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Lysosomal flux assay

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ABSTRACT

This protocol details the assessment of the autophagic flux in cells, by evaluating LC3II and p62 amount before and after bafilomycin treatment.

ATTACHMENTS

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Lysosomal flux assay, Autophagic flux, LC3II, p62, Bafilomycin, ASAPCRN

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MATERIALS TEXT

MATERIALS AND REAGENTS:

- Western blot running tank
- iBlot transfer system
- LI-COR Clx scanner
- bench centrifuge
- 4-12 % Bis-Tris gel
- iBlot nitrocellulose stacks
- LDS 4X buffer
- Protease/phosphatase inhibitors
- Bafilomycin
- PBS
- Odyssey blocking buffer
- tween20

LC3B Antibody **Cell Signaling**

- **Technology Catalog #2775**

Anti-SQSTM1 Antibody clone 20F1.1 **Merck**

- **Millipore Catalog #MABN130**
- Actin antibody (Sigma A2066)

Anti-Actin antibody produced in rabbit **Sigma**

Aldrich Catalog #A2066

- Secondary antibodies (800 anti Rb, 800 anti Ms, 680 anti Rb)

iBlot™ 2 Transfer Stacks, nitrocellulose, mini **Thermo**

- **Fisher Catalog #IB23002**

Odyssey® Blocking Buffer

- (PBS) **Licor Catalog #927-40000**

Bafilomycin treatment

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1 Change medium to complete medium + bafilomycin (eg [M]**50 nanomolar (nM)** , [M]**100 nanomolar (nM)** , [M]**200 nanomolar (nM)**).

2 Treat control wells with complete medium + DMSO (eg 500X, to match bafilomycin dilution) (bafilomycin aliquots are resuspended in DMSO).

3 Keep at **37 °C** until designated collection timepoint(s) (e.g. 12 h).

4 Collect cell pellet.

Protein extraction 15m 15s

5 Keep samples **On ice** throughout extraction.

6 Dilute 4X blue LDS buffer (Cat. no. B0007, Life Tech) in water plus protease inhibitors to get 1X LDS buffer.

7 Resuspend each pellet in 1X LDS buffer (**100 µL** , but reduce or increase the volume according to the pellet size).

8 Sonicate twice for **00:00:15** at 50% power, keeping sample **On ice** . 15s

9 Boil for **00:05:00** at **100 °C** ; return directly to ice. 5m

10  10m

Centrifuge for **00:10:00** at **850 x g** .

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11 Retain the supernatant.

12 Perform BCA assay on protein samples; dilute the standards and the blank in 1X LDS buffer.

13 Store lysates at **-80 °C** .

Western blot 35m

14 

Calculate the volume of each sample containing **30 µg** of proteins; add 1X LDS to bring volume to **22.5 µL** ; add **2.5 µL** Thermo Fisher reducing reagent per sample (if using a 10-wells gel).

15 Boil samples at **100 °C** for **00:05:00** . 5m

16 

Load **25 µL** per well into 10-well mini Bis-Tris 4-12% gel(s).

17 Dilute protein ladder in 1X LDS (e.g. **4 µL** protein ladder + **16 µL** 1X LDS buffer).

18 Run in MES buffer (1X), **00:30:00** , at 200V. 30m

19 Cut off wells and bottom of gel; move gel directly to transfer stack.

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20 Transfer using iBlot P0 program to nitrocellulose membrane (Cat. no. IB23002, Thermo).

21 Do not touch membrane with gloves-use forceps and razor.

22 Cut off edges (perimeter of gel).

23 Cut across sample section between 2nd and 3rd (100kDa) ladder bands from top (or somewhere else above 75 KDa).

24 Cut across samples at 25kDa (lower red) band (middle of the band).

25 Rehydrate membrane in PBS for **00:05:00** on orbital shaker. 5m

26 Block **01:00:00** at **Room temperature** in Licor Odyssey Buffer PBS (Cat. no. 927-40000, Licor). 1h

27 Prepare primary antibody solutions in Odyssey plus 0.1% Tween:

- HMW: rabbit-anti-actin 1:1200 (**-20 °C**) + mouse-anti-p62 1:1000 (labeled “SQSTM1”, at **4 °C**).
- LMW: rabbit-anti-LC3 1:1000.

28 Recover blocking solution to be used for secondary antibodies—keep **On ice** or at **4 °C** .

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29 3h

Incubate in primary antibody solutions for **02:00:00** at **Room temperature** on orbital shaker or at **4 °C** **Overnight** .

30 5m

Wash 4x **00:05:00** with PBS-T (0.05% Tween).

31 1h

Incubate secondary antibodies at 1:10,000 dilution in Licor Odyssey plus 0.1% Tween, **01:00:00** at **Room temperature** in black box or aluminium foil:

- HMW: 680-anti-rabbit, 800-anti-mouse.
- LMW: 800-anti-rabbit.

32 5m

Wash 4x **00:05:00** with PBS-T (0.05%), in black box or aluminium foil.

33 5m

Wash 1x **00:05:00** with PBS.

34 Change to fresh PBS.

35 Immediately before acquisition, dry membranes on kimwipe.

36 Reassemble membrane and scan with Licor Clx scanner.

- Flip membranes so that lower left corner is in upper left.

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37 Analysis: LC3 II normalized over Actin (or LC3 I), then divided by baseline (DMSO condition).
- LC3 II is larger than LC3 I, but charge makes it run faster: ratio is lower band divided by upper band (or Actin).
- Normalize p62 over Actin to corroborate LC3.

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