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Immunostaining using Notch2 (D76A6) XP Rabbit mAb #5732 (Cell Signaling Technologies):

- For paraffin sections, remove paraffin and hydrate following a standard protocol -

1. Perform antigen retrieval using a pressure cooker for 2h

   Use citrate buffer pH 6.3. The pH of this buffer is critical for the detection of N2ICD. It will need to be optimized in each case since staining depends on the fixation and embedding protocols used for tissue handling. In our hands, a pH of 6.3 yields proper results on sections that have been fixed for 1-2h with 4% PFA on a rocker at 4C. After this time, tissues are washed with PBS for 5 minutes, 3 times. Then, tissues were incubated o/n on a rocker at 4C with 30% sucrose in PBS. To embed, the tissues are transferred to OCT, left for 30 min at RT, and then frozen using dry ice.

   For paraffin, after PFA, wash similarly with PBS and transfer the tissues to 70% EtOH. Keep at 4C and embed in paraffin.

   Once the procedure has started the sections must never dry out. This could result in non-specific staining.

2. Wash once with PBS
3. Block endogenous peroxidases by incubating 10 min at RT with 3% H_2O_2
4. Wash with PBS 5 min each, 3x
5. Block with PBS-0.1% Triton 1% BSA for 10-15 min RT
6. Incubate with primary antibody 1:2000 in PBS-0.1% Triton 1% BSA o/n at 4C
7. The next day, wash with PBS, 5 min each, 3x
8. Incubate with secondary antibody, donkey anti-rabbit HRP 1:500 – 1:1000 in PBS-0.1% Triton 1% BSA for 2 hours at RT
9. Wash with PBS, 5 min each, 3x
10. Incubate with TSA diluted 1:100 - 1:200 in PBS 0.1% Triton for 15 min at RT
    TSA™ Biotin Tyramide Reagent Pack from PerkinElmer (SAT700001EA)
11. Wash with PBS, 5 min each, 3x
12. Incubate with Streptavidin-594 diluted 1:300 - 1:500 in PBS-0.1% Triton 1% BSA for 30 min at RT
13. Wash with PBS, 5 min each, 3x

    If desired, incubate with other primary antibodies, followed by their respective secondary antibodies. If not, continue to # 14

If another primary antibody from the same species is going to be used, block with goat serum anti-rabbit 5% in PBS for 1h followed by incubation with an excess of the Fab fragment Donkey anti-Rabbit IgG (H+L) (711-007-003, Jackson Immunoresearch) for 1h at RT.

If another primary antibody that also needs amplification is going to be used, block using the "Endogenous Biotin-Blocking Kit" (E21390, Life technologies) following the manufacturer instructions.

14. Add DAPI 1:1000 in PBS for 5 min, wash with PBS and mount.