Protocol: Inhibition of Autophagy and LC3B Antibody (NB100-2220) Western Blot

Materials

- Chloroquine diphosphate (CQ) (10 mM) in dH_2O
- 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
- RIPA buffer: 150 mM NaCl, 1% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0, 20 mM Tris-HCl, pH 7.5
- 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-LC3 primary antibody (NB100-2220) in blocking buffer (~2 μg/mL)

Methods

Tip: For more information on Western Blotting, see our Western Blot handbook at novusbio.com/wb-handbook.

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

2. Add CQ to culture dishes to a final concentration of 50 μM and incubate overnight (16 hours). Remember to include an untreated sample as a negative control.

   Note: Validated autophagy inducers should be included as positive controls.

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

   Note: LC3-I and LC3-II are sensitive to degradation, although LC3-I is more labile. These proteins are sensitive to freeze-thaw cycles and SDS sample buffers. Fresh samples should be analyzed quickly to prevent protein degradation.

4. Sonicate and incubate cells for 5 minutes at 95^{o}C.

   Tip: Cells are lysed directly in sample buffer or may be lysed in RIPA buffer.

5. Load samples of Chloroquine-treated and -untreated cell lysates 40 μg/lane on a 4-20% polyacrylamide gradient gel (SDS-PAGE).

   Tip: For detection of LC3 it is particularly important to monitor the progress of the gel as this protein is relatively small (~14kDa).

Tip: Alternatively, for non-gradient gels, use a 20% polyacrylamide gel. https://www.novusbio.com/support/support-by-application/western-blot/protocol.html

6. Transfer proteins to a 0.2 μm PVDF membrane for 30 minutes at 100V.

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

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

9. Block the membrane using blocking buffer solution (5% non-fat dry milk in TBST) for 1 hour at room temperature.

10. Rinse the membrane with TBST for 5 minutes.

11. Dilute the rabbit anti-LC3 primary antibody (NB100-2220) (~2 μg/mL) in blocking buffer and incubate the membrane for 1 hour at room temperature.

12. Rinse the membrane with dH_2O.

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

14. Incubate the membrane with diluted secondary antibody, according with product's specifications, (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.

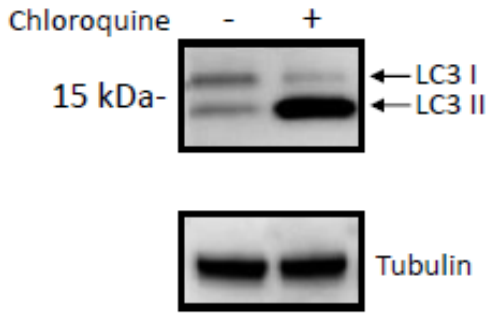
15. Rinse the membrane with TBST, 3 times for 10 minutes each.

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

17. Image the blot.

    Tip: LC3-I and it's lipidated form LC3-II have different electrophoretic mobility properties, with the lipidated form moving faster in an SDS-PAGE gel, albeit its larger molecular weight. LC3-II runs at 14-16 kDa while LC3-I runs at 16-18kDa.

    Note: This assay measures the difference in the LC3-II signal in the presence and absence of inhibitors (e.g., lysosomotropic agents). When autophagic flux is present or induced in a system an increase in the LC3-II signal should be observed with the inhibitor.



Western Blot: Neuro2a Chloroquine Treated / Untreated Cell Lysate [NBP2-49688] - Mouse Neuoblast cells (Neuro2A) were treated with (+) and without (-) 50 μM Chloroquine overnight. Whole cell protein lysates were prepared in 1x Laemmli sample buffer and approximately 10 μg of each lysate (NBP2-49688) was separated on a 4-15% gel by SDS-PAGE, transferred to 0.2 μm PVDF membrane and blocked in 5% non-fat milk in TBST. The membrane was probed with 1 μg/mL anti-LC3 (NB100-2220) and 1 μg/mL anti-alpha tubulin (NB100-690) as a loading control, and detected with the appropriate secondary antibodies using chemiluminescence.