LC3 flux assay

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METHOD

Preparation of samples

1. Seed cells (e.g. MEF cells) in 12-well plates in DMEM supplemented with 10% (v/v) heat-inactivated FBS and penicillin-streptomycin, and maintain the cells at 37°C with 5% CO_2.
2. Wash the cells twice with PBS and culture them in the regular medium or EBSS with or without 125 nM Bafilomycin A1 for 2 h at 37°C.
3. Wash the cells with PBS, and lyse them with 1x Laemmli sample buffer (100-200 µl), sonicate them, and then, boil them for 5 min.

NOTE Prepared samples can be stored at -20°C.

Immunoblot

4. Run the standard SDS-PAGE using 15% polyacrylamide gels.

NOTE We usually load 10 µl of samples prepared above for 1 lane.

5. After electrophoresis, the gels are electrotransferred to PVDF membrane for 1 h with constant current (400 mA). Staining the membrane with 0.1% (w/v) Ponceau S in 1% acatic acid for 5 min with constant agitation is advisable to verify the electrotransfer efficiency.

NOTE We use not only a semi-dry transfer, but also wet transfer system.

6. Incubate the membrane with 1% (w/v) skim milk in TBS/T at room temperature for 30 min with constant agitation.
7. Incubate the membrane with polyclonal anti-LC3 antibody (1:2000) in 1% (w/v) skim milk in TBS/T at room temperature for 1 h with constant agitation.

NOTE Incubation can be carried out at 4°C overnight.

8. Wash the membrane three times with TBS/T for 5 min at room temperature.
9. Incubate the membrane with HRP-conjugated anti-rabbit IgG (1:10000) in 1% (w/v) skim milk in TBS/T at room temperature for 1 h with constant agitation.
10. Wash the membrane three times with TBS/T for 5 min at room temperature.
11. Using an HRP-based Western blotting detection system such as ECL,

develop the signal according to the manufacture's instructions.

NOTE We usually develop signal for 1 min just before detect the signal.

12. Using an equivalent apparatus such as LAS-3000, detect the luminescent signals on the membrane.

MATERIALS

REAGENTS

- Mouse embryonic fibroblasts (MEF)
- Fetal bovine serum (FBS) (Gibco), heat inactivated (56 ˚C, 45 min)
- Dulbecco's modified eagle's medium (DMEM) (Sigma, D6429)
- Earle's balanced salt solution (EBSS) (Sigma, E2888)
- Penicillin-Streptomycin (Sigma, P4333)
- 30% Acrylamide/Bis solution (BIO-RAD, 161-0158)
- Ammonium Peroxodisulfate (nacalai tesque, 02627-34)
- *N,N,N',N'*-tetramethyl-ethylenediamine (Wako, 205-06313)
- Sodium chloride (Wako, 191-01665)
- Potassium chloride (Wako, 163-03545)
- Disodium hydrogenphosphate (Wako, 197-02865)
- Potassium dihydrogenphosphate (Wako, 169-04245)
- Glycine (Wako, 077-00735)
- Sodium lauryl sulfate (SDS) (nacalai tesque, 31607-65)
- Trizma base (Tris) (Sigma, T6066)
- Skim milk for immunoassay (nacalai tesque, 31149-75)
- Polyoxyethylene sorbitan monolaurate (Tween-20) (nacalai tesque, 28353-85)
- Hydrochloric acid (nacalai tesque, 18321-05)
- Glycerol (Wako, 075-00616)
- Bromophenol blue (Wako, 021-02911)
- (±)-Dithiothreitol (Wako, 041-08976)
- Dimethyl sulfoxide (DMSO) (Wako, 046-21981)
- Bafilomycin A1 (Wako, 029-11643)
- Methanol (Kishida Chemical Co., Ltd, 000-48666)
- Acetic acid (nacalai tesque, 00211-95)
- Luminata Forte Western HRP Substrate (Millipore, WBLUF0100)

• Ponceau S (Merck, 115927)
• Anti-LC3 polyclonal antibody (MBL, PM036)
• Anti-Rabbit IgG, HRP-Linked Whole Ab Donkey (GE Healthcare, NA934)

EQUIPMENT

• Immobilon-P (Millipore, IPVH 00010)
• Chromatography paper (Whatman, 3030-917)
• 12 well plate (Thermo, 150628)
• Semidry Transfer apparatus (BIO CRAFT, BE-300)
• Sonifier 250 (Branson)
• Dry Thermo Unit (TAITEC, DTU-1B)
• CO_2 incubator (Thermo, HERACELL 150i)
• LAS-3000 (FUJIFILM)

REAGENT PREPARATION

PBS

For 10 × PBS stock solutions, dissolve 400 g of sodium chloride, 10 g of potassium chloride, 72 g of disodium hydrogenphosphate, 12 g of potassium dihydrogenphosphate in 5 L of distilled water. Dilute 10 × PBS 1:10 with distilled water. This reagent can be stored at room temperature.

250 μM Bafilomycin A1

Dissolve 100 μg of bafilomycin A1 in 640 μl of DMSO. Prepare aliquots of the stock solution and the aliquots can be stored at -20°C. Avoid repeated freeze-thaw cycles.

0.1% Ponceau S

Dissolve 0.1 g of Ponceau S in 1ml of acetic acid and 99 ml of distilled water. This reagent can be stored at room temperature.

SDS-PAGE running buffer

For 10 × running buffer, dissolve 151 g of Tris, 720 g of Glycine, 50 g of SDS in 5 L of distilled water. For 1 × working solutions, dilute 10 × running buffer 1:10 with

distilled water. This reagent can be stored at room temperature.

TBS/T (pH 7.4)

For 10 × TBS/T, dissolve 400 g of sodium chloride, 10 g of potassium chloride, 151.25 g of Tris, 70-80 ml of hydrochloric acid (to adjust pH 7.4), 50 ml of Tween-20 in 5 L of distilled water. For 1 × working solutions, dilute 10 × TBS/T 1:10 with distilled water. This reagent can be stored at room temperature.

Transfer buffer

For 50 × Transfer buffer, dissolve 15.1 g of Tris, 71.3 g of glycine in 500 ml of distilled water. This reagent can be stored at room temperature. For 1 × working solutions, dilute 50 × Transfer buffer 1:50 with distilled water (80%) and methanol (20%).

0.5 M Tris-HCl (pH 6.8)

Dissolve 30.3 g of Tris in 400 ml of distilled water, and then, add HCl (about 25 ml) to adjust pH 6.8, and dilute to 500 ml with distilled water. This reagent can be stored at room temperature.

Laemmli sample buffer

For 6 × Laemmli sample buffer, dissolve 6 g of SDS, 4.6 g of DTT, 30 ml of 0.5 M Tris-HCl (pH 6.8), 3 mg of bromophenol blue, 15 ml of glycerol, and dilute to 50 ml with distilled water. This stock solution can be stored at -20°C. For 1 × Laemmli sample buffer, dilute 6 ×Laemmli sample buffer 1:6 with distilled water.

TROUBLESHOOTING TIPS

1. No LC3 bands can be detected

   There might be some possibilities. a) Low amount of proteins. If so, the volume of sample to load should be increased. b) Insufficient transfer efficiency. PVDF membrane, but not nitrocellulose membrane, should be used because LC3 is very small protein (about 15 kDa). Also, transfer efficiency is dependent on the apparatus. The transfer conditions (buffer, time, current) should be examined to detect LC3 bands exactly. c) Incubation time for

anti-LC3 antibody. Incubation with anti-LC3 antibody at 4°C would be better than incubation at room temperature.

2. High background.

   1% skim milk in TBS/T is used as a standard blocking and antibody dilution buffer. However, if high background is detected, the concentration of skim milk can be increased (up to 5 %), or washing time with TBS/T can be expanded (10 ~ 20 min).

3. No increase of LC3-II bands by starvation.

   FBS might be remained in dish. Culture medium containing FBS should be removed completely to replace starvation medium (EBSS). Even when LC3-II bands seem to be unchanged by treatment of some reagents or siRNAs compared to control treatment, LC3 flux might be changed. LC3 flux assay using lysosomal inhibitors should be examined.

4. No increase of LC3-II bands by bafilomycin A1 treatment.

   Bafilomycin A1 treatment is usually performed in a concentration of 100-200 nM for 2 h. If the increase of LC3-II bands is not observed even under bafilomaycin A1 treatment, other lysosomal inhibitors such as chloroquine or E64d (cysteine protease inhibitor) and pepstatin A (aspartyl protease inhibitor), can be used.