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NB400-135 Protocol

Protocol specific for CHREBP Antibody (NB400-135)

Procedure for Western blot

1. Wash human hepatocytes grown in a culture dish with PBS twice and lyse the cells by Pierce M-Per reagent.

2. Measure protein concentration in lysates by Bio-rad protein assay.

3. Mix 20 ug of protein with Bio-rad loading buffer and 5% 2-mercaptoethanol. Boil for 5 minutes.

4. Place Bio-rad 4-15% Tris-Hcl gel. Load and run at 125V for 1 hour.

5. Transfer the proteins to nitrocellulose membrane. Run at 100V for 1 hour.

6. Block the membrane by 1% nonfat dry milk in Tris-buffered saline-Tween (TBS-T) for 1 hour.

7. Rinse the membrane twice by TBS-T.

8. Incubate with rabbit anti-ChREBP antibody (NB 400-135) 1:1,000-1:3,000 in TBS-T with 0.02% BSA overnight at 4 degrees C.

9. Wash the membrane twice by TBS-T.

10. Incubate with Goat anti-rabbit AP conjugated antibody 1:3000 in TBS-T for 1 hour at room temperature.

11. Wash the membrane twice by TBS-T.

12. Detect the signal by Bio-rad Immun-star kit and develop film.

Procedure for immunofluorescent staining

1. Wash human hepatocytes grown on cover-slips with PBS twice.

2. Fix cells with 10% buffered formalin for 25 minutes at room temperature.

3. Wash cells with PBS 3 times.

4. Incubate with 0.5% Triton X-100 in PBS for 5 minutes.

5. Wash cells with PBS 3 times.

6. Block with 1% BSA in PBS for 15 minutes at 37 degrees C.

7. Incubate with rabbit anti-ChREBP antibody (NB 400-135) 1:100-1:500 in PBS with 1% BSA for 30 minutes at 37 degrees C.

8. Wash cells with PBS 3 times.

9. Incubate with Goat anti-rabbit Alexa Fluor 568 antibody 1:2,000 in PBS with 1% BSA for 30 minutes at 37 degrees C.

10. Wash cells with PBS 3 times.

11. Mount by Vectashield mounting medium with DAPI.

12. Take pictures by using Rhodamine and DAPI filters.

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 C.

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 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.

-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).