



NCL Method GTA-11

Autophagic Dysfunction Assay:

Qualitative Analysis of MAP LC3-I to LC3-II Conversion By Western Blot

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

Lysosomal dysfunction is recognized as a potential toxic mechanism for xenobiotics that can result in various pathological states (1). There is concern that nanoparticles, in particular, may cause lysosomal pathologies, since they are likely to accumulate within lysosomes (2).

Lysosomal dysfunction could potentially result from nanoparticle biopersistence, or inhibition of lysosomal enzymes, such as inhibition of phospholipase resulting in phospholipidosis, or inhibition of lysosomal protein degradation resulting in lysosomal overload (1). Possibly related to lysosomal dysfunction, nanoparticle exposure has also been shown to cause autophagic dysfunction (3), resulting in accumulation of autophagic vacuoles. Common methods used to detect autophagic dysfunction include microscopy and protein modification assays, such as microtubule associated protein light chain 3-I (MAP LC3-I) lipidation (4).

2. Principles

This assay measures lipidation of MAP LC3-I to LC3-II by immunoblot. The amount of LC3-II subunit expression is used as a surrogate marker of autophagy (5). As other conditions could potentially result in LC3-II expression, treatment-related changes in expression should be further evaluated by morphological assessment, using techniques such as electron microscopy, to confirm autophagosome involvement.

3. Reagents, Materials, Cell Lines, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

3.1 Reagents

- 3.1.1 Cell Extraction Buffer (Invitrogen, FNN0011)
- 3.1.2 Protease Inhibitor Cocktail (Sigma, P-2714)
- 3.1.3 phenylmethylsulphonyl fluoride (PMSF) (Sigma, P7626)
- 3.1.4 4-20% Tris-Glycine gels (Invitrogen, EC6025)
- 3.1.5 Tris-Glycine Running Buffer (10x) (Invitrogen, LC2675)
- 3.1.6 NuPAGE LDS 4X sample buffer (Invitrogen, NP0007)

- 3. 1.7 Reducing agent (10X) (Invitrogen, NP0004)
- 3. 1.8 Westran S, polyvinylidene fluoride (PVDF) protein blotting membrane (Schleicher&Schuell, 10 413 052)
- 3. 1.9 Blotting paper (Schleicher&Schuell, CB-03)
- 3.1.10 Tris-Glycine Transfer Buffer (25X) (Invitrogen, LC3675)
- 3.1.11 Methanol (Sigma-Aldrich, 179337)
- 3.1.12 Tris-Buffered Saline (TBS) (25X) (Amresco, J640-4L)
- 3.1.13 Tween 20 (Sigma, P7949)
- 3.1.14 StartingBlock Blocking Buffer (Pierce, 37538)
- 3.1.15 Mouse Monoclonal Antibody anti-LC3 antibody (NanoTools, 0231-100/LC3-5F10)
- 3.1.16 Peroxidase-conjugated AfiniPure Donkey Anti-Mouse IgG (Jackson ImmunoResearch Labs, 715-035-151)
- 3.1.17 ECL Western Blotting Substrate (Pierce, 32106)
- 3.1.18 Hyperfilm ECL (Amersham Biosciences, RPN 2103K)
- 3.1.19 SeeBlue® Plus2 Pre-Stained Standard (Invitrogen, LC5925)
- 3.1.20 BCA Protein Assay (Pierce, 23235)
- 3.1.21 Pelikan Fount India-17 Black drawing ink (221143)
- 3.1.22 Hank's Balanced Salt Solution (with calcium and magnesium) (HBSS) (Invitrogen, 14025)
- 3.1.23 Dulbecco's phosphate buffered saline (PBS), Ca/Mg free (Sigma, D8537)
- 3.1.24 Dimethyl sulfoxide (DMSO) (Aldrich, 154938)
- 3.1.25 M199 Cell Culture Media (Cambrex, 12-109-F)
- 3.1.26 Fetal Bovine Serum (Hyclone, SH30070.03)
- 3.2 Materials
 - 3.2.1 Pipettes covering the range from 0.05 to 1 mL
 - 3.2.2 Microcentrifuge tubes 1.5 mL
 - 3.2.3 Pipet tips 0.5 µL – 1.0 mL
 - 3.2.4 Gel-loading tips
 - 3.2.5 Hybridization bags
 - 3.2.6 Saran Wrap

- 3.2.7 Scissors
- 3.2.8 Ruler
- 3.2.9 Film cassette
- 3.2.10 15 mL conical tubes
- 3.2.11 T75 tissue culture flasks
- 3.3 Cell Lines
 - 3.3.1 LLC-PK1 (pig kidney cells) (ATCC, CL-101)
- 3.4 Equipment
 - 3.4.1 Microcentrifuge
 - 3.4.2 Refrigerator, 2-8°C
 - 3.4.3 Freezer, -20°C
 - 3.4.4 Vortex
 - 3.4.5 Incubator set at 37°C
 - 3.4.6 Mini-gel protein electrophoresis system
 - 3.4.7 Mini-gel blotting system
 - 3.4.8 Rocking platform

4. Reagent and Control Preparation

- 4.1 Tris-Glycine Running Buffer:

Prepare working solution by diluting 10X concentrated stock with distilled water. For example, mix 100 mL of stock with 900 mL of water. Use fresh.
- 4.2 Tris-Glycine Transfer Buffer with 20% Methanol:

Prepare working buffer from 25X stock solution by diluting 40 mL of stock in 800 mL of distilled water; then add 200 mL of methanol. Mix well. Chill before use. Use fresh.
- 4.3 Cell Extraction Buffer – Stable for 24 h at 4°C:
 - 4.3.1 Add 17 μ L of 0.3 M PMSF stock in DMSO to 5 mL of Cell Extraction Buffer, to make 1 mM PMSF solution.
 - 4.3.2 Add 250 μ L of reconstituted protease inhibitor cocktail to 5 mL of sample prepared in step 4.3.1.
- 4.4 TBST (TBS + 0.01% Tween 20):

Dilute 25X TBS stock in distilled water by mixing 40 mL of the stock with 960 mL of water. Then, add 100 μ L of Tween 20 and mix well. Unused buffer can be stored at room temperature overnight or up to 1 week at a nominal temperature of +4°C.

4.5 StartingBlock Blocking Buffer:

Add 5 μ L of Tween 20 to 50 mL of StartingBlock blocking buffer and mix well. Use fresh.

4.6 Primary Antibody Solution:

Thaw an aliquot of anti-LC3 antibody and dilute 1:200 in 5 mL of the StartingBlock blocking buffer. Use freshly prepared.

Note: If antibody from a source other than that tested in validation is used, the final dilution of this antibody can be adjusted to provide more optimal assay performance (i.e., minimum background, high signal-to-noise ratio). The mouse monoclonal antibody (see 3.1.15 in Reagents List) exhibits cross-reactivity with the following species: human, mouse, rat, dog, and hamster.

4.7 Secondary Antibody Solution:

Dilute donkey Anti-Mouse IgG(H+L) HRP conjugate 1:50,000 in 50 mL of StartingBlock blocking buffer. Use freshly prepared. Discard after use.

Note: If antibody from a source other than that tested in validation is used, the final dilution of this antibody can be adjusted to provide more optimal assay performance (i.e., minimum background, high signal-to-noise ratio).

4.8 India Ink Stain:

Add 100 μ L India ink to 100 mL of PBS that contains Tween 20 (0.3% v/v).

4.9 BCA Standard Curve Samples:

Preparation of Standards

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA concentration (µg/mL)
A	900	100 stock	200
B	800	200 vial A	40
C	400	400 vial B	20
D	400	400 vial C	10
E	400	400 vial D	5
F	400	400 vial E	2.5
G	480	320 vial F	1
H	400	400 vial G	0.5
I	800	0	blank

Note: Diluent: Add an equivalent ratio of cell extraction buffer to H₂O to match the dilution buffer of the cell lysate samples. (Cell lysate samples (from step 5.3) will have to be diluted in water to fit the BSA standard concentration range.)

4.10 Preparation of the Micro BCA™ Working Reagent (WR)

Formula for total volume of WR required:

$$[(\# \text{ standards} + \# \text{ unknowns}) \times (\# \text{ replicates}) \times (150 \mu\text{L})] + 300 \mu\text{L} = \text{total volume WR required}$$

Prepare WR by mixing 25 parts of Micro BCA™ Reagent MA and 24 parts Reagent MB with 1 part of Reagent MC (25:24:1, Reagent MA:MB:MC).

5. Cell Lysate Preparation

5.1 Cell Preparation (or as recommended by supplier)

5.1.1 Harvest cryopreserved cells from prepared flasks (**limit to 20 passages**).

5.1.2 Count cell concentration using a coulter counter or hemocytometer.

5.1.3 Dilute cells to a density of 2.5×10^5 cells/mL in M199 (3% FBS) cell culture media.

5.1.4 Place 20 mL cells/T75 flask. Prepare T75 flasks for media negative control, positive starvation control and nanomaterial samples for each timepoint tested.

- 5.1.5 Incubate flasks for 24 hours at 5% CO₂, 37°C and 95% humidity (**cells are grown to approximately 80% confluence**) (Figure 1).
- 5.2 Cells are treated in T-75 flasks with positive control starvation buffer (HBSS), media negative control, or nanomaterial, for the desired time period. Nanomaterial concentrations and time periods are based on results from the Lysotracker Autophagic Dysfunction Assay, NCL Method GTA-12. Cells are washed three times with cold PBS (1X) and scraped into 1 mL cold PBS (1X), transferred to a 15 mL conical tube, and centrifuged at 700 x g for 3 min at 4°C.
- 5.3 Supernatant is discarded, and cells are then lysed with 200 µL of Cell Extraction Buffer containing protease inhibitors (from step 4.3.2). Lysed cells are placed on ice for 30 min, vortexing every 10 min. Centrifuge lysate at 15,000 x g for 10 min at 4°C. Aliquot the clear lysate to clean microcentrifuge tubes.
- 5.4 Lysate samples can be used immediately or stored at -80°C until use.
- 5.5 Protein content in cell lysate samples should be determined by the BCA protein assay.

Note: At this stage samples can be either used for further analysis or frozen at a nominal temperature of -80°C. If frozen, samples should be thawed at room temperature, vortexed and briefly spun down before analysis.

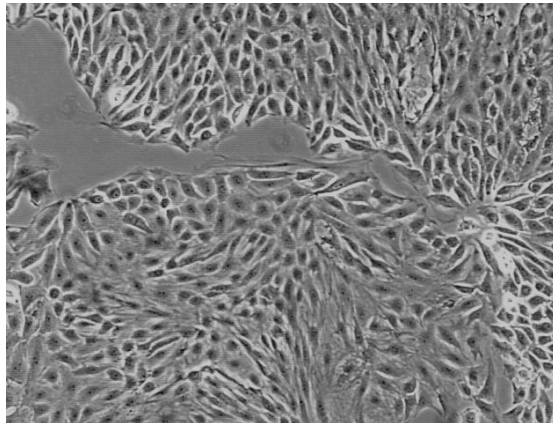


Figure 1. Example of LLC-PK1 Cell Culture Appearance

Image was taken with a phase contrast microscope at 225X magnification. LLC-PK1 cells are approximately 80% confluent at this stage.

6. BCA Protein Assay

- 6.1 Pipette 150 μL of each standard or unknown sample replicate (diluted with 0.5N NaOH to fit the BSA standard concentration range) into a microplate well.
- 6.2 Add 150 μL of the WR to each well and mix plate thoroughly on a plate shaker for 30 s.
- 6.3 Cover plate and incubate at 37°C for 2 h.
- 6.4 Cool plate to room temperature (RT).
- 6.5 Measure the absorbance at or near 562 nm on a plate reader.
- 6.6 Subtract the average 562 nm absorbance reading of the Blank standard replicates from the 562 nm reading of all other individual standard and unknown sample replicates.
- 6.7 Prepare a standard curve by plotting the average Blank-corrected 562 nm reading for each BSA standard vs. its concentration in $\mu\text{g}/\text{mL}$. Use the standard curve to determine the protein concentration of each unknown sample.

7. Experimental Procedure

- 7.1 Dilute aliquots of all sample cell lysates to the lowest sample protein concentration determined by the BCA assay, in order to obtain equal protein loading and greatest assay sensitivity (i.e., if 0.8 μg protein/ μL is the lowest cell lysate protein concentration, dilute all samples to 0.8 $\mu\text{g}/\mu\text{L}$ in H_2O). Add 10 μL of 4X NuPAGE buffer and 4 μL reducing agent to 30 μL of diluted sample. Vortex and heat at a nominal temperature of 95°C for 5 min. Spin in a microcentrifuge at a maximum speed for 30 min and carefully transfer supernatants to clean tubes.
Note: At this stage samples can be either used for further analysis or frozen at a nominal temperature of -80°C. If frozen, samples should be thawed at room temperature, vortexed and briefly spun down before analysis.
- 7.2 Assemble gel running system. Prime wells with running buffer, then load 3 μL of pre-stained MW standard, and 30 μL of test samples and controls in duplicate.
- 7.3 Run gel at 125 V for approximately 2 h or until dye reaches bottom of the gel.

- 7.4 Rinse the gel with deionized water and assemble protein transfer sandwich.
- 7.5 Perform protein transfer overnight at 30 mA.
- 7.6 Wash membrane 3 times with 100 mL of TBST for approximately 15 min each, with rocking.
- 7.7 Block the membrane with 50 mL StartingBlock blocking buffer (0.01% Tween-20) at room temperature for approximately 1 h, with rocking.
- 7.8 Incubate membrane with primary antibody solution for 2 h at room temperature, using hybridization bags cut to size, with rocking.
- 7.9 Wash the membrane twice with 100 mL of TBST, for 15 min each, with rocking.
- 7.10 Incubate the membrane with the secondary antibody solution for 1 h at room temperature, with rocking.
- 7.11 Wash the membrane twice with 100 mL of TBST, for 15 min each, with rocking.
- 7.12 Incubate membrane with 3 mL ECL peroxidase substrate solution (1:1 peroxidase substrate to luminol enhancer solution) for approximately 1 min and proceed to develop blot for 5 and 8 min (Figure 2).

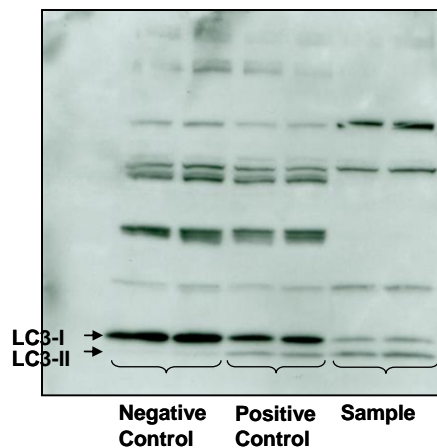


Figure 2. LC3 Immunoblot

LLC-PK1 cells were treated for 6 h with media (negative control), starvation buffer (positive control), and nanoparticle sample in duplicate. Cell lysate proteins were separated by SDS-PAGE, transferred to PVDF membrane, and probed for LC3 reactive proteins. LC3-I and the LC3-II lipidation products are labeled on the immunoblot.

8. India Ink Staining

- 8.1 Wash blot in 100 mL of PBS/Tween 20 (0.3 %), with two changes at 5 min each.
- 8.2 Place the blot in ~100 mL of India ink suspension.
- 8.3 Incubate at room temperature for 15 min to 18 h. *Longer incubations will increase sensitivity.*
- 8.4 Destain by washing the blot in multiple changes of PBS.
- 8.5 Let blot air dry; place in saran wrap to archive.

9. Acceptance Criteria

- 9.1 Run is acceptable if both replicates of the positive control demonstrate acceptable performance, i.e., evident conversion of LC3-I to LC3-II in comparison to negative media control.
- 9.2 If one of the replicates of the positive control fails to meet acceptance criterion 9.1, entire run should be repeated.
- 9.3 If both replicates of a study sample demonstrate evident conversion of LC3-I, or one replicate is positive and the other replicate demonstrates intermediate conversion, the sample is considered positive.
- 9.4 If one replicate of a study sample demonstrates positive response and second replicate is negative, this sample should be re-analyzed.
- 9.5 If both replicates of a study sample demonstrate no obvious conversion of LC3-I, the sample is considered negative and no further analysis is required.

10. References

1. Schneider, P., Korolenko, T.A., and Busch, U. (1997). A review of drug-induced lysosomal disorders of the liver in man and laboratory animals. *Microsc Res Tech.* **36**:253-275.
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3. Zabirnyk, O., Yezhelyev, M., and Seleverstov, O. (2007). Nanoparticles as a novel class of autophagy activators. *Autophagy* **3**:278-281.

4. Klionsky, D.J., Cuervo, A.M., Seglen, P.O. (2007) Methods for monitoring autophagy from yeast to human. *Autophagy*. **3**:181-206.
5. Mizushima N, Yoshimori T. How to Interpret LC3 Immunoblotting (2007). *Autophagy*. **3**:542-5.

11. Abbreviations

BCA	bicinchoninic acid
BSA	bovine serum albumin
DMSO	dimethyl sulfoxide
IgG(H+L) HRP	immunoglobulin G (high + low chains) horseradish peroxidase
LLC-PK1 cells	renal epithelial cell line, porcine kidney
mA	milliamp
MAP LC	microtubule associated protein light chain
PBS	phosphate buffered saline
PMSF	phenylmethanesulphonyl fluoride
PVDF	polyvinylidene fluoride
rpm	revolutions per minute
TBS	tris-buffered saline
TBST	tris-buffered saline with Tween
WR	working reagent