Western Blot protocol for ATG5 Antibody (NB110-53818)

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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-Atg5 primary antibody (NB110-53818) in blocking buffer (1:500)
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 95oC.

Tip: Cells are lysed directly in sample buffer.

4. Load 10-40 ug/lane of sample on a 12% polyacrylamide gel (SDS-PAGE).
5. Transfer proteins to a PVDF 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 dH2O and stain with Ponceau S for 1-2 minutes to confirm efficiency of protein transfer.
7. Rinse the membrane in dH2O 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 anti-Atg5 primary antibody (NB110-53818) in blocking buffer (1:500) and incubate the membrane for 1 hour at room temperature.

11. Rinse the membrane with dH2O.
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.

Deparaffinization:
1. Treat slides with Xylene: 3 changes for 5 minutes each. Drain slides for 10 seconds between changes. 2. Treat slides with 100% Reagent Alcohol: 3 changes for 5 minutes each. Drain slides for 10 seconds between changes.

Quench Endogenous Peroxidase:
1 .Place slides in peroxidase quenching solution: 15-30 minutes. To Prepare 200ml of Quenching Solution: Add 3ml of 30% Hydrogen Peroxide to 200ml of Methanol. Use within 4 hours of preparation.
2. Place slides in distilled water: 2 changes for 2 minutes each.

Retrieve Epitopes:
1. Preheat Citrate Buffer. Place 200ml of Citrate Buffer Working Solution into container, cover and place into steamer. Heat to 90-96 degrees C.
2. Place rack of slides into hot Citrate Buffer for 20 minutes. Cover.
3. Carefully remove container with slides from steamer and cool on bench, uncovered, for 20 minutes.
4. Slowly add distilled water to further cool for 5 minutes.
5. Rinse slides with distilled water. 2 changes for 2 minutes each.

Immunostaining Procedure:
1. Remove each slide from rack and circle tissue section with a hydrophobic barrier pen (e.g. Liquid Blocker-Super Pap-Pen).
2. Flood slide with wash solution. Do not allow tissue sections to dry for the rest of the procedure.
3. Drain wash solution and apply 4 drops of blocking reagent to each slide and incubate for 15 minutes.
4. Drain Blocking Reagent (do not wash off the Blocking Reagent), apply 200ul of primary antibody solution to each slide, and incubate for 1 hour.
5. Wash slides with wash solution: 3 changes for 5 minutes each.
6. Drain wash solution, apply 4 drops of secondary antibody to each slide and incubate for 1 hour.
7. Wash slides with wash solution: 3 changes for 5 minutes each.

10. Drain wash solution, apply 4 drops of Hematoxylin to each slide and stain for 1-3 minutes. Increase time if darker counterstaining is desired.
11. Wash slides with wash solution: 2-3 changes for 2 minutes each.
12. Drain wash solution and apply 4 drops of Bluing Solution to each slide for 1-2 minutes.
13. Rinse slides in distilled water.
14. Soak slides in 70% reagent alcohol: 3 minutes with intermittent agitation.
15. Soak slides in 95% reagent alcohol: 2 changes for 3 minutes each with intermittent agitation.
16. Soak slides in 100% reagent alcohol: 3 changes for 3 minutes each with intermittent agitation. Drain slides for 10 seconds between each change.
17. Soak slides in Xylene: 3 changes for 3 minutes each with intermittent agitation. Drain slides for 10 seconds between each change.
18. Apply 2-3 drops of non-aqueous mounting media to each slide.
19. Lay slides on a flat surface to dry prior to viewing under microscope.

NOTES:
-Use treated slides (e.g. HistoBond) to ensure adherence of FFPE sections to slide.
-Prior to deparaffinization, heat slides overnight in a 60 degrees C oven.
-All steps in which Xylene is used should be performed in a fume hood.
-For Epitope Retrieval, a microwave or pressure cooker may be substituted for the steamer method. Adjust times as necessary depending on conditions.
-For the initial IHC run with a new primary antibody, test tissues with and without Epitope Retrieval. In some instances, Epitope Retrieval may not be necessary.
-200ul is the recommended maximum volume to apply to a slide for full coverage. Using more than 200ul may allow solutions to wick off the slide and create drying artifacts. For small tissue sections, less than 200ul may be used.
-5 minutes of development with DAB substrate should be sufficient. Do not develop for more than 10 minutes. If 5 minutes of development causes background staining, further dilution of the primary antibody may be necessary.
-Hematoxylin should produce a light nuclear counterstain so as not to obscure the DAB staining. Counterstain for 1-2 minutes for nuclear antigens. Counterstain for 2-3 minutes for cytoplasmic and membranous antigens. If darker counterstaining is desired, increase the time (up to 10 minutes).

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