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NB100-105 Protocol

Western Blot Protocols specific for HIF-1 alpha Antibody (NB100-105)

Western Blot Protocol 1 (used to produce the image on the datasheet)

1. Perform SDS-PAGE (3-8%) on samples to be analyzed, loading 40ug of total protein per lane (COS-7 treated and untreated lysates.
2. Transfer proteins to Nitrocellulose 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 hour.
6. Dilute the mouse anti-HIF-1 alpha primary antibody (NB 100-105) in blocking buffer and incubate 2 hours at room temperature.
7. Wash the membrane in water for 5 minutes and apply the diluted mouse-IgG HRP-conjugated secondary antibody in blocking buffer (as per manufacturer's instructions) and incubate 1 hour 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 manufacturers 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 Procedure 2

1) Resolve aliquots (25-30 ug) of induced nuclear protein extracts on a Tris-HCl gel.
2) Transfer to nitrocellulose membranes in 20 mM Tris-HCL (pH 8.0)/150 mM glycine/20% (vol/vol) methanol
3) Block membranes for 1 hour with 1X western wash buffer containing 5% non-fat dry milk (NFDM).
4) Incubate membranes overnight at 4C in NB 100-105 diluted 1:500 in 1X western wash/5% NFDM.
5) Wash with 1X western wash for 35 minutes at RT (1 X 15 minutes, 2 X 10 minutes).
6) Incubate membranes with 1:2,000 dilution of HRP conjugated anti-mouse IgG for 1 hour (RT) in 1X western wash/5% NFDM
7) Wash with 1X western wash for 35 minutes at RT (1 X 15 minutes, 2 X 10 minutes).
8) Drain membrane and place on saran wrap
9) Using Amersham ECL Kit, mix equal volumes of two reagents. Pour over membrane (protein side facing up). Let solution sit on membrane for 15-20 seconds.
10) Drain membrane and place on new saran wrap.
11) Wrap up membrane and expose to film.
12) Develop accordingly. 10X Western wash 24.2g Tris 80g NaCl Tween-20 to 1% Ph 7.6 and QS to 4L Stripping buffer 100 mM BME 2% SDS 62.5 mM Tris (pH 6.7) Incubate membrane for 30 minutes at 56C
13) Wash membrane for 15 minutes with several changes of 1X western wash.

Notes: If hypoxia treatment is not hypoxic enough (less than 2% oxygen to get an induction), signal will be absent. Also, if the harvest time is too slow or there are not enough protease inhibitors, etc., the induced protein will be rapidly lost as HIF-1alpha has a very short half-life.
Nuclear Extract Preparation Reference: Wang and Semenza. Purification and Characterization of Hypoxia-Inducible Factor. Journal of Biological Chemistry. 270(3): 1230-1237, 1995