Long-lived protein degradation assay

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METHOD

Pulse and chase

1. Seed cells in 24-well plates in regular medium supplemented with 10% (v/v) heat-inactivated FBS and appropriate supplements, and maintain the cells overnight at 37°C with 5% CO_2.

NOTE The regular medium should be changed to appropriate medium for used cell line. Most cell lines are cultured in DMEM.

NOTE Make more than 3 samples in the same condition for statistical analysis.

2. Remove the medium completely and add 500 μl of pulse-labeling medium (regular medium supplemented with 0.5 microCi ml^{-1} of L-leucine-U-14C) to the cells.

NOTE In some cases, ^{14}C-valine is used substitute for ^{14}C-leucine.

3. Maintain the cells for 18-24 hours.
4. Wash the cells twice with 1ml of PBS.
5. Add 500 μl of chase medium (regular medium supplemented with 2 mM leucine) to the cells.
6. Maintain the cells for 2 hours.
7. Wash the cells once with PBS.
8. Add 500 μl of assay medium (i.e. KRB buffer, EBSS, or HBSS with 15 mM HEPES) supplemented with 2 mM leucine or DMEM with or without lysosomal inhibitor to the cells.

NOTE Addition of 2 mM leucine in the assay medium is essential to avoid free amino acids utilized for newly synthesized protein.

NOTE The volume of culture medium should be precisely added.

9. Maintain the cells for 4 hours.
10. Place the plate on ice to stop the chase by cooling.

Calculate the ratio of the acid-soluble radioactivity in the medium

1. Dispense 50 μl of 100% (w/v) TCA to a new 1.5 ml-tube.
2. Transfer 400 μl of the culture supernatant to the above tube.

3. Add 50 μl of DW or serum to the non-starved or starved sample, respectively.

NOTE After this step, wash the plate once with PBS and store at -20°C.

4. Vortex the tube vigorously and place on ice for 5 min.
5. Centrifuge the tube for 5min at 12,000 rpm at r.t.
6. Transfer 250 μl of the supernatant to a scintillation vial.
7. Add 5 ml of scintillator.
8. Determine radioactivity by liquid scintillation counting (cpm released).

NOTE Measurement condition: nuclear species; ^{14}C, measurement time; 1 min, cycle; 1, mode; CPM (non-DPM correction)

Calculate the ratio of the acid-precipitable radioactivity

1. Take the plate from -20°C and place on ice.
2. Gently add 500 μl of 10% (w/v) TCA to the plate and incubate for 5 minutes.
3. Remove TCA solution completely from the plate.
4. Add 500 μl of 1M NaOH into the plate.

NOTE The amount of reagent should be added exactly.

5. Incubate for 20 min at 37°C in a humidified chamber.
6. Transfer 250 μl of the supernatant to a scintillation vial.
7. Add 250 μl of 10% TCA.
8. Add 5 ml of scintillator.
9. Determine radioactivity by liquid scintillation counting (cell cpm).
10. NOTE Measurement condition: nuclear species; ^{14}C, measurement time; 1 min, cycle; 1, mode; CPM (non-DPM correction)

Calculate the rate of the long-lived protein degradation

1. Total cpm released = cpm released x2 x1.25
2. Total cell cpm = cell cpm x2
3. Degradation rate=(total cpm released)/{(total cpm released)+(total cell cpm)} x100

MATERIALS

REAGENTS

- Mouse embryonic fibroblasts (MEFs)
- Regular medium
  - Dulbecco's modified eagle's medium - high glucose (Sigma, D5796)
  - William's E medium, no Glutamine (Life technologies, 12551-032)
  - RPMI 1640 medium (Life technologies, 11875-093)
- MEM Non-Essential Amino Acid Solution, 100x (Life technologies, 11140-050)
- Sodium Pyruvate (100 mM) (Life technologies, 11360-070)
- Trypsin-EDTA (0.25%) (Life technologies, 25200-056)
- Fetal bovine serum (Equitech-Bio, Inc), heat inactivated (56 _C, 30 min)
- DPBS (Nissui Pharmaceutical, 08190)
- Earle's balanced salt solution (EBSS; SIGMA, E2888)
- Leucine (Leu; Wako, 124-00852)
- 100% (w/v) Trichloroacetic acid Solution (Wako, 206-08081)
- NaOH (Wako, 198-13765)
- Leucine L- $[^{14}C(U)]$ (PakinElmer, NEC-279E)
- Valine, L- $[^{14}C(U)]$ (PakinElmer, NEC-291E)
- E64d (Peptide institute, 4321)
- Pepstatin A (Peptide institute, 4397)
- OptiPhase SuperMix Cocktail, 1X5L (Perkin Elmer, 1200-439)
- NaCl (Wako, 191-01665)
- KCl (Wako, 166-17945)
- KH_2PO_4 (Wako, 166-04255)
- $NaHCO_3$ (Wako, 199-05985)
- Glucose (Wako, 049-31165)
- $CaCl_2$ (Wako, 036-00485)
- $MgSO_4$ (Wako, 138-00415)

EQUIPMENT

- Costar® 24 Well Clear TC-Treated Multiple Well Plate (CORNING, #3524)
- Liquid aspirator setup (ULVAC, DAP-15)
- CO_2 incubator (Panasonic, MCO-20AIC)

˝ Liquid scintillation analyzer (Aloka, LSC-6100)
˝ Low diffusion PE vial, 20 ml, anti-static, case of 100 (Perkin Elmer, 6000477)

REAGENT PREPARATION

PBS for cell culture

Dissolved 9.6 g of DPBS in 1000 ml of distilled water and autoclave (121 _C, 30 min). This reagent can be stored at room temperature.

1M NaOH

Dissolve 2 g of sodium hydroxide in 50 ml of distilled water. This reagent can be stored at room temperature.

Labeling medium

Regular medium supplemented with 10% (v/v) heat-inactivated and dialysed FBS, 50 U ml^{-1} penicillin-streptomycin, and 2 mM L-glutamine. Add Leucine L-［^{14}C(U)］ to the medium at the final concentration of 0.5 μCi/ml.

100 mM leucine

Dissolve 1.31 g of leucine in 100 ml of distilled water and filtrate by using a syringe filter system. This solution can be stored at 4°C.

Chase medium

DMEM supplemented with 10% (v/v) heat-inactivated FBS and 50 U ml^{-1} penicillin-streptomycin, 2 mM L-glutamine, and 2 mM leucine.

Assay medium

Add 10 μl of 10 mg/ml E64d and 10 mg/ml pepstatin A to 10 ml of regular medium or starvation-medium (EBSS or KRB buffer).

Krebs-Ringer bicarbonate (KRB) buffer

˝ 118.5 mM NaCl
˝ 4.74 mM KCl
˝ 1.18 mM KH_2PO_4

˝ 23.4 mM $NaHCO_3$
˝ 6 mM Glucose
˝ 2.5 mM $CaCl_2$
˝ 1.18 mM $MgSO_4$

Dissolve 6.93g of NaCl, 353 mg of KCl, 161 mg of KH_2PO_4, 1.97 g of $NaHCO_3$, 1.08 g of Glucose, 368 mg of $CaCl_2$, and 290 mg of $MgSO_4$ in 500 ml of distilled water. pH 7.4-7.6.

10 μg/ml E64d

Dissolve 5 mg of E64d in 500 μl of DMSO. This reagent can be stored at . 30°C.

10 μg/ml Pepstatin A

Dissolve 25 mg of Pepstatin A in 2.5 ml of DMSO. This reagent should be stored at . 30°C.

TROUBLESHOOTING TIPS

1. No or weak induction of autophagy.
   1. PBS wash may be incomplete. Make sure that culture medium is completely washed away before starvation.
   2. ^{14}C-valine is used instead of ^{14}C-leucine. Leucine may inhibit induction of autophagy.

2. No inhibition of autophagy by treatment of protease inhibitor.
   This may be caused by inactivation of lysosomal inhibitors, E64d and pepstatin A. Prepare these reagents before use. 20 mM NH_4Cl or 100nM bafilomycin A1 can be used as a substitute reagent of lysosomal inhibitor.

3. The obtained results are not replicable.
   In the process of sample preparation, the number of the cells or the quantity of the reagent may be different among samples. The number of the cells exactly and take the reagent of accurate volume. As other possibility, cell death may be occurred during starvation. Check the cell state at each step.