Protocol: Western Blot Protocol for p62/SQSMT1 Antibody (NBP1-48320)

Materials

- 1X PBS
- Sample buffer, 2X Laemmli buffer: 4% SDS, 5% 2-mercaptoethanol (BME), 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl, pH 6.8
- 1X Running Buffer: 25 mM Tris-base, 192 mM glycine, 0.1% SDS. Adjust to pH 8.3
- 1X Transfer buffer (wet): 25 mM Tris-base, 192 mM glycine, 20% methanol Adjust to pH 8.3
- TBS
- TBST, TBS and 0.1% Tween
- Blocking solution: TBST, 5% non-fat dry milk
- rabbit anti-p62/SQSMT1 primary antibody (NBP1-48320) in blocking buffer (~2 μg/mL)

Methods

1. Grow cells (e.g. HeLa or Neuro2A) *in vitro* to semi-confluency (70-75%).

2. Rinse cells with ice-cold 1X PBS and lyse cells with sample buffer.

3. Sonicate and incubate cells for 5 minutes at 95°C.

   Tip: Cells are lysed directly in sample buffer.

   Note: For cell lysis, an SDS containing buffer is recommended to identify the entire cellular pool of p62/SQSMT1.

4. Load 10-40 μg/lane of sample on a 12% polyacrylamide gel (SDS-PAGE).

   Note: To determine autophagic flux based on p62/SQSTM, immunoblot analysis should include samples treated with autophagy inducers and inhibitors.

5. Transfer proteins to a Nitrocellulose membrane for 60 minutes at 100V.

   Tip: For more information on Western Blotting, see our Western Blot handbook: https://images.novusbio.com/design/BR_westernblotguide_042816b.pdf

6. After transfer, rinse the membrane with dH_2O and stain with Ponceau S for 1-2 minutes to confirm efficiency of protein transfer.

7. Rinse the membrane in dH_2O to remove excess stain and mark the loaded lanes and molecular weight markers using a pencil.

8. Block the membrane using blocking buffer solution (5% BSA in TBST) for 1 hour at room temperature.

9. Rinse the membrane with TBST for 5 minutes.

10. Dilute the rabbit anti-p62/SQSMT1 primary antibody (NBP1-48320) in blocking buffer (~2 μg/mL) and incubate the membrane for 1 hour at room temperature.

11. Rinse the membrane with dH_2O.

12. Rinse the membrane with TBST, 3 times for 10 minutes each.

13. Incubate the membrane with diluted secondary antibody, according with product's specification, (e.g. anti-rabbit-IgG HRP-conjugated) in blocking buffer for 1 hour at room temperature.

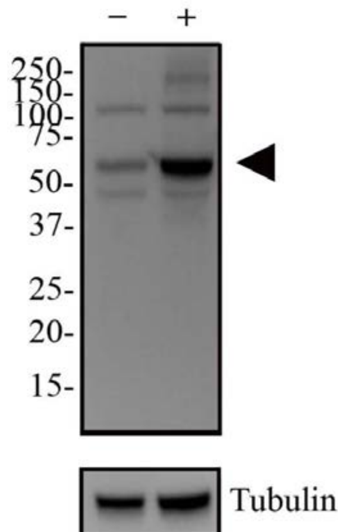
    Note: Tween-20 may be added to the blocking or antibody dilution buffer at a final concentration of 0.05-0.2%, provided it does not interfere with antibody-antigen binding.

14. Rinse the membrane with TBST, 3 times for 10 minutes each.

15. Apply the detection reagent of choice (e.g. BioFX Super Plus ECL) in accordance with the manufacturer's instructions.

16. Image the blot.

    Note: p62/SQSTM1 is primarily degraded by autophagy, therefore its presence and amount in autophagosomes inversely correlates with autophagic activity. Inhibition of autophagosome content degradation increases the levels of p62/SQSTM1 detected by immunoblot.



Western Blot: p62/SQSTM1 Antibody [NBP1-48320] - Cultured HeLa cells were treated with or without 50 μM chloriquine for 24 hours as indicated. Cell lysates were prepared and separated on a 12% gel by SDS-PAGE. Protein was transferred to PVDF membrane and blocked in 5% non-fat milk. The membrane was then probed with 1 μg/mL anti-p62/SQSMT1 in 1% milk and detected with an anti-rabbit HRP secondary antibody using chemiluminescence. Note the upregulation of p62 (arrowhead) in response to chloroquine treatment and the blockage of autophagy.