GFP-Atg8 Cleavage Assay

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METHOD

For yeast strain construction, culture, and induction of non-selective autophagy by starvation or rapamycin treatment, also see the protocols "Cultivation of Yeast Cells and Induction of Autophagy" and "Fluorescence Microscopy of GFP-Atg8 Transport into the Vacuole and Observation of Autophagic Bodies".

Sample preparation

1. Culture yeast cells expressing GFP-Atg8 to mid-log phase (OD_{600}=~1.5) at 30°C, and induce autophagy as described in the protocol "Cultivation of Yeast Cells and Induction of Autophagy".
2. Take 1 ml of the culture, and harvest cells by centrifugation at 15,000 *g* for 1 min at 4°C using a centrifuge (MX-105, TOMY). Store the cell pellets at −30°C until all samples are obtained.

NOTE GFP fragments generated by vacuolar degradation of GFP-Atg8 become detected in immunoblotting analysis after nitrogen starvation/rapamycin treatment for ~2 h under our experimental conditions. We usually take samples at 0, 2, and 4 h.

NOTE An *atg* mutant should be included as a control in order to judge whether GFP-Atg8 cleavage occurs in an autophagy-dependent manner under your experimental conditions.

NOTE Cell pellets can be stored at −30°C for a few weeks.

3. Suspend the cells in 100 µl of ND solution. Stay on ice for 15 min.
4. Mix with 100 µl of 20% TCA. Stay on ice for 15 min.
5. Centrifuge at 15,000 *g* for 5 min at 4°C.
6. Remove the supernatants, and suspend the pellets in 0.5 ml of cold acetone using a bath sonicator containing iced water.
7. Centrifuge at 15,000 *g* for 5 min at 4°C.
8. Remove the supernatants, and dry the pellets at room temperature for 10 min with the lids of the test tubes opened.
9. Dissolve the pellets in [the value of OD_{600} units × 50] µl of SDS sample buffer by mixing at 65°C for 10 min using a mixer (Thermomixer Comfort, Eppendorf),

and then boil for 3 min.

NOTE Some proteins, at least Ape1, can disappear in immunoblotting detection, if the pH of the sample buffer becomes acidic due to the carry-over of TCA. Thus, we recommend using SDS-PAGE sample buffer of the composition described below.

10. Centrifuge at 15,000 *g* for 1 min at room temperature, and use the supernatants for immunoblotting analysis.

SDS-PAGE and Immunoblotting

11. To prepare 10 ml of 12% acrylamide gel, mix 4 ml of 30% acrylamide/bisacrylamide (37.5:1), 2.5 ml of 1.5 M Tris–HCl (pH 8.8), 100 µl of 10% (w/v) SDS, and 3.3 ml of H_2O.

12. Add 50 µl of 20% (w/v) APS and 4 µl of TEMED, and gently mix.

13. Cast the mixture into assembled glass plates (10 cm × 16 cm× 1 mm).

14. Gently overlay a few milliliters of 70% EtOH. Stay at room temperature for 15 min.

15. Remove 70% EtOH thoroughly.

16. Mix 2.5 ml of Stacking gel mix with 12.5 µl of 20% (w/v) APS and 2.5 µl of TEMED, overlay the mixture onto the separate gel, insert a comb, and stay at room temperature for 15 min.

17. Remove the comb and flush lanes with distilled water.

18. Load 10 µl of the samples and molecular weight markers (Precision Plus Protein™ Dual Color Standards, Bio-Rad, 1610374), and perform electrophoresis at a constant current of 40 mA for ~90 min using Gel electrophoresis apparatus (BE-260/BE-250, Bio Craft, and PowerPac HC, BioRad), and Running buffer for SDS-PAGE (Nacalai Tesque) (until the dye front reaches the bottom of the gel).

19. Set filter papers (Blot Paper, Bio Craft), a PVDF membrane (Immobilon-P PVDF membranes, Millipore), and the gel on a semidry transfer cell (Trans-Blot Turbo System, Bio-Rad), and perform transfer at a constant voltage of 25 V for 25 min using Transfer buffer.

20. Shake the membrane in Blocking buffer at room temperature for 30 min using a seesaw shaker (BC-700, Bio Craft).

21. Exchange Blocking buffer and add anti-GFP antibody to a dilution of 1:2,000. Shake at 4°C overnight.
22. Shake the membrane in TBS-T for 5 min at room temperature. Repeat this wash step three times.
23. Shake the membrane in Blocking buffer containing peroxidase-conjugated rabbit anti-mouse IgG (1:5,000 dilution) at room temperature for 30 min.
24. Wash the membrane in TBS-T for 5 min at room temperature three times.
25. Detect the bands of GFP-Atg8 and GFP fragments on the membrane using chemiluminescent reagent for horseradish peroxidase (FEMTOGLOW^{Plus}, Michigan Diagnostics LLC) and a imager system (LAS4010 ImageQuant System, GE Healthcare). The bands of GFP-Atg8 and GFP fragments will appear near the molecular weight markers of 37 kD and 25 kD, respectively.

MATERIALS

REAGENTS

- NaOH (Wako, 198-13765)
- Dithiothreitol (DTT) (Nacalai Tesque, 14112-52)
- Trichloroacetic acid (TCA) (Nacalai Tesque, 34637-85)
- Acetone (Nacalai Tesque, 00310-53)
- Tris(hydroxymethyl)aminomethane (Tris) (Nacalai Tesque, 35434-21)
- HCl (Nacalai Tesque, 18321-05)
- Sodium dodecyl sulfate (SDS) (Nacalai Tesque, 31607-65)
- Glycerol (Nacalai Tesque, 17018-83)
- Bromophenol blue (Wako, 021-02911)
- 30% acrylamide and bisacrylamide (37.5:1) solution (Bio-Rad, 06144-05).
- Ammonium persulfate (APS) (Nacalai Tesque, 02627-34)
- N, N, N', N' -tetramethylethylenediamide (TEMED) (Nacalai Tesque, 33401-72)
- Glycine (Wako, 073-00737)
- Precision Plus Protein™ Dual Color Standards (Bio-Rad, 1610374)
- Running Buffer Solution (10x) for SDS-PAGE, Tris-Glycine (Nacalai Tesque, 30329-74)
- Methanol (Nacalai Tesque, 21915-93)
- Immobilon-P PVDF membranes (Millipore, IPVH00010).

• Semidry Gel Pad (filter paper) (Bio Craft, 7002)
• Grade 3MM Chr Blotting Paper (GE Healthcare, 3030-917)
• NaCl (Wako, 191-01665)
• KCl (Wako, 163-03545)
• Tween-20 (Nacalai Tesque, 28353-85)
• Meiji Step skim milk (Meiji)
• Anti-GFP monoclonal antibody (Clontech, JL-8, 632381)
• Peroxidase-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch, 315-035-003)
• FEMTOGLOW^{Plus} (Funakoshi/Michigan Diagnostics LLC, SHRPE21008)

EQUIPMENT

• Centrifuge MX-105 (TOMY)
• Thermomixer Comfort (Eppendorf)
• Gel electrophoresis apparatus BE-260, BE-250 (Bio Craft)
• PowerPac HC (BioRad)
• Trans-Blot Turbo System (Bio-Rad)
• Seesaw Shaker BC-700 (Bio Craft).
• LAS4010 ImageQuant System (GE Healthcare)

REAGENT PREPARATION

ND solution

• 0.2 M NaOH
• 0.1 M DTT

To prepare 1 ml of this reagent, mix 0.2 ml of 1 M NaOH, 0.1 ml of 1 M DTT (store at −20°C), and 0.7 ml of distilled water, and chill on ice, immediately before use.

SDS sample buffer

• 0.1 M Tris–HCl (pH 7.5)
• 2% (w/v) SDS
• 10% (v/v) glycerol
• 20 mM DTT

• bromophenol blue (trace amount)

To prepare 50 ml of this buffer, mix 5 ml of 1 M Tris–HCl (pH 7.5) with 10 ml of 10% (w/v) SDS, 5 ml of glycerol, 0.154 g of dithiothreitol, 30 ml of distilled water, and a trace amount of bromophenol blue. Store aliquots at −20°C.

Stacking gel mix

• 5% acrylamide and bisacrylamide (37.5:1) solution

• 125 mM Tris–HCl (pH 6.8)

• 0.1% (w/v) SDS

To prepare 300 ml of this buffer, mix 50 ml of 30% acrylamide and bisacrylamide solution (37.5:1) with 75 ml of 0.5 M Tris–HCl (pH 6.8), 3 ml of 10% (w/v) SDS and 169 ml of ultrapure distilled. Store at 4°C.

Transfer buffer

Lower Buffer (for top thick papers)

• 0.3 M Tris

• 10% (v/v) methanol

To prepare 1 L of this buffer, mix 200 ml of 1.5 M Tris, 100 ml of methanol, and 700 ml of ultrapure distilled. Store at room temperature.

Middle Buffer (for middle thin papers and PVDF membranes)

• 25 mM Tris

• 0.02% (w/v) SDS

• 10% (v/v) methanol

To prepare 1 L of this buffer, mix 16.8 ml of 1.5 M Tris, 100 ml of methanol, 2 ml of 10% (w/v) SDS, and 881.2 ml of distilled water. Store at room temperature.

Upper Buffer (for bottom thick papers)

• 25 mM Tris

• 0.02% (w/v) SDS

• 10% (v/v) methanol

• 40 mM 6-aminohexanoic acid

To prepare 1 L of this buffer, mix 16.8 ml of 1.5 M Tris, 100 ml of methanol, 2 ml of 10% (w/v) SDS, 5.2 g of 6-aminohexanoic acid, and 881.2 ml of distilled water. Store at room temperature.

Put papers and PVDF membranes in the following order (from the bottom to the top): a thick paper, a thin paper, a PVDF membrane, a gel, and a thick paper.

TBS-T (10×)

• 250 mM Tris

• 138 M NaCl

• 27 mM KCl

• 0.01% (v/v) Tween 20

To prepare 1 L of this 10× stock solution, mix 30.25 g of Tris, 80 g of NaCl, 2 g of KCl, and 10 ml of Tween-20 in 900 ml of distilled water. Add 12.5 ml of 1 M HCl to adjust the pH to 7.4, and top up to 1 L with distilled water. For a 1× solution, mix 100 ml of this stock solution with 900 ml of distilled water.

Blocking buffer

• 2% skim milk in TBS-T buffer

To prepare 50 ml of this buffer, dissolve 1 g of skim milk in TBS-T.