Orders: orders@novusbio.com
Support: technical@novusbio.com
Web: www.novusbio.com
Protocols, Publications, Related Products, Reviews and more:
www.novusbio.com/NB100-479

NB100-479 Protocol

Western Blot protocol for HIF-1 alpha Antibody (NB100-479)

[[URL:https://www.novusbio.com/products/hif-1-alpha-antibody_nb100-479]][[Caption:HIF-1 alpha Antibody]]
Western Blot Protocol I

1. Perform SDS-PAGE on samples to be analyzed, loading 10ug of total protein per lane.
2. Transfer proteins to Nitrocellulose membrane according to the instructions provided by the manufacturer of the transfer apparatus.
3. Stain the blot using ponceau S for 1-2 minutes to access the transfer of proteins onto the nitrocellulose membrane. Rinse the blot in water to remove excess stain and mark the lane locations and locations of molecular weight markers using a pencil.
4. Rinse the blot in TBS for approximately 5 minutes.
5. Block the membrane using 5% non-fat dry milk in TBS for 1.5 hours.
6. Dilute the mouse anti-HIF-1 alpha primary antibody (NB 100-479) 1:500 in blocking buffer and incubate overnight at 4C.
7. Wash the membrane in water for 5 minutes and apply the diluted rabbit-IgG HRP-conjugated secondary antibody in blocking buffer (as per manufacturer's instructions) and incubate 1.5 hours at room temperature.
8. Wash the blot in TBS containing 0.05-0.1% Tween-20 for 10-20 minutes.
9. Wash the blot in type I water for an additional 10-20 minutes (this step can be repeated as required to reduce background).
10. Apply the detection reagent of choice in accordance with the manufacturer's instructions (Amersham ECL is the standard reagent used at Novus Biologicals).
Note: Tween-20 can be added to the blocking buffer at a final concentration of 0.05-0.2%, provided it does not interfere with antibody-antigen binding.

Western Blot Protocol II (MEF Nuclear Extracts) Nuclear Extract Preparation for HIF 1 alpha

1. Wild type and HIF1a -/- MEF cells, (initially plated at 6X10 6 cells/plate), are grown to near confluence in two 15-cm plates in 20 ml of media appropriate to cell type.
2. For hypoxic induction, 20 ml of fresh medium is added to all plates. One plate is sealed in modulator incubator chamber, flushed with 1% 02 gas for 2-3 min. at 2 psi, and incubated at 37C for 6 hrs.
3. The cells are washed once with ice cold PBS (10 mls/plate) and scraped with a rubber policeman into 5 mls of PBS. Work as quickly as possible. Cells are collected by centrifugation at 4°C for 5 minutes at 1500 X g. Control cells were incubated at 37C at 20% 02 are harvested in the same way.
4. At this point, cells may be frozen at - 80C after removing PBS.
5. For nuclear extract preparation, the cells are resuspended in 4 PCV of Buffer A. Cells are kept on ice for 10 minutes.
6. Cell suspension is homogenized with 25 strokes in a glass douncer with type b pestle (loose) and transferred to 1.5 ml Eppendorf tube.
7. Nuclei are pelleted by centrifugation for 10 minutes at 12,000 X g and resuspended in 3.5 packed cell volumes of Buffer C, and rotated for 45 minutes in the cold room.
8. The samples are centrifuged for 10 minutes at 20,000 X g, (or full speed for 90 min. in a refrigerated tabletop microfuge) aliquotted, and stored at - 80C.
Buffer A: 10 mM Tris (pH 7.5) 1.5 mM MgCl2 10 mM KCl freshly supplemented with 1:500 (v/v) of 1M DTT, 0.2M PMSF, 1 mg/ml Leupeptin, 1 mg/ml Aprotinin, 1 mg/ml Pepstatin, and 0.5M Na3VO4. Buffer C: 0.42M KCl 20 mM Tris (pH 7.5) 20% glycerol 1.5 mM Mg Cl2 freshly supplemented with 1:500 (v/v) of 1M DTT, 0.2M PMSF, 1 mg/ml Leupeptin, 1 mg/ml Aprotinin, 1 mg/ml Pepstatin, and 0.5M Na3VO4. Buffer A and C may be supplemented with Complete Protease Inhibitor Tablets (Roche) in lieu of individual inhibitors. However, DTT and sodium vanadate must still be added separately

Western Blotting
1.Cast 7.5 % SDS-PAGE gel and separate 50 ug nuclear protein and transfer to nitrocellulose membrane.
2. Block the membrane with 5% skim milk for 1 hour at room temperature.
3. Incubate with HIF1a antibody (NB 100-479, Novus Biologicals) 1:2000 for 2 hours at RT.
4. Wash 3 times with 1X TBST (Tween 0.1%) for 15 min. each
5. Incubate with Rabbit secondary antibody (HRP) 1:10000 (sc-2004, Santacruz Biotech) for 1hour at RT.

6. Wash 3 times with 1X TBST (Tween 0.1%) for 15 min. each
7. Use Amersham ECL kit for detection.

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

II. Quench Endogenous Peroxidase:
A. Place slides in peroxidase quenching solution: 15-30 minutes.

To Prepare 200 ml of Quenching Solution:
Add 3 ml of 30% Hydrogen Peroxide to 200 ml of Methanol. Use within 4 hours of preparation
B. Place slides in distilled water: 2 changes for 2 minutes each.

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

IV. Immunostaining Procedure:
A. Remove each slide from rack and circle tissue section with a hydrophobic barrier pen (e.g. Liquid Blocker-Super Pap Pen).
B. Flood slide with Wash Solution.
Do not allow tissue sections to dry for the rest of the procedure.
C. Drain wash solution and apply 4 drops of Blocking Reagent to each slide and incubate for 15 minutes.
D. Drain Blocking Reagent (do not wash off the Blocking Reagent), apply 200 ul of Primary Antibody solution to each slide, and incubate for 1 hour.
E. Wash slides with Wash Solution: 3 changes for 5 minutes each.
F. Drain wash solution, apply 4 drops of Secondary antibody to each slide and incubate for 1 hour.
G. Wash slides with Wash Solution: 3 changes for 5 minutes each.
H. Drain wash solution, apply 4 drops of DAB Substrate to each slide and develop for 5-10 minutes.
Check development with microscope.
I. Wash slides with Wash Solution: 3 changes for 5 minutes each.
J. Drain wash solution, apply 4 drops of Hematoxylin to each slide and stain for 1-3 minutes.
Increase time if darker counterstaining is desired.
K. Wash slides with Wash Solution: 2-3 changes for 2 minutes each.
L. Drain wash solution and apply 4 drops of Bluing Solution to each slide for 1-2 minutes.
M. Rinse slides in distilled water.
N. Soak slides in 70% reagent alcohol: 3 minutes with intermittent agitation.
O. Soak slides in 95% reagent alcohol: 2 changes for 3 minutes each with intermittent agitation.
P. Soak slides in 100% reagent alcohol: 3 changes for 3 minutes each with intermittent agitation. Drain slides for 10 seconds between each change.
Q. Soak slides in Xylene: 3 changes for 3 minutes each with intermittent agitation. Drain slides for 10 seconds between each change.
R. Apply 2-3 drops of non-aqueous mounting media to each slide and mount coverslip.
S. Lay slides on a flat surface to dry prior to viewing under microscope.

NOTES:
-Use treated slides (e.g. HistoBond) to assure adherence of FFPE sections to slide.
-Prior to deparaffinization, heat slides overnight in a 60 degrees celcius 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.
-200 ul is the recommended maximum volume to apply to a slide for full coverage. Using more than 200 ul may allow solutions to wick off the slide and create drying artifacts. For small tissue sections less than 200 ul 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 1-1 ½ minutes for nuclear antigens. Counterstain for 2-3 minutes for cytoplasmic and membranous antigens. If darker counterstaining is desired increase time (up to 10 minutes).

HIF-1alpha blots with normox and hypoxed Hepa 1-6 cells
1. Urea buffer for Protocol I -
2. Homogenization Buffer A: 20 mM HEPES (pH 7.5), 1.5 mM MgCl , 0.2 mM EDTA, 100 mM NaCl
3. 4 M NaCl
4. Dilution buffer B: 20mM HEPES (pH 7.5), 1.5 mM MgCl , 0.2 mM EDTA, 0.45M NaCl, 40% vol/vol glycerol, 2mM DTT, 0.4mM PMSF and 1mM sodium vanadate
5. 1 M DTT
6. Protease inhibitor cocktail tablets (Complete Mini, EDTA free) from Roche
7. 250 mM sodium orthovanadate (45.98 mg/ml in water) - Sigma
8. 13 mM deferoxamine (8.54 mg/ml – freshly made) - Sigma (cat# D9533)
9. Nonfat dry milk
10. PBST
11. SDS-PAGE gel, running buffer, sample loading buffer, markers
12. Hybond-P membrane (Amersham)
13. Electrophoresis and transfer buffers (NuPAGE/Invitrogen
14. Primary antibodies HIF-1alpha (mouse and human Novus) and beta-actin (Sigma)
15. Secondary antibody HRP conjugated goat anti-rabbit IgG (Pierce)
16. Amersham ECL Plus reagent

Cell Culture:
1. Plate Hepa cells (4 X 100 mm plates) one day before the experiment.
2. Culture O/N at 37C in 20% O2+ 5% CO2 incubator.
3. On the morning of the experiment, aspirate media and add 10ml fresh media (DMEM-HG + 10% FCS) equilibrated O/N at 1% O2 to two plates. Add 100 ul of 13 mM deferoxamine freshly resuspended in sterile water (final conc = 130 uM)
4. Leave the other two plates at 20% O2+ 5% CO2 incubator with fresh media.
5. Make the urea and HEPES buffers 15 min before the 4 h are up.
6. After 4h, take the plates out and work quickly.
7. Aspirate media.
8. Wash plate with PBS and aspirate. Add the lysis buffers at per protocol.

Protein extraction - Protocol I (urea):
Buffer prep just prior to use:
1. To 10 ml urea buffer add 1 protease inhibitor tablet.
2. Remove 1.5 ml 8M urea, 10mM Tris pH 6.8, 1% SDS + protease cocktail into a new tube.
3. Add 7.5 ul of 1M solution - 5 mM DTT final

Processing of cells.

1. Process one plate at a time as quickly as possible.
2. Add 500 ul of the above buffer to hypoxed (1%) and normox (20%) plate.
3. Scrape cells, collect to end side of the plate by tilted and transfer to a 1.7 ml microfuge tube. Pass the lysate 6-7X through a 1 ml syringe with 27 gauge needle to shear genomic DNA.
4. Remove a 50 ul aliquot and dilute it 1:10 to measure protein by Bradford reagent (BioRad).
5. Freeze the extract on dry ice and keep at -80C till the following day

Protein extraction - Protocol II (HEPES buffer)

1. Add 1 protease inhibitor tablet to 10 ml of buffer A and 1 tablet to 10 ml Buffer B.
2. Add 20 ul of 1 M DTT (final conc 2 mM) to each tube.
3. Add 40 ul of 0.25M sodium vanadate (final 1 mM) to each tube.
4. Add 250 ul of buffer A (20 mM HEPES (pH 7.5), 1.5 mM MgCl2, 0.2 mM EDTA, 100 mM NaCl), 2 mM DTT, protease cocktail and 1 mM Na vanadate
5. Add 21.87 ul of 4M NaCl (final conc of 0.45M).
6. Spin at 10,000g for 30 min at 4C.
7. Mix supernatant with an equal volume (270 ul) of 20mM HEPES (pH 7.5), 1.5 mM MgCl2, 0.2 mM EDTA, 0.45M NaCl, 40% vol/vol glycerol, 2mM DTT, 0.4mM PMSF and 1mM sodium orthovanadate.
8. Remove a 50 ul aliquot and dilute it 1:10 to measure protein by Bradford reagent (BioRad).
9. Freeze 450 ul supernatant at -80C.
10. Run 50 ug of protein on a 7% Tris acetate gel (NuPAGE/Invitrogen).

Gel and Blot:

1. Run 50 ug of protein on a 7 % Tris acetate gel (NuPAGE/Invitrogen).
2. Transfer to Hybond-P membrane (Amersham).
3. Block with 5% non-fat milk in PBST for O/N at 4C.
4. Incubate with HIF1alpha antibody 1:1000 for 1 h at RT.
5. Wash 3X with 1X PBST (Tween 0.1%) for 15 min each.
6. Incubate with HRP coupled goat anti-rabbit secondary antibody (Pierce cat# 1858415) 1:10,000 for 1h at RT.
7. Wash 3X with 1X PBST (Tween 0.1%) for 15 min each.
8. Use Amersham ECL Plus kit for detection. (from Kong, et al. JBC. 282: 15498-15505, 2008; with modifications by Rashmi Chandra, Duke University)