

GFAP IMMUNOHISTOCHEMISTRY

Optional: heat slides to 68 C for 2 hours

I. Deparaffin (in hood):

- a. HistoClear I 5 min or dip 50 X
- b. HistoClear II 5 min.
- c. 100% EtOH I 5 min.
- d. 100% EtOH II 5min.
- e. 95% EtOH 3 min.
- f. 70 % EtOH 3 min.
- g. 35% EtOH 3min.
- h. PBS 5 min.

*slides can be stored @ 4C in PBS o/n at this point

- i. (I prefer use ABC-AP substrate) Optional: if you will use elite (peroxidase) as substrate later then Quench the slides in 3% H₂O₂ in MeOH for 10 min. (Stored @ 4 C) (it seems all of H₂O, PBS (freshly made) work according to the experience of other staining, such as MAP Kinase.)
- j. Wash in PBS 3 X 5 min.

II. Blocking

If GFAP is anti-rabbit use goat serum to block

- a. set up hydration chamber (Rubbermaid with lid, sponge, H₂O)
- b. drain off excess PBS and dab slides dry (!! don't let the section totally dry out)
- c. use Pap pen to circle samples
- d. using a pipette tip, apply 5% goat serum in PBS to each sample (150 ul/sample)
- e. incubate for 30 min. @ rt in chamber

III. Primary antibody incubation

- a. drain off blocking solution
- b. using a pipette tip (!! for any plastic small bottle in the kits, don't use pipette, but use the bottle to drop the solution directly as possible as you can to reduce the contaminate) , apply 5% goat serum in PBS, 1:100 GFAP ab-1 incubate 1 hour @ rt or o/n @ 4 C in chamber (overnight is not good for GFAP, which is sensitive and easy to get overstaining)

IV. Secondary antibody incubation

- a. Wash in PBS 2 X 5 min.
- b. Apply 2% goat serum in PBS, 1:333 secondary antibody (anti-rabbit) to each sample.

- c. Incubate @ rt for 30 min in chamber
- d. Go to step Va-b while incubating (because the ABC reagent prepared later needs to stand in RT for 30 min before use)

V. ABC-AP substrate kit

- a. Prepare ABC-AP (Vector Co.) reagent are 1:100 dilution in TS-T (one drop to 5 ml) and allow it to stand at R.T for about 30 min before use.

(Optional (for peroxidase/elite substrate) ABC Elite vectrastain reagent

Add 2 drops of bottle A and 2 drops of B from Elite Vectrastain ABC kit to 5 ml TS-Tincubate reagents 30 min @ rt (during secondary antibody incubation))

- b. Wash slides 2 X in TS-T 5 min
- c. Add ABC-AP (optional: Elite AB) prepared reagent to samples via dropper
- d. Incubate 30 min. @ rt in chamber
- e. Wash 2 X in TS-T 5 min

f. Substrate Incubation

- a. Mix AP substrate (Vector Co.) immediately before use. Add one drop of reagent 1 to 2.5 ml 0.1 M Tris-HCl pH8.2 and mix well, then one drop of reagent 2, mix well, and then one drop of reagent 3, mix well.

Optional: if you use elite/peroxidase, mix solutions from Vector Substrate (peroxidase) kit as instructed (in H₂O) just before use *If using DAB: filter solution through Drummond filter using syringe

- b. Apply filtered substrate to samples and incubate no more than 5 min.
- d. Wash 5 min in H₂O; color depends on substrate; DAB=brown, Novared=red (Novared works well)

g. Counterstain

- a. if use AP substrate, the staining is blue, then red counterstaining is preferred. Nuclear Fast Red 12 min in R.T.
- b. Optional: Counter stain slides with Hematoxylin (Vector) for 2-5 min when using a red or brown substrate (usually elite/peroxidase kit). Rinse 1X in H₂O. If use Hematoxylin then the slide needs to Rinse in Li₂CO₃ (counter staining will change from brown to blue)
- c. Rinse in H₂O

h. Dehydration (in hood)

- a. 35% EtOH 3 min.

- b. 70 % EtOH 3 min.
 - c. 95% EtOH 3 min.
 - d. 100 % EtOH 5 min.
 - e. 100 % EtOH 5 min.
 - f. histoclear II 5 min.
 - g. histoclear I 5 min.
- If color is faint: skip a and b, 95%, 100%, 100% for 20 sec each.

i. Mount slides

- a. apply a drop of cytooseal to sample while slide is still wet
- b. coverslip immediately
- c. dry at least 30 min. before using microscope

TS-T Buffer

50 ml	1 M tris pH 7.6
30 ml	5 M NaCl
10 ml	10% tween 20 or 1 ml tween 20
910 ml	ddH ₂ O

*if primary antibody is made from rabbit, and biotinylated anti rabbit IgG is made from goat, use 5% goat serum in PBS to block

*if primary antibody is mouse, use serum because biotinylated anti mouse IgG is from goat