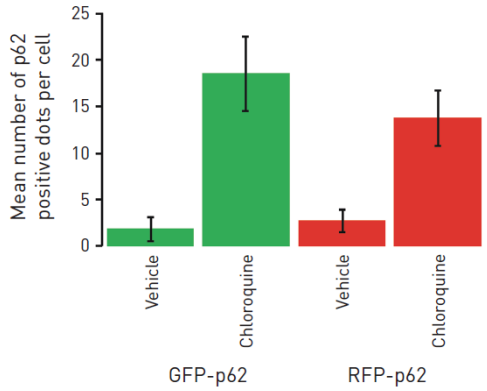

Figure 4 Blockade of autophagy leads to an accumulation of p62-positive vesicles.
A549 cells were transduced with either the Premo™ Autophagy Sensor GFP-p62 or the Premo™ Autophagy Sensor RFP-p62 and cultured for 24 hours. Cells were then treated with either vehicle (water) or 60 µM chloroquine for an additional 16 hours. A549 cells were then labeled with Hoechst™ 33342 and imaged using standard DAPI/FITC/Texas Red™ filter sets. The p62-positive spots were counted for each condition and for each Premo™ Autophagy Sensor for 20 cells from two separate experiments.

Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B Kit

Single FP (fluorescent protein) chimeras such as the Premo™ Autophagy Sensor LC3B-GFP (Cat. No. P36235) or Premo™ Autophagy Sensor 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 allows an enhanced dissection of the maturation of the autophagosome to the autolysosome (Geetha and Wooten, 2002; Pankiv et al., 2007). 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™ Autophagy Tandem 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 5 and Figure 6).

Each Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B Kit includes the BacMam RFP-GFP-LC3B and 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.

Combining the Premo™ Autophagy Tandem Sensor with the far-red emitting LysoTracker™ Deep Red allows for a three-color analysis of the autophagosomal/autolysomal/lysosomal dynamics.

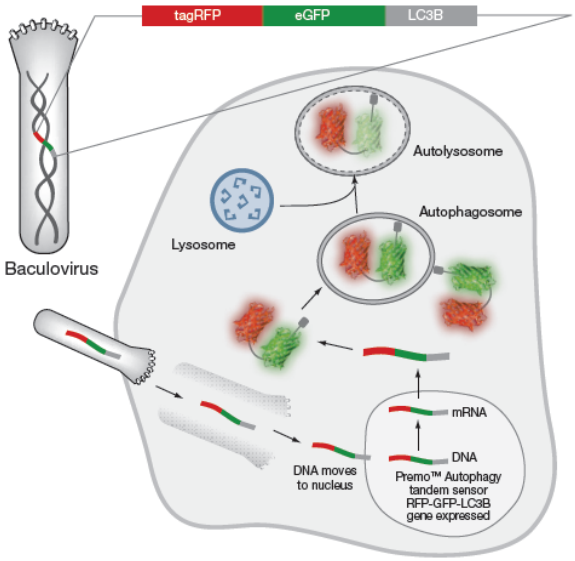

Figure 5 The Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B Kit allows for an enhanced dissection of the maturation of the autophagosome to the autolysosome.
By combining an acid-sensitive GFP with an acid-insensitive RFP, the change from autophagosome (neutral pH) to autolysosome (with an acidic pH) can be visualized by imaging the specific loss of the GFP fluorescence, leaving only red fluorescence.

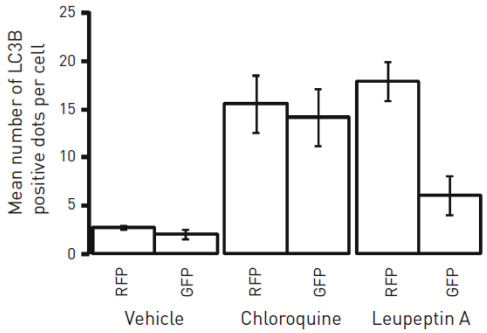

Figure 6 The Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B Kit can be used to discriminate between acidic and neutral LC3B-positive vesicles.
HeLa cells were transduced with 40 particles per cell of the Premo™ Autophagy Tandem Sensor and cultured for 24 hours. Cells were then incubated in either Vehicle, 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.

Contents and storage

Table 1 Premo™ Autophagy Sensor LC3B-FP Kits

Component	Cat. No. P36235	Cat. No. P36236	Concentration	Storage[1]
LC3B-FP (Component A)	1 mL (LC3B-GFP)	1 mL (LC3B-RFP)	~1 × 10^8 viral particles/mL	2°C to 8°C Protect from light DO NOT FREEZE
Control LC3B(G120A)-FP (Component B)	200 µL (LC3B(G120A)-GFP)	200 µL (LC3B(G120A)-RFP)	~1 × 10^8 viral particles/mL	
Chloroquine diphosphate (Component C)	1 mL	1 mL	30-mM aqueous solution	≤25°C Protect from light
Approximate fluorescence excitation/emission maxima: GFP = 485/520 nm; RFP = 555/584 nm.				

[1] When stored as directed, this kit is stable for at least 6 months.

Table 2 Premo™ Autophagy Sensor p62-FP Kits

Component	Cat. No. P36240	Cat. No. P36241	Concentration	Storage[1]
p62-FP (Component A)	1 mL (GFP-p62)	1 mL (RFP-p62)	~1 × 10^8 viral particles/mL	2°C to 8°C Protect from light DO NOT FREEZE
Chloroquine diphosphate (Component B)	1 mL	1 mL	30-mM aqueous solution	≤25°C Protect from light
Approximate fluorescence excitation/emission maxima: GFP (TagGFP) = 483/506 nm; RFP (mKate2) = 588/633 nm.				

[1] When stored as directed, this kit is stable for at least 6 months.

Table 3 Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B Kit (Cat. No. P36239)

Component	Amount	Concentration	Storage[1]
RFP-GFP-LC3B (Component A)	1 mL	~1 × 10^8 viral particles/mL	2°C to 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: GFP (Emerald GFP) = 488/509 nm; RFP (TagRFP) = 555/584 nm.			

[1] When stored as directed, this kit is stable for at least 6 months.

Before you begin

Required materials not supplied

- 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 one well of a 6-well culture plate, with a particles per cell (PPC) 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.
- If transduction efficiency is low, you can alter the PPC, cell density, temperature, and incubation time or use cells at a lower passage.
- We recommend transducing cells at a confluency of ~70% for best results.
- For first-time users of BacMam reagents, we recommend exceptionally well-transduced cells like U-2 OS.
- BacMam reagents typically do not efficiently transduce hematopoietic-derived cells or cell lines.

Transduce cells using BacMam 2.0 reagent

Transduce and image cells

Cells can be transduced with BacMam reagents while in suspension or after plating. For best results, add BacMam reagents to cells in suspension at the time of plating.

1. Calculate the volume of the BacMam 2.0 LC3B-FP, p62-FP, or tandem reagent using the equation below:

$$\text{mL of BacMam 2.0 reagent} = \frac{(\text{number of cells})\ (\text{PPC})}{(1 \times 10^8\ \text{particles/mL})}$$

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:

$$\text{mL of BacMam 2.0 reagent} = \frac{(40{,}000)\ (30)}{(1 \times 10^8\ \text{particles/mL})} = 0.012\ \text{mL}\ (12\ \mu\text{L})$$

2. Mix each BacMam 2.0 LC3B-FP, p62-FP, or tandem reagent by inversion to ensure a homogenous solution.

IMPORTANT! Do not vortex the BacMam 2.0 reagent.

Note: Overexpression of LC3B has been shown to promote aggregation of this protein in a non-autophagic dependent manner (Hailey and Lippincott-Schwartz, 2009); for this reason, we recommend titrating the expression levels by varying the PPC.

3. Add the BacMam 2.0 LC3B-FP, p62-FP, or tandem reagent directly to the cells in complete cell medium and mix gently.

4. Plate the cells at the desired density if the BacMam 2.0 reagent was added to cells in suspension.

5. Incubate the cells overnight (≥16 hours).

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

Note: 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 when using the Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B Kit. 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.

7. Image the cells using the appropriate filters for GFP or RFP. Autophagosomes are typically located in the perinuclear region.

Note: The Premo™ Autophagy Sensor Kits 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 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.

Note: Expression of exogenous autophagy markers can cause aggregation and false positives (Hailey and Lippincott-Schwartz, 2009); this is especially true for p62 (Bjørkøy et al., 2009). Exogenous p62 is prone to form cytoplasmic bodies; however, these bodies clear over time (Bjørkøy et al., 2009; Klionsky et al., 2012). Therefore, we recommend that the experiments are conducted 48 hours post-transduction.

Note: When using the Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B Kit, be aware of bleed-through from one fluorescent protein into the detection channel of another (Pankiv et al., 2007), especially with the excitation of TagRFP by GFP filter sets and the

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

Product list

Product	Cat. No.	Unit size
Premo™ Autophagy Sensor LC3B-GFP *BacMam 2.0*	P36235	1 kit
Premo™ Autophagy Sensor LC3B-RFP *BacMam 2.0*	P36236	1 kit
Premo™ Autophagy Sensor GFP-p62	P36240	1 kit
Premo™ Autophagy Sensor RFP-p62	P36241	1 kit
Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B Kit	P36239	1 kit

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 at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

References

Klionsky DJ. (2007) Autophagy: from phenomenology to molecular understanding in less than a decade. *Nat Rev Mol Cell Biol.* 8:931–937.

Mizushima N. (2007) Autophagy: process and function. *Genes Dev.* 21:2861–2873.

Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky, DJ. (2007) Potential therapeutic applications of autophagy. *Nat Rev Drug Discov.* 6:304–312.

Bjørkøy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H, Johansen T. (2005) p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol.* 171:603–614.

Geetha T, Wooten MW. (2002) Structure and functional properties of the ubiquitin binding protein p62. *FEBS Lett.* 512:19–24.

Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Øvervatn A, Bjørkøy G, Johansen T. (2007) p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem.* 282:24131–24145.

Hailey DW, Lippincott-Schwartz J. (2009) Using Photoactivatable Proteins to Monitor Autophagosome Lifetime, *Methods Enzymol.* 452:25-45,

Bjørkøy G, Lamark T, Pankiv S, Øvervatn A, Brech A, Johansen T. (2009) Monitoring Autophagic Degradation of p62/SQSTM1, *Methods Enzymol.* 452:181-197

Klionsky DJ et al. (2012) Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy.* 8:445–544.

Life Technologies Corporation | 29851 Willow Creek Road | Eugene, Oregon 97402 USA

For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: Pub. No. MAN0026683

Revision	Date	Description
A.0	15 June 2022	The format was updated and content from MAN0002600, MAN0007576, and MAN0007580 was combined.