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NBP2-42213 Protocol

Immunohistochemistry-Paraffin protocol for Thrombomodulin/BDCA-3 Antibody (NBP2-42213)

Thrombomodulin/BDCA-3 Antibody (6C8.2F9): https://www.novusbio.com/products/thrombomodulin-bdca-3-antibody-6c82f9_nbp2-42213
Reagents Required
Wash Buffer: 1X PBS (0.145 M NaCl, 0.0027 M KCl, 0.0081 M Na2HPO4, 0.0015 M KH2PO4, pH 7.4)
Incubation Buffer: 1% bovine serum albumin, 1% normal donkey serum, 0.3% Triton X-100, and 0.01% sodium azide in PBS
Primary Antibodies
Cell and Tissue Staining Kits: Kits include Biotinylated Secondary Antibodies, Serum Blocking Reagent, Peroxidase Blocking Reagent, Avidin Blocking Reagent, Biotin Blocking Reagent, High Sensitivity Streptavidin-HRP Conjugate (HSS-HRP), and Chromogen Solution. Kits are available with chromogenic substrates 3,3' Diaminobenzidine (DAB, brown precipitate) or 3-amino-9-ethylcarbazole (AEC, red precipitate).
DAB Enhancer (Catalog # CTS010)
Hematoxylin Counterstain
Aqueous Mounting Medium (Catalog # CTS011)
Antigen Retrieval Reagents (if required; Protocol for Heat-induced Epitope Retrieval (HEIR))
Materials
Gelatin-coated Slides (Protocol for Gelatin-coated Slides for Histological Tissue Sections)
Coverslips

Procedure
This staining protocol has been developed and optimized for use with R&D Systems Cell and Tissue Staining Kits.

1. Tissue must be rehydrated before commencing staining protocol.
   a. Immerse the slides in xylene (mixed isomers) 2 times for 10 minutes each.
   b. Immerse the slides in 100% alcohol 2 times for 10 minutes each.
   c. Immerse the slides in 95% alcohol for 5 minutes.
   d. Immerse the slides in 70% alcohol for 5 minutes.
   e. Immerse the slides in 50% alcohol for 5 minutes.
   f. Rinse the slides with deionized H2O.
   g. Rehydrate the slides with wash buffer for 10 minutes. Drain the excess wash buffer.

Note: Excessive fixation may result in the masking of an epitope and strong non-specific background signal that can obscure specific labeling. If necessary, an antigen retrieval protocol can be performed at this time.

Note: Endogenous peroxidase and biotin can react with secondary reagents and cause non-specific background staining. R&D Systems Cell and Tissue Staining Kits contain reagents to address these potential artifacts (applied in protocol steps 3 - 7).

2. Surround the tissue with a hydrophobic barrier using a barrier pen.

3. To quench endogenous peroxidase activity, incubate the sample with 1-3 drops peroxidase blocking reagent (3% H2O2 in water or methanol) for 5-15 minutes.

4. Rinse the sample, then gently wash in wash buffer for 5 minutes.

5. To reduce non-specific hydrophobic interactions between the primary antibodies and the tissue, block the section with 1-3 drops of serum blocking reagent for 15 minutes. Drain the slides and wipe away any excess blocking reagent before proceeding to the next step. Do not rinse.

6. To block binding to endogenous biotin, incubate the sample with 1-3 drops of avidin blocking reagent for 15 minutes. Rinse the sample with wash buffer, drain slides, and wipe away any excess wash buffer.

7. To block subsequent binding to the avidin applied in step 6, incubate the sample with 1-3 drops of biotin blocking reagent for 15 minutes. Rinse with wash buffer, drain the slides, and wipe away any excess wash buffer.

8. Incubate the sample with primary antibodies in Incubation Buffer. Follow manufacturer's recommendations regarding working dilution for the primary antibody. For chromogenic IHC staining of paraffin-embedded tissue sections using R&D Systems antibodies, it is recommended to incubate overnight at 2-8 degrees C. This incubation regime allows for optimal specific binding of antibodies to tissue targets and reduces non-specific background staining. These variables may need to be optimized for your system.

Note: Appropriate controls are critical for the accurate interpretation of IHC/ICC results. All IHC/ICC experiments should include a negative control using the incubation buffer with no primary antibody to identify non-specific staining of the secondary reagents. Additional controls can be employed to support the specificity of staining generated by the primary antibody. These include absorption controls, isotype matched controls (for monoclonal primary antibodies), and tissue type controls.

9. Rinse the sample with wash buffer. Wash 3 times with wash buffer for 5 minutes each, and drain the slides.

10. Incubate the sample with 1-3 drops of biotinylated secondary antibodies for 30-60 minutes, adjusting the incubation time depending on the thickness of the section (approximately 30 minutes for 5-10 um thick sections and 60 minutes for 10-20 um thick sections).

11. Rinse with wash buffer 3 times for 15 minutes each and drain the slides.

12. Incubate the sample with 1-3 drops of High Sensitivity Streptavidin-HRP conjugate (HSS-HRP) for 30 minutes. This signal amplification technique is referred to as the labeled streptavidin-biotin (LSAB) method.

Note: High Sensitivity Streptavidin is a chemical analog of Streptavidin that has little net positive charge at neutral or slightly alkaline pH and will interact only with biotin attached to secondary antibodies. HSS-HRP shows little or no non-specific binding to phospholipids, nucleic acids, and carbohydrate binding proteins.

13. Rinse and wash 3 times in wash buffer for 2 minutes each.

14. Calculate the required working volume of DAB/AEC Chromogen Solution given that 100-200 uL is required to cover the entire tissue section on a single slide. Add 1-5 drops of DAB/AEC Chromogen Solution to cover the entire tissue section and incubate for 3-20 minutes. Monitor the intensity of the tissue staining under a light microscope. Colored precipitate will localize to the sites of antigen expression as the chromogenic substrate is converted by HRP enzyme into insoluble end product.

Note: DAB and AEC are hazardous materials. Gloves and safety glasses should be worn and all steps performed inside a fume hood. Please refer to the MSDS for safe deactivation.

Note: If required, DAB Enhancer (Catalog # CTS010) can be used to intensify the DAB Chromogen solution.

15. Rinse the sample with wash buffer 3 times for 10 minutes each.

16. Rinse in deionized H2O and drain the slides.

17. Stained tissue can be mounted either without nuclear counterstaining or counterstained with nuclear counterstain hematoxylin for better visualization of the tissue morphology.

Note: Hematoxylin counterstain can obscure visualization of targets localized in cell nuclei.

18. Cover stained tissue with a coverslip of an appropriate size, place slides vertically on filter paper or a towel to drain excess mounting medium, and allow them to dry.

Note: Unlike DAB, AEC is soluble in alcohols and xylene. Tissue sections subjected to an HRP-AEC protocol should be coverslipped using only aqueous mounting media.

19. Visualize staining of tissue under a microscope using a bright-field illumination.

Note: Initial IHC/ICC studies often require further optimization and/or additional troubleshooting steps.

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