Important Product News

Date: September 25, 2019

Product Change Notification

Dear Valued Customer,

The standard used in the Human LC3B AlphaLISA Detection Kit has changed. The AlphaLISA assays with the new analyte show greater sensitivity compared to the original product.

Should you have any questions or require additional support please contact our global technical support team at Global.TechSupport@PERKINELMER.COM

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Human Microtubule-associated Protein 1 Light Chain 3 Isoform B (LC3B) Kit

Product No.: AL306 C/F/L

Lot No.: 2822440

Material Provided

Format: AL306C: 500 assay points AL306F: 5 000 assay points
The number of assay points is based on an assay volume of 50 µL in 96- or 384-well assay plates using the kit components at the recommended concentrations.

Manufacturing date: **January 22, 2021**

Product Information

Kit content: The kit contains 6 components: AlphaLISA Acceptor beads coated with an Anti-Analyte Antibody, Streptavidin-coated Donor beads, Biotinylated Anti-Analyte Antibody, lyophilized analyte, 5X AlphaLISA Lysis Buffer, and 10X AlphaLISA Immunoassay Buffer.
Assay microplates (96-, 384- or 1536-well plates) must be purchased separately (see page 3 for more details).

Storage: Store kit in the dark at +4˚C. Store reconstituted analyte at -20°C.

Stability: This product is stable for at least 12 months from the manufacturing date when stored in its original packaging and the recommended storage conditions. Note: Once reconstituted, the human LC3B analyte is stable for at least 75 days at -20°C (see page 2: Reagents and Materials).

Application: This kit is designed for the quantitative determination of human LC3B in buffered solution (biochemical assay) or cell lysates using a homogeneous AlphaLISA assay (no wash steps). The antibodies in the kit detect both LC3B type I and type II. The analyte in this kit consists of recombinant LC3B fused to His-tag at the N-terminus.

Sensitivity: Lower Detection Limit (LDL): 7.9 ng/mL - Lower Limit of Quantification (LLOQ): 19.5 ng/mL

Dynamic range: 7.9 – 3 000 ng/mL (see page 11: Assay Performance Characteristics).

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Quality Control

Lot to lot consistency is confirmed in an AlphaLISA assay. Maximum and minimum signals, EC_{50} and LDL were measured on an EnVision® HTS instrument using the High sensitivity protocol described in this technical data sheet. We certify that these results meet our quality release criteria. Maximum counts may vary between bead lots and depending on assay conditions with no impact on LDL measurement.

Maximum signal:	356,852 counts
Minimum signal:	440 counts
EC_{50}:	2197.000 ng/mL
LDL:	6182.000 pg/mL

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Precautions

- Only the AlphaScreen® Donor beads are light-sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (LEE 090 filters (preferred) or Roscolux filters #389 from Rosco) can be applied to light fixtures.
- All blood components and biological materials should be handled as potentially hazardous. Some analytes are from human source.
- Some analytes are present in saliva. Take precautionary measures to avoid contamination of the reagent solutions.
- The Biotinylated Anti-Analyte Antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Reagents and Materials

The reagents provided in the AlphaLISA kit are listed in the table below:

Kit components	AL306C (500 assay points)	AL306F (5 000 assay points)
AlphaLISA Anti-LC3B Acceptor beads stored in PBS, 0.05% Kathon, pH 7.2	50 µL @ 5 mg/mL (1 brown tube, white cap)	500 µL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon, pH 7.4	200 µL @ 5 mg/mL (1 brown tube, black cap)	2 X 1 mL @ 5 mg/mL (2 brown tubes, black caps)
Biotinylated Antibody Anti-LC3B stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	50 µL @ 500 nM (1 tube, black cap)	500 µL @ 500 nM (1 tube, black cap)
AlphaLISA human LC3B (3 µg), lyophilized analyte *	1 tube, clear cap	1 tube, clear cap
AlphaLISA Lysis Buffer (5X) **	10 mL, 1 small bottle	100 mL, 1 large bottle
AlphaLISA Immunoassay Buffer (10X) ***	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute human LC3B in 100 µL Milli-Q® grade H_2O. The reconstituted analyte should be used within 60 minutes, if possible, or aliquoted into screw-capped polypropylene vials and stored at -20°C for further experiments. Avoid multiple freeze-thaw cycles. It has been demonstrated that reconstituted human LC3B is stable for at least 75 days at -20°C. One vial contains an amount of human LC3B sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL306S).

** Extra AlphaLISA Lysis buffer can be ordered separately (cat # AL003C: 10 mL, cat # AL003F: 100 mL).
Note: 5X buffer might be yellow. However, this does not affect the assay results.

*** Contains 250 mM HEPES, pH 7.4, 1% Casein, 10 mg/mL Dextran-500, 5% Triton X-100 and 0.5% Kathon. Extra buffer can be ordered separately (cat # AL000C: 10 mL, cat # AL000F: 100 mL).
Note: 10X buffer might be slightly yellow. However, this does not affect the assay results.

Once diluted, 1X AlphaLISA Immunoassay Buffer contains 25 mM HEPES, pH 7.4, 0.1% Casein, 1 mg/mL Dextran-500, 0.5% Triton X-100 and 0.05% Kathon.

Sodium azide should **not** be added to the stock reagents. High concentrations of sodium azide (> 0.001 % final in the assay) might decrease the AlphaLISA signal. Note that sodium azide from the Biotinylated Antibody stock solution will not interfere with the AlphaLISA signal (0.0001% final in the assay).

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Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Adhesive Sealing Film	PerkinElmer Inc.	6050195
EnVision®-Alpha Reader	PerkinElmer Inc.	-

Protocols have been optimized for 50 µL assays in white OptiPlate™-384 microplates. Other assay volumes can be used with similar protocols and identical final AlphaLISA reagent concentrations:

Format	# of data points	Total assay volume	Sample volume	AlphaLISA beads / Biotin Antibody MIX volume *	SA-Donor beads volume *	Plate recommendation
AL306C	250	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290)
	500	50 µL	5 µL	20 µL	25 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 250	20 µL	2 µL	8 µL	10 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	1 µL	4 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL306F	5 000	50 µL	5 µL	20 µL	25 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	12 500	20 µL	2 µL	8 µL	10 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	1 µL	4 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)

* Volumes based on the Quick protocol.

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Specific additional required reagents and materials for a cell-based assay or cell lysates preparation:

The following materials are recommended:

Item	Suggested source	Catalog #
Proteinase Inhibitor Cocktail	Sigma	P2714
Medium MEM/EBSS	Hyclone	SH30024.02
Orbital Shaker	Bellco Glass	7744-01000

Protocols have been optimized for 50 µL cell-based assays in white CulturPlate™-384 microplates. Other assay volumes can be used with similar protocols and identical final AlphaLISA reagent concentrations.

Format	# of data points	Total assay volume	Cell lysate volume	AlphaLISA beads / Biotin Antibody MIX volume	SA-Donor beads volume	Plate recommendation
AL306C	500	50 µL	10 µL	5 µL	35 µL	CulturPlate-384 (cat # 6007680)
AL306F	5 000	50 µL	10 µL	5 µL	35 µL	CulturPlate-384 (cat # 6007680)

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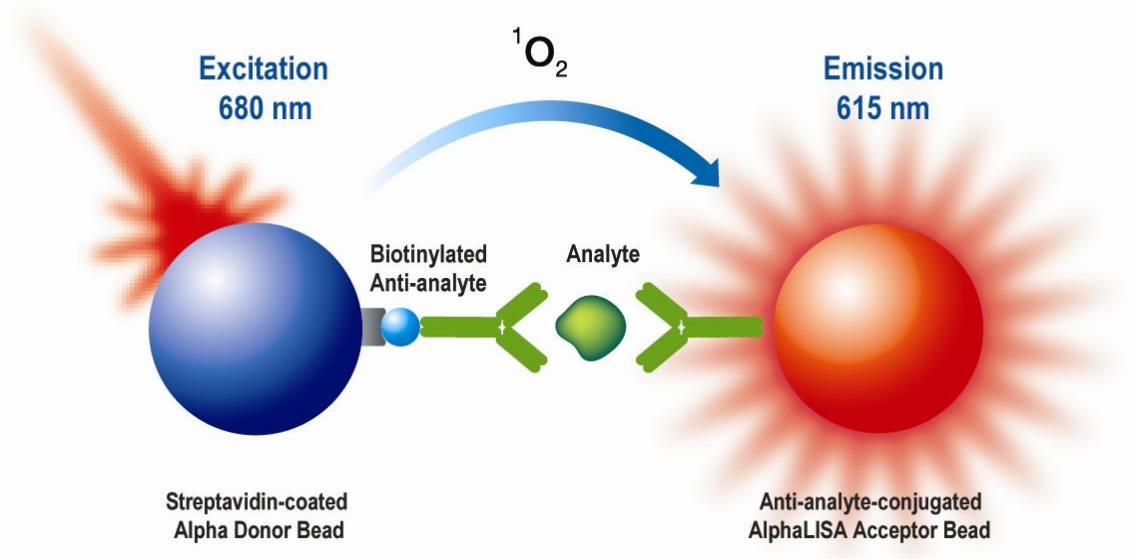
Analyte of Interest

LC3 represents a mammalian homologue of the yeast autophagy related gene ATG8. It was originally characterized as light chain 3 of the microtubule associated protein 1 (MAP1LC3). The protein family consists of LC3 A, B, and C and the GABARAP subfamily. Human LC3B is 125 amino acids long. After synthesis, it is cleaved by ATG4B to expose a C-terminal glycine, representing the cytosolic form LC3B I. During autophagy the C-terminus is covalently linked to autophagosomal vesicle membranes via a phospholipid anchor and this form is called LC3B II. The transformation of LC3B I to II is mediated by a ubiquitination-like process involving ATG7 (E1), ATG3 (E2) and the ATG16L complex (E3). To date, LC3B is considered as the most persistent marker of the autophagy pathway.

Description of the AlphaLISA Assay

AlphaLISA technology allows the detection of molecules of interest in buffer, cell culture media, serum and plasma in a highly sensitive, quantitative, reproducible and user-friendly mode. In an AlphaLISA assay, a Biotinylated Anti-Analyte Antibody binds to the Streptavidin-coated Donor beads while another Anti-Analyte Antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (see figure below).



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Recommendations

General recommendations:

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to prewet the tip.
- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2 000 g, 10-15 sec). Resuspend all reagents by vortexing before use.
- Use Milli-Q® grade H_2O (18 MΩ•cm) to dilute 10X AlphaLISA Immunoassay Buffer and to reconstitute the lyophilized analyte.
- When diluting the standard or samples, change tips between each standard or sample dilution. When loading reagents in the assay microplate, change tips between each standard or sample addition and after each set of reagents.
- When reagents are added in the microplate, make sure the liquids are at the bottom of the well.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal-A Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film.
- The AlphaLISA signal is detected with an EnVision Multilabel Reader equipped with the ALPHA option using the AlphaScreen standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).
- AlphaLISA signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperature should be used for each plate.
- The standard curves shown in this technical data sheet are provided for information only. A standard curve must be generated for each experiment. The standard curve should be performed in a similar matrix as the samples (e.g. FBS for serum samples).

Specific recommendations for the cell-based assay:

- Cells should be maintained at low passages in full growth media without antibiotics.
- Cells should not be allowed to grow to confluence. Follow manufacturer instructions for cell-line specific splitting conditions and media recommendations. Useful guides can be found at the ATCC website (http://www.atcc.org/).
- For cell-based assays, it is highly recommended to use a medium free of biotin for the treatment of cells as lower counts and lower sensitivity are expected in the presence of biotin.
- Adherent cells should be seeded the day before performing the AlphaLISA assay to let them adhere adequately to the surface.
- The addition of proteinase inhibitors to the AlphaLISA Lysis Buffer just prior to use is recommended.
- For quantitative analysis of LC3B in cell lysates, samples can be interpolated from a standard curve. Furthermore, dilutional linearity experiments should be performed to confirm that LC3B can be accurately quantified.
- As the results generated with the AlphaLISA LC3B kit are highly dependent on the optimal cell culture conditions, the following parameters need to be investigated:
    - Cell density: A cell titration assay should be performed to determine the optimal assay performance.
    - Inducer dose-response curve: A dose-response curve with the inducer compound should be performed to determine the concentration that will elicit the optimal assay window.
    - Time course: A time course experiment for the chosen cell line and treatment is highly recommended to determine the optimal incubation time.
    - Lysis buffer: The lysis step may need to be optimized when using other cell lines than HeLa. Other lysis buffers (eg. RIPA) might be used. However, the assay performance in the presence of those buffers was not determined.

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The kit has been optimized for analysis of LC3B in biochemical assays or cell lysates. The suggested protocols, typical results, and assay performance characteristics have been therefore divided into two sections: Biochemical Assays and Cell-based Assays.

Biochemical Assays

Protocol 1: Quick protocol (2 incubation steps) – Dilution of standards in 1X AlphaLISA Immunoassay Buffer

Protocol 2: High sensitivity protocol (3 incubation steps) – Dilution of standards in 1X AlphaLISA Immunoassay Buffer

IMPORTANT: PLEASE READ THE RECOMMENDATIONS ABOVE BEFORE USE

Common Steps for Preparing Reagents (Protocols 1 & 2)

If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.

1) Preparation of 1X AlphaLISA Immunoassay Buffer:
Add 2.5 mL of 10X AlphaLISA Immunoassay Buffer to 22.5 mL H_2O.

2) Preparation of human LC3B analyte standard dilutions:
Reconstitute lyophilized human LC3B (3 µg) in 100 µL H_2O.
Prepare standard dilutions as follows (change tip between each standard dilution):

Tube	Vol. of human LC3B (µL)	Vol. of diluent (µL) *	[human LC3B] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted human LC3B	90	3E-06	3 000 000
B	60 µL of tube A	120	1E-06	1 000 000
C	60 µL of tube B	140	3E-07	300 000
D	60 µL of tube C	120	1E-07	100 000
E	60 µL of tube D	140	3E-08	30 000
F	60 µL of tube E	120	1E-08	10 000
G	60 µL of tube F	140	3E-09	3 000
H	60 µL of tube G	120	1E-09	1 000
I	60 µL of tube H	140	3E-10	300
J	60 µL of tube I	120	1E-10	100
K	60 µL of tube J	140	3E-11	30
L	60 µL of tube K	120	1E-11	10
M ** (background)	0	100	0	0
N ** (background)	0	100	0	0
O ** (background)	0	100	0	0
P ** (background)	0	100	0	0

* Dilute standards in diluent (e.g. 1X AlphaLISA Immunoassay Buffer).
At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.

** Four background points in triplicate (12 wells) are used when LDL/LLOQ is calculated. If LDL/LLOQ does not need to be calculated, one background point in triplicate can be used (3 wells).

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Protocol 1: Quick Protocol (2 Incubation Steps)

The protocol described below is for one standard curve (48 wells) and samples (452 wells). Dilution of standards can be done in 1X AlphaLISA Immunoassay Buffer.

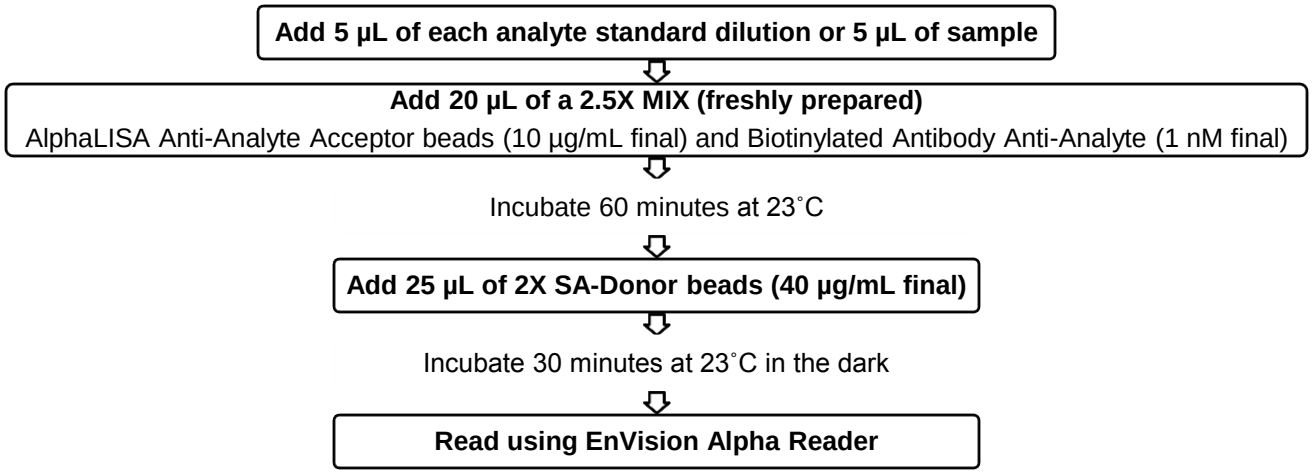
If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.

3) Preparation of 2.5X AlphaLISA Anti-LC3B Acceptor beads + Biotinylated Antibody Anti-LC3B MIX (25 µg/mL / 2.5 nM):
Add 50 µL of 5 mg/mL AlphaLISA Anti-LC3B Acceptor beads and 50 µL of 500 nM Biotinylated Antibody Anti-LC3B to 9 900 µL of 1X AlphaLISA Immunoassay Buffer. Prepare just before use.

4) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL): Keep the beads under subdued laboratory lighting.
Add 200 µL of 5 mg/mL SA-Donor beads to 12 300 µL of 1X AlphaLISA Immunoassay Buffer.

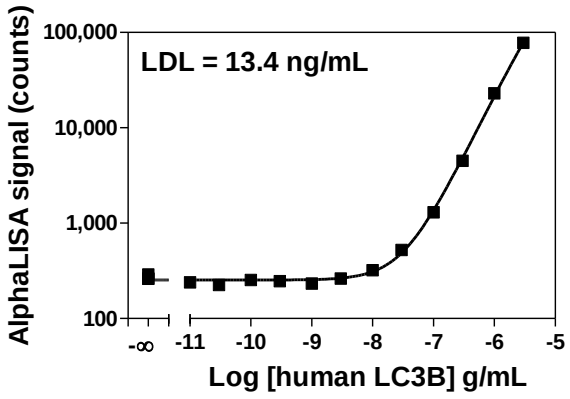
5) Samples: If applicable, dilute samples to be tested in diluent (e.g. 1X AlphaLISA Immunoassay Buffer).

6) In a 96- or 384-well microplate:

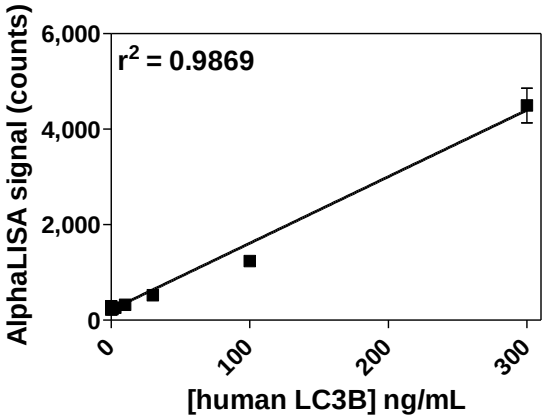


Protocol 1 - Typical results in 1X AlphaLISA Immunoassay Buffer

Log-Log scale:



Linear-Linear scale (linear range):



The data was generated using a white Optiplate-384 microplate and an EnVision-Alpha Reader 2102.

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Protocol 2: High Sensitivity Protocol (3 Incubation Steps)

The protocol described below is for one standard curve (48 wells) and samples (452 wells). Dilution of standards can be done in 1X AlphaLISA Immunoassay Buffer.

If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.

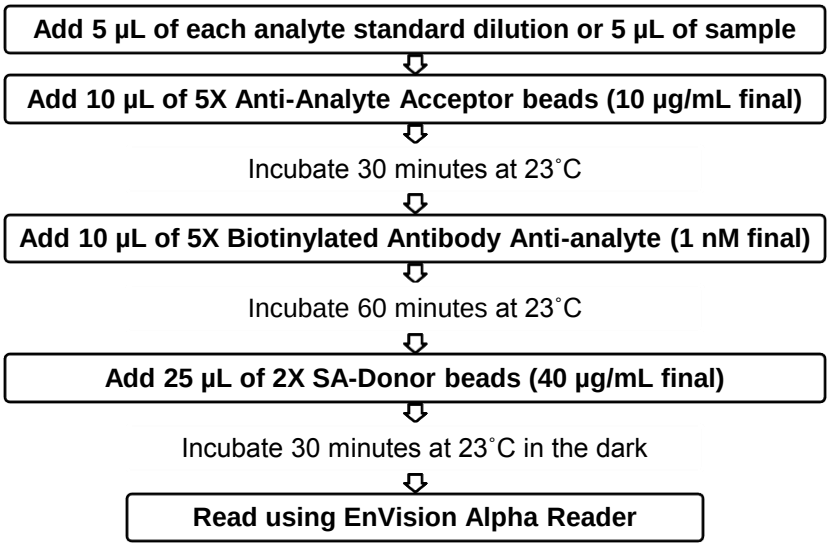
3) Preparation of 5X AlphaLISA Anti-LC3B Acceptor beads (50 µg/mL):
Add 50 µL of 5 mg/mL AlphaLISA Anti-LC3B Acceptor beads to 4 950 µL of 1X AlphaLISA Immunoassay Buffer.

4) Preparation of 5X Biotinylated Antibody Anti-LC3B (5 nM):
Add 50 µL of 500 nM Biotinylated Antibody Anti-LC3B to 4 950 µL of 1X AlphaLISA Immunoassay Buffer.

5) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL): Keep the beads under subdued laboratory lighting.
Add 200 µL of 5 mg/mL SA-Donor beads to 12 300 µL of 1X AlphaLISA Immunoassay Buffer.

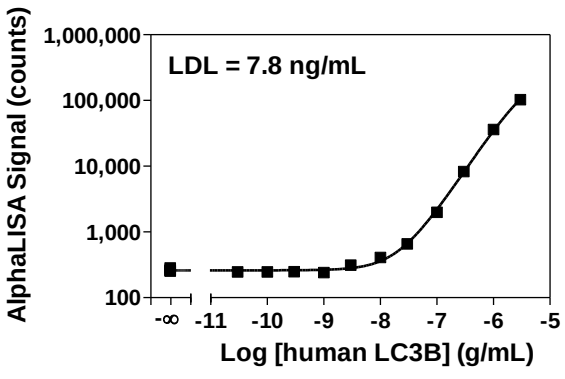
6) Samples: If applicable, dilute samples to be tested in diluent (e.g. 1X AlphaLISA Immunoassay Buffer).

7) In a 96- or 384-well microplate:

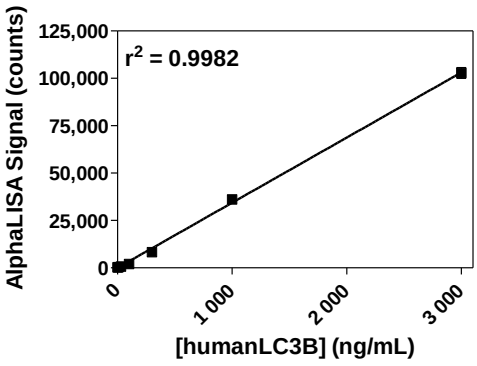


Protocol 2 - Typical results in 1X AlphaLISA Immunoassay Buffer

Log-Log scale:



Linear-Linear scale (linear range):



The data was generated using a white Optiplate-384 microplate and an EnVision-Alpha Reader 2102.

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Protocols 1 & 2 - Interpreting the Data

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaLISA counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting (the values at maximal concentrations of analyte after the hook point should be removed for correct analysis).
- The LDL is calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + (3xSD)) on the standard curve.
- The LLOQ is calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + (10xSD)) on the standard curve.
- Read from the standard curve the concentration of analyte contained in the samples.
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Assay Performance Characteristics

AlphaLISA assay performance described below was determined using the High sensitivity protocol.

Sensitivity:

The LDL/LLOQ were calculated as described above. The values correspond to the lowest concentration of analyte that can be detected/quantified in a volume of 5 µL using the recommended assay conditions.

- Average LDL is 7.9 ng/mL* (using 5 µL of analyte in AlphaLISA Immunoassay Buffer) (mean of 18 independent experiments).
- Average LLOQ is 19.5 ng/mL* (using 5 µL of analyte in AlphaLISA HiBlock Buffer) (mean of 18 independent experiments).

* Note that LDL can be decreased (i.e. sensitivity increased) by increasing the volume of analyte in the assay (e.g. use 10 µL of analyte in a final assay volume of 50 µL).

Dynamic range: 7.9 – 3 000 ng/mL (in AlphaLISA Immunoassay Buffer)

Assay precision:

The following assay precision data were calculated from a total of 18 assays. Two operators performed three independent assays using three different kit lots. Each assay consisted of one standard curve and two control samples of high (A), and low (B) concentration, assayed in triplicate. The assays were performed in 384-well format using AlphaLISA Immunoassay Buffer.

- Intra-assay precision:

The intra-assay precision was determined using a total of 18 independent determinations in triplicate for each control sample.

Sample	Mean (ng/mL)	SD (ng/mL)	% CV (n = 18)
A	1 086	60	5.5
B	297	20	6.8

- Inter-assay precision:

The inter-assay precision was determined using a total of 6 independent determinations with 9 measurements for each control sample.

Sample	Mean (ng/mL)	SD (ng/mL)	% CV (n = 6)
A	1 086	115	10.6
B	297	31	10.5

Specificity:

Cross-reactivity of the AlphaLISA LC3B Kit was tested using the following proteins at 3 µg/mL in AlphaLISA Immunoassay Buffer.

Protein	% Cross-reactivity
LC3A	0
LC3C	0
Gamma-aminobutyric acid receptor-associated protein (GABARAP)	0

The possible interference from p62, fused to glutathione S-transferase, was investigated. The LC3B was kept at a constant concentration (EC_{50} value of the standard curve). The binding protein was titrated into the assay. No interference was observed up to 10 µg/mL, which is the maximum concentration tested.

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Cell-based Assays

All-in-one Well Assays

The assay was optimized using HeLa cells and 50 µM Chloroquine overnight to arrest the turnover of autophagic vesicles.

Adherent Cells

⇩

Plate Cells Overnight

⇩

Remove Culture Medium

⇩

Add Chloroquine in Medium to Cells and Incubate

⇩

Remove Culture Medium

⇩

Lyse Cells

⇩

Add Mix: AlphaLISA Anti-Analyte Acceptor Beads and Biotinylated Antibody Anti-Analyte

⇩

Add SA-Donor Beads

⇩

Read

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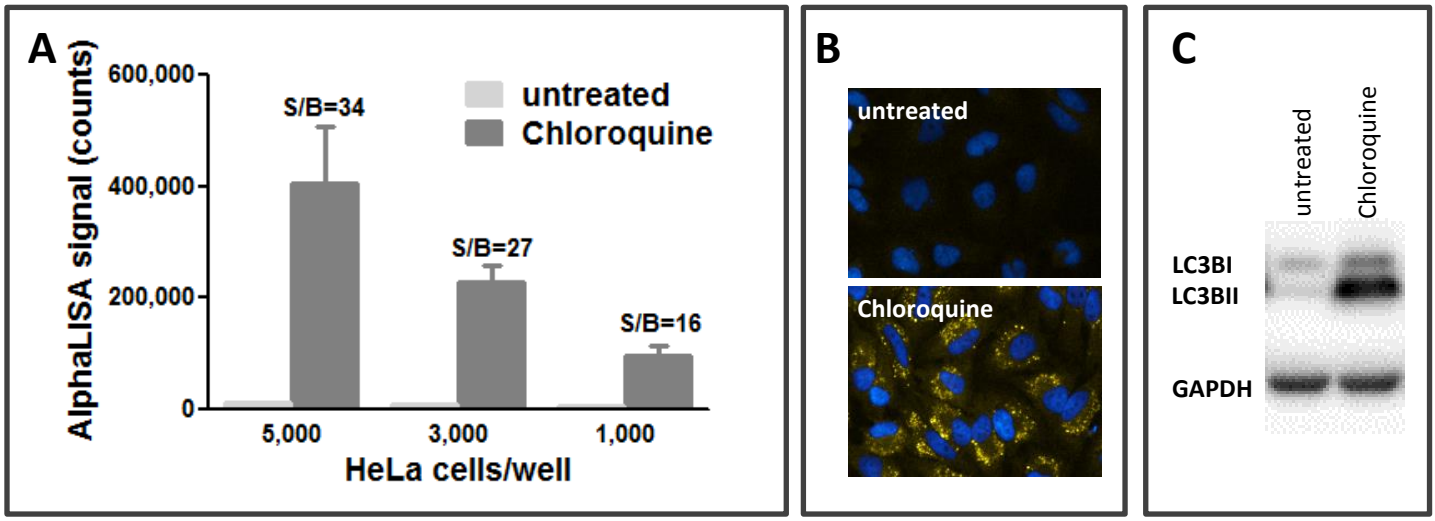
Detailed Protocol

1) Remove medium from the flasks.
2) Gently wash attached cells with PBS.
3) Trypsinize and resuspend the cells in medium supplemented with FBS.
4) Centrifuge, discard the supernatant and resuspend the cells in medium supplemented with FBS.
5) Count cells and concentrate/dilute at the desired concentration.
6) Seed 50 µL of the cell suspension into a white CulturPlate-384.
7) Incubate overnight (16-20 hours) at 37˚C, 5% CO_2.
8) Remove medium and treat the cells with 50 µM Chloroquine, prepared in medium supplemented with FBS.
9) Incubate cells at 37˚C, 5% CO_2 overnight (16-20 hours).
10) Remove medium.
11) Add 10 µL of AlphaLISA Lysis Buffer supplemented with proteinase inhibitors and agitate at approximately 350 rpm on a plate shaker for 10 minutes at room temperature.
12) Add 5 µL of a 10X MIX (freshly prepared and supplemented with proteinase inhibitors) AlphaLISA Anti-Analyte Acceptor beads (10 µg/mL final) and Biotinylated Antibody Anti-Analyte (1 nM final).
13) Incubate 60 minutes at 23˚C.
14) Add 35 µL of 1.43X SA-Donor beads (40 µg/mL final, supplemented with proteinase inhibitors).
15) Incubate 30 minutes at 23˚C in the dark.
16) Read using EnSpire or EnVision-Alpha Reader.

Typical results

HeLa cells were cultured in MEM/EBSS medium supplemented with 10% FBS. Cells were incubated with 50 µM Chloroquine diluted in MEM/EBSS medium overnight. Cells in medium without Chloroquine were used as controls. For the AlphaLISA assay, cells were lysed with 1X AlphaLISA Lysis Buffer. Figure A shows that Chloroquine treatment increases the AlphaLISA LC3B signal. The amount of signal and the signal ratio between treated and untreated cells increases with higher cell numbers per well. The data was generated using a white CulturPlate-384 microplate and an EnVision-Alpha Reader 2103.
Figures B and C: To confirm the autophagy-specific increase in LC3B, Chloroquine-treated cells were fixed and stained for LC3B (B) or extracted and subjected to SDS-PAGE and Western blotting (C). Chloroquine treatment clearly leads to a perinuclear accumulation of LC3B positive spots and an increase in the amount of LC3B-II.



Assay precision:

HeLa cells were grown, induced, and assayed for LC3B as detailed in the All-in-One-Well-Assays protocol.

The assay variability was evaluated by performing a Z' factor value determination experiment.

Z' factor value was calculated using the following equation:

$$Z' = 1 - \frac{(3 \times SD\ (Chloroquine\ treated)) + (3 \times SD\ (Untreated))}{Mean\ (Chloroquine\ treated) - Mean\ (Untreated)}$$

The signal to background (S/B) ratio value was calculated using counts generated in the presence of Chloroquine-treated cells over counts obtained with non-treated cells. The assay precision was determined using a total of 2 independent determinations with 48 measurements for each control sample.

Assay	5,000 cells + 50 µM Chloroquine			
	Z'	S/B	% CV Chloroquine	% CV untreated
1	0.51	12.4	14.1	11.8
2	0.59	17.5	12.4	5.8

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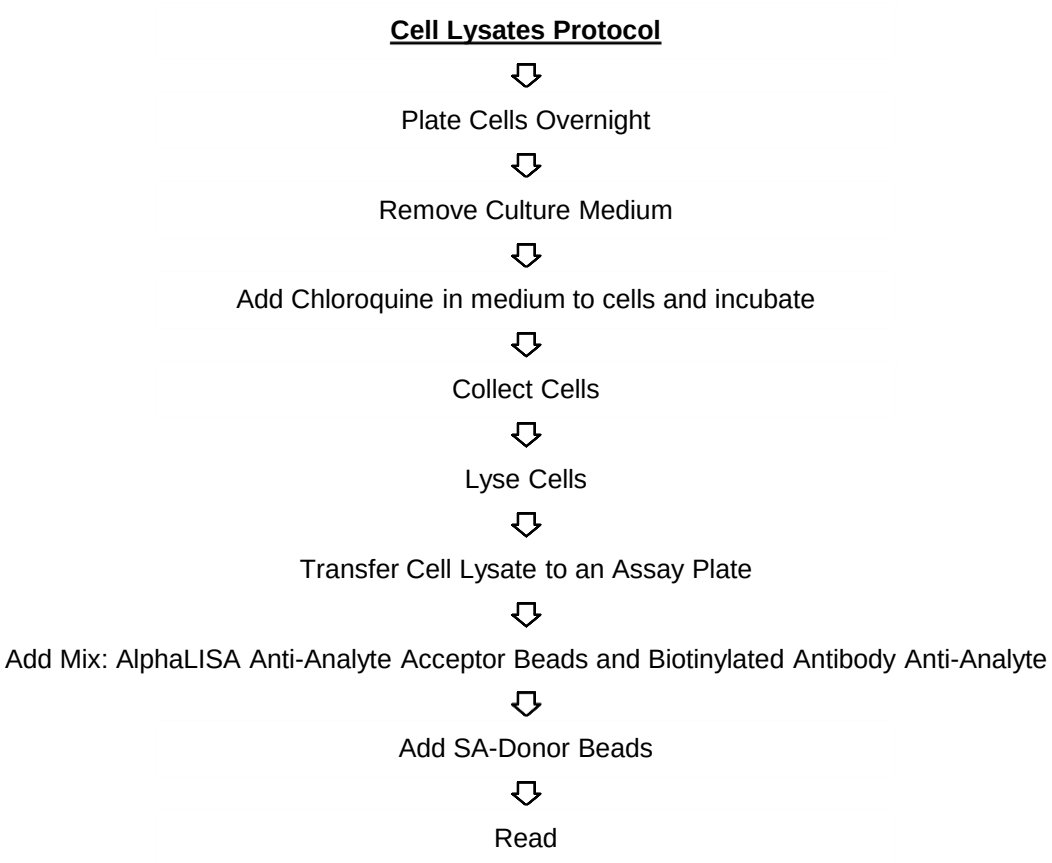
Cell Lysates Assay

Protocol Overview:



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Detailed Protocol

1) Remove medium from the flasks.
2) Gently wash attached cells with PBS.
3) Trypsinize and resuspend the cells in medium supplemented with FBS.
4) Centrifuge, discard the supernatant and resuspend the cells in medium supplemented with FBS.
5) Count cells and concentrate/dilute them to a density of 20 000 cells/cm^2 (e.g. $2x10^5$ cells/well in a 6-well plate).
6) Incubate overnight at 37˚C, 5% CO_2.
7) Remove medium and treat the cells with 50 µM Chloroquine, prepared in medium supplemented with FBS.
8) Incubate cells at 37˚C, 5% CO_2 overnight.
9) Remove medium.
10) Wash cells with ice-cold PBS.
11) Scrape cells in ice-cold PBS and collect cells (e.g. 1 mL/well in a 6-well plate).
12) Pellet cells (4°C, 1 minute, 2 000 g in microcentrifuge).
13) Remove supernatant and add 1X AlphaLISA Lysis Buffer (e.g. 0.1 mL/$2x10^5$ initially plated cells), supplemented with proteinase inhibitors.
14) Resuspend pellet and incubate 10 minutes on ice with occasional vortexing.
15) Spin lysate to remove cell debris (4°C, 5 minutes, 14 000 g in microcentrifuge). At this point, samples can be frozen at -80˚C or used for the AlphaLISA assay.
16) Transfer 10 µL of each lysate sample in triplicate to the wells of a 384-Optiplate. If necessary, dilute the sample in the appropriate amount of AlphaLISA Immunoassay Buffer.
17) Add 5 µL of a 10X MIX (freshly prepared) AlphaLISA Anti-Analyte Acceptor beads (10 µg/mL final) and Biotinylated Anti-Analyte Antibody (1 nM final)
18) Incubate 60 minutes at 23˚C.
19) Add 35 µL of 1.43X SA-Donor beads (40 µg/mL final).
20) Incubate 30 minutes at 23˚C in the dark.
21) Read using EnSpire or EnVision-Alpha Reader.

Typical results

- Dilutional linearity:

The dilutional linearity was determined by serial dilutions of HeLa cell lysates in AlphaLISA Immunoassay Buffer. The samples were interpolated on a standard curve prepared using AlphaLISA Immunoassay Buffer. The recovery was calculated using the neat sample as the 100% value. The experiment was performed twice with very similar results. The 8 and 16-fold dilutions of the untreated cell lysates gave concentrations of LC3B below the LDL of the assay.

Dilution Factor	% Recovery		Interpolated concentration of LC3B (µg/mL)	
	Untreated	Chloroquine	Untreated	Chloroquine
1	100	100	0.02	0.16
2	106	93	0.03	0.15
4	98	80	0.02	0.13
8	-	88	-	0.14
16	-	95	-	0.15

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