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NB200-145 Protocol

Serum protocol for TRPM8 Antibody (NB200-145)

TRPM8 Antibody: https://www.novusbio.com/products/trpm8-antibody_nb200-145
General Western Blot Protocol

1. Polyacrylamide gel electrophoresis and blotting
a. Add an appropriate amount of electrophoresis sample buffer (1X 125mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol, 0.003% bromophenol blue, and 1% beta-mercaptoethanol) to all samples.
b. Heat to 95o C for 3-5 minutes.
c. Load 5-100 ug total protein in a volume that is appropriate for the size of the wells.
d. Electrophorese proteins for the appropriate time according to the manufacturers instructions.
e. Transfer proteins from the gel to a suitable membrane (e.g. nitrocellulose, PVDF, etc.) following the manufacturers protocol for transfer.
f. High molecular weight proteins (>120 kDa) can be wet transferred more efficiently if transfer time is increased and 0.05% SDS is included in the transfer buffer.

2. Blocking
a. Remove the filter from the transfer apparatus and rinse in PBST/TBST to remove loose acrylamide.
b. Transferred proteins can be visualized by staining the membrane for 15-30 minutes with Ponceau S.
c. Remove stain from filter by washing with PBST/TBST.
d. Place filter into blocking solution.
e. Block for 30 minutes at 37o C, 1 hour at room temperature, or overnight at 4o C.

3. Incubation with primary antibody
a. Decant the blocking buffer and add the antibody, diluted in blocking buffer as suggested in the product description sheet.
b. Incubate with agitation for 30 minutes at 37o C, 1 hour at room temperature, or overnight at 4o C.

4. Incubation with secondary antibody
a. Wash for 30 minutes with agitation in wash buffer (TBS or PBS with 0.1% Tween 20), changing the wash buffer every 5 minutes.
b. Decant the wash solution and add AffiniClear HRP-conjugated secondary antibody, diluted in 5% non-fat dry milk in wash buffer.
c. Incubate for 30 minutes at 37o C, 1 hour at room temperature, or overnight at 4o C.
d. Decant the antibody conjugate and wash for 30 minutes with agitation in wash buffer (TBS or PBS with 0.1% Tween 20), changing the wash buffer every 5 minutes.

5. Substrate incubation (ECL)
a. Decant washing buffer and place the blot in a plastic bag or clean tray containing the development working solution (0.125 ml/cm2) for 1-5 minutes.
b. Agitate bag or tray to cover the surface of the membrane.
c. Remove the blot from the bag or tray and place it between two pieces of write-on transparency film. d. Smooth over the covered blot to remove air bubbles and excess substrate.
e. Expose to X-ray film or any sensitive screen. (Check manufacturer's instructions for specific ECL reagents and procedures.)

IHC-FFPE sections

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 90-96 degrees Celcius.
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 1/2 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).