

## **Proteinase K protection assay**

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### **METHOD**

#### **Preparation of cells**

1. Seed cells (e.g. MEF cells) in 6-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<sub>2</sub>.
2. Wash the cells twice with PBS and culture them in the regular medium or EBSS for 2 h at 37°C.
3. Wash the cells with PBS and harvest them using a solution of trypsin-EDTA.

#### **Fractionation**

4. Wash the harvested cells with ice-cold PBS in 1.5 ml tube, and suspend them in 200 µl of homogenization buffer.

**NOTE** Cells harvested using trypsin-EDTA should be washed with PBS as soon as possible. Also, experimental procedure after this step should be carried out on ice.

5. Homogenize the cells with 10 strokes using a syringe with 27-gauge needle.
6. Centrifuge the sample at 300 × g for 5 min at 4°C to recover the postnuclear supernatant (PNS).
7. Centrifuge the PNS at 7,700 × g for 5 min at 4°C to separate the low-speed pellet (LSP) fraction.

**NOTE** LSP is suspended in 200 µl of homogenization buffer.

8. Centrifuge the supernatant obtained at step 7 at 100,000 × g for 30 min at 4°C to generate the high-speed pellet (HSP) and the high-speed supernatant (HSS).

**NOTE** HSP is suspended in 200 µl of homogenization buffer.

#### **Proteinase K treatment**

9. Add proteinase K (final conc. 100 µg/ml) to the LSP and HSP fractions with or without 0.5% TritonX-100 on ice for 30 min.

**NOTE** The samples added to the proteinase K or TritonX-100 should be

suspended well.

10. The LSP and HSP fractions treated with the reagents are precipitated with 10% trichloroacetic acid on ice for 30 min, and then, centrifuged at 12,000 × g for 5 min at 4°C.

11. Aspirate the supernatant, and wash the pellet with ice-cold acetone twice.

NOTE The pellet after acetone wash is quite loose. Be careful aspirating the supernatant.

12. Remove acetone from the pellet completely, and resuspend it in 50 µl of 1 × Sample buffer with 3 M Urea.

NOTE The color of samples turns yellow, but not blue, when acetone is not washed out completely.

13. After boil samples for 5 min, analyze by SDS-PAGE and immunoblot using anti-LC3 and anti-p62 antibodies.

## **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)
- Trypsin-EDTA (Sigma, T4174)
- Sodium chloride (Wako, 191-01665)
- Potassium chloride (Wako, 163-03545)
- Disodium hydrogenphosphate (Wako, 197-02865)
- Potassium dihydrogenphosphate (Wako, 169-04245)
- Sodium lauryl sulfate (SDS) (nacalai tesque, 31607-65)
- Trizma base (Tris) (Sigma, T6066)
- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma, H4034)
- Sucrose (Sigma, 28-0010-5)
- Urea (Wako, 217-00615)
- Acetone (Wako, 016-00346)
- D-mannitol (Sigma, M4125)

- Potassium hydroxide (Wako, 168-21815)
- Calcium chloride dihydrate (Wako, 031-00435)
- Trichloroacetic acid (TCA) (nacalai tesque, 34603-15)
- Proteinase K (Novagen, 70663-4)
- TritonX-100 (nacalai tesque, 35501-15)
- 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)
- Anti-LC3 polyclonal antibody (MBL, PM036)
- Anti-p62 polyclonal antibody (MBL, PM045)

## **EQUIPMENT**

- 1 ml syringe (Terumo, SS-01T)
- 27-gauge needle (Terumo, NN-2719S)
- 6 well plate (Thermo, 140675)
- Optima MAX Ultracentrifuge (Beckman Coulter, TLS-55T)
- TLA-100 rotor (Beckman Coulter)
- Dry Thermo Unit (TAITEC, DTU-1B)
- CO<sub>2</sub> incubator (Thermo, HERACELL 150i)

## **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.

### **5 N KOH**

Dissolve 5.6 g of potassium hydroxide in 20 ml of distilled water. This reagent can be stored at room temperature.

### **1 M HEPES-KOH (pH 7.5)**

Dissolve 119 g of HEPES in 400 ml of distilled water, and then, add 5N KOH to adjust pH 7.5, and dilute to 500 ml with distilled water. This reagent can be stored at room temperature.

### **Homogenization buffer (10 mM HEPES-KOH (pH 7.5), 0.22 M mannitol, 0.07 M sucrose)**

Dissolve 1 ml of 1 M HEPES-KOH (pH 7.5), 4 g of D-mannitol, 2.4 g of sucrose, and dilute to 100 ml with distilled water. This reagent can be stored at 4°C.

### **1 M Tris-HCl (pH 8.0)**

Dissolve 60.6 g of Tris in 400 ml of distilled water, and then, add HCl to adjust pH 8.0, and dilute to 500 ml with distilled water. This reagent can be stored at room temperature.

### **1 M CaCl<sub>2</sub>**

Dissolve 1.47 g of calcium chloride dihydrate in 10 ml of distilled water. This reagent can be stored at room temperature.

### **20 mg/ml proteinase K**

Dissolve 100 mg of proteinase K in 5 ml of buffer (20 mM Tris-HCl (pH 8.0), 1 mM CaCl<sub>2</sub>). Prepare aliquots of the stock solution and the aliquots can be stored at -20°C. Avoid repeated freeze-thaw cycles.

### **10% TritonX-100**

Dilute 100% TritonX-100 1:10 with distilled water. This reagent can be stored at room temperature.

### **6 M Urea**

Dissolve 36 g of Urea in 100 ml of distilled water. This reagent can be stored at room temperature.

### **Laemmli sample buffer with 3 M Urea**

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 with 3 M Urea, dilute 6 × Laemmli sample buffer 1:6 with 6 M Urea and distilled water.

## **TROUBLESHOOTING TIPS**

1. The band signal from the LSP and HSP fractions is quite weak.

The homogenization procedure might be insufficient. The number of strokes using a syringe with needle can be increased. Also, the amount of proteins obtained in 6-well plate might be low. The number of cells for this assay should be increased. When protein degradation is suspected, protease inhibitors should be used.

2. What should we use as the positive control?

We usually use TritonX-100 as simple positive control because TritonX-100 can solubilize the membrane. The expression of Atg4B mutant (Atg4B C74A) can also be used as positive control because the sealing end to end of autophagosome is suppressed in Atg4B mutant expressing cells (Mol. Biol. Cell. 19; 4651-4659, 2008).

3. Protein degradation cannot be occurred even under TritonX-100 treatment condition.

The proteinase K might be inactivated by repeating freeze-thaw cycles. Replace proteinase K batch a new one. Also, the concentration of proteinase K might not be sufficient. The concentration can be increased up to 500 µg/ml.

4. The sample cannot be solubilized after TCA precipitation.

Acetone wash might not be properly performed. Make sure that TCA and acetone are completely removed. If acetone cannot be washed away by aspiration, acetone would be removed by air drying.